Studies on the Biological Activity of Nicotinylalanine, an Analogue of Kynurenine*

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Considerable evidence has been obtained that 3-hydroxyanthranilic acid and quinolinic acid can be converted to niacin in the intact rat (2-9). The usually low yield of niacin from these compounds suggested that another pathway from tryptophan to niacin might exist. Thus, kynurenine or hydroxykynurenine might be converted to niacin through an intermediate such as \( \gamma \)-3-pyridyl-\( \gamma \)-oxo-\( \alpha \)-aminobutyrate (referred to as nicotinylalanine) via ring oxidation and rearrangement similar to that known to occur with 3-hydroxyanthranilic acid (Fig. 1). To test this hypothesis, nicotinylalanine was synthesized and tested as a possible precursor of niacin in the intact rat and in tissue preparations.

Nicotinylalanine is relatively nontoxic, and its intraperitoneal injection was followed by an increase in urinary excretion of N-methyl nicotinamide. When nicotinylalanine was incubated with rat liver homogenates, it appeared to be converted to nicotinic acid by a kynureninase like enzyme. In studies with tryptophan-3-\( ^{14} \)C, data were obtained indicating that nicotinylalanine is not an intermediate in the conversion of tryptophan to niacin. However, it was found that nicotinylalanine is an effective inhibitor of kynureninase and kynurenine hydroxylase in vivo and in vitro.

EXPERIMENTAL PROCEDURE

Reagents—\( \gamma \)-\( \alpha \)-\( \beta \)-tryptophan-3-\( ^{14} \)C \( (0.25 

\[ \text{C}_{10} \text{H}_{12} \text{O}_{7} \text{N}_{2} \text{S} \]

Calculated: C 36.95, H 4.14, O 38.32, N 9.59, S 10.97

Found: C 36.85, H 4.30, O 38.51, N 9.24, S 11.29

Animals—Male albino rats weighing 150 to 200 g were fed a purified diet (11) and housed in stainless steel metabolism cages. Urine was collected in flasks containing 0.15 ml of xylene and 0.15 ml of glacial acetic acid for each 24-hour volume. Tryptophan and nicotinylalanine were administered by intraperitoneal injection of an aqueous solution. Nicotinylalanine was administered free of sulfate ion, which was precipitated by the addition of an equivalent of powdered barium carbonate to an aqueous solution of the compound.

Excretion of N-Methyl nicotinamide after Injection of Tryptophan and Nicotinylalanine—L-Tryptophan, nicotinylalanine, or both were injected into groups of four rats (2.5 \( \mu \)moles per g of body weight). Urine was collected from the animals for 24 hours before and after injection of the supplements. Samples of urine equivalent to approximately 3% of a 24-hour collection from one rat were used to determine N-methyl nicotinamide by the procedure of Vivian, Reynolds, and Price.

Incubation of Nicotinylalanine with Rat Liver Preparations—Nicotinylalanine was incubated with 20% rat liver homogenate prepared in 0.9% sodium chloride with a Potter-Elvehjem homogenizer. The reaction mixtures contained 2 ml of the homogenate, 5 \( \mu \)moles of nicotinylalanine, 40 \( \mu \)g of pyridoxal phosphate, and 0.1 ml of 1.5 \( \mu \)m phosphate buffer at pH 7.2 in a 3-ml final volume. Controls contained 5 \( \mu \)moles of L-kynurenine in place of the nicotinylalanine. Incubation was carried out at 37°C, and reactions were stopped by addition of 1 ml of 1 \( \mu \)m trichloroacetic acid at the times indicated in Fig. 3. The clear supernatant solutions were diluted to 15 ml and placed on Dowex 50W columns, 1 X 11 cm. The columns were washed with 50 ml of 1 \( \mu \)m HCl, and the nicotinic acid was eluted with 120 ml of 2.4 \( \mu \)m HCl. The eluates were taken to dryness on a rotary evaporator and dried on the aliquots for microbiological assay of nicotinic acid with \( Lactobacillus plan- 

\[ \text{L. arabinosus} \]

(formerly named \( L. arabinosum \)). The extent of growth was determined by measurement of turbidity at 650 \( \mu \)m.

Influence of Nicotinylalanine on Nicotinamide Adenine Dinucleotide Levels in Rat Liver—Nicotinylalanine sulfate was

1 Elemental analyses were made by Hufmann Microanalytical Laboratories, Wheatridge, Colorado.

2 V. M. Vivian, M. S. Reynolds, and J. M. Price, unpublished observations.

3 These data were kindly provided by Dr. Frederick N. Minard
administered at the level of 0.27 μmole per g of body weight to
seven rats 1 to 4 hours before death. The NAD in the liver
was extracted from the frozen tissue with perchloric acid and
determined by the method of Jedeikin and Weinhouse (13).

Metabolism of dL-Tryptophan-3-C14 in Presence of Nicotinyl-
alanine in Vivo—The method of Weinhouse and Friedmann (14)
was employed in the metabolite overloading experiment with
nicotinylalanine. dL-Tryptophan-3-C14 (3.9 μmoles; 14.6 με)
was administered to each of two 170-g rats. One rat also re-
ceived 0.9 mmole of nicotinylalanine in addition to the labeled
tryptophan, and the urine was collected from the animals for
12 hours. Carrier kynurenine and nicotinylalanine were added
to the urines and were separated by gradient elution from a
Dowex 50W column, 15 × 1.5 cm, with HCl. Kynurenine and
nicotinylalanine were recrystallized to constant specific activity
from ethanol as the sulfate salts before the radioactivity was
counted. The C14 content of solid samples was determined with
a Nuclear-Chicago thin window gas flow counter.

Assays of Enzymes Involved in Tryptophan Metabolism—
Tryptophan pyrrolase, kynureninase, kynurenine hydroxylase,
and kynurenine transaminase were determined in rat liver
preparations by the method of DeCastro, Brown, and Price
(15). Nicotinylalanine, 10 pmoles (twice the substrate con-
centrations), was added to determine its effect on the enzyme
activity. Incubations were carried out at 37° for 1 hour.

The effect of nicotinylalanine on the adaptation of hepatic
tryptophan pyrrolase was determined in two experiments. In
the first study, nicotinylalanine sulfate was administered in-
traperitoneally to a group of four rats at a dose of 2.5 μmoles per g
of body weight 3 hours before death and assay of enzyme activity.
A second group of four animals received, per g of body weight,
2.5 μmoles of L-tryptophan and 2.5 μmoles of nicotinylalanine.
Control groups of animals were given either L-tryptophan at
the location of the postulated intermediate, nicotinylalanine.

The second study was the same in design, but groups of five rats were given
7.5 μmoles of nicotinylalanine (with the sulfate removed by BaCO3 treatment) and 5.0 μmoles of L-tryptophan per g of body
weight. The compounds were neutralized and suspended or
dissolved in 2.0 ml of NaCl solution before administration.

Influence of Nicotinylalanine on Urinary Excretion of Trypto-
phan Metabolites—Loading doses of L-tryptophan (2.5 μmoles
per g of body weight) were given to two groups of five rats;
one group also received 2.5 μmoles of nicotinylalanine per g of
body weight. Urine was collected from each group for 24 hours.
Samples of rat urine equivalent to approximately 50% of a
24-hour collection from one rat were resolved into five fractions
on columns of Dowex 50W by the method of Brown and Price
(16) for the determination of the diazotizable aromatic amines
with a modified procedure of Bratton and Marshall (16, 17).
3-Hydroxykynurenine was determined as described by Brown
(18). Kynurenic and xanthurenic acids were determined by the
method of Satoh and Price (19). 3-Hydroxyxanthurenic acid
was isolated and measured by the method of Panmuke, Brown,
and Price (20).

RESULTS

Excretion of N-Methylnicotinamide after Injection of Tryptophan
and Nicotinylalanine The data in Fig. 2 are the average of four
studies. Administration of dL-nicotinylalanine and L-trypto-
phan separately produced 4-fold increases in the urinary excretion
of N-methylnicotinamide over the basal level. Simultaneous
administration of the two compounds revealed that they were
not additive in their effects on the urinary excretion of N-methyl-
nicotinamide.

Conversion of Nicotinylalanine to Nicotinic Acid by Rat Liver
Preparations Incubation of nicotinylalanine with the rat liver
homogenates resulted in a linear increase in nicotinic acid activity
for L. planarum (Fig. 3). The liver preparations catalyzed this
reaction at a rate of 0.45 μmole per g of tissue, wet weight, per
hour. The control reaction mixture containing kynurenine
produced an insignificant increase in niacin activity. Nicotinyl-
alanine itself was inactive as a source of niacin in the microbio-
logical assay and did not inhibit the bacterial utilization of
nicotinic acid.
Influence of Nicotinylalanine on NAD Levels in Rat Liver—Nicotinylalanine produced a 20% increase in NAD levels of rat liver over a period of 1 to 4 hours after administration of 0.27 μmoles of the compound per g of body weight. The mean value of the controls was 0.79 ± 0.020 μmole per g of liver, wet weight, whereas the mean of the test group was 0.94 ± 0.024. The two means were significantly different (p < 0.001).

Metabolism of α-Tryptophan 3-Cl4 in Presence of Nicotinylalanine in Vitro—The injected nicotinylalanine was intended to act as an overloading dose that would be excreted in the urine with compound labeled from tryptophan, if present. The radioactivities of the kynurenine and nicotinylalanine isolated from the urine of the two rats are shown in Table I. A small amount of radioactivity appeared in the nicotinylalanine fraction in both experiments, but a second chromatographic separation showed that the isotope peak did not correspond with the peak of the compound and was an impurity. Kynurenine radioactivity was increased 5.5 times when the rat was given nicotinylalanine with the labeled tryptophan.

Effect of Nicotinylalanine on Enzymes Involved in Tryptophan Metabolism—Table II shows typical data obtained when nicotinylalanine was incubated in the assays of four enzymes involved in the first stages of tryptophan degradation. The compound did not influence the activities of tryptophan pyrrolole or kynurenine transaminase, but kynureninase and kynurenine hydroxylase were inhibited 63 and 53%, respectively, in the presence of a substrate to inhibitor ratio of 1:2. Nicotinylalanine had no effect on the NADPH-generating system of the hydroxylating reaction. Inhibition could be reversed in both the kynureninase and hydroxylase reactions by increasing the substrate concentration.

The data in Table III indicate that administration of nicotinylalanine sulfate produced a 2-fold increase in the activity of liver tryptophan pyrrolase compared with the controls that received NaCl solution, whereas l-tryptophan at the same concentration resulted in a 4-fold increase in enzyme activity. When both tryptophan and nicotinylalanine were injected there was a small increase in pyrrolase activity as compared with the effect produced by tryptophan alone. However, nicotinylalanine administered free from the sulfate ion (Table III) did not show a significant effect on tryptophan pyrrolase, even when the dose was 3 times that used in the first experiment.

Influence of Nicotinylalanine on Urinary Excretion of Tryptophan Metabolites—Nicotinylalanine produced marked changes in the urinary excretion of some tryptophan metabolites. The results in Fig. 4 are from one of four experiments of this type. Considerable variation was observed from one experiment to another in the levels of urinary metabolites excreted, depending upon animal size and dosages given, but the qualitative results were much the same. Although moderate increases were observed in anthranilic acid glucuronide, o-aminohippurate, kynurenic acid, and hydroxykynurenic acid, the greatest increases were noted with N-methylnicotinamide, kynurenic acid, and xanthurenic acid. That the apparent change in kynurenic and xanthurenic acid levels was not the result of interference in the assay by metabolites of nicotinylalanine was shown by measuring these tryptophan metabolites in the 24-hour urines from rats.
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DISCUSSION

Since N-methyl nicotinamide is a major urinary metabolite of niacinamide in the rat (21), the level of its excretion was used as a guide to niacin production in vivo. dl-Nicotinylalanine was approximately as effective as L-tryptophan in increasing the excretion of N-methyl nicotinamide. If only the L isomer of nicotinylalanine was utilized, as is the case with kynurenine (22), it appears that the compound was twice as efficient as tryptophan as a source of niacin. The formation of nicotinic acid from nicotinylalanine by a liver enzyme seems likely to have occurred by a hydrolytic reaction, much like that catalyzed by kynureninase. In view of the limited specificity of this enzyme (23), such a reaction is not surprising.

The rise in the NAD levels of the rat livers provided further evidence that nicotinylalanine was converted to niacin. Although weilker experiment ruled out the apparent production of N-methyl nicotinamide or NAD by interfering metabolites of nicotinylalanine, the fact that nicotinic acid was produced in vitro gives strong support to the possibility that it was formed in vivo as well.

Although these experiments established that nicotinylalanine could give rise to niacin, they did not prove that nicotinylalanine is a metabolite of tryptophan in the rat. Observations made with labeled tryptophan did not support its role as an intermediate, since no radioactive nicotinylalanine could be detected in the urine after injection of tryptophan-C14 either with or without loading doses of nicotinylalanine. The analogue functioned in a different manner, however, causing an increased excretion of labeled kynurenine and other radioactivity in the urine. Because of the similarities in structure between kynurenine and nicotinylalanine, a logical explanation was that the latter inhibited metabolism of kynurenine to niacin. This would explain why the administration of tryptophan and nicotinylalanine did not cause an additive increase in N-methyl nicotinamide excretion. Both kynureninase and kynurenine hydroxylase were, in fact, inhibited by nicotinylalanine in vitro. Since hydroxykynureninase and kynurenine are presumably the same enzyme (23), a "sequential block" (24) in the pathway would result if this occurred in vivo. The increased accumulation of kynurenine metabolites in urine after tryptophan was given with nicotinylalanine supported the conclusion that inhibition of these enzymes occurred in vivo as well. The possibility that the increase in metabolite excretion was caused by tryptophan pyrroline adaptation, which resulted in more kynurenine being produced, was ruled out when it was found that nicotinylalanine and tryptophan injection did not increase induction of enzyme activity appreciably over the level obtained when tryptophan was given alone. The 2-fold adaptation caused by nicotinylalanine sulfate (Table III) probably was caused by the stress arising from the salts accompanying the compound (25), since this adaptation did not occur in a similar experiment (Table III) when the salt was removed, even when the dose of nicotinylalanine was increased 3-fold.

Results of the quantitative determination of several tryptophan metabolites in the urine are not easily interpreted, since both kynurenine and inhibitor concentrations probably were in a fluctuating state. Kynurenine and kynurenic acid were expected to accumulate, since kynurenine hydroxylase and kynureninase would be inhibited. Inhibition of hydroxykynureninase would also result in an increased excretion of hydroxykynurenine and xanthurenic acid. Xanthurenic acid and kynurenic acid accounted for more than 85% of the total increase in the tryptophan metabolites measured in nearly all experiments. The observation that nicotinylalanine produced only moderate increases in urinary kynurenine and hydroxykynurenine would be expected if the excess of these metabolites were adequately shifted to the uninhibited transaminase reactions, effecting the increases in the production of kynurenine and xanthurenic acids.

Although the anthranilic acid conjugates, o-aminohippuric acid and anthranilic acid glucuronide, are products of the kynureninase reaction, their excretion was increased when nicotinylalanine was administered with tryptophan. The accumulation of kynurenine resulting from the sequential block would tend to shift the equilibrium of the kynureninase reaction in favor of anthranilic acid. The decreased urinary level of 3-hydroxyanthranilic acid, the product of hydroxykynureninase, is in accord with the evidence for a sequential block.

SUMMARY

Intraperitoneal administration of nicotinylalanine to rats caused a 4-fold increase in urinary N-methyl nicotinamide, and incubation of nicotinylalanine with rat liver homogenates produced a linear increase in nicotinic acid with time. Data obtained in isotope carrier and "overloading" studies with tryptophan-3-C14 indicated that nicotinylalanine is not a metabolite of tryptophan; instead, it acted as an antimetabolite of kynurenine by inhibiting kynureninase and kynurenine hydroxylase in vitro. Evidence was obtained to indicate that inhibition occurred in vivo as well, resulting in a sequential block of the major route of kynurenine degradation.

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