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HEPATOTOXICITY OF CHEMICALS IN ISOLATED HEPATOCYTES

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by

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C O N T E N T S

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APPENDICES 1-5

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 in the text. Material from this thesis has formed part of the following publications:

Stacey, N. H. and Priestly, B. G. (1977). The bicarbonate ion concentration of Krebs-Henseleit solution. Aust. J. exp. Biol. med. Sci., 54, 103-105.

Stacey, N. H., Priestly, B. G. and Hall, R. C. (1978). Toxicity of halogenated volatile anesthetics in isolated rat hepatocytes. Anesthesiology, 48, 17-22.

Stacey, N. H. and Priestly, B. G. (1978). Dose dependent toxicity of CCl_4 in isolated rat hepatocytes and the effects of hepatoprotective treatments. Toxicol. Appl. Pharmacol., in press.

Stacey, N. H. and Priestly, B. G. (1978). Lipid peroxidation in isolated rat hepatocytes. Relationship to toxicity of CCl_4, ADP/Fe^{+++} and diethyl maleate. Toxicol. Appl. Pharmacol., in press.

Stacey, N. H., Cook, R. and Priestly, B. G. (1978). Comparative stability of alanine aminotransferase in rat plasma and hepatocyte suspensions. Aust. J. exp. Biol. med. Sci., in press.

Results of this thesis have also been presented to meetings of the Australasian Society of Clinical and Experimental Pharmacologists in Adelaide (November, 1976) (2 papers) and Sydney (November, 1977), to the Australian Biochemical Society in Armidale (May, 1976) and to the Australian Physiological and Pharmacological Society in Hobart (May, 1977).

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ABSTRACT

Isolated rat hepatocytes have been shown to be sensitive to the toxin CCl_4 in a dose and time related manner. There was a loss of intracellular K^+ and ALT, an increase in L/P and an inhibition of ureogenesis on incubation with CCl_4. Changes in the ultrastructure included effects on the RER similar to those seen after *in vivo* administration of the toxin. Simultaneous incubation with several chemicals associated with protection against CCl_4 induced liver injury failed to give any consistent sign of amelioration of the toxic response. Incubation at 4°C was able to inhibit injury to the cell.

Lipid peroxidation was found not to be associated with the toxicity of CCl_4 to the suspensions of isolated hepatocytes, even though evidence for such a relationship was found for diethyl maleate. Lipid peroxidation was also shown to occur in response to ADP/Fe^{+++} in the isolated hepatocytes, confirming a similar observation by Hogberg *et al.* (1975).

Phenobarbitone pre-treatment did not produce isolated hepatocytes which were more sensitive to CCl_4 than those from non-induced rats.

Thus the toxic response to CCl_4 found in the isolated hepatocytes does not appear to be occurring by a mechanism similar to that *in vivo*. A similar toxic response to benzene and loss of K^+ from Erlich Ascites Tumor cells on treatment with CCl_4 support this conclusion. The finding that toxicity in isolated hepatocytes due to bromobenzene also occurs in a manner dissimilar to that *in vivo*, and the lack of toxicity with paracetamol can also be taken to indirectly support the idea that the response to CCl_4 in isolated hepatocytes occurs via a mechanism different to that in the intact animal.

However, the more specific observation that changes in the RER occur without demonstrable lipid peroxidation does question the link that has been drawn between these two events during the CCl_4 hepatotoxic response in the whole animal.

The toxic response of isolated hepatoyctes to a group of halogenated anaesthetics gave an order of cytotoxic potency similar to the incidence of clinical hepatotoxic response. This indicates that isolated hepatocytes, like cultured hepatic cells, could be useful in predicting which drugs are likely to display idiosyncratic hepatotoxicity in the human patient. A similar conclusion was reached by Zimmerman *et al.* (1974).

In conclusion, the results of this thesis have shown that even though a toxic response can be elicited from the isolated hepatocytes, the mechanism by which it is brought about is not necessarily the same as that *in vivo*. The evaluation of the isolated rat hepatocyte suspension as a system for toxicological studies has, therefore, indicated that care is required in the interpretation of results, particularly when extrapolating to the whole animal.

ABBREVIATIONS

A.A.	abdominal aorta
ALT	alanine aminotransferase (L-alanine: 2-oxoglutorate aminotransferase 2.6.1.2.)
ATP	adenosine triphosphate
$BrCCl_3$	Bromotrichloromethane
BSA	bovine serum albumin
$CBrCl_3$	bromotrichloromethane
CCl_4	carbon tetrachloride
CO	carbon monoxide
DDT	1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane
DPPD	N,N'-diphenyl-p-phenylenediamine
EAT cells	Erlich Ascites Tumor cells
EDTA	ethylenediaminetetraacetate
EM	electron microscopy
ER	endoplasmic reticulum
xg	times gravity; relative centrifugal force
G-6-P'ase	glucose-6'-phosphatase
GSH	reduced glutathione
$[HCO_3^-]$	concentration of bicarbonate ion
HPV	hepatic portal vein
I.U./L	international units per litre
i.p.	intraperitoneal
Kp/cm^2	kilopascals per centimetere squared
LDH	lactate dehydrogenase
L/P	lactate to pyruvate ratio
3-MC	3-methylcholanthrene
MDA	malonic dialdehyde

n	number of experiments
NADPH	nicotine adenine dinucleotide phosphate (reduced form)
NIH	National Institute of Health (U.S.A.)
nm	nanometres
OPT	o-phthalaldehyde
PB	phenobarbitone
PCA	perchloric acid
PCN	pregnenolone-16α-carbonitrile
RER (rer)	rough endoplasmic reticulum
RNA	ribonucleic acid
r.p.m.	revolutions per minute
s.e.m.	standard error of the mean
SER (ser)	smooth endoplasmic reticulum
SKF-525A	2-diethylaminoethyl-2,2-diphenylvalerate
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEM	transmission electron micrograph
TPB	tetraphenylboron
w/v	weight per volume
% w/v	g per 100 millilitres

GENERAL INTRODUCTION

(a) Hepatocyte isolation procedures

Early methods of obtaining isolated hepatocytes were primarily mechanical. These are well documented by Schreiber and Schreiber (1973) and Berry (1976). Cells prepared by such methods are damaged to the extent that they even fail the more insensitive viability tests (David *et al.*, 1975). Attempts to disjoin the intercellular matrix with chelating agents such as citrate, ethylenediaminetetracetate (EDTA) and tetraphenylboron (TPB), also yielded cells with low levels of viability (Wagle and Ingebretsen, 1975). However, some success has been claimed with such agents (Pertoft, 1969; Casanello and Gerschenson, 1970; Suzangar and Dickson, 1970). Experiments carried out in the same laboratory using different preparative methods have enabled a more direct and objective comparison (Gallai-Hatchard and Gray, 1971; Lipson *et al.*, 1972; Muller *et al.*, 1972).

Attempts have been made to disrupt the intercellular matrix with various enzymes some of which have resulted in cells with poor viability (Schreiber and Schreiber, 1973; Jeejeebhoy and Phillips, 1976; Berry, 1976). An isolation procedure employing lysozmye has given good yields of rat hepatocytes with a higher trypan blue exclusion (Hommes *et al.*, 1970), but with low ATP levels. The same procedure was also successful for mice in the hands of other workers (Pretlow and Williams, 1973). However, inadequacies have also been reported (Muller *et al.*, 1972; Crisp and Pogson, 1972).

Use of collagenase and hyaluronidase for isolating rat hepatocytes was first described by Howard *et al.* (1967) and later by Howard and Pesch (1968). Although hepatocyte yields were low, viability characteristics of the cells showed improvement. As pointed out by Krebs

et al. (1974) and Berry (1976), these enzymes had been used previously for isolation of other cell types. The procedure of Howard and Pesch (1968) was modified by Berry and Friend (1969), who introduced a recirculating perfusion of the liver with the enzymes, resulting in a much higher yield of cells without compromising viability.

These improvements are now recognised as major steps in this area (Jeejeebhoy and Phillips, 1976; Schreiber and Schreiber, 1973). This is borne out by the fact that most workers, although they may have introduced minor modifications, now use this basic technique for preparation of isolated hepatocytes (Berry, 1976). The continued development of the poor yield non-recirculating technique is in order, as it is applicable to samples such as human biopsy material (Fry *et al.*, 1976; Krebs, 1976).

(b) Applications of isolated hepatocytes

The isolated hepatocyte system has a wide applicability in studies of liver function, as is evident from the number of areas already under investigation. Carbohydrate metabolism has been widely studied (Jeejeebhoy and Phillips, 1976). More specifically, areas examined have included gluconeogenesis (Berry and Friend, 1969; Veneziale and Lohmar, 1973; Seglen, 1973a) including hormonal modulation (Zahlten and Stratman, 1974; Johnson *et al.*, 1972; Clark *et al.*, 1974), glycogen synthesis and sensitivity to hormones (Seglen, 1973b; Wagle *et al.*, 1973), glycolysis (Berry and Friend, 1969; Crisp and Pogson, 1972) and activity in the pentose phosphate pathway (Baquer *et al.*, 1973). Protein synthesis, both general (Schreiber and Schreiber, 1973; Ingebretsen *et al.*, 1972) and specific (Weigand *et al.*, 1971; East *et al.*, 1973, Phillips *et al.*, 1974) have been investigated. Studies on lipid metabolism have been summarized recently, as have those studies on enzyme synthesis and induction

(Jeejeebhoy and Phillips, 1976). Other areas studied have included RNA synthesis (Seglen, 1973a), bile acid secretion and synthesis (Anwer *et al.*, 1975), uptake of taurocholic acid (Schwarz *et al.*, 1975) and protein metabolism (Seglen, 1977; Seglen, 1976; Seglen and Reith, 1977). Drochmans *et al.* (1975) have fractionated hepatic parenchymal cells with a view to correlating cell size and enzyme activity and relating these to the lobular distribution of hepatocyte function. The possibility of attaining a culture of mature viable adult rat hepatocytes has also been the subject of investigations with these cells (Bissell *et al.*, 1973; Iype, 1971; Iype *et al.*, 1972).

Studies with isolated hepatocytes which bear a more direct relation to the subject matter of this thesis include investigations into drug metabolism (Henderson and Dewaide, 1969; Holtzman *et al.*, 1972; Moldeus *et al.*, 1974; Grundin, 1975; Inaba *et al.*, 1975; Wiebkin *et al.*, 1976; Vadi *et al.*, 1975; Morland and Olsen, 1977; Billings *et al.*, 1977; Yih and van Rossum, 1977; Aarbakke *et al.*, 1977), lipid peroxidation (Hogberg *et al.*, 1975; Hogberg *et al.*, 1975a; Hogberg *et al.*, 1975b; Remmer *et al.*, 1977), and toxicity (Zimmerman *et al.*, 1974; Corona *et al.*, 1973; Abernathy *et al.*, 1975; Hofmann *et al.*, 1976; Abernathy and Zimmerman, 1975; Hogberg and Kristoferson, 1977; Frimmer *et al.*, 1976; Hegner *et al.*, 1976; Frimmer and Kroker, 1973; Frimmer *et al.*, 1974; Weiss *et al.*, 1973; Fry and Bridges, 1977; Gravela and Poli, 1977). Aspects of these will be discussed in greater depth later (Section (e)).

(c) Cell viability

Two types of biochemical tests have been used to assess the viability of isolated hepatocytes, these being tests of membrane integrity and tests of metabolic function.

Perhaps the most common index in use as an assessment of viability is the exclusion of, from all parts of the cell, the vital dye, trypan blue. Cells which take up the stain are considered to have damaged membranes (Howard *et al.*, 1973). As pointed out by Baur *et al.*, (1975), however, this test can only reflect severe irreversible damage. Care is required in the interpretation of trypan blue results when albumin is present because this protein binds trypan blue, such that some damaged cells may not take up the dye (Berry, 1976). It was suggested by this same author that the roundness of the cells may give a better index of viability.

As with trypan blue, leakage of intracellular enzymes, such as alanine aminotransferase and lactate dehydrogenase, is suitable only as an indication of severe structural damage. Other more subtle changes in membrane structure require more sensitive tests. Among these, according to Baur *et al.* (1975), are membrane potential and intracellular Na^+ and K^+ concentrations. The greater sensitivity of K^+ as an index of cellular viability is supported by a study with other cell types (Medzihrzadsky and Marks, 1975).

Cellular respiration has been measured by several workers, as an assessment of viability of their hepatocyte suspensions (Berry and Friend, 1969; Krebs *et al.*, 1974; La Brecque *et al.*, 1973; Moldeus *et al.*, 1974). In particular, the ability of the membrane to exclude succinate, and thus prevent stimulation of oxygen utilization by the succinate, is taken as a stringent test of membrane integrity (Baur *et al.*, 1975).

Even though it is not a sensitive test, trypan blue exclusion was used as an initial test of viability of fresh cells in this thesis because

it is quick and easy. Intracellular K^+ was monitored routinely in the experiments of this thesis, as a more sensitive parameter. Concentrations in freshly prepared cells, and cells incubated with drugs, were determined as indices of initial viability, and adverse effects of the drugs, respectively. Loss of the intracellular enzyme, alanine aminotransferase (ALT), was also routinely assessed, as an index of severe membrane damage, particularly in response to drugs added to the incubated suspensions.

Several of the metabolic functions mentioned in section (b) have been used as an indication of the functional status of the cell preparation by various laboratories. In particular, energy consuming metabolic processes such as gluconeogenesis and ureogensis are useful indices of metabolic viability (Hems *et al.*, 1966; Biebuyck *et al.*, 1972). Abnormality in urea synthesis is recognised as a reflection of hepatic injury in experimental animals (Zimmerman, 1976). Ureogenesis was therefore assessed as an index of metabolic function in the experiments of this thesis.

A second metabolic parameter, the lactate to pyruvate ratio (L/P), which is recognised as a sensitive indicator of liver function (Graf *et al.*, 1973), was also used as an index of cellular status. This can reflect the function of the respiratory mechanisms of the cell (Abouna *et al.*, 1969). Linked therein is the ATP status of the hepatocytes, also a sensitive index of cell viability (Baur *et al.*, 1975). Levels of ATP in the cells of experiments of the present work have been estimated, but not in a routine manner.

The morphological appearance of the cells under the electron

microscope has been another widely used method of assessing cellular integrity (Berry and Friend, 1969; Schreiber and Schreiber, 1973; Drochmans *et al.*, 1975; Jeejeebhoy and Phillips, 1976; Frimmer *et al.*, 1976). Morphologically intact cells should be spherical with numerous microvilli and have a substructure of normal appearance (Jeejeebhoy and Phillips, 1976).

Eagle's basal medium, which contains both amino acids and vitamins (see Appendix 3) was chosen as the incubation medium for hepatocytes in experiments described in this thesis for two primary reasons. Firstly, cells tend to lose some of their amino acid content during the isolation procedures (Schreiber and Schreiber, 1973; Krebs *et al.*, 1974), and it may be advisable to add amino acids to the medium in the physiological range (Krebs, 1976a). Isolated hepatocytes have been shown to be able to concentrate amino acids by Jeejeebhoy *et al.* (1975). In fact, the ability to do so has been deemed a further index of membrane integrity (Jeejeebhoy and Phillips, 1976). Secondly, it was observed empirically in initial experiments of this thesis that higher rates of ureogenesis were obtained with an amino acid supplemented medium.

Apart from providing a sensitive and comprehensive appraisal of cell viability, the parameters chosen for assessment had the added advantage that, unlike trypan blue exclusion, the assessment did not have to be made immediately after the incubation step.

(d) Comparison of isolated hepatocytes with other liver preparations

Krebs *et al.* (1974) have made a detailed comparison of isolated hepatocytes to other liver preparations such as the *in vivo* liver, isolated perfused liver and liver slices. They indicate that biochemical

justification in comparison to other liver systems is necessary to enable appropriate interpretation of results and the ultimate extrapolation to the *in vivo* situation. They report that the isolated cells are very comparable to perfused liver and superior to liver slices. In fact, ATP levels of the cells are closer to freeze clamped liver than ATP content of perfused liver. Other reports have indicated levels of ATP in isolated cells similar to that found *in vivo* (Phillips *et al.*, 1974; Jeejeebhoy *et al.*, 1975; Williamson *et al.*, 1974; Hofman *et al.*, 1976; Baur *et al.*, 1975). Some have found lower values (Baquer *et al.*, 1973; Veneziale and Lohmar, 1973) but this may reflect the quality of their cells. The sensitivity of K^+ as an index of viability has been discussed and it is noteworthy that cells, from more recent reports in particular, contain intracellular K^+ at a level similar to that *in vivo* (Krebs *et al.*, 1974; Baur *et al.*, 1975; Hems *et al.*, 1975; Barnabei *et al.*, 1974; Frimmer *et al.*, 1976). Krebs *et al.* (1974) concluded that there was, at that time, no serious fault with the cells biochemically, but pointed out that only a few liver functions had been assessed in any depth, and that limitations could arise.

Having established the biochemical equivalence of isolated hepatocytes, the advantages of this system can be more fully exploited. A major advantage concerns the quantity of information obtainable from the biological preparation. For the type of experimental work of this thesis, isolated cells yield about twenty times the information when compared to the isolated perfused liver per preparation. This advantage is not offset by the additional time required to analyse the increased number of samples generated. Liver slices possess a similar advantage, but their markedly inferior functional state, related to damaged cells at the

slice perimeters and lack of adequate oxygenation of the innermost cells, puts a limit on their use (Johnson *et al.*, 1972). Also, as with the perfused liver, the system contains more than one cell type, which disallows a direct study of function of a particular cell population. It should be remembered, however, that isolation of hepatocytes is accompanied by loss of lobular structure, and hence concentration and oxygen gradients.

Cultured cells of hepatic origin, too, possess the potential for a large yield of information, but suffer from the drawback of de-differentiation, which refers to the inability of the cells to fully retain their specific differentiated functions (Bissell *et al.*, 1973; Jeejeebhoy and Phillips, 1976). This can be of particular importance in toxicity studies where toxicity may be due to a metabolite rather than the parent compound. Cultured cells often lose or exhibit dissimilar pathways for drug metabolism (Fry and Bridges, 1977; Owens and Nebert, 1975). This helps to illustrate an advantage of the whole cell over sub-fractionated particles in this field too. In cellular sub-fractions a second or follow up pathway may be lost (Yih and van Rossum, 1977). Such a pathway may detoxify the initial toxic metabolite, which may reflect more accurately the situation *in vivo* than any straight toxicity observed in the sub-fraction (Fry and Bridges, 1977). The advantages of a preparation involving whole cells rather than sub-cellular fractions for the study of lipid peroxidation, too, has been discussed by Remmer *et al.* (1977).

Use of isolated cells also enables a direct control of the external environment with subsequent comparisons between control and otherwise treated cells from the same homogeneous parent suspension. As pointed

out by Jeejeebhoy and Phillips (1976) this produces a sensitive test of the effects of the treatment in question.

Walton and Buckley (1975) have discussed the advantages of *in vitro* systems over the whole animal. In particular, they mention the complexity of the organism with subsequent difficulties in interpretation of results. The inherent limitations of *in vitro* models are also discussed by these authors, with emphasis on the relationship to toxicity studies. Elliot *et al.* (1976) have discussed the lack of maintenance of steady-state concentrations in closed incubation systems, which is a disadvantage in comparison to the perfused organ. Disadvantages such as this and others can be overcome by using a flow through system similar to that described by van der Meer *et al.* (1976).

Although further advances in the technology of isolating cells from non-perfused liver are required to increase yields of viable hepatocytes, an advantage of the isolated cells becomes apparent when considering hepatic studies overall. That is, this technique could allow experimental studies on liver physiology, biochemistry and pathology from human sources (biopsy and possibly cadaver) in a manner and depth not before possible. In fact, a recent report has appeared suggesting a good yield, high viability preparation may well be available (Belleman *et al.*, 1977). Further biochemical characterization of cells prepared in this way will determine their future value.

(e) Toxicity and liver cells

Various systems (*in vivo* and *in vitro*) have been used in the investigation of hepatotoxicity (Zimmerman, 1976). The advent of methods for isolation of viable hepatocytes has, in recent years, seen this

technique applied to the study of drug-induced hepatotoxicity. Most studies have been concerned with the comparative toxicity of potential hepatotoxins by determination of dose-response relationships. Indices of damage have been primarily those associated with membrane integrity. Cellular sub-fractions have been used more for studying mechanisms of toxicity, whereas the intact animal has been used from both aspects.

It should also be remembered that normal cell function is often studied by using toxins as metabolic probes (Farber, 1971).

To enable evidence of cell toxicity to be placed in perspective relevant to the *in vivo* situation, information regarding the mechanism of drug-induced toxicity in isolated hepatocytes is essential. Investigation into mechanisms is deemed one of the main objectives of toxicological research (Walton and Buckley, 1975). It is of paramount importance to show that the mechanisms operating in the isolated hepatocyte preparation (for direct acting hepatotoxins) are comparable to those operating *in vivo* if the use of this *in vitro* technique, with all its associated advantages, is to be validated.

To investigate the use of isolated hepatocytes in toxicity studies, a chemical whose hepatotoxicity *in vivo* is dose related, and whose mechanism has been extensively investigated would seem a logical choice for initial studies, where some standardization of the technique would be necessary. Carbon tetrachloride is such a compound, and the vast amount of study already documented about its effects and mechanisms makes its choice relatively simple. It is recognised by others as valuable in the study of hepatotoxicity (Zimmerman, 1968). However, there are inconsistencies in some of the data, particularly with respect

to the protective actions of some chemicals, and this has led to some disagreement over the proposed mechanisms of CCl_4-induced hepatotoxicity. This leaves the opportunity for investigations with a new preparation to provide further information in such 'grey' areas and perhaps lead to a better understanding of the events occurring *in vivo*. A particular advantage of isolated cells in studies on mechanism is that any toxic response can be monitored simultaneously with other changes within the cell. For example, monitoring lipid peroxidation concurrently with cell damage may help to elucidate further the relationship between these two, this being an area of some dispute (Plaa and Witschi, 1976; Recknagel and Glende, 1973; de Ferreyra *et al.*, 1975).

On the other hand, some aspects of CCl_4-induced hepatotoxicity are less contentious, such as enhancement of toxicity after phenobarbitone pre-treatment. Therefore, if the mechanism of CCl_4-induced toxicity in isolated hepatocytes reflects the mechanism operating *in vivo*, studies with hepatocytes isolated from phenobarbitone pre-treated liver donors should help to resolve the applicability of the method.

For comparative purposes, studies with other known hepatotoxins such as dimethylnitrosamine or bromobenzene should be carried out. The toxicity of all three of these compounds has been linked to their metabolism by the mixed function oxidase system (McLean and Day, 1975; Reid *et al.*, 1971). Thus the necessity for a test system with this capacity is reiterated, and isolated hepatocytes have been reported to metabolize drugs at rates comparable to *in vivo* or isolated microsomes without the requirement of added cofactors (Inaba *et al.*, 1975; Wiebkin *et al.*, 1976; Holtzman *et al.*, 1972; Moldeus *et al.*, 1974; Billings *et al.*, 1977). Apparent advantages of isolated cells over microsomal

preparations are indicated by the studies of Yih and van Rossum (1977) and Billings *et al.* (1977). In particular, the isolated hepatocytes are able to carry out a range of metabolic reactions (e.g. oxidation, conjugation) whereas microsomes are limited by substrate and cofactor requirements. This is especially shown by the work of Wiebkin *et al.* (1976) and Aarbakke *et al.* (1977). In establishing the validity of a new system, such comparisons (i.e. bromobenzene with CCl_4) are necessary in order to reduce the possibility of misinterpretation of the cause of differences, or a false acceptance of apparent similarities.

Drugs which have been described as idiosyncratic hepatotoxins, such as the erythromycins and phenothiazines, have been examined in several *in vitro* models (Zimmerman, 1976). The development of a satisfactory method of testing for the potential of a drug to produce this idiosyncratic response is important for two reasons. Firstly, this type of toxic response is rarely reproducible in experimental animals *in vivo*, and secondly, it probably accounts for a high incidence of drug-related human hepatic injury (Berthelot, 1973). Studies with cultured cells of hepatic origin have shown, within a particular group of drugs, cytotoxicity in an order similar to their clinically reported hepatotoxicities (Dujovne, 1975; Goto *et al.*, 1976). As mentioned previously, and by Zimmerman *et al.* (1974), the cultured cells can be criticised on the basis of having become de-differentiated and therefore being less representative of fully functioning liver cells. Chang cells, which have been used in several of these studies, are of human embryonic origin, which in an added disadvantage. This is especially so in relation to toxic metabolites, as the mixed function oxidase system is not fully developed until after birth. In the rat it is at least seven

days after birth before complete development of this system (Zimmerman, 1976; Vesell, 1972). Holtzman *et al.* (1972) also comment on the use of cells cultured from embryonic hamster liver in relation to drug metabolism studies. Hepatoma cells, which have also been used in such drug studies, also do not fully represent normal adult liver. Gillette (1974) has discussed reactive metabolite studies in relation to toxicity, pointing out inadequacies similar to those mentioned above. Suspensions of isolated hepatocytes, however, are not subject to such criticisms and are probably, therefore, likely to be a more appropriate model for such investigations.

Studies have appeared in the literature (Zimmerman *et al.*, 1974; Abernathy *et al.*, 1975; Abernathy and Zimmerman, 1975) using isolated hepatocytes for investigating idiosyncratic hepatotoxins. The results showed a response similar to that found with the cell cultures. An extension of such studies would not only provide further information, particularly on metabolic effects, with such a group of drugs, but would also provide a basis for comparison of the techniques used in this laboratory with those of other workers.

(f) Aims of the thesis

The overall aim of this thesis, then, was an appraisal of the isolated hepatocyte preparation as a tool in the study of hepatotoxicity of drugs. Parameters reflecting both structural and metabolic integrity of the cell were to be used to assess toxicity. The plan included investigations into the mechanisms of toxic responses.

GENERAL METHODS

Methods described in this section are those used generally throughout the thesis. Any alteration from these will be documented in the appropriate place. Methods relevant to only a single chapter will be outlined in that chapter.

Animals used in these studies were male Wistar rats in the weight range 200-300 g. They were obtained from the University's 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 or water deprivation. Constituents of the solid diet are documented in Appendix 1, as are the sources of the chemicals and drugs used in this study. Experiments were carried out on a similar time sequence throughout this thesis, surgery commencing between 8.45 - 9.00 a.m.

(a) Isolation of rat hepatocytes

The procedure followed was basically that of Berry and Friend (1969) with some modifications. The rat to be used as liver donor was anaesthetized with ether. Surgery, which was based on the procedures described by Miller (1973), was commenced with laparotomy and the liver was exposed. Connective tissue between the liver and surrounding tissues was disjoined, taking care not to tear the liver itself. The oesophagus and accompanying blood vessels were then doubly ligated and cut between the two ties. The intestines were moved to the left of the animal so as to expose the portal vein. The small vessel running close to the bile duct and joining the portal vein close to the liver was ligated. Loose ligatures were placed about the portal vein in preparation for cannulation. At this stage the animal received heparin (750 - 1,000 units) via the inferior vena cava. After approximately two minutes the distal ligature around the portal vein was tied. Tension was placed on the vessel via this tie

so as to hold the portal vein taut. The proximal ligature was lifted slightly to provide better access to the length of portal vein required for cannulation and also to reduce back flow of blood from the liver, so as not to obscure the field of operation. The portal vein was then snipped across about half its diameter and the cannula inserted, pushed past the proximal ligature and tied into place with that ligature. The inferior vena cava was then cut just distal to the renal veins and perfusion of the liver was commenced from a constant pressure head of 20 cm. The composition of the perfusion medium is listed in Appendix 2. The effluent flowed into a waste container thereby flushing the blood from the liver. Failure to achieve rapid and complete perfusion of all hepatic lobes led to a poor preparation of hepatocytes. Perfusion was maintained during the subsequent excision of the liver. Flow was re-interrupted for approximately a further 15 seconds as the liver was transferred to a humidified perfusion cabinet thermostatically maintained at 37°C. Perfusion was re-instituted under recirculating conditions with the same calcium free physiological medium. After approximately 12 minutes of perfusion (including the time while the liver was still *in situ*) 10 ml of a concentrated solution of collagenase 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 the collagenase containing medium was continued for 10-15 minutes, after which time appreciable swelling of the liver indicated that digestion of the intercellular matrix was adequate.

The liver was then placed in a beaker containing 25 ml of fresh collagenase buffer, the capsule disrupted with a blunt spatula, and the liver tissue pulled apart with blunt dissection. Any strands of remaining

tissue were cut into small pieces with scissors. The remaining fibrous tree, to which the cannula was still attached, was discarded. Volume was made up to 50-60 ml with collagenase buffer from the recirculation, and the suspension dispensed equally into 2 x 250 ml Erlenmeyer flasks and incubated for a further 10 minutes in a metabolic shaker bath at 80 oscillations per minute and maintained at 37°C.

Physiological media used throughout the isolation of hepatocytes and for their subsequent incubation were equilibrated with humidified carbogen (O_2:CO_2, 95:5).

Bovine serum albumin (BSA) to a final level of 1.2% (w/v) was added and the hepatocytes were then sieved through two layers of nylon mesh (250 and 61 micron pore size from Henry Simon Ltd, Stockport, England). The resulting cell suspension was centrifuged at 50xg for one minute and washed twice with this same medium. A final wash was carried out in Eagle's Basal Medium continaing 1.2% (w/v) BSA (see Appendix 3 for composition), in which the cells were finally resuspended for ensuing incubations at a concentration of approximately 4-5 x 10^6 cells/ml. The cells were counted with a haemocytometer (Improved Neubauer) and a trypan blue index performed by adding a drop of trypan blue solution (0.2% (w/v) in physiological saline) to a drop of cell suspension and comparing the number of those cells taking up the dye to those excluding it. Cell preparations with a trypan blue exclusion index of less than 90% were rarely obtained and never used in any of the studies of this thesis. This index was generally 92-95% and the yield of cells usually between 3-6 x 10^8 cells.

(b) Incubations of hepatocyte suspensions

Erlenmeyer flasks (25 ml) were pre-gassed with carbogen before and

addition of aliquots (usually 2 ml) of cell suspension. After addition of the cells, gassing was continued for a further five mintues. The relevant drugs were then added (never more than 0.2 ml to 2 ml cell supension) and the flasks were immediately stoppered with rubber bungs just as the gas source was removed. This was shown to be satisfactory for maintenance of pH and the partial pressures of oxygen and carbon dioxide for the duration of the incubation procedures.

After stoppering, flasks were immediately placed in a Gallenkampf Metabolic shaking bath where they were incubated at 37°C and 80 oscillations per minute for the appropriate duration. Flasks were then removed from the bath and samples taken for the appropriate assays.

(c) Potassium ion (K^+) and alanine aminotransferase (ALT)

For estimation of intracellular K^+ and supernatant ALT (L-alanine: 2 - oxoglutarate aminotransferase 2.6.1.2.), an aliquot (usually 0.5 ml) of cell suspension was centrifuged at 50xg for one minute. The resulting supernatant was decanted, and a proportion of this used for ALT analysis by the method of Reitman and Frankel (1957). This assay was generally performed on fresh samples as a decrease in activity was observed with frozen storage. This is documented in detail in Appendix 4. The remaining cell pellet was resuspended in 0.5 ml of water and precipitated with 0.5 ml of 6% (w/v) perchloric acid (PCA). Centrifugation at 3,500 r.p.m. for 10 minutes then followed. A suitably diluted portion of the resulting supernatant was used for estimation of K^+ with an Eel Model 150 Clinical Flame Photometer.

(d) Lactate, pyruvate and urea

Aliquots (1.1 ml) of cell suspension were added to an equal volume of 6% (w/v) PCA and subsequently homogenised with a Potter-Elvenhjem

homogeniser and a motor driven teflon pestle for three strokes. The samples were then centrifuged at 3,500 r.p.m. for ten minutes. The resulting supernatant was neutralized with 1 M potassium hydroxide and allowed to stand on ice for twenty minutes, after which a second centrifugation at 3,500 r.p.m. for three minutes was carried out. This final supernatant was used for determination of lactate by the method of Hohorst (1965), pyruvate following Bücher *et al.* (1965) and urea by Kaplan (1965), using his suggested modifications for samples with low levels of this compound. The development of colour due to background ammonia was checked in samples assayed for urea.

(e) Concentration of administered agents: Carbon tetrachloride, chloroform, halothane, methoxyflurane, enflurane and bromobenzene

The procedure for estimation of concentration in the cell suspension was essentially the same for all of these agents. A 1 ml sample of hepatocyte suspension was added to 1 ml of zinc reagent (100 g $ZnSO_4.7H_2O$ + 40 ml 6 M H_2SO_4, made to 1 litre with water), followed by 0.75 ml of 1 M sodium hydroxide solution and then mixed by inversion. Sample tubes were well filled and kept closed with tightly fitting lids except during the necessary additions. Standards were prepared by adding known amounts of the chemical under assay to 1 ml of hepatocyte suspension, followed by 1 ml zinc reagent and sodium hydroxide (0.75 ml). Treatment of the standards was identical to samples. After being allowed to stand for at least ten minutes, all tubes were centrifuged at 3,500 r.p.m. for fifteen minutes. Supernatant (2 µl samples, usually) was injected directly on to the column for analysis of these agents on a Becker 409 gas chromatograph equipped with a flame ionization detector. The glass column, two feet in length, contained 5% OV 210 on Varaport 30. Operating conditions

included an oven temperature of 45°C, injection port and detector temperatures of 150°C and nitrogen carrier gas at a pressure of 0.3 Kp/cm^2. A trace obtained with CCl_4 standards is shown in Fig. 1.1. All samples were analysed at least in duplicate, with the usual determination being in triplicate.

(f) Reduced glutathione (GSH)

The fluorometric method of Cohn and Lyle (1966) was used to estimate GSH content in the hepatocyte suspensions. Aliquots (0.2 ml) of cell suspension were added to 0.5 ml ice cold, 30 µM ethylenediaminetetra-acetic acid (EDTA) followed by 0.2 ml 25% (w/v) metaphosphoric acid. After centrifugation at 3,500 r.p.m. for ten minutes portions of the supernatant were estimated for GSH content by the reaction with o-phthalaldehyde (OPT). Tris buffer (0.25 M) was used to adjust the pH of the reaction mixture to 8.0.

(g) Lipid peroxidation

Estimation of lipid peroxidation in hepatocytes was via two procedures. Firstly, thiobarbituric acid (TBA)- reacting products of lipid peroxidation (commonly referred to as malonic dialdehyde - MDA) were determined by addition of 0.4 ml of hepatocyte suspension of 1 ml of 20% (w/v) trichloroacetic acid (TCA), followed by 2 ml of 0.67% (w/v) TBA, and then incubation at 100°C for 15 minutes. After centrifugation, the absorbance of the supernatant at 532 nm was measured. Results were expressed simply in terms of absorbance change due to TBA reactants (i.e. TBA reacting substances). This procedure was derived essentially from the methods of Wills (1966) and Ottolenghi (1959).

Secondly, diene conjugation, which is reflected by an increase in

absorbance of the lipids at 234 nm, was examined in cell pellets derived by centrifugation (50xg for 1 min) of 1 ml of cell suspension. The method followed was essentially that of Hogberg *et al.* (1975), except that 6 ml of chloroform : methanol was used instead of 10 ml. The absorbance of each sample was read against solvent which had been treated similarly, except for addition to the cell pellet.

(h) Aminopyrine metabolism in hepatocyte suspensions

Metabolism of aminopyrine was determined by measuring the formation of formaldehyde by the procedure of Nash (1953). Incubations were as previously described and were carried out over twenty minutes, with an initial aminopyrine concentration of 5 mM. Semi-carbazide (4.1 mM) was also added with the aim of trapping the formaldehyde. The reaction was terminated at 20 minutes by addition of 0.5 ml 20% (w/v) TCA. The resulting precipitate was spun down at 3,500 r.p.m. for ten minutes. To 1.5 ml of Nash reagent was added 1.5 ml of the supernatant followed by mixing with a vortex mixer and incubation at 50°C for 15 minutes. Absorbance of the samples was measured at 412 nm with a Unicam SP1800 within 15 minutes of the end of incubation.

(i) Adenosine triphosphate

Cellular levels of adenosine triphosphate (ATP) were determined by the firefly method described by Stanley and Williams (1969), with the modifications described by Head *et al.* (1977). Samples were prepared by addition of 0.5 ml of cell suspension to 0.5 ml 6% (w/v) PCA. After standing on ice for approximately fifteen minutes the samples were centrifuged at 3,50C r.p.m. for ten minutes. Aliquots of 20 µl of the final supernatant were added to the reagents to start the reaction. The resulting luminescence was measured with a Packard Tricarb Scintillation

Spectrometer with the circuitry set out of coincidence.

(j) Microscopy

Examination of hepatocytes for the purpose of cell counting, for appearance and for assessment of trypan blue exclusion was performed using an Olympus light microscope at 100 times magnification.

Electron microscopy* was carried out by standard methods (Glauert, 1974). Briefly, a 1 ml aliquot of cell suspension was centrifuged at 50xg for 45 seconds. The supernatant was decanted and the remaining cell pellet resuspended in Karnovsky's (1965) fixative (diluted 1:4.5 with sodium cacodylate buffer) for 4 hours at 4°C. The cells were then washed in buffer (sodium cacodylate 0.1 M adjusted to 330 m.osmols/l with sodium chloride) overnight. Post fixation for 1 hour with 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer was then carried out. Uranyl acetate (0.5%, w/v) was used for block staining. The blocks were dehydrated in alcohol and embedded in Spurr's epoxy resin. Sections of 1 μm were cut and stained with methylene blue-Azure II for light microscopy (Richardson *et al.*, 1960). Thin sections were cut with a microtome from all portions (bottom, middle and top) of the block, mounted on uncoated grids, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300.

(k) Expression of results and statistics

Results have generally been given as the mean ± s.e.m. when the number of experiments (n) used in obtaining the figure was 3 or more. If n=2, then the average of the two figures has been presented. It should also be noted that the n refers to the number of actual cell preparations

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* Electron microscopic procedures carried out by Dr J. Fanning (Department of Pathology) and Dr W. Breed (Department of Anatomy).

used in obtaining the particular value and not replicate incubations from the one preparation of hepatocytes from a single rat.

The reference point generally used for the results has been gram wet weight. Appendix 5 should be consulted for details concerning the reference and for considerations related to calculations from the raw data.

To evaluate the results once calculated, statistical procedures were used. If parametric statistics were applicable, as determined by the F-test, then data was analysed by a student's t-test (Clarke, 1969). Either a paired or an unpaired test was used, depending on which was appropriate for the particular evaluation. Similarly, the test was two-tailed if the nature of the change was not predicted, but one-tailed if a shift in a particular direction was included in the working hypothesis.

If non-parametric statistics were indicated, a Rank Sum test was used (Hoel, 1960).

Any other more specific tests used will be documented in the appropriate place.

A significance level of $p<0.05$ was used as the probability that an observed difference was not due to chance.

CHAPTER ONE

TOXICITY OF CARBON TETRACHLORIDE IN ISOLATED RAT HEPATOCYTES

INTRODUCTION

Carbon tetrachloride is the prototype of direct hepatotoxins (Zimmerman, 1968) and the most studied of hepatotoxic agents (Zimmerman, 1976; Recknagel, 1967). For these reasons CCl_4 was chosen for initial toxicity studies with the isolated hepatocyte preparation.

Manifestations of CCl_4 toxicity seen clinically include hepatic and renal failure, neurologic and gastrointestinal effects, a degree of vascular collapse, cardiac arrhythmias, cardiac failure and pulmonary oedema (Zimmerman, 1976). Renal failure may well be the cause of death after CCl_4 in some cases (Editorial, 1970). It has been suggested that the kidney and liver lesions are independent (Wolman, 1975; Editorial, 1970). This illustrates the danger of taking the death of an animal as the biological end point measurement in hepatotoxic studies with CCl_4.

The historical aspects of hepatotoxins in general, and CCl_4, itself, have been reviewed in recent years (Reckangel, 1967; Zimmerman, 1968; Farber, 1971; Zimmerman, 1976). The original evidence of hepatic damage was the histological appearance of lipid accumulation and necrosis. The characteristics of these changes have been discussed in detail by Rouiller (1964).

The time sequence of events occurring in response to CCl_4 has been summarized by Recknagel (1967) and Zimmerman (1976). The earliest changes detectable include a disturbance in lipid metabolism, a decrease in protein synthesis and a number of aspects of microsomal metabolism (e.g. glucose-6-phosphatase, cytochrome P_{450}, aminopyrine demethylase) and alterations in the ultrastructure of the endoplasmic reticulum (ER). Such alterations in the ER include a dilation and vesiculation of the

cisternae of the rough endoplasmic reticulum (RER), a loss of orderly ribosomal arrays and a dispersal of ribosomes from the membrane surface (Smuckler and Arcasoy, 1969). There is also a marked alteration in the polysomal profile after CCl_4, as mentioned by Smuckler and Arcasoy (1969) and others (de Ferreyra *et al.*, 1975). The changes in polyribosomes and protein synthesis seen with CCl_4 treatment (Tilzer *et al.*, 1975; Gravela and Dianzani, 1970; Gravela *et al.*, 1971) are consistent with the ultrastructural alterations in the RER. Similarly, changes in the appearance of SER under the EM are consistent with the alterations in the biochemical function of this organelle (Reynolds and Ree, 1971).

Following the early changes, an increase in Ca^{++} content of liver becomes apparent 2 to 4 hours after dosage. This is accompanied by a disturbance in electrolyte balance (loss of intracellular K^+) and a swelling of treated livers as water accumulates. Liver glycogen is depleted. Lysomal disruption occurs between 5 and 10 hours and intracellular enzymes (e.g. ALT) appear in the plasma. Mitochondrial damage is seen at 10 to 20 hours. Centrilobular pre-necrotic change is evident at about 12 hours with marked centrilobular necrosis having developed by 24 hours.

Zimmerman (1976) has discussed some of the more general factors related to CCl_4 induced toxicity such as species, age and sex of the animal, and dietary effects. Others have reported on various effects of these parameters (Sasame *et al.*, 1968; Cawthorne *et al.*, 1970; McLean, 1967; Taylor and Tappel, 1976).

The mechanism by which CCl_4 leads to its injurous effects on hepatocytes has been the subject of numerous investigations. The

development and eventual disproving of earlier hypotheses for toxic mechanism has been discussed in detail by Recknagel (1967). These included the phopholipid, mitochondrial and catecholamine hypotheses. The same author has also presented valid reasons for the observed decrease in protein synthesis not being causative in the eventual necrosis. Zimmerman (1976) has also discussed some of the earlier areas of research, including the idea that necrosis may have resulted from centrizonal ischemia, this having been caused by swollen parenchymal cells. Studies with isolated perfused liver were instrumental in disproving such a hypothesis, and also in studies involving the effects of catecholamines. This provides an example of how the application of a relatively new biological preparation to an area of research can provide definitive answers to current problems.

Another of the theories of CCl_4 toxicity related to the lipid solvent properties of this chemical (Recknagel, 1967). Slater (1966) put forward several questions concerning the necrogenic activity of CCl_4 which are very difficult to answer if toxicity is to be explained by solvent action alone. This has been discussed by other authors as well (Recknagel and Glende, 1973; Zimmerman, 1976). Such questions along with subsequent studies have been the focal point in development of the idea that it is a metabolite of CCl_4, and not the parent compound itself, which is responsible for the toxic actions of CCl_4. In fact, Recknagel and Glende (1973) and Zimmerman (1976) state that it is 'abundantly clear' and 'conclusive', respectively, that it is a metabolite of CCl_4 responsible for hepatic injury. Zimmerman (1976) also points out, however, that non-metabolized CCl_4 may contribute to the injury. Studies with isolated membranes have also shown a direct effect of CCl_4 itself and it has

been suggested that the observed effects may account for, in part, the toxic effects of CCl_4 observed *in vivo* (Dorling and Le Page, 1972; Rufeger and Frimmer, 1976).

The subsequent studies referred to above in relation to toxicity via a metabolite include potentiation of toxicity with agents that increase activity of the smooth endoplasmic reticulum (e.g. phenobarbitone, DDT - see Chapter 4) and reduction in toxicity by agents which reduce microsomal metabolism (e.g. cobalt, dibenamine, CCl_4 itself - see Chapter 2). Studies of this nature have also provided information on the actual locus of metabolism. It appears likely that this is at the cytochrome P_{450} site (Recknagel and Glende, 1973; Sipes *et al.*, 1977) and that the process is reductive rather than oxidative (Sipes *et al.*, 1977; D'Acosta *et al.*, 1975). Earlier workers had indicated that the site of activation may have been the NADPH cytochrome c reductase (Slater and Sawyer, 1971a). Others have suggested that metabolism of CCl_4 may occur at the two sites on the NADPH cytochrome P_{450} chain (Shah and Carlson, 1975). The nature of the reactive metabolite is thought to be a free radical. This was originally proposed by Butler (1961) and the evidence for this comprehensively reviewed by Recknagel and Glende (1973).

The events which occur after formation of the active metabolite and which are responsible for the toxic effects form an area of some dispute.

Steatosis and necrosis have come to be recognised as independent manifestations of CCl_4 hepatotoxicity (Zimmerman, 1976). The development of steatosis is due to a blocking of movement of fat out of the cell. The mechanism for coupling triglycerides to the appropriate apoprotein to form lipoprotein carrier molecule is disrupted. This toxic mechanism has

been clearly elucidated (Recknagel, 1967; Zimmerman, 1976). The pathogenesis of necrosis, however, is still unclear. Difficulties associated with studies in this area are documented by Judah *et al.* (1970).

More recent studies have been concentrated in the area of early injury, in an effort to determine initial events responsible for eventual cell damage. Such studies have shown that lipid peroxidation and binding of ^{14}C from labelled CCl_4 to microsomal and mitochondrial lipids and proteins occur very early (Recknagel and Glende, 1973). Gordis (1969) found that metabolites associated with binding of ^{14}C and ^{36}Cl, from doubly labelled CCl_4, into microsomal lipids comprised a heterogeneous group of branched long chain chlorinated fatty acids. Such changes occur prior to other detectable biochemical alterations, suggesting an involvement of these processes in the initiation of toxic response. This relates to the specific area of dispute with respect to the mechanism of toxicity. Some workers postulate lipid peroxidation as causative in the toxic response while others suggest that it only accompanies toxicity rather than having a pathogenic role of importance (Zimmerman, 1976). This area will be covered in greater detail in Chapter 3.

The initial aims of the thesis, and those of this chapter, were to determine whether or not there was a qualitative toxic response of the isolated hepatocytes to carbon tetrachloride and if so to quantitate the response from the dose and temporal viewpoints. The quantitative data would also serve to allow selection of an appropriate dose of CCl_4 and incubation time for planned subsequent studies.

METHODS

Rat liver parenchymal cells were isolated from rats weighing between 200 and 260 g. Incubation conditions were as described in the General Methods, with CCl_4 doses (10, 15 or 20 µl) being added to side arms which had been previously attached to the 25 ml Erlenmeyer flasks (Kenco glassblowers). This method of addition allowed the CCl_4 to enter the cell suspension via the vapour phase, which produced a less variable response at lower dose levels than when CCl_4 was added directly to the suspension (empirical observation from preliminary experiments).

Duration of incubation was generally up to 20 min. At the appropriate times, samples were taken for assessment fo toxicity by measurement of K^+, ALT, ureogenesis and L/P. Owing to the indirect method of CCl_4 addition some samples were also assayed for actual concentration of this chemical. Hepatocytes from some incubations were also prepared for electron microscopy (EM). Samples incubated under some other conditions (5 µl dose for 15 or 60 min, 10 µl for 60 min and 15 µl for 30 min) were carried out to provide samples for EM. Assessment of K^+ and ALT was also performed on such samples.

RESULTS

The level of CCl_4 in the suspension was found to increase with the dose over the range used in these experiments (Table 1.1). Rapid partitioning between gas and medium is also indicated by these results, as equilibration had apparently been reached within 5 min of incubation. A trace obtained from the gas chromatographic assay of CCl_4 standards (2.5-0.5 µl/ml) is illustrated by Fig. 1.1.

TABLE 1.1

CONCENTRATION OF CCl_4 [a] IN HEPATOCYTE SUSPENSIONS

DOSE (µl)	MINUTES OF INCUBATION			
	5	10	15	20
10	8.9 ± 0.9	10.0 ± 1.2	8.8 ± 0.3	9.7 ± 1.2
15	11.8 ± 2.7	14.1 ± 0.2	14.0 ± 0.3	14.5 ± 2.4
20	17.0 ± 5.0	18.0 ± 2.6	17.8 ± 1.7	16.6 ± 0.7

[a] CCl_4 was added to the side arm of the incubating flasks at 0 min incubation. Values shown are the mean ± s.e.m. (n=3) of the CCl_4 concentration (mM) in the cell suspension.

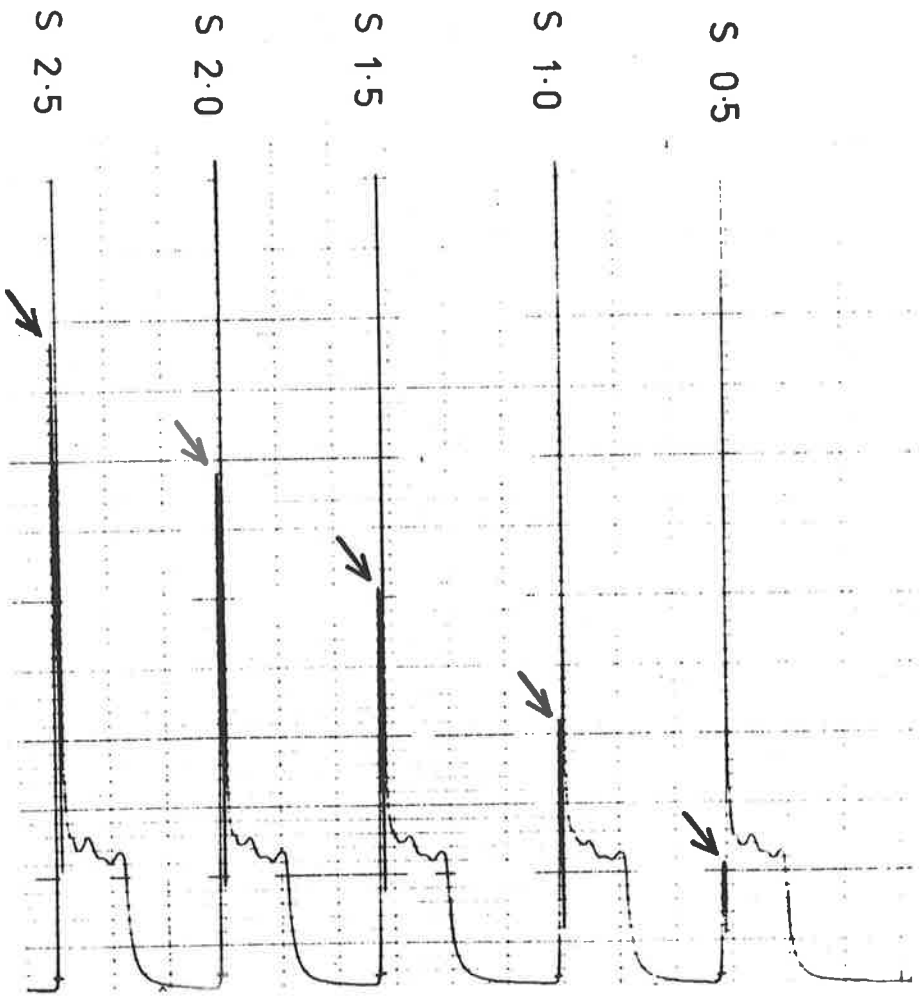


FIG. 1.1 Tracing from gas chromatograph showing peaks (arrowed) obtained from CCl_4 standards (0.5 → 2.5 µl/ml, shown as S 0.5 → S 2.5).

The loss of K^+ from the cell pellet and ALT into the suspending medium is indicated in Figs. 1.2 and 1.3, respectively. All treatment regimes yielded a significant (statistically) loss of K^+. No ALT was released at 5 min incubation or with the 10 µl dose (5-20 min); all other time/dose combinations showed a significant increase in supernatant ALT. The release of ALT was clearly dose and time related and, although not as marked, a similar trend was seen with the K^+ response.

Metabolic function of the hepatocyte suspensions was disturbed by CCl_4, as indicated by the decrease in ureogenesis (Fig. 1.4) and the increase in L/P (Fig. 1.5). A virtually superimposable pattern of decrease in urea production was seen with each of the doses used. None of the doses at 5 min and only the 20 µl dose at 10 min incubation caused a significant reduction in ureogenesis. A dose and time relation similar to the ALT response was seen with L/P. The 10 µl doses at 15 and 20 min incubation caused a significant rise in L/P as compared to the relevant controls. The 15 and 20 µl doses caused an increase at the three longer incubation times, but not at 5 min.

Incubation with a 5 µl dose of CCl_4 caused no change in structural parameters even after 60 min incubation. A dose of 10 µl gave an increased loss of K^+ (30.7 µmol/g wet wt more than controls) and a loss of ALT (control 31, treated 125 I.U./L) over this 60 min period in the single experiment where these conditions were employed. Changes in structural parameters after 30 min with a 15 µl dose of CCl_4 are presented in Chapter 3.

In a single experiment, ATP was found to drop from 2.69 to 0.16 µmol/g wet wt on treatment with 15 µl CCl_4 over a 15 min incubation,

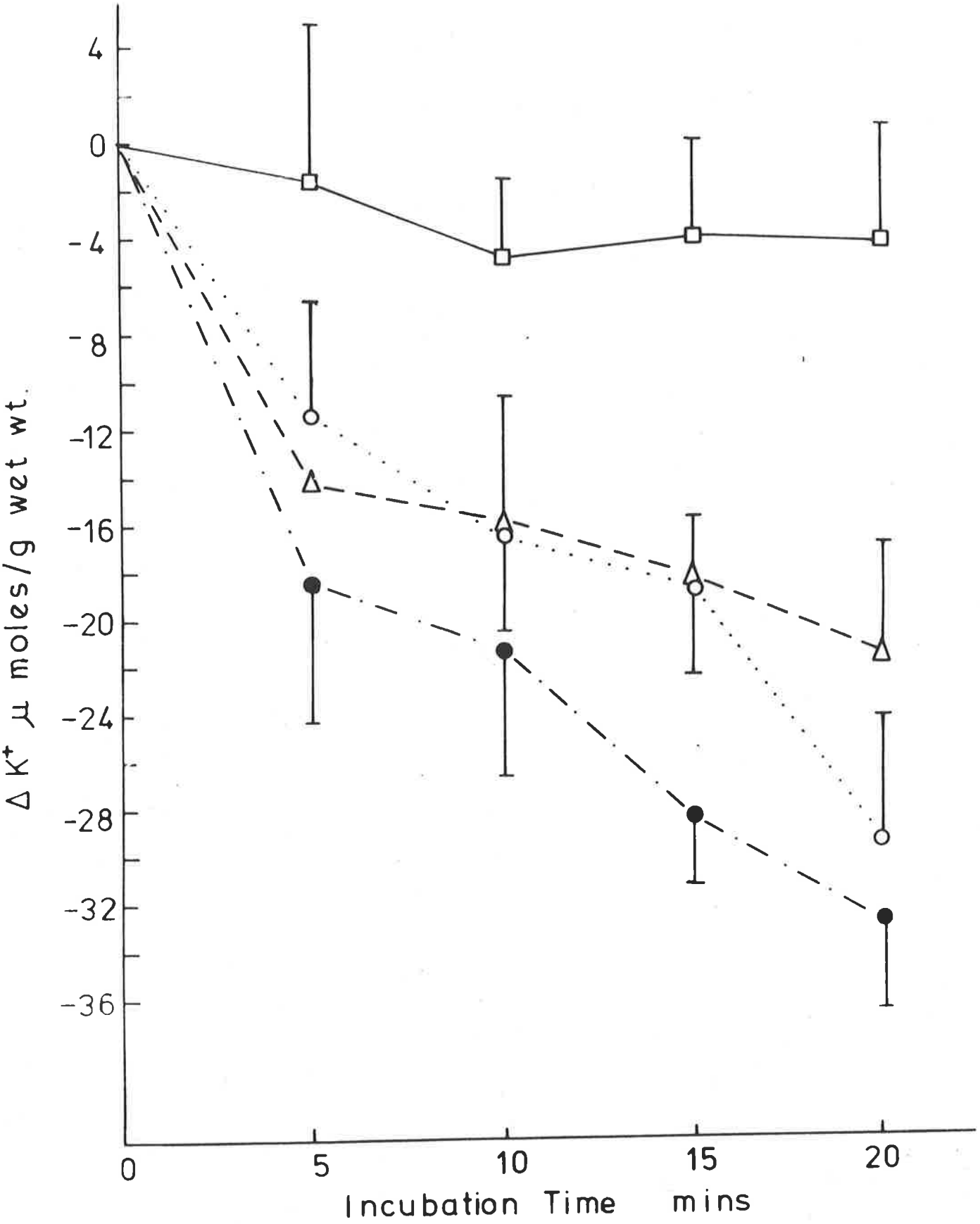


FIG. 1.2 Loss of intracellular K^+ in hepatocyte suspensions exposed to CCl_4 (10 µl --Δ--; 15 µl ··O··; 20 µl ·-●-·; controls -□-). Each point represents the mean of 4 experiments, and the s.e.m. is indicated by bars (some have been omitted for the sake of clarity).

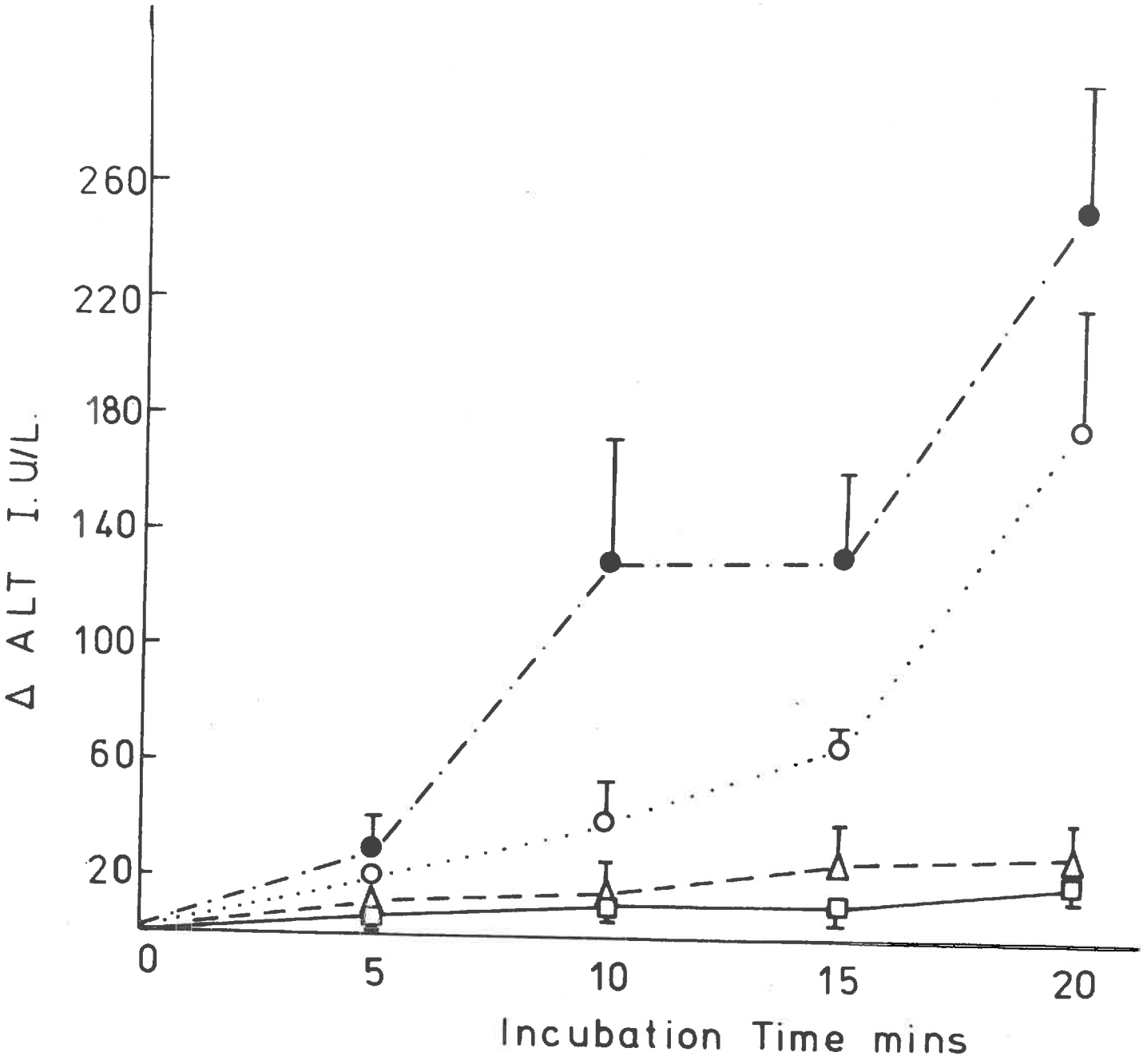


FIG. 1.3 Release of ALT into the medium from hepatocyte suspensions exposed to CCl_4 (10 µl --Δ--; 15 µl ··O··; 20 µl ·-●-·; controls -□-). Each point represents the mean of 4 experiments, and the s.e.m. is indicated by bars (some have been omitted for the sake of clarity).

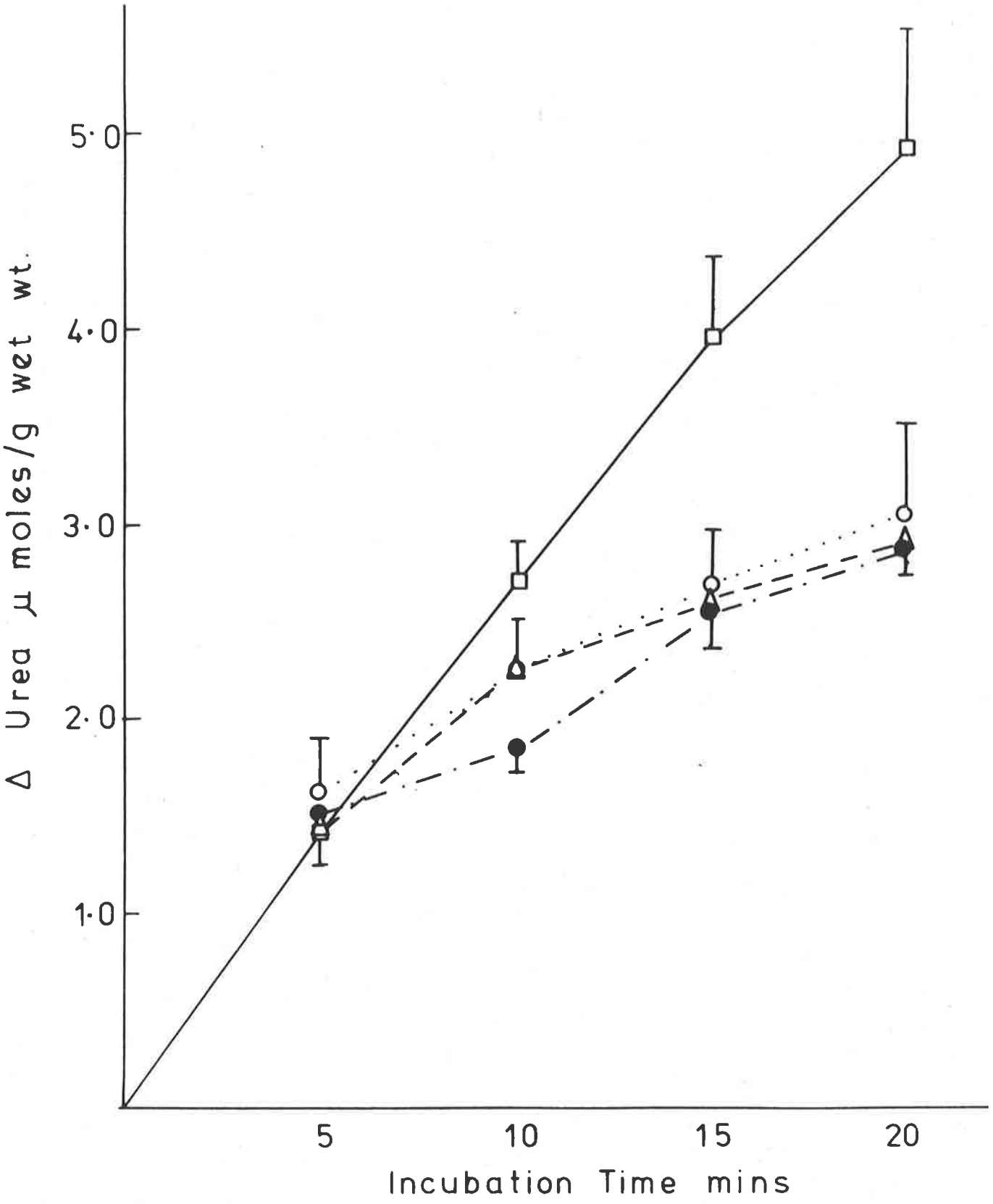


FIG. 1.4 Ureogenesis in hepatocyte suspensions exposed to CCl_4 (10 µl --Δ--; 15 µl ··○··; 20 µl ·-●-·; controls -□-). Each point represents the mean of 4 experiments, and the s.e.m. is indicated by bars (some have been omitted for the sake of clarity).

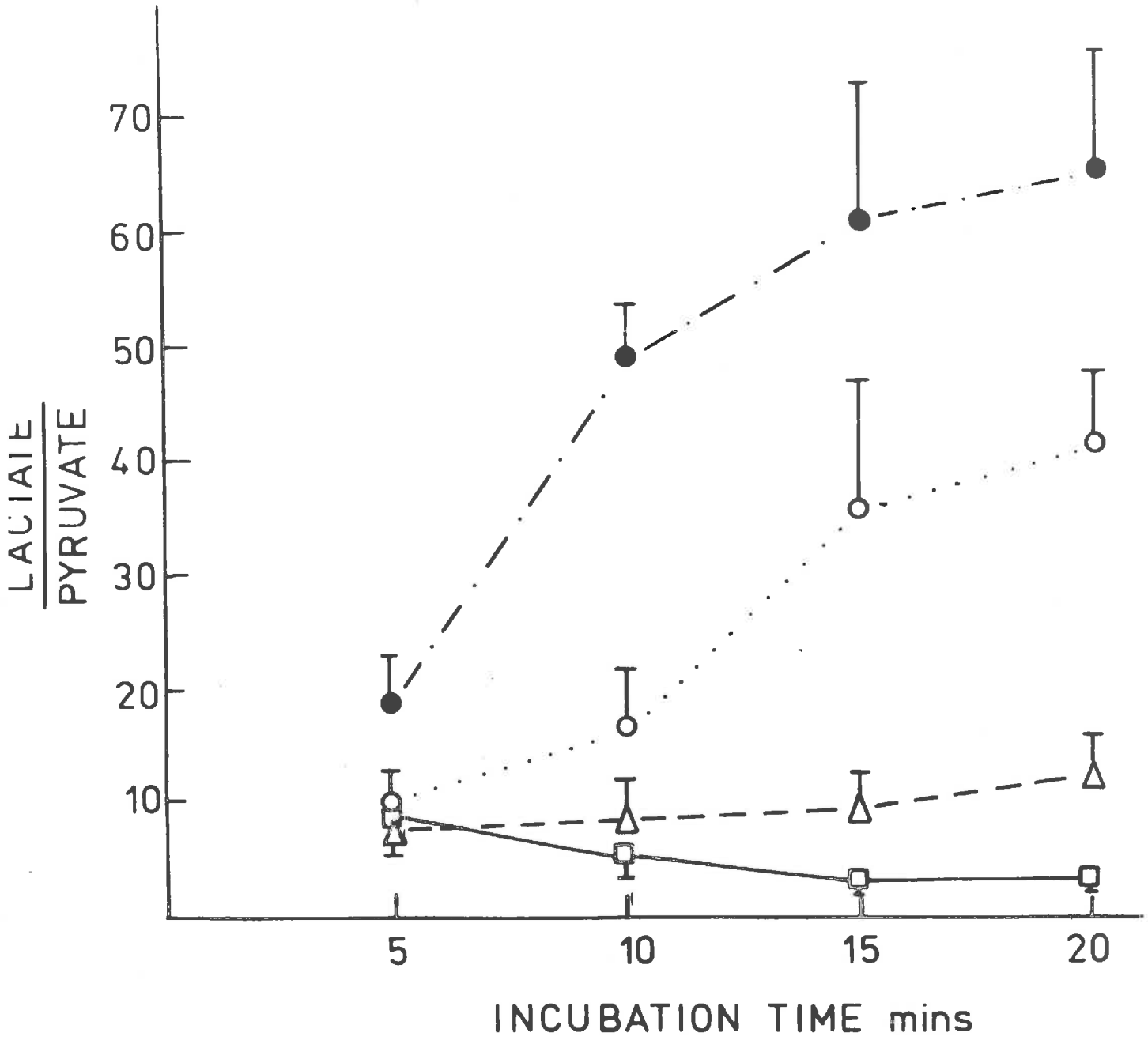


FIG. 1.5 Lactate/pyruvate ratio in hepatocyte suspensions exposed to CCl_4 (10 µl --Δ--; 15 µl ··O··; 20 µl ·-●-·; controls -□-). Each point represents the mean of 4 experiments, and the s.e.m. is indicated by bars (some have been omitted for the sake of clarity).

while the controls remained stable.

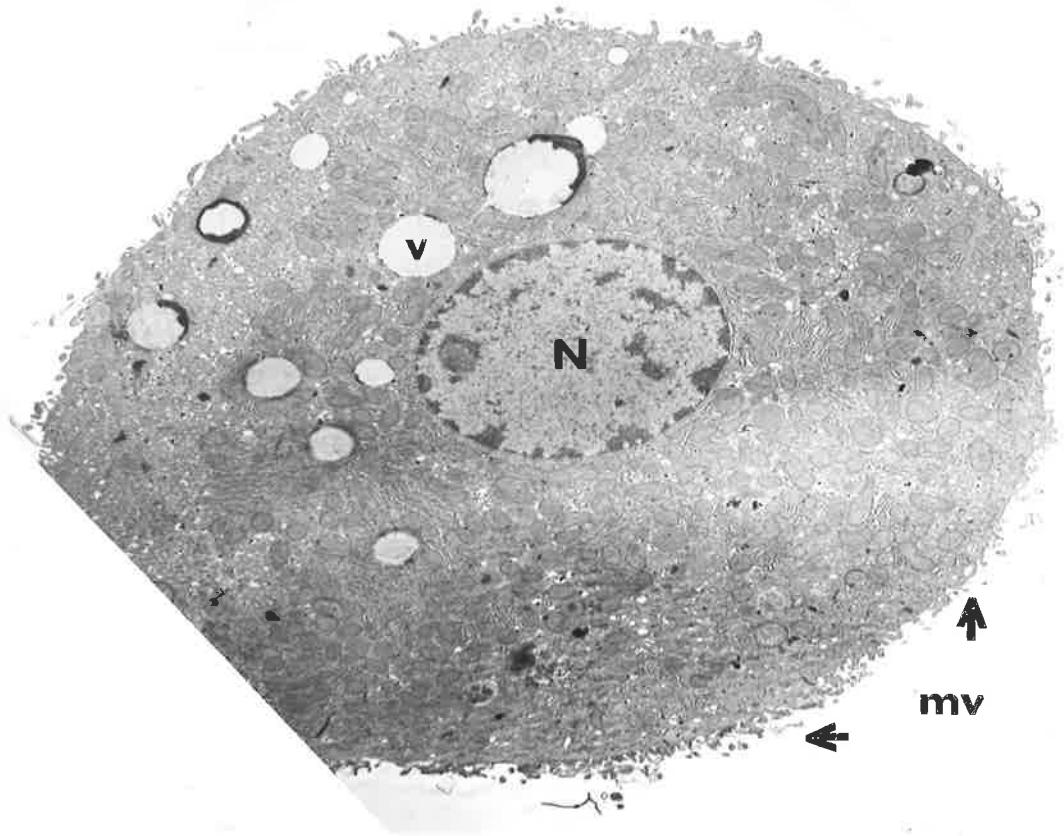
Electron microscopic examination of cells incubated with and without CCl_4 shows, generally, differences in, the number of microvilli along the cell membrane, the appearance of rough endoplasmic reticulum (RER) and mitochondrial appearance. Figs. 1.6 and 1.7 show cells incubated as controls for 15 min. Non-incubated cells are not shown because they are quite similar to those incubated as controls. Indeed, cells that had been incubated seemed to have a slightly better overall appearance. Extension of the period of incubation to 60 min did not cause any outstanding changes in the appearance of control hepatocytes (Figs. 1.8, 1.9).

The effects of 15 min incubation with CCl_4 (15 µl) are demonstrated in Fig. 1.10. Most noticeable in comparison to Fig. 1.7, the relevant control, is the loss of microvilli and apparent regions of discontinuation of the outer membrane, mitochondrial changes (especially the vacuolization), the formation of lamellar structures, which are associated with some of the mitochondrial vacuoles as well as those seen extramitochondrially, and the dilation, loss of orderly array and degranualtion of the RER. There is also a dilation of the nuclear double membrane. Continuation of incubation through to 30 min with CCl_4 leads to many more cells showing a very marked degeneration as in Fig. 1.11. The largest structure in Fig. 1.11 is a cytoplasmic protrusion, many of which have appeared after this 30 min period with CCl_4 (15 µl). More detailed examination of such a process (Fig. 1.12) reveals that it may contain large numbers of ribosomes which had apparently become detached from the RER.

After only 5 min incubation with 15 µl CCl_4 the changes are not as

FIG. 1.6 Transmission electron micrograph (TEM) of a rat hepatocyte isolated and then incubated for 15 min under control conditions (see General Methods). Note the nucleus (N), the microvilli (mv) covering the perimeter of the cell and the vacuoles (v) in the cytoplasm. Regular arrays of rough endoplasmic reticulum (rer) and mitochondria of normal appearance can be seen. These are better illustrated under higher magnification, as shown in Fig. 1.7. x 6,600.

FIG. 1.7 TEM of part of an isolated rat hepatocyte under similar conditions to Fig. 1.6. Areas of smooth endoplasmic reticulum (ser) and rer are shown. Mitochondria (M) are indicated. x 30,000.


v
N
mv

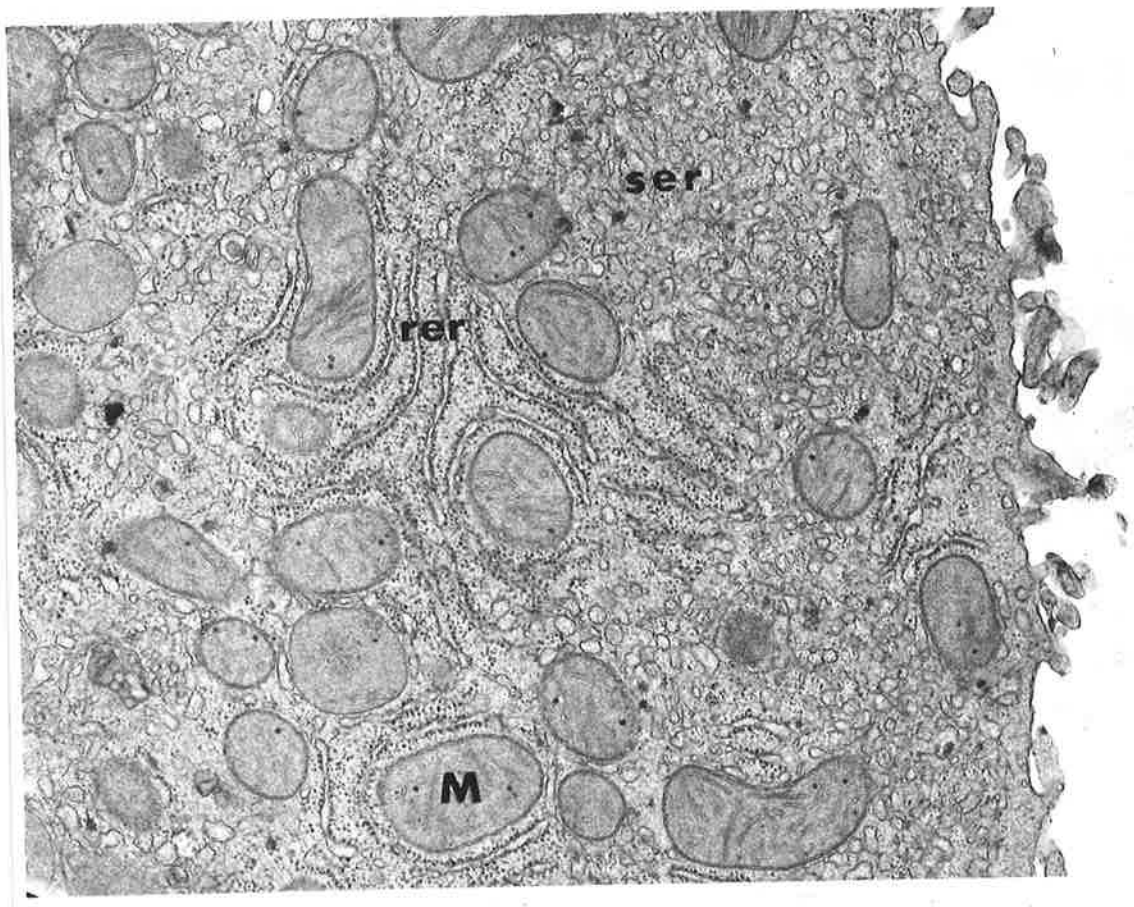
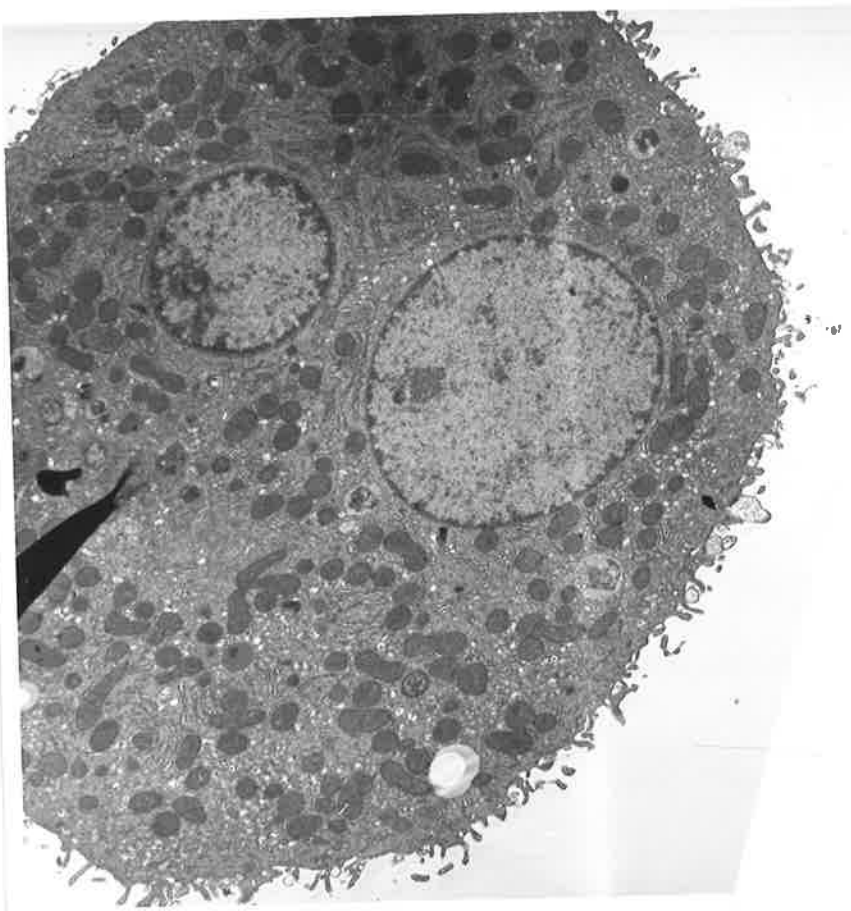

ser
rer
M

FIG. 1.8 TEM of an isolated rat hepatocyte under conditions as in Fig. 1.6 except that incubation was over 60 min. The substructure is still well preserved. Note the disappearance of vacuoles. x 6,600.

FIG. 1.9 TEM of part of an isolated rat hepatocyte under conditions as in Fig. 1.8. Note the nuclear double membrane (arrowed). x 30,000.



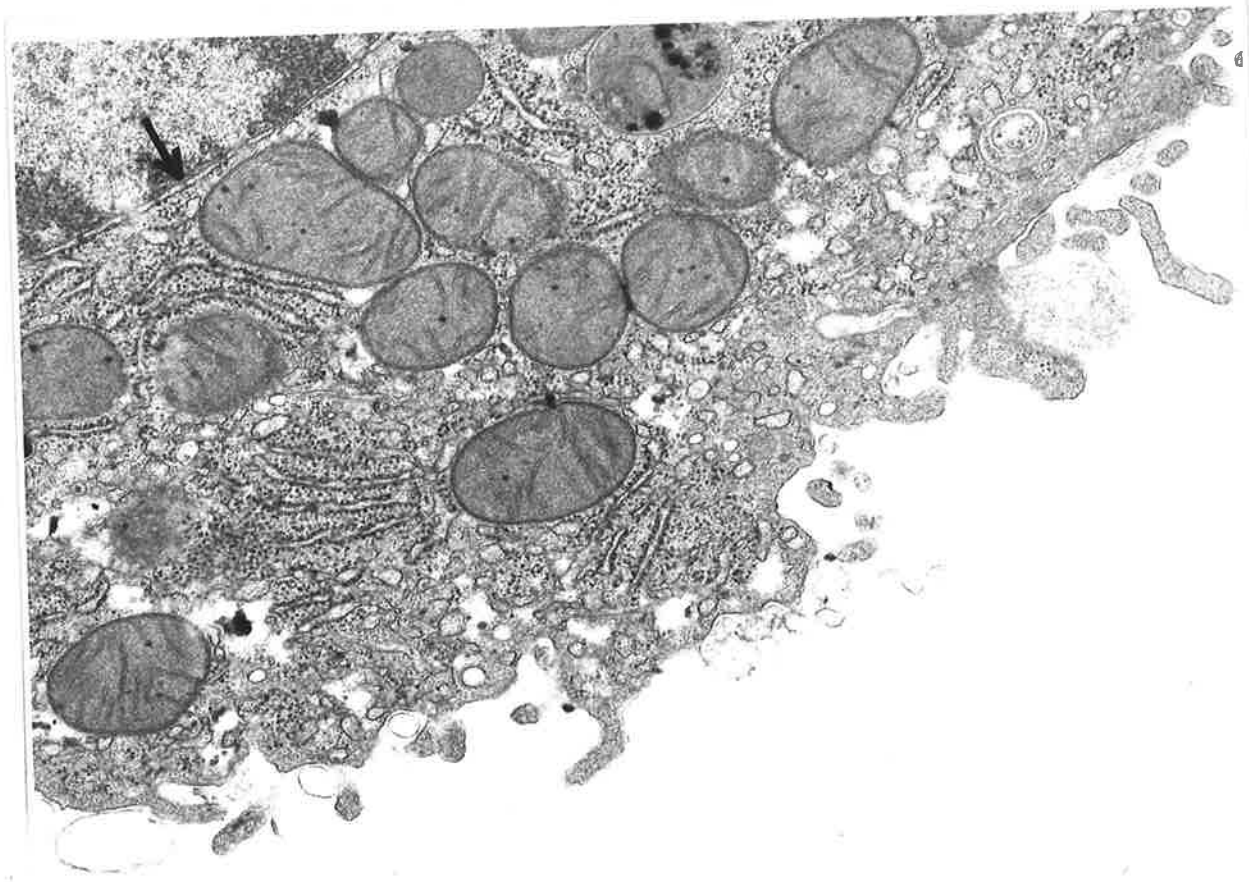
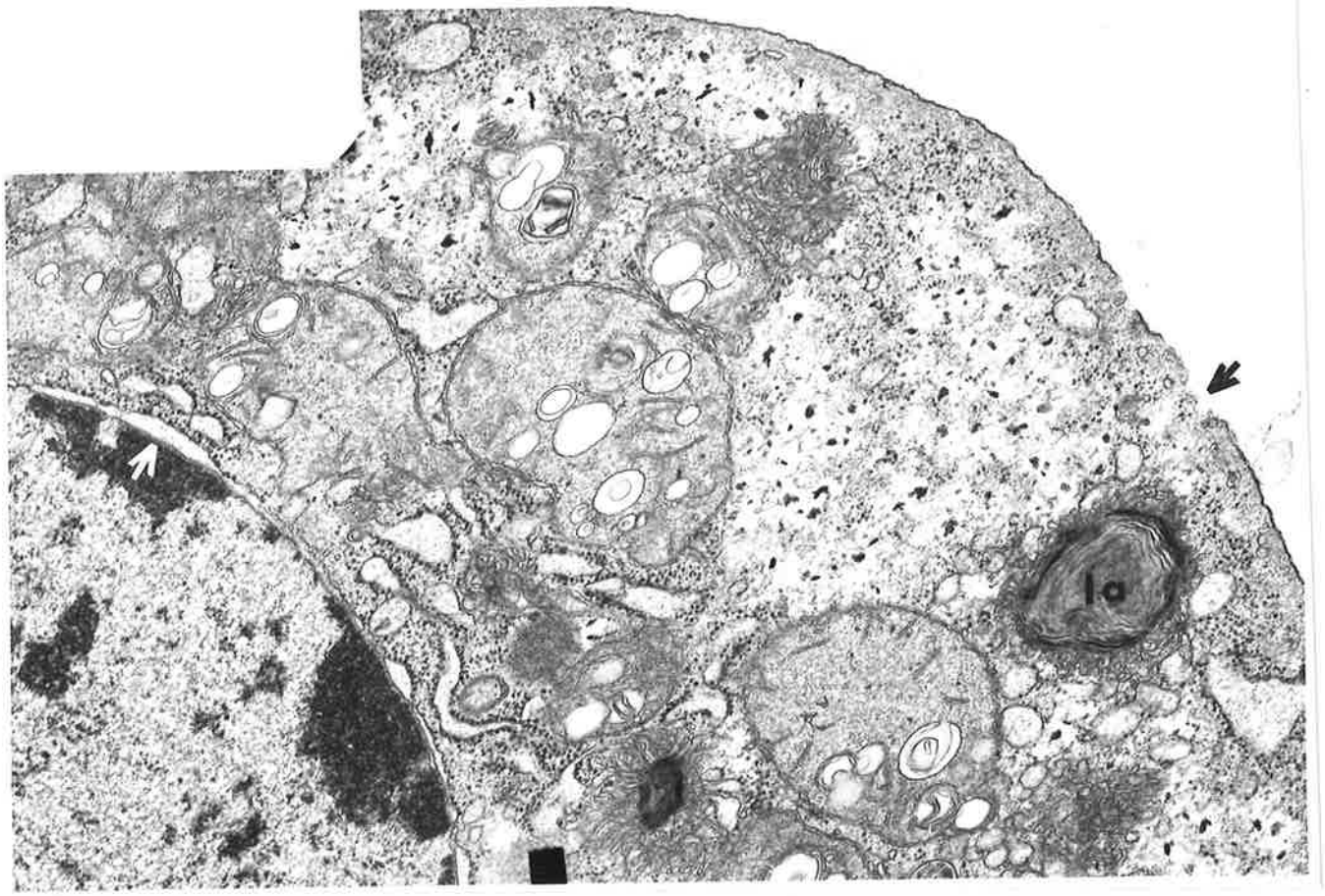


FIG. 1.10 TEM of part of an isolated rat hepatocyte incubated for 15 min with 15 µl CCl_4 added to the side arm of the vessel. Note a loss of microvilli, areas of discontinuity of the cell membrane (dark arrows), dilation, degranulation and loss of organization of rer, dilation of the nuclear double membrane (white arrows), appearance of lamellar formations (la) and vacuoles in the mitochondria. x 30,000.

FIG. 1.11 TEM of parts of four separate hepatocytes incubated as in Fig. 10 except that incubation with CCl_4 was over 30 min. Note the marked degeneration of three cells and the large granular structure which is a protrusion from the fourth hepatocyte. x 6,600.


la

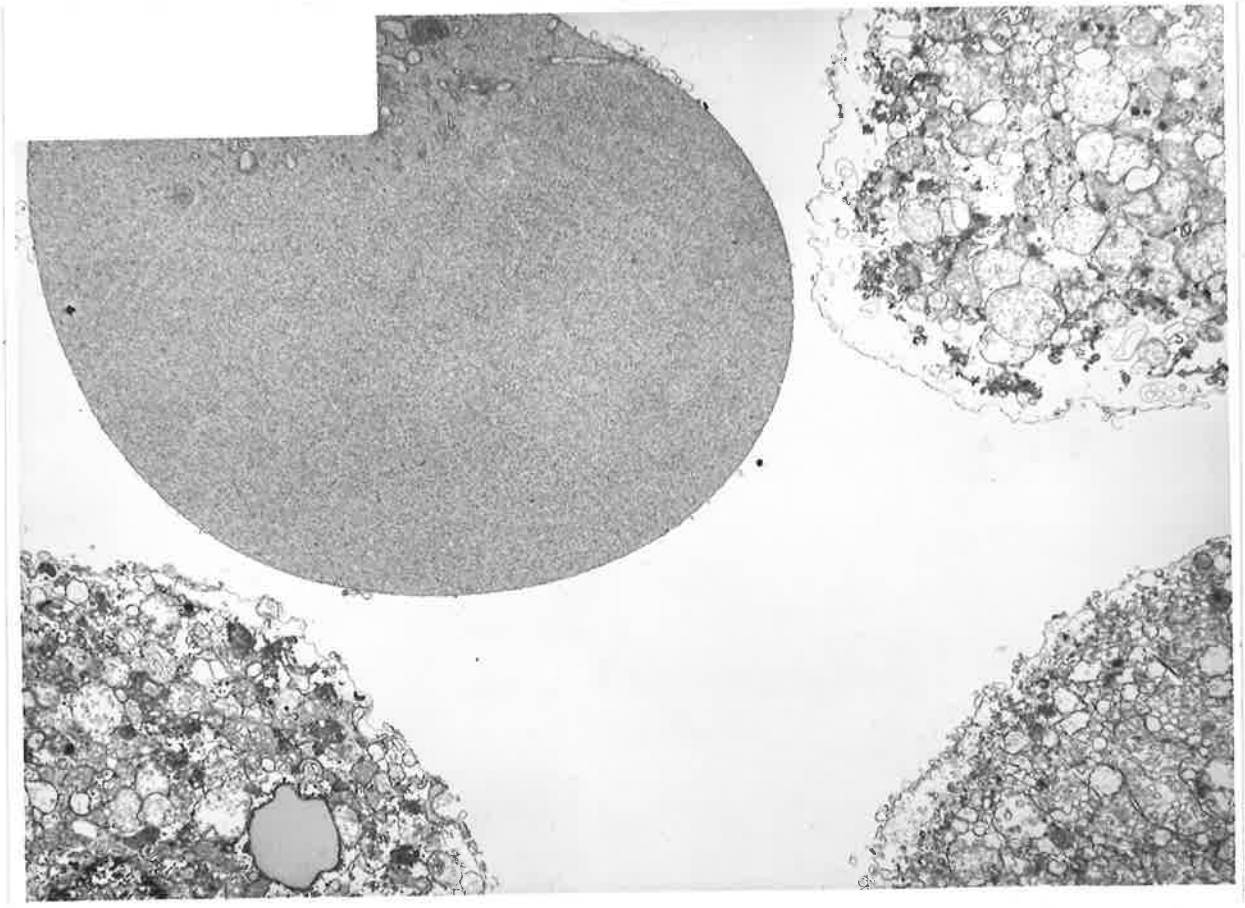
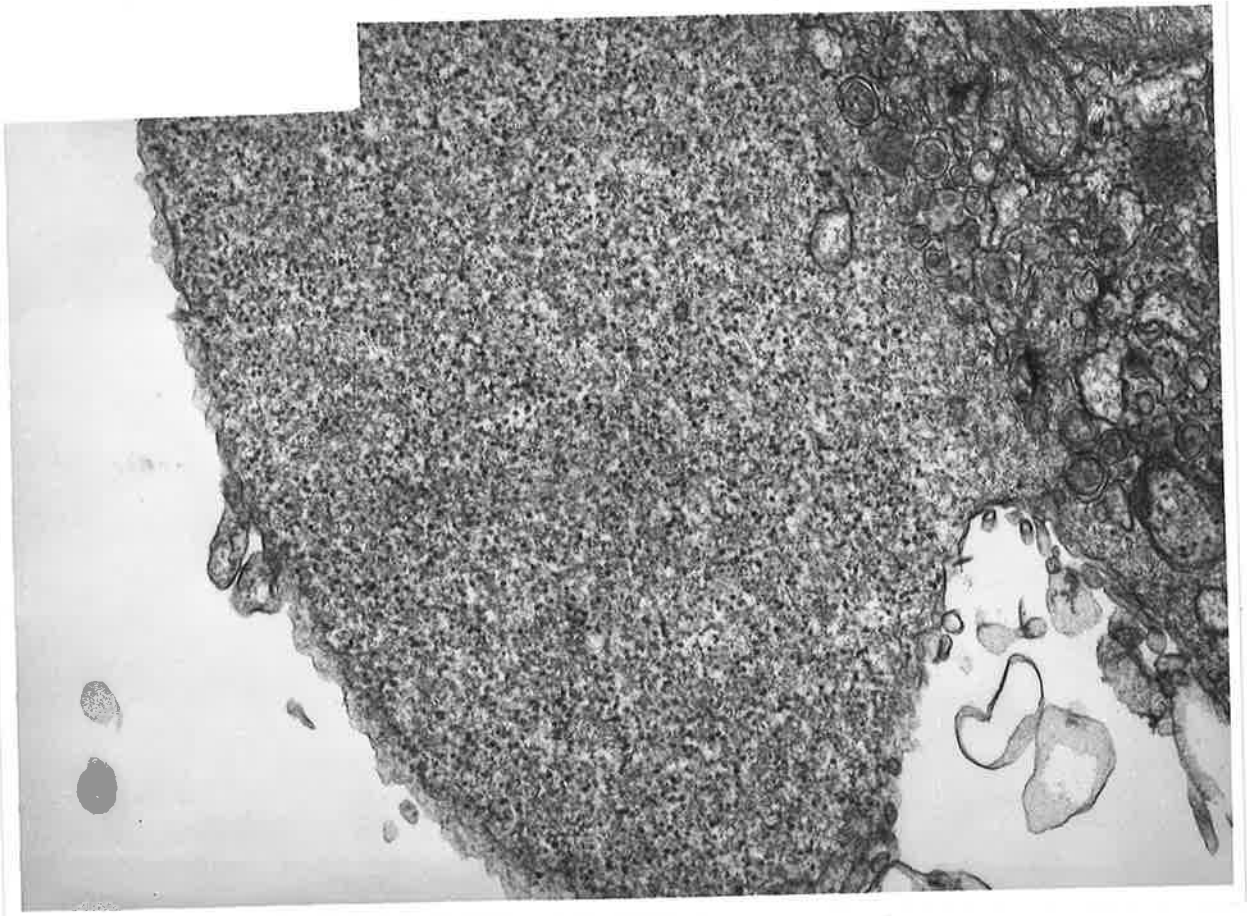


FIG. 1.12 TEM of a cytoplasmic protrusion produced in response to 15 µl CCl_4 over 30 min incubation. x 40,000.

FIG. 1.13 TEM of an isolated rat hepatocyte treated as in Fig. 1.10 except that incubation was for 5 min only with CCl_4. Note the loss of microvilli. x 6,600.



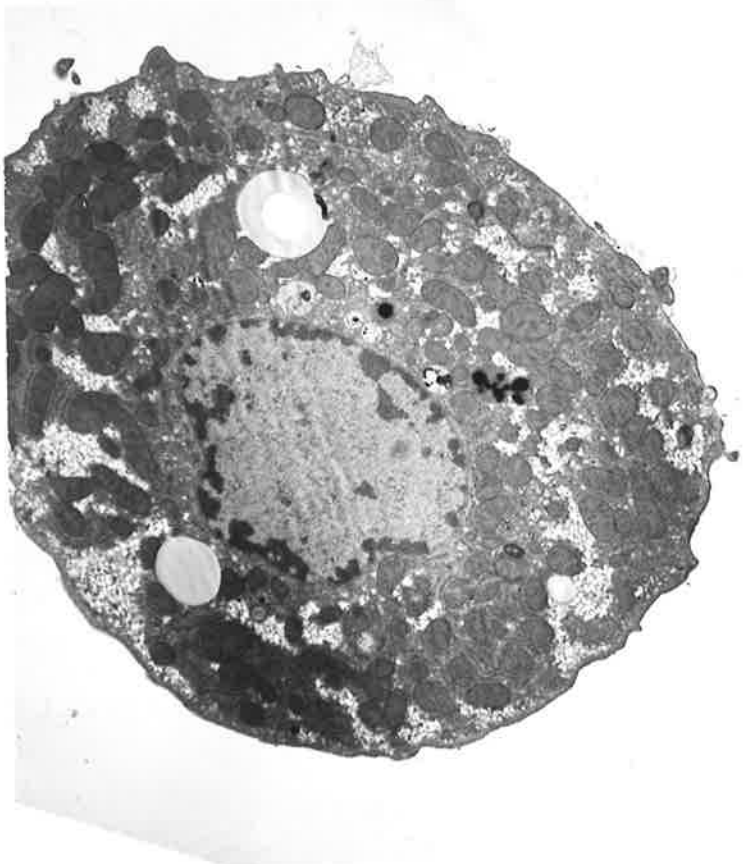
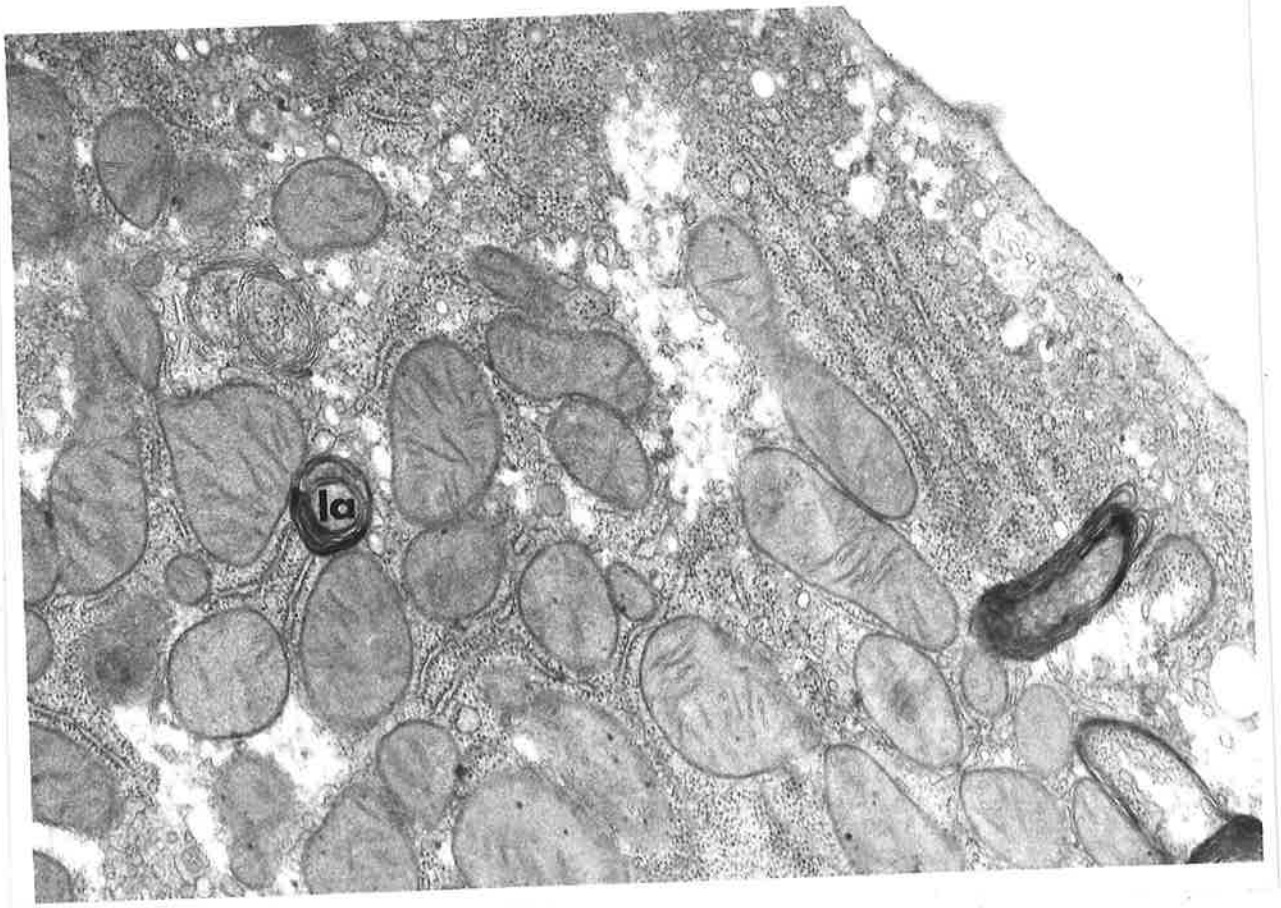
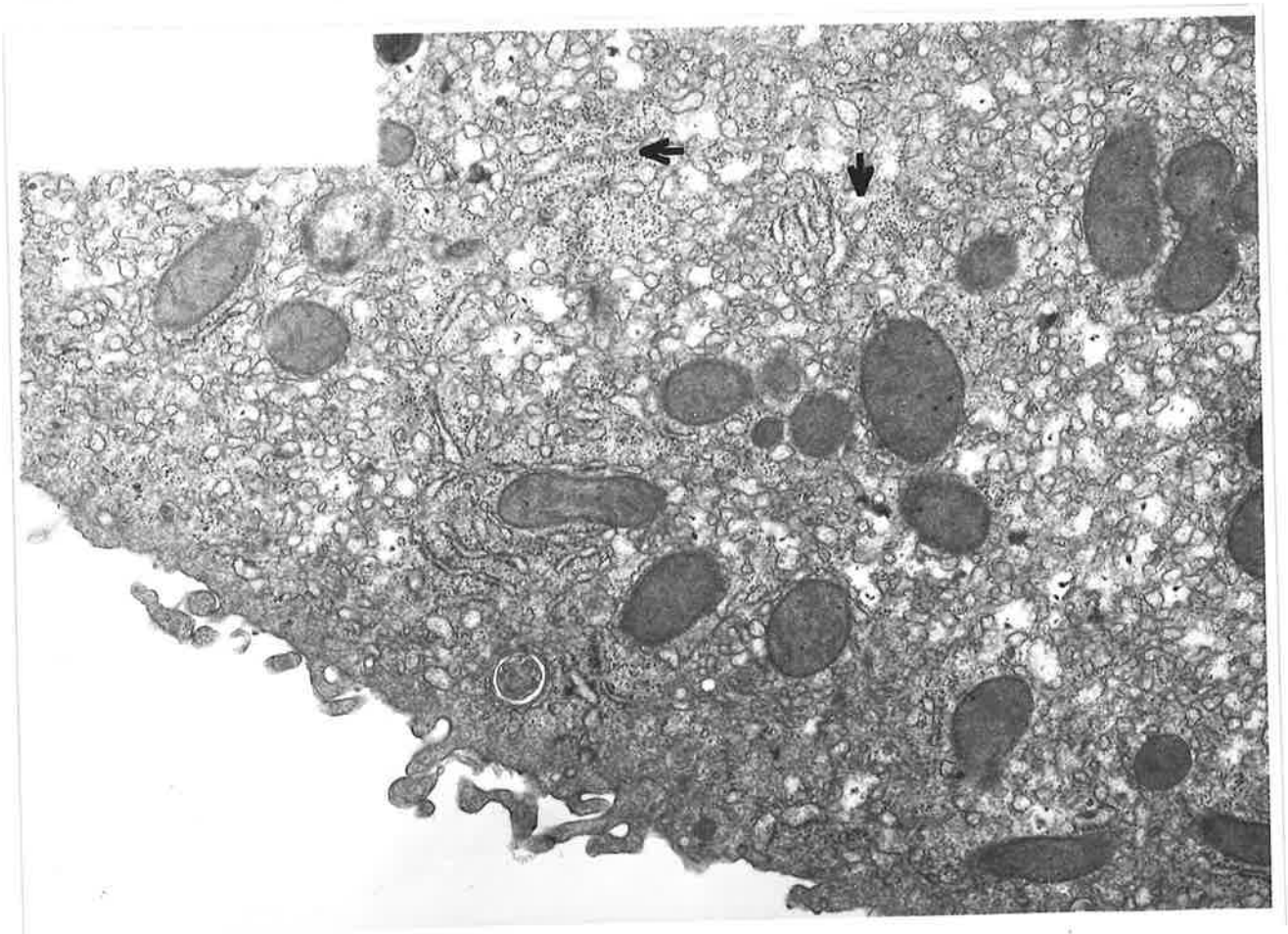


FIG. 1.14 TEM of part of an isolated rat hepatocyte under the same conditions as Fig. 1.13. Loss of microvilli is again apparent. Note also lamellar formations (la) and degranulation of the rer. x 30,000.

FIG. 1.15 TEM of part of an isolated rat hepatocyte after 60 min incubation with 5 µl CCl_4. Note that microvilli are still present, but there is loss of orderly array of the rer (arrows). x 20,000.


la



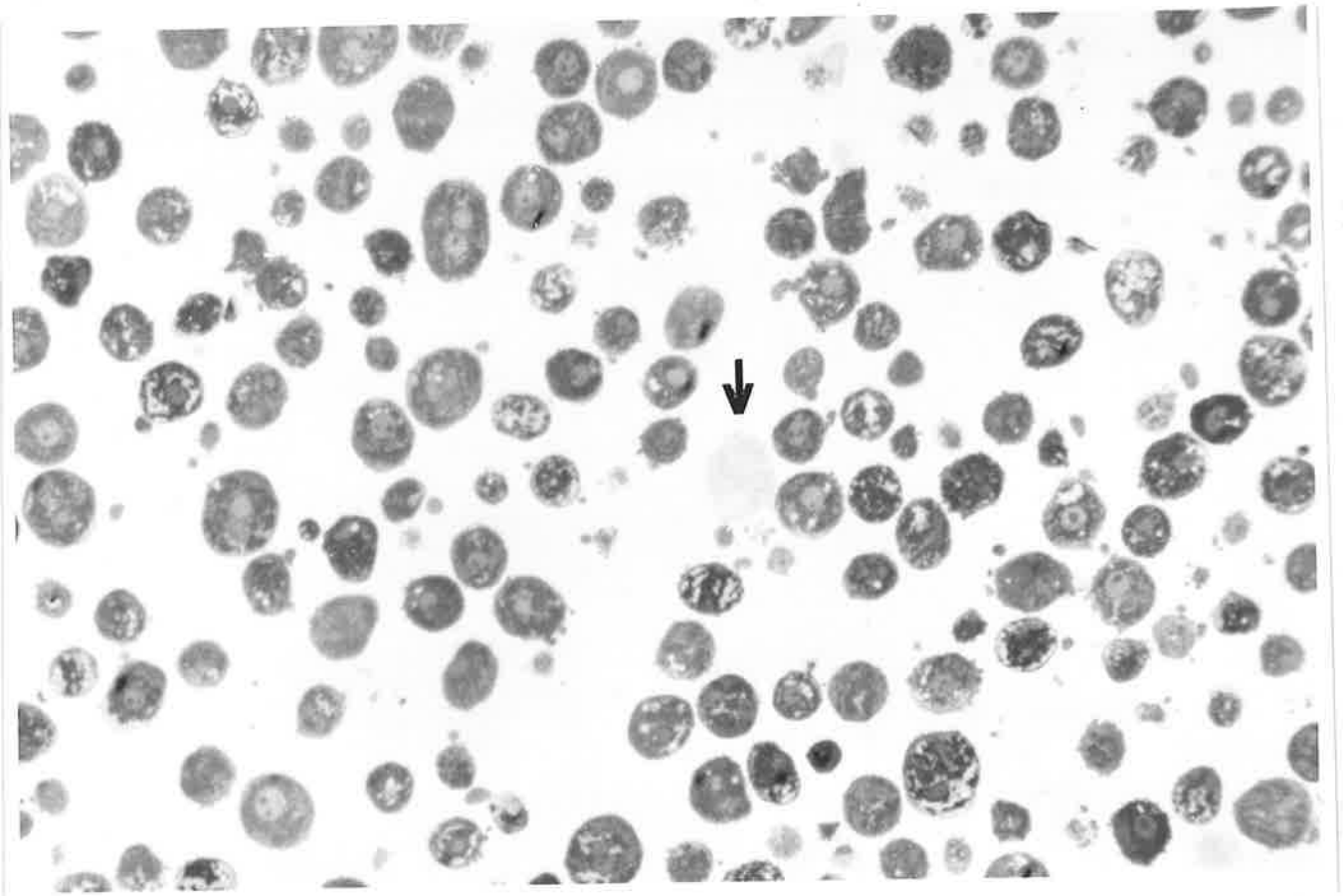


FIG. 1.16 Light micrograph of a section of isolated rat hepatocytes incubated under control conditions for 15 min. Note the rounded appearance of the cells. The arrow indicates a non-viable cell.

marked. However, there is a loss of microvilli, formation of lamellar structure, some ribosomal detachment, but no vacuolization of the mitochondria (Figs. 1.13, 1.14). Incubation with a 5 µl dose of CCl_4 over 60 min, which does not give biochemical evidence of structural damage, shows evidence of ultrastructural damage to the RER (Fig. 1.15). This figure also shows the possible beginning of a lamellar structure.

A section of cells under the light microscope after incubation under control conditions for 15 minutes is shown in Fig. 1.16.

DISCUSSION

A toxic response to CCl_4 has been demonstrated in isolated rat hepatocytes by the experimental results of this chapter, both biochemically and morphologically. The changes observed in the structural parameters are analogous to some changes observed *in vivo*. The loss of K^+ from the hepatocytes would correspond with the disturbances in electrolyte balance and swelling of liver cells occurring in the intact animal (Recknagel, 1967; Zimmerman, 1976). The resultant increase in liver weight has been used as an index of hepatic damage (Castro, 1977; Garner and McLean, 1969). The mutual involvement of K^+ loss and cellular swelling in cell suspensions has also been pointed out by Frimmer *et al.* (1976). Electron microscopic examination has, as pointed out by Castro and Diaz Gomez (1972) shown swelling at an early stage along with changes in the ER. This would be consistent with the early changes seen in intracellular K^+ here. A decrease in the ability to reaccumulate K^+ after cooling has been reported in liver slices from rats pre-treated with CCl_4 as compared to slices from control rats (Rees, 1964). This

may be related to alterations in K^+ here, but the time scale of the response is quite different.

Release of the cytoplasmic enzyme into the surrounding medium is a response to CCl_4 closely related to the measurement of serum ALT clinically (Zimmerman, 1976) and in whole animal studies as a test for injury to the liver (Paquet and Kamphausen, 1975; Hasumura *et al.*, 1974; Maling *et al.*, 1975). It is apparent from the results that changes in ALT occur after changes in K^+, which is consistent with their physical characteristics.

The inhibition of urea synthesis seen in the isolated hepatocytes has also been demonstrated in isolated perfused liver from CCl_4 pretreated animals (Heimberg *et al.*, 1962). Large doses were required to produce the same effect when CCl_4 was added *in vitro*, however (Heimberg *et al.*, 1964). The finding that ureogenesis is decreased is not surprising considering its energy requirements (Krebs *et al.*, 1974a) and the uncoupling of oxidative phosphorylation and impairment of the mitochondrial respiratory chain that occurs with CCl_4 (Lyachovich *et al.*, 1971; Brabec *et al.*, 1974). Such effects are also consistent with the rise in L/P observed in the isolated rat hepatocytes on incubation with CCl_4. Large increases in L/P may also be related in part to membrane changes, since a homogenate L/P of 22 has been reported (Schimassek *et al.*, 1974). The possibility of a more specific nature of the change is indicated by an increase in L/P on incubation with ethanol and the inhibition of this with concurrent addition of alprenolol (Grundin, 1975). These respiratory interferences may also account for the observation that treatment of hepatocyte suspension with 15 µl CCl_4 over a 15 min incubation period caused a drop in ATP levels.

Morphological changes observed in the RER of isolated hepatocytes are similar to changes seen on treatment of the intact animal with CCl_4, as reviewed by Smuckler and Arcasoy (1969) and Zimmerman (1976). In these same papers, the production of whorls or lamellar formations on treatment with CCl_4 has been mentioned. Zimmerman (1976) indicates that the significance of such formations is unclear. These may be similar to the condensed membranous areas seen in CCl_4 treated cell suspensions (Fig. 1.10). The formation of vacuoles in mitochrondria, however, is not an observation associated with *in vivo* changes in the ultrastructure, and it is difficult to account for this difference. It is of interest to note that Weiss *et al.* (1973) found a lack of vacuolization in isolated hepatocytes in contrast to *in vivo* and *in situ* results when preparations were treated with phalloidin. It appears that with some of these vacuoles in the CCl_4 treated cells from this thesis, lamellar structures are present. Lamellar structures were also found in response to CCl_4 by Hubner (1965). They were associated with mitochondrial and ER membranes. It was concluded that they formed in response to a direct effect of CCl_4 on the membranes.

Somewhat similar alterations in the RER to those seen on incubation with CCl_4 were reported on incubation of isolated hepatocytes with cumene hydroperoxide (Hogberg *et al.*, 1975b). They found irregularly shaped ER with distended cisternae. Disrupted plasma membranes were also reported by this group.

It should be appreciated that ribosomal detachment could be related to the reduced levels of ATP seen in the isolated cells after CCl_4 treatment. The possible involvement of ATP with ribosomal detachment has been discussed by Judah *et al.* (1970).

The loss of microvilli in response to CCl_4, can be associated with the swelling of liver cells, both directly, in isolated hepatocytes, and indirectly, in the whole tissue. The disappearance of microvilli has been associated with an increase in cellular volume in a study on the cytotoxicity of leucocidin (Frimmer *et al.*, 1976). Another cytotoxic chemical of biological origin, phalloidin, has been found to produce cytoplasmic protrusions (Frimmer *et al.*, 1974; Weiss *et al.*, 1973). Such protrusions or pseudopodia have been reported in isolated hepatocytes incubated for 3-6 hours (Phillips *et al.*, 1974). The contents of protrusions from these other reports are different to the mass of ribosomes seen in those of this thesis. Phillips *et al.* (1974) point out that such structures may be explained on the basis of cell injury, which would be consistent with their appearance in these cells treated with CCl_4. It would be interesting to see if cytochalasin B inhibited the formation of these protrusions in response to CCl_4 as it inhibited protrusions in the 3-6 hour incubated cells.

Doses of CCl_4 (1-2.5 ml/kg) which produce frank hepatic necrosis *in vivo* generally achieve peak concentrations in liver tissue of around 1000-1200 µg/g (Recknagel and Littera, 1960; Castro *et al.*, 1972; Castro *et al.*, 1974). Lower concentrations have been reported with similar dose levels by other workers (Marchand *et al.*, 1970; Maling, Eichelbaum *et al.*, 1974) as have higher concentrations (Bini *et al.*, 1975; Castro *et al.*, 1977). There was no apparent consistent effect of different routes of administration on levels of CCl_4 attained between studies, but investigation of this aspect in the one study clearly indicated that intraperitoneal administration gave higher levels in the liver than oral dosage (Nadeau and Marchand, 1973).

Levels of 1000-1200 µg/g would correspond approximately to a level of 7-8 mM measured in these experiments. Thus the *in vivo* peak liver level (with supramaximal hepatotoxic doses) is just less than the level reached with a 10 µl dose, and this dose produces toxicity (especially evident after 60 min incubation with respect to structural parameters) in the isolated hepatocytes. A dose of 5 µl, however, did not give evidence of structural damage and the concentration reached with this dose (4.4 mM at 20 min incubation - average of two experiments) has been found to produce the hepatotoxic effects of CCl_4 in many studies with intact animals. It should be remembered that the levels of CCl_4 quoted in the experiments of this thesis were determined in the hepatocyte suspension rather than hepatocytes or medium only. It is quite conceivable that the concentration of CCl_4 in the hepatocytes themselves might be higher than the suspension overall, when considering that the liver levels are higher than corresponding blood levels in the studies cited above. The most meaningful comparison to *in vivo* concentrations would require this additional information of concentration within the actual hepatocytes.

The method of addition of CCl_4 to the hepatocyte suspensions has been used by other workers. Dianzani *et al.* (1966), who were using lysosomes, found that a 10 µl dose equilibrated into 5 ml of suspension in a Warburg flask by 15 min, when a 20 µl dose had not. They found that a 10 µl dose gave a concentration of about 700 µg/ml which would correspond to about 4.5 mM in the experiments of this chapter. Slater and Sawyer (1971b) also added CCl_4 to their microsomal suspensions via a side arm. They found that addition of 2 µl led to a CCl_4 concentration of about 50 nl/ml which is roughly comparable to 0.5 mM in the experiments

of this thesis. There are apparent differences in time of equilibration of dose and levels attained between these two papers and the results from this laboratory. However, several factors may explain these differences, such as volume and configuration of the incubation flasks, contents of the flask and method of assay of CCl_4.

The experiments of this chapter have shown that CCl_4 is injurous to isolated rat hepatocytes and that the response is related to both dose and time of incubation. The toxic responses generally appear to be similar to those that occur *in vivo*. Conditions of incubation for further experiments are indicated, such that any change in toxic response, either increase or decrease could be appreciated.

CHAPTER TWO

EFFECTS OF HEPATOPROTECTIVE TREATMENTS ON CARBON TETRACHLORIDE TOXICITY IN ISOLATED RAT HEPATOCYTES

INTRODUCTION

Many treatments have been shown to protect against carbon tetrachloride hepatotoxicity. The actions are very diverse, some agents protecting against some aspects of toxicity while others of a similar group of chemicals may protect against some other aspect or perhaps not at all (Cignoli and Castro, 1971; Green, 1972; Reynolds and Moslen, 1974a). Furthermore, as Reynolds and Moslen (1974a) point out, there are numerous, often contradictory reports in the literature with respect to protective properties.

Compounds that exert some protection are both endogenous and exogenous, the former indicating the relevance of dietary considerations. These protective chemicals have also been recognised as possessing one or more of a group of properties such as: free radical scavenger, antioxidant, membrane stablizer, microsomal enzyme inhibitor (general) or inducer of less toxic pathways of metabolism (Reynolds and Moslen, 1974a). By this recognition of the protective agents possessing such properties, hypotheses concerning the mechanism of toxicity have been put forward. Toxicity via a reactive metabolite (free radicals) then via peroxidation of lipids and/or lipid and protein binding has received most attention over recent years (see Chapter 3). Experiments have been carried out both *in vivo* and *in vitro*.

Much of the earlier work with these protective treatments has been reviewed by Recknagel (1967). Those compounds with free radical scavenging and/or antioxidant activity include both exogenous and endogenous compounds. Exogenous compounds of this group used extensively include DPPD (de Ferreyra *et al.*, 1975; Cawthorne *et al.*, 1970; Hartman *et al.*, 1968; Reynolds and Moslen, 1974a; Slater and Sawyer, 1970; Castro *et al.*,

1968), EDTA (Recknagel and Glende, 1973; Glende *et al.*, 1976; Slater and Sawyer, 1971) and promethazine (Rees and Spector, 1961; Serratoni *et al.*, 1969; Slater and Sawyer, 1971; Reynolds and Moslen, 1974a; de Ferreyra *et al.*, 1975). It has been suggested that promethazine may exert its protective effects by its membrane stabilizing properties (Judah *et al.*, 1970; Zimmerman *et al.*, 1966). Interaction with the drug metabolising enzymes has also been suggested to help account for promethazine protection (McLean, 1967). We have been made aware of the effect of promethazine on the absorption of CCl_4 (Nadeau and Marchand, 1973), which indicates that a reduced liver level may be associated with the protective nature of promethazine treatment. The data of Castro *et al.* (1974) are at variance with that of the previous authors.

Vitamin E (α-tocopherol) is a naturally occurring antioxidant which has been much studied in relation to dietary necrosis and liver damage due to CCl_4 (Slater and Sawyer, 1971; Reynolds and Moslen, 1974a; Alpers *et al.*, 1968; Hafeman and Hoekstra, 1977; Ugazio *et al.*, 1976; de Ferreyra *et al.*, 1975). Some reports involved with vitamin E protection have presented conflicting evidence (McLean, 1967; Cawthorne *et al.*, 1970; Green, 1972; Recknagel and Glende, 1973). More recent studies have indicated that the situation is complex. Although the problem remains unsolved, these studies lend some idea as to how such discrepancies can arise (Goshal, 1976; Taylor and Tappel, 1976).

Another endogenous antioxidant is reduced glutathione (GSH) (Glende and Recknagel, 1969), one of the sulphur containing compounds which have been known for some time to protect against CCl_4 liver injury (Recknagel and Glende, 1973). It is thought that GSH might exert its action by 'mopping up' free radicals (Judah *et al.*, 1970; Docks and

Krishna, 1976; McLean and Day, 1975). Pre-treatment with diethyl maleate, which reduces markedly hepatic GSH levels (Boyland and Chasseaud, 1970), increases the toxicity of CCl_4, suggesting an interaction of GSH with injury (Gillette *et al.*, 1974). Some aspects of CCl_4 hepatotoxicity are afforded protection by GSH (Ugazio *et al.*, 1976; Sipes *et al.*, 1977; Gravela and Dianzani, 1970; Gravela *et al.*, 1971; Glende and Recknagel, 1974). The protective mechanism of GSH is probably linked with the soluble enzyme glutathione peroxidase, which catalyses the rapid conversion of lipid hydroperoxides to corresponding alcohols (Recknagel and Glende, 1973; Plaa and Witschi, 1976). The mechanism of action of another protective agent, selenium (a dietary factor) is also involved here. It is reported that it exerts its effect as a cofactor for the glutathione peroxidase (Plaa and Witschi, 1976; Hafeman and Hoekstra, 1977). Activity of glutathione peroxidase (and reductase) in isolated rat hepatocytes has been reported (Sies *et al.*, 1977).

Other sulphur containing chemicals found to protect against CCl_4-induced injury include methionine (Hove, 1948; Hafeman and Hoekstra, 1977), cysteine (de Ferreyra *et al.*, 1974), cystamine (Castro *et al.*, 1972; Castro *et al.*, 1972a; Castro *et al.*, 1973) and diethyldithiocarbamate (DEDTC) (Lutz *et al.*, 1973). It has been suggested that the sulphur containing amino acids may exert their protective effects by supplying cysteine for synthesis of intracellular GSH (Hafeman and Hoekstra, 1977). Both cysteine and methionine have recently been shown to be intimately involved in the synthesis of GSH in isolated rat hepatocytes (Reed and Orrenius, 1977). Docks and Krishna (1976) have suggested that cysteine may act directly as a nucleophilic agent, at least in chloroform induced liver injury. It is noteworthy that cysteine and GSH have both

been reported to be able to chelate iron (Thompson and Reitz, 1975).

Treatments protecting against CCl_4-induced liver injury that also affect the microsomal mixed function oxidase system can be divided into two groups. Firstly, there are animal pre-treatments which cause a general downturn in overall function of the drug metabolising system and secondly, those treatments which presumably require the presence of the chemical simultaneously with the noxious agent.

Treatments which have been studied and can be put into the first group include the effects of diet (primarily low protein) (McLean and McLean, 1966; Seawright and McLean, 1967). Not all workers have been able to demonstrate this diet protection (Nayak *et al.*, 1970; Chopra *et al.*, 1972). The exact diet is important in relation to the previously discussed endogenous antioxidants. Starvation for only 24 hours prior to CCl_4 treatment alters toxicity and this may be related to levels of these endogenous protectors (Slater and Sawyer, 1970; Diaz Gomez *et al.*, 1975).

Other treatments to be included in this first group include pre-treatment with cobaltous chloride (Suarez and Bhonsle, 1976), methyl mercury hydroxide (Carlson, 1975), lead nitrate (Pani *et al.*, 1975) and carbon tetrachloride itself (small doses) (Recknagel and Glende, 1973). These treatments are generally associated with a drop in cytochrome P_{450}. A recent report has indicated a lack of protection with cobaltous chloride (Suriyachan and Thithapandha, 1977). However, the rate of reduction of cytochrome P_{450} by NADPH was not affected, which, as the authors conclude, is compatible with CCl_4 activation at the hemoprotein site.

Spironolactone also has been shown to give protection against injury due to CCl_4 (Carlson *et al.*, 1974). This chemical causes an increase in

levels of cytochrome c reductase but does not change cytochrome P_{450} levels (Carlson *et al.*, 1974). Other workers, however, found no protection on treatment with spironolactone (Tuchweber and Kovacs, 1971).

Studies with some chemicals that enhance aspects of the drug metabolising system may appear paradoxical because they have been associated with a decrease in CCl_4 toxicity. These include 3-methylcholanthrene and pregnenolone-16α-carbonitrile, which are discussed in detail in Chapter 4.

Chemicals which can be listed in the second group include the much studied 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A), which inhibits oxidative microsomal metabolism of type I substrates, of which CCl_4 is one (Recknagel and Glende, 1973; D'Acosta *et al.*, 1975). Some earlier work on protective effects of this compound has been discussed by Recknagel and Glende (1973). Other reports relevant to SKF-525A inhibition of CCl_4 injury have appeared (Recknagel *et al.*, 1974; Reynolds and Moslen, 1974a; Castro *et al.*, 1974; Rao *et al.*, 1970). Several papers have dealt with a suppression of lipid peroxidation *in vitro* by SKF-525A (Ugazio *et al.*, 1976; Recknagel and Glende, 1973; Slater and Sawyer, 1971a). It now appears as if the protective effects of this compound are due to antioxidant and some other properties, rather than its inhibitory action on oxidative drug metabolism (D'Acosta *et al.*, 1975; Shah and Carlson, 1975; Sipes *et al.*, 1977). Not all studies with SKF-525A have been able to demonstrate protective effects (Nayak *et al.*, 1970; Tuchweber and Kovacs, 1971; Chopra *et al.*, 1972; Suriyachan and Thithapandha, 1977). The effects of SKF-525A on the absorption and distribution of CCl_4 have been described (Marchand *et al.*, 1970; Marchand *et al.*, 1971).

Several other inhibitors of drug metabolism have been tested and were found to have protective effects which varied in nature and degree (Cignoli and Castro, 1971; Castro *et al.*, 1974). Administration of dibenamine has been found to ameliorate CCl_4 induced toxicity (Maling, Highman *et al.*, 1974; Sohel *et al.*, 1974) and this has been related to an impairment of the drug metabolising system (Stripp *et al.*, 1974), perhaps mediated by a metabolite of dibenamine (Maling, Eichelbaum *et al.*, 1974).

Several substrates of the drug metabolising enzymes such as aminopyrine, aniline and pyrazole inhibit the *in vitro* pro-oxidant effect of CCl_4, probably by 'tying up cytochrome P_{450}' (Recknagel and Glende, 1973; Ugazio *et al.*, 1976). Pyrazole has been shown to reduce some aspects of CCl_4 liver injury (de Toranzo *et al.*, 1975; Recknagel and Glende, 1973). Aminopyrine, too, has been shown to exert some protection against CCl_4 injury, with a competition for factors involved in lipoperoxidation and drug metabolism being suggested to account for its action (Cignoli and Castro, 1971). Another microsomal substrate, hexobarbitone, does not inhibit endogenous microsomal peroxidation (Wolman, 1975; Gram and Fouts, 1966), but, as pointed out by Recknagel and Glende (1973), is able to reverse the effect of CCl_4 on triglyceride secretion in the perfused liver. It has also been suggested that the protective effect of cystamine may be exerted via its effects on the drug metabolising system (Diaz Gomez *et al.*, 1973).

Other compounds which have an interaction with the mixed function oxidase system that have been investigated include carbon monoxide (Suarez *et al.*, 1972; Slater and Sawyer, 1970; Sipes *et al.*, 1977; Villarruel *et al.*, 1975), p-chloromercuribenzoate (Slater and Sawyer, 1971a), cytochrome c (Slater and Sawyer, 1971a; Glende and Recknagel,

1969; Ohnishi *et al.*, 1974) and nicotinamide (Slater and Sawyer, 1971; Gibb and Brody, 1967). These last few treatments have been mainly concerned with *in vitro* studies aimed at determining the locus of CCl_4 metabolism.

The protective treatments covered have been primarily chemical. It has been suggested that the protective effects of some of these may be mediated by reducing body temperature (Slater, 1966). Larson and Plaa (1963; 1965) showed that the protection afforded by spinal cord transection was, in fact, due to lowered body temperature of the treated animal. Temperatures of 0-1°C have also been used for control incubations with an *in vitro* preparation of microsomes, where activation of CCl_4 was under investigation (Glende *et al.*, 1976).

This discussion of reported protective treatments against CCl_4-induced toxicity has not been exhaustive. There are several other protective agents documented which have not been listed here.

Having found that the isolated rat hepatocyte preparation showed toxic responses to CCl_4, it was of interest to see if some of the treatments shown to protect against CCl_4 injury in other systems showed a similar pattern with the isolated hepatocytes. It was anticipated that further evidence for the protective nature of such compounds might be obtained. Hexobarbitone, SKF-525A, dibenamine, 0-4°C incubation, promethazine, GSH, methionine and cysteine were chosen for the investigation. All the treatments chosen, except hexobarbitone and GSH have been previously shown to reduce CCl_4-induced necrosis. Hexobarbitone was selected because it has been shown to yield a type I spectral change with cytochrome P_{450} in isolated rat hepatocytes (Moldeus *et al.*, 1973).

Reduced glutathione was incorporated into the study because of its potentially important role in CCl_4 toxicity (as discussed above) and its involvement with other hepatotoxins (Mitchell and Jollow, 1975).

METHODS

Rats of between 200 and 260 g were used as liver donors in the experiments of this chapter. Suspensions of cells were incubated with 15 µl CCl_4 added to the side arm for a period of 15 minutes. The results presented in Chapter 1 indicated that this would allow either an increase or a decrease in the toxic response to be demonstrated. Cells, with and without CCl_4, were incubated with: saline vehicle (0.1 ml) as control, SKF-525A, promethazine or dibenamine (0.1 mM), temperature between 0-4°C (ice in incubating bath - temperature monitored intermittently), reduced glutathione (0.25 mM), hexobarbitone, methionine or cysteine (1 mM). Concentrations are those attained after addition of the cell suspension.

Samples were taken for estimation of the four parameters of cellular performance K^+, ALT, ureogenesis and L/P. The concentration of CCl_4 attained in those incubates at low temperature were monitored in some experiments.

The ability of the cells to produce formaldehyde from aminopyrine was investigated in some preparations and the effect on this production by SKF-525A (0.1 mM) was examined. Suspensions were also examined for any signs of toxicity due to aminopyrine or semicarbazide used in the procedure. The interation of hexobarbitone (5 mM) with aminopyrine metabolism was also studied.

RESULTS

None of the chemical treatments stopped CCl_4 from causing a loss of K^+ from the isolated hepatocytes, as shown in Fig. 2.1. Ice temperature incubation, however, prevented appearance of a statistically significant difference between control and CCl_4-treated cells. Although most of the chemical treatments themselves produced a small decrement in intracellular K^+, none of them significantly modified (even partially) the loss due to CCl_4. A similar pattern was observed with release of ALT into the supernatant (Fig. 2.2). Again CCl_4 caused a significant increase in ALT over and above respective controls with all treatments except ice incubation, and again none of the chemical treatments altered this response at all.

Fig. 2.3 shows urea formed over the 15' incubation with the treatments as indicated. In all cases except ice temperature and cysteine treatment, the CCl_4 treated cells showed a statistically significant decrease in urea formation over the incubation period (1-tailed, paired t-test, $p<0.05$). Similarly, only ice and cysteine showed a statistical amelioration of the CCl_4 related drop in ureogenesis. It is also seen in this diagram that the hypothermic incubation markedly reduces urea formation, which is quite expected. In the case of the second metabolic parameter, L/P (Fig. 2.4) all of the chemical treatments appeared to ameliorate the CCl_4 response without changing the control value, but this achieved statistical significance only in the cases of hexobarbitone, promethazine, dibenamine and cysteine. Again there was no effect on the ice temperature control response by CCl_4.

The concentration of CCl_4 in hepatocyte suspensions incubated at 0-4°C was monitored, because the reduced temperature might have affected

Fig. 2.1 Changes in intracellular K^+ content during 15 minutes incubation of control hepatocyte suspensions (open bars) or those exposed to 15 µl CCl_4 added to the side arm (hatched bars). Hepatoprotective treatments were:- C - saline vehicle only; S - SKF-525A 0.1 mM; H - hexobarbitone 1 mM; I - incubation at 4^oC; P - promethazine 0.1 mM; D - dibenamine 0.1 mM; M - methionine 1 mM; Cy - cysteine 1 mM; G- reduced glutathione 0.25 mM. Data shown are means and s.e.m. (n=4 or 5).

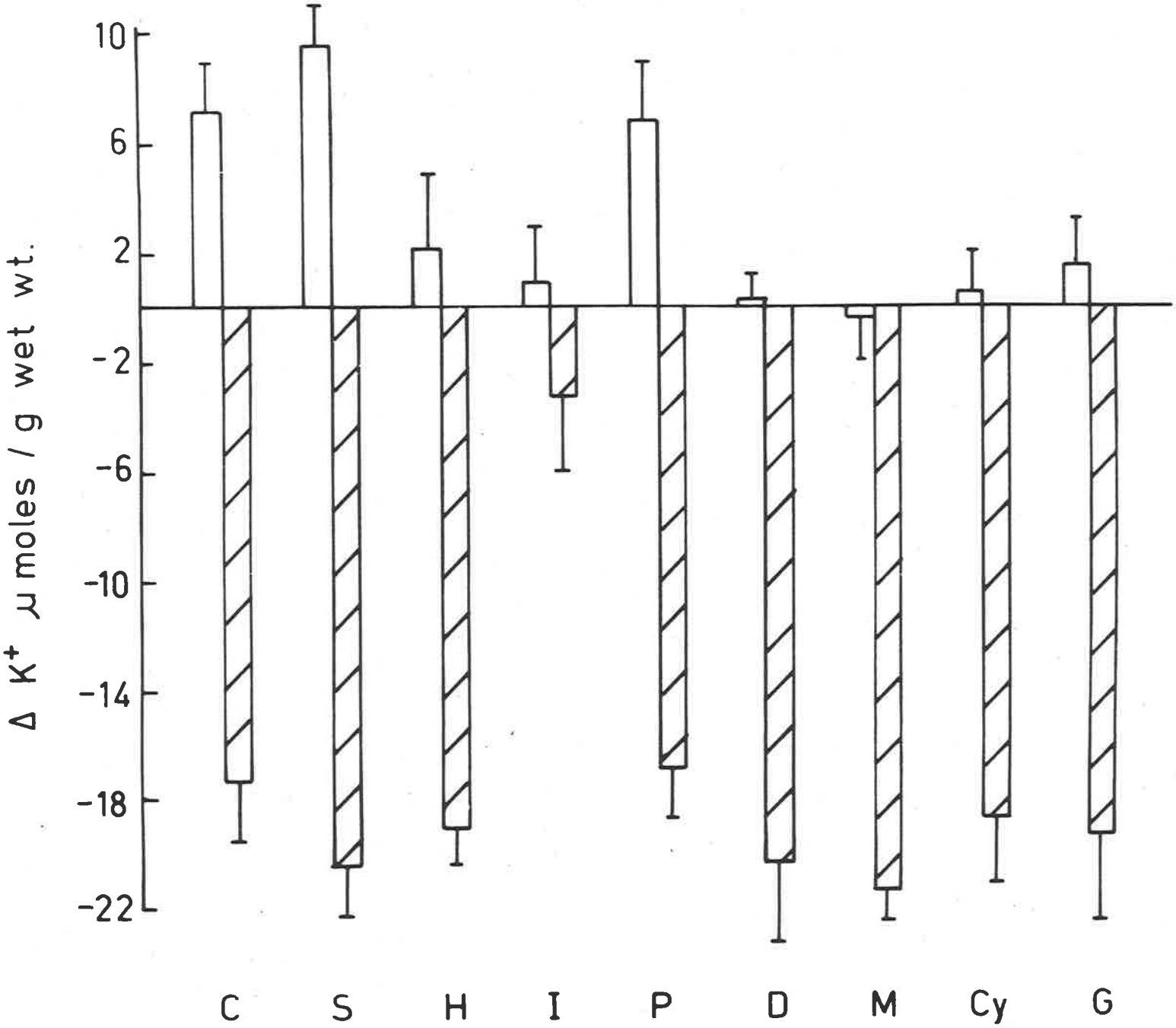

10
6
2
-2
-6
-10
-14
-18
-22
Δ K+ μ moles / g wet wt.
C
S
H
I
P
D
M
Cy
G

Fig. 2.2 Changes in ALT concentration in the medium during 15 minutes incubation of control hepatocyte suspensions (open bars) or those exposed to 15 µl CCl_4 added to the side arm (hatched bars). Hepatoprotective treatments were:- C - saline vehicle only; S - SKF-525A 0.1 mM; H - hexobarbitone 1 mM; I - incubation at 4°C; P - promethazine 0.1 mM; D - dibenamine 0.1 mM; M - methionine 1 mM; Cy - cysteine 1 mM; G - reduced glutathione 0.25 mM. Data shown are means and s.e.m. (n=4 or 5).

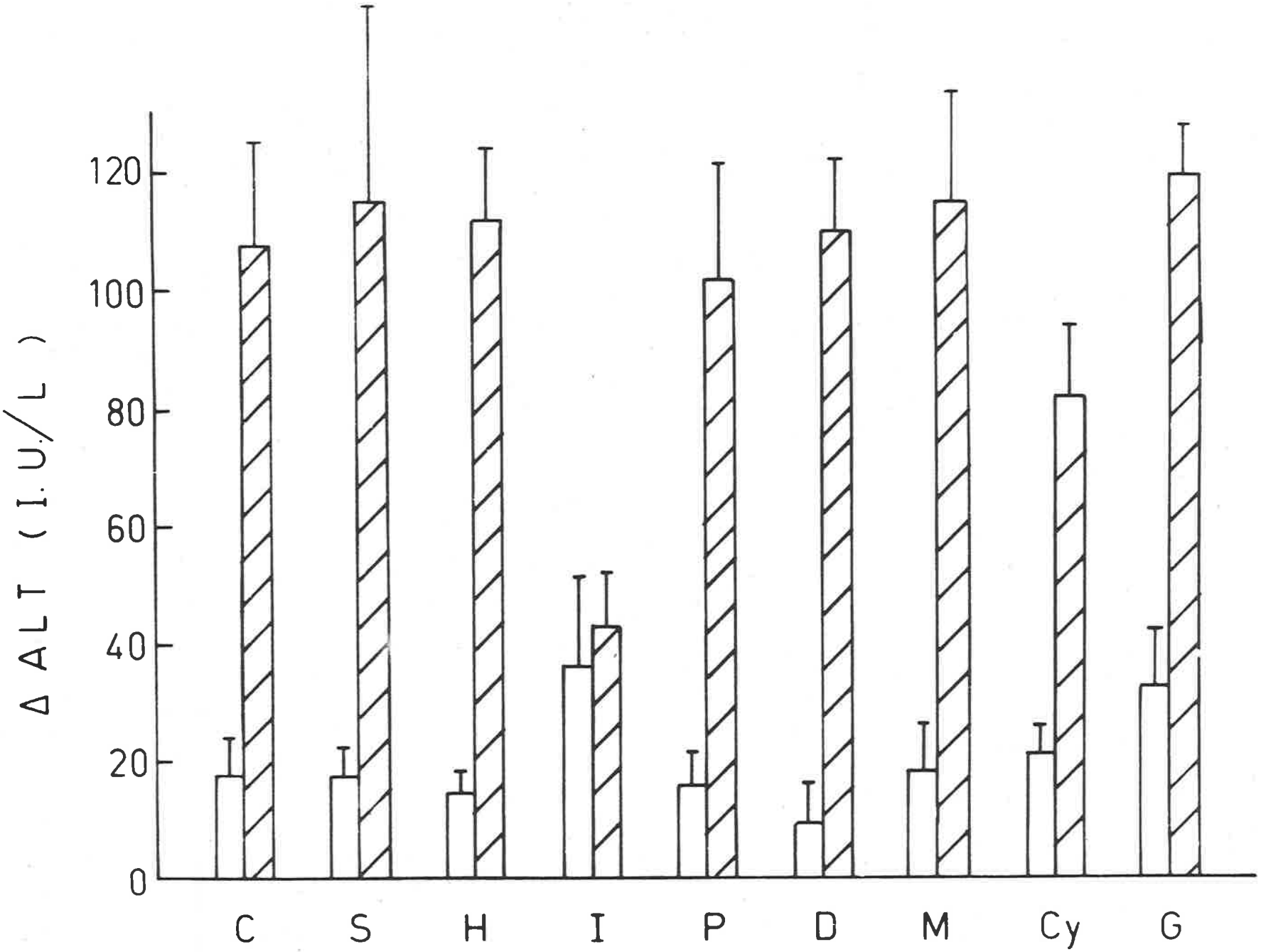

Δ ALT (I.U./L)
120
100
80
60
40
20
0
C
S
H
I
P
D
M
Cy
G

Fig. 2.3 Ureogenesis during 15 minutes incubation of control hepatocyte suspensions (open bars) or those exposed to 15 µl CCl_4 added to the side arm (hatched bars). Hepatoprotective treatments were:- C - saline vehicle only; S - SKF-525A 0.1 mM; H - hexobarbitone 1 mM; I - incubation at 4°C; P - promethazine 0.1 mM; D - dibenamine 0.1 mM; M - methionine 1 mM; Cy - cysteine 1 mM; G - reduced glutathione 0.25 mM. Data shown are means and s.e.m. (n=4 or 5).

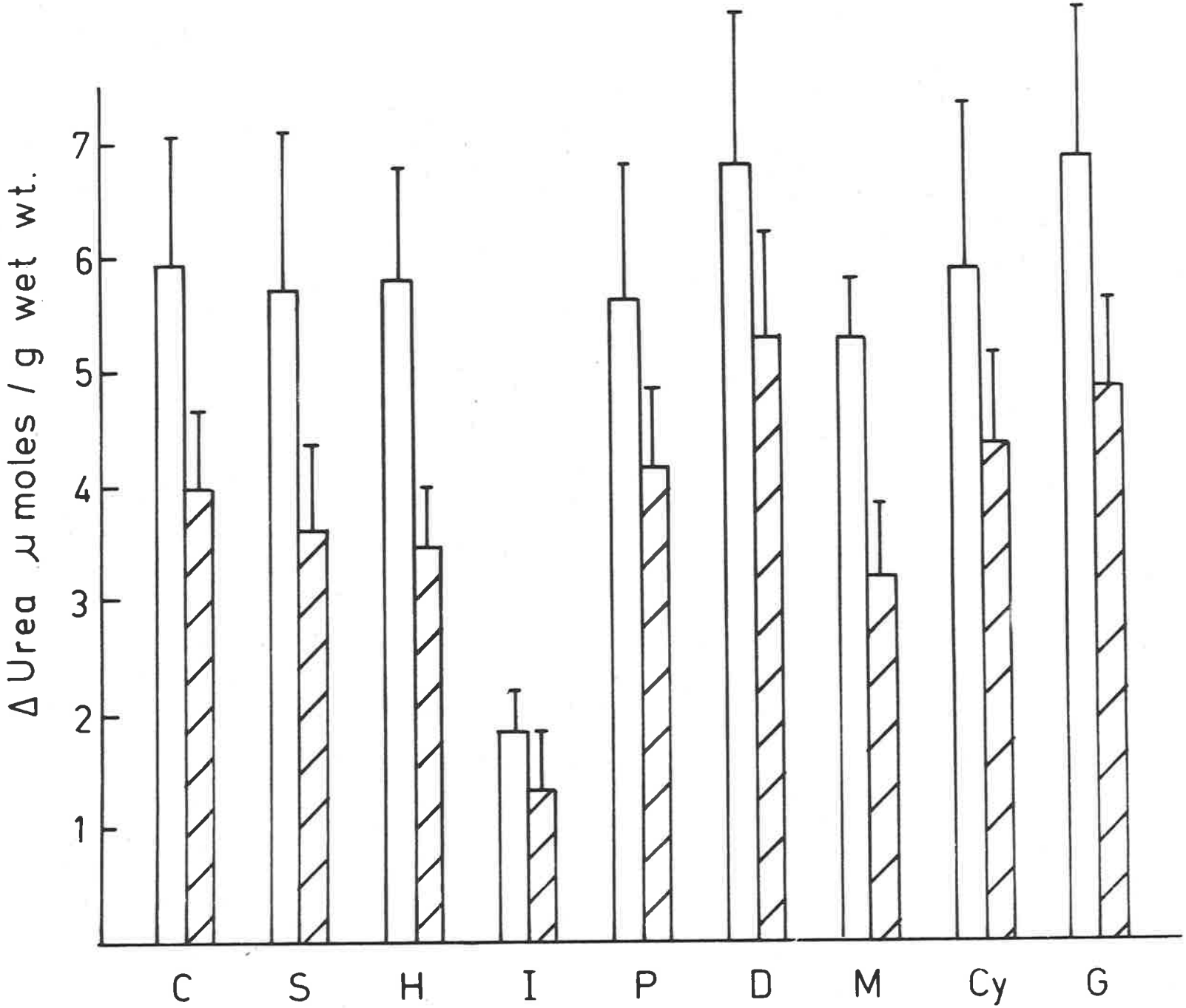

Δ Urea μ moles / g wet wt.
7
6
5
4
3
2
1
C
S
H
I
P
D
M
Cy
G

Fig. 2.4 Lactate/pyruvate ratio after 15 minutes incubation of control hepatocyte suspensions (open bars) or those exposed to 15 µl CCl_4 added to the side arm (hatched bars). Hepatoprotective treatments were:- C - saline vehicle only; S - SKF-525A 0.1 mM; H - hexobarbitone 1 mM; I - incubation at 4°C; P - promethazine 0.1 mM; D - dibenamine 0.1 mM; M - methionine 1 mM; Cy - cysteine 1 mM; G - reduced glutathione 0.25 mM. Data shown are means and s.e.m. (n=4 or 5).

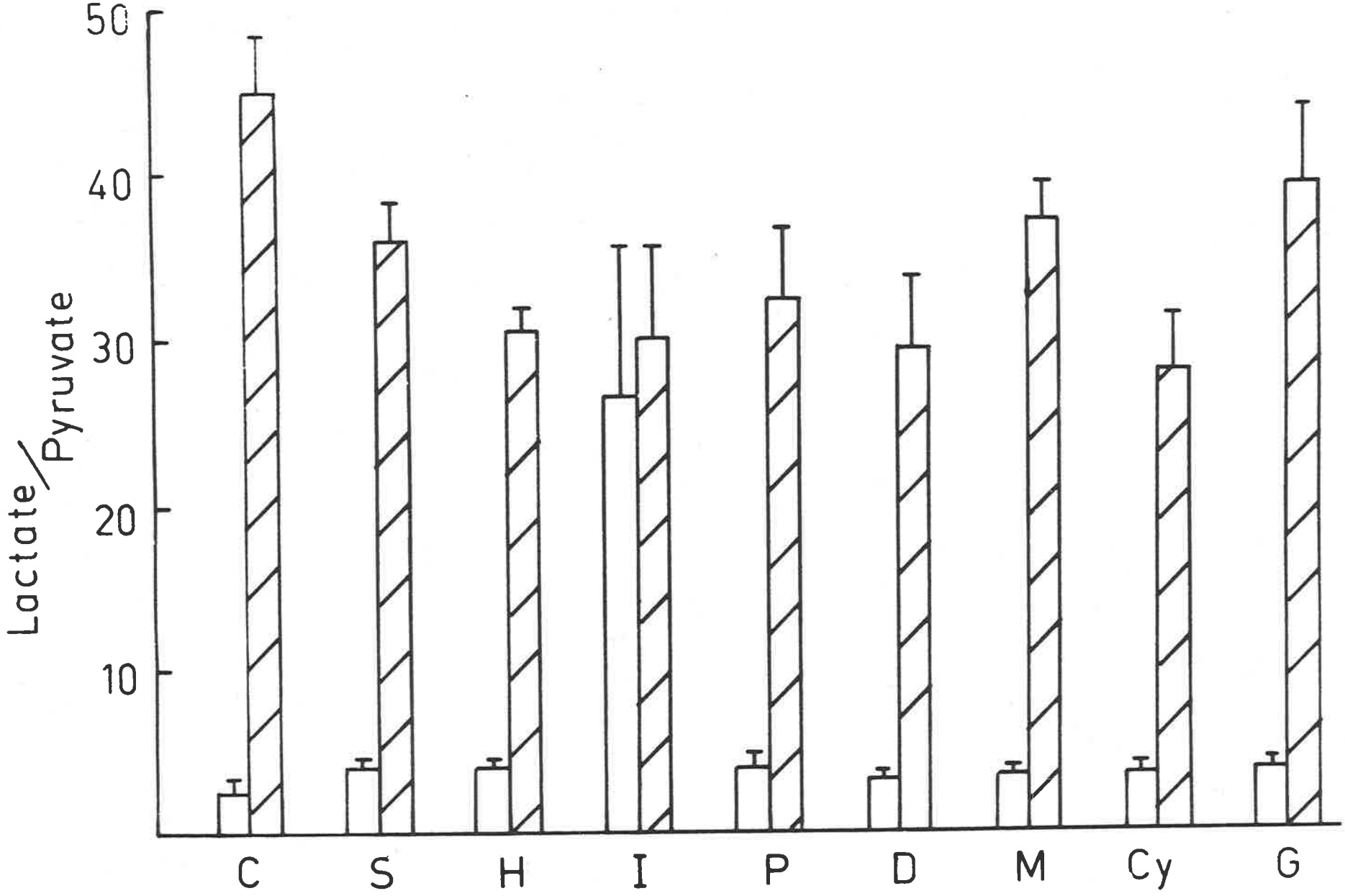

50
40
30
20
10
Lactate/Pyruvate
C
S
H
I
P
D
M
Cy
G

TABLE 2.1

CONCENTRATION OF CCl_4 IN HEPATOCYTE SUSPENSIONS INCUBATED AT 0-4°C WITH 15 µl ADDED TO THE SIDE ARM

MINUTES OF INCUBATION	CONCENTRATION[a] mM	
5	12.2	(1)
10	17.5	(1)
15	31.3 ± 3.2	(3)
20	29.0	(1)

[a] Numbers in parentheses are the number of cell preparations from which samples were assayed. Value where n=3 is mean ± s.e.m.

TABLE 2.2

AMINOPYRINE METABOLISM IN ISOLATED RAT HEPATOCYTES AND THE EFFECTS OF SKF-525A (0.1 mM)

TREATMENT	RATE OF[a] METABOLISM	% INHIBITION
Control	21.3 ± 2.8 (3)	-
+ SKF (0.1 mM)	4.1 ± 1.3[b] (3)	82

[a] As assessed by rate of formaldehyde formation in nmoles/min/g wet wt. Values represent mean ± s.e.m. Numbers in parentheses are number of cell preparations used.

[b] Statistically significantly different to value for control.

the partitioning of CCl_4 from the side arm into the suspension. The results are given in Table 2.1.

The capability of the isolated hepatocytes to metabolise the chemical, aminopyrine, and of SKF-525A (0.1 mM) to inhibit this metabolism by 82% is shown in Table 2.2. In one experiment, hexobarbitone (5 mM) was found to cause a 53% inhibition of aminopyrine metabolism.

The possibility that aminopyrine and semicarbazide might be toxic to the isolated hepatocytes was found not to be so. There was absolutely no change in the parameters K^+ and ALT during incubation with these chemicals over the 20' period.

DISCUSSION

The response of the isolated rat hepatocytes to a dose of 15 µl over 15 min incubation was the same as found in the previous chapter, except for some minor quantitative differences. None of the chemical treatments was found to have any consistent protective effects on the parameters of membrane integrity that were assessed.

The protective effects of SKF-525A were originally thought to be related to its effect of inhibiting oxidative drug metabolism. Further studies indicated that this was unlikely because the activation of CCl_4 was found to be a reductive process (Sipes *et al.*, 1977). The nature of protective properties, although not fully understood, is thought to relate, in part, to antioxidant properties (D'Acosta *et al.*, 1975; Shah and Carlson, 1975). In fact, examination of some of the data of earlier

publications indicates an inhibitory effect by SKF-525A on lipid peroxidation *in vitro* (Rao *et al.*, 1970; Slater and Sawyer, 1971a). This may account for a lack of effect in the present experiments since inhibition of CCl_4-induced lipid peroxidation may require concentrations of SKF-525A higher than those used here. Thus, although we demonstrated a substantial inhibition of aminopyrine demethylation by 100 μM SKF-525A, this concentration may have been inappropriate to display any protective effect operative by other mechanisms. A comparison to *in vivo* in this regard is difficult because there is no information on the levels of SKF-525A attained in the hepatic tissue. Use of the isolated hepatocytes does overcome, however, the problem of action via an effect on the absorption or distribution of CCl_4 in studies on the toxicity of this chemical.

Comparisons among studies carried out in the whole animal are difficult due to differences in experimental protocol. The lack of uniformity in the results from such studies may be related to the differences in dose of both SKF-525A and CCl_4 and the temporal aspects of their administration. Although not entirely so, studies which show protection against CCl_4 generally use a higher dose of SKF-525A and a lower dose of the toxin than studies where protection is lacking.

The lack of protection by hexobarbitone would be consistent with the finding by Chopra *et al.* (1972) that pentobarbitone gave no amelioration of the hepatotoxic effects of CCl_4. Perhaps a substrate better associated with protection would have been more appropriate in an initial study, however. Consideration of its concentratior is necessary, too, because, although inhibitory effects of 5 mM were found on the metabolism of a second type I substrate (aminopyrine) the influence of 1 mM (the

concentration used with CCl_4) was not investigated. However, Rubin and Kosorog (1970) reported complete reversal of the inhibitory effects of CCl_4 on hepatic lipid accumulation in the isolated perfused rat liver at a hexobarbitone:CCl_4 molar ratio of .07:1, which is very similar to the ratio of the present experiments. Nevertheless, the lack of protection with the barbiturates suggests that type I substrates of the microsomal drug metabolising enzymes do not exert protective effects simply by tying up the system as suggested by Recknagel and Glende (1973).

The lack of protection in the present experiments by dibenamine may be accounted for by temporal factors. The studies of the group from N.I.H. (Maling, Highman *et al.*, 1974) found that pre-treatment with dibenamine was required at least 24 hours before dosage with CCl_4 to see inhibition of toxicity. Dibenamine and CCl_4 were given simultaneously in the isolated cell experiments. However, Sohel *et al.* (1974) found protection when dibenamine was given only 1 hour before CCl_4. This latter group used lower doses of dibenamine and of the toxin. The dose of dibenamine used in the isolated hepatocytes was selected on the basis of the *in vivo* treatment.

Incubation of hepatocytes at low temperature (0-4^{o}C) was the only treatment found to consistently modify the damaging effects of CCl_4. Although hypothermia is a relatively non-specific treatment, one of the possibilities of explanation of its protective nature would be an inhibition of microsomal metabolism to reduce formation of the proposed reactive metabolite. Glende *et al.* (1976) found temperatures of 0-1^{o}C capable of inhibiting metabolism by microsomal preparations. The kinetics of the free radical chain reaction, which is postulated to

follow formation of the reactive metabolite of CCl_4, is also dependent on temperature (Castro *et al.*, 1977). Inhibition of metabolism, however, would seem an unlikely explanation in view of the negative results obtained with the three chemical treatments described above. Some of the findings discussed in other chapters of this thesis would also indicate that active metabolites are not responsible for the toxic reaction. A study over a range of temperatures may well prove fruitful when it is considered that the hypothermic protection in the study of Larson and Plaa (1963) was associated with a rectal temperature of about 28°C. A simultaneous assessment of microsomal metabolism (of aminopyrine and CCl_4) would provide valuable information. It should also be remembered that CCl_4 causes loss of G-6-P'ase activity in isolated rat liver microsomes at 30°C as well as 38°C (Goshal and Recknagel, 1965).

Although the integrity of the hepatocytes is protected by hypothermic conditions, the biological significance of this result with respect to metabolic status is questionable. When the control levels become reduced as in ureogenesis, then the disappearance of the apparent protective effect is much less meaningful. Similarly with L/P, the actual levels of lactate and pyruvate under hypothermic conditions were at the extreme lower limit of the assay system, such as to question even the control values of L/P. This makes a statement concerning the effects of CCl_4 on this parameter of even less significance.

A lowering of the amount of CCl_4 partitioning into the hepatocyte suspension with hypothermic incubation cannot be used as an explanation of protection. The data in Table 2.1 show that there is no reduction in CCl_4 levels at 15 min at 0-4°C as compared to 37°C (Table 1.1). In fact, there is an increase. Measurement at other times of incubation show no

apparent delay in attaining the level reached under normothermic conditions.

In the experiments of Zimmerman *et al.* (1966) using carcinoma laryngeal cells, promazine and chlorpromazine were found to protect against the cytotoxic effects of large doses of CCl_4. Concentrations of the phenothiazines lower than the 10^{-4} M used in the present study for promethazine were required if the protection was to be displayed. This may account for the lack of protection seen with promethazine in the isolated rat hepatocytes. However, McLean (1960) found 10^{-4} M promethazine to be effective in restoring the ability of liver slices (pre-treated for 35 min with 0°C temperature) from rats fed necrogenic diets for 5 days, to reaccumulate K^+. The inhibition of the stimulation of lipid peroxidation by CCl_4 in microsomal preparations is seen with levels of promethazine around 0.1-10 μM (Slater and Sawyer, 1970). In view of a lack of effect of promethazine, however, an investigation of concentrations similar to those found to be protective in the study of Zimmerman *et al.* (1966) may prove informative. This is perhaps particularly so in view of the comment by Judah *et al.* (1970) that promethazine, even though a potent antioxidant, is not much more effective as a protective agent than other non-antioxidant stabilizers. An investigation of such stabilizers in the isolated rat hepatocytes, over a range of concentrations, might also provide some data of interest.

There are factors which can be used as possible explanations for the lack of protective effects of the sulphur containing chemicals. For example, GSH has been reported not to readily enter cells (Macdonald *et al.*, 1977). Direct evidence for accumulation of effective concentrations of chemicals within the hepatocytes has only been obtained with

SKF-525A. A quantitative determination of penetration of all the chemicals into the cells is desirable for more adequate interpretation of the results. In spite of the reported poor entry into cells, however, protection of aspects of hepatic injury by GSH administered intraperitoneally has been reported (Gravela and Dianzani,1970; Gravela *et al.*, 1971).

The uptake of cysteine and methionine into isolated hepatocytes has been demonstrated (Reed and Orrenius, 1977). The results indicate that incubation for an hour rather than 15 min gives higher levels of amino acid taken up and a larger amount of GSH synthesised from these (hepatocytes were isolated from diethyl maleate pre-treated rats). This indicates that pre-incubation with the amino acids before treatment of the hepatocytes with CCl_4 may be worthy of investigation. However, de Ferreyra *et al*, (1974) found cysteine to be protective against CCl_4-induced necrosis and fatty liver when the amino acid was administered 30 min before or 60 min after the hepatotoxin. Nevertheless, an extension of the isolated hepatocyte study with cysteine in particular is warranted because this treatment has given a hint of a protective action in the results. It is one of the treatments which reduces the increase in L/P due to CCl_4, although the cells treated with CCl_4 alone may have given an abnormally high L/P (reference to L/P data of Chapter 1 would support this). Cysteine also was the only chemical treatment which showed a statistical amelioration of the CCl_4 related inhibition of ureogenesis, even though the magnitude of the change was small. Observation of the pattern of loss of ALT into the supernatant (Fig. 2.2) indicates a smaller change in cysteine treated cells but this is not statistically significant. A similar situation is apparent for loss

of K^+.

In comparing the results from all the chemicals to *in vivo* data and, in fact, in comparing reports of *in vivo* studies themselves, several factors require careful attention. These include, (i) the dose and liver concentrations of the protective agent and the toxin, CCl_4; (ii) the time of dosage of both; (iii) the actual parameters measured - for example, one study might conclude a drug has no protective properties and a second study find amelioration when in fact both studies have measured different indices of CCl_4-induced toxicity; (iv) the time after treatment that samples are taken for assay for signs of toxicity; (v) the diet the animals have been on especially with respect to anti-oxidant levels and protein content. Studies such as those of McLean and Day (1975) and Hafeman and Hoekstra (1977) indicate the importance of this consideration.

The lack of protection with the chemical treatments seen in the present study could be related to various factors. One is the already mentioned possible inappropriate concentrations of the chemicals. Secondly, the mechanism of toxic response to CCl_4 might be different to that *in vivo* such that these treatments are not effective. A third possibility is that the methods of assessing toxicity were too insensitive. This would appear unlikely since in the case of ALT and L/P there was a clear discrimination between CCl_4 doses over a two-fold range (Chapter 1) and the conditions of treatment with CCl_4 and the chemicals were such that a shift of toxic response in either direction would not have been masked (as indicated by hypothermic incubation).

Overall, even though criticisms can be made about most of the

treatments used, it seems reasonable to expect some indications of protection, at least with some of the chemicals, if the toxic response is occurring in a manner similar to *in vivo*. No consistent pattern of amelioration of the toxic responses to CCl_4 was seen, however, with any of the chemicals used. All of these have been associated with protection against one or more aspects of CCl_4 toxicity in the whole animal. Some of these protective actions have been the subject of conflicting reports, however. Nevertheless, further experiments incorporating dose and temporal changes in the protocol have been suggested and are recommended.

CHAPTER THREE

LIPID PEROXIDATION IN ISOLATED RAT HEPATOCYTES. RELATIONSHIP TO TOXICITY OF CCl_4, ADP/Fe^{3+} AND DIETHYL MALEATE

INTRODUCTION

Over the past 10-15 years the lipid peroxidation theory, which has been proposed to explain the toxicity of CCl_4, has been subject to many studies. The earlier aspects of the development of this theory have been fully reviewed by Recknagel (1967). Briefly, the theory was first invoked when it became evident that compounds with antioxidant properties afforded protection against aspects of injury due to CCl_4. The realization that toxicity was related to a metabolite and that the metabolite was likely to be a free radical also supported the possibility of the involvement of lipid peroxidation. Finally, it was demonstrated that lipid peroxidation did occur in response to dosage with CCl_4. Lipid peroxidation has subsequently been shown to be one of the earliest dectable events after such dosage (Rao and Recknagel, 1968). These authors also present a diagramatic representation of the molecular events likely to be involved in lipid peroxidation (Rao and Recknagel, 1969). The rapid and devastating effects associated with CCl_4 are said to be associated with the autocatalytic nature of the lipid peroxidation response.

Studies by other workers have also indicated the involvement of lipoperoxidation in the pathogenesis of CCl_4-induced liver injury. Di Luzio and Hartman (1969) studied levels of liver lipid antioxidant in response to CCl_4 and their results support the lipoperoxidation theory. The results of Gravela *et al.* (1971) on studies with protein synthesis indicated to them a relationship with lipoperoxidation. More recently, a protection study by Carlson (1975) supported the metabolism of CCl_4 in the initiation of lipid peroxidation, a reduction here accompanying a reduction in toxicity. A new technique, that of ethane evolution, for indicating lipoperoxidation, has also provided a statement concerning a role of

lipoperoxidation with toxic effects of CCl_4 (Hafeman and Hoekstra, 1977). Care is required in interpretation of results, however, because it is noted that there is less mortality associated with a higher ethane evolution in their cod liver oil (CLO) basal group as compared to CCl_4 treated basal (non-CLO) group. The authors' comments about ethane levels being underestimates in groups where animals died is important here. Relevant here, too, is that a larger fraction of fatty acids undergoing peroxidation in the animals fed the cod liver oil diet are of the ω-3-unsaturated variety, which lead to production of the ethane. Perhaps their results would be more meaningful if expressed in a time form as well. It is also noted that these authors infer protection by vitamin E, methionine and selenium against CCl_4-induced lipid peroxidation. This is so if the absolute levels are used. However, if the CCl_4 peroxidation to basal peroxidation ratio is used, a relative drop in CCl_4 peroxidation is evidenced only with selenium. It should also be remembered that these studies measuring ethane evolution are in the whole animal, such that the site of the lipid peroxidation cannot be directly and immediately assumed as being the liver. Nevertheless, PB has been shown to increase the ethane evolution due to CCl_4 (Riely *et al.*, 1974) suggesting a major involvement of the liver in this peroxidation response. It is noted that these experiments were with mice, a species which had been previously associated with a lack of lipid peroxidative response to CCl_4 (Diaz Gomez *et al.*, 1975a). This indicates that more sensitive estimates of a lipid peroxidation response may prove useful in demonstrating evidence of this, where it was previously thought not to occur.

The lipid peroxidation theory has not gone unchallenged. Some of the earlier criticisms arose from further antioxidant studies by McLean (1967),

Green *et al.* (1969), Cawthorne *et al.* (1970), Green (1972) and Cignoli and Castro (1971a). These studies in particular have received attention from the proponents of the lipid peroxidation theory, with answers to the criticisms being put forward (Recknagel and Glende, 1973; Recknagel *et al.*, 1974). It has already been mentioned (Chapter 2) that more recent studies in this area have helped to illustrate how discrepancies in such studies may arise (Taylor and Tappel, 1976).

Other studies presenting data which do not support this theory have been reported. Sasame *et al.* (1968) decided that the CCl_4-induced destruction of cytochrome P_{450} was probably not mediated by lipid peroxidation, since ethanol, which enhanced lipoperoxidation, had no effect on the cytochrome. Klaassen and Plaa (1969) found that the dosage relationship between CCl_4 and lipid peroxidation was weak and that other chemicals which induced liver necrosis showed no evidence of lipid peroxidation (e.g. chloroform). Judah *et al.* (1970) point out that promethazine, which is a potent antioxidant, has little more protective effect than non-antioxidant stabilizers. Studies by Cawthorne *et al.* (1971) on kidney and liver glucose-6-phosphatase (G-6-P'ase) led them to conclude (with results of other studies) that lipoperoxidation and G-6-P'ase changes were secondary and had little to do with the initial development of the lesion. A lack of correlation between lipid peroxidation and CCl_4-induced inhibition of protein synthesis was reported by Alpers *et al.* (1968). Cignoli and Castro (1971) found that imipramine, although ineffective as a protector, was the most potent inhibitor of lipid peroxidation from a series of chemicals they tested. Another compound, Sch 5706, was the most effective protector, but did not inhibit CCl_4-induced lipid peroxidation. Further studies from this South American group with cystamine and

cysteine showed protection by both these agents without effect on measurable lipid peroxidation (Castro *et al.*, 1972; de Ferreyra *et al.*, 1974). Studies with the microsomal inducer, 3-methylcholanthrene, which protects against CCl_4-induced toxicity, showed no alteration in lipid peroxidation (Pani *et al.*, 1973). More recently, a species study has indicated that susceptibility to CCl_4 is not directly related to amount of lipid peroxidation (Diaz Gomez *et al.*, 1975a). The same laboratory found that some antioxidants, although decreasing other aspects of CCl_4 toxicity, could not prevent lipid peroxidation (de Ferreyra *et al.*, 1975). Lack of evidence of lipid peroxidation in mice as cytochrome P_{450} was destroyed and CCl_4 activated led de Toranzo *et al.* (1975) to conclude that the P_{450} destruction was mainly mediated by a direct attack rather than through lipid peroxidation.

This direct attack on subcellular components by metabolites of CCl_4 has also been investigated by several workers. It has been put forward as a possible alternative to the lipid peroxidation explanation by some authors (Reynolds, 1967; Castro *et al.*, 1972; de Toranzo *et al.*, 1975). Maling, Eichelbaum *et al.* (1974) support this idea by saying that the covalent binding of CCl_4 derivatives to liver lipids and proteins presumably initiates the processes responsible for CCl_4 hepatotoxicity. This does not necessarily exclude an involvement of lipid peroxidation.

Other reports have indicated a lack of correlation on a quantitative basis between CCl_4 injury and both lipid peroxidation and covalent binding of the CCl_4 metabolites (Reynolds and Ree, 1971; Reynolds *et al.*, 1972).

The relationship between covalent binding and toxicity in general has been discussed at length by Gillette (1974, 1974a).

Recknagel and Glende (1973) have discussed the covalent binding aspects of CCl_4 toxicity in depth, indicating drawbacks in this theory. It is interesting to note that part of the evidence against this theory is that the incorporation of ^{14}C from the non-necrotizing ^{14}C-labelled Cl_2CH_2 is only slightly less in the microsomal fraction than with CCl_4. A very similar line of reasoning is used in disputing the lipid peroxidation theory. Ethylene dibromide exhibits toxicity much less than that of CCl_4 but still produces *in vivo* lipid peroxidation. Another study with 1,1-dichlorethylene found evidence of toxicity comparable to that of CCl_4, but without detectable lipid peroxidation. This compound does not exert a pro-oxidant effect *in vitro* either (Plaa and Witschi, 1976). These authors discuss a similar situation with chloroform ($CHCl_3$). However, $CHCl_3$ can exhibit lipid peroxidation if the animal has been pre-treated with phenobarbitone. Other compounds, such as bromobenzene, produce centrilobular necrosis, as does CCl_4, but without any evidence of associated lipid peroxidation (Gillette, 1974). As pointed out, it is difficult to reconcile these findings with the lipid peroxidation theory. Plaa and Witschi (1976) go on to discuss the idea that lipid peroxidation may be produced but in quantities too small to measure with present techniques. That is, when lipid peroxidation is measurable, it would represent an amount much greater than that necessary to produce liver injury. Evidence which is quoted in support of lipid peroxidation being causative is protection afforded against injury concurrent with a decrease in measurable lipid peroxidation. The question that arises is, "can both of these explanations be used in overall explanations of the lipid peroxidation and both still hold true?" It would appear to the author of this thesis that the two are somewhat conflicting.

In other words, if one proposes that undetectable lipid peroxidation is responsible for massive damage can we then say that a decrease in measurable peroxidation (presumably a larger amount) is directly related to a decrease in damage (presumably a lesser amount).

Several of the studies mentioned above, and others, have been discussed in recent reviews (Wolman, 1975; Plaa and Witschi, 1976). The evidence both for and against a causative role of lipid peroxidation in CCl_4-induced liver injury has been presented by these authors. They leave the reader with the conclusion that the theory is neither fully substantiated nor totally disproven. Other reviewers of experimental hepatotoxicity have, with respect to lipid peroxidation, been drawn to a similar conclusion (Farber, 1971; Zimmerman, 1976).

The two hypotheses, covalent binding and lipid peroxidation, are further discussed in a more recent study by Glende *et al.* (1976). Here evidence has been presented that strongly supports a causative role of lipid peroxidation in the *in vitro* loss of microsomal aminopyrine demethylase, cytochrome P_{450} and glucose-6-phosphatase. A worrying feature of this work is that malonic dialdehyde (MDA) production has been used to estimate the extent of lipid peroxidation. In their anaerobic incubations (when activity of these microsomal parameters was retained) no evidence of MDA was found. In the scheme of molecular events of lipid peroxidation presented by this laboratory previously (Rao and Recknagel, 1969) the incorporation of molecular oxygen is seen as a step necessary for the eventual production of MDA. This incorporation occurs after formulation of organic free radical and diene conjugation. Thus, the lipid membrane structure could still have been disarranged to some extent, but their assay system not detecting it because of the lack of molecular oxygen. Nevertheless, their

results support an association between lipid peroxidation and loss of these microsomal functions, but are not unequivocal evidence of a causative relationship. The other important aspect from this study, however, is that, under these anaerobic incubation conditions, CCl_4 is still metabolized and found covalently bound to the microsomal fraction, indicating that covalent binding of products of CCl_4 metabolism is not the mechanism responsible for loss of microsomal G-6-P'ase, cytochrome P_{450} or aminopyrine demethylase.

Other treatments besides CCl_4 can result in lipid peroxidation. Adenosine diphosphate complexed with ferrous ion, other pyrophosphates and Fe^{++} and ascorbic acid are the most commonly used experimentally. These have been covered in the review of Plaa and Witschi (1976). The mechanism of the reaction has been outlined by Recknagel and Glende (1973).

Lipid peroxidation has been shown to occur in isolated rat hepatocytes in response to ADP/Fe^{3+} and cumene hydroperoxide (Hogberg *et al.*, 1975; Hogberg *et al.*, 1975a; Hogberg *et al.*, 1975b). Remmer *et al.* (1977) have demonstrated an increased level of lipid peroxidation in isolated hepatocytes from ethanol pre-treated rats as compared to control rats. They also found lipid peroxidation in the cells in response to $CBrCl_3$.

Due to the amount of evidence involving lipid peroxidation with CCl_4 toxicity and an apparent lack of protection for the cells by treatments protective in other systems it was important to determine the involvement of lipid peroxidation in the toxicity of CCl_4 to the isolated rat hepatocytes. As mentioned by Hogberg *et al.* (1975) the isolated cells provide advantages in studying intracellular lipid peroxidation, and it was hoped that this system would provide more definitive data regarding the nature of

the role of lipid peroxidation in the pathogenesis of CCl_4 toxicity. As GSH is involved with the CCl_4 toxic response (Chapter 2) and CCl_4-induced lipid peroxidation (Wills, 1966; Glende and Recknagel, 1969; Hogberg *et al.*, 1975) it was also of interest to determine if any changes in the levels of this tripeptide were evident. As ADP/Fe^{3+} had already been shown to cause lipid peroxidation in isolated rat hepatocytes, concurrent incubations with this mediator, along with CCl_4 incubations, would be useful in such a study, particularly if a lipid peroxidation response to CCl_4 was not demonstrable.

The aims of the work of this chapter then, were, firstly, to investigate lipid peroxidation in response to CCl_4 in the cell system, along with treatment (ADP/Fe^{3+}) already shown to induce lipid peroxidation. Some initial experiments, where the effect of diethyl maleate on GSH content of the cells was being examined (with a view to investigating the response to CCl_4 in cells pre-treated with the diethyl maleate), indicated that lipid peroxidation was promoted by this agent. For this reason this treatment was also incorporated in the present study as a pro-oxidant. The second aim was to determine whether or not any change in GSH could be linked to the toxic response to CCl_4 and to any possible lipid peroxidation.

METHODS

Hepatocytes were isolated from the livers of rats weighing between 200 and 260 g.

In the present series of experiments, 2.5 ml aliquots of cell suspension were incubated with 10, 15 or 20 µl of CCl_4 as described in Chapter 1.

Other 2.5 ml aliquots from the same stock suspension were incubated with ADP/Fe^{3+} (40 or 80 μM with respect to Fe^{3+}; 3.3 and 6.7 mM with respect to ADP - final concentrations). This followed the incubation procedure of Hoberg *et al.* (1975). Further incubations incorporating diethyl maleate (2, 4 or 6 μl directly to the cell suspension) were carried out. The doses of this fluid treatment were selected by empirical observation.

All incubations were over a period of 15 or 30 min (to give moderate and extensive damage with CCl_4). At the end of this time, samples were taken for estimation of K^+ and ALT (toxicity assessment), TBA-reacting products and diene conjugation (lipid peroxidation) and reduced glutathione.

RESULTS

Control incubations showed no significant change in cell viability (K^+ retention and ALT release) over 15 or 30 minutes (Table 3.1). Lipid peroxidation, as measured by the absorbance of TBA reactants, did occur and increased with time. This effect was not seen with diene conjugation of extracted lipids, which appeared to be a less sensitive estimate in this preparation. In experiments with CCl_4, ADP/Fe^{3+} and diethyl maleate, the data are presented as mean differences between treated and control incubates in each experiment.

Fig. 3.1 shows that CCl_4 produced a dose- and time-dependent increase in ALT release. The ability of the hepatocytes to retain K^+ was markedly reduced at all doses tested. In spite of the extensive reduction in cell viability, lipid peroxidation as measured by malondialdehyde formation or diene conjugation did not increase beyond control values.

TABLE 3.1

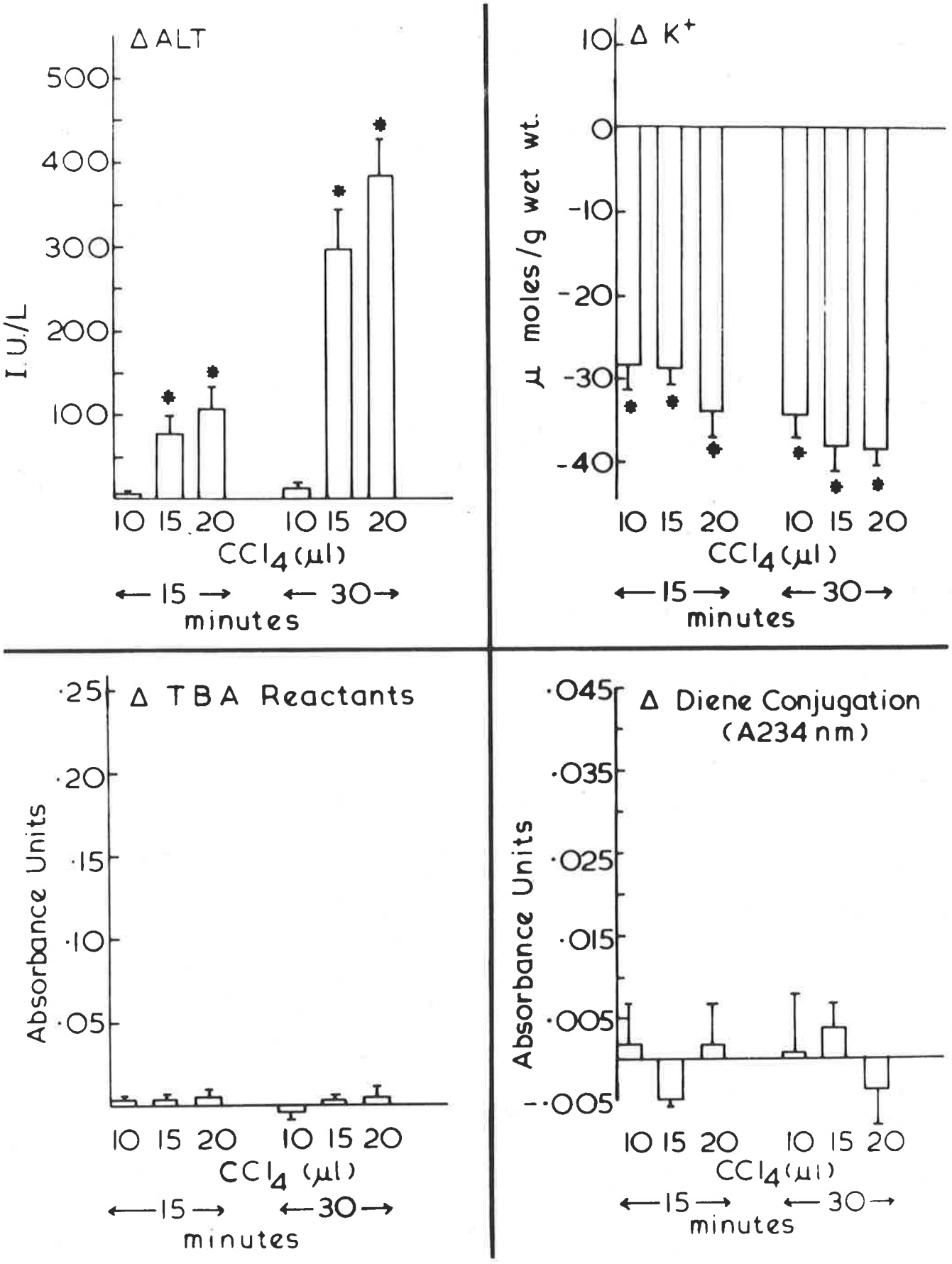
PARAMETERS[a] OF CELL MEMBRANE INTEGRITY AND LIPID PEROXIDATION IN CONTROL HEPATOCYTE SUSPENSIONS AFTER 0, 15 OR 30 MINUTES INCUBATION

PARAMETER	INCUBATION TIME (MIN)		
	0	15	30
K^+ retention µmoles/g wet wt cells	75.9 ± 3.5	80.4 ± 2.4	79.0 ± 3.0
ALT release I.U./L	52.2 ± 4.2	58.2 ± 2.0	62.7 ± 4.3
TBA-reactants Absorbance Units (532 nm)	0.117 ± 0.003	0.131 ± 0.002[b]	0.159 ± 0.005[b]
Diene conjugation Absorbance Units (234 nm)	0.042 ± 0.004	0.039 ± 0.003	0.036 ± 0.004

[a] Data expressed as mean ± s.e.m. (n=6).

[b] Statistically significantly different from zero time.

Fig. 3.1 Parameters of cell membrane integrity (K^+ retention and ALT release) and lipid peroxidation (TBA-reactants and lipid extract diene conjugation) in hepatocytes exposed to CCl_4 (10, 15 or 20 µl) for 15 or 30 minutes. Bars represent the mean and s.e.m. (n=5 or 6) of differences from control cells incubated for the same period. An asterisk denotes that the difference is significant.


Δ ALT
I.U./L
500
400
300
200
100
10 15 20 10 15 20
CCl4 (μl)
← 15 → ← 30 →
minutes
Δ K+
μ moles/g wet wt.
10
0
-10
-20
-30
-40
10 15 20 10 15 20
CCl4 (μl)
← 15 → ← 30 →
minutes
Δ TBA Reactants
Absorbance Units
·25
·20
·15
·10
·05
10 15 20 10 15 20
CCl4 (μl)
← 15 → ← 30 →
minutes
Δ Diene Conjugation
(A234 nm)
Absorbance Units
·045
·035
·025
·015
·005
-·005
10 15 20 10 15 20
CCl4 (μl)
← 15 → ← 30 →
minutes

Incubation of cells with ADP/Fe^{3+} produced nearly the opposite effect (Fig. 3.2). No change in ALT release was noted, and the drop in intracellular K^+ was small. A dose- and time-dependent increase in lipid peroxidation was noted, with increased TBA-reacting material and an apparent increase in diene conjugation, although this failed to achieve statistical significance except for the highest dose, and longest incubation. The increase in lipid peroxidation was apparently related to the Fe^{3+} component. Incubation of cells with similar doses of the carrier ADP without iron, produced no changes in lipid peroxidation, although the effect on K^+ and ALT was similar (Table 3.2).

The effects of diethyl maleate (Fig. 3.3) had features of both of the previous responses. The extent of cell damage after 15 minutes incubation was very small at any of the three doses. However, by 30 minutes, a dose-dependent pattern of cell damage was beginning to emerge. The extent of lipid peroxidation was more marked than that seen with ADP/Fe^{3+}. TBA-reacting material increased with both dose and incubation time, although the trend was not quite so clear in the diene conjugation response.

Diethyl maleate produced a significant fall in GSH content (Table 3.3). Incubation with the highest dose of CCl_4 for the full 30 minutes was the only other treatment which caused a significant fall in GSH content.

DISCUSSION

Lipid peroxidation has been shown to occur in the rat hepatocytes isolated in this laboratory, both endogenously and in response to chemical treatments. The occurrance of lipid peroxidation in untreated cells was also seen by Remmer *et al.* (1977) and the data found with ADP/Fe^{3+} are

Fig. 3.2 Parameters of cell membrane integrity (K^+ retention and ALT release) and lipid peroxidation (TBA-reactants and lipid extract diene conjugation) in hepatocytes exposed to ADP/Fe^{3+} (40 or 80 μM with respect to Fe^{3+}) for 15 or 30 minutes. Bars represent the mean and s.e.m. (n=5 or 6) of differences from control cells incubated for the same period. An asterisk denotes that the difference is significant.

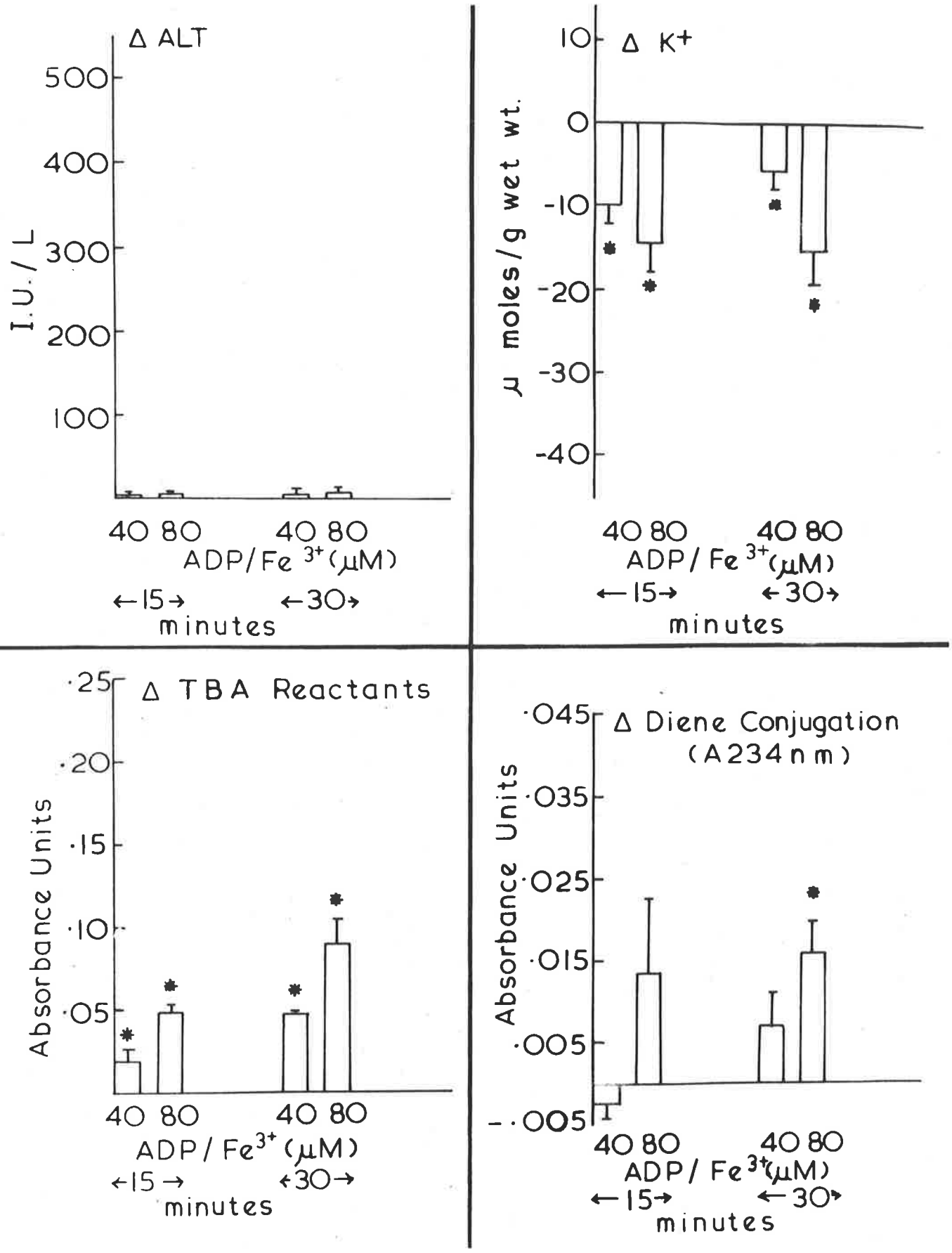

Δ ALT
500
400
300
200
100
I.U./L
40 80 40 80
ADP/Fe 3+ (μM)
←15→ ←30→
minutes
Δ K+
10
0
-10
-20
-30
-40
μ moles/g wet wt.
40 80 40 80
ADP/Fe 3+ (μM)
←15→ ←30→
minutes
Δ TBA Reactants
·25
·20
·15
·10
·05
Absorbance Units
40 80 40 80
ADP/Fe3+ (μM)
←15→ ←30→
minutes
Δ Diene Conjugation
(A234nm)
·045
·035
·025
·015
·005
-·005
Absorbance Units
40 80 40 80
ADP/Fe3+(μM)
←15→ ←30→
minutes

TABLE 3.2

EFFECT OF ADP WITHOUT IRON ON ISOLATED HEPATOCYTES

ADP CONCENTRATION AND INCUBATION TIME	$\Delta^a K^+$ (μMOLE/G WET WT CELLS)	Δ^aALT (I.U./LITRE)	Δ^aMDA ABSORBANCE UNITS
3.3 mM 15'	- 9.8 ± 1.8[b]	- 4 ± 7	-0.005 ± 0.005
30'	- 4.3 ± 5.3	-10 ± 6	0.002 ± 0.021
6.7 mM 15'	-14.1 ± 4.1[b]	- 2 ± 7	-0.003 ± 0.005
30'	- 8.8 ± 2.5[b]	-20 ± 6[b]	0.007 ± 0.005

[a] Mean ± s.e.m. (n=4) of differences between suspensions incubated with and without added ADP.

[b] Difference statistically significant from relevant controls.

Fig. 3.3 Parameters of cell membrane integrity (K^+ retention and ALT release) and lipid peroxidation (TBA-reactants and lipid extract diene conjugation) in hepatocytes exposed to diethyl maleate (2, 4 or 6 µl) for 15 or 30 minutes. Bars represent the mean and s.e.m. (n=5 or 6) of differences from control cells incubated for the same period. An asterisk denotes that the difference is significant.

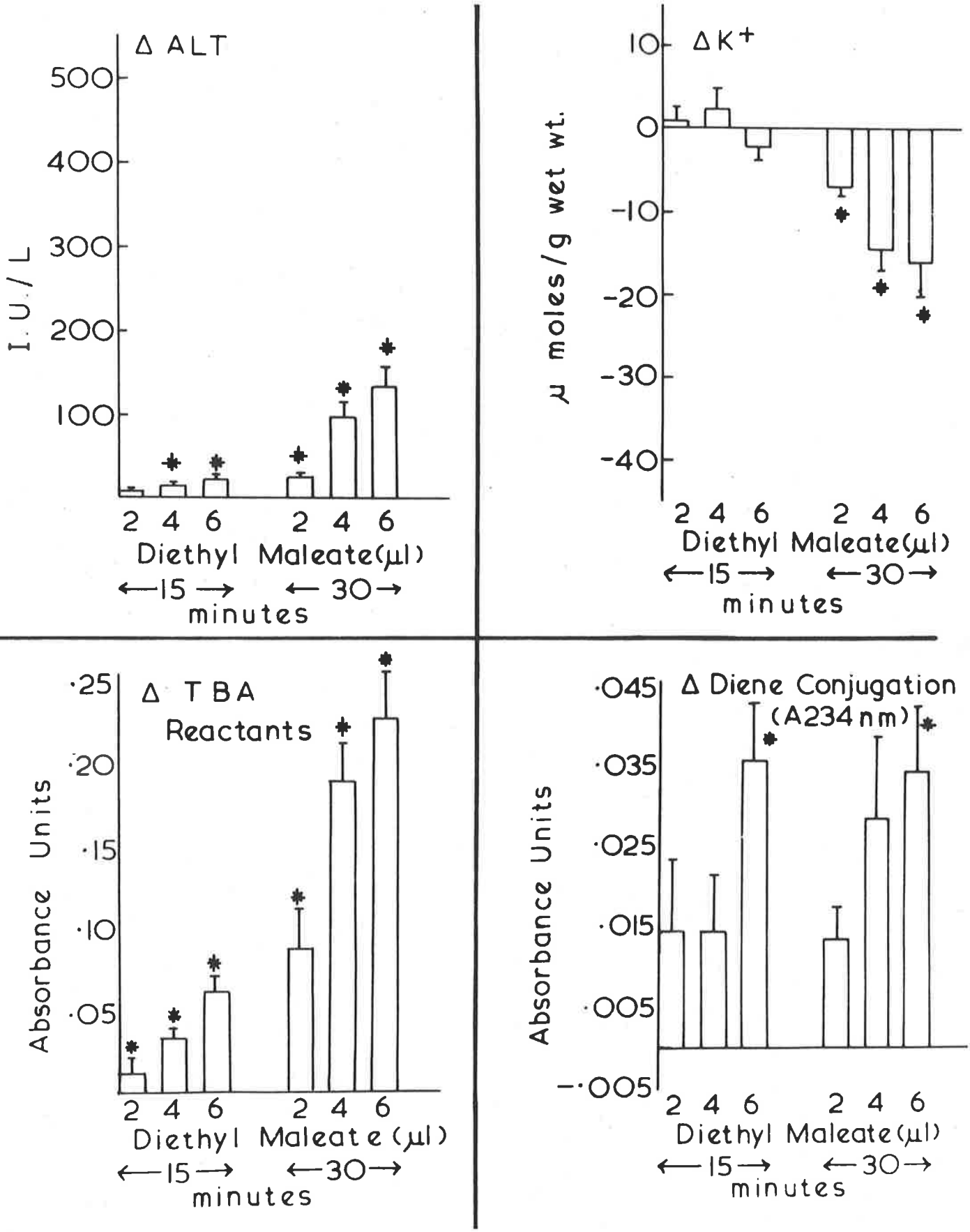

Δ ALT
I.U./L
500
400
300
200
100
2 4 6 2 4 6
Diethyl Maleate(μl)
←15→ ←30→
minutes
ΔK+
μ moles/g wet wt.
10
0
-10
-20
-30
-40
2 4 6 2 4 6
Diethyl Maleate(μl)
←15→ ←30→
minutes
Δ TBA
Reactants
Absorbance Units
·25
·20
·15
·10
·05
2 4 6 2 4 6
Diethyl Maleate (μl)
←15→ ←30→
minutes
Δ Diene Conjugation
(A234 nm)
Absorbance Units
·045
·035
·025
·015
·005
-·005
2 4 6 2 4 6
Diethyl Maleate(μl)
←15→ ←30→
minutes

TABLE 3.3

REDUCED GLUTATHIONE CONTENT[a] OF HEPATOCYTES INCUBATED WITH CCl_4, ADP/Fe^{3+} OR DIETHYL MALEATE

TREATMENT[b]		INCUBATION TIME	
		15 min	30 min
Control		4.65 ± 0.23	4.85 ± 0.25
CCl_4	10 µl	4.96 ± 0.21	4.90 ± 0.34
CCl_4	15 µl	4.91 ± 0.18	4.09 ± 0.44
CCl_4	20 µl	4.98 ± 0.24	3.47 ± 0.37[c]
ADP/Fe^{3+}	40 µM	4.87 ± 0.22	4.54 ± 0.37
ADP/Fe^{3+}	80 µM	4.40 ± 0.24	4.39 ± 0.20
Diethyl maleate	2 µl	3.31 ± 0.17[c]	3.24 ± 0.22[c]
Diethyl maleate	4 µl	3.23 ± 0.13[c]	3.34 ± 0.20[c]
Diethyl maleate	6 µl	3.35 ± 0.14[c]	3.35 ± 0.17[c]

[a] µmoles/g wet weight.

[b] CCl_4 (10-20 µl) was added to the side arm of the incubation flasks. The concentration of ADP/Fe^{3+} is in terms of the Fe^{3+} content.

[c] Significantly different from controls.

similar to those of Hogberg *et al.* (1975) except that their levels of TBA reactants were higher (by about a factor of 3 when expressing results/ 10^6 cells). There was no change in structual integrity of the hepatocytes directly related to this lipid peroxidation. Similar changes in K^+ were found on incubation of the hepatocytes with ADP alone without evidence of lipid peroxidation (Table 3.2). The effect on K^+ could possibly be related to a specific interaction with the ion pump responsible for maintenance of high intracellular K^+ levels. Hogberg *et al.* (1975b) found an apparent leakage of cellular constituents in response to ADP/Fe^{3+}. A high leakage from control cells, lack of statistical evaluation and no mention of response to ADP alone make their results difficult to interpret, however, especially in relation to lipid peroxidation. Their results with cumene hydroperoxide are more suggestive of a link between lipid peroxidation and cellular damage. There is also an inhibition of alprenolol metabolism due to ADP/Fe^{3+} in their experiments, while cytochrome P_{450} remains stable. The authors suggest that a loss of NADPH-cytochrome c reductase may be responsible, but it should also be remembered that a simple loss of co-factors (NADPH) could also explain the inhibition (loss of cytoplasmic enzymes from the cells would be accompanied by loss of smaller molecular weight substances).

Nevertheless, the response to cumene hydroperoxide is probably similar to the present results with diethyl maleate. To infer damage to cells as a result of lipid peroxidation, however, a demonstration of evidence of lipid peroxidation before that of significant leakage is required. This has been indicated with diethyl maleate (Fig. 3.3) where a marked lipid peroxidative response is seen prior to significant evidence of structural damage to the cells. The data of Hogberg *et al.* (1975)

indicated a rise in TBA reactants due to 50 µl of diethyl maleate per 8 ml of cell suspension (their Fig. 5) quite similar to that seen with the largest dose of the present experiments. They indicated in the text that diethyl maleate did not stimulate microsomal malondialdehyde production but it is not clear if this relates to the reported data or that found elsewhere in actual microsomal preparations. The changes in their isolated cell data may not have achieved statistical significance.

Unlike the response to diethyl maleate, CCl_4 was found to produce cellular damage in the absence of demonstrable lipid peroxidation. If the injury to the isolated cells is occurring similar to that *in vivo*, then these results would be definite evidence against lipid peroxidation being causative in toxic responses, as put forward by Recknagel and Glende (1973). The experimental data are consistent with other reports showing a lack of such a relationship, as documented in the introduction to this chapter.

Other studies have indicated that the lipid peroxidation response *in vivo* is virtually complete within 15 min of dosing an animal with CCl_4 (Rao and Recknagel, 1968; Reynolds and Ree, 1971). The response was about half maximal by 5 min. Studies with the microsomal fraction have indicated a maximal lipid peroxidation after 15 min (Glende *et al.*, 1976) or about 30 min (Goshal and Recknagel, 1965a; Recknagel and Goshal, 1966). Thus there would appear to be sufficient time to see an analagous change in the present isolated hepatocyte experiments. However, the methods used to assess lipid peroxidation are both open to criticism such that an absence of this response does not necessarily mean it is not occurring. It may be useful to employ other assays of lipid peroxidation. As well as the insensitivity of the assay methods, the lack of evidence of lipid

peroxidation may be due to leakage of essential cofactors through the more permeable cell membrane. These aspects are further discussed in the General Discussion. It may be of interest to add cofactors to the incubating media in concentrations similar to those used in experiments with microsomal preparations, when leakage has occurred, and re-examine the suspension for lipid peroxidation. Even if leakage was to be a contributing factor, the damage to the cell membrane would still be occurring prior to lipid peroxidation. The possibility that CCl_4-induced lipid peroxidation may occur at a highly localised site so as to escape detection when total cell lipids are analysed, must also be considered. Goshal (1976) has suggested that such a specificity may account for the apparent lack of efficacy of vitamin E in preventing lipid peroxidation.

Apart from the many studies in other systems demonstrating lipid peroxidation in response to CCl_4, the finding that bromotrichloromethane ($CBrCl_3$) elicits formation of TBA reactants in isolated hepatocytes (Remmer *et al.*, 1977) suggests that evidence for a similar response to CCl_4 in isolated hepatocytes may yet be demonstrable. The longer incubation time (120 min) used by Remmer *et al.* (1977) with this more potent hepatotoxin suggests that increased duration of incubation with CCl_4 could be useful.

An investigation incorporating benzoyl peroxide and CCl_4 (which can produce $CCl_3^{\cdot}$ and $Cl^{\cdot}$ radicals (Villarruel *et al.*, 1975)) on the structure and function of the isolated hepatocytes could perhaps yield some applicable data. The nature of the chemicals and reaction conditions involved suggest that technical problems may be considerable, however.

The lack of evidence of lipid peroxidation associated with the toxic

response of the isolated hepatocytes to CCl_4 is perhaps not surprising on consideration of the lack of protection afforded by the antioxidant chemical, promethazine (Chapter 2). An investigation of the response to diethyl maleate in the presence of antioxidant compounds could well provide valuable information into the role of lipid peroxidation in the leakage of cellular contents with this treatment. This approach is discussed in relation to the thesis overall in the General Discussion.

Hogberg *et al.* (1975) found that pre-treatment with diethyl maleate, which lowered cellular GSH, enhanced the lipid peroxidation induced by ADP/Fe^{3+}. Their higher levels of MDA production (as compared to this laboratory) in control cells may be related to the GSH status of the hepatocytes, those of the present series of experiments retaining approximately double those of the former study. Furthermore, the cells used in this thesis were more resistant to the lowering effects of diethyl maleate and did not show a loss of GSH during control incubations. Hogberg *et al.* (1975a) reported smaller changes in GSH during control and ADP/Fe^{3+} incubations. The importance of initial GSH levels and their maintenance is recognised and discussed further by Hogberg and Kristoferson (1977). They found that use of an enriched medium allowed production rather than loss of GSH over prolonged incubation periods. Such a consideration could account for maintenance of GSH levels in the controls of the present experiments since a supplemented incubation medium was being used (as compared to the loss in simple salt solution in the study of Hogberg *et al.*, 1975). The presence of methionine and cystine (which is convertible to cysteine) is probably particularly relevant here in view of the findings of Reed and Orrenius (1977). They showed both methionine and cysteine to be precursors for GSH biosynthesis in

isolated rat hepatocytes. These points illustrate the importance of an appropriate consideration not only of the original nutritional status of the animal, but also of the incubation medium being used during studies involving lipid peroxidation and associated responses.

The fall in hepatocyte GSH on treatment with diethyl maleate is analagous to that seen *in vivo* (Boyland and Chasseaud, 1970), but the drop is not as marked. The response appears to be maximal since all doses and both incubation times gave a similar [GSH]. The only other significant change in GSH was after 30 min incubation with 20 µl CCl_4. The lack of response with other CCl_4 doses suggests that this result is due to hepatocyte damage rather than a specific interaction between CCl_4 and GSH. Macdonald *et al.* (1977) concluded that GSH depletion following ethanol treatment in mice occurred as a consequence rather than a cause of lipid peroxidation and hepatic damage. Other drug treatments have been associated with a GSH fall prior to liver injury, however (Mitchell and Jollow, 1975). Discussion of these aspects of GSH levels in the isolated hepatocytes illustrates the point made by Hogberg *et al.* (1975) that the cells have an advantage over other *in vitro* systems in that potential protective mechanisms are retained.

In summary, the data presented in this chapter indicate that lipid peroxidation can occur in isolated hepatocytes without associated damage (as with ADP/Fe^{3+}), that cellular leakage can occur subsequent to evidence of lipid peroxidation (diethyl maleate) and that hepatocytic injury can occur in the absence of demonstrable lipid peroxidation (CCl_4). These observations suggest that lipid peroxidation is not an important factor in the initiation of CCl_4-induced damage to isolated rat hepatocytes. By extrapolation, the involvement of this process in hepatic necrosis after treatment with CCl_4 can also be questioned.

CHAPTER FOUR

CARBON TETRACHLORIDE TOXICITY IN HEPATOCYTES ISOLATED FROM PHENOBARBITONE PRE-TREATED RATS

INTRODUCTION

Most treatments that potentiate the toxicity of CCl_4 correlate with reported changes in microsomal metabolism (Reynolds and Moslen, 1974a). This correlation contrasts the situation noted in Chapter 2 with protective agents. There is certainly not the discrepancy in the literature for the effects of phenobarbitone (PB) on CCl_4 toxicity as there is with several of the protectors. Various laboratories have reported an increased toxicity with CCl_4 in animals pre-treated with PB. Garner and McLean (1969) found pre-treatment with PB to decrease the LD_{50} of CCl_4, along with an accentuation of other aspects of CCl_4 toxicity. A decreased LD_{50} for CCl_4 in PB treated animals has also been reported by Cawthorne *et al.* (1970) and Tuchweber and Kovacs (1971). Enhancement of CCl_4-induced necrosis, among other parameters has also been reported in PB pre-treated animals (Nayak *et al.*, 1970; Chopra *et al.*, 1972; Pani *et al.*, 1973). Potentiation of CCl_4 effects on endoplasmic reticulum and protein synthesis by PB pre-treatment was demonstrated by Shah and Carlson (1975). Changes in other parameters of the endoplasmic reticulum due to CCl_4 have also been shown to be enhanced after PB pre-treatment (Sasame *et al.*, 1968). Increased levels of lipid peroxidation have been found in animals pre-treated with this barbiturate and subsequently dosed with CCl_4 as compared to dosage with CCl_4 alone (Rao *et al.*, 1970). Such an enhanced lipid peroxidation has been associated with increased toxicity within the one study (Suarez *et al.*, 1975). The effects of PB pre-treatment on lipid peroxidation have been further reported to depend on the antioxidant status of the animal (Taylor and Tappel, 1976). Covalent binding of ^{14}C from labelled CCl_4 has also been shown to be increased in animals pre-treated with PB (Diaz Gomez *et al.*, 1973). Reynolds *et al.* (1972) decided that their experiments showed no quantitative relationship

between PB increased toxicity and either covalent binding or lipid peroxidation.

A longer term study by Gans *et al.* (1976) indicated that PB did not accelerate or promote cirrhosis due to CCl_4 in mice but did enhance the effects on liver weight and other parameters. It should be noted that an increase in protein synthesis with CCl_4 treatment alone was observed in this study.

Stenger *et al.* (1970) have also reported on the increased toxicity of CCl_4 in PB pre-treated rats, studying mortality and histological effects. They found that the barbiturate pre-treatment delayed the onset of, but eventually increased the liver injury due to CCl_4. In an important follow-up study (Stenger and Johnson, 1971) evidence was presented which indicated that proliferated hepatic smooth surfaced membranes *per se* were not responsible for enhanced CCl_4 toxicity. When increased activity of microsomal metabolism was associated with the electron microscopic proliferation, then enhanced toxicity due to CCl_4 was displayed.

A study using pregnenolone-16α-carbonitrile (PCN) pre-treatment in some animals and PB pre-treatment in others showed CCl_4 toxicity to be enhanced with PB but decreased by PCN pre-treatment (Tuchweber *et al.*, 1974). This is an interesting observation because both PB and PCN increase proliferation of SER, hexobarbitone oxidation, ethylmorphine N-demethylation, cytochrome P_{450} and NADPH-cytochrome c reductase activity. This indicates the observation of a general increase in microsomal parameters does not necessarily foreshadow an enhanced CCl_4 hepatotoxicity, but that some specific aspects require induction, or

that PCN and PB may induce the above moieties with different catalytic properties, as put forward by the authors.

A somewhat similar situation exists with 3-Methylcholanthrene (3-MC). Studies have shown a protective effect of this chemical even though it is recognised as an inducer of microsomal metabolism (Reid *et al.*, 1971a; Suarez *et al.*, 1972a; Stripp *et al.*, 1972; Pani *et al.*, 1973; Suarez *et al.*, 1975). However, Shah and Carlson (1975) have found an increase in the disruption of the hepatic polyribosomal profile with CCl_4 on 3-MC pre-treatment. These authors point out that 3-MC is a different class of inducer to PB, and this may account for some observed differences. We are reminded by Tuchweber *et al.* (1974) that benzpyrene, which increases cytochrome P_{448} levels (like 3-MC), enhances CCl_4 hepatotoxicity, however.

Several studies have also demonstrated a potentiating effect of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane (DDT) on the hepatotoxicity due to CCl_4 (Seawright and McLean, 1967; Judah *et al.*, 1970; Cawthorne *et al.*, 1970). The inducing properties of DDT seem to be very similar, qualitatively, to those of PB.

The potentiating effects of ethanol and other alcohols have been well studied, since an increased predisposition of alcoholics to CCl_4 poisoning has been recognised for some time (Hasumura *et al.*, 1974; Maling *et al.*, 1975; Traiger and Plaa, 1971; Traiger and Bruckner, 1975). It has been suggested that an interaction with microsomes is involved (Plaa and Traiger, 1973) but not all evidence supports this (Zimmerman, 1976).

Reports of CCl_4 hepatotoxicity potentiation with other compounds probably linked to microsomal metabolism have appeared. These include

chlordane (Stenger *et al.*, 1975), aroclor 1254, 1260 and 1221, which are polychlorinated biphenyls (Carlson, 1975a), progesterone and triamcinolone (Tuchweber and Kovacs, 1971) and carbon monoxide (CO) (Suarez *et al.*, 1972). The potentiating effect of CO would seem to agree with the *in vitro* work of Slater and Sawyer (1971a) where CO increased lipid peroxidation due to CCl_4. The same data would appear to conflict with the decrease of *in vitro* covalent binding of ^{14}C from labelled CCl_4 (Sipes *et al.*, 1977).

Methemoglobinemia in response to sodium nitrite has also been described as increasing the toxic response to CCl_4 (Pankow and Ponsold, 1976). Induction of microsomal metabolism by steroid hormones released by the adrenals in response to this stress was suggested as a possible explanation of the effect. The authors also point out that other stress conditions such as CO or cold exposure enhance microsomal metabolism, which may, in part, account for the above mentioned confliction in CO studies.

Due to the consistency of the reported *in vivo* potentiating effects of PB on CCl_4 induced hepatotoxicity, it could well be expected that hepatocytes isolated for PB pre-treated rats would exhibit a similar increased sensitivity to the toxic actions of CCl_4. This is particularly so if toxicity is being elicited by similar mechanisms in the two systems. A primary aim of the work of this chapter, therefore, was to establish whether or not CCl_4 was more toxic to hepatic parenchymal cells isolated from PB pre-treated rats as compared to cells from non-treated rats. The increased levels of lipid peroxidation in response to CCl_4 in PB pre-treated animals (discussed above) and the increase in lipid peroxidation by PB treatment alone (Hahn *et al.*, 1976) made it of interest

to investigate this response in these hepatocytes in relation to the toxic response.

METHODS

Rats weighing 230-300 g were pre-treated with sodium phenobarbitone (80 mg/kg - dissolved in physiological saline at 80 mg/ml) intraperitoneally at approximately 10 a.m. on each of the three days prior to hepatocyte isolation.

Experimental conditions were as in Chapter 1 except that an extra low dose of 5 µl CCl_4 was incorporated in the full study and only two incubation times (10 and 15 minutes) were used. Incubation times of 60 min were used with doses of 5 and 10 µl of CCl_4 in some instances, as an extension of the study.

Incubations were run in duplicate to allow several parameters to be examined in cells from the same stock suspension. The toxicity of CCl_4 to these cells was assessed in one series by measuring K^+, ALT, ureogenesis and L/P. In the duplicates the CCl_4 concentration was monitored and samples were taken for assay for lipid peroxidation (TBA reactants) and reduced glutathione.

The metabolism of aminopyrine to formaldehyde was also examined in these hepatocytes from phenobarbitone pre-treated rats.

RESULTS AND DISCUSSION

Fig. 4.1 depicts the loss of K^+ in response to CCl_4. The data from

non-induced hepatocyte preparations that were presented in Chapter 1 are included for comparison. There is no difference in the pattern of response. Incubation over 60 min also gave similar responses to those in non-induced cells. A dose of 5 µl caused no loss of K^+ and 10 µl gave an increased loss to 32.9 µmol/g wet wt as compared to the 60 min control. Data were obtained from two experiments.

The second structural parameter, ALT, also showed no marked difference in its response to CCl_4 (Fig. 4.2) when compared to the data in Chapter 1 (see Fig. 1.3). The two experiments over 60 min also gave a response similar to that seen in Chapter 1. There was no loss of ALT into the medium with the 5 µl dose and the 10 µl dose showed a rise of 115 as compared to 12 IU/L in the control cells. The levels of ALT release at the 20 µl dose, 10 min incubation show the widest spread on comparing PB pre-treated to non-induced results, but even this difference does not attain statistical significance. Large variability in the data reduces the possibility of seeing statistical significance in this comparison. Even so, the direction of the difference is different to that predicted by theoretical considerations (i.e. it would be predicted that more ALT would be released from hepatocytes of PB pre-treated rats).

The effects of CCl_4 on hepatocytes isolated from PB pre-treated rats with respect to the metabolic parameters, ureogenesis and L/P, are shown in Table 4.1. The pattern of inhibition of ureogenesis by CCl_4 was much the same in these experiments as it was in those of Chapter 1 (Fig. 1.4) except the 10 and 15 µl doses caused a significant drop at the 10 min incubation (the small decrease seen in Fig. 1.4 was not statistically significant). The levels of urea formation over the incubation periods seem to be higher in cells from PB animals (especially at

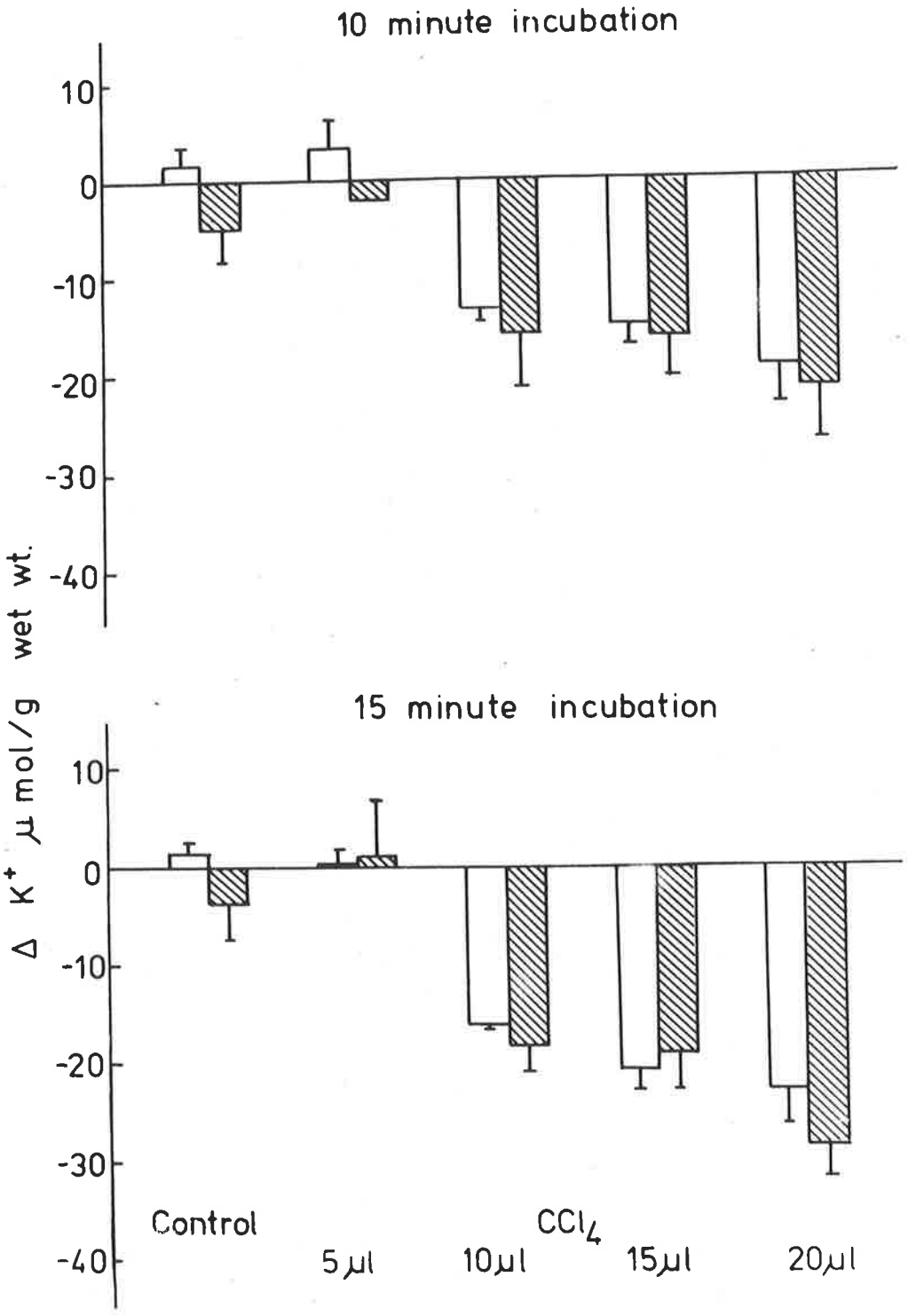


Fig. 4.1 Loss of intracellular K^+ from hepatocytes isolated from phenobarbitone (80 mg/kg, i.p.) pre-treated rats (open bars) and untreated rats (hatched bars) (from Chapter 1, Fig. 1.2) after 10 min (top panel) and 15 min (bottom panel) incubation with CCl_4 or in control cells. Bars represent the mean ± s.e.m. of 4 experiments (except 5 μl hatched bar, 10 min incubation, which is the average of 2 experiments only).

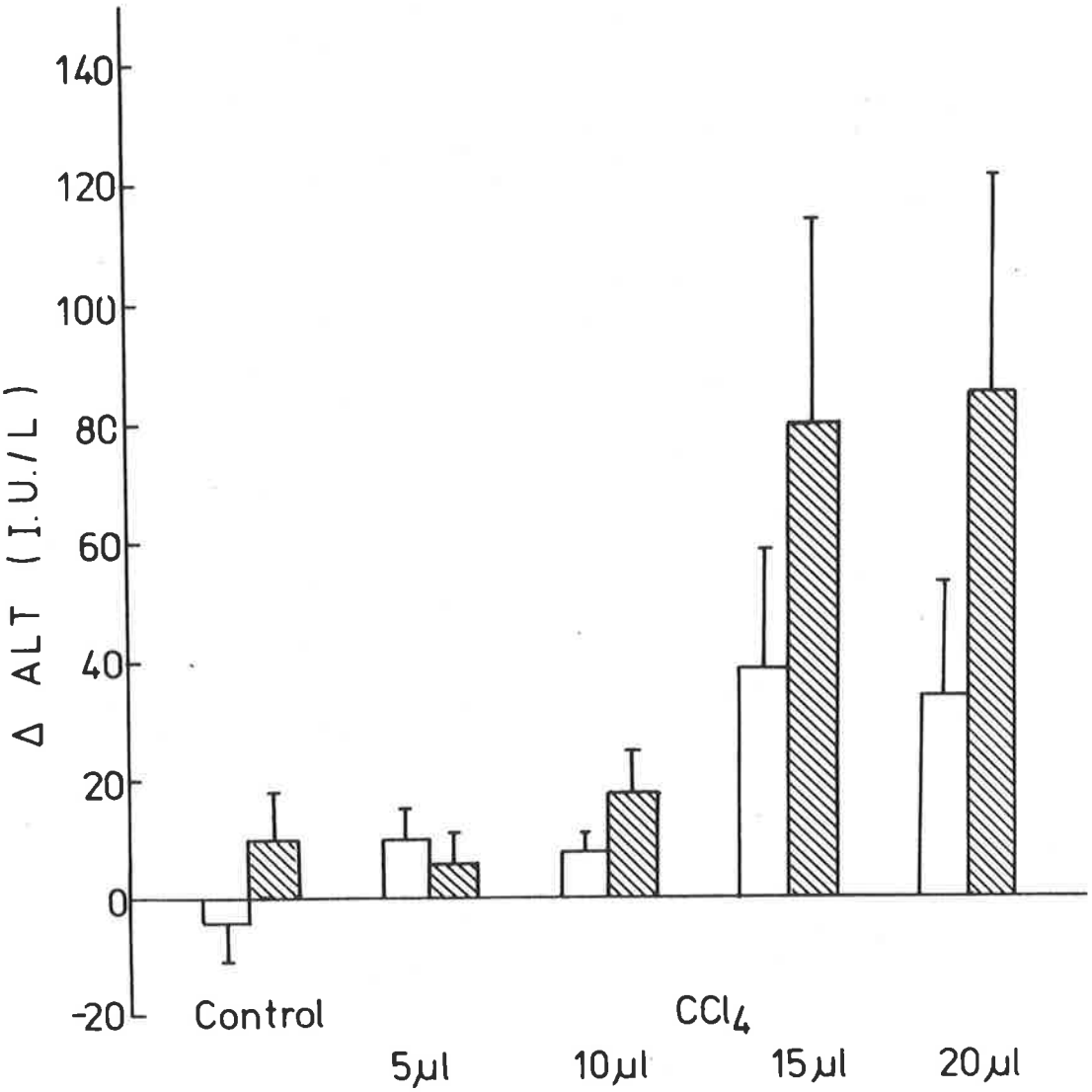


Fig. 4.2 Release of ALT into the medium from hepatocytes isolated from phenobarbitone (80 mg/kg, i.p.) pre-treated rats after 10 min (open bars) and 15 (hatched bars) incubation with CCl_4 or in control cells. Bars represent the mean ± s.e.m. of 4 experiments.

TABLE 4.1

EFFECTS OF CCl_4 ON METABOLIC PARAMETERS, UREOGENESIS AND LACTATE/PYRUVATE RATIO (L/P), ON HEPATOCYTES ISOLATED FROM PHENOBARBITONE PRE-TREATED RATS[a]

TREATMENT	INCUBATION TIME (MIN)	Δ UREA[b]	L/P[c]
Control	10	4.0 ± 0.82	3.1 ± 0.8
CCl_4 5 µl	10	3.26 ± 0.70[d]	3.3 ± 0.4
CCl_4 10 µl	10	3.01 ± 0.70[d]	6.1 ± 0.9[d]
CCl_4 15 µl	10	3.11 ± 0.76[d]	11.7 ± 2.4[d]
CCl_4 20 µl	10	3.10 ± 0.61[d]	16.2 ± 3.5[d]
Control	15	4.53 ± 0.67	1.9 ± 0.3
CCl_4 5 µl	15	3.65 ± 0.60[d]	3.1 ± 0.3[d]
CCl_4 10 µl	15	3.26 ± 0.57[d]	7.7 ± 1.0[d]
CCl_4 15 µl	15	3.62 ± 0.82[d]	21.8 ± 2.4[d]
CCl_4 20 µl	15	3.33 ± 0.73[d]	26.9 ± 2.9[d]

[a] 80 mg/kg i.p. on each of 3 days prior to hepatocyte isolation.

[b] Change in urea content of cell suspension (µmol/g wet wt) over the period of incubation. Values represent the mean ± s.e.m. (n=4).

[c] Values represent the mean ± s.e.m. (n=4).

[d] Statistically significantly different to respective controls.

10 min incubation) but the relevance of this observation is questioned when considering that nearly 6 µmol/g wet wt was formed over 15 min in the series of experiments shown in Fig. 2.3. An increase in L/P was observed on treatment with CCl_4 at all dose/time combinations except 5 µl, 10 min incubation. Again this was qualitatively similar to the results of Chapter 1 (Fig. 1.5) except that 10 µl, 10 min was not significantly different in the data from non-induced hepatocytes. The magnitude of the values obtained is somewhat higher in the results from cells of the non-induced animals as compared to those that had undergone PB pre-treatment.

Table 4.2 gives the CCl_4 concentrations actually measured in the hepatocyte suspensions prepared from PB pre-treated rats. Comparison with Table 1.1 indicates that there is no consistent difference between the concentration of CCl_4 measured in the suspensions from PB pre-treated and untreated rats.

Intracellular GSH content is given in Table 4.3, as are changes in the levels of TBA reactants. At 10 min incubation GSH is significantly higher in CCl_4 treated cells (except the 15 µl dose which just fails to reach significance) than in respective controls. At 15 min, however, there is no change in GSH, which is similar to the finding with hepatocytes from non-induced animals (Table 3.3). A comparison of GSH in 15 min incubated control cells from induced and non-induced animals (i.e. Table 4.3 and 3.3) showed that there was a significantly higher concentration of GSH from animals that had not been pre-treated, as compared to those that had received PB. Such an observation is not apparent in other studies *in vivo* where animals have been pre-treated with PB (Docks and Krishna, 1976; McLean and Day, 1975). There is a slight drop seen in the

TABLE 4.2

CONCENTRATION OF CCl_4 [a] IN HEPATOCYTE SUSPENSIONS ISOLATED FROM PHENOBARBITONE PRE-TREATED RATS [b]

DOSE µl	TIME OF INCUBATION	
	10	15
5	5.4 ± 0.4	5.0 ± 0.7
10	8.4 ± 1.0	8.9 ± 0.7
15	13.6 ± 1.4	11.6 ± 0.6
20	16.9 ± 1.1	16.0 ± 1.9

[a] CCl_4 was added to the side arm of the incubating flasks at t = zero. Values shown are mean ± s.e.m. (n=4) of CCl_4 concentration (mM) in the cell suspension.

[b] 80 mg/kg i.p. on each of 3 days prior to hepatocyte isolation.

TABLE 4.3

EFFECTS OF CCl_4 ON REDUCED GLUTATHIONE (GSH) CONTENT AND TBA REACTING PRODUCTS IN HEPATOCYTES ISOLATED FROM PHENOBARBITONE PRE-TREATED RATS[a]

TREATMENT	INCUBATION TIME (MIN)	GSH[b]	Δ TBA REACTANTS[c]
Control	10	3.16 ± 0.27	0.022 ± 0.007
CCl_4 5 µl	10	3.53 ± 0.31[d]	0.023 ± 0.007
CCl_4 10 µl	10	3.67 ± 0.37[d]	0.029 ± 0.005
CCl_4 15 µl	10	3.64 ± 0.42	0.028 ± 0.006
CCl_4 20 µl	10	3.90 ± 0.33[d]	0.027 ± 0.006
Control	15	3.49 ± 0.32	0.026 ± 0.005
CCl_4 5 µl	15	3.68 ± 0.45	0.035 ± 0.006[d]
CCl_4 10 µl	15	3.79 ± 0.56	0.034 ± 0.007[d]
CCl_4 15 µl	15	3.52 ± 0.61	0.037 ± 0.008[d]
CCl_4 20 µl	15	3.59 ± 0.70	0.038 ± 0.003[d]
Control	60	-	0.092 (2)
CCl_4 5 µl	60	-	0.088 (2)
CCl_4 10 µl	60	-	0.087 (2)

[a] 80 mg/kg i.p. on each of 3 days prior to hepatocyte isolation.

[b] Reduced glutathione concentrations of hepatocyte suspensions in µmol/g wet wt. Values represent the mean ± s.e.m. (n=4). Concentration at zero time was 3.79 ± 0.42 µmol/g wet wt (n=4).

[c] Change in levels of TBA reactants in cell suspensions (absorbance units) over the incubation period. Values represent the mean ± s.e.m. (n=4), except for 60 min incubation where n is in parentheses.

[d] Statistically significantly different to respective controls.

data of Jollow *et al.* (1974) but this was probably not statistically significant. This finding in the cells could be related to the calculation and expression of results, but this is doubtful since there was no corresponding decrease in control K^+ levels.

There was no significant effect of CCl_4 on the amount of TBA reacting substance produced over 10 min incubation, but each dose of CCl_4 at 15 min incubation caused a statistically significant rise in this product. This is in contrast to the results with cells from non-induced animals, which yielded no significant increase in TBA reactants over 15 min (or 30 min) incubation with 10, 15 or 20 µl CCl_4. However, the biological significance is of some doubt since this increase with PB cells was not maintained over 60 min incubation with the two doses tested (5 and 10 µl). The observed differences may be due to an abnormally low control level. Consideration of change in TBA reactants per unit time from the 10 and 60 min incubations, as compared to that from 15 min, would support this.

A comparison of the amounts of TBA reactants formed in 15 min incubated control cells from induced (Table 4.3) and non-induced (Table 3.1) animals showed a statistically significant difference between them. With induced cells there was a change of 0.026 ± 0.005 absorbance units (532 nm) over the 15 min as compared to 0.015 ± 0.001 absorbance units with hepatocytes from the untreated animals. This observation is consistent with the work of Hahn *et al.* (1976) who showed increases in lipid peroxidation in livers from PB pre-treated animals.

The metabolism of aminopyrine was measured in hepatocytes isolated from the PB pre-treated rats, the results showing about a 3-fold increase over rate of formaldehyde formation in cells from non-induced animals

TABLE 4.4

AMINOPYRINE METABOLISM[a] IN HEPATOCYTE SUSPENSIONS

HEPATOCYTES	METABOLISM[a]
Non-induced[b]	21.3 ± 2.8 (3)
Phenobarbitone[c]	57.7 ± 17.8 (4)[d]

[a] Measured by the rate of formation of formaldehyde in nmol/min/g wet wt. Values are mean ± s.e.m. (n=number in parentheses).

[b] Data as presented in Table 2.2 for control hepatocytes.

[c] Animals pre-treated with phenobarbitone (80 mg/kg, i.p.) on each of 3 days prior to hepatocyte isolation.

[d] Statistically significantly different from metabolism of non-induced hepatocytes.

(Table 4.4). This finding demonstrates an increase in function of the microsomal drug metabolising enzymes after PB treatment, as seen *in vivo* (Gillette *et al.*, 1972; Lauterburg and Bircher, 1976). Thus, it can be reiterated that if CCl_4 is producing cellular damage similar in mechanism to *in vivo* toxic responses, then it is quite reasonable to expect evidence of an increase in the toxic response in the isolated hepatocytes, since this has been shown without dispute in several studies in the whole animal (see Introduction to this chapter). However, this is not the case, there being no consistent pattern of differences in response between hepatocytes isolated from untreated or PB pre-treated rats over the range of parameters determined. The doses of CCl_4 and incubation times used allow the scope for any increase in toxic response to be demonstrated. That is, evidence of toxicity is below maximal. One of the characteristics of PB pre-treatment on CCl_4 hepatotoxicity *in vivo* is a lowering of dose required to produce injury (Garner and McLean, 1969), which is not consistent with the observation that toxicity to the 5 µl dose is still absent.

In conclusion, the data presented in this chapter suggest that the toxicity shown in the isolated rat hepatocytes in response to CCl_4 is not manifest via a reactive metabolite. This is in direct contrast to the situation *in vivo*.

CHAPTER FIVE

TOXICITY OF BROMOBENZENE TO THE ISOLATED HEPATOCYTES

INTRODUCTION

Since about 1970 several reports on the *in vivo* toxicity of bromobenzene have appeared, many of which have been summarized by Gillette (1974) and Gillette *et al.* (1974). Biochemical events which characterise the toxic response *in vivo* have been well defined and include the following. The role of biotransformation, which includes both production and inactivation of a toxic metabolite, has been extensively evaluated. The pattern of metabolism has been given by Jollow *et al.* (1974) and is shown in Fig. 5.1. An epoxide is formed via the mixed function oxidase system (subject to inhibition by SKF-525A) and this is the active metabolite which can combine covalently with tissue macromolecules. Detoxification pathways of the epoxide are shown, the conjugation step with GSH being the major pathway. The amount of GSH substrate available can determine the extent of this conjugation, which is of importance in the toxicity of bromobenzene. It was found by Jollow *et al.* (1974) that necrosis (and arylation of cellular macromolecules) only occurred when levels of GSH had been markedly reduced. The involvement of the mixed function oxidase system had been previously demonstrated by a species study (Mitchell *et al.*, 1971) and experiments incorporating treatments which alter the level of function of the metabolizing enzymes (Reid *et al.*, 1971). In the species study, those animals which showed greater metabolism of bromobenzene also exhibited a greater toxic response. The latter study showed that pre-treatment of animals with PB, which increases microsomal enzyme activity, increased the hepatic injury, while prior administration of SKF-525A lessened toxicity.

The importance of the interaction of the various steps in the overall pathway of bromobenzene has been pointed out by Zampaglione *et al.* (1973). They found that 3-methylcholanthrene was protective against toxic effects

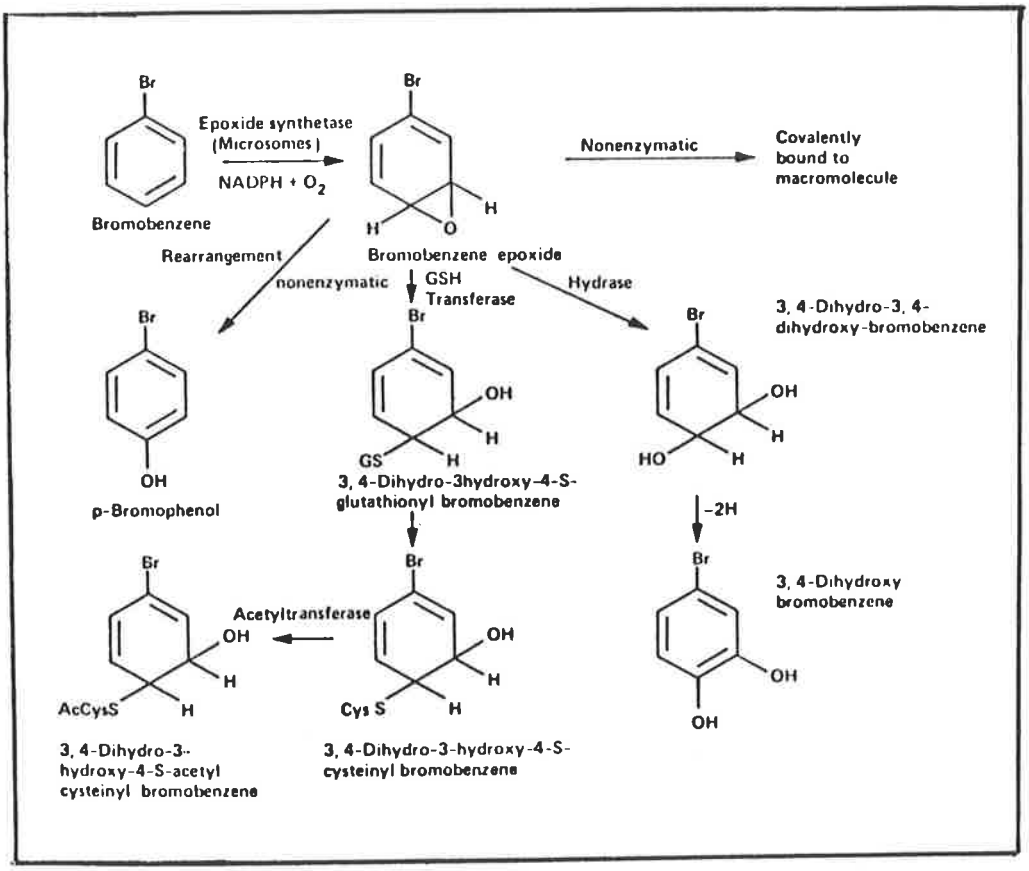


Fig. 5.1 Pathway of bromobenzene metabolism *in vivo*.

of bromobenzene. The evidence indicated that protection was due to an increase in hydrase activity and diversion of metabolism into a comparatively non-toxic pathway resulting in production of some 2-bromophenol (as opposed to 4-bromophenol).

It can be appreciated that toxicity of bromobenzene is similar to that of CCl_4 with respect to requirement of metabolite formation. Bromobenzene, like CCl_4, produces centrilobular necrosis *in vivo*. However, this occurs without any evidence of lipid peroxidation (Gillette, 1974). Microsomal parameters also respond differently (as compared to CCl_4) to bromobenzene (Reynolds, 1972). The similarities and differences between toxicities of these two chemicals, together with the undisputed biochemical events associated with bromobenzene injury, made it of great interest to investigate the effects of this second treatment on isolated hepatocytes. This would provide for a comparison of a second chemical treatment in the cell suspension to the *in vivo* situation. Such a comparison would provide further information as to the nature of the apparent differences in mechanism of toxicity of CCl_4 in isolated hepatocytes and *in vivo*. A finding of bromobenzene toxicity with a mechanism different in single cells to *in vivo* would suggest the anomalies found with CCl_4 to be due to the *in vitro* system used rather than a direct disagreement with the nature of the toxic response *in vivo*. On the other hand, demonstration of similarity in mechanism for bromobenzene could suggest the reverse.

An investigation such as this may also provide further insight into how both chemicals produce centrilobular necrosis, while associated with some different biochemical responses. This is of special interest when considering the theory that lipid peroxidation is responsible for the eventual necrosis in response to CCl_4 (Chapter 3).

The direct aims of the experiments documented in this chapter were two fold. Firstly, could a toxic response to bromobenzene be shown in the isolated hepatocytes? Secondly, was there a similarity of mechanism of toxicity in the isolated cells as compared to *in vivo*? The levels of GSH and sensitivity to protection by SKF-525A were investigated to provide information with respect to this second aim.

METHODS

Rats used in the experiments of this chapter weighed between 220 and 300 g. Bromobenzene (1-9 µl) was added directly to 2 ml of cell suspensions using a microsyringe; SKF-525A was dissolved in physiological saline and 0.1 ml added to the relevant flasks giving a final concentration of 0.1 mM.

Incubations were of 10 or 20 minutes' duration after which time samples were taken for the assessment of toxicity by measurement of K^+, ALT, ureogenesis and L/P. Samples were also taken for assay of GSH and in some experiments the bromobenzene concentration itself was monitored.

RESULTS

Figs 5.2 and 5.3 show that bromobenzene produced a dose related loss of K^+ from the cell pellet, and release of ALT into the supernatant. The release of ALT produced by the highest doses of bromobenzene was 70-80% of that achieved by physical disruption of the cells. The effect was time-related in that incubation with bromobenzene for twenty minutes produced a larger response than that for ten minutes, while controls

Fig. 5.2 Loss of intracellular K^+ in hepatocyte suspensions incubated with bromobenzene with and without SKF-525A (0.1 mM). Each point represents the mean hepatocyte K^+ (n=4 experiments) and the bars represent the s.e.m.
Bromobenzene: ○——○ 10 min; □——□ 20 min.
Bromobenzene + SKF-525A: ●-----● 10 min; ■-----■ 20 min.

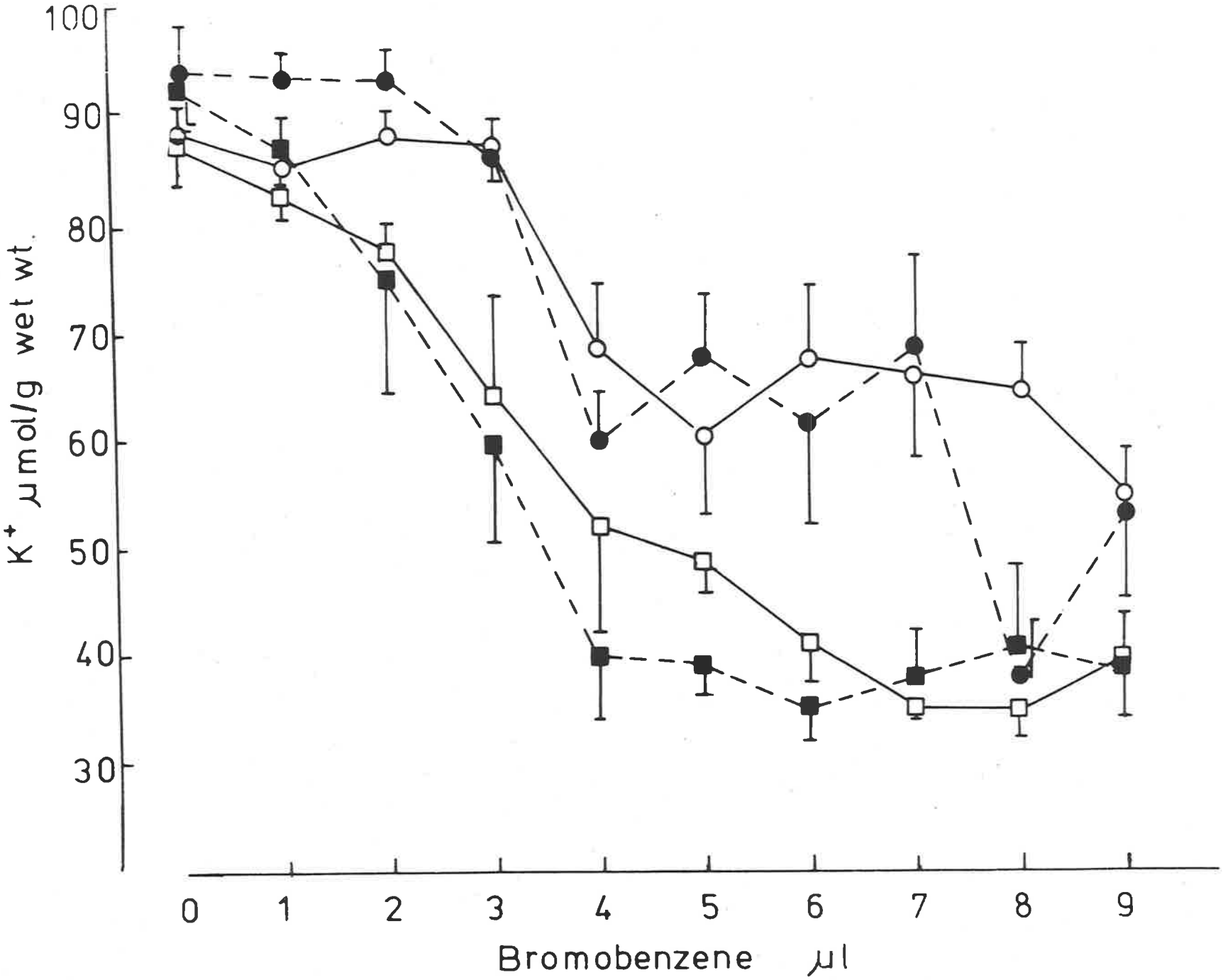
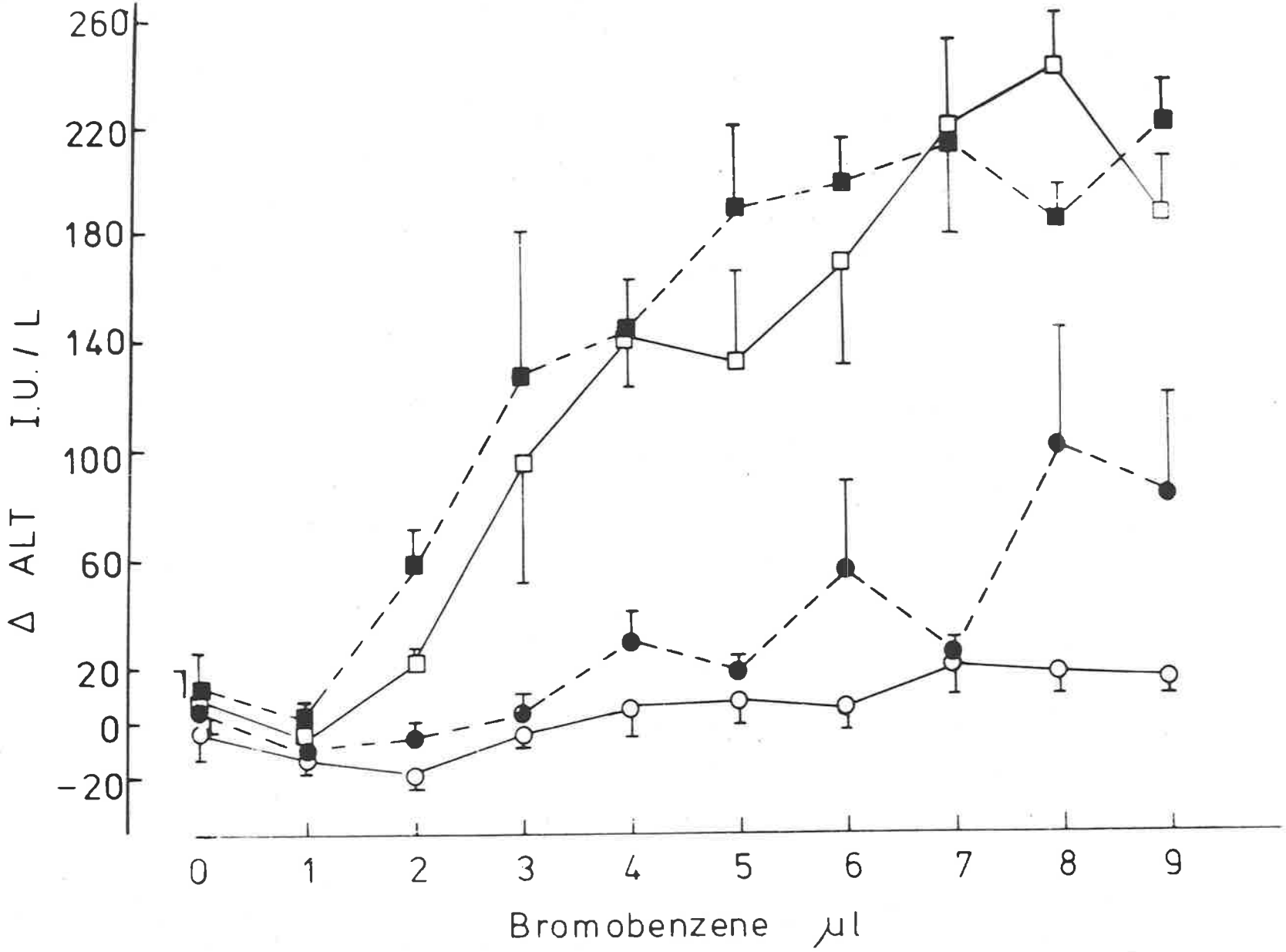

100
90
80
70
60
50
40
30
K+ µmol/g wet wt.
0
1
2
3
4
5
6
7
8
9
Bromobenzene µl

Fig. 5.3 Release of ALT into the medium from hepatocytes incubated with bromobenzene with and without SKF-525A (0.1 mM). Each point represents the mean change in supernatant ALT from the beginning of incubation (n=4 experiments) and the bars represent s.e.m.

Bromobenzene: ○——○ 10 min; □——□ 20 min.

Bromobenzene + SKF-525A: ●-----● 10 min; ■-----■ 20 min.


260
220
180
140
100
60
20
0
-20
Δ ALT I.U./L
0
1
2
3
4
5
6
7
8
9
Bromobenzene µl

remained stable over these periods. The addition of SKF-525A produced no protective effect on cells incubated with bromobenzene. If anything, there was an increase in toxic response at the occasional dose level, but this was certainly not a consistent response.

The time related course of urea synthesis in control cells is shown in Fig. 5.4, the rate being about 0.5-0.7 µmol/min/g wet weight. The effect of bromobenzene as shown in Fig. 5.4 was to inhibit this formation of urea. Fig. 5.5, which includes the data from all the bromobenzene doses tested shows that the effect was maximal at the lowest dose (1 µl). The rate of ureogenesis was increased by SKF-525A in control cells, but this agent did not modify the bromobenzene-induced inhibition. Fig. 5.6 shows that bromobenzene produced a dose related increase in the L/P ratio. A great variability in response, particularly at the higher doses, was associated with this effect. SKF-525A had no consistent effect on the response since the apparent differences achieved statistical significance only at the lower dose levels and the direction of the change appeared to differ at some higher dose levels. The data at the lower dose levels are shown on an expanded scale in Fig. 5.7, where it can be appreciated that this significant alteration of the bromobenzene response is an increase in this parameter of toxicity.

The levels of reduced glutathione (GSH) in the cell suspension are shown in Table 5.1. Incubation for ten or twenty minutes did not affect GSH in control cells and bromobenzene produced only a small drop, showing statistical significance generally at the higher doses and longer incubation time, as indicated in the table. There was no consistent modification of response by SKF-525A.

Fig. 5.4 Ureogenesis in hepatocyte suspensions incubated with and without 1 µl bromobenzene in the presence or absence of SKF-525A (0.1 mM). Each point represents the mean change in urea concentration (n=4 experiments) and the bars represent the s.e.m.

Controls (no bromobenzene): Δ——Δ;

Controls + SKF-525A (0.1 mM) Δ-----Δ;

Bromobenzene 1 µl (▲——▲);

Bromobenzene 1 µl + SKF-525A (0.1 mM) ▲-----▲.

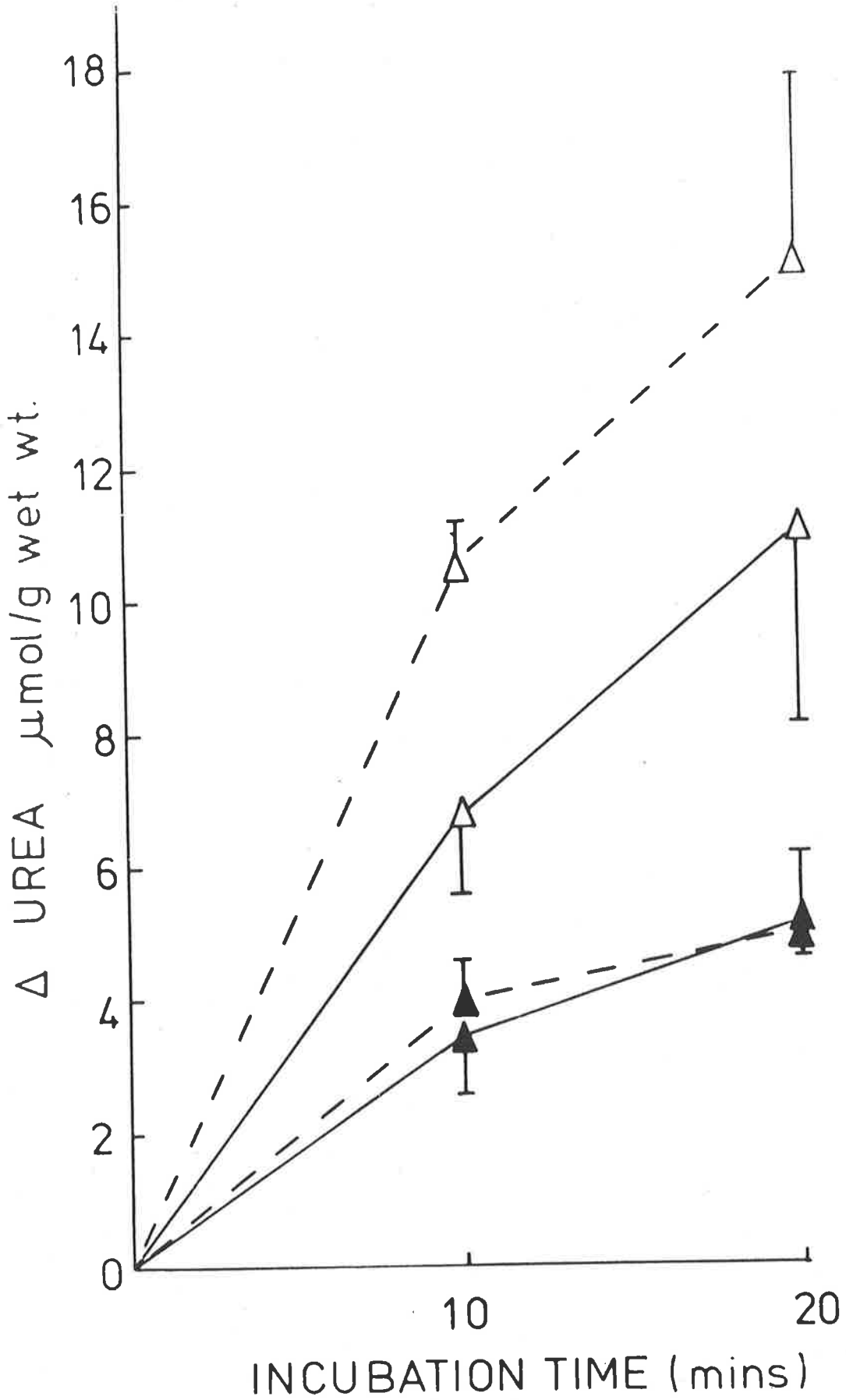

18
16
14
12
10
8
6
4
2
0
Δ UREA µmol/g wet wt.
10
20
INCUBATION TIME (mins)

Fig. 5.5 Ureogenesis in hepatocyte suspensions incubated with bromobenzene with and without SKF-525A (0.1 mM). Each point represents the mean change in urea concentration from the beginning of incubation (n=4 experiments) and the bars represent the s.e.m.

Bromobenzene: ○——○ 10 min; □——□ 20 min.

Bromobenzene + SKF-525A: ●-----● 10 min; ■-----■ 20 min.

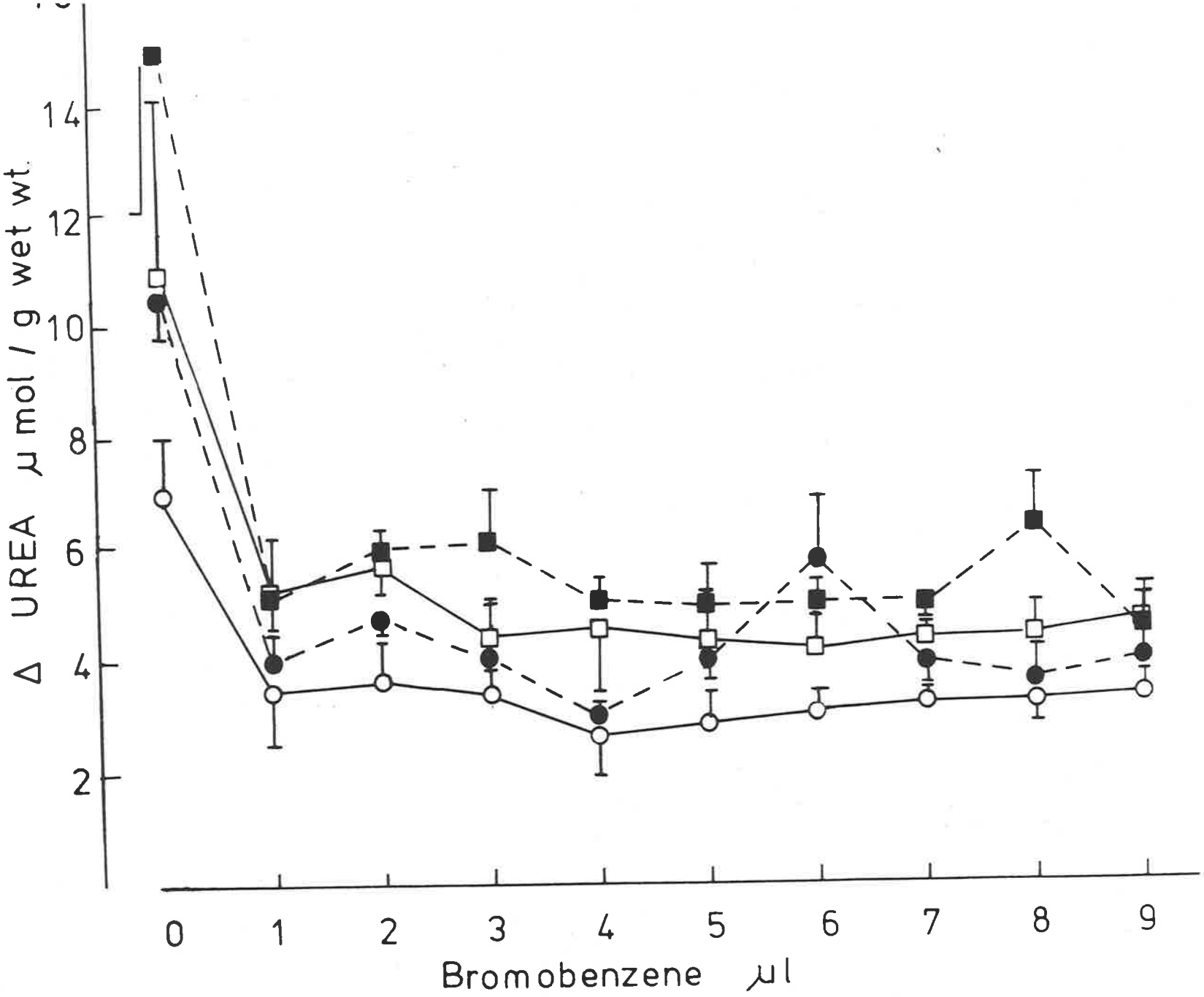

Δ UREA µ mol / g wet wt.
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
Bromobenzene µl

Fig. 5.6 Lactate/pyruvate ratio in hepatocyte suspensions incubated with bromobenzene with and without SKF-525A (0.1 mM). Each point represents the mean L/P ratio (n=4 experiments) and the bars represent the s.e.m.
Bromobenzene: ○——○ 10 min; □——□ 20 min.
Bromobenzene + SKF-525A: ●----● 10 min; ■----■ 20 min.

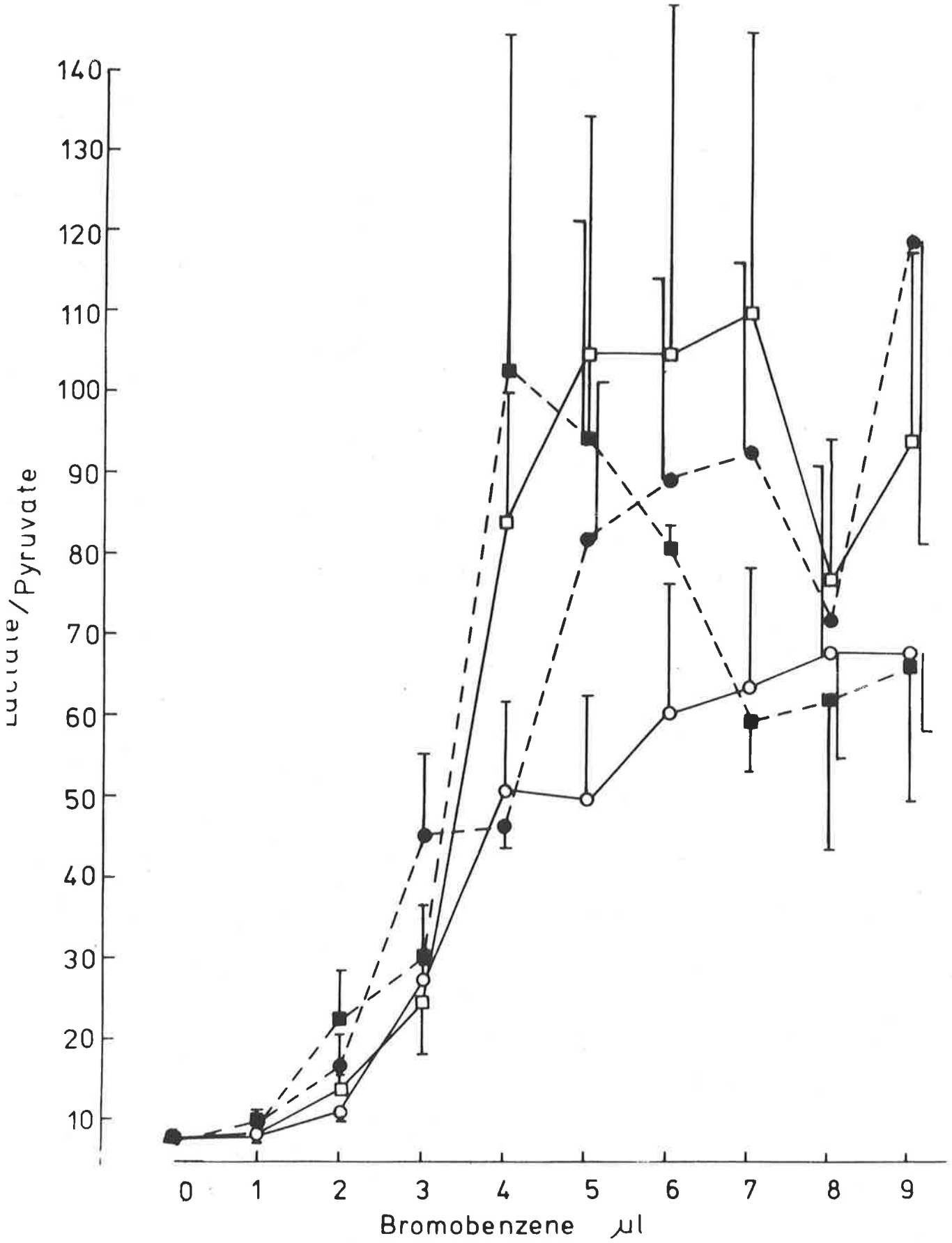

Lactate/Pyruvate
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
Bromobenzene µl

Fig. 5.7 Lactate/pyruvate ratio in hepatocyte suspensions incubated with the lower doses of bromobenzene (0, 1, 2, and 3 µl) with and without SKF-525A (0.1 mM) Each point represents the mean L/P ratio (n=4 experiments) and the bars represent the s.e.m.
Bromobenzene: ○——○ 10 min; □——□ 20 min.
Bromobenzene + SKF-525A: ●-----● 10 min; ■-----■ 20 min.

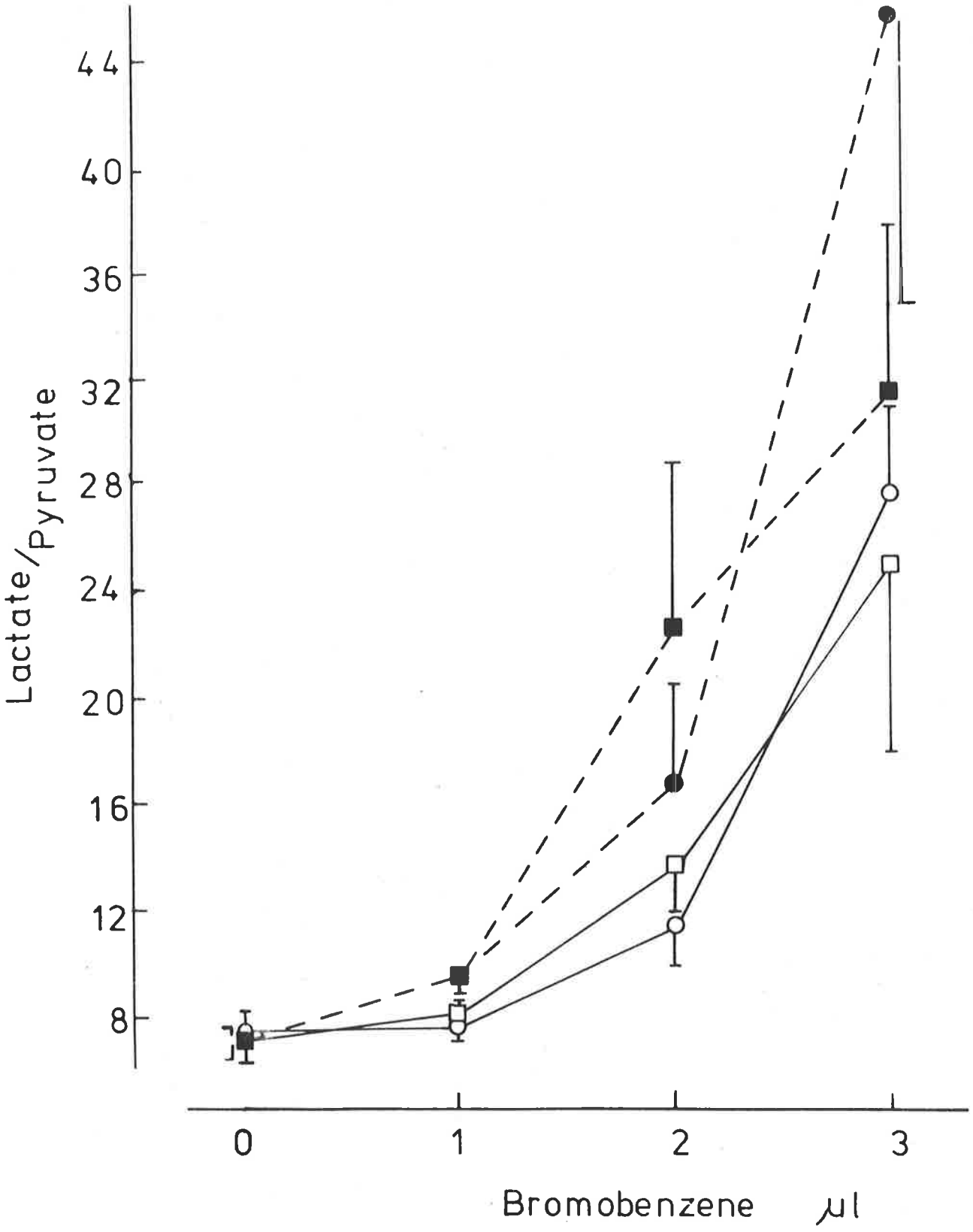

44
40
36
32
28
24
20
16
12
8
Lactate/Pyruvate
0
1
2
3
Bromobenzene µl

TABLE 5.1

REDUCED GLUTATHIONE CONCENTRATIONS IN HEPATOCYTES[a]

BROMOBENZENE DOSE (µl)	CONTROLS		SKF-525A	
	10'	20'	10'	20'
0	5.06 ± 0.54	5.15 ± 0.57	5.17 ± 0.53	5.10 ± 0.62
1	4.31 ± 0.27[b]	4.32 ± 0.26[b]	5.05 ± 0.40	4.87 ± 0.76
2	4.58 ± 0.24	4.57 ± 0.23	5.12 ± 0.38	4.76 ± 0.40
3	4.73 ± 0.22	4.26 ± 0.26	5.14 ± 0.32	4.39 ± 0.46
4	4.71 ± 0.21	4.25 ± 0.20[b]	4.99 ± 0.35	4.27 ± 0.55[b]
5	4.64 ± 0.14	4.47 ± 0.18	5.13 ± 0.43	4.14 ± 0.58
6	4.76 ± 0.18	4.36 ± 0.20	5.00 ± 0.36	4.03 ± 0.56[b]
7	4.84 ± 0.26	3.97 ± 0.04[b]	5.17 ± 0.37	3.99 ± 0.58[b]
8	4.81 ± 0.25	4.13 ± 0.27[b]	4.73 ± 0.40[b]	4.18 ± 0.57[b]
9	4.75 ± 0.23	4.12 ± 0.32[b]	4.93 ± 0.37	4.06 ± 0.55[b]

[a] Values shown are the mean ± s.e.m. (n=4) reduced glutathione concentrations (µmol/g wet wt) in hepatocytes incubated 10 or 20 minutes with 0-9 µl bromobenzene, with and without SKF-525A (0.1 mM). The mean ± s.e.m. (n=4) reduced glutathione concentrations prior to any treatment was 5.00 ± 0.50.

[b] Significantly lower than controls (no bromobenzene added) after the same period of incubation.

The actual concentrations of bromobenzene measured in the hepatocyte suspensions after incubation for 10 min are given in Table 5.2. Similar levels were found at the 20 min incubation mark in one experiment. It is apparent that doses in excess of 4 µl exceed the maximum solubility of bromobenzene in the suspension. In fact, small droplets could be seen at the bottom of the incubation vessels given the higher doses. Nevertheless, this undissolved bromobenzene may provide a consistent medium concentration in the event that there was any loss due to metabolism.

DISCUSSION

Bromobenzene has been shown to elicit a toxic response from the isolated hepatocytes. The changes in parameters of membrane integrity, K^+ and ALT, were both time and dose related. Inhibition of ureogenesis was maximal at the lowest dose of bromobenzene, whereas L/P increased with the increase in dose used in these experiments. These results are similar in pattern to those observed with CCl_4 (Chapter 1).

Although not totally consistent, there appeared to be a trend to a levelling off in response after about the 4 µl dose. This observation is consistent with the actual concentrations of bromobenzene measured in the cell suspension (Table 5.2).

The presence of SKF-525A did not ameliorate the response, and this is clearly different to the effect seen in the intact animal (Reid *et al.*, 1971; Mitchell *et al.*, 1971). This indicates that the mechanism of toxicity in the isolated cells may be different to that *in vivo*. In particular, these data cast doubt on the involvement of toxic metabolite formation in isolated hepatocytes. The metabolism of bromobenzene itself

TABLE 5.2

CONCENTRATION OF BROMOBENZENE[a] IN HEPATOCYTE SUSPENSIONS AFTER 10 MIN INCUBATION

DOSE (µl)	CONCENTRATION (mM)
1	6.6 ± 1.7
2	13.6 ± 1.8
3	22.1 ± 5.5
4	26.9 ± 6.7
5	25.3 ± 5.1
7	23.1 ± 4.3
9	25.5 ± 7.2

[a] Bromobenzene was added by microsyringe to 2 ml of the cell suspension at 0 min incubation. Values shown are the mean ± s.e.m. (n=3) of bromobenzene concentration (mM) in the cell suspension.

has not been investigated in these experiments, but the capacity of the isolated hepatocytes for metabolism through the mixed function oxidase pathway has been extensively documented (see General Introduction). The data demonstrating such a capability of cell suspensions prepared in this laboratory have been presented in Chapters 1 and 4. Examination of the cells for the presence of the epoxide metabolite of bromobenzene and binding of ^{14}C from labelled bromobenzene to tissue macromolecules would allow for a more direct statement on the involvement (or lack of) of a toxic metabolite in the experiments of this chapter. Experiments with PB treated cells could also be used to obtain data to support, or otherwise, the apparent non-involvement of a reactive metabolite. The likelihood of an increased toxic response is perhaps reduced on consideration of the lack of such an effect with CCl_4.

Toxicity occurring through a metabolite is dependent on several kinetic factors. These include rate of formation, inherent reactivity and rate of detoxification of the particular metabolite and the rate of injury repair (Mitchell and Jollow, 1975). Studies with inhibitors or inducers of microsomal enzymes require care in interpretation of results. For example, if evidence of toxicity is looked at after it has become maximal, inhibitory effects of a treatment may be missed, the inhibition only having delayed the toxicity. The importance of the temporal aspects involved in such toxicity studies has been discussed by Gillette (1974). The lack of a protective effect of SKF-525A in the experiments of this chapter cannot be explained by such considerations, since its effects on non-maximal response and over the time period of the response have been examined.

The data concerning GSH found with the isolated hepatocytes, too,

show marked differences to that in the intact animal (Jollow *et al.*, 1974). The *in vivo* studies showed that GSH had to fall markedly (from about 7 to 2 μmol/g wet wt) before toxicity became evident. In the present study GSH fell by only about 1 μmol/g wet wt, and this generally only achieved statistical significance after obvious signs of toxicity had become apparent. It is possible that a loss of activity of the enzyme responsible for conjugation, glutathione epoxide transferase, could account for this finding in the isolated cells. This would seem unlikely for two reasons. Firstly, the cells have retained their K^+ and ALT suggesting that physical loss of the enzyme would not have occurred, and secondly, the isolated rat hepatocytes have been reported to retain the related enzymes, glutathione peroxidase and glutathione reductase (Sies *et al.*, 1977). The activity needs to be directly determined, however, before this possibility can be discounted.

There was no consistent alteration of the small changes in GSH by SKF-525A. It is interesting to note, however, that at the 1 μl dose both the 10 and 20 minute incubations showed a significant fall in GSH and that SKF-525A appeared to protect against this (SKF-525A versus non-SKF-525A incubates: $p<0.05$, 10 minute incubation; $0.1>p>0.05$, 20 minute incubation). This suggests that longer term incubations with this and even lower doses may yield information more analagous to that found in the *in vivo* studies (Zampaglione *et al.*, 1973).

Nevertheless, the fact remains that toxicity has been demonstrated to occur in isolated rat hepatocytes in response to bromobenzene, and that there are differences in the biochemical events associated with this toxicity when compared to those seen in the whole animal.

CHAPTER SIX

TOXICITY OF HALOGENATED VOLATILE ANAESTHETICS IN ISOLATED RAT HEPATOCYTES

INTRODUCTION

Drugs found to possess hepatotoxic properties in a limited portion of the human population are often described as idiosyncratic. This is because the reaction is only seen in the small number of patients who can thus be described as having an idiosyncrasy to display hepatotoxic effects to the drug. Some of these drugs have also been described as being of a hypersensitivity group because a number of them are associated with reactions found with a hypersensitivity response (for example, serum antibodies, eosinophilia, rash, urticaria). This is seen to occur in a number of the patients displaying the hepatotoxic reaction, but not necessarily all patients with all such drugs. It is probably therefore more appropriate to classify these agents as idiosyncratic hepatotoxins, with a subdivision of hypersensitivity, as indicated by Zimmerman (1976). A major problem with the toxicology of such agents is that their toxicity is not reproducible in animal experiments. Consequently their toxicity is not predictable nor can the mechanism be more thoroughly investigated.

It has been suggested, however, that the toxic potential of some idiosyncratic toxins may be determined in *in vitro* experiments using isolated or cultured liver cells. Cultured cells have been used to test the cytotoxicity of the phenothiazines (Dujovne and Zimmerman, 1969; Zimmerman and Kendler, 1970), erythromycins (Zimmerman *et al.*, 1973; Dujovne, 1975), the laxative, oxyphenisatin (Dujovne and Shoeman, 1972) and some general anaesthetics (Goto *et al.*, 1976a; Goto *et al.*, 1976b). The isolated perfused liver has also been used in toxicity studies on some of these drugs (Kendler *et al.*, 1971; Kendler *et al.*, 1972). The outcome of such studies has been that the order of cytotoxic potency was found to parallel the *in vivo* incidence of hepatotoxic reaction.

Some inhalational anaesthetics have been associated with liver injury. In particular, halothane can be classed as an idiosyncratic hepatotoxin (Conn, 1974). The actual existence of a causal relationship has not gone unchallenged, however, as discussed by Conn (1974), Van Stee (1976) and Sherlock (1972). Reves (1974) and Van Stee (1976) both conclude that the mechanism remains to be established. Conflicting reports concerning mechanism have appeared. In particular, the effects of phenobarbitone pre-treatment on halothane response (Brown *et al.*, 1974; Reynolds and Moslen, 1974; Reynolds and Moslen, 1975; Stenger and Johnson, 1972) and also evidence concerning a possible inter-relationship with reduced glutathione (Brown *et al.*, 1974; Rosenberg, 1971; Reynolds and Moslen, 1977). Data have also been presented which dispute cell mediated hypersensitivity to a halothane metabolite being the pathogenic agent in hepatic dysfunction after halothane anaesthesia (Walton *et al.*, 1976).

It has been suggested that the mechanism of clinical hepatitis after halothane may be similar to that of CCl_4 (Gillette *et al.*, 1974). Furthermore, halothane is from the same family of halogenated hydrocarbon drugs as CCl_4 (Reves, 1974). Points such as these add to the relevance of incorporating an investigation of halothane in the experiments presented in this thesis.

Methoxyflurane, another inhalational anaesthetic has also been associated with liver injury (Conn, 1974), but this has been somewhat overshadowed by its nephrotoxic effects (Cousins *et al.*, 1974). Enflurane, too, has been linked with nephrotoxicity in patients with decreased preoperative renal function. The potential risk, however, is not as great with enflurane as methoxyflurane, as indicated by serum fluoride levels attained on treatment with these anaesthetics (Cousins *et al.*, 1976).

Only isolated case reports have associated enflurane with a toxic response to the liver (Van der Reis *et al.*, 1974; Sadove and Kim 1974; Denlinger *et al.*, 1974) and this is from some 10 million exposures (Corall *et al.*, 1977). A study by Stevens *et al.* (1977) showed histological evidence of liver injury on exposure to enflurane in mice but not rats or guinea pigs. The reaction in the mice was less than that seen with a five fold lower dose of halothane. These authors mention, however, that there is no convincing evidence of hepatic injury with enflurane in man. The studies of Dobkin *et al.* (1968) and Black *et al.* (1977) indicate some of the other possible advantages of enflurane as a clinical anaesthetic. It was therefore of interest to investigate methoxyflurane and enflurane along with halothane to see how the toxicities compared. A fourth anaesthetic, chloroform, was also included in this study because its hepatotoxic (and nephrotoxic) effects are well known (Little and Wetstone, 1964). It is also quite clearly hepatotoxic in experimental animals (Brown *et al.*, 1974).

The primary aims of the experiments of this chapter were to investigate the relative toxicities of halothane, methoxyflurane, enflurane and chloroform in the isolated hepatocytes, to see how this compared to other *in vitro* studies and to determine if this correlated with present knowledge of hepatotoxic reactions encountered in the clinical situation.

METHODS

Rats weighing between 200 and 260 g were used as liver donors in the experiments of this chapter. Volatile anaesthetics (2.5-20 µl) were added directly to 2 ml of cell suspensions, which, after sealing, were incubated

for 20 or 60 minutes. Samples were taken for assessment of toxicity by measurement of K^+, ALT, ureogenesis and L/P. The actual concentrations of dissolved anaesthetics were also monitored during some experiments.

Comparative potency of the four drugs was assessed by tabulating treatment differences in each of the four hepatocyte preparations and determining the statistical significance of these differences using the Wilcoxon matched-pairs signed-rank test (Siegel, 1956).

RESULTS

Chloroform, methoxyflurane and halothane produced a dose-dependent loss of K^+ from the cell pellet, (Fig. 6.1) and release of ALT into the supernatant (Fig. 6.2) when compared with control cells. Enflurane did not alter the cell permeability to K^+ or ALT over 20 minutes incubation even at the highest dose level (Figs. 6.1 and 6.2). Extension of the incubation period to 60 minutes did not cause a significant change in these parameters, either, on incubation with 20 µl of enflurane as shown in Fig. 6.3. In contrast, the toxic effects of halothane were greater with the longer incubation period (Fig. 6.3) on treatment with the 15 µl and 20 µl doses. By reference to Table 6.1 it can be appreciated that the 15 µl dose of halothane results in a concentration similar to the concentration achieved with a 20 µl dose of enflurane. The concentrations attained in the cell suspensions at 20' and with the other anaesthetics are also presented in Table 6.1

Enflurane produced a dose-related increase in the concentrations of both lactate and pyruvate measured in cell suspensions after 20 minutes' incubation (Fig. 6.4). The effects of the other anaesthetics were more

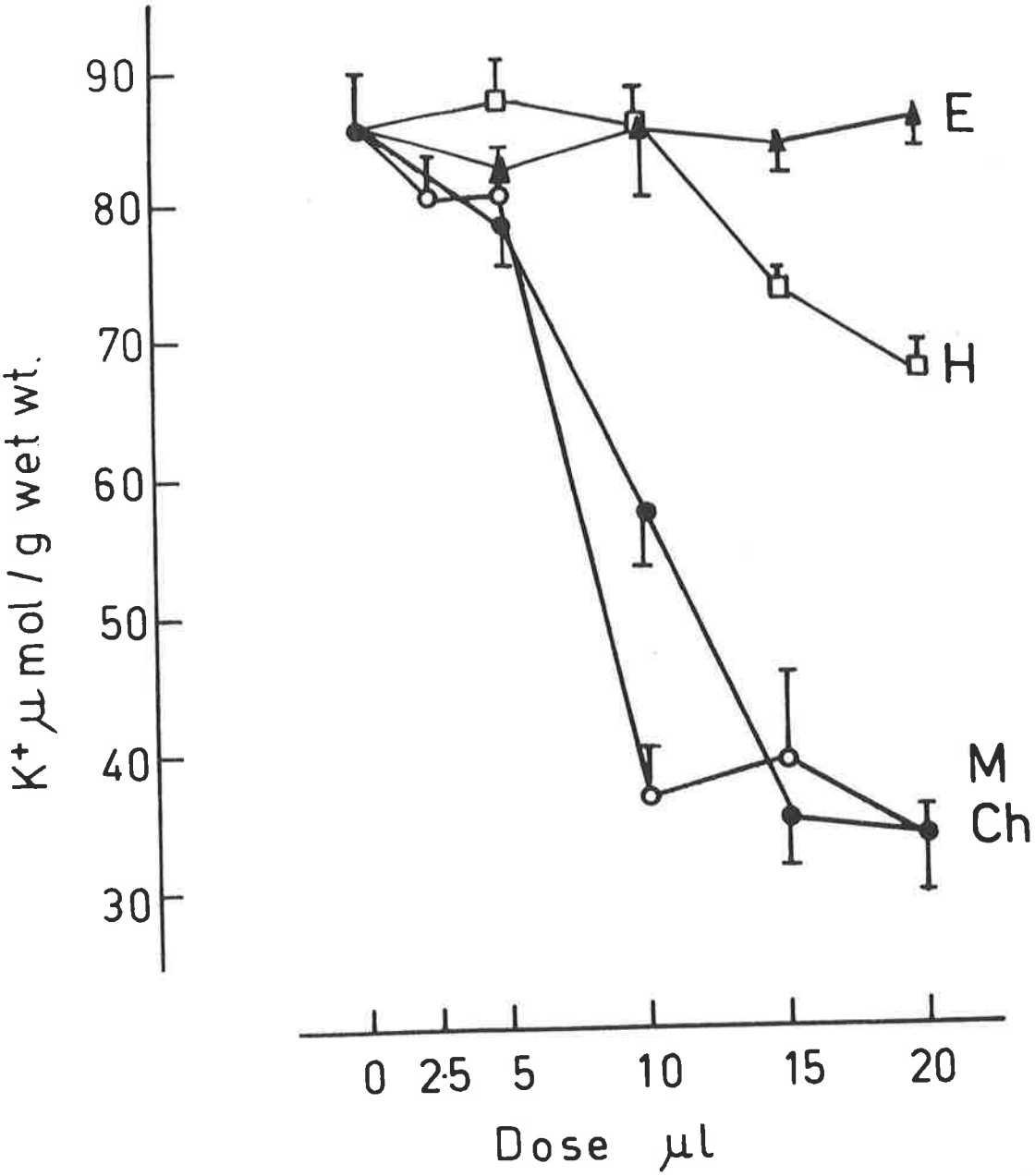


Fig. 6.1 Intracellular potassium ion content after incubation of hepatocyte suspensions (20 minutes, 37°C) with varying doses of anaesthetics. Halothane —□— H; enflurane —▲— E; Methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the s.e.m. Comparative potency: Ch = M > H = E $p<0.01$, n=15 (doses 5-20 µl). For doses 10-20 µl, H > E $p<0.01$ n=12.

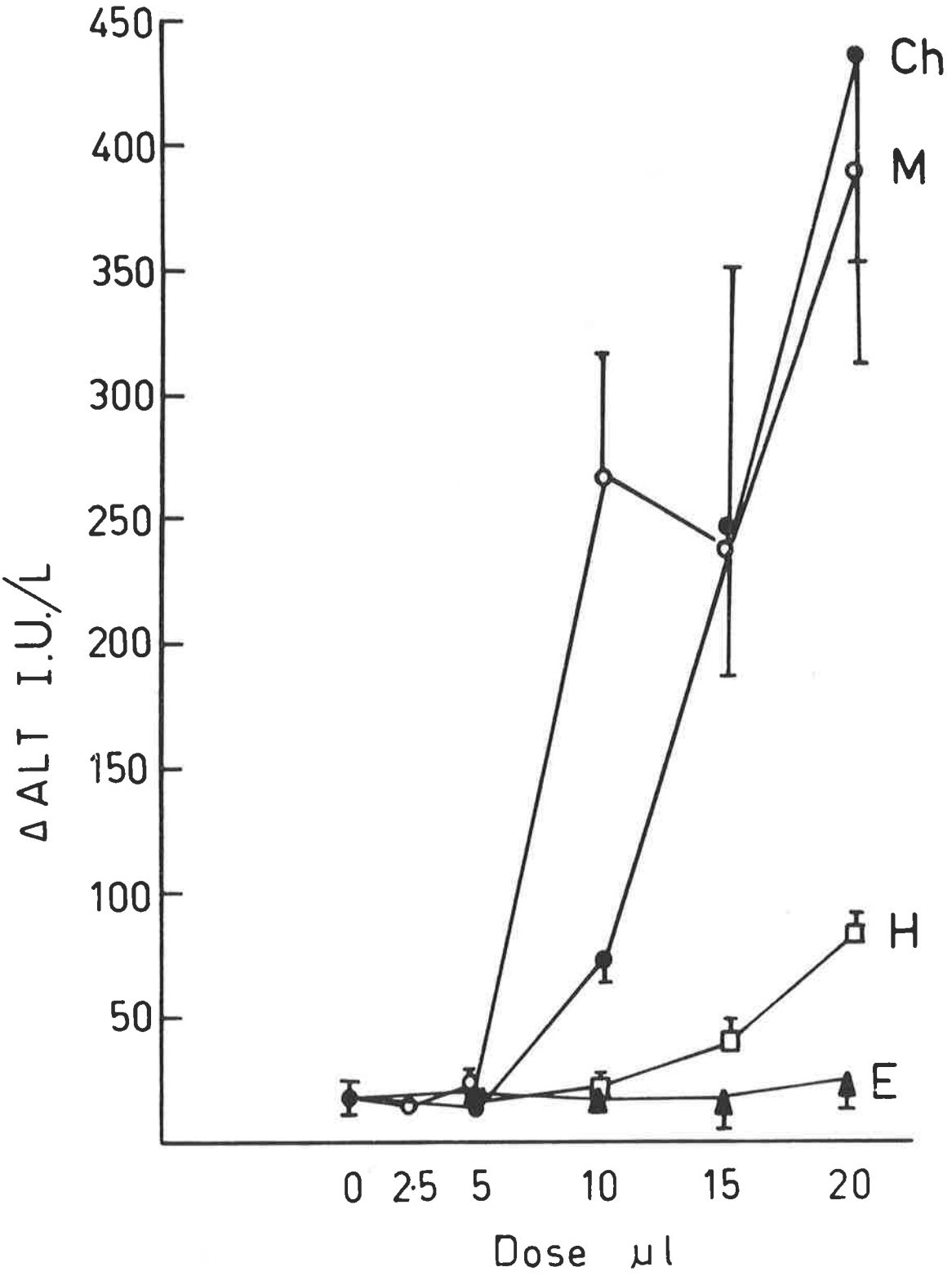


Fig. 6.2 Alanine aminotransferase activity in the supernatant after incubation of hepatocyte suspensions (20 minutes, 37°C) with varying doses of anaesthetics. Halothane —□— H; enflurane —▲— E; Methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the s.e.m. Comparative potency: Ch = M > H > E $p<0.01$, $n=15$ (doses 5-20 µl).

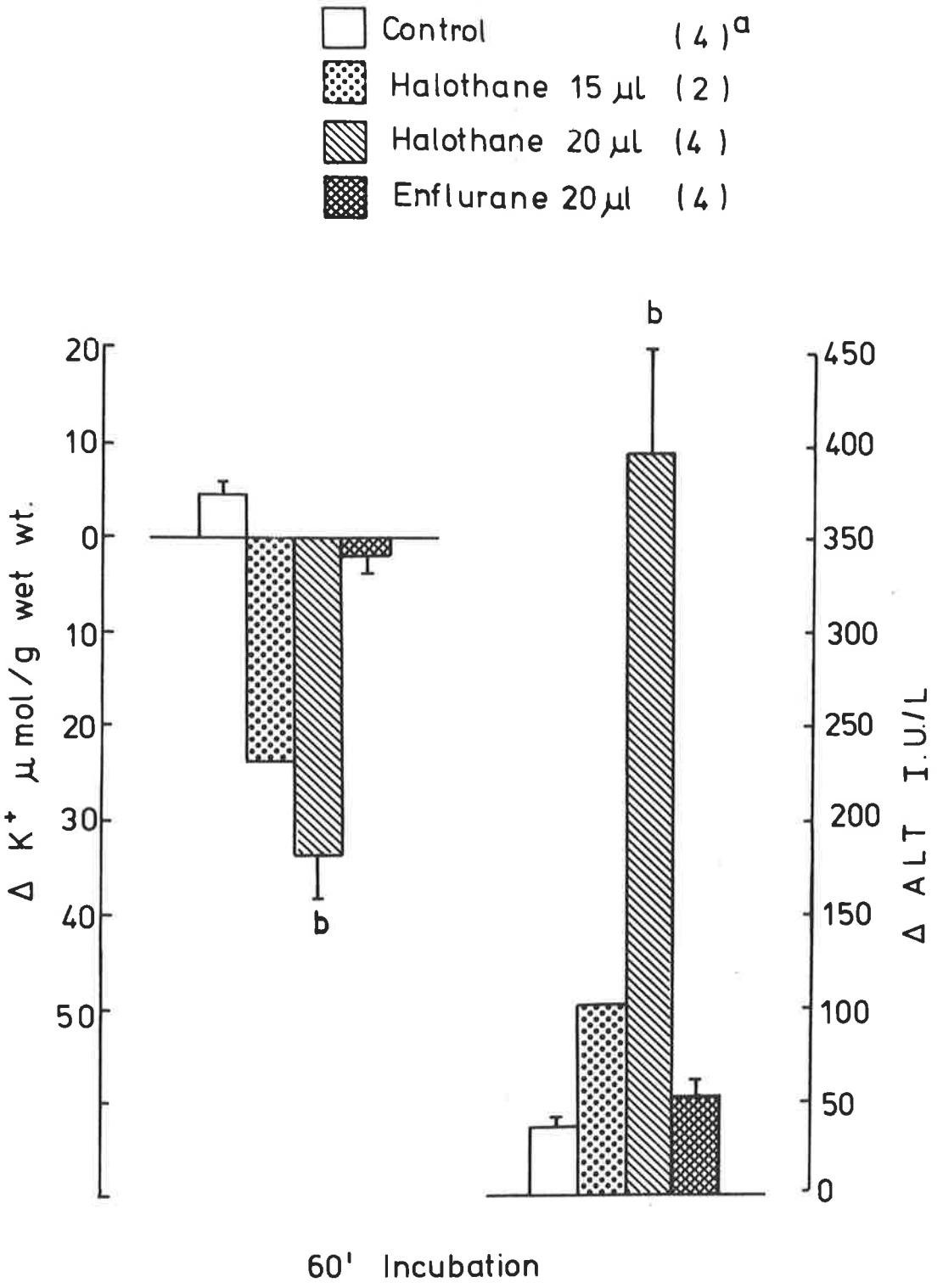


Fig. 6.3 Loss of intracellular potassium ion from the cells (left panel) and alanine aminotransferase into the supernatant (right panel) after 60 minutes incubation (37°C) with halothane (15 or 20 µl) or enflurane (20 µl) compared with controls. Bars represent the mean (n=2) or mean ± s.e.m. (n=4).

[a] Numbers in parentheses are the number of experiments.

[b] Significantly different from controls.

TABLE 6.1

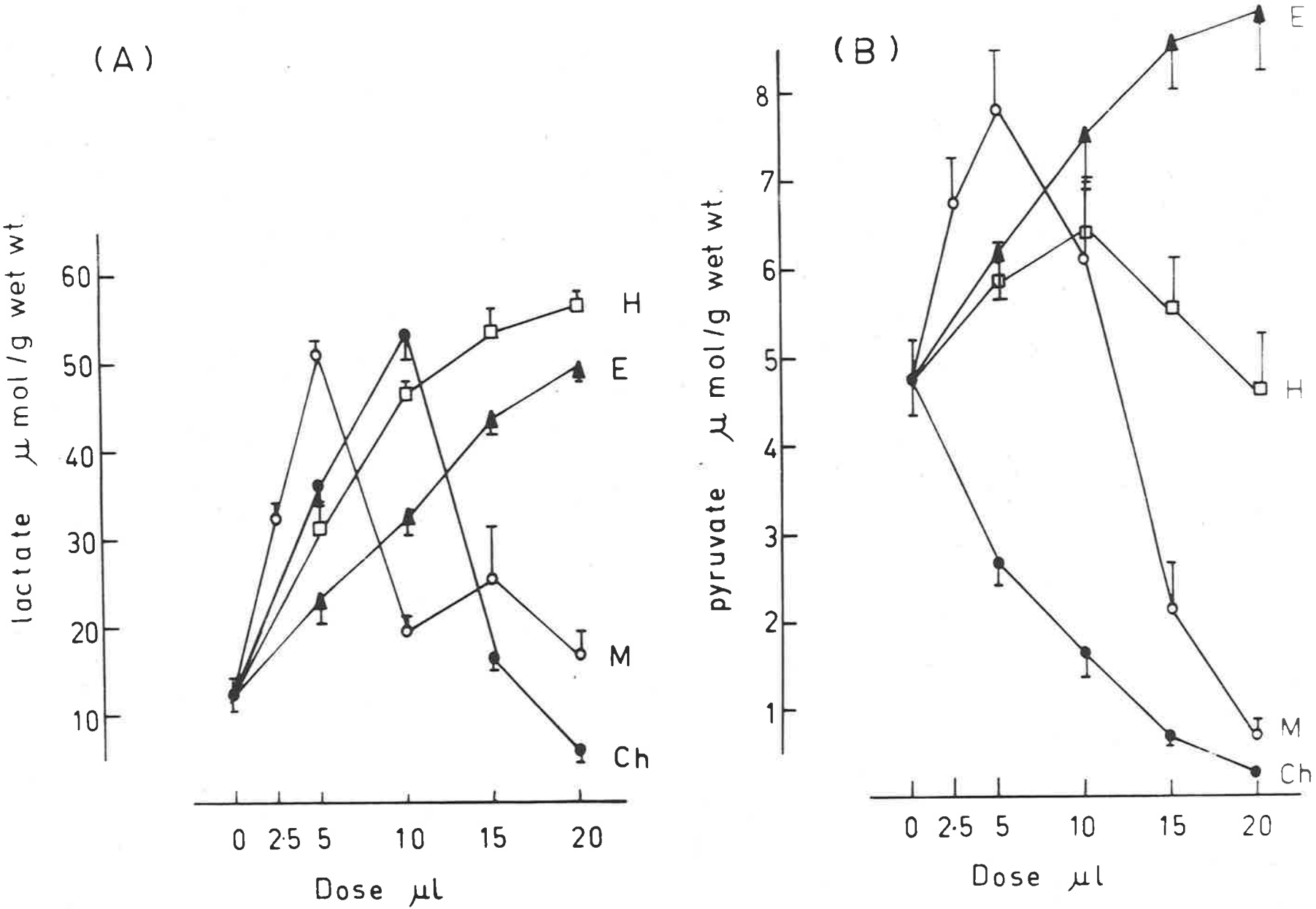
CONCENTRATION OF INHALATIONAL ANAESTHETICS IN HEPATOCYTE SUSPENSIONS

ANAESTHETIC	DOSE (µl)	TIME[a] (min)	CONCENTRATION[b] (mM)
Halothane	5	20	2.9 (2)
	10	20	6.8 (2)
	15	20	9.4 (2)
	15	60	10.2 (1)
	20	20	14.1 ± 0.7 (3)
	20	60	14.8 (2)
Enflurane	5	20	3.2 (2)
	10	20	5.2 ± 0.3 (3)
	15	20	6.7 (2)
	20	20	10.1 ± 3.1 (3)
	20	60	9.3 (1)
Methoxyflurane	2.5	20	1.4 (2)
	5	20	3.8 (2)
	10	20	8.4 (2)
	15	20	9.9 (2)
	20	20	13.2 (2)
Chloroform	5	20	5.9 (2)
	10	20	11.7 ± 2.0 (3)
	15	20	15.9 (2)
	20	20	19.8 ± 2.3 (3)

[a] Refers to the time of incubation at which sample taken for assay of anaesthetic concentration.

[b] Values are mear (± s.e.m. where n>2) with the number of experiments in parentheses.

Fig. 6.4 Lactate (left figure) and pyruvate (right figure) content of hepatocytes after incubation (20 minutes, 37°C) with varying doses of anaesthetics. Halothane —□— H; enflurane —▲— E; Methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the s.e.m.


(A)
lactate µ mol/g wet wt
60
50
40
30
20
10
H
E
M
Ch
0 2·5 5 10 15 20
Dose µl
(B)
pyruvate µ mol/g wet wt
8
7
6
5
4
3
2
1
E
H
M
Ch
0 2·5 5 10 15 20
Dose µl

complex. A stimulatory effect was generally seen with lower doses, with no apparent change, or a reduction in levels seen with some of the higher doses. Exceptions were an increase in lactate production over the entire dosage range with halothane and a decrease in pyruvate production over a similar range with chloroform. The meaning of these changes with respect to the metabolic status of the cells is demonstrated more adequately when the results are expressed as the L/P ratio, as in Fig. 6.5. Treatment with each of the anaesthetic agents caused an increase in this entity, with enflurane causing the smallest and chloroform the greatest shift.

The rate of urea synthesis was decreased by all of the anaesthetics and enflurane again had the least effect, as illustrated in Fig. 6.6. However, the effect of enflurane on ureogenesis was greater than its effects on the other variables measured. The effects of halothane, methoxyflurane and chloroform were very similar, to the extent that there was no clear differentiation between them. This was especially so with doses of 10 µl and greater where depression of ureogenesis appeared to be maximal.

DISCUSSION

The experiments of this chapter have clearly demonstrated cytotoxicity of halogenated volatile anaesthetics in the isolated rat hepatocytes, with enflurane having no effect on the structural parameters. The relative order of cytotoxic potency was found to be:

chloroform = methoxyflurane > halothane > enflurane

These results complement those of Goto *et al.* (1976a, 1976b) obtained in hepatocyte cell cultures. By finding these results, the cells isolated

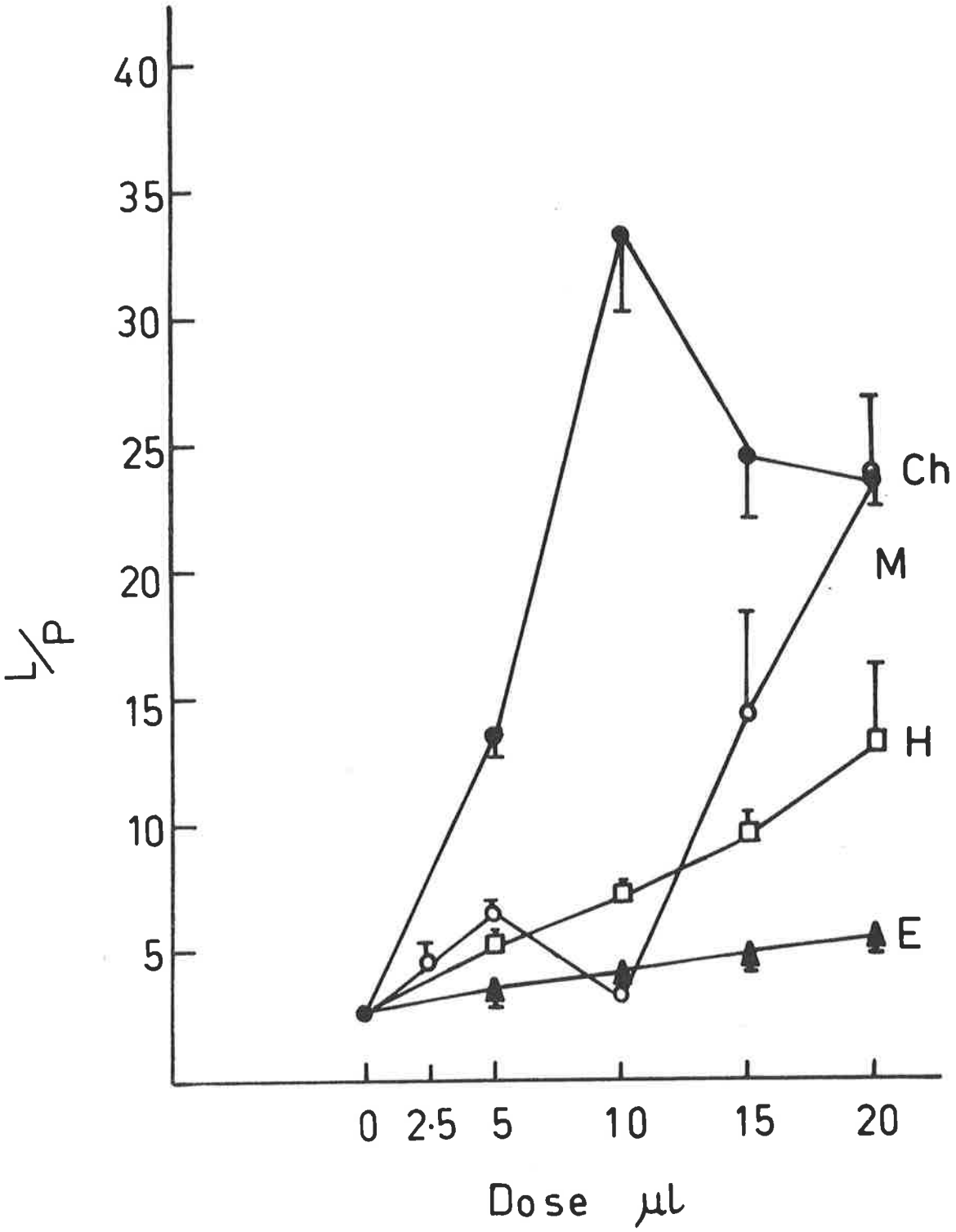


Fig. 6.5 Lactate/pyruvate ratios (L/P) after incubation of hepatocyte suspensions (20 minutes, 37°C) with varying doses of anaesthetics. Halothane —□— H; enflurane —▲— E; Methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the s.e.m. Comparative potency: Ch > M = H > E $p<0.01$, $n=16$ (doses 5-20 µl).

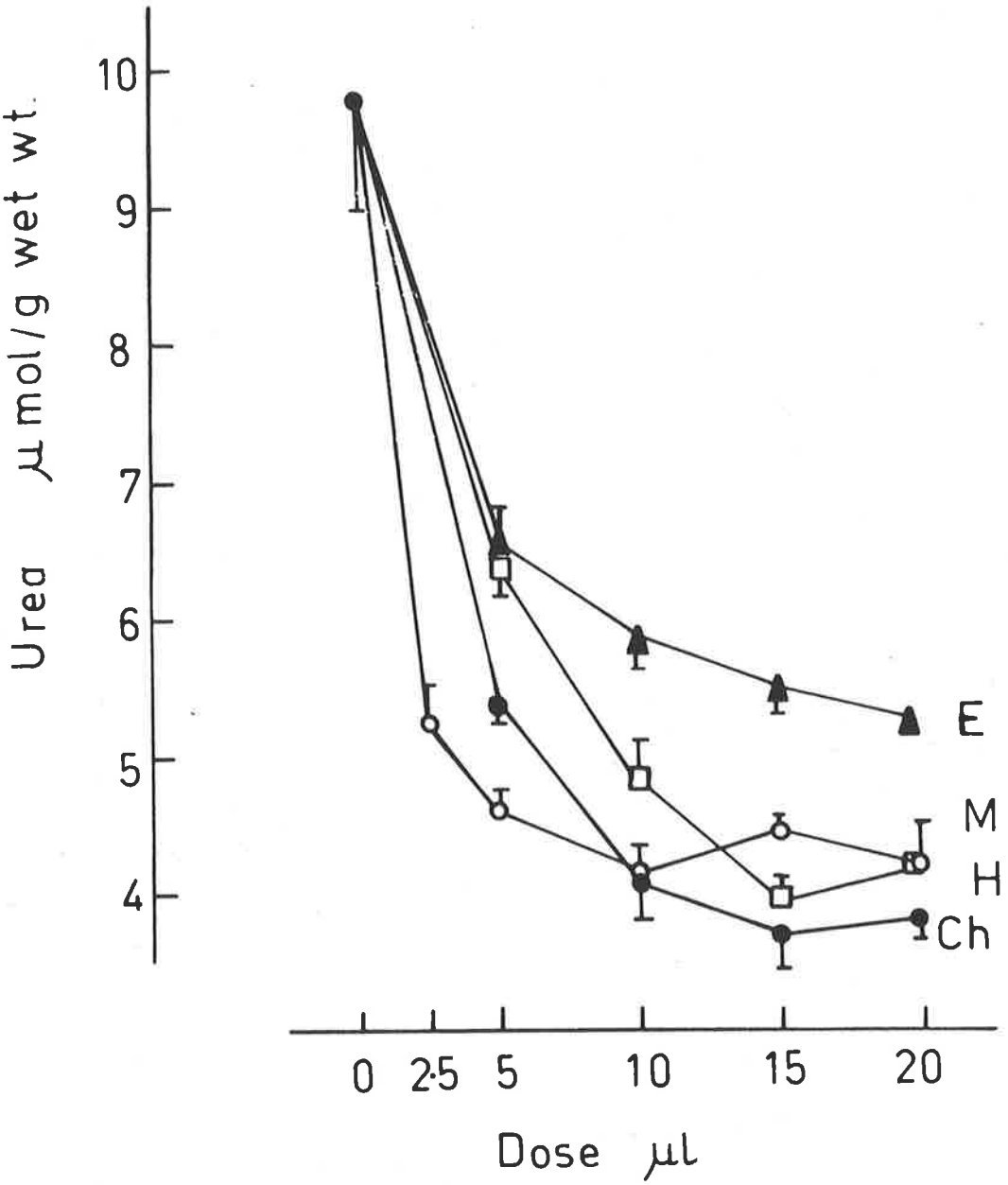


Fig. 6.6 Urea formed over 20 minutes during incubation (37°C) of hepatocyte suspensions with varying doses of anaesthetics. Halothane —□— H; enflurane —▲— E; Methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the s.e.m. Comparative potency: Ch > M = H > E $p<0.02$, n=12 (doses 10-20 µl). For doses 5-20 µl Ch = M $p<0.05$, n=16.

in this laboratory have also been shown to be capable of detecting cytotoxicity of drugs which can be classed as idiosyncratic hepatotoxins. Similar findings have been found with other drug groups by other workers (Zimmerman *et al.*, 1974; Abernathy *et al.*, 1975). Furthermore, as with other *in vitro* studies, comparative cytotoxicity is in good agreement with the clinical presentation of hepatotoxic reactions.

Extrapolation of the results presented in this chapter to the clinical situation infers that enflurane has a lower potential for hepatotoxicity than halothane, a point which, at this stage, seems justified on the basis of clinical experience (Black *et al.*, 1977). Although nephrotoxicity is the major side effect with methoxyflurane, hepatotoxicity is not unknown and liver dysfunction is generally acknowledged as a contraindication to its use (Conn, 1974). The well established injurious effects of chloroform are consistent with the high degree of toxicity found in these experiments.

It should be noted that the different anaesthetics do not achieve the same medium concentrations at comparable dose levels. The concentration achieved is a function of dose, the solubility and partition coefficients for exchange between liquid and vapour phases, and the configuration of the incubation vessels. These factors have been discussed by Goto *et al.* (1976a). However, it is apparent that the differences in medium concentration do not account for the differences in toxicity. For example, the range of concentrations for methoxyflurane and halothane are very similar, yet the evidence of toxicity with methoxyflurane is much greater. It is unlikely that a greater concentration of methoxyflurane in the cells themselves would account for differences in toxicity because the liver:blood partition coefficients for methoxyflurane and halothane are quite similar (Goodman and Gilman, 1975). A second

important example relates to lack of structural damage in response to enflurane. At 20 minutes' incubation the 20 µl dose of enflurane shows a concentration similar to that of the 15 µl dose of halothane, where loss of K^+ is evident. This is more pronounced with the 60 minute incubation data where the enflurane 20 µl dose and halothane 15 µl dose again yield similar concentrations and there is an increased toxicity to halothane (as compared to 20 minute incubation) but still no significant toxicity with enflurane.

Goto *et al.* (1976a) also measured anaesthetic concentrations attained. Their doses, which were lower than those used in experiments reported here, resulted in medium concentrations which they claimed to be similar to those found in the hepatic vein during clinical anaesthesia. Anaesthetic concentrations in the experiments of this chapter generally fell outside this range. However, the lowest dose produced a concentration very close to the upper levels of the purported clinical range. This means that evidence of structural damage was seen at concentrations greater than those usually found during anaesthesia (for example, halothane 15 µl dose gives a concentration about three times the upper anaesthetic level).

Another factor to be considered is the duration of exposure associated with the toxic response. Evidence of cell damage was seen within 20 minutes' exposure to the more potent agents and this is relatively short in comparison with *in vivo* effects. An investigation over longer incubation periods with doses in the clinical range might prove beneficial in further studies with these anaesthetics.

The effects of these anaesthetic agents on the metabolic parameters, L/P and ureogenesis, are, in general, qualitatively similar to those

seen with CCl_4 (Chapter 1). Halothane has been shown to increase L/P both *in vivo* (Stier *et al.*, 1972) and in the isolated perfused rat liver (Biebuyck *et al.*, 1972). Mapes (1977) has recently published data also showing an increased L/P in isolated hepatocytes. The effect of halothane on ureogenesis was also examined in the study of Biebuyck *et al.* (1972). They found a marked reversible inhibitory effect by halothane on this biosynthetic process.

The results presented here do not allow any conclusions to be drawn about the mechanism of anaesthetic toxicity in hepatocytes. More than one possibility exists. For example, lipid solvent effects may account for observed changes. Anaesthetics can modify cell membrane characteristics in a reversible manner, which could account for a loss of K^+. Release of ALT, however, is not consistent with such a reversible change in cell membrane permeability.

Toxicity via a toxic metabolite in other systems has been discussed by Van Stee (1976). Trifluoroacetate, a metabolite of halothane, was found to produce similar effects to its parent compound, only more immediate, suggesting the involvement of biotransformation (Stier *et al.*, 1972). Similarity between findings on inhibition of the electron transport chain in isolated perfused liver and isolated mitochondria led Biebuyck *et al.* (1972) to the opposite conclusion. Toxicity through a metabolite must be considered as a possibility in these studies, but the findings with CCl_4 and bromobenzene in this thesis would suggest such a mechanism to be unlikely. It would be of interest to investigate the effects of trifluroacetate in the isolated hepatocytes. The short period of incubation used, together with the studies of Goto *et al.* (1976a, 1976b) tend to favour a conclusion that a direct cytotoxicity may be responsible.

CHAPTER SEVEN

TOXICITY OF BENZENE, PARACETAMOL AND CHLORPROMAZINE IN ISOLATED RAT HEPATOCYTES AND RELATIONSHIP TO CCl_4 TOXICITY. TOXICITY OF CCl_4 TO ERLICH ASCITES TUMOR CELLS

INTRODUCTION

Several other aspects of toxic responses in cell suspension systems can yield further information on the use of isolated hepatocytes in toxicity studies, or the way in which other poisons are operating. Four brief experiments were designed to perform such a function and they are presented as sections of this chapter.

(i) Studies with benzene

Benzene is a widely used industrial chemical which causes depression of the bone marrow on chronic exposure. This aspect of benzene toxicity and its mechanism has been recently reviewed (Snyder and Kocsis, 1975). A further study on the mechanism of benzene toxicity with respect to the role of a metabolite has recently appeared (Timbrell and Mitchell, 1977).

Hepatic injury in response to benzene, however, is not recognised as being an important toxic effect, especially in comparison to CCl_4 (Recknagel *et al.*, 1974). Snyder and Kocsis (1975) indicate a similar opinion when they say that 'benzene toxicity is manifested in the bone marrow rather than the liver'. The experiments of Wirtschafter and Cronyn (1964) indicated a release of hepatic cytoplasmic enzyme into the blood and somewhat mild histological changes in the liver in response to benzene. The same study did indicate a much more dramatic response to CCl_4, however. This lack of marked hepatocellular damage in response to benzene has been used in interpretations of the mechanism of CCl_4 toxicity. The lipid solvent effects of CCl_4 and benzene are similar, and this is taken, along with the wide differences in toxicity, as evidence against lipid solvent properties being directly responsible for the liver injury by CCl_4 (Reynolds, 1967). Benzene has also been shown not to share the deleterious properties of CCl_4 upon the endoplasmic

reticulum (Reynolds, 1972). In contrast to the actions of CCl_4 again, it has been shown that benzene is not a pro-oxidant *in vitro* (Glende and Recknagel, 1969). Lipid peroxidation in microsomes obtained from benzene pre-treated rats has been shown to be less than controls (Pawar and Mungikar, 1975). The same authors also found aminopyrine N-demethylase to be decreased (similar to CCl_4) but acetanilide hydroxylase activity was increased.

To further study the mechanism of the CCl_4 induced toxic response in isolated hepatocytes, then, the toxicity to benzene was investigated. Consideration of the *in vivo* events would lead to the prediction that damage to the hepatocytes would be much less evident with benzene as compared to CCl_4.

(ii) Studies with paracetamol

Paracetamol is a widely used analgesic which can, on large overdose, produce massive hepatic necrosis and death (McLean and Day, 1975). The pattern of production of hepatic injury by paracetamol is quite similar to that of bromobenzene. Both treatments appear to be toxic via a metabolite of the mixed function oxidase system, after sufficient reduction of intracellular reduced glutathione levels (Mitchell and Jollow, 1975). Since toxicity due to bromobenzene or CCl_4 does not appear to occur in the isolated hepatocytes as it does *in vivo*, it was of interest to investigate a compound such as paracetamol. As well as the similarities between paracetamol and bromobenzene toxicities *in vivo*, paracetamol has different physical properties to CCl_4 and bromobenzene. In particular, paracetamol is a solid at room temperature, whereas the others are volatile liquids, and a significant lipid solvent effect would not be expected.

An investigation of the toxic aspects of paracetamol in the isolated hepatocytes, then, may provide further indications as to the nature of the toxicities due to these other compounds.

(iii) Studies with cell suspensions of non-hepatic origin

As the *in vivo* toxicity of CCl_4 is due to a metabolite produced by the mixed function oxidase system found predominantly in the liver (Chapter 1 - introduction), it could be considered unlikely that a cell system lacking in this metabolic capacity would be subject to the toxic actions of CCl_4. This assumes, again, that toxicity is manifest in the cells as it is *in vivo*.

It was therefore decided to examine the response of such a cell population to CCl_4. Observation of a damaging effect by CCl_4 would indicate a direct cytotoxicity, rather than one via a metabolite, in the cells. Such a response has been reported in laryngeal carcinoma cells at high CCl_4 levels (Zimmerman and Mao, 1965). Erlich Ascites Tumor (EAT) cells were used as the non-hepatic cell system for investigation of CCl_4 effect under the conditions used in this laboratory.

(iv) Studies with chlorpromazine under normothermic and hypothermic conditions

Incubation in an ice water bath was the only treatment used in this thesis which gave hepatocytes protection against the toxic nature of CCl_4. It was of interest to see if this protective effect was unique to CCl_4 or whether it occurred with other drugs toxic to the cells. Such information could be helpful in further determining aspects of the mechanism of the toxic response to CCl_4 in the hepatocytes. For such an investigation a liver toxin from a different class (e.g. idiosyncratic

rather than direct acting) seemed appropriate. Chlorpromazine was chosen because it met the above criterion and had been shown to cause leakage of cytoplasmic enzymes in isolated rat hepatocytes (Zimmerman *et al.*, 1974) and because initial experiments in this laboratory showed damage to structural parameters by chlorpromazine.

The overall aim of the experiments of this chapter was to provide further indications as to the usefulness of the isolated hepatocytes in toxicity studies, generally by further investigation of the mechanisms underlying CCl_4 toxicity in the hepatocytes as compared to *in vivo*.

Specifically

(i) Does benzene cause a change in parameters of structural integrity in isolated hepatocytes?

(ii) Does paracetamol damage isolated liver cells - especially as compared to bromobenzene?

(iii) Does a suspension of non-hepatic cells also exhibit signs of toxicity in response to CCl_4 under incubation conditions similar to those used with hepatocytes?

(iv) Is the protective nature of ice temperature incubation unique to CCl_4?

METHODS

Hepatocytes isolated from either untreated or sodium phenobarbitone pre-treated rats (80 mg/kg - dissolved in physiological saline at 80 mg/ml - intraperitoneally at approximately 10 a.m. on each of the three days prior

to experimental procedures) were used in the experiments of this chapter. Donor rats weighed between 230-300 g.

Incubation conditions were as in the General Methods. Benzene (20 µl) was added either directly to the cell suspension or to a side arm to allow a vapour phase transition to the incubation medium. Incubations were of 10 min duration. Paracetamol and chlorpromazine were both added directly to the cell suspension as a concentrate dissolved in physiological saline. The vehicle was added to the appropriate control vessels. Chlorpromazine incubations were over 30 and 60 min while incubations of the latter time are reported for paracetamol. Samples for estimation of K^+ and ALT were taken for all the above treatments. A sample from benzene treated cells was taken for EM studies. The change in TBA reactant levels was monitored for paracetamol.

Erlich Ascites Tumor (EAT) cells* were used at a cell concentration of approximately 5 x 10^6/ml, and 99% of these excluded the vital dye, trypan blue. Conditions of incubation were much the same as in Chapter 1. Aliquots of 2 ml of stock EAT cell suspension (after storage on ice for approximately 30 min after preparation) were placed in the incubation flasks, gassed with carbogen for 10-15 min, dosed with 10, 15 or 20 µl CCl_4 via the side arm and incubated for 15 or 20 min. A 1 ml sample was taken and centrifuged for 2½ min at 1000 r.p.m. The supernatant was removed and the cell pellet assayed for K^+ as described for hepatocyte pellets (General Methods). A trypan blue exclusion test was also performed on control cells and cells incubated with the 20 µl dose of CCl_4, after 20 min of incubation.

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* EAT cells provided by Mrs Jane Sawyer and Dr E. Kotlowski, Department of Microbiology, University of Adelaide.

RESULTS

(i) The effects of a 10 min incubation with benzene on the structural parameters, K^+ and ALT are shown in Table 7.1. In hepatocytes isolated from PB pre-treated rats 20 µl benzene added to the side arm of the flask caused a statistically significant loss of K^+, but not of ALT. A larger drop in K^+ and an appreciable increase in supernatant ALT was observed in the two experiments where 20 µl benzene was added directly to the cell suspension. These were not tested statistically. A similar pattern of response was seen in the single experiment where cells were isolated from untreated rats. Electron microscopic examination of cells (from untreated rats) incubated with 20 µl benzene added to the side arm showed loss of microvilli and formation of cytoplasmic protrusions (Fig. 7.1). Vacuolization of mitochondria is also apparent and the formation of intramitochondrial lamellar structures is illustrated in Fig. 7.2. This micrograph also indicates some dilation and perhaps some ribosomal detachment of the RER in some areas. The structure of some of the RER is well preserved, however. When the 20 µl dose of benzene is added directly to the cell suspension there is a marked degeneration of the hepatocytes after 10 min incubation (Fig. 7.3).

(ii) Paracetamol was found not to be associated with any significant changes in K^+ and ALT over a 60 min incubation period in cells isolated from PB pre-treated rats. These data are shown in Table 7.2. Also shown are similar results found in two experiments using hepatocytes from untreated animals. A significant inhibition of lipid peroxidation (production of TBA-reactants) was observed in cells from the PB treated rats.

(iii) There was a loss of K^+ from EAT cells on incubation with CCl_4 as

TABLE 7.1

EFFECTS OF BENZENE (20 µl)[a] ON STRUCTURAL PARAMETERS[b] IN ISOLATED RAT HEPATOCYTES AFTER 10 MIN INCUBATION

TREATMENT[a]	ANIMAL[c] PRE-TREATMENT	ΔK^+[d] µmol/g wet wt		ΔALT I.U./L	
Control	None	1.7	(1)	15	(1)
Benzene (s.a.)	None	-23.7	(1)	13	(1)
Benzene (c.s.)	None	-68.3	(1)	408	(1)
Control	PB	0.5 ± 2.2	(3)	-9 ± 6	(3)
Benzene (s.a.)	PB	-19.7 ± 4.7[e]	(3)	31 ± 24	(3)
Benzene (c.s.)	PB	-46.6	(2)	368	(2)

[a] Benzene (20 µl) was added either to the side arm (s.a.) of a flask or directly to the cell suspension (c.s.) at zero time. Control incubations received no additions.

[b] Values given are mean ± s.e.m. (n=3), mean (n=2) or a single figure (n=1). They represent the difference between end of and prior to start of incubation levels of the parameters. Numbers in parentheses are the number of cell preparations used to determine the particular value.

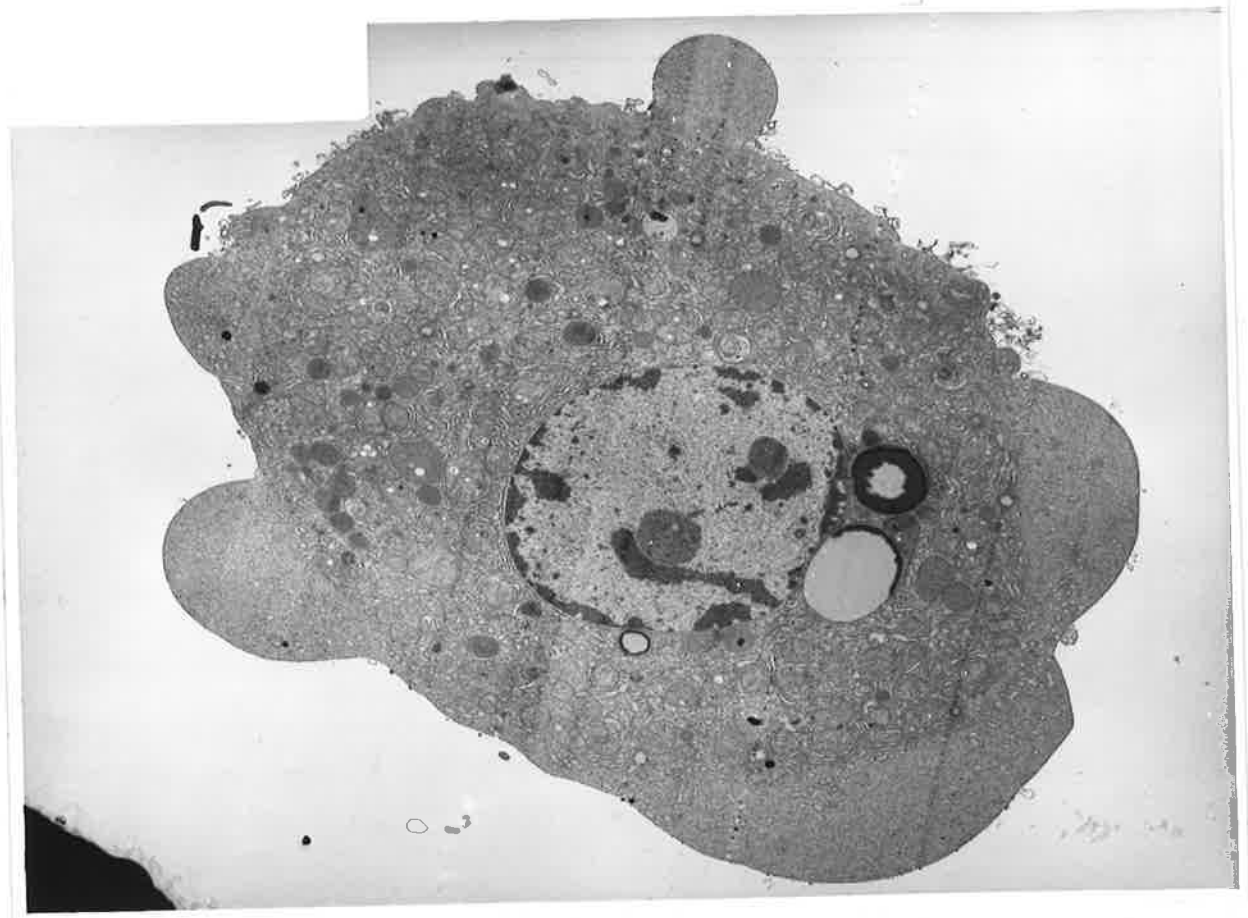
[c] Rats pre-treated with sodium phenobarbitone (PB) received 80 mg/kg, i.p., on each of 3 days prior to hepatocyte isolation.

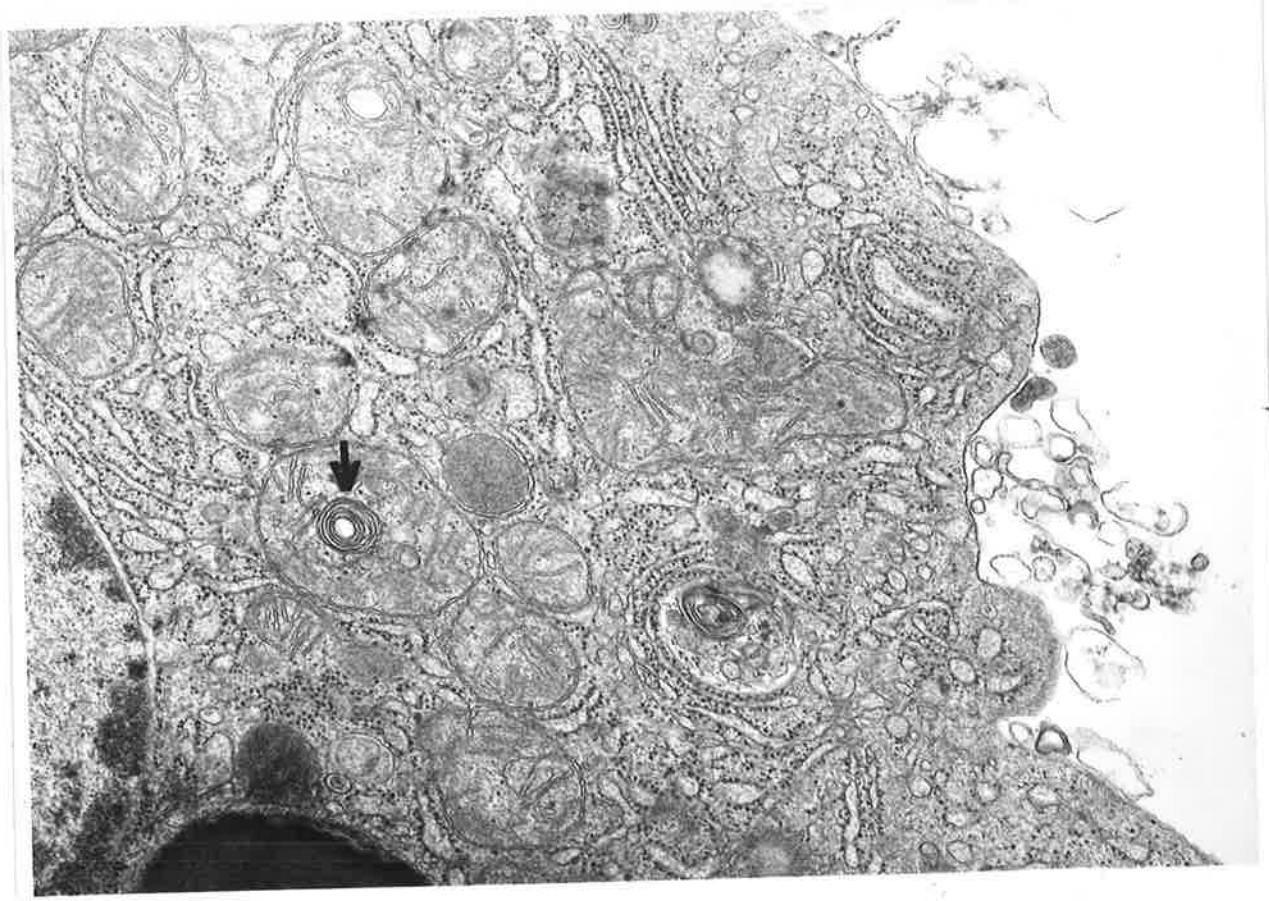
[d] K^+ calculated from pellet weight (see Appendix 5).

[e] Statistically significantly different from control.

FIG. 7.1 TEM of an isolated hepatocyte treated with 20 µl benzene (added to vessel side arm) over 10 min incubation period. Note the appearance of cytoplasmic protrusions, vacuoles in the mitochondria and a loss of microvilli. x 6,600.

FIG. 7.2 TEM of part of an isolated hepatocyte treated as in Fig. 7.1. Loss of microvilli and mitochondrial vacuoles (associated with lamellar formations - arrowed) are apparent. Cisternae of the RER show some dilation. x 30,000.





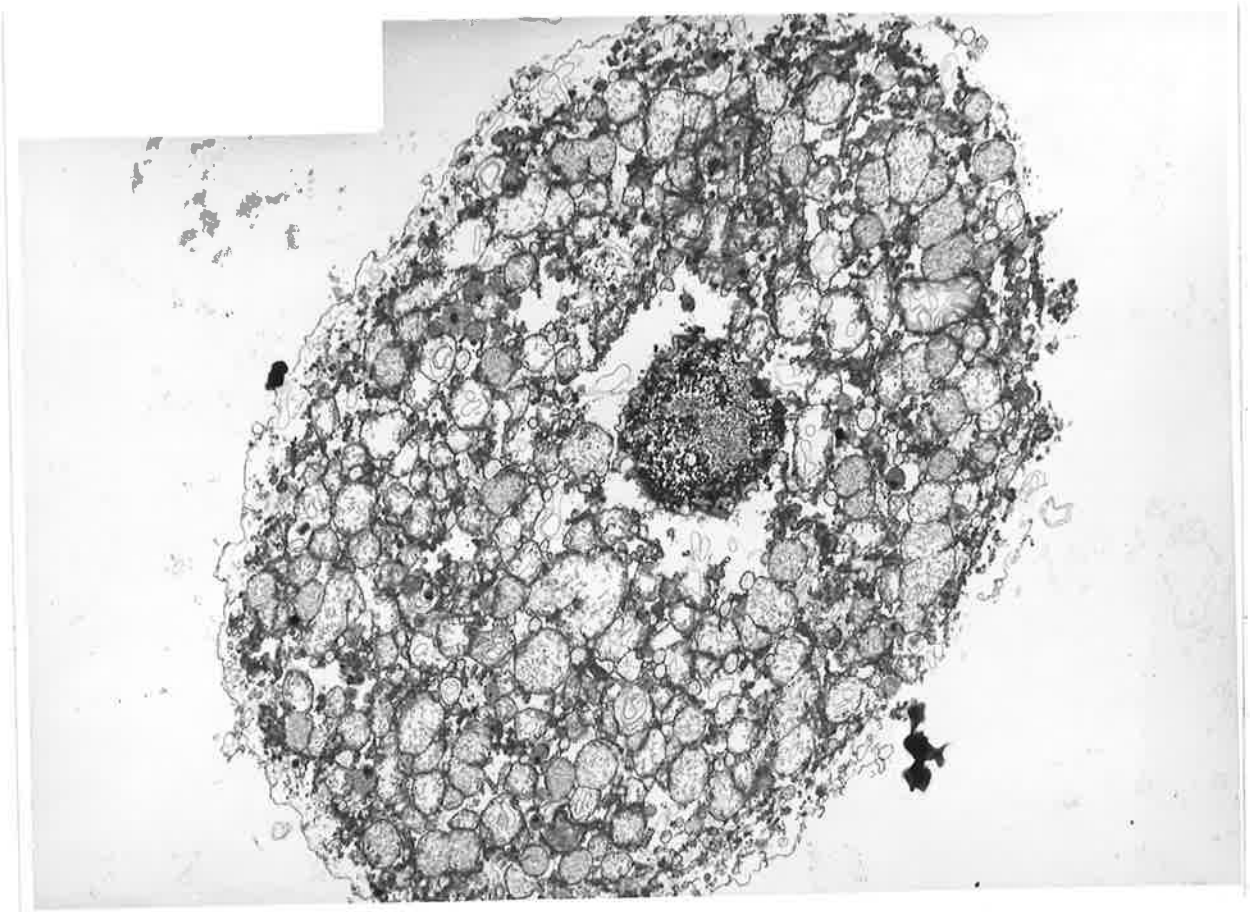


FIG. 7.3 TEM of an isolated hepatocyte incubated for 10 min with 20 µl benzene (added directly to the cell suspension). The hepatocyte shows marked degeneration. x 6,600.

TABLE 7.2

EFFECTS OF PARACETAMOL ON STRUCTURAL PARAMETERS[a] AND LIPID PEROXIDATION[a] IN ISOLATED RAT HEPATOCYTES AFTER 60 MIN INCUBATION

TREATMENT	ANIMAL[b] PRE-TREATMENT	ΔK^+[c] µmol/g wet wt	ΔALT I.U./L	ΔTBA REACTANTS (O.D. UNITS)
Control	None	-4.9 (2)	32 (2)	
Paracetamol (10 mM)	None	-6.7 (2)	26 (2)	
Control	PB	1.7 ± 4.3 (4)	18 ± 7 (4)	0.087 ± 0.005 (3)
Paracetamol (10 mM)	PB	-5.6 ± 6.2 (4)	31 ± 11 (4)	0.010 ± 0.011[d] (3)

[a] Values given are mean ± s.e.m. (no s.e.m. where n=2) of the difference between end of incubation levels and those before start of incubation. Numbers in parentheses are the number of cell preparations used to determine the particular value.

[b] Rats pre-treated with sodium phenobarbitone (PB) received 80 mg/kg, i.p., on each of 3 days prior to hepatocyte isolation.

[c] K^+ calculated from pellet weight (see Appendix 5).

[d] Statistically significantly different from control.

compared to controls (Table 7.3). This is particularly evident after the 20 min incubation period. These results are from a single experiment. Trypan blue exclusion of control cells incubated over 20 min remained unchanged to the 99% found prior to incubation. Cells treated with 20 µl CCl_4 over this period, however, showed a drop in exclusion of this dye to 48%.

(iv) The loss of K^+ and ALT from hepatocytes in response to incubation at 37°C with chlorpromazine (10^{-3} M) is indicated in Fig. 7.4. This was over 30 or 60 min incubation with the drug. All results are the average of two experiments with cells isolated from untreated rats. Ice temperature incubation prevented the loss of ALT seen at 37°C with chlorpromazine and reduced the loss of K^+, particularly at 30 min incubation. Controls showed no evidence of dramatic shift in either K^+ or ALT.

DISCUSSION

(i) Benzene, which has negligible hepatoxic effects *in vivo* (Recknagel *et al.*, 1974) has been shown to elicit a change in parameters of structural integrity of isolated rat hepatocytes. The change in intracellular K^+ for the 20 µl of benzene added to the side arm is quite similar to that found with CCl_4 in hepatocytes from both untreated and PB pre-treated rats. The increase in toxicity seen when benzene is added directly to the cell suspension probably reflects a higher medium concentration. The actual concentrations of benzene need to be measured to support this. Strictly, similar information should be available to allow an accurate comparison with CCl_4, when doses are given via the side arm. Although the boiling points of the two chemicals are very similar there is some

TABLE 7.3

EFFECTS OF CCl_4[a] ON POTASSIUM CONTENT[b] OF ERLICH ASCITES TUMOR CELLS

SAMPLE	INCUBATION TIME	K^+ µmol/10^9 CELLS
Stock suspension (0-4°C)	0	104.8
Control	15	127.8
CCl_4 10 µl	15	124.7
CCl_4 15 µl	15	120.1
CCl_4 20 µl	15	94.7
Control	20	149.4
CCl_4 10 µl	20	107.8
CCl_4 15 µl	20	83.2
CCl_4 20 µl	20	64.7

[a] CCl_4 added to the side arm of the flask (General Methods).

[b] Values from a single experiment.

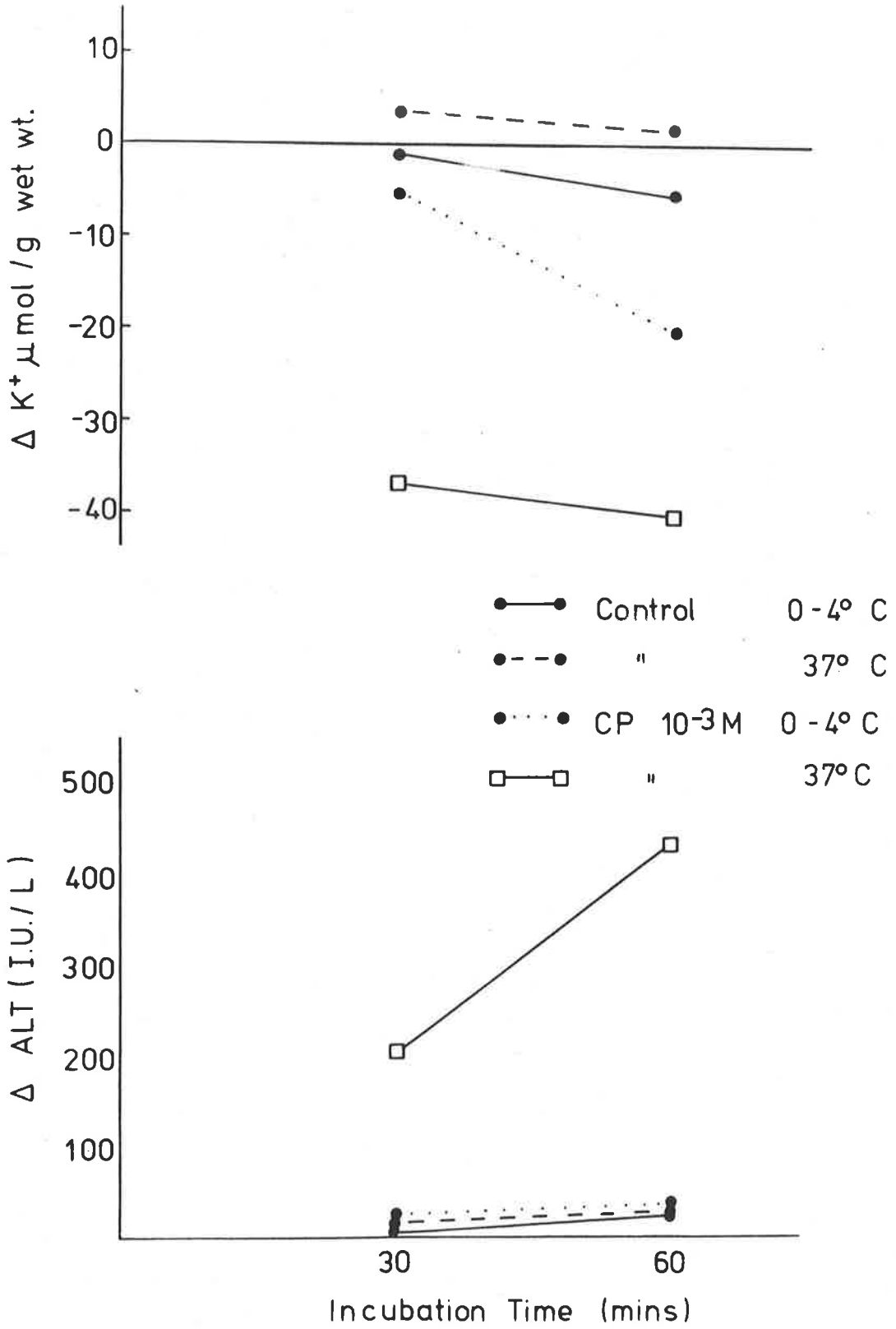


FIG. 7.4 Loss of intracellular K^+ (top panel) and release of ALT into the medium (bottom panel) from isolated rat hepatocytes (untreated rats) after incubation with chlorpromazine (CP) (10^{-3} M) at 0-4°C or 37°C. Each point is the average of two experiments.

difference in their water solubilities (Reynolds, 1972) which would probably allow benzene to reach higher concentrations. However, the *in vivo* study of Wirtschafter and Cronyn (1964) used similar doses in their experiments and are therefore subject to similar criticisms. Thus a finding of similar toxicities in our experiments is in contrast to the wide difference reported in the above *in vivo* study. This suggests that the toxicity seen with CCl_4 may not be occurring in the same manner as it does *in vivo*. Furthermore, the results with benzene suggest that toxicity may be due to some property similar to both benzene and CCl_4.

A direct comparison to CCl_4 of effects on the ultrastructure of the hepatocytes by benzene is difficult, because a 20 µl dose at 10 min of incubation was not examined with CCl_4. However, it is the impression of the author that changes in the RER were much less with benzene than CCl_4 while other changes (e.g. mitochondrial, loss of microvilli) were similar. If a more intensive investigation substantiated this impression it would be in agreement with the findings of Reynolds (1972) that benzene did not affect microsomal parameters at a time when CCl_4 did.

(ii) Even though the lack of evidence of toxicity with paracetamol is disturbing, little inference can be drawn from it. Hogberg and Kristoferson (1977) also report lack of toxic effects in cells from non-induced animals with paracetamol, but a depletion of GSH in cells from PB pre-treated rats by 6 hr incubation. This suggests that longer incubation times may be required, as does the fact that, *in vivo*, 3-6 hours is required for evidence of toxicity (Thorgeirsson *et al.*, 1976). However, in a comparison to studies with bromobenzene, it might be reasonably expected that paracetamol would cause damage to the hepatocytes. This assumes that the observed bromobenzene toxicity *in vitro*

is occurring similarly to *in vivo*. The studies with bromobenzene and paracetamol *in vivo* both gave peak liver concentrations of around 2 mM. At a concentration of 6-7 times this, bromobenzene showed evidence of toxicity after 20 min incubation. The time relationship of the response suggests that this would have been greater over a 60 min incubation period. Paracetamol, at 10 mM, over 60 min incubation, gave no evidence of toxicity, however. The *in vivo* studies of the group at NIH have indicated that the mechanism by which these two chemicals induce toxicity bear marked similarities (Mitchell and Jollow, 1975). Consequently, it can be reiterated that a toxic response to paracetamol might reasonably be expected since bromobenzene produced a toxic response. Lack of toxicity with paracetamol may be taken as an indication that bromobenzene is not acting as *in vivo* to cause toxicity, but caution is required in this interpretation. Further studies with more accurate dose and time similarities should help to illuminate this point.

The marked inhibition of lipid peroxidation (occurring spontaneously) in suspensions of isolated hepatocytes is an interesting observation, suggesting anti-oxidant properties of paracetamol. This may be linked with the observation that paracetamol does not lead to an increase in diene conjugation after administration of a toxic dose (Thorgeirsson *et al.*, 1976). That is, if toxicity is occurring via the metabolite, which may normally elicit changes in diene conjugation, residual parent compound may be acting to mask evidence of this response.

(iii) The loss of K^+ and the decreased ability to exclude trypan blue from EAT cells on incubation with CCl_4 indicate a direct injury to the outer membrane. Presumably, then, this could also account for changes in parameters of the integrity of isolated hepatocytes. This finding

is in agreement with the studies of Zimmerman and Mao (1965), although their concentrations are reportedly higher. As pointed out by Zimmerman (1968), the role of an effect on the outer membrane by the parent CCl_4 cannot be totally disregarded, although the significance of such an effect *in vivo* is certainly obscure.

Other preparations of cell suspensions could have been used for this investigation, with reasoning on a similar basis. The review of Recknagel and Glende (1973) suggests some likely preparations - for example, chicken hepatocytes (known to show marked resistance to CCl_4 *in vivo*), heart cells (resistant to high concentrations of CCl_4 during studies on hepatotoxic effects in the whole animal), hepatocytes from rats pre-treated with low doses of CCl_4, which makes them less susceptible to subsequent higher doses. Hepatocytes isolated from female rather than male rats could possibly be investigated, as some reports indicate the female to be less affected by CCl_4 (Cawthorne *et al.*, 1970). The benefit of female hepatocytes is questioned by Zimmerman (1976) where it is reported that there is no significant or consistent sex difference. As well as this, Castro and Diaz Gomez (1972) report no difference in CCl_4 activating ability between the sexes.

(iv) Damage of isolated rat hepatocytes by chlorpromazine was effectively inhibited by incubation at 0-4°C rather than 37°C. Thus the protection seen with CCl_4 is not unique to this compound and may be related to a general effect on cells rather than a more specific one (such as, inhibition of microsomal metabolism). Chlorpromazine is regarded as a surfactant hepatotoxin (Dujovne, 1975), but it has also been pointed out that toxicity to Chang cells may be related to stability of a free radical intermediate formed during metabolism (Zimmerman and Kendler,

1970). Thus it is possible, but by no means conclusive, that hypothermia inhibits toxicity via a membrane effect. Similar experiments with erythromycin estolate in the hepatocytes or CCl_4 and chlorpromazine in EAT cells would indicate more clearly the likely nature of this protective effect.

Overall, then, the experiments reported in this chapter have yielded information that can be used to further interpret the results already obtained with CCl_4, and have suggested continuation of some of the experimental lines.

GENERAL DISCUSSION

Discussion of the results of this thesis will initially be concerned with the suspensions of isolated hepatocytes in comparison to those of other laboratories, and the biochemical competence of these cells in comparison to other liver systems. The ultrastructure of the isolated hepatocytes will also be compared to *in vivo*. The nature of cellular damage in response to CCl_4 will then be discussed in relation to the injury produced *in vivo* and changes found to occur in other *in vitro* systems. The bearing on CCl_4-induced toxicity of results from experiments using other potentially toxic chemicals will also be discussed. Incorporated into this discussion will be some relevant theoretical considerations and some suggestions for further experimentation.

Retention of K^+ by isolated hepatocytes was found to be a sensitive parameter of cell viability, which is in agreement with the observations of Baur *et al.* (1975). In particular, the K^+ content was consistently a more reliable determinant of the inital cell viability than was the trypan blue index. Cell batches, with a similar and acceptable trypan blue exclusion index (90%-95%) were found to have intracellular K^+ levels anywhere between, approximately, 60 and 100 µmol/g wet wt. It is noted that Berry and Friend (1969) found low K^+ levels with a high trypan blue exclusion. It seems that higher K^+ levels in hepatocytes are obtained as the experience of the operator increases. Also from the author's experience with the isolated hepatocyte preparation, it became apparent that cell suspensions with a trypan blue exclusion index of less than 90% indicated a poor quality batch of cells. Preparations with a percentage lower than this were not used in any of the experiments reported in this thesis.

The K^+ levels found in the studies of this thesis (60-100 µmol/g

wet weight) correspond well with other reports using isolated hepatocytes. Several reports of values in the range 80-100 µmol/g wet weight have appeared (Frimmer *et al.*, 1976; Krebs *et al.*, 1974; Hems *et al.*, 1975; Baur *et al.*, 1975). These correspond adequately with levels of about 100 µmol/g wet weight found for homogenates of perfused or fresh liver tissue (Baur *et al.*, 1975; Howard *et al.*, 1973; Barnabei *et al.*, 1974; Berry and Friend, 1969). A direct comparison is complicated by slight variations in preparative procedures. For example, Barnabei *et al.* (1974) found about 100 µmol/g wet wt for cells prepared by the method of Seglen (1972) and about 50 µmol/g wet wt for cells isolated by the method of Berry and Friend (1969). Furthermore, the method used in the present studies, although not identical with either of those above, consistently yielded K^+ levels of 90-100 µmol/g wet wt in the later stages of the experimental work.

Other studies reporting K^+ levels include Howard *et al.* (1973) (70 µmol/g wet wt), Dubinsky and Cockrell (1974) (21 µmol/g wet wt) and Campbell and Hales (1971) (82 µmol/g wet wt for cultured liver cells). It is noted that the K^+ levels in the latter study fall off more rapidly than in isolated hepatocytes of this laboratory.

It should be noted that some of the figures from other publications required recalculation to allow direct comparison (i.e. by expression with a similar reference). Factors from the literature were used where necessary (e.g. wet wt:dry wt - 3.7:1; 200 mg protein/g wet wt hepatocytes).

The levels of K^+ were also valuable in the assessment of changes in structural integrity on incubation of hepatocytes with drugs.

Release of the cytoplasmic enzyme, ALT, generally occurred after

some loss of K^+ had been observed, on incubation with toxins. This, too, is an indication of the sensitivity of K^+ as a structural parameter.

Levels of ALT in the suspension medium of fresh cells were generally between 5 and 10% of the total activity measurable after physical disruption of the hepatocyte membranes. A similar value was found by Hofman *et al.* (1976) for ALT. Other studies have shown retention of this enzyme in isolated cells by investigating specific activities (Berry and Friend, 1969; Fry *et al.*, 1976). Solyom *et al.* (1972) have reported no significant disruption of isolated cells as evidenced by retention of ALT and lactate dehydrogenase (LDH). This second enzyme, LDH, is also cytoplasmic and has been used by others to assess viability characteristics of cells. Values between 7-10% of total activity have been reported for this enzyme in supernatant from suspensions of fresh cells (Sies *et al.*, 1977; Baur *et al.*, 1975; Hofman *et al.*, 1976). Increase in supernatant enzyme activity has also been used by other authors as an assessment of toxicity by added agents (Wiebkin *et al.*, 1976; Hofman *et al.*, 1976). It is of interest to note that retention of alkaline phosphatase activity (a membrane bound enzyme) has also been reported in isolated hepatocytes (David *et al.*, 1975).

Ureogenesis by isolated liver cells has been reported as being similar to or greater than the rate of formation in the isolated perfused liver (Jeejeebhoy *et al.*, 1975; Krebs *et al.*, 1974). Other reports have indicated rates of ureogenesis by perfused livers similar to that reported by the latter of the previously cited studies (Heimberg *et al.*, 1964; Oomen and Chamalaun, 1971). A truly direct comparison is made difficult by considerations such as differential cell type in the whole liver and slight variations in suspending or perfusing media

between laboratories. The rates of urea formation reported in this thesis ranged from 0.25-0.6 μmol/g wet wt/min. The higher rates were found with later experiments and were associated with increased K^+ levels. This was taken as an indication that ureogenesis, like K^+ content, is very sensitive to preparative technique, such that gradual refinement through operator experience yields cells of higher metabolic as well as structural quality. Nevertheless, the results of this thesis are in good agreement with other reports, such as Krebs *et al.* (1974) (0.27 μmol/g wet wt/min) and Williamson *et al.* (1974) (0.2 μmol/g wet wt/min). Rognstad and Clark (1974) report a level of about 0.15 μmol/g wet wt/min (recalculated), using an isotopic method with ^{14}C bicarbonate as the biosynthetic precursor. Since CO_2 is produced metabolically close to the site of urea formation, unlabelled CO_2 might be responsible for a relatively higher proportion of ureogenesis. This could give a falsely low rate of urea production. Seglen (1977) has reported that enough CO_2 is generated by cellular respiration to support urea formation in non-bicarbonate buffers at rates similar to those utilizing this physiological buffer system. A rate of 0.29 μmol/g wet wt/min is reported by Seglen (1977) with no added amino acids. Data from other laboratories reported thus far have been under control conditions with a basic physiological salt solution as the incubating medium. As mentioned in the introduction, Eagle's Basal Medium gave higher ureogenic rates. Increasing amino acid levels was also found to increase ureogenesis by Seglen (1977). The level at normal amino acid concentrations (Seglen's Fig. 3) was seen to be approximately 0.38 μmol/g wet wt/min which is very similar to the data of this thesis. However, Eagle's Basel Medium lacks ornithine and citrulline, which have been shown to further stimulate ureogenesis (Williamson *et al.*, 1974). This point emphasizes the difficulty in direct comparisons among laboratories.

It is evident from the dose-response data in Chapters 1, 5 and 6 that inhibition of ureogenesis is very sensitive to chemically-induced disruption of cell metabolism. With several of the treatments the lowest dose gave a maximal inhibition even in the absence of significant structural damage. It can therefore be appreciated that a metabolic disturbance is not necessarily associated with cell death, even in suspensions of isolated hepatocytes.

The second parameter of metabolic function used extensively throughout the thesis, L/P, gave control values a little lower than other liver systems. Values from isolated perfused livers (after equilibration) and *in vivo* have generally been about 10 (Schimassek, 1963; Schimassek, 1963a; Williamson *et al.*, 1967; Graf *et al.*, 1973; Stier *et al.*, 1972; Schimassek *et al.*, 1974). Levels of about 3 were found for L/P in control incubated isolated hepatocytes throughout the experiments of this thesis. This was a remarkably consistent value, especially in comparison to other parameters. As this value is a ratio it would not be so susceptible to variations in cell count as would the other parameters, and this may account for the apparent consistency. Indeed, the actual levels of lactate and pyruvate showed much more variability than the final ratio. It was considered that a reported loss of glycogen from cells on isolation (Drochmans *et al.*, 1975) may account for the lower value in isolated hepatocytes since Stier *et al.* (1972) reported a value of 5.3 with isolated perfused livers from starved rats. However, the reported values of 6.6 from fed rats by Biebuyck *et al.* (1972) and 17 from starved rats by Williamson *et al.* (1967) (in comparison to their 12.5 from fed rats) question this explanation.

Other studies using isolated hepatocytes have shown L/P's similar

to the *in vivo* values (Grundin, 1975; Anwer *et al.*, 1975). However, reports have also appeared where values similar to those of this thesis have been documented (Clark *et al.*, 1974; Otto and Ontko, 1974; Mapes, 1977). Even though there is this seemingly inexplicable difference in the control values of this parameter, a similar qualitative shift was found in response to halothane (see Chapter 6), this being the only appropriate comparison found by the author in the literature.

Cellular levels of ATP have also been indicated as an index of cellular viability, but changes in the levels of this energy rich compound lag behind changes in membrane characteristics (Baur *et al.*, 1975). These authors report an ATP level of 15 nmol/mg protein (about 3 µmol/g wet wt), indicating this to be similar to the isolated perfused liver. Hems (1966) and Biebuyck *et al.* (1972) have reported levels of 2-2.5 µmol/g wet wt for the isolated perfused liver. Comparisons among preparations have been presented by Krebs *et al.* (1974) and Phillips *et al.* (1974) where quite similar levels for isolated perfused liver are shown. Values for ATP concentration in freeze clamped liver are given as 2.53 and 3.37 µmol/g wet wt in the above publications. A third figure of about 2 µmol/g wet wt has been presented by Baquer *et al.* (1973). Several reports besides Baur *et al.* (1975) have given data on ATP levels in isolated hepatocytes as around 2.4-3.3 µmol/g wet wt (Williamson *et al.*, 1974; Krebs *et al.*, 1974; Phillips *et al.*, 1974; Jeejeebhoy *et al.*, 1975; Hofman *et al.*, 1976). The ATP content of isolated hepatocytes found in one experiment in this laboratory (2.69 µmol/g wet wt) is comparable to the above studies and the levels *in vivo*. Consideration of the calculation being based on cell count (as discussed in Appendix 5) would put this figure with the higher reported values. Some studies

have reported lower levels of ATP in isolated hepatocytes (around 1 µmol/g wet wt) (Bissell *et al.*, 1973; Baquer *et al.*, 1973; Veneziale and Lohmar, 1973). These low values may reflect poor integrity of the cells, similar to the low levels found with the biochemically inferior liver slices (Krebs *et al.*, 1974).

Levels of GSH found in the hepatocytes used during the experiments of this thesis were between 4 and 5 µmol/g wet wt. Values for this tripeptide in the literature for fresh rat liver are generally between 5 and 8 µmol/g wet wt (Hissin and Hilf, 1976; Boyland and Chasseaud, 1970; Docks and Krishna, 1976; Jollow *et al.*, 1974; McLean and Day, 1975). Thus the levels of GSH in hepatocytes appear to be slightly lower than *in vivo*. However, consideration of calculations (see Appendix 5) would indicate that this value may fall well within this range. Even so, the value obtained would seem to be acceptable and could be taken as an indication of retention of cellular integrity throughout the isolation process. It would be expected that if membranes were damaged there would be a leakage of GSH which would be lost during washing of the cell pellets.

Other values of GSH levels in isolated hepatocytes have been presented. Hogberg *et al.* (1975) reported a value of about 2 µmol/g wet wt. A subsequent study from the same laboratory found improved isolation and incubation conditions gave a higher level of GSH very similar to that found in this laboratory (about 5 µmol/g wet wt using conversion factor of 1.3×10^8 hepatocytes/g wet wt) (Hogberg and Kristoferson, 1977). This emphasises the benefit of using an enriched incubation medium (as compared to a basic physiological salt solution), particularly in toxicity studies where intracellular protective mechanisms (e.g. GSH concentration)

require close attention. A further study incorporating isolated hepatocytes from diethyl maleate pre-treated rats showed that incubation with cysteine or methionine resulted in synthesis of GSH to a level of around 4-5 μmol/g wet wt (Reed and Orrenius, 1977).

Henderson and Dewaide (1969) reported a rate of formaldehyde formation from aminopyrine in isolated rat hepatocytes similar to that for microsomal preparations. However, they used a cofactor containing medium in their incubations and the difference between their intact and sonicated cells was small, so as to suggest that the barrier due to cell membranes was not great. It is noted that the hepatocytes were isolated by citrate chelation, which is not recognised as producing high quality cells (see Introduction).

The rate of formation of formaldehyde from aminopyrine by isolated rat hepatocytes in this laboratory (Table 2.2) is lower than that found with *in vitro* microsomal preparations (Drew, 1976; Gram *et al.*, 1968). The V_{max} for $^{14}CO_2$ production from ^{14}C aminopyrine (Lauterburg and Bircher, 1976) is greater than the rate of formaldehyde formation in the experiments of this thesis. The fact that CO_2 is produced by further metabolism of formaldehyde could well explain the lower rates observed in the isolated hepatocytes. Firstly, the cells retain, presumably, the capacity to metabolize formaldehyde to CO_2, which would lead to a reduced amount of formaldehyde to be measured in the assay. Secondly, there is no guarantee that semicarbazide, which is used to trap the formaldehyde, enters the cells. This could also lead to less measurable formaldehyde. Thus it is not altogether surprising that there is an apparent quantitative difference in metabolism.

Similarities in the metabolism of hexobarbitone have, however, been found in systems prepared by the author and with other published data. Oxidation of hexobarbitone in both isolated rat hepatocytes* and isolated perfused rat livers† occurred at about 100 nmol/min/g wet wt (taken from 20 min incubation or perfusion). The inital concentration in the latter preparation was, however, about three times that of the former. Junge and Brand (1975), at a similar initial concentration of hexobarbitone, found a very comparable rate of metabolism of 120 nmol/min/g wet wt in isolated hepatocytes. They indicated that their findings with respect to maximal velocity of hexobarbitone metabolism were quite similar to measurements in microsomal fractions.

Another study concerning metabolism of barbiturates found that kinetic values for heptabarbital were similar in isolated hepatocytes and perfused liver, and that metabolites formed for barbiturates were similar in the cells, isolated perfused and intact liver (Yih and van Rossum, 1977).

Further observations of similarity of the mixed function oxidase system in isolated hepatocytes and *in vivo* is seen by the inhibitory effects of SKF-525A (Table 2.2) and the potentiating effects of PB pre-treatment (Table 4.4). Moldeus *et al.* (1974) found similar responses.

Thus, even though there are some apparent quantitative differences, the hepatocytes used in this thesis retained activity of the microsomal enzymes. The lower rates of aminopyrine metabolism may be related to decreased access to the site of metabolism (of aminopyrine and/or

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* Hexobarbitone metabolism performed by A. Harman.

† Hexobarbitone metabolsim performed by R. Drew.

semicarbazide), further metabolism of formaldehyde or availability of some alternate metabolic pathway. Similar arguments have been used by Wiebkin *et al.* (1976) to account for a lower rate of biphenyl metabolism in isolated hepatocytes as compared to microsomal preparations. Further considerations which could account for such differences, in general, have been discussed by Billings *et al.* (1977). It should be remembered that incubation with aminopyrine and semicarbazide caused no structural damage to the isolated hepatocytes, such that leakage of cofactors essential to the metabolism cannot explain the lower rates.

The morphology of rat hepatocytes isolated in this laboratory is comparable to that seen by other workers by both light and transmission electron microscopy. The spherical appearance with well defined cell outline shown by light microscopy is in accord with a good preparation of cells (Berry, 1976). Such preparations are shown by other authors such as Seglen (1973a). The presence of microvilli is noted by several laboratories (Drochmans *et al.*, 1975; Howard *et al.*, 1967; Berry and Friend, 1969; Phillips *et al.*, 1974) and Jeejeebhoy and Phillips (1976) indicate that isolated hepatocytes should show microvilli in the cell membrane. They also say that the substructure should appear normal. The ultrastructure of control hepatocytes from this thesis is quite comparable to that found by others where there are no marked differences to *in vivo* structure and distribution. The condensed mitochondria reported by Schreiber and Schreiber (1973) were not often observed in the hepatocytes of this thesis. It is noted that there is a lack of microvilli in the electron micrographs of hepatocytes presented by Schreiber and Schreiber (1973). As with Phillips *et al.* (1974) large vacuoles were seen in the hepatocytes, especially those freshly prepared

or incubated over short periods. They found recovery within 6 hours of incubation and suggested that their initial formation may be a consequence of the separation procedure.

Taking the data obtained for control isolated hepatocytes overall, it can be appreciated that the cell suspensions of this thesis compare quite well with those of other laboratories, and with other liver systems (including the whole animal). This stems from consideration of the parameters of viability and the appearance of the ultrastructure. This is not to say that changes in the protocol would not result in even better viability. For example, gravity sedimentation, rather than centrifugation, could improve the final suspension of cells (Jeejeebhoy *et al.*, 1975). Enrichment of the incubation medium, especially for long term incubations, could also be advantageous.

The initial studies with CCl_4 (Chapter 1) did not dispute the applicability of isolated hepatocytes for investigation of injurous chemicals. Indeed, structural and functional changes were analagous to those seen *in vivo*, although they occurred more rapidly *in vitro*, requiring slightly higher concentrations.

However, several of the subsequent experiments described in this thesis cast doubt on the comparability of the mechanism *in vitro* and *in vivo*. These studies include a lack of amelioration of toxicity by agents found to be protective *in vivo* (Chapter 2), lack of evidence of lipid peroxidation associated with CCl_4-induced toxicity (especially when lipid peroxidation was shown in response to ADP/Fe^{3+} and diethyl maleate, the latter with ensuing evidence of structural damage (Chapter 3)), lack of potentiation of the toxic response in hepatocytes isolated from PB

pre-treated rats (Chapter 4), an apparent dissimilarity in mechanism of toxicity of bromobenzene (Chapter 5), a finding of similar toxicity for benzene as for CCl_4 in isolated hepatocytes (Chapter 7), a lack of toxicity in response to paracetamol, which is in contrast to *in vivo* (Chapter 7) and the finding that CCl_4 also produces a toxic response in EAT cells (Chapter 7).

Thus, even though toxicity in isolated hepatocytes initially appears similar to that *in vivo*, the manner in which it is brought about may not necessarily be the same in both systems. Studies using the isolated hepatocytes for investigation of preventative or curative treatments, then, would probably be rather futile. At the very least, great care would be required in the interpretation of any results obtained.

Although the data found with the anaesthetics do not directly support, or otherwise, the implications of the other experiments with respect to mechanism of CCl_4 toxicity, it is important to remember that the results complement those in tissue culture (Goto *et al.*, 1976a; Goto *et al.*, 1976b). A similar situation was found for the erythromycins by Zimmerman *et al.* (1974) on comparison of their isolated hepatocyte experiments to those carried out in tissue culture (Zimmerman *et al.*, 1973; Dujovne, 1975). In other words, the cells isolated in this laboratory have been found to act in response to a group of toxins in a manner similar to that found in other laboratories, which helps to justify the hepatocytes used in this thesis. It is perhaps paradoxical to note that the isolated cells may well be useful in testing for these indirect (idiosyncratic) toxins, whereas doubt has been raised about their use for studies on the mechanism of action of well known direct acting hepatotoxins.

The possibility that CCl_4 is toxic to suspensions of single cells in a manner different to the hepatic injury seen in the whole animal has also been indicated by the studies of Zimmerman and Mao (1965), who found a release of intracellular enzymes from laryngeal carcinoma cells on incubation with high levels of CCl_4 (reportedly 20 mM or more). A similar response was found in rat hepatoma cells with lower concentrations (0.2 mM) by Watanabe *et al.* (1977). The direct effects of non-metabolized CCl_4 *in vivo* (in comparison to the metabolite) have been discussed by Zimmerman (1976), where it was indicated that the parent molecule may play some, albeit small, part in the toxic response in the whole animal. In particular, rapid loss of intracellular enzymes and electrolytes was mentioned. Appearance of enzymes in the plasma after *in vivo* CCl_4 administration, however, is not appreciable until 2-4 hours (see Introduction, Chapter 1). The 10-fold greater toxicity to CCl_4 in rats as compared to chickens is also mentioned by Zimmerman (1976), this being used to infer a much reduced toxicity to the CCl_4 molecule itself. It should be remembered that the interpretation of hepatotoxicity from LD_{50} values is subject to criticism, however. For example, there is no guarantee that death is due to hepatotoxicity. The cause of death due to CCl_4 may be related to depression of the central nervous system or renal lesions.

From consideration of rapid electrolyte shifts and the species differences, it would be interesting to carry out experiments on liver swelling in both rats and chickens over a time course similar to that used in this thesis (e.g. 5, 10, 20, 30 and 60 min). If unchanged CCl_4 does exert such effects *in vivo*, it might be expected that livers from both species would show some similar degree of swelling within minutes after CCl_4 administration.

It has been pointed out that large shifts in K^+ and Na^+, although disturbing the cell, do not necessarily lead to its death in the whole tissue (Farber, 1971). McLean *et al.* (1965) indicated that such ion shifts and associated swelling are usually reversible, but that they could be responsible for setting in train other changes (mitochondrial). Recknagel and Glende (1973) state that the idea that CCl_4 is a generalised cytotoxic agent is not borne out by recent studies. This is certainly indicated for the injury occurring in the intact rat, as is made clear by Zimmerman (1976). However, the present and above mentioned studies deny this categorical statement in relation to *in vitro* preparations of cells.

In studies on maintaining viable cells in organ culture of mature rat liver, Campbell and Hales (1971) found that change in ion content was an early event preceding necrosis. Their studies indicated to them that failure of the sodium pump rather than damage to the cell membrane was the likely cause of observed changes. It was mentioned in Chapter 1 that CCl_4 has been shown to inhibit membrane ATP'ases (Dorling and Le Page, 1972; Rufeger and Frimmer, 1976) and there is the possibility that this may account for the observed shifts in ion concentration. A comparative study with the known inhibitor of the sodium pump, ouabain, could yield information into the liklihood of such a possibility. Indeed, a similar approach was used in the studies of Campbell and Hales (1971), who found a decrease in intracellular K^+ on incubation of their cell cultures with ouabain. A similar response to ouabain was found in isolated rat hepatocytes by Barnabei *et al.* (1974). The effects of ouabain on L/P and ureogenesis could give an indication as to the nature of the CCl_4-induced effects on these metabolic parameters.

Perhaps a study over longer incubation periods (4-8 hours) with lower doses (approximately 1 µl) would provide evidence of a toxicity more related to that seen *in vivo*. The lack of evidence of lipid peroxidation with 5 µl CCl_4 over 60 min incubation might suggest this to be unlikely (further discussion below). On the other hand, however, extension of the incubation times may yield further information on the relationship *in vivo* between lipid peroxidation and tissue damage.

In further experiments such as those described above, measurement of CCl_4 concentrations would be required to enable the most appropriate comparisons to *in vivo*, since toxicty in isolated hepatocytes is seen at concentrations higher than in the intact rat. Concentrations higher than those reached in *in vivo* studies were also required to produce damage to the hepatocytes with other chemicals in the experiments of this thesis. It is therefore stressed that care is required in the interpretation of *in vitro* data in relation to *in vivo*, particularly on consideration of concentrations of the toxins. Furthermore, the *in vivo* concentrations referred to are most often peak levels, which are not necessarily those concentrations present when necrosis is occurring.

Overall, it would appear that the toxicity shown by changes in the parameters of cellular integrity may well be due to the parent compound. On the other hand, ultrastructural changes similar to those seen *in vivo* and which have been attributed to lipid peroxidation initiated by an active metabolite, have been observed in isolated hepatocytes incubated with CCl_4. These changes include degranulation and loss of organised structure of the RER. Although not all studies are consistent with lipid peroxidation being responsible for changes in the RER (see below), perhaps we are observing two types of toxicity, as suggested by Zimmerman

(1976) for the *in vivo* situation. Toxicity due to the parent compound could be dominating in the *in vitro* system, which is opposite to the proposal for *in vivo*. Reversed domination could be related to the physical characteristics of the two systems. Perhaps a mechanism of protection of the cell membrane to the effects of CCl_4 has been lost during isolation of the hepatocytes.

Consideration of CCl_4-induced changes in RER and the apparent lack of lipid peroxidation in the isolated hepatocytes does, however, show a definite disagreement with studies *in vivo* where it has been shown that changes in both the RER and lipid peroxidation occur early in the time sequence of hepatic response to CCl_4. As the lipid peroxidation develops just prior to reduction of protein synthesis and polyribosomal dispersion, it has been suggested that peroxidation of the ER membranes is causative of the other alterations (Recknagel and Glende, 1973).

At doses and times when there was evidence of gross disturbance of RER structure, there was no evidence of lipid peroxidation. It is important to note that 5 µl CCl_4 over 60 min incubation gave no lipid peroxidation, while there were changes in the appearance of the RER. There was no evidence of structural damage to the cell membrane under these conditions and the measured level of CCl_4 was similar to that found when lipid peroxidation has been measured *in vivo*. A possible explanation for the discrepancy between cell damage and lipid peroxidation is that cofactors required for the process of lipid peroxidation could have been lost through the damaged membrane. This is unlikely to be so, however, when cells have retained their full complement of K^+.

A second possible explanation is that lipid peroxidation has

occurred but not been detected. Estimation of lipid peroxidation by measurement of TBA reacting substances in the whole cell is subject to the criticism that MDA is metabolized further by mitochondria (Goshal and Recknagel, 1965a). In this respect, it may be productive to investigate the conditions with chloral hydrate present, as this has been reported to inhibit the further mitochondrial metabolism (Hogberg *et al.*, 1975). However, these authors indicate that the TBA reaction only underestimates lipid peroxidation by about 50%, suggesting that this aspect, *per se*, is unlikely to account for the lack of demonstrable lipid peroxidation with CCl_4.

Diene conjugation measurement is also subject to criticism because the extraction of total lipids from the cells could well mask a small shift in absorbance at 233 nm occurring only in the microsomal lipids (Recknagel and Goshal, 1966a).

Other assays might provide further information with regard to lipid peroxidation. For example, the fluorometric technique of Yagi (1976) is about 10 times more sensitive in estimating TBA reactants than the colorimetric method. This may be of particular use in a re-appraisal of the lipid peroxidation at 15 min in response to CCl_4 in hepatocytes isolated from PB pre-treated rats (Table 4.1). Other assays of fluorometric products (perhaps in individual membrane systems) as outlined by Plaa and Witschi (1976) could be worthy of investigation. Measurement of fatty acid composition of the lipid fraction from treated hepatocytes could also be worthwhile in looking for evidence of lipid peroxidation. The production of ethane as a result of lipid peroxidation appears to be a sensitive estimate, and measurement of ethane trapped in the stoppered incubation flasks is a possibility for further study. Lipid peroxidation

in response to ethanol has been shown by this method, as has lipid peroxidation in mice (Koster and Remmer, 1977). It is interesting to note that the relative proportions of ethane production between rats and mice make it difficult to appreciate the lack of evidence for lipid peroxidation previously associated with CCl_4 administration to mice.

A study by Scheig and Klatskin (1969) indicated that unfasted animals did not show stimulation of MDA production on dosage with CCl_4. They also found that rats between 180-220 g gave absolutely no evidence of lipid peroxidation under any conditions. Their findings suggest that experiments using fasted animals of perhaps a different weight range could be useful. However, inconsistencies in their own data (e.g. difference in stimulatory effect of CCl_4 on MDA production when 1.0 ml rather than 0.4 ml of 9,000 x g supernatant was used) and with other studies would question the above suggestion.

In spite of these technical deficiencies, lipid peroxidation was detected using the TBA reactant method in control incubated cells (Table 3.1). Furthermore, a dose related increase in TBA reactants was observed with chemical treatments other than CCl_4 (Chapter 3). It would therefore be a logical experimental follow up to determine the effect of these treatments on the RER. If changes in RER were found then the possibility of a direct relationship with lipid peroxidation could be investigated. If there was no apparent effect on the RER then this could be taken as evidence in support of lipid peroxidation not being responsible for changes in the RER ultrastructure. A similar type of argument has been put forward by Recknagel (1967) to dispute a drop in protein synthesis being responsible for the eventual necrosis on treatment with CCl_4. Other chemical treatments (e.g. ethionine) also

cause a drop in protein synthesis but no necrosis, and hence CCl_4 necrosis is not manifest by its inhibitory effects on protein synthesis. This tends to support the validity of using the above argument for lipid peroxidation not being responsible for changes in the RER. In such a discussion the localisation of lipid peroxidation is important because the measured lipid peroxidation with one treatment may not necessarily be occurring at the same place as with a second treatment. However, lipid peroxidation with both ADP/Fe^{3+} (Hochstein and Ernster, 1964) and CCl_4 (Introduction, Chapter 3) has been found to occur in microsomes.

An investigation using isolated hepatocytes into the effects of antioxidants on the lipid peroxidation response and any changes in the RER (including those due to CCl_4) could provide further information about the possible connection between these two events. The effect of antioxidant compounds on endogenous lipid peroxidation could also prove interesting, especailly with respect to preservation of structure and function over extended periods of incubation. An investigation of microsomal parameters (such as glucose-6-phosphatase, cytochrome P_{450} etc.) in response to CCl_4 with and without antioxidants could prove helpful in studies involving ER. Results from experiments of this nature could allow for a differentiation between changes seen in the ER and biochemical dysfunction. This may then indicate independence of the two events. Alternatively changes similar to those *in vivo* may be observed, which would be consistent with ultrastructural changes. Such experiments may help to explain some of the apparent differences in CCl_4 toxicity between isolated hepatocytes and *in vivo* while simultaneous similarities are seen to exist.

Since lipid peroxidation due to CCl_4 is reportedly subject to a prior

metabolism of the toxin (Recknagel and Glende, 1973) it would be of interest to determine whether CCl_4 itself is metabolized in the isolated cells and if this bears any relation to alterations in the RER. Similarly, the response of the RER of isolated chicken hepatocytes to the same concentrations of CCl_4 would be of interest, as these animals are reported not to metabolize CCl_4 and not to peroxidize lipids to any significant extent (Recknagel, 1967).

The time course of lipid peroxidation *in vivo* and in microsomal preparations is short enough to suggest that longer term incubations (4-6 hr) with a 5 µl (or lower) dose of CCl_4 would be of little benefit in extending the search for CCl_4-induced lipid peroxidation in isolated hepatocytes. On the other hand, as pointed out in Chapter 3, the work of Remmer *et al.* (1977) indicates that longer incubations could be useful. The controls in such long term incubations might also provide information concerning lipid peroxidation and cell leakage in view of the small endogenous production of TBA reactants. If associated with a loss of K^+, the effects of antioxidants and dietary factors could be used to further investigate the response. The ability to demonstrate quite low levels of lipid peroxidation in control cells suggests that an increase in cell number per assay would be unlikely to lead to measurable TBA reactants in response to CCl_4. That is, a simple increase in material capable of forming (or containing) TBA reactants would probably not lead to an appropriate increase in sensitivity.

Other studies have indicated that RER alterations are not necessarily directly caused by lipid peroxidation. Alpers *et al.* (1968) concluded from their studies that there may not be a direct relationship between inhibition of protein synthesis and lipid peroxidation. Part of their

evidence included the *in vitro* finding that ribosomes remained bound to microsomal membranes despite large changes in lipid peroxidation. However, their findings of lack of protection of protein synthesis by antioxidants have been disputed by Gravela and Dianzani (1970) who used a different dosage regimen. These authors also found, however, that their *in vitro* experiments did not support a direct link between lipid peroxidation and ribosomal changes. In a further study from this laboratory the conclusion is reached that there is a direct relationship between lipid peroxidation and protein synthesis (Gravela *et al.*, 1971). Still from the same laboratory, Gravela (1973) in a study involving the life-span of messenger RNA on treatment with CCl_4, concluded that there was no evidence that detachment of ribosomes from RER was due to membrane changes induced by CCl_4. From a study on the effects of PB and 3-MC pre-treatment on response to CCl_4, Shah and Carlson (1975) concluded that the effects of CCl_4 on lipid peroxidation and protein synthesis are independent of each other and that both processes may be involved in the hepatotoxicity attributed to CCl_4. De Ferreyra *et al.* (1975) found that promethazine, although not reducing the lipid peroxidation, did protect against the change in polyribosomal profile by CCl_4. Investigation of responses to CCl_4 in animals with and without PB pre-treatment indicated that although lipid peroxidation could be demonstrated there was no apparent quantitative link between this and extent of injury to the ER (Reynolds and Ree, 1971; Reynolds *et al.*, 1972).

In summary of this aspect of CCl_4 toxicity, the results of experiments of this thesis do not support the theory that lipid peroxidation is responsible for changes in RER. Further experimental approaches have been suggested to gain further insight into this intriguing area. Thus,

although CCl_4 toxicity overall shows differences between *in vivo* and this *in vitro* preparation, investigation of certain aspects of the toxic response are not to be discounted. Apart from this, it should be remembered that findings of apparent differences between two different biological systems can lead to discovery of why they are different, which can, in turn, increase knowledge of the response in the whole animal.

It has been mentioned in conjunction with RER changes that it would be interesting to determine if CCl_4 itself is metabolized in isolated hepatocytes. This could easily be incorporated into a study to see whether or not ^{14}C from labelled CCl_4 is binding covalently to cellular macromolecules, this itself being taken as evidence of metabolism to free radicals. At the same time the presence of other metabolites ($^{14}CO_2$ and $^{14}CHCl_3$) could be monitored. A study of the covalent binding would provide further information for comparison to *in vivo*. Incubations over extended time periods (and perhaps doses lower than 5 µl) could be used as well as the shorter times, especially in view of the findings of more appropriate similarities to *in vivo* in the 120 min incubations of Remmer *et al.* (1977) with $BrCCl_3$.

No evidence of metabolism was seen from measurement of total CCl_4 levels in the cell suspensions. This is not surprising since only a very small proportion of a dose administered *in vivo* is metabolized through the microsomal system (McLean and Day, 1975). Besides this, the time for potential metabolism is not long, and the CCl_4 in the vapour phase would continue to re-equilibrate, thus helping to mask any small amount lost through metabolism.

Some interesting points arise from the consideration of covalent

binding of reactive metabolites. It has been reported that anaerobic conditions enhance the binding of ^{14}C from $^{14}CCl_4$ (Villarruel *et al.*, 1975; Sipes *et al.*, 1977). Without oxygen lipid peroxidation cannot proceed (Glende *et al.*, 1976), at least not to its full extent, as described earlier. Thus the increase in binding under anaerobiosis would suggest that ^{14}C is bound only at the site of initiation of a peroxidizing fatty acid. However, the work of Gordis (1969) found incorporation of label from ^{14}C $^{36}Cl_4$ into a heterogeneous group of branched long chain fatty acids, suggesting incorporation of label into many different sites. An interpretation of this could, perhaps, be that CCl_4 activation occurs at many different sites. Alternatively, some lipid peroxidation (the initial stages) could be occurring. However, a loss of some sites for potential incorporation of the label would suggest that a lower level of ^{14}C binding would be observed. Thus there is a further apparent anomaly between lipid peroxidation and covalent binding studies.

If binding of ^{14}C was only at an initiation (or activation) site, then the action of the antioxidants at multiple sites within the peroxidizing membranes might well be in question. The marked decrease of ^{14}C binding when GSH is present (Sipes *et al.*, 1977) suggests that the GSH may act only to bind a radical from split CCl_4 or the radical within the fatty acid at this site. Such a localized action would be consistent with the importance of antioxidant concentrations in very specific areas, as discussed by Goshal (1976) with respect to vitamin E. However, this could again be taken to indicate activation at multiple sites.

Studies with labelled antioxidants could provide valuable information in this area. If such chemicals are acting to interrupt lipid peroxidation at all stages of the process as well as the radicals of

the initiating agent, then a heterogeneous picture of covalent binding should emerge. On the other hand, a more specific pattern should be encountered if binding occurs only with the reactive metabolite or at a single initiation site.

In relation to binding at various sites within the macromolecules of the tissue, it has been tempting to speculate that unchanged CCl_4 might be cleaved at the site of ongoing lipid peroxidation, with immediate incorporation of the chemical free radical at that site. This could be used to explain the appearance of covalently bound label in mitochondria. The lack of significant incorporation under conditions of endogenous peroxidation (in tissues such as brain (Villarreul *et al.*, 1977)) could be taken as evidence against this. Perhaps there is a requirement of large scale lipid peroxidation first, however, to overcome intracellular protective mechanisms or whatever. Interestingly, the above mentioned observation that ^{14}C binding increases under conditions inhibitory to lipid peroxidation (anaerobiosis) is against this non-cytochrome P_{450} mediated incorporation.

While considering the rather speculative hypothesis above it is appropriate to mention some other ideas which may need consideration for future studies with CCl_4. Firstly, there may be some protective mechanisms associated with tissues other than the liver which help to account for the greater toxicity to this organ. Secondly, some repair type process might be found in other tissues to help them overcome the injury due to CCl_4. There is a possibility that the protective effects of cysteine observed by de Ferreyra *et al.* (1974) could be related to a repair type process. The formation of a protein deleterious to the cell is a third possibility which may be involved with CCl_4 toxicity. Castro

et al. (1977) have recently shown cycloheximide to protect against some aspects of CCl_4 toxicity (including necrosis). Other studies involving the relationship between protein synthesis and cellular damage were discussed in this paper. The depressant effects of CCl_4 on protein synthesis might suggest production of a deleterious protein to be an unlikely mechanism. General depression of protein synthesis does not necessarily mean that production of all proteins is reduced, however.

There are other areas of toxicity relevant to this thesis which could be investigated. Several other protective agents could be examined for effect on the CCl_4 response. Combinations of treatments could be investigated - an example would be vitamin E, selenium and the sulphur containing amino acids, which have been described as inter-related in their hepatoprotective activity (Wolman, 1975). In view of the overall findings of this thesis, continuation of this approach is probably unlikely to yield results enabling conclusions different to those already reached. Pre-treatment of animals with diethyl maleate to reduce GSH levels could be worthwhile, especially in studies with paracetamol. Hogberg and Kristoferson (1977) found some evidence of damage on incubation of hepatocytes with diethyl maleate and paracetamol together. The results in Chapter 3 would suggest that this could well be related to the diethyl maleate, rather than the paracetamol.

Considering the involvement of oxygen with both CCl_4 activation and the lipid peroxidation process, investigation of toxicity at varying concentrations of oxygen is suggested. Care would be required to ensure adequate oxygenation for maintenance of normal cellular function. Levels of ureogenesis and L/P in control cells would indicate the achievement (or otherwise) of this.

Other treatments associated with hepatotoxicity could also be investigated. Similarities to the *in vivo* situation could be found with some of these. Indeed, the studies of Hofman *et al.* (1976) showed similarity of toxicity between isolated hepatocytes and *in vivo*, and it was concluded that isolated hepatocytes are suitable for studies on the pathogenic sequence of events elicited by D-galactosamine. Other hepatotoxic chemicals such as ethionine, furosemide, thioacetamide, dimethylnitrosamine, α-methyl dopa, isoniazid and iproniazid could also be examined in the isolated hepatocytes. Several of these treatments exert their toxic effects through reactive metabolites.

The findings in Chapter 7 of a toxic response to benzene and lack of the same in response to paracetamol suggest that the organic solvent properties may be playing a role in the toxicity seen in the isolated hepatocytes. In this respect a more detailed comparison, experimentally, of chemicals known to possess lipid solvent properties with those which do not could be advantageous. Selection of a non-solvent group would require omission of surface active agents, as demonstrated by the toxic effects seen with chlorpromazine (Chapter 7). From the same point of view, determination of the solvent properties of diethyl maleate would indicate whether such an effect needs to be considered for involvement in this response.

An investigation of hepatotoxins over periods of days (or weeks) in cells cultured from isolated hepatocytes could provide the basis of a 'chronic' *in vitro* exposure. This could be compared to the acute effects examined in hepatocytes isolated from the same rat. Improvements in the maintenance of cultured cells in a state similar to *in vivo* would be required before the potential value of such a system could be fully

exploited.

In conclusion, the results of this thesis have shown that a toxic response to CCl_4 can be elicited from suspensions of isolated hepatocytes. However, further investigation has indicated the mechanism of the toxic response to be different to hepatic injury seen after administration of CCl_4 to the whole animal. A similar situation was found with bromobenzene. Thus it is apparent that care is required in the interpretation of toxicity studies using isolated hepatocytes when attempting to extrapolate to *in vivo*. Experiments with some halogenated anaesthetics have indicated that the isolated hepatocytes could be useful in predicting idiosyncratic hepatotoxins, in a similar manner to cultured cells. On a more specific basis, the involvement of lipid peroxidation with disarray of the RER is not supported by experimental results found in this laboratory. Experiments have been suggested to help obtain further information into this and several other aspects of the hepatotoxic response.

APPENDICES 1-5

APPENDIX 1

RAT SOLID DIET

Charlick's M & V 164 Mouse Cubes

	%	Additives	
Wheat	40	Vitamin A	1780 IU per lb
Barley	19	Vitamin D_3	440 IU per lb
Bran	6	Vitamin E	7.5 mg per lb
Pollard	6	Vitamin B_2	0.75 mg per lb
Meat (50%)	10	Pantothenic Acid	0.24 mg per lb
Soya (50%)	6	Vitamin B_{12}	1.55 mg per lb
Fish	5	Vitamin K	0.25 mg per lb
Buttermilk	3	Vitamin B_6	0.09 mg per lb
Salt	1	Vitamin B_1	0.28 mg per lb
Molasses	3	Choline	11 mg per lb
Yeast	1	Manganese Oxide	5.5 mg per lb
	100	Folic Acid	0.5 mg per lb

SOURCES OF DRUGS AND CHEMICALS

The common chemicals used were of reagent or pharmaceutical grade quality, and supplied by local distributors. Most biochemical reagents were obtained from Sigma Chemical Co., U.S.A. Certain specific substances were obtained from the sources set out below.

Collagenase Type CLS II - Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Eagle's Basal Medium - Commonwealth Serum Laboratories, Melbourne, Australia.

Some drugs were donations from various sources, as follows:

Paracetamol	-	Royal Adelaide Hospital
Methoxyflurane	-	Royal Adelaide Hospital
Enflurane	-	Abbott Laboratories
Chlorpromazine	-	May & Baker
Promethazine	-	May & Baker
SKF-525A	-	Smith, Kline & French
Dibenamine	-	Smith, Kline & French

APPENDIX 2

COMPOSITION OF PERFUSION MEDIUM

The composition of the physiological solution used during the isolation of rat hepatic parenchymal cells is documented in Table A.2.1. This formula was based on that of Krebs and Henseleit (1932) (which is also set out in Table A.2.1.). Modifications to this formula were based on measured values of some of the relevant constituents in rat blood drawn from the hepatic portal vein (HPV) and abdominal aorta (AA), with allowance made for the protein binding, which would alter the levels of free ions. Sodium gluconate, an impermeant anion, was included to allow the chloride ion level to be adjusted to a more physiological level (Bretag, 1969). The absence of Ca^{++} is a requirement for the dissociation of the hepatocytes (Berry, 1976).

METHODS

Measurement of plasma parameters

Blood samples were withdrawn from the hepatic portal veins and abdominal aortae of anaesthetised rats (intraperitoneal sodium pentobarbitone, 50 mg/kg). Animals were heparinized (5 U/g, i.v.) about 5 minutes prior to sampling and blood was placed into heparinized tubes for centrifugation at 4,000 r.p.m. for 10 minutes. The plasma was then assayed for content of sodium and potassium ion by flame photometry with an Eel Model 150 Clinical Flame Photometer, calcium* and magnesium* ion by atomic absorption spectrophotometry (Varian AA5) by the method of Savory

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*Analyses by the Department of Chemical Pathology, Adelaide Children's Hospital, courtesy of Drs W. Carey and A. Pollard.

TABLE A.2.1

COMPOSITION OF PHYSIOLOGICAL MEDIA USED

CONSTITUENT	NOMINAL CONCENTRATION (mM)	
	KREBS-HENSELEIT	SOLUTION FOR ISOLATION OF RAT HEPATOCYTES
Na^+	143	147.7
K^+	5.9	3.9
Ca^{++}	2.54	-
Mg^{++}	1.18	0.74
Cl^-	128	97.4
HCO_3^-	24.9	30
Phosphate	1.18	2.5
$SO_4^=$	1.18	0.74
Gluconate anion	-	21.7

et al. (1969), inorganic phosphorus by the methods of Fiske and Subbarow (1925) and Hohenwallner and Wimmer* (1973), chloride ion* with a Radiometer chloridometer (CMT 10 Chloride Titrator), and sulphate ion by a procedure adapted from the technicon autoanalyser manual, as follows. An aliquot (0.2 ml) of sample or standard was added to 3.4 ml of water, followed by 1 ml of the barium chloride ($BaCl_2$) reagent ($BaCl_2$ 20 g, gelatin 5 g, thymol 0.5 g made to one litre with water). Absorbance was then measured at 520 nm on a Unicam SP 1800 spectrophotometer. Reference cuvettes contained the same as the sample cell with the exception of $BaCl_2$.

Osmolarities of plasma samples were determined with a Knauer Halbmikro osmometer.

Determination of similar variables in artificial physiological media were performed using these same techniques.

Analysis of bicarbonate ion concentration ($[HCO_3^-]$) was also carried out, using whole blood. Partial pressure of carbon dioxide (CO_2) was measured using a Radiometer BMS3 Mk 2 Blood Micro system at 37°C and room atmospheric pressure. Allowance was made for daily variations in the latter in the standard calibration procedure of the instrument. Analysis of the pH of the sample with this instrument allowed for a determination of the $[HCO_3^-]$ by the Henderson-Hasselbalch equation, according to the instructions supplied with the instrument.

This, and three other methods were used to estimate $[HCO_3^-]$ in artificial physiological solutions. The other three were as follows:

(1) Estimation of CO_2 evolved in the closed manometric Van Slyke

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*Analyses by the Department of Chemical Pathology, Adelaide Children's Hospital, courtesy of Drs W. Carey and A. Pollard.

apparatus according to the method for urine (Peters and Van Slyke, 1932). Samples were gassed with carbogen and corrections for dissolved CO_2 and temperature variations were applied as described in the reference text.

(2) Using method (b) of Siggaard-Andersen (1962). Samples (with and without addition of hydrochloric acid to a final concentration of 10 mmol/l) were equilibrated with gases of known CO_2 content, and $[HCO_3^-]$ calculated after measuring the pH of the solutions, at 37°C.

(3) A similar estimate of $[HCO_3^-]$ was performed by an independent laboratory* employing the Astrup apparatus as used for routine clinical specimens.

RESULTS AND DISCUSSION

Constituents in the physiological solution of Krebs and Henseleit (1932) were found to be similar to the amounts added except for $[HCO_3^-]$. This apparent discrepancy between the amount of bicarbonate added and that which could be measured led to a more detailed investigation of this point. Other estimates of $[HCO_3^-]$ were used to support the initial findings, the results being shown in Table A.2.2. It was found that a nominal concentration of 30 mM was required to produce a measurable concentration comparable to the 24.9 ± 1.3 mM found in rat plasma (determined in HPV and AA samples).

The difference between nominal and measurable $[HCO_3^-]$ of Krebs-Henseleit solution was supported by evidence gained from further experimentation with the Van Slyke apparatus. Krebs-Henseleit medium was prepared by mixing an

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*Analyses by the Department of Chemical Pathology, Adelaide Children's Hospital, courtesy of Drs W. Carey and A. Pollard.

TABLE A.2.2

BICARBONATE ION CONCENTRATION (mM)[a]

METHOD	KREBS-HENSELEIT	MODIFIED[b] KREBS-HENSELEIT	RAT PLASMA
Radiometer	20.2 ± 0.3 (6)	25.1 (2)	24.9 ± 1.3 (12)
Van Slyke	21.5 ± 0.21 (4)	25.7 (2)	
Siggaard-Andersen	20.0 (1)		
Astrup	20.5 (1)		

[a] Values are mean ± S.D. with the number of different samples shown in parentheses. At least five replicate determinations were made on each sample.

[b] As for Krebs-Henseleit except that nominal concentration of bicarbonate ion increased by 5 mM to 30 mM.

appropriate amount of a solution of sodium bicarbonate with a solution of the remaining electrolytes in the chamber of the Van Slyke apparatus. The CO_2 evolved during this mixing process averaged 5.2 mmol/l in the four determinations which were carried out. This value is similar to the difference between the nominal and measured $[HCO_3^-]$'s of Krebs-Henseleit solution.

A similar requirement for increased nominal $[HCO_3^-]$ over measurable $[HCO_3^-]$ was also found to apply to the solution used for isolation of hepatocytes. Other inorganic constitutents of this solution were assayed by the methods detailed above, but there were no discrepancies from the calculated nominal value.

Values obtained from plasma determinations other than $[HCO_3^-]$ are shown in Table A.2.3.

An interesting observation, which bears no relevance to the actual formulation of the physiological medium, was made during the course of measurement of constituent ion levels. At one stage animals which had received a laparotomy as a sham operation 24 hours previously were used as blood donors. As shown in Table A.2.4, the K^+ of the HPV was significantly elevated over that of the AA, while there was no difference in Na^+ levels. This may be related to release of K^+ from ruptured cells, the K^+ being absorbed into the blood in the gut region and passing into the liver via the HPV.

From the results presented in this appendix the physiological medium shown in Table A.2.1 was derived, this showing some differences to Krebs-Henseleit solution.

TABLE A.2.3

MEASURED LEVELS OF PLASMA PARAMETERS[a]

PARAMETER		SITE OF BLOOD SAMPLE	
		HEPATIC PORTAL VEIN	ABDOMINAL AORTA
Na^+	(10)	146.8 ± 2.7 mM	144.00 ± 3.3 mM
K^+	(8)	4.00 ± 0.14 mM	3.75 ± 0.13 mM
Ca^{++}	(10)	2.67 ± 0.02 mM	2.71 ± 0.02 mM
Mg^{++}	(10)	0.88 ± 0.04 mM	0.83 ± 0.04 mM
P_i	(8)	2.62 ± 0.07 mM	2.79 ± 0.08 mM
Cl^-	(10)	101.10 ± 1.1 mM	104.6 ± 1.4 mM
$SO_4^=$	(3)	0.73 ± 0.17 mM	0.73 ± 0.12 mM
Osmolality	(5)	297.00 ± 0.5 mosmol/kg	293.2 ± 1.9 mosmol/kg

[a] Values are mean ± s.e.m. with the number of samples from different animals given in parentheses in the parameter column.

TABLE A.2.4

LEVELS OF Na^+ AND K^+ AT DIFFERENT SAMPLING SITES 24 HOURS AFTER LAPAROTOMY[a]

ION		SITE OF BLOOD SAMPLE	
		HEPATIC PORTAL VEIN	ABDOMINAL AORTA
Na^+	(5)	146.4 ± 0.6 mM	148.0 ± 1.8 mM
K^+[b]	(5)	5.2 ± 0.4 mM	4.0 ± 0.1 mM

[a] Values are mean ± s.e.m. with the number of samples from different animals given in parentheses in the ion column.

[b] HPV $[K^+]$ significantly different from AA $[K^+]$

APPENDIX 3

COMPOSITION OF INCUBATION MEDIUM

Eagle's Basal Medium (Eagle, 1955; Eagle *et al.*, 1956) was prepared as set out below.

For 1 litre:

Eagle's Basal Medium 10 x concentrate (see next page)	100 ml
Glutamine solution (100 mM or 14.6 mg/ml)	20 ml
Distilled water	to 800 ml
Dissolve BSA	12 g
Equilibrate with carbogen	(O_2 + CO_2 : 95 + 5)
$NaHCO_3$ (2.8% w/v) (carbogen gassing maintained)	as required to pH 7.4
Distilled water	to 1 litre

Eagle's Basal Medium 10 x concentrate

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 Pencillin G	100 000 Units
l-Methionine	7.5 mg	Streptomycin Sulphate	100 000 mcg
l-Phenylalanine	16.5 mg	i-Inositol (Meso)	1.8 mg

APPENDIX 4

STABILITY OF ALT ON FROZEN STORAGE

Measurements of ALT in initial experiments led to some doubt as to the stability of activity of this enzyme under the conditions of storage being used. A more detailed study was therefore carried out so that any loss of activity could be defined. The stability of this enzyme in hepatocyte-derived samples was compared with that of plasma stored under identical conditions.

High activity plasma samples were obtained by centrifugation of blood samples drawn from the abdominal aorta of rats injected (i.p.) 24 hours previously with 1 ml/Kg CCl_4 in a 1:1 peanut oil vehicle.

Samples from isolated hepatocytes were taken from control incubated cells (conditions as in General Methods; 20 minute incubation of 2.5 ml cell suspension), cells incubated under similar conditions but exposed to 20 µl of CCl_4 added to a side arm of the flask (as in Chapter 1) and from cells disrupted by 20 strokes with a Potter-Elvenhjem homogeniser. Samples were run in quadruplicate and 0.3 ml aliquots taken for subsequent assays.

Storage of the samples was at -25°C in a deep freeze for up to 56 days. Assays were performed on samples that had been thawed once only, immediately prior to enzyme analysis (except for day 0, which had not been frozen).

The stability on storage of the samples is indicated in Table A.4.1. ALT activity was noticeably more stable in plasma. Activity in controls was maintained at the same level throughout the study. The higher activity in the CCl_4-treated rat samples was stable up to 14 days, but declined thereafter to approximately 60% of initial levels. In contrast,

TABLE A.4.1

ALT ACTIVITIES (I.U./LITRE) IN FRESH AND FROZEN-STORED SAMPLES OF RAT PLASMA AND ISOLATED HEPATOCYTE PREPARATION.

MEAN ± S.E.M. OF 4 SAMPLES.

	DAYS OF STORAGE						
	0	1	2	7	14	28	56
Plasma							
Controls	10.6 ± 0.5	11.8 ± 0.9	9.4 ± 1.0	8.8 ± 0.8	9.7 ± 0.8	6.3 ± 1.4	10.6 ± 1.2
CCl_4-treated	99 ± 26	95 ± 30	81 ± 21	84 ± 31	97 ± 32	59 ± 21*	63 ± 18*
Hepatocytes							
Control supernatants	51.8 ± 5.3	37.0 ± 1.8	28.3 ± 3.9*	13.5 ± 2.6*	11.3 ± 1.2*	5.8 ± 2.1*	7.7 ± 2.9*
CCl_4-supernatants	261 ± 19	278 ± 24	172 ± 10*	103 ± 10*	48 ± 6*	14 ± 21*	29 ± 3*
Cell homogenates	340 ± 16	376 ± 7	241 ± 2*	145 ± 9*	100 ± 10*	24 ± 4*	27 ± 6*

* Significantly lower than day 0

ALT activity in the hepatocyte preparations was stable for only one day, and declined markedly thereafter. After 28-56 days storage, the ALT activities were approximately 8-16% of the initial levels and there was relatively little difference between the control and CCl_4-treated cells.

The requirement for an early assay of ALT samples has been illustrated by this study. Immediate assay for ALT on fresh samples from hepatocyte suspension incubations was therefore carried out routinely in the experiments of this thesis unless otherwise indicated.

APPENDIX 5

CALCULATIONS AND EXPRESSION OF RESULTS

As a comparison to other laboratories and other liver systems was required for the cells isolated in this laboratory, it was decided to relate measured parameters to unit weight. Similar reasoning led Krebs *et al.* (1974) to express their results using the same reference.

Reference points used by others have included cell number, dry weight, cell protein (TCA insoluble material) and DNA. There are interconversion factors available for these, as discussed (in part) by Krebs *et al.* (1974). The use of DNA has been criticised (Knook, 1976) in this regard.

Calculations were based on the number of cells (as counted in a Neubauer haemocytometer) per unit volume with a subsequent conversion of unit wet weight, using a factor derived from the literature.

Several figures have been published relating hepatocyte number to unit wet weight, ranging from just under 10^8 hepatocytes/g wet weight (Zahlten and Stratman, 1974) to over 2×10^8 hepatocytes/g wet weight (Suzangar and Dickson, 1970) (recalculated from 20×10^6 cells/25-30 mg dry weight using a factor of 3.7 (Krebs *et al.*, 1974) to convert dry weight to wet weight). The majority of papers, however, report a figure close to $1.2 - 1.4 \times 10^8$ hepatocytes/g wet weight (Ingebretsen *et al.*, 1972; Ingebretsen and Wagle, 1972; Krebs *et al.*, 1974; Junge and Brand, 1975; Hofman *et al.*, 1976). Jeejeebhoy *et al.* (1975) and Seglen (1973a) have reported similar figures but a figure of 10^8 cells/g wet weight has also been reported by the same laboratories (Phillips *et al.*, 1974; Seglen, 1973b). Wiebkin *et al.* (1976) have used the figure of Daoust

(1958) (1.2×10^8 hepatocytes/g liver) in their calculations. The findings of Weibel *et al.* (1969) also have been used by others (East *et al.*, 1973; Hommes *et al.*, 1970), but it appears that the figure of 1.69×10^8 hepatocyte/g liver used had not been adjusted to allow for the binucleate cells that exist in this tissue.

As $1.2 - 1.4 \times 10^8$ hepatocytes/g wet weight is the most common figure, 1.3×10^8 was chosen as the conversion factor for cell number to wet weight in this thesis.

A factor which requires some attention is that results from isolated hepatocytes expressed as per g wet weight generally relate to the weight of the hepatocytes and not the original parent liver tissue. This is important from the aspect of extracellular space (see below) and the point that liver contains cells other than hepatocytes, even though the parenchymal cells account for a large portion of liver mass (Seglen, 1973a). That is, even though both are expressed per g wet weight, that gram in the first case may refer only to hepatocytes whereas in the latter the gram generally refers to hepatocytes, Kupffer cells, intercellular components, cells of the biliary tree and so on.

In expressing the results per number of cells before conversion to wet weight, the count related to was that of viable cells only (that is, trypan blue excluding cells). When drugs were added to the cell suspension in a vehicle, this dilution was taken into account in the calculations. It should also be noted that no correction was incorporated for K^+ content of residual fluid in the cell pellet. As the extracellular space is around 16% and the levels of K^+ in this fluid only $^1/_{20}$th that in the cells, this is a negligible factor.

In some instances K^+ has been determined directly from the weight of the cell pellet - this will be documented with the relevant data. When the cell pellet weight is used for the direct calculation, an allowance for extracellular space must be made. A figure of 15.8% was determined for extracellular space of a pellet of cells from this laboratory by measuring concentrations of ^{14}C sorbitol in cell suspension and an equivalent volume of homogenised cell suspension. Albumin labelled with ^{125}I was also used, giving a figure of 13.5%. The sorbitol figure of 16% was used in the relevant calculations since the difference between the figures is minimal and the sorbitol exclusion is more applicable to smaller molecules such as hydrated K^+ ion. The figures of extracellular space given above compare favourably with most other published values (Berry and Friend, 1969; Krebs *et al.*, 1974; Frimmer *et al.*, 1976). One group of workers has reported a contamination figure, which presumably is an estimate of extracellular space, of only 2% (Inaba *et al.*, 1975).

The point made by Frimmer *et al.* (1976) in relation to the fruitlessness of inclusion of extracellular space factors in calculations where cells are damaged is of particular relevance to this thesis. However, control cell levels require this factor in their calculation to give the most appropriate comparison to *in vivo* and other liver systems, and this is of importance, especially in initial preparations, to justify the hepatocytes biochemically.

It was noted that calculation of K^+ from pellet weight resulted in K^+ levels about 20 µmol/g wet wt higher than when calculated from cell count.

In one experiment where both methods were used, cell count based

calculations gave 71 µmol/g wet wt whereas use of pellet weight gave 95 µmol/g wet wt. Only a few final experiments employed pellet weight based calculations such that the majority of K^+ levels may be lower than the true value. This may question the method of cell counting in this laboratory, or the literature figure of 1.3×10^8 cells/g wet wt used in the calculations.

It should also be noted that such considerations with respect to calculations would also apply to other parameters assayed in the hepatocytes. These would include urea formation, aminopyrine metabolism, and the levels of GSH and ATP.

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