Pyridine nucleotide synthesis by rat adipose tissue in vitro

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ABSTRACT The synthesis of NAD and NADP by rat adipose tissue was measured in vitro. Nicotinamide-7-^{14}C and $NaH_2{}^{32}PO_4$ were incorporated together into NAD with a $^{32}P/^{14}C$ ratio of 1.82 and nicotinic-7-^{14}C acid and $NaH_2{}^{32}PO_4$ with a ratio of 1.94. Nicotinic acid stimulated, by 90%, lipogenesis from glucose-U-^{14}C by rat adipose tissue in vitro. Glucose plus insulin and refeeding for 48 hr after a 48 hr fast markedly increased the incorporation of nicotinic-7-^{14}C into NAD in rat epididymal fat pads in vitro, but neither fructose, L-glutamine, nor insulin alone increased the synthesis of NAD in this tissue.

Glucose-1-^{14}C, ribose-1-^{14}C, and to a greater extent glucose-6-^{14}C are incorporated into the NAD of rat adipose tissue. Fasting followed by refeeding sharply increased the radioactivity of NAD-^{14}C formed from glucose-1-^{14}C and glucose-6-^{14}C but not from ribose-1-^{14}C. Increasing the ribose concentration from 2 mM to 10 mM increased its incorporation into adipose tissue NAD twofold.

The nicotinic-7-^{14}C acid incorporation into NAD increased over the 1st hr of incubation and remained constant for the next 3 hr. The concentration of NAD in the fat pads showed a similar response to the time of incubation. NADP concentrations increased over the entire 4 hr incubation period as did the incorporation of nicotinic-7-^{14}C acid into NADP.

The results of this study suggest that NAD is synthesized de novo by rat adipose tissue in vitro and that this synthesis is increased by factors which stimulate lipogenesis.

KEY WORDS adipose tissue · NAD · NADP · biosynthesis · incorporation · nicotinamide · $^{32}P_i$ · nicotinic acid · effect · lipogenesis · coenzyme synthesis · fasting–refeeding · rat

THERE HAS BEEN an increasing awareness of the importance of pyridine nucleotides as factors in the control of metabolic processes. The demonstration of Langdon (1) in 1957 of the NADPH requirement[1] for lipogenesis, and the work of Wakil and associates (2, 3) in identifying the specific NADP-linked dehydrogenase system involved in lipogenesis established an important link between the level of reduced pyridine nucleotides and the synthesis of fatty acids. The current concept holds that the formation of NADPH via the pentose pathway (4, 5) and the malic enzyme system (6, 7) and the generation of NADH by the oxidation of triose phosphates in glycolysis (4) are important factors in controlling lipogenesis. Studies by Greenbaum, Clark, and McLean (8, 9) and Glock and McLean (10) have shown changes in the hepatic concentration of pyridine coenzymes in rats in response to treatment with thyroxine and growth hormone and in alloxan diabetes. Greengard, Quinn, and Reid (11, 12) reported an elevated level of NAD and NADP in response to nicotinamide challenge in the livers of adrenalectomized and (or) hypophysectomized rats. These and other studies (13, 14) suggest that alterations in cellular metabolism are often reflected in alterations in the concentration of pyridine nucleotides.

In the present paper the relationship between the synthesis of NAD and NADP and lipogenesis in adipose tissue is explored. The results show that NAD and NADP are synthesized by this tissue, and that this synthesis is increased by conditions which increase lipogenesis.

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Abbreviation: DEAE, diethylaminoethyl.

[1] *Convention for coenzyme nomenclature.* NAD^+ and $NADP^+$ refer to the oxidized, and NADH and NADPH to the reduced forms of the pyridine nucleotides. NAD and NADP are used for the coenzymes in general without reference to their state of oxidation.

MATERIALS AND METHODS

Materials

Nicotinic-7-^{14}C acid, nicotinamide-7-^{14}C, ribose-1-^{14}C, glucose-1-^{14}C, glucose-6-^{14}C, and $NaH_2{}^{32}PO_4$ were purchased from New England Nuclear Corp., Boston, Mass. D-Glucose, D-ribose, L-glutamine, and D-fructose were obtained from Calbiochem, Los Angeles, Calif. NAD, NADP, NADH, NADPH, ADP ribose, and nicotinamide mononucleotide were obtained from P-L Laboratories, Milwaukee, Wis. N-Methylnicotinamide was prepared by the method of Huff, Perlzweig, and Sharp (15) and used as the iodide.

Diethylaminoethyl (DEAE) cellulose, type 40, with a capacity of 0.81 meq/g was purchased from Brown Co., Berlin, N.H., and cellulose powder MN-300 (particle size $< 10\ \mu$) from Brinkmann Instruments, Inc., Westbury, N. Y.

Highly purified porcine insulin was generously supplied by Dr. O. K. Behrens of the Eli Lilly Research Laboratories, Indianapolis, Ind.

General Methods

Male Wistar rats, 350–400 g, were killed by a sharp blow to the head. The epididymal fat pads were removed, trimmed of vascular tissue, and transferred to 60 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin (Fraction V) and appropriate radioactive substrates with specific activities as indicated in the tables. The tissue was incubated for 3 hr at 38°C in a shaking water bath (90 strokes/min) in an atmosphere of 95% O_2–5% CO_2. After incubation, the fat pads were rinsed in 0.9% NaCl and cut in small pieces directly into 5.0 ml of boiling 0.01 M glycylglycine buffer, pH 8.4. and heated for 3 min. The tissue suspension was transferred to an ice bath and rapidly cooled, then homogenized in a Virtis blender and centrifuged for 20 min at 1000 *g*. Adipose tissue, handled in this manner, yielded a homogenate which separated into three layers upon centrifugation: an upper fat cake, an intermediate layer containing soluble cell components, and a bottom layer consisting of fibrous stroma and cell debris. The intermediate layer was removed and a portion was taken for nitrogen determination by micro-Kjeldahl digestion followed by nesslerization (16). The remainder of the tissue extract was treated with crystalline trichloroacetic acid to give a final concentration of 5%. The trichloroacetic acid was removed by three extractions with 3 volumes of diethyl ether. The ether layer was drawn off and the remaining traces of ether were removed by immersion of the aqueous solution in a 60°C water bath with continual swirling. The pH of the aqueous phase was adjusted to 7.4 with 0.5 N NaOH and the extract was then chromatographed on a DEAE cellulose column. Extraction of the pyridine nucleotides by this procedure gave a recovery of about 90% of the NAD and 105% of the NADP added to the defatted extract of epididymal adipose tissue.

Chromatography

Approximately 3–4 ml of the adipose tissue extract was applied to a DEAE cellulose column 1.0 × 15 cm and chromatographed by gradient elution with 0.3 M NaCl in 0.01 M glycylglycine buffer, pH 7.4 as described previously (17, 18). Fractions were collected at a flow rate of 2 ml/min by use of a peristaltic pump (Buchler Instruments Inc., Fort Lee, N.J.).

The pyridine nucleotide concentration in each fraction was determined by fluorescence in strong base according to Lowry, Roberts, and Kapphahn (19). Fluorescence was measured in a fluorometer (G. K. Turner, Associates, Palo Alto, Calif., Model 110) equipped with a high sensitivity cuvette holder. The primary filter was a Corning combination filter No. 7–33 with a maximum transmission at 365 mμ, and the secondary filter was a Corning No. 5–75 with a maximum transmission at 465 mμ. Standard NAD and NADP added to the column were quantitatively recovered after chromatographic separation as described above (18).

Fractions containing NAD or NADP were rechromatographed on thin layers of cellulose. The layers (250 μ) were prepared by spreading a mixture of 15 g of cellulose powder and 90 ml of water on glass plates. Plates were heated at 105°C for 2 hr and stored over a desiccant in a closed chamber until used. Pyridine nucleotides and other compounds were separated by ascending thin-layer chromatography in isobutyric acid (redistilled)–NH_4OH (concd)–water 96:1:22 for 2–3 hr.

After development, the plates were exposed to cyanogen bromide vapor for 10 min, sprayed with a benzidine solution (0.5% w/v in ethanol), and again exposed to CNBr vapors for an additional 10 min (20). Nicotinic acid appeared red and nicotinamide a light yellow and both fluoresced strongly under ultraviolet light (254 mμ). Pyridine nucleotides were easily visible when the plates were viewed under ultraviolet light (254 mμ). Inorganic phosphate was detected by the method of Hanes and Isherwood (21). All sprays were applied with the Universal Aerosol Spray Kit obtained from Nutritional Biochemicals Corporation, New York, N. Y.

Spots corresponding to standard nucleotide coenzymes were scraped from the thin-layer plates into liquid scintillation vials that contained 2 ml of water. The cellulose containing the nucleotide was suspended in the water and 15 ml of Bray's scintillation mixture (22) was added. This procedure completely removed the radioactive coenzymes from the cellulose. The fractions containing NADP had been eluted from the DEAE cellulose

column with high concentrations of NaCl (Fig. 1). Because the NaCl interfered with the thin-layer chromatographic procedures, the NADP-^{14}C was measured directly in each fraction that had been shown by fluorometric assay to contain NADP. Portions of these fractions were transferred to liquid scintillation vials containing 15 ml of Bray's liquid scintillation medium (22). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (model 314 EX) at 10°C and the simultaneous measurement of ^{14}C- and ^{32}P-labeled NAD was done in a Packard Tri-Carb model 574. The counting efficiency, as determined by internal standards, was 38–49% for ^{14}C. In the experiments in which the incorporation of both ^{14}C and ^{32}P was measured, the efficiency was determined by external standardization. All radioactivity measurements involving ^{32}P were corrected for spontaneous decay of this isotope.

Fig. 1 shows the elution pattern, from a DEAE cellulose column, of a mixture of NAD and NADP added to an extract of rat adipose tissue. This procedure effectively separates NAD and NADP from nicotinamide, *N*-methylnicotinamide, ribose, glucose, and nicotinamide mononucleotide as reported previously (18). It did not, however, separate nicotinic acid and inorganic phosphate (NaH_2PO_4) from NAD (18). Fractions containing NAD were rechromatographed on cellulose thin layers in isobutyric acid–NH_4OH–water 96:1:22 to separate these compounds further. The relative position of standards applied to cellulose thin layers is shown in Fig. 2. Nicotinic acid and inorganic phosphate were separated from NAD and NADP and each spot was easily distinguishable under ultraviolet light (254 mμ)

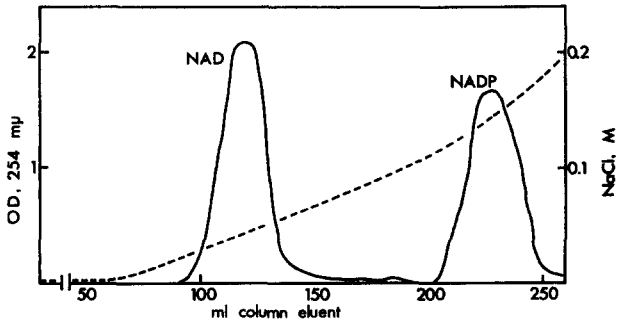

FIG. 1. Elution pattern of standard NAD and NADP from a 15 cm × 1 cm DEAE cellulose column treated according to Pastore and Friedkin (17). Approximately 7 μmoles of NAD and NADP were added to the trichloroacetic acid extract of six epididymal fat pads prepared as outlined in Methods. The ultraviolet absorption of the column eluent was measured at 254 mμ with an ultraviolet analyzer (Instrumentation Specialties Co., Inc., Lincoln, Nebr.). The nucleotide coenzymes were chromatographed by gradient elution with 0.3 M NaCl in 0.01 M glycylglycine buffer, pH 7.4. A flow rate of 2.0 ml/min was maintained by use of a peristaltic pump. Dashed line is the concentration of NaCl.

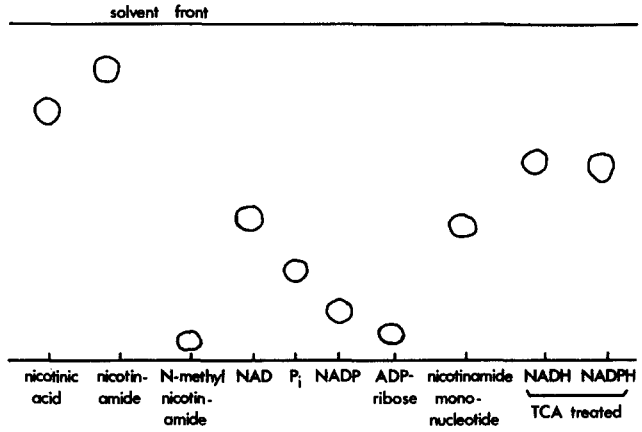

FIG. 2. Cellulose thin-layer chromatogram of tissue components possibly related to NAD biosynthesis. Approximately 40 μl of each compound in a concentration of 1 mg/ml was applied and the plates were developed for 2–3 hr in a solvent system of isobutyric acid–NH_4OH–water 96:1:22. Compounds were detected as outlined in Methods. TCA, trichloroacetic acid.

after treatment with cyanogen bromide and benzidine. It was therefore possible to separate chromatographically NAD and NADP from the ^{14}C- or ^{32}P-labeled precursors used in the incubation studies.

No attempt was made to measure the incorporation of labeled precursors into either NADH or NADPH. Strong acid is known to cause the decomposition of reduced pyridine nucleotides, possibly owing to an acid-catalyzed opening of the pyridine ring at the 6-position as discussed by Kaplan (23). In the thin-layer chromatographic system employed in the present study, the compound formed by treatment with strong acid (5% trichloroacetic acid) separates from NAD (Fig. 2). A spot corresponding to acid-treated NADH–NADPH was found in extracts of tissues studied. However, no detectable fluorescence which might be attributed to NADH or NADPH was noted in any of the fractions from the DEAE cellulose column after trichloroacetic acid extraction of adipose tissue (Fig. 3).

Fatty Acid Synthesis

Pieces of rat epididymal fat pad, weighing about 250 mg, were incubated for 3 hr in 5.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, under the same conditions as outlined above. Glucose, glucose-U-^{14}C, nicotinic acid, and insulin were added at concentrations given in Table 2. After incubation the tissue was rinsed in 0.9% NaCl and transferred to 10 ml of chloroform–methanol 2:1 and the lipid was extracted overnight, with shaking, at room temperature. The lipid extract was washed 3 times by the method of Folch, Lees, and Sloane Stanley (24) and the fatty acid was isolated as previously described (25). The radioactivity was determined by dissolving the isolated fatty acid in 10 ml of toluene con-

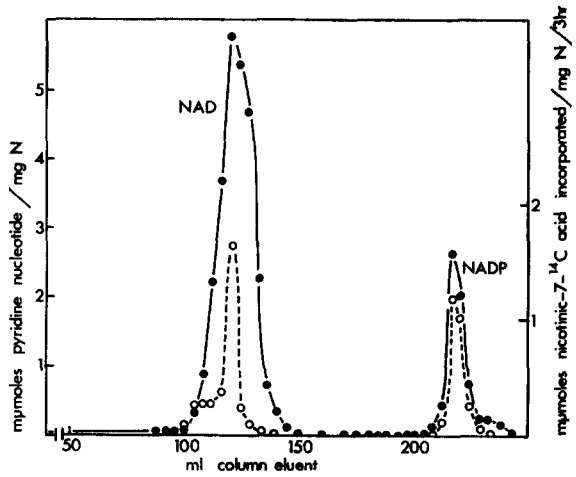

FIG. 3. Elution from a DEAE cellulose column (15 cm × 1 cm) of NAD and NADP from rat adipose tissue following incubation in a medium containing nicotinic-7-^{14}C acid. Six epididymal fat pads were incubated in 60 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 2 g of bovine serum albumin (Fraction V), 100 μc of nicotinic-7-^{14}C acid, and 10 μmoles of nicotinic acid. The concentration of pyridine nucleotides was determined fluorometrically and the radioactivity of NAD separated from other labeled contaminants by cellulose thin-layer chromatography was measured.

taining, per liter, 4 g of 2,5-diphenyloxazole (PPO), 0.15 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene, (POPOP) and 230 ml of absolute ethanol. The counting efficiency, as determined by internal standardization, was 40–50% and all measurements were corrected accordingly.

RESULTS

The chromatographic isolation of NAD and NADP from adipose tissue after incubation in buffer containing nicotinic-7-^{14}C acid is shown in Fig. 3. The elution pattern of NAD and NADP from adipose tissue extracts on a DEAE cellulose column as measured fluorometrically (Fig. 3) closely corresponds to the elution pattern of standard NAD and NADP as measured spectrophotometrically (Fig. 1). The NAD was further separated from possible ^{14}C-containing intermediates by cellulose thin-layer chromatography and isolated, and its radioactivity measured. The amount of NAD-^{14}C varied directly with the concentration of NAD in each fraction (Fig. 3). A similar pattern was noted for the NADP containing fractions. Under these conditions we were able to separate pyridine nucleotides from precursors that contain ^{14}C and ^{32}P.

Time Course of Nicotinic-7-^{14}C Acid Incorporation into NAD and NADP

The effect of the length of the incubation period on the NAD and NADP concentration and the incorporation of nicotinic-7-^{14}C acid into NAD and NADP by rat epididymal fat pads is shown in Table 1. The incorporation of nicotinic-7-^{14}C acid into NAD increased during the 1st hr of incubation and remained constant during the next 3 hr, whereas the radioactivity present in NADP increased over the 4 hr incubation period. The concentration of NAD in adipose tissue increased during the 1st hr of incubation and remained constant (except for a lower value of 3 hr) over the next 3 hr. NADP concentrations increased with increasing length of incubation. The specific activity of the isolated NAD increased over the 1st hr of incubation and did not change for the next 3 hr, whereas the NADP specific activity increased up to the 4th hr of incubation (Fig. 4).

TABLE 1 EFFECT OF INCUBATION TIME ON NAD AND NADP SYNTHESIS FROM NICOTINIC-7-^{14}C ACID BY RAT ADIPOSE TISSUE IN VITRO

	Coenzyme Measured	Incubation Time (Hr) 0.5	1	2	3	4
		mμmoles				
Nicotinic-7-^{14}C incorporated/mg N	NAD	1.57	3.53	3.74	3.85	4.36
	NADP	0.755	1.29	5.54	3.70	7.51
Coenzyme/mg N	NAD	19.80	32.82	31.50	23.77	30.77
	NADP	6.47	7.92	9.01	7.24	9.66

The experimental conditions were the same as those outlined in Table 3. Substrate concentrations, per 60 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, were 2 g of bovine serum albumin, 100 μc of nicotinic-7-^{14}C acid, and 10 μmoles of nicotinic acid.

Effect of Nicotinic Acid on Lipogenesis from Glucose

Epididymal fat pads incorporated more glucose-U-^{14}C in vitro into fatty acids in the presence than in the ab-

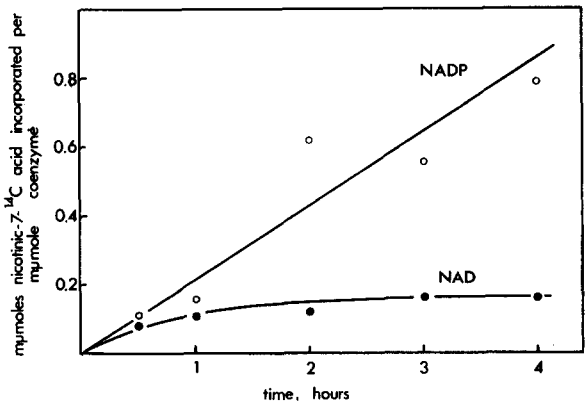

FIG. 4. The specific activity of NAD and NADP from rat adipose tissue incubated for various periods of time in 50 ml of buffer containing nicotinic-7-^{14}C acid. The incubation medium contained 100 μc of nicotinic-7-^{14}C acid and 10 μmoles of nicotinic acid. The specific activity is expressed as mμmoles of nicotinic-7-^{14}C acid incorporated into pyridine nucleotide coenzymes per mμmole of coenzyme.

sence of nicotinic acid (Table 2). Insulin had the customary stimulatory effect on lipogenesis from glucose-U-^{14}C in adipose tissue and nicotinic acid further increased this effect.

TABLE 2 EFFECT OF NICOTINIC ACID ON FATTY ACID SYNTHESIS FROM GLUCOSE-U-^{14}C BY RAT ADIPOSE TISSUE IN VITRO

Glucose	Nicotinic Acid + Glucose	Insulin + Glucose	Nicotinic Acid + Insulin + Glucose
	μmoles glucose incorporated/g tissue/3 hr		
0.327* ±0.011 (5)	0.579 ±0.018 (5)	2.24 ±0.350 (5)	3.92 ±0.750 (5)

Approximately 200–300 mg of epididymal fat pad was incubated in 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 15 μmoles of glucose, 2.5 μc of glucose-U-^{14}C, 10 μmoles of nicotinic acid, and 0.1 unit of insulin as indicated.

* Mean ± SEM for the number of animals in parentheses.

Incorporation of NAD Precursors

NAD synthesis by adipose tissue from normal fed and 48-hr fasted–48-hr refed rats, using as precursors nicotinic-7-^{14}C acid, nicotinamide-7-^{14}C, and $NaH_2{}^{32}PO_4$, is shown in Table 3. Both nicotinic-7-^{14}C acid and $^{32}P_i$ were simultaneously incorporated into NAD by adipose tissue in vitro. The specific activity of the isolated NAD was greater with nicotinamide than with nicotinic acid although the $^{32}P/^{14}C$ ratio remained almost the same. Adipose tissue taken from fasted–refed animals and incubated in a medium containing glucose plus insulin showed a fourfold increase in specific activity in NAD with nicotinic-7-^{14}C acid as a precursor and a twofold increase with $^{32}P_i$ as a precursor. The $^{32}P/^{14}C$ ratio for the simultaneous incorporation of $NaH_2{}^{32}PO_4$ and nicotinic-7-^{14}C acid was 1.1.

TABLE 3 SIMULTANEOUS INCORPORATION OF NICOTINIC-7-^{14}C ACID PLUS $NaH_2{}^{32}PO_4$ AND NICOTINAMIDE-7-^{14}C PLUS $NaH_2{}^{32}PO_4$ INTO NAD BY RAT ADIPOSE TISSUE IN VITRO

Nutritional State	Specific Activity* Nicotinic-7-^{14}C Acid	Nicotinamide-7-^{14}C	$NaH_2{}^{32}PO_4$	$^{32}P/^{14}C$
Normal fed	0.120	—	0.232	1.9
Normal fed	—	0.164	0.305	1.8
48 Hr fasted–48 hr refed	0.529	—	0.592	1.1

Six epididymal fat pads were incubated in 60 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 2 g of bovine serum albumin and 100 μc each of nicotinic-7-^{14}C acid, nicotinamide-7-^{14}C, and $NaH_2{}^{32}PO_4$. The concentration of each substrate was 10 μmoles of nicotinic acid, 10 μmoles of nicotinamide, and 77 μmoles of NaH_2PO_4. Glucose, 0.36 mmole, and 0.1 unit of insulin were added to the medium for the incubation of adipose tissue from fasted–refed rats.

* Specifically labeled substrate, in mμmoles, incorporated into NAD per 3 hr per mμmole of NAD.

Effect of Added Substrates and Insulin on NAD Synthesis

A number of substrates involved in lipogenesis or in the synthesis of NAD in other tissues were tested for their ability to stimulate NAD synthesis in adipose tissue in vitro (Table 4). Glucose, fructose, and L-glutamine had no effect on the incorporation of nicotinic-7-^{14}C acid into NAD of adipose tissue from normal fed rats, although the specific activity of NAD was higher in the presence of fructose because of a lowered concentration of NAD. Glucose plus insulin markedly stimulated nicotinic-7-^{14}C acid incorporation into NAD but also increased the total amount of NAD in the tissue with the result that the specific activity was similar to that for NAD from tissue incubated with nicotinic acid alone.

Fasting and refeeding increased the incorporation of nicotinic-7-^{14}C acid into NAD, whereas the addition of glucose and insulin to the incubation medium caused a sixfold increase in incorporation of labeled nicotinic acid.

TABLE 4 EFFECT OF VARIOUS SUBSTRATES ON NICOTINIC-7-^{14}C ACID INCORPORATION INTO NAD BY RAT ADIPOSE TISSUE IN VITRO

	Additions to the Incubation Medium						
	None	L-Glutamine	Fructose	Glucose	Insulin + Glucose	None	Insulin + Glucose
	Normal Fed					48 Hr Fasted–48 Hr Refed	
	mμmoles						
Nicotinic-7-^{14}C acid incorporated/mg N/3 hr	3.32	3.09	3.08	3.41	5.31	6.63	20.94
NAD/mg N	25.48	25.74	14.40	25.42	35.00	16.49	58.57
Specific activity*	0.120	0.112	0.214	0.134	0.151	0.402	0.355

Experimental conditions were the same as those given in Table 3. Substrate concentration per 60 ml of Krebs Ringer bicarbonate buffer, pH 7.4, were 100 μc of nicotinic-7-^{14}C acid, 0.1 unit of insulin, 0.36 mmole of glucose, 0.36 mmole of fructose, 4.9 mmoles of L-glutamine, and 10 μmoles of nicotinic acid.

* Nicotinic-7-^{14}C acid, in mμmoles, incorporated per mμmole of NAD per 3 hr.

TABLE 5 EFFECT OF FASTING 48 HR AND REFEEDING 48 HR ON NAD SYNTHESIS FROM GLUCOSE-1-^{14}C, GLUCOSE-6-^{14}C, AND RIBOSE-1-^{14}C BY RAT ADIPOSE TISSUE IN VITRO

	Normal Fed				48 Hr Fasted–48 Hr Refed		
	Gl-1-^{14}C	Gl-6-^{14}C	Ribose-1-^{14}C	Ribose-1-^{14}C (10 mM)	Gl-1-^{14}C	Gl-6-^{14}C	Ribose-1-^{14}C
				mμmoles			
Specifically labeled substrate incorporated/mg N/3 hr	9.90	20.90	14.60	31.34	25.95	44.60	12.09
NAD/mg N	19.94	24.38	31.56	27.11	26.91	43.56	39.82
Specific activity	0.496	0.860	0.464	1.11	0.960	1.01	0.303

The experimental conditions were the same as those in Table 3. The final substrate concentrations in Krebs-Ringer bicarbonate buffer, pH 7.4, were 100 μc each of glucose-1-^{14}C, glucose-6-^{14}C, and ribose-1-^{14}C per 60 ml; and 2 mM glucose, 2 mM ribose (except 10 mM where indicated), 0.1 unit insulin per 60 ml, and 0.2 mM nicotinic acid.

Incorporation of Glucose and Ribose into NAD

Glucose-1-^{14}C, glucose-6-^{14}C, and ribose-1-^{14}C were incorporated into NAD by rat adipose tissue in vitro (Table 5). There was more than twice as much glucose-6-^{14}C incorporated into NAD as glucose-1-^{14}C and nearly a twofold difference in the specific activity of the NAD. The specific activity of NAD after incubation with 2 mM ribose-1-^{14}C was almost the same as that of NAD after incubation with a similar concentration of glucose-1-^{14}C. Fasting and refeeding caused an increase in the incorporation of both glucose-1-^{14}C and glucose-6-^{14}C into adipose tissue NAD. This increase in NAD-^{14}C was accompanied by a 100% increase in NAD concentration with glucose-6-^{14}C as substrate, but a far smaller increase was noted with glucose-1-^{14}C. It is not clear why glucose-1-^{14}C and -6-^{14}C should have different effects on NAD concentration, although we have noted this result in two separate experiments. The specific activity of NAD isolated from adipose tissue of fasted–refed animals after incubation increased twofold with glucose-1-^{14}C and only 20% with glucose-6-^{14}C. Ribose-1-^{14}C incorporation into NAD by adipose tissue was not greatly affected by fasting and refeeding and the specific activity of the isolated NAD was similar for normal and fasted–refed animals. Increasing the ribose concentration from 2 mM to 10 mM caused a sharply increased ribose-1-^{14}C incorporation into NAD. The specific activity of the NAD after incubation of the adipose tissue in 10 mM ribose was 1.11 as compared with a specific activity of 0.464 with 2 mM ribose.

DISCUSSION

Synthesis of NAD

It is difficult to assess the rate of NAD synthesis by measuring the incorporation of nicotinamide-7-^{14}C into NAD, because of the possibility of labeling of the NAD molecule due to the activity of NAD glycohydrolase (EC 3.2.2.5). Zatman, Kaplan, and Colowick (26) have demonstrated that this enzyme catalyzes the direct exchange of nicotinamide with the nicotinamide moiety of NAD. The incorporation of inorganic ^{32}P into NAD would, however, be a true measurement of NAD synthesis. We have measured the incorporation of $^{32}P_i$ and nicotinamide-7-^{14}C, and of $^{32}P_i$ and nicotinic-7-^{14}C acid, into NAD of rat adipose tissue in vitro. The ^{32}P/^{14}C ratio found in the NAD was 1.82 and 1.94 respectively and showed a portion of the NAD to be newly synthesized. The significance of the ratio ^{32}P/^{14}C of approximately 2 can be better understood by considering the pathway of NAD synthesis. One of the phosphate groups is incorporated into the pyridine nucleotide through 5-phosphoribosyl pyrophosphate which is in turn derived from ribose-5-phosphate. The other phosphate is from the α-phosphate of ATP. A ^{32}P/^{14}C ratio≃2 thus suggests: (*a*) a negligible exchange of nicotinamide and nicotinic acid, at a concentration of 0.2 mM, directly into the NAD molecule by NAD glycohydrolase activity, (*b*) that both ribose-5-phosphate and the α-phosphate of ATP contained ^{32}P, and (*c*) that portions of the ribose-5-phosphate and AMP were synthesized during the 3 hr incubation period.

The incorporation of ribose-1-^{14}C, glucose-1-^{14}C, and glucose-6-^{14}C into NAD by adipose tissue further supports this suggestion. The specific activity of the isolated NAD after incubation of adipose tissue for 3 hr with 10 mM ribose-1-^{14}C was 1.11, whereas the specific activity of NAD resulting from incubation with glucose-1-^{14}C and glucose-6-^{14}C under conditions of rapid glucose uptake (fasting and refeeding) was 0.960 and 1.01 respectively. This indicates that at least half of the ribose of NAD was newly synthesized during the 3-hr incubation. Shuster and Goldin (27) have demonstrated a nicotinamide-induced increase in the net synthesis or mobilization of AMP for the formation of NAD in mouse liver. The incorporation of nicotinic-7-^{14}C acid and inorganic ^{32}P into the nicotinamide mononucleotide portion of NAD was studied in Ehrlich-Lettre ascites cells by Dietrich and Fuller (28). They found a correlation between nicotinic acid-7-^{14}C and inorganic ^{32}P uptake into NAD which indicated a rapid synthesis de novo.

The results of the present study show that NAD is synthesized de novo by rat adipose tissue in vitro, from precursor nicotinic acid and nicotinamide. These findings offer a basis for interpreting the reports of an effect of nicotinic acid and nicotinamide on lipid metabolism in adipose tissue. Hanson and Ziporin (29) previously noted that nicotinamide stimulated the oxidation in vitro of β-hydroxybutyric acid to CO_2 by mouse adipose tissue, presumably by increasing the concentration of NAD. Lee, Ellis, and Segal (30) observed an increased lipogenesis from glucose and acetate by rat adipose tissue in vitro in the presence of nicotinic acid. In the present paper we have noted a similar nicotinic acid-stimulated increase in lipogenesis from glucose-U-^{14}C by rat adipose tissue. It is likely that nicotinic acid increases lipogenesis by increasing the concentration of pyridine coenzymes within the fat cell. Several other effects of nicotinic acid on lipid metabolism in adipose tissue, which may not be related to coenzyme synthesis, have been reported by Carlson (31), who showed that nicotinic acid, but not nicotinamide, lowered the free fatty acid mobilization induced by norepinephrine in vivo and ACTH and glucagon in vitro.

Relation of NAD Synthesis to Lipogenesis

It was our aim in these studies not only to establish a de novo synthesis of NAD by adipose tissue, but also to link this synthesis with lipogenesis. We therefore sought to determine whether metabolic conditions that favor lipogenesis also favor NAD synthesis. Glucose plus insulin (32) and fasting–refeeding (33), both of which stimulate fatty acid synthesis in adipose tissue, also enhance NAD synthesis from nicotinic-7-^{14}C acid and nicotinamide-7-^{14}C.

A sharp increase in the activity of the pentose pathway in adipose tissue and liver is associated with increased lipogenesis (4). This pathway generates NADPH for reductive synthesis of fatty acids as well as being a major source of the ribose-5-phosphate used for NAD synthesis. The supply of precursors such as the ribose-5-phosphate produced via the pentose pathway may limit the synthesis of NAD in adipose tissue. Therefore, the incorporation of glucose-1-^{14}C and glucose-6-^{14}C into NAD was studied. More than twice as much glucose-6-^{14}C as glucose-1-^{14}C was found in the isolated NAD of adipose tissue incubated with insulin. The fact that any ^{14}C from glucose-1-^{14}C was incorporated into NAD suggests a synthesis of ribose-5-phosphate by a pathway which bypasses the initial oxidative reactions of the pentose pathway. Carruthers and Winegrad (34), measuring the incorporation of glucose-1-^{14}C and glucose-6-^{14}C into the pentose moiety of RNA by rat adipose tissue in vitro, observed a similar incorporation of glucose-1-^{14}C into RNA. In the presence of insulin they found 0.97 μmole of glucose-1-^{14}C carbon against 1.46 μmoles of glucose-6-^{14}C per 100 μmoles of RNA ribose. This ratio C-1/C-6 = 0.660 is in close agreement with the ratio of 0.577 that we observed for incorporation into NAD. A C-1/C-6 ratio of 0.856 was reported by Shuster and Goldin (27) for the incorporation of glucose-1-^{14}C and glucose-6-^{14}C in vivo into mouse liver NAD. The close agreement in the pathway of glucose conversion to ribose-5-phosphate in the synthesis of both NAD and RNA in adipose tissue suggests a nonoxidative route of considerable magnitude.

The increased activity of the pentose pathway in adipose tissue of fasted–refed rats is due, in part, to an increased activity of the enzymes glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) (33). If fasting and refeeding stimulates NAD synthesis from glucose-^{14}C chiefly by enhancing the conversion of glucose-6-phosphate to ribose-5-phosphate, no stimulation by fasting and refeeding on NAD synthesis from ribose-1-^{14}C should be noted. Our data, indeed, demonstrate that the incorporation of ribose-1-^{14}C into NAD by rat adipose tissue is not affected by fasting and refeeding. We were able, however, to stimulate the synthesis of NAD-^{14}C by increasing the concentration of ribose from 2 mM to 10 mM.

Greenbaum et al. (9) have measured the activity of nicotinamide mononucleotide adenylyltransferase, (EC 2.7.7.1), NAD kinase (EC 2.7.1.23), and NAD glycohydrolase (EC 3.2.2.5), along with the hepatic concentration of NAD and NADP in response to adrenalectomy, diabetes, thyroxine, growth hormone, and glucagon. They found a lack of correlation between the activity of these key enzymes of NAD synthesis and coenzyme concentration in each of the hormonal conditions and suggested that other factors play a role in regulating the rate of NAD synthesis, such as the availability of ATP, nicotinamide, or ribose. Bosch and Harper (35) have reported a drop in the rate of nicotinamide-induced NAD synthesis in hyperthyroid rats due, presumably, to a depletion of a precursor required for NAD synthesis. In the present study, conditions such as fasting and refeeding or glucose plus insulin, which would increase the availability of substrates for NAD synthesis, stimulated coenzyme synthesis from nicotinic acid. These findings are consistent with the proposal of Greenbaum et al. (9) that the availability of precursor products determines, in part, the rate of NAD synthesis.

Dietrich and Fuller (28) noted a sharp increase in NAD synthesis from nicotinic acid in Ehrlich-Lettre ascites cells in the presence of L-glutamine. No such effect on coenzyme synthesis was observed in the present study. Adipose tissue is permeable to glutamine as

demonstrated by the conversion of D,L-glutamine-^{14}C to fatty acids (36). It is possible that sufficient intracellular L-glutamine is already present or that the pathway of NAD synthesis from nicotinic acid in adipose tissue may not require L-glutamine as an intermediate.

NADP Synthesis

Although there was an enhanced incorporation of nicotinic-7-^{14}C acid into NADP with length of incubation, there was less marked a change in the concentration of NADP after the first 30 min. Such a finding suggests a synthesis and breakdown of NADP. Stoller and Kaplan (37) have reported a more rapid turnover of the monoester phosphate of NADP than of the phosphate groups of NAD in rat liver because of the high activity of cleavage enzymes. NADP is synthesized from NAD and ATP by the enzyme NAD kinase. In liver this enzyme has an activity exceeding the observed rate of NADP synthesis and Greenbaum et al. (9) have demonstrated a lack of correlation between its activity and the NADP concentration. If there is a similar lack of correlation between enzyme activity and the rate of NADP synthesis in adipose tissue, the formation of NADP would depend on the concentration of precursor NAD and ATP.

We have not, in the present study, attempted to measure the incorporation of labeled precursors into NADH or NADPH because of the difficulty of their isolation. Until such experiments are carried out an exact balance sheet of total pyridine nucleotide coenzymes synthesized by adipose tissue cannot be calculated. Of the four nicotinamide coenzymes, NAD is present in the greatest concentration in adipose tissue, (29, 38) so that changes in the labeling pattern of NAD would probably reflect general changes in the total coenzyme pool. These preliminary studies indicate a possible link between the synthesis of pyridine coenzymes and lipogenesis and reveal the coenzyme pool to be dynamic in nature and dependent on the availability of cofactors and substrates.

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