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NICOTINAMIDE ADENINE DINUCLEOTIDES IN ACUTE LIVER INJURY: EFFECTS OF AZASERINE AND PUROMYCIN IN THE RAT

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Abstract—The effects of single doses of azaserine or puromycin on the nicotinamide adenine dinucleotides (NADs) in rat liver have been studied.

Azaserine produced a very rapid decrease in NAD + $NADH_2$ such that 30 min after dosing the sum was only 36 per cent of the control value. The sum NADP + $NADPH_2$ was not affected until considerably later in the injury process. Since NADP is synthesised via NAD, the relative rate of decrease of NADP + $NADPH_2$ compared to NAD + $NADH_2$ provides some indication of the turnover rate of NADP in the affected liver. The estimate for the "turnover time" of NADP in rat liver was 5 hr.

Puromycin had no significant effect on the levels of NAD, $NADH_2$, NADP and $NADPH_2$ in rat liver although the dose used is known to inhibit protein synthesis almost completely *in vivo*. It is concluded that enzymes concerned in the biosynthesis of NAD and NADP do not "turn-over" so rapidly that suppression of their biosynthesis for a 1 hr period significantly affects the overall content of nicotinamide adenine dinucleotides in the liver.

PREVIOUS reports from this laboratory have been concerned with changes in the hepatic concentrations of the NADs in experimentally induced acute liver injury.[1–3] The administration to rats of a variety of toxic materials that ultimately produce necrosis and/or fatty degeneration was found to produce early alterations in the levels and relative properties of the NADs. However, the pattern of the changes observed varied considerably with the particular toxic agent under consideration.

Administration of azaserine (*o*-diazoacetyl-L-serine) to *rats* produces hepatic necrosis together with accumulation of fat.[4] No data has, however, been published concerning the levels of NAD, $NADH_2$, NADP and $NADPH_2$ in *rat* liver during the development of the injury, although rapid changes in NAD and $NADH_2$ have been shown to occur in *mouse* liver.[5–7] Data on the changes occurring in these nucleotides in rats poisoned with azaserine would be of interest for two main reasons. Firstly it would enable a comparison of such changes to be made with previous studies on the rat using other hepatotoxins.[1–3] Secondly, since NADP is synthesised in liver via the cytoplasmic enzyme NAD-kinase,[8–11] and providing that the level of NAD in the rat liver falls precipitously as it does in the mouse, it is of interest to see how rapidly the level of NADP falls in comparison to the very rapid decrease in the substrate NAD; such data could provide information concerning the "turnover time" of NADP in rat liver.

Another substance that causes severe hepatic malfunction following parenteral administration is the antibiotic puromycin. This material inhibits protein synthesis

at the ribosomal level[12, 13] and, as a consequence, produces a fatty liver through the resulting failure in lipoprotein excretion.[14] The effect on protein synthesis in the rat following intraportal injection is extremely rapid and reproducible; almost complete inhibition was found within 15 min.[15] We have therefore studied the effects of puromycin administration on the levels of the nicotinamide adenine dinucleotides to see whether an almost complete and very rapid suppression of protein synthesis *per se* has any influence on those levels through an effect on the turnover of the enzymes involved in nucleotide biosynthesis.

METHODS

Adult female albino rats were used; body wt. approx 130 g. They were killed by cervical dislocation and pieces of liver immediately removed and used for the estimation of NADs by the method of Slater *et al.*[16]

Azaserine was dissolved in 0·9% saline and injected intraperitoneally with the rat under light ether anaesthesia; the dose was 20 mg/100 g body wt. Puromycin hydrochloride was dissolved in 0·9% saline; 10 mg/100 g body wt. was given intraportally as a neutral solution with the rat under deep ether anaesthesia which was maintained for the duration of the experiment. In each case, control rats received an equivalent volume of solvent under identical conditions.

RESULTS AND DISCUSSION

Azaserine

Tables 1 and 2 show the results found using azaserine. It can be seen that there was a very rapid decrease in the sum of NAD + $NADH_2$ following the administration of

TABLE 1. EFFECT OF THE ADMINISTRATION OF AZASERINE ON THE NAD AND $NADH_2$ CONTENT OF RAT LIVER

Time (hr)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt. (g)}} \times 100$	NAD + $NADH_2$ (μg/whole liver/100 g body wt.)	$\frac{\text{NAD}}{NADH_2}$
0·5	Control	2	5·49	2010	3·7
	Azaserine	2	5·33	734	2·8
1·0	Control	2	5·62	2545	4·2
	Azaserine	2	4·72	532	2·2
2·0	Control	4	5·60 ± 0·65	2257 ± 310	4·2 ± 0·34
	Azaserine	4	5·07 ± 0·27	522 ± 101	7·2 ± 2·33

the toxic agent (Table 1): this value had decreased to 36 per cent of the control group mean value after only 30 min of intoxication. The sum of NADP + $NADPH_2$, on the other hand, was not significantly changed 1 hr after azaserine dosing but then began to decrease quite rapidly; by 3 hr the sum had decreased to 53 per cent of the control group mean value. The overall picture just described for azaserine is similar to that found after dimethylnitrosamine administration[2] which also induces liver necrosis. Dosing with carbon tetrachloride,[1] however, produced an early drop in the sum of NADP + $NADPH_2$ with no immediate change in NAD + $NADH_2$.

The very rapid decrease found in *rat* liver NAD + $NADH_2$ following the administration of azaserine is in good agreement with previous studies in the *mouse*.[5–7] NAD is a substrate of the NAD-kinase reaction and it might be expected therefore that rapid depeletion of NAD would decrease the rate of synthesis of NADP that would subsequently show up as an overall decrease in the sum NADP + $NADPH_2$. But, as already mentioned, there is a period of approx 1 hr in the early phase of azaserine poisoning during which the liver has a much decreased content of NAD + $NADH_2$ and yet a more or less normal level of NADP + $NADPH_2$. Possible contributory effects to the observed lag between the fall in NAD + $NADH_2$ and the decrease in NADP + $NADPH_2$ are discussed below.

Firstly, the normal concentration of NAD in rat liver could be considerably in excess of normal requirements for NADP synthesis so that a significant decrease in NAD could be tolerated without overmuch affecting the rate of synthesis of NADP. This would seem unlikely however, since the concentration of NAD in rat liver cytoplasm is approx 0·6 mM whereas the apparent K_m(NAD) for the NAD-kinase reaction, calculated from the data of Clark *et al.*[17], is 5 mM. The cytoplasmic concentration of the other substrate in the NAD-kinase reaction, ATP, is approx 1 mM[18] in our rat colony and the apparent K_m(ATP) is 6 mM.[19] From this data the NAD-kinase reaction appears almost equally poised around the concentrations of both substrates NAD and ATP, and a significant decrease in either would be expected to decrease the rate of NADP synthesis.

TABLE 2. EFFECT OF THE ADMINISTRATION OF AZASERINE ON THE NADP AND $NADPH_2$ CONTENT OF RAT LIVER

Time (hr)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt. (g)}} \times 100$	NADP + $NADPH_2$ μg/whole liver/100 g body wt.	$\frac{NADPH_2}{NADP}$
0·5	Control	4	4·84 ± 0·31	869 ± 50	5·8 ± 0·28
	Azaserine	4	4·98 ± 0·27	809 ± 35	5·5 ± 0·83
1·0	Control	4	5·36 ± 0·25	924 ± 115	7·9 ± 1·02
	Azaserine	4	4·88 ± 0·29	933 ± 99	6·7 ± 0·96
2·0	Control	4	5·60 ± 0·65	1100 ± 36	7·9 ± 1·03
	Azaserine	4	5·07 ± 0·27	815 ± 76	5·7 ± 0·48
				P = 0·005	
3·0	Control	3	3·63 ± 0·31	932 ± 110	9·0 ± 0·30
	Azaserine	4	4·32 ± 0·10	490 ± 20	4·2 ± 0·49
				P < 0·005	

On the other hand, the normal rate of synthesis of NADP in rat liver may be relatively slow so that changes in the rate would take a considerable period to produce significant changes in the overall level of NADP + $NADPH_2$. In fact, using the data given in the previous paragraph and simple 2-substrate kinetics,[20] it can be calculated that the *in vivo* rate of NAD-kinase is unlikely to exceed one-sixtieth of the maximum *in vitro* rate obtained under optimal conditions. The maximal *in vitro* rate is capable of synthesising 2·5 mg NADP/g liver/hr[21] and the liver's content of NADP + $NADPH_2$ is approx 200 μg/g liver (Table 2). Thus a period of some 5 hr would be required to

synthesise the total amount of NADP + $NADPH_2$ in the liver under normal *in vivo* conditions. Such a value means that even if NADP synthesis via the NAD-kinase reaction were largely abolished, as is probably the case with azaserine due to NAD-depletion, a lag-period would be observed before a significant drop in NADP + $NADPH_2$ could be detected. After the lag-period, and assuming that catabolism proceeds unchanged whilst synthesis is greatly depressed, the rate of decrease in the sum NADP + $NADPH_2$ should be an approximate measure of the rate of synthesis under normal conditions. In fact from the data in Table 2 it can be seen by a limited extrapolation of the linear rate of decrease that the sum NADP + $NADPH_2$ originally present in the liver would have been half-depleted in $2\frac{1}{4}$ hr giving a total time for synthesis of liver NADP + $NADPH_2$ of some 4–5 hr, a figure of a similar order of magnitude to that derived by the above calculation.

From such discussion it appears that the synthesis of NADP *in vivo* is relatively slow compared to the maximal rate of the NAD-kinase measured *in vitro*. This relatively slow rate of synthesis *in vivo* of NADP appears to be the most likely cause of the time-lag in the decrease in NADP + $NADPH_2$ compared to the very rapid drop in NAD + $NADH_2$ observed after dosing with azaserine.

Puromycin

The results found for liver NAD + $NADH_2$ and for NADP + $NADPH_2$ following the intraportal administration of puromycin are given in Table 3 for time periods

TABLE 3. EFFECT OF PUROMYCIN ON THE NAD CONTENT OF RAT LIVER

Time (min)	Group	No. of rats	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$ (g)	NADP + $NADPH_2$	$\frac{NADPH_2}{NADP}$
30	Control	3	4·58 ± 0·40	941 ± 73	6·6 ± 1·0
	Puromycin	3	4·35 ± 0·22	914 ± 130	5·9 ± 0·9
60	Control	4	4·90 ± 0·06	1044 ± 45	9·2 ± 0·6
	Puromycin	4	4·51 ± 0·09	992 ± 59	7·4 ± 0·4
				NAD + $NADH_2$	$\frac{NAD}{NADH_2}$
30	Control	4	4·67 ± 0·29	1882 ± 252	4·1 ± 0·4
	Puromycin	4	4·38 ± 0·15	1764 ± 234	3·3 ± 0·2
60	Control	2	4·88	2207	3·3
	Puromycin	2	4·64	2009	5·1

30 min and 60 min after dosing. No evidence was obtained to suggest that significant decreases in the levels of the nicotinamide adenine dinucleotides occurred during the first hour of puromycin intoxication. During this period, and after an identical dose, protein synthesis has been repeatedly shown to be virtually completely abolished by puromycin administration.[15] It would seem reasonable to assume, therefore, as a working hypothesis, that the enzymes involved in nicotinamide

adenine dinucleotide (phosphate) synthesis and catabolism do not "turn over" so rapidly that inhibition of protein synthesis for 1 hr significantly affects the overall levels of the nucleotides.

The administration of carbon tetrachloride to rats produces centrilobular necrosis and fatty degeneration of the liver.[22] Rapid changes in the level of NADP + $NADPH_2$ in the liver following dosing with carbon tetrachloride have been reported.[1] It had previously been suggested that these changes in NADP + $NADPH_2$ are associated with the early reactions leading to necrosis rather than to the accumulation of fat The results of this investigation and of the previous study involving dosing with. ethionine[3] favour that view. Both puromycin and ethionine produce a fatty liver but no consistent early disturbances in liver nicotinamide adenine dinucleotides have been found.

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