METABOLISM AND TOXICITY OF PARACETAMOL IN ISOLATED RAT AND MOUSE HEPATOCYTES

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by

PORNPEN PRAMYOTHIN

(B.Sc. Hons, M.S.)

Department of Clinical and Experimental Pharmacology,

The University of Adelaide,

South Australia.

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DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge contains no material previously published by any other person, except where due reference is made.

Results of this thesis have been presented to meetings of the Australasian Society of Clinical and Experimental Pharmacologists in Sydney (August, 1979) and at a symposium, Drugs and the Liver, jointly organised by the Australasian Society of Clinical and Experimental Pharmacologists and the Gastroenterological Society of Australia (Melbourne, October, 1980).

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* * *

ABSTRACT

The aim of the thesis was to evaluate the use of isolated hepatocytes for studying the relationships between metabolism and toxicity, using paracetamol as a model drug.

The study of paracetamol activation in isolated rat and mouse hepatocytes confirmed the involvement of microsomal cytochrome P-450. The formation of reactive metabolite(s) was quantitated by products of secondary reactions, namely, irreversible binding to hepatic protein and glutathione conjugation.

Treatment of the hepatocytes with metabolic inducers and inhibitors resulted in the modification of both the irreversible protein binding and the formation of glutathione conjugate. Although there were some discrepancies between drug-induced changes in binding and glutathione conjugation, it is possible to generalise the finding that microsomal enzyme inducers, particularly benz(α)pyrene, enhanced both the binding and paracetamol-glutathione conjugation, while the microsomal enzyme inhibitors (e.g., α-naphthoflavone, metyrapone) reduced both of them.

Furthermore, prior depletion of reduced glutathione by diethyl maleate in rat hepatocytes greatly enhanced the irreversible protein binding. Sulphydryl compounds, particularly N-acetylcysteine, decreased the binding and increased the glutathione conjugation in isolated mouse hepatocytes whereas only a reduction in binding was found in rat hepatocytes. The antidotal mechanism of N-acetylcysteine was not resolved, but the evidence favoured the facilitation of glutathione synthesis and/or action as an alternative nucleophile.

Species difference was also demonstrated in the isolated hepatocyte system. Isolated mouse hepatocytes activated paracetamol to a greater extent than hepatocytes from rats and this accords with species difference observed in vivo.

Neither glutathione depletion nor toxicity were noted in both rat and mouse hepatocytes after incubating with paracetamol (250 μM) for 3 hours, but after incubation over 24 hours in primary hepatocyte cultures, some drug-related loss of cell membrane integrity was revealed, particularly in hepatocytes from benz-(α)pyrene-induced rats. This demonstrates that latency, as shown in vivo, is required for the expression of toxicity.

In conclusion, isolated hepatocytes appear to have potential for studying drug metabolism and drug-induced toxicity.

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ABBREVIATIONS

ALT	alanine aminotransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
EDTA	ethylenediamine tetraacetic acid
GLDH	glutamate lactate dehydrogenase
GSH	reduced glutathione
HPLC	high performance liquid chromatography
MEM	Minimum Essential Medium
NADH	dihydronicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	dihydronicotinamide adenine dinucleotide phosphate
OPT	o-phthaldehyde
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PCA	perchloric acid
SGOT	serum glutamate oxaloacetate transaminase
SGPT	serum glutamate pyruvate transaminase
TCA	trichloroacetic acid
TPB	tetraphenylboron
UDP	uridine nucleotide

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GENERAL INTRODUCTION

(a) **The preparation of isolated hepatocytes:**

Early methods used mechanical force to dissociate the cells from the liver. Cells prepared by these techniques usually exhibited marked changes in fine structure and lack of respiration when suspended in typical extracellular media (Berry, 1976). They were severely damaged and failed even relatively insensitive viability tests (David et al., 1975). Chelating agents, such as citrate, tetraphenylboron (TPB) and ethylenediamine tetraacetic acid (EDTA), were later used to remove Ca^{+2} and K^{+} from the intercellular bonds. These methods also resulted in poor yields of relatively damaged cells (Jeejeebhoy and Phillips, 1976; Wagle and Ingebretsen, 1975; Moldeus et al., 1978). Attempts to dissolve intercellular junctions using digestive enzymes (trypsin, papain, lysozyme, pepsin, etc.), which also digest and damage the plasma membrane of hepatocytes, also resulted in cells with poor viability (Berry, 1976; Jeejeebhoy and Phillips, 1976).

A major development was the introduction of collagenase and hyaluronidase for isolation of rat hepatocytes. This technique was first described by Howard et al. (1967, 1968). These enzymes digest intercellular junctions without seriously affecting the plasma membrane and had been used previously for isolation of adipocytes (Rodbell, 1964). Although yields obtained by Howard's technique were very low, liver cells appeared to be morphologically intact and metabolically active (Berry, 1976). Howard's method was improved and modified by Berry and Friend (1969), who introduced a recirculating perfusion technique. This modification greatly increased cell yields and improved the subcellular integrity of the isolated liver cells.

The basic technique for isolation of hepatocytes by Berry and Friend (1969) has been modified by several investigators. Wagle and Ingebretsen (1975) simplified the procedure by using collagenase as the only digestive enzyme. Seglen (1973) introduced preperfusion of the liver with calcium-free medium prior to the perfusion with collagenase in a calcium-containing medium. These modifications decreased the perfusion time and increased viable cell yields.

It appears that any successful method must involve three critical steps; namely, exposure of the tissue to a calcium-free medium, digestion with collagenase and gentle mechanical treatment (Berry, 1976; Jeejeebhoy and Phillips, 1976).

Although most of the current methods are based on the perfusion of liver with digestive enzymes, non-perfusion techniques are still in use, particularly when perfusion is not feasible; e.g., when cells are to be isolated from biopsy material (Fry et al., 1976; Fry and Bridges, 1977; Bellemann, 1977).

(b) **Cell viability:**

Isolated hepatocytes vary in both yield and quality, even when the same isolation procedures are used; therefore, it has been necessary to develop criteria of viability to verify that the results from cell preparations are representative of normal hepatocyte function (Jeejeebhoy and Phillips, 1976). The criteria used most widely are based upon morphological features, particularly plasma membrane integrity, cell respiration, and the capacity to maintain biosynthetic and catabolic reactions (Jeejeebhoy and Phillips, 1976; Baur et al., 1975).

The morphology of isolated hepatocytes under the electron microscope has been used by many investigators (Berry and Friend, 1969; Schreiber and Schreiber, 1973; Howard et al., 1973; Wagle and Ingebretson, 1975; Jeejeebhoy and Phillips, 1976). The electron micrographs should show cells with spherical shape, whose cell membranes show numerous microvilli and whose organelles show normal structure and distribution (Jeejeebhoy and Phillips, 1976).

For measurement of plasma membrane integrity, suitable tests include the ability of cells to exclude substances (e.g., trypan blue or succinate) which should normally be impermeable or the ability to retain cytoplasmic enzymes (e.g., lactate dehydrogenase) (Seglen, 1976). Amongst those in common use are tests such as: trypan blue exclusion, leakage of intracellular enzymes, membrane potential, intracellular K^+, respiratory stimulation by succinate, ATP level, and penetration of NADH into cells (Berry and Friend, 1969; Baur et al., 1975; Jeejeebhoy and Phillips, 1976; Moldeus et al., 1978).

With regard to sensitivity, the trypan blue exclusion test, and tests of intracellular enzyme leakage appear to detect only severe, probably irreversible, damage to the plasma membrane. Retention of intracellular K^+ appears to be more sensitive, in that it shows effects not detectable in the above tests (Baur et al., 1975; Stacey and Priestly, 1978).

Among the tests of metabolic capacity in common use are: gluconeogenesis, ketogenesis, ureogenesis and protein synthesis (Krebs, 1976; Schreiber and Schreiber, 1973; Zimmerman, 1976). This metabolic capacity should be comparable to that of intact

animal (Jeejeebhoy and Phillips, 1976). Drug metabolism (both phase I and phase II reactions) in isolated hepatocytes should also be correlated with in vivo drug metabolism (Billings et al., 1977; Yih and Rossum, 1977). Cytochrome P-450 (monooxygenase system) is largely present in the oxidized, non-substrate bound state and the addition of various drugs to the cell suspension produces the type I spectral change as the result of the formation of the cytochrome P-450 substrate complex. Although the rate of formation of the spectral change may be slower, the magnitude should be the same when compared to results with isolated microsomes (von Bahr et al., 1974; Moldeus et al., 1973, 1978).

The factors involved in selecting suitable viability tests include their simplicity and sensitivity. Different laboratories seem to have developed their own test battery. In experiments described in this thesis, the trypan blue exclusion test was used routinely to establish the viability of freshly prepared hepatocytes, while K^+ retention and ALT leakage were used to monitor the plasma membrane integrity during the course of an experiment. The focus on plasma membrane integrity is justified by the hypothesis (Farber and El Mofty, 1975) that derangement of this membrane is a critical step in the sequence of events leading to cell death.

(c) Comparison of isolated hepatocytes with other liver preparations:

Isolated liver cells have an intermediary level of function between that of isolated organelle fractions and that of isolated perfused livers, liver slices, or the intact animal (Moldeus et al.,

1978). Each of these other systems have inherent disadvantages in the study of hepatic metabolism and toxicity.

Liver slices are a non-uniform and complex system. They consist mainly of intact cells in which oxygen diffusions to cells on the inside of the slice is limited and mechanically damaged cells on the outer surface. Essential components of liver metabolism become greatly impaired on slicing, thus a large proportion of the capacity for gluconeogenesis, oxidising fatty acids and maintaining normal concentrations of adenine nucleotide is lost (Krebs, 1970; Krebs et al., 1974; Johnson et al., 1972; Cornell and Filkins, 1974).

Liver homogenates and their fractionated organelles have been used extensively for studies on hepatic metabolism, but they commonly require supplementation with essential cofactors. They lack the adenine nucleotides and glycogen necessary for maintaining a variety of metabolic reactions (Schimassek et al., 1974). Furthermore, although microsomal preparations catalyse the oxidative reactions important in the metabolism of drugs and endogenous liposoluble substances, the associated conjugative reactions may be either deficient or out of balance when compared with the in vivo state. This can lead to serious problems of interpreting the rate and significance of drug metabolic reactions.

Isolated perfused livers have the advantage of being an in vitro system with function close to that of the in vivo state, but subtle differences in technique may result in substantial differences in interpretation. For example, in clearance studies, the size of perfusate volume compared to the normal plasma volume may result in quantitatively different results (Rowland, 1972).

In studies on glycogen and amino acid metabolism, results differed when recirculating perfusate systems were used instead of non-recirculating systems (Shimazu, 1971; Schimassek et al., 1974).

The intact animal, although it is the most relevant model for assessing metabolic and toxicologic reactions in vivo, may suffer from difficulties in interpreting the contribution of hepatic processes when extra-hepatic factors contribute substantially to the response.

The isolated hepatocytes can overcome many of the disadvantages mentioned in other systems. They permit a direct evaluation of regulation and integration of hepatic metabolism and function. They allow the study of many variables while also providing simultaneous control samples. Many homogenous samples can be rapidly withdrawn from an incubation mixture and incubation conditions can be adjusted to the need of the experiment (Jeejeebhoy and Phillips, 1976; Cornell and Filkins, 1974).

It is important to show that isolated hepatocytes compare favourably in terms of their membrane integrity and metabolic function with other standardised experimental models. It has been shown that both ATP and intracellular K^+ concentrations in isolated cells are similar to that in vivo (Jeejeebhoy et al., 1975; Krebs et al., 1974; Baur et al., 1975). Furthermore, it has been shown that gluconeogenesis, cholesterogenesis and plasma protein synthesis occur at a rate comparable to that in the perfused liver (Jeejeebhoy and Phillips, 1976; Krebs et al., 1974; Nilsson et al., 1973). Thus, isolated hepatocytes seem to retain many essential properties of intact tissue, including similar permeability characteristics and metabolic activity.

A potential limitation to the use of isolated hepatocyte suspensions is their relatively short duration of viability in typical incubation experiments using relatively simple media. In order to extend the experimental period, primary monolayer hepatocyte cultures have been used. These have demonstrated several functions for at least three days of incubation, namely, albumin synthesis, gluconeogenesis and microsomal enzyme activity (Jeejeebhoy and Phillips, 1976; Bissell et al., 1973; Bonney et al., 1974). Although the phenotypic expression of differential liver functions seems to be modified by culture conditions, the culturing of adult liver parenchymal cells may yet be of great interest for studies on metabolic regulation, growth control and carcinogenesis (Bonney et al., 1974; Seglen, 1979).

(d) **Applications of isolated hepatocytes:**

Suspension of isolated hepatocytes are particularly suitable for biochemical studies on the regulation of liver function. The technique has found application in studies on cell respiration and the metabolism of carbohydrates: gluconeogenesis (Cornell and Filkins, 1974; Story et al., 1976; Berry and Kun, 1972; Johnson et al., 1972; Allan and Sneyd, 1975); glycogenesis (Bernaert et al., 1974; Wagle et al., 1973); glycolysis (Berry and Friend, 1969; Crisp and Pogson, 1972); the metabolism of lipids (Jeejeebhoy and Phillips, 1976); bile acid synthesis (Yousef et al., 1978) and secretion (Gardner and Chenouda, 1978); the metabolism of protein (Seglen, 1976a; van Bezooijen et al., 1976; Jeejeebhoy et al., 1975; Feldhoff et al., 1977; Seglen, 1977; Crane and Miller, 1974); the metabolism of nucleic acids and their precursors (Seglen, 1973);

membrane transport (Berg and Iversen, 1976), and the dynamics of intracellular lipid peroxidation (Hogberg et al., 1975; Hogberg et al., 1975a; Hogberg et al., 1975b; Remmer et al., 1977).

Studies with isolated hepatocytes which are more related to the matter of this thesis include drug metabolism (Moldeus et al., 1974; Moldeus et al., 1976; Grundin, 1975; Wiebkin et al., 1976; Billings et al., 1977; Holtzman, 1972; Yih and Rossum, 1977; Jones et al., 1978; Wiebkin et al., 1978) and drug-induced toxicity (Zimmerman, 1976; Abernathy et al., 1975; Zimmerman et al., 1974; Yasuhara et al., 1979; Stacey et al., 1978; Jollow and Smith, 1977; Mitchell and Jollow, 1975; Stacey and Priestly, 1978; Seglen, 1979; Walton and Buckley, 1975).

(e) **Metabolism, toxicity and isolated hepatocytes:**

Most of hepatic drug metabolism is dependent on two metabolic reactions, namely, oxidation (phase I) and conjugation (phase II). Oxidation is catalysed by a microsomal mixed function oxidase system which requires NADPH, molecular oxygen and cytochrome P-450. This system converts a number of lipid soluble compounds of both endogenous and exogenous origin into more polar products which may be subsequently conjugated with glucuronic acid or sulphate (Moldeus et al., 1976; von Bahr and Bertilsson, 1971).

Metabolism of xenobiotics by isolated hepatocytes in suspension closely resembles the situation in vivo, largely because the biochemical and structural organisation at the cellular level in isolated cells is intact (Fry et al., 1976; Fry and Bridges, 1977). Therefore, isolated hepatocytes are likely to be particularly suit-

able for the study of coupled oxidative/conjugative drug metabolism (Jones et al., 1978; Wiebkin et al., 1978). They have been shown to maintain a sufficiently high NADPH/$NADP^+$ ratio to support drug oxidation in the absence of exogenously added cofactors such as NADPH or UDP-glucuronic acid (Grundin, 1975; Moldeus et al., 1974, 1976). They are capable of carrying out a number of typical reactions catalysed by the microsomal mixed function oxidase system (Billings et al., 1977; Holtzman et al., 1972; Yih and van Rossum, 1977). Dealkylation and hydroxylation reactions (Moldeus et al., 1974; Vadi et al., 1975; Moldeus et al., 1976; Wiebkin et al., 1976), conjugations (Moldeus et al., 1976; Wiebkin et al., 1976), cytochrome P-450 and its type I binding of drug substrates (Moldeus et al., 1973; Moldeus et al., 1974; von Bahr et al., 1974) have all been measured with isolated hepatocytes.

Preparations of isolated hepatocytes also provide a convenient in vitro system for studying other factors which determine the rate of hepatic metabolism. These factors include cell membrane permeability for xenobiotics and/or metabolites, the overall metabolite pattern and inter-relationship between phase I and phase II metabolism, and the subsequent rate of release of free and conjugated metabolites from the cell into the extracellular environment (Jones et al., 1978; Wiebkin et al., 1978).

Metabolism of drugs or other xenobiotics by hepatic monooxygenase system linked to cytochrome P-450 usually leads to the formation of pharmacologically inert, readily excretable products. However, it is now recognised that cytochrome P-450 catalysed oxidation can result in the formation of metabolites which are chemically highly reactive. Covalent binding of these reactive

metabolites to cellular macromolecules may initiate events leading to cell death. A number of serious toxicities, including allergic reactions, blood dyscrasias, haemolytic anaemia, teratogenicity, mutagenicity and carcinogenesis, have been linked with this mechanism (Jollow and Smith, 1977; Mitchell and Jollow, 1975). There is now little doubt that hepatic damage induced by carbon tetrachloride, halobenzenes, paracetamol, furosemide and isoniazid is mediated by chemically reactive metabolites (Gillette, 1974; Jollow and Smith, 1977).

By itself, the finding that covalent binding of radiolabel to tissue macromolecules occurs after the administration of radiolabelled foreign compound would not be sufficient proof that a reactive metabolite mediated the toxicity under investigation or any other toxicity. The hypothesis would be strengthened if treatments of animals which alter the pattern of metabolism of the toxicant caused parallel changes in the amount of covalent binding to macromolecules and in the incidence and severity of toxicity. According to this view, it would not be necessary to identify either the reactive metabolite or target macromolecule in order to determine whether the toxicity was mediated by a chemically reactive metabolite (Gillette, 1974).

(f) **Isolated hepatocytes and hepatotoxicity:**

Many chemicals have been reported to cause liver damage. Some hepatotoxins or their metabolic products produce direct injury to the hepatocyte and its organelles, especially the endoplasmic reticulum. These are classified as intrinsic or predictable hepatotoxins (e.g., carbon tetrachloride). Others

which cause hepatic injury, unpredictably, in a small proportion of the recipients are considered to depend upon an idiosyncratic response of the individual, rather than the toxic effects of the drug. One form of idiosyncratic response is suggested to be a hypersensitivity reaction or drug allergy (Zimmerman, 1976; Abernathy et al., 1975).

A variety of in vivo and in vitro experimental models have been used to assess drug-induced hepatotoxicity. Isolated hepatocytes may be said to offer potential advantages over in vivo systems in that they permit the exclusion of influencing factors, such as hormonal regulation, blood flow, and extrahepatic factors, which may determine the disposition of the toxicant. Furthermore, they may more readily provide insight into cellular mechanisms that mediate the toxic response (Walton and Buckley, 1975; Zimmerman, 1976).

Other advantages of isolated hepatocytes include: economy relative to in vivo studies requiring large numbers of animals; their suitability for studying dose-response relationships; drug interactions; and the comparative effects of different drugs (in the same cell preparation). These advantages are well illustrated by studies on drugs whose hepatotoxic potential in vivo ranges from idiosyncratic to intrinsic. Such studies include those on chlorpromazine and erythromycin estolate (Zimmerman et al., 1974); tricyclic antidepressants (Abernathy et al., 1975; Yasuhara et al., 1979), volatile anaesthetics (Stacey et al., 1978), carbon tetrachloride (Stacey and Priestly, 1978; Gravela et al., 1979) and bromobenzene (Thor et al., 1978, 1978a).

Possible limitations to the use of isolated hepatocytes in

studies of drug-induced hepatotoxicity are their relatively short duration of viability and their inability to excrete metabolites and unchanged drug. These limitations may be particularly important where there is a relatively long latency period in the development of the hepatic lesion. It is possible that this problem may be overcome by reverting to primary hepatocyte culture techniques.

Long-term maintenance of non-dividing adult liver parenchymal cells in primary cultures can be achieved by three major methods: monolayer culture (Bissell et al., 1973; Bonney et al., 1974), suspension culture (Jeejeebhoy et al., 1975), and culture on floating collagen membranes (Michalopoulos and Pitot, 1975). It has been shown that a variety of liver associated functions (e.g., albumin synthesis and secretion, gluconeogenesis, haeme catabolism, drug metabolism, etc.) are maintained in primary cultured cells in vitro at the levels comparable to those of liver in vivo for 3-4 days as monolayer cultures and 14-21 days as the free-floating cultures on the collagen membranes (Jeejeebhoy and Phillips, 1976; Michalopoulos et al., 1976). A major defect of hepatocytes in primary culture is the deficiency of cytochrome P-450, which rapidly breaks down and decreases to 20% of the level in vivo by 24 hours after plating (Guzelian and Bissell, 1974). However, treatment of adult hepatocytes on floating collagen membranes with either phenobarbital or 3-methylcholanthrene induces up to a two-fold increase in cytochrome P-450 and P-448 (Michalopoulos et al., 1976). Alternatively, supplementation of the medium with hormones and cofactors may preserve the content of cytochrome P-450 in monolayer cultures at levels close to those

found in vivo (Decad et al., 1977). Preservation of microsomal activation systems is clearly imperative if cultured cells are to be used in the investigation of hepatocarcinogenesis by chemicals in vitro or as a screening system for the detection of carcinogenic or procarcinogenic substances (Seglen, 1979; Michalopoulos et al., 1976).

(g) Aims of the thesis:

The overall aim of this thesis was to evaluate the use of isolated hepatocyte preparations for studying the relationships between metabolism and toxicity. Paracetamol was chosen to test the toxicological application of the model on the basis that the relationship between metabolic activation/inactivation and hepatotoxicity has been extensively investigated in the intact animal and many of the regulatory factors have been identified.

If isolated hepatocytes represent a toxicological model from which valid extrapolation of toxic mechanisms and the effects of drug interactions and other modifying factors can be made, then the following criteria will need to be satisfied:

1. The effect of paracetamol should be dose-related.
2. Species differences in paracetamol metabolism and toxicity should reflect the in vivo state.
3. Induction or inhibition of the formation of reactive metabolites should be shown to occur as a result of manipulation of microsomal mixed-function oxidase activity, or of associated conjugative metabolic pathways.
4. The GSH status of the hepatocytes should be a factor in the extent of reactive metabolite formation. It should

be possible to potentiate covalent binding of ^{14}C-paracetamol by the use of drugs which reduce hepatic GSH, or to inhibit covalent binding by the use of sulphydryl compounds which augment hepatic GSH, or act as the alternative substrates.

* * *

GENERAL METHODS

Methods used generally throughout the thesis are described in this section. Experimental design and specific methods are elaborated in individual chapters and appendices.

Adult male Porton rats (200-250 g) and male LACA Albino mice (20-30 g) were used as the liver donors. They were obtained from the University Central Animal House and maintained on Charlick's M and V 164 mouse cubes and Adelaide tap water, which were allowed ad libitum. No experiments involved food and water deprivation. Chemicals and drugs used in this study are documented in Appendix 1. All experiments followed a similar time sequence, with surgery commencing between 8.30-9.00 a.m. each day.

(a) **Isolation of rat hepatocytes:**

The method of Berry and Friend (1969), as modified by Stacey and Priestly (1978), was used for the preparation of isolated hepatocytes.

Under ether anaesthesia, the rat peritoneal cavity was opened by a midventral incision and the liver exposed. Connective tissue surrounding the liver was carefully disjoined. The oesophagus and accompanying blood vessels were doubly ligated and cut between the two ties. Intestines were then removed to the left of the animal to expose the portal vein. The small blood vessel joining the portal vein and running close to the bile duct was ligated. Heparin (750-1000 units in 0.15 ml) was injected in the inferior vena cava. Two loose ligatures were applied around the portal vein; the distal ligature was then tied and tension was placed to the vessel via this tie. The proximal ligature was

lifted slightly to reduce back flow of blood from the liver; an oblique incision was then made, and the cannula was immediately inserted, pushed past the proximal ligature and then tied into place with that ligature. The inferior vena cava was cut, and perfusion of the liver was commenced from a constant pressure head of about 20 cm of water with calcium-free physiological solution (see Appendix 2). When the perfusion of all hepatic lobes was rapid and complete (1 minute), the liver was excised and transferred to a humidified temperature controlled chamber maintained at 37°C. Perfusion was then re-instituted under recirculating conditions (flow rate 30-35 ml/min) with the same calcium-free physiological solution equilibrated with Carbogen (95% O_2 : 5% CO_2). Collagenase (Sigma, type IV) was added to give a final perfusate concentration of 70 units/ml. The final volume of the recirculating collagenase medium was 90 ml. Perfusion with collagenase containing medium was continued (10-12 minutes) until the liver appeared swollen and pale, indicating an adequate digestion of the intracellular matrix.

The liver was then placed in a beaker containing 25 ml of fresh collagenase buffer (70 units/ml), the capsule was disrupted gently with a blunt spatula, and the volume was made up to 50-60 ml with collagenase buffer from the recirculation. The dispersed liver was incubated for a further 10 minutes in a metabolic shaking bath at 80 oscillations per minute and maintained at 37°C in 2 x 250 Erlenmeyer flasks under an atmosphere of Carbogen (95% O_2 : 5% CO_2).

Bovine serum albumin (BSA) was added to give a final concentration of 12 mg/ml and cells were harvested by sieving

through nylon mesh (250 and 61 μm). Cells were centrifuged (50 g, 1 min) and washed twice with this medium, and then once with Eagle's Basal Medium (see Appendix 3) containing 12 mg/ml BSA, and finally resuspended in this medium at the concentration of approximately 5-6 x 10^6 cells/ml.

Cells were counted with a haemocytometer and a trypan blue exclusion test was performed. Cell preparations with a trypan blue exclusion index of less than 90% were never used in any studies of this thesis. Cell yields of 4-6 x 10^8 cells were routinely obtained with a trypan blue exclusion index of 95-99%.

(b) **Isolation of mouse hepatocytes:**

The procedure used for isolation of mouse hepatocytes was similar to that used for preparation of rat hepatocytes. For each experiment, the hepatocytes of four mouse livers were pooled. The portal vein of each mouse was cannulated under ether anaesthesia with one of four catheters connected in parallel. Perfusion of each liver was continuous from the time of cannulation to the time when all four excised livers were transferred to the perfusion cabinet. The interval between cannulation of the first and fourth livers was usually 10-15 minutes. After transfer, perfusion was re-instituted under recirculating conditions. Collagenase digestion and cell harvesting were carried out as described for rat hepatocytes.

Yields of 1-2 x 10^8 cells were routinely obtained with a trypan blue exclusion index of 93-96%. Cell preparations with a trypan blue exclusion index of less than 90% were never used.

(c) Incubation of hepatocyte suspensions:

Erlenmeyer flasks (25 ml) were used for the incubation. The relevant drugs (never more than 0.3 ml to 2.5 ml cell suspension) and aliquots (usually 2.5 ml) of cell suspension were added to the flasks which had been pregassed with Carbogen (95% O_2 : 5% CO_2). The flasks were then stoppered with rubber bungs, but gassing continued throughout the incubation via a gas manifold of fine polythene tubes supplying each flask. Flasks were incubated for up to 3 hours in a metabolic bath at 37° C with reciprocal shaking at 80 oscillations per minute.

(d) Potassium ion (K^+) and alanine aminotransferase (ALT):

Aliquots (usually 0.5 ml) of cell suspensions were centrifuged at 50 g for 1 minute. ALT activity in the supernatant was determined by the method of Reitman and Frankel (1957). Enzyme activity was always measured on the day of the experiment (Stacey, Cook and Priestly, 1978). The cell pellet was extracted with 1 ml 3% (w/v) perchloric acid (PCA), then centrifuged at 3500-5000 r.p.m. for 5-10 minutes. The resulting supernatant was diluted with distilled water and the K^+ concentration determined by flame photometry (Eel Model 150 Clinical Flame Photometer).

(e) Reduced glutathione (GSH):

A fluorometric assay for glutathione based upon that described by Cohn and Lyle (1966) was used. Aliquots (0.2 ml) of cell suspension were added to 0.5 ml ice cold 30 µM ethylenediamine tetraacetic acid (EDTA). Proteins were removed by precipitation with 0.2 ml 25% (w/v) metaphosphoric acid followed

by centrifugation in the cold at 5000 g for 5-10 minutes. A 0.5 ml aliquot of the protein free supernatant was then diluted to 2.5 ml with distilled water. Tris buffer (0.25 M) was used to adjust the pH of the solution to 8, then 0.1 ml (0.1% w/v) of o-phthalaldehyde (OPT) was added with thorough mixing. After a 15-20 minutes reaction period at room temperature, the reaction mixture was transferred to a cuvet and the fluorescence at 420 mμ resulting from activation at 350 mμ was determined. The GSH content in hepatocyte suspension was quantitated by reference to a standard curve of GSH in Appendix 4.

(f) **Irreversible protein binding of ^{14}C-paracetamol:**

The procedure followed was basically that of Jollow et al. (1973). Quantitation was based upon the amount of non-extractable ^{14}C bound to proteins after incubation of hepatocyte suspensions with ^{14}C-paracetamol (0.33 μCi ^{14}C-paracetamol diluted with non-radiolabelled paracetamol to the final concentration of 0.25 mM). The (ring - ^{14}C) paracetamol (specific activity 14.6 mCi/mmole) was 98% pure when analysed by thin layer chromatography on cellulose in butan-2-one : acetone : formic acid : water (40 : 2 : 1 : 6) and was used without further purification. This radiolabelled paracetamol was obtained from the Radiochemical Centre (Amersham). After the appropriate period of incubation, cells were resuspended in 2 ml ice cold 0.1 M phosphate buffer, pH 7.4, and then 1 ml 0.9 M trichloroacetic acid (TCA) was added to precipitate the protein, followed by centrifugation at 1500 g for 5 minutes. The supernatant was discarded. The protein precipitate was extracted once with 0.6 M TCA and six times with

acetone. Preliminary experiments established that this procedure exhaustively extracted unbound radioactivity. The extracted protein was dissolved in 1 ml soluene-350 and 0.5 ml aliquot was added to Aquasol scintillation fluid (Triton-toluene) and counted in a Packard Tri-Carb scintillation counter. Radioactivity was corrected for background and for quench (external standard).

The protein concentration of the resuspended cell preparations prior to addition of 0.9 M TCA was determined by the method of Lowry et al. (1951). The calculation of irreversible protein binding of ^{14}C-paracetamol as nmole/mg protein is shown in Appendix 5.

(g) Quantitative determination of paracetamol and its metabolites by high performance liquid chromatography:

Paracetamol and paracetamol metabolites were determined using high performance liquid chromatography by the method of Howie et al. (1977) and Moldeus (1978). Aliquots (1 ml) of cell suspension were added to 0.5 ml 3 N perchloric acid (PCA) and protein removed by centrifugation in the cold at 5000 g for 10 minutes. The protein free supernatant was used for paracetamol and metabolite analysis. A 15 µl aliquot of the supernatant was chromatographed on a reverse phase µ-Bondapak C_{18} column (Waters Associates Inc., Massachusetts, U.S.A.) with the solvent system containing water : glacial acetic acid : ethyl acetate (98 : 1 : 1). The flow rate was 1.4 ml/min (Waters Model 45 solvent pump) and the detector was a Waters Model 440 UV detector operating at 254 nM.

Quantitation of paracetamol and its metabolites was

estimated by reference to peak height ratios with 3-hydroxy phenacetin as internal standard. The internal standard and reference metabolites were kindly provided by Dr R. S. Andrews of Winthrop Laboratories, Sterling Winthrop, Newcastle, England. The standard chromatogram and standard curve of paracetamol and paracetamol metabolites are shown in Appendix 6.

(h) Primary monolayer tissue cultures of isolated adult rat liver cells:

Throughout the preparation of rat hepatocyte cultures aseptic precautions (including the use of laminar flow hood) were taken whenever possible. Cells were prepared as previously described (section a.) by perfusion with a sterile recirculating physiological solution containing collagenase and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin sulphate). After the digestive step with collagenase under recirculating conditions, the dispersed liver was shaken gently (by hand) in the same medium and filtered through sterile nylon mesh (250 and 61 µm). Cells were centrifuged (50 g, 1 min) and washed twice with sterile Eagle's MEM (see Appendix 7) containing 100 units/ml penicillin G and 100 µg/ml streptomycin sulphate. They were then washed again and resuspended at a concentration of 0.5-1 x 10^6 cells/ml in sterile Eagle's MEM containing 10% foetal calf serum and antibiotics (same concentrations).

Aliquots of 5 ml of this cell suspension were dispensed into Falcon tissue culture flasks with 25 cm^2 growth area and canted neck (Falcon, California, U.S.A.). The flasks were gassed with Carbogen (95% O_2 : 5% CO_2) for 2-3 minutes and immediately

sealed. The flasks were incubated in a humidified chamber at 37 °C for 24 hours. Although a controlled-atmosphere incubator was not available, this procedure was shown to be adequate to maintain the pH and, by inference, the CO_2 partial pressure, since this is a component of the bicarbonate buffer system in the incubates.

(i) **Microscopy:**

Cell counting, cell appearance and trypan blue exclusion tests of isolated hepatocytes were performed using an Olympus light microscope at 100x magnification.

Photomicrography of hepatocyte monolayer cultures was done using an Olympus Vanox light microscope with camera attachment. Aliquots of 2 ml cell suspension (from section h.) were put into Leighton tubes, with flat surface well containing fitted slides (Bello Glass Inc., Vineland, N.J.). These tubes were gassed with Carbogen (95% O_2 : 5% CO_2) for 2-3 minutes and immediately sealed with their screw caps, then incubated in a humidified incubator at 37 °C for 24 hours. After 24 hours, slides with attached hepatocytes were carefully removed from the tubes and covered with small cover slide prior to photomicrography.

(j) **Expression of results and statistics:**

Values of cell viability and metabolite formation have been expressed using the wet weight of cells as a reference point (see Appendix 8).

The results have been generally expressed as mean ± s.e.m. The number of cell preparations (n) was usually 3, but within

each experiment, replicates (x = 2-3) were used to generate mean values.

Differences between treated and control groups were analysed by student t-test (probability p = 0.05) using either a paired or unpaired test according to the experimental design.

* * *

CHAPTER I

PARACETAMOL METABOLISM IN ISOLATED RAT HEPATOCYTES

Introduction

Paracetamol appears to be a very safe minor analgesic and antipyretic drug when taken in normal therapeutic doses. Large overdoses of paracetamol, however, can produce fatal hepatic necrosis in man and animals (Prescott et al., 1977; Davis et al., 1974, 1976). Thus, the pattern of paracetamol metabolism can change with the dose which may be toxic when a certain threshold is exceeded.

Metabolism of paracetamol primarily includes conjugation reactions at the 4-hydroxy group forming the non-toxic glucuronide and ethereal sulphate derivatives, which account for about 80% of the clearance of this drug in all species studied (Jollow et al., 1974). In addition, paracetamol is oxidized by cytochrome P-450-dependent drug metabolising enzymes to a reactive intermediate. This was initially believed to be the N-hydroxy derivative, which then underwent spontaneous degradation to N-acetyl-benzoquinone (Jollow et al., 1973; Mitchell et al., 1974; Jollow et al., 1974). Although the identity of the toxic metabolite(s) is currently a matter of some dispute in the literature, there is consensus to the view that it reacts with hepatic glutathione, forming a glutathione conjugate, and after further metabolism, is excreted in the urine as mercapturic acid and cysteine derivatives. The clearance of this pathway varies from 3-5% in rats and man, to 12-15% in mice and hamsters (Jollow and Smith, 1977; Jollow et al., 1974; Mitchell et al., 1974). After a non-hepatotoxic dose of paracetamol, glutathione is capable of protecting the cell from the reactive intermediate; but after paracetamol overdose, with subsequent glutathione depletion, considerable

covalent binding of toxic metabolites to hepatic macromolecules occurs (Gillette et al., 1974). This may be the initiating step in the process of liver necrosis. (Fig. 1.1 summarises the metabolism of paracetamol and the proposed toxic pathway.)

Since the reactive metabolite is detoxified by conjugation with glutathione and appears in the urine as a mercapturic acid, the urinary excretion of paracetamol mercapturate may be regarded as an index of the activity of the metabolic pathway leading to the formation of the toxic metabolite (Jollow et al., 1974). This principle has been applied to the assessment of paracetamol toxicity in man and it has been used to show that phenobarbital induction increases the formation of the mercapturate conjugate (Gillette et al., 1974). Thus, studies of the pattern of paracetamol metabolites after modifying cytochrome P-450 mixed function oxidase activity by metabolic inducers (e.g., phenobarbital) and inhibitors (e.g., metyrapone) may grant further insight into the role of paracetamol metabolism in the production of liver damage in man and animals.

Metabolic inducers can broadly be categorised into two groups, one group being exemplified by phenobarbital and the other by the polycyclic aromatic hydrocarbons (e.g., 3-methylcholanthrene, benz(α)pyrene, benzanthracene, etc.). The cytochrome P-450 content, as well as the metabolism of numerous drugs in isolated liver microsomes, has been shown to increase after phenobarbital induction. These two groups of inducers may be distinguished by differences in the types of reaction induced and sensitivity to inhibitors, and by spectrophotometrically, or electrophoretically, determined differences in the haemoprotein

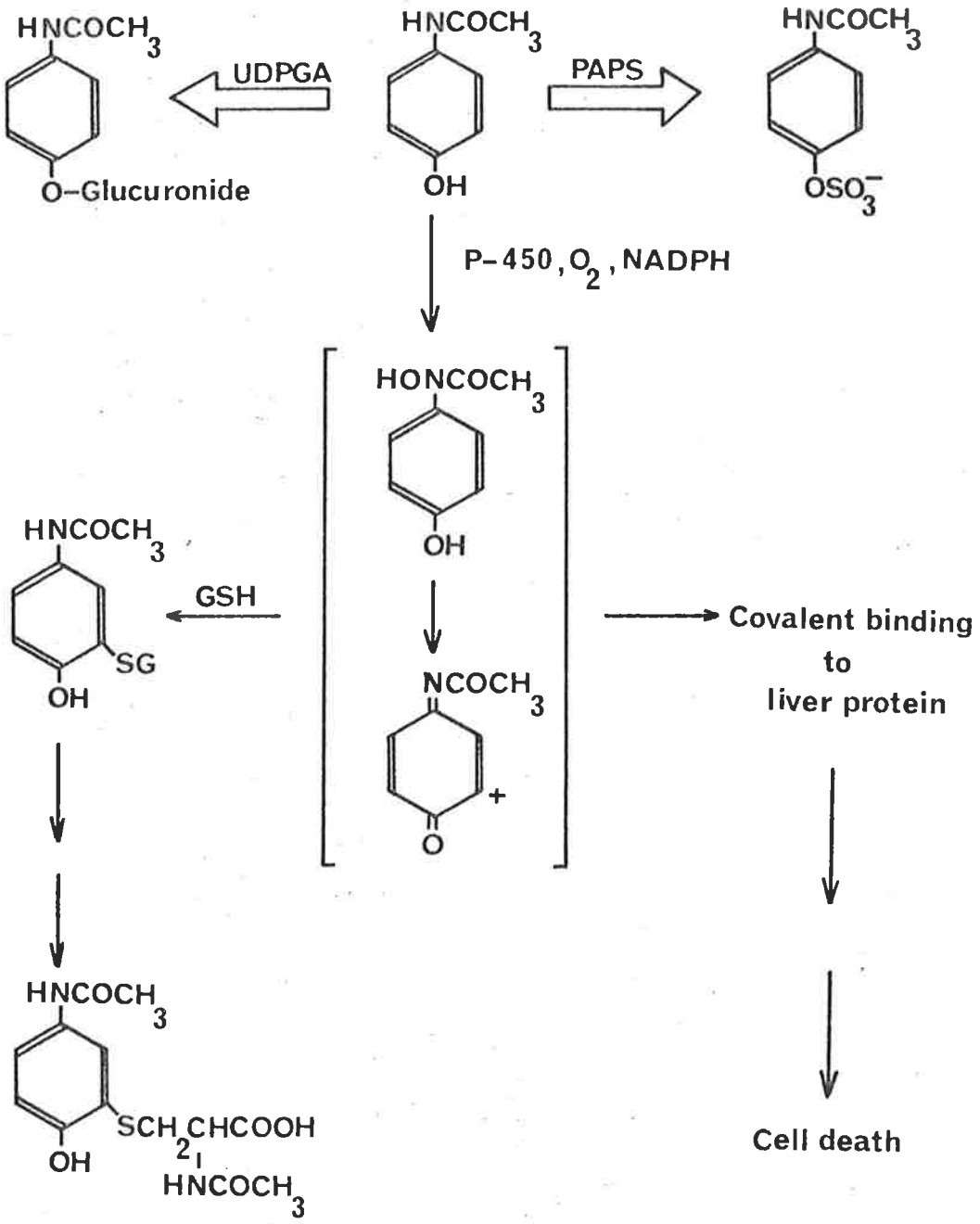


Fig. 1.1

Pathways of paracetamol metabolism.

(From Jollow and Smith, 1977.)

(cytochrome P-450) induced. Phenobarbital induction is by far the more general and important induction type, particularly in the clinical situation (Fry et al., 1979).

Metabolic inhibitors differ in the selectivity of their inhibitory effects. For example, SKF 525-A and α-naphthoflavone have been shown, respectively, to be the specific inhibitors of the microsomal cytochrome P-450 induced by phenobarbital and 3-methylcholanthrene (Ullrich et al., 1973); metyrapone is an inhibitor of cytochrome P-450 mediated hydroxylation for both type I and type II substrates (Mull et al., 1977; Moldeus, 1978a); and salicylamide is an inhibitor of the secondary conjugation of 7-ethoxycoumarin metabolites, and does not affect the primary oxidative diethylation (Moldeus et al., 1978).

Fresh isolated hepatocytes catalyse various phase I and phase II reactions involved in drug metabolism at rates comparable to those in vivo or in the isolated perfused liver (Billings et al., 1977). This experimental model may therefore be particularly suitable for elucidating the role of various conjugation reactions, notably the formation of glucuronide, sulphate and glutathione conjugates (Orrenius et al., 1978).

Sulphation and glucuronidation are catalysed by a soluble sulphotransferase and glucuronyl transferase, respectively. UDP-glucuronyl transferase is located in the endoplasmic reticulum, as is cytochrome P-450, and the two reactions, oxidation and glucuronidation, have been suggested to exist in a multi-enzyme complex (von Bahr and Bertilsson, 1971). However, glucuronidation often does not respond to the induction in the same manner as oxidation. This indicates that these two reactions may function

independently (Moldeus et al., 1977). The formation of a glutathione conjugate may be catalysed by glutathione S-transferase or may occur non-enzymatically. Hepatic glutathione S-transferase activities have been shown to be inducible by some metabolic inducers, such as phenobarbital, 3-methylcholanthrene or benz-(α)pyrene, as well as by isomers of hexachlorophenyl and tetrachlorodibenzo-p-dioxin (Younes et al., 1980).

With polar substrates and at low substrate concentration, the sulphate conjugation pathway is generally dominant. Increased lipophilicity or higher substrate concentration results in a shift toward producing a higher proportion of glucuronides. This suggests that glucuronidation is less easily saturable than sulphation, although both pathways may operate in parallel for the conjugation of various drug substrates (Orrenius et al., 1978).

There have been a number of recent reports of HPLC techniques which separate paracetamol metabolites. The sulphate, glucuronide, cysteine and mercapturic acid conjugates have been identified in human urine samples (Howie et al., 1977). Moldeus (1978) identified paracetamol metabolites from isolated hepatocyte incubations.

The interpretation of the toxicological consequences of metabolic activation of paracetamol in isolated hepatocytes necessitates the quantitation of the metabolites produced. This chapter describes the HPLC techniques used to quantitate the metabolic pathways of paracetamol and variations in these pathways caused by various chemical interactants. The aim was to modify the activity of the cytochrome P-450-dependent activation and conjugative pathways using selective inducers and inhibitors. The

correlation of these changes with changes in irreversible protein binding and consequent toxicity will be discussed in later chapters.

Methods

Hepatocytes were isolated from untreated control, phenobarbital and benzy(α)pyrene treated male rats weighing between 200-250 g. Pretreated animals received phenobarbital sodium and benz(α)pyrene intraperitoneally at the daily dose of 80 mg/Kg and 20 mg/Kg, respectively, for 4 days.

Incubation conditions were as described in the General Methods; 2.5 ml aliquots of cell suspension (5-6 x 10^6 cells/ml) were incubated with 0.1 ml paracetamol (1 mg/ml), 0.1 ml of physiological saline for control or 0.1 ml of various metabolic inhibitors. The final concentrations of substrate and inhibitors in the cell suspension were: paracetamol 250 μM, metyrapone 120 μM, salicylamide 200 μM and α-naphthoflavone 66 μM (Moldeus et al., 1978).

Duration of incubation was varied from half to 3 hours. At the end of incubation procedure, 1 ml samples were taken for metabolite analysis by HPLC.

Various doses of paracetamol (250 μM to 2000 μM) were also studied in control isolated rat hepatocytes using a similar procedure with an incubation time of one hour.

Results

Three major metabolites (glucuronide, sulphate and glutathione conjugates) were found after incubation of isolated rat hepatocytes with paracetamol (Fig. 1.2). Paracetamol cysteine and

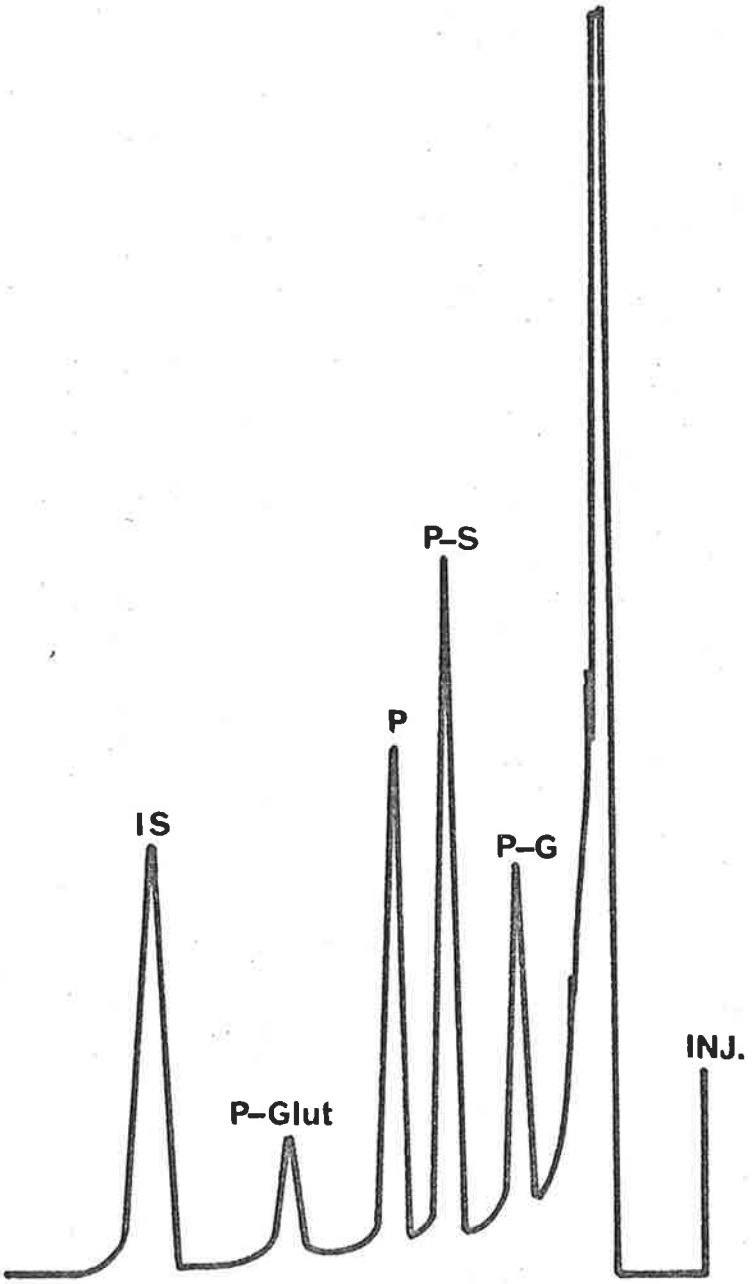


Fig. 1.2

High performance liquid chromatogram of an extract from isolated rat hepatocytes after incubation (1 hr) with paracetamol.

Inj	injection (15 µl)	P-G	paracetamol glucuronide
P-S	paracetamol sulphate	P	paracetamol
P-Glut	paracetamol glutathione	IS	internal standard

paracetamol N-acetylcysteine were not detected. At an initial paracetamol concentration of 250 µM, conjugation with sulphate exceeded that with glucuronide. However, the sulphate pathway became saturated at relatively low paracetamol concentration, and as the concentration increased, the proportion of glucuronide conjugate increased (Fig. 1.3). The time course of the depletion of substrate and the formation of three conjugates at an initial concentration of 250 µM is shown in Figure 1.4.

After phenobarbital pretreatment, the formation of glucuronide conjugate was enhanced nearly two-fold, whereas the sulphate and glutathione conjugates were minimally affected (Table 1.1). In contrast, the formation of glucuronide and sulphate conjugates was unchanged in hepatocytes isolated from benz(α)pyrene-treated rats, whereas the formation of the glutathione conjugate was increased approximately two-fold (Table 1.1).

In untreated (control) rat hepatocytes, metyrapone decreased the formation of paracetamol glucuronide and this was accompanied by a relatively late increase in sulphate conjugation. The formation of glutathione conjugate was unchanged (Fig. 1.4). In hepatocytes isolated from phenobarbital-treated rats, metyrapone still inhibited the glucuronide formation, but there was no change in the sulphate conjugate. Paradoxically, the glutathione conjugate was increased (Fig. 1.5). A further difference was noted in benz(α)pyrene-treated hepatocytes. Both glucuronide and glutathione conjugation were inhibited while the sulphate showed the same late rise as in the controls (Fig. 1.6).

Salicylamide inhibited the formation of sulphate conjugate in hepatocytes isolated from control, phenobarbital and benz(α)-

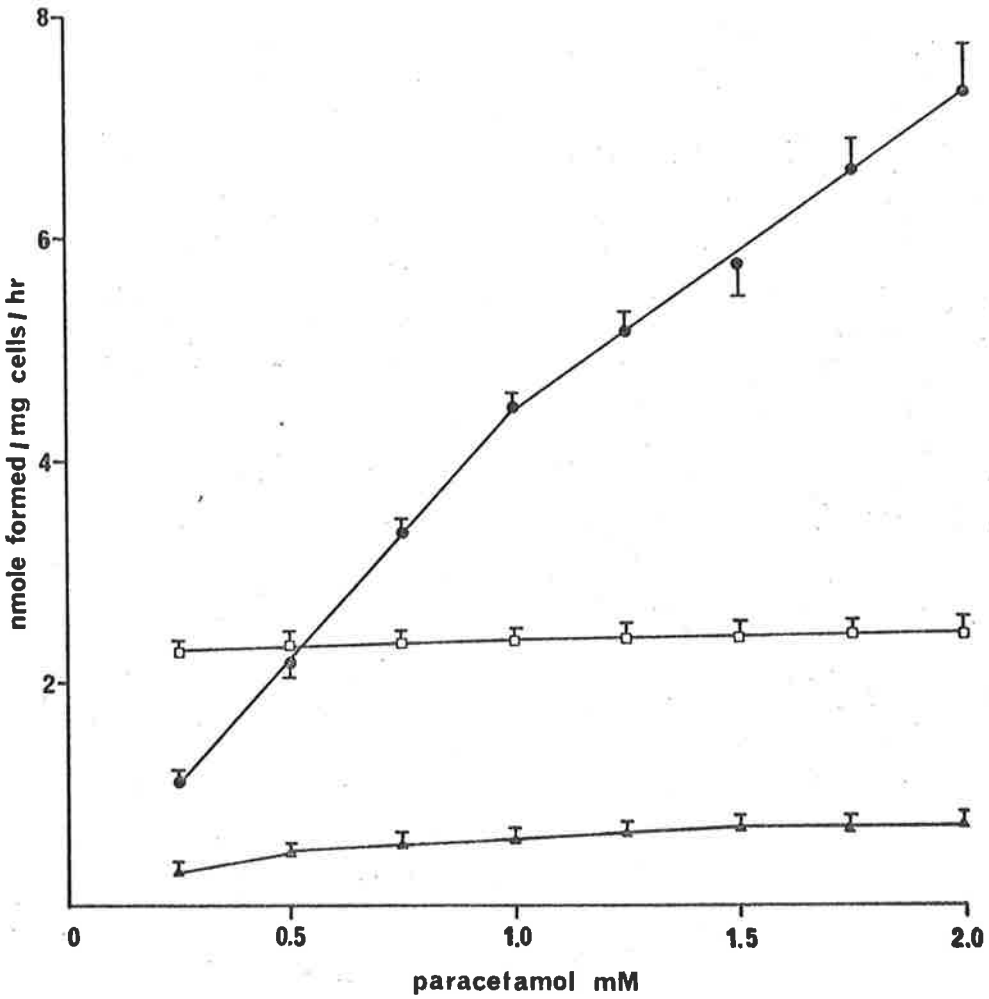


Fig. 1.3

Conjugates of paracetamol in isolated rat hepatocytes (1 hour incubation).

●—● paracetamol glucuronide

□—□ paracetamol sulphate

▲—▲ paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

TABLE 1.1

Effects of metabolic inducers on paracetamol disposition in isolated rat hepatocytes.

Treatment	Incubation time (hr)	Metabolite formed (nmole/mg cells) P-G	P-S	P-Glut
Control	$\frac{1}{2}$	0.79 ± 0.10	1.29 ± 0.04	0.20 ± 0.03
	1	1.29 ± 0.13	2.40 ± 0.14	0.37 ± 0.06
	2	1.37 ± 0.17	3.14 ± 0.17	0.37 ± 0.06
	3	1.46 ± 0.13	3.32 ± 0.07	0.36 ± 0.06
Phenobarbital	$\frac{1}{2}$	1.11 ± 0.05*	1.15 ± 0.06	0.15 ± 0.03
pretreated	1	1.79 ± 0.11*	2.04 ± 0.12	0.24 ± 0.01*
	2	2.34 ± 0.11*	3.12 ± 0.13	0.28 ± 0.01
	3	2.52 ± 0.12*	3.13 ± 0.18	0.30 ± 0.03
Benz(α)pyrene	$\frac{1}{2}$	0.69 ± 0.06	1.18 ± 0.21	0.38 ± 0.05*
pretreated	1	1.17 ± 0.05	2.22 ± 0.25	0.75 ± 0.07*
	2	1.34 ± 0.07	3.01 ± 0.19	0.94 ± 0.09*
	3	1.39 ± 0.08	3.30 ± 0.16	0.95 ± 0.08*

Values shown are mean ± s.e.m. (n=3)

P-G paracetamol glucuronide

P-S paracetamol sulphate

P-Glut paracetamol glutathione

* significantly different from control ($p<0.05$, t-test)

Fig. 1.4

Time course of metabolite formation in hepatocytes isolated from untreated rats incubated with paracetamol (250 μM) and various metabolic inhibitors.

□ saline control

▨ salicylamide (200 μM)

▥ metyrapone (120 μM)

■ α-naphthoflavone (66 μM)

P-G paracetamol glucuronide

P-S paracetamol sulphate

P paracetamol

P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

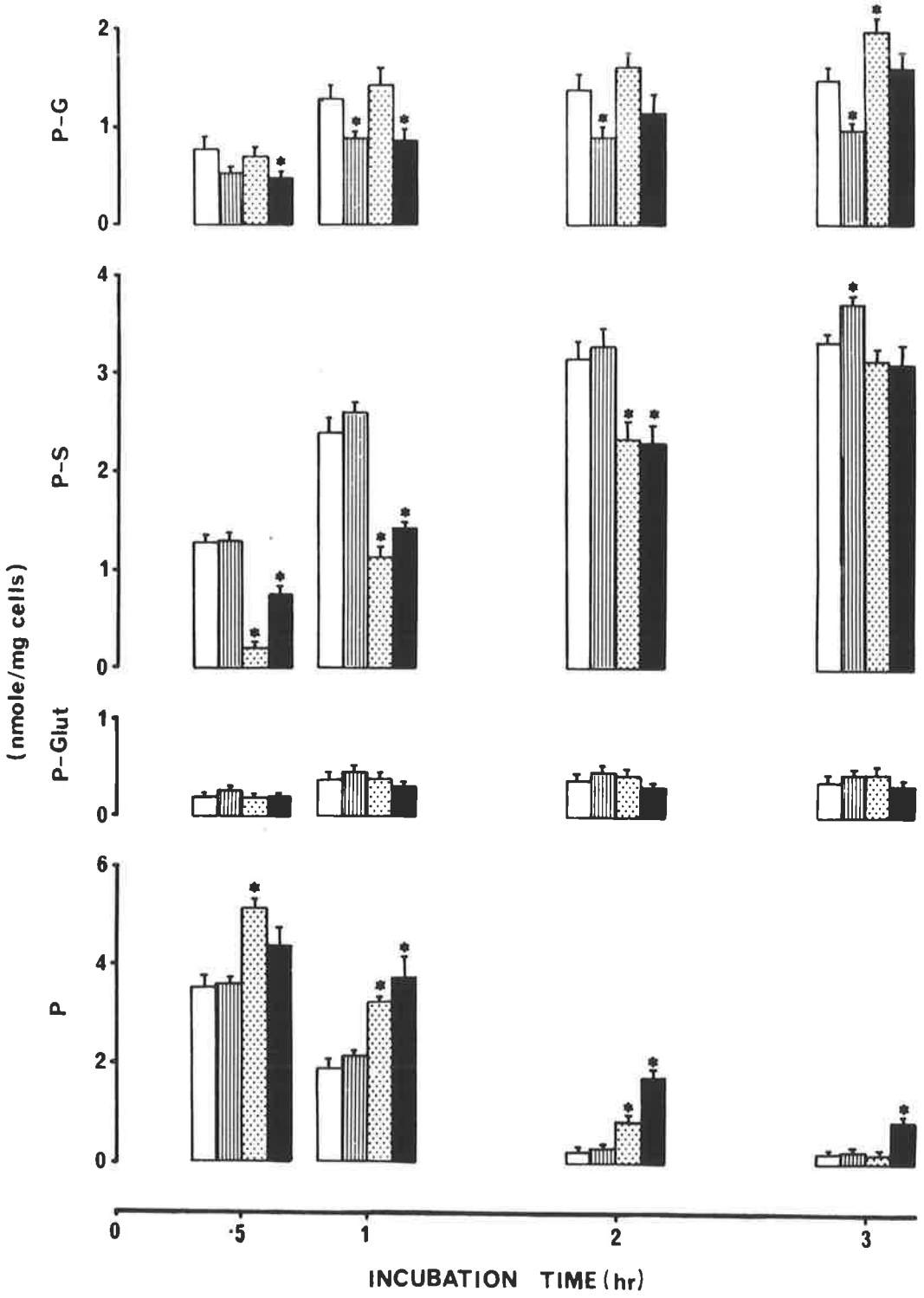

P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
5
6
·5
INCUBATION TIME (hr)

Fig. 1.5

Time course of metabolite formation in hepatocytes isolated from phenobarbital-treated rats incubated with paracetamol (250 μM) and various metabolic inhibitors.

□ saline control

⊡ salicylamide (200 μM)

▥ metyrapone (120 μM)

■ α-naphthoflavone (66 μM)

P-G paracetamol glucuronide

P-S paracetamol sulphate

P paracetamol

P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

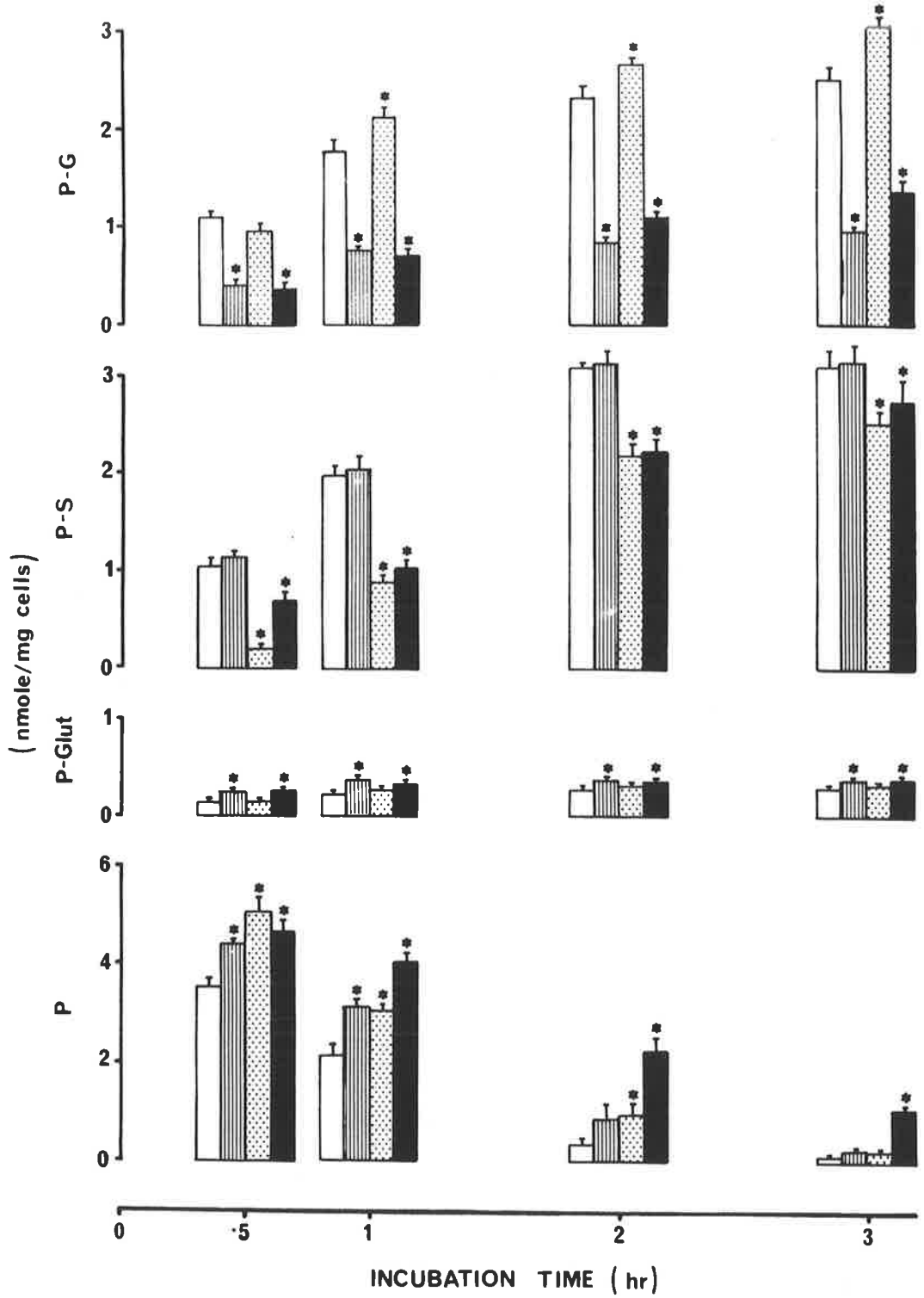

P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
6
·5
INCUBATION TIME (hr)

Fig. 1.6

Time course of metabolite formation in hepatocytes isolated from benz(α)pyrene-treated rats incubated with paracetamol (250 μM) and various metabolic inhibitors.

□ saline control

▨ salicylamide (200 μM)

▥ metyrapone (120 μM)

■ α-naphthoflavone (66 μM)

P-G paracetamol glucuronide

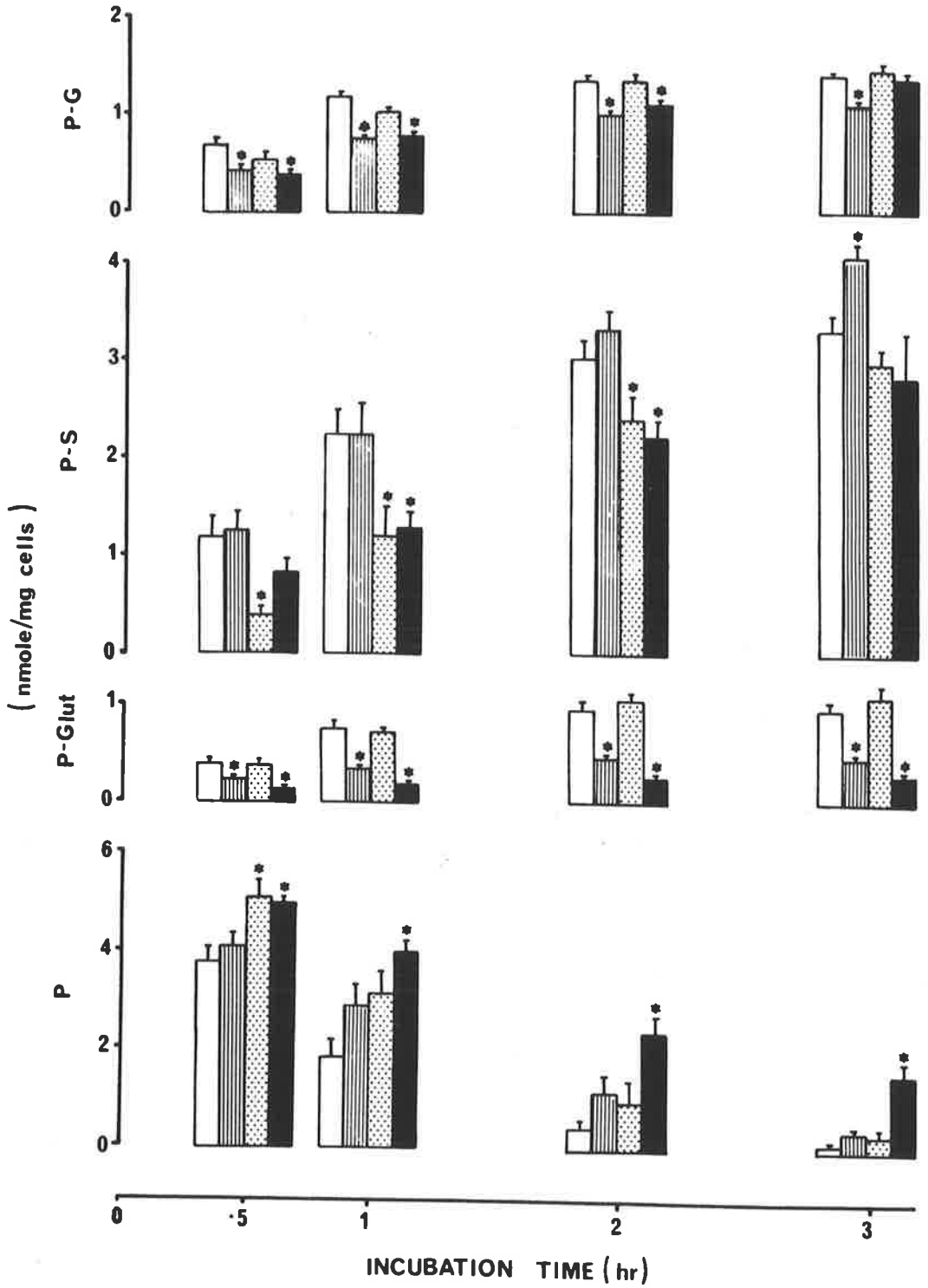
P-S paracetamol sulphate

P paracetamol

P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).


P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
6
·5
INCUBATION TIME (hr)

pyrene-treated rats without any effect on glutathione conjugate (Figs 1.4-1.6). However, it increased the formation of the glucuronide conjugate in phenobarbital-treated hepatocytes, with a similar but smaller effect in the controls (Figs 1.4 and 1.5).

α-Naphthoflavone inhibited both glucuronidation and sulphation in hepatocytes from all three groups. Formation of the glutathione conjugate was unaffected in control hepatocytes, slightly enhanced in hepatocytes from phenobarbital-treated rats and inhibited approximately two-fold in benz(α)pyrene-treated hepatocytes (Figs 1.4-1.6).

Discussion

In isolated rat hepatocytes, paracetamol formed three detectable metabolites, the glucuronide, sulphate and glutathione conjugates. Similar results have been reported by Moldeus (1978). The fact that cysteine and mercapturic acid conjugates were not detected, even after 3-hour incubation, suggests that the secondary metabolism of the glutathione conjugate is primarily extra-hepatic. Thus, the formation of glutathione conjugate probably reflects the formation of cytochrome P-450 mediated paracetamol reactive metabolite(s).

In agreement with in vivo data (Jollow et al., 1974), sulphate and glucuronide conjugates were about 90% of total metabolites formed while glutathione conjugate was less than 10%. This elucidates the resistance of rat hepatocytes to paracetamol induced hepatotoxicity. The sulphate pathway was saturated at relatively low doses of paracetamol, and this is consistent with the plateau of excreted sulphate and glucuronide conjugates after

administration of high doses of paracetamol to rats in vivo (Davis et al., 1976a). This has been previously ascribed to limitation of active sulphate (3'-phosphoadenosine 5'-phosphosulphate, PAPS) formation due to diminished hepatic levels of cysteine (Jollow et al., 1974).

The formation of glutathione conjugate increased with increasing paracetamol concentration and appeared to approach saturation over the range 1.5-2 mM paracetamol. Moldeus (1978) reported saturation of this pathway at 25 mM using the same experimental model. The discrepancy may be related to differences in the GSH status of the hepatocytes and the fact that phenobarbital-induced hepatocytes were used in Moldeus's study.

Among the metabolic inhibitors used, salicylamide appeared to be the only one which retained selectivity towards conjugative metabolism (particularly sulphate conjugation). Metyrapone and α-naphthoflavone both had non-specific inhibitory effects, in that glucuronide and sulphate conjugative pathways were variably susceptible. The expected effect on the oxidative pathway, as determined by glutathione conjugation, was not uniformly inhibitory; in fact, inhibition was only seen in benz(α)pyrene-treated hepatocytes, and in phenobarbital-treated hepatocytes there was a paradoxical apparent increase in this pathway. These results indicate that metabolic induction by phenobarbital may either have little effect on the cytochrome P-450 monooxygenase and/or glutathione S-transferase activities, or that the increase in glucuronidation indirectly decreased the amount of substrate cleared by the minor oxidative pathway.

In conclusion, although the oxidative pathway in rat

hepatocytes is a minor one as it is in vivo, the pattern of paracetamol metabolism can still be modified by metabolic inducers (particularly benz(α)pyrene) and inhibitors (e.g., metyrapone, α-naphthoflavone) even at relatively low paracetamol concentrations. This confirms the participation of both cytoplasmic enzymes and the microsomal cytochrome P-450 monooxygenase system in metabolic activation/inactivation of paracetamol. However, it appears that, where inhibition of both oxidative and conjugative pathways is possible (e.g., with metyrapone and α-naphthoflavone), the amount of paracetamol metabolised via the reactive metabolite pathway is difficult to predict.

* * *

CHAPTER 2

PARACETAMOL METABOLISM IN ISOLATED MOUSE HEPATOCYTES

Introduction

The susceptibility to paracetamol-induced liver necrosis varies among different species. Hamsters and mice are quite susceptible, whereas rats, guinea pigs and rabbits are relatively resistant. It has been suggested that these species differences are associated with differences in the formation of reactive metabolites of paracetamol (Davis et al., 1974).

Both dose- and species-dependent variations in urinary elimination of paracetamol metabolites have been shown in hamsters, mice and rats (Jollow et al., 1974). After relatively low doses of paracetamol, the major metabolites (sulphate and glucuronide) were approximately equally proportioned in the hamsters, but mice excreted more glucuronide, and rats excreted more sulphate. Paracetamol mercapturate represented a constant proportion of the total urinary metabolites over a wide dose range, indicating that the synthetic activity in vivo is a first-order process which is not readily saturable. Furthermore, mercapturate conjugation was increased by pretreatment of animals with cytochrome P-450 inducers, phenobarbital and 3-methylcholanthrene, and decreased by the treatment with cytochrome P-450 inhibitor, piperonylbutoxide. Further, within a species, any treatments that alter the susceptibility to paracetamol-induced liver necrosis were also found to have parallel effect on the activity of this minor pathway (Jollow et al., 1974).

The aim of this chapter was to investigate paracetamol metabolite patterns in hepatocytes isolated from the mouse, a species more sensitive to paracetamol-induced hepatic necrosis than the rat. It was envisaged that the contrast between

paracetamol metabolism in rat and mouse hepatocytes, both with and without modification of the cytochrome P-450-catalysed mono-oxygenase system, would enable a more meaningful interpretation of the toxic effects of irreversible binding of paracetamol in isolated hepatocytes.

Methods

Hepatocytes were isolated from LACA Albino male mice weighing 20-30 g. For induction, the animals were treated daily with intraperitoneal doses of phenobarbital sodium (80 mg/Kg) or benz-(α)pyrene (20 mg/Kg) for 4 days.

Aliquots 2.5 ml of hepatocyte suspension (5-6 x 10^6 cells/ml) were incubated with 0.1 ml paracetamol (250 μM) and 0.1 ml physiological saline (control) or 0.1 ml of various metabolic inhibitors including metyrapone (120 μM), salicylamide (200 μM) and α-naphthoflavone (66 μM).

After half to 3 hour incubation, 1 ml samples were taken for metabolite analysis using the HPLC technique.

Results

As in rat hepatocytes, after incubating paracetamol with hepatocytes isolated from mice, three major metabolites were detected by HPLC separation. These were the glucuronide, sulphate and glutathione conjugates. Glucuronide formation was the major conjugative pathway while sulphate conjugation was less. The glutathione conjugate was produced at a faster rate than observed in similar experiments with rat hepatocytes (Fig. 2.1 cf Fig. 1.2).

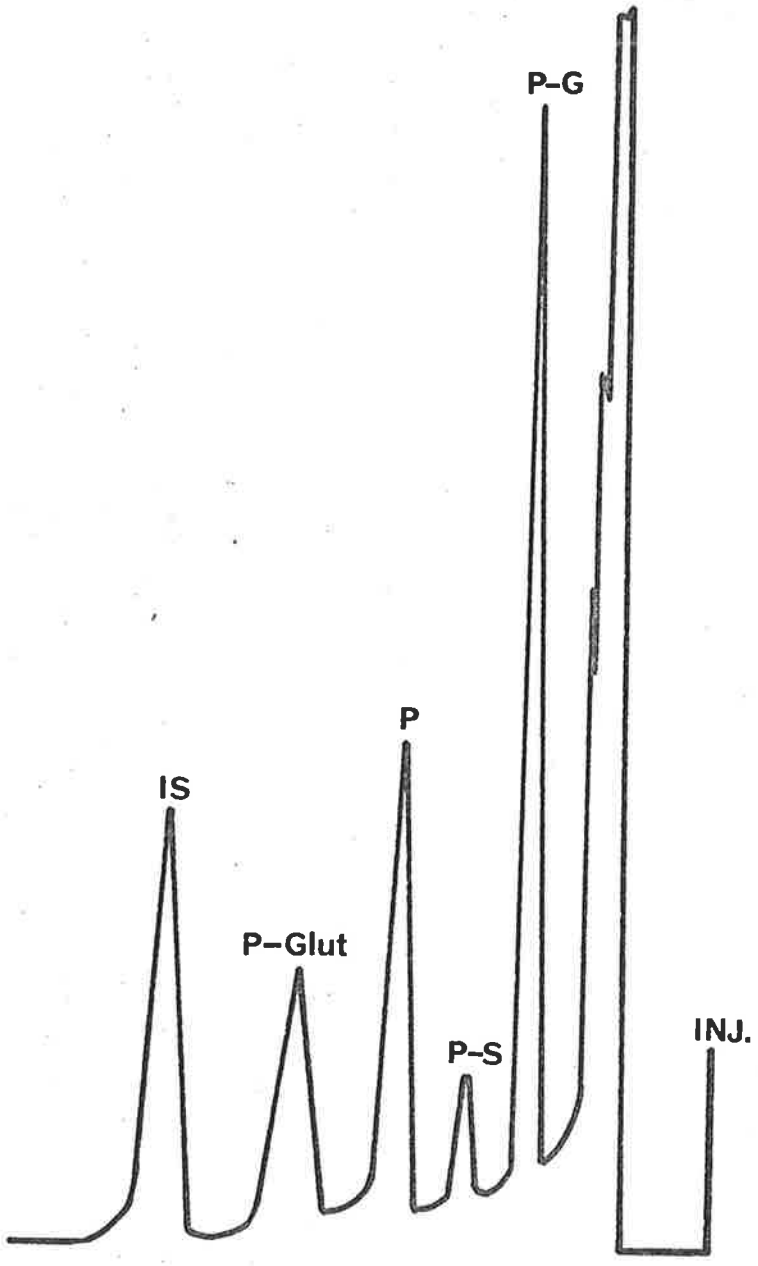


Fig. 2.1

High performance liquid chromatogram of an extract from isolated mouse hepatocytes after incubation (1 hr) with paracetamol.

Inj	injection (15 µl)	P-G	paracetamol glucuronide
P-S	paracetamol sulphate	P	paracetamol
P-Glut	paracetamol glutathione	IS	internal standard

Phenobarbital induction resulted in an increase in sulphate conjugation, but only a small, early rise in glucuronide conjugation. On the other hand, benz(α)pyrene induction resulted in a decrease in both sulphation and glucuronidation of paracetamol. Glutathione conjugation was enhanced by both enzyme-inducing pretreatments, but the increase after benz(α)pyrene (approximately 60%) was greater than that observed after phenobarbital induction (approximately 25%) (Table 2.1).

Metyrapone did not alter paracetamol metabolism in hepatocytes from either control or phenobarbital-treated mice (Figs 2.2 and 2.3). However, in benz(α)pyrene-treated hepatocytes, glutathione conjugation was inhibited and a delayed increase in glucuronide and sulphate formation was noted (Fig. 2.4).

Salicylamide inhibited the conjugation of both glucuronide and sulphate, but had no effect on glutathione conjugate in mouse hepatocytes irrespective of the pretreatment (Figs 2.2, 2.3 and 2.4).

α-Naphthoflavone inhibited the formation of all three conjugates in all three groups of mouse hepatocytes (Figs 2.2, 2.3 and 2.4). However, the inhibition of glutathione conjugation was greatest in hepatocytes from benz(α)pyrene-treated, and this was accompanied by an apparent reversal of the inhibitory effect on glucuronide formation (Fig. 2.4).

Discussion

Hamsters and mice are more sensitive than rats to paracetamol-induced hepatotoxicity in vivo (Davis et al., 1974). Since hamsters are prohibited in Australia, isolated hepatocytes

TABLE 2.1

Effects of metabolic inducers on paracetamol metabolism in isolated mouse hepatocytes.

Treatment	Incubation time (hr)	Metabolites (nmole/mg cells) P-G	P-S	P-Glut
Control	$\frac{1}{2}$	1.73 ± 0.04	0.47 ± 0.04	0.65 ± 0.03
	1	2.55 ± 0.06	0.58 ± 0.04	0.82 ± 0.05
	2	3.25 ± 0.06	0.71 ± 0.04	0.92 ± 0.04
	3	3.44 ± 0.05	0.76 ± 0.04	0.95 ± 0.07
Phenobarbital	$\frac{1}{2}$	2.19 ± 0.08*	0.51 ± 0.04	0.82 ± 0.04*
pretreated	1	3.04 ± 0.08*	0.73 ± 0.03*	1.01 ± 0.05*
	2	3.48 ± 0.09	0.84 ± 0.01*	1.07 ± 0.05
	3	3.60 ± 0.11	1.13 ± 0.04*	1.20 ± 0.07*
Benz(α)pyrene	$\frac{1}{2}$	1.53 ± 0.15*	0.29 ± 0.05*	1.04 ± 0.07*
pretreated	1	1.94 ± 0.08*	0.35 ± 0.07*	1.27 ± 0.08*
	2	2.07 ± 0.14*	0.31 ± 0.01*	1.36 ± 0.04*
	3	2.33 ± 0.12*	0.43 ± 0.01*	1.56 ± 0.05*

Values shown are mean ± s.e.m. (n=3)

P-G paracetamol glucuronide

P-S paracetamol sulphate

P-Glut paracetamol glutathione

* significantly different from control ($p<0.05$, t-test)

Fig. 2.2

Time course of metabolite formation in hepatocytes isolated from untreated mice incubated with paracetamol (250 μM) and various metabolic inhibitors.

☐	saline control	▥	metyrapone (120 μM)
⚄	salicylamide (200 μM)	■	α-naphthoflavone (66 μM)
P-G	paracetamol glucuronide	P-S	paracetamol sulphate
P	paracetamol	P-Glut	paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

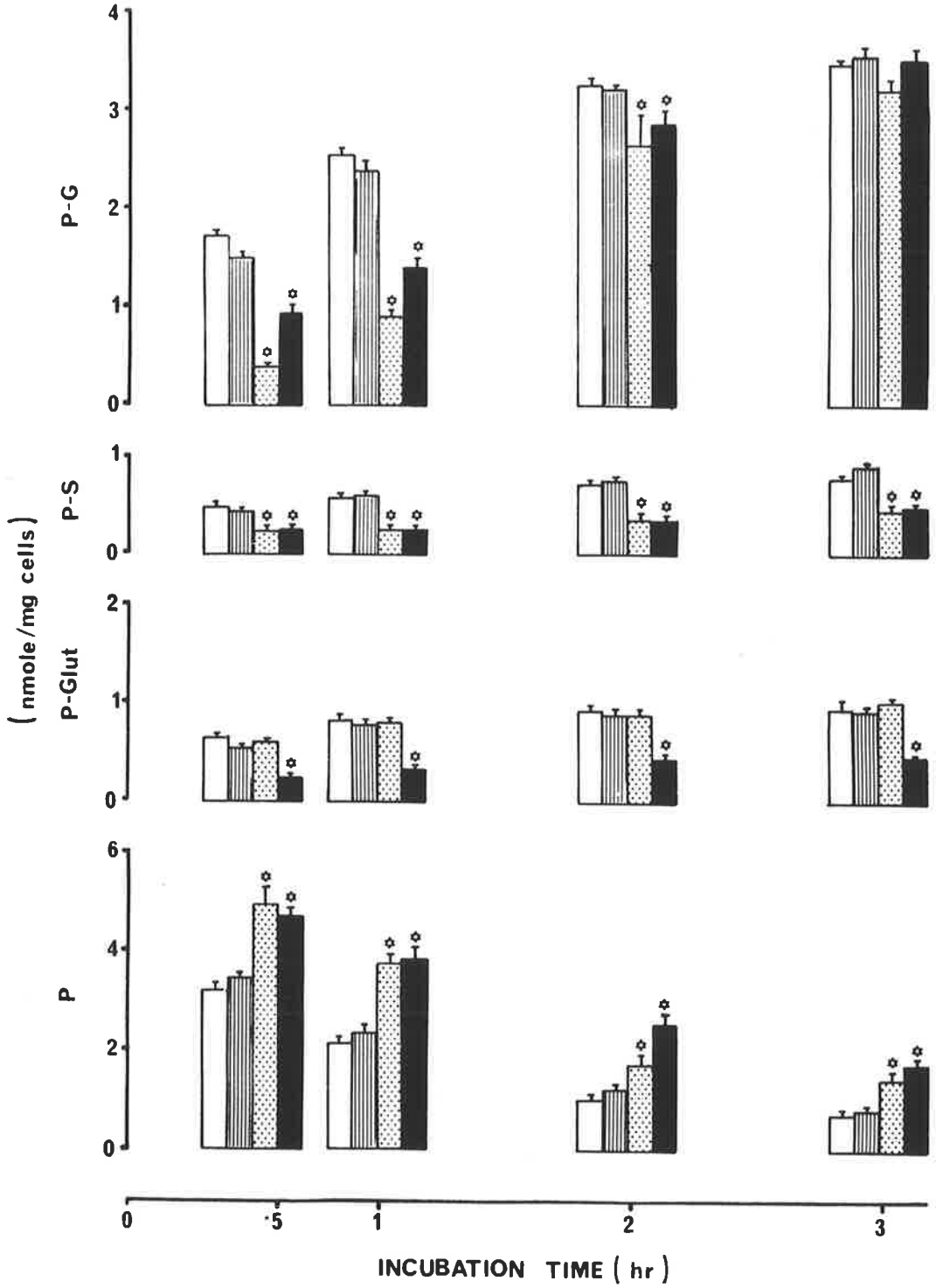

P-G
P-S
P-Glut
P
(nmole/mg cells)
INCUBATION TIME (hr)
0
·5
1
2
3

Fig. 2.3

Time course of metabolite formation in hepatocytes isolated from phenobarbital-treated mice incubated with paracetamol (250 μM) and various metabolic inhibitors.

□	saline control	▥	metyrapone (120 μM)
⊡	salicylamide (200 μM)	■	α-naphthoflavone (66 μM)
P-G	paracetamol glucuronide	P-S	paracetamol sulphate
P	paracetamol	P-Glut	paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

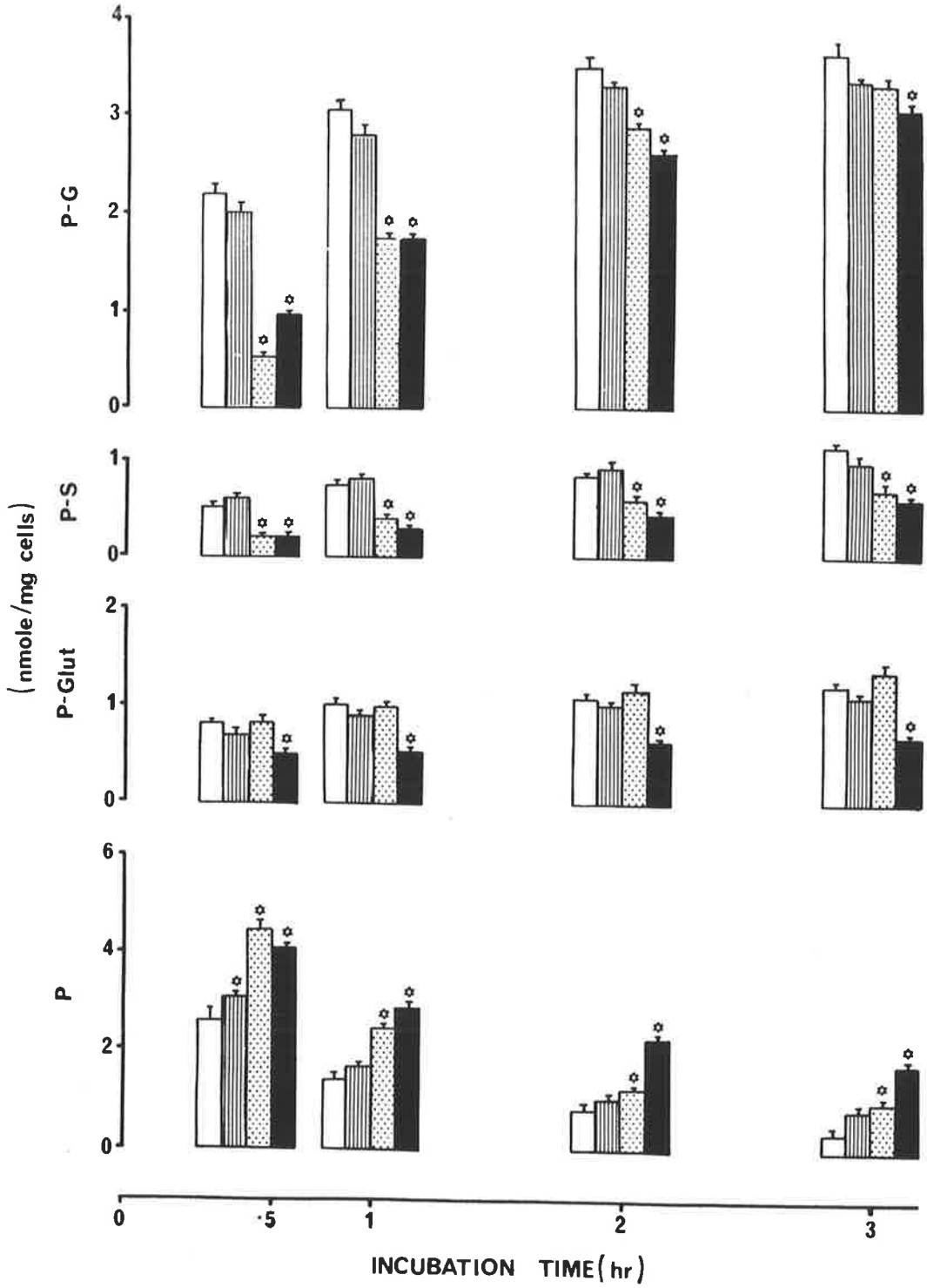

P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
6
0
·5
1
2
3
INCUBATION TIME(hr)

Fig. 2.4

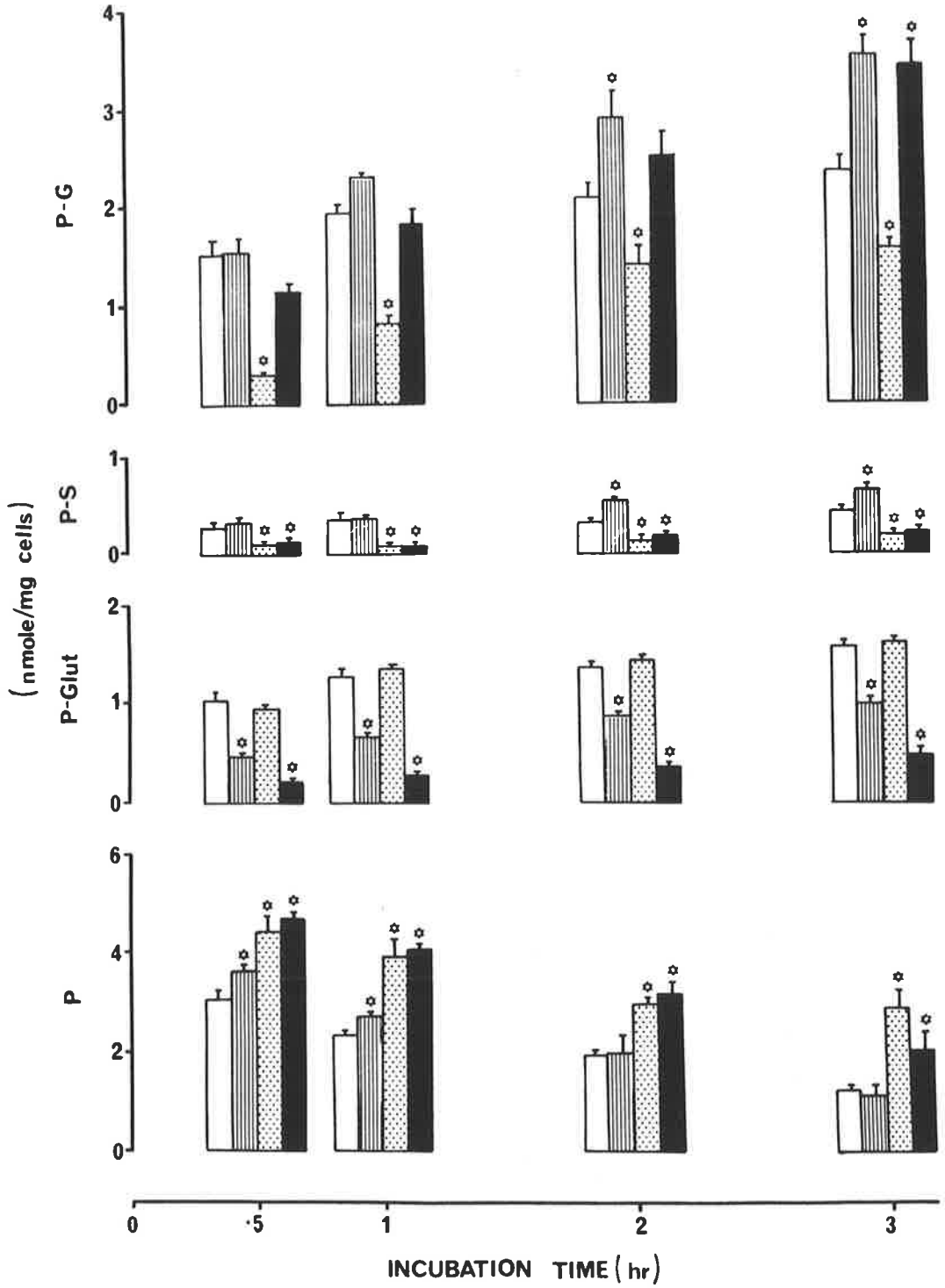
Time course of metabolite formation in hepatocytes isolated from benz(α)pyrene-treated mice incubated with paracetamol (250 μM) and various metabolic inhibitors.

- saline control
- salicylamide (200 μM)
- metyrapone (120 μM)
- α-naphthoflavone (66 μM)

P-G paracetamol glucuronide
P-S paracetamol sulphate
P paracetamol
P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).


P-G
P-S
P-Glut
P
(nmole/mg cells)
INCUBATION TIME (hr)
0
·5
1
2
3

from mice were used to make comparisons with isolated rat hepatocytes in this study. At relatively low dose of paracetamol (0.25 mM) in isolated mouse hepatocytes, about 80% of total metabolites formed were glucuronide and sulphate conjugates and 20% was glutathione conjugate. These values were comparable to those found in vivo (Jollow et al., 1974).

There were at least three differences between isolated mouse and rat hepatocytes concerning paracetamol metabolism. Sulphation in mouse hepatocytes was only 30% of that found in hepatocytes from control rats, while glucuronidation was 50% and 25%, respectively, higher than controls and phenobarbital-treated rat hepatocytes. The formation of glutathione conjugate which reflected the formation of paracetamol reactive metabolites mediated by cytochrome P-450 was more than double in mouse hepatocytes. This confirms the sensitivity of mouse hepatocytes to the oxidative pathway implicated in paracetamol-induced hepatotoxicity.

In contrast to rat hepatocytes, phenobarbital induction appeared to have little effect on glucuronidation but showed the inductive effect over the formation of reactive metabolite(s) as determined by glutathione conjugate. This result, combined with those in phenobarbital-treated rat hepatocytes, strengthened the hypothesis (Moldeus et al., 1977) that oxidation and glucuronidation are independently operated. The inductive effect of benz-(α)pyrene is apparently more selective than phenobarbital. It induced only the formation of reactive metabolite(s) as shown by the increase in glutathione conjugate in both rat and mouse hepatocytes (cf Table 1.1 and 2.1).

Salicylamide has been shown to be a competitive inhibitor

of the conjugation of paracetamol with both sulphate and glucuronide in hamster liver cell suspensions with a K_i about 1×10^{-5} M and 3×10^{-5} M, respectively (Jollow and Smith, 1977). The present study confirms the extensive blockade of these major conjugative pathways in isolated mouse hepatocytes too.

Inhibiting the activation of paracetamol by metyrapone in vivo has been shown to protect mice from toxic doses of paracetamol and increase paracetamol LD_{50} by 30% (Goldstein and Nelson, 1979; Fossa et al., 1979; Nelson et al., 1980). In the present study, glutathione conjugation was inhibited by metyrapone only in hepatocytes isolated from benz(α)pyrene-treated mice, but not in the controls or in phenobarbital-treated mouse hepatocytes. Moldeus (1978a) has reported an inhibitory effect of metyrapone on the activation of paracetamol in hepatocytes isolated from diethylmaleate-treated mice. It should be noted, however, that his doses of paracetamol and metyrapone (2 mM and 1 mM, respectively) were higher than those used in the present study (0.25 mM and 0.12 mM, respectively).

Although α-naphthoflavone was relatively non-selective in its inhibitory effect, and decreased sulphation and glucuronidation as well as glutathione conjugation, its effect on the activation of paracetamol was greatest in benz(α)pyrene-treated hepatocytes.

In summary, the metabolism of paracetamol via an oxidative pathway appeared to be greater in mouse than in rat hepatocytes. Furthermore, the oxidative pathway was more sensitive to induction and inhibition by drugs.

In common with the results found in isolated rat hepatocytes,

the non-specific inhibition of glucuronide and sulphate conjugation can complicate the prediction of drug effect. However, in mouse hepatocytes the effects of metyrapone were more straightforward, since the inhibitory effect on glucuronidation was absent.

* * *

CHAPTER 3

PARACETAMOL TOXICITY IN ISOLATED RAT HEPATOCYTES

Introduction

Evidence from in vivo studies suggests that paracetamol-induced liver necrosis is mediated through the formation of a chemically reactive metabolite whose formation is catalysed by the cytochrome P-450 monooxygenase system (Mitchell and Jollow, 1975, Jollow and Smith, 1977). The metabolite appears to be strongly electrophilic, since it combines readily with nucleophiles in hepatocyte macromolecules (e.g., protein). Thus, irreversible protein binding may be a useful index of the formation of the metabolite and it might be the most reliable estimate for the availability of toxic metabolite in situ (Mitchell and Jollow, 1975; Gillette, 1977).

It has been shown that the severity of paracetamol-induced liver necrosis often correlates well with the magnitude of irreversible protein binding of the reactive metabolite and the decrease in hepatic glutathione level (Potter et al., 1974; Mitchell and Jollow, 1975; Labadarios et al., 1977). At moderate doses of paracetamol the cell is protected by its content of reduced glutathione, but after high doses, glutathione is depleted. When the depletion exceeds 70-85% of the initial hepatic levels, a considerable amount of irreversible protein binding occurs. This may initiate the events leading to cell death and finally liver necrosis (Gillette, 1974; Gillette et al., 1974; Jollow and Smith, 1977).

Since the formation of reactive metabolite by cytochrome P-450 monooxygenase system may be regarded as the oxidative toxic pathway and the protective mechanism by hepatic glutathione as the defensive pathway, modification of these two should result in the modification of paracetamol toxicity. There is much

experimental evidence to support this hypothesis. Pretreatment of animals (rats, mice) with metabolic inducers such as phenobarbital increases the extent of irreversible protein binding and severity of liver necrosis. Conversely, pretreatment of animals (rats, mice) with metabolic inhibitors markedly decreases the metabolism of paracetamol, the irreversible protein binding and the hepatic necrosis (Jollow et al., 1973; Mitchell et al., 1973; Potter et al., 1973). Furthermore, prior administration of either diethyl maleate, which depletes hepatic glutathione without causing necrosis (Boyland and Chasseaud, 1970), or cysteine, which leads to the synthesis of reduced glutathione, respectively, increases or decreases the irreversible protein binding and the severity of paracetamol-induced necrosis (Mitchell et al., 1973).

The aim of this investigation was to show that irreversible protein binding mediated by microsomal monooxygenase can occur in isolated rat hepatocytes, and that this binding may be modified by inducers and inhibitors of these enzymes. In addition, changes in intracellular K^+ and ALT release were examined as indices of cell damage, in order to examine possible correlative effects between irreversible protein binding and toxicity.

Methods

Hepatocytes were isolated from male rats weighing 200-250 g. The pretreated animals were given phenobarbital sodium or benz(α)pyrene intraperitoneally at a daily dose of 80 mg/Kg or 20 mg/Kg for 4 days.

Incubations were carried out using 2.5 ml aliquots of cell suspension (5-6 x 10^6 cells/ml) incubated with 0.1 ml paracetamol

(1 mg/ml), 0.1 ml (ring-^{14}C)-paracetamol (3.3 µCi/ml), 0.1 ml physiological saline for control or 0.1 ml of various metabolic inhibitors. The final concentrations of substrate and inhibitors were: paracetamol 250 µM, metyrapone 120 µM, salicylamide 200 µM and α-naphthoflavone 66 µM.

After half to 3 hours incubation, various samples were taken: 0.5 ml for K^+ and ALT assay, 0.2 ml for the estimation of reduced glutathione (GSH) and the rest for the determination of irreversible protein binding.

The dose-response relationship was also studied in control rat hepatocytes using paracetamol (250 µM - 1250 µM) and one hour incubations.

Results

Irreversible protein binding of ^{14}C-paracetamol in isolated rat hepatocytes increased with doses (Fig. 3.1) and time of incubation (Fig. 3.3). Figure 3.2 shows double reciprocal plots of the irreversible protein binding (A) and glutathione conjugation (B). Binding appeared to follow Michaelis-Menten kinetics with Km and Vmax estimated at 1.33 mM and 4.35 nmoles/mg protein/hr. In contrast, the kinetics of the formation of the glutathione conjugate appeared to have a lower Km (0.63 mM) and higher Vmax (1.05 nmoles/mg cells/hr, or 6.56 nmoles/mg protein/hr when converted using the factor for protein concentration of isolated rat hepatocytes obtained from preliminary experiments, 16 mg protein/g cells).

After phenobarbital pretreatment, the amount of binding increased, particularly toward the end of a 3 hour incubation

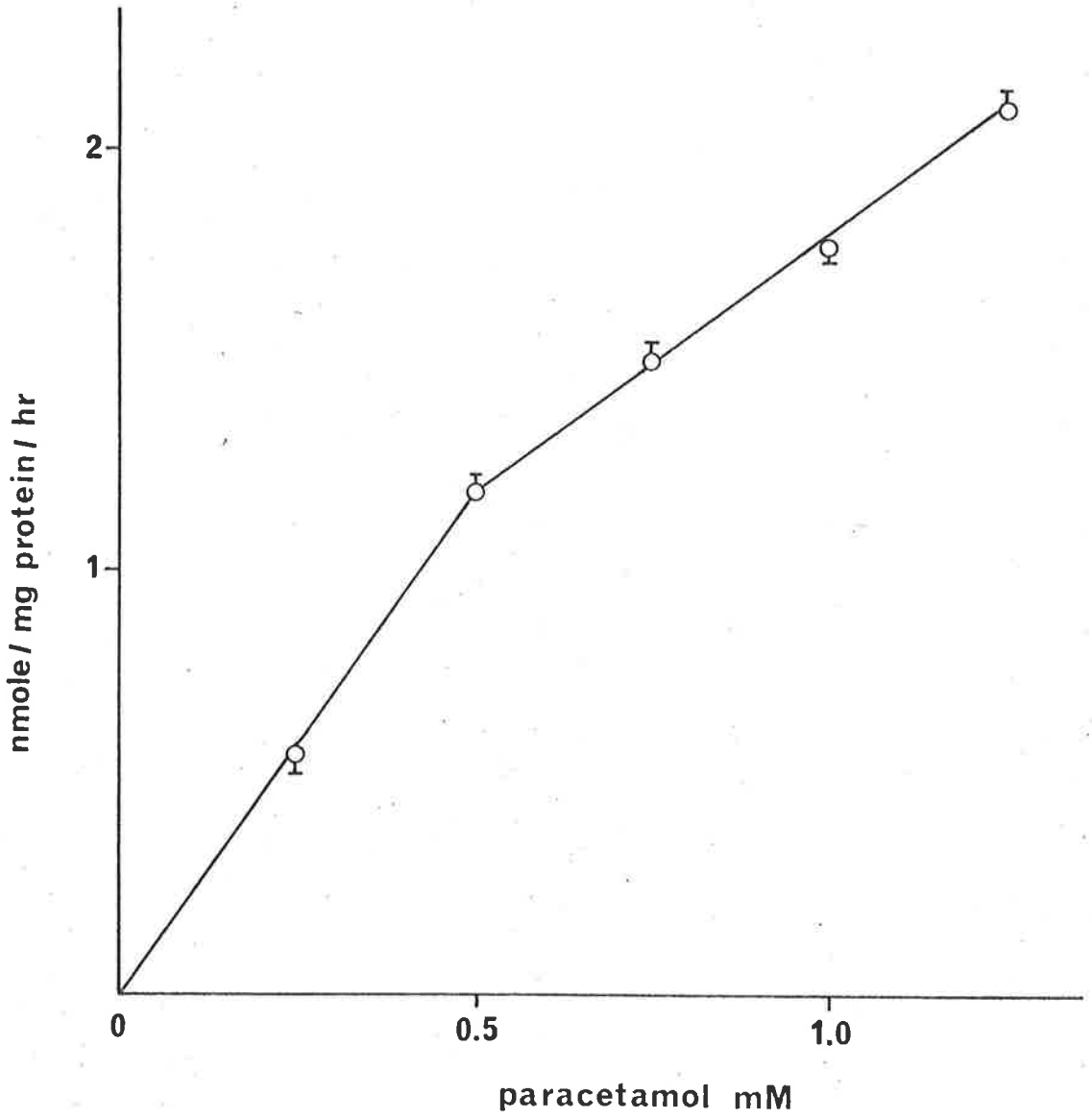


Fig. 3.1

Irreversible protein binding in control isolated rat hepatocytes with various doses of paracetamol.

Values shown are mean ± s.e.m. (n=3).

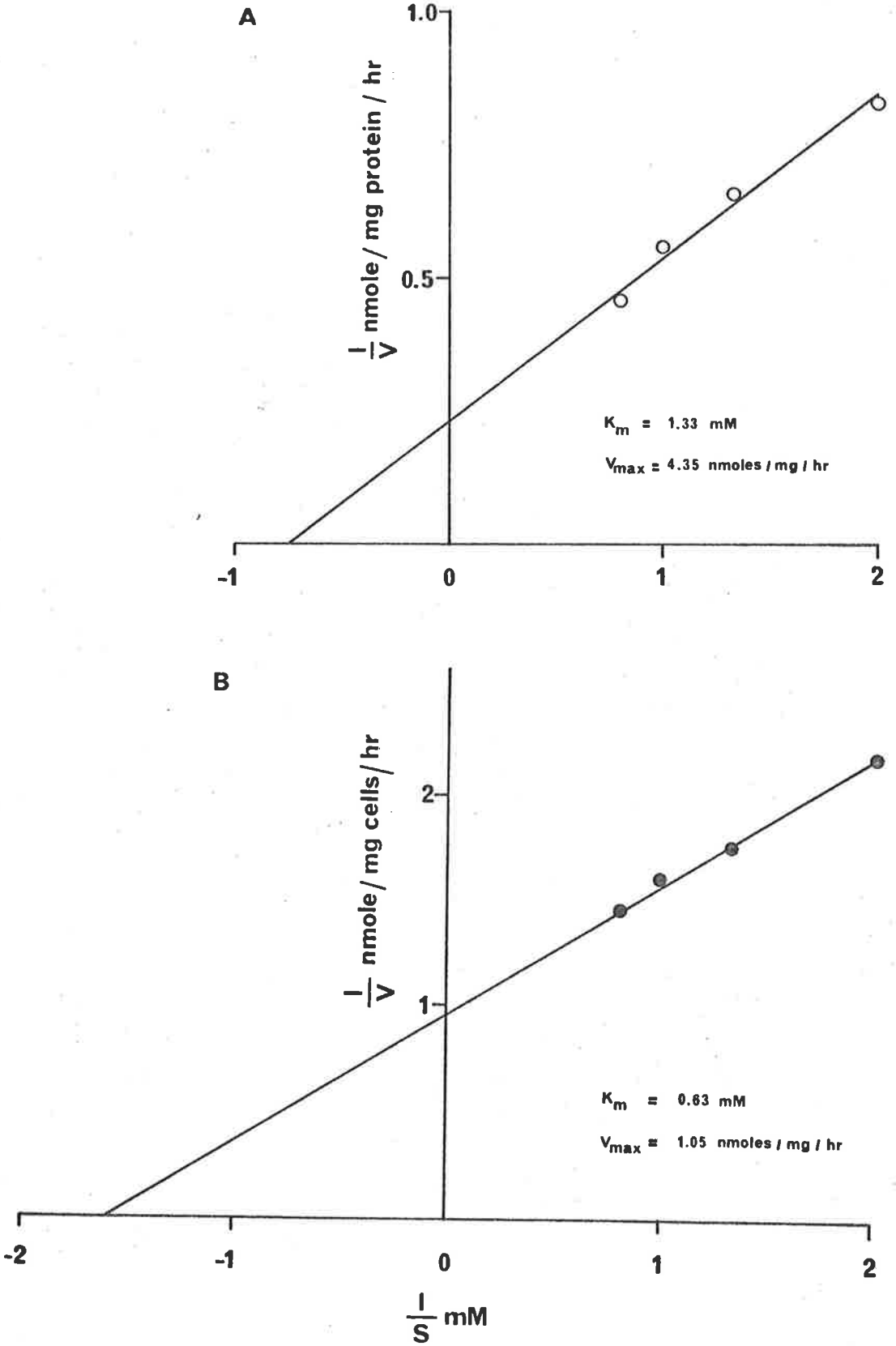
Fig. 3.2

Lineweaver-Burk plots of irreversible protein binding and glutathione conjugation in isolated rat hepatocytes after incubating with various doses of paracetamol for 1 hour.

A, plot for irreversible protein binding; values shown are means of 3 cell preparations.

B, plot for glutathione conjugation; values shown are means of 3 cell preparations.

Linear regression coefficients > 0.98 in each case.


A
1.0
0.5
$\frac{1}{V}$ nmole / mg protein / hr
K_m = 1.33 mM
V_{max} = 4.35 nmoles / mg / hr
-1
0
1
2
B
2
1
$\frac{1}{V}$ nmole / mg cells / hr
K_m = 0.63 mM
V_{max} = 1.05 nmoles / mg / hr
-2
-1
0
1
2
$\frac{1}{S}$ mM

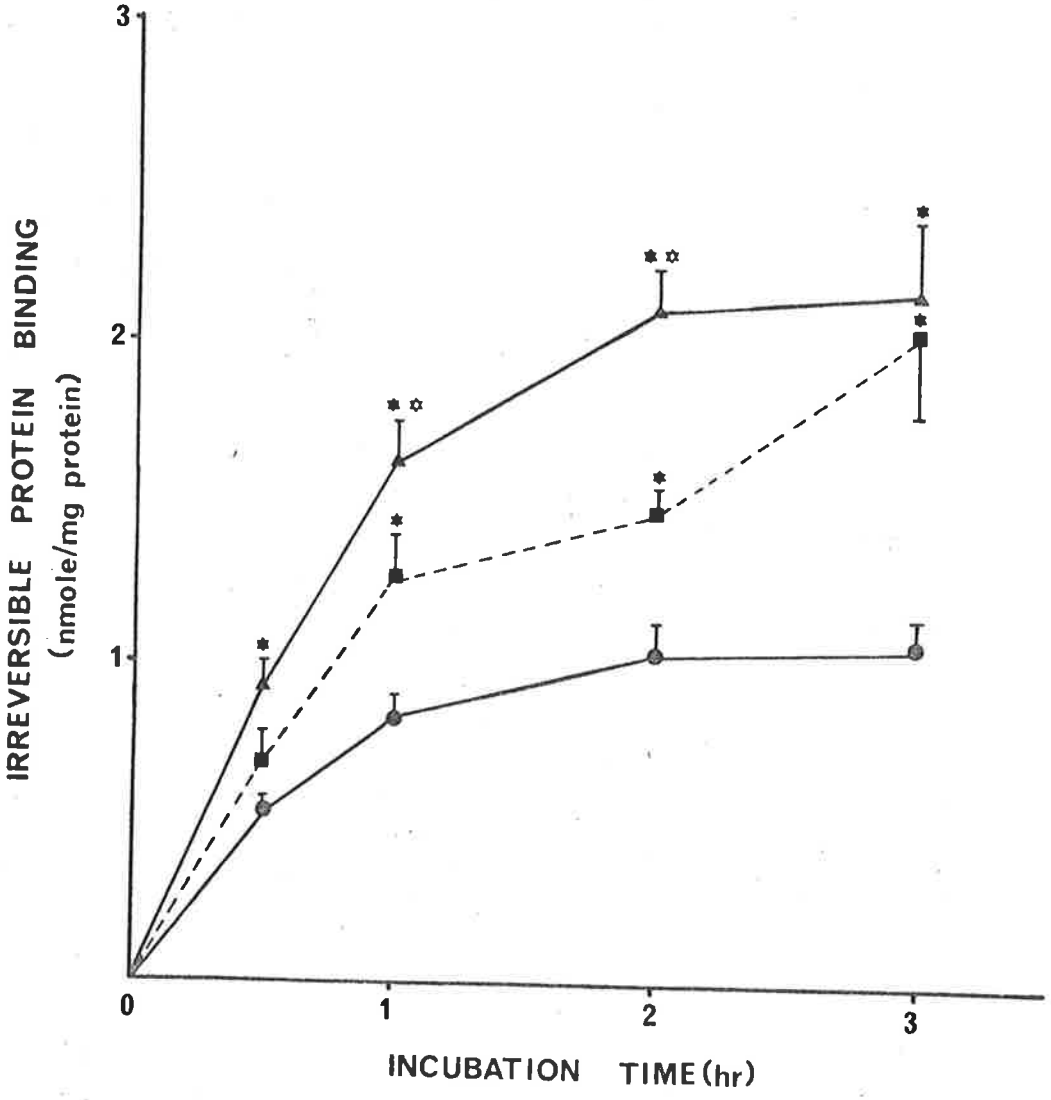


Fig. 3.3

Irreversible protein binding in hepatocytes isolated from untreated control (●——●), phenobarbital (■- -■) and benz(α)pyrene - (▲——▲) treated rats after incubating with ^{14}C-paracetamol. Values shown are mean ± s.e.m. (n=3).

*significantly different from control hepatocytes ($p<0.05$, t-test).

✡significantly different from phenobarbital-treated hepatocytes ($p<0.05$, t-test).

period. Similar results were found in benz(α)pyrene-treated hepatocytes, but the increase in binding was more marked (Fig. 3.3).

The effect of metabolic inhibitors on the binding was variable. Although metyrapone had no effect in the controls (Fig. 3.4), it decreased the amount of binding nearly by half in benz(α)pyrene-treated hepatocytes (Fig. 3.6), with a similar, but smaller, effect in hepatocytes from phenobarbital-treated rats (Fig. 3.5). In contrast, α-naphthoflavone had a small inhibitory effect in the controls (Fig. 3.4), with a greater inhibitory effect in benz(α)pyrene-treated hepatocytes (Fig. 3.6), while it increased the binding in hepatocytes from phenobarbital-treated rats (Fig. 3.5). Salicylamide had no effect on the binding in any of the three groups of hepatocytes (Figs 3.4-3.6).

Paracetamol-induced glutathione depletion was not observed in hepatocyte suspensions after any of the enzyme inductive or inhibitory treatments. There were sampling times when some of the differences imposed by the treatments did reach statistical significance, but there was no discernably consistent trend to these changes (Table 3.1).

There were no paracetamol-induced changes in cell viability, as measured by K^+ retention and ALT release, associated with any of the treatments (Figs 3.7-3.9). Actual values of intracellular K^+ and ALT activity from which the differences were calculated are shown in Table 3.2.

Discussion

Although the identity of the reactive metabolite(s) of

Fig. 3.4

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in control isolated rat hepatocytes.

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

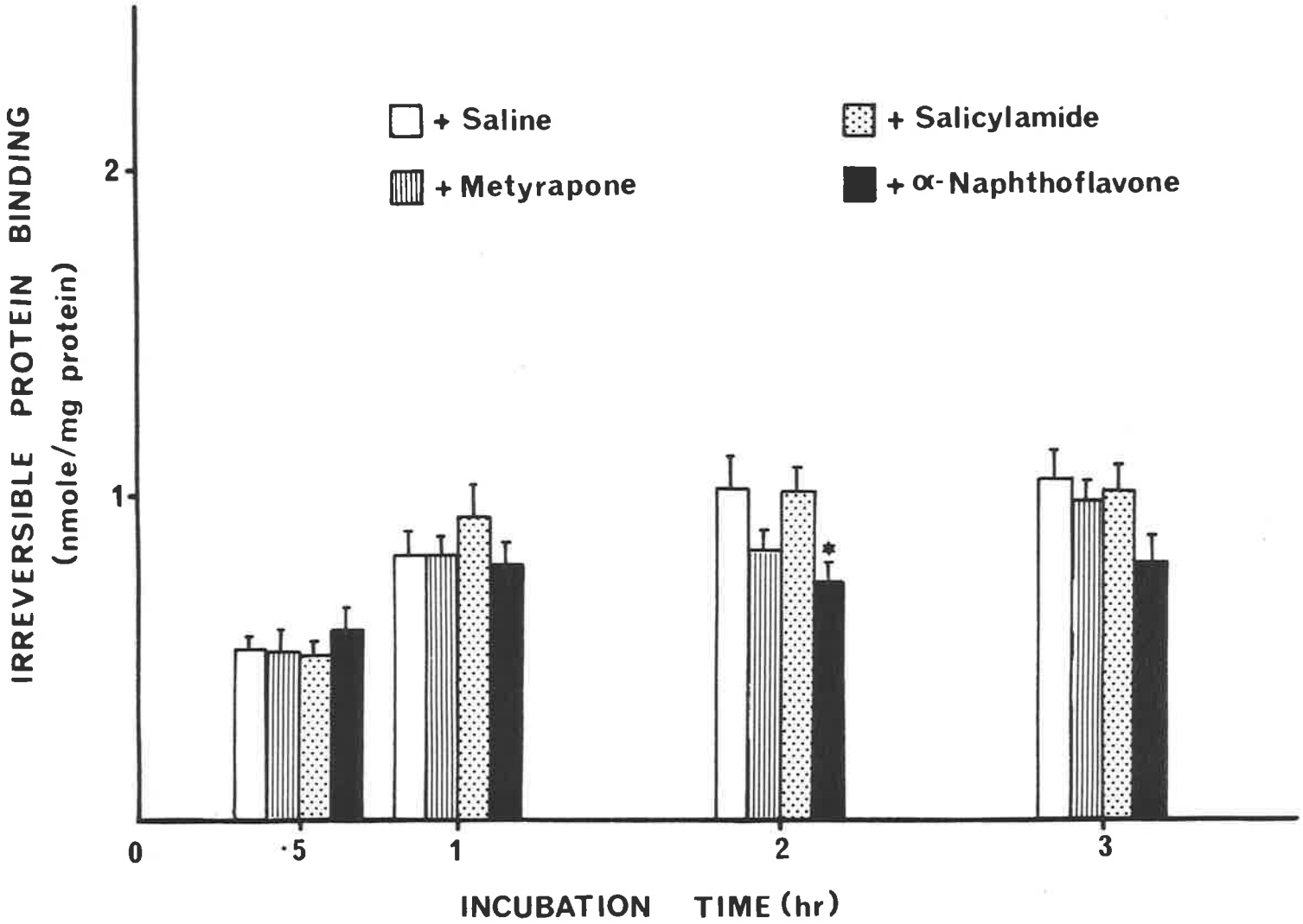

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
·5
1
2
3
+ Saline
+ Metyrapone
+ Salicylamide
+ α-Naphthoflavone
*
INCUBATION TIME (hr)

Fig. 3.5

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in hepatocytes isolated from phenobarbital-treated rats.

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

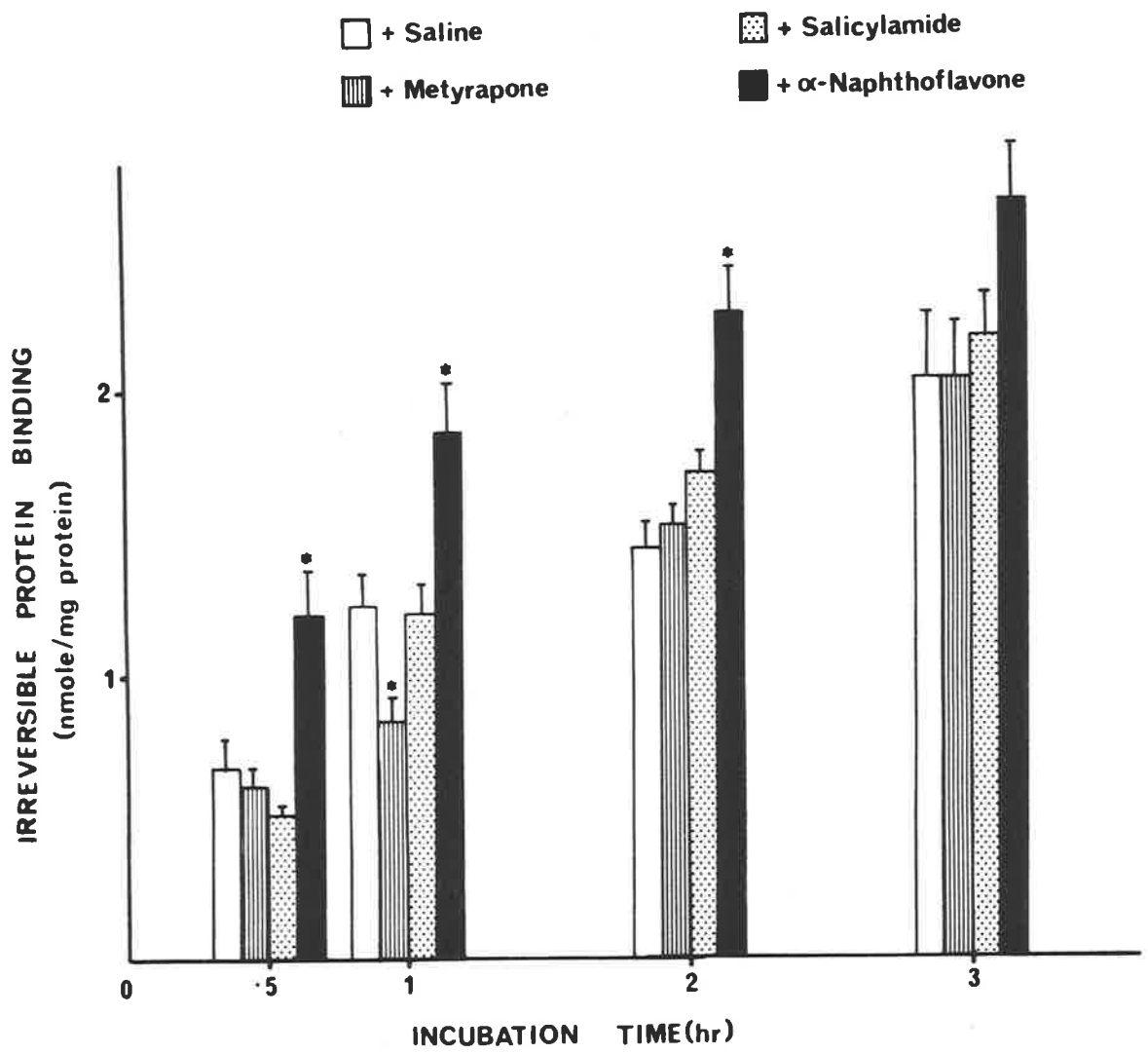

+ Saline
+ Salicylamide
+ Metyrapone
+ α-Naphthoflavone
IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
·5
1
2
3
INCUBATION TIME(hr)

Fig. 3.6

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in hepatocytes isolated from benz(α)pyrene-treated rats.

Values shown are mean ± s.e.m. (n=3).

*significantly different from saline control ($p<0.05$, t-test).

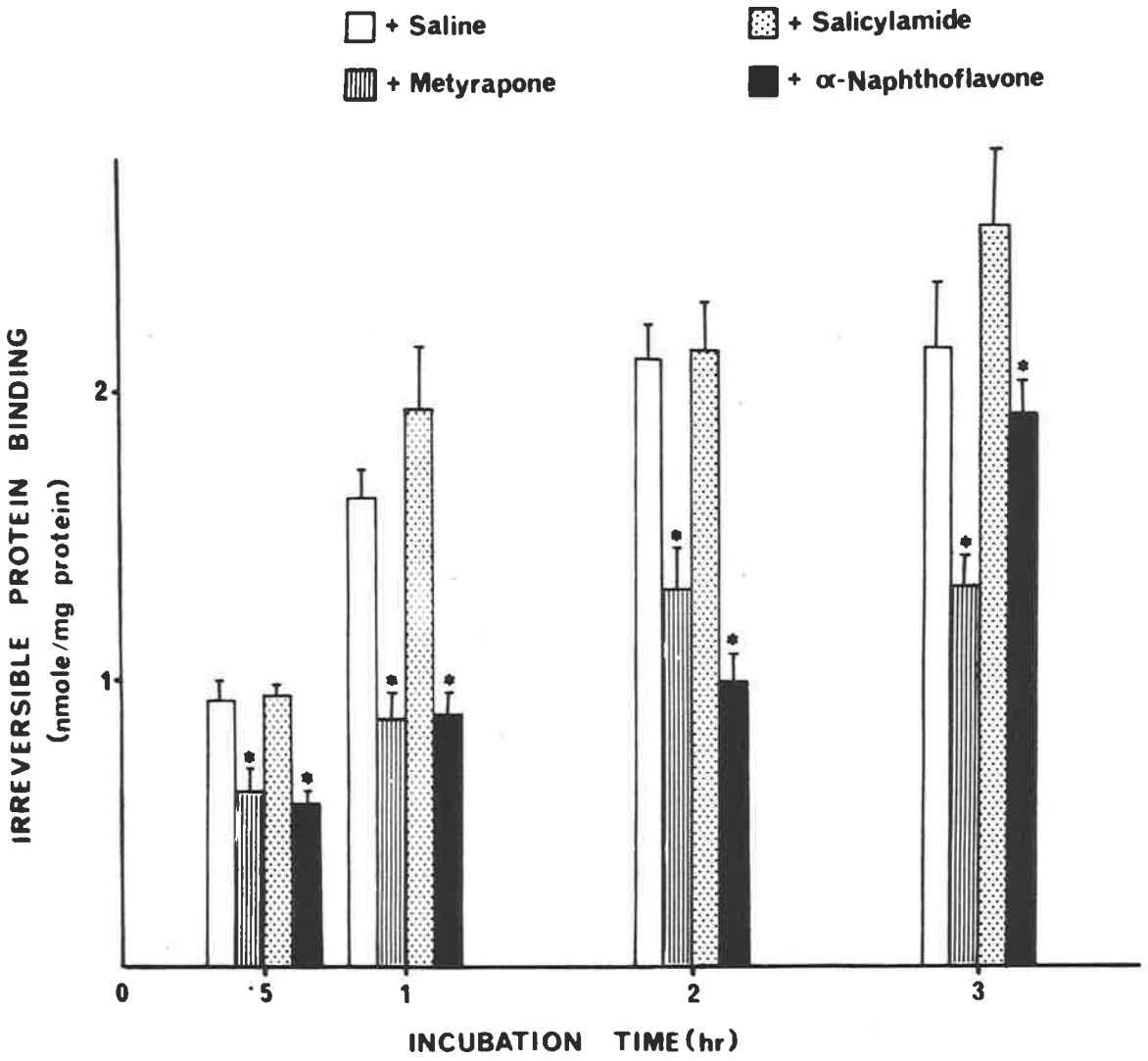

+ Saline
+ Salicylamide
+ Metyrapone
+ α-Naphthoflavone
IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
·5
1
2
3
INCUBATION TIME (hr)

TABLE 3.1

Estimation of reduced glutathione level in isolated rat hepatocytes.

Treatment	Incubation time (hr)	Control (No paracetamol)	GSH (μmole/g cells) Paracetamol			
			Saline	Metyrapone	Salicylamide	α-Naphthoflavone
Control	0	5.35 ± 0.16	5.61 ± 0.22	5.30 ± 0.20	6.05 ± 0.28	4.86 ± 0.13
	½	5.20 ± 0.12	5.15 ± 0.15	5.19 ± 0.25	5.39 ± 0.24	4.32 ± 0.22*
	1	4.92 ± 0.13	4.88 ± 0.29	4.89 ± 0.18	5.61 ± 0.32	4.46 ± 0.22
	2	5.13 ± 0.20	5.37 ± 0.26	5.24 ± 0.25	5.50 ± 0.24	4.42 ± 0.16*
	3	5.25 ± 0.13	4.73 ± 0.13	5.05 ± 0.21	5.63 ± 0.27*	4.35 ± 0.11
Phenobarbital	0	5.13 ± 0.12	5.14 ± 0.27	5.28 ± 0.24	5.49 ± 0.23	4.83 ± 0.24
pretreated	½	4.61 ± 0.81	4.21 ± 0.16	4.42 ± 0.14	4.67 ± 0.18	3.96 ± 0.28
	1	4.56 ± 0.20	4.34 ± 0.12	4.29 ± 0.23	4.43 ± 0.07	3.76 ± 0.16*
	2	4.38 ± 0.18	4.44 ± 0.14	4.25 ± 0.09	4.83 ± 0.11*	3.99 ± 0.21
	3	4.34 ± 0.29	4.17 ± 0.14	3.99 ± 0.07	4.65 ± 0.11*	3.85 ± 0.24
Benz(α)pyrene	0	4.99 ± 0.33	4.92 ± 0.57	4.76 ± 0.62	5.34 ± 0.42	4.58 ± 0.42
pretreated	½	4.47 ± 0.16	4.15 ± 0.45	4.30 ± 0.60	4.89 ± 0.69	3.78 ± 0.43
	1	4.59 ± 0.36	3.81 ± 0.67	4.24 ± 0.64	4.82 ± 0.54	3.35 ± 0.45
	2	4.53 ± 0.23	4.01 ± 0.61	4.58 ± 0.64	5.00 ± 0.75	3.53 ± 0.56
	3	4.56 ± 0.21	4.10 ± 0.76	4.10 ± 0.76	4.75 ± 0.80	3.49 ± 0.49

Values shown are mean ± s.e.m. (n=3)

* significantly different from saline ($p<0.05$, t-test)

TABLE 3.2

Normal values of intracellular K^+ and ALT activity in isolated rat hepatocytes.

Treatment	Treatment time (hr)	Intracellular K^+ (µmole/g cells) Control (No paracetamol)	Intracellular K^+ (µmole/g cells) Paracetamol	ALT (IU/l) Control (No paracetamol)	ALT (IU/l) Paracetamol
Control	0	90.20 ± 3.05	92.98 ± 3.41	32.69 ± 3.84	36.67 ± 4.04
	½	87.90 ± 3.66	90.18 ± 4.34	43.99 ± 1.86	42.78 ± 6.69
	1	89.44 ± 4.46	90.17 ± 4.88	54.27 ± 5.29	53.33 ± 2.98
	2	86.40 ± 3.54	87.72 ± 1.22	55.21 ± 2.82	55.56 ± 6.06
	3	85.37 ± 3.93	88.42 ± 4.55	50.48 ± 2.61	48.89 ± 4.01
Phenobarbital	0	79.59 ± 5.50	73.34 ± 2.10	73.33 ± 3.86	73.34 ± 6.67
pretreated	½	74.01 ± 3.33	71.93 ± 1.49	102.69 ± 5.66	120.00 ± 16.67
	1	73.57 ± 2.43	71.93 ± 2.32	112.27 ± 5.61	117.78 ± 14.57
	2	72.01 ± 4.51	72.63 ± 4.30	125.12 ± 3.04	126.45 ± 10.33
	3	76.54 ± 3.43	74.23 ± 4.03	131.91 ± 5.80	145.00 ± 22.67
Benz(α)pyrene	0	86.77 ± 3.50	84.88 ± 3.28	46.39 ± 2.78	53.34 ± 7.70
pretreated	½	88.17 ± 4.71	88.43 ± 4.64	68.05 ± 2.50	71.67 ± 9.95
	1	88.14 ± 4.93	87.04 ± 3.59	72.91 ± 5.05	74.17 ± 3.43
	2	87.37 ± 4.48	88.77 ± 5.17	71.21 ± 4.01	70.00 ± 6.09
	3	90.05 ± 3.73	92.61 ± 5.94	74.02 ± 6.69	75.84 ± 5.34

Values shown are mean ± s.e.m. (n=3)

Fig. 3.7

Changes in intracellular K^+ and ALT activity in the medium in control isolated rat hepatocytes incubated with or without paracetamol and various metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. (n=3) of the differences from paracetamol/saline values at zero time.

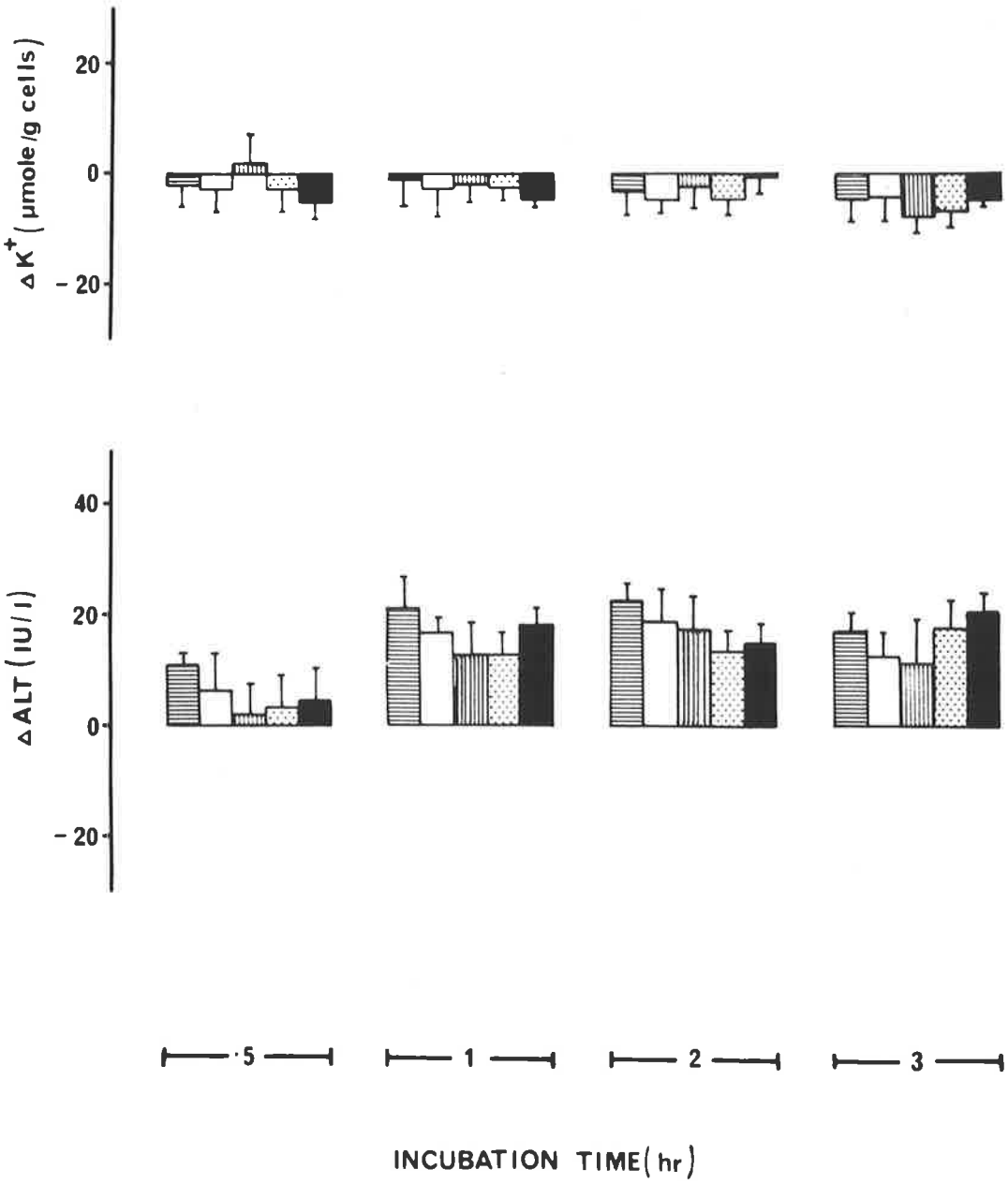

20
0
- 20
ΔK+ (μmole/g cells)
40
20
0
- 20
ΔALT (IU/l)
5
1
2
3
INCUBATION TIME(hr)

Fig. 3.8

Changes in intracellular K^+ and ALT activity in the medium in hepatocytes isolated from phenobarbital-treated rats incubated with or without paracetamol and various metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. (n=3) of the differences from paracetamol/saline values at zero time.

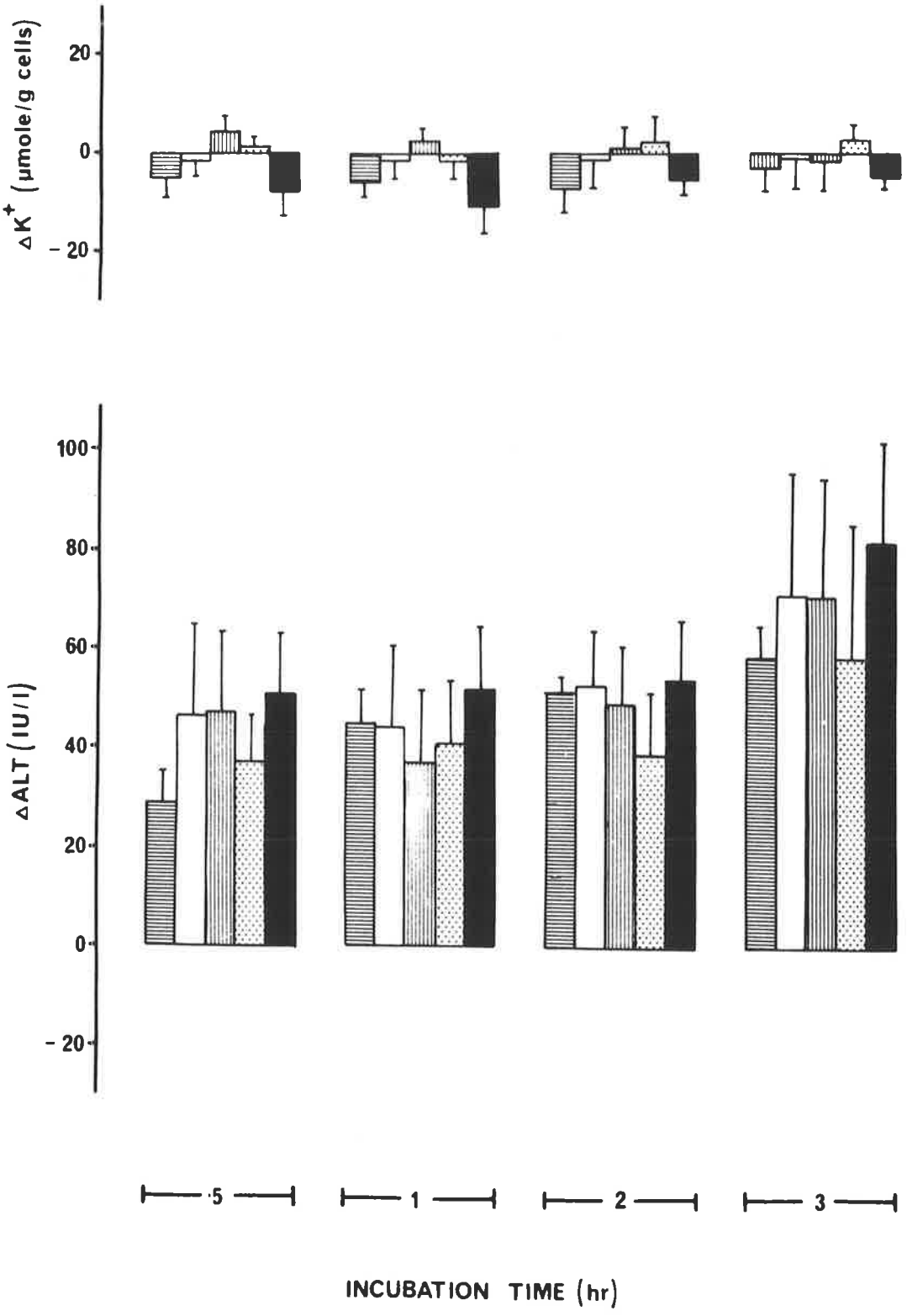
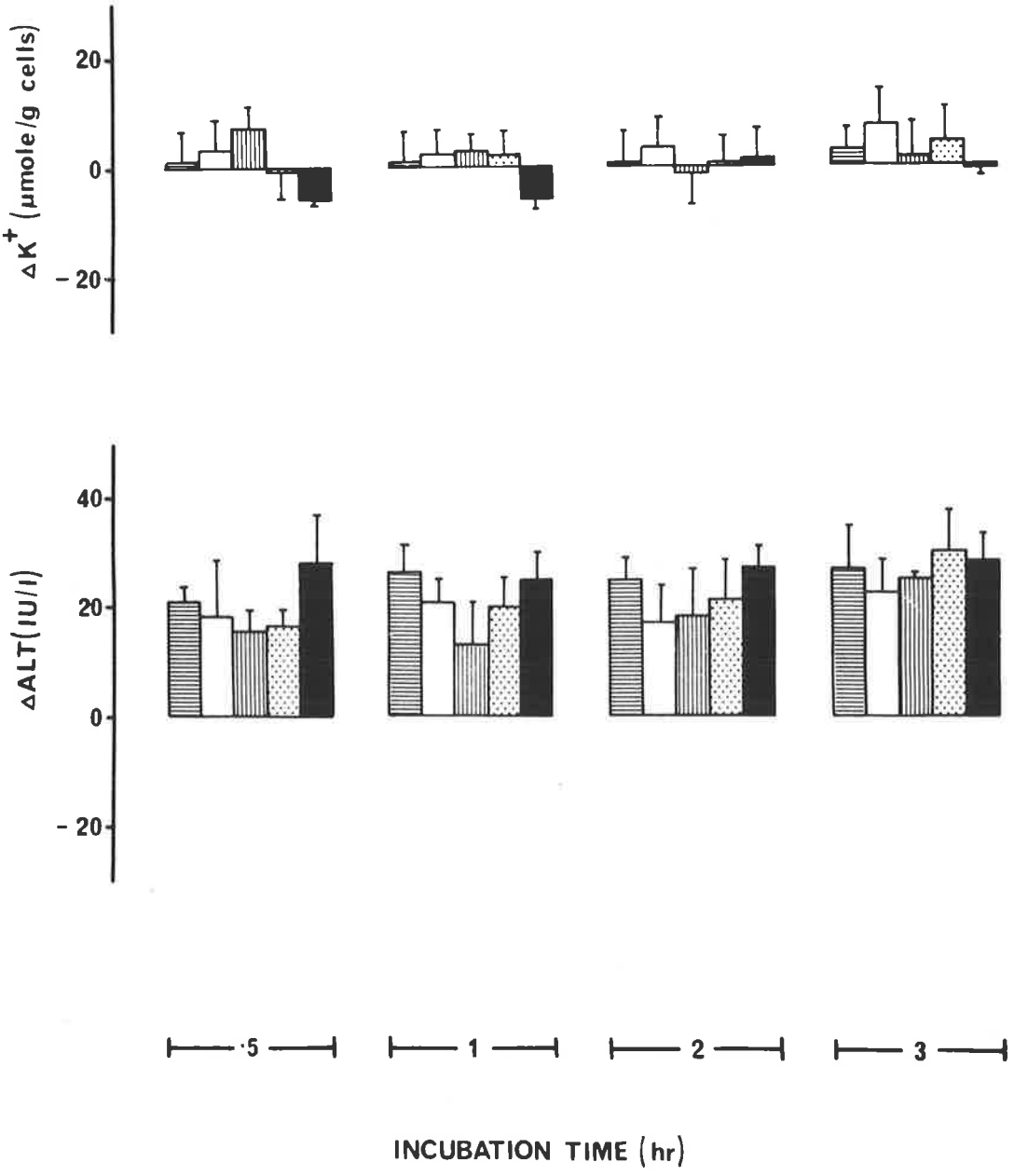

ΔK+ (μmole/g cells)
20
0
- 20
ΔALT (IU/l)
100
80
60
40
20
0
- 20
5
1
2
3
INCUBATION TIME (hr)

Fig. 3.9

Changes in intracellular K^+ and ALT activity in the medium in hepatocytes isolated from benz(α)pyrene-treated rats incubated with or without paracetamol and various metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. (n=3) of the differences from paracetamol/saline values at zero time.


20
0
- 20
ΔK+ (μmole/g cells)
40
20
0
- 20
ΔALT(IU/l)
·5
1
2
3
INCUBATION TIME (hr)

paracetamol has still not been firmly established, their formation is usually quantitated either by determining the extent of their irreversible protein binding, or by the formation of paracetamol-glutathione conjugate. Binding is a more direct method of quantitation, since it is believed to be a non-enzymatic process and occurs spontaneously. The formation of glutathione conjugate depends upon additional factors, such as the availability of GSH and the catalytic activity of glutathione-s transferase. This enzyme plays an important role in the detoxification of many xenobiotics and/or metabolites and is induced by many xenobiotics (Younes et al., 1980).

Irreversible protein binding in isolated rat hepatocytes was found to be increased with both dose and time, in a manner similar to that reported in microsomal preparations in vitro (Potter et al., 1973). The extent of binding is, however, higher in isolated hepatocytes, and this may be attributable to the greater availability of protein targets in intact cells. On the other hand, kinetic studies indicated that the reactive metabolite(s), once formed, are preferentially conjugated with reduced glutathione, rather than cell protein. This is basically in agreement with in vivo studies (Gillette, 1974; Gillette et al., 1974; Jollow and Smith, 1977).

There were some correlations between the extent of irreversible protein binding and the formation of glutathione conjugate in isolated rat hepatocytes. For example, the increase in binding after benz(α)pyrene induction correlated well with the enhancement of formation of glutathione conjugate. However, phenobarbital pretreatment increased the binding, but slightly

decreased formation of the glutathione conjugate. This paradoxical effect of phenobarbital cannot be readily explained. Although Jollow et al. (1974) observed a small decrease in mercapturic acid clearance in phenobarbital-treated rats after very high doses of paracetamol (800-1200 mg/Kg), they suggested that this may be due to diuresis-enhanced clearance of unchanged paracetamol and paracetamol glucuronide. This explanation is not appropriate for the present study. It would be necessary to invoke either an inhibitory effect of phenobarbital on GSH conjugation, or a large change in the relative kinetics of metabolic activation and glutathione conjugation.

The effect of metabolic inhibitors on the binding and glutathione conjugate were complex. Salicylamide was the only one which clearly inhibited the major conjugative pathway (sulphation), but there were no effects on binding or the formation of glutathione conjugate. Cytochrome P-450-mediated paracetamol activation was inhibited by metyrapone as shown in benz(α)pyrene-treated hepatocytes with the decrease in both binding and the formation of glutathione conjugate. However, in hepatocytes from phenobarbital-treated rats, metyrapone decreased binding, but slightly increased the formation of glutathione conjugate.

Moldeus et al. (1977) demonstrated the selectivity of the inhibitory effect of α-naphthoflavone in isolated rat hepatocytes using the substrate 7-ethoxycoumarin. They reported that α-naphthoflavone strongly inhibited the metabolism of 7-ethoxycoumarin in 3-methylcholanthrene-treated hepatocytes, but had no effects on its metabolism in hepatocytes from phenobarbital-treated rats. In the present study, an inhibitory effect of α-naphthoflavone on

the irreversible protein binding of paracetamol was noted, particularly in benz(α)pyrene-induced, but not in phenobarbital-induced, hepatocytes. Therefore, although it clearly has some non-specific inhibitory effects on glucuronidation and sulphation, it is relatively specific in its inhibitory effects on cytochrome P-450 induced by polycyclic aromatic hydrocarbons.

Values of reduced glutathione in isolated rat hepatocytes were comparable to those reported by other investigators (Hogberg and Kristoferson, 1977; Moldeus, 1978), but there was no evidence of GSH depletion in any treatments of the present study. Furthermore, there were no changes in K^+ retention or ALT release, both of which have been shown to be sensitive indicators of cell damage induced by hepatotoxins (Stacey and Priestly, 1978; Stacey et al., 1980). This may be attributed to the fact that the rat is relatively resistant to paracetamol-induced hepatotoxicity in vivo and the dose used in this in vitro system may not have been comparable to the high paracetamol doses required in vivo. In support of this hypothesis, Moldeus (1978) found that in hepatocytes isolated from phenobarbital-treated rats, very high paracetamol concentrations (10 mM) were needed to demonstrate GSH depletion and toxicity.

In conclusion, metabolic activation of paracetamol was found to be dose-dependent in this in vitro system, and could be modified by inducers and inhibitors of cytochrome P-450. However, demonstration of a correlation between irreversible protein binding and the formation of glutathione conjugate of paracetamol appeared to depend mainly upon the selectivity of the interactive drugs used.

* * *

CHAPTER 4

PARACETAMOL TOXICITY IN ISOLATED MOUSE HEPATOCYTES

Introduction

Rather than attempting to isolate the toxic metabolite of paracetamol directly, the covalent binding of radiolabelled paracetamol to hepatic protein has been used as a convenient indicator of the extent of formation of this presumed toxic metabolite (Potter et al., 1973). A direct correlation between the extent of covalent binding in vitro and the susceptibility to paracetamol-induced hepatotoxicity in different species has been found. The binding is greatest in hamsters and mice, which are the most susceptible species, and markedly less in rabbits, guinea pigs and rats, which are relatively resistant. It is likely that these differences reflect the relative activities of the metabolic activating and inactivating systems in these species (Davis et al., 1974).

Mitchell et al. (1973) demonstrated the relationship between the rate of glutathione depletion and the severity of liver necrosis after a toxic dose of paracetamol. The most rapid fall in hepatic glutathione after a single dose of paracetamol was found in the most susceptible species. At a dose of 300 mg/Kg in vivo, paracetamol depleted hepatic glutathione by 80% in hamsters and mice, but only 30% in guinea pigs and 10% in rats. This dose of paracetamol caused liver necrosis in hamsters and mice, but none in guinea pigs and rats (Davis et al., 1974). The extent of glutathione depletion also correlated well with the rate of in vivo and in vitro covalent binding and with the amount of mercapturic acid excreted in the urine (Davis et al., 1974; Potter et al., 1974).

The fact that mice are more susceptible to the hepatotoxic effects of paracetamol than rats and that consistent, dose-

dependent liver damage can be produced at the same dose levels that cause damage in human beings (Walker et al., 1980) means that studies of paracetamol metabolism and toxicity in mice may be more relevant to understanding the mechanism of paracetamol-induced hepatotoxicity in man.

Since the main aim of this thesis was to investigate the use of isolated hepatocytes as an in vitro toxicological model, it seemed logical to compare paracetamol metabolism and irreversible protein binding in hepatocytes from two species with different susceptibility. Since there are no experimental animal species with proven high susceptibility and a sufficiently large size to enable easy preparation of isolated hepatocytes by the perfusion technique, the necessary surgical skills were developed to prepare isolated mouse hepatocytes. Experiments similar to those described in Chapter 3 were therefore carried out using hepatocytes isolated from mice.

Methods

Hepatocytes were isolated from LACA Albino male mice (20-30 g). Pretreatments, incubation and data analysis were the same as those described in Chapter 3.

Results

The amount of irreversible protein binding of ^{14}C-paracetamol in isolated mouse hepatocytes was about two-fold higher than that in rat hepatocytes, and benz(α)pyrene pretreatment produced a further strong increase of approximately two times. However, in contrast with rat hepatocytes, the effect of pheno-

barbital induction was to produce only a small early increase in irreversible binding (Table 4.1).

The effects of metabolic inhibitors on the binding of ^{14}C-paracetamol in mouse hepatocytes resembled those in rat hepatocytes, with some subtle differences. Metyrapone decreased the binding in hepatocytes isolated from control (Fig. 4.1), as well as from phenobarbital (Fig. 4.2) and benz(α)pyrene-treated mice (Fig. 4.3). α-Naphthoflavone inhibited binding in control and benz(α)pyrene-induced hepatocytes, but failed to enhance binding in phenobarbital-induced hepatocytes (Figs 4.1, 4.2 and 4.3). Salicylamide, now revealed an inhibitory effect on binding in control (Fig. 4.1) and benz(α)pyrene-induce hepatocytes (Fig. 4.3), but had no effect in hepatocytes from phenobarbital-pretreated mice (Fig. 4.2).

As in rats, a consistent pattern of drug-induced glutathione depletion was not observed in any of the experiments. However, the glutathione levels were generally less than half those found in rat hepatocytes (Table 4.2). The pattern of changes in cell viability, as determined by the ability of cells to retain intracellular K^+ or to release ALT activity into the medium, was also unchanged after incubating all three groups of hepatocytes with paracetamol alone or with paracetamol and inhibitors (Figs 4.4-4.6). Actual values of intracellular K^+ and ALT activity in mouse hepatocytes are shown in Table 4.3.

Discussion

The irreversible protein binding and glutathione conjugation of paracetamol has been shown to occur in isolated mouse hepato-

TABLE 4.1

Effect of metabolic inducers on the irreversible protein binding of ^{14}C-paracetamol in isolated mouse hepatocytes.

Treatment	Incubation time (hr)	Irreversible protein binding (nmole/mg protein)
Control	$\frac{1}{2}$	0.84 ± 0.01
	1	1.49 ± 0.12
	2	2.06 ± 0.25
	3	2.34 ± 0.32
Phenobarbital pretreated	$\frac{1}{2}$	1.11 ± 0.12✡
	1	1.60 ± 0.15
	2	2.27 ± 0.23
	3	2.71 ± 0.31
Benz(α)pyrene pretreated	$\frac{1}{2}$	1.14 ± 0.07✡
	1	2.20 ± 0.19✡✱
	2	4.16 ± 0.83✡✱
	3	3.78 ± 0.30✡

Values shown are mean ± s.e.m. (n=3)

✡ significantly different from control hepatocytes ($p<0.05$, t-test)

✱ significantly different from phenobarbital-treated hepatocytes ($p<0.05$, t-test)

Fig. 4.1

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in untreated control isolated mouse hepatocytes.

●——● saline; O---O metyrapone;

■——■ salicylamide; □---□ α-naphthoflavone.

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

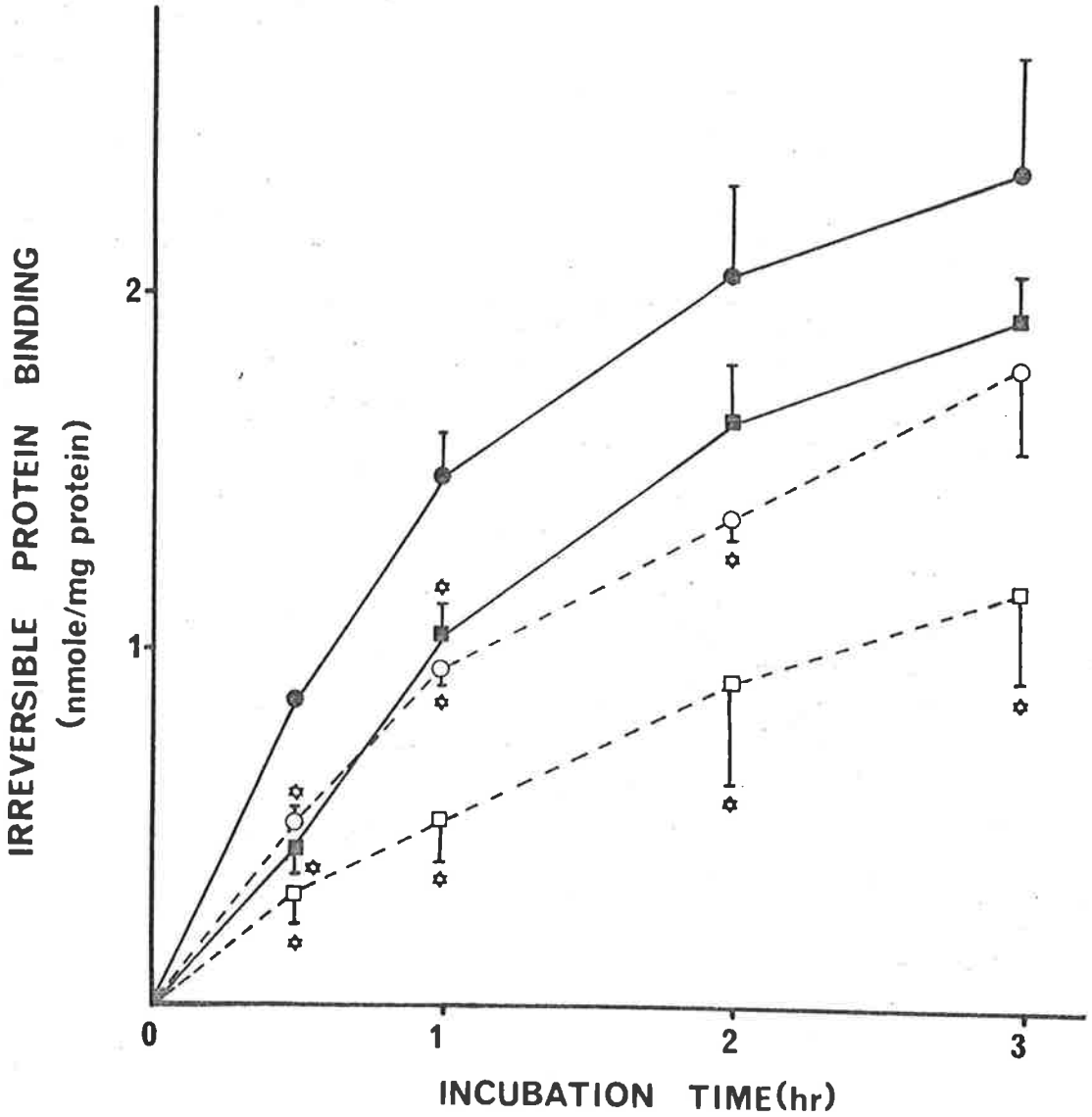

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
1
2
3
INCUBATION TIME(hr)

Fig. 4.2

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in hepatocytes isolated from phenobarbital-treated mice.

●——● saline; O---O metyrapone;

■——■ salicylamide; □---□ α-naphthoflavone.

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

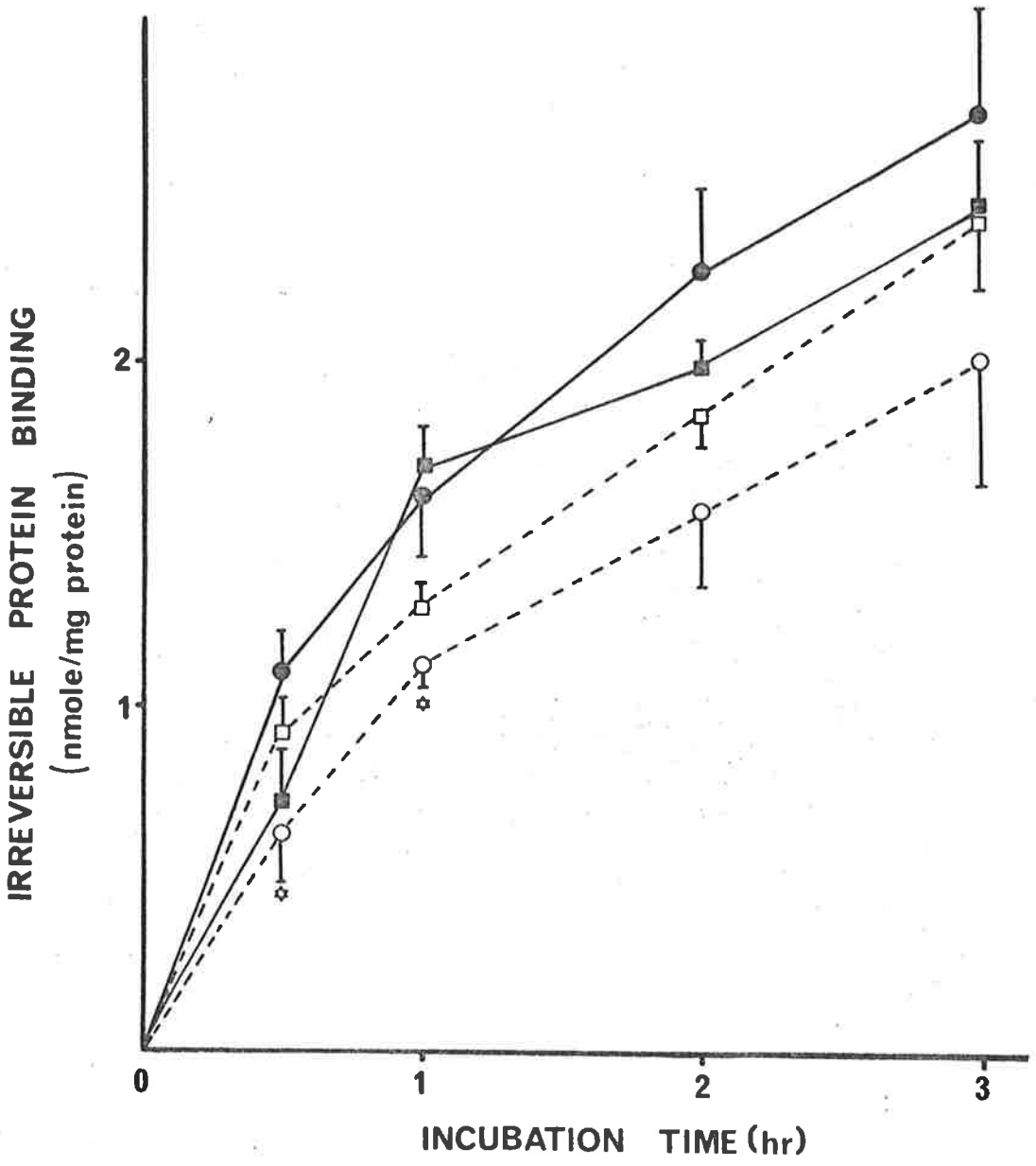

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
1
2
3
INCUBATION TIME (hr)

Fig. 4.3

Effect of metabolic inhibitors on the irreversible protein binding of ^{14}C-paracetamol in hepatocytes isolated from benz(α)pyrene-treated mice.

●——● saline; ○---○ metyrapone;

■——■ salicylamide; □---□ α-naphthoflavone.

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

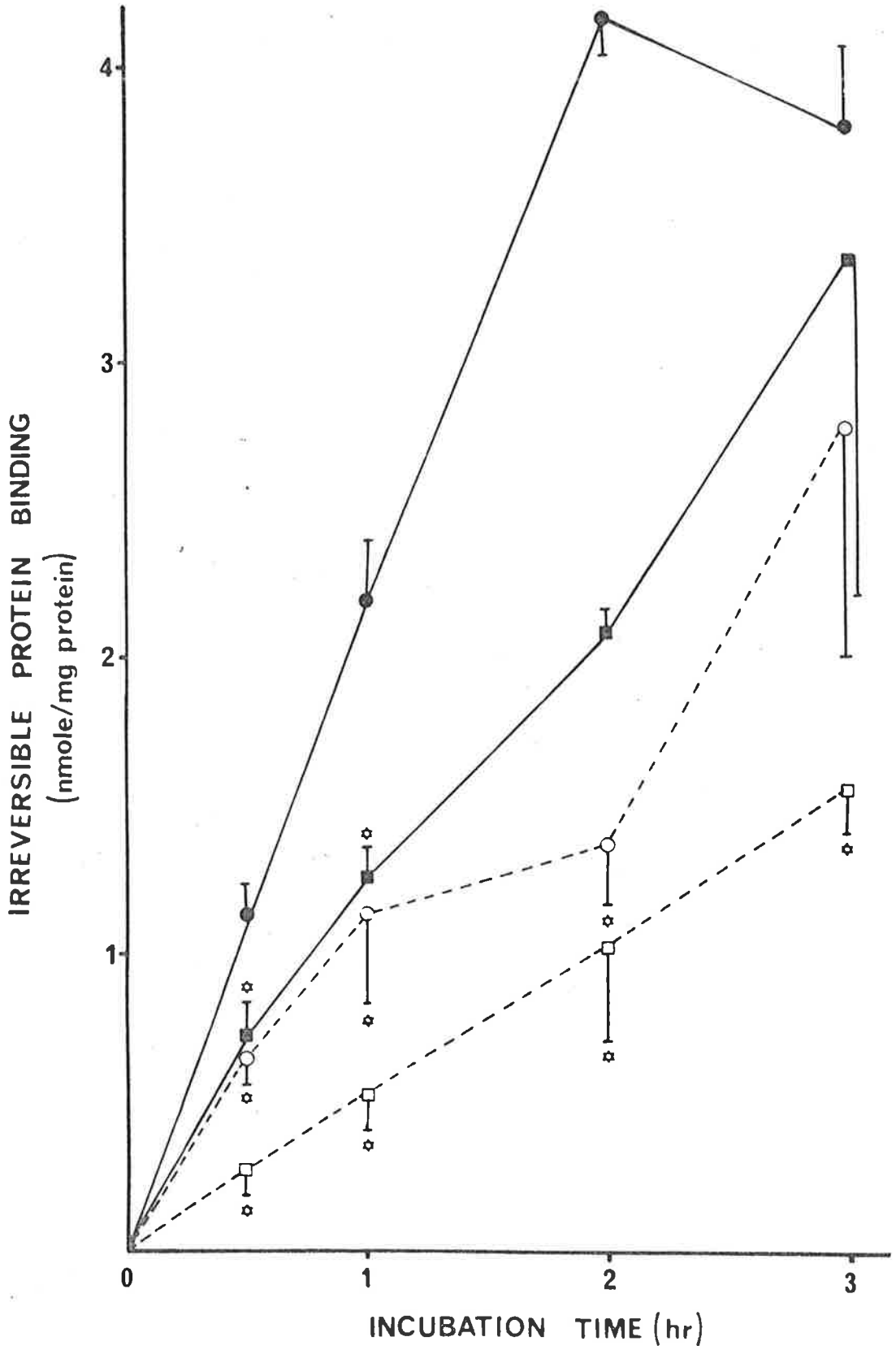

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
4
3
2
1
0
1
2
3
INCUBATION TIME (hr)

TABLE 4.2

Estimation of reduced glutathione level in isolated mouse hepatocytes.

Treatment	Incubation time (hr)	Control	GSH (µmole/g cells) Paracetamol			
			Saline	Metyrapone	Salicylamide	α-Naphthoflavone
Control	0	2.37 ± 0.02	2.45 ± 0.11	2.49 ± 0.11	2.82 ± 0.13	2.51 ± 0.09
	½	2.34 ± 0.01	2.30 ± 0.04	2.30 ± 0.10	2.69 ± 0.01	2.32 ± 0.11
	1	2.45 ± 0.05	2.31 ± 0.19	2.41 ± 0.15	2.64 ± 0.12	2.43 ± 0.13
	2	2.35 ± 0.05	2.14 ± 0.26	2.42 ± 0.34	2.58 ± 0.18	2.25 ± 0.31
	3	2.69 ± 0.12	2.62 ± 0.21	2.21 ± 0.35	2.48 ± 0.34	2.21 ± 0.35
Phenobarbital	0	2.42 ± 0.15	2.54 ± 0.13	2.64 ± 0.05	2.85 ± 0.15	2.55 ± 0.08
pretreated	½	2.09 ± 0.21	2.10 ± 0.19	2.24 ± 0.20	2.67 ± 0.13	1.98 ± 0.24
	1	2.25 ± 0.11	2.16 ± 0.08	2.12 ± 0.08	2.52 ± 0.11	2.20 ± 0.33
	2	2.31 ± 0.05	2.17 ± 0.21	2.15 ± 0.18	2.50 ± 0.35	2.04 ± 0.16
	3	2.33 ± 0.06	2.13 ± 0.20	2.13 ± 0.20	2.31 ± 0.27	2.10 ± 0.20
Benz(α)pyrene	0	2.41 ± 0.10	2.45 ± 0.06	2.44 ± 0.14	2.87 ± 0.15	2.48 ± 0.17
pretreated	½	2.16 ± 0.14	2.15 ± 0.08	2.40 ± 0.21	2.76 ± 0.23	2.56 ± 0.28
	1	2.15 ± 0.23	2.10 ± 0.14	2.44 ± 0.19	2.54 ± 0.13	2.47 ± 0.13
	2	2.07 ± 0.21	1.95 ± 0.20	2.17 ± 0.12	2.45 ± 0.32	2.04 ± 0.04
	3	1.86 ± 0.14	1.72 ± 0.18	1.99 ± 0.15	1.86 ± 0.13	1.78 ± 0.18

Values shown are mean ± s.e.m. (n=3).

TABLE 4.3

Normal values of intracellular K^+ and ALT activity in isolated mouse hepatocytes.

Treatment	Incubation time (hr)	Intracellular K^+ (µmole/g cells)		ALT (IU/l)	
		Control (No paracetamol)	Paracetamol	Control (No paracetamol)	Paracetamol
Control	0	78.03 ± 5.69	77.57 ± 8.38	15.67 ± 0.99	17.10 ± 1.50
	½	75.02 ± 1.91	75.85 ± 2.95	19.85 ± 1.22	20.40 ± 2.10
	1	69.10 ± 3.95	66.54 ± 2.67	21.68 ± 3.15	21.65 ± 2.20
	2	61.36 ± 1.07	58.42 ± 1.97	25.10 ± 1.65	29.60 ± 2.55
	3	59.23 ± 1.09	57.94 ± 0.65	32.91 ± 5.30	34.60 ± 8.65
Phenobarbital	0	78.95 ± 3.53	79.31 ± 2.31	19.70 ± 2.20	20.85 ± 3.72
pretreated	½	71.10 ± 6.87	70.83 ± 5.96	23.11 ± 2.60	24.15 ± 3.65
	1	61.23 ± 5.95	62.82 ± 5.80	25.16 ± 3.95	28.35 ± 5.00
	2	62.19 ± 5.80	61.46 ± 6.31	29.60 ± 2.75	30.40 ± 2.75
	3	60.17 ± 6.16	61.35 ± 9.00	33.67 ± 3.77	38.75 ± 4.00
Benz(α)pyrene	0	79.96 ± 4.22	78.99 ± 9.74	17.65 ± 0.22	17.10 ± 1.50
pretreated	½	70.18 ± 3.95	69.28 ± 6.51	20.96 ± 3.95	20.40 ± 2.55
	1	62.28 ± 4.45	60.06 ± 5.30	22.25 ± 2.50	23.35 ± 1.50
	2	60.95 ± 4.25	54.80 ± 2.72	29.90 ± 3.40	30.50 ± 5.50
	3	60.15 ± 4.98	60.22 ± 7.53	36.87 ± 5.10	37.90 ± 4.25

Fig. 4.4

Changes in intracellular K^+ and ALT activity in control isolated mouse hepatocytes after incubating with or without paracetamol and metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. (n=3) of the differences from saline values at zero time.

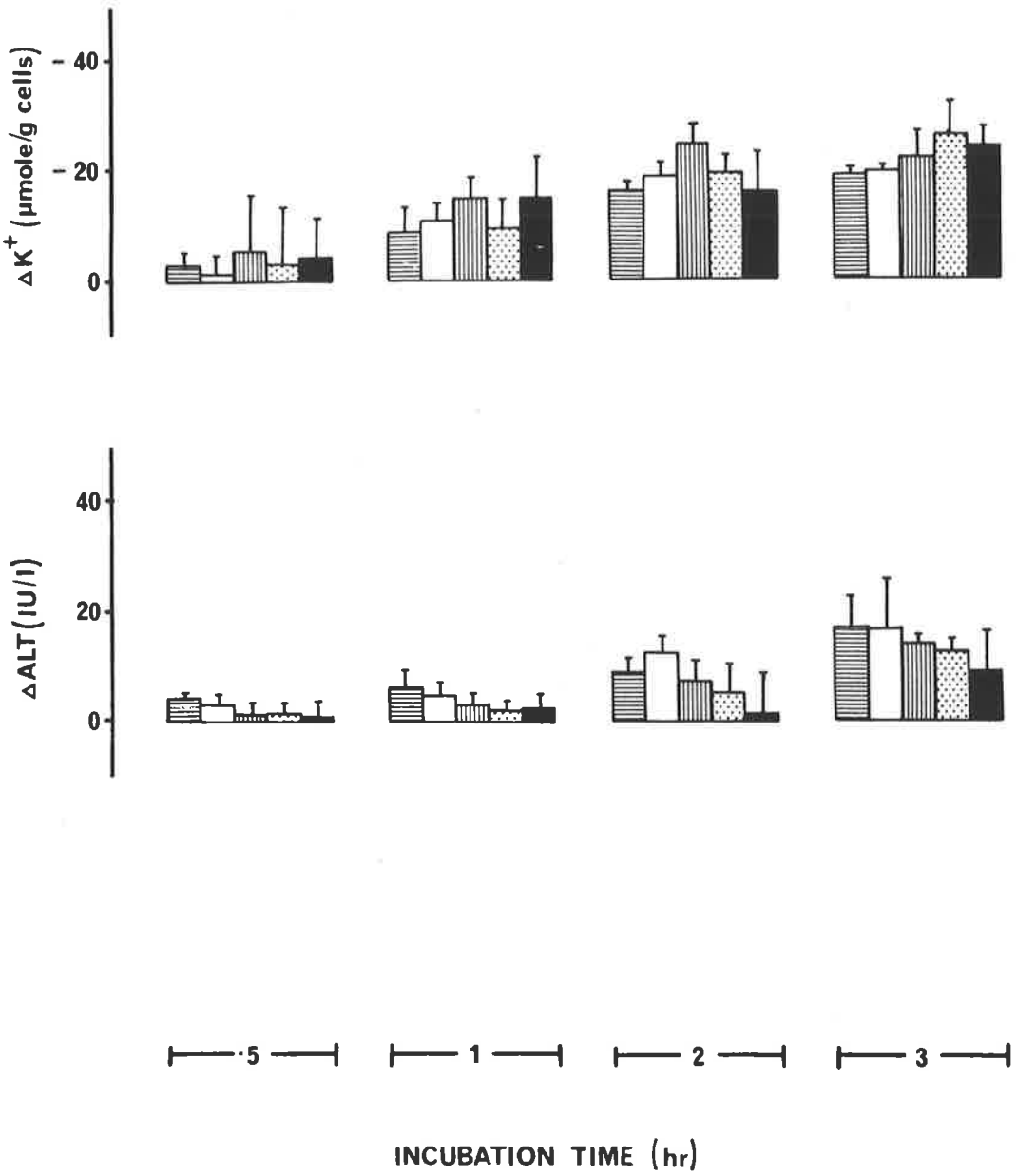

ΔK+ (μmole/g cells)
40
20
0
ΔALT(IU/l)
40
20
0
·5
1
2
3
INCUBATION TIME (hr)

Fig. 4.5

Changes in intracellular K^+ and ALT activity in hepatocytes isolated from phenobarbital-treated mice after incubating with or without paracetamol and metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. (n=3) of the differences from saline values at zero time.

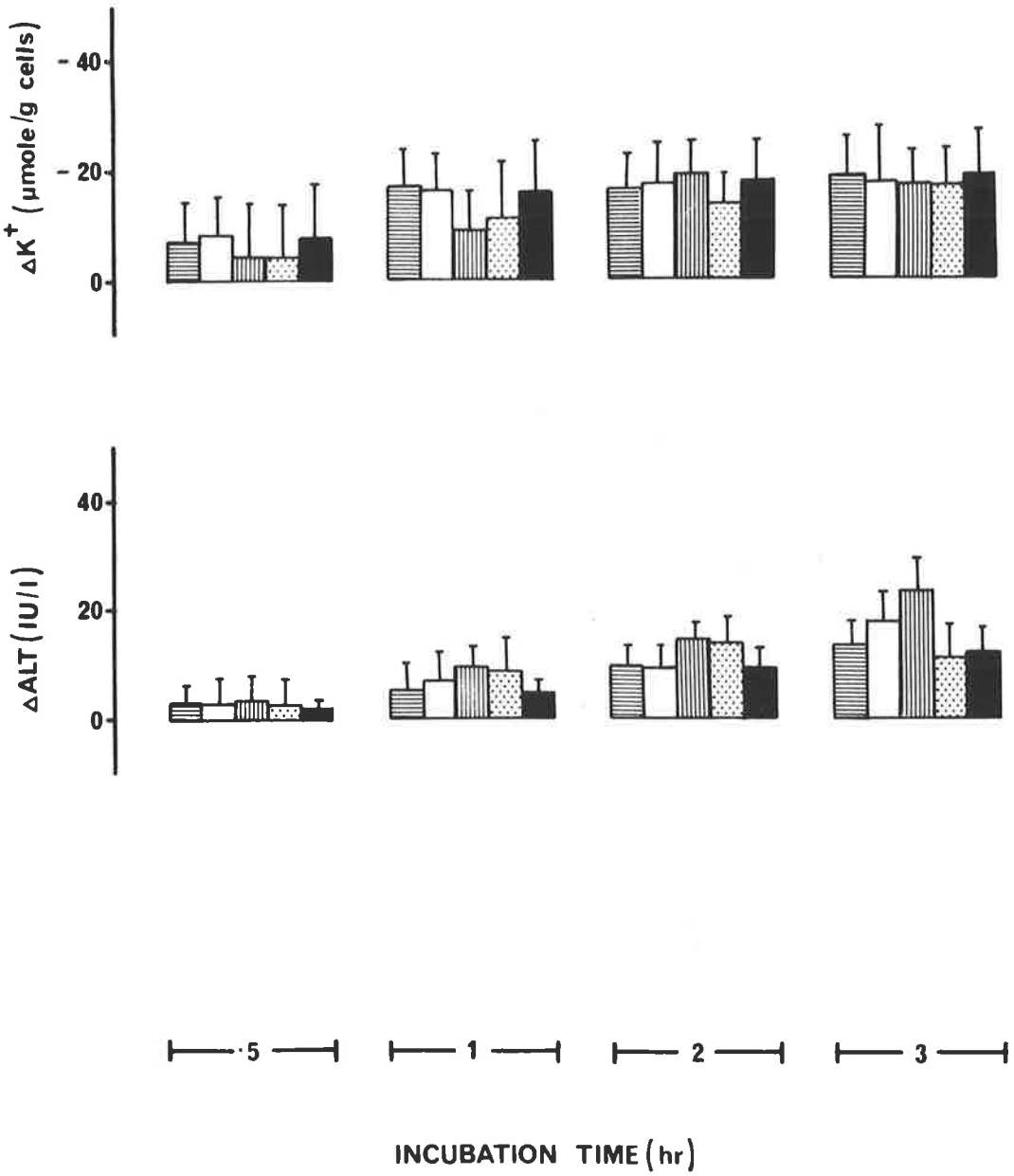
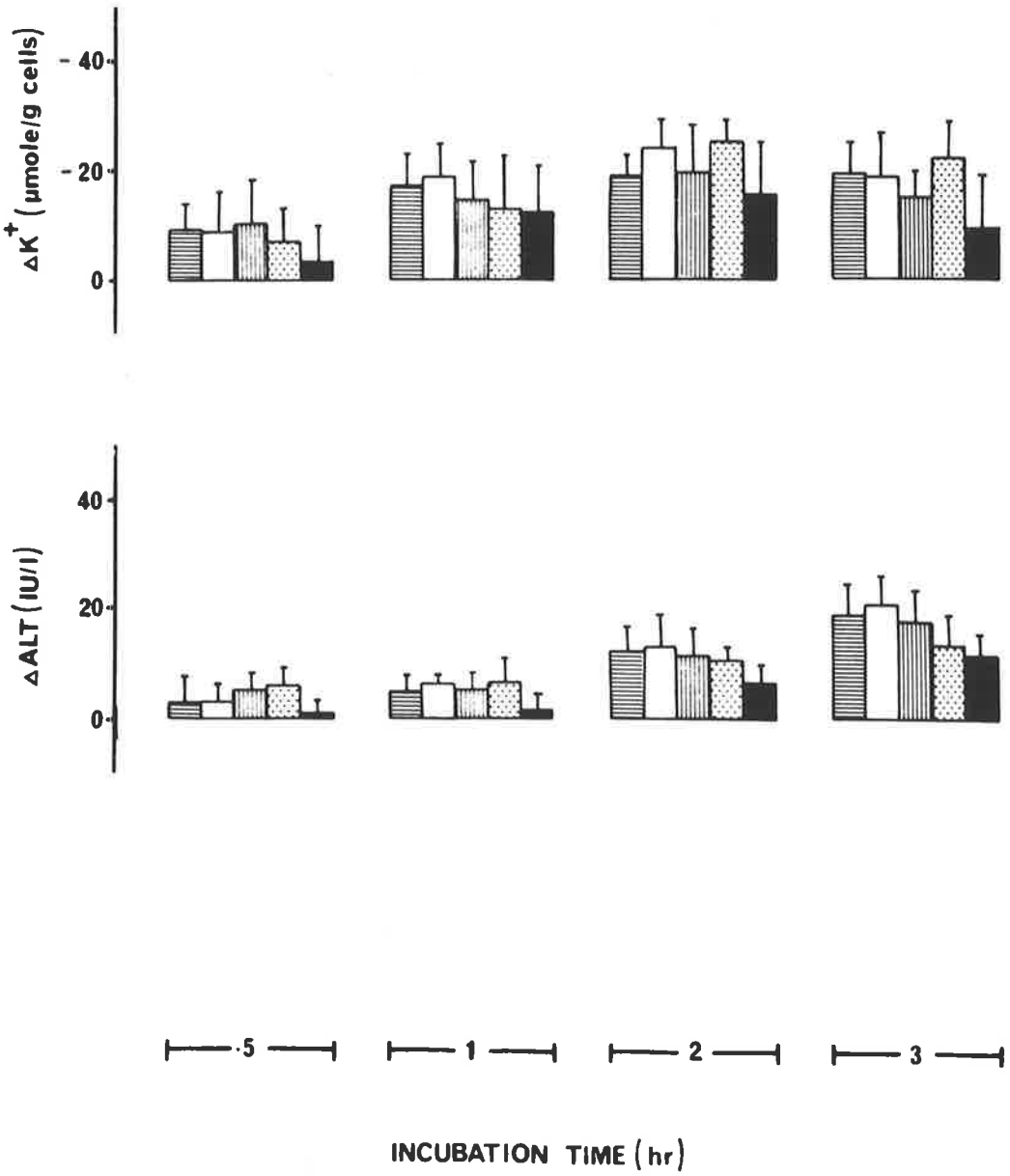

ΔK+ (μmole/g cells)
40
20
0
ΔALT (IU/l)
40
20
0
5
1
2
3
INCUBATION TIME (hr)

Fig. 4.6

Changes in intracellular K^+ and ALT activity in hepatocytes isolated from benz(α)pyrene-treated mice after incubating with or without paracetamol and metabolic inhibitors.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + metyrapone
- paracetamol + salicylamide
- paracetamol + α-naphthoflavone

Values shown are mean ± s.e.m. of the differences from saline values at zero time.


ΔK^+ (μmole/g cells)
40
20
0
ΔALT (IU/l)
40
20
0
·5
1
2
3
INCUBATION TIME (hr)

cytes. The extent of metabolic activation was approximately double that found in the experiments with rat hepatocytes and was potentiated to a greater extent by benz(α)pyrene induction. This is consistent with the hypothesis that enhanced metabolic activation explains the higher susceptibility of mice to paracetamol-induced hepatotoxicity.

Pretreatment of mice with phenobarbital potentiates both the incidence and severity of paracetamol-induced hepatic necrosis (Mitchell et al., 1973) and markedly increases covalent binding of paracetamol in vivo (Jollow et al., 1973). Similar enhancement of the binding has also been found in microsomal preparations isolated from phenobarbital-treated mice (Potter et al., 1973), and kinetic analyses indicated that this was associated with an increase in the Vmax (0.3 nmole/mg protein/min, compared to control value of 0.2 nmole/mg protein/min), while the apparent Km (3.2×10^{-4} M) was unchanged. In the present study, using isolated mouse hepatocytes, phenobarbital pretreatment showed little effect on binding, although it did increase glutathione conjugation. These results are in direct contrast to those found in isolated rat hepatocytes (Chapters 1, 3) and indicate an interesting, although inexplicable, species difference in the inductive effect of phenobarbital.

As in rat hepatocytes, the effects of inhibitors on the activation of paracetamol in mouse hepatocytes were variable. Metyrapone appeared to be a strong cytochrome P-450 inhibitor. It decreased binding in all hepatocytes, irrespective of pretreatment, although the effect was strongest in the benz(α)pyrene-treated group. The specificity of α-naphthoflavone towards cytochrome

P-450 induced by benz(α)pyrene noted in rat hepatocytes was also seen in mouse hepatocytes.

Salicylamide has been shown to competitively inhibit the formation of reactive metabolite(s) in hamster microsomes, as determined by the covalent binding of radiolabelled paracetamol to microsomal protein (Jollow and Smith, 1977). The K_i for this reaction (1.5×10^{-4} M) was greater than those found for inhibition of sulphation and glucuronidation (K_i about 1×10^{-5} M and 3×10^{-5} M, respectively). Thus, salicylamide is a more effective inhibitor of the major conjugative detoxifying pathways for paracetamol metabolism than for the activating oxidative pathway. Nevertheless, in mouse hepatocytes, salicylamide revealed an inhibitory effect on paracetamol activation which was not seen in rat hepatocytes. This occurred in spite of the fact that it also inhibited sulphate and glucuronide conjugation (Chapter 2), and suggests that regulation of these latter pathways may play a relatively minor role in determining paracetamol-induced hepatotoxicity.

The level of reduced glutathione in isolated mouse hepatocytes was lower than those found in rats. This differs from the observation of Moldeus (1978, 1978a), who showed similar GSH levels in both rat and mouse hepatocytes. The length of the perfusion time during preparation of hepatocytes is known to have a strong influence on the GSH levels. Hogberg and Kristoferson (1977) showed that prolonged perfusion time did not lower the yield of viable hepatocytes, but resulted in a continuous release of glutathione. It is unlikely that this relatively low level of GSH is responsible for the high rate of paracetamol activation in

isolated mouse hepatocytes, since no further paracetamol-induced GSH depletion and/or toxicity were noted, even in benz(α)pyrene-induced hepatocytes in which a considerable amount of binding and glutathione conjugation occurred.

In conclusion, species differences have an important influence on the variations of the effects of metabolic inducers and inhibitors and, in particular, on the correlation between the irreversible protein binding of paracetamol reactive metabolite(s) and the formation of glutathione conjugate.

* * *

CHAPTER 5

PARACETAMOL METABOLISM AND TOXICITY IN ISOLATED RAT AND MOUSE HEPATOCYTES: PROTECTIVE EFFECTS OF SULPHYDRYL COMPOUNDS

Introduction

Glutathione (L- γ -glutamyl-L-cysteinyl-glycine) plays an important role in hepatic drug detoxification. It may form water soluble and excretable conjugates with xenobiotics or their metabolically activated intermediates, or act as a co-factor for the selenium-dependent GSH-peroxidase, which reduces lipid peroxidases and hydrogen peroxide and has been implicated in the defence against lipid peroxidation (Anundi et al., 1979). Compounds or their reactive metabolites which deplete the hepatic GSH reservoir promote lipid peroxidation and/or covalent binding with subsequent cellular damage (Hogberg and Kristoferson, 1977).

The electrophilic metabolite which mediates paracetamol-induced hepatic necrosis reacts alternatively with cell macromolecules or with glutathione. Pretreatment of mice with diethyl maleate, which depletes hepatic glutathione, has been shown to increase the covalent binding of the reactive metabolite and to potentiate paracetamol-induced hepatic necrosis, whereas pretreatment with cysteine, a glutathione precursor, has been shown to decrease the covalent binding and to prevent hepatic damage (Mitchell et al., 1973).

A wide variety of sulphydryl-containing compounds, including cysteine, cysteamine, methionine, N-acetylcysteine and dimercaprol have been used in attempts to prevent or reduce the hepatotoxic effects of paracetamol both in man and experimental animals (Mitchell et al., 1973 and 1974; Prescott et al., 1976; Hughes et al., 1977; Moldeus, 1978a; Prescott et al., 1979). The exact mechanism of their actions has not yet been clarified. Possibilities include the facilitation of reduced glutathione synthesis,

direct conjugation as an alternative substrate and inhibition of cytochrome P-450-dependent activation of paracetamol. Intravenous administration of N-acetylcysteine is favoured as a treatment for paracetamol poisoning in man, because it is more effective than cysteamine and methionine and has fewer adverse effects (Prescott et al., 1979).

The aim of experiments described in this chapter was to determine whether irreversible protein binding, metabolism and toxicity of paracetamol in isolated rat and mouse hepatocytes are susceptible to changes in sulphydryl status. This was manipulated by administration of diethyl maleate prior to rat hepatocyte preparation, using a dose (0.69 ml/Kg) shown previously to deplete hepatic GSH by approximately 90% (Boyland and Chasseaud, 1970), or by concurrent incubation of hepatocytes with paracetamol and N-acetylcysteine, cysteamine or N-acetylmethionine.

Methods

Hepatocytes were isolated from male rats (200-250 g), diethyl maleate pretreated rats (0.69 ml/Kg, i.p., 20 min prior to hepatocyte isolation) and male mice (20-30 g) as described in the General Methods.

Metabolism study:

Aliquots of 2.5 ml cell suspension (5-6 x 10^6 cells/ml) were incubated with 0.1 ml paracetamol (1 mg/ml) and 0.1 ml saline or 0.1 ml of sulphydryl compounds (N-acetyl-l-cysteine or cysteamine HCL or N-acetyl-dl-methionine) for half to 3 hours. The final concentrations were 0.25 mM for paracetamol and 5 mM for

each of the sulphydryl substances.

At the end of the incubation procedure, 1 ml samples were taken for metabolite analysis by HPLC method.

Toxicity study:

Aliquots of 2.5 ml cell suspension (5-6 x 10^6 cells/ml) were incubated with 0.1 ml paracetamol (1 mg/ml), 0.1 ml (ring ^{14}-C) paracetamol (3.3 µCi/ml) and 0.1 ml physiological saline or 0.1 ml sulphydryl compounds (N-acetyl-l-cysteine or cysteamine HCL or N-acetyl-dl-methionine) for half to 3 hours. The final concentrations of paracetamol and sulphydryl compounds were 0.25 mM and 5 mM, respectively.

At the end of the incubation procedure, 0.5 ml samples were taken for intracellular K^+ and ALT activity assay, 0.2 ml for the estimation of reduced glutathione (GSH) and the rest for the determination of irreversible protein binding.

In hepatocytes isolated from diethyl maleate-treated rats the concentration of paracetamol was 0.13 mM.

Results

Diethyl maleate pretreatment lowered the level of reduced glutathione in rat hepatocytes from 5.56 ± 0.16 to 2.94 ± 0.17 µmoles/g cells and increased the amount of binding, particularly toward the end of 3 hours incubation period (Fig. 5.1). Neither paracetamol nor diethyl maleate and paracetamol altered the pattern of changes in cell viability as determined by K^+ retention and ALT release (Fig. 5.2).

Cysteamine was a potent inhibitor of paracetamol metabolism

Fig. 5.1

Irreversible protein binding and reduced glutathione in hepatocytes isolated from control (●—●) and diethyl maleate-treated rats (■—■).

Values shown are mean ± s.e.m. (n=3).

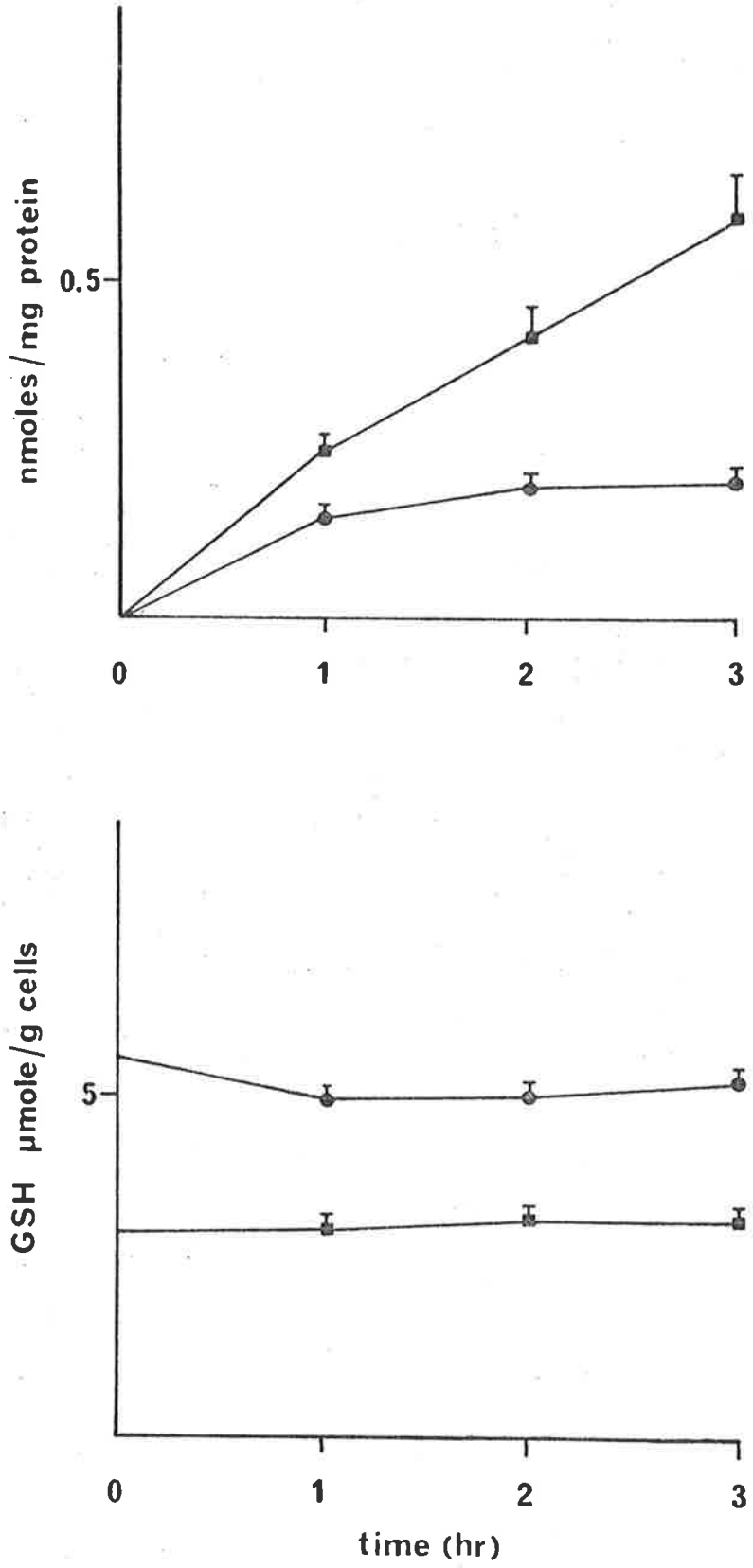
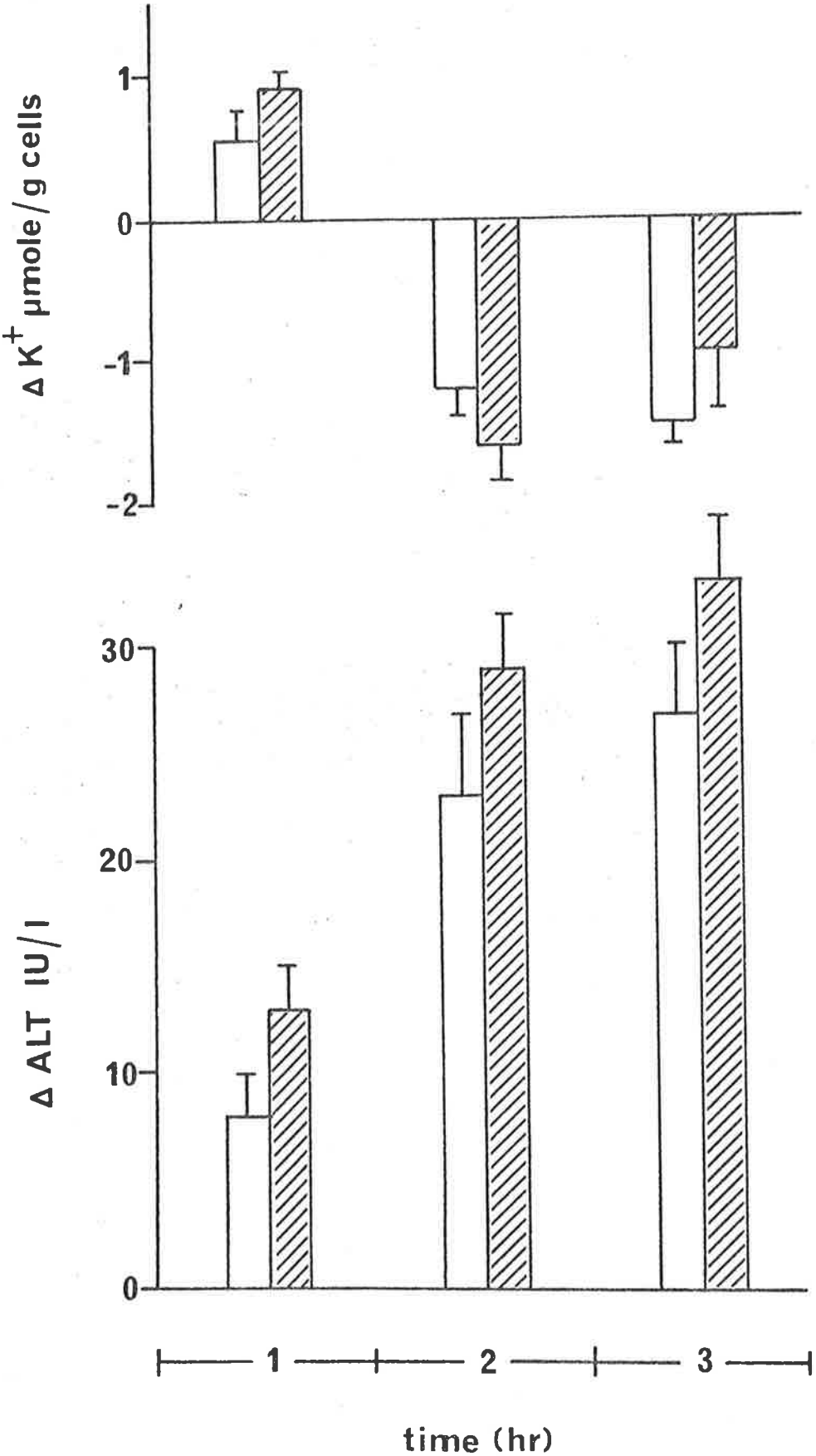

nmoles/mg protein
0.5
0
1
2
3
GSH µmole/g cells
5
0
1
2
3
time (hr)

Fig. 5.2

Changes in the intracellular potassium (ΔK^+) and ALT activity (Δ ALT) in control (□) and diethyl maleate-treated rat hepatocytes (▨) incubated with paracetamol (0.13 mM).

Values shown are mean ± s.e.m. (n=3).


1
0
-1
-2
Δ K+ µmole/g cells
30
20
10
0
Δ ALT IU/l
1
2
3
time (hr)

and reduced the formation of all three conjugates in both species. N-acetylmethionine had no consistent effect on paracetamol metabolism. However, N-acetylcysteine, while having no effect on conjugation with sulphate or glucuronide in either rat or mouse hepatocytes, did cause a significant increase in glutathione conjugation in mouse hepatocytes. This effected an enhancement of paracetamol clearance from the mouse hepatocyte suspensions (Figs 5.3 and 5.4).

Both N-acetylcysteine and cysteamine decreased the amount of irreversible protein binding in both isolated rat and mouse hepatocytes, while the binding was unchanged in the presence of N-acetylmethionine (Figs 5.5 and 5.6).

There was no alteration in the reduced glutathione (GSH) level in hepatocytes from both species after incubating with paracetamol or paracetamol and N-acetylmethione. N-acetylcysteine and cysteamine interfered with the method used to estimate the level of reduced glutathione, so the apparent GSH presented in Table 5.1 was not the true estimation. However, the GSH level in the presence of cysteamine dropped nearly to zero in both hepatocytes isolated from rats and mice.

In isolated rat hepatocytes, only cysteamine caused the reduction in cell viability as determined by the decrease in intracellular K^+ and the increase in ALT activity (Fig. 5.7).

In isolated mouse hepatocytes, cysteamine had a similar but smaller effect compared to that seen in rats. N-acetylcysteine decreased intracellular K^+ loss without any change in ALT activity, while the converse was observed with N-acetylmethionine (Fig. 5.8).

Fig. 5.3

Time course of metabolite formation in hepatocytes isolated from rats incubated with paracetamol (0.25 mM) and sulphydryl compounds.

saline

N-acetyl cysteine (5 mM)

cysteamine (5 mM)

N-acetyl methionine (5 mM)

P-G paracetamol glucuronide

P-S paracetamol sulphate

P paracetamol

P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

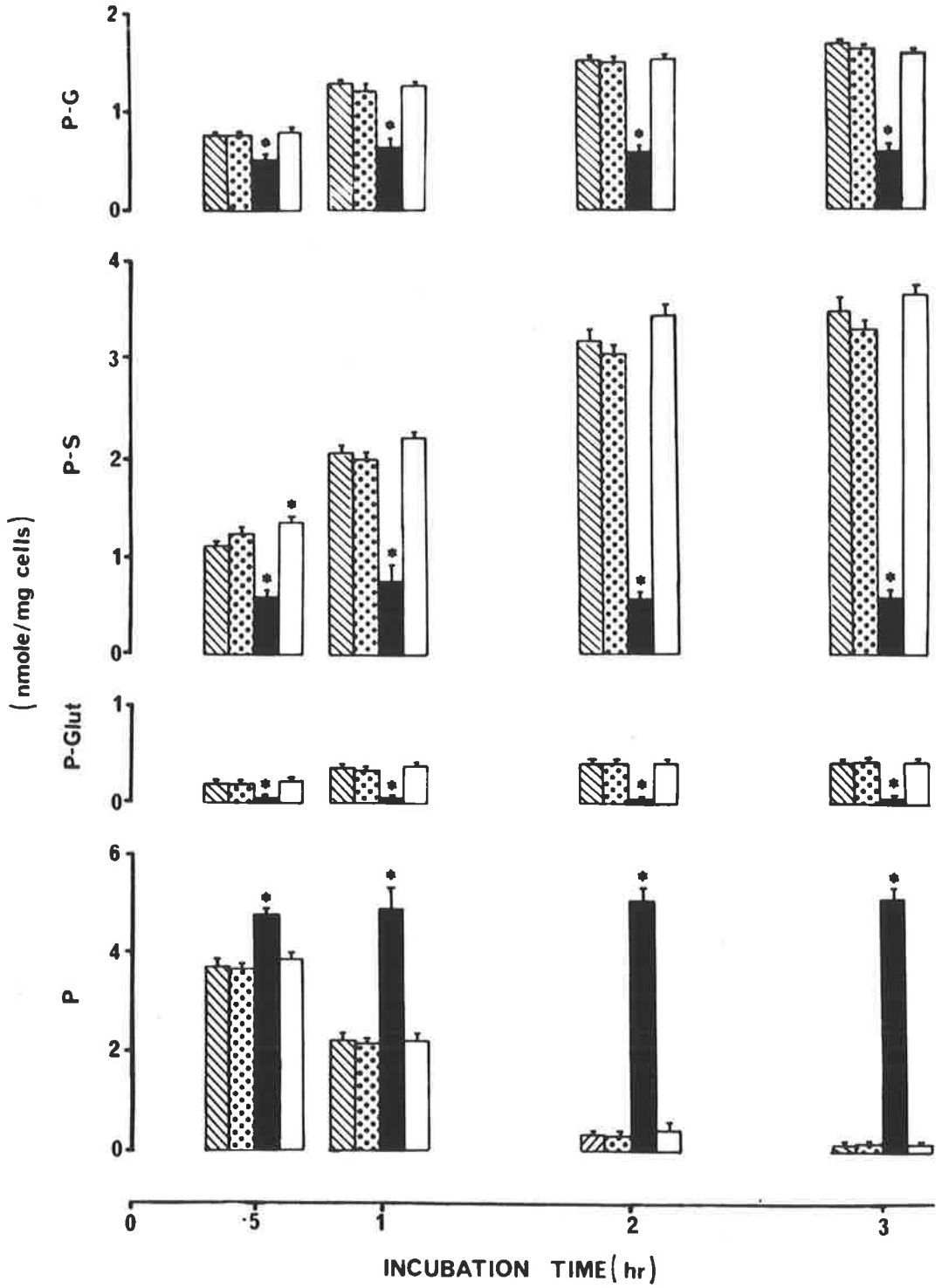

P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
6
·5
INCUBATION TIME (hr)

Fig. 5.4

Time course of metabolite formation in hepatocytes isolated from mice incubated with paracetamol (0.25 mM) and sulphydryl compounds.

saline

N-acetyl cysteine (5 mM)

cysteamine (5 mM)

N-acetyl methionine (5 mM)

P-G paracetamol glucuronide P-S paracetamol sulphate

P paracetamol P-Glut paracetamol glutathione

Values shown are mean ± s.e.m. (n=3).

*significantly different from saline control ($p<0.05$, t-test).

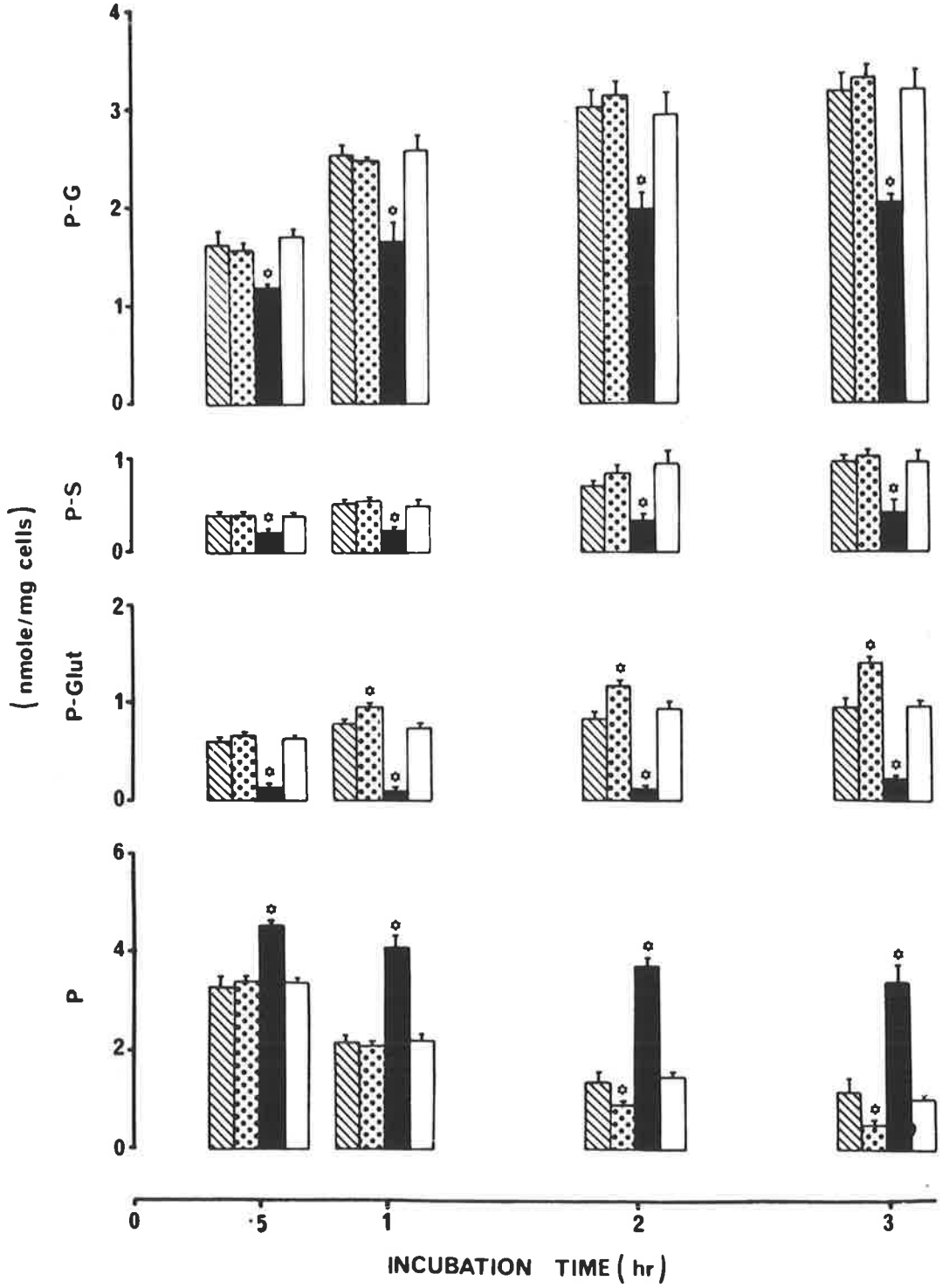

P-G
P-S
P-Glut
P
(nmole/mg cells)
0
1
2
3
4
5
6
·5
INCUBATION TIME (hr)

Fig. 5.5

Irreversible protein binding of ^{14}C-paracetamol in isolated rat hepatocytes in the presence or absence of sulphydryl compounds.

▨ saline; ⊡ N-acetyl cysteine;

■ cysteamine; □ N-acetyl methionine.

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

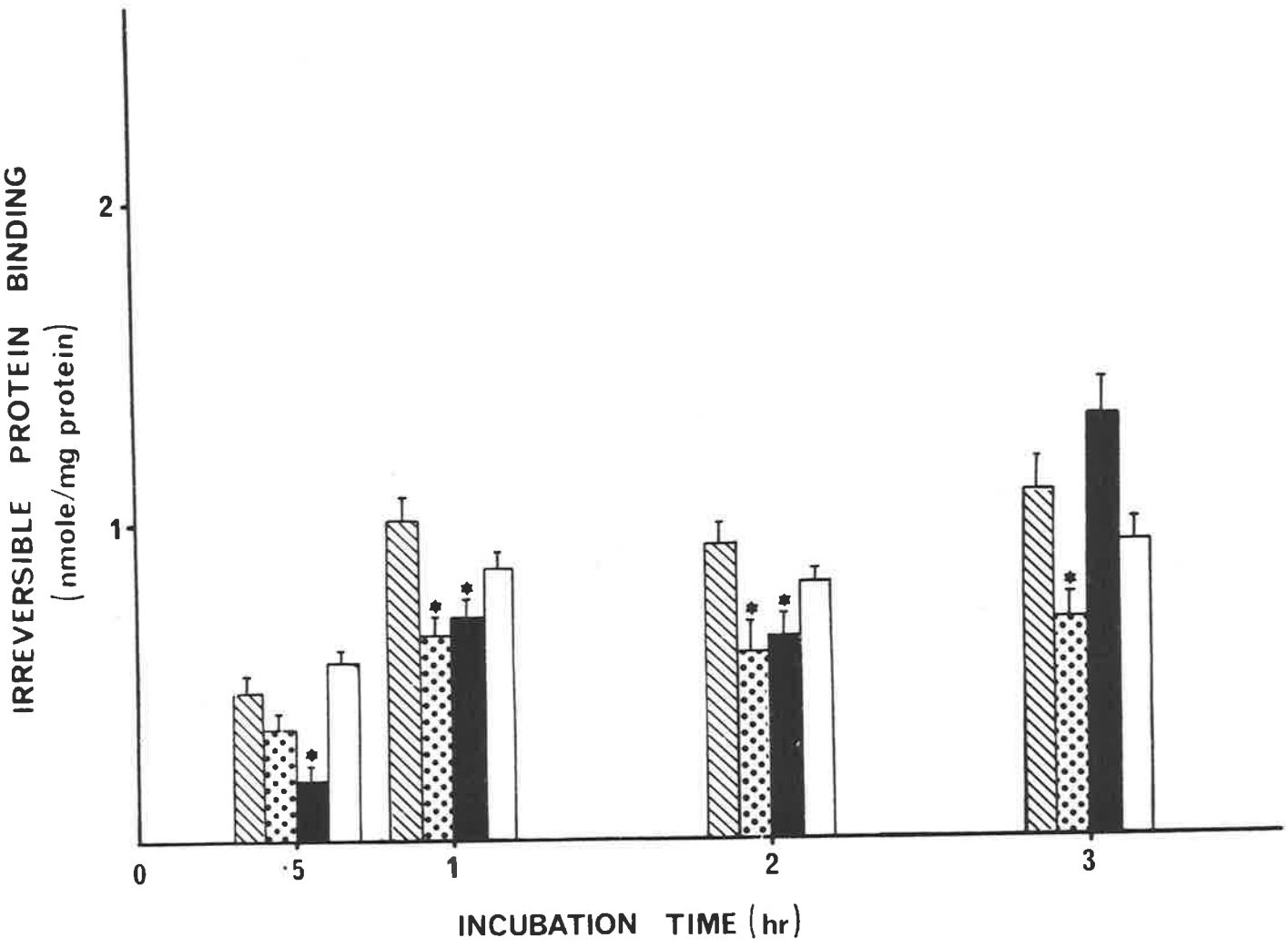

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
·5
1
2
3
INCUBATION TIME (hr)

Fig. 5.6

Irreversible protein binding of ^{14}C-paracetamol in isolated mouse hepatocytes in the presence or absence of sulphydryl compounds.

▨ saline; ▩ N-acetyl cysteine;

■ cysteamine; □ N-acetyl methionine.

Values shown are mean ± s.e.m. (n=3).

✡ significantly different from saline control ($p<0.05$, t-test).

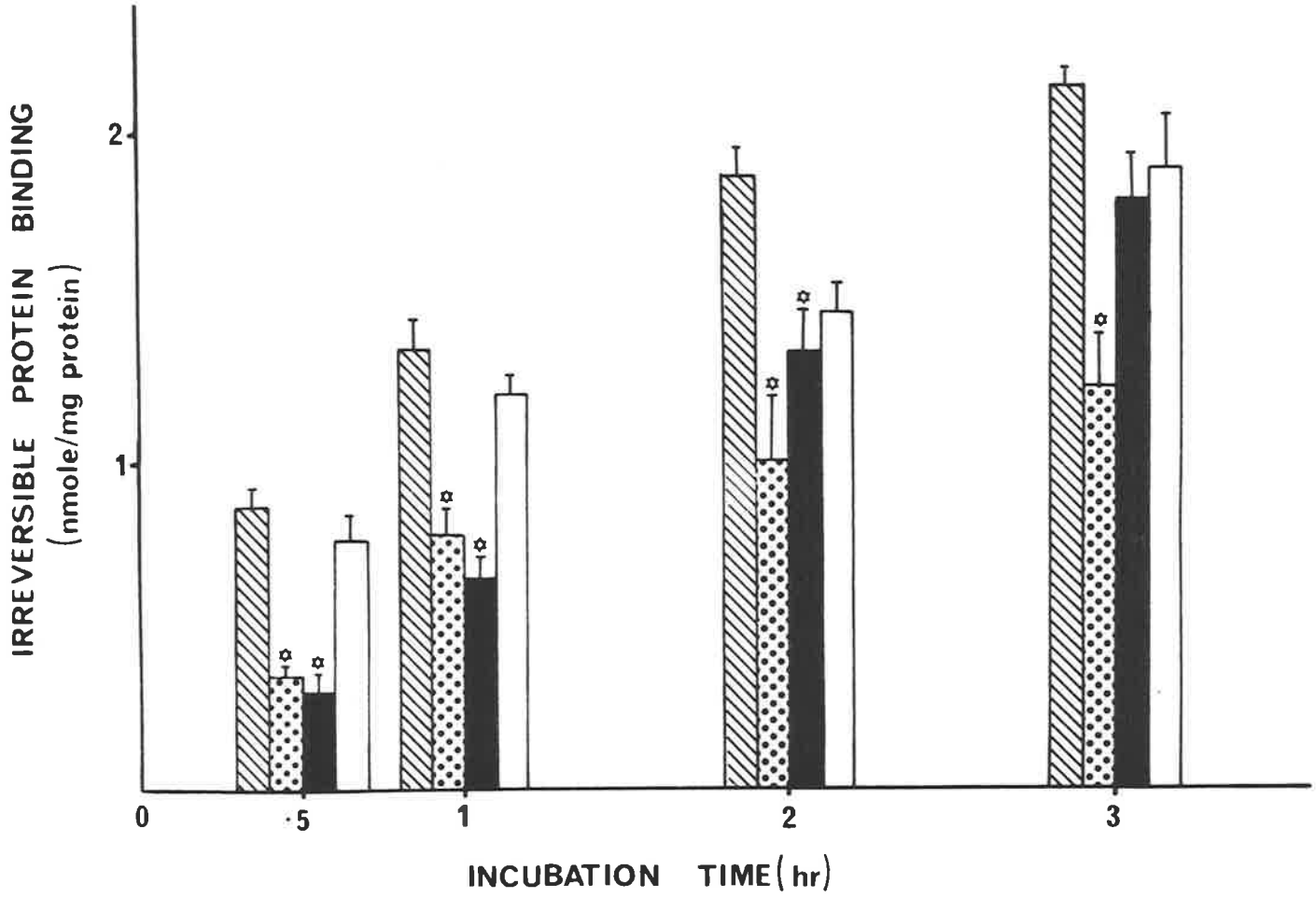

IRREVERSIBLE PROTEIN BINDING
(nmole/mg protein)
2
1
0
·5
1
2
3
INCUBATION TIME (hr)

TABLE 5.1

Estimation of reduced glutathione (GSH) level in isolated rat and mouse hepatocytes.

Animal	Incubation time (hr)	Control (No paracetamol)	GSH (µmole/g cells) Paracetamol			
			Saline	N-acetyl-cysteine	Cysteamine HCl	N-acetyl-methionine
Rats	0	5.35 ± 0.16	4.29 ± 0.14	66.93 ± 2.00*	13.27 ± 0.66*	4.30 ± 0.13
	½	5.20 ± 0.12	4.13 ± 0.23	59.36 ± 1.10*	0.61 ± 0.02*	4.41 ± 0.27
	1	4.92 ± 0.13	4.30 ± 0.33	35.28 ± 2.26*	0.62 ± 0.03*	4.59 ± 0.30
	2	5.13 ± 0.20	4.18 ± 0.15	12.69 ± 0.91*	0.65 ± 0.03*	5.28 ± 0.24
	3	5.25 ± 0.13	4.34 ± 0.13	6.89 ± 0.30*	0.21 ± 0.09*	5.34 ± 0.25
Mice	0	2.37 ± 0.02	2.66 ± 0.23	50.98 ± 3.12*	5.44 ± 1.77*	3.36 ± 0.36
	½	2.34 ± 0.01	2.56 ± 0.11	34.34 ± 3.47*	0.65 ± 0.04*	2.81 ± 0.27
	1	2.45 ± 0.05	2.35 ± 0.19	12.71 ± 1.12*	0.58 ± 0.02*	2.55 ± 0.18
	2	2.35 ± 0.05	2.42 ± 0.12	3.06 ± 0.09*	0.49 ± 0.07*	2.49 ± 0.12
	3	2.69 ± 0.12	2.13 ± 0.04	2.26 ± 0.09	0.34 ± 0.13*	2.29 ± 0.19

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control with paracetamol ($p<0.05$, t-test)

Fig. 5.7

Changes in intracellular K^+ and ALT activity in isolated rat hepatocytes incubated with or without paracetamol and sulphydryl compounds.


control (without paracetamol)


paracetamol + saline


paracetamol + N-acetyl cysteine


paracetamol + cysteamine


paracetamol + N-acetyl methionine

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).

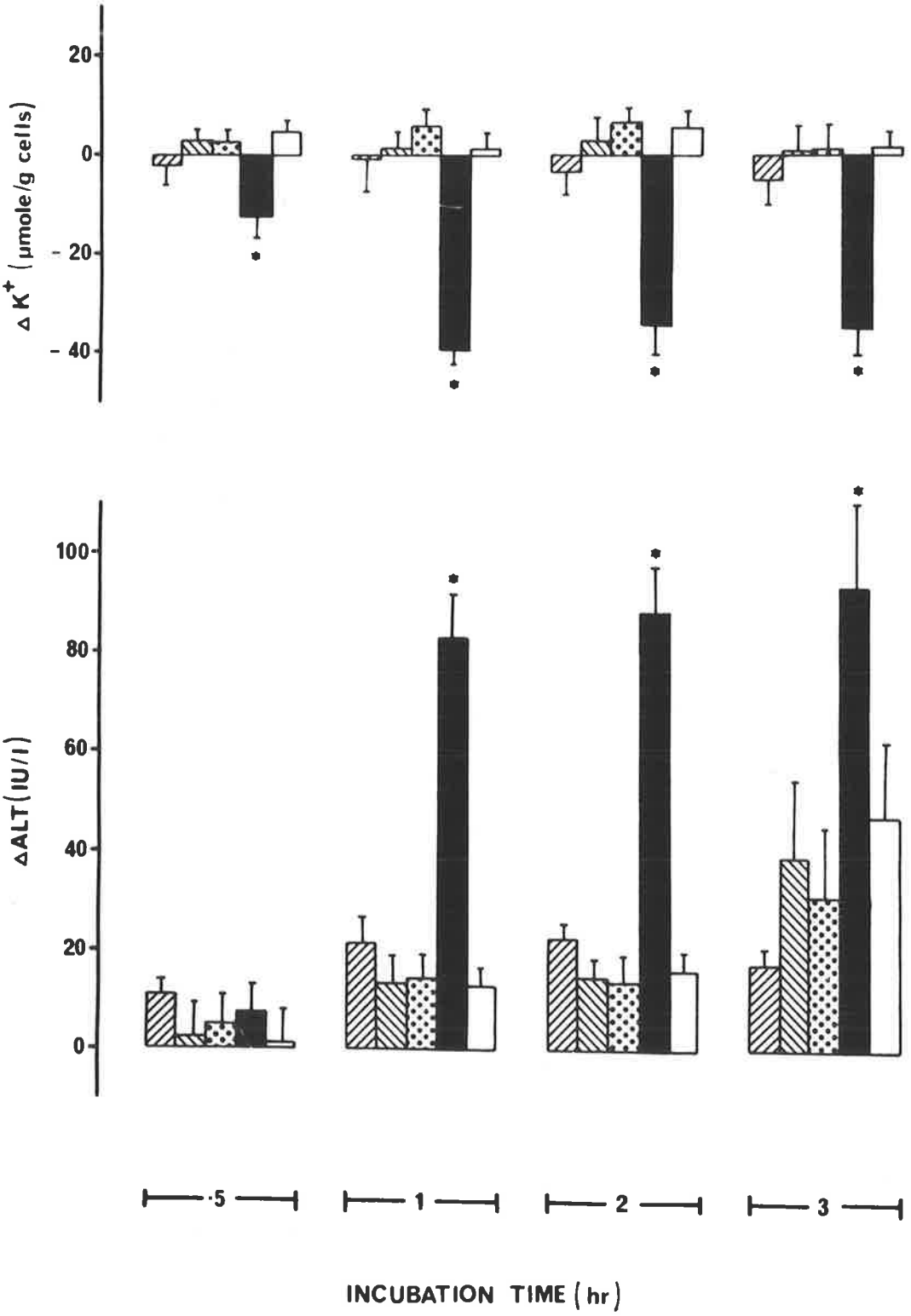

20
0
- 20
- 40
Δ K+ (μmole/g cells)
100
80
60
40
20
0
ΔALT(IU/l)
·5
1
2
3
INCUBATION TIME (hr)

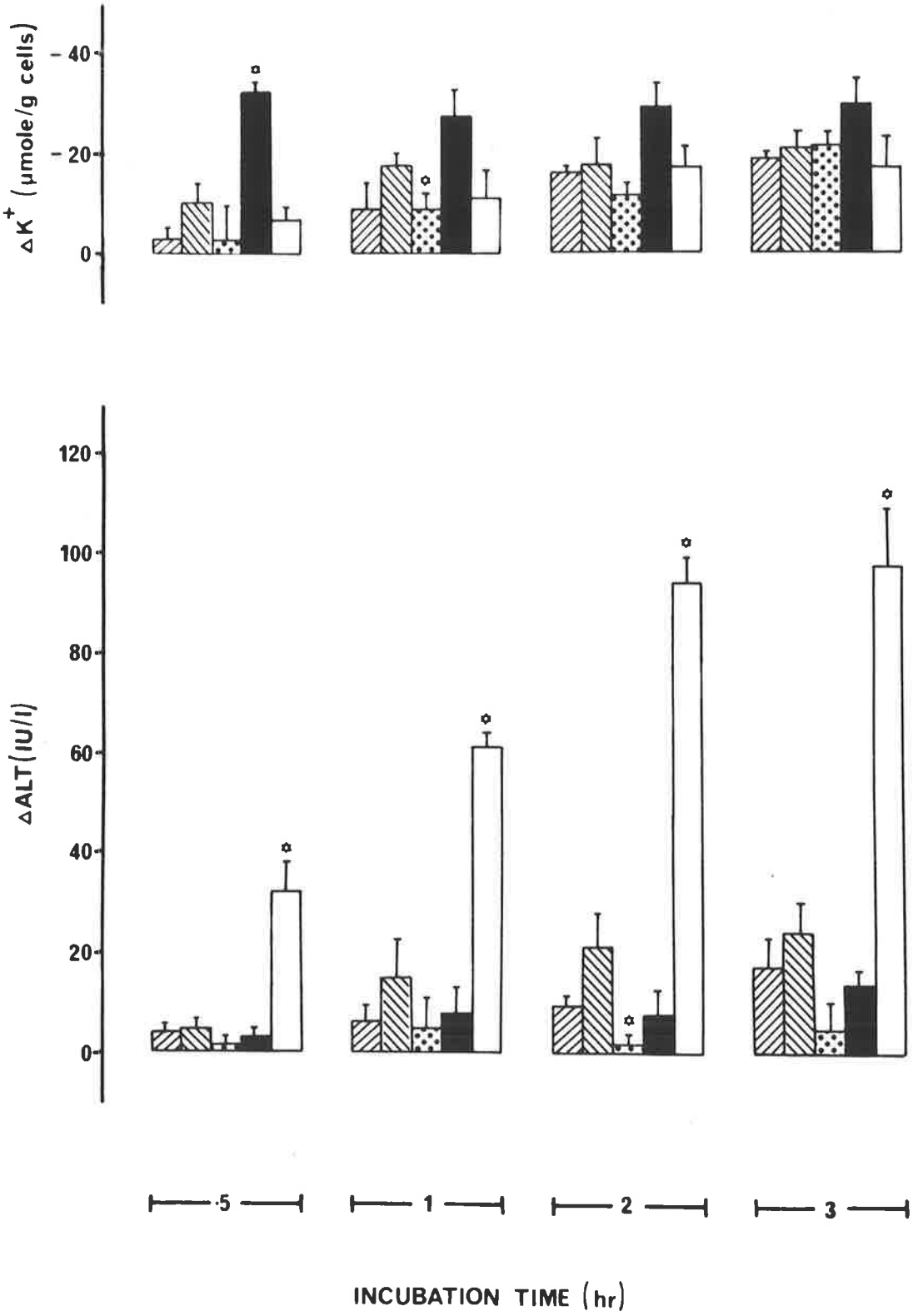
Fig. 5.8

Changes in intracellular K^+ and ALT activity in isolated mouse hepatocytes incubated with or without paracetamol and sulphydryl compounds.

- control (without paracetamol)
- paracetamol + saline
- paracetamol + N-acetyl cysteine
- paracetamol + cysteamine
- paracetamol + N-acetyl methionine

Values shown are mean ± s.e.m. (n=3).

* significantly different from saline control ($p<0.05$, t-test).


ΔK^+ (μmole/g cells)
40
20
0
ΔALT(IU/l)
120
100
80
60
40
20
0
5
1
2
3
INCUBATION TIME (hr)

Discussion

Depletion of reduced glutathione by diethyl maleate increased the amount of irreversible protein binding in rat hepatocytes even with a relatively low dose of paracetamol (0.13 mM). The results accord with those seen in vivo (Mitchell et al., 1973) and suggest that GSH also plays a protective role against binding of reactive paracetamol metabolite(s) in this in vitro system. Furthermore, Moldeus (1978a) has shown that isolated mouse hepatocytes are more sensitive to paracetamol-induced hepatotoxicity after previous depletion of reduced glutathione with diethyl maleate, but that added cysteine, methionine and N-acetylcysteine protected against this.

Although the concentration of paracetamol used was relatively low (nearly ten times lower than the dose reported by Moldeus) and did not, in itself, lead to glutathione depletion or cell damage, there was, nevertheless, evidence of a protective effect by added sulphydryl compounds against irreversible protein binding, although this was not necessarily accompanied by changes in paracetamol conjugation with glutathione. N-acetylcysteine was the only one which increased the formation of glutathione conjugate and decreased irreversible protein binding in mouse hepatocytes. However, in rat hepatocytes, it decreased only the binding with no effect on glutathione conjugation. This result in the rat could be interpreted as an inhibitory effect on oxidative activation. There is no confirmatory evidence for this hypothesis from in vivo experiments; and Moldeus (1978a), after studying the protective effect of this compound against paracetamol toxicity in hepatocytes isolated from diethyl maleate-treated mice,

strongly supported the hypothesis that N-acetylcysteine facilitates the biosynthesis of GSH and stimulates the formation of glutathione conjugate.

In the present study, in both rat and mouse hepatocytes, experiments with N-acetylcysteine invariably showed an unknown peak on HPLC chromatograms. Its retention time was close to that of paracetamol-sulphate and it increased in magnitude during the course of incubation. In an attempt to identify this peak, a different solvent (methanol : 0.1% acetic acid : ethyl acetate, 60 : 940 : 1) was also used. As shown in Figure 5.9, the unknown peak did not co-elute with the mercapturate reference standard, and although its retention time was very close to that of the cysteine conjugate, it could not be positively identified as such. Further evidence against this unknown metabolite being the mercapturate formed in vivo is that the mercapturate has been shown recently to be formed extra-hepatically in rats, primarily in the kidney (Jones et al., 1979). It is possible, then, that the unknown peak may be a paracetamol mercapturate or glutathione conjugate whose configuration differs from that normally formed in vivo. In support of this hypothesis, Buckpitt et al. (1977 and 1979) have shown that a paracetamol mercapturate conjugate may be formed by mouse microsomes in vitro after concurrent incubation with paracetamol and N-acetylcysteine.

N-acetylmethionine has been shown to be almost equi-effective with N-acetylcysteine in its protective effect in vivo against paracetamol-induced hepatotoxicity (Stock, 1979). However, it has no protective efficacy against paracetamol activation in rat or mouse hepatocytes in vitro. This may indicate a different

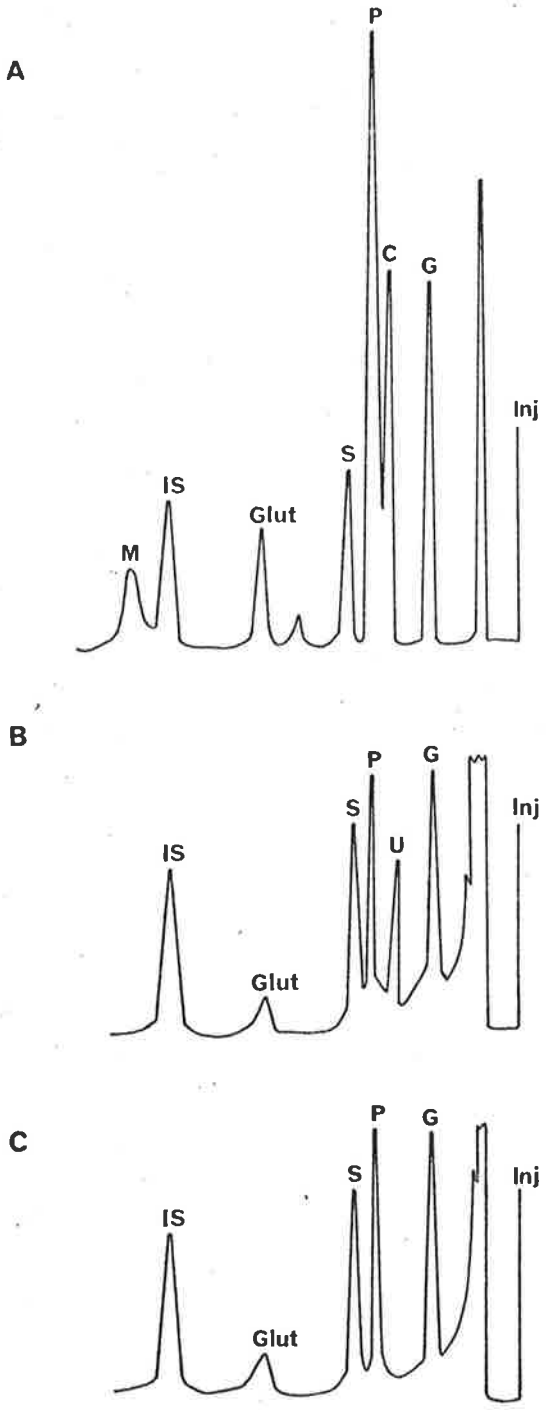


Fig. 5.9

A. Chromatogram of paracetamol and standard metabolites.

B. Chromatogram of an extract from rat hepatocytes incubation (1 hr) with paracetamol and N-acetyl cysteine.

C. Chromatogram of an extract from rat hepatocytes incubation (1 hr) with paracetamol.

Inj injection; G paracetamol glucuronide; S paracetamol sulphate; C paracetamol cysteine; P paracetamol; Glut paracetamol glutathione; IS internal standard; M paracetamol mercapturate; U unknown.

protective mechanism for N-acetylmethionine, or merely that dose-response relationships in vitro are different from those in vivo.

Cysteamine appeared to inhibit not only the activation of paracetamol, but also the detoxifying conjugative pathways. However, since there was also a rapid loss of K^+, GSH, and ALT from the cells, it is more likely that this effect is attributable to a non-specific toxic effect of cysteamine itself.

In conclusion, the activation of paracetamol can be modified by both diethyl maleate and sulphydryl compounds, and this confirms the involvement of reduced glutathione in this process. The mechanism of the protective effects of sulphydryl compounds is still not clear, but direct conjugation and/or facilitation of reduced glutathione synthesis are the most likely explanations.

* * *

CHAPTER 6

PARACETAMOL TOXICITY IN PRIMARY MONOLAYER CULTURES OF ADULT RAT HEPATOCYTES

Introduction

Although freshly isolated hepatocyte suspensions have been found to be quite suitable for studying drug metabolism and toxicity, they tend to manifest severe degenerative changes within several hours after isolation. This disadvantage limits the usefulness of this preparation for some studies, such as protein synthesis, metabolic regulation, growth control, mutagenicity and carcinogenesis (Jeejeebhoy and Phillips, 1976).

Many attempts have been made recently to develop a system for culturing adult liver parenchymal cells. The original reports by Bissell et al. (1973) and Bonney et al. (1974) described two slightly different procedures for the preparation of primary monolayer cultures in which liver parenchymal cells capable of expressing differentiated function have been maintained for 4-6 days. The former workers used albumin synthesis, gluconeogenesis and microsomal enzyme activity as criteria of differentiated liver cell function, while the latter report described steroid induction of tyrosine aminotransferase. Since the life span of even these preparations was inadequate to complete certain experiments, attempts have been made to increase the functional longevity of differentiated liver parenchymal cells in culture by modifying the media (Laishes and Williams, 1976; Williams et al., 1977, 1978). Most modifications met with rather limited success. The most successful approach was introduced by Michalopoulos and Pitot (1975) whereby hepatocytes were maintained on floating collagen membranes. The culture of hepatocytes by this technique retains epithelial morphology and viability for a period of more than 20 days. Savage and Bonney (1978) simplified this method

by replacing the collagen membrane with millipore filters and maintained the cells as free-floating monolayer cultures.

The culture method can markedly influence cell morphology. Cells cultured by the method of Bonney et al. (1974) become well attached and flattened on the plastic plates, and retain their epitheloid shape even when not in contact with other cells. Cells cultured by the Savage and Bonney (1978) method assume a rounded, ball-like appearance, and form large clusters in monolayer on millipore filters.

The levels of cytochrome P-450 and drug metabolising enzymes in non-replicating adult hepatocytes maintained as monolayer on regular petri dishes or on floating collagen membranes have been examined by several investigators. The level of cytochrome P-450 drops within 24 hours after isolation and plating of hepatocytes (Michalopoulos et al., 1976; Guzelian et al., 1979; Guzelian and Barwick, 1979). However, the microsomal monooxygenase activity in primary hepatocyte culture can still be induced by both phenobarbitone and polycyclic aromatic hydrocarbons (e.g., benzanthracene, 3-methylcholanthrene) (Michalopoulos et al., 1976, 1976a; Fry et al., 1979). The kinetics of these inductions are very similar to the kinetics of induction seen in the liver in vivo. Rapid induction is effected by polycyclic aromatic hydrocarbons within 1-2 days after addition to the culture, whereas phenobarbitone induction is slower and continues for 3-5 days after the addition of the inducer (Michalopoulos et al., 1976, 1976a; Stenberg and Gustafsson, 1978; Fry et al., 1979). Although the cytochrome P-450 activity of primary hepatocyte cultures is usually evanescent, it can be maintained at the levels close to

those found in vivo by using a defined medium containing hormones (Michalopoulos, 1976; Decad et al., 1977; Guzelian et al., 1979) or a source of vitamin C (Bissell and Guzelian, 1979).

The fragility of the mixed-function oxygenase system contrasts sharply with the robust behaviour of other cell metabolic functions. For example, albumin synthesis and secretion, gluconeogenesis, etc., are easily maintained in cell cultures for several days at levels comparable to those found in vivo (Jeejeebhoy and Phillips, 1976).

Recent applications for primary monolayer cultures of adult hepatocytes (mainly from rats) have included studies on protein degradation (Hopgood et al., 1980), uptake of bile salt (taurocholate) (Schwarz and Barth, 1979), hepatotoxicity of various drugs (Anuforo et al., 1978; Tolman et al., 1978; Acosta et al., 1980) and carcinogenesis (Michalopoulos et al., 1976; Lorentzen et al., 1979; Lowing et al., 1979).

The duration of the experiment may be a critical factor when studying drug-induced toxicity. The development of toxicity in vivo commonly occurs over a time span of 24-48 hours even for potent hepatotoxins such as carbon-tetrachloride and paracetamol. It was evident from the earlier chapters that toxicity did not develop within the 3 hours incubation with freshly isolated hepatocytes even though metabolic activation and covalent binding to cell macromolecules were demonstrated. Studies with primary hepatocyte cultures exposed to paracetamol were therefore undertaken in an effort to extend the period in which toxicity could develop. Since induction and inhibition of paracetamol metabolism was shown in the earlier chapters (3 and 4) to modify irreversible

protein binding, the experiments were also designed to determine whether such short-term changes would be reflected by changes in the development of toxic responses over a longer time span.

Methods

The preparation of isolated rat hepatocytes and culturing procedure have been described in the General Methods (sections a and h).

Triplicate cell cultures were prepared from each of two separate experiments using untreated rats, phenobarbital-induced or benz(α)pyrene-induced rats.

Aliquots of 5 ml suspension (0.5-1 x 10^6 cells/ml) were incubated with paracetamol and various metabolic inhibitors which were added to the cell suspension prior to dispensation of the cells into Falcon tissue culture flasks. Controls were supplemented with appropriate volumes of saline and/or metabolic inhibitors. The final concentrations of paracetamol and metabolic inhibitors were: paracetamol (250 μM), metyrapone (120 μM), salicylamide (200 μM) and α-naphthoflavone (66 μM).

At the end of 24 hours incubation (37°C) in the presence of added drugs, ALT activity was determined in the decanted supernatant. Cultured cells were harvested from the flask, using a pasteur pipette, after addition of 2 ml of 0.25% Triton X-100 and vigorous mixing with the help of a blunt spatula. Protein was precipitated by adding 1 ml 3% PCA, and intracellular K^+ was determined by flame photometry using a 1:40 dilution of the supernatant after centrifugation.

Results

The morphology of control isolated adult rat hepatocytes before and after culturing in Eagle's MEM containing 10% foetal calf serum is shown in Figures 6.1 and 6.2, respectively. After 24 hours in culture, nearly all of the cells had become attached to the bottom of the flasks or the tubes. Only 60% of them excluded trypan blue. Although the cells were attached, they retained a rounded appearance, and there was an abundance of normal-appearing intracellular organelles as shown in Figure 6.2.

The analysis of cell viability by K^+ retention and ALT release showed some interesting trends. Values before and after 24 hours culture (Table 6.1) showed a reduction in cell viability in both control and drug-treated hepatocytes. Paracetamol imposed no additional effect in cells from untreated rats; however, in cells from phenobarbital- and benz(α)pyrene-induced rats paracetamol caused an excess loss of viability. Furthermore, combination of paracetamol with metabolic inhibitors revealed effects consistent with enhanced toxicity (Figs 6.3-6.5).

In untreated cells, all three metabolic inhibitors (metyrapone, α-naphthoflavone and salicylamide) appeared to potentiate paracetamol-induced K^+ loss, although the effects on ALT release were complicated by an effect in the absence of paracetamol as well, as shown by salicylamide and α-naphthoflavone (Fig. 6.3).

In phenobarbital- and benz(α)pyrene-induced cells, where a toxic effect of paracetamol by itself was revealed, the additional effect of the inhibitors was variable. For example, metyrapone enhanced paracetamol-induced toxicity in phenobarbital-induced hepatocytes, but protected in benz(α)pyrene-induced hepatocytes.

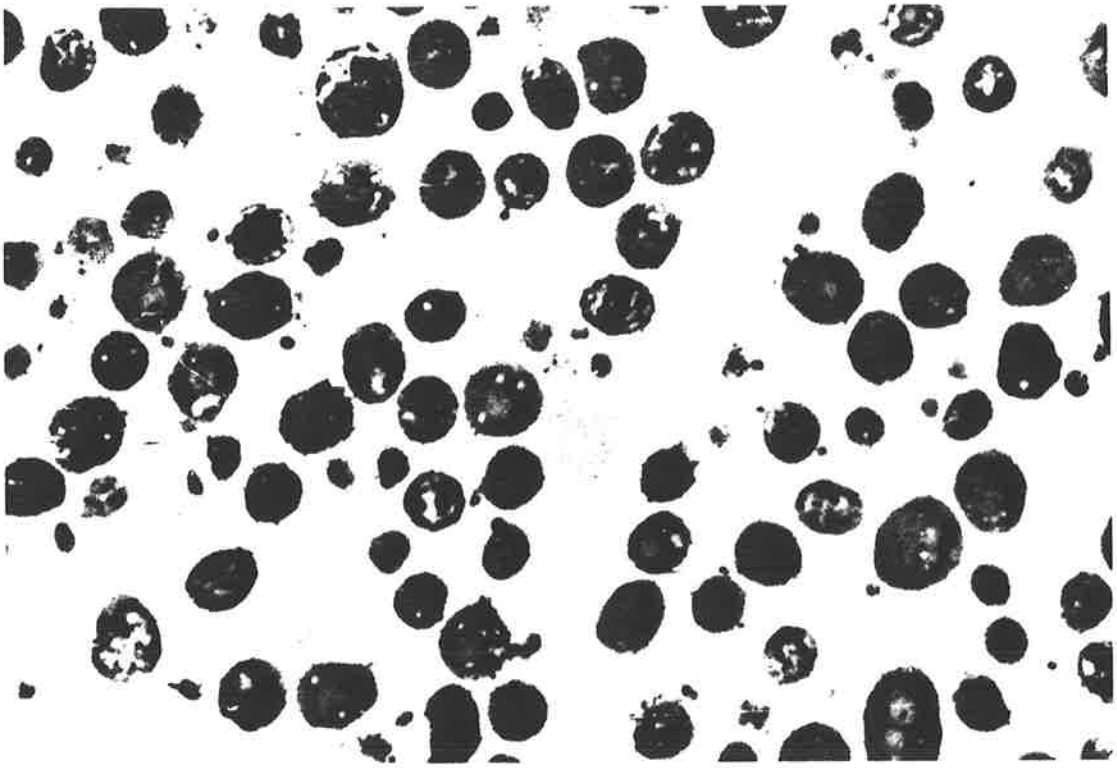


Fig. 6.1

Suspension of freshly isolated rat parenchymal cells in Eagle's MEM containing 10% of foetal calf serum and antibiotics (X 100).

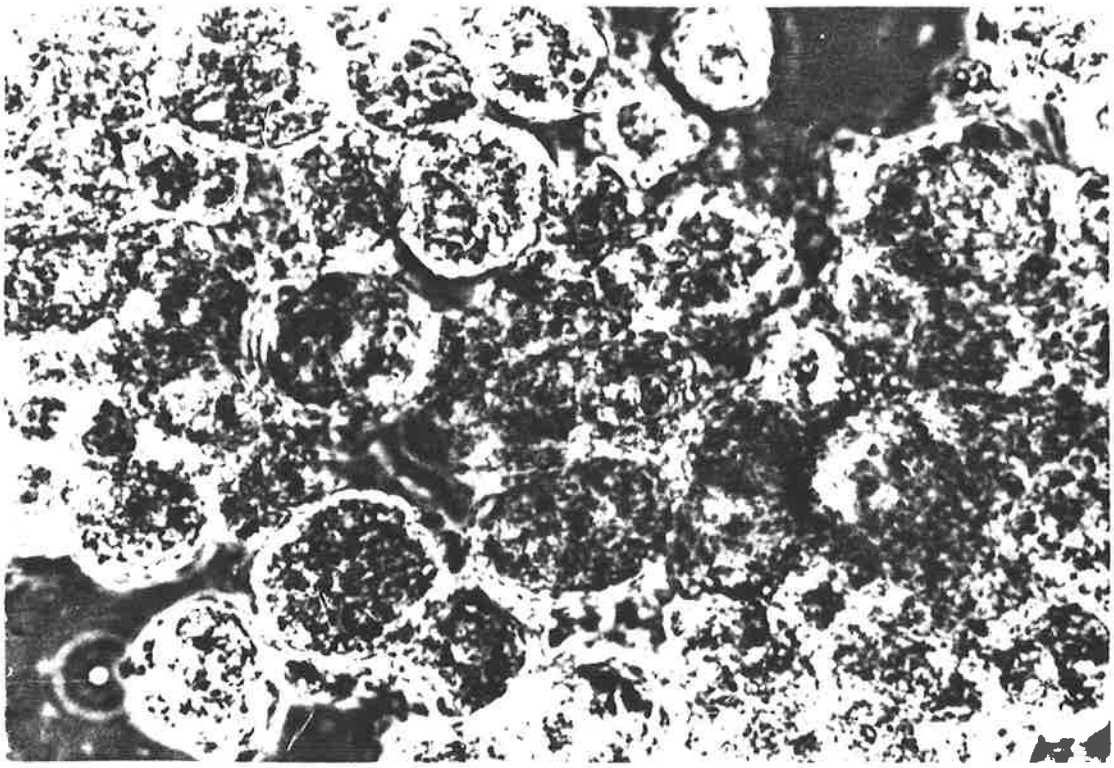


Fig. 6.2

Cells after 24 hours in culture in Eagle's MEM containing 10% foetal calf serum and antibiotics (X 500).

TABLE 6.1

Normal values of intracellular K^+ and ALT activity in adult rat hepatocyte primary monolayer cultures.

Treatment	Incubation time (hr)	Intracellular K^+ (µmole/g cells) Saline	Paracetamol	ALT activity (IU/l) Saline	Paracetamol
Control	0	103.34 ± 4.59		40.50 ± 2.95	
	24	68.30 ± 1.09	71.30 ± 3.27	72.00 ± 3.43	75.93 ± 3.05
Phenobarbital	0	103.67 ± 1.36		37.80 ± 1.32	
pretreated	24	60.38 ± 1.59	56.74 ± 0.67*	86.33 ± 6.80	90.00 ± 2.88
Benz(α)pyrene	0	105.67 ± 3.93		36.17 ± 2.20	
pretreated	24	64.64 ± 0.95	55.07 ± 0.66*	79.00 ± 2.94	108.00 ± 4.23*

Values shown are mean ± s.e.m. (n=2, triplicate samples for each cell culture).

* significantly different from control saline ($p<0.05$, t-test)

Fig. 6.3

Changes in intracellular K^+ and ALT activity in control isolated rat hepatocytes after 24 hours in culture in the absence or presence of paracetamol and/or metabolic inhibitors.

□ saline control (without paracetamol) ■ paracetamol

S saline M metyrapone

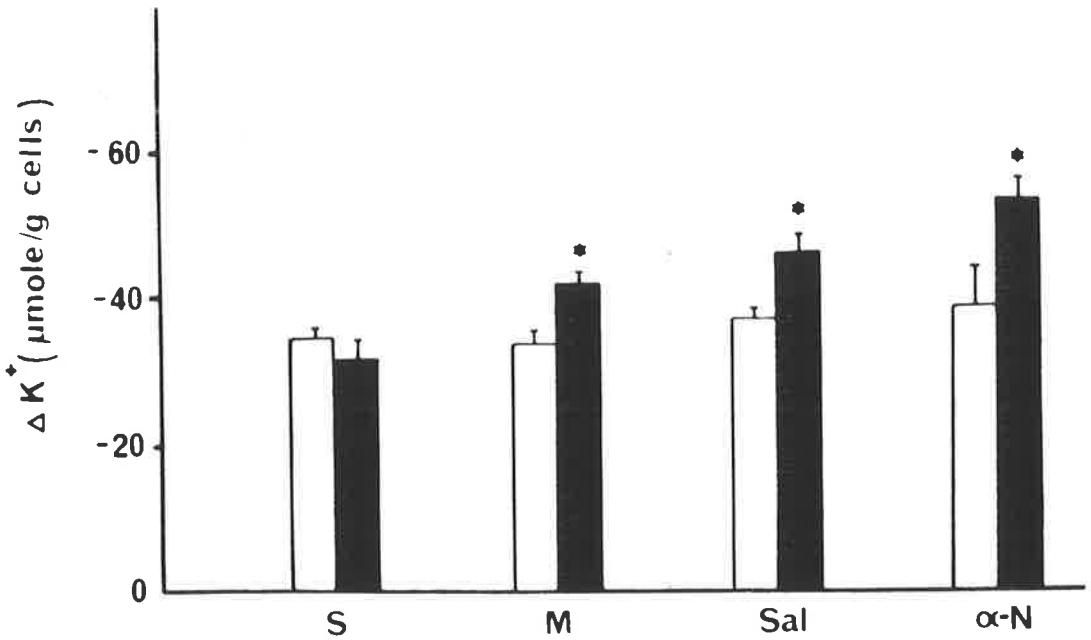
Sal salicylamide α-N α-naphthoflavone

Values shown are mean ± s.e.m.

(n=2 experiments, 3 replicates per experiment).

✡ significantly different from control containing no paracetamol or other drugs (S - open bar) ($p<0.05$, t-test).

✱ significantly different from the relevant paracetamol-free control (open bar).


Δ K+ (μmole/g cells)
-60
-40
-20
0
S
M
Sal
α-N

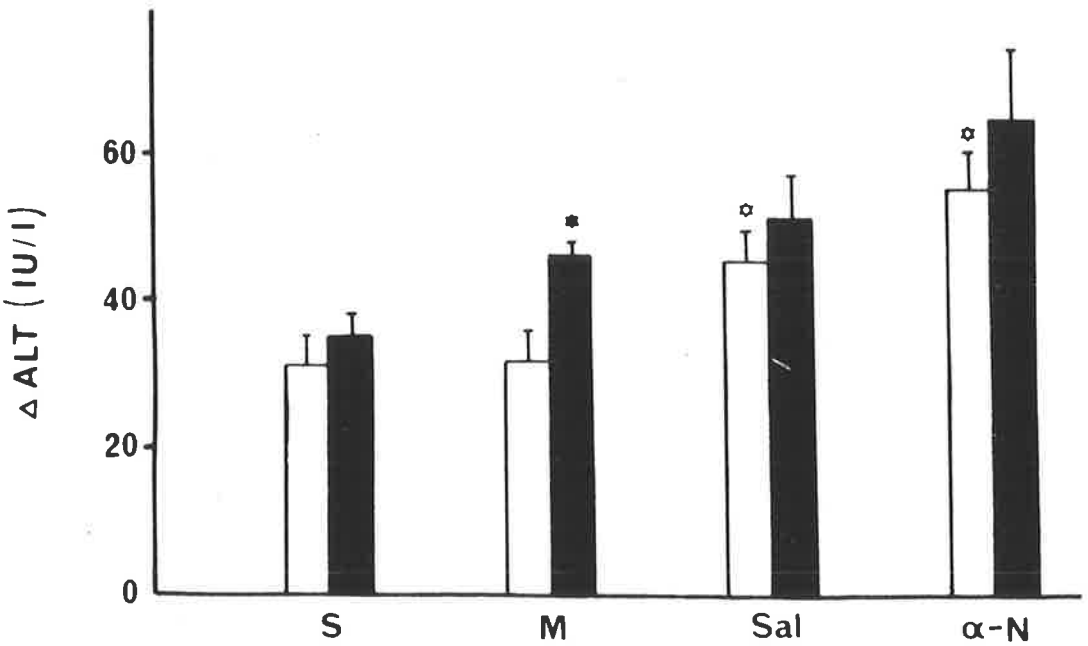

Δ ALT (IU/l)
60
40
20
0
S
M
Sal
α-N

Fig. 6.4

Changes in intracellular K^+ and ALT activity in phenobarbital-treated rat hepatocytes after 24 hours in culture in the absence or presence of paracetamol and/or metabolic inhibitors.

□ saline control (without paracetamol) ■ paracetamol

S saline M metyrapone

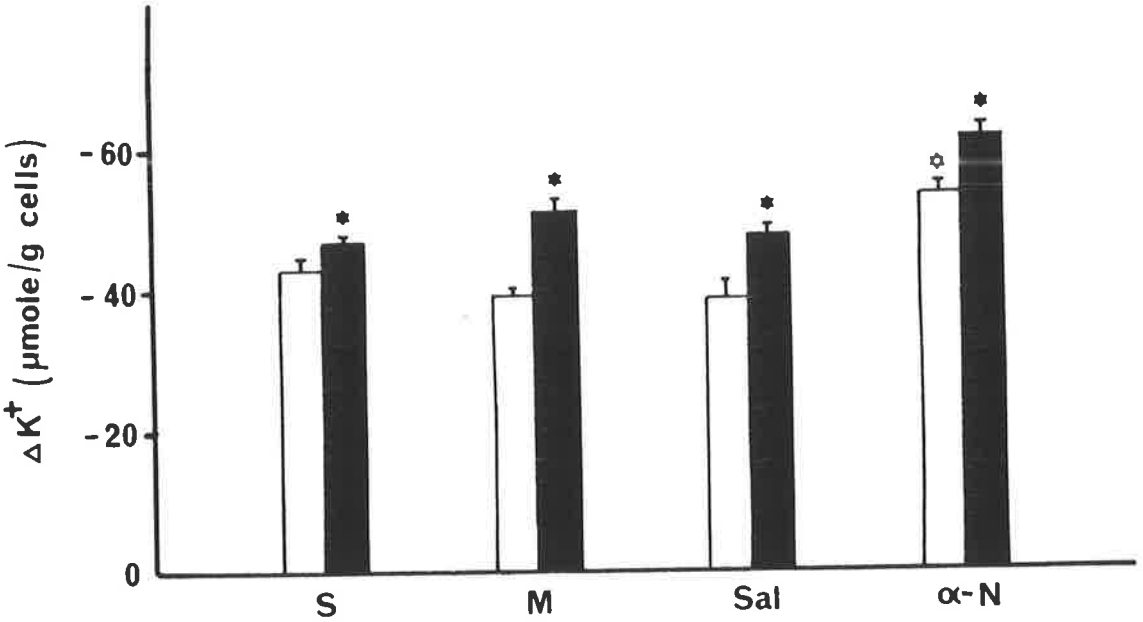
Sal salicylamide α-N α-naphthoflavone

Values shown are mean ± s.e.m.

(n=2 experiments, 3 replicates per experiment).

✡ significantly different from control containing no paracetamol or other drugs (S - open bar) ($p<0.05$, t-test).

✱ significantly different from the relevant paracetamol-free control (open bar).


ΔK+ (μmole/g cells)
-60
-40
-20
0
S
M
Sal
α-N

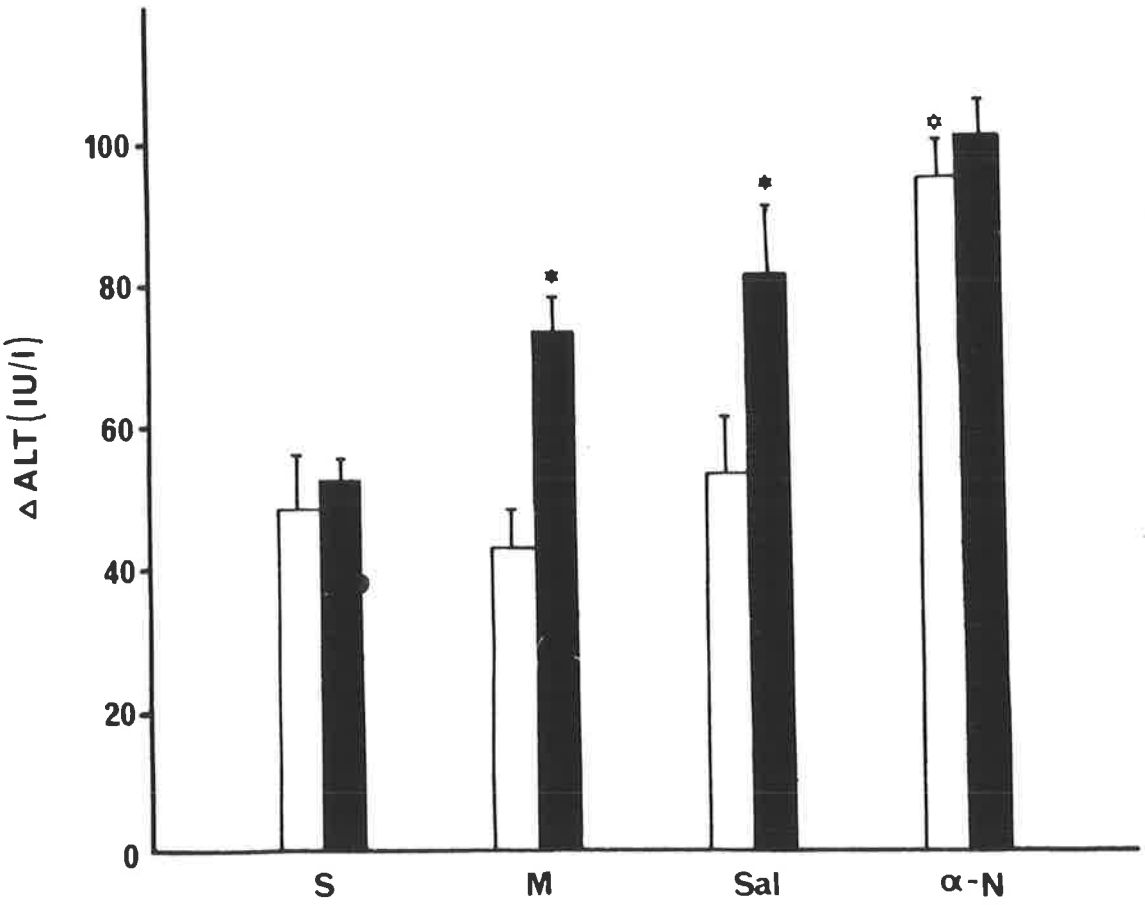

ΔALT(IU/l)
100
80
60
40
20
0
S
M
Sal
α-N

Fig. 6.5

Changes in intracellular K^+ and ALT activity in benz(α)pyrene-treated rat hepatocytes after 24 hours in culture in the absence or presence of paracetamol and/or metabolic inhibitors.

□ saline control (without paracetamol) ■ paracetamol

S saline M metyrapone

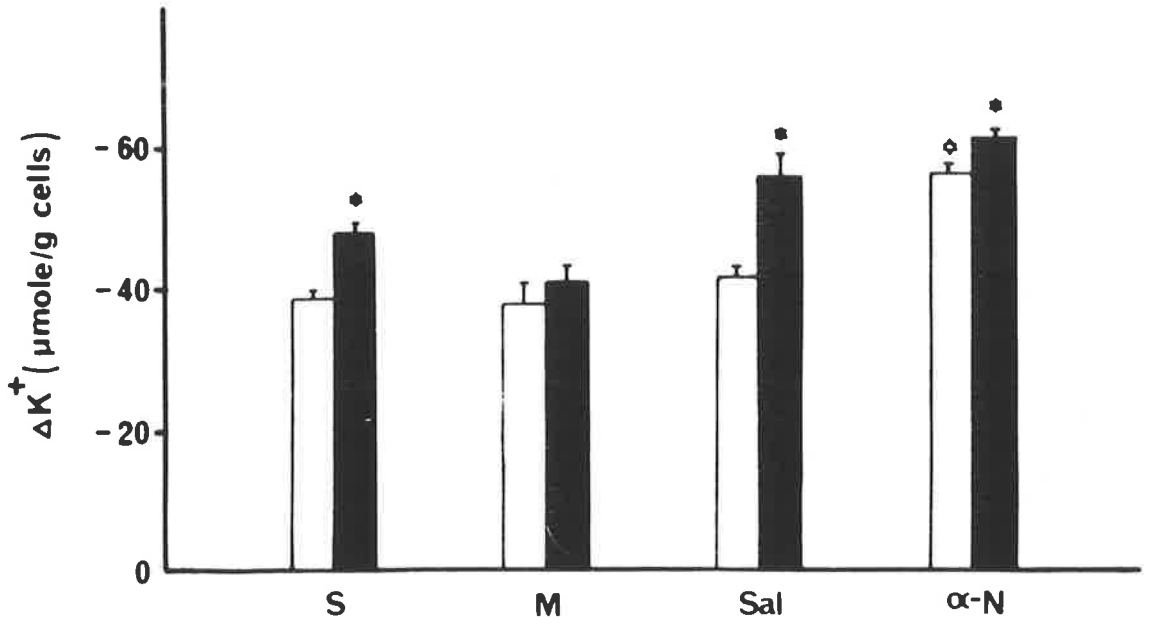
Sal salicylamide α-N α-naphthoflavone

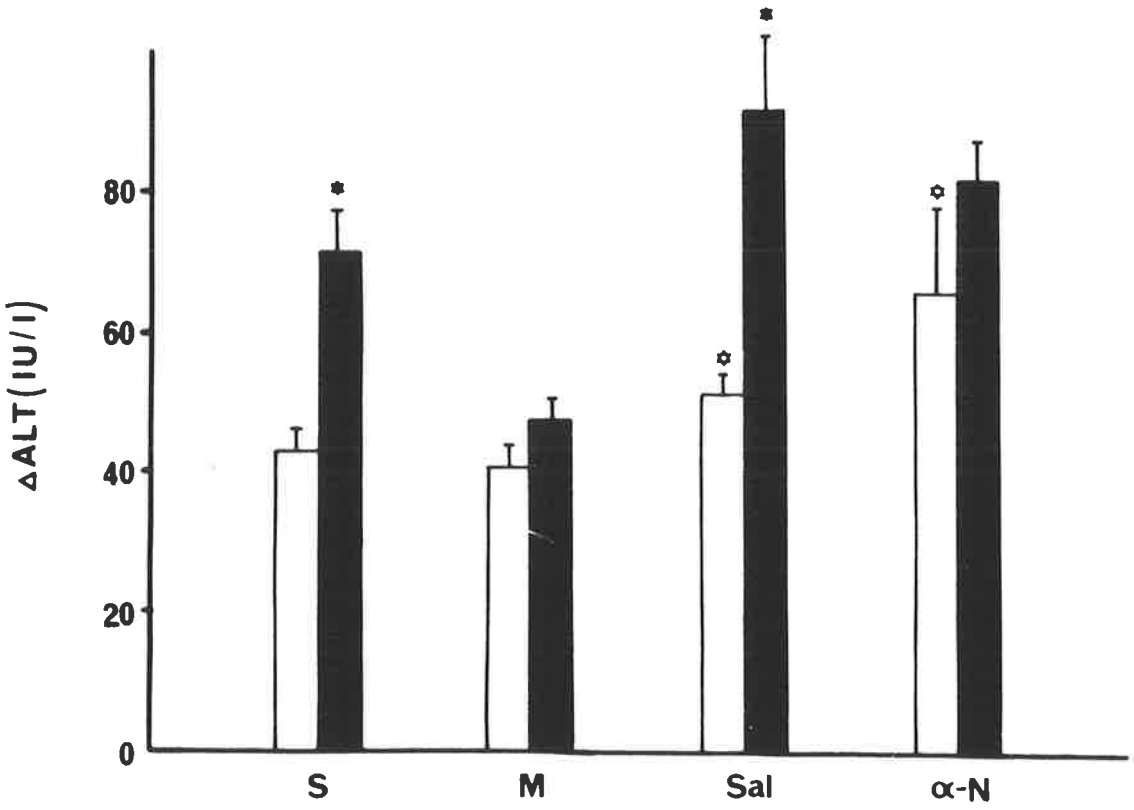
Values shown are mean ± s.e.m.

(n=2 experiments, 3 replicates per experiment).

✡ significantly different from control containing no paracetamol or other drugs (S - open bar) ($p<0.05$, t-test).

* significantly different from the relevant paracetamol-free control (open bar).


ΔK+ (μmole/g cells)
60
40
20
0
S
M
Sal
α-N


ΔALT(IU/l)
80
60
40
20
0
S
M
Sal
α-N

It was itself non-toxic. Salicylamide caused only an enhancement of paracetamol-induced toxicity with minimal toxic effects by itself. The effects of α-naphthoflavone on paracetamol-induced toxicity were minimal and could be attributed mainly to its own toxicity in this system.

Discussion

The concept that drug-induced hepatotoxicity may be initiated by reactive metabolites which bind covalently to cell macromolecules is well established. In an earlier chapter it was shown that the amount of irreversible protein binding in freshly isolated rat hepatocytes is capable of modification by many factors. For example, binding was increased by both phenobarbital and more specifically by benz(α)pyrene induction. Metyrapone and α-naphthoflavone decreased the binding in benz(α)pyrene-treated rat hepatocytes, but only α-naphthoflavone increased it in hepatocytes from phenobarbital-treated rats.

Some correlations between irreversible protein binding in freshly isolated hepatocytes and toxicity in hepatocyte cultures were noted; for example, the effects of microsomal enzyme inducers were consistent. Benz(α)pyrene induced more binding and caused more toxicity than phenobarbital (Table 6.1). In uninduced cells, no toxicity was seen with this low dose of paracetamol. The effects of metabolic inhibitors were not so consistent; for example, salicylamide, which had no effect on binding, enhanced toxicity. Metyrapone, which inhibited binding in induced, but not control, hepatocytes, caused both inhibition and enhancement of toxicity. The effects of α-naphthoflavone did not

correlate with its relatively strong potentiation of binding in phenobarbital-induced hepatocytes or its inhibition of binding in hepatocytes from benz(α)pyrene-induced rats.

In conclusion, these preliminary experiments suggest that, under certain circumstances, the extent of metabolic activation during short-term incubation in isolated hepatocytes indicates the extent of toxicity to be expected from extended exposure. However, several mitigating factors must also be taken into account, as follow. The rat is a species relatively resistant to paracetamol-induced hepatotoxicity, and the dose used in these experiments was relatively low. The interactive drugs themselves exhibited different degrees of toxicity, and were also shown to inhibit the conjugative pathways primarily responsible for paracetamol clearance. There is a growing body of evidence from in vivo studies that the relationship between covalent binding and hepatotoxicity is not a simple one; for example, the paracetamol analogue 3-hydroxyacetanilide is not hepatotoxic, yet still binds appreciably to protein after metabolic activation (Nelson, 1980).

Therefore, these data point to further studies which may help to obtain further information into this and several other aspects of hepatotoxic response.

* * *

GENERAL DISCUSSION

The present study has shown that freshly isolated hepatocytes metabolise paracetamol by pathways similar to those by which this drug is cleared metabolically in vivo. Saturable pathways involving sulphate and glucuronide conjugation account for most of the metabolic clearance, but there was evidence that a minor pathway was present in which the production of a reactive intermediary metabolite was inferred by the products of its secondary reactions; viz, its conjugation with reduced glutathione, and the presence of irreversibly bound radiolabel, after incubation with ^{14}C-paracetamol.

Mouse hepatocytes catalysed the activation of paracetamol to a greater extent than did rat hepatocytes, and this accords with species differences observed in vivo. Evidence for the involvement of microsomal cytochrome P-450 in this activation was obtained using classical microsomal inducers and inhibitors.

The overall effects of these modifiers are summarised in Figures 1 and 2. Although the interpretation of these changes has been discussed in more detail in previous chapters, it is evident that few of the compounds have acted selectively. It is possible to generalise the findings to the extent that, the microsomal enzyme inducers, phenobarbital and, particularly, benz-(α)pyrene, irrespective of their effects on other pathways, enhanced the irreversible binding of reactive metabolite(s) to protein. Furthermore, the microsomal enzyme inhibitors, α-naphthoflavone and metyrapone, reduced the formation of both the irreversibly protein bound and glutathione conjugated metabolites, although the effects were not necessarily uniform for each pathway, or in each species.

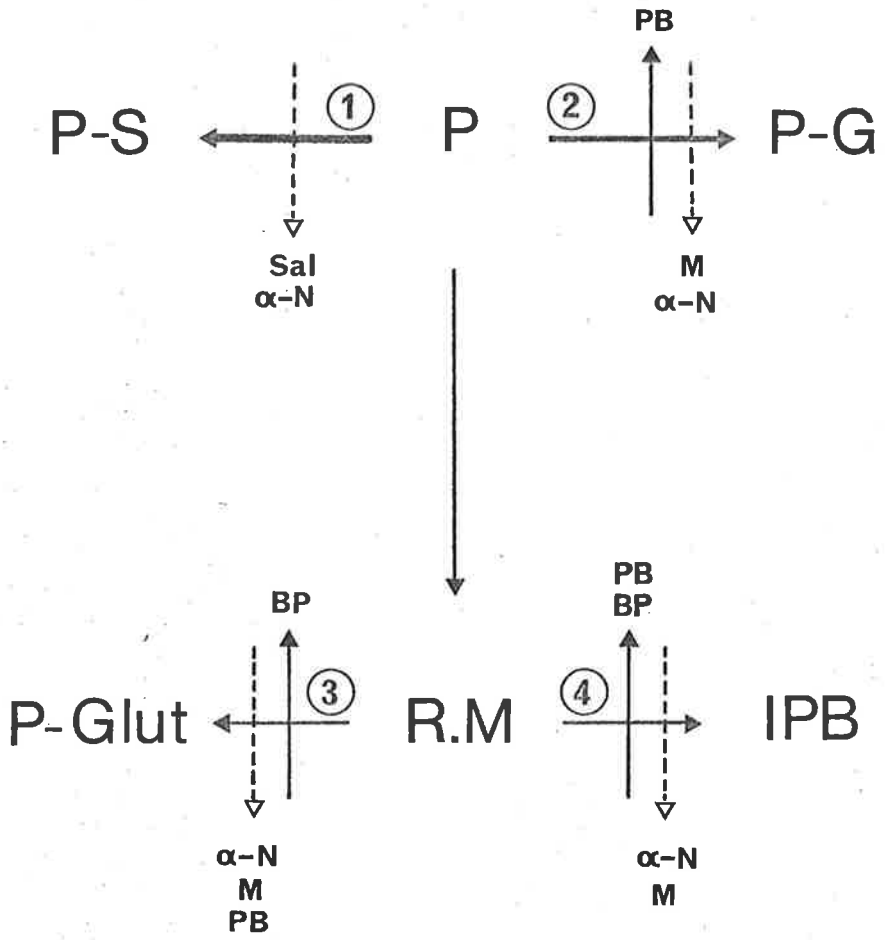


Fig. 1

Schematic representation of paracetamol (250 μM) metabolism in isolated rat hepatocytes. Effects of metabolic inducers (BP and PB) and inhibitors (α-N, M, and Sal).

P	paracetamol	P-G	paracetamol glucuronide
P-S	paracetamol sulphate	P-Glut	paracetamol glutathione
R.M	reactive metabolite	IPB	irreversible protein binding

① ② ③ ④ Sequence of reactions (according to the amount of metabolite formed)

↑ induction ↓ inhibition

PB	phenobarbital	BP	benz(α)pyrene
α-N	α-naphthoflavone (66 μM)	M	metyrapone (120 μM)
Sal	salicylamide (200 μM)		

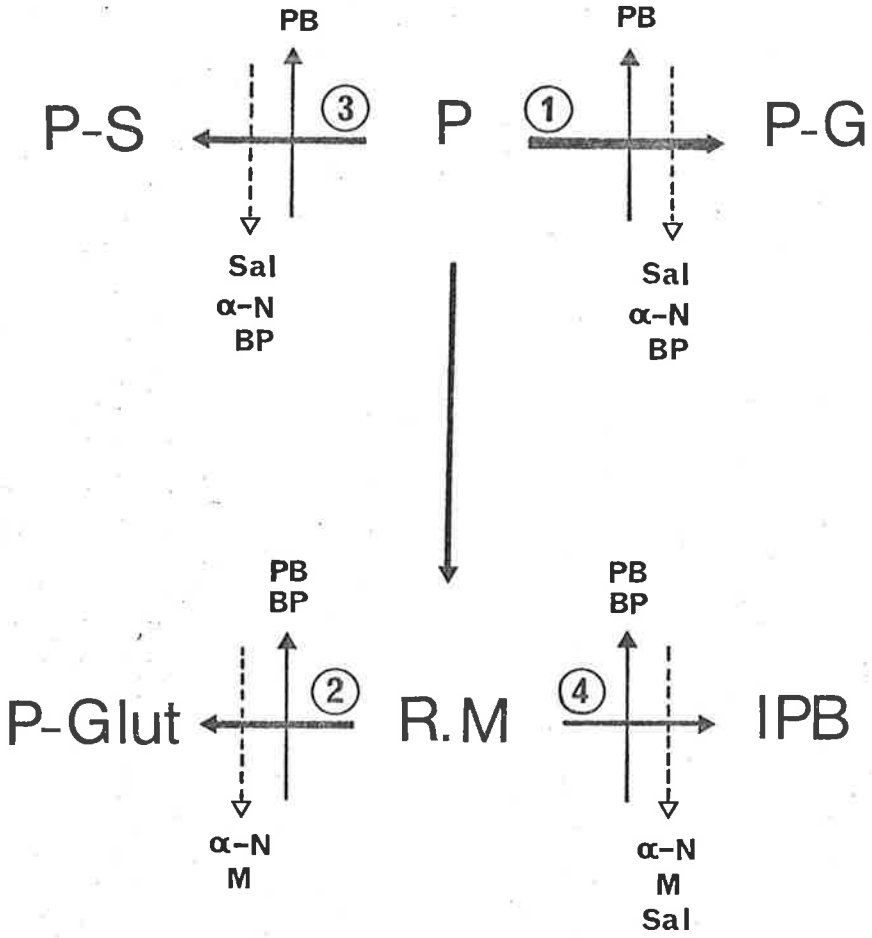


Fig. 2

Schematic representation of paracetamol (250 μM) metabolism in isolated mouse hepatocytes. Effects of metabolic inducers (BP and PB) and inhibitors (α-N, M, and Sal).

P	paracetamol	P-G	paracetamol glucuronide
P-S	paracetamol sulphate	P-Glut	paracetamol glutathione
R.M	reactive metabolite	IPB	irreversible protein binding

① ② ③ ④ Sequence of reactions (according to the amount of metabolite formed)

↑ induction ↓ inhibition

PB	phenobarbital	BP	benz(α)pyrene
α-N	α-naphthoflavone (66 μM)	M	metyrapone (120 μM)
Sal	salicylamide (200 μM)		

These findings are also generally consistent with the effects of the various drug treatments on paracetamol-induced hepatotoxicity in vivo (Jollow et al., 1973, 1974; Potter et al., 1973, 1974). Hepatoprotective effects have also been demonstrated in rat liver slices for some antioxidants (McLean and Nuttall, 1978). The mechanism presumably involves scavenging of peroxidative free radicals, but the possibility that inhibition of paracetamol's metabolic activation may complement this action cannot be ruled out. It would therefore be interesting to extend the present studies of drug interactions with paracetamol activation in isolated hepatocytes to include this group of drugs.

It was unfortunate that in the present series of experiments the reactive metabolite could not be identified, although it must be pointed out that they were not specifically designed to do so. There is currently a great deal of controversy surrounding the metabolic pathway(s) involved in paracetamol activation. Initially, it was postulated (Jollow et al., 1974) that the initial step is N-hydroxylation, followed by dehydration to form a reactive imidoquinone. A number of publications have reported data which supports this hypothesis (Jollow et al., 1974; Healey et al., 1977; Hinson et al., 1977; Nelson et al., 1978; Miner and Kissinger, 1979). However, there have also been studies (Hinson et al., 1979a, b, c) which suggest that the reactive metabolite(s) is/are not derived from N-hydroxyparacetamol. For example, it has been shown in hamsters that N-hydroxyparacetamol can be formed from phenacetin but not from paracetamol. Furthermore, studies of ^{18}O incorporation into oxidative metabolites show that, although 3-hydroxyparacetamol can be formed from paracetamol in hamster

microsomes, presumably via a 2,3-epoxide intermediate, this pathway does not appear to be important for covalent binding, since glutathione and ascorbate, both of which inhibited the covalent protein binding, did not appear to mutually inhibit the 3-hydroxylation of paracetamol. It was considered unlikely that further oxidation of this catechol, e.g., by a superoxide pathway, contributed significantly to covalent binding either. The finding that 3-hydroxyparacetamol is not hepatotoxic in mice (Nelson, 1980) supports the view that this pathway is relatively unimportant for metabolic activation. A recent study, in which ^{14}C-ring and ^{14}C-acetyl-labelled paracetamol were incubated with hamster microsomes (Hinson and Gillette, 1980), suggests that more than one metabolite capable of covalently binding is formed and that one of these pathways involves cleavage of the N-acetyl side chain to form acetamide. These recent investigations on the mechanisms of paracetamol and phenacetin activation have been carried out in microsomal preparations in which primary conjugative pathways with glucuronide and sulphate are likely to be deficient. Whether the activity of these pathways has any modifying effect on the oxidative activation pathway is questionable. On the one hand, it has been shown that sodium sulphate administration ameliorates paracetamol hepatotoxicity in both rats and mice (Slattery and Levy, 1977; Galinsky et al., 1979), presumably by enhancing paracetamol sulphate conjugation; on the other hand, studies in this thesis showed that neither enhancement nor inhibition of the glucuronide or sulphate conjugation in isolated hepatocytes consistently, or predictably, altered paracetamol activation. Nevertheless, it might be useful to carry out studies complementary to

those of Gillette's group using isolated hepatocytes, rather than microsomes.

There is a significant body of evidence which links susceptibility to paracetamol-induced hepatic necrosis with GSH status, and it is now well established that conjugation of the reactive metabolite(s) with glutathione confers a hepatoprotective effect (Mitchell et al., 1973). Experiments in this thesis, using diethylmaleate to deplete hepatocyte GSH, and using N-acetylcysteine to augment sulphydryl conjugative capacity, have confirmed the importance of this mechanism for determining the amount of reactive metabolite which becomes covalently bound to cell protein. Moldeus (1978, 1978a) demonstrated a similar relationship between paracetamol activation and GSH status in isolated rat and mouse hepatocytes.

The status of N-acetylcysteine as an antidotal therapy in paracetamol intoxication is worthy of further comment. Injected intravenously in man, it is claimed to be the treatment of choice (Prescott et al., 1979), mainly on the grounds of adequate efficacy and reduced incidence of side effects. In isolated rat and mouse hepatocytes, N-acetylcysteine was clearly more efficacious than N-acetylmethionine in inhibiting the irreversible protein binding of paracetamol. However, Gerber et al. (1977) have shown that the in vivo hepatoprotective effect in mice is not associated with a decrease in covalent binding of paracetamol. Since the present study was not able to resolve whether N-acetylcysteine forms an adduct directly with activated paracetamol or whether it acts by augmenting or maintaining the intracellular GSH pool, it appears that further studies on this compound are warranted.

One of the key issues in this project was to show, not only that isolated hepatocytes can carry out the metabolic reactions involved in paracetamol activation and inactivation, but that changes in metabolic activation are predictive of toxicity to the cell. It is clear that, while relatively short-term (3 hour) incubations of freshly isolated hepatocytes with paracetamol can reveal the presumed initiating step in necrosis (irreversible binding to cell macromolecules), the expression of the toxicity may require a longer period of observation. Some preliminary data were obtained using cell culture techniques, which demonstrated that paracetamol-induced impairment of cell viability does occur in the longer term, and that these effects are capable of modification by drugs. Furthermore, the latency of these effects is comparable to the time over which hepatotoxic reactions develop in vivo.

On the other hand, Moldeus (1978) has been able to show, not only metabolic activation of paracetamol in isolated rat and mouse hepatocytes, with enhancement by phenobarbital induction, but also a reduction in cell viability within 3-5 hours incubation. The doses used (2 mM mouse, 10 mM rat) were much higher than those used in the present study (250 μM). This raises the whole question of interpreting the dose-response relationships. One of the putative advantages of using isolated hepatocytes as a toxicological model is that it is possible to regulate drug concentrations to which cells are exposed. But what "dose" in an isolated hepatocyte preparation is relevant to the in vivo situation?

In rats, lethal oral doses of paracetamol have been estimated by Boyd and Bereczky (1966) to range from: LD_0 0.9±0.8 g/Kg, LD_{50} 3.71±0.83 g/Kg and LD_{100} 6.5±0.8 g/Kg (all mean ± s.e.m.).

An oral dose of 1 g/Kg produces significant hepatic damage, as determined by elevated SGOT, SGPT and GLDH (Siegers et al., 1978). Plasma paracetamol concentrations associated with this dose ranged from 150-100 μg/ml (approximately 1000-670 μM) from 1-8 hours after dosing. In man, at 4 hours after an intoxicating dose, a plasma concentration of >200 μg/ml (approximately 1.3 mM) is considered to be an "action level" at which antidotal therapy is indicated (Prescott et al., 1977), although the setting of such an arbitrary threshold level for the initiation of therapy has been considered to be unwise by others (Gilligan et al., 1980). Although most cases of paracetamol-induced liver damage in man are associated with frank overdosage, there has been one case report (Bonkowsky et al., 1978) in which hepatic damage has been associated with chronic ingestion of doses in the "therapeutic" range.

Although it is difficult to directly relate "plasma levels" in vivo with incubation concentrations in vitro, the above figures suggest that, for isolated rat hepatocytes, 250 μM paracetamol may well be a sub-toxic dose. This is not necessarily true, however, in the mouse hepatocytes. The mouse is sensitive to paracetamol-induced liver damage at doses approximately 8-10 fold lower than the rat. Furthermore, it is unwise to classify it as a sub-toxic dose for either species when microsomal cytochrome P-450 has been induced, particularly by benz(α)pyrene. Therefore, while further exploration of the short-term dose-response relationships for paracetamol and other metabolically activated hepatotoxins in isolated rat and mouse hepatocytes would appear to be warranted, additional studies over longer periods of

incubation with the relatively low doses, such as those used in the present study, may also be useful in evaluating mechanistic aspects of drug-induced hepatotoxicity.

* * *

APPENDICES

APPENDIX 1

Sources of Drugs and Chemicals

The common chemicals used were analytical grades and supplied by local distributors. Certain specific substances were obtained from the following sources:

Sigma Chemical Co., U.S.A.	N-Acetyl-l-cysteine
	N-Acetyl-dl-methionine
	Cysteamine HCl (mercaptoethylamine HCl)
	α-Naphthoflavone (7,8 Benzoflavone)
	Metyrapone (α methyl-1,2,-dl-3 pyridyl-1-propanone)
	Salicylamide
	Collagenase Type IV
	Bovine serum albumin
Commonwealth Serum Laboratories, Melbourne, Australia.	Eagle's Basal Medium
	Eagle's MEM
	Foetal calf serum
	Sodium Penicillin G
	Streptomycin sulphate
Royal Adelaide Hospital	Paracetamol B.P.
The Radiochemical Centre, Amersham.	p-hydroxy[ring-3,5-^{14}C] acetanilide
Chemical Division, Sterling Winthrop, Newcastle, England.	Reference paracetamol metabolites
	Internal standard (3-hydroxy phenacetin)

APPENDIX 2

Composition of Perfusion Medium

The composition of physiological solution used for liver perfusion during the isolation of hepatocytes was the modified formula of Krebs and Henseleit (1932). Sodium gluconate was incorporated to allow the chloride ion to be adjusted to a more physiological level (Bretag, 1969). Calcium was omitted to promote the dissociation of hepatocytes (Berry, 1976). The original and the modified formula is set out below.

Constituent	Normal concentration (mM)	
	Krebs-Henseleit	Modified formula
Na^+	143.0	147.7
K^+	5.9	3.9
Ca^{++}	2.45	-
Mg^{++}	1.18	0.74
Cl^-	128.0	97.4
HCO_3^-	24.9	30.0
Phosphate	1.18	2.5
$SO_4^=$	1.18	0.74
Gluconate ion	-	21.7

These modifications were based on measured values of the relevant constituents in rat blood drawn from the hepatic portal vein and abdominal aorta (Stacey and Priestly, 1976).

APPENDIX 3

Composition of Eagle's Basal Medium

Eagle's Basal Medium (Eagle, 1955; Eagle et al., 1956) 10x

Ingredients per 100 ml

NaCl	6.8 g	l-Threonine	23.8 mg
KCl	0.4 g	l-Valine	23.4 mg
$NaH_2PO_4.2H_2O$	0.16 g	l-Tryptophane	4.1 mg
$CaCl_2$	0.2 g	Biotin	1.0 mg
$MgCl_2.6H_2O$	0.17 g	Choline chloride	1.0 mg
Glucose	1.0 g	Folic acid	1.0 mg
l-Tyrosine	18.0 mg	Nicotinamide	1.0 mg
l-Cystine	12.0 mg	Pantothenic acid	1.0 mg
l-Arginine HCl	21.0 mg	Pyridoxal HCl	1.0 mg
l-Histidine HCl H_2O	10.5 mg	Thiamin HCl	1.0 mg
l-Isoleucine	26.2 mg	Riboflavin	0.1 mg
l-Leucine	26.2 mg	Phenol Red	20.0 mg
l-Lysine HCl	36.5 mg	Sodium Penicillin G	100,000 units
l-Methionine	7.5 mg	Streptomycin sulphate	100,000 µg
l-Phenylalanine	16.5 mg	i-Inositol (meso)	1.8 mg

Preparation of Eagle's Basal Medium

For 1000 ml

Eagle's Basal Medium 10x concentration	100 ml
Glutamine solution (14.6 mg/ml)	20 ml
Bovine serum albumin (BSA)	12 g
Distilled water to	800 ml
Sodium bicarbonate solution (2.8% w/v)	as required to pH 7.4
Equilibrate with carbogen (95% O_2:5% CO_2)	
Distilled water to	1000 ml

APPENDIX 4

Standard Curve of Reduced Glutathione (GSH)

Solutions of standard reduced glutathione were prepared fresh daily with ice cold distilled water and kept in ice until immediately before use. Stock standard solution of 100 µg/ml (solution A) was diluted according to the following schedule:

Tube	Solution A (ml)	Distilled Water (ml)	Concentration (µg/ml)	µg/Sample (in 0.2 ml)
1	10	-	100	20
2	5	5	50	10
3	2.5	7.5	25	5
4	0.5	9.5	5	1
5	0.25	9.75	2.5	0.5

An aliquot of 0.2 ml from each standard was added to 0.5 ml ice cold, 30 µM EDTA. The same procedure was carried out as previously described in General Method (section e).

Fluorescence of the GSH-OPT fluorophore as a function of concentration (µg/sample) is shown in Figure A.4.1.

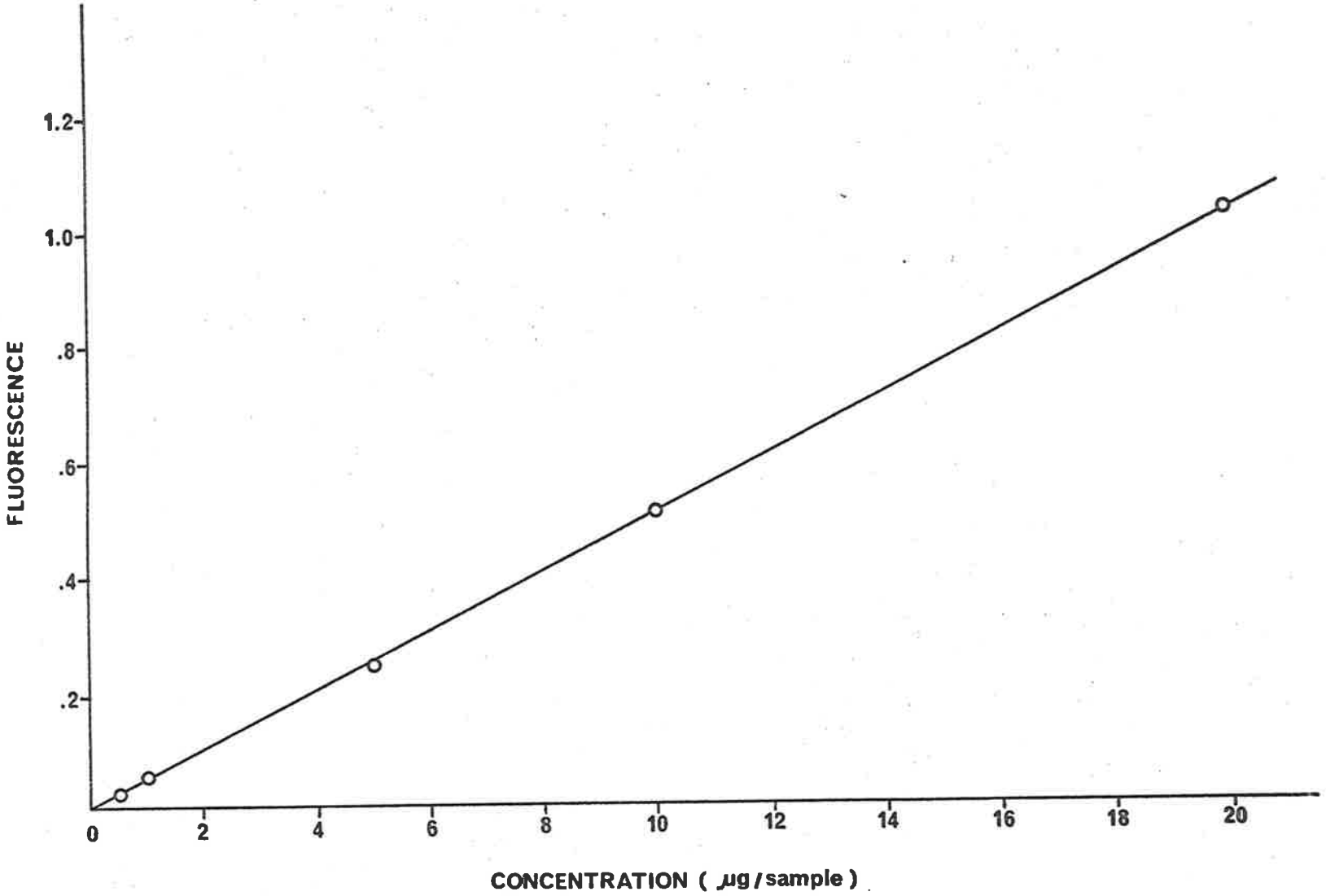


Fig. A.4.1

Standard curve of reduced glutathione. Values shown are mean from 7 experiments.

APPENDIX 5

Irreversible Protein Binding of ^{14}C-Paracetamol

Calculation:

Irreversible protein binding

$$= \frac{dpm \times 10^3}{2.22 \times 106 \times 14.6 \times protein} \times \text{dilution factor} \quad \text{nmole/mg protein}$$

nett cpm = cpm (after incubation) - cpm (zero time) (e.g., 9821 - 2740)

dpm - from nett cpm (using 70% efficiency of standard ^{14}C or cpm = 1.42 dpm)

$^1/2.22 \times 10^6$ - change dpm to μCi (1 μCi = 2.22×10^6 dpm)

$^1/14.6$ - change μCi to μmole (specific activity of ^{14}C-paracetamol = 14.6 μCi/μmole)

$\times 10^3$ - change μmole to nmole (1 μmole = 10^3 nmole)

$^1/$protein - $^1/$ protein concentration in mg (in ^{14}C-bound precipitate)

dilution factor - ratio of non-radiolabelled paracetamol to radiolabelled paracetamol (in experiments with 0.25 mM total paracetamol, this factor was 30)

Since the protein extract containing the counts was dissolved in soluene, it was decided that the protein concentration should be determined upon an aliquot of cells resuspended in phosphate buffer before the initial precipitation step. A series of experiments were done to verify that the recovery of protein after precipitation and exhaustive extraction was consistent (coefficient of variation 8.7%).

APPENDIX 6

Chromatogram and Curve of Paracetamol and Standard Metabolites

Stock solution of paracetamol and standard metabolites (in distilled water) at the concentration of 1 mg/ml were diluted as follows:

Tube	P-G (µl)	P-C (µl)	P-S (µl)	P-Glut (µl)	P (µl)	Compensate Water (µl)	Concentration (µg/ml)
1	10	10	10	10	10	950	10
2	15	15	15	15	15	925	15
3	25	25	25	25	25	875	25
4	30	30	30	30	30	850	30
5	40	40	40	40	40	800	40

Aliquots of 20 µl internal standard (1 mg/ml) and 0.5 ml 3N PCA were added into each tube. After thorough mixing, 15 µl aliquots were chromatographed on a reversed phase µ-Bondapak C_{18} column (Waters Associates Inc.) with the solvent system containing water : glacial acetic acid : ethyl acetate (98 : 1 : 1). The flow rate was 1.4 ml/min (Waters Model 45 solvent pump) using UV detector (Waters Model 440) operating at 254 nM. The standard chromatogram and ratio of peak height (metabolite : internal standard) as the function of concentration (µg/ml) are shown in Figure A.6.1 and A.6.2.

It should be noted that the paracetamol glutathione reference standard contained an unidentified impurity (U in Fig. A.6.1) whose retention time did not coincide with any other identifiable

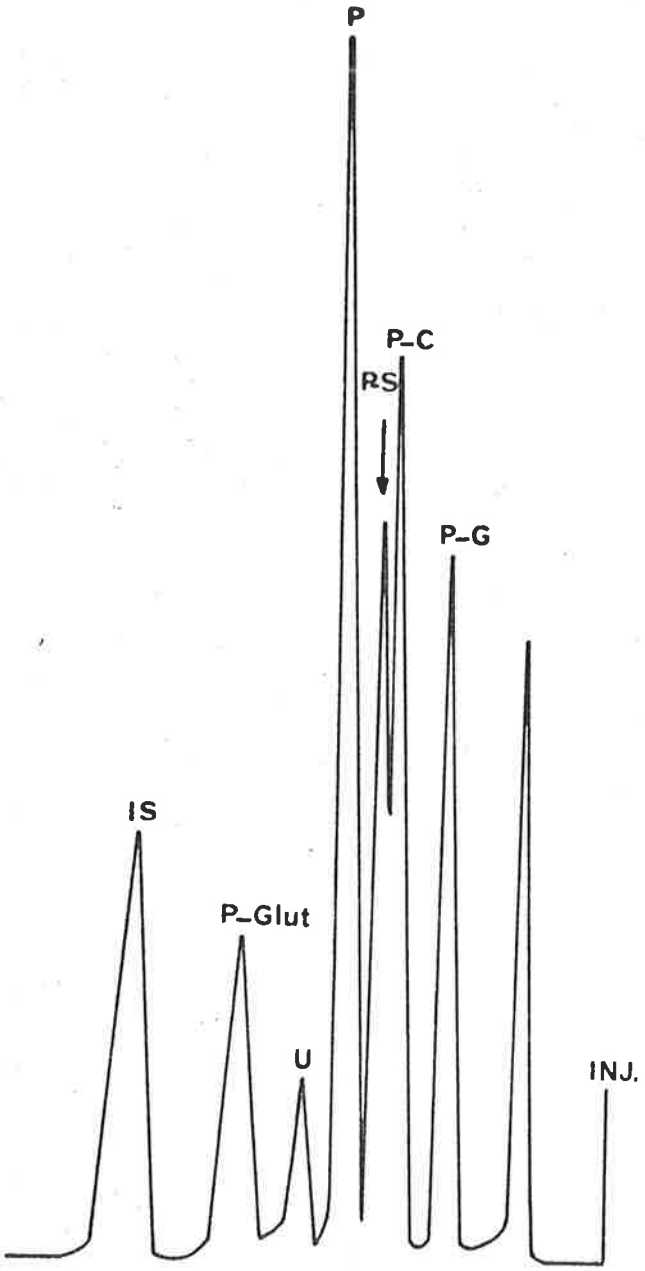


Fig. A.6.1

Chromatogram of paracetamol and standard metabolites (15 µl, 30 µg/ml).

Inj	injection	P-G	paracetamol glucuronide
P-C	paracetamol cysteine	P-S	paracetamol sulphate
P	paracetamol	P-Glut	paracetamol glutathione

U unknown (in standard P-Glut)

IS internal standard

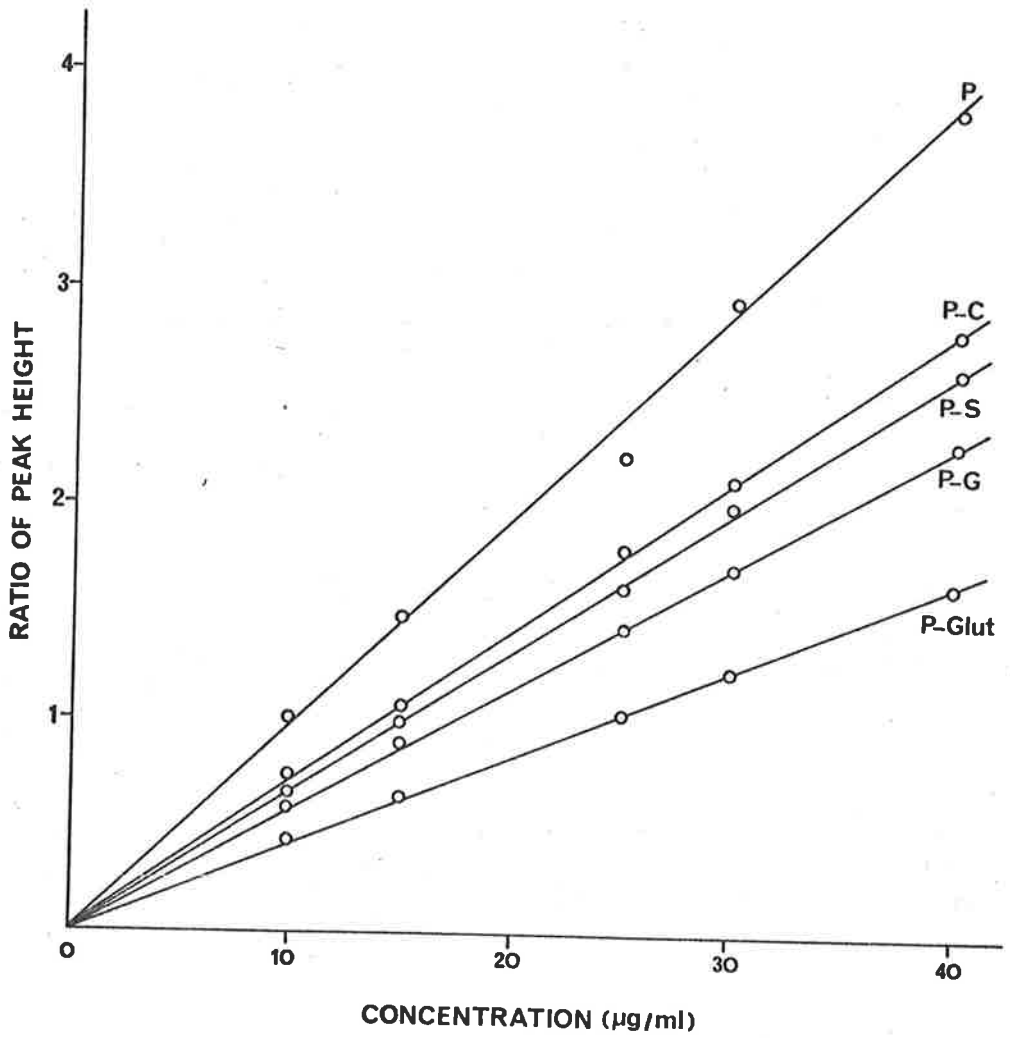


Fig. A.6.2

Standard curve of paracetamol and metabolites.

P paracetamol

P-C paracetamol cysteine

P-S paracetamol sulphate

P-G paracetamol glucuronide

P-Glut paracetamol glutathione

Values shown are mean from triplicate samples.

standards. A correction factor was applied to this peak based upon relative peak heights from a chromatogram of the individual reference standard.

APPENDIX 7

Composition of Eagle's Minimum Essential Medium (MEM)

Eagle's Minimum Essential Medium (Dried Powder) (Eagle, 1959)

Ingredients per 1000 ml equivalent

NaCl	6.8 g	l-Valine	46.0 mg
KCl	0.4 g	l-Cystine	24.0 mg
$MgCl_2.6H_2O$	0.2 g	l-Tyrosine	24.0 mg
$NaH_2PO_4.2H_2O$	0.15 g	Choline Cl	1.0 mg
$CaCl_2$	0.2 g	Folic acid (cryst.)	1.0 mg
l-Arginine HCl	105.0 mg	i-Inositol (meso)	2.0 mg
l-Histidine HCl H_2O	31.0 mg	Nicotinamide	1.0 mg
l-Isoleucine (Allo free)	52.0 mg	Calcium Pantothenate	1.0 mg
l-Leucine	52.0 mg	Pyridoxal HCl	1.0 mg
l-Lysine HCl	58.0 mg	Riboflavin	0.1 mg
l-Methionine	15.0 mg	Thiamine HCl	1.0 mg
l-Phenylalanine	32.0 mg	l-Glutamine	0.292 g
l-Threonine (Allo free)	48.0 mg	Glucose	1.0 g
l-Tryptophane	10.0 mg	Phenol Red	15.0 mg

Preparation of Eagle's Minimum Essential Medium (MEM)

For 1000 ml

Eagle's MEM (powdered for 1 litre)	1 bottle
Sterile deionized water	800 ml
Sodium bicarbonate	2.2 g/200 ml water

Filter immediately after mixing with bicarbonate solution (0.22 µm, millipore filter)

APPENDIX 8

Expression of Results

In the literature, many different reference points have been used for the expression of data from isolated hepatocytes. These include: cell number, protein, DNA, dry weight and wet weight. Wet weight of cells is perhaps the most convenient unit and has been used quite extensively. It was chosen as the basis for expressing results in this thesis.

Cell wet weight can be determined in two ways. Firstly, the weight of cells sedimented by centrifugation in a tared tube provides a preliminary estimate which must be corrected for entranced extracellular fluid. This method was used for determination of K^+ in centrifuged cell pellets. An extracellular fluid correction value of 16% was used (Krebs et al., 1974; Berry and Friend, 1969; Stacey, 1978). The alternative method is based upon a conversion factor from cells counted in a haemocytometer. The majority of papers report the value close to $1.2 - 1.4 \times 10^8$ cells/g wet weight (Krebs et al., 1974; Ingebretsen and Wagle, 1972; Hofmann et al., 1976), so 1.3×10^8 cells/g wet weight was chosen as the conversion factor in this thesis. The validity of this conversion factor was confirmed in a series of preliminary experiments. It should be noted that the number of cells used for the conversion to wet weight was only that of the viable cells (trypan blue excluding cells).

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