Changes in Lysosomal Enzymes in Acute Experimental Liver Injury

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1. An investigation has been made of the changes occurring in lysosomal enzyme activities during the early development of experimentally produced liver injury in the rat. Three enzymes have been studied: acid phosphatase, acid ribonuclease and β -glucuronidase. Four different methods of inducing liver injury have been used: administration of carbon tetrachloride, thioacetamide, dimethylnitrosamine and the fungal toxin sporidesmin. 2. The majority of the data presented concern alterations produced by carbon tetrachloride. Despite the extensive central necrosis and accompanying fat accumulation which this poison produced in the liver, only small changes in the activity and latency of lysosomal enzymes could be detected. In the early (pre-necrotic) period of injury these changes were insignificant. At a late stage of injury, when extensive centrilobular necrosis was present, there were indications of lysosomal rupture. 3. The results obtained with the other three hepatotoxins were similar to those described for carbon tetrachloride in that no evidence of early lysosomal rupture was obtained during the pre-necrotic period. It is concluded that lysosomes probably play no role in the early development of the four types of liver injury studied but, instead, are involved in later scavenging processes.

The existence in rat liver of a class of intracellular particles (the lysosomes) containing a variety of acid hydrolases, first recognized in 1955 by de Duve and his colleagues (see de Duve, 1959), drew attention to the possibility that the segregation of such hydrolytic enzymes might be a mechanism to control and restrict autolysis. Early work on lysosomal function (for references see de Duve, 1959, 1961) favoured the view that damage to the lysosomal membrane *in vivo* led to the liberation of the degradative enzymes with subsequent cell destruction. There is much evidence (de Duve & Beaufay, 1959; Beaufay, Van Camphenout & de Duve, 1959) that lysosomal integrity is modified during liver injury in the rat, although only the later stages of this process have been examined. Experimental ligation of the vasculature supplying one lobe of the liver, with consequent liver anoxia, for instance, resulted in a large increase in the proportion of total acid-hydrolase activity present appearing in the free form in the soluble fraction (de Duve & Beaufay, 1959). These previous studies were, however, concerned with periods at which much irreversible damage to the tissue had already occurred; no attempt was made to establish whether lysosomal rupture was part of the early stages of tissue injury or whether it was secondary to this damage, i.e. involved as a type of scavenging mechanism.

We have attempted to answer two questions: (i) are lysosomal changes an important part of the earliest biochemical perturbations occurring in experimentally induced acute liver injury?; (ii) are they of a relatively late occurrence in these forms of cellular injury so that they might be regarded as part of the secondary consequences of an initial lesion rather than as part of the lesion itself?

Evidence is presented on the extent of lysosomal changes in the early stages of experimentally produced acute liver injury. The hepatotoxins used have been carbon tetrachloride, thioacetamide, dimethylnitrosamine and the fungal toxin, sporidesmin. In each case the time-course of the developing injury at the dose used has been well established by previous work. Thus, for each toxin, lysosomal changes were studied over a period immediately before the onset of necrosis. If lysosomal particles play a part in the establishment of necrosis, rather than in the autolysis which follows, it is in this pre-necrotic period that lysosomal changes might be expected.

Another approach has been made possible by the observations of Gallagher, Gupta, Judah & Rees (1956) and of Rees, Sinha & Spector (1961) that the necrosis which normally develops after administration of carbon tetrachloride or thioacetamide

can be prevented by concomitant treatment with promethazine (Phenergan; May and Baker Ltd., Dagenham), although this treatment has little or no effect on other aspects of poisoning with carbon tetrachloride, e.g. the development of a fatty liver. Experiments involving protection by Phenergan have therefore been included.

The results show that no lysosomal damage can be detected in the earliest stages of liver injury induced by any of the hepatotoxins used. A preliminary account of this work has been published (Slater, Greenbaum & Wang, 1963).

METHODS

Animals. The rats used were adult female albinos of body weight approx. 160g. They were maintained on stock diet with free access to food and water.

Administration of hepatotoxins. (a) Carbon tetrachloride was given by stomach tube, under light ether anaesthesia, as a 1:3 (v/v) mixture in liquid paraffin (dose 1.25 ml. of carbon tetrachloride/kg. body wt.). Control animals received an appropriate volume of pure liquid paraffin. (b) Sporidesmin was administered by intraperitoneal injection of 6mg./kg. body wt. in a suspension (made by dissolving 5 mg. in 0.3 ml. of ethanol and then adding 4.7 ml. of 0.9% NaCl). (c) Thioacetamide was injected intraperitoneally as an aq. 2% solution (dose 200 mg./kg. body wt.). (d) Dimethylnitrosamine was injected intraperitoneally as an aq. 2% solution (dose 100 mg./kg. body wt.). (e) Phenergan was injected intraperitoneally at the same time as the toxin (dose 25 mg./kg. body wt.). Where the animal was kept for more than 6 hr. after administration of the toxin, a second dose of Phenergan (12.5 mg./kg. body wt.) was given 5 hr. after the first.

Collection of serum. Blood was obtained from the carotid artery from rats under ether anaesthesia. In a few instances the animals were killed by cervical dislocation and the blood was quickly taken by cardiac puncture.

Preparation of tissue suspensions. The rats were killed by cervical dislocation and the livers were rapidly removed and weighed on a torsion balance. Each liver was homogenized in ice-cold 0.25M-sucrose by three up and down motions in a Potter-Elvehjem homogenizer with a plastic pestle having a diameter of 0.8 in. and a clearance of 0.015 in.

The volume was made up with ice-cold 0.25M-sucrose to give a final tissue concentration of 1:10. In most instances portions of the homogenate were centrifuged at 100000g for 30 min. and the supernatant so obtained was used to assay the unsedimentable activity of the enzymes.

Histology. Samples were taken from each liver, fixed in formol saline and stained with haematoxylin and eosin. The conclusions agreed with the previously reported pathology and acted as a check on the extent and degree of liver injury present.

Enzyme assays. The 'free activity' of a lysosomal enzyme is that activity measured under conditions known to favour lysosomal integrity, i.e. the incubation media were maintained at 0.25M in respect to sucrose and the pH was 5.0 or greater. The 'total activity' was that obtained after freezing and thawing (β -glucuronidase) or by the addition of a non-ionic detergent Triton X-100 (acid ribonuclease, acid phosphatase). The difference in activity between the 'total activity' and the 'free activity' is the 'bound activity' (see de Duve, 1959).

Acid ribonuclease was assayed in liver suspensions by the method described by Slater (1961), the detergent Triton X-100 being used to burst the lysosomal particles (Wattiaux & de Duve, 1956). Incubation was for 10 min. at 37°; after the reaction had been stopped with 2 ml. of uranyl acetate reagent (MacFadyen, 1934) the suspension was filtered and diluted to 20 ml. and the extinction at 260 m μ measured. Acid-ribonuclease activity in serum was measured by the same procedure but with 0.1 ml. of serum in place of tissue suspension.

β -Glucuronidase activity was assayed in liver suspensions by the method of Gianetto & de Duve (1955), phenolphthalein glucuronide being used as substrate with 20 min. incubation at 37°. Serum β -glucuronidase activity was measured in a similar fashion but 0.1 ml. of serum was used and the incubation period at 37° was 1 hr.

Acid-phosphatase activity was assayed in liver suspensions by the method of Berthet & de Duve (1951), and in serum by the method of King (1951).

DNA and protein were measured in liver suspensions by the methods of Burton (1956) and of Lowry, Rosebrough, Farr & Randall (1951) respectively.

DNA values are expressed in terms of phosphorus content (DNA P); a calibration curve was constructed with a DNA sample purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., P content 8.3%. Protein values were calculated from a calibration curve

Table 1. *Effects of administration of carbon tetrachloride on liver weight and composition in normal rats and rats treated with Phenergan*

Results are given as mean values $\pm$ S.E.M.; numbers of rats used are given in parentheses.

Status	Liver wt. (g.)		Liver wt. (g./100 g. body wt.)		DNA P content (mg./100 g. of liver)		Protein content (g./100 g. of liver)	
	Normal	P*	Normal	P*	Normal	P*	Normal	P*
Control	5.8 $\pm$ 0.4 (6)	—	3.8 $\pm$ 0.3 (6)	—	10.9 $\pm$ 1.4 (5)	—	—	—
3 hr.	6.1 $\pm$ 0.4 (10)	6.2 $\pm$ 0.4 (8)	3.7 $\pm$ 0.2 (10)	3.6 $\pm$ 0.2 (8)	11.1 $\pm$ 1.6 (4)	11.9 $\pm$ 0.8 (4)	18.3 $\pm$ 1.8 (4)	20.5 $\pm$ 1.0 (4)
5 hr.	5.6 $\pm$ 0.3 (8)	5.3 $\pm$ 0.2 (6)	3.5 $\pm$ 0.4 (8)	2.9 $\pm$ 0.1 (6)	11.5 $\pm$ 1.3 (6)	9.0 $\pm$ 1.0 (4)	14.6 $\pm$ 0.6 (6)	17.4 $\pm$ 1.2 (4)
14 hr.	5.4 $\pm$ 0.2 (6)	6.3 $\pm$ 0.2 (6)	3.2 $\pm$ 0.1 (6)	3.6 $\pm$ 0.03 (6)	13.4 $\pm$ 2.4 (3)	11.6 $\pm$ 1.1 (3)	21.8 $\pm$ 0.4 (3)	20.1 $\pm$ 0.8 (3)
24 hr.	6.2 $\pm$ 0.3 (6)	6.5 $\pm$ 0.6 (6)	3.8 $\pm$ 0.2 (6)	3.6 $\pm$ 0.02 (6)	—	10.4 $\pm$ 1.2 (4)	—	16.9 $\pm$ 0.8 (4)

* Rats treated with 2.5 mg. of Phenergan/100 g. body wt. (intraperitoneally) at the same time as administration of carbon tetrachloride. In the groups treated with carbon tetrachloride for 14 and 24 hr., a second dose of 1.25 mg. of Phenergan/100 g. body wt. was given 5 hr. after the first.

Table 2. *Effect of administration of carbon tetrachloride to normal and Phenergan-treated rats on the activity of liver β -glucuronidase*Results are given as mean values $\pm$ S.E.M. ($10^{-3} \times \mu$ g. of phenolphthalein liberated/hr.).

Status	No. of animals		Total activity/g. of liver		Total activity/liver/100g. body wt.		Total free activity/liver/100g. body wt.		Bound activity Free activity	
	Normal	P*	Normal	P*	Normal	P*	Normal	P*	Normal	P*
Control	5	—	6.85 $\pm$ 0.67	—	24.01 $\pm$ 2.68	—	13.46 $\pm$ 1.32	—	0.72 $\pm$ 0.06	—
3hr.	6	6	6.25 $\pm$ 0.60	6.78 $\pm$ 0.89	21.66 $\pm$ 2.76	23.76 $\pm$ 3.50	12.31 $\pm$ 1.43	15.04 $\pm$ 2.27	0.77 $\pm$ 0.06	0.61 $\pm$ 0.06
5hr.	6	4	5.78 $\pm$ 0.71	6.96 $\pm$ 0.61	19.96 $\pm$ 2.33	19.30 $\pm$ 1.94	11.91 $\pm$ 1.67	10.42 $\pm$ 1.08	0.78 $\pm$ 0.07	0.86 $\pm$ 0.09
14hr.	6	6	7.01 $\pm$ 0.97	7.24 $\pm$ 0.14	23.15 $\pm$ 3.44	25.69 $\pm$ 1.48	15.10 $\pm$ 2.29	15.39 $\pm$ 1.25	0.55 $\pm$ 0.06	0.70 $\pm$ 0.09
24hr.	4	6	6.90 $\pm$ 0.37	5.77 $\pm$ 0.43	25.12 $\pm$ 1.16	20.61 $\pm$ 1.46	13.11 $\pm$ 0.44	11.30 $\pm$ 0.87	0.91 $\pm$ 0.07	0.84 $\pm$ 0.07

* Rats treated with Phenergan at the same time as with carbon tetrachloride, as described in the Methods section.

based on ox-plasma albumin fraction V (Armour Pharmaceutical Co. Ltd.).

In Tables 1-3, Student's *t* test was used to evaluate the differences between the control group and the treated groups in each case (*, $0.1 > P > 0.05$; **, $0.05 > P > 0.001$; ***, $P < 0.001$; where no *P* value is indicated, $P > 0.10$ for the pair of means concerned).

RESULTS

Carbon tetrachloride

Liver. The effect of administration of carbon tetrachloride (with and without Phenergan) on the liver weight, and on the liver contents of protein and DNA, are shown in Table 1. During the period 0-5 hr. no significant change in the values of any of these quantities occurred.

Liver enzymes. Table 2 shows the results obtained for β -glucuronidase activity in the liver after administration of carbon tetrachloride. No significant alterations in the total β -glucuronidase activity, expressed either as activity/g. or as total liver activity/100g. body wt., or in the ratio bound activity/free activity, were found.

The much smaller ratios of bound activity/free activity found for β -glucuronidase than, for instance, acid ribonuclease (Table 4) are not due in the main to an increased disruption of the lysosomal particles during the incubation procedures for β -glucuronidase. This point was checked by incubating liver suspension with mixed substrates (i.e. phenolphthalein glucuronide, RNA and β -glycerophosphate), and incubating at pH 5.0. Samples were removed at 10 and 30 min. and the respective procedures for β -glucuronidase, ribonuclease and acid phosphatase carried through to measure the substrate breakdown that had occurred. Although, under these conditions, the bound activity/free activity ratio for acid ribonuclease (2.5) was lower than that usually found after incubation at pH 5-8, this value and that for acid phosphatase (3.34) were still much higher than that found for β -glucuronidase (0.83). Thus a large part of this enzyme is present *in situ*

in a free form, as demonstrated by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).

Table 3 gives the results for acid phosphatase. Administration of carbon tetrachloride produces a decrease in acid phosphatase, expressed either as activity/g. of tissue or as total liver activity/100g. body wt., over the 24 hr. period studied (Table 3, columns 3 and 4). Over the first 5 hr., however, the fall in activity is not significant. Corresponding to this decrease in total activity there is an increase in the free activity of the whole liver expressed per 100g. body wt. (column 6), which results in a considerable fall in the ratio bound activity/free activity (column 8). Once again, this change is not significant over the early time-intervals. Phenergan appears to exert a small effect in reducing the magnitude of these changes.

Changes in acid ribonuclease are shown in Table 4. Carbon tetrachloride produces a net increase in this enzyme (units/whole liver/100g. body wt.) over the 24 hr. period (Table 4, column 3), and this increase is completely prevented by Phenergan. Although there is a rise in the total free activity (column 4) this is not sufficient to produce a significant alteration in the bound activity/free activity ratio.

Results in Tables 2-4 indicate that there are only minor changes in lysosomal enzyme activities (and bound activity/free activity ratios) during the early stages of carbon tetrachloride poisoning. Identical conclusions were reached when the dose of carbon tetrachloride was trebled, and when the rats were treated with adrenocorticotrophin hormone at the same time as with carbon tetrachloride (1 unit of the hormone was injected intraperitoneally 18 hr. before the animals were killed).

Serum enzymes. Table 5 gives the results obtained for the effects of carbon tetrachloride (with and without simultaneous treatment with Phenergan) on the serum activities of β -glucuronidase, acid phosphatase and acid ribonuclease. The activity of β -glucuronidase rises sharply after treatment and

Table 3. *Effect of administration of carbon tetrachloride to normal and Phenengan-treated rats on the activity of liver acid phosphatase*

Results are given as mean values $\pm$ s.e.m. ($10^{-3} \times \mu\text{g. of P liberated/hr.}$).

Status	No. of animals		Total activity/g. of liver		Total activity/liver/100 g. body wt.		Total free activity/liver/100 g. body wt.		Bound activity/Free activity	
	Normal	P†	Normal	P†	Normal	P†	Normal	P†	Normal	P†
Control	5	—	5.95 $\pm$ 0.27	—	21.60 $\pm$ 1.54	—	5.57 $\pm$ 0.65	—	2.9 $\pm$ 0.4	—
3 hr.	6	6	5.78 $\pm$ 0.44	6.31 $\pm$ 0.76	20.28 $\pm$ 2.33	22.77 $\pm$ 1.09	6.30 $\pm$ 1.36	7.05 $\pm$ 1.27	2.5 $\pm$ 0.3	2.4 $\pm$ 0.3
5 hr.	8	6	5.53 $\pm$ 0.40	5.42 $\pm$ 0.66	19.72 $\pm$ 2.53	15.81 $\pm$ 2.73	6.18 $\pm$ 0.99	4.58 $\pm$ 1.53	2.4 $\pm$ 0.2	2.7 $\pm$ 0.4
14 hr.	6	6	4.80 $\pm$ 0.51	5.63 $\pm$ 0.61	15.39 $\pm$ 1.53**	19.86 $\pm$ 1.88	5.42 $\pm$ 1.24	6.85 $\pm$ 0.55	2.3 $\pm$ 0.2	2.0 $\pm$ 0.2
24 hr.	4	6	3.93 $\pm$ 0.20***	4.31 $\pm$ 0.22***	14.33 $\pm$ 0.85**	15.46 $\pm$ 0.92	7.39 $\pm$ 1.00	6.33 $\pm$ 0.36	1.1 $\pm$ 0.09**	1.6 $\pm$ 0.1**

* , 0.10 > P > 0.05; ** , 0.05 > P > 0.001; *** , P < 0.001.
† Rats treated with Phenengan at the same time as with carbon tetrachloride.

Table 4. *Effect of administration of carbon tetrachloride to normal and Phenengan-treated rats on the activity of liver acid ribonuclease*

Results are given as mean values $\pm$ s.e.m. (unit: change in $E_{260\text{m}\mu}$ /hr. under the standard conditions described).

Status	No. of animals		Total activity/g. of liver		Total activity/liver/100 g. body wt.		Total free activity/liver/100 g. body wt.		Bound activity/Free activity	
	Normal	P†	Normal	P†	Normal	P†	Normal	P†	Normal	P†
Control	6	—	62.4 $\pm$ 1.8	—	247.2 $\pm$ 31.8	—	39.6 $\pm$ 9.0	—	5.40 $\pm$ 0.79	—
3 hr.	10	8	65.4 $\pm$ 4.2	57.6 $\pm$ 1.2	251.4 $\pm$ 31.8	216.0 $\pm$ 23.4	48.6 $\pm$ 12.8	30.0 $\pm$ 4.2	5.28 $\pm$ 0.48	5.94 $\pm$ 0.27
5 hr.	8	6	52.8 $\pm$ 3.6	52.8 $\pm$ 1.2	187.8 $\pm$ 20.4	152.6 $\pm$ 21.6	31.2 $\pm$ 5.4	24.0 $\pm$ 3.6	5.46 $\pm$ 0.69	6.10 $\pm$ 1.12
14 hr.	3	3	75.0 $\pm$ 5.4	49.8 $\pm$ 9.0	225.0 $\pm$ 9.6	172.2 $\pm$ 34.2	33.0 $\pm$ 6.0	—	6.20 $\pm$ 1.22	—
24 hr.	6	6	93.6 $\pm$ 16.2**	57.0 $\pm$ 9.0	356.4 $\pm$ 50.4	204.6 $\pm$ 33.0	62.4 $\pm$ 6.6	37.8 $\pm$ 7.2	4.80 $\pm$ 0.67	4.55 $\pm$ 0.45

** , 0.05 > P > 0.001.
† Rats treated with Phenengan at the same time as with carbon tetrachloride.

by 3 hr. the activity/ml. of serum has increased some 20-fold. After this, the activity rapidly declines and is back to normal by 24 hr. Phenergan appears to delay as well as reduce the appearance of the enzyme in the blood so that the peak activity is attained only after 5 hr., after which it declined, but was still elevated over control activity at 24 hr.

Acid-phosphatase activity also increases after administration of carbon tetrachloride although the rise is slower and more sustained than with β -glucuronidase. Again Phenergan showed a delaying effect but this was not so marked as with β -glucuronidase. Acid-ribonuclease activity in serum increased sharply after administration of carbon tetrachloride and this rise was not modified by Phenergan.

A surprising finding in the course of these experiments is illustrated in Table 5. In general, carbon tetrachloride and Phenergan were administered under ether anaesthesia but in some instances

no anaesthesia was used. Although this variation of the routine procedure had no effect on the development of activity of the serum enzymes after carbon tetrachloride alone, omission of the ether anaesthesia greatly increased the protection afforded by Phenergan. The β -glucuronidase content of serum, for instance, 3 hr. after injection of carbon tetrachloride was 690 units whether the injection was made under ether anaesthesia or not. If carbon tetrachloride and Phenergan are administered together under ether anaesthesia then the protective action of the drug reduced the serum β -glucuronidase activity to 450 units. If carbon tetrachloride and Phenergan were administered without prior treatment with ether the enzyme activity in the serum was only 95 units. This ether effect diminished as the time between dosing with Phenergan and killing the animal increased. Acid ribonuclease, the one enzyme unaffected by Phenergan treatment, did not display this ether effect.

Table 5. Serum enzyme activities after administration of carbon tetrachloride to rats

Results are given as mean values $\pm$ s.e.m. (units: β -glucuronidase, μ g. of phenolphthalein liberated/hr./ml. of serum; acid phosphatase, μ g. of phosphorus liberated/hr./ml. of serum; acid ribonuclease, change in $E_{260m\mu}$ /hr./ml. of serum) with the numbers of animals given in parentheses.

Time after dosing (hr.)	β -Glucuronidase		Acid phosphatase		Acid ribonuclease	
	Normal	P†	Normal	P†	Normal	P†
0	34 $\pm$ 7 (5)	—	54 $\pm$ 2 (4)	—	13.0 $\pm$ 1.5 (7)	—
3	690 $\pm$ 140 (10)**	450 $\pm$ 130 (14)* 95 $\pm$ 19 (6)†***	90 $\pm$ 6 (10)***	75 $\pm$ 6 (14) 57 $\pm$ 4 (6)†	26.4 $\pm$ 3.0 (10)**	26.5 $\pm$ 3.2 (14)** 24.2 $\pm$ 3.3 (6)†***
5	290 $\pm$ 43 (8)***	550 $\pm$ 40 (9)** 375 $\pm$ 95 (3)†	101 $\pm$ 11 (8)**	98 $\pm$ 9 (9)** 82 $\pm$ 7 (3)†***	15.9 $\pm$ 3.4 (8)	29.4 $\pm$ 3.2 (9)** 29.4 $\pm$ 0.1 (3)†***
14	215 $\pm$ 53 (6)**	72 $\pm$ 9 (6)**	160 $\pm$ 11 (6)***	123 $\pm$ 5 (6)***	19.4 $\pm$ 5.1 (6)	19.4 $\pm$ 3.2 (6)
24	62 $\pm$ 22 (6)	175 $\pm$ 26 (6)**	118 $\pm$ 12 (6)**	158 $\pm$ 11 (6)***	22.2 $\pm$ 4.3 (6)*	17.2 $\pm$ 4.8 (6)

*, 0.1 > P > 0.05; **, 0.05 > P > 0.001; ***, P < 0.001.

† Rats treated with Phenergan at the same time as with carbon tetrachloride.

‡ No ether anaesthesia used.

Table 6. Effect of administration of different hepatotoxins on the activity of β -glucuronidase of rat liver

Results are given as mean values $\pm$ s.e.m. ($10^{-3} \times \mu$ g. of phenolphthalein liberated/hr.).

Treatment	Period of treatment (hr.)	No. of animals	Total activity/g. of liver	Total activity/liver/100 g. body wt.	Total free activity/liver/100 g. body wt.	Bound activity Free activity
Control	—	5	6.85 $\pm$ 0.67	24.91 $\pm$ 2.68	13.46 $\pm$ 1.32	0.72 $\pm$ 0.06
Thioacetamide	4	2	9.73	38.92	20.43	0.91
	18	4	6.58	25.30	10.57	1.40
	42	2	5.77	20.20	9.33	1.18
Dimethylnitrosamine	3	2	5.63	29.78	15.75	0.87
	24	2	6.14	28.31	18.57	0.52
Sporidesmin	48	2	7.67	30.09	15.36	0.92

Table 7. *Effect of administration of different hepatotoxins on the activity of acid phosphatase of rat liver*

Results are given as mean values $\pm$ s.e.m. ($10^{-3} \times \mu\text{g. of P liberated/hr.}$).

Treatment	Period of treatment (hr.)	No. of animals	Total activity/g. of liver	Total activity/liver/100g. body wt.	Total free activity/liver/100g. body wt.	Bound activity Free activity
Control	—	5	5.95 $\pm$ 0.27	21.60 $\pm$ 1.54	5.57 $\pm$ 0.65	2.9 $\pm$ 0.4
Thioacetamide	4	2	5.64	22.64	6.69	2.35
	18	4	5.90	20.37	8.08	1.58
	42	2	3.35	11.75	4.72	1.49
Dimethylnitrosamine	3	2	7.89	42.12	10.67	3.12
	24	2	6.53	30.00	5.93	4.08
Sporidesmin	48	2	6.77	24.89	3.72	7.0

Table 8. *Effect of administration of different hepatotoxins on the activity of acid ribonuclease of rat liver*

Results are given as mean values $\pm$ s.e.m. (unit: change in $E_{280\text{m}\mu}/\text{hr.}$ under the standard conditions described).

Treatment	Period of treatment (hr.)	No. of animals	Total activity/g. of liver	Total activity/liver/100g. body wt.	Total free activity/liver/100g. body wt.	Bound activity Free activity
Control	—	6	62.4 $\pm$ 1.8	247.2 $\pm$ 31.8	39.6 $\pm$ 9.0	5.40 $\pm$ 0.79
Thioacetamide	4	5	67.2 $\pm$ 13.8	279.6 $\pm$ 27.2	41.4 $\pm$ 6.0	6.06 $\pm$ 1.02
	18	8	81.0 $\pm$ 8.4	318.6 $\pm$ 38.4	52.8 $\pm$ 9.0	6.10 $\pm$ 1.08
	42	2	114.6	405.0	46.4	8.60
Dimethylnitrosamine	3	5	82.2 $\pm$ 9.6	396.0 $\pm$ 53.4	77.4 $\pm$ 6.6	4.34 $\pm$ 0.32
	24	6	45.6 $\pm$ 6.6	204.0 $\pm$ 30.6	51.0 $\pm$ 10.2	3.10 $\pm$ 0.45
Sporidesmin	48	4	67.8 $\pm$ 8.9	303.0 $\pm$ 40.1	63.0 $\pm$ 11.1	4.10 $\pm$ 0.92

Thioacetamide, dimethylnitrosamine and sporidesmin

Liver enzymes. The effects of these hepatotoxins on the activity of liver β -glucuronidase at various times after treatment are shown in Table 6. Thioacetamide, at a dose which causes extensive centrilobular necrosis in 12–24 hr., appears to cause a transient increase in total β -glucuronidase/whole liver/100g. body wt. at 4 hr., after which there is a progressive decrease in activity to below the control value. However, there seems to be no evidence for an increased fragility or disruption of the lysosomal particles during this period as the bound activity/free activity ratio was, if anything, higher than that of the control. With both dimethylnitrosamine and sporidesmin, likewise, there seems no evidence from the given data that lysosomal β -glucuronidase is liberated during the phase of the injury preceding the appearance of necrosis.

Tables 7 and 8 show the changes in acid phosphatase and ribonuclease in the livers of rats treated with the three hepatotoxins. The changes in the total content of acid phosphatase as well as changes in the ratio bound activity/free activity appear to differ in extent and in time-sequence for different hepatotoxins. Similar variations can be seen for the effects of the toxins on acid ribonuclease.

Fig. 1. Changes in the serum activities of (A) β -glucuronidase, (B) acid ribonuclease and (C) acid phosphatase with time after dosing rats with thioacetamide. Methods of assay and other details are given in the Methods section. Results are expressed as percentages of the respective normal rat serum activities (see Table 5).

Serum enzymes. Changes in the activities of acid phosphatase and β -glucuronidase in the blood serum after treatment of the animals with thioacetamide are shown in Fig. 1. The activities of β -glucuronidase and acid ribonuclease decrease

during the first few hours after poisoning with thioacetamide, which contrasts with the response to administration of carbon tetrachloride (Table 5) where, for example, β -glucuronidase activity increased some 20-fold. Although all three enzymes studied after administration of thioacetamide (Fig. 1) increase in activity during the later phases of liver injury, the increase found for β -glucuronidase was still very much smaller than was found with carbon tetrachloride-poisoned rats. Both acid phosphatase and acid ribonuclease, however, showed a twofold increase in activity similar to that found after carbon tetrachloride poisoning.

DISCUSSION

Serum changes. There are rapid and substantial increases in the activities of several lysosomal enzymes in the serum within a few hours of administration of carbon tetrachloride (Table 5). For example, β -glucuronidase increases 20-fold in activity in the first 3 hr. Since administration of carbon tetrachloride to rats is known to affect primarily the liver, and leakage of enzymes from this organ into the blood is well documented, it might be thought that such rapid and unequivocal changes are evidence of lysosomal rupture in the liver, with consequent leakage of these enzymes into the serum.

However, the hepatic origin of the serum enzymes in this investigation has proved difficult to establish. The increases in the serum activities for both β -glucuronidase and acid phosphatase represent but a very small proportion of the total liver activity. Thus it was not possible to demonstrate a decreased liver-enzyme content concomitant with an increased serum activity as has been shown in several other cases of hepatic injury (Rees & Sinha, 1960). With acid ribonuclease, the rise in serum activity is so large that it is unlikely to be hepatic in origin; further, unlike the early rises in acid phosphatase and β -glucuronidase, the rise in acid-ribonuclease activity in the serum was unaffected by prior dosing with Phenergan. The origin of the increased serum acid-ribonuclease activity is unknown; histologically, little evidence of pancreatic damage in carbon tetrachloride poisoning was found and no rise in serum amylase occurred during the injury.

It is important to remember that even if the serum increases in β -glucuronidase and acid phosphatase represent a leakage from a damaged liver, the leakage could be from a non-lysosomal site. Some β -glucuronidase activity is present in the endoplasmic reticulum (de Duve *et al.* 1955) which is known to be disrupted early in the course of carbon tetrachloride injury (Recknagel & Lombardi, 1961), and a soluble form of acid phos-

phatase with properties similar to, but not identical with, the lysosomal bound enzyme is present in rat liver (Shibko & Tappel, 1963). In fact, the increased serum acid phosphatase which appears in carbon tetrachloride poisoning appears to be non-lysosomal in origin since no serum activity was detectable when sodium β -glycerophosphate was used in place of sodium phenyl phosphate as substrate (T. F. Slater & A. L. Greenbaum, unpublished work). The 'soluble' acid phosphatase of rat liver, as with the acid phosphatase of milk (Bingham & Zittle, 1963), is known to be virtually unreactive towards β -glycerophosphate (Shibko & Tappel, 1963).

Thus although it is possible that the increases in serum acid-phosphatase and β -glucuronidase activities are a consequence of liver injury, the present data cannot be used to implicate lysosomal damage during the early period of carbon tetrachloride poisoning. In thioacetamide poisoning, the serum activities of these three acid hydrolases actually fell during the first few hours of the injury. We are thus led to conclude that the results obtained with serum provide no direct and unequivocal evidence for any substantial rupture of lysosomes in the two types of liver injury studied by these means.

Liver changes. There are numerous reports showing that carbon tetrachloride, thioacetamide and dimethylnitrosamine cause profound disturbances in the liver during the first 3 hr. after their administration to rats. Each poison eventually produces extensive central necrosis, which is maximal 18–24 hr. after the doses used in this study. Further, both carbon tetrachloride and dimethylnitrosamine also cause a fatty liver, the early signs of which are easily detectable 5–8 hr. after administration of the poison. Sporidesmin, on the other hand, causes essentially a much more slowly developing type of periportal injury (Slater, Sträuli & Sawyer, 1964).

From such considerations one can conclude that if lysosomal changes are an important early manifestation of the changes leading to central necrosis then such changes should be clearly detectable during the first 3 hr. after poisoning. If, on the other hand, lysosomal changes begin to show only 12–18 hr. after dosing then they may be considered to be secondary in nature rather than primary events in the sequence of steps leading to the establishment of necrosis.

The results of Tables 2–4 and 6–8 do not give any clear indication of lysosomal changes during the first 3 hr. after administration of carbon tetrachloride, thioacetamide or dimethylnitrosamine. There was no unequivocal increase either in the total enzyme activity/liver/100 g. body wt. or in the relative proportion of 'free' enzyme to that bound within the particles in a latent form.

Even at late stages of liver injury (18–24 hr.) there were no consistent increases in the total enzyme activities or in the bound activity/free activity ratios. That some lysosomal changes were found at this time is not surprising since during this period appreciable volumes of dead liver cells are undergoing autolysis or phagocytosis by histiocytes. Further, the picture at this time is even more complex than usual for superimposed on these destructive elements is active regeneration.

The failure to detect a general increase in total activities of the enzymes indicates that there is no appreciable contribution to the measured enzyme activities studied resulting from a macrophage invasion as occurs, for example, in muscular dystrophy (Zalkin *et al.* 1962). During this late period of injury, the changes found were very variable from case to case. For example, the bound activity/free activity ratio for acid ribonuclease is substantially increased after thioacetamide but is decreased after dimethylnitrosamine; the ratio for acid phosphatase is strongly decreased after thioacetamide but increased after dimethylnitrosamine; for β -glucuronidase the ratio increased slightly after thioacetamide but decreased after dimethylnitrosamine.

In view of these findings, the magnitude and the direction of change of this bound activity/free activity ratio appear to have little relevance to the degree of necrosis produced by these three hepatotoxins.

The relatively few experiments performed with rats poisoned with sporidesmin also fail to provide evidence of lysosomal rupture before the onset of necrosis. With sporidesmin the necrosis which develops is peripheral in nature, being around the bile ducts as in sheep poisoned with this fungal toxin (Mortimer & Taylor, 1962).

In fact, results of this investigation point to the conclusion that during the early phase of experimental liver injury, induced by four different toxic substances, there is little change in the overall level or degree of latency of the three lysosomal enzymes studied. Significant changes are detectable in these enzymes only at a late stage of the injury when necrosis is well established. Thus although many severe and extensive metabolic perturbations have been detected in the liver during the first few hours

after administration of carbon tetrachloride, dimethylnitrosamine or thioacetamide, lysosomal damage cannot be included in this group.

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