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CCl_4 cirrhosis in rats: irreversible histological changes and differentiated functional impairment

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Cirrhosis of the rat liver was induced by a 12 week individualized CCl_4/phenobarbital treatment. After treatment, all surviving animals (81%) showed cirrhosis of the liver. The cirrhosis induced was irreversible when evaluated 24 weeks after cessation of treatment. Quantitative liver function measurements were reduced in a differentiated manner. Ranked according to the most pronounced changes they are: capacity of urea-N synthesis (CUNS), galactose elimination capacity (GEC) and antipyrine clearance (APC). Hepatic glutathione concentrations were only slightly decreased after the CCl_4 treatment. It is possible to produce a high incidence of irreversible cirrhosis with differentiated functional impairment in the rat.

A reliable experimental model to produce severe, decompensated micronodular cirrhosis in the rat has been described by Proctor et al. (1). The procedure is based on a dosing scheme of CCl_4, taking into account individual sensitivity to the hepatotoxin, as assessed by daily weight measurements. A high frequency of cirrhosis and a low death rate has been reported by this method.

The purpose of the present work was to investigate this model of cirrhosis for irreversibility with regard to histological changes and impairment of liver cell functions.

Material and Methods

Experimental design. Six-week-old male Wistar rats with an initial weight of 145–155 g were kept under constant temperature and humidity in a 12 h controlled dark/light cycle with stock pellets and tap water ad libitum.

Cirrhosis was induced by exposing 80 rats to phenobarbital in the drinking water and weekly oral CCl_4 administration according to Proctor et al. (1), as described in detail below. The CCl_4-treated rats were examined in groups of 6–18 animals after 12 weeks of CCl_4 treatment and 12 and 24 weeks after cessation of the CCl_4 treatment. Twenty-eight control rats were untreated litter mates and were examined in groups of 5–12 animals at ages of 8, 20 and 44 weeks. Thus, the age of cirrhotic rats and controls corresponded. The data from the control rats have been published in a previous study (17), but the measurements were performed in the same experimental series setup.

The rats were subjected to liver function measurements in vivo with subsequent liver tissue measurements and light microscopic evaluation of liver histology, as described below. The procedure was performed between 9 a.m. and 1 p.m. with two animals simultaneously. Due to the extensive technical procedure, three animals were prepared for each investigation of two rats. Animals not used were killed.

Induction of cirrhosis. CCl_4 dissolved in corn oil to a total volume of 2 ml was given by gastric tube once a week.

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The initial CCl_4 dose used was from a preceding experiment described by Proctor et al. (1). Twenty rats were divided into four groups of five rats. A single dose of CCl_4 was given to each group. The doses were 0.04, 0.08, 0.16 and 0.32 ml CCl_4, respectively. The initial dose in the present experiment was then defined as half the dose at which death began to occur which, in this case, was 0.08 ml CCl_4. Several months later when additional rats began CCl_4 treatment the initial dose was increased to 0.12 ml CCl_4.

Subsequent weekly doses of CCl_4 were adjusted from the weight responses of previous doses. The intended weight change was a weight loss of 6–9% 2–3 days after each CCl_4 administration and a weight gain on the 7th day. A total weight loss of approx. 25% was intended in the CCl_4-treated animals compared to controls. In general, the CCl_4 dose had to be increased week by week (1), due to both the age-related increase in rat body weights, and due to a reduced sensitivity to CCl_4 from reduced cytochrome *P*-450 activation (2).

In rats achieving the intended weight changes, weekly doses of CCl_4 were increased by 1.5-, 2-, 3-, 4-, etc., times the initial dose (standard scheme). In rats with a weight loss of less than approx. 4% at day 2–3 after CCl_4 administration, the dose was increased by twice the initial dose. In rats with a weight loss of 4–6%, the dose was increased by 1.5-times the initial dose. When the weight loss was 6–9%, the CCl_4 dose was increased as described in the standard scheme. If the body weight loss was more than 9%, the dose was maintained when there was a clear weight gain on the 7th day. Doses were reduced by the initial dose if the body weight was just regained or increased a little on the 7th day. Doses were reduced by double the initial dose if the body weight was not regained. After some time, a clinical impression of the animals' condition also helped to determine the adjustment of dosage. The doses of CCl_4 ranged from 0.04 to 0.96 ml. The CCl_4 treatment schedule lasted for 12 weeks (i.e., 12 doses).

CCl_4-treated animals had free access to drinking water containing phenobarbital (350 mg/l) starting about 2 weeks (10–14 days) before the first CCl_4 dose and during the entire period of CCl_4 administration. The measurements following 12 weeks of CCl_4 treatment were carried out 6–7 days after the last CCl_4 dose. Phenobarbital administration had also stopped. This procedure ensured regression of the enzyme inducing effect of phenobarbital (3).

Among the 80 rats receiving CCl_4 treatment, 15 (19%) died following 4–12 doses of CCl_4.

Liver function measurements. The animals were given pentothal anaesthesia (87.5 mg/kg body weight). Tracheostomy and retroperitoneal nephrectomy were performed to avoid extrahepatic galactose and urea elimination during the procedure. Polyethylene catheters were placed in the left internal jugular vein for infusion and into the right common carotid artery for blood sampling (4).

The maximum volume injected during the experiments was 27 ml per kg body weight, which has no influence on the formula for total body water or volume of distribution for galactose.

Galactose elimination capacity (GEC). Galactose (Kabi, Sweden) was given as an injection of 0.15–0.25 ml of a 50% w/v solution in water, followed by an infusion of 1.2 ml/h of a 2% solution.

Blood samples (100 μl) were taken in duplicate after 20 min of equilibration every 10 min for 50 min for the determination of galactose concentration by the galactose dehydrogenase method (5).

The galactose elimination capacity (GEC) was calculated as

$$GEC = I - (dc/dt \cdot 0.40\ b.w.)$$

where I is the galactose infusion rate, dc/dt is the linear slope of galactose blood concentration-time curve, b.w. is the body weight and 0.40 b.w. is the estimated volume of distribution for galactose (6).

Capacity of urea-N synthesis (CUNS). Alanine (Sigma, St. Louis, MO) was administered as an intravenous injection of 0.6–1.8 ml of a 10% w/v solution in water, followed by an infusion for 70 min of 2–9 ml/h of a 2% solution to a steady-state amino acid concentration between 7.3–11.6 mmol/l adjusted from rapid analysis of total α-amino-N as described in detail earlier (7). Blood samples (150 μl) were taken after an equilibration period of 20 min every 10 min for 50 min for the determination of urea and total α-amino-N concentrations.

Total blood α-amino-N concentration was measured by the dinitrofluorobenzene method (4), blood urea concentration by the urease-Berthelot method (8).

The capacity of urea-N synthesis (CUNS) was calculated as

$$CUNS = dc/dt \cdot 0.63\ b.w. \cdot 1.25$$

where dc/dt is the slope of the linear regression of arterial blood urea concentration over time during α-amino-N steady-state (defined as less than 10% change during a period of 50 min or longer), 0.63 b.w. is the volume of distribution for urea (9), and 1.25 a correction for intestinal urea hydrolysis (10).

Antipyrine plasma clearance (APC). Antipyrine (Sigma, St. Louis, MO) 2 mg/100 g b.w. was given intragastrically 4–6 h before the start of the determination of GEC

and CUNS. A blood sample of 1 ml was taken before starting the infusions mentioned above.

Antipyrine concentration in plasma was measured by high-pressure liquid chromatography (11).

The antipyrine plasma clearance (APC) was calculated according to the one sample method (11) as

$$APC = 0.66\,b.w.\cdot(\ln(D/0.66\,b.w.) - \ln(c_t))/t$$

where D is the antipyrine dose, 0.66 b.w. is the antipyrine apparent volume of distribution (11) and c_t is the concentration at the sampling time t (11).

Liver tissue measurements. Immediately following the above procedures, the rat was exsanguinated by puncture of the aorta, and the liver was quickly excised, blotted on filter paper, sliced with scissors in chilled isotonic KCl and homogenized in a Potter-Elvehjem glass-teflon homogenizer with 10 ml of chilled isotonic KCl. Liver homogenate was assayed for cytochrome *P*-450 (12), total glutathione concentration (13) and protein (14). The microsomal fraction of liver homogenate (15) was assayed for protein (14), cytochrome *P*-450 (12) and *para-N*-anisole demethylase activity (16) by the methods indicated. There was no difference in the recovery of protein and cytochrome *P*-450 in homogenate vs. microsomes for any of the groups, and consequently only microsomal data are given. Due to the abundant sampling and the necessity for immediate analysis of the liver tissue samples, these analyses were performed on a maximum of 12 animals at each time point. Due to technical difficulties, only five animals had the capacity of urea-N synthesis estimated in the various groups. In addition, a few technical failures occurred, identifiable in Table 1 where the initial number of animals can be read in the column with body weight.

Liver histology. Samples of liver tissue were fixed in 10% neutral buffered formalin immediately after removal of the liver from the animals. Sections were cut from paraffin-embedded blocks and stained with haematoxylin-eosin, van Gieson's stain for collagen, Gordon and Sweet' silver impregnation for reticulin fibres, the ferrocyanide reaction for iron and periodic acid-Schiff (PAS) +/– diastase for glycogen. Histological examination was performed blind, i.e., without knowledge of the treatment or measurements performed.

Statistical analysis. Data were compared with Student's *t*-test, correlation analysis was performed by standard parametric methods, p values less than 0.05 were considered statistically significant.

Results

After a 12 week CCl_4 treatment all 18 rats showed micronodular liver cirrhosis (Fig. 1), eight with very discrete signs of acute inflammation. Ascites were found in four rats with a volume of 20–43 ml. After cessation of CCl_4 treatment all twelve (after 12 weeks) and twelve rats (after 24 weeks), respectively, had micronodular liver cirrhosis. A more marked fibrosis was found immediately after cessation of CCl_4 treatment (Fig. 1) than after 24 weeks, where the septae had transformed into dense, more mature connective tissue (Fig. 2). Only one rat had ascites (11 ml) at the age of 44 weeks and the liver in this rat showed activity in the cirrhosis, i.e., portal inflammation, and reduced liver weight (7.6 g) in contrast to the remaining livers in this group.

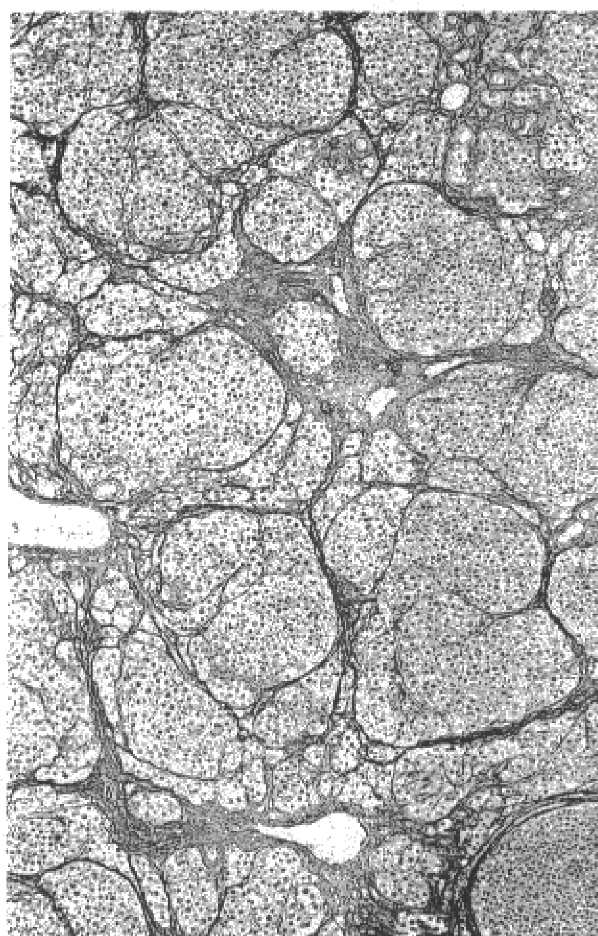


Fig. 1. Light microscopy 6–7 days after cessation of 12 weeks' CCl_4 treatment showing hepatic cirrhosis.

Table 1 gives the initial values of quantitative liver function measurements and liver tissue analyses, i.e., for rats 8 weeks of age and having had no CCl_4 treatment (17). Table 2 and Fig. 3a–c show the relative values for CCl_4 rats and control rats after 12 weeks of treatment, and 12 weeks and 24 weeks following cessation of treatment.

As mentioned above, a total weight loss was intended for the CCl_4-treated animals in comparison to controls.

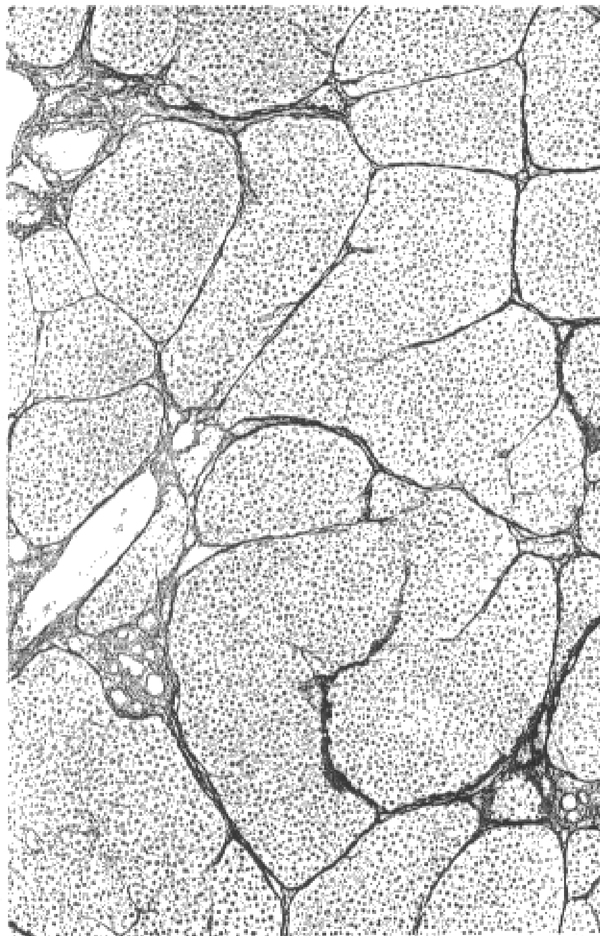


Fig. 2. Light microscopy 24 weeks after cessation of 12 weeks' CCl_4 treatment showing hepatic cirrhosis.

TABLE 1

Initial (control) values at an age of 8 weeks of galactose elimination capacity (GEC), capacity of urea-N synthesis (CUNS), antipyrine clearance (APC) and liver biopsy measurements, and the number of rats investigated

Body weight (g) (6)[a]	200 ± 4.96	(10,18,12,12,12)[b]
Liver weight (g) (6)[a]	8.03 ± 0.512	(10,18,12,12,12)[b]
Quantitative liver function measurements		
GEC (5)[a]		
(μmol/min)	2.33 ± 0.231	(9,13,12,10,8)[b]
(μmol/min per 100 g)	1.18 ± 0.109	(9,13,12,10,8)[b]
CUNS (5)[a]		
(μmol/min)	14.3 ± 1.88	(8,8,6,5,8)[b]
(μmol/min per 100 g)	7.21 ± 0.908	(8,8,6,5,8)[b]
APC (5)[a]		
(ml/min)	1.28 ± 0.112	(6,14,12,10,11)[b]
(ml/min per 100 g)	0.625 ± 0.052	(6,14,12,10,11)[b]
Liver tissue measurements		
Microsomal protein (5)[a]		
(mg/g liver)	41.9 ± 3.95	(6,10,8,6,12)[b]
Microsomal *P*-450 (5)[a]		
(nmol/g liver)	28.8 ± 2.53	(6,10,8,6,12)[b]
Glutathione (5)[a]		
(μmol/g liver)	4.41 ± 0.706	(6,10,8,6,12)[b]
PNA (5)[a]		
(nmol/mg micr. prot. per 10 min)	7.08 ± 0.506	(6,10,8,6,12)[b]

Values are given as mean ± S.E.

[a] Indicates the number of animals in initial (control) experiment.

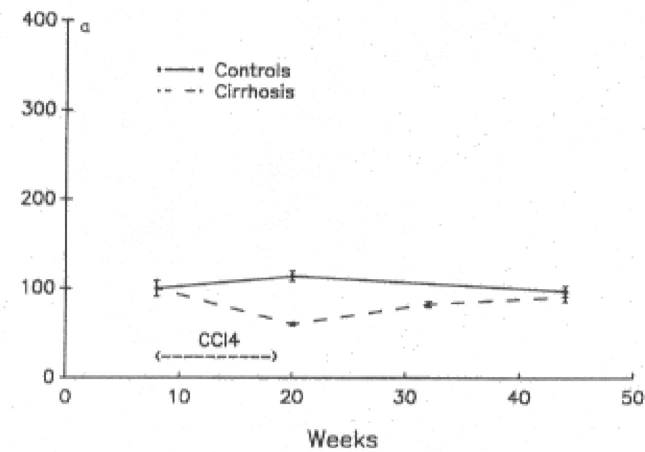
[b] Indicates the number of animals on which Tables 2 and 3 and Fig. 3a–c are based (20 weeks control, 20 weeks cirrhotics, 32 weeks cirrhotics, 44 weeks control, 44 weeks cirrhotics).

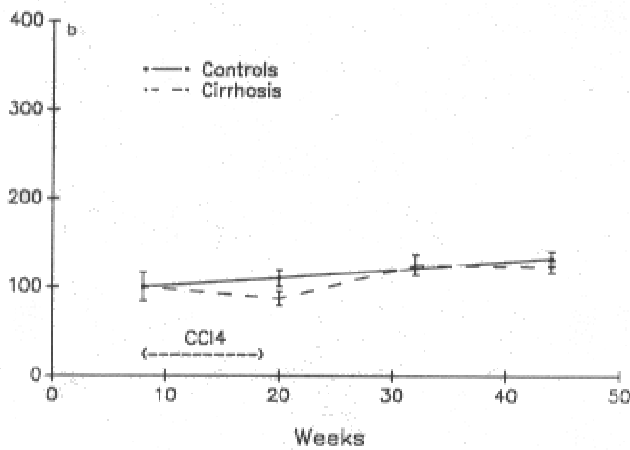
After 12 doses of CCl_4 the mean body weight was 71% (Table 2) of the control value ($p < 0.01$). At this stage the mean liver weight had also increased less in cirrhotic animals and was 78% (Table 2) of the control value ($p < 0.01$). After discontinuation of CCl_4 treatment, cirrhotic rats compensated their previous relative body and liver weight loss and at the age of 44 weeks the mean body weight was 91% ($p < 0.05$) and the mean liver weight 99% (N.S.) of the control values. The liver weight/body weight ratio was approximately the same for cirrhotic (data not given) and control animals at all ages. There was, however, a decrease with age from about 4.0% at the age of 8 weeks to about 2.7% at 44 weeks of age (17).

The quantitative liver function measurements of galactose elimination capacity and capacity of urea-N synthesis were decreased after 12 weeks of treatment, with mean values of 72 and 76%, respectively ($p < 0.05$), of controls (Table 2). Twenty-four weeks after cessation of the CCl_4 treatment a further deterioration was seen, with values of 64 and 63%, respectively, of controls ($p < 0.01$). When expressed per 100 g body weight (Table 3) there was no significant difference between cirrhotic and control animals at the age of 20 weeks (12 weeks' treatment) for either of the two function measurements, whereas 24 weeks after cessation galactose elimination capacity was reduced to 70% ($p < 0.01$) and capacity of urea-N synthesis to 73% ($p < 0.01$) in cirrhotic animals versus controls.

The antipyrine clearance was only slightly, and not significantly ($p > 0.05$), decreased after 12 weeks' treatment. Relative to body weight (Table 3) the mean value after induction of cirrhosis was 133% in cirrhotic rats versus controls ($p < 0.01$). Twenty-four weeks after induction of cirrhosis there was no statistically significant difference in antipyrine clearance between cirrhotic animals and controls.

After 12 weeks of CCl_4 treatment the hepatic microsomal protein, microsomal cytochrome *P*-450 and total glutathione were reduced to 72, 53 and 79%, respectively ($p < 0.01$), of controls when expressed per g liver. These differences were diminished in the weeks following the cessation of the CCl_4 treatment. Twenty-four weeks after cessation of CCl_4 values of cirrhotic animals were similar to the control values (Fig. 3).





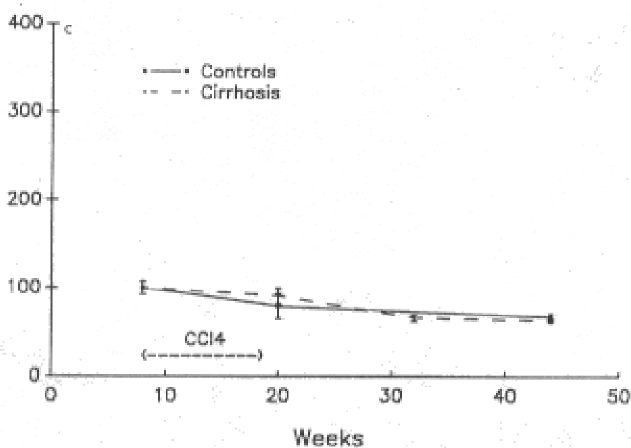
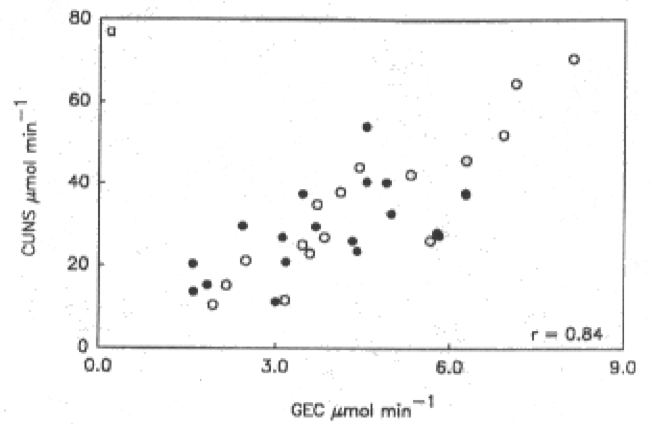
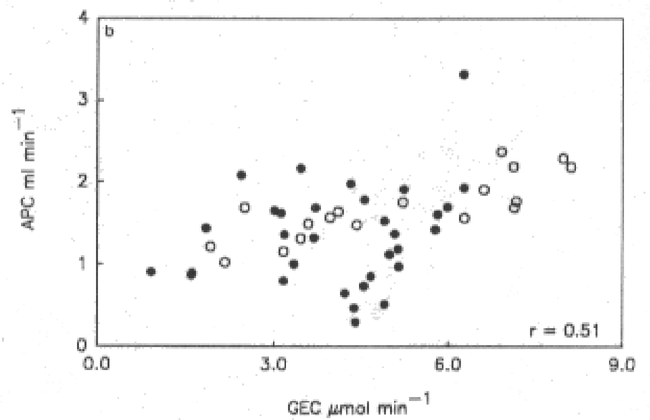


Fig. 3. Changes in liver biopsy measurements; a, microsomal cytochrome *P*-450 (nmol/g liver); b, total glutathione (μmol/g liver); and c, *para-N*-anisole demethylase activity (nmol/mg microsomal protein per 10 min) in controls and cirrhotic animals. The number of animals of each group can be found in Table 1.





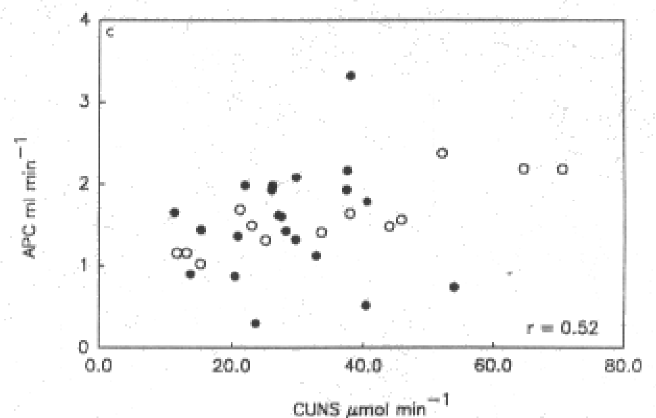


Fig. 4. The relation between the galactose elimination capacity (μmol/min), the capacity of urea-N synthesis (μmol/min) and the antipyrine clearance (ml/min) in controls and cirrhotic animals. Only experiments where both the respective functions were estimated are included in this figure. ○, control rats; ●, CCl_4-cirrhotic rats.

TABLE 2

Changes in body weight (g), liver weight (g), galactose elimination capacity (GEC) (μmol/min), capacity of urea-N synthesis (CUNS) (μmol/min) and antipyrine clearance (APC) (ml/min) relative to initial values

	Before treatment	6–7 days after 12 weeks' CCl_4	12 weeks after 12 weeks' CCl_4	24 weeks after 12 weeks' CCl_4
Body weight				
Control	100 ± 3	224 ± 5		271 ± 6
Cirrhotic		159 ± 4[a]	220 ± 4	247 ± 8
Liver weight				
Control	100 ± 6	167 ± 7		182 ± 6
Cirrhotic		130 ± 8[a]	162 ± 8	180 ± 10
GEC				
Control	100 ± 10	187 ± 15		267 ± 14
Cirrhotic		134 ± 16	211 ± 10	184 ± 22
CUNS				
Control	110 ± 13	219 ± 18		384 ± 38
Cirrhotic		165 ± 18	221 ± 19	243 ± 25
APC				
Control	100 ± 9	124 ± 6		148 ± 8
Cirrhotic		115 ± 8	91 ± 19	123 ± 13

Values are given as mean ± S.E. with initial mean values set to 100, the number of animals in each group can be found in Table 1.
[a] Designates $p < 0.05$.

Para-N-anisole demethylase activity expressed relative to microsomal protein showed no differences compared to controls (Fig. 3).

Fig. 4a–c shows the relations between the three quantitative liver function measurements. The r values for capacity of urea-N synthesis versus galactose elimination capacity, antipyrine clearance versus galactose elimination and antipyrine clearance versus capacity of urea-N synthesis were 0.84, 0.51 and 0.52, respectively ($p < 0.05$). A small negative intercept (N.S.) was found between capacity of urea-N synthesis and galactose elimination capacity, whereas antipyrine clearance versus either of the two other liver function measurements showed a statistically significant positive intercept.

TABLE 3

Galactose elimination capacity (GEC), capacity of urea-N synthesis (CUNS) and antipyrine clearance (APC) relative to body weight in controls and cirrhotic animals

	6–7 days after 12 weeks' CCl_4	24 weeks after 12 weeks' CCl_4
GEC (μmol/min per 100 g)		
Control	0.963 ± 0.065 (9)	1.25 ± 0.065 (10)
Cirrhotic	0.972 ± 0.093 (13)	0.870 ± 0.092 (8)[a]
CUNS (μmol/min per 100 g)		
Control	7.08 ± 0.443 (8)	9.79 ± 0.740 (5)
Cirrhotic	7.82 ± 0.777 (8)	7.18 ± 0.624 (8)[a]
APC (ml/min per 100 g)		
Control	0.348 ± 0.012 (6)	0.352 ± 0.020 (10)
Cirrhotic	0.464 ± 0.035 (14)[a]	0.320 ± 0.034 (11)

Values are given as mean ± S.E., the number of animals is given in brackets.
[a] Designates $p < 0.05$.

Discussion

This study demonstrates that cirrhosis produced by CCl_4 and phenobarbital is histologically irreversible, and that certain quantitative liver function measurements are persistently decreased.

The CCl_4/phenobarbital treatment procedure as suggested by Proctor et al. (1) produced cirrhosis in all surviving animals with a mortality of 19% ($n = 15$ animals). The death of 12 animals occurred a few days after CCl_4 administration and was probably due to acute CCl_4 toxicity rather than cirrhosis. Two animals died 4–5 weeks after CCl_4 treatment was discontinued, probably due to a histologically verified cirrhosis. One animal died due to technical failure.

In the present study we chose a fairly low CCl_4 dose to minimize selection bias from death due to acute toxicity. A higher dose would probably produce more severe cirrhosis. Although Proctor et al. (1) used CCl_4 doses that were approximately the same or slightly lower than doses in the present study, the cirrhosis in the study of Proctor et al. was severe and decompensated in 74% of the rats of identical sex and strain.

From the present quantitative liver function measurements, a moderate reduction occurred in galactose elimination capacity and capacity of urea-N synthesis, falling to about 63–76% of control rats. When expressed per 100 g body weight no difference was found 6–7 days after a 12 week CCl_4 treatment, but 24 weeks following the cessation of CCl_4, the values in cirrhotic rats were about 70% of control values. The toxicity of CCl_4 affects, therefore, both the liver and the entire body. After cessation of the toxin there is an increase in both body and liver weight. Since this is not accompanied by a similar increase in cytosolic liver function the findings point at an irreversible hepatic toxicity histologically recognised as micronodular cirrhosis. As demonstrated in an earlier study there is an age-related increase in cytosolic functions (17) which is impaired in CCl_4-treated rats.

In contrast, antipyrine plasma clearance, interpreted as a measure of some of the hepatic microsomal functions, did not differ significantly from controls, immediately af-

ter CCl_4 treatment or later. While galactose elimination capacity reflects cytosolic liver function (6) capacity of urea-N synthesis reflects both cytosolic and mitochondrial function (7). The results thus demonstrate that in this model of cirrhosis different liver functions are unequally affected, with the microsomal functions involved in antipyrine clearance only slightly affected, if at all. Such differentiated functional impairment has been described in other types of liver damages, for example chemically induced damage and in the early phases of hepatic regeneration which follow partial hepatectomy (18–21). In earlier studies a linear relationship between each of the three quantitative functions was found in rats of different ages (17). This is illustrated in Fig. 4a–c, control rats. In cirrhotic rats we found a similar relationship, although with much wider variation, especially between antipyrine clearance and the other two liver function measurements.

The aminopyrine breath test in cirrhotic rats generally has not been found to differ statistically significantly from controls (22), the cirrhosis induced by CCl_4/phenobarbital. For the increases in antipyrine clearance relative to controls when exposed per 100 g body weight in our study, decreased body weight combined with maintained total hepatic antipyrine metabolising capacity seems the most likely explanation. Normally phenobarbital-dependent enzyme induction should be completely regressed at the time of experiment performance (3), and maintained induction is therefore an unlikely explanation. The age-related increase found in control animals in galactose elimiation capacity and capacity of urea-N synthesis relative to body weight is not seen for antipyrine clearance; by contrast this function decreases with age (17). This could be a partial contribution to the differences in functional impairment. Interestingly, the severe reduction in microsomal cytochrome *P*-450 was not accompanied by a reduction in antipyrine clearance suggesting a differential of damage to the different cytochrome *P*-450 isoenzymes.

The values of *para-N*-anisole demethylase activity in cirrhotic animals were similar to those found in controls when related to microsomal protein. This suggests a functional integrity of a reduced amount of endoplasmic reticulum. Early after the CCl_4 treatment, total glutathione concentrations were found decreased in cirrhotic rats compared to controls. Thereafter the values in cirrhotic rats increased and reached the control values. This could perhaps be explained by an acute toxic effect of CCl_4. However, as reported by others this acute toxicity should have disappeared at day 7 after the last CCl_4 dose (23,24).

In contrast to our results Villeneuve et al. (23) found no decrease in liver protein content in cirrhotic rats. Farrell and Zaluzny (24) found that the microsomal protein synthesis in cirrhotic rats was similar to controls. The decrease in cytochrome *P*-450 found at day 6–7 after CCl_4 treatment in the present study is consistent with reports from other studies concerning phenobarbital/CCl_4-induced liver cirrhosis in rats (23–25). Moreover, Marshall and McLean (25) found that in mildly cirrhotic animals control values may be reached within 3 or 4 weeks, whereas in severely cirrhotic animals no recovery was found within 8 weeks after CCl_4 treatment had stopped.

According to a personal communication from E. Proctor core temperature taken under controlled conditions within 4 min of leaving the animal house, and recovery time from a standardised dose of halothane anaesthesia may also be used as a guide to liver damage. We have not yet examined this in our laboratory.

We conclude that the present phenobarbital/CCl_4 treatment produces a high incidence of irreversible cirrhosis with a differentiated functional impairment, and a low mortality.

Acknowledgements

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