ROLE OF ESTERASES IN THE DETOXIFICATION OF PESTICIDES

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by

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ABSTRACT

Occupational exposure of pesticides occurs via inhalation through the lungs or by absorption through the skin. The assessment of the possible importance of these extrahepatic tissues in the hydrolysis of pesticides is important.

Although the liver has been shown to be the most metabolically active tissue, both the skin and lung have the ability to metabolise pesticides. Therefore, the possibility exists that pesticides which are absorbed through the lung and skin may undergo first pass metabolism in these tissues.

In the study, the esterase enzymes responsible for the hydrolysis and subsequent detoxification of a number of pesticide substrates were identified and quantified. Esterases which hydrolysed the pesticides fluazifop-butyl and carbaryl and phenylacetate, a marker substrate for esterase activity, were found to be distributed in the microsomal fraction of the liver and lung and in the blood. In vitro studies in the rat show that lung, skin and plasma have an important role to play in the first pass metabolism of the pesticides fluazifop-butyl and carbaryl. In paraoxon hydrolysis, the plasma plays an important role in first pass metabolism, whereas the lung and skin have little effect.

With the use of inhibitors and inducers these esterase enzymes were characterised as 'A' or 'B' esterases. Fluazifop-butyl and carbaryl were hydrolysed by carboxylesterase, a 'B' esterase, whereas paraoxon was hydrolysed by paraoxonase, an 'A' esterase. Phenylacetate was found to be hydrolysed by both 'A' and 'B' esterases.

Parallel studies were carried out in human liver and blood to establish whether the rat was an appropriate model for study of the detoxification of pesticides by esterases. Studies have shown that there is considerable similarity in the nature of human and rat esterase enzymes, although there are significant differences in absolute activities.

PUBLICATIONS

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EXPERIMENTAL AIMS

Occupational exposure to pesticides can occur mainly via inhalation through the lungs or by dermal absorption. Many pesticides are esters and undergo hydrolysis by esterases following absorption into the body. Following dermal absorption and inhalation, absorbed compounds may be hydrolysed in skin, lung and circulation before the major site of hydrolysis, the liver Most current knowledge on esterase hydrolysis of xenobiotics has been obtained through studies in the liver of animals where esterase activity is found to be at its greatest. Although studies on esterase activity have been carried out in the plasma of animals, little information is available on possible esterase activity in the extra-hepatic tissues of the lung and skin.

The aim of the study was to determine the distribution of the esterase enzyme in the microsomal and cytosolic fractions of the rat liver, lung and skin as well as in the plasma and erythrocytes, to establish the importance of these tissues in the detoxification by hydrolysis of a number of pesticides. Using specific inhibitors and inducers the nature of the esterases involved in the hydrolysis of these pesticides being studied was elucidated. To compare rat and human tissues where possible, experimental studies were carried out in liver and plasma to assess the rat as a model for extrapolation to man.

SECTION I : GENERAL INTRODUCTION

CHAPTER 1 : XENOBIOTIC METABOLISM

1.1 INTRODUCTION

The body is continually exposed to a number of foreign compounds (xenobiotics). They may be absorbed by a variety of routes including ingestion, inhalation and dermal absorption. Most xenobiotics that are absorbed undergo metabolic transformation in the body to enable elimination by excretion. Excretion often follows conversion of the xenobiotic to a more soluble metabolite. The ability of the body to metabolise xenobiotics was first reported by Wohler in 1824, who showed that benzoic acid ingested in food converted to a metabolite which was excreted into the urine.

Enzymes responsible for xenobiotic metabolism form a diverse group, which biotransform many exogenous compounds, and some endogenous substrates. Drugs and xenobiotics are transformed by a variety of pathways classified as phase I and phase II reactions. Phase I reactions or functionalisation reactions serve to introduce a functional group into the xenobiotic thereby changing it to a more polar and hence more excretable form. Products from phase I reactions may act as substrates for phase II metabolism.This involves conjugation with endogenous substrates, resulting in increased water solubility and polarity of the compound. The increases in the solubility and polarity of the xenobiotic aid in its elimination or excretion from the body. Examples of types of phase I and phase II reactions can be seen on table 1.1. The enzyme system responsible for metabolic transformation of xenobiotics are located principally in the liver, although enzymes are present to a lesser extent in other tissues. However, most of the fundamental knowledge of xenobiotic metabolism has been derived from studies in the rat liver.

Most current knowledge has been obtained by disruption of the liver by differential centrifugation to release subcellular organelles. The enzymes found in the endoplasmic reticulum and the cytoplasmic fraction of the subcellular components are responsible for xenbiotic metabolism. Phase I oxidative enzymes are oxidases localised in the endoplasmic reticulum with the phase II enzyme glucuronyl transferase.

Table 1.1. Examples of phase I and phase II drug metabolism reactions

Phase I	Phase II
Hydrolysis	Sulphation
Oxidation	Glucuronidation
Reduction	Glutathione conjugation

Hydrolytic enzymes are present in both endoplasmic reticulum and cytosol. Other phase II enzymes are predominantly located in the cytosol. Intact endoplasmic reticulum consists of a network of filamentous membrane bound channels which, on disruption, form microsomes. These microsomes retain most of their enzymatic activity.

1.2 PHASE I XENOBIOTIC METABOLISM

1.2.1. Hepatic Metabolism

The liver is the largest single organ, constituting about 2% of the mass of the body. It is enclosed in a connective tissue sheath known as Gilssons capsule. The functional mass of the liver contains four main components : (1) the parenchymal cells or hepatocytes, which constitute about 60% of the mass of liver; (2) reticuloendothelial cells (Kupffer cells); (3) the biliary tracts; and (4) blood vessels.

The portal vein and hepatic artery enter the liver at the porta hepatis and the intrahepatic portion of the bile duct emerges through the same orifice. Branches of all the three vessels run together within the liver, constituting Gilssons triad, together with connective tissue and lymphatic channels. The simple liver acinus (SLA), the stuctural and functional hepatic unit is arranged around the Gilssons triad. The smallest structural units of the SLA are the lobules, which number 50 to 60 thousand. Each lobule is roughly a hexagonal prism, several millimeters long and with a cross sectional area of about $2mm^2$. Within each lobule, hepatocytes are arranged in sheets or plates to form a labyrinth, the walls of which are normally only one cell thick.

Each hepatocyte is polyhedral, having 5-12 sides, and a diameter of 12-25µm. The cytoplasm typically displays copious amounts of both granular and agranular endoplasmic reticulum, high concentrations of mitochondria, lysosomes and

a well developed Golgi complex- all features indicating a high metabolic activity.

The hepatocytes carry out a multitude of metabolic activities, including the synthesis and release into the blood of various plasma proteins including albumins, clotting factors and complement components. Hepatocytes also eliminate endogenous and exogenous substances from the blood stream, and are thus important in detoxification of the blood.

1.2.1.i. Hydrolysis

Hydrolysis by esterases is a major route for the metabolism of endogenous compounds as well as xenobiotics. The liver is a major site for esterases but there is a wide distribution in other body tissues and fluids. Esters, amides, hydrazides and carbamates can be hydrolysed by various esterase enzymes. The basic hydrolysis of xenobiotic esters catalysed by esterases is as follows

$$RCOOR + H_2O \text{ ---------> } RCOOH + R\,OH$$

where RCOOR represents a xenobiotic ester, RCOOH and R OH the products.

In 1953 Aldridge classified these esterases into three groups on the basis of their interaction with organophosphates: 'A' esterases which hydrolyse organophosphates and include arylesterase and paraoxonase; 'B' esterase which are inhibited by organophosphates and include carboxylesterase, cholinesterase and acetylcholinesterase and 'C' esterases which do not interact with organophosphates (see Table 1. 2.).

Table 1.2. Classification of 'A' and 'B' type esterases.

Type of Esterase	Enzyme Nomenclature	Active centre
A-Esterase	arylesterase, paraoxonase (E.C.3.1.1.2.)	cysteine amino acid residue
B-Esterase	carboxylesterase (E.C.3.1.1.1.)	serine amino acid
	cholinesterase (E.C.3.1.1.8.)	residue
	acetylcholinesterase (E.C.3.1.1.7.)	

Classification of these esterases is by the use of specific inhibitors. Organophosphate compounds are non-selective inhibitors of 'B' esterases. Inhibition is as a result of phosphorylation of the serine amino acid residue at the active centre of the enzyme. Inhibitors of carboxylesterase and cholinesterase include physostigmine, neostigmine, pyridostigmine and edrophonium. However, bis- (4-nitrophenyl) phosphate (BNPP) is a more selective inhibitor of carboxylesterase. The organophosphate bis- (4-cyanophenyl) phosphate and 4-nitrophenyl phosphoriate can be used to selectively inhibit the carboxylesterase lysophospholipase and medium chain monoacylglycerol lipase (Brandt et al, 1980).

1.2.1.i.a. A Esterases

The A esterase enzyme has a cysteine amino acid residue at its active centre and is at present classified under arylesterase (E.C.3.1.1.2.; A esterase, paraoxonase) (Enzyme Nomenclature, 1984). The term arylesterase was introduced by Augustinsson (1958) with respect to an enzyme activity of human plasma which rapidly hydrolysed aromatic ester compounds (Whitaker and Mounter, 1953). Enzymes of this class are not inhibited by bis-nitrophenol phosphate (BNPP) or physostigmine and rapidly hydrolyse aromatic esters such as phenylacetate and organophosphates, whereas butyrates are slowly hydrolysed (La Du and Eckerson, 1984). The designation of A esterase for arylesterase was based on the capacity of the enzyme to hydrolyse the organophosphate insecticide paraoxon, however, there is some doubt concerning the capacity of purified serum arylesterase to hydrolyse paraoxon, therefore, paraoxonase is currently classified as a separate enzyme. Ca^{2+} ions are known to increase the activity of the A esterase enzyme.

Human paraoxonase activity has been found to vary considerably among individuals. This may be of considerable importance in protection against acetylcholinesterase inhibition by organophosphate compounds (Williams, 1987). It has been reported that phenylacetate hydrolysis in human serum (caucasian population)

is unimodally distributed (Simpson, 1977; Loreentz et al, 1979; Reiner et al, 1987), whereas paraoxon hydrolysis has a triphasic distribution (Reiner et al, 1987; Geldmachen-V Mallinckrodt et al, 1973; Eckerson et al, 1983 a and b). Published data, however, concerning the specificity and number of enzymes are still ambiguous and even controversial (Skrinjaric-Spoljar and Reiner, 1968; Geldmachen-V Mallinckrodt et al, 1973; Eckerson and La Du, 1984; Mackness et al, 1987). A lack of correlation between paraoxon and phenylacetate hydrolysis in the total serum population and in any of the modes based upon paraoxon hydrolysis has been reported (Geldmachen-V Mallinckrodt, 1988). This however is contrary to that found by Eckerson et al, (1983 a and b), who found high correlation for the hydrolysis of paraoxon and phenylacetate in all three phenotypes, suggesting that these activities might be the property of the same enzyme. However, gel-filtration experiments carried out by Mackness et al, (1987) suggest that paraoxon and phenylacetate hydrolysis can be partially separated, supporting that different enzymes hydrolyse paraoxon and phenylacetate.

High A esterase activity is found in the serum of a wide variety of mammalian species but the activity is low or absent in avian species (Brealey et al, 1980; Mackness et al, 1987). The hydrolysis of the toxic metabolites is extremely rapid in mammalian serum compared to that of the avian sera (Machin et al, 1978, 1975; Brealy, 1981).This difference has been shown to responsible for the selective toxicity of organophosphates to mammals compared to birds. The hydrolysis of paraoxon by paraoxonase was found to be 7-fold greater in rabbit serum than in rat serum, with the dose of paraoxon required to produce similar signs of toxicity and similar degrees of cholinesterase inhibition in rats and rabbit differing by 4-fold. In the house mouse four distinct enzymes for arylesterase have been found, noted genetically as ES-8, ES-16, ES-19 and ES-24, whereas in the rat only one has been identified, ES-17 (Chapman et al, 1974; von Deimling and Taylor, 1987).

1.2.1.i.b. B Esterases

Carboxylesterase

Carboxylesterases (E.C.3.1.1.1.) have a serine amino acid residue at its active centre and are subclassified according to their isoelectric points (Mentlein et al, 1987). The term carboxylesterase gradually replaced the term aliesterase which was first used by Richter and Croft (1942).

Carboxylesterase comprises the majority of tissue esterase in mammals. This class predominates in mammals both qualitatively and quantitatively, and posses a high degree of multiplicity and a large number of isoenzymes (Hedrich et al, 1987). Similarly, clusters of closely linked carboxylesterase gene loci have been located in the mouse (Peters, 1982) and at least six chromasomal gene loci have been located in the rabbit (Zutphen et al, 1987).Carboxylesterase is also widely distributed in the tissues of vertebrates and insects (Brooks, 1979; Beckendorf and Stephen, 1970).Among various tissues of animals, the highest esterase activity with various substrates is found in the liver (Junge and Krisch, 1975; von Deimling and Bocking, 1976). Rat kidney has moderate carboxylesterase activity in the proximal tubules (Echobichon, 1973; Bocking et al, 1976) as well as being found in the small intestine epithelial cells of rats (De Jong et al, 1978; Bocking et al, 1976). Besides these tissues, tissues carboxylesterase has been found in the lung (Milz and Budd, 1978), testis (Beckendorf and Stephen, 1970), adipose tissue (Okuda and Fujii, 1967; Tsujita et al, 1982), the central nervous system (Rumsby et al, 1973; Paul and Halaris, 1976) and rat brain (Kishimoto, 1973).

As in experimental animals, humans have carboxylesterase in muscle (Ecobichon and Kalow, 1965), kidney (Ecobichon and Kalow, 1964), brain (Hojring and Svensmark, 1976; 1977), liver (Junge et al, 1974) and serum (Brogren and Boeg-Hansen, 1975).

Rat carboxylesterase have been widely studied (Mentlein et al, 1980; Robbi et al, 1983). Using isoelectric focussing, six carboxylesterases have been purified from rat liver microsomes. Initially they were identified by their isoelectric points (esterase pI 5.0, 5.2, 5.6, 6.0 and 6.4). Although closely related in their molecular properties, four of the esterases have different primary structures and different substrate specificities to drugs and lipids (Mentlein et al, 1984a and b, 1985). All the esterases examined are in multiple forms. These micro heterogenous forms (eg. pI 6.2 and 6.4) seem to have almost identical properties. Thus carboxylesterases are currently named according to their action on intracellular substrates, with at least one endogenous ester found to be hydrolysed in each case.

The microsomal carboxylesterase, showing about five bands in isoelectric focussing around a pI of 5.6, hydrolyses simple aromatic esters very effectively, representing the acetanilide cleaving hydrolase of the liver. This corresponds to ES-3 in genetic nomenclature for rat liver esterases (Van Zutphen, 1983), has also been termed butyrylesterase L-1 (Keneka et al, 1979) or esterase EA (Arndt et al, 1978). This has been shown to be identical with the ß-glucuronidase-complexing protein of the rat (Medda et al, 1987). Mentlein et al, (1984a and 1985) showed that the esterase cleaves at least three different types of endogenous substrate: lysophospholipids, monoglycerides and long chain acylcarnitines.

The carboxylesterases with isoelectric points of 6.2 and 6.4 was found to have nearly identical specificity and chemical properties (Mentlein et al, 1984a and b). This esterase hydrolyses aspirin, propanidid, malathion, pyrethroid esters and many other esters. This corresponds to ES-4 in genetic nomenclature (Mentlein et al, 1987) and has been named cholesterol ester hydrolase (Nilson, 1976), ethanol acyltransferase (Polokoff and Bell, 1978), monoacylglycerol lipase (Ikeda et al, 1977; Mentlein et al, 1985b) or palmitoyl CoA endogenous substrates (Jamdar, 1979; Berge, 1980; Mentlein et al, 1985a).These esterases cleave long chain glycerol and CoA at high rates, while action on retinoyl, palmitate and cholesterol esters is much slower (Mentlein and Heymann, 1987).

The liver carboxylesterase most active against short chain aliphatic esters is identified as both ES-8 and ES-10 in genetic nomenclature (Mentlein, 1987). This enzyme may exist as a monomer or a trimer. This enzyme shows one or more bands around pI 6.0 on isoelectric focussing, also being described as ES-1 by Arndt et al, (1973) or as monoglyceride by Oerlemans et al, (1977). The enzyme hydrolyses procaine, clofibrate and other xenobiotics (Mentlein and Heymann, 1984), and is found in other organs besides liver, including small intestine, lung, testis, brown fat and heart (Mentlein et al, 1987). This esterase cleaves acylglycerols of medium chain length at a faster rate than long or short chain derivatives. The term medium chain monoacylglycerol lipase is used to describe this enzyme.

The carboxylesterase termed pI 5.2 (Mentlein and Heymann, 1981) or esterase pI by Robbi and Beaufy, (1983) is active with mono and diacyl glycerols and is currently termed diacylglycerol lipase (Mentlein et al, 1985). It is found in multiple forms (Mentlein et al 1984a; Robbi and Beaufy, 1986) in the liver, although no activity has been found in the liver of new born rats (Mentlein et al, 1987). Diacylglycerol lipase cleaves phorbol esters that interfere with the regulatory function of diacylglycerols in vivo.

The four groups of carboxylesterase represent the so called non-specific esterase activity of rat liver. The four esterases exhibit the highest activity in the liver, except for ES-4 which is equally active in the kidney. The carboxylesterases appears to be found in the microsomal and cytosolic fractions of the cell.

Hosokawa et al, (1990) purified seven carboxylesterase isoenzymes to electrophoretic homogoneity from liver microsomes of mouse, hamster, guinea-pig, rabbit and monkey. The substrate specificity and immunological reactivity of liver microsomal carboxylesterase from pig, cow, beagle dog and human were also examined. It was found that the ten purified preparations had similar subunit weight (57,000-64,000 daltons), but that their isoelectric points varied widely (pI 4.7-6.5).

Carboxylesterase activity is similar in the livers of the rat and chicken, but different in the adrenal glands, with chicken carboxylesterase activity lower in the rat.

Species variation in carboxylesterase are responsible for differences in the hydrolysis of xenobiotics. Sertkaya and Gorrod, (1988) investigated the hydrolysis of three isomeric arylamides; 2-acetamidobiphenyl (2-AABP), 3-acetoamidobiphenyl (3-AABP) and 4-acetamidobiphenyl (4-AABP) by microsomal carboxylesterase from mouse, hamster, guinea-pig, rat and rabbit livers. The 2-AABP was hydrolysed the fastest rate in all species except the mouse, the rate of 3 and 4 isomers was similar. The mouse arylamide hydrolysing activity was uniformly less sensitive to both carboxylesterase inhibitors BNPP and BCPP (bis- (4-cyanophenyl) phosphate) suggesting an enzyme distinct from the ES-3 carboxylesterase may be involved. Luttrel and Castle, (1988) found that meperidine hydrolysis was not detected in the guinea-pig, very low in human and extremely high in the dog. Phenylacetate hydrolysis was also measured in liver microsomal preparations, with guinea-pig showing the greatest hydrolytic activity and rat showing the least. It was therefore thought that liver microsomes from different species may contain different carboxylesterases, having different affinities for meperidine.

Acetylcholinesterase

Acetylcholinesterase (E.C.3.1.1.7.) has a serine amino acid residue at its active centre and its function is to hydrolyse acetylcholine (see Figure 1.1.). Acetylcholineesterase has the same type of binding site as cholinesterase, however, cholinesterase is a less specialized enzyme than acetylcholinesterase. Unlike acetylcholinesterase, it lacks an anionic site in a position that specially adapts it to react with acetylcholine, therefore, the acetylcholinesterase hydrolyses acetylcholine at a greater rate than cholinesterase.

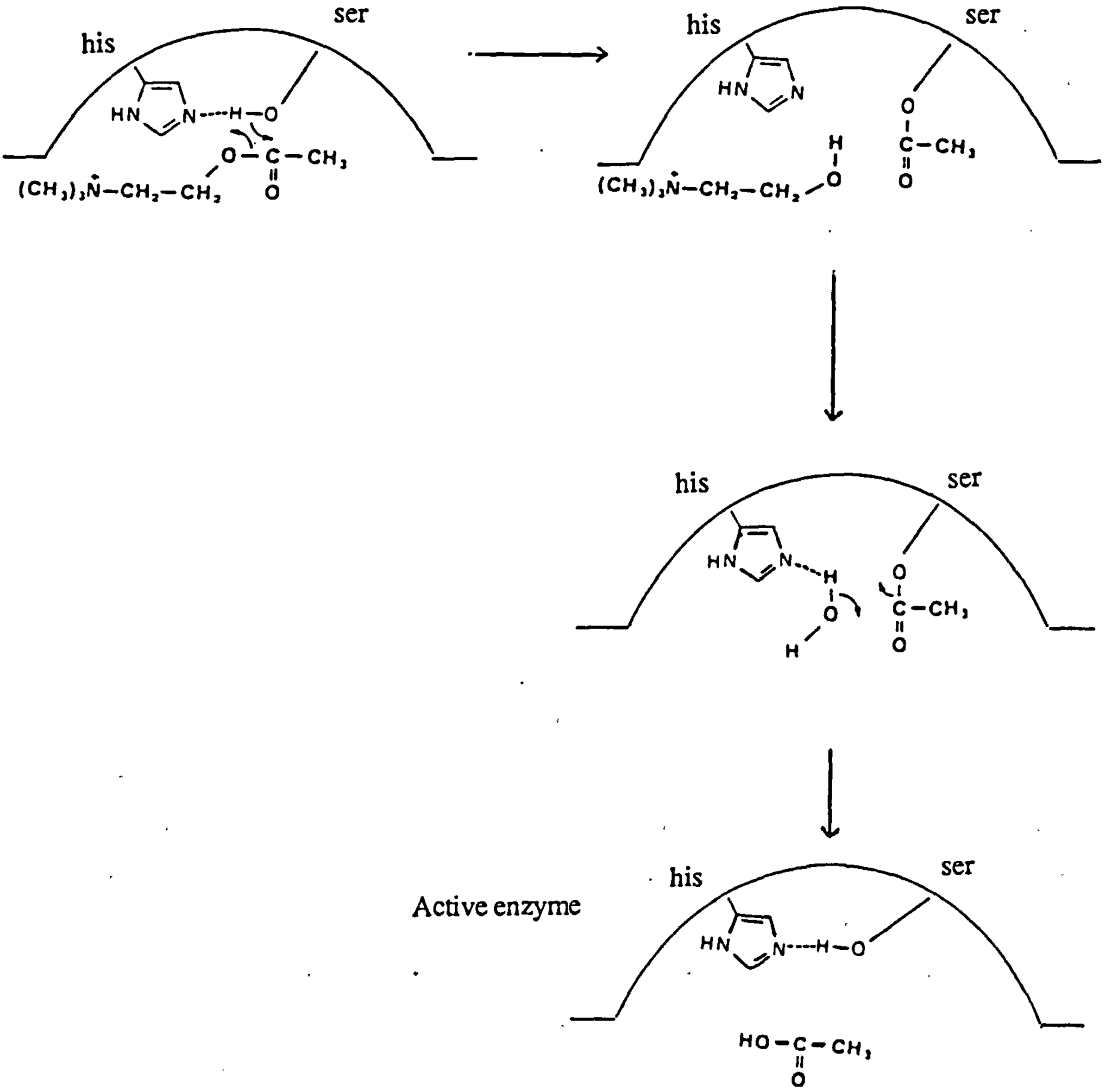


Figure 1.1.

Hydrolysis of acetylcholine at the esteratic site of acetylcholinesterase.

Acetylcholinesterase is found in nervous tissue, striated muscle and red blood cells. Monitoring AchE in the blood is a fairly sensitive method for detecting exposure to organophosphates or carbamate pesticides (Gage, 1967; Wills, 1972). The enzyme acetylcholinesterase hydrolyses the neurotransmitter acetylcholine at the cholinergic nerve synapses (Hayes, 1982). Organophosphate and N-methyl carbamate pesticides inhibit acetylcholinesterase, causing first excitation, then depression of the parasympathetic nervous system (Miller and Satoh, 1982). Acetylcholinesterase at the nerve synapses is the most meaningful index of the risk of poisoning, although this would be difficult to measure, therefore, monitoring usually involves measuring the acetylcholinesterase activity of red blood cells (rbc's) and plasma (Wills, 1973; Roberts, 1979) from blood samples.

Cholinesterase

Cholinesterase is at present classified as E.C.3.1.1.8. (Enzyme Nomenclature, 1984). In the serum, cholinesterase has been referred to as pseudocholinesterase (Augustinsson, 1948). Other names are butyrylcholinesterase or non-specific cholinesterase. The configuration of cholinesterase at the active centre is different from other esterases and this results in specificity of the enzyme. The active centre consists of two binding sites; an anionic and esteratic serine site, whereas other esterases contain only an esteratic serine site. Charged N- and methyl groups of choline esters bind to the anionic sites and hydrolysis occurs as a two stage process. Firstly, the acyl group binds to the serine residues of the esteratic site, and choline is released. Secondly, the transiently acylated enzyme reacts with water to yield the acid and regenerate the enzyme. Butyrylcholine and benzoylcholine are prefered substrates. Cholinesterase hydrolyses drugs such as suxamethonium (succinyl choline), acetyl salicyclic acid, diacetyl morphine, procaine and other local anaesthetics as well as steroid esters.

Suxamethonium, in most individuals is hydrolysed very rapidly to choline

by plasma cholinesterase with a half life of about 4 minutes (Durrant and Katz, 1982). This duration of reaction is related to plasma cholinesterase (Viby-Morgenson, 1980). Genetically atypical cholinesterase has a half-life of elimination of suxamethonium from plasma in 4 hours. Use of suxamethonium in these individuals would therefore be life threatening (Whittaker, 1980). Procaine, another type of local anaesthetic is rapidly hydrolysed to p-aminobenzoic acid and diethylamino ethanol mainly by plasma cholinesterase (Reidenberg et al, 1972; Myers et al, 1982). Rapid hydrolysis of procaine to the inactive products results in its relatively short time of action.

Heroin is rapidly hydrolysed by serum esterases to 6-mono acetylmorphine and the more slowly to morphine following an intravenous dose (Way et al, 1960; 1965). Lockridge et al, (1980) found cholinesterase to be the major esterase involved in the hydrolysis of heroin in serum. Owen and Nakatsu, (1983) have identified three further esterases involved in heroin hydrolysis within the red blood cells with at least one to these being similar to that responsible for hydrolysing aspirin.

Aspirin (acetylsalicyclic acid) is rapidly hydrolysed to salicylate and free acetate by cholinesterases following absorption and can be detected in plasma for only 60-90 minutes after an oral dose (Rowland and Reigelman, 1968). The rate of hydrolysis of aspirin is important in determining the pharmalogical and toxic effects of the drug since aspirin has different properties to salicylate.It was found that plasma and liver aspirin esterase activities in samples from a group of patients varied over a two fold range and there was a significant correlation between interindividual plasma and liver activities (Williams et al, 1989). Serum aspirin-esterase activity has also been studied in a group of epileptic patients under treatment with a number anti-epileptic drugs (Puche et al, 1989). These findings suggest that antiepileptic dugs may induce plasma esterase activity, thus leading to an increase in serum hydrolytic activity in epileptic subjects.

Many steroid esters have been shown to be substrates for serum

cholinesterase. Either hydrolysis of the ester pro-drug liberates the active steroid or serum hydrolysis may be employed to hydrolyse active locally applied steroid esters to inactive compounds and prevent unwanted side effects. In the same way pro-drugs have been developed to overcome problems in absorption and toxicity, with plasma esterases used to activate many pro-drugs.

The human plasma cholinesterase consists of four main molecular forms designated as C1, C2, C3 and C4 according to their electrophoretic mobility in gels (Masson, 1989). The major component, C4, is the tetrameric form; C1 and C3 are the monomeric and trimeric forms respectively. The C2 dimeric form, which has an apparent free electrophoretic mobility higher than that of the other three isomers, and moreover, a higher isoelectric point, was found to be a covalent conjugate between the cholinesterase monomer and the serum albumin.

Serum cholinesterase has a broader specificity than acetylcholinesterase which is found in nervous tissue, striated muscle and red blood cells. Carbamate pesticides produce cholinesterase inhibition which is rapidly reversible within 24 hours. Due to the wide range of normal levels, effective cholinesterase monitoring requires the periodic comparison of blood cholinesterase activity values to an individual cholinesterase activity baseline established prior to exposure to cholinesterase inhibiting pesticides. (Wolfsie, 1957; Vandekar, 1980). Inhibition of cholinesterase to levels of 60-25% of an individuals baseline (ie. depression from 40-75% below baseline) may result in symptoms such as nausea, vomiting, diarrhea, increased sweating, constriction of pupils, blurred vision and chest tightness. Still greater inhibition may result in respiratory difficulty, unconciousness, pulmonary oedema and death due to respiratory arrest (Namba et al, 1971; Kalovanova, 1982).

Cholinesterase activity has been found in the homogenates of the gut mucosal cells from seven mammal species and differs from one species to another (Sine et al, 1988). Except rabbit , all the other species contain a (64) globular tetrameric form

and either a (61) monomeric form (pig and ox) or a (62) dimeric form (mouse, rat, sheep). Both (61) and (62) forms are found with the (64) form in the mucosal cells of kittens and cat. The mucosal cells from the studied species essentially possess either acetylcholinesterase (rabbit, kitten, cat) or cholinesterase (ox, pig, sheep, rat, mouse).

1.2.1.ii. Oxidation

Oxidation is the most common reaction of xenobiotic metabolism and occurs in a number of subcellular sites. Enzymes in the endoplasmic reticulum are the most important and these enzymes are known commonly as mixed-function oxidases (Mason, 1957, 1965) or monoxygenases. These enzymes are complex multicomponent systems consisting of reduced nicotinamide adenine dinucleotide phosphate (NADP), phospholipid, the flavoprotein NADPH cytochrome P450 (c) reductase and the haemoprotein cytochrome P450 (Lu and Coon, 1968, Lu et al., 1969, Strobel, 1970).

1.2.1.ii.a. Components of MFO system

Cytochrome P450

The occurance in liver of a carbon monoxide binding pigment was first reported independently by Kligenberg in 1958. Studies carried out by Omura and Sato (1964) provided evidence that this novel pigment was a haemoprotein. The enzyme consists of a family of closely related iso-enzymes embedded in the membrane of the endoplasmic reticulum (ER). The name cytochrome P450 is derived from the specral absorbance maximum exhibited at 450nm when the enzyme is reduced and complexed with carbon monoxide. The haemoprotein serves as an oxygen and substrate binding site for the MFO reaction, and, in association with the flavoprotein reductase NADPH cytochrome P450 reductase, it undergoes cyclic oxidation and reduction of the haem

iron. The general reaction in which cytochrome P450 serves as an oxygenating catalyst is as follows:

$$R\text{-}H + NADPH + O_2 + H^+ \text{----------} > R\text{-}OH + NADPH^+ + H_2O$$

where R-H represents the substrate and NADPH the electron donor.

NADPH Cytochrome P450 Reductase

NADPH cytochrome P450 reductase is a flavin containing enzyme (flavoprotein), consisting of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) prosthetic group. It is responsible for the transfer of reducing equivalents from NADPH and H^+ to cytochrome P450 and is closely associated with cytochrome P450 on the ER membrane. NADPH and H^+ donates two electrons, while cytochrome P450 accepts one. It is thought the NADPH cytochrome P450 acts as a tranducer of reducing equivalents by accepting electrons from NADPH and transferring them sequentially to cytochrome P450.

Lipid

Since the successful isolation of the components (Lu and Coon, 1968; Lu et al. , 1969) and their reconstitution to an enzymatically active system (Lu et al, 1969b; Strobel et al, 1970) the protein-lipid interaction associated with cytochrome P450 has been extensively studied. The catalytic activity of cytochrome P450 systems is phospholipid dependant, with acetylated phosphatidylcholines, mono and dilaurylphosphatidylcholine appearing to be most effective (Strobel et al, 1970). The formation NADPH cytochrome P450 reductase and the cytochrome P450 complex is regulated by different lipid species. The ratio of cytochrome P450 reductase to

cytochrome P450 on the ER memrane is 20:1. The role of lipid appears to be the favourable formation of a 1:1 protein complex (Blanck et al, 1989).

1.2.1.ii.b. Catalytic cytochrome P450 cycle

The mechanism by which cytochrome P450 mediates monoxygenase reactions is not fully understood. The following scheme represents the current understanding of the microsomal cytochrome P450 cycle.

The initial step of the cycle begins with the substrate binding to the enzyme. Cytochrome P450, like many other haemoproteins exists in an equilibrium of high and low spin states, with the low state predominating (Schenkman and Gibson, 1981). Binding of substrate results in the shift of this equilibrium from low spin state to high spin state (Tsai et al, 1970), therefore causing an increase in the redox potential of the haemoprotein. This facilitates electron transfer from NADPH to the cytochrome (Ullrich, 1978).

Since oxygen can only bind to the ferrous cytochrome P450, the reduction of the ferric cytochrome P450-substrate complex is a very important stage in the reaction process. The oxidised P450-substrate complex is reduced by an electron originating from NADPH and transferred to the complex by the flavoprotein, NADPH cytochrome P450(c) reductase. This is followed by an oxygen activation process. The reduced substrate-enzyme complex reacts with oxygen to form a complex characterized by an absorption peak at 448nm (Eastabrook et al, 1971). The exact nature of the oxycytochrome is unclear. It is believed to exist in at least two equilibrating forms, including superoxide anion bound ferric haemoprotein ($Fe^{3+}O_2^-$) and oxygen bound ferrous haemoprotein ($Fe^{2+}O_2$) (Schenkman and Gibson, 1981).

The oxycytochrome P450 is further reduced by a second electron originating from NADPH via the reductase. The reductase can also donate the second electron indirectly via cytochrome b_5 (Peterson and Holtzman, 1980). Finally, oxygen bound cytochrome P450 dissociates to yield water and one oxygen atom, the latter being inserted into the substrate.

1.2.1.ii.c. Multiplicities of cytochrome P450

Cytochrome P450 has the capacity to metabolise a wide range of compounds, whose only common feature appears to be the degree of lipid solubility. This capacity has been attributed at least in part, to the existence of more than one drug-metabolising enzyme. This was first postulated by Axelrod in 1956 as a result of his observation that there was a marked interspecies variation in the rate of N-demethylation of morphine, methadone and meperidine. Conney et al, 1959 not only suggested interspecies variation in drug metabolising enzymes but also that the liver of a single species contains several enzymes. They observed that in the rat benz(o)pyrene increased the rate of microsomal oxidation of some substrates while others were decreased or remained unaffected.

A series of in vitro experiments using inducers of cytochrome P450 demonstrated the existence of distinct forms of the enzyme in microsomes prepared from the livers of treated animals. The polycyclic hydrocarbon 3-methylcholanthrene (3-MC) induced a different form of cytochrome P-450 in rat liver, which exhibited different spectral and substrate specificities from cytochrome P450 present in untreated rats (Sladek and Mannering, 1966; Alvares et al, 1967; Gnosspelius et al, 1969; Kuntzman et al, 1969; Mannering, 1971). This was designated cytochrome P448 (Alvares et al, 1967) due to a reduced carbon monoxide complex absorbance maxima at 448nm. This change in the spectral characteristics was not due to the binding of the inducer due to the haemoprotein but due to the synthesis of new protein since the change could be prevented by pretreatment with inhibitors of protein and nucleic acid

synthesis such as actinomycin D (Kantzman et al, 1969).

Weibel et al, (1971) showed that 7,8-benzoflavone inhibited the hydroxylation of benzo(a)pyrene by liver microsomes from 3-methylcholanthrene treated rats, but not by microsomes from phenobarbitone treated rats.

Definite proof for the existence of multiple forms of cytochrome P450 followed the pioneering work of Lu and Coon, (1968), who successfully solubilized and characterised the individual compounds of the microsomal mono oxygenase system. This system, when constituted was later shown to be capable of catalysing a number of substrates (Lu and Levin, 1974). Imai and Sato, (1974 b) purified cytochrome P450 which was electrophoretically homogenous. Using a modified version of this technique, Guengerich et al, (1982a) purified eight isoenzymes of cytochrome P450 from rat liver microsomes to electrophoretic homogeneity. The different forms were distinguished by certain criteria including reduced carbon monoxide difference spectrum properties, monomeric molecular weights, peptide mapping, immunochemical reactivity and catalytic activity towards different substrates.

Studies using cDNA probes to cytochrome P450 and amino acid sequencing methology has enabled recognition of cytochrome P450 gene structure and evolution of the gene family. The cytochrome P450 gene superfamily consists of 10 known gene families, eight of which are present in mammalian tissues, consisting of variable numbers of gene subfamilies (Nerbert and Gonzalez, 1987; Nerbert et al, 1987). Currently, twenty different forms of cytochrome P450 have been isolated and identified from rat liver microsomes (Guengerich, 1989).

Nomenclature of cytochrome P450 genes has been varied, but recently they have been standardized, based on the divergent evolution of the gene superfamily, using alignment of amino acid sequence to determine gene families and subfamilies

(Nerbert et al, 1987). Gene families are recommended to be designated by Roman numerals and subfamilies by capital letters. The form of cytochrome P450 inducible by poly aromatic hydrocarbons in rat, rabbit, mouse and man resides in the P450I gene family (Nerbert and Gonzalez, 1987). The cytochrome P450 previously identified as PB-inducible are in the cytochrome P450II family, with P450IIB the rat PB-inducible form. The P450III gene family are steroid-inducible forms of cytochrome P450. The microsomal enzymes responsible for 17-alpha and C21 hydroxylation of steroids are coded for by genes in the XVII and XVI families respectively and mitochondrial enzymes responsible for 11ß-hydroxylation and cholesterol side-chain cleavage are encoded in the XI and XXII families respectively. A unique P450 family, P450XIX, encodes the enzyme involved in oestrogen synthesis.

Some of the members of the first four families (I-IV), are inducible by xenobiotic compounds in laboratory animals (Gonzalez, 1989). In all cases there is evidence for the existence of at least one representative of each of the inducible families of cytochrome P450 in man (Nerbert and Gonzalez, 1989), although whether all such isoenzymes are the true orthologues of the inducble forms in animals has still to be determined. In many cases the human analogue is similarly inducible to the isoenzymes in laboratory animals. Examples of this inducible form are both members of the P450IA subfamilies, P450IIE1 and representatives of the P450IIC and P450IIIA subfamilies.

1.3. PHASE II XENOBIOTIC METABOLISM

1.3.i. Conjugation

Conjugation reactions involve the enzyme catalysed combination of a foreign or endogenous compound or its metabolite with a conjugating agent. This foreign compound or metabolite may be chemically stable or a reactive electrophilic species arising from phase I metabolism, such as an epoxide or N-oxidation product of

an aromatic amine, amide or nitrosamine, or a free radical.

Glucuronic acid

Conjugation with glucuronic acid or sulphuric acid are quantitatively the most important phase II biotransformations (Dutton, 1966; Dutton and Burchell, 1977). Glucuronide conjugation involves the UDP glucuronyltransferase and utilises the co-substrate UDP glucuronic acid (UDPGA). UDPGA is synthesised from glucose in four steps. First, phosphorylation to glucose-6-phosphate, followed by isomerisation to glucose-1-phosphate, which is then urylated to form UDP glucose. This is then oxidised by NAD^+ dependant UDP glucuronic acid dehydrogenase to form UDP glucuronic acid. ATP is required for the phosphorylation of glucose for the rephosphorylation of UDP to UTP (Dills et al, 1987).

UDP glucuronyltransferases exists as a multi-gene family in the rat. Individual UDPGT rat liver isoenzymes tend to differ in terms of regulation and xenobiotic substrate specifity (Burchell and Coughtrie, 1989). Bock et al, 1984a reported that in the rat, glucuronidation is catalysed by at least five independant forms of UDPGT, with substrates nitrophenol, morphine, bilirubin, testosterone and oestrone being representative of their specificities. The multiplicity of human liver UDPGT is also now accepted on the basis of results from microsomal kinetic and inhibitor studies (Bock et al, 1978, 1984b; Miners et al, 1988a and b, 1990), the cloning of UDPGT cDNA's (Fournel-Gigleux et al, 1989; Harding et al, 1988; Jackson et al, 1987) and the isolation of purified enzymes (Irshaid and Tephly, 1987).

Two groups of glucuronyl transferase activities have been identified in the rat liver, based on differential induction by either phenobarbital (PB) or 3-methylcholanthrene (3-MC). 3-MC induction led to an increase in activity towards benzo (a) pyrene-3,6-quinol and 1-naphthol (Bock Bock-Hennig, 1987). PB inducible

forms preferentially metabolise the substrates 4-nitrophenol, 1-naphthol, 4-methylumbelliferone, 2-aminophenol and 3-hydroxybiphenyl (a) pyrene. 3-MC inducible forms conjugate morphine and 4-hydroxyphenyl. The enzymes from rat liver may also be classified on the basis of perinatal developement, termed clusters of glucuronyltransferase activity. The late foetal cluster is similar to the PB inducible group of enzymes (Bock et al, 1983). Differential induction of enzymes has been demonstrated in human liver microsomes using PB and 3-MC. PB induction led to a 3-fold increases in bilirubin conjugation, with moderate increases in activity towards quinol, 1-naphthol and 4-methylumbelliferone. Available evidence suggests the 4-methylumbelliferone activity comprises at least two closely related isoenzymes with similar kinetic properties and broadly comparable chemical substrate specificities (Miners et al, 1988).

Sulphate

Conjugation with sulphate requires phosphadenosine phosphate (PAPS) as the sulphate donor. PAPS biosynthesis is fueled by two molecules of ATP which probably accounts for its generation only when required and explains its low intercellular concentration (Wang and Yeo, 1979; Hazelton et al, 1985). Its formation is catalysed by ATP-sulfurylase (EC 2.7.7.4.) and APS-kinase (EC 2.7.1.25.). Conjugation of PAPS with endogenous compounds is catalysed by one or more sulphotransferase, present in most tissues (Mulder, 1981).

Though glucuronidation is quantitively the most important conjugation reaction, many substrates which are glucuronidated are also sulphated (Dodgson and Rhodes, 1970), therefore these reactions compete for the same substrates. For example phenolic substrates undergo both glucuroidation and sulphation (Mulder, 1983).

Extrahepatic conjugation is significant. When 4-nitrophenol was

administered to the rat via the jugular or portal vein, 50% of the resulting conjugate formation was found to extra-hepatic and conjugation by the kidney also provide a major contribution (Elbers et al, 1980). Conjugation activity has also been detected in the lungs, intestines and skin (Moloney et al, 1982).

Sulphation activity varies with age and sex and exhibits a marked organ distribution. Liver has the highest sulphotransferase activity in the rat, man and in human foetal tissue.

Glutathione

The other type of reaction involves the formation of an activated xenobiotic followed by conjugation of the conjugated ligand by a transferase enzyme. The most important reaction involves conjugation with glutathione. This type of conjugation provides a major defence mechanism against toxicities formed inadvertantly by phase I oxidation reactions. Substrates for glutathione conjugation possess the common feature of having an electrophillic centre that can react with the nucleotide glutathione. These conjugates can be excreted in the bile or further metabolised to cysteine or N-acetylcysteine (mercapturic acid) conjugates which are then excreted in the bile (Jerina and Bend, 1977).

1.4. INDUCTION

The activity of microsomal MFO system and concentration of cytochrome P450 can be increased by a variety of xenobiotics, including polycyclic aromatic hydrocarbons, steroids and pesticides. The inducibility of some P450's observed after exposure to different drugs and chemicals is a main feature of these systems (Distlerath and Guengerich, 1988). Investigations have revealed the existence of a multigenic

superfamily (Nerbert and Gonzalez, 1987; Nerbert et al, 1989) and made possible a classification of the inducers into five different groups, according to the form(s) of P450 specifically induced: (a) polycyclic aromatic hydrocarbon (P450IA); (b) phenobarbitone (P450IIB); (c) ethanol, acetone, benzene,imidazole and derivatives (P450IIE); (d) glucocortioids and macrolide antibiotics (P450IIIA); (e) clofibrate (P450IVA) (Gedet al, 1989).

Polycyclic hydrocarbons and phenobarbitone induce P450 in different ways. 3-methylcholanthrene and other polycyclic aromatic hydrocarbons, the first step in the induction process is the binding to cytosolic receptors which are further translocated into the nucleus and stimulate gene transcription (Okey and Vella, 1982). Once in the nucleus the inducer-receptor complex binds to the genome and initiates transcription and translocation of genes coding for cytochrome P448, UDP glucuronyltransferase and glutathione-S-transferase (Bend, 1980). Induction by polycyclic hydrocarbons is regulated by the Ah locus at the genome (aromatic hydrocarbon responsiveness). The main product of the Ah regulatory genes is the cytosolic receptor essential for induction.

The mechanism of induction of phenobarbitone is not fully elucidated but suggests activation of gene transcription for P450B and e, epoxide hydrolase and glutathione transferase (Hardwick and Gonzalez, 1983; Ding and Pickett, 1985). A 30-fold increase in cytosolic concentration of mRNA encoding the PB-inducible form of cytochrome P450 has been observed (Adesnik et al, 1981).

Phenobarbital induces hepatic carboxylesterase activities (Nousiainen and Hanninen, 1981; Beohr et al, 1966; Kaur and Ali, 1983). Satoh and Moroi, (1973) demonstrated a species difference in phenobarbital-induced increases in carboxylesterase activity towards isocarboxazid. It has also been shown that the toxicity of procaine was modified by pretreatment of rats with phenobarbital, tri-o cresylphosphate (Satoh and Moroi, 1977) and ethyl p-nitrophenyl

phenylphosphorothioate (EPN) (Satoh and Moroi, 1973; 1975). DDT (Read et al, 1965), PCB (Andersson and Tegelstrom, 1979) and transtilbene oxide also induce liver carboxylesterase activity. Kidney and lung carboxylesterase activity cannot be induced by phenobarbital (Raftell et al, 1977). Raftell et al, (1977) also reported that 3-methylcholanthrene had no inductive effect on microsomal carboxylesterase. Nousainen et al, (1984) showed that, in dose- and time-dependant studies, benz(a)anthracene, benzo(a)pyrene and 3-methylcholanthrene moderately induced the hepatic cyosolic and kidney microsomal carboxylesterase activities. The hepatic microsomal and kidney cytosolic enzyme activities were not altered by the polycyclic aromatic hydrocarbons investigated.

Clofibrate (ethyl-ʎ-p-chlorophenoxyisobutyrate) and several other structurally diverse hypolipidemic agents have been shown to induce hepatic responses. These include hepatomegaly, proliferation of smooth endoplasmic reticulum and induction of cytochrome P450IVA-1-dependant fatty acid omega hydroxylase activity, peroxisome proliferation with associated changes in enzyme composition and alteration in mitochondrial number and structure with concomitant increases in certain enzyme levels (Orton and Higgins, 1979; Gibson et al, 1982; Reddy et al, 1982; Sharma et al, 1988). These subcellular changes are toxicologically important as sustained proliferation of peroxisomes in rodents is frequently associated with the developement of hepatocellular carcinomas. This has led to suggestions that peroxisome proliferarors constitute a novel class of non-genotoxic hepatocarcinogens (Reddy et al, 1983; Lalwani et al, 1983). The induction of cytochrome P450IVA is mirrored by a concomitant parallel increase in lauric acid hydroxylase activity (Sharma et al, 1988).

Kawashima et al, (1973) reported the induction of hepatic long-chain acyl-CoA hydrolase in the cytosolic fraction by administration of clofibric acid to rats, mice, or guinea-pigs. Hosokawa et al, (1987) reported that administration of clofibrate caused a significant increase in the activities of p-nitrophenylacetate, isocarboxazid, butanilicaine and palmitoyl-CoA hydrolases in rat liver microsomes. p-Nitrophenylacetate and isocarboxazid hydrolase activities were not induced by

clofibrate, whereas palmitoyl-CoA hydrolase activity was significantly induced. These results were consistent with the findings on the amounts of three carboxylesterase isoenzymes in liver microsomes and cytosol by radial immunodiffusion assay. It was found that clofibrate induced all three isoenzymes of carboxylesterase in hepatic microsomes, but not in the cytosol.

CHAPTER 2 : FACTORS AFFECTING XENOBIOTIC METABOLISM

2.1 GENDER

In 1953, Nicholas and Barron showed that there was a marked sex difference in amylobarbitone induced sleeping time in the rat. Plasma levels of hexabarbitone were found to be significantly higher and duration longer in female rats than in male rats (Quinn et al, 1958). These differences were found to be reversed by castration of the male or by administration of tetosterone to the females. It is now known that these differences are due to variations in the activities of the drug metabolising enzymes between male and female (especially in the properties of cytochrome P450 in liver microsomes (Kato, 1974)).

The results of Quinn et al, (1958) suggest that the androgenic actions of testosterone are involved in maintaining this difference. This has been confirmed by Kitagawa et al, (1985) who showed that sex differences in hepatic metabolism of aminopyrine, hexabarbitone, aniline and p-nitroanisole between male and female rats reach a peak at twelve months of age (males having higher activities than females). This fall is due to the loss of male-specific forms of cytochrome P450 (Fujita et al, 1985; Kamataki et al, 1985) which coincides with an age related fall in plasma testosterone levels (Kitigawa et al, 1985). Testosterone has an indirect effect on the liver by influencing the secretion of growth hormone from the pituitary gland which in turn regulates liver mixed function oxidase enzymes (Gustafsson and Stenberg, 1976; Mode et al, 1982).

Purified forms of cytochrome P450 from livers of male and female rats show a marked difference both immunologically and electrophoretically (Kamataki et al, 1982; 1985). One of the most widely studied sexually differentiated cytochrome P450 dependant mono oxygenase reactions is testosterone 16-alpha- hydoxylase activity. A form of this has been purified from male rat which is highly specific and which could not be purified from female rats (Morgan et al, 1980).

A variety of carboxylesterase isoenzymes, having different substrate specificities have been found in liver microsomes of male and female rats (Mentlein et al, 1980; Blomberg and Raftell, 1974). The overall hydrolytic activity of the liver is similar in both sexes, though quantitative differences in the isoenzymes have been reported (Raftell et al , 1977). Sex hormones have been shown to effect the activities of esterases in mouse liver and kidney (Andersson and Tegelstrom, 1979; Tegelstrom and Ryttman, 1981). It has also been reported that microsomal carboxylesterase was altered in castrated male rats, with enzyme activities towards p-nitrophenylacetate and malathion being decreased (Hosokawa et al, 1984). Administration of testosterone propionate to the castrated rats reversed the observed decreases. However, removal of the ovaries in the female resulted in substrate dependant changes in p-nitrophenylacetate and malathion hydrolase activity, and a decrease in acetanilide and isocarboxazid hydrolysis. Oestradiol benzoate administration led to an increase in acetanilide and isocarboxazid hydrolysis and a decrease in p-nitrophenylacetate and malathion hydrolysis. It would therefore seem that rat hepatic carboxylesterase activities may be, at least in part, affected by sex hormones, which exert different effects on the isoenzymes. Hosokawa et al, (1985) demonstrated that adrenalectomy in castrated male rats slightly decreased activity towards p-nitrophenylacetate and malathion. Following adrenalectomy the magnitude of testosterone-induced changes in the hydrolase activity of hepatic microsomes in castrated male rats was markedly decreased. However, when 5-alpha dihydrotestosterone was administered to adrenalectomized castrated male rats, malathion and p-nitrophenylacetate esterase activities were increased. Kidneys of male mice have been shown to possess several testosterone-dependant esterases (Bocking et al, 1976; Weinker, 1973). Human serum has been shown to possess an oestrogen dependant esterase, which is present in relatively high levels in males and is dependant in males and is independant of age, while in women the low level increases with age (Fabian et al, 1977).

Sex differences in human metabolism are less generally observed, than the environmental and genetic factors. Miners et al, (1986) found that there was a 61% salicylic acid clearance in males compared to a control female group, an effect due largely to enhanced activity of the glycine conjugation pathway (salicyluric acid

formation) in males. These results were consistent with previous studies which have demonstrated higher salicyclic acid clearance in males than in females (Graham et al, 1977; Ho et al, 1985) or higher plasma salicylate concentrations in males compared to females (Coppe et al, 1981; Kelton et al, 1981) after administration of aspirin. These results however, were not found by Williams et al, (1984). Antipyrine clearance has been shown to be significantly higher in men than in women (Blain et al, 1982), but not by others.

2.2 AGE

In animals, age has a significant influence on the specific activity of xenobiotic metabolising enzymes. Low hepatic MFO activity was found in new-born rodents (Jondor et al, 1958; Fouts and Adamson, 1959), increasing rapidly up to 6-10 weeks when adult MFO activities are reached (Fouts and Devereux, 1972). In man a number of foetal tissues are known to contain cytochrome P450 dependant MFO activities (Juchan et al, 1980). The activities of these enzymes rise during gestation and at birth are approximately 50% adult levels (Pelkonen and Karti, 1973).

Age related changes in the response of a number of enzymes activities, in the liver of old rats compared to young was reported by Adelman, (1971). They observed a slower rate of enzyme induction in old rats (24 months) compared to the young (2 months) and postulated that this latency represented a biochemical index of ageing. Schmucker and Wang (1980) reported an age related decline in non-induced NADPH cytochrome P450 reductase activity and cytochrome P450 levels, however, PB treatment caused marked increases in these parameters in young (1 month) and mature (16 months) but not in old rats (27 months). The rate of enzyme and haemoprotein induction, and maximum induced levels reached after 6 days were significantly greater in young and mature but not in old rats.

In the rat, multiple activities of glucuronyltransferase can be divided into two developmental groups, one of which has activities towards indoleamines and planar phenolic compounds and which developes prenatally during later gestation. Adult levels are reached at birth. The second group includes glucuroyltransferase with activities towards complex compounds such as steroid hormones and bilirubin, which develops postnatally (Leaky and Burchell, 1987). A single foetal isoenzyme is thought to be responsible for all the activities in the first group (Scragg et al, 1985).

Animal studies have revealed a fall in the activity of drug metabolising enzymes with age, however, studies in man have failed to show this (Woodhouse, 1984; Wynne et al, 1988; 1989). Williams et al, (1989) found plasma aspirin esterase to be similar in a group of healthy elderly adults, a group of young adults and a group of frail young adults, but was lower in a sample of frail elderly subjects. Plasma albumin levels in healthy elderly were lower than in the young subjects and these levels lower still in frail elderly subjects. In the frail elderly reduced plasma cholinesterase activities were also seen.

The clearance of many drugs, including antipyrine, diazepam, theophylin and propranolol is reduced in elderly human subject (Greenblatt et al, 1982). The body's ability to remove or eliminate a drug or xenobiotic (clearance) is affected by age. The ageing process affects the clearing organs, principally the liver and kidneys. Hepatic blood flow, a major determinant of the hepatic clearance of drugs and xenobiotics (Wilkinson and Shand, 1975) declines with age, partly because of reduced cardiac output. An estimated 40-45% reduction in total liver flow may be observed in elderly compared to young (Geokas and Haverback, 1969). Likewise, liver size decreases with age, Both in absolute terms and as a percentage of body weight. Studies on the influence of the menstrual cycle on serum cholinesterase have seen significant differences in values of cholinesterase with age (Fairbrother et al, 1989). It was found that cholinesterase was lowest in 36-40 year olds and highest in 30-35 years old

2.3 GENETIC INFLUENCES

Major differences in metabolism exist between species and such differences can also occur within one species, although not to such a marked extent. These differences can be seen in the various inbred strains of rats and mice and are due to the different genetic make-up of the strains. These differences also include differences in the induction of xenobiotic metabolising enzymes. Cross breeding of the strains has shown that the inheritance of inducibilty is an autosomal dominant trait. In rats a marked difference exists in the Gunn rat, which is genetically deficient and thereby unable to form many of the glucuronides produced by other strains of rat (Scragg et al, 1985). Interbreeding of Gunn and normal strains leads to glucuronidation capacities intermediate between the two, indicating that neither trait is dominant.

Often in man, interfering influences from environmental sources mask genetic differences on xenobiotic metabolism. Cytochrome P450 isoenzyme activities appear to vary widely in humans and the consequence of inter-individual variations are of pharmacological importance. Those who metabolise certain drugs at slower rates than normal, " poor metabolisers", are likely to accumulate the potent drug and therefore any adverse effects will be enhanced (Maghoub et al, 1977; Kupfer and Preisig, 1983). Poor metabolisers, however, may be at decreased risk from some types of cancer (Ayesh et al, 1984; Kaisary et al, 1987).

The first genetic polymorphism to be recognised among hepatic microsomal P450 was a deficiency in the 4-hydroxylation of the anti-hypertensive agent debrisoquine (Maghoub et al, 1977). 3-10% of Caucasians are poor metabolisers and may suffer side effects from this drug. Polymorphism of P450DB6 was discovered following observations of exaggerated hypotensive responses to debrisoquine and the occurance of an adverse side effect of perhexiline treatment, peripheral neuropathy. Health is not generally impaired as long as these drugs are not taken (Guengerich, 1989).

Other genetic polymorphisms in human cytochrome P450 include variation in the cytochrome P450MP, involved in the 4-hydroxylation of the anti-epileptic drug mephenytoin variation in cytochrome P450NF, involved in nifedipine oxidation and cytochrome P450 enzyme involved in metabolism of ethanol phenacetin and adrenal steroids (Guengerich, 1989).

Most individuals possess the typical form of cholinesterase in serum (Lehman and Liddell, 1964). Inter-individual variations in cholinesterase activity have been reported, and may be a consequence of both genetic and environmental factors which give rise to a range of activities. Differences in activities may be important in metabolising drugs such as succinyl choline and thereby altering its pharmalogical effect. 0.5% of Caucasian population possess an atypical inactive form of cholinesterase. The typical and atypical forms are as a result of the homozygous gene expression. Heterozygotes of the usual and atypical exhibit intermediate cholinesterase activities.

Inter-ethnic variation can also be seen in plasma paraoxonase activity between Caucasians and other ethnic groups. Player et al, (1976) found differences in oriental and black African populations. Williams et al, (1986) found lower paraoxonase, aspirin esterase and arylesterase activity in a Ghanaian population compared to a Caucasian population, whereas the cholinesterase activities were similar.

Recently a new liver specific rat carboxylesterase enzyme (E.C.3.1.1.1.) designated esterase-18 (ES-18) has been described in a new strain of rat (Rattus norvegieus) (Kluge et al, (1990)). Nakamura et al, (1989) also found strain differences in rat carboxylesterase, related to the phenotype differences of esterase-3.

CHAPTER 3 : PESTICIDES USED IN XENOBIOTIC METABOLISM STUDIES

3.1. INTRODUCTION

For centuries biocidal chemicals have been used by man to control those plants and animals that are considered to be pests. Major developements recently in synthetic organic chemistry and biology have enabled more selective pesticides to be produced that can be targeted at particular species of plants or insects, but, being 'bioactive' these compounds still retain some potential for toxicity to mammals.

Direct toxic interference with the function of the insect nervous system has been a common underlying mechanism of action of many of the traditional insecticides. Safer insecticides have been developed that utilize specific differences in metabolic pathways between mammals and insects; neurotoxic metabolites are formed in greater amounts in insects than in mammals (Vohida et al, 1964; Krueger et al, 1960). Unfortunately, insects may eventually develop tolerance or other pathways for detoxification and hence develop resistance to the pesticides (Townsend and Busvine, 1969).

3.2. ORGANOPHOSPHATES

Many organophosphate compounds are pesticides widely used for the control of insect vectors. They are not ideal agents because they lack vector selectivity, and have caused severe toxicity and death in humans and domestic animals. Their toxicity has been recognised since the 1930's, when they were also developed for use as chemical warfare agents. A unique feature of all OP's is their structural fit with the ChE enzyme. OP's mimic the molecular shape of AChE. AChE has two active sites (see chapter 1.2.), the esteratic and anionic site (see Figure 3.1.). The inhibition of AChE is very similar to the early stages of AChE hydrolysis. The phosphorus moiety (RO_2-$\ddot{P}$- OR-) of organophosphates bind to the esteratic site the amino acid serine, while the remainder of the molecule attaches to the anionic site. The OP is hydrolysed

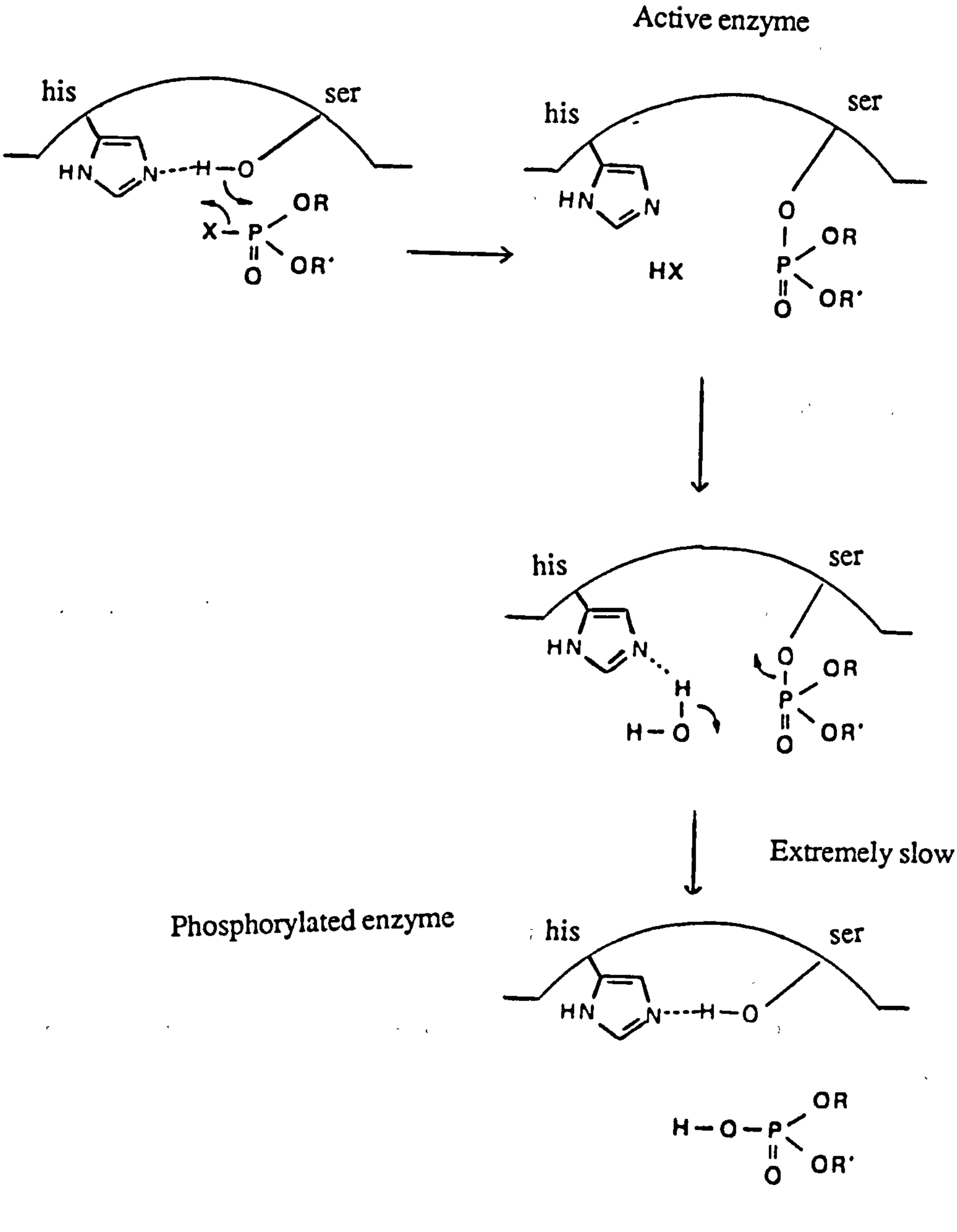


Figure 3.1.

Reaction of an organophosphorus compound with the esteratic site of acetylcholinesterase.

so that the alcohol fragment (HOR') leaves the reaction and the phosphorus remains attached to the serine,phosphorylating the enzyme.

A phosphorothionate insecticide such as parathion is an inheritantly weak anticholinesterase,which must be activated via a cytochrome P450-dependant desulfuration reaction to the reactive phosphate (oxon) metabolite i.e. paraoxon (0,0-diethyl-0- (4-nitrophenyl) phosphate) (Gage, 1953; Nakatsugawa and Dahm, 1967; Neal, 1967).

The usual cause of death in mammals from acute organophosphate poisoning is respiratory failure as a result of inhibition of acetylcholinesterase at the nerve endings and in the respiratory control centre of the brain (O' Brien, 1960). The I50 to brain acetylcholinesterase (AChE) is reduced by this reaction about three orders of magnitude, from about 10^{-5} to about 10^{-8}M (Forsyth and Chambers, 1989). OP's are capable of inhibiting other serine esterases, such as carboxylesterase, at low inhibitor concentrations (Chambers et al, 1989). Since the liver absorbs parathion so readily (Nakatsugawa et al, 1980; Tsuda et al, 1987), since it has such a high concentration of parathiondesulphurationactivity (Neal, 1967), and since it would be responsible for the first- pass metabolism following an oral exposure a large fraction of paroxon produced is expected to be generated in the liver. However the liver also possesses a high specific activity of carboxylesterase (Chambers et al, 1990). Likewise the plasma in some species, such as rat, contains apprecable levels of carboxylesterase, which could also sequester hepatically generated paraoxon. Thus, carboxylesterases may serve a protective role in phosphorothionate insecticide or ortho phosphate intoxication by removing a significant amount of the activated metabolite prior to its reaching the main target, AChE in the nervous system.

Generally, it has been assumed that the majority of the active metabolite is produced in the liver and is released into the circulation to find its target, AChE. While the liver is typically the most active organ in the body for xenobiotic activation and

detoxification reactions, Neal and coworkers suggested that extrahepatic activation of parathion to paraoxon may be important to its toxicity (Neal, 1972). While isolated hepatocytes absorbed parathion rapidly but reversibly, perfusion studies indicated that intact parathion exists in the liver (Tsuda et al, 1987). This parathion released into the circulation could play a major role in phosphorothionate poisoning if it were activated at a critical target site.

Phosphorothionate absorbed following dermal exposure, a more likely route of occupational and many accidental poisonings, could circulate directly to extrahepatic tissues, including the target tissues. Topical application of parathion results in a first-pass bioactivation to paraoxon and the production of p-nitrophenol (Carver et al, 1990). It was also reported that cutaneous metabolite profiles can be altered by both physical (occlusion) and chemical (1-aminobenzotriazole) means.

Sultatos et al, (1984) found that albumin could serve as a means of transport for parathion in the blood. They reported an esterase-like activity of bovine serum albumin for paraoxon. Further, the oxons can phosphorylate other blood components such as AChE, carboxylesterase and other B-esterases. Thus, because of the potential degradation of the oxons by the blood proteins and phosphorylation of the blood proteins by the oxons, relatively small amounts of oxon produced in the liver could reach the brain AChE.

The major route of detoxification of paraoxon, the active metabolite of parathion is by hydrolysis (see Figure 3.2.). Oxon analogues of organophosphate insecticides are cleaved by Ca^{2+} dependant paraoxonase.

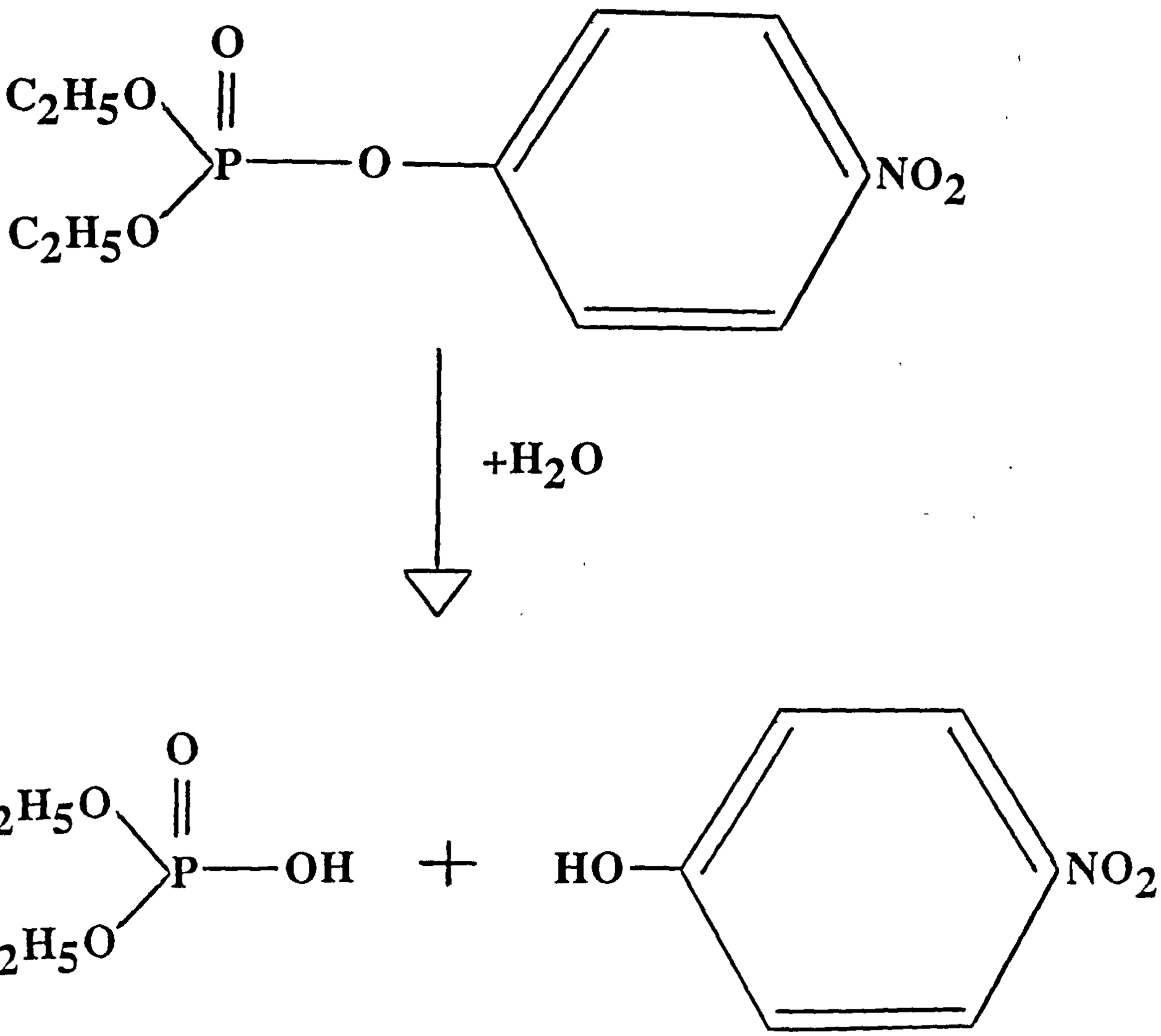


Figure 3.2. Hydrolysis of paraoxon to p-nitrophenol and diethyl phosphoric acid.

3.3 CARBARYL

Carbaryl (1-naphthyl methylcarbamate) belongs to the carbamate family of pesticides (see Figure 3.3.). It is recomended for use against caterpillars of the winter moth, as well as earwigs and capsid bugs. It was introduced by Union Carbide Company as a broad spectrum insecticide acting by reversible inhibition of acetylcholinesterase (Kolbezen et al, 1954).

Carbamates have a high affinity for acetylcholinesterase and formation of a reversible complex, is therefore, favourable which results in the formation of a carbamoylated enzyme complex. Dissociation of the complex is by hydrolysis which proceeds at a slower rate than carbamoylated resulting in a build up of inhibited enzyme (Kuhr and Dorough, 1976). It is widely used due to its relatively low anticholinesterase activity in mammals, as a result of the more rapid metabolising capacity of mammalian liver carboxylesterase compared to the metabolic activity of the insect (Kolbezen et al, 1954; Best and Murray, 1962; Dorough, 1970) and the reversible nature of the enzyme inhibition.

The metabolic fate of carbaryl is important in the determination of its potential toxic hazard. Following oral or dermal exposure, carbaryl has been shown to be rapidly absorbed and distributed in various tissues and organs throughout the body.

Carbaryl is metabolised by rats and excreted in the urine as the hydrolysed product 1-naphthol, and as conjugates of 1-naphthol (Carpenter, 1961).The liver has been shown to be a major site for carbaryl metabolism in the mammal. Following intra venous administration of carbaryl, first pass metabolism by the liver lowered plasma carbaryl levels considerably (Houston et al, 1974). Human liver fractions have been shown to possess less metabolic activity toward carbaryl than rat, with ring hydroxylation the major route of metabolism (Strother,1972). The extent of hydrolysis

(see Figure 3.3.) that acts to detoxify carbaryl is the major difference in carbaryl metabolism between species (Knaak et al, 1965).

Carbaryl metabolism by other tissues have been studied, with liver activity the greatest followed by kidney and then lung (Chin et al,1974). It is also readily hydrolysed by serum albumin (Casida and Augustinsson, 1959) and by blood. Carbaryl metabolism by rat liver and skin post mitochondrial fraction has been studied by MacPherson et al, (1991). It was shown that carbaryl hydrolysis took place in both the liver and skin post mitochondrial fractions, with skin esterase hydrolysis accounting for less than 3% of liver when comparing specific activities. Urinary metabolites present following exposure to carbaryl by any of the possible routes, represents a composite of metabolic activity in a number of tissues, therefore, it is important to assess the contribution of local hydrolysis in the skin and lung following absorption through the skin and lung as well as the type of esterases causing the hydrolysis of carbaryl to 1-naphthol.

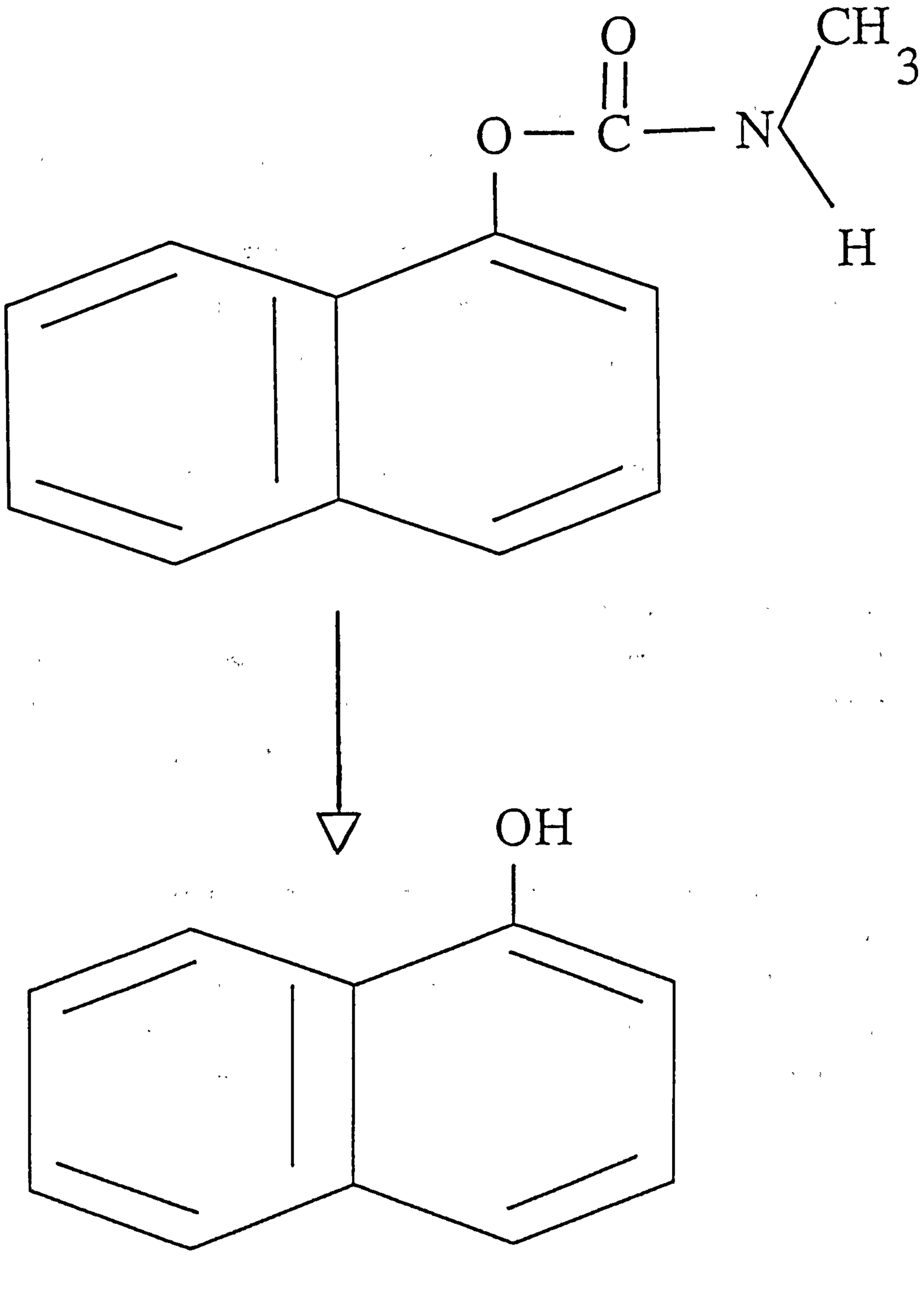


Figure 3.3. Hydrolysis of carbaryl to 1-naphthol.

3.4. FLUAZIFOP-BUTYL

Fluazifop-butyl [butyl 3-[4-(5-trifluoromethyl-2-pyridyloxy phenoxy] propionate] is an aryloxyphenoxy propionate herbicide and is the active ingredient of FUSILADE herbicides which are used to control grass weeds in broadleaved crops (see Figure 3.4.). At room temperature fluazifop-butyl is an oily yellow liquid with a melting point of 5^{o}C. It is soluble in most organic solvents but only very slightly soluble in water. It has low acute toxicity via oral or percutaneous routes; rat oral MLD 3300 mg kg^{-1}, rabbit dermal MLD > 5000 mg kg^{-1}, and it is not carcinogenic in rats or micein lifetime dietary studies (UK M.A.F.F., 1988). Fluazifop-butyl is a racemic mixture.

In the rat fluazifop-butyl excretion is rapid following oral administration and the majority of the dose is recovered in urine as the acid metabolite fluazifop (Hart et al, 1982) (see Figure 3.4.). There is a sex difference in the rate of elimination; male rats $t_{1/2}$ 33-38 hours, female rats $t_{1/2}$ 2.5 hours (Batten et al, 1981).

Woollen et al, (1991) in humans have shown that following oral dosing, fluazifop-butyl is rapidly absorbed and hydrolysed to its acid metabolite fluazifop. The estimated half-life of elimination of fluazifop from male human blood (12-37h) was found to be slower than that from the blood of female rats (2.5h) but faster than from male rats (33-38h). Batten et al, (1981) in the dog indicates a half- life of approximately 20 hours with no sex differences.

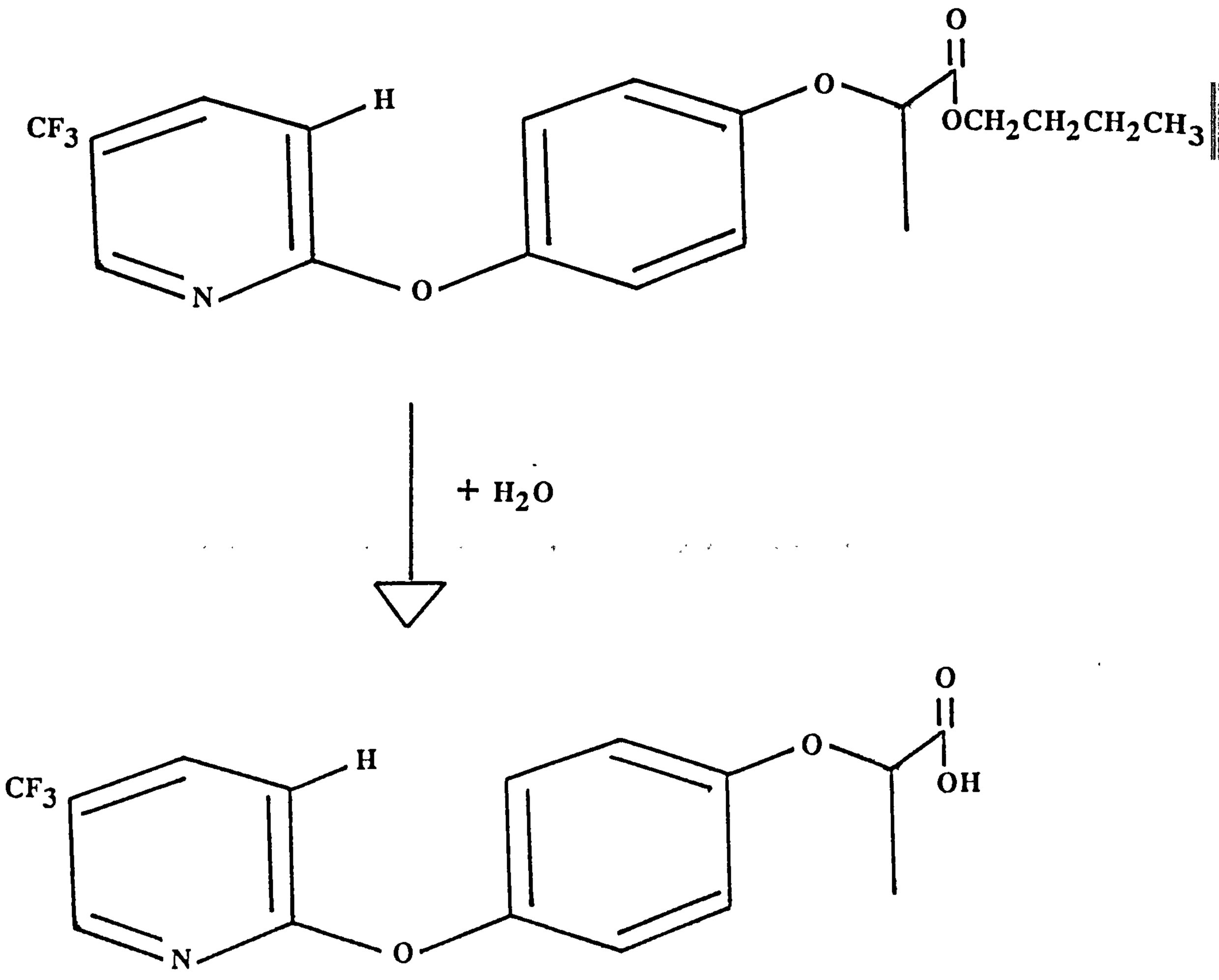


Figure 3.4. Hydrolysis of fluazifop-butyl to fluazifop.

CHAPTER 4 : XENOBIOTIC METABOLISM IN THE LUNG

4.1. INTRODUCTION

The respiratory system has two main components: a conducting system, designed to process, deliver and remove air; and a gaseous exchange system by which blood is oxygenated and decarbonated. The latter, the respiratory acinus consists mainly of alveoli.

The conducting system is divided into the nasopharynx, and tracheobronchiolar tree or alternatively described as the upper and lower airways (Figure 4.1.). An important factor in respiratory tract toxicology is the physiochemical nature of the material. Inhaled substances may be gases, vapours, liquids or solid and particles. Three areas of depositiion of aerosols can be defined broadly within the respiratory tract. In the extrathoratic region, comprised of the nasopharynx and larynx, particles of more than 6μm aerodynamic diameter, deposit, whereas in the tracheobronchial tree particles of 5-7μm aerodynamic diameter come to rest. Particles of 3-4μm settle in the pulmonary compartment, comprised of bronchioles and alveoli. Only the very small particles 1μm aerodynamic diameter or less, are likely to diffuse into the alveolar region in large quantities, whereas particles <0.5μm may avoid deposition altogether and be subsequently exhaled (Yu et al, 1972; Itkin and Anand, 1970; Cockcroft et al, 1978). Gases and vapours on the other hand may diffuse throughout the respiratory tract . Factors influencing absorption through the alveoli include lipophilicity, surface area and blood supply.

4.2. METABOLISM

4.1.i. Introduction

The respiratory tract, being a major portal of entry of xenobiotics into the

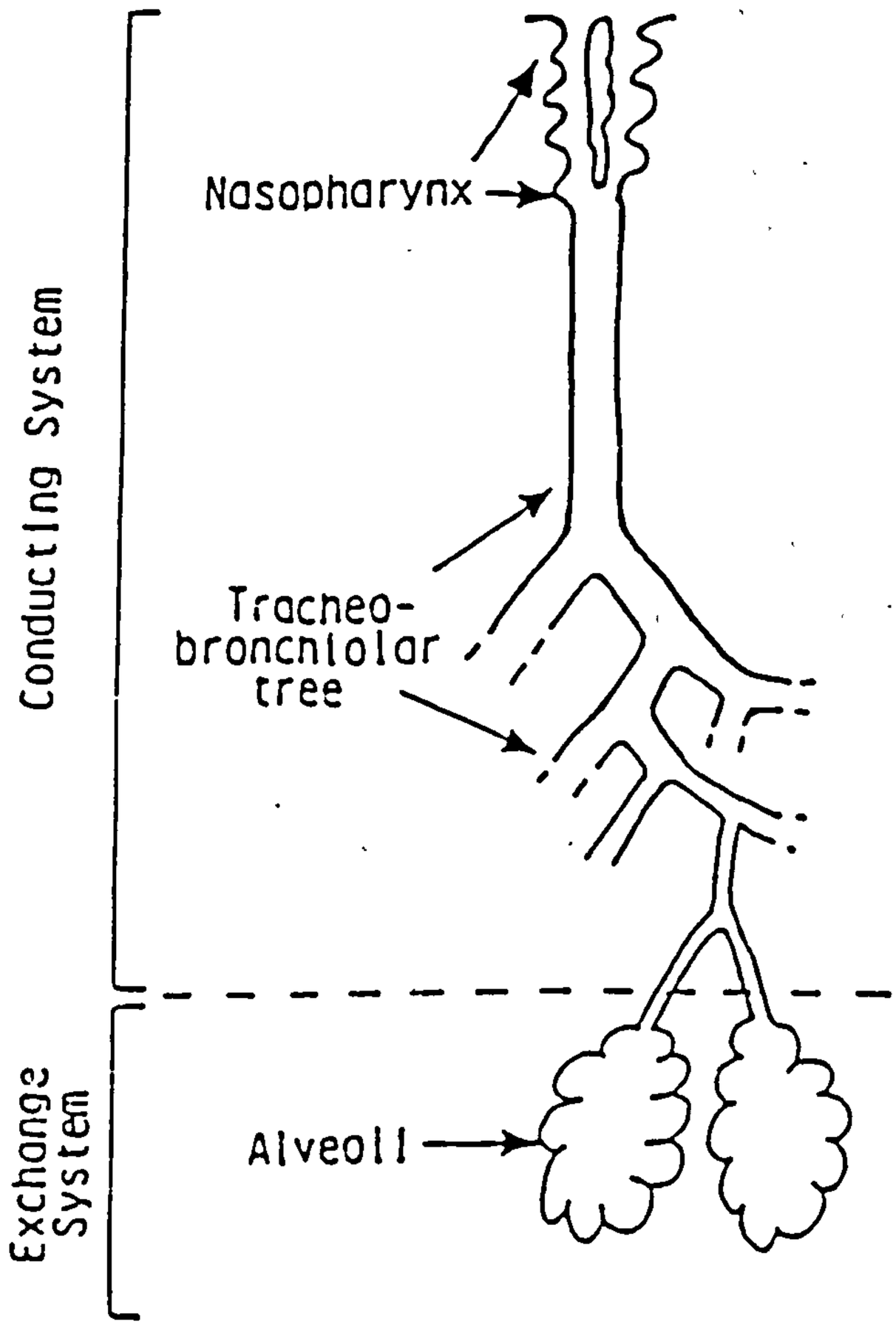


Figure 4.1. Gross anatomy of the respiratory system

body, is exposed continuously to airborne environmental chemicals as well as to those substances which are present in the general circulation. Additionally, xenobiotics which are locally biotransformed into reactive metabolites usually exert relatively selective toxic effects within the difference segments of the respiratory tract and frequently damage either a specific morphological cell type or groups of morphological similar cells which are located within a selected area or region of the tissue (Boyd et al, 1974; Baron and Kawabata, 1983; Minchin and Boyd, 1983).

The nasal mucosa is found in the nasopharynx region of the conducting system. It is a relatively complex and heterogenous tissue which consists of olfactory and respiratory regions. The nasal mucosa contains cytochrome P450 and other xenobiotic-metabolism of inhaled chemicals. Moreover it is damaged by many xenobiotics which must undergo a cytochrome P450 mediated biotransformation prior to exerting their cytotoxic actions (Hecht et al, 1980; Brittebo et al, 1983; Hashek et al, 1983).

Histologically, the trachea is a much less complex and heterogenous tissue than is the nasal mucosa. Its epithelium consists of ciliated cells, goblet, or mucous secreting cells and basal cells, all of which rest on a well-defined basement membrane. The tracheal epithelium has been reported to be capable of supporting cytochrome P450 dependant monoxygenase activities (Kaufman et al, 1973; Autrup et al, 1980) and represents a major target for the carcinogenic actions of xenobiotics, especially polycyclic aromatic hydrocarbons (Palekar et al, 1968; Trump et al, 1984).

The bronchus, bronchiole, and alveolar wall represents the major sites for xenobiotic metabolism within the lung (Wattenberg and Leong, 1963; Machin and Boyd, 1983). Bronchial epithelial cells (Kahn et al, 1981), non-ciliated bronchiolar epithelial or clara cells (Boyd, 1977; 1980; Devereux, 1984), and type II pneumocytes (Teel and Douglas, 1980; Devereux, 1984) have all been demonstrated to be capable of oxidatively metabolizing xenobiotics. Clara cells contain the greatest concentrations of cytochrome P450PB-B, NADPH cytochrome P450 reductase, and epoxide hydrolase

with respect to findings on cytochrome P450PB-B, it should be recognised that this cytochrome P450 appears to be the principle enzyme responsible for catalyzing the bioactivation of pulmonary toxicants such as 4-ipomeanal in the rat (Guengerich et al, 1982a).

4.2.ii. Esterase Hydrolysis in Lung

The mammalian lung is rich in esterase activity. In various species, high esterase activity has been detected histochemically in the bronchial mucosa and, to a lesser extent in the alveolar lining cells (Nachlas and Seligman, 1949; Barrnet, 1952; Ches ick, 1953). The multiple nature of these esterase enzymes was demonstrated by electrophoretic seperation (Market and Hunter, 1959).

Lung esterase again received attention when a relationship between esterase activity and the lamellated bodies (LB), considered to store the surfactant factor, was established (Vatter et al, 1968). The identification of the LB-associated esterase and its biological significance was attempted (Hichcock-O'Hare et al, 1971; 1976; Bocking et al, 1981). At least 13 different esterases were distinguished and identified in the house mouse, mainly by their catalytic properties, susceptibility to inhibition, developemental patterns and phenotypic variation amongst different strains (von Deimling et al, 1983). Each of the 13 esterases of the lung also occurs in one or several other tissues. At least 8 esterases were found which belong to the isozyme system of carboxylesterase under the control of genes located on chromosome 8. These esterases account for about 90% of the esterase activity in the lung. It is thought that the main sites of carboxylesterase isoenzymes are the columnar epithelium of the bronchioles, the alveolar septal cells and the type II alveocytes. These sites have been shown to be associated with organophosphate-sensitive esterases in the rat (Milz and Budd, 1978).

CHAPTER 5 : XENOBIOTIC METABOLISM IN THE SKIN

5.1. STRUCTURE

5.1.i. Introduction

The skin together with its appendages, effectively comprise the outer surface of the vertebrate animal. The skin protects the internal body structure from the external environment, for example, pollution, radiation and temperature variation by providing physical protection and by limiting the passage of substances, most importantly water, into and out of the body. Although the skin possess selective barrier properties many chemicals are able to penetrate the barrier and, once absorbed, enter the systemic circulation.

The skin is divided into three distinct layers; epidermis, statum corneum and dermis (Figure 5.1.)

5.1.ii. Epidermis

The epidermis consists of several layers, including the basal (stratum germinativum) layer, keratinocytes, the granular layer (stratum gerulosum) and the stratum corneum. The epidermis is a proliferating layer of actively metabolising and dividing cells. In man the epidermis is 25µm thick, compared to the total skin thickness of 2mm. This compares to a thickness of 18µm and a total skin thickness of 800µm in the rat (Scott et al, 1991). The epidermis is constantly renewed as basal cells gradually migrate outwards, undergoing morphological and histochemical changes, to form the stratum corneum. This process is called keratinization and leads to the formation of keratinocytes. As keratinocytes migrate to the surface they form keratin, which forms the outer layer of the skin, the stratum corneum. The process of cell division, migration and differentiation takes 28 days in normal adult skin and serves as

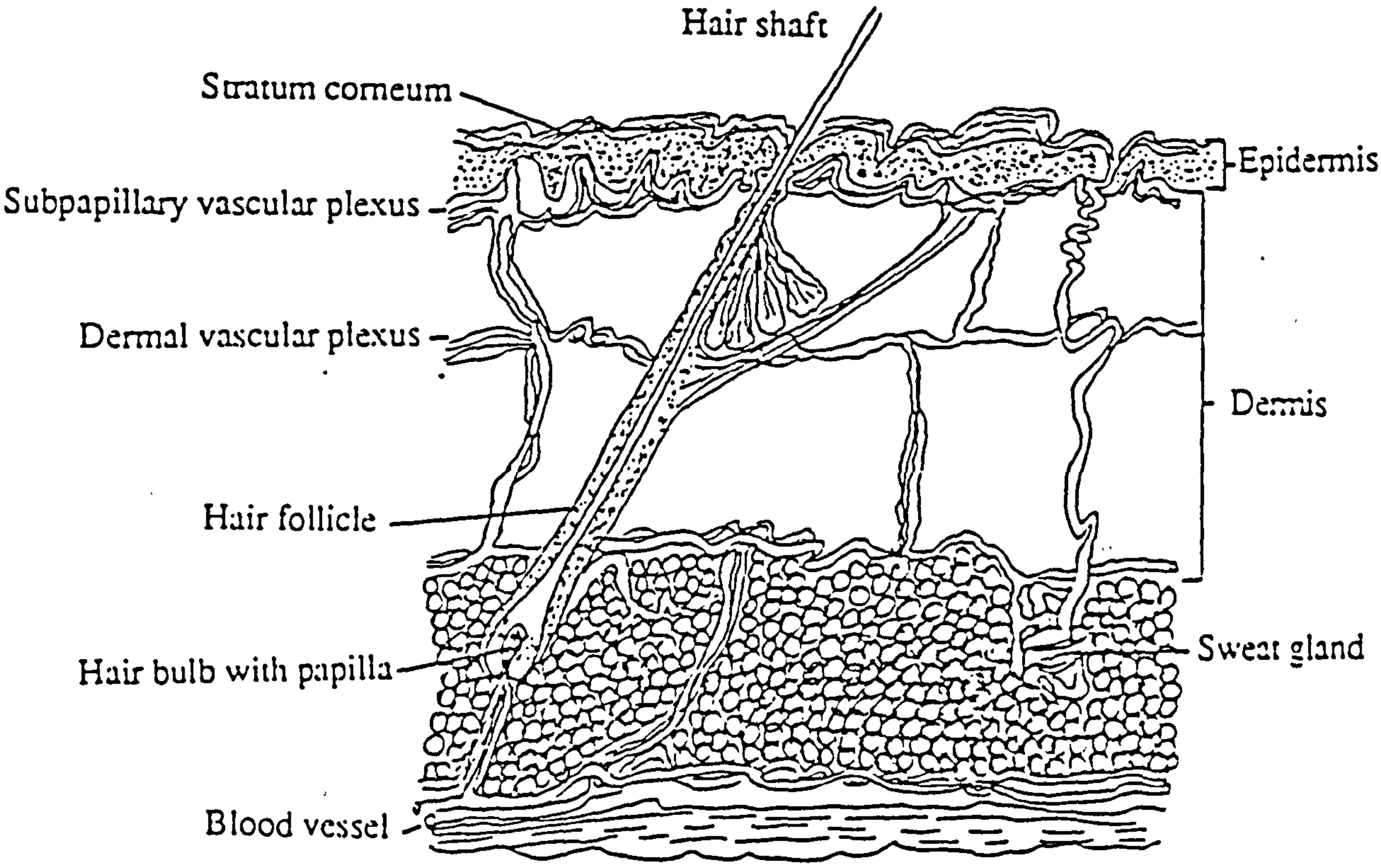


Figure 5.1. Structure of skin.

a protection against damage to the skin by friction and against foreign objects which may have penetrated the outer layers.

5.1.iii. Stratum Corneum

The stratum corneum consists of 10-15 layers of dead keratinized cells in humans. The stratum corneum in man is about 20μm, compared to 16μm in the rat. These are laterally arranged with the lateral edges interdigitating with adjacent cells. This arrangement forms alternate layers of hydrophobic and hydrophillic regions, with the keratin outer cell layers forming lipophilic regions and the intercellular contents forming hydrophilic regions. This arrangement is thought to be important in the selective permeability properties of the stratum corneum (Schaffer, 1982).

5.1.iv. Dermis

The dermal layer represents the bulk of the skin. Dermis consists of a matrix of connective tissue, collagen, elastin and reticulin embedded in mucopolysaccarides. Blood vessels, lymphatics and nerves cross the matrix and skin appendages such as sebaceous glands and hair follicles penetrate it.

The dermal layer in man is divided into the upper papillar layer and the deeper reticular layer. The papillar layer is well supplied by capillaries which enables the skin to regulate body temperature, to remove waste products from and nutrients to the skin. The tough resilient qualities of the skin are provided by the reticular layer. It is composed of coarse collagen bundles, elastic fibres and mucopolysaccarides. Most substances penetrating the stratum corneum are absorbed into the blood via the vessels located in the dermis and therefore enter the systemic circulation.

5.1.v. Skin Appendages

Skin appendages include eccrine and apocrine sweat glands, hair follicles, sebaceous glands and nails. Hair follicles develop from invaginations of epidermis into the dermis. One or more sebaceous glands are attached to a hair follicle to form a pilosebaceous unit. These glands are holocrine and synthesise lipids which are expelled onto the skin surface as sebum, which is thought to play a role in protection from dehydration and infection and to keep skin supple. Apocrine and eccrine sweat glands arise as secretory coils in the dermis and form the ducts into the epidermis. They may open into the hair follicle or onto the skin surface to secrete sweat onto the skin surface to dissipate excessive body heat by evaporation.

5.2. METABOLISM

5.2.i. Introduction

Skin has traditionally has been seen as an inert structural barrier preventing entry of toxic substances and loss of critical body constituents. The skin posses a range of metabolic activity, including the ability to metabolise endogenous substances such as lipids, carbohydrates and proteins (Rongone, 1977; Bickers and Kappas, 1980) and exogenous xenobiotic compounds (Bickers and Kappas, 1980; Noonan and Wester, 1985).

5.2.ii. Esterase Hydrolysis in the Skin

Esterases which split various esters, including esters of drugs,are measurable in all parts of the skin. Fredriksson, (1958) showed in vitro, that guinea-

pig skin contains an enzyme belonging to the group of phosphorylphosphatases, capable of hydrolysing the anticholinesterase agent sarin (isopropoxy-methyl-phosphonyl-fluoride). In vivo studies in the cat suggested that sarin may undergo cutaneous first pass metabolism during percutaneous absorption, thereby inactivating the compound before it enters the systemic circulation. Fredriksson, (1961) investigated the capacity of skin from man, cat, rabbit and rat to hydrolyse paraoxon and metabolise parathion. Paraoxon was hydrolysed to p-nitrophenol in all species tested, apart from the rat, whilst parathion was not metabolised by the skin from any of the species tested. Fredriksson et al, (1969) showed that the organophosphate pesticide soman (pincoloxy-methyl-phosphonyl fluoride) and tabun (dimethyl-amido-ethoxy-phosphonyl-cyanide) were hydrolysed in vitro, by guinea-pig skin. The hydrolysis of diflucortone valerate in the rat, guinea-pig and human skin has been demonstrated in vivo, with a marked species difference in the rate of metabolism exhibited, rat and guinea-pig skin metabolising the compound at a greater rate than human skin (Tauber and Toda, 1976). MacPherson et al, (1991) found that carbaryl hydrolysis took place in the skin post mitochondrial fraction.

Based on the presence of esterases in the skin, a number of ester prodrugs have been developed which can be topically applied to the skin where they are hydrolysed. The pharmalogical concept is relatively new and can be used to treat skin diseases or for systemic circulation. Work by Cheung et al, (1985) demonstrated the presence of esterase activity in the skin, very similar to that found in the liver. It was also discovered that the betamethasone-17 steroid ester was resistant to esterase hydrolysis, whereas the 21-steroid ester was rapidly hydrolysed. By being more resistant to the cutaneous esterases, the 17-steroid ester, upon application to the skin would tend to form better resevoirs in the skin and therefore act for longer periods of time. Using alkyl p-nitrobenzoate esters as substrates Pannatier et al, (1981) found considerable hydrolytic activity in the skin preparations from mice. Using ethyl p-nitrobenzoate as a substrate, it was found that the mouse liver homogenate is approximately 10 times more active than the skin on a weight of tissue basis.

Esterase activity has been used as a means of activating or inactivating prodrugs following topical application. For example, Yu et al, (1979) showed that the prodrug vidaribine 5' valerate is metabolised by skin esterases to form the antiviral agent vidarabine in vivo following topical application to human skin. Loftsson et al, (1982) showed that acetyl salicyclic acid was similtaneously transported and hydrolysed in vitro in hairless mouse. Studies using metroidazole and 5-fluorouracil prodrugs show that leaching of esterases takes place rapidly during permeation, using excised human skin preparations. The receptor phase drug metabolism due to leaching enzymes may be of great importance in evaluating permeation studies of hydrolysable drugs or prodrugs such as esters (Bundgaard et al, 1982).

CHAPTER 6 : XENOBIOTIC METABOLISM IN THE BLOOD

6.1. Introduction

Blood is an opaque fluid with a viscosity somewhat greater than that of water. When oxygenated, as in the systemic arteries, it is bright scarlet, and when deoxygenated, as in systemic veins, it is dark red to purple. Blood is a heterogenous fluid consisting of a clear liquid, plasma, and formed elements, corpuscles.

Plasma is a clear, slightly yellow fluid which contains many substances in solution or suspension. Plasma is rich in sodium and chloride ions and also contains potassium, calcium, magnesium, phosphate, bicarbonate and many other ions, glucose and amino acids. The colloids include the high molecular weight plasma proteins, composed chiefly of those associated with clotting, particularly prothrombin, the immunoglobulins, and compliment proteins, involved in immunological defence and glycoproteins, polypeptides and steroids concerned with hormonal activities. Since most of the metabolic activities of the body are reflected in the composition of the plasma, routine chemical analysis of this fluid has become of great importance.

The red blood corpuscles (erythrocytes or red blood cells) form the greater proportion of the blood cells (99% of the total number), with a count of 4.1-6 x 10^6 / mm^3 in adult males and 3.9-5.5 x 10^6 / mm^3 in adult females. Each cell is a biconcave disc with a diameter of 6.3-7.9 μm and a rim thickness of 1.9 μm; in wet preparations the mean diameter is 8.6 μm. Erythrocytes lack nuclei and are pale red by transmitted light with paler centres because of their biconcavity. Erythrocytes are bounded by a plasma membrane and consist internally of a single protein, haemoglobin, apart from a few remnants from their initial developement. The plasma membrane is about 60% lipid and glycolipid, and 40% protein and glycoprotein.

6.2. Esterase Hydrolysis in Blood (see chapter 1.2.)

SECTION II : GENERAL METHODS

CHAPTER 7 : PREPARATION OF ANIMAL TISSUE

7.1. ANIMALS

Male Wistar rats weighing 180g were obtained from the Comparative Biology Centre, The Medical School, Newcastle University. The rats were housed in steel cages and subjected to an artificial light cycle of twelve hours duration. The rats had free access to water and standard rodent chow (lab. diet 41D).

7.2. REMOVAL OF RAT TISSUE

Male Wistar rats were killed by cervical dislocation. With the use of scissors and forcep, the liver and lung were removed. The whole of the dorsal area was shaved using electric clippers (Ostler) and the skin removed. The tissues were then placed in plastic bags and put immediately on ice. Tissues were later frozen and stored at -80^{o}C.

10ml of blood was removed from anaesthetized rats by cardiac puncture and placed in heparinised tubes. Plasma and blood were separated by centrifugation using a Mistral 3000 centrifuge, at 880 x g for 5 minutes. The plasma white cells were removed and stored at -80^{o}C before analysis. The red blood cells were washed using an equal volume of isotonic saline. After centrifugation at 880 x g for 10 minutes the upper saline layer was removed and discarded. This procedure was again repeated. The red blood cells were then stored at -80^{o}C before analysis.

7.3. PREPARATION OF TISSUE HOMOGENATE

Liver and lung tissues were washed in ice cold 0.34M sucrose buffer,

pH7. The skin was placed in a petri dish on ice and the subcutaneous fat and vascular tissue was removed from the dermis. 1 g portions of each of the tissues were weighed and finely minced using scissors and forceps in 10mls of 0.34M sucrose buffer, pH7.0.

Tissue homogenates were prepared using a cooled polytron homogenizer (Kinematic) using two bursts of 10 seconds duration at full power, with cooling between the bursts for skin and two bursts at 2 seconds duration at full power for liver and lung. Tissue homogenates were centrifuged in a Sorvall RC 5B refrigerated centrifuge at 1,800 x g for 5 minutes at 4^{o}C to remove tissue debris. The supernatant was further centrifuged at 10,000 x g for 10 minutes at 4^{o}C to remove mitochondria and nuclei.

7.4. PREPARATION OF SUBCELLULAR FRACTIONS

The post-mitochondrial fraction was then centrifuged in a Sorval ultra-centrifuge at 100,000 x g for one hour and ten minutes. The supernatant (cytosol) was removed and the resulting pellet (microsomes) was resuspended by homogenizing the pellet in 50mM trisma HCl buffer pH 7.5, using a glass to glass homogenizer. The resulting pellet from the second spin was resuspended into 1ml of 50mM trisma HCl pH 7.5 for lung and skin and 5 mls for liver. The microsomal and cytosolic fractions were stored in aliquot tubes at -80^{o}C before analysis.

7.5. PROTEIN ESTIMATIONS

Microsomal and cytosolic protein estimations were made using a variation on the Lowry et al, (1956) method, by Peterson, (1977).

To analyse the protein concentrations a calibration curve had to be set up. From a neat protein stock BSA(0.6mg/100ml)in 100 dilution was made. This gave a final stock solution of 0.6µg/µl. The following protein standards were used: 0, 6, 30, 60 and 90µg. These standards were placed into 10mls round bottomed tube. This was made up to 1ml with distilled water. 0.1ml of deoxy-cholic acid (D.O.C.) was added , mixed and allowed to stand for 10 minutes. After the 10 minutes, 0.1ml of T.C.A. (72%) was added and then mixed. The tubes were then placed in a centrifuge and vortexed at 5000 x g for 15minutes. At the end of this the supernatant was discarded and the tubes were turned upside down to dry. 1ml of distilled water was added followed by 1ml of reagent A (equal volumes of the following: copper-tartrate-carbonate (C.T.C.), 0.8M NaOH, 10% S.D.S. and water). This was all mixed together and allowed to stand for 10 minutes. 0.5mls of reagent B (20ml folin-ciocalteau phenol reagent + 100mls of distilled water) was added and then alowed to stand for 30 minutes. The absorbance at each concentration was read at 750nm on a UV Kontron spectrophotometer.

Having established the calibration curve, the protein recoveries for the different tissue fractions were calculated by taking a fixed amount of either microsomal or cytosolic tissue and repeating the above proceedure.

CHAPTER 8 : ENZYME KINETICS

8.1. INTRODUCTION

The kinetic feature that most distinguishes enzyme-catalysed reactions from simple chemical reactions is that they show saturation. Nearly all enzyme-catalysed reactions show a first-order dependance of rate on substrate concentration at very low concentrations, but instead of increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no dependance of rate on concentration and the reaction becomes a zero order with respect to substrate (see Figure 8.1.). Work by Michaelis and Menton, (1913) is usually taken as the starting point when discussing enzyme kinetics. Their mechanism supposes that the first step in the reaction is the binding of the substrate (A) to the enzyme (E) to form an enzyme-substrate complex (EA) which then reacts to give the product (P) with the regeneration of the free enzyme: $E + A \xrightarrow{k1} EA \xrightarrow{k2} E + P$ where k_1 is the initial rate of conversion of E + A to EA and k_2 is the first order conversion of EA to E + P. From this fundamental equation the' Michaelis-Menton equation' was derived:

$$v = \frac{k_o e_o a}{K_m + a} \qquad (8.1)$$

From this equation two kinetic parameters can be measured: (1) V max- the maximum rate of hydrolysis at enzyme saturation. and (2) Km- substrate concentration enzyme has at 1/2 the Vmax. Before these kinetic parameters could be measured conditions of linearity for protein and time had to be established. Once these had been established a ten fold range in substrate concentrations was used to obtain values for Km and Vmax.

In the experiments two types of plot were used to determine the kinetic parameters: double-reciprocal plot and direct line plot .

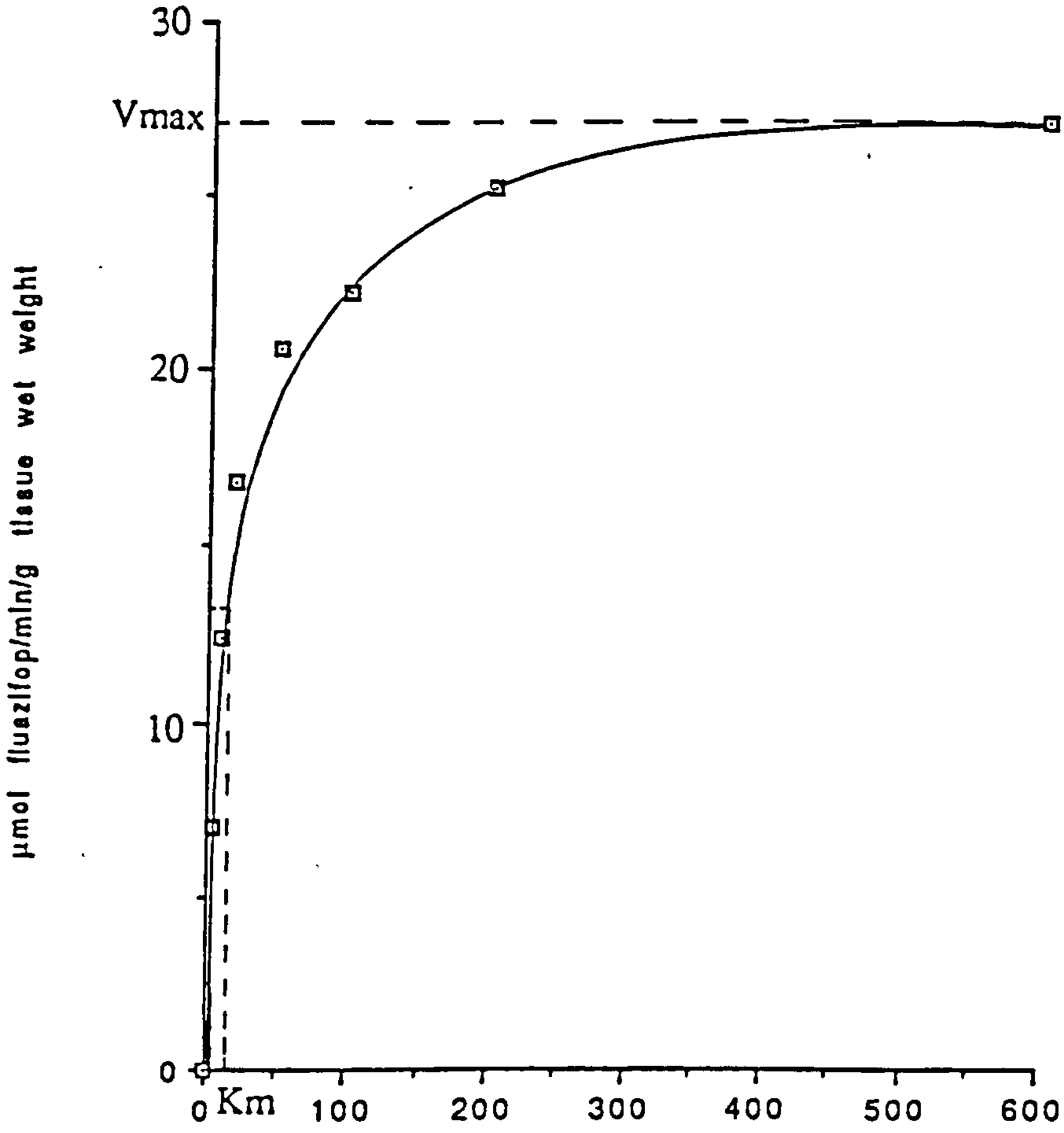


Figure 8.1. Hydrolysis of fluazifop-butyl by rat liver microsomes. Calculation of Vmax (µmol/ min/g wet weight) and Km (µM) by simple Michaelis Menton Kinetics.

8.2. DOUBLE-RECIPROCAL PLOT

The most natural way of plotting steady-state kinetic data is to plot v against the substrate concentration s, as in Figure 8.1, and this is certainly the clearest way to display the behaviour. However, this is not a good way to determine kinetic parameters, because the line is curved and it does not approach the limit fast enough to judge accurately where the limit is. To overcome this the Michaelis-Menton equation was transformed into the equation for a straight line by taking reciprocals of both sides of equation 9.1.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{Km \cdot 1}{V_{max}\, s} \qquad (8.2)$$

A plot of 1/v against 1/s is a straight line, with a slope of $1/k_a e_o$ which is equal to Km/V, an intercept on the ordinate (1/v) axis of $1/k_a e_o$ which equal to 1/V, and an intercept on the abscissa (1/s) axis of $-k_a/k_o$ equal to -1/Km (Figure 8.2.). The objection to the double-reciprocal plot, as it is commonly known, is that it distorts the appearance of any experimental error in the primary observations of v, so that one cannot judge which points are most accurate when drawing a straight line through the set of points.

In the experimental studies described here the double-reciprocal plot was initially used, however, this proved to be inaccurate at times especially at lower substrate concentrations (larger deviation), therefore the direct line plot was favoured.

8.3. DIRECT LINE PLOT

The direct linear plot (Eisenthal and Cornish-Bowden, 1974) requires each observation to be plotted as a straight line and the parameter values appear as a point rather than a slope and intercept of a line. The Michaelis-Menton equation is

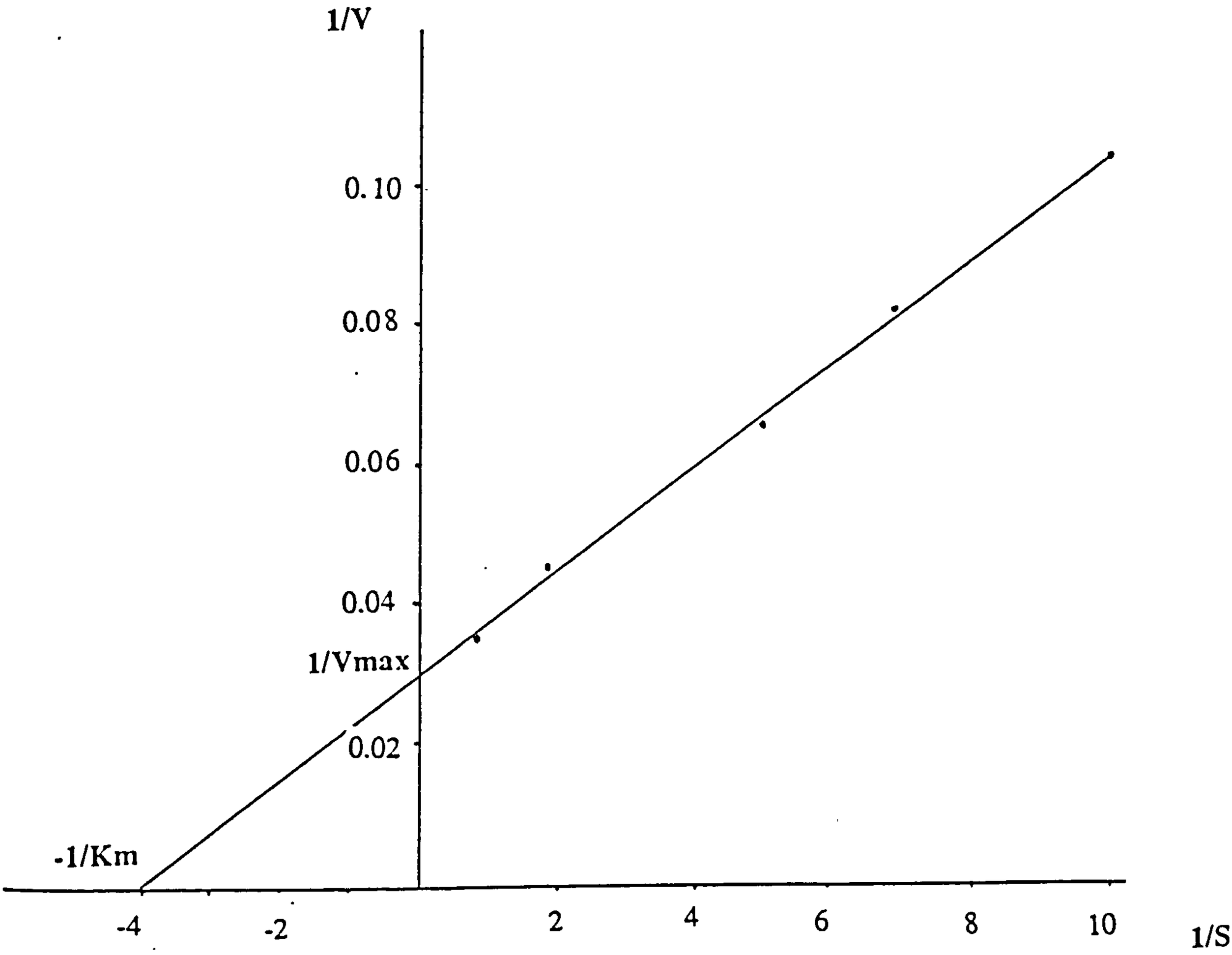


Figure 8.2. Hydrolysis of fluazifop-butyl by ratliver microsomes. Calculation of Vmax (µmol/ min/g wet weight) and Km (µM) by double reciprical plot.

rearranged as such:

$$V = v + (v/s)\,Km \qquad (8.3.)$$

If V and Km are treated as variables and v and a as constants, this equation defines a straight line with intercepts v on the V axis and -s on the Km axis.This line relates all possible pairs of (Km,V) values that exactly satisfy an observation of rate v at substrate concentration s. A second line drawn in the same way for a second observation relates all parameter values that satisfy this second observation. Only one point is on both lines, their point of intersection, and its coordinates define the unique pair of parameter values that satisfy both observations exactly.

In the absence of experimental error, we should expect that n such lines for a set of n observations would all intersect at a unique point, the coordinates of which would give the values of Km and V. A real experiment, however, is subject to error, and gives a family of intersection points, as seen on Figure 8.3. It is nonetheless possible to estimate the parameter values because each intersection point provides one estimate of Km and one estimate of V. These can be marked on the axes and the best estimate of each parameter can be taken as the median (middle) value of the set. The use of non-parametric assessment does not assume a normal distribution of the data. In taking the median value the plot is insensitive to outliners.As well as this the direct linear plot is independant of weighting.

Initially in the study all three approaches were used to determine the apparent Km and Vmax values, however, the direct line plot was favoured because it was found to be more accurate at lower substrate concentrations than the double-reciprocal plot.

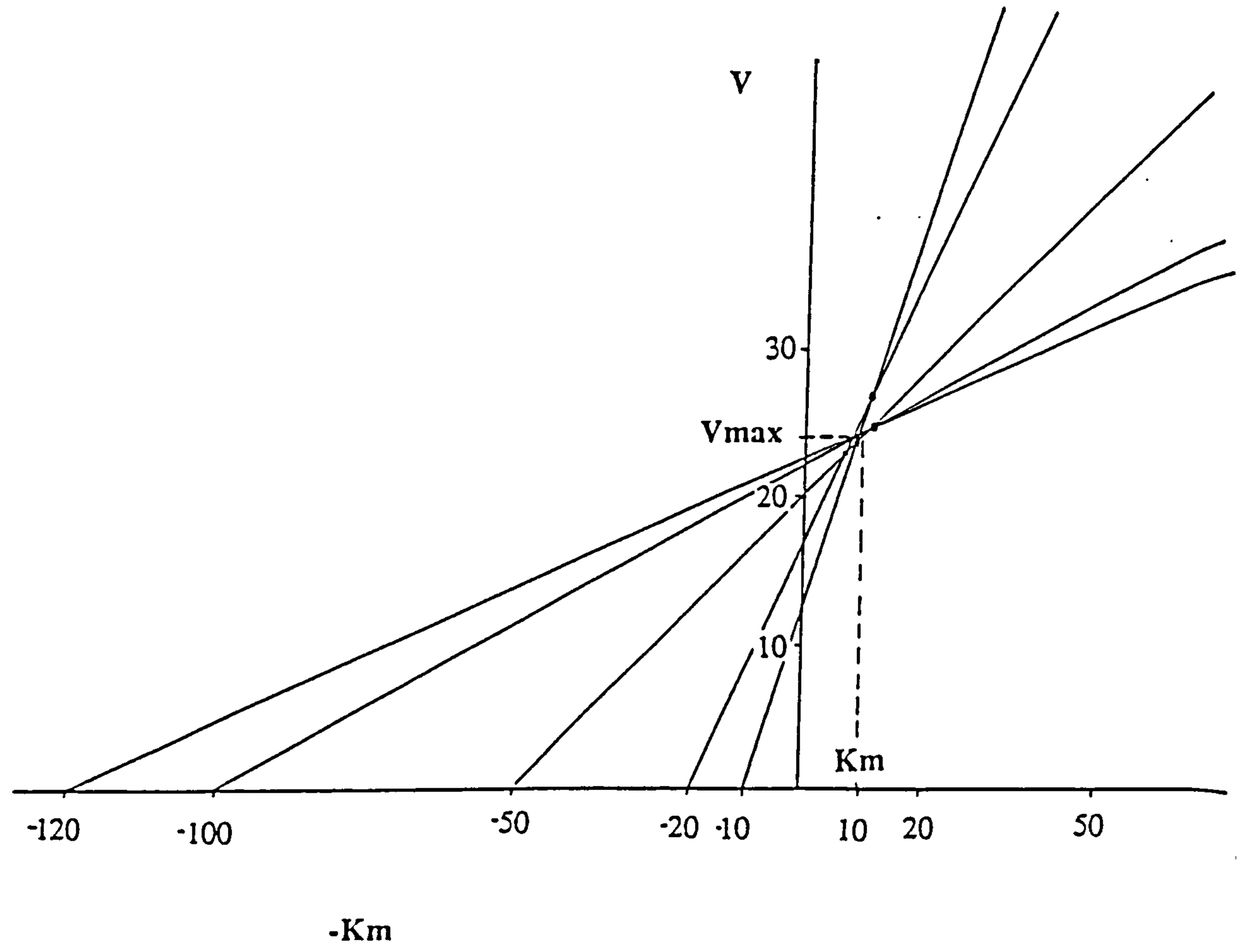


Figure 8.3. Hydrolysis of fluazifop-butyl by ratliver microsomes. Calculation of Vmax (µmol/ min/g wet weight) and Km (µM) by direct linear plot.

CHAPTER 9 : ENZYME ASSAYS

9.1. INTRODUCTION

The following enzymatic assays were set up to examine the hydrolysis, by esterase enzymes, of a number of xenobiotic esters to establish localisation and distribution of esterase activity in the liver, lung, skin and blood of the rat. Activities for skin were a combination of activities from the dermis and epidermis.

Using the analytical techniques of HPLC and spectrophotometry, the formation of product was measured over a range of substrate concentrations to establish the Km and Vmax (see chapter 9) for the hydrolytic reactions, after conditions of linearity with protein and time had been established.

9.2. FLUAZIFOP-BUTYL ESTERASE

9.2.i. Chemicals

Fluazifop-butyl [butyl 3-[4-(5-trifluoromethyl-2-pyridyloxy phenoxy] propionate] has the empirical formula of $C_{19}H_{20}F_3NO_4$ and a molecular weight of 383.4 . Fluazifop-COOH has the empirical formula $C_{14}H_{11}F_3NO_2COOH$ and a molecular weight of 308 . Both chemicals were gifts from Central Toxicology Laboratory, ICI.

9.2.ii. Incubation

Microsomal and cytosolic protein, between 0.1-30mg of liver, lung and skin original wet weight or 10-50µl of plasma and red blood cells were incubated with fluazifop-butyl (0.05-1mM). Incubations were carried out in a final volume of 500µl

50mM trisma buffer pH 8, containing 0.1mM calcium chloride at 37^{o} C.

Reactions were started by the addition of fluazifop-butyl (5-20µl of a 10mM and 1mM stock in acetonitrile) and stopped with the addition of 500µl of 6% perchloric acid (w/v in H_2O), containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes .80µl of the clear supernatant was injected onto HPLC.

Using a fixed concentration of fluazifop-butyl (1mM) and time of incubation (10 mins), varying amounts of protein from liver (see Figure 9.2.1.), lung (see Figure 9.2.2.) and skin (see Figure 9.2.3.) were used in the incubations to establish conditions of linearity for protein. The amount of plasma was also varied, with a constant time and constant fluazifop-butyl concentration (see Figure 9.2.4.). Having established the appropriate amount of protein for the incubation the time of hydrolysis was varied (0-30 minutes) with a fixed fluazifop-butyl concentration (1mM), to establish conditions of linearity for time for the following tissues: liver (see Figure 9.2.5.); lung (see Figure 9.2.6.) ; skin (see Figure 9.2.7.) and plasma (see Figure 10.2.8.).

The conditions of linearity for protein, with constant time (10 mins) and fluazifop-butyl concentration (1mM) were 0.4, 5 and 30mg of liver, lung and skin original wet weight or 10µl of plasma. The conditions of linearity for time, with constant protein and fluazifop-butyl concentration were 4 minutes for liver and plasma and 10 minutes for lung and skin. The sensitivity of the fluazifop-butyl assay was 1nmol/ 10 min. No spontaneous hydrolysis of fluazifop-butyl was seen.

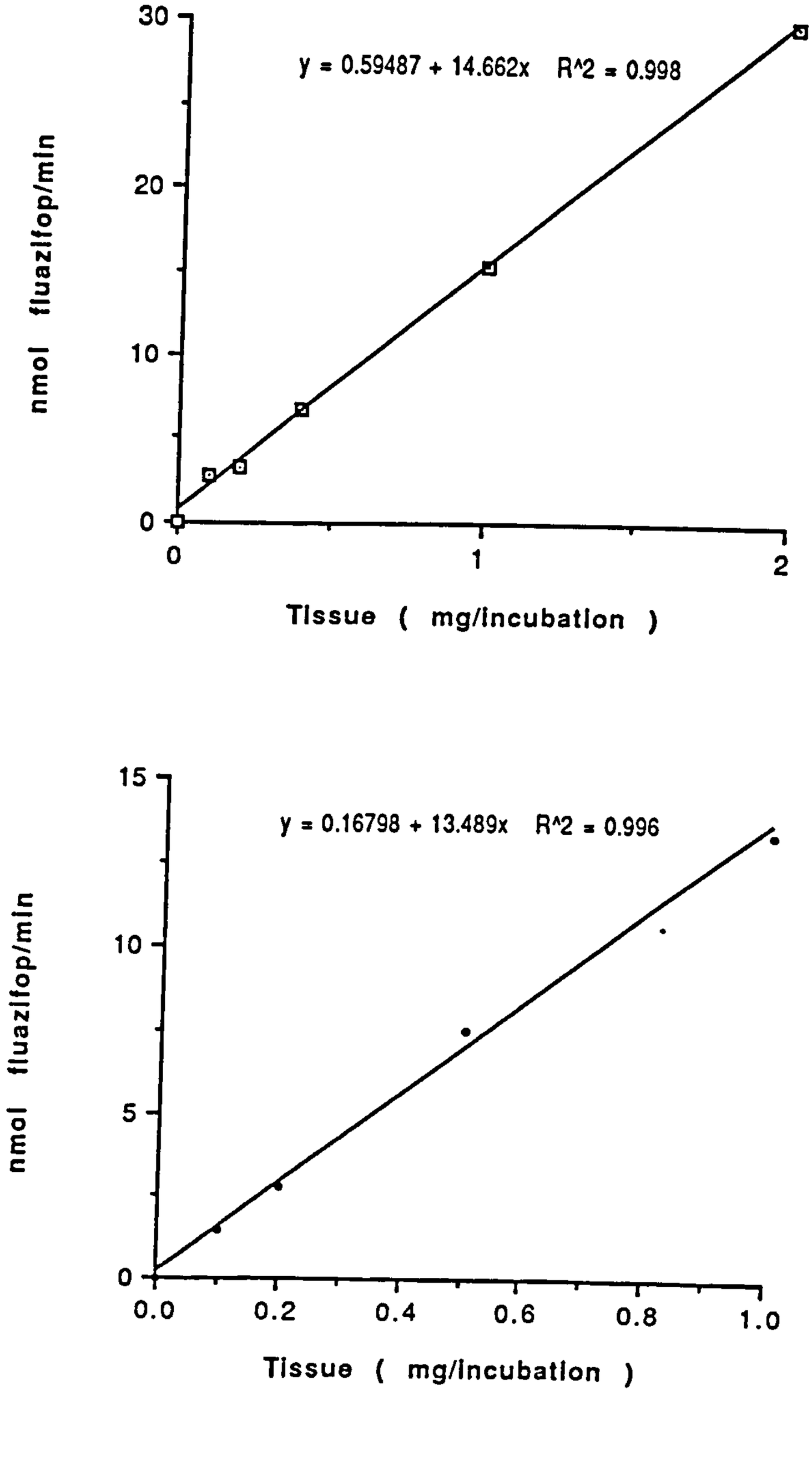
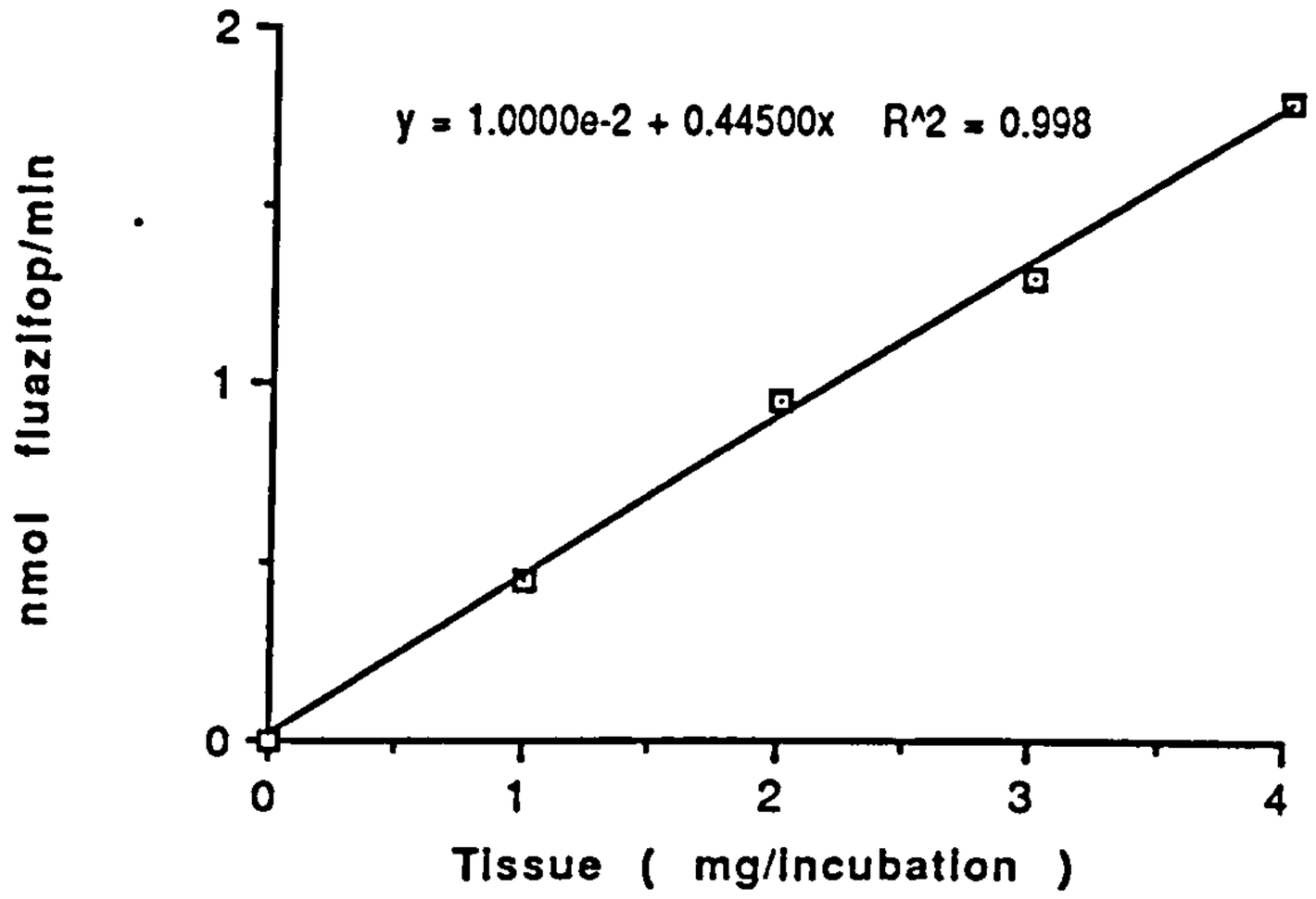


Figure 9.2.1.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying rat liver microsomes (⊡) and cytosol (•) (0-2 mg tissue wet weight/incubation).

Each point represents a mean of duplicate determinations.



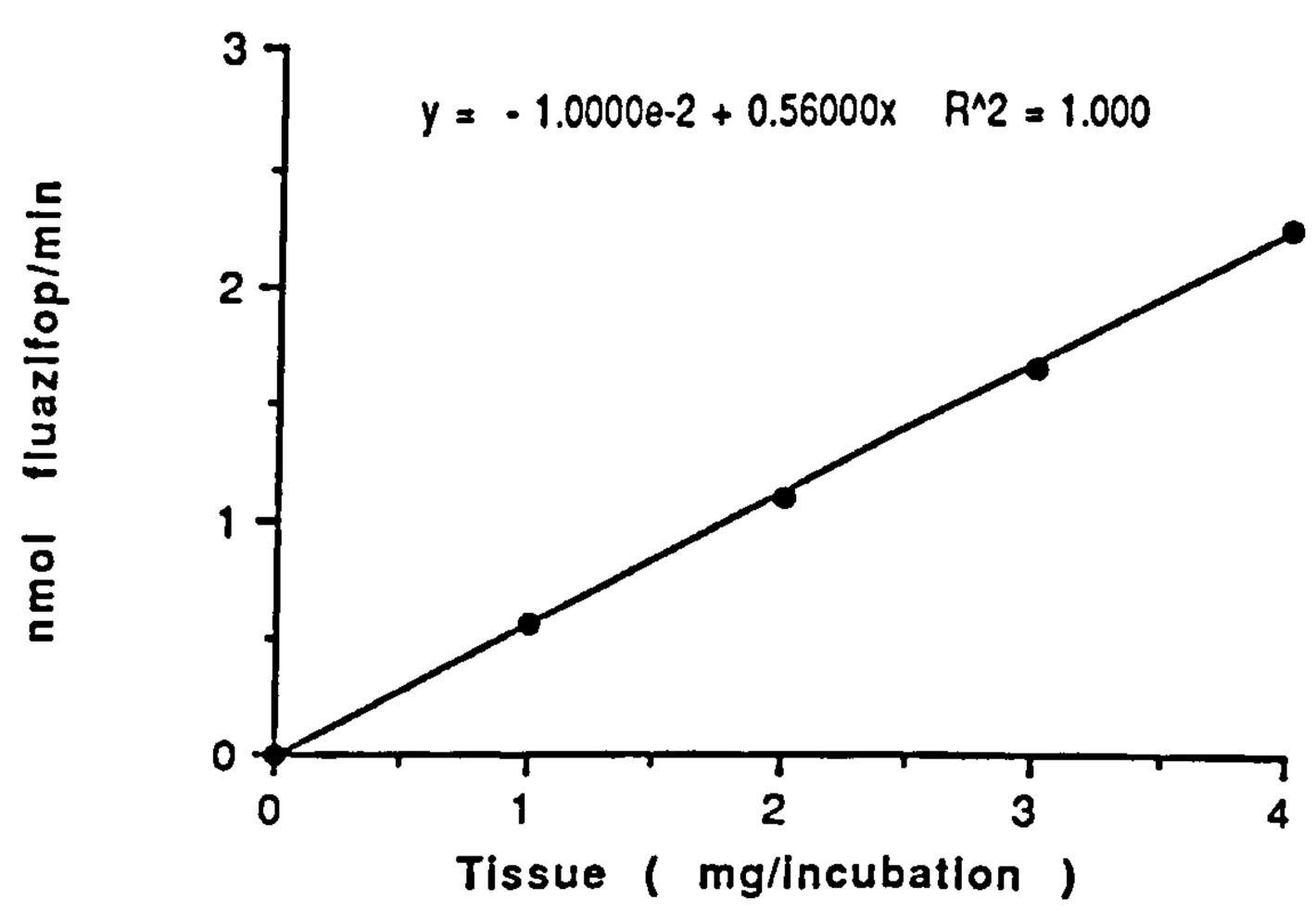
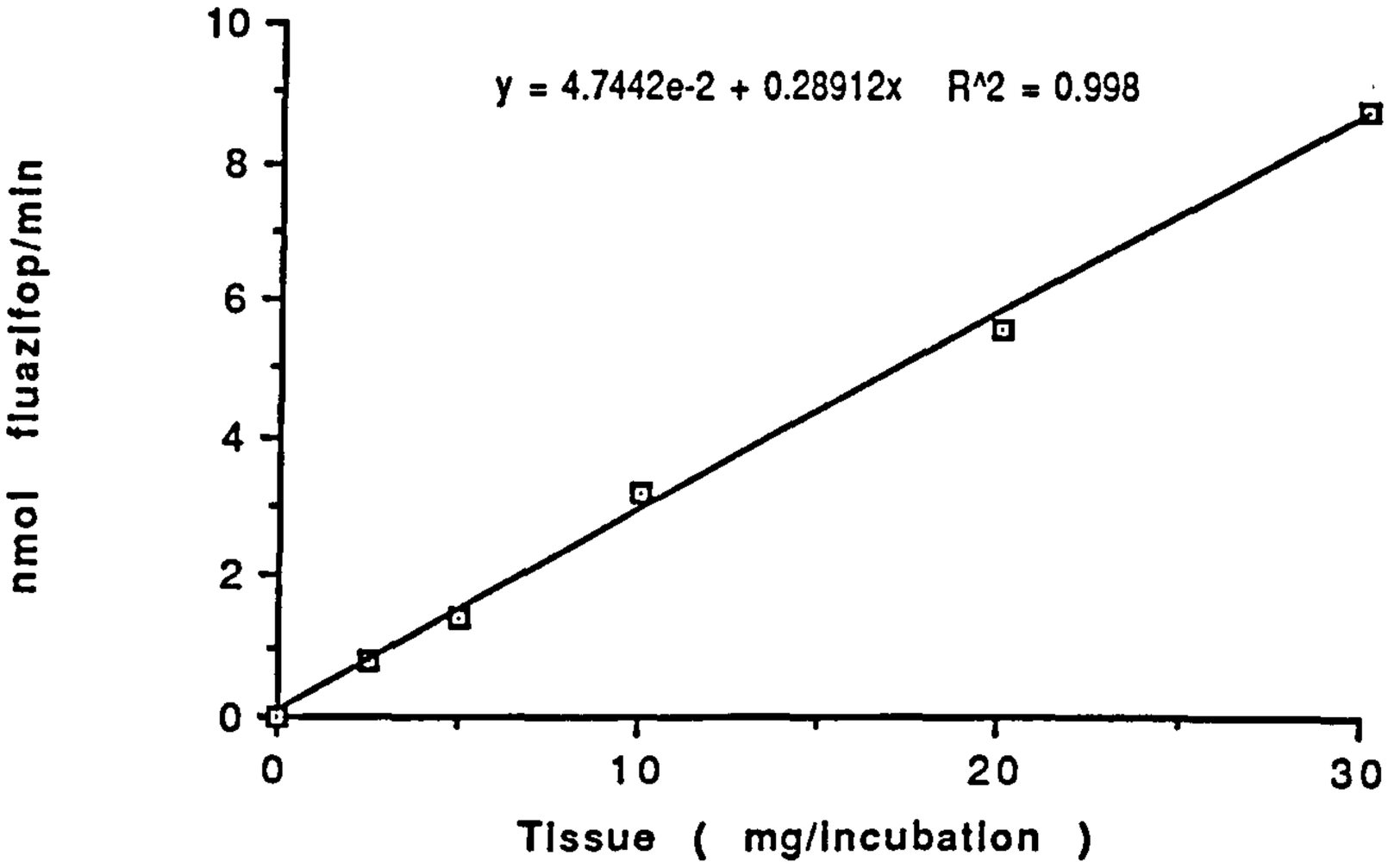


Figure 9.2.2.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying rat lung microsomes (⊡) and cytosol (•) (0-4 mg tissue wet weight/incubation).

Each point represents a mean of duplicate determinations.



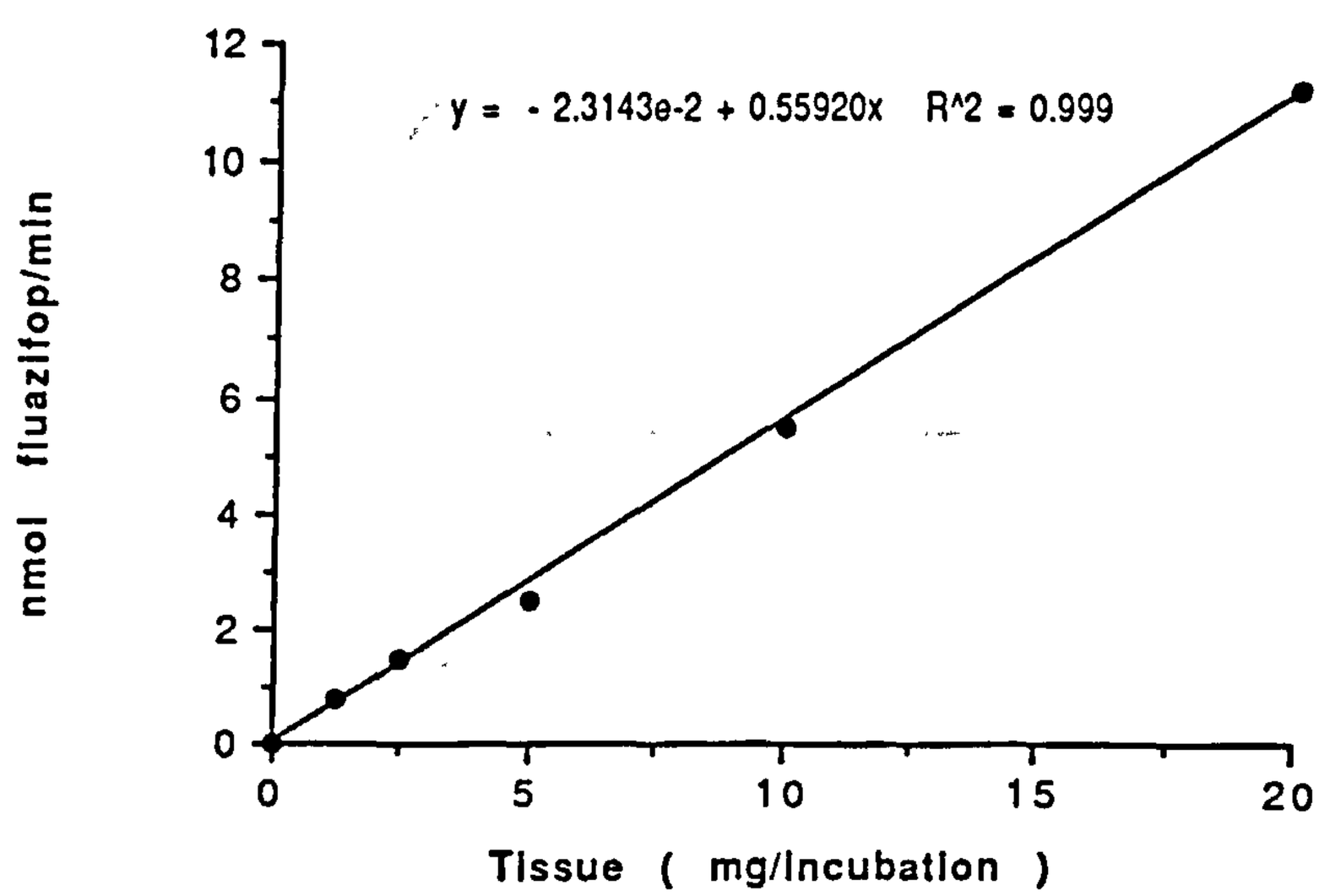


Figure 9.2.3.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying rat skin microsomes (⊡) and cytosol (•) (0-30 mg tissue wet weight/incubation).

Each point represents a mean of duplicate determinations.

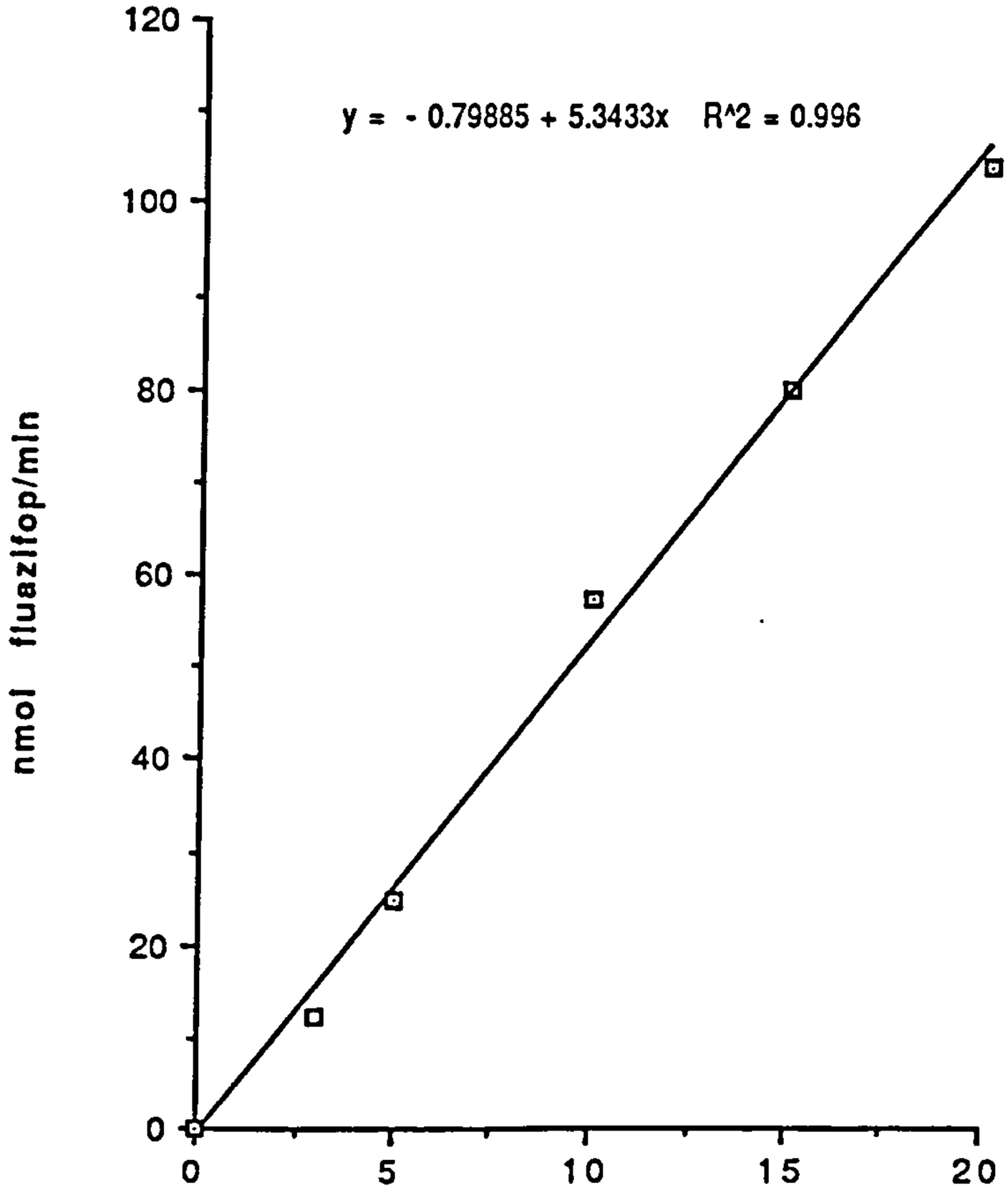
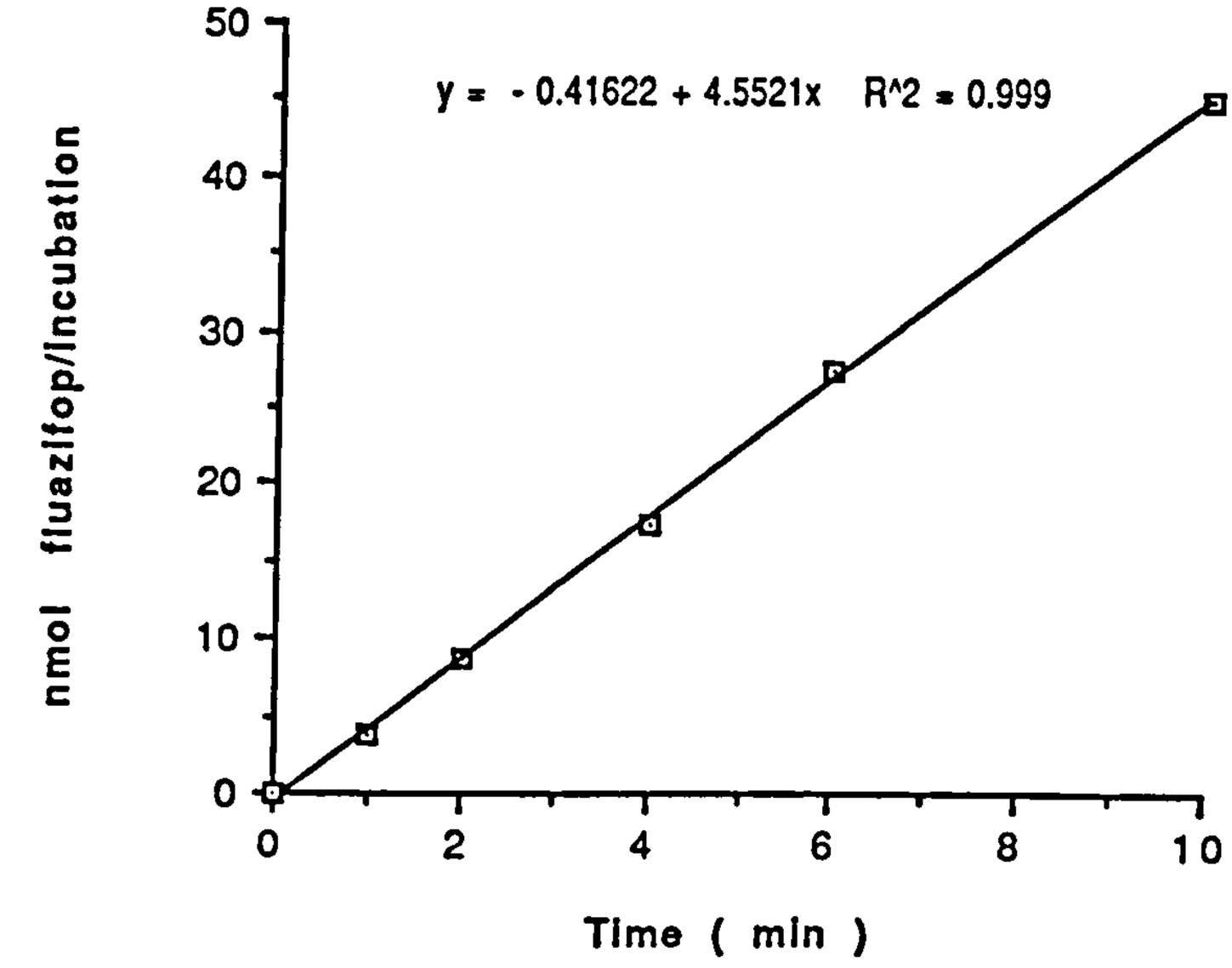


Figure 9.2.4.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying rat plasma (0-20 µl plasma/incubation).
Each point represents a mean of duplicate determinations.



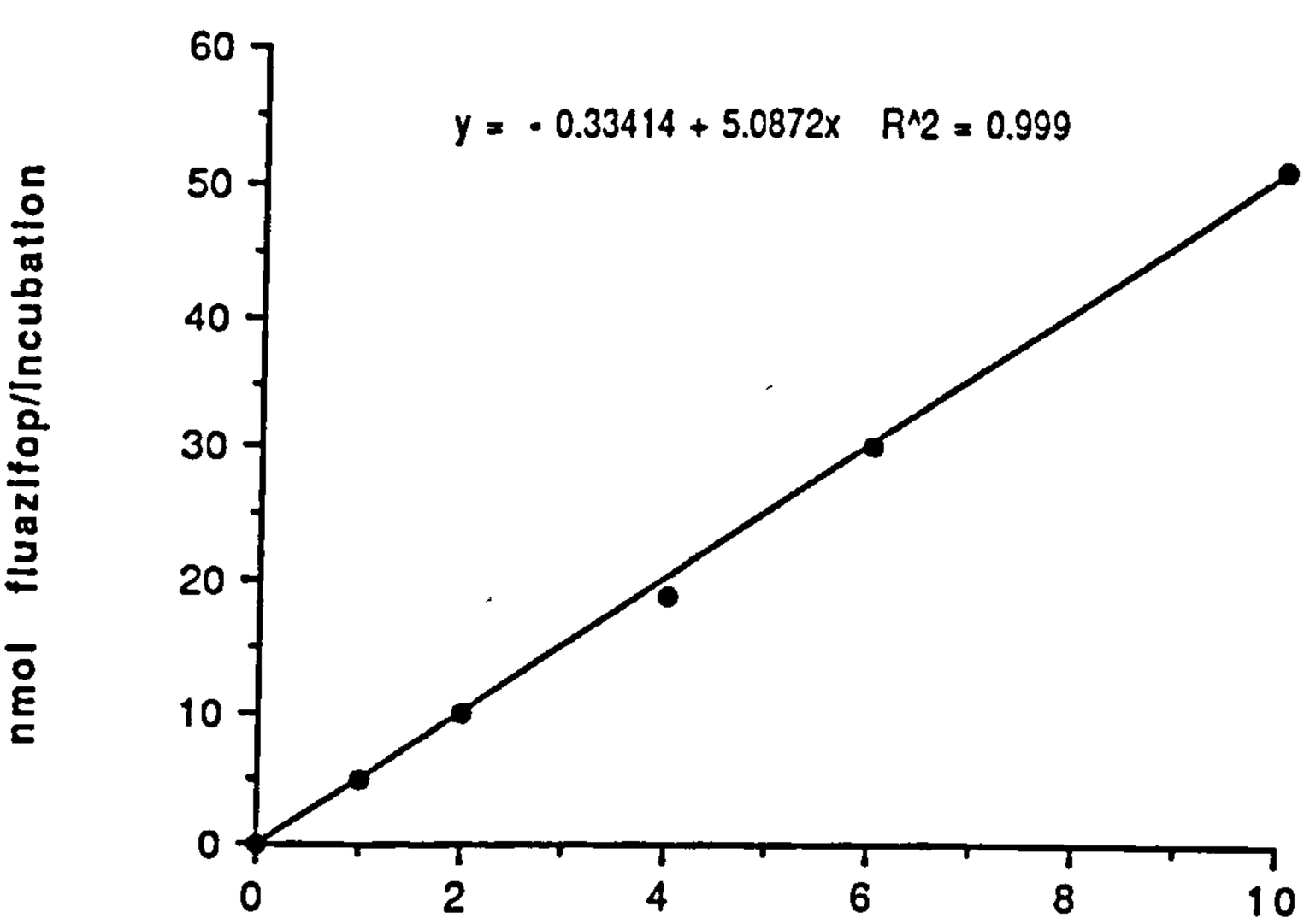


Figure 9.2.5.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by rat liver microsomes (⊡) and cytosol (•) (0.4mg/incubation) for varying times of hydrolysis . Each point represents a mean of duplicate determinations.

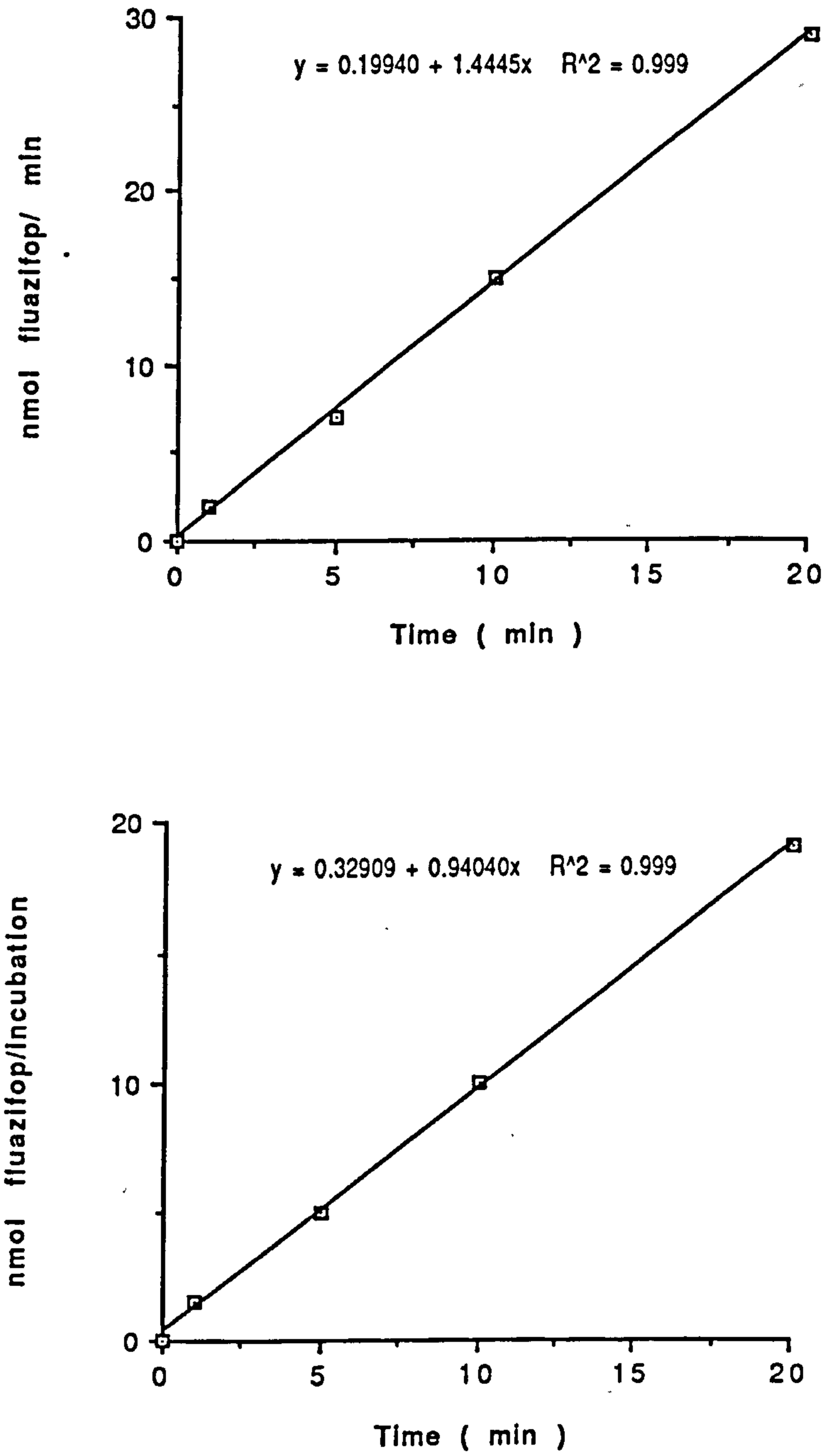
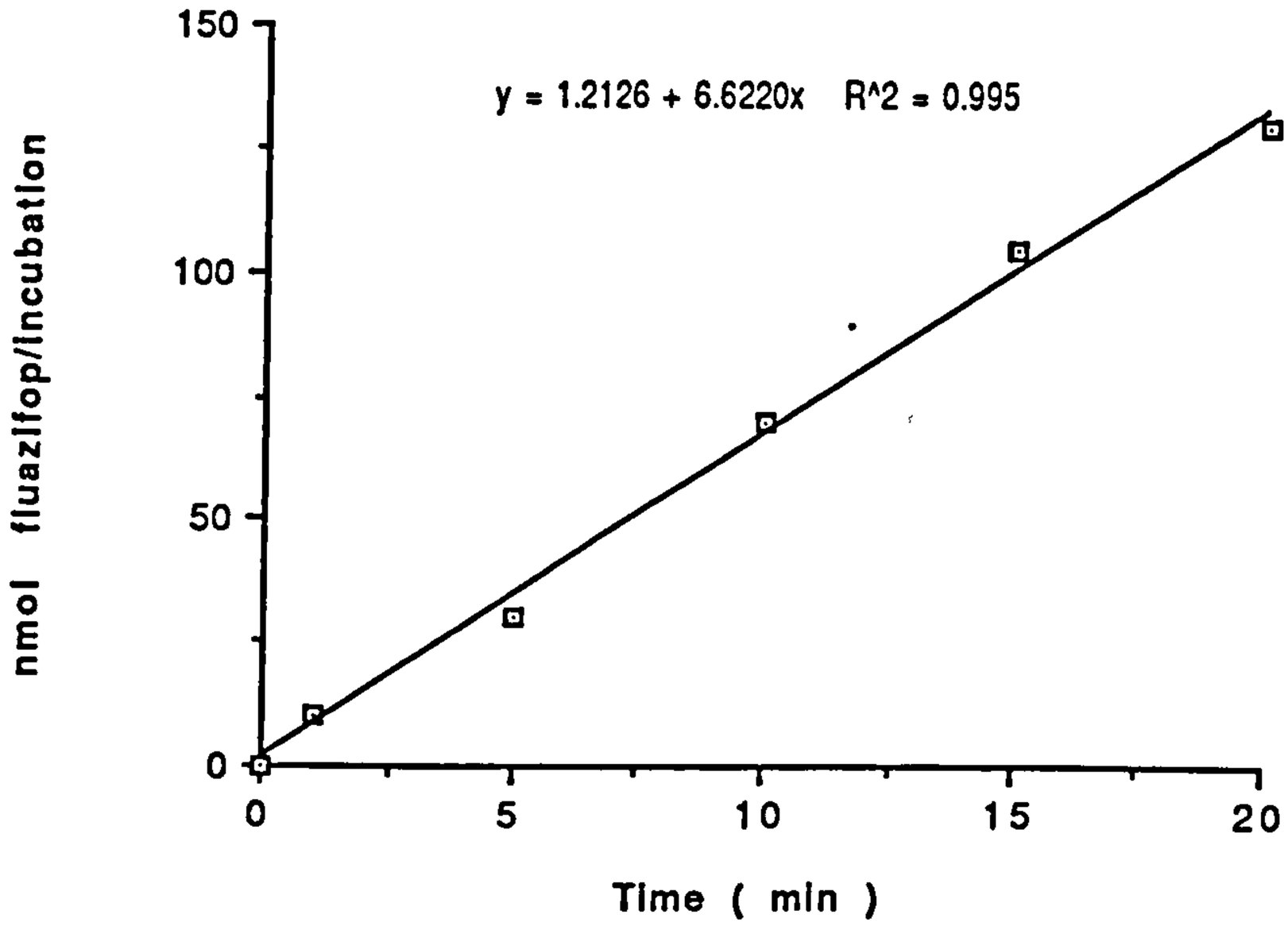


Figure 9.2.6.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by rat lung microsomes (⊡) and cytosol (•) (5mg/incubation) for varying times of hydrolysis. Each point represents a mean of duplicate determinations.



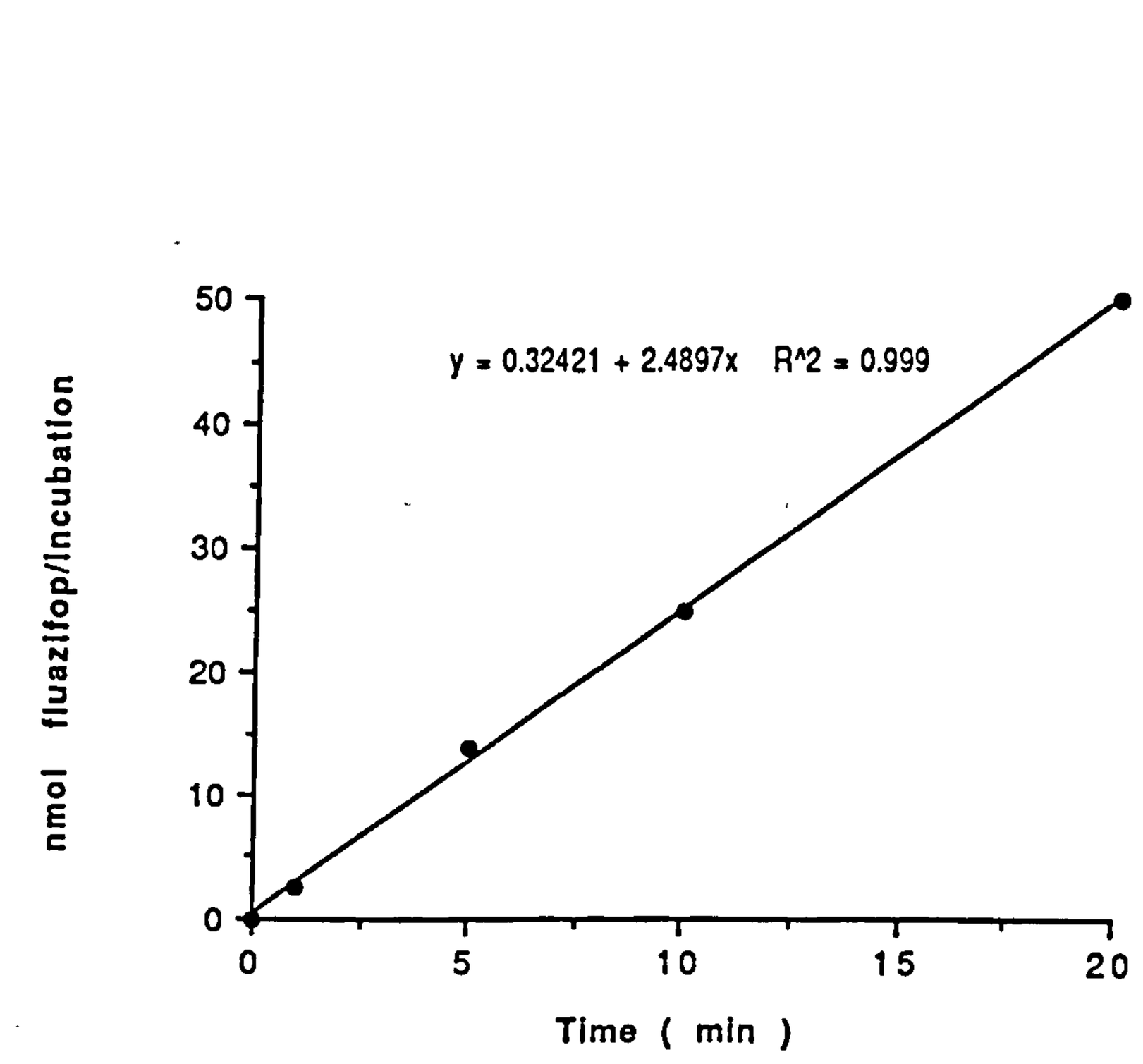


Figure 9.2.7.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by rat skin microsomes (□) and cytosol (•) (30mg/incubation) for varying times of hydrolysis. Each point represents a mean of duplicate determinations.

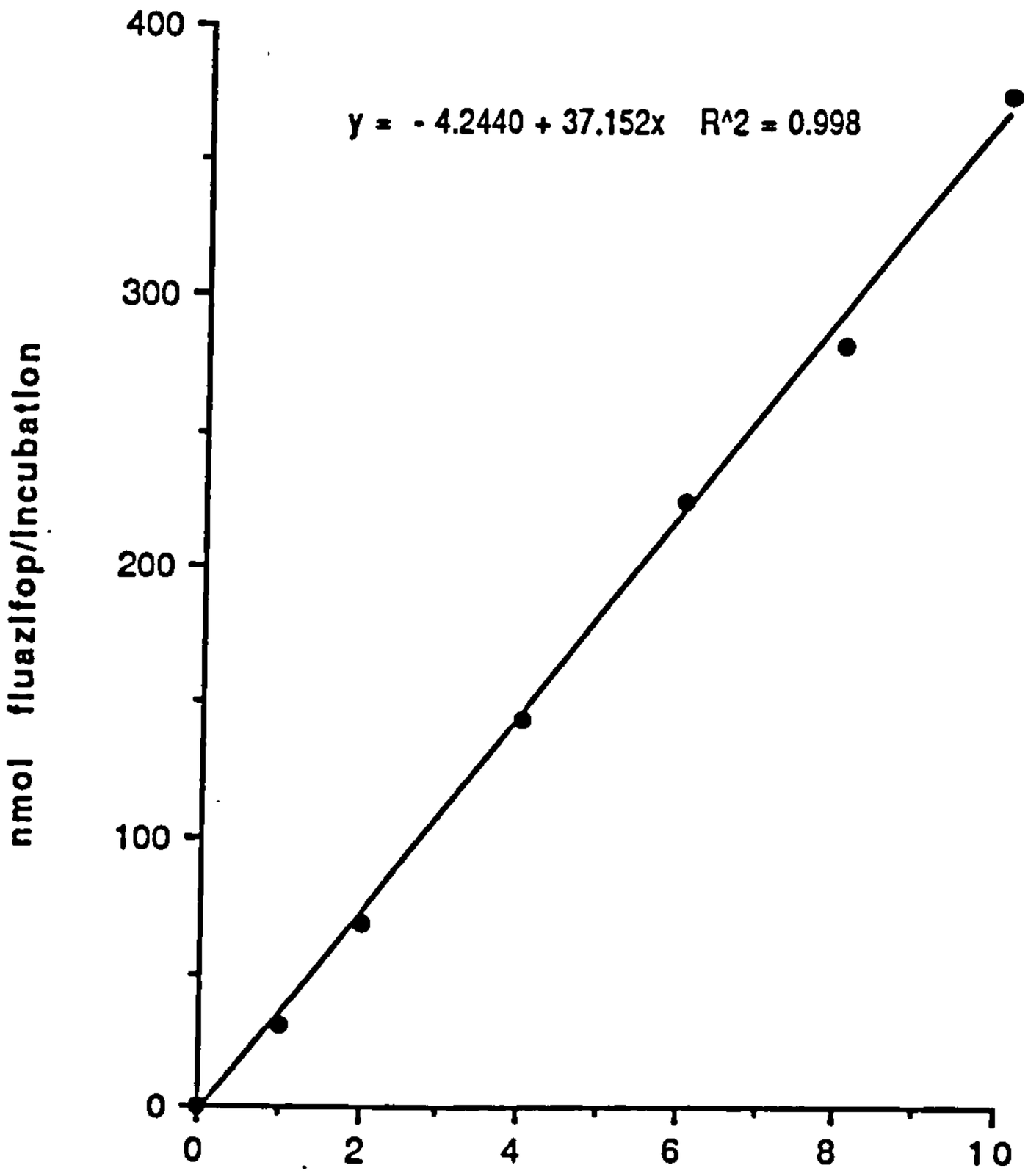


Figure 9.2.8.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by rat plasma (10 µl/incubation) for varying times of hydrolysis .

Each point represents a mean of duplicate determinations.

9.2.iii. HPLC separation of Fluazifop-butyl and Fluazifop-COOH

A Kontron system with dual wavelength UV detection fitted with a Spherisorb ODS (5µ, 15cm) C18 reverse phase HPLC column was used for the fluazifop-butyl assay with a mobile phase of orthophosphoric acid (0.072% w/v) / acetonitrile (40/60) at a rate of 1ml/min. Fluazifop-butyl and fluazifop-COOH were measured at 270nm, with the internal standard being measured at 250nm. The retention time of internal standard 2.5 minutes, fluazifop-COOH 3.5 minutes and fluazifop-butyl 12.5 minutes (see Figure 9.2.11.).

Calibration curves for fluazifop-butyl and fluazifop-COOH were established. Fluazifop-COOH and fluazifop-butyl standards (0-120µM final concentration in H_2O) mixed with an equal volume of perchloric acid, containing 10µg/ml p-toluic acid (internal standard) were injected onto the column (80µl) without neutralisation to enable the calculation of fluazifop-COOH (see Figure 9.2.9.) and fluazifop-butyl (see Figure 9.2.10.) in the assay.

9.3. CARBARYL ESTERASE

9.3.i. Chemicals

Carbaryl (1-naphthyl methylcarbamate) has an empirical formula of $C_{12}H_{11}NO_2$ and a molecular weight of 201.2 . 1-Naphthol has an empirical formula of $C_{10}H_8O$ and a molecular weight of 144.2 . Both chemicals were gifts from Central Toxicology Laboratory, ICI.

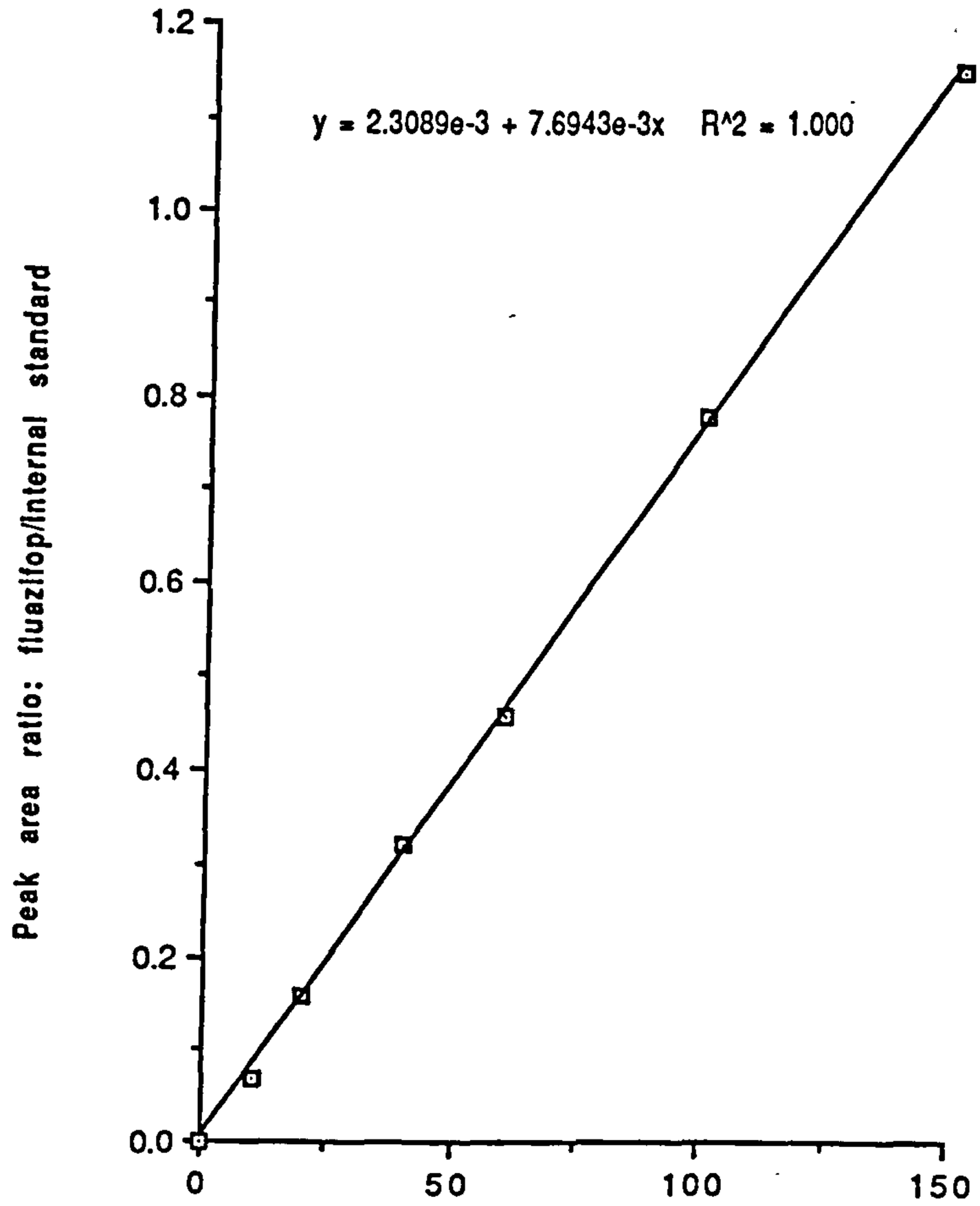


Figure 9.2.9.

A typical standard curve of fluazifop following direct injection (80µl) of standards (0-150µM).

Each point represents a mean of duplicate determinations.

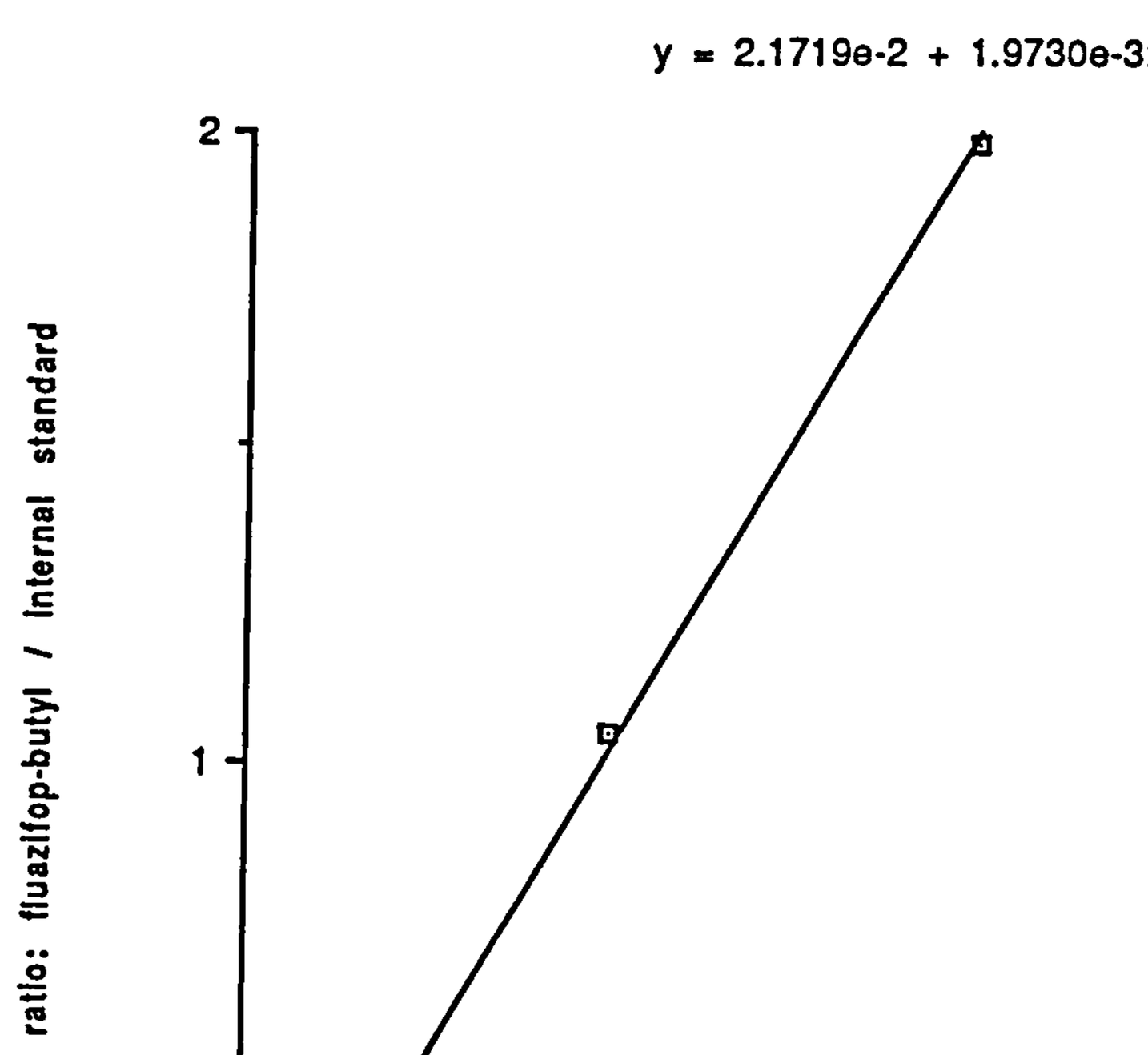


Figure 9.2.10.

A typical standard curve of fluazifop-butyl following direct injection (80µl) of standards (0-1000µM).

Each point represents a mean of duplicate determinations.

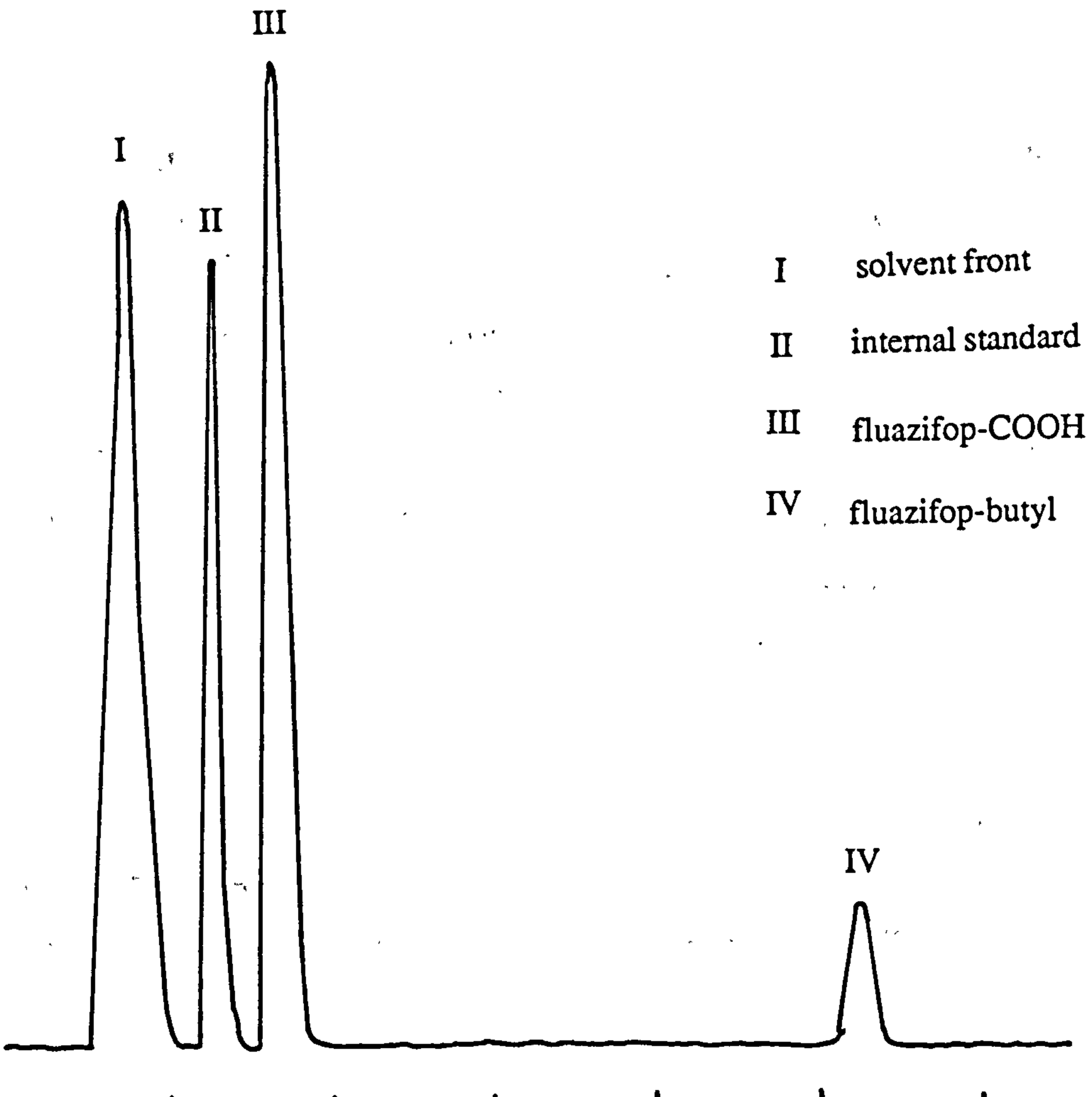


Figure 9.2.11. Typical chromatogram showing seperation of fluazifop-butyl, fluazifop and internal standard by reverse phase HPLC, using a mobile phase of 60:40 acetonitrile: orthophosphoric acid (0.072w/v) pumped at a rate of 1ml/minute. Detection is by UV spectrophotometry at a wavelength of 250nm for internal standard and 270nm for fluazifop and fluazifop-butyl..

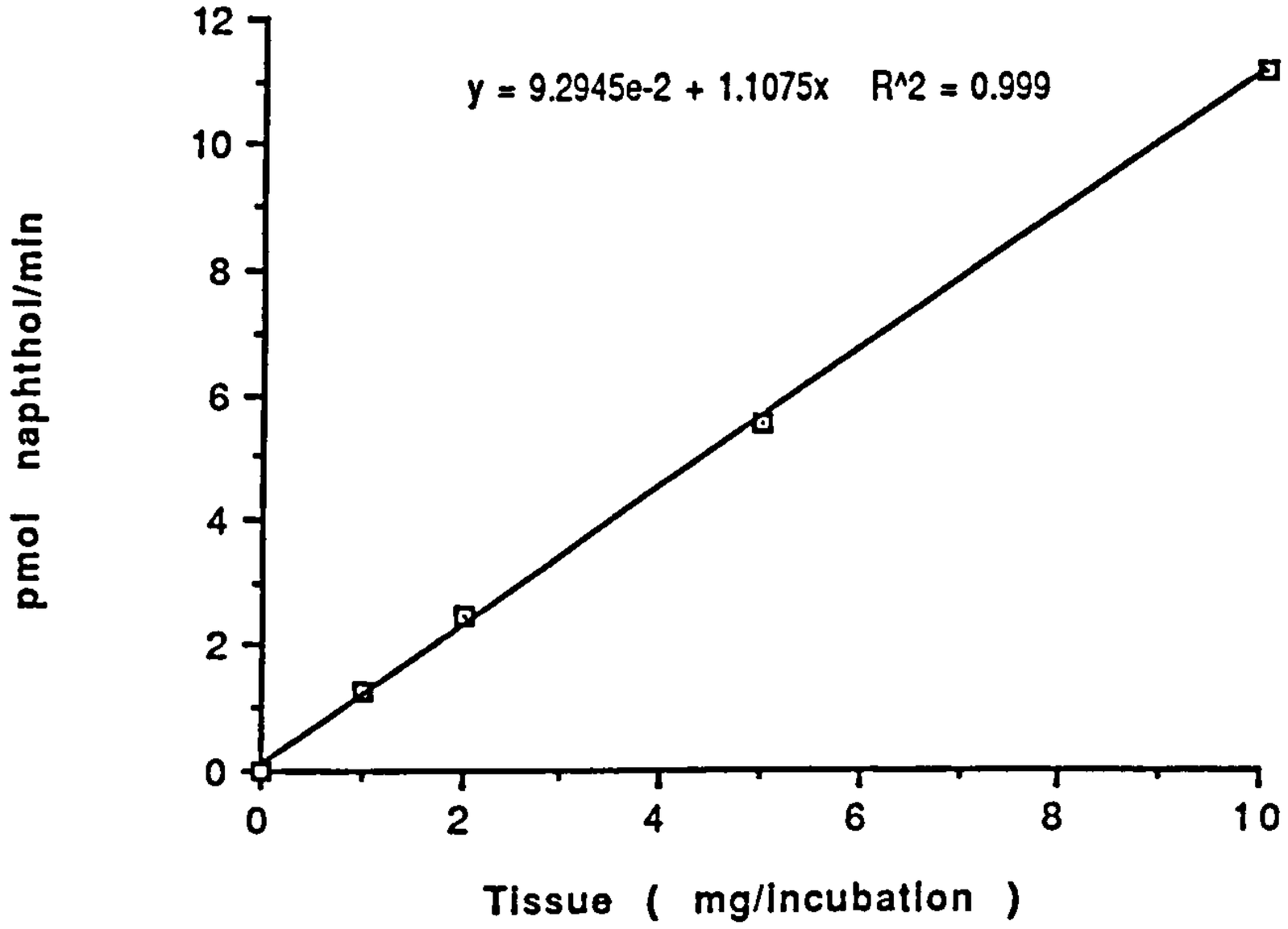
9.3.ii. Incubations

Microsomal and cytosolic protein, between 0.1-30mg of liver, lung and skin original wet weight or 10-50µl of plasma and red blood cells were incubated with carbaryl. Carbaryl (5-50µM) was added to eppendorf tubes in 15µl methanol, because it would not dissolve in H_2O. The methanol was evaporated off under nitrogen before the protein was added to the tube to prevent the methanol having an inhibitory effect on carbaryl hydrolysis. Incubations were carried out in a final volume of 1ml of 0.1M phosphate buffer pH 7.25, at 37°C. Control incubations containing 0.1M phosphate buffer pH 7.25 and carbaryl, were conducted in parallel to access spontaneous hydrolysis.

Reactions were started by the addition of tissue and stopped with the addition of 500µl absolute ethanol. Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 50µl of supernatant was injected onto reverse phase HPLC.

Using a fixed concentration of carbaryl and time of incubation, varying amounts of protein from liver (see Figure 9.3.1.), lung (see Figure 9.3.2.) and skin (see Figure 9.3.3.) were used in the incubations to establish conditions of linearity for protein. The amount of plasma was also varied, with a constant time and constant carbaryl concentration (see Figure 9.3.4.). Having established a fixed amount of protein for the incubation, the time of hydrolysis was varied (0-30 minutes) with a fixed carbaryl concentration, to establish conditions of linearity for time for the following tissues: liver (see Figure 9.3.5.); lung (see Figure 9.3.6.); skin (see Figure 9.3.7.) and plasma (see Figure 9.3.8.).

The conditions for linearity for protein were 5, 10 and 30mg for liver, lung and skin original wet weight or 20µl of plasma with constant time (20 mins) and carbaryl concentration (25µM). The conditions of linearity for time were 20 minutes for



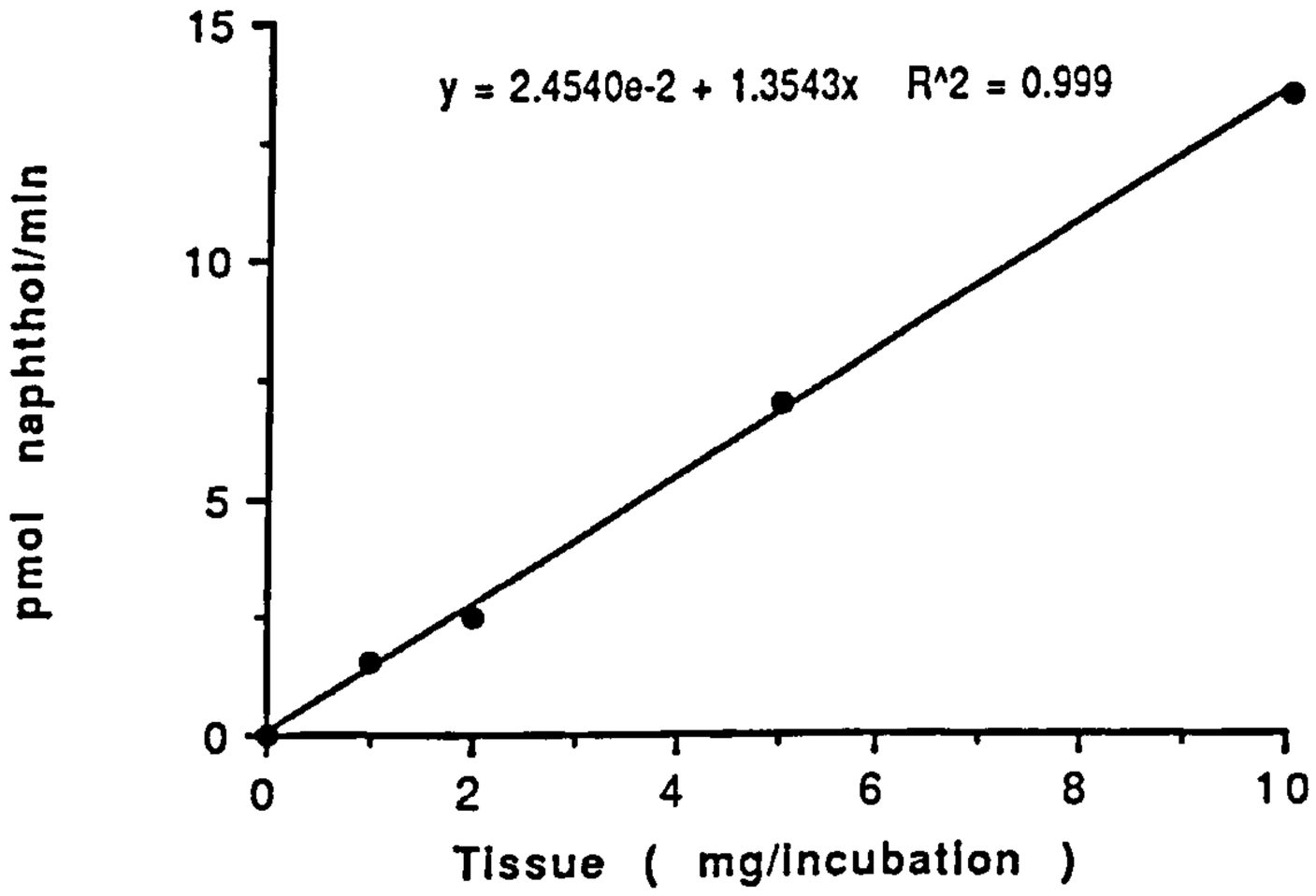
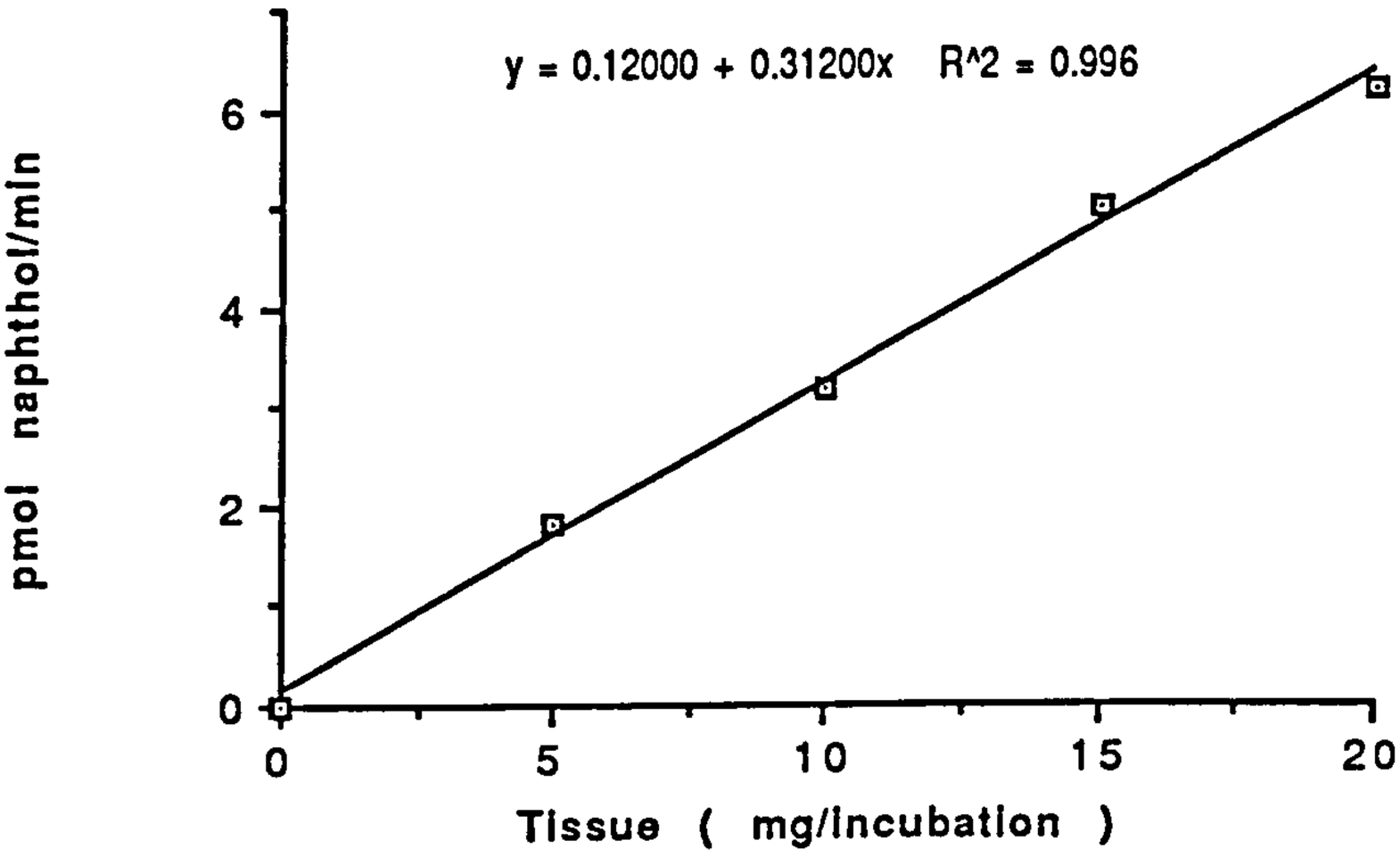


Figure 9.3.1.

Rate of carbaryl (1mM) hydrolysis to 1-naphthol (pmol/minute) in the presence of varying concentrations of rat liver microsomes (⊡) and cytosol (•) (0-10 mg/incubation).

Each point represents a mean of duplicate determinations.



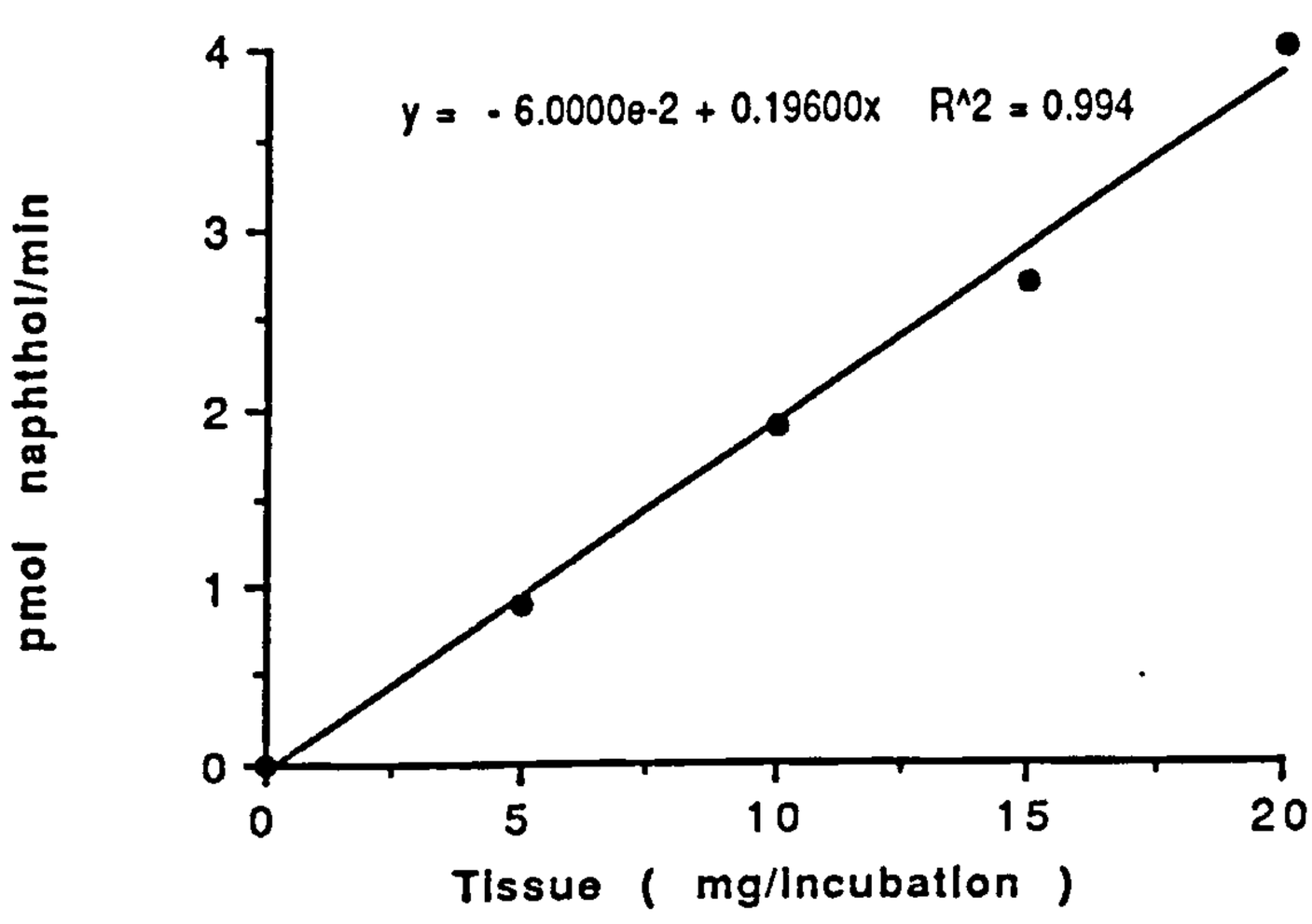


Figure 9.3.2.

Rate of carbaryl (25μM) hydrolysis to 1-naphthol (pmol/minute) in the presence of varying concentrations of rat lung microsomes (⊡) and cytosol (•) (0-20 mg/incubation).

Each point represents a mean of duplicate determinations.

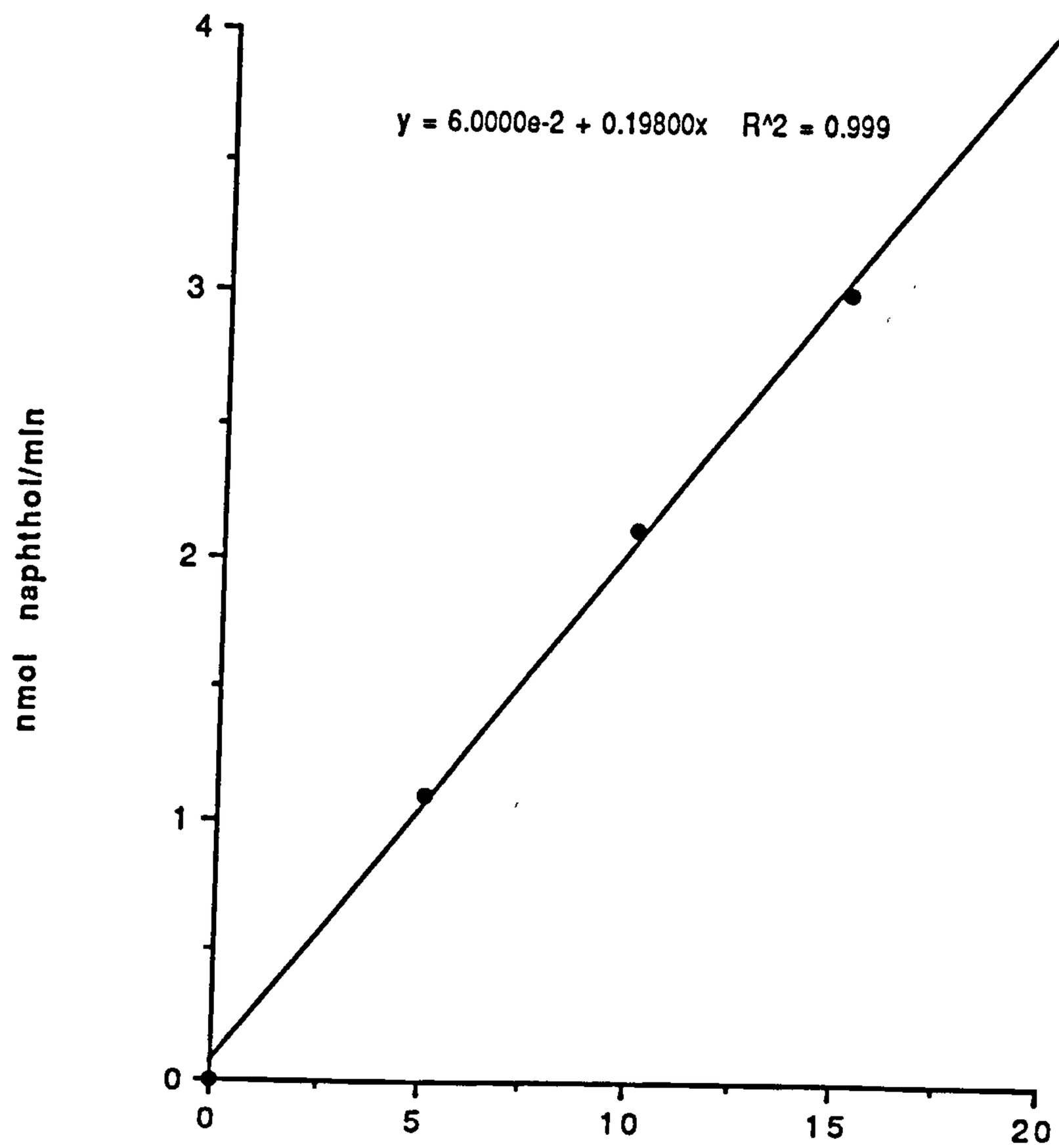


Figure 9.3.3.

Rate of carbaryl (25μM) hydrolysis to 1-naphthol (pmol/minute) in the presence of varying concentrations of rat skin cytosol (•) (0-20 mg/incubation).
Each point represents a mean of duplicate determinations.

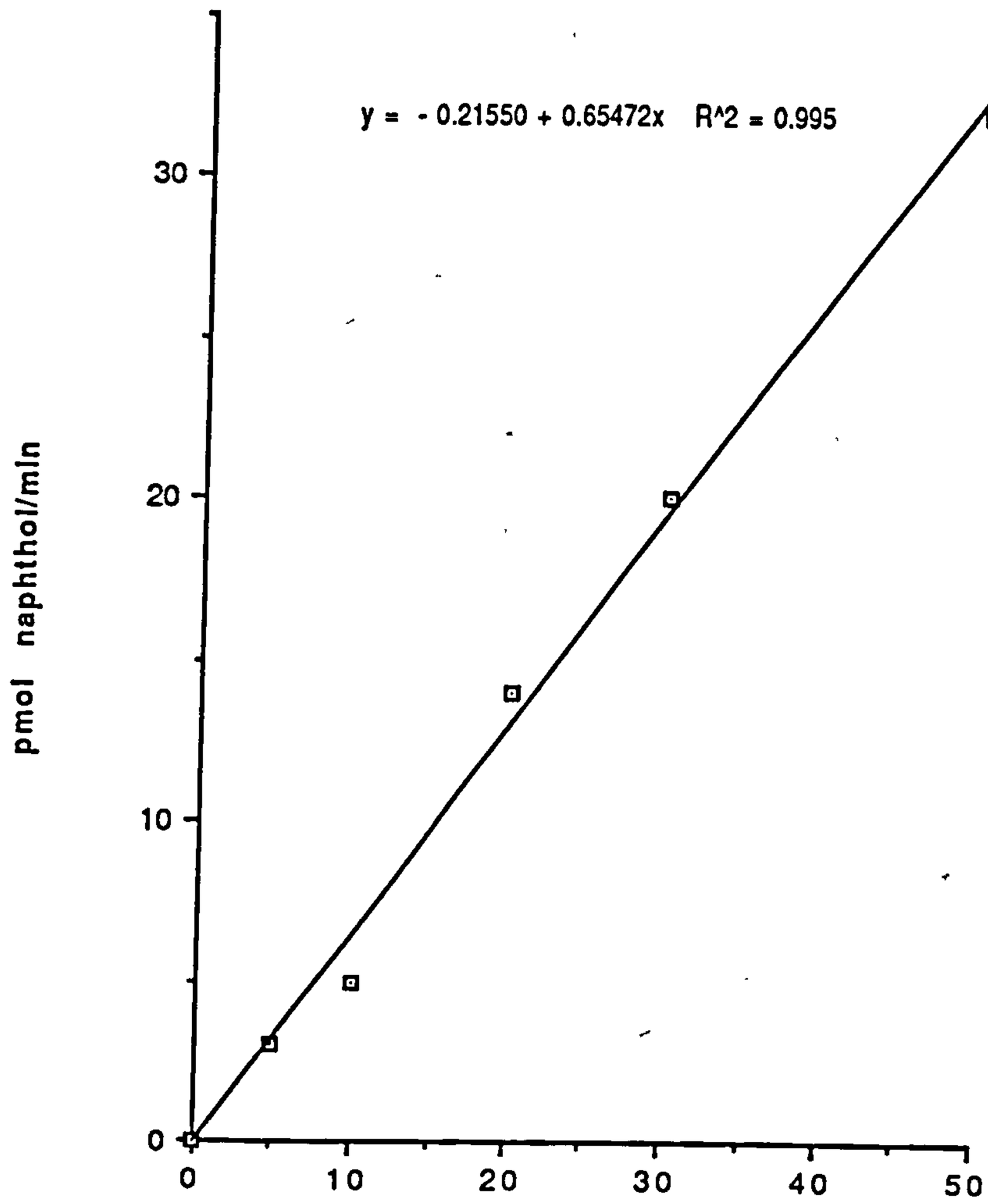


Figure 9.3.4.

Rate of carbaryl (25µM) hydrolysis 1-naphthol (pmol/minute) in the presence of varying amounts of rat plasma (0-50 µl/incubation).
Each point represents a mean of duplicate determinations.

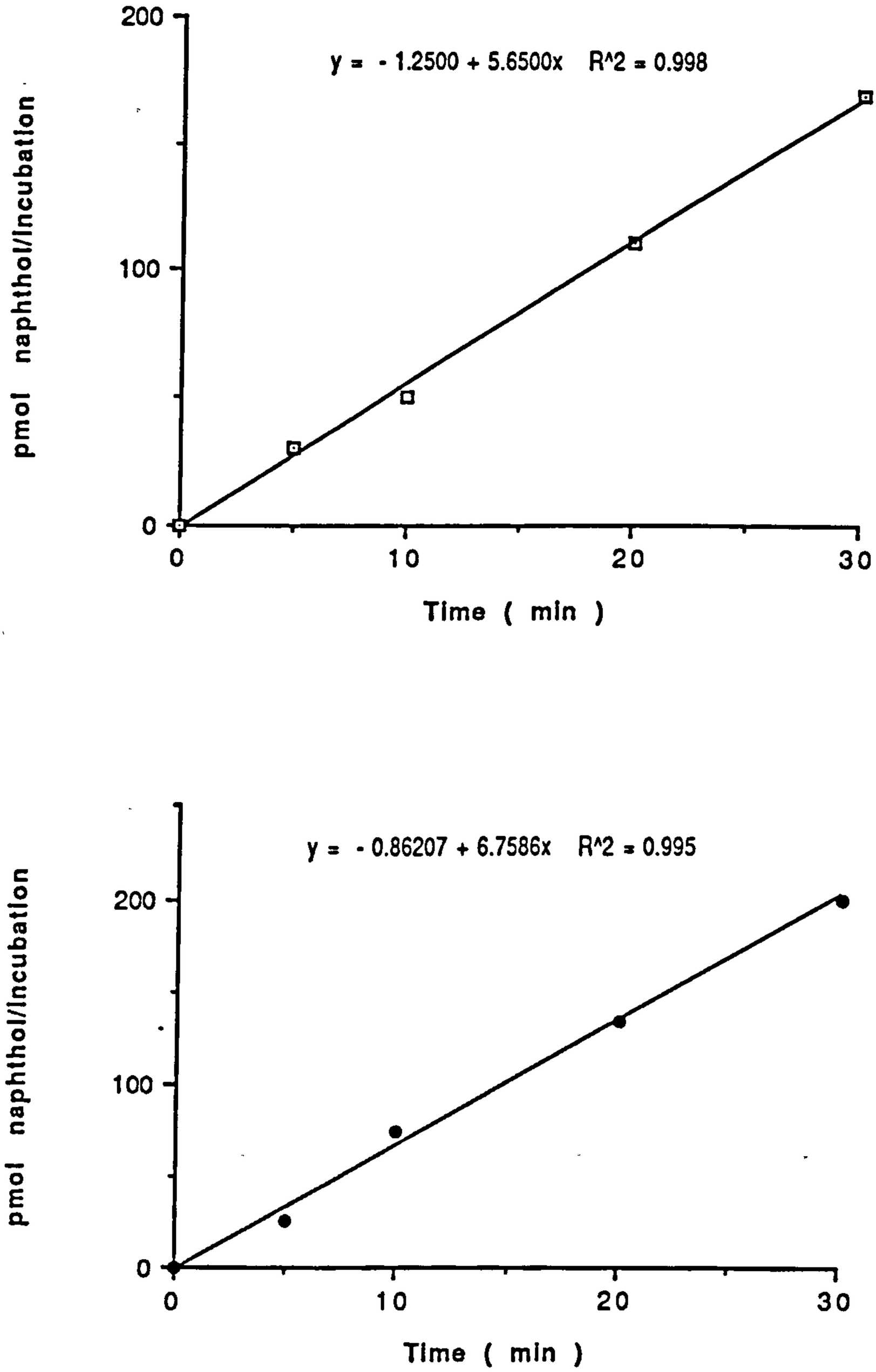
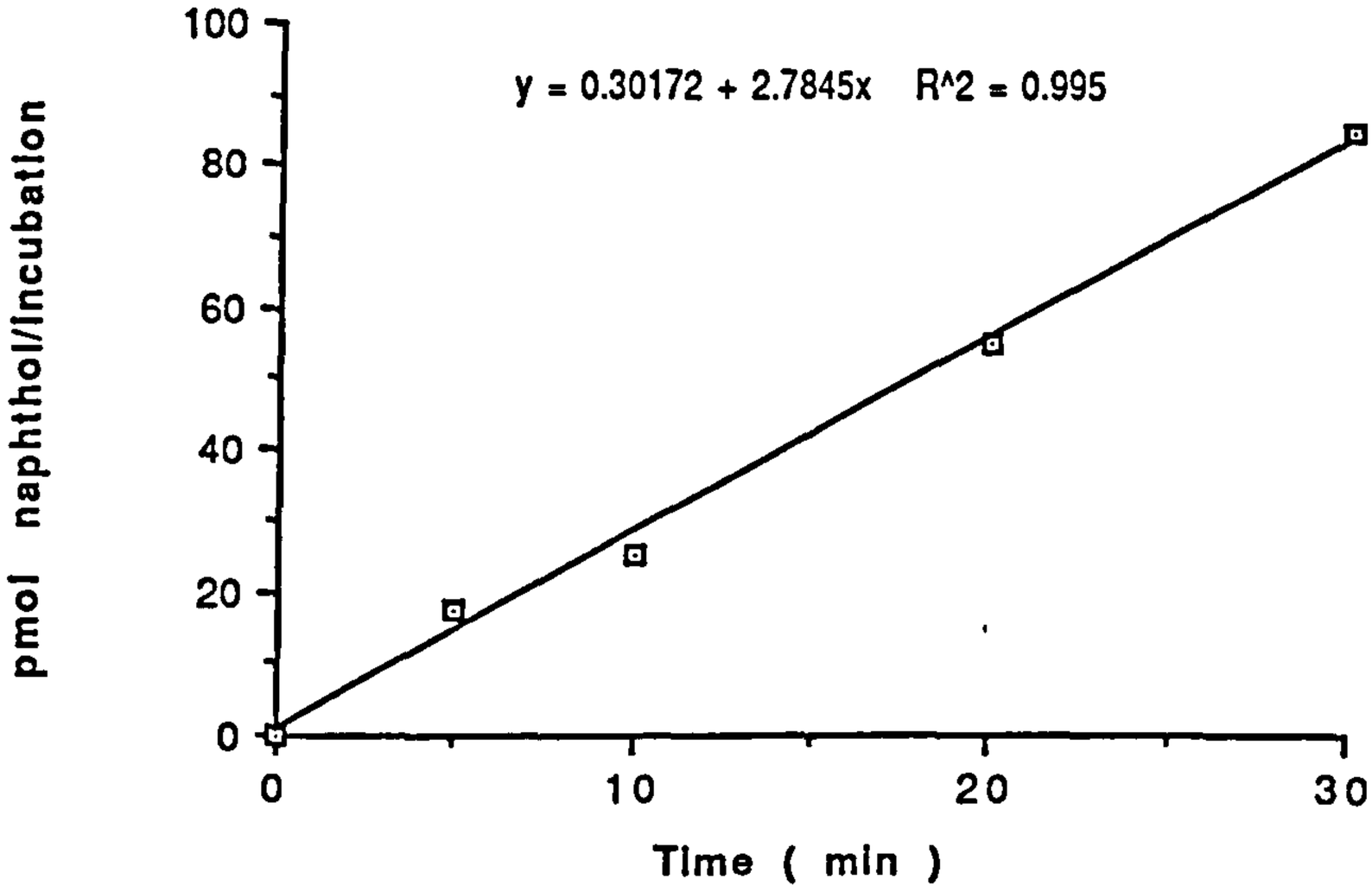


Figure 9.3.5.

Carbaryl (25μM) hydrolysis to 1-naphthol (pmol/incubation) by rat liver microsomes (⊡) and cytosol (•) (5mg/incubation) for varying times of hydrolysis .
Each point represents a mean of duplicate determinations.



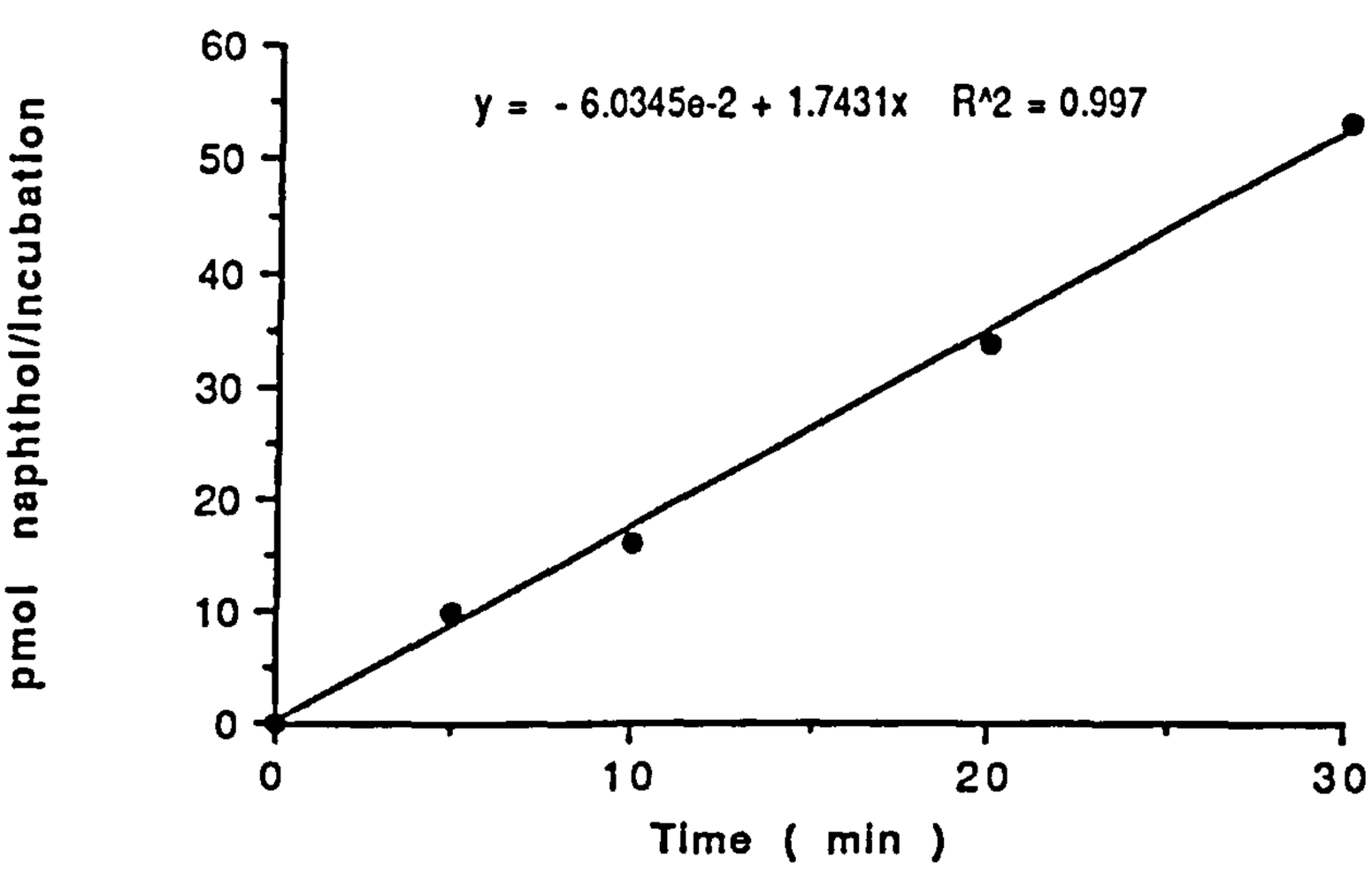


Figure 9.3.6.

Carbaryl (25μM) hydrolysis to 1-naphthol (pmol/incubation) by rat lung microsomes (⊡) and cytosol (•) (10mg/incubation) for varying times of hydrolysis.
Each point represents a mean of duplicate determinations.

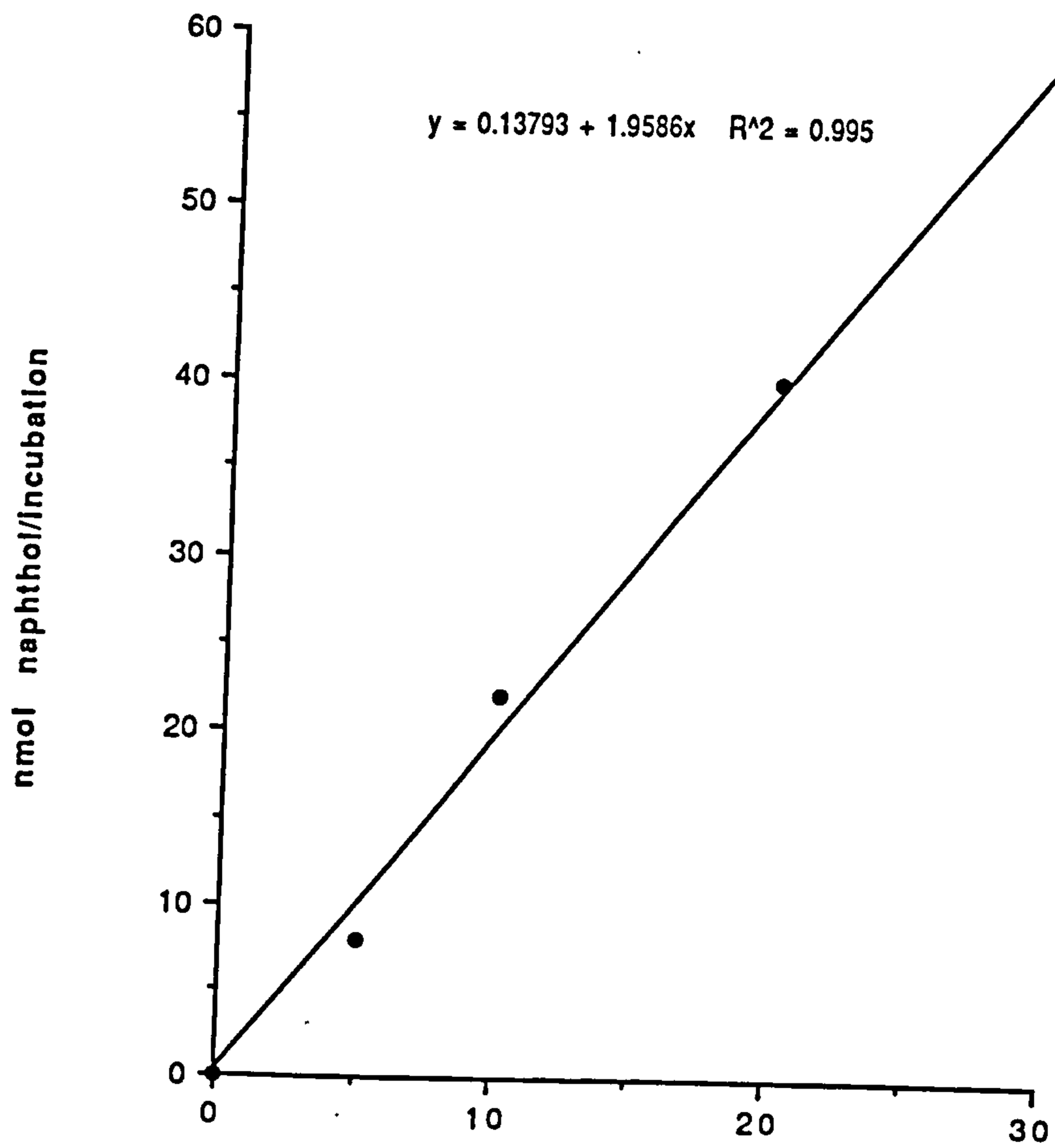


Figure 9.3.7.

Carbaryl (25μM) hydrolysis to 1-naphthol (pmol/incubation) by rat skin microsomes ( ) and cytosol (•) (30mg/incubation) for varying times of hydrolysis .
Each point represents a mean of duplicate determinations.

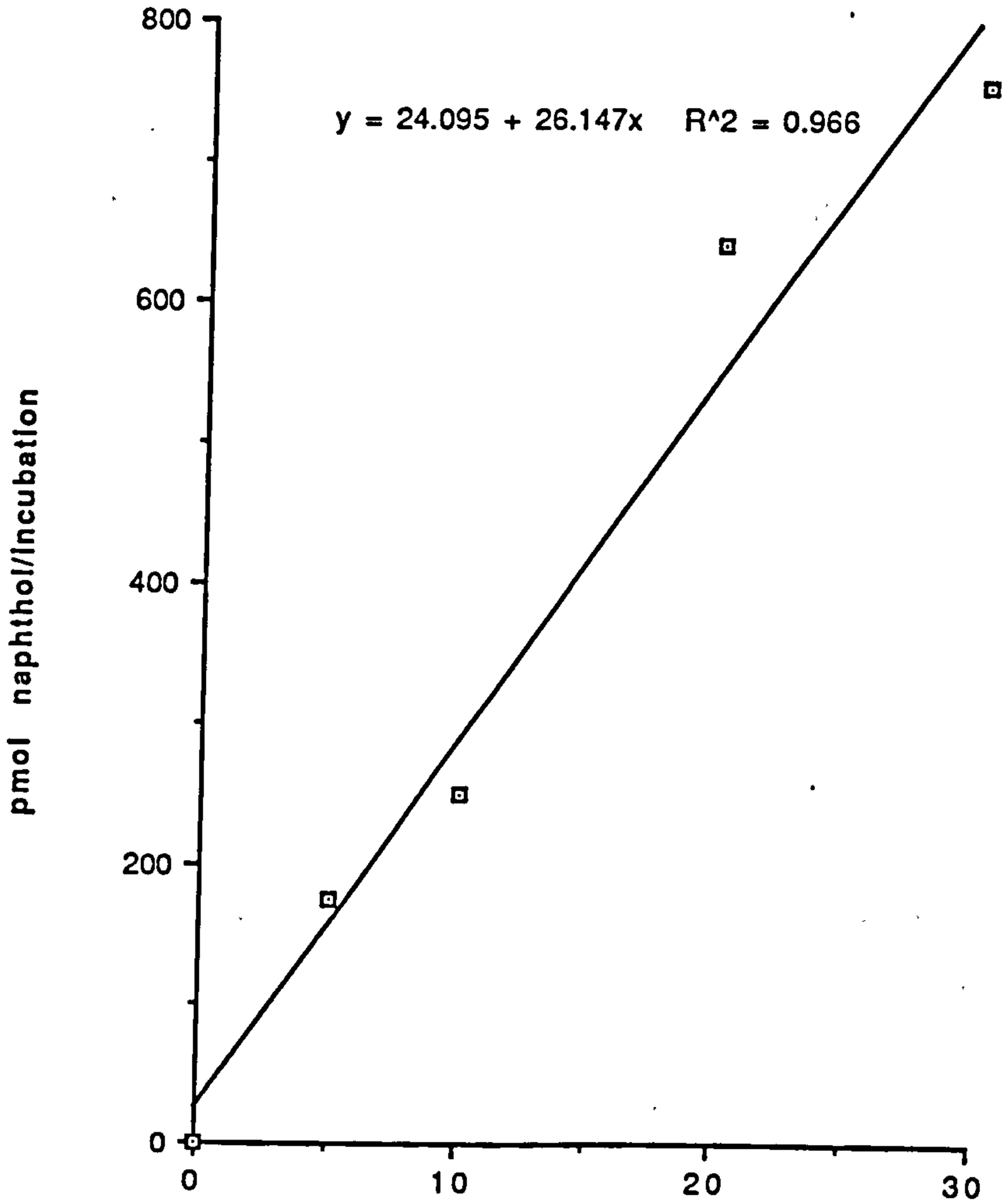


Figure 9.3.8.

Carbaryl (25μM) hydrolysis to 1-naphthol (pmol/incubation) by rat plasma (20μl/incubation) for varying times of hydrolysis .

Each point represents a mean of duplicate determinations.

liver, lung, skin and plasma with constant protein and carbaryl concentration.

The results for the spontaneous hydrolysis of carbaryl can be seen on Table 9.1. The sensitivity of the assay was 20pmol.

9.3.iii. HPLC separation of Carbaryl and 1-Naphthol

A Spherisorb ODS (5µ, 15cm) C18 reverse phase HPLC column was used for the carbaryl assay with a mobile phase of water / acetonitrile (60/40) at a rate of 1.4ml/min, pumped by a Waters 510 HPLC pump. 1-naphthol was measured in the eluent using a fluoresence spectrophotometer (Perkin-Elmer 3000) set at an excitation wavelength of 296nm and emission wavelength of 460nm. The retention time of 1-naphthol was 6.2 minutes (see Figure 9.3.10.).

A calibration curve for 1-naphthol was established. 1-Naphthol standards (0-2nmoles) in 15µl methanol was added to eppendorf tubes and the methanol was evaporated off under nitrogen. Incubations were carried out in a final volume of 1ml 0.1M phosphate buffer ph7.25. 500µl of absolute ethanol was mixed to the incubation and this was injected onto the column without neutralization to enable the calculation of 1-naphthol (see Figure 9.3.9.).

Table 9.1. Spontaneous hydrolysis of carbaryl

Substrate concentration (μM)	Activity (pmol/incubation)
5	28±5
10	60±7
25	132±10
50	372±25

mean±SEM (n=2)

limit of sensitivity > 0.3nmol/min

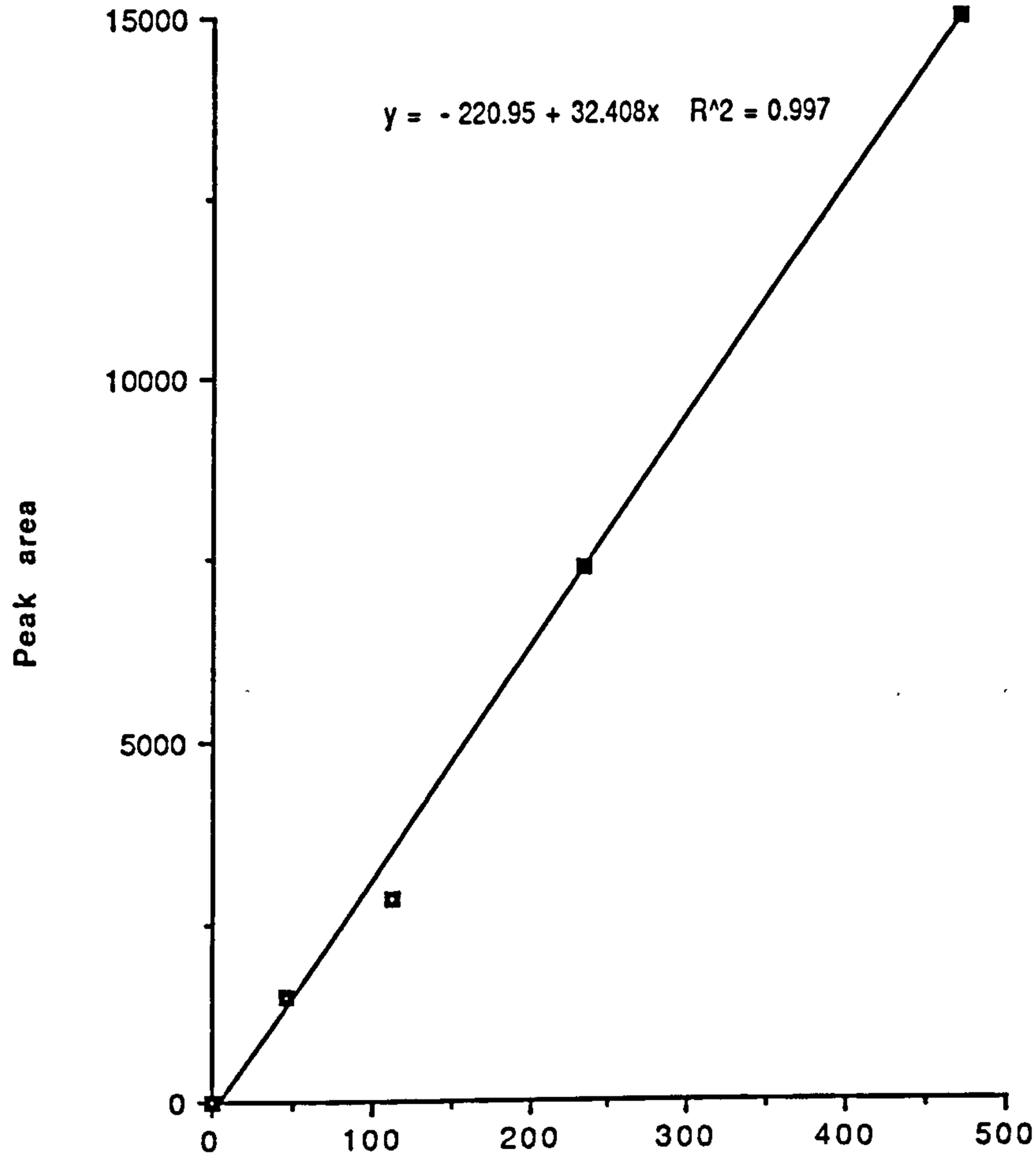


Figure 9.3.9.

A typical standard curve of 1-naphthol following direct injection (50µl) of standards (0-500pmol).

Each point represents a mean of duplicate determinations.

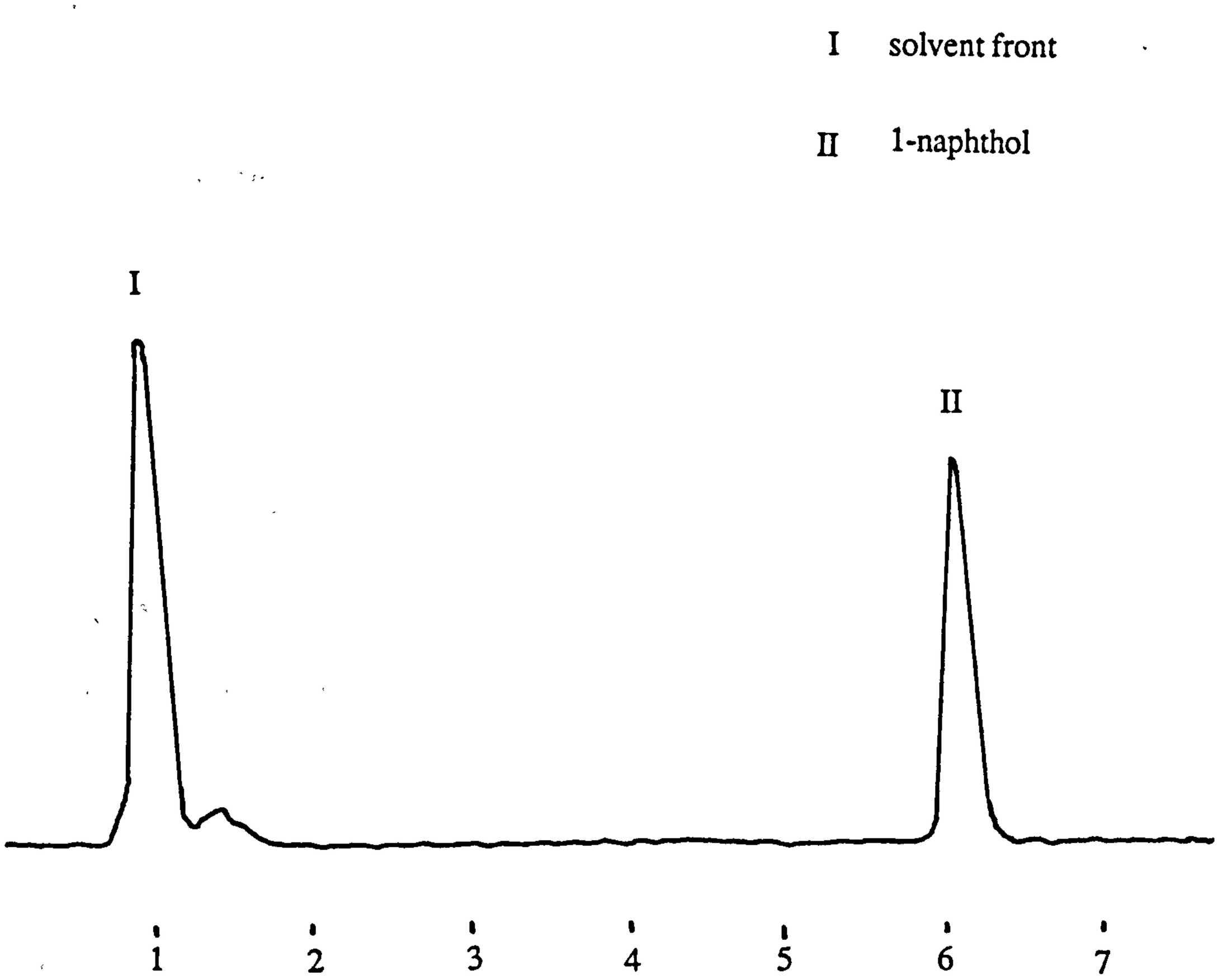


Figure 9.3.10 Typical chromatogram showing seperation of 1 naphthol by reverse phase HPLC, using a mobile phase of 40:60 acetonitrile: water pumped at a rate of 4ml/minute. Detection is by fluorescence spectrophotometry at an excitation wavelength of 296nm and an emission wavelength of 460nm.

9.4. PARAOXONASE

9.4.i. Chemicals

Paraoxon [0,0-diethyl-0- (4-nitrophenyl) phosphate] has an empirical formula of $C_{10}H_{14}NO_6P$ and a molecular weight of 275.2 . p-Nitrophenol has an empirical formula of $C_6H_5NO_3$ and a molecular weight of 139.1 . Both chemicals were obtained from Sigma Chemical Company.

9.4.ii. Incubation

Microsomal and cytosolic protein, between 0.1-30mg of liver, lung and skin, original wet weight, or 10-50µl of plasma or red blood cells was incubated along with paraoxon (0.05-1mM). Incubations were carried out in a final volume of 500µl of 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C.

Reactions were started by the addition of paraoxon (5-50µl of 10mM stock , in freshly prepared trisma buffer) and stopped with the addition of 500µl of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of supernatant was injected onto reverse phase HPLC. Control incubations containing 50mM trisma buffer and paraoxon were conducted in parallel to assess spontaneous hydrolysis.

Using a fixed concentration of paraoxon and time of incubation, varying amounts of microsomal protein from liver (see Figure 9.4.1.), lung (see Figure 9.4.2.) and skin (not detected) were used in the incubations to establish conditions of linearity for protein. The amount of plasma was also varied, with a constant time and paraoxon

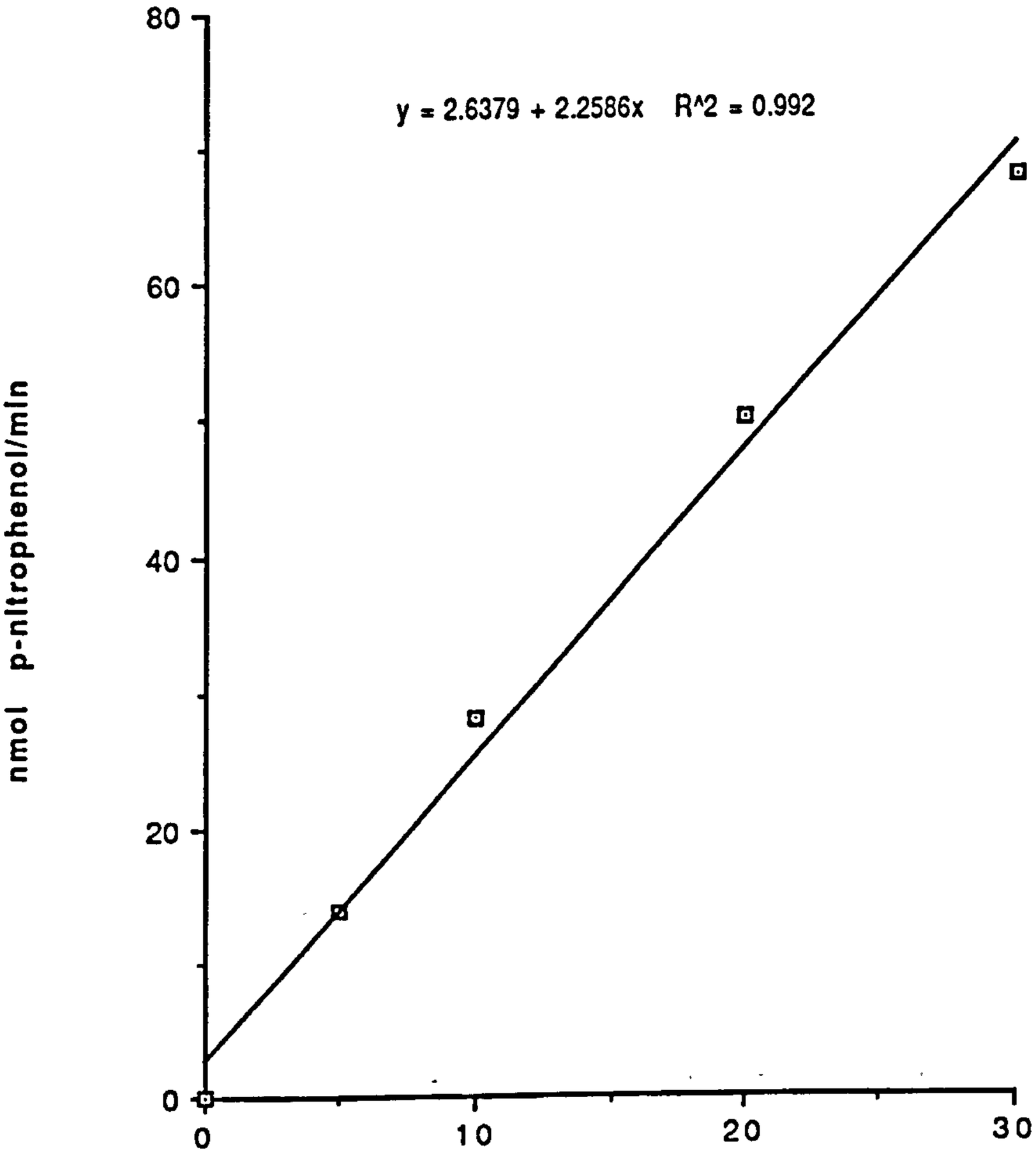


Figure 9.4.1.

Rate of paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/minute) in the presence of varying concentrations of rat liver microsomes (⧈) (0-30 mg/incubation).
Each point represents a mean of duplicate determinations.

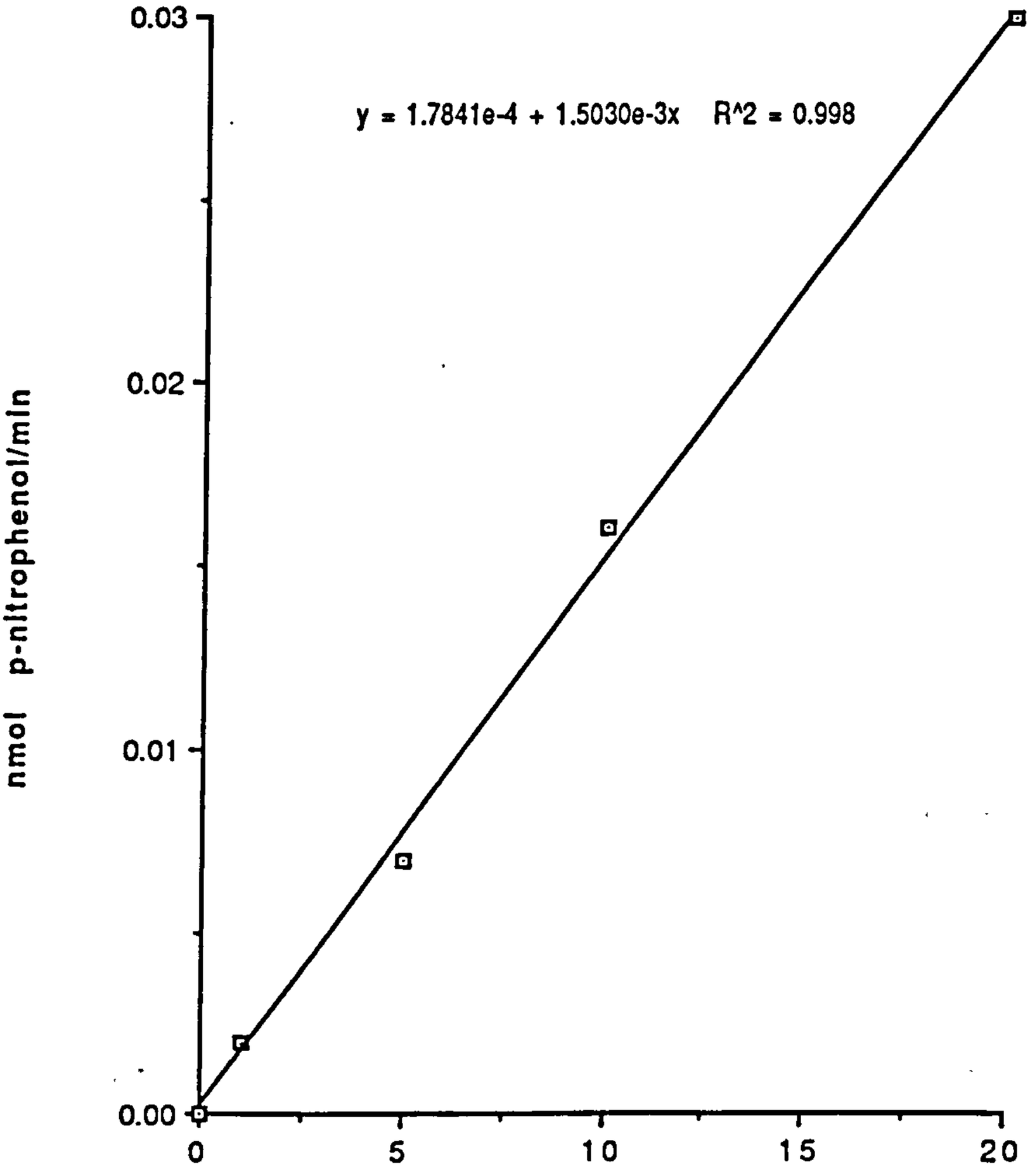


Figure 9.4.2.

Rate of paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/minute) in the presence of varying concentration of rat lung microsomes (⊡) (0-20 mg/incubation).
Each point represents a mean of duplicate determinations.

concentration (see Figure 9.4.3.). Having established a fixed amount of protein for the incubation the time of hydrolysis was varied (0-30 minutes) with a fixed paraoxon concentration, to establish conditions of linearity for time for the following tissues: liver (see Figure 9.4.4.); lung (see Figure 9.4.5.); skin (not detected) and plasma (see Figure 9.4.6.).

The conditions for linearity for protein were 0.5, 20 and 30mg for liver, lung and skin original wet weight or 10µl of plasma with constant time (10 mins) and paraoxon concentration (1mM). The conditions of linearity for time were 15 minutes for liver, lung, skin and plasma with constant protein and paraoxon concentration.

The results for the spontaneous hydrolysis of paraoxon can be seen on Table 9.2. The sensitivity of the assay was 0.5nmol/incubation.

9.4.iii. HPLC separation of Paraoxon and p-Nitrophenol

A Kontron system with dual wavelength UV detection fitted with a Spherisorb ODS (5µ, 15cm) C18 reverse phase HPLC column was used for the paraoxon assay, with a mobile phase of orthophosphoric acid (0.072% w/v) / methanol (45/55) at a flow rate of 1ml/min. Paraoxon and p-nitrophenol was measured at 330nm, with internal standard being measured at 250nm. The retention times: p-nitrophenol 4.5 minutes; internal standard 6.4 minutes and paraoxon 10 minutes (see Figure 9.4.9.).

Calibration curves for paraoxon and p-nitrophenol were established. Paraoxon and p-nitrophenol standards (0-120µM final concentration) mixed with an equal volume of perchloric acid was injected onto the column (80µl) without neutralisation to enable the calculation of p-nitrophenol (see Figure 9.4.7.) and paraoxon (see Figure 9.4.8.).

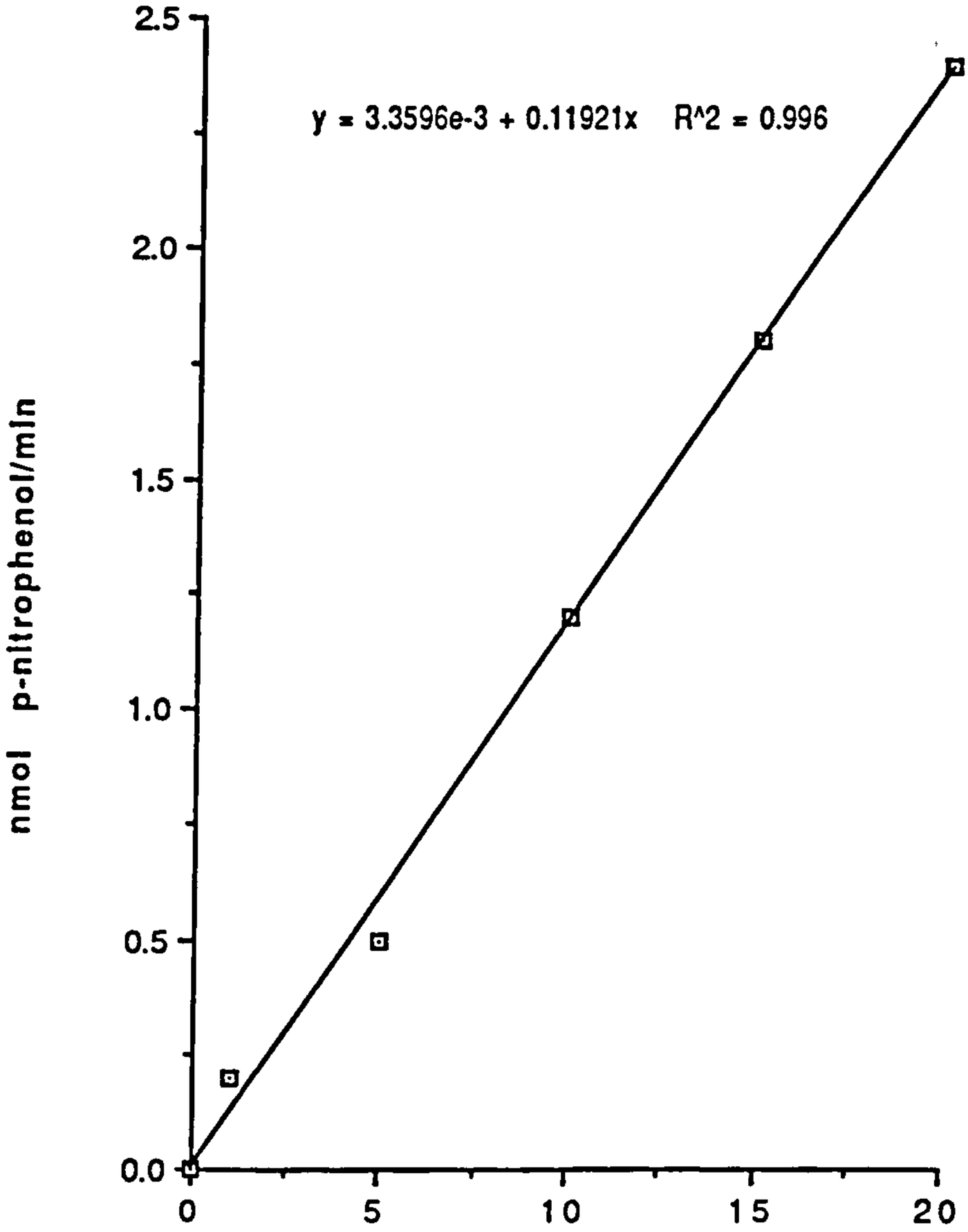


Figure 9.4.3.

Rate of paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/minute) in the presence of varying amount of rat plasma (0-20 µl/incubation).
Each point represents a mean of duplicate determinations.

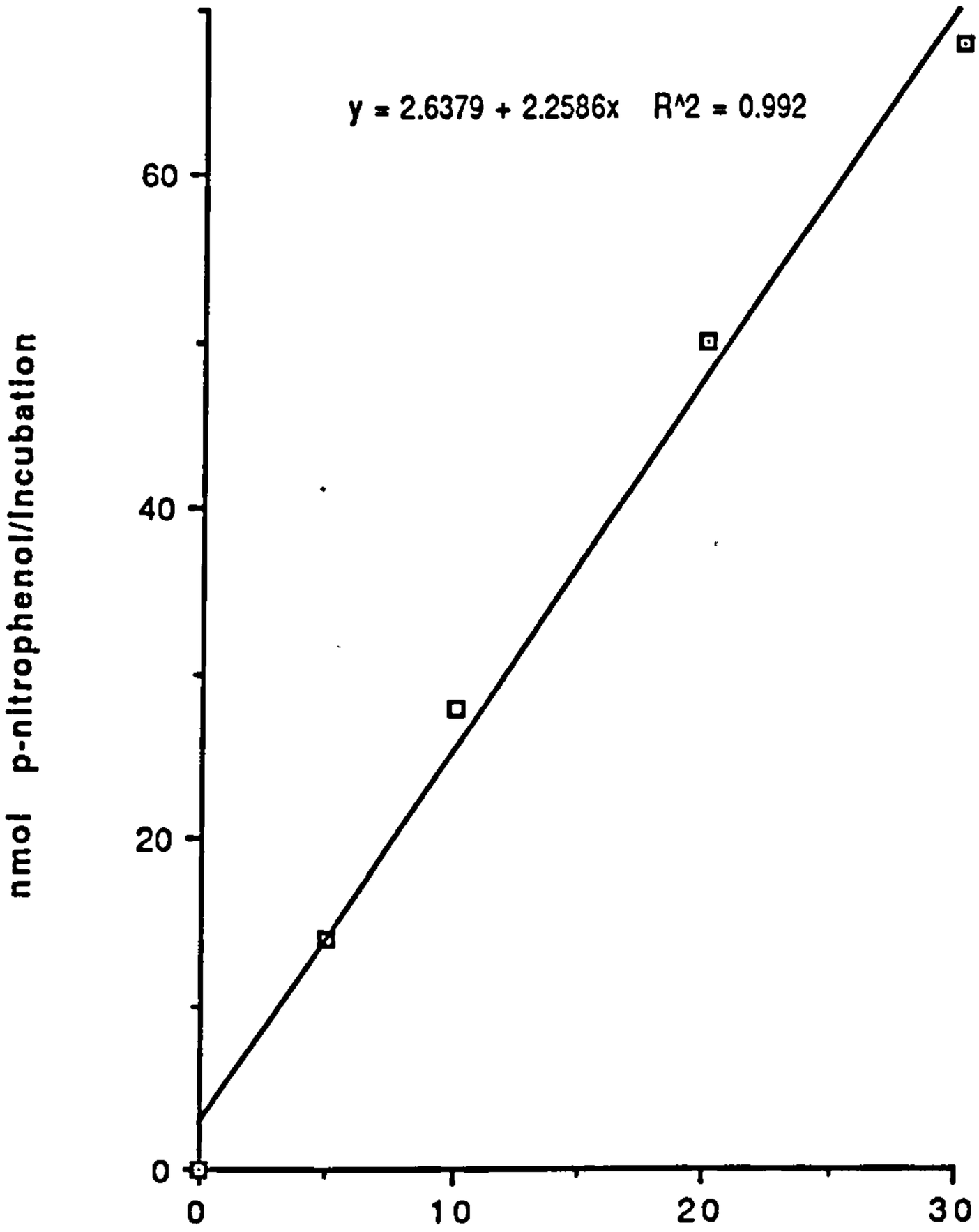


Figure 9.4.4.

Paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/incubation) by rat liver microsomes (⧈) (0.5mg/incubation) for varying times of hydrolysis .
Each point represents a mean of duplicate determinations.

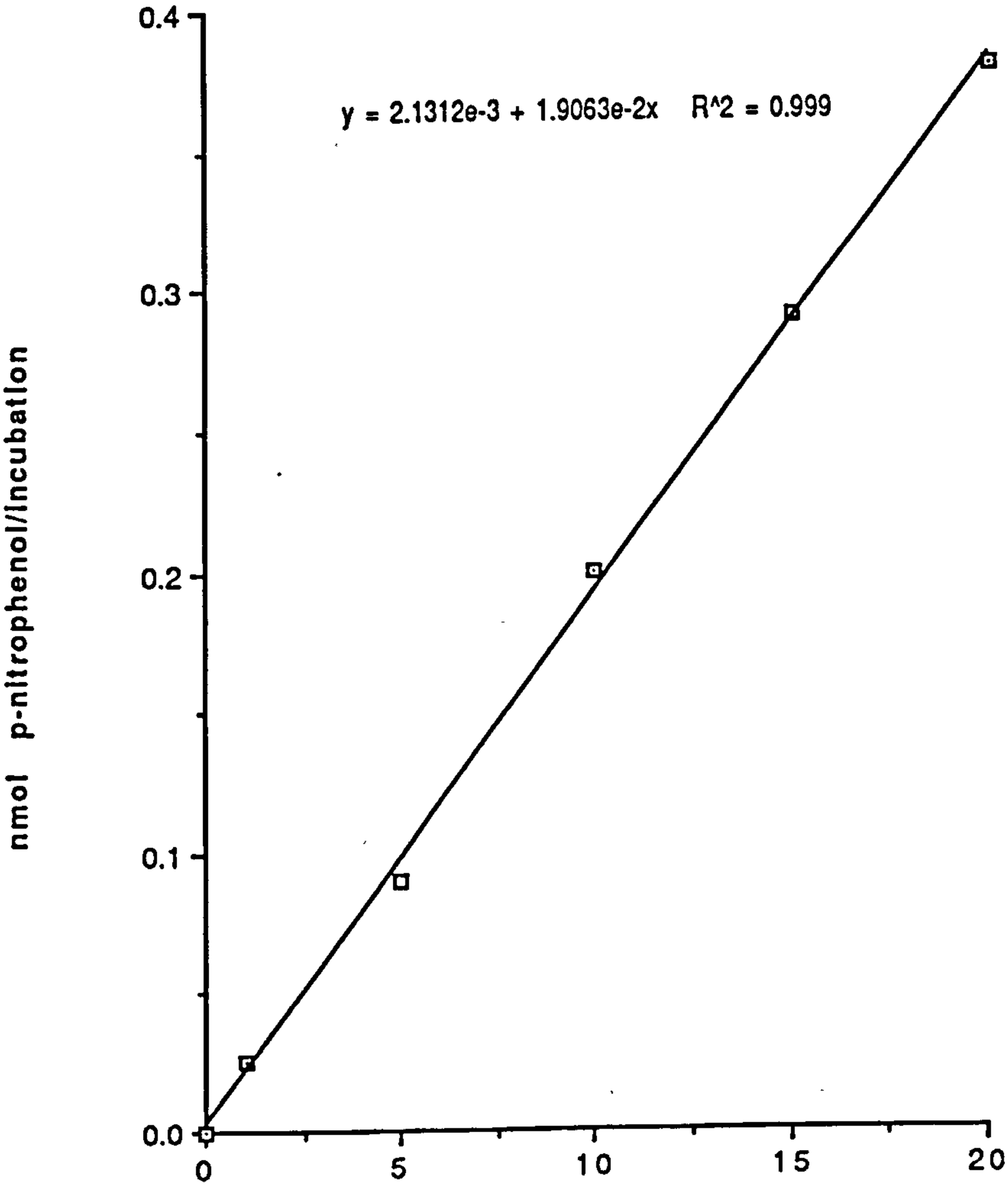


Figure 9.4.5.

Paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/incubation) by rat lung microsomes (⊡) (20mg/incubation) for varying times of hydrolysis .
Each point represents a mean of duplicate determinations.

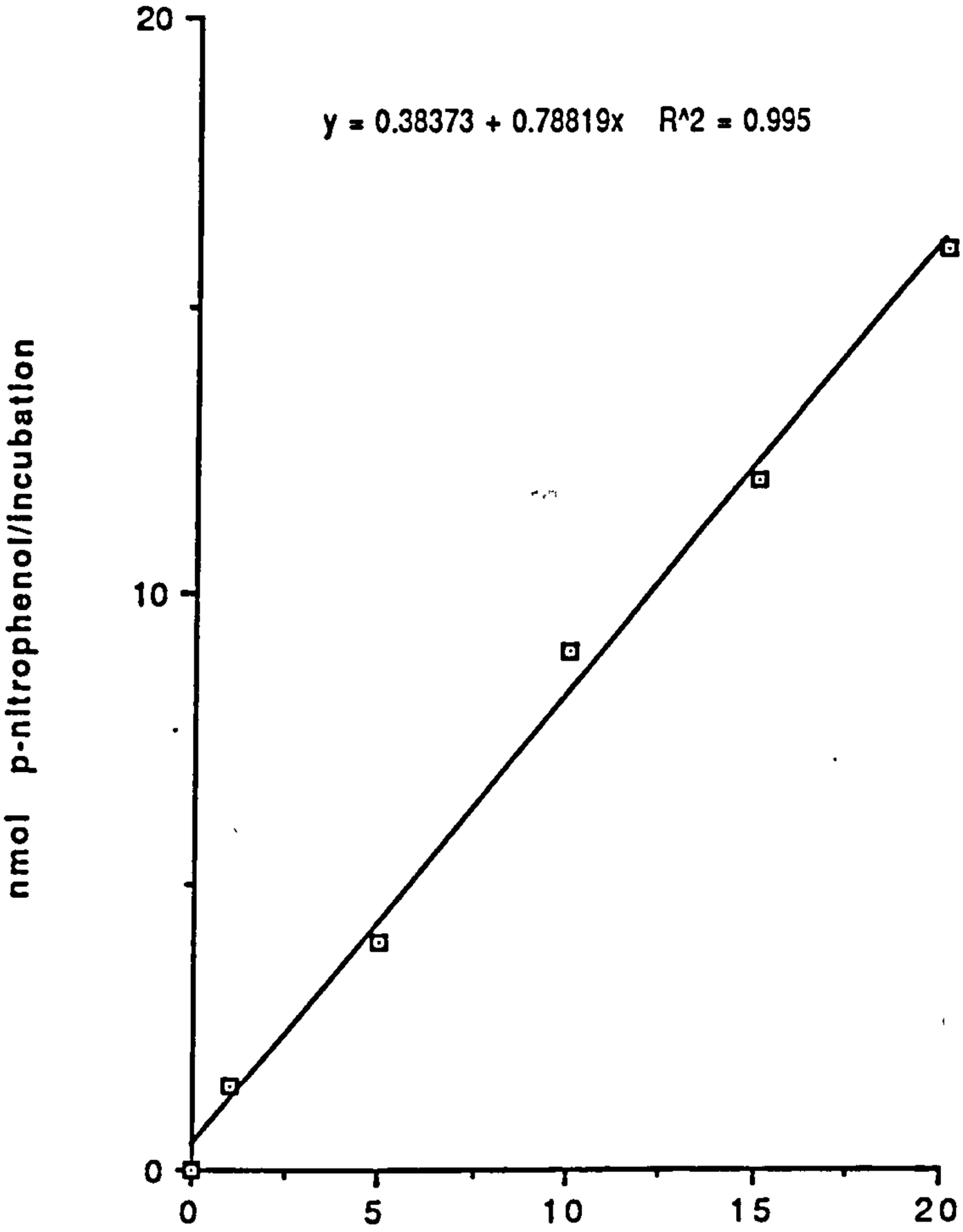
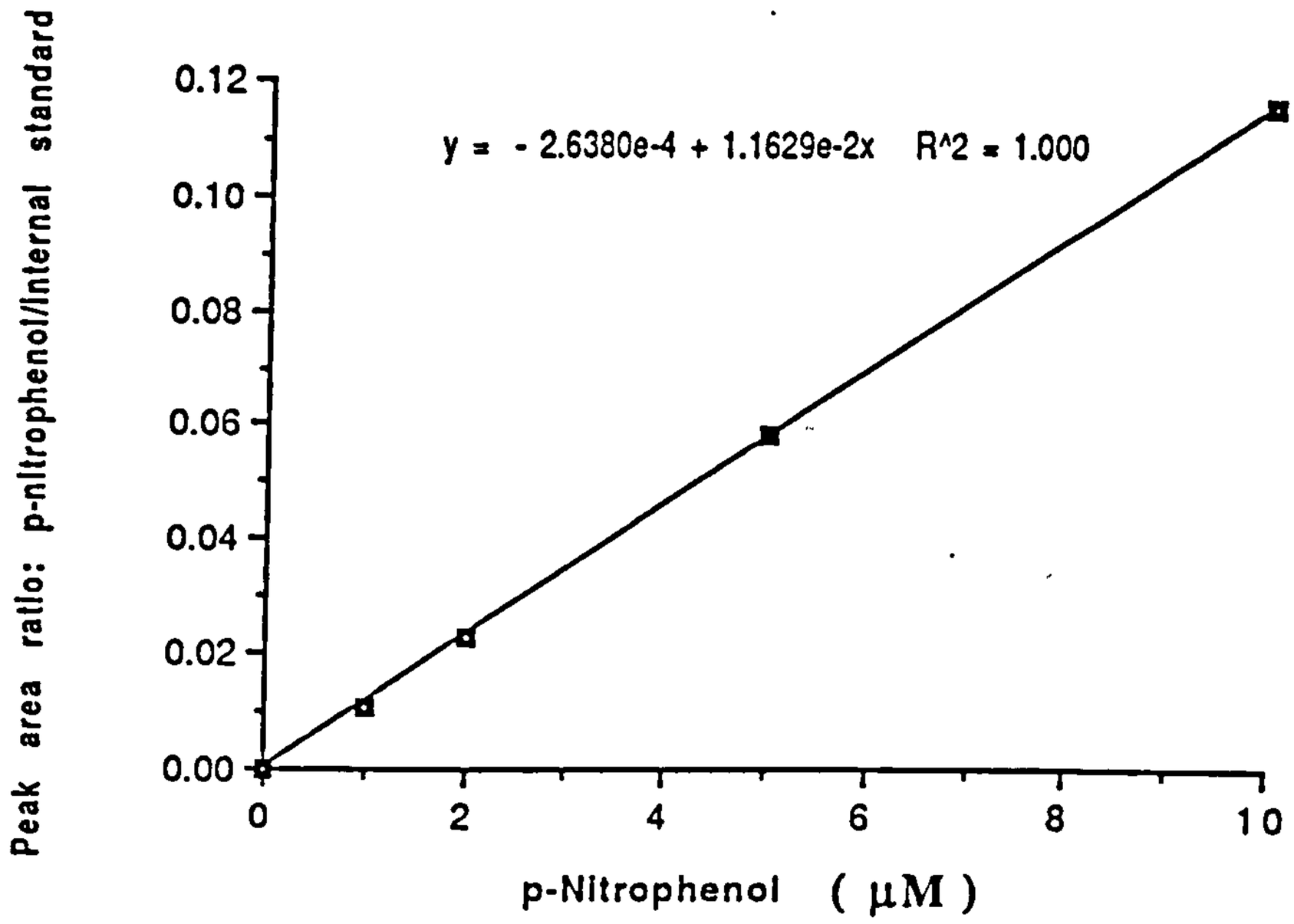


Figure 9.4.6.

Paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/incubation) by rat plasma (10µl/incubation) for varying times of hydrolysis .

Each point represents a mean of duplicate determinations.



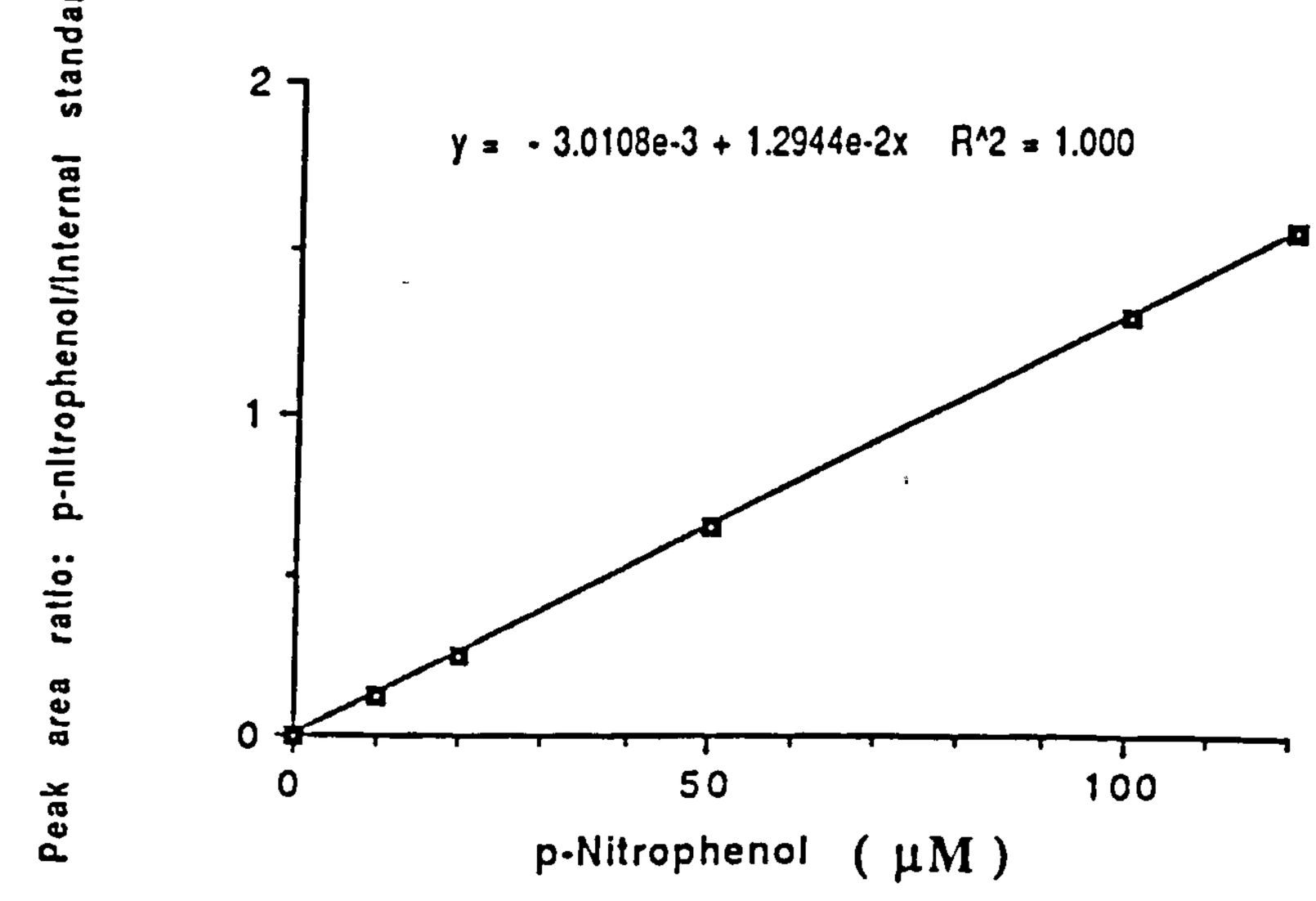


Figure 9.4.7.

A typical standard curve of p-nitrophenol following direct injection (80µl) of standards (0-10µM and 0-120µM).

Each point represents a mean of duplicate determinations.

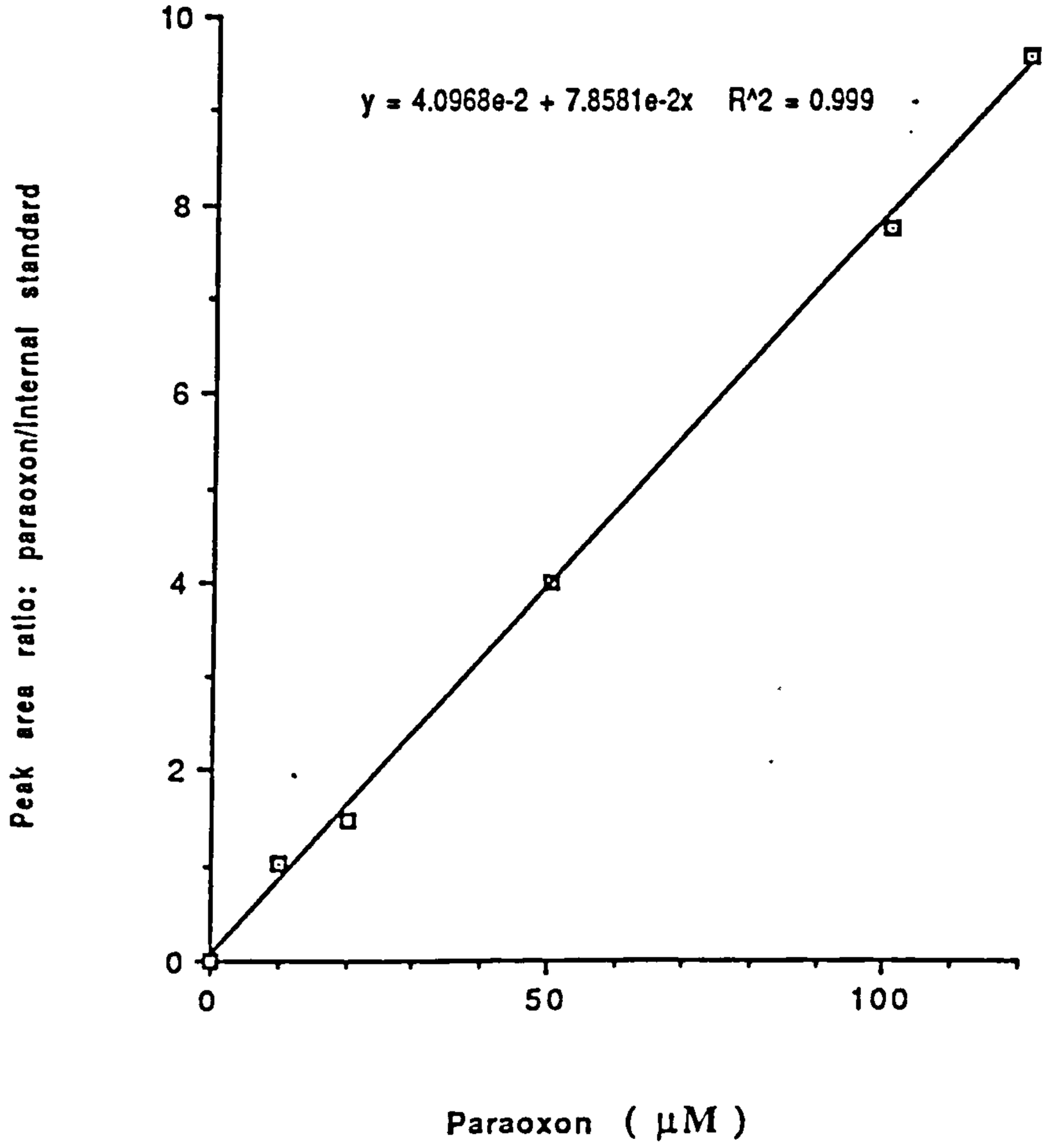


Figure 9.4.8.

A typical standard curve of paraoxon following direct injection (80µl) of standards (0-120µM).

Each point represents a mean of duplicate determinations.

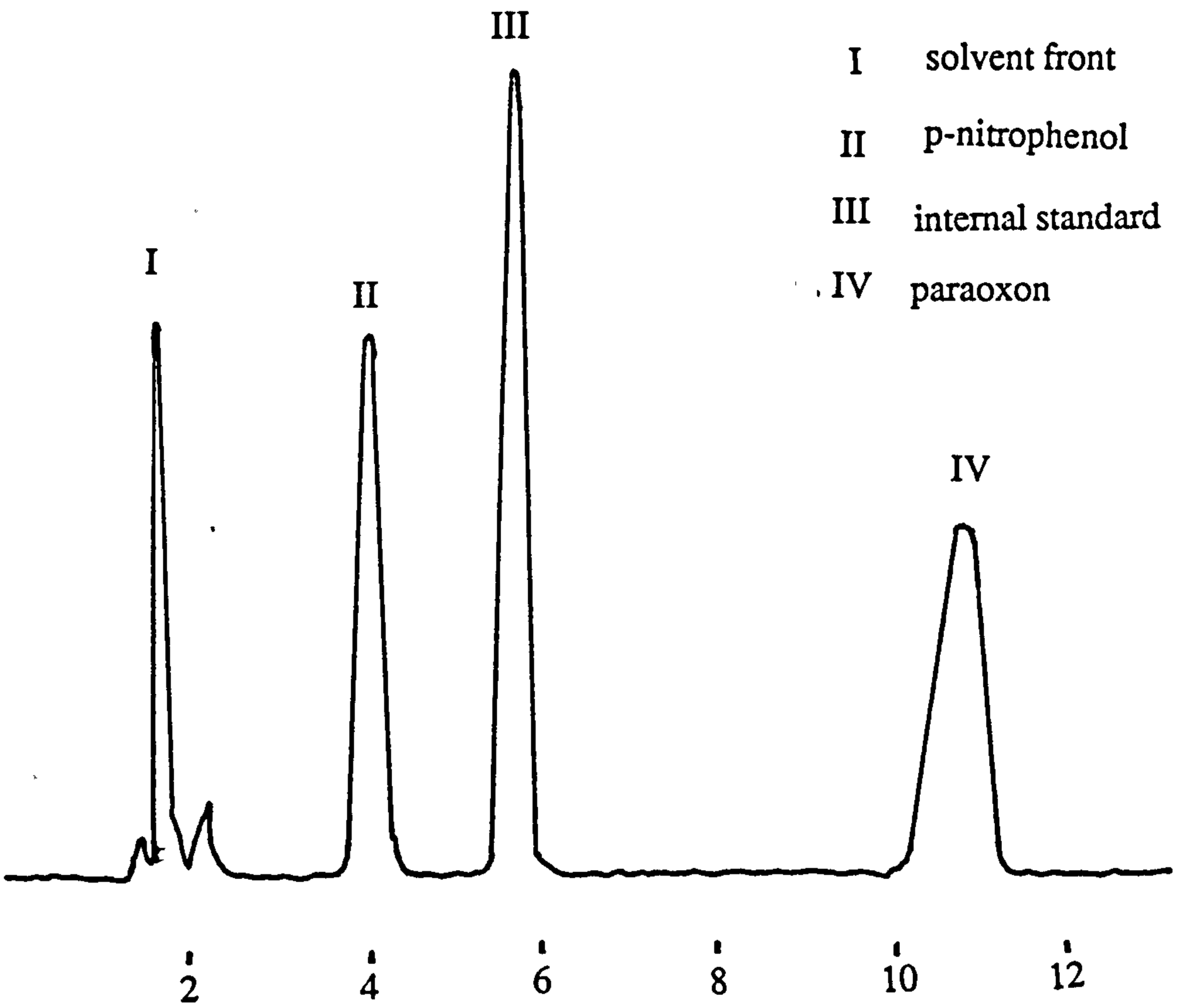


Figure 9.4.9. Typical chromatogram showing seperation of paraoxon, p-nitrophenol and internal standard by reverse phase HPLC, using a mobile phase of 50:50 methanol: orthophosphoric acid (0.072w/v) pumped at a rate of 1ml/minute. Detection is by UV spectrophotometry at a wavelength of 250nm for paraoxon and internal standard and 310nm for p-nitrophenol.

Table 9.2. Spontaneous hydrolysis of paraoxon

Substrate concentration (μM)	Activity (nmol/min)
20	0.53±0.02
50	1.10±0.03
80	1.90±0.1
1000	2.5±0.15

mean±SEM (n=2)

limit of sensitivity > 0.3nmol/min

9.5. PHENYLACETATE ESTERASE

9.5.i. Chemicals

Phenyl acetate (empirical formula $C_8H_8O_2$ and molecular weight 136.1) and phenol (empirical formula C_6H_6O and molecular weight 94.1) were obtained from Sigma Chemical Company.

9.5.ii Incubations

The phenylacetate method of Kitchen et al, (1983) was used in the experiment. Microsomal and cytosolic protein, between 0.1-30mg of liver, lung and skin, original wet weight or 10-50µl of plasma or red blood cells was incubated along with phenylacetate (0.05-1mM final concentration). Incubations were carried out in a final volume of 3mls of 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C. Reactions were started by the addition of phenylacetate. The cuvettes were inverted and placed in the spectrophotometer. Control incubations containing 50mM trisma buffer, but no tissue fraction were conducted in parallel.

The amount of protein or plasma was varied until a linear plot was obtained over 3 minutes. The reason for this was to stop all the hydrolysis of the phenylacetate by the arylesterase enzyme taking place at the begining of the reaction.

The conditions for linearity for protein were 2, 20 and 30mg for liver, lung and skin original wet weight or 10µl of plasma).

The results for the spontaneous hydrolysis of phenylacetate can be seen on Table 9.3. The sensitivity of the assay was 0.25µmol/incubation.

9.5.iii. Monitoring of Phenol

The formation of phenol was monitored continuously at a wavelength of 272nm on a Kontron UV spectrophotometer with temperature control, set at 37°C. Measurements were converted from absorbance units / min to nmoles phenol / min / cuvette by the absorption coefficient for phenol.

Table 9.3. Spontaneous hydrolysis of phenylacetate

Substrate concentration (mM)	Activity (nmol/min)
0.4	3.4±0.5
1.0	6.1±0.6
2.0	11.4±0.8
3.0	17.5±1.0
4.0	24.2±1.6

mean±SEM (n=2)

limit of sensitivity > 0.23nmol/min

SECTION III : EXPERIMENTAL STUDIES

CHAPTER 10: PRELIMINARY STUDIES OF ESTER HYDROLYSIS IN RAT

10.1. INTRODUCTION

Many studies of esterase activity have been restricted to the microsomal fraction of the cell. However, recent studies have indicated, for a number of substrates that esterases are also present in the cytosol. The subcellular distribution of esterases involved in the hydrolysis of the pesticides of interest has not been defined.

Preliminary experiments were therefore carried out using the substrate phenylacetate (PhAc) to determine the distribution of phenylacetate esterase in the microsomal and cytosolic fractions of a number of tissues from the rat.

10.2. SUBCELLULAR DISTRIBUTION OF ESTERASE

10.2.i. Distribution of phenylacetate esterase during subcellular fractionation of rat liver

The following experiment was designed to determine the distribution of phenylacetate esterase activity in the subcellular fractions of the rat liver as well as to establish enzyme assay reproducibility within a single rat tissue. Esterase values in the following liver tissue fractions were examined: post mitochondrial , microsomes, cytosol and pre-mitochondrial sediment (containing mitochondria, nuclear fraction and cell wall debris). Esterase activity was examined in the pre-mitochondrial sediment of the rat tissue to establish how much activity, if any is trapped when the mitochondria, nuclear fraction and cell wall debris are initially spun down when preparing the post-mitochondrial fraction from the tissue homogenate.

Methods

Liver tissue was obtained from one male Wistar rat (190g). Four 1g portions of liver were taken from the same rat liver. Homogenates and subcellular fractions were prepared as previously described (see chapter 7).

In all cases 1mg original wet weight of liver was taken from each of the fractions and incubated with a final concentration of 0.4-4mM phenylacetate. Incubations were in a final volume of 3ml in 0.1M trisma buffer ph8.0, containing 0.1mM calcium chloride at 37^{o}C, in a cuvette. Reactions were started with the addition of phenylacetate (5µl-20µl of 600mM phenylacetate in DMSO). Cuvettes were inverted and then placed in the spectrophotometer.

Results were expressed in µmol/min/g wet weight of tissue. Vmax and Km values were calculated by double reciprocal plot for liver sample. To determine the inter-assay variability of phenylacetate esterase in the subcellular fractions the coefficient of variation, over a range of substrate concentrations was were determined. This involved calculating the mean and standard deviation for the 4 liver samples at each of the substrate concentrations used in the experiment. Having calculated the coefficient of variation for the 4 liver samples at each of the substrate concentrations, these values were then the meaned. These calculations were carried out for each of the subcellular fractions.

Results

Results show that phenylacetate esterase is distributed in both microsomal and cytosolic fractions of the liver. In the liver it can be seen that 90% of the post-mitochondrial fraction esterase activity is due to the microsomal fraction, with the other

10% of esterase activity being found in the cytosol. High phenylacetate esterase activity was also seen in the pre-mitochondrial sediment. The apparent Km's were found to be similar in the post-mitochondrial and microsomal fractions, whereas it was found to be lower in the cytosolic and pre-mitochondrial fractions.These results can be seen on Table 10.1.

Coefficients of variation were carried out to determine the inter-assay variability for each of the tissue fractions. Coefficient of variations were found to be lower in the post-mitochondrial and cytosolic fractions and higher in the microsomal and pre-mitochondrial sediment fractions. These can be seen on Table 10.1.

Discussion

The results show that most of the phenylacetate esterase activity in the liver is found in the microsomal fraction of the cell, with the cytosol having a 1/10 of the activity of the microsomes. It was also found that there was a great amount of phenylacetate esterase activity in the pre-mitochondrial fraction of the liver samples, indicating that a lot of the esterase activity from the microsomal or cytosolic esterase enzymes was being trapped down with the initial spins to produce the post-mitochondrial fraction.

Apparent Km's seem to vary between tissue fractions. The apparent Km for microsomal arylesterase is more similar to the homogenate Km than the Km for cytosol. This is not surprising considering the majority of arylesterase activity is found in the microsomal fraction. The Km value of the pre-mitochondrial fraction cannot really be compared to the other Km values because incorporated into the fraction are a combination of both microsomal and cytosolic esterase enzymes.

Table 10.1. The hydrolysis of phenylacetate to phenol by rat liver post-mitochondrial, microsomal, cytosolic and pre mitochondrial sediment fractions. Results were expressed at the Vmax (μmol/min/g wet weight tissue) and the apparent Km (mM) by the double reciprocal plot.

Tissue fraction	Vmax (μmol / min / g)	K_m (mM)	CV for assay (%)
Post-mitochondrial	949±28	1.92±0.06	3.2
Microsomes	780±52	1.33±0.04	14.5
Cytosol	101±3.2	0.40±0.04	2.7
Pre-mitochondrial	518±88	0.93±0.2	18

mean±SEM (four 1g portions from same rat)

CV: coefficient of variation.

In using the experimental assay on one single day basis there will be a 3.2%, 14.5% and a 2.7% difference in phenylacetate esterase activities in the tissue post mitochondrial fraction, microsomes and cytosol respectively in liver tissue. The results also show that a lot of phenylacetate esterase activity is in fact trapped when the tissue debris and mitochondria are spun down. A variation of esterase activity in the order of 18% between assays was seen. This result could go to explain why there is a 14% difference in arylesterase activities seen in the microsomal fraction, indicating that a proportion of microsomal esterase is being trapped down on the first spin.

10.2.ii. NADPH cytochrome c(P450) reductase

NADPH cytochrome c(P450) reductase was also measured in these studies as a marker for microsomal protein contamination. Measurements were carried out to determine whether there was any microsomal contamination of the cytosolic fraction when the subcellular fractions are spun down from tissue homogenate, to eliminate microsomal contamination as a source of cytosolic esterase activity.

Method

Liver and lung was obtained from 4 male Wistar rats (190g). Homogenates were prepared in 50mM trisma buffer, pH 7.4 and from this microsomal and cytosolic fractions were prepared as previously described.

The method of Gibson and Skett, (1986) was used in the experiment. 50µl of the rat microsomal fraction from liver (0.28mg protein) and lung (0.1mg protein) were incubated with 250µl of cytochrome c (50mg to 10mls water) and 2.15mls of 0.1M trisma buffer pH 7.4, in a 3 mls cuvette. This was run in parallel with 50µl of the rat cytosolic fraction from liver (0.34mg protein) and lung (0.26mg

protein). 50µl of cytosol was incubated with 250µl of cytochrome c and 2.15 mls of 0.1M trisma buffer pH 7.4, in a 3 mls cuvette.

Reactions were started with the addition of 2% NADPH (10mg to 0.5ml H_2O, prepared fresh) solution. The cuvettes were inverted and placed in a UV Kontron spectrophotometer and the rate of cytochrome c reduction was measured.

Results

The rate of cytochrome c reduction was expressed in nmol/min/mg of microsomal protein (see Table 10.2.). From the results it can be seen that cytosolic NADPH cytochrome c reductase was 4% of the microsomal value in the liver and 3.3% for the lung.

Discussion

Results suggest the esterase activities found in the cytosolic fraction of the tissues studied previously is in fact as a result of cytosolic esterase activity, rather than as a result of microsomal esterase contamination of the cytosolic fraction.

10.2.iii. Microsomal and cytosolic esterase distribution in rat tissues

Having already established that phenylacetate esterase activity is distributed in the microsomal and cytosolic fractions of the rat liver, other tissues in the rat were studied to determine the localisation of phenylacetate esterase activity in the rat.

Table 10.2. The NADPH cytochrome c(P450) reductase activity in rat liver and lung microsomal and cytosolic fractions. Results were expressed in μmol/min/mg tissue protein.

	Tissue	NADPH cytochrome c reductase activity (μmol / min / mg protein)
Microsomes	Liver	52.4±26
	Lung	32.018
Cytosol	Liver	2.0±0.6
	Lung	0.5±0.1

mean±SEM (n=4)

Methods

Liver, lung, kidney, skin, brain, muscle and gut were removed from the same rat. 1g portions of each tissue were homogenized in 10ml of 0.1M trisma buffer. Microsomal and cytosolic fractions were prepared as previously described (see chapter 7). Microvilli where scrapped off from the 1g portion of the gut and used in the experiment.

Tissue homogenate equivalent to 1.5mg (kidney, lung, muscle and gut), 3mg (brain and liver) and 15mg of liver original wet weight was incubated with a final concentration of 0.4-4mM phenylacetate.Microsomal protein equivalent to 1.5mg (liver, kidney, lung and muscle), 15mg (skin and brain) and 5mg (gut) original wet weight and cytosol protein equivalent to 1.5mg (liver, kidney and lung) and 15mg (skin, muscle, brain and gut) original wet weight was incubated with a final concentration of 0.4-4mM phenylacetate. Incubations were carried out in a volume of 3mls of 50mM trisma buffer pH 8.0, containing 0.1mM calcium chloride, in a cuvette. Reactions were started by the addition of phenylacetate (5µl-20µl of 600mM phenylacetate in DMSO). The cuvettes were inverted and placed in the spectrophotometer. Phenol production was measured as previously described (see chapter 9.5.)

Results were expressed in µmol/min/g wet weight of tissue. Vmax and Km values were calculated by double reciprocal plot for the tissue samples.

Results

Post-mitochondrial, microsomal and cytosolic phenylacetate esterase activity were measured in all the tissues studied. Phenylacetate esterase was expressed at Vmax and can be seen on Table 10.3. The hydrolysis of phenylacetate in the post-

Table 10.3. The hydrolysis of phenylacetate to phenol by rat post-mitochondrial, microsomal and cytosolic fractions in the tissues of the liver, lung, kidney, skin, brain and gut. Results were expressed at the Vmax (μmol/min/g wet weight tissue or ml) and the apparent Km (mM) calculated from the double reciprocal plot.

	Tissue	Vmax (μmol / min / g)	K_m (μM)
Post-mitochondrial	Liver	947	1.96
	Kidney	52	0.65
	Lung	35	1.20
	Skin	17	2.63
	Muscle	4	2.10
	Brain	5	1.80
	Gut	181	1.50
Microsomes	Liver	865	2.4
	Kidney	24	0.62
	Lung	17	2.5
	Skin	13	0.65
	Brain	4	1.8
	Gut	0.9	1.2
Cytosol	Liver	102	2.2
	Kidney	17	0.34
	Lung	17	2
	Skin	12	0.4
	Brain	0.9	1.4
	Gut	154	1.96

mitocondrial fraction of the liver was 5 times that of gut, 18 times that of kidney, 27 times that of lung, 50 times that of skin, 200 times that of brain and 230 times that of muscle. Highest microsomal esterase activity was found in the liver and this was greater than 400 times that of the kidney. Esterase activity was similar in the skin and lung and lower in the brain and gut. In the cytosol the liver had the greatest amount of activity. The kidney, lung and skin cytosolic esterase activity was similar with lower activity in the brain. In the gut cytosol esterase activity was found to be very high. Vmax ratio's of esterase activity between the microsomal and cytosolic fractions were found to be different in the tissues studied. In the liver and brain most activity was found in the microsomes, whereas in the skin and gut most activity was found in the cytosol. In the lung and kidney there was equal distribution of esterase between the microsomes and cytosol.

Apparent Km's for the post-mitochondrial fractions varied between 2.6 to 0.65mM. The apparent Km's were found to be highest in the skin and similar in the liver, lung, skin and gut but lower in the kidney. The apparent Km's for the microsomal fractions varied between 2.4 and 0.65. There was a similar spread of Km values in the liver, lung, brain and gut and apparently lower in the skin and kidney. A similar spread of apparent Km's for cytosolic esterases was seen as in the microsomal fractions with similar values in the liver, lung, brain and gut and lower values in the skin and kidney. These results can be seen on Table 10.3.

Discussion

The hydrolysis of phenylacetate by phenylacetate esterase occurs mainly in the post-mitochondrial fraction of the liver, although significant amounts of hydrolysis occured in the extrahepatic tissues of the kidney, lung, skin, brain, muscle and the gut. The fact that most esterase activity is found in the liver is not surprising, considering the function of the liver is to metabolise exogenous and endogenous compounds in the body.

The gut arylesterase activity was high because of the contamination due to other enzymes, such as proteases, in the lumen of the gut. To be able to compare arylesterase activities with the other tissues trypsin would have to be incubated with the gut post-mitochondrial fraction. The trypsin would inhibit the protease enzymes but not the hydrolysis of phenylacetate.

Most reported information on esterase activity has been centred on the microsomal fraction of the tissue, whereas little is available on cytosolic esterase activity in different tissues of the rat. Results presented here show that there is indeed esterase activity in both the microsomal and cytosolic fraction of the tissue studied. In the case of the skin and gut the majority of arylesterase activity was found in the cytosolic fraction, rather than the microsomal fraction. In the gut the majority of activity in the cytosol would be as a result of proteases rather than esterase activity. Results from the tissues studied would seem to indicate that the cytosol fraction contains an appreciable amount of esterase activity. When considering the overall contribution of esterases to the hydrolysis of phenylacetate both the cytosol and microsomal fractions contribute.

Phenylacetate is a non-specific substrate for arylesterase activity. Results from the apparent Km's show that the arylesterase enzymes in the different fractions have a low affinity for phenylacetate in the liver, lung, brain and gut tissues, with a higher affinity in the skin and kidney.

CHAPTER 11 : XENOBIOTIC METABOLISM IN THE RAT

11.1. INTRODUCTION

Having already established distribution and subcellular localisation in various tissues of the rat using the non-specific esterase substrate phenylacetate, the aim was to determine esterase activity in tissues of the liver, lung, skin and plasma for the pesticide substrates of interest.

There are three main routes of exposure to pesticides: via the alimentary canal, the respiratory tract and through percutaneous absorption. Absorption of pesticides via the alimentary canal is small and and first pass metabolism of the pesticide would involve the liver, therefore this route of exposure was not considered further. As has already been stated one of the livers function is to metabolise exogenous and endogenous compounds in the body. Most esterase hydrolysis has been reported in the liver, however, little information is available on esterase hydrolysis in the tissues of the lung and skin. Exposure to pesticides occurs via inhalation or through dermal absorption and so the skin and lung would be exposed to the pesticide first. Experiments were therefore set up to determine whether local hydrolysis of the pesticides studied in the tissues of the lung and skin took place or if in fact hydrolysis of these pesticides was entirely due to esterase enzymes in the plasma and liver.

Once initial exposure to a pesticide has taken place, by absorption through lung or skin, it is then transported via the systemic circulation to the liver. The proposed routes are as follows:

Intra-dermal: skin ------> blood ------> liver

Inhalation: lung ------> blood ------> liver

The following pesticides were selected for study: paraoxon an organophosphate pesticide (see chapter 3.2.); fluazifop-butyl an alkoxy phenoxy

propionate herbicide (see chapter 3.4.); carbaryl an anticholinesterase insecticide (see chapter 3.3.) and phenylacetate. Using these pesticides, attempts will be made to establish the extent of pesticide metabolism within the liver, lung, skin and blood. Furthermore what role they play in the overall detoxification of the pesticides.

11.2. PROTEIN ESTIMATION IN RAT TISSUE

Protein estimations in the microsomal and cytosolic fractions of the liver, lung and skin were carried out to determine the recoveries of protein in the experiment after the tissue subcellular fractions were prepared from the initial tissue homogenate.

Methods

Liver, lung, skin and blood were obtained from 6 male Wistar rats (190g). Homogenates, subcellular fractions and separation of blood were prepared as previously described in general methods (see chapter 7).

Protein recoveries were made using a variation on the Lowry et al , (1956) method, by Peterson, (1977) (see chapter 7.5.).

Results

Microsomal content of the skin was found to be a 1/10 and lung a 1/4 that of liver. The cytosolic protein content of the liver was similar to that of the lung, but lower in the skin. Microsomal protein recoveries were 3% liver, 1% lung and 0.2% skin, with cytosolic protein recoveries 3% liver, 2% lung and 1% skin . These results

can be seen on Table 11.1.

11.3. FLUAZIFOP-BUTYL HYDROLYSIS

Fluazifop-butyl was selected for study because it is known to be readily hydrolysed by esterases to fluazifop acid. Little information however is available on what actual type of esterase acts to hydrolyse fluazifop-butyl to fluazifop-COOH (see chapter 3.4.). In the studies presented localisation and subcellular distribution of esterase activity was examined in the liver, lung, skin and plasma of the rat.

Methods

Liver, lung, skin and blood were obtained form 6 male Wistar rats (190g). Preparation of microsomal and cytosolic fractions as well as blood separation was as previously described (see chapter 7).

Microsomal and cytosolic protein equivalent to 0.4, 5 and 30mg of liver, lung and skin original wet weight (or 10µl of plasma and 50µl of lysed red blood cells) were incubated with a final concentration of 0.02-1mM fluazifop-butyl. Incubations were carried out in a final volume of 500µl in 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C. Reactions were started by the addition of fluazifop-butyl (5µl-20µl of 1 and 10mM stock, in acetonitrile) and stopped after 4 minutes for liver and plasma and 10 minutes for lung, skin and red blood cells by the addition of an equal volume of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes to remove protein. 80µl of the supernatant was injected onto reverse phase HPLC (see chapter 9.2.).

Table 11.1. Protein recoveries for rat liver, lung and skin subcellular fractions. Results were expressed mg/g tissue protein.

	Tissue	Protein recovery (mg / g)
Microsomes	Liver	27.8±2.25
	Lung	8.65±1.5
	Skin	2.2±0.15
Cytosol	Liver	32.5±1.0
	Lung	49.2±4
	Skin	8.8±1.25

mean±SEM (n=6)

Results were expressed in terms of wet weight tissue, so that the results in the microsomal and cytosolic fractions could be compared. Values for Vmax and apparent Km were calculated by direct linear plot for each tissue (see chapter 9.2.).

Results

Fluazifop-butyl was hydrolysed to fluazifop by microsomal and cytosolic fractions from liver, lung and skin as well as by plasma and erythrocytes (see Table 11.2 and Figure 11.1).Hydrolysis by liver microsomal esterase was 16 times that of lung and 300 times that of skin. Cytosolic esterase was greatest in the liver and similar to that of the microsomal activity. Liver cytosolic esterase was found to be 4 times that of lung and 17 times that of skin. Considering microsomal and cytosolic esterase activities together, the rat liver contains most fluazifop-butyl esterase activity followed by the plasma and then the lung, skin and erythrocytes.

Apparent Km's for microsomal esterase varied between 14 and 33μM, with Km values in the liver and the skin similar and slightly higher in the lung. In the cytosol Km values varied between 22 and 63μM, with Km values lowest in the liver and highest in the lung (see Table 11.2.).

Discussion

Fluazifop-butyl was hydrolysed by microsomal and cytosolic esterases in the liver, lung and skin as well as in the plasma and erythrocytes. Although most activity was found in the liver and plasma there was significant amounts of activity in the lung and skin.

Table 11.2. The hydrolysis of fluazifop-butyl to fluazifop by the rat liver, lung and skin subcellular fractions as well as by plasma and erythrocytes. Results were expressed as the Vmax (μmol/min/g wet weight tissue or ml) and the apparent Km (μM) by direct linear plot.

	Type of Tissue	Vmax (μmol / min / g)	K_m (μM)
Microsomes	Liver	6.29±0.4	14.4±2
	Lung	0.38±0.1	33±5.5
	Skin	0.02±0.0015	14±2.8
Cytosol	Liver	6.84±0.85	22±4.5
	Lung	1.5±0.32	63±14
	Skin	0.4±0.06	42±2.8
	Plasma	5.8±0.48	18.2±2.2
	Erythrocyte	0.03±0.015	33±1.2

mean±SEM (n=6)

limit of sensitivity > 0.5nmol/min/g(ml)

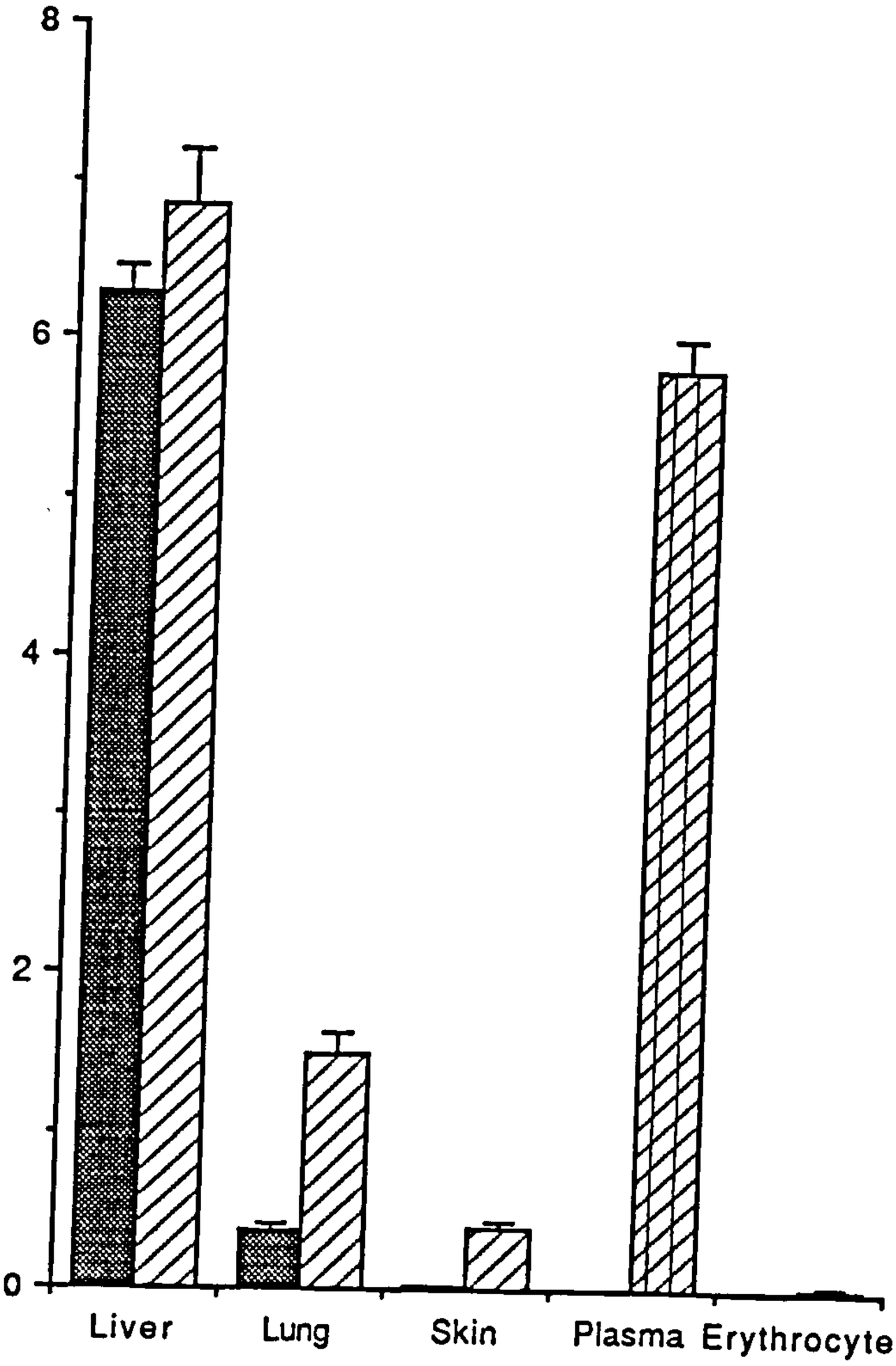


Figure 11.1.

The hydrolysis of fluazifop-butyl to fluazifop in the presence of varying amounts of substrate by rat liver, lung and skin microsomal (▩) and cytosolic (▧) fractions as well as by plasma (▨) and erythrocytes (□). Activities are presented as the Vmax (µmol/min/g wet weight tissue) determined by direct linear plot.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=6).

Fluazifop-butyl is applied in spray form and would therefore potentially come into contact with both uncovered skin and lung following inhalation. Exposure through inhalation is not thought to be a major route of exposure due to the spray particle size of fluazifop-butyl (Chester and Hart, 1986). The spray particles would be deposited in the upper airways of the lung rather than passing into the lung and therefore be absorbed orally. Following oral absorption first pass metabolism would take place in the liver. Therefore following exposure to fluazifop-butyl, local hydrolysis in the skin would play an important role in the hydrolysis of fluazifop-butyl to fluazifop before it reached the systemic circulation and liver.

The apparent Km's of the esterase enzyme for the fluazifop-butyl substrate were found to vary by 2-3 fold, being measured in μM. Previous calculated Km values for the arylesterase enzyme were measured in the mM range. The fact that the esterase enzyme is 5-10 fold more specific for fluazifop-butyl than for phenylacetate could indicate that either the esterase enzymes have different affinities for the same enzyme or that the enzymes acting on the two substrates are different. To determine the classification of the esterases hydrolysing fluazifop-butyl in the different tissues specific inhibitory studies need to be carried out (see later).

11.4. CARBARYL HYDROLYSIS

Carbaryl is a reversible anticholinesterase type insecticide belonging to the carbamate family of insecticides (see chapter 3.3.). Carbaryl is thought to be hydrolysed to 1-naphthol by the 'B' esterase, carboxylesterase (Kolbezen et al, 1954; Best and Murray, 1962). In ths study the localisation and subcellular distribution of carbaryl esterase hydrolysis was measured in the tissues of the liver, lung and skin as well as the plasma and erythrocytes.

Methods

Liver, lung, skin and blood were obtained from 6 male Wistar rats (190g). Microsomal and cytosolic fractions as well as seperation of blood into plasma and erythrocytes were prepared as previously described (see chapter 7).

A final concentration of 0-50μM of carbaryl in 15μl of methonol was added into glass conical bottomed tubes. The methanol was evaporated off under nitrogen. Microsomal and cytosolic protein euivalent to 5, 10 and 30mg of liver, lung and skin original wet weight or 20μl of plasma and 50μl of erythrocytes was added to the tubes. Samples were then vortexed for 5 seconds. Incubations were carried out in a final volume of 1ml in 0.1M phosphate buffer pH7.25 at 37^{o}C. Reactions were started with the addition of tissue to the carbaryl and stopped after 20 minutes by protein precipitation following addition of ethanol (500μl per incubation) and then centrifuged at 5400 x g for 5 minutes. 50μl of supernatant was injected onto reversed phase HPLC (see chapter 9.3.).

Results were expressed in nmol/min/g of tissue wet weight or ml of plasma or red blood cells. Values for Vmax or apparent Km's were calculated by direct linear plot.

Results

In all the tissues studied increases in activity with substrate concentration was seen over the range studied. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and apparent Km were calculated and can be seen on Table 11.3.

Table 11.3. The hydrolysis of carbaryl to 1-naphthol by the rat liver, lung and skin subcellular fractions as well as by plasma and erythrocytes. Results were expressed as the Vmax (nmol/min/g wet weight tissue or ml) and the apparent Km (μM) by direct linear plot.

	Type of Tissue	Vmax (μmol / min / g)	K_m (μM)
Microsomes	Liver	2.07±0.25	20±3.5
	Lung	1.64±0.25	30±3.5
	Skin	0.19±0.035	32±4.2
Cytosol	Liver	6.70±0.75	53±4.5
	Lung	1.36±0.25	26±1.2
	Skin	0.50±0.12	36±3.8
	Plasma	2.98±0.25	64±4
	Erythrocyte	nd	nd

mean±SEM (n=6)

limit of sensitivity > 0.3nmol/min/g(ml)

Carbaryl was hydrolysed to 1-naphthol by microsomal and cytosolic esterase enzymes from the liver, lung and skin as well as by the plasma. The Vmax for liver microsomal esterases was similar to that of lung and 10 times that of skin. Liver cytosolic esterase activity was 2 times that of plasma, 5 times that of lung and 12 times that of skin. Cytosolic esterase activity was found to be greater than microsomal esterase activity in the liver and skin and similar in the lung. Values for Vmax can be seen on Figure 11.2.

The apparent Km's for microsomal esterase varied between 20 and 32μM. Values were found to be similar in skin and lung but lower in liver. Apparent Km values in the cytosolic esterases varied between 26 and 64μM. Cytosolic esterases had higher Km values than the microsomes, indicating a lower affinity. In the cytosol values were found to be similar in the liver and skin and lower in the lung. The apparent Km in the plasma was found to be be the same as that of the lung cytosol. These results can be seen on Table 11.3.

Discussion

Carbaryl was hydrolysed by microsomal and cytosolic fractions in the liver, lung and skin as well as the plasma. Most activity was found in the liver and plasma, however, there was also significant hydrolysing activity in the tissue of the lung and skin. Therefore, following exposure to carbaryl in rat, first pass metabolism would be expected to occur in the lung and skin before the carbaryl reached the systemic circulation and the liver . The skin is a more important route of exposure to carbaryl than the lung. Carbaryl in its spray form is 15-20μm in diameter (Johnstone, 1969; 1971) and so would be deposited in the upper airways, rather than being absorbed through the lungs. The deposited carbaryl in the upper airways would then be absorbed through the gut and be taken to the liver where it would be hydrolysed.

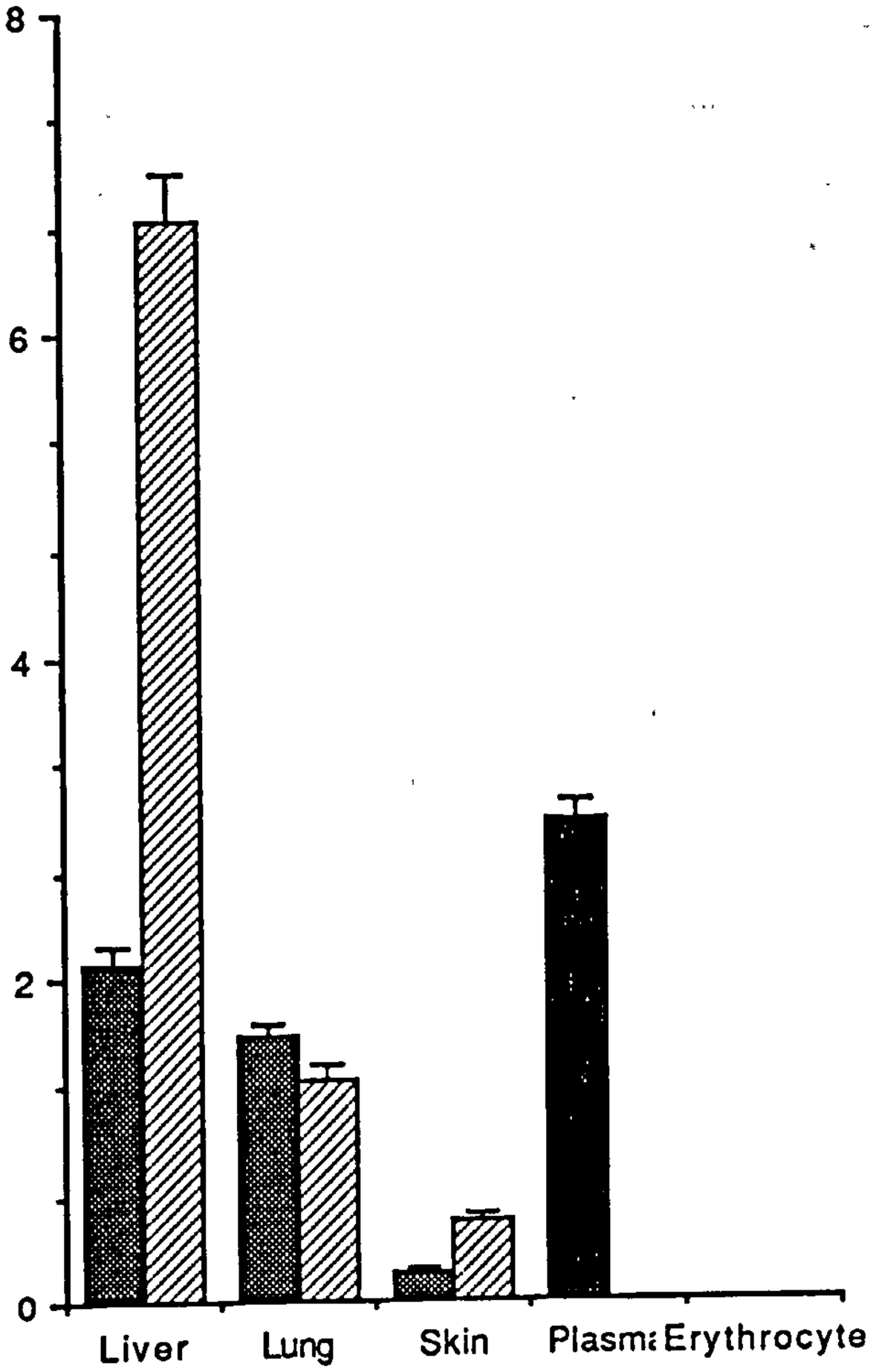


Figure 11.2.

The hydrolysis of carbaryl to 1-naphthol in the presence of varying amounts of substrate by rat liver, lung and skin microsomal (▩) and cytosolic (▨) fractions as well as by plasma (■) and erythrocytes (□).Activities are presented as the Vmax (nmol/min/g wet weight tissue) determined by direct linear plot. Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=6).

Carbaryl hydrolysis was found to be greatest in the cytosolic fraction of the liver. A possible reason for this is that there is competition for carbaryl metabolism in the microsomal fraction of the liver. Carbaryl is metabolised by the oxidative pathway as well as being hydrolysed by the esterase in the liver microsomes, whereas in the liver cytosol metabolism is entirely due to hydrolysis. Following hydrolysis of the carbaryl to 1-naphthol, the 1-naphthol is removed by phase II conjugation. The oxidative metabolism of carbaryl or the phase II conjugation of 1-naphthol was not investigated in this study. However, work has been carried out by MacPherson et al, (1991) on oxidative metabolism of carbaryl in the liver homogenate of the male Wistar rat. In the cytosol however, metabolism of carbaryl is due to hydrolysis by the esterase enzyme.

The apparent affinities of the esterase enzyme for carbaryl are very similar to those found for fluazifop-butyl, in the μM range. Previous work (Kolbezen et al, 1954; Best and Murray, 1962; Dorough, 1970) has suggested that the low anti-cholinesterase activity of carbaryl in mammals compared to insects is as a result of the capacity of the mammalian liver carboxylesterase to rapidly metabolize the carbaryl. Specific inhibitory studies using the carboxylesterase inhibitor BNPP would need to be carried out to determine whether in fact carboxylesterase is responsible for hydrolysing carbaryl to 1-naphthol.

11.5. PARAOXON HYDROLYSIS

Paraoxon is an organophosphate pesticide developed after the second world war as a nerve gas. It is the active oxon of the pesticide parathion formed following activation by oxidative metabolism (see chapter 3.2.). Paraoxon is hydrolysed to p-nitrophenol and diethylphosphoric acid by the 'A' esterase, paraoxonase. In this study the localisation and subcellular distribution of paraoxonase was studied in the tissues of the liver, lung and skin as well as in the plasma and erythrocytes.

Method

Liver, lung, skin and blood were obtained from the same 6 male Wistar rats (190g). Microsomal and cytosolic fractions as well as separation of blood to plasma and erythrocytes was carried out as previously described.

Microsomal and cytosolic protein equivalent to 0.5, 20 and 30mg of liver, lung and skin original wet weight or 10µl plasma and 50µl of lysed erythrocytes was added with a final concentration of 0.1-1mM paraoxon. Incubations were carried out in a final volume of 500µl in 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37^{o}C. Reactions were started with the addition of paraoxon (5-50µl of a 10mM stock, freshly prepared in trisma buffer) and stopped after 15 minutes by the addition of an equal volume of 6% perchloric acid, containing 10µg/ml p-toluic acid. Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of supernatant was injected onto reverse phase HPLC (see chapter 9.4.iii.). Results were expressed in µmol p-nitrophenol/min/g of tissue wet weight or ml of plasma or erythrocytes. Values for Vmax and apparent Km were calculated from direct linear plot (see chapter 8).

Results

In all the tissues studied increases in activity with substrate concentration were seen over the range of concentrations studied. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and the apparent Km's were calculated and presented in Table 11.4.

Most paraoxonase activity was found in the liver microsomes and the plasma of the rat. There was 150 times more activity in the liver microsomes than in the lung, whereas in the skin microsomes or the erythrocytes no activity was detected (limit

Table 11.4. The hydrolysis of paraoxon to p-nitrophenol by the rat liver, lung and skin and subcellular fractions as well as by plasma. Results were expressed as the Vmax (μmol/min/g wet weight tissue or ml) and the apparent Km (μM) by direct linear plot.

Tissue		Vmax (μmol / min / g tissue)	K_m (μM)
Microsome	Liver	0.333±0.025	200±8
	Lung	0.002±0.00025	380±30
	Skin	nd	nd
Plasma		0.246±0.012	225±15
Erythrocyte		nd	nd

mean±SEM (n=6)

limit of sensitivity > 0.3nmol/min/g(ml)

of detection > 0.3nmol/min/g(ml)). In the cytosolic fractions of the liver, lung and skin no paraoxonase activity was detected (limit of detection > 0.3nmol/min/g(ml)) (see Figure 11.3.).

The apparent Km's for microsomal paraoxonase varied between 200 and 380μM, with the liver having a lower apparent Km value than the lung. The apparent Km value in the plasma was found to be 225μM and this value was similar to that found in the liver microsomes. These results can be seen on Table 11.4.

Discussion

It is known that parathion is readily absorbed both through the lungs and skin. It was observed that paraoxon hydrolysis only took place in the microsomal fraction of the liver and lung tissue. No paraoxonase activity was found in any cytosolic fraction. In these tissues the parathion is activated to the active oxon paraoxon (Norman and Neal, 1975). It is this paraoxon which irreversibly inhibits cholinesterase and other 'B' esterases. Hydrolysis of paraoxon by paraoxonase occurs mainly in the liver microsomes and plasma. There was some activity found in the lung microsomes but none was found in the skin microsomes. In the rat, following exposure, little paraoxon hydrolysis would be expected to take place in the extra-hepatic tissues of the lung and the skin. Although there is some paraoxonase activity in the lung microsomes this would not be sufficient to hydrolyse the paraoxon before it started to inhibit acetylcholinesterase in the body. Plasma paraoxonase activity has an important role to play in paraoxon hydrolysis. Respiratory failure occurs as a result of acetyl cholinesterase inhibition at the lung and the respiratory centre of the brain (Chambers et al, 1989). The more paraoxon hydrolysis taking place in the plasma during first pass metabolism would reduce the effect of paraoxon on the respiratory centre of the brain Although there is substantial paraoxonase activity in the liver microsomes, it would only play an important role in detoxification of paraoxon if in fact the absorbed paraoxon was taken to the liver for detoxification before it reached the respiratory centre

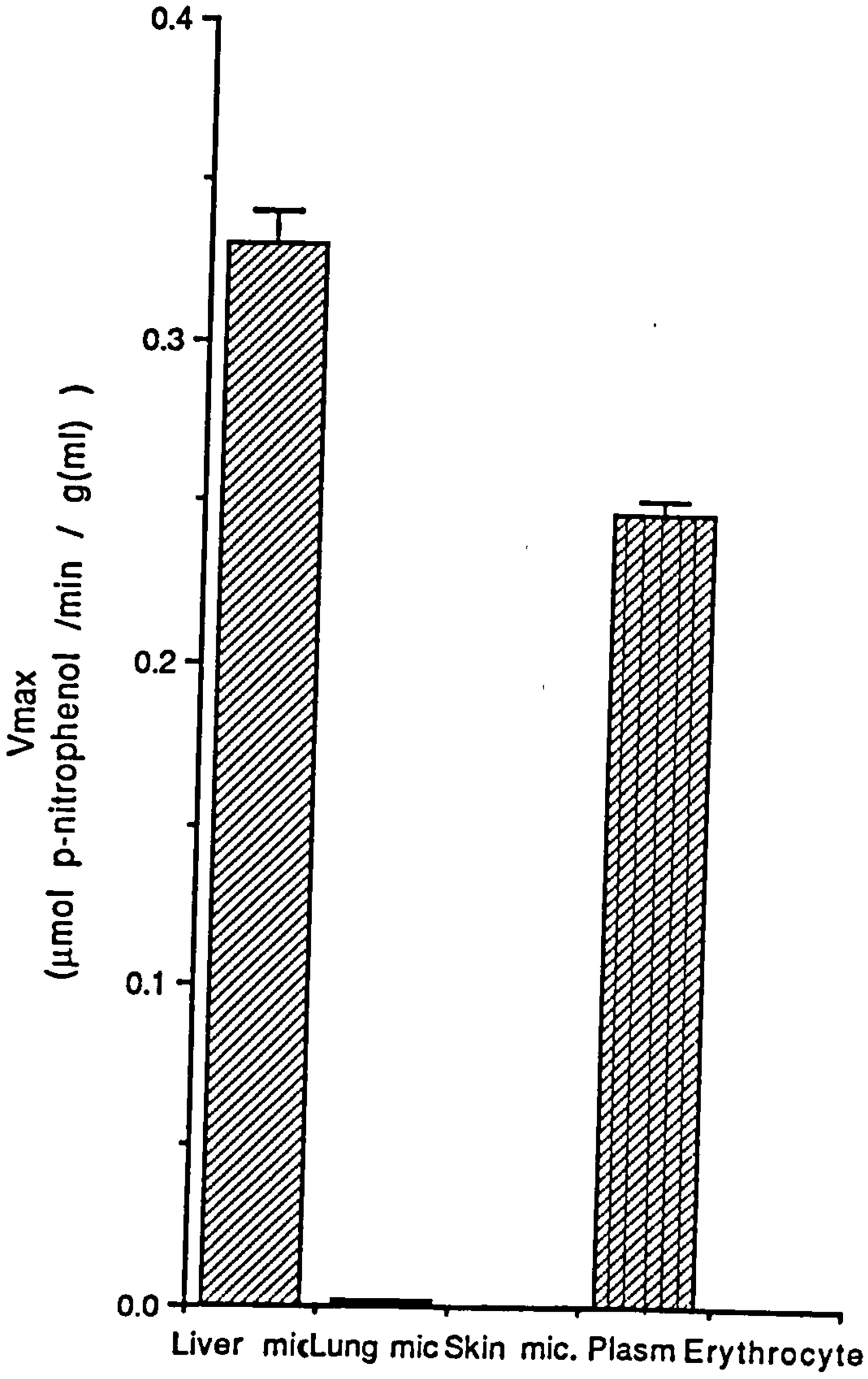


Figure 11.3.

The hydrolysis of paraoxon to p-nitrophenol in the presence of varying amounts of substrate by rat liver (▨), lung (■) and skin (□) microsomes as well as by plasma (▨) and erythrocytes (▩). Activities are presented as the Vmax (µmol/min/g wet weight tissue) determined by direct linear plot.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=6).

in the brain, however, following initial exposure the absorbed paraoxon is taken straight to the brain, by-passing the liver. Only on subsequent passes would the liver play an important role in the detoxification, or if in fact the paraoxon was ingested orally in which case first pass metabolism would take place in the liver.

The absence of paraoxon hydrolytic activity in the cytosolic fractions indicates the involvement of different esterases compared to those hydrolysing fluazifop-butyl and carbaryl. The apparent Km's of the paraoxonase enzyme for the paraoxon are around 6-10 fold greater than those for carbaryl and fluazifop-butyl. Although the distribution of paraoxonase and phenylacetate esterase is very similar, there is however, a small amount of phenylacetate esterase activity taking place in the cytosolic fractions of the liver, lung and skin (see later). Inhibitory studies of phenylacetate hydrolysis will be able to determine if in fact the esterase enzymes acting to hydrolyse paraoxon and phenylacetate are similar.

11.6. PHENYLACETATE HYDROLYSIS

As has already been discussed phenylacetate is a non-specific substrate for esterase. Phenylacetate is hydrolysed by arylesterase to phenol and acetic acid. Localisation and subcellular distribution of arylesterase activity in the tissues of the liver, lung and skin as well as in the plasma and erythrocytes of the blood.

Methods

Liver, lung, skin and blood was obtained from the same 6 male Wistar rats (190g). Microsomal and cytosolic fractions as well as separation of blood into plasma and erythrocytes was prepared as previously described (see chapter 7).

Microsomal and cytosolic protein equivalent to 2, 20 and 30mg of liver, lung and skin original wet weight or 10µl of plasma and 50µl of lysed red blood cells were incubated with a final concentration of 0.5-4mM phenylacetate. Incubations were carried out in a volume of 3mls in 50mM trisma buffer pH8, containing 0.1mM calcium chloride at 37°C, in a cuvette. Reactions were started with the addition of phenylacetate (5µl-20µl of 600mM phenylacetate stock in DMSO). The cuvettes were inverted and then placed in the spectrophotometer (see chapter 9.5.iii.). Results were expressed in terms of µmol/min/g wet weight of tissue or ml of plasma or erythrocytes. Values for Vmax and apparent Km were calculated using the direct linear plot.

Results

In all the tissues studied increases in activity with substrate concentration were seen over the range of concentrations studied. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and the apparent Km's were calculated and can be seen on Table 11.5.

Phenylacetate was hydrolysed to phenol by microsomes and cytosol from liver, lung and skin as well as by plasma. Hydrolysis by liver microsomal arylesterase was 160 times that of lung and 200 times that of skin. Liver cytosolic arylesterase was 2 times that of lung and 9 times that of lung (see Figure 11.4.). Although liver cytosol contains the greatest cytosolic arylesterase activity, it was found to be 20 times less active than liver microsomal arylesterase. The plasma contains a high amount of arylesterase activity compared to that of the lung and skin.

The apparent Km's for microsomal esterases varied between 0.95 and 1.46mM. Apparent Km values were similar in the liver and lung and lower in the skin. The apparent Km values for cytosolic esterase varied between 0.34 and 1.33mM. Apparent Km values were found to be lowest in the skin cytosol and highest in the lung

Table 11.5. The hydrolysis of phenylacetate to phenol by rat liver, lung and skin subcellular fractions as well as by plasma and erythrocytes. Results were expressed as the Vmax (µmol/min/g wet weight tissue or ml) and the apparent Km (mM) by direct linear plot.

Type of Tissue		Vmax (µmol / min / g tissue)	K_m (mM)
Microsomes	Liver	800±75	1.43±1.4
	Lung	4.86±3.1	1.46±0.58
	Skin	1.13±0.025	0.95±0.1
Cytosol	Liver	30.7±3.0	0.9±0.5
	Lung	14.2±5.5	1.33±0.55
	Skin	3.44±1.4	0.34±0.05
Plasma		290±40	2.34±0.4
Erythrocytes		nd	nd

mean±SEM (n=6)

limit of sensitivity of assay > 0.3µmol/min/g(ml)

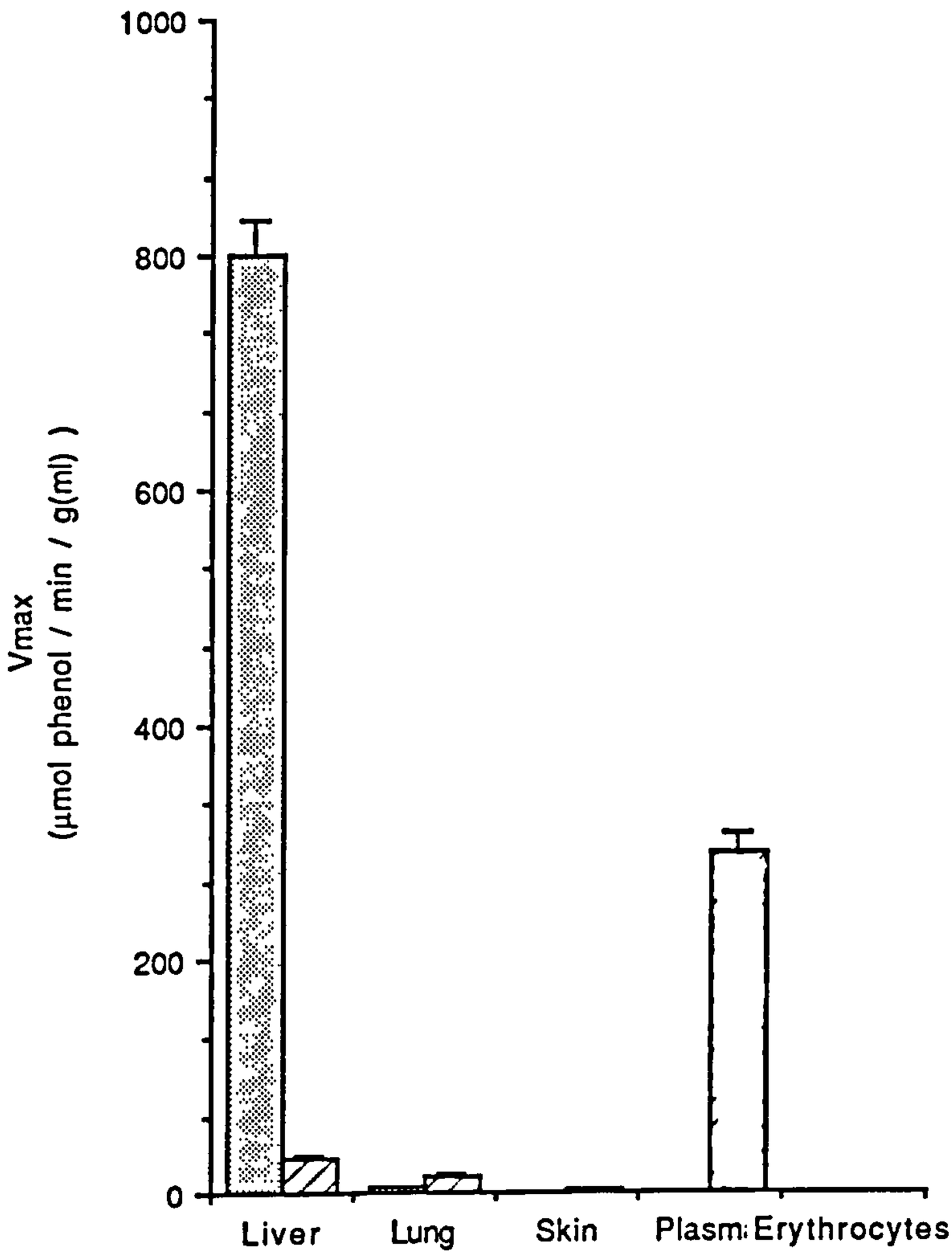


Figure 11.4.

The hydrolysis of phenylacetate to phenol in the presence of varying amounts of substrate by rat liver, lung and skin microsomal (▩) and cytosolic (▧) fractions as well as by plasma (□) and erythrocytes (▨). Activities are presented as the Vmax (µmol/min/g wet weight tissue) determined by direct linear plot.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=6).

cytosol. The apparent Km value for plasma was found to be apparently higher than any Km value in either the microsmal or cytosolic tissues studied. These results can be seen on Table 11.5.

Discussion

Liver arylesterase activity was very much greater than activities in the extra-hepatic tissues of the lung, skin and plasma. These results confirm preliminary studies carried out with phenylacetate as substrate. The distribution of microsomal arylesterase is very similar to that of paraoxonase for the microsomal component and supports the involvement of 'A' esterases in both reactions.

The apparent affinity of arylesterase for phenylacetate was about 5 fold less than the affinity of paraoxonase for paraoxon. Reported information on arylesterase and paraoxonase is conflicting. Some believe that arylesterase and paraoxonase belong to the same enzyme (ie. Eckerson and La Du, 1983), whereas others believe that they are in fact different 'A' esterase enzymes (ie. Reiner et al, 1987; Mackness et al, 1987). Although there was a 5 fold difference in Km values this was not enough to indicate a difference in enzymes acting on phenylacetate and paraoxon, particularly when considering the contrasting studies in the literature. Studies carried out in this study would seem to point to a more specific enzyme acting to hydrolyse paraoxon than phenylacetate, however, until purification and separation of the 'A' esterase enzyme have been carried out and the 'A' esterase fully characterized no real conclusion can be made.

CHAPTER 12 : INHIBITORY STUDIES IN THE RAT

12.1. INTRODUCTION

Inhibitory studies were carried out to confirm the type of esterase enzymes acting to hydrolyse fluazifop-butyl, carbaryl and phenylacetate in the tissues of the liver, lung and skin as well as in the plasma. The classical inhibitors of paraoxon, BNPP, mercury chloride and physostigmine were used to establish what type of esterase was responsible for hydrolysing the different substrates. Paraoxon inhibits 'B' esterases including cholinesterase and carboxylesterase, BNPP is a differential inhibitor for carboxylesterase activity and physostigmine, an anticholinesterase, inhibits cholinesterase and to some extent carboxylesterase. Mercury chloride inhibits enzymes which contain an -SH group (cysteine) at the active centre of the enzyme i.e. an 'A' esterase (Brandt et al, 1980).

12.2. FLUAZIFOP-BUTYL ESTERASE

Studies were therefore designed to determine whether fluazifop-butyl was hydrolysed by carboxylesterase or by cholinesterase. Inhibition studies on fluazifop-butyl esterase were carried out in both microsomal and cytosolic fractions of the liver, lung and skin as well as in the plasma.

Methods

Liver, lung, skin and plasma were obtained from the same 6 male Wistar rats (190g). Microsomal and cytosolic fraction as well as plasma separation from blood was were prepared as previously described.

Incubations were carried out in a final volume of 500µl in 50mM trisma

buffer pH8.0, at 37°C .Microsomal and cytosolic protein equivalent to 0.4, 5 and 30mg of liver, lung and skin original wet weight or 10µl of plasma were incubated along with 0.1mM paraoxon, 0.1mM BNPP, 0.1mM physostigmine (final concentration) and fluazifop-butyl. 0.1mM 4-hydroxymercuribenzoate was incubated along with 10µl of plasma and fluazifop-butyl. Final concentrations of 300µM for liver, 50µM for lung, 30µM for skin and 200µM for plasma of fluazifop-butyl were used in the incubations. Control incubations without any inhibitors in the incubation were carried out in parallel. Reactions were started by the addition of fluazifop-butyl (10-20µl of a 1 and 10 mM stock in acetonitrile) and stopped after 10 minutes by the addition of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of the supernatant was injected onto reversed phase HPLC (see chapter 9.2.).

Incubations containing the inhibitors of paraoxon, BNPP physostigmine and 4-hydroxymercuribenzoate were compared directly to control incubations. Conversion to product in the presence of inhibitor was determined as previously described (see chapter 9.2.ii.). Inhibition was estimated by expressing remaining activity in the presence of the inhibitor as a percentage of the control activity.

Results

Using the inhibitor paraoxon the percentage of remaining activities of the control were 2±0.25, 10±0.75 and 17±3 for liver, lung and skin microsomes and 2±1, 9±6.5 and 2±2 for liver, lung and skin cytosol as well as 4±0.25 for plasma.Using the inhibitor BNPP the percentage of remaining activities of control were 33±8, 67±6.5 and 40±4.5 for liver, lung and skin microsomes and 20±2, 41±2.5 and 26±2.5 for liver, lung and skin cytosol as well as 63±3 for plasma. Using the inhibitor physostigmine the percentage of remaining activities of the control were 64±3.5, 100 and 80±2 for liver, lung and skin microsomes and 69±4.8, 95±3.5 and 86±2.5 for liver, lung and skin cytosol, as well as 84±2 for plasma. Using the inhibitor 4-

hydroxymercuribenzoate, the % of remaining activity of the control was 100±0 for plasma. These results can be seen on Table 12.1.

Inhibition of the microsomal fraction by paraoxon varied from complete inhibition in the liver to around 85% inhibition in the skin. Inhibition of the microsomal fraction by BNPP varied between 70% in the liver and skin to 35% in the lung. Inhibition of the microsomal fraction by physostigmine varied from 30% in the liver to no inhibition in the lung (see Figure 12.1.). Results of inhibition of the cytosolic fraction by paraoxon, BNPP and physostigmine were found to be similar to those found in the microsomes (see Figure 12.2.)

Discussion

Paraoxon caused almost complete inhibition in the tissues studied, in both microsomal and cytosolic fractions as well as in the plasma. Paraoxon inhibits 'B' esterases by irreversibly phosphorylating the serine residues at the active centre of the enzyme. Results indicate that the microsomal and cytosolic esterases responsible for fluazifop-butyl hydrolysis are 'B' esterases. Results using mercuric chloride, an 'A' esterase inhibitor seem to back up the results seen with paraoxon. In the plasma no inhibition of fluazifop-butyl hydrolysis was seen, indicating that no 'A' esterases are acting to hydrolyse fluazifop-butyl. However, high inhibition was seen in the liver cytosol (93%) which do not seem to fit with the other inhibitory results.

BNPP also phosphorylates the serine residue at the active centre of the 'B' esterase, but less efficiently than paraoxon. BNPP is a differential inhibitor of carboxylesterase.There is very little information on the classification of cytosolic esterases, most information dealing with carboxylesterase activity has been centred on the microsomal fraction of the cell (Junge and Krisch, 1975). Results in this study, however, indicate that carboxylesterase activity is also found in the cytosolic as well as microsomal fraction of the cell. Mentlein et al, (1986) found a differential effect on

Table 12.1. Inhibition of fluazifop-butyl hydrolysis to fluazifop in rat liver, lung and skin subcellular fractions as well as plasma by 0.1mM BNPP, physostigmine, $HgCl_2$ and paraoxon. Results were expressed as the % remaining activity of control.

	Tissue	Substrate (μM)	% Remaining activity				
			control	+BNPP	+Physostigmine	+$HgCl_2$	+Paraoxon
Microsomes	Liver	300	100	33±8	64±5	60±7.5	2±0.25
	Lung	30	100	67±6.5	100	-	10±0.75
	Skin	20	100	40±4.5	80±2	-	17±3
Cytosol	Liver	300	100	20±2	69±4.8	7±1.2	2±1
	Lung	60	100	41±2.5	95±3.5	-	9±6.5
	Skin	40	100	26±2.5	86±2.5	-	2±2
	Plasma	200	100	63±3	84±2	100±0	4±0.85

mean±SEM (n=6)

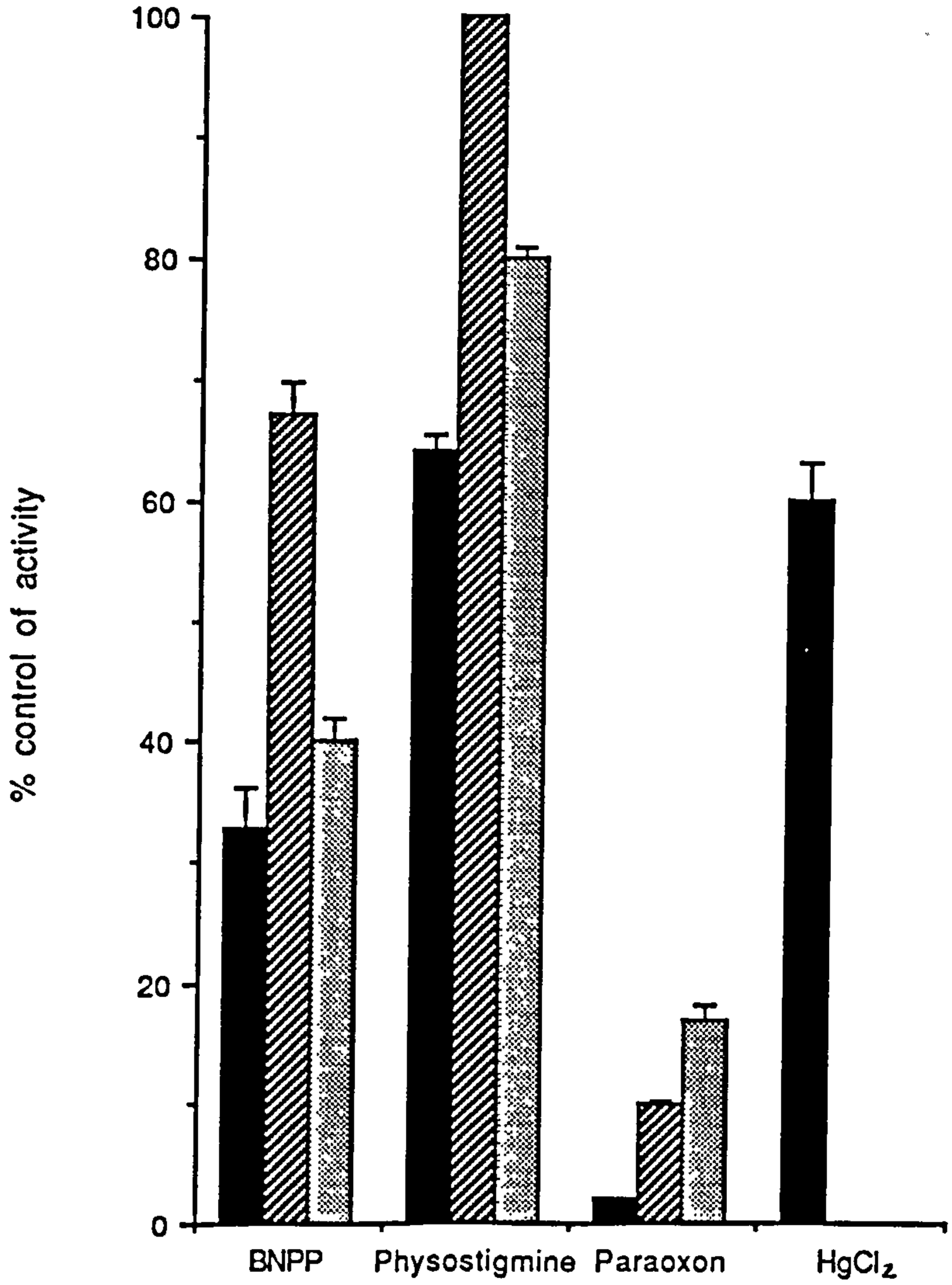


Figure 12.1.

Inhibition of fluazifop-butyl hydrolysis to fluazifop in rat liver (■), lung (▨) and skin (▩) microsomes by 0.1mM BNPP, physostigmine, $HgCl_2$ and paraoxon. Results were expressed as the % remaining activity of the control. Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

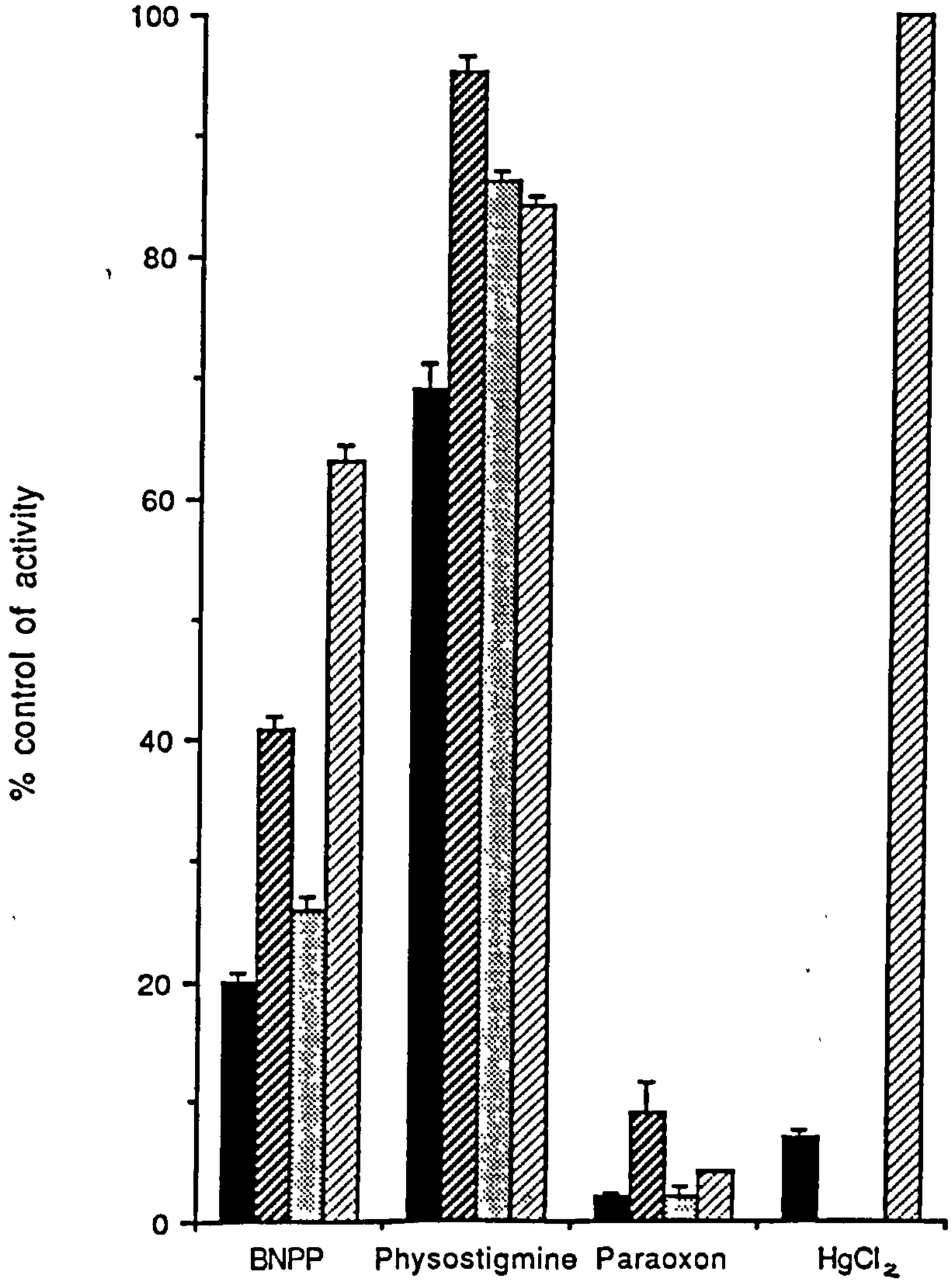


Figure 12.2.

Inhibition of fluazifop-butyl hydrolysis to fluazifop in rat liver (■), lung (▨) and skin (▩) cytosol as well as by plasma (▨) by 0.1mM BNPP, physostigmine, $HgCl_2$ and paraoxon. Results were expressed as the % remaining activity of the control.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

carboxylesterase isoenzymes in rat liver microsomes. Results show that there was between 60-80% inhibition in the tissues of the skin and liver and 40% inhibition in the plasma of fluazifop-butyl hydrolysis. In the lung microsomes there was only about 20% inhibition, whereas in the lung cytosol, inhibition was about 60%. A possible reason for the low inhibition of carboxylesterase in the lung microsomes could be as a result of a different population of carboxylesterase isoenzymes which contribute to fluazifop-butyl hydrolysis. The more specific phosphate inhibitors such as 4-cyano bis-nitrophenol phosphate would have been used to further assess the carboxylesterase, but it was not possible to obtain this. These results were similar to those found by Gaustad et al, (1991). They found that in the lung of the guinea-pig, BNPP had little effect on a specific carboxylesterase isoenzyme (pI<4.2). Hydrolysis was a two phase reaction with two different first order rate constants for BNPP inhibition. This indicated two different forms of the isoenzyme with different sensitivities towards the enzyme. Plasma was around 40% inhibited by BNPP. This seems to agree with the fact that the rat , unlike the human contains carboxylesterase in the plasma.

Physostigmine is a specific inhibitor of cholinesterase but also carboxylesterase to a lesser extent. Results show that there was between 0-30% inhibition of the liver and skin microsomal and cytosolic esterase, but no inhibition in the lung fractions. It would therefore seem that taking into consideration the BNPP and physostigmine results, although there is cholinesterase in the tissues of the liver and skin it is not important in the metabolism of this substrate. What inhibition that is seen would be more likely to be inhibition of the carboxylesterase.

It would therefore seem that the esterase enzyme acting to hydrolyse fluazifop-butyl to fluazifop in the microsomal and cytosolic fractions of the rat liver, lung, and skin as well as in the plasma are the carboxylesterases.

12.3. CABARYL ESTERASE

Previous studies of carbaryl metabolism suggested that it was hydrolysed to 1-naphthol by carboxylesterase enzyme (Kolbezen et al, 1954; Best and Murray, 1962). Inhibitory studies were therefore designed to look at inhibition of carbaryl hydrolysis in the microsomal and cytosolic subcellular fractions of the liver, lung and skin as well as in the plasma to determine whether fluazifop-butyl was hydrolysed by carboxylesterase or if in fact it was also hydrolysed by cholinesterase.

Methods

Liver, lung and skin as well as plasma was obtained from the same 4 male Wistar rats (190g). 1g portions of tissue was homogenized into 10mls of 50mM trisma buffer, ph8.0. Microsomal and cytosolic fraction as well as plasama separation from blood was prepared as previously described (see chapter 7).

A final concentration of 25µM carbaryl in 15µl of methanol was added to round bottomed conical tubes. The methanol was evaporated off under nitrogen. Microsomal and cytosolic protein equivalent to 5, 10 and 30mg of liver, lung and skin original wet weight or 20µl of plasma were incubated along with , 0.1mM BNPP and physostigmine (final concentration). Incubations were carried out in a final volume of 1ml in 0.1M phosphate buffer pH7.25, at 37^{o}C. Control incubations without any inhibitors in the incubation were carried out in parallel. Reactions were started with the addition of the tissue and stopped after 20 minutes by protein precipitation following the addition of ethanol (500µl per incubation) and then centrifuged at 5440 x g for 5 minutes. 50µl of supernatant was injected onto reversed phase HPLC as described (see chapter 9.3.iii.).

Incubations containing the inhibitors of BNPP and physostigmine were compared directly to control incubations. Conversion to product in the presence of inhibitor was determined as previously described (see chapter 9.3.ii.). Inhibition was estimated by expressing remaining activity in the presence of the inhibitor as a percentage of the control activity.

Results

Using the inhibitor BNPP, the percentage of remaining activities of control were 72±3, 59±4 and 76±7.5 for liver, lung and skin microsomes and 79±3.2, 70±6.2 and 64±8.8 for liver, lung and skin cytosol as well as 56±3 for plasma. Using the inhibitor physostigmine the percentage of remaining activities of control were 96±2, 74±5 and 89±5 for liver, lung and skin microsomes and 97±1.8, 98±1.5 and 88±7 for liver, lung and skin cytosol as well as 93±2.5 for plasma.These results can be seen on Table 12.2.

Inhibition of the microsomal fraction by BNPP varied between 30-40% for the liver, lung and skin. Inhibition of the microsomal fraction by physostigmine varied between 20-40% for the liver, lung and skin (see Figure 14.3.).Inhibition of the cytosol fraction by BNPP varied between 5-25% for the liver, lung and skin. Inhibition of the cytosolic fraction by physostigmine varied between 2-10% for the liver, lung and skin as well as by 7% in the plasma (see Figure 12.4.).

Discussion

From the results it can be seen that physostigmine seems to have little inhibitory effect on carbaryl hydrolysis in the skin, liver, lung and plasma except for the lung microsomes which is inhibited by 26%. Apart from the lung it would seem that

Table 12.2. Inhibition of carbaryl hydrolysis to 1-naphthol in rat liver, lung and skin subcellular fractions as well as plasma by 0.1mM BNPP and physostigmine. Results were expressed as the % remaining activity of control.

			% Remaining activity		
	Tissue	Substrate (μM)	control	+BNPP	+Physostigmine
Microsomes	Liver	25	100	72±3	96±2
	Lung	25	100	59±4	74±5
	Skin	25	100	24±7.5	89±5
Cytosol	Liver	25	100	80±4.2	97±1.8
	Lung	25	100	70±6.2	97±1.5
	Skin	25	100	64±8.8	88±7
	Plasma	25	100	56±3.5	93±2.5

mean±SEM (n=4)

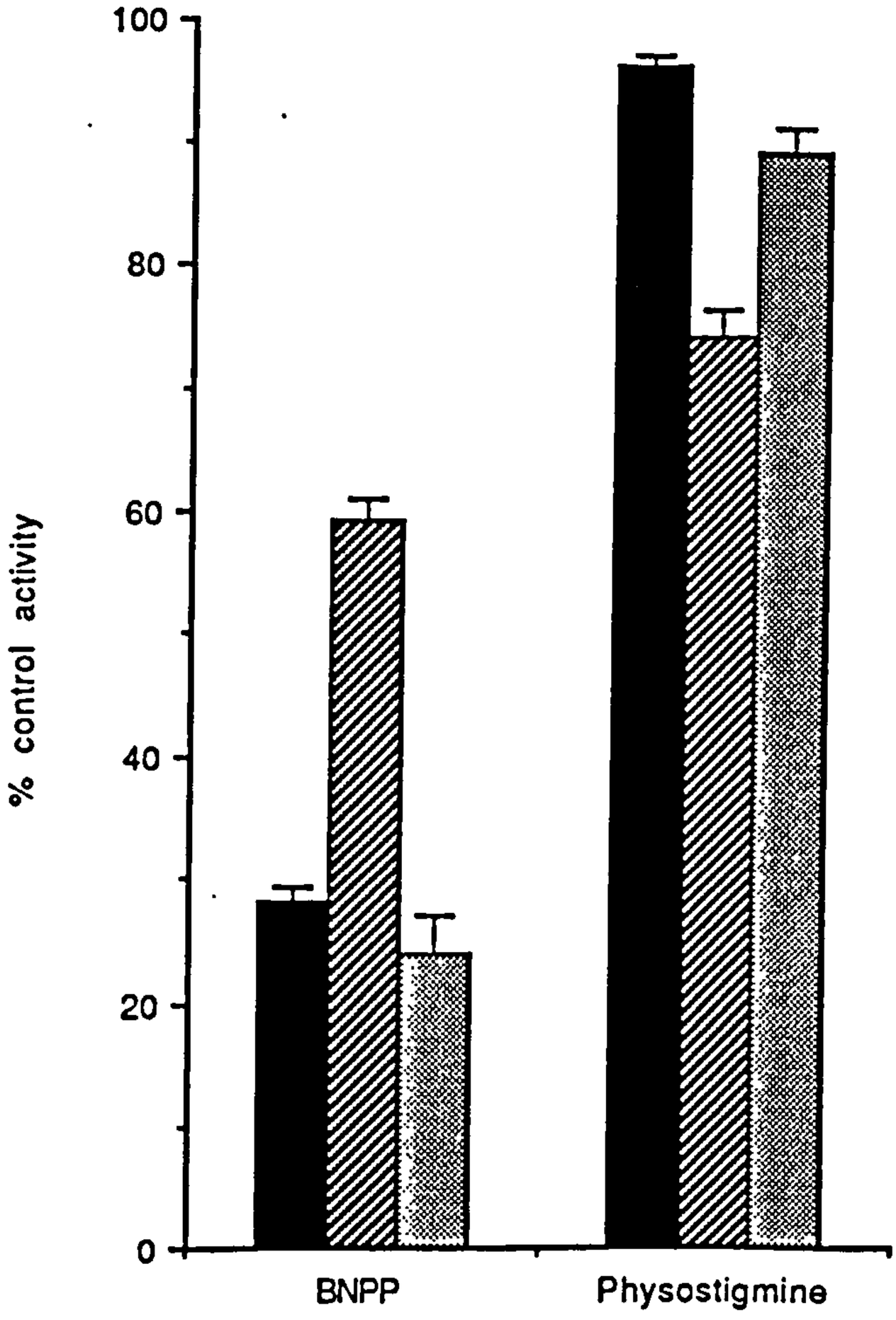


Figure 12.3.

Inhibition of carbaryl hydrolysis to 1-naphthol in rat liver (■), lung (▨) and skin (▩) microsomes by 0.1mM BNPP and physostigmine. Results were expressed as the % remaining activity of the control.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

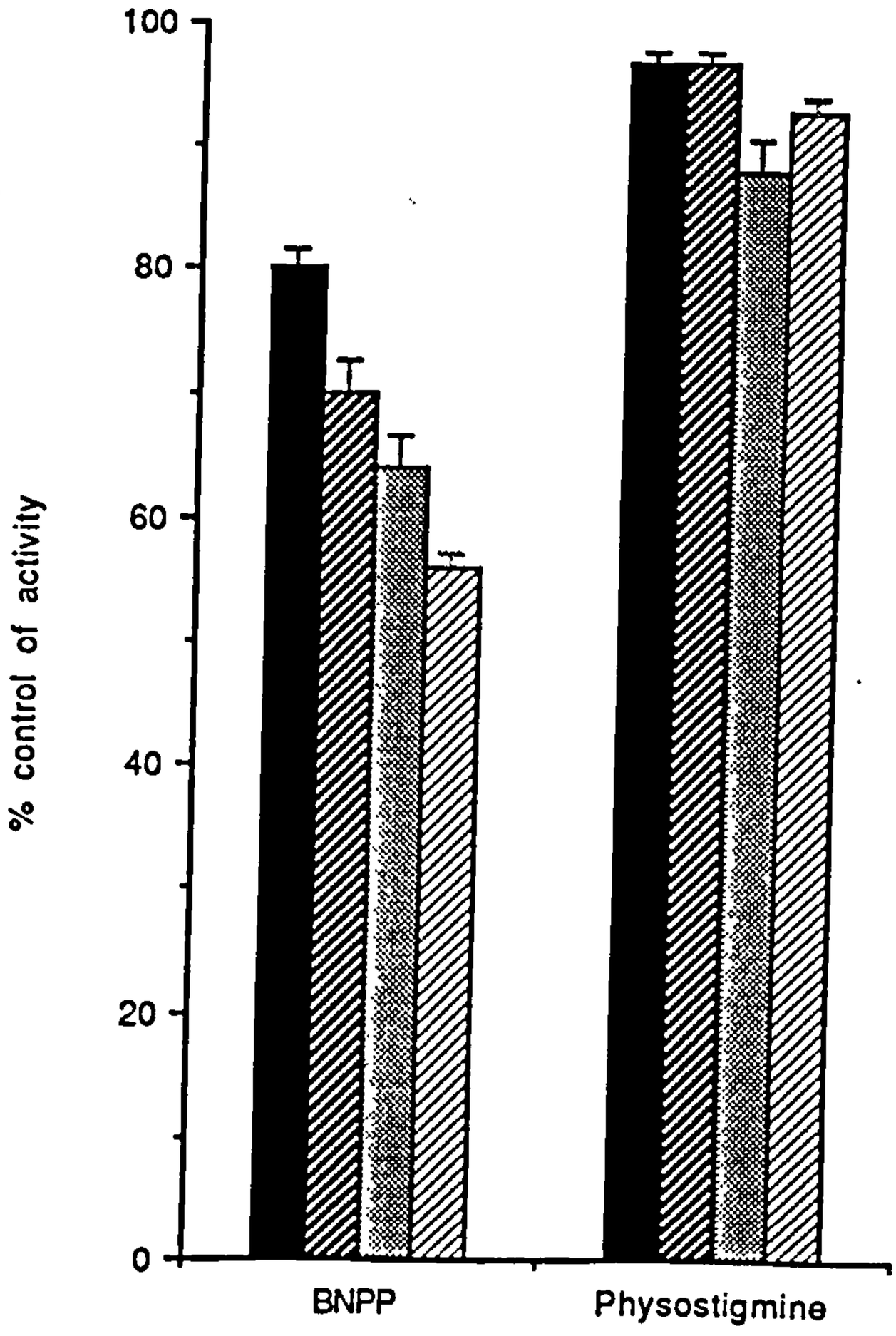


Figure 12.4.

Inhibition of carbaryl hydrolysis to 1-naphthol in rat liver (■), lung (▨) and skin (▩) cytosol as well as by plasma (▨) by 0.1mM BNPPand physostigmine. Results were expressed as the % remaining activity of the control.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

cholinesterase has little effect on carbaryl hydrolysis. There is between 20-36% inhibition of carbaryl hydrolysis by BNPP with the microsomal and cytosolic fractions of the liver, lung and skin as well as by the plasma. It would therefore seem that carbaryl was hydrolysed by carboxylesterases.

12.4. PHENYLACETATE ESTERASE

Inhibitory studies were conducted to determine the nature of esterases hydrolysing phenylacetate in the subcellular fractions of the liver, lung and skin tissues as well as the plasma.

Methods

Liver, lung and skin as well as plasma were obtained from the same 6 male Wistar rats (190g). 1g portions of tissue were homogenized in 10mls of 50mM trisma buffer, ph8.0. Microsomal and cytosolic fractions as well as plasma separation were prepared as previously described (see chapter 7).

Incubations were carried out in a final volume of 3mls in 50mM trisma buffer pH8, at 37^{o}C. Microsomal and cytosolic protein equivalent to 2, 20 and 30mg of liver, lung and skin original wet weight or 10µl of plasma with a final concentration of 0.1mM paraoxon, 0.1mM mercury chloride, 0.1mM BNPP or 0.1mM physostigmine. A final concentration of 2mM phenylacetate was used in the incubation. Control incubations without any inhibitors in the incubations were carried out in parallel. Reactions were started with the addition of phenylacetate (10µl of 600mM phenylacetate stock). The cuvettes were inverted and then placed in the spectrophotometer (see chapter 9.5.).

Incubations containing the inhibitors of paraoxon, BNPP, mercury chloride and physostigmine were compared directly to control incubations. Conversion to product in the presence of inhibitor was determined as previously described (see chapter 10.5.ii.). Inhibition was estimated by expressing remaining activity in the presence of the inhibitor as a percentage of the control activity.

Results

Table 12.3. presents the results of phenylacetate esterase inhibition in the rat liver, lung and skin subcellular fractions as well as plasma by 0.1mM paraoxon, BNPP, physostigmine and mercuric chloride.

Inhibition of microsomal arylesterase by BNPP varied from 16% in the liver to 0% in the skin microsomes, whereas inhibition due to physostigmine varied from 13-19% in the liver and lung to 40% in the skin microsomes (see Figure 12.5.). Inhibition of cytosolic arylesterase by BNPP varied from 25-35% in the liver and skin to 0% in the lung cytosol, whereas inhibition due to physostigmine varied from 20-40% in the liver and skin to 96% in the lung (see Figure 12.6.). Inhibition by mercury chloride, an inhibitor of cysteine containing groups ('A' esterases) was found to be 40%, 15% and 27% in the liver, lung and skin microsomes and 7%, 38% and30% in the liver, lung and skin cytosol.

In the plasma there was 19% inhibition of phenylacetate hydrolysis by BNPP and only 11% inhibition due to physostigmine.

Table 12.3. Inhibition of phenylacetate hydrolysis to phenol in rat liver, lung and skin subcellular fractions as well as plasma by 0.1mM BNPP, $HgCl_2$, physostigmine and paraoxon. Results were expressed as the % remaining activity of the control.

	Tissue	Substrate (μM)	% Remaining activity				
			control	+BNPP	+$HgCl_2$	+Physostigmine	+Paraoxon
Microsomes	Liver	2	100	84±3.5	60±16	87±1	50±3.8
	Lung	2	100	94±5	85±12	81±5	37±8.5
	Skin	2	100	100	73±14	60±10	38±8.6
Cytosol	Liver	2	100	75±12	93±1.2	57±14	33±7.5
	Lung	2	100	100	62±17	4±2.5	48±5
	Skin	2	100	65±5	30±10	78±1.8	35±5
	Plasma	2	100	81±4	59±2.5	89±4	83±5

mean±SEM (n=6)

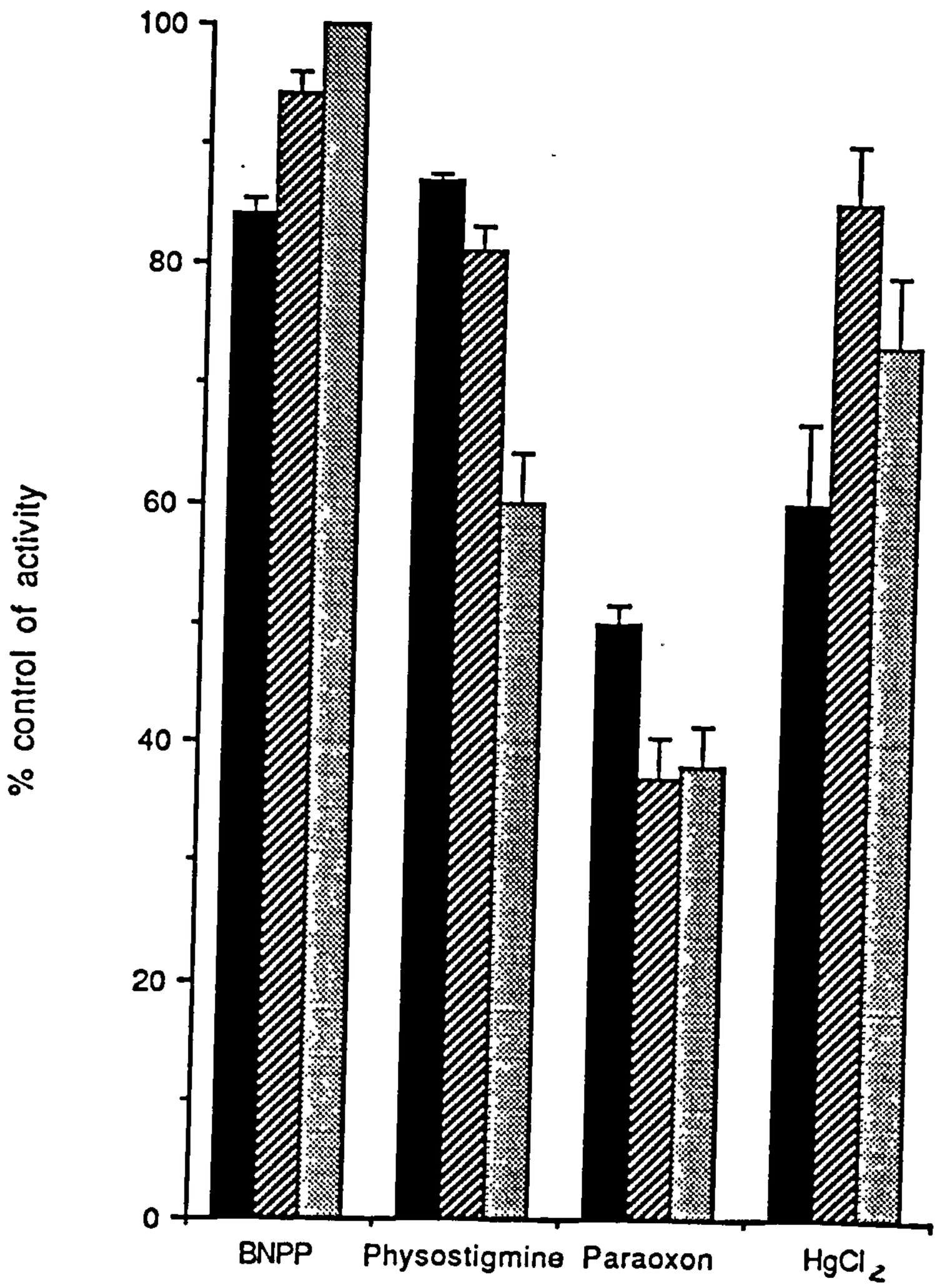


Figure 12.5.

Inhibition of phenylacetate hydrolysis in rat liver (■), lung (▨) and skin (▩) microsomes by 0.1mM BNPP, physostigmine, $HgCl_2$ and paraoxon. Results were expressed as the % remaining activity of the control.

Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

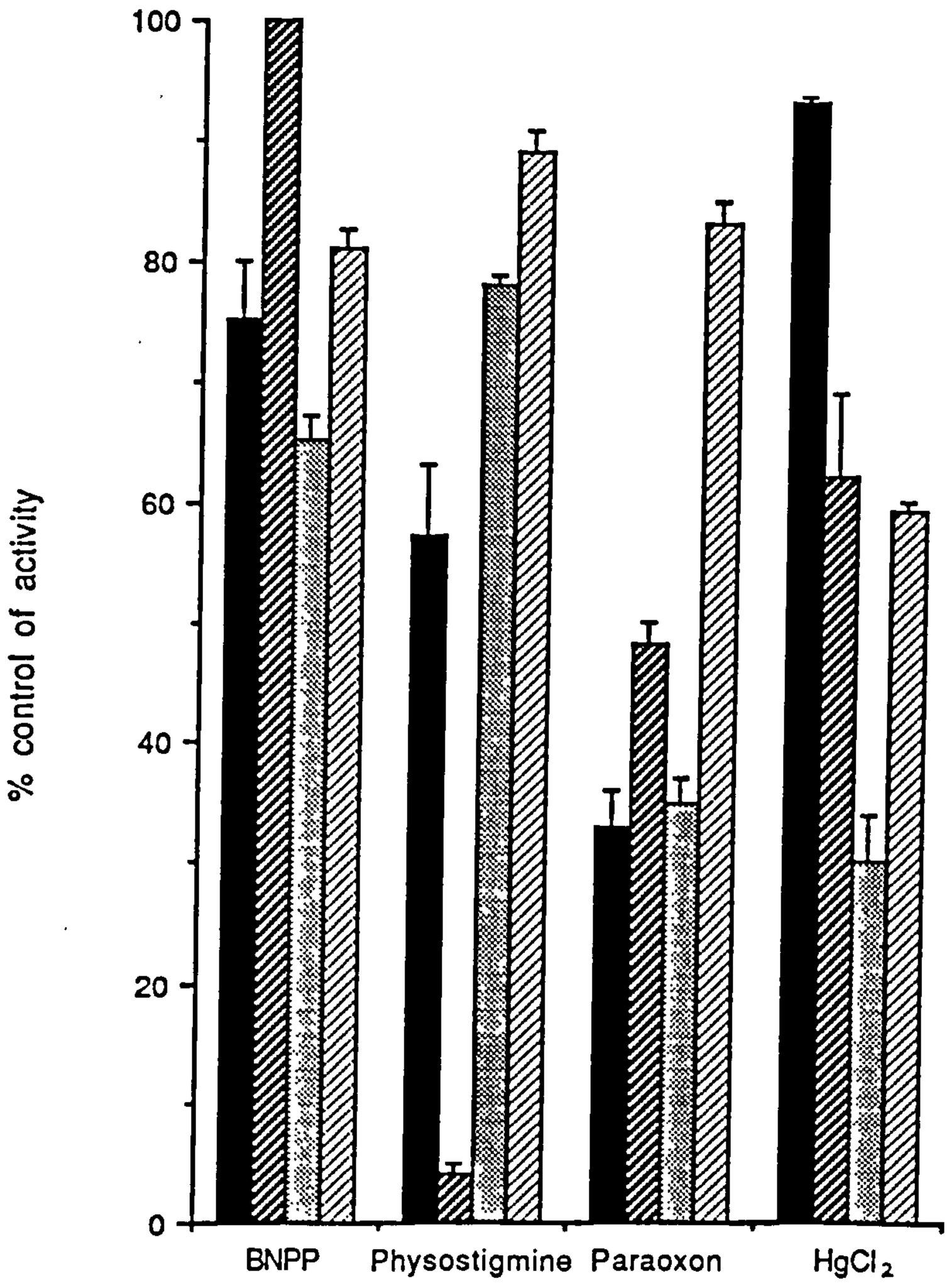


Figure 12.6.

Inhibition of phenylacetate hydrolysis in rat liver (■), lung (▧) and skin (▩) cytosol as well as by plasma (▨) by 0.1mM BNPP, physostigmine, $HgCl_2$ and paraoxon . Results were expressed as the % remaining activity of the control. Each point represents the mean of duplicate determinations and vertical lines represent mean±SEM (n=4).

Discussion

Paraoxon caused between 50-60% inhibition of phenylacetate hydrolysis in the liver, lung and skin microsomes and between 50-80% inhibition in the cytosolic fractions . It would therefore seem that a lot of phenylacetate hydrolysis is taking place as a result of 'B' esterases. Inhibition of phenylacetate hydrolysis by mercury chloride was seen in microsomal and cytosolic fractions from the liver, lung and skin, indicating the presence of the arylesterase enzyme. Results using paraoxon and mercury chloride seem to compliment each other i.e. a high paraoxon inhibition of phenylacetate hydrolysis in liver cytosol (67%) is complimented by a low inhibition by mercury chloride (7 %).

Inhibition of hydrolysis by BNPP or physostigmine was very low, varying from 0-19% between the different tissues. The skin microsomes, however had 40% inhibition of the phenylacetate esterase indicating that a lot of phenylacetate hydrolysis in the skin microsomes is due to cholinesterase. If phenylacetate esterase in the skin microsomes is due to cholinesterase rather than 'A' esterase activity, the microsomal esterase distribution in paraoxon and phenylacetate hydrolysis is very similar, with esterase activity being found in the liver, lung and plasma.

In the liver cytosol there was little inhibition of phenylacetate esterase by mercury chloride, an 'A' esterase inhibitor, but inhibition by paraoxon. It would therefore seem that in the liver cytosol hydrolysis of phenylacetate was due to 'B' type esterases. In the lung and skin cytosol inhibition of phenylacetate esterase seems to be due to a combination of both 'A' and 'B' type esterase.

In the plasma there was not much inhibition of phenylacetate hydrolysis by either BNPP or physostigmine. This would seem to indicate that most of the phenylacetate hydrolysis in the plasma is due to arylesterase activity rather than by 'B'

esterase.

CHAPTER 13 : INDUCTION STUDIES IN RAT

13.1. INTRODUCTION

The effects of the classical inducers, phenobarbitone, ß-naphthoflavone and clofibric acid on the hydrolysis of paraoxon, fluazifop-butyl , carbaryl and phenylacetate by esterase in rat liver, lung and skin was investigated. Most work on the induction has involved the monoxygenases and conjugating enzymes, whereas there has been little investigation of esterase induction.

It has been reported by Satoh and Moroi, (1973) that hepatic microsomal carboxylesterases are induced by phenobarbital, whereas 3-methylcholanthrene type inducers have no effect. Work by Raftell et al, (1977) found no induction of the lung carboxylesterase by phenobarbital. Therefore experiments were set up to examine these findings as well as to establish if carboxylesterase induction took place in the skin microsomes as well as in the cytosolic fractions of the liver, lung and skin.

Animal Induction

Liver, lung, skin and blood were obtained from 18male Wistar rats .Male Wistar rats were treated by intraperitoneal injection with phenobarbitone (80mg/kg in 0.9% aqueous NaCl for 3 days), ß-naphthoflavone (50mg/kg in corn oil for 3 days), clofibric acid (200mg/kg in H_2O adjusted to ph8.5 with NaOH for 3 days) or saline (for 3 days). Animals were killed approximately 24 hours after the last treatment and liver, lung and skin were obtained.

Preparation of liver and lung tissues from each of the induced tissues were weighed. Microsomal and cytosolic fractions of the liver, lung and skin were prepared as previously described (see chapter 7.3.). Hydrolysis of fluazifop-butyl, carbaryl, paraoxon and phenylacetate was measured.

Statistical Analysis

In the study results were statistically analysed by analysis of variance and then further by Tukey's HSD (Honestly Significant Difference) test (Tukey, 1949). This is used for testing the null hypotheses that all possible pairs of treatment means are equal when the samples are all of the same size.

Tukey's test, which is usually referred to as the HSD test, makes use of a single value against which all differences are compared. This value called the HSD, is given by

$$HSD = q_{\alpha},k,N-k \quad \frac{MSE}{n}$$

where α is the chosen level of significance, k is the number of means in the experiment, N is the total number of observations in the experiment, n is the number of observations in a treatment, MSE is the error mean square from the analysis of variance table, and q is obtained by entering the values of α, k and N-k into the 5% points of the studentized range.

All possible differences between pairs of means are computed and any difference which yields an absolute value that exceeds HSD is declared to be significant.

13.2. PROTEIN ESTIMATION

Protein estimations in the microsomal and cytosolic fractions of the liver,

lung and skin were carried out in the presence of the classical inducers of phenobarbitone, ß-naphthoflavone and clofibric acid to determine whether hyperplasia or hypertrophy took place in the tissues studied.

Methods

Protein estimations were determined by the method of Peterson et al, (1977) (see chapter 7.5.).

Results

The tissue weights following the addition of phenobarbitone, ß-napthoflavone and clofibric acid can be seen on Table 13.1. Although the results show that there is an increase in size of liver (hypertrophy) and lung with the inducers this increase was not satistically significant for any treatment.

Results for protein recoveries were measured in mg/g of tissue protein. The protein estimations following the addition of phenobarbitone, ß-napthoflavone and clofibric acid can be seen on Table 13.2. The results of the protein recoveries show that although there was some increase tissue protein (hyperplasia) it was not statistically significancant for any of the treatments. The greatest increase in protein recovery was seen in the liver microsomes with phenobarbitone, as would be expected. See appendix (Table 17.8-10.) for raw data.

Table 13.1. Induction of rat liver and lung tissues by phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed in grammes.

	Inducers				
Tissue	saline (n=4)	phenobarbitone (n=5)	ß-naphthoflavone (n=4)	clofibric acid (n=5)	HSD
Liver	12.2±0.8	12.5±0.7	14.0±0.3	14.4±0.56	2.65
Lung	1.28±0.2	1.62±0.08	1.50±0.02	1.56±0.1	0.52

mean±SEM

* significantly different $p < 0.05$

Table 13.2. Protein recoveries for rat liver, lung and skin subcellular fractions after induction with phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed as mg/g of tissue protein.

		Inducers				
Tissue		saline (n=4)	phenobarbitone (n=5)	ß-naphthoflavone (n=4)	clofibric acid (n=5)	HSD
Microsomes	Liver	16.4±1.5	19.6±3.4	16.6±0.9	14.6±1.1	15.8
	Lung	12.1±1.0	9.60±1.1	9.20±0.3	11.3±0.3	2.6
	Skin	1.50±0.12	1.10±0.18	1.33±0.26	1.26±0.12	0.54
Cytosol	Liver	49.1±1.8	49.3±3.6	43.3±2.6	52.5±5.2	15.8
	Lung	51.3±2.6	56.2±3.4	58.7±8.8	58.7±4.4	23.7
	Skin	19.3±0.1	21.0±0.2	21.5±0.08	19.4±0.2	5.8

mean±SEM

* significantly different $p < 0.05$

13.3. FLUAZIFOP-BUTYL ESTERASE

The induction of fluazifop-butyl hydrolysis by phenobarbitone, ß-naphthoflavone and clofibric acid was studied using the microsomal and cytosolic fractions of the liver, lung and skin.

Methods

Microsomal and cytosolic protein from the induced tissues, equivalent to 0.1, 5 and 30mg of liver, lung and skin original wet weight was incubated with fluazifop-butyl. Final concentrations of fluazifop-butyl of 250, 30 and 40µM for liver, lung and skin microsomes and 300, 60 and 20µM for liver, lung and skin cytosol were used in the incubations. The fluazifop-butyl concentrations used were the values at the Vmax, calculated from the previous studies of fluazifop-butyl (see chapter 11.3.). Incubations were carried out in final volume of 500µl in 50mM trisma buffer pH8.0, at 37°C. Reactions were started by the addition of fluazifop-butyl (10-30µl of a 1 and 10mM stock in acetonitrile) and stopped after 10 minutes by the addition of an equal volume of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of the supernatant was injected onto reversed phase HPLC.

Measurements for fluazifop formation were as previously described (see chapter 9.2.iii.). Activities were expressed as µmol/min/g wet weight tissue. The effects of inducers were compared by analysis of variance and then by Tukey's HSD test (see earlier).

Results

Analysis of variance, followed by Tukeys HSD test were used to determine any significant difference between the control activities and activities for the induced tissues. Any values which yields an absolute value that exceeds HSD was declared to be significantly different. These results can be seen on Table 13.3. It was found that phenobarbitone significantly induced ($p<0.05$) fluazifop-butyl hydrolysis only in the liver microsomes and the lung cytosol. ß-naphthoflavone did not induce fluazifop-butyl hydrolysis in any of the tissues, whereas the only effect of clofibric acid was to significantly reduce ($p<0.05$) fluazifop-butyl hydrolysis in the skin microsomes. See appendix (Table 17.11-13.) for raw data.

13.4. CARBARYL ESTERASE

The results of the inhibitory studies previously presented have suggested that carbaryl hydrolysis is hydrolysed by carboxylesterases in liver, lung and skin. It has also been suggested that carboxylesterase is responsible for the hydrolysis of fluazifop-butyl, therefore induction of both carbaryl and fluazifop-butyl hydrolysis should be similar, although different isoenzymes could be involved.

Methods

Carbaryl esterase activity was measured in the same tissue fractions as fluazifop-butyl esterase (see chapter 13.3.).

A final concentration of 25µM of carbaryl in 15µl of methanol was added

Table 13.3. Induction of fluazifop-butyl hydrolysis in rat liver, lung and skin subcellular fractions by phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed in µmol fluazifop/min/g wet weight.

		Inducers				
	Tissue	saline (n=4)	phenobarbitone (n=5)	ß-naphthoflavone (n=4)	clofibric acid (n=5)	HSD
Microsomes	Liver	1.10±0.8	1.94±0.26*	0.96±0.08	1.10±0.06	0.52
	Lung	0.28±0.012	0.27±0.03	0.28±0.03	0.30±0.012	0.083
	Skin	0.0024±0.0004	0.0016±0.0002	0.002±0.0002	0.0113±0.0002*	0.001
Cytosol	Liver	0.86±0.14	0.97±0.1	0.66±0.1	1.10±0.12	0.79
	Lung	1.31±0.048	1.46±0.028*	0.98±0.16	1.31±0.06	0.09
	Skin	0.60±0.012	0.58±0.048	0.58±0.028	0.59±0.022	0.11

mean±SEM

* significantly different $p < 0.05$

into glass conical bottomed tubes. The methanol was evaporated under nitrogen. Microsomal or cytosolic protein equivalent to 5, 10 or 30mg of liver, lung or skin original wet weight was added to the tubes. Samples were then vortexed for 5 seconds. Incubations were carried out in a final volume of 1ml in 0.1M phosphate buffer ph7.25 at 37°C. Reactions were started by the addition of the tissue fraction to the carbaryl and stopped after 20 minutes by protein precipitation following addition of ethanol (500µl per incubation) and then centrifuged at 5400 x g for 5 minutes. 50µl of supernatant was injected onto reverse phase HPLC (see chapter 9.3.iii.). Activities were expressed as nmol/min/g wet weight tissue. The effects of inducers were compared by analysis of variance and then by Tukey's HSD test (see earlier).

Results

Analysis of variance, followed by Tukeys HSD test were used to determine any significant difference between the control activities and activities for the induced tissues. Any values which yields an absolute value that exceeds HSD was declared to be significantly different. These results can be seen on Table 13.4. The results show that although phenobarbitone and clofibric acid increased liver and lung microsomal esterase, the difference did not reach statistical significance at the 5% level by analysis of variance. There was no statistical significant in cytosolic esterase hydrolysis in any of the tissues studied. No significant difference was found in esterase hydrolysis of carbaryl, between the saline and induced skin. See appendix (Table 17.14-16.) for raw data.

13.5. PARAOXON HYDROLYSIS INDUCTION

The induction of paraoxon hydrolysis by paraoxonase was examined to see how phenobarbitone, ß-naphthoflavone and clofibric acid type inducers, induce 'A' esterase. Induction of paraoxon hydrolysis was examined in the microsomal and

Table 13.4. Induction of carbaryl hydrolysis in rat liver, lung and skin subcellular fractions by phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed in nmol 1-naphthol/min/g wet weight.

		Inducers				
	Tissue	saline (n=4)	phenobarbitone (n=4)	ß-naphthoflavone (n=4)	clofibric acid (n=4)	HSD
Microsomes	Liver	1.14±0.12	1.90±0.28	1.04±0.12	1.66±0.3	0.93
	Lung	0.72±0.1	0.90±0.2	0.66±0.06	0.90±0.16	0.6
	Skin	0.06±0.014	0.029±0.008	0.037±0.01	0.029±0.0002	0.04
Cytosol	Liver	1.30±0.06	0.90±0.32	0.99±0.26	0.77±0.1	0.9
	Lung	0.68±0.12	0.48±0.1	0.76±0.22	0.83±0.08	0.58
	Skin	0.30±0.04	0.28±0.02	0.26±0.04	0.19±0.04	0.136

mean±SEM

* significantly different $p < 0.05$

cytosolic fractions of the liver and lung.

Methods

Paraoxonase activity was measured in the same tissue fractions as fluazifop-butyl esterase (see chapter 13.3.).

Microsomal protein equivalent to 0.5 and 20mg of liver and lung original wet weight was incubated with a final concentration of paraoxon of 0.2mM for liver and 0.3mM for lung. Incubations were carried out in a final volume of 500µl in 50mM trisma buffer ph8.0, containing 0.1mM calcium chloride, at 37°C. Reactions were started with the addition of paraoxon (10 or 15µl of 10mM paraoxon stock in 50mM trisma buffer ph8.0, freshly prepared) and stopped after the addition of an equal volume of 6% perchloric acid, containing 10µg/ml p-toluic acid. Tubes were vortexed and centrifuged at 5440xg for 5 minutes. 80µl of the supernatant was injected onto reverse phase HPLC, as described (see chapter 9.4.iii.). Activities were expressed as nmol/min/g wet weight tissue. The effects of inducers were compared by analysis of variance and then by Tukey's HSD test (see earlier).

Results

Analysis of variance, followed by Tukeys HSD test were used to determine any significant difference between the control activities and activities for the induced tissues. Any values which yielded an absolute value that exceeds HSD was declared to be significantly different. These results can be seen on Table 13.5.

Results show that there was significant induction of paraoxonase activity

Table 13.5. Induction of paraoxon hydrolysis in rat liver and lung fraction by phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed in nmol p-nitrophenol/min/g wet weight.

		Inducers				
Tissue		saline (n=4)	phenobarbitone (n=5)	ß-naphthoflavone (n=4)	clofibric acid (n=5)	HSD
Microsomes	Liver	70±0.6	130±16*	80±0.4	95.5±0.6	32
	Lung	8.2±0.6	9.1±0.4	9.6±0.4*	9.8±0.2*	1.2

mean±SEM

* significantly different $p < 0.05$

by phenobarbitone in the liver microsomes. Although both ß-naphthoflavone and clofibric acid increased paraoxonase activity in the liver microsomes , the increase was not statistically significant. In the lung microsomes there was a significance increase in paraoxonase activity with ß-naphthoflavone and clofibric acid ($p<0.05$). Phenobarbitone also increased activity compared to the control, but this difference did not achieve significant difference at the 5% level. See appendix (Table 17.17-18.) for raw data.

13.5. PHENYLACETATE ESTERASE INDUCTION

The induction of phenylacetate hydrolysis by phenobarbitone, ß-naphthoflavone and clofibric acid was also studied using the same microsomal and cytosolic fractions of the liver, lung and skin.

Methods

Phenylacetate activity was measured in the same tissue fractions as fluazifop-butyl esterase (see chapter 13.3.).

Microsomal and cytosolic protein equivalent to 2mg liver and 10mg lung and skin original wet weight was incubated with a final concentration of 2mM phenylacetate. Incubations were carried out in a final volume of 3mls in 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C, in a cuvette. Reactions were started by the addition of phenylacetate (10µl of 600mM phenylacetate stock in DMSO). The cuvettes were inverted and then placed in the spectrophotometer (see chapter 9.5.iii.). Activities were expressed as nmol/min/g wet weight tissue. The effects of inducers were compared by analysis of variance and then by Tukey's HSD test (see earlier .).

Results

Analysis of variance, followed by Tukeys HSD test were used to determine any significant difference between the control activities and activities for the induced tissues. Any values which yields an absolute value that exceeds HSD was declared to be significantly different. These results can be seen on Table 13.6.

Results show that there was a significant induction by phenobarbitone pre-treatment of arylesterase activity in the liver microsomes and lung cytosol. No other arylesterase induction was seen in any microsomal or cytosolic fractions of any of the other tissues studied. However, there was a reduction in arylesterase activity in the skin cytosol following ß-naphthoflavone and clofibric acid pre-treatment, however, this was not statistically significant at the 5% level (analysis of variance). See appendix (Table 17.19-21.) for raw data.

13.7. DISCUSSION

The observed results seem to indicate that in the microsomal fraction of the tissues studied only phenobarbitone significantly induced fluazifop-butyl hydrolysis. This agrees with work carried out by Satoh and Morio, (1973), who using the substrate isocarboxazid, found that only microsomal hepatic carboxylesterase was induced by phenobarbitone. In the cytosolic fraction the lung cytosol carboxylesterase was also induced by phenobarbitone. Previous work done on fluazifop-butyl hydrolysis (see chapter 11.3.) has shown that most hydrolysis takes place in the lung cytosol, rather than the microsomal fraction of the tissue. Earlier inhibitory studies using BNPP also showed that 60% of carboxylesterase activity was inhibited in the cytosol compared to 33% in the microsomal fraction of the lung. The fact that there seems to be induction of carboxylesterase in the lung cytosol could be as a result of a population of inducible isoenzymes found in the lung cytosol whichare not present in

Table 13.6. Induction of phenylacetate hydrolysis in rat liver, lung and skin subcellular fractions by phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed in µmol phenol/min/g wet weight.

			Inducers			
	Tissue	saline (n=4)	phenobarbitone (n=5)	ß-naphthoflavone (n=4)	clofibric acid (n=5)	HSD
Microsomes	Liver	180±8.4	226±20*	155±5	172±6.6	44.9
	Lung	4.3±0.32	4.1±0.26	3.8±0.32	4.1±0.2	0.94
	Skin	0.135±0.12	0.12±0.01	0.127±0.1	0.18±0.036	0.07
Cytosol	Liver	58±4	53±8	51±6	64±4	24.8
	Lung	15±0.6	21.4±2*	10.8±2	13.5±1.6	5.9
	Skin	6.9±0.28	7.3±0.86	5.7±0.24	5.2±0.14	1.66

mean±SEM

* significantly different $p < 0.05$

the lung microsomes.

No induction of fluazifop-butyl hydrolysis by ß-naphthoflavone was found to take place in any of the tissues studied. These results agree with previous work carried out by Raftell et al, (1977), who found that the 3-methylcholanthrene type inducer had no effect on hepatic microsomal carboxylesterase. No induction of fluazifop-butyl hydrolysis took place in any of the tissues studied using clofibric acid as an inducer. These results were contrary to those found by Kawashima et al, (1973) and Hosokawa et al, (1987). Kawashima et al, (1973) reported the induction of hepatic long-chain acyl-CoA hydrolase in the cytosolic fraction by administration of clofibric acid to rats, mice, or guinea-pigs. Hosokawa et al, (1987) reported that administration of clofibrate caused a significant increase in the activities of p-nitrophenylacetate, isocarboxazid, butanilicaine and palmitoyl-CoA hydrolases in rat liver microsomes. p-Nitrophenylacetate and isocarboxazid hydrolase activities were not induced by clofibrate, whereas palmitoyl-CoA hydrolase activity was significantly induced. These results suggest that fluazifop-butyl is hydrolysed by a different esterase isoenzyme from those studied by Kawashima et al, (1973) and Hosokawa et al, (1987). Another possible reason for the lack of induction of carboxylesterase activity by clofibric acid could be that the dosage schedule may have not completely induced the enzymes.

It was observed that no induction of carboxylesterase took place in the skin tissue. This could indicate that either there was no induction taking place in the skin or that the inducers were not taken up by the skin tissue efficiently enough from the circulation. This could be as a result of a reduced blood supply to the skin compared to that of the lung or liver.

Although carbaryl hydrolysis is apparently induced by phenobarbitone and clofibric acid in the liver microsomes, the large inter-animal resulted in the differences not achieving statistically significant at the 5% level. See appendix for individual data. The reproducibility of the carbaryl assay was poor due to the high

spontaneous carbaryl hydrolysis. The coefficient of variation between the assays was 21% (see chapter 9.3.ii.). For fluazifop-butyl hydrolysis, the inter-assay variation was much smaller and so, only small variations in the sample populations were seen (see appendix).

If in fact carbaryl hydrolysis was induced by phenobarbitone in the liver microsomes, the effects on carbaryl and fluazifop-butyl hydrolysis would be very similar. In the future a larger group of animals should be used for induction studies in carbaryl hydrolysis due to the large variation between rat samples.

Induction of paraoxonase activity by phenobarbitone was seen in the liver microsomes. In the lung, ß-naphthoflavone and clofibric acid induced paraoxonase activity . This could be as a result of ß-naphthoflavone and clofibric acid inducing a number of specific isoenzymes not present, previously before induction.

Induction of arylesterase activity was seen in the liver microsomes following phenobarbitone pre-treatment. Phenobarbitone also induced phenyl acetate esterase activity in the lung cytosol. Previously, inhibitory studies have shown that the esterase enzyme hydrolysing arylesterase in the lung cytosol and liver microsomes are in fact both 'A' and 'B' type esterase (phenylacetate esterase activity was inhibited 52% by paraoxon and 38% by $HgCl_2$ in lung cytosol and inhibited 50% by paraoxon and 40% by $HgCl_2$ in the liver microsomes). This result is in agreement with fluazifop-butyl esterase induction where it was also found that phenobarbitone induced fluazifop-butyl esterase, a 'B' esterase in the cytosol of the lung and microsomes of the liver.

No other induction of phenylacetate esterase following pre-treatment with the classical inducers in the microsomal or cytosolic fractions of any of the other tissues studied was observed. A possible reason for the lack of induction of phenylacetate esterase in the lung and skin microsomes by phenobarbitone could be as a result of the

lack of inducible carboxylesterase isoenzymes compared to that of the liver microsmes.

The use of inducers has helped to identify the esterases involved in the hydrolysis of fluazifop-butyl, carbaryl, paraoxon and phenylacetate.

CHAPTER 14 : XENOBIOTIC METABOLISM IN THE HUMAN

14.1. INTRODUCTION

Having established the nature of the esterases in the detoxification of a number of pesticides in the rat, studies using human liver and blood were carried out for comparison. Species differences exist between rat and human in the nature and specificity of xenobiotic metabolising enzymes. For extrapolation it was possible to study human liver and blood samples. The aim of the experiments were to establish human liver and blood hydrolytic activity for paraoxon, fluazifop-butyl and phenylacetate and to determine the distribution of hydrolytic activity in the liver microsomal and cytosolic fractions and plasma and erythrocytes.

Having established the distribution of hydrolytic activity for paraoxon, fluazifop-butyl and phenylacetate inhibitory studies were then carried out to distinguish the enzymes involved in their hydrolysis, to compare with those found in the rat.

14.2. SUBJECTS STUDIED

Samples of human liver were given by Dr F. Kumali, Wolfson Unit of Clinical Pharmacology, Newcastle. Samples had been obtained from transplant donors from a number of hospitals in the North East of England by collaboration with the transplant coordinator. Clinical details of the human subjects can be seen on table 14.1. None of the liver donors were receiving medication except number 3 who was receiving ampicillin. The smoking status was not available. Liver samples were stored in liquid nitrogen as soon as possible before analysis.

Venous blood samples were obtained from 10 healthy male volunteers who were drawn from scientific staff of the Division of Environmental and Occupational Medicine, University of Newcastle upon Tyne. Clinical details of the

Table 14.1. Human liver information

Liver sample number	Sex	Age	Liver function test	Cause of death
1	male	55	normal	head injury
2	female	63	normal	cerebral haemorrhage
3	female	47	-	subarachnoid haemorrhage
4	male	35	normal	subarachnoid haemorrhage
5	male	55	normal	head injury
6	female	45	normal	subarachnoid haemorrhage
7	female	47	normal	subarachnoid haemorrhage

human subjects can been seen on table 14.2. The study had the approval of the relevant Hospital Ethics commitee. The nature and purpose of the studies were explained in detail to all subjects.

Separation of Blood

10ml of blood was obtained from 10 healthy male volunteers by intravenous puncture. This blood was put into heparinized tubes and placed on a Luckham rolling mixer. Plasma and red blood cells were separated by centrifugation using a Mistral 3000 centrifuge, at 880 x g for 5 minutes. The plasma was removed and stored at -80^{o}C before analysis. The red blood cells were washed using an equal volume of isotonic saline. After centrifugation at 880 x g for 10 minutes the upper layer was removed and discarded. This procedure was again repeated. The red blood cells were then stored at -80^{o}C before analysis.

Preparation of Liver Homogenate and Subcellular Fractions

The liver was washed in ice cold 50mM trisma HCl buffer pH 7.5. 0.4 g portions of liver were weighed and finely minced using scissors and forceps in 4mls of 50mM trisma HCl buffer pH 7.5. Tissue homogenate was prepared in a glass to glass homogenizer, cooled on ice.

Tissue homogenates were centrifuged in a Sorvall RC 5B refrigerated centrifuge at 1,800 x g for 5 minutes at 4^{o}C to remove tissue debris. The supernatant was further centrifuged at 10,000 x g for 10 minutes at 4^{o}C to remove mitochondria and nuclei.

Table 14.2. Human plasma information

Plasma sample number	Sex	Age	Smoker
1	male	23	no
2	male	22	no
3	male	24	no
4	male	25	no
5	male	19	no
6	male	39	no
7	male	28	no
8	male	30	no
9	male	31	no
10	male	24	no

The post mitochondrial fraction was centrifuged in a Kontron ultra-centrifuge at 100,000 x g for 1 hour and 10 minutes. The supernatant was removed (4mls) and the resulting pellet was resuspended by glass to glass homogenization in 50mM trisma HCl buffer pH 7.5. The resulting pellet from the second spin was resuspended into 1ml of 50mM trisma HCL buffer, pH 7.5. Cytosolic protein was equivalent to 0.1g tissue/ml and microsomal enzymes equivalent to 0.4g tissue/ml. The microsomal and cytosoic fractions of the liver were stored in ependorf tubes at -80^{o}C before analysis.

14.3. GENERAL HUMAN ASSAY METHODS

The following enzymatic assays were set up to examine the hydrolysis of a number of xenobiotic esters by the human liver and blood. Using the analytical techniques of HPLC and spectrophotometry, the formation of product was measured over a range of substrate concentrations to establish the Vmax and apparent values for the esterase enzyme, after conditions of linearity had been established.

The methods applied to determine the enzyme activity in rat subcellular fractions were adapted for measurement of activity in human microsomes and cytosol, where activities were generally lower.

14.3.i. Fluazifop-butyl esterase assay

Chemicals (see chapter 9.2.i.)

Incubations

Microsomal or cytosolic protein, equivalent to 1-5mg of liver original wet weight or 10-50µl of plasma or red blood cells was incubated with fluazifop-butyl (0.05-1mM final concentration). Incubations were carried out in a final volume of 500µl of 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C.

Reactions were started by the addition of fluazifop-butyl (5-50µl of a 1mM and 10mM stock in acetonitrile) and stopped with the addition of 500µl of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of supernatant was injected onto reverse phase HPLC. Conditions for the HPLC assay of fluazifop-butyl and fluazifop were as previously described (see chapter 9.2.iii.).

Using a fixed concentration of fluazifop-butyl (1mM) and time of incubation (10 min), varying amounts of microsomal and cytosolic protein (see figure 14.3.1.) were used in the incubations to establish conditions of linearity for protein. The amount of plasma was also varied, with a constant time (10 min) and constant fluazifop-butyl concentration (1mM) (see Figure 14.3.2.). Having established the optimal conditions for incubation, the time of hydrolysis was varied (0-30 minutes) with a fixed fluazifop-butyl concentration (1mM) to establish conditions of linearity for time for the following tissues: liver (see Figure 14.3.3.) and plasma (see Figure 14.3.4.).

The optimal conditions selected for the study with a constant time of (10 mins) and fluazifop-butyl concentration (1mM) for microsomal and cytosolic protein were 2mg of liver original wet weight or 50µl of plasma. The conditions of linearity for time, with constant protein and fluazifop-butyl concentration were 4 minutes for liver and plasma. The lower limit of detection of the fluazifop-butyl assay was 1nmol/

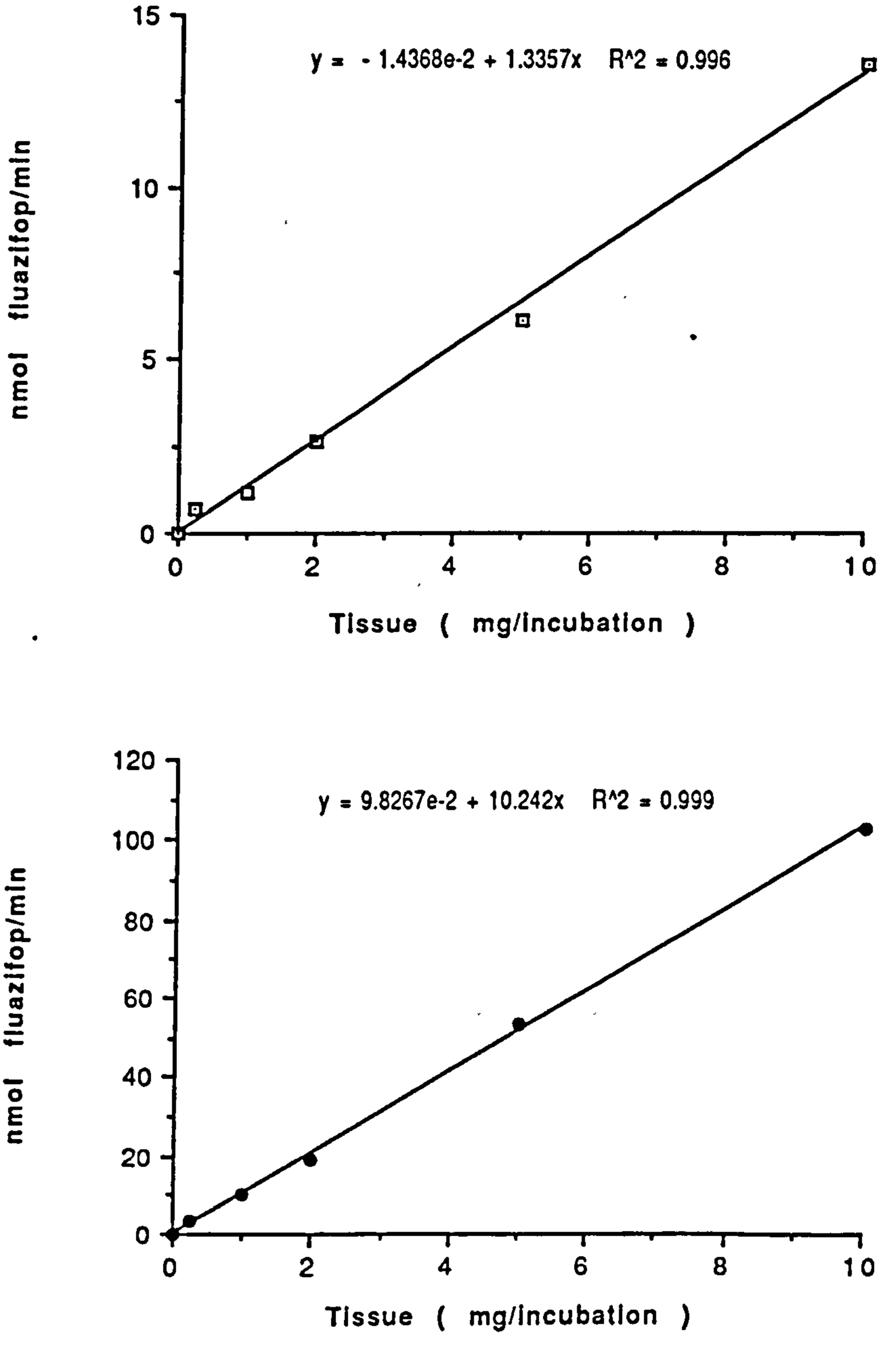


Figure 14.3.1.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying human liver microsomes (⧈) and cytosol (●) (0-10 mg/incubation). Each point represents a mean of duplicate determinations.

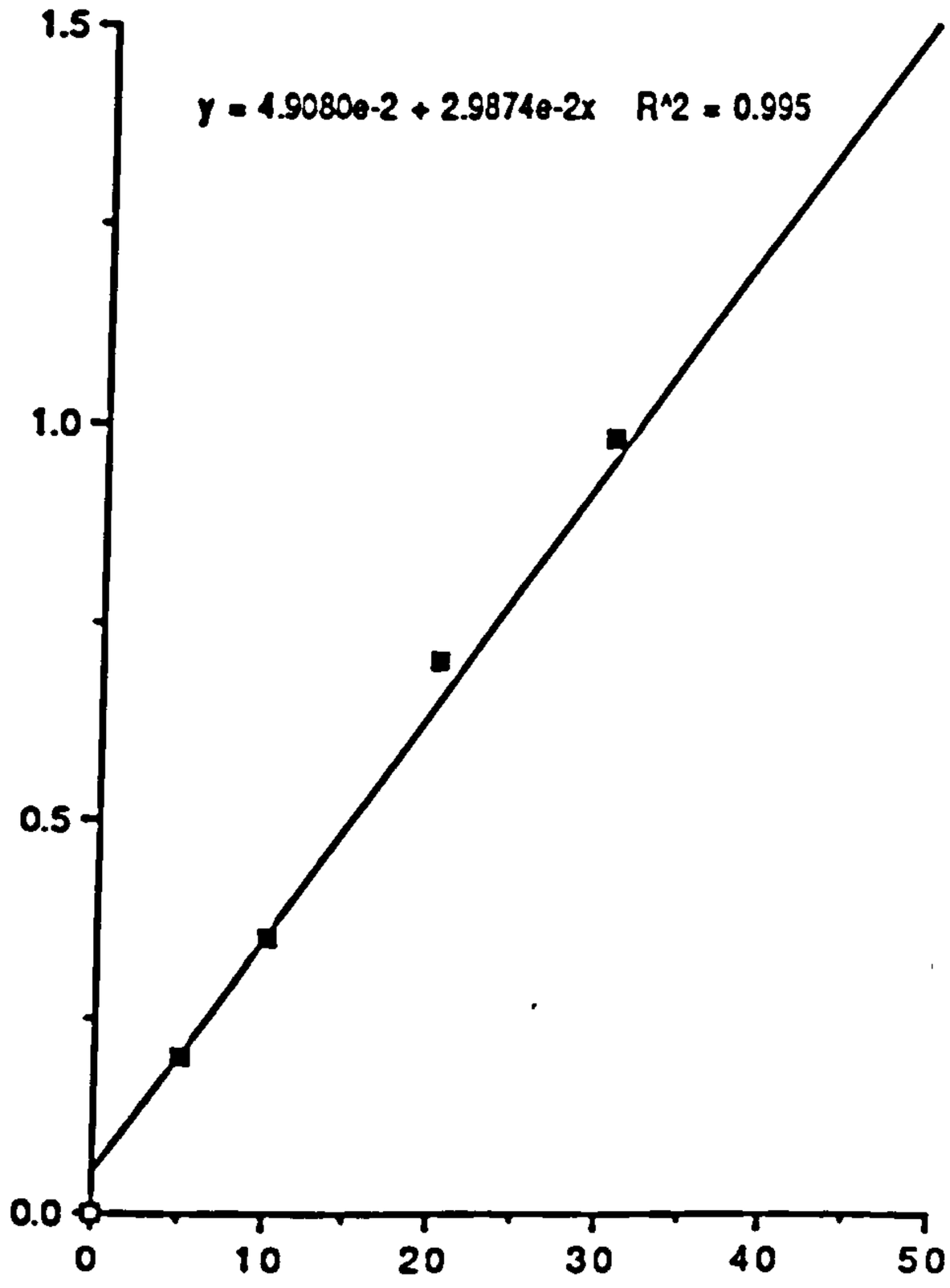
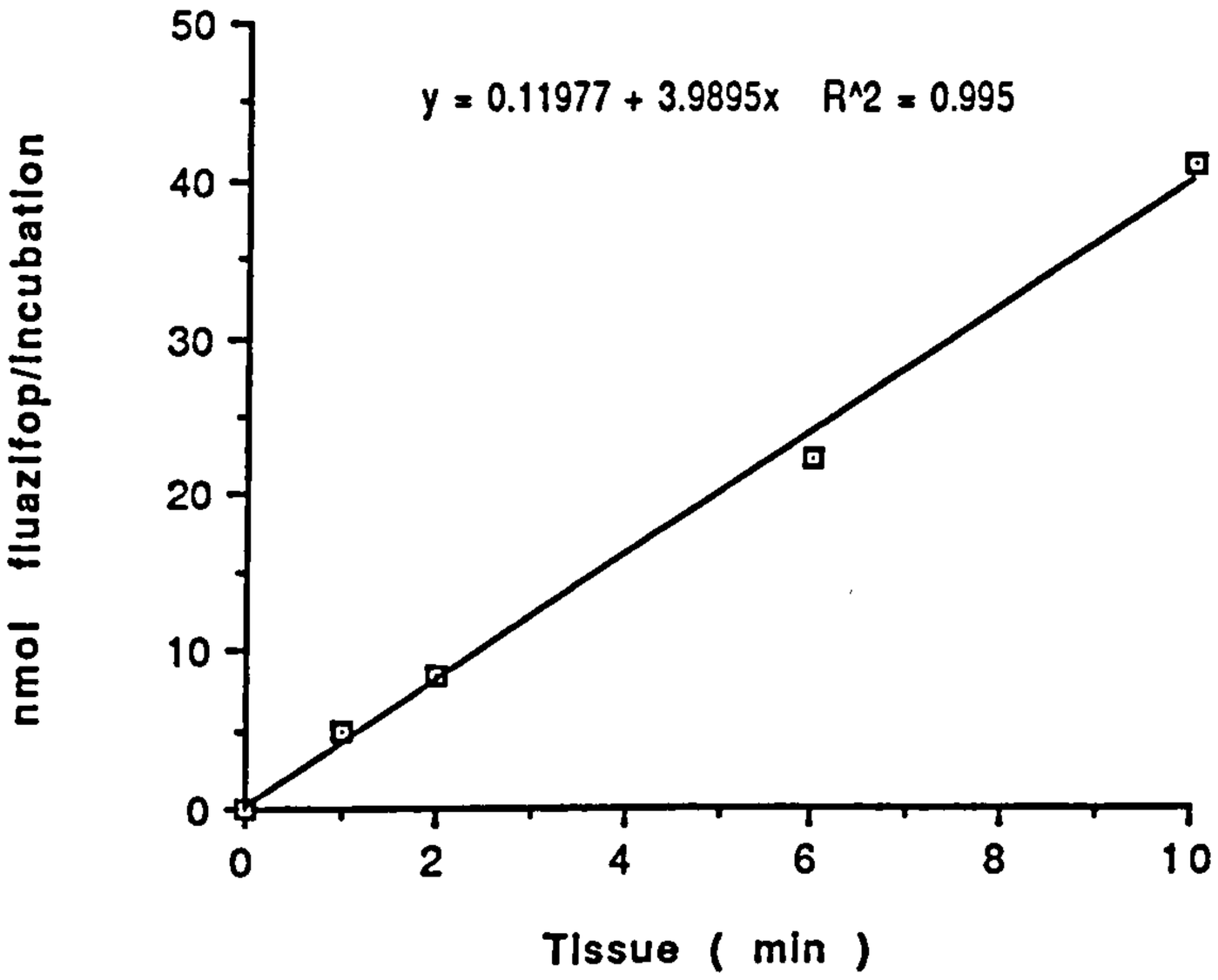


Figure 14.3.2.

Rate of fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/minute) in the presence of varying human plasma (■) (0-50 µl/incubation).
Each point represents a mean of duplicate determinations.



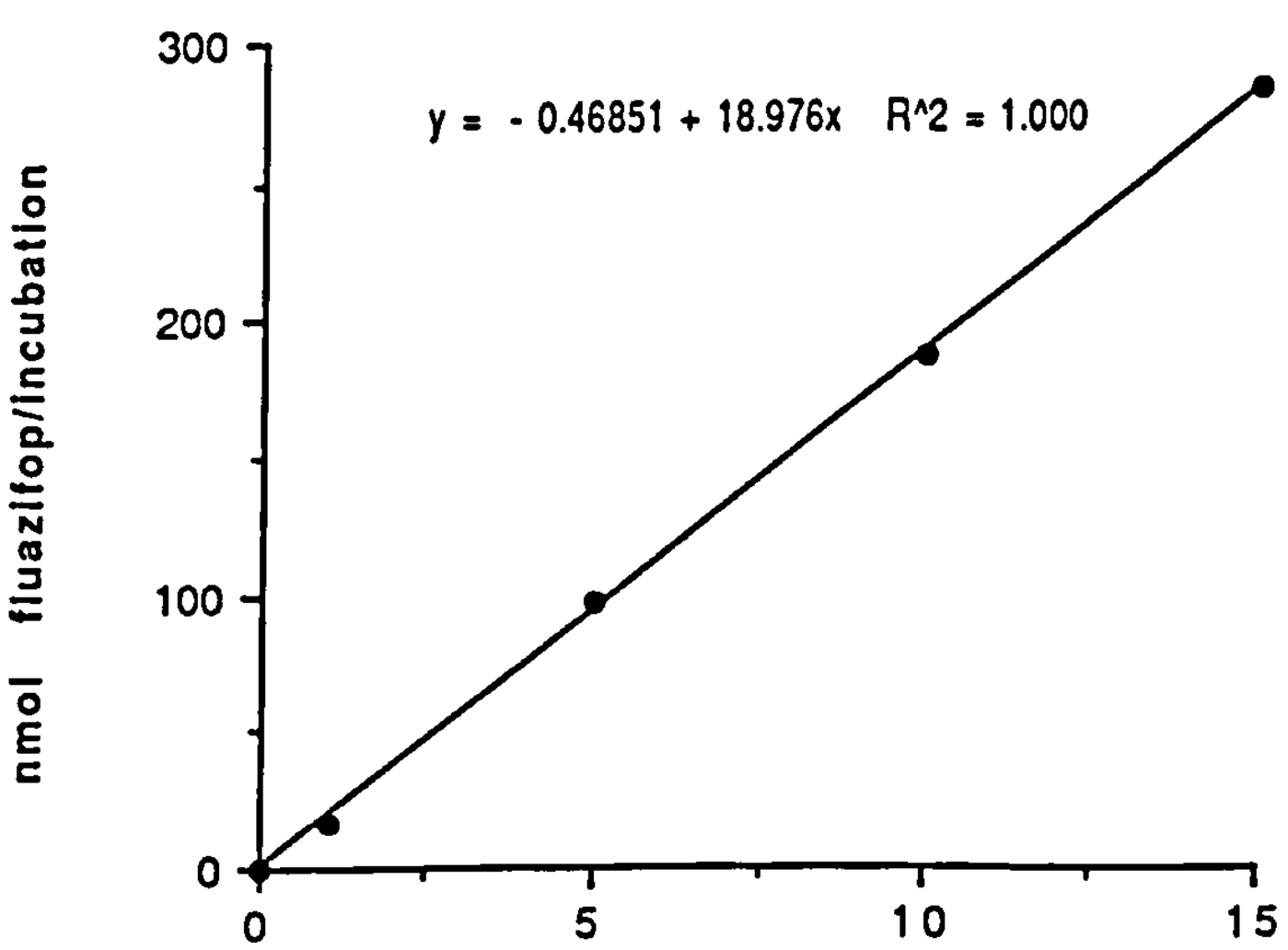


Figure 14.3.3.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by human liver microsomes (⧈) and cytosol (●) (2mg/incubation) for varying times of hydrolysis

Each point represents a mean of duplicate determinations.

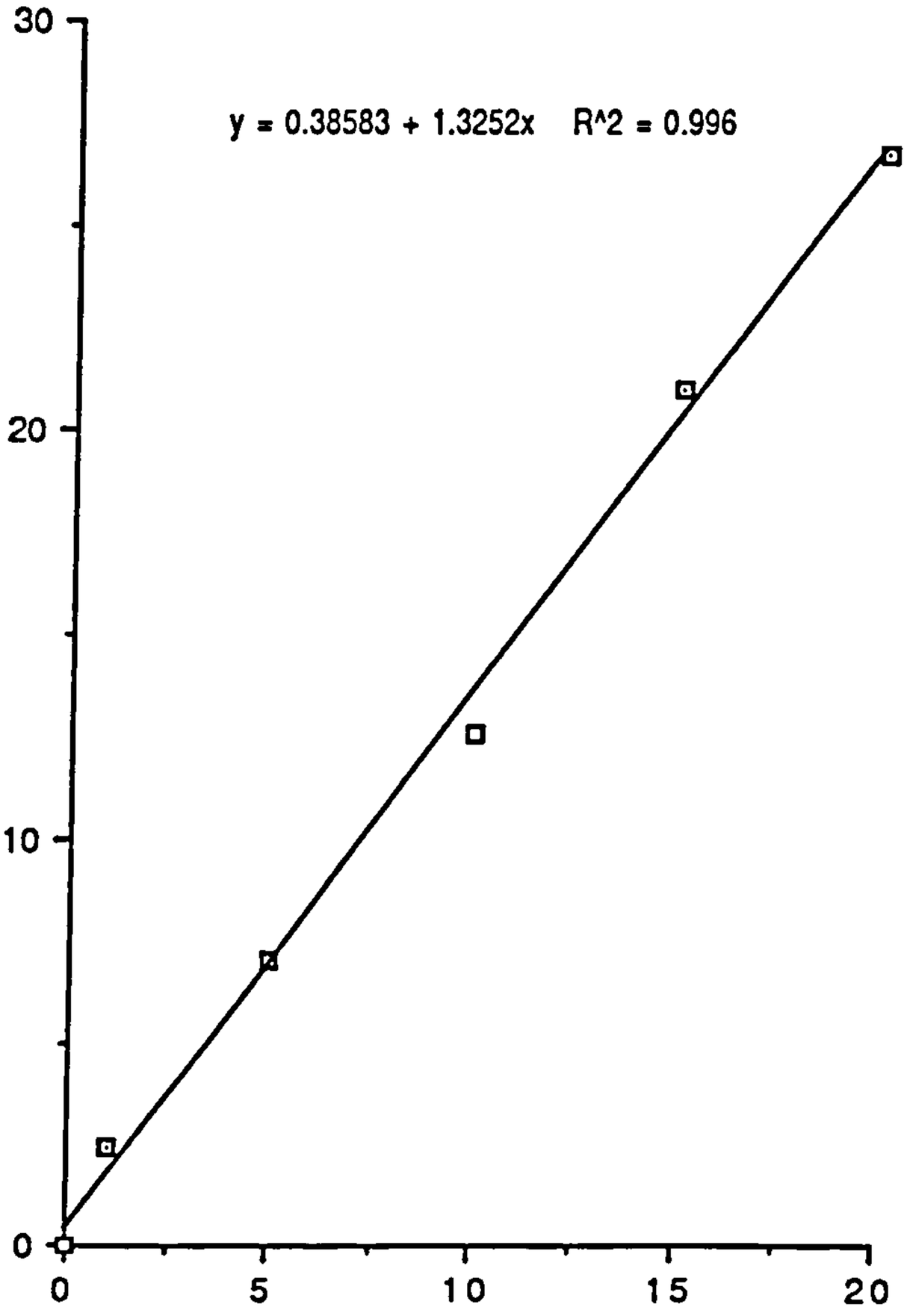


Figure 14.3.4.

Fluazifop-butyl (1mM) hydrolysis to fluazifop (nmol/incubation) by human plasma (50µl/incubation) for varying times of hydrolysis

Each point represents a mean of duplicate determinations.

incubution.

14.3.ii. Paraoxonase assay

Chemicals (see chapter 9.4.i.)

Incubations

Microsomal or cytosolic protein, between 1-20mg of liver original wet weight was incubated with paraoxon (0.05-1mM). Incubations were carried out in a final volume of 500µl of 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37°C.

Reactions were started by the addition of paraoxon and stopped with the addition of an equal volume of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of supernatant was injected onto reverse phase HPLC. Control incubations containing 50mM trisma buffer pH 8.0 and paraoxon, but no tissue were carried out in parallel to access spontaneous hydrolysis. Conditions for the HPLC assay of paraoxon and p-nitrophenol were as previously described (see chapter 9.4.iii.).

Using a fixed concentration of paraoxon and time of incubation, varying amounts of microsomal protein from liver microsomes (see Figure 14.3.5.). were used in the incubation to establish conditions of linearity for protein. Having established a fixed amount of protein for the incubation, the time of hydrolysis was varied (0-30 minutes) with a fixed paraoxon concentration to establish conditions of linearity for time with liver microsomes (see Figure 14.3.6.). No cytosolic paraoxonase activity

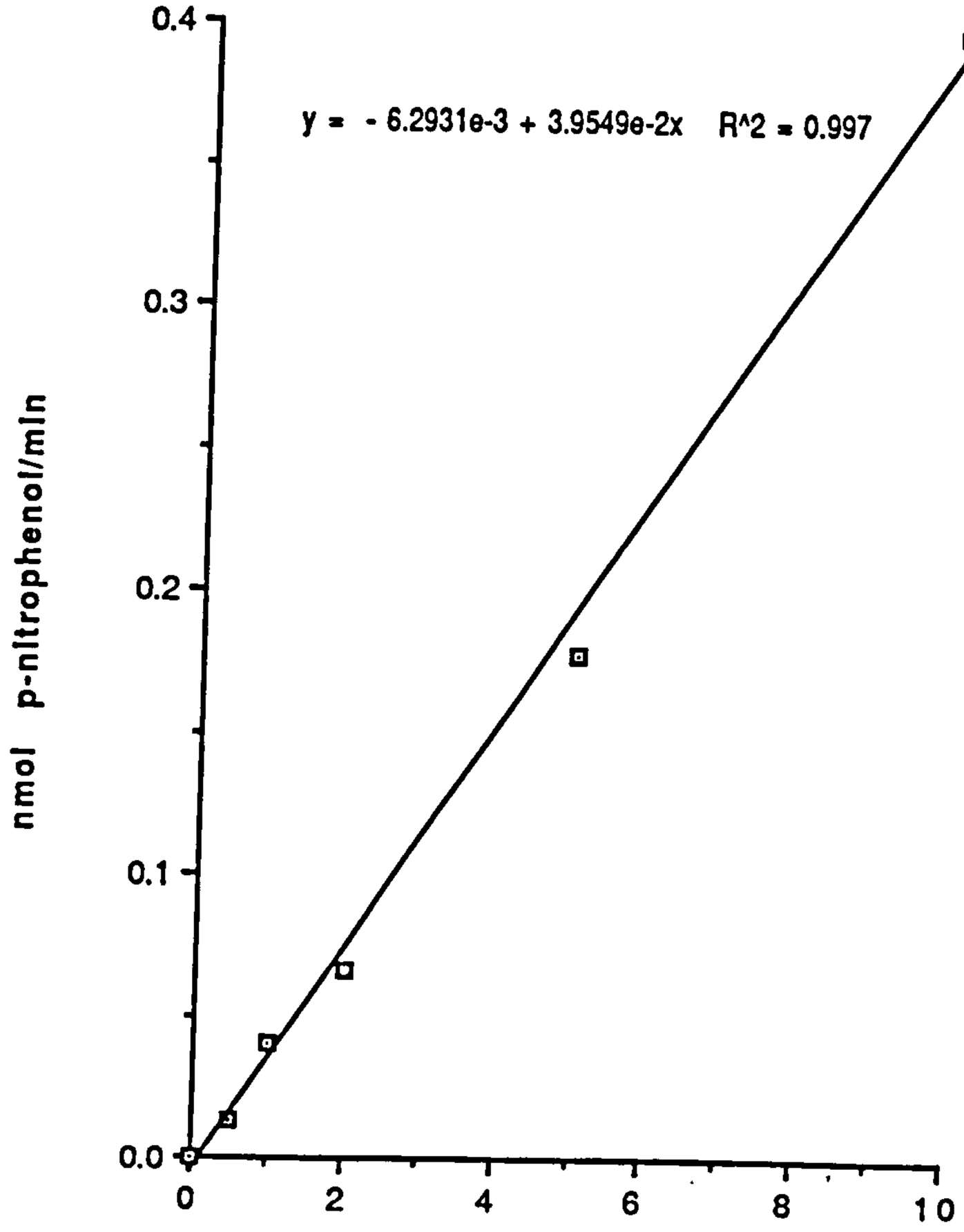


Figure 14.3.5.

Rate of paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/minute) in the presence of varying human liver microsomes (0-10 mg/incubation).
Each point represents a mean of duplicate determinations.

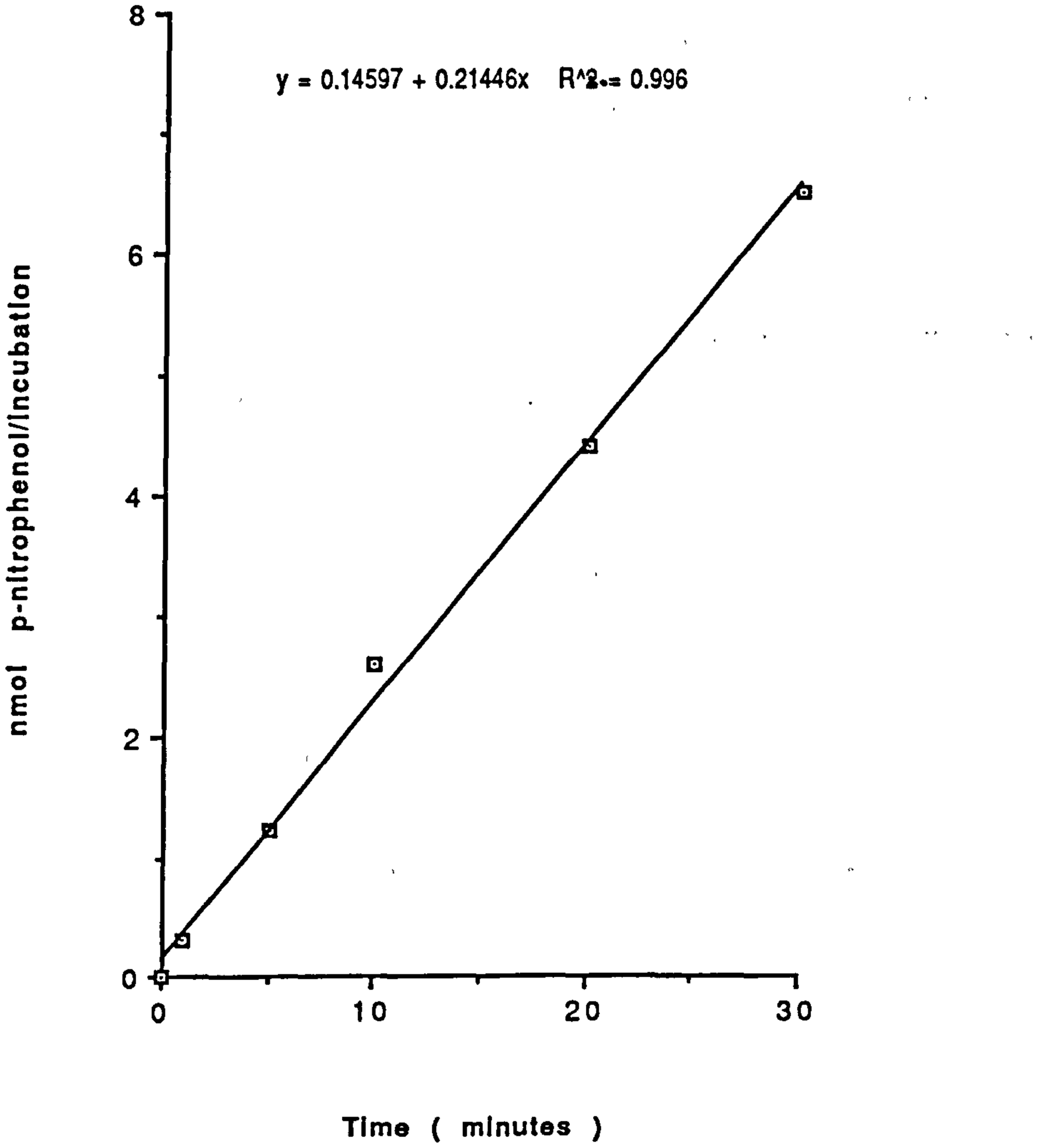


Figure 14.3.6.

Paraoxon (1mM) hydrolysis to p-nitrophenol (nmol/incubation) by human liver microsomes (5mg/incubation) for varying times of hydrolysis
Each point represents a mean of duplicate determinations.

was detectable.

The conditions of linearity for liver tissue, with constant time (10 mins) and paraoxon concentration (1mM) were 2mg of liver original wet weight or 50µl of plasma. The conditions of linearity for time, with constant protein and paraoxon concentration were 15 minutes for liver and plasma. The sensitivity of the paraoxon assay was 1nmol/ incubation.

The results for the spontaneous hydrolysis of paraoxon were the same as those found in the rat studies (see Table 14.3.).

Incubation of Human Plasma and Red Blood Cells

The hydrolysis of paraoxon to p-nitrophenol in the plasma was measured using a modification of the Eckerson et al, (1983) method. 10-50µl of plasma or red blood cells were incubated with paraoxon. Incubations were carried out in a final volume of 3ml 50mM glycine buffer pH10.5, containing 1mM calcium chloride at 30°C. Reactions were started by the addition of paraoxon. Cuvettes were inverted and placed in the spectrophotometer.

The formation of p-nitrophenol was monitored continuously at a wavelength of 412nm on a Kontron UV spectrophotometer. Control incubations containing 50mM glycine/ 1mM calcium chloride buffer pH 10.5, but no plasma were conducted in parallel. Measurements were converted from absorbance units / min to nmol p-nitrophenol / min / ml by using the absorbance coefficient for p-nitrophenol.

The amount of plasma was varied until a linear plot was obtained over 3

Table 14.3. Spontaneous hydrolysis of paraoxon

Substrate concentration (μM)	Activity (nmol/min)
50	0.33±0.06
100	1.33±0.14
250	7.70±1.0
500	17.0±2.1
1000	35.0±2.8

mean±SEM (n=2)

limit of sensitivity > 0.3nmol/min

minutes. The reaction rate was detected on the linear portion of the curve. Spontaneous hydrolysis rates for paraoxon are shown on Table 14.3.

14.3.iii. Phenylacetate esterase assay

Chemicals (see chapter 9.5.i.)

Incubations

The method of Kitchen et_al, (1983) was used in the experiment. Microsomal or cytosolic protein, between 0.5-10mg of liver original wet weight or 10-50µl of plasma or red blood cells was incubated with phenylacetate (0.5-4mM). Incubations were carried out in a final volume of 3ml of 50mM trisma buffer pH8.0, containing 0.1mM calcium chloride at 37^{o}C. Reactions were started with the addition of phenylacetate. The cuvettes were inverted and placed in the spectrophotometer. Control incubations containing 50mM trisma buffer pH8.0 and phenylacetate were carried out in parallel to access spontaneous hydrolysis. The monitoring of phenol formation was the same as described in chapter 9.5.iii. The amount of protein or plasma was varied until a linear plot was obtained over 3 minutes. The reaction rate was detected on the linear portion of the curve. The spontaneous hydrolysis results for phenylacetate are shown on Table 14.4.

14.4. PROTEIN ESTIMATION IN HUMAN LIVER FRACTIONS

Protein estimations in the microsomal and cytosolic fractions of the human liver were carried out to determine the recoveries of protein in the experiment after tissue subcellular fractions were prepared from the initial tissue homogenate.

Table 14.4. Spontaneous hydrolysis of phenylacetate

Substrate concentration (mM)	Activity (nmol/min)
0.2	3.9±0.42
0.5	9.8±1.0
1.0	19.5±1.6
2.0	38.2±2.0
3.0	54.1±2.4
4.0	65.9±4.3

mean±SEM (n=2)

limit of sensitivity > 0.23nmol/min

Methods

Protein recoveries were made using a variation on the Lowry et al , (1956) method, by Peterson, (1977) (see chapter 7.5.).

Results

Protein content of the liver microsomal fraction was found to be lower than the cytosol.. The protein recoveries were approximately 1% for liver microsomes (see Table 14.5.) and 5% for liver cytosol (see Table 14.6.).

Discussion

When comparing the protein recoveries from the rat and human liver it can be seen that there is a greater recovery of microsomal protein in the rat (rat: 27.8±2mg/g compared to human: 11.4±0.68mg/g) while in the cytosol there was more cytosolic protein in the human than in the rat (rat: 32.5±1mg/g and human: 46.6±5.3mg/g).

14.5. FLUAZIFOP-BUTYL ESTERASE

Human volunteer studies carried out by Wollen et al, (1990) have shown that following absorption fluazifop-butyl is rapidly hydrolysed to fluazifop acid and only the acid is detectable in the blood and urine. Experiments were carried out to determine what role liver and blood esterases play in its metabolism.

Table 14.5. Protein recoveries for human liver microsomal fraction. Results were expressed as mg/g of tissue protein.

Tissue number	Protein recovery (mg/g)
1	12.2
2	12.4
3	13.9
4	10.6
5	12.4
6	10.4
7	8.2
mean±SEM	11.4±0.68

Table 14.6. Protein recoveries for the human liver cytosolic fraction. Results were expressed as mg/g of tissue protein.

Tissue number	Protein recovery (mg/g)
1	59
2	60
3	47
4	49
5	56
6	30
7	25
mean±SEM	46.6±5.3

Methods

Microsomal and cytosolic protein equivalent to 0.8mg original wet weight or 50µl of plasma and 50µl of lysed red blood cells were incubated with a final concentration of 0.02-1mM fluazifop-butyl. Incubations were carried out in a final volume of 500µl in 50mM trisma buffer pH8, containing 0.1mM calcium chloride at 37°C. Reactions were started by the addition of fluazifop-butyl (5-20µl of a 1mM and 10mM fluazifop-butyl stock in acetonitrile) and stopped after 4 minutes by the addition of 6% perchloric acid, containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of the supernatant was injected onto reversed phase HPLC.

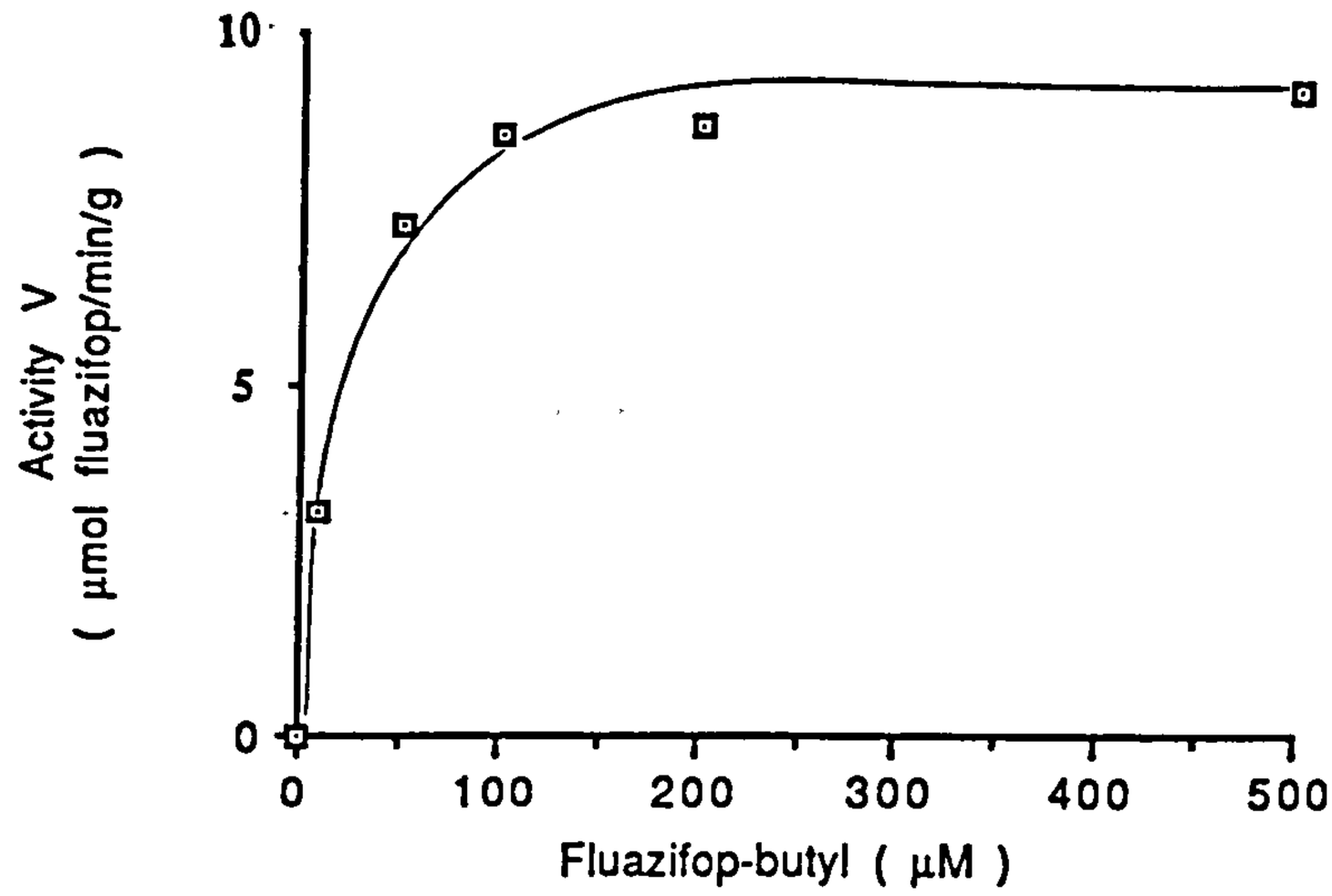
Results were expressed as of µmol/min/g wet weight for liver microsomes and cytosol, µmol/min/ml for plasma and µmol/min/ml lysed cell volume for erythrocytes. Values for Vmax and apparent Km were calculated using the direct linear plot.

Results

In all the tissues studied increases in activity with substrate concentration were seen over the range of concentrations studied. Figure 14.5.1. shows the hydrolysis of fluazifop-butyl by liver cytosolic esterases.Substrate activity curves were similar for liver microsomal and plasma esterase hydrolysis of fluazifop-butyl. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and the apparent Km's were calculated for liver microsomes (see Table 14.7.), liver cytosol (see Table 14.8.), plasma (see Table 14.9.) and erythrocytes (see Table 14.10.).

Results show that fluazifop-butyl hydrolysis is equally distributed in the

(i)



(ii)

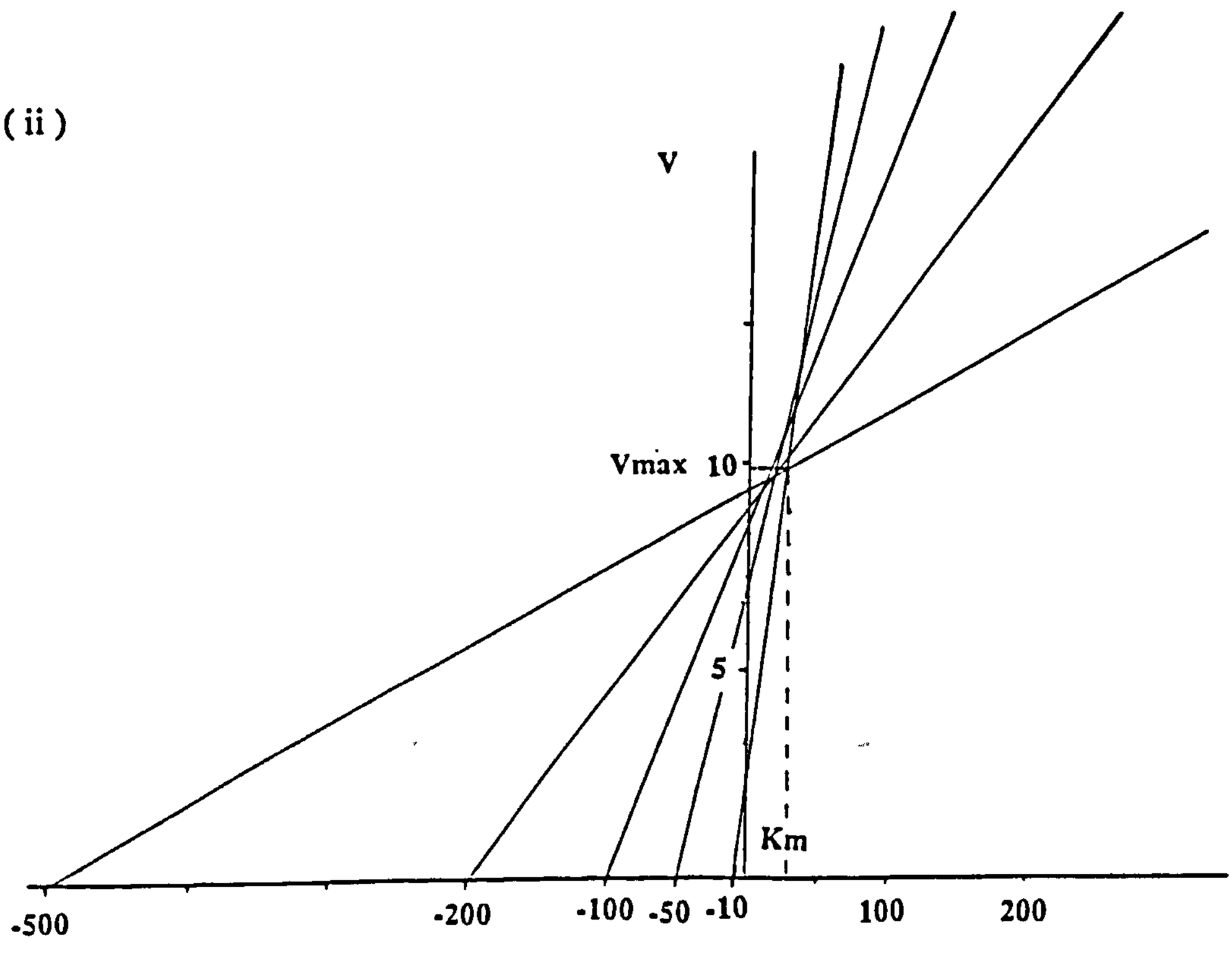


Figure 14.5.1.

Hydrolysis of fluazifop-butyl in human liver cytosol (number 1). Calculation of Vmax (µmol/min/g wet weight) and Km (µM) by (i) plot of activity versus substrate concentration and (ii) direct linear plot.

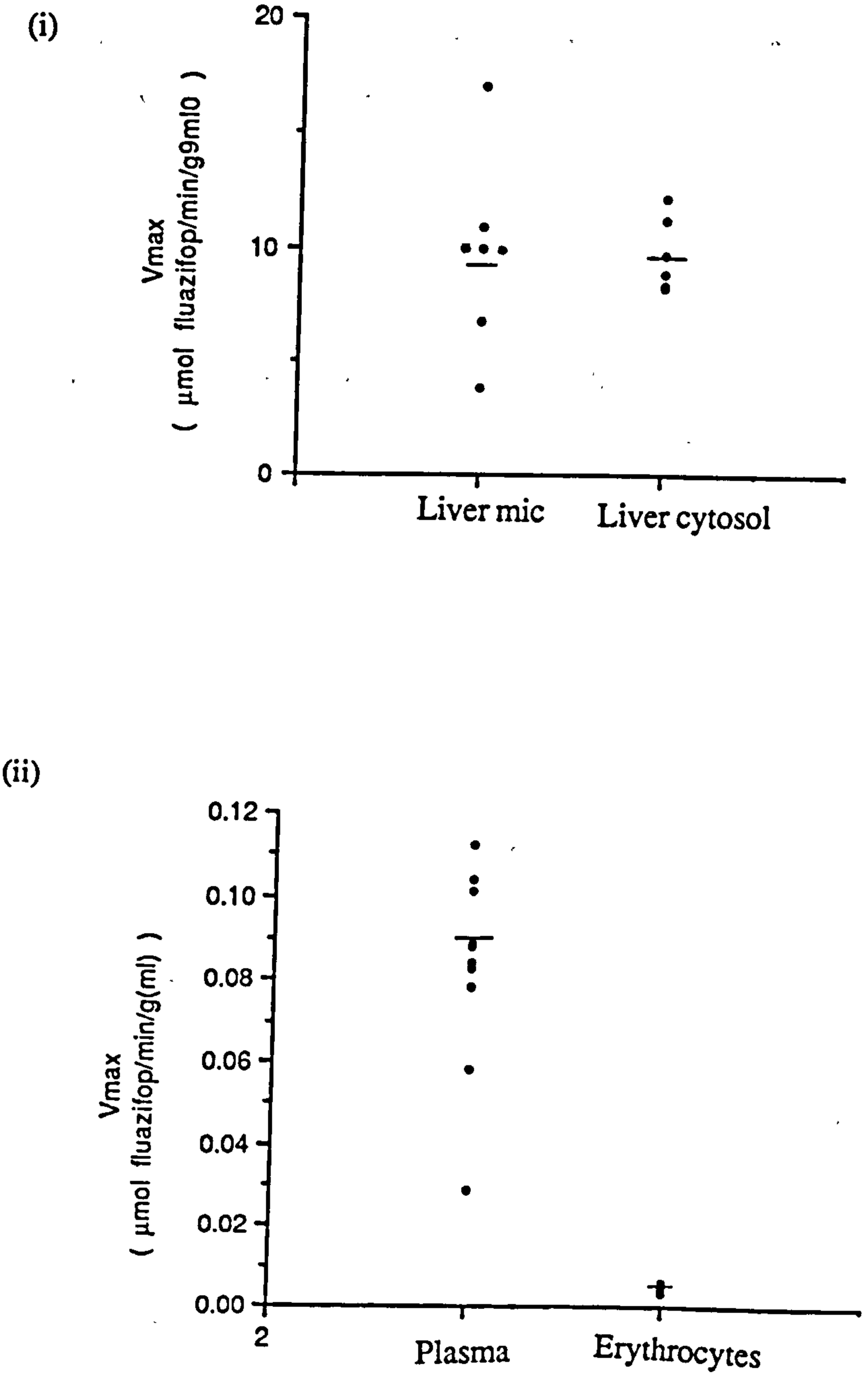


Figure 14.5.2.

Vmax values of fluazifop-butyl hydrolysis for comparison between:

(i) Liver microsomal and cytosolic fractions

(ii) Plasma and erythrocytes

Table 14.7. The hydrolysis of fluazifop-butyl to fluazifop by human liver microsomes. The activities at the substrate concentrations were measured in μmol/min/g wet weight. results were expressed as the Vmax (μmol/min/g wet weight) and the apparent Km (μM) were detected by direct linear plot.

Sample no.	Substrate concentration (μM)					Vmax	K_m
	10	50	100	200	500		
1	2.0	7.8	9.1	11.2	13.8	17	45
2	1.8	5.0	5.3	6.4	7.0	10.0	40
3	1.3	5.6	6.5	6.6	5.9	10.0	45
4	1.8	6.9	7.2	7.5	8.4	10.0	40
5	1.9	5.8	6.6	6.9	8.8	10.9	40
6	1.2	2.2	2.6	2.8	2.7	3.8	20
7	4.7	5.0	5.3	5.6	5.4	6.8	20
mean±SEM						9.8±1.6	36±4.2

limit of sensitivity > 1nmol/min/g wet weight

Table 14.8. The hydrolysis of fluazifop-butyl to fluazifop by human liver cytosol. The activities at the substrate concentrations were measured in μmol/min/g wet weight. Results were expressed as the Vmax (μmol/min/g wet weight) and the apparent Km (μM) were detected by direct linear plot.

Sample no.	Substrate concentration (μM)					Vmax	K_m
	10	50	100	200	500		
1	3.0	6.8	8.1	8.2	8.6	9.7	25
2	1.4	6.1	7.2	7.3	7.8	8.3	30
3	1.5	5.8	6.9	7.4	8.0	8.9	25
4	2.1	8.9	14	11.6	12.2	12.2	30
5	1.6	6.9	8.4	9.1	11.1	11.3	50
6	1.9	7.3	7.9	8.9	10.1	11.2	40
7	1.6	5.3	6.6	7.5	8.1	8.4	25
mean±SEM						10±0.52	32±3.7

limit of sensitivity > 1nmol/min/g wet weight

Table 14.9. The hydrolysis of fluazifop-butyl to fluazifop by human plasma. The activities at the substrate concentrations were measured in nmol/min/ml plasma. Results were expressed as the Vmax (nmol/min/ml plasma) and the apparent Km (μM) were detected by direct linear plot.

Sample no.	Substrate concentration (μM)						Vmax	K_m
	20	50	100	200	400	600		
1	8.2	12.8	17.4	29.0	25.6	25.4	29	65
2	20.0	25.2	73.5	87.0	65.4	65.6	82	65
3	17.0	22.4	66.2	72.5	72.5	81.2	101	65
4	12.8	16.4	37.5	65.0	61.0	65.0	78	110
5	9.0	18.5	34.0	85.0	90.0	80.0	89	140
6	18.0	23.0	52.5	60.0	72.5	70.0	84	80
7	11.0	17.5	35.0	46.2	43.8	45.0	58	90
8	15.0	22.5	48.8	57.5	63.8	75.0	88	110
9	17.5	26.2	65.0	90.0	104	72.5	104	90
10	21.2	32.5	72.5	83.8	95.0	95.0	112	65
mean±SEM							88.3±5.6	88±7.8

limit os sensitivity > 1nmol/min/ml plasma

Table 14.10. The hydrolysis of fluazifop-butyl to fluazifop by human red blood cells .The activity in nmol/min/ml was measured at a concentration of 400µM,by direct linear plot.

Tissue number	Activity at 400µM (nmol / min / ml)
1	3.31
2	4.23
3	5.36
4	3.86
5	5.58
6	4.76
7	3.26
8	4.38
9	3.56
10	4.1
mean±SEM	4.35±0.25

limit of sensitivity > 1nmol/min/ml

microsomal and cytosolic fraction of the liver. The liver seems to contain around a 100 times more esterase activity than the plasma. The red blood cells contain a very small amount of esterase activity . The activity of the fluazifop-butyl esterase expressed in mg of protein were 0.86±0.12 for liver microsomes and 0.21±0.01µmol/min/mg protein in cytosol

The apparent Km's for the liver microsomal (see Table 14.7.)and cytosolic (see Table 14.8.) esterase were found to be very similar. The apparent Km for plasma esterase (see Table 14.9.) was found to be around 2 fold greater than that of the liver fractions.

Discussion

As was found in the rat, esterase activity was equally distributed between the microsomes and cytosol. Comparing the activities it was found that human liver was more active at hydrolysing fluazifop-butyl than the rat esterase. This was found to be the case in both the microsomal (human: 9.8±1.57 and rat: 6.29±0.4µmol fluazifop/min/g) and cytosol (human: 10±0.52 and 6.84±0.85µmol fluazifop/min/g) fractions of the liver. Expressing the results in terms of mg of protein, activities in the rat and human liver cytosol were the same (0.21µmol fluazifop/min/mg protein), whereas they were found to be 4 times as high in the human liver microsomes as in the rat (rat: 0.22±0.005 and human: 0.86±0.13µmol fluazifop/min/mg protein).

Earlier studies carried out in the rat suggested that the enzyme responsible for the hydrolysis of fluazifop-butyl was the 'B' esterase, carboxylesterase. Hydrolysis of fluazifop-butyl in rat by cholinesterase was found to play a minor role. Fluazifop-butyl hydrolysis in human plasma was found to be much lower than that of the rat (human:0.09±0.02 and rat: 5.8 ±0.5µmol fluazifop/min/ml). This is not surprising considering it has been reported that there is very little, if no carboxylesterase activity in the human plasma. Rat plasma does contain a large amount

of carboxylesterase activity, and this is why the fluazifop-butyl hydrolysis activity is greater than in the human plasma. To determine whether carboxylesterase or cholinesterase is responsible for this reduced hydrolytic activity inhibitory studies will need to be carried out.

The apparent Km's of the esterase enzyme for the fluazifop-butyl substrate in the human and rat liver varied by 2 fold in both microsomal (human: 36±4.2 and rat: 14.4±2μM) and cytosol (human: 32±3.7 and rat: 22±5μM) but were similar in the plasma (human: 88±6.6 and rat:182±2.2μM).

14.6. PARAOXON HYDROLYSIS

Paraoxon hydrolysis in the human plasma has been well documented, however, there is not much information on how much hydrolysis takes place in human liver. Experiments were carried out to compare paraoxonase activities in the liver microsomes and cytosol and the plasma and red blood cells of humans.

Method

Liver microsomal and cytosolic protein equivalent to 10mg original wet weight or 50μl of plasma was incubated with a final concentration of 0.1-1mM paraoxon. Incubations were carried out in a final volume of 500μl in trisma buffer pH8, containing 0.1mM calcium chloride at 37°C.

Reactions were started with the addition of paraoxon (5-50μl of 10mM paraoxon stock, freshly prepared in trisma buffer) and stopped after 15 minutes by the

addition of 6% perchloric acid, containing 10µg/ml p-toluic acid. Tubes were vortexed and centrifuged at 5440 x g for 5 minutes. 80µl of the supernatant was injected onto reversed phase HPLC as described (see chapter 9.4.iii.).

Results were expressed as µmol/min/g wet weight for liver microsomes and cytosol, µmol/min/ml for plasma and µmol/min/ml lysed red blood cells. Values for Vmax and apparent Km were calculated using the direct linear plot.

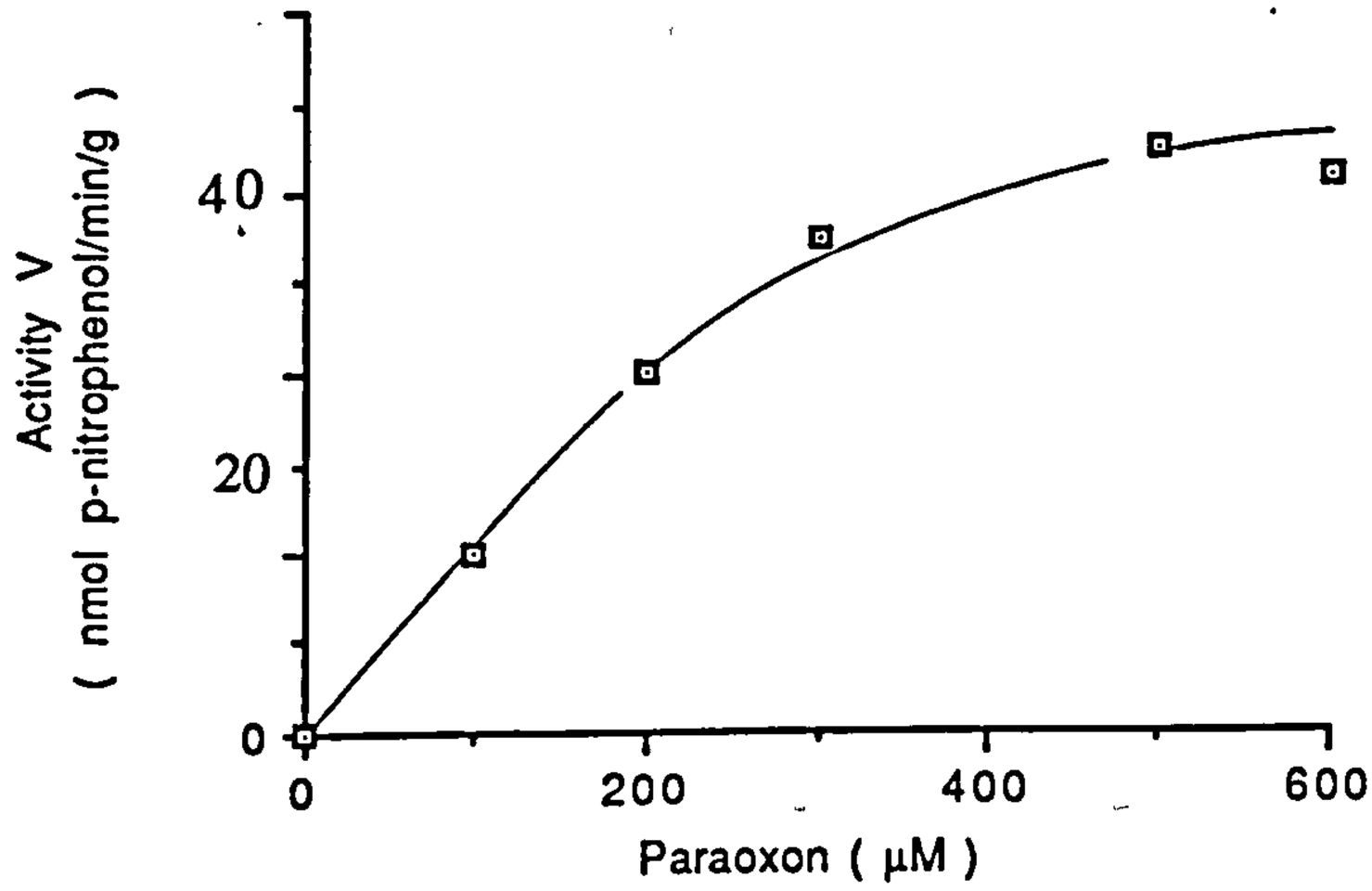
Results

In all the tissues studied increases in activity with substrate concentration were seen over the range of concentrations studied. Figure 14.6.1. shows the hydrolysis of paraoxon by human liver microsomal paraoxonase. This figure was representative of plasma paraoxonase hydrolysis of paraoxon. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and the apparent Km's for were calculated.

Results show that in the human there is paraoxonase activity in the liver microsomes (see Table 14.11.) and plasma (see Table 14.12.). No paraoxonase activity was found in the cytosolic fraction of the liver or in the red blood cells. The plasma was found to contain 4 times more paraoxonase activity than the liver microsomes. When expressed on a weight/volume basis. These results can be seen on Figure 14.6.2. The liver microsomal paraoxonase, expressed in nmol/min/mg of protein was found to be 4.2±1.

The apparent Km for liver microsomal paraoxonase (see Table 14.11.) and plasma paraoxonase (see Table 14.12.) for paraoxon was found to be similar.

(i)



(ii)

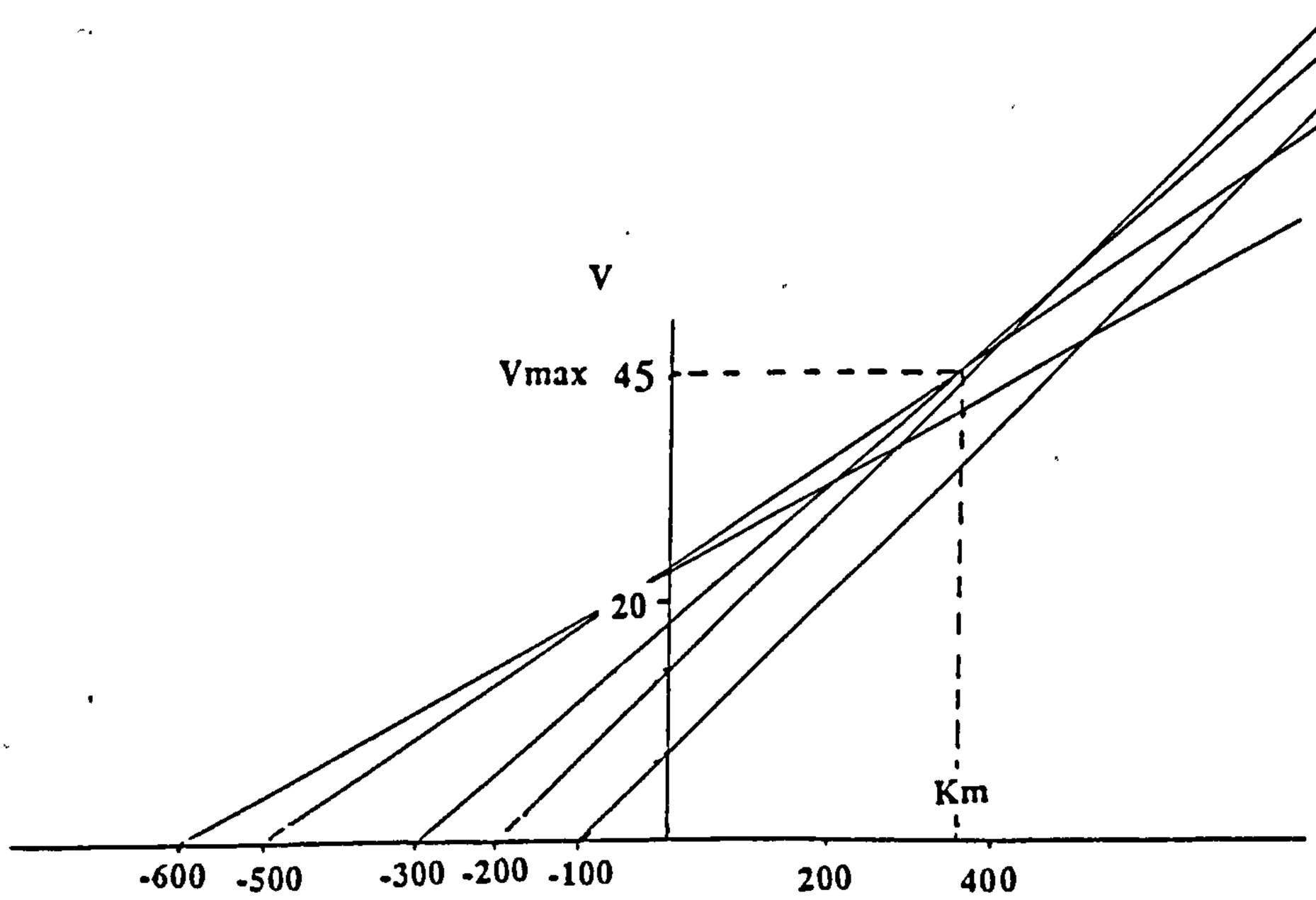


Figure 14.6.1.

Hydrolysis of paraoxon in human liver microsomes (number 4). Calculation of Vmax (nmol/min/g wet weight) and Km (μM) by (i) plot of activity versus substrate concentration and (ii) direct linear plot.

Table 14.11. The hydrolysis of pararoxon to p-nitrophenol by human liver microsomes. The activities at the substrate concentrations were measured in µmol/min/g wet weight. Results were expressed at the Vmax (nmol/min/g wet weight) and the apparent Km (µM) by direct linear plot.

Sample no.	Substrate concentration (µM)					Vmax	K_m
	100	200	300	500	600		
1	16	10.0	13.1	16.3	22.5	28	340
2	7.3	10	14.7	16.3	23.3	48	280
3	5.3	10.6	11.3	16.7	28.3	45	360
4	6.7	13.3	18.3	21.7	20.7	42	260
5	22.5	39.0	43.5	47.0	58.4	74	180
mean±SEM						47.4±7.5	280±23

limit of sensitivity > 0.5nmol/min/g wet weight

Table 14.12. The hydrolysis of pararoxon to p-nitrophenol by human plasma. The activities at the substrate concentrations were measured in µmol/min/ml plasma. Results were expressed at the Vmax (nmol/min/ml plasma) and the apparent Km (µM) by direct plot.

Sample no.	Substrate concentration (µM)					Vmax	K_m
	50	100	200	500	1000		
1	23	31	116	143	166	190	200
2	30	39	149	188	207	245	170
3	27	35	135	171	198	245	220
4	20	32	84	112	132	170	260
5	28	36	153	185	202	230	140
6	21	28	98	128	141	190	230
7	31	44	194	234	263	295	140
8	22	32	106	137	161	200	220
9	12	17	52	78	89	135	400
10	17	27	74	109	130	200	440
mean±SEM						210±14	240±31

limit of sensitivity > 0.5nmol/min/ml plasma

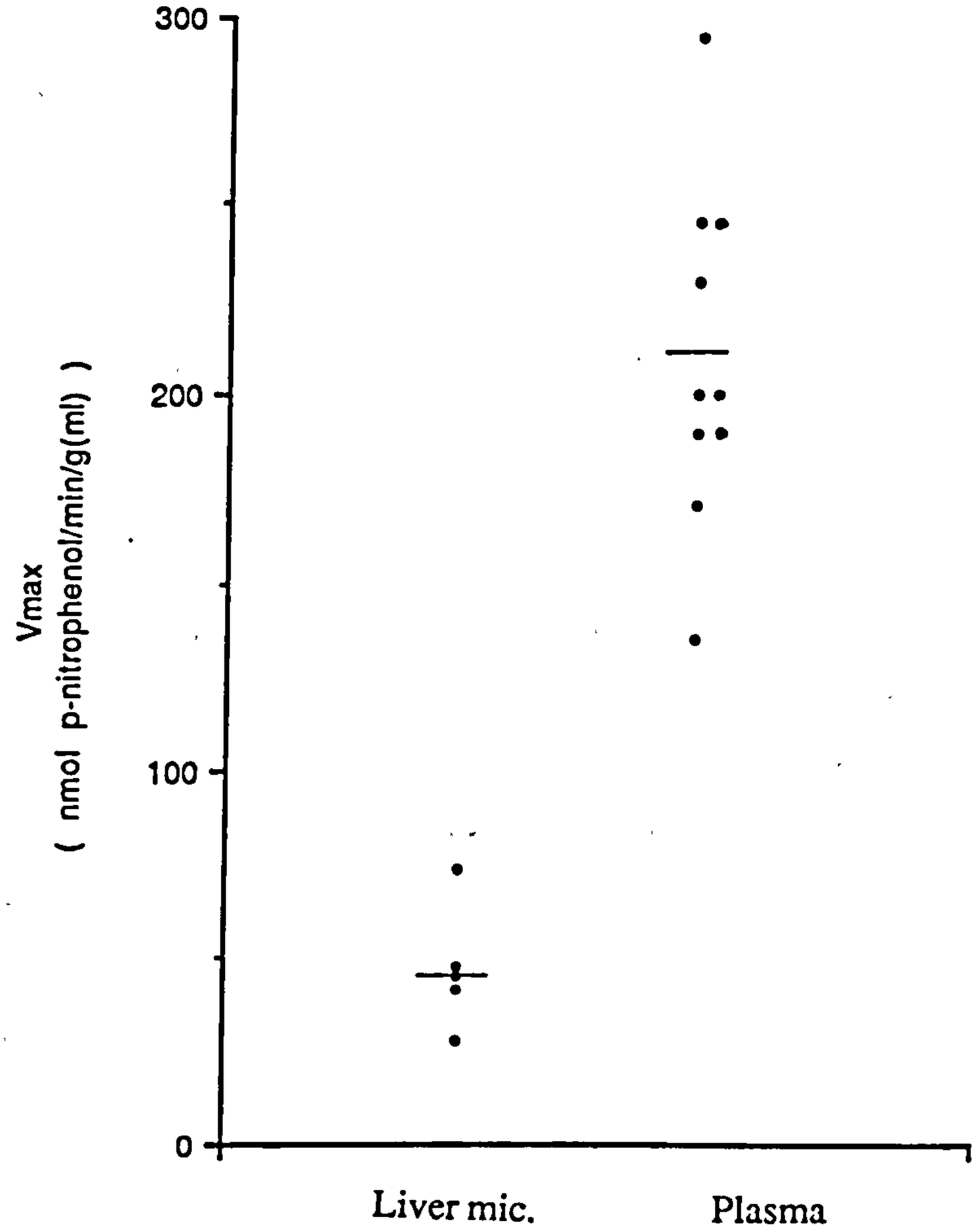


Figure 14.6.2.

Vmax values of paraoxon hydrolysis for comparison between human tissues.

Discussion

Paraoxon hydrolysis in the plasma of the rat and the human was found to be similar (human: 0.21±0.014 and rat: 0.25±0.012µmol p-nitrophenol/min/ml). The apparent Km's for rat and human plasma paraoxonase for the paraoxon substrate were found also to be similar (human: 240±31 and rat: 225±15mM). These results suggest that human and rat plasma contain the same sort of paraoxonase enzyme.

However, paraoxon hydrolysis in the liver microsomes of the rat was around 8 times that of human (human: 0.047±0.0075 and rat: 0.33±0.25 µmol p-nitrophenol/min/g tissue). When this was expressed as mg of protein the rat liver microsomes was found to contain only 3 times as much activity as the human (rat: 11.8±0.68 and human: 4.2±1nmol/min/mg protein). A possible contribution to the large difference in paraoxonase activity between liver microsomal paraoxonase and plasma paraoxonase activity is due to the pH used in the paraoxon assays. In the plasma paraoxonase assay a pH of 10.5 was used, whereas in the liver paraoxonase assay the pH was 8.0. Work carried out by Mallinckrodt and Diepgen, (1988) and Ortigoza-Ferado et al, (1984) showed that using pH 10.5 there was an increase of 60% in paraoxonase activity compared to the activity at pH8.0. The apparent Km's for rat and human liver microsomal paraoxonase for the paraoxon substrate were found to be similar (human: 280±23 and rat: 200±7.5mM). Human and rat liver contain a similar paraoxonase enzyme with different activities.

As was found in the rat liver cytosolic fraction, no paraoxonase activity was found in the human liver cytosolic fraction of the liver. These results seem to indicate that the distribution of paraoxonase in the rat and human liver and plasma is very similar. Work carried out by Fredricksson et al, (1961) had found that although no paraoxonase activity was detected in the skin of the rat activity was detected in the skin of the human. These results, however, do not seem to fit in to the general picture of esterase activity in the human compared to that of the rat. Throughout the study with

the exception of fluazifop-butyl esterase in the liver microsomes, esterase activity in the human has been lower than in the rat. If this is in fact the case we would not expect to see any paraoxonase activity in the skin of the human.

14.7. PHENYLACETATE HYDROLYSIS

Phenylacetate hydrolysis was monitored in the microsomal and cytosolic fractions of the liver and in the plasma and erythrocytes to determine the amount and distribution of arylesterase activity in the human.

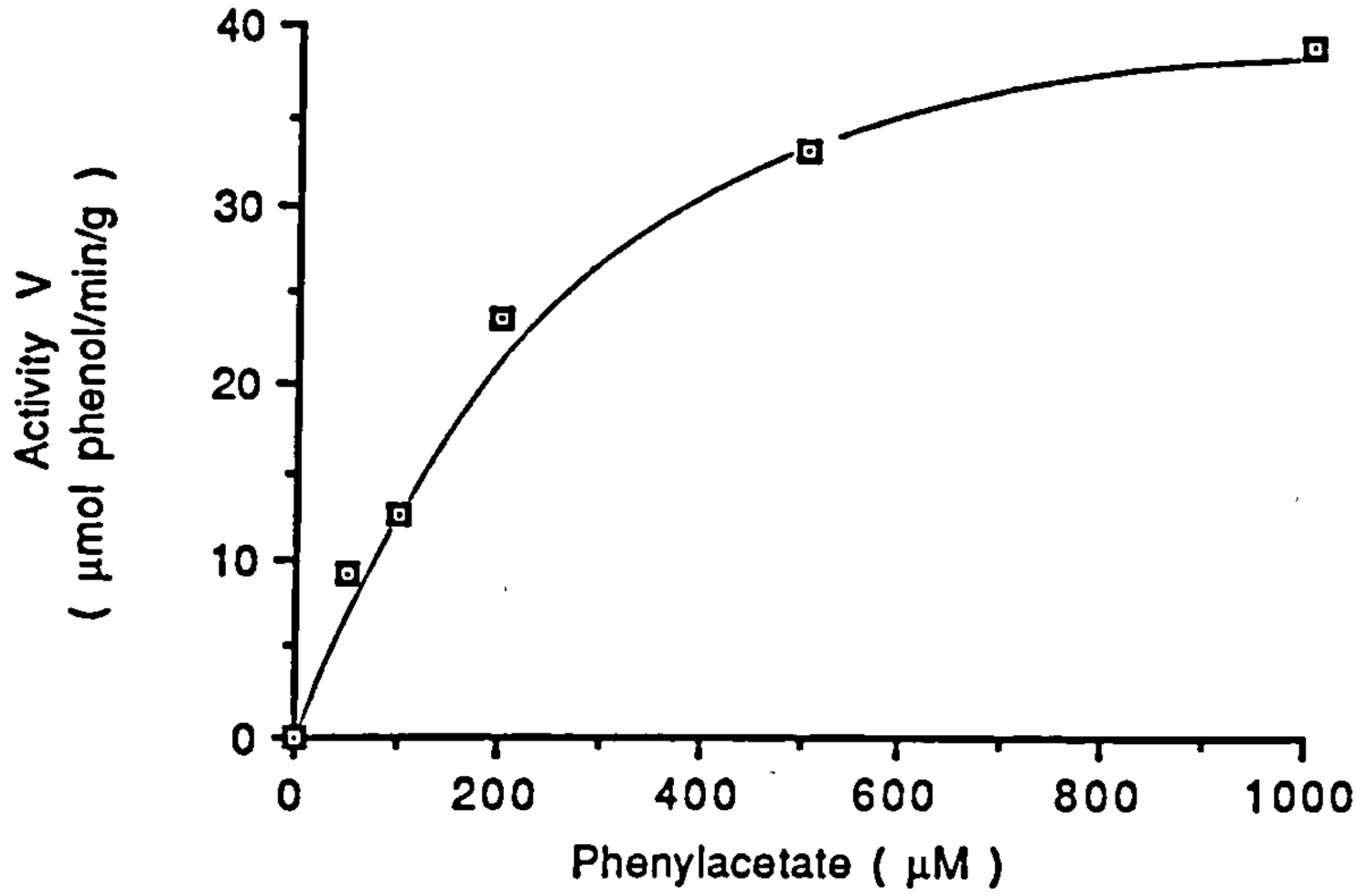
Methods

Microsomal and cytosolic protein equivalent to 3mg or 50µl of plasma and 50µl of red blood cells were incubated with a final concentration of 0.5-4mM phenylacetate. Incubations were carried out in a final volume of 3mls in 50mM trisma buffer pH8, containing 0.1mM calcium chloride at 37^{o}C. Reactions were started with the addition of phenylacetate (5-20µl of a 600mM phenylacetate stock in DMSO). The cuvettes were inverted and then placed in the spectrophotometer. Results were expressed in terms of µmol/min/g wet weight for liver microsomes and cytosol, µmol/min/ml for plasma and µmol/min/ml for lysed red blood cells. Values for Vmax and apparent Km were calculated using the direct linear plot.

Results

In all the tissues studied increases in activity with substrate concentration were seen over the range of concentrations studied. Figure 14.7.1. shows the hydrolysis of phenylacetate by human liver microsomes. This figure is representative of

(i)



(ii)

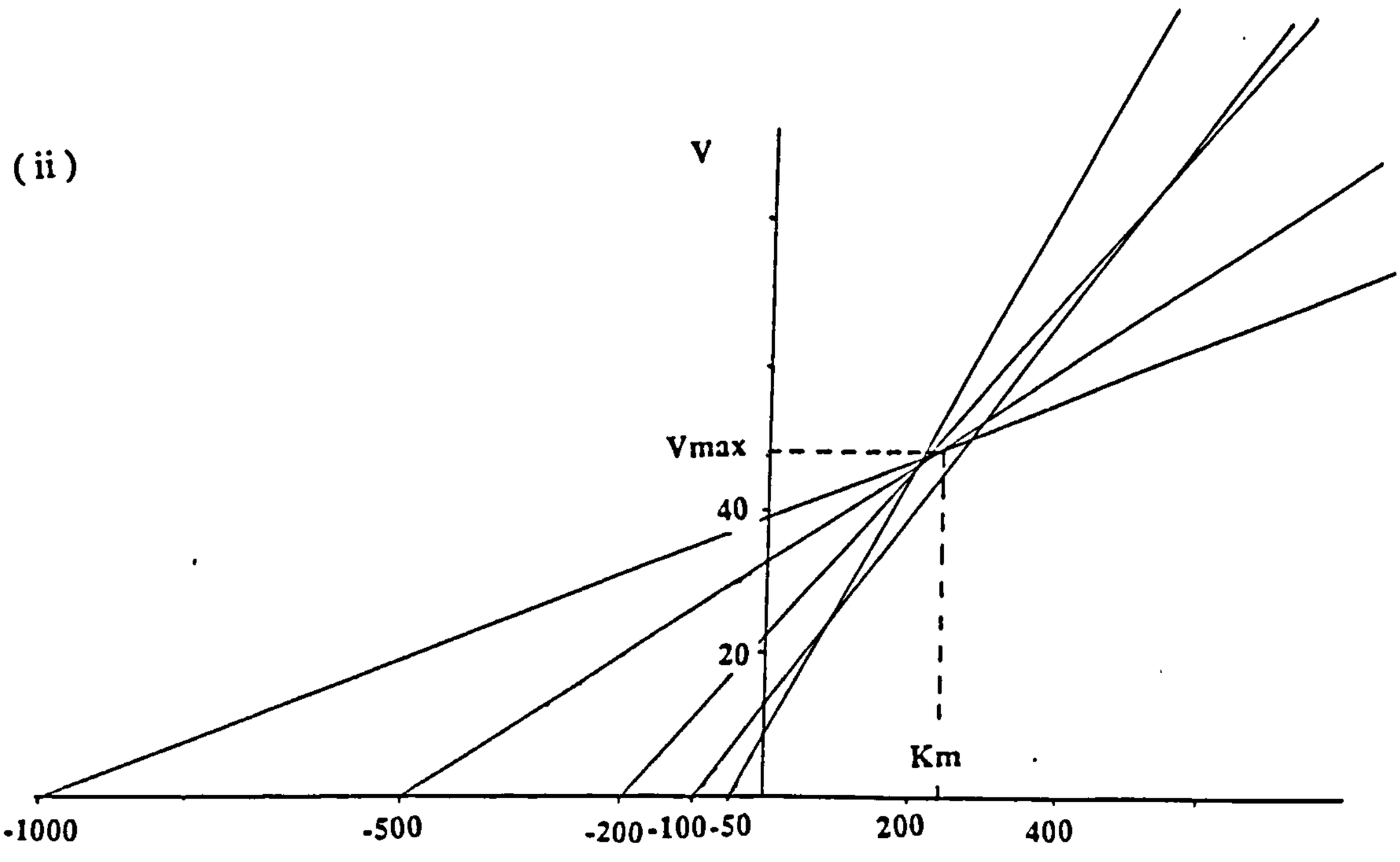


Figure 14.7.1.

Hydrolysis of phenylacetate in human liver microsomes (number 7). Calculation of Vmax (µmol/min/g wet weight) and Km (µM) by (i) plot of activity versus substrate concentration and (ii) direct linear plot.

liver cytosol and plasma esterase hydrolysis of phenylacetate. Activity increases could be described by Michaelis Menton Kinetics. Values for Vmax and the apparent Km's for liver microsomes (see Table 14.13.), cytosol (see Table 14.14.) and plasma (see Table 14.15.) were calculated.

Results seem to indicate that arylesterase is equally distributed in the liver microsomes and cytosol. Expressed in terms of mg of protein the activities of phenylacetate esterase in the liver cytosol were 0.8±0.13µmol/min/mg and liver microsomes 5.0±1.0µmol/min/mg of protein. Plasma arylesterase was found to be much greater than that found in the liver (see Figure 14.7.2.).

The apparent Km's for liver microsomal (see Table 14.13.) and cytosolic (see Table 14.14.) esterase for phenylacetate were found to be very similar. In the plasma (see Table 14.15.) the apparent Km value of plasma esterase for phenylacetate was found to be much greater (8 fold) than that found in the liver fractions.

Discussion

Arylesterase activities in the human liver microsomes were much less than those found in the rat (human: 57± 8 and rat: 800±75µmol phenol/min/g), whereas in the liver cytosol activities for human and rat arylesterase were similar (human: 37±2.9 and 30.7±3.0µmol phenol/min/g).When these results were expressed in mg of protein results in the liver cytosol were similar (rat: 0.85±0.1 and human: 0.8±0.13µmol/min/mg), whereas in the microsomes there was 5 times as much activity in the rat as in the human (rat: 28.6±4.5 and human: 5±1.0µmol/min/mg). Arylesterase in the human and rat plasma were similar (human: 250±17 and rat: 290±40µmol phenol/min/ml).

Table 14.13 The hydrolysis of phenylacetate to phenol by human liver microsomes. The activities at the substrate concentrations were measured in μmol/min/g wet weight. Results were expressed as the Vmax (μmol/min/g wet weight) and the apparent Km (μM) were detected by direct linear plot.

Sample no.	Substrate concentration (μM)					Vmax	K_m
	50	100	200	500	1000		
1	10.0	16.2	23.5	40.0	62.6	72	380
2	8.7	15.0	21.1	32.7	40.5	50	240
3	11.2	19.2	25.7	40.4	56.4	66	350
4	11.0	17.2	27.1	46.7	55.8	74	340
5	10.4	18.6	27.6	42.9	54.8	72	340
6	6.9	8.6	10.7	13.4	16.9	16	100
7	9.3	12.6	23.5	33.0	39.0	48	240
mean±SEM						57±8	280±35

limit of sensitivity > 0.3μmol/min/g wet weight

Table 14.14. The hydrolysis of phenylacetate to phenol by human liver cytosol. The activities at the substrate concentrations were measured in μmol/min/g wet weight. Results were expressed as the Vmax (μmol/min/g wet weight) and the apparent Km (μM) were detected by direct linear plot.

Sample no.	Substrate concentration (μM)					Vmax	K_m
	50	100	200	500	1000		
1	12.0	18.0	25.0	28.5	39.0	39	200
2	8.0	12.6	19.2	29.0	30.0	40	220
3	7.5	12.2	11.0	26.0	29.0	33	170
4	8.2	14.0	24.4	36.0	40.0	52	280
5	8.2	12.0	17.5	29.4	30.0	36	220
6	9.5	14.6	17.8	26.0	29.4	32	130
7	9.4	12.4	14.8	24.7	26.0	28	140
mean±SEM						37±2.9	190±25

limit of sensitivity > 0.3μmol/min/g wet weight

Table 14.15. The hydrolysis of phenylacetate to phenol by human plasma. The activities at the substrate concentrations were measured in µmol/min/ml plasma. Results were expressed as the Vmax (µmol/min/ml plasma) and the apparent Km (mM) were detected by direct linear plot.

Sample no.	Substrate concentration (µM)						Vmax	K_m
	0.2	0.5	1	2	3	4		
1	26	43	107	166	176	181	320	1.55
2	30	50	129	176	194	232	250	1.55
3	27	43	126	139	110	150	190	1.10
4	19	32	88	119	131	146	195	1.35
5	32	50	116	153	204	240	310	1.70
6	21	33	85	134	146	177	240	1.60
7	34	54	143	180	187	227	300	1.20
8	24	37	93	145	163	183	280	1.70
9	21	43	101	151	175	186	145	1.10
10	25	40	95	145	165	185	275	1.90
mean±SEM							250±17	1.48±0.09

limit of sensitivity > 0.3µmol/min/ml plasma

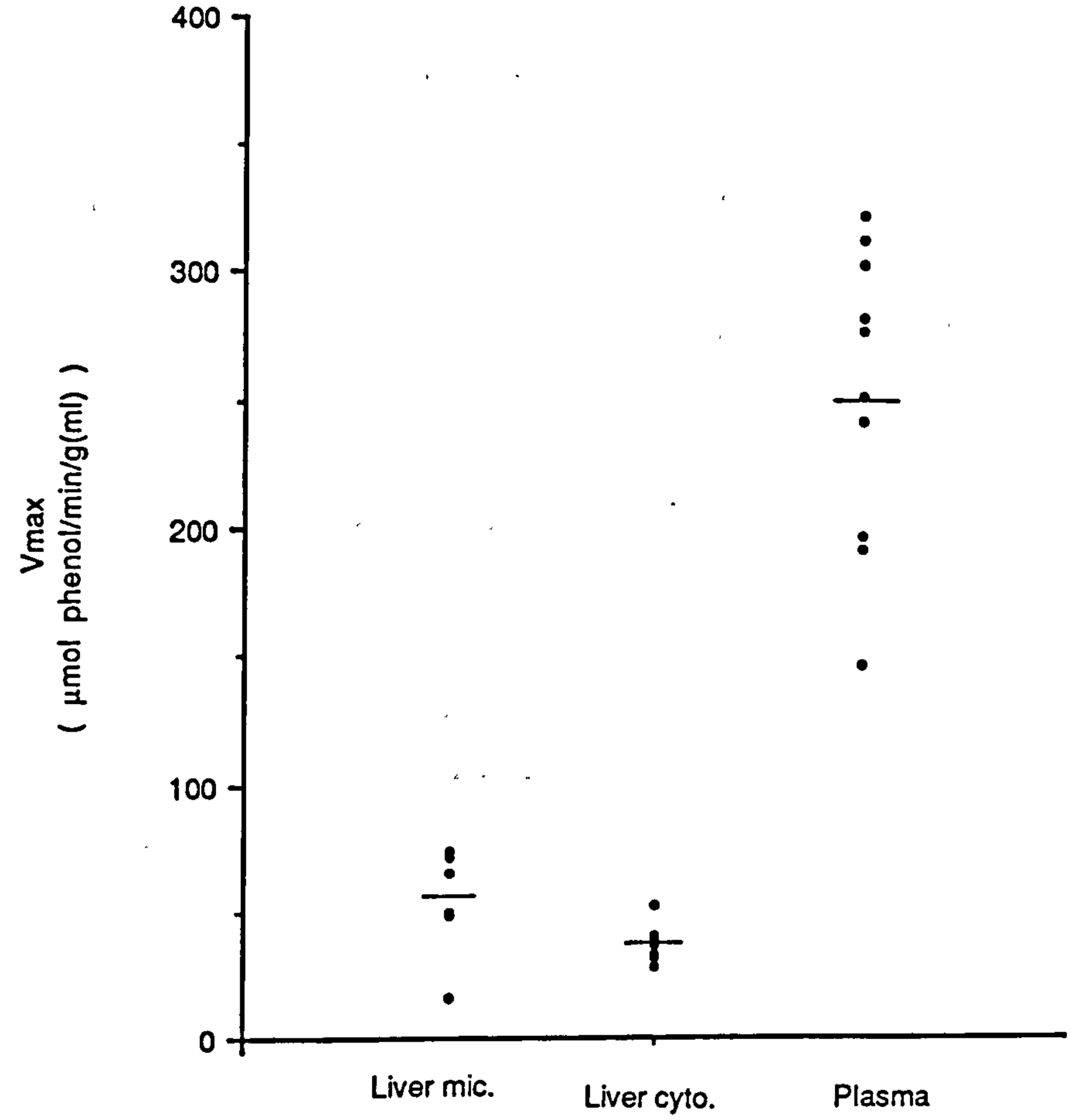


Figure 14.7.2.

Vmax values of phenylacetate hydrolysis for comparison between human tissues.

The apparent Km's for liver microsmal arylesterase in the human and rat are very different (human: 0.28±0.035 and rat: 1.43±1.4mM) as are values for liver cytosol (human: 0.19±0.025 and rat: 0.9±0.5mM). Apparent Km values for human and rat plasma arylesterase were similar (human: 1.48±0.09 and rat: 2.34±0.4mM).

The results indicate that the enzymes hydrolysing phenylacetate in the liver microsomes are different in the human and rat. The apparent affinities of the enzymes for phenylacetate vary 5 fold between the human and rat. Enzymatic activities also show less arylesterase in the human sample compared to the rat, with the rat having more than 15 times the activity of the human. In the cytosol the activities were found to be the same in the human and rat, although apparent affinities varied by 4 fold between the human and rat. Plasma arylesterase activities were similar in the human and rat with apparent affinities only varying slightly.

Inhibitory studies should be carried out to distinguish the actual enzymes acting to hydrolyse phenylacetate in the liver microsomal and cytosolic fractions as well as the plasma.

CHAPTER 15 : INHIBITORY STUDIES IN THE HUMAN

15.1. INTRODUCTION

Having already established that there is esterase hydrolysis of the fluazifop-butyl, paraoxon and phenylacetate substrates in the human liver and plasma, inhibitory studies were carried out using paraoxon, BNPP, $HgCl_2$ and physostigmine.

15.2. FLUAZIFOP-BUTYL ESTERASE INHIBITION

Inhibitory studies in the rat indicated that fluazifop-butyl hydrolysis to fluazifop was due to carboxylesterase. Using the inhibitors paraoxon, BNPP, mercury chloride and physostigmine the esterase enzymes responsible for fluazifop-butyl hydrolysis in the human liver microsomal and cytosolic fraction as well as the plasma were determined.

Method

Incubations were carried out in a final volume of 500µl in 50mM trisma buffer pH8.0, at 37°C. Microsomal or cytosolic protein equivalent to 2mg or 50µl of plasma were incubated along with a final concentration of 0.1mM paraoxon, 0.1mM mercuric chloride, 0.1mM BNPP or 0.1mM physostigmine and 25µM fluazifop-butyl as substrate. Control incubations without any inhibitors in the incubation were carried out in parallel.

Reactions were started by the addition of fluazifop-butyl (12.5µl of 1mM stock in acetonitrile) and stopped after 10 minutes by the addition of 6% perchloric acid containing 10µg/ml p-toluic acid (internal standard). Tubes were vortexed and

centrifuged at 5440 x g for 5 minutes. 80µl of the supernatant was injected onto reversed phase HPLC (see chapter 9.2.iii.).

Incubations containing the inhibitors of paraoxon, BNPP, mercuric chloride and physostigmine were compared directly to control incubations. Conversion to product in the presence of inhibitor was determined as previously described (see chapter 9.2.ii.). Inhibition was estimated by expressing remaining activity in the presence of the inhibitor as a percentage of the control activity.

Results

Paraoxon completely inhibited fluazifop-butyl hydrolysis in the human liver microsomes, cytosol and plasma (see Figure 15.1.). Inhibition of the liver microsomal an cytosolic fractions and plasma were very similar to those found with paraoxon (inhibition of the liver microsomal and cytosolic fraction by BNPP was 86% and 92%, whereas in the plasma it was 66%). Mercuric chloride did not significantly inhibit the hydrolysis of fluazifop-butyl by esterases in the human liver microsomal and cytosolic fractions (inhibition of the liver microsomal and cytosolic fractions by mercury chloride was 3%). Inhibition of the liver microsomal and cytosolic esterase by physostigmine was lower than that of BNPP inhibition (Inhibition of the liver microsomes and cytosol fraction by physostigmine was 14% and 11%), whereas in the plasma the inhibition was found to be greater (inhibition was 41%). These results can be seen on Table 15.1. and Figure 15.1.

Discussion

Paraoxon is an inhibitor of 'B' esterases, and the results show that there is complete inhibition of fluazifop-butyl hydrolysis in the liver microsomal and

Table 15.1. Inhibition of fluazifop-butyl hydrolysis to fluazifop in human liver microsomal and cytosolic fractions and plasma by 0.1mM paraoxon, BNPP, physostigmine and $HgCl_2$. Results were expressed as the remaining activity as a % of the control.

Tissue	Substrate	% Remaining activity			
	(μM)	control	+Pararoxon	+BNPP	+Physostigmine	+$HgCl_2$
Liver microsomes	25	100	0±0	14±1.3	86±5.2	97±1.8
Liver cytosol	25	100	4±1.0	8±0.52	89±3.1	97±2.4
Plasma	100	100	0±0	44±2.4	59±4.2	-

liver: mean±SEM (n=7)

plasma: mean±SEM(n=10)

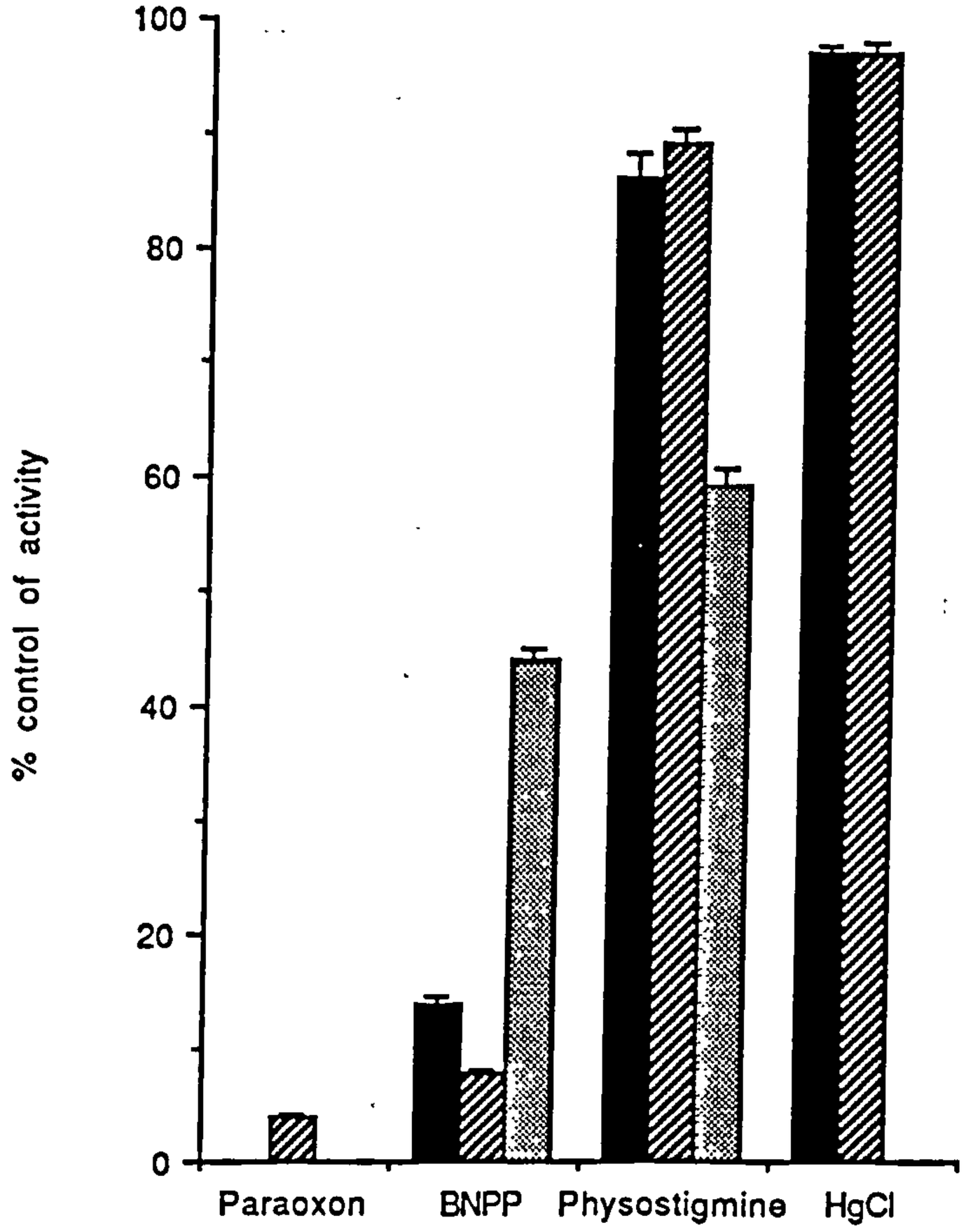


Figure 15.1.

Inhibition of fluazifop-butyl hydrolysis to fluazifop in human liver microsomes (■) and cytosol (▨) and by plasma (⊡) by 0.1mM paraoxon, BNPP, physostigmine and mercuric chloride ($HgCl_2$). Results were expressed as the % remaining activity of the control. Each point represents the mean of duplicate determinations and the vertical lines represent mean±SEM (n=7 for liver and n=10 for plasma).

cytosolic fractions of the liver and the plasma. These results indicate that the esterase hydrolysing fluazifop-butyl is a 'B' esterase. Mercuric chloride is an inhibitor of SH containing enzymes (containing the cysteine amino acid at the active centre) and therefore acts to inhibit 'A' type esterases. Results from the experiments show that little (3%) if no inhibition of fluazifop-butyl hydrolysis took place in the liver microsomal and cytosolic fractions by mercuric chloride. These results indicated that 'A' esterase was not responsible for fluazifop-butyl hydrolysis in the liver.

As has already been stated, BNPP is a specific inhibitor for carboxylesterase. The results show that in the human liver microsomes 86% inhibition of carboxylesterase takes place, whereas in the liver cytosol the value was 92%. The results indicate that human fluazifop-butyl hydrolysis is due to carboxylesterase. These results agree with inhibitory studies carried out in the rat liver. Inhibition due to physostigmine was very small in the microsomal (14%) and cytosol (11%) fractions of the lung and this small amount of inhibition is most probably due to inhibition of the carboxylesterase enzyme.

Physostigmine is a highly specific inhibitor of cholinesterase. In the plasma, inhibition of fluazifop-butyl was only 50%, suggesting the contribution of other esterases in the plasma.This hydrolysis of fluazifop-butyl could be as a result of either carboxylesterase or albumin. Work carried out by Mackness et al, (1987) seem to agree with this. Using the substrateα-naphthylacetate the presence of carboxylesterase in human plasma was characterized by gel-filtration. These esterase activities were then further separated from the 'A' esterases by anion-exchange chromatography.

15.3. ARYLESTERASE INHIBITION

After arylesterase activity had been identified in the human liver microsomal and cytosolic fractions and plasma, inhibitory studies were carried out to

determine the type of esterase acting to hydrolyse phenylacetate to phenol.

Method

Incubations were carried out in a final volume of 500µl in 50mM trisma buffer pH8.0, at 37°C. Microsomal and cytosolic protein equivalent to 3mg original wet weight or 30µl of plasma were incubated with a final concentration of 0.1mM paraoxon, 0.1mM mercuric chloride, 0.1mM BNPP or 0.1mM physostigmine and 2mM phenylacetate. Control incubations without any inhibitors in the incubation were carried out in parallel.

Reactions were started with the addition of phenylacetate (10µl of a 600mM stock in DMSO). The cuvettes were inverted and then placed in a spectrophotometer (see chapter 9.5.iii.).

Incubations containing the inhibitors of paraoxon, BNPP, mercury chloride and physostigmine were compared directly to control incubations. Conversion to product in the presence of inhibitor was determined as previously described (see chapter 9.5.ii.). Inhibition was estimated by expressing remaining activity in the presence of the inhibitor as a percentage of the control activity.

Results

Using the inhibitor paraoxon there is 62% inhibition of phenylacetate hydrolysis in the liver microsomes, 68% in liver cytosol and 7% inhibition in the plasma. Inhibition due to mercuric chloride was found to be 41% in the liver microsomes and 10% in the liver cytosol. Inhibition due to BNPP was very similar to

that of paraoxon with high inhibition of the liver microsomal (52%) and cytosolic esterases (76%), whereas little inhibition of the plasma esterase (3%). In the liver microsomes inhibition of arylesterase due to physostigmine was 52%, whereas in the cytosol it was 26%. It was found that no physostigmine inhibition of the plasma arylesterase took place. These results can be seen on Table 15.2. and Figure 15.2.

Discussion

The inhibitory studies in the human were very similar to those found in the rat. Inhibition due to paraoxon , a 'B' esterase inhibitor was significant in the liver microsomal and cytosolic fractions, whereas in the plasma there was little inhibition. It would seem that in the liver cytosol and microsomes most phenylacetate hydrolysis is due to the carboxylesterase and not the arylesterase. The lack of arylesterase activity would explain why in the human the activity of phenylacetate esterase was lower in the liver microsomal fraction than the liver microsomes of the rat. Since the enzymes in the microsomal fraction of the human and rat liver are different (in rat phenylacetate is hydrolysed mainly by arylesterase, whereas in the human this is due to mainly carboxylesterase) this could explain the low phenylacetate hydrolysis and low apparent Km values that were seen in the human liver microsomes compared to that of the rat liver microsomes. Results from mercuric chloride, an 'A' type inhibitor, showed that there was hydrolysis of phenylacetate by arylesterase in the liver microsomes, whereas in the liver cytosol hydrolysis due to arylesterase was very small. It would therefore seem that in the liver microsomes hydrolysis of phenylacetate is due to both 'A' and 'B' type esterases, whereas in the cytosol of the liver hydrolysis seems to be as a result of 'B' esterases.

As would be expected most if not all phenylacetate hydrolysis in the human plasma was due to the arylesterase, since very little if no inhibition was seen with either paraoxon, BNPP or physostigmine inhibitors. This result is not surprising considering there is very little if any carboxylesterase activity contained in the human

Table 15.2. Inhibition of phenylacetate hydrolysis to phenol in human liver microsomal and cytosolic fractions and plasma by 0.1mM paraoxon, BNPP, physostigmine and $HgCl_2$. Results were expressed as the remaining activity as a % of the control.

Tissue	Substrate (mM)	% Remaining activity				
		control	+Paraoxon	+BNPP	+Physostigmine	+$HgCl_2$
Liver microsomes	2	100	38±10	48±2.9	48±2.9	59±6.3
Liver cytosol	2	100	32±4.7	24±1.8	74±1.8	90±3.1
Plasma	2	100	94±2.1	97±2.6	99±1.0	-

liver: mean±SEM (n=7)
plasma: mean±SEM(n=10)

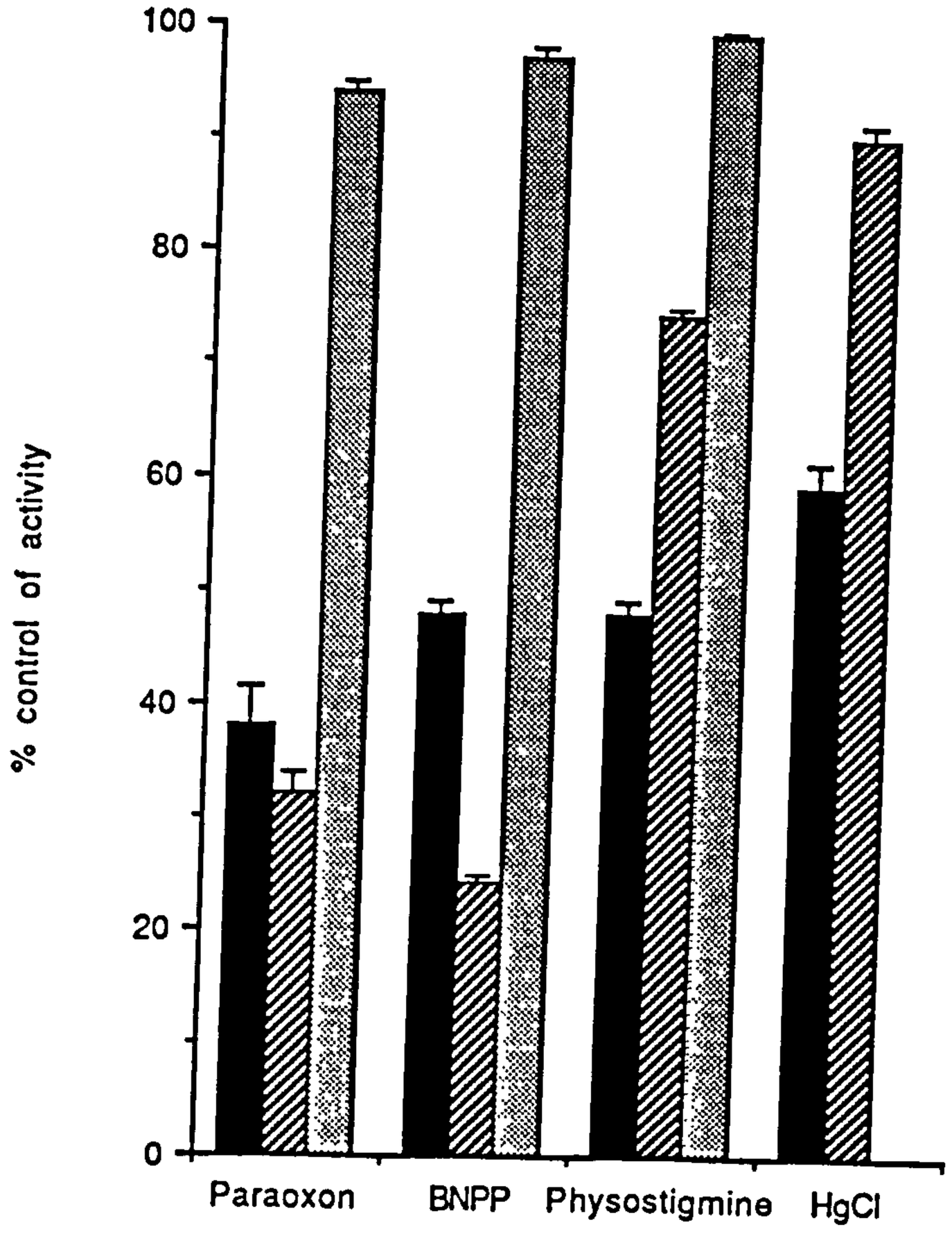


Figure 15.2.

Inhibition of phenylacetate hydrolysis to phenol in human liver microsomes (■) and cytosol (▧) and by plasma (▩) by 0.1mM paraoxon, BNPP, physostigmine and mercuric chloride ($HgCl_2$). Results were expressed as the % remaining activity of the control. Each point represents the mean of duplicate determinations and the vertical lines represent mean±SEM (n=7 for liver and n=10 for plasma).

plasma. This result confirms earlier inhibitory studies that hydrolysis of fluazifop-butyl in the plasma of the human is as a result of the carboxylesterase enzyme.

CHAPTER 16 : GENERAL DISCUSSION

The main aim of these studies was to identify and quantify the esterase enzymes responsible for hydrolysis and subsequent detoxification of a number of pesticide substrates. Having initially determined the extent of esterase activity in the liver, lung, skin and blood of the rat for the pesticides fluazifop-butyl, carbaryl, paraoxon and, the marker substrate phenylacetate the aim was to characterize these enzymes as 'A' or 'B' esterases by the use of specific inhibitors and inducers. Having initially characterised and quantitated esterase activity in the rat, parallel studies were then to be carried out in human tissue to establish whether the rat was an approprite model for study of the detoxification of pesticides by esterases.

Fluazifop-butyl, an alkoxyphenoxy herbicide was found to be hydrolysed in all the tissues from the rat studied. Most hydrolysis took place in the liver and plasma, however, there was significant hydrolysis in the extra-hepatic tissues of the skin and lung. Fluazifop-butyl esterase activity was equally distributed between the microsomal and cytosolic fraction of the liver, whereas in the lung and skin most activity was found in the cytosol.

The distribution of carbaryl esterase activity was found to be very similar to that of fluazifop-butyl esterase distribution. Most activity was found in the liver and plasma and again there was significant activity in the skin and lung. In the liver most hydrolysis of carbaryl was found in the cytosol, with the microsomal esterase having only a 1/3 the activity of the cytosol. As has already been discussed, in the microsomal fraction of the liver, carbaryl is metabolised both by hydrolysis and oxidation, therefore competition may exist for the substrate. In the cytosol, however, carbaryl metabolism is entirely due to hydrolysis. The apparent Km values for the microsomal and cytosolic esterase enzyme hydrolysing fluazifop-butyl and carbaryl in the tissues studied were similar, suggesting the involvement of similar types of esterase isoenzyme.

To determine whether the iso zymes responsible for the hydrolysis of carbaryl and fluazifop-butyl were similar, inhibitory studies were carried out. Paraoxon

was used in the study, because it inhibits 'B' esterases and mercuric chloride because it inhibits 'A' esterases. The differential inhibitor for carboxylesterase (BNPP) and the specific cholinesterase inhibitor physostigmine were also used to attempt to identify the specific isoenzyme type of 'B' esterase responsible for hydrolysis.

With rat liver, lung and skin tissues and with plasma there was complete inhibition of fluazifop-butyl hydrolysis by paraoxon, indicating the involvement of serine hydrolases. Mercuric chloride had little effect; thus the enzyme acting on fluazifop-butyl bears no SH (cysteine) group essential for hydrolysis. BNPP caused widespread inhibition of both fluazifop-butyl and carbaryl hydrolysis in the tissues studied, whereas physostigmine had little effect.

Results therefore showed that both fluazifop-butyl and carbaryl were hydrolysed by carboxylesterase in the rat microsomal and cytosolic fractions of the liver, lung and skin and the plasma. In the lung microsomes inhibition of carboxylesterase by BNPP was low compared to the other tissues studied, using both fluazifop-butyl and carbaryl as substrates. This could be as a result of the involvement of specific carboxylesterase isoenzymes in the lung microsomes which were not susceptible to inhibition by BNPP, but which were still inhibited by paraoxon.

The distribution of paraoxon hydrolysing enzymes was different between rat tissue was different from the distribution of carbaryl and fluazifop-butyl esterase. Most paraoxonase activity was found in the liver microsomes and the plasma. A very small amount of paraoxonase activity was found in the lung microsomes and none was detected in the skin. No activity was found in any cytosolic fraction of any of the tissues studied. Differences in metabolism might be expected as paraoxonase is an 'A' esterase and fluazifop-butyl and carbaryl esterases are 'B' esterases.

Most phenylacetate esterase activity was found in the liver microsomes

and the plasma of the rat, with a smaller amount in the lung and skin. There was however some activity in the cytosolic fractions of the tissues studied. The distribution of phenylacetate esterase activity had similarities to both carbaryl and fluazifop-butyl esterase and to paraoxonase. Phenylacetate has been used in the past as a marker for arylesterase activity. If this was the case then the distribution of paraoxonase and arylesterase would be expected to be similar. These studies have shown that although distribution in the microsomal fraction of tissues and plasma is similar there is a marked difference in the cytosolic fractions. Phenylacetate hydrolysis took place in the cytosol of the liver, lung and skin, whereas no paraoxonase activity was found in these cytosolic fractions. Phenylacetate appears to be a non-specific substrate for esterase enzymes. The esterase activity in the cytosolic fractions of the tissues studied could be due to isoenymes of 'A' esterase not not involved in paraoxon hydrolysis, or or due to phenylacetate hydrolysis by carboxylesterase. The inhibitory studies carried out showed that phenylacetate hydrolysis in the cytosolic fractions of the liver, lung and skin was as a result of carboxylesterases rather than arylesterase, whereas in the microsmal fraction hydrolysis was due to arylesterase rather than 'B' esterases. These results shed doubt on using phenylacetate as a marker enzyme for arylesterase activity as can clearly be seen that phenylacetate was a substrate for cytosolic carboxylesterase.

Having established the type of esterase enzyme involved in hydrolysing a range of pesticides in the rat, studies were carried out to establish whether esterase activity was inducible.The classical inducers phenobarbitone, ß-naphthoflavone and clofibric acid were used in thestudies. Previous work by Satoh and Moroi, (1973), using the substrate isocarboxazid reported induction of hepatic microsomal carboxylesterase by phenobarbital, but not by the 3-methylcholanthrene type inducers. Using the substrate naphthyl propionate, Raftell et al, (1977) found no induction of lung microsomal carboxylesterase by phenobarbital. The results presented here were similar to these earlier studies. All the esterase enzymes studied were induced by phenobarbitone in the liver microsomes. The treatment with phenobarbitone has been shown to activate gene transcription of a range of enzymes including P450b and e, epoxide hydrolase and glutathione transferase (Hardwick and Gonzalez, 1983; Ding and Pickett, 1985). These results suggest that esterases may be induced in a similar

manner, however, the mechanism remains to be fully elucidated. ß-naphthoflavone on the other hand has a more specific effect involving the binding to a specific cytosolic receptor followed by translocated into the nucleus to stimulate gene transcription (Okey and Vella, 1982). Induction bf ß-naphthoflavone is therefore more specific for mono-oxygenase isoenzymes and appears to have little effect on the esterase activity in the liver microsomes using any of the substrates.

Hosokawa et al, (1987) found that after administration of clofibrate, there was a significant increase in the activities of the p-nitrophenylacetate, isocarboxazid and palmitoyl-CoA hydrolases in the rat liver microsomes, whereas in the liver cytosol only palmitoyl-CoA hydrolase was induced. Results from these studies indicated no induction of these three carboxylic acid isoenzymes in either of the microsomal or cytosolic fractions of the liver, lung and skin. Phenobarbitone did not induce cytosolic esterase activity in the liver or skin, but was found to induce fluazifop-butyl and phenylacetate hydrolysis in the lung cytosol. These results show that in the liver microsomes phenobarbitone induces both 'A' and 'B' type esterases, whereas in the lung cytosol the carboxylesterase activity is induced. Although phenylacetate esterase activity in the lung cytosol was induced, previous inhibitory studies have indicated that in the lung cytosol phenyl acetate hydrolysis is entirely due to carboxylesterase and not arylesterase. The induction in the lung microsomes could be as a result of the activation of a specific lung paraoxonase ß-naphthoflavone and clofibric acid inducible isoenzyme not expressed in liver at significant levels. In the skin no activation was found in microsomal or cytosolic fractions with any of the inducers. This could be as a result of low sensitivity of esterase activity in the skin to inducers or that inadequate concentrations of the inducers reached the esterase enzymes of the skin. Studies in which inducers were locally applied to the skin, thus ensuring high concentrations would indicate the absolute induction potential of the skin esterases.

The relevance of the induction studies were to aid in the classification of esterases and to determine the implications in vivo following prior exposure to inducers. Atmospheric induction generally occurs with the polycyclic hydrocarbons,

whereas phenobarbital and clofibric acid induction would only take place following drug therapy. Although induction by phenobarbital and clofibric acid have little implication in vivo, differential induction in the extra-hepatic tissues might influence the relative prehepatic metabolism.The effect of the inducers on the levels of circulating plasma enzymes probably parallel the liver levels (site of synthesis of plasma enzymes) although this was not confronted here.

One of the overall aims of the study in the rat is to obtain information which can be extrapolated to man, in this case to man exposed to chemicals such as pesticides in the work place. On this project it was possible to study the equivalent esterases in human blood and human liver samples. As was found in the rat, fluazifop-butyl esterase activity was equally distributed between the microsomal and cytosolic fractions of the human liver. Expressing specific activities in terms of µmol/min/mg protein fluazifop-butyl esterase activity was similar in the human and rat liver cytosol (i.e. approximately 0.21µmol/min/mg) and 4 times higher in human liver microsomes (rat: 0.22 and human: 0.86µmol/min/mg). In the rat however, there was far greater fluazifop-butyl esterase activity in the plasma than in the human (rat: 5.8 and human: 0.088µmol/min/ml plasma). This low plasma fluazifop-butyl esterase activity was consistent with low levels of carboxylesterase, for which fluazifop-butyl is a preferential substrate. Lack of inhibition with physostigmine indicated that cholinesterase did not hydrolyse fluazifop-butyl. These results compare to studies conducted by Clark, 1991 (personal communication) who found a 5 fold range in fluazifop-butyl esterase activity (0.04-0.15µmol/min/g wet weight tissue) in the human skin. This was a 1/10 the fluazifop-butyl activity of the rat skin. The apparent Km values for the microsomal and cytosolic fluazifop-butyl esterase in the liver and plasma of the rat and human were very similar (1-2 fold difference), again suggesting the existence of a similar enzyme in the human and rat liver and plasma responsible for fluazifop-butyl hydrolysis.

Paraoxonase activity in human plasma was similar to that of the rat (rat: 0.244 and human: 0.210µmol/min/ml plasma), however, in the liver microsomes there

was 3 times more paraoxonase activity in the rat (11.8nmol/min/mg) compared to the human (4.2nmol/min/mg) when the activity was expressed in terms of mg protein. Values for human paraoxonase activity agree with previous work carried out by Mutch et al, (1991), who found that in a population of 127 males paraoxonase activity varied between 90-300nmol/min/ml plasma. These results however are different from those found by Losch et al, (1982) who found human paraoxonase activity to be 30nmol/min/ml plasma, whereas, in the liver it was 350nmol/min/g wet weight tissue. There is controversy as to whether the enzymes hydrolysing paraoxon and phenylacetate in the human plasma are different or part of the same enzyme. A lack of correlation between paraoxon and phenylacetate hydrolysis in a serum population was reported by Mallinckrodt, (1988). This however, is contrary to that found by Eckerson et al, (1983 a and b), who found high correlation for the hydrolysis of paraoxon and phenylacetate by human plasma and subdivision into 3 modes representing 3 phenotypes, suggesting that these activities might be the property of the same enzyme. The apparent Km values for paraoxonase in human plasma in this study indicate that the apparent affinities of the 'A'esterase for the paraoxon and phenylacetate substrates are very different. A possible explanation for this could be the involvement of different esterase isoenzymes.

Apparent Km values for paraoxonase in liver microsomes and plasma were similar for both the human and the rat, indicating the possible involvement of the same enzyme.

Phenylacetate esterase activity in human liver microsomes was much lower than the rat (rat: 25.6 and human: 5μmol/min/mg of protein), whereas the liver cytosol and plasma activities for human and rat were similar. Apparent Km values in human and rat livers differed in both microsomal and cytosolic fractions. A possibly reason for this could be the involvement of different enzymes. Inhibitory studies in the rat supported this. From the study it would seem that in human liver microsomes phenylacetate is hydrolysed by mostly 'B' esterases, whereas in the rat hydrolysis was due to arylesterase. In the cytosol of the liver phenylacetate hydrolysis in both human

and rat is due to 'B' esterases. It is only in the plasma of the human that the arylesterase is entirely responsible for the hydrolysis of phenylacetate.

One of the overall aims of these studies was to predict whether or not following exposure to xenobiotic pesticide via dermal or inhalation the tissues of the lung, skin or plasma would play an important role in the first pass detoxification.

Following exposure to fluazifop-butyl it was seen that in the rat a significant amount of hydrolysis took place in the lung and the skin. A degree of hydrolysis would therefore take place in these tissues before the fluazifop-butyl was transported to the blood supply where further hydrolysis could take place. Finally the fluazifop-butyl would then be transported to the liver where most hydrolysis has been shown to take place. In practice the most important route of exposure to fluazifop-butyl is through absorption through the skin, because if fluazifop-butyl was inhaled, the fluazifop-butyl would be deposited as droplets in the upper airways of the lung and so would not be absorbed (Chester and Hart, 1986). The reason for the lack of absorption is that the particle size of the fluazifop-butyl used in its spray form exceeds 5µm in diameter and so would deposits in the upper airways before reaching the alveoli. This same picture would apply to exposure to carbaryl. Following exposure local hydrolysis would be expected to occur in the lung and the skin tissues. Since the carbaryl particle in its spray form is between 15-20µm in diameter (Johnstone, 1969; 1970) the absorption through the skin is the more important route of exposure compared to that of inhalation.

The rat skin and lung tissue have little capacity to hydrolyse paraoxon. Therefore following initial exposure to parathion and activation to paraoxon little first pass metabolism would be expected to occur in skin or lung. The plasma paraoxonase activity would play the most important role in the detoxification of paraoxon. The pesticide generally used parathion, is an inherently weak organophoshate anticholinesterase, as it must be activated via a cytochrome P450-dependant

desulphuration reaction to the reactive phosphate (oxon) metabolite i.ie. paraoxon (Gage, 1953; Neal, 1967). Activation of parathion to paraoxon has not been shown but it is activated in the lung (Norman and Neal, 1975) and liver (Neal, 1967). Parathion is known to be absorbed easily through the lung and skin (Neal, 1967). It is known that paraoxon irreversible inhibits acetylcholinesterase in the nervous system and respiratory centre of the brain, therefore following exposure, paraoxon would be transported from the lung by the blood through the circulation to the nervous system and respiratory centre of the brain to have its effect. Only after inhibition the onset of acetylcholinesterase inhibition at the target sites would there be any hydrolysis of paraoxon due to paraoxonase in the liver microsomes. In the case of parathion exposure through the lung it would therefore, seem that the plasma has an important role to play in the detoxification of the paraoxon. This may be even more important in man due to the lower paraoxonase activity in the liver microsomes, compared to plasma. Following exposure through the dermal absorption, if there was no activation of the parathion in skin, the liver would be the major site for activation of parathion and detoxification of paraoxon. No activation would take place in the plasma. Therefore following paraoxon exposure through the lung, the liver would play a far less important role in detoxification, because the onset of action of the paraoxon on the target tissues would appear before second pass metabolism would occur, however, if the paraoxon was orally ingested or absorbed through the skin then the liver microsomes be involved in the first pass metabolism of the pesticide. The brain has the ability to activate parathion to paraoxon due to the presence of the cytochrome P450-desulphation reaction so that parathion absorbed through the skin could be transported by the blood to the brain causing inhibition of the brain acetylcholinesterases at the respiratory centre before it reached the liver for detoxification. The extra-hepatic tissues of the lung and skin and blood do indirectly play a role in preventing the irreversible inhibition of the acetylcholinesterase. Carboxylesterase in the lung and skin and plasma is inhibited by paraoxon, which binds irreversibly, therefore decreasing the amount of intact paraoxon in the circulation.

The studies have shown that there is considerable similarity in the nature of human and rat esterase enzymes, although there are significant differences in

absolute activities. The rat therefore, seems to be a good model for studying pesticide detoxification by esterase hydrolysis and extrapolation of results to the human would be possible.

CHAPTER 17 : APPENDIX

Table 17.1. The hydrolysis of fluazifop-butyl to fluazifop in rat liver, lung and skin microsomes. Results were expressed at: (i) Vmax (μmol/min/g wet weight or ml) and (ii) the apparent Km (μM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin
1	6.25	0.7	0.021
2	7.5	0.21	0.019
3	5.3	0.21	0.031
4	6.52	0.24	0.023
5	5.7	0.14	0.014
6	6.1	0.21	0.0173
mean±SEM	6.29±0.4	0.38±0.1	0.02±0.0015

(i) Apparent Km:

Rat number	Liver	Lung	Skin
1	20	43	8
2	12	60	8
3	15	43	17
4	10	30	11
5	15	25	14
6	13	29	15
mean±SEM	14.4±2.8	33±5.5	14±2.8

Table 17.2. The hydrolysis of fluazifop-butyl to fluazifop in rat liver, lung and skin cytosol and plasma and erythrocytes. Results were expressed at: (i) Vmax (μmol/min/g wet weight or ml) and (ii) the apparent Km (μM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	5.8	1.34	0.35	5.25	0.028
2	9.7	1.13	0.34	5.5	0.048
3	6.6	1.16	0.46	5.2	0.026
4	6.9	1.92	0.38	6.5	0.033
5	5.2	0.67	0.58	6.0	0.048
6	6.8	0.60	0.34	5.7	0.038
mean±SEM	6.84±0.85	1.5±0.32	0.4±0.06	5.8±0.48	0.03±0.05

(i) Apparent Km:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	10	120	36	15	38
2	20	80	44	17	43
3	20	54	13	14	30
4	25	60	40	25	34
5	35	42	30	20	40
6	23	20	47	19	23
mean±SEM	22±4.5	63±14	42±2.8	18.2±2.2	33±1.2

Table 17.3. The hydrolysis of carbaryl to 1-naphthol in rat liver, lung and skin microsomes . Results were expressed at: (i) Vmax (nmol/min/g wet weight) and (ii) the apparent Km (μM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin
1	2.64	1.6	0.132
2	2.15	2.36	0.167
3	2.38	1.68	0.26
4	1.58	1.35	0.188
5	2.3	1.84	0.182
6	1.9	1.97	0.20
mean±SEM	2.07±0.25	1.64±0.25	0.19±0.035

(i) Apparent Km:

Rat number	Liver	Lung	Skin
1	11	34	33
2	24	22	22
3	22	34	36
4	18	28	38
5	15	31	30
6	20	27	31
mean±SEM	20±3.5	30±3.5	32±4.2

Table 17.4. The hydrolysis of carbaryl to 1-naphthol in rat liver, lung and skin cytosol and plasma and erythrocytes. Results were expressed at: (i) Vmax (nmol/min/g wet weight or ml) and (ii) the apparent Km (μM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	5.7	0.96	0.82	2.40	0
2	8.4	1.65	0.78	3.60	0
3	6.1	0.78	0.38	2.36	0
4	6.4	1.79	0.38	3.47	0
5	6.6	1.10	0.40	3.40	0
6	7.0	1.40	0.54	3.00	0
mean±SEM	6.7±0.75	1.36±0.25	0.5±0.12	2.98±0.25	0.0±0.0

(i) Apparent Km:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	64	25	21	54	0
2	44	23	28	77	0
3	50	28	18	52	0
4	53	27	30	63	0
5	55	24	22	68	0
6	51	18	32	61	0
mean±SEM	53±4.5	26±1.2	36±3.8	64±4	0±0

Table 17.5. The hydrolysis of paraoxon to p-nitrophenol in rat liver, lung and skin microsomes and plasma and erythrocytes . Results were expressed at: (i) Vmax (μmol/min/g wet weight) and (ii) the apparent Km (mM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	0.37	0.0021	0	0.22	0
2	0.34	0.0029	0	0.215	0
3	0.33	0.0018	0	0.25	0
4	0.32	0.0016	0	0.25	0
5	0.35	0.0024	0	0.24	0
6	0.31	0.0015	0	0.30	0
mean±SEM	0.33±0.025	0.002±0.00025	0±0	0.246±0.012	0±0

(i) Apparent Km:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	0.18	0.33	0	0.26	0
2	0.33	0.43	0	0.22	0
3	0.19	0.28	0	0.22	0
4	0.19	0.46	0	0.22	0
5	0.24	0.44	0	0.26	0
6	0.22	0.34	0	0.17	0
mean±SEM	0.2±0.035	0.38±0.013	0±0	0.225±0.015	0±0

Table 17.6. The hydrolysis of phenylacetate to phenol in rat liver, lung and skin microsomes . Results were expressed at: (i) Vmax (μmol/min/g wet weight) and (ii) the apparent Km (mM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin
1	745	6.2	1.75
2	1100	6.6	0.74
3	845	4.5	1.24
4	800	4.2	1.10
5	530	4.0	0.84
6	780	3.6	1.09
mean±SEM	800±75	4.86±3.1	1.13±0.025

(i) Apparent Km:

Rat number	Liver	Lung	Skin
1	1.5	1.33	1.0
2	2.0	1.71	1.25
3	1.4	1.64	0.85
4	1.4	1.3	0.70
5	0.9	1.67	0.95
6	1.4	1.14	0.90
mean±SEM	1.43±0.11	1.46±0.58	0.95±0.1

Table 17.7. The hydrolysis of phenylacetate to phenol in rat liver, lung and skin cytosol and plasma and erythrocytes. Results were expressed at: (i) Vmax (µmol/min/g wet weight or ml) and (ii) the apparent Km (mM) by direct linear plot.

(i) Vmax values:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	34.5	11.5	4.0	230	0
2	20.5	12.6	4.0	230	0
3	37	16.7	3.7	268	0
4	30	13.7	2.9	294	0
5	24	13.4	3.4	244	0
6	38	17.0	2.7	425	0
mean±SEM	30.7±3	14.2±5.5	3.44±1.4	290±40	0.0±0.0

(i) Apparent Km:

Rat number	Liver	Lung	Skin	Plasma	Erythrocytes
1	1.0	1.2	0.15	1.6	0
2	1.0	1.6	0.3	1.8	0
3	0.7	1.4	0.6	2.2	0
4	1.0	1.6	0.4	2.7	0
5	0.8	1.3	0.38	1.8	0
6	0.9	1.0	0.2	4.1	0
mean±SEM	0.90±0.5	1.33±0.55	0.34±0.05	2.34±6.4	0±0

Table 17.8. Protein recoveriers from rat liver subcellular fractions after induction with phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed as mg/g of tissue protein.

(i) Liver Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	14.8	16.5	16.0	13.8
2	18.0	29.1	14.6	12.9
3	19.0	18.6	18.0	15.8
4	13.7	13.8	18.2	16.8
mean±SD	16.4±1.5	19.6±3.4	16.6±0.96	14.6±0.12

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	51.0	45.5	40.8	43.2
2	44.0	59.6	41.0	42.0
3	49.3	47.6	40.5	60.8
4	52.2	43.0	50.5	64.1
mean±SD	49.1±1.8	49.3±3.6	43.3±2.6	52.5±5.0

Table 17.9. Protein recoveriers from rat lung subcellular fractions after induction with phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed as mg/g of tissue protein.

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	11.3	9.3	10.4	11.3
2	12.2	12.0	9.2	11.7
3	10.6	8.1	9.2	11.7
4	14.2	9.0	7.9	11.9
mean±SD	12.1±1.0	9.60±1.1	9.20±3.0	11.3±3.0

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	57.8	57.2	80.3	56.6
2	46.8	53.8	45.0	75.9
3	52.6	58.6	40.8	64.0
4	48.2	62.9	68.3	50.3
mean±SD	51.3±2.6	56.2±3.4	58.7±9.0	58.7±4.0

Table 17.10. Protein recoveriers from rat skin subcellular fractions after induction with phenobarbitone, ß-naphthoflavone and clofibric acid. Results were expressed as mg/g of tissue protein.

(i) Skin Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	1.45	1.0	1.80	1.28
2	1.83	0.92	1.39	1.06
3	1.26	0.88	0.92	1.23
4	1.36	1.32	1.22	1.48
mean±SD	1.50±0.12	1.10±0.2	1.33±0.26	1.26±0.12

(i) Skin Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	19.8	22.0	21.1	19.8
2	20.0	27.3	23.7	20.0
3	19.2	14.7	20.8	19.0
4	17.2	20.2	20.2	19.0
mean±SD	19.3±0.1	21.0±0.2	21.5±0.08	19.4±0.2

Table 17.11. Induction of fluazifop-butyl hydrolysis to fluazifop in rat liver . Results expressed in µmol/min/g wet weight.

(i) Liver Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.93	2.52	0.82	1.10
2	1.34	2.10	0.95	0.98
3	1.30	1.96	0.91	1.56
4	1.28	1.3	1.16	1.07
5	-	1.91	-	1.09
mean±SD	1.21±0.18	1.94±0.53	0.96±0.14	1.10±0.14

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	1.07	1.06	0.70	1.24
2	0.63	1.21	0.72	0.91
3	1.12	0.87	0.67	1.30
4	0.65	0.73	0.36	0.80
5	-	0.33	-	0.26
mean±SD	0.86±0.27	0.97±0.21	0.66±0.19	1.06±0.22

sensitivity > 1nmol/incubation

Table 17.12. Induction of fluazifop-butyl hydrolysis to fluazifop in rat lung . Results expressed in µmol/min/g wet weight.

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.28	0.13	0.32	0.33
2	0.30	0.30	0.29	0.30
3	0.26	0.27	0.19	0.32
4	0.30	0.33	0.30	0.30
5	-	0.33	-	0.25
mean±SD	0.28±0.019	0.27±0.03	0.27±0.08	0.30±0.03

(i) Lung Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	1.33	1.52	1.40	1.45
2	1.33	1.50	0.57	1.21
3	1.31	1.42	0.85	1.32
4	1.30	1.45	1.08	1.10
5	-	1.41	-	1.46
mean±SD	1.31±0.09	1.47±0.07	0.98±0.32	1.31±0.14

sensitivity > 1nmol/incubation

Table 17.13. Induction of fluazifop-butyl hydrolysis to fluazifop in rat skin . Results expressed in µmol/min/g wet weight.

(i) Skin Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.28	0.13	0.32	0.33
2	0.30	0.30	0.29	0.30
3	0.26	0.27	0.19	0.32
4	0.30	0.33	0.30	0.30
5	-	0.33	-	0.25
mean±SD	0.28±0.019	1.94±0.53	0.27±0.08	0.30±0.03

(i) Skin Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.59	0.64	0.60	0.66
2	0.62	0.45	0.54	0.56
3	0.62	0.59	0.58	0.57
4	0.57	0.65	0.59	0.6
5	-	0.57	-	0.59
mean±SD	0.60±0.026	0.58±0.9	0.58±0.09	0.59±0.046

sensitivity > 1nmol/incubation

Table 17.14. Induction of carbaryl hydrolysis to 1-naphthol in rat liver . Results expressed in nmol/min/g wet weight.

(i) Liver Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	1.4	2.2	0.76	1.5
2	1.1	1.97	1.08	1.8
3	1.25	2.33	1.01	0.93
4	0.81	1.12	1.33	2.4
mean±SD	1.14±0.25	1.90±0.54	1.04±0.23	1.66±0.61

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	1.26	0.44	0.53	0.66
2	1.13	0.39	1.58	0.80
3	1.44	1.78	1.55	1.02
4	1.38	0.99	1.30	0.60
mean±SD	1.30±0.14	0.90±0.65	0.99±0.51	0.77±0.19

sensitivity > 20pmol/incubation

Table 17.15. Induction of carbaryl hydrolysis to 1-naphthol in rat lung . Results expressed in nmol/min/g wet weight.

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.65	0.49	0.49	0.66
2	1.01	0.74	0.68	1.01
3	0.72	1.39	0.65	1.34
4	0.50	1.0	0.83	0.61
mean±SD	0.72±0.21	0.90±0.38	0.66±0.14	0.90±0.34

(i) Lung Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.49	0.23	0.60	0.80
2	0.82	0.62	0.22	1.04
3	0.42	0.43	1.13	0.73
4	0.91	0.62	1.09	0.74
mean±SD	0.69±0.26	0.48±0.19	0.76±0.43	0.83±0.14

sensitivity > 20pmol/incubation

Table 17.16. Induction of carbaryl hydrolysis to 1-naphthol in rat skin . Results expressed in nmol/min/g wet weight.

(i) Skin Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.059	0.051	0.008	0.032
2	0.074	0.011	0.049	0.033
3	0.086	0.037	0.037	0.025
4	0.023	0.018	0.055	0.027
mean±SD	0.06±0.027	0.029±0.018	0.037±0.02	0.029±0.004

(i) Skin Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.21	0.22	0.22	0.17
2	0.39	0.38	0.34	0.28
3	0.29	0.28	0.18	0.16
4	0.3	0.31	0.28	0.13
mean±SD	0.30±0.07	0.28±0.05	0.26±0.07	0.19±0.07

sensitivity > 20pmol/incubation

Table 17.17. Induction of paraoxon hydrolysis to p-nitrophenol in rat liver and lung . Results expressed in µmol/min/g wet weight.

(i) Liver Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.073	0.129	0.075	0.078
2	0.081	0.136	0.084	0.107
3	0.074	0.09	0.085	0.098
4	0.058	0.171	0.075	0.099
5	-	0.134	-	0.098
mean±SD	0.07±0.011	0.132±0.032	0.080±0.008	0.096±0.012

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	.ß-naphthoflavone	Clofibric acid
1	0.0082	0.0095	0.0092	0.0094
2	0.009	0.0094	0.0094	0.0097
3	0.0074	0.0095	0.01	0.01
4	0.0088	0.0079	0.0098	0.0099
5	-	0.009	-	0.0099
mean±SD	0.0082±0.01	0.0091±0.0008	0.0096±0.0005	0.0098±0.0004

sensitivity > 1nmol/incubation

Table 17.18. Induction of phenylacetate hydrolysis to phenol in rat liver. Results expressed in µmol/min/g wet weight.

(i) Liver Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	177	222	154	182
2	190	171	158	184
3	161	269	142	162
4	196	244	166	159
5	-	228	-	169
mean±SD	179.5±16	226.5±42	155±10	171.8±13

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	63	68	31	69
2	61	62	53	65
3	49	43	67	53
4	87	38	56	67
5	-	53	-	63
mean±SD	65±16	52.8±14	50.2±13	63.5±7

sensitivity > 0.3µmol/incubation

Table 17.19. Induction of phenylacetate hydrolysis to phenol in rat lung. Results expressed in µmol/min/g wet weight.

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	4.43	4.79	2.85	3.75
2	3.82	3.58	3.67	4.88
3	4.10	4.11	4.38	3.63
4	5.05	4.75	4.16	4.23
5	-	3.14	-	4.06
mean±SD	4.38±0.58	4.08±0.68	3.77±0.64	4.11±0.5

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	14.5	22.2	7.6	10.0
2	16.4	19.3	11.2	13.4
3	13.3	27.4	16.6	11.1
4	15.7	14.4	7.8	20.1
5	-	21.0	-	12.2
mean±SD	14.95±1.27	21.37±4.8	10.76±4.0	13.49±3.8

sensitivity > 0.3µmol/incubation

Table 17.20. Induction of phenylacetate hydrolysis to phenol in rat skin. Results expressed in µmol/min/g wet weight.

(i) Lung Microsomes:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	0.126	0.111	0.111	0.239
2	0.130	0.136	0.128	0.243
3	0.136	0.10	0.147	0.096
4	0.148	0.134	0.124	0.135
5	-	0.122	-	0.182
mean±SD	0.135±0.024	0.12±0.022	0.127±0.021	0.179±0.07

(i) Liver Cytosol:

Rat number	Saline	Phenobarbitone	ß-naphthoflavone	Clofibric acid
1	7.75	7.25	5.72	5.67
2	6.38	7.17	5.75	5.05
3	6.85	5.04	6.36	5.04
4	6.75	9.58	5.12	5.15
5	-	7.31	-	5.23
mean±SD	6.93±0.54	7.26±1.73	5.74±0.47	5.20±0.27

sensitivity > 0.3µmol/incubation

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