Influence of Hormones on the Nicotinamide Nucleotide Coenzymes of Adipose Tissue

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(Received 31 March 1967)

The concentrations of the oxidized and reduced forms of the nicotinamide nucleotides were measured in the epididymal fat pads of normal, alloxan-diabetic and hypophysectomized rats. In both alloxan-diabetic rats and hypophysectomized rats the weight of the adipose tissue fell, as did the total content of NADH and NADPH; in addition, NAD^+ was decreased in the alloxan-diabetic group. Of these changes the most marked was in NADPH and this was the only significant difference when the results were expressed as nicotinamide nucleotides/mg. of tissue protein. The concentration of NADPH in the hypophysectomized rats was not altered by treatment with growth hormone but was restored to normal by treatment with thyroxine. These results are discussed in relation to the known effect of these hormonal conditions on lipid synthesis in adipose tissue.

There is, at present, relatively little information on the concentration and oxidoreduction state of the nicotinamide nucleotides in adipose tissue. The few reports so far published have been concerned mainly with the concentration in normal rat adipose tissue (Eichel, 1959; Pastan, Wills, Herring & Field, 1963; Greenbaum, Clark & McLean, 1965*a*). Since adipose tissue is particularly responsive to changes in hormonal conditions or dietary regime it seemed a very suitable tissue in which to study hormonal control mechanisms in relation to the nicotinamide nucleotides.

There is ample evidence for a close association of the pentose phosphate pathway with fat synthesis. However, recent studies with labelled glucose have stressed the limitation of this pathway in providing all the NADPH needed for fat synthesis in adipose tissue, particularly in conditions of rapid lipogenesis (Flatt & Ball, 1964; Katz, Rognstad & Kemp, 1965; Katz, Landau & Bartsch, 1966; Rognstad & Katz, 1966). The contribution of hydrogen from NADH and NADPH to fatty acid synthesis has been studied by using ^{14}C - and ^3H -glucose, and it would seem that as much as one half of the hydrogen equivalents for fatty acid synthesis may originate from non-mitochondrial NADH (Katz & Rognstad, 1966). For this reason it seemed of interest to measure the concentration of the oxidized and reduced NAD and NADP in hormonal conditions where lipogenesis is altered.

The present results report the effect of hypo-

physectomy and of replacement treatment with growth hormone or thyroxine on the nicotinamide nucleotide concentrations of adipose tissue; the effect of alloxan-diabetes was also studied. These results emphasize the importance of thyroxine as well as insulin in controlling fatty acid synthesis in adipose tissue.

METHODS

Materials. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and alcohol dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Phenazine methosulphate and L-thyroxine were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Growth hormone (bovine) was a gift from the Endocrinology Study Section of the National Institutes of Health, Bethesda, Md., U.S.A.

Animals and hormonal treatment. Hypophysectomized and control male rats were purchased from the Charles River Co. (Wilmington, Mass., U.S.A.) and were fed on a stock diet, supplied *ad lib.* and supplemented by 5% (w/v) glucose in the drinking water, until used 8 days after hypophysectomy. The rats were then divided into four groups: (1) normal control rats; (2) hypophysectomized rats; (3) hypophysectomized rats treated with 0.5 mg. of growth hormone/day; (4) hypophysectomized rats treated with 10 μg . of L-thyroxine/day; treatment was continued for 8 days, during which period the food intake of each group was matched, by limit feeding, to the amount consumed by the hypophysectomized group.

Adult male albino rats (body wt. 200–220 g.) were injected subcutaneously with a single dose of 20 mg. of alloxan/100 g. body wt. These animals were then given 2 units of

Table 1. *Comparison of methods of extraction on the estimation of nicotinamide nucleotides of adipose tissue*

One fat pad was clamped immediately in metal tongs cooled in liquid N₂ and then powdered and extracted with 0.5N-HClO₄ as described by Bücher, Krejci, Rüssman, Schnitger & Weseman (1964). The other fat pad from the same rat was extracted by dropping it immediately into boiling 0.1N-HCl as described by Glock & McLean (1955) and Greenbaum *et al.* (1965a). Similarly the reduced nicotinamide nucleotides were extracted by the cold and hot extraction method with 0.1N-NaOH. The oxidized and reduced forms of the nicotinamide nucleotides of the neutralized extracts were determined as described in the Methods section.

	Nicotinamide nucleotides ($\mu\text{g./fat pad}$)			
	Cold extraction		Hot extraction	
	0.5N-HClO ₄	0.1N-NaOH	0.1N-HCl	0.1N-NaOH
NAD ⁺ , NADH	22.0	6.7	22.6	6.5
NADP ⁺ , NADPH	2.6	14.2	2.2	12.1

protamine-zinc-insulin/day for 1 week after the administration of the alloxan; insulin treatment was then stopped and the rats were killed 2 weeks later for determination of the nicotinamide nucleotides in the epididymal fat pads.

Determination of the nicotinamide nucleotide coenzymes. The concentrations of the oxidized and reduced forms of NAD and NADP were measured in adipose tissue by the method of Greenbaum *et al.* (1965a). This method was chosen since it is the most convenient extraction technique and experimental evidence has been obtained (Table 1) to show that it gave results almost identical with those obtained when the more elaborate 'rapid fixation by freezing' method was used. One epididymal fat pad was used for acid extraction in 0.1N-HCl and one for alkaline extraction in 0.1N-NaOH for the oxidized and reduced forms of the nicotinamide nucleotides respectively. The results are given as $\mu\text{g.}$ of coenzyme/epididymal fat pad or as $\mu\text{g.}$ of coenzyme/mg. of tissue protein. The protein was determined with the whole-tissue homogenate essentially by the method of Lowry, Rosebrough, Farr & Randall (1951).

Distribution of NAD(H) + NADP(H) in adipose tissue. The distribution of the nicotinamide nucleotides in the mitochondrial and microsome + supernatant fraction was studied by homogenizing two fat pads (approx. 1.4g.) in 10ml. of 0.25M-sucrose and centrifuging for 160000g-min. The supernatant from this centrifugation was used directly for the estimation of the oxidized and reduced forms of the nucleotides, whereas the mitochondrial pellet was resuspended in 10ml. of 0.25M-sucrose before being so used. Perhaps a more complete fractionation would have been desirable, but it was considered that the much greater times of centrifugation would have led to more uncertainty in the validity of the results obtained. Although the absolute values found may not reflect the true picture of the redox state, they nevertheless almost certainly reveal that, in adipose tissue, the vast bulk of the NADP(H) is in the supernatant fraction. The molecular weights of NAD and NADP were taken as 663 and 745 respectively.

Preparation of tissue extracts. The two fat pads were homogenized with an all-glass Potter-Elvehjem homogenizer in 10ml. of a medium containing 150mM-KCl, 5mM-MgCl₂, 5mM-EDTA and 10mM- β -mercaptoethanol and adjusted to pH7.4 by adding KHCO₃. The homogenates were centrifuged at 100000g for 45min. and the

supernatant was dialysed against the extracting medium for 1 hr. in the cold. The dialysed preparation was used for the determination of the enzyme activities.

Determination of enzyme activities. Hexokinase (D-hexose-ATP phosphotransferase; EC 2.7.1.1), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate-NADP oxidoreductase; EC 1.1.1.44) and isocitrate dehydrogenase [α -isocitrate-NADP oxidoreductase (decarboxylating); EC 1.1.1.42] were measured as described previously (McLean & Brown, 1966). The unit of enzyme activity is defined as that amount of enzyme forming 1 μmole of product/min. at 25°; for convenience the adipose-tissue values are expressed as milliunits, i.e. μmoles of products produced/min./mg. of protein at 25°. The rate of reduction of NADP⁺ was measured in a Unicam SP.800 recording spectrophotometer with constant-temperature cell housing and scale-expansion accessory.

RESULTS

The content of nicotinamide nucleotides in adipose tissue has been measured in several different Laboratories by a variety of techniques (Eichel, 1959; Pastan *et al.* 1963; Greenbaum *et al.* 1965a). In accord with the general pattern found in most tissues NAD was present largely in the oxidized form and NADP in the reduced form.

The distribution of the nicotinamide nucleotides between the soluble and the particulate fractions of adipose tissue shows that whereas NAD⁺ + NADH is distributed approximately equally between these two fractions the NADP⁺ + NADPH was located largely in the supernatant fraction, which contained about 80% of the total NADP(H) (Table 2). The oxidation-reduction state of the nicotinamide nucleotides altered during the fractionation procedure and a greater proportion was found in the oxidized form.

The effect of hypophysectomy on the content of the oxidized and reduced forms of NAD and

Table 2. *Distribution of nicotinamide nucleotides between the soluble and particulate fraction of adipose-tissue homogenates*

Adipose-tissue homogenates were prepared in 0.25 M-sucrose with the proportions of two fat pads (1.4 g. of tissue) to 10 ml. of sucrose. These were spun at 8000g for 20 min. The top layer of fat was discarded, the supernatant layer and the particulate fraction, which was resuspended in 10 ml. of 0.25 M-sucrose, were used for the determination of the oxidized and reduced forms of the nicotinamide nucleotides by heating with a final concentration of 0.1 N-HCl or 0.1 N-NaOH as described by Glock & McLean (1955) and Greenbaum *et al.* (1965a).

	Nicotinamide nucleotides ($\mu\text{g./fat pad}$)			Whole fat pad
	Supernatant fraction	Particulate fraction	Total (supernatant+particulate)	
NAD ⁺	11.0	8.5	19.5	22.6
NADH	3.6	6.7	10.3	6.7
NAD ⁺ +NADH	14.6	15.2	29.8	29.3
NADP ⁺	4.2	1.5	5.7	2.2
NADPH	5.4	1.1	6.5	12.1
NADP ⁺ +NADPH	9.6	2.6	12.2	14.3

Table 3. *Effect of hypophysectomy and growth hormone on the content of nicotinamide nucleotide coenzymes of epididymal fat pad*

Results are expressed as means $\pm$ s.e.m. Fisher's *P* values are given and are considered significant if *P* is less than 0.05; where this value is greater than 0.2 the values are given as N.S. (not significant).

Treatment ...	Control	Hypo-	Fisher's <i>P</i>	Hypo-	Fisher's <i>P</i>	Fisher's <i>P</i>
Group no. ...	(1)	physectomy	(1) versus (2)	physectomy + growth hormone	(2) versus (3)	(1) versus (3)
No. of animals	6	6		6		
Wt. of fat pad (mg.)	542 $\pm$ 59	433 $\pm$ 51		450 $\pm$ 53		
Protein content (mg./fat pad)	12.3 $\pm$ 1.0	10.3 $\pm$ 0.9		12.5 $\pm$ 0.8		
Body wt. (g.)						
Initial	162 $\pm$ 6	151 $\pm$ 5		155 $\pm$ 5		
Final	172 $\pm$ 6	154 $\pm$ 4		170 $\pm$ 6		
Coenzymes ($\mu\text{g./fat pad}$)						
NAD ⁺	14.40 $\pm$ 2.26	11.80 $\pm$ 1.50	N.S.	10.0 $\pm$ 1.40	N.S.	N.S.
NADH	4.57 $\pm$ 0.51	2.67 $\pm$ 0.36	0.015	3.14 $\pm$ 0.46	N.S.	0.06
NADP ⁺ *	1.3	1.6		1.1		
NADPH	5.66 $\pm$ 0.77	2.48 $\pm$ 0.33	0.003	2.53 $\pm$ 0.22	N.S.	0.002
Coenzymes ($\mu\text{g./mg. of protein}$)						
NAD ⁺	1.31 $\pm$ 0.18	1.14 $\pm$ 0.07	N.S.	0.90 $\pm$ 0.13	N.S.	0.09
NADH	0.40 $\pm$ 0.06	0.29 $\pm$ 0.07	N.S.	0.27 $\pm$ 0.06	N.S.	0.12
NADP ⁺ *	0.14	0.17		0.09		
NADPH	0.46 $\pm$ 0.07	0.25 $\pm$ 0.04	0.013	0.21 $\pm$ 0.03	N.S.	0.004

* The NADP⁺ values are for pooled samples since adipose tissue contains very low concentrations of this coenzyme.

NADP is shown in Table 3. When the results are expressed as content per fat pad there is a depression in the reduced forms of both coenzymes, more marked with NADPH; however, when calculated on the basis of tissue protein, only the NADPH

showed a significant fall. Treatment with growth hormone failed to restore the NADPH content to the normal value; however, thyroxine was effective in raising the NADPH content to the normal range (Table 4).

Table 4. *Effect of hypophysectomy and treatment with thyroxine on the NADPH content and activities of certain NADP-linked enzymes in adipose tissue*

A unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mole of product/min. at 25°. Results, in milliunits, are given as means $\pm$ s.e.m. Fisher's *P* values are given for the comparison of each of the experimental groups with the control; where *P* is greater than 0.1 the values are quoted as N.S. (not significant).

No. of animals	Treatment ...	Control 9	Hypophysectomy 9	Hypophysectomy + thyroxine 6
NADPH (μ g./mg. of protein)		0.48 $\pm$ 0.06	0.27 $\pm$ 0.03 (<i>P</i> = 0.01)	0.43 $\pm$ 0.03 (N.S.)
Enzymes (milliunits/mg. of protein)				
Hexokinase		50 $\pm$ 4.5	14 $\pm$ 2.7 (<i>P</i> < 0.001)	53 $\pm$ 5.6 (N.S.)
Glucose 6-phosphate dehydrogenase		74 $\pm$ 9	74 $\pm$ 14 (N.S.)	103 $\pm$ 24 (N.S.)
6-Phosphogluconate dehydrogenase		74 $\pm$ 8	71 $\pm$ 15 (N.S.)	116 $\pm$ 15 (<i>P</i> = 0.035)
Isocitrate dehydrogenase		58 $\pm$ 6	59 $\pm$ 6 (N.S.)	64 $\pm$ 7 (N.S.)

Table 5. *Effect of alloxan-diabetes on the nicotinamide nucleotide coenzymes of epididymal fat pad*

Results are given as means $\pm$ s.e.m. Fisher's *P* values are given and are considered significant if *P* is less than 0.05; where this value is greater than 0.1 the result is given as N.S. (not significant). The NADP⁺ values were too low to measure.

No. of animals	Control group 10	Alloxan-diabetic group 9	Fisher's <i>P</i>
Body wt. (g.)	232 $\pm$ 8	212 $\pm$ 5	
Wt. of 1 fat pad (mg.)	800 $\pm$ 78	320 $\pm$ 55	
Protein content (mg./fat pad)	20 $\pm$ 3.2	10 $\pm$ 1.7	
Coenzymes (μ g./fat pad)			
NAD ⁺	16.2 $\pm$ 2.1	8.8 $\pm$ 0.9	0.006
NADH	4.35 $\pm$ 0.35	2.2 $\pm$ 0.20	< 0.001
NADPH	7.3 $\pm$ 1.6	1.3 $\pm$ 0.13	0.002
Coenzymes (μ g./mg. of protein)			
NAD ⁺	0.82 $\pm$ 0.11	0.87 $\pm$ 0.09	N.S.
NADH	0.22 $\pm$ 0.02	0.21 $\pm$ 0.02	N.S.
NADPH	0.37 $\pm$ 0.08	0.13 $\pm$ 0.17	0.01

Comparison of the activities of three NADP-linked dehydrogenases in adipose tissue, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase, showed that in hypophysectomized rats there was little or no change in the activities of these enzymes expressed on the basis of activity/mg. of protein, i.e. these enzymes decreased in parallel with the loss of protein from the adipose tissue. Hexokinase was a striking exception to this pattern, and was greatly decreased in the hypophysectomized rats.

The effect of alloxan-diabetes on the nicotinamide nucleotides of epididymal fat pad is shown in Table 5. When considered as total coenzyme in the fat pad it is clear that NAD⁺, NADH and NADPH all decrease markedly. However, both

the weight and the protein content of the epididymal fat pad decrease in the diabetic group, and when the results are expressed on a protein basis then only NADPH is significantly decreased in the diabetic group. This pattern of change is very similar to that found in the hypophysectomized rats.

Measurements of the enzymes hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase show that all four enzymes were decreased on the basis of the total adipose-tissue content, and this difference was still apparent for the first three of these enzymes when the results were expressed on a protein basis. The activities of these enzymes/mg. of adipose-tissue protein relative

to the control values were 52%, 43%, 77% and 98% respectively for hexokinase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase (J. Brown & P. McLean, unpublished work). In this respect the pattern of change is different from the hypophysectomized rats, where only hexokinase seemed to be specifically decreased faster than the tissue protein.

DISCUSSION

The most striking change found in the present experiments was the decrease of the NADPH content of adipose tissue both in hypophysectomized rats and in alloxan-diabetic animals. In the hypophysectomized groups the NADPH concentration was returned to the control value by treatment with thyroxine.

Although it is not at present known how this change affects the nicotinamide nucleotides of different cell fractions, from Table 2 it is clear that since nearly 80% of the NADP of adipose cells is in the 'supernatant' fraction any change in the total NADP content greater than 20% must affect the concentration in the supernatant fraction.

The lowered NADPH content could have been caused either by an increased rate of degradation or by a decreased rate of synthesis. The former is unlikely since it has been shown that the NAD glycohydrolase activity of the liver of either alloxan-diabetic or hypophysectomized rats was unaffected by these treatments (Greenbaum, Clark & McLean, 1965*b*; Greengard, Petrack & Kalinsky, 1967). The rate of synthesis would depend on any of three factors, the enzyme NAD kinase and its two substrates NAD and ATP. The values given above for the concentration of NAD, expressed as $\mu\text{g.}$ of NAD/mg. of protein, make it clear that the NAD concentration does not decrease in the adipose tissue of either hypophysectomized or alloxan-diabetic animals and it is therefore unlikely that the fall in NADP concentration is the result of a primary effect on NAD. It is also unlikely that an ATP deficiency is the causative agent, as Denton, Yorke & Randle (1966) have shown that alloxan-diabetes and insulin have no significant effect on the concentrations of the adenine nucleotides in adipose tissue, although it may be noted that their values were obtained in tissues incubated *in vitro*. These considerations would seem to point to the enzyme NAD kinase as being the rate-limiting factor in the synthesis of NADP in the adipose tissue of alloxan-diabetic rats. The activity of this enzyme has not been measured in adipose tissue, but it has been observed that the NAD kinase activity of liver does not decrease in alloxan-diabetic rats (Greenbaum *et al.* 1965*b*).

The second aspect of these changes is their relation to the metabolic state of the tissue.

There is now ample evidence for the view that there is a close gearing between the pentose phosphate pathway and reductive synthetic processes such as fatty acid synthesis, the link being the supply of NADP⁺ and NADPH. The rate of re-oxidation of NADPH appears to be an important factor in regulating the flow of glucose through the pentose phosphate pathway. This has been shown by experiments in which the rate of formation of ¹⁴CO₂ from [1-¹⁴C]glucose was stimulated by adding an artificial electron acceptor such as phenazine methosulphate (McLean, 1960). Studies of the effect of acetate on glucose utilization by epididymal fat pad have shown that the flow of glucose carbon through the pentose phosphate cycle increased 83% in the presence of acetate (Flatt & Ball, 1966). It has been shown by using [³H]glucose that NADPH derived from the pentose phosphate pathway transfers hydrogen nearly quantitatively to fatty acids, although the pentose phosphate pathway may not supply all the hydrogen required in conditions where lipid synthesis proceeds at a high rate, as in insulin-treated animals, or in starved-re-fed rats (Katz *et al.* 1965; Katz & Rognstad, 1966; Rognstad & Katz, 1966; Flatt & Ball, 1964).

Two things must be considered in this present context. These are the fall in the total amount of NADPH available and the NADP⁺/NADPH ratio. Since there is a depression in the amount of NADPH without a concomitant rise in NADP⁺ it would seem that the rate of reduction of NADP⁺ by enzymes of the pentose phosphate cycle is not rate-limiting despite the fall in hexokinase and, on a whole-organ basis, in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The fall in the total available NADPH, of which the greater part must occur in the soluble fraction of the cell, may be correlated with the known depression in lipid synthesis in alloxan-diabetic rats and hypophysectomized rats and the restoration of this process, in the latter case, with thyroxine (see Masoro, 1962; Bates, Zomzely & Mayer, 1955; Dayton, Dayton, Drimmer & Kendall, 1960; Fain & Wilhelmi, 1962). Treatment with growth hormone over a period of several days has been shown to depress lipid synthesis in liver (Brady, Lukens & Gurin, 1951; Greenbaum & Glascock, 1957; Greenbaum & Bunyan, 1965) and adipose tissue (Goodman, 1963), and in this context it may be noted that in the present experiments growth hormone did not cause any restoration of the NADPH content of adipose tissue.

We thank the British Empire Cancer Campaign for financial support, the U.S. Public Health Service for the award of a Fellowship to J.B. while working at The

Middlesex Hospital Medical School, and the Medical Research Council for the purchase of the Unicam spectrophotometers (P. McL. and A. L. G.).

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