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REPORT NO: CTL/R/1091

# COMPARATIVE TOXICOLOGY RESEARCH REVIEW, 1991

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Date of Issue: 12. 11. 1991

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## SUMMARY

The Comparative Toxicology Research Project is dominated by problem solving research programmes on ICI products or development chemicals of commercial value to ICI. The majority of the work described in this report has been directly funded by the appropriate business, and as such is often reported directly to them. The scope of projects, their size and complexity makes it challenging to pull them all together in a cohesive report, but broadly the work can be divided into two categories.

One comparative toxicology where an acute or subacute toxicological response is observed which appears to have some species specificity. We then try and identify the biochemical basis for this toxic response and endeavour to establish its relevance to man. Current programmes in this area include the production of hepatic porphyria in the mouse with tralkoxydim, the occurrence of corneal lesions in rats and dogs with various triketones and the production of renal damage in the rat with p-aminophenol. In cases where there is no obvious species difference in toxicity, an alternative strategy of formulating the product in such a way as to markedly reduce its toxicity has been undertaken, see the report on paraquat.

The second more complex area is investigating comparative carcinogenesis issues, in this area the team has considerable expertise in mechanisms of non-genotoxic carcinogenesis. As with the former area the approach adopted is to endeavour to identify key events (biochemical and pathological) that lead to the carcinogenic process and then to determine whether these occur in non responsive species and whether or not they are likely to be relevant to man. Current programmes in this area include rodent specific induction of liver tumours following methylene chloride, certain triketones, and products known as safeners which are put in herbicide formulations. In addition the production of rat specific nasal tumours with acetochlor and liver, kidney and thyroid tumours with chlorinated paraffins.

For convenience a summary of the progress to date has been arranged under these two areas. The individuals involved are named under each separate sub-section of the report.

## Comparative Toxicology

Tralkoxydim is a potent porphyrinogenic agent in mice. However, the insensitivity of several other species of experimental animals to this hepatic porphyria questions the relevance of this toxicity to man. The remit of the tralkoxydim research programme is to evaluate the potential of tralkoxydim to cause porphyria in exposed humans.

A key objective in achieving this aim, has been the elucidation of the mechanism by which tralkoxydim causes porphyria in mice. Previous data has led to the proposals that the porphyrinogenicity of the tralkoxydim molecule is due to the way in which it is metabolised in the mouse liver and that the species differences in porphyria are due to species differences in this metabolic pathway. Recent studies have confirmed that this metabolic pathway does exist in mice. In the mouse liver tralkoxydim has been shown to be metabolised by a mechanism which leads to a specific methyl group - the terminal methyl group of the C-methyl moiety - being lost and incorporated into haem. This methyl group becomes covalently bound to the pyrrole nitrogens of haem and is not incorporated into the porphyrin macrocycle. Two desmethyl metabolites of tralkoxydim have also been identified. The haem which is methylated by tralkoxydimn is believed to be that of cytochrome P-450, by analogy with other agents which elicit the production of N-alkylated haems in the liver. Whilst the majority of mouse liver cytochrome P-450 isoenzymes have now been shown to be unaffected by tralkoxydim, a marked inhibition of the microsomal metabolism of testosterone to androstenedione has been discovered. The significance of this is currently being evaluated.

The mechanism elucidated so far has implicated species differences in metabolism of tralkoxydim as being responsible for the species differences in porphyria, and comparison of this toxic metabolic pathway will provide a reliable means of confirming this hypothesis, and may also provide an alternative, non-invasive, means of investigating the porphyrinogenicity of tralkoxydim in exposed humans.

An assessment of the porphyrinogenic activity of tralkoxydim in man has been made possible by the development of an in vitro model. Using inhibition of ferrochelatase as an end point, it can be concluded that tralkoxydim is unlikely to cause hepatic porphyria in man. However, the inhibition of ferrochelatase in hepatocytes, as in the liver should be associated with accumulation of porphyrin and recent studies have been directed towards understanding the basis for the difference in porphyrinogenic response due to the dihydropyridines between human and rat or mouse hepatocytes in culture.

Significant differences in the behaviour of aminolaevulinic acid synthetase, the rate limiting enzyme of the haem biosynthetic pathway, between human and mouse hepatocytes in culture have been identified and could well account for the differences in porphyrinogenic response. Further progress in this area is likely to be dictated by the availability of human liver samples.

The triketones are a novel series of herbicides which have been shown to produce corneal injury in the rat and with some analogues an increased incidence of liver tumours in rodents (see later). The aim of the ocular programme has been to try and understand the mechanism whereby the model triketone SC-0735 produces corneal injury in the rat. This may then enable us to understand the basis for the species difference in susceptibility and hence the likely response of man. The dog and rat develop eye lesions following continuous administration while the mouse, rabbit and rhesus monkey do not. The triketone SC-0735 is a potent inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) which is responsible for the conversion of 4-hydroxyphenylpyruvate to homogentisic acid in the catabolism of tyrosine. Inhibition of this enzyme located primarily in the liver and kidney leads to a build up of 4-hydroxyphenylpyruvate and in particular its precursor tyrosine. An elevation in tyrosine in plasma leads to a concomitant increase in tyrosine in ocular fluid. We now know that a sustained elevation in plasma tyrosine of greater than 1000nmol/ml at the end of six weeks exposure in rats leads to the onset of corneal injury, whereas at values below this no corneal lesions are seen. Recent work has focused on the kinetics of inhibition of HPPD by various

triketones, development of a mathematic model of the triketone-induced eye lesions, using our experimental database. Studies on the toxicity of tyrosine to the eye and the use of SC-0735 for the treatment of tyrosinaemia type I in man.

Enzyme kinetics have established that, for a series of triketones the mechanism of inhibition of HPPD is similar. Differences in the kinetics of inhibition, across the series, is governed by the rate of dissociation of the chemical from the enzyme. Analysis of kinetic data and the use of molecular modelling suggests a two-stage mechanism of inhibition involving the initial formation of an E-I complex that undergoes rearrangement to a secondary complex ($E\text{-}I^*$). The rearrangement is postulated to result from a pseudorotation of the triketone bound to the iron at the active site and is driven by conformational changes and/or favourable binding interactions between the inhibitor and the protein. The stability of the $E\text{-}I^*$ complex is dependent upon the magnitude of these binding interactions that effect the rate of dissociation of the inhibitor. Upon dissociation the enzyme is released in an active form.

Mathematical modelling using the experimental database has helped develop our scientific understanding of how triketones cause eye lesions and to apply this knowledge to predict the likelihood of harmful effects in man (ie risk assessment). We now believe the overall toxic effect is the result of several factors: (i) The pharmacokinetics of the triketone molecule. (ii) Its pharmacodynamics - ie its inhibitory effect on the enzyme HPPD. (iii) The consequences of this upon tyrosine kinetics - leading to elevated plasma tyrosine. (iv) The effect of this tyrosinaemia on the eye. A mathematical model has been developed that describes these factors for the triketone SC-0735, the effects of tyrosine in the eye were not modelled, as the mechanism by which tyrosine causes eye lesions is not known. The model brings together a large database of pharmacokinetic, toxicity and <u>in vitro</u> data from our own studies, and from the scientific literature and shows that elevation of plasma tyrosine is a necessary precondition for the development of eye lesions.

The model shows which factors are likely to be important in explaining the differences in toxicity observed between different triketones and in different species. It also supports the hypothesis that the major difference between responding and non-responding species is in their ability to eliminate excess tyrosine by routes other than through HPPD. To date a quantitative description of tyrosine kinetics has been developed for one species, the rat, this is a springboard for thinking about tyrosine kinetics in man, and the data that would be required to decide whether man is likely to develop sustained elevations of tyrosine and eye lesions in response to triketone exposure. This work represents a substantial development in our experience with mathematical modelling. For the first time we have used pharmacokinetic and pharmacodynamic data together, to predict a toxic effect - in this case tyrosinaemia.

In vitro studies using either red blood cells or a $3T_3$ cell line in culture has shown that tyrosine crystals can cause haemolysis or cytotoxicity respectively in these systems. Tyrosine in true solution was inactive. These findings add further support to the suggestion(s) in the literature that it is formation of tyrosine crystals, occurring at the saturating concentrations in ocular fluid that are likely to form the basis for the cytotoxicity.

Previously studies have shown that the rate of tyrosine elimination from the body is is a critical factor in determining the extent of tyrosinaemia. For example, the mouse is able to clear tyrosine from the plasma and keep the concentration below 1000nmol/ml and does not develop eye lesions. The opportunity to use SC-0735 in man to treat a rare inborn error in metabolism also enabled us to assess the extent of tyrosinaemia produced in man at doses of SC-0735 that were designed to completely inhibit HPPD. After treatment the degree of tyrosinaemia was mild with plasma values raised to 500-600nmol/ml below the known threshold for ocular lesions in man. Ocular toxicity was not seen in the five patients treated. Inhibition of HPPD in these patients reversed the clinical chemical changes caused by inborn error of metabolism and their general clinical condition improved with no signs of side effects to date.

These findings on the therapeutic use of SC-0735, plus data available in the literature on inborn errors of tyrosine metabolism in man form the basis of our case that inhibition of HPPD in man will not produce a marked or sustained enough tyrosinaemia that will cause ocular lesions. In this regard we believe man more closely resembles the rhesus monkey, rabbit and mouse. Work on the ocular toxicity of the triketones has now stopped having developed a position with regard to the relevance of this finding to man.

4-Aminophenol (p-aminophenol, PAP) causes selective necrosis to the pars recta of the proximal tubule in Fischer 344 rats. The basis for this selective toxicity is not known, but PAP can undergo oxidation in a variety of systems to form the 4-aminophenoxy free radical. Oxidation or disproportionation of this radical will form 1,4-benzoquinoneimine which can covalently bind to tissue macromolecules. Recent studies have shown that certain benzoquinol-glutathione conjugates can cause renal necrosis in rats. We have synthesized a putative glutathione conjugate of PAP. The effect on the kidney of this conjugate and the sulphate and N-acetyl conjugates, known metabolites of PAP, have been examined in Fischer 344 rats. 4-Amino-3-S-glutathionylphenol produced a dose-dependent (92-920$\mu$mol/kg) necrosis of the proximal tubular epithelium and altered renal excretory function. The lesion at the low dose was restricted to the pars recta of the proximal tubule in the medullary rays, while at the higher doses it affected the pars recta region of all nephrons. In contrast, PAP-O-sulphate and N-acetyl-4-aminophenol (paracetamol) caused no histological or functional alteration to the kidney at 920$\mu$mol/kg. The renal necrosis produced by 4-amino-3-S-glutathionylphenol was very similar to that produced by PAP (367-920$\mu$mol/kg), both functionally and histologically, except that smaller doses of the glutathione conjugate were required. These studies indicate that glutathione conjugation of PAP generates a metabolite that is more toxic to the kidney than the parent compound.

Studies with various modifiers of xenobiotic metabolism have shown that prior treatment of rats with acivicin, a potent inhibitor of $\gamma$-glutamyltranspeptidase protects against the nephrotoxicity produced

by PAP-GSH. In addition, co-administration of ascorbic acid with either PAP or PAP-GSH affords protection. These studies support findings by others that PAP requires oxidation to form 1,4-benzoquinoneimine which then readily covalently binds to proteins. In addition, we suggest that a possible mechanism of toxicity could involve glutathione conjugate formation, which analogous to that reported for glutathione conjugates of certain quinones, could involve oxidation to form a 1,4-benzoquinoneimine thioether that may covalently bind to protein and/or redox cycle and lead to cytotoxicity.

During 1991, effort has been devoted towards optimizing a new less toxic formulation of paraquat. A formulation known unofficially as MAGNOXONE has been patented by CTL. This formulation is based on the product GRAMOXONE but contains additives with known pharmacological properties. The primary additive in MAGNOXONE is magnesium trisilicate, an antacid which gels on contact with gastric acid. Gelling traps paraquat and slows gastric emptying. An emetic is also added to remove this gel. Finally, a purgative, magnesium sulphate is present which has a synergistic effect on emesis plus an osmotic cathartic effect on the small intestine to rapidly remove non-absorbed paraquat. MAGNOXONE has been found to have identical herbicidal efficacy to GRAMOXONE in extensive Field trials. In animals there are marked differences in oral toxicity between the two formulations. A dose of 12mg/kg paraquat as GRAMOXONE is lethal to dogs. Doses of 32, 64 and 128mg/kg paraquat as MAGNOXONE are tolerated without any signs of paraquat toxicity. The use of magnesium salts in MAGNOXONE was based on studies carried out on the mechanism of paraquat absorption. Paraquat is principally absorbed in the small intestine by an energy dependent, saturable process which can be stimulated with luminal calcium or inhibited with calcium chelators or magnesium. We have explored the effect of parenteral magnesium sulphate on target cell toxicity of paraquat in the rat. A dose of 50mg/kg magnesium sulphate i.p. following a lethal dose of paraquat reduced concentration of paraquat in lung from 18.6 ± 0.3 to 2.1 ±0.8$\mu$g/g at 15min and kidney 12.7 ± 1.1 to 5.44 ± 0.3$\mu$g/g at 24hours. Plasma clearance of creatinine and urea nitrogen were also normal following i.p. magnesium. In separate ten day studies in rats magnesium sulphate (50-300mg/kg i.p) was an effective antidote to paraquat poisoning. It is

suggested that the calcium-dependent paraquat uptake into the enterocyte, a process blocked by magnesium, may occur beyond the level of the gastrointestinal tract in specific cells in the lung and kidney. Further work using in vitro preparations from these tissues would more clearly address this hypothesis.

## Comparative Carcinogenesis

R29148, R29187 and dichlormid are dichloracetamide compounds used as 'safeners' in herbicide formulations. Although the results of short term studies suggested that the safeners did not present any significant toxicological concerns, a recent bioassay with R29148 concluded it to be an hepatocarcinogen in rats. The objectives of the safener research programme are (1) to elucidate the mechanism by which R29148 is hepatocarcinogenic and (2) to identify early biochemical or morphological markers of this toxicity which can be used to evaluate and discriminate the potential carcinogenicity of other dichloracetamide compounds.

Although R29148 is not overtly hepatotoxic, it does produce a number of changes in liver biochemistry at high doses. Several of these changes, including elevation of non-protein sulphydryl and induction of $\gamma$-glutamyl transpeptidase and glutathione S-transferase (GS-t) activities appear to be functionally related. Experimental pathology studies have added support to this idea by demonstrating that each of these effects is localised in the liver, being restricted to the zone 1 hepatocytes. It is proposed that conjugation of R29148 with glutathione in the liver may be the critical event which relates these hepatic changes together. This conjugation reaction is likely to be catalysed by cytosolic GS-ts and R29148 has been shown to produce marked changes in the profile of GS-t isoenzymes in the liver. A comparison of the effects of R29187 and dichlormid with R29148 have so far revealed remarkably similar changes in the liver, suggesting that this toxicity is imparted by the dichloracetamide moiety, a structural feature common to all three compounds. R29148 is non-genotoxic. Current studies are aimed at evaluating the significance of the various biochemical changes seen in short term studies, in the development of liver tumours. Experimental

pathology studies will be used to identify foci of phenotypically altered hepatocytes, an interim stage in the development of tumours, after long term administration of R29148. Phenotypic characterisation of these foci may enable a relationship between the short term effects and neoplastic transformation to be identified.

Acetochlor is a chloroacetanilide pre-emergence herbicide active against grasses, sedge and broadleaf weeds in corn. A number of chronic studies have demonstrated an oncogenic potential in rodents with a variety of targets dependent upon dose and species. Of the cancers observed, tumours in the nasal turbinates of rats were considered to be the most significant finding. These tumours, which are mainly polypoid adenomas, were not seen in mice. Species differences in response to nasal toxins or carcinogens are not uncommon and it is generally believed that the rat is a poor model for man for this type of effect. Several such chemicals have now been studied in detail and considerable information is available about the capacity of nasal tissues to activate chemicals. Thus, not only is there a clear species difference in laboratory animals in response to acetochlor, it is also in an area which is now amenable to experimental investigation.

Administration of acetochlor to male rats either by daily gavage or in the diet failed to have any consistent or reproducible effect on nasal morphology. The dietary concentration was 2000ppm (10 days), the highest single gavage dose was 2000mg/kg, and in repeat dose studies 1000mg/kg was given for up to 30 days. A number of putative metabolites, including 2-ethyl-6-methylaniline, were also dosed to rats without effect on the nasal tissues. The highest dose of 2-ethyl-6-methylaniline was 500mg/kg for 20 days. Whole body autoradiography and covalent binding studies failed to detect any selective accumulation or localisation of radioactivity in nasal tissues following either a single or repeated dose. The pharmacokinetics of acetochlor and its metabolites in blood following administration of C-14 acetochlor revealed that most of the radioactivity in blood was bound to the red blood cell. Acetochlor itself could not be detected in blood. It is concluded that extensive first pass metabolism of acetochlor in the liver and/or binding of metabolites to red blood

cells protect the nasal tissues from exposure to acetochlor or its metabolites in the short-term. Studies of the in vitro metabolism of acetochlor in hepatic and nasal tissue fractions suggest that nasal tumours seen in rats may be due to the metabolism of acetochlor to formaldehyde.

Previous studies in CTL had offered an explanation for the lung and liver tumours produced in mice, but not in rats or hamsters exposed to methylene chloride. The explanation was based on a species differences in pharmacokinetics of methylene chloride which correlated with the reported cancer incidences. Metabolism by the glutathione S-transferase pathway correlating well with the tumour incidence while metabolism by the alternative pathway involving cytochrome P-450 did not. The precise mechanism whereby methylene chloride is carcinogenic to the mouse has not been resolved. Recent studies have shown that the isoenzyme responsible for the metabolism of methylene chloride is glutathione S-transferase 5-5. This, plus recent publications showing that glutathione S-transferase(s) including 5-5 are present in the nucleus where they are thought to be involved in the protection or function of the genome, stimulated further mechanistic studies. The hypothesis are (1) methylene chloride interferes with the normal function of nuclear transferases or (2) nuclear metabolism of methylene chloride has lead to the interaction of a metabolite with the genome. Studies are underway to see whether nuclear metabolism of methylene chloride occurs in mouse liver and to isolate and characterise the glutathione S-transferase(s) responsible.

Comparison of the alkaline unwinding and alkaline elution assays for the detection of DNA single strand breakage (SSB) revealed that the elution method appeared to be the more sensitive assay in mouse liver cells. Methylene chloride (DCM) induced DNA-SSB in hepatic cells from $B_6C_3F_1$ mice following single oral administration. In contrast, no effects were observed in liver DNA from Fischer F344 rats after oral dosing of DCM. Liver DNA from mice exposed to DCM by inhalation for 6h showed a small statistically significant increase in SSB compared with the concurrent control but not with the aggregated controls; longer periods of exposure (6x6h and 65x6h) produced no detectable increases in SSB. Exposure of cultured human lymphocytes to DCM resulted in significantly increased

frequencies of cells with chromosomal aberrations. Since it has been shown that DCM does not bind covalently to DNA and does not induce DNA repair (UDS), the above observations suggest that DCM, or a metabolite, may interact with chromatin rather than with DNA itself and hence induce SSB and chromosomal aberrations by a mechanism involving an indirect interaction with DNA. Although methylene chloride is metabolised to formaldehyde, experiments both in vivo and in vitro failed to detect an increase in DNA-protein crosslinking in mouse liver. In the search for the glutathione-S-transferase responsible for the metabolism of methylene chloride in mouse liver, a number of proteins have been isolated which will shortly be sequenced and further characterised.

Carcinogenicity studies with certain chlorinated paraffins have shown that they produce an increased tumour incidence in the liver thyroid and kidney of rats. Studies have been designed to try and identify the early events taking place in these target organs following exposure.

Male and female Fischer 344 rats were gavaged daily for 90 days with 312 or 625mg/kg of Chlorowax 500C (short chain CP with 58% chlorination) or Chlorparaffin 40G (medium chain CP with 40% chlorination) in corn oil. The liver, kidney, and thyroid were investigated using a combination of approaches involving pathology, immunohistochemistry and biochemistry. Our findings in this study, in conjunction with earlier studies has allowed the following hypothesis to be proposed. (1) Short chain CPs, such as Chlorowax 500C, are probably producing liver tumours due to their induction of hepatic peroxisome proliferation and stimulation of replicative DNA synthesis. (2) Both the short and medium chain CP appear to have the potential to induce kidney neoplasia in the male rat only. There was a small $\alpha$2u stimulation, but, more importantly, a sustained increase in replicative DNA synthesis, probably due to a reparative process. (3) The thyroid tumours observed in the 2 year bioassay, with a short chain CP, are probably due to the induction of $T_4$ glucuronidation in the liver and hence increased clearance of $T_4$ from the plasma. The fall in $T_4$ plasma levels induced synthesis of TSH in order to return hormone levels to normal, causing hypertrophy and hyperplasia of the thyroid, which was then maintained throughout the study.

The objectives of the triketone liver project are to establish a mechanism for the differential nongenotoxic hepatocarcinogenicity of triketones SC0774 and ICIA0051 and to predict the carcinogenic potential of future development triketones (eg. ICIA0179 and its bisenamine and Cu-chelate complexes, R-250936 and SC0456). The hepatocarcinogenicity of SC0774 in mice and rats is thought to result from enhancement of liver growth (SER proliferation and centrilobular hypertrophy) and phenotypic change demonstrated by induction of cytochrome P-450 IIB1/2 and IIIA1. These changes did not occur with ICIA0051. Other compounds such as phenobarbitone and cyproterone acetate, which are major inducers of cytochrome P-450s IIB1/2 and IIIA1 respectively, also stimulate liver growth and cause increased incidences of liver tumours. ICIA0179 also induced liver growth and cytochrome P-450 IIIA1 in the mouse and is therefore thought to have hepatocarcinogenic potential in this species, although the effect was less pronounced than with SC0774. In the rat only limited liver growth occurred with ICIA0179 but cytochrome P-450 IA1 was strongly induced. Cytochrome P-450 IA1 is regulated by the Ah receptor which is known to be associated with carcinogenesis, although the potency of ICIA0179 in eliciting this response compared with the classical planar polyclic aromatic hydrocarbons is unknown. The Cu-chelate of ICIA0179 offered no advantage over ICIA0179 itself in terms of hepatotoxicity although the bisenamine complex did have a more favourable hepatotoxicity profile. R-250936 and SC0456 caused only mild SER proliferation and cytochrome P-450 IIB1/2 and/or IIIA1 induction are therefore not predicted to be hepatocarcinogenic. Cultured hepatocytes were a good model for P-450s IA1 and IIIA1 induced by triketones and appropriate positive controls. They are being used as a primary screen for these compounds and for structure-activity studies.

A further aim of this project is to identify the structural features of a chemical endowing that molecule with the potential to induce one of the major forms of P-450. An understanding of these parameters and an estimate of the potency of induction may allow us to predict which structures may be most likely to be non-genotoxic carcinogens. This characterisation of P-450 induction in primary hepatocytes and comparison with complementary <u>in vivo</u> data for the closely related chemical series

of triketone herbicides, is coincident with Agrochemical Business needs, forming part of a battery of toxicology screens for potential triketone development candidates. Therefore, the triketone series of chemicals forms the basis of this report, but this work may also be viewed as a first step towards assessing the feasibility of the wider goal of accurate prediction of non-genotoxic carcinogens.

The encouraging, initial, results from this work suggest that a planar face of the appropriate size and electronic configuration, of a larger non-planar structure, may be able to induce P450IA1, and that the current literature theories of structure activity relationships in this area could be expanded. Diketone molecules seem to be more potent inducers than equivalent triketone structures, and triketones with ortho nitro substituents, in turn, appear to be more potent than those with chlorine in this position. The possible reasons for these differences are discussed. This work also highlights the usefulness of conformationally restricted molecules in this type of structure activity work, helping define active conformations, and gives us confidence that, at least for the triketone series, we may be laying some foundations which will afford us greater predictive confidence in induction of P4500IA1 and cancer by these chemicals.

# TRALKOXYDIM (PP604) RESEARCH

A M BRADY

## INTRODUCTION

The mechanism of tralkoxydim-induced hepatic porphyria in mice has been largely elucidated. This mechanism provides a rational explanation for the observed species differences in porphyria, where mice appear to be unique in their sensitivity to this hepatotoxicity. The data obtained so far supports the proposal that the porphyrinogenicity of the tralkoxydim molecule is due to the way in which it is metabolised in the mouse liver and that the species differences in porphyria result from species differences in this metabolic route. The precise details of this metabolic route are unknown at present and our work over the last year has been directed towards resolving this toxic pathway.

In addition, we have previously developed an in vitro model of porphyria using primary cultures of hepatocytes, which has enabled the porphyrinogenic activity of tralkoxydim in man to be determined. In contrast to studies in mouse and rat hepatocytes, however, human hepatocytes exposed to a 'positive control' agent, DDC, failed to accumulate porphyrin. This in vitro modelling forms an important part of the registration database for tralkoxydim and it was considered important to try and resolve or understand the basis for this lack of response. The limited availability of human tissue has impeded studies in this area, but some progress has been possible.

## PROGRESS - MECHANISM OF ACTION

### ORIGIN OF METHYL GROUP

The porphyrinogenic activity of tralkoxydim in mice has been shown to be due to its ability to inhibit the enzyme ferrochelatase in the liver.

Previous studies have demonstrated that tralkoxydim does not inhibit this enzyme directly, but rather through eliciting the production of N-methyl protoporphyrin IX (N-$CH_3$ PPIX) - a previously characterised, potent inhibitor of the enzyme. Using [$^{14}$C]-radiolabelled analogues of tralkoxydim we had identified that the N-$CH_3$ substituent of this porphyrin, which confers its inhibitory activity, originated from the C-ethyl moiety of the tralkoxydim molecule. In order to define the nature of this methyl group transfer from the C-ethyl moiety, two further [$^{14}$C]-radiolabelled analogues were synthesised, in which the internal (analogue II, Figure 1) and terminal (analogue III, Figure 1) methyl groups were labelled. These analogues were administered to mice and the specific activity of the N-$CH_3$ PPIX produced in the liver was determined.

The N-$CH_3$ PPIX isolated following administration of the internal [$^{14}$C-methyl]-labelled analogue (II) was found to contain only traces of radioactivity, confirming that this internal methyl group is not involved in the formation of N-$CH_3$ PPIX. In contrast, the N-$CH_3$ PPIX isolated following administration of the terminal [$^{14}$C-methyl]-labelled analogue (III) was found to contain significant radioactivity. The ratio of tralkoxydim (from $^{14}$C radioactivity) to N-$CH_3$ PPIX present was approximately 1 (0.93) indicating that one molecule of tralkoxydim is utilised per molecule of N-$CH_3$ PPIX formed. This confirms that the terminal methyl group of the C-ethyl moiety alone is transferred during the formation of N-$CH_3$ PPIX by tralkoxydim.

Copper catalysed dealkylation of the $^{14}$C[N-$CH_3$ PPIX] purified from mice dosed with analogue III has been shown to result in the formation of unlabelled protoporphyrin IX, confirming that the methyl group from tralkoxydim is indeed bound to the pyrrole nitrogens of this porphyrin and has not been incorporated into the porphyrin macrocycle.

## METABOLISM OF TRALKOXYDIM

The transfer of a methyl group from tralkoxydim during the formation of N-$CH_3$ PPIX must result in the production of one or more desmethyl metabolites in which this 'active' methyl group is missing. The major

metabolites of tralkoxydim had been identified previously and from their structures it is clear that they play no part in this toxic pathway. Several minor metabolites had also been found and although their structures have not been determined, as they appeared to be unique to the mouse, it seemed likely that they could be the products of this toxic pathway.

We proposed that metabolism of the terminal [$^{14}$C-methyl] analogue of tralkoxydim (analogue III, Figure 1) by this toxic pathway would yield desmethyl metabolites which were non-radioactive. It was forseeable that such non-radioactive metabolites could then be identified by comparison of equivalent metabolite profiles from mice dosed with [$^{14}$C-methyl]phenyl ring-labelled tralkoxydim or the internal [$^{14}$C-methyl]-labelled analogue (analogue II Figure 1).

Following this principle we have confirmed that the minor metabolites found previously do not in fact correspond to desmethyl metabolites and these unidentified, mouse-specific metabolites are, therefore, unlikely to be products of this toxic pathway.

Several methodologies have since been evaluated to enable resolution of further metabolites, the relevance of which have been analysed using the differential labelling principle described above.

Two structurally different desmethyl metabolites have now been found in acetone liver extracts; the first by TLC of lyophylised DEAE cellulose column fractions and the second by HPLC. Effort is currently being employed in defining isolation systems for these desmethyl metabolites in order to purify sufficient material for mass spectrometric characterisation. Once the structures of these metabolites are confirmed, it should be possible to define a rational chemical mechanism for this reaction.

## ORIGIN OF HAEM

The formation of N-$CH_3$ PPIX in the liver by tralkoxydim is a consequence of direct methylation of pre-existing haem. The effects of tralkoxydim on the major haemoprotein pools in the liver - microsomal cytochrome(s) P-450 and $b_5$, the mitochondrial cytochromes and catalase - have been determined, but a clear target pool was not apparent (Figures 2 and 3).

Cytochrome(s) P-450 constitutes the largest of the haem pools in the liver (36% of total) and has been confirmed as the target for other agents which elicit the production of N-alkylated porphyrins eg. dihydropyridines, allylisopropylacetamide and ethynyl-substituted steroids. Although several studies, such as that in Figure 2, have failed to demonstrate any significant loss of total cytochrome P-450, it is unlikely that such gross measurements would detect small changes incurred by selective destruction of a specific isoenzyme, for example. The effects of tralkoxydim on specific isoenzymes of cytochrome P-450 have now been determined by measuring microsomal activities towards isoenzyme-specific substrates. The marker substrates selected are based on the data for rat liver isoenzymes as relevant data on mouse liver isoenzymes was not available.

Following administration of porphyrinogenic doses of tralkoxydim to mice, P-450 dependent metabolism of EROD (IA1), PROD (IIB1) and DMN (IIE1), and site specific metabolism of testosterone to 7$\alpha$-OH (IIA1), 16-$\beta$OH (II B1), 16$\alpha$OH and 2$\alpha$OH (IIC11), 2$\beta$-OH (IIIA1) and 6$\beta$-OH (IIIA2) testosterone were unchanged (Figure 4). The only microsomal activity which was significantly affected by tralkoxydim was the metabolism of testosterone to androstenedione (Figure 4). The metabolism of testosterone to androstenedione was rapidly inhibited (within 1hr of dosing), was dose-dependent and was sustained for the duration of the study (8hr) (Figure 5). This short time course was chosen for study because N-$CH_3$ PPIX has been shown to accumulate in the liver within 1hr of dosing.

Testosterone is metabolised by several isoenzymes of cytochrome P-450, but the major products differ significantly and are considered characteristic of the isoenzyme (see Figure 4). In control mouse liver

microsomes the metabolism of testosterone to androstenedione was significant, accounting for 26% of the total metabolism and androstenedione was the second most abundant metabolite produced.

Despite the observation that androstenedione has been shown to be a product of testosterone metabolism for several purified rat liver P-450 isoenzymes, whether this apparently simply oxidation reaction (Figure 5) is in fact cytochrome P-450-dependent is questionable. Further studies are in progress to determine whether cytochrome P-450 or another microsomal haemoprotein is involved, which may clarify whether this marked inhibition is associated with the target haem pool from which N-$CH_3$ PPIX is derived.

## PROGRESS - IN VITRO MODEL

The dihydropyridines DDC and E-DDC have been shown to inhibit ferrochelatase activity in mouse, rat, hamster and human hepatocytes. Although in cultures of mouse, rat and hamster hepatocytes this inhibition was accompanied by a marked accumulation of porphyrin, studies in human hepatocytes have consistently demonstrated a lack of porphyrin accumulation, even though ferrochelatase is markedly inhibited.

Previous studies have evaluated the competency of human hepatocytes in synthesising porphyrins by adding a biosynthetic intermediate - 5-aminolaevulinic acid (ALA) - to the culture medium. Such studies have repeatedly demonstrated that addition of ALA to human hepatocytes in culture results in a rapid and marked accumulation of porphyrin. These studies with ALA imply that all of the enzymes of the haem biosynthetic pathway, with the exception of ALA synthetase itself, are indeed functional in human hepatocytes in culture. However, as ALA synthetase is both the first, and the rate-limiting enzyme of the haem biosynthetic pathway, its activity must be demonstrated before the haem biosynthetic pathway can be deemed fully competent.

The activity of ALA synthetase and its inducibility by E-DDC have been compared in primary cultures of mouse and human hepatocytes. In mouse

hepatocytes ALA synthetase activity was found to decline steadily over the 4 day culture period but it was rapidly induced by exposure to 25$\mu$M E-DDC and this induced activity was maintained for the duration of the exposure period (Figure 6). A significant increase in porphyrin was also noted.

In isolated human hepatocytes the activity of ALA synthetase was only one third of that in isolated mouse hepatocytes and it increased significantly with time in culture (Figure 7). Exposure to 25$\mu$M E-DDC had no effect on ALA synthetase activity in human hepatocytes over the first 60hr of exposure but a small significant increase was noted after 84 hours (Figure 7). As in previous experiments, exposure of human hepatocytes to E-DDC did not result in accumulation of porphyrin (Figure 7).

Thus, although the enzymatic activity of ALA synthetase is not remarkably different in isolated mouse and human hepatocytes, the development of this activity with time in culture differs considerably. It will be important to differentiate whether the steady increase in ALA synthetase activity in human hepatocytes is due to induction or depression - the latter perhaps offering a rational explanation for its lack of inducibility by compounds such as E-DDC.

## FUTURE WORK

1. The desmethyl metabolites which have been found will be isolated and identified.

2. The above information will be used to define a chemical mechanism for this reaction.

3. This toxic metabolic pathway will be compared in mice, rats and hamsters and also in human hepatocytes.

4. The significance of inhibition of testosterone metabolism to androstenedione will be determined by:

   i) comparing effects in rat liver, and
   ii) determining whether this reaction is cytochrome P-450 dependent.

5. Studies will be carried out on further human hepatocyte cultures to determine the activity of ALA synthetase and its inducibility by E-DDC and other agents.

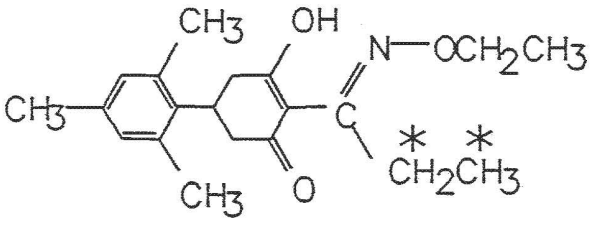
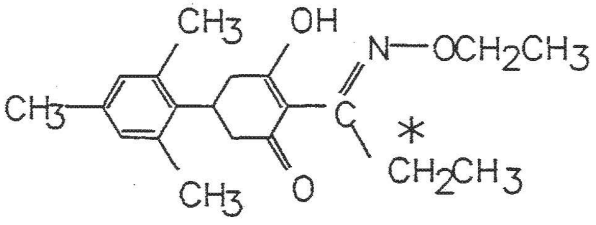
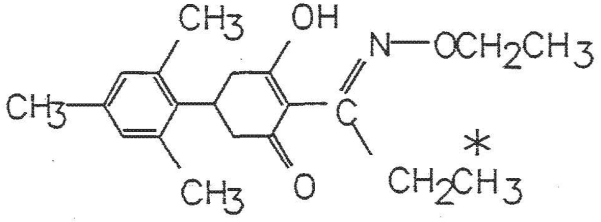
## CONTRIBUTORS

A Brady
B Elcombe
R Hasmall
M Prout
A Gledhill

## RESOURCES 1991

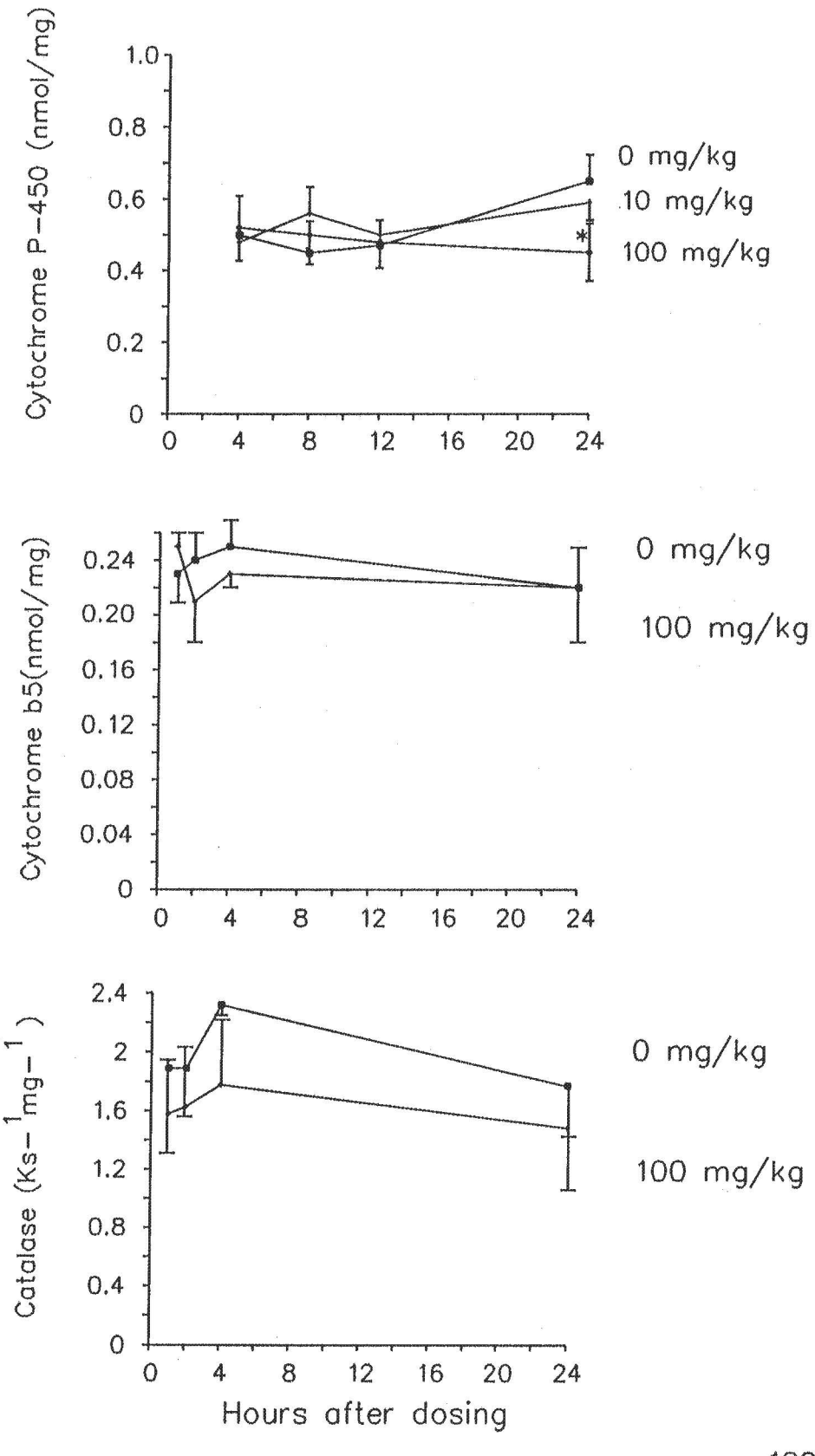
Corporate : 1.5 man-years
Agrochemical : 2.0 man-years

FIG 1.[14-C] labelling of N-methyl PPIX following administration of different radiolabelled forms of tralkoxydim

| Analogue | Position of [14-C]-label | $N-CH_3$ PPIX nmol (A) | 14C-radioactivity nmol tralkoxydim (B) | B/A RATIO |
|---|---|---|---|---|
| I |  | 15.96 | 11.91 | 0.75 |
| II |  | 9.84 | 0.056 | 0.006 |
| III |  | 9.60 | 8.95 | 0.93 |

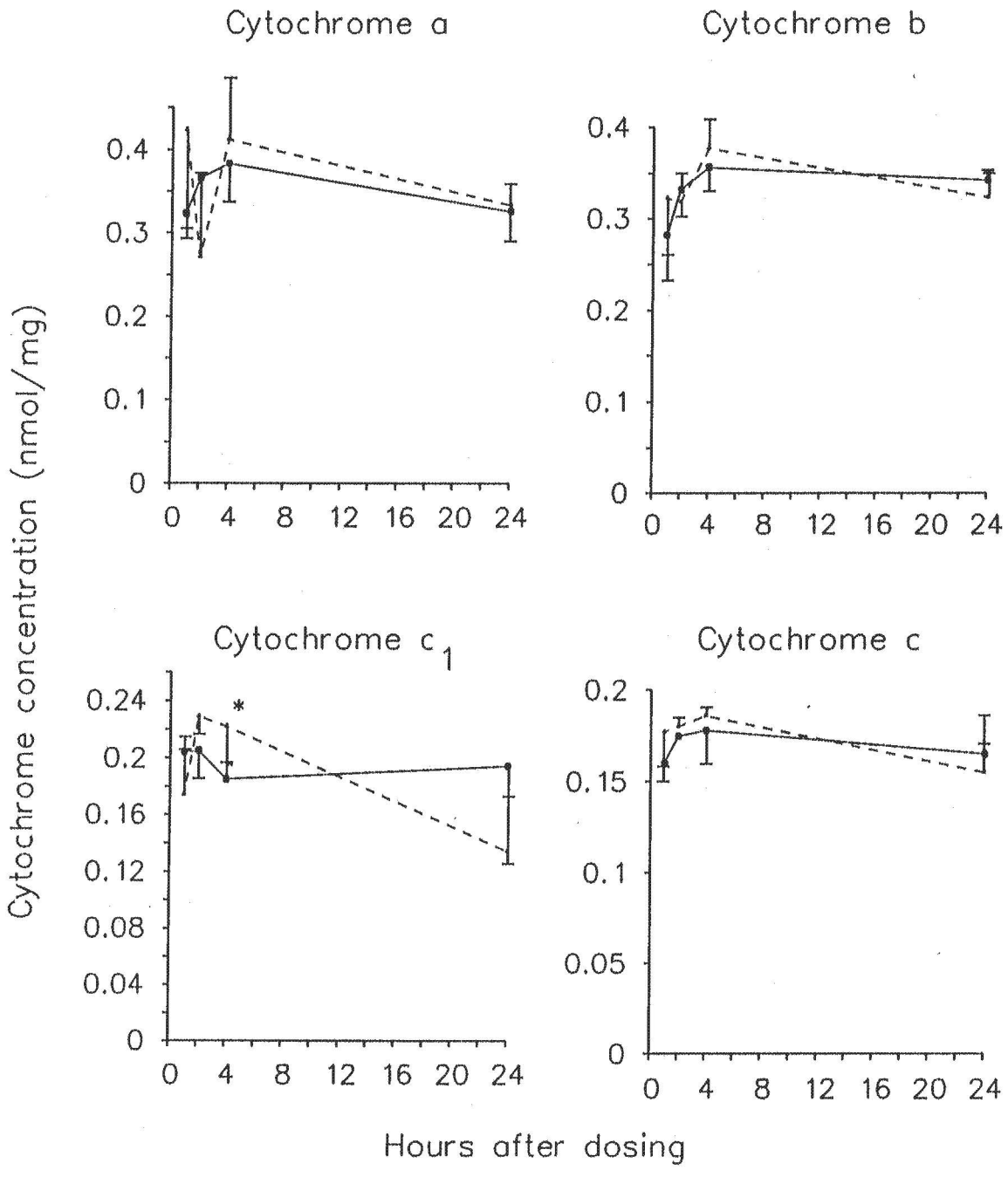
0291(1)

FIG 2.Effects of tralkoxydim on hepatic haemoproteins




1091()

FIG 3.Effects of tralkoxydim on mouse liver mitochondrial cytochromes




1090(8)

Fig 4 Effect of tralkoxydim on mouse liver cytochrome P-450 isoenzyme activities

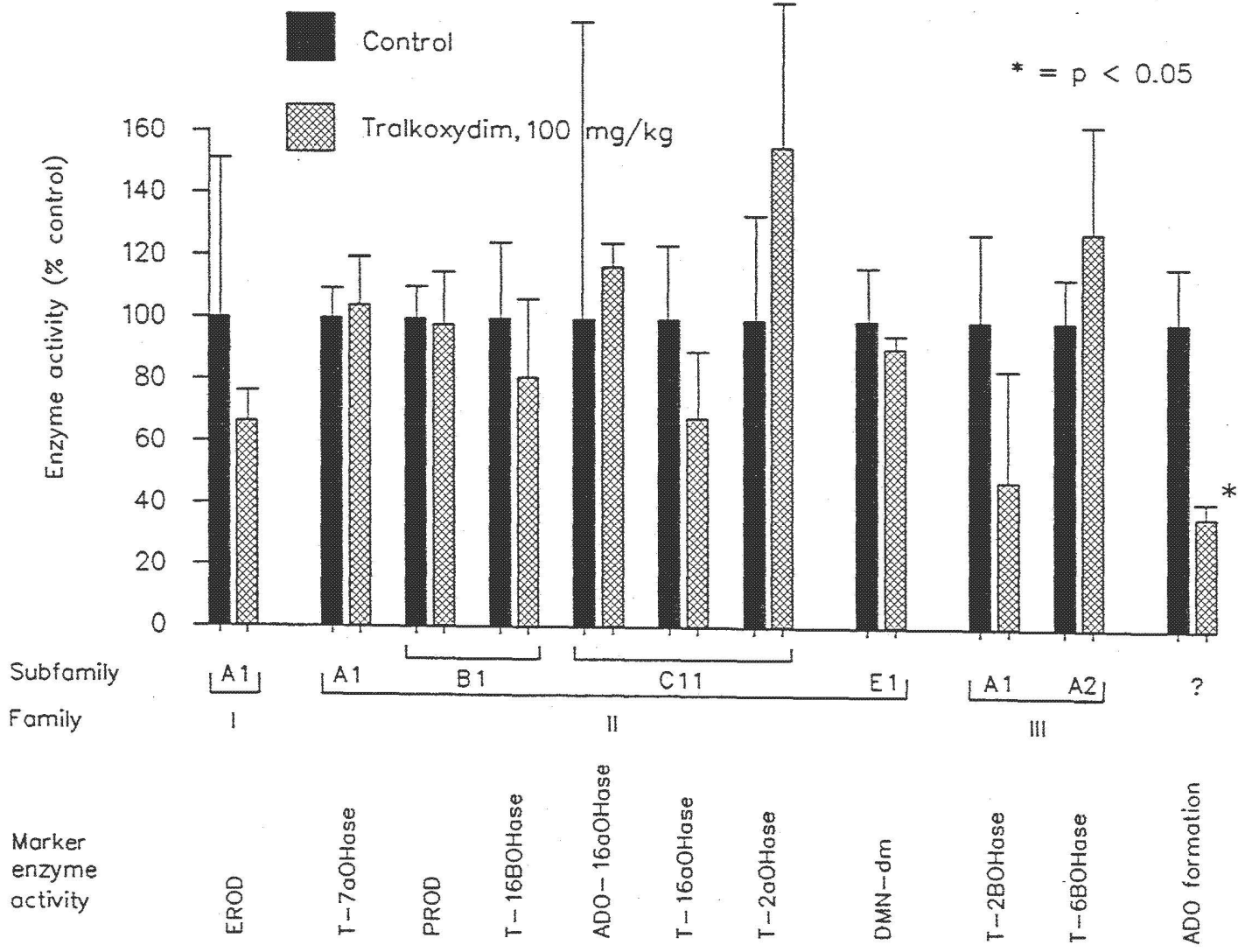
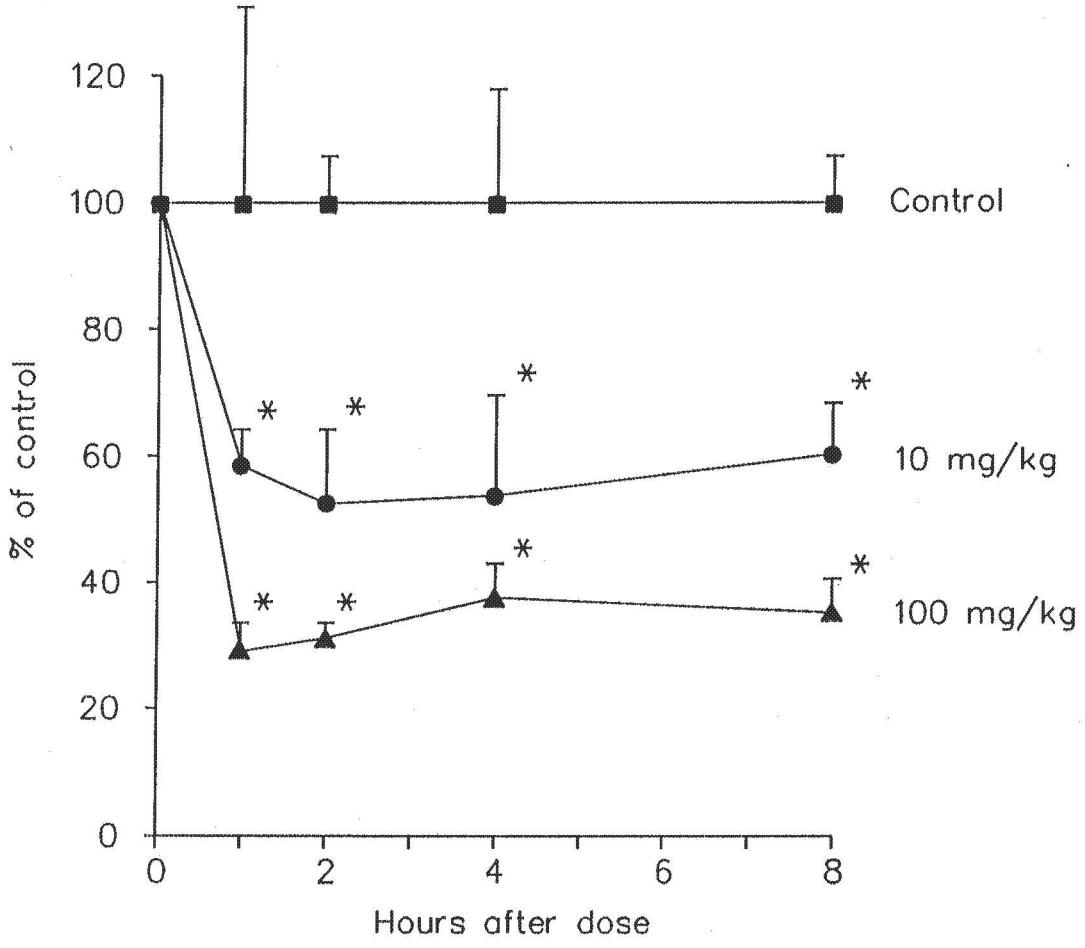
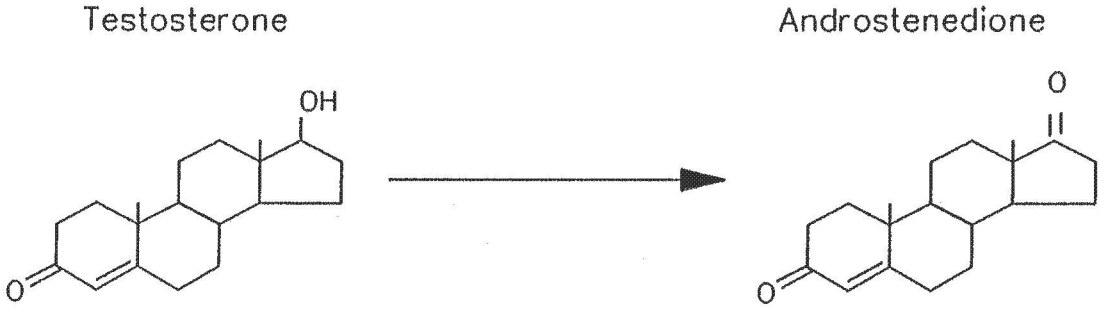


Fig 5 The effect of tralkoxydim on the metabolism testosterone to androstenedione by mouse liver microsomes




1091(5)

FIG 6. ALA synthetase activity and accumulation of porphyrin in mouse hepatocyte cultures exposed to E-DDC.

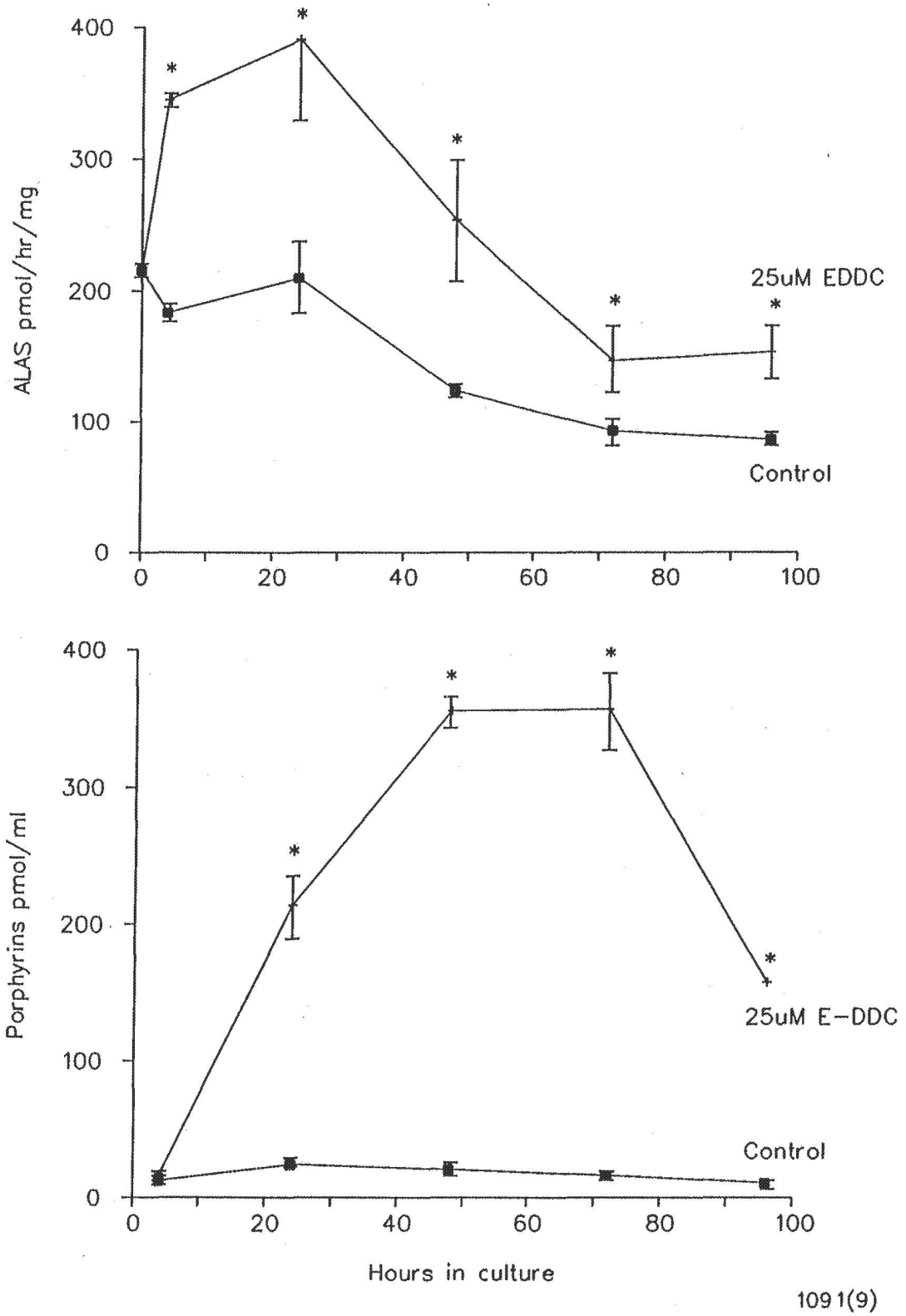
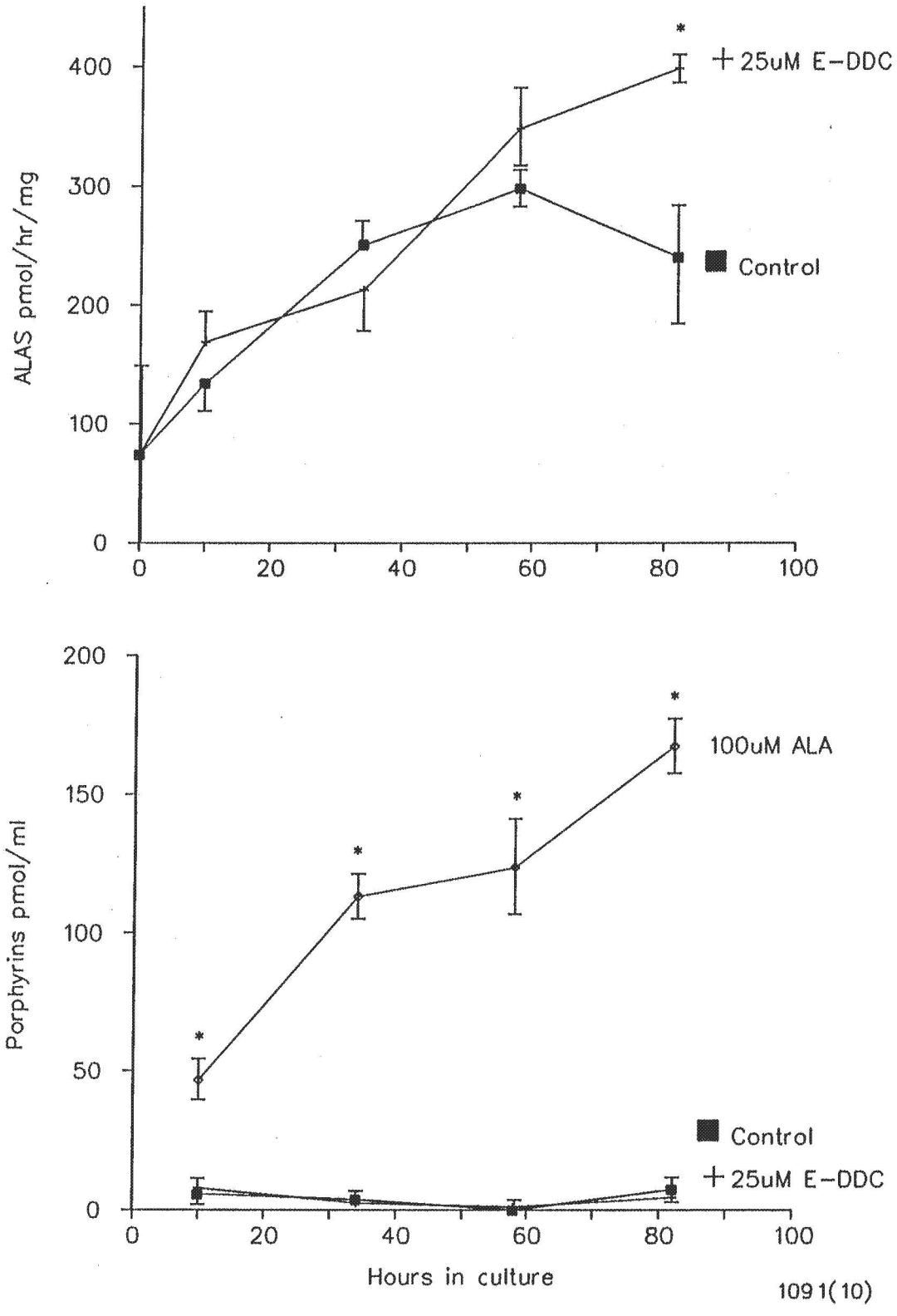


FIG 7. ALA synthetase activity and accumulation of porphyrin in human hepatocyte cultures exposed to E-DDC.



# MECHANISM OF INHIBITION OF 4-HYDROXYPHENYLPYRUVATE DIOXYGENASE BY THE TRIKETONE HERBICIDES

M K ELLIS, L GOWANS, P PHILLIPS AND A MULHOLLAND

## INTRODUCTION

Our aim has continued to be targeted towards understanding the mechanism of inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD) by the triketones. This has been focused on establishing whether, following the inactivation of HPPD by a series of tight-binding triketones, the process is reversible. The findings have established that the triketone family of herbicides inhibit HPPD by a similar mechanism and that differences in the kinetics is governed by the rate of dissociation of the chemical from the enzyme-inhibitor complex.

To aid the understanding of how the triketones interact with HPPD, molecular modelling techniques have been adopted to elucidate possible modes of binding of the triketones to the enzyme. A hypothesis for the mechanism of inhibition of HPPD by the triketones is discussed.

Polyclonal antibodies have been raised against purified rat HPPD in the rabbit. These antibodies were employed to determine the presence of HPPD in the liver, kidneys, ocular humour and the whole eyes of rats and the livers of mice, dogs and rabbits. The possibility that isoforms of this enzyme may exist in all four species is discussed.

## MECHANISM OF INHIBITION OF RAT HPPD BY THE TRIKETONES

Kinetic analyses have established that the triketones inhibit HPPD by one of three mechanisms:

1. There are those triketones that are competitive inhibitors of the enzyme.

2. Triketones that bind to the enzyme and slowly dissociate from the enzyme-inhibitor complex to attain an equilibrium rate of enzyme activity, termed slow-binders.

3. Triketones that bind to the enzyme and are presumed to dissociate from the enzyme-inhibitor complex extremely slowly, termed tight-binders.

Those triketones that have an acceptable herbicidal efficacy are, to date, potent tight-binding inhibitors of rat HPPD (Smith et al, 1989). Their kinetics of inhibition is dominated by a rapid inactivation of the enzyme. Attempts to isolate the enzyme with triketone bound were unsuccessful leading to the conclusion that a covalently bound enzyme-inhibitor complex is not formed.

To establish that the tight-binding inhibitors do dissociate from the inactive enzyme-inhibitor complex, samples of rat liver cytosol were treated individually with the triketones SC-0735, ICIA0051 and R-90101 (37°C/15min). Once it had been determined that HPPD is completely inactivated in these cytosols, the unbound inhibitor was removed by dialysis (0°C, 1h) into 0.2M Tris/HCl buffer, pH 7.2 (2 litres). To determine the rate of dissociation of the inhibitor from the inactive enzyme-inhibitor complex, the resulting cytosols, containing HPPD with triketone bound, were dialysed (25°C) against 0.2M Tris/HCl buffer pH 7.2 (total, 5 litres) and aliquots removed at time intervals and analysed for HPPD activity. The rate of dissociation of the bound triketone was determined by measuring the rate of decarboxylation of [1-$^{14}$C]-HPPA by the freed enzyme (Figure 1). It was determined that the tight-binding inhibitors SC-0735, ICIA0051 and R-90109 dissociate slowly from the enzyme-inhibitor complex with half-lives of ~63h, 10h and ~63h, respectively. These results are consistent with the hypothesis that the differences in the kinetics expressed by the triketones in their inhibition of HPPD is largely dependent upon the rate at which the chemical dissociates from the enzyme-inhibitor complex. The enzyme, in all studies to date, is released in an active state.

To further substantiate this hypothesis, the effect of temperature upon the kinetics of inhibition was studied. It was demonstrated that those triketones that were slow-binders at 37°C (R-86113, R-81528 and R-99796) expressed a tight-binding kinetics at 25°C (Figure 2). Likewise, R-243356 a competitive inhibitor at 37°C became a slow-binder at 25°C. These observations are consistent with a marked temperature effect upon the rate of dissociation and establishes that the differences in the kinetics expressed by the triketone series of herbicides is dependent upon the rate of dissociation of the chemical from the enzyme-inhibitor complex.

The screening of novel, non-triketone, HPPD inhibitors within CTL has ceased and this service, to the chemists at WRC, is now undertaken by the toxicologists at WRC (Dr L Mutter). The input from CTL is at the advisory level.

## TISSUE DISTRIBUTION OF HPPD

HPPD is present in the livers and kidneys of mammals. Inhibition of HPPD by the triketones causes an elevation in plasma and ocular tyrosine levels that is a pre-requisite for ocular toxicity. Though this pre-requisite exists, it has long been thought that other underlying processes may also be involved. To establish whether the ocular effects of the triketones may, in part, be associated with the inhibition of low levels of HPPD within the eye, the ocular humour and whole eyes of rats were examined to determine the presence of this enzyme in these tissues.

Polyclonal antibodies were raised against purified rat HPPD in rabbits. These antibodies are highly specific towards rat HPPD and were used to determine the presence of this enzyme in ocular tissue. Analyses of rat liver, kidney, ocular humour and whole eye homogenates identified HPPD in the liver and kidney whereas the enzyme was not detected in ocular humour or whole eye (Figure 3). To improve the sensitivity of the assay, a second series of antibodies are being raised against rat HPPD in sheep. These antibodies were to be used in the development of a sandwich ELISA assay. This assay is capable of detecting protein at very low concentrations (typically, nmolar) and should establish the presence of HPPD in the eye.

The polyclonal antibodies cross react with HPPD from other species. Incubation of the antibodies with electrophoretically separated proteins derived from the livers of mice, dogs and rabbits identified HPPD in these tissues (Figure 4).

Analysis of the crude liver homogenates from rats, mice, dogs and rabbits by 2D-electrophoresis established that two major and at least one minor isoform of the enzyme may exist (Figure 5). Though rat HPPD has been purified to homogeneity, isoforms have not previously been reported (Lin et al, 1976). Our observations are consistent with more recent data derived from purified human HPPD where three isoforms have been reported (Lindblad, 1977; Rundgren, 1977a and Rundgren, 1977b). Whether our initial observations are fact or whether the observed different forms are artifacts of the purification procedure remains to be determined. Interestingly, but not yet conclusive, differences in the isoform distribution may also exist across mammalian species.

## MOLECULAR MODELLING

Molecular modelling techniques have been employed to elucidiate how the triketones bind to the active site of HPPD. An acceptable model for the binding must fulfil a number of criteria based upon existing knowledge of the kinetics and SAR for binding of the inhibitors and the postulated mechanism of product formation from substrates. These criteria include:

1) The triketone moiety of the inhibitor is postulated to mimic the $\alpha$-keto acid functionality of the substrate (HPPA). Can we rationalise this?

2) Substituents on the aromatic ring of the triketone strongly influence the binding characteristics of the inhibitor. Can these effects be accounted for in the model?

3) To date, no triketone tested against HPPD is a substrate for the enzyme. Is there a plausible explanation for this observation?

Several mechanisms have been proposed for the reaction catalysed by HPPD. Goodwin and Witkop (1957) postulated initial oxygenation of the aromatic ring, followed by decarboxylation and rearrangement. Alternatively, to avoid the difficulty of oxygen attacking at a position on the ring that is not highly activated Hamilton (1971) and Jefford and Cadby (1981) proposed a mechanism in which oxidative decarboxylation occurs first. Such mechanisms invoke the involvement of a reactive oxoiron (IV) species similar to that postulated in the oxidation of xenobiotics by the cytochrome P-450s.

In the present work, computational methods, for the determination of preferred molecular structures (molecular mechanics and semi-empirical molecular orbital methods) for the substrate, product and some known inhibitors of HPPD, were used. The accuracy of these techniques have been extensively tested (eg. against X-ray data) and have been found to be reliable for structural determination. Such methods provide a means of calculating lowest energy structures and other minima close to that conformation. It must be noted that, whilst the conformation of any chemical bound to the active site of an enzyme may differ significantly from its minimum energy conformation, the triketones are conformationally restricted (ie. they have a fixed geometry). Any conformation change that can occur on binding of the triketone, with respect to its calculated minimum energy conformation, will be minimal.

Enzyme inhibitors may resemble the substrate, product or act as transition state analogues. Molecular modelling can give insight into possible transition state structures and therefore into possible mechanisms of inhibition.

Mechanistically, if the triketone moiety mimics the $\alpha$-keto acid of the substrate then these groups can be presumed to occupy a similar binding domain at the enzyme active site. Overlaying $\alpha$-keto acid functionalities of HPPD and synthetic substrates showed marked similarities in these structures in respect to the sites of oxidation (Figure 6a and 6b). This suggests that the $\alpha$-keto acids occupy the same binding domain at the enzyme active site. In comparison, overlaying the triketone moiety (endocyclic enol form) of SC-0735 and the $\alpha$-keto acid of HPPD showed the two chemicals

to be structurally dissimilar (as judged by the poor overlay of the aromatic rings) (Figure 7). This dissimilarity suggests that these two functionalities do not occupy the same enzyme binding pocket upon inhibition and that the triketones are not structural analogues of HPPA.

The mechanism of homogentisic acid formation from HPPA is postulated to involve an initial chelation of the $\alpha$-keto acid moiety of the substrate to an iron at the enzyme active site followed by oxidative decarboxylation. In an attempt to build up a rough picture of this binding, model iron-ligand complexes were constructed. The geometry of the ferric iron in native HPPD was shown to be rhombic, high spin Fe (111) (Bradley et al, 1986). The geometry of the iron [Fe(11)] centre in the active form is likely to also be octahedral with some rhombic distortion. Tris-hexafluoroacetylacetonate iron (111) complex was used to model the iron in HPPD. The iron in this model is octahedral and does not allow for distortions of the iron coordination at the active site, but it does, at least, allow some estimate of binding geometries. Not surprisingly, the $\alpha$-keto acid function of HPPA, is able to coordinate with the iron (Figure 8). With HPPA bound in this way, its aromatic ring lies almost perpendicular to the plane of the $\alpha$-keto acid. It is possible to imagine an oxygen molecule coordinated to the iron at the position closest to the aromatic ring facilitating oxygen attack at the 2-position on the substrate (Figure 8). Similarly, triketones (endocyclic end form) are able to chelate through a $\beta$-diketonate functionality (Figure 9).

Aligning the HPPA and SC-0735 bound to the same iron coordinates gives, as expected, a poor overlay of the aromatic rings for the two chemicals (Figure 10).

The iron bound at the active site of HPPD has three sites available for coordination, two are occupied by the substrate and the third by oxygen. Models were constructed where HPPA is bound to the substrate binding iron coordinates whereas SC-0735 was bound to one of the substrate binding coordinates and the oxygen binding site. Overlaying the two conformations (Figure 11) shows the aromatic ring of substrate and triketone to occupy similar spatial orientations. The close similarities are consistent with

both rings occupying the same hydrophobic pocket at the enzyme active site and suggests that the triketones may bind to HPPD in this fashion.

It is postulated that the mechanism of inhibition of HPPD by the triketones occurs through a two stage process. The initial stage involves a weak binding of the triketone to the iron-substrate binding coordinates. This accounts for the requirement of the triketone moiety for inhibition and its mimicing of the $\alpha$-keto acid of the substrate. Secondly, once bound, the triketone shifts its iron binding coordinates, by a pseudorotation, to occupy the oxygen binding site. This second stage is driven by binding forces associated with the occupation of the substrate aromatic ring binding domaine or by subtle conformational changes of the protein at the active site. Once bound in this conformation the inhibitor is held by hydrophobic and non-covalent interactions (hydrogen bonding) of the aromatic ring and substituents with amino acid residues on the protein. Rates of dissociation of the triketone will depend upon the magnitude of these interactions.

This model has been used to design novel, non-triketone inhibitors of HPPD. These compounds await synthesis at WRC and testing against the enzyme.

## CONTRIBUTORS

L A Gowans
P Phillips
A Mulholland (pre-postgraduate student)

## RESOURCES

| | | | |
|---|---|---|---|
| M K Ellis | - Biochemical Toxicology | : | 0.4 man-years. |
| L A Gowans | - Biochemical Toxicology | : | 0.3 man-years. |
| P Phillips | - Biochemical Toxicology | : | 0.3 man-years. |
| A Mulholland | | : | 0.2 man-years. |

## REFERENCES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Hamilton J A (1971). Progress in Bioinorganic Chemistry, 1, 83-157.

Jefford C W and Cadby P A (1981). Experimentia, 37, 1134-1137.

Lin K-T and Crawhall J C (1976). Can J Biochem, 54, 423-431.

Lindblad B, Lindstedt G, Lundstedt S and Rundgren M (1977). J Biol Chem, 252, 5073-5084.

Rundgren M (1977a). J Biol Chem, 252, 5085-5093.

Rundgren M (1977b). J Biol Chem, 252, 5094-5099.

Smith L L, Robinson M, Lock E A, Ellis M K and Provan W M (1989). ICI CTL Report No. CTL/R/1042. Triketone Project Team Progress Report.

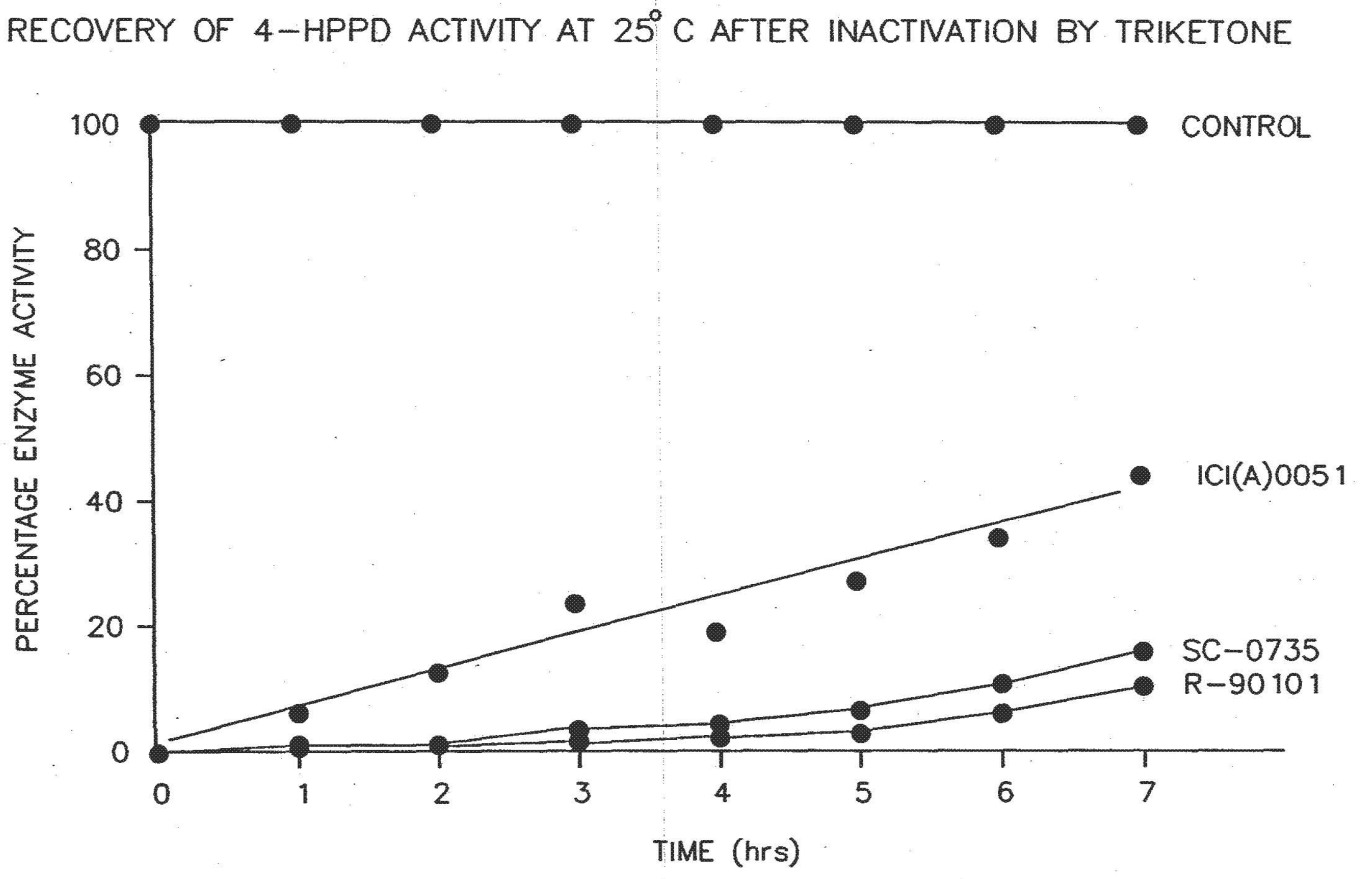
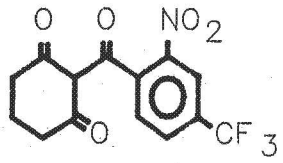
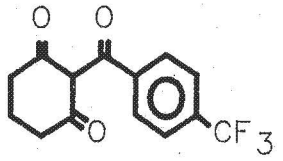
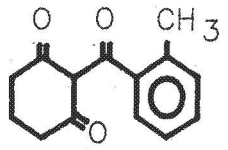
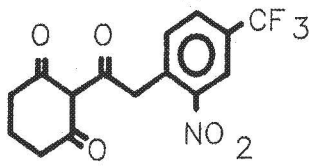
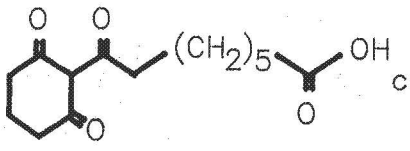

RECOVERY OF 4-HPPD ACTIVITY AT 25° C AFTER INACTIVATION BY TRIKETONE
PERCENTAGE ENZYME ACTIVITY
100
80
60
40
20
0
0
1
2
3
4
5
6
7
TIME (hrs)
CONTROL
ICI(A)0051
SC-0735
R-90101

Figure 1

**Figure 2**

THE EFFECT OF TEMPERATURE ON THE INHIBITION OF HPPD BY THE TRIKETONES

| | TEMPERATURE | |
|---|---|---|
| | 37 °C | 25 °C |
|  SC–0735 | tight binding | tight binding |
|  R–86113 | slow binding | tight binding |
|  R–81528 | slow binding | tight binding |
|  R–99796 | slow binding | tight binding |
|  R–243356 | competitive | slow–binding |

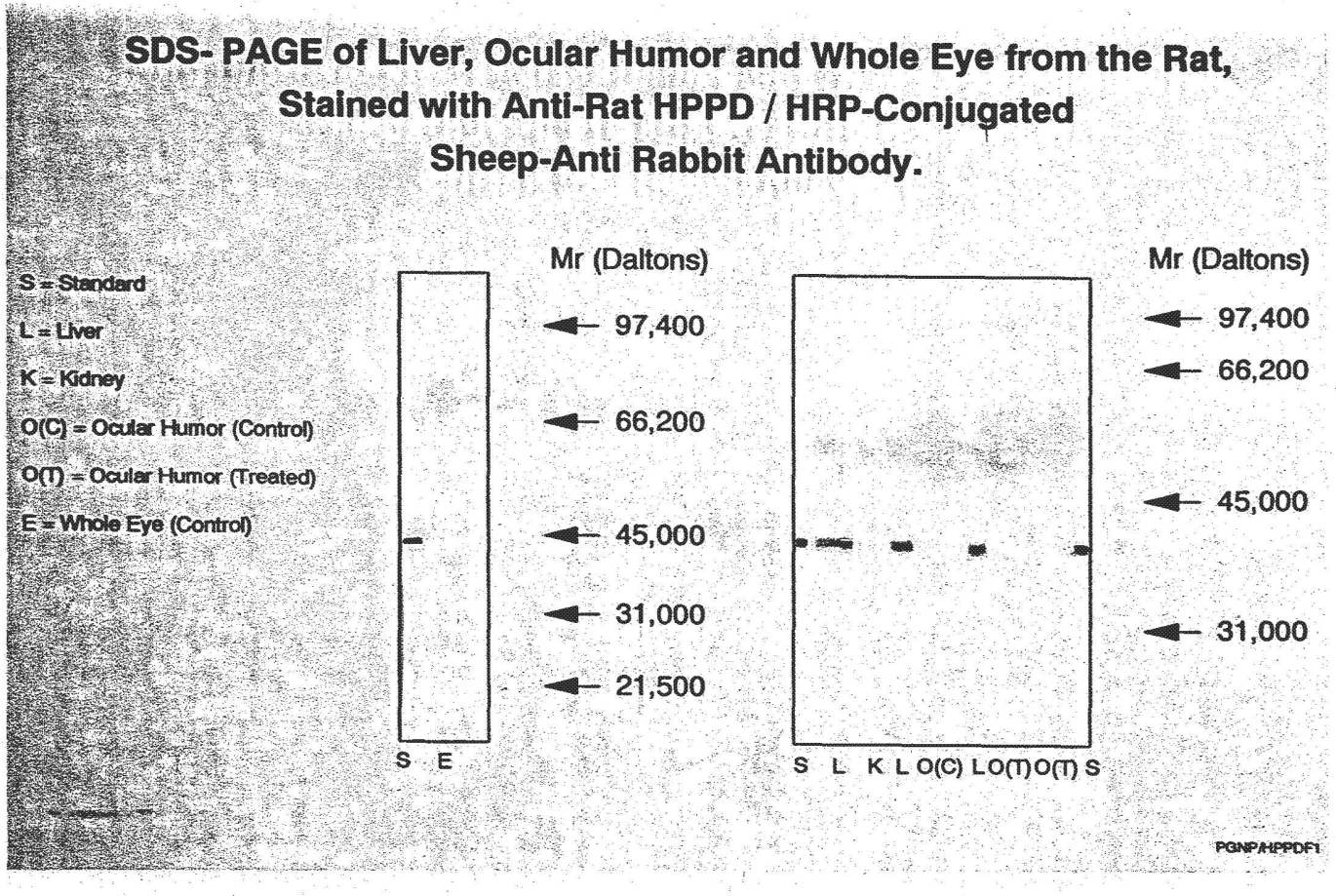
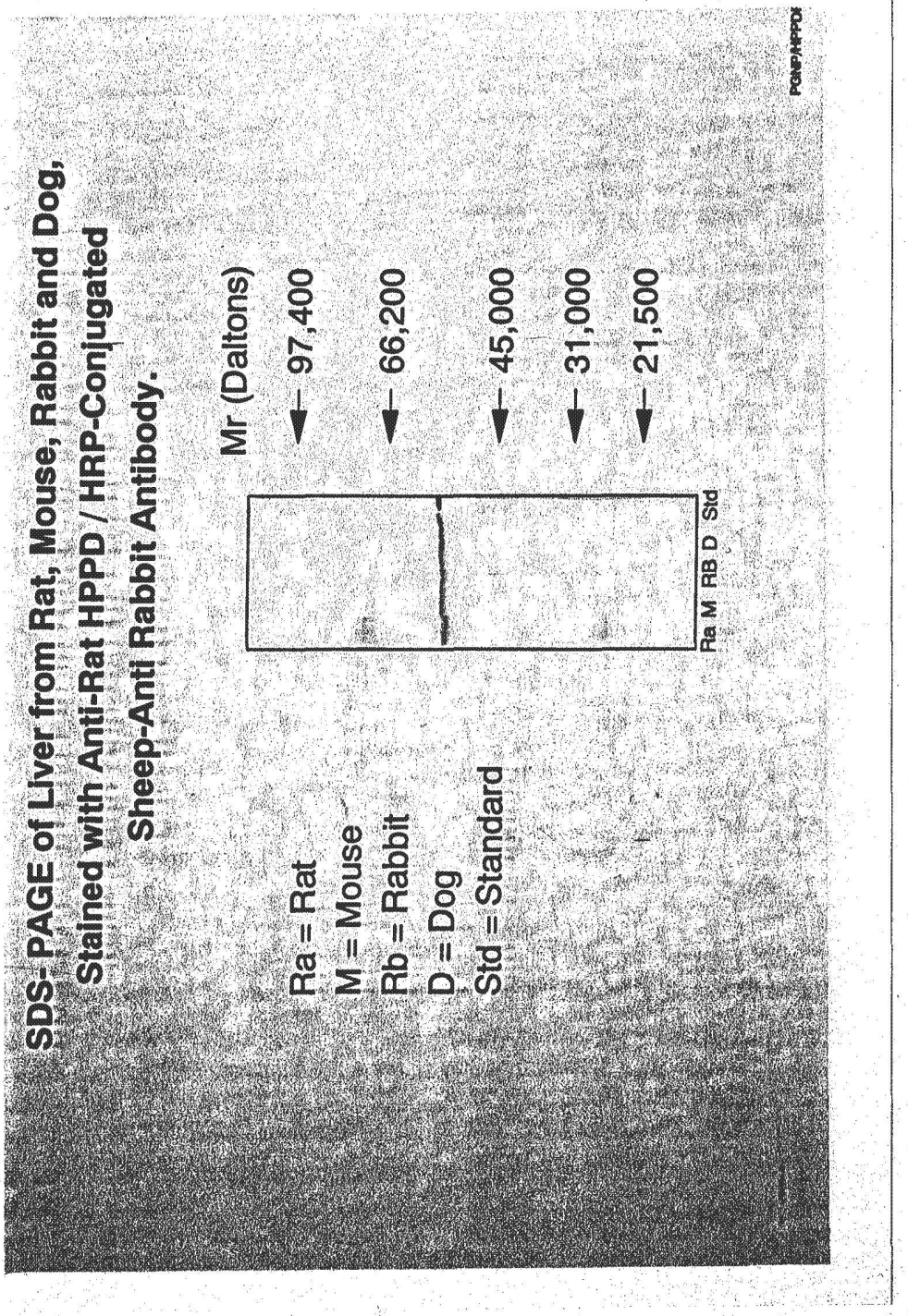

SDS- PAGE of Liver, Ocular Humor and Whole Eye from the Rat, Stained with Anti-Rat HPPD / HRP-Conjugated Sheep-Anti Rabbit Antibody.
S = Standard
L = Liver
K = Kidney
O(C) = Ocular Humor (Control)
O(T) = Ocular Humor (Treated)
E = Whole Eye (Control)
Mr (Daltons)
97,400
66,200
45,000
31,000
21,500
S E
Mr (Daltons)
97,400
66,200
45,000
31,000
S L K L O(C) L O(T) O(T) S
PGNPAHPPDF1

Figure 3

## Figure 4


SDS- PAGE of Liver from Rat, Mouse, Rabbit and Dog,
Stained with Anti-Rat HPPD / HRP-Conjugated
Sheep-Anti Rabbit Antibody.
Ra = Rat
M = Mouse
Rb = Rabbit
D = Dog
Std = Standard
Mr (Daltons)
97,400
66,200
45,000
31,000
21,500
Ra M RB D Std
PGNPAHPPD

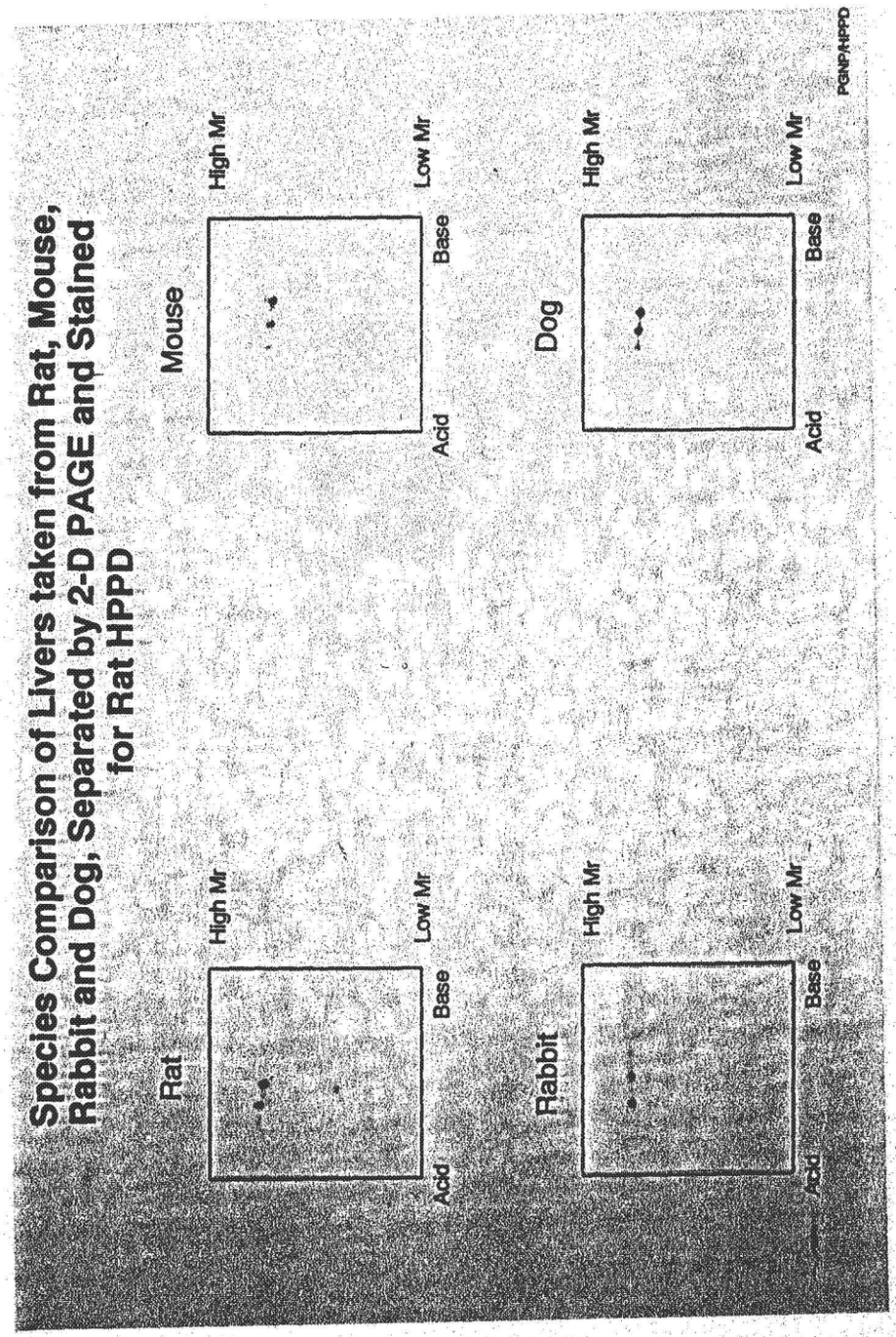
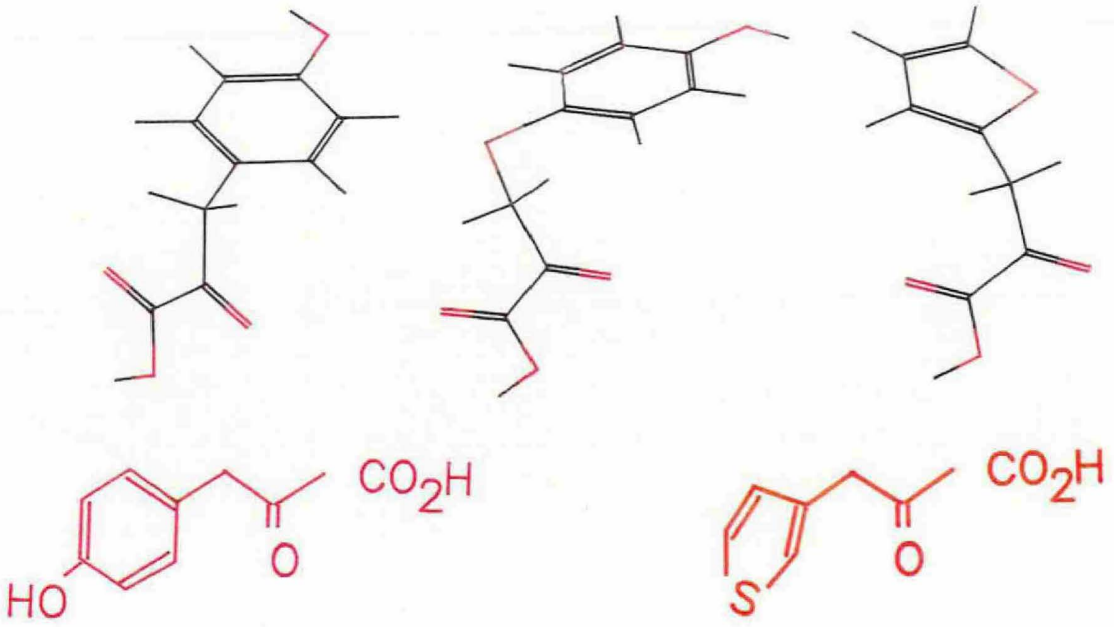
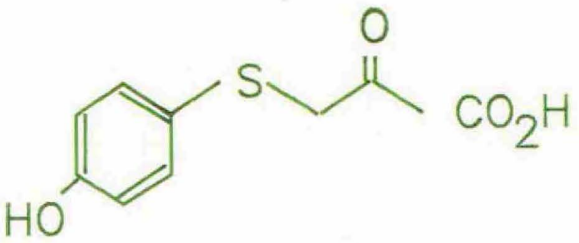


Figure 6a




HPPA

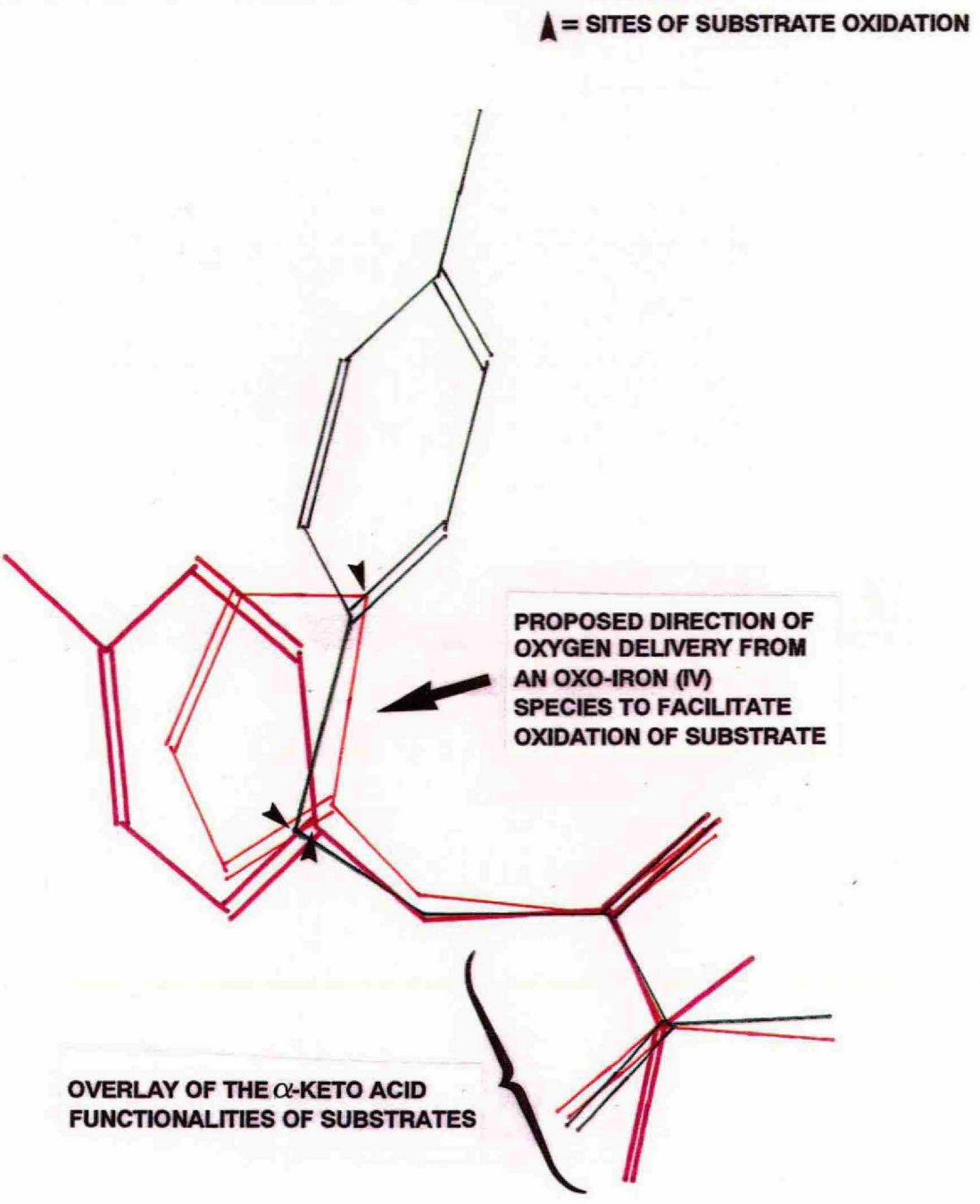
2–THIENYLPYRUVIC ACID




4–HYDROXYPHENYLTHIOPYRUVATE

ENERGY MINIMISED STRUCTURES OF THE SUBSTRATES
HPPA,4–HYDROXYPHENYLPYRUVATE AND
2–THIENYLPYRUVATE

Figure 6b




RED: 4-HYDROXYPHENYLPYRUVATE (HPPA)
GREEN: 4-HYDROXYPHENYLTHIOPYRUVATE
ORANGE: 2-THIENYLPYRUVATE

Figure 7

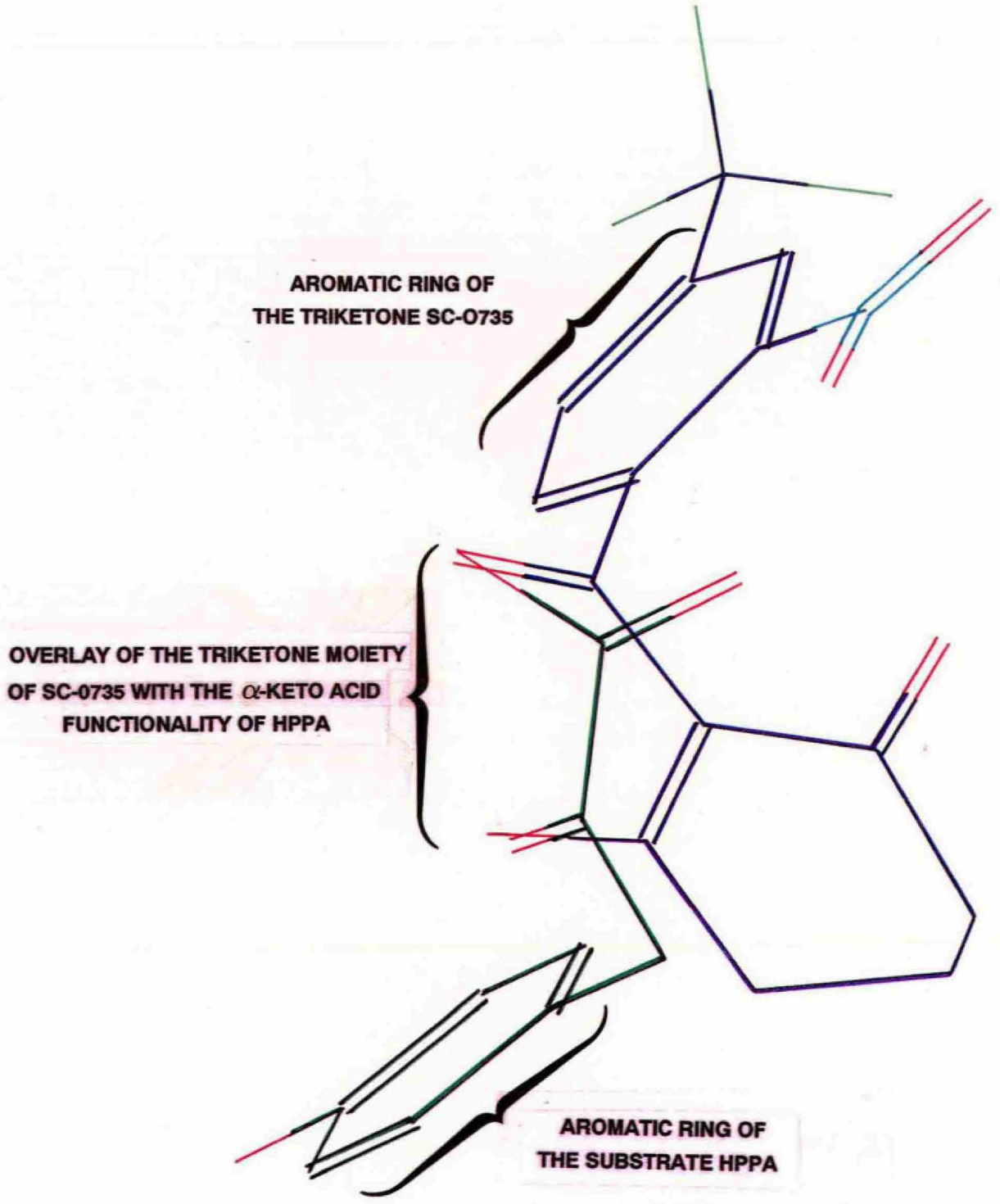


**Figure 8**

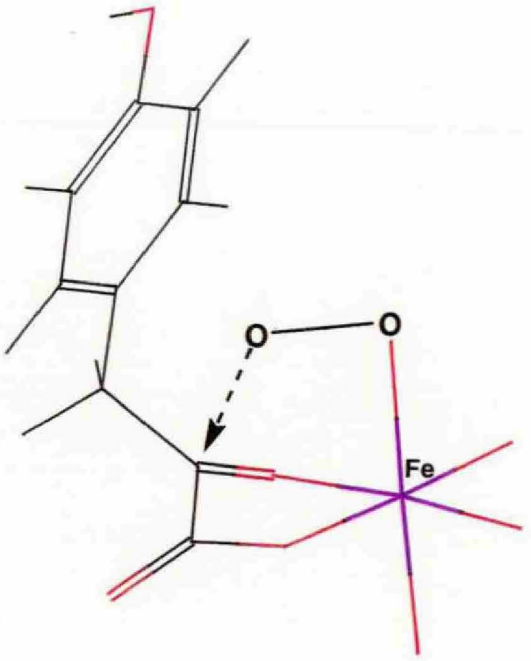



**Figure 9**

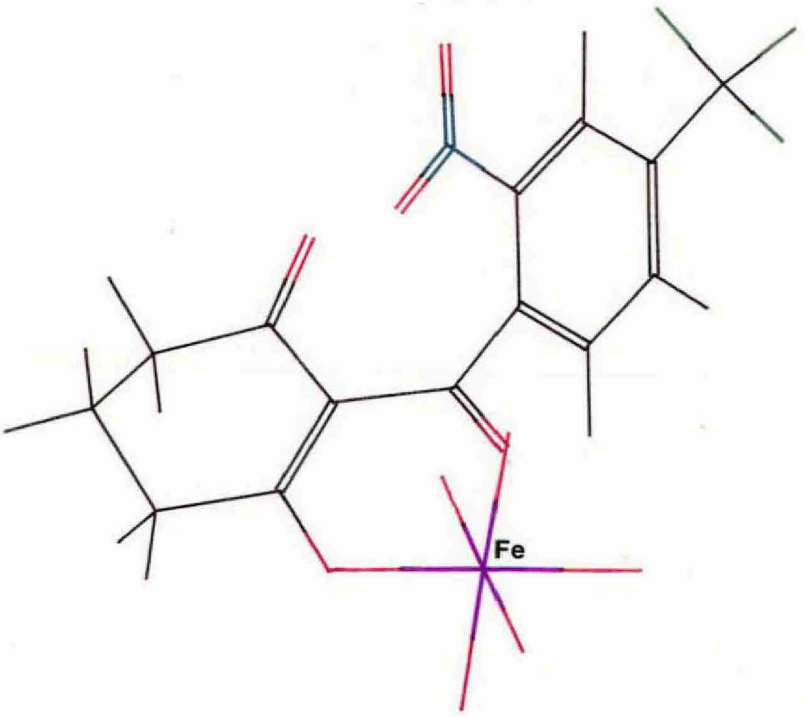


Figure 10

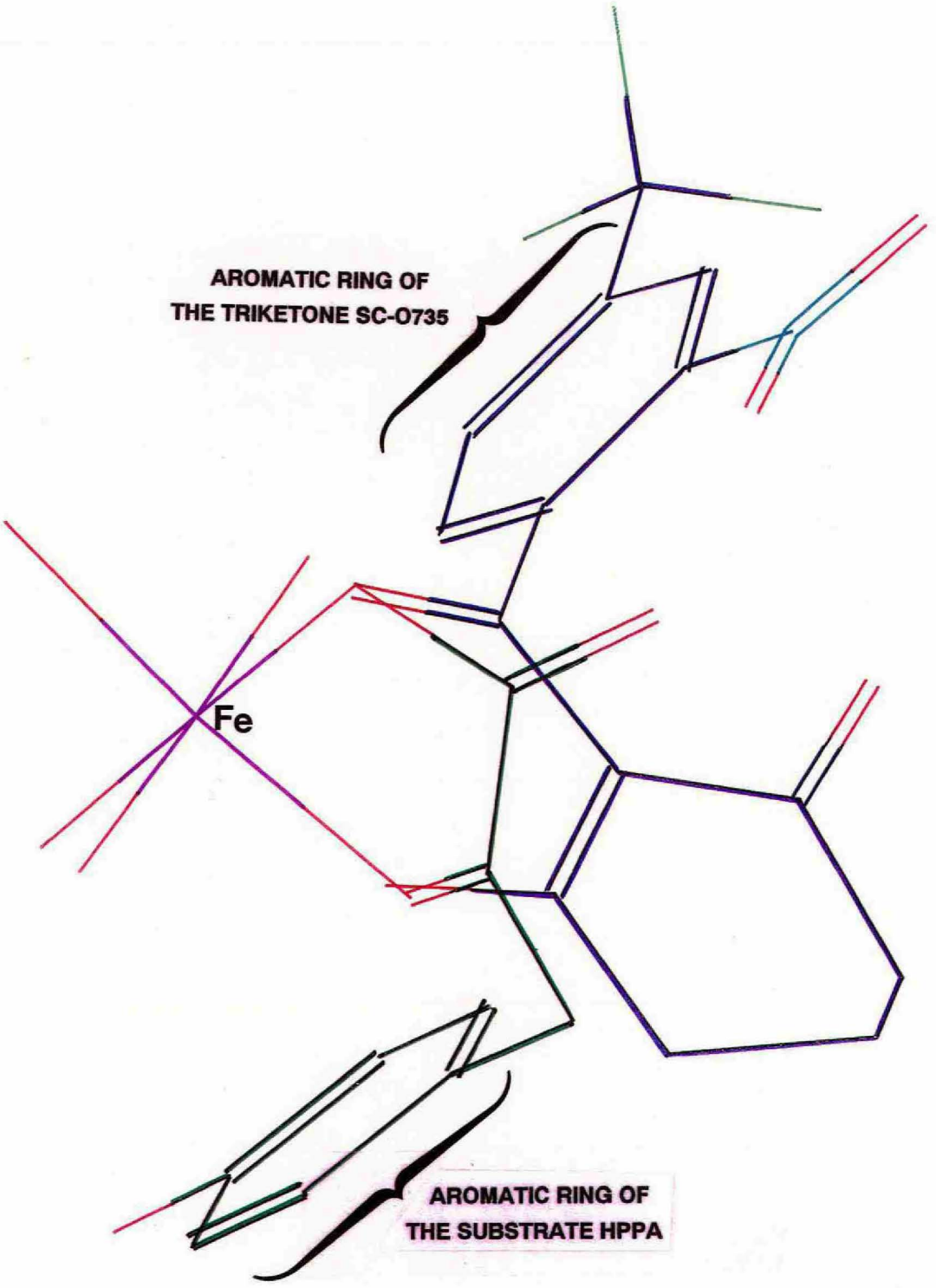
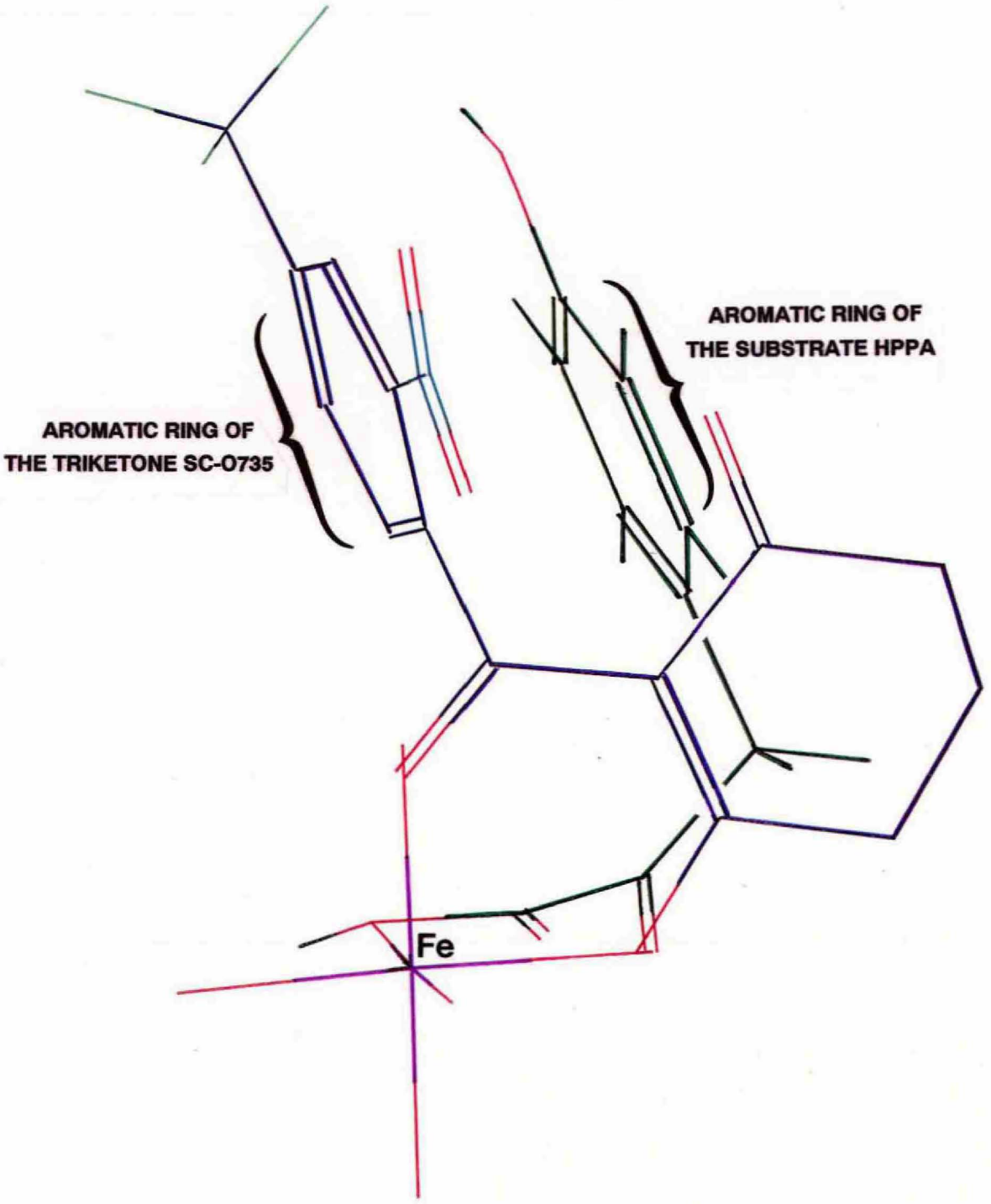


Figure 11



# MATHEMATICAL MODELLING OF TRIKETONE PHARMACOKINETICS AND TYROSINE CATABOLISM

T R AUTON

## INTRODUCTION

The aim of this work is to improve our understanding of how the triketone SC-0735 causes tyrosinaemia. A substantial database of experimental results has been accumulated on the pharmacokinetics of SC-0735, including plasma and tissue concentrations and amounts excreted in urine and faeces, and its effects on plasma tyrosine and the enzyme 4-hydroxyphenylpyruvate dioxygenase (4-HPPD). This report shows how this diverse data has been used to build a mathematical model, which provides a concise and consistent description of the whole database. The model embodies a detailed set of assumptions or hypotheses about the mechanism of action. Starting from this detailed statement of assumptions it is possible to judge each in turn to see how well it is justified by the data or consistent with other knowledge. This process develops understanding of the key mechanisms and shows up gaps or inconsistencies in the data.

## PROGRESS

A brief description of the model is provided below. Full details of the data used to estimate model parameters and validation of model predictions against experiment are given in a CTL report (Auton et al, 1991). The kinetics of tyrosine aminotransferase (TAT) were derived from in vitro data from the scientific literature (Rosenberg and Litwack, 1970) as described in Auton 1991.

Figure 1 shows a schematic of the model describing the pharmacokinetics of SC-0735. The triketone is absorbed readily from the gut following oral dosing and eliminated into both urine and faeces. Tissue concentration data show that the triketone is retained in liver and other organs (Figure 2). Comparing tissue retention after different dose levels

suggests that it binds to a saturable binding site. A consequence of this is that elimination is much slower and a larger fraction of the dose is retained following a low dose (0.3$\mu$mol/kg) than a high dose which exceeds the binding capacity (30.0$\mu$mol/kg).

The major pharmacodynamic effect of the triketone is to inhibit 4-HPPD. Activities of this enzyme have been measured ex vivo. A single oral dose of 30$\mu$mol/kg SC-0735 causes substantial inhibition of 4-HPPD for at least 7 days (Figure 3). SC-0735 binds tightly to this enzyme in vitro (Auton and Ellis, 1989). Combining this information with the pharmacokinetic and pharmacodynamic data suggests that the tissue binding site is the HPPD enzyme. This is assumed in the model. The success of the model in fitting the available data provides evidence that this assumption is correct.

Figure 4 shows a simplified model of tyrosine kinetics. Tyrosine is absorbed from the diet, some is used for synthesis of proteins or other biochemicals. Some may be recycled from denatured protein. It is assumed that these processes leave a constant net flux of tyrosine (cTyin) to be eliminated. The major route of elimination is via the TAT enzyme to form 4-hydroxyphenylpyruvate (HPP), which is then oxidised by 4-HPPD to homogentisic acid. When 4-HPPD is inhibited other pathways come into play to eliminate the excess HPP or tyrosine. In the model it is assumed that there is an alternative elimination pathway for HPP, probably by urinary excretion of HPP and 4-hydroxyphenyl lactate. Consequently, inhibition of 4-HPPD causes an increase in HPP until there is adequate clearance by this other pathway. Rosenberg and Litwack (1970) have shown that TAT from rat liver cytosol is inhibited by its product, HPP. This in turn causes an increase in plasma tyrosine. Experimental and model fitted values of the time course of plasma tyrosine are shown in Figure 5, and the dose response in Figure 6. A complete list of the model parameter values used to calculate these results is given in Table 1.

It is important in developing a mathematical model to validate the model by comparing its predictions against experimental data. This must be additional data to the values used to derive model parameters. The model

has been validated by comparing predictions of the model for plasma tyrosine with additional data (Table 2). The results show that the model reliably predicts tyrosine concentrations under a range of dosing conditions with SC-0735.

A detailed model has been developed and validated for SC-0735 in the rat. It had been hoped to extend the model to other species, and in particular the mouse, which does not develop eye lesions. This work had not been completed by the time the project was stopped. Nevertheless, modelling the mechanism of toxicity in the rat was useful in a number of ways. Firstly, it served to confirm, quantitatively, the qualitative interpretation of the experiments. Secondly, it highlighted the factors which are likely to be important in assessing the risk from triketones to man. The most important of these is the kinetics of tyrosine elimination by pathways not dependant on 4-HPPD.

## FUTURE WORK

As the research project has stopped future work will be limited to completing internal reports and preparing external publications.

## CONTRIBUTORS

All the modelling work was done by T R Auton. None of this would have been possible, however, without the efforts of the rest of the triketone team in providing experimental results and their interpretation.

## RESOURCES

1991 - T R Auton : 2 man-months

## REPORTS

Auton T R (1991). Inhibition of tyrosine aminotransferase by 4-hydroxyphenyl pyruvate: Kinetic analysis. ICI CTL Report No. CTL/R/1093 (in preparation).

Auton T R, Gaskin P and Lock E A (1991a). Mathematical modelling of tyrosine catabolism in weanling rats. ICI CTL Report No. CTL/L/4299 (in preparation).

Auton T R, Robinson M, Provan W M and Lock E A (1991b). Mathematical modelling of the pharmacokinetics of SC-0735 in the rat and its effect on tyrosine catabolism. ICI CTL Report No. CTL/R/1092 (in preparation).

## REFERENCES

Auton T R and Ellis M K (1989). Inhibition of rat liver 4-hydroxyphenyl pyruvate dioxygenase by SC-0735 in vitro: Characterisation and kinetic parameter estimation. ICI CTL Report No. CTL/R/983.

Rosenberg J S and Litwack G (1970). Liver cytosol tyrosine aminotransferase: product inhibition and interaction with substrates. J Biol. Chem. 245, 5677-5684.

Table 1

MODEL PARAMETER VALUES:
ESTIMATES AND COEFFICIENTS OF VARIATION

| Parameter Estimates | Estimate ± C.V. [1] | Units | Description |
|---|---|---|---|
| ka | 1.69 ± 12.0% | h-1 | Absorption rate const |
| V | 0.314 ± 21.6% | l/kg | Volume of distribution |
| Clu | 0.00998 ± 21.3% | l/kg/h | Clearance rate into urine |
| Clf | 0.0146 ± 21.3% | l/kg/h | Clearance rate into faeces |
| kb | 1.22 ± 66.1% | l/μmol/h | Binding rate constant |
| KD | 0.0115 ± 32.6% | μmol/l | Dissociation constant |
| KDr | 0.0427 ± 49.7% | μmol/l | Reversible binding diss const. |
| dbsl | 5.62 ± 7.2% | μmol/kg | Density of binding sites in liver |
| dbsk | 1.73 ± 3.6% | μmol/kg | Density of binding sites in kidney |
| dbs | 0.491 ± 7.1% | μmol/kg | Average density of binding sites over whole body |
| PL | 0.561 ± 24.5% | | Liver:plasma partition cft |
| PK | 0.409 ± 21.5% | | Kidney:plasma partition cft |
| cTyin | 1060 ± 29.5% | μmol/l/h | Scaled net flux of tyrosine |
| VmTAT | 2600 ± 30.0% | μmol/l/h | Vmax for TAT |
| KmTAT | 221 | μM | Km for tyrosine in TAT |
| KmHTAT | 1.97 | μM | Inhibition constant for HPP inhibition |
| CTHTAT | 0.17 | | Interaction constant for tyrosine and HPP - |
| cHPPO | 8.01 | μM | Threshold for HPP inhibition |
| VmHPPD | 24900 ± 44.3% | | Scaled Vmax for HPPD enzyme |
| KmHPPD | 20.9 | | Km for HPPD enzyme |
| cTymax | 2300 ± 16.7% | μM | Maximum conc of tyrosine (due to complete inhibition of HPPD) |
| HPPDiv | 2.2 ± 12.7% | [2] | Normal <u>in vitro</u> activity of 4 HPPD enzyme |

[1] CV = coefficient of variation = standard error/parameter estimate.
[2] Units: μL O2/min/mg cytosolic protein.

Table 2

COMPARISON OF MODEL PREDICTIONS AND EXPERIMENTAL MEASUREMENTS OF PLASMA TYROSINE CONCENTRATION

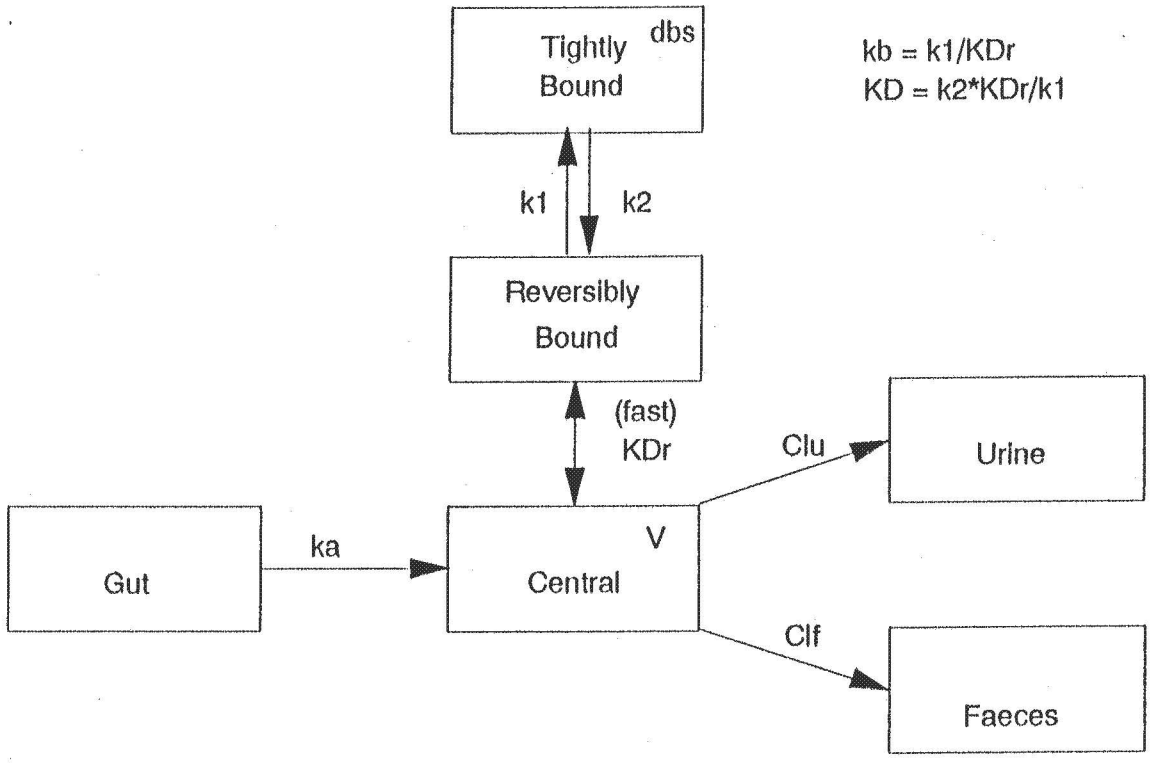
| Study | Dosing Conditions | Equivalent Daily Dose | Time | Plasma Tyrosine | |
|---|---|---|---|---|---|
| | | | | Measured | Predicted |
| | [1] | (μmol/kg/d) | (days) | (μmol/l) | (μmol/l) |
| XR1782 | 0.2 mg/kg | 0.6 | 24 h | 1946 | 824 |
| XR1782 | 10 mg/kg | 30 | 24 h | 3173 | 1961 |
| XR1844 | 10 mg/kg | 30 | 2 h | 221 | 701 |
| | | | 4 h | 442 | 1011 |
| | | | 8 h | 662 | 1388 |
| | | | 12 h | 1490 | 1616 |
| | | | 16 h | 1876 | 1771 |
| | | | 24 h | 2373 | 1961 |
| | | | 36 h | 1987 | 2101 |
| | | | 48 h | 1601 | 2162 |
| XR1560 | 0.1 mg/kg/d | 0.3 | 42 | 2042 | 1897 |
| XR1560 | 0.5 mg/kg/d | 1.5 | 42 | 2318 | 2201 |
| XR1560 | 10 mg/kg/d | 30 | 42 | 2428 | 2220 |
| XR1560 | 80 mg/kg/d | 240 | 42 | 2594 | 2221 |
| XR1560 | 160 mg/kg/d | 480 | 42 | 2428 | 2221 |
| XR1561 | 2 mg/kg/d | 6 | 42 | 2539 | 2219 |
| XR1561 | 10 mg/kg/d | 30 | 42 | 2870 | 2220 |
| XR1561 | 40 mg/kg/d | 120 | 42 | 2649 | 2221 |
| XR1499 | 10 mg/kg/d | 30 | 42 | 1711 | 2220 |

Table 2 - continued

COMPARISON OF MODEL PREDICTIONS AND EXPERIMENTAL MEASUREMENTS OF PLASMA TYROSINE CONCENTRATION

| Study | Dosing Conditions | Equivalent Daily Dose | Time | Plasma Tyrosine | |
|---|---|---|---|---|---|
| | | | | Measured | Predicted |
| | [1] | (μmol/kg/d) | (days) | (μmol/l) | (μmol/l) |
| XR1676 | 10 mg/kg/d | 30 | 6 h | 573 | 1225 |
| | | | 18 h | 2479 | 1830 |
| | | | 24 h | 2722 | 1961 |
| | | | 36 h | 3102 | 2101 |
| | | | 48 h | 2740 | 2164 |
| | | | 72 h | 2417 | 2208 |
| | | | 96 h | 2461 | 2217 |
| XR1799 | 1 ppm | 0.15 | 42 | 1588 | 1731 |
| | 5 ppm | 0.75 | 5 | 2507 | 2191 |
| | | | 28 | 2110 | 2193 |
| | | | 42 | 1855 | 2193 |
| | 10 ppm | 1.5 | 42 | 2455 | 2213 |
| XR1878 | 5 ppm | 0.75 | 42 | 2233 | 2193 |
| | 5 ppm | 0.75 | 42 | 2272 | 2193 |
| XR2002 | 5 ppm | 0.75 | 42 | 2234 | 2193 |
| | 5 ppm | 0.75 | 42 | 2151 | 2193 |

[1] The units chosen are appropriate to the method of dosing.
For single oral doses by gavage - mg/kg.
For daily oral dosing by gavage - mg/kg/d.
For dietary admixture - ppm.




See Table 1 for explanation of parameters.

Figure 1. Block diagram of pharmacokinetic model for triketone SC-0735.

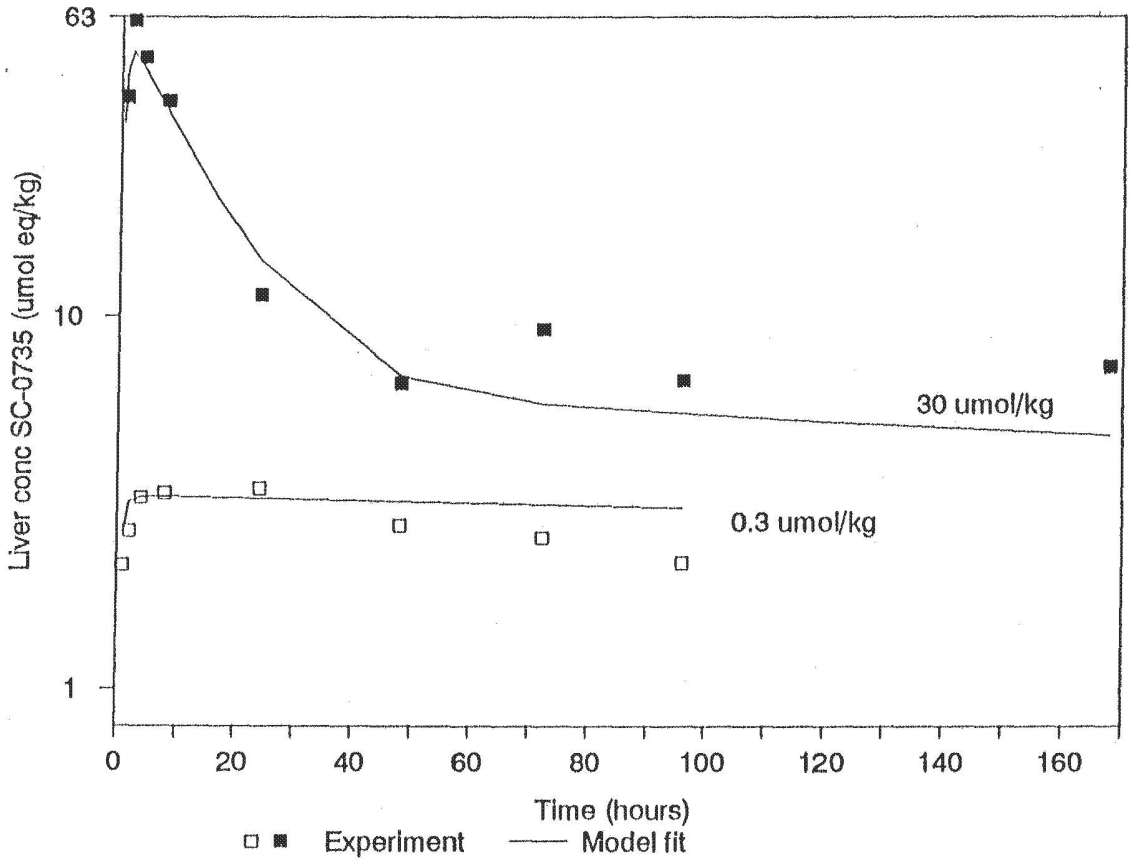



Figure 2. Concentration of radioactivity in liver following single oral dose of 30 or 0.30$\mu$mol/kg 14C SC-0735. Comparison of model fitted values (solid line) and experimental data (squares).

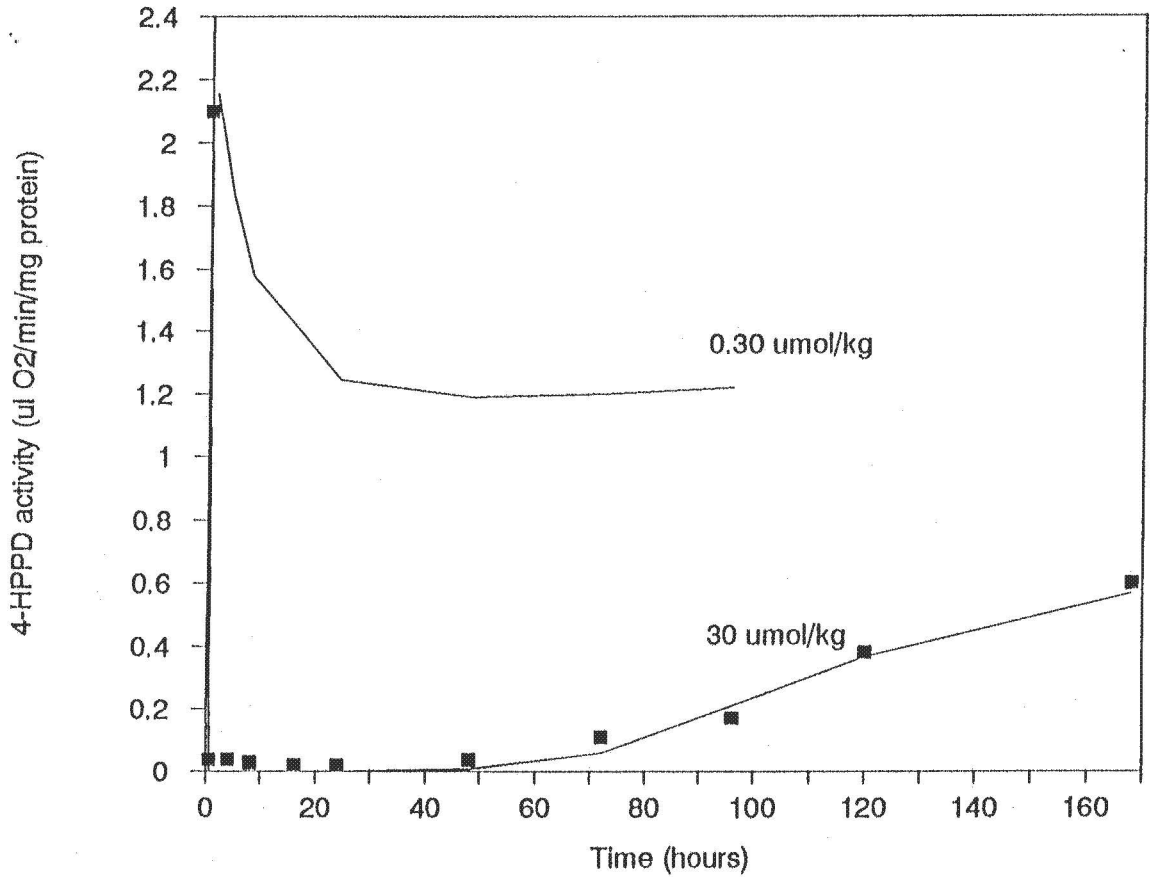
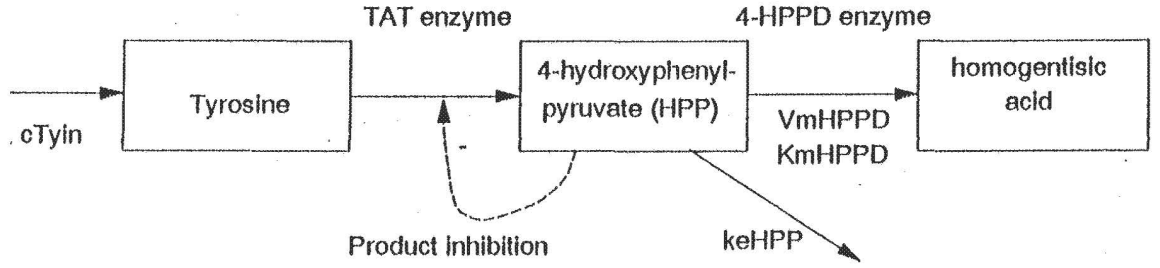



Figure 3. Ex vivo activity of 4-HPPD following single oral dose of 30$\mu$mol/kg SC-0735. Comparison of model fitted values (solid line) and experimental data (squares). The figure also shows model predictions of activity following a 0.30$\mu$mol/kg dose.




See Table 1 for explanation of parameters.

**Figure 4.** Block diagram of model of tyrosine pharmacokinetics.

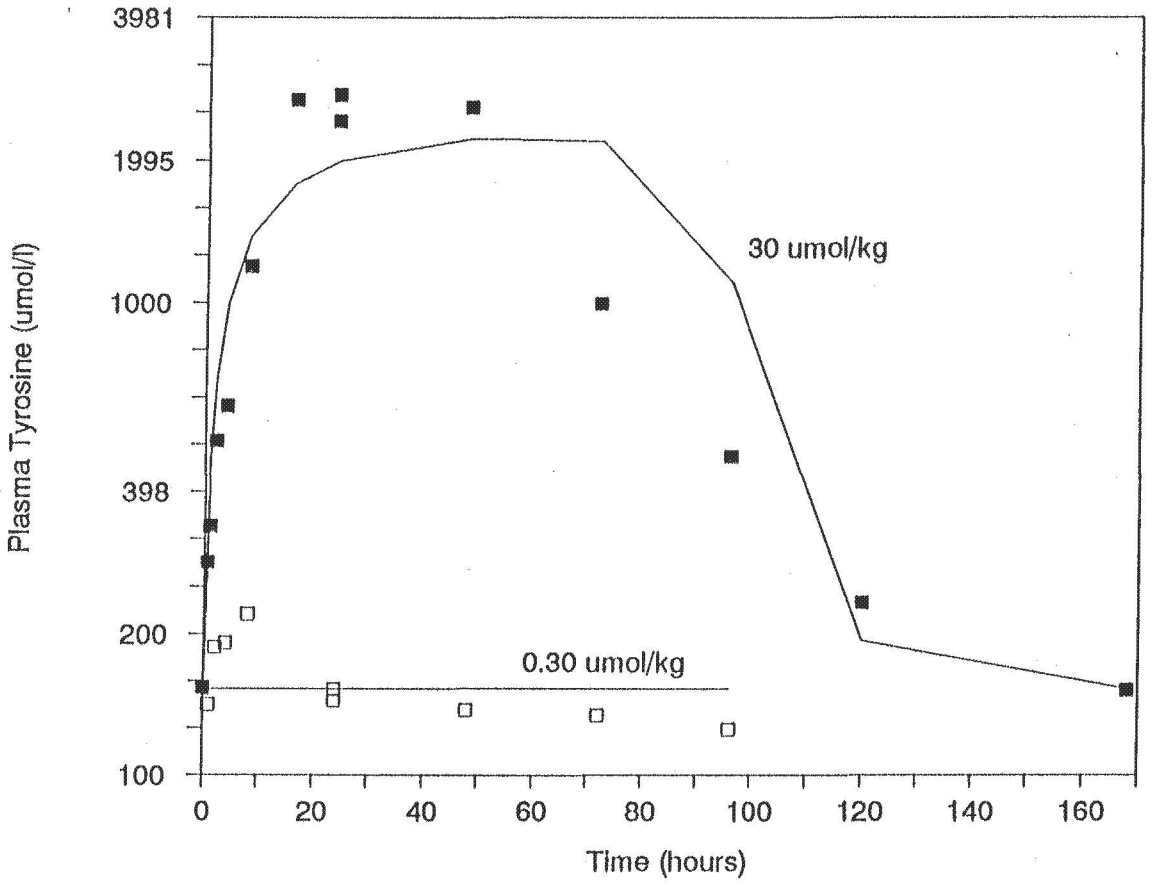



**Figure 5.** Concentration of tyrosine in plasma following single oral dose of 30 or 0.30$\mu$mol/kg SC-0735. Comparison of model fitted values (solid line) and experimental data (30$\mu$mol/kg dose - filled squares, 0.30$\mu$mol/kg - empty squares).

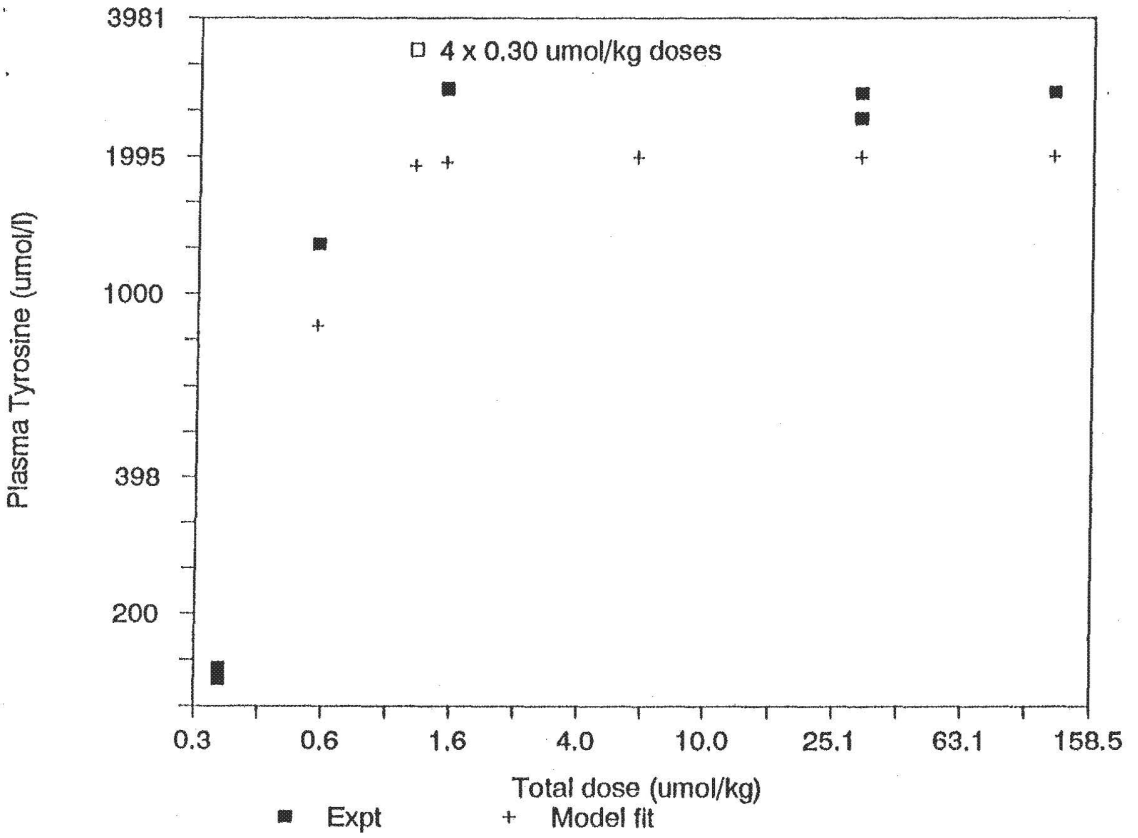



Figure 6. Concentration of tyrosine in plasma 24 hours after various single doses or 4 daily doses of 0.30μmol/kg SC-0735. Comparison of model fitted values (+) and experimental data (squares).

# STUDIES ON THE MECHANISM OF TRIKETONE-INDUCED OCULAR LESIONS

E A LOCK AND P GASKIN

## INTRODUCTION

The aim of this work is to understand the mechanism whereby the model triketone SC-0735 produces corneal injury in the rat. This may then enable us to understand the basis for the species difference in susceptibility and hence the likely response in man. The rat and dog develop corneal lesions following continuous administration while the mouse, rabbit and rhesus monkey are refractory.

Work on this aspect of the project has now stopped as a position on the relevance of this finding to man has been developed. This information has been used to support the application for registration of the triketone ICI(A)0051 and will be of use for other triketones that may follow.

The triketone SC-0735 is a potent inhibitor of the enzyme 4-hydroxyphenyl pyruvate dioxygenase (HPPD) which is responsible for the conversion of 4-hydroxyphenylpyruvate to homogentisic acid in the tyrosine catabolism pathway. Inhibition of this enzyme, located primarily in the liver and kidney, leads to a build up of 4-hydroxyphenylpyruvate and in particular its precursor tyrosine. Elevation in plasma tyrosine leads to a concomitant increase in the concentration of tyrosine in ocular fluid. A sustained and marked elevation in plasma tyrosine of greater than 1000nmol/ml at the end of a six week study in the rat leads to the onset of ocular lesions, whereas below this level ocular lesions are not seen. It is now clear that a sustained and marked elevation in tyrosine in the eye is responsible for the ocular damage. This is supported by our own work on feeding tyrosine in a low protein diet to rats (Smith et al, 1991) and by earlier studies reported in the literature (Harper et al, 1970; Rich et al, 1973; Goldsmith, 1983).

## PROGRESS

During the year studies have concentrated on three aspects:

1) Comparison studies on the extent of tyrosinaemia produced by ICI(A)0051 and SC-0735 in the rat, mouse, dog and rhesus monkey.

2) Working with Professor S Lindstedt to seek approval for the use of SC-0735 for the treatment of tyrosinaemia type I in man.

3) Looking at the cytotoxicity of tyrosine in two *in vitro* systems to try and further understanding why tyrosine is toxic to the cornea.

These areas will be discussed in more detail below.

### 1) Comparative Toxicology of Tyrosinaemia-Induced by Triketones and its Relationship to Ocular Toxicity

The extent of tyrosinaemia induced by ICI(A)0051 was compared with that produced by SC-0735 following a single oral dose of 10mg/kg to rats, mice, or dogs. In essence the extent and duration of tyrosinaemia with ICIA(A)0051 was considerably less than that seen with SC-0735. This is entirely consistent with the difference in potency for inhibition of HPPD *in vitro* (see Ellis *et al*, this report). This data plus an overview on the pathology of the eye lesion, inhibition of HPPD by the triketones and the relationship between tyrosinaemia and ocular toxicity in the rat are available in CTL report number CTL/R/1051.

### 2) Use of SC-0735 for the Treatment of Tyrosinaema Type I

Hereditary tyrosinaemia type I is an inborn error of metabolism. The patients suffer from multi-organ symptoms and may develop severe liver failure at a very early age, or more progressively liver disease which leads to nodular cirrhosis and the development of primary hepatic

carcinoma. Damage to the kidneys and blood forming organs may also occur. Death usually occurs before the age of 20 years. Some patients develop symptoms characteristic of acute porphyria with episodes of acute abdominal pain and generalised paresis. A porphyric episode may be the cause of death. The incidence in Scandinavia and probably many European countries is about 1 in 100,000 births, however in certain regions of Canada the incidence is higher eg. in the Lac-St Jean area of Quebec, 1 in 685 births.

In 1977 Professor Lindstedt and co-workers at the University of Gothenburg, Sweden, demonstrated that the primary defect in hereditary tyrosinaemia type I is a deficiency of the enzyme fumarylacetoacetase (see Figure 1). This results in a build up and increased excretion of succinylacetoacetate and succinylacetone. Succinylacetone is a powerful inhibitor of porphobilinogen synthase which catalyses the formation of porphobilinogen from 5-aminolevulinic acid in the haem biosynthetic pathway (see Figure 1). This offers an explanation for the excretion of 5-aminolevulinic acid and the porphyric symptoms. The plasma concentration of $\alpha$-fetoprotein is often high and a dramatic increase in this protein may indicate the development of liver cancer.

A possible therapeutic strategy for this disease would be to prevent the formation of maleylacetoacetate ie. the compound which is thought to be the primary toxic substance. Candidates for such a strategy are inhibitors of the enzyme HPPD or homogentisate-1,2-dioxygenase. With the discovery that SC-0735 is a potent inhibitor of HPPD, and the finding in collaboration with Professor S Lindstedt that SC-0735 is a potent inhibitor of human liver HPPD (see Smith et al, 1991), he suggested that SC-0735 may have some benefit in treating patients with tyrosinaemia type I.

In collaboration with Professor Lindstedt we have sought approval for its use in patients with this disease to investigate where SC-0735 can suppress the formation of maleyl and fumarylacetoacetate and improve their clinical condition. Following submission to the CTL Human Investigation Panel, the Ethical Committee at Salhgren's Hospital in Gothenburg and the Medical Product Agency in Uppsala approval was given for its use in one severely ill child and subsequently for a further five patients.

Five cases have so far been treated with SC-0735 at 0.1-0.4mg/kg/day. The compound does prevent or markedly reduce the formation of maleyl and fumarylacetoacetate as indicated by the rapid decrease in the urinary excretion of succinylacetone and succinylacetoacetate (see Figure 2). The almost complete inhibition of porphobilinogen synthase was abolished and the excretion of 5-aminolevulinic acid decreased to within the normal range or slightly above (Figure 3). In four patients the concentration of $\alpha$-fetoprotein decreased to values approaching normal over a four to six month treatment period, see Figure 4 for an example of one case. One patient developed rachitis six months before treatment and had gross excretion of markers of renal tubular dysfunction. After three weeks of treatment this excretion had stopped.

The clinical condition of these patients has generally improved and no side effects of the treatment with SC-0735 have been encountered to date. The longest treatment is now approaching 8 months. SC-0735 produces a mild tyrosinaemia with values rising from around 100nmol/ml of plasma to 400-500nmol/ml (see Figure 5 for a typical example). These values are below the threshold for the production of ocular toxicity in man and no ocular effects have been seen in these patients. The basis for a threshold phenomena in man comes from clinical data on inborn errors in tyrosine metabolism, and is discussed in detail in CTL report number CTL/R/1058.

The data available on these inborn errors in tyrosine metabolism in man plus the therapeutic use of SC-0735 in hereditary tyrosinaemia type I form the basis of our opinion that inhibition of HPPD in man will not produce a marked and sustained tyrosinaemia that will cause ocular lesions. In this regard we believe man more closely resembles the rhesus monkey, rabbit and mouse.

### 3) Mechanism of Tyrosine-Induced Cytotoxicity

Goldsmith (1975) proposed that tyrosine-induced toxicity in rats was due to the fact that it concentrated to such a marked extent in ocular fluid that this poorly soluble amino acid came out of solution forming

crystalline deposits. This observation was consistent with a report by Gipson et al (1975) where they observed crystals, presumably tyrosine, in the cornea of rats fed a tyrosine-supplemented low protein diet. This mechanism is similar to that implicated in the toxicity of a number of other compounds such as $\alpha$-quartz-silicosis of the lung (Nash et al, 1966) and monosodium urate monohydrate - gout (Weismann and Rita, 1972).

We have examined the ability of tyrosine crystals to cause haemolysis of erythrocyte membranes, as originally outlined by Goldsmith (1975) and as used previously in CTL to investigate the fibrinogenicity of dusts. In addition the toxicity of tyrosine crystals has been examined in cultures of mouse 3T3 fibroblasts.

## Studies with Rat Erythrocytes

Fresh rat erythrocytes were washed three times with ice-cold phosphate buffer saline and diluted to 1% (v/v) for use. Tyrosine was hand-crushed in a pestle and mortar to give a reasonably uniform crystal size range and various concentrations of tyrosine suspensions were then incubated with the erythrocytes at 37°C for 1hr with gentle shaking. After 1hr the solution was centrifuged (1500xg or at 4° for 15min) to sediment the cellular components and crystals and the amount of haemoglobin released into the supernatant determined. The extent of haemolysis was compared to that produced by osmotic shocking erythrocytes in distilled water.

Suspensions of L-p-tyrosine produce a dose-dependent haemolysis of erythrocytes over 1hr (Figure 6) with no effect at 1, 2 or 4mg/ml but an increasing response at 20mg/ml and above. This effect appears to require an interaction between the crystals and the erythrocyte membrane as when the tyrosine suspension is confined to a dialysis membrane, such that only tyrosine in solution comes into direct contact with the erythrocytes, no haemolysis is seen (Figure 6). This rather coarse index of membrane damage occurs at much higher concentrations than are present in the eye (ocular fluid) where a maximum concentration would be around 1mg/ml (5525nmol/ml). Nevertheless it does indicate that L-p-tyrosine

crystals can cause membrane damage. Interestingly crystals of DL-tyrosine, a racemic mixture of the two stereoisomers, is much more potent than the L-isomer suspensions of 1mg/ml causing a marked and significant haemolysis (Figure 7). The basis for this difference probably lies with the different crystal structures of the L verus the DL-mixture. X-ray crystallographic analysis of the structure of L-p-tyrosine shows regular cleavage planes exposing surfaces exhibiting the hydrophobic portion (phenol ring) of the molecule (Marti, 1969). While the crystal structure of DL-p-tyrosine (Mostad and Romming, 1973) has cleavage planes which exposure charged groups on both surfaces. Charged groups are believed to be important in a number of crystal-mediated toxic mechanisms (Mandel, 1976).

The charged surfaces of tyrosine crystals if formed *in vivo* are likely to interact readily with soluble intracellular proteins, in this regard the addition of proteins such as bovine serum albumin or rat plasma will protect the erythrocyte from haemolysis by L-p-tyrosine (see Figure 8 as an example). Certain amino acids will afford some protection against the haemolysis produced by L-p-tyrosine to erythrocytes (Figure 9). This may represent competition between tyrosine and the amino acid for binding to the membrane surface. Of particular interest is the finding that threonine will afford some protection at concentrations of 5 to 10mM (Figure 10). The inclusion of high levels of threonine in the diet of rats affords protection against ocular lesion produced by L-p-tyrosine (Alam *et al*, 1967) and SC-0735 (Smith *et al*, 1991). The concentration of threonine measured in the ocular fluid in these studies was about 10mM which is not dissimilar from that used *in vitro*.

## Studies With 3T3 Fibroblasts

Swiss mouse 3T3 fibroblasts were plated out onto 6 well plates (for microscopy) or 24 well plates (for LDH leakage) as described by Duffy *et al* (1987) and incubated overnight at 37°C to plate down. The following morning the cells were exposed to medium containing L-p-tyrosine or one of the putative protective agents for 4hr at 37°C with gentle shaking. The medium was then removed, briefly centrifuged to remove tyrosine

crystals and large cellular debris and the supernatant assayed for LDH. The data are expressed as a percentage of that released by Triton X-100 a membrane solubilising agent. The cells attached to the wells were then fixed in ethanol, and stained with haematoxylin and Papanicolaou, and the number of viable cells counted by microscopy.

Exposure of these cells to L-p-tyrosine crystals as a suspension at a concentration of 1-4mM (1000-4000nmol/ml) over a 4hr period caused no increase in LDH leakage into the medium (Figure 11). However at 6mM there was a marked increase in LDH leakage which increased with increasing tyrosine concentration (Figure 11). The onset of enzyme leakage was reflected in the morphological findings where there was a progressive increase in cellular damage with increasing tyrosine concentration. However in many cases the damage was focal with badly damaged cells lying next to apparently unaffected cells. These findings in viable cells, albeit a model system, indicate that L-p-tyrosine is toxic in the range 4000-6000nmol/ml which is close to the concentration detected in the eye after sustained triketone or tyrosine exposure in rats.

Further studies were made to ascertain whether the addition of protein (bovine serum albumin, 0.5mg/ml) or L-threonine (50mM) would protect the 3T3 against the cytotoxicity produced by L-p-tyrosine (10mM). Both agents afforded some protection but it was very small. At this time the project was stopped.

In summary these <u>in vitro</u> measures of cytotoxicity confirm and extend the studies of Goldsmith (1975) showing that L-p-tyrosine crystals do interact with membranes and can cause cytotoxicity. These effects occur at concentrations that are relevant to the <u>in vivo</u> situation and lend support to the hypothesis that tyrosine crystals in the eye could cause the lesion. However, to date we have been unable to obtain any evidence from ophthalmoscopy or microscopy of tyrosine crystals in SC-0735-induced eye lesions in rats.

## FUTURE DIRECTION

All experimental work on this aspect of the programme has stopped and any remaining effort will be directed towards writing the work up for internal reports and external publication. Collaboration with Professor Lindstedt will continue to find ways of making the chemical available for the treatment of tyrosinaemia type I.

## CONTRIBUTORS

Peter Gaskin and Liz Prescott were primarily involved with the tyrosine cytotoxicity studies. We are grateful to Dr Paul Duffy, Safety of Medicines, Pharmaceuticals, for help and advice with the 3T3 studies.

Other members of the project team who gave advice and assistance were Mac Provan, Mervyn Robinson, Martin Ellis and Lewis Smith.

The studies with Professor Lindstedt would not have taken place without the support of the Directors of Agrochemicals, Pharmaceuticals and CTL.

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Figure 1

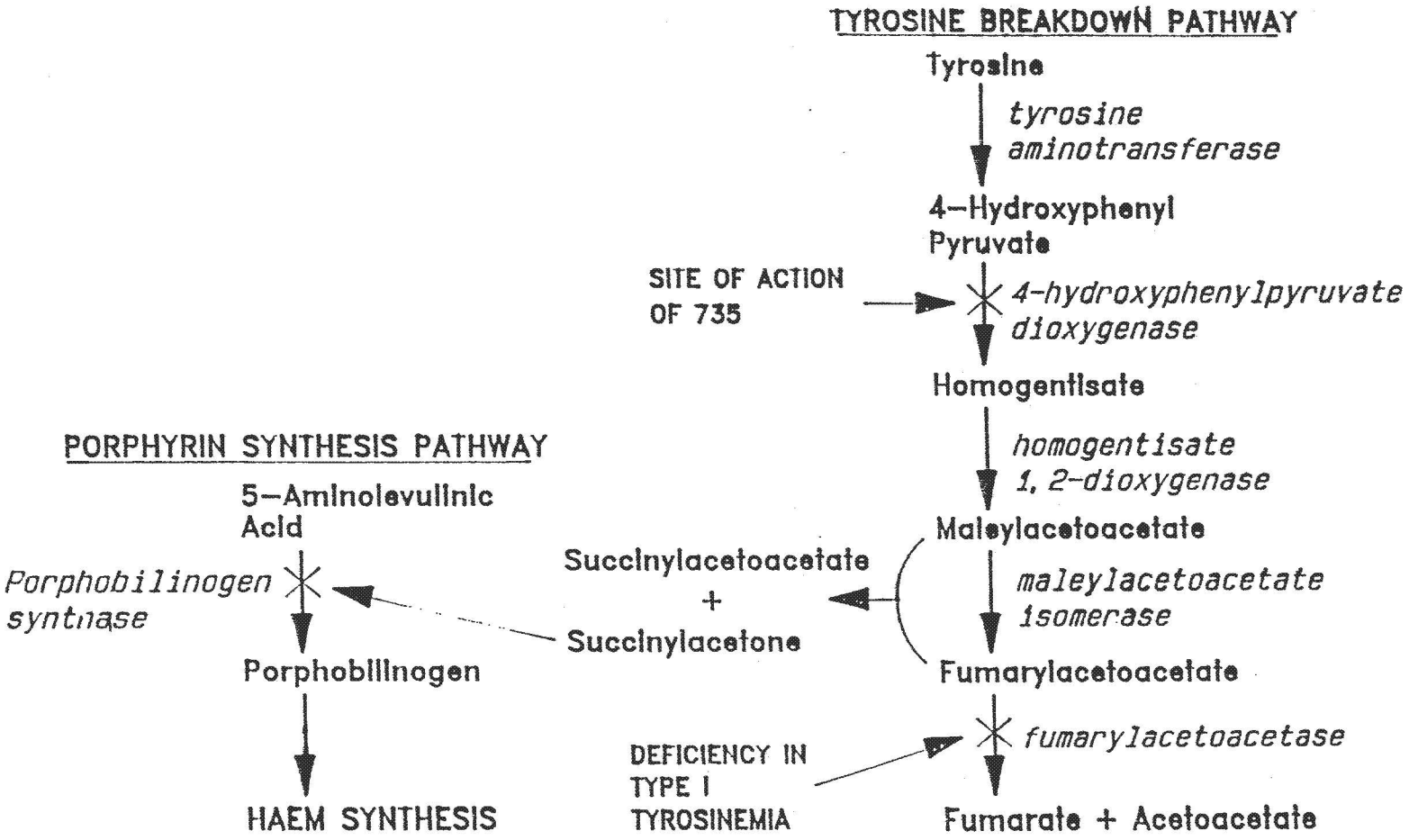


Figure 2

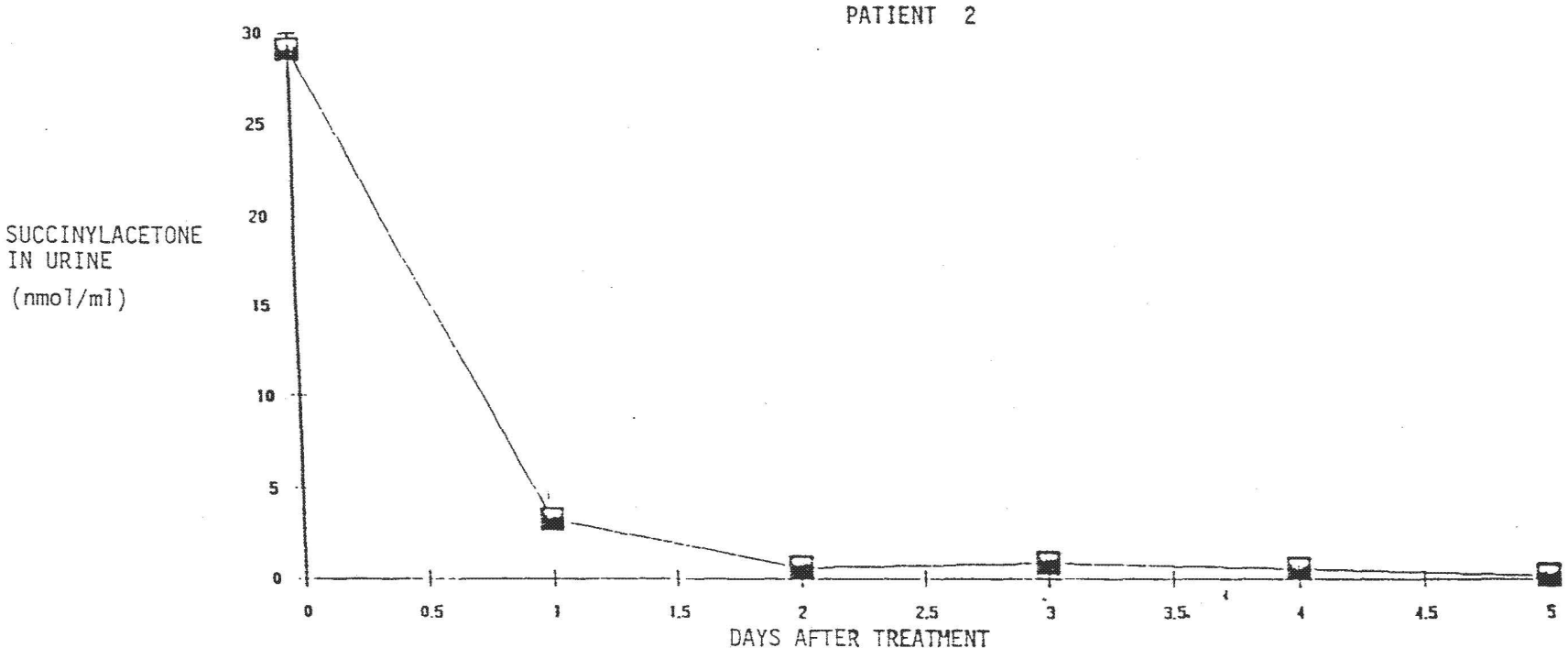

PATIENT 2
SUCCINYLACETONE
IN URINE
(nmol/ml)
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
DAYS AFTER TREATMENT

Figure 3

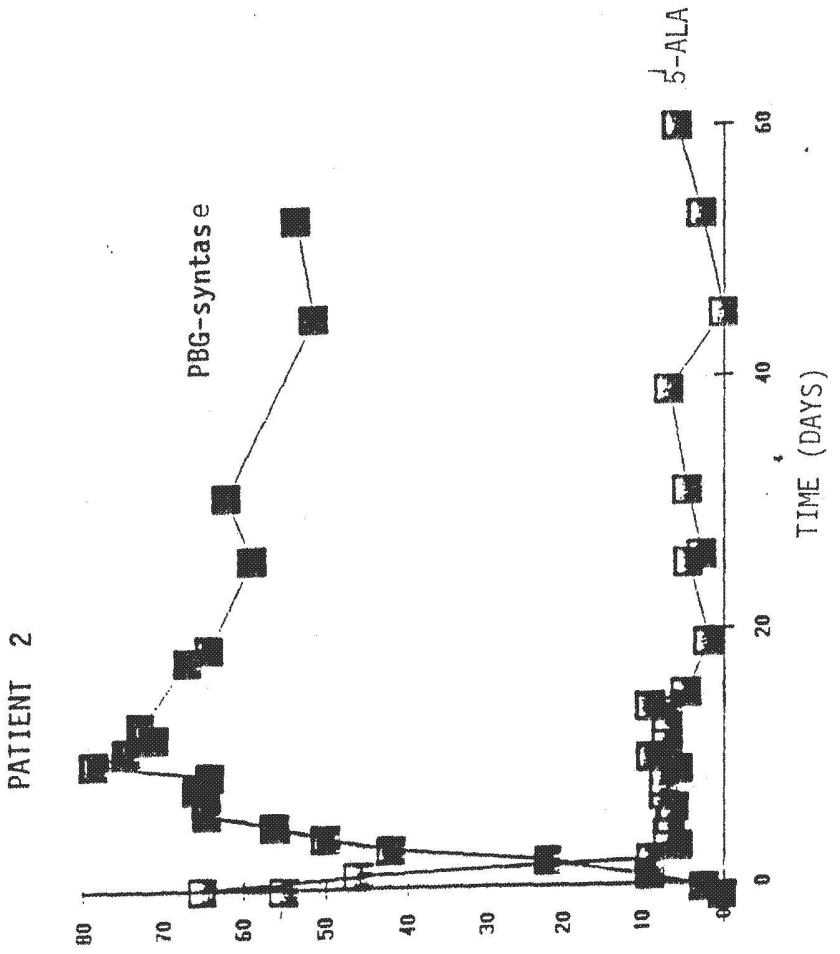


Figure 4

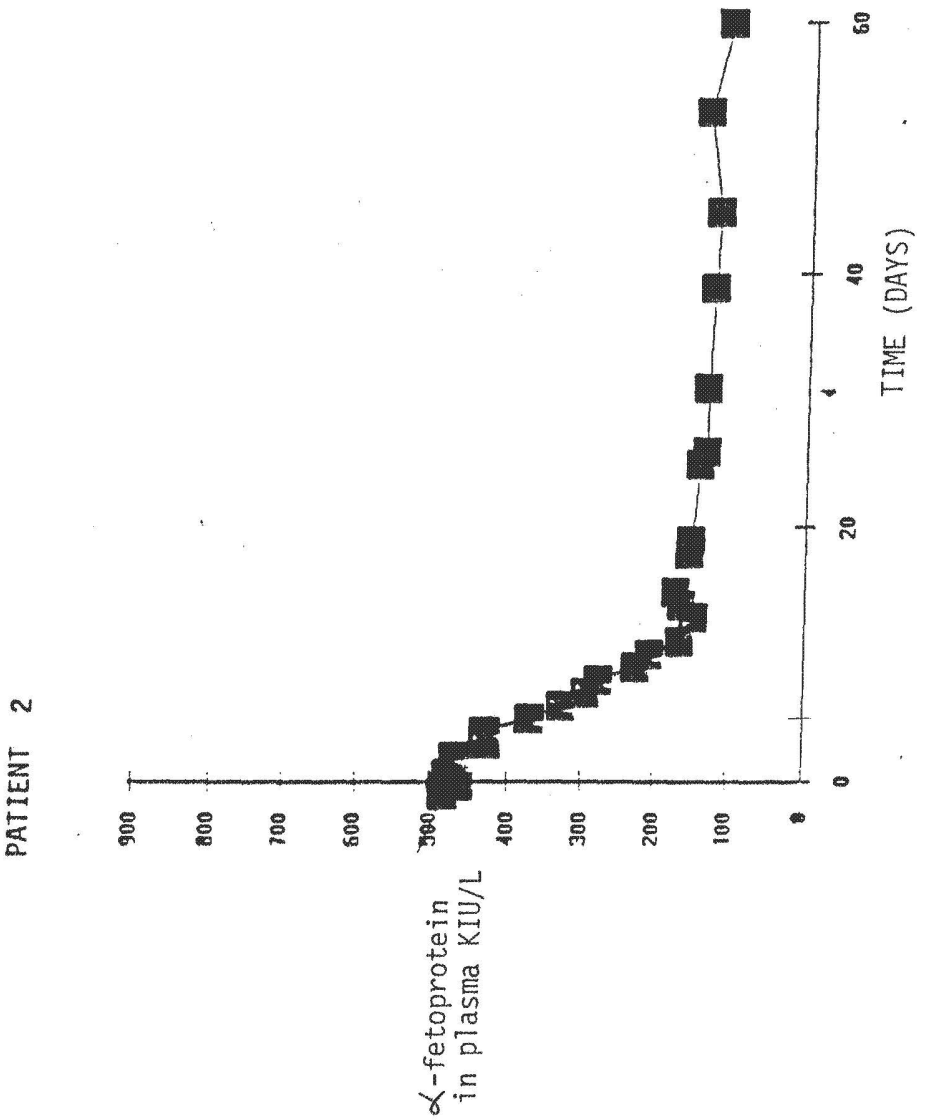


Figure 5

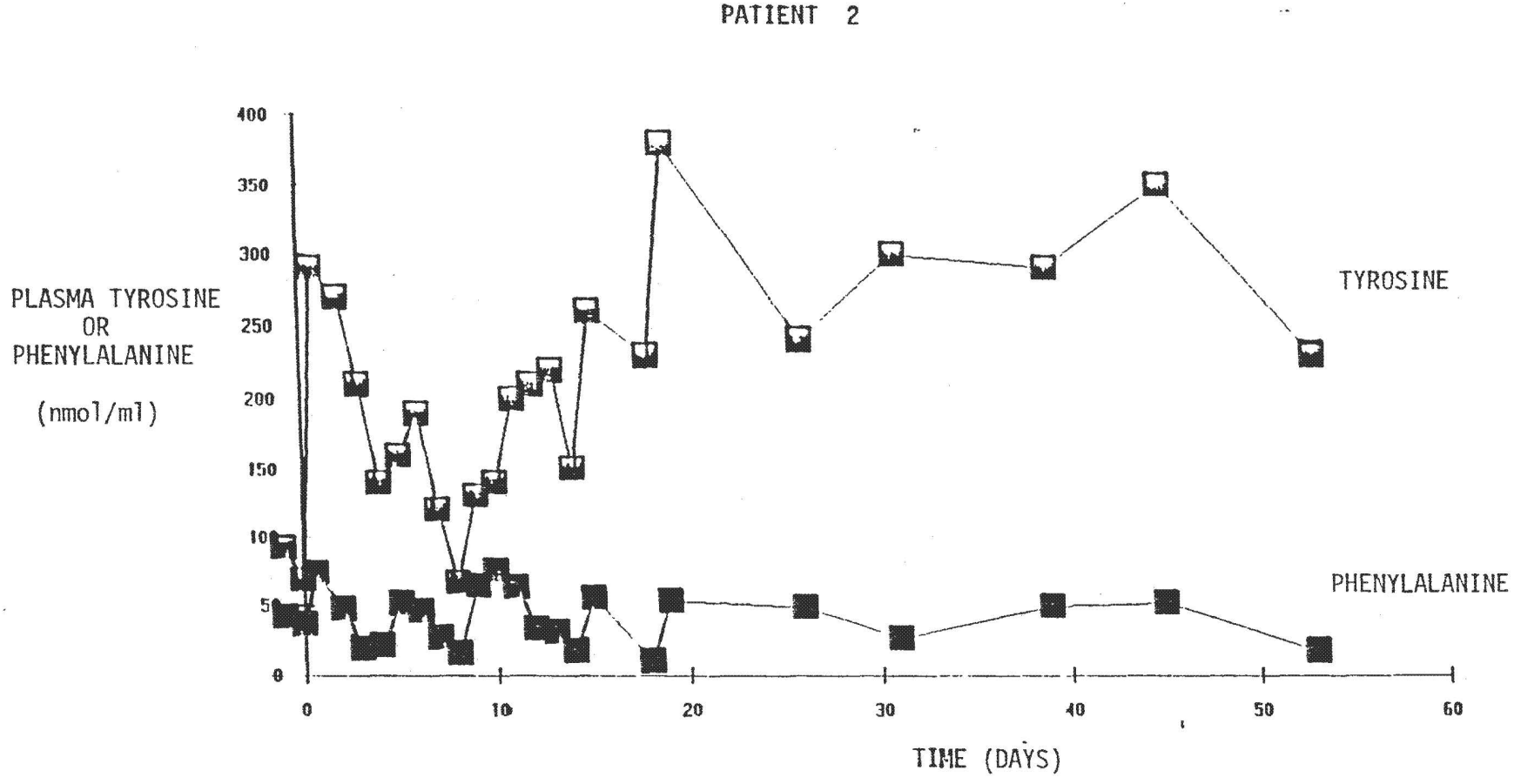


Figure 6

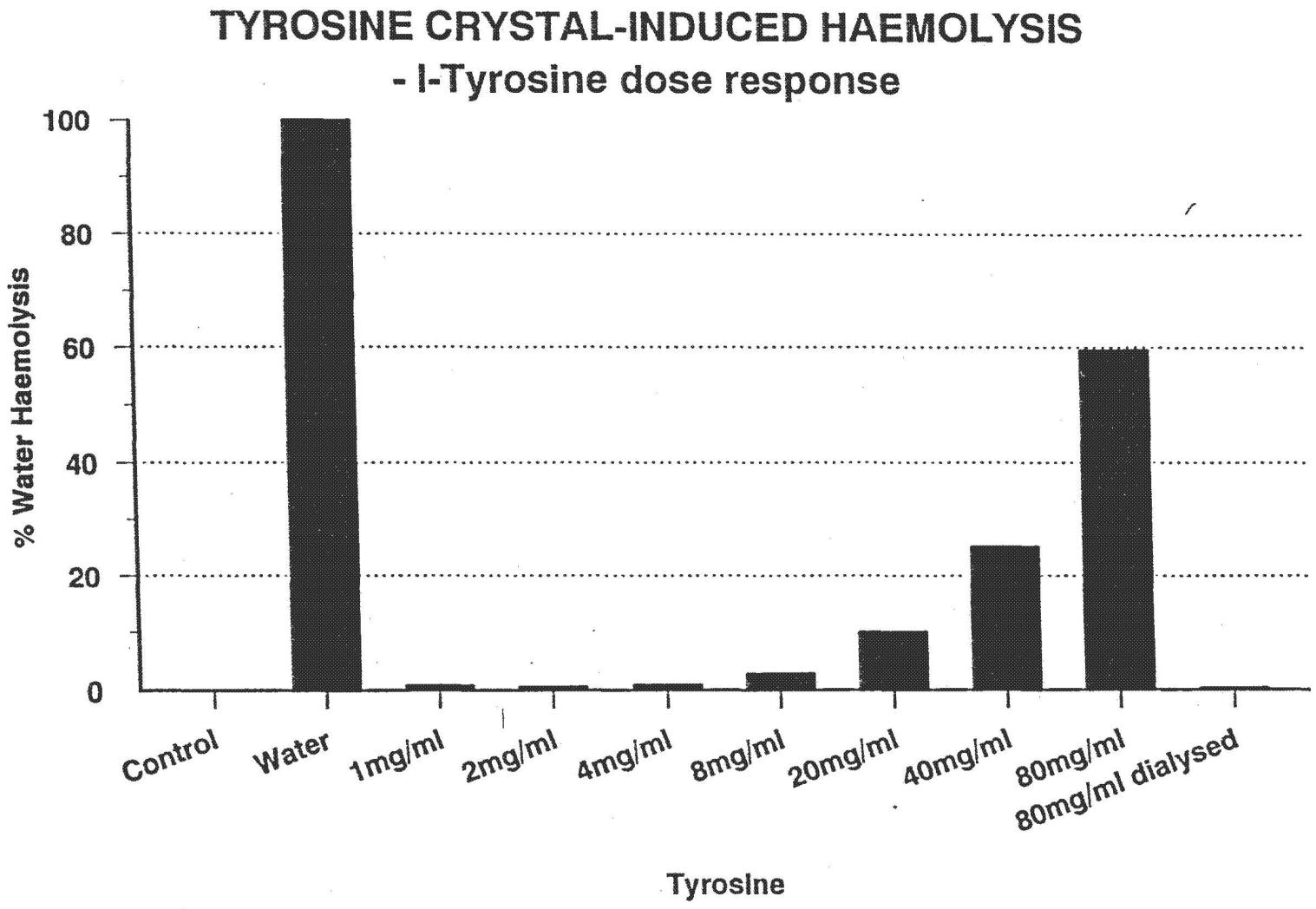


**Figure 7**

% water haemolysis
0
20
40
60
80
100
control
water
0.005mg/ml BSA
32mg/ml tyr
0.01mg/ml BSA
32mg/ml tyr
0.05mg/ml BSA
32mg/ml tyr
0.1mg/ml BSA
32mg/ml tyr
0.5mg/ml BSA
32mg/ml tyr
1mg/ml BSA
32mg/ml tyr
2mg/ml BSA
32mg/ml tyr
haemolysis of rat erythrocytes by l-p tyrosine
effect of bovine serum albumin
n=3
mean= +/- SD

Figure 8

**Figure 9**

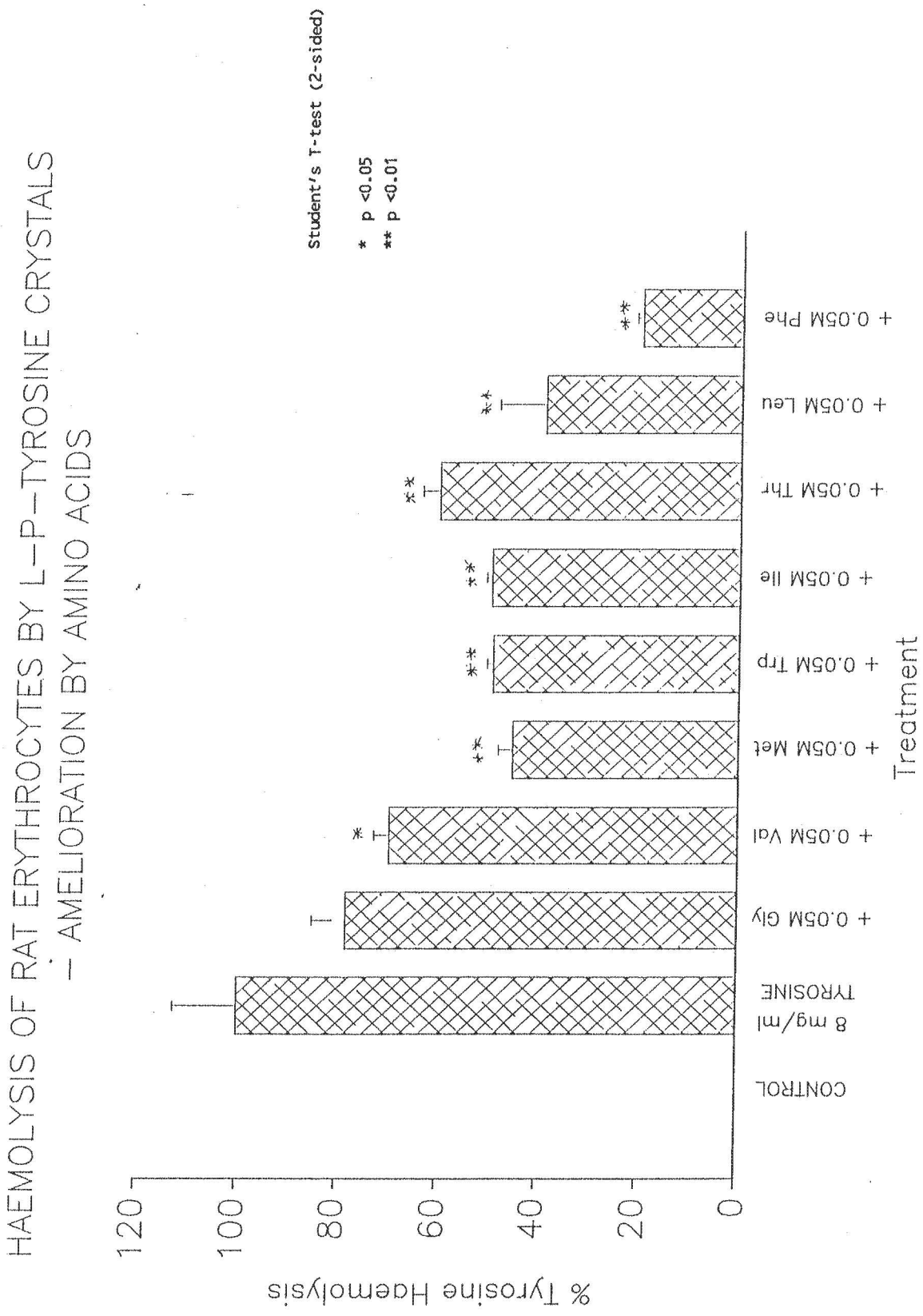
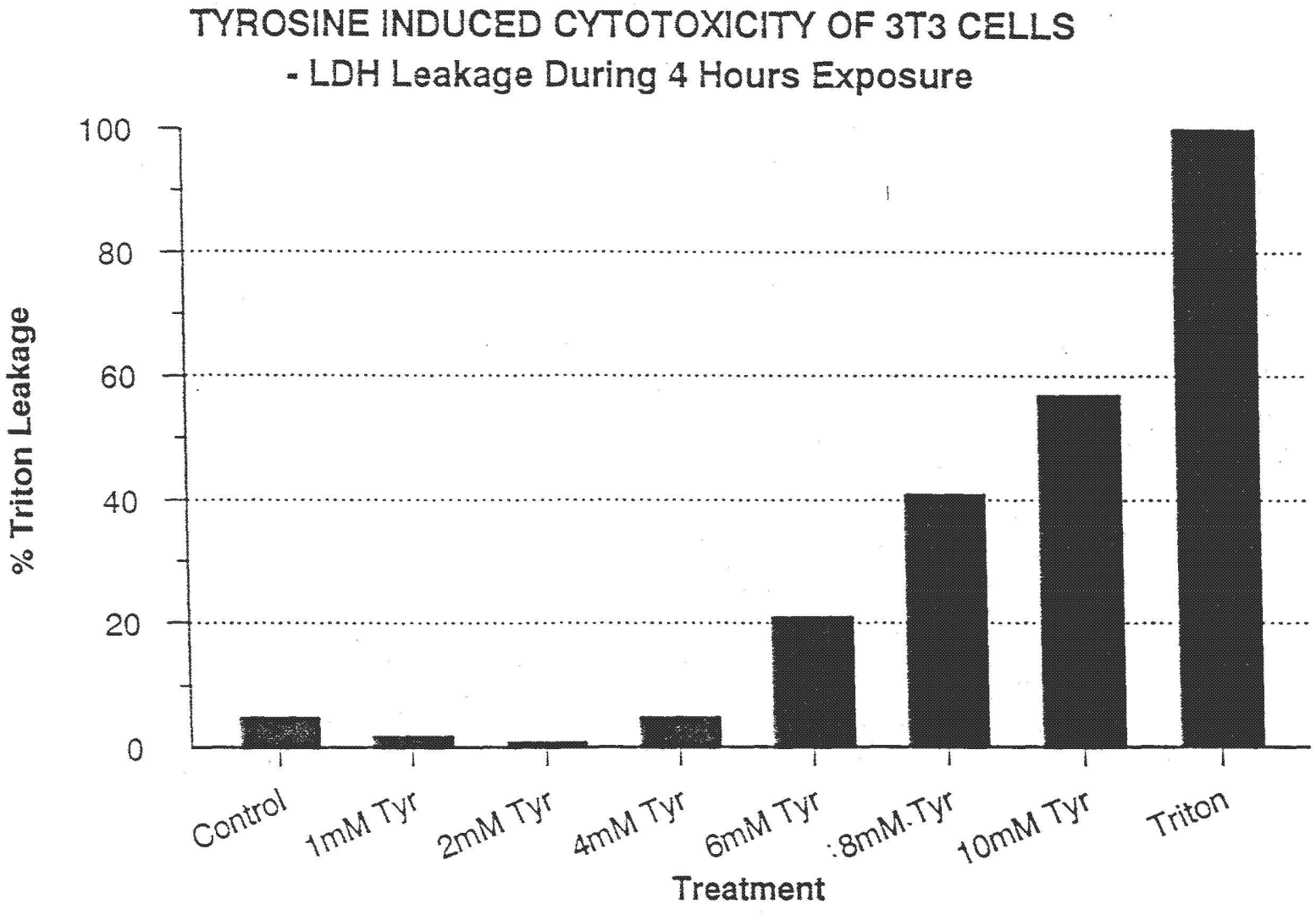


**Figure 10**

Figure 11



# NEPHROTOXICITY RESEARCH

L M FOWLER, J R FOSTER AND E A LOCK

## INTRODUCTION

This programme of work forms the basis of a PhD programme registered with the Council for National and Academic Awards for Ms Lynn Fowler under the supervision of Ted Lock (Biochemical Toxicology) and John Foster (Experimental Pathology); Lynn is just starting her final year. The project is aimed at trying to understand why certain chemicals are selectively toxic to the kidney and the chemical 4-aminophenol (PAP) was selected as a model compound for these studies.

PAP is used in photographic processing and along with other aminophenols as hair dyes. It is also formed during the metabolism of aniline, pesticides such as isopropyl carbanilate and the common analgesics paracetamol and phenacetin. PAP is acutely nephrotoxic, producing necrosis of pars recta of the proximal tubule in the rat following a single injection (Green et al 1969, Kiese et al 1975, Davis et al 1983). Associated with the morphological damage, elevation in blood urea nitrogen and the excretion of known indications of renal dysfunction in urine are observed (eg. see Gartland et al 1989). The mechanism by which PAP exerts its nephrotoxic action is not understood. It has been suggested that like paracetamol-induced hepatotoxicity, PAP-induced nephrotoxicity could result from formation of the reactive metabolite 1,4-benzoquinoneimine, that covalently binds to thiol groups of proteins and glutathione. This hypothesis is supported by the finding that PAP produces a dose-related decrease in renal glutathione (Crowe et al 1979).

Oxidative metabolism of PAP seems to be a prerequisite for toxicity and this can be readily demonstrated in vitro using horseradish peroxidase, prostaglandin synthase or rat liver microsomes, resulting in the formation of phenoxy radicals and 1,4-benzoqinoneimine (Josephy et al 1982). Quinonimines and their hydrolysis products quinones are particularly prone

to reductive 1,4-addition reactions of the Michael-type with sulphydryl groups. Recent groups in Germany (Eckert et al 1990) and Japan (Eyangi et al 1991) have studied the reaction products of 1,4-benzoquinoneimine with glutathione. They identified the formation of 4-amino-3-S-glutathionylphenol and 4-amino-2-S-glutathionylphenol and the corresponding formation of a 3,5-disubstituted thio-adduct and a 2,3,6-trisubstituted thio-adduct, respectively. These thio-adducts can autoxidise more readily in vitro than PAP itself, suggesting that thioether formation is not a detoxification process. Based on these latter findings and the fact that glutathione conjugation of several haloalkenes (studies done in CTL) and bromobenzene (Monks et al 1985) leads to selective toxicity to the kidney, the project has concentrated on the relevance of metabolism to the toxicity. The nephrotoxicity of known PAP metabolites such as paracetamol, PAP-O-sulphate and the putative glutathione metabolite 4-amino-3-S-glutathionylphenol (PAP-GSH) have been studied in rats. In addition, the effects of various modifiers of PAP metabolism or transport have been studied with regard to their influence on the nephrotoxicity.

## PROGRESS

We have confirmed previous observations in the literature showing that PAP produces a dose-related nephrotoxicity to Fischer 344 rats both using functional and morphological criteria (Table 1). A significant morphological finding, which had not been previously clearly documented was the discovery that at low doses of PAP the lesion is restricted to the pars recta of the proximal tubules in the medullary rays (cortical nephrons). As the dose of PAP increases so the lesion spreads to the entire length of the pars recta, affecting both cortical and juxta-glomerular nephrons (see Figure 1 for a diagrammatic representation). The basis for this nephron selectivity is not known. PAP is known to undergo metabolism primarily by N-acetylation to give paracetamol and by O-sulphation and O-glucuronidation (see Figure 2). There is also some indirect evidence that a minor pathway could involve conjugation with glutathione. We have administered two of the major metabolites and one putative minor metabolite to rats at similar molar doses to PAP to

determine whether they are nephrotoxic. A single dose of paracetamol at 920$\mu$mol/kg or PAP-O-sulphate at 920 or 1150$\mu$mol/kg produced no functional or morphological evidence of renal injury (data not shown). By contrast, administration of PAP-GSH produced a dose-related nephrotoxicity that was similar both functionally (Table 2) and morphologically to that seen with PAP. However the injury occurred at much lower doses of PAP-GSH, compared to PAP (see Table 2 versus Table 1 and Figure 3).

These findings report for the first time that conjugation of PAP with glutathione could be a toxic pathway of metabolism producing renal tubular necrosis and altered renal function at lower molar doses than PAP itself.

Recent work has focused on attempting to modify the metabolism of PAP or PAP-GSH in vivo to see whether this could influence the extent of nephrotoxicity. Three separate treatments have been used (1) co-administration of ascorbic acid to keep the chemical(s) in the reduced form and slow or prevent the formation of the reactive metabolite 1,4-benzoquinoneimine (2) prior treatment with acivicin, a potent inhibitor of renal $\gamma$-glutamyltranspeptidase, this enzyme is important in the processing of glutathione conjugates by the kidney and (3) prior administration of probenecid, an inhibitor of renal organic anion transport systems to see whether the selective necrosis to the pars recta of the proximal tubule could be related to a specific transport function.

Co-administration of ascorbic acid (3:1 molar excess) with PAP (458 or 687$\mu$mol/kg) protected against the nephrotoxicity (Figure 4). Additional studies with [$^3$H]PAP showed that this treatment reduced the total concentration of radiolabel in the kidney as well as that covalently bound to renal proteins (data not shown). Similarly ascorbic acid afforded partial protection against the nephrotoxicity produced by PAP-GSH (92-286$\mu$mol/kg) (data not shown). These findings support the hypothesis that further oxidation of these chemicals is required to produce the nephrotoxic response. Treatment with acivicin prior to PAP (458 or 687$\mu$mol/kg) did not afford protection, in fact it potentiated the nephrotoxicity (Figure 5). This finding is in agreement with the concentration of radiolabel from PAP in the renal cortex where the total

amount and that covalently bound to renal cortical proteins was increased. The basis for this potentiation is not currently understood.

In contrast, acivicin afforded total protection against the nephrotoxicity produced by PAP-GSH at doses less than 286$\mu$mol/kg as determined both morphologically and functionally (Figure 6). This latter finding strongly supports the view that further metabolism of the glutathione conjugate, presumably to the cysteine conjugate (Figure 2) is required to generate the proximate nephrotoxic metabolite. Prior treatment with probenecid had no effect on the nephrotoxicity of PAP or PAP-GSH (data not shown) indicating that probenecid sensitive transport systems do not appear to be involved in this kidney-specific toxicity.

## FUTURE PLANS

Future work will focus primarily in two areas which are outlined below. The first depends entirely on the availability of synthetic chemistry support to try and synthesise the cysteine or N-acetylcysteine conjugate of PAP. Assessment of the nephrotoxicity of these metabolites and attempts to inhibit their further metabolism *in vivo* should help pinpoint the proximate metabolite and provide some insight into the likely mechanism of toxicity. An exciting possibility is that our findings could have implication to paracetamol nephrotoxicity, a common form of poisoning in man. Is the glutathione conjugate of paracetamol (known to be formed in man) deacetylated to PAP-GSH and if so could it account for the renal impairment seen in some of these poisoning cases? Synthesis of this metabolite is planned to test this notion.

The second major area to be explored is the toxicity of these conjugates to isolated rat renal cortical cells. This *in vitro* system should enable some insight to be gained with respect to the likely mechanism of toxicity and determine some of the biochemical changes that occur in the kidney prior to cytotoxicity. The methodology for isolation of renal cells is currently being established in the laboratory and the system characterised prior to its use with these chemicals.

## CONTRIBUTORS

Lynn M Fowler full time, plus 0.5 man-year Corporate support from synthetic chemistry (Dick Moore), experimental pathology and clinical chemistry. Supervision and advice from John Foster and Ted Lock.

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1. Fowler L M, Moore R B, Foster J R and Lock E A. (1991). Nephrotoxicity of 4-aminophenol glutathione conjugate. Poster BTS. Edinburgh 18-20 September.

2. Fowler L M, Moore R B, Foster J R and Lock E A (1991). Nephrotoxicity of 4-aminophenol glutathione conjugate. Human and Exptal Toxicol. In press.

## Table 1

## The effect of 4-aminophenol (PAP) on urine volume, specific gravity and the excretion of urinary glucose, protein and γ-glutamyltransferase in rats over 24h.

| Dose ($\mu mol\ kg^{-1}$) | Dose ($mg\ kg^{-1}$) | Volume (ml) | Specific gravity | Glucose ($mg\ dl^{-1}$) | Total protein ($mg\ dl^{-1}$) | γ-Glutamyltransferase ($IU\ l^{-1}$) |
|---|---|---|---|---|---|---|
| | 0 | 6 ± 0.4 | 1.059 ± 0.002 | 154 ± 51 | 364 ± 12 | 1685 ± 267 |
| 92 | 10 | 5 ± 0.9 | 1.066 ± 0.004 | 138 ± 112 | 411 ± 26 | 1628 ± 581 |
| 183 | 20 | 6 ± 0.8 | 1.059 ± 0.003 | 148 ± 104 | 458 ± 24* | 1756 ± 543 |
| 367 | 40 | 9 ± 1.2 | 1.056 ± 0.005 | 1727 ± 148* | 443 ± 22* | 4892 ± 768* |
| 458 | 50 | 11 ± 0.7* | 1.052 ± 0.003 | 2448 ± 93* | 555 ± 34* | 5776 ± 486* |
| 687 | 75 | 12 ± 0.8* | 1.055 ± 0.003 | 2547 ± 104* | 601 ± 24* | 6994 ± 543* |
| 920 | 100 | 15 ± 0.6* | 1.046 ± 0.002* | 2835 ± 76* | 828 ± 18* | 8287 ± 397* |

PAP was administered ip at the doses shown. Results are mean ± s.e. with at least four animals per dose.
* Statistically significantly different from controls using two-way ANOVA, $P < 0.05$.

## Table 2

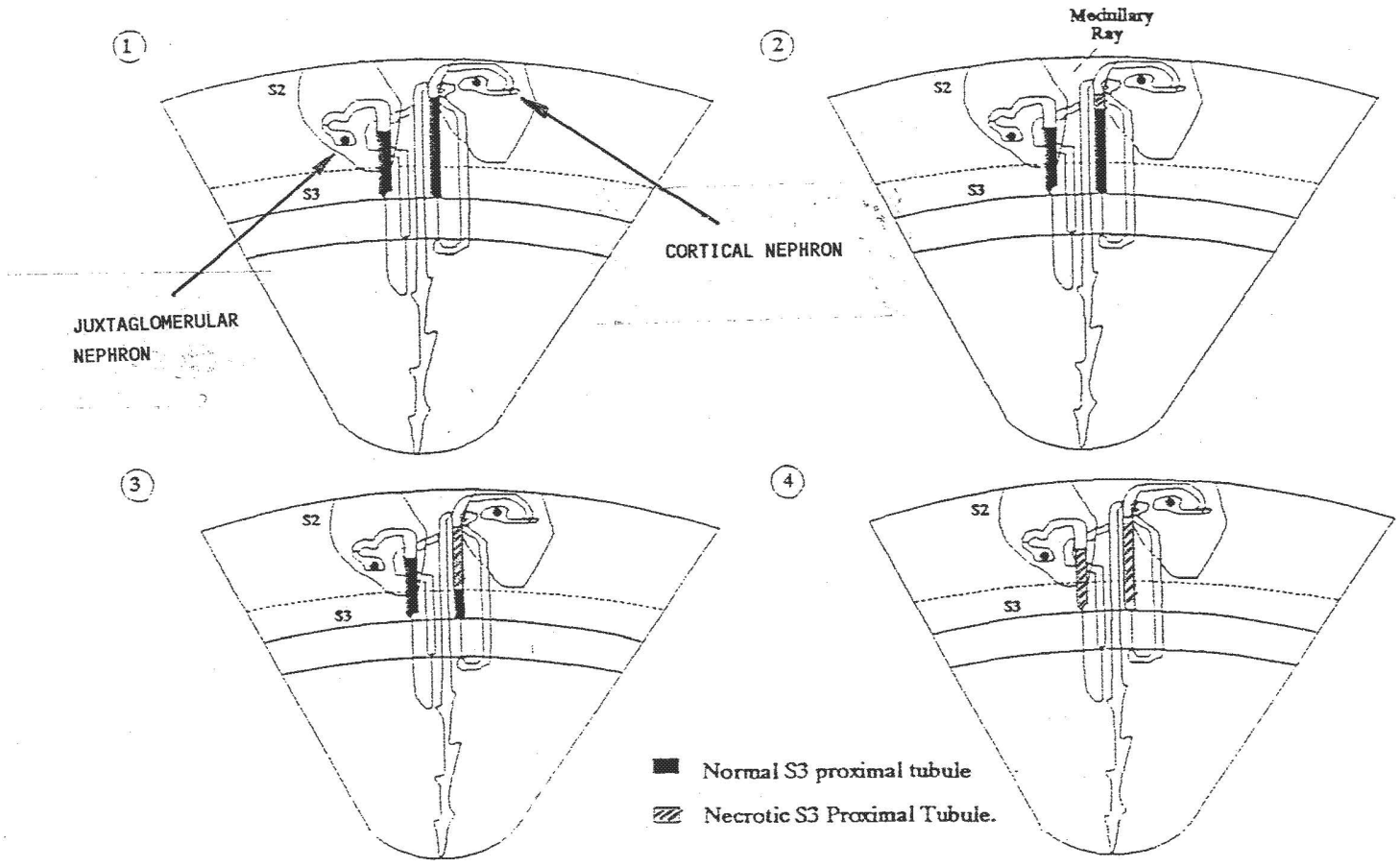
## The effect of 4-amino-3-S-glutathionylphenol (PAP-GSH) on urine volume, specific gravity and the excretion of urinary glucose, protein and γ-glutamyltransferase in rats over 24h.

| Dose ($\mu mol\ kg^{-1}$) | Volume (ml) | Specific gravity | Glucose ($mg\ dl^{-1}$) | Total protein ($mg\ dl^{-1}$) | γ-Glutamyltransferase ($IU\ l^{-1}$) |
|---|---|---|---|---|---|
| 0 | 6 ± 0.4 | 1.063 ± 0.003 | 178 ± 51 | 453 ± 22 | 1943 ± 410 |
| 92 | 4 ± 0.8 | 1.070 ± 0.005 | 189 ± 88 | 560 ± 38* | 3413 ± 710* |
| 183 | 8 ± 0.8 | 1.058 ± 0.005 | 1620 ± 88* | 695 ± 38* | 4852 ± 710* |
| 276 | 12 ± 0.8* | 1.050 ± 0.005 | 1615 ± 88* | 728 ± 38* | 5797 ± 710* |
| 367 | 12 ± 1.1* | 1.053 ± 0.007 | 1628 ± 125* | 895 ± 54* | 9314 ± 1005* |
| 458 | 5 ± 0.8 | 1.057 ± 0.005 | 2066 ± 88* | 1188 ± 38* | 15418 ± 710* |
| 920 | 0.4 ± 0.2* | N.D. | 4143, 5076 | 3162, 2941 | N.D. |

PAP-GSH was administered ip at the doses shown. Results are mean ± s.e. with four animals per dose.
* Statistically significantly different from controls using two-way ANOVA, $P < 0.05$. N.D. not determined.

# Figure 1

Dose-dependent Necrosis of the S3 Proximal Tubules of the Kidney following Exposure of Rats to p-aminophenol.




Diagrammatic representation of renal damage 24hr after dosing
1. Control; 2. PAP, 183$\mu$mol/kg; 3. PAP, 367$\mu$mol/kg; 4. PAP, 920$\mu$mol/kg.

Figure 2

**Proposed nephrotoxic pathway of metabolism of PAP in the rat**

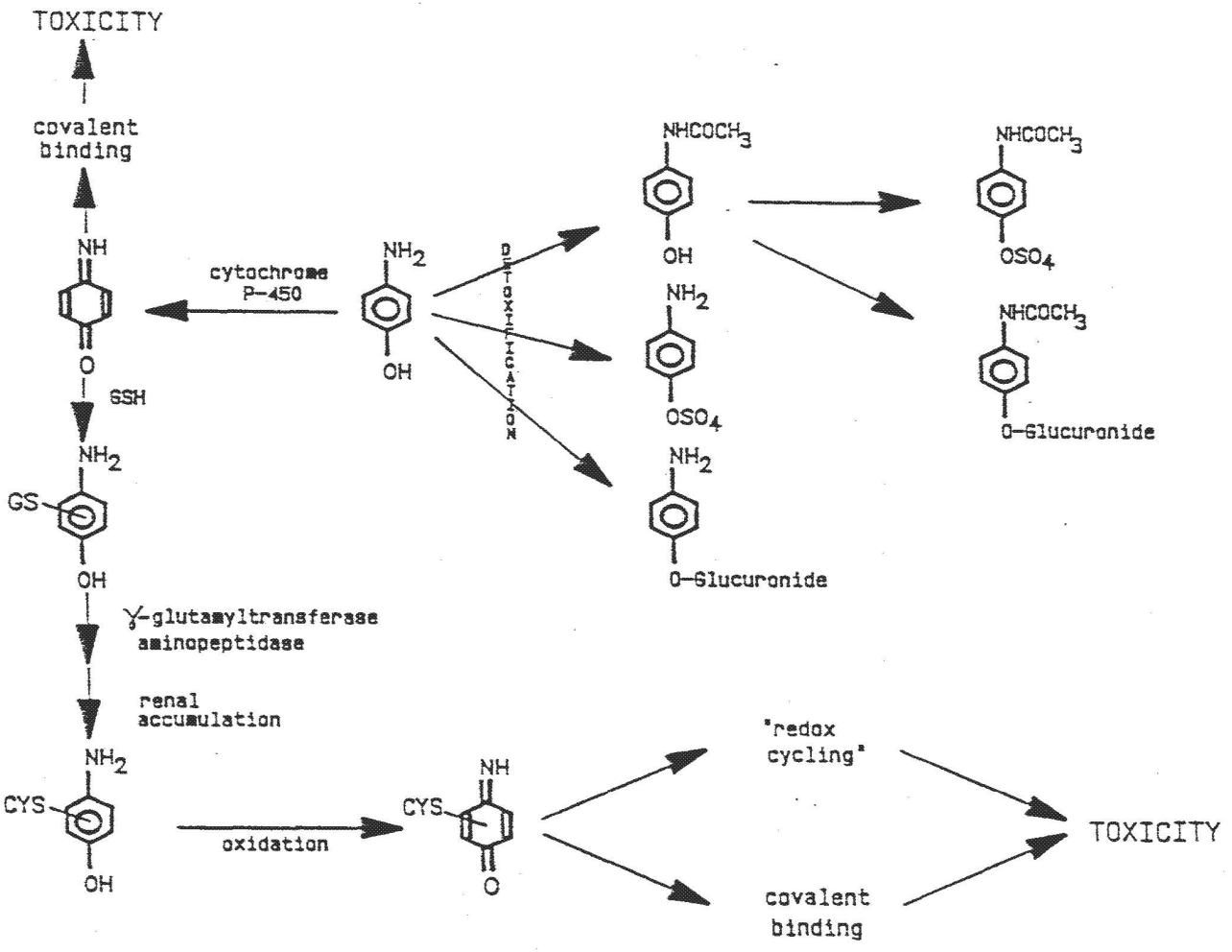


Figure 3

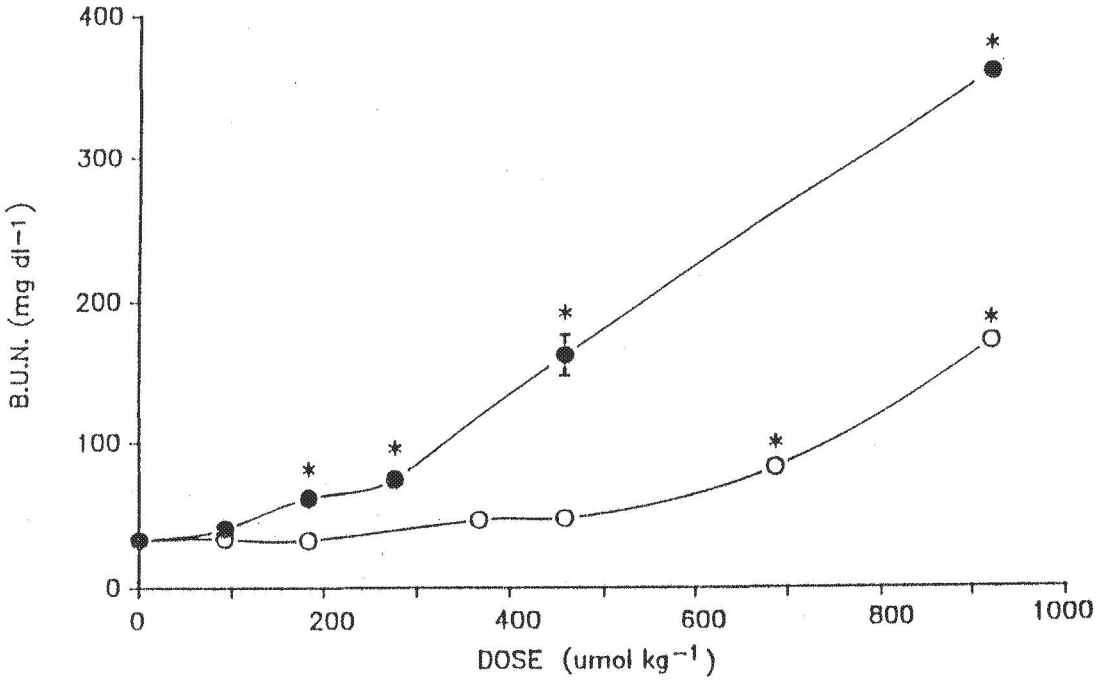
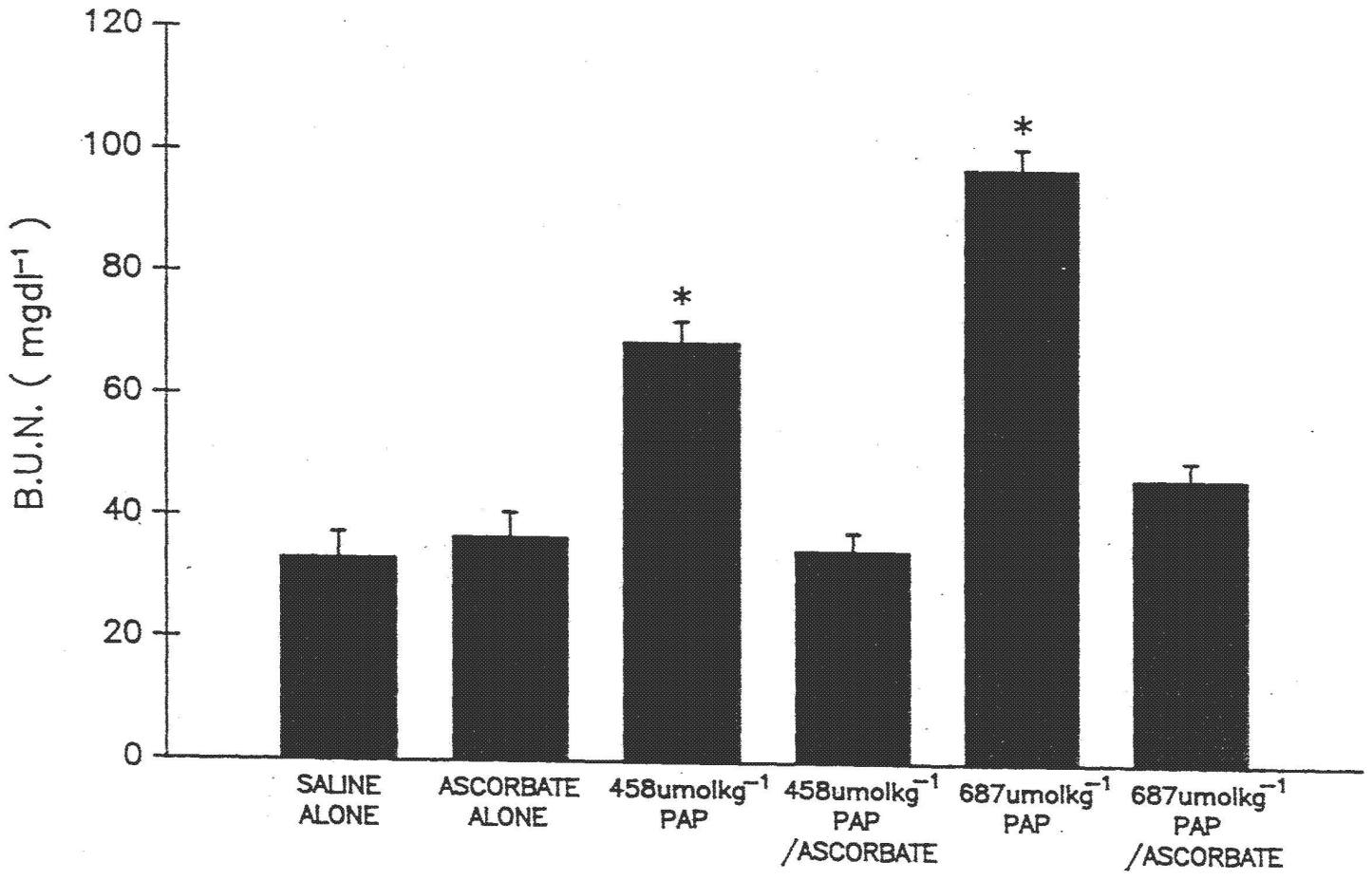



Figure 3. The effect of 4-aminophenol (PAP) and 4-amino-3-S-glutathionylphenol (PAP-GSH) on blood urea nitrogen levels in the rat. Rats were given a single i.p. dose of PAP (o) or PAP-GSH (●) at the doses shown and blood urea nitrogen measured 24h after dosing. Results are mean ± S.E. with at least four animals per dose. In most cases the error bars lie within the symbol. * Statistically significantly different from controls $P < 0.05$.

Figure 4

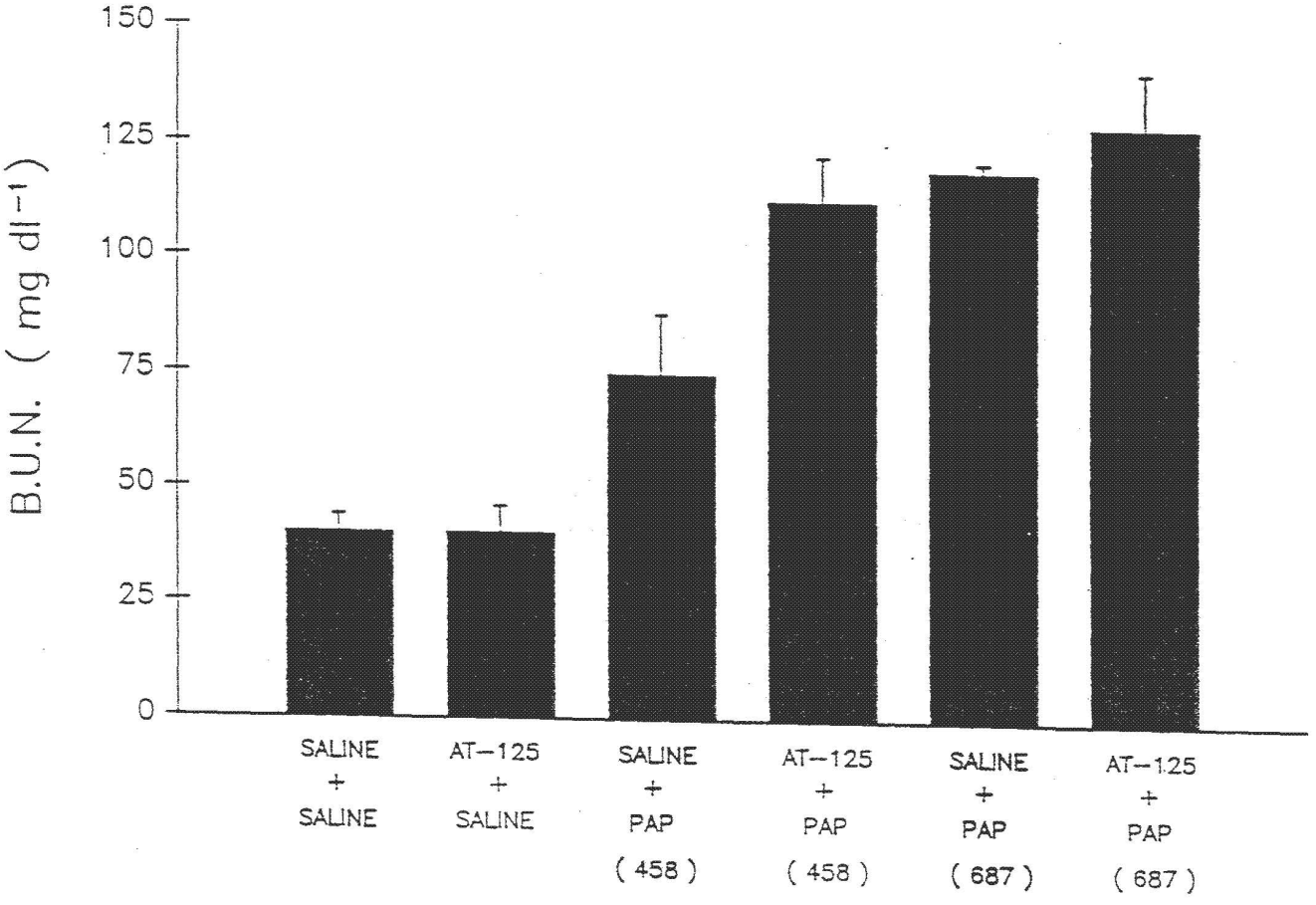
The effect of ascorbic acid on 4-aminophenol (PAP) induced elevation in blood urea nitrogen in the rat.




Ascorbic acid was co-administered (3:1) molar excess) with PAP, ip, at the doses shown and blood urea nitrogen determined 24hr after dosing. * Statistically significantly different from control. $P < 0.05$.

Figure 5

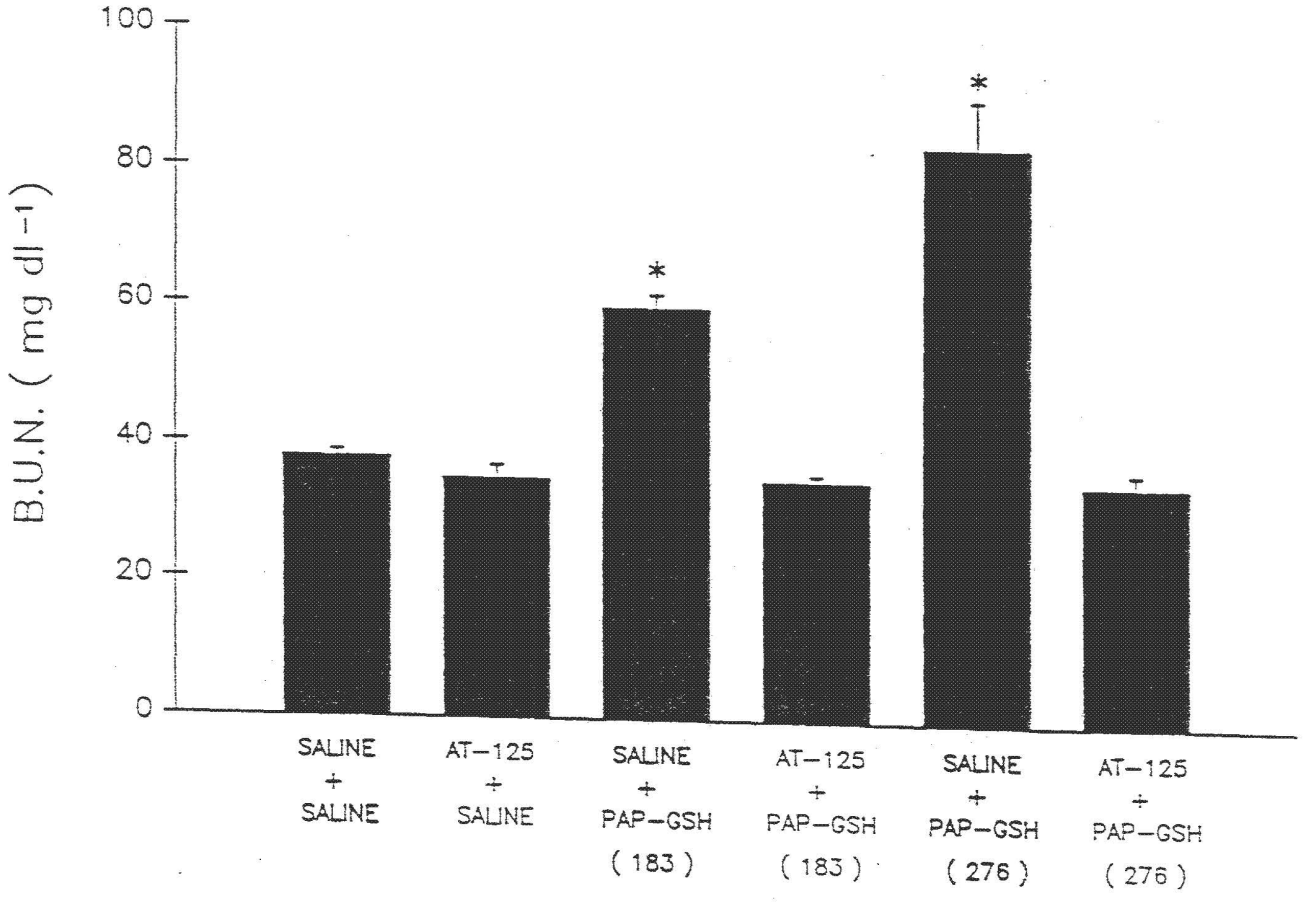
The effect of acivicin (AT-125) on 4-aminophenol (PAP) induced elevation in blood urea nitrogen in the rat.




Acivicin was administered at 10mg/kg 1hr before PAP and blood urea nitrogen determined 24hr after dosing.

Figure 6

**The effect of acivicin (AT-125) on 4-amino-3-S-glutathionylphenol (PAP-GSH) induced elevation in blood urea nitrogen in the rat**




Acivicin was administered at 10mg/kg 1hr before PAP-GSH and blood urea nitrogen determined 24hr after dosing.
* Statistically significantly different from control $P<0.05$.

# GASTROINTESTINAL ABSORPTION OF PARAQUAT

JON R HEYLINGS

## INTRODUCTION

The sales of paraquat are forecast to rise through the 1990s within an expanding non-selective contact herbicide market. However, GRAMOXONE and other paraquat based products continue to face pressure from Regulatory authorities which is often centred around the incidence of human paraquat poisonings, mainly suicides. Commercial assessment by ICI Agrochemicals indicates that a toxicologically safer formulation paraquat is required to provide a strategic response to de-regulation. Collaborative research between Biochemical Toxicology here at CTL and ICI Agrochemicals at Jealott's Hill over the last four years has been directed towards devising such a new formulation which meets the criteria of (i) a 10-fold reduction in paraquat oral toxicity and (ii) at least 90% biological efficacy relative to standard paraquat.

The majority of the research effort at CTL during 1991 has centred on the optimization of a paraquat formulation containing additives which have specific pharmacological properties designed to reduce the absorption of paraquat from the gastrointestinal tract. These additives include two magnesium salts, one a purgative (magnesium sulphate) and the other an antacid/gelling agent (magnesium trisilicate). The formulation which was first prepared at CTL in 1988 is known unofficially as MAGNOXONE. Most of the research studies have been carried out in the dog since this animal has been shown to be very similar to man in terms of paraquat pharmacokinetics. Over the last twelve months a total of 26 variants of MAGNOXONE have been examined in the dog at several dose levels. The progress with the MAGNOXONE research programme over the last year is reported.

In addition to the work on new potentially safer formulations of paraquat, the GI Tract Research group have explored new aspects of paraquat toxicity. This research stems from the observation in 1990 that calcium-dependent

paraquat uptake from the gastrointestinal tract can be inhibited by magnesium. During 1991, calcium absorption across rat isolated duodenum has been studied. The objective here was to compare the mechanism of absorption of this nutrient divalent cation with the toxic divalent cation paraquat in the same in vitro duodenal preparation, in order to examine the nature of calcium-dependent paraquat uptake. A second research objective this year was to determine how calcium increased the systemic toxicity of paraquat, particularly in lung and kidney, and whether magnesium given parenterally could reduce such damage and therefore have potential antidote properties for paraquat in man.

## PROGRESS

### TOXICOLOGY OF NEW FORMULATIONS OF PARAQUAT IN THE DOG

At the end of 1990 the GI Tract Research group had made a new 200g/l formulation of paraquat containing the antacid magnesium trisilicate (100g/l), the purgative magnesium sulphate (100g/l) and a thickening agent, kelzan (3g/l). In addition, the concentration of emetic PP796 was optimized in extensive dog studies at 1.5g/l. This formulation, MAGNOXONE M19 was tolerated in dogs at 32, 64 and 128mg/kg paraquat doses. The safety factor of the formulation is estimated at around 15-fold compared to GRAMOXONE, which is lethal to dogs at 12mg/kg.

Following a Technical Review Committee meeting at ICI Agrochemicals in November 1990 (TMF 32821C), it was decided to continue research on MAGNOXONE at CTL with a view to optimizing a new paraquat formulation which also met the commercial requirements of the Agrochemicals Business. Since MAGNOXONE had been invented at CTL (UK Patent Application No: 9015134.1), and each new formulation variant had been made up in our own laboratory, the first phase of the research programme involved transferring our technology to Agrochemicals Formulation Research group at Yalding. The formulation chemists at Yalding produced a larger scale version of MAGNOXONE M19 according to our specifications containing the ingredients shown in Table 1.

This formulation (YF7981) was tested in dogs at 32 and 64mg/kg. At these doses, which are equivalent to 3 and 6 lethal doses of paraquat respectively, there was no toxicity in the dog. A new version of MAGNOXONE containing commercial grade trisilicate, YF7981A, was also tested at 32 and 64mg/kg paraquat doses. Again, the plasma paraquat area-under-curve (AUC) and clinical observations were identical to CTL's original version of MAGNOXONE M19, thus confirming the robustness of the system on larger scale formulation.

Simultaneous studies were carried out for biological efficacy in the glasshouse, plus extensive storage stability trials at a range of temperatures. Several versions of MAGNOXONE have been tested in field trials around the world during 1991 against the local paraquat product. In all cases MAGNOXONE has performed equally to control against a wide range of plant species. In addition there were no problems with dilution, sprayability, cleaning of equipment etc in these trials. However, during the course of storage studies it was found that the magnesium sulphate crystallized out of the formulation after 12 weeks at 40 and 50°C. As a result of this we are currently examining MAGNOXONE using lower levels of magnesium sulphate as well as testing the more soluble magnesium chloride as an alternative purgative in the formulation. Table 2 summarizes the data in the dog at different dose levels for those variants of MAGNOXONE. The data is presented as mean plasma paraquat area-under-curve (AUC) over 24 hours following a single oral dose in three animals.

To date, reduction of $MgSO_4$ to 10g/l (YF8003) or, alternatively, switching to 100g/l $MgCl_2$ does not affect the safening properties of MAGNOXONE. Figure 1 shows the effect of 64mg/kg paraquat in the dog. MAGNOXONE formulations containing 10 or 50g/l $MgSO_4$ gave comparable plasma profiles and are non toxic in this species. In addition, replacing $MgSO_4$ for $MgCl_2$ does not compromise safening. At 4 hours there is very little paraquat in plasma. GRAMOXONE given at 64mg/kg would give plasma values above 10$\mu$g/ml at 4 hours and would be invariably fatal. Separate studies using GRAMOXONE containing different levels of $MgSO_4$ have demonstrated that the purgative action of this salt only occurs with 50g/l $MgSO_4$ when a single lethal dose of GRAMOXONE is given to dogs. The recommendation to the Business is that YF8004 (which contains 50g/l $MgSO_4$) is chosen at the 'lead' formulation at this time.

## EFFECT OF THE DIVALENT CATIONS CALCIUM AND MAGNESIUM ON PARAQUAT TOXICITY

### INTRODUCTION

During the course of the Agrochemicals' Research programme using Multiple Emulsion formulations of paraquat, we observed that when the external water phase of the emulsion contained calcium chloride that these emulsions were surprisingly more toxic than sodium chloride based systems. As a result we examined the potential for calcium to enhance paraquat absorption by adding it to the liquid paraquat concentrate GRAMOXONE. These studies demonstrated that calcium increased the toxicity of paraquat quite markedly. Plasma levels of paraquat were seven times higher in rats within 15 min after oral dosing and the median lethal dose was reduced from 100mg/kg to 50mg/kg in this species. We also reported last year (CTL/R/1054) that magnesium could inhibit calcium-dependent paraquat uptake in the rat both *in vitro* and *in vivo*.

### PROGRESS

During 1991, we have further explored the role of both calcium and magnesium in paraquat toxicity in terms of absorption and target cell toxicity. We have demonstrated this year that our Ca/Mg hypothesis for paraquat absorption extends to the dog. Thus, when animals were dosed with GRAMOXONE containing either 1.5M $CaCl_2$ or 1.5M $MgCl_2$ there was a significant difference in the plasma paraquat AUC values following a non-lethal paraquat dose of 8mg/kg. GRAMOXONE containing $CaCl_2$ gave an AUC of 23.3 ± 5.3$\mu$g/ml.h compared to 13.4 ± 3.9$\mu$g/ml.h for $MgCl_2$. Since the amount of $MgCl_2$ was below a dose which would cause emesis and/or purgation in dogs, it is presumed that Mg has an additional effect on reducing paraquat uptake into the systemic circulation.

There is also the possibility that magnesium may protect vital organs involved in the systemic toxicity of paraquat. For instance, if paraquat

uptake into lung or kidney cells is dependent on calcium, and the process is inhibited by magnesium (as found in the gastrointestinal tract) perhaps lung and kidney function can be maintained following magnesium dosing, even with high plasma paraquat concentrations. This has been one of our main research activities during 1991, and has been examined in the rat in vivo by dosing magnesium parenterally. The mechanism by which calcium enhances paraquat toxicity probably involves enhanced gut absorption. However, this has not been conclusively demonstrated to date. We therefore have conducted a number of in vitro studies with calcium and paraquat in order to more fully examine the role of Ca and Mg in the paraquat absorption process.

## (i) Duodenal Uptake of Paraquat In Vitro

Paraquat is absorbed in the small intestine of rats by a saturable, energy dependent process up to luminal levels of 100mg/ml. Above this dose, paraquat is absorbed by passive diffusion through a damaged epithelial cell membrane (Heylings, J R. Toxicol. Appl. Pharmacol., 1991). We therefore have examined the effect of Ca on this process using the rat isolated duodenal mucosa. Initially, we examined Ca absorption using $^{45}Ca$ in our previously developed in vitro model (Heylings J R. The Toxicologist, 1991). In the absence of paraquat, Ca absorption across rat duodenum was energy dependent, being inhibited by both anoxia and at 4°C. Over a luminal calcium concentration range of 0.5-10mM, the absorption of calcium from lumen to blood side was saturable (Figure 2). Luminal concentrations of 1.25-2.5mM calcium were used as a submaximal doses for further studies with magnesium and paraquat. As shown in Figure 3, addition of 1-10mM Mg to the luminal solution inhibited mucosal uptake of calcium in rat duodenum.

Paraquat absorption was then followed in the presence of calcium. The luminal solution was spiked with $^{3}H$-paraquat and $^{45}Ca$ in order to detect both calcium and paraquat absorption by dual label counting. A 20mg/ml luminal concentration of paraquat was used, which is around the Km value for paraquat uptake in this model. Paraquat uptake into the duodenal mucosa was significantly increased by almost 4-fold when calcium (2.5mM)

was added to the luminal solution bathing the duodenum (Figure 4). This was also the case with a lower calcium concentration (1.25mM) as shown in Figure 5 over a full time course. Hence, when paraquat total absorption from lumen to blood side was measured over 4 hours, significant increases in paraquat absorption occur as early as 10 minutes after exposure to calcium.

(ii) Distribution of Paraquat In Vivo

During 1991, we have also explored the role of parenteral magnesium on paraquat toxicity in the rat. This has involved both a biochemical and pathological assessment of paraquat toxicity in the lung and kidney. The objective of this research was to determine whether the 'safening' properties afforded by MAGNOXONE extend beyond the level of the gastrointestinal tract, as suspected from early dog studies. We therefore embarked on a number of dosing studies in rats where paraquat was given orally as GRAMOXONE at a predicted lethal dose (200mg/kg). In addition, various other treatments including intraperitoneal dosing with magnesium sulphate were examined.

In previous reports we have shown that oral dosing of GRAMOXONE formulations containing high doses of $MgSO_4$ increases survival in rats (CTL/R/1009). This is undoubtedly due to a reduction in paraquat absorption caused by the purgative action of this salt. This has been confirmed recently where faecal mass and paraquat excretion were significantly higher over 48 hours in $MgSO_4$-treated rats. However, it was also noted in this study that the urinary excretion of paraquat was also more effective despite there being less bipyridyl in plasma over this time period. As shown in Figure 6 the rate of urinary excretion of paraquat, especially over 3-7 hours is much higher in the $MgSO_4$-treated rats. This is probably due to more efficient renal excretion of paraquat over this time. It is therefore possible that Mg may have inhibited the uptake of paraquat into the renal tubular cells. We therefore examined the effect of Mg given intraperitoneally on paraquat toxicity in the rat.

A single i.p. dose of $MgSO_4$ immediately following 200mg/kg paraquat (as GRAMOXONE) resulted in a significant increase in survival from paraquat intoxication in rats. Initial studies were conducted with 100, 200 and 300mg/kg Mg i.p. This was later reduced to 50mg/kg i.p. which proved to be the lowest effective antidote dose of Mg. It was found that intraperitoneal dosing with $MgSO_4$ was even more effective at reducing both lung and kidney pathology induced by paraquat compared to oral dosing of $MgSO_4$. Tissue levels of paraquat at both 15 min (Table 3) and 24 hours (Table 4) after paraquat dosing were reduced following i.p. $MgSO_4$. Furthermore the plasma creatinine and blood urea nitrogen (BUN) levels were normal in the i.p. $MgSO_4$ dosed groups which fits well with observed reduction in kidney damage assessed pathologically. Plasma Mg levels were significantly raised in the first hour following i.p. dosing but within a normal range by 24 hours.

The addition of calcium chloride (1.5M) to the GRAMOXONE dosing solution had quite profound effects on paraquat toxicity. It significantly increased the extent and degree of histopathological lesions in both the kidney and lung over that seen with any other formulation at 24 hours. In the kidney, the calcium chloride paraquat formulation progressed the mild hydropic changes seen with GRAMOXONE alone to a much more extensive lesion which also included frank necrosis and calcification of the pars recta. In addition, the plasma creatinine and BUN levels were markedly increased, suggesting impaired renal function by 24 hours. Lung and kidney levels of paraquat were also much higher than the GRAMOXONE controls. All these features comply with the enhanced acute oral toxicity of paraquat formulations containing calcium chloride.

The next stage of the research programme was to determine whether intraperitoneal Mg could prevent the extensive renal damage caused by paraquat plus calcium when given after the initial exposure. Groups of rats were dosed with 200mg/kg paraquat as GRAMOXONE plus 1.5M $CaCl_2$, followed immediately after by saline or $MgSO_4$ (50mg/kg) given intraperitoneally. Groups of animals were sacrificed at 15 min and 24 hours. As shown in Tables 3 and 4, quite different values for paraquat distribution were obtained between the two groups of animals.

At 15 min (Table 3) there was no difference between plasma paraquat, creatinine or BUN levels between groups. However, plasma Mg levels were significantly raised (as expected) following i.p. $MgSO_4$. Lung levels of paraquat were raised but this effect was not statistically significant.

At 24 hours (Table 4), the plasma paraquat concentration in the i.p. Mg group was about half that of the control GRAMOXONE/$CaCl_2$ group, a reduction which was significant. Plasma creatinine and BUN were also lower at 24 hours in the Mg-treated animals suggesting improved kidney function. Plasma Mg concentrations were within the normal physiological range by 24 hours. The most interesting change was in tissue paraquat concentration. The lung level of paraquat was reduced from 25.8 ± 5.9$\mu$g/g to 6.9 ± 1.4$\mu$g/g at 24 hours. Kidney paraquat levels were half that observed in the GRAMOXONE/$CaCl_2$ control group. It would appear therefore that parenteral dosing of magnesium can reduce systemic paraquat toxicity in rats. Separate 10 day studies in rats have now shown that a dose as low as 50mg/kg $MgSO_4$ i.p. is an effective antidote when given after GRAMOXONE. The implications of this to treatment of paraquat poisoning in man should be further explored.

## FUTURE STUDIES

Over the next six months the 'lead' MAGNOXONE formulation (YF8004) plus the back up formulations (YF8003, YF781A and YF8034) are to be tested up to their limit doses in the dog. As new storage stability data becomes available, the most promising MAGNOXONE systems will be examined at high paraquat doses in naive dogs to determine whether we can achieve a greater than 20x safety factor compared to GRAMOXONE.

Additional studies with a large scale batch of the 'lead' formulation are planned for next year prior to introduction of MAGNOXONE into Suriname in 1992. CTL have also been asked to evaluate a chemical or immunological marker which will be added to MAGNOXONE in order to determine which product has been involved in human poisonings. To this end the GI Tract Research group will investigate potential markers which can be measured in dog

plasma up to 24 hours after paraquat ingestion. This work will commence over the next few months. Plans are also in hand for CTL research to explore similar technology to MAGNOXONE for other products containing the herbicide diquat in 1992. Decisions as to the support of this new research programme are to be made at Agrochemicals TRC meeting in December.

Throughout the 'safer' formulation research programme, the GI tract workgroup has also conducted fundamental research directed at furthering our knowledge on paraquat absorption and toxicity. It is hoped to continue this area in 1992 in order to provide additional scientific information which is of importance to the Agrochemicals Business. This information, especially around the antidote properties of magnesium, once published, can be used to support the Regulatory submissions required during the hopeful introduction of MAGNOXONE into ICI's product range.

## REFERENCES

Heylings J R (1990). ICI Agrochemicals Technical Review Committee Report TMF 3821C (November 1990).

Heylings J R (1991). The Toxicologist 11(1), 193.

Heylings J R (1991). Toxicology and Applied Pharmacology 107, 482-493.

Heylings J R (1991). UK Patent Application No: 9015134.1.

## CONTRIBUTORS

J R Heylings
M J Farnworth
A J Foster
A Whim
J R Foster

## RESOURCES 1991

| | | |
|---|---|---|
| J R Heylings | : | 1.0 man-year |
| M J Farnworth | : | 1.0 man-year |
| A J Foster | : | 0.1 man-year |
| Toxicity Support | : | 0.5 man-year |

## Table 1

| MAGNOXONE M19 (YF7981) | Property | g/l |
|---|---|---|
| Paraquat (GRAMOXONE) | Herbicide | 200 |
| Magnesium trisilicate | Antacid | 100 |
| Magnesium sulphate | Purgative | 100 |
| Kelzan | Thickener | 3 |
| PP796 | Emetic | 1.5 |

## Table 2

MAGNOXONE

PLASMA PARAQUAT AUC ($\mu$g/ml.h)

Paraquat Dose (mg/kg)

| Formulation | 16 | 32 (2.5X) | 64 (5X) | 128 (10X) | 256 (20X) |
|---|---|---|---|---|---|
| GRAMOXONE | LD | | | | |
| MAGNOXONE M19 (CTL, 100g/l $MgSO_4$) | - | 8 | 14 | 24 | (LD) |
| YF7980 (Unstenched) | - | 16 | 15 | | |
| YF7981 (Stenched) | - | 20 | 20 | | |
| YF7981A (100g/l $MgSO_4$ PDM 15) | - | 9 | 13 | ? | |
| YF8003 (10g/l $MgSO_4$) | - | 9 | 15 | ? | |
| YF8004 (50g/l $MgSO_4$) | - | 19 | 17 | ? | |
| MAGNOXONE M24 (CTL, 100g/l $MgCl_2$) | - | 15 | 18 | ? | |
| YF8034 (100g/l $MgCl_2$) | - | ? | | | |

GRAMOXONE $LD_{50}$ = 12mg/kg or AUC = 40$\mu$g/ml.h
(X) Safety factor compared to GRAMOXONE.

## Table 3

Effect of Intraperitoneal Magnesium on Paraquat Distribution in the Rat

1) 15 minutes after dosing

| Biochemical markers at 15 min | Gramoxone | Gramoxone $MgSO_4$ i.p. | Gramoxone/ $CaCl_2$ | Gramoxone/ $CaCl_2$ $MgSO_4$ i.p. |
|---|---|---|---|---|
| Plasma Paraquat ug/ml | 4.36 ± 1.2 | 4.66 ± 0.5 | 6.55 ± 0.3 | 6.72 ± 0.2 |
| Plasma Mg mg/dl | 1.92 ± 0.03 | 2.54 ± 0.2* | 2.06 ± 0.08 | 3.02 ± 0.2† |
| Lung Paraquat ug/g wet wt. | 18.62 ± 0.3 | 2.08 ± 0.8* | 16.67 ± 3.5 | 23.14 ± 2.9 |
| Kidney Paraquat ug/g wet wt. | 11.85 ± 5.0 | 9.35 ± 2.4 | 195.33 ± 87.8 | 173.34 ± 23.8 |
| Plasma creatinine mg/dl | 0.5 ± 0.03 | 0.4 ± 0.0* | 0.7 ± 0.07 | 0.68 ± 0.04 |
| Blood urea nitrogen mg/dl | 24.8 ± 0.9 | 21.6 ± 0.8 | 29.0 ± 1.6 | 24.4 ± 1.2 |

Gramoxone dosed orally (200mg/kg paraquat).
$CaCl_2$ dosed orally in Gramoxone at 1.5 M (equivalent to 220 mg/kg).
$MgSO_4$ dosed i.p. at 50mg/kg.
Mean values ± SEM, n = 5 each group.
* Significant versus Gramoxone control.
† Significant versus Gramoxone $CaCl_2$.

## Table 4

Effect of Intraperitoneal Magnesium on Paraquat Distribution in the Rat

2) 24 hours after dosing

| Biochemical markers at 24 hours | Gramoxone | Gramoxone $MgSO_4$ i.p. | Gramoxone/ $CaCl_2$ | Gramoxone/ $CaCl_2$ $MgSO_4$ i.p. |
|---|---|---|---|---|
| Plasma Paraquat ug/ml | 3.08 ± 0.9 | 2.18 ± 0.1 | 2.94 ± 0.6 | 1.39 ± 0.3† |
| Plasma Mg mg/dl | 2.12 ± 0.07 | 2.08 ± 0.08 | 2.48 ± 0.2 | 2.26 ± 0.04 |
| Lung Paraquat ug/g wet wt. | 9.54 ± 1.9 | 6.86 ± 1.1 | 25.78 ± 5.9 | 6.87 ± 1.4† |
| Kidney Paraquat ug/g wet wt. | 12.7 ± 1.1 | 5.44 ± 0.3* | 38.43 ± 9.5 | 18.33 ± 2.0 |
| Plasma creatinine mg/dl | 0.46 ± 0.03 | 0.48 ± 0.02 | 0.66 ± 0.08 | 0.54 ± 0.02 |
| Blood urea nitrogen mg/dl | 35.75 ± 3.7 | 23.4 ± 1.5* | 71.6 ± 14.9 | 30.0 ± 2.7† |

Gramoxone dosed orally (200mg/kg paraquat).
$CaCl_2$ dosed orally in Gramoxone at 1.5 M (equivalent to 220 mg/kg).
$MgSO_4$ dosed i.p. at 50mg/kg.
Mean values ± SEM, n = 5 each group.
* Significant versus Gramoxone control.
† Significant versus Gramoxone $CaCl_2$.

Figure 1

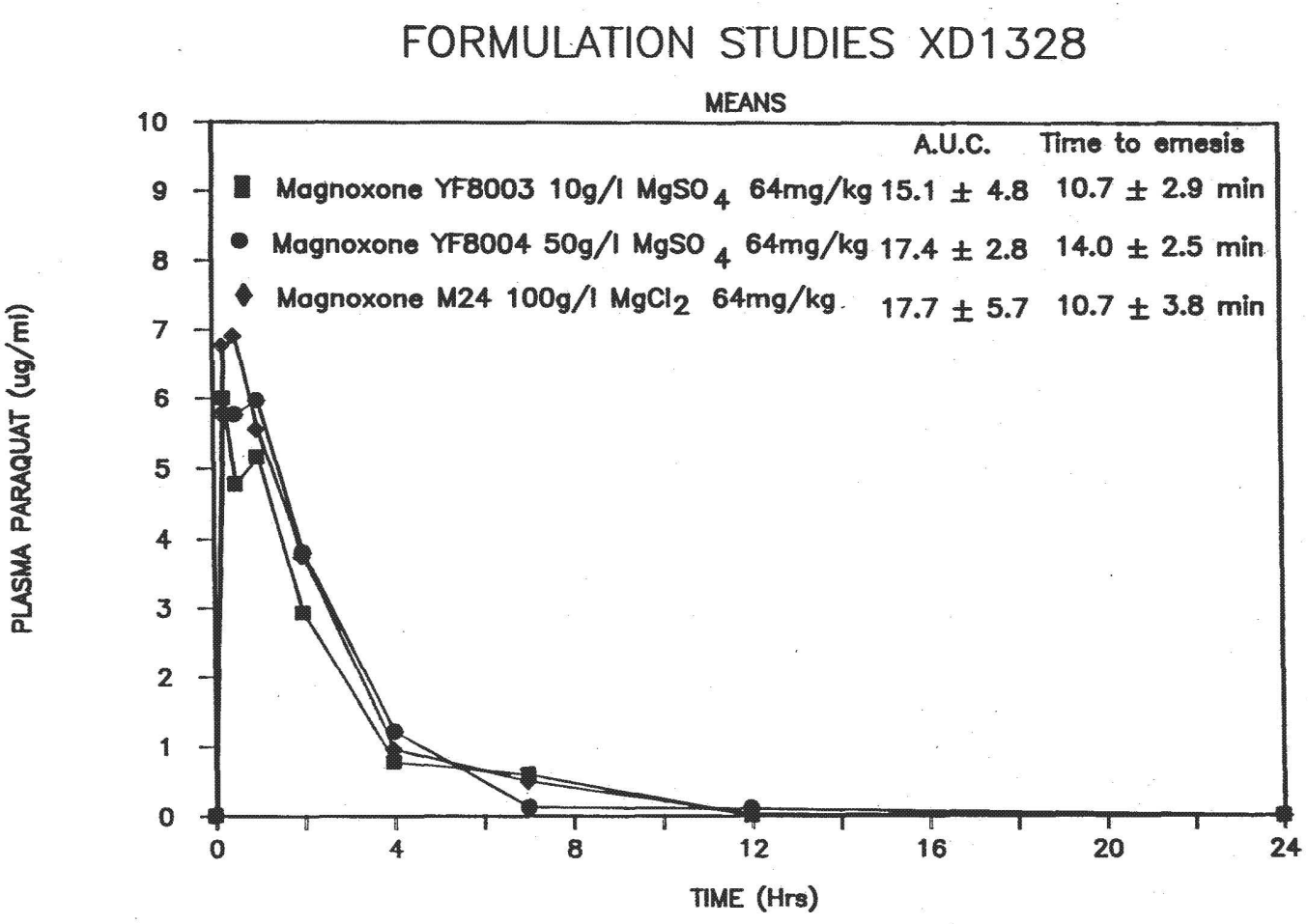

FORMULATION STUDIES XD1328
MEANS
A.U.C. Time to emesis
Magnoxone YF8003 10g/l MgSO4 64mg/kg 15.1 ± 4.8 10.7 ± 2.9 min
Magnoxone YF8004 50g/l MgSO4 64mg/kg 17.4 ± 2.8 14.0 ± 2.5 min
Magnoxone M24 100g/l MgCl2 64mg/kg 17.7 ± 5.7 10.7 ± 3.8 min
PLASMA PARAQUAT (ug/ml)
TIME (Hrs)
0
1
2
3
4
5
6
7
8
9
10
0
4
8
12
16
20
24

Figure 2 - Rate of calcium absorption by the rat duodenum (bathing solution contained 2.5mM $CaCl_2$ in all cases).

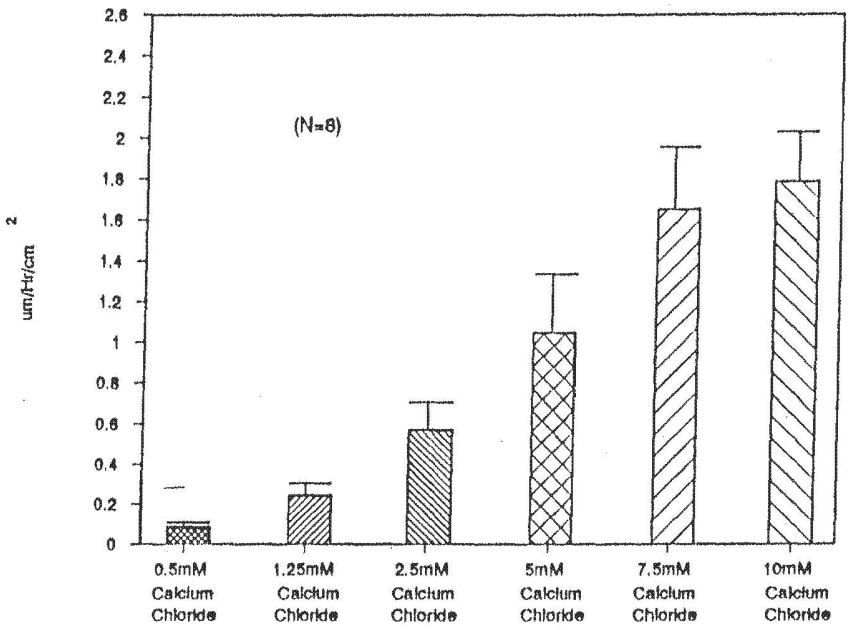



Figure 3 - Effect of magnesium on the tissue uptake of 1.25mM $CaCl_2$ into the rat duodenum (bathing solution contained 2.5mM $CaCl_2$ in all cases).

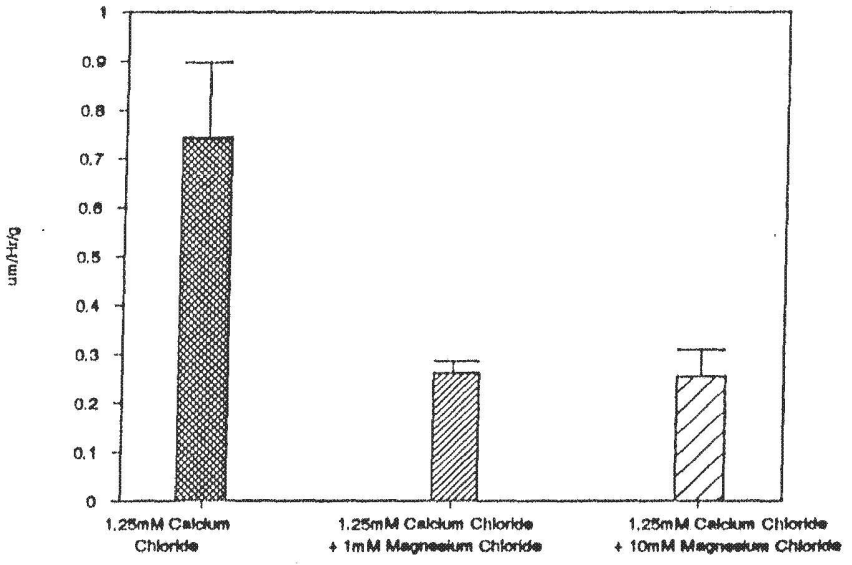


Figure 4 - Effect of calcium on the tissue uptake of paraquat by the rat duodenum (bathing solution contained 2.5mM $CaCl_2$ unless stated).

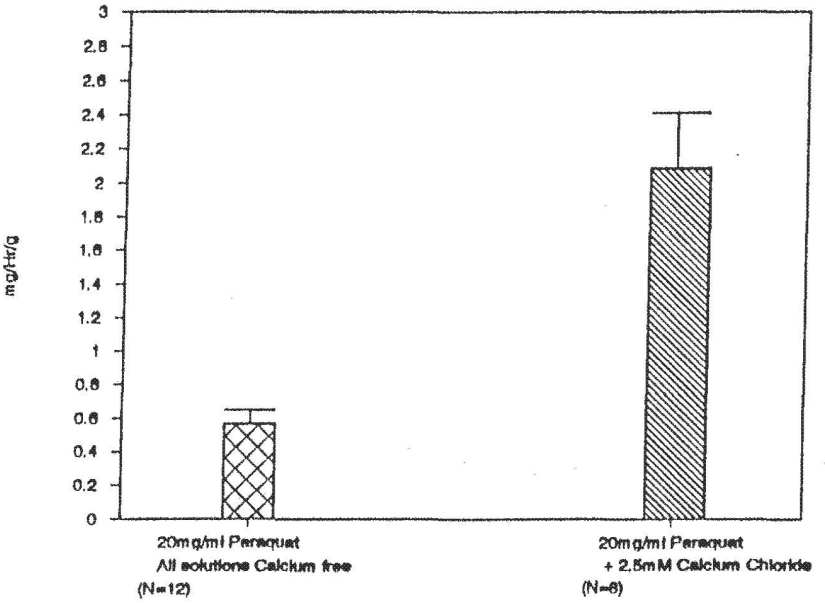



Figure 5 - Effect of 1.25mM $CaCl_2$ on the absorption of paraquat by the rat duodenum (bathing solution contained 2.5mM $CaCl_2$ in all cases).

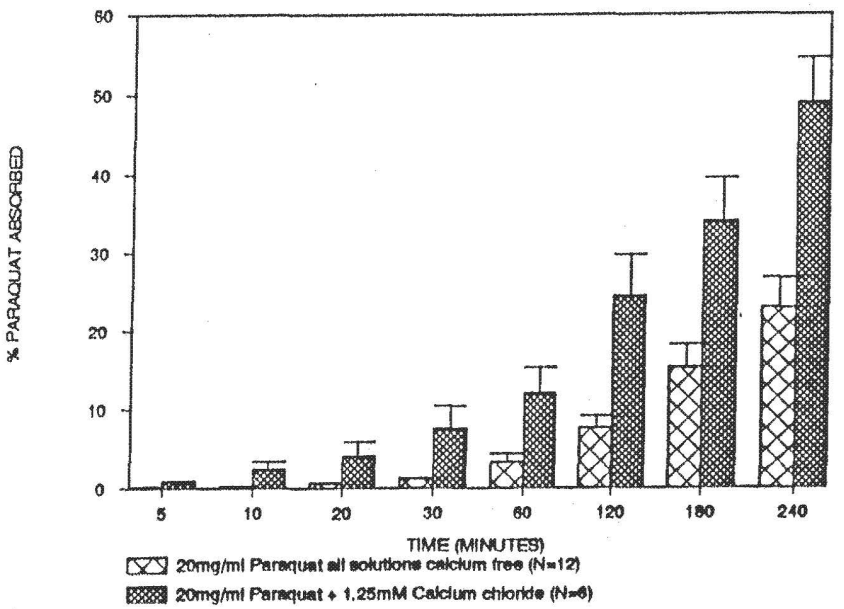
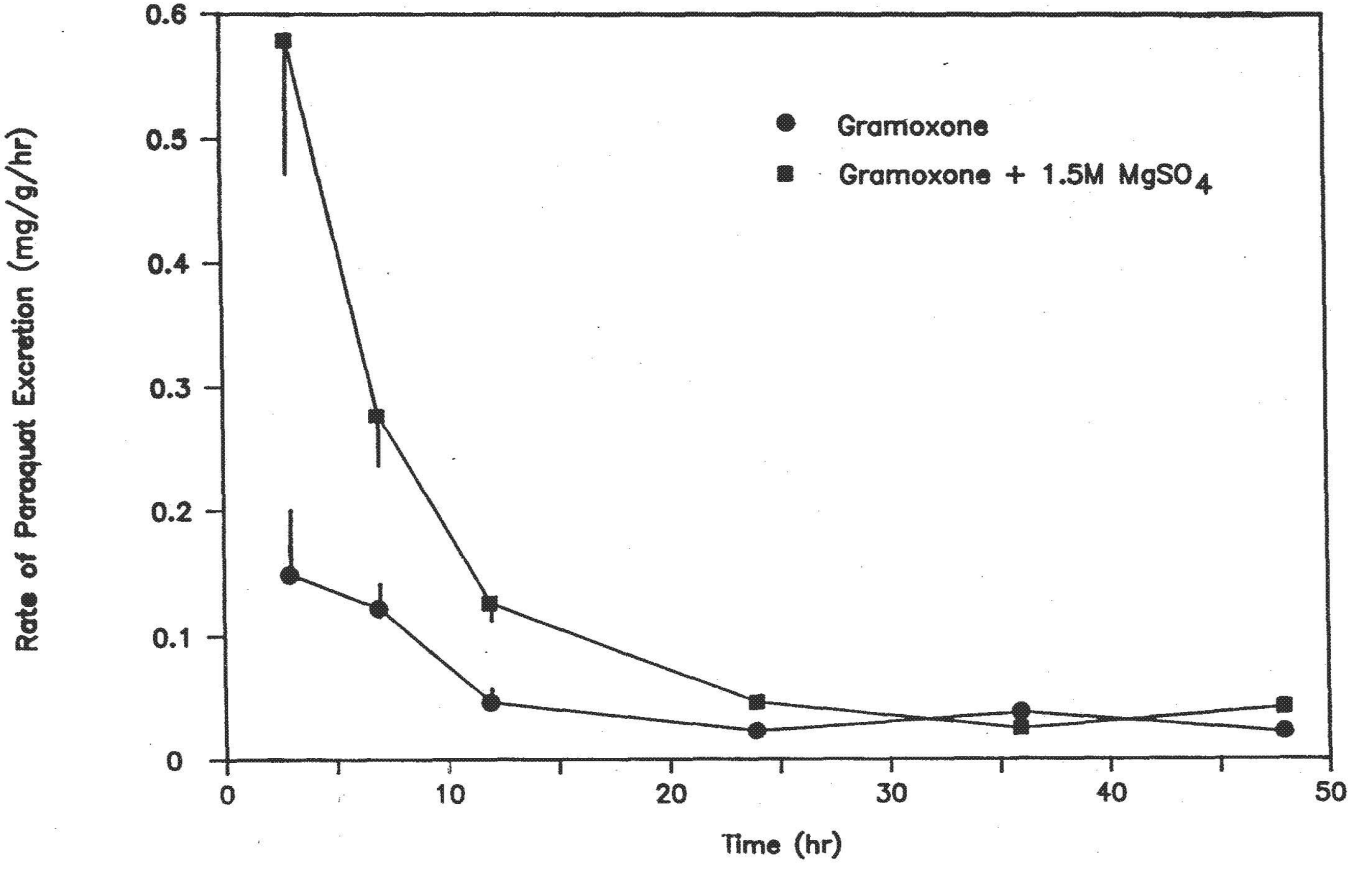


Figure 6

The Effect of Gramoxone and Magnesium Sulphate on the Rate of Excretion of Paraquat in the Urine following an Oral dose at 200mg/kg PQ



# SAFENERS RESEARCH

A M BRADY

## INTRODUCTION

R29148, R29187 and dichlormid are dichloracetamide compounds used as 'safeners' in herbicide formulations. Safeners are currently classified as 'inerts' for registration purposes and, in contrast to the 'active' herbicide with which they will be used, are required to undergo only minimal toxicological evaluation. Short term studies suggest that the safeners are only mildly hepatotoxic, but concern has been raised recently following the discovery that R29148 significantly increased the incidence of hepatocellular carcinomas in a life-time feeding study in rats.

## OBJECTIVES

The objectives of the safener research programme are:

1) To elucidate the mechanism by which R29148 is hepatocarcinogenic.

2) To identify early biochemical or morphological markers of this toxicity which can be used to evaluate and discriminate the potential carcinogenicity of other dichloracetamide compounds.

## PROGRESS

### CHARACTERISATION OF HEPATIC EFFECTS

Previous studies indicated that the dichloracetamide safeners were not overtly hepatotoxic in rats and mice but at very high doses they did induce liver enlargement and several changes in hepatic and blood biochemistry;

hepatic non-protein sulphydryl content (NPSH), glutathione S-transferase activity (GS-t), γ-glutamyl transpeptidase activity (γGT) and hepatic DNA synthesis were significantly elevated and the concentration of bile acids in the plasma was increased. The doses used in these preliminary studies greatly exceeded the highest dose used in the previous R29148 carcinogenicity bioassay (800ppm). Thus, as a first step towards defining whether any of the changes observed at such high doses are causally related to the induction of tumours, the dose-response relationships for the effects at R29148 on the liver were determined.

Administration of R29148 to male rats or mice for 28 days resulted in significant, dose-related increases in NPSH, GS-t and γGT (Figure 1). In rats, significant increases in NPSH were noted at all doses, whereas the increases in GS-t, γGT and plasma bile acids were apparent only at doses of 1000ppm and above. A similar pattern was observed in mice, but 100ppm was a clear no effect level for all effects (Figures 1 and 2). The most marked effect of R29148 on the liver was the induction of γGT activity, which was increased by 8-10-fold at the highest doses.

Dichlormid and R29187 produced similar dose-related increases in hepatic γGT activity in rats and in mice (Figure 3). Dichlormid and R29187 also produced similar dose-related increases in NPSH and GS-t (data not shown).

## Significance of γGT Induction

The elevation in plasma bile acids and induction of γGT activity by the safeners was suggested to be indicative of intrahepatic cholestasis. However, it is noteworthy that other plasma markers of cholestasis such as ALP, bilirubin, cholesterol and γGT were not significantly affected by administration of R29148 (Figure 2).

In the liver γGT is a membrane-bound enzyme which is localised almost exclusively in the biliary epithelium. In intrahepatic cholestasis the induction of hepatic γGT activity is associated with proliferation of the biliary epithelium. Cytochemical staining for γGT in sections of

liver from animals dosed with R29148 confirm the marked induction of this enzyme in the liver (Figure 4). This induction was clearly not associated with proliferation of the biliary epithelium but rather to induction of hepatocyte activity.

Thus, the elevation of bile acids in the plasma does not appear to be due to intrahepatic cholestasis and may reflect a specific effect of R29148 on bile acid metabolism.

It is noteworthy that the induction of γGT in the liver by R29148 is localised, occurring almost exclusively in the zone 1 (periportal) hepatocytes (Figure 4). In this respect it is interesting that the pathological changes produced by R29148 including single cell necrosis, enlarged nuclei and cellular hypertrophy have also been shown to be confined to zone 1 hepatocytes.

The physological function of γGT is unknown but several of the chemical reactions it catalyses support a role for this enzyme in the synthesis and utilisation of glutathione (GSH). The structure of the safeners suggests that they may be metabolised by conjugation with glutathione and the roles of γGT in the synthesis of GSH and export of GSH-conjugates into bile may be causally related to its induction. In this respect, it is noteworthy that the conjugation of compounds with GSH is a reaction which predominates in zone 1 hepatocytes due to the higher levels of GSH in these cells.

Further experimental pathology studies have been designed to investigate the relationships between the proposed conjugation of R29148 with GSH and the morphological and biochemical changes in the liver.

## GLUTATHIONE S-TRANSFERASES

The safeners have been shown to induce cytosolic GS-t activity in the livers of rats and mice and also in plants. If the safeners are metabolised by conjugation with GSH, then this reaction would most likely

be mediated by GS-transferases. The GS-transferases are a family of isoenzymes exhibiting marked differences in substrate specificity. Studies have thus been carried out to characterised the profile of isoenzymes induced by R29148.

The cytosolic GS-transferases are dimeric proteins consisting of two homologous (mainly) or heterologous subunits. In control rat liver the major GS-t subunits were found to be 1a, 1b and 2 ($\alpha$ class) and 3 and 4 ($\mu$ class) which is in good agreement with similar studies described in the Literature. Adminisration of R29148 to rats was found to have profound effects on the profile of GS-t subunits in the liver (Figure 5). The $\alpha$-class subunits 1a and 1b, and to a lesser extent 2, were markedly reduced, whereas subunits 3 ($\mu$ class) and 7 ($\pi$ class) were induced (Figure 5).

Comparative studies with dichlormid and R29187 revealed remarkably similar changes in the profile of GS-t subunits in the liver (Figures 6 and 7). There were no significant differences in potencies between the three different safeners.

Immunocytochemical staining for the different GS-t isoenzymes is being employed to investigate the distribution of these effects in the liver. Preliminary studies have indicated, for example, that induction of subunit 7 ($\pi$ class) by R29148 is localised, as with the induction of $\gamma$GT, to the zone 1 hepatocytes (Figure 8).

Long Term Effects

Mechanism of non-genotoxic carcinogenesis are poorly understood. By studying the effects of the safeners on the liver after long term administration we may be better able to evaluate the significance of the short term effects observed so far.

Following administration of R29148 to male rats for 1 year significant increases in $\gamma$GT and GS-t were noted (Figure 9). Although the degree of induction of $\gamma$GT in liver homogenates appeared small, cytochemical

staining again localised the induction to the zone 1 hepatocytes of the liver, suggesting that locally this induction is far greater than indicated by biochemical measurements. Marked changes in the profile of $\alpha$ class GS-t subunits were apparent (Figure 10); subunits 1a and 1b were significantly decreased, as had been noted in short-term studies. The concentration of subunits 3 ($\mu$ class) and 7 (pi class) were largely unchanged.

Further cytochemical and immunochemical staining techniques will be used to characterise changes in $\gamma$GT, GSH and GS-transferase isoenzymes. These experimental pathology studies will also be used to clarify (i) how and where these biochemical changes are manifest in the liver, (ii) how such lesions develop with time and (iii) to identify whether foci of phenotypically altered hepatocytes are present, the characteristics of which may allow a relationship between the early changes and development of preneoplastic hepatocytes to be defined.

## PORPHYRIA - DICHLORMID

Chemicals containing allyl groups such as allylisopropyacetamide and allyl-containing barbiturates and steroids induce hepatic porphyria in rodents. This porphyrinogenic activity is imparted by the allyl group which can inactivate cytochrome P-450 by covalently binding to its haem moiety, thereby stimulating haem biosynthesis. Dichlormid contains two allyl groups in its structure, and studies in rats have confirmed its porphyrinogenic activity (Figure 11a). Mice appeared to be far less sensitive to the porphyrinogenicity activity of dichlormid than rats (Figure 11a). Detailed time course studies in rats (Figure 11b) have shown that the hepatic porphyria takes several days to develop, exhibits marked inter-animal variation and is relatively mild when compared to tralkoxydim.

R29148 and R29187 did not elicit hepatic porphyria in rats or mice (data not shown).

## FUTURE WORK

1. To evaluate the biochemnical and morphological relationships between γGT induction, NPSH levels, GS-t isoenzymes and induced DNA synthesis in the liver.

2. To determine whether any phenotypic changes are apparent in hepatocytes after longer term administration of R29148 particularly with regards to γGT, NPSH and GS-t isoenzymes.

3. To study the metabolism of R29148 by GS-transferase isoenzymes in the liver.

4. To evaluate the significance of the changes in GS-t isoenzymes with regards to hepatotoxicity and carcinogenicity of the safeners.

## CONTRIBUTORS

A Brady
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## RESOURCES 1991

Biochemical Toxicology - 2.2 man-years
Pathology - 0.7 man-years
Toxicity - 0.1 man-years

## RESOURCES 1992

Fig. 1 Effect of R-29148 on hepatic enzyme activities in rats and mice

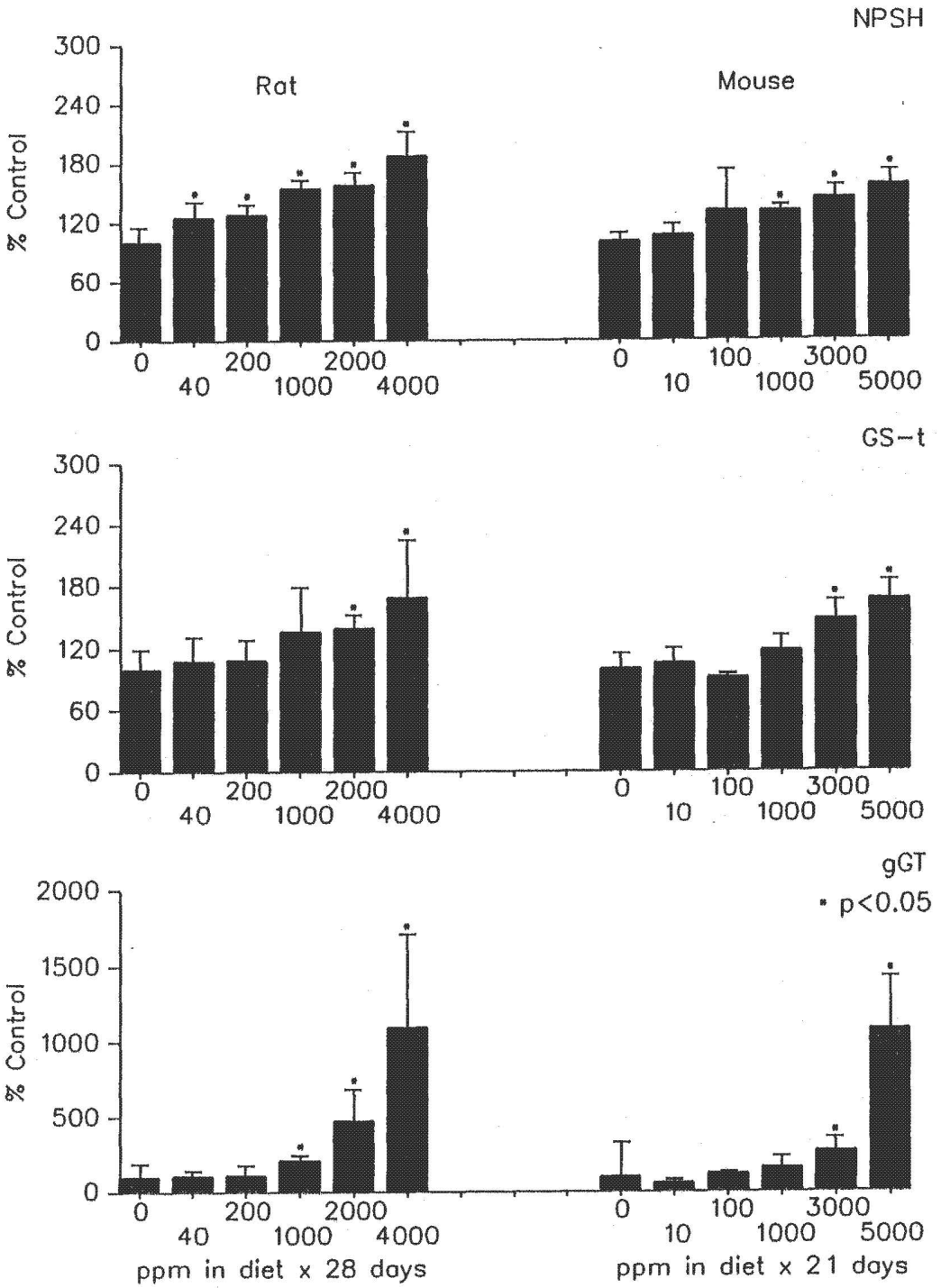


Fig. 2 Effect of R-29148 on plasma parameters in rats and mice

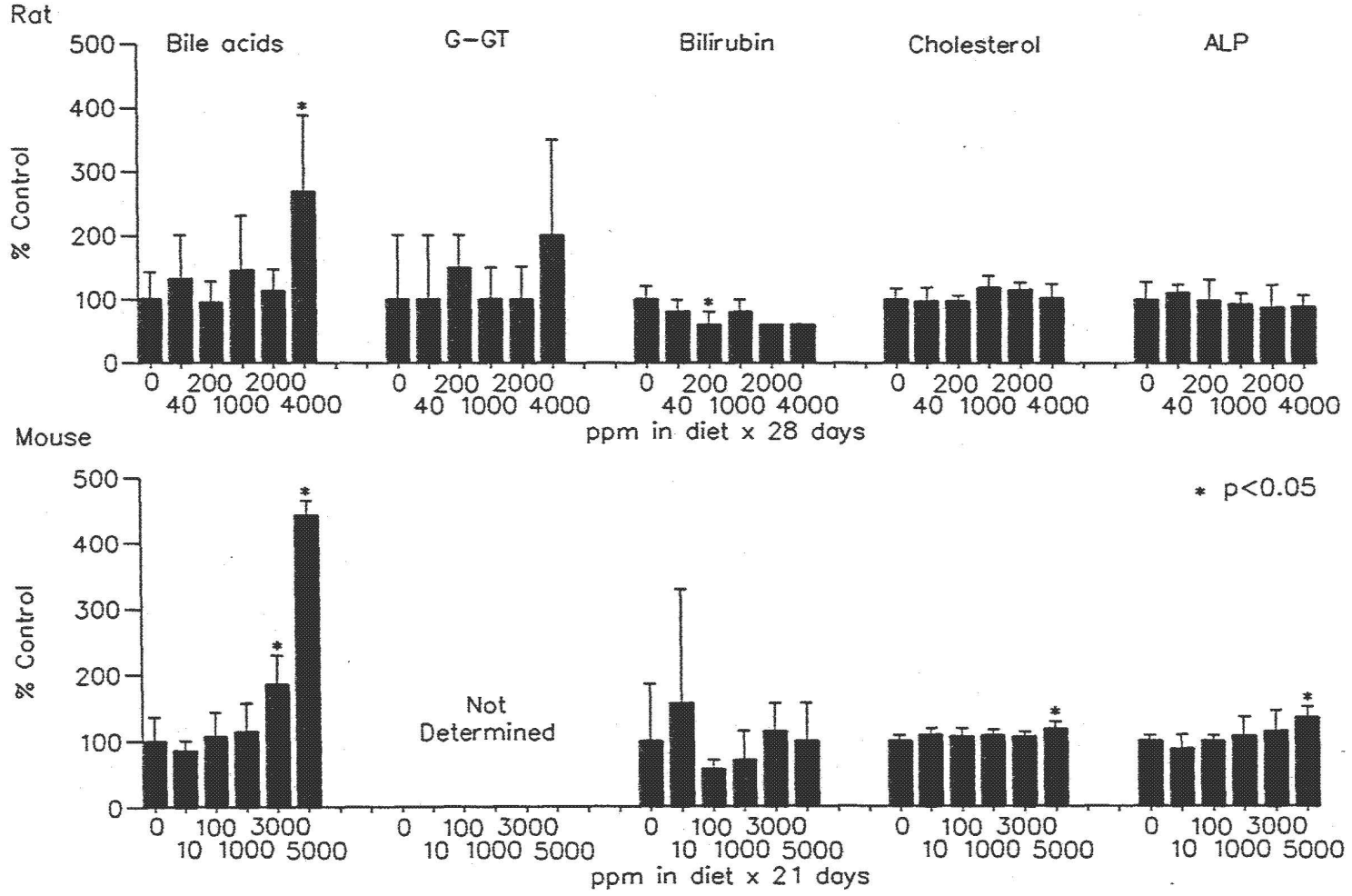
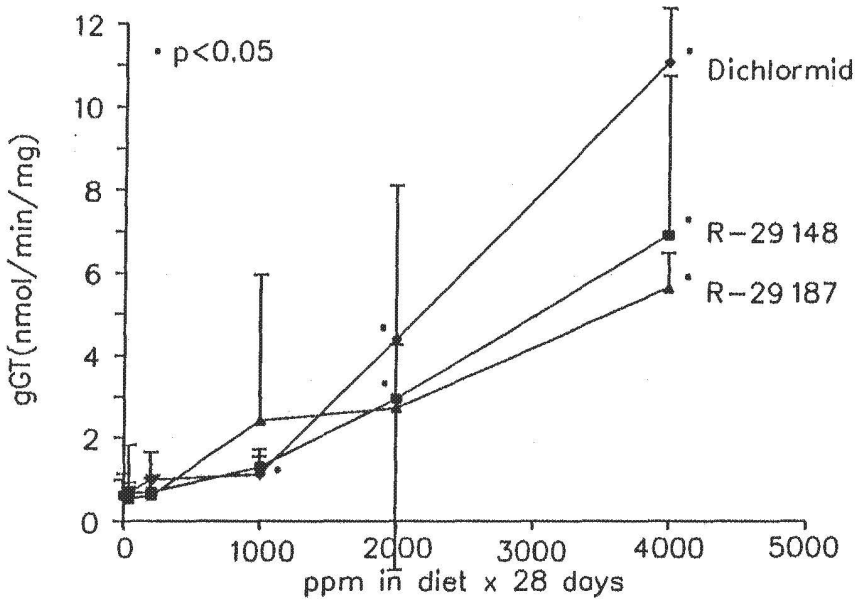


Fig. 3 Effects of R-29148, R-29187 and Dichlormid on hepatic gGT activity in rats and mice

(a) Rat




(b) Mouse

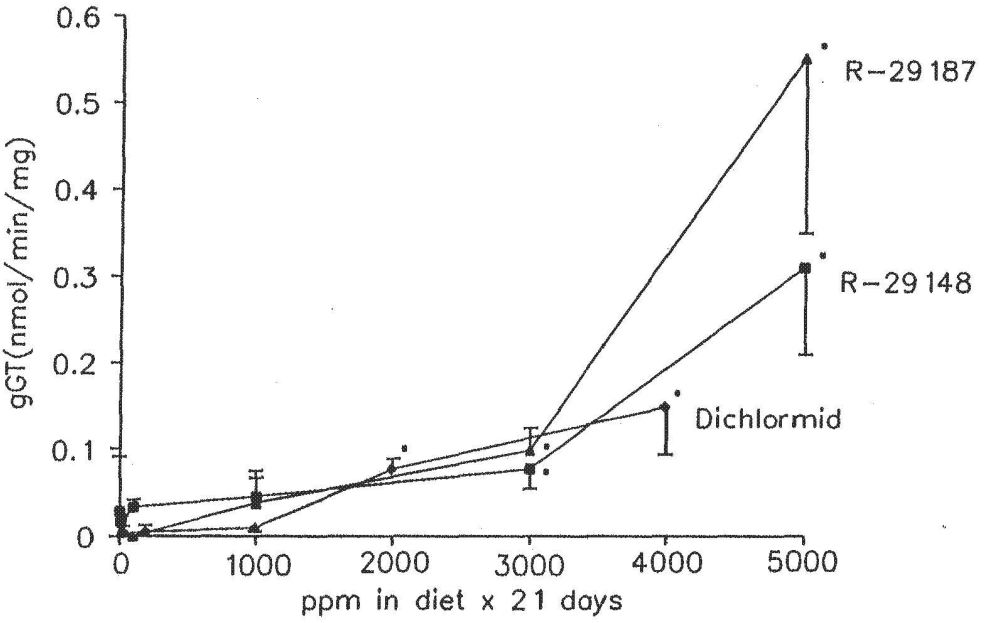
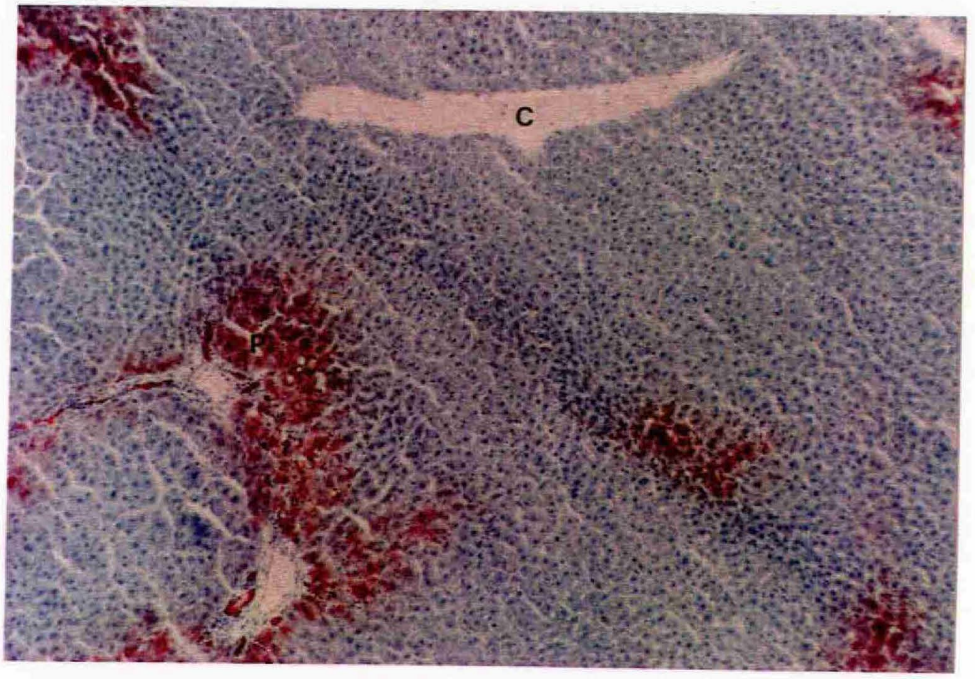


Fig.4 Effects of R29148 on rat liver:Cytochemical staining for gamma glutamyl transpeptidase

Control


R29148 x 4000 ppm x 28 days




P = periportal (zone 1)
C = centrilobular (zone 3)

**Fig.5 Effects of R29148 on rat liver GS-transferase subunits**

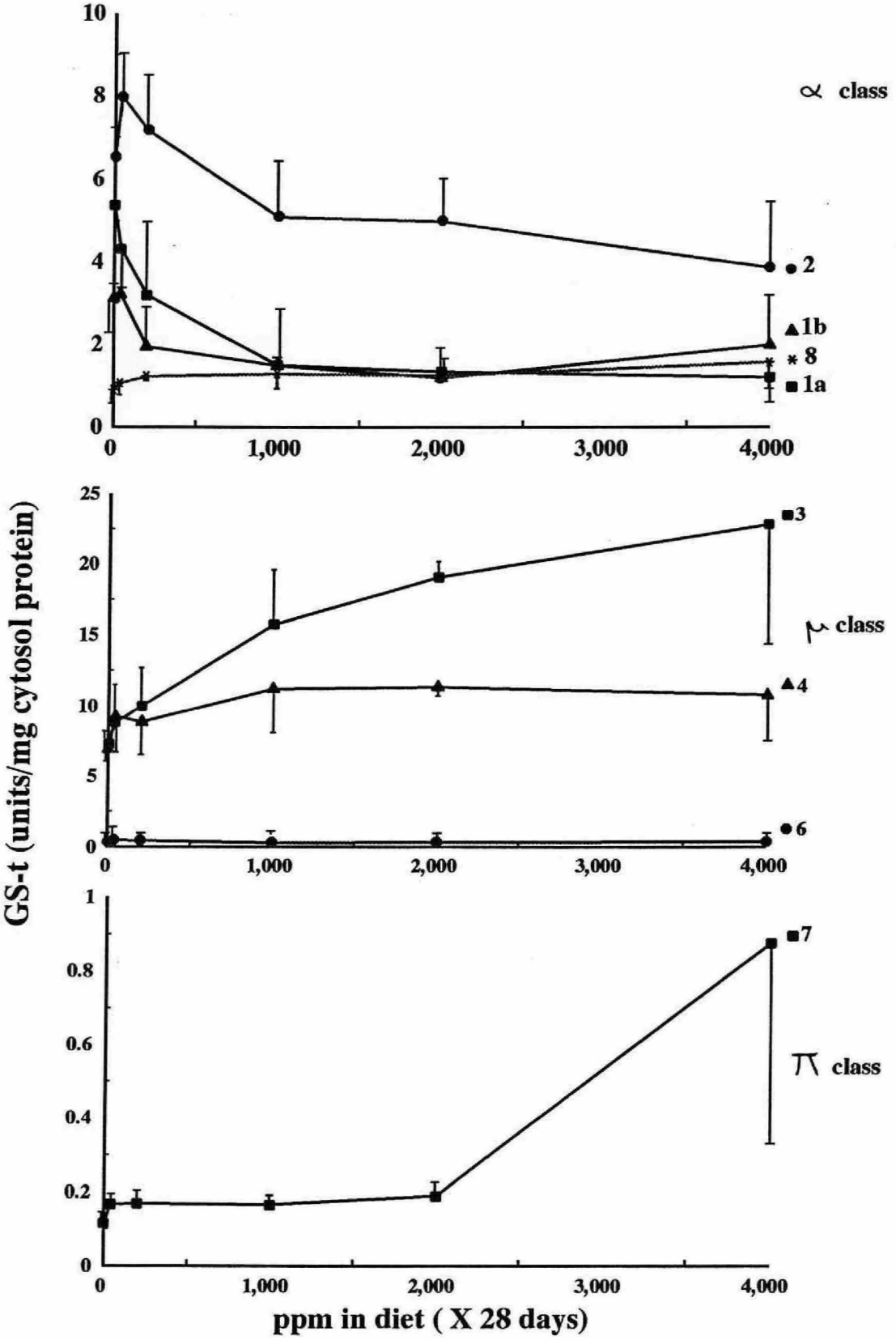


**Fig.6 Effects of Dichlormid on rat liver GS-transferase subunits**

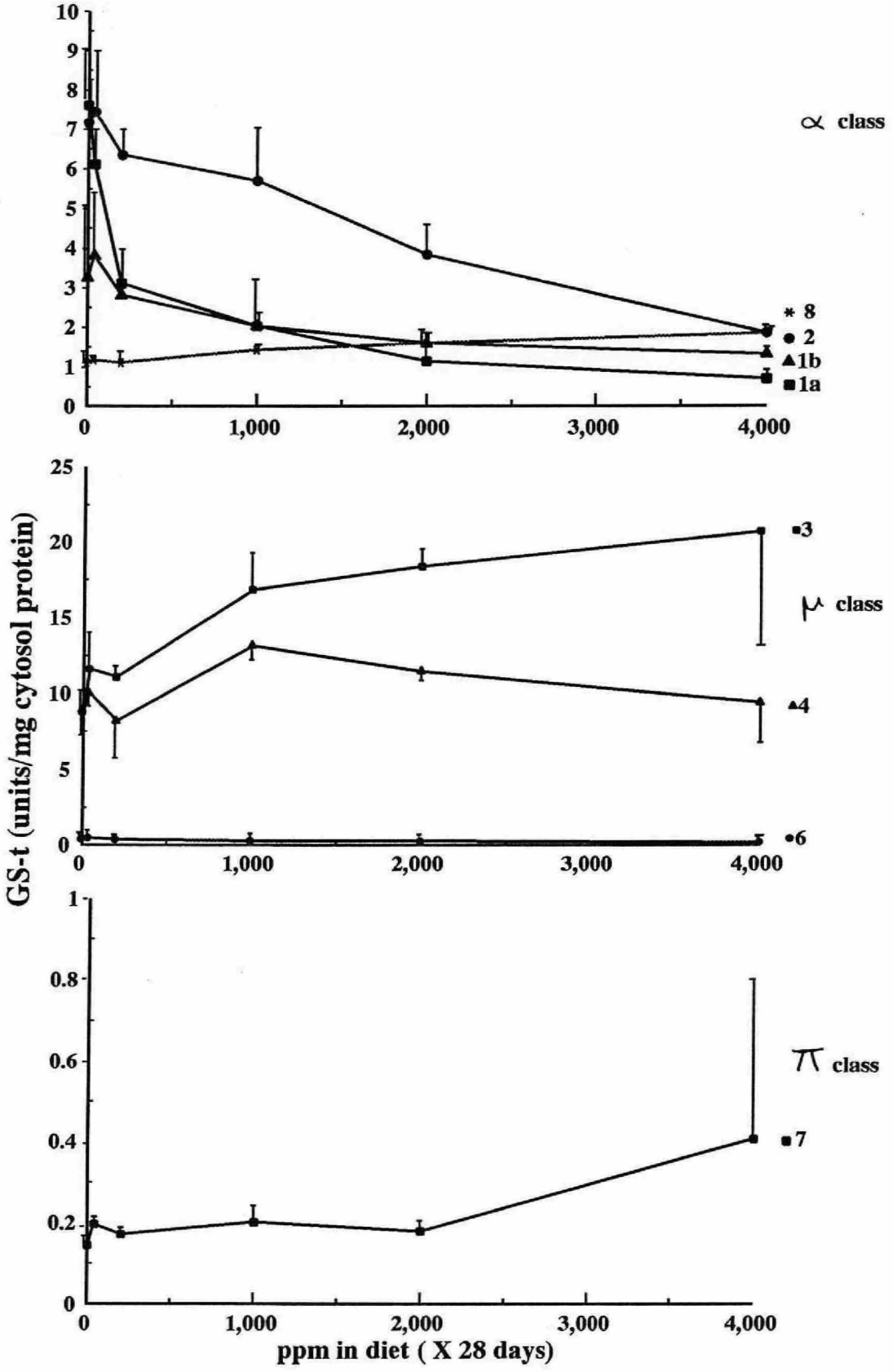


**Fig.7 Effects of R29187 on rat liver GS-transferase subunits**

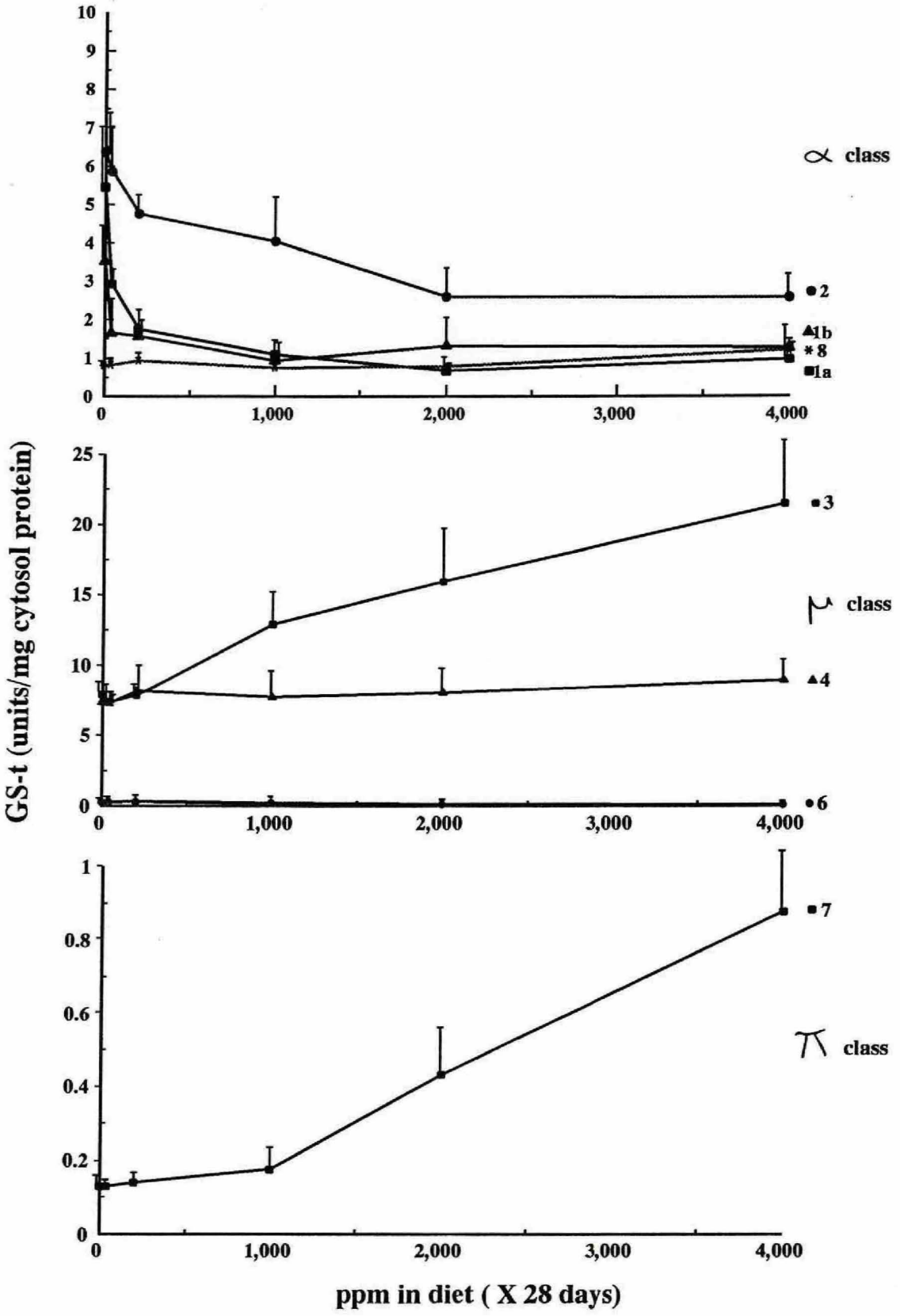
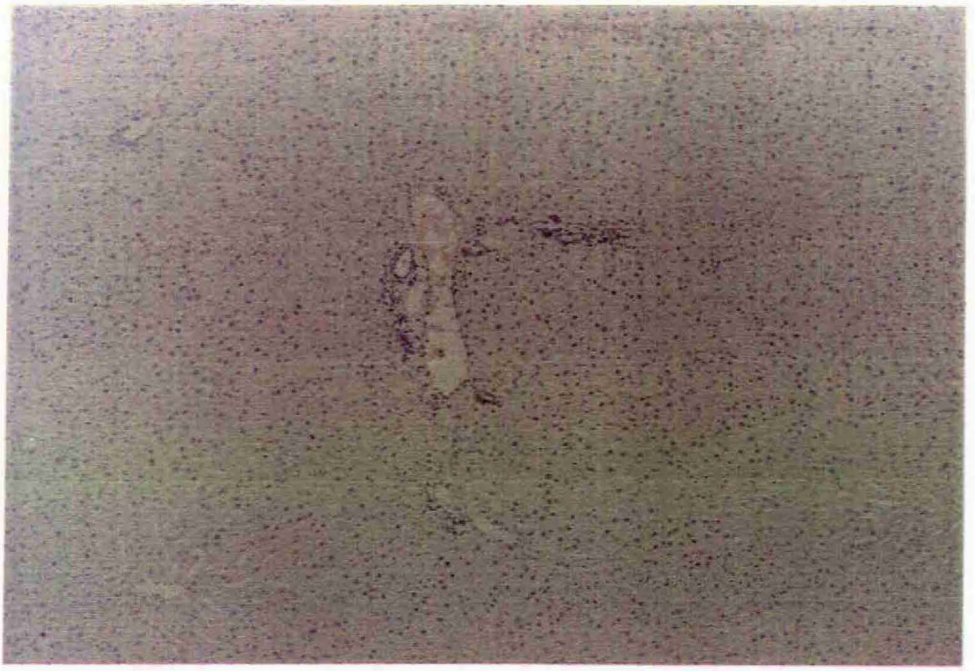
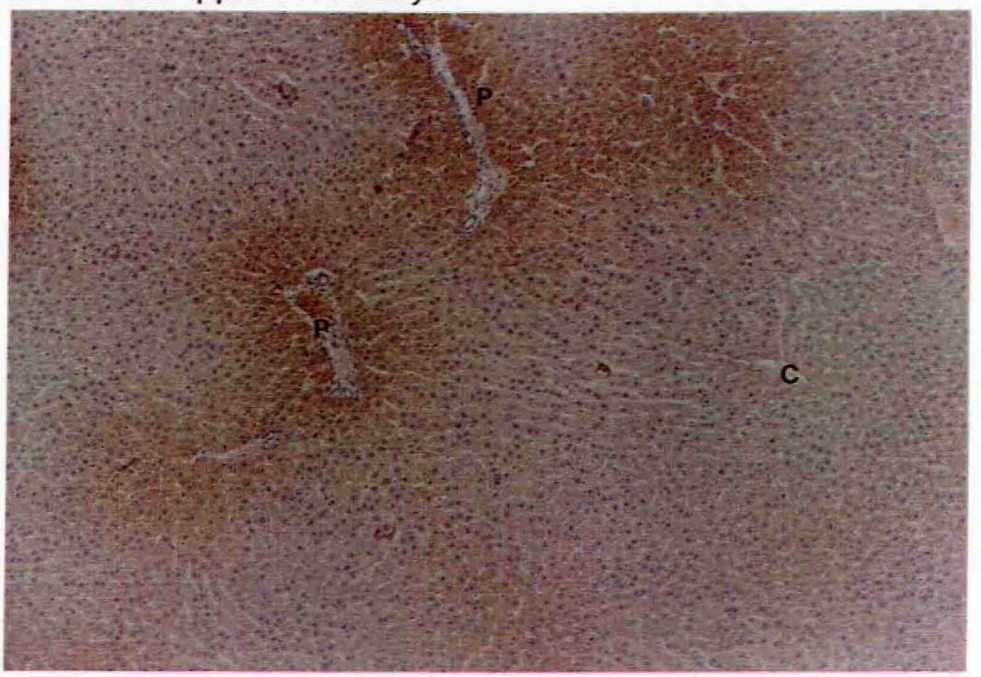


Fig.8 Effects of R29148 on GS-transferase isoenzyme 7-7 in rat liver:immunocytochemical staining with anti-rat 7-7

Control



R29148 x 4000 ppm x 21 days




P = periportal(zone 1)

C = centrilobular(zone 3)

Fig.9 Effects of long term administration (1 year) of R29148 on rat liver biochemistry

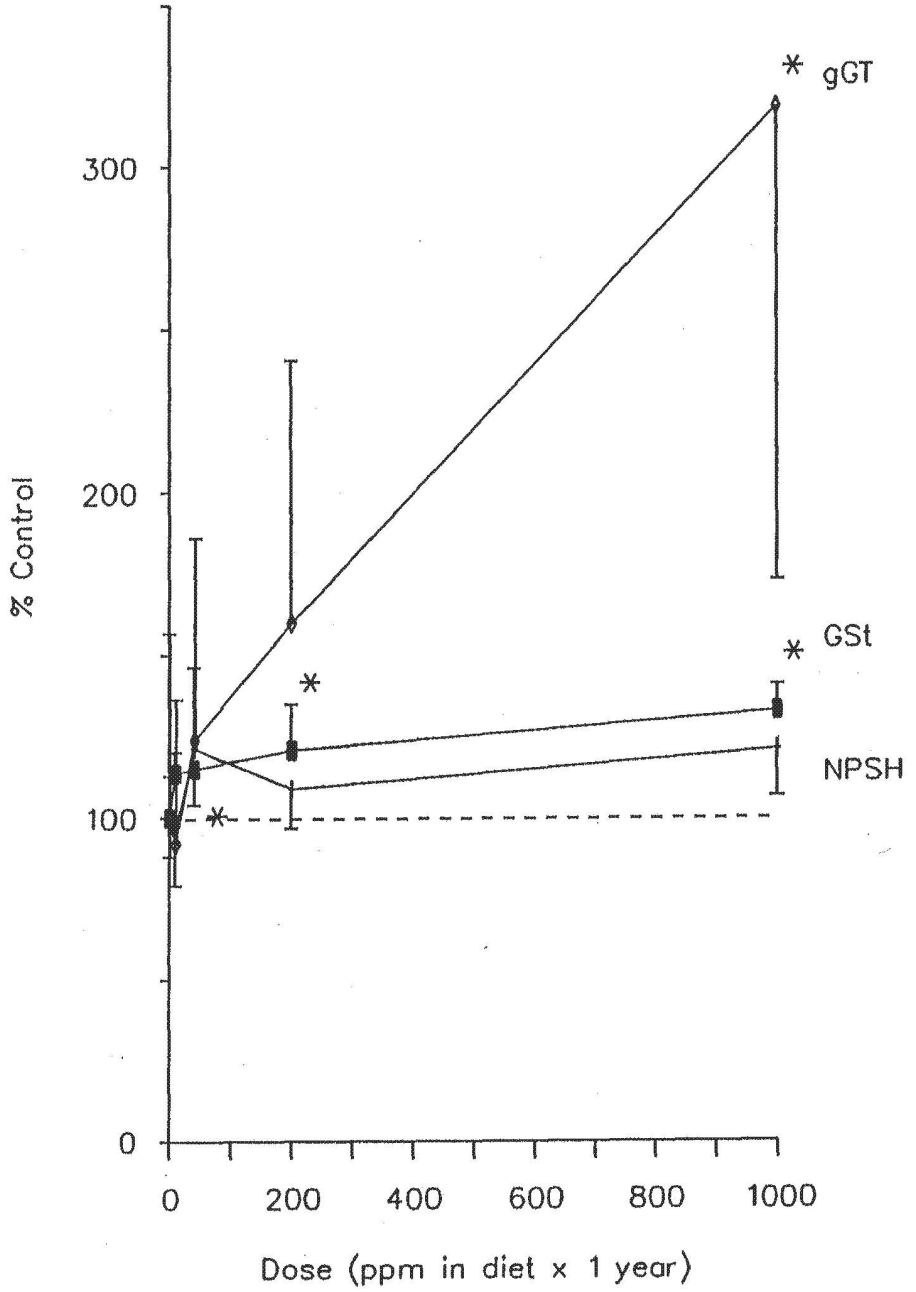


**Fig.10 Effects of R29148 on rat liver GS-transferase subunits after long term administration**

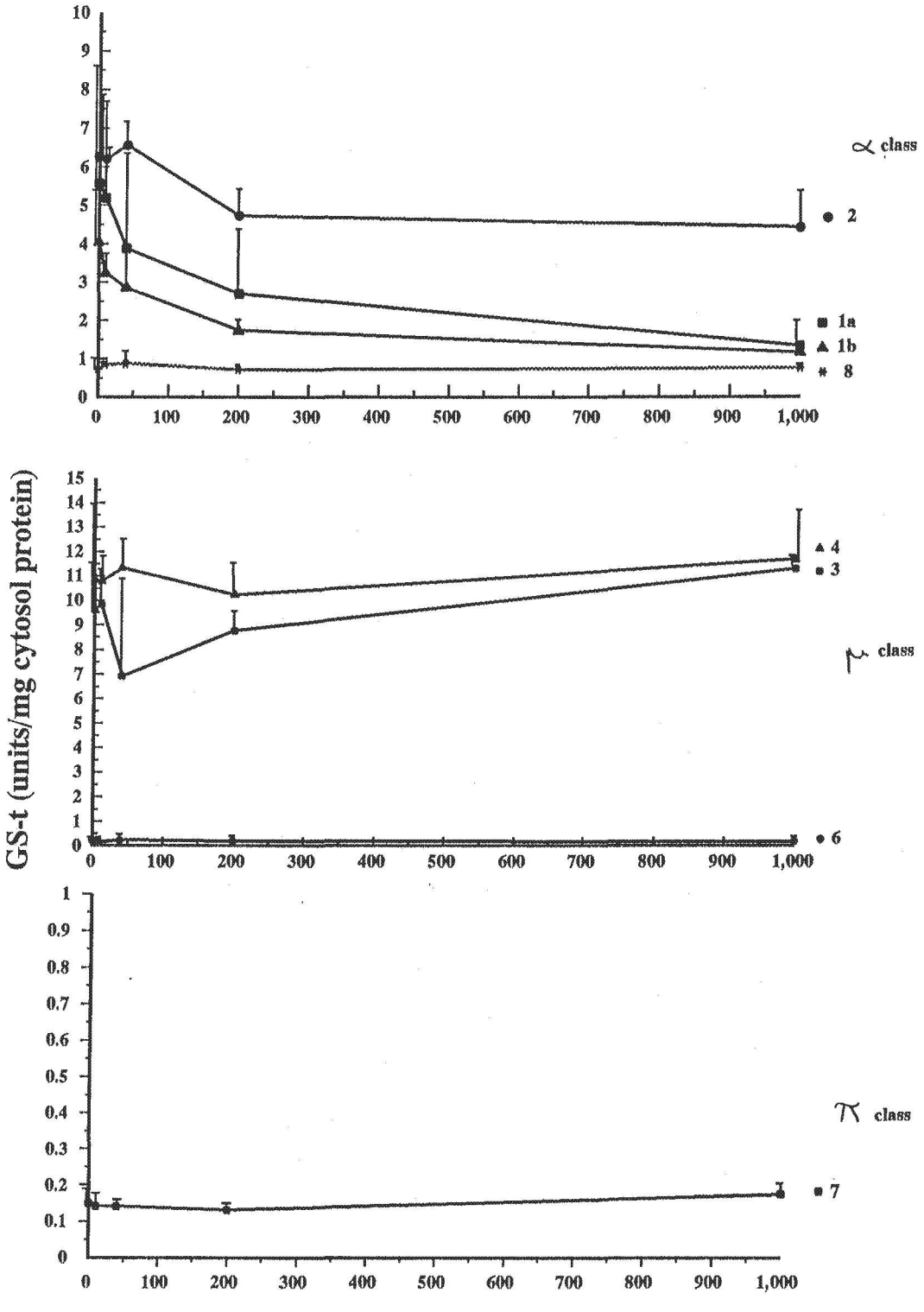
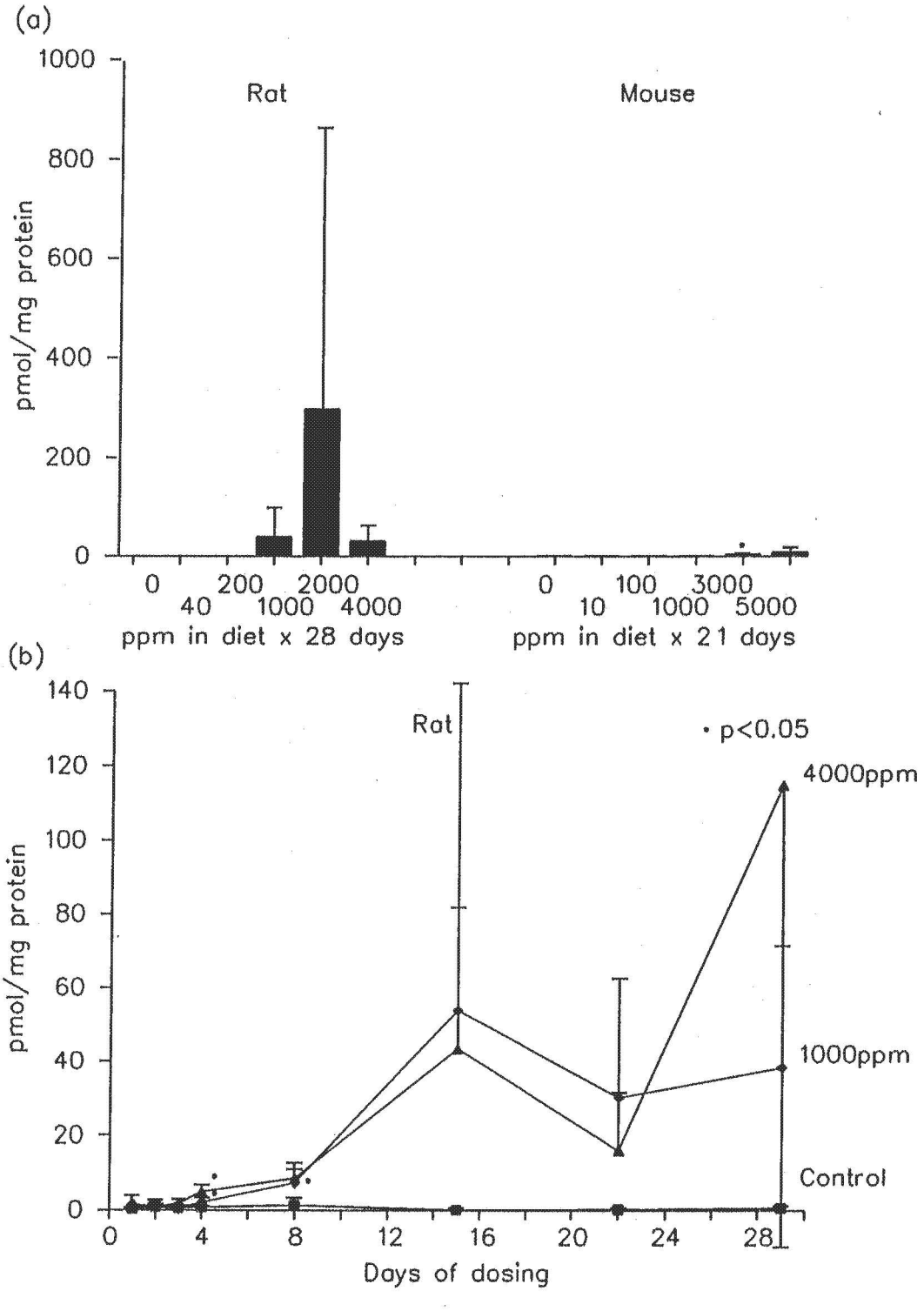


Fig. 11 Effect of Dichlormid on liver porphyrin levels in rats and mice



# ACETOCHLOR RESEARCH

T GREEN

## INTRODUCTION

Acetochlor is a chloroacetanilide pre-emergence herbicide active against grasses, sedge and broadleaf weeds in corn. Two year feeding studies in rodents have shown that dietary administration of acetochlor is associated with an increased incidence of tumours in the nasal turbinates of rats. These tumours which were mainly polypoid adenomas were not seen in mice. The objectives of this research are to identify the mechanism of tumour formation in the rat and to determine the relevance to humans exposed to acetochlor.

## PROGRESS

The work has been divided into two main areas:

1. The effects of acetochlor and its metabolites on nasal tissue morphology and the selective accumulation of the same in nasal tissues in vivo.

2. The activation of acetochlor to its carcinogenic form.

1. Cytotoxicity and increase cell division are well characterised events in the development of chemically-induced nasal tumours. These events are normally detectable in the short-term either as tissue damage or by selective uptake and binding of the chemical or its metabolites to the target tissues. In order to characterise the effects of acetochlor on nasal tissues a number of studies have looked for changes in nasal morphology after dosing acetochlor, for the binding of radioactivity in nasal tissues, and at the distribution of radioactivity by whole body autoradiography.

In summary, rats have been given a single dose of 2000mg/kg acetochlor or, in repeat dose studies, up to 1000mg/kg for up to 30 days. These dose levels were all significantly higher than the 79.5mg/kg (1750ppm) dose which had caused tumours in the 2 year studies. Both technical (89.4%) and analytical grade (99.5%) acetochlor were used.

Administration of acetochlor in all of these studies, or in a previous study in which it was given in the diet (2000ppm:10 days), failed to have any consistent or reproducible effect on nasal morphology. When histological changes were noted they were either seen in both control and dosed groups or when they were seen only in the dosed group they were not seen in subsequent studies at the same dose level or they were not seen at higher dose levels.

A number of putative metabolites of acetochlor also failed to have an effect on nasal tissues. One of them, 2-ethyl-6-methylaniline was given at dose levels of up to 500mg/kg daily for 20 days. All of the dose levels used were significantly higher than the level of metabolites that could be derived from the metabolism of acetochlor itself.

Whole body autoradiography studies with C-14 acetochlor at dose levels from 10-1000mg/kg and with kills up to 5 days after dosing failed to show any selective accumulation of radioactivity in nasal tissues. In one of the studies, rats were dosed daily for 10 days and both longitudinal and sagittal sections taken through the nasal regions, again without effect. Nasal tissues were also taken in a number of other studies and assayed for total radioactivity and covalently bound radioactivity. There was no evidence of accumulation after daily dosing for up to 30 days at dose levels of 200 and 1000mg/kg.

Two explanations have been considered for the lack of effect on nasal tissues. Firstly, acetochlor cannot be detected in plasma over the duration of these experiments and secondly, the majority of the

radioactivity in blood is covalently bound to the red blood cell (reported previously CTL/R/1054). It is believed therefore that acetochlor or certain key metabolites are not available to the nasal tissues in the short-term, hence there are no short term acute effects that can be detected either as morphological change or accumulation of radioactivity.

2. In considering the mechanism of action of acetochlor, there are four possible sites of electrophilic activity that could lead to cytotoxicity, DNA damage or both. The first two are derived from the ethoxymethyl side chain which produces formaldehyde and acetaldehyde by oxidative metabolism (Figure 1). The third site is the chloroacetyl N-substituent with a potentially replaceable chlorine and the fourth, the formation of quinone imine as a result of para-hydroxylation. Short-term test data suggest that the chlorine is not directly reactive, leaving aldehyde formation and the quinone imine as the more likely reactive metabolites of acetochlor.

A significant number of pharmacokinetic and metabolic studies are in progress both in vivo and in vitro. The overall objective is to look at the availability of acetochlor and its hepatic metabolites to nasal tissues and their subsequent further metabolic activation in situ in the target tissues.

The major hepatic and nasal metabolite in vitro is 2-ethyl-6-methyl chloroacetanilide formed by oxidative metabolism of the ethoxymethyl side chain. Formaldehyde and acetaldehyde are products of this oxidation process (Figure 1). Trace amounts of 2-ethyl-6-methyl-aniline were also detected in the incubation mixtures at the end of these experiments. Preliminary experiments specifically measuring formaldehyde formation in liver and nasal tissues have shown higher rates in the nasal olfactory microsomes than in hepatic microsomes. Furthermore the levels of formaldehyde detoxifying enzymes, formaldehyde dehydrogenase and aldehyde dehydrogenase, were lower in nasal tissues than in the liver (Table 1). The conversion of

acetochlor to formaldehyde was at least 3 orders of magnitude greater than the conversion of acetochlor to 2-ethyl-6-methylaniline.

Overall the data to date suggest that in situ formation of formaldehyde is the more likely cause of nasal tumours in rats than the formation of a quinone imine. Formaldehyde is formed during one of the initial and major metabolic transformations of acetochlor. It requires only a single metabolic enzyme, cytochrome P-450. Acetaldehyde, also a known nasal carcinogen, is also formed in the same reaction. However the potency of acetaldehyde is several orders of magnitude lower than that of formaldehyde. Quinone imine formation on the other hand is a multi-step process involving a number of enzymes with several opportunities for competing detoxification reactions. Furthermore, dosing of 2-ethyl-6-methylaniline, a precursor of the quinone imine, to rats at high dose levels and for long periods had no effect on the nasal epithelium. Thus on balance the evidence favours the formaldehyde hypothesis.

## FUTURE WORK

Attempts are still being made to understand the reasons for the lack of effect of acetochlor on nasal tissues in the short term. This is being approached by an analysis of the metabolites in plasma and red blood cells in order to define what the nasal tissues are actually exposed to. The mechanistic work is continuing with the synthesis of radiolabelled acetochlor with the radiolabel in the ethoxymethyl side chain rather than the phenyl ring. This should permit us to monitor the release of formaldehyde and acetaldehyde in vivo and in vitro in nasal tissues. Comparisons between the phenyl and ethoxymethyl labelled forms should ultimately allow us to distinguish between the formaldehyde and benzoquinone hypothesis.

All of the studies summarised here have recently been published in two CTL reports, CTL/R/1078 and CTL/P/3511. Further details of this work can be obtained from these reports.

## CONTRIBUTORS

John Nash
Keith Thornley
Barbara Gaskell
William Laird
Trevor Green

The above contributed to the work in 1991 with funding from the Agrochemicals Business. The same resource is expected to be available in 1992. John Nash and Keith Thornley are employed full-time on the project, the other contributors to varying degrees up to 0.3 man-year.

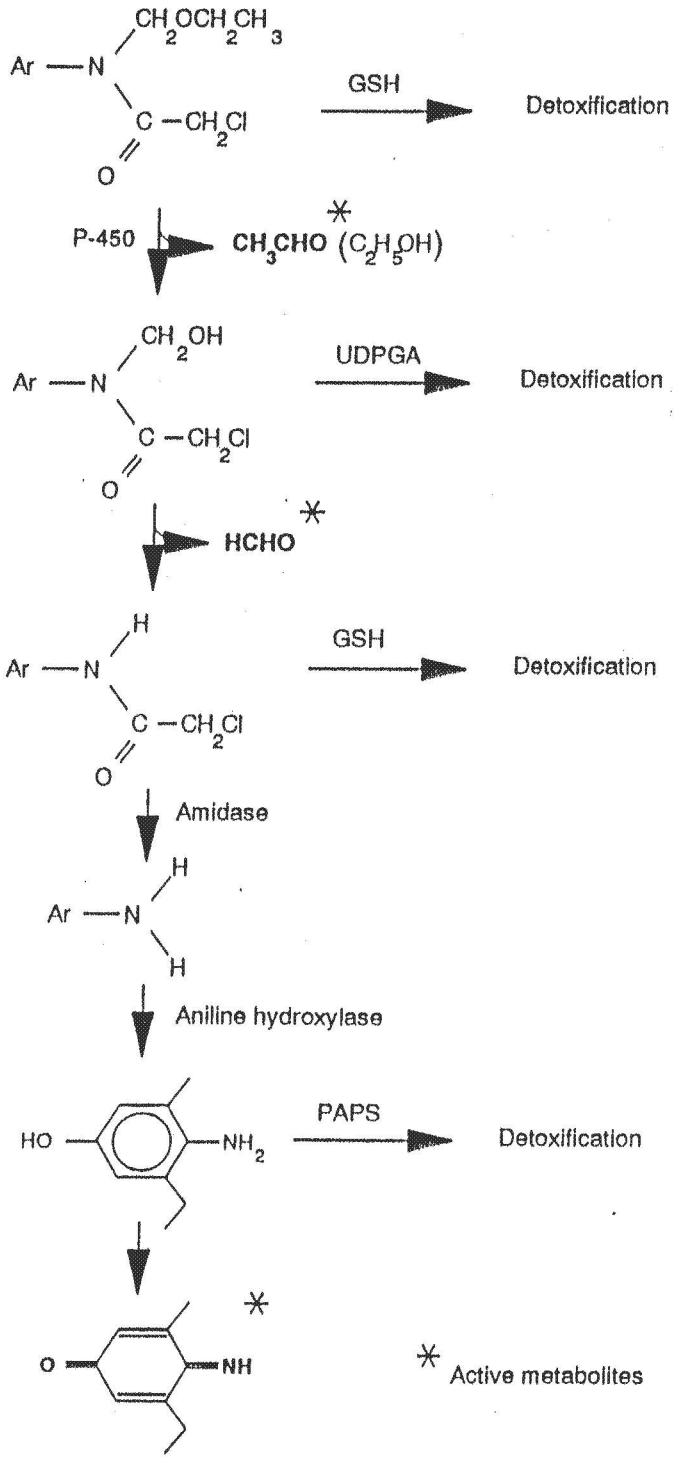
## Table 1

**The *In Vitro* Metabolism of Acetochlor to Formaldehyde in Hepatic and Nasal Fractions**

| Assay | Rate (nmol/min/mg protein) | | |
|---|---|---|---|
| | Microsomal fraction | | |
| | Liver | Olfactory | Respiratory |
| Acetochlor Formaldehyde | 8.7 | 19.6 | 12.5 |
| Formaldehyde dehydrogenase | 2.45 | 1.12 | 0.41 |
| Aldehyde dehydrogenase | 0.94 | 0.05 | 0.02 |

Figure 1

POSSIBLE MECHANISMS OF ACTIVATION OF ACETOCHLOR
Ar=2-ethyl-6-methyl phenyl



# METHYLENE CHLORIDE

J A STYLES AND T GREEN

## INTRODUCTION

It has been concluded from previous studies that the species differences in carcinogenicity of methylene chloride (DCM) are associated with the relatively higher rate of metabolism of the chemical via the glutathione-S-transferase pathway in mice compared with other species, including man. However the mechanism of interaction with genome in the mouse remains unknown.

The programme is being tackled in two ways. The glutathione-S-transferase enzyme is believed to be a nuclear transferase whose function may be relevant to the mechanism of action, and secondly, work is in progress to detect and characterise any interaction between methylene chloride and the genome in the mouse liver.

A profile of the genetic toxicology of DCM (Figure 1) showed, at the outset of our investigations, that the chemical appears to be active as a gene mutagen in prokaryotes, lower eukaryotes and insects (but not in mammalian cells), and that it induced chromosomal aberrations in cultured mammalian cells, but had no detectable clastogenic action in the mouse micronucleus assay. Studies in rats and mice exposed to radiolabelled DCM showed no evidence that the chemical alkylated the DNA in the liver or lung. The only manifestation of genotoxicity *in vivo* was the induction of liver and lung tumours in $B_6C_3F_1$ mice, but no increase in tumour incidence was observed in F344 or Sprague-Dawley rats or in Syrian hamsters. The investigations during 1991 were aimed at determining whether or not DCM was capable of causing damage to hepatic DNA specifically in mice. Although previous studies using the liver UDS assay had shown no evidence of DNA repair in hepatic cells following exposure of mice or rats to DCM, there is evidence that UDS is not an inevitable consequence of damage to DNA, and that some liver carcinogens may induce damage to DNA that is not recognized or repaired by the mechanisms operating in UDS.

PROGRESS

## 1. DNA AND CHROMOSOMAL INTERACTIONS

### a) Comparison of DNA-SSB assays

Two assays for the detection of DNA-SSB were compared: alkaline unwinding and alkaline elution. Reference carcinogens and genotoxins were administered to mice and the amount of SSB measured. The results are summarized in Tables 1 and 2 and show that the alkaline elution assay is more sensitive than the unwinding assay for the detection of SSB in mouse liver cells. The unwinding assay (Table 1) only detected dimethylnitrosamine (DMN) as positive, whereas mitomycin C (MMC), as an example of an alkylating and cross-linking agent, gave a negative response; likewise benzo(a)pyrene (BaP) and formaldehyde elicited no response. These data suggested that the phenomenon of SSB was organ-specific, since DMN was the only liver carcinogen tested. However, no increase in DNA-SSB was detected in the liver DNA from mice given DCM by gavage (Table 1), suggesting that the assay might be lacking in sensitivity. In contrast, the alkaline elution assay (Table 2) detected positive responses from DMN, MMC, ethylmethane-sulphonate (EMS), and 2-acetylaminofluorene (2AAF).

### b) DNA-SSB in mouse liver after oral administration of DCM

After administration of a single oral dose of DCM to mice the livers were removed after 4h and the DNA assayed for SSB by alkaline elution. The results, given in Table 2, show that there was a small but significant induction of SSB in the hepatic DNA by DCM.

### c) DNA-SSB in rat liver after oral administration of DCM

DCM was given to rats by gavage and the amount of SSB assayed in liver DNA after 4h. The results are given in Table 3 and show that there was no significant increase in SSB.

d) DNA-SSB in mouse livers after inhalation of DCM

Frozen livers from an inhalation study were assayed for SSB using the alkaline elution method. The results are given in Table 4 and show a small statistically significant increase in SSB in the livers from animals after a single 6h period of exposure when the results are compared with the concurrent control value. The effect is not significantly different if the statistical comparison is made with the aggregated controls which showed an increase at the later sampling times. No increases in SSB were detected following exposure for longer periods to DCM (6x6h and 65x6h).

e) Clastogenic effects of DCM on cultured human lymphocytes

Cultures of human peripheral lymphocytes were exposed to DCM and analysed for the occurrence of chromosomal aberrations. The results are presented in Table 5 and show that DCM caused a significant increase in the incidence of cells with chromosomal damage.

f) DNA protein crosslinking

Methylene chloride is metabolised by the glutathione-S-transferase pathway to formaldehyde. DNA-protein crosslinking is believed to be a fundamental feature of formaldehyde carcinogenicity. Consequently crosslinking has been investigated with methylene chloride in vitro using mouse liver nuclei and in vivo using mouse liver that had been stored from the 90-day study conducted in the laboratory previously. All of these investigations have failed to detect any significant increase in DNA-protein crosslinking.

## 2. THE GLUTATHIONE-S-TRANSFERASE ISOENZYME

The glutathione-S-transferase pathway isoenzyme (GST) responsible for the metabolism of methylene chloride has been isolated from rat liver in this laboratory and characterised by others as GST 5-5.

Subsequently work has been underway isolating the enzyme from mouse liver. Despite being a very minor and highly unstable transferase with isolation characteristics atypical of the rest of the family, it is hoped that one or more proteins will be available for sequencing within the next few weeks. A knowledge of the sequence of these enzymes, there appears to be more than one form, may help us to determine their true function in the nucleus and its relevance to the mechanism of action.

## FUTURE WORK

Studies planned for the future are aimed at investigating the following:

1. Whether formaldehyde evolved from DCM by the GST metabolic pathway can induce DNA-SSB in mouse liver. Our previous attempts to show SSB after oral administration of formaldehyde gave negative results. This may have been due to the reaction of formaldehyde with non-critical macromolecules resulting in an insignificant amount remaining to react with the liver DNA. We shall attempt to increase the concentration of formaldehyde in the liver by perfusing it into the hepatic circulation in situ.

2. The $B_6C_3F_1$ mouse has a high spontaneous incidence of liver tumours. In order to investigate whether or not this is a significant factor in the liver carcinogenicity of DCM, studies on the induction of liver cell DNA-SSB will be carried out in $C_{57}$ mice, a strain that has a very low spontaneous incidence of liver tumours.

3. Since it has been shown that DNA-SSB can be elicited in the liver by DCM, and the lung is the other main target organ for DCM-induced carcinogenicity in the mouse, the induction of DNA-SSB in mouse Clara cells following inhalation of DCM will be investigated.

4. With the exception of DNA-SSB, DCM does not appear to have any genotoxic activity in vivo to presage carcinogenicity, and in vitro no genotoxic activity has been detected in mammalian cells except

for clastogenicity. This suggests that SSB in DNA may not always be caused by direct interaction with DNA but may involve interaction with chromatin. The relationship between chromosomal damage and SSB in cultured human lymphocytes will be studied.

5. To investigate further the bacterial mutagenicity of methylene chloride. Previous studies have utilized the standard Ames tester strains of Salmonella typhimurium which contain glutathione. To investigate the involvement of the GST pathway in the mutagenicity of DCM, glutathione deficient strains of Salmonella typhimurium will be exposed to DCM.

6. Since the Ames assay is the only known unequivocal interaction between methylene chloride and DNA, an attempt will be made to characterise that interaction. The role of glutathione will be determined with S-35 labelled GSH to study its binding to DNA in the presence of methylene chloride. If possible an attempt will be made to characterise the adduct formed.

7. To facilitate 5 and 6, an effort is being made to make the glutathione conjugate of methylene chloride. This intermediate has never been isolated and synthesised and a knowledge of the stability and reactivity may help us to understand its role in methylene chloride carcinogenesis.

8. Work will continue to isolate, sequence and characterise the glutathione-S-transferase from mouse liver. By raising an antibody to this enzyme we will be able to study its cellular and sub-cellular distribution in the species of interest.

## CONTRIBUTORS

Hugh Eyton-Jones, Andrew Gyte, Ian Wilder, Colin Coutts, James Mackay and Ginny Randall have contributed to the DNA and chromosome studies. Jacky Naylor has undertaken the DNA-protein crosslinking studies and Guy Mainwaring the isolation of the transferase enzyme.

## RESOURCES

The work is funded by a consortium of 15 methylene chloride manufacturers from the USA, Europe and Japan together with two man-years of Corporate effort. This resource will continue in 1992.

Table 1

## ALKALINE UNWINDING OF LIVER DNA FROM MICE TREATED WITH VARIOUS CHEMICALS

| CHEMICAL | DOSE (mg/kg) | EXPOSURE TIME (hrs) | % UNWOUND t = 0 mins | % UNWOUND t = 80 mins |
|---|---|---|---|---|
| DIMETHYLNITROSAMINE | 10 | 4 | 11.29 +/- 4.53(6) | 46.09 +/- 2.92(7) *** |
| MITOMYCIN C | 10 | 4 | 12.58 +/- 3.03(10) | 22.34 +/- 4.17(10) |
| BENZO(A)PYRENE | 250 | 4 | 12.91 +/- 6.80(10) | 23.63 +/- 4.17(10) |
| FORMALDEHYDE | 25 | 4 | 8.79 +/- 5.53(9) | 19.08 +/-3.03(10) + |
| METHYLENE CHLORIDE | 1275 | 4 | 15.54 +/- 7.47(10) | 20.14 +/- 6.0(10) + |
| AGGREGATED CONTROLS | | — | 11.99 +/- 4.65(24) | 26.17 +/- 6.36(24) |

VALUES ARE MEAN +/- S.D WITH NUMBER OF ANIMALS EXAMINED IN PARENTHESES
*** P < 0.0005 COMPARED WITH CONTROLS
+ FORMALDEHYDE AND METHYLENE CHLORIDE CAUSED A SIGNIFICANT REDUCTION IN SINGLE STRAND BREAKS ANALYSED BY A TWO-SIDED t TEST

## TABLE SUMMMARISING RESULTS OF ALKALINE ELUTION ASSAY

| CHEMICAL | EXPOSURE TIME (hrs) | K ($cm^{-3}$) |
|---|---|---|
| DIMETHYLNITROSAMINE (10 mg/kg ) | 4 | 0.173 +/- 0.05(9) *** |
| MITOMYCIN C (10 mg/kg ) | 4 | 0.052 +/- 0.02(9) ** |
| BENZO(A)PYRENE (50 mg/kg ) | 4 | 0.012 +/- 0.004(7) + |
| BENZO(A)PYRENE (250 mg/kg ) | 4 | 0.021 +/- 0.003(7) + |
| ETHYL METHANESULPHONATE (300 mg/kg ) | 4 | 0.049 +/- 0.02(10) * |
| ETHYL METHANESULPHONATE (600 mg/kg ) | 4 | 0.108 +/- 0.02(4) *** |
| METHYLENE CHLORIDE (1275 mg/kg ) | 4 | 0.042 +/- 0.01(7) * |
| 2-(ACETYLAMINO) FLUORENE (1000 mg/kg) | 4 | 0.064 +/- 0.008(5) *** |
| AGGREGATED CONTROLS | | 0.032 +/- 0.01(30) |

K VALUES ARE MEAN +/- S.D. WITH THE NUMBER OF ANIMALS ANALYSED IN PARENTHESES.

* P < 0.01 COMPARED WITH CONTROLS

** P < 0.005 COMPARED WITH CONTROLS

*** P < 0.0005 COMPARED WITH CONTROLS

+ BENZO(A)PYRENE CAUSED A SIGNIFICANT REDUCTION IN SINGLE STRAND BREAKAGE AS ANALYSED BY A TWO-SIDED t-TEST

Table 3

## ALKALINE ELUTION OF LIVER DNA FROM RATS TREATED WITH METHYLENE CHLORIDE

| DOSE (mg/kg) | EXPOSURE TIME (hrs) | K ($cm^{-3}$) |
|---|---|---|
| 1275 | 4 | CON 0.041 +/- 0.006(5)<br>TEST0.031 +/- 0.016(10) |

VALUES ARE MEAN +/- S.D. WITH NUMBER OF ANIMALS EXAMINED IN PARENTHESES

Table 4

## ALKALINE ELUTION OF MOUSE LIVER DNA FROM 90-DAY INHALATION STUDY WITH METHYLENE CHLORIDE(PMO 753)

| DAY OF POST MORTEM | EXPOSURE TIME (hrs) | K ($cm^{-3}$) |
|---|---|---|
| 2 | 6 | CON 0.219 +/- 0.037(4)<br>TEST 0.285 +/- 0.01(4)* |
| 9 | 36 | CON 0.365 +/- 0.057(4)<br>TEST 0.390 +/- 0.052(4) |
| 89 | 390 | CON 0.432 +/- 0.106(4)<br>TEST 0.374 +/- 0.065(5) |

VALUES ARE MEAN +/- S.D. WITH NUMBER OF ANIMALS EXAMINED IN PARENTHESES
EXPOSURE CHAMBERS CONTAINED EITHER 4,000 ppm METHYLENE CHLORIDE(TEST) OR CLEAN,DRY AIR(CONTROLS)

* $P < 0.025$ COMPARED WITH CONTROLS

Table 5

## CYTOGENETIC EFFECTS OF METHYLENE CHLORIDE ON CULTURED HUMAN LYMPHOCYTES

| TREATMENT | DOSE LEVEL | % ABNORMAL CELLS EXCLUDING GAPS | | MEAN % MITOTIC INDEX | |
|---|---|---|---|---|---|
| | | -S9 | +S9 | -S9 | +S9 |
| VEHICLE | 5 ul/ml | 2.5 | 0.5 | 12.1 | 12.9 |
| METHYLENE CHLORIDE | 3325 ug/ml | 15.0** | 15.0** | 8.2 | 7.5 |
| MITOMYCIN C | 1 ug/ml | 37.0** | - | 6.9 | - |
| CYCLOPHOSPHAMIDE | 50 ug/ml | - | 35.0** | - | 6.6 |

** P < 0.01

Figure 1

# GENETIC TOXICOLOGY OF METHYLENE CHLORIDE

| in vitro | | in vivo | |
|---|---|---|---|
| GENE MUTATION | | GENE MUTATION | ? |
| non-mammalian | + | | |
| mammalian cell | - | | |
| CHROMOSOMAL MUTATION | + | CHROMOSOMAL MUTATION | - |
| UDS | - | UDS | - |
| DNA BINDING | ? | DNA BINDING | - |
| SSB | ? | SSB | ? |

# CHLORINATED PARAFFINS

I WYATT, J R FOSTER AND C R ELCOMBE

## INTRODUCTION

Chlorinated paraffins (CPs) are a group of industrially important chemicals ranging in chain length from 10 to 30 carbon atoms ($C_{10}$-$C_{13}$ short, $C_{14-17}$ medium and $C_{20-30}$ long chain) and chlorinated from 40-70% by weight. CPs are frequently used as plasticizers, fire retardants, extreme pressure additives for gear and cutting oils, paint additives and electrical insulators.

The CPs are chemically relatively inert and have a low mammalian toxicity (Birtley et al, 1980; Serrone et al, 1987). They have also been shown to be non-genotoxic in conventional assays (Birtley et al, 1980; Ashby et al, 1990). In a 2 year bioassay, male and female $B_6C_3F_1$ mice and Fischer 344 rats were gavaged daily for 5 days per week with 312mg/kg or 625mg/kg of a short chain CP (58% chlorinated) in corn oil. This regime led to an increased incidence of liver tumours in both sexes of each species, follicular cell neoplasms in the thyroids of females of both species, and adenocarcinoma in the kidneys of male rats only (NTP, 1986a). Two 90 day studies with a similar short chain CP (58% chlorinated), given in the diet in one study or by gavage in corn oil for the other study, at 100 or 625mg/kg/day to male and female Fischer 344 rats showed similar pathological trends to that shown in the two year study. The livers had hepatocellular hypertrophy in both sexes, the male kidneys showed chronic nephritis, whilst hypertrophy and hyperplasia of the thyroid was seen in both sexes (Serrone et al, 1987). In contrast a long chain CP in a 2 year study did not produce tumours in rats or mice (NTP, 1986b).

CPs have been shown to induce liver enzymes and to cause liver peroxisomal proliferation in the rat (Nilsen et al, 1981). The no effect level for peroxisomal proliferation in the male rat and mouse after daily gavage in corn oil over 14 days was 184, 600 and 473mg/kg and 180, 120 and 252mg/kg

respectively for short chain CPs (58 and 56% chlorinated) and a medium chain CP (40% chlorinated)(Wyatt et al, unpublished observations). The compounds were found to be more potent in the mouse than in the rat. The short chain CPs were more potent peroxisome proliferators than the medium chain CP (Wyatt et al, unpublished observations). In a preliminary study from this laboratory a long chain CP daily gavaged at 1g/kg for 14 days to male and female rats and mice gave no increase in hepatic peroxisomal proliferation. All these data suggest that certain CPs may elicit liver tumours by non-genotoxic mechanisms.

The current series of studies were designed to characterise some of the early events which occur in the liver, thyroid and kidneys of male and female Fischer 344 rats gavaged daily, 7 days per week for up to 90 days, with Chlorowax 500C (short chain CP with 58% chlorination) and Chlorparaffin 40G (medium chain CP with 40% chlorination) at 312 and 625mg/kg/day in corn oil, using a combination of approaches involving pathology, immunohistochemistry and biochemistry (Figure 1). The objective being to try and identify, more completely, the early events in the liver, thyroid and kidney which might lead to cancer in these tissues following dosing with CPs.

## PROGRESS

The following lists the pathological findings in the current study:

The groups referred to in the pathology tables are:

Group 1 - Control
Group 2 - 312mg/kg Chlorowax 500C
Group 3 - 625mg/kg Chlorowax 500C
Group 4 - 312mg/kg Chlorparaffin 40G
Group 5 - 625mg/kg Chlorparaffin 40G

1. Exposure to both Chlorowax 500C (C500C) and Chlorparaffin 40G (C40G) induced centrilobular hypertrophy in the livers of both male and female rats (Table 1), which was sustained for the duration of the study. The staining of the DNA in the nuclei with BrdU showed a decrease in the number of nuclei/$mm^2$ of sectioned liver for both compounds, a consequence of the hypertrophy (Table 2). However there was no major increase in liver DNA replication (Table 2). Peroxisome proliferators generally produce an increased incidence of hepatic DNA replication within the first few days of dosing (Styles et al 1988), thus this study will have missed this event.

2. Exposure to both C40G and C500C induced proliferative changes in the kidneys of male rats only and promoted the evolution of regenerative tubular basophilia which was dose dependent, and for which C500C was more effective than C40G (Table 3). Hyperplasia was only detected in the kidneys of the male rats (Table 4). Hypertrophy, as determined by the number of nuclei/$mm^2$ of kidney section, was observed in both males and females on Day 29 (Table 4), whilst only the top doses of C500C and C40G in male rats showed a marginal increase in $\alpha$2u levels (Table 5) on Day 91.

3. Exposure to both C40G and C500C induced a sustained induction of hypertrophy and hyperplasia in the thyroid follicular cells, although C500C was the more potent at inducing the effect (Table 6). C500C also produced a greater incidence of DNA replication in the thyroid of both male and female rats (Table 7).

Similarly the following lists the biochemical findings for the current study:

1. By day 15 of the study the liver weight to bodyweight ratio had already reached its peak level for both C500C and C40G in both sexes, with C500C producing the largest effect (Table 8). C500C was the better hepatic peroxisome proliferator as monitored by $CN^-$insensitive palmitoyl CoA $\beta$-oxidation (Table 8).

2. C500C treatment resulted in an initial depression in free thyroxine ($T_4$) on day 8, with a concomitant increase in thyroid stimulating hormone levels (TSH) in the male rat. The female rat showed a decrease in $T_4$ levels at the top dose for both compounds on day 8 (Table 9).

3. C40G depressed triiodothyronine ($T_3$) levels in the male and female on day 15 (Table 10).

4. The glucuronidation of $T_4$ by hepatic microsomes from C500C and C40G dosed animals was stimulated above that of microsomes from control livers at all time points studied (Table 11).

An increasing amount of evidence indicates that chemically induced cell proliferation may play a key role in chemical carcinogenesis (Loury et al, 1987; Butterworth, 1990). For many of the non-genotoxic carcinogens, induction of cell proliferation in the target tissue appears to be associated with tumour formation (Butterworth, 1990). A study, using chemicals which were hepatic peroxisome proliferators, suggested that the osmotic pumping of the DNA precursor [$^3$H]thymidine gave a better prediction of hepatocarcinogenicity (Marsman et al, 1988). A strong correlation was observed between hepatocarcinogenicity and the ability to induce a persistent increase in replicative DNA synthesis. This present study did not show the persistent increase in hepatic replicative DNA synthesis, but there was an increase in the level of hepatic peroxisome proliferation with C500C being the more potent compound. Thus it is probable that C500C would elicit liver tumours, as seen in the NTP bioassay (1986a) by non-genotoxic mechanisms.

The liver has been shown to play a major role in the metabolism of the thyroid hormones, thyroxine ($T_4$) and triodothyronine ($T_3$) (Sheridan, 1983; Kohrle et al, 1987). Compounds which have the ability to induce hepatic microsomal activity, such as phenobarbital, have been shown to increase the clearance of $T_4$ by hepatic glucuronidation with a concomitant lowering of plasma $T_3$ and $T_4$ levels but increasing plasma thyroid stimulating hormone level (TSH) (McClain et al, 1989). However, the depression of

the thyroidal hormones with concomitant increase in TSH was most marked during the early part of the study (the first 2 weeks). Similar claims and observations have been made for the hepatic peroxisome proliferators, nafenopin (Kaiser et al, 1988), phthalic acid esters (Hinton et al, 1986) and recently CPs (Wyatt et al, unpublished observations). The depression in plasma thyroid hormones ($T_3$ and $T_4$) with a concomitant increase in TSH levels is the classical response of the thyroid to a thyroid hormone imbalance. The depression of plasma thyroid hormones triggers the pituitary to increase TSH in order that the thyroid will release/synthesise more thyroid hormones to return the plasma levels of $T_3$ and $T_4$ to normal. However, continuously increased levels of TSH leads to hypertrophy and hyperplasia of the thyroid with eventual formation of follicular cell carcinoma (Hill et al, 1989). Recently there have been suggestions in the literature that the initial process of rectifying the hormonal balance ie. decreased $T_3$ and $T_4$ increased TSH, with concomitant hypertrophy/ hyperplasia of the thyroid, might be resulting in the thyroid becoming hypersensitive to TSH stimulation. Thus, apparently normal levels of TSH could result in the eventual formation of follicular cell carcinoma. The implication has been made, using phenobarbital, that the induction of hepatic microsomal enzymes which effect thyroid hormone clearance and alter thyroid function in rats might be part of the process that will eventually lead to thyroid gland neoplasia (McClain, 1989). This hypothesis has been proposed for CPs in a previous study where two short chain CPs and a medium chain CP were daily gavaged with 1g/kg in corn oil to male rats for 14 days (Wyatt et al, unpublished observations). The present study shows the anticipated alterations in the thyroid following a stimulation by TSH; that is an increase in hypertrophy and hyperplasia of the organ which lasted for the duration of the study. Thus, the follicular cell adenomas or carcinomas of the thyroid seen with the short chain CP in the 2 year study (NTP, 1986a) could be due initially to the induction of microsomal enzymes in the liver involved in the clearance of thyroid hormones ($T_3$ and $T_4$). Therefore, for the short chain CP, the liver and thyroid carcinogenic events observed in the NTP study (1986a), may be explained by non-genotoxic mechanisms.

The kidneys of male rats showed a marginal increase in staining for $\alpha$2u-globulin on day 91 for the top dose of both C500C and C40G, and

a concomitant increase in 'S' phase. The female kidneys were completely unaffected. α2u-Globulin nephropathy is recognised as an important toxicologic syndrome that occurs only in male rats following exposure to a variety of industrial and environmental chemicals and a correlation has been observed between cells in 'S' phase and eventual neoplasia of the kidney (Swenberg et al, 1989). However, more detailed studies with animals chronically exposed to unleaded gasoline (UG) or 2,2,4-trimethylpentane (TMP) suggest that the chronic cell proliferation associated with α2u nephropathy and chronic progressive nephrosis (CPN) in male rats may be responsible for the sex and species specific nephrocarcinogenicity seen with these compounds (Short et al, 1989). Thus the CPs could be behaving in a similar manner, which would indicate that the kidney tumours observed in male rats only in the 2yr bioassay, were due to a sex specific sustained stimulation of DNA synthesis and cell division - ie. a non-genotoxic mechanism.

In conclusion, these data suggest that:

1. Short chain CPs, such as Chlorowax 500C, are probably producing liver tumours due to their induction of hepatic peroxisome proliferation and stimulation of replicative DNA synthesis.

2. Both the short and medium chain CP appear to have the potential to induce kidney neoplasia in the male rat only. There was a small α2u stimulation, but, more importantly, a sustained increase in replicative DNA synthesis, probably due to a reparative process.

3. The thyroid tumours observed in the 2 year bioassay, with a short chain CP, are probably due to the induction of $T_4$ glucuronidation in the liver and hence increased clearance of $T_4$ from the plasma. The fall in $T_4$ plasma levels induced synthesis of TSH in order to return hormone levels to normal, causing hypertrophy and hyperplasia of the thyroid, which was then maintained throughout the study.

## FUTURE WORK

1. A more detailed investigation of the initial events a) in the thyroid, b) on $T_3$, $T_4$ and TSH plasma levels and c) in the induction of liver enzymes, following dosing with CPs.

2. Determine the no effect level for CPs on thyroidal events.

## CONTRIBUTORS IN 1990/1991

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Figure 1

# 90 DAY PROTOCOL

Male/female Fischer 344 rats

Daily gavage - 2 compounds

a) Chlorowax 500C ($C_{12}$ 58% Cl)
b) Chlorparaffin 40G ($C_{14-17}$ 40% Cl)

Four time points (14, 28, 56 + 90 days)

## Proposed Investigations

1) Pathology :-

a) 28 + 90 days - 5 day minipump BrdU

Cell proliferation in Thyroid
Kidney
Liver
Small intestine

b) Kidney $\alpha$ 2u globulin
c) Liver
d) Thyroid

} H + E

2) Biochemistry :-

a) Plasma $T_3$ + $T_4$ Free and Total
TSH

b) Liver 2000g supernatent
i) Peroxisome (PCoA)
ii) Microsomal glucuronidation

3) Body and organ weights

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 1a: Summary of Histopathology in the Livers of MALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| **DAY 15** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) slight | | 3 | | 3 | |
| b) moderate | | 2 | | 1 | |
| c) marked | | | 3 | | 3 |
| d) panlobular | | | 2 | | 2 |
| **DAY 29** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 1 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) marked | | 5 | 2 | 5 | 2 |
| b) panlobular | | | 3 | | 3 |
| **DAY 55** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 1 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) slight | | | | 4 | |
| b) moderate | | 4 | 1 | | 3 |
| c) marked | | 1 | 3 | 1 | 2 |
| d) panlobular | | | 1 | | |
| **DAY 91** | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 8 | 1 | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) marked | | 7 | | 8 | 9 |
| b) panlobular | | 3 | 10 | 2 | 1 |

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 1b: Summary of Histopathology in the Livers of FEMALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| DAY 15 | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) slight | | 1 | | | |
| b) moderate | | 2 | | | |
| c) marked | | 2 | | 5 | |
| d) panlobular | | | 5 | | 5 |
| DAY 29 | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) marked | | | | 1 | 1 |
| b) panlobular | | 5 | 5 | 4 | 4 |
| DAY 55 | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) slight | | 5 | | | |
| b) moderate | | | | | |
| c) marked | | | 1 | | 2 |
| d) panlobular | | | 4 | 5 | 3 |
| DAY 91 | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 10 | | | | |
| Centrilobular Hypertrophy: | | | | | |
| a) marked | | 10 | 10 | 10 | 10 |

Study Title: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

Study Number: XR2143

Table 2: Nuclear Density and BRDU incorporation data in LIVER.

| | Day 29 | | Day 91 | |
|---|---|---|---|---|
| | Nuclei/mm$^2$ | Labelled Cells (%) | Nuclei/mm$^2$ | Labelled Cells (%) |
| MALE: | | | | |
| Corn Oil | 2379+447 | 1.1+0.5 | 2318+285 | 0.6+0.1 |
| C500C (312.5) | 2225+222 | 0.7+0.3 | 1870+397 * | 0.5+0.1 |
| C500C (625) | 1423+146 ** | 2.1+1.2 | 1903+179 * | 0.3+0.1 ** |
| C40G (312.5) | 1978+338 | 1.4+0.5 | 2256+83 | 0.3+0.1 ** |
| C40G (625) | 1947+291 | 0.8+0.3 | 2287+182 | 0.9+0.2 * |
| FEMALE | | | | |
| Corn Oil | 2181+202 | 2.5+1.1 | 3231+397 | 1.2+0.8 |
| C500C (312.5) | 2024+497 | 3.7+1.8 | 2735+219 * | 0.9+0.3 |
| C500C (625) | 1577+619 * | 6.5+3.9 * | 1684+288 ** | 1.7+1.1 |
| C40G (312.5) | 1993+255 | 2.0+1.0 | 1885+315 ** | 2.2+1.3 |
| C40G (625) | 1792+358 * | 3.5+3.1 | 2194+252 ** | 1.6+0.6 |

Notes: * significant at the 5% level (Students' t test).

** significant at the 1% level.

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 3a: Summary of Histopathology in the Kidney of MALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| **DAY 15** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | 1 | 1 | 1 | |
| Cortical Eosinophilia | | | | | |
| a) Slight | | 4 | 2 | 4 | 1 |
| b) Moderate | | | 2 | | 4 |
| **DAY 29** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 1 | | | 2 | 2 |
| Cortical Eosinophilia | | | | | |
| a) Slight | 4 | 1 | 2 | 3 | 2 |
| b) Moderate | | 4 | 3 | | 1 |
| Tubular Basophilia | | | | | |
| a) Focal | | | 1 | | |
| **DAY 55** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 1 | | | | |
| Cortical Eosinophilia | | | | | |
| a) Slight | 3 | | 1 | | |
| b) Moderate | | 3 | | 5 | 5 |
| c) Marked | | 2 | 4 | | |
| Tubular Basophilia | | | | | |
| a) Focal | | 1 | 1 | 3 | |
| b) Multifocal | | 1 | 2 | | |
| Focal Tubular Dilatation | | | 1 | | |
| **DAY 91** | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 8 | 1 | | | |
| Cortical Eosinophilia | | | | | |
| a) Slight | | | | | |
| b) Moderate | | 7 | 9 | 10 | 9 |
| c) Marked | | 2 | | | |
| Tubular Basophilia | | | | | |
| a) Focal | 2 | 3 | 4 | 5 | 6 |
| b) Multifocal | | 4 | 6 | | 1 |
| Tubular Dilatation | | | 1 | | |

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 3b: Summary of Histopathology in the Kidney of FEMALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| **DAY 15** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | 5 | 5 | 5 | 5 |
| **DAY 29** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 1 | 5 | 4 | 5 | 5 |
| Cortical Eosinophilia | | | | | |
| a) Slight | | | 1 | | |
| **DAY 55** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | 4 | 5 | 5 | 5 |
| Tubular Basophilia | | | | | |
| a) Focal | | 1 | | | |
| **DAY 91** | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 9 | 9 | 10 | 10 | 9 |
| Tubular Basophilia | | | | | |
| a) Focal | 1 | 1 | | | 1 |

Study Title: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

Study Number: XR2143

Table 4: Nuclear Density and BRDU incorporation data in KIDNEY.

| | Day 29 | | Day 91 | |
|---|---|---|---|---|
| | Nuclei/mm$^2$ | Labelled Cells (%) | Nuclei/mm$^2$ | Labelled Cells (%) |
| MALE: | | | | |
| Corn Oil | 4967+293 | 2.0 + 0.6 | 4689+705 | 1.2 + 0.2 |
| C500C (312.5) | 4105+407 ** | 3.2 + 0.7 * | 5192+188 | 1.5 + 0.5 |
| C500C (625) | 3285+84 ** | 3.6 + 0.4 ** | 6318+848 ** | 3.0 + 1.0 ** |
| C40G (312.5) | 3391+285 ** | 3.7 + 0.5 ** | 4397+510 | 1.8 + 0.5 * |
| C40G (625) | 3761+183 ** | 3.7 + 1.0 ** | 4556+302 | 2.2 + 0.5 ** |
| FEMALE | | | | |
| Corn Oil | 4013+338 | 1.4 + 0.3 | 4079+172 | 0.7 + 0.2 |
| C500C (312.5) | 3708+1033 | 1.3 + 0.3 | 3788+361 | 0.5 + 0.1 |
| C500C (625) | 2860+317 ** | 1.4 + 0.6 | 3165+313 ** | 0.8 + 0.3 |
| C40G (312.5) | 3695+414 | 1.6 + 0.7 | 4860+685 * | 0.5 + 0.2 |
| C40G (625) | 3311+294 ** | 1.1 + 0.4 | 4026+275 | 0.3 + 0.1 |

Notes: * significant at the 5% level (Students' t test).

** significant at the 1% level.

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

Table 5: Summary of immunohistochemical results for alpha 2u globulin localisation in male and female kidney.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| MALES | | | | | |
| DAY 15 | 3.6+0.9 | 3.6+0.5 | 3.8+0.4 | 4.2+0.8 | 4.4+0.5 |
| DAY 29 | 3.0+0.0 | 3.0+0.0 | 3.6+0.9 | 3.2+0.4 | 4.0+0.0 |
| DAY 55 | 4.0+0.0 | 4.0+0.0 | 4.2+0.8 | 4.0+0.7 | 4.2+0.4 |
| DAY 91 | 3.9+0.7 | 4.3+0.5 | 4.8+0.8$^{XX}$ | 4.0+0.0 | 4.5+0.5 $^{X}$ |
| FEMALES | | | | | |
| DAY 15 | 2.0+0.0 | 2.0+0.0 | 2.0+0.0 | 2.0+0.7 | 2.4+0.5 |
| DAY 29 | 2.0+0.0 | 2.2+0.4 | 2.0+0.0 | 2.2+0.4 | 1.8+0.4 |
| DAY 55 | 1.4+0.6 | 1.4+0.6 | 1.4+0.6 | 2.0+0.7 | 2.0+0.0 |
| DAY 91 | 2.3+0.9 | 2.4+0.8 | 2.8+0.8 | 2.8+0.4 | 2.1+0.9 |

NOTES:

The figures are calculated from the histochemical scoring system as follows:

- = 1
+/-- = 2
+/- = 3
+ = 4
++ = 5
+++ = 6

All groups contained 5 animals per group with the exception of day 91 which contains 10 animals per group.

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 6a: Summary of histopathology in the Thyroid Glands of MALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| DAY 15 | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | 1 | | | |
| b) moderate | | 3 | 2 | 4 | 2 |
| c) marked | | 1 | 3 | 1 | 3 |
| DAY 29 | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | 3 | | | |
| b) moderate | | 2 | 2 | 4 | 2 |
| c) marked | | | 3 | 1 | 3 |
| DAY 55 | | | | | |
| No Examined | 4 | 5 | 5 | 5 | 5 |
| NAD | 4 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | | | | |
| b) moderate | | 1 | 1 | 1 | 1 |
| c) marked | | 4 | 4 | 4 | 4 |
| Follicular Cell Hyperplasia | | 5 | 5 | 5 | 5 |
| DAY 91 | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 10 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | | | 3 | |
| b) moderate | | 3 | | 2 | |
| c) marked | | 7 | 10 | 5 | 10 |
| Follicular Cell Hyperplasia | | 10 | 10 | 10 | 10 |

STUDY TITLE: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

STUDY NO: XR2143

TABLE 6b: Summary of histopathology in the Thyroid Glands of FEMALE animals.

| | GROUP | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| **DAY 15** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | 1 | | 2 | |
| b) moderate | | 3 | 3 | 3 | 3 |
| c) marked | | 1 | 2 | | 2 |
| **DAY 29** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | | | 5 | 2 |
| b) moderate | | 3 | 4 | | 3 |
| c) marked | | 2 | 1 | | |
| **DAY 55** | | | | | |
| No Examined | 5 | 5 | 5 | 5 | 5 |
| NAD | 5 | | | 1 | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | 1 | | 3 | 1 |
| b) moderate | | 3 | 2 | | 4 |
| c) marked | | 1 | 3 | | |
| Follicular Cell Hyperplasia | | 5 | 5 | 2 | 5 |
| **DAY 91** | | | | | |
| No Examined | 10 | 10 | 10 | 10 | 10 |
| NAD | 10 | | | | |
| Follicular Cell Hypertrophy: | | | | | |
| a) mild | | | | 9 | |
| b) moderate | | 4 | 3 | 1 | 3 |
| c) marked | | 6 | 7 | | 7 |
| Follicular Cell Hyperplasia | | 10 | 10 | 10 | 10 |

Study Title: Chlorinated Paraffins: 90 day study with C500C and C40G in male and female F344 rats.

Study Number: XR2143

Table 7: Nuclear Density and BRDU incorporation data in THYROID.

| | Day 29 | | Day 91 | |
|---|---|---|---|---|
| | Nuclei/mm$^2$ | Labelled Cells (%) | Nuclei/mm$^2$ | Labelled Cells (%) |
| MALE: | | | | |
| Corn Oil | 6835+637 | 1.0 + 0.3 | 8297+943 | 7.7 + 4.0 |
| C500C (312.5) | 6737+296 | 2.0 + 1.4 | 9076+582 | 15.8 + 2.8 ** |
| C500C (625) | 7692+216 * | 1.3 + 0.1 | 11124+406 ** | 14.0 + 1.9 * |
| C40G (312.5) | 7469+1796 | 2.0 + 0.8 * | 11416+1806 * | 7.7 + 4.0 |
| C40G (625) | 7645+1839 | 1.9 + 0.9 | 9424+612 | 5.4 + 3.0 |
| FEMALE | | | | |
| Corn Oil | 8224+731 | 1.3 + 0.3 | 9724+571 | 6.5 + 1.4 |
| C500C (312.5) | 7167+1108 | 2.1 + 0.5 * | 10114+910 | 14.1 + 2.4 ** |
| C500C (625) | 7632+443 | 1.5 + 0.2 | 10755+1371 | 14.2 + 2.7 ** |
| C40G (312.5) | 8028+1816 | 3.2 + 0.8 ** | 12022+569 ** | 3.5 + 0.7 ** |
| C40G (625) | 7455+590 | 3.4 + 0.8 ** | 10502+704 | 4.3 + 2.7 |

Notes: * significant at the 5% level (Students' t test).

** significant at the 1% level.

Table 8

# Effect of Chlorinated Paraffins on Liver/body Weight Ratios and Peroxisome Proliferation

| | Day 15 | | Day 29 | | Day 57 | | Day 91 | |
|---|---|---|---|---|---|---|---|---|
| *MALE* | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg |
| *Control* | 4.40 ±0.37 | 3.94 ±3.54 | 4.17 ± 0.22 | 3.71 ± 0.91 | 3.96 ± 0.23 | 3.40 ± 0.83 | 3.64 ± 0.31 | 2.48 ± 0.37 |
| *Chlorowax 500C* | | | | | | | | |
| *312.5mg/kg* | 6.00 ± 0.23 ** | 7.55 ± 2.46 | 6.18 ± 0.13 ** | 9.71 ± 1.26 ** | 6.28 ± 0.06 ** | 9.85 ± 0.54 ** | 5.75 ± 0.16 ** | 9.18 ± 2.01 ** |
| *625mg/Kg* | 7.33 ± 0.16 ** | 9.41 ± 2.20 * | 7.37 ± 0.10 ** | 13.17 ± 1.39 ** | 7.67 ± 0.10 ** | 14.74 ± 0.94 ** | 6.73 ± 0.26 ** | 13.40 ± 2.11 ** |
| *Chlorparaffin 40G* | | | | | | | | |
| *312.5mg/kg* | 5.19 ± 0.29 ** | 4.87 ± 0.85 | 5.28 ± 0.28 ** | 6.30 ± 1.01 ** | 4.86 ± 0.10 ** | 7.84 ± 1.92 ** | 4.54 ± 0.20 ** | 7.99 ± 1.60 ** |
| *625mg/Kg* | 5.68 ± 0.21 ** | 6.35 ± 1.40 | 5.51 ± 0.25 ** | 9.37 ± 0.48 ** | 5.53 ± 0.19 ** | 5.67 ± 1.17 * | 5.97 ± 0.42 ** | 9.05 ± 1.69 ** |
| *FEMALE* | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg | % Liver/body weight | PCoA nmol/min/mg |
| *Control* | 4.07 ± 0.24 | 4.80 ± 2.46 | 4.01 ± 0.18 | 2.40 ± 0.35 | 3.51 ± 0.11 | 2.33 ± 0.37 | 3.36 ± 0.17 | 2.81 ± 0.82 |
| *Chlorowax 500C* | | | | | | | | |
| *312.5mg/kg* | 5.56 ± 0.26 ** | 10.01 ± 2.99 * | 6.16 ± 0.10 ** | 8.06 ± 1.15 ** | 5.85 ± 0.09 ** | 9.05 ± 1.13 ** | 5.51 ± 0.24 ** | 9.49 ± 2.05 ** |
| *625mg/Kg* | 7.20 ± 0.21 ** | 14.34 ± 3.21 ** | 7.92 ± 0.36 ** | 13.18 ± 1.88 ** | 7.28 ± 0.25 ** | 14.94 ± 1.74 ** | 7.12 ± 0.36 ** | 14.27 ± 3.82 ** |
| *Chlorparaffin 40G* | | | | | | | | |
| *312.5mg/kg* | 4.80 ± 0.25 ** | 5.72 ± 1.46 | 5.15 ± 0.08 ** | 5.89 ± 0.84 ** | 4.43 ± 0.16 ** | 6.72 ± 0.96 ** | 4.49 ± 0.32 ** | 6.04 ± 0.91 ** |
| *625mg/Kg* | 5.56 ± 0.32 ** | 9.63 ± 6.26 | 5.80 ± 0.35 ** | 6.63 ± 1.47 ** | 5.11 ± 0.11 ** | 7.29 ± 1.14 ** | 5.87 ± 0.25 ** | 10.33 ± 2.61 ** |

* $p < 0.05$ ** $p < 0.01$

Table 9

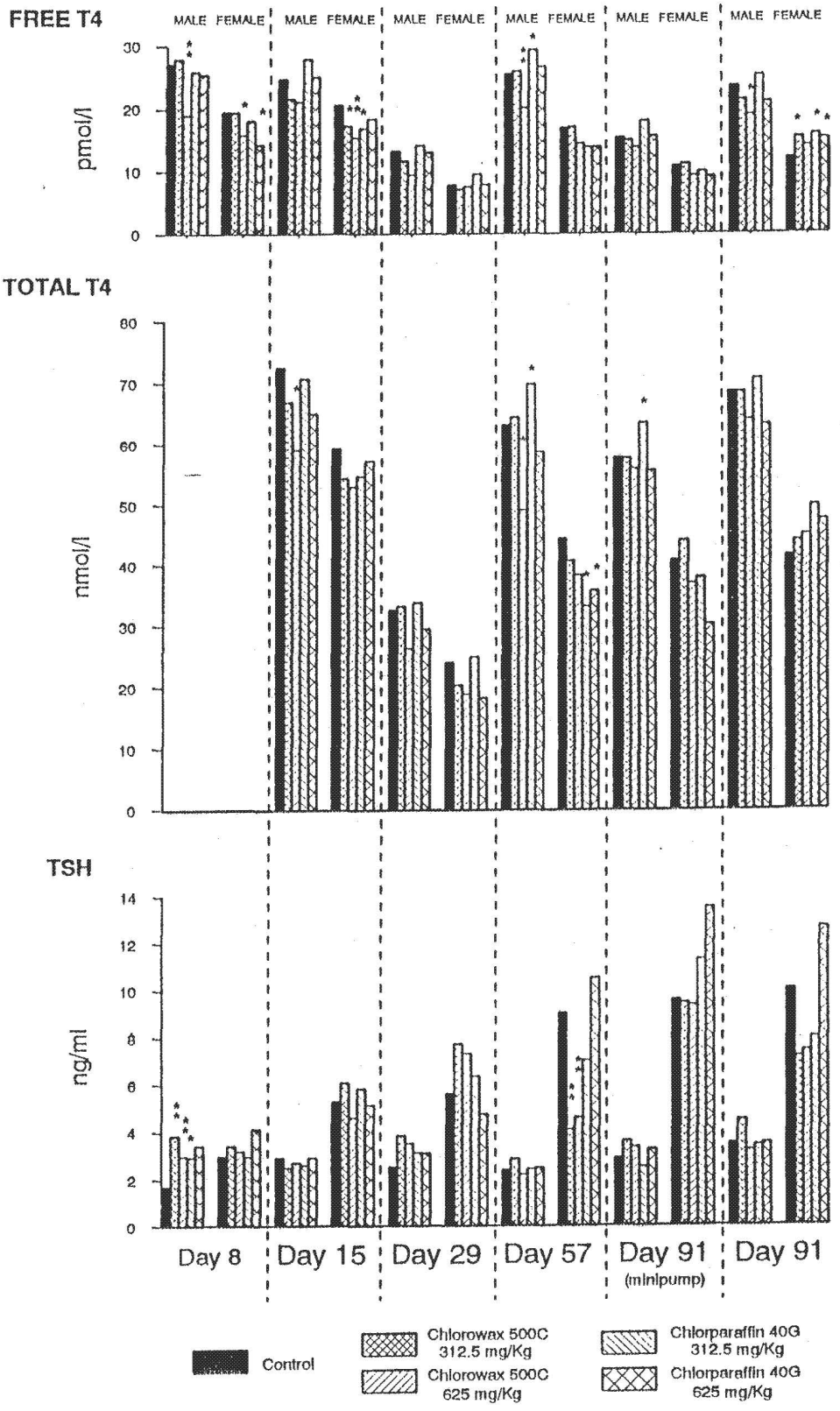


Table 10

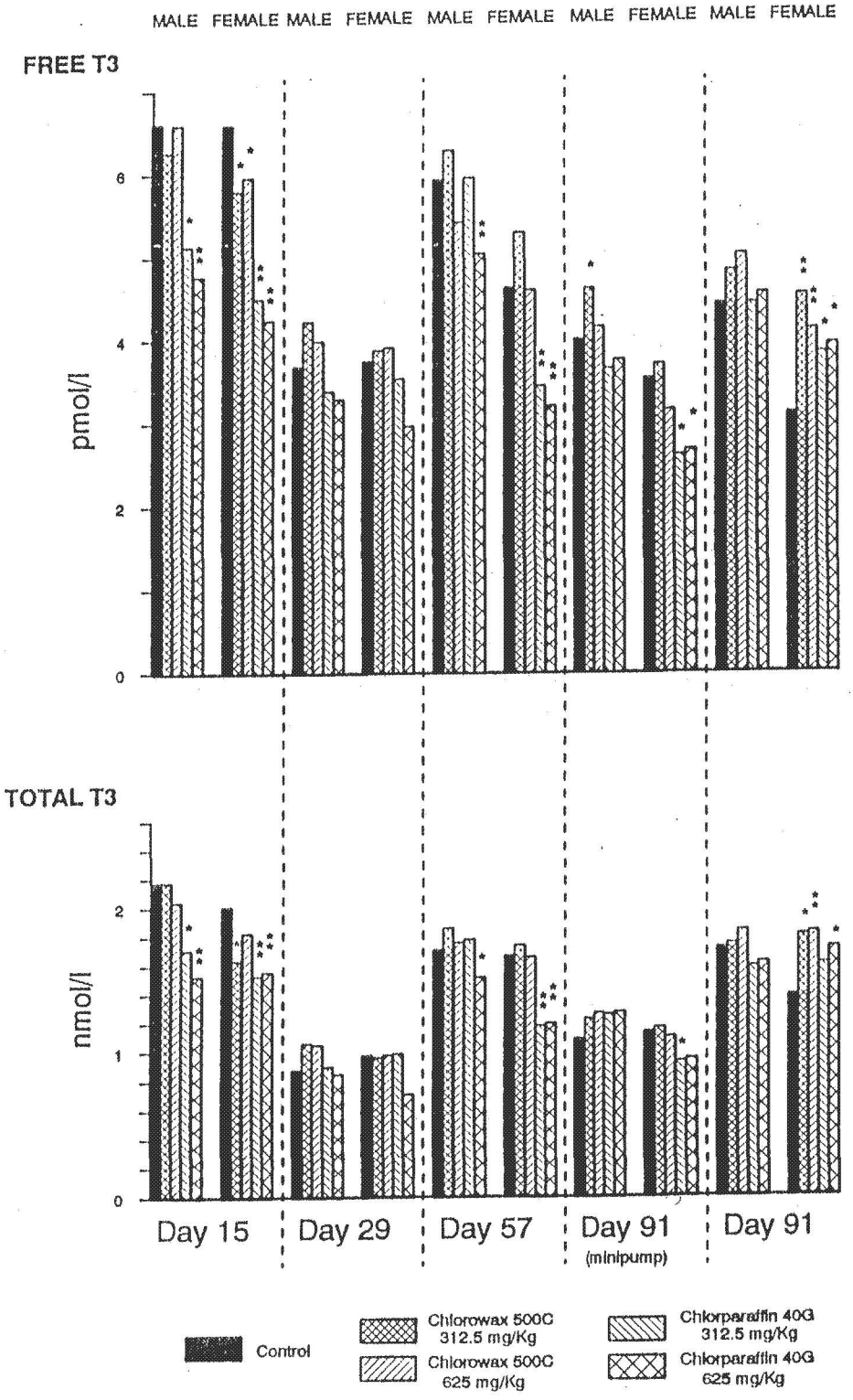


Table 11

| | | $T_4$ GLUCURONIDATION | | PARA-NITROPHENOL GLUCURONIDATION | |
|---|---|---|---|---|---|
| | | % increase (control = 100%) | | % increase (control = 100%) | |
| | | Male | Female | Male | Female |
| Day 15 | Chlorowax 500C 312.5mg/Kg | 155 ± 16** | 172 ± 32** | 200 ± 64* | 225 ± 25** |
| | 625mg/Kg | 167 ± 7** | 223 ± 40** | 226 ± 62* | 275 ± 25** |
| | Chlorparaffin 40G 312.5mg/Kg | 125 ± 12** | 166 ± 18** | 147 ± 51 | 133 ± 19** |
| | 625mg/Kg | 114 ± 11* | 150 ± 13** | 128 ± 26 | 161 ± 24** |
| Day 29 | Chlorowax 500C 312.5mg/Kg | 163 ± 21** | 180 ± 67* | 164 ± 81 | 154 ± 41* |
| | 625mg/Kg | 202 ± 21** | 228 ± 79** | 163 ± 43* | 170 ± 53* |
| | Chlorparaffin 40G 312.5mg/Kg | 182 ± 66* | 161 ± 74 | 104 ± 20 | 102 ± 21 |
| | 625mg/Kg | 158 ± 13** | 131 ± 28* | 135 ± 65 | 140 ± 65 |
| Day 57 | Chlorowax 500C 312.5mg/Kg | 158 ± 33** | 173 ± 24** | 207 ± 20** | 225 ± 23** |
| | 625mg/Kg | 177 ± 57* | 214 ± 57** | 279 ± 69** | 313 ± 50** |
| | Chlorparaffin 40G 312.5mg/Kg | 139 ± 17** | 144 ± 29* | 164 ± 12** | 237 ± 113* |
| | 625mg/Kg | 112 ± 24 | 133 ± 15* | 173 ± 33** | 193 ± 33** |
| Day 91 (minipump) | Chlorowax 500C 312.5mg/Kg | 166 ± 41** | 161 ± 48* | 249 ± 42** | 162 ± 36** |
| | 625mg/Kg | 178 ± 51* | 147 ± 33* | 295 ± 35** | 215 ± 53** |
| | Chlorparaffin 40G 312.5mg/Kg | 134 ± 33 | 143 ± 30* | 180 ± 23** | 150 ± 48* |
| | 625mg/Kg | 169 ± 34** | 147 ± 34* | 365 ± 39** | 213 ± 38** |
| Day 91 | Chlorowax 500C 312.5mg/Kg | 225 ± 50** | 170 ± 16** | 224 ± 57** | 169 ± 43** |
| | 625mg/Kg | 205 ± 43 | 176 ± 28 | 222 ± 41 | 204 ± 59 |
| | Chlorparaffin 40G 312.5mg/Kg | 186 ± 55** | 132 ± 22* | 164 ± 26** | 123 ± 37** |
| | 625mg/Kg | 191 ± 27 | 165 ± 45 | 299 ± 44 | 210 ± 58 |

* $p < 0.05$ ** $p < 0.01$

# INDUCTION OF P450 ISOENZYMES IN PRIMARY RAT HEPATOCYTES - MOLECULAR STRUCTURE VERSUS INDUCTION PROFILE

M PROVAN

## INTRODUCTION

The previous paper addressed the relationship between P450 induction and cancer, this complementary research is centred around the earliest stages of that process - the binding of the chemical to the target site, and attempts to correlate molecular structure with P450 induction. The table below details nomenclature and benchmark inducers of the seven major inducible forms of P450.

Table 1

| | |
|---|---|
| P450IA1 P450IA2 | Polycyclic aromatics |
| P450IIB1 P450IIB2 | Phenobarbital |
| P450IIIA1 | Pregnenolone 16 alpha carbonitrile |
| P450IIE1 | Ethanol |
| P450IVA1 | Peroxisome Proliferators |

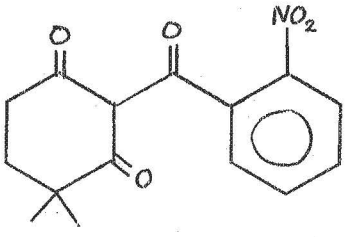
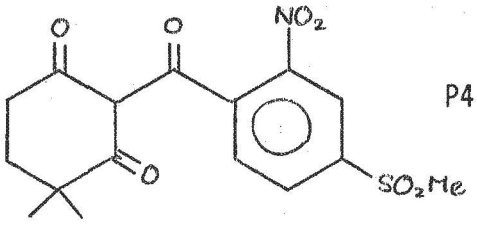
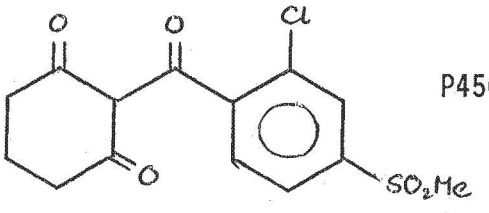
Our aim is to identify the structural features of a chemical endowing that molecule with the potential to induce one of the major forms of P450. An understanding of these parameters and an estimate of the potency of induction may allow us to predict which structures may be most likely to be non-genotoxic carcinogens. This characterisation of P450 induction in primary hepatocytes, for the closely related chemical series of triketone herbicides, is coincident with Agrochemicals Business needs, forming part of a battery of toxicology screens for potential triketone development candidates. Therefore, the triketone series of chemicals forms the basis of this report, but this work may also be viewed as a first step towards assessing the feasibility of the wider goal of accurate prediction of non-genotoxic carcinogens.

PROGRESS

A good correlation has so far been found between in vivo and in vitro induction profiles for a variety of chemicals including triketones, suggesting that results from induction studies in primary hepatocytes can be valuable in assessing the potential of chemicals to induce P450 in vivo.

It is also clear, Table 2, that small changes in triketone structure can substantially alter this P450 induction profile in primary hepatocytes.

Table 2

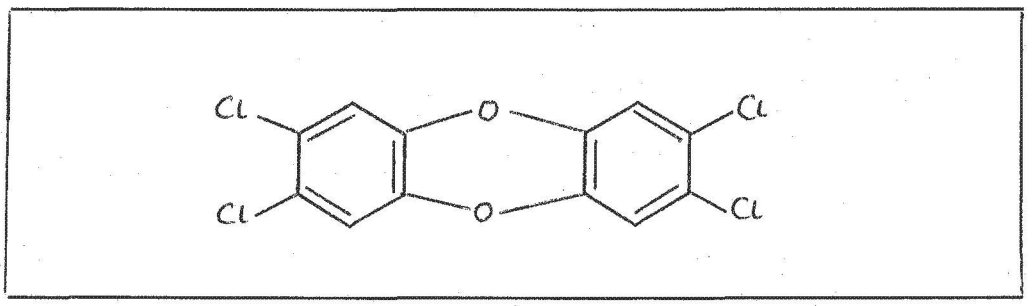
| Structure | Induction Profile in Primary Rat Hepatocytes | Magnitude of Response |
|---|---|---|
|  | P450IIA1 | Zero |
| | P450IIB1 P450IIB2 | Strong |
| | P450IIIA1 | Strong |
|  | P450IA1 | Strong |
| | P450IIB1 P450IIB2 | Moderate |
| | P450IIIA1 | Marginal |
|  | P450IA1 | Marginal |
| | P450IIB1 P450IIB2 | Zero |
| | P450IIIA1 | Zero |

To date, we have discovered that triketone herbicides can induce, in primary rat hepatocytes, three of the five major families of P450, namely, P450IA1; P450IIB1 and P450IIB2; P450IIIA1. The major thrust of our studies

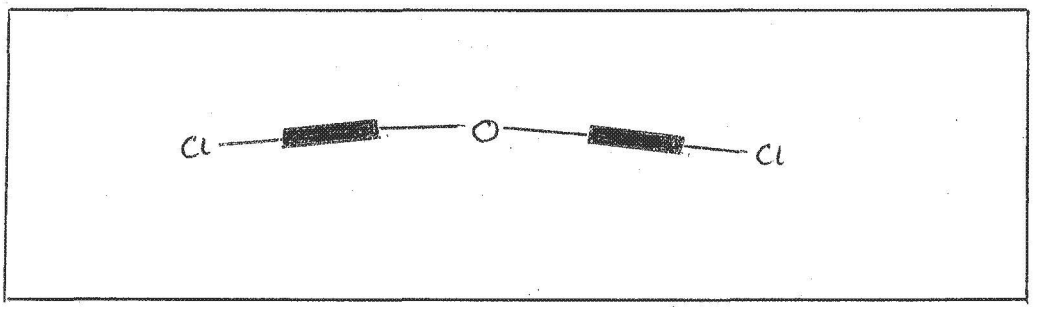
has been directed towards the measurement of induction of P450IA1 and P450IIIA1 in primary rat hepatocyte cultures by SDS gel electrophoresis and Western Blotting using antibodies for detection. Complementary methodology for the measurement of induction of P450IIB1 P450IIB2 is still in the development phase.

The results of the studies attempting to define the structural requirements for P450IA1 induction are shown in Table 3.

It has been reported many times in the literature that molecules which induce P450IA1 should be planar or very near planar and would fit inside an imaginary box of length 8 Angstroms and breadth 3 Angstroms. Typically, such a molecule will comprise of two coplanar phenyl rings, substituted in the lateral positions with polarisable groups, eg. chlorine. Heteroatoms joining the coplanar phenyl rings appear to increase potency, the most potent inducer of P450IA1 found to date is tetrachlorodibenzodioxin, (TCDD).

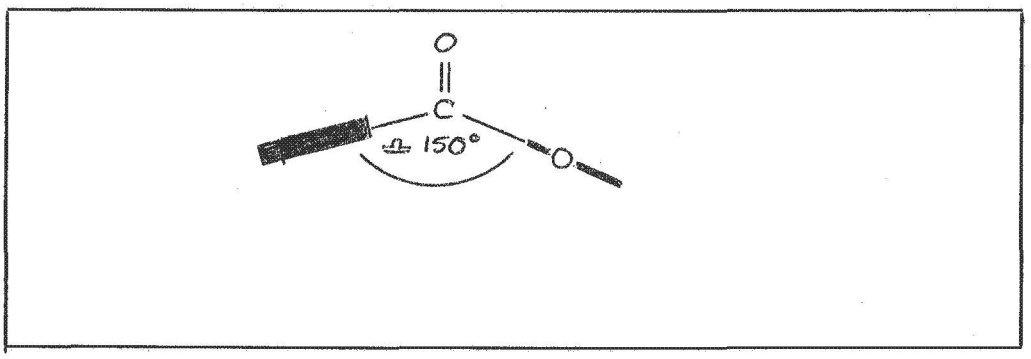



Then, viewing this molecule from the lower edge of the coplanar phenyl rings allows an appreciation of the planarity of the molecule.



The mechanisms by which chemicals induce these forms of P450 is poorly understood, however, it seems likely that the induction process is mediated by the binding of the chemical to a cellular receptor. Groups of chemicals which induce enzymes by a receptor mediated mechanism have been shown to share common structural features. Therefore to fit the currently accepted theory of structural requirements for P450IA1 inducers, triketones, which have been shown to induce P450IA1, must be planar.

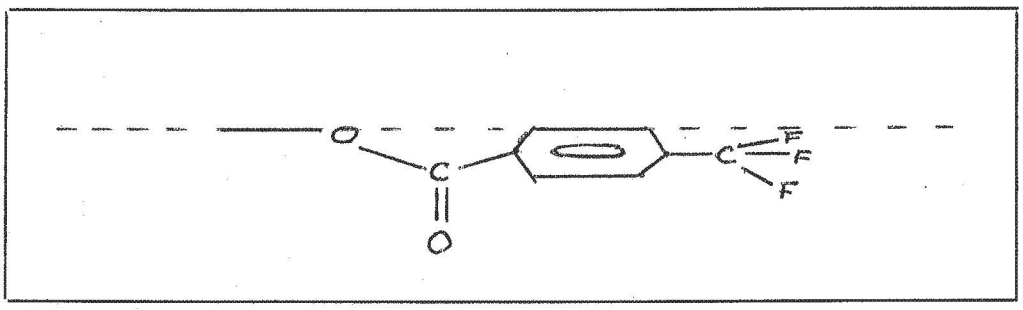
However, the minimum energy conformations of the triketones, studied by molecular modelling techniques at Western Research Center, Jealott's Hill and at Pharmaceuticals, have the two rings held in a skewed conformation. The energy differences between these skewed minimum energy conformations, and approximately 'coplanar' triketone conformation is considerable. Furthermore, even in this unfavoured 'coplanar' conformation the two ring systems are not totally coplanar and lie at about 150 degrees to each other.




The question then arises - if the two ring systems and the three oxygen atoms of a triketone are unlikely to become coplanar, then how can such a molecule interact with the same receptor as for example TCDD?

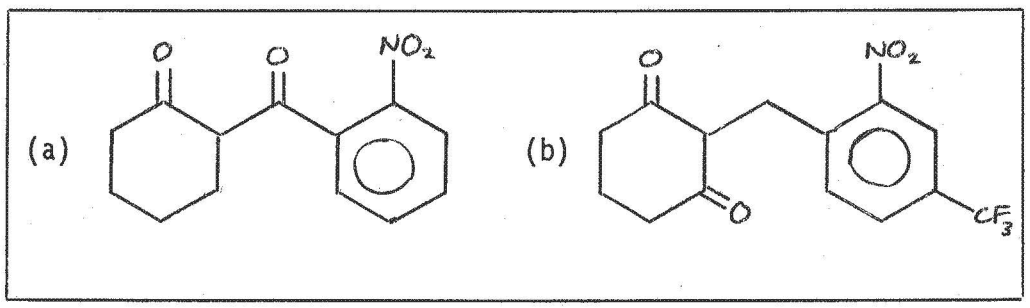
If one builds a spacefilling model of these compounds with the rings in the orthogonal arrangement, a 'planar surface' is apparent. This surface comprises of the cyclohexanedione ring, which is in the 'same plane' as the edge of the orthogonal phenyl ring, with the bulk of the phenyl ring

lying beneath this plane. Any substituent in the 4 position of the phenyl ring, eg. chlorine or CF3 would also lie in this planar surface. The optimum area of this surface may still be 8x3 Angstroms.



One of the important features of this conformation is that the phenyl ring is orientated below, rather than bisecting, this planar surface.

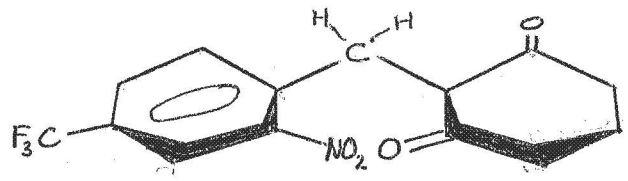
There are two areas of discussion from the results, Table 3, of our induction on the triketone herbicides, that this interim report will address. Firstly, the two most potent compounds studied to date are diketones. Structure (a) can be described as having an endocyclic and an exocyclic carbonyl group, whilst in structure (b) both carbonyl functions are endocyclic.




Both compounds (a) and (b) induce P450IA1 to a similar extent at the three concentrations used 1, 0.5 and 0.1mM. With compound (b), the two

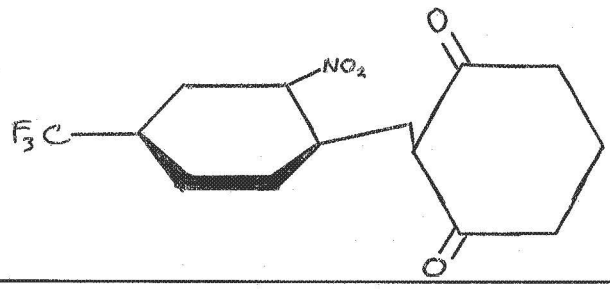
ring systems, being linked by an SP3 carbon, cannot lie in the same plane, the angle between the two rings must be about 109 degrees, conformation b1.

b1




With the lower energy orthogonal arrangement between the rings, the endocyclic carbonyl groups lie above and below the plane of the phenyl ring in a line almost at right angles to the plane of the phenyl ring, conformation b2.

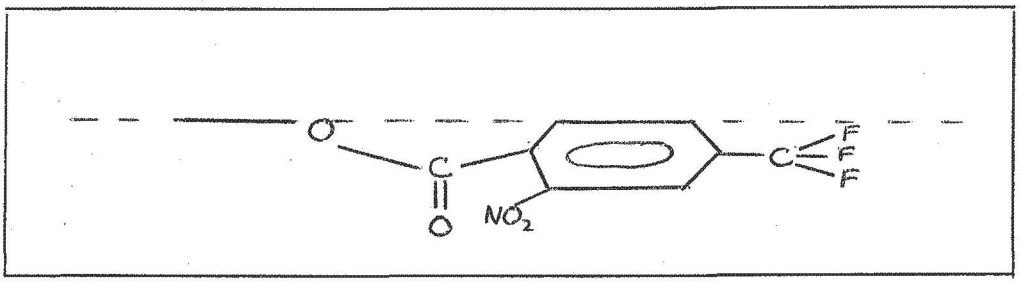
b2



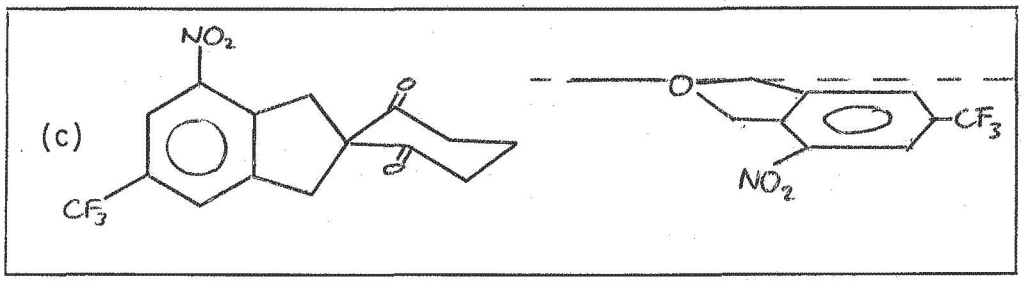

Conformation b1 is must less preferred due to the adverse interactions between the dione oxygen atoms with the 2 and 6 (H and NO2) positions of the phenyl ring.

A similar conformation is likely to be found in compound (a) where the steric interactions between the carbonyls, and the 2 and 6 position of the phenyl ring will be much greater than in the previous example, as one of the carbonyl functions is now exocyclic and so lies closer to the phenyl ring.

Both of these chemicals, the most active P450IA1 inducers tested from the triketone general series, have similar low energy conformations, and this conformation is consistent with the conformation proposed to bind the receptor.

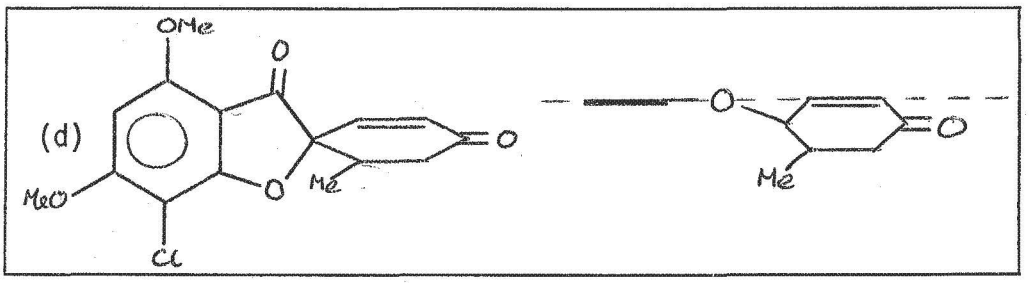


To further test this hypothesis, synthesis of the following chemical (c) was requested.



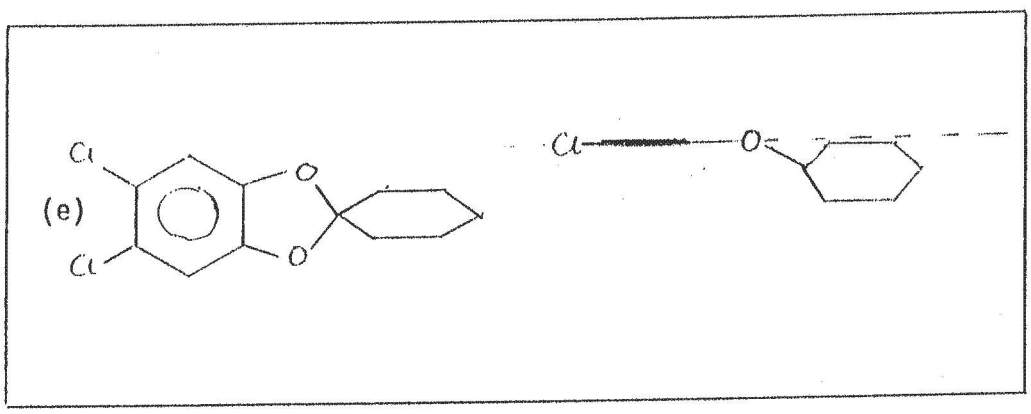

The third ring introduced into this compound will restrict the conformational freedom of the molecule, locking the two rings into an orthogonal arrangement. Unfortunately, the synthesis of this compound may be difficult, but other conformationally restricted compounds, such as spiro compounds, may be more readily accessible, yet still of value in induction studies.

One such compound may be desmethoxy griseofulvin, (d).



The phenyl ring, carbonyl and the oxygen are coplanar, whilst the cyclohexenone ring is held orthogonal. Space filling models suggest that the edge of this cyclohexenone ring is in the same plane as the phenyl, carbonyl and oxygen system. I have tested this compound in the primary hepatocyte system but it does not give a decisive result - a marginal increase in P450IA1 with solubility problems above about 0.1mM. Based on structure - receptor binding relationships, reported by Safe and Co-workers, for TCDD, exploring the effect of position and nature of phenyl ring substituents upon the strength of binding, perhaps the weak induction response for this compound is not surprising considering the phenyl ring substitution pattern. Clearly, a potential P450IA1 inducer must not only have a suitably sized planar surface, but in addition the potency of induction will be modulated by the electronic configuration of that surface.

I am currently exploring the synthesis of compound (e), which I hope will be complete early November.




The left hand portion is obviously modelled on TCDD to optimise induction of P450IA1 but I have introduced a SP3 carbon, which means the cyclohexane ring is held orthogonal to this plane, with the bulk of the cyclohexane ring lying below the plane and the upper edge lying in the plane of the rest of the molecule.

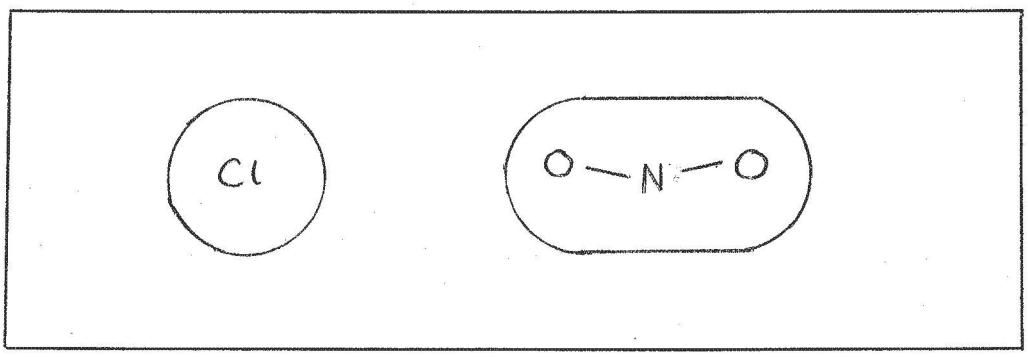
From the above discussion, it appears that:

1. Induction of P450IA1 may be dependent upon the likelihood of each triketone molecule to maintain a particular conformation, and modulated by electronic effects.

2. The diketones seem more potent than triketones.

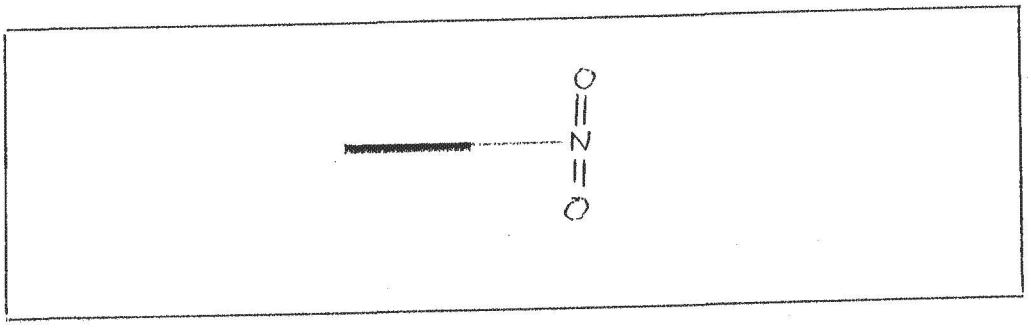
The second striking observation from Table 3, is that the potency of induction of P450IA1 appears to depend upon the nature of the ortho substituent of the phenyl ring. If the ortho substituent is chlorine, then induction is minimal, however, the potency of induction is increased by the substitution of chlorine by a nitro function.

A comparison of nitro and chlorine as substituents in aromatic systems suggests that the two groups may behave rather differently. The difference is based upon the shapes of the chlorine and nitro substituents. The chlorine may be envisaged as a roughly spherical substituent with a strong inductive effect upon the phenyl ring. The nitro group, however, is not spherical but rather more of a 'sausage' shape, and can exert both inductive and mesomeric effects on the aromatic ring.




As a substituent of a phenyl ring the nitro group adopts the energetically preferred conformation of having the phenyl ring and the oxygens of the nitro group in the same plane, thereby allowing the favourable interaction

between the pi orbitals of the ring and the nitro group. However, if the nitro group is sterically hindered, a more favourable conformation is sometimes found with the oxygen atoms out of the plane of the ring.



The optimum triketone conformation suggested above for P450IA1 induction requires the ortho substituent to be very close to the exocyclic carbonyl and therfore displays considerable steric crowding. It may be that this particular conformation, required for induction, may be more readily achievable by ortho nitro, where the oxygens can move out of the plane of the ring, relieving steric crowding, than by the, roughly spherical, ortho chlorine substituted triketones. However, we cannot rule out the possibility that there is simply a more favourable interaction for nitro than chlorine at the binding site.

## CONCLUSIONS

This work suggests that a planar face of the appropriate size and electronic configuration, of a larger non planar structure, may be able to induce P450IA1, and that the current theories of structure activity relationships in this area could be expanded. Diketone molecules seem to be more potent inducers than equivalent triketone structures, and triketones with ortho nitro substituents, in turn, appear to be more potent than those with chlorine in this position. The work also highlights the usefulness of conformationally restricted molecules in this type of structure activity work to help define active conformations and gives us confidence that, at least for the triketone series, we may be laying some foundations which will afford us greater pedictive confidence in induction of P450IA1 by these chemicals.

## FUTURE WORK

1. The ideas discussed in this paper relate to P450IA1 induction. We are currently considering correlations between the molecular structure of a chemical and its ability to induce P4350IIIA1, again using the triketone series as model ligands and comparing with benchmark inducers of this class.

2. We intend to investigate, within the triketone series, the effect of substitution in the cyclohexanedione ring, upon induction of P450IA1 and P450IIIA1.

3. The methodology problems associated with P450IIB1 and P450IIB2 appear to be superficial, some effort could be directed towards overcoming these problems and generating a similar triketone based dataset for these equally important P450 isoenzymes.

4. We could explore the synthesis of analogues of structure (e), to define the effect of bulky substituents on P450IA1 induction.

5. Measurement of the binding affinites of some triketones to the P450IA1 receptor could further enhance our understanding of this process of enzyme induction for this class of chemical.

## CONTRIBUTORS

Alison Whitfield
Cliff Elcombe
Mac Provan

## Table 3

## Induction of P450IA1 in Primary Rat Hepatocytes

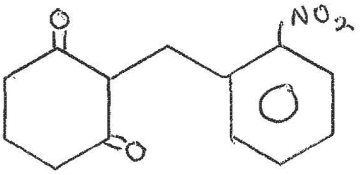
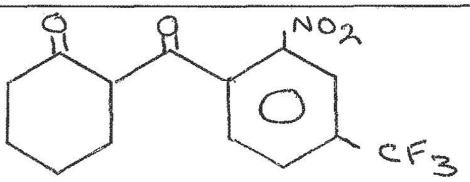
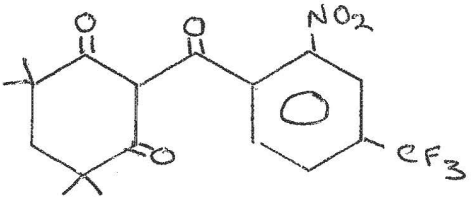
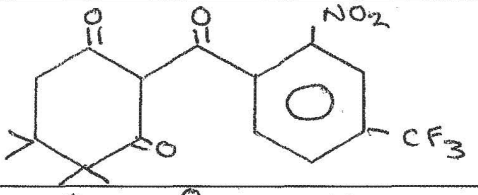
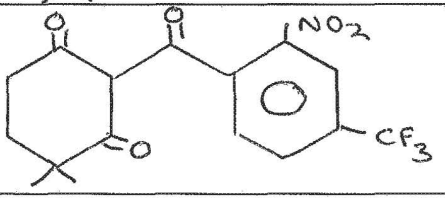
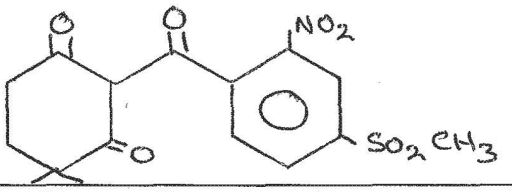
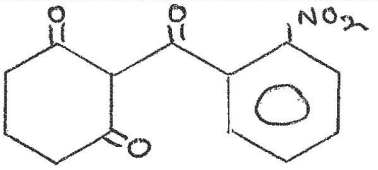
| | |
|---|---|
| **GROUP 1** | POTENCY = +++ |
| R240280 |  |
| R-68150 |  |
| **GROUP 2** | POTENCY = ++ |
| R-96734 |  |
| R-97392 |  |
| R-82734 |  |
| ICIA0179 |  |
| R-81775 |  |

Table 3 - continued

Induction of P450IA1 in Primary Rat Hepatocytes

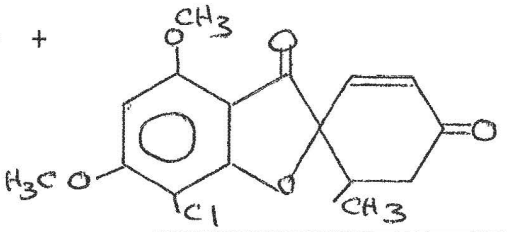
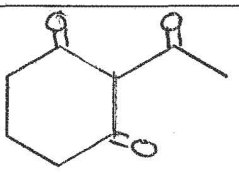
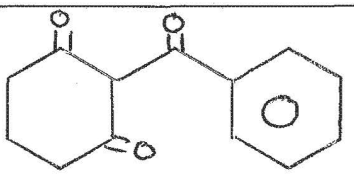
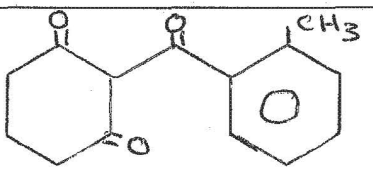
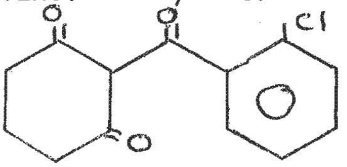
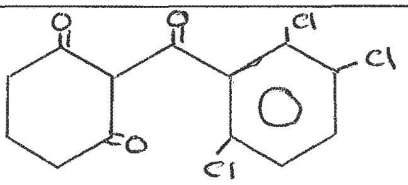
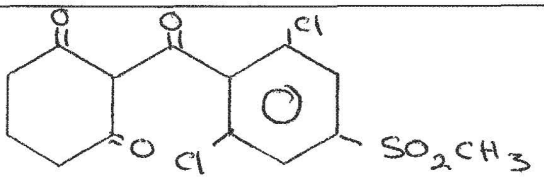
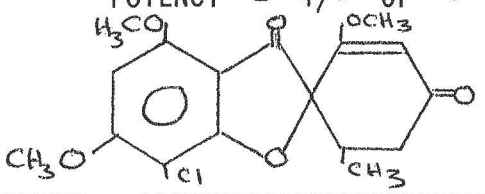
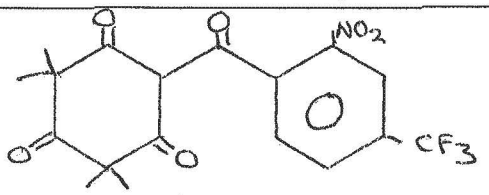
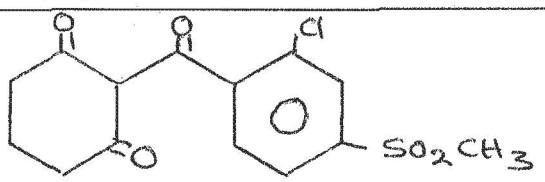
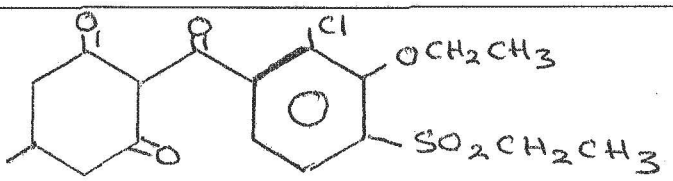
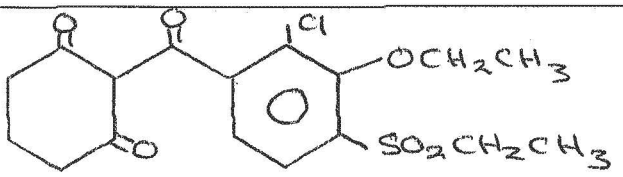
| | |
|---|---|
| **GROUP 3** DESMETHOXYGRISEOFULVIN | POTENCY = +  |
| R-86113 |  |
| R-62582 |  |
| R-81528 |  |
| **GROUP 4** R62785 | POTENCY = +/- or -  |
| R-63532 |  |
| R-87363 |  |

Table 3 - continued

Induction of P450IA1 in Primary Rat Hepatocytes

| | |
|---|---|
| **GROUP 4** (cont)<br>GRISEOFULVIN | POTENCY = +/- or -<br>  |
| R-90101 |  |
| ICIA0051 |  |
| R250936 |  |
| R25006 |  |

# TRIKETONE - LIVER

J ODUM, J R FOSTER, C R ELCOMBE

## INTRODUCTION

The triketone herbicide SC0774 is a non-genotoxic mouse and rat liver carcinogen whilst the triketone ICIA0051 is not carcinogenic. The objectives of this project are to establish a mechanism for the differential hepatocarcinogenicity of SC0774 and ICIA0051 and to predict carcinogenic potential for other development triketones. Previous studies in the mouse have indicated that SC0774 may be hepatocarcinogenic through a mechanism involving liver growth and cytochrome P-450 induction (IIB1/2) similar to that described for agents like phenobarbitone. The development compound ICIA0179 was also an inducing agent in the mouse but of the "steroid class" (P-450 IIIA1). In the present studies the P-450 isoenzyme profiles induced by SC0774, ICIA0179 and ICIA0051 have been further characterised in mouse and rat. No effect levels have been established for SC0774 and ICIA0179 and possible sex differences investigated. The hepatotoxicity of the triketone development compounds ICIA0179-bisenamine, ICIA0179-Cu chelate (ICIA0179 complexes), R-250936 and SC0456 have been investigated. A standard protocol of 28 day dietary administration has been used for all the in vivo studies. At termination histopathology, ultrastructural morphometry and biochemical analysis were carried out on the livers. An in vitro system for studying P-450 isoenzyme induction by triketones, using isolated rat hepatocytes has also been established.

A study to investigate the ability of triketones SC0774 and ICIA0179 to elicit preneoplastic foci in rats and mice has been initiated to test the predictions which have been made based on results from 28 day studies. This also provides an opportunity to study the relationship between liver growth, enzyme induction and hepatocarcinogenesis. SC0774 and ICIA0179 are being administered in the diet at both P450-inducing and non-inducing doses. The preneoplasia study also includes the positive controls phenobarbitone and cyproterone acetate. Liver growth, enzyme induction, histopathology and clinical chemistry are being monitored over a 12 month period which terminates in April 1992.

PROGRESS

## 1. The Effect of SC0774, ICIA0179 and ICIA0051 on Mouse and Rat Liver

SC0774 and ICIA0179 induced liver growth and P-450 isoenzymes in both species but the effects differed qualitatively and quantitatively. In contrast, ICIA0051 had very little effect. In male and female mice both compounds increased relative liver weight and caused SER proliferation and centrilobular hypertrophy. In both sexes SC0774 had a greater effect on these parameters of liver growth than ICIA0179 when administered at similar dose levels. SDS-PAGE-Western blot analysis showed that SC0774 was a strong inducer of P-450 IIB1/2 and IIIA1 whilst ICIA0179 was a moderate inducer of P-450 IIIA1 with only marginal induction of P-450 IIB1/2. This was also confirmed by selective P-450 catalytic activities. The no-effect level for ICIA0179 was lower in females than males (100ppm compared to 1000ppm), similar to other P-450 IIIA1 inducers, although the effect was similar at the top doses. SC0774 showed no sex differences, both males and females having a no-effect level of 100ppm (Tables 1 and 2).

In the rat SC0774 and ICIA0179 also increased relative liver weight, caused SER proliferation and centrilobular hypertrophy but to a lesser extent than in the mouse. Female rats were more sensitive to both compounds giving no-effect levels of 100ppm compared to 1000ppm in males. The P-450 isoenzyme induction profile for IIB1/2 and IIIA1 was also similar to mouse (Tables 3 and 4). A major species difference however was a strong induction of P-450 IA1 by ICIA0179 in male rats. P-450 IA1 induction was not seen in mice given ICIA0051, ICIA0179 or SC0774 although a marginal response was also seen in male rats given ICIA0051 and female rats given ICIA0179. P-450 IA1 induction is part of the pleiotrophic response mediated by the Ah receptor which binds planar polycyclic aromatic hydrocarbons.

## 2. The Effect of ICIA0179-bisenamine and Cu-chelate on Mouse and Rat Liver

These complexes of ICIA0179, which are thought to degrade to ICIA0179, are less toxic to the eye than the parent compound but their hepatotoxicity had not been investigated. Mice and rats, however, showed much less tolerance to ICIA0179-bisenamine than they had to ICIA0179 itself. The highest doses of bisenamine which could be administered to mice and rats were 1000 and 5000ppm respectively compared with 7000 and 16000ppm respectively of parent compound. At these dose levels the bisenamine had little effect on liver growth and P-450 isoenzyme induction in rat and mouse which was unsurprising as ICIA0179 itself had a no-effect level of 1000ppm for these parameters in both species. ICIA0179-Cu chelate appeared to have a similar effect on liver growth to the parent compound and hence represented no alleviation of toxicity. Therefore P-450 izoenzyme profiles were not determined with this complex (Table 5).

## 3. The Effect of R-250936 and SC0456 on Mouse and Rat Liver

These compounds are structurally very similar, but quite different, biologically, from ICIA0179 and SC0774. Both compounds had a small effect on mouse and rat liver causing slight liver growth and SER proliferation. Some induction of P-450 isoenzymes IIB1/2 and/or IIIA1 occurred but the effect was only weak (Tables 6 and 7).

## 4. Comparison of Cytochrome P-450 Induction In Vitro and In Vivo

Cultured hepatocytes incubated with triketones SC0774, ICIA0179, ICIA0051, R-250936, SC0456 and appropriate positive controls gave a similar response to that seen in vivo with respect to P-450s IA1 and IIIA1 (Table 8). Induction was characterised by Western blot analysis and P-450 substrates. This system is now being used for primary screening of potential triketone herbicides and for determining structure activity relationships.

## 5. SC0774 and ICIA0179 Preneoplasia Study

Some results from the 3 and 6 month terminations on this study are available. Mice (CD1 and C57B1/10J) and rats (CD) given SC0774 and ICIA0179 (at doses which induced P-450 after 28 days) and phenobarbitone and cyproterone acetate showed a sustained increase in relative liver weight after 3 and 6 months. Concomitant with this was a moderate to very marked centrilobular hypertrophy and portal hyperplasia in these groups (both species), and karyomegaly (mice) after 3 months. These effects were not seen in groups given non-P-450-inducing doses. Mice given cyproterone acetate for 3 months also showed portal fatty vaculation which correlated with changes in clinical chemistry (increased plasma cholesterol and triglycerides). This group also had increased plasma transaminases and bilirubin although there was no histological evidence of necrosis. Clinical chemistry in the other groups was largely unremarkable. SC0774 treatment resulted in very marked liver growth in both species which after 6 months resulted in the development of macroscopic liver tumours in the CD1 mouse. Although these results are not yet complete SC0774 appears to be an extremely potent non-genotoxic liver carcinogen.

## CONCLUSIONS

The hepatocarcinogenicity of SC0774 in mice and rats is still thought to result from stimulation and possibly altered regulation of liver growth along with phenotypic change in hepatocytes as demonstrated by P-450 isoenzyme induction. This occurs in both species although is most marked in the mouse which is also the most responsive species in terms of hepatocarcinogenicity. ICIA0179 also induces liver growth and cytochrome P-450 in the mouse and is therefore thought to have hepatocarcinogenic potential in this species, although the effect is less pronounced than with SC0774. In the rat liver, growth is much less with ICIA0179 than with SC0774, however ICIA0179 induced cytochrome P-450 IA1 which is frequently associated with receptor (Ah) mediated carcinogenesis. Although the bisenamine complex of ICIA0179 had a more favourable hepatotoxicity profile than ICIA0179 itself, no further work will be be carried out as

development of ICIA0179 has since stopped due to unfavourable soil retention characteristics. R-250936 and SC0456 have only mild liver effects and therefore are not predicted to be hepatocarcinogenic.

## FUTURE WORK

1. Continuation of the preneoplasia study.

2. Identification of triketone induced P-450 isoenzymes by Western blots does not always correlate with changes seen in substrate activities. The possibility that triketones may both induce and inhibit P-450 will be investigated.

3. Investigate the hepatoxicity of further development triketones.

## CONTRIBUTORS

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F Smith
J R Foster
E Wheeldon
M Simpson
C W Gore

## RESOURCES 1991

Biochemical Toxicology : 3.5 man-years

Support for 28 day and preneoplasia studies : 2 man-years

## Table 1

## The Effect of ICIA0179 and SC0774 on Mouse Liver (Male)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | ER | MR | BR | PR |
| ICIA0179 | | | | | | | | | | |
| 100 | 97 | 96 | - | - | - | 92 | 105 | 109 | 106 | 87 |
| 1000 | 100 | 102 | - | - | +/- | 99 | 107 | 81 | 119 | 89 |
| 2000 | 111* | 122* | - | - | +/- | 129 | 110 | 127 | 135 | 106 |
| 3500 | 121* | 183* | - | - | + | 138 | 85 | 83 | 130 | 239* |
| 5000 | 131* | 161* | - | - | ++ | 156 | 115 | 94 | 247* | 120 |
| 7000 | 129* | 297* | - | +/- | ++ | 146 | 101 | 80 | 650* | 161 |
| SC0774 | | | | | | | | | | |
| 100 | 104 | 96 | - | - | - | 97 | 105 | 78 | 143 | 101 |
| 1000 | 109 | 174* | - | + | ++ | 210 | 111 | 52* | 606 | 120 |
| 3500 | 162* | ND | - | ++ | ++ | 497* | 168 | ND | ND | 225* |
| 7000 | 251* | 306* | - | ++ | ++ | 625* | 125 | 74 | 4236* | 255* |

Compounds were administered in the diet for 28 days.
All values are expressed as % of control, * statistically significant P<0.01.
SER = smooth endoplasmic reticulum proliferation.
Severity classification for SDS-PAGE: +/- minimal, + mild, ++ moderate, +++ marked.
P450 substrates: EC ethoxycoumarin; ER, MR, BR and PR ethoxy, methoxy, benzoxy and pentoxy resorufin.
The dotted line indicates no-effect levels.

## Table 2

## The Effect of ICIA0179 and SC0774 on Mouse Liver (Female)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | ER | MR | BR | PR |
| ICIA0179 | | | | | | | | | | |
| 100 | 111* | 99 | - | - | - | 104 | 64 | 89 | 52 | 70 |
| 1000 | 115* | 127* | - | - | ++ | 161* | 83 | 127 | 125 | 108 |
| 2000 | 110* | 171* | - | - | ++ | 161* | 96 | 124 | 143 | 109 |
| 5000 | 117* | 213* | - | +/- | ++ | 199* | 124 | 102 | 102 | 110 |
| 7000 | 127* | 259* | - | +/- | ++ | 224* | 106 | 113 | 258* | 105 |
| SC0774 | | | | | | | | | | |
| 100 | 104 | 105 | - | - | - | 156* | 70 | 67 | 82 | 64 |
| 1000 | 113* | 197* | - | + | ++ | 219* | 109 | 66 | 358* | 113 |
| 7000 | 176* | 293* | - | ++ | ++ | 561* | 114 | 43 | 757* | 128 |

For explanation see Table 1.

## Table 3

## The Effect of ICIA0179 and SC0774 on Rat Liver (Male)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | ER | MR | BR | PR |
| ICIA0179 | | | | | | | | | | |
| 100 | 105 | 83 | - | - | - | 80 | 72 | 71 | 88 | 157 |
| 1000 | 107 | 116 | - | - | - | 86 | 105 | 111 | 90 | 277 |
| 2000 | 107 | 124* | +/- | - | - | 94 | 125 | 113 | 138 | 260* |
| 4000 | 107 | 135* | + | - | +/- | 111 | 204* | 142 | 146 | 177* |
| 6000 | 112* | ND | ++ | - | + | 164* | 340* | 297* | 198* | 167* |
| 16000 | 111* | 143* | ++ | - | ++ | 187* | 816* | 741* | 352* | 1058* |
| SC0774 | | | | | | | | | | |
| 100 | 105 | 97 | - | - | - | 80 | 96 | 55 | 89 | 88 |
| 1000 | 118* | 149* | - | - | - | 94 | 134 | 55 | 174 | 92 |
| 4000 | 133* | 163* | - | ++ | +++ | 135 | 180* | 53* | 1099* | 441* |

For explanation see Table 1.

## Table 4

## The Effect of ICIA0179 and SC0774 on Rat Liver (Female)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | ER | MR | BR | PR |
| ICIA0179 | | | | | | | | | | |
| 100 | 100 | 121 | - | - | - | 103 | 113 | 96 | 105 | 100 |
| 1000 | 102 | 185* | - | - | - | 97 | 107 | 268 | 99 | 91 |
| 2000 | 104 | 202* | - | - | - | 112 | 116 | 123 | 105 | 96 |
| 4000 | 102 | 236* | - | - | +/- | 110 | 107 | 101 | 107 | 85 |
| 16000 | 115* | 248* | +/- | +/- | ++ | 164* | 263* | 247* | 223* | 114 |
| SC0774 | | | | | | | | | | |
| 100 | 100 | 160* | - | - | - | 67 | 87 | 107 | 68 | 79 |
| 1000 | 108 | 205* | - | + | +/- | 85 | 127 | 81 | 370* | 77 |
| 4000 | 145* | 289* | - | ++ | ++ | 113 | 142 | 62* | 1393* | 284* |

For explanation see Table 1.

# Table 5

## The Effect of ICIA0179-bisenamine and Cu-chelate on Mouse and Rat Liver (Male)

**ICIA0179-bisenamine**

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | Enzyme Activities ER | Enzyme Activities MR | Enzyme Activities BR | Enzyme Activities PR |
| **MOUSE** | | | | | | | | | | |
| 1000 | 109 | 116 | - | - | - | 145 | 137 | 119 | 89 | 95 |
| **RAT** | | | | | | | | | | |
| 1000 | 110 | ND | - | - | - | 93 | 153 | 145 | 105 | 191 |
| 5000 | 107 | ND | - | - | +/- | 149 | 216* | 283 | 154 | 336* |

**ICIA0179-Cu chelate**

| Dose (ppm) | Liver Growth | |
|---|---|---|
| | Relative Liver Weight | SER Proliferation |
| **MOUSE** | | |
| 1000 | 105 | 98 |
| 5000 | 118 | 145* |
| 10000 | 139* | 190* |
| 15000 | 258* | 220* |
| **RAT** | | |
| 1000 | 108 | ND |
| 5000 | 117* | ND |
| 10000 | 114* | ND |
| 15000 | 116* | ND |

For explanation see Table 1.

## Table 6

## The Effect of R-250936 and SC0456 on Mouse Liver (Male)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | ER | MR | BR | PR |
| R-250936 | | | | | | | | | | |
| 1000 | 94 | 98 | - | +/- | - | 143* | 123* | 103* | 180* | 93 |
| 3000 | 112* | 127 | - | +/- | +/- | 200* | 110 | 108 | 158* | 85 |
| 7000 | 103 | 204* | - | +/- | + | 333* | 211* | 116 | 238* | 88 |
| SC0456 | | | | | | | | | | |
| 1000 | 109 | 112 | - | +/- | +/- | 105 | 101 | 88 | 177* | 83 |
| 3000 | 116* | 149* | - | +/- | + | 210* | 140* | 113 | 209* | 95 |
| 7000 | 120* | 228* | - | + | + | 394* | 196* | 108 | 406* | 173* |

For explanation see Table 1.

## Table 7

## The Effect of R-250936 and SC0456 on Rat Liver (Male)

| Dose (ppm) | Liver Growth | | P450 Isoenzyme Induction | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Relative Liver Weight | SER Proliferation | SDS-PAGE IA | SDS-PAGE IIB | SDS-PAGE IIIA | Enzyme Activities EC | Enzyme Activities ER | Enzyme Activities MR | Enzyme Activities BR | Enzyme Activities PR |
| R-250936 | | | | | | | | | | |
| 1000 | 112* | 91 | - | - | + | 93 | 72 | 78 | 84 | 71 |
| 3000 | 120* | 133 | - | - | + | 105 | 93 | 78 | 72 | 93 |
| 7000 | 119* | 76 | - | - | + | 107 | 85 | 75 | 93 | 98 |
| SC0456 | | | | | | | | | | |
| 1000 | 110 | 88 | - | - | +/- | 123 | 92 | 75 | 129 | 121 |
| 3000 | 116* | 92 | - | + | +/- | 109 | 91 | 55 | 88 | 113 |
| 7000 | 107 | 94 | - | + | +/- | 69 | 131 | 129 | 99 | 136 |

For explanation see Table 1.

## Table 8

### P-450 Isoenzyme Induction - In Vivo : In Vitro Comparison (Rat)

| | | P-450 isoenzyme IA1 | IIIA1 |
|---|---|---|---|
| Phenobarbitone | mic | - | ++ |
| | hep | + | ++ |
| Dexamethasone | mic | - | +++ |
| | hep | - | +++ |
| β-Naphthoflavone | mic | +++ | - |
| | hep | ++ | - |
| ICIA0051 | mic | +/- | - |
| | hep | +/- | - |
| ICIA0179 | mic | ++ | ++ |
| | hep | ++ | ++ |
| SC0774 | mic | - | ++ |
| | hep | - | ++ |
| R-250936 | mic | - | + |
| | hep | +/- | +/- |
| SC0456 | mic | - | +/- |
| | hep | +/- | + |

Compounds are compared at maximum inducing doses.
mic: liver microsomes, hep: hepatocytes cultured for 4 days.
Isoenzyme induction was determined by Western blotting.
(see Table 1).