METABOLISM OF PHENACETIN IN THE RAT

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SUMMARY

The abuse of phenacetin-containing analgesic mixtures has been linked epidemiologically with nephrotoxicity and carcinogenicity in man.

In addition, clinical and histopathological tests after chronic administration of phenacetin in man and animals have indicated that it is nephrotoxic and carcinogenic. Furthermore, it has a chemical similarity to other known carcinogenic arylamides. However, the induction of toxicity with phenacetin remains a controversial subject.

A number of chronic dosing studies with phenacetin have been carried out to demonstrate the ability of the drug to induce carcinogenesis and nephropathy. However, none have sought to explain the reasons for the chronic nature of phenacetin toxicity on the basis of the increased formation of toxic metabolites after continued administration of the drug. In the present chronic daily-dosing study with phenacetin in the rat, the metabolism of the drug was monitored by analysing urine samples at regular weekly intervals. Particular attention was paid to the formation of N-hydroxyphenacetin, which has been implicated in phenacetin carcinogenicity.

The metabolism of phenacetin was monitored in five groups of Hooded Wistar rats. Each group was subjected to a different treatment. The purpose was to elucidate the effects of the size of the dose, duration of treatment, influence of commonly co-
administered drugs (aspirin, caffeine) and the influence of a sulfation inhibitor (pentachlorophenol) on the metabolism of phenacetin.

The metabolic trends indicated auto-induction of N-hydroxylation, evidenced by the increased formation of N-hydroxyphenacetin in all treatments. The induction was most pronounced with the large dose of phenacetin and, significantly, was prominent with the co-administration of aspirin at the lower dose of phenacetin.

Paracetamol-sulfate was the major metabolite of phenacetin in the rat, while paracetamol-glucuronide and free paracetamol were the other products of the deethylation pathway of phenacetin. The mercapturate and cysteinyl conjugates were not detected.

Pentachlorophenol, a known inhibitor of sulfation, did not block sulfation completely. The partial suppression of sulfation with pentachlorophenol resulted in the increased formation of 2-hydroxy-p-phenetidine and yielded a larger fraction of unchanged phenacetin in the urine.

The metabolism of p-phenetidine, a deacetylated metabolite of phenacetin, was followed in freshly isolated rat hepatocytes.

The biotransformation of p-phenetidine to phenacetin and 2-hydroxyphenetidine indicated that N-acetylation was significant in the Hooded Wistar rat. However, N-acetylation and aromatic hydroxylation did not fully account for the disappearance of p-phenetidine from the in vitro system.
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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

(C.A.D'SOUZA)
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CHAPTER 1. INTRODUCTION

1.1. ANALGESIC ABUSE-ASSOCIATED TOXICITY AND PHENACETIN

Phenacetin, a widely used antipyretic-analgesic, was first introduced for the treatment of pain in 1887. It continued to be extensively used, chiefly as a constituent in analgesic mixtures, until recently (Flower et al., 1980). Analgesic nephropathy has been recognized as a frequent clinical, radiological and autopsy finding in Scandinavia (Lindgren et al., 1959; Bengtsson, 1962; Harvald, 1963), Australia (McCutcheon, 1962; Jacobs and Morris, 1962; Dawborn et al., 1966; Kincaid-Smith, 1969), Britain (Jacobs, 1964; Sanerkin and Weaver, 1964), United States of America (Reynolds and Edmondson, 1963) and Canada (Lakey, 1961). However, controversy persists with regard to the involvement of phenacetin in the development of analgesic nephropathy (Freeland, 1975; Nanra, 1976).

1.1.1. ANALGESIC-INDUCED NEPHROPATHY IN MAN

Analgesic abuse, now defined as an intake in excess of 1 g of an analgesic per day, for one year, by Bengtsson et al. (1978), was first identified as a problem in Sweden, with particular reference to phenacetin, in 1918 (Grimlund, 1963). However, it was not until 1953 that Spuhler and Zollinger (1953) pointed out that analgesic drugs could cause chronic renal disease. It has since been an area of concern demanding intensive research effort to explain the induction of toxicity by analgesics.

Dubach and co-workers conducted an epidemiological study of the
abuse of analgesics in a Swedish population, over a period of ten years from 1968-1979, and concluded that heavy users of analgesic mixtures over the course of a decade exhibited a higher incidence of both abnormal kidney function and kidney-related mortality, though the absolute incidences remained small even among heavy users (Dubach et al., 1968; 1971; 1975; 1983). Further evidence implicating analgesics in nephrotoxicity was provided by the studies of Duggin (1977), Bengtsson et al. (1978), Kincaid-Smith (1978), Bengtsson and Angervall (1979) and Prescott (1966; 1982), who examined the relationship between ingestion of analgesics and the development of renal disease.

Analgesic abuse-associated nephropathy, as the condition is known and described today, is affected by addiction or abuse of alcohol, cigarettes, barbiturates, hypnotics, tranquilizers and laxatives (Prescott, 1976; Kincaid-Smith, 1978; Nanra et al., 1978).

1.1.2. ANALGESIC-INDUCED CARCINOMA IN MAN

Phenacetin has also been implicated as a cause of carcinoma of the renal pelvis. Hultengren et al. (1965) were the first to suggest the association of analgesic abuse with carcinoma in Sweden. They were followed by researchers in other countries (Taylor, 1972; Liu et al., 1972; Bengtsson et al., 1978; Lornoy et al., 1979), who provided further evidence on the relationship between analgesic abuse and tumors of the renal pelvis.
1.1.3. PHENACETIN-INDUCED TOXICITY IN MAN

The decline in the use of phenacetin is also attributed to the methemoglobinemia, sulfhemoglobinemia and hemolytic anemia it produces on chronic administration (Flower et al., 1980), as well as nephropathy (Spuhler and Zollinger, 1953) and neoplasia (Liu et al., 1972). Although the adverse effects produced by phenacetin are reversed in most instances on withdrawal of the drug, they still remain an alarming testimony of drug-induced pathophysiological disorders. The fact that phenacetin was the one common ingredient in all combination analgesics responsible for renal impairment (Koutsaimanis and de Wardener, 1970), was used to incriminate it as the compound responsible for the increased incidence of tumors of the renal pelvis (Hultengren et al., 1965; Bengtsson et al., 1968; Angervall et al., 1969; Johansson et al., 1974; Johansson and Wahlqvist, 1977) interstitial nephritis and renal papillary necrosis (Nordenfelt and Ringertz, 1961). It was also seen that the withdrawal of phenacetin from analgesic preparations in Sweden in 1961 resulted in fewer deaths from renal failure among analgesic abusers in subsequent years (Nordenfelt, 1972), an observation that added credence to the alleged toxicity of phenacetin. However, recent data suggests that phenacetin may not be the only toxic analgesic (Sec. 1.1.5).

1.1.4. PHENACETIN-INDUCED TOXICITY IN ANIMALS

In earlier animal experiments, phenacetin failed to manifest any noteworthy toxicity. Neither phenacetin nor most of its metabolites were proven to be significantly nephrotoxic in
animals (Calder et al., 1971). Only minimal renal papillary necrosis has been produced in animals by administration of phenacetin alone, even in large doses (Clausen, 1964; Fordham et al., 1965). Although renal pelvic tumors in laboratory animals have not been encountered after long-term administration of phenacetin, it must be borne in mind that several bladder carcinogens exhibit species specificity, and failure to produce carcinoma in animals is not sufficient proof of their safety in man (Nery, 1971a). For example, 2-naphthylamine, a known carcinogen in man, does not produce malignancies in rats, rabbits, cats and mice (Bonser et al., 1959). However, in recent years phenacetin has been shown to cause hepatic necrosis in hamsters (Mitchell et al., 1975; 1976; Nelson et al., 1978). Chronic administration of phenacetin to rats in the diet induced urothelial hyperplasia of the renal papillae, earduct tumors and mammary adenocarcinomas (Johansson and Angervall, 1976), nasal carcinomas and urinary tract tumors (Isaka et al., 1979) and had a carcinogenic effect on most tissues (Johansson, 1981). These studies suggested a more general carcinogenic effect of phenacetin in the rat. The rat therefore has been chosen as a suitable animal model for the investigation of the metabolism of phenacetin to putative toxic metabolites.

1.1.5. TOXICITY INDUCED BY OTHER ANALGESICS

It should be noted, however, that renal papillary necrosis has been produced by aspirin, phenacetin and caffeine mixtures and by aspirin alone, more readily than by phenacetin itself (Abrahams et al., 1964; Saker and Kincaid-Smith, 1969; Nanra and
Kincaid-Smith, 1970; 1972a; 1973b; Nanra et al., 1970). Lesions were seen to develop more frequently at low dose levels with aspirin than with phenacetin (Axelsen, 1976). The substantial increase in excretion of renal tubular cells (a gauge of renal damage) observed in healthy volunteers receiving aspirin, compared with those receiving phenacetin, was taken as further evidence of the greater nephrotoxic risk of aspirin (Prescott, 1965) in comparison to phenacetin. Cognisance must also be taken of the renal papillary necrosis reportedly induced in animals with other analgesics and nonsteroidal anti-inflammatory drugs including phenazone (Nanra and Kincaid-Smith, 1973a) indomethacin and phenylbutazone (Nanra et al., 1970; Arnold et al., 1974), amidopyrine (Brown and Hardy, 1968), mefenamic acid (Nanra et al., 1970) and the sole responsibility of phenacetin for such toxicity diminished.

Although in no cases reported have patients consumed phenacetin alone, the incrimination of phenacetin as the nephrotoxic and carcinogenic entity in analgesic mixtures has been made on the presumption that its metabolism is similar to that of known carcinogenic amines (Miller and Miller, 1966a; 1966b; Bengtsson et al., 1978). The metabolism of phenacetin has been investigated in several species, including rats, hamsters, rabbits and human subjects (Smith and Griffiths, 1976; Kuntzman et al., 1977; Prescott, 1980; Vaught et al., 1981). However, there remains the need to elucidate, in greater depth, its metabolism after chronic administration.
1.2. METABOLITES OF PHENACETIN

Several studies have been carried out to identify the metabolites of phenacetin since Brodie and Axelrod (1949) and Smith and Williams (1949) independently found N-acetyl-p-aminophenol (paracetamol, APAP) as the major and p-phenetidine (PN) as the minor metabolite of phenacetin. Jagenburg and Toczko (1964) isolated S-(1-acetamido-4-hydroxyphenyl)cysteine as a urinary metabolite of phenacetin in man, while Klutch et al. (1966) identified 2-hydroxyphenacetin in the urine of humans, dogs and cats. Buch et al. (1966,1967) detected 2-hydroxyphenetidine (2HPN), in human and rat urine and 3-hydroxyphenacetin in the urine of rats given phenacetin.

N-hydroxyphenacetin (NHP), whose conjugates are postulated to be potential carcinogens, was reportedly first detected in the urine of cats and dogs treated with phenacetin (Klutch et al., 1966). It was further found in the urine of humans and dogs by Klutch and Bordun (1968), but had not been indisputably demonstrated to be a metabolite of phenacetin in vivo (Weisburger and Weisburger, 1973; Hinson and Mitchell, 1976), until 1981 in the rat (McLean et al., 1981). Kiese and Lenk (1969) detected 4-ethoxyglycolanilide as a phenacetin metabolite in the urine of rabbits. Nery (1971b) found N-acetyl-S-ethylcysteine, acetamide and quinol to be metabolites of phenacetin in the rat. Focella et al. (1972) identified 4-hydroxy-3-methylthio-acetanilide, while Fischbach et al. (1977) found N-[4-(2-hydroxyethoxy)phenyl]-acetamide in the urine of rats and rabbits after administration of phenacetin.
4-Acetaminophenoxyacetic acid was detected as a new metabolite of phenacetin by Dittman and Renner (1977) and 3-methylthio-4-hydroxyacetanilide by Klutch et al. (1978).

Recently there has been considerable interest in the disposition of phenacetin and its metabolites in animals and in man (Prescott et al., 1968; Kampffmeyer, 1974; Raafflauub and Dubach, 1975; Welch et al., 1976) with relevance to enzyme induction, which increased the metabolism of phenacetin (Pantuck et al., 1974). Habitual smoking is reported to enhance the metabolism of phenacetin (Pantuck et al., 1972) and dietary habits such as the consumption of charcoal broiled beef have been known to increase the metabolism of phenacetin (Conney et al., 1976). The potential interaction of reactive metabolites of phenacetin with biological macromolecules has also been investigated in recent years (Mulder et al., 1977; Hinson et al., 1977).
FIG. 1: Biotransformation of phenacetin
1.3. BIOTRANSFORMATION OF PHENACETIN

The metabolic biotransformations by which phenacetin is hydroxylated, oxidatively deethylated, N-deacetylated and conjugated by hepatic microsomal enzymes (Brodie and Axelrod, 1949; Smith and Williams, 1949; Klutch et al., 1966; Buch et al., 1967; Prescott, 1969; Nery, 1971b; Focella et al., 1972; Mrochek et al., 1974) are depicted in Fig. 1.

1.3.1.1. PARACETAMOL: METABOLITE-MEDIATED TOXICITY

Paracetamol is the major, immediate metabolite of phenacetin (Brodie and Axelrod, 1949) and is a widely used antipyretic-analgesic in its own right. It is known to be hepatotoxic in massive overdosage (Boyd and Bereczky, 1966; Prescott et al., 1971; Mitchell et al., 1973a; Kleinman et al., 1980) and nephrotoxic following prolonged abuse (Duggin and Mudge, 1976; Mitchell et al., 1977; Mudge et al., 1978) due to metabolic activation to a highly reactive toxic metabolite (Mitchell et al., 1973a; 1973b; Jollow et al., 1973; Potter et al. 1973; Hinson et al., 1980; Hinson and Gillette, 1980).

The implication of a reactive metabolite in paracetamol-induced toxicity was first disclosed by the studies of Mitchell et al. (1973a), who showed that paracetamol produced an increased incidence of hepatic necrosis in rats in which drug metabolizing enzymes were induced by prior treatment with phenobarbitone or 3-methylcholanthrene, and that a decrease in the incidence and severity of toxicity followed the use of inhibitors of drug metabolism such as piperonyl butoxide or cobaltous chloride.
(Potter et al., 1974) and recently, cimetidine (Mitchell et al., 1981). This reactive metabolite is formed by a microsomal cytochrome P-450 mixed function oxidase and is detoxified by conjugation with glutathione (Mitchell et al., 1973b; Jollow et al., 1973).

Paracetamol has also been recognized as a nephrotoxic metabolite of phenacetin by other workers such as Nanra et al. (1980) and Margetts (1976). Further observations that patients who persisted in abusing analgesic mixtures in which phenacetin was replaced with salicylamide or paracetamol still presented with typical analgesic nephropathy and renal failure (Krishnaswamy and Nanra, 1976; Nanra et al., 1978) corroborates the nephrotoxic potential of paracetamol.

In animal studies Nanra et al. (1970) and Nanra and Kincaid-Smith (1970; 1972b) established a similarity between the nephrotoxicity caused by phenacetin and that produced by paracetamol when administered alone or in combination with other analgesic constituents. It seems apparent, therefore, that if the toxicity caused by the chronic ingestion of phenacetin alone is accepted, then it is metabolite-mediated and could be at least partly due to one or more of the highly reactive metabolites of paracetamol (Sec. 1.3.1.2).
1.3.1.2. PARACETAMOL: POSTULATED REACTIVE METABOLITES

Jollow et al. (1973) and Mudge et al. (1978) established that acute overdosage of paracetamol in rats and mice ultimately resulted in covalent binding of the reactive metabolite to tissue macromolecules in the liver and kidney, after the depletion of glutathione. Although a study of urinary metabolites has indicated that reactive intermediates had reacted with cellular glutathione (Hinson et al., 1980; Jollow et al., 1974a) none of the postulated toxic intermediates have been identified as being solely responsible for the toxicity, during the course of in vivo and in vitro (microsomal) experimentation.

1.3.1.2.1. N-HYDROXYPARACETAMOL

Among the proposed toxic metabolites (Fig. 2) was N-hydroxyparacetamol (NHAPAP). This had been earlier shown to dehydrate to N-acetyl-p-benzoquinoneimine (NAQ), a compound known to react with cellular glutathione and protein and postulated to be toxic (Jollow et al., 1974a). Therefore NHAPAP was believed to be the toxic reactive intermediate of paracetamol (Mitchell et al., 1973a; 1973b; Jollow et al., 1973; Potter et al., 1973; 1974). This concept was questioned because NHAPAP was not detected as a metabolite of paracetamol although it is formed from phenacetin (Hinson et al., 1979a; Nelson et al., 1980). At a physiological pH NHAPAP did not rapidly dehydrate to NAQ (Healey et al., 1978; Gemborys et al., 1980) and was only slightly more toxic than APAP (Healey et al., 1978), hence could not be regarded as its toxic intermediate.
1.3.1.2.2. 3-4, EPOXIDE-PARACETAMOL

The implication of 3,4-epoxy-paracetamol as the toxic metabolite (Andrews et al., 1976) was negated by the investigations of Hinson et al. (1980) and Hinson and Gillette (1980). They demonstrated through mass spectral studies that atmospheric oxygen was not incorporated into the paracetamol-glutathione adduct, which would otherwise be expected were paracetamol to form an arene oxide in the 3,4-position followed by rearrangement to NAQ prior to conjugation (Hinson et al., 1977). Also when p-18-0-paracetamol was used as a substrate, all of the 18-0 was retained in the paracetamol-glutathione complex (Hinson et al., 1979c). The evidence does not indicate 3,4-epoxidation of paracetamol, but similar experiments with phenacetin did reveal 3,4-epoxidation as one mechanism of microsomal activation of phenacetin to a reactive metabolite (Hinson et al., 1979a).

A theory of free radical mediated toxicity has been proposed but is yet to be proved (Andrews et al., 1976).

1.3.1.2.3. N-ACETYL-p-BENZOQUINONEIMINE

The other proposed toxic intermediate for paracetamol in the literature to date is the electrophile N-acetyl-p-benzoquinoneimine (NAQ). NAQ, which covalently binds with protein (Mulder et al., 1978) and glutathione (Hinson et al., 1979c), has been generally accepted as the most likely ultimate toxic intermediate of paracetamol (Hinson et al., 1979a; Nelson et al., 1980; Calder et al., 1981). Though it was supposedly formed from NHAPAP, a toxic reactive species in itself (Jollow
et al., 1974a), the evidence (Hinson et al., 1979a; Nelson et al., 1980) indicates that it must be formed from still another reactive source because NHAPAP, with a relatively slow decomposition (Hinson et al., 1979a) which would enable it to be detected if it were formed, is not an intermediate involved in the metabolism of paracetamol in vitro (Hinson et al., 1979a; Nelson et al., 1980) or in vivo (Gemborys and Mudge, 1981). Calder et al. (1981) further confirmed that no N-hydroxylated metabolites resulted from paracetamol administration and postulated the concept of hepatotoxicity and nephrotoxicity being directly mediated by an oxidation of paracetamol to the toxic reactive intermediate NAQ by the cytochrome P-450-dependent mixed function oxidase. However, NHAPAP could still be a reactive toxic metabolite of phenacetin, as it has been found to be present as a metabolite of phenacetin and though believed earlier to be formed by the N-hydroxylation of paracetamol, it has since been shown to be formed by the subsequent de-ethylation of N-hydroxylated phenacetin (Hinson et al., 1979a). An in vivo measurement of NHP formation would therefore reflect the extent to which postulated toxic metabolites of paracetamol could be formed from phenacetin.

1.3.1.3. p-AMINOPHENOL

p-Aminophenol (PAP), identified as a urinary metabolite of paracetamol and NHAPAP in the hamster (Gemborys and Mudge, 1981) and known to be highly nephrotoxic though relatively non-hepatotoxic (Green et al., 1969; Calder et al., 1971; Crowe et al., 1979; Newton et al., 1982) could be of toxicological interest.
as well. It has of late been considered to be an immediately reactive nephrotoxic compound (Calder et al., 1979).

1.3.1.4. CONJUGATES OF PARACETAMOL

The major urinary metabolites of paracetamol, paracetamol-glucuronide, paracetamol-sulfate and 3-mercapturate-paracetamol, occur in all species (Gemborys and Mudge, 1981), with other metabolites being identified uniquely to a particular species. These are 4-hydroxyglycoanilide in rats (Smith and Griffiths, 1976), 3-cysteinyl-paracetamol in man and mice (Mrochek et al., 1974; Whitehouse et al., 1977), 3-sulfoxymethyl-paracetamol in hamsters (Wong et al., 1976), 3-thiomethyl-paracetamol in man and dog (Klutch et al., 1978), 3-methoxyparacetamol in man (Andrews et al., 1976) and 3-hydroxyparacetamol in man (Andrews et al., 1976; Mrochek et al., 1974).

1.3.2. p-PHENETIDINE

N-deacetylation is considered to be an essential step in the precipitation of haemotoxicity by acetanilide analogues (Mitchell et al., 1973c). The N-deacetylation of phenacetin gives p-phenetidine (PN) as shown in Fig. 1. This is the second major direct metabolite of phenacetin. The subsequent metabolism of PN has not been adequately reported, although it has been suggested that it undergoes oxidation to quinones and aromatic nitrosoamines and it is known to cause methemoglobinemia by the formation of haemotoxic metabolites NHPN, 2HPN and PAP (Kiese, 1966; Uehleke, 1973). Evidence of its
existence as the second major metabolite of phenacetin and its disappearance in the metabolic process has been recorded using isolated hepatocyte systems (McLean, 1978). The de-ethylated, N-hydroxylated, nitrosated and 2-hydroxylated metabolites of PN have been reported (Brodie and Axelrod, 1949), but their contribution to nephrotoxicity has not been determined.

1.3.3. 2-HYDROXYPHENACETIN, 3-HYDROXYPHENACETIN and 2-HYDROXYPHENETIDINE

The hydroxylated products of phenacetin, 2-hydroxyphenacetin (2HP), 3-hydroxyphenacetin (3HP) and 2-hydroxyphenetidine (2HPN) have been reported (Buch et al., 1966; 1967; Klutch et al., 1966) earlier. 2HP was devoid of antipyretic activity in rats and did not cause methemoglobinemia in dogs and in rats no abnormal gross or histological changes in kidney function or structure were produced on oral administration for a prolonged period (Burns and Conney, 1965). No toxicity has been reported with 2HP in the concentrations at which it is usually present. However, isolated cases of toxicity have been reported for 2HPN. Shahidi and Hemaidan (1969) presented a case where large amounts of 2HPN were present in the urine of a female patient who developed a severe hemolytic reaction. These abnormalities in response were rare and familial.
1.3.4. N-HYDROXY METABOLITES

Many toxic substances are generally detoxified within biological systems by conjugation with glucuronic acid by UDP-glucuronyltransferase, or sulfation by sulfotransferase (Mulder and Scholtens, 1977), or combination with glutathione (GSH) (Mitchell et al., 1973b; 1974; Jollow et al., 1974b). Whether a compound would eventually prove to be toxic, would therefore depend on the extent to which the conjugating enzymes were active and the quantity of glutathione available for electrophilic "mopping up".

In recent years attention has been focussed on the toxicity caused by arylamines. The arylamines have earned notoriety as compounds capable of undergoing hydroxylation by hepatic mixed function oxidases to yield the proximal carcinogenic N-arylhydroxylamines and N-arylhydroxamic acids (Baldwin and Smith, 1965; Miller and Miller, 1966a; Kiese, 1966; Miller, 1970; Weisburger and Weisburger, 1973; Miller, 1978).

Several compounds possessing an N-hydroxyl group in their molecules, have been reported as carcinogenic compounds. N-arylhydroxylamines such as N-hydroxy-2-naphthylamine, N-hydroxy-4-aminobiphenyl (Radomski and Brill, 1970; 1971; Radomski et al., 1977) and N-arylhydroxamic acids such as N-hydroxy-2-acetylaminofluorine (Miller et al., 1961; Razzouk et al., 1977), whose activated esters such as the sulfate, acetate and glucuronide, are strong electrophiles. These compounds have been shown to possess carcinogenic character reflected in their ability to bind to the nucleophilic groups in cellular
macromolecules (Scribner and Naimy, 1973; Miller et al., 1974; Miller and Miller, 1976).

In specific cases of N-arylhydroxylamines and N-arylhydroxamic acids such as N-hydroxy-2-acetylaminofluorene (DeBaun et al., 1970), N-hydroxy-N-methyl-4-aminobenzene (Kadlubar et al., 1976), N-hydroxy-4-acetylaminobiphenyl (Kriek, 1971), N-hydroxy-N,N-diacetylbenzidine (Morton et al., 1980), N-hydroxy-2-acetylaminophenanthrene (Scribner and Naimy, 1973) and N-hydroxyphenacetin (Mulder et al., 1977), sulfation increased the toxicity of the parent compounds.

The sulfate conjugates produced were highly reactive with tissue nucleophiles. Contrary to expectations of the detoxification role of sulfation in biological processes, sulfation of certain N-arylhydroxylamines and N-arylhydroxamic acids results in the formation of their reactive entities.

The esterification of N-arylhydroxylamines and N-arylhydroxamic acids has been therefore regarded as an activation pathway leading to carcinogenesis for N-hydroxy compounds (Weisburger, 1978). However, the carcinogenic character of a compound is not entirely explained by the occurrence of an N-hydroxyl group within its molecular structure (Weisburger, 1978). Phenylhydroxylamine and N-hydroxysuccinimide are examples of such non-carcinogenic compounds.
1.3.4.1. **N-HYDROXYPHENACETIN : A PROXIMAL CARCINOGEN**

NHP is an important metabolite of phenacetin, in terms of its carcinogenicity. It has been suggested to be the metabolite of phenacetin most likely to induce tumors due to its chemical similarity to the known carcinogenic N-arylhydroxamic acids (Calder and Williams, 1975). It has also been suggested as the metabolic product of phenacetin which could best account for the covalent binding of phenacetin to cell protein (Nery, 1971a) and as an intermediate in the formation of other products of phenacetin metabolism (Nery, 1971b; Calder et al., 1974). The mechanism of the renal and hepatic toxicity of phenacetin has been proposed to occur in two steps, N-hydroxylation followed by conjugation and subsequent decomposition to a reactive intermediate (Mulder et al., 1977; 1978). The same metabolic sequence activates the carcinogen, 2-acetylaminofluorene. Evidence of **in vitro** N-hydroxylation by liver microsomes has been demonstrated in hamsters (Hinson and Mitchell, 1976), rabbits (Fischbach et al., 1977), mice (Kapetanovic et al., 1979) and **in vivo** experiments in rats (McLean et al., 1981). Evidence of conjugation with sulfate and glucuronide **in vitro** by rat liver enzymes, and subsequent decomposition has been contributed by Mulder et al. (1977, 1978). Calder et al. (1976) showed that, in chronically treated rats NHP induced neoplasia and tumors of the liver were a direct manifestation of its carinogenicity. A quantitative estimation of NHP over a prolonged study period would therefore provide information on the increased formation or accumulation of this metabolite, which may ultimately relate to the toxicity of the parent analgesic compound, phenacetin.
1.4. CHRONIC DOSING STUDIES

Since toxicity from phenacetin is only seen after prolonged administration of the drug, progressive monitoring of metabolic changes over extended periods, accompanied by pathophysiological surveillance and toxicokinetic evaluation would prove helpful in determining the cause of phenacetin-induced carcinogenicity and nephrotoxicity.

The effect of the size of the chronic dose on metabolism and or on the development of carcinoma or nephropathy could also be included in such investigations. The development of tolerance or increased susceptibility to phenacetin-toxicity could further be closely examined as there have been reports of related analogues of phenacetin being able to protect animals from paracetamol-induced hepatotoxicity (Kapetanovic, 1979). Chronic dosing with phenacetin itself has been shown to cause tolerance and protection from hepatotoxicity (Boyd and Hottenroth, 1968; Boyd, 1971; Carro-Ciampi, 1971; 1972; Kapetanovic and Mieyal, 1979). The metabolic interference likely to be caused by other drugs or compounds co-administered with phenacetin could also be investigated.

1.4.1. ALTERED METABOLISM WITH CHRONIC DOSING

Carro-Ciampi (1972), in chronic studies with phenacetin, further demonstrated tolerance to phenacetin-induced hypothermia in both albino rats and guinea pigs, after repeated daily administration. Irrespective of the daily dose used, tolerance developed in guinea pigs more slowly than in albino rats. In
acute phenacetin toxicity death is due to hypothermic coma as well as cyanosis, respiratory depression and cardiac arrest and therefore, when tolerance develops to phenacetin hypothermia, animals are able to survive normally lethal doses of the drug (Boyd, 1959; 1960; Boyd et al., 1969; Carro-Ciompi, 1971).

These chronic-dose studies (Carro-Ciampi, 1972) indicated a halving of plasma levels of phenacetin within 10 days of initiation of phenacetin administration. This effect developed more slowly in guinea pigs, and was accompanied by a marked increase in PN levels. This would account for the chronic hemolytic anemia (Schnitzer and Smith, 1966) and methemoglobinemia (Brodie and Axelrod, 1949) seen more commonly in guinea pigs.

One hundred day LD 50 studies provide further examples of how chronic dosing studies (Boyd and Hottenroth, 1968; Boyd and Carro-Ciampi, 1970; Boyd, 1971) undertaken to stress the importance of toxicity caused by prolonged administration of phenacetin, could be beneficial.

1.4.2. **INFLUENCE OF DOSE SIZE, ADMINISTRATION ROUTE, SPECIES VARIATION AND DURATION OF TREATMENT**

Few investigations have been carried out to examine factors such as dose, administration route and species which could probably affect the metabolism of phenacetin and its alleged toxicity with chronic use. Smith and Timbrell (1974) carried out such investigations and found the drug to be largely metabolized in the rat, rabbit, guinea pig, ferret and man by oxidative
deethylation and deacetylation. Minor pathways of aromatic hydroxylation and cysteine conjugation were also present.

In species-related metabolism of phenacetin (Smith and Timbrell, 1974), deacetylation was highest in the rat (21% of dose), aromatic hydroxylation to 2-hydroxyphenacetin was highest in the ferret (6% of dose) and formation of the 3-cysteine conjugate was highest in the rabbit (8% of dose). The pattern of conjugation was such that glucuronidation was predominant in the rabbit, guinea pig and ferret while sulfate conjugation was the major route of metabolism in the rat. The metabolic profile differed between large and small oral doses but showed no appreciable differences whether the drug was given orally or intraperitoneally.

Different species of animals are affected by methemoglobinemia caused by phenacetin to varying degrees (Lester, 1943; Welch et al., 1966). This is probably because several deacetylated derivatives of phenacetin, (NHPN, 2HPN and PAP), which are known to oxidize hemoglobin (Uehleke, 1973), are formed to varying degrees in the different species (Smith and Timbrell, 1974).

In studies of Smith and Griffiths (1976) metabolism of 14-C-phenacetin in rats fed the drug in their diet over a 3 month period, was examined and compared with that in a control group of rats receiving only a single dose of the drug. The major metabolite was APAP-SULF in both groups of animals. Variance was noted in glucuronidation between the groups. Other metabolites seen were APAP, p-hydroxyglycoanilide, p-ethoxyglycoanilide and 2HP. Excretion of total radioactivity
was proportionally reduced when larger doses of phenacetin were given.

1.4.3. INFLUENCE OF CO-ADMINISTERED DRUGS

More recently chronic dosing studies with phenacetin, caffeine and aspirin singly or in combination were undertaken by Macklin and Szot (1980) in mice. Histopathological changes indicating mild progressive renal papillary necrosis occurred in the urinary tract with earliest changes observed in those animals on the highest dose of phenacetin. Sulfhemoglobinemia was induced in all animals subjected to treatment with phenacetin alone or in combination with the other agents. Failure to demonstrate carcinogenicity even at these toxic doses of the drugs was in agreement with the negative results obtained in similar studies in mice described in the NCI Technical report (No. 67, 1978) cited by Macklin and Szot (1980), rats (Woodard et al., 1965; Schmahl and Reiter 1954, NCI Tech. report, No. 67, 1978) and dogs (Schmahl and Reiter, 1954; Woodard et al., 1965). Yet, other chronic dosing studies with phenacetin in rats did present evidence of carcinogenicity (Johannson and Angervall, 1976; 1979; Isaka et al., 1979).

The disagreement in these results of very similar experiments was suggested to be due to a formulation shortcoming (Macklin and Szot, 1980). Administration of the dose through the diet in the studies which produced tumours involved pelletization of the drug along with the other food ingredients. This process requires high temperatures of about 80°C, during which the formation of N-oxidation reactive products was claimed to occur.
Nitrosocompounds have been known to induce tumours, particularly of the nasal cavities (Magee et al., 1976) as well as the urinary tract, urinary bladder, renal and mammary glands (Magee and Barnes, 1967; Magee et al., 1976; IARC, 1978; Ito et al., 1971).

Johannsson (1981) studied long term treatment with phenacetin, phenazone and caffeine, individually and in combination. Renal pelvic tumours occurred only in rats treated with phenacetin, or phenazone alone or in combination with caffeine. Half of the rats treated with phenacetin, phenazone and caffeine in combination developed hepatomas which were considered to be a result of the altered metabolism of phenacetin caused by phenazone and caffeine. It was postulated that this altered metabolism ultimately increased the production of N-hydroxyphenacetin, a known liver carcinogen (Johansson, 1981).

1.4.4. INFLUENCE OF AGE AND SEX

Factors of age- and sex-related differences within a species are also likely to affect the metabolism of phenacetin. The hepatotoxicity of paracetamol has been investigated with reference to age of the experimental mice by Hart and Timbrell (1979) who found paracetamol to be less toxic in neonatal mice than in adult animals. This suggests that the development of the ability to detoxify reactive metabolites precedes the development of the enzyme systems producing them, because glutathione levels have been shown to be higher than the levels of P-450 in neonates. Green and Fischer (1981) established from similarly oriented research that age-related changes in
paracetamol metabolism in rats, especially in the extent of glucuronidation or sulfation, are complex and depend on the dose of the drug and sex of the animal. The influence of age and sex on phenacetin metabolism have not been investigated in the present study.

1.4.5. INFLUENCE OF SULFATION INHIBITION

Interference with metabolism by the administration of compounds capable of inhibiting metabolic pathways in order to study the ensuing metabolic changes has been carried out previously (Meerman et al., 1980; Meerman and Mulder 1981; Mulder and Scholtens, 1977). The sulfation pathway, which is a predominant metabolic pathway for phenacetin in the rat, has been selectively inhibited by 2,6-dichloro-4-nitrophenol, salicylamide and pentachlorophenol with a simultaneous increase in glucuronidation of the substrate, harmol (Mulder and Scholtens, 1977). It has been previously postulated that N-O-sulfation, following N-hydroxylation, has been responsible for the production of the reactive metabolites of phenacetin which combine with tissue macromolecules (Mulder et al., 1977; 1978). The analogous N-O-sulfation product of N-hydroxy-2-acetylamino-fluorene has been similarly implicated in the precipitation of toxicity (DeBaun et al., 1970). Inhibition of sulfation would therefore be expected to obviate toxicity claimed to be caused by the N-O-sulfate conjugate of NHP. This, therefore, warrants investigation with a further study of any accompanying metabolic changes that occur when sulfation is inhibited.
FIG. 2: Postulated mechanisms of phenacetin-induced toxicity
1.5. POSTULATED MECHANISMS OF PHENACETIN-INDUCED TOXICITY

Although phenacetin is referred to as a toxic drug, the precise mechanism of its toxicity is still to be determined. Studies in hamsters (Mitchell et al., 1975) have postulated conversion to chemically reactive metabolites via several routes of metabolism (Hinson et al., 1979b; 1979c; Nelson et al., 1981). At least three different methods of activation have been suggested by which phenacetin is expected to be converted to chemically reactive metabolites responsible for its toxicity (Fig. 2).

i) Phenacetin undergoes oxidative deethylation by hepatic enzymes to form paracetamol, which in turn is converted to a chemically reactive nephrotoxic and hepatotoxic metabolite, probably NAQ (Calder et al., 1981).

ii) Hepatic enzymes convert phenacetin to an arylating metabolite: phenacetin-3,4-epoxide (Hinson et al., 1977).

iii) Hepatic enzymes convert phenacetin to N-hydroxyphenacetin (Hinson and Mitchell, 1976), which could be conjugated as the N-O-sulfate and N-O-glucuronide, both reactive electrophiles (Mulder et al., 1977, 1978). Additionally or alternatively NHP could be converted to NAQ (Calder et al., 1974).

In the light of recent research, NHP has been considered to be the metabolite of phenacetin most likely to induce nephropathy and carcinogenesis (Calder et al., 1973; 1976; Calder and Williams, 1975; Nery, 1971c). The urinary excretion of NHP could therefore be indicative of the probable carcinogenicity and nephrotoxicity of phenacetin.
1.6. SCOPE OF THE PRESENT IN VIVO CHRONIC DOSING STUDY

The present chronic study was therefore undertaken to elucidate more clearly the metabolism of phenacetin and any changes in metabolism after prolonged administration of the drug. Such factors could be indicative of the mechanism of phenacetin-induced toxicity.

An in vivo chronic study in rats was undertaken to monitor the excretion levels of the various metabolites of phenacetin, during a period of prolonged administration of the drug, at regular (weekly) intervals.

NHP has been implicated as a likely proximal carcinogen of phenacetin. Levels of this probably carcinogenic, reactive intermediate, which were not determined in other chronic studies (Smith and Griffiths, 1976; Smith and Timrell, 1974), were the focus of the present study.

The effect of the size of dose has already been investigated with reference to the extent of metabolism of the drug (Smith and Timrell, 1974) and its toxicity (Boyd and Hottenroth, 1968; Boyd, 1971), but these studies had not measured the significant metabolite, NHP. The present study also sought to examine the influence of size of dose on the formation of NHP.

The influence of aspirin and caffeine on the metabolism of phenacetin was examined. It was of particular interest to detect any alteration in metabolism of phenacetin when co-administered with these drugs.
The effect of chemicals likely to interfere with certain major pathways of phenacetin metabolism (such as sulfation) was of interest in this study. The intention was to infer the extent of saturation of the existing alternative pathways by suppressing the sulfation pathway. The possibility of direct interference with the metabolism of phenacetin by these chemicals was also of interest. An inhibition of sulfation would also be expected to result in diminished formation of the N-O-sulfate conjugate of NHP, a known reactive metabolite.

1.7. ELUCIDATION OF THE METABOLISM OF p-PHENETIDINE IN VITRO

The toxicity caused by PN, which is known to be hemolytic, could be direct or through a subsequent metabolite.

Although in vitro systems using microsomes have been used to study the metabolism and toxicity of several compounds, including phenacetin and paracetamol (Hinson et al., 1979a; Nelson et al., 1980), metabolic studies using PN as a substrate (Buch et al., 1967) which indicated its conversion to 2-HPN, did not reveal details of its further metabolism. A study involving an in vitro examination of PN metabolism was therefore also undertaken in the present work.
1.8. SELECTION OF AN IN VITRO SYSTEM

Berry and Friend (1969) successfully developed enzymatic techniques for the isolation of viable hepatocytes and thereby introduced a reliable *in vitro* system for studying the metabolism or toxicity of xenobiotics.

Several substrates have been studied using isolated hepatocytes. Benzpyrene (Cantrell and Bresnick, 1972), alpranolol (Moldeus et al., 1974), biphenyl (Wiebkin et al., 1976), ethyl morphine (Erickson and Holtzman, 1976), amylopyrene, dansylamide, quinine (Hayes and Brendel, 1976), barbiturates (Yih and van Rossum, 1977), amphetamine (Hirata et al., 1977) and methotrexate (Horne et al., 1976) are a few examples.

Isolated hepatocytes were chosen as the *in vitro* technique for the present study because this system has already proven its suitability for studies of drug metabolism and toxicity (Thor et al., 1978a; 1978b; Moldeus, 1978). This is because more cellular properties are retained by isolated hepatocytes in comparison with microsomal preparations. Both cytochrome P450-dependent oxidation reactions (Moldeus et al., 1974; Yih and van Rossum, 1977) and subsequent conjugation reactions (Wiebkin et al., 1976; Billings et al., 1977) are possible. Most importantly, better correlation with *in vivo* results of drug metabolism has been established (Billings et al., 1977; Yih and van Rossum, 1977) by using isolated hepatocytes in comparison with 900xg supernatant.
1.9. AIMS OF THE PRESENT STUDY

i) Study the metabolism of chronically administered phenacetin in the rat.

ii) Examine the influence of dosage size on metabolism.

iii) Investigate the effect of sulfation inhibition on phenacetin metabolism and comparatively assess the metabolic alterations, if any, seen on administration of phenacetin acutely and chronically.

iv) Detect and determine any alteration in phenacetin metabolism when phenacetin and aspirin are chronically co-administered.

v) Detect and determine any alteration in phenacetin metabolism when phenacetin and caffeine are chronically co-administered.

vi) Follow the further metabolism of deacetylated phenacetin (p-phenetidine) in an in vitro system and to account for its disappearance.
2.1. MATERIALS

p-Phenetidine (Hopkin & Williams Ltd, England) was redistilled, b.p. 251 C. p-Nitrosophenetole was synthesized (Sec. 2.1.1.). N-Hydroxyphenacetin and deuterated N-hydroxyphenacetin were gifts from Dr. S. McLean (School of Pharmacy, University of Tasmania). 2-Hydroxyphenetidine, N-acetyl-4-aminobenzoic acid and N-butyryl-4-aminobenzoic acid were gifts of Mr. M. Veronese (School of Pharmacy, University of Tasmania).

The glucuronide, sulfate, cysteiny1 and mercapturate conjugates of paracetamol were gifts from Dr. K. Henderson (Sterling Winthrop, Newcastle-upon-Tyne, Great Britain).

Extract of Helix pomatia (beta-glucuronidase plus arylsulfatase) was obtained from Boehringer, Mannheim, Germany. Fluorescent silica gel (Schleicher and Schull type G7, size 10-40) was coated (0.25 mm thick) on to glass thin layer chromatography plates (20 x 20cm).

Collagenase Type IV and Bovine Serum Albumin (BSA) essentially fatty acid free (Sigma Chemical Company, U.S.A.) were employed in hepatocyte isolation.

Diazomethane was prepared fresh when required for methylation from p-tosylsulfonylmethylnitrosamide by the method of Vogel (1956), and used as the ethereal solution.

Drugs were of B.P. grade. All other chemicals and solvents were
of A.R. grade and purchased commercially.

2.1.1. SYNTHESIS OF p-NITROSOPHENETOLE

p-Nitrosophenetole was synthesized by a modification of the method of Vogel (1959). p-Nitrophenetole (500mg, 0.003 mole) was dissolved in ethanol (99.5% v/v, 16ml). Ammonium chloride (640 mg, 0.012 mole) dissolved in water (3ml) and zinc powder (780mg, 0.012 mole) was added.

The reaction mixture was stirred vigorously at room temperature for 90 min and checked for completion by thin layer chromatography on a silica gel coated miniplate developed in ether and visualized with ferric chloride (2.5% w/v in M/2 HCl). The reaction mixture was filtered and the precipitate washed with ice cold ethanol. The filtrate was transferred to a separating funnel. A saturated solution of sodium chloride was added and the mixture shaken thoroughly.

Chloroform (2x25 ml) was used to extract the salted-out p-nitrosophenetole and the pooled chloroform extract washed with ice cold water (2x25 ml). The chloroform extract was dried over anhydrous magnesium sulfate for 1 hour with frequent agitation, and evaporated off under vacuum in a rotary evaporator. This left a residue of p-nitrosophenetole which was later recrystallized from benzene and characterized by mass spectrometry. The recrystallized p-nitrosophenetole was pure. It produced a single spot when thin layer chromatographed (silica gel-ether) and one peak when gas chromatographed under conditions described in Fig. 6.
2.2. **ANIMALS**

A Hooded Wistar strain of rats fed on a standard laboratory diet with water *ad libitum* were used in all present studies. The rats weighed approximately 200 g and were 4 weeks old when used.

2.3. **IN VITRO STUDY**

An *in vitro* system using freshly isolated hepatocytes from the rat was employed.

2.3.1. **HEPATOCELLULAR ISOLATION**

A modified method incorporating the techniques of Berry & Friend (1965) and Seglen (1972; 1973a; 1973b) was adopted (Fig. 3).

The rat was anaesthetised with pentobarbitone (65 mg/kg; I.P.) prior to surgery. After regular respiration was established, a mid-ventral incision was made to expose the liver and heparin (0.1 ml of 25,000 units/ml) was injected into the inferior vena cava to prevent blood coagulation.

The hepatic portal vein was cannulated with a tube delivering a flow of 5 ml/min of calcium-free Krebs-Henseleit buffer (glucose 10mM, carbogen equilibrated, pH 7.4, 37 °C). Immediately after cannulation the buffer flow was increased to 30 ml/min, the liver was flushed of all blood and the superior vena cava was cut open to allow for the drainage of the buffer, *in situ*. 
FIG. 3: Scheme for the isolation and use of hepatocytes
The liver was excised from its connective tissue and mounted in a perforated plastic receptacle. After 12 min the buffer was replaced with Krebs-Henseleit buffer (glucose 10mM, carbogen equilibrated, pH 7.4, 37 C).

After 5 min, collagenase (25 mg in 5 ml buffer) was added to give a final concentration of 0.05 % in the perfusate (50 ml), which was circulated for a further 45 min. The liver showed visible signs of disruption after this period.

The liver was subsequently transferred into Krebs-Henseleit buffer containing BSA (0.1%w/v). The capsule was removed and hepatocytes gently shaken free from the connective tissue.

The suspension of cells and tissue was sieved through nylon gauze which retained the connective tissue. The cell suspension was allowed to stand for a few minutes and the sedimented cells were harvested by decanting off the supernatant liquid.

After reconstituting with BSA-Krebs Henseleit buffer, the cells were allowed to recover under carbogen, in an orbital shaker (37 C, 30 min, 120 osc/min).

Trypan blue (0.2%) was used to estimate the viability count which was done in a Neubauer chamber. Viability was between 68% and 75%. Cell yield was between $1.3 \times 10^8$ to $1.6 \times 10^8$. 
2.3.2. INCUBATION AND SAMPLE COLLECTION

The isolated hepatocytes from healthy untreated rats were reconstituted to give a viable cell concentration of $2.5 \times 10^6$ ml in Krebs-Henseleit buffer (BSA 1%, pH 7.4). An aliquot (5 ml) was collected to serve as the blank sample. The substrate of $p$-phenetidine (3.4 mg in 100 ul ethanol/water:30/70) was added to the cell suspension (50 ml) in an incubation flask, giving a PN concentration of 0.5 mM, found suitable for metabolic studies by McLean (1978).

A zero time sample aliquot (5 ml) was removed, carbogen flushed through the incubation flask and the flask replaced in the orbital shaker bath (180 osc/min, 37°C), until the next collection was due. Samples were collected as scheduled in Table 1, and the carbogen replenished in the flask after each sample removal.

The aliquots were collected in 15 ml centrifuge tubes kept in ice. The aliquot suspensions were centrifuged immediately (2500 rpm for 10 min) and 2 ml duplicate samples of supernatant were transferred to centrifuge tubes, frozen immediately in liquid nitrogen and stored for assay at -20°C.

The samples were analysed as outlined in the analytical scheme for metabolites of $p$-phenetidine (Sec. 2.4. and Fig. 4) and the results of the analyses are presented in Table 1.
FIG. 4: Analytical scheme for the in vitro metabolites of p-phenetidine
2.4. **ANALYSIS OF THE METABOLITES OF p-PHENETIDINE**

Analytical methods for the extraction and analysis of the metabolites of p-phenetidine present in the supernatant of the incubate were developed.

2.4.1. **DETECTION AND MEASUREMENT OF PHENACETIN, PARACETAMOL, p-AMINOPHENOL AND p-PHENETIDINE**

2.4.1.1. **HYDROLYSED SAMPLES**

An aliquot (2 ml) of incubate supernatant was buffered to a pH of 5.2 with 200 ul acetate buffer (1.1M, pH 5.2), in a 15 ml centrifuge tube. Helix pomatia extract (100 ul) and the internal standard, p-toluidine (TDN, 50 ug in 50 ul methanol), were added. After vortexing briefly (10 sec) the sample was hydrolysed (37 C, 16 hr).

Sodium bicarbonate (120 mg) was added to the sample after hydrolysis and vortexed until dissolved to obtain a neutral pH. Butyric anhydride (25 ul) was added and the sample vortexed frequently over a period of 1 hour after which butyrylation was complete.

The sample was then partitioned with dichloromethane (3 ml), vortexed (30 sec) and centrifuged (2500 rpm for 15 min). The aqueous upper layer was discarded and the dichloromethane extract decanted off after freezing with liquid nitrogen. The dichloromethane extract was further concentrated to 50 ul under a gentle stream of nitrogen at room temperature. Finally, 1 ul of the concentrated extract was gas chromatographed using
conditions shown in Fig. 5. Detection of the metabolites was by comparison with authentic standards. The concentrations of the metabolites were determined by reference to the standard curves for phenacetin (linearity: 0.5 ug/ml - 10 ug/ml), paracetamol (linearity: 0.5 ug/ml - 10 ug/ml), p-aminophenol (linearity: 0.5 ug/ml - 5 ug/ml) and p-phenetidine (linearity: 5 ug/ml - 150 ug/ml), using peak height ratios to internal standard.

2.4.1.2. UNHYDROLYSED SAMPLES

The enzyme incubation step was omitted. p-Toluidine was added and samples were butyrylated and extracted for analysis as above.

2.4.2. DETECTION AND MEASUREMENT OF p-NITROSOPHENETOLE AND N-HYDROXYPHENETIDINE.

An aliquot of incubate supernatant (2 ml), was transferred into a 15 ml centrifuge tube. p-Bromoaniline (BA, 50 ug in 50 ul methanol), the internal standard, was added. The method of Kiese and Renner (1963) using potassium ferricyanide to oxidize the N-hydroxyphenetidine to p-nitrosophenetole was employed. Potassium ferricyanide (25 ug in 25 ul water) was added and the sample vortexed for 10 sec. Carbon tetrachloride (1 ml) was vortexed for 60 sec with the sample, to extract the required metabolites. The sample mixture was then centrifuged (2500 rpm for 15 min) and the upper aqueous layer discarded. The sample was frozen and the organic phase decanted and concentrated under a stream of nitrogen. Finally 1 ul of the extract was gas
chromatographed using conditions shown in Fig. 6. Detection was by comparison with the authentic standard and concentrations could be determined by reference to the standard curve for p-nitrosophenetole (linearity: 0.5 ug/ml - 10 ug/ml) using peak height ratios to internal standard.

2.4.3. DETECTION AND MEASUREMENT OF 2-HYDROXYPHENETIDINE

A method based on that of Shahidi and Hemiaidan (1969) was used to measure 2-hydroxyphenetidine. An aliquot of incubate supernatant (4 ml) in a 30 ml stoppered centrifuge tube, was acid hydrolysed (1 ml 10 M HCl, 100 C, 1 hr). The hydrolysed sample was neutralized with 5M sodium hydroxide (2 ml) and the pH adjusted to 8-9 with sodium bicarbonate. After the sample was vortexed for 30 sec it was kept at room temperature for 3 hr to allow for the formation of the phenoxazone. The tubes were cooled in ice and extracted sequentially with chloroform (2x20 ml). The chloroform extracts were pooled and evaporated to dryness under vacuum in a rotary evaporator. Color development was effected through the addition of preheated (70 C) 1M hydrochloric acid (2 ml). The absorbance was read at 580 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. Concentrations were determined by reference to a standard curve for 2-hydroxyphenetidine (linearity: 0.5 ug/ml - 25 ug/ml).
FIG. 5: GC trace for the metabolites of p-phenetidine in isolated hepatocytes

Metabolites were derivatized (Section 2.4.1.).

Hewlett Packard gas chromatograph 5700A;
Column : OV210 (3% on 100/120 diatomite MQ, glass, 90cm x 2mm ID);
col : 200°C; inj : 250°C; det. : 250°C;
Carrier gas : N₂ (30 ml/min)
FID gases : Air (240 ml/min), H₂ (30 ml/min).
FIG. 6: GC trace for the metabolites of p-phenetidine in isolated hepatocytes

Hewlett Packard gas chromatograph 5700A:
Column: OV-225 (3% on 100/120 diatomite MQ, GLT, 1.0m x 1.8mm ID);
col.: 132°C; inj.: 150°C; det.: 200°C;
Carrier gas: N₂ (20 ml/min);
FID gases: Air (240 ml/min), H₂ (30 ml/min).
2.5. IN VIVO STUDY

2.5.1.1. CHOICE OF DOSE

Chronic oral daily doses of phenacetin (50 mg/kg), aspirin (50 mg/kg) and caffeine (10 mg/kg) were chosen on the basis of relevance to present day human consumption of the drugs. The dose of 100 μmol/kg pentachlorophenol, a known inhibitor of sulfation, was the same as that used by Mulder and Scholtens (1977) to block the sulfation of harmol. An additional, higher dose of phenacetin, 500 mg/kg daily, less than half the chronic LD 50 dose of 1.12g/kg (Boyd and Hottenroth, 1968), was used to examine the effect of chronic dosing on the metabolism of phenacetin.

2.5.1.2. TIME AND ROUTE

All the drugs in the respective experiments were administered orally before midday. Pentachlorophenol was administered 45 min prior to the phenacetin dose. Aspirin and caffeine were administered with phenacetin.

2.5.1.3. FREQUENCY AND DURATION

With the exception of acute pentachlorophenol dosing, which was co-administered with phenacetin on the first and seventeenth day of treatment, all other drugs were administered daily for the duration of the experiments.

2.5.1.4. FORMULATION

Caffeine was administered in aqueous solution. Phenacetin,
aspirin and pentachlorophenol were formulated as suspensions in 0.25% sodium carboxymethylcellulose. Aspirin and pentachlorophenol were prepared fresh before each dose.

2.5.2.1. **DRUG TREATMENTS**

Five different treatments were investigated. A fresh group of six rats was used for each investigation except in the instances where phenacetin was co-administered with either caffeine or aspirin. In these instances each group comprised three rats.

1. **P500** : Phenacetin (500 mg/kg), administered daily for 29 days.

2. **P50** : Phenacetin (50 mg/kg) administered daily for 17 days. Pentachlorophenol (100 umol/kg) administered on day 17.

3. **P50/PCP** : Phenacetin (50 mg/kg) co-administered with pentachlorophenol (100 umol/kg) for one day.

4. **P50/A** : Phenacetin (50 mg/kg) co-administered with aspirin (50 mg/kg) daily for 15 days.

5. **P50/C** : Phenacetin (50 mg/kg) co-administered with caffeine (50 mg/kg) daily for 15 days.

2.5.2.2. **URINE COLLECTION**

During the 24 hr period of urine collection food was withheld and the rats were only provided with water *ad libitum*. Urines were collected at intervals of 7 days beginning from day 1.
using urine collection cages. These cages were of galvanized iron with mesh bottoms and mounted over plastic funnels to facilitate urine collection. The urine was allowed to run into a measuring cylinder, immersed in a Dewar flask containing a freezing mixture of ice and salt.

A minimal amount of distilled water was used to wash the cage mesh bottom and the washings were allowed to collect in the receiver.

Urines and washings were collected, the volumes noted and the urines filtered separately. Aliquots (2 ml) were removed when required from each sample for the respective assays, which were routinely done after each collection.
**FIG. 7:** Analytical scheme for the urinary metabolites of phenacetin

**ENZYMATIC HYDROLYSIS**
- β-glucuronidase, 37°C,
- + arylsulfatase, 16 hr

**EXTRACTION**
- CH₂Cl₂

**METHYLATION**
- CH₃N₂ in CH₃OH

**TLC**
- Silica gel/CHCl₃

**LOWER BAND**
- GC: P

**UPPER BAND**
- MS: NHP
- GC: 2HP

**DILUTION**

**ACID HYDROLYSIS**
- 10N HCl, 100°C, 1 hr

**PHENOXAZONE FORMATION**
- pH 8-9, room temp.

**EXTRACTION**
- CHCl₃

**COLOR DEVELOPMENT**
- 1N HCl, 70°C

**SPECTROPHOTOMETRY**
- λ 580 nm

**INTERNAL STANDARDS**
2.6. ANALYSIS OF THE METABOLITES OF PHENACETIN

The analytical scheme for the metabolites of phenacetin is presented in Fig. 7.

2.6.1. DETECTION AND QUANTITATION OF PHENACETIN, 2-HYDROXYPHENACETIN AND 3-HYDROXYPHENACETIN

Detection and quantitation of phenacetin, 2-hydroxyphenacetin and 3-hydroxyphenacetin was by gas chromatography (Figs. 8 and 9). Analytical procedures were essentially the same as those of McLean et al. (1981) as modified by Veronese (1982).

2.6.2. DETECTION AND QUANTITATION OF PARACETAMOL AND ITS CONJUGATES

Detection and quantitation of paracetamol, and its sulfate, glucuronyl, mercapturate and cysteinyll conjugates was by high pressure liquid chromatography (Fig. 10). Analytical procedures were essentially those of Rumble as cited by Veronese (1982).

2.6.3. DETECTION AND QUANTITATION OF 2-HYDROXYPHENETIDINE

Detection and quantitation of 2-hydroxyphenetidine was carried out spectrophotometrically. The method was the same as that used in the hepatocyte experiments (Sec. 2.4.3.).
FIG. 8: GC trace of 2-methoxyphenacetin (upper band-TLC)

Hewlett Packard gas chromatograph 5700A;
Column: SCOT-OV17 (0.45mm ID x 23.65m);
col.: 205°C; inj.: 200°C; det.: 250°C;
Carrier gas: H₂ (55 cm/sec); FID gases: Air (20 ml/min), H₂ (30 ml/min).
FIG. 9: GC trace of phenacetin (lower band-TLC)

Hewlett Packard gas chromatograph 5700A;
Column: SCOT-OV17 (0.45 mm ID x 23.65 m);
col.: 205°C; inj.: 200°C; det.: 250°C;
Carrier gas: H₂ (55 cm/sec); FID gases: Air (20 ml/min), H₂ (30 ml/min).
FIG. 10: HPLC ANALYSIS OF THE METABOLITES OF PHENACETIN: PARACETAMOL AND CONJUGATES OF PARACETAMOL

- COLUMN: μBondapak C₁₈ (Waters Associates, 3.9mm ID x 30cm).
- SOLVENT: CH₃CN/phosphate buffer (10mM, pH 5.0) : 3/97.
- FLOW RATE: 2ml/min (Waters Associates model M45 solvent delivery system).
- DETECTOR: UV:254nm (Waters Associates model 441 absorbance detector).
- SENSITIVITY: 0.02 - 1.0 AUFS.
- CHART SPEED: 15″/hr (Houston Instruments Omniscribe Recorder).
2.6.4. ASSAY FOR N-HYDROXYPHENACETIN

NHP was detected and quantitated by a modification of an earlier method (McLean et al., 1981). An aliquot of urine (2 ml) was mixed with acetate buffer (200 ul, 1.1 M, pH 5.2), extract of Helix pomatia (100 ul) and the internal standard, deuterated N-hydroxyphenacetin (DNHP, 10 ug in 10 ul methanol).

The urine mixture was incubated at 37 C overnight to hydrolyse conjugated metabolites. The incubate was extracted into dichloromethane, methylated with diazomethane and separated by thin layer chromatography on silica gel as described before (McLean et al., 1981). Methylated derivatives of NHP (N-methoxyphenacetin, NMP), DNHP (deuterated N-methoxyphenacetin, DNMP) and 2-HP (2-methoxyphenacetin, 2MP) and 3-HP (3-methoxyphenacetin, 3-MP) migrated together (with Rf 0.26) after two developments in chloroform. The corresponding urine zone was eluted and NMP identified by combined gas chromatography and mass spectrometry (GC/MS). The mass spectrum of NMP was identical to that reported earlier (McLean et al., 1981). A method based on multiple metastable peak monitoring (Gaskell and Millington, 1978) after direct insertion of the sample into a double focussing mass spectrometer, set up and operated as described by Davies et al. (1982) in a similar assay for warfarin, was used for quantifying NHP in rat urine.

DNMP and NMP produce significant first field free region metastable peaks for the successive loss of the elements of the acetyl and methoxyl groups (DNMP: m/z 213->139; NMP: m/z 209->135, Fig.11). These metastable peaks are not found in any of
the ring-methoxylated metabolites and so could be used to directly measure DNMP and NMP in the presence of the other methoxylated metabolites of phenacetin. Quantitation was by area of the ion current versus time curve during the distillation at 120 C from the probe (Fig. 12). Blank urine gave no peaks and the calibration curve (ratio NHP/DNHP) was linear over the range of 0-50 ug NHP added to urine.

In urine samples collected after the administration of pentachlorophenol on day 17 of chronic phenacetin dosing, an interfering peak for the 209->135 reaction was encountered. This is evident from the disparity in the distillation profiles, shown in Fig. 12. A GC/MS analysis was carried out to determine the source of interference. Two additional components present in the sample were detected and tentatively identified as ethyl hippurate and ethyl phenyl-acetyl-glycine. The molecular weight of ethyl hippurate was 207 and it gave a large fragment ion at 134, with simultaneous isotope peaks at 208 and 135.

It therefore appeared that the interference could have originated from a 208->135 reaction, as interference peaks are known to occur in first field free region metastable studies due to reactions from adjacent precursors to the same daughter ion or reactions within fields (Lacey and Macdonald, 1977).

The effect this interfering reaction has on the quantitation of NMP could be excluded if the accelerating voltage was changed to monitor a hypothetical 210->135 reaction, thereby allowing the detection of a 209->135 reaction, but not a 208->135 reaction. This was carried out and the samples were then found to have the
FIG. 11: Mass spectra of N-methoxyphenacetin and deuterated N-methoxyphenacetin

Arrows show reactions monitored for quantitation of NHP.
FIG. 12: N-methoxyphenacetin

Profile of ion current vs. time for standard and metabolite containing urine samples.
FIG. 13: N-methoxyphenacetin

Elimination of interference by increasing the accelerating voltage to ion-monitor the 210→135 reaction.
expected distillation profile, indicating the exclusion of the interference (Fig. 13).

2.7. STATISTICAL ANALYSIS OF DATA

A students paired t test was performed for each group of rats in order to determine the significance of the observed changes in the metabolism of phenacetin with the different treatments. The change was considered significant when the value of p was less than 0.05.
CHAPTER 3. RESULTS AND DISCUSSION

3.1. METABOLITES OF p-PHENETIDINE IN VITRO

Results of the metabolite analysis obtained from the incubation of 0.5 mM p-phenetidine (3.4 mg in 50 ml) with freshly isolated hepatocytes are presented in Table 1.

The expected metabolites, paracetamol, p-aminophenol, nitrosophenetole and N-hydroxyphenetidine were not detected although the sensitivity of the assays used provided detection limits of 0.5 ug/ml for the compounds.

As shown in Table 1 and Fig. 14 the PN was further metabolized, indicated by its decreasing concentration over the period of incubation. However, the decrease in concentration was inadequately accounted for by the formation of phenacetin and 2-hydroxyphenetidine, the acetylated and hydroxylated metabolites respectively. The N-acetylation of the deacetylated metabolite of phenacetin in the rat, namely the N-acetylation of PN, is not considered significant, because the N-acetylated metabolites of PN are not excreted in the urine of Chester Beatty rats gavage fed [ethyl-14-C]-p-phenetidine (Nery, 1971b). However, from the in vitro experiments performed (using freshly isolated hepatocytes from Hooded Wistar rats) in the present work, N-acetylation of PN was significantly demonstrated. The unaccounted for PN was probably converted to certain other unknown metabolites or conjugates.

The relative instability of PN metabolites makes their isolation
and investigation difficult. Nevertheless, the tendency of isolated hepatocytes to hydroxylate and acetylate the xenobiotic suggested the elimination of PN via these pathways and explained in some measure the increased 2HPN formed \textit{in vivo} by inhibition of sulfation or stimulation of aromatic hydroxylation (Sec. 3.2.2.).
### Table 1: Metabolism of p-phenetidine in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Time (min)</th>
<th>Control (without hepatocytes)</th>
<th>Hepatocyte Incubate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-phenetidine</td>
<td>p-phenetidine</td>
<td>phenacetin</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>64.35</td>
<td>52.78</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>-</td>
<td>51.62</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>61.58</td>
<td>49.36</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>-</td>
<td>40.87</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>60.81</td>
<td>34.79</td>
</tr>
</tbody>
</table>

1. Typical of 4 experiments.

2. Paracetamol, p-aminophenol, p-nitrosophenetole and N-hydroxyphenetidine were not detected.
FIG. 14: Metabolism of p-Phenetidine in isolated rat hepatocytes.
3.2. METABOLITES OF PHENACETIN IN VIVO

Buch et al. (1967) demonstrated that the major fraction (58%) of metabolized phenacetin, in the rat, is accounted for as paracetamol and its sulfate and glucuronide conjugates. From the results presented in Table 2 and the graphic representations, Figs. 15 to 21, it is seen that a major fraction of the phenacetin dose was deethylated to paracetamol which was excreted in urine either as free paracetamol or as conjugated paracetamol-sulfate and paracetamol-glucuronide. This finding was compatible with the findings of earlier in vivo studies (Brodie and Axelrod, 1949; Nery 1971b). The cysteinyl and mercapturate conjugates reportedly found in albino rats of the Birmingham Wistar strain, when treated with phenacetin (Smith and Griffiths, 1976) were not detected in the Hooded Wistar strain of rats used in these experiments.

Paracetamol-sulfate (APAP-SULF) was the major metabolite found in the present experiments, while paracetamol-glucuronide (APAP-GLUC) levels were higher than those reported by Buch et al. (1967).

Hydroxylation of phenacetin was in evidence in the present work. N-hydroxyphenacetin (NHP) was found present up to 0.3% of the dose of phenacetin. 2-Hydroxyphenacetin (2HP), measured as 0.07% of the dose of phenacetin in other earlier studies was present up to a maximum of 0.06% of the dose of phenacetin in the present study. 3-Hydroxyphenacetin (3HP) was not detected. 2-Hydroxyphenetidine (2HPN) was also formed to the maximum extent of 0.8% of the dose of phenacetin as compared to 6% of the
phenacetin dose reported as the sulfate conjugate of 2HPN (Buch et al., 1967). The N-deacetylated metabolite 2HPN comprised only a minor fraction of the administered dose of phenacetin, an observation which has been similarly demonstrated in earlier experiments (Brodie and Axelrod, 1949; Smith and Williams, 1949), further indicating the metabolically inert nature of the acetyl group of phenacetin as previously suggested by Nery (1971b). Unchanged phenacetin was found up to 0.8% of the dose administered.
Table 2: Metabolites of phenacetin in the rat with different treatments

<table>
<thead>
<tr>
<th>Days</th>
<th>TREATMENT</th>
<th>PERCENTAGE DOSE OF METABOLITES OF PHENACETIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APAP</td>
<td>APAP-SULF</td>
</tr>
<tr>
<td>1</td>
<td>4.05</td>
<td>61.03</td>
</tr>
<tr>
<td></td>
<td>(0.31)</td>
<td>(2.50)</td>
</tr>
<tr>
<td>8</td>
<td>5.98</td>
<td>58.95</td>
</tr>
<tr>
<td></td>
<td>(0.31)</td>
<td>(7.48)</td>
</tr>
<tr>
<td></td>
<td>P500</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>6.64</td>
<td>52.99</td>
</tr>
<tr>
<td></td>
<td>(0.61)</td>
<td>(0.88)</td>
</tr>
<tr>
<td>29</td>
<td>6.98</td>
<td>48.51</td>
</tr>
<tr>
<td></td>
<td>(0.61)</td>
<td>(3.42)</td>
</tr>
<tr>
<td>1*</td>
<td>2.72</td>
<td>76.27</td>
</tr>
<tr>
<td></td>
<td>(0.16)</td>
<td>(1.70)</td>
</tr>
<tr>
<td>8</td>
<td>4.13</td>
<td>88.72</td>
</tr>
<tr>
<td></td>
<td>(0.29)</td>
<td>(1.61)</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>5.14</td>
<td>102.29</td>
</tr>
<tr>
<td></td>
<td>(0.27)</td>
<td>(6.77)</td>
</tr>
<tr>
<td>17*</td>
<td>4.43</td>
<td>75.32</td>
</tr>
<tr>
<td></td>
<td>(0.37)</td>
<td>(8.89)</td>
</tr>
</tbody>
</table>
Table 2: (continued)

<table>
<thead>
<tr>
<th>Days</th>
<th>TREATMENT</th>
<th>PERCENTAGE DOSE OF METABOLITES OF PHENACETIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>APAP</td>
</tr>
<tr>
<td>1</td>
<td>3.62</td>
<td>78.7</td>
</tr>
<tr>
<td></td>
<td>(0.57)</td>
<td>(1.33)</td>
</tr>
<tr>
<td>8</td>
<td>P50/A</td>
<td>4.78</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(5.64)</td>
</tr>
<tr>
<td>15</td>
<td>4.77</td>
<td>77.3</td>
</tr>
<tr>
<td></td>
<td>(0.50)</td>
<td>(3.05)</td>
</tr>
<tr>
<td>1</td>
<td>3.92</td>
<td>80.22</td>
</tr>
<tr>
<td></td>
<td>(0.68)</td>
<td>(1.53)</td>
</tr>
<tr>
<td>8</td>
<td>P50/C</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td>(0.57)</td>
<td>(4.91)</td>
</tr>
<tr>
<td>15</td>
<td>4.80</td>
<td>73.33</td>
</tr>
<tr>
<td></td>
<td>(0.33)</td>
<td>(12.06)</td>
</tr>
</tbody>
</table>

(*) pentachlorophenol (100 μmol/kg) administered.

Each value is the mean (± SE) of 6 rats, except for P50/A and P50/C where 3 rats were used.
FIG. 15: Paracetamol sulfate

Profile of the percentage of phenacetin dose vs. time

P500 : Phenacetin (500mg/kg) daily.
P50  : Phenacetin (50mg/kg) daily,
     : (▲) with pentachlorophenol (100μmol/kg) on day 17,
     : (■) with pentachlorophenol (100μmol/kg) for one day
     (another group of rats was used for this point only).
P50/A : Phenacetin (50mg/kg) and aspirin (50mg/kg) daily.
P50/C : Phenacetin (50mg/kg) and caffeine (10mg/kg) daily.

(*) p < 0.05; (**) p < 0.01 versus day 1.
FIG. 16: Paracetamol-glucuronide

Profile of the percentage of phenacetin dose vs. time.

Refer to the legend of Fig. 15 for particulars of treatments.
Profile of the percentage of phenacetin dose vs. time.

Refer to the legend of Fig. 15 for particulars of treatments.
FIG. 18: 2-hydroxyphenacetin
Profile of the percentage of phenacetin dose vs. time.
Refer to the legend of Fig. 15 for particulars of treatments.
FIG. 19: N-hydroxyphenacetin
Profile of the percentage of phenacetin dose vs. time.
Refer to the legend of Fig. 15 for particulars of treatments.
FIG. 20: 2-hydroxyphenetidine

Profile of the percentage of phenacetin dose vs. time.

Refer to the legend of Fig. 15 for particulars of treatments.
FIG. 21: Phenacetin

Profile of the percentage of phenacetin dose vs. time.

Refer to the legend of Fig. 15 for particulars of treatments.
3.2.1. P500 TREATMENT

In rats dosed with 500 mg/kg of phenacetin daily, between 48% and 61% of the dose was excreted as APAP-SULF (Fig. 15). The levels of APAP-SULF in the present work were higher than those reported by Smith and Timbrell (1974) and declined over the 29-day period of study. A progressive depletion, with time, of cytosolic sulfotransferase sulfation was therefore inferred. The progressive decrease in sulfation was compensated for with a simultaneous increase in glucuronidation. Markedly increased levels of APAP-GLUC were witnessed during the experimental period (Fig. 16). The fraction of dose excreted as APAP-GLUC increased from 23% to 55% during the dosing period, higher than the maximum of 18% of the dose reported previously (Smith and Timbrell, 1974).

The glucuronidation of phenacetin with UDP-glucuronyltransferase and its sulfation with cytosolic sulfotransferase enzymes of phenacetin are competitive pathways of metabolism (Mulder and Meerman, 1978). Their precedence in the conjugation process are inversely related, as demonstrated by Kadiubar et al. (1980), Meerman et al. (1980) and Mulder and Scholtens (1977) in studies on the regulatory role of sulfation in some biological processes. The results of the present work were consistent with this inverse relationship and it was seen that glucuronidated levels of the drug were notably increased when sulfation was suppressed or impaired.

Smith and Timbrell (1974) found that free APAP is excreted to the extent of 5% of the dose of phenacetin. In the present
study the fraction of unconjugated APAP increased from 4% to 7% of the dose of phenacetin, over the experimental period (Fig. 17). This indicated that not all of the decreased sulfation was compensated for by an increase in glucuronidation alone.

Smith and Timbrell (1974) found 2HP present to the extent of 3.0% of the dose of phenacetin, but did not measure the metabolites NHP and 2HPN in rat urine. NHP and 2HPN are significantly relevant to the toxicity of phenacetin. The significant increase observed in the formation of 2HP and NHP over the experimental period (Figs. 18,19) could be a result of induction of the hydroxylation pathway. Auto-induction of 2HP formation was obvious in the first fortnight but did not increase further in the next. This resulted in its continued high level of formation. The induction of formation of NHP continued for the entire 29-day period of study and was evidenced by the increasingly higher levels of NHP formed.

NHP had first been reported as an in vivo metabolite in the rat by McLean et al. (1981). The arylhydroxamic acids (Miller and Miller, 1966a; Weisburger and Weisburger, 1973) including NHP (Calder et al., 1976) are known to be carcinogenic. The toxicity of phenacetin seen on chronic administration could therefore be due to the increased formation of NHP.

However, irrespective of the reason for the increased hydroxylated products formed, the increased levels of NHP seen on administration of the 500 mg/kg dose daily, could well be a reason for the chronically induced toxicity of phenacetin.
The amount of deacetylated metabolites excreted in the urine of rats treated with phenacetin is increased when larger doses of phenacetin are administered (Raaflaub and Dubach, 1969). A higher percentage of the dose of phenacetin present as 2HPN was seen on this treatment (P500) in comparison with that seen at the lower dose of phenacetin treatments (P50; P50/A; P50/C). Formation of 2HPN exhibited a decreasing trend from 0.8% to 0.5% of the phenacetin dose (Fig. 20). This decrease was most prominent in the first week and was ostensibly related to the precedence of the glucuronidation pathway and the increased direct hydroxylation of phenacetin to 2HP and NHP. An increase or return to normality after the first week was seen in which 2HPN reached a maximum of 0.75% of the dose on day 29 (Table 2).

The percentage of the dose of phenacetin found unchanged in urine increased in the first week from 0.002% to 0.011% and then decreased over the next three weeks (Fig. 21). It appeared logical to assume that the kinetics of metabolism permitted the accumulation of phenacetin until steady state had been attained. Subsequently induced metabolism converted more free phenacetin to its respective metabolites.
3.2.2. P50 TREATMENT

For rats on this dosage regimen a larger percentage of the dose was excreted as APAP-SULF, in comparison to the P500 treated rats. A significant increase in APAP-SULF formation was evidenced over the 15 day experimental period (Fig. 15). The sulfation pathway coped with this dose of phenacetin and its induction resulted in increased sulfation. Pentachlorophenol (PCP, 100uM)) a known inhibitor of sulfation (Mulder and Meerman, 1978; Meerman et al., 1980) given orally on day 1 of the experiment did not alter the level of APAP-SULF in rats significantly (Fig. 15), contrary to expectations held on the basis of the earlier experiments in acutely dosed rats (Mulder and Meerman, 1978). Administered on day 17 of the study, PCP inhibited sulfation significantly (p<0.05) though incompletely (Fig. 15). The inhibition of sulfation was 27%, compared to 100% found by Mulder and Scholtens (1977) in their experiments using the same dose of PCP intraperitoneally. Therefore, PCP administered orally exerts only partial sulfation inhibition in rats treated chronically with phenacetin.

The APAP-GLUC levels were much lower in comparison to the P500 treated rats and remained unaltered at 15% of the phenacetin dose (Fig. 16) during the course of the experiment. This was not in accordance with the expectation that the glucuronidation activity would be reduced as a consequence of the induction of the competitive sulfation pathway (Mulder and Meerman, 1978). Contrary to expectations therefore, the progressive increase in sulfation proceeded with no apparent effect on glucuronidation
at this dose. Mulder and Scholtens (1977) have demonstrated the suppression of glucuronidation with PCP (100uM, IP) in the rat. In the present work PCP (100uM, po) did not affect the glucuronidation of APAP. The percentage of the dose of phenacetin excreted as APAP-GLUC remained unaffected, thereby further establishing the selective sulfation inhibitory effect of PCP.

The percentage of dose excreted as APAP was lower than that seen in the P500 treated animals. The APAP fraction increased from 2.3% to 5.1% of the dose of phenacetin over the study period (Fig. 17). PCP did not alter the percentage of the dose of phenacetin excreted as APAP when given on day 1, but significantly (p<0.05) lowered it when co-administered on day 17 of the chronic treatment with phenacetin (Fig. 17).

A marked decrease in 2HP formation in the first week was followed by a recovery in the next (Fig. 18). PCP did not alter the percentage dose of phenacetin converted to 2HP when given on day 1 but lowered it (p<0.05) when co-administered with phenacetin on day 17 (Fig. 18).

Formation of NHP was marginally decreased in the first week and then significantly increased in the next (Fig. 19). Induction of the N-hydroxylating pathway was noticed in the second week of the experiment.

The levels of NHP were not affected on day 1, but were lowered (p<0.05) on day 17 by the co-administration of PCP and phenacetin. PCP therefore, apparently inhibited the
hydroxylation of phenacetin to NHP and 2HP in rats treated chronically with phenacetin.

The percentage of dose excreted as 2HPN diminished sharply in the first week and partially recovered in the second week of the experiment (Fig. 20). PCP did not affect the formation of 2HPN significantly on day 1, but caused a steep rise in 2HPN levels on day 17 of the experiment when co-administered with phenacetin (Fig. 20).

The partial block of sulfation by PCP resulted in an increased formation of 2HPN. This suggested a metabolic shift towards increased indirect hydroxylation of phenacetin via PN or increased deacetylation of 2HP or both. Phenacetin excretion was also increased. However, the total increase in excretion of 2HPN and phenacetin was less than 1% of the dose of phenacetin administered. This did not account for the large decrease (27% of dose) in sulfation caused by PCP on day 17.
3.2.3. P50/A TREATMENT

Aspirin has been shown to be nephrotoxic (Prescott, 1970; Nanra and Kincaid-Smith, 1972b; 1973b) but its possible potentiation of phenacetin-induced carcinogenicity and nephrotoxicity require further investigation. Altered metabolism of phenacetin resulting from an aspirin-phenacetin (Thomas et al., 1973; 1974) or paracetamol-phenacetin (Whitehouse et al., 1977) metabolic interaction have been reported earlier. In the present instance when aspirin and phenacetin were co-administered the percentage dose of phenacetin excreted as APAP-SULF showed no increase over the 15 day period (Fig. 15) as compared to the increased levels seen on the administration of the same dose of phenacetin alone. The induction of sulfation was interfered with and as a consequence APAP-SULF levels were marginally depressed in the first week and only partially recovered in the second week. These results were in accordance with those of Thomas et al. (1974) for phenacetin-aspirin co-administered to rats, Whitehouse et al. (1977) for paracetamol-aspirin co-administered to mice and Wong et al. (1976) for paracetamol-aspirin co-administered to hamsters. In these studies the significant metabolic effect of aspirin is the reduction of APAP sulfation.

The suppression of sulfation accounted for the increased compensatory glucuronidation which yielded higher levels of APAP-GLUC in the first week and subsequently slightly lower elevated levels (Fig. 16). An increase in glucuronidation was also indicative of the enhanced UDP-glucuronyltransferase activity that salicylates are reported to cause (Hanninen and
Free APAP levels increased from 3.6% to 4.8% of the phenacetin dose during the experiment (Fig. 17), a trend similar to that observed in the P50 treated rats.

The hydroxylation of phenacetin to 2HP was increased in the first week and lowered marginally in the next (Fig. 18). The N-hydroxylated product NHP was increasingly formed in the first week of treatment and continued a gradual escalation in the next week (Fig. 19). The 2HPN fraction increased over the 15 day period, most of the increase occurred in the first week of the experiment.

The co-administration of phenacetin and aspirin therefore resulted in increased aromatic- and N-hydroxylation. This could be a result of the direct induction of the hydroxylation pathway or a consequence of the partial sulfation inhibition. Analgesic mixtures containing aspirin in combination with phenacetin are known to be more nephrotoxic than analgesic mixtures in which aspirin is excluded or is replaced with phenazone (Nanra et al., 1980). Johansson (1981) explains the occurrence of renal pelvic tumors (only seen in rats treated with phenacetin or phenazone alone or in combination with caffeine) as a consequence of the altered metabolism of phenacetin, increasing the production of NHP, a postulated liver carcinogen. The induced N-hydroxylation of phenacetin by aspirin was therefore a noteworthy observation of significant interest in the context of potentiated phenacetin-induced carcinogenicity and nephrotoxicity.
The unchanged phenacetin levels declined over the period of study (Fig. 21). The decline was mainly seen in the second week.
3.2.4. P50/C TREATMENT

Caffeine continues to be included in analgesic mixtures today. The contribution of caffeine to phenacetin-induced toxicity needs to be investigated. Research into the mutagenic activity and carcinogenic character of caffeine has indicated its mutagenic activity in lower organisms and its association with bladder cancer (Kuhlmann et al., 1968; Cole, 1971), while other investigations have found that caffeine seemed to decrease the risk of malignancy in rats treated with phenacetin (Granberg-Ohman et al., 1980).

In the present work, when phenacetin and caffeine were co-administered a trend similar to that observed in the P50/A treated rats was witnessed. The APAP-SULF levels showed no increase over the 15 day period of study (Fig. 15), quite unlike the increased levels of APAP-SULF seen when the same dose of phenacetin was administered alone.

The induction of sulfation was inhibited to a degree by caffeine and consequently conversion of phenacetin to APAP-SULF in the first week was marginally depressed and only fractionally recovered in the second week.

Increased glucuronidation, apparent in the first week, compensated for the partial inhibition of sulfation (Fig. 16). Free APAP levels increased from 3.9% to 4.8% of the phenacetin dose over the duration of the experiment (Fig. 17). This observation was common to the P50 and P50/A treated rats.
The hydroxylation of phenacetin to 2HP decreased in the first week and then recovered in the next (Fig. 18) an observation similar to that seen in the P50 treated rats. Thus caffeine did not affect the formation of 2HP.

A progressive increase in the percentage dose of NHP was observed over the 15 day experiment (Fig. 19). The increase was more evident in the second week. Contribution to induction of N-hydroxylation by caffeine was only marginal, as compared to that of aspirin when co-administered with phenacetin. However, it still was significantly relevant to the potentiation of phenacetin-induced toxicity.

An increase in the percentage dose of 2HPN occurred over the entire 15 day period of study (Fig. 20). Caffeine therefore noticeably induced the hydroxylation of phenacetin to 2HPN. The unchanged phenacetin levels remained unaltered during the period of study (Fig. 21).
CONCLUSION

Sulfation plays an important role in the metabolism of phenacetin in the rat. Most of the drug was accounted for as the major metabolite, paracetamol-sulfate. Partial suppression of the sulfation pathway with pentachlorophenol increased only the fraction of phenacetin metabolized to 2-hydroxyphenetidine. It did not increase the formation of 2-hydroxyphenacetin or N-hydroxyphenacetin.

As a result of sulfation inhibition, more phenacetin was excreted unchanged. The phenacetin metabolized via deethylation and the sulfation pathway was not alternatively accounted for through any other metabolic pathway. This may indicate a limited capacity of the alternative pathways of phenacetin metabolism in the rat.

Autoinduction of hydroxylating enzymes was seen at both high and low doses of phenacetin used, though to a lesser degree with the lower dose. Induction of N-hydroxylation was enhanced by aspirin and caffeine. This significant observation could explain the potentiated toxicity encountered in the chronic abuse of aspirin-phenacetin-caffeine combinations. The increase in N-hydroxylation was more likely a result of direct stimulation of the hydroxylating enzymes rather than an effect of the accompanying partial suppression of sulfation resulting when these drugs are co-administered with phenacetin.
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