THE EFFECT OF DIURNAL RHYTHMS ON THE HEPATOTOXICITY OF THIOACETAMIDE IN MALE AND FEMALE RATS

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SUMMARY

Single 200mg/Kg body weight i.p. injections of thioacetamide administered to litter mate male and female rats at 09.00, 13.00, 17.00 and 21.00 h caused body weight losses, elevated plasma glutamate pyruvate transaminase (GPT) levels, decreases in hepatic glycogen, zone 3-specific necrosis and leucocyte infiltration. All of these changes were more marked in males. The effects of thioacetamide on females were more severe at later injection times while no diurnal variations were apparent in males. Decreases in hepatic glycogen were most obvious in necrotic perivenous hepatocytes and correlated with increases in active glycogen phosphorylase which were probably caused by raised cytosolic calcium concentrations resulting from thioacetamide induced damage to cell membranes.

I. INTRODUCTION

The acute hepatotoxicity of thioacetamide is believed to be due to an active metabolite produced through the action of microsomal mixed function oxidases /1,2/. Flavin monooxygenase and cytochrome P450 have both been implicated in the bioactivation of thioacetamide /1-4/. The enzymes are believed to catalyse a series of reactions whereby thioacetamide is converted to thioacetamide-S-oxide which is then further oxidised to acetamide and other polar products that bind irreversibly to tissue macromolecules /3,5/. Cellular necrosis occurs predominantly in cells surrounding the terminal hepatic venule and seems to be primarily caused by increased cytosolic calcium concentrations which arise as a result of the interaction of toxic metabolites with lipids in cellular membranes /6-9/. The effects of thioacetamide can be prevented by simultaneous administration of calcium channel blocking agents /10/ and are potentiated in male rodents by fasting /9,11/ and by pretreatment with ethanol /12,13/ and with microsomal enzyme inducers /1,3/.

Recently, diurnal variations in the hepatotoxicity of compounds such as paracetamol /14/ and chloroform /15/ have been linked with rhythmic alterations in mixed function oxidase activity /16/. Similarly, sex-specific variations in microsomal mixed function oxidase activity are thought to account for the differing susceptibilities of male and female animals to various hepatotoxins /17/. In the

present study we have therefore used biochemical and histochemical techniques to examine whether rhythmic changes also affect thioacetamide hepatotoxicity and to determine whether sex specific differences are apparent.

II. MATERIALS AND METHODS

Thioacetamide, bovine liver glycogen, amyloglucosidase, amylase, periodic acid, Schiff's reagent, DL aspartate, AMP, α-ketoglutarate, 2,4-dinitrophenylhydrazine, polyvinyl alcohol (type II, water soluble, low molecular weight) and glucose l-phosphate were obtained from Sigma Chemical Company Ltd. (UK). Sudan Black and OCT embedding compound were purchased from Raymond A Lamb (UK), Sagatal from May and Baker (UK) and glucose test-combination kits from Boehringer Mannheim (UK). All other reagents were of AnalaR or of best available grade from Fisons (UK).

2.1 Treatment of animals

Litter mate male and female Sprague Dawley rats were kept from birth in a constant light/dark cycle (light on 08.00-20.00 h) with food and water available *ad libitum* at all times. Groups of 3 rats aged between 37 and 39 days, were either given a single 200mg/Kg body weight i.p. injection of thioacetamide in water (50mg/ml) at 09.00, 13.00, 17.00 and 21.00 h or an equivalent volume of water. 48 h later, the animals were anaesthetised with Sagatal (0.4ml/100g body weight of a 20% (v/v) solution in 10% (v/v) ethanol). 5ml blood samples were taken by cardiac puncture into EDTA-coated tubes. The animals were then killed by cervical dislocation and their livers removed and weighed. Pieces of liver 3-5mm thick were snap-frozen in liquid nitrogen-cooled isopentane as previously described /18/ or fixed in Carnoy's 2 fixative for 24 h prior to paraffin embedding. The remaining liver was chopped into 1-2cm cubes for storage in liquid nitrogen.

2.2 Biochemical assays

Plasma glutamate pyruvate transaminase (GPT) activities were

determined by the method of Reitman and Frankel /19/. The glycogen content of samples of snap-frozen liver was determined as described in /20/. Homogenates (10% (w/v)) in 0.03M HCl were heated for 5 min at 100°C and then centrifuged at 15,000g for 20 min at 5°C. The glucose formed by the degradation of tissue glycogen and bovine liver glycogen standards with amyloglucosidase (1 h at 37°C) was determined using a Boehringer Mannheim glucose test-combination kit.

2.3 Histochemical procedures

Glycogen was visualised using the periodic acid Schiff (PAS) technique /21/ on 8 m sections fixed for 10 min in Carnoy's l fluid. Glycogen phosphorylase activity was visualised in unfixed 8μm frozen sections using the method described by Jepson et al. /22/: staining in the presence and absence of AMP being used to identify inactive phosphorylase b and active phosphorylase a. Sudan black staining was carried out as previously described /23/.

2.4 Statistical analyses

Levels of statistical significance were assessed by Student's t-tests and by Mann-Whitney tests. Significant differences had P values < 0.05.

III. RESULTS

3.1 Sex differences in responses to thioacetamide

48h after administration of a single 200mg/Kg body weight injection of thioacetamide, marked body weight losses, elevated plasma glutamate pyruvate transaminase (GPT) levels and decreases in hepatic glycogen were apparent in all animals compared with age and time matched controls (Table 1 and Figs. 1 and 2). The interpretation of changes in hepatic glycogen concentration is complicated by the diurnal variations that are normally found in control rats /24-26/ (Fig. 2), with levels at 21.00 h being significantly lower than at 9.00 h in males and females. However, despite these natural variations, glycogen concentrations in male thioacetamide-

TABLE 1

Time of injection (h)	% body weight loss		liver weight as % body weight	
	male	female	male	female
09.00	8.26±0.18	3.18±0.85*	4.43±0.15	4.69±0.19**
13.00	8.37±0.17	1.95±0.61*	3.76±0.20	4.23±0.08**
17.00	7.71±0.74	6.33±1.53	3.64±0.09	4.05±0.09**
21.00	6.49±0.49	4.44±1.42	4.08±0.09	4.35±0.14**

Results are given as means ± S.E.

Footnotes to Table 1

* Body weight loss in female rats significantly less than in male rats ($P < 0.05$ in Students' t test)
Control rats showed an average increase in body weight over the same time period of 2.35±0.51% (male) and 2.82±1.13% (female)

** Liver weights significantly greater than in time, age and sex matched controls ($P < 0.05$ in Students' t test) where male control = 3.86±0.20%, female control = 3.71±0.08%.

treated liver were significantly lower than in control male liver at 9.00, 13.00 and 17.00 h and those in female thioacetamide-treated liver were significantly lower than in control female liver at 13.00 and 17.00 h.

There were, however, marked sex differences in the response of litter mate male and female rats. Body weight losses were consistently smaller in females than in males (Table 1) and plasma GPT levels were significantly lower at 9.00, 13.00 and 17.00 h (Fig. 1). Similarly, hepatic glycogen concentrations were less at a given time of injection in males than in females (Fig. 2); the differences being significant at 9.00 and 13.00 h. These results suggested that female rats were less affected by thioacetamide treatment than male rats. Liver weights (expressed as % body weight) were, however, higher in female thioacetamide-treated rats than in controls (Table 1) while there was no equivalent significant increase in liver weight in thioacetamide-treated male rats.

Haematoxylin and eosin-stained sections from all animals showed the morphological changes typically associated with thioacetamide-

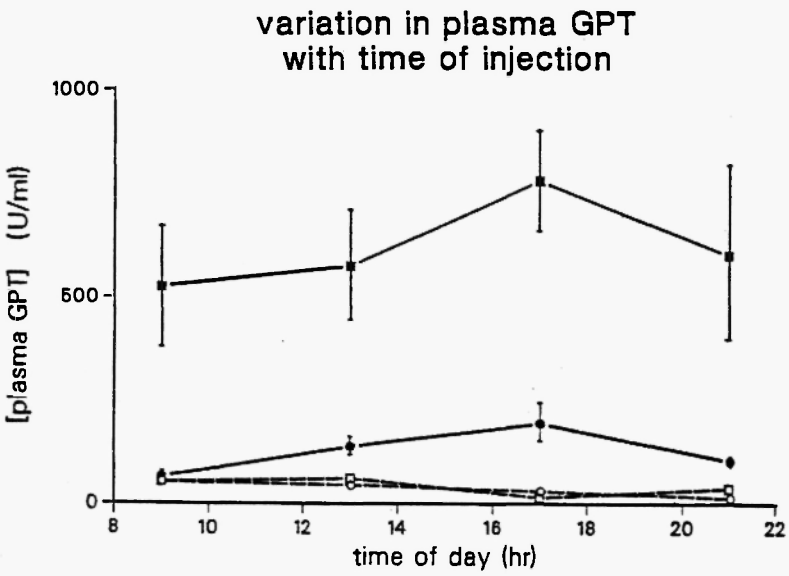

Fig. 1: Variation in plasma glutamate pyruvate transaminase activity with time of injection 48 h after the i.p. administration of 200 mg/Kg body weight thioacetamide or an equivalent volume of water.
Results are given as means ± S.E.
○---○ control females
●---● thioacetamide injected females
□---□ control males
■---■ thioacetamide injected males

induced liver damage /27/. Necrosis was confined to hepatocytes near terminal hepatic venules and was accompanied by the appearance of groups of infiltrating white cells in perivenous and mid-zone regions. Periportal cells were relatively unaffected but were characterised particularly in male rats by extensive cytoplasmic basophilia. Histochemical staining for glycogen confirmed the biochemical results showing considerably less staining in male than in female treated rats. Glycogen was in each case confined to non-necrotic, periportal hepatocytes, with a rim of necrotic perivenous cells surrounding each infiltration zone being completely devoid of staining. The reason for this lack of staining became clear on examination of sections stained for active glycogen phosphorylase

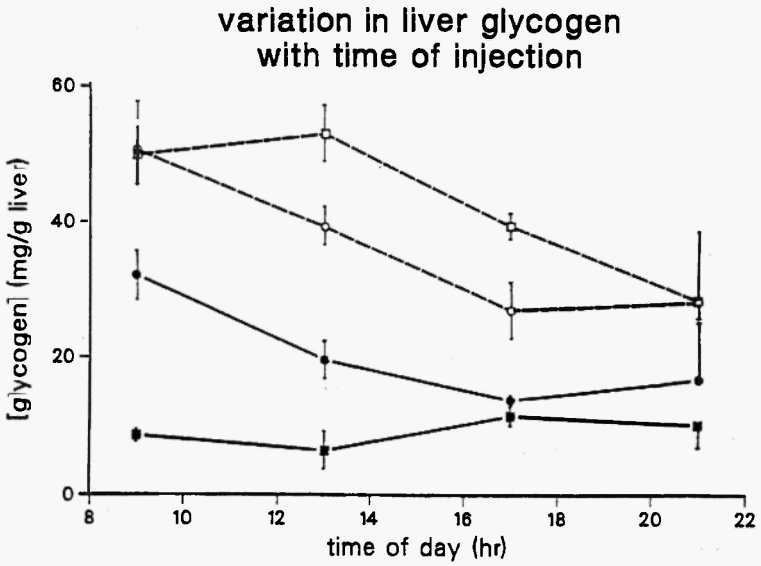

Fig. 2: Variation in liver glycogen concentration with time of injection 48 h after the i.p. administration of 200 mg/Kg body weight thioacetamide or an equivalent volume of water.
Results are given as means ± S.E.
○---○ control females
●---● thioacetamide injected females
□---□ control males
■---■ thioacetamide injected males

since the highest levels of the enzyme were to be found in the same rim of necrotic hepatocytes. In females, hepatocytes surrounding and within the infiltration zones stained darkly with sudan black indicating that they contained large amounts of intracellular lipid.

3.2 Diurnal variations in responses to thioacetamide

Female rats showed a marked diurnal variation in response to thioacetamide with body weight losses, decreases in hepatic glycogen concentration and increases in plasma GPT levels all being significantly more marked at later times of injection (Figs. 1 and 2 and Table 1). Histological changes were also more extensive in

tissues taken from female rats injected at 17.00 and 21.00 h. Leucocyte infiltration zones were larger and were surrounded by wider rims of necrotic hepatocytes devoid of glycogen and containing high concentrations of active glycogen phosphorylase. Accumulations of intracellular lipid tended, however, to be less marked in these animals and to bear more resemblance to the levels seen in male rats.

Similar significant diurnal variations were not apparent in male rats and responses in individual animals were less consistent at all times of injection than in females.

IV. DISCUSSION

Toxin-dependent depletion of hepatic glycogen has previously been demonstrated following the administration of paracetamol /22,28/ and thioacetamide /29/ to rodents. Reduced incorporation of 14C-labelled glucose into glycogen has also been reported in thioacetamide-treated mice /30/ as has increased glycogen breakdown in hepatocytes isolated from rats injected with paracetamol /31/. The sex-specific differences in glycogen depletion demonstrated in the present study do not, however, seem to have been investigated by previous workers who have generally restricted their studies to various strains of male rats. Glycogen depletion has recently been linked to increased active glycogen phosphorylase in damaged hepatocytes and the correlations between histochemical staining for active glycogen phosphorylase a and glycogen depletion seen in the present study have also been reported in paracetamol treated rats /22/. Biochemical studies /32,33/ have shown that active glycogen phosphorylase can be used as an indicator of increased cytosolic Ca^{2+} concentration and such increases have been implicated as the major cause of paracetamol-induced hepatotoxicity /34/. The high levels of active glycogen phosphorylase presently demonstrated in perivenous hepatocytes are thus consistent with previous biochemical studies implicating increased cytosolic Ca^{2+} concentrations in thioacetamide-induced hepatocellular damage /6-10/. The advantage of the present histochemical study being that it demonstrates that the increased active glycogen phosphorylase is restricted to necrotic perivenous hepatocytes, thus reinforcing the concept that selective hepatotoxicity of thioacetamide may result from localised increases in cytosolic Ca^{2+} concentration.

Diurnal variations in response to thioacetamide were evident in female but not male rats. Body weight losses, plasma GPT levels, staining for active glycogen phosphorylase in perivenous hepatocytes and leucocyte infiltration were all greater in female animals dosed at the end of the light phase of the light-dark cycle. As hepatic glycogen levels show marked diurnal variations in untreated rats (Fig. 2 and references 24-26,35) with minimum concentrations occurring at the end of the light phase, this effect may have been due to loss of the protective effect of glycogen on microsomal lipid peroxidation /36/. It may also be related to diurnal variations in flavin monooxygenase activity. This enzyme can carry out the critical conversion of thioacetamide-S-oxide to thioacetamide dioxide from which polar intermediates that bind irreversibly to cellular macromolecules are derived /2/. Flavin monooxygenase activity is believed to peak at 17.00 h (J.R. Cashman, personal communication), corresponding to the injection time resulting in maximal plasma GPT levels in female thioacetamide-treated rats.

The hepatotoxicity of thioacetamide was greater in male than in female rats when assessed in terms of % body weight loss, plasma GTP elevation, hepatic glycogen depletion and degree of leucocyte infiltration. As hepatic flavin monooxygenase activity is higher in female rats, the increased toxicity of thioacetamide in male rats suggests that in these animals, cytochrome P450 may also have played an important role in the bioactivation process. This interpretation would be consistent with the higher levels of hepatic cytochrome P450 in male rats and with studies demonstrating the potentiation of thioacetamide toxicity in male rodents by fasting /9,11/ and by pretreatment with ethanol /12,13/ and with microsomal enzyme inducers /1,3/.

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