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Effect of a Liver Extract on Hepatic Injury Produced by Carbon Tetrachloride.* (27508)

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It has recently been stated(1) that a protein-free, water soluble fraction of the liver of cattle ("Ripason")‡ can significantly modify the hepatotoxic effects of carbon tetrachloride (CCl_4). If experimental animals are treated with this extract for some time before, and also during, the period of administration of CCl_4, it is stated that sections of their livers show considerably less damage on histological examination than those of their controls not given liver extract. The liver preparation in question apparently also accelerates the disappearance of the fatty deposits in the damaged liver during the period of convalescence following cessation of injections of CCl_4(2), prevents impairment of liver function by this agent(3), and is stated to exhibit lipotropic properties in other experimental situations as well(3,4). To some extent these claims are similar to older observations in which a favorable effect of liver preparations on hepatic damage caused by CCl_4 or by deficient diets has been reported (5,6,7). The possibility that the liver may contain one or several protective principles which prevent liver damage caused by various hepatotoxic factors in other species is of great theoretical, and possibly of practical, interest and has found its clinical expression in the notion of "organotherapy". There are available in the older, as well as in the more recent literature(8,9), reports describing favorable effects produced in patients with a variety of hepatic disorders by liver extracts, including the preparation to be studied here. It was, therefore, of interest to see whether some of the claims made for this particular preparation could be confirmed experimentally. Carbon tetrachloride was chosen as the most suitable hepatotoxic agent, not only because it is still one of the most widely used poisons for this purpose, but also because it was hoped that an understanding of the reported protective effects of liver extracts against this agent might lead to a better understanding of the mode of action of CCl_4 itself.

Methods. A total of 58 Sprague-Dawley rats was used. Because of the death of some animals only 50 rats, or 25 pairs, were included in the final analysis. Twelve of these animals, all belonging to the first experiment, were females; the remainder were males. The weight of the animals at the start of experiment ranged from 42 to 152 g. This large variation in age and weight of the animals was offset by the use of littermates of the same sex, so that each treated animal was compared only to its untreated brother or sister. It was assumed that the variations in sex, age and weight of the experimental rats, controlled by the littermate arrangement adhered to throughout, would help to define the dependence of any effects to be demonstrated

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‡ "Ripason", Robapharm (Canada) Ltd.

on the factors of age and sex. All animals were kept in individual cages, and had free access to the stock rat cake diet.

Three separate experiments were undertaken.

In *Experiment I,* six pairs of female and 3 pairs of male littermate rats were used. The experiment lasted for 20 days. During the first 10 days one rat of each pair was given a daily subcutaneous injection of 0.3 ml/100 g body weight of liver extract, ("Ripason"); their controls received the same amount of saline. On the eleventh day the dose of "Ripason" was increased to 1 ml per 100 g body weight, and in addition 5 *subcutaneous* injections of CCl_4, one injection every other day, were given to all animals in a dose of 0.1 ml/100 g body weight. All animals were killed 24 hours after the last injection of CCl_4. This experimental design was based on the experiments described by Tanyol and Friedman(1).

In *Experiment II,* 7 pairs of male rats were used. The experimental design was similar to that used in Exp. I, except that the daily dose of "Ripason" during the first 10-day period was increased to 0.5 ml/100 g body weight. During the second period of 10 days 1 ml of Ripason/100 g body weight was used as before, but CCl_4 was given *intraperitoneally* every other day in 5 doses of 0.1 ml/100 g body weight. All animals were killed 36 hours after the last injection of CCl_4.

In *Experiment III,* 9 pairs of male rats were used. Each animal was given a daily subcutaneous injection of 0.5 ml of CCl_4 per 100 g body weight for 5 days. The control animals were then allowed a rest period of 4 days, during which no more injections were given, but the test animals received during this rest period, as well as on the last day of treatment with CCl_4, a daily subcutaneous injection of "Ripason" (1 ml/100 g body weight). All animals were killed 24 hours after the last injection. This experimental design was based on the experiments reported by Hartmann(2).

At the end of the experimental period all animals were exsanguinated under light ether anesthesia and the liver was rapidly excised and weighed. Weighed samples of the liver were dried to constant weight (2 weighings) in the oven at a temperature of 104°C, and the lipid was extracted in a Soxhlet apparatus by a hot mixture of methanol and chloroform (2:1). The difference between dry weight and weight of the fat-free residue represented the lipid extracted, expressed as grams of lipid per 100 g wet liver weight. For statistical analysis of the data, Wilcoxon's ranking test(10) was used.

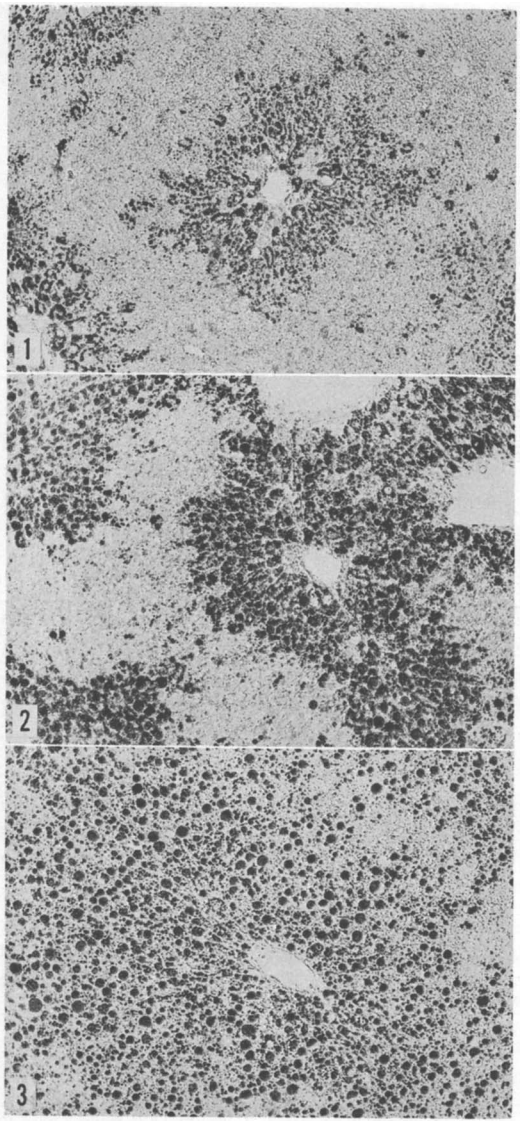
TABLE I. Comparison of Histological Assessment of Degree of Fatty Change with Level of Lipids (g per 100 g Wet Liver Weight) Extracted from the Liver of Rats Treated with Carbon Tetrachloride.

Histological grading	Mean level of lipids (with range)
1+	6.10 (5.8 – 6.3)
2+	8.25 (6.6 – 10.2)
3+	12.22 (8.9 – 16.3)
4+	16.42 (13.2 – 18.3)

The histological changes were studied in paraffin sections of liver fixed in 10% formol saline and stained with hematoxylin-eosin, or in frozen sections stained for lipids with Oil Red O. The hepatic damage was assessed by observing the extent of hepatocellular necrosis, hydropic vacuolation and degree of sinusoidal cell proliferation. The amount of fatty change was graded on the scale described by Hall and Drill(7), in which the sign of + was given to sections containing isolated large fatty droplets in every lobule; the sign of ++++ represented sections of liver that contained almost no fat-free cells. Representative pictures are shown in Fig. 1-3.

Results. The effects of CCl_4 on the liver are well known. For our purposes here attention is directed mainly to the level of lipids in the liver, since "Ripason" is considered to have a lipotropic effect.

In this connection the relation of the amount of liver fat extracted to its histological assessment is of particular interest. It is apparent from Table I that some degree of correlation exists between the chemical values and the histological finding in that an increased level of liver fat could frequently also be demonstrated histologically. This agrees to some extent with the findings of Billing, Conlon, Hein and Schiff(11). However, there is some degree of overlap between these



Representative examples of varying degrees of fatty change in the liver of rats injected with carbon tetrachhloride (Formalin fixed, frozen sections, Oil Red O).

FIG. 1. Grade ++.
FIG. 2. Grade +++.
FIG. 3. Grade ++++.

values, which becomes more pronounced with the increasing severity of the fatty change. The grade of "+++" for instance, was allocated to livers with a chemically determined fat content ranging from 8.9% to 16.3%. It is, therefore, questionable whether the histological assessment of the fatty changes, unsupported by quantitative data, should be used for comparative studies of the type reported by Tanyol and Friedman(1), unless very gross changes only are to be evaluated. In conjunction with chemical determinations, however, histological studies can be of great help, since the approximate parallelism found not only serves as corroborating evidence, but can also direct attention to occasional discrepancies due to technical errors. That histological studies are a condition *sine qua non* for localization of the fatty change is not relevant for our purposes here.

The results of Exp. I-III are presented in Table II. Treatment with "Ripason" had no significant effect on the level of lipids in the liver. The liver extract could neither prevent the fatty change following treatment with CCl_4 (Exp. I and II) nor could it accelerate its rate of disappearance during a period of rest (Exp. III). The outcome of Exp. II could even be interpreted to the effect that treatment with "Ripason" might raise the level of lipids in the liver. This failure of "Ripason" to influence the course of events in the manner claimed by Tanyol and Friedman (1) and by Hartmann(2) was not affected by differences in age or sex (compare Exp. I and II) of experimental animals. While this conclusion is mainly based on the quantitative and histological evaluation of the level of liver lipids, it was confirmed by a study of the degree of liver cell damage as seen in sections stained with hematoxylin and eosin, when the degree of "ballooning" and vacuolation of the liver cells, and of proliferation of the sinusoidal cells, was taken into account.

Discussion. The results presented here are at variance with those reported by Forbes *et al.*(5,6), Tanyol and Friedman(1), and Hartmann(2). The experiments of the older workers are not strictly comparable to the present series, since differences in the liver preparations used may account for the difference in results. This explanation, however, does not account for the failure to confirm the more recent observations of Tanyol and Friedman(1) and of Hartmann(2) who used the same type of liver preparation as was injected in the present series. The fact that Tanyol and Friedman(1) used cats, whereas rats were the experimental animals used here,

TABLE II. Mean Level (S.D.) of Lipids (g per 100 g Wet Liver Weight) Extracted from the Liver of Rats Treated with Carbon Tetrachloride (CCl_4) with and without "Ripason."

		CCl_4 only		CCl_4 plus "Ripason"		
No. of exp.	No. and sex of animal pairs	% liver lipids	No. of rats with grade 1+ to 4+	% liver lipids	No. of rats with grade 1+ to 4+	"P"
I (all pairs)	6♀ 3♂	11.1± 3.9	2+=5 3+=2 4+=2	12.3± 3.0	2+ =2 3+ =5 4+ =2	N.S.
I (female only)	6♀	12.1± 4.2	2+=2 3+=2 4+=2	13.0± 3.4	2+ =1 3+ =3 4+ =2	N.S.
II	7♂	6.9± .9	1+=3 2+=3 3+=1	8.1± .7	1+ =0 2+ =6 3+ =1	P<0.05
III	9♂	10.5± 3.6	1+=0 2+=7 3+=2	9.2± 2.2	1+ =1 2+ =5 3+ =3	N.S.

N.S. = Not significant.

is also of questionable significance. At best it could be said that the liver extract acted differently in cats than in rats. However, Hartmann(2), whose results and conclusions also differ from those reported here despite our adherence to his experimental design, used rats as the experimental animals. This makes it unlikely that the discrepancies observed will be determined by species differences, and further experimental work may be required to answer the question. It should, however, be pointed out that the combination of histological assessment and quantitative determination of degree of fatty change used here would appear to be less subject to error, and more suitable for comparative purposes, than the cytological examination devised and used by Tanyol and Friedman(1), which relies on a fat droplet count only (no mention is made of size of the droplets).

Finally, it may be pertinent to underline again the fact that littermate pairs were used in this series, in contrast to the other experiments, the outcome of which led to the claims of a lipotropic effect of "Ripason". This arrangement, used previously in a somewhat similar reinvestigation of the alleged protective effect of Vit. B_{12} on hepatotoxic action of CCl_4(12), appears to be particularly well suited for comparative studies of the type reported here, and deserves to be more widely used than it is at present.

Summary. The claim that a protein-free, water-soluble extract of the liver of cattle exerts a lipotropic and protective effect on the liver of rats has been reinvestigated. The degree of fatty change following injection of CCl_4 was assessed by quantitative estimations and by histological grading, and these criteria were compared with each other. Although an approximate parallelism was obtained, there was some degree of overlap, which led to the suggestion that both methods should be used in conjunction with each other. No effect of the liver extract in question could be demonstrated. The discrepancy between these findings and those of other workers suggests a need for further investigations.

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