The Effect of Different Hormonal Conditions on the Concentration and Oxidoreduction State of the Nicotinamide Nucleotides of Rat Liver

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1. The concentration and oxidoreduction state of the liver nicotinamide nucleotides of rats subjected to a number of hormonal treatments have been measured. 2. Adrenalectomy decreases the NADP⁺ content by 80% but has little effect on NAD⁺, NADH or NADPH. High doses of cortisone produce similar changes, but more physiological doses (5 µg. daily) tend to increase the NADP⁺ content. 3. Glucagon treatment of normal rats lowered the NADH and NADP⁺ concentrations but did not affect the total amounts present. Growth hormone increased the concentrations and total amounts of NAD⁺ and NADH but significantly decreased the concentrations and total amounts of NADP⁺ and NADPH. 4. Measurements have been made of a number of enzymes in the livers of adrenalectomized and glucagon-treated rats that could affect the oxidoreduction state of NADP. The activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are not affected by adrenalectomy or treatment with cortisone or glucagon. Nor does adrenalectomy affect the activity of NADPH-cytochrome c oxidoreductase or NADPH-glutathione oxidoreductase. The hepatic content of glutathione is, however, decreased 50% by adrenalectomy. 5. Measurements of the oxidation of [1-¹⁴C]glucose and [6-¹⁴C]glucose by liver slices from adrenalectomized rats showed that glucose oxidation was substantially normal, although phenazine methosulphate caused a smaller stimulation of the oxidation of C-1 of [1-¹⁴C]glucose in slices from the livers of adrenalectomized rats than it did with slices from controls. The hepatic synthesis of lipids from [1-¹⁴C]glucose was marginally increased in adrenalectomized rats. 6. The additional NADP⁺ found when liver is extracted with 0.02N-sulphuric acid-0.1M-sodium sulphate is less affected than the NADP⁺ extracted with 0.1N-hydrochloric acid in adrenalectomized or glucagon-treated rats. Hooded Norway rats appear to have less of this extra form of NADP⁺ than albino rats. 7. An attempt has been made to correlate the observed changes in the nicotinamide nucleotides with metabolic patterns prevailing in different hormonal conditions.

Endocrine imbalance leads to changes in the enzymic and cofactor constitution of the tissues of the target organs and such changes become manifest as considerable alterations in the metabolic pattern of these tissues. Knowledge of the nature and sequence of such changes could throw considerable light on the mechanism of action of hormones.

In the present study three hormones have been investigated, the steroid hormones of the adrenal cortex, glucagon and the growth hormone of the anterior pituitary. All three hormones are known to influence profoundly the protein metabolism of

the organism; cortical hormones (Long, Smith & Fry, 1960) and glucagon (Tybergheim, 1953; Best, 1959; Miller, 1960) both cause an increased rate of protein breakdown, whereas growth hormone causes a retention of nitrogen and decreased protein catabolism (Young, 1945; Li, Simpson & Evans, 1948; Greenbaum, 1953). In addition, the first two accelerate gluconeogenesis (Long, Katzin & Fry, 1940; Tilton, Torralba & Ingle, 1955; Foá & Galansino, 1962), whereas growth hormone appears to depress carbohydrate oxidation in favour of lipid oxidation (Young, 1945; Greenbaum, 1953).

A common feature of the metabolic patterns for most substrates is their requirement for cofactors such as the nicotinamide nucleotides, and competition may exist between the various metabolic pathways for these compounds. Availability of such cofactors could play a determining role in the rate and direction of competing pathways, both the concentration and oxidoreduction state being involved in this control. Further, cofactors can play a part in controlling metabolic pathways by obligatory linking via a common nucleotide. The linking of respiratory control to the processes of oxidative phosphorylation via ADP is a classic example of such a mechanism, as is the interaction of lipid synthesis and glucose oxidation by way of the pentose phosphate pathway with NADP⁺ as a link (Winegrad & Renold, 1958, Tepperman & Tepperman, 1958; Abraham & Chaikoff, 1959; McLean, 1960).

There have been a number of attempts to relate prevailing metabolic patterns to the nicotinamide nucleotides of the liver in a number of hormonal conditions. Diabetes has been studied by Helreich, Holzer, Lamprecht & Goldschmidt (1954), Glock & McLean (1955), Jedeikin & Weinhouse (1955) and Greenbaum & Graymore (1956), growth hormone by Glock & McLean (1955) and Greenbaum & Graymore (1956), and thyroxine by Maley & Lardy (1955) and Glock & McLean (1955). In most of these investigations only the concentrations and oxidoreduction states of the nucleotides were measured, and usually only NAD⁺ and NADH. The present study attempts to extend our knowledge of the role of the nicotinamide nucleotides in the changes of metabolic pattern induced by hormones in three ways: (1) by determining, in a wide variety of conditions, not only NAD⁺, NADH, NADP⁺ and NADPH, but also the acid-labile form of NADP⁺ described by Burch, Lowry & Von Dippe (1963); (2) by adding two further hormones to the list of those investigated; (3) with the adrenal hormones, which cause striking changes in the concentration of NADP⁺, by studying some of the enzymes that require this coenzyme, i.e. glucose 6-phosphate dehydrogenase [D-glucose 6-phosphate-NADP⁺ oxidoreductase (EC 1.1.1.49)], 6-phosphogluconate dehydrogenase [6-phosphogluconate-NADP⁺ oxidoreductase (decarboxylating) (EC 1.1.1.44)], NADPH-glutathione oxidoreductase (EC 1.6.4.2) and NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.3).

The incorporation of ¹⁴C from specifically labelled glucose into the respiratory carbon dioxide and into lipid has also been measured in this condition.

METHODS

Animals. Unless otherwise stated albino rats (body wt. 140–160g.) were used. They were fed on a stock diet

compounded of 1500g. of powdered M.R.C. diet no. 41 B plus 300 ml. of 0.01% sodium alginate to bind the diet.

Maintenance of adrenalectomized animals. After bilateral adrenalectomy the rats were given 1% NaCl to drink. Paired-feeding was begun immediately after the operation. Two weeks later the rats were killed by cervical dislocation and parts of the liver taken for estimation of the nicotinamide nucleotides, as described by Greenbaum, Clark & McLean (1965), for the preparation of liver slices for the isotope experiments and for the preparation of tissue homogenates for enzyme assays.

Treatment of rats with adrenal-cortical steroids. (a) Long-term treatment. Male rats were injected with 5mg. of microcrystalline cortisone acetate daily for 3 days. On the third day a supplementary dose of 2mg. was injected at 5 p.m. and the rats were killed at 10 a.m. on the fourth day. The rats were pair-fed throughout the period of treatment.

(b) Short-term treatment. (1) Cortisone acetate. Doses of either 5mg. (intramuscularly) or 50 µg. (intraperitoneally) were given and the rats killed 24hr. later. (2) Hydrocortisone hemisuccinate. Rats were given a single injection of between 5 µg. and 5mg. and were killed at various times after the injection (2.5–24hr.)

Glucagon treatment. Male rats were injected subcutaneously with 100 µg. of glucagon three times a day for 3 days. A last injection of 100 µg. was administered 1hr. before the rats were killed on the fourth day. All rats were pair-fed throughout the period of treatment.

Treatment with growth hormone. Female hooded Norway rats (body wt. approx. 200g.) were treated as follows: (a) 1mg. of pituitary growth hormone (gift from Armour Pharmaceutical Co., Eastbourne, Sussex, and the Endocrinological Study Section of the National Institutes of Health) was injected intraperitoneally daily for 3 days and the rats were killed on the fourth day; these animals were also pair-fed during the period of treatment; (b) 1mg. of pituitary growth hormone was administered as a single dose 12hr. before the rats were killed.

Diabetic animals. Male rats (body wt. approx. 220–250g.) were starved for 48hr. and then injected with alloxan monohydrate (19mg./100g. body wt.) (Kass & Waisbren, 1945). The alloxan was dissolved in sodium acetate buffer, pH 4.5, to prevent its decomposition (Klebanoff & Greenbaum 1954). The following day 2 units of insulin (protamine-zinc-insulin; Boots Pure Drug Co. Ltd., Nottingham) were injected subcutaneously and a single dose of 2 units of insulin was given daily for a further 6 days. Throughout this period food was provided *ad lib*. Then the insulin was discontinued and the rats were maintained on a limit-feeding regime (50g./day, the amount normally eaten by control rats of the same age and body weight). They were killed 3 weeks after the cessation of the insulin treatment. These rats excreted 8–10g. of sugar/day and the blood sugar concentrations of the fed rats were always over 500mg./100ml.

Control rats. In view of the results of Yasin & Bergel (1963) on the effect of the injection procedure on the concentrations of the nicotinamide nucleotides, control rats for each group were given equivalent injections of 0.9% NaCl.

Isotope experiments. Batches (300mg.) of liver slices were incubated in modified Warburg flasks for 2hr. in 4.5ml. of Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1957) containing 100 µmoles of unlabelled glucose

and 0.4 μ C of either [1- 14 C]glucose or [6- 14 C]glucose. The gas phase was O₂ + CO₂ (95:5). At the end of the incubation 1.0 ml. of 5N-KOH was injected into the centre well and 0.5 ml. of 5N-H₂SO₄ into the outer compartment. The 14 CO₂ content was determined after precipitation with BaCl₂ (containing NH₄Cl), the infinitely thick technique

being used. The radioactivity of the labelled glucose used in the reaction vessels was similarly determined after complete oxidation to 14 CO₂ with potassium persulphate (Abraham & Hassid, 1957). In some experiments phenazine methosulphate was added to give a final concentration of 0.1 mM. Incorporation of the 14 C into lipid was measured as

Table 1. *Effect of adrenalectomy and of treatment of normal rats with cortisone acetate on the concentration of the nicotinamide nucleotides in the liver*

For the adrenalectomized group, female animals were adrenalectomized 2 weeks before being killed and were maintained on 1% NaCl throughout this period. For the cortisone acetate-treated group, normal male rats were injected with 5 mg. of cortisone acetate daily for 3 days; a supplementary dose of 2 mg. was injected on the evening of the third day and the rats were killed on the fourth day. The control groups were pair-fed with the treated groups. Nucleotides were extracted and assayed as described in the Methods section. The values given are the means $\pm$ s.e.m. Fisher's *P* values represent the probability of difference between the means (corresponding pairs). Values of *P* greater than 0.1 are shown as N.S., i.e. not significantly different.

	No. of animals.....	Control group 8	Adrenalectomized group 8	Fisher's <i>P</i> value
Initial body wt. (g.)		154 $\pm$ 3.3	150 $\pm$ 9.5	
Final body wt. (g.)		156 $\pm$ 3.7	159 $\pm$ 12.3	
Liver wt. (g.)		7.22 $\pm$ 0.3	7.24 $\pm$ 0.3	
Concn. of nucleotide (μ g./g. of liver)				
NAD ⁺		269 $\pm$ 24	236 $\pm$ 20	N.S.
NADH		126 $\pm$ 6	134 $\pm$ 19	N.S.
NAD ⁺ /NADH ratio		2.13	1.8	
NADP ⁺		32 $\pm$ 4	7 $\pm$ 2	< 0.001
NADPH		205 $\pm$ 13	214 $\pm$ 17	N.S.
Total liver nucleotide (μ g.)				
NAD ⁺		1940 $\pm$ 178	1699 $\pm$ 184	N.S.
NADH		900 $\pm$ 42	957 $\pm$ 127	N.S.
NADP ⁺		238 $\pm$ 36	48 $\pm$ 14	< 0.001
NADPH		1466 $\pm$ 88	1541 $\pm$ 130	N.S.
Total liver nucleotide (μ g./100 g. body wt.)				
NAD ⁺		1251 $\pm$ 125	1071 $\pm$ 96	N.S.
NADH		580 $\pm$ 31	605 $\pm$ 84	N.S.
NADP ⁺		151 $\pm$ 21	31 $\pm$ 9	< 0.001
NADPH		944 $\pm$ 58	972 $\pm$ 84	N.S.
	No. of animals.....	Control group 5	Cortisone acetate-treated group 5	Fisher's <i>P</i> value
Initial body wt. (g.)		158 $\pm$ 8.7	159 $\pm$ 9.3	
Final body wt. (g.)		163 $\pm$ 5.1	158 $\pm$ 10.5	
Liver wt. (g.)		8.72 $\pm$ 0.3	9.20 $\pm$ 0.7	
Concn. of nucleotide (μ g./g. of liver)				
NAD ⁺		511 $\pm$ 19	467 $\pm$ 18	N.S.
NADH		116 $\pm$ 12	137 $\pm$ 13	N.S.
NAD ⁺ /NADH ratio		4.4	3.4	
NADP ⁺		22 $\pm$ 4	12 $\pm$ 1	0.039
NADPH		266 $\pm$ 18	229 $\pm$ 19	N.S.
Total liver nucleotide (μ g.)				
NAD ⁺		4431 $\pm$ 166	4318 $\pm$ 387	N.S.
NADH		1001 $\pm$ 81	1262 $\pm$ 149	N.S.
NADP ⁺		195 $\pm$ 33	109 $\pm$ 11	0.067
NADPH		2292 $\pm$ 151	2131 $\pm$ 289	N.S.
Total liver nucleotide (μ g./100 g. body wt.)				
NAD ⁺		2744 $\pm$ 176	2722 $\pm$ 139	N.S.
NADH		623 $\pm$ 70	794 $\pm$ 65	N.S.
NADP ⁺		122 $\pm$ 22	69 $\pm$ 6	0.059
NADPH		1410 $\pm$ 84	1330 $\pm$ 101	N.S.

follows: the slices and medium were extracted together by the method of Bligh & Dyer (1959), and the extracted lipid was washed and then counted in a Nuclear-Chicago scintillation counter.

Enzymic methods. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured by the method of Glock & McLean (1953). NADPH-cytochrome *c* oxidoreductase was measured as described by McLean (1958). NADPH-glutathione oxidoreductase was measured by the method of Racker (1955).

Chemical methods. The extraction and the estimation of the nicotinamide nucleotides was carried out as described by Greenbaum *et al.* (1965). In addition, samples of liver were taken for the extraction of the 'acid-labile NADP⁺' as described by Burch *et al.* (1963). For this form of NADP⁺ a piece of liver (approx. 500mg.) was frozen in liquid nitrogen and then extracted in the 0.02N-H₂SO₄-0.1M-Na₂SO₄ reagent of Burch *et al.* (1963). The NADP⁺ so extracted is referred to below as 'NADP⁺ extractable in weak acid'.

The tissue content of non-protein thiol groups was determined by the method of Ellman (1959) by using the colour reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Deproteinized tissue extracts were prepared by the method of Hunter (1957). Since the major component of the non-protein thiols of tissues is glutathione (see Thompson & Martin, 1959) the substances reacting with the above reagent are referred to below as 'glutathione'. Glucose was estimated by the method of Nelson (1944).

Materials. Glucagon was a gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A. Cortisone acetate (from E. Merck, Darmstadt, Germany), hydrocortisone hemisuccinate (from Boots Pure Drug Co. Ltd.), glucose 6-phosphate, 6-phosphogluconate, NADP⁺ and NADPH (all from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), phenazine methosulphate (from L. Light and Co. Ltd., Colnbrook, Bucks.) and 5,5'-dithiobis-(2-nitrobenzoic acid) (from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) were obtained from the commercial sources indicated. Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks. Glutathione was a gift from The Distillers Co. Ltd., Speke, Liverpool, and oxidized glutathione was prepared by the copper oxidation method of Pirie (1931).

RESULTS

The effects of adrenalectomy and of the treatment of intact rats with either adrenal cortical steroid, glucagon or growth hormone on the concentrations and oxidation-reduction state of NAD and NADP are shown in Tables 1-3. There were considerable variations between the concentrations of NAD⁺, and to a smaller extent of NADPH, among the different control groups. This reflects the dietary regime on which the control groups were maintained. Since all groups were pair-fed with the appropriate experimental group, wide variations in food intake occur, particularly in controls pair-fed with the adrenalectomized animals where the food intake had to be decreased from a normal average of 30-35g. to 25g. Restriction of food intake is

known to decrease the concentration of the nucleotides (Helmreich *et al.* 1954; Glock & McLean, 1955). Adrenalectomy decreased the concentration of NADP⁺ in the liver to about 20% of the control value, but had no appreciable effect on those of NAD⁺, NADH and NADPH (Table 1). Calculation of the results as total nucleotide in the liver or as total hepatic nucleotide/100g. body wt. did not alter this picture. These results are in substantial agreement with those of Reid (1961), who, in a survey of hormonal effects on liver ribonucleotides by column chromatography, also reported a fall in NADP⁺ and an unchanged NAD⁺ concentration in adrenalectomized rats.

The converse of this experiment, namely the treatment of normal intact rats with cortisone acetate, gave a similar result in that the concentrations and amounts of NAD⁺, NADH and NADPH were unaffected by the treatment, whereas NADP⁺ again was decreased both in concentration and amount (Table 1). In view of this unexpected response, the effect of different doses of cortisone acetate (50 µg.-5mg.) and of hydrocortisone, either as the microcrystalline form or as the soluble succinate (5-500 µg.), was determined for different lengths of time (2.5-24hr.). At the lowest dose, given for 24hr., there is a tendency towards an elevation of the NADP⁺ concentration, but the response was variable and not significant [5 µg. of hydrocortisone hemisuccinate, 29.3 ± 6.8 µg./g.; controls, 20.4 ± 2.4 µg./g. (ten pairs)]. Thus at the more physiological dose of 5 µg. of steroid there was a tendency to reverse the effect of adrenalectomy.

Treatment of rats with glucagon (100 µg./day for 3 days) decreased the concentrations of NADH, NADP⁺ and NADPH by 20-30%, whereas the NAD⁺ concentration remained unchanged (Table 2). The changes in NADH and NADP⁺, but not of NADPH, were statistically significant when calculated on the concentration basis. However, when the results are calculated on the basis of total hepatic nucleotide or total hepatic nucleotide/100g. body wt., these differences disappear.

The effects of acute (12hr.) and chronic (3-day) treatment of rats with pituitary growth hormone are shown in Table 3. The concentration of NAD⁺ shows a distinctly diphasic response: 12hr. after administration of the hormone the NAD⁺ concentration is lowered significantly but by 3 days it has increased to a value significantly greater than that of the control. The NADH concentration is also lowered after 12hr., although not as much as that of NAD⁺, and this also rises to supernormal values at 3 days. The sum of the total hepatic NAD⁺ and NADH at 3 days is 5690 µg. for the treated rats and 4170 µg. for controls. NADP⁺, on the other hand, is decreased both at 12hr. and at 3 days, the decline being statistically significant

Table 2. *Effect of glucagon treatment on the concentrations of the nicotinamide nucleotides of rat liver*

Rats were treated with three doses, each of 100 μ g. of glucagon, daily for 3 days. The control group was pair-fed with the treated group. Nucleotides were extracted and assayed as described in the Methods section. Statistical treatment was as in Table 1.

	No. of animals ...	Control group 6	Glucagon-treated group 6	Fisher's <i>P</i> value
Initial body wt. (g.)		150 $\pm$ 3.8	152 $\pm$ 4.5	
Final body wt. (g.)		163 $\pm$ 6.4	155 $\pm$ 8.5	
Liver wt. (g.)		7.50 $\pm$ 0.5	8.75 $\pm$ 0.6	
Concn. of nucleotide (μ g./g. of liver)				
NAD ⁺		485 $\pm$ 36	463 $\pm$ 27	N.S.
NADH		131 $\pm$ 18	92 $\pm$ 6	0.012
NAD ⁺ /NADH ratio		3.6	5.0	
NADP ⁺		24 $\pm$ 3	19 $\pm$ 2	0.020
NADPH		255 $\pm$ 30	182 $\pm$ 17	0.032
Total liver nucleotide (μ g.)				
NAD ⁺		3608 $\pm$ 256	4017 $\pm$ 287	N.S.
NADH		994 $\pm$ 155	795 $\pm$ 46	0.090
NADP ⁺		181 $\pm$ 25	166 $\pm$ 20	N.S.
NADPH		1926 $\pm$ 308	1598 $\pm$ 204	N.S.
Total liver nucleotide (μ g./100 g. body wt.)				
NAD ⁺		2216 $\pm$ 161	2586 $\pm$ 199	0.10
NADH		602 $\pm$ 83	516 $\pm$ 40	N.S.
NADP ⁺		110 $\pm$ 15	106 $\pm$ 13	N.S.
NADPH		1169 $\pm$ 147	1021 $\pm$ 116	N.S.

Table 3. *Effect of acute (12 hr.) and chronic (3-day) treatment of rats with growth hormone on the concentration of nicotinamide nucleotides in the liver*

For the group receiving acute treatment, rats were injected intraperitoneally with 1 mg. of growth hormone 12 hr. before being killed. For the group receiving chronic treatment, rats were injected daily with 1 mg. of growth hormone intraperitoneally for 3 days and killed on the fourth day. Nucleotides were extracted and assayed as described in the Methods section. Statistical treatment was as in Table 1.

	No. of animals ...	Control group 5	Growth hormone-treated group (acute treatment for 12 hr.) 6	Fisher's <i>P</i> value	Growth hormone-treated group (chronic treatment for 3 days) 6	Fisher's <i>P</i> value
Final body wt. (g.)		206 $\pm$ 12	209 $\pm$ 2		224 $\pm$ 1	
Liver wt. (g.)		8.4 $\pm$ 0.4	7.0 $\pm$ 0.1		7.9 $\pm$ 0.2	
Concn. of nucleotide (μ g./g. of liver)						
NAD ⁺		391 $\pm$ 20	334 $\pm$ 20	0.090	539 $\pm$ 36	0.008
NADH		105 $\pm$ 14	113 $\pm$ 9	N.S.	158 $\pm$ 8	0.007
NAD ⁺ /NADH ratio		3.7	3.0		3.4	
NADP ⁺		22 $\pm$ 3	19 $\pm$ 2	N.S.	14 $\pm$ 2	0.044
NADPH		170 $\pm$ 17	195 $\pm$ 7	N.S.	141 $\pm$ 13	N.S.
Total liver nucleotide (μ g.)						
NAD ⁺		3300 $\pm$ 280	2350 $\pm$ 151	0.013	4240 $\pm$ 165	0.015
NADH		868 $\pm$ 89	796 $\pm$ 72	N.S.	1250 $\pm$ 74	0.009
NADP ⁺		188 $\pm$ 25	135 $\pm$ 12	N.S.	109 $\pm$ 11	0.030
NADPH		1420 $\pm$ 125	1370 $\pm$ 41	N.S.	1110 $\pm$ 94	N.S.
Total liver nucleotide (μ g./100 g. body wt.)						
NAD ⁺		1600 $\pm$ 74	1125 $\pm$ 73	0.001	1890 $\pm$ 72	0.020
NADH		430 $\pm$ 50	381 $\pm$ 33	N.S.	560 $\pm$ 34	0.055
NADP ⁺		90 $\pm$ 13	65 $\pm$ 5	N.S.	48 $\pm$ 5	0.012
NADPH		691 $\pm$ 45	657 $\pm$ 24	N.S.	495 $\pm$ 42	0.010

for all three modes of calculation at 3 days. The NADPH is also lowered by growth hormone treatment, but the decline is only significant at 3 days when expressed on the basis of total liver nucleotide/100g. body wt. The sum of the total hepatic NADP⁺ and NADPH at 3 days is 1608 μ g. for the control rats and 1220 μ g. for the treated animals.

Table 4 compares the NADP⁺ concentrations found when the liver is extracted with 0.1N-hydrochloric acid with the concentrations found when the liver is extracted with 0.02N-sulphuric acid-0.1M-sodium sulphate. The existence of the 'acid-labile' form of NADP⁺ (Burch *et al.* 1963) has been confirmed, although our values for the ratio (NADP⁺ extractable in weak acid)/(NADP⁺ extractable in 0.1N-hydrochloric acid) are rather less than theirs. Contrary to the finding of Burch *et al.* (1963) we could find no differences in the responses of males and females; in both cases a value of approx. 2.7 was obtained for the ratio. Adrenalectomy affects the concentration of the 0.1N-hydrochloric acid-stable form of NADP⁺ to a

greater degree than the NADP⁺ extractable in weak acid, so leading to a higher ratio, and the same is true for glucagon-treated rats.

NADP-linked enzymes. The effect of adrenalectomy on the activities of some NADP-linked enzymes is shown in Table 5. Of the enzymes measured only 6-phosphogluconate dehydrogenase shows a tendency to be decreased, and in this case only when measured at pH 9.0 (the optimum pH) and expressed as units/g. of tissue. The failure to find any change in the activities of the two reductases is somewhat surprising in view of the finding of a decreased amount of NADP⁺ in the tissue (Table 1). This appears to indicate that the activities of these enzymes are not the only factors that regulate the NADP⁺/NADPH ratio.

Other factors that could be involved here would be the concentrations of substrates and electron acceptors such as cytochrome *c* and glutathione. In fact, adrenalectomy leads to a 50% fall in the concentration of glutathione in the liver (Table 5).

Since estimations of enzyme activities are carried

Table 4. *Effects of various hormonal treatments on two forms of NADP⁺ (differing with respect to their stability in 0.1N-hydrochloric acid) in rat liver*

For the adrenalectomized group, rats were maintained on 1% NaCl and were killed 2 weeks after the operation. For the glucagon-treated groups, rats received 100 μ g./day for 3 days and 100 μ g. of glucagon on the fourth day 1 hr. before being killed. For the alloxan-diabetic group, rats were killed 4 weeks after being made diabetic. For the growth hormone-treated group, rats received 1 mg. of pituitary growth hormone daily for 3 days and were killed on the fourth day. The nucleotides were extracted and assayed as described in the Methods section, and expressed as μ g./g. of liver. Statistical treatment was as in Table 1.

Treatment	No. of animals	NADP ⁺ extracted in 0.1N-HCl (a)	NADP ⁺ extracted in 0.02N-H ₂ SO ₄ -0.1M-Na ₂ SO ₄ (b)*	b/a ratio	'Extra' NADP ⁺ (b-a)	(b-a)/a ratio
Control group	5	21 $\pm$ 2	59 $\pm$ 2	2.80	38 $\pm$ 1.1	1.81
Adrenalectomized group (males)	5	13 $\pm$ 1.3	46 $\pm$ 4	3.54	33 $\pm$ 1.9	2.54
Fisher's <i>P</i> value	...	0.009	0.070		N.S.	
Control group	6	20 $\pm$ 1.7	54 $\pm$ 2.9	2.70	34 $\pm$ 0.9	1.70
Adrenalectomized group (females)	6	12 $\pm$ 0.7	60 $\pm$ 3.3	5.0	48 $\pm$ 2.2	4.00
Fisher's <i>P</i> value	...	0.001	N.S.		< 0.001	
Control group	6	24 $\pm$ 3.4	69 $\pm$ 4.5	2.85	45 $\pm$ 3.4	1.87
Glucagon-treated group	6	19 $\pm$ 2.0	59 $\pm$ 2.1	3.11	40 $\pm$ 1.0	2.10
Fisher's <i>P</i> value	...	0.020	0.021		N.S.	
Control group	5	23 $\pm$ 4.4	73 $\pm$ 7.1	3.20	50 $\pm$ 5.3	2.17
Alloxan-diabetic group	5	28 $\pm$ 1.7	81 $\pm$ 4.9	2.87	53 $\pm$ 2.7	1.89
Fisher's <i>P</i> value	...	N.S.	N.S.		N.S.	
Control group	5	22 $\pm$ 3.5	42 $\pm$ 3.5	1.89	20 $\pm$ 1.6	0.91
Growth hormone-treated group	6	14 $\pm$ 1.6	27 $\pm$ 1.9	1.94	13 $\pm$ 0.6	0.93
Fisher's <i>P</i> value	...	0.044	0.004		< 0.001	

* Estimated by the method of Burch *et al.* (1963).

Table 5. *Effect of adrenalectomy on the activities of NADP-linked enzymes*

Rats were adrenalectomized 2 weeks before use and were pair-fed throughout this period. The adrenalectomized rats were maintained on 1% NaCl. Enzymes were extracted and assayed as described in the Methods section. One unit is defined as the amount of enzyme oxidizing or reducing 1 μ mole of NADPH or NADP⁺/hr. at 20°. Statistical treatment was as in Table 1.

No. of animals ...	Control group 10	Adrenalectomized group 10	Fisher's P value
Initial body wt. (g.)	157 $\pm$ 5	159 $\pm$ 5	
Final body wt. (g.)	158 $\pm$ 4	159 $\pm$ 4	
Liver wt. (g.)	6.80 $\pm$ 0.44	6.85 $\pm$ 0.38	
Glucose 6-phosphate dehydrogenase (pH 7.6)			
(units/g. of liver)	112 $\pm$ 5	107 $\pm$ 4	N.S.
(total units/liver)	793 $\pm$ 55	736 $\pm$ 54	N.S.
(total units in liver/100g. body wt.)	478 $\pm$ 37	460 $\pm$ 29	N.S.
6-Phosphogluconate dehydrogenase (pH 7.6)			
(units/g. of liver)	70 $\pm$ 8	62 $\pm$ 7	0.097
(total units/liver)	473 $\pm$ 36	420 $\pm$ 26	N.S.
(total units in liver/100g. body wt.)	298 $\pm$ 19	236 $\pm$ 14	N.S.
6-Phosphogluconate dehydrogenase (pH 9.0)			
(units/g. of liver)	141 $\pm$ 22	119 $\pm$ 13	N.S.
(total units/liver)	957 $\pm$ 80	814 $\pm$ 45	N.S.
(total units in liver/100g. body wt.)	603 $\pm$ 49	508 $\pm$ 23	0.097
NADPH-cytochrome <i>c</i> oxidoreductase*			
(units/g. of liver)	69 $\pm$ 5	59 $\pm$ 4	N.S.
(total units/liver)	429 $\pm$ 39	382 $\pm$ 39	N.S.
(total units in liver/100g. body wt.)	273 $\pm$ 22	240 $\pm$ 24	N.S.
NADPH-glutathione oxidoreductase*			
(units/g. of liver)	186 $\pm$ 8	198 $\pm$ 17	N.S.
(total units/liver)	1108 $\pm$ 81	1259 $\pm$ 74	N.S.
(total units in liver/100g. body wt.)	713 $\pm$ 46	793 $\pm$ 54	N.S.
Non-protein thiol†			
(μ moles/g. of liver)	6.60 $\pm$ 0.40	3.33 $\pm$ 0.43	< 0.001
(total μ moles/liver)	44.5 $\pm$ 2.6	24.7 $\pm$ 2.7	< 0.001
(total μ moles in liver/100g. body wt.)	28.8 $\pm$ 1.8	15.1 $\pm$ 1.9	< 0.001

* These groups contained six pairs of animals only.

† These groups contained seven pairs of animals only.

out in the presence of excess of substrate and co-factor, the results do not necessarily reflect the activities of the enzymes *in vivo*. To approximate more closely to the conditions obtaining *in vivo* the isotope experiments listed in Table 6 were carried out. It is apparent that adrenalectomy does not affect the partition of glucose between the two oxidative pathways as judged by the incorporation of ¹⁴C into carbon dioxide and by the ratio (¹⁴CO₂ from [1-¹⁴C]glucose)/(¹⁴CO₂ from [6-¹⁴C]glucose), which is about 1.6 for both sets of animals. Neither does it affect incorporation of ¹⁴C into lipids. However, the presence of phenazine methosulphate in the incubation mixture caused the anticipated rise in the oxidation of C-1 of glucose in normal rats but a smaller rise in the oxidation of C-1 in adrenalectomized rats. Results of the converse experiment, the treatment of normal rats with adrenal steroids (high dosage, cortisone acetate; low dosage, hydrocortisone hemisuccinate), are shown in

Table 7. The only observed change is that the high dose of cortisone acetate decreased the activity of glucose 6-phosphate dehydrogenase.

In the light of the changes of nicotinamide nucleotides found after treatment with glucagon, the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were also measured, but no significant changes were detected.

DISCUSSION

The evaluation of the part played by the nicotinamide nucleotides as regulators of metabolic processes is complicated because it is always possible to interpret the changes found as either a cause or a result of metabolic events. For this reason, hormonal conditions were chosen that affected each of the major metabolic pathways, certain of them causing the same end effect through obviously different mechanisms. Adrenalectomy

Table 6. *Effect of adrenalectomy on the metabolism of specifically labelled glucose by rat liver*

Tissue slices (300 mg.) were incubated in 4.5 ml. of Krebs-Ringer bicarbonate medium with 100 μ moles of glucose containing 0.4 μ C of [1-¹⁴C]glucose or [6-¹⁴C]glucose or in the presence of [1-¹⁴C]glucose + phenazine methosulphate (final concn. 0.1 mM). The gas phase was O₂ + CO₂ (95:5), the time of incubation was 3 hr. and the temperature was 37°. Rats were killed 2 weeks after bilateral adrenalectomy. The control group were pair-fed with the adrenalectomized group. Details of the isotopic procedures are given in the Methods section. Statistical treatment was as in Table 1.

	No. of animals	Control group	Adrenalectomized group	Fisher's <i>P</i> value
Initial body wt. (g.)	17	150 ± 3	150 ± 4	
Final body wt. (g.)	17	156 ± 3	160 ± 5	
Liver wt. (g.)	17	6.79 ± 0.31	7.13 ± 0.27	
Incorporation of ¹⁴ C into CO ₂ (μ moles/g./hr.)				
From [1- ¹⁴ C]glucose (<i>a</i>)	17	4.78 ± 0.26	4.35 ± 0.23	N.S.
From [6- ¹⁴ C]glucose (<i>b</i>)	8	2.49 ± 0.20	2.57 ± 0.30	N.S.
<i>a/b</i> ratio		1.92	1.69	
From [1- ¹⁴ C]glucose + phenazine methosulphate	12	8.87 ± 0.68	6.96 ± 0.39	0.016
Incorporation of ¹⁴ C into lipid (μ moles/g./hr.)				
From [1- ¹⁴ C]glucose	14	1.19 ± 0.17	1.68 ± 0.23	0.082
From [6- ¹⁴ C]glucose	8	1.24 ± 0.20	1.37 ± 0.32	N.S.
Residual glucose (μ moles/flask)				
[¹⁴ C]Glucose	6	111 ± 4	122 ± 9	N.S.
[¹⁴ C]Glucose + phenazine methosulphate	6	113 ± 2	128 ± 5	N.S.

Table 7. *Effect of treatment of normal male rats with either cortisone acetate or hydrocortisone hemisuccinate on the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase*

The rats were given a daily dose of 5 mg. of cortisone acetate for 3 days or a single dose of 5 μ g. of hydrocortisone hemisuccinate. Enzymes were extracted and assayed as described in the Methods section. One unit is the amount of enzyme reducing 1 μ mole of NADP⁺/hr. at 20°. Statistical treatment was as in Table 1.

	No. of animals.....	Control group 8	Cortisone acetate-treated group 8	Fisher's <i>P</i> value
Initial body wt. (g.)		162 ± 6	163 ± 6	
Final body wt. (g.)		168 ± 4	159 ± 7	
Liver wt. (g.)		8.51 ± 0.23	9.26 ± 0.42	
Glucose 6-phosphate dehydrogenase (pH 7.6)				
(units/g. of liver)		61 ± 6	42 ± 3	0.014
(total units/liver)		525 ± 59	384 ± 28	0.045
(total units in liver/100 g. body wt.)		312 ± 35	245 ± 17	N.S.
6-Phosphogluconate dehydrogenase (pH 9.0)				
(units/g. of liver)		107 ± 5	99 ± 4	N.S.
(total units/liver)		907 ± 43	916 ± 55	N.S.
(total units in liver/100 g. body wt.)		541 ± 27	578 ± 29	N.S.
	No. of animals.....	Control group 5	Hydrocortisone hemisuccinate-treated group 5	Fisher's <i>P</i> value
Body wt. (g.)		151 ± 5	147 ± 5	
Liver wt. (g.)		8.51 ± 0.29	7.66 ± 0.23	
Glucose 6-phosphate dehydrogenase (pH 7.6)				
(units/g. of liver)		97 ± 2	93 ± 5	N.S.
(total units/liver)		830 ± 85	717 ± 64	N.S.
(total units in liver/100 g. body wt.)		550 ± 60	516 ± 39	N.S.

and growth hormone both depress urea formation, whereas cortisone, diabetes and glucagon treatment have the opposite effect. All of these hormones have powerful effects on carbohydrate metabolism, and on glycogen synthesis and storage in particular; there are many similarities between diabetes, glucagon and growth hormone in this respect. There is evidence for increased oxidation of fat in both growth hormone-treated and diabetic rats accompanied by an inhibition of fatty acid synthesis, whereas glucagon and cortisone both promote ketone-body formation. It was thought that a consideration of the changes in nicotinamide nucleotide concentrations in each of these conditions, where the flow of metabolites may be so widely different, could contribute to an understanding of the role of these nucleotides in control mechanisms.

One of the most striking changes that have emerged from the present study is the change in the oxidation state of the hepatic NADP⁺. Three of the four treatments used lead to a considerable decrease in the tissue concentration of NADP⁺ where, because of its low concentration, a change is more readily detectable than in the concentration of the NADPH. Only diabetes caused no change in this nucleotide. Among the many factors that can influence the concentration of NADP⁺ in a tissue is the rate of reoxidation of NADPH by enzymes such as NADPH-cytochrome *c* oxidoreductase, NADPH-glutathione oxidoreductase, transhydrogenase, the malic enzyme bringing about the NADPH-linked carboxylation of pyruvate (EC 1.1.1.40), which is important in the formation of glycogen from non-glucose precursors, and enzyme systems such as that achieving fatty acid synthesis, as well as many microsomal hydroxylation and desaturation processes. The concentration of NADP⁺ can also be influenced by the rate of its reduction by enzymes such as NADP⁺-linked isocitrate dehydrogenase (EC 1.1.1.42) and the enzymes of the hexose monophosphate pathway.

Effect of adrenalectomy on the concentration of the nicotinamide nucleotides. To account for the decline in the concentration of NADP⁺ measurements have been made of the activities of two of the enzymes that bring about its reduction (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and two of the enzymes that cause the reoxidation of NADPH (cytochrome *c* reductase and glutathione reductase). However, in no case was there a significant decrease. Our results are in agreement with many previous reports (Huggins & Fung-o Yao, 1959; Ashmore, 1959; Weber, Banerjee & Bronstein, 1961; Freedland & Barnes, 1963) that also show that the removal of these glands has little or no effect on the activity of the dehydrogenase. The isotope experiments with

liver slices (Table 6) give a much closer approximation to conditions *in vivo* and therefore present a picture more closely reflecting normal tissue function and enzyme activity. Such results must, however, be interpreted with caution. Thus Katz & Wood (1960) have drawn attention, in particular, to the need for information on the changes in glucose concentration during the experiment. In the present study it has been shown that, despite possible differences in initial glycogen content of the livers from control and adrenalectomized rats, the final concentration of glucose in the incubation medium was the same in both groups of animals, even when phenazine methosulphate was present (Table 6). (A liver glycogen concentration of 4% could contribute up to 12mg. of glucose compared with the 18mg. initially added to the medium.) Thus quantitative interpretation has not been attempted. Nevertheless, some general inferences may be drawn from the results of the isotope experiments. In particular, the difference between ¹⁴CO₂ from [1-¹⁴C]glucose and ¹⁴CO₂ from [6-¹⁴C]glucose (referred to below as C-1 - C-6) may be taken to be related to the activity of the first two enzymes of the pentose phosphate pathway. Table 6 shows that the activities of these two dehydrogenases are comparatively unchanged by adrenalectomy (C-1 - C-6 or controls, 2.29; C-1 - C-6 for adrenalectomized group, 1.78), and the overall activity of the systems producing ¹⁴CO₂ from either form of labelled glucose is virtually the same in control and experimental animals. Thus the rate of reduction of NADP⁺ is essentially the same in both groups.

The ratio (C-1 - C-6)/C-6 may, as a first approximation, be taken to relate the activities of the pentose phosphate pathway to carbon dioxide production via the glycolytic and tricarboxylic acid route. Thus in the adrenalectomized animals there is a smaller contribution from the pentose phosphate pathway, the ratio being 0.92 for control animals and 0.69 for adrenalectomized animals. The shift is shown more clearly when slices are incubated in the presence of phenazine methosulphate. The addition of artificial electron acceptors such as phenazine methosulphate, pyocyanine or methylene blue is generally believed to stimulate the pentose phosphate pathway by regenerating NADP⁺, and the smaller stimulation, compared with control rats, found in the adrenalectomized group in the presence of such an acceptor (Table 6) reveals a smaller capacity of the pentose phosphate pathway in these animals.

It seems unlikely that the rate of reoxidation of NADPH by lipogenesis is much altered since the incorporation of ¹⁴C from labelled glucose into lipid is only very slightly increased by adrenalectomy. In similar studies Fain & Wilhelmi (1962) reported that adrenalectomy does not

affect the incorporation of ^3H from $^3\text{H}_2\text{O}$ into fats, although Perry & Bowen (1956a,b) found a slight decrease in this synthetic pathway under similar conditions. The balance of evidence, however, seems to indicate only minor changes in the rate of lipogenesis. In the present results the slightly increased rate of lipogenesis would, if anything, tend to increase the concentration of NADP^+ . The evidence of the isotope experiments therefore provides no explanation of the lowered concentration of NADP^+ actually found.

The bulk of evidence showing that the rate of reoxidation of NADPH to NADP^+ in a wide variety of tissues is an important factor controlling the overall activity of the pentose phosphate pathway [corneal epithelium (Kinoshita, 1957); muscle (Rossi, Zatti & Greenbaum, 1963); liver (Cahill, Hastings, Ashmore & Zottu, 1958; Hers, 1957); ascites-tumour cells (Wenner, Hackney & Moliterno, 1958); mammary gland (McLean, 1960)] makes it rather surprising that the low concentration of NADP^+ found *in vivo* does not have a more striking effect on the metabolism of glucose by liver slices from adrenalectomized rats. It could be that the change in concentration of NADP^+ does not take place in the soluble fraction of the cell in which the dehydrogenases of the pentose phosphate pathway are localized. An examination of the intracellular distribution of the oxidized and reduced forms of the nicotinamide nucleotides after adrenalectomy could throw further light on this problem.

Other systems that could lead to a reoxidation of NADPH are the malic enzyme, NADPH -cytochrome *c* oxidoreductase and NADPH -glutathione oxidoreductase. The activity of the malic enzyme has been measured by Shrago, Lardy, Nordlie & Foster (1963), who found it unchanged by adrenalectomy. The two reductases also appear to be unchanged by adrenalectomy (Phillips & Langdon, 1956, and Table 5). However, control of a metabolic reaction can be achieved not only by variation in the activity of the enzyme concerned but also by the availability of its substrate, a point well illustrated by the work of Lowry & Passonneau (1964). Tipton (1944) and Tipton, Leath, Tipton & Nixon (1946) have found a fall in both the cytochrome *c* content and the cytochrome-oxidase activity in adrenalectomized rats, which suggests that the overall activity of this route could be decreased. Also in the same category is the tissue concentration of glutathione. On the evidence of the activity of the NADPH -glutathione oxidoreductase this enzyme could be of considerable importance in the reoxidation of NADPH , since it has an activity approximately equal to that of the pentose phosphate pathway (Table 5). Although the biochemical route of hydrogen transfer from

glutathione is not entirely clear (see Knox, 1960), the association of a marked fall in the concentration of GSH, both in adrenalectomized rats (Table 5) and in those treated with large doses of cortical steroids (Jocelyn, 1959, and Table 5), with a decreased concentration of NADP^+ appears to indicate a significant linking of these cofactors. The parallelism is all the more striking in that adrenalectomy and treatment with corticoids normally produce opposing changes.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been measured in both adrenalectomized and cortisone-treated rats and, though only glucose 6-phosphate dehydrogenase is significantly decreased, there is a trend towards a fall in activity of both enzymes in both conditions (Tables 5 and 7).

Finally, the influence of adrenalectomy on other NADP -linked reactions may be considered. Remmer (1958) and Gillette (1963) have both studied the NADP^+ -linked microsomal hydroxylation reactions and report that such reactions are decreased in adrenalectomized rats, and this also would limit the rate of re-formation of NADP^+ . Ammonia fixation by glutamate dehydrogenase is also an NADPH -linked reaction, and the activity of this enzyme is probably decreased by adrenalectomy (see Knox, Auerbach & Lin, 1956; McLean & Gurney, 1963); this, again, would result in a lowered rate of NADPH oxidation.

The design of experiments to investigate the effects of restoration of adrenal influence by replacement therapy is exceedingly difficult in that the gland normally secretes a complex of steroids and replacement is usually with only a single steroid. It seemed more useful therefore to study the effect of a single steroid superimposed on the existing pattern in a normal animal. The injection of a physiological dose for a relatively short period of time ($5\text{ }\mu\text{g.}$ for 24 hr.) tended to produce the reverse of the adrenalectomy effect, i.e. a raised NADP^+ concentration. Larger doses (up to 5 mg.) for longer times (up to 3 days) of cortisone acetate produced a pattern of change similar to that found after adrenalectomy; it could be that at this high dose the steroid has a 'toxic' effect, producing secondary changes via the pituitary. The decreased NADP^+ concentration found both after adrenalectomy and in the presence of excess of steroid points to the existence of some common features in these two conditions, although many facets of the metabolic pattern change in opposite directions. A striking feature of the similarity between adrenalectomized and cortisone-treated animals is the remarkable change in the $\text{NADP}^+/\text{NADPH}$ ratio. This is about doubled in both experimental groups.

Treatment with glucagon. Although glucagon causes decreases in the NADH , NADP^+ and

NADPH concentrations, the decreases are probably of minor physiological significance since, when calculated either as total hepatic nucleotide or as total hepatic nucleotide/100g. body wt., these differences disappear. The activities of the first two enzymes of the pentose phosphate pathway also show no response to glucagon treatment. Perhaps more interesting is the change in the NAD^+/NADH ratio. There are many metabolic parallels between a glucagon-treated and a diabetic animal, in the sense that in both cases there is an accelerated protein breakdown, increased glycogenolysis (Best, 1959; de Duve, 1953) and ketosis (see Salter, 1960). This resemblance is also shown by the pattern of change of the nucleotides. Thus the NAD^+/NADH ratio of glucagon-treated rats increases from the control value of 3.7 to 5.0, largely owing to a fall in the NADH content. Similar falls in NADH content have been reported for diabetic animals by Helmreich *et al.* (1954) and Greenbaum & Graymore (1956), although Glock & McLean (1955) found the opposite change in this ratio in their diabetic rats, as did Hohorst, Kreutz & Reim (1961) although their method of determining the ratio was entirely different.

Treatment with pituitary growth hormone. At 12 hr. after the injection of growth hormone the liver has a high rate of fat oxidation (Greenbaum & McLean, 1953) and a depressed rate of lipogenesis (Greenbaum & Glascock, 1957), whereas after 3 days of treatment the rate of oxidation has returned to normal (Greenbaum & McLean, 1953) although lipogenesis is still very depressed (P. J. Bunyan & A. L. Greenbaum, unpublished work). The decreased content of NADP^+ and the high $\text{NADPH}/\text{NADP}^+$ ratio found at both times could well be explained in terms of a decline in the activities of the enzymes involved in fatty acid synthesis, evidence for which has been obtained from a study of acetyl-CoA carboxylase and from the incorporation of malonyl-CoA into palmitate in similarly treated animals (P. J. Bunyan & A. L. Greenbaum, unpublished work). After treatment with growth hormone for 3 days, a different pattern is established with respect to both the nicotinamide nucleotides and lipid metabolism. At this stage there is an increase in the total liver content of NAD^+ and NADH (the sum of these nucleotides is increased some 35% over the control level) and a fall of 25% in the total available NADP^+ and NADPH. There is also at this time a normal rate of fatty acid oxidation, an NAD-requiring process, and a depressed rate of lipogenesis, an NADP^+ -requiring process. It appears possible that changes in both enzyme activity and coenzyme content may play a part in the decreased fatty acid synthesis that follows growth hormone treatment. The implications of these changes in relation to the

known alterations in cell structure must also be considered. There is evidence for a striking increase in mitochondrial numbers after treatment with growth hormone (Reid, Slater & Greenbaum, 1955) and, here again, the distribution of the nicotinamide nucleotides in the subcellular compartments would clearly be of interest. The intracellular distribution of the nucleotides is such that only about 13% of the total NAD is in the mitochondria, whereas about one-third of the total NADP is in this fraction (Glock & McLean, 1956; Jacobson & Kaplan, 1957; Birt & Bartley, 1960). The three-fold elevation in mitochondrial numbers in conjunction with the altered concentrations of nicotinamide nucleotides will surely cause alteration in the relative concentrations of the nicotinamide nucleotides in the soluble fraction or in the mitochondria themselves.

The fact that the sum total of all four nucleotides is unchanged by growth hormone treatment and that the increase in total NAD is approximately balanced by the decline of NADP may indicate a competition for a common precursor that is preferentially available for NAD synthesis. The facts could also be interpreted as showing an enzymic lesion in the NAD^+ -kinase step or a change in the relative activities of enzymes causing the breakdown of NAD or NADP.

Form of NADP^+ extractable in weak acid. The physiological significance of this form of NADP^+ is not known. In three of the treatments applied (glucagon, alloxan-diabetes and growth hormone) the two forms show parallel changes. In adrenalectomy, however, the ratio (NADP^+ extractable in weak acid)/(NADP^+ extractable in 0.1N-hydrochloric acid) is increased, more strikingly so in females than in males; and in fully developed primary hepatomas the NADP^+ extractable in weak acid disappears altogether although some of the 0.1N-hydrochloric acid-stable NADP^+ still remains (Clark, Greenbaum, McLean & Reid, 1964 and Table 4). The resemblance between the labile form of NADP^+ described by Burch *et al.* (1963) and the 'extra- NADP^+ ' described by Purvis (1960) emphasizes the possible metabolic significance of this NADP^+ in mitochondrial metabolism. Some preliminary studies (A. L. Greenbaum & S. Pinder, unpublished work) have shown that mitochondria have by far the highest (NADP^+ extractable in weak acid)/(NADP^+ extractable in 0.1N-hydrochloric acid) ratio.

Clearly, information on the relation between mitochondrial numbers and content of the two forms of NADP^+ in a variety of metabolic states is desirable in evaluating the metabolic function of the extra NADP^+ . Such information as is available on mitochondrial numbers does not show any very close correlation with this extra form of NADP^+

extracted in weak acid. For example, only slight changes occur in mitochondrial numbers in adrenalectomized rats (Reid *et al.* 1955), where the highest proportion of this form of NADP⁺ is found, whereas in growth hormone-treated rats, which have a large increase in mitochondrial numbers (Reid *et al.* 1955), the NADP⁺ extractable in weak acid decreases. Clearly, these interrelationships are not simple and further interpretation will depend on information on the intracellular distribution, together with more knowledge of the changes of the subcellular structure of the cell as a result of hormonal treatment.

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