Changes in Liver Nucleotide Concentrations in Experimental Liver Injury

2. ACUTE ETHANOL POISONING

BY T. F. SLATER, BARBARA C. SAWYER AND URSULA D. STRÄULI

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(Received 19 December 1963)

A single administration of ethanol to rats increases the liver lipid content (Mallov & Bloch, 1956; Di Luzio, 1958); the increase is detectable as early as 5 hr. after dosing, but the maximum effect occurs some 12 hr. later. An explanation for this effect has been based on the alteration in the NAD:NADH₂ ratio (Lieber & Schmid, 1961). This ratio is decreased in the liver 3 hr. after the intraperitoneal injection of ethanol (Smith & Newman, 1959; see also Forsander, Riih & Suomalainen, 1958) and 16 hr. after the stomach-tube administration of a much higher dose (Rebouas & Isselbacher, 1961). On the basis of these studies it has been suggested that the elevated concentration of NADH₂ creates a favourable environment for increased lipid synthesis (Lieber & Schmid, 1961). However, although rather elaborate explanations of several of the metabolic effects of ethanol on the rat have been constructed from this hypothesis (see Lieber & Davidson, 1962), no systematic study of the changes in NAD and NADH₂ concentrations over the whole period of ethanol poisoning has been made and no consideration has been taken of possible changes in NADP and NADPH₂ concentrations, although it is known that cytoplasmic lipid synthesis is NADPH₂-linked (Wakil, 1961).

The provision of data on these two points was the major aim of the present paper, which is concerned with the effects of a single intraperitoneal injection of ethanol on the concentrations and relative proportions of the nicotinamide-adenine dinucleotides (NAD, NADH₂, NADP and NADPH₂) in rat liver.

METHODS

Female albino rats (body wt. approx. 130 g.) were used in this investigation. The ethanol-treated rats received 1.5 ml. of 20% (v/v) ethanol in 0.9% NaCl/100 g. body wt. by intraperitoneal injection while under light ether anaesthesia. The saline-treated controls received 1.2 ml. of 0.9% NaCl/100 g. body wt. by the same route. In a few cases ethanol was given by stomach tube as an aqueous 50% solution at a dose of 1.56 ml./100 g. body wt. (equiv-

alent to 0.6 g. of ethanol/100 g. body wt.). Animals were killed by cervical dislocation at the appropriate time after dosing; pieces of liver (approx. 0.6 g.) were rapidly removed, weighed on a torsion balance and used for the estimation of the nicotinamide-adenine dinucleotides by the method of Slater, Sawyer & Struli (1964). Results were calculated both in terms of the 'concentration' of nucleotide ($\mu\text{g./g.}$ wet wt. of liver), and as the amount of nucleotide in the whole liver after correction to an arbitrary 100 g. body wt. basis. This latter procedure is abbreviated in the text as $\mu\text{g.}$ of nucleotide/whole liver/100 g. body wt. [see Slater, Struli & Sawyer (1964) for a discussion of this notation]. The mean values obtained for the ethanol- and saline-treated groups were tested for significant differences by Student's *t* test. Where this procedure indicated a statistically significant difference (i.e. $P < 0.05$) the values are marked *** in Tables 1 and 2; where $0.10 > P > 0.05$ the values are marked *. Values for $P > 0.10$ are not marked in either Table.

RESULTS

Oxidized and reduced nicotinamide-adenine dinucleotide. The intraperitoneal administration of 0.9% sodium chloride to rats resulted in no significant disturbance of the NAD and NADH₂ contents in liver compared with the values obtained from untreated female rats. After the administration of the ethanol-saline mixture, however, there was an extremely rapid and extensive alteration in the concentrations and relative proportions of NAD and NADH₂ (Table 1). Within 30 min. of dosing with ethanol there was a significant decrease in the NAD:NADH₂ ratio that resulted from an accumulation of NADH₂ (the total NAD + NADH₂ content was unaltered throughout the 24 hr. period studied). The ratio reached a minimal value approx. 90 min. after the administration of ethanol, but by 18 hr. after dosing there was no significant difference between the ethanol- and saline-treated groups.

The limited number of experiments performed with the high enteral dose of ethanol are also included in Table 1. This dose (approx. 2.5 times that generally used in the present study) appeared

Table 1. *Effect of the administration of ethanol on the contents of oxidized and reduced nicotinamide-adenine dinucleotide in rat liver*

Female rats received 0.3 ml. of ethanol/100 g. body wt. (E) or an equivalent volume of saline (S) by intraperitoneal injection or 0.75 ml. of ethanol/100 g. body wt. (EST) by stomach tube. For further details see the Methods section. The results are expressed as means $\pm$ S.E.M.

Time (hr.)	Substance administered	No. of rats	NAD			NADH ₂			NAD + NADH ₂			NAD:NADH ₂ ratio
			μ g./g.	μ g./liver	μ g./g.	μ g./g.	μ g./liver	μ g./g.	μ g./g.	μ g./liver	μ g./g.	
0	—	7	330 $\pm$ 12	1830 $\pm$ 58	79 $\pm$ 8	411 $\pm$ 43	416 $\pm$ 23	2254 $\pm$ 165	4.1 $\pm$ 0.47	—	—	—
0.25	E	2	345	1670	153	743	499	2413	2.25	—	—	—
0.5	E	4	320 $\pm$ 21	—	148 $\pm$ 14	—	468 $\pm$ 33	—	2.16 $\pm$ 0.14	—	—	—
0.5	S	3	374 $\pm$ 22	2165†	85 $\pm$ 5***	502†	459 $\pm$ 19	2667†	4.40 $\pm$ 0.43***	—	—	—
1.0	E	6	296 $\pm$ 57	1482 $\pm$ 133	178 $\pm$ 17	869 $\pm$ 65	475 $\pm$ 21	2351 $\pm$ 93	1.78 $\pm$ 0.26	—	—	—
1.0	S	5	363 $\pm$ 17	1746 $\pm$ 114	86 $\pm$ 7***	411 $\pm$ 27***	449 $\pm$ 14	2157 $\pm$ 108	4.20 $\pm$ 0.44***	—	—	—
1.5	E	3	270 $\pm$ 9	1255†	201 $\pm$ 18	1040†	472 $\pm$ 16	2295†	1.37 $\pm$ 0.20	—	—	—
1.5	S	3	361 $\pm$ 4***	1803 $\pm$ 102	81 $\pm$ 5***	406 $\pm$ 6	442 $\pm$ 7	2206 $\pm$ 98	4.50 $\pm$ 0.31***	—	—	—
2.0	E	4	284 $\pm$ 13	1320 $\pm$ 80	191 $\pm$ 13	839 $\pm$ 84	474 $\pm$ 30	2159 $\pm$ 57	1.52 $\pm$ 0.17	—	—	—
2.0	S	3	369 $\pm$ 26***	1733 $\pm$ 63***	89 $\pm$ 5***	407 $\pm$ 22***	458 $\pm$ 30	2140 $\pm$ 81	4.26 $\pm$ 0.08***	—	—	—
5.0	E	3	295 $\pm$ 11	1915†	128 $\pm$ 19	732†	422 $\pm$ 27	2647†	2.37 $\pm$ 0.24	—	—	—
5.0	S	2	344	1880	104	569	448	2448	3.32	—	—	—
13.0	E	4	344 $\pm$ 15	1685†	73 $\pm$ 12	272†	417 $\pm$ 24	1957†	5.05 $\pm$ 0.70	—	—	—
13.0	EST	2	317	1220	104	397	420	1617	3.10	—	—	—
18.0	E	4	416 $\pm$ 16	—	81 $\pm$ 10	—	497 $\pm$ 26	—	5.32 $\pm$ 0.54	—	—	—
18.0	EST	2	330	2000	68	415	398	2415	4.95	—	—	—
24.0	S	3	385 $\pm$ 33	—	82 $\pm$ 13	—	466 $\pm$ 38	—	4.90 $\pm$ 0.75	—	—	—
24.0	E	3	331 $\pm$ 29	—	80 $\pm$ 5	—	411 $\pm$ 24	—	4.23 $\pm$ 0.64	—	—	—
24.0	S	4	386 $\pm$ 10*	—	84 $\pm$ 3	—	470 $\pm$ 13	—	4.15 $\pm$ 0.20	—	—	—

* 0.10 > P > 0.05; *** P < 0.05.

† Two animals only in this group.

to prolong the depression of the NAD:NADH₂ ratio since the value found 13 hr. after dosing (3.1) was considerably lower than that found after the lower dose of ethanol (5.05 $\pm$ 0.70), whereas both doses produced similar ratios (4.95 and 5.32 $\pm$ 0.54 respectively) 18 hr. after dosing.

Oxidized and reduced nicotinamide-adenine dinucleotide phosphate. As found for NAD and NADH₂, the injection of saline alone caused no significant alterations in the contents of NADP and NADPH₂ in rat liver. In contrast with the rapid and extensive changes produced by the administration of ethanol on NAD and NADH₂ (Table 1), the only significant change shown in Table 2 was a temporary decrease in the NADP content (in μ g./whole liver/100 g. body wt.) in the ethanol-treated group compared with the saline-treated group some 60–90 min. after dosing.

DISCUSSION

Even with a higher dose of ethanol than that used in the present work, Mallov & Bloch (1956) found that the increase in liver lipid was not maximal until some 18 hr. after administration. There is no reason to suppose that the lower ethanol dose used in the present investigation produces fat accumulation more rapidly. However, the alteration found in the NAD:NADH₂ ratio is extremely rapid (15–30 min. being required for a dramatic effect); further, the ratio is approaching the normal range less than 13 hr. after dosing at a time when the accumulation of fat is, at the most, about 50 % of the maximum deposition (Mallov & Bloch, 1956). This disparity between the time-sequences of the nucleotide changes and the accumulation of fat suggests that there is no close and direct connexion between the two events. Rebouças & Isselbacher (1961) reached a similar conclusion after finding that the administration of sorbitol decreased the NAD:NADH₂ ratio to a similar extent as did ethanol but, in contrast, did not produce fat accumulation. A further factor that makes it difficult to reconcile the decreased NAD:NADH₂ ratio with the increased fat synthesis after the administration of ethanol is that alcohol dehydrogenase is located in the soluble cytoplasmic compartment of the cell (Nyberg, Schuberth & Åuggård, 1953). Thus the rapid changes in the NAD:NADH₂ ratio that follow the administration of ethanol are presumably changes in the 'soluble NAD' of the liver cell. However, lipid synthesis associated with NAD-linked enzymes is mitochondrial in location (for references see Green & Gibson, 1960), whereas cytoplasmic fat synthesis is NADP-linked (Wakil, 1961); the results of the present investigation show that the NADPH₂:NADP ratio is not significantly altered by the

Table 2. Effect of the administration of ethanol on the contents of oxidized and reduced nicotinamide-adenine dinucleotide phosphate in rat liver

Female rats received 0.3 ml. of ethanol/100 g. body wt. (E) or an equivalent volume of saline (S) by intraperitoneal injection. For further details see the Methods section. The results are expressed as means $\pm$ S.E.M.

Time (hr.)	Substance administered	No. of rats	NADP		NADPH ₂		NADP + NADPH ₂		NADPH ₂ :NADP ratio
			μg./g.	μg./liver	μg./g.	μg./liver	μg./g.	μg./liver	
0-0	—	10	27 ± 2	142 ± 8	190 ± 6	1017 ± 71	219 ± 7	1160 ± 48	7.2 ± 0.56
0-50	E	4	33 ± 2	143 ± 7	246 ± 24	1044 ± 51	279 ± 26	1186 ± 41	7.3 ± 0.46
	S	4	26 ± 2*	130 ± 23	194 ± 15	935 ± 128	220 ± 17*	1065 ± 150	7.4 ± 0.49
1-0	E	8	20 ± 2	91 ± 9	201 ± 10	898 ± 67	221 ± 14	988 ± 71	10.4 ± 1.10
	S	7	26 ± 2***	123 ± 9***	196 ± 8	913 ± 62	223 ± 6	1035 ± 56	7.7 ± 0.78*
1-5	E	6	25 ± 5	103 ± 16	223 ± 21	913 ± 67	248 ± 23	1006 ± 69	10.1 ± 1.85
	S	7	34 ± 2	167 ± 12***	216 ± 12	1044 ± 71	265 ± 14	1212 ± 76*	6.7 ± 0.61*
2-0	E	6	26 ± 1	111 ± 6	209 ± 14	876 ± 73	238 ± 13	985 ± 71	8.5 ± 0.66
	S	6	26 ± 3	130 ± 21	211 ± 82	1013 ± 49	236 ± 8	1143 ± 73	8.9 ± 1.30
4-0	E	4	34 ± 3	138 ± 13	213 ± 8	917 ± 84	246 ± 12	1059 ± 95	6.6 ± 0.52
	S	2	31	146	197	936	228	1082	6.5
13-0	E	2	35	—	243	—	279	—	6.8
18-0	E	5	28 ± 3	149 ± 15	209 ± 32	1041 ± 116	237 ± 35	1191 ± 119	7.4 ± 0.70
	S	3	28 ± 1	152†	172 ± 11	897†	200 ± 12	1049†	6.2 ± 0.31
24-0	E	2	37	—	246	—	282	—	6.6
	S	2	31	159	199	946	230	1105	6.0

* 0.10 > P > 0.05; *** P < 0.05.
† Two animals only in this group.

† Two animals only in this group.

* 0.10 > P > 0.05; *** P < 0.05.

administration of ethanol. It is therefore unlikely that the disturbances in the nicotinamide-adenine dinucleotides produced by a single dose of ethanol have any direct relationship to the establishment of the fatty liver, although, of course, the decreased ratio might set in motion other secondary processes that themselves lead to fat accumulation.

Large-scale changes in the NAD:NADH₂ ratio can occur without affecting in any significant fashion the NADPH₂:NADP ratio (e.g. by transhydrogenation). The reverse was found to be true in carbon tetrachloride poisoning (Slater *et al.* 1964), where extensive alterations in total liver NADP and NADPH₂ concentrations were found, whereas NAD and NADH₂ concentrations showed only minor fluctuations. These findings suggest that extensive alterations in the NAD:NADH₂ or NADPH₂:NADP ratio are not by themselves sufficient to energize transhydrogenase-type reactions *in situ*.

SUMMARY

1. Changes in the concentrations of NAD, NADH₂, NADP and NADPH₂ in rat liver after a single intraperitoneal injection of ethanol were determined at frequent stages during the ensuing poisoning.

2. The concentrations of NAD and NADH₂ in the liver show very rapid changes, such that the NAD:NADH₂ ratio decreases from a control value of 4.1 $\pm$ 0.47 to 2.16 $\pm$ 0.14 within 30 min. of the administration of ethanol. The ratio is still very low 5 hr. later but has returned to normal approx. 13 hr. after the injection of ethanol. Dosing rats with a much higher amount of ethanol (6.0 g./kg. body wt. instead of 2.4 g./kg. body wt.) prolonged the effect; the NAD:NADH₂ ratio was still decreased 13 hr. after dosing, but was in the normal range 5 hr. later.

3. In contrast, no significant changes were found in the liver's content of NADP and NADPH₂ during the entire period of poisoning studied, other than a slight fall in the NADP concentration 60-90 min. after dosing with ethanol.

4. The relationship of the changes in NAD and NADH₂ concentrations to the developing fatty liver are discussed, as are the implications of the lack of change in the NADP and NADPH₂ contents. It is concluded that the changes in the NAD:NADH₂ ratio bear no apparent or direct relationship to the increased lipid content that occurs in the liver after treatment with ethanol.

REFERENCES

- Di Luzio, N. R. (1958). *Amer. J. Physiol.* **194**, 453.
 Forsander, O., Rih, N. & Suomalainen, H. (1958).
Hoppe-Seyl. Z. **312**, 243.

- Green, D. E. & Gibson, D. M. (1960). In *Metabolic Pathways*, vol. 1, p. 330. Ed. by Greenberg, D. M. New York: Academic Press Inc.
- Lieber, C. S. & Davidson, C. S. (1962). *Amer. J. Med.* **33**, 319.
- Lieber, C. S. & Schmid, R. (1961). *J. clin. Invest.* **40**, 394.
- Mallov, S. & Bloch, J. L. (1956). *Amer. J. Physiol.* **184**, 29.
- Nyberg, A., Schuberth, J. & Ånggård, L. (1953). *Acta chem. scand.* **7**, 1170.
- Rebouças, G. & Isselbacher, K. J. (1961). *J. clin. Invest.* **40**, 1355.
- Slater, T. F. & Sawyer, B. C. & Sträuli, U. D. (1964). *Arch. int. Physiol. Biochim.* **72**, 427.
- Slater, T. F., Sträuli, U. D. & Sawyer, B. C. (1964). *Biochem. J.* **93**, 260.
- Smith, M. E. & Newman, H. W. (1959). *J. biol. Chem.* **234**, 1544.
- Wakil, S. (1961). *J. Lipid Res.* **2**, 1.

Biochem. J. (1964), **93**, 270

Synthesis of Porphyrin *c*-Type Compounds from Protoporphyrinogen

BY SEIYO SANO AND NORIAKI NANZYŌ

Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto, Japan

AND C. RIMINGTON

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(Received 22 January 1964)

The porphyrin component of cytochrome *c* was first prepared by Hill & Keilin (1931). They named it porphyrin *c* and found it to be insoluble either in ether containing acetic acid or in chloroform. It could be transformed into haematoporphyrin. Theorell (1939) prepared natural porphyrin *c* with a molecular weight of 890 ± 10 by hydrolysis of cytochrome *c* with hydrochloric acid. On heating protoporphyrin or haematoporphyrin with cysteine in hydrochloric acid, he also prepared a product resembling porphyrin *c*. He concluded that the prosthetic group of cytochrome *c* is bound to the apoprotein by thio ether bonds uniting cysteine residues to the vinyl side chains of protohaem (Theorell, 1938). Paul (1951) confirmed this result and prepared an optically active haematoporphyrin from cytochrome *c* through cleavage of these thio ether bonds with silver sulphate (Paul, 1950).

Zeile & Meyer (1939) hydrolysed cytochrome *c* with sulphuric acid under conditions that were presumed to exclude the possibility of addition between cysteine and the vinyl groups in the porphyrin. They obtained a dicysteine-porphyrin adduct and assumed it to be preformed in cytochrome *c*. Zeile & Meyer (1939) synthesized a porphyrin *c* type of compound by melting together L-cysteine hydrochloride and dibromoporphyrin. The natural and synthetic porphyrins *c* agreed in analytical data, absorption spectra and partition of their esters between ether and acid buffers, but their optical activities were very different.

Neilands & Tuppy (1960) prepared a crystalline synthetic porphyrin *c* by a modification of Zeile &

Meyer's (1939) method. This was split at a negligible rate by an enzyme from *Albizzia lophanta* (Hansen, Kjaer & Schwimmer, 1959) which cleaves a number of *S*-alkylcysteines having straight-chain alkyl groups but which has little action on those with branched chains. Neilands & Tuppy's (1960) finding would favour an α - rather than a β -linkage in their synthetic product.

Although the above studies indicate that porphyrin *c* is a dicysteine-protoporphyrin adduct, they do not afford incontrovertible evidence on the point of attachment of the sulphur atoms to the side chains in positions 2 and 4 of the porphyrin ring (Fig. 1). Different isomers would result according to whether reaction of cysteine was with the α - or β -carbon atoms of the vinyl side chains of protoporphyrin. Rigorous proof of the structure of the haematoporphyrin derived by cleavage of the C—S linkage would settle the issue but this has not been forthcoming. Paul's (1951) report of an optically active haematoporphyrin suggests the α -isomer.

Recent biochemical evidence shows that the actual intermediates in the biosynthesis of haem are not porphyrins, but their reduction products, the porphyrinogens, which are hexahydroporphyrins. Sano & Granick (1961) demonstrated that protoporphyrinogen is an intermediate in the biosynthesis of protoporphyrin from coproporphyrinogen *in vitro*. They also observed that thiol compounds reacted with the vinyl side chains of protoporphyrinogen during its autoxidation in an acid medium under the influence of light, yielding a porphyrin *c* type of compound. This discovery opened up the possibility of synthesizing cyto-