The Activities of Nicotinamide Mononucleotide Adenylyltransferase and of Nicotinamide-Adenine Dinucleotide Kinase in the Livers of Rats Subjected to Different Hormonal Treatments

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1. The activities of NMN adenylyltransferase and of NAD⁺ kinase have been measured in the livers of adrenalectomized or alloxan-diabetic rats and in the livers of rats treated with glucagon, pituitary growth hormone or thyroxine. 2. The activities of these enzymes have been compared with the effects of the same treatments on the nicotinamide nucleotide concentrations in the liver. 3. Alloxan-diabetes (+37%) and thyroxine (+27%) both increased the activity of NMN adenylyltransferase. The other treatments were without effect on this enzyme. 4. Only thyroxine increased the activity of NAD⁺ kinase significantly (+26%) although both adrenalectomy and glucagon tended to increase its activity. 5. The activity of NAD⁺ glycohydrolase was measured in the livers of diabetic rats, and in the livers of rats treated with either growth hormone or thyroxine. Of these treatments, only growth hormone altered the enzyme activity (+26%, calculated on a total hepatic activity basis). 6. Female rats had a greater hepatic NAD⁺-kinase activity than males but there was no sex difference with respect to NMN adenylyltransferase. 7. The lack of correlation between the maximum potential activity of these three enzymes and the known changes of the nicotinamide nucleotides in each of the hormone conditions is discussed.

The importance of the nicotinamide nucleotides as regulators of cellular metabolism is now recognized (see Dickens, 1959; Kaplan, 1961; Morton, 1961; Chance, 1961). Much of the work on this subject has been concerned with the concentration and oxidoreduction state of these compounds and less attention has been devoted to the enzymic factors concerned in maintaining the amounts of NAD and NADP in the cell, that is the enzymes involved in the synthesis and degradation of the nucleotides.

It seems that, in general, the concentration and relative amounts of the oxidized and reduced nicotinamide nucleotides remain fairly steady; for example, NADP is found in most cells in the reduced form and NAD mostly in the oxidized form. However, certain treatments and, in particular, hormonal factors, will cause changes in both the total amount and the ratios of the oxidized and reduced forms of these coenzymes. Where such shifts in concentration have been found it seemed important to measure enzymes concerned with both the synthesis and degradation of these co-

enzymes; even where steady amounts were maintained and therefore the balance between synthesis and degradation was constant, it was possible that the turnover rate had been changed. It was therefore decided to determine the rates of these processes. Although there are divergent views about the exact mode of NAD synthesis (see Kaplan, 1961), the pathway from nicotinamide mononucleotide is well established in liver and this is the route we have studied. The mechanism of synthesis of NADP, on the other hand, is generally agreed and consists of the single step catalysed by NAD⁺ kinase which phosphorylates NAD by ATP (Mehler, Kornberg, Grisolia & Ochoa, 1948; Wang & Kaplan, 1954).

The enzymes measured are NMN adenylyltransferase (EC 2.7.7.1), NAD⁺ kinase (EC 2.7.1.23) and NAD⁺ glycohydrolase (EC 3.2.2.5).

The conditions we have studied are the effects of adrenal cortical hormones, the pancreatic hormones insulin and glucagon, thyroxine and pituitary growth hormone, all of which have been previously studied for their effects on the concentration and

oxidoreduction state of the nicotinamide nucleotides in liver (Glock & McLean, 1955; Greenbaum & Graymore, 1956; Greenbaum, Clark & McLean, 1965b). It is concluded that there is no obvious correlation between the amounts of the nicotinamide nucleotides in the liver in these conditions and the activities of the enzymes involved in their synthesis. It seems that control may be exercised by other factors, e.g. the nucleotide precursors and, in particular for NADP, by ATP.

METHODS

Animals. Unless otherwise stated the rats used were an inbred strain of albinos with body weights of 140–160 g. and were pair-fed on a stock diet made by mixing 1.5 kg. of powdered Medical Research Council diet no. 41b with 300 ml. of 0.01% sodium alginate.

Adrenalectomized group. Immediately after bilateral adrenalectomy paired-feeding was begun and the rats were maintained on 1% NaCl throughout the experiment. Two weeks after the operation the animals were killed and samples of liver taken for the measurement of NMN-adenylyltransferase and NAD⁺-kinase activities.

Glucagon-treated group. Male rats were injected subcutaneously with three daily doses of glucagon (kindly given by Eli Lilly and Co., Basingstoke, Hants.), each of 100 µg., for 3 days. On the fourth day a final dose of 100 µg. of glucagon was given 1 hr. before killing. The rats were pair-fed throughout the experiment.

Growth-hormone-treated group. Normal female hooded Norway rats, body weight approx. 200 g., were used. The first group were given a single injection of 1 mg. of the hormone in 0.5 ml. of saline intraperitoneally and were killed 12 hr. later. These rats were not pair-fed. The second group were given intraperitoneal injections of 1 mg. of hormone, dissolved in 0.5 ml. of saline, daily for 3 days and were killed on the fourth day. The hormone was an ox-pituitary preparation kindly supplied by Armour and Co., Eastbourne, Sussex, and the Endocrinological Study Section of the National Institutes of Health, Bethesda, Md., U.S.A.

Diabetic animals. Male rats, with body weights of 220–250 g., were made diabetic by a single subcutaneous injection of alloxan monohydrate (19 mg./100 g. body wt.; Kass & Waisbren, 1945) after they had been starved for 48 hr. The alloxan was administered as a 4% solution in 0.1 M-sodium acetate buffer, pH 4.5 (Klebanoff & Greenbaum, 1954). Two units of insulin (protamine-zinc-insulin; Boots Pure Drug Co. Ltd.) were given 6 hr. later and then a further 2 units of insulin were injected daily for 6 days. During this period the rats had free access to food. Seven days after the alloxan injection the insulin treatment was stopped and a limit-feeding regime instituted (50 g./day). The rats were killed 3 weeks later. The rats had fed blood sugars of over 500 mg./100 ml. and were excreting between 8 and 11 g. of glucose a day.

Thyroxine-treated group. Male rats were injected subcutaneously with 1 mg. of DL-thyroxine daily for 5 days. The rats were pair-fed during the treatment on the stock diet supplemented by vitamin B complex (one tablet of Becosyn/300 g. of diet) in view of the results of Tipton (1950)

showing an extra vitamin B requirement in thyrotoxic rats. They were killed on the sixth day.

Enzymic procedures

Chemicals. NAD⁺, NADH, NADP⁺, NADPH, ATP and NMN were all obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Triethanolamine hydrochloride was obtained from Eastman-Kodak Ltd., Kirkby, Liverpool. All other chemicals and enzymes used were of AnalaR grade or from the same sources as used previously (Greenbaum *et al.* 1965a,b).

Estimation of NMN adenylyltransferase. The activity of this enzyme was estimated in liver homogenates prepared in 0.25 M-sucrose (1 g. of liver to 4 ml. of 0.25 M-sucrose) essentially according to the method of Kornberg (1950a). The reaction mixture used for the assay contained the following components: glycylglycine buffer, pH 7.6, 50 mM; NMN, 2 mM; ATP, 6 mM; MgCl₂, 4 mM; nicotinamide, 0.2 M. Conditions of the assay: 0.15 ml. of the assay mixture described above was added to 0.1 ml. of liver homogenate in 0.25 M-sucrose (1:5, w/v) and the whole incubated at 37° for 10 min. At the end of this time 0.5 ml. of 0.15 N-HCl was added and the whole mixture heated at 100° for 2 min. The mixture was cooled on ice, 0.2 ml. of 0.25 M-glycylglycine buffer, pH 7.6, added and the mixture neutralized with 0.05 ml. of 1.5 N-NaOH. The mixture was then centrifuged at 2000g for 5 min. The NAD⁺ formed was estimated by the method described by Greenbaum *et al.* (1965a). With these conditions the assay is linear for at least 15 min. and is directly proportional to enzyme concentration up to, at least, 30 mg. of tissue. The addition of nicotinamide to the assay system effectively blocked the breakdown of the NAD⁺ formed. In a model system in which the NMN was replaced by NAD⁺, i.e. 40 µg. of NAD⁺ was added at the beginning of the incubation period, not more than 3% of the NAD was lost during a 15 min. incubation.

The concentrations of NMN and ATP used here are the optimum concentrations described by Kornberg (1950a) and are, in fact, greater than those he usually used. We have investigated the effect of higher concentrations of these substances and found that increasing the NMN concentration from 2 to 4 mM causes a fall in the rate of NAD⁺ synthesis of some 35% and increasing the ATP concentration from 6 to 20 mM also decreases the rate of formation by some 30%. In all experiments where the ATP concentration was changed the Mg²⁺/ATP ratio was kept constant at 2:3. The two factors which could alter the apparent rate of NAD⁺ synthesis are the conversion of NAD⁺ into NADH and the further phosphorylation of NAD⁺ by ATP to NADP⁺. The conversion of NAD⁺ into the reduced form could occur by the action of dehydrogenases present in the whole liver homogenates, particularly since these preparations were not dialysed. Any NADH so formed would not normally be measured since it is very labile in dilute acid and would be destroyed by the extraction described above. Measurements of NADH produced during the incubation procedure were made by stopping the reaction with 0.1 N-NaOH, followed by neutralization. Since the NADH formed was only 6–10% of the NAD⁺, this was not normally measured. It was also found that very little NADP⁺ was formed under the conditions of the NAD⁺ assay, which may be a reflection of the higher *K_m* for ATP for NAD⁺ kinase (see below).

NMN adenylyltransferase is thought to be entirely nuclear in distribution (Hogeboom & Schneider, 1952). The studies of Hogeboom & Schneider (1952) and of Branster & Morton (1956) show that a better recovery of activity can be obtained with full homogenates than with a purified nuclear fraction. Liver homogenates in 0.25M-sucrose were therefore used directly without any further treatment. Further, such extracts are remarkably stable and lose very little of their activity even over 10 days when stored at -15° .

Estimation of NAD⁺ kinase. The procedure adopted for the assay of this enzyme is a modification of that described by Wang & Kaplan (1954). NAD⁺ kinase is located exclusively in the soluble fraction (Vilée & Hagerman, 1958; Stollar & Kaplan, 1961). In the present study the supernatant fraction was isolated either by the centrifugation of liver homogenates in 0.15M-KCl-0.16M-KHCO₃ at 135000g-min. or from homogenates in 0.25M-sucrose at 2.4×10^6 g-min. These two supernatant fractions were compared for their content of NAD⁺ kinase by the method given below. Both the activity and stability of the two preparations were essentially the same. As a routine procedure therefore the supernatant fraction used in these experiments was prepared from KCl-KHCO₃ homogenates, since lower centrifugal speeds were required.

Such preparations lose only about 10% of their activity when stored at -15° for 10 days, in agreement with the observations of Wang & Kaplan (1954), who found little loss of activity after 4 months at -15° .

In investigating the assay procedure we confirmed the observations of Wang & Kaplan (1954) on the optimum conditions and we used the same concentrations for NAD⁺, ATP, Mg²⁺ ions and nicotinamide. We substituted 100 μ -moles of triethanolamine buffer (pH 7.6) for the tris used by Wang & Kaplan (1954), since this consistently gave a small, but definite, increase in rate of NADP⁺ synthesis. The system was also modified by adding NaF immediately after the enzyme addition to inhibit adenosine triphosphatases. Wang & Kaplan measured the amount of NADP⁺ synthesized by estimating the amount of NADP⁺ present in a neutral extract of the incubation mixture. Since we used supernatant fractions in place of the purified enzyme system employed by Wang & Kaplan (1954), we thought that there could be appreciable amounts of substrate present for NADP⁺-linked dehydrogenases and this could lead to a disappearance of NADP⁺ and the formation of some NADPH. The incubated medium of a NAD⁺-kinase assay was therefore divided into two. One part was extracted in 0.1N-HCl for the NADP⁺ and the other part was extracted with 0.1N-NaOH for the NADPH. Approx. 25% of the NADP⁺ present was in the form NADPH and, furthermore, this figure was somewhat variable, depending on the metabolic state of the animal from which the liver was taken. To obviate this complication glucose 6-phosphate was added to the incubation mixture to ensure total conversion of the NADP⁺ into the reduced state. Although complete conversion occurs in the liver owing to the high activity of the glucose 6-phosphate-dehydrogenase content, this might not be true of other tissues and here this enzyme might have to be added as well. Thus it was by the amount of NADPH which accumulated, rather than by the amount of NADP⁺, that the NAD⁺ kinase was measured.

That this was a valid procedure can be deduced from the results of two assays, identical but for the fact that one

contained 2.5 μ moles of added glucose 6-phosphate and the other did not. After 15 min. incubation, acid and alkaline extracts were prepared from each of the assay mixtures. In the absence of glucose 6-phosphate 803 μ g. of NADP⁺/g. of liver and 207 μ g. of NADPH/g. of liver were formed, i.e. a total of 1010 μ g. of NADP/g. of liver. In the presence of glucose 6-phosphate no NADP⁺ was found but 957 μ g. of NADPH/g. of liver was formed.

In view of these results, which show a 95% recovery with only a single extraction, assays were always performed in the presence of added glucose 6-phosphate. Conversion of NADP⁺ into the reduced form may explain the increase in activity recovered which was observed by Wang & Kaplan (1954) after the first step of their purification procedure. The removal of substrates would prevent the loss of NADP⁺ through this conversion.

The final assay procedure therefore was as follows. Assay mixture: 6.7 μ moles of NAD⁺; 8 μ moles of ATP; 10 μ moles of nicotinamide; 10 μ moles of Mg²⁺; 100 μ moles of triethanolamine buffer, pH 7.6; 2.5 μ moles of glucose 6-phosphate; total vol., 0.85 ml. To this mixture was added 0.1 ml. of a supernatant fraction prepared as above, i.e. from a 20% (w/v) liver homogenate in KCl-KHCO₃ and, immediately afterwards, 0.05 ml. of 0.9M-NaF. This mixture was incubated at 37° for 10 min. and the reaction stopped by the addition of 0.1 ml. of N-NaOH. The mixture was heated at 100° for 2 min., cooled on ice, 0.1 ml. of 0.25M-glycylglycine buffer, pH 7.6, added and the whole neutralized by the addition of 0.1 ml. of N-HCl. After centrifuging at 600g for 5 min. to remove the precipitated protein, the NADPH was estimated by the method of Greenbaum *et al.* (1955a). Under these conditions the assay is linear for at least 20 min. with 30 mg. of tissue and is proportional to the amount of enzyme added up to the equivalent of at least three times the amount normally used.

Effect of NADH on NAD⁺ kinase. Wang & Kaplan (1954) demonstrated a competitive inhibition of NADP⁺ synthesis by NADH. Since some of the NAD⁺ added as substrate for the NAD⁺-kinase reaction might be converted into NADH by enzymes and endogenous substrates in the supernatant fractions used, and so inhibit the formation of NADP⁺, some experiments to measure this conversion were carried out. The alkaline extract made after an NAD⁺-kinase incubation contained measurable quantities of NADH. The amount formed was remarkably constant and amounted to 110 ± 8 μ g. (eight estimations) out of the 4.4 mg. of NAD⁺ added, i.e. a conversion of 2.5%. It can be calculated from the data of Wang & Kaplan (1954) that with an NAD⁺/NADH ratio 40:1 the inhibition of NADP⁺ synthesis would be no more than 5%.

The possibility that NADPH could also inhibit the rate of formation of NADP⁺, either by feed-back control or by other mechanisms, was also considered; but the figures given above in the experiment showing that the addition of glucose 6-phosphate to the reaction mixture allows the formation of an amount of NADPH equal to 95% of the sum of NADP⁺ + NADPH formed in the absence of this substrate demonstrate that such an inhibition, if it exists, is negligible.

Estimation of NAD⁺ glycohydrolase. The estimation of the cleavage of NAD⁺ was carried out by two procedures: (a) The disappearance of NAD⁺ as measured by the alcohol-dehydrogenase reaction and reading at 340 m μ . (b) By the

procedure of Colowick, Kaplan & Ciotti (1951) based on the cyanide reaction of NAD⁺ and measured at 325 m μ . The rate of cleavage of NAD⁺ as measured by both of these methods gave essentially similar results and it may be concluded, in agreement with Jacobson & Kaplan (1957), that rat liver contains virtually no NAD⁺-pyrophosphatase activity and that the measurements made in the present study with the assay of residual NAD⁺ by alcohol dehydrogenase is, in fact, a measurement of NAD⁺ glycohydrolase. The assay system used was: 50 μ moles of glycylglycine buffer, pH 7.6; 0.3 μ mole of NAD⁺; 0.2 ml. of liver homogenate (1:100 in 0.15 M-KCl-0.16 M-KHCO₃); total vol., 1 ml. The mixture was incubated for 20 min. at 37° and the reaction stopped by the addition of 1 ml. of N-HClO₄. After cooling and removal of the protein precipitate, the excess of HClO₄ was removed as KClO₄ by neutralization with 5 N-KOH and 1 ml. of the supernatant was used for NAD⁺ assay with alcohol dehydrogenase.

The unit of enzyme activity used throughout is defined as that amount of enzyme which synthesizes or degrades 1 μ mole of NAD or NADP/hr. at 37° under the conditions of the assay.

The results are presented as the means $\pm$ S.E.M. The values for *P* are given when this value is less than 0.1; above this value the term N.S. is given to signify 'not statistically significant'. Where controls and experimental

animals were pair-fed the results have been analysed by the 'paired-*t*' test.

RESULTS

For the results of the effect of hormonal treatments on the activity of enzymes in the liver it is probably more meaningful to consider changes only of the total hepatic enzymic activity or possibly of the hepatic activity/100 g. body wt., since changes in the degree of hydration of the liver or movements of the lipid into or out of this tissue could appreciably affect the concentration without affecting the overall activity.

NMN adenylyltransferase. The results showing the changes in activity of this enzyme as a result of treatment of the rats with different hormones are shown in Table 1. The decreased rate of synthesis of NAD⁺ found after adrenalectomy when the results are calculated on a concentration basis is probably not important biologically, since no difference between experimental and control can be seen when the results are calculated either as total hepatic activity or as activity/100 g. body wt.

Table 1. Activity of NMN adenylyltransferase in the livers of rats subjected to a variety of hormonal treatments

Hormonal treatment of the animals was as described in the Methods section. Enzymes were extracted and estimated as described in the Methods section and the values given are means $\pm$ S.E.M. Fisher's *P* represents the probability of the difference between the means. Values of *P* greater than 0.1 are shown as N.S., i.e. not significantly different. The adrenalectomized, thyroxine-treated and glucagon-treated groups were analysed by the method of corresponding pairs.

Treatment	No. of animals	Sex	Body wt. (g.)		Liver wt. (g.)	NAD synthesized		
			Initial	Final		(μmoles/hr./g.)	(μmoles/hr./total liver)	(μmoles/hr./100g. body wt.)
Control	6	♂	142 ± 2.3	150 ± 2.9	6.67 ± 0.25	5.56 ± 0.16	36.77 ± 2.01	24.58 ± 1.19
Adrenalectomized	6	♂	140 ± 1.8	158 ± 3.8	7.67 ± 0.38	4.93 ± 0.17	37.85 ± 2.44	23.84 ± 1.24
Fisher's <i>P</i>						0.002	N.S.	N.S.
Control	6	♂	152 ± 3.9	164 ± 5.2	7.26 ± 0.46	5.13 ± 0.52	36.65 ± 2.99	22.42 ± 1.83
Glucagon	6	♂	154 ± 4.2	156 ± 7.1	8.69 ± 0.47	4.42 ± 0.29	37.89 ± 3.55	24.53 ± 1.33
Fisher's <i>P</i>						N.S.	N.S.	N.S.
Control	6	♀	—	206 ± 12.5	8.4 ± 0.36	4.90 ± 0.25	41.16 ± 1.74	19.98 ± 0.94
Growth hormone (12 hr.)	6	♀	—	209 ± 1.8	7.02 ± 0.08	5.79 ± 0.31	40.66 ± 2.18	19.51 ± 1.09
Fisher's <i>P</i>						0.07	N.S.	N.S.
Control	6	♀	208 ± 11.1	207 ± 11.0	8.3 ± 0.30	4.96 ± 0.25	41.32 ± 1.71	19.96 ± 0.91
Growth hormone (3 days)	6	♀	217 ± 8.7	224 ± 1.0	7.93 ± 0.23	4.66 ± 0.35	36.69 ± 2.31	16.36 ± 1.02
Fisher's <i>P</i>						N.S.	N.S.	0.004
Control	5	♂	—	310 ± 7.8	11.7 ± 0.63	4.54 ± 0.31	52.91 ± 4.41	17.03 ± 1.13
Diabetic	5	♂	—	205 ± 17.7	9.7 ± 0.66	4.86 ± 0.40	46.80 ± 4.03	23.03 ± 2.20
Fisher's <i>P</i>						N.S.	N.S.	0.04
Control	6	♂	182 ± 3	208 ± 10	9.73 ± 0.30	5.29 ± 0.49	51.04 ± 3.65	24.53 ± 1.90
Thyroxine	6	♂	182 ± 3	181 ± 5	8.0 ± 0.40	8.04 ± 0.61	65.08 ± 5.05	35.90 ± 4.19
Fisher's <i>P</i>						0.005	0.05	0.03

No change was found after treatment with glucagon. When growth hormone was administered no change could be detected after 12 hr. treatment but after 3 days the rate of synthesis was depressed, significantly so when calculated as NAD⁺ synthesized in total liver/100 g. body wt.

In the diabetic group there is an increase of the rate of synthesis of NAD⁺ which is only significant when calculated on the basis of total liver activity/100 g. body wt. In this particular group this mode of expressing results has been chosen because of the great disparity of body weights in the control and experimental groups. The controls here are 'age controls', i.e. the two groups were identical in age and weight initially but while the control animals continued to grow slowly the experimental groups lost weight as a result of their diabetic condition. In rats treated with thyroxine there was an increased activity of the enzyme, which was statistically significant with all three methods of expressing the results. The results given in this Table also show that there was no difference in the rate of synthesis of NAD⁺, measured under optimum conditions, between male and female rats (cf. the control groups of the adrenalectomized and

glucagon-treated with the growth-hormone-treated groups; the difference between the thyroxine-treated group and the rest may be due to the dietary supplementation given to the thyroxine group).

NAD⁺ kinase. No change in the activity of this enzyme is apparent in the adrenalectomized group, in the group treated with glucagon or in the diabetic group (Table 2). Acute growth-hormone administration (12 hr.) causes a decrease of activity but this is largely reversed if the treatment is prolonged to 3 days. Thyroxine stimulates the activity of NAD⁺ kinase significantly as shown by all three modes of calculation. The potential activity of NAD⁺ kinase appears to be higher in the female rats (30.56 ± 1.37 units total liver activity; 12 animals) than in the male (16.64 ± 1.78 units total liver activity; 14 animals; Fisher's $P < 0.001$), although part of this difference may be due to the strain difference between the groups.

NAD⁺ glycohydrolase. Although the lack of change of activity of NMN adenylyltransferase and NAD⁺ kinase in the livers of adrenalectomized rats or in rats treated with glucagon is in keeping with the very minor changes in nucleotide concentrations induced by these hormonal treatments

Table 2. Activity of NAD⁺ kinase in the livers of rats subjected to a variety of hormonal treatments

Hormonal treatment of the animals was as described in the Methods section. Enzymes were extracted and estimated as described in the Methods section and the values given are means $\pm$ s.e.m. Fisher's P represents the probability of difference between the means. Values of P greater than 0.1 are shown as N.S., i.e. not significantly different. The adrenalectomized, thyroxine-treated and glucagon-treated groups were analysed by the method of corresponding pairs.

Treatment	No. of animals	Sex	Body wt. (g.)		Liver wt. (g.)	NADP synthesized		
			Initial	Final		(μmoles/hr./g.)	(μmoles/hr./total liver)	(μmoles/hr./100g. body wt.)
Control	8	♂	164±3.8	159± 2.3	5.86±0.21	2.80±0.36	16.92±2.19	10.49±1.48
Adrenalectomized	8	♂	165±4.0	159± 2.7	6.29±0.19	2.92±0.28	18.54±2.07	11.58±1.21
Fisher's <i>P</i>						N.S.	N.S.	N.S.
Control	6	♂	152±3.9	164± 5.2	7.26±0.46	2.25±0.46	16.21±3.18	9.88±1.97
Glucagon	6	♂	154±4.2	156± 7.1	8.69±0.47	2.13±0.38	19.17±4.21	11.91±2.24
Fisher's <i>P</i>						N.S.	N.S.	N.S.
Control	6	♀	—	196± 3.2	6.88±0.20	4.43±0.11	30.52±1.37	15.59±0.62
Growth hormone (12 hr.)	6	♀	—	209± 1.8	7.02±0.08	3.67±0.23	25.79±1.66	12.34±0.71
Fisher's <i>P</i>						0.02	0.08	0.01
Control	6	♀	—	198± 3.6	6.96±0.30	4.53±0.14	31.55±1.20	15.92±0.62
Growth hormone (3 days)	6	♀	—	224± 1.0	7.93±0.20	3.98±0.14	31.46±0.99	14.03±0.40
Fisher's <i>P</i>						0.05	N.S.	0.09
Control	6	♂	—	218±15.6	9.09±0.62	3.1 ±0.30	28.26±4.89	12.49±1.07
Diabetic	6	♂	—	206± 9.29	9.75±0.33	2.78±0.26	26.97±2.18	13.34±1.24
Fisher's <i>P</i>						N.S.	N.S.	N.S.
Control	6	♂	154±3.6	164± 3.3	7.71±0.30	3.81±0.26	29.56±2.62	17.97±1.47
Thyroxine	6	♂	158±3.8	152± 3.1	7.02±0.18	5.30±0.17	37.20±1.42	24.52±1.92
Fisher's <i>P</i>						0.004	0.018	0.005

(Greenbaum *et al.* 1965b), the changes in activity of these enzymes in rats treated with either growth hormone or thyroxine is in sharp contrast with the nucleotide changes in the livers of similarly treated rats previously reported by Glock & McLean (1955) and by Greenbaum *et al.* (1965b). This led to a consideration of other factors which might affect the concentration of the nicotinamide nucleotides. Measurements were therefore made of the activity of NAD⁺ glycohydrolase in the three hormonal treatments which produced the greatest changes in the amount of NAD⁺ and NADH, i.e. thyroxine and growth-hormone treatment and in alloxan-diabetes. The results of these experiments are shown in Table 3, which also shows the changes in the liver content of NAD⁺ + NADH and of NADP⁺ + NADPH. Of the animals treated in the three ways, only the growth-hormone-treated rats had an NAD⁺-glycohydrolase activity different from controls, and in this treatment the activity of the enzyme had increased by some 25%. Thus it is apparent that the measurements of enzyme activity, both synthetic and degradative, do not correlate very well with the observed changes of NAD. In diabetic rats there is a considerable decrease in the amount of NAD present in the liver and only slight changes in the activities of NMN adenylyltransferase and NAD⁺ glycohydrolase. In growth-hormone-treated

animals the NAD content increases by 30%, the transferase remains relatively unchanged and the cleavage enzyme is also increased by some 25%. Thyroxine-treated animals lose about a quarter of their NAD but under the same conditions the NMN adenylyltransferase increases by 27% and the glycohydrolase decreases by approx. 10%. In the last two conditions therefore the changes of enzyme activity might be expected to produce changes in nucleotide content opposite to those actually observed.

DISCUSSION

The rapidity with which the nicotinamide nucleotide contents respond to metabolic change or change imposed by a number of hormonal agents, together with isotopic studies which reveal a high turnover number for these compounds, both point to the fact that the tissue concentration of the nucleotide coenzymes is a dynamic phenomenon dependent, in part, on the activities of the enzymes involved in synthesis which are, in turn, dependent on the concentration of enzymes and the availability of substrates and cofactors. The present work examines how closely changes in the concentrations of the nicotinamide nucleotides can be correlated with changes in the maximum potential activities of two of the enzymes concerned with their syn-

Table 3. *Effect of different hormonal treatments on the activity of NMN adenylyltransferase, NAD⁺ kinase and NAD⁺ glycohydrolase compared with the effect of the same treatments on the hepatic content of nicotinamide nucleotides*

Condition	Percentage change of nicotinamide nucleotide*		Percentage change of activity of enzyme*			
	NAD ⁺ + NADH	NADP ⁺ + NADPH	NMN adenylyl- transferase	NAD ⁺ kinase	NAD ⁺ glycohydrolase	Glucose 6-phosphatase
Adrenalectomy	94†	93†	103	110	—	80 (Ashmore <i>et al.</i> 1956)
Alloxan-diabetes	68‡	76‡	135§	107§	97	290 (Ashmore <i>et al.</i> 1956)
Glucagon	104†	84†	103	118	—	—
Growth hormone (12 hr.)	76†	94†	99	84	—	—
Growth hormone (72 hr.)	132†	75†	88	103	126	100 (Harper & Young, 1959)
Thyroxine	78‡	65‡	127	126	92	143 (Tata <i>et al.</i> 1963)
Mean control values	600†	210	3.8¶	2.5¶	130¶	81¶ (Ashmore <i>et al.</i> 1956)

* Calculated as percentage of control value (total hepatic nucleotide or enzyme activity).

† Calculated from the data of Greenbaum *et al.* (1965b).

‡ Calculated from the data of Glock & McLean (1955).

§ Calculated as total hepatic activity/100 g. body wt.

|| μ g. of nucleotide/g. of liver.

¶ mg. of nucleotide or substrate formed or destroyed/g. of liver/hr.

thesis: NAD⁺ kinase and NMN adenylyltransferase. For the former enzyme this represents the whole enzymic machinery responsible for the conversion of NAD into NADP. The latter enzyme, although only the final step in the chain leading to the synthesis of NAD⁺, was studied because Branster & Morton (1956) found a relationship between the activity of this enzyme and the NAD content in developing tissue and tumours, and because Preiss & Handler (1958) suggest that this same enzyme also catalyses the step from nicotinic acid mononucleotide to deamido-NAD in their pathway of NAD synthesis. If this is true, the exact nature of the pathway leading to NAD synthesis is not relevant, since assay of NMN adenylyltransferase measures either the final or penultimate enzymic step in the two different pathways. The activity of NAD⁺ glycohydrolase was also measured as this appears to be the most important enzyme cleaving NAD (Kaplan, 1961).

In considering these results the most meaningful parameter is generally, as stated above, the total hepatic activity. This is not true, however, of the diabetic animals. Here the controls were of the same physiological age and by the end of the experiment there was a wide disparity in body weight between the diabetic animals and their controls. The most realistic parameter is therefore probably the total hepatic activity/100g. body wt. It is also more useful to consider total NAD (NAD⁺ + NADH) and total NADP (NADP⁺ + NADPH) rather than each of the oxidized and reduced forms separately since this paper is concerned with the relationship between the synthesizing systems and the total amount of nucleotide present rather than the distribution of the nucleotides between the oxidized and reduced forms. To facilitate this comparison, data taken from the work of Glock & McLean (1955) and Greenbaum *et al.* (1965b) have been summarized, together with the results of the present study, in Table 3.

This Table shows that the changes in the enzyme activities found here and the changes in the amounts of nucleotide that have been found under the same conditions bear little relation to one another. The most striking example, perhaps, is the thyroxine-treated group. Here, the activity of NMN adenylyltransferase is significantly increased, whereas the NAD content falls by some 22%. At the same time the activity of the NAD⁺ glycohydrolase remains unchanged. A similar pattern is apparent for NADP; NAD⁺ kinase activity is again significantly increased, whereas the actual NADP content falls by almost 40%.

Again, a lack of correlation is found in the growth-hormone-treated groups. There is a diphasic response in the NAD content in that 12 hr. after administration of the hormone the total NAD

content decreases by over 20% and after 3 days of treatment the amount rises to concentrations as much as one-third above normal. At neither of these times is there a significant change of the activity of the synthesizing enzyme and, indeed, there is a tendency to change in the opposite direction to that shown by the nucleotide. Here also there is no evidence that the NAD⁺ glycohydrolase is responsible for the longer-term increase of NAD since this enzyme remains unchanged. Also, the decline of NADP found after 3 days of treatment is not paralleled by the change of NAD⁺ kinase activity, which again shifts, if at all, in the opposite direction.

In diabetic rats there are striking decreases in both the NAD and NADP content of liver. Neither NAD⁺ kinase activity, which is virtually unchanged, nor NMN-adenylyltransferase activity, which is increased by about 35% when expressed as total hepatic activity/100g. body wt., correlates well with this finding. No conclusions are drawn from the data obtained in the two remaining hormonal treatments, i.e. adrenalectomy and glucagon treatment, where the changes, if any, are very small and the total liver values are nowhere significant at *P* 0.05.

The lack of correlation between the observed concentrations of the nicotinamide nucleotides and the activities of the NMN adenylyltransferase and NAD⁺ kinase revealed in Table 3 directs attention to other factors, such as the activity of the nucleotide-cleaving enzymes or substrate concentrations, which might act as regulators of the nucleotide concentration.

NAD breakdown and synthesis

Cleavage enzymes. The cleavage enzymes are many times more active than the two synthetases measured in this paper (see also Jacobson & Kaplan, 1957) and relatively small changes in their activity would be sufficient to account for the changes in the nicotinamide nucleotide contents that have been found. Indeed, several reports have implicated the cleavage enzymes as regulators of the cellular concentrations of NAD (Roitt, 1956; Green & Bodansky, 1963; Greengard, Kalinsky & Petrack, 1961b). Green & Bodansky (1963), for instance, found an inverse relationship between the NAD content in ascites-tumour cells and their NAD⁺ glycohydrolase activity; Hilz, Hlavica & Bertram (1963) reached similar conclusions in their study of the effect of radiation on the NAD content of the same tissue, and Märki & Greengard (1964) also concluded that the low NAD content of Ehrlich ascites-tumour cells was related to the cleavage enzymes.

Nevertheless, the potential activities of the cleavage enzymes as measured *in vitro* may not

reflect their activity *in vivo*. They may well be 'latent' enzymes *in vivo*, since iso-osmotic homogenates have about 10% of the activity of homogenates that have been diluted with water. NAD⁺ glycohydrolase in an iso-osmotic homogenate cleaves 20 μ moles of NAD⁺/g. of liver/hr. (Jacobson & Kaplan, 1957), whereas a water homogenate cleaves about 130 μ moles of NAD⁺/g. of liver/hr. (Table 3); a similar disparity has been reported by Roitt (1956) in ascites-tumour cells and extracts and by Gore, Ibbott & McIlwain (1950) for brain slices and dispersions.

In the present experiments the activity of NAD⁺ glycohydrolase has been measured in three hormonal conditions: thyroxine-treated, growth-hormone-treated and alloxan-diabetic rats, i.e. in those conditions where significant changes were found in the NAD content. The results in Table 3 show that in thyroxine-treated and growth-hormone-treated rats the NAD⁺-glycohydrolase activity changes in the same direction as the NAD content; it seems unlikely therefore that this enzyme is the main controlling mechanism in these conditions. Bosch & Harper (1959) also concluded, as a result of their investigations on the rate of disappearance of newly synthesized hepatic NAD⁺ after the injection of nicotinamide, that the activity of the hydrolase is the same in both normal and hyperthyroid animals; but Greengard, Quinn & Landrau (1961a) and Greengard, Quinn & Reid (1964), in similar studies on hypophysectomized rats, have implicated pituitary hormones as agents regulating the NAD concentrations via glycohydrolase activity.

Synthesis. The activity of the NMN adenylyltransferase is sufficient to turn over the entire hepatic NAD (i.e. 3600 μ g.) six times in an hour. Turnover times of NAD in the intact liver can be calculated from the data of Gordon (1963), about 140 μ g./hr., and of Purvis & Lowenstein (1961), about 350 μ g./hr., and the maximum capacity of the enzyme is greatly in excess of these figures. Further evidence that the enzyme is present in great excess can be seen in the threefold increase of hepatic NAD⁺ after the injection of nicotinamide (Greengard & Quinn, 1962; Kaplan, 1961, see p. 649) and even under these conditions the rate of synthesis is only about one-twentieth of the maximum potential activity of the enzyme.

There was no significant change in the activity of the NMN adenylyltransferase in any of the hormonal conditions studied in the present paper except for thyroxine, where the *P* value for the rise in activity was just significant (i.e. 0.05) on the total liver basis, and in the alloxan-diabetic group where the activity is also significantly increased. Maley & Lardy (1955), however, found no change in the activity of this enzyme in prepara-

tions from liver nuclei from hyperthyroid rats. Although there are no changes in the activity of NMN adenylyltransferase in growth-hormone-treated or adrenalectomized rats, the pituitary and adrenal gland may regulate NAD synthesis, as shown by the effects of hypophysectomy and adrenocorticotrophic hormone on the induced synthesis of NAD *in vivo* after injection of nicotinamide, possibly via their effect on the deamidation of nicotinamide (Greengard *et al.* 1961a, 1964).

Another enzyme which could affect the rate of synthesis of NAD is 'inorganic pyrophosphatase'. The equilibrium position for the NMN adenylyltransferase $[NMN][ATP]/[NAD^+][PP_i]$ is 0.45 (Kornberg, 1950a) and removal of the pyrophosphate formed will clearly favour NAD⁺ formation. Although direct measurements of pyrophosphatase activity in hormonal conditions are few, the probable identity of this enzyme with glucose 6-phosphatase (Nordlie & Arion, 1964) permits the extrapolation of the great bulk of data on the hormonal control of glucose 6-phosphatase to the regulation of NAD⁺ synthesis by the pyrophosphatase. There should therefore be a positive correlation between the activity of inorganic pyrophosphatase and the NAD content if there are no other limiting factors. Table 3 shows that, although this may be true for growth-hormone treatment, it is not so for the other hormonal conditions studied. For example, there is a significant fall of NAD in diabetic animals, a condition where the glucose 6-phosphatase (i.e. by inference inorganic pyrophosphatase) is known to increase markedly (Ashmore, Hastings, Nesbett & Renold, 1956).

These results show that other factors probably play an important role in regulating the rate of NAD synthesis: in particular, the role of substrates must be considered. The administration of nicotinamide, nicotinic acid or tryptophan increases the amount of NAD in the liver; this may indicate that the supply of the precursor of the nicotinamide moiety of NAD can be rate-limiting. In their study of the nicotinamide-induced NAD synthesis in normal and hyperthyroid rats Bosch & Harper (1959) observed that, although the initial rates of synthesis of NAD were the same in the two groups, the rate fell off much more rapidly in the hyperthyroid rats than in the normal controls, and they suggested that this could be attributed to the depletion of some component required for the synthesis, possibly ATP.

NADP breakdown and synthesis

Cleavage enzymes. The rapid turnover of the monoester phosphate of NADP, compared with the relatively slow turnover of the phosphate

groups of the NAD moiety of the molecule (Stollar & Kaplan, 1961), is evidence for the existence of cleavage enzymes of high activity. There are, as yet, no quantitative measurements of these enzymes in the type of condition studied here.

Synthesis. The control of the synthesis of NADP is particularly amenable to investigation since the step from NAD is achieved by a single enzyme which is located in the soluble fraction of the cell (Villem & Hagerman, 1958; Stollar & Kaplan, 1961). This is in contrast with the synthesis of NAD, which is located in the nuclei and mitochondria (see Morton, 1961), and the inorganic pyrophosphatases, which occur in the microsomes (Jacobson & Kaplan, 1957; Nordlie & Arion, 1964; see also Kaplan, 1961). There is, moreover, evidence that NADP synthesis is under hormonal control (Villem & Hagerman, 1958).

As with NMN adenylyltransferase, NAD⁺ kinase appears to have a potential activity greatly exceeding the actual rate of synthesis of NADP; moreover, there appears to be no correlation between the activity of the enzyme as measured and the NADP concentration (Table 3). In no case was a significant decline of the activity of the kinase found, but the liver content of NADP fell in both diabetes and in thyroxine-treated rats. The substrates for this reaction are NAD⁺, ATP and Mg²⁺ ions. The optimum concentration of Mg²⁺ ions is 9mM (Kornberg, 1950b), a high value and about 50 times as great as that required for NAD synthesis, and it is possible that ion shifts could exert an influence on the phosphorylation. It seems unlikely that NAD⁺ limits the rate of synthesis of NADP as injection of nicotinamide can increase the concentration of hepatic NAD about tenfold without appreciably affecting the NADP content (Kaplan, Goldin, Humphreys & Ciotti, 1956), although Wintzerith, Klein, Mandel & Mandel (1961) and Greengard *et al.* (1964) in similar experiments have both described a small rise in the NADP. The failure to obtain increased NADP synthesis can hardly be ascribed to an adverse localization of the new NAD since the newly synthesized NAD is mainly found in the extramitochondrial compartment, i.e. in the compartment where the NAD⁺ kinase is located.

The second factor which might operate to limit the rate of NADP formation is the availability of ATP. Although ATP is necessary for the formation of both NAD and NADP the Michaelis constants for these two phosphorylations are such that a decrease in the ATP content would primarily affect the NAD⁺ kinase. The K_m for NMN adenylyltransferase is 0.46mM (Kornberg, 1950a; Morton, 1961) and the apparent K_m for NAD⁺ kinase is 18mM (calculated from the data of Wang & Kaplan, 1954). It might be expected, therefore,

that in conditions where a decrease in the content of ATP is found an associated decline in NADP would also occur. This appears to be true for rats treated with thyroxine, where there is evidence for a fall in ATP (Chatagner & Gautheron, 1960; Fletcher & Myant, 1961; Reid, 1961) and of NADP (Glock & McLean, 1955). Growth hormone also decreases the hepatic NADP (Greenbaum *et al.* 1965b) and possibly the ATP content as well (Melhuish & Greenbaum, 1961). In diabetes there is a fall of total NADP (Glock & McLean, 1955) and Kaplan & Greenberg (1944) reported that the ATP content is also affected by insulin treatment. A similar parallelism exists in precancerous liver where the ATP content falls (Reid, 1962) and there is a decline of the NADP at a time when the activity of the NAD⁺ kinase is unaffected (Clark, Greenbaum, McLean & Reid, 1964).

Of the two remaining hormonal conditions studied, adrenalectomy and treatment with glucagon, neither showed any striking change in the NADP content of the liver. Adrenalectomy causes a transient rise of ATP (Reid, 1961) and there is no information on the effect of glucagon on liver ATP.

It would seem therefore that a study of hormonal effects on the concentration of substrates such as nicotinamide mononucleotide or nicotinic acid mononucleotide, ATP or phosphoribose 1-pyrophosphate in liver might throw useful light on the mechanism of the regulation of nicotinamide nucleotide synthesis.

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