STRUCTURAL REQUIREMENTS FOR THE FORMATION OF CARBINOLAMINES BY OXIDATIVE METABOLISM OF N-METHYL CONTAINING COMPOUNDS

by

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A study was made of the structural requirements in an N-methyl containing molecule which predisposes it to form a characterizable N-hydroxymethyl compound during metabolic oxidative N-demethylation.

The metabolism by mouse liver microsomes of hexamethylmelamine and derivatives, N,N-dimethylaminoantipyrine and its N-desmethyl derivative, and compounds of the general formula Aryl-X-N-$(CH_3)_2$ where X is either -N=N- (3-aryl-1,1-dimethyltriazenes, -N=CH- (N'-aryl-N,N-dimethyl-formamidines), -NH-CO- (N'-aryl-N,N-dimethylureas) or is absent (aryldimethylamines) was studied using a colourimetric technique (Nash assay). Whereas the N-methyl moieties of the aryldimethyltriazenes, formamidines, amines and aminoantipyrine derivatives were metabolized to formaldehyde, aryldimethylureas and the N-methyl melamine derivatives except 2-azido-4, 6-bis-(dimethylamino)-1,3,5-triazine formed stable formaldehyde precursors during metabolism.

The metabolism of the herbicide monuron [N'-(4-chlorophenyl)-N,N-dimethylurea] was investigated using high pressure liquid chromatography. Two metabolites were observed on incubation of monuron with microsomes. One metabolite was characterized as the N-desmethyl derivative whilst the other was tentatively identified as N'-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea. Certain N-hydroxymethyl compounds were so stable that they did not produce a positive response in the Nash assay. Two such N-methylols were N-hydroxymethylformamide, which was tentatively identified as a urinary metabolite of N-methylformamide in mice and man, and N-hydroxymethylbenzamide which was characterized as a metabolite of N-methylbenzamide *in vitro* and as a urinary metabolite. N-Hydroxymethyl-N-methylbenzamide was identified as a microsomal metabolite of N,N-dimethylbenzamide but, unlike N-hydroxymethyl-benzamide, was a relatively unstable species and produced a positive response in the Nash assay.

Both N-methylbenzamide and N-hydroxymethylbenzamide were metabolized to N-formylbenzamide *in vivo* and in hepatocytes. The conversion of N-hydroxymethylbenzamide to N-formylbenzamide *in vitro* was also catalyzed by 9000g and microsomal supernatants and by horse liver alcohol dehydrogenase, and was inhibited by pyrazole. N-Formylbenzamide was unstable and degraded to produce benzamide in Earl's buffer (pH=7.4, 37°C) with a half-life of 7.8 minutes. This suggests that N-demethylation need not be synchronous with formaldehyde production.

Electron density calculations were consistent with metabolic evidence which indicated that the formation of stable N-hydroxymethyl compounds during metabolic N-demethylation was favoured if the nitrogen bearing the methyl group was situated in an electron withdrawing environment.

Key words:

Metabolic N-demethylation; N-hydroxymethyl stability; structure-metabolism relationships; N-formyl metabolites; electron density calculations.

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ABBREVIATIONS

G.C.	Gas chromatography
G.C.-M.S.	Gas chromatography - mass spectrometry
H.P.L.C.	High pressure liquid chromatography
i.p.	Intra-peritoneally
M.S.	Mass spectrometry
p.o.	By mouth
s.d.	Standard deviation
T.L.C.	Thin layer chromatography
U.V.	Ultra-violet
BSTFA	N,O-bis-(trimethylsilyl)-trifluoroacetamide
DMSO	Dimethylsulphoxide
G6P	Glucose-6-phosphate
GPDH	Glucose-6-phosphate dehydrogenase
HFAA	Heptafluoracetic acid anhydride
HLAD	Horse liver alcohol dehydrogenase
MBTH	3-methyl-2-benzothiazolone hydrazone hydrochloride
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
TMS	Trimethylsilyl
TSIM	Trimethylsilylimidazole

SECTION 1

INTRODUCTION

1.I Historical Perspective

It has been recognized for over a century that drugs can be transformed during their passage through the body but until approximately thirty years ago in-vitro methods to study the mechanism of a particular metabolic pathway were lacking. It was then that Mueller and Miller observed that N-methyl substituted aminoazo dyes were N-demethylated by rat liver homogenates and that this biotransformation did not involve transfer of the intact N-methyl group but its oxidation to produce formaldehyde (Mueller & Miller, 1948, 1953).

In the early 1950s progress in the area of drug metabolism was rapid. La Du et al. (1955) and Axelrod (1955, 1956) showed that the enzymes responsible for oxidative N-dealkylation required both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. These enzymes were located in the microsomal fraction of liver which could be obtained from the endoplasmic reticulum of liver parenchymal cells. The same workers also showed that a wide variety of structurally diverse compounds could be metabolized by this microsomal system (Brodie et al, 1955). This demonstrated that N-dealkylation was only one of a number of enzyme activities located in the microsomal fraction and the requirements for a reducing agent and molecular oxygen caused the enzyme systems to be classified as mixed function oxidases (Mason, 1957). This classification implies that during the course of oxidation one atom of an oxygen molecule is transferred to the substrate -

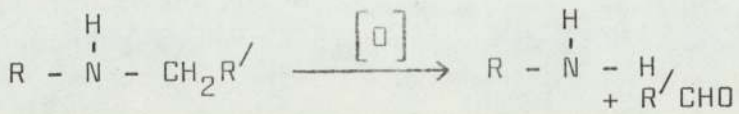
which was confirmed by Posner et al. (1961) - whilst the second atom undergoes a two electron reduction and appears as water.

A haemoprotein was also found to be an essential part of this system (Garfinkel, 1958; Klingenberg, 1958) and was given the name of cytochrome P450 because of its spectral properties after exposure to carbon monoxide (Omura & Sato, 1964). This haemoprotein is only one of a number of closely related enzymes (Coon et al., 1977) and is involved in the transfer of an activated oxygen species to the substrate. The nature of this activated oxygen complex is still uncertain but it is thought to involve an oxene species, the oxygen atom of which is transferred from molecular oxygen via cytochrome P450 to the substrate in a multi-step reaction (Estabrook & Werringloer, 1977; Trager, 1980).

1.II Mechanism of N-dealkylation

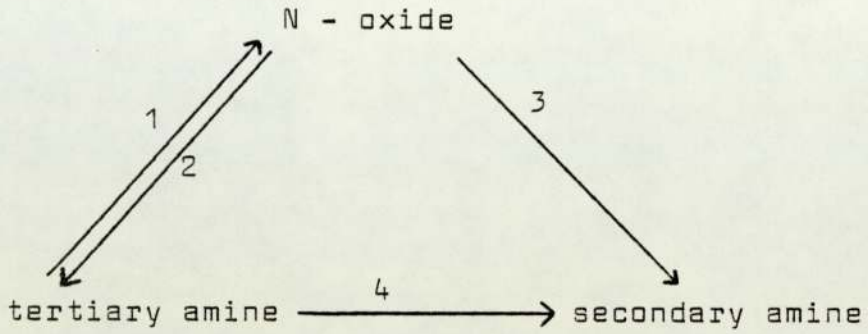
The overall oxidative N-dealkylation reaction as proposed by McMahon (1966) is shown in scheme 1.1.

Scheme 1.1



The precise mechanism of N-dealkylation is not fully understood. It is not known for example in the case of tertiary amines if initial carbon oxidation is involved to generate a carbinolamine, or whether nitrogen oxidation occurs followed either by rearrangement to a carbinolamine or by direct N-oxide demethylation. The problem is not as complex in the case of secondary amines as N-oxidation yields a hydroxylamine and not an N-oxide (Gorrod, 1978).

It was first proposed by Fish and co-workers that the initial oxidative step in the N-demethylation of tertiary amines could be the formation of an N-oxide. They demonstrated that N-oxides of dimethylglycine, dimethyltryptophan and other biogenic amines could be N-demethylated to yield the respective N-desmethyl compounds and formaldehyde. (Fish et al. 1955, 1956a, 1956b; Sweeley & Horning, 1956). A scheme showing the intermediary position of N-oxides in the N-demethylation of tertiary amines was proposed by Bickel (1969) and is shown in Fig. 1.1. The scheme postulates four enzymic reactions; N-oxidation, N-oxide reduction, N-oxide demethylation and tertiary amine N-demethylation, and these reactions have all been shown to occur simultaneously during the metabolism of dimethylaniline in-vitro using rat liver



1 - N-oxidation
2 - N-oxide reduction
3 - N-oxide demethylation
4 - tertiary amine demethylation

Fig. 1.1 The possible involvement of N-oxides in the N-demethylation of tertiary amines

(From Bickel, 1969)

microsomes (Willi & Bickel, 1973). The possible rearrangement of an N-oxide to yield a carbinolamine was also considered by Bickel (1969) as originally suggested by Wenkert (1954) when studying the oxidation of alkaloids. Wenkert proposed that an N-oxide could be converted to a carbinolamine by a Polonovski reaction mechanism (Polonovski & Polonovski, 1926) involving an electron transfer from biologically available ions or enzyme surfaces. The electronic structures of both N-oxides and carbinolamines have been investigated using molecular orbital calculations by Nelson et al. (1973a). These results suggest that N-oxides may be converted to carbinolamines but definitive conclusions regarding the relative involvement of the two species in N-dealkylation could not be made.

The involvement of N-oxides in N-dealkylation has been the subject of much debate and the early evidence has been summarized by McMahon (1966), who suggested that N-oxidation and N-dealkylation represent alternative pathways for the metabolism of tertiary amines. This seems probable as N-dealkylation is inhibted by carbon monoxide and SKF525-A whereas N-oxidation is not (Bickel, 1971) and N-dealkylation can occur with some substrates which cannot form N-oxides, e.g. tertiary amides (Testa & Jenner, 1976; Gorrod, 1978). Some substrates which undergo microsomal N-demethylation can be N-oxidized in microsomes but subsequent N-oxide demethylation is not catalyzed by microsomal enzymes (Bickel, 1972). This also supports the view that N-oxidation and N-dealkylation are separate biotransformations.

A general mechanism of N-dealkylation for all tertiary amines seems unlikely but the majority of evidence favours the formation of a carbinolamine at some stage during the process. In the case of N-demethylation this carbinolamine is a N-hydroxymethyl compound, and these species have indeed been isolated as intermediates in N-demethylation reactions (sec. 1.III).

It is also still uncertain whether the reaction of the activated oxygen species - the proposed oxene intermediate (see sec. 1.I) - proceeds via direct insertion of oxygen, initial hydrogen abstraction, or a combination of both mechanisms. The observation of small isotope effects during N-demethylation (Abdel-Monem, 1975) suggests that some degree of hydrogen abstraction does occur. In fact hydrogen abstraction itself can proceed by a heterolytic mechanism leading to the generation of an iminium ion (A, Fig.1.2) or via the generation of a stabilized methylene radical (B, Fig.1.2) (Hansch et al., 1965). There is evidence to suggest that iminium ions are produced in N-demethylation reactions (Breck & Trager, 1971; Nelson et al., 1973b; Hucker, 1973; Murphy, 1973; Nguyen et al., 1976, 1979). Whether they are formed by hydrogen abstraction or by dehydration of an N-hydroxymethyl compound - as proposed by Barker et al. (1980) in the case of dimethyltryptophan metabolism - remains to be elucidated. The possibility that a free radical mechanism may be involved in mixed function oxidase catalyzed N-demethylation is supported by the observations that chemical oxidation in systems which generate

free radicals also leads to N-demethylation (Needles & Whitfield, 1964; Tanaka & Wien, 1979; Sayo & Hasokawa, 1980). Indeed McMahon (1966) in a review of the mechanism of microsomal N-dealkylation suggested that this mechanism could be best understood in terms of a free radical hydroxylation reaction. It has been shown recently that free radicals are involved in biotransformation in certain biological systems. Lasker et al. (1981) and Sivarajah et al.(1982) have observed that N-dealkylation of a variety of substrates can occur during prostaglandin biosynthesis in-vitro. They and other workers have shown that this type of biotransformation involves a peroxidase enzyme which functions via a mechanism of one electron oxidation resulting in the generation of free radicals (Lasker et al. 1981; Sivarajah et al., 1982; Moldeus et al., 1982).

In summary it appears that N-dealkylation can occur by one or a combination of mechanisms and the possible enzymic pathways for the N-demethylation of tertiary amines are shown in Fig. 1.2.

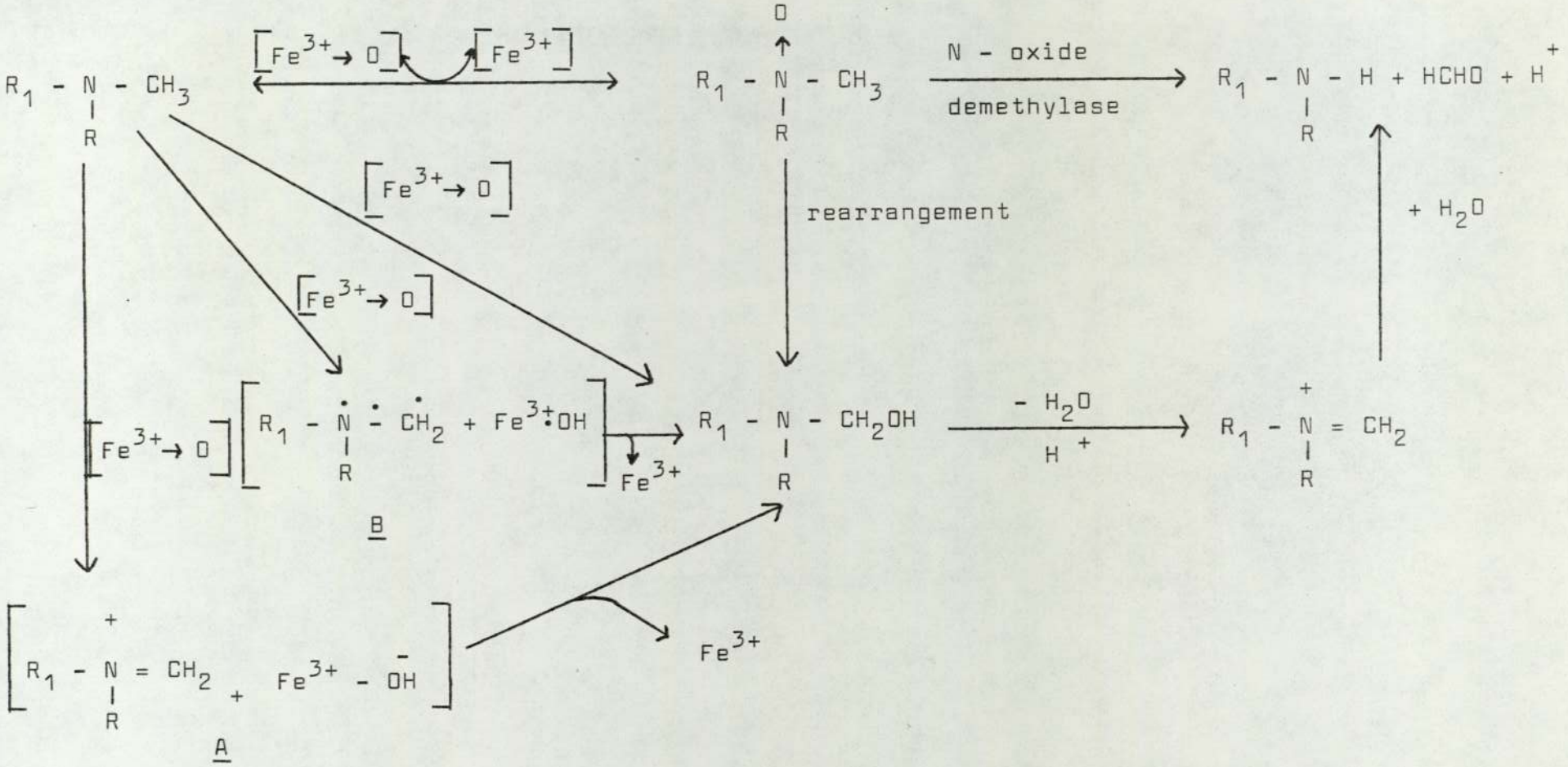
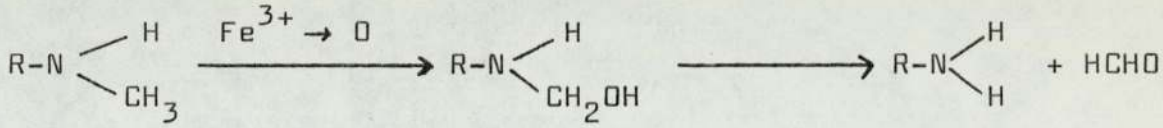


Fig. 1.2 Possible enzymic pathways for the N-demethylation of tertiary amines

1.III The synthesis of stable N-hydroxymethyl compounds and evidence for their production during the N-demethylation of N-methyl containing xenobiotics

Oxidative N-demethylation of N-methyl containing xenobiotics is considered to proceed via the generation of an N-hydroxymethyl intermediate or N-methylol (sec. 1.II). N-hydroxymethyl derivatives of amines or amides can be synthesized by reaction of the appropriate amine or amide with formaldehyde (Einhorn, 1905). This reaction is readily reversible in aqueous media (Kallen & Jenck, 1966). It is not surprising therefore that N-methylols generated during the metabolism of N-methyl containing compounds have been considered to be transient species which degrade chemically to yield the N-desmethyl derivative and formaldehyde (Brodie et al., 1958; Hucker, 1973; Testa & Jenner, 1976). This is represented in scheme 1.2.

Scheme 1.2



It was realised as early as 1953 in one of the first studies of N-demethylation *in-vitro* that N-hydroxymethyl compounds produced during metabolism may have sufficient stability to undergo further chemical reaction (Mueller & Miller, 1953). This interpretation was based on the production of formaldehyde during the N-demethylation of 3′-methyl 4-(methylamino)-azobenzene and on the ability of glutathione to react with an intermediate of metabolism to yield a water-soluble azo dye. The

latter compound could be hydrolyzed in acid to yield the water-insoluble N-demethylated aminoazo dye. It was proposed that the water-soluble dye was an N-methylene-glutathione adduct formed by the reaction of an N-hydroxymethyl intermediate with glutathione. Although these conclusions were tentative, unequivocal evidence has since been published which questions the assumption that N-hydroxymethyl derivatives are unstable species and this evidence is summarized below.

There are examples of synthetic N-hydroxymethyl derivatives of hexamethylmelamine (Borkovec & De Milo, 1967), dimethylphenyltriazenes (Gescher et al., 1978), and certain N-methyl amines, amides and imides (Einhorn, 1905; Koskikallio, 1956; Johansen & Bundgaard, 1979) which are stable compounds. Indeed the formation of N-hydroxymethyl derivatives of certain cyclic amides and imides has been used to increase the water solubility of the parent compounds (Bansal, 1976; Bansal et al., 1981; Bundgaard & Johansen, 1980a). These pro-drugs subsequently decompose in aqueous solution to yield the parent compound and one such pro-drug, N-hydroxymethylnitrofurantoin has been used clincially (Sorel & Roseboom, 1979). The release of formaldehyde during the decomposition of N-hydroxymethyl compounds could have toxicological implications (sec. 1.IV) but this release can also be advantageous. This is demonstrated by the therapeutic use of the urinary antiseptics such as noxythiolin (N′-hydroxymethyl-N-methylthiourea) whose antiseptic activity

is due to the release of formaldehyde in aqueous solution (Kingston, 1965).

Gescher et al. (1979) observed that stable precursors of formaldehyde were produced during the N-demethylation of hexamethylmelamine in-vitro using a mouse liver microsomal system, and subsequently characterized N-hydroxymethylpentamethylmelamine as a major metabolite of hexamethylmelamine in this system (Gescher et al., 1980). A stable N-methylol has also been isolated in the urine of rats after the administration of the chemotherapeutic agent DTIC (Kolar et al., 1980). Likewise N-hydroxymethyl compounds have been identified as in-vitro metabolites of the antineoplastic agent procarbazine (Weinkam & Shiba, 1978) and N-methylcarbazole (Gorrod & Temple, 1976). Barker et al. (1980) have suggested that the formation of tetrahydro-β-carbolines as metabolites of dimethyltryptophan in rat brain may occur via dehydration of an N-hydroxymethyl intermediate followed by cyclization of the resultant iminium ion. It remains a possibility that direct cyclization of an N-hydroxymethyl intermediate may also occur. This has been proposed as the mechanism whereby bromhexine forms a substituted tetrahydroquinazoline in-vivo (Schraven et al., 1967).

The early observation of Mueller & Miller (1953) that N-hydroxymethyl derivatives of N-methyl substituted aminoazo dyes may have sufficient stability to undergo further chemical reaction was supported by the findings of Roberts & Warwick (1963). They observed greater covalent binding of 4-aminoazobenzene to nucleic acids in an in-vitro

system when formaldehyde was present than when it was omitted, and suggested the greater binding was a result of the formation of an N-hydroxymethyl compound. Ketterer et al. (1982) have shown recently that an N-methylene-glutathione adduct could be isolated in the bile of rats after the administration of N,N-dimethylaminoazobenzene. They suggested, in agreement with Mueller & Miller (1953), that an N-hydroxymethyl intermediate may be the metabolite which reacts with glutathione to form this adduct. A depletion in hepatic glutathione levels in rats has been shown to inhibit the rate of biliary excretion of metabolites of N,N-dimethylaminoazobenzene in-vivo and to decrease the N-demethylation of this compound in-vitro (Levine & Finkelstein, 1978). These workers and others have also demonstrated that the in-vitro metabolism of N,N-dimethylaminoantipyrine is not affected by glutathione depletion (Levine & Finkelstein, 1978; Brindley et al., 1982a) and it has been suggested that N,N-dimethylaminoantipyrine forms formaldehyde rather than an N-hydroxymethyl intermediate during metabolism in-vitro (Gescher et al., 1979). A tentative explanation of the findings described above may be that glutathione affects the N-demethylation of substrates depending on the extent to which they are biotransformed to N-hydroxymethyl metabolites or formaldehyde.

N-hydroxymethyl compounds have also been shown to be metabolites both in plants and animals of the methylamide insecticides bidrin, azodrin (Menzer & Casida, 1965), and dimethoate (Lucier & Menzer, 1970), of carbaryl (Dorough &

Casida, 1964) and of many other carbamate insecticides (Fukuto, 1972). The N-methylol derived from N/ -(4-chlorophenyl)-N-methylurea has been isolated in a microsomal system prepared from cotton plants (Tanaka et al., 1972), and the N-hydroxymethyl derivative of the insecticide schradan has been tentatively identified as an oxidation product of the parent compound in plants, animals and chemical systems (Heath et al., 1955; Spencer et al., 1957).

In the examples quoted above the synthesis of N-methylols, and their isolation or the isolation of their further reaction products during metabolism has been described. Conjugation of N-hydroxymethyl compounds formed during metabolism with endogenous compounds other than glutathione may also occur. O-glucuronide derivatives of N-methylols have been isolated as urinary metabolites of 3-(2,4,6-trichlorophenyl)-1,1-dimethyltriazene (Kolar & Carubelli, 1979), diphenamid (McMahon & Sullivan, 1965), carbaryl (Hassan et al., 1966) oxadiazole (Allen et al., 1971) and N-methyl-α-ethyl-glutarimide (Keberle et al., 1963) in rats. The O-glucoside and α-glycoside conjugates of the N-hydroxymethyl metabolites of monuron (Frear & Swanson, 1972) and carbaryl (Kuhr & Casida, 1967) respectively have been isolated in plants.

It has been suggested that the metabolic conversion of an N-methyl group to an N-formyl group may occur in a two step process involving an N-hydroxymethyl intermediate (McMahon, 1966). The isolation of such N-formyl compounds

as metabolites of the N-methyl containing compounds chlorotoluron (Muecke et al., 1976) and N-methylaminoantipyrine (Noda et al., 1976; Nigam et al., 1980) suggests that these compounds may have been metabolized to N-methylols with sufficient stability to undergo further oxidative biotransformation.

In many of the examples of the synthesis of N-hydroxymethyl compounds and their isolation during metabolism the N-methyl group is situated adjacent to a carbonyl group. This is in accordance with the view of Hucker (1973) that carbinolamides are more stable than carbinolamines although no explanation of this has been given. It may be relevant that in all cases where N-hydroxymethyl intermediates have been isolated the N-methyl group is attached to molecules of electronegative character. This is in agreement with the suggestion of Gorrod et al. (1970) that the basicity of the nitrogen bearing the N-methyl group may determine whether complete N-demethylation or the generation of a characterizable N-methylol occurs.

1.IV Possible implications of the formation of N-hydroxymethyl intermediates during metabolism

Many N-methyl compounds do form N-hydroxymethyl intermediates during oxidative N-demethylation (see sec.1.III) including many herbicides and insecticides. The N-demethylation of these compounds in plants and pests appears to be a detoxification process (Menzer & Casida, 1965; Casida & Lykken, 1969; Tanaka & Wien, 1979) although in some cases the biotransformation products have equal or greater toxicity than the parent compounds. It has been proposed for example that the active inhibitor of choline esterases produced during the metabolism of the insecticide schradan is its N-hydroxymethyl derivative (Heath et al., 1955; Spencer et al., 1957).

The generation of N-hydroxymethyl metabolites of N-methyl containing xenobiotics with sufficient stability to reach sites distal from the liver may have toxicological significance. This potential toxicity could result from reactions of the intact N-hydroxymethyl compound or be associated with its degradation products. The evidence pertaining to both of these possibilities is discussed below.

Weitzel et al. (1963) have shown that some N-hydroxymethyl amines and amides can amino- or amidomethylate the nucleophiles cysteine and glutathione in aqueous solution. These thiols, which are present in high concentrations in the liver, have a protective function and are able to react with potentially toxic electrophiles (Orrenius & Jones, 1978). They may therefore be able to detoxify N-hydroxymethyl

compounds if these are generated within the liver. However if N-methylols are able to reach extrahepatic sites with only low thiol levels, they may react with essential nucleophilic sites of enzymes or nucleic acids rather than undergo detoxification by reaction with thiols.

It could also be speculated that N-hydroxymethyl compounds may induce a toxic effect by interference with the synthesis and utilization of one carbon units within the cell. These are required for various synthetic reactions including the synthesis of purines and thymidine (Harper, 1975). A cyclic series of reactions in which methyl groups are oxidized and one carbon units generated has been described by Mackenzie & Frisell (1958) and is shown in Fig. 1.3. Dimethylglycine is demethylated by mitochondrial enzymes in a two step process to produce sarcosine and glycine. Mackenzie and co-workers have shown that this biotransformation is accompanied by the generation of one carbon units with the oxidation level of formaldehyde at each demethylation step. These one carbon units are able to condense with glycine to form serine whereas formaldehyde is not incorporated into this pathway. The authors called these one carbon units 'active formaldehyde' and demonstrated that they could be converted to formaldehyde in an irreversible reaction, (Mackenzie, 1955; Frisell & Mackenzie, 1955; Mackenzie & Abeles, 1956). 'Active formaldehyde' has been shown to be N_5-N_{10}-methylene-tetrahydrofolate (Osborn et al., 1960).

The N-demethylation of N,N-dimethylglycine has been suggested to occur via an N-oxide (Fish et al., 1955,

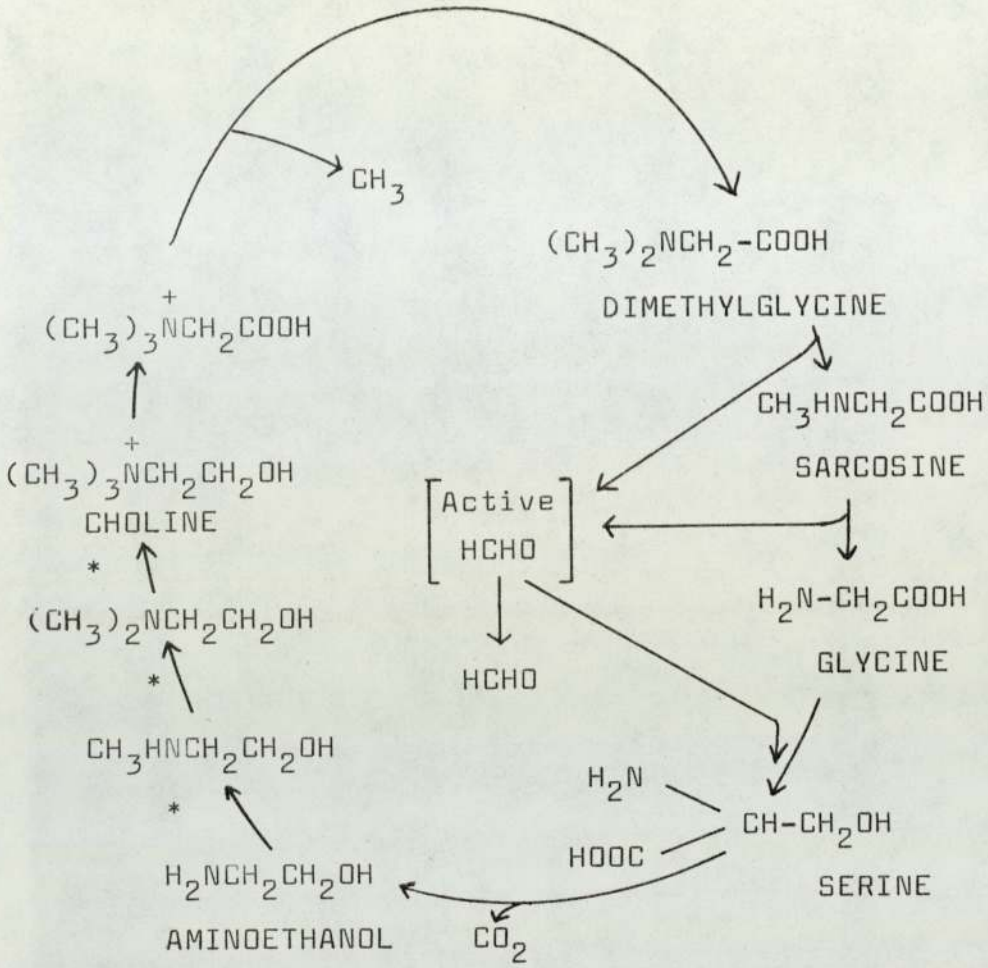


Fig. 1.3 The one-carbon cycle

From Mackenzie and Frisell (1958)

* = + S - adenosylmethionine

1956a, 1956b) which may then rearrange to an N-hydroxymethyl intermediate (Sweeley & Horning, 1956). Gaudette & Brodie (1959) showed that the microsomal and mitochondrial enzyme systems which catalyze oxidative N-demethylation had widely differing substrate specificity dependent mainly on the lipid solubility of the substrate. They suggested that although N-demethylation by the two enzyme systems proceeds by different mechanisms the generation of an N-hydroxymethyl intermediate may be common to both processes. It may therefore be speculated that N-hydroxymethyl compounds which are generated in microsomes may be able to inhibit or act as substrates for the enzymes responsible for the metabolism of dimethylglycine in mitochondrial systems. Miller et al. (1952) have shown that the N-methyl groups of substituted aminoazo dyes, which it has been proposed form N-hydroxymethyl intermediates during N-demethylation, (see sec. 1.III), are incorporated into the one carbon pool. Any connection however between microsomal generation of N-hydroxymethyl metabolites and incorporation into the one carbon pool is very difficult to establish as formaldehyde can also act as a source of one carbon units when injected into animals (Sakami 1948; Siekevitz & Greenberg, 1949; Plaut et al., 1950; Neely, 1964). This occurs by conversion of formaldehyde via formate to N_{10}-formyltetrahydrofolate (Waydhas et al., 1978) which can then be converted to N_5,N_{10}-methylenetetrahydrofolate (Krebs et al., 1976) which can then enter the one carbon pool. It remains a possibility that there is a quantitative difference in the extent to which N-hydroxymethyl compounds or formaldehyde

are incorporated into the one carbon pool. This may be one explanation of the findings of Gescher & Raymont (1981) who observed quantitative differences in the metabolism of the N-methyl groups of a series of substrates to carbon dioxide in-vivo.

It is conceivable that if N-hydroxymethyl intermediates (or conjugates) have sufficient stability to leave the liver they may deposit formaldehyde and the N-des-(hydroxymethyl) compound in extrahepatic tissues.

Formaldehyde is mutagenic (Rapoport, 1946; Auerbach et al., 1977) and carcinogenic (Nature, 1979; Swenberg et al., 1980). These properties have been associated with the ability of formaldehyde to form dimers of adenylic acid linked through methylene bridges (Alderson, 1960, 1964). Formaldehyde has also been shown to cause DNA-protein cross links (Ross & Shipley, 1980), transfer RNA cross links (Axelrod et al., 1969), altered ribosomal RNA transcription (Nocentini et al., 1980) and to interact with amino groups in amino acids to produce N-hydroxymethyl derivatives and amino acid dimers (Fraenkel-Conrat & Olcott, 1948; Kallen & Jenck, 1966; Kitamoto & Maeda, 1980). Usually the potential toxicity of formaldehyde is not expressed when it is formed as a result of the metabolism of a xenobiotic in the liver. This is a result of the rapid hepatic oxidation of formaldehyde to formate which is catalyzed mainly by dehydrogenase enzymes (Tephly et al., 1974). If formaldehyde was deposited in extrahepatic tissues as a degradation product of an N-methylol efficient detoxification of the formaldehyde generated would depend on the level of

formaldehyde dehydrogenase activity in these tissues.

The generation of the N-des-(hydroxymethyl) compound may also be of toxicological significance in certain cases, e.g. with the carcinogens N,N-dimethyltriazenes, N,N-dimethylnitrosamine and azoxymethane. The monomethyl derivatives of these compounds have very short half lives (Preussman et al., 1969; Barbin & Bartsch, in Kuroki & Drevon, 1978; Nagasawa et al., 1972) and it has been shown that they or their decomposition products are capable of methylating nucleophiles in biological systems, (Preussman & von Hodenberg, 1970; Magee et al., 1975; Matsumoto & Higa, 1966). N-hydroxymethyl intermediates may therefore be regarded as transport forms of methylating agents, and the formation of N-hydroxymethyl compounds during metabolism has been implicated in the formation of carcinogenic species derived from N,N-dimethyltriazenes (Preussman et al., 1969) N,N-dimethylnitrosamine (Druckrey et al., 1967; Fahmy & Fahmy, 1975; Gold & Linder, 1979) and azoxymethane, a metabolite of N,N-dimethylhydrazine (Druckrey, 1970; Fiala, 1977).

The generation of N-hydroxymethyl compounds during the metabolism of some N-methyl containing xenobiotics may also have implications for antitumour activity. It has been shown that certain N-methyl containing antitumour agents possess absolute requirements for the N-methyl group and bioactivation in order to exert antitumour activity, e.g. hexamethylmelamine (Rutty & Connors, 1977) aryldimethyltriazenes (Audette et al., 1973; Connors et al., 1976) and procarbazine (Bollag, 1964; Oliverio, 1973). N-methylols have been implicated in the mechanism of action of these N-methyl

containing antitumour agents (Hickman, 1978) and this activity may be mediated through processes similar to those discussed previously, i.e. the reactions of intact N-methylols or their degradation products (Weitzel et al., 1963; Rutty & Connors, 1977; Hickman, 1978).

The hypothesis that intact N-methylols may be involved in the antitumour activity of N-methyl containing antitumour agents is supported by the findings of Weitzel et al. (1963). They showed that the same N-hydroxymethyl compounds which were found to react with glutathione and cysteine also inhibited the growth of mouse ascites carcinoma cells in-vitro. This inhibition could not be reversed by semicarbazide, a compound which combines with free formaldehyde (Mann & Saunders, 1960a). As discussed with respect to toxicity N-methylols may interfere with the enzymes responsible for one carbon unit utilization within the cell and this may also have implications for antitumour activity.

As formaldehyde is cytotoxic (Kini & Cooper, 1962) its generation during the metabolism of N-methyl containing antitumour agents may be related to their mode of antineoplastic activity. However it can be argued that not all N-methyl compounds which generate formaldehyde on metabolism are antitumour agents. If metabolites derived from the N-methyl group are important for antitumour activity then it has to be assumed that there are differences in the metabolism of the N-methyl moiety of antitumour agents and of other N-methyl containing compounds. The varying stabilities of N-hydroxymethyl compounds generated during metabolism may explain this difference.

1.V Aim and Scope of present work

The metabolic generation of N-hydroxymethyl compounds from N-methyl containing xenobiotics may have toxicological consequences and their formation may also be involved in the mechanism of action of antitumour agents which contain the N-methyl moiety (see 1.IV). The aim of this study was to characterize the types of N-methyl containing compound which form N-hydroxymethyl intermediates rather than formaldehyde during metabolism, and to investigate the stability of these intermediates. An understanding of the structural characteristics necessary in an N-methyl containing molecule which predisposes it to form a characterizable N-hydroxymethyl intermediate during metabolism may be important in predicting toxicity and may lead to novel antitumour agents.

There are many examples of N-methyl containing compounds which form relatively stable N-hydroxymethyl intermediates during metabolism (sec.1.III), but these intermediates have been isolated in widely differing biological systems. It was therefore considered relevant to conduct a structure-metabolism study to investigate the metabolism of N-methyl containing compounds in an *in-vitro* model system which would allow comparisons of the metabolism of the substrates used. The method used for initial studies was a colourimetric assay, previously described by Gescher et al. (1979). This method is able to distinguish between free formaldehyde and its stable precursors such as N-hydroxymethyl compounds.

The rationale for the choice of substrate was based on previous studies with hexamethylmelamine and aryldimethyltriazenes in which stable N-hydroxymethyl derivatives of these compounds had been synthesized, and isolated as metabolites of the parent compounds (sec.1.III). Derivatives of hexamethylmelamine (V, Fig.1.4) and aryldimethyltriazenes (II, Fig.1.4) were therefore included in this study as examples of compounds which could form stable N-methylols during N-demethylation. The analgesic agent N,N-dimethylaminoantipyrine (VI, Fig.1.4) was also included as it had been shown to produce free formaldehyde during metabolism in the in-vitro system used in this study (Gescher et al., 1979). The other model compounds used in this study were of the general formula $Ar-X-N(CH_3)_2$ shown in Fig.1.4 where X equals -NHCO- (aryldimethylureas, IV Fig.1.4), X equals - N=CH- (aryldimethylformamidines, III, Fig.1.4), or where X was absent (aryldimethylamines, I, Fig.1.4). The ureas and formamidines are structurally similar to the triazenes but the nitrogen bearing the methyl groupings is in different electronic and steric environments. The arylamines were chosen as simpler models and were considered appropriate to this study as evidence had been presented which suggests that substituted arylamines such as N-methyl substituted aminoazo dyes may be metabolised to produce N-methylols (sec. 1.III).

The formation of N-hydroxymethyl compounds during

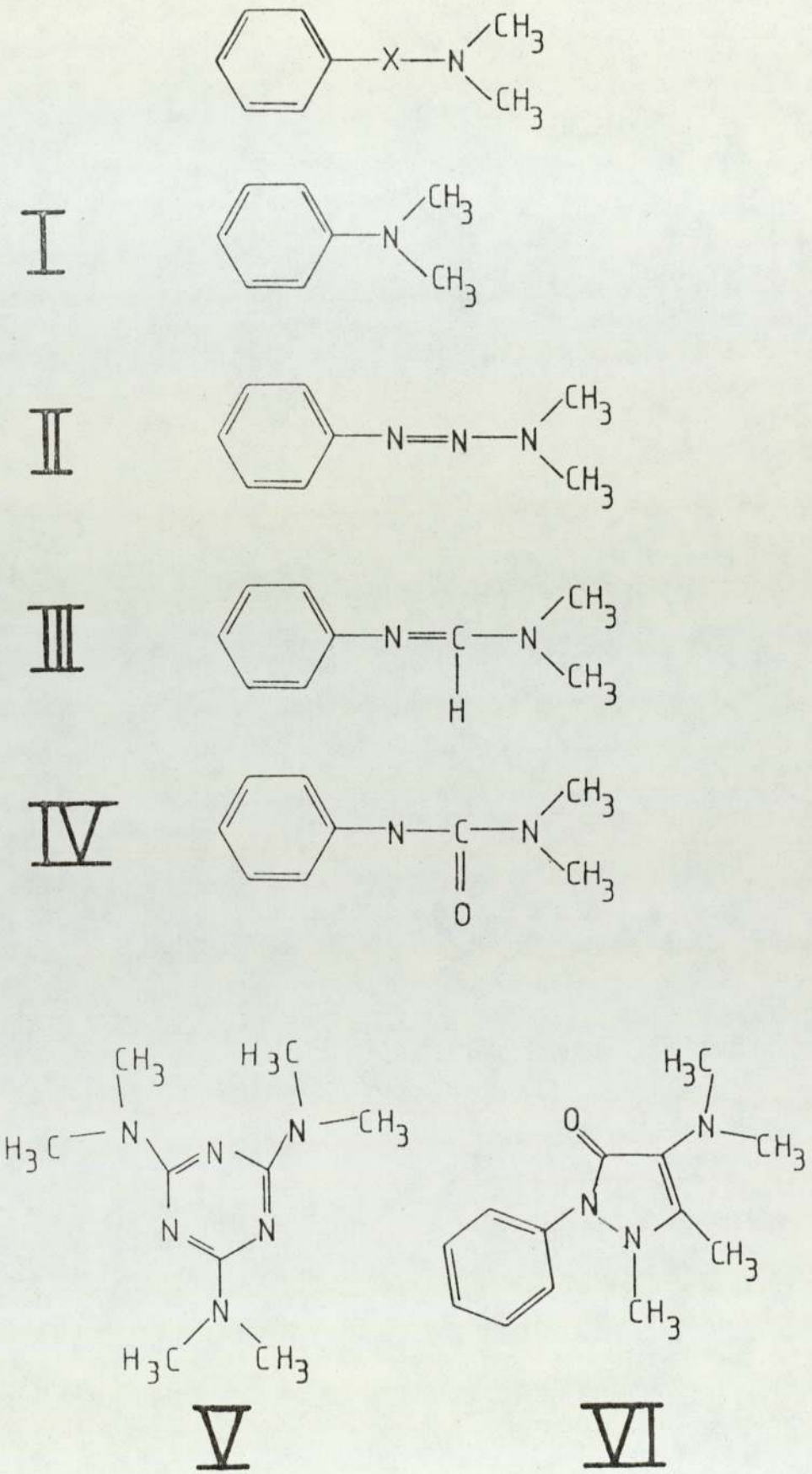


Fig. 1.4 Types of substrates used in structure-metabolism study

the metabolism of N-methyl containing xenobiotics may depend on the basicity of the nitrogen atom bearing the N-methyl group (Gorrod et al., 1970). The nitrogen atom in the model compounds Ar-X-$N(CH_3)_2$ is situated in varying electronic environments which therefore allows investigation of this hypothesis. It was also of interest to determine if electronic changes within a particular class of compounds could qualitatively affect the products of N-demethylation, i.e. whether an N-hydroxymethyl intermediate or formaldehyde was produced. Derivatives of the aromatic model compounds (I to IV, Fig. 1.4) were therefore used in this investigation with both electron withdrawing and electron donating substituents in the 4-position of the ring.

Physical properties such as lipid solubility and basicity have been shown to correlate with the rate of N-demethylation of certain N-methyl containing compounds both in-vitro and in-vivo (McMahon, 1966; Hansch et al., 1965; Gaudette & Brodie, 1959). It was therefore considered relevant to assess whether the generation of an N-hydroxymethyl metabolite rather than formaldehyde during N-demethylation could be correlated with these physical properties and the electron density distribution of the parent N-methyl compounds.

It was also proposed to investigate the metabolism of selected compounds in more detail. The choice of these compounds depended on the results of the initial structure-

metabolism study and on stability studies of various synthetic N-methylols using colourimetric techniques. In these further investigations more definitive analytical techniques such as high pressure liquid chromatography (H.P.L.C.) and gas chromatography (G.C.) were utilized to characterize proposed N-hydroxymethyl intermediates and identify any products of their further biotransformation.

SECTION 2

MATERIALS

2.I Substrates and derivatives used in metabolism studies

2.I.1 Purchased

The following compounds were purchased from the sources indicated.

2.I.1.a B.D.H. Chemicals Limited, Atherstone

4-dimethylaminoazobenzene

Paraformaldehyde

2.I.1.b Aldrich Chemical Company, Gillingham

2-dimethylaminopyridine

4-dimethylaminobenzonitrile

4-dimethylaminopyridine

N,N-dimethylaminoantipyrine

N,N-dimethylaniline

N,N-dimethylformamide

N,N-dimethyl-p-toluidine

Formamide

N-methyformamide

2.I.2 Gifts

N-hydroxymethylbenzamide, N-hydroxymethylphthalimide, and N-hydroxymethyltrichloroacetamide were gifts from Dr. H. Bundgaard, Royal Danish School of Pharmacy, Copenhagen. N-methylaminoantipyrine was kindly provided by Hoechst Pharmaceuticals, Frankfurt.

2.I.3 Synthesized

The compounds listed in this section were synthesized in our laboratories by:

1) Dr. M. D. Threadgill
2) Prof. M. F. G. Stevens
3) Dr. K. Vaughan
4) Mr. S. P. Langdon
5) Mr. E. N. Gate

2.I.3.a N′-phenylurea derivatives[1]

The following compounds were prepared by addition of a solution of the corresponding aryl isocyanate in diethylether or tetrahydrofuran to a large excess of the appropriate ethereal amine essentially according to published procedures (Ross et al., 1982).

4-chlorophenylurea

N,N-dimethyl-N′-phenylurea

N′-(4-bromophenyl)-N,N-dimethylurea

N′-(4-chlorophenyl)-N,N-dimethylurea

N′-(4-chlorophenyl)-N-methylurea

N′-(4-cyanophenyl)-N,N-dimethylurea

N′-(4-methoxyphenyl)-N,N-dimethylurea

N′-(4-methylphenyl)-N,N-dimethylurea

N′-(4-trifluoromethylphenyl)-N,N-dimethylurea

N′-(4-chlorphenyl)-N-formyl-N-methylurea and N′-(4-chlorophenyl)-N-formylurea were prepared according to the method of Crosby & Tang (1969).

2.I.3.b N′-phenyl-N,N-dimethylformamidines[1,2].

These compounds were prepared by condensation of the appropriate aniline with dimethylformamide dimethylacetal essentially according to the method of Zupan et al. (1974).

N′-(4-cyanophenyl)-N,N-dimethylformamidine

N′-(4-sulphonamidophenyl)-N,N-dimethylformamidine.

The following compounds were prepared according to the above method and isolated as their tosylate salts:

N′-phenyl-N,N-dimethylformamidine

N′-(4-chlorophenyl)-N,N-dimethylformamidine

N′-(4-methylphenyl)-N,N-dimethylformamidine

N′-(4-trifluoromethylphenyl)-N,N-dimethylformamidine

2.I.3.c 3-phenyl-1,1-dimethyltriazenes [2,3]

The following compounds were prepared by treatment of the appropriate aryl diazonium salt with aqueous dimethylamine essentially according to the method of Connors et al. (1976).

3-phenyl-1,1-dimethyltriazene

3-(4-acetylphenyl)-1,1-dimethyltriazene

3-(4-carboxymethylphenyl)-1,1-dimethyltriazene

3-(4-chlorophenyl)-1,1-dimethyltriazene

3-(4-trifluoromethylphenyl)-1,1-dimethyltriazene

2.I.3.d Melamine derivatives [2,4]

N-hydroxymethylpentamethylmelamine was prepared according to the method of Borkovec & De Milo (1967). The following compounds were prepared essentially according to the methods of Thurston et al. (1951) and Paget & Hamner (1958).

Hexamethylmelamine

Pentamethylmelamine

N_2,N_2,N_4,N_4-tetramethylmelamine

N_2,N_2,N_4,N_6-tetramethylmelamine

N_2,N_4,N_6-trimethylmelamine

2-azido-4,6-bis-(dimethylamino)-1,3,5-triazine

2-chloro-4,6-bis-(dimethylamino)-1,3,5-triazine

2.I.3.e Benzamide derivatives [1]

All N-methyl and unsubstituted benzamides were

prepared by reaction of the appropriate benzoyl chloride with the corresponding amine by the Schotten-Baumann technique (Mann & Saunders, 1960b).

All N-hydroxymethylbenzamides were prepared by treatment of the corresponding benzamide with excess aqueous formaldehyde (37%) in boiling tetrahydrofuran in the presence of potassium carbonate.

N-formylbenzamide was synthesized according to the method of Finkbeiner (1965).

2.I.3.f Formamide derivatives [5]

N-hydroxymethylformamides were prepared from the corresponding amine and paraformaldehyde according to published methods (Grady & Stott, 1965).

N-ethoxymethylformamide was prepared by the reaction of N-hydroxymethylformamide with ethanol under acidic conditions (Finkelstein & Ross, 1972).

N-(N′-N′-dimethylaminomethyl)-formamide, N-acetyloxymethyl-N-methylformamide and its N-benzoyloxymethyl derivative were synthesized according to published procedures (Bohme & Fuchs, 1970; Ross et al., 1966; Bamford & White, 1959). N-ethylformamide was prepared according to the method of Saegusa et al. (1969).

2.II Miscellaneous chemicals

The following compounds were purchased from the sources indicated.

2.II.1 B.D.H. Chemicals Limited, Atherstone

Acetylacetone

Ammonium acetate

Benzoic acid

4-chloroaniline

2.II.2 Aldrich Chemical Company, Gillingham

N,N-dimethylacetamide

3-methyl-2-benzothiazolone hydrazone hydrochloride

4-nitrobenzamide

Pyrazole

2.II.3 Sigma Chemical Company, Poole

Bovine serum albumin - A8022

Glucose-6-phosphate (G6P) - - G-7879

Heparin - H7005

Nicotinamide adenine dinucleotide (NAD) - N0632

Nicotinamide adenine dinucleotide phosphate (NADP) - N0505

Nicotinamide adenine dinucleotide phosphate reduced form (NADPH) - N1630

Trizma base - T3253

2.III Chromatographic materials

2.III.1 Columns, plates and packing materials

H.P.L.C.columns were purchased from Anachem Limited, Luton, apart from radial compression columns which were purchased from Waters Limited, Northwitch. Reverse phase

thin layer chromatography (T.L.C) plates (K-C_{18}) were obtained from Whatman Lab Sales Limited, Maidstone. Packing materials for G.C. columns were obtained from Phase Separations Limited, Queensferry.

2.III.2 Derivatizing agents

N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), trimethylsilylimidazole (TSIM) and heptafluoracetic acid anhydride (HFAA) were purchased from Phase Separations Limited, Queensferry.

2.III.3 Solvents

Liquid chromatography grade methanol, hexane and analar grade ethanol were obtained from B.D.H. Chemicals Limited Atherstone.

2.IV Enzymes

The following enzymes were obtained from Sigma Chemical Company Limited, Poole.

B-glucuronidase	- G0251
Collagenase type IV	- C5138
Glucose-6-phosphate dehydrogenase (GPDH)	- G8878
Horse liver alcohol dehydrogenase (HLAD)	- 340-L2
Yeast alcohol dehydrogenase	- A-7011
Yeast alcohol oxidase	- A-0763

2.V Animals and their diets

Animals were obtained from Bantin and Kingman Limited, Hull, and were maintained in an animal house for one week to acclimatise. They were fed on water and Heygates modified 41B breeding diet ad libitum.

2.VI Human body fluid samples

These were obtained from patients who were under the medical supervision of Dr. G. McVie, Netherlands Cancer Institute, Plesmanlaan, Amsterdam.

2.VII Buffer solutions and serum

2.VII.1 Earl's buffer

Sodium chloride	-	6.80g
Sodium bicarbonate	-	2.20g
Glucose	-	1.00g
Potassium chloride	-	0.40g
Sodium dihydrogen phosphate dihydrate		0.14g
Distilled water	-	to 1L.

This buffer was adjusted to pH=7.4, unless otherwise stated, by the addition of hydrochloric acid.

2.VII.2 Krebs-Henseleit buffer

Sodium chloride	-	6.80g
Sodium bicarbonate	-	2.10g
Glucose	-	2.00g
Potassium chloride	-	0.40g
Magnesium sulphate heptahydrate	-	0.14g
Calcium chloride	-	0.28g
Sodium dihydrogen phosphate dihydrate		0.14g
Distilled water	-	to 1L.

This buffer was adjusted to pH-7.4 by the addition of hydrochloric acid.

2.VII.3 Tris buffer 0.01M

Trizma base	1.21g
Distilled water	to 1L

This buffer was adjusted to the required pH using hydrochloric acid.

2.VII.4 Acetate buffer

This was prepared from 0.1M acetic acid and 0.1M sodium acetate solution as described in Documenta Geigy (1962).

2.VII.5 Foetal calf serum

This was purchased from Gibco Limited, Glasgow.

SECTION 3

METHODS

Male Balb c mice (20 - 25g) were used for all experiments unless otherwise stated.

3.I In-vitro structure - metabolism study using a colourimetric method which distinguishes between free formaldehyde and its precursors.

3.I.1 The Nash colourimetric assay for formaldehyde.

This method was first described by Nash (1953) and is based on a reaction for the synthesis of pyridines (Hantzsch, 1882). The assay employs acetylacetone which has been described as the most highly selective reagent for the determination of formaldehyde (Sawicki & Sawicki, 1975) and the principle of the reaction is shown in Fig.3.1.

The assay was used in this study as described by Werringloer (1978). Nash reagent (1 ml) was added to the test solution (2 ml) and the mixture was heated at $60^{o}C$ for 20 minutes. The absorbance at 412nm. of the resultant chromophore was determined using a Cecil CE5095 ultra-violet (U.V.) spectrophotometer. Standard solutions were prepared by dissolving paraformaldehyde in 1N NaOH solution (1 ml) and adjusting to a final volume of 100 ml with distilled water. Formaldehyde solutions were prepared by dilution of standard solutions with Earl's buffer and calibration curves were constructed from the absorbance values at 412nm corrected using control mixtures without added formaldehyde, using linear regression analysis.

3.I.2 The in-vitro N-demethylation of N-methyl containing compounds

Livers were excised between 8 and 10 a.m. each morning and rinsed in ice cold sucrose 0.25M. A 20% $^{w}/_{v}$ homogenate in ice cold sucrose was then prepared using 8

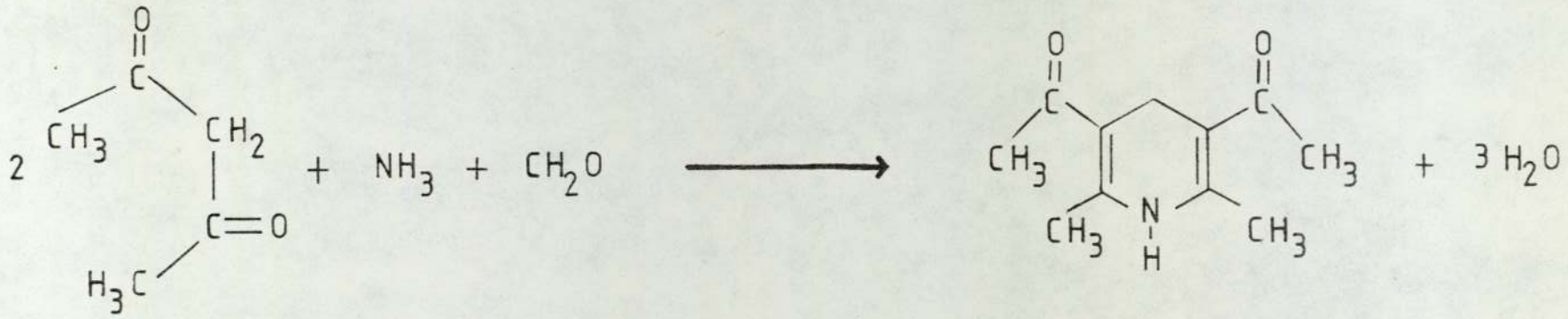


Fig. 3.1 The Principle of the Nash reaction

strokes of a Camlab 563C homogeniser (speed 6) fitted with a teflon pestle.

Microsomes were prepared by differential centrifugation according to the method of Schenkman & Cinti (1972) which involves the use of calcium chloride to increase the rate of sedimentation of microsomes. It has been shown that the activities of hepatic mixed function oxidase enzymes in calcium-sedimented microsomes and in microsomes prepared by conventional methods are similar (Cinti et al., 1972). The resultant microsomal pellet was suspended in Earl's buffer.

Reaction mixtures consisted of varying amounts of microsomal suspension, magnesium chloride ($MgCl_2$) 5mM, and sufficient G6P, GPDH and NADP to produce NADPH 0.5mM in a final volume of Earl's buffer (2.5ml). Reactions were performed in beakers (25ml), and were started by the addition of substrate in a maximum volume of 0.1ml of a suitable solvent (Table 3.1.) Reactions were incubated for thirty minutes in a shaking water bath (80 strokes/min) at $37^{o}C$, and were stopped by the addition of 20% $^{w}/v$ trichloroacetic acid (0.25ml) or in the case of acid labile triazenes by 20% $^{w}/v$ zinc sulphate solution (0.6ml) and saturated barium hydroxide solution (0.6ml). Protein was removed by centrifugation and the products of demethylation were measured as described in sec. 3.I.1.

In experiments where N,N-dimethylaminoazobenzene was used as substrate, 1N NaOH solution (0.3ml) was added to the aqueous layer which was then extracted twice with ether (5ml) to remove residual azo dyes prior to the addition of Nash reagent.

SUBSTRATE	SOLVENT
3-aryl-1,1-dimethyltriazenes	acetone
Arylamines	acetone
$N^{/}$-aryl-N,N-dimethylureas except 1) $N^{/}$-(4-cyanophenyl)-N,N-dimethylurea	acetone DMSO ≠
$N^{/}$-aryl-N,N-dimethylformamidines 1) free bases 2) tosylate salts	 acetone buffer
Melamine derivatives except 1) $N_2N_2N_4N_4$-tetramethylmelamine	acetone DMSO
Aminoantipyrine derivatives	acetone

≠ - Dimethylsulphoxide

Table 3.1 Solvents used to dissolve substrates

Control incubations without an NADPH generating system, and with solvent replacing substrate solution were used and all incubations were performed in duplicate.

3.I.3 The colourimetric assay to distinguish between free formaldehyde and its precursors produced during N-demethylation in-vitro

This assay was performed essentially as described by Gescher et al. (1979) and an outline of the method is given in Fig. 3.2. Substrates were incubated with hepatic microsomes using the conditions described in sec. 3.I.2 except that a final volume of Earl's buffer (12.5ml) was used in beakers (50ml). At the end of a thirty minute incubation period microsomes were precipitated by centrifugation and aliquots of the supernatant were incubated with microsome-free homogenate equivalent to 50mg liver and fortified with NAD 1mM in a final volume of 2.5ml Earl's buffer in beakers (25ml). At time = 0 and after 5 and 15 minutes incubation duplicate samples were deproteinized and assayed as described in sec. 3.I.1.

Substrates were added in a maximum volume of 0.5ml of the appropriate solvent (Table 3.1). Substrate concentrations varied between 0.5 and 5mM, and the amount of microsomal suspension used varied between the equivalent of 0.8g and 1.6g of wet liver weight per 12.5ml incubation according to the degree of N-demethylation of the substrate (see sec. 4.I.2.b).

Control incubations were performed without an NADPH generating system, and with solvent replacing the

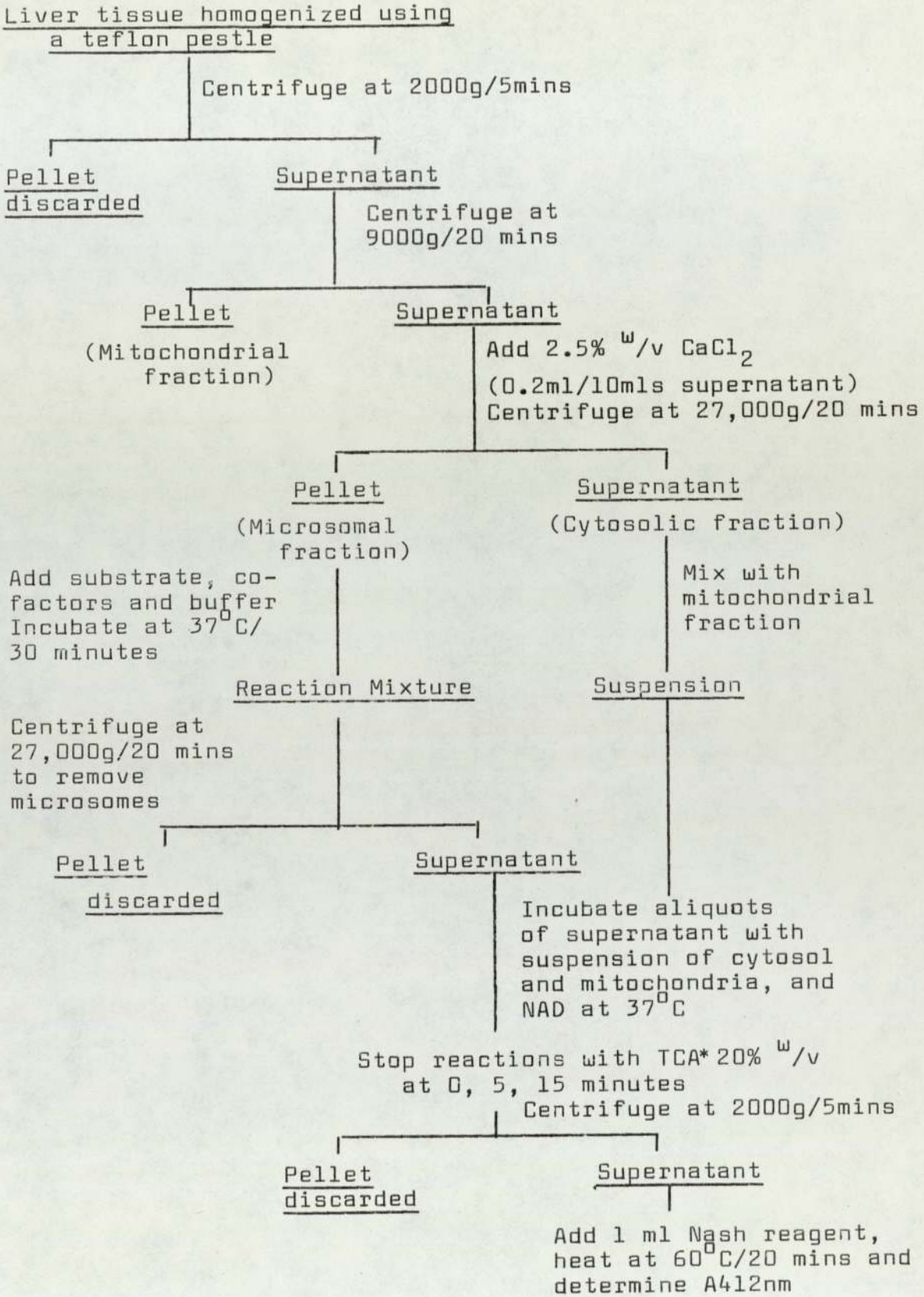


Fig. 3.2 Method to distinguish between free formaldehyde and its precursors produced during N-demethylation

* = trichloroacetic acid

substrate solution. All substrates were also incubated with microsome-free liver homogenate and NAD in the presence and absence of formaldehyde to ensure that the substrates did not inhibit the removal of formaldehyde and were not N-demethylated to Nash positive species by the microsome-free homogenate. The spectra of the products of the Nash reaction were also determined to ensure that the chromophore was due to a dihydrolutidine adduct with a λ max at 412nm and was not the result of other interfering chromophores.

After subtraction of control absorbance values obtained from mixtures without added substrate or without added co-factors, the amount of Nash positive species present after 5 and 15 minutes incubation with microsome-free homogenate and NAD was expressed as a percentage of the amount of Nash positive species present at the start of this incubation i.e., at time = 0.

3.II The stability of synthetic N-hydroxymethyl compounds in aqueous solution

3.II.1 Measured by their ability to form a coloured complex in the Nash reaction

Substrates were added in a maximum volume of 0.1 ml acetone to yield a maximum concentration of 0.2mM in Earl's buffer (2 ml). Earl's buffer (0.25 ml), 1N HCl (0.25ml), or 1N NaOH solution (0.25ml) was then added and the solutions were left at room temperature for 5 minutes. The pH values of the resultant solutions were 7.4, 1.5 and 10.4 respectively. When intermediate pH values were required the following buffer systems were used: pH = 5, - acetate buffer; pH = 6 - 8, - Earl's buffer; pH = 7.4 - 11.4, - Tris buffer 0.01M. At the end of the 5 minute period solutions were assayed as described in sec. 3.I.1.

3.II.2 The stability of N-hydroxymethylbenzamides measured by H.P.L.C.

N-hydroxymethylbenzamide, its 4-chloro and 4-t-butyl derivatives were added to Earl's buffer (50ml) in methanol to give a final concentration of 400µg/ml. The appropriate internal standards (see sec.3.VI.1) were added in methanol to give a final concentration of 100µg/ml. Samples (0.1 ml) were removed after varying periods of incubation at 37^{o}C and the amount of N-hydroxymethyl compound remaining was measured using the appropriate H.P.L.C.method (sec. 3.VI.1).

3.III The stability of formaldehyde precursors generated during the metabolism of hexamethylmelamine in-vivo

3.III.1 Development of a colourimetric method to distinguish between formaldehyde and its precursors

The colourimetric method used was a modification of the method of Sawicki et al. (1961).

Solutions (0.25ml) containing either formaldehyde or N-hydroxymethylpentamethylmelamine were added to a 0.4% $^{w}/_{v}$ 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) solution (0.25ml) in quadruplicate at each concentration. Duplicate samples were then left at either 0-4^{o}C, or at 60^{o}C for 30 minutes when a 0.12% $^{w}/_{v}$ solution of anhydrous iron III chloride (1.25ml) was added. The mixtures were left for a further 5 minutes and the reactions were terminated by the addition of acetone (3.2ml). The optical density of the resultant tetraazopentamethinecyanine complex was measured at 670nm using a U.V. spectrophotometer.

3.III.2 Injections

Hexamethylmelamine was injected intraperitoneally (i.p.) as a suspension in 10% DMSO/arachis oil (0.1ml) to give a dose of 100mg/Kg. Control animals received vehicle only.

3.III.3 Preparation of plasma samples

Animals were anaesthetized using a mixture of halothane, nitrous oxide and oxygen. Blood was obtained by cardiac puncture using a disposable syringe (1ml) fitted with a $\frac{3}{8}$", 26G, needle which had been rinsed previously with heparin 2,500u/ml (0.05ml). Plasma was obtained by centrifugation of the blood samples for one minute in an

Eppendorf 5412 centrifuge (speed 7). Plasma samples were then assayed as described (sec.3.III.1), except that only one determination was possible at each temperature because of the small volume of plasma obtained.

3.IV Investigation of the metabolism of N'-(4-chlorophenyl) -N,N-dimethylurea

3.IV.1 Development of chromatographic methods

Gas chromatography - mass spectrometry (G.C.-M.S.) was performed using a Pye 204 gas chromatograph equipped with a fused silica SE52 capillary column linked to a VG7070 mass spectrometer. The injector temperature was 250°C and the column temperature was 100°C initially increasing by 32°C/minute for 5 minutes. A 1:10 split of carrier gas was used and the mass spectrometer source temperature was 220°C.

T.L.C. was performed using reverse phase T.L.C. plates (Whatman - K C_{18}) which were developed with methanol/water.

H.P.L.C. was performed using an Altex 421 gradient programmer in conjunction with two Altex 100A pumps and a Cecil 2112 U.V. detector (λ = 247nm). Separation of reference compounds was achieved using two methods:

1) A reverse phase method

An Ultrasphere - O.D.S., 4.6mm x 150mm column was used with a linear gradient elution system from 10% methanol/water to 100% methanol over twenty minutes, starting two minutes after sample injection. The mobile phase flow rate was 1ml/min.

2) A normal phase method

A Spherisorb 5µm column (25cm) was used with a programmed elution system of ethanol 4% in hexane for six minutes, increasing to 8% ethanol over eight minutes, and after sixteen minutes increasing to 10% ethanol over two minutes. The mobile phase flow rate was 2ml/min.

Calibration curves were obtained by determining ratios of peak areas of standard compounds to the peak area of internal standard used.

3.IV.2 In-vitro metabolism studies

3.IV.2.a Incubations

Incubations were performed as described in sec. 3.I.2. N/-(4-chlorophenyl)-N,N-dimethylurea was added in acetone (0.1ml) to give a final concentration of 1mM.

Hepatocytes were prepared according to the method of Renton et al. (1978) and incubations were carried out in Krebs-Henseleit buffer (2.5ml) containing 1% $^{w}/_{v}$ bovine serum albumin and 10% $^{v}/_{v}$ foetal calf serum. Incubations were performed in an atmosphere of oxygen containing 5% carbon dioxide. Under these conditions the hepatocytes maintained unchanged viability for at least two hours.

3.IV.2.b Preparation for H.P.L.C. analysis

Reactions were terminated by the addition of an equal volume of cold methanol containing internal standard (N,N-dimethyl-N/-phenylurea, 20µg/ml) and were then centrifuged at 2000g for 5 minutes to remove protein. The supernatant was then injected directly onto the column for analysis by reverse phase H.P.L.C.. For analysis by normal phase H.P.L.C. methanol was removed by evaporation under a stream of nitrogen at room temperature and the remaining solution was extracted with ethyl acetate (2.5ml). The extract was then injected directly onto the column.

3.IV.3 In-vivo metabolism studies

3.IV.3.a Injections

N′-(4-chlorophenyl)-N,N-dimethylurea was injected i.p. into mice as a suspension in 10% DMSO/ arachis oil to give a dose of 200mg/Kg. Control animals received vehicle only.

3.IV.3.b Collection and treatment of urine

Urine was collected in metabolic cages (Jencons, U.K.), centrifuged at 2000g for 5 minutes to remove any sediment and prepared for H.P.L.C.analysis as described in sec.3.IV.2.b.

Enzymatic hydrolysis of urine samples was performed using urine (0.2ml) diluted with acetate buffer pH = 5 (1.8ml) containing either β-glucuronidase (5,000u) or sulfatase (150u sulphatase + 5000u β-glucuronidase). The samples were incubated at 37°C for 17 hours and prepared for H.P.L.C.analysis as described in sec. 3.IV.2.b.

3.IV.4 Isolation and concentration of metabolites

H.P.L.C. eluates corresponding to peaks with the retention times of authentic standards were collected from the column and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in acetone (0.025ml) and subjected to mass spectral analysis.

3.IV.5 Determination of mass spectra

Mass spectra were determined using a VG7070 mass spectrometer at a scan rate of 1 second/decade using the direct insertion probe and were processed using a VG2035 data system. Isobutane was used as reagent gas and an electron energy of 50eV was employed for determinations in the chemical ionization mode. The electron energy for spectra determined using the electron impact method was 70eV.

3.V Investigations of the metabolism of formamide derivatives

3.V.1 Animals

Male CBA Ca mice (20-25g) were used for all in-vivo experiments whilst both these mice and male Balb c mice (20-25g) were used for in-vitro experiments.

3.V.2 Development of chromatographic methods

A G.C.method for the simultaneous estimation of N-methylformamide and formamide was used which was a modification of the method of Kimmerle & Eben (1975). Separation was performed using a Pye 204 gas chromatograph fitted with a selective nitrogen-phosphorus detector and a glass column (4mm x 2m) packed with 100-120 mesh Chromosorb W17 AWDMCS. The following conditions were used; injector temperature - 200^{o}C, column temperature - 190^{o}C, detector temperature - 250^{o}C. Flow rates were nitrogen - 40ml/minute, hydrogen - 30ml/minute, air - 300ml/minute. Calibration curves were obtained by determining ratios of peak areas of standard compounds to the peak area of internal standard used.

Derivatization of N-hydroxymethyl-N-methylformamide for G.C analysis was attempted using a 10mg/ml solution of this compound in either pyridine or acetonitrile (0.1ml) and an equal volume of either BSTFA or TSIM at room temperature and at 60^{o}C for 5 and 30 minutes. G.C.analysis was performed as described above except a flame ionization detector was used and the flow rates were nitrogen - 40ml/minute, hydrogen - 300ml/minute, air - 30ml/minute.

Derivatization of N-hydroxymethylformamide for G.C. analysis was attempted using a 10mg/ml solution (0.1ml) of N-hydroxymethylformamide in either pyridine, acetonitrile,

or ethyl acetate and an equal volume of either TSIM, BSTFA or HFAA. The mixtures were left at varying temperatures (0 - 60°C) for time periods up to 30 minutes. Combined G.C.- M.S. was performed on the products of BSTFA derivatization as described in sec. 3.IV.1.

3.V.3 In-vitro metabolism studies

3.V.3.a Incubations

Incubations were performed essentially as described in sec.3.IV.2.a. Concentrations of formamides used varied between 0.2 and 6.5mM added in a maximum volume of 0.1ml acetone. 9000g and 2000g supernatant fractions of liver were used at a concentration equivalent to 0.25g of wet liver weight per incubation. The concentration of microsomes used varied between the equivalent of 0.25g and 1g of wet liver weight per incubation.

3.V.3.b Preparation for analysis

For colourimetric analysis microsomes were removed by centrifugation and the supernatant (2ml) was treated with either Earl's buffer (0.25ml) or 1NNaOH solution (0.25ml). The solutions were left for five minutes at room temperature and then assayed as described in sec.3.I.1.

For G.C. analysis incubation mixtures were deproteinized by the addition of an equal volume of cold acetone containing internal standard (N,N-dimethylacetamide 30µg/ml), followed by centrifugation at 2000g for 5 minutes. Aliquots of the supernatant were injected directly into the gas chromatograph and analyzed using the nitrogen-phosphorous detector and conditions as described in sec.3.V.2 .

3.V.4 In-vivo metabolism studies

3.V.4.a Injections

N,N-dimethylformamide, N-methylformamide and N-ethylformamide were injected i.p. in saline (0.1ml) to give a dose of 495mg/Kg (N,N-dimethylformamide and N-ethylformamide) and 400mg/Kg (N-methylformamide). Control animals received vehicle only.

3.V.4.b Collection and treatment of plasma and urine samples

Plasma and urine were obtained as described in sec.3.III.3 and sec.3.IV.3.b respectively.

For colourimetric analysis urine from mice (0.1ml) was diluted with Earl's buffer (1:20), whilst human urine and both mouse and human plasma samples were diluted with an equal volume of Earl's buffer. These solutions were then treated as described in sec.3.V.3.b.

For G.C.analysis urine samples from mice were diluted with 9 parts of cold acetone containing internal standard (N,N-dimethylacetamide 30µg/ml) whilst plasma samples from mice were diluted with 5 parts of the same solution. The samples were centrifuged at 2000g for 5 minutes and the supernatant was injected directly into the gas chromatograph. The area under the plasma concentration of N-methylformamide versus time curve was calculated by the trapezoidal rule. The plasma half-life of N-methylformamide was calculated from this data using linear regression analysis.

Deconjugation of urine samples was performed as described in sec.3.IV.3.b.

3.VI Investigations of the metabolism of benzamide derivatives

3.VI.1 Development of chromatographic methods

H.P.L.C.methods were developed for the separation and quantitation of the following compounds:

a) N-methylbenzamide, N-hydroxymethylbenzamide, benzamide and N-formylbenzamide

b) 4-chloro-N-methylbenzamide, 4-chloro-N-hydroxymethylbenzamide and 4-chlorobenzamide

c) N-methyl-4-t-butylbenzamide, N-hydroxymethyl-4-t-butylbenzamide and 4-t-butylbenzamide

d) Benzoic acid

Separation was performed using a Waters radial compression system with radial compression columns complete with guard columns. An Altex 100A pump and a Pye L.C.-U.V. detector were also used. The conditions of the elution systems are shown in Table 3.2. Calibration curves were obtained using these methods by determining the ratio of peak height of reference compound to the peak height of the internal standard.

3.VI.2 In-vitro metabolism studies

3.VI.2.a Incubations

Incubations were performed essentially as described in sec.3.IV.3.a. Substrate concentrations used were; N-methylbenzamides - 10mM, except in incubations containing hepatocytes where concentrations of 1mM were used; N,N-dimethylbenzamide - 5mM; N-hydroxymethylbenzamide and benzamide - 1mM. Substrates were added in acetone (0.1ml) except in the case of N-methylbenzamides (10mM) which were added in methanol (0.1ml).

H.P.L.C. * SYSTEM	a	b	c	d
COLUMN	C_{18}- 5µm	C_{18}- 5µm	C_{18}- 5µm	C_{18}- 10µm
MOBILE PHASE	30% methanol/water	50% methanol/water	57% methanol/water	35% methanol/65% -5% acetic acid
FLOW RATE (ml min^{-1})	2	1.5	2	1.5
DETECTION WAVELENGTH (nm)	254	254	254	240
INTERNAL STANDARD	4-nitrobenzamide 200µg/ml in methanol	4-nitrobenzamide 200µg/ml in methanol	4-chlorobenzamide 200µg/ml in methanol	-

* see sec. 3.VI.1

Table 3.2 Conditions of the H.P.L.C. elution systems used for the analysis of benzamide derivatives

Incubation mixtures were fortified with either NAD 1mM, NADPH 0.5 - 1mM and $MgCl_2$ 5mM, or with $MgCl_2$ 5mM and sufficient G6P, GPDH and NADP to produce NADPH 0.5 - 1mM. Microsomes were added to give a concentration equivalent to 1g and 0.7g wet liver weight per incubation in the case of N-methylbenzamides and N,N-dimethylbenzamide respectively. In all other experiments the amount of each liver fraction used was equivalent to 0.25g wet liver weight.

When purified horse liver alcohol dehydrogenase (HLAD) was used as an enzyme source 1.8 units were used per incubation with or without the addition of pyrazole 1mM. Incubations were performed in a final volume of Earl's buffer pH = 8.8 (2.5ml) for 30 minutes at $37^{o}C$ with a 3 minute incubation period prior to the addition of substrate. Incubations were performed under an atmosphere of nitrogen using Erlenmeyer flasks (25ml).

3.VI.2.b Preparation for analysis

Incubations were prepared for colourimetric analysis as described in sec.3.V.3.b. For H.P.L.C.analysis the reactions were stopped by immersing the incubation mixtures in ice, the appropriate internal standard (0.15ml) was added (Table 3.2), and the mixtures were extracted twice with ethyl acetate (4ml). The extracts were evaporated to dryness under a stream of nitrogen at room temperature, re-dissolved in 30% methanol/water (0.3ml) and subjected to H.P.L.C analysis as described in sec. 3.VI.1.

3.VI.3 In-vivo metabolism studies

3.VI.3.a Injections

N-methylbenzamide and N-hydroxymethylbenzamide were injected i.p. into mice as a suspension in 10% DMSO/arachis oil (0.1ml) to give a dose of 200mg/Kg. Control mice received vehicle only.

3.VI.3.b Collection and treatment of urine

Urine was collected and subjected to enzymatic deconjugation as described in sec. 3.IV.3.b.

For H.P.L.C. analysis internal standard (0.04ml 4-nitrobenzamide; 500µg/ml) was added to urine samples (0.5ml), which were then extracted with ethyl acetate (2 x 1.5ml). The extracts were then prepared for analysis as described in sec. 3.VI.2.b.

3.VI.4 Isolation and concentration of metabolites

This was performed essentially as described in sec. 3.IV.4 except that in the case of N-formylbenzamide column eluates were collected and extracted with ethyl acetate. The extracts were then evaporated to dryness and subjected to mass spectral analysis (sec. 3.IV.5).

3.VI.5 Derivatization of isolated metabolites

Metabolites were dissolved in pyridine (0.05ml) and BSTFA (0.05ml) was added. The mixtures were then heated at 60^{o}C for 15 minutes and subjected to combined G.C.-M.S. analysis (sec. 3.IV.1).

3.VII Determination of Physical Constants

3.VII.1 Determination of electron densities

Molecular wave functions were calculated for various molecules from data obtained from crystal structure determinations using the method of Pulay (1969), adapted for Gaussian 70 by Schlegel (1975) as amended by Mallinson (1981). A minimal basis set was used for all calculations (STO - 3G).

This method enables the calculation of energies and forms of molecular orbitals and the distribution of charges within a molecule. The position of the atoms in a molecule may also be refined by the use of a force-relaxation option of the programme, until the forces on the atoms reach zero.

While the absolute values of electron density calculated using this method are unlikely to be accurate at this level of calculation (STO - 3G), the relative values for similar molecules are likely to be much more accurate.

SECTION 4

RESULTS AND DISCUSSION

4.I In-vitro structure-metabolism study using a colourimetric method which distinguishes between free formaldehyde and its precursors

4.I.1 Introduction

The rationale for the choice of compounds used in this study has been discussed in sec. 1.V. The structural formulae of the compounds used are shown in Fig. 4.1.

The method of Gescher et al. (1979) was used to distinguish between free formaldehyde and its precursors produced during N-demethylation and is described in sec.3.1. In this method metabolically generated formaldehyde is oxidized to formic acid by formaldehyde dehydrogenases which are abundant in mitochondria and liver cell cytosol but which are virtually absent in microsomes (Koivula & Koivusalo, 1975; Savenije - Chapel & Noordhoek, 1980). This method utilizes the Nash assay to detect formaldehyde (sec. 3.I.1) and as formic acid is not detected in this assay the enzymic removal of formaldehyde can be observed.

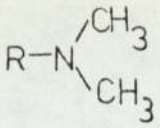
The use of the method of Gescher et al. (1979) depends on the assumptions that:

1) Formaldehyde precursors react like formaldehyde in the Nash reaction using the conditions described in sec. 3.I.

2) Formaldehyde precursors, such as N-hydroxymethyl compounds, are not metabolized by microsome-free homogenate which is used in the assay of Gescher et al. (1979) as a source of formaldehyde dehydrogenase activity.

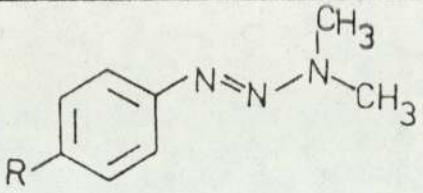
Both of these assumptions have been shown to be true in the case of hexamethylmelamine and the primary product of its oxidative N-demethylation,

Aryldimethylamines I



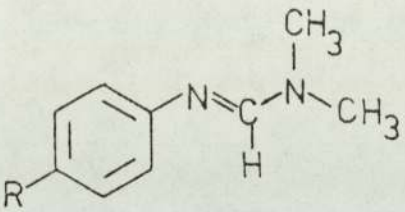
Ia: R = phenyl
b: R = 4-methylphenyl
c: R = 4-cyanophenyl
d: R = pyridin-2-yl
e: R = pyridin-4-yl

Aryldimethyltriazenes II



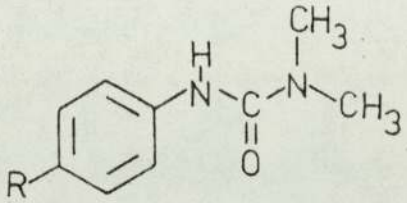
IIa: R = H
b: R = CF_3
c: R = Cl
d: R = $COCH_3$
e: R = CO_2CH_3

Aryldimethylformamidines III



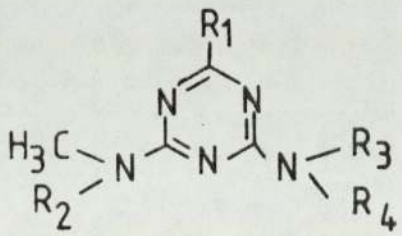
IIIa: R = H
b: R = CH_3
c: R = CN
d: R = CF_3
e: R = Cl
f: R = SO_2NH_2

Aryldimethylureas IV



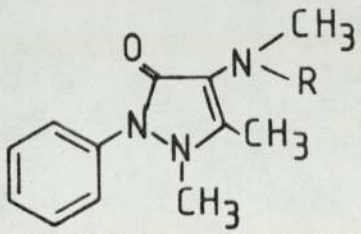
IVa: R = H
b: R = CH_3
c: R = CN
d: R = CF_3
e: R = Cl
f: R = Br
g: R = OCH_3

Melamine derivatives V



	R_1	R_2	R_3	R_4
Va:	$N(CH_3)_2$	CH_3	CH_3	CH_3
b:	$N(CH_3)_2$	CH_3	CH_3	H
c:	$N(CH_3)_2$	CH_3	H	H
d:	$N(CH_3)_2$	H	CH_3	H
e:	$NHCH_3$	H	CH_3	H
f:	N_3	CH_3	CH_3	CH_3
g:	Cl	CH_3	CH_3	CH_3

Aminoantipyrine derivatives VI



	R
VIa:	CH_3
b:	H

Fig. 4.1 Formulae of compounds used in the structure metabolism study

N-hydroxymethylpentamethylmelamine (Gescher et al., 1979). It was however not feasible to synthesize the N-hydroxymethyl compounds required to test the validity of these two assumptions for every N-methyl containing substrate. It is therefore possible that some N-methylols formed during N-demethylation may not react like formaldehyde in the Nash test or be further metabolized by microsome free homogenate. In spite of this the assay described by Gescher et al. (1979) was used as a preliminary test but the use of more precise analytical techniques was required to characterize any proposed metabolites.

4.I.2 Results

4.I.2.a Calibration curve for the colourimetric determination of formaldehyde according to the method of Werringloer (1978)

An example of such a calibration curve is shown in Fig. 4.2. The correlation coefficient (r) of this curve was 0.9996 and the standard deviation of three determinations of the amount of formaldehyde in a sample at five different concentrations was a maximum of 9% of the mean. The detection limit of this method is approximately 15µM formaldehyde or formaldehyde equivalents.

4.I.2.b In-vitro N-demethylation

In order to determine if free formaldehyde or its precursors were produced during N-demethylation it was necessary to define conditions under which sufficient N-demethylation of each substrate to formaldehyde equivalents took place. The concentration of a particular substrate and the amount of microsomal suspension used were varied until a concentration of at least 80µM formaldehyde equivalents was produced during a thirty minute incubation period. Using this criterion the conditions described in Table 4.1 were suitable for the investigation of the metabolism of the substrates listed.

Three of the substrates listed in Fig. 4.1 were not biotransformed to species forming 3,5-diacetyl-1,4-dihydrolutidine, the coloured chromophore in the Nash reaction; 4-dimethylaminopyridine (Ie), N′-(4-sulphonamidophenyl)-N,N-dimethylformamidine (IIIf) and N′-(4-cyanophenyl)-N,N-dimethylurea (IVc). In the case of

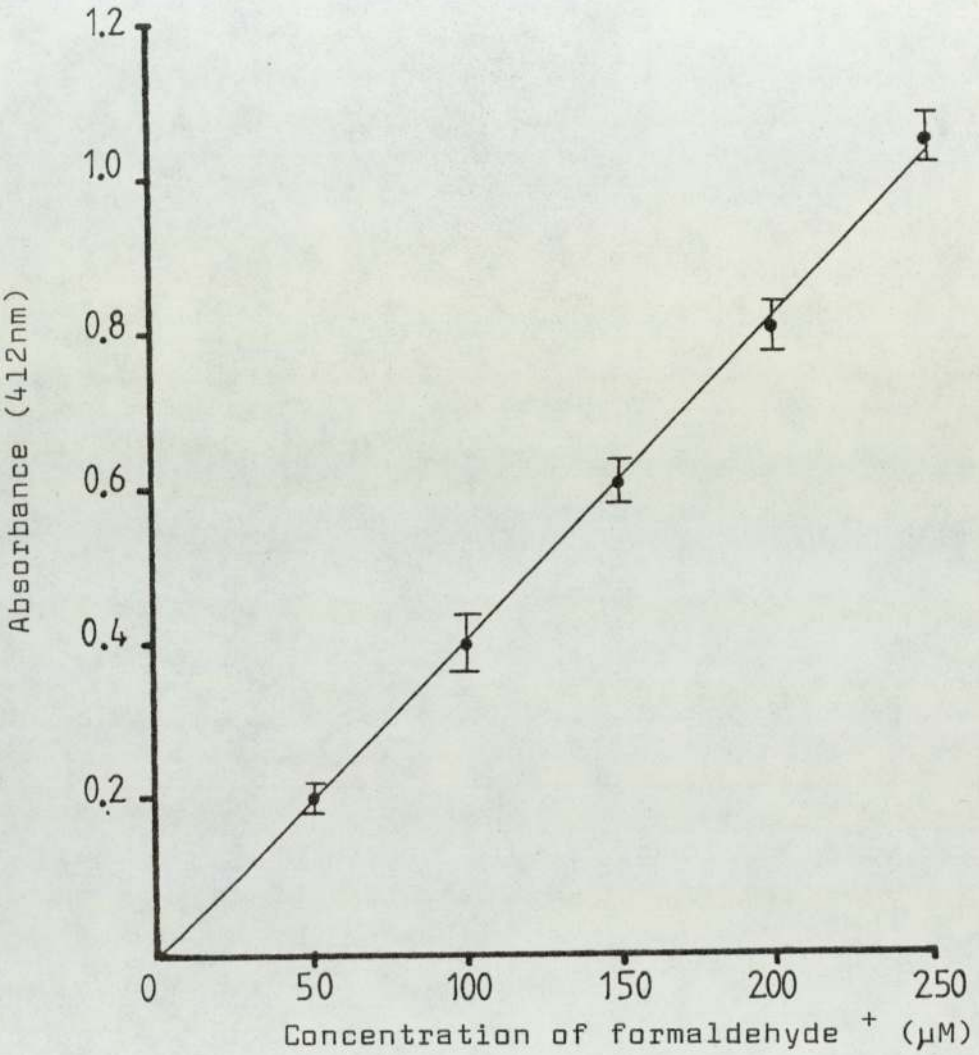


Fig. 4.2 Calibration curve for the determination of formaldehyde using the method of Nash (1953) as modified by Werringloer (1978)

Each value represents the mean ± s.d. of at least three determinations.

+ - concentration of formaldehyde in original 2.5ml solution

Substrate		Amount of microsomal suspension per incubation (12.5ml), expressed as equivalent wet liver weight (g)	Concentration of Substrate (mM)
N,N-dimethylarylamines	(I)	0.8	5
N,N-dimethylaryltriazenes	(II)	0.8	0.5
N,N-dimethylarylformamidines	(III)	0.8	1
N,N-dimethylarylureas	(IV)	1.6	1
Melamine derivatives	(V)	0.8	0.5
except			
N_2,N_2,N_4,N_4-tetramethylmelamine	(Vc)	0.8	1
N_2,N_4,N_6-trimethylmelamine	(Ve)	0.8	5
N,N-dimethylaminoantipyrine	(VIa)	0.8	5
N-methylaminoantipyrine	(VIb)	1.6	5

time period of incubation = 30 min. at 37°C

Table 4.1 Conditions used for the investigation of the in-vitro N-demethylation of various substrates

4-dimethylaminoazobenzene sufficient levels of N-demethylation were only produced by using high microsomal concentrations (≡ 1g wet liver weight/2.5ml) and incubation periods of up to one hour. Extractions to remove residual azo dyes using these conditions were irreproducible even between duplicate incubations. This was presumably due to residual protein preventing efficient extraction and therefore results obtained using this substrate are not included here.

4.I.2.c Assay to distinguish between free formaldehyde and its precursors produced during the N-demethylation of N-methyl containing xenobiotics

The time course of removal of exogenous formaldehyde by microsome-free homogenate fortified with NAD is shown in Fig. 4.3. The metabolism of Nash positive microsomal metabolites of substrates (I —— VI) by microsome-free homogenate is shown in the following figures:

N,N-dimethylarylamines (I)	Fig. 4.4
N,N-dimethylaryltriazenes (II)	Fig. 4.5
N,N-dimethylarylformamidines (III)	Fig. 4.6
N,N-dimethylarylureas (IV)	Fig. 4.7
N-methyl substituted melamines (V)	Fig. 4.8
N-methyl substituted aminoantipyrines (VI)	Fig. 4.9

4-cyano-N,N-dimethylaniline (Ic) inhibited the removal of formaldehyde by microsome-free homogenate in control incubations. 78% of formaldehyde was removed in 15 min. by microsome -free homogenate and NAD whereas when (Ic) was added only 33% of formaldehyde was removed in the same time period. 4-cyano-N,N-dimethylaniline itself was not Nash positive.

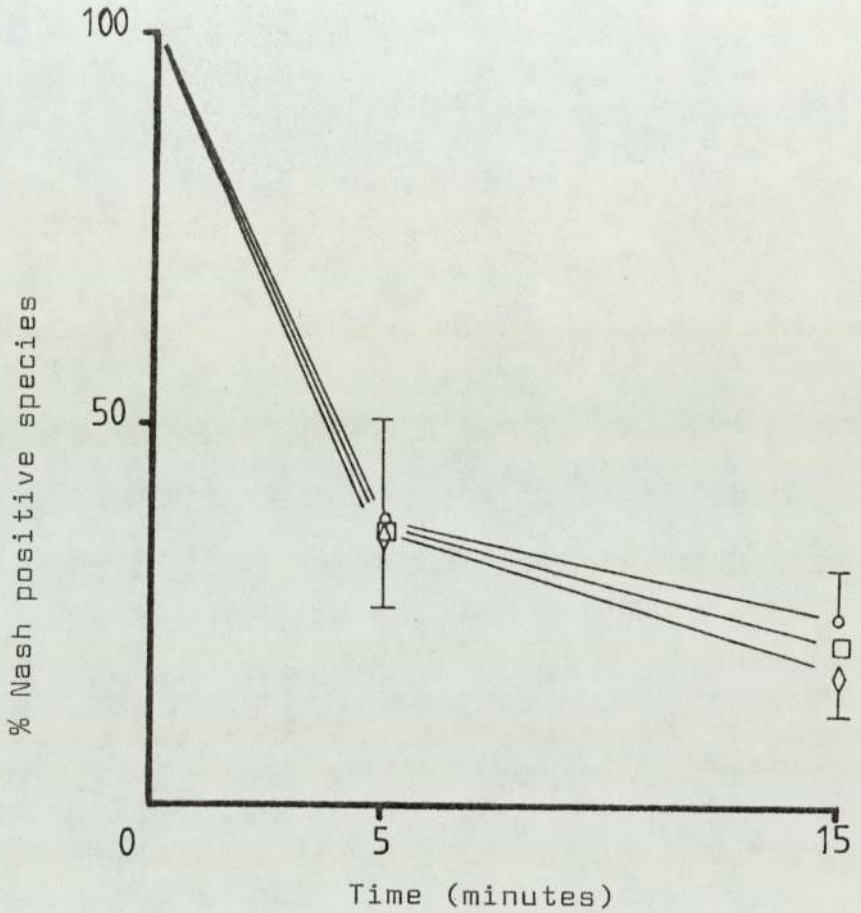


Fig. 4.3 The removal of formaldehyde 100μM (∘), 150μM (□) and 200μm (◊) by mouse liver homogenate free from microsomes.

Each point represents the mean ± s.d. of at least six experiments.

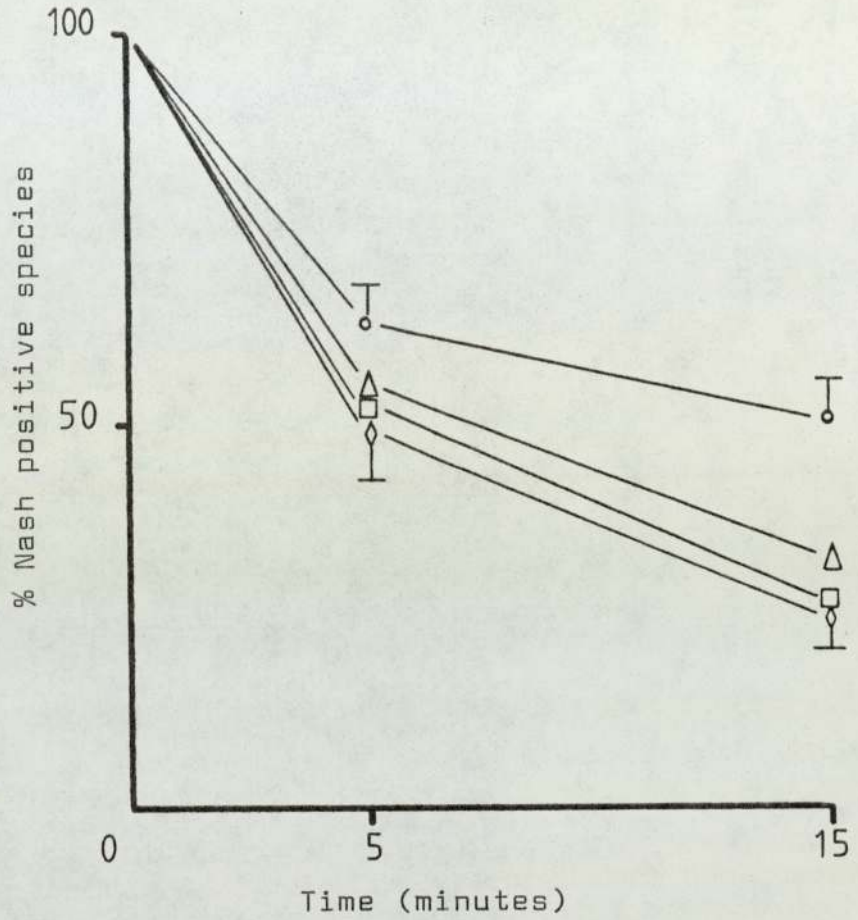


Fig. 4.4 Metabolism of Nash positive microsomal metabolites of N,N-dimethylaniline (□,Ia), N,N-dimethyl-p-toluidine (◊,Ib), 4-cyano-N,N-dimethylaniline (○,Ic) and 2-dimethylaminopyridine (Δ,Id) by mouse liver homogenate free from microsomes. Each point represents the mean ± s.d. of at least three experiments.

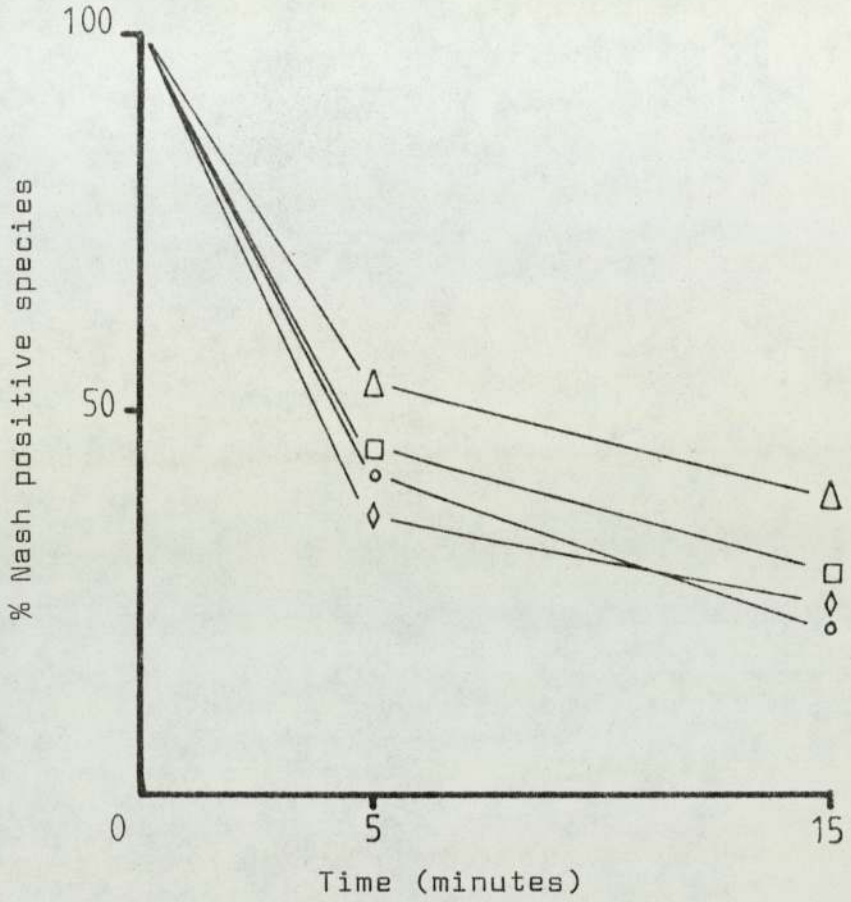


Fig. 4.5 Metabolism of Nash positive microsomal metabolites of 3-phenyl-1,1-dimethyltriazene (□, IIa) 3-(4-trifluoromethylphenyl)-1,1-dimethyltriazene (◊, IIb) 3-(4-chlorophenyl)-1,1-dimethyltriazene (Δ, IIc) and 3-(4-acetylphenyl)-1,1-dimethyltriazene (o, IId) by mouse liver homogenate free from microsomes

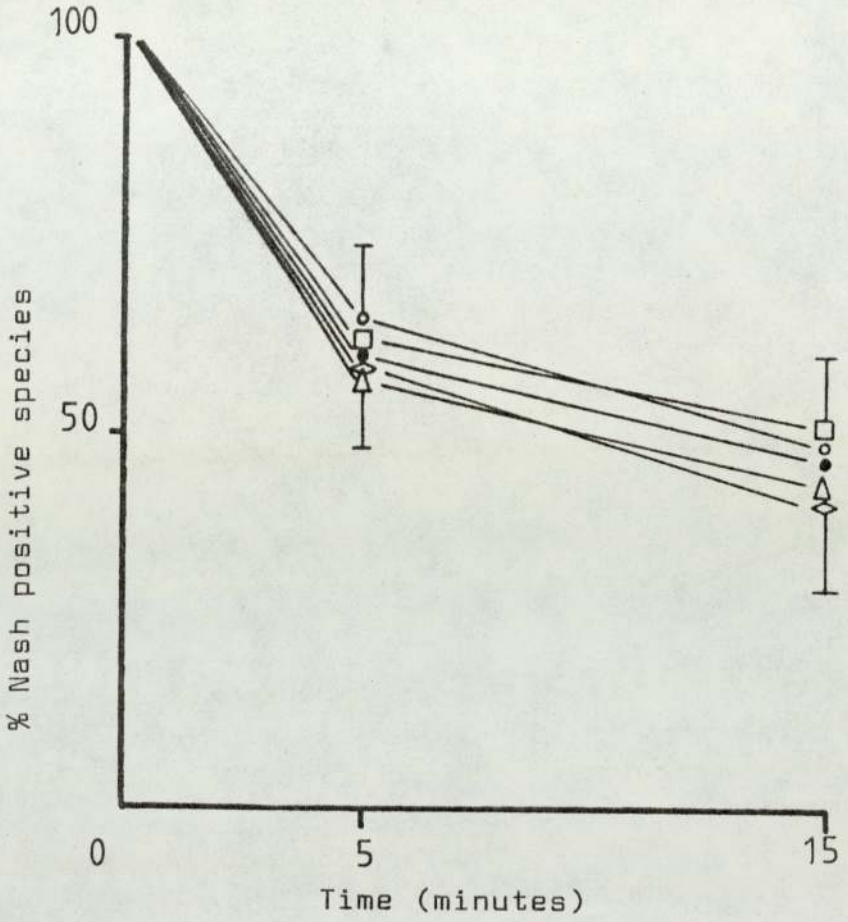


Fig. 4.6

Metabolism of Nash positive microsomal metabolites of N/-phenyl-N,N-dimethylformamidine (• , IIIa) N/-(4-methylphenyl)-N,N-dimethylformamidine (□, IIIb) N/-(4-cyanophenyl)-N,N-dimethylformamidine (Δ , IIIc) N/-(4-trifluoromethylphenyl)-N,N-dimethylformamidine (○ , IIId) N/-(4-chlorophenyl)-N,N-dimethylformamidine (◇, IIIe) by mouse liver homogenante free from microsomes

Each point represents the mean ± s.d. of at least three experiments.

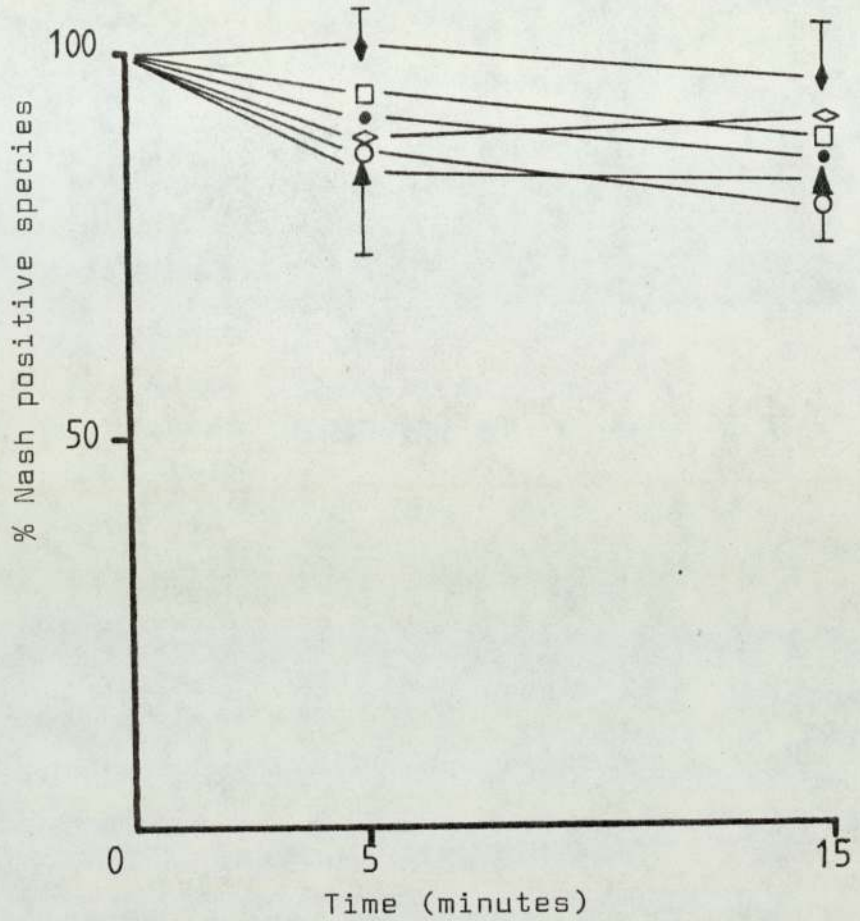


Fig. 4.7

Metabolism of Nash positive microsomal metabolites of N/-phenyl-N,N-dimethylurea (▲, IVa), N/-(4-methylphenyl)-N,N-dimethylurea (●, IVb), N/-(4-trifluoromethylphenyl)-N,N-dimethylurea(□, IVd), N/-(4-chlorophenyl)-N,N-dimethylurea (♦, IVe), N/-(4-bromophenyl)-N,N-dimethylurea (○, IVf), and N/-(4-methoxyphenyl)-N,N-dimethylurea (◇, IVg) by mouse liver homogenate free from microsomes. Each point represents the mean ± s.d. of at least three experiments.

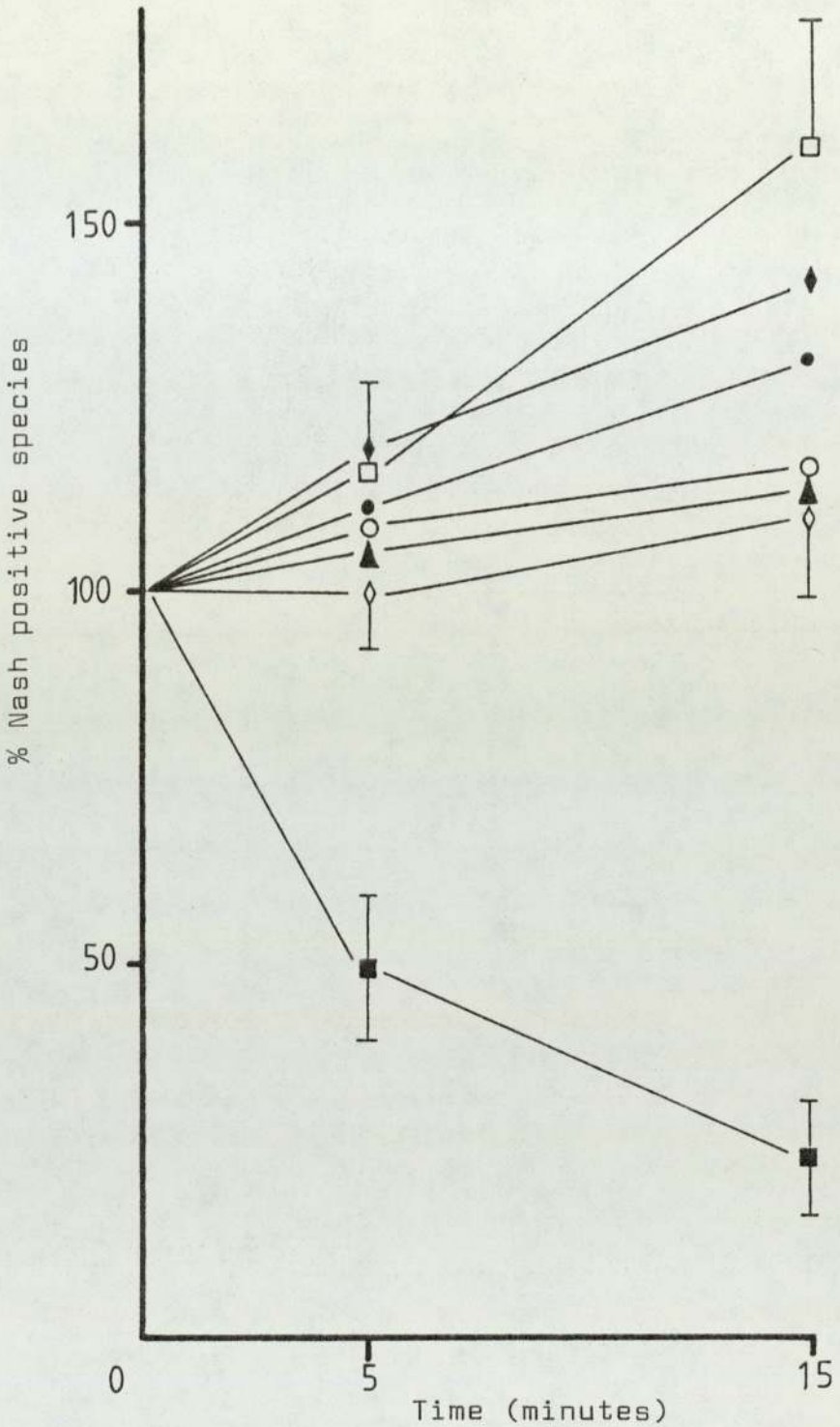


Fig. 4.8

Metabolism of Nash positive microsomal metabolites of hexamethylmelamine (○, Va), pentamethylmelamine (◊ ,Vb), N_2,N_2,N_4,N_4-tetramethylmelamine (□,Vc), N_2,N_2,N_4,N_6-tetramethylmelamine (▲,Vd), N_2,N_4,N_6-trimethylmelamine (♦ ,Ve), 2-azido-4,6-bis-(dimethylamino)-1,3,5-triazine (■,Vf) and 2-chloro-4,6-bis-(dimethylamino)-1,3,5-triazine (●, Vg) by mouse liver homogenate free from microsomes

Each point represents the mean ± s.d. of at least three experiments.

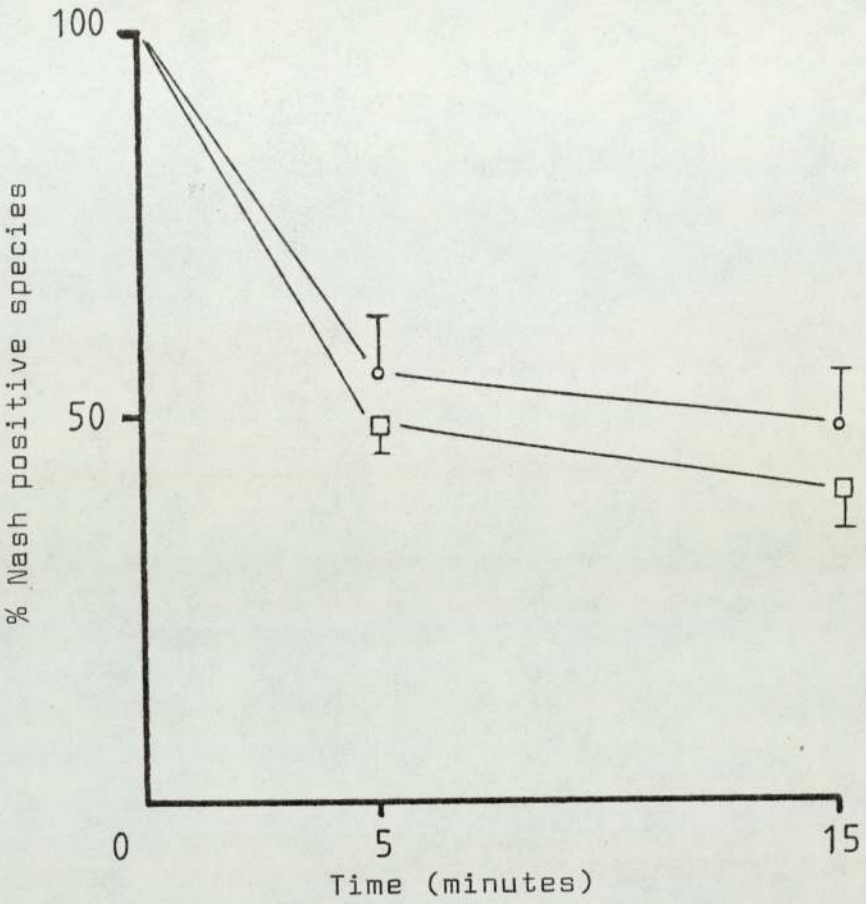


Fig. 4.9 Metabolism of Nash positive microsomal metabolites of N,N-dimethylaminoantipyrine (o, VIa), and N-methylaminoantipyrine (□, VIb) by mouse liver homogenate free from microsomes

Each point represents the mean ± s.d. of at least three experiments.

Composite absorbance spectra were obtained in the cases of 3-phenyl-1,1-dimethyltriazene (IIa) and 3-(4-acetylphenyl)-1,1-dimethyltriazene (IId) which were caused by a direct interaction of these substrates with Nash reagent. Interpretation of the results was possible by the use of appropriate control incubations containing substrates. In the case of 3-(4-carboxymethylphenyl)-1,1-dimethyltriazene (IIe) high absorbance values were obtained in control incubations lacking NAD. This was found to be due to a temperature dependent interaction of this substrate with liver homogenate which led to high absorbance values only after the addition of Nash reagent. Interpretation of results obtained using this substrate was therefore not possible.

N-demethylation of N,N-dimethylaminoantipyrine and pentamethylmelamine can occur in buffer systems of up to pH = 10.4 without a significant change in rate of metabolism from that which occurs at pH = 7.4 (Fig. 4.10). Figs. 4.11 - 4.14 show the effect of microsome-free homogenate on the Nash positive species generated during metabolism of pentamethylmelamine (Vb), N,N-dimethylaminoantipyrine (VIa), 2-dimethylaminopyridine (Id), and N$^{/}$-(4-cyanophenyl)-N,N-dimethylformamidine (IIIc) respectively at pH = 10.4 in Tris buffer 0.01M. The profiles obtained are similar to those obtained during metabolism at pH = 7.4 in Earl's buffer which are also included in Figs. 4.11 - 4.14.

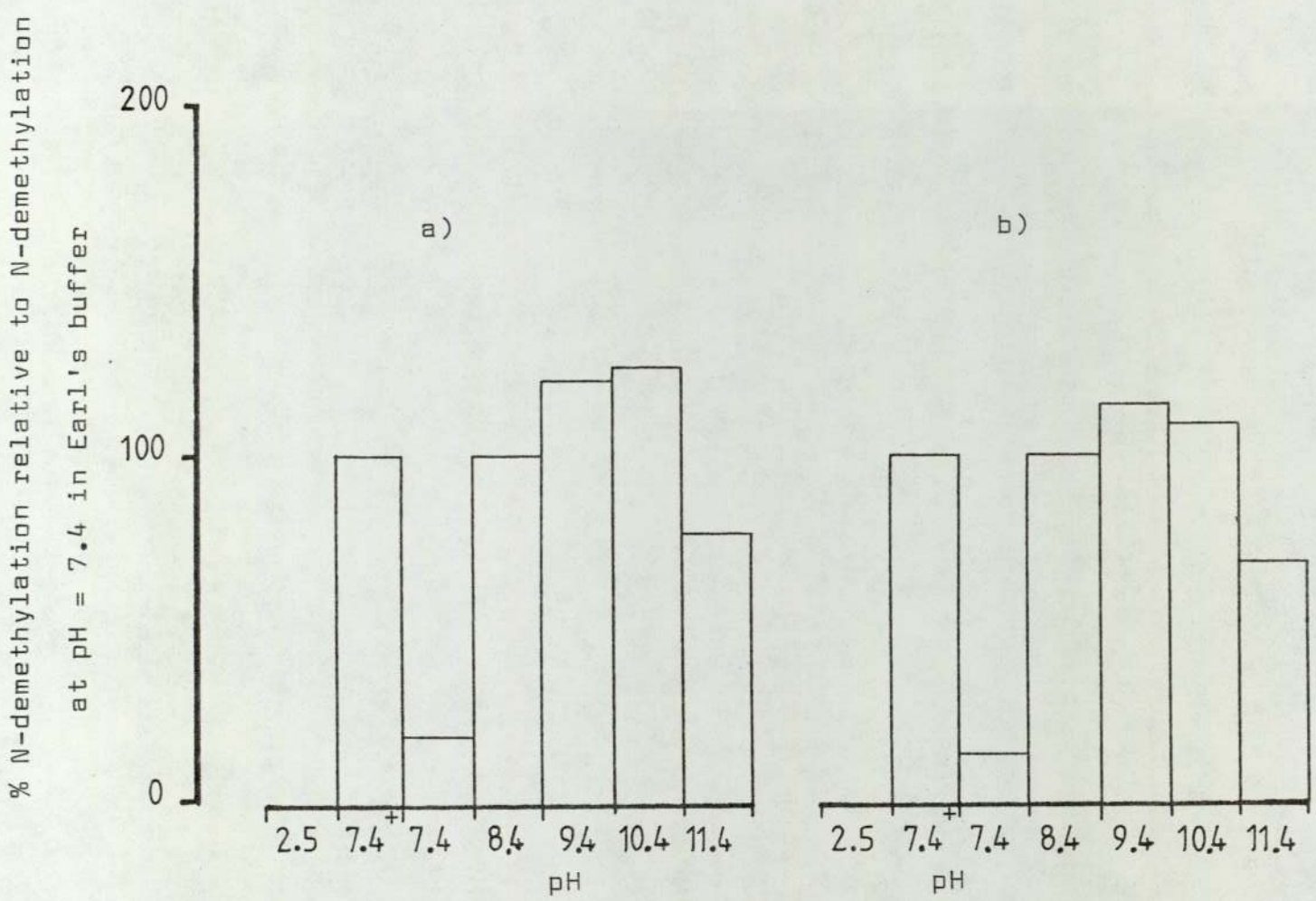


Fig. 4.10 The N-demethylation of a)N,N-dimethylaminoantipyrine (5mM) and b) pentamethylmelamine (0.5mM) at various pH values

Buffer systems used: pH = 2.5 (acetate), pH = 7.4^{+} (Earl's), pH = 7.4 - 11.4 (Tris 0.01M).

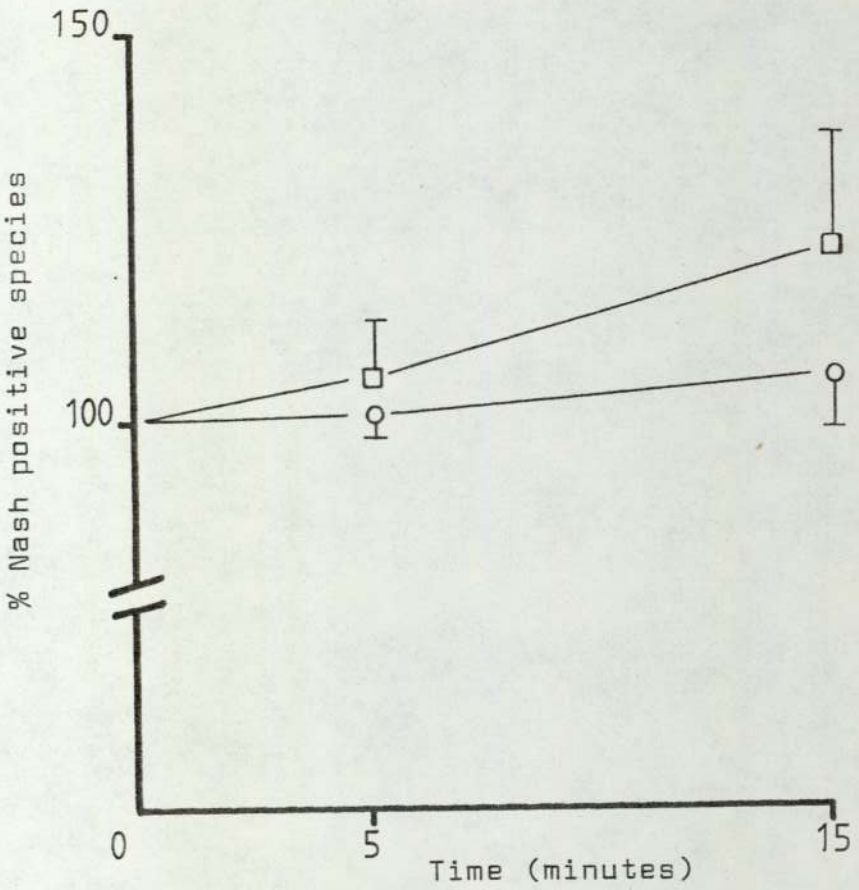


Fig. 4.11 Metabolism of Nash positive microsomal metabolites of pentamethylmelamine (Vb) generated at pH=7.4 (□) and pH=10.4 (○) by mouse liver homogenate free from microsomes.

Each point represents the mean ± s.d. of at least three experiments.

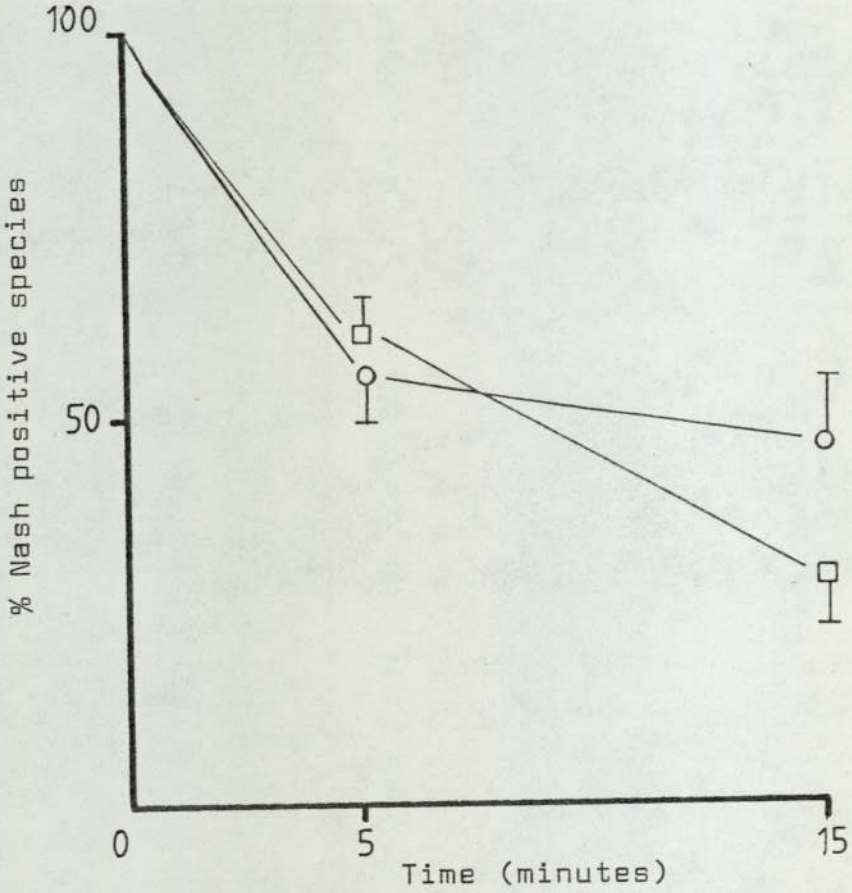


Fig 4.12 Metabolism of Nash positive microsomal metabolites of N,N-dimethylaminoantipyrine (VIa) generated at pH=7.4 (□) and pH=10.4 (○) by mouse liver homogenate free from microsomes.

Each point represents the mean ± s.d. of at least three experiments.

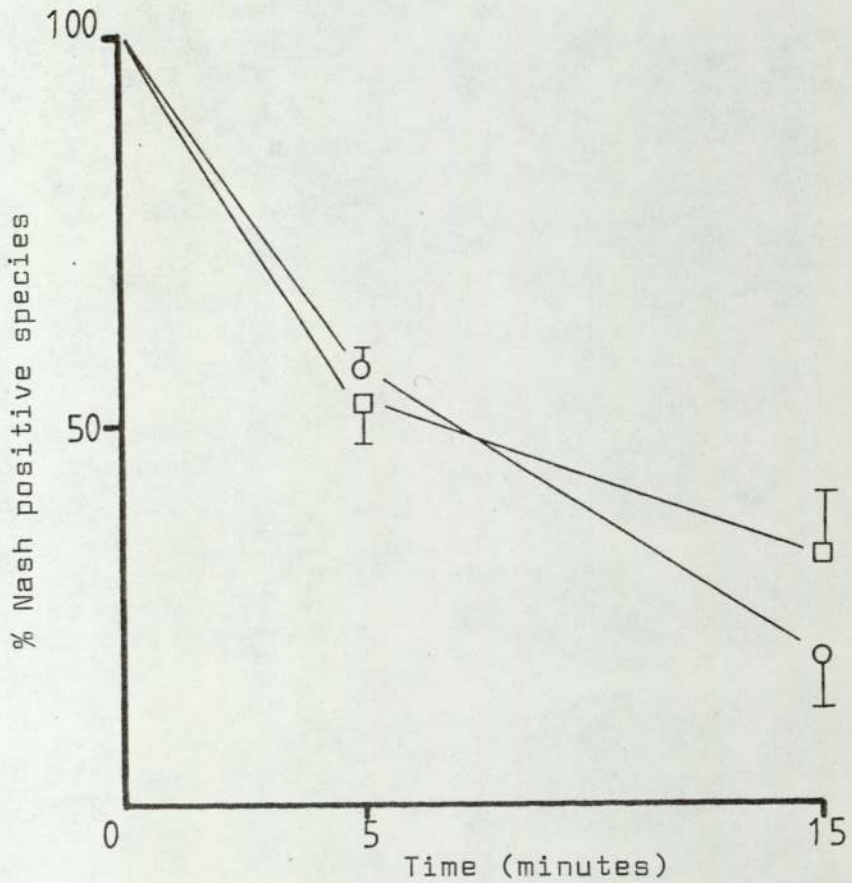


Fig. 4.13 Metabolism of Nash positive microsomal metabolites of 2-dimethylaminopyridine (Id) generated at pH=7.4 (□) and at pH=10.4 (○) by mouse liver homogenate free from microsomes.

Each point represents the mean ± s.d. of at least three experiments.

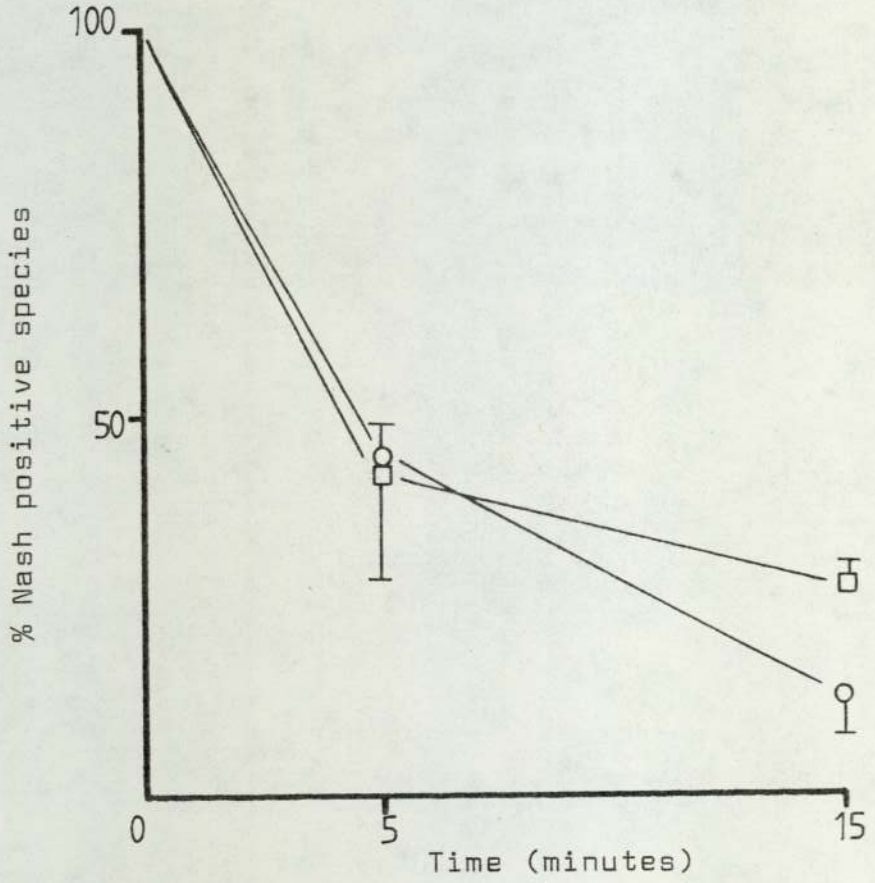


Fig. 4.14 Metabolism of Nash positive microsomal metabolites of N′-(4-cyanophenyl)-N,N-dimethylformamidine (IIIc) generated at pH=7.4 (□) and at pH=10.4 (○) by mouse liver homogenate free from microsomes.

Each point represents the mean ± s.d. of at least three experiments.

4.I.3 Discussion

The results presented in sec. 4.I.2.c show that the Nash positive species generated during the microsomal metabolism of N,N-dimethylarylamines (I, Fig. 4.4), N,N-dimethylaryltriazenes (II, Fig. 4.5), N,N-dimethylarylformamidines (III, Fig. 4.6), and N-methyl substituted aminoantipyrine derivatives (VI, Fig. 4.9) behaved like free formaldehyde on exposure to microsome-free homogenate.

4-cyano-N,N-dimethylaniline (Ic) partially inhibited the oxidation of formaldehyde in microsome-free homogenate (sec. 4.I.2.c), which explains the decreased removal of Nash positive species generated during the metabolism of this compound (Fig. 4.4). 4-cyano-N,N-dimethylaniline has been shown to be mutagenic after metabolic activation in-vitro (Ashby et al., 1980). One could argue that as formaldehyde itself is mutagenic (sec. 1.IV), inhibition of the efficient removal of formaldehyde after its production during the N-demethylation of Ic may be associated with the in-vitro mutagenic activity of Ic. The yellow colour of aminoazobenzene derivatives interfered with the colourimetric assay used in this study and prevented the investigation of the metabolism of N,N-dimethylaminoazobenzene.

The failure of the assay to detect stable N-hydroxymethyl derivatives of N,N-dimethylaryltriazenes is surprising in view of the isolation of a glucuronide of an N-hydroxymethyl-N-methyltriazene as a urinary metabolite of

an N,N-dimethylaryltriazene in the rat (Kolar & Carubelli, 1979). This discrepancy may be due to species differences in metabolism or may be a result of the degradation of N-hydroxymethyl compounds derived from N,N-dimethylaryltriazenes under the conditions used in this assay. Alternatively the lack of conjugating enzymes in the microsomal system used may prevent the stablization of any N-hydroxymethyltriazene produced as a metabolite.

The detection of Nash positive microsomal metabolites of N,N-dimethylaminoantipyrine which were removed by microsome-free homogenate (VIa, Fig. 4.9) confirms the results of Gescher et al. (1970). The similar behaviour of the Nash positive species produced during the microsomal metabolism of N-methylaminoantipyrine (VIb, Fig. 4.9) suggests that this compound is also metabolized to free formaldehyde in this system rather than to N-hydroxymethyl intermediates.

Gescher et al. (1979) also showed that hexamethylmelamine produced stable precursors of formaldehyde during microsomal metabolism and this was confirmed in this study (Va, Fig. 4.8). Similar results were obtained with all of the melamine derivatives tested except 2-azido-4,6-bis-(dimethylamino)-1,3,5-triazine (Vf), which formed metabolites during microsomal metabolism that behaved like free formaldehyde upon exposure to microsome-free homogenate (Fig. 4.8). 2-chloro-4,6-bis-(dimethylamino)-1,3,5-triazine (Vg) is structurally identical to Vf except that a chloro substituent in Vg replaces the azido substituent in Vf. The 2-chloro substituted compound (Vg), unlike the

2-azido derivative (Vf) forms stable formaldehyde precursors during microsomal metabolism (Fig. 4.8). As the chloro substituent has a similar electron withdrawing effect to the azido substituent (Perrin, 1980), it is unlikely that the behaviour of Vf in this test, as compared to Vg, can be explained by electronic effects.

It is puzzling that the amount of Nash positive species obtained upon microsomal metabolism of many of the melamine derivatives increased during incubation with microsome-free homogenate (Fig. 4.8). This could not be explained but it may reflect the metabolism of a Nash negative species formed by microsomal enzymes to a Nash positive species by mitochrondrial and cytosolic enzymes.

The Nash positive species produced during the microsomal metabolism of the N,N-dimethylarylformamidines behaved like free formaldehyde (Fig. 4.6), whereas those produced from N,N-dimethylarylureas were not metabolized by microsome-free homogenate (Fig. 4.7). This suggests that N,N-dimethylarylureas (IV) may produce stable N-methylols during microsomal metabolism.

As N,N-dimethylarylformamidines are relatively strong bases (pKa, N$^{/}$-phenyl-N,N-dimethylformamidine = 8.35 - Table 4.20, sec. 4.VII.3), they would be expected to be predominately in a protonated form, probably on the imino nitrogen atom rather than the amino nitrogen atom (Foubert & Huyskens, 1976), at physiological pH. In an attempt to determine if this ionization of N,N-dimethylarylformamidines influenced whether formaldehyde or its more stable precursors, such as N-hydroxymethyl compounds, were produced during

N-demethylation, the metabolism of N′-(4-cyanophenyl)-N,N-dimethylformamidine (IIIc) was also performed at pH = 10.4. At this pH greater than 99% of IIIc would be expected to be in the unionized form. The results presented in Fig. 4.14 show that there was little difference between the metabolism of Nash positive species, generated during the metabolism of IIIc at pH = 7.4 and at pH = 10.4, by microsome-free homogenate. These results suggest that protonation of IIIc does not affect whether formaldehyde or more stable precursors of formaldehyde are produced during N-demethylation. Similarly the generation of microsomal metabolites of 2-dimethylaminopyridine (Id), pentamethylmelamine (Vb), and N,N-dimethylaminoantipyrine (VIa) at pH = 10.4 rather than at pH 7.4 did not influence whether formaldehyde or its precursors were produced during the N-demethylation of these substrates (Figs. 4.11 - 4.13). These results were expected as these compounds are all weak bases (pKa < 7, see sec. 4.VII.3) and would already be predominately in the unionized form at pH = 7.4.

Substitution in the 4 position of the aromatic model compounds (I - IV) did not alter the metabolism of the products of microsomal N-demethylation by mitochondrial and cytosolic enzymes when compared to their unsubstituted congeners (Figs. 4.4 - 4.7). It is conceivable therefore that of the factors involved in the stabilization of N-hydroxymethyl compounds produced during metabolism of the aromatic model compounds (I - IV), the electronic environment as influenced by 4-substitution in the phenyl ring is only of minor importance.

4.II The stability of synthetic N-hydroxymethyl compounds in aqueous solution

4.II.1 Introduction

The stability of N-hydroxymethyl compounds which are generated during the metabolism of N-methyl containing xenobiotics has possible implications for both toxicity and antitumour activity (sec. 1.IV). In order to predict the stability of metabolically generated N-methylols it was considered relevant to investigate the chemical stability of authentic N-hydroxymethyl derivatives under the conditions used for in-vitro metabolism studies.

4.II.2 Results

4.II.2.a The ability of N-hydroxymethyl compounds to form a coloured complex in the Nash reaction

The Nash assay is a colourimetric assay used for the determination of formaldehyde and the development of the chromophore requires heating at 60°C for twenty minutes (see sec. 3.I.1). If an N-hydroxymethyl compound produces a positive Nash reaction then it must be assumed that either the N-methylol itself, or formaldehyde produced from its breakdown under the conditions used, reacts with Nash reagent.

The results of the reaction of synthetic N-methylols and related compounds with Nash reagent (Table 4.2) show that certain N-hydroxymethylamides do not react with Nash reagent in the absence of added alkali. These results are analogous to those of Johansen & Bundgaard (1979) who showed that the same N-hydroxymethylamides produced a positive reaction in the colourimetric method of Sawicki et al. (1961), which is also used for the determination of formaldehyde, only under strongly alkaline conditions. They assumed that the appearance of colour reflected the breakdown of N-methylolamides to release formaldehyde. It is very difficult to decide whether N-methylols themselves or formaldehyde released during their degradation reacts in the Nash or Sawicki colourimetric assays. An indication that the latter is the case was given by mass spectral investigation of extracts of the Nash positive adducts of formaldehyde and N-hydroxymethylbenzamide solutions adjusted to pH = 12. Both extracts contained a compound with a molecular ion of $^{m}/z = 193$ which is presumably

COMPOUND	% Formaldehyde equivalents * obtained in Nash reaction after adjustment of solutions containing substrates to pH -		
	7.4	12.4	1.4
Formaldehyde	100	100	100
Hexamethylmelamine	0	0	0
N-hydroxymethylpentamethylmelamine	100	100	98
Pentamethylmelamine	0	0	0
N,N-dimethylformamide	0	0	0
N-hydroxymethyl-N-methylformamide	13	55	9
N-methylformamide	0	0	0
N-hydroxymethylformamide	3	80	2
Formamide	0	0	0
N-ethoxymethylformamide	0	6	n.d
N-acetoxymethyl-N-methylformamide	0	20	n.d
N-benzoyloxymethyl-N-methylformamide	0	15	n.d
N-(N/,N/-dimethylaminomethyl)-formamide	67	77	n.d
N-hydroxymethylbenzamide	0	93	0
N-hydroxymethyl-4-t-butylbenzamide	0	89	0
4-chloro-N-hydroxymethylbenzamide	0	90	0
N-hydroxymethyltrichloroacetamide	100	n.d	n.d
N-hydroxymethylphthalimide	100	n.d	n.d

n.d = not determined

* = amount of Nash positive species quantified using authentic formaldehyde standards.

Table 4.2 The reaction of N-methylols and related compounds in the Nash assay, after adjustment of the pH of solutions containing substrates to pH = 7.4, 12.4 or 1.4

3,5-diacetyl-1,4-dihydrolutidine (see Fig. 3.1). Furthermore alkaline conditions which are required for the reaction of certain N-methylolamides with Nash reagent (Table 4.2) are likely to produce the N-oxymethyl anion (see scheme 4.1) and as the species which reacts with the Nash or Sawicki colourimetric reagents is an electrophilic species (Nash, 1953; Sawicki et al., 1961) it seems unlikely that this anion would react directly in these colourimetric assays.

Therefore it was assumed in further experiments that the positive colourimetric reaction obtained with N-hydroxymethylamides under alkaline conditions was due to formaldehyde release.

The conditions under which N-hydroxymethylformamide liberates formaldehyde and hence produces a positive Nash reaction were investigated in more detail and the results are shown in Fig. 4.15. The decomposition of 4-chloro-N-hydroxymethylbenzamide was investigated using this method and half-lives together with correlation coefficients calculated from the decay curves obtained in various buffer systems at 37°C are shown in Table 4.3.

4.II.2.b The stability of N-hydroxymethylbenzamides measured by H.P.L.C.

The decomposition of N-hydroxymethylbenzamide, 4-chloro-N-hydroxymethylbenzamide and N-hydroxymethyl-4-t-butylbenzamide were measured in Earl's buffer pH = 7.4 at 37°C. This was performed by H.P.L.C. analysis of the amount of N-hydroxymethyl compound remaining after various periods of incubation and the results are summarized in Table 4.4.

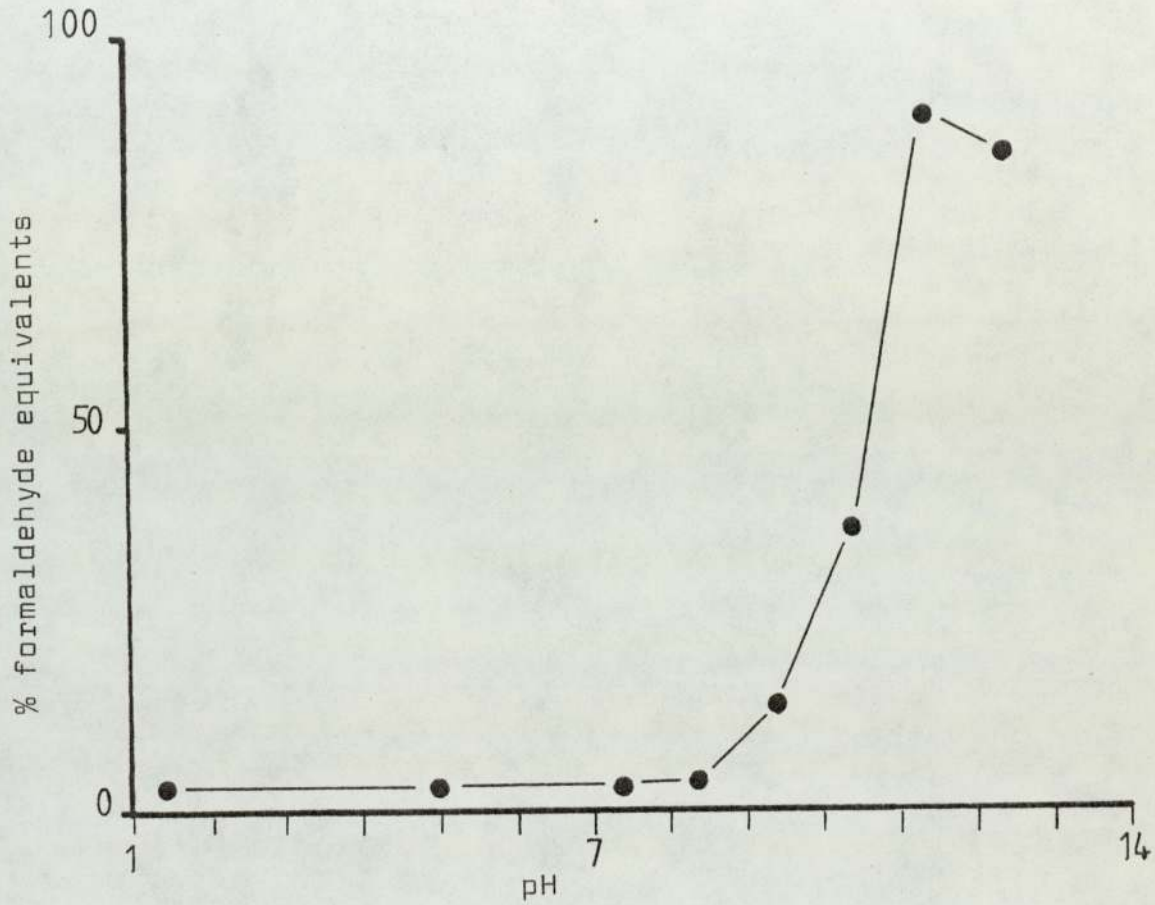


Fig. 4.15 The stability of N-hydroxymethylformamide as a function of pH

BUFFER SYSTEM	TEMPERATURE (^{o}C)	pH	HALF-LIFE ($t_{\frac{1}{2}}$) (h)	FIRST-ORDER RATE CONSTANT (K) (h^{-1})	CORRELATION COEFFICIENT OF LOG $CONC^{N}$./TIME PLOT (r)
Earl's	37	7.4	13.2	0.052	-0.9970
Earl's	60	7.4	0.6	1.203	-0.9780
Tris 0.01M	60	7.4	15.1	0.046	-0.9931
Tris 0.01M	37	8.4	16.7	0.041	-0.9990
Tris 0.01M	60	8.4	1.8	0.378	-0.9998
Tris 0.01M	37	9.4	2.5	0.277	-0.9996
Tris 0.01M	60	9.4	0.6	1.214	-0.9610

Table 4.3 The decomposition of 4-chloro-N-hydroxymethylbenzamide in various buffer systems at 37°C and at 60°C, measured by the liberation of formaldehyde using the Nash assay.

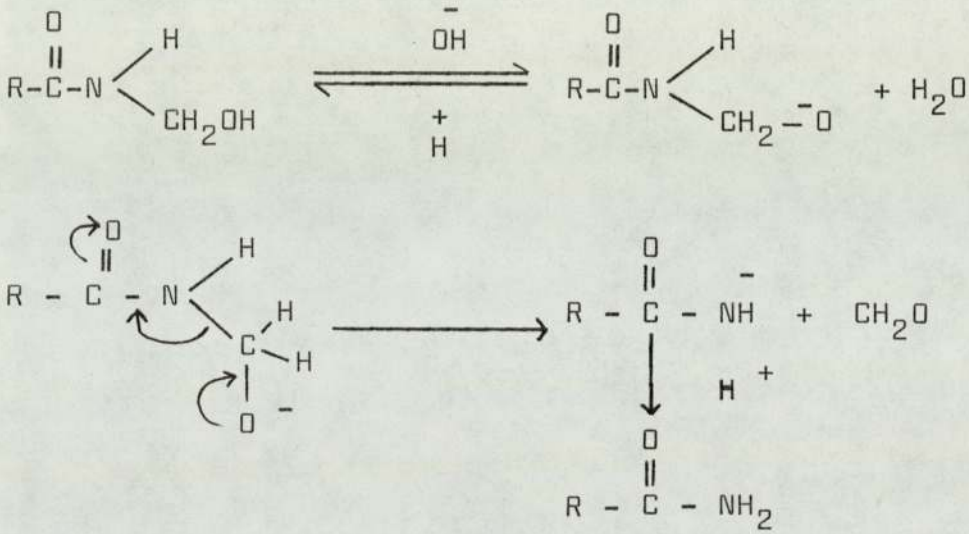
	N-hydroxymethylbenzamide	4-chloro-N-hydroxymethylbenzamide	N-hydroxymethyl 4-t-butylbenzamide
Half-life-$t_{\frac{1}{2}}$(h.)	21.1	15.8	12.1
1st order rate constant-k ($h.^{-1}$)	0.033	0.044	0.057
Correlation coefficient of log Conc^{n}/time plot	-.9982	-.9974	-.9955

Table 4.4 The decomposition of N-hydroxymethylbenzamides in Earl's buffer pH = 7.4, measured using H.P.L.C.

4.II.3 Discussion

The results presented in sec.4.II.2.a and those obtained by Johansen & Bundgaard (1979) suggest that N-hydroxymethylamides degrade under alkaline conditions to produce formaldehyde. The mechanism of this breakdown as proposed by Johansen & Bundgaard (1979) is shown in scheme 4.1.

Scheme 4.1



This mechanism involves the generation of an N-hydroxymethylamide anion under basic conditions which then undergoes N - C bond cleavage resulting in the formation of an amide anion and formaldehyde. This decomposition is favoured by electron withdrawing R groups as such groups will both favour the formation of an N-hydroxymethyl anion, and increase the leaving ability of the amide anion facilitating N - C bond cleavage. Of these two parallel effects the latter is considered to be predominant and a good correlation has been observed between the rate of breakdown of N-hydroxymethylamides under alkaline conditions and the acidity of the parent amide (Johansen & Bundgaard, 1979).

The formation of stable N-hydroxymethyl compounds appears to be favoured by the position of the nitrogen bearing the hydroxymethyl group in an electron-withdrawing environment (sec. 1.III). However interpretation of the reaction mechanism in scheme 4.1 suggests that in the case of N-hydroxymethylamides decomposition is favoured under alkaline conditions with an electron-deficient nitrogen compared with other N-hydroxymethylamides possessing less acidic nitrogen atoms.

The results in Table 4.2 show that N-hydroxymethylphthalimide and N-hydroxymethyltrichloroacetamide react like formaldehyde with Nash reagent at pH = 7.4 whereas other N-hydroxymethylamides such as N-hydroxymethylbenzamide do not. This can be explained in terms of the acidity of their nitrogen atoms which influences their rates of degradation at pH = 7.4. Phthalimide (pKa = 8.30) and trichloroacetamide (pKa = 12.42) are stronger acids than benzamide (pKa = 14-15) (Johansen & Bundgaard, 1979). Thus on the basis of the reaction mechanism in scheme 4.1, the N-hydroxymethyl derivatives of phthalimide and trichloroacetamide would be expected to degrade more rapidly to produce formaldehyde.

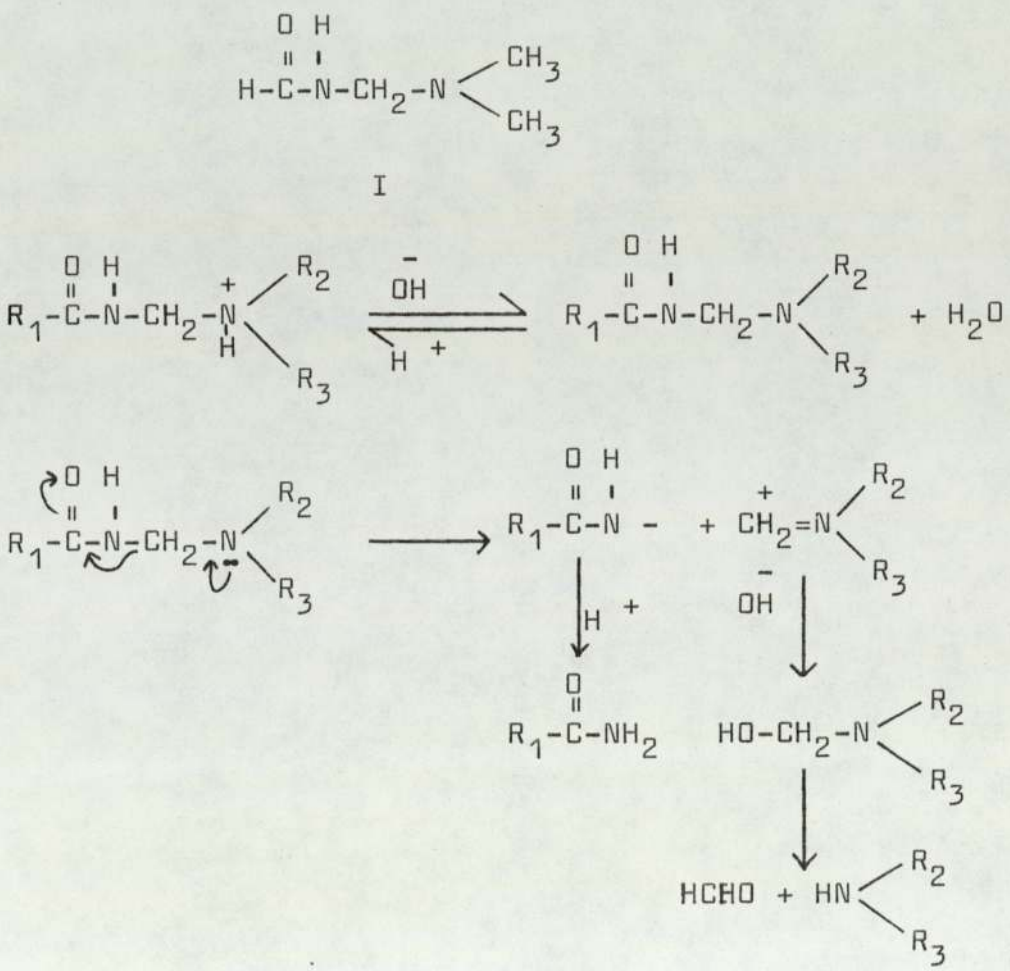
The half-lives of N-hydroxymethylbenzamide and its 4-chloro derivative in dilute sodium hydroxide solution (pH = 7.4, μ = 0.5) observed by Johansen & Bundgaard (1979) were greater than those observed in this study using Earl's buffer pH = 7.4. For example the half-life of 4-chloro-N-hydroxymethylbenzamide was measured as 83.3h by Johansen & Bundgaard (1979), whereas in the present study the half-life of this compound was measured as 15.8h by H.P.L.C.(Table 4.4).

The half-life of 4-chloro-N-hydroxymethylbenzamide measured using H.P.L.C. compares with a value of 13.2h as measured by the amount of formaldehyde liberated using the Nash reaction (Table 4.3). The half-life values shown in Table 4.4 indicate that the 4-chloro derivative of N-hydroxymethylbenzamide has a shorter half-life than N-hydroxymethylbenzamide itself. This can be explained in terms of the mechanism of degradation of N-hydroxymethylamides in scheme 4.1. The 4-chloro group is electron-withdrawing and 4-chloro-N-hydroxymethylbenzamide would therefore be expected to degrade more rapidly than N-hydroxymethylbenzamide itself. On the other hand the 4-t-butyl substituent pushes electrons into the aromatic ring system. Thus 4-t-butylbenzamide is likely to be a weaker acid than benzamide and on the basis of the mechanism outlined in scheme 4.1 one would predict that 4-t-butyl-N-hydroxymethylbenzamide would have a greater half-life than N-hydroxymethylbenzamide. This was however not the case; the half-life of 4-t-butyl-N-hydroxymethylbenzamide was shorter than those of both N-hydroxymethylbenzamide and its 4-chloro derivative (Table 4.4).

N-hydroxymethylformamide and N-hydroxymethyl-N-methylformamide are examples of N-hydroxymethyl derivatives which do not react with Nash reagent at pH = 7.4 (Table 4.2). It is interesting to note that the ether and ester derivatives of N-hydroxymethylformamides did not react with Nash reagent at pH = 7.4 whereas N-($N^{/}$-$N^{/}$-dimethylaminomethyl)-formamide (I, scheme 4.2) did. This may be explained by the

fact that the latter compound is presumably a relatively strong base and as such would be predominately in a protonated form at pH = 7.4. The resultant protonated dimethylamino cation would be a good leaving group and this may favour the reaction of the methylene carbon with nucleophiles such as those present in Nash reagent. Alternatively the positive reaction of N-(N′-N′-dimethylaminomethyl)-formamide in the Nash assay can be explained in terms of the mechanism of degradation of N-Mannich bases proposed by Bundgaard & Johansen (1980b) which is shown in scheme 4.2.

Scheme 4.2



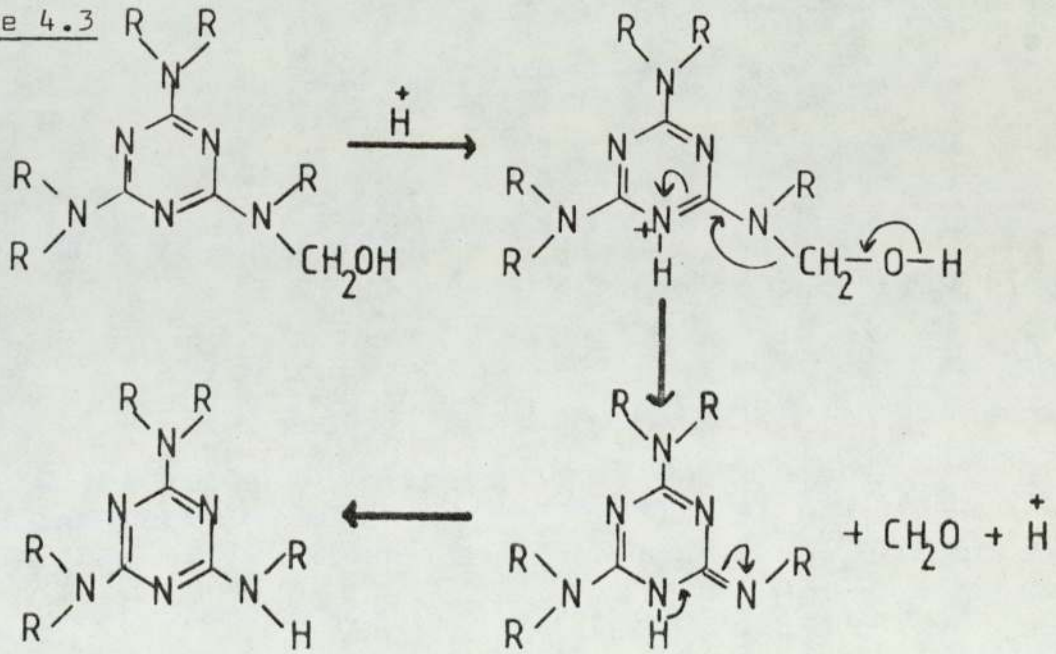
The mechanism involves a unimolecular N-C bond cleavage which generates an amide anion and an immonium cation.

An hydroxide ion is then transferred from a solvent molecule to the immonium ion to produce an N-methylolamine which rapidly decomposes to yield formaldehyde and the respective amine.

Like N-hydroxymethylbenzamide, N-hydroxymethylformamide only liberates formaldehyde under alkaline conditions (pH > 9) and not under acidic conditions (Fig.4.15). This is typical of N-hydroxymethylamides which have been shown to degrade to produce formaldehyde at a rate which is proportional to the hydroxide ion activity up to pH = 12 (Johansen & Bundgaard, 1979). There are however other N-hydroxymethyl compounds which exhibit different stability characteristics. Newell (1980) has shown that N-hydroxymethylpentamethylmelamine was more stable in phosphate buffer under alkaline conditions (pH = 8, 37^{o}C, $t\frac{1}{2}$ = 26.3 minutes) than under acidic conditions (pH = 5, 37^{o}C, $t\frac{1}{2}$ = 5.1 minutes). N-hydroxymethylpentamethylmelamine was also shown to be more stable at 37^{o}C in Tris buffer 0.1M at pH = 10.4 ($t\frac{1}{2}$ = 105.0 minutes) than at pH = 7.4 ($t\frac{1}{2}$ = 35.9 minutes). It is relevant to note that as N-hydroxymethylpentamethylmelamine reacts like formaldehyde in the Nash assay under acidic, basic or neutral conditions (Table 4.2) studies of the stability of this N-methylol were performed using H.P.L.C.. The fact that N-hydroxymethylpentamethylmelamine does not degrade to liberate formaldehyde as readily under alkaline conditions as under acidic conditions suggests that it may undergo a different pH dependent mechanism of breakdown to the N-hydroxymethylamides. The site of protonation of an

N-alkylmelamine under acidic conditions is uncertain but it is possible that protonation occurs on a ring nitrogen rather than an exocyclic nitrogen atom because of the greater delocalization of the positive charge that is possible in the resultant melaminium cation. In addition calculations of the electron density distribution of hexamethylmelamine (sec. 4.VII.2) have shown that the ring nitrogen atoms are more basic than the exocyclic nitrogen atoms. Assuming that protonation occurs on the ring and not on the amine functions of N-hydroxymethylpentamethylmelamine the degradation of this N-methylol under acidic conditions to release formaldehyde, as described in scheme 4.3, would be favoured.

Scheme 4.3



There are clearly different types of N-methylols which exhibit different pH dependent mechanisms of degradation. Therefore it is conceivable that N-methylols produced in-vivo during the metabolism of different N-methyl containing compounds may degrade at different sites

depending on the chemical environment of that site. It may be speculated that this degradation or the further reactions of an intact N-methylol may be involved in the varying pharmacological and toxic activites exerted by different N-methyl containing compounds.

4.III The stability of formaldehyde precursors produced during the metabolism of hexamethylmelamine in-vivo

4.III.1 Introduction

Rutty et al. (1978) have shown that formaldehyde equivalents were present in the plasma of mice which had received hexamethylmelamine. However these authors were uncertain whether these formaldehyde equivalents represented free formaldehyde or formaldehyde produced from the break-down of a more stable precursor during the colourimetric assay used in their study.

Gescher et al. (1979) have shown that hexamethylmelamine produces stable precursors of formaldehyde during metabolism in-vitro. It was the purpose of this investigation to determine if hexamethylmelamine produced stable precursors of formaldehyde rather than free formaldehyde in-vivo and to investigate the stability of these precursors.

4.III.2 Results

4.III.2.a Development of analytical method

The colourimetric method of Sawicki et al. (1961) was modified to distinguish between free formaldehyde and N-hydroxymethylpentamethylmelamine. Calibration curves for the assay of N-hydroxymethylpentamethylmelamine and formaldehyde at $0^{o}C$ and at $60^{o}C$ are shown in Figs. 4.16 and 4.17 respectively. The correlation coefficients of these calibration lines were formaldehyde $60^{o}C$, $r = 0.9997$; formaldehyde $0^{o}C$, $r = 0.9990$; N-hydroxymethylpentamethylmelamine $60^{o}C$, $r = 0.9972$. The curves show that formaldehyde can be determined in the presence of N-hydroxymethylpentamethylmelamine at $0^{o}C$ presumably because the N-methylol does not degrade to release formaldehyde under these conditions. At $60^{o}C$ however both formaldehyde and N-hydroxymethylpentamethylmelamine are detected by this assay and the colourimetric response was shown to be additive. The quantities of N-hydroxymethylpentamethylmelamine and formaldehyde in a mixture can therefore be determined by performing this colourimetric assay at $0^{o}C$ and at $60^{o}C$ using the same sample.

4.III.2.b Determination of the plasma profile of formaldehyde precursors after the administration of hexamethylmelamine to mice

Fig. 4.18 shows the plasma profile of formaldehyde precursors after the intraperitoneal (i.p.) injection of hexamethylmelamine 100mg/Kg to mice. No free formadehyde was detected in the plasma. Peak plasma levels of formaldehyde precursors of 108 nmol/ml formaldehyde equivalents were obtained one hour after the administration of hexamethylmelamine and the elimination half-life of these precursors was 1.9h, calculated by linear regression analysis.

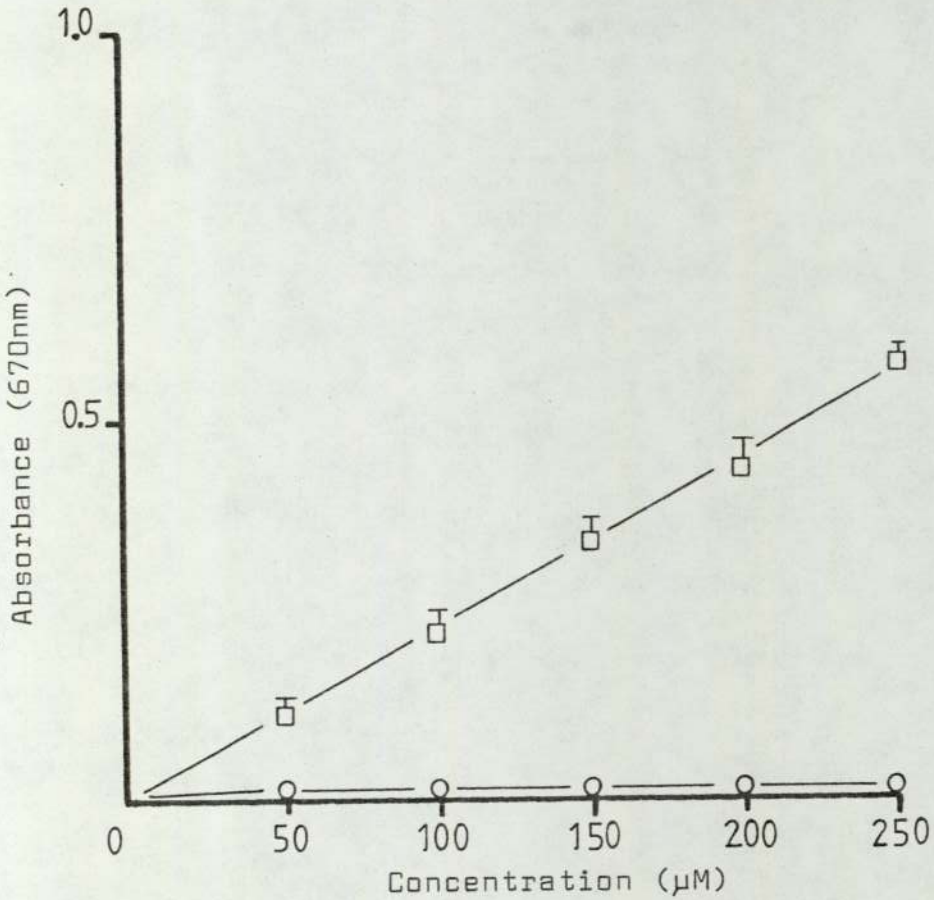


Fig. 4.16 Calibration curve for the determination of formaldehyde (□) and N-hydroxymethylpentamethylmelamine (○) using the method of Sawicki et al. (1961) at 0°C.

Each point represents the mean ± s.d. of at least three determinations.

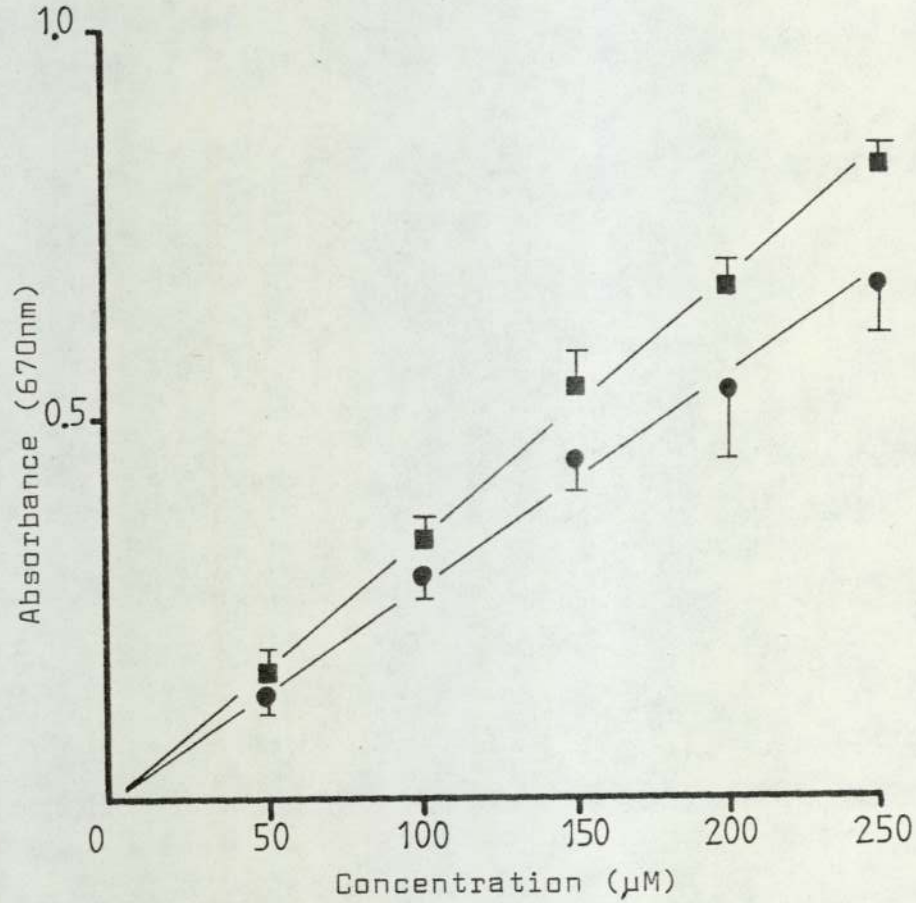


Fig. 4.17 Calibration curve for the determinatio of formaldehyde (■) and N-hydroxymethylpentamethylmelamine (● using the method of Sawicki et al. (1961) at 60°C

Each point represents the mean ± s.d. of at least three determinations.

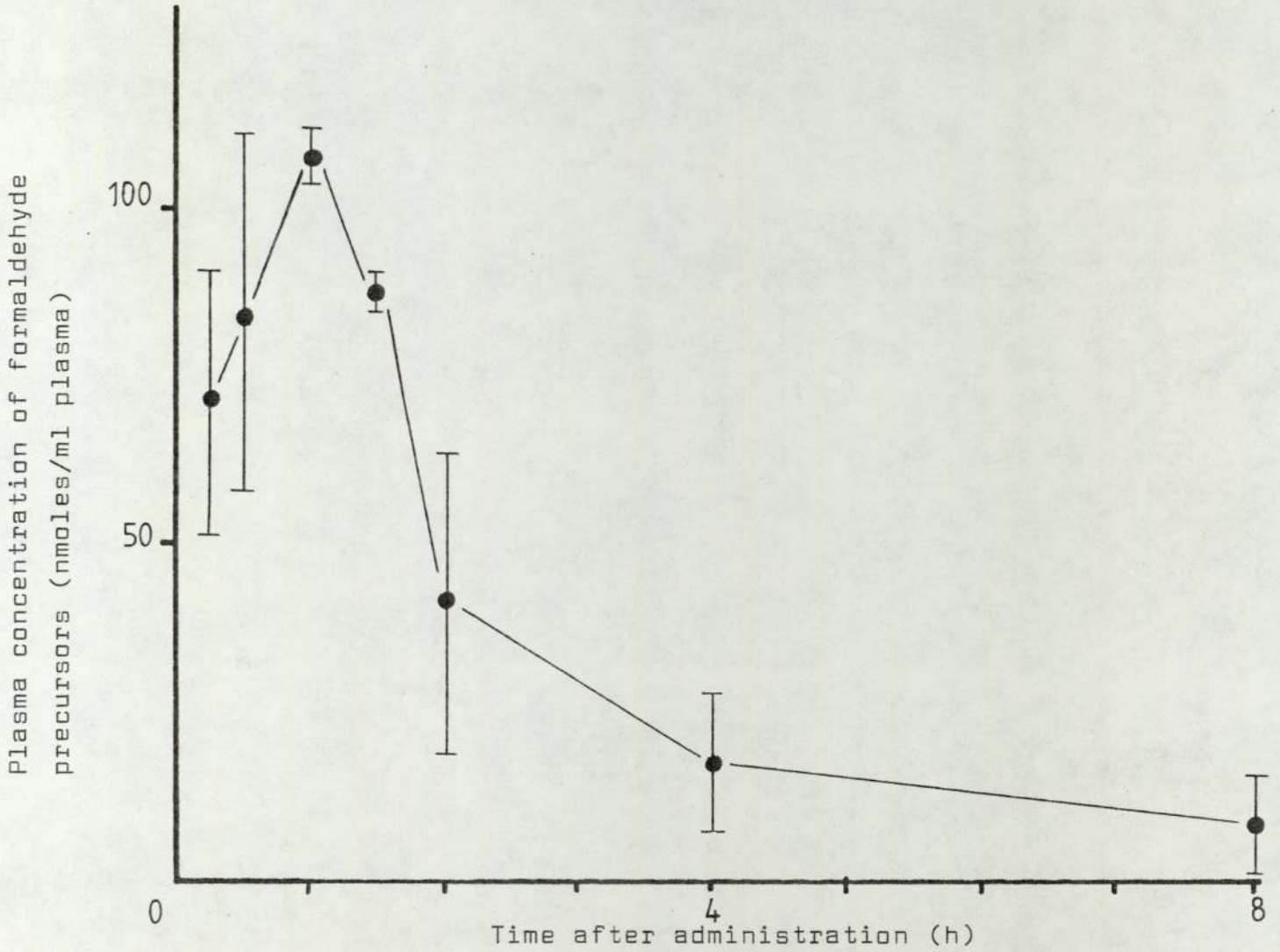


Fig. 4.18 Time course of the appearance of formaldehyde precursors in the plasma of mice which had received hexamethylmelamine 100mg/Kg i.p.

Each point represents the mean ± s.d of at least three determinations.

4.III.3 Discussion

The results presented in Fig.4.18 show that formaldehyde precursors rather than free formaldehyde were present in the plasma of mice which had received hexamethylmelamine. The plasma profile of formaldehyde precursors (Fig.4.18) is similar to the profile observed by Rutty et al. (1978) who measured total blood formaldehyde by means of the Nash reaction after the administration of hexamethylmelamine to mice. It is probable therefore that the formaldehyde measured by Rutty et al. (1978) arose from the decomposition of formaldehyde precursors during the Nash assay and was not free formaldehyde.

Rutty & Connors (1977) have shown that hexamethylmelamine requires metabolic activation in order to express significant cytotoxic activity in-vitro. In an attempt to clarify the mechanism of this activation Rutty & Abel (1980) have compared the cytotoxicities of formaldehyde, N-hydroxymethylpentamethylmelamine, hexamethylmelamine and its N-demethylated metabolite pentamethylmelamine to PC6A plasmacytoma, L1210 leukaemia, and Walker 256 ascites cells in-vitro. They proposed that the toxicity of N-methylols derived from N-methylmelamines to L1210 and Walker 256 cells was due to formaldehyde, whereas the intact N-methylols appeared to be cytotoxic to PC6A cells. However Ross et al. (1981) have investigated the DNA damage caused by hexamethylmelamine and its potential metabolites in L1210 cells in-vitro and have suggested that the cytotoxicity of N-methylols is unlikely to be mediated via free formaldehyde.

These results do not agree with those of Rutty & Abel (1980) but both studies do suggest that the in-vitro cytotoxicity of N-methylolmelamines to certain tumour cell lines may be mediated by the intact N-methylol and not by free formaldehyde liberated from its breakdown. It has been proposed by Newell et al. (1981) that the in-vivo antitumour activity of N-methylmelamines in mice, rats, and man may be related to the rates of formation and disappearance of N-methylols in the plasma of these species.

As the major pathway for the metabolism of hexamethylmelamine in-vivo is N-demethylation to lower melamines such as N_2,N_4-dimethylmelamine and monomethylmelamine (Worzalla et al., 1973; 1974; Rutty et al., 1978) it seems probable that the formaldehyde precursors measured in this study represent N-hydroxymethyl derivatives of a number of N-demethylated metabolites of hexamethylmelamine. These precursors had a plasma elimination half-life of 1.9 hours (Fig. 4.18) which would render them sufficiently stable to reach extrahepatic sites. In view of the proposed importance of N-methylols to the in-vitro and in-vivo antitumour activity of N-methylmelamines the stability of these metabolically generated precursors may be a determinant of the in-vivo antitumour activity of hexamethylmelamine.

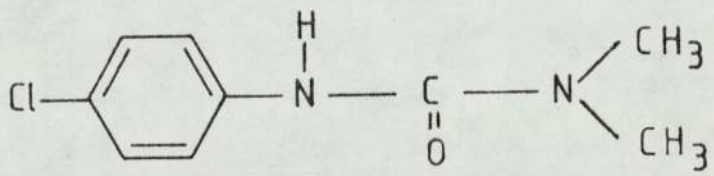
4.IV Investigations of the metabolism of N/-(4-chlorophenyl)-N,N-dimethylurea

4.IV.1 Introduction

N,N-dimethylarylureas were metabolized in mouse hepatic microsomal systems to stable precursors of formaldehyde (sec. 4.I.2.c). The aim of this investigation was to study the metabolism of one of these ureas in more detail in an attempt to characterize these precursors.

The compound chosen for a more detailed investigation was the 4-chloro derivative (scheme 4.4) which is a herbicide called monuron (Dupont).

Scheme 4.4



N/-(4-chlorophenyl)-N,N-dimethylurea
(IVe, monuron)

This compound was chosen mainly for analytical reasons as molecules which contain chlorine atoms when subjected to mass spectrometry produce a characteristic isotope splitting pattern in all chlorine containing fragments. This facilitates identification of peaks which are derived from the parent compound in the mass spectrum.

The activity of phenylurea herbicides is thought to be mediated via inhibition of one of the enzymic steps involved in photosynthesis and metabolism of the herbicide by the plant is not essential for this activity (Albert, 1973). Monuron has been shown to be mutagenic (Wuu and Grant, 1966; Seiler, 1978) and has also been classed as a carcinogen (IARC Monographs, 1976). It has been suggested by Seiler (1979) that

biotransformation of monuron may be required in order for it to exert mutagenic and carcinogenic effects. Thus it was considered that characterization of the products of the N-demethylation of monuron may help explain its mutagenic and carcinogenic potential.

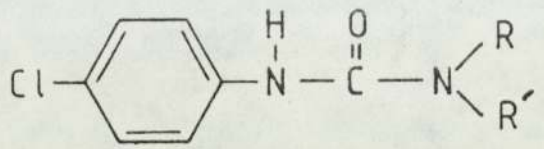
4.IV.2 Results

4.IV.2.a Development of chromatographic methods

Monuron produced a single peak upon G.C.analysis which was identified as 4-chlorophenylisocyanate by M.S. ($^{m}/z$ 153, $^{35}ClM^{+}$; $^{m}/z$ 155 $^{37}ClM^{+}$), irrespective of the thermal conditions used for the analysis.

The authentic standards available for this investigation are shown in Fig. 4.19. Although separation of monuron, its N-desmethyl derivative and 4-chlorophenylurea could be achieved using reverse phase T.L.C, the low sensitivity of this method (sensitivity limit for monuron $>$ 25µg/ml) was considered inappropriate for metabolism studies.

Separation of all reference compounds was achieved using both a reverse phase H.P.L.C.method (Fig. 4.20) and a normal phase H.P.L.C.method (Fig. 4.21). The reverse phase method was used as the method of choice because of the ease of sample preparation (sec. 3.IV.2.b). Calibration curves for monuron, its N-desmethyl derivative and 4-chlorophenylurea obtained using both methods had correlation coefficients of $r > 0.9980$. Using the reverse phase method the s.d of six determinations of the amount of monuron in a sample was less than 7% of the mean and the limit of sensitivity for monuron was $<$ 0.1 µg/ml.



R	R´
$-CH_3$	$-CH_3$
$-CH_3$	-H
-H	-H
-CHO	$-CH_3$
-CHO	-H

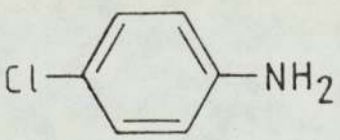


Fig. 4.19 Reference compounds available for the study of the metabolism of monuron

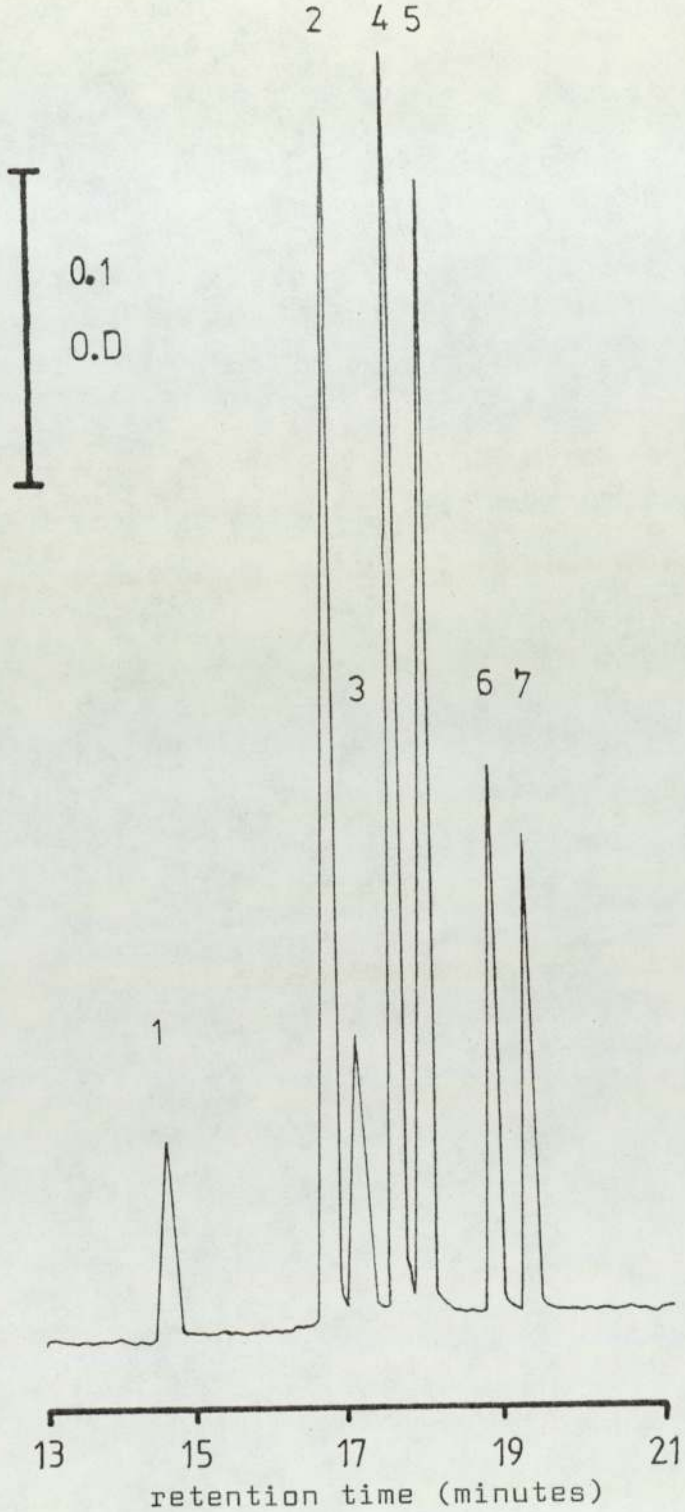


Fig. 4.20 High pressure liquid chromatogram of a mixture of internal standard (1), 4-chlorophenylurea (2), 4-chloroaniline (3), N/-(4-chlorophenyl)-N-methylurea (4), monuron (5), N/-(4-chlorophenyl)-N-formylurea (6), and N/-(4-chlorophenyl)-N-formyl-N-methylurea (7), obtained using reverse phase H.P.L.C.

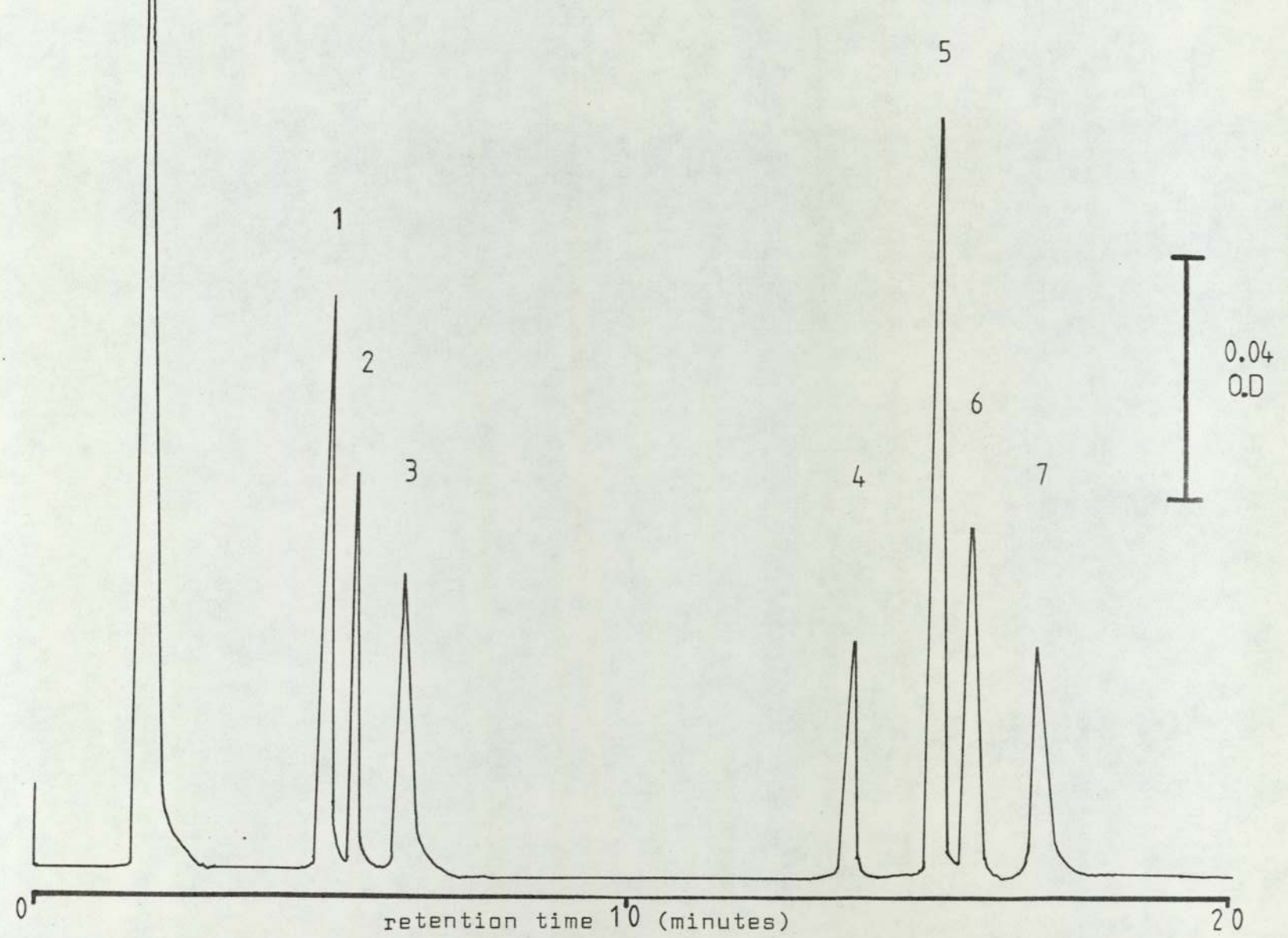


Fig. 4.21 High pressure liquid chromatogram of a mixture of 4-chloroaniline (1), N/-(4-chlorophenyl)-N-formyl-N-methylurea (2), N/-(4-chlorophenyl)-N-formylurea(3), N/-(4-chlorophenyl)-N-methylurea (4), internal standard (5), monuron (6) and 4-chlorophenylurea (7) obtained using normal phase H.P.L.C.

4.IV.2.b In-vitro metabolism studies

Chromatograms of authentic standards and the products of the microsomal metabolism of monuron are shown in Fig. 4.22. The two metabolites detected after the incubation of monuron with microsomes had similar retention times to 4-chlorophenylurea (metabolite A - Fig.4.22) and N/-(4-chlorophenyl)-N-methylurea (metabolite B - Fig.4.22). These metabolites were also found in incubations of monuron with whole liver homogenate, 9000g supernatant and isolated hepatocytes.

The time course of appearance of metabolites A and B during the microsomal metabolism of monuron is shown in Fig. 4.23. The amount of metabolite A in the incubation mixture initially increases and then subsequently decreases during the microsomal incubation. Fig. 4.24 shows the chromatogram produced by adding authentic 4-chlorophenylurea to the metabolism mixture and demonstrates that metabolite A has a different retention time to that of 4-chlorophenylurea. When the products of metabolism were measured by normal phase H.P.L.C no 4-chlorophenylurea could be detected. When the microsomal mixture was heated at 60^{o}C (Fig. 4.25), when acid or alkali was added to the mixture, or upon incubation of aliquots of the mixture in acetate buffer pH = 5 at 37^{o}C the amount of metabolite A present decreased with a corresponding increase in the amount of metabolite B present in the mixture Metabolite A was not a substrate for yeast alcohol dehydrogenase or yeast alcohol oxidase, and was not removed during a 15 minute incubation with microsome free homogenate

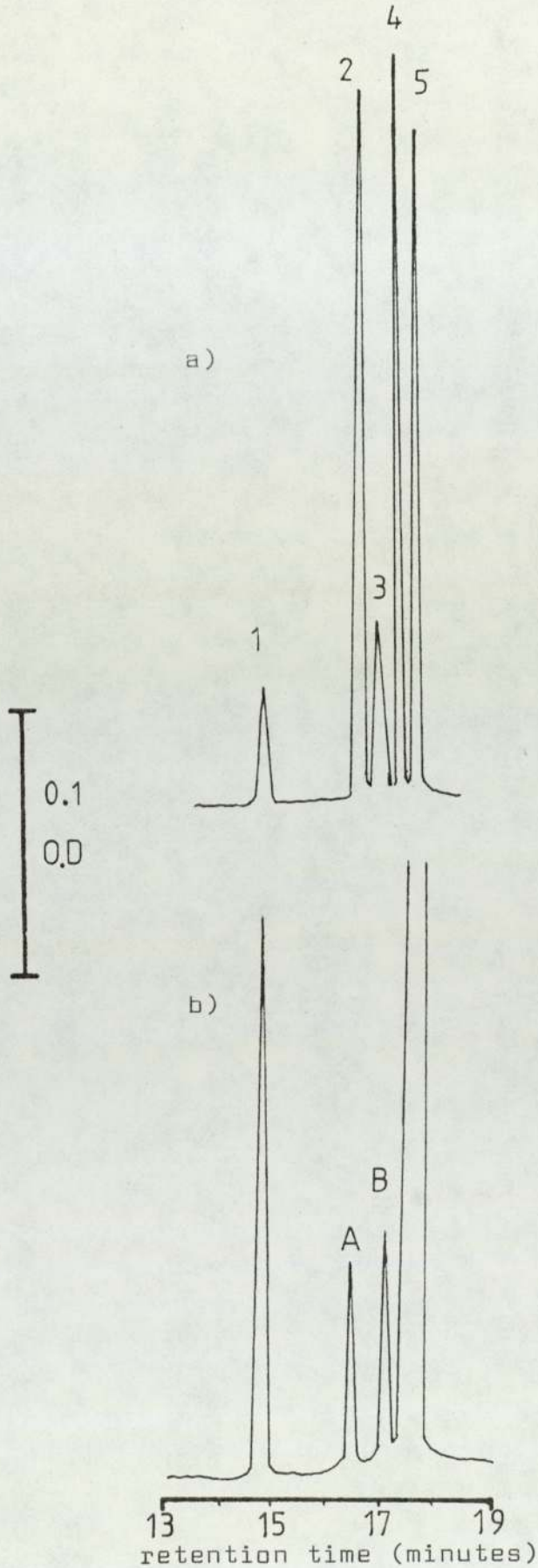


Fig. 4.22 High pressure liquid chromatograms of, a) a mixture of internal standard (1), 4-chlorophenylurea (2), 4-chloraniline (3), N'-(4-chlorophenyl)-N-methylurea (4) and monuron (5) and b) an incubation mixture of monuron with mouse liver microsomes fortified with an NADPH generating system.

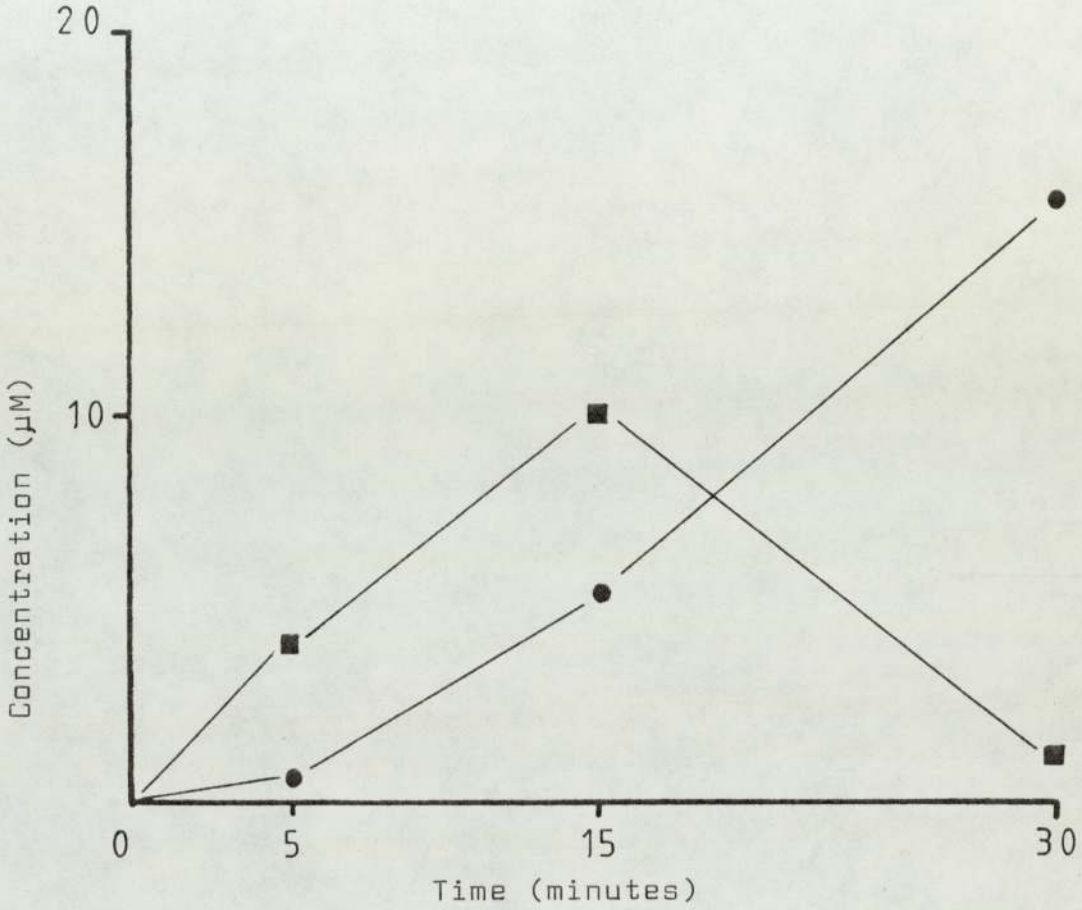


Fig. 4.23 Time-course of appearance of microsomal metabolites of monuron

(■) - a metabolite of monuron with a similar retention time to 4-chlorophenylurea (metabolite A), (●) - a metabolite of monuron with a similar retention time to N/-(4-chlorophenyl)-N-methylurea (metabolite B)

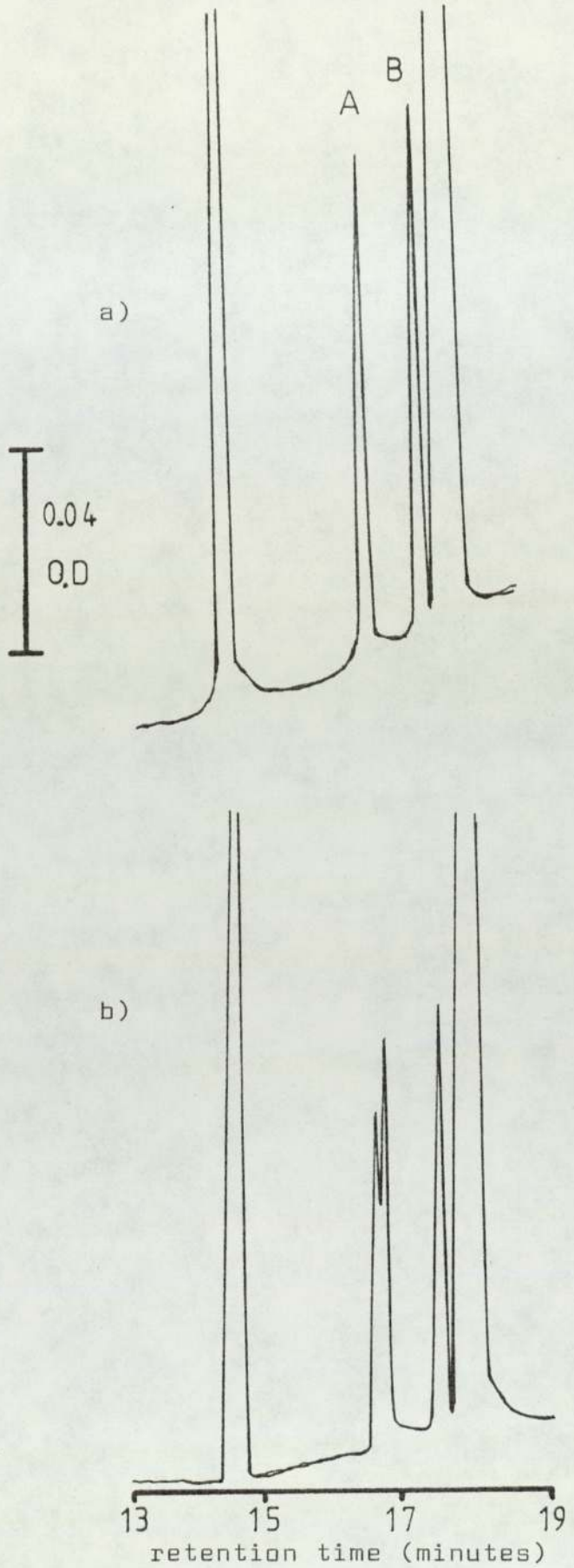


Fig. 4.24 High pressure liquid chromatograms of an incubation mixture of monuron with mouse liver microsomes before (a) and after (b) the addition of authentic 4-chlorophenylurea.

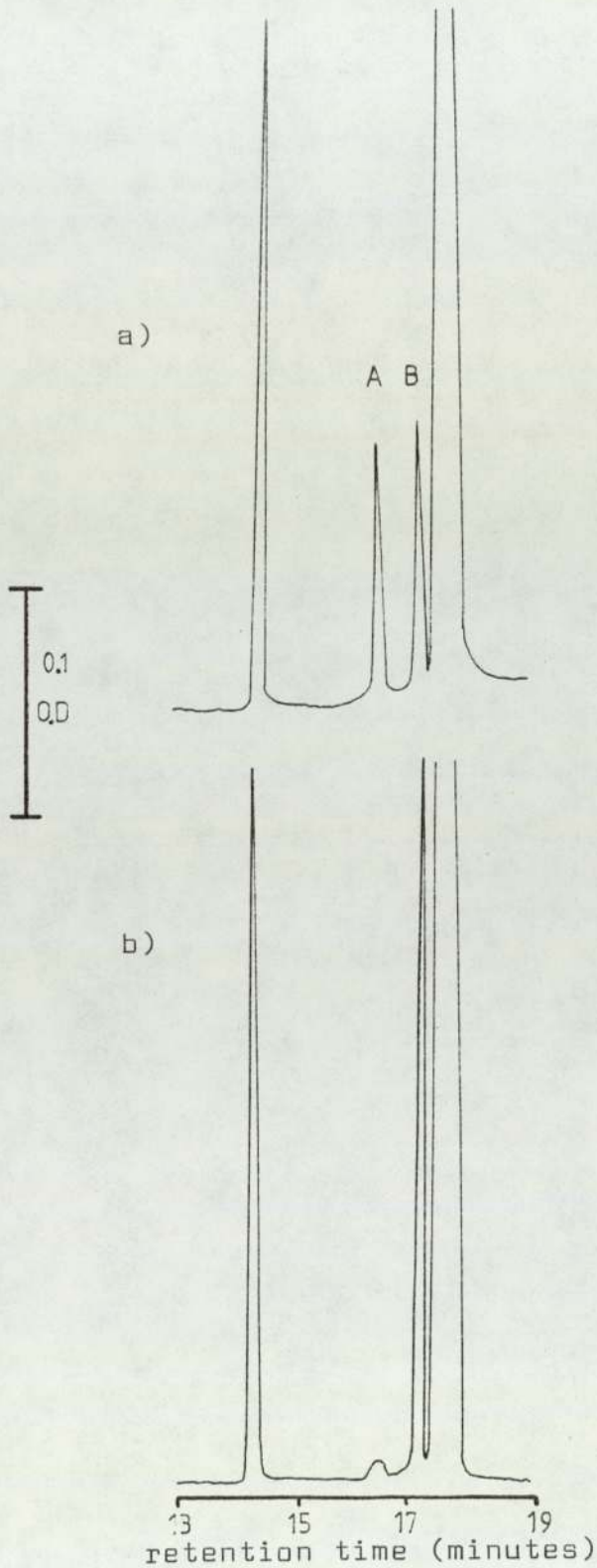


Fig. 4.25 High pressure liquid chromatograms of an incubation mixture of monuron with mouse liver microsomes before (a) and after (b) heating at 60°C for 30 minutes.

and NAD. The mass spectra of the concentrated H.P.L.C. eluates corresponding to the retention times of authentic 4-chlorophenylurea and metabolite A, and of authentic N/-(4-chlorophenyl)-N-methylurea and metabolite B are shown in Figs. 4.26 and 4.27 respectively.

There was no change in the retention time of the metabolites generated during microsomal incubation of monuron when the incubates were deproteinized with cold ethanol instead of cold methanol.

Formaldehyde could not be detected during the thermal decomposition of metabolite A using the colourimetric methods of Nash (1953), Sawicki et al. (1961), the semicarbazide method as described by Johansen & Bundgaard (1979), and the 2,4-dinitrophenylhydrazine method as adapted for H.P.L.C. by Fung and Grosjean (1981).

The products of microsomal N-demethylation of monuron were measured using various analytical methods and deproteinization techniques. The results are summarized in Table 4.5.

4.IV.2.c In-vivo metabolism studies

Chromatograms of the urine of mice which had received vehicle and the urine of mice which had received monuron 200mg/Kg i.p. are shown in Fig.4.28. The major metabolite (2, Fig.4.28) in the urine of mice which had received monuron was characterized as 4-chlorophenylurea by M.S. Small peaks due to metabolites were also present in the chromatogram (Fig. 4.28) which had identical retention times to N/-(4-chlorophenyl)-N-methylurea (5, Fig.4.28) and

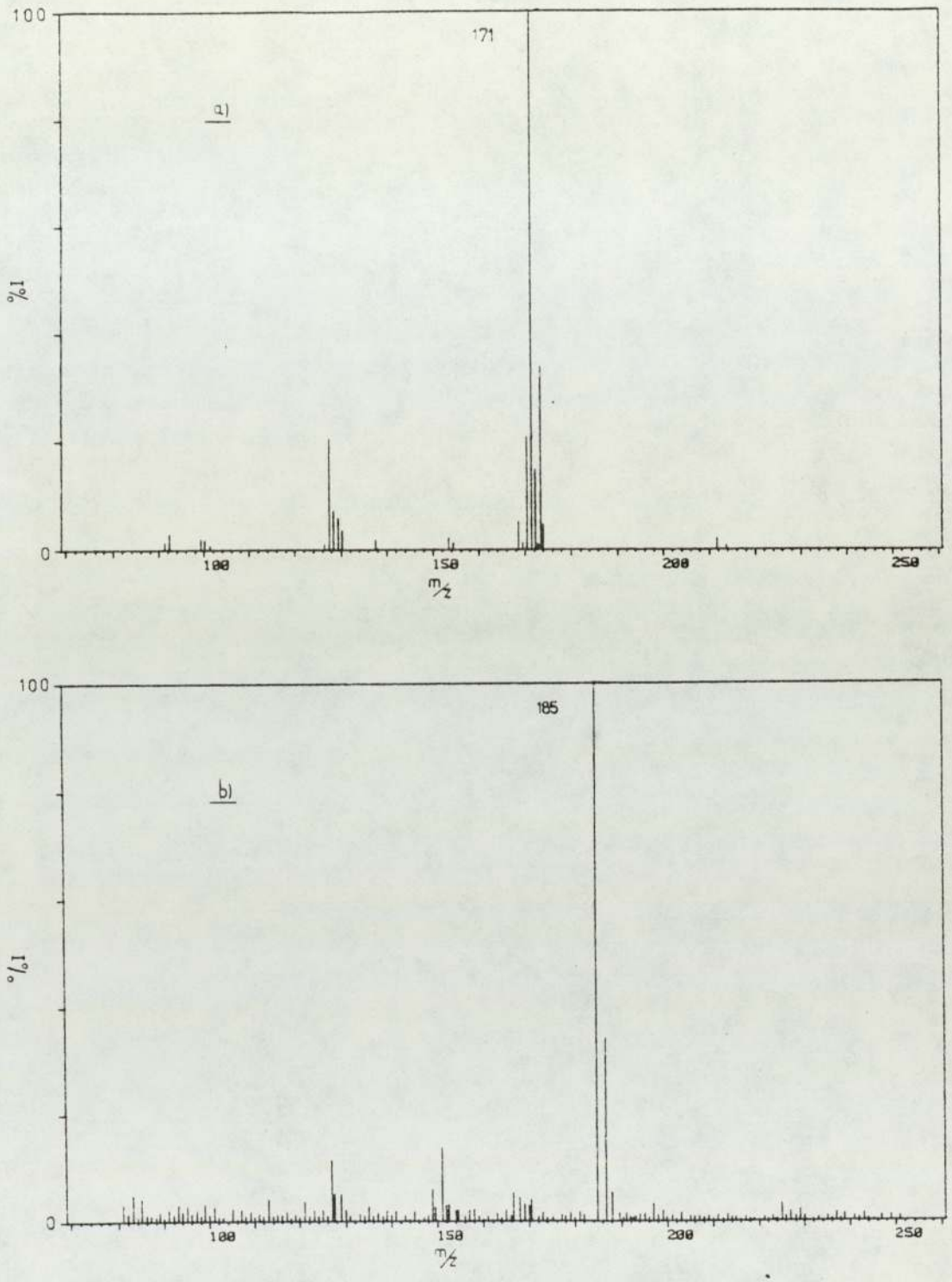


Fig. 4.26 Chemical ionization mass spectra of a) 4-chlorophenylurea, and b) an in-vitro metabolite of monuron with a similar retention time (metabolite A)

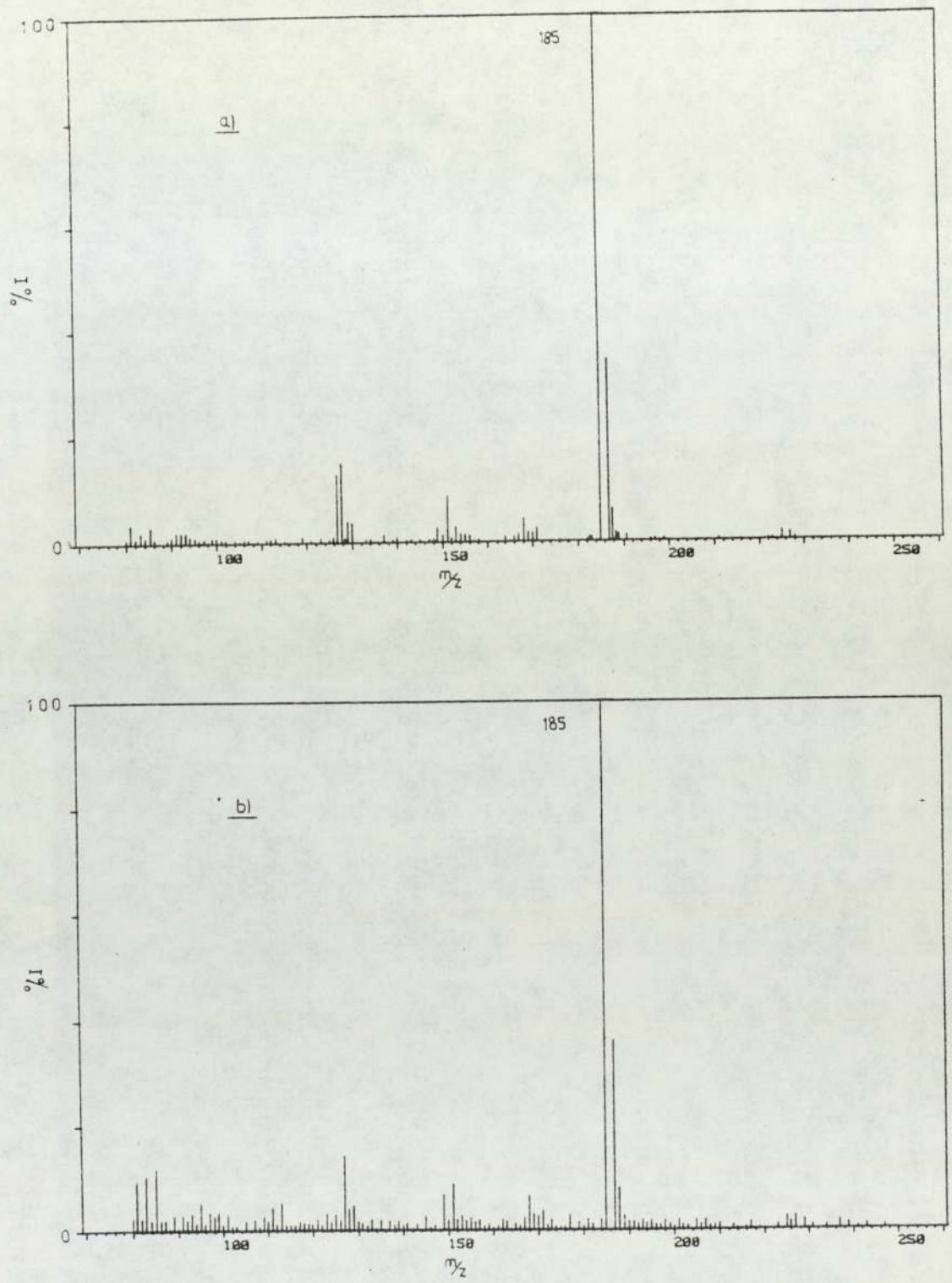


Fig. 4.27 Chemical ionization mass spectra of a) N'-(4-chlorophenyl)-N-methylurea and b) an in-vitro metabolite of monuron with a similar retention time (metabolite B)

Deproteinization technique	Total products of the N-demethylation of monuron (mM) measured using the method of		
	Nash (1953)	Sawicki et al.(1961)	H.P.L.C.*
Trichloroacetic acid 20% $^{w}/_{v}$ (0.25ml)	270	n.d	93
Zinc sulphate 20% $^{w}/_{v}$ (1ml) Barium hydroxide sat'd (1ml)	335	350	63
Cold methanol (2.5ml)	n.d	n.d	66

n.d - not determined

* - assuming metabolite A has a similar extinction coefficient to N'-(4-chlorophenyl)-N-methylurea

Table 4.5 The products of the microsomal N-demethylation of monuron using different methods

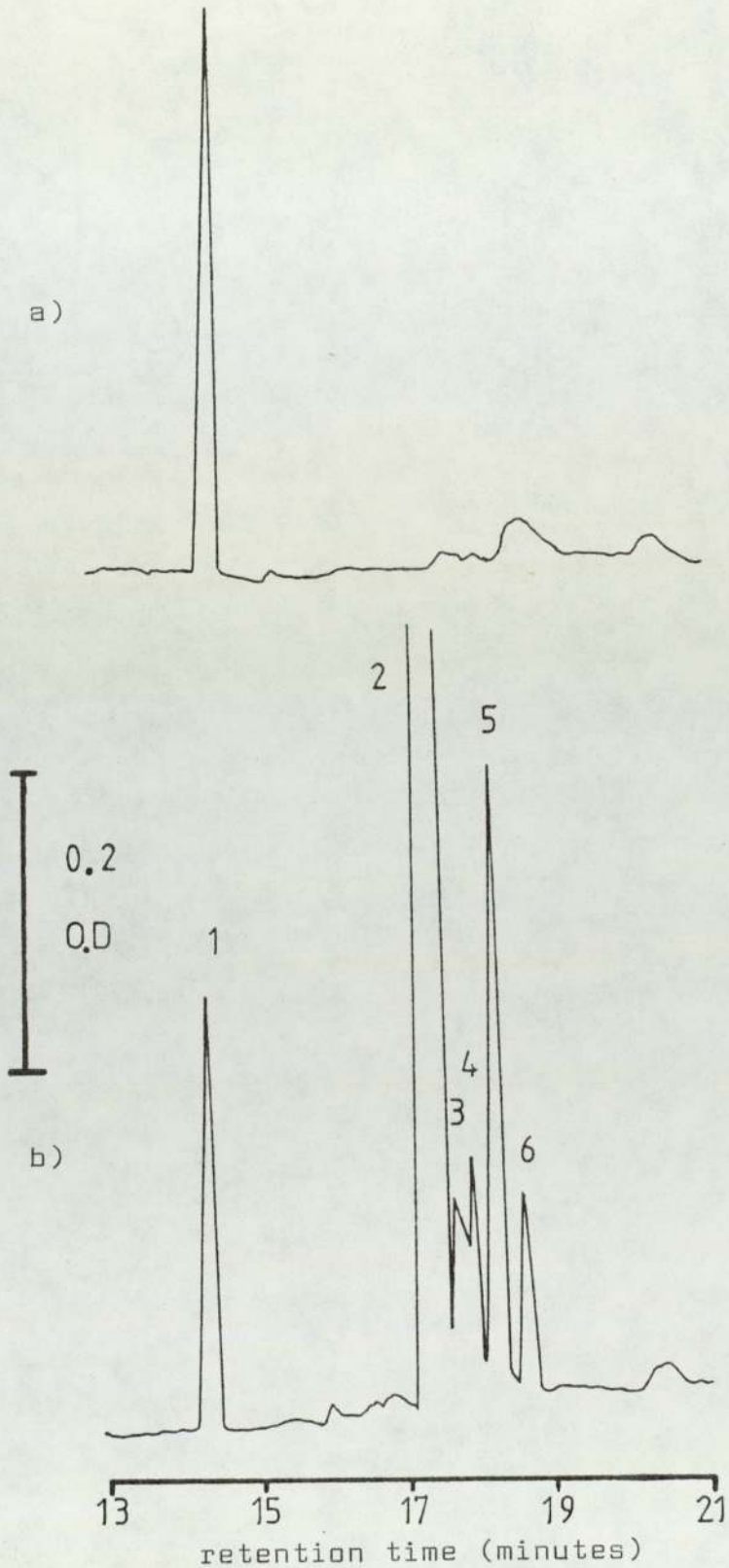


Fig. 4.28 High pressure liquid chromatograms of the urine of mice which had received vehicle (a) and monuron 200mg/Kg i.p. (b)

For peak identification - see text.

monuron (6, Fig. 4.28). There were also two minor peaks in the chromatogram (3 and 4, Fig. 4.28) with retention times intermediate between 4-chlorophenylurea and N′-(4-chlorophenyl)-N-methylurea, one of which may have been due to 4-chloroaniline (see Fig. 4.22).

After incubation of urine samples of mice which had received monuron with deconjugating enzymes the amount of a metabolite with an identical retention time to N′-(4-chlorophenyl)-N-methylurea present in the sample increased (3, Fig. 4.29). That this metabolite was N′-(4-chlorophenyl)-N-methylurea was confirmed by M.S..

The metabolites of monuron found in the urine of mice both before and after deconjugation were quantified and the results are summarized in Table 4.6.

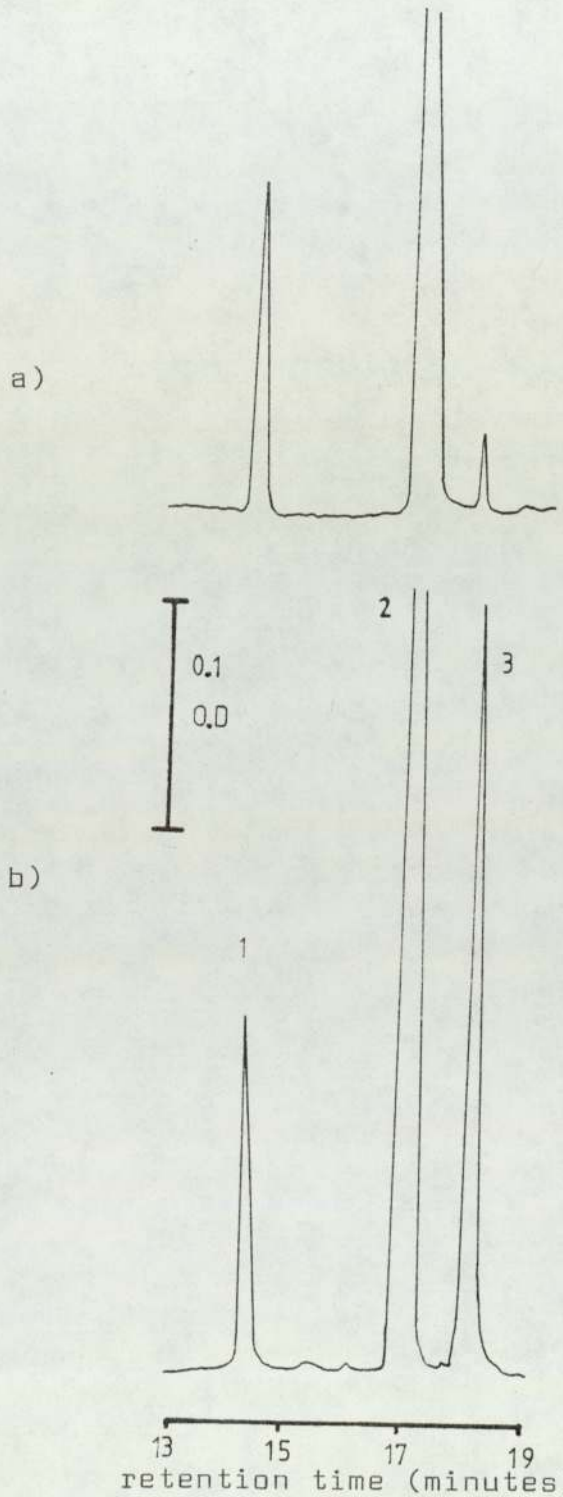


Fig. 4.29 High pressure liquid chromatograms of the urine of mice which had received monuron 200mg/Kg i.p. before (a) and after (b) enzymatic deconjugation. (1 = internal standard, 2 = 4-chlorophenylurea, 3 = N/-(4-chlorophenyl)-N-methylurea.

Metabolite	Amount in urine expressed as % of dose		
	FREE	CONJUGATED*	TOTAL
4-chlorophenylurea	9.0	0	9.0
N′-(4-chlorophenyl)-N-methylurea	0.3	5.9	6.2
N′-(4-chlorophenyl)-N,N-dimethylurea	0.1	0	0.1

Table 4.6 Metabolites in the urine of mice 24 hours after the administration of monuron 200mg/Kg i.p.

* - measured after enzymatic deconjugation with glucuronidase and sulfatase.

4.IV.3 Discussion

Phenylureas are thermolabile and on G.C.analysis degrade to produce the corresponding phenylisocyanate (Spengler & Hamroll, 1970). Buser & Grolimund (1974) however have shown that by the elimination of catalytic surface effects a number of phenylureas including monuron remain intact on gas chromatographic analysis. In a study using capillary G.C.Grob Junr. (1981) classed monuron as 'difficult' but not 'impossible' with respect to its ease of analysis by this method. The results obtained using combined capillary G.C.- M.S.(sec. 4.II.2.a) confirm that monuron is thermolabile and show that it degrades to 4-chlorophenylisocyanate under the conditions used for the analysis. The thermal instability of the phenylureas is thought to be due to the presence of an amide hydrogen adjacent to the phenyl ring (Buchert & Lokke, 1975). It was probable therefore that all of the phenylurea reference compounds in Fig. 4.19 would produce an identical peak on G.C.analysis, rendering this technique unsuitable for a study of monuron metabolism. As T.L.C.analysis was not sufficiently sensitive H.P.L.C.was the method of choice for an investigation of the metabolism of monuron.

The results described in sec. 4.II.2.b demonstrate that two metabolites were formed during the metabolism of monuron in-vitro using whole liver homogenate, microsomal and 9000g supernatant fractions of liver and isolated hepatocytes. One metabolite (metabolite B) was identified

as N/-(4-chlorophenyl)-N-methylurea on the basis of chromatographic (Fig. 4.22) and mass spectral evidence (Fig. 4.27). The other metabolite (metabolite A) had a retention time similar to 4-chlorophenylurea upon H.P.L.C. analysis (Fig. 4.22), but experiments using normal phase chromatography and the addition of authentic 4-chlorophenylurea (Fig. 4.24) proved that this metabolite was not the latter compound. On treatment with acid, alkali, or upon heating the sample metabolite A decomposed with a corresponding increase in the amount of N/-(4-chlorophenyl)-N-methylurea present in the sample. These results suggested that this metabolite was a precursor of N/-(4-chlorophenyl)-N-methylurea and this was confirmed by M.S. (Fig. 4.26).

Therefore it seems reasonable to assume that this unstable metabolite may be N/-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea (II, Fig. 4.30). The fact that formaldehyde could not be detected during the thermal decomposition of this unstable metabolite (4.IV.2.b) is not in agreement with the proposed structure. However it is likely that the amount of formaldehyde present in H.P.L.C. eluates after heating was below the detection limit of the assays used.

It remained a possibility that the unstable metabolite could be the methyl ether of II (Fig. 4.30) formed by reaction of II with methanol which was used for deproteinization. It has been shown that N/-(4-chlorophenyl)-N-hydroxymethylurea is a metabolite of N/-(4-chlorophenyl)-N-methylurea in a microsomal system derived from cotton plants

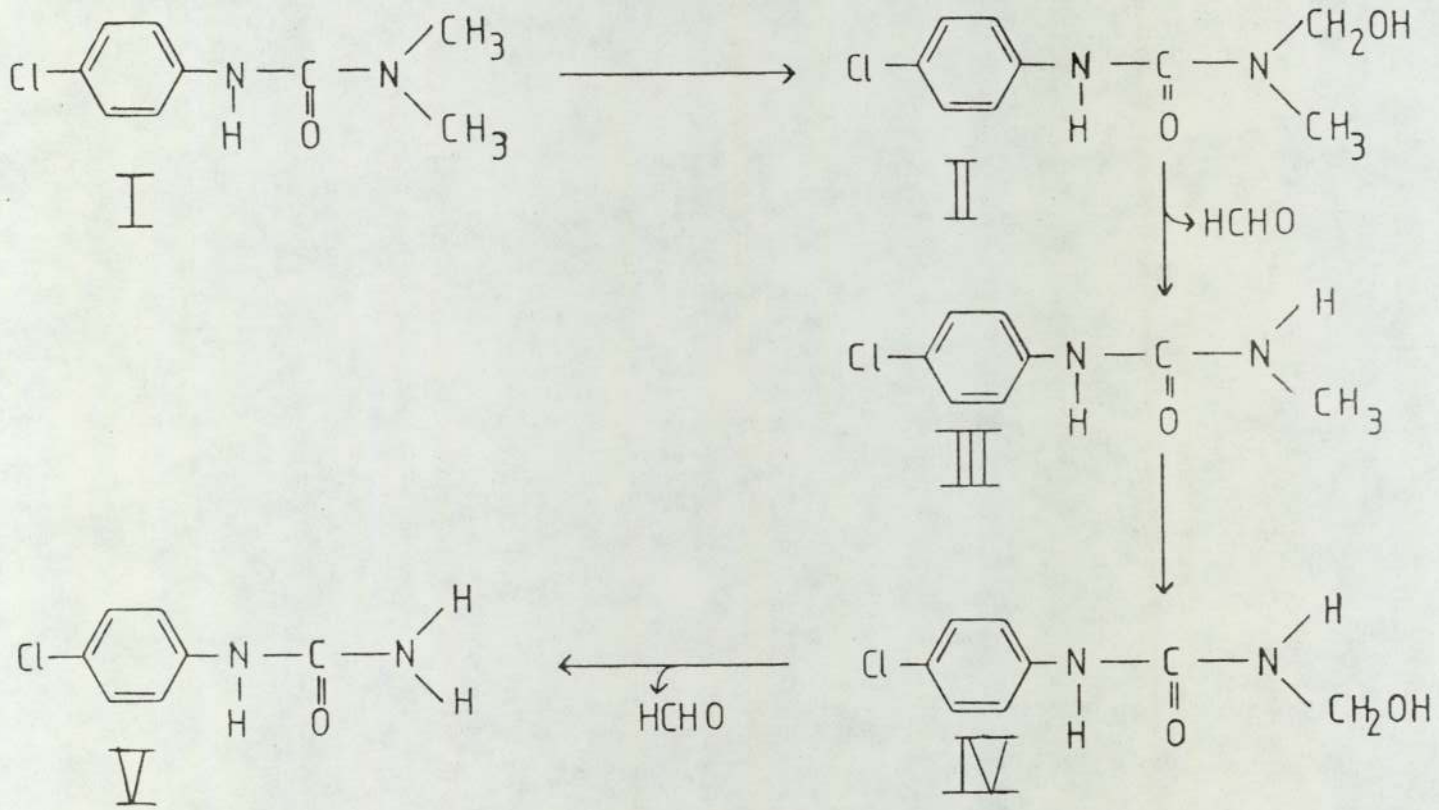


Fig. 4.30 Metabolism of monuron

(Tanaka et al., 1972). The same authors demonstrated that if N′-(4-chlorophenyl)-N-hydroxymethylurea was stored overnight in methanol or ethanol the corresponding alkoxy derivative of the N-hydroxymethyl metabolite was formed. When deproteinization of the microsomal incubate in this study was performed with ethanol rather than methanol there was no change in the H.P.L.C.retention time of the unstable metabolite. The ethoxy derivative of II (Fig. 4.30) would reasonably be expected to have a different H.P.L.C.retention time to the methoxy derivative. This suggests therefore that the formation of alkoxy derivatives of this unstable metabolite does not occur spontaneously in this system. Also on the basis of T.L.C.Rf values of N′-(4-chlorophenyl)-N-methylurea and its N-methoxymethyl derivative (Tanaka et al., 1972) it would be expected that the methyl ether of II (Fig. 4.30) would not be sufficiently polar to have a retention time upon H.P.L.C.analysis similar to 4-chlorophenylurea.

Thus the evidence presented in sec. 4.II.2.b favours the suggestion that the unstable metabolite isolated during microsomal metabolism (metabolite A, Fig.4.22) is N′-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea. The glucoside conjugate of this compound has been reported as a product of the metabolism of monuron in cotton plants (Frear & Swanson, 1972) but this is the first report of its formation in animals.

The products of microsomal metabolism were measured by the methods of Nash (1953), Sawicki (1961) and by H.P.L.C,

assuming that the proposed metabolite (II, Fig. 4.30) had a similar extinction coefficient to N/-(4-chlorophenyl)-N-methylurea, after varying deproteinization procedures. The results, summarized in table 4.5, reveal large differences in the amounts of N-demethylation measured using different analytical methods even when the same deproteinization technique was employed. It is doubtful that such differences can be explained by a different extinction coefficient of the proposed N-hydroxymethyl-N-methyl compound as compared to N/-(4-chlorophenyl)-N-methylurea. One possible explanation is that other, as yet unidentified, formaldehyde precursors which produce a positive colourimetric response but which are not detected by H.P.L.C. may also be present in the incubation mixture. Alternatively the differences observed may be a function of the methods used. Bast & Noordhoek (1981) have observed that the amount of N-demethylation of N,N-dimethylaminoantipyrine measured was dependent on the analytical method used. These authors suggested that an H.P.L.C. method was unsuitable for assessing the N-demethylation of N,N-dimethylaminoantipyrine as it underestimated formaldehyde formation when compared to the Nash colourimetric assay. Thus whether the stable formaldehyde precursors observed during the in-vitro metabolism of monuron as measured by the Nash assay (Fig. 4.7) were due to the formation of the proposed N/-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea metabolite as detected by H.P.L.C. is uncertain. The fact that the unstable metabolite observed using H.P.L.C. analysis was unaffected by a fifteen minute

incubation with microsome-free homogenate and NAD (4.IV.2.b) supports the view that this metabolite may be at least one of the stable formaldehyde precursors observed during the microsomal metabolism of monuron. However the possibility that these stable formaldehyde precursors observed using the colourimetric assay (4.I.2.c, Fig. 4.7) are due to as yet unidentified metabolites of monuron cannot be excluded.

The major metabolite found in the urine of mice which had received monuron was characterized as 4-chlorophenylurea by M.S.. This is in agreement with the observation of Ernst & Bohme (1965) who found that 4-chlorophenylurea was the major urinary metabolite of monuron in the rat. These workers also demonstrated that, as in this study, N′-(4-chlorophenyl)-N-methylurea was a urinary metabolite of monuron although it was present in insufficient amounts to quantify in rat urine. Ernst & Bohme (1965) did find small quantities of various ring hydroxylated metabolites in the urine of rats which had received monuron. These reference compounds were not available for this investigation but it is possible that the unidentified peaks in the chromatogram (3 and 4, Fig.4.28) may represent such metabolites.

That a urinary metabolite of monuron after enzymatic deconjugation in acetate buffer pH = 5 was identified as the N-desmethyl derivative of monuron (Fig. 4.20) is consistent with the suggestion that its precursor may be N′-(4-chlorophenyl)-N-hydroxymethyl-N-methylurea (II, Fig. 4.30). After hydrolysis of its glucuronide or sulphate derivative the free N-hydroxymethyl compound would be liberated. It has been shown

that the unstable metabolite obtained on incubation of monuron with microsomes decomposes during incubation in acetate buffer pH = 5 to yield the N-desmethyl derivative of monuron (sec. 4.IV.2.b). Thus if one assumes that this unstable microsomal metabolite is II (Fig. 4.30) then it is feasible that the compound which gives rise to N′-(4-chlorophenyl)-N-methylurea upon deconjugation is an O-linked conjugate of II, Fig. 4.30. Alternatively this precursor may be a conjugate of N′-(4-chlorophenyl)-N-methylurea linked to the conjugating species by either of the two nitrogen atoms. Ernst &Bohme (1965) found increased amounts of ortho and meta ring-hydroxylated products after enzymatic deconjugation of the urine of rats which had received monuron. This observation and the fact that they did not find increased amounts of N′-(4-chlorophenyl)-N-methylurea in the urine after deconjugation are probably the results of species differences in the metabolism of monuron by mice and rats.

Muecke et al. (1976) investigated the metabolism of a compound closely related to monuron, N′-(3-chloro-4-methylphenyl)-N,N-dimethylurea (chlorotoluron), in the rat. They tentatively identified an N-formyl derivative of the parent compound as a urinary metabolite and suggested that it may be an intermediate in the demethylation process. A similar metabolite was isolated following the metabolism of chlorotoluron in post mitochondrial supernatant fractions prepared from the livers of japanese quail (Hinderer & Menzer, 1976a) although no significant metabolism could be detected

when liver fractions were prepared from rats (Hinderer & Menzer, 1976b). Monuron is known to form N-formyl derivatives upon photolysis (Crosby & Tang, 1969; Tanaka et al., 1977) and upon oxidation in chemical systems (Tanaka & Wien, 1979). However N-formyl metabolites of monuron were not isolated in-vitro or in-vivo in this study.

4.V Investigations of the metabolism of formamide derivatives

4.V.1 Introduction

The antitumour activity of N-methylformamide against murine tumours was first described in the early 1950s by Clarke et al. (1953) and Furst et al. (1955). In structure-activity studies of formamide derivatives on the S180 sarcoma and Ehrlich ascites murine tumour systems N-methylformamide was found to be the most potent inhibitor of tumour growth. Small changes in the molecular structure of N-methylformamide such as substitution of the N-methyl group with an N-ethyl group resulted in a significant decrease in antitumour activity. These observations led to a clinical trial of N-methylformamide in man (Myers et al., 1956). However, the hepatotoxicity exhibited by N-methylformamide in this clinical trial was considered to be an unacceptable side effect and interest in the clinical use of N-methylformamide was lost.

The mechanism whereby N-methylformamide exerts its antitumour activity is not known but there is evidence that it requires metabolism to produce a selective cytotoxic effect in-vivo (Gescher et al., 1982). It appears that N-methylformamide requires both the N-methyl moiety and bioactivation for antitumour activity. These requirements N-methylformamide has in common with hexamethylmelamine, procarbazine and aryldimethyltriazenes (sec.1.IV), which have all been shown to form characterizable N-hydroxymethyl compounds during metabolism in varying systems (sec. 1.III). As N-hydroxymethylformamide is stable in aqueous solution at physiological pH (4.II.2.a) it was one of the aims of this study to determine if this compound was a metabolite of

N-methylformamide. The metabolism of N-alkylformamides was also investigated in order to elucidate possible mechanisms of cytotoxicity and hepatotoxicity.

4.V.2 Results

4.V.2.a Development of chromatographic method

Fig. 4.31 shows a gas chromatogram of a mixture of N-methylformamide, formamide and N,N-dimethylacetamide (internal standard). The detection limits using this method were 2μg/ml for formamide and 0.5μg/ml for N-methylformamide and N-ethylformamide. Calibration curves obtained for N-methylformamide, N-ethylformamide and formamide had correlation coefficients > r = 0.9988.

The N-methylol derivatives of N-methylformamide and N,N-dimethylformamide decomposed upon G.C. analysis to produce formamide and N-methylformamide respectively. Derivatization of N-hydroxymethyl-N-methylformamide with BSTFA and TSIM was unsuccessful. Derivatization of N-hydroxymethylformamide with BSTFA, TSIM or HFAA resulted in at least three products in each case. Combined G.C.-M.S. analysis was performed on the products of the derivatization of N-hydroxymethylformamide with BSTFA. Five products were observed with the following molecular ions: $^{m}/z$ = 219, 219, 234, 234, and 321. The two products with $^{m}/z$ = 219 were presumably di-TMS derivatives of N-hydroxymethylformamide whereas the identity of the other three products was not known.

4.V.2.b In-vitro metabolism studies

The metabolism of N,N-dimethylformamide and N-methylformamide was investigated in-vitro using the Nash method after the addition of alkali to detect stable formaldehyde precursors (sec. 3.V.3.b), and the G.C. method described in sec. 3.V.

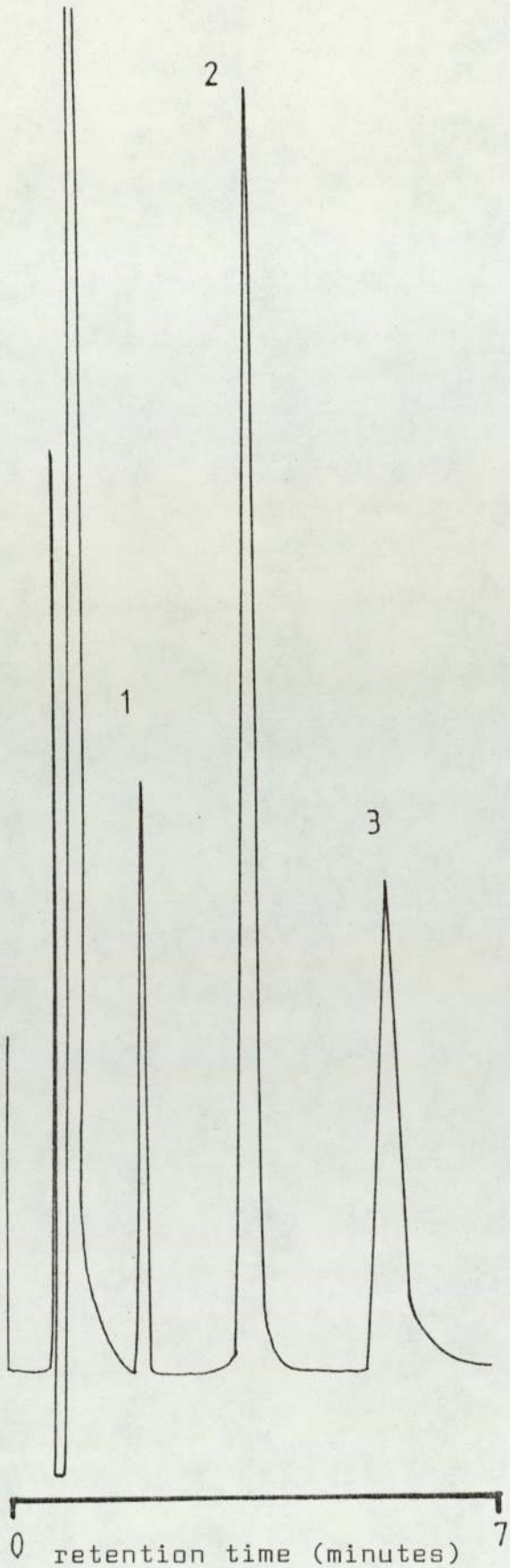


Fig. 4.31 Gas chromatogram of a mixture of the following reference compounds: 1, N,N-dimethylacetamide; 2, N-methylformamide; 3, formamide

Formaldehyde precursors equivalent to 60μM and 43μM formaldehyde were detected as products of the microsomal metabolism of N,N-dimethylformamide 6.5mM in two separate experiments. The spectra of the adducts obtained by reaction of the hydrolysis products of microsomal metabolites with Nash reagent are shown in Fig. 4.32. When the products of the microsomal metabolism of N,N-dimethylformamide were analyzed by G.C, N-methylformamide was detected at a concentration of 65μM in the incubation mixture.

Metabolism of N-methylformamide to formaldehyde precursors could not be detected in either whole liver homogenate or 9000g supernatant and microsomal fractions of liver. No appearance of formamide or disappearance of substrate could be detected by G.C.analysis when N-methylformamide was incubated at concentrations of 5mM and 200μM respectively with hepatocytes, whole liver homogenate and 9000g supernatant and microsomal fractions of liver. There was also no metabolism of N-methylformamide, as measured by formamide appearance and N-methylformamide disappearance, during incubation with microsomes under an atmosphere of 95% O_2/5% CO_2.

4.V.2.c In-vivo metabolism studies

Recoveries of standard compounds from plasma and urine as measured by G.C.analysis are shown in Table 4.7.

Fig. 4.33 shows the plasma concentration versus time profile of N-methylformamide in mice which had received N-methylformamide, as measured by G.C.. The plasma elimination

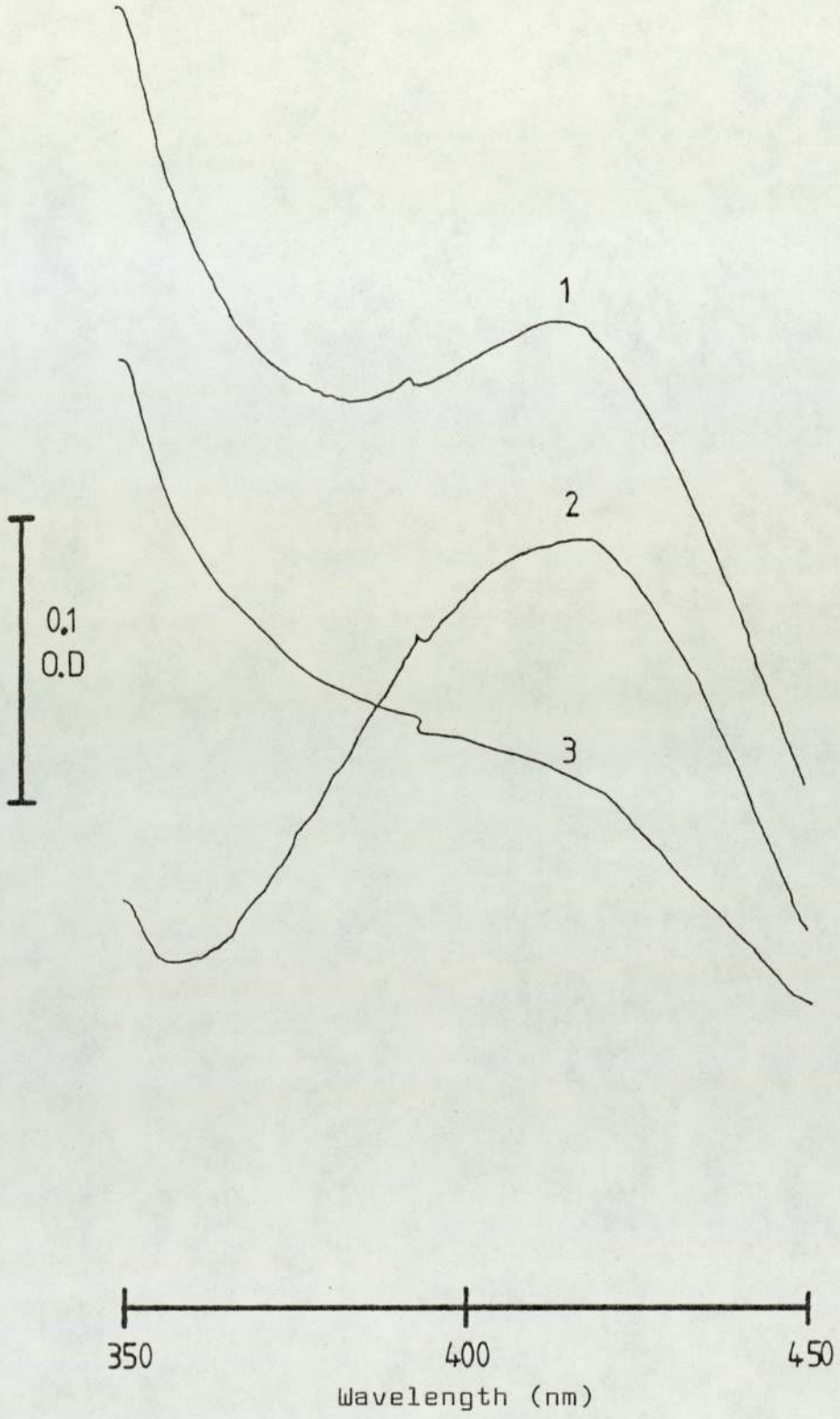


Fig. 4.32 U.V. spectra of the adducts produced by reaction of Nash reagent with formaldehyde 60μM (2), and the products of the microsomal metabolism of N,N-dimethylformamide before (3) and after (1) alkaline hydrolysis.

	N-methylformamide	Formamide	N-ethylformamide
% recovery of compound from plasma	106 ± 10	109 ± 12	101 ± 6
% recovery of compound from urine	98 ± 7	101 ± 8	112 ± 10

Table 4.7 Recoveries of standard compounds from plasma and urine

Each value represents the mean ± s.d.of three determinations

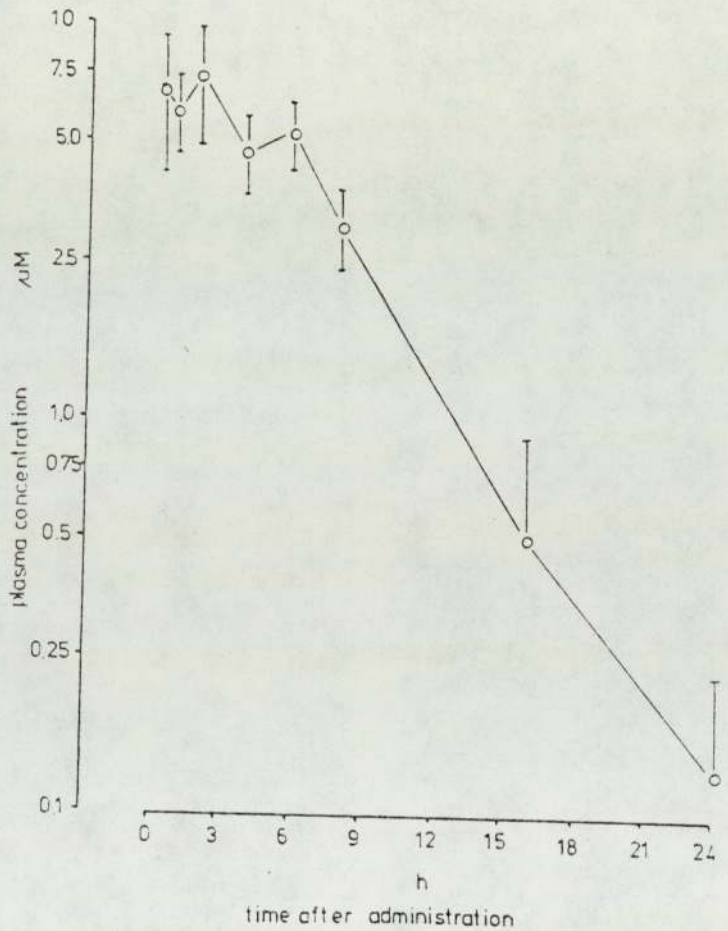


Fig. 4.33 Disappearance of N-methylformamide from mouse plasma after the administration of N-methylformamide 400mg/Kg i.p.

Each point represents the mean ± s.d. of determinations from four animals.

half-life assuming first order kinetics was calculated as 3.6h ($r = -0.9124$). The area under the plasma concentration versus time curve was 62umol.h/ml.

The excretion of unchanged drug and formamide in the urine of mice which had received N-methylformamide and N-ethylformamide, as measured by G.C.analysis, is shown in Fig. 4.34. When the urine of mice which had received N-methylformamide was subjected to enzymatic deconjugation no increases in the amounts of N-methylformamide or formamide in the urine were observed.

Formaldehyde precursors were excreted in the urine of mice which had received either N-methylformamide or N,N-dimethylformamide. The quantities excreted, expressed as both formaldehyde equivalents and as percentage of dose administered are shown in Table 4.8. The spectra of the adducts produced by reaction of Nash reagent with the products of the alkaline hydrolysis of the urine of mice which had received N-methylformamide and N,N-dimethylformamide are shown in Figs. 4.35 and 4.36 respectively. Formaldehyde precursors were not detected in the urine of mice which had received N-ethylformamide or vehicle only, or in the plasma of mice which had received any N-alkylformamide.

Formaldehyde precursors were also observed in the urine of a patient who had received a dose of $2.4g/m^2$ orally and the results are summarized in Table 4.9. The spectra obtained by reaction of these urine samples with Nash reagent after the addition of base are shown in Fig. 4.37.

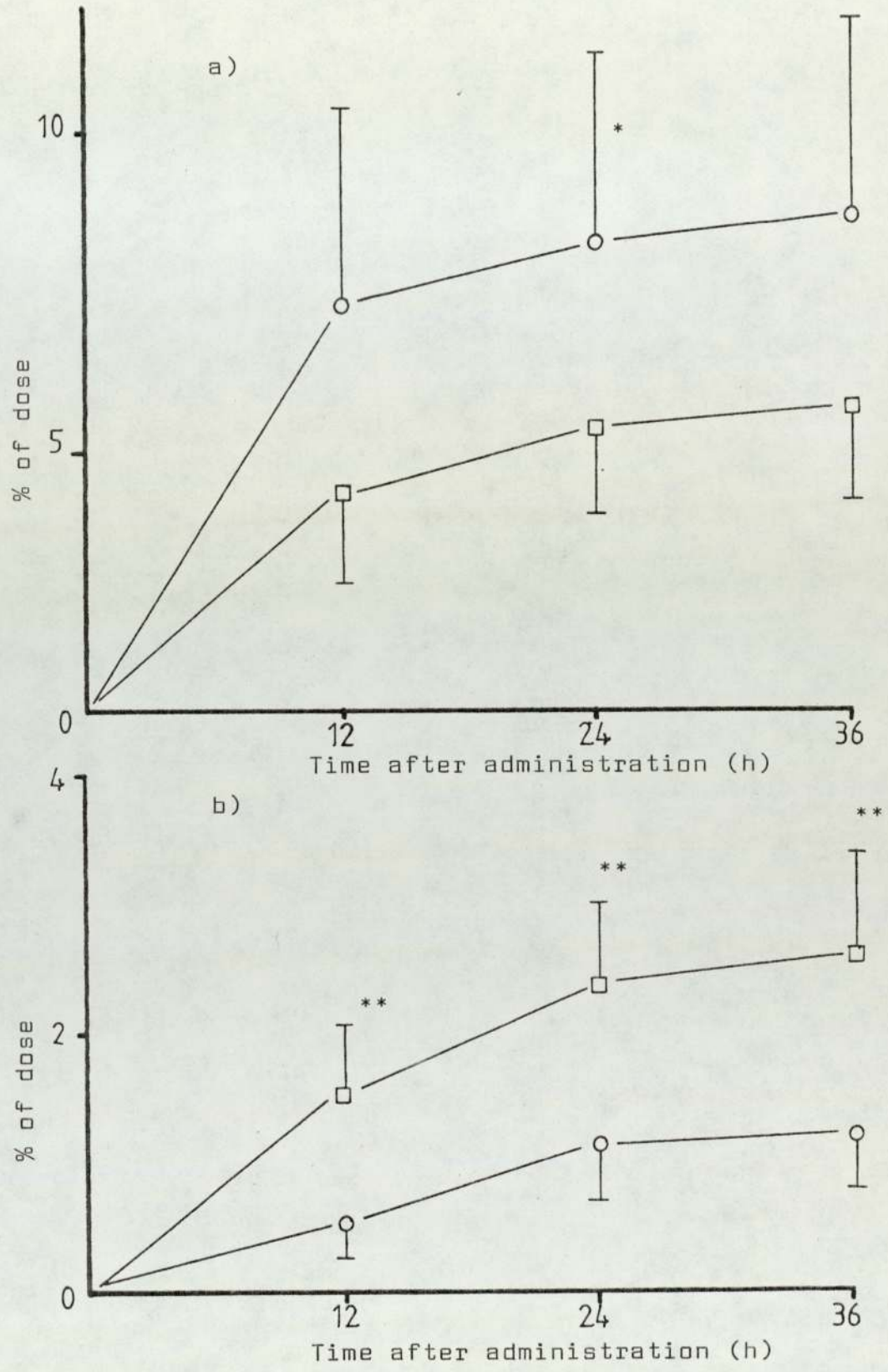


Fig. 4.34 Cumulative urinary excretion of unchanged drug (a) and formamide (b) as measured by G.C, after the administration of N-methylformamide 400mg/Kg i.p. (○) or N-ethylformamide 495mg/Kg i.p. (□) to mice. Each value represents the mean ± s.d. of six experiments.

* = $P < 0.05$
** = $P < 0.01$

Compound administered	Time period of urine collection (h after administration	Formaldehyde precursors in urine (μmoles)	% of dose excreted in urine as formaldehyde precursors
N-methylformamide	0-12	2.4	1.8
	12-24	1.1	0.8
	24-36	0.9	0.7
	TOTAL	4.4	3.3
N,N-dimethylformamide	0-12	35.5	26.1
	12-24	13.7	10.1
	24-36	0.4	0.3
	TOTAL	49.6	36.5

Table 4.8 Quantities of formaldehyde precursors liberated upon the alkaline hydrolysis of the urine of mice which had received N,N-dimethylformamide 495mg/Kg i.p., or N-methylformamide 400mg/Kg i.p. Values are the results of one experiment, typical of two.

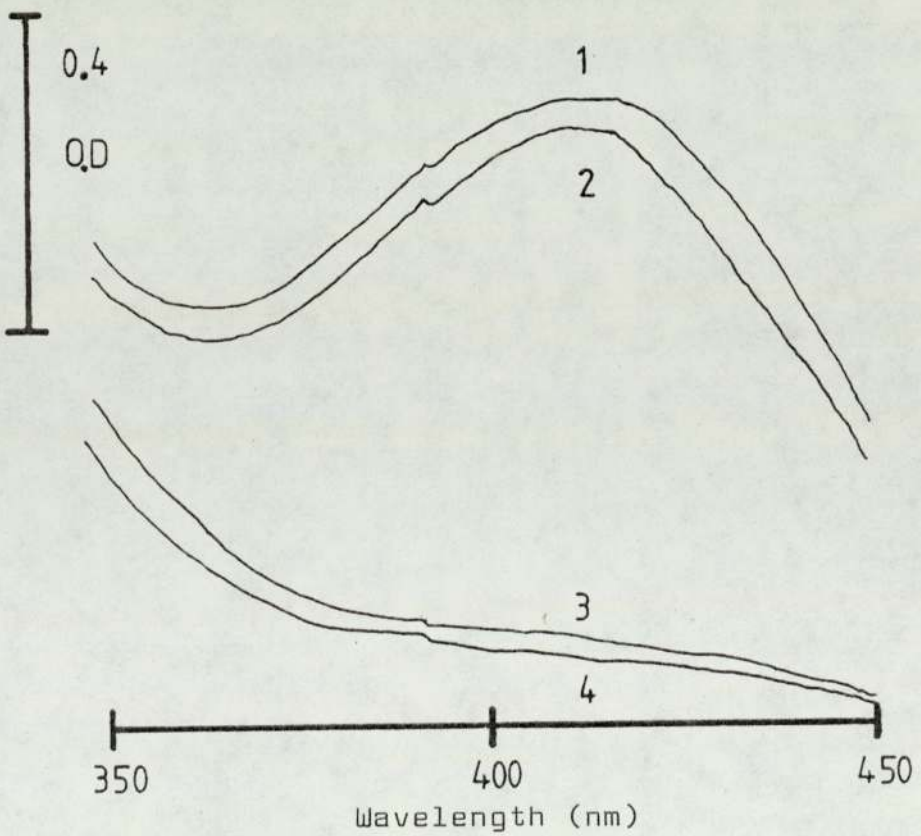


Fig. 4.35 U.V. spectra of the adducts produced by reaction of Nash reagent with formaldehyde 150μM (2), the urine of mice which had received N-methylformamide 400mg/Kg i.p. before (3) and after (1) alkaline hydrolysis, and the urine of mice which had received vehicle after alkaline hydrolysis (4).

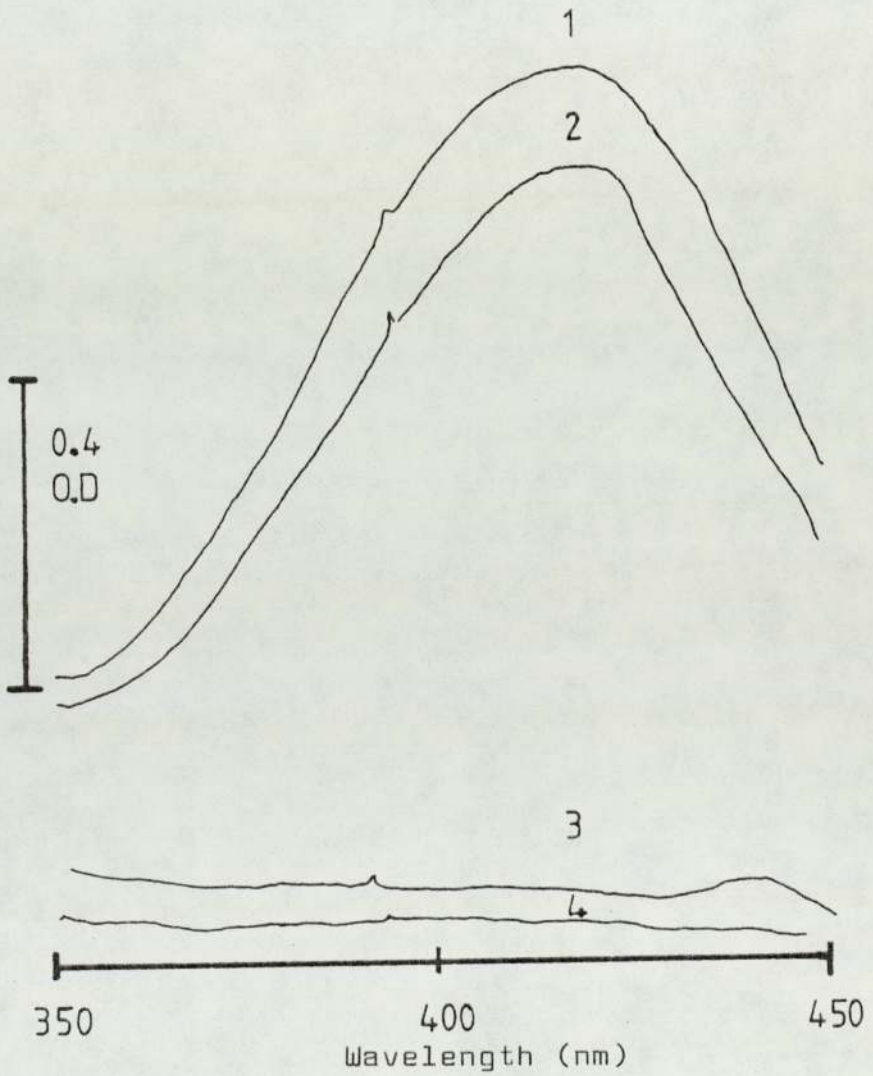


Fig. 4.36 U.V. spectra of the adducts produced by reaction of Nash reagent with formaldehyde 200µM (2), the urine of mice which had received N,N-dimethylformamide before (4) and after (1) alkaline hydrolysis, and the urine of mice which had received vehicle after alkaline hydrolysis (3)

Time period of urine collection (h after administration)	Formaldehyde precursors excreted in urine (μmoles)	% of dose administered excreted in urine as formaldehyde precursors
0-12	5.5	0.01
12-18	59.6	0.09
18-24	20.0	0.03
24-30	18.4	0.03
30-36	10.3	0.02
Total	113.8	0.18

Table 4.9 Amount of formaldehyde precursors liberated upon alkaline hydrolysis of the urine of a patient (Hoiberg) who had received N-methylformamide 2.4 g/m^2 p.o.

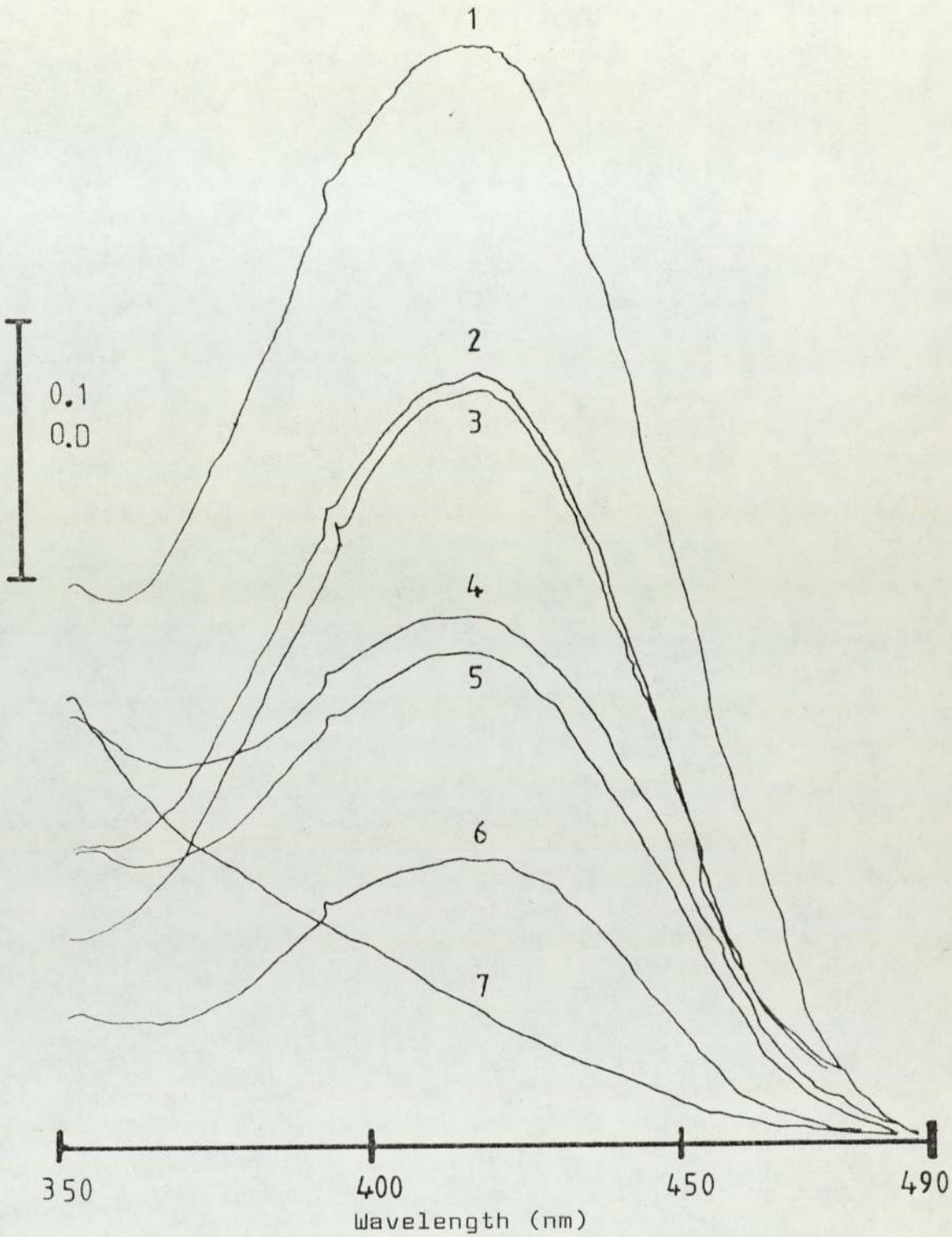


Fig. 4.37 U.V. spectra of the adducts produced by reaction of Nash reagent with formaldehyde 100μM (1), and the alkaline hydrolysis products of the urine of a patient before (7), 0-12h (2), 12-18h (3), 18-24h (4), 24-30h (5) and 30-36h (6) after, the administration of N-methylformamide 2.4g/m^2 p.o.

The quantities of formaldehyde precursors found in the urine of mice after the administration of N-alkylformamides were greater than the quantity that would be expected from the results of the G.C. analysis of the metabolites present in urine. This was not the case in man and the results are summarized in Table 4.10. These comparisons are based on the assumptions that one molecule of formaldehyde is produced from one molecule of formaldehyde precursor, and that one molecule of N-methylformamide or N,N-dimethylformamide both form one molecule of formaldehyde precursor upon oxidative metabolism.

Compound/Species	Time period of urine collected (h) after administration	Conc^{n}. of ≠ formaldehyde precursors in urine (mM)	Conc^{n}. of N-methyl ≠ formamide (N) or formamide (F) in urine (mM)
N,N-dimethylformamide /mice	0-12	71.1	*N - 41.2
	12-24	39.7	22.5
	24-36	3.2	3.0
N-methylformamide /mice	0-12	4.2	F - 0.8
	12-24	13.0	6.8
	24-36	2.8	0.3
N-methylformamide /man	0-12	0.03	*F - 0.17
	12-18	0.08	0.19
	18-24	0.08	0.27
	24-30	0.05	0.28
	30-36	0.05	0.17

≠ - measured using the Nash assay

≠ - measured using G.C.

* - from Brindley (1982)

Table 4.10 Concentrations of urinary metabolites of N-alkylformamides in mice and man, measured using the Nash reaction and G.C.

4.V.3 Discussion

Formamide and its derivatives such as N-methylformamide do not possess a chromophore which absorbs at wavelengths greater than 200nm. Thus analytical methods which utilize U.V detection systems cannot be used to quantify these compounds. The most suitable method to study the metabolism of formamide derivatives was G.C.. However, N-hydroxymethylformamide and N-hydroxymethyl-N-methylformamide degraded upon G.C. analysis to produce formamide and N-methylformamide respectively. Derivatization of these N-methylols to stable volatile species was attempted but was not successful for N-hydroxymethyl-N-methylformamide, whilst the derivatization of N-hydroxymethylformamide resulted in more than one product (4.V.2.a). Therefore the detection and quantitation of N-hydroxymethyl compounds by derivatization and subsequent G.C analysis was not possible.

An alternative method used for the detection of N-hydroxymethylformamides had its origins in the work described in sec. 4.II. N-hydroxymethylformamides did not produce Nash positive species unless alkaline hydrolysis was performed prior to reaction with Nash reagent. This method could therefore be used to detect formaldehyde precursors, presumably N-hydroxymethyl derivatives, formed upon metabolism of N-methyl substituted formamides.

Using this colourimetric method N,N-dimethylformamide was shown to undergo metabolism to a formaldehyde precursor in microsomal fractions in-vitro. In separate incubations under identical conditions N-methylformamide was detected as a

metabolite of N,N-dimethylformamide as measured by G.C. analysis. N-hydroxymethylformamide was however unstable on G.C. analysis (sec. 4.V.2.a) and as the quantities of formaldehyde precursors and N-methylformamide measured by the two methods were similar (sec. 4.V.2.b), it is probable that the N-methylformamide measured by G.C. arose from degradation of N-hydroxymethyl-N-methylformamide.

N-methylformamide was not metabolized in-vitro to formaldehyde precursors as measured by the colourimetric method, to formamide as measured by G.C., or as measured by substrate removal using G.C., in liver fractions and hepatocytes (sec. 4.V.2.b). These observations contradict those of Barnes & Ranta (1972) who reported 10% biotransformation of N-methylformamide after incubation with a rat liver homogenate for two hours. The amount of formaldehyde produced in this study was however close to the detection limit of the Nash assay, and comparable experiments using mouse liver preparations showed that incubation for such long periods of time leads to significant control absorbances. Barnes & Ranta (1972) also reported that N-methylformamide was N-demethylated to a greater extent than N,N-dimethylformamide in-vitro. The results of the investigation performed as a part of this thesis led to the opposite conclusion (sec. 4.V.2.b). This contradiction may be explained by species differences in the metabolism of N-methylamides. Alternatively the validity of the results of Barns & Ranta may be questioned on the basis of the small amount of formaldehyde found.

N-methylformamide was found to have a plasma elimination half-life of 3.6 hours in mice when plasma samples were obtained from a number of mice by cardiac puncture and assuming first order kinetics. Subsequent studies (Brindley et al., 1982b) using repeated sampling from the same animal have shown that the plasma concentration versus time curves obtained after injection of N-methylformamide to mice exhibit a concave downward curvature and do not fit models based on first order elimination kinetics. The fact that a more complex pharmacokinetic profile was observed in the study of Brindley et al. (1982b) may reflect the smaller standard errors involved in a single animal rather than a multi-animal study. Alternatively it has been shown that varying pharmacokinetic profiles of the same drug may be observed if different sampling techniques are used (Bazare Jnr. et al., 1981). It is therefore conceivable that the differences between the plasma elimination kinetics of N-methylformamide observed in this study and those observed by Brindley et al. (1982b) may be explained by the use of different sampling methods.

Formamide, as measured by G.C., was identified as a urinary metabolite of both N-methylformamide and N-ethylformamide. The formamide detected as a metabolite of N-methylformamide may have arisen in part or completely from the thermal degradation of N-hydroxymethylformamide on the G.C. column. It remains a possibility that the formamide detected as a urinary metabolite of N-ethylformamide may

also have arisen from the decomposition of an N-hydroxyalkyl precursor. That the formamide found in the urine after the administration of N-methylformamide to mice was formed from the degradation of an N-methylol was supported by the finding that formaldehyde precursors were also present in the same urine samples (Table 4.8). Such precursors were also identified as urinary metabolites of N,N-dimethylformamide in mice, where they represented a much higher percentage of the dose (Table 4.8), and of N-methylformamide in man (Table 4.9). The quantities of formaldehyde precursors found in the urine of mice after the injection of N-methylformamide and N,N-dimethylformamide were consistently higher than those expected on the basis of the G.C.analysis of the N-desmethyl species in the urine (Table 4.10). This observation suggests that formaldehyde precursors other than N-hydroxymethyl compounds, which are not detectable by G.C.analysis, may have been present in the urine of mice which had received N-methyl substituted formamides. The amount of formamide measured by G.C in the urine of one patient who had received N-methylformamide was greater than the quantity of formaldehyde precursors measured in the same urine sample (Table 4.10). This suggests that formamide, as well as N-hydroxymethylformamide, may have been a urinary metabolite of N-methylformamide in this patient.

Increases in the amount of formamide and N-methylformamide after enzymatic deconjugation of the urine samples of mice which had received N-methylformamide were not observed. Therefore conjugates of

N-hydroxymethylformamide, formamide or N-methylformamide were either not present in the urine, were not substrates for these enzymes, or degraded to produce N-methylformamide and formamide on the G.C.column.

One of the aims of this study was to investigate a possible metabolic basis for the in-vivo antitumour activity and hepatotoxicity of N-methylformamide. Some cytotoxic and most hepatotoxic drugs have been shown to exert toxicity via reactive metabolites (Connors, 1976; Gillette, 1979). By analogy one could argue that metabolites of N-methylformamide may be responsible for its toxic activity. Both N-methylformamide and the weakly antineoplastic N-ethylformamide were metabolized in mice to what was analytically detected as formamide (Fig. 4.34). Formamide itself possesses only marginal antitumour activity against a number of murine tumour systems - the TLX5 lymphoma, the sarcoma 180 and the M5076 ovarian sarcoma - (Gescher et al., 1982). The amount of formamide in the urine of mice which had received N-methylformamide was significantly different to that found in the urine of mice which had received an equimolar dose of N-ethylformamide (Fig. 4.34). However as the quantity of formamide excreted in both cases represents less than 2.5% of the dose administered, it is considered unlikely that the difference in antitumour activity between N-methylformamide and N-ethylformamide is attributable to the metabolic production of formamide

If the formamide measured in the urine of mice which had received N-alkylformamides was due to the degradation of N-hydroxyalkyl compounds then it is conceivable

that the N-hydroxymethyl metabolite derived from N-methylformamide may have exerted cytotoxic activity which the N-hydroxyethyl metabolite derived from N-ethylformamide could not. It could be argued that if the generation of formaldehyde precursors was important for antitumour activity then N,N-dimethylformamide, which produces much greater amounts of formaldehyde precursors during metabolism in-vivo than N-methylformamide, would be an effective antitumour agent. But, it is not (Gescher et al., 1982). Alternatively the antitumour activity associated with N-methylformamide may be specifically due to the formation of N-hydroxymethylformamide and other formaldehyde precursors such as N-hydroxymethyl-N-methylformamide may not exert such activity. Recent results (Cooksey et al., 1982) have suggested that the formation of N-hydroxymethylformamide during the metabolism of N-methylformamide represents a deactivation pathway with respect to antitumour activity.

The mechanism of hepatoxicity of N-methylformamide has not yet been elucidated. It has been observed however that both N-hydroxymethylformamide and formamide do not deplete hepatic glutathione whereas N-methylformamide does (Cooksey et al., 1982; Gescher et al., 1982). This suggests that the formation of N-hydroxymethylformamide and formamide during the metabolism of N-methylformamide may not be relevant to the hepatotoxic effect exhibited by the latter compound.

4.VI Investigations of the metabolism of benzamide derivatives

4.VI.1 Introduction

The N-hydroxymethyl derivatives of benzamide, 4-chlorobenzamide and 4-t-butylbenzamide are relatively stable compounds which degrade to produce formaldehyde with half-lives of greater than twelve hours (sec. 4.II.2.b). It was therefore of interest to determine if these N-methylols were produced during the metabolism of their respective parent N-methylamides and to investigate whether such stable N-methylols could undergo further biotransformation. In addition the metabolism of N,N-dimethylbenzamide and its 4-chloro derivative was studied in order to determine if N-methylols were produced during the metabolism of these N,N-dimethyl substituted benzamides.

4.VI.2 Results

4.VI.2.a Development of chromatographic methods

Fig. 4.38 shows a high pressure liquid chromatogram of a mixture of N-methylbenzamide, N-hydroxymethylbenzamide, benzamide, 4-nitrobenzamide (internal standard) and N-formylbenzamide. High pressure liquid chromatograms of mixtures of 4-chloro and 4-t-butyl substituted reference compounds are shown in Figs. 4.39 and 4.40 respectively. Calibration curves with correlation coefficients greater than r = 0.9986 were obtained for all standard compounds using these H.P.L.C. methods. The correlation coefficient of the calibration curve for benzoic acid was 0.9980. The standard deviation of five determinations of the amount of a reference compound in a sample was less than 5% of the mean for all standard compounds. The limit of detection calculated for N-methylbenzamide was 1µg/ml.

4.VI.2.b In-vitro metabolism studies

The in-vitro metabolism of N-methylbenzamide N,N-dimethylbenzamide, and 4-chloro-N,N-dimethylbenzamide by microsomal fractions of liver was investigated using the Nash colourimetric assay, modified by the use of sodium hydroxide as described in sec. 3.V.3.b. The amount of formaldehyde equivalents detected during the microsomal metabolism of the latter three substrates before and after alkaline hydrolysis is shown in Table 4.11.

The in-vitro metabolism of various N-methyl substituted benzamides and N-hydroxymethylbenzamide was also investigated using the H.P.L.C.methods previously

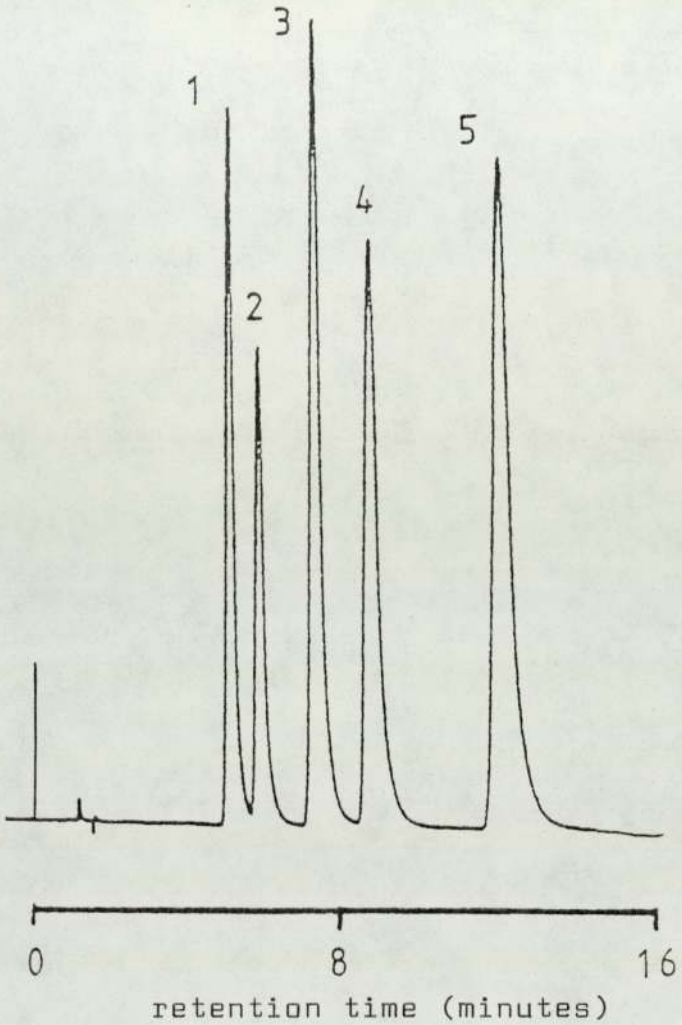


Fig. 4.38 High pressure liquid chromatogram of a mixture of the following reference compounds: 1, N-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 4, N-methylbenzamide; 5, N-formylbenzamide.

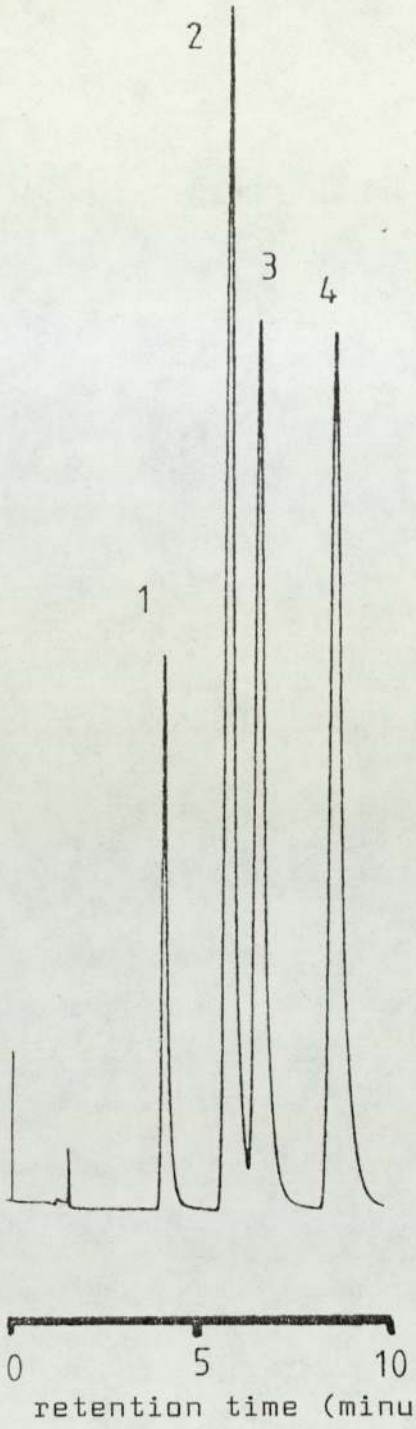


Fig. 4.39 High pressure liquid chromatogram of a mixture of the following reference compounds: 1, internal standard; 2, 4-chloro-N-hydroxymethylbenzamide; 3, 4-chlorobenzamide; 4, 4-chloro-N-methylbenzamide.

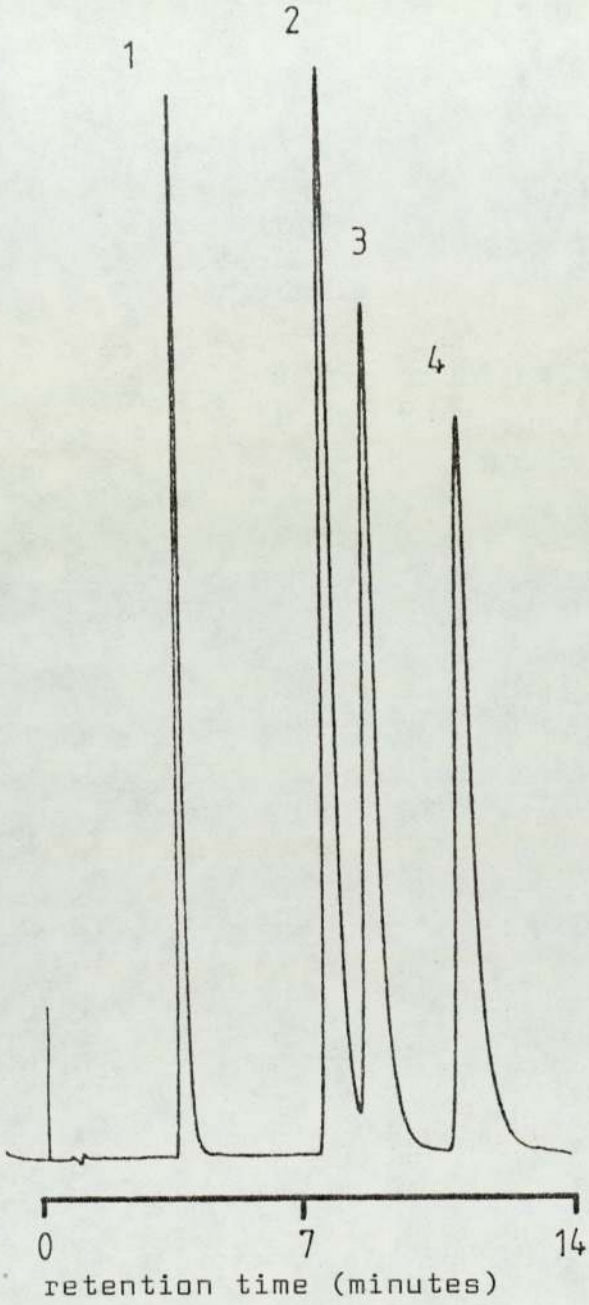


Fig. 4.40 High pressure liquid chromatogram of a mixture of the following reference compounds: 1, internal standard; 2, N-hydroxymethyl-4-t-butylbenzamide; 3, 4-t-butylbenzamide; 4, N-methyl-4-t-butylbenzamide.

Substrate for microsomal metabolism	Formaldehyde equivalents measured before alkaline hydrolysis (μM)	Formaldehyde equivalents measured after alkaline hydrolysis (μM)
N,N-dimethylbenzamide (5mM)	195	349
4-chloro-N,N-dimethyl-benzamide (5mM)	319	414
N-methylbenzamide (10mM)	0	90

Table 4.11 Formaldehyde equivalents generated during the microsomal metabolism of N,N-dimethylbenzamide (5mM) 4-chloro-N,N-dimethylbenzamide (5mM) and N-methylbenzamide (10mM) measured using the Nash reaction before and after alkaline hydrolysis.

described (sec. 3.VI.1). The recoveries of standard compounds from incubation mixtures is shown in Table 4.12.

Fig. 4.41 shows a chromatogram of an extract of the products of the microsomal metabolism of N-methylbenzamide. A metabolite was produced which had an identical retention time to that of authentic N-hydroxymethylbenzamide (1, Fig. 4.41). The production of this metabolite was dependent upon the presence of oxygen and NADPH. Upon treatment with 1N NaOH this metabolite degraded irreversibly with a corresponding increase in the amount of benzamide present in the mixture (Table 4.13). The amount of benzamide liberated upon alkaline hydrolysis of the extract was in agreement with the amount of Nash positive species produced (Table 4.13). The chemical ionization mass spectrum of this unstable metabolite was identical with that of authentic N-hydroxymethylbenzamide (Fig. 4.42), but a molecular ion was not observed for either the reference compound or the isolated metabolite. The peak in the spectrum at $^{m}/z = 134$ (Fig. 4.42) is presumably due to a fragment produced by the dehydration of N-hydroxymethylbenzamide. That the unstable metabolite was N-hydroxymethylbenzamide was confirmed by combined G.C.- M.S.of the metabolite and authentic N-hydroxymethylbenzamide, after derivatization with BSTFA (Fig. 4.43). The peak in the spectrum at $^{m}/z = 223$ corresponds to a mono-trimethylsilyl derivative of N-hydroxymethylbenzamide. Di-trimethylsilyl derivatives ($^{m}/z = 295$) of both authentic standard and metabolite were also obtained (not shown).

Compound	% recovery from incubation mixtures
N,N-dimethylbenzamide	90 ± 8
N-methylbenzamide	87 ± 12
N-hydroxymethylbenzamide	88 ± 8
benzamide	89 ± 7
4-nitrobenzamide	90 ± 8
4-chloro-N-methylbenzamide	92 ± 2
4-chloro-N-hydroxymethylbenzamide	96 ± 3
4-chlorobenzamide	96 ± 4
N-methyl-4-t-butylbenzamide	96 ± 4
N-hydroxymethyl-4-t-butylbenzamide	98 ± 4
4-t-butylbenzamide	98 ± 5

Table 4.12 Recoveries of standard compounds from incubation mixtures

Results shown are the mean ± s.d of at least three determinations

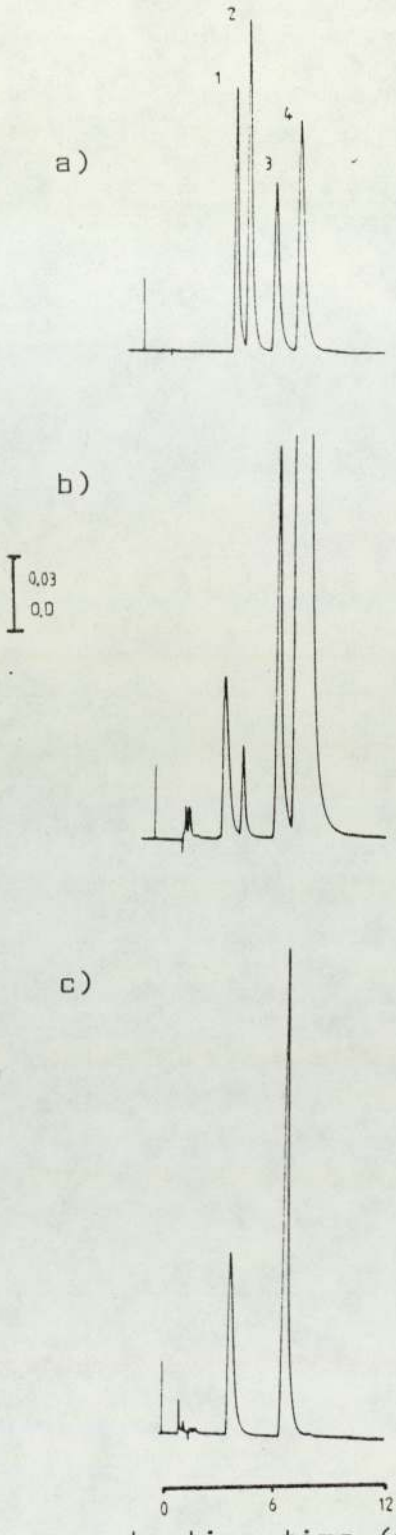


Fig. 4.41 High pressure liquid chromatograms of extracts of a) a mixture of the following reference compounds: 1, N-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 4, N-methylbenzamide; b) a mixture of N-methylbenzamide (10mM) with microsomes and an NADPH generating system incubated for 30 min. at 37°C, c) an incubation mixture omitting N-methylbenzamide.

	Concentration of N-hydroxymethylbenzamide in incubation mixture measured by H.P.L.C. (μM)	Concentration of benzamide in incubation mixture measured by H.P.L.C. (μM)	Concentration of formaldehyde equivalents in incubation mixture measured by the Nash assay (μM)
Before alkaline hydrolysis	70	0	0
After alkaline hydrolysis	0	72	85

Table 4.13 Products of the microsomal metabolism of N-methylbenzamide measured by H.P.L.C. and by the Nash assay before and after alkaline hydrolysis.

The results shown are from one experiment but are typical of three.

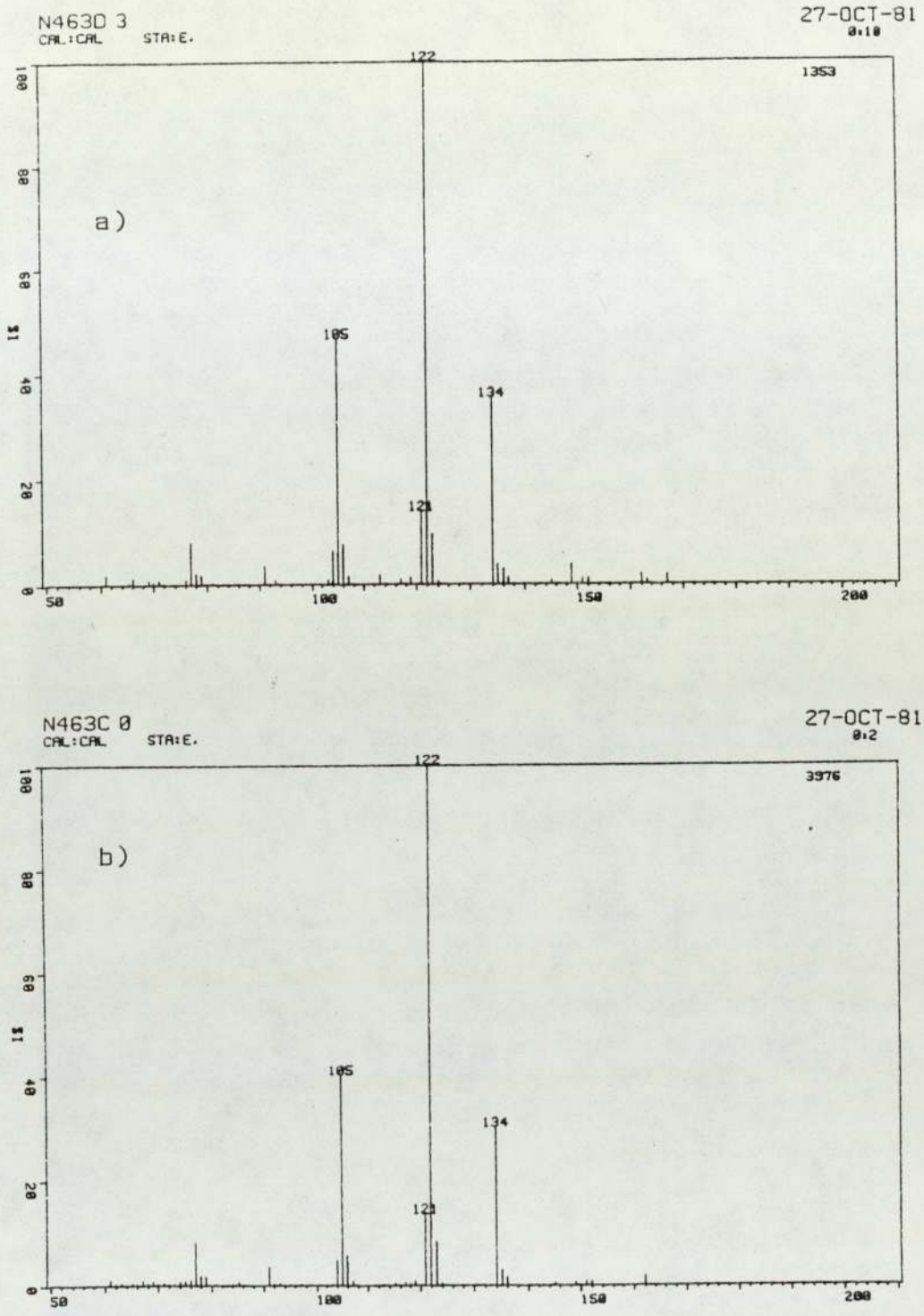


Fig. 4.42 Chemical ionization mass spectra of authentic N-hydroxymethylbenzamide (a) and an in-vitro metabolite of N-methylbenzamide with an identical retention time(b).

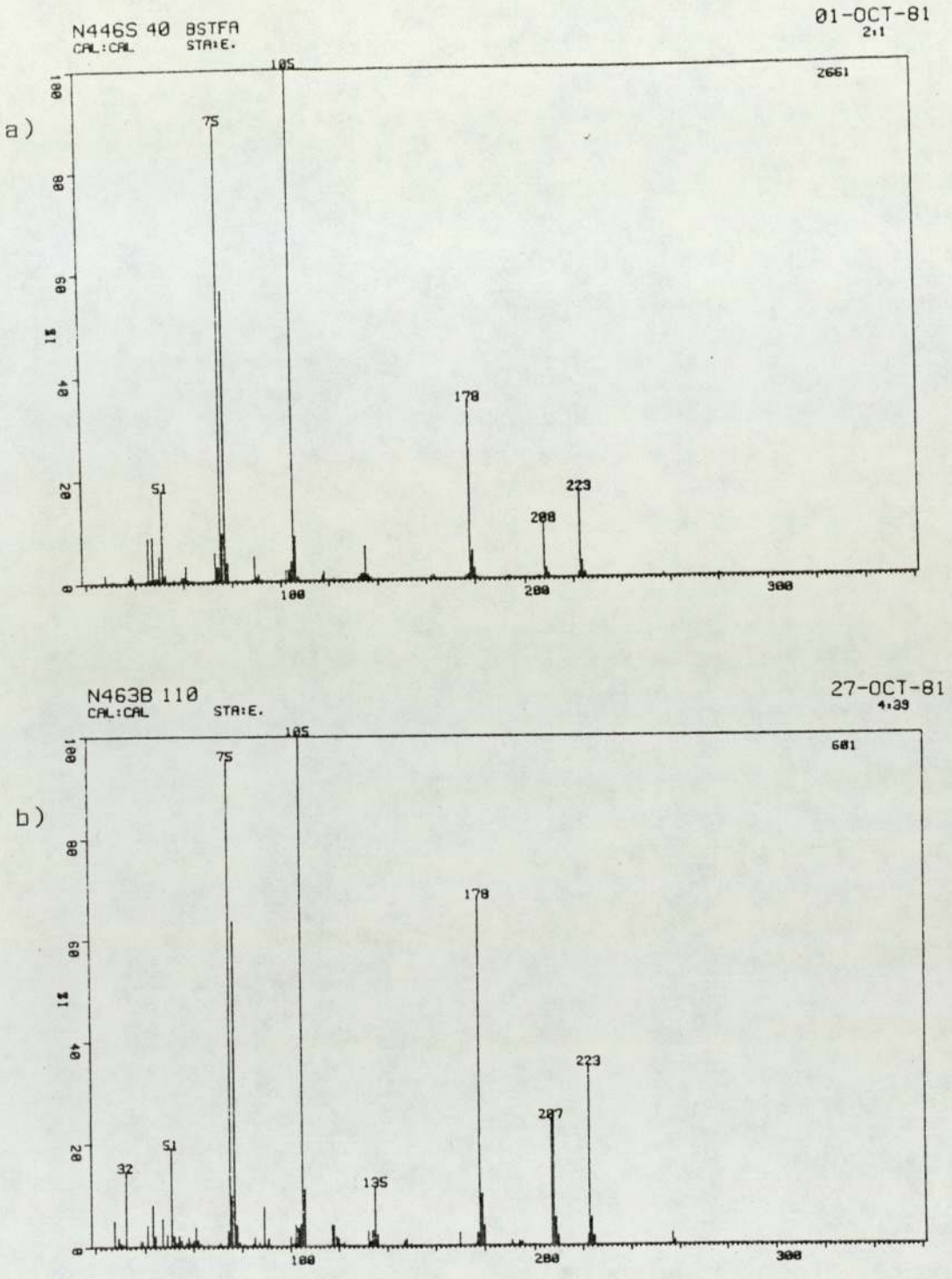


Fig. 4.43 Mass spectra of a peak (t_R = 8.8 min.) produced during the combined G.C-M.S analysis of authentic N-hydroxymethylbenzamide (a), and an in-vitro metabolite of N-methylbenzamide with an identical retention time on H.P.L.C (b), after derivatization with BSTFA.

Chromatograms of extracts of the products of microsomal metabolism of 4-chloro-N-methylbenzamide and N-methyl-4-t-butylbenzamide are shown in Figs. 4.44 and 4.45 respectively. Both substrates formed metabolites with identical retention times to their corresponding N-hydroxymethyl reference compounds. These metabolites decomposed upon alkaline hydrolysis to yield corresponding amounts of the appropriate amide. The chemical ionization mass spectrum of the proposed N-hydroxymethyl metabolite of 4-chloro-N-methylbenzamide (2, Fig. 4.44) was identical to that of authentic 4-chloro-N-hydroxymethylbenzamide (Fig. 4.46). Derivatization of this metabolite with BSTFA however was unsuccessful. An unidentified polar metabolite (5, Fig. 4.45) and metabolites with identical retention times to N-hydroxymethyl-4-t-butylbenzamide (2, Fig. 4.45) and 4-t-butylbenzamide (3, Fig. 4.45) were formed during the microsomal metabolism of 4-t-butyl-N-methylbenzamide.

Similar results using all three substrates were obtained using 9000g supernatant fractions of liver. Table 4.14 summarizes the extent of N-demethylation of N-methylbenzamide and its 4-substituted derivatives in microsomal and 9000g supernatant fractions of liver.

The microsomal metabolism of N,N-dimethylbenzamide produced a metabolite with an identical retention time to N-methylbenzamide (3, Fig. 4.47) and another metabolite (1, Fig. 4.47). The identity of metabolite 3 (Fig. 4.47) was confirmed as N-methylbenzamide by M.S.. Metabolite 1 (Fig. 4.47) decomposed upon alkaline hydrolysis and an increase in the amount of N-methylbenzamide was observed.

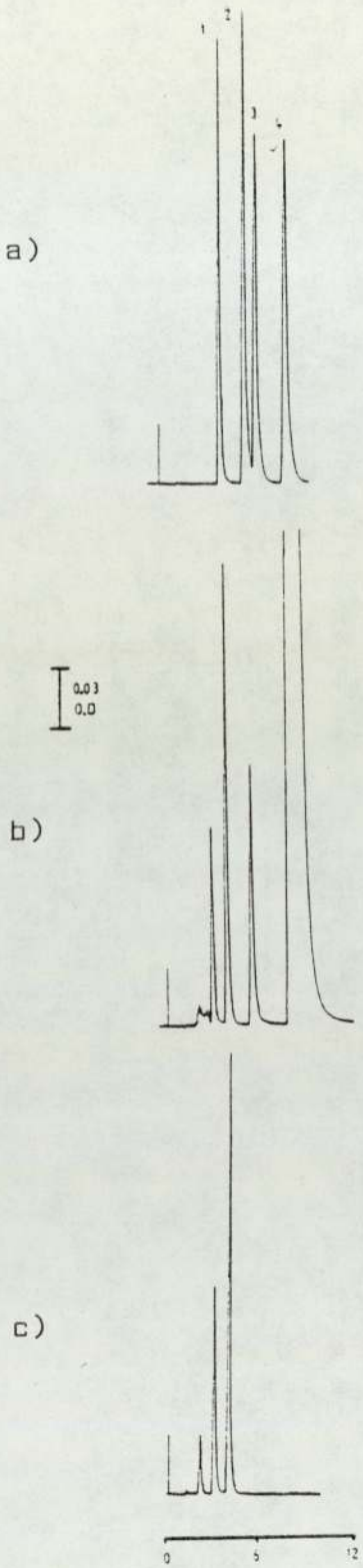


Fig. 4.44 High pressure liquid chromatograms of extracts of a) a mixture of the following reference compounds: 1, internal standard; 2, 4-chloro-N-hydroxymethylbenzamide; 3, 4-chlorobenzamide; 4, 4-chloro-N-methylbenzamide, b) a mixture of 4-chloro-N-methylbenzamide (10mM) with microsomes and an NADPH generating system incubated for 30 min. at 37°C, c) an incubation mixture omitting 4-chloro-N-methylbenzamide.

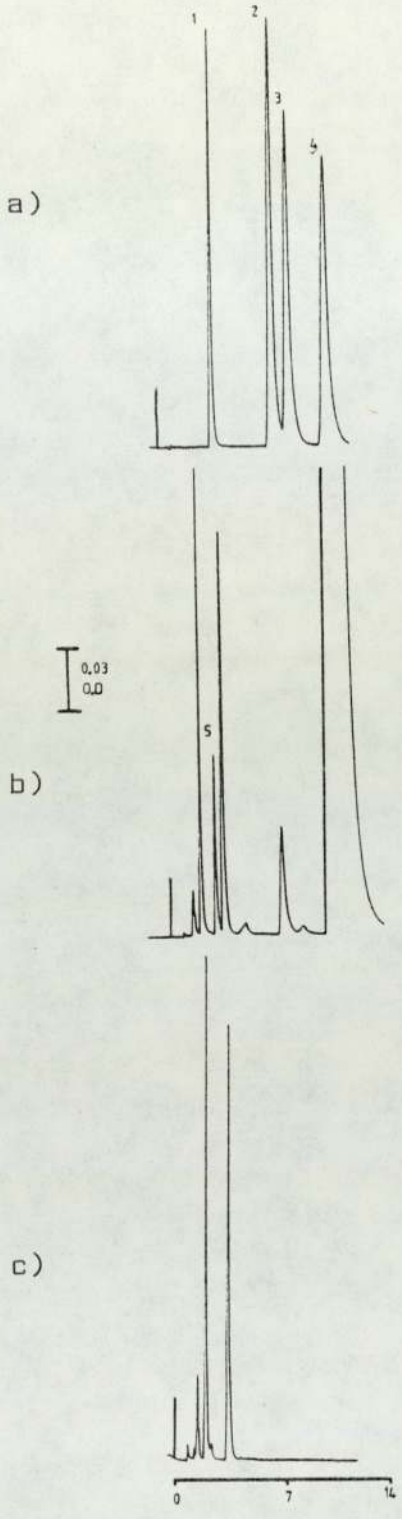


Fig. 4.45 High pressure liquid chromatograms of extracts of a) a mixture of the following reference compounds: 1, internal standard; 2, N-hydroxymethyl-4-t-butylbenzamide; 3, 4-t-butylbenzamide; 4, N-methyl-4-t-butylbenzamide, b) a mixture of N-methyl-4-t-butylbenzamide (10mM) with microsomes and an NADPH generating system incubated for 30 min. at 37°C; 5, is an unidentified metabolite, c) an incubation mixture omitting N-methyl-4-t-butylbenzamide.

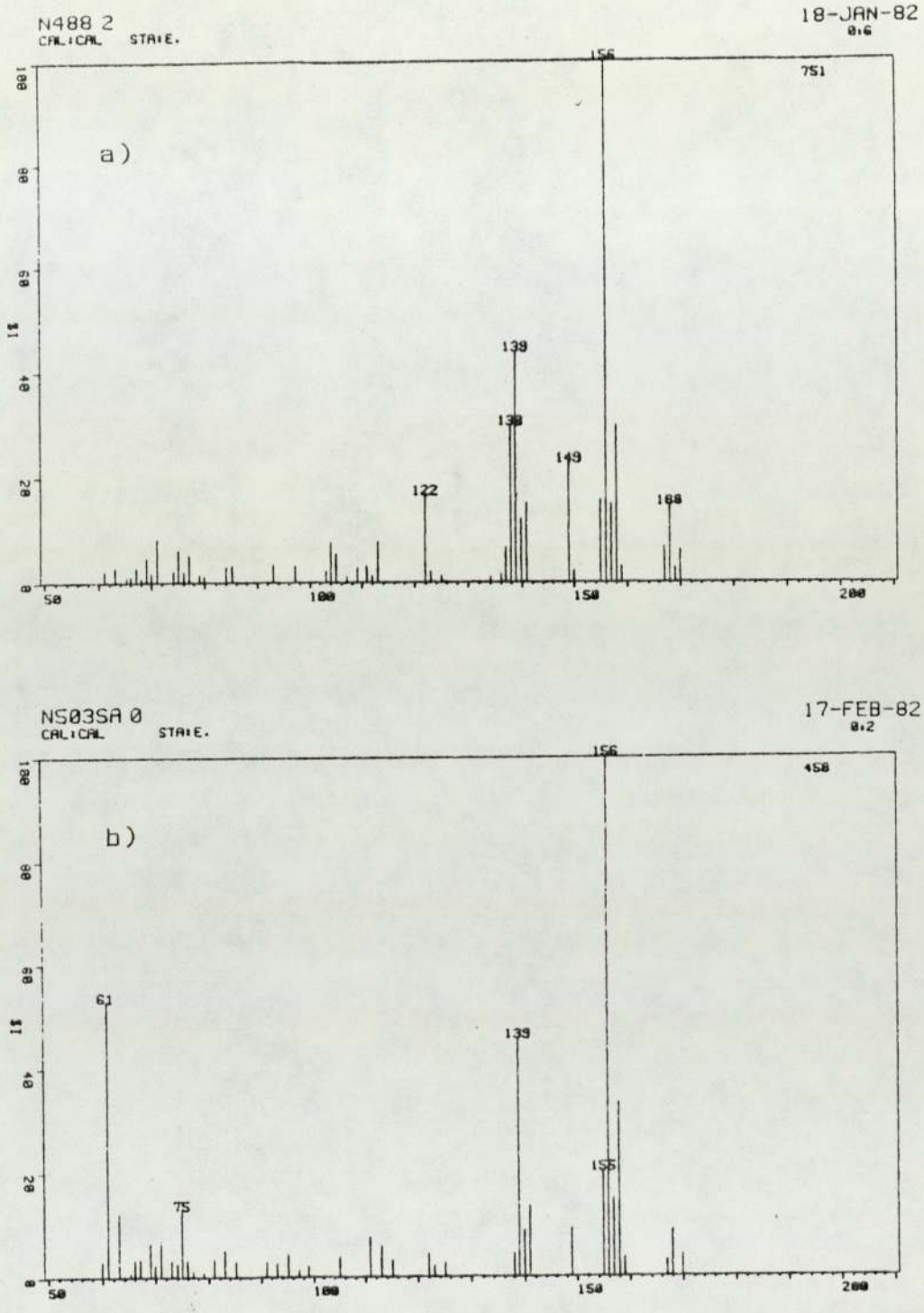


Fig. 4.46 Chemical ionization mass spectra of authentic 4-chloro-N-hydroxymethylbenzamide (a), and an in-vitro metabolite of 4-chloro-N-methylbenzamide with an identical retention time (b)

Compound	% metabolism* to			
	N-hydroxymethyl derivative		N-desmethyl derivative	
	Microsomes	9000g sup^{nt}	Microsomes	9000g sup^{nt}
N-methylbenzamide	2.81	2.94	0	0
4-chloro-N-methylbenzamide	1.68	1.65	0	0.02
N-methyl-4-t-butylbenzamide	0.60	0.45	0.02	0.16

* - mixtures contained either microsomes equivalent to 1g wet liver weight or 9000g supernatant equivalent to 0.25g wet liver weight, and sufficient G6P, GPDH and NADP to yield NADPH 1mM. Mixtures were incubated at 37^{o}C for 30 minutes.

Table 4.14 The extent of N-demethylation of 4-substituted N-methylbenzamides (10mM) in microsomal and 9000g supernatant fractions of liver.

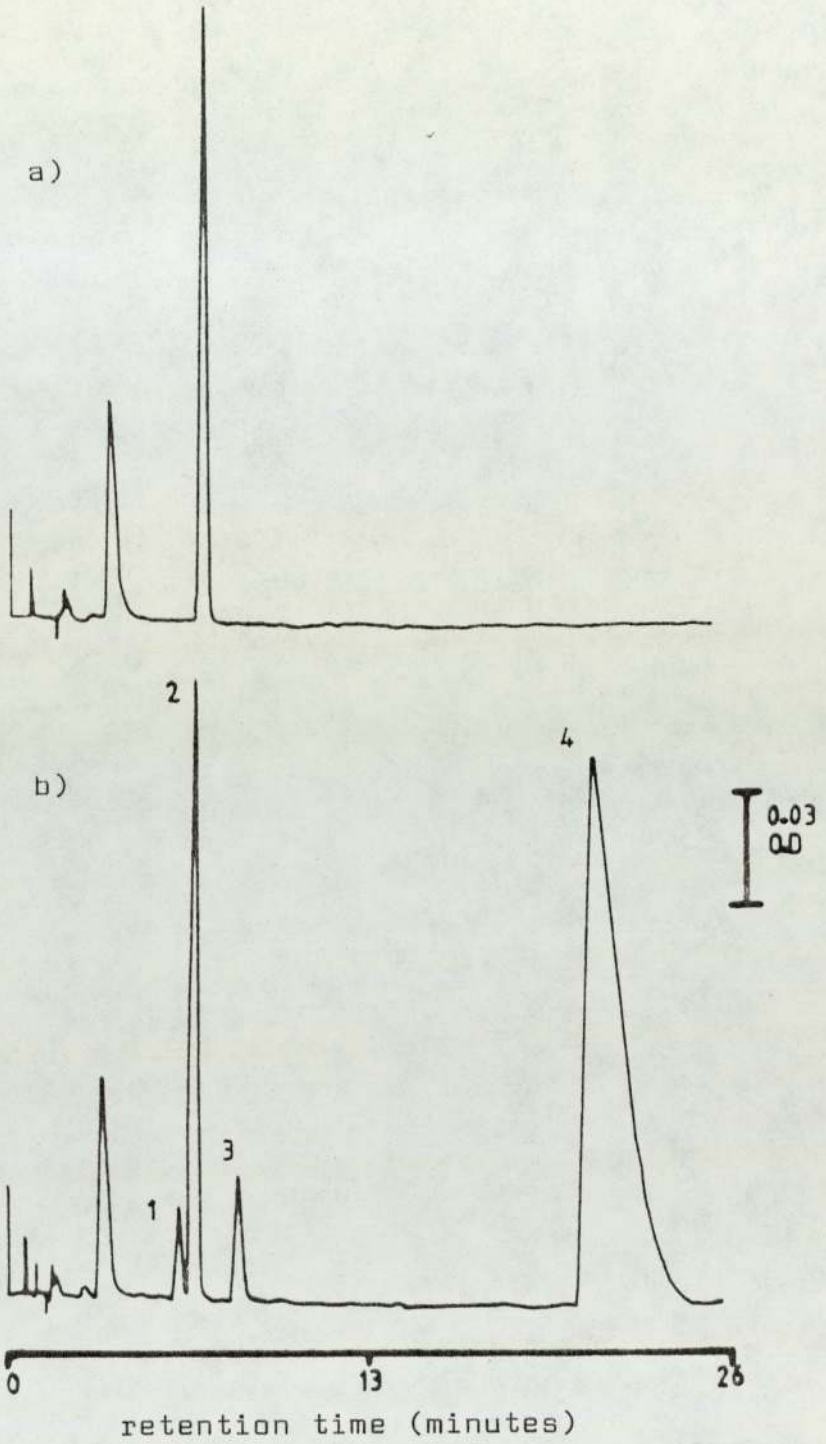


Fig. 4.47 High pressure liquid chromatograms of
a) an extract of a mixture of microsomes and an NADPH generating system incubated for 30 min. at 37°C,
b) an extract of an identical incubation mixture which also contained N,N-dimethylbenzamide (5mM)

(1, unidentified metabolite; 2, internal standard; 3, N-methylbenzamide; 4, N,N-dimethylbenzamide).

The time course of production of these two metabolites (Fig. 4.48) shows that the amount of metabolite 1 in the incubation mixture first increased and then decreased. Metabolite 1 (Fig. 4.47) was derivatized using BSTFA and subjected to combined G.C.-M.S. analysis. The mass spectrum obtained (Fig. 4.49) indicates that metabolite 1 (Fig. 4.47) is N-hydroxymethyl-N-methylbenzamide [$^{m}/z$ 105 - $C_6H_5CO^{+}$, 100%; $^{m}/z$ 237 - $C_6H_5CON(CH_3)CH_2OSi^{+}(CH_3)_3$, 61.2%; $^{m}/z$ 192 - $C_6H_5C\langle OSi(CH_3)_2\rangle NCH_3^{+}$, 40.7%; $^{m}/z$ 222 - $C_6H_5CON(CH_3)CH_2OSi^{+}(CH_3)_2$, 37.5%]. Authentic N-hydroxymethyl-N-methylbenzamide could not be synthesized using a similar method to that which furnished N-hydroxymethylbenzamide (sec. 2.I.3.e). The stabilities of authentic N-hydroxymethylbenzamide and N-hydroxymethyl-N-methylbenzamide produced during metabolism were compared in different buffer systems at 37°C and the results are shown in Fig. 4.50.

A chromatogram of an extract of the products of metabolism of N-methylbenzamide in isolated hepatocytes is shown in Fig. 4.51. N-hydroxymethylbenzamide (1, Fig. 4.51), benzamide (2, Fig. 4.51) and N-formylbenzamide (5, Fig. 4.51) were identified as metabolites of N-methylbenzamide (4, Fig. 4.51) on the basis of their chromatographic retention times. 4-chloro-N-hydroxymethylbenzamide was identified as a product of the metabolism of 4-chloro-N-methylbenzamide in isolated hepatocytes. Another metabolite with a retention time greater than that of the substrate itself was also observed. The biotransformation of N-methyl-

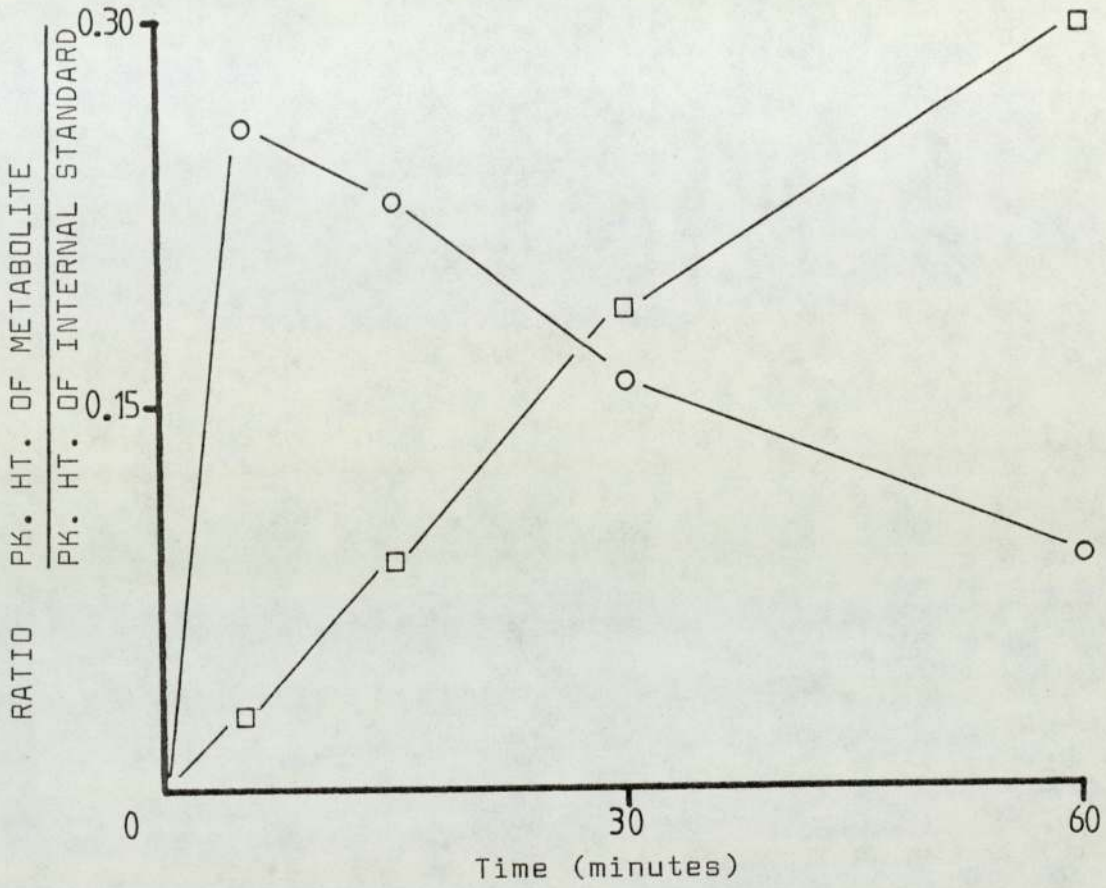


Fig. 4.48 Time-course of production of N-methylbenzamide (□), and an unidentified metabolite (○), during the metabolism of N,N-dimethylbenzamide (5mM) in microsomes.

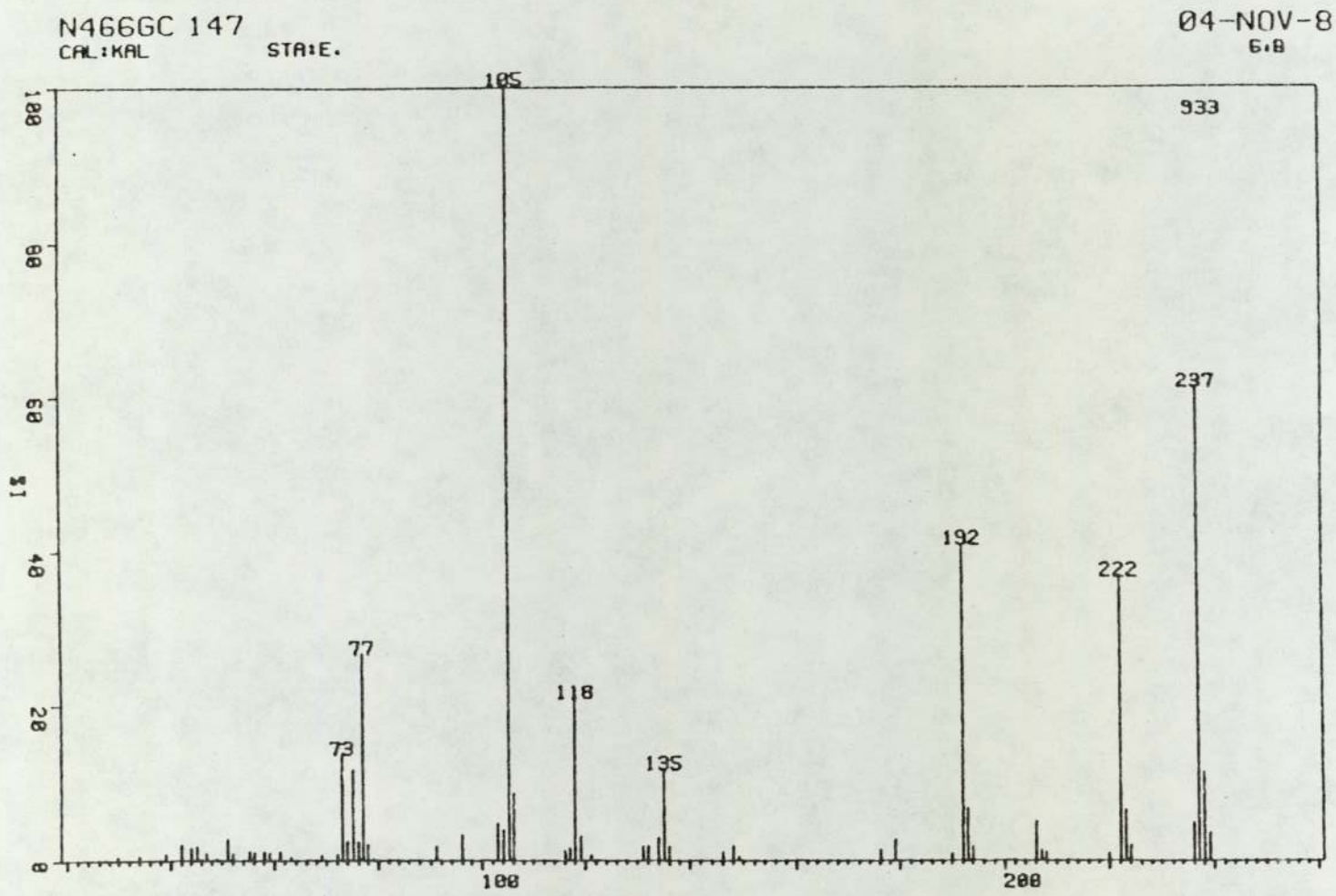


Fig. 4.49 Mass spectrum of a peak (t_R = 6.0 minutes) produced during the combined G.C-M.S. analysis of metabolite 1, Fig. 4.47, after derivatization with BSTFA.

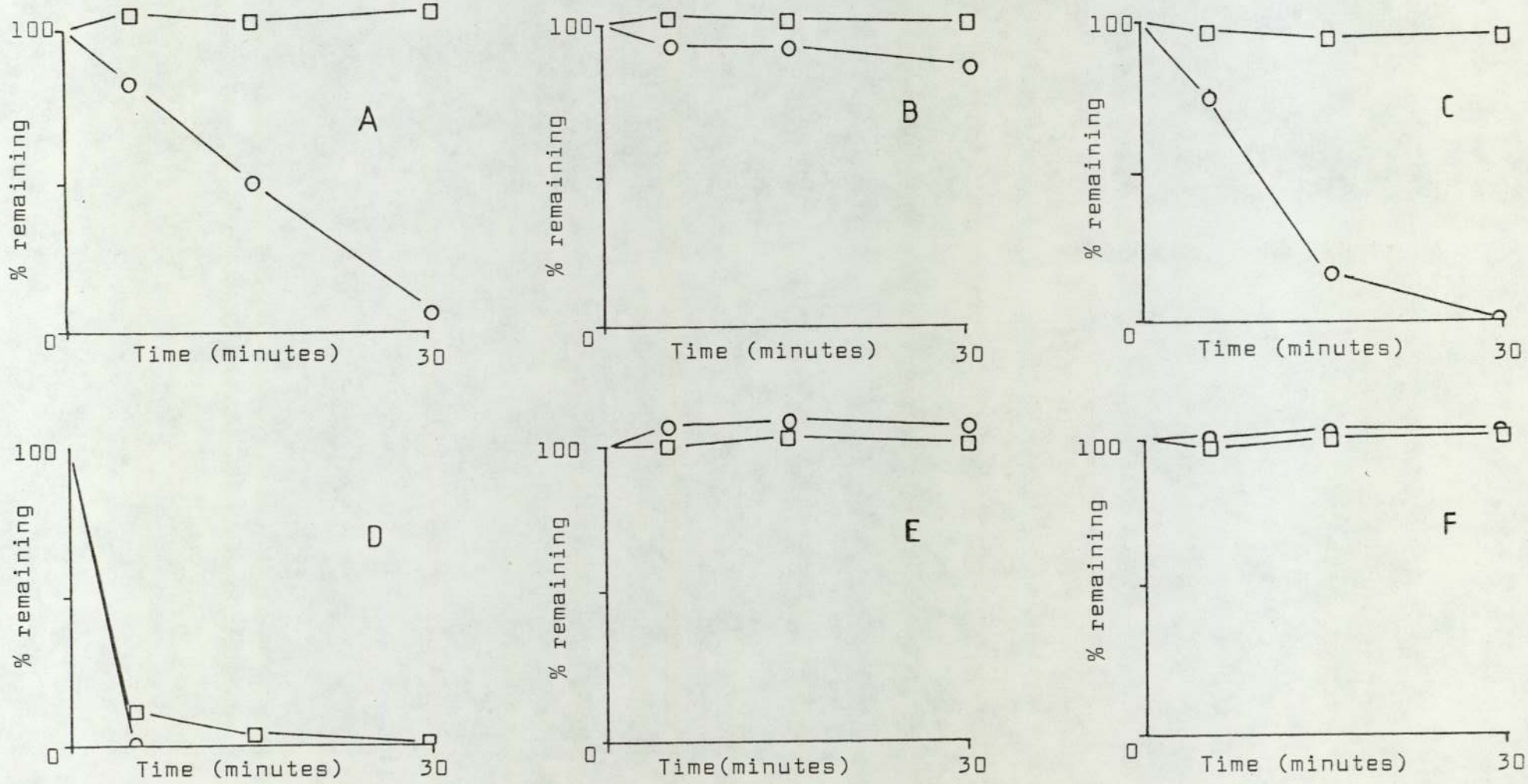


Fig. 4.50 The stability of authentic N-hydroxymethylbenzamide (□), and metabolically generated N-methyl-N-hydroxymethylbenzamide (○), at 37°C in the following buffer systems: A- Earl's pH=7.4, B - Tris 0.01M pH=7.4, C - Tris 0.01M pH=9, D - Tris 0.01M pH=12, E - Acetate pH=5, F - Acetate pH=2.8.

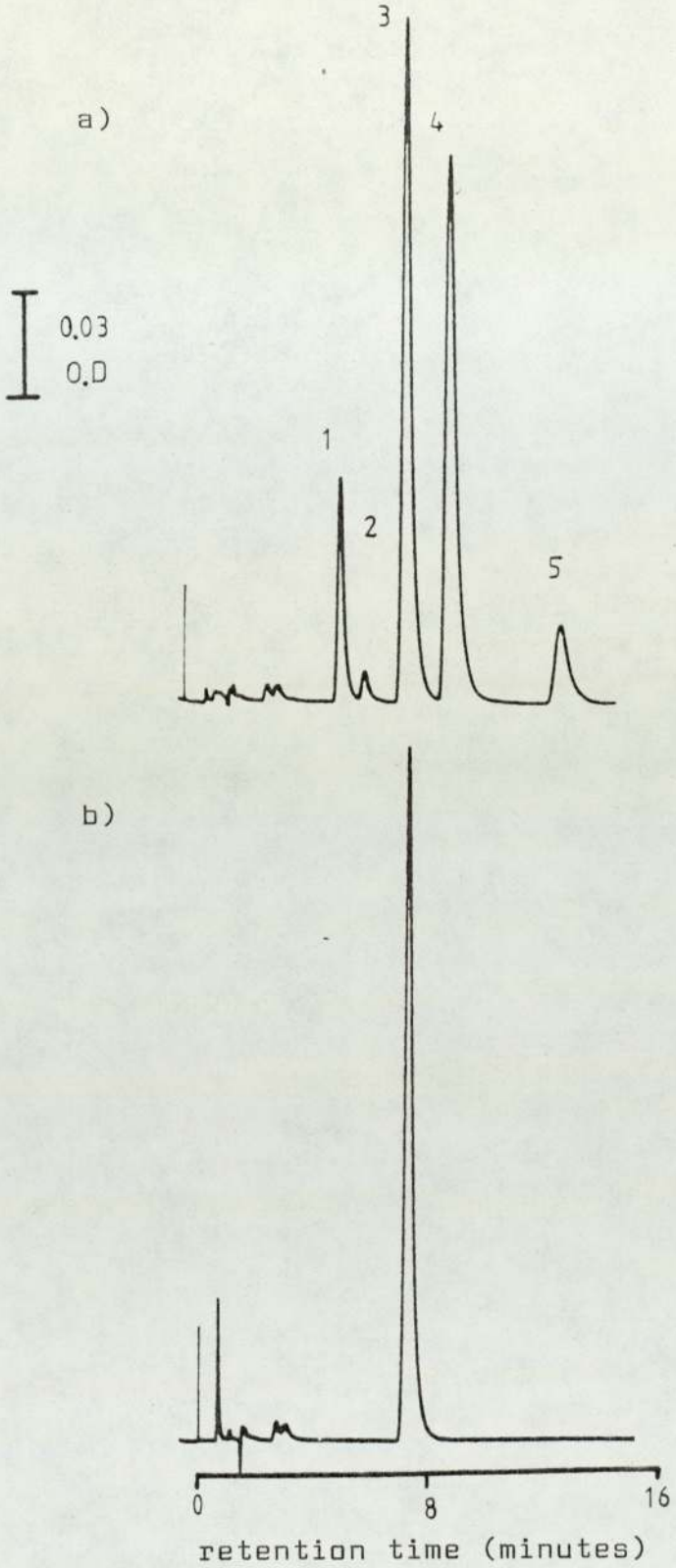


Fig. 4.51 High pressure liquid chromatograms of a) an extract of a mixture of N-methylbenzamide (1mM) with hepatocytes incubated at 37°C for 90 minutes b) an extract of an incubation mixture omitting N-methylbenzamide (1, N-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 4, N-methylbenzamide; 5, N-formylbenzamide)

4-t-butylbenzamide in isolated hepatocytes produced the N-hydroxymethyl derivative, the N-desmethyl derivative, a metabolite with a retention time greater than the substrate itself and a polar metabolite with an identical retention time to metabolite 5 (Fig. 4.45). The quantities of metabolites isolated during the biotransformation of N-methylbenzamide and its 4-chloro and 4-t-butyl derivatives by isolated hepatocytes are shown in Table 4.15. The viability of the hepatocytes (initially greater than 75%) was unchanged after incubation with these substrates for 30 minutes.

When N-hydroxymethylbenzamide was incubated with 9000g supernatant fortified with NAD two metabolites were observed which had identical retention times to authentic samples of benzamide and N-formylbenzamide (Fig. 4.52). The identity of N-formylbenzamide was confirmed by chemical ionization M.S.(Fig. 4.53). The time course of the metabolic generation of N-formylbenzamide and benzamide from N-hydroxymethylbenzamide using isolated hepatocytes and 9000g supernatant fortified with NAD is shown in Fig. 4.54. Authentic N-formylbenzamide hydrolyzed quantitatively to yield benzamide (and presumably formic acid) with a half-life of 7.8 $\pm$ 1.9 minutes (n = 3) in Earl's buffer pH = 7.4. Benzoic acid could not be detected during this decomposition. The half-life of N-formylbenzamide was not altered by the addition of 9000g supernatant and NAD indicating that the degradation is purely a chemical process.

Substrate	% metabolism to *		
	N-hydroxymethyl derivative	N-desmethyl derivative	N-formyl derivative
N-methylbenzamide	10.5	2.5	2.2
4-chloro-N-methylbenzamide	8.6	6.9	?
N-methyl-4-t-butylbenzamide	≠	≠	I

≠ = below 0.1%

? = unidentified peak present but standard not available

* = after thirty minutes incubation at 37°C with 5×10^5 cells/ml.

Table 4.15 The metabolism of 4-substituted N-methylbenzamides (1mM) by isolated hepatocytes

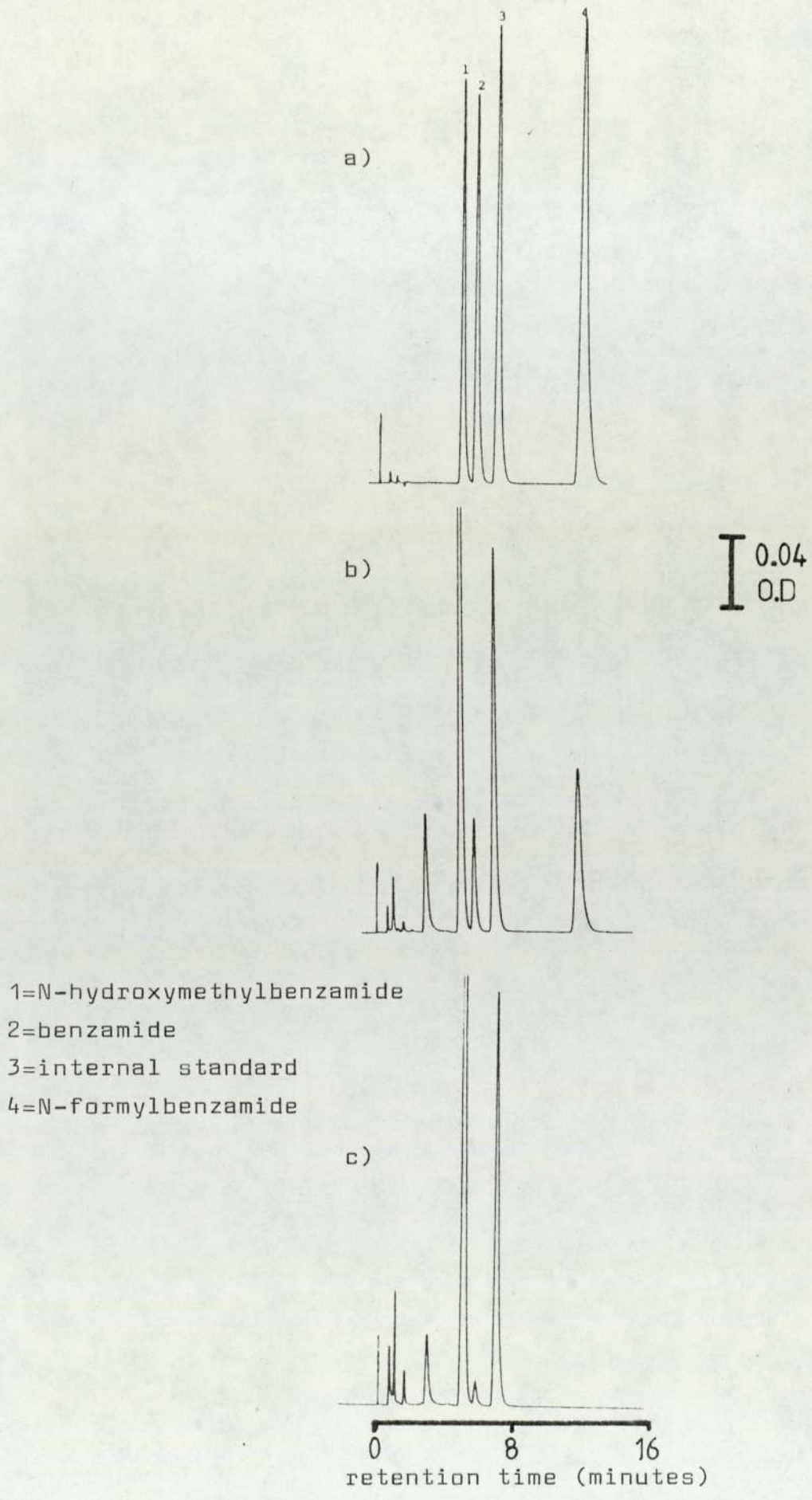


Fig. 4.52 High pressure liquid chromatograms of a) reference compounds b) a mixture of N-hydroxymethylbenzamide (1mM) with 9000g supernatant and NAD (1mM) c) an incubation mixture without NAD.

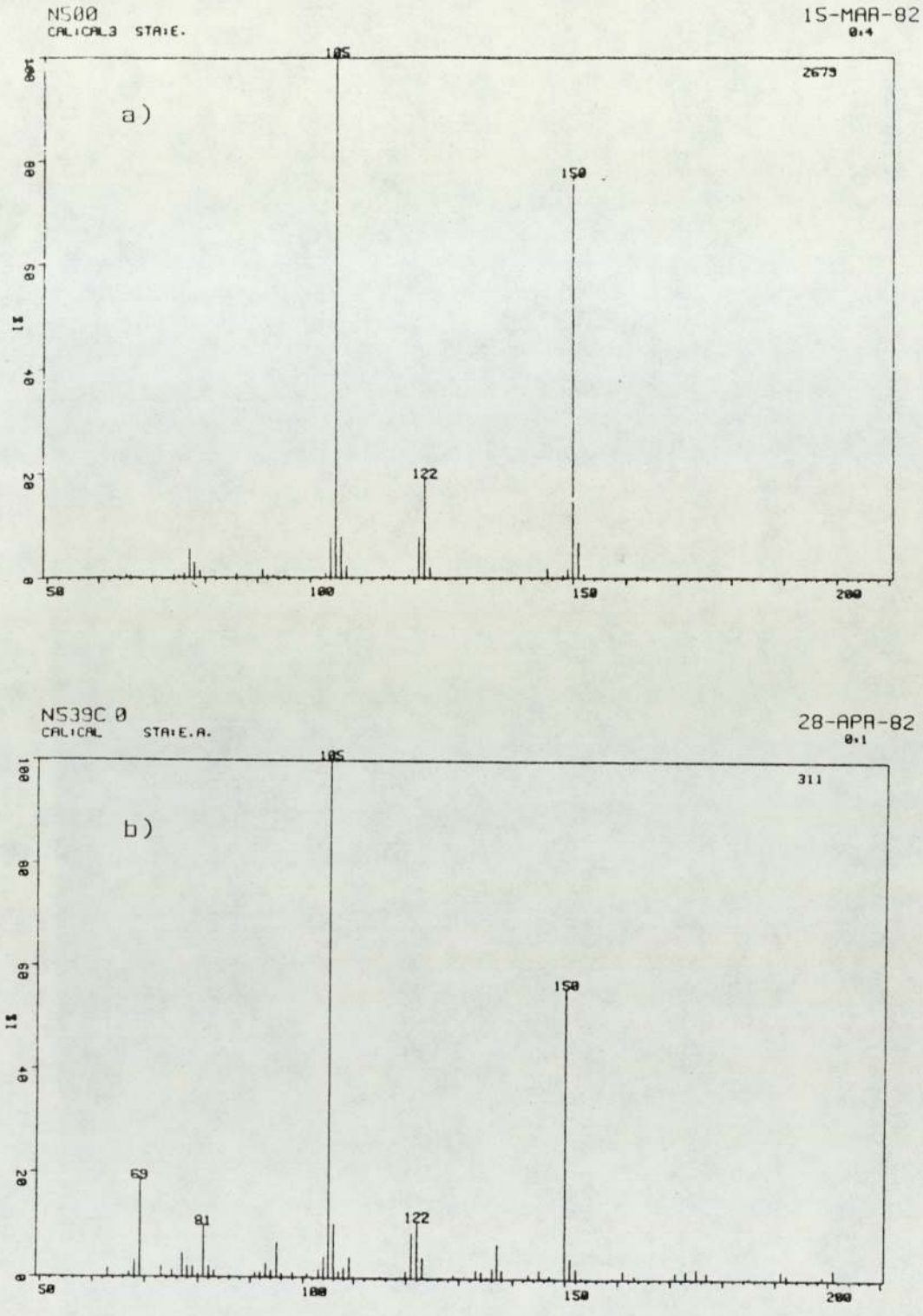


Fig. 4.53 Chemical ionization mass spectra of authentic N-formylbenzamide (a) and an in-vitro metabolite of N-hydroxymethylbenzamide with an identical retention time (b)

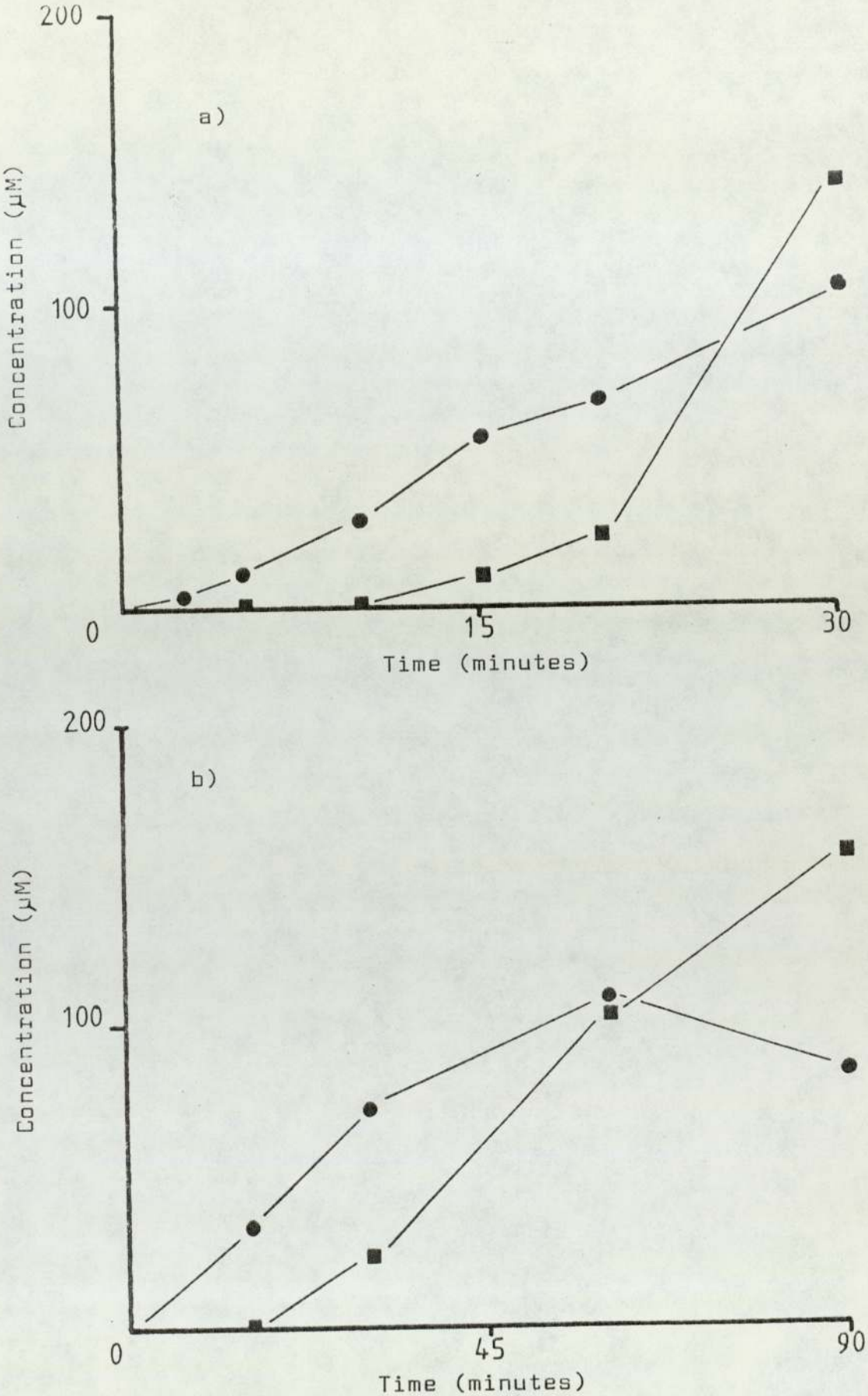


Fig. 4.54 Time-course of the metabolic generation of N-formylbenzamide (●) and benzamide (■) during incubation of N-hydroxymethylbenzamide (1mM) with 9000g supernatant fortified with NAD (a), and in isolated hepatocytes (b)

The biotransformation of N-hydroxymethylbenzamide to N-formylbenzamide and benzamide was also observed in microsomal supernatant and to a minor extent in microsomal and mitochondrial fractions (Table 4.16). The rate of metabolism in the liver supernatant preparations was greatly enhanced by the addition of either NAD, NADPH, or an NADPH generating system. This biotransformation could also be catalyzed by purified horse liver alcohol dehydrogenase (HLAD) and was inhibited by pyrazole and nitrogen (Table 4.17). When benzamide (1mM) was used as a substrate N-formylbenzamide could not be detected in incubations with 9000g and microsomal supernatants fortified with NAD, and in hepatocytes.

In all in-vitro incubations no other metabolites were identified when the pH of the incubation mixtures were adjusted to pH = 2 and re-extracted, after the normal extraction process had been performed.

4.VI.2.c In-vivo metabolism studies

The recoveries of standard compounds from urine are shown in Table 4.18.

Figs. 4.55 and 4.56 show chromatograms of extracts of urine from mice which had received N-methylbenzamide and N-hydroxymethylbenzamide respectively. In both cases N-hydroxymethylbenzamide (1, Figs. 4.55, 4.56), benzamide (2, Figs. 4.55, 4.56) and N-formylbenzamide (5, Figs 4.55, 4.56) were identified in the urine on the basis of chromatographic retention times. In addition N-methylbenzamide (4, Fig. 4.55) was found in the urine of mice which had received that compound. When the urine samples of mice which had received N-methylbenzamide and

Liver Fraction* (= 250mg liver)	Amount of metabolites formed, μM (Benzamide + N-formylbenzamide)
Mitochondria	22 ± 29
9000g supernatant	249 ± 53
Microsomes	25 ± 21
Microsomal supernatant	152 ± 55
Hepatocytes	273 ± 31

* - Liver fractions, but not hepatocytes, were fortified with 1mM NAD and incubated at $37^{o}C$ for 30 min. $5x10^{5}$ hepatocytes/ml were incubated for 90 min.

Table 4.16 Metabolism of N-hydroxymethylbenzamide (1mM) in various liver fractions and in isolated hepatocytes

The values represent the mean ± s.d of at least three experiments

Enzyme source *	Amount of metabolites formed μM. (Benzamide + N-formylbenzamide)
9000g supernatant	211 ± 11
9000g supernatant + Pyrazole	0
9000g supernatant + Nitrogen	97 ± 38
HLAD	309 ± 22
HLAD + Pyrazole	0
HLAD + Nitrogen	364 ± 15

* - Mixtures were fortified with 1mM NAD and incubated at 37°C for 90 min.

Table 4.17 The effect of pyrazole (1mM) and nitrogen on the metabolism of N-hydroxymethylbenzamide (1mM) by 9000g supernatant and horse liver alcohol dehydrogenase (HLAD)

The values represent the mean ± s.d of at least three experiments.

Compound	% recovery
N-methylbenzamide	97
N-hydroxymethylbenzamide	96
Benzamide	98
4-nitrobenzamide	102
N-formylbenzamide	98

Table 4.18 Recoveries of standard compounds from urine

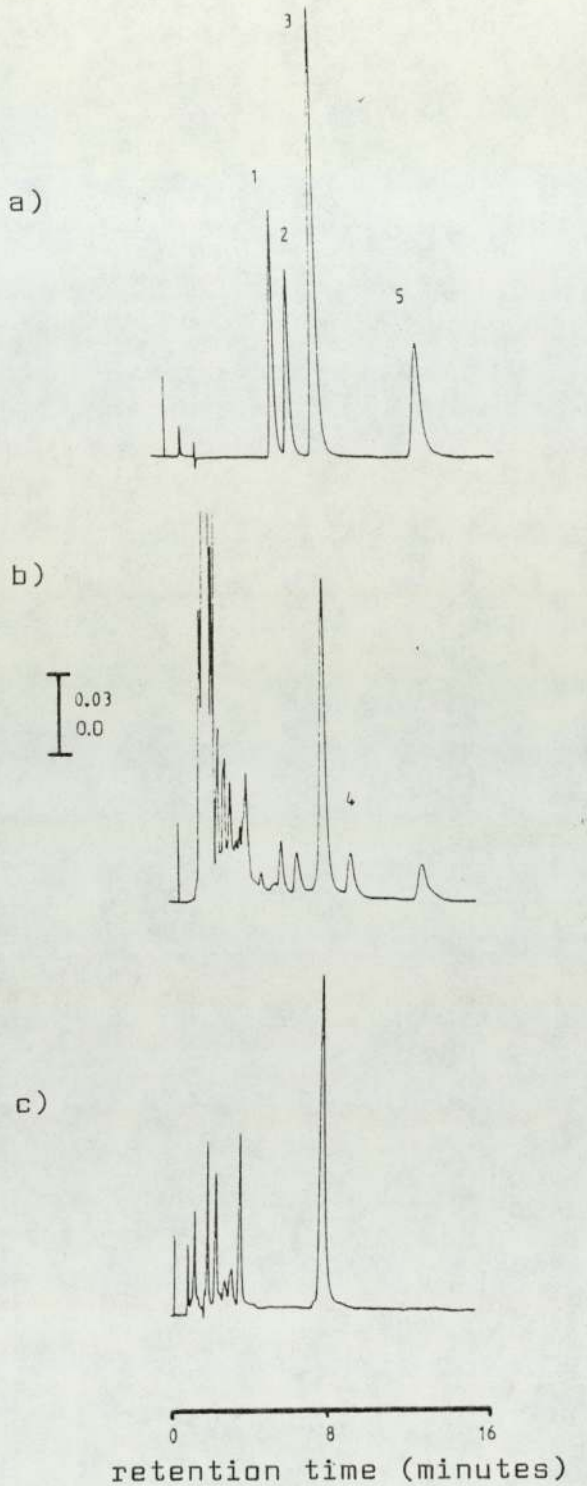


Fig. 4.55 High pressure liquid chromatograms of extracts of a) a mixture of reference compounds 1, N-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 5 N-formylbenzamide; b) urine from mice which had received N-methylbenzamide 200mg/Kg i.p; (4=N-methylbenzamide) c) control urine.

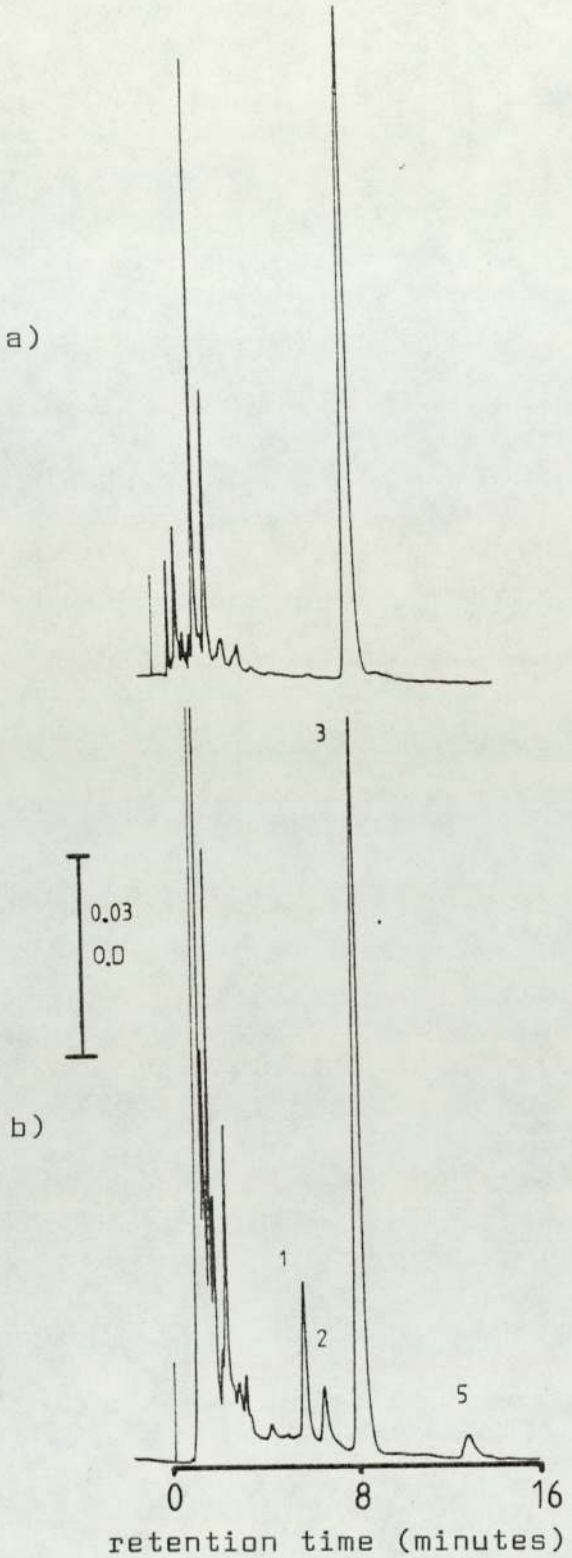


Fig. 4.56 High pressure liquid chromatogramsof extracts of a) control urine and b) urine from mice which had received N-hydroxymethylbenzamide 200mg/kg i.p. (1, N-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 5, N-formylbenzamide)

N-hydroxymethylbenzamide were adjusted to pH = 2 and re-extracted, two major polar peaks were observed which were not extracted from control urine samples adjusted to pH = 2. The chemical ionization mass spectrum of one these metabolites suggested that it may be hippuric acid ($^{m}/z$ 180 - MH^{+}, 100%; $^{m}/z$ 105 - $C_6H_5CO^{+}$, 66%; $^{m}/z$ 135 - $C_6H_5C(OH)N^{+}HCH_2$, 15%). No major products were observed when the urine of mice which had received either N-methylbenzamide or N-hydroxymethylbenzamide was subjected to enzymatic deconjugation.

4.VI.3 Discussion

The biotransformation of N-methylbenzamide and its 4-chloro and 4-t-butyl derivatives to their respective N-hydroxymethyl compounds was shown to occur in microsomes, 9000g supernatant and in suspensions of isolated hepatocytes. That this process was catalyzed by cytochrome P450 was suggested by the dependence of the microsomal metabolism of N-methylbenzamide to N-hydroxymethylbenzamide upon oxygen and NADPH (Brodie et al., 1955). In the case of N-methylbenzamide an N-formyl derivative was also characterized as a metabolite in isolated hepatocytes. Unidentified peaks were also present in the chromatograms of the extracts of incubation mixtures of 4-chloro-N-methylbenzamide and N-methyl-4-t-butyl benzamide with isolated hepatocytes (Table 4.15). It is possible that these peaks were caused by the formation of N-formyl species during metabolism, but this could not be confirmed due to the absence of reference compounds.

The metabolism of N-methyl-4-t-butylbenzamide in microsomal and 9000g supernatant fractions of liver, and in isolated hepatocytes resulted in the formation of a polar metabolite (5, Fig. 4.45). Such a polar species was not found in metabolic incubations containing N-methylbenzamide and its 4-chloro derivative. It is therefore conceivable that this metabolite may have arisen as a result of hydroxylation of the t-butyl moiety.

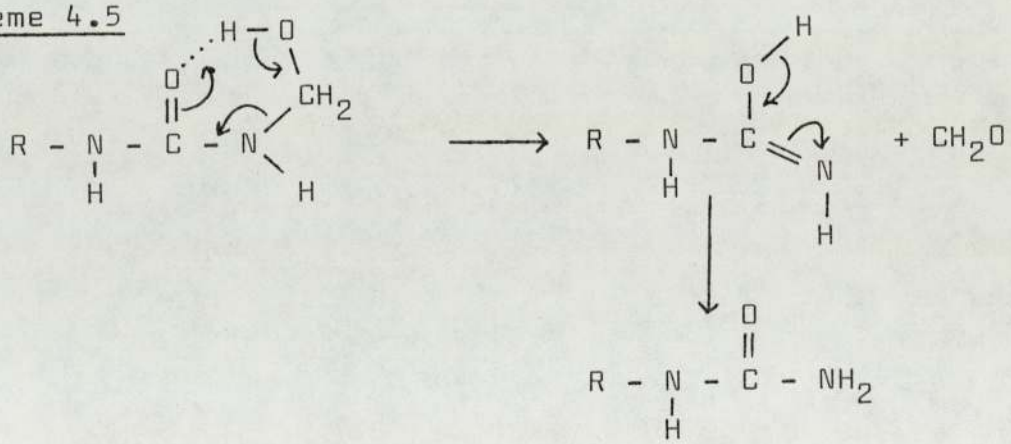
It has been shown that 4-substitution in the phenyl ring of N-hydroxymethyl benzamides does alter their rate of

breakdown to produce formaldehyde (sec. 4.II.2.b). However N-hydroxymethylbenzamide and its 4-chloro and 4-t-butyl derivatives all have half-lives in Earl's buffer at 37°C of greater than 12 hours (4.II.2.b) and would be expected to be sufficiently stable to be isolated after incubations of up to ninety minutes at 37°C in Earl's buffer. This was confirmed by the characterization of N-methylols during the in-vitro metabolism of N-methylbenzamide and its 4-chloro and 4-t-butyl derivatives. Thus, although 4-substitution in the phenyl ring of N-methylbenzamides does result in quantitative differences in metabolism (Tables 4.15, 4.16), it does not alter the stability of the respective N-methylols sufficiently to cause qualitative differences in metabolism.

The metabolism of N,N-dimethylbenzamide in microsomes resulted in the formation of N-hydroxymethyl-N-methylbenzamide (I, Fig. 4.57) which was characterized by combined G.C.-M.S. after derivatization with BSTFA (Fig. 4.49). The metabolism of N,N-dimethylbenzamide and its 4-chloro derivative, unlike N-methylbenzamide resulted in the generation of Nash positive species. The amount of such species produced during the metabolism of these N,N-dimethyl substituted benzamides increased upon treatment of the incubation mixtures with alkali prior to the colourimetric reaction (Table 4.11). That the production of Nash positive species without the addition of alkali was due to the degradation of N-hydroxymethyl-N-methylbenzamide to produce formaldehyde was suggested by the time course of production of metabolites of N,N-dimethylbenzamide during

incubation with microsomes (Fig. 4.48). This hypothesis was confirmed by an investigation of the stability of metabolically generated N-hydroxymethyl-N-methylbenzamide in Earl's buffer pH = 7.4 (Fig. 4.50). The results presented in Fig. 4.50 demonstrate that N-hydroxymethyl-N-methylbenzamide is less stable under alkaline conditions than N-hydroxymethylbenzamide. This may be a result of the electron donating effect of the additional N-methyl group. Alternatively hydrogen bonding may account for the difference in stabilities. Tanaka et al. (1972) have suggested that intramolecular hydrogen bonding of N-hydroxymethyl-N′-phenylureas leads to their degradation as described in scheme 4.5.

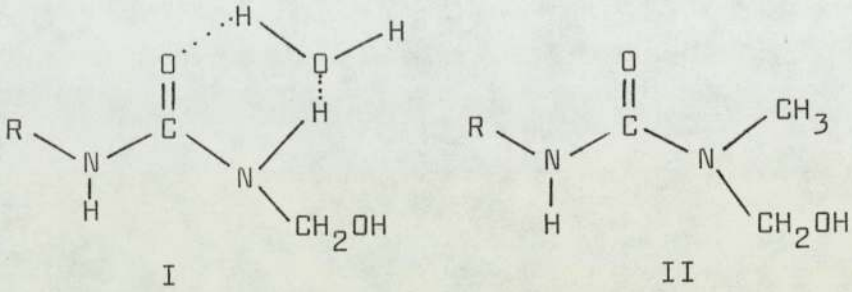
Scheme 4.5



This mechanism involves the transfer of the hydroxyl proton to the carbonyl oxygen via a six membered cyclic transition state resulting in the elimination of formaldehyde. Tanaka et al. (1972) proposed that the greater stability of the N-hydroxymethyl derivative (I, scheme 4.6) as compared to the N-hydroxymethyl-N-methyl derivative (II, scheme 4.6) may be due to the fact

that the N-hydroxymethyl group in I can be held preferentially in a configuration trans to the carbonyl group by a water molecule.

Scheme 4.6



I II

In the case of the N-hydroxymethyl-N-methyl derivative (II, scheme 4.6) this hydrogen bonding is not possible as a proton is not available on the nitrogen atom. Consequently the formation of the unstable transition state involving intramolecular hydrogen bonding to the carbonyl group would be more probable in the case of II than I, and would result in the more rapid degradation of II as compared to I. This explanation of the differential stability of N-hydroxymethyl and N-hydroxymethyl-N-methyl derivative of N/-phenylureas may also be applicable to N-hydroxymethyl substituted benzamides.

N-hydroxymethylbenzamide is a stable species (sec. 4.II.2.b) and was metabolized to benzamide and N-formylbenzamide (sec. 4.IV.2.b). N-formyl derivatives have been isolated as metabolites of N,N-dimethylaminoantipyrine (Noda et al., 1976; Nigam et al., 1980) and chlorotoluron (Muecke et al., 1976). It has also been shown that in molecules which possess a heteroalicyclic ring system, such as nicotine, ring carbon atoms adjacent to a nitrogen atom bearing a methyl group can be hydroxylated

and then further oxidized to yield the corresponding lactam (Hucker et al., 1960; Cho et al., 1961). However the isolation of N-formyl compounds during the metabolism of N-hydroxymethyl containing substrates has not been reported.

The enzymes which catalyzed the conversion of N-hydroxymethylbenzamide to N-formylbenzamide and benzamide were localized predominately in the soluble fraction of the cell (Table 4.16). That dehydrogenase enzymes may be involved was suggested by the observation that this biotransformation could be catalyzed by HLAD (Table 4.17). In addition pyrazole, an inhibitor of alcohol dehydrogenase (Reynier, 1969) totally inhibited the conversion of N-hydroxymethylbenzamide to N-formylbenzamide and benzamide by both 9000g supernatant and HLAD (Table 4.17). However the effect of nitrogen on the biotransformation catalyzed by HLAD and 9000g supernatant (Table 4.17) and the non-specific co-factor requirement (sec. 4.VI.2.b) indicates that more than one enzyme system may be involved.

The observation that N-formylbenzamide was not detected when benzamide was used as a substrate in metabolic incubations suggests that any N-formylbenzamide produced arises from the biotransformation of the N-hydroxymethyl group and is not a result of metabolic N-formylation. Authentic N-formylbenzamide was shown to undergo quantitative hydrolysis to benzamide. Therefore any benzamide produced during the metabolism of N-hydroxymethylbenzamide was presumably a product of the degradation of N-formylbenzamide,

and the time course of production of N-formylbenzamide and benzamide supports this hypothesis (Fig. 4.54).

In view of these results and the observation that N-formylbenzamide has been shown to be a metabolite of N-methylbenzamide in isolated hepatocytes (Fig. 4.51), the metabolic scheme in Fig. 4.57 is proposed for the in-vitro metabolism of the N-methyl group in N-methyl substituted benzamides. Although N-hydroxymethylbenzamide (IV, Fig. 4.57) does not degrade chemically to benzamide under the conditions used for in-vitro incubations enzymic production of benzamide cannot be ruled out. N-hydroxymethyl compounds are able to aminomethylate nucleophiles such as glutathione (Weitzel et al., 1963) and this process has recently been implicated as the mechanism whereby an N-methylene-glutathione conjugate was formed after the administration of 4-dimethylaminoazobenzene to rats (Ketterer et al., 1982).

N-hydroxymethylbenzamide, benzamide and N-formylbenzamide were identified in the urine of mice which had received either N-methylbenzamide (Fig. 4.55) or N-hydroxymethylbenzamide (Fig. 4.56). This implies that the metabolic scheme proposed for the in-vitro metabolism of the N-methyl group in N-methylbenzamide (Fig. 4.57) may also be applicable in-vivo. Two metabolites of both N-methylbenzamide and N-hydroxymethylbenzamide could only be extracted from urine when the urinary pH was adjusted to pH = 2. On the basis of this observation and mass spectral evidence (sec. 4.VI.2.c) one of these metabolites was tentatively identified as hippuric acid. It has been known for over a century that benzoic acid can be converted to hippuric acid

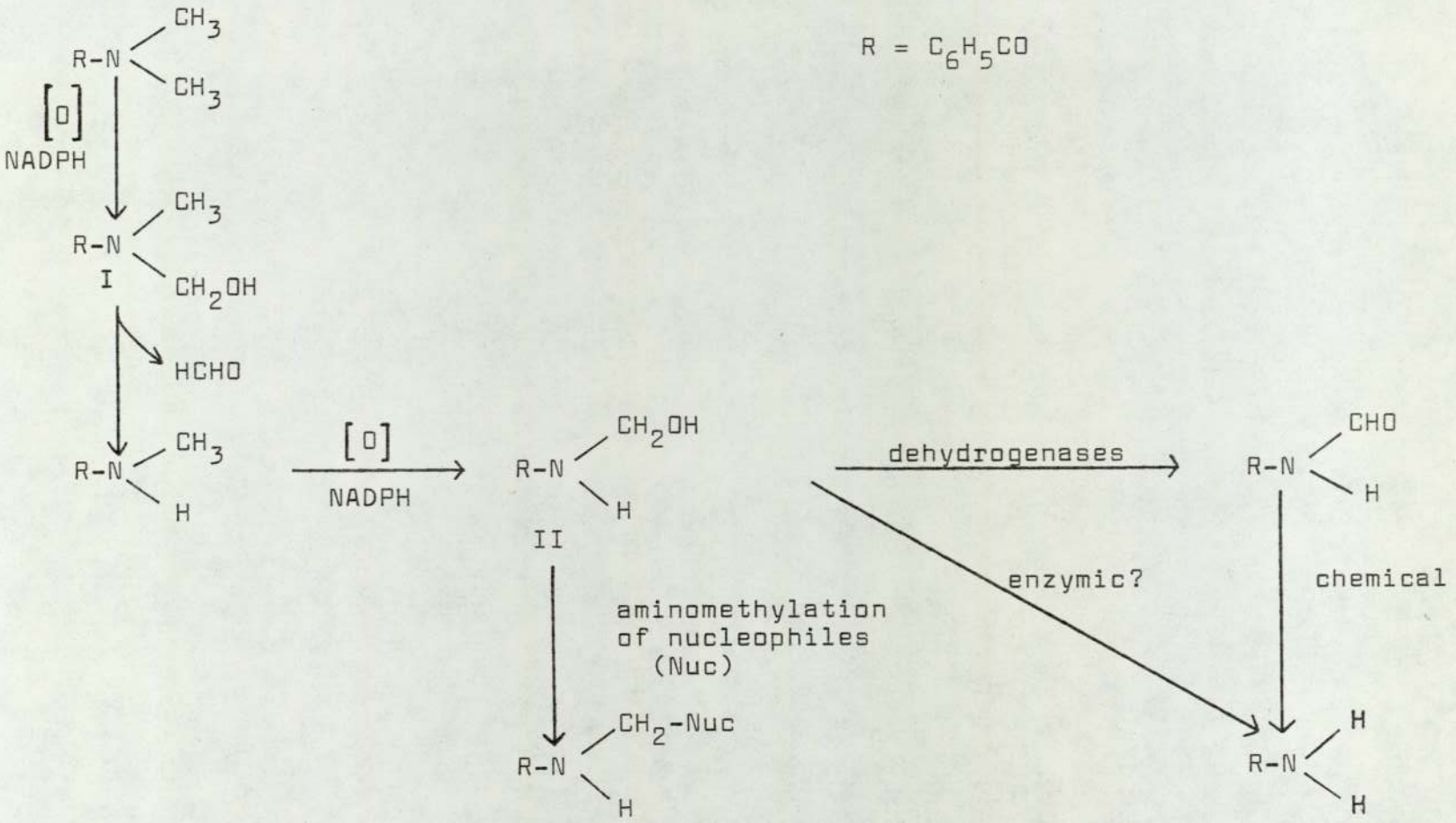


Fig. 4.57 Metabolic scheme for the metabolism of the N-methyl groups of N,N-dimethylbenzamide.

in animals (Keller, 1842). It therefore seems probable that this metabolite was formed in-vivo by enzymic hydrolysis of the amide linkage in the benzamide molecule to yield benzoic acid, which was then conjugated with endogenous glycine to produce hippuric acid.

4.VII Correlation of metabolism with physico-chemical constants

4.VII.1 Introduction

Gaudette & Brodie (1959) have shown that only N-methyl containing molecules that are sufficiently lipophilic were N-demethylated by the hepatic microsomal system. These results were confirmed by the observation of a correlation between lipid solubility and rate of N-demethylation of a series of tertiary amines both in-vitro and in-vivo (McMahon, 1961; McMahon & Easton, 1961). Hansch et al. (1965) extended the analysis of the results of McMahon (1961) and McMahon & Easton (1961) and concluded that the rate of N-demethylation in this series of tertiary amines could be correlated both with lipid solubility, as measured by partition coefficient, and with the electron density of the nitrogen bearing the methyl group as measured by the pKa of the amine. Further quantitative correlations between electron density of heteroatoms and the extent of their metabolism have also been demonstrated in the following cases: amine acetylation (Perault & Pullman, 1963a), O-demethylation (Perault & Pullman, 1963b), N-demethylation (Ishidate & Hanaki, 1961) and conjugation with glucuronic acid or sulphate (Pullman, 1964).

It was the purpose of this investigation to determine if the qualitative aspects of N-demethylation, i.e - whether a characterizable N-hydroxymethyl compound or formaldehyde was formed, could be interpreted using physical constants such as partition coefficients, pKa values and electron densities.

4.VII.2 Results

4.VII.2.a Determination of electron density distributions

Calculations to determine electron density distributions were performed on hexamethylmelamine (Fig. 4.58), N,N-dimethylaniline (Fig. 4.59) and its 4-cyano and 4-nitroso derivatives (Table 4.19), and N,N-dimethylnitrosamine (Fig. 4.60). The initial structural data were obtained from crystal structure determinations of hexamethylmelamine (Bullen et al., 1972), N,N-dimethyl-4-nitrosoaniline (Romming & Talberg, 1973) and N,N-dimethylnitrosamine (Krebs & Mandt, 1975). In the case of the latter molecule the structure was refined using force-relaxation (maximum force = 0.026). A comparison of the input geometry of N,N-dimethylnitrosamine with that produced by force relaxation showed a maximum variation in bond length of 9.0% with a mean variation of 4.7% $\pm$ 3.0%. A similar comparison of electron density values showed a maximum variation of 2.2% with a mean variation of 1.2% $\pm$ 0.9%.

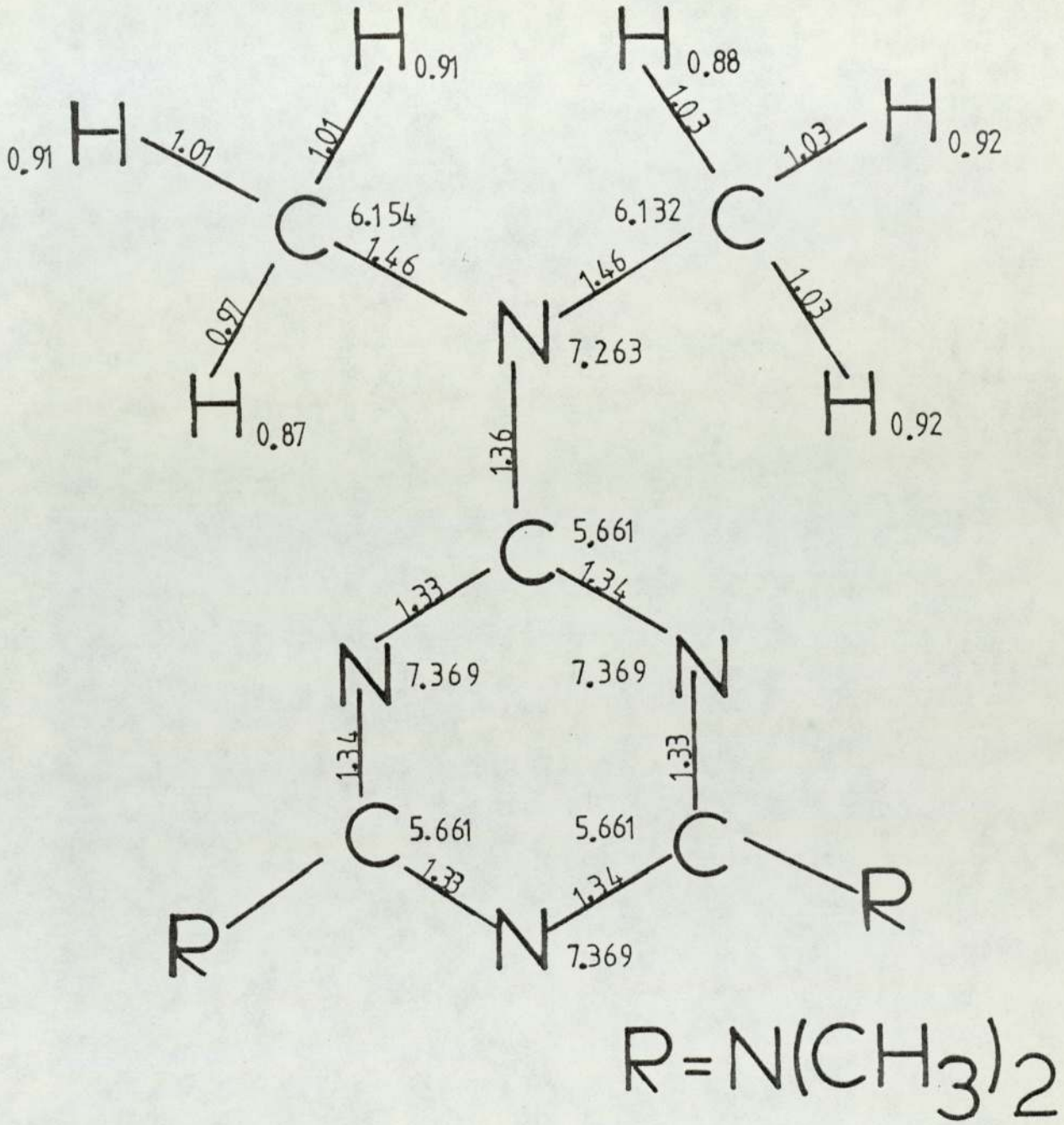


Fig. 4.58 Electron density distribution of hexamethylmelamine

Electron densities are represented as numbers of electrons and bond lengths are given in angstroms.

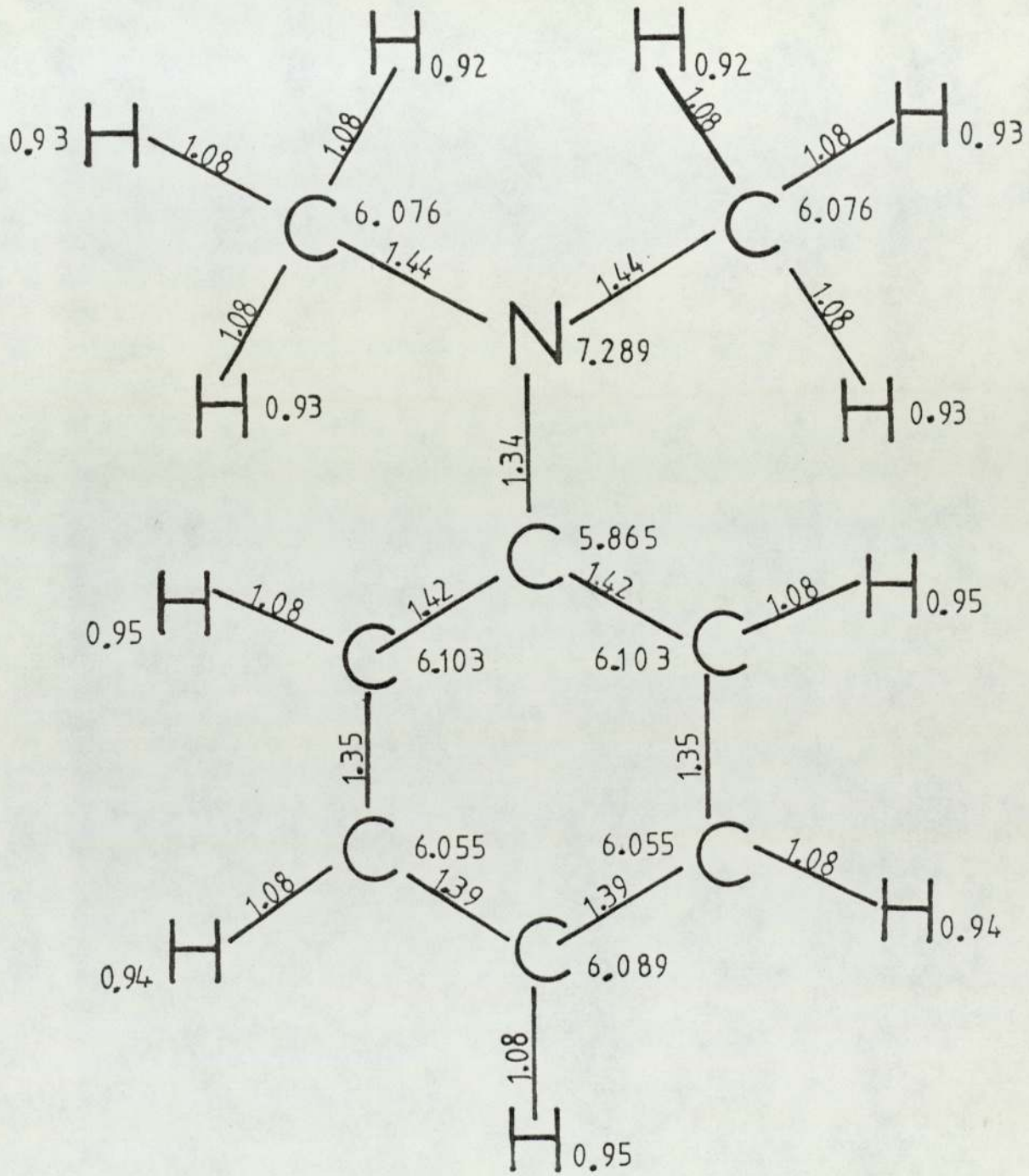


Fig. 4.59 Electron density distribution of N,N-dimethylaniline

Electron densities are represented as numbers of electrons and bond lengths are given in angstroms.

Compound	Electron density of the amino nitrogen atom
N,N-dimethylaniline	7.289
4-cyano-N,N-dimethylaniline	7.284
N,N-dimethyl-4-nitrosoaniline	7.283

Table 4.19 The electron density of the amino nitrogen atom in substituted N,N-dimethylanilines

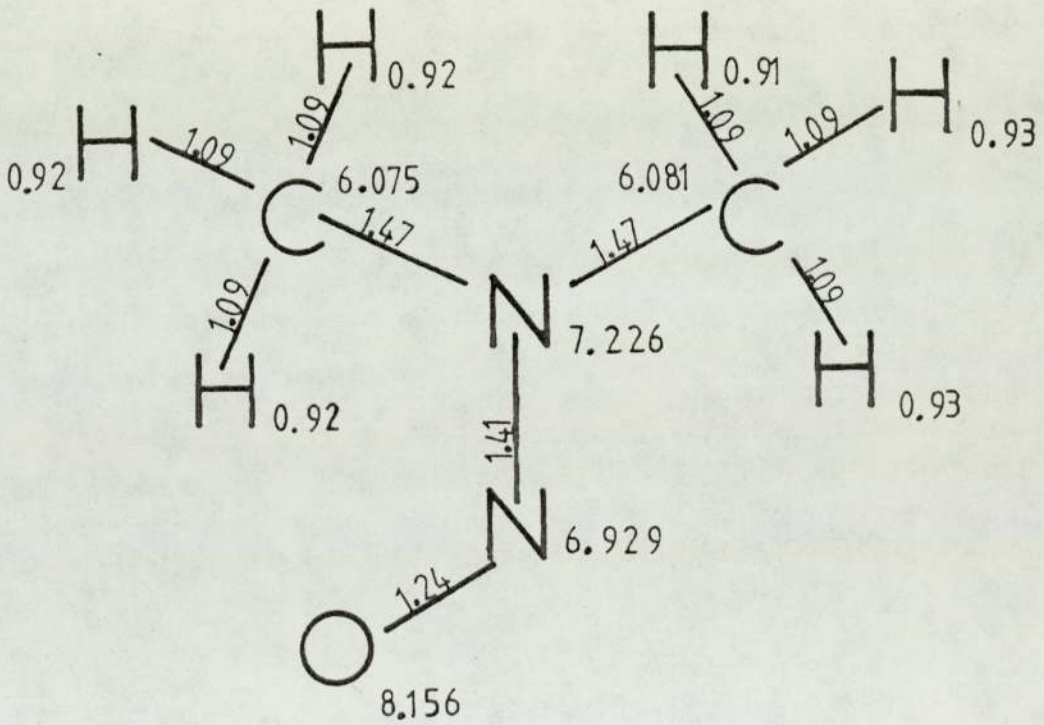


Fig. 4.60 Electron density distribution of N,N-dimethylnitrosamine

Electron densities are represented as numbers of electrons and bond lengths are given in angstroms.

Compound	$\log_{10} P^{+}$
a)	
Hexamethylmelamine	2.73,2.63
N-methylbenzamide	0.86
N'-(4-chlorophenyl)-N,N-dimethylurea	1.94
b)	
N,N-dimethylaniline	2.31,2.31,2.29,2.61
N,N-dimethylaminoantipyrine	0.80

+ data taken from Hansch & Leo (1979)

Table 4.20 Octanol-water partition coefficient (P) of N-methyl containing compounds which either form characterizable N-hydroxymethyl intermediates (a) or formaldehyde (b) during N-demethylation

Compound	pKa	Reference
a)		
4-chloro-N-methylbenzamide	-1.72	Barnett & O'Connor (1973)
N/-(4-chlorophenyl)-N,N-dimethylurea	-1.21	Barnett & O'Connor (1973)
N,N-dimethylformamide	-0.70	Adelman (1964)
hexamethylmelamine	4.92	Langdon (1982)
b)		
N,N-dimethylaminoantipyrine	5.0	Shore et al. (1957)
N,N-dimethylaniline	5.07	Folkers & Runquist (1964)
2-dimethylaminopyridine	6.99	Cruège et al. (1970)
N,N-dimethyl-N/-phenylformamidine	8.35	Foubert & Huyskens (1976)

Table 4.21 pKa values of N-methyl containing compounds which either form characterizable N-hydroxymethyl intermediates (a) or formaldehyde (b) during N-demethylation

4.VII.3 Discussion

The metabolism studies presented as a part of this thesis and other evidence (sec. 1.3) suggest that the formation of stable N-hydroxymethyl compounds during N-demethylation is favoured if the nitrogen bearing the methyl group is relatively acidic. This hypothesis is borne out by the data presented in Table 4.21. Although the metabolism of only a limited number of compounds has been studied a correlation between the pKa value of the parent N-methyl compound and the metabolic generation of a characterizable N-hydroxymethyl species is evident. The substrates which form stable N-methylols during metabolism are all very weak bases (pKa < 0) except hexamethylmelamine (pKa = 4.92), whereas those compounds which have been shown to form formaldehyde during metabolic N-demethylation under the conditions used in this study have pKa values $\geq$ 5 (Table 4.20). A correlation between the partition coefficient of the substrate and the metabolic production of a stable N-methylol was not apparent (Table 4.20).

In general there is a correlation between the pKa values of amines and the electron density of the nitrogen atoms of those amines (Sykes, 1975). This is not necessarily true when there is more than one site where protonation may occur. In the case of hexamethylmelamine it is probable that the ring nitrogen atom rather than the exocyclic nitrogen atom is protonated (sec. 4.II.3) and the results of the electron density calculation of hexamethylmelamine (Fig.4.58)

support this view. The electron density at the ring nitrogen atoms of hexamethylmelamine is calculated to be greater than that at the exocyclic nitrogen atoms and therefore the former are more likely to be protonated. The pKa value of hexamethylmelamine suggests that it is a weaker base than N,N-dimethylaniline (Table 4.21). However the electron density calculations indicate that the ring nitrogen atoms of hexamethylmelamine (electron density = 7.369, Fig. 4.58) have a greater negative charge than the nitrogen atom of N,N-dimethylaniline (electron density = 7.289, Fig. 4.59). Thus the pKa values of hexamethylmelamine and N,N-dimethylaniline do not correlate with the electron densities at their respective sites of protonation. The pKa value of an amine is determined not only by the electron availability on the nitrogen atom but also by the extent to which the cation formed by addition of a proton can undergo solvation and thus become stabilized (Sykes, 1975). Thus it is a possibility that the lack of correlation between the pKa values of hexamethylmelamine and N,N-dimethylaniline and the electron densities at their respective sites of protonation may be explained by differences in the extent of cation solvation. It is also conceivable that the pKa value of hexamethylmelamine may reflect a combination of the electron densities of both the ring and the exocyclic nitrogen atoms.

The results of the electron density calculations (Figs. 4.58, 4.59) show that the nitrogen atoms bearing the methyl groups in hexamethylmelamine (electron density = 7.263, Fig. 4.58) are less basic than the nitrogen atom in N,N-

dimethylaniline (electron density = 7.289, Fig. 4.59). This is in agreement with the hypothesis that the formation of relatively stable N-methylols during N-demethylation may depend on the electron deficiency of the nitrogen bearing the methyl group in the parent compound. From the results presented in Figs. 4.58 and 4.59 it could be argued that an electron density between 7.263 and 7.289 on the nitrogen atom bearing the methyl group predisposes the molecule to form a characterizable N-methylol during N-demethylation under the conditions used in this study. Assuming this to be the case it is conceivable that N,N-dimethylnitrosamine may also form an N-methylol during N-demethylation as the electron density of the nitrogen bearing the methyl groups in this molecule (electron density = 7.226, Fig. 4.60) is less than that of the exocyclic nitrogen atoms of hexamethylmelamine (electron density = 7.263, Fig. 4.58). Indeed that the N-hydroxymethyl derivative of N,N-dimethylnitrosamine; N-hydroxymethyl-N-methylnitrosamine; has been shown to be sufficiently stable to be synthesized, (Mochizuki et al., 1980) further supports this hypothesis.

It must be stressed that interpretation of the results of these electron density calculations is speculative as only three types of compound have been studied. In addition the initial structural data originates from crystal structure determinations and the geometry of a particular molecule may well be different in solution or at the active site of the enzyme(s) responsible for N-demethylation.

SECTION 5

GENERAL DISCUSSION

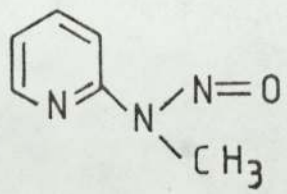
The method used to differentiate between formaldehyde and its more stable precursors produced during the in-vitro N-demethylation of N-methyl containing compounds (sec. 4.II) has many disadvantages. The major problem inherent in the method was that characterization of a particular metabolite was not possible as the assay was only able to differentiate between formaldehyde and its precursors. In addition the use of the method depended upon two assumptions. The first of these was that formaldehyde precursors such as N-hydroxymethyl compounds could be detected analytically as formaldehyde using the Nash reaction. That this assumption did not have general applicability was demonstrated in sec. 4.II. Some N-hydroxymethylamides, for example, when in solution at pH= 7.4 do not produce a positive reaction in the Nash test, presumably because they are sufficiently stable under these conditions not to degrade to produce formaldehyde. This suggests that the Nash test is inadequate when used to assess the N-demethylation of those N-methyl containing compounds which form stable N-hydroxymethyl compounds during metabolism which do not react with Nash reagent. This was confirmed in in-vitro studies of the N-demethylation of N-methylbenzamide (sec. 4.VI.2.b) and N,N-dimethylformamide (sec. 4.V.2.b), where the stability of the respective N-hydroxymethyl compounds resulted in a negative Nash test even though detectable metabolism had occurred.

It could therefore be argued that the three

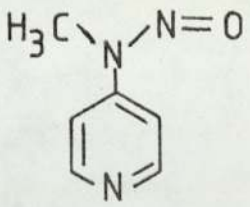
substrates which did not produce Nash positive species during microsomal metabolism (sec. 4.I.2.b); 4-dimethylaminopyridine (Ie), N′-(4-sulphonamidophenyl)-N,N-dimethylformamidine (IIIf), and N′-(4-cyanophenyl)-N,N-dimethylurea (IVc) were N-demethylated but produced stable N-methylols which did not react with Nash reagent. However there is evidence which suggests that this was not the case and this is summarized below.

The pKa value of 4-dimethylaminopyridine is 9.60 whilst that of its 2-dimethylamino isomer, which was N-demethylated to Nash positive species by microsomal enzymes, is 6.99 (Cruège et al., 1970). Thus greater than 99% of the 4-dimethylaminopyridine would be in the protonated form at pH = 7.4, whereas 2-dimethylaminopyridine would be predominately in an unionized form at pH = 7.4. As microsomal N-demethylation has been shown to depend on lipid solubility (Gaudette & Brodie, 1959) the high degree of protonation of 4-dimethylaminopyridine would presumably decrease the extent of its metabolism compared with that of 2-dimethylaminopyridine. In addition Heydt et al. (1982) have shown, using H.P.L.C, that 2-nitrosomethylaminopyridine (I, scheme 5.1) was N-demethylated by rat liver microsomes to a much greater extent than its 4-nitrosomethylamino isomer (II, scheme 5.1). An analogous situation may also exist for 2- and 4-substituted dimethylaminopyridines.

Scheme 5.1



I



II

N/-(4-sulphonamidophenyl)-N,N-dimethylformamidine (IIIf) did not produce Nash positive species during microsomal metabolism, whereas other N,N-dimethylarylformamidines with stronger electron withdrawing substituents in the 4-position of the phenyl ring e.g. cyano (Perrin, 1980) did. It therefore seems unlikely that the substitution of the electron-withdrawing sulphonamido substituent in the phenyl ring rendered the N-hydroxymethyl group in IIIf such a stable species that it did not react in the Nash assay. It is however conceivable that IIIf may have a decreased affinity for the active site of the N-demethylase enzyme(s) because of the steric effect of the 4-sulphonamido substituent. In the case of N/-(4-cyanophenyl)-N,N-dimethylurea (IVc) its poor solubility may have prevented sufficient substrate from entering into solution and thus coming into contact with the microsomal suspension. However, that the three substrates, Id, IIIf and IVc, may have formed stable N-methylols during metabolism which did not react in the Nash assay cannot be totally excluded.

The second assumption on which the method used in the structure-metabolism study was based was that formaldehyde precursors, such as N-hydroxymethyl compounds, would not be

metabolized by mitochondrial and cytosolic enzymes. This was shown not to be true in the case of N-hydroxymethylbenzamide which was metabolized to an N-formyl derivative by enzymes localized in the soluble fraction of the cell (sec. 4.VI.2.b). It could therefore be argued that the removal of Nash positive species observed in the structure-metabolism study did not represent the removal of free formaldehyde but the metabolism of an N-hydroxymethyl compound to a Nash negative species such as the N-formyl derivative. This is unlikely however as the time course of removal of Nash positive species generated during the microsomal metabolism of the model substrates (I — III, VI, Fig. 4.1) was very similar to the time course of removal of free formaldehyde in the same system (Figs 4.3 - 4.6, 4.9). In addition the time course of the generation of the N-formyl derivative from N-hydroxymethylbenzamide in 9000g supernatant fortified with NAD (Fig. 4.54) was not as rapid as the removal of formaldehyde (Fig. 4.3).

Thus both assumptions upon which the colourimetric assay used in the structure-metabolism study was based have been shown not to be applicable in the case of certain N-methylamides and their N-methylol derivatives. However all of the substrates used in the structure-metabolism study, unlike these N-methylamides, produced Nash positive species during metabolism in a microsomal system at pH = 7.4 (sec. 4.I.2.c) apart from the three compounds discussed above which were not metabolized at all. Therefore it could be argued that the two assumptions described in sec. 4.I.1 may still be valid for those N-methyl containing substrates which produce

Nash positive species during metabolism. The results of the structure-metabolism study were, however, interpreted with caution and in the case of the aryldimethylureas further investigations were performed using more specific analytical techniques.

The results obtained using the preliminary colourimetric assay suggested that N′-phenyl-N,N-dimethylureas formed stable precursors of formaldehyde during microsomal metabolism (sec. 4.I.2.c). An investigation of the metabolism of one of these compounds, N′-(4-chlorophenyl)-N,N-dimethylurea (monuron), using H.P.L.C. indicated that the N-hydroxymethyl-N-methyl derivative may have been formed during microsomal metabolism (sec. 4.IV.2.b). This observation supports the hypothesis that the stable formaldehyde precursors found using the colourimetric assay were N-hydroxymethyl compounds formed during metabolism. However the possibility that other stable precursors of formaldehyde were also formed during the in-vitro metabolism of monuron cannot be excluded (sec. 4.IV.3).

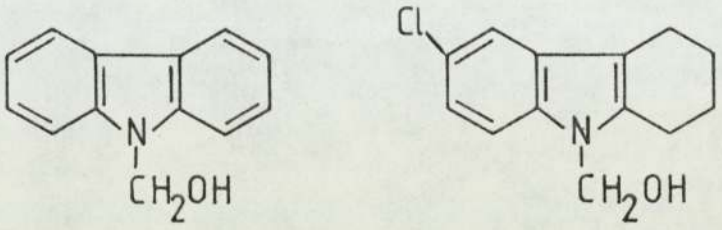
The results presented in this thesis support the view of Gorrod et al. (1970) and Gorrod & Temple (1976), that the formation of a stable N-hydroxymethyl species during the metabolism of an N-methyl containing compound is favoured if the nitrogen bearing the methyl group is in an electron-deficient environment, e.g. - in the N-methyl substituted melamines, N′-phenylureas and amides. Gorrod (1978) has also suggested that the enzyme system which catalyzes the N-oxidation of amines is determined by the pKa

of the nitrogen atom of the substrate. It could therefore be argued that a low basicity of a nitrogen atom which bears a methyl group predisposes the substrate to be N-demethylated by an enzyme system which functions via the generation of N-hydroxymethyl compounds. An alternative pathway for the N-demethylation of relatively basic nitrogen atoms bearing methyl groups may involve N-oxidation and N-oxide demethylation as described in sec. 1.II. However the observation that synthetic N-hydroxymethyl compounds were stable only when the nitrogen bearing the hydroxymethyl moiety was relatively acidic, suggests a purely chemical rather than an enzymic explanation for the formation of stable N-methylols as metabolites of N-methyl containing compounds. One could argue therefore that if a synthetic N-hydroxymethyl compound is sufficiently stable it would be formed during the N-demethylation of the appropriate N-methyl compound.

Electron density calculations (sec. 4.VII) suggest that an electron density value between that of the nitrogen atom in N,N-dimethylaniline (electron density = 7.289) and that of the exocyclic nitrogen atoms of hexamethylmelamine (electron density = 7.263) is required on a nitrogen atom bearing a methyl group to confer stability upon an N-hydroxymethyl intermediate produced during N-demethylation. Therefore changes in structure which affect the electron density distribution around a nitrogen atom may affect the stability of an N-hydroxymethyl compound. This is illustrated by N-hydroxymethylcarbazole (I, scheme 5.2) which is a stable

species and can be isolated from aqueous buffer systems (sec. 1.3), and its 6-chloro-1,2,3,4-tetrahydrocarbazole derivative (II, scheme 5.2).

Scheme 5.2



I II

The nitrogen atom in I is electron deficient, the pKa of N-methylcarbazole being -8.3 (Gorrod & Temple, 1976). However when only one ring is unsaturated as in II, the pKa of the compound is presumably greater than that of I, and this synthetic N-methylol (II) is a very unstable species even in non-polar solvents such as carbon tetrachloride (Murakami & Ishii, 1981).

In this study 4-substitution in the phenyl ring of the aromatic model compounds used in the structure-metabolism study did not affect whether formaldehyde or its precursors were formed during N-demethylation (sec. 4.I.2.c). Likewise 4-substitution in the phenyl ring of N-methylbenzamide did not influence the stability of the respective N-methylolbenzamides sufficiently to influence whether an N-hydroxymethyl compound or formaldehyde was produced during N-demethylation. Substitution on the nitrogen atom of N-methylbenzamide however, did markedly affect the stability of the N-methylol produced during N-demethylation. The

N-hydroxymethyl compound produced during the metabolism of N,N-dimethylbenzamide (II, Fig. 4.57) was an unstable species and degraded partly to produce formaldehyde under the conditions used for in-vitro metabolism studies (sec. 4.VI.2.b). This N-methylol (II, Fig. 4.57) also produced a positive response in the Nash test under the conditions used whereas the N-hydroxymethyl derivative of N-methylbenzamide (IV, Fig. 4.57) did not. Therefore in the metabolic scheme proposed for the N-methyl groups in N,N-dimethylbenzamide (Fig. 4.57) the first N-demethylation produces formaldehyde liberated from the degradation of an unstable N-methylol, whereas the second N-demethylation yields a stable N-methylol. The explanation of the inherent instability of the N-hydroxymethyl-N-methyl derivative as compared to the N-hydroxymethyl species may be because of the electronic effects of the second methyl moiety or because of the lack of an amide hydrogen atom which prevents solvent participation in hydrogen bonding (sec. 4.VI.3). Whatever the reason it is conceivable that N-methylols of differing stabilities may be generated during the successive N-demethylations of other N,N-dimethyl containing substrates.

Clearly as well as N-methylols produced during successive N-demethylations of the same substrate, N-methylols generated during the metabolism of different types of N-methyl containing compound have differing stabilities. There are those obtained from N,N-dimethylaminoantipyrine which decompose rapidly to formaldehyde and the N-desmethyl compound, those of an intermediate stability such as derived from N-methyl substituted melamines and N′-phenylureas, and those which are

so stable that they only react partly or not at all with Nash reagent in the absence of added alkali. In addition different types of N-methylol may have different pH dependent mechanisms of breakdown (sec. 4.II.3) and these observations may have toxicological implications. For example Negishi & Hayatsu (1980) have proposed that the greater stability of N-hydroxymethyl-N-methylnitrosamine in acidic solution (pH = 2-4) as compared to its stability in neutral or basic solution (Mochizuki et al., 1980) may explain the increased mutagenic activity of N,N-dimethylnitrosamine observed in an in-vitro test when acidic conditions were used. It is therefore conceivable that a particular type of N-methylol may be responsible for the antitumour activity seen with certain N-methyl containing compounds; e.g. - N-methyl substituted melamines, and the toxicity and carcinogenicity of other N-methyl containing compounds such as monuron, N,N-dimethylnitrosamine and N,N-dimethylaminoazobenzene.

N-hydroxymethyl compounds may also be further metabolized. In this study an N-formyl species was characterized both in-vitro and in-vivo as a metabolite of both N-methylbenzamide and N-hydroxymethylbenzamide. The results suggest that alcohol dehydrogenases sensitive to inhibition by pyrazole may be the enzymes responsible for this conversion. The metabolic generation of an N-formyl species from an N-hydroxymethyl containing substrate may be of toxicological significance as this conversion has been suggested as a possible route of activation of the carcinogens N,N-dimethylnitrosamine and methylazoxymethanol (Schoental,

1973). It has also been proposed that this conversion is responsible for the organ specific carcinogenicity of 1,2-dimethylhydrazine and its derivatives such as methylazoxymethanol. In a study using the cytosol of various rat tissues Grab & Zedeck (1977) showed that only the cytosols derived from those organs in which tumours arose after the administration of methylazoxymethanol - colon, caecum and liver - were able to catalyze the reduction of NAD using methylazoxymethanol as substrate. These authors showed that this reduction of NAD using methylazoxymethanol could also be catalyzed by horse liver alcohol dehydrogenase and inhibited by pyrazole. It has recently been shown that pyrazole inhibited the formation of intestinal tumours in rats which had received methylazoxymethanol, although paradoxically the rats developed skin and kidney tumours (Notman et al., 1982). The authors suggested that pyrazole-sensitive alcohol dehydrogenase does play a role in the tumourigenic effects of methylazoxymethanol, but that other non pyrazole-sensitive enzymes exist in other organs which can also activate this carcinogen. Prival & Mitchell (1981) have also demonstrated that cytosol in addition to the microsomal fraction of liver is required for the activation of N,N-dimethylnitrosamine to a mutagenic species in-vitro. Although the effect of pyrazole was not evaluated in this study it may be speculated that the generation of an N-formyl species may be involved in the mechanism of mutagenicity of N,N-dimethylnitrosamine observed in-vitro.

Finally it is pertinent to note that the N-formyl derivative produced during the metabolism of N-hydroxymethylbenzamide was less stable than the parent N-methylol and decomposed to yield the corresponding amide (sec. 4.VI.2.b). This shows that in certain cases both N-hydroxymethyl and N-formyl species can be intermediates in N-demethylation, and that N-demethylation itself need not be synchronous with formaldehyde production (see Fig. 4.57).

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