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NICOTINAMIDE ADENINE DINUCLEOTIDES IN THE LIVER IN EXPERIMENTALLY INDUCED PORPHYRIAS

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Abstract—The concentrations of the nicotinamide adenine dinucleotides in rat liver following the administration of substances known to induce acute hepatic porphyria have been studied. The substances investigated were allyl isopropyl acetamide (AIA), Sedormid, 1,2,4-trichlorobenzene, 1,2,3,4-tetrachlorobenzene and Griseofulvin. Two major points were considered: is there any significant decrease in the total concentration of these nucleotides? and is there any gross disturbance in their redox state in whole liver samples?

Although AIA and the chlorinated benzenes produced an overall decrease in the sum NAD + $NADH_2$ + NADP + $NADPH_2$ in the livers of rats exhibiting porphyria this was not true of rats poisoned with Sedormid. There was no consistently significant change in the ratios of NAD:$NADH_2$ and $NADPH_2$:NADP with the various agents tested although the ratio NADP + NAD:$NADPH_2$ + $NADH_2$ tended to be elevated with all the drugs studied. Long-term Griseofulvin treatment produced enormous increases in liver size with concomitant increases in the total amount of nucleotide/whole liver. These changes are discussed.

THE administration to rats and/or mice of a variety of compounds including allyl isopropyl acetamide, "Sedormid", chlorinated benzenes, and the fungistatic drug "Griseofulvin" results in the appearance of disturbances in porphyrin synthesis (for refs. see reviews by Granick,[1] Rimington,[2] and Tschudy[3]). These experimentally induced porphyrias have close similarities to the hepatic porphyrias seen in clinical medicine. Of the numerous suggestions offered in explanation of these drug-induced liver disturbances there have been two which provided the stimulus for the work reported here.

It has become apparent that the large increases in porphyrins and/or their precursors which are found in experimental porphyria result from an over production rather than under utilization or decreased catabolism.[4, 5] The biosynthetic route to the porphyrin (ogens) involves, at an early stage, the coupling of glycine with succinyl-CoA to yield δ-aminolaevulic acid. This substance, as well as undergoing condensation to produce PBE can, by conversion to α-ketoglutaraldehyde, donate one-carbon units to the purine biosynthetic pathway. Thus, a large increase in porphyrin synthesis might be expected to reduce purine synthesis by draining away both glycine and one-carbon units.[6] In fact, evidence has been found for a depleted purine biosynthesis in chick embryos when incubated in the presence of AIA and Sedormid.[6]

Abbreviations: allylisopropylacetamide—AIA; deoxyribonucleic acid phosphorus—DNA-P; porphobilinogen—PBE; δ-aminolaevulic acid—ALA.

However, in a previous study of this interesting point it has been shown[7] that there is no overall deficiency of acid-soluble purine nucleotides in liver samples obtained from rats exhibiting experimental hepatic porphyria although the ratio of ATP/AMP was disturbed in certain instances. In this investigation we have looked specifically at the levels of hepatic nicotinamide adenine dinucleotides to see if there is any decrease in content which can be correlated with the degree of porphyria and to provide additional data on the above speculation concerning purine deficiency.

The second main stimulus for this investigation came from the very interesting suggestion of Rimington[2] that, in certain types of experimental porphyria, and in cutaneous porphyria associated with chronic alcoholism, there is a disturbance in the redox level within the parenchymal cells. This might influence the balance between porphyrinogens and porphyrins. Only the former pass along the direct biosynthetic pathway; the latter cannot be further metabolized and find their way into the excreta. We have estimated the ratios of $NAD/NADH_2$ and $NADPH_2/NADP$ in liver samples obtained from rats suffering from experimentally induced porphyria to see if any consistent change compared to untreated rats is apparent.

METHODS

The rats used were adult male albinos, body wt. approx. 170 g; mice were albino females, body wt. approx. 20 g. The rats and mice were killed by cervical dislocation and pieces of liver immediately removed for estimation of the nicotinamide adenine dinucleotide content by the method of Slater *et al.*[8]

AIA, Sedormid and chlorinated benzenes were administered to rats by stomach tube as solutions or suspensions in propylene glycol. The doses were: AIA, 250 mg/kg body wt.; Sedormid, 250 mg/kg body wt.; 1,2,4-trichlorobenzene, 625 mg/kg body wt. Griseofulvin was fed to mice as a 2·5% mixture in normal ground-up diet; the Griseofulvin was very kindly donated by Imperial Chemical Industries Ltd.

Faeces and urine were collected and coproporphyrin, uroporphyrin, protoporphyrin, PBE and ALA acid were estimated as described by Rimington and Ziegler.[9] DNA was estimated by Burton's method;[10] RNA was estimated by the orcinol procedure.[11] Acid-soluble nucleotides in liver samples taken from rats poisoned with Sedormid were separated on Dowex-1 resin by the method of Hurlbert *et al.*[12] as described previously.[7]

RESULTS

In the majority of rats used for the assay of the NADs, determinations were also carried out on the urinary and liver levels of porphyrins and porphyrin precursors and on liver DNA and RNA. The mean values so obtained for the 24-hr urinary excretion levels of coproporphyrin, uroporphyrin, PBE and ALA, and the liver levels of coproporphyrin, uroporphyrin and protoporphyrin are shown in Table 1. These values confirm the nature of the disturbance affecting the rats used in the nucleotide assays; the porphyrin values shown in Table 1 are in broad agreement with values previously published.[9] No significant changes were found to occur in liver RNA-P during the conditions studied; these RNA-P estimations were kindly performed by Dr. C. W. I. Owens and have been previously reported by him.[20]

Table 2 gives the results obtained for the NAD levels in liver samples obtained from rats treated with 1,2,4-trichlorobenzene; 1,2,3,4-tetrachlorobenzene; AIA; and

Sedormid. Values obtained from untreated adult male rats are given for comparison.

It can be seen that AIA produced little change in NAD and $NADH_2$ under the conditions used, but did produce a significant drop in the sum NADP + $NADPH_2$. AIA treatment had no effect on the ratios NAD : $NADH_2$ or $NADPH_2$: NADP but slightly increased the combined ratio NAD + NADP : $NADH_2$ + $NADPH_2$.

TABLE 1. EFFECTS OF TREATMENT WITH ALLYL ISOPROPYLACETAMIDE, SEDORMID, 1,2,4-TRICHLOROBENZENE AND 1,2,3,4-TETRACHLOROBENZENE ON THE URINARY EXCRETION AND HEPATIC CONCENTRATIONS OF COPROPORPHYRIN, UROPORPHYRIN, PORPHOBILINOGEN, δ-AMINOLAEVULIC ACID, AND PROTOPORPHYRIN

Group	Urinary				Liver		
	copro-porphyrin	uro-porphyrin	PBG	ALA	copro-porphyrin	uro-porphyrin	proto-porphyrin
Control	4·3–6·8	0·1–0·3	2·5–6·5	38·7–51·6	4·5	1·3	9·7
1,2,4-trichloro-benzene	117 (3)	5·9 (2)	71 (3)	206 (3)	18 (2)	18 (2)	35 (2)
1,2,3,4-tetrachloro-benzene	37 (2)	7(1)	593 (2)	355 (2)	9 (2)	19 (2)	16 (2)
AIA	19 (3)	5·0 (3)	880 (3)	626 (3)	36 (3)	14 (3)	51 (3)
Sedormid	10 (4)	3·6 (3)	686 (4)	389 (4)	9 (2)	17 (2)	12 (2)

The control values are taken from Rimington and Ziegler (1964).[9] The number of animals used in each estimation are shown in parenthesis. Mean values are given as μg/24 hr urine volume and as μg/100 g wet wt. liver.

Treatment with Sedormid produced no significant changes in NAD, $NADH_2$, NADP or $NADPH_2$. Further, in acid extracts from pooled liver samples obtained from two rats treated with 250 mg/kg body wt. of Sedormid for 6 days, no significant differences were noticed in the acid-soluble nucleotide pattern. The results obtained (in μ-moles nucleotide/100 g wet wt. liver) were: AMP, 57; ADP, 102; ATP, 92; total of AMP + ADP + ATP = 251; ratio ATP : AMP = 1·6. These values are similar to values obtained on untreated control rats.[7]

1,2,3-trichlorobenzene produced a considerable decrease in the sum NAD + $NADH_2$ and an associated increase in the ratio NAD : $NADH_2$. This substance also reduced both the overall level of NADP + $NADPH_2$ and the ratio $NADPH_2$: NADP. Thus, the net effect was a general increase in oxidized compared to reduced nucleotide and this is shown by a considerable increase in the ratio NAD + NADP : $NADH_2$ + $NADPH_2$.

Rats treated with 1,2,3,4-tetrachlorobenzene behaved similarly to those dosed with the trichlorobenzene. If anything, the shift to more oxidized conditions was accentuated with the higher chlorinated benzene; the ratio NAD + NADP/$NADH_2$ + $NADPH_2$ was 1·44 compared to 1·28 for the trichlorobenzene and 1·09 for untreated rats.

Table 3 gives results obtained using mice treated with Griseofulvin. It can be seen that there is a tendency for the sums NAD + $NADH_2$ and NADP + $NADPH_2$ to drop shortly (20 hr) after putting the mice onto the Griseofulvin diet. However this response is not significant in contrast to the results found with mice fed the diet for

TABLE 2. EFFECT OF TREATMENT WITH ALLYL ISOPROPYLACETAMIDE, SEDORMID, 1,2,4-TRICHLOROBENZENE AND 1,2,3,4-TETRACHLOROBENZENE ON THE HEPATIC LEVELS OF NAD, $NADH_2$, NADP AND $NADPH_2$

Treatment	Period of dosing (days)	Dose mg/kg	No. of rats	NAD	$NADH_2$	$\frac{NAD+NADH_2}{\mu g/g}$	$\frac{NAD+NADH_2}{\mu g/\mu g\ DNAP}$	$\frac{NAD}{NADH_2}$	NADP	$NADPH_2$	$\frac{NADP+NADPH_2}{\mu g/g}$	$\frac{NADP+NADPH_2}{\mu g/\mu g\ DNAP}$	$\frac{NADPH_2}{NADP}$	$\frac{NAD+NADH_2+}{NADP+NADPH_2}$	$\frac{NAD+NADP}{NADH_2+NADPH_2}$
Untreated	—	—	7	358±14	89±3	447±10	2·50*±0·28	4·1±0·3	47±4	270±29	317±31	1·57*±0·21	5·9±0·7	765±36	1·16±0·08
AIA	3–13	250 or 500	7	313±12	83±3	396±15	2·63*±0·18	3·8±0·3	38±4	195±7	232±10	1·72*±0·07	5·6±0·8	631±15	1·29±0·10
Sedormid	5–15	250	5	347±17	77±9	424±22	2·42±†0·11	4·5±0·5	51±5	286±20	339±21	2·16†±0·04	5·7±0·6	770±20	1·14±0·06
1,2,4-Trichlorobenzene	6–23	500	3	301±12	48±5	349±20	2·20±0·10	6·3±0·4	56±8	232±31	288±29	1·80±0·15	4·4±1·1	637±46	1·31±0·14
1,2,3,4-Tetrachlorobenzene	6–10	640	2	346	63	409	2·48	5·8	44	207	251	1·49	5·5	660	1·44

The values are given as μg/g wet wt. tissue and in some instances as μg/μg DNA-P. Mean values are given ±S.E.M.
* Means of 3 rats only. † Means of 4 rats only.

TABLE 3. EFFECT OF FEEDING GRISEOFULVIN ON THE LEVELS OF NAD, $NADH_2$, NADP AND $NADPH_2$ IN MOUSE LIVER

Group	Sex	No. of mice	$\frac{\text{liver wt.}}{\text{body wt.}} \times 20$ (g)	NAD+ $NADH_2$	$\frac{NAD}{NADH_2}$	NADP+ $NADPH_2$	$\frac{NADPH_2}{NADP}$	NAD+$NADH_2$+ NADP+$NADPH_2$	$\frac{NAD+NADP}{NADH_2+NADPH_2}$
Control	F	4	1·02	406	3·02	176	4·9	582	1·41
20 hr	F	4	0·92	350	3·26	134	4·6	484	1·52
44 hr	F	2	1·20	322	2·54	200	5·2	522	1·03
67 hr	F	2	1·18	356	3·03	192	4·1	548	1·22
20–26 day	F	2	4·10	1630	4·7	834	4·7	2464	1·54
26 day	M	1	2·50	1620	4·9	750	4·9	2370	1·84

Mean values are given as μg nucleotide/whole liver/20 g body wt. The liver values were corrected to correspond to an arbitrary body wt. of 20 g to allow for small variations in body wt. of individual mice about the mean of approx 20 g; such variations, if uncorrected, produce increased variability in values calculated on a whole liver wt. basis.

approx 3 weeks. In these animals the liver was extremely large and the μg nucleotide/whole liver was increased approx 3-fold.

DISCUSSION

The very interesting suggestion has been made by Rimington[2, 13] that the redox-environment in the liver may play an important role in determining both the pattern of excretion and extent of accumulation of porphyrins in certain types of porphyria. The reasoning behind this suggestion is as follows. It is known that the components of the biosynthetic sequence linking PBE and protoporphyrin are not uro- and copro-porphyrin but the corresponding reduced forms, the porphyrinogens. Under oxidizing conditions these porphyrinogens are transformed to their corresponding porphyrins which appear incapable of undergoing the reverse reaction (i.e. reduction to porphyrinogens) under normal physiological conditions. Thus oxidation to the porphyrin can be regarded as an escape route from the biosynthetic chain leading to protoporphyrin and heme;[13] if of significant proportions, this escape would result in the accumulation of porphyrins in the liver and increased excretion. Further, it has been shown that the renal excretion of these various porphyrins and porphyrinogens depends both on the side-chain substituents on the pyrrole ring and on the state of oxidation of the central ring system.[14, 15] as a consequence it can be seen that the *in vivo* oxidation of porphyrinogens to their respective porphyrins would affect in an important manner both their accumulation and route of excretion.

Two major redox systems in the liver cell are the couples NAD : $NADH_2$ and $NADPH_2$: NADP. In normal rat liver these have values of approx 3·0 and 7·0 respectively.[8] Thus the $NADPH_2$: NADP couple is poised on the side of reduction. Previous studies from this and many other laboratories have shown that these ratios can be considerably disturbed by various experimental treatments e.g. administration of ethanol.[16]

In the light of the preceding discussion, it was of considerable interest to see if extensive changes occurred in these ratios during experimental porphyria induced by AIA, Sedormid, chlorinated benzenes or Griseofulvin. However, the results of this investigation show that no dramatic or consistent changes occur in these ratios in the various examples of experimental porphyria studied. The halogenated benzenes for example produced increases in the ratio NAD : $NADH_2$ and decreases in $NADPH_2$: NADP whereas Sedormid administration was without effect on these parameters. With AIA, and the chlorinated benzenes, however, there was a tendency for the ratio of total oxidized nucleotide : total reduced nucleotide to be increased compared to normal (Table 2, column 13). In these cases the tendency would appear more favourable to oxidation processes. It is important to note that concomitant porphyrin analyses performed on most of the rats used for nucleotide experiments confirmed the presence of serious disturbances in porphyrin metabolism.

The analyses reported here were on whole liver samples. It is therefore possible to argue that they hide very large fluctuation in NAD : $NADH_2$ or $NADPH_2$: NADP in small compartments of the cell in the same way as has been found to occur with ATP.[17] However, the enzymes concerned with the synthesis of uroporphyrinogen and coproporphyrinogen are almost exclusively in the soluble fraction of the cell.[1, 3] Since this fraction contains the majority of the NAD + $NADH_2$ and a substantial part of the NADP + $NADPH_2$ of the cell[18, 19] it is unlikely that "compartmentalization" is

of significance in this context. This assumes that the conversion of the porphyrinogens to the porphyrins occurs also in the soluble fraction but this is as yet unknown. The failure in this investigation to find any consistent relationship between the induction of experimental porphyria and the redox levels of the couples NAD : $NADH_2$ and $NADPH_2$: NADP was disappointing in view of the attractive hypothesis previously mentioned.[2, 13]

However, that hypothesis was principally developed in terms of human porphyria associated with chronic alcoholism, which most probably involves a different biochemical disturbance than does, for example, the porphyria induced by AIA. Despite this fact, the finding here that AIA and the chlorinated benzenes do produce a small disturbance in the ratio NAD + NADP : $NADH_2$ + $NADPH_2$ in the direction of increased oxidation, a tendency consistent with the speculation of Rimington,[2, 13] makes it all the more important to test the hypothesis respectively on human chronic alcoholics who do not suffer from disturbed porphyrin metabolism and those who have also developed a cutaneous porphyria.

The results of Table 2 further show that there is no apparent shortage of purine nucleotides available for the biosynthesis of NADs. The total sum NAD + $NADH_2$ + NADP + $NADPH_2$ is not substantially depressed in any of the cases studied on a μg/g wet wt. basis. Since the liver size is known to increase in experimental porphyria[20, 21] the overall change in μg nucleotide/whole liver would be minimal as is indeed illustrated by the values in Table 2 for μg nucleotide/μg DNAP. This conclusion is in agreement with previous results indicating that there is no apparent disturbance in purine levels as evidenced by: (i) allantoin excretion[22]; (ii) acid-soluble ribonucleotides[7] and (iii) no decrease in liver nucleic acid content.[7, 20, 21, 23]

The relatively few results obtained in this study for mice fed a diet rich in Griseofulvin also fail to indicate any close connection between the onset of hepatic porphyria and appreciable changes in the NAD ratios. Griseofulvin administration is known to produce extensive alterations in porphyrin metabolism in the liver and bone marrow; there is a large-scale urinary excretion of porphyrin precursors within 2 days of commencing treatment.[24, 25] With prolonged treatment the liver becomes greatly enlarged and heavily pigmented with protoporphyrin.[24, 25] The very small number of experiments reported here for mice on a prolonged course of Griseofulvin also showed a very much increased liver weight and also that this was accompanied by a similarly extensive net synthesis of NAD.

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REFERENCES

1. S. Granick, *Trans. N.Y. Acad. Sci.* **25**, 53 (1962).
2. C. Rimington, *Ann. N.Y. Acad. Sci.* **104**, 658 (1963).
3. D. P. Tschudy, *J. Am. Med. Ass.* **191**, 718 (1965).
4. A. Goldberg and C. Rimington, *Proc. R. Soc.* B **143**, 257 (1955).
5. R. Schmid in *The Biosynthesis of Porphyrins and Porphyrin Metabolism* (Ed. G. E. W. Wolstenholme) p. 262, Churchill, London (1955).

6. R. F. LABBE, E. L. TALMAN and R. A. ALDRICH *Fedn. Proc.* **14**, 241 (1955).
7. F. DE MATTEIS, T. F. SLATER and D. Y. WANG, *Biochim. Biophys. Acta.* **68**, 100 (1963).
8. T. F. SLATER, B. SAWYER and U. STRAULI, *Archs int. Physiol. Biochim.* **72**, 427 (1964).
9. C. RIMINGTON and G. ZEIGLER, *Biochem. Pharmac.* **12**, 1387 (1963).
10. K. BURTON, *Biochem. J.* **62**, 315 (1956).
11. A. L. GREENBAUM and T. F. SLATER, *Biochem. J.* **66**, 151 (1959).
12. R. B. HULBERT, H. SCHMITZ, A. F. BRUMM and V. R. POTTER, *J. biol. Chem.* **209**, 23 (1954).
13. C. RIMINGTON, *Panminerva med.* **4**, 307 (1962).
14. S. SANO and C. RIMINGTON, *Biochem. J.* **86**, 203 (1963).
15. A. J. M. REESE and C. RIMINGTON, *Br. J. exp. Path.* **45**, 30 (1964).
16. T. F. SLATER, B. SAWYER, and U. STRAULI, *Biochem. J.* **93**, 267, (1964).
17. H. FRUNDER, E. BLUME, K. THIELMANN and H. BORNIG, *Hoppe-Seylers Z. physiol. Chem.* **325**, 146 (1961).
18. G. E. GLOCK and P. MCLEAN, *Expl. Cell Res.* **11**, 234 (1956).
19. T. F. SLATER, *Biochem. J.* In press (1966).
20. C. W. I. OWENS, Ph.D. Thesis, University of London (1965).
21. F. I. LOTTSFELDT and R. F. LABBE, *Proc. Soc. exp. Biol. Med.* **119**, 228 (1965).
22. F. DE MATTEIS and B. E. PRIOR, *Biochem. J.* **83**, 1 (1962).
23. A. MERCHANTE, B. L. WAJCHENBERG and S. SCHWARTZ, *Proc. Soc. exp. Biol. Med.* **95**, 221 (1957).
24. F. DE MATTEIS and C. RIMINGTON, *Br. J. Derm.* **75**, 91 (1963).
25. C. W. I. OWENS, *Br. J. Derm.* **76**, 81 (1964).
26. L. L. BARICH, J. SCHWARZ and D. L. BARICH, *Cancer Res.* **22**, 53 (1962).