Liver Nucleotides in Acute Experimental Liver Injury induced by Dimethylnitrosamine and by Thioacetamide

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1. The concentrations of the nicotinamide-adenine dinucleotides in rat liver have been determined at intervals during the period 1–24 hr. after feeding adult female rats with dimethylnitrosamine or thioacetamide. 2. The administration of dimethylnitrosamine resulted in a rapid decrease in the sum of NAD + NADH₂. This sum was decreased by 40% 3 hr. after dosing. 3. Dimethylnitrosamine administration also produced an overall decrease in the NADP + NADPH₂ but this decrease was not so early nor as marked as that found for NAD + NADH₂. 4. The changes produced by thioacetamide were quite different from those obtained with dimethylnitrosamine. Thioacetamide produced a temporary rise in the NAD + NADH₂ followed by a small fall. The NADP + NADPH₂ was little changed in the early hours after dosing with thioacetamide but had decreased by approx. 15% 18 hr. after administration. 5. These changes are discussed in terms of the known hepatotoxic actions of dimethylnitrosamine and thioacetamide, and are compared with previously reported changes found after the administration of carbon tetrachloride.

It has previously been shown (Slater, Sträuli & Sawyer, 1964b) that there is a rapid decrease in NADP + NADPH₂ in the liver within 60 min. of dosing rats with carbon tetrachloride. This decrease was prevented by concomitant treatment with the antihistamine drug promethazine (Phenergan; May and Baker Ltd., Dagenham, Essex), which also inhibits the onset of necrosis (Rees, Sinha & Spector, 1961) while not affecting greatly the accumulation of fat in the liver. As a consequence it has been suggested (Slater *et al.* 1964b) that the rapid decrease in NADPH₂ in the liver after the administration of carbon tetrachloride is part of the early reactions that proceed to necrosis.

After a single dose of dimethylnitrosamine (Barnes & Magee, 1954) or thioacetamide (Ambrose, De Eds & Rather, 1949; Gupta, 1956) extensive centrilobular necrosis develops in the liver at approximately the same rate as observed after a single dose of carbon tetrachloride (Cameron & Karunaratne, 1936). Certain histological features, however, distinguish the appearances of the liver lesions: dimethylnitrosamine, for example, produces a much more haemorrhagic lesion than is usual after carbon tetrachloride, and, in contrast with the other two toxic agents, thioacetamide produces only a very small disturbance in the fat content of the liver. In view of the histological similarities and differences of the liver damage produced by the three toxic agents the effect of the administration of dimethylnitrosamine and

thioacetamide on the concentration of the nicotinamide-adenine dinucleotides in the liver was of interest. The present study was undertaken to answer two main questions: (1) are there very early changes (i.e. within the first 2 hr.) in the concentrations of the nicotinamide-adenine dinucleotides in rat liver after administering dimethylnitrosamine and thioacetamide similar to those previously reported to occur after the administration of carbon tetrachloride (Slater *et al.* 1964b)?; (2) is there any broad correlation between the changes found in the amounts of the nicotinamide-adenine dinucleotides in the liver and the onset of centrilobular necrosis induced by carbon tetrachloride, dimethylnitrosamine and thioacetamide?

METHODS

The rats used were adult albino females of the Wistar strain, body wt. approx. 120 g.; they were fed on M.R.C. diet 41B. Dimethylnitrosamine (2%, w/v, in water) was injected intraperitoneally with the rat under light ether anaesthesia, dose 10 mg. of dimethylnitrosamine/100 g. body wt. Thioacetamide (2%, w/v, in water) was similarly injected, dose 20 mg./100 g. body wt. Control rats received an equivalent volume of water.

At various times after dosing, rats were killed by cervical dislocation and pieces of liver immediately removed for assay of NAD, NADH₂, NADP and NADPH₂ by the method of Slater, Sawyer & Sträuli (1964a). The results are expressed in terms of $\mu\text{g. of nucleotide/whole liver/100 g. body wt.}$ (see Slater *et al.* 1964b); these values may be converted into $\mu\text{g./g. wet wt.}$ upon division by the corresponding liver wt./100 g. body wt. Student's *t* test was

Table 1. *Effect of dimethylnitrosamine on NAD and NADH₂ in rat liver*

Nucleotides are given as $\mu\text{g./whole liver/100 g. body wt.}$ Mean values $\pm$ s.e.m. are given. Mean values for NAD+NADH₂ in the groups treated with dimethylnitrosamine (DMN) were compared with the corresponding control by using Student's *t* test; values for *P* so obtained are given.

Time (hr.)	Treatment	No. of rats	Liver wt. Body wt. $\times 100$ (g.)	NAD+NADH ₂	<i>P</i>	$\frac{\text{NAD}}{\text{NADH}_2}$
1	Control	5	5.82 $\pm$ 0.45	2539 $\pm$ 159	0.05	3.5 $\pm$ 0.7
	DMN	7	5.23 $\pm$ 0.36	2102 $\pm$ 112		3.2 $\pm$ 0.2
2	Control	5	5.03 $\pm$ 0.08	2867 $\pm$ 166	< 0.02	3.2 $\pm$ 0.3
	DMN	5	5.72 $\pm$ 0.33	2016 $\pm$ 225		2.5 $\pm$ 0.1
3	Control	4	4.25 $\pm$ 0.24	1968 $\pm$ 70	< 0.05	3.1 $\pm$ 0.5
	DMN	4	4.50 $\pm$ 0.44	1186 $\pm$ 287		2.3 $\pm$ 0.3
5	Control	2	4.85	2288	0.001	3.8
	DMN	4	4.44 $\pm$ 0.18	526 $\pm$ 82		3.6 $\pm$ 1.5
18	Control	3	5.03 $\pm$ 0.37	2473 $\pm$ 320	0.001	3.0 $\pm$ 0.3
	DMN	4	4.35 $\pm$ 0.19	655 $\pm$ 41		2.6 $\pm$ 0.7

Table 2. *Effect of dimethylnitrosamine on NADP and NADPH₂ in rat liver*

Nucleotides are given as $\mu\text{g./whole liver/100 g. body wt.}$ Mean values are given $\pm$ s.e.m. Mean values for NADP+NADPH₂ in the groups treated with dimethylnitrosamine (DMN) were compared with the corresponding control group by using Student's *t* test; values for *P* so obtained are given (N.S., not significant). Other details are given in the Methods section.

Time (hr.)	Treatment	No. of rats	Liver wt. Body wt. $\times 100$ (g.)	NADP+ NADPH ₂	<i>P</i>	$\frac{\text{NADPH}_2}{\text{NADP}}$
1	Control	5	5.81 $\pm$ 0.45	1069 $\pm$ 69	0.10	7.3 $\pm$ 0.34
	DMN	7	5.28 $\pm$ 0.36	958 $\pm$ 18		7.3 $\pm$ 0.34
2	Control	5	4.88 $\pm$ 0.12	1127 $\pm$ 39	N.S.	8.4 $\pm$ 0.87
	DMN	5	5.20 $\pm$ 0.20	1164 $\pm$ 48		6.2 $\pm$ 0.67
3	Control	6	4.51 $\pm$ 0.22	897 $\pm$ 56	0.10	8.7 $\pm$ 0.88
	DMN	8	5.11 $\pm$ 0.40	747 $\pm$ 64		6.1 $\pm$ 0.38
5	Control	3	4.38 $\pm$ 0.52	905 $\pm$ 77	0.05	7.1 $\pm$ 0.26
	DMN	5	4.80 $\pm$ 0.39	652 $\pm$ 48		4.7 $\pm$ 0.42
18	Control	5	5.27 $\pm$ 0.57	1078 $\pm$ 60	< 0.001	8.3 $\pm$ 0.91
	DMN	4	4.19 $\pm$ 0.11	490 $\pm$ 54		3.6 $\pm$ 0.45
24	DMN	2	4.23	367		2.8

used to evaluate the significance of differences between the means of control groups (injected with water only) and the groups treated with dimethylnitrosamine or thioacetamide; the values for *P* so obtained are given in Tables 1-4. Other pieces of liver were fixed in formalin-saline, sectioned, stained with haematoxylin and eosin, and then examined microscopically to check the extent of damage.

RESULTS

Dimethylnitrosamine. Table 1 gives the results found for NAD and NADH₂; Table 2 gives the corresponding results for NADP and NADPH₂. It was not always possible to estimate both pairs of nucleotides on each animal and this accounts for the differences in liver wt./100g. body wt. given for corresponding times in Tables 1 and 2.

It can be seen (Table 1) that dimethylnitrosamine produces a rapid decrease in the NAD+NADH₂; this is depressed 17% at 1hr., 29% at 2hr., 40% at 3hr. and 73% at 18hr. after dosing. Despite the rapid loss of NAD and NADH₂, dimethylnitrosamine poisoning does not greatly affect the ratio NAD/NADH₂ (Table 1) although there is a tendency for the ratio to be depressed throughout; the decrease is significant in the 2hr. group.

Although (Table 2) dimethylnitrosamine eventually produces a substantial decrease in the NADP+NADPH₂ (50% at 18hr.), the overall effect is less rapid than found for NAD+NADH₂, and there is no sustained difference between the control and dimethylnitrosamine-treated groups until 3hr. after dosing. There is some indication of

Table 3. *Effect of the administration of thioacetamide on the NAD and NADH₂ content of rat liver*

Mean values are given $\pm$ S.E.M. Other details are given in the Methods section.

Time (hr.)	Treatment	No. of rats	Liver wt. Body wt. $\times 100$ (g.)	NAD+NADH ₂ (μ g./whole liver/ 100g. body wt.)	NAD NADH ₂
2	Control	6	5.08 $\pm$ 0.53	2093 $\pm$ 197	4.0 $\pm$ 0.34
	Thioacetamide	4	5.35 $\pm$ 0.33	2491 $\pm$ 399	3.8 $\pm$ 0.13
5	Control	3	4.77 $\pm$ 0.12	2376 $\pm$ 185	3.5 $\pm$ 0.27
	Thioacetamide	5	4.95 $\pm$ 0.10	2744 $\pm$ 147	3.7 $\pm$ 0.23
18	Control	2	6.57	2626	3.9
	Thioacetamide	2	6.09	2091	4.8

Table 4. *Effect of the administration of thioacetamide on the NADP and NADPH₂ content of rat liver*

Mean values are given $\pm$ S.E.M. Mean values for the thioacetamide groups were compared with the corresponding control group mean value by using Student's *t* test; values for *P* so obtained are given in column 5. Other details are given in the Methods section.

Time (hr.)	Treatment	No. of rats	Liver wt. Body wt. $\times 100$ (g.)	NADP+NADPH ₂ (μ g./whole liver/ 100g. body wt.)	<i>P</i>	NADPH ₂ NADP
1	Control	6	5.35 $\pm$ 0.36	905 $\pm$ 78	N.S.	7.6 $\pm$ 0.80
	Thioacetamide	5	5.01 $\pm$ 0.12	947 $\pm$ 63		7.3 $\pm$ 0.4
2	Control	5	4.72 $\pm$ 0.19	876 $\pm$ 40	0.01	7.2 $\pm$ 0.8
	Thioacetamide	6	4.50 $\pm$ 0.20	719 $\pm$ 29		6.1 $\pm$ 0.5
5	Control	4	4.59 $\pm$ 0.21	1001 $\pm$ 110	N.S.	8.0 $\pm$ 0.3
	Thioacetamide	8	4.50 $\pm$ 0.23	917 $\pm$ 67		7.4 $\pm$ 0.6
18	Control	3	5.98 $\pm$ 0.70	1052 $\pm$ 74	N.S.	9.1 $\pm$ 1.3
	Thioacetamide	5	5.40 $\pm$ 0.33	887 $\pm$ 41		4.6 $\pm$ 0.4

a transient decrease at 1 hr. followed by recovery, which is reminiscent of the similar though much more pronounced effect occurring with carbon tetrachloride (Slater *et al.* 1964b). In contrast with the values given in Table 1, it can be seen in Table 2 that dimethylnitrosamine produces a marked decrease in the ratio $\text{NADPH}_2/\text{NADP}$ during most of the period studied.

Histological studies of sections taken from poisoned rats were in agreement with many previously published reports and showed that extensive central necrosis and fatty degeneration were present in the 18 hr. and 24 hr. groups.

Thioacetamide. Table 3 gives the results found after the administration of thioacetamide. There is no decrease in the liver's content of $\text{NAD} + \text{NADH}_2$ during the first 5 hr. after poisoning. Indeed, the groups treated with thioacetamide show an early tendency towards an increased $\text{NAD} + \text{NADH}_2$ content, compared with their corresponding controls. Later, however, this tendency is reversed so that by 18 hr. the thioacetamide group mean is approx. 20% lower than its control group mean. With the $\text{NADP} + \text{NADPH}_2$ (Table 4) there is a tendency for the values to be depressed after thioacetamide administration, but this effect is not statistically significant, except in the 2 hr. group. Histological examination of stained sections prepared from rats dosed 18 hr. previously with thioacetamide confirmed that the liver was extensively damaged.

DISCUSSION

After the administration of a single dose of carbon tetrachloride to rats very early changes have been observed in the liver, particularly in components of the endoplasmic reticulum (Smuckler, Iseri & Benditt, 1962; Reynolds, 1963; Slater, 1966) and in the amount of $\text{NADP} + \text{NADPH}_2$ (Slater *et al.* 1964b). Both dimethylnitrosamine and thioacetamide likewise produce very early disturbances in the endoplasmic reticulum, as indicated, for example, by the rapid decreases in protein synthesis after administration of single doses of these toxic materials (Magee, 1958; Barker, Smuckler & Benditt, 1963). The interactions of dimethylnitrosamine and thioacetamide with the endoplasmic reticulum appear to involve conversion of each material into a more toxic derivative through the participation of the enzyme systems responsible for the metabolism of foreign compounds (see Magee, 1964; Clifford & Rees, 1966; Rees, Rowland & Varcoe, 1965). A similar 'activation' of carbon tetrachloride to a more toxic form has been suggested to occur in rat liver (Butler, 1961; Ghoshal & Recknagel, 1965; Slater, 1966).

Despite the apparent similarities between the early actions of dimethylnitrosamine, thioacetamide and carbon tetrachloride on the endoplasmic reticulum, the results given in Tables 1-3 (and previously for carbon tetrachloride; Slater *et al.* 1964b) clearly show that there is no common pattern of change in the concentrations of the nicotinamide-adenine dinucleotides in rat liver after dosing with these three hepatotoxic agents. With dimethylnitrosamine the decrease in the $\text{NAD} + \text{NADH}_2$ is rapid and extensive and the results for the later stages are in broad agreement with previous investigations, which, however, did not consider either the very early stage of injury or changes in $\text{NADP} + \text{NADPH}_2$ at all, features of particular interest to this study (Gallagher & Rees, 1960; Emmelot, Bos & Reyers, 1960).

As shown in Table 2, the changes found to occur in the $\text{NADP} + \text{NADPH}_2$ were much less marked than as mentioned above for $\text{NAD} + \text{NADH}_2$. In fact, there appears to be a lag phase of approx. 2 hr. between the commencement of the rapid fall in $\text{NAD} + \text{NADH}_2$ and the appearance of significant changes in $\text{NADP} + \text{NADPH}_2$. This lag phase is of considerable interest by itself and provides a further example of considerable changes occurring in the whole-liver concentration of NAD without necessarily greatly affecting the concentrations of NADP and NADPH_2 (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Slater & Sawyer, 1966).

The nucleotide changes found here after the administration of thioacetamide exhibit a third type of behaviour, different in time-pattern both from the previous findings with carbon tetrachloride and from those outlined above for dimethylnitrosamine. The results given in Table 3 show that there is no statistically significant decrease in the $\text{NADP} + \text{NADPH}_2$ during the first 18 hr. of poisoning except in the 2 hr. group. The $\text{NAD} + \text{NADH}_2$ actually shows a tendency to rise during the first few hours after dosing: in this same period dosing with dimethylnitrosamine decreased the $\text{NAD} + \text{NADH}_2$ by 49% (Table 1). The failure to observe any marked decrease in nucleotide content after treatment with thioacetamide is in broad agreement with the work of Gallagher & Rees (1960) based on measurements of $E_{260\text{ m}\mu}$ of acid extracts of damaged liver. Since many of the parenchymal cells are frankly necrotic by 18 hr. after dosing with thioacetamide, the very small decrease found in the total nucleotide content of whole-liver samples implies that the cells in regions of the lobule other than the central necrotic zone must have increased their rate of synthesis of both types of nucleotide and, moreover, the content per cell in these peripheral areas of the lobules must be increased. This reasoning is based on the assumption that necrotic cells in advanced stages

of deterioration (e.g. 18hr. after dosing with thioacetamide) have lost many of the intracellular concentration gradients that characterize the living state. This is illustrated by the increased activity of liver enzymes appearing in the serum as necrosis develops and a concomitant decrease in many soluble enzymes of the liver cell itself (Rees *et al.* 1961). If, however, the above assumption is invalid in the sense that necrotic cells are able to retain substantial amounts of nucleotides and yet allow enzymes to be released into plasma then ideas on the non-specificity of such leakage phenomena in necrosis will have to be revised. The results obtained with thioacetamide give an indication of the difficulties inherent in investigations where a hepatotoxin may affect different regions of the lobule in qualitatively as well as quantitatively different degrees. Consequently, estimations on samples of whole liver obtained from poisoned animals may not always reflect in a proportional manner the extent of damage localized in a particular zone of the liver lobule.

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