BILE FORMATION IN RATS WITH ACUTE LIVER DAMAGE FROM CARBON TETRACHLORIDE

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Summary: Effects of CCl_4 on bile formation and on the hepatic bilirubin metabolism were studied in rats by recording the intrabiliary pressure and flow rate, BSP and bilirubin clearances and by estimating the activity of the hepatic enzyme, Uridine diphosphate (UDP) glucuronyl transferase. From the results of these studies it was concluded that : (i) CCl_4 reduced the rate of bile secretion by the lever cells of the rats, (ii) spontaneous bile flow and choleretic response to dehydrocholate declined in the CCl_4 treated rats, (iii) CCl_4 reduced the clearance of BSP and bilirubin (UCB or BG) at low plasma concentrations as well as the absolute rate of BSP and bilirubin (UCB or BG) excretion when plasma levels were above those required to saturate active transport of the dye or hepatic excretory mechanisms of bilirubin, (iv) CCl_4 produced a specific bilirubin conjugatory defect by inhibiting the activity of hepatic UDP-glucuronyl transferase and that (v) all these hepatotoxic effects of CCl_4 appeared as early as 2-3 hours after its administration.

Key words : rat bile bile flow bile pressure carbon tetrachloride bilirubin bilirubin clearance BSP clearance

INTRODUCTION

Elevated total plasma bilirubin levels (18) and a defective clearance of sulphobromophthalein, BSP (16) in rats with acute carbon tetrachloride (CCl_4) toxicity, point to a possible association of CCl_4 and altered bile formation. Studies done in the recent years (4,12,17,19,22,) have revealed that the primary site of hepatotoxic action of CCl_4 is the endoplasmic reticulum of liver cells which gets damaged both structurally and enzymatically, as early as 2-3 hrs after intoxication, with the resultant serious derangements in lipid metabolism. The enzymes of the smooth endoplasmic reticulum are also known to regulate hepatic bile pigment metabolism (1) which could also be affacted by carbon tetrachloride. The present investigation was, therefore, undertaken to evaluate the effects of CCl_4 on bile formation and on the hepatic transport of bilirubin.

MATERIALS AND METHODS

Normal albino rats weighing between 280 to 400 *g* were used. Carbon tetrachloride was administered by stomach tube under light ether anesthesia and on empty stomach. The dose of CCl_4 was 0.25 *ml* (diluted with an equal amount of liquid paraffin) per 100 *g* bodyweight. Experiments on these rats were performed 2-3 hrs after dosing. Veterinary pentobarbital sodium (Sagatal) was injected intraperitoneally in the dose of 3 *mg*/100 *g* body weight with a maximum of 12 *mg*. Supplemental ether was administered by inhalation and the animals were kept under light anesthesia throughout the experiment. The common bile duct was cannulated with a poly-

thene tubing and the collecting tube was changed at intervals of 3 to 5 minutes. Blood samples were obtained from the cannulated right jugular vein.

Bile flow rate and pressure studies:

For the bile flow rate and the intrabiliary pressure studies, the common bile duct was cannulated by a special cannula as designed by Barber-Riley (2). The rate of bile flow was recorded by means of a drop counter and the collected volumes estimated by weight. The stability of the intrabiliary pressure following the obstruction of the common bile duct was monitored continuously by a Model 7 Grass Polygraph using a Stathem pressure-transducer.

The choleretic response to dehydrocholate was studied after a continuous infusion of sodium dehydrocholate at a rate of 0.6 *mg/min* and lasting for 45 minutes.

BSP clearance:

A single intravenous injection of BSP, in the dose of 60 *mg/kg* was given to the rats and the biliary excretion of the dye was measured at approximately 5 min intervels for 50 minutes.

Steady-state BSP excretion was measured by a continuous infusion of BSP, dissolved in 0.9% NaCl, at a rate of 0.1 *mg/min* for 45 minutes.

Plasma and bile BSP levels were measured by the method of Goodman and kingsley (8). Hepatic BSP centent was determined by the method of Whelan *et al.* (24).

Bilirubin clearance studies:

In general, the technique of Weinbren and Billing (23) was used for the study of bilirubin clearance. 100 *mg* of unconjugated bilirubin (Sigma) was dissolved in 50 *ml* of isotonic solution containing 0.52 g Na_2CO_3 and 0.52 g NaCl/100 *ml*. The solution was prepared freshly for each experiment and kept in the dark at 4°C. Single dose of unconjugated bilirubin (2 *mg*/100 *g* body weight) was given intravenously to rats and its clearance from blood was determined over the next 45 minutes. Blood samples were obtained at approximately 5 min intervals for the estimation of conjugated, unconjugated and total bilirubin. The bile was collected continuously and the collecting tube changed every 5 minutes.

Conjugated bilirubin was obtained from the bile which was collected from the cannulated common bile duct of rats receiving infusions of unconjugated bilirubin. The bile was centrifuged at 15,000 rev/min at 4°C for 15 minutes and the concentration of conjugated bilirubin in the clear supernatant was estimated (15). Single dose of approximately 3 *mg*/100 *g* body weight of conjugated bilirubin was injected intravenously to the rats and its biliary excretion rate was studied at apprpximately 5 min intervals for 45 minutes.

Steady-state bilirubin clearance was determined by a constant intravenous infusion of unconjugated (UCB) and conjugated bilirubin (BG). A priming dose of 2 *mg*/100 *g* rat of UCB or

BG was injected intravenously followed by a continuous intravenous infusion at a rate of 1 *mg* of UCB/3 min or 2.5 *mg* BG/3 min respectively and continued for 45 minutes in each case.

The conjugated and the unconjugated bilirubin levels of the plasma were determined by the micromodification (11) of the method of Malloy and Evelyn (15). The method described by Weinbren and Billing (23) was adopted for the estimation of pigments in bile.

Assay of the enzyme Uridine diphosphate (UDP) glucuronyl transferase in the liver tissue of the rats was done by the method of Heirwegh *et al.* (6,10).

RESULTS

Effect on bile flow rate and intrabiliary pressure:

There was a 40% reduction in the spontaneous bile flow rate and a 30% decline in the choleretic response to dehydrocholate in the CCl_4 treated rats (3 hours after intoxication) when compared with the controls (Table I).

TABLE I: Bile flow and intrabiliary pressure changes in CCl_4 posioning (Mean $\pm$S.D.).

Rats	*Bile flow (ul/100 g/min)*		*Intrabiliary pressure*		
	Spontaneous	*Change with dehydrocholate*	*Time taken for initial rise to 12.5 cm H_2O (min)*	*Optimum level reached (cm H_2O)*	*Time taken to reach optimum level (min)*
Control (n=11)	5.5 $\pm$0.5	12.0 $\pm$1.8	1.19 $\pm$0.62	15.45 $\pm$1.46	10.75 $\pm$1.73
CCl_4-treated (n=8)	3.31 $\pm$1.5**	8.2 $\pm$4.2*	2.53 $\pm$0.43**	13.50 $\pm$0.83**	14.55 $\pm$1.43**

*P < 0.05
**P < 0.001 when compared with control rats.

In experimental obstruction of the common bile duct, the initial rate of rise in the intrabiliary pressure was slower in the CCl_4 treated rats (3 hrs after intoxication) when compared with the controls. Thus a pressure of 12.5 cm H_2O was reached in 2.53 $\pm$0.43 min after obstruction in the treated rats compared with 1.19 $\pm$0.62 min in the controls; the time taken to reach maximum pressure being 14.55 $\pm$1.43 min and 10.75 $\pm$1.73 min respectively. The final optimum pressure recorded in the treated rats was 13.50 $\pm$0.83 cm H_2O compared with 15.45 $\pm$1.46 cm H_2O in the controls, a reduction of 1.95 cm H_2O or 10% of the mean control values (Table I).

Effect on BSP excretion:

(a) *Clearance after single injection of BSP:* Fig. 1 shows the time course of BSP excretion following a single intravenous injection of BSP (60 *mg/kg*) in five control and in five CCl_4 treated rats (3 hrs after intoxication), in order to saturate the excretory transport of the dye.

Peak biliary excretion of the dye occurred in both groups after 25 minutes of the injection and remained almost constant for the next 15 minutes. Peak excretion of the dye

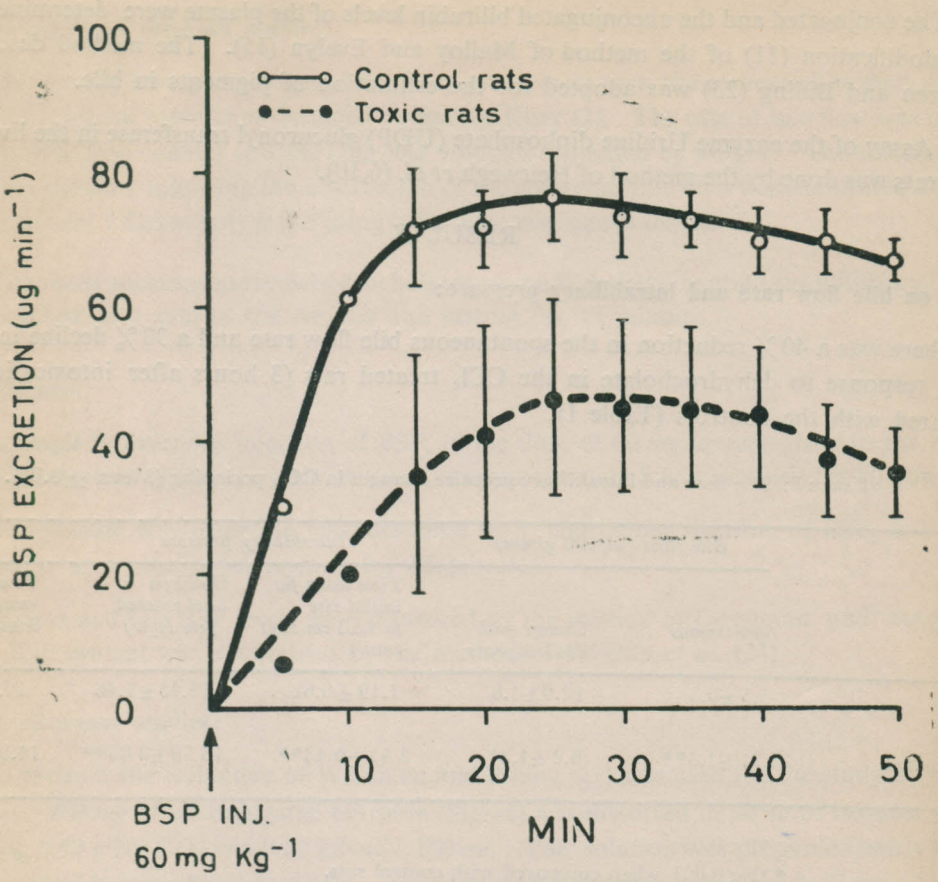

Fig. 1: The time course of BSP excretion after intravenous injection of 60 *mg/kg* to saturate excretory transport. Mean values $\pm$ S.D.

in the CCl_4 treated rats was 83% lower when compared with the controls. This change was associated with a mean decrement of 64% in the hepatic content of BSP; 2.25 0.51 *µg/g* wet weight in the treated rats as compared with 6.31 ± 2.9 *µg/g* wet weight in the control rats.

(b) *Steady-state BSP clearance:* Table II shows the steady state BSP excretion in the control and CCl_4 treated rats (3 hrs after intoxication). There was a reduced clearance of the dye from the plasma of the treated rats. The decline in clearance was associated with mean decrements of 33% in the Bile: Plasma concentration ratios and 63% in the hepatic content of BSP.

TABLE II: Steady-state sulphobromophthalein (BSP) excretion (Mean ±S.D.).

Rats	*Plasma BSP (mg %)*	*Bile: Plasma ratio*	*BSP clearance ml/min*	*Hepatic BSP content µg/g wet wt.*
Control (n=5)	1.55±0.54	326.4±95.2	4.33±1.48	3.55±1.59
CCl_4—treated (n=5)	3.30±1.1**	216.6±50.0*	2.47±1.30*	1.31±0.63**

*P <0.05

**P <0.01 when compared with control rats.

Effect on bilirubin clearance :

(*a*) *Clearance after single injection of unconjugated bilirubin:* Fig. 2 shows the clearance of injected unconjugated bilirubin from the plasma in a representative normal rat and in a representative CCl_4 treated rat (3 hrs after intoxication). The mean values of plasma bilirubin levels, studied 45 min post-injection, are given in Table III. The CCl_4 treated rats were unable to clear the injected unconjugated bilirubin from the plasma at a normal rate and the plasma level of unconjugated bilirubin remained very much elevated even after 45 minutes. This was accompanied by a significant ($P<0.001$) increase in the plasma conjugated bilirubin levels of these rats.

The rate of bile pigment excretion (µg/100 g body weight/min) in each of the collecting periods and for both the representative normal and CCl_4 treated rats is shown in Fig. 3. The curve of biliary excretion, when studied in a representative treated rat, 2 hrs after intoxication, was found to follow closely the normal pattern though it was shifted to later time periods and increase in biliary excretion could start only after 5 minutes of the injection. The curve was almost flat when studied, three hrs after intoxication. The maximal pigment excretion rate in the bile (Table IV) decreased by 22% and 90% of the mean control values in the treated rats, 2 hrs and 3 hrs after intoxication respectively.

(*b*) *Clearance after single injection of conjugated bilirubin*: Fig. 4 shows the rate of pigment excretion in the cannulated bile duct in the control and the treated rats. A seen, the pattern of pigment excretion following single injection of BG is almost identical to that following a single injection of UCB (Fig. 3). The maximal pigment excretion rate (Table IV) was also very much reduced and did not exceed 9 *µg/100 g* body weight/min in the treated rats, three hours after intoxication. Plasma total bilirubin levels, when studied 45 min post-injection, were found to be 1.55 *mg%* and 21 *mg%* in the control and in the treated rats (3 hrs after intoxication) respectively.

(c) *Steady-state bilirubin clearance*: Hepatic excretory mechanisms were saturated by a constant intravenous infusion of UCB and BG in six control and in six CCl_4 treated rats (3 hrs after intoxication) respectively. The maximal biliary excretion of the

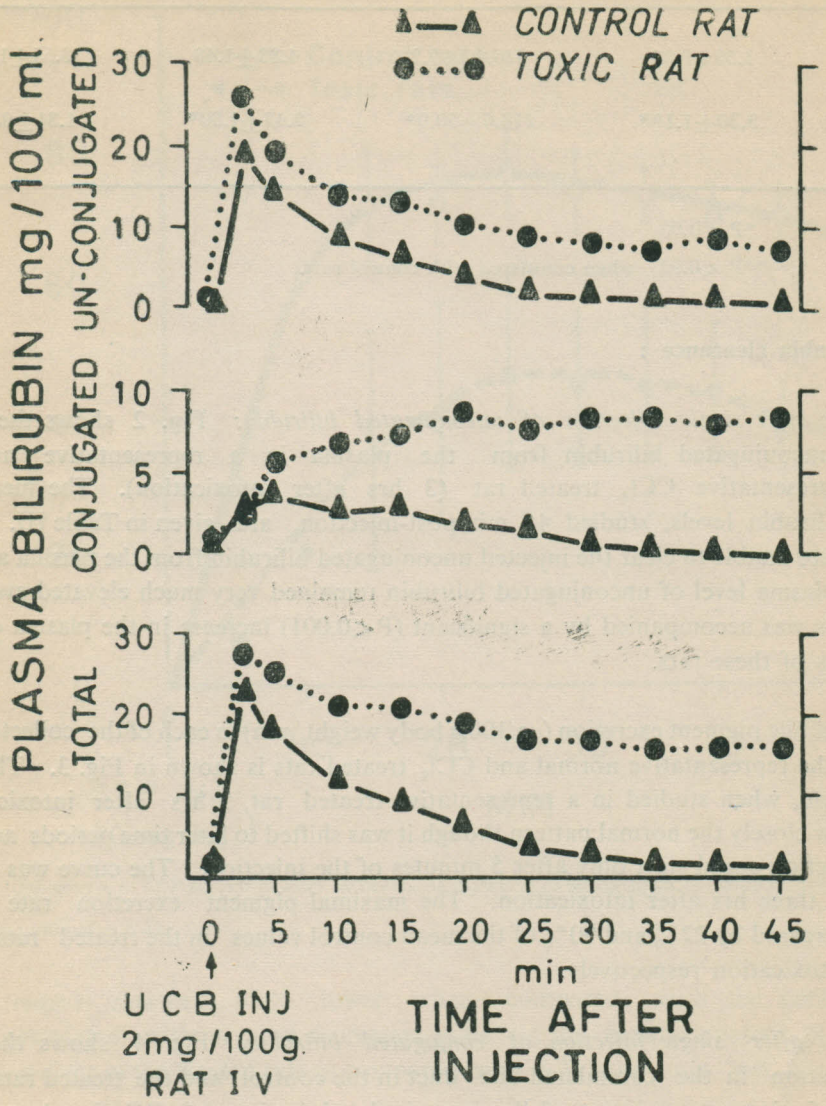

Fig. 2: Clearance of injected unconjugated bilirubin from plasma in representative normal and CCl_4-treated (3 hrs) rats.

pigment in the control rats following infusion of UCB was 114.3 ± 22.9 $\mu g/100\ g$ rat/min compared with 10.7±1.5 $\mu g/100\ g$ rat/min in the treated rats. The maximum pigment excretion in the control rats following the administration of BG was 121±19.7 $\mu g/100\ g$

TABLE III: Plasma clearance of unconjugated bilirubin (*mg%*, Mean ± S.D.)

Rats	*UCB*	*BG*	*Total bilirubin*
Control (n=8)	1.25±0.71	0.57±0.35	1.82±0.75
CCl_4-treated (n=8)	7.54±2.67*	8.48±1.27*	16.02±1.97*

*P < 0.001

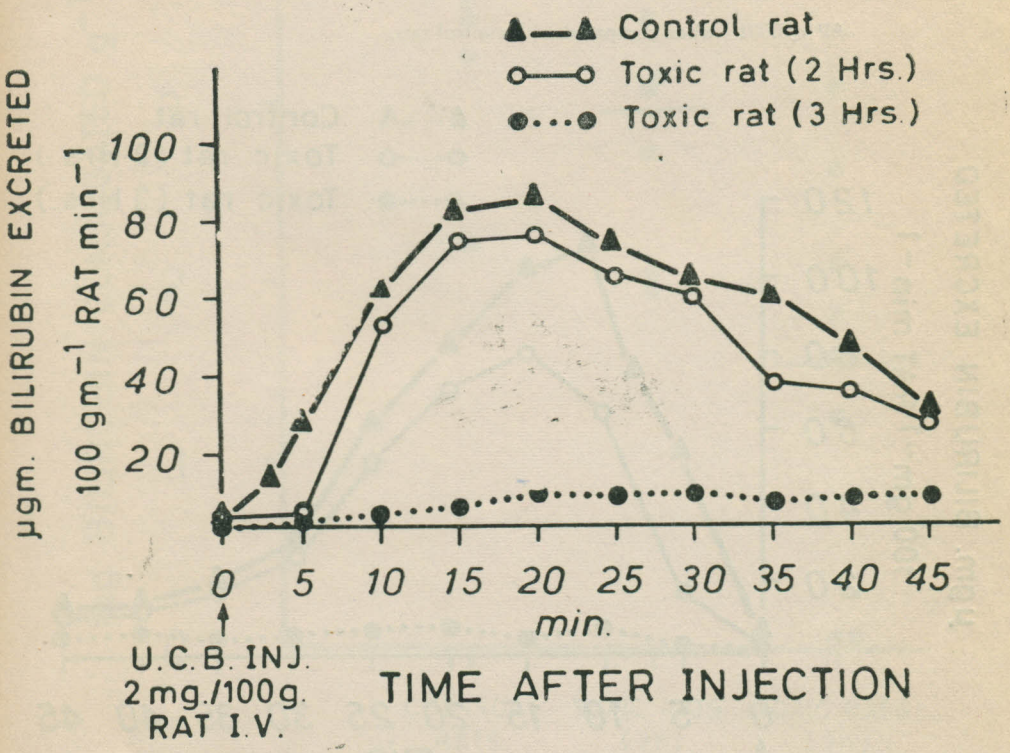

Fig. 3: Rate of pigment excretion in bile following a single intravenous injection of unconjugated bilirubin in representative normal and CCl_4-treated rats.

rat/min compared with 11.3 ± 2.9 µg/100 *g* rat/min in the treated rats. Thus both the UCB and BG excretion rates in the bile decreased by the mean value of 90 and 91% respectively in the treated rats when compared with the controls.

TABLE IV: Maximal pigment excretion rate in the bile following a single i.v. injection of unconjugated and conjugated bilirubin (μg/100 g rat/min, Mean ±S.D.)

Rats	*UCB injection*	*BG injection*
Control (n=8)	88.7±5.7	115.6±9.5
CCl_4-treated (2 hr) (n=8)	68.9±5.8*	88.8±7.3*
CCl_4-treated (3hr) (n=8)	8.61±0.55*	8.98±1.08*

*P <0.001 when compared with control rats.

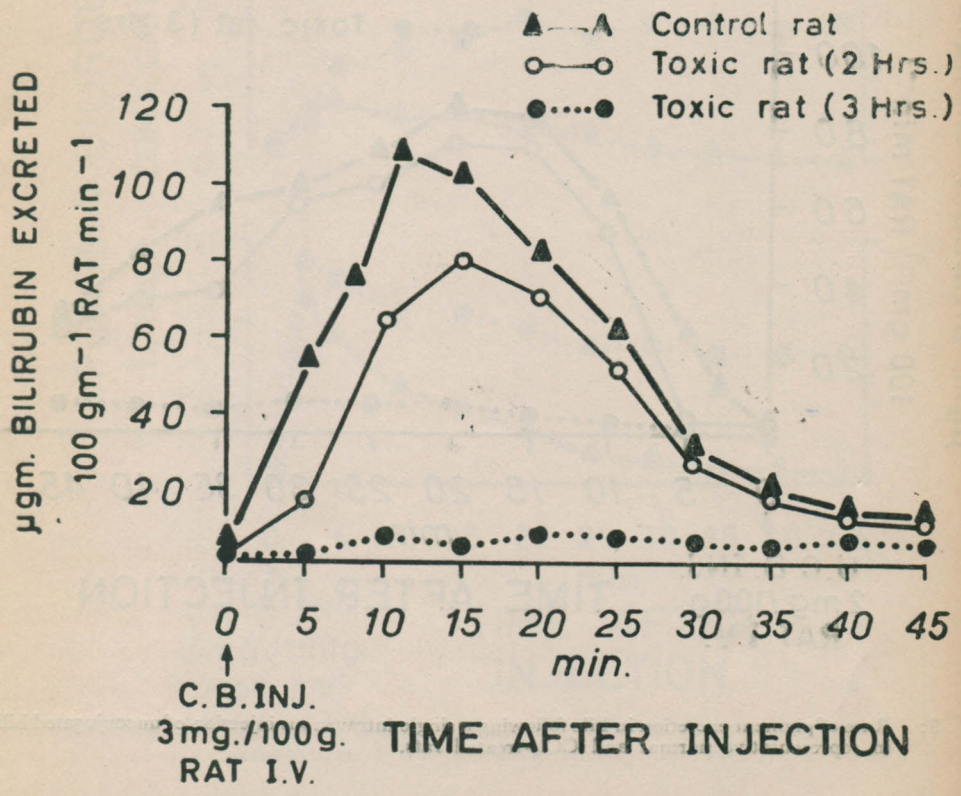

Fig. 4: Rate of pigment excretion in bile following a single intravenous injection of conjugated bilirubin in respresentative normal and CCl_4-treated rats.

Fig. 5 shows a marked decrease in the hepatic UDP glucuronyl-transferase activity of the CCl_4 treated rats. A 22% fall in the activity was found, 2 hrs after intoxication as compared to a 60% fall, 3 hrs after intoxication.

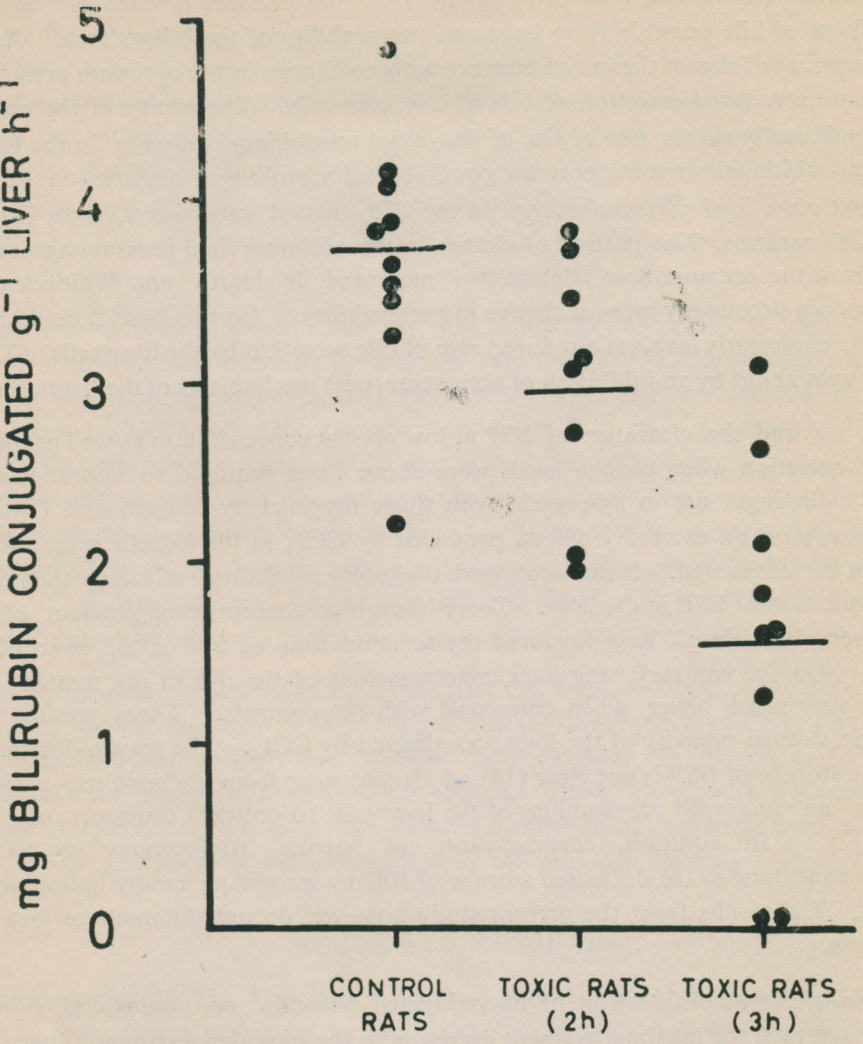

Fig. 5: Hepatic bilirubin UDP-glucuronyl transferase activity in control and CCl_4-treated rats (mean values indicated by the bar).

DISCUSSION

Bile flow appears to be largely determined by the active canalicular secretion of bile salts (7). The reduction in the spontaneous bile flow and a decline in the choleretic response to dehydrocholate may, therefore, be ascribed to the inhibition of active transport mechanisms of the liver cells

by CCl_4. The cholestatic effect of CCl_4 due to a decreased bile secretion could be distinguished from the one due to increased permeability or constriction of the biliary tree by recording the intrabiliary pressure changes during mechanical obstruction to bile flow (3). A normal rate of rise of the initial pressure and a lowering of the optimum pressure reached should indicate an increase leakage of bile possibly from increased permeability of the biliary tree. On the other hand, a slower rate of rise of the initial pressure with no change in the optimum pressure reached should indicate a reduced secretion of bile by the liver cells. The results of the present study showed a significantly slower rate of rise of the initial intrabiliary pressure in the CCl_4-treated rats, a finding which does not support the possibility of a prolonged constriction of the biliary tree. The optimum final pressure reached in the CCl_4-treated rats was 1.95 cm H_2O lower as compared with controls. This pattern of change in the optimum final pressure agrees with that calculated from the pressure/flow relationship measured by Harth and Waldeck (3, 9) and, threfore, does not necessarily mean a change in permeability. On this basis it could be concluded that CCl_4 cholestasis involves a reduced rate of bile secretion by the liver cells. This change could be brought about by an inhibition of active transport mechanisms of the liver cells by CCl_4.

CCl_4 reduced the clearance of BSP at low plasma concentration as well as the absolute rate of BSP excretion when plasma levels were above those required to saturate active transport. These findings are in agreement with those reported by Maggio and Fujimoto (14) for mice. However, an excretory defect, produced by CCl_4 in the hepatic transport of BSP (5) could not be confirmed. If excretion were the major mechanism affected, then one would expect accumulation of BSP in the liver. There was a high concentration gradient of BSP from plasma to liver which should have favoured the accumulation of BSP in the liver of the CCl_4 treated rats. On the contrary, the liver concentrations of the dye in the treated rats were found to be very much lower when compared with the controls. These results suggested that the active storage capacity of the liver was affected by CCl_4. The reason for this decrease in the hepatic storage of BSP is not clear (14). It may arise from the impaired uptake of the dye or from an increased permeability of the liver cells to outward transport of BSP and its conjugates (7). In addition, accumulation of hepatic triglycerides in CCl_4-treated rats (12) may contribute to the decreased storage of BSP by providing a more liphophilic hepatic environment. The results from the present study however, do not differentiate between these possible effects.

Following the administration of unconjugated bilirubin or conjugated bilirubin to the CCl_4-treated rats, the maximal pigment excretion in the cannulated common bile duct, three hours after the intoxication, did not exceed 9 μg/100 g body weight/min (Fig. 3, Fig. 4, Table IV). This was accompanied by a gradual rise in the plasma conjugated bilirubin levels (Table III). The clearance of unconjugated bilirubin from the plasma was also defective in these rats (Fig. 2, Table III). These observations revealed that the toxic effects of CCl_4 are directed both on the hepatic uptake and on the hepatic bilirubin conjugatory and excretory mechanisms.

CCl_4 has been shown to interfere with the transfer of unconjugated bilirubin from the blood to the liver (13). The activity of the hepatic enzyme, uridine diphosphate (UDP)

glucuronyl transferase which regulates the conjugation of bilirubin by the liver, decreased by 22% and 60% after 2 hours and 3 hours of the intoxication respectively (Fig. 5). Efficient hepatic mechanisms responsible for conjugation of bilirubin are found to be located in the endoplasmic reticulum (1). Activities of various enzymes of the endoplasmic reticulum have been shown to undergo changes when CCl_4 was found to be at its peak concentration in the liver during the first few hours of the intoxication (20). The enzyme bilirubin UPD-glucuronyl transferase is located in the smooth endoplasmic reticulum of the liver cells (1). Swelling and disruption of the endoplasmic reticulum following CCl_4 administration (4,22), is likely to affect appreciably or to completely destroy the integrity of this enzyme resulting in a defective hepatic conjugation of bilirubin. Furthermore, increased lipid peroxidation, produced by CCl_4 (21), is known to depress the activities of various endoplasmic enzymes (21, 25).

Under normal circumstances, once the unconjugated bilirubin is conjugated with glucuronic acid, it is rapidly excreted by the liver into the bile and apparently there is little if any intracellular storage of conjugated bilirubin (1). A significant increase in the plasma conjugated bilirubin levels following the injection of UCB in the CCl_4-treated rats (Fig. 2, Fig. 3, Table III), therefore, suggest a block in the excretion of bilirubin from the liver into the bile resulting in the regurgitation of conjugated bilirubin from the liver into the blood. A defective clearance of conjugated bilirubin (Fig. 4, Table IV), supported these conclusions. These effects of CCl_4 on the hepatic bilirubin metabolism appeared between 2-3 hours after intoxication. This time schedule for the apperance of functional liver damage could be well correlated with the structural and enzymatic liver damage produced by CCl_4 by (4, 17, 19, 22).

From these studies it was concluded that (i) CCl_4 treatment affects the hepatic uptake, storage, conjugation and excretion of bilirubin and that (ii) these effects of CCl_4 on the hepatic bilirubin metabolism start as early as 2 hours of the poisoning. Furthermore, CCl_4 reduced the rate of bile secretion by the liver cells of the rats. These findings are consistent with a postulate that CCl_4 cholestasis may involve enhanced diffusion of materials from bile to blood in addition to the inhibition of active transport in the opposite direction.

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