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RELATIONSHIP OF NICOTINAMIDE AND NICOTINIC ACID TO HYPOLIPIDEMIA

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Abstract—Acute administration of nicotinamide-like nicotinic acid caused a decrease of serum cholesterol, triglycerides and free-fatty acids in the fasted rat. However, the dose of nicotinamide required for these effects was much larger than that of nicotinic acid. The serum half-life of nicotinamide was found to be three times greater than nicotinic acid. The duration of hypolipidemic activity of nicotinamide was longer than that of nicotinic acid.

Nicotinic acid was found to accumulate in the serum in amounts proportional to the injected dose of nicotinamide. The concentration of nicotinic acid in the serum after nicotinamide treatment was larger than minimal serum concentrations necessary to produce free-fatty acid lowering. It was concluded that the lipopenic action of nicotinamide was indirect and was dependent upon deamidation to nicotinic acid.

THE EFFICACY of nicotinic acid (NA) as a hypolipidemic agent has been known for several years,[1] and the clinical value of this compound as an agent for lowering serum cholesterol content has been demonstrated.[2,3] Nicotinamide (NAM) has been extensively tested in man, in comparison with nicotinic acid, but in no case was lipid lowering observed.[2,3] This failure to demonstrate serum cholesterol lowering in man has caused nicotinamide to be ignored as a lipid-lowering agent. In contrast to the common findings, we have found nicotinamide to be an effective lipopenic agent in rats and would like to report our observations.* These experiments will show that nicotinamide becomes deamidated to nicotinic acid and maintains levels of nicotinic acid in the blood sufficient to support prolonged hypolipidemia.

METHODS

Male Charles River rats, weighing 180–240 g, were deprived of food for 16 hr, but were allowed drinking water *ad lib*. Dosage of drugs, route of administration and time intervals before sacrifice are indicated in the tables and figures. Appropriate sham-injected rats were sacrificed as control for all experimental time intervals. Rats were anesthetized with carbon dioxide before sacrifice and blood was obtained by heart puncture.

Serum was extracted with isopropanol and shaken with an activated zeolite mixture (200 g zeolite, 20 g $Ca(OH)_2$, 10 g $CuSO_4 \cdot 5H_2O$) prior to analysis for cholesterol

* A preliminary communication of some of these data has appeared in C. Dalton, *Nature, Lond.* **216**, 825 (1967).

using the Technicon Autoanalyzer method of Block *et al.*,[4] and triglycerides utilizing the automated fluorometric technique of Kessler and Lederer.[5] Free-fatty acids were extracted from 0·3 ml serum by the procedure of Itaya and Ui[6] and determined colorimetrically by the method of Dalton and Kowalski.[7]

Antilipolytic activity *in vitro* was determined using an isolated fat-cell system prepared from rat epididymal adipose tissue according to Rodbell.[8] Cells were incubated in a Krebs–Ringer bicarbonate buffer,[9] but with half the recommended concentration of calcium and 4% bovine serum albumin (Armour Pharmaceutical Co., Fraction V). Theophylline (5×10^{-4}M) was used to stimulate free-fatty acid mobilization. NA and NAM were added in graded concentrations. The cell suspension was incubated for 1 hr at 37° and the reaction was terminated by adding 3 ml of phosphate buffer (pH 6·3) to each flask followed by 20 ml of chloroform. Vials were capped, shaken, the upper layer aspirated and the lower layer filtered through Whatman No. 1 filter paper. For triglyceride analysis, 1-ml aliquots from each filtrate were pipetted into separate tubes, evaporated to dryness, dissolved in 10 ml of aldehyde-free isopropanol, and analyzed by the automated method of Kessler and Lederer.[5] Analysis for free-fatty acids was conducted directly on the chloroform extract by the automated procedure described above.[7] The rate of lipolysis was expressed as: μequiv. of free fatty acids (FFA) per g triglyceride per hr. The IC_{50} (concentration of inhibitor which reduced maximal lipolytic activity of theophylline by 50 per cent) was obtained by inspection of a plot of log concentration versus per cent inhibition.

Nicotinic acid and nicotinamide were extracted from serum with acetone (6 vol.), centrifuged, and the supernatant was quantitatively transferred to a test tube containing chloroform (7 vol.). The mixture was recentrifuged and 0·5 ml of the upper aqueous layer was removed for analysis. Extracted samples were qualitatively analyzed for NA, NAM, nicotinuric acid and *n*-methylnicotinamide. A standard mixture of these 4 compounds was successfully resolved by thin-layer chromatography on silica gel (Gelman, DF-5), using distilled water as the developing solvent and short-wave u.v. light as the mode of visualization. Quantitative analysis of NA and NAM was performed colorimetrically by the König reaction,[10] as modified by Hever,[11] with KCN as the color developing agent, after TLC separation on silica gel with *n*-propanol–10% ammonia (95:5, v/v) and elution with water, as reported by Carlson.[12] Standard amounts of NA and NAM ranging from 5 to 100 μg/ml and 5 to 300 μg/ml, respectively, were taken through the same extraction, TLC separation and colorimetric procedure as the experimental samples.

RESULTS

A single subcutaneous injection of NA or NAM caused a lowering of serum FFA in the fasted rat (Fig. 1). Free-fatty acids were lowered maximally by NA at the earliest sampling point and remained depressed for 6 hr, after which there was a "rebound" of FFA levels. Nicotinamide also caused a maximal lowering of FFA after 15 min, but levels were depressed for more than 8 hr and no "rebound phenomenon" was observed in this case. Serum triglycerides were depressed by NA in a parallel fashion to the FFA levels except a rebound effect was not observed. Triglycerides were reduced more slowly by NAM treatment and remained lowered during the 12 hr of sampling. Serum total cholesterol levels were lowered in a similar manner upon treatment with both NA and NAM. Nicotinamide under these experi-

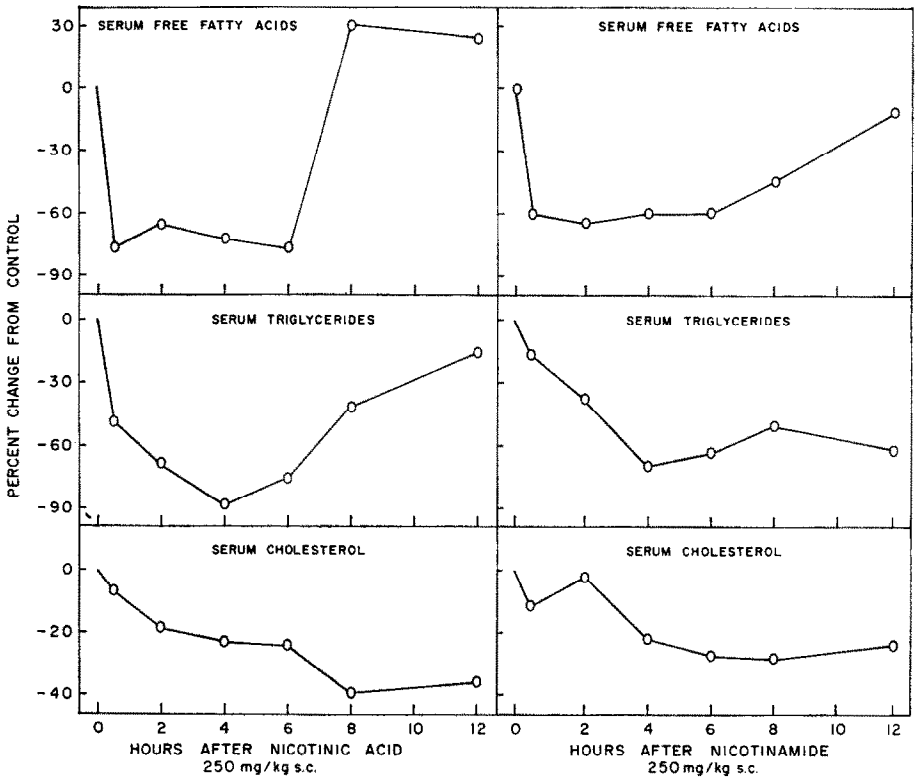

FIG. 1. Effect of nicotinic acid and nicotinamide on serum free-fatty acids, triglycerides and cholesterol in the fasted rat. Eight rats per group. Zero time control values were: free-fatty acids, 556 ± 26 μequiv./l. ± S. E.; triglycerides, 72 ± 7·9 mg/100 ml ± S. E.; cholesterol, 87 ± 3·4 mg/100 ml ± S. E. All treated mean values were significantly different from the control, $P < 0{\cdot}01$, with the exception of serum cholesterol concentration at 2 hr and serum free-fatty acid at 12 hr after nicotinamide injection and serum triglyceride levels 12 hr after nicotinic acid administration.

mental conditions appeared to have more prolonged antilipidemic activity than nicotinic acid (Fig. 1).

The antilipolytic activity of NA and NAM on theophylline-induced lipolysis in isolated fat cells is shown in Fig. 2. Nicotinic acid was a very potent inhibitor of lipolysis with a IC_{50} of 3×10^{-7}M. Nicotinamide which has previously been reported to lack antilipolytic properties in fat pads[13] was found to have anti-lipolytic properties in the isolated fat cell preparation if added in high concentrations (IC_{50}, 2×10^{-4}M).

It has been generally considered that the cholesterol-lowering activity of nicotinic acid was unrelated to its known vitamin role as a precursor of pyridine nucleotides, because nicotinamide, which is more readily incorporated into pyridine nucleotides, had little effect on serum cholesterol levels in man. With the demonstration above of serum lipid-lowering properties of NAM in the rat, the function of nicotinamide and other pyridine nucleotide precursors and their relationship to lipid lowering were re-opened. Tryptophan and quinolinic acid, however, were found to have no effect on serum FFA levels in the rat (Table 1). Nicotinic acid and NAM, under the same conditions, caused FFA lowering.

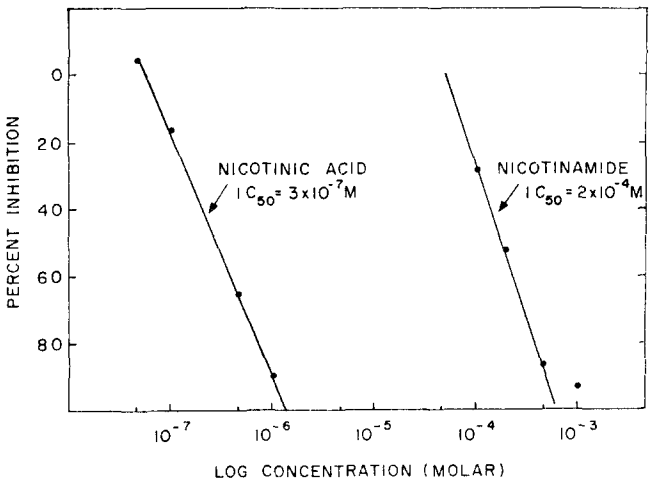

FIG. 2. Comparison of the inhibitory effects of nicotinic acid and nicotinamide on theophylline-induced lipolysis in an isolated fat-cell preparation. IC_{50}, is the concentration of drug which caused 50 per cent inhibition. Rate of theophylline-induced lipolysis, 147 ± 4·2 μequiv. free-fatty acid/g triglyceride/hr ± S. E.

TABLE 1. EFFECT OF PRECURSORS ON PYRIDINE NUCLEOTIDE SYNTHESIS ON SERUM FREE-FATTY ACID LEVELS IN THE FASTED RAT*

Agent	Serum FFA (μequiv./l. ± S. E.)
Control	689 ± 25
Nicotinic acid	188 ± 10†
Nicotinamide	256 ± 16†
Tryptophan	796 ± 39
Quinolinic acid	690 ± 24

* Compounds were injected subcutaneously at 250 mg/kg and rats were sacrificed 2 hr later.

† Significance between treated and control group, $P < 0.001$.

The relative efficiency of NA and NAM as hypolipidemic agents was assessed in the fasted rat. The drugs were injected intraperitoneally and the animals sacrificed 2 hr later. Changes in serum FFA are shown in Fig. 3 and in Tables 2 and 3. The dose–response curve for nicotinic acid showed a precipitous drop in FFA levels and maximal effects were obtained at 50 mg/kg. An increase in FFA levels was obtained after a 10 mg/kg injection of NA which may be a "rebound effect" resulting from earlier inhibition of free-fatty acids. A rebound of FFA levels at higher concentrations of NA might be expected to occur after a longer time interval than 2 hr. The dose–response curve for nicotinamide was more gradual and maximal lowering of FFA levels was not attained until 500 mg/kg was injected (Fig. 3, Table 3). Decrease in serum triglyceride concentration was observed with doses of NA as low as 10 mg/kg and maximum lowering occurred after 25 mg/kg (Table 2). Nicotinamide treatment resulted in a dose–response curve that was almost parallel to NA, but larger doses of

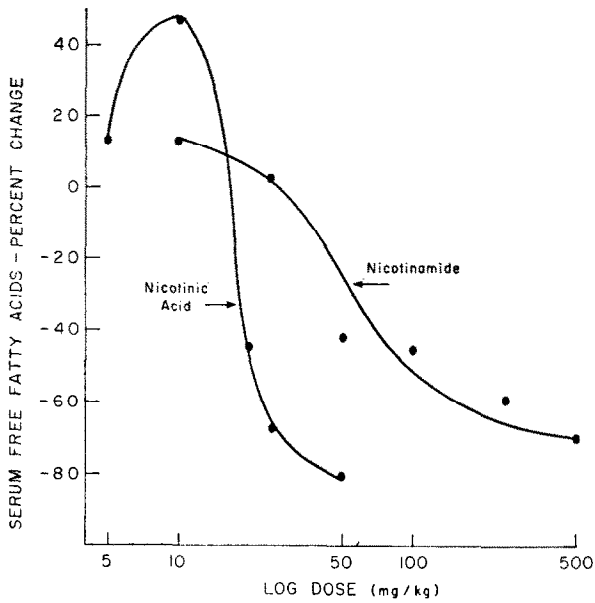

FIG. 3. Effect of nicotinic acid and nicotinamide on serum free-fatty acid levels in the fasted rat. Drugs were injected intraperitoneally and animals sacrificed after 2 hr. Each point represents the mean of eight to 14 rats. Control serum free-fatty acid levels were, 665 ± 23 μequiv./l. ± S. E. and 553 ± 18 μequiv./l. ± S. E. respectively.

TABLE 2. CHANGES IN SERUM LIPID COMPONENTS IN FASTED RATS TREATED WITH NICOTINIC ACID*

Dose (mg/kg, i.p.)	FFA (μequiv./l. ± S. E.)	Triglycerides (mg % ± S. E.)	Cholesterol (mg % ± S. E.)
Control	665 ± 23	57·1 ± 10	74·7 ± 3·3
5	755 ± 25†	69·5 ± 4·6	73·1 ± 3·8
10	981 ± 57§	50·6 ± 4·6	74·2 ± 2·2
20	365 ± 91‡	26·8 ± 2·7†	75·6 ± 2·6
25	214 ± 66§	19·1 ± 2·3‡	67·2 ± 3·1
50	129 ± 9§	20·3 ± 1·9‡	68·8 ± 3·8

* Values are mean of eight to 14 rats. Rats sacrificed 2 hr after drug injection.
† Significance between treated and control group, $P < 0.05$.
‡ Significance between treated and control group, $P < 0.01$.
§ Significance between treated and control group, $P < 0.001$.

the NAM were needed to bring about maximum lowering (Table 3). No quantitative changes in serum total cholesterol levels were obtained under these conditions (Tables 2 and 3).

The slow onset of nicotinamide hypolipidemic activity and the need for large doses suggest that a metabolism or conversion product may be the cause of hypolipidemic activity. Techniques were established to look for all known metabolic products of NA and NAM in the serum of NAM-treated rats. No metabolic product was found in the serum which was considered of any consequence, but relatively large amounts of

TABLE 3. CHANGES IN SERUM LIPID COMPONENTS IN FASTED RATS TREATED WITH NICOTINAMIDE*

Dose (mg/kg, i.p.)	FFA (μequiv./l. ± S. E.)	Triglycerides (mg % ± S. E.)	Cholesterol (mg % ± S. E.)
Control	553 ± 18	47·2 ± 3·9	76·9 ± 2·8
10	626 ± 27	62·5 ± 7·7	90·8 ± 6·5†
25	572 ± 56	35·2 ± 5·6	73·3 ± 4·6
50	320 ± 31‡	37·6 ± 4·2	75·6 ± 4·4
100	304 ± 39‡	34·2 ± 3·9†	82·5 ± 2·6
250	226 ± 34‡	21·9 ± 3·3‡	73·1 ± 3·5
500	169 ± 14‡	21·9 ± 3·8‡	75·5 ± 4·4
750	238 ± 20‡	16·0 ± 1·8‡	78·3 ± 3·0

* Values are mean of eight to 14 rats. Rats sacrificed 2 hr after drug injection.
† Significance between treated and control group, $P < 0.05$.
‡ Significance between treated and control group, $P < 0.001$.

TABLE 4. RELATIONSHIP BETWEEN INJECTED DOSE OF NICOTINAMIDE AND SERUM LEVELS OF NICOTINAMIDE AND NICOTINIC ACID*

Dose (mg/kg, i.p.)	Nicotinamide (μg/ml ± S. E.)	Nicotinic acid μg/ml ± S. E.)
0	3·4 ± 0·5	0·8 ± 0·2
10	6·4 ± 0·2	1·3 ± 0·3
25	20·1 ± 1·1	2·1 ± 0·3
50	33·5 ± 1·9	2·3 ± 0·6
100	93·6 ± 2·8	2·9 ± 0·2
250	295·0 ± 7·0	3·5 ± 0·3
500	462·4 ± 24·8	5·3 ± 0·4

* Values are mean of six rats per group. Rats sacrificed 2 hr after nicotinamide injection.

TABLE 5. RELATIONSHIP BETWEEN INJECTED DOSE AND SERUM NICOTINIC ACID LEVELS*

Dose (mg/kg, i.p.)	Serum nicotinic acid (μg/ml ± S. E.)
0	Trace
5	Trace
10	0·05 ± 0·05
20	2·20 ± 0·70
25	4·60 ± 1·40
50	41·50 ± 3·10

* Values are mean of six rats per group. Rats sacrificed 2 hr after nicotinic acid injection.

nicotinic acid were found. Experiments were established to test the assumption that deamidation of NAM was taking place at rates which could account for the hypolipidemic activity reported for nicotinamide. Data recorded in Table 4 show the concentrations of NA and NAM present in the serum 2 hr after NAM injection. Serum NA levels were found to be proportional to the dose of NAM injected. The concentrations of NA present in serum after hypolipidemic doses of NA were measured and are shown in Table 5. Serum levels as low as 2·2 μg/ml (20 mg/kg, i.p.)

had very pronounced lipid-lowering activity, and levels of this magnitude were readily reached in animals injected with nicotinamide. When the serum FFA changes, after NAM injection, are plotted as a function of nicotinic acid levels measured in the same rat, a typical sigmoid inhibitory response curve is obtained (Fig. 4). Corresponding values of NA concentrations determined in NA-treated rats can be fitted perfectly on this curve of serum NA concentration versus serum FFA lowering.

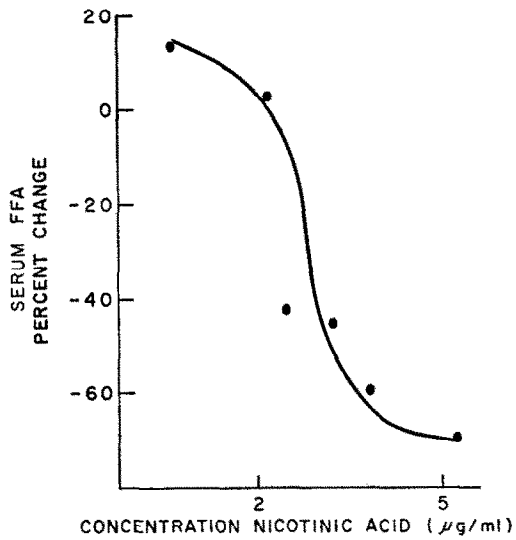

FIG. 4. Changes in serum free-fatty acid levels plotted as a function of serum nicotinic acid levels in nicotinamide-treated rats. Nicotinamide was injected intraperitoneally and the animals sacrificed after 2 hr. Each point represents the mean of six rats. Control serum free-fatty acid levels were 553 ± 18 μequiv./l. ± S. E.

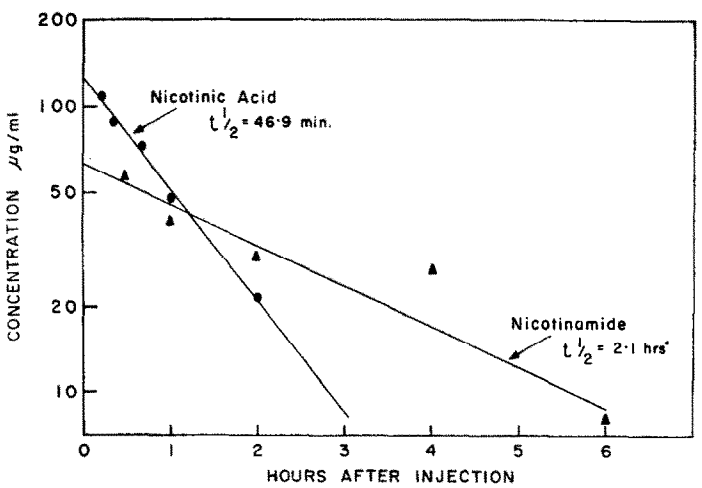

FIG. 5. Logarithmic plot of nicotinic acid and nicotinamide concentrations in fasted rat serum as a function of time. Nicotinic acid and nicotinamide were each injected intravenously at 50 mg/kg Each point represents mean of two to three rats. Apparent serum half-life, $T_{1/2}$.

Demonstration of the fact that the NA levels measured at a fixed time interval in the experiments described above could be applied to events occurring over a longer time period was obtained by determination of serum half-lives ($T_{1/2}$) of NA and NAM in the rat. A plot of the NA and NAM concentrations obtained in rat serum after intravenous injection of 50 mg/kg of NA and NAM is shown in Fig. 5. Serum $T_{1/2}$ for NA was 47 min and $T_{1/2}$ for NAM was 2·1 hr. It is clear from these half-life data that NA has a duration in rat serum long enough to account for the hypolipidemic activity observed.

DISCUSSION

In contrast to the usual clinical and experimental findings, we have found NAM to have hypolipidemic properties in the rat. Injected nicotinamide was found at sufficiently high doses to give an equivalent response to nicotinic acid in decreasing the concentration of cholesterol, triglycerides and free-fatty acids in the serum of the fasted rat. The experiments described here show that nicotinamide had a slower onset of lipopenic activity than nicotinic acid. It took longer for NAM than NA to reduce serum FFA and triglycerides, and NAM had a longer duration of action than nicotinic acid. Throughout these experiments, NAM has been compared with the more thoroughly studied NA and the spectra of activity obtained, although limited, are almost identical qualitatively. This similarity suggested a common mechanism of action. We felt it incumbent, in view of the unanticipated nature of our observation, to demonstrate a possible mechanism of hypolipidemic activity for nicotinamide. Possible pathways taken into consideration included: pyridine nucleotide synthesis; direct antilipolytic activity; metabolic conversion to nicotinic acid and metabolic conversion along with nicotinic acid to some other active substance.

It has generally been agreed that the cholesterol lowering activity of nicotinic acid is unrelated to this compound's known vitamin role as a precursor of pyridine nucleotides because much larger doses are needed for hypolipidemia than are usually associated with vitamin activity. Nicotinamide, which is readily incorporated into pyridine nucleotides, has been reported inactive as a hypolipidemic agent. Demonstration herein that NAM does possess lipid lowering properties re-opened the possibility that NAD^+ levels may be connected with cholesterol lowering. Fontenot *et al.*,[14] in a study of cholesterol fed rabbits, indicated that a direct relationship existed between elevated pyridine nucleotide levels of packed erythrocytes and hypocholesterolemia in animals dosed with NA or NAM. It was postulated that NAD^+ production could be the primary cause of the hypolipidemic effects of NA or NAM treatment. In our hands, a time-course study of whole blood NAD^+ levels in rats treated with NA or NAM revealed no evidence of such a correlation.[15] It was further demonstrated in this laboratory that tryptophan and quinolinic acid, both precursors of NAD^+, had no antilipidemic effect in the rat (Table 1). It has been concluded that the effects of NA and NAM on serum lipid levels are not related to their roles as precursors of pyridine nucleotides.

It seemed unlikely that NAM was hypolipidemic because of direct antilipolytic properties. Carlson has reported a lack of antilipolytic activity of NAM in rat epididymal fat pads *in vitro*.[13] Using an isolated fat cell preparation, we have shown that NAM does possess antilipolytic activity, but at about 1000 times the concentration of NA (Fig. 2). This separation of activity appears to be unrelated to lipid lowering

activity because effective doses *in vivo* of NA and NAM are more closely related. It should be noted that a 0·1 per cent contamination of NAM with NA could account for the antilipolytic activity observed *in vitro*.

Indirectly, NA and NAM may well be related to a common antilipolytic action. Both NA and NAM caused maximal reduction of FFA much earlier than the decrease in serum triglycerides; this was paralleled, after a short lag period, by a slow decrease in serum cholesterol. This temporal relationship of serum lipid levels has been observed by Carlson and Nye,[16] and Dalton *et al.*,[17] after NA administration. The effect of nicotinic acid on FFA concentrations in the serum is so pronounced that it has been suggested that the primary activity of nicotinic acid is to inhibit the release of FFA from adipose tissue depots, and the effect on blood and liver lipid constituents is an indirect consequence of this activity.[16,17] It appears from the data reported here that the same arguments might be applied to account for the hypolipidemic action of nicotinamide.

The contention that both NA and NAM might be converted to some common metabolic product with lipid-lowering properties was investigated. The sera from NAM-treated animals, when extracted and examined by silica gel TLC, showed no appreciable amounts of nicotinic acid metabolites, but measurable amounts of nicotinic acid were present. Serum from NA-treated rats contained little nicotinamide, *n*-methyl nicotinamide or nicotinuric acid, but nicotinic acid was readily detectable. It was clear from these data that the only candidate material detectable in both sera which could account for the pronounced hypolipidemic activity was nicotinic acid. The nicotinic acid was presumably being derived by deamidation of NAM in NAM-treated animals.

Ricci and Pallini have reported an increase in free-NA levels in the livers of rats injected with large amounts of nicotinamide.[18] Enzyme systems have been demonstrated that could account for NA accumulation in liver and serum after NAM injection. Petrack *et al.*[19,20] have partially purified a liver enzyme which catalyzes the deamidation of NAM to NA, and suggest that this reaction represents a rate-limiting step in the synthesis of pyridine nucleotides from nicotinamide. Further characterization of this enzyme has been obtained by Kirchner *et al.*,[21] who confirm the low activity of deamidase in the liver, the difficulty of detection reported by Petrack *et al.*,[19,20] and show that deamidase activity is not found in other tissues. Accumulation of free-nicotinic acid in the liver and plasma can be explained by assuming that deamidation of NAM occurs at a higher rate than the reaction of nicotinic acid with 5-phosphoribosyl-1-pyrophosphate, the initial stage of conversion of nicotinic acid to NAD^+ via the Preiss–Handler pathway.[22] Ijichi *et al.*[23] have also demonstrated the presence of a deamidase enzyme but with a low K_m, in the small intestine of mice, and suggest that following parenteral administration of NAM, large amounts of NAM are secreted from the liver, accumulate in the gastrointestinal tract, are deamidated to nicotinic acid, and are reabsorbed as such into the liver to serve as precursors of NAD^+ over a long period of time. It seemed clear from our data that an enzyme with a high K_m for NAM was involved in the deamidation we were observing, since large amounts of NAM were required to give rise to measurable serum levels of nicotinic acid. Increased NAM administration caused increased serum NA levels. Least squares regression analysis of a plot of log blood concentration of nicotinic acid against log of injected dose of nicotinamide produced a good fit for a straight-line

relationship (coefficient of determination = 0·967, Table 4). After elucidation of a relationship between injected dose of NAM and plasma NA concentration, it was necessary to demonstrate that NA levels attained were sufficient to account for hypolipidemia.

Serum NA concentration, determined in NA-injected rats exhibiting hypolipidemia, showed that serum levels of 2 μg/ml or greater were accompanied by FFA lowering. Similar levels of NA compatible with FFA lowering have been reported by Nye and Buchanan[24] in sheep, and by Carlson *et al.*[25] in man. Serum NA levels measured in NAM-treated rats were higher than these minimal requirements and a clear relationship existed between serum levels of NA resulting from deamidation of NAM and serum-lipid lowering (Fig. 4). It is concluded from this study that the hypolipidemic activity of NAM should be attributed to the NA generated in these animals by deamidation of nicotinamide.

Half-life studies, in which NAM was found to have a much longer serum $T_{1/2}$ than NA in the rat (Fig. 5), appeared to account for the longer duration of action of NAM when compared to nicotinic acid. Levels of NAM are maintained in serum providing substrate for deamidation activity over a long period of time. It seems from these observations that NAM might offer some therapeutic advantage over NA as a hypolipidemic agent. Clinically, 3–6 g/day of nicotinic acid are used. At these doses, it appears to lower all serum lipid constituents. Equimolar doses of NAM have been found to be ineffective. Considering the high concentration of NAM necessary to saturate the deamidase enzyme, it is possible that high enough doses of NAM have not been tried in man. Hypolipidemic data *in vivo* showed an effective dose ratio for NAM to NA of about three. Extrapolation of this ratio to man would necessitate a dose of 9–18 g/day. It is of interest to note that the dosage of either NA and NAM recommended by Hoffer[26] is 3–18 g/day for treatment of schizophrenia. Such a large dose, if tolerated, would have the advantage of less frequent administration because of the longer half-life of nicotinamide. Nicotinamide administration may also eliminate many of the distressing side effects observed after nicotinic acid therapy.

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