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Control of the Steady-State Concentrations of the Nicotinamide Nucleotides in Rat Liver

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1. The effects of injecting nicotinamide, 5-methylnicotinamide, ethionine, nicotinamide + 5-methylnicotinamide and nicotinamide + ethionine on concentrations in rat liver of NAD, NADP and ATP were investigated up to 5hr. after injection. 2. Nicotinamide induced three- to four-fold increases in hepatic NAD concentration even in the presence of 5-methylnicotinamide or ethionine, whereas 5-methylnicotinamide or ethionine alone did not cause marked changes in hepatic NAD concentration. 3. Nicotinamide alone also induced a twofold increase in hepatic NADP concentration. However, in the presence of 5-methylnicotinamide + nicotinamide, the NADP concentration decreased by 25% after 5hr., and in the presence of nicotinamide + ethionine by 30% in the same time. In the presence of 5-methylnicotinamide or ethionine alone hepatic NADP concentrations fell by 50% after 5hr. 4. 5-Methylnicotinamide inhibited the microsomal NAD^+ glycohydrolase (EC 3.2.2.6) by 60% at a concentration of 1mM and the $NADP^+$ glycohydrolase by 40% at the same concentration. 5. The rat liver NAD^+ kinase (EC 2.7.1.23) was found to have $V_{max.}$ 4·83 μmoles/g. wet wt./hr. and K_m (NAD^+) 5·8mM. This enzyme was also inhibited by 5-methylnicotinamide in a 'mixed' fashion. 6. The results are discussed with respect to the control of NAD synthesis. It is suggested that *in vivo* the $NAD(P)^+$ glycohydrolases are effectively inactive and that the increased NAD concentrations induced by nicotinamide are due to increased substrate concentration available to both the nicotinamide and nicotinic acid pathways of NAD formation.

The nicotinamide nucleotides (NAD and NADP) may be important in mediating changes in the metabolic flux of a cell under varying conditions. They provide links between different metabolic pathways (Dickens, 1959), and by virtue of their redox state in particular cell compartments may influence the direction of metabolism of certain intermediates in competing pathways (e.g. glycolysis and gluconeogenesis; see Krebs, 1967). NAD has also been implicated in the control of cell division (Morton, 1958), particularly as one of the enzymes involved in its synthesis is located exclusively within the nucleus (Hogeboom & Schneider, 1952; Branster & Morton, 1956), and Kun, Langer, Ulrich, Holzer & Grunicke (1964) have suggested that certain carcinostatic agents act by decreasing the NAD content of the tumour cells.

Knowledge of the control of the biosynthesis of the nicotinamide nucleotides is therefore pertinent to an understanding of these functions of NAD and NADP. As with most cell constituents the steady-state concentration is the resultant of the synthetic and breakdown activities of the enzymes associated with the metabolism of these nucleotides. The synthesis of NAD, and by extension NADP, may occur by any of three routes; from nicotinamide directly (Kornberg, 1950; Dietrich, Fuller, Yero & Martinez, 1965, 1966; Grunicke *et al.* 1966); from nicotinamide via nicotinic acid and its derivatives (Preiss & Handler, 1957, 1958*a*,*b*); from tryptophan via quinolinic acid (Nishizuka & Hayaishi, 1963; Nakamura, Ikeda, Tsuji, Nishizuka & Hayaishi, 1963; Gholson, Ueda, Ogaswara & Henderson, 1964). The degradation of both NAD and NADP is carried out mainly by a $NAD(P)^+$ glycohydrolase (EC 3.2.2.6), although a small amount of the nicotinamide nucleotides may be destroyed by a non-specific pyrophosphatase (EC 3.6.1.9) (Kaplan, 1961).

It has been known for some time that the injection of suitable quantities of nicotinamide leads to a striking increase in the NAD content of the livers of mice and rats (Kaplan, Goldin, Humphreys & Stolzenbach, 1957; Petrack, Greengard & Kalinsky, 1966). This has led to the suggestion that the nicotinamide inhibits the normal breakdown of NAD by the NAD^+ glycohydrolases, thus implicating the latter enzymes in the control of

NAD concentration (Greengard, Kalinsky & Petrack, 1961; Green & Bodansky, 1963).

In the present study the role of nicotinamide in elevating the hepatic NAD concentrations was investigated by the use of the analogue 5-methylnicotinamide, which is known to be an inhibitor of the NAD^+ glycohydrolases (Kun *et al.* 1964) (cf. nicotinamide), but which does not act as a substrate for NAD synthesis. Further studies were carried out on the cellular ATP concentrations under various conditions to establish whether ATP has an important controlling function in the maintenance of nicotinamide nucleotide concentrations in rat liver. The results suggest that the NAD^+ glycohydrolases are unlikely to be significantly active *in vivo* and that the elevated NAD concentrations induced by injection of nicotinamide are most likely due to increased substrate availability. The experiments carried out in the presence of an ethionine-induced ATP deficiency indicate that the nicotinamide-induced NAD synthesis does not appear to be inhibited by the overall cellular ATP deficiency whereas the formation of NADP is diminished.

A preliminary communication on part of this work has appeared (Pinder & Clark, 1968).

METHODS

Animals. Female albino rats of Wistar strain (body wt. 140g.) were used in all these experiments. They were given stock diet 41B (Bruce & Parkes, 1949) *ad lib.* All animals were deprived of food but not water overnight (16hr.) before use except where stated otherwise.

Animals were injected intraperitoneally with 6ml. of 0·9% NaCl containing the following compound or combination of compounds: 0·57m-mole of nicotinamide (i.e. 500mg./kg. body wt.); 0·57m-mole of 5-methylnicotinamide (i.e. 557mg./kg. body wt.); 0·86m-moles of DL-ethionine (1g./kg. body wt.); 0·57m-mole of nicotinamide + 0·86m-mole of DL-ethionine; 0·57m-mole of 5-methylnicotinamide + 0·86m-mole of DL-ethionine. Control animals were injected intraperitoneally with 6ml. of 0·9% NaCl solution only.

Reagents. NAD^+, NADH, $NADP^+$, NADPH, phenazine methosulphate and DL-ethionine were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Yeast alcohol dehydrogenase (EC 1.1.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), 3-phosphoglycerate kinase (EC 2.7.2.3), glucose 6-phosphate and 3-phosphoglyceric acid were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). ATP was obtained from the Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Nicotinamide was purchased from Hopkin and Williams (Chadwell Heath, Essex) and 5-methylnicotinamide was a gift from Eli Lilly and Co. (Indianapolis, Ind., U.S.A.). All other chemicals were of A.R. grade where possible.

Assay of NAD, NADP and ATP. Liver tissue was extracted and assayed for content of NAD^+, NADH, $NADP^+$ and NADPH by the method of Greenbaum, Clark & McLean (1965*a*) and for ATP content by the method of Adams (1963).

Assay of $NAD(P)^+$ glycohydrolase (EC 3.2.2.6) and nucleotide pyrophosphatase (EC 3.6.1.9) activities. These enzyme activities have been localized in the microsomal fraction (Blake, Blake & Kun, 1967). The microsomes were prepared in 0·25M-sucrose–1mM-EDTA by centrifuging a 1:5 (w/v) liver homogenate at 10000*g* for 20min. at 2° in an MSE 18 centrifuge and then centrifuging the supernatant from this low-speed run at 105000*g* for 60min., also at 2°, in a MSE Superspeed 50 ultracentrifuge. The pellet formed from the high-speed centrifugation was made up 1:1 (w/v) in 0·25M-sucrose–1mM-EDTA giving a concentration of approx. 25mg. of microsomal protein/ml. The total amount of $NAD(P)^+$ destroyed by both $NAD(P)^+$ glycohydrolase and the nucleotide pyrophosphatase was assayed as follows. A tube containing 0·3ml. of 0·1M-sodium phosphate buffer, pH7·2, and 0·6ml. of microsomal preparation was preincubated at 37° for 3min. The reaction was started by adding 0·1ml. of 10mM-$NAD(P)^+$ and 0·1ml. samples of the reaction mixture were removed after 15sec., 45sec., 90sec., 2min., 4min., 6min. and 8min. and placed into tubes containing 0·5ml. of 0·15M-HCl. These tubes were then boiled at 100° for 2min. and the contents were neutralized with 0·2ml. of 0·25M-glycylglycine buffer, pH7·6, and 0·05ml. of 1·5M-NaOH. After suitable dilution the $NAD(P)^+$ content of the samples was assayed by the method of Greenbaum *et al.* (1965*a*).

The amount of $NAD(P)^+$ destroyed by the $NAD(P)^+$ glycohydrolase alone was assayed at similar times by the method of Kaplan (1955), based on monitoring the production of the NAD(P)–cyanide complex at 325nm.

The difference in the amounts of $NAD(P)^+$ destroyed as measured by these two methods gave an estimate of the nucleotide pyrophosphatase activity alone.

Assay of NAD^+ kinase (EC 2.7.1.23) activity. This enzyme was prepared as a supernatant fraction in 0·25M-sucrose–1mM-EDTA by centrifuging a 1:5 (w/v) liver homogenate at 105000*g* for 60min. in a MSE Superspeed 50 ultracentrifuge. The assay was carried out by the method of Greenbaum, Clark & McLean (1965*b*).

RESULTS

Choice of experimental conditions. As Blake, Blake, Loh & Kun (1967) reported that starvation augments the nicotinamide-induced hepatic NAD concentrations, all animals used were starved overnight. As shown in Tables 1 and 2, this led to a fall of almost 50% in the hepatic ATP content expressed per g. of tissue but only to a small, statistically insignificant, fall in the total NAD and NADP content expressed per g. of tissue.

It was also found that the injection procedure did not significantly alter the hepatic nicotinamide nucleotide concentrations or redox states (Table 2) when starved animals that had been injected with saline and killed immediately or injected and left for 1hr. and then killed were compared with non-injected starved animals. This is in conflict with the report of Yasin & Bergel (1963). However, it was found that the hepatic ATP content expressed

per g. wet wt. of tissue of starved rats injected with saline fell by 30% on injection but that it returned to the value of the non-injected starved animals by 1hr. (Table 1). To nullify any effects due to injection alone our control animals in all cases were injected with 6ml. of saline and left for 1hr., when they were killed and the hepatic nicotinamide nucleotide and ATP contents assayed.

Each group of animals with the appropriate controls was injected with one of five different compounds or combinations of compounds and the hepatic contents of NAD^+, NADH, $NADP^+$, NADPH and ATP were assayed at hourly intervals for the first 5hr. after injection. Figs. 1–5 show the changes in total NAD, NADP and ATP contents (expressed as μmoles/g. wet wt. of tissue) under these different conditions and Table 3 indicates the variation in the redox states of NAD and NADP.

Injection of nicotinamide. Fig. 1 shows the changes in total hepatic NAD, NADP and ATP content that

Table 1. *Effect of various treatments on hepatic ATP concentrations*

All animals used were female albino rats of approx. 140g. initial body wt. The fed animals were kept on a stock diet *ad lib.* and had mean body wt. 140±4·0g. and mean liver wt. 6·4±0·20g. when killed. The starved animals were deprived of food overnight (16hr.) and when killed had mean body wt. 131±2·8g. and mean liver wt. 4·2±0·1g. Those animals that were injected were given 6ml. of 0·9% NaCl intraperitoneally; one group were then killed immediately; the other group were left and killed 1hr. after the injection. ATP was assayed as indicated in the Methods section. The results are expressed as the means ± S.E.M. for at least six animals.

Treatment	ATP concentration (μmoles/g. wet wt.)	(μmoles/liver)
Fed	1·75±0·09	11·2±0·56
Starved	0·941±0·03	3·33±0·33
Starved and injected (used immediately)	0·635±0·06	2·66±0·39
Starved and injected (used after 1hr.)	0·958±0·07	3·96±0·27

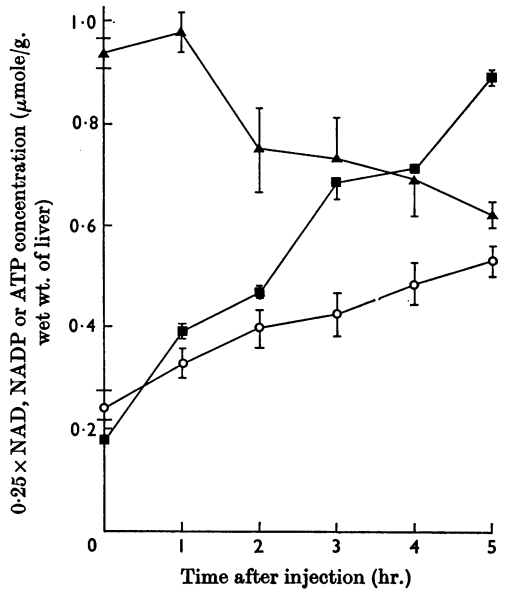

Fig. 1. Effect of nicotinamide on total hepatic concentrations of NAD (■), NADP (○) and ATP (▲). Female albino rats were starved overnight and injected intraperitoneally with 0·57m-mole of nicotinamide in 6ml. of 0·9% NaCl. The controls (zero time) were injected with 6ml. of 0·9% NaCl alone. The NAD, NADP and ATP contents of the liver were assayed as indicated in the Methods section at hourly intervals after injection, up to a maximum of 5hr. The results are expressed in μmoles/g. wet wt., each point being the average for six animals. The vertical bars represent twice the S.E.M.

Table 2. *Effect of various treatments on hepatic NAD and NADP concentrations*

The animals and treatments were as described in Table 1. The nicotinamide nucleotides were assayed as indicated in the Methods section. The results are expressed as means ± S.E.M. for at least six animals.

Treatment	NAD concentration (nmoles/g. wet wt.)				NADP concentration (nmoles/g. wet wt.)			
	NAD^+	NADH	ΣNAD	NAD^+/NADH	$NADP^+$	NADPH	ΣNADP	NADPH/$NADP^+$
Fed	699±17	243±24	943±10	3·2±0·34	26±4·4	330±28	355±30	15·7±3·3
Starved	680±36	164±12	823±30	4·0±0·4	24±2·7	286±10	310±11	12·9±2
Starved and injected (used immediately)	642±32	176±21	813±48	3·7±0·3	22±2·7	315±36	336±35	14±2·8
Starved and injected (used after 1hr.)	632±47	158±15	784±57	4·0±0·4	20±3·1	339±13	358±14	14·8±1·8

Table 3. *Effect of various agents on the redox state of the hepatic nicotinamide nucleotides*

All animals were female albino rats of initial body wt. 140±4g. They were starved overnight (16hr.) and when killed had mean body wt. 131±2·8g. and mean liver wt. 4·2±0·1g. The animals were treated as described in the Methods section and the controls were injected with saline and used 1hr. after injection. The nicotinamide nucleotides were assayed as indicated in the Methods section. The values are expressed as the means for at least six animals.

Time after treatment (hr.)	Nicotinamide	5-Methylnicotinamide	Nicotinamide+5-methylnicotinamide	Ethionine	Ethionine+5-methylnicotinamide
	NAD+/NADH ratio				
0	4·2	4·6	3·2	3·6	3·2
1	7·5	9·6	7·5	6·2	9·1
2	5·8	6·8	7·4	3·7	8·7
3	7·4	7·1	8·1	3·3	11·2
4	6·2	4·5	6·4	3·7	8·3
5	7·7	5·6	7·6	4·1	10·7
	NADPH/NADP+ ratio				
0	15·5	15·6	12·6	17·2	11·1
1	16·4	15·1	13·0	10·0	12·8
2	14·1	13·8	19·3	9·7	6·1
3	17·6	12·2	12·5	10·5	3·6
4	16·9	11·7	14·8	10·5	5·3
5	18·5	12·9	12·4	9·4	4·9

occurred during the first 5hr. after injection of nicotinamide (see the Methods section for details). The total NAD content rose continuously at an average rate of approx. 550nmoles/g. of tissue/hr., so that at 5hr. the total hepatic NAD content was about five times that of the control, whereas the NADP content only increased marginally at a rate of approx. 55nmoles/g./hr. to just over twice the control value (cf. Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Greengard, Quinn & Reid, 1964). Thus the rate of increase of NAD appeared to be an order of magnitude greater than that of NADP. The major part of the NAD formed after injection of nicotinamide was in the oxidized form, leading to an increase in the NAD+/NADH ratio for the cell (Table 3). The slightly increased amount of NADP was, however, mainly in the reduced form, leading to only a small increase in the NADPH/NADP+ ratio (Table 3). The ATP concentration, however, fell after the first hour and at 5hr. was only 65% of the zero-time value (cf. Dietrich & Yero, 1964).

Injection of 5-methylnicotinamide. As the increase in NAD induced by nicotinamide may be attributed either to increase in substrate availability or to inhibition of the NAD-breakdown enzymes or to a combination of the two, it was decided to inject an equivalent quantity of 5-methylnicotinamide, an analogue that is as potent an inhibitor of the NAD-breakdown enzymes but that is inactive as a substrate for NAD formation (Kun *et al.* 1964).

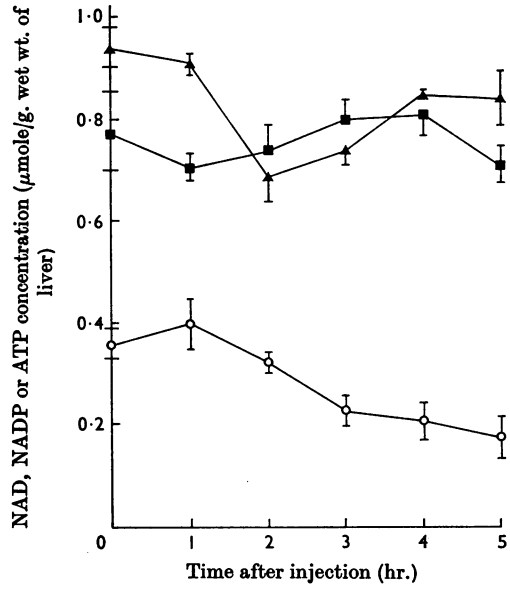

Fig. 2. Effect of 5-methylnicotinamide on total hepatic concentrations of NAD (■), NADP (○) and ATP (▲). Female albino rats were starved overnight and injected intraperitoneally with 0·57m-mole of 5-methylnicotinamide in 6ml. of 0·9% NaCl. The controls (zero time) were injected with 6ml. of 0·9% NaCl alone. All other conditions were as described in Fig. 1. The results are expressed in μmoles/g. wet wt., each point being the average for six animals. The vertical bars represent twice the S.E.M.

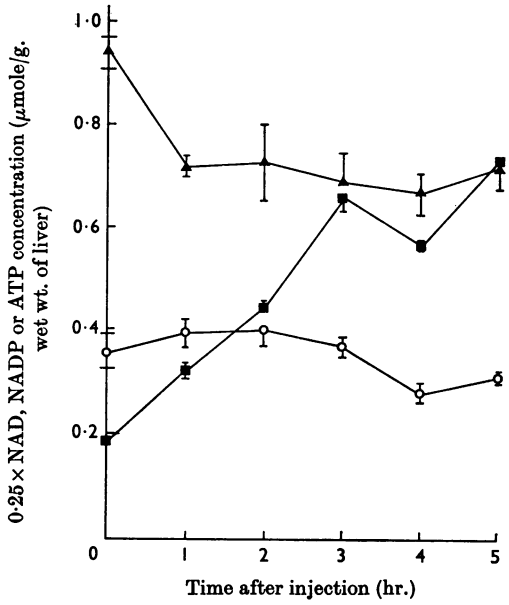

Fig. 3. Effect of nicotinamide + 5-methylnicotinamide on total hepatic concentrations of NAD (■), NADP (○) and ATP (▲). Female albino rats were starved overnight and injected intraperitoneally with 0·57m-mole of nicotinamide + 0·57m-mole of 5-methylnicotinamide in 6ml. of 0·9% NaCl. All other conditions, and the controls, were as described in Fig. 1. The results are expressed in μmoles/g. wet wt., each point being the average for six animals. The vertical bars represent twice the S.E.M.

Fig. 2 shows the results obtained. The total hepatic NAD content varied slightly over the 5hr. but by no more than ±8% of the control value; hence effectively the NAD concentrations remained constant. The total NADP concentration, however, fell markedly until at 5hr. it was about 50% of the control value. 5-Methylnicotinamide did not affect the $NAD^+/NADH$ ratio very significantly although there was a slight increase (Table 3), whereas the fall in NADP content was mainly in the form of NADPH, leading to a decreased $NADPH/NADP^+$ ratio (Table 3). In the presence of 5-methylnicotinamide the cellular ATP concentration initially fell to 75% of the control value at 2hr., but then recovered and at 5hr. was about 90% of the control value (Fig. 2).

Injection of nicotinamide + 5-methylnicotinamide. 5-Methylnicotinamide may also act as an inhibitor of one of the enzymes of NAD synthesis, NMN pyrophosphorylase (EC 2.4.2.12) (Grunicke *et al.* 1966), at the same time as acting as an inhibitor of the NAD^+ glycohydrolases. As this could account for the lack of effect on the hepatic NAD concentrations, an equimolar mixture of nicotinamide and 5-methylnicotinamide was injected. These results are shown in Fig. 3. The total hepatic NAD concentrations increased from 0·75 μmole/g. to approx. 2·9 μmoles/g. at a rate of 470nmoles/g. of tissue/hr. This increase in NAD was about 15% less than that which occurred in the presence of nicotinamide alone, suggesting the possibility of a slight inhibition. There was, however, no net increase in the total NADP concentration in the first 3hr., but it then fell by 20–25% of the control value during the next 2hr. It appeared therefore that the increased NAD concentrations induced by nicotinamide to some extent protected the NADP concentration against the effects of 5-methylnicotinamide. As far as the $NAD^+/NADH$ ratios are concerned, there appeared to be little difference from that when nicotinamide alone was injected, i.e. the increased amount of NAD was mainly in the oxidized form, leading to an increased $NAD^+/NADH$ ratio (Table 3). The $NADPH/NADP^+$ ratio increased temporarily at 2hr. but in general remained unchanged, following the pattern set by 5-methylnicotinamide alone. There was, however, a significant 25% fall in the ATP concentration after only 1hr., this fall being maintained over the next 4hr. (Fig. 3). This depletion of ATP was similar in degree and extent to that caused by injection of nicotinamide alone (Fig. 1).

Injection of ethionine. Since the fall in ATP concentration seemed to be an effect that occurred on injection of nicotinamide it was decided to study the effects of nicotinamide-induced elevation of NAD concentration in the presence of a cellular ATP deficiency induced by another agent, ethionine. Animals were therefore injected with ethionine alone to serve as a control to those injected with nicotinamide + ethionine. The animals injected with ethionine alone showed similar effects to those observed previously (Shull, 1962; Greenbaum, Clark & McLean, 1964; Clark & Pinder, 1966) (Fig. 4). The total hepatic NAD concentrations remained essentially constant over the 5hr. and, although a transient decrease occurred after 3hr., the values returned to the control value at 5hr. The total NADP concentration, however, decreased by about 50% over the same time. There was little change in the redox state of NAD as indicated by the $NAD^+/NADH$ ratios (Table 3), whereas the decrease in the $NADPH/NADP^+$ ratio (Table 3) indicated that most of the decrease in NADP content occurred from the reduced form. The ATP concentration fell rapidly to 50% of the control value after 2hr. and remained in the region of 50–55% of the control value up to 5hr. after injection.

Injection of nicotinamide + ethionine. When both nicotinamide and ethionine were injected at the same time, the total hepatic NAD concentrations rose to about four times the control value. The total

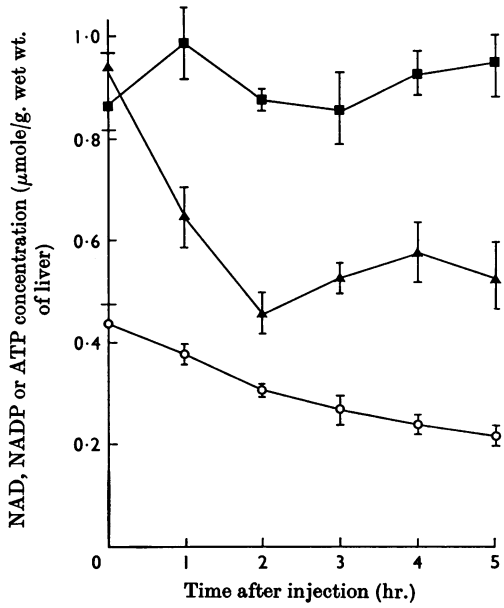

Fig. 4. Effect of ethionine on total hepatic concentrations of NAD (■), NADP (○) and ATP (▲). Female albino rats were starved overnight and injected intraperitoneally with 0·86 m-mole of DL-ethionine in 6 ml. of 0·9% NaCl. All other conditions, and the controls, were as described in Fig. 1. The results are expressed in μmoles/g. wet wt., each point being the average for six animals. The vertical bars represent twice the S.E.M.

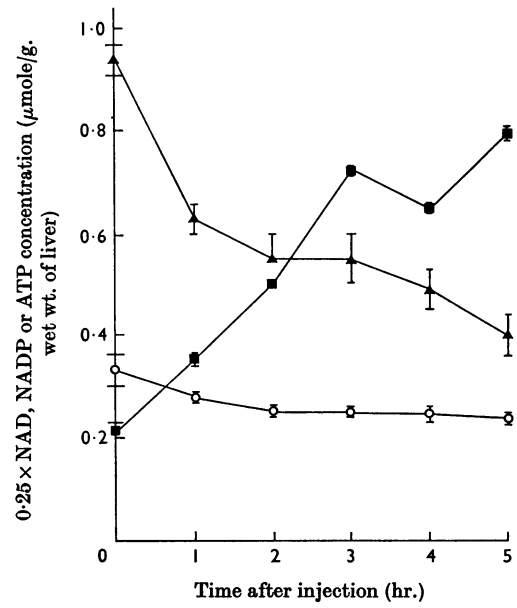

Fig. 5. Effect of ethionine + nicotinamide on total hepatic concentration of NAD (■), NADP (○) and ATP (▲). Female albino rats were starved overnight and injected intraperitoneally with 0·86 m-mole of DL-ethionine + 0·57 m-mole of nicotinamide in 5 ml. of 0·9% NaCl. All other conditions, and the controls, were as described in Fig. 1. The results are expressed in μmoles/g. wet wt., each point being the average for six animals. The vertical bars represent twice the S.E.M.

NAD concentration at 5 hr., 3·2 μmoles of NAD/g. of tissue, and the rate of NAD synthesis, 500 nmoles/g./hr., were both marginally (10%) less than the same parameters measured after the injection of nicotinamide alone. However, the NADP concentrations fell only by 30% in the presence of nicotinamide + ethionine (Fig. 5), compared with 50% in the presence of ethionine alone (Fig. 4). The NAD^+/NADH ratios showed a marked increase in the presence of nicotinamide + ethionine whereas the NADPH/$NADP^+$ ratio showed a distinct decrease (Table 3). The hepatic ATP concentration showed a more marked decrease (60% decrease at 5 hr.) than that caused by either nicotinamide (37% decrease at 5 hr.) or ethionine (50% decrease at 5 hr.) alone (Fig. 5).

Enzymic studies with 5-methylnicotinamide. The fact that the hepatic NAD concentration was unaffected by 5-methylnicotinamide suggested the following possibilities: (*a*) the NAD-breakdown enzymes were not inhibited by 5-methylnicotinamide as previously suggested (Kun *et al.* 1964); (*b*) the NAD-breakdown enzymes were not active *in vivo* and hence 5-methylnicotinamide would not be expected to have an effect; (*c*) the 5-methylnicotinamide was not getting into the cell at the correct place and concentration for inhibition to occur. 5-Methylnicotinamide appears to get into the cell, since an effect was observed on the NADP concentrations that was not apparent in the controls. The effect of 5-methylnicotinamide was therefore studied *in vitro* on the $NAD(P)^+$ glycohydrolases and on NAD^+ kinase (the enzyme responsible for NADP formation). This was to check the reported inhibition of the $NAD(P)^+$ glycohydrolase by 5-methylnicotinamide (Kun *et al.* 1964) and also to test whether the NAD^+ kinase was inhibited by 5-methylnicotinamide, since on injection of this compound the hepatic NADP concentrations decreased by 50%.

Effect of 5-methylnicotinamide on $NAD(P)^+$ glycohydrolase. Initial studies confirmed the observations by Jacobson & Kaplan (1957) and Greenbaum *et al.* (1965*b*) that rat liver contains only a very small nucleotide pyrophosphatase activity, contributing only about 5% of the total NAD(P)-destruction capability of the cell. The studies on the effect of 5-methylnicotinamide on destruction of NAD(P)

Table 4. *Effect of 5-methylnicotinamide on NAD(P)+ glycohydrolase activity*

The enzyme source in each case was a microsomal preparation as outlined in the Methods section. The NAD(P)+ glycohydrolase activity (expressed as initial velocity, v) was assayed as indicated in the Methods section.

Substrate	v (μmoles of substrate destroyed/g. wet wt. of liver/hr.)	Final concn. of 5-methylnicotinamide (mM)	Inhibition (%)
NAD	12·0	0	0
	4·65	1·3	61
	3·25	13	73
	0	130	100
NADP	6·72	0	0
	3·83	1·3	43
	2·89	13	57
	0	130	100

Table 5. *Effect of 5-methylnicotinamide on NAD+ kinase activity*

The enzyme source was in each case a supernatant fraction prepared as outlined in the Methods section. The NAD+ kinase activity was measured at each inhibitor concentration. The Michaelis parameters and their standard errors were calculated for each set of results by a weighted regression analysis run on the University of London Atlas computer.

Final concn. of 5-methylnicotinamide (mM)	Michaelis parameters of NAD+ kinase: K_m (mM)	$V_{max.}$ (μmoles of NADP+ formed/g. wet wt. of tissue/hr.)
0	5·8±1·3	4·83±0·64
1·3	3·36±0·96	3·67±0·58
13	3·60±0·8	2·62±0·49
130	3·80±1·32	2·86±0·58

were therefore confined to the NAD(P)+ glycohydrolase as assayed by the cyanide method of Kaplan (1955).

It was found that 5-methylnicotinamide was a potent inhibitor of both NAD+ and NADP+ glycohydrolase activities, 61% inhibition occurring of the NAD+ glycohydrolase at an inhibitor concentration of approx. 1mM and 43% inhibition occurring of the NADP+ glycohydrolase activity at the same inhibitor concentration (Table 4). Complete inhibition of both activities occurred at an inhibitor concentration of 100mM. From assays of the NAD(P)+ glycohydrolase activities of microsomal preparations from control animals it also became apparent that NAD was split about twice as rapidly as NADP (cf. Swislocki, Kalish, Chasalow & Kaplan, 1967; Bock, Gang, Beer, Kronau & Grunicke, 1968). Also, there was no difference in the NAD(P)+ glycohydrolase activity of microsomes that had been isolated from animals treated with 5-methylnicotinamide, compared with non-treated animals. It was therefore apparent that a differential effect by 5-methylnicotinamide on the NAD(P)+ glycohydrolase with respect to its two substrates, NAD and NADP, was an unlikely explanation of why NAD concentration remained unchanged and NADP concentration fell *in vivo* after injection of 5-methylnicotinamide. Accordingly the effects of 5-methylnicotinamide on the NADP-synthesizing enzyme, NAD+ kinase, were the subject of some preliminary investigations.

Effect of 5-methylnicotinamide on NAD+ kinase. The initial velocity of a NAD+ kinase preparation at various substrate (NAD) concentrations was assayed at four different inhibitor concentrations. The results were assessed for the Michaelis–Menten parameters, K_m and $V_{max.}$, by a computer programme that fitted the results to the best Lineweaver–Burk plot and then by a weighted regression analysis calculated the best Michaelis parameters and their standard errors.

The results are shown in Table 5, which shows that the inhibition of NAD+ kinase by 5-methylnicotinamide appears to be of a 'mixed' nature. In the presence of any of the concentrations of the inhibitor the K_m falls to a value that is about 60% of the K_m of the uninhibited enzyme, whereas the $V_{max.}$ falls to 75% of the uninhibited value in the presence of 1mM inhibitor and to 55–60% of the value for uninhibited enzyme at inhibitor concentrations of 10–100mM. Hence, although the nature and mechanism of this inhibition is unclear from these preliminary results, it is apparent that some inhibition of the NAD+ kinase activity may occur at inhibitor concentrations that *in vitro* inhibit the NAD(P)+ glycohydrolase by 50% (i.e. 1mM).

DISCUSSION

On theoretical grounds it must be assumed that the NAD(P)+ glycohydrolase cannot be very active *in vivo*, at least not based on the optimum activities measured *in vitro*. From the activities measured in the present experiments (Table 4) and also from other work (Roitt, 1956; Jacobson & Kaplan, 1957; Greenbaum *et al.* 1965*b*) it was calculated that it would take the NAD(P)+ glycohydrolase only a few minutes to destroy the total liver content of NAD and NADP should it be active at anywhere near $V_{max.}$. This is not proved by the few preliminary estimates that are available for the turnover of

NAD and NADP. Slater & Sawyer (1966) have estimated that NADP has a turnover time of about 4–5 hr. *in vivo*, which would be accounted for by about 1% of the $NADP^+$ glycohydrolase activity *in vitro*. If in fact the $NAD(P)^+$ glycohydrolase were only active to this minute extent (1%), this would explain why injection of 5-methylnicotinamide does not cause an increase in the NAD concentration (Fig. 2), since it is probably only at fairly high inhibitor concentrations that total (100%) inhibition is achieved (Table 4).

A second possible explanation of the lack of increase in NAD concentrations is that the inhibition of the $NAD(P)^+$ glycohydrolase is offset by an equal inhibition of the enzymes synthesizing NAD, particularly as Grunicke *et al.* (1966) have reported a 50% inhibition of NMN pyrophosphorylase by 5-methylnicotinamide at a concentration of 50 mM in a cell-free extract of ascites-tumour cells, this concentration of 5-methylnicotinamide also inhibiting the NAD^+ glycohydrolase by 95%. This seems unlikely in this experiment since, when 5-methylnicotinamide and nicotinamide are injected simultaneously, the rate and extent of nicotinamide-induced elevation of NAD concentration (Fig. 3) is only about 15% below that observed when nicotinamide alone is injected (Fig. 1), suggesting only a very slight inhibition of NAD synthesis, if any at all.

A further possible explanation of the lack of effect of 5-methylnicotinamide on NAD concentrations is that the 5-methylnicotinamide is unable to traverse the cell membrane, thus rendering it inoperative. This appears unlikely on the basis of the effects on NADP content of the injection of 5-methylnicotinamide alone, where a marked decrease in the NADP concentrations is apparent. This effect is most likely due to an inhibition of NAD^+ kinase, the enzyme responsible for forming NADP, since 5-methylnicotinamide was shown to be an effective inhibitor of the $NADP^+$ glycohydrolase *in vitro* (Table 2), which by itself would be expected to increase the NADP concentrations if anything. Preliminary studies *in vitro* of inhibition of NAD^+ kinase by 5-methylnicotinamide (Table 5) suggest that this compound inhibits NAD^+ kinase in a 'mixed' fashion, lowering the $V_{max.}$ in a concentration-correlated fashion but also decreasing the K_m in an uncorrelated way. The fact that the fall in NADP concentration is not so large when nicotinamide and 5-methylnicotinamide are injected together suggests that the increased NAD concentrations induced by nicotinamide protect NAD^+ kinase from inhibition by 5-methylnicotinamide, either because nicotinamide is a much less effective inhibitor of NAD^+ kinase than 5-methylnicotinamide or because the increased NAD concentrations induced by the nicotinamide enable the NAD kinase to synthesize NADP at a greater rate and hence give a net decrease in the fall in NADP concentration.

If, as these experiments appear to indicate, the effect of injecting nicotinamide is merely one of increasing the availability of substrate for NAD synthesis and not one of inhibiting NAD breakdown, it is important to establish how nicotinamide does this. The rate of increase of NAD content after injection of nicotinamide is approx. 550 nmoles/g. of tissue/hr. If the maximal activities *in vitro* of all the enzymes for biosynthesis of NAD from nicotinamide by either route are considered (see Greenbaum & Pinder, 1968) it becomes apparent that the two rate-limiting enzymes in each pathway are the NMN pyrophosphorylase and nicotinic acid mononucleotide pyrophosphorylase with activities of 165 nmoles/g./hr. and 336 nmoles/g./hr. respectively (see Dietrich *et al.* 1966; Shimoyama, Kori, Usuki, Lan & Gholson, 1965). Accepting these values as approximations to the maximum activity *in vivo*, it is apparent that the maximum rate of NAD synthesis by both pathways is about 500 nmoles/g./hr. Allowing for enzyme destroyed on extraction and experimental error this agrees reasonably closely with the observed rate of NAD formation in the presence of excess of nicotinamide, of 550 nmoles/g./hr. It has also been found (Greengard *et al.* 1964) that nicotinamide in excess of the dose used in these experiments (500 mg./kg. body wt.) does not increase the rate of NAD augmentation. It may be suggested therefore that in the presence of large amounts of nicotinamide both the nicotinamide (i.e. via the amidated derivatives) and nicotinic acid (i.e. via the deamidated derivatives) pathways of NAD formation are operative, whereas under normal physiological conditions only one of these pathways is functioning.

Chaykin (1967) has suggested several points that support the contention that the pathway operative under normal conditions is the pathway that proceeds via NMN. The conclusions arrived at from the experiments reported here would fit in well with this suggestion. As NAD^+ glycohydrolase is not normally very active *in vivo*, the increased NAD concentrations induced by nicotinamide would then be due to the fact that the intracellular nicotinamide concentrations would be sufficiently high not only to saturate the normally active pathway via NMN but also to allow a reasonable activity of the nicotinamide deamidase to provide saturating concentrations of nicotinic acid for the alternative pathway. The increased NAD concentrations would then be of a magnitude established by the maximum rates of the two rate-limiting enzymes in each pathway, i.e. NMN pyrophosphorylase and nicotinic acid mononucleotide pyrophosphorylase.

Under such circumstances of high concentrations of nicotinamide the only other substrate that could

become rate-limiting is ATP or phosphoribosyl pyrophosphate and it is noteworthy that on the injection of nicotinamide there is a fall in ATP concentration and a subsequent elevation of NAD concentration (Fig. 1) (cf. Dietrich & Yero, 1964; Blake *et al.* 1967). It is possible that this decrease in cellular ATP concentration caused by the increased rate of NAD formation may in part be responsible for the only slight increase in NADP content. The fact that the NAD-biosynthetic system appears to be able to maintain its activity in spite of a cellular ATP deficiency is evident from the results of the experiments after injection of ethionine alone and ethionine + nicotinamide (Figs. 4 and 5). These results have important reflections on the availability of ATP within the different cell compartments. Ethionine causes a generalized cellular ATP deficiency and in these circumstances NADP synthesis is drastically impaired whereas the ability of nicotinamide to induce increased NAD synthesis is in no way affected. The enzymic step in NAD synthesis that requires ATP directly as a substrate in either pathway (NMN adenylyltransferase, EC 2.7.7.1) is exclusively located in the nucleus, whereas the NAD^+ kinase step, which also requires ATP for the formation of NADP from NAD, is located in the cytoplasm. It appears therefore either that nuclear systems draw their ATP from different sources to the cytoplasmic systems and ethionine only affects the cytoplasmic system, or that the nucleus is able to maintain its ATP concentrations most efficiently even in the presence of a 50% fall in total cellular ATP concentration. Okazaki, Shull & Farber (1968) have reported that nuclei of the livers of rats treated with ethionine show a 50% decrease in ATP content as compared with a total cell decrease in ATP of about 75%. G. Siebert (personal communication), however, has found that similarly prepared nuclei do not show any decrease in ATP content, all the loss occurring in the cytoplasmic fraction. From the present results it is obvious that the NAD-synthesizing system can maintain full activity in spite of the large decrease in cellular ATP concentration. This suggests that the nucleus may play an important part in the control of NAD synthesis over and above its function as the site of the NMN adenylyltransferase. In particular, *in vivo* it may possess the ability to maintain sufficient ATP concentrations to allow its activities (e.g. DNA formation, messenger RNA formation, NAD synthesis) to proceed unimpaired in the presence of generalized cellular ATP depletion.

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