

THE ISOLATION AND IDENTIFICATION OF THE ANTI-BLACK TONGUE FACTOR*

BY C. A. ELVEHJEM, ROBERT J. MADDEN, F. M. STRONG, AND
D. W. WOOLLEY

(From the Department of Agricultural Chemistry, University of
Wisconsin, Madison)

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The preparation of concentrates of the antipellagra vitamin which were active in the prevention of pellagra-like symptoms in chicks and the cure of black tongue in dogs was described previously (1). In this paper we wish to report additional work on the purification of the vitamin which led to the demonstration of the activity of nicotinic acid and nicotinic acid amide in the cure of canine black tongue.

EXPERIMENTAL

Dogs have been used exclusively for assay purposes. Several different breeds have been used but in each case the animals were brought to the laboratory shortly after weaning. They were given a complete diet for about 2 weeks, during which time they were kept under observation, and were then placed on the modified Goldberger diet¹ described previously (1). Of the entire group, two have been adult dogs, but the time required to produce black tongue in the older animals makes them rather unsatisfactory for this work. In order to reduce the loss of dogs to a minimum, the animals were used for the assays before severe

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¹ The casein was purified by washing crude casein with water eight times and then dissolving in ammonia and precipitating with hydrochloric acid.

symptoms developed. However, in every case, the supplement was not given until the dog showed drastic loss of weight and the early but definite symptoms of black tongue. Usually the animal refused its food for at least 2 days before the test material was administered. Many of the dogs were used for several assays. In each case the dog was continued on the basal ration until typical symptoms reappeared, at which time a new supplement was given. The extent of the growth response and the time required for the symptoms to reappear gave a fair indication of the quantitative potency of the material tested.

In our previous studies the purification had been carried through a final charcoal clarification step. Since the majority of the activity was recovered in the charcoal filtrate, it was concluded that the vitamin was not adsorbed on charcoal. It was planned, therefore, to continue to use this procedure for the preparation of relatively large amounts of the concentrate for isolation purposes. However, our original supply of charcoal was exhausted and our new material consisted of norit A (Pfanstiehl). When the activated norit was used, the filtrate proved inactive in the cure of black tongue (Fig. 1, Curves 1 and 2). When the same procedure was repeated with a sample of crude vegetable charcoal, the filtrate again contained a definite amount of activity (Fig. 1, Curves 3 and 4). It is evident that the concentrate which had been treated with this charcoal was almost as active as the untreated material. This observation emphasizes again the variable results which may be obtained with different samples of charcoal and that conclusions should be made only for the specific type used.

The use of the activated norit was continued because it was hoped that the adsorption and possible elution would offer a means of concentration of the vitamin. The following method was used. An aliquot of the concentrate carried down to the charcoal step according to the method described previously (1) equivalent to 400 gm. of liver extract² and containing about 6 gm. of solids was diluted to 200 cc. 4 gm. of norit A were added and the solution made to pH 3 with HCl. The suspension was stirred at 85° for 5 minutes, after which the norit was filtered off. The

² We are indebted to Dr. C. Nielsen of Abbott Laboratories and Dr. David Klein of The Wilson Laboratories, who supplied generous samples of liver extract.

norit removed approximately 1 gm. of the original solids. The norit was fed directly to the dogs in order to determine whether the total activity could be recovered. The norit showed activity, Fig. 1, Curves 5 and 6, but did not account for the entire potency of the extract before adsorption. If we assume that the vitamin was adsorbed more or less quantitatively without destruction, we may conclude that the dogs were able to elute only about one-third of the adsorbed vitamin.

The norit was treated with a number of reagents and combinations of reagents in an attempt to effect a quantitative removal of the vitamin. 4 gm. of norit carrying about 1 gm. of adsorbed solids equivalent to 400 gm. of liver extract were treated with 200 cc. of a mixture containing 2 parts of acetone, 1 part of water, and 1 part of pyridine. The mixture was heated to boiling for 3 minutes and the charcoal filtered off. This procedure was repeated twice. The eluate was concentrated to dryness *in vacuo* and the residue dissolved in water. About one-half of the solids adsorbed on the charcoal was recovered in the eluate. The activity of this eluate is shown in Fig. 1, Curves 7 and 8. From a rough estimate of the potency we may conclude that the elution was fairly quantitative.

Since this charcoal treatment aided materially in the removal of inert material and concentration of the active substance, it was made an additional step in our method. The following procedure was adopted as the routine method. Solutions containing 1 per cent solids were treated with sufficient norit A to give a 2 per cent suspension and the mixture adjusted to pH 4.0. The suspension was stirred for 1 hour at room temperature, after which the norit was filtered off and washed with distilled water. The norit was then added to a sufficient quantity of a mixture of 4 parts of methyl alcohol and 1 part of pyridine to form a 10 per cent suspension and the mixture was stirred for 15 minutes at room temperature. This procedure was repeated twice. The alcohol and pyridine were removed by vacuum distillation and the residue made up to a definite volume with water. This method of adsorption and elution was no more efficient than the method described above, but it was considerably more convenient. An assay of the concentrate carried through the above procedure is shown in Fig. 1, Curve 9.

Certain attempts to esterify the active compound were made

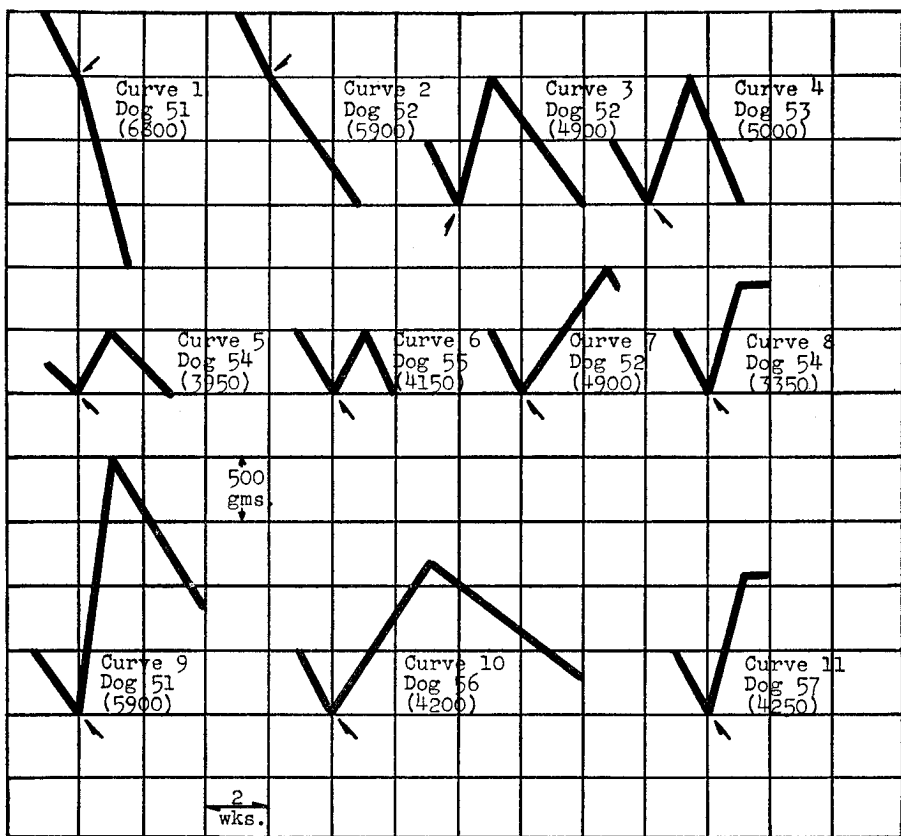


FIG. 1. Growth responses obtained in dogs maintained on basal black tongue diet when given various liver fractions. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, norit A filtrate fed in 10 cc. doses daily for 5 days. 1 cc. \cong 80 gm. of fresh liver. Curve 2, norit A filtrate fed in 10 cc. doses daily for 8 days. 1 cc. \cong 58 gm. of fresh liver. Curve 3, concentrate before treatment with a crude vegetable charcoal fed in 10 cc. doses daily for 4 days. 1 cc. \cong 50 gm. of fresh liver. Curve 4, concentrate after treatment with a crude vegetable charcoal fed in 10 cc. doses daily for 4 days. 1 cc. \cong 50 gm. of fresh liver. Curve 5, norit A with vitamin adsorbed fed in 1 gm. doses daily for 8 days. 1 gm. \cong 200 gm. of fresh liver. Curve 6, norit A with vitamin adsorbed fed in 1 gm. doses daily for 8 days. 1 gm. \cong 200 gm. of fresh liver. Curve 7, vitamin eluted from norit A with acetone, water, and pyridine fed in 10 cc. doses daily for 7 days. 1 cc. \cong 40 gm. of fresh liver. Curve 8, vitamin eluted from norit A with acetone, water, and pyridine fed in 10 cc. doses daily for 7 days. 1 cc. \cong 40 gm. of fresh liver. Curve 9, vitamin eluted from norit A with methyl alcohol and pyridine fed in 10 cc. doses daily for 4 days. 1 cc. \cong 80 gm. of fresh liver. Curve 10, concentrate after treatment with NaOH fed in 10 cc. doses daily for 4 days. 1 cc. \cong 60 gm. of fresh liver. Curve 11, concentrate extracted with acetone at pH 9.4 fed in 10 cc. doses daily for 4 days. 1 cc. \cong 80 gm. of fresh liver.

at this point. In general these experiments were unsuccessful and the details will not be included in this paper. However, this work did lead to a more complete study of the stability of the vitamin to alkali, since it was necessary to hydrolyze the ester before feeding. The stability which was observed was truly remarkable. It is necessary to mention only one experiment, in which a concentrate containing 100 mg. of solids was treated with 50 cc. of 1 N sodium hydroxide and heated at 100° for 10 hours. The response obtained (Fig. 1, Curve 10) demonstrates that the activity is not reduced by this treatment. It was also found that the majority of the potency remained after refluxing a concentrate with 40 per cent hydrobromic acid.

After this stability was established, we were interested in the possibility of changing the solubility of the vitamin or its contaminants in organic solvents by forming sodium salts. At this stage we were fortunate in obtaining from Dr. H. W. Rhodehamel of Eli Lilly and Company concentrates prepared from both liver and liver extract according to our original procedure, except that the charcoal adsorption was omitted. The availability of these concentrates greatly facilitated our work and we wish to express our appreciation to Dr. Rhodehamel for this gift. An aliquot of a concentrate equivalent to 17 kilos of fresh liver was carried through the charcoal adsorption and elution, made to pH 9.5 with NaOH, and evaporated to dryness. The dry material was extracted with a total of 100 cc. of acetone. A determination of the solids showed that 0.8 gm. was left in the residue and 0.7 gm. was obtained in the extract. The activity of the acetone extract is shown in Fig. 1, Curve 11. It is evident from the response obtained and the equivalent amount fed that most of the activity was obtained in the acetone. This step gave no clear cut idea of the chemical properties of the compound but it did add another step which would remove about one-half of the total solids as inert material.

A considerable quantity of the liver extract concentrate was carried through the acetone extraction step. The final preparation gave no precipitate with alcoholic mercuric chloride or phosphotungstic acid. Several attempts were made to obtain crystals from different solvents without success. The material gave a negative test for sulfur and phosphorus. A nitrogen determination on the crude dried material showed about 10 per cent N.

While this work was in progress other studies in our laboratory (Frost and Elvehjem (2)) showed that nicotinic acid had some growth-stimulating effects in rats reared on certain purified diets. Upon comparison of the properties of nicotinic acid with those observed for the vitamin, we decided to test nicotinic acid itself

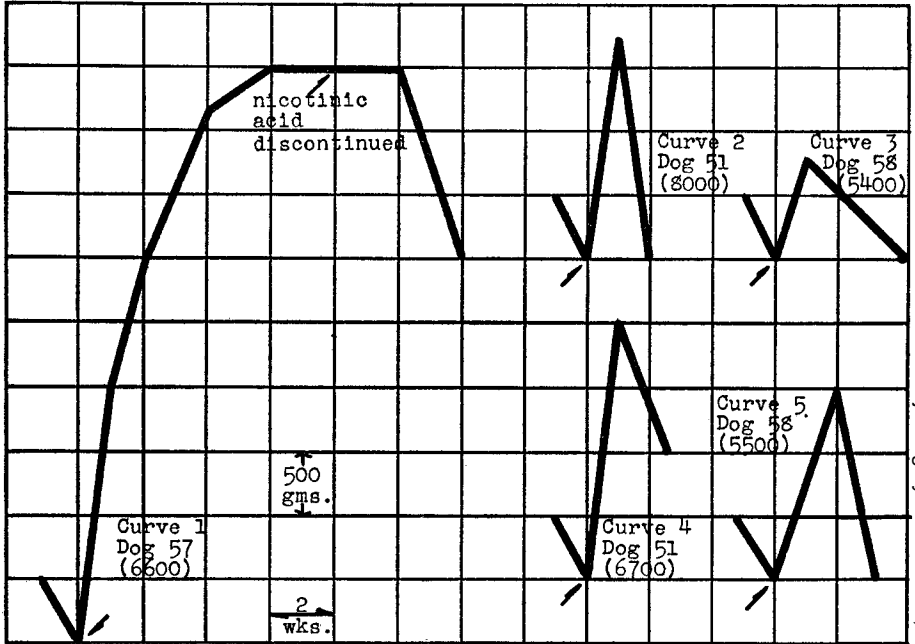


FIG. 2. Growth responses obtained in dogs maintained on basal black tongue diet when given nicotinic acid. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, 30 mg. of nicotinic acid every other day for 56 days. Curve 2, 40 mg. of synthetic nicotinic acid in one dose. Curve 3, 25 mg. of nicotinic acid in one dose. Curve 4, 60 mg. of nicotinic acid in three equal doses given on 3 successive days. Curve 5, 25 mg. of nicotinic acid in one dose.

on dogs. A dog showing all the symptoms of black tongue was given a single dose of 30 mg. of nicotinic acid (Eastman Kodak Company) and a phenomenal response was obtained. The appetite improved in a very short time, the mouth lesions disappeared in less than 2 days, and the growth response was very similar to that obtained with active concentrates. The responses ob-

tained in five different dogs are shown in Fig. 2. Dog 57 was kept on the basal diet plus 30 mg. of nicotinic acid every other day for 2 months, during which time the dog grew well and appeared normal in every way. Of the five dogs, four received commercial nicotinic acid, which is prepared by the oxidation of nicotine. Dog. 51 received nicotinic acid prepared from quinolinic acid merely to show that the commercial preparation did not carry impurities which might account for its activity.

The concentration of the vitamin from liver was continued through the aid of a molecular still. An aliquot of one of the

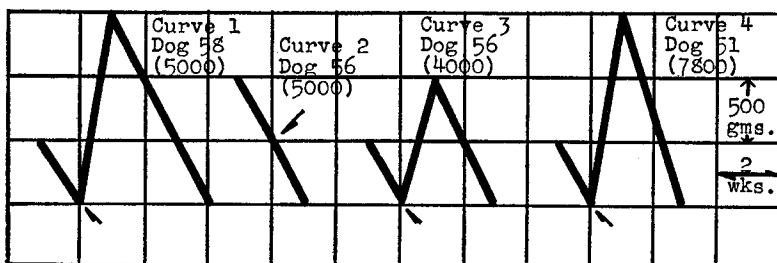


FIG. 3. Growth responses obtained in dogs maintained on basal black tongue diet when given nicotinic acid amide and the filtrate from which the amide had been removed. The arrow indicates the point of supplementation. The number in parentheses denotes the weight of the dog when the supplement was added. Curve 1, 108 mg. of a high vacuum distillate fed in one dose. 1 mg. \cong 20 gm. of fresh liver. Curve 2, 58 mg. of the filtrate after nicotinic acid amide had been removed fed in one dose. Curve 3, 30 mg. of nicotinic acid amide in one dose. Curve 4, 50 mg. of nicotinic acid amide isolated from liver fed in one dose.

concentrates obtained from Dr. Rhodehamel equivalent to $2\frac{1}{2}$ kilos of fresh liver was carried through the alkaline acetone extraction and 120 mg. of solids were obtained.

The entire material was placed in a molecular still, the condenser of which was cooled with solid CO_2 , and held at $160\text{--}165^\circ$ and approximately 0.0001 mm. pressure for 3 hours. At the end of this time nearly all of the material had distilled, yielding a pale yellow, sticky solid. The entire distillate (108 mg.) was fed to a dog showing typical symptoms and an immediate response was obtained, Fig. 3, Curve 1.

Another aliquot equivalent to 10 kilos of liver was carried

through the acetone extraction and 450 mg. of solids were obtained. The entire preparation was distilled under similar conditions and the distillate was dissolved in about 4 cc. of alcohol and treated with an excess of saturated alcoholic HgCl_2 . Almost immediately a white crystalline precipitate formed which was filtered off, washed, dissolved in dilute HCl , and decomposed with H_2S . When the resulting filtrate was concentrated to dryness, 175 mg. of long white needles were obtained. 50 mg. of these crystals when fed proved to be active, Fig. 3, Curve 4, while 58 mg. of the sirup resulting when the mercury was removed from the HgCl_2 filtrate proved to be inactive, Fig. 3, Curve 2. The remainder of the crystals was recrystallized from alcohol-benzene, and melted at $227\text{--}228^\circ$ (uncorrected). When mixed with nicotinic acid amide hydrochloride, the melting point was $227\text{--}228^\circ$.

$\text{C}_6\text{H}_5\text{ON}_2\cdot\text{HCl}$. Calculated. C 45.5, H 4.45, N 17.67
 Found.³ " 45.6, 45.9, H 4.9, 4.6, N 17.13, 16.97

The free base was prepared by removing HCl with Ag_2O from 16 mg. of the hydrochloride, and was crystallized from ethyl acetate. It melted at $126\text{--}127^\circ$, and when mixed with nicotinic acid amide, at $127\text{--}127.5^\circ$.

The chloroaurate was made by adding excess AuCl_3 to a solution of 16 mg. of the hydrochloride in dilute HCl , and recrystallizing the yellow plates which formed from dilute HCl . The melting point was 205° , and was not changed when nicotinic acid amide chloroaurate was mixed with it. The compound did not decompose when melted.

$\text{C}_6\text{H}_5\text{ON}_2\cdot\text{HAuCl}_4$. Calculated, Au 42.6; found, Au 42.4

It was observed that the concentrate before distillation did not yield precipitates with alcoholic HgCl_2 or with phosphotungstic acid, and for this reason it was at first thought that the nicotinic acid amide in the concentrate was not free, but that it perhaps was formed by decomposition during distillation of some more complex substance present in the concentrate. That this was not true, however, was shown by isolation of the amide from the concentrate before distillation. 25 mg. of the alkaline acetone ex-

³ We wish to thank Mr. H. A. Campbell and Dr. K. P. Link for performing these analyses.

tract were dissolved in about 3 cc. of alcohol acidified with HCl and to this solution an excess of alcoholic H_2PtCl_6 was added. The resulting precipitate was filtered off, washed with alcohol, dissolved in dilute HCl, and decomposed with H_2S . The filtrate from the PtS_2 was concentrated to dryness, whereupon 13 mg. of crystals, melting at 228° , were obtained. From these crystals a chloroaurate was prepared which melted at 205° . It is thus apparent that 40 per cent of the concentrate before distillation could be isolated as free nicotinic acid amide in the form of its hydrochloride.

Nicotinic acid amide prepared from ethyl nicotinate was also fed to a few dogs to see whether it had the same activity as the amide isolated from liver. The results are given in Fig. 3, Curves 3 and 4. From the results obtained it appears that the activity of the synthetic amide and isolated amide is very similar. Nicotinic acid has been injected intramuscularly in two cases with results very similar to those obtained through oral administration. It is impossible at present to give any figures for the exact ratio of activity of the same compound when administered by mouth or by injection. It is interesting to mention that one dog showed such severe symptoms of black tongue that we did not expect to save the animal; however, a single dose of nicotinic acid injected intramuscularly brought about great improvement and it continued to improve upon additional oral feeding.

DISCUSSION

The results presented in this paper demonstrate conclusively that nicotinic acid and nicotinic acid amide are active in the cure and prevention of canine black tongue and that the activity of liver in the treatment of this disease is undoubtedly due to its content of nicotinic acid amide. Whether the majority of the nicotinic acid amide occurs in liver as such or in a more complex form cannot be answered at present. In any case the entire activity of liver may be correlated with its potential supply of nicotinic acid amide. Although it has been possible to isolate but a small amount of the total nicotinic acid amide in liver, we can estimate the original amount in a general way. We have used for isolation work liver extract preparations made in different laboratories but all preparations have shown about equal potency

when fed in amounts equivalent to the same quantity of fresh liver. A single dose of liver extract equivalent to 200 gm. of fresh liver fed to a dog suffering from black tongue cures the symptoms and gives a continued growth response for about 1 week. A single dose of 50 mg. of nicotinic acid amide gives a very similar response. If there has been no appreciable loss during the preparation of liver extract from liver, we may conclude that 100 gm. of fresh liver contain about 25 mg. of potential nicotinic acid amide.

In our final isolation 175 mg. of nicotinic acid amide crystals were isolated from liver extract equivalent to 10 kilos of fresh liver, which would contain about 2500 mg. of the amide. This is a recovery of only 5 per cent of the original material. The assays indicate that about 35 per cent of the potency was recovered in the concentrates carried up to the charcoal stage and that the recovery was reduced to 5 per cent after the acetone extraction step.

It is also possible from the few results given in this paper to estimate the nicotinic acid requirement of dogs. Since Dog 57 grew from 7 to 11 kilos in 5 weeks when receiving 30 mg. every other day, the intake would be about 1.5 mg. per kilo per day. The dogs weighing about 5 kilos grew for a little over a week when given one dose of 25 mg. and those weighing 8 to 9 kilos grew for about the same period when given 50 mg. of nicotinic acid. The requirement under the conditions in which we have worked may be tentatively set as 0.5 to 1.5 mg. per kilo per day.

Although there are no figures available for the amount of nicotinic acid in various foodstuffs, this compound and its derivatives are evidently rather widely distributed in nature. Nicotinic acid was first isolated from naturally occurring materials in 1912 by Suzuki, Shimamura, and Otake (3). Trigonelline, the methyl betaine of nicotinic acid, was isolated as early as 1885 by Jahns (4). In 1913 Funk (5) isolated nicotinic acid from the vitamin fraction of rice polishings. The activity of nicotinic acid in polyneuritis was very variable, but it was thought that it might be a decomposition product of the vitamin curing polyneuritis, or that some closely related compounds might show greater activity. These possibilities were also investigated by Williams (6) but

again variable and indeterminate results were obtained. Except for a paper by Szymańska and Funk (7), who attributed a food-sparing and weight-preserving action to nicotinic acid and the amide, very little interest was shown in the possible rôle of pyridine derivatives in living systems until the work of Warburg and Christian in 1935. They (8) characterized nicotinic acid amide as one of the hydrolysis products from the coenzyme which they had isolated from red corpuscles of horse blood. Kuhn and Vetter (9) isolated nicotinic acid amide from heart muscle and von Euler, Albers, and Schlenk (10) isolated the amide of nicotinic acid from cozymase.

This work gave new impetus to the application of these findings in the field of nutrition. Von Euler and Malmberg (11), using a ration very similar to the Sherman-Bourquin diet supplemented with a vitamin B₁ concentrate and flavin, found no growth response with nicotinic acid or nicotinic acid amide at a level of 1 mg. daily. However, the rats receiving the nicotinic acid lived longer than the controls. Funk and Funk (12) have reported recently that rats and pigeons on certain rations showed a larger food intake and better growth when given nicotinic acid and especially nicotinic acid amide. Frost and Elvehjem (2) obtained a very definite growth response in rats on purified rations through the addition of adenine nucleotides and nicotinic acid. It is also interesting to note that nicotinic acid and its derivatives play a part in the nutrition of lower organisms (Lwoff and Lwoff (13), Knight (14), and Mueller (15)). Thus nicotinic acid seems to have important functions in various types of organisms.

It is impossible to conclude definitely from the activity of nicotinic acid in canine black tongue that it will prove useful in the treatment of human pellagra. However, Spies has used nicotinic acid in four cases of classical pellagra and reports (personal communication) that the fiery red color associated with pellagrous dermatitis, stomatitis, and vaginitis improved promptly.

The use of nicotinic acid in the treatment of human pellagra brings up the possible toxicity of this compound. His in 1887 (16), Cohn in 1893 (17), and Abderhalden, Brahm, and Schittenhelm in 1909 (18) found that dogs fed 1 gm. daily of pyridine hydrochloride or pyridine acetate excreted equivalent amounts of methyl pyridinium hydroxide. The similar ability of humans to

methylate pyridine is indicated from the work of Kutscher and Lohmann (19), who isolated small amounts of methyl pyridinium hydroxide from the urine of humans. The complete failure of rabbits to detoxicate pyridine was shown in the same work.

Cohn (17) isolated α -pyridinuric acid, the dipeptide of picolinic acid and glycine, from the urine of rabbits injected with α -picoline. Thus rabbits, lacking the ability to methylate pyridine compounds, are able to oxidize α -picoline to picolinic acid. The conjugation of picolinic acid with glycine is analogous to the synthesis of hippuric acid from benzoic acid and glycine, a well known detoxication process in many vertebrates. In 1912 Ackermann (20) presented evidence that a dog fed large amounts of nicotinic acid (9 gm. of nicotinic acid as sodium nicotinate over a 5 day period) excreted about equal amounts of trigonelline and nicotinuric acid, the dipeptide of nicotinic acid and glycine. No change in the condition of the dog was observed during the administration of these large amounts of nicotinic acid along with a normal ration. Ackermann performed the experiment with only one dog which he described as middle-sized. Upon duplication of Ackermann's work in our laboratory⁴ with a small dog (4.6 kilos) definite toxicity was observed after 5 days of nicotinic acid administration (2 gm. per day). The dog failed to eat and showed spasmodic vomiting. Upon discontinuing the nicotinic acid, the dog returned to normal.

In 1926 Tomita, Komori, and Sendju (21) fed nicotinuric acid prepared according to the method of Ackermann to polyneuritic pigeons without effect. However, they reported that 0.5 gm. of sodium nicotinate by injection, or 1 gm. by stomach tube, proved toxic to pigeons. Thus the ability of dogs to handle large amounts of nicotinic acid is undoubtedly dependent upon their ability to conjugate at least part of that absorbed with glycine. Animals that are not able to bring about this conjugation so readily will probably show greater toxicity to nicotinic acid. However, since humans are undoubtedly similar to dogs in this respect, no difficulty should be encountered from the use of reasonable amounts of nicotinic acid in humans.

⁴ We are indebted to D. V. Frost for the availability of these data.

SUMMARY

1. The factor necessary for the cure and prevention of black tongue in dogs produced on a modified Goldberger diet has been isolated from liver and identified as nicotinic acid amide.

2. Both nicotinic acid and nicotinic acid amide are effective in curing black tongue in dogs and in maintaining dogs in a normal condition on the basal black tongue-producing diet.

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