The Effect of Nicotinamide and Homologs on the Activity of Inducible Enzymes and NAD Content of the Rat Liver

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(Received February 13, 1967, and in revised form May 16, 1967)

SUMMARY

The activity of tryptophan pyrrolase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12) and tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) of the rat liver increases after intraperitoneal injection of nonphysiological amounts of nicotinamide. Actinomycin D (2 mg/kg), injected 30 minutes prior to nicotinamide, inhibited this effect of nicotinamide.

Certain pyridine derivatives related to nicotinamide, such as nicotinic acid, 5-fluoronicotinamide, isonicotinic acid hydrazide, and nikethamide (*N,N*-diethylnicotinamide) had similar effects by increasing tryptophan pyrrolase activity.

Hypophysectomy abolished the induction of enzymes caused by nicotinamide, 5 mmoles/kg (within 6 hr after injection), while hydrocortisone (5.2×10^{-2} mmoles/kg) in hypophysectomized rats increased enzyme activity 10-fold during the same period.

Feeding inhibited by 50% the increase of tryptophan pyrrolase by nicotinamide as compared to the rate of induction in fasted animals. On the other hand, induction of tryptophan pyrrolase by hydrocortisone, 5.2×10^{-2} mmoles/kg, did not depend on the nutritional state of the animals.

Augmentation of NAD in rat liver following injection of nicotinamide precedes enzyme induction. Starvation increases NAD accumulation from nicotinamide. Actinomycin D has no effect on NAD augmentation from precursors. Newly formed NAD following injection of nicotinamide is almost exclusively localized in the cytoplasmic cell fraction of rat liver. The rate and degree of NAD accumulation following injection of various metabolic precursors of NAD exhibit marked tissue dependent variation.

A working hypothesis is proposed, predicting that nicotinamide causes enzyme induction in liver by augmenting NAD levels of adrenals. Increased pyridine nucleotide content of adrenals may then cause an increased synthesis and release of cortical hormones; thus the latter would be the true inducers of liver enzymes. Toxic nicotinamide homologs that do not augment NAD levels, but act as enzyme inducers, may release cortical hormones directly, without contributing to their continued biosynthesis.

INTRODUCTION

Factors influencing the biosynthesis and degradation of pyridine nucleotide coenzymes are likely to have significantly modifying effects on metabolism of various animal tissues. Since the rate-limiting role of specific pyridine nucleotide linked dehydrogenases in metabolic flux appears to vary from tissue to tissue within the same animal (1, 2), alteration of pyridine nucleotide levels in various tissues may be expected to elicit tissue specific metabolic responses. There are indications that relatively selective inhibition of metabolism of cancer cells by certain carcinostatic agents (cf. 3) may be mediated by an effect on cellular NAD levels. The question arises

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whether or not this model of metabolic regulation can provide molecular mechanisms for selective metabolic effects of a variety of drugs and hormones. As a preliminary to more direct testing of this hypothesis, we have first designed experiments to identify possible relationships between substrate level and hormonal control of NAD metabolism.

According to present information, NAD levels of animal tissues are controlled in part by availability of various substrates for enzymes involved in NAD biosynthesis, such as nicotinic acid (4), quinolinic acid (5) (the latter derived metabolically from tryptophan), or nicotinamide (5, 6). Nicotinamide can enter as a precursor directly (5, 6), presumably competing for PRPP with quinolinic and nicotinic acids, or may be first deamidated to nicotinic acid (7). Some uncertainty prevails concerning the metabolic significance of deamidation of nicotinamide, a reaction which takes place in the liver (7), since direct synthesis of NAD from nicotinamide has also been shown to occur in this tissue (5) or in Ehrlich ascites tumor cells (6). A possible participation of NAD glycohydrolase (EC 3.2.2.5) (cf. 8) in the biosynthesis of NAD further complicates this picture.

Various hormonal effects influencing NAD content of rat liver have been described by Greengard *et al.* (9–11). Hypophysectomy or adrenalectomy increases the NAD augmentation in rat liver which follows the injection of nicotinamide, while glucocorticoids and ACTH have an opposite effect. More recently, Greengard *et al.* have reported that hypophysectomy increases the biological half-life of injected nicotinamide (12) and also increases nicotinamide deamidase activity of rat liver (13).

Both availability of enzyme substrates and variation in enzyme content can influence metabolic rates. Postulation of causal relationship between metabolic regulation by changes in substrate levels and direct alterations of rates of biosynthesis and degradation of specific enzymes of a metabolic pathway are feasible if the temporal organization of the observed metabolic reaction and the rates of enzyme biosynthesis or degradation do not show obvious discrepancies. As discovered by Berlin and Schimke (14), the rate of enzyme induction by glucocorticoids is determined by the biological half-life of each particular enzyme protein. This observation predicts that tissue-specific metabolic effects can be mediated by factors influencing enzyme induction provided the biological half-life of enzymic components of the same pathway is different in various tissues. It is interesting that tryptophan, conspicuously a potential metabolic precursor of pyridine-containing substances (15) commonly used in experimental studies dealing with NAD biosynthesis, is also well known to augment tryptophan pyrrolase and tyrosine-α-oxoglutarate transaminase of rat liver (16–18). The mechanism of this effect of tryptophan on tryptophan pyrrolase, as clearly shown by Schimke *et al.* (19), is a stabilization of the enzyme protein, not a direct stimulation of its biosynthesis. It is yet uncertain whether or not the same mechanism is responsible for the augmenting effect of tryptophan on tyrosine-α-oxoglutarate transaminase (17, 18). One of the missing links connecting hormonal and substrate level control of NAD metabolism is the effect of NAD precursors metabolically derived from tryptophan on levels of those enzymes which are increased by tryptophan as well as cortical hormones. The present work deals with this question. Results indicate sequential temporal relationship between rates of augmentation of certain liver enzymes and the rate of increase of NAD content of rat liver following nicotinamide injection. Possible mechanisms of this phenomenon as well as certain experimental factors involved in the regulation of NAD and enzyme biosyntheses were also analyzed.

METHODS AND MATERIALS

A. Methods Employed in Studies of Liver Enzymes

General procedure. Sprague-Dawley male rats weighing 200 ± 20 g (purchased from

the Berkeley Pacific Laboratories) were used. When fasted, food but not water was withheld for 15 hr prior to experiments. Fed rats were maintained on a Wayne-Lab Blox diet ad libitum. All compounds tested were injected intraperitoneally in neutralized 0.9% NaCl solution. Hypophysectomized male rats weighing 180 ± 10 g were a gift from the Laboratory for Hormone Research, University of California, Berkeley. Experiments with hypophysectomized rats were performed 1 week after the operation.

Livers were rapidly excised (within 1–2 min after cervical dislocation), washed in an ice-cold 0.14 M KCl solution (adjusted to pH 8.5 with dilute KOH), and homogenized in a Teflon-glass homogenizer. (The ratio of liver to homogenizing medium was 1:7, w/v.) The homogenate was centrifuged for 20 min at 12,800 *g* in the 9RA rotor of a Lourdes refrigerated centrifuge at 0°. Tryptophan pyrrolase and tyrosine transaminase activities were determined in the centrifugal supernatant solution.

Enzyme assays. Tryptophan pyrrolase activity was measured by the procedure of Knox (20) by spectrophotometric analysis of kynurenine formation, read at 365 mμ in a Zeiss PQ II spectrophotometer (5-cm light path). The reaction mixture contained 1.0 ml of 0.3 M phosphate buffer (pH 7.0), 0.3 ml of 0.03 M L-tryptophan, 1.0 or 2.0 ml of the liver supernatant, and deionized water to make up a final volume of 4.0 ml. Blanks contained all components except L-tryptophan. The tryptophan pyrrolase activities reported in Tables 2–5 were assayed in the presence of added hematin in a final concentration of 10 μM. Incubations were carried out in a Dubnoff shaker at 37° for 1 hr in an atmosphere of oxygen. The reaction was stopped by 2.0 ml of 5% trichloroacetic acid added to the vessels, which were chilled in ice. Aliquots of the trichloroacetate extract (after centrifugation) were assayed for kynurenine spectrophotometrically. Enzyme activities were defined as micromoles of kynurenine accumulated per gram of liver (wet weight) per hour.

Tyrosine transaminase activity (assayed at 25° in 1-cm quartz cuvettes over a period of 20 min) was determined by continuous spectrophotometric recording of the rate of formation of the enol-borate complex of *p*-hydroxyphenyl pyruvate (21), read at 310 mμ. Each reaction mixture contained 0.57 M borate (pH 8.0), 30 μg pyridoxal phosphate, 0.1 ml of the liver supernatant, 80 μmoles α-oxoglutarate, and deionized H_2O to a total volume of 3.5 ml. The solution of α-oxoglutarate was added last to initiate the reaction. Reaction blanks contained all components except L-tyrosine. Since tyrosine transaminase is a better characterized enzyme than tryptophan pyrrolase, enzyme activities were expressed in micromoles of *p*-hydroxyphenylpyruvate formed per milligram protein per hour, while pyrrolase activity was expressed on a tissue weight basis. Enzyme activities between two series, one calculated on a tissue weight, the other on a milligrams of protein basis, are not strictly comparable unless the protein:tissue weight ratio remains constant, which was the case. Relative changes of enzyme activities were the same regardless of the basis on which they were expressed. Protein was determined by the modified biuret procedure of Beisenherz *et al.* (22).

Materials. Hydrocortisone sodium succinate (Solu-Cortef) was purchased from the Upjohn Company; *N,N*-diethylnicotinamide (nikethamide) from CIBA Pharmaceutical Products, Inc., and isonicotinic acid hydrazide from Calbiochem. 5-Fluoronicotinamide and actinomycin D were gifts from Eli Lilly and Company and Merck and Company, respectively.

B. Method Used for the Determination of Rates of NAD Accumulation

Male Sprague-Dawley rats (200 ± 20 g) were fasted for 15 hr prior to experiments. NAD precursors were injected in neutralized 0.9% NaCl solution intraperitoneally. Tissues were removed (within 1 min after cervical dislocation), frozen in liquid N_2, weighed and homogenized in 0.6 *N* $HClO_4$ (at 0°). NAD was determined spectrophotometrically in neutralized extracts (after removal of ClO_4^- as K^+ salt) by the

alcohol dehydrogenase test (23) using cuvettes of 5-cm light path in a Zeiss PMQ spectrophotometer. Results were expressed as %△NAD, which is the increment measured above control values (i.e., NAD content of livers of rats receiving only 0.9% NaCl) and calculated on a tissue protein basis (determined by the biuret method). This method of recording the effect of NAD precursors is justified since NAD content of livers and other tissues of control animals was found to be quite constant (e.g., 2.72 ± 10% μmoles NAD per gram of liver protein). Each experimental point was obtained by analysis of tissues pooled from 4–6 animals.

Subcellular distribution of NAD in liver homogenates was determined by a simplified procedure of differential centrifugation of homogenates prepared in 0.25 M sucrose containing 5×10^{-2} M nicotinamide (ratio of tissue to homogenizing medium was 2:8). Homogenates were separated into a pellet (containing the nuclear and mitochondrial fraction, sedimented at 12,800 *g* for 10 min at 4°) and a supernatant fraction containing soluble cell fraction and microsomes. Separation of microsomes by prolonged ultracentrifugation resulted in variable loss of NAD due to NAD-glycohydrolase activity of microsomes, a loss that could not be completely prevented by nicotinamide. For this reason, the abbreviated cell fractionation was adopted. Omission of nicotinamide in the homogenizing medium resulted in 75% loss of NAD during centrifugation of homogenates for 10 min.

RESULTS

As summarized in Table 1, tryptophan pyrrolase activity of livers of fasted rats increased approximately 10-fold in 6 hr after intraperitoneal injection of a dose of 5 mmoles of nicotinamide per kilogram of body weight. This increase of tryptophan pyrrolase activity could also be demonstrated by the intraperitoneal injection of several structurally related pyridine derivatives, indicating that this effect is not specific for nicotinamide (Table 2). Although optimal doses were not determined, it appears that 5-fluoronicotinamide and isonicotinic acid hydrazide were more effective than nicotinamide itself. Niketh-amide was lethal in these studies at the dose of 2.5 mmoles per kilogram of body weight, and thus had to be used in smaller amounts.

TABLE 1

Time course of the effect of nicotinamide on rat liver tryptophan pyrrolase activity

The results were determined in three separate experiments with 10 fasted rats per experiment (i.e., a total of 30 rats). In each experiment, measurements were made at 0, 2, 4, 6, and 8 hr with 2 rats at each time interval. Nicotinamide was injected intraperitoneally at a dose of 5 mmoles/per kilogram body weight. Control groups (at 0 time) received only 0.9% NaCl solution (no nicotinamide). The animals were sacrificed at 2-hr intervals after the injection. Each value represents the average of duplicate determinations on individual livers. The data from the three experiments were combined as the mean ± the standard error.

Time (hr)	Experiments			
	No. 1	No. 2	No. 3	Mean ± SE
0	0.5	1.1	0.6	
	0.5	0.7	2.5	1.0 ± 0.3
2	1.6	2.6	2.6	
	1.9	2.3	3.5	2.4 ± 0.8
4	3.0	5.1	7.7	
	2.8	3.6	9.1	5.2 ± 1.1
6	7.8	14.0	8.9	
	8.5	18.3	9.2	11.1 ± 1.7
8	4.2	12.6	4.8	
	4.9	11.9	10.1	8.1 ± 1.6

Additional characteristics of the increase of activity of rat liver adaptive enzymes by nicotinamide are presented in Table 3. Both pyrrolase and transaminase activities increased after injection of nicotinamide. Intraperitoneal injection of actinomycin D (2 mg/kg) 30 min prior to nicotinamide prevented the increase in tryptophan pyrrolase activity at 6 and 8 hr. However, if the administration of actinomycin D (2 mg/kg) was delayed 4 hr after the injection of nicotinamide, tryptophan pyrrolase activity measured at 8 hr was induced to levels comparable to those found in livers of rats treated only with nicotinamide.

TABLE 2

Augmentation of tryptophan pyrrolase activity by nicotinamide and related pyridine derivatives

Pyridine derivatives were administered by intraperitoneal injection to fasted rats which were sacrificed 6 hr subsequently. The livers in each experimental group, indicated by numbers in parentheses, were combined for enzyme analysis. Each value represents the average of duplicate determinations on the combined extract. Tryptophan pyrrolase activity was calculated as micromoles of kynurenine accumulated per gram of liver (wet weight) per hour. Results were also expressed as the increase above the control values (net increase).

Compound injected	Dose (mmoles/kg body wt.)	Tryptophan pyrrolase activity	
		Total activity	Net increase
None	—	3.4 (4)	—
Nicotinamide	1.25	9.4 (4)	6.0
	2.50	10.2 (4)	6.8
	5.00	17.0 (4)	13.6
	10.00	11.0 (4)	7.6
Nikethamide	0.25	7.4 (4)	4.0
	0.50	9.5 (4)	6.1
	1.25	12.7 (4)	9.3
5-Fluoronicotinamide	2.50	28.5 (2)	25.1
Isonicotinic acid hydrazide	0.25	8.2 (4)	4.8
	2.50	21.3 (2)	17.9
Nicotinic acid	5.00	10.9 (2)	7.5

TABLE 3

Effect of actinomycin D on augmentation of tryptophan pyrrolase and tyrosine transaminase by nicotinamide

Fasted rats were injected intraperitoneally with nicotinamide at a dose of 5 mmoles per kilogram body weight. In No. 1, enzyme assays were done prior to nicotinamide injection. In No. 2 and No. 3, actinomycin (2 mg/kg) was injected 30 min prior to nicotinamide (−30 min), and in No. 4, 4 hr after nicotinamide. TPO = tryptophan pyrrolase activity (in μmoles kynurenine/1 g liver/1 hr); TTA = tyrosine transaminase activity (in μmoles *p*-hydroxyphenylpyruvate/mg protein of the centrifugal supernatant fluid/hour). A = actinomycin D. Each value is the average of duplicate assays on individual rat livers.

No.	Experimental conditions	Time of enzyme assay (hr)	Enzyme activity			
			TPO		TTA	
			No A	+ A	No A	+ A
1	Control (i.e., assays done prior to injections)	0 (TPO, TTA)	5.3	—	0.7	—
			4.2	—	0.4	—
			3.4	—	0.6	—
					0.4	—
2	Nicotinamide, 5 mmoles/kg, at 0 time;	6 (TPO)	13.8	5.1	2.7	0.9
	actinomycin at −30 min	4 (TTA)	16.0	4.2	1.8	1.3
			27.0	3.8	2.1	0.7
3	Nicotinamide, 5 mmoles/kg, at 0 time;	8 (TPO)	17.4	4.9	3.8	1.1
	actinomycin at −30 min	6 (TTA)	27.8	3.0	4.6	1.9
			22.8	—	3.6	—
			—	—	4.5	—
			—	—	2.1	—
4	Nicotinamide, 5 mmoles/kg, at 0 time;	8 (TPO)	17.4	16.8	—	—
	actinomycin 4 hr after nicotinamide		27.8	22.1	—	—
			22.8	—	—	—

Liver tyrosine transaminase activity at 4 and 6 hr following the intraperitoneal injection of nicotinamide at a level of 5 mmoles/kg was increased approximately 4- and 7-fold, respectively, above the 0 time control values. Pretreatment with actinomycin D resulted in about 50% reduction of enzyme activity at 4 hr, and caused 60–71% diminution 6 hr after injection of nicotinamide.

Tryptophan pyrrolase activity of livers of hypophysectomized rats 6 hr following intraperitoneal injection of nicotinamide (5 mmoles/kg) or hydrocortisone (5.2×10^{-2} mmoles/kg) is shown in Table 4. Nicotinamide was not effective in hypophysectomized rats, whereas hydrocortisone caused a nearly 10-fold increase in enzyme activity during an experimental period of 6 hr.

Results presented in Table 1–4 were obtained with rats fasted overnight for approximately 15 hr prior to experiments. The effect of starvation itself on tryptophan pyrrolase activity, 6 hr following the intraperitoneal injection of either nicotinamide (5 mmoles/kg) or hydrocortisone (5.2×10^{-2} mmoles/kg), is shown in Table 5. It is evident that starvation resulted in significant elevation of enzyme activity caused by nicotinamide injection. The absolute increase in enzyme activity caused by nicotinamide (determined by subtraction of pyrrolase activity found prior to nicotinamide injection, a value close to 4.0) was 14.3 in fasted and 7.3 in fed rats, clearly indicating that nicotinamide is far more effective in fasted rats. On the other hand, an increase in enzyme activity after injection of 5.2×10^{-2} mmoles of hydrocortisone per kilogram is uninfluenced by fasting.

A possible temporal relationship between rates of increase in pyrrolase and transaminase activities of rat liver and rates of NAD synthesis from pyridine-containing precursors was sought. The time function of NAD formation after injection of nicotinamide, nicotinic acid, and quinolinic acid (at a dose of 0.1 mmole per kilogram body weight) is shown in Fig. 1. Rates of NAD augmentation as a function of nicotinamide concentration is illustrated in Fig. 2. It is important to note that in liver the rate of NAD augmentation after nicotinamide injection is very rapid, even at a dose level one-tenth of that employed in experiments dealing with the effect of nicotinamide injections on pyrrolase and transaminase. Maximal rates of NAD augmentation were obtained at dose levels which cause 5- to 10-fold increase of enzymic activities in liver tissue. Quinolinic acid elicits an increase in NAD content of liver tissue only. Apparent tissue-specific responses in terms of marked differences in rates of NAD accumulation were also observed.

TABLE 4

Effect of hypophysectomy on the increase of tryptophan pyrrolase by nicotinamide and hydrocortisone

Hypophysectomized male rats, 1 week post operative and fasted overnight, were injected intraperitoneally with either nicotinamide or hydrocortisone and then sacrificed 6 hr subsequently. Each value represents the average of duplicate determinations on individual rat livers. Tryptophan pyrrolase activity was calculated as micromoles of kynurenine accumulated per gram of liver (wet weight) per hour.

Experimental conditions	Tryptophan pyrrolase activity
0 Hour, no injections	4.0
	4.3
	1.7
	2.5
	Mean = 3.1 ± 0.6
Nicotinamide, 5 mmoles/kg	2.7
	3.2
	2.7
	2.8
	Mean = 2.8 ± 0.1
Hydrocortisone, 5.2×10^{-2} mmoles/kg	21.1
	32.3
	30.1
	32.0
	Mean = 28.9 ± 2.6

Since tryptophan pyrrolase and tyrosine-α-oxoglutarate transaminase were determined in the soluble cytoplasmic cellular fraction of liver tissue, it was of interest to identify the subcellular localization of

TABLE 5

Comparison of augmentation of tryptophan pyrrolase activity by nicotinamide and hydrocortisone in fasted and fed rats

For experimental details see "General Procedure." Tryptophan pyrrolase activities were determined 6 hours after injection of either nicotinamide or hydrocortisone.

Injected substance	Tryptophan pyrrolase activity	
	Fasted	Fed
Nicotinamide, 5 mmoles/kg	22.1	9.8
	18.3	19.7
	15.8	7.0
	19.9	8.0
	17.9	11.6
	16.0	11.4
	Mean = 18.3 ± 1.0	Mean = 11.3 ± 1.8
Hydrocortisone, 5.2×10^{-2} mmoles/kg	13.6	27.8
	23.6	20.5
	26.3	26.0
	19.9	21.3
	20.3	18.4
	17.3	12.9
	Mean = 20.2 ± 1.8	Mean = 21.2 ± 2.1

NAD formed after injection of nicotinamide. As described above (see Materials and Methods, Section B), high NAD glycohydrolase activity of microsomes interferes with the analysis for NAD in subcellular fractions prepared by prolonged differential centrifugation. However, the abbreviated fractionation of liver homogenates (see Section B above) yielded useful information. Since recovery of added NAD to liver homogenates in the supernatant fraction (containing microsomes) was about 80–90%, this method gives a reasonably accurate subcellular localization of newly formed NAD, which is predominantly in the supernatant fraction (see Table 6). No definite conclusion can be drawn from these results as to the localization of NAD biosynthesis itself, since intracellular transfer processes may significantly contribute to this picture. It is well known that extramitochondrial NAD does not enter readily into mitochondria (cf. 24); thus the understanding of the metabolic significance of NAD accumulation in extramitochondrial space poses further interesting problems.

Actinomycin D in doses employed (2 mg/kg, injected 30 min prior to nicotinamide) had no influence on rates of NAD accumulation caused by nicotinamide. On the other hand, fasting for 15 hr (prior to nicotinamide injection) doubled the rate of NAD accumulation which followed the injection of nicotinamide. All experiments reported here were performed on fasted

TABLE 6

Subcellular distribution of NAD in rat liver

Freshly removed rat livers were homogenized in 0.25 M sucrose containing 5×10^{-3} M nicotinamide at 0° (ratio of tissue to homogenizing medium was 2 to 8). Homogenates were separated by centrifugation at 12,800 *g* for 10 min into a pellet (containing nuclei and mitochondria) and supernatant fluid (containing microsomes and soluble cell fraction). Omission of nicotinamide in the homogenizing medium results in 75% loss of NAD. Results shown in this table were obtained from 6 rats livers analyzed individually and expressed as the mean ± the standard error.

Experimental conditions	NAD (μmoles/g protein)	
	Pellet	Supernatant containing microsomes
Control	1.16 ± 0.08	4.27 ± 0.2
6 hr after injection of 5 mmoles/kg nicotinamide	2.2 ± 0.4	16.4 ± 4.0

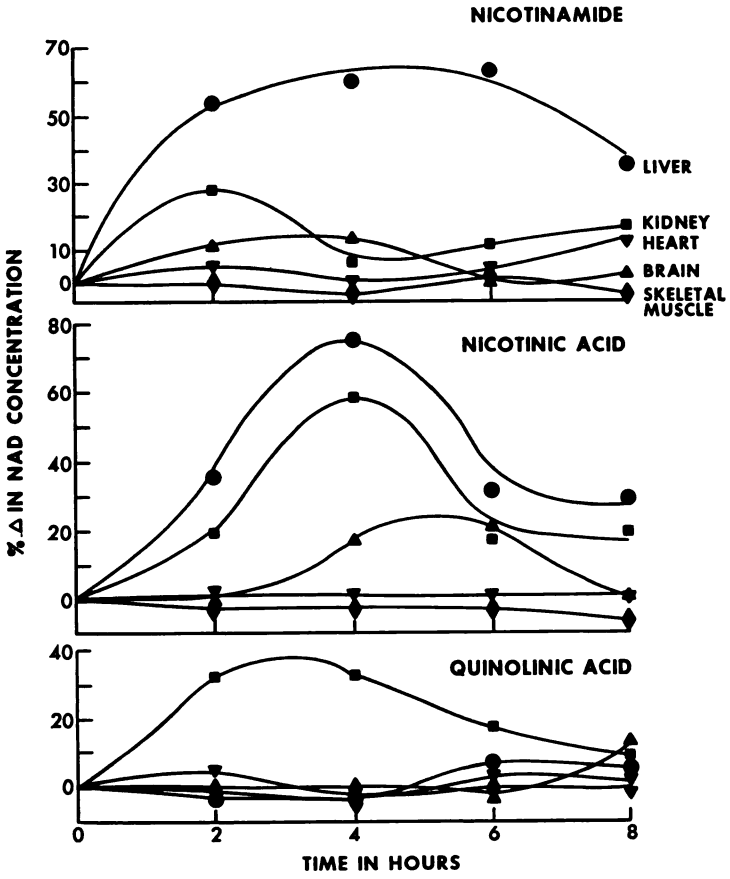

FIG. 1. *The effect of nicotinamide, nicotinic acid, and quinolinic acid on NAD content of various rat tissues*

The compounds were injected intraperitoneally to starved rate at a dose of 0.1 mmole/kg and analyzed at hourly intervals after injection. Each experimental point represents NAD content of tissues pooled from 4 to 6 rats, expressed as percent change of NAD levels, compared to values obtained by analysis of pooled tissues (4 to 6 rats) of control animals, receiving only saline injections, sacrificed at time intervals indicated on abscissa.

rats. It is of interest that ATP, ADP, and AMP levels of rat livers did not change during NAD accumulation following injection of nicotinamide.

DISCUSSION

In vivo effects of nicotinamide on rates of increase of certain inducible enzymes and on NAD content exhibit a readily observable sequential kinetic relationship. There is a rapid rate of increase in NAD content, almost maximal in 2 hr, which is followed by a slower rate of augmentation of both inducible enzymes. NAD synthesis clearly precedes the accumulation of these enzymes. Further analysis of these two phenomena may eventually yield more comprehensive details of regulatory mechanisms, but from results presently available only outlines of a working hypothesis are possible.

The following arguments provide the basis for this hypothesis. Actinomycin D was found to inhibit augmentation of both inducible enzymes following injection of nicotinamide, but does not influence the rate of NAD accumulation. According to Reich *et al.* (25) and Hurwitz *et al.* (26), actinomycin D inhibits DNA-directed RNA synthesis, a reaction essential for

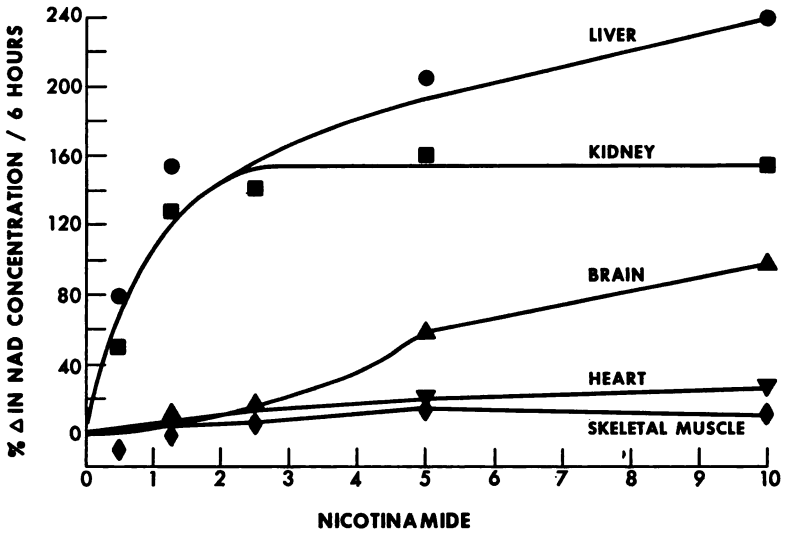

FIG. 2. *The effect of the dose of nicotinamide on the rate of NAD augmentation in various rat tissues*

Rates are expressed as percent change of NAD content, analyzed 6 hours after intraperitoneal injection of varying doses of nicotinamide to starved rats. Abscissa indicates the dose of nicotinamide, expressed as millimoles of nicotinamide/kilogram body weight. In obtaining experimental points, the same procedure was followed as described in the legend to Fig. 1.

de novo enzyme synthesis. This mechanism was extensively dealt with by many investigators and shown to play a central role in the mode of action of hydrocortisone (27) and other steroid hormones (28) on enzyme biosynthesis. The time course of the inhibitory effect of actinomycin D on the enzyme-augmenting action of nicotinamide indicates that this substance may regulate enzyme content by a mechanism similar to that of hydrocortisone. On the basis of present experiments, it cannot be rigorously excluded that nicotinamide has a "stabilizing" effect on both enzymes studied; however, this mechanism appears to be unlikely on the following grounds. *In vivo* enzyme stabilization by a substrate analog has definite stereospecific chemical requirements (cf. 19). It is difficult to imagine how nicotinamide fulfills these requirements for both tryptophan pyrrolase and tyrosine transaminase. It seems more probable that the effect of nicotinamide on tryptophan pyrrolase and tyrosine transaminase content of liver is actually mediated by a hormonal mechanism. Hypophysectomy, as shown here, completely abolishes the enzyme-inducing effect of nicotinamide, while it increases the augmenting influence of nicotinamide on NAD content of liver (cf. 9–12). These observations, together with the differential effect of actinomycin D on NAD and enzyme biosynthesis, distinguish the two processes, but do not exclude a sequential coupling of both events, either in liver or in other tissues such as the adrenal gland.[2] It is pro-

[2] The arguments based on effectivity of actinomycin D are, strictly speaking, pragmatic, i.e., based on analogies (25–28), and thus have limited value inasmuch as they do not define exact mechanisms. However, effectivity of actinomycin D very probably does indicate involvement of *de novo* enzyme synthesis. In a recent note, Kim *et al.* (29) described that actinomycin D under *in vitro* conditions (maximally at pH 8–10) interacts with thyroxine. Extrapolation of this observation to physiological conditions is difficult at present, particularly since—as the authors themselves point out—actinomycin D reacts 10–20 times more effectively with DNA than with thyroxine; thus the generally accepted *in vivo* use of actinomycin D at moderate doses is not invalidated by these observations.

posed that NAD precursors in adrenal tissue, similarly to their effect in liver, may augment pyridine nucleotide content. Increased coenzyme content may directly or indirectly augment hormone synthesis and mobilization, which subsequently induces enzyme synthesis in liver. It is probable that either pyridine nucleotide synthesis in adrenals or subsequent reactions leading to an increased synthesis or mobilization of glucocorticoids require a functional pituitary gland, since hypophysectomy abolishes the effect of nicotinamide on levels of inducible enzyme of the liver.

The proposed mechanism allows for the interference of nutritional status with enzyme biosynthesis initiated by nicotinamide, but not by hydrocortisone. Alteration of intermediary metabolism by starvation (which stimulates gluconeogenesis, cf. 30) may modify the pathway leading from NAD precursors to cortical hormone release. On the other hand, the direct effect of hydrocortisone on enzyme biosynthesis (27) is probably much less sensitive to changes in intermediary metabolism. An interesting but more complicated involvement of glucagon, simulating starvation, on enzyme induction by glucocorticoids has recently been discovered by Greengard and Baker (31).

The obvious defect of this working hypothesis lies in the ignorance of the mechanism of biosynthesis of pyridine nucleotides in adrenal gland and its connections to hormone biosynthesis, an experimental problem presently pursued in this laboratory. Further arguments against the probability of this hypothesis can be found in Table 2, where it is shown that fluoronicotinamide, which may form NAD homologs not active in most dehydrogenase systems, is very effective in augmenting tryptophan pyrrolase. It cannot be excluded that fluoronicotinamide may augment pyridine nucleotide content of certain tissues, such as adrenals, by inhibiting NAD catabolism. This possibility is suggested by the observation that 5-fluoronicotinamide, the most potent inducer of pyrrolase, is a potent inhibitor of NAD glycohydrolase of Ehrlich ascites tumor cells (cf. 3). Inhibition of this enzyme in a tissue with high rate of NAD turnover is likely to increase the steady state level of NAD. It was also found in preliminary experiments that intraperitoneally injected 5-fluoronicotinamide (at 2.5 mmoles/kg dose level) increases NAD content of rat liver by 61%, which is about half the effect caused by nicotinamide. Fluoronicotinamide (unpublished experiments) and nicotinic acid hydrazide are both inhibitors of NAD-glycohydrolase, and can serve as substrates for this enzyme which is known to catalyze the biosynthesis of various NAD homologs (32). It is presently not known whether or not the fluoro homolog of NAD can serve as coenzyme in dehydrogenase or transhydrogenase reactions.

Nikethamide and nicotinic acid hydrazide were found to have no effect on NAD levels of liver, yet increase pyrrolase activity. It is therefore necessary to postulate a different mechanism for their effect on the level of inducible enzymes. While nicotinamide is doses used shows no toxicity (as measured by lethal effect), nikethamide and nicotinic acid hydrazide are highly toxic for the rat (20–30% mortality at doses employed). It cannot be excluded that these pyridine derivatives elicit enzyme induction by stimulating cortical hormone release, a mechanism fundamentally different from the more physiological effect of nicotinamide, which according to the proposed hypothesis should indirectly augment the biosynthesis of cortical hormones by raising NAD levels of the adrenal tissue. Fluoronicotinamide in doses employed also shows significant toxicity (mortality of rats was approximately 15–20%); thus an effect similar to nikethamide may be superimposed on its possible nicotinamide-like action (presumably on NAD levels of the adrenal gland). Direct experimental testing of this hypothesis, a work presently pursued, should provide answers to these questions. It is of significance that injection of nicotinamide in mice does not alter glutamate, lactate, or glucose-6-phosphate dehydrogenase content of the liver (cf. 33); thus as shown in this paper, its effect seems to be restricted to

enzymes that are rapidly augmented by glucocorticoids.

The apparent tissue specificity of NAD precursors causing markedly different rates of NAD accumulation in various tissues raises further questions related to possible tissue specific regulatory mechanisms, which cannot be defined on the basis of available information. Gholson (34) recently proposed a cyclic metabolic scheme for pyridine nucleotide pathways. It is possible that this more complex multienzymic organization of enzymes involved in NAD metabolism and specific mechanisms of substrate transfer in various tissues may be the basis of presently obtained observations.

ACKNOWLEDGMENTS

This work was supported by research grants of the American Heart Association, Inc. (66-652), the National Science Foundation (GB-3488), and the United States Public Health Service (R01-01239-11 and R01-CA-07955-03), and in part by USPHS training grant HE-5251.

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