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Nicotinamide–Adenine Dinucleotides in Acute Liver Injury induced by Ethionine, and a Comparison with the Effects of Salicylate

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1. The effects of single doses of ethionine or sodium salicylate on the nicotinamide–adenine dinucleotide content of rat liver have been studied. 2. There was no significant change in the sum of NAD + $NADH_2$ during the early period (0–2hr.) of the liver injury induced by ethionine but there was a decrease in this value of approx. 30% by 4hr. after administration. 3. Ethionine had no significant effect on the NADP + $NADPH_2$ during the first 2hr. period after administration. The sum then decreased to a value approx. 70% of the control by 3hr. after dosing but showed a partial recovery at the 4hr. period before decreasing again in later stages of the poisoning. 4. Salicylate produced a very rapid decrease in the NADP + $NADPH_2$ in the liver after intraperitoneal injection. After 1hr. the decrease was approx. 30% of the initial value; the sum slowly returned towards the normal range during the following 4hr. 5. A high parenteral dose of salicylate was found to cause only a small depression in the concentration of ATP in rat liver in contrast with the rapid depletion produced by ethionine. 6. These results are discussed in terms of the liver disturbances produced by ethionine and salicylate.

A single dose of ethionine produces a fatty liver (Farber, Simpson & Tarver, 1950) and it has been suggested that this results from the reduction in hepatic ATP concentration that occurs shortly after ethionine treatment (for references see Farber, Schull, Villa-Trevino, Lombardi & Thomas, 1964). Although it is known (Greenbaum, Clark & McLean, 1964) that a single dose of ethionine causes a substantial fall in NADP + $NADPH_2$ 5hr. after administration of the toxic agent, no data are available concerning the very early period (0–3hr.) after ethionine administration, a period when changes have been observed with liver poisons that produce necrosis with or without a concomitant accumulation of fat (Slater, Sträuli & Sawyer, 1964*b*; Slater & Sawyer, 1966*a*,*b*). In this paper the early effects of ethionine on the amounts of the nicotinamide–adenine dinucleotides in liver are considered, and the relationship of these changes to the rapid decrease that occurs in liver ATP is examined.

Some data are also included here on the effects of a high parenteral dose of sodium salicylate on the nicotinamide–adenine dinucleotide content of rat liver. This substance is known to inhibit ATP formation *in vitro* (for references see Smith, 1963) and, at the dose used here, suppresses long-term inflammatory changes under a variety of conditions (for references see Collier, 1963).

METHODS

The rats used were adult female albinos of the Wistar strain, body wt. approx. 130g. Rats were fed on M.R.C. diet 41B and were killed by cervical dislocation. Immediately after killing, pieces of liver were removed for the determination of the nicotinamide–adenine dinucleotides by the method of Slater, Sawyer & Sträuli (1964*a*). ATP was estimated in liver samples frozen in liquid N_2 by the procedure described by Lamprecht & Trautschold (1963). NAD(P) glycohydrolase was measured in liver suspensions (1:100 in water) by the cyanide method of Colowick, Kaplan & Ciotti (1951).

DL-Ethionine (chromatographically pure material; Calbiochem, Los Angeles, Calif., U.S.A.) was dissolved in water and neutralized with KOH; the solution (25mg./ml.) was injected intraperitoneally with the rat under ether anaesthesia. The dose of ethionine was 100mg./100g. body wt. as used in previous studies (Schull, 1962; Greenbaum *et al.* 1964). Sodium salicylate was dissolved in 0·9% NaCl soln. and the solution was injected intraperitoneally with the rat under ether anaesthesia. The dose was 40mg. of sodium salicylate/100g. body wt. Control rats received injections of water or saline corresponding to the amounts administered with the ethionine or sodium salicylate respectively. After dosing, the control rats were caged separately from those treated with ethionine or salicylate.

Liver sections were taken from rats which had received sodium salicylate; the sections were fixed in formalin–saline and stained with haematoxylin–eosin and with neutral red.

RESULTS

Tables 1 and 2 give the results found for the concentrations of (1) NADP+$NADPH_2$ and (2) NAD+$NADH_2$ in liver after the administration of ethionine. Estimations were not carried out at later periods than 6hr. after dosing since this investigation was primarily concerned with the early biochemical changes occurring before the onset of definite histological lesions.

The results given in Table 1 show that there is no significant decrease in NADP+$NADPH_2$ until more than 2hr. after dosing with ethionine. There is a significant decrease of approx. 28% in the mean value obtained for the 3hr. group but this is partially restored towards the normal range by 4hr. The NADP+$NADPH_2$ is further depressed at later stages of the injury. The partial recovery noticed between 3 and 4hr. after injection of ethionine is similar to that found 1–2hr. after administering carbon tetrachloride (Slater *et al.* 1964*b*).

The results given in Table 2 show that the NAD+$NADH_2$ does not decrease significantly until several hours after administration of ethionine.

Table 1. *Effect of ethionine administration on the concentrations of NADP and $NADPH_2$ in rat liver*

Mean values are given ±S.E.M. The ethionine group means were compared with their corresponding control groups by using Student's *t* test; value for *P* so obtained is given (N.S., not significant). Liver samples from rats in the 6hr. group were treated differently from other groups in this study in that they were frozen immediately in liquid nitrogen and kept overnight at −195° before assay; this procedure is known not to affect NADP+$NADPH_2$ but does alter the ratio $NADPH_2$/NADP (Slater, 1966). Other details are given in the Methods section.

Time (hr.)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$ (g.)	NADP+$NADPH_2$ (μg./whole liver/ 100g. body wt.)	*P*	$\frac{NADPH_2}{NADP}$
1	Control	5	4·88±0·15	992±65	N.S.	8·8±0·53
	Ethionine	6	5·04±0·17	938±48		7·4±0·52
2	Control	4	5·10±0·39	982±86	N.S.	7·3±0·61
	Ethionine	4	4·64±0·30	954±23		7·0±0·75
3	Control	6	4·96±0·24	923±67	0·01	7·0±0·28
	Ethionine	6	4·50±0·16	660±37		6·4±0·60
4	Control	3	5·31±0·07	1029±17		8·7±1·50
	Ethionine	4	5·06±0·27	842±64		5·1±0·60
6	Control	2	4·45	1009		4·1
	Ethionine	5	3·75±0·10	600±53		2·2±0·25

Table 2. *Effect of ethionine administration on the concentrations of NAD and $NADH_2$ in rat liver*

Mean values are given ±S.E.M. Liver samples from rats in the 6hr. group were treated differently from other groups in this study in that they were frozen immediately in liquid nitrogen and kept overnight at −195° before assay; this procedure is known not to affect NADP+$NADPH_2$ but does alter the ratio $NADPH_2$/NADP (Slater, 1966). Other details are given in the Methods section.

Time (hr.)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$ (g.)	NAD+$NADH_2$ (μg./whole liver/ 100g. body wt.)	$\frac{NAD}{NADH_2}$
1	Control	5	4·88±0·15	1880±77	3·1±0·4
	Ethionine	6	5·04±0·17	1750±93	3·6±0·3
2	Control	2	4·47	1657	3·1
	Ethionine	2	4·17	1816	3·4
4	Control	3	5·31±0·07	1886±30	2·8±0·1
	Ethionine	4	5·06±0·27	1374±166	2·2±0·2
6	Control	2	4·45	1836	3·7
	Ethionine	5	3·75±0·10	1320±66	3·5±0·2

The mean value was depressed approx. 30% in both the 4hr. and 6hr. groups compared with the normal control mean values. The activity of NAD(P) glycohydrolase was measured in rat-liver suspensions 2hr. after giving water (control group) or ethionine (treated group). No significant difference was found between the two groups. The mean values ± S.E.M. were (a) control (3), 50 ± 4, (b) ethionine (5), 40 ± 3 μmoles/g./hr. The mean value obtained from untreated female rats was 53 ± 5 μmoles/g./hr. (9); the numbers of rats used are in parentheses. ATP estimations on liver samples taken from rats poisoned with ethionine showed that by 3hr. after dosing the ATP was depressed 70% (mean of four rats) compared with the control group of four rats that had received an injection of saline alone. The values obtained were (a) control group, 2·14 ± 0·10, (b) ethionine group 0·64 ± 0·05 μ-moles of ATP/g. wet wt. The depression in the amount of ATP obtained 2hr. after dosing with ethionine was 45% (mean value 1·17 ± 0·07 μ-moles/g., mean of four rats).

Table 3 gives the results found for the liver amounts of NADP and $NADPH_2$ after dosing with sodium salicylate. It can be seen that there is a rapid decrease in the NADP + $NADPH_2$ so that 1hr. after dosing the treated group is depressed 27% below the control value. There is then a slow return to the normal value. During the course of salicylate poisoning there is a considerable decrease in the ratio $\frac{NADPH_2}{NADP}$ (Table 3). Table 4 gives corresponding results for NAD and $NADH_2$ after salicylate administration. Unlike the results reported for NADP and $NADPH_2$ (Table 3) there is no rapid fall in the NAD + $NADH_2$ and, in fact, no significant decrease was observed during the period studied.

Table 3. *Effect of the administration of sodium salicylate on the concentrations of NADP and $NADPH_2$ in rat liver*

Mean values are given ± S.E.M. The salicylate group means were compared with their corresponding control groups by using Student's *t* test; values for *P* so obtained are given. Other details are given in the Methods section.

Time (hr.)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$ (g.)	NADP + $NADPH_2$ (μg./whole liver/ 100g. body wt.)	*P*	$\frac{NADPH_2}{NADP}$
0·5	Control	4	5·68 ± 0·17	992 ± 91	N.S.	7·05 ± 1·07
	Salicylate	4	4·54 ± 0·20	936 ± 94		6·1 ± 1·13
1·0	Control	7	4·84 ± 0·20	945 ± 44	< 0·01	8·7 ± 0·48
	Salicylate	4	4·37 ± 0·33	691 ± 44		4·9 ± 0·42
2·0	Control	4	5·01 ± 0·28	971 ± 85	0·10	8·0 ± 0·31
	Salicylate	4	4·28 ± 0·16	781 ± 58		4·3 ± 0·45
3·0	Control	4	4·91 ± 0·23	994 ± 62	< 0·05	9·1 ± 1·45
	Salicylate	6	4·55 ± 0·46	788 ± 58		5·8 ± 0·24
5·0	Control	2	5·30	1130		9·4
	Salicylate	2	4·10	985		7·6

Table 4. *Effect of the administration of sodium salicylate on the concentrations of NAD and $NADH_2$ in rat liver*

Mean values are given ± S.E.M. Other details are given in the Methods section.

Time (hr.)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$ (g.)	NAD + $NADH_2$ (μg./whole liver/ 100g. body wt.)	$\frac{NAD}{NADH_2}$
0·5	Control	4	5·68 ± 0·15	2267 ± 72	4·07 ± 0·32
	Salicylate	4	4·54 ± 0·20	2064 ± 77	3·10 ± 0·33
1·0	Control	5	5·31 ± 0·23	2572 ± 192	3·44 ± 0·28
	Salicylate	5	4·71 ± 0·26	2342 ± 210	3·10 ± 0·24
2·0	Control	4	5·01 ± 0·28	2063 ± 200	2·86 ± 0·19
	Salicylate	4	4·28 ± 0·16	1866 ± 117	3·00 ± 0·22
3·0	Control	4	4·91 ± 0·22	2592 ± 233	2·69
	Salicylate	4	4·21 ± 0·43	2404 ± 353	2·48

ATP estimations were carried out on liver samples obtained from (a) rats dosed 1hr. previously with sodium salicylate in saline solution (40mg. of salicylate/100g. body wt.), (b) control rats given an equivalent volume of saline and (c) untreated female rats. The mean values obtained ± S.E.M. (no. of rats used in parentheses) were: (a) 2·20 ± 0·12 μ-moles of ATP/g. wet wt. of liver (4); (b) 2·23 (2); (c) 2·46 ± 0·11 (7). Histological examination of liver sections taken from rats 18hr. and 24hr. after administering sodium salicylate showed no significant liver-cell necrosis and no appreciable accumulation of fat.

DISCUSSION

The administration of a single dose (100mg./100g. body wt.) of DL-ethionine to female rats is known to produce an extremely rapid fall in the concentration of ATP in the liver (Stekol, Mody, Bedrak, Keller & Perry, 1960; Schull, 1962; Farber *et al.* 1964). The decrease was so rapid that within 3hr. of dosing the ATP concentration was depressed 70–80% (Villa-Trevino, Schull & Farber, 1963). The results obtained in this study confirm that liver ATP decreases rapidly after ethionine although the rate of decrease found here is rather lower in the first 2hr. than that reported by Villa-Trevino *et al.* (1963). However, by 3hr. after dosing the value found here (70% depression) is similar to that previously given (86% depression) by Villa-Trevino *et al.* (1963).

It is clear from the results given in Table 1 that there is no very early decrease in the NADP + $NADPH_2$ of a similar magnitude to the decrease that occurs with ATP. For example, the corresponding percentage decreases in the NADP + $NADPH_2$ (Table 1) and in the ATP concentration (by using for illustrative purposes the more extensive results of Villa-Trevino *et al.* 1963) are respectively: 1hr., −8% and −50%; 2hr., −3% and −80%; 3hr., −29% and −86%; 4hr., −18% and −85%.

It would appear therefore that the concentration of NADP + $NADPH_2$ in rat liver does not necessarily show immediate and proportional alterations to large-scale changes in ATP content. Yet ATP is obligatory for the accepted scheme of NADP synthesis utilizing NAD kinase (for references see Wang, 1955). Further, of the substrates and enzymes involved in the synthesis of NADP, both NAD itself and NMN adenyltransferase have been shown to be unaltered during this early phase (Stirpe & Aldridge, 1961; Greenbaum *et al.* 1964; this paper, see the Results section). NAD kinase is also unchanged 5hr. after ethionine administration (Greenbaum *et al.* 1964) and NADP glycohydrolase does not appear to be significantly decreased at 2hr. after dosing (see the Results section).

The most likely explanation for the finding that the decrease in the content of NADP + $NADPH_2$ lags behind the large decrease in ATP by about 2hr. is that the turnover time of nicotinamide–adenine dinucleotide phosphate is relatively long, measuring hours rather than minutes, and that *in vivo* its synthesis is considerably slower than the maximal rate for NAD kinase measured under optimum conditions *in vitro*. A rough calculation has been made of the turnover time of NADP in normal rat liver from previously published data for the NAD-kinase reaction; the value found was approx. 5hr. (Slater & Sawyer, 1966*b*; Slater, 1966). Such a value is compatible with the pattern of results found in this investigation where, despite a large decrease in ATP, the rate of decrease in the NADP + $NADPH_2$ is relatively low. The explanation of the time-lag of approx. 2hr. before a decrease occurs in the sum of NADP + $NADPH_2$ despite a large decrease in ATP could be that the major part of the ATP decrease occurs in a compartment of the cell other than the soluble fraction where NAD kinase is localized. Thus the concentration of ATP in the kinase compartment could be maintained despite a considerable decrease in the whole-liver ATP value.

The results obtained with salicylate (Tables 3 and 4) contrast quite sharply with those with ethionine described above. It has been found that the large dose of salicylate used (40mg./100g. body wt.) produces a rapid and sustained decrease in the NADP + $NADPH_2$. The fall at 1hr. is larger than found with ethionine and, moreover, is maintained for the succeeding 2–3hr. There is no evidence that the NAD + $NADH_2$ is depressed after salicylate (Table 4).

Despite the well-documented effect of salicylate in uncoupling phosphorylation in isolated rat-liver mitochondria (Brody, 1956; Smith, 1963; Miyahara & Karler, 1965) there are apparently no data concerning its uncoupling action *in vivo*. The values presented here for hepatic ATP after salicylate indicate that at the dose administered this substance does not significantly decrease liver ATP. This finding is similar to the result obtained with a high dose (7·5mg./100g. body wt.) of 2,4-dinitrophenol, which also had little effect *in vivo* on the ATP concentration in rat liver (Miss V. B. Delaney, unpublished work).

Salicylate is known to inhibit many enzyme reactions utilizing nicotinamide–adenine dinucleotides by a form of direct competition with the coenzyme (Euler & Ahlstrom, 1943; Bryant, Smith & Hines, 1963). However, the possibility that the decrease in NADP + $NADPH_2$ could be due to the salicylate displacing the nucleotide from a binding site into free solution, thereby making it more susceptible to enzymic breakdown by the glycohydrolase, would seem unlikely: the NAD + $NADH_2$

changes more slowly than the NADP + $NADPH_2$, whereas the glycohydrolase is several times more active with NAD than NADP in rat liver (Slater & Sawyer, 1966*b*). The mechanism by which salicylate causes the observed changes in the nicotinamide–adenine dinucleotides in rat liver is at present unknown.

In attempting to interpret data obtained from samples of whole liver from rats treated with hepatotoxic agents it should not be forgotten that many hepatotoxins affect zones of the liver lobule to different extents; ethionine, for instance, produces fat accumulation primarily in the periportal region in the early stage of the injury (Koch-Weser, Farber & Popper, 1951). Superimposed on this lobular heterogeneity is the possibility of intracellular shifts in substrates and enzymes that cannot readily be resolved by whole-tissue analysis: for example, the shifts in hepatic ATP observed by Frunder, Blume, Thielmann & Bornig (1961) after carbon tetrachloride poisoning. One approach to such problems is the development of adequate histochemical procedures for the localization of enzymes involved in the synthesis and breakdown of NAD and NADP. Such studies are at present in progress.

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REFERENCES

Brody, T. M. (1956). *J. Pharmacol.* **117**, 39.

Bryant, C., Smith, M. J. H. & Hines, W. J. W. (1963). *Biochem. J.* **86**, 391.

Collier, H. O. J. (1963). In *Salicylates*, p. 120. Ed. by Dixon, A. S. J., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. London: J. and A. Churchill Ltd.

Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951). *J. biol. Chem.* **191**, 447.

Euler, H. von & Ahlstrom, L. (1943). *Hoppe-Seyl. Z.* **279**, 175.

Farber, E., Schull, K. H., Villa-Trevino, S., Lombardi, B. & Thomas, M. (1964). *Nature, Lond.*, **203**, 34.

Farber, E., Simpson, M. V. & Tarver, H. (1950). *J. biol. Chem.* **182**, 91.

Frunder, H., Blume, E., Thielmann, K. & Bornig, H. (1961). *Hoppe-Seyl. Z.* **325**, 146.

Greenbaum, A. L., Clark, J. B. & McLean, P. (1964). *Biochem. J.* **93**, 17C.

Koch-Weser, D., Farber, E. & Popper, H. (1951). *A.M.A. Arch. Path.* **51**, 498.

Lamprecht, W. & Trautschold, I. (1963). In *Methods of Enzymatic Analysis*, p. 543. Ed. by Bergmeyer, E. London: Pergamon Press Ltd.

Miyahara, J. T. & Karler, R. (1965). *Biochem. J.* **97**, 194.

Schull, K. H. (1962). *J. biol. Chem.* **237**, PC1734.

Slater, T. F. (1966). *Biochem. J.* **99**, 2P.

Slater, T. F. & Sawyer, B. C. (1966*a*). *Biochem. Pharmacol.* (in the Press).

Slater, T. F. & Sawyer, B. C. (1966*b*). *Biochem. Pharmacol.* (in the Press).

Slater, T. F., Sawyer, B. C. & Sträuli, U. (1964*a*). *Arch. int. Physiol. Biochim.* **72**, 427.

Slater, T. F., Sträuli, U. & Sawyer, B. (1964*b*). *Biochem. J.* **93**, 260.

Smith, M. J. H. (1963). In *Salicylates*, p. 47. Ed. by Dixon, A. S. J., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. London: J. and A. Churchill Ltd.

Stekol, J. A., Mody, U., Bedrak, E., Keller, S. & Perry, J. (1960). *Fed. Proc.* **19**, 37.

Stirpe, F. & Aldridge, W. N. (1961). *Biochem. J.* **80**, 481.

Villa-Trevino, S., Schull, K. & Farber, E. (1963). *J. biol. Chem.* **238**, 1757.

Wang, T. P. (1955). In *Methods in Enzymology*, vol. 2, p. 652. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.