Cirrhosis of the rat liver was induced by a 12 week individualized CCl₄/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 antipyrene clearance (APC). Hepatic glutathione concentrations were only slightly decreased after the CCl₄ treatment. It is possible to produce a high incidence of irreversible cirrhosis with differentiated functional impairment in the rat.

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 CCl₄ administration according to Proctor et al. (1), as described in detail below. The CCl₄-treated rats were examined in groups of 6–18 animals after 12 weeks of CCl₄ treatment and 12 and 24 weeks after cessation of the CCl₄ 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 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. CCl₄ 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 CCl₄ 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 CCl₄ was given to each group. The doses were 0.04, 0.08, 0.16 and 0.32 ml CCl₄, 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 CCl₄. Several months later when additional rats began CCl₄ treatment the initial dose was increased to 0.12 ml CCl₄.

Subsequent weekly doses of CCl₄ 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 CCl₄ administration and a weight gain on the 7th day. A total weight loss of approx. 25% was intended in the CCl₄-treated animals compared to controls. In general, the CCl₄ 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 CCl₄ from reduced cytochrome P-450 activation (2).

In rats achieving the intended weight changes, weekly doses of CCl₄ 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 CCl₄ 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× the initial dose. When the weight loss was 6–9%, the CCl₄ 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 CCl₄ ranged from 0.04 to 0.96 ml. The CCl₄ treatment schedule lasted for 12 weeks (i.e., 12 doses).

CCl₄-treated animals had free access to drinking water containing phenobarbital (350 mg/l) starting about 2 weeks (10–14 days) before the first CCl₄ dose and during the entire period of CCl₄ administration. The measurements following 12 weeks of CCl₄ treatment were carried out 6–7 days after the last CCl₄ dose. Phenobarbital administration had also stopped. This procedure ensured regression of the enzyme inducing effect of phenobarbital (3).

Among the 80 rats receiving CCl₄ treatment, 15 (19%) died following 4–12 doses of CCl₄.

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

\[ \text{GEC} = \frac{\text{dc/dt}}{0.40 \, \text{b.w.}} \]

where \( \text{dc/dt} \) is the galactose infusion rate, \( \text{dc/dt} \) 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

\[ \text{CUNS} = \frac{\text{dc/dt}}{0.63 \, \text{b.w.} \cdot 1.25} \]

where \( \text{dc/dt} \) 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

$$APC = rac{0.66 \text{ b.w.} \cdot (\ln(D/0.66 \text{ b.w.}) - \ln(c))}{t}$$

where $D$ is the antipyrine dose, 0.66 b.w. is the antipyrine apparent volume of distribution (11) and $c$ 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-teslin 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 microcute 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.

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 in comparison to controls.
After 12 doses of CCl₄ 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 CCl₄ 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 control (Table 2). Twenty-four weeks after cessation of the CCl₄ 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 CCl₄ 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 CCl₄ treatment. Twenty-four weeks after cessation of CCl₄ values of cirrhotic animals were similar to the control values (Fig. 3).

![Table 1](image)
Fig. 3. Changes in liver biopsy measurements: a, microsomal cytochrome P-450 (nmol/g liver); b, total glutathione (nmol/g liver); and c, para-N-anisidine 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; ●, CCl₄-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

<table>
<thead>
<tr>
<th></th>
<th>Before treatment</th>
<th>5–7 days after 12 weeks CCl₄</th>
<th>12 weeks after 12 weeks CCl₄</th>
<th>24 weeks after 12 weeks CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 3</td>
<td>159 ± 4</td>
<td>220 ± 4</td>
<td>247 ± 8</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 6</td>
<td>167 ± 7</td>
<td>150 ± 8</td>
<td>182 ± 6</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td></td>
<td>134 ± 16</td>
<td>211 ± 10</td>
<td>184 ± 22</td>
</tr>
<tr>
<td>GEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 10</td>
<td>187 ± 15</td>
<td>257 ± 14</td>
<td>184 ± 22</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td></td>
<td>134 ± 16</td>
<td>211 ± 10</td>
<td>184 ± 22</td>
</tr>
<tr>
<td>CUNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>110 ± 13</td>
<td>219 ± 18</td>
<td>243 ± 25</td>
<td>243 ± 25</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td></td>
<td>165 ± 18</td>
<td>221 ± 19</td>
<td>243 ± 25</td>
</tr>
<tr>
<td>APC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 9</td>
<td>124 ± 6</td>
<td>148 ± 8</td>
<td>148 ± 8</td>
</tr>
<tr>
<td>Cirrhotic</td>
<td></td>
<td>115 ± 8</td>
<td>91 ± 19</td>
<td>123 ± 13</td>
</tr>
</tbody>
</table>

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.

### Discussion

This study demonstrates that cirrhosis produced by CCl₄ and phenobarbital is histologically irreversible, and that certain quantitative liver function measurements are persistently decreased.

The CCl₄/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 CCl₄ administration and was probably due to acute CCl₄ toxicity rather than cirrhosis. Two animals died 4–5 weeks after CCl₄ 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 CCl₄ 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 CCl₄ 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 CCl₄ treatment, but 24 weeks following the cessation of CCl₄, the values in cirrhotic rats were about 70% of control values. The toxicity of CCl₄ 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 CCl₄-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 CCl₄ 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 anti-
pyrine clearance only slightly affected, if at all. Such dif-
erentiated functional impairment has been described in
other types of liver damages, for example chemically in-
duced damage and in the early phases of hepatic regen-
eration which follow partial hepatectomy (18–21). In ear-
erlier 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 cir-
 rhotic 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 CCl₄/phenobarbi-
tal. 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 repressed at the
time of experiment performance (3), and maintained in-
duction is therefore an unlikely explanation. The age-
related increase found in control animals in galactose elim-
nation 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 micro-
somal cytochrome P-450 was not accompanied by a
reduction in antipyrine clearance suggesting a differential
damage to the different cytochrome P-450 isoenzymes.

The values of para-N-arisole demethylase activity in
cirrhotic animals were similar to those found in controls
when related to microsomal protein. This suggests a func-
tional integrity of a reduced amount of endoplasmic retic-
ulum. Early after the CCl₄ 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 CCl₄.
However, as reported by others this acute toxicity should
have disappeared at day 7 after the last CCl₄ dose (23,24).

In contrast to our results Villeneuve et al. (23) found no
decrease in liver protein content in cirrhotic rats. Farrell
and Zalusny (24) found that the microsomal protein syn-
thesis in cirrhotic rats was similar to controls. The de-
crease in cytochrome P-450 found at day 6–7 after CCl₄
 treatment in the present study is consistent with reports
from other studies concerning phenobarbital/CCl₄-in-
duced 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, where-
as in severely cirrhotic animals no recovery was found
within 8 weeks after CCl₄ treatment had stopped.

According to a personal communication from E. Proc-
tor 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/CCl₄ treat-
ment produces a high incidence of irreversible cirrhosis
with a differentiated functional impairment, and a low
mortality.

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