benzyl groups were removed by the action of anhydrous hydrogen bromide in glacial acetic acid at 2° for 65 hours.[13] Copoly-(L-glutamyl, L-cyclohexylalanyl) gelatin was precipitated with ether, dissolved in water at *p*H 7 (1 *N* sodium hydroxide was added for the neutralization), dialyzed against distilled water for 3 days, and lyophilized.

In order to determine the content of glutamic acid and of cyclohexylalanine the gelatin derivative described was hydrolyzed with 12 *N* hydrochloric acid at 105° for 48 hours. The hydrolysate was subjected to paper electrophoresis on Whatman No. 1 filter paper in a phthalate buffer, 0.025, *M* *p*H 5.92, at a potential gradient of 10 volts/cm., at 25°, for 2 hours. It was then chromatographed in the second dimension in *n*-butyl alcohol–glacial acetic acid–water (50:12:50 v./v.) for 48 hours. Glutamic acid and cyclohexylalanine were quantitated by ninhydrin colorimetry.[22] The gelatin derivative described contained 19.3% glutamic acid residues and 18.3% cyclohexylalanine residues. Taking into consideration that the original gelatin contained 10.1% glutamic acid residues (Eastoe[25] reports 10.2% glutamic acid residues for bone gelatin), this corresponds to an attachment of 114 moles of glutamic acid and 192 moles of cyclohexylalanine per 100,000 g. of gelatin.

Copoly-(L-glutamyl, L-cyclohexylalanyl) gelatin was dinitrophenylated and analyzed for dinitrophenylamino acids, similarly to polycyclohexylalanyl gelatins. Dinitrophenyl-L-glutamic acid accounted for 10% of the amino groups, dinitrophenyl-L-cyclohexylalanine for 43%, and ε,N-dinitrophenyl-L-lysine for 47%.

Polycyclohexylalanyl Egg Albumin.—N-Carboxy-L-cyclohexylalanine anhydride (III) (0.2 mg. in 20 ml. dry dioxane) was treated with egg albumin (1 g.) in aqueous solution buffered at *p*H 7 with 0.05 *M* phosphate (40 ml.) at 5°, and treated as described for polycyclohexylalanyl gelatin. The lyophilized final product is soluble in water. The material contained 9.7% cyclohexylalanine residues. Dinitrophenylation analysis showed that only 35% of the amino groups of the original protein served as initiators in the polymerization reaction (*i.e.*, dinitrophenyl-L-cyclohexylalanine accounted for 35% of the total dinitrophenylamino acids).

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[CONTRIBUTION FROM THE CHEMISTRY AND BIOLOGY DEPARTMENTS, BROOKHAVEN NATIONAL LABORATORY AND THE BOTANY DEPARTMENT, COLUMBIA UNIVERSITY]

The Biosynthesis of Nicotine from Isotopically Labeled Nicotinic Acids[1]

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The four specific ring hydrogen-labeled nicotinic acids have been prepared and fed to tobacco root cultures in sterile media, then the nicotine produced by the roots has been isolated and analyzed. Recoil tritium and carbon-14 labeled nicotinic acid have been similarly employed. The nicotine from all of these except nicotinic acid-6-*t* has shown similar and substantial incorporation into the nicotine. Oxidation of nicotinic acid, obtained from the nicotine, to the corresponding 2- and 6-pyridones has indicated that the position of hydrogen label is conserved during the conversion to nicotine. The 6-labeled acid gave less than 10% of the amount of incorporation shown by the other acids, indicating the probability of enzymatic attack on the 6-position of nicotinic acid during its conversion to nicotine by the tobacco roots. The conversion probably does not proceed *via* oxidation at the 6-position, since both 6-hydroxynicotinic acid-N^{15} and 1-methyl-6-oxynicotinamide-2-*t* failed to be incorporated. The possibility that the acid is incorporated into nicotine *via* a 1,6-dihydro intermediate is being investigated. Nicotinamide is incorporated to at least as great an extent as is the corresponding labeled acid.

Nicotinic acid was proposed as a likely precursor of nicotine in the tobacco plant by Trier[2] almost thirty years ago. At that time, there had been one reported isolation of nicotinic acid from a plant source.[3] However, the prevailing concepts of biogenesis emphasized structural relationships between natural products and those plant constituents not only of simpler nature but also of relatively widespread occurrence. Trier was led, therefore, to propose a derivation of nicotinic acid from proline (pyrrolidine-2-carboxylic acid). In his Weizmann lectures, Robinson[4] accepted the plausibility of the latter suggestion on chemical grounds, but he raised strong objections to the proposed relation between nicotinic acid and nicotine. These objections involved (a) the inertness of the 3 position of pyridine and (b) the absence of a recorded case of displacement of the nicotinic acid carboxyl group.

Following its introduction, Trier's hypothesis was subjected to repeated tests.[5] Owing to a necessary dependence upon quantitative analytical procedures and to the absence of suitable experimental systems, however, these tests yielded inconclusive results. Recently, the development of techniques for the use of isotopic tracers and for the study of nicotine production by sterile cultures of isolated tobacco roots[6] have made possible the reexamination of several hypotheses[7] including that of Trier.

The present paper offers evidence that nicotinic acid can function as a precursor of the pyridine ring of nicotine. Some chemical details of the transformation are described which eventually may afford an answer to the objections raised by Robinson.[4] Leete and others[8] have shown that proline can act as a precursor of the pyrrolidine, but not of the pyridine, ring of nicotine. We have confirmed this finding. Since, in his scheme, Trier had also

(1) Research performed under the auspices of the U. S. Atomic Energy Commission at Brookhaven and under contract No. AT (30-1)-1778 at Columbia. A grant from the Rockefeller Foundation aided the initial stages of the work.

(2) E. Winterstein and G. Trier, "Die Alkaloide," 2nd Ed., Borntraeger, Berlin, 1931, p. 1031.

(3) U. Suzuki, T. Shimamura and S. Odake, *Biochem. Z.*, **43**, 89 (1912).

(4) R. Robinson, "Structural Relations of Natural Products," Clarendon Press, Oxford, 1955, pp. 67–71.

(5) G. Klein and H. Linser, *Planta*, **20**, 470 (1933); A. Gorter, *K. akad. Wetensch. Amsterdam. Proc. Sec. Sci.*, **39**, 87 (1936); R. Dawson, *Plant Physiol.*, **14**, 479 (1939). *Cf.* R. Dawson, *Ann. Rev. Biochem.*, **17**, 541 (1948).

(6) P. White, *Am. J. Bot.*, **25**, 348 (1938); R. Dawson, *ibid.*, **29**, 813 (1942); M. L. Solt, *Plant Physiol.*, **32**, 480 (1957).

(7) A. A. Bothner-By, R. F. Dawson and D. R. Christman, *Experientia*, **12**, 151 (1956).

(8) (a) E. Leete, THIS JOURNAL, **80**, 2162 (1958); (b) E. Leete, *Chem. and Ind.*, 537 (1955); (c) L. J. Dewey, R. V. Byerrum and C. D. Ball, *Biochim. Biophys. Acta*, **18**, 141 (1955).

TABLE I

INCORPORATION INTO NICOTINE OF NICOTINIC ACID LABELED BY NUCLEAR RECOIL

Isotope	Acid sp. act., d.p.m./mg. (H or C)	Acid concn. in culture fluid, mg./ml.	Duration feeding period, days	Yield nicotine picrate, mg.	Nicotine picrate sp. act., d.p.m./mg. H or C	Sp. act. ratio, nicotine/acid	Radiochem. yield
Tritium	5.34×10^5	0.01	22	24.5	1.28×10^5	0.10	0.06
	5.34×10^6	.01	22	30.8	1.28×10^5	.10	.08
	5.34×10^6	.01	29	55.1	1.02×10^5	.08	.10
	5.34×10^6	.01	29	57.7	8.96×10^4	.07	.08
	7.26×10^6	.02	28	148	1.83×10^5	.10	.10
	7.26×10^6	.02	28	127	1.56×10^5	.09	.08
	7.26×10^6	.02	35	80.8	1.71×10^5	.09	.06
	7.26×10^6	.02	35	80.2	2.52×10^5	.14	.09
Carbon-14	1.95×10^3	.03	32	69.6	59	.13	.09
	1.91×10^3	..	36	91	47	.11	..

derived the pyrrolidine ring of nicotine from proline, present information offers a remarkable validation of most of Trier's speculations. Preliminary accounts of this work have been published elsewhere.[9,10]

Results

For exploratory purposes, nicotinic acid was ring-labeled with carbon-14 and with tritium, respectively, by nuclear recoil methods.[11] Acids so prepared are considered to be randomly labeled for the purposes of this paper, although the actual degree of randomization of the activity remains to be established. When a suitable degree of radiochemical purity was achieved, such preparations gave consistent results. Activity appeared in the nicotine in every instance where one of these labeled acids was supplied to the tobacco root cultures (Table I). Whether expressed as radiochemical yields or as ratios of specific activities (of pyridine ring hydrogens in the case of tritium labeling), the extent of incorporation of label was substantial and of the same order of magnitude in both cases.

The tritium-labeled nicotine prepared by feeding recoil tritium-labeled nicotinic acid was oxidized with neutral permanganate to nicotinic acid and the product assayed. The result, 4.74×10^6 d.p.m./mg. H, corresponds to 98% of the calculated specific activity of the nicotine, 4.84×10^6 d.p.m./mg. H as nicotinic acid, assuming that all of the tritium occurred in the pyridine ring of the alkaloid.

Similar results were obtained by supplying specifically labeled nicotinic acid-2-t to the root cultures. Nicotine produced by these cultures contained tritium in amounts comparable with those obtained when the recoil labeled acid was used (Table II). The nicotine from several such experiments was pooled and oxidized first with neutral permanganate to nicotinic acid and subsequently with potassium ferricyanide to a mixture of the 2- and 6-pyridones of 1-methylnicotinamide. Separation of the two pyridones was accomplished on an alumina column and was followed by taking the ultraviolet absorption spectra of successive collections of eluate. The specific activity of the 2-pyridone (311 d.p.m./mg. H) was negligible compared to that of the 6-pyridone (4.68×10^4 d.p.m./mg. H). The activity of the 2-pyridone is probably due to the presence of positional isomers of 2-bromopicoline in the starting material.

TABLE II

INCORPORATION OF SPECIFICALLY LABELED NICOTINIC ACIDS INTO NICOTINE[a]

Acid supplied	No. of exp.	Mean isotope yield
Nicotinic acid-2-*t*	6	0.113 ± 0.0115[b]
Nicotinic acid-4-*d*	2	$.130 \pm .000$
Nicotinic acid-5-*t*	4	$.142 \pm .017$
Nicotinic acid-6-*t*	4	$.011 \pm .0035$
Nicotinic acid-*t*[c]	8	$.080 \pm .014$

[a] Conditions: nicotinic acid concentration in culture fluid below 0.032 mg./ml.; duration of feeding period more than 20 days. [b] Standard deviation. [c] Nuclear recoil labeling. Isotope yield is a mean of values listed in Table I.

Recoveries of isotopes of hydrogen in nicotine obtained by feeding other specifically hydrogen-isotope-labeled nicotinic acids are given in Table II. The figures for incorporation of 4-deuterium and 5-tritium nicotinic acids are similar to those already described for the 2-tritium acid. However, the incorporation of nicotinic acid-6-*t* was only about one-tenth of the above amount. The recovery of label from the 6-tritium acid was not affected by the strength of base used in isolating the nicotine. Further, nicotinic acid of the same specific activity (188,000 d.p.m./mg.) as that initially supplied (186,200 d.p.m./mg.) was isolated from the root cultures after four weeks of incubation. It seems likely, therefore, that loss of tritium from the 6 position in the conversion of nicotinic acid to nicotine occurs as a specific step in this conversion and not merely as an exchange reaction depending upon an unusual lability of the 6-hydrogen of nicotinic acid in the experimental system or of nicotine in the process of isolation.

Since tritium in the 6 position of nicotinic acid is lost during conversion of the latter to nicotine, the recovery of isotope from recoil tritium-labeled nicotinic acid may be corrected by multiplying by the fraction 4/3, to a first approximation. The mean radiochemical yield (0.080) then becomes 0.108. The latter figure is virtually identical with the mean figure for recovery of label in the experiments utilizing nicotinic acid-2-*t* (Table II).

(9) R. F. Dawson, D. R. Christman, R. C. Anderson, M. L. Solt, A. F. D'Adamo and U. Weiss, THIS JOURNAL, **78**, 2645 (1956).

(10) R. F. Dawson, D. R. Christman, A. F. D'Adamo, M. L. Solt and A. P. Wolf, *Chem. & Ind. (London)*, 100 (1958).

(11) (a) A. P. Wolf and R. C. Anderson, THIS JOURNAL, **77**, 1609 (1955); (b) R. C. Anderson, E. Penna-Franca and A. P. Wolf, Brookhaven National Laboratory Quarterly Progress Report, October 1–December 31, 1954; (c) R. Wolfgang, F. S. Rowland and C. N. Turton, *Science*, **121**, 715 (1955).

Likewise, the figures for isotope yield and ratio of specific activities in experiments involving recoil C^{14}-labeled nicotinic acid may be corrected for loss of the carboxyl carbon. Thus, by multiplying the figures in lines 10 and 11 of Table I by the fraction 6/5, a radiochemical yield of 0.11 and specific activity ratios of 0.16 and 0.13, respectively, are obtained.

The amounts of nicotinic acid supplied to the cultures in these experiments ranged from 1 to 3 or more times the calculated requirement for nicotine production. Since not more than 16% of the acid supplied was actually used for nicotine production in any one instance, it is apparent that there existed a potential excess of precursor at all times. It is remarkable, therefore, that no increase in nicotine yield has been detected in any experiment where nicotinic acid was supplied to the root cultures.

The relations between isotope recovery and concentration of nicotinic acid in the culture medium are of interest. Below 0.032 mg. of acid per ml., radiochemical yields were independent of nicotinic acid concentration in all cases. The ratio of specific activities of pyridine ring hydrogen, however, were linearly related to acid concentration. The proportionality constant relating concentration of acid in mg. per ml. of culture medium to ratio of specific activities in the experiments utilizing nicotinic acid-2-t was 6.91. The standard deviation of observed from calculated ratios in the 7 instances recorded was 0.030. In four instances involving nicotinic acid-5-t, the proportionality constant was 6.94 and the standard deviation of observed from calculated ratios was 0.013. In both cases, the proportionality constants are the slopes of lines of least squares, passing through the origin, and standard deviations are the indices of variation of actual data around these lines.

Nicotinamide labeled with tritium by the nuclear recoil method was also supplied to root cultures. Both radiochemical yield and ratio of specific activities (Table III) were higher than those obtained by feeding the free acid, similarly labeled, to separate cultures of the same passage. However, none of the figures was higher than the mean values given in Tables I and II. Similar experiments with 6-hydroxynicotinic acid-N^{15} and with the 6-pyridone of 1-methylnicotinamide-2-t indicated no appreciable transfer of label from these compounds to nicotine.

TABLE III

TRANSFER OF LABEL FROM VARIOUS PYRIDINE DERIVATIVES TO NICOTINE

Compound supplied	Concn. in culture fluid, mg./ml.	Isotope yield
Nicotinamide-t^a	0.011	0.13
		.12
6-Hydroxynicotinic acid-N^{15}	.034	.000
6-Oxo-1-methylnicotinamide-2-t	.018	.001

[a] Nuclear recoil labeling.

Discussion

The evidence presented herein supports the Trier hypothesis of nicotine biogenesis by showing that nicotinic acid, ring-labeled with either C^{14} or H^3, can be utilized by the tobacco root for nicotine production. Although the introduction of H^3 into nicotine from recoil tritium-labeled nicotinic acid might be interpreted as a result of isotope exchange, all evidence indicates a transfer of molecular dimensions. For example, there may be cited the virtual identity of radiochemical yields (or of ratios of specific activities) obtained in experiments where recoil tritium-labeled and recoil C^{14}-labeled nicotinic acids were supplied to the root cultures. Furthermore, the magnitude of incorporation of label between the pyridine rings of nicotinic acid and nicotine would appear very unlikely not only in the case of C^{14} but also in the case of tritium. Hydrogen atoms attached to the pyridine ring are reasonably stable even to mineral acids.

The over-all nature of the utilization of nicotinic acid in nicotine biosynthesis has also been substantially clarified in these and related experiments. Earlier, we have shown that the carboxyl carbon of the acid is not incorporated into nicotine.[12] Jeffrey and Tso[13] have reported that the nitrogen atom is retained and the present paper demonstrates retention of hydrogen atoms in positions 2, 4 and 5. However, the hydrogen atom in position 6 is apparently lost. It is altogether likely that the carbon atoms in positions 2, 4 and 5 are retained, since to postulate otherwise would require virtually quantitative removal from and restoration to these positions of the hydrogen isotopes as used in our experiments. It is equally probable that the carbon atom in position 3 is retained owing to the complex nature of bonding at that site. Whether the carbon atom in position 6 is conserved cannot be positively determined from present evidence; the probability certainly favors retention. A complete accounting of the immediate origins of the atoms of the pyridine ring of nicotine is thus near at hand.

Some light is thrown upon mechanism. Clearly, nicotinic acid is not converted to nicotine *via* a symmetrical intermediate, for, with such an intermediate, the retention of label in position 5 would be approximately one-half that in position 2 or 4 in the absence of an isotope effect during nicotine formation. In any case, it would be appreciably less. Furthermore, the retention of label in nicotine would likely be the same whether tritium were contained in position 2 or 6 of the acid. Robinson speculated that the 3-substitution of pyridine in nicotine "indicates the site of an active position in a reduced pyridine precursor." It is now quite clear that this reduced pyridine precursor cannot be derived from lysine.[7] It is probable, however, that nicotinic acid may be reduced at the 6 position and subsequently reoxidized with loss of the original 6-hydrogen at one stage or the other. Two intermediates would seem most likely within the framework of contemporary biochemical knowledge. One would be a 6-pyridone.[14] The other would be a 1,6-dihydro derivative analogous, perhaps, to the

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(13) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, **80**, 46 (1959).

(14) W. E. Knox and W. I. Grossman, *J. Biol. Chem.*, **166**, 391 (1946); *idem.*, THIS JOURNAL, **70**, 2172 (1948); W. E. Knox, *J. Biol. Chem.*, **163**, 699 (1946).

1,4-dihydro pyridinium derivative of the naturally occurring coenzymes.[15] The first possibility is rendered unlikely by the failure of label transfer from 6-hydroxy nicotinic acid to nicotine, the second remains to be examined.

Experimental

Tobacco Root Cultures.—Excised roots of one clone of *Nicotiana tabacum L., var.* Turkish, were subcultured through repeated passages in 125 ml. Erlenmeyer flasks containing 30 ml. each of White's medium modified by additions of 0.01 p.p.m. of copper and 0.003 p.p.m. of molybdenum. Yeast extract (DIFCO) was added to the culture medium in the amount of 25 mg./l. The standard inoculum was a single lateral root with from 1 to 3 growing tips. Incubation temperature was 29 ± 0.5°. The cultures were grown for one week prior to addition of labeled compounds.

Addition of Labeled Compounds to Cultures.—The labeled compounds were dissolved in water and sterilized by autoclaving or by filtration through fritted glass disks (Pyrex UF). The sterilized solutions were pipetted aseptically into the culture flasks. All microbially contaminated cultures were discarded. The period of feeding was usually 4 weeks.

Prior to experimenting with a given labeled compound, the appropriate concentration range for use with root cultures was determined with nonlabeled material. Concentrations which reduced growth rate (in terms of dry weight increase) were consequently avoided.

Isolation of Nicotine.—The cultures were harvested by separating root masses from spent culture fluids on a Büchner funnel. The roots were ground with sharp sand in the presence of a little trichloroacetic acid and subsequently twice extracted with boiling water. The filtered aqueous root extracts were combined with the spent culture fluids, and the total volume reduced *in vacuo*. After making alkaline with NaOH or MgO, these solutions were continuously extracted for 48 hr. with ether. The ethereal extracts were twice shaken out with 10 ml. of 1 *N* HCl. The acid extracts were steam distilled from excess MgO or NaOH into aqueous picric acid solution. The distillates were taken to dryness on a rotating evaporator under reduced pressure. Sufficient boiling water was added to dissolve the picrates, care being taken to avoid an excess. After cooling, the crystals were filtered, washed with methanol and ether dried.

Oxidation of Nicotine to Nicotinic Acid.—The nicotine picrates were dissolved in warm 2.5 *N* hydrochloric acid and shaken out with ether until colorless. The resulting solutions were neutralized and treated with small portions of potassium permanganate with stirring until the solution no longer decolorized. After refluxing 4 hr., the solution was treated with hydrogen peroxide to destroy excess permanganate and then with sufficient concentrated hydrochloric acid to dissolve the precipitated manganese dioxide. The acid solution was extracted continuously with ether. The *p*H of the aqueous layer was then adjusted to 3.0. If care was used, little manganese dioxide precipitated at this point. Ether extraction was then continued for 3 days. The ether was removed and the nicotinic acid was purified by sublimation and finally by recrystallization from ethanol.[16]

Preparation of the Pyridones.—Nicotinic acid was converted to the corresponding 1-methylnicotinamide and then oxidized with ferricyanide to a mixture of the 2- and 6-pyridones of 1-methylnicotinamide.[17] The components were separated as follows. After exactly 30 min. in contact with the oxidizing agent, the reaction mixture was passed through a bed containing 45 ml. of Dowex-1 (OH form) and 15 ml. of Dowex 50 (H form). The effluent together with 500 ml. of wash water was concentrated to dryness in a small r.b. flask under reduced pressure. The residue was dissolved in 2 ml. of hot methanol. Benzene and chloroform were added to a final ratio of benzene 4, chloroform 4 and methanol 1 part. Using the same solvent, the solution of pyridones was passed through an alumina column (80–200 mesh, column dimensions, 2.5 × 30 cm.). Fractions were collected in 10 ml. portions and each was evaporated to dryness on a water-bath using an airjet. The center cuts were monitored on a Beckman spectrophotometer and the obvious mixtures as well as the first pure cuts on each side of the center cut were discarded. Appropriate combinations of eluates were made and the solutions were evaporated to dryness. The residues were dissolved in hot methanol and crystallized at −10°. The supernatant fluids were removed and the solids were crystallized a second time from methanol. The over-all procedure was suggested to us by Professor Fausto Ramirez.

Isotope Analysis.—Samples of the alkaloid picrates, the various labeled nicotinic acids and the degradation products were assayed for carbon-14 and for tritium by combustion and proportional gas counting.[18] For deuterium analysis, the alkaloid was isolated as the binoxalate. The method of deuterium analysis is described in a succeeding section. For N^{15} assay, nicotine was converted to the zinc chloride double salt and the nitrogen collected from the combustion of this salt was analyzed mass spectrometrically.

Results are expressed in two ways. Radiochemical (or isotope) yield is the ratio of total isotope recovered in nicotine to total supplied to the cultures and represents the proportion of labeled compound supplied to the cultures which was converted to nicotine. The ratio of specific activities of pyridine ring hydrogen of nicotine to nicotinic acid represents the proportion of nicotine that arose from labeled acid during the culture period.

Nicotinic Acid-C^{14}.[11b]—Nicotinamide was irradiated in the Brookhaven reactor at about 30° for two weeks at a flux of about 1.8×10^{12} neutrons/cm.^{2}/sec. The amide was dissolved in ethanol, treated with charcoal, filtered, benzamide carrier added and the ethanol removed under vacuum. The mixed amides were hydrolyzed by refluxing with 10% NaOH. The solution was taken to a *p*H of <1 and continuously extracted with ether to remove benzoic acid; then the nicotinic acid was similarly extracted at a *p*H of 3. The nicotinic acid at this point had a specific activity of 4,700 d.p.m./mg. C. Then 3.8 g. of this material was recrystallized from water, dissolved again in ethanol, charcoal treated, filtered and allowed to crystallize to give 1.8 g. of acid with a specific activity of 2375 d.p.m./mg. C. After two more crystallizations from ethanol, the acid was esterified with diazomethane, while suspended in ether, and the ether was removed to leave an oil which was refluxed for 2 hr. with conc. ammonium hydroxide. The aqueous portion was removed under vacuum and the residue refluxed with 4 *N* sodium hydroxide for 2 hr. The solution was continuously extracted with ether for 4 hr., then made strongly acid and similarly extracted for 4 hr. Extraction at a *p*H of 3 gave 400 mg. of nicotinic acid which was sublimed under vacuum and recrystallized from ethanol and which had a specific activity of 2000 d.p.m./mg. C. This was again crystallized from water to give material showing an analysis of 1951 d.p.m./mg. C. for the second feeding.

Nicotinic Acid-d_4.—A typical preparation of this material involved the intimate mixing of 20.1 g. of nicotinic acid with 1.11 g. of lithium carbonate by grinding in a mortar, then the irradiation of the mixture in the Brookhaven reactor at 30° for one day by 1.8×10^{12} neutrons/cm.^{2}/sec.[19] The brown material was submitted to vacuum sublimation and to recrystallization from 100 ml. of water to give 13.5 g. of acid with a specific activity of 6.69×10^6 d.p.m./mg. H. Recrystallization from ethanol gave acid with an activity of 7.20×10^6 d.p.m./mg. H. This material was used for some feeding experiments.[9] However, 11.1 g. of the material was esterified by refluxing with 80 ml. of methanol and 12 ml. of conc. sulfuric acid. The solution was concentrated somewhat under vacuum and slowly made basic with solid sodium carbonate. Continuous extraction with ether, drying with Drierite, removal of the ether under vacuum, dissolving the oil in 50 ml. of hexane and 2 ml. of methanol (hot) and cooling overnight in a refrigerator gave 6.9 g. of methyl nicotinate, m.p. 36–38°; specific activity 4.21×10^6 d.p.m./mg. H. About 6.5 g. of the ester was refluxed 3 hr. with 60 ml. of conc. ammonium hydroxide, taken to dryness under vacuum and crystallized from benzene twice to

(15) A. San Pietro, N. O. Kaplan and S. P. Colowick, *J. Biol. Chem.*, **212**, 941 (1955).

(16) For details of this procedure we are indebted to the late W. G. Frankenburg, formerly Director of Research of the General Cigar Company.

(17) M. E. Pullman and S. P. Colowick, *J. Biol. Chem.*, **206**, 121 (1954).

(18) (a) D. R. Christman, N. E. Day, P. R. Hansell and R. C. Anderson, *Anal. Chem.*, **27**, 1935 (1955); (b) D. R. Christman and A. P. Wolf, *ibid.*, **27**, 1939 (1955); (c) K. E. Wilzbach, L. Kaplan and W. G. Brown, *Science*, **118**, 522 (1953); (d) D. R. Christman, *Chemist Analyst*, **46**, 5 (1957).

(19) F. S. Rowland and R. Wolfgang, *Nucleonics*, **14**, #8, 58 (1956).

give 2.7 g. of nicotinamide, m.p. 117–121°; specific activity 5.65×10^6 d.p.m./mg. H. About 2.5 g. of the amide was hydrolyzed with 50 ml. of 20% sodium hydroxide by refluxing for 15 hr.; then the basic solution was continuously extracted with ether for 4 hr. After acidifying with 50 ml. of 6 *N* hydrochloric acid, it was extracted overnight. The solution was extracted at *p*H 3 for three days with ether, the ether removed and the residue crystallized from water to give nicotinic acid with a specific activity of 7.16×10^6 d.p.m./mg. H. Most of the root feedings were done with this acid and with the amide immediately preceding it in the purification scheme. It should also be noted that in this case about 19.4% of the tritons produced in the reactor were present in the 13.5 g. of acid after the first purification steps.

Nicotinic Acid-2-*t*.—2-Bromo-3-picoline was prepared by diazotizing 2-amino-3-picoline in the presence of hydrobromic acid, b.p. 218–219°. A solution of 12.6 g. of 2-bromo-3-picoline in 50 ml. of ether was dried for 3 hr. over anhydrous magnesium sulfate. It was then added slowly to 0.09 mole of butyl lithium in 200 ml. of ether at −18°. The reaction mixture was stirred for 7 min. The solution was treated with 1 ml. of tritium oxide dissolved in 1 ml. of ethanol and then allowed to come to room temperature. Bases were extracted with 3 *N* hydrochloric acid. The acid extract was extracted continuously with ether for 12 hr. After adjustment to *p*H 8.5, ether extraction was renewed for 24 hr. Finally, the ether from the last extraction was removed by distillation and the residue fractionally distilled. A fraction boiling at 140–143° was collected (3-picoline-2-*t*).

3-Picoline-2-*t* (3.2 g.) was oxidized with excess neutral potassium permanganate at 90° for 4 hr. The oxidation mixture was cooled, decolorized with hydrogen peroxide and acidified with hydrochloric acid until all precipitated manganese dioxide was redissolved. The resulting solution was extracted continuously with ether for 24 hr. Acidity was then adjusted to *p*H 3.2 and extraction with ether resumed for another 24 hr. The last ethereal extract was taken to dryness and the residue sublimed at 3 mm., m.p. 230–235° (subl.). The product was recrystallized twice from ethanol.

Anal. Found: 36.45×10^6 d.p.m./mg. H.

This preparation was diluted with inactive nicotinic acid for experimentation.

Anal. Found: 6.34×10^6 d.p.m./mg. H.

The acid was diluted with carrier and converted to the corresponding 1-methylnicotinamide iodide. *Anal.* Found: 0.142×10^6 d.p.m./mg. H. The 2-pyridone prepared by ferricyanide oxidation of the iodide was assayed. *Anal.* Calcd: 0.00 mμc./mg. H. Found: 0.00872×10^6 d.p.m./mg. H. (5.5% of specificity activity of the iodide when the latter is multiplied by 9/8 to compensate for loss of 1 H in pyridone). The 6-pyridone was also assayed. *Anal.* Calcd: 0.160×10^6 d.p.m./mg. H. Found: 0.1704×10^6 d.p.m./mg. H. (107% of adjusted specific activity of iodide).

Nicotinic acid-2-*t* was also prepared by decarboxylating tritiated quinolinic acid. Quinolinic acid (1.0 g.) was refluxed for 3 hr. with 1 ml. of tritium oxide. The mixture was allowed to stand overnight at room temperature. Water was removed by distillation and the compound heated at 110°. The solid residue was dissolved in base and extracted with ether for 24 hr. The aqueous solution was then adjusted to *p*H 1.0 and ether extraction was continued for another 24 hr. Finally, the *p*H was adjusted to 3.0 and nicotinic acid was extracted with ether for a 24-hr. period. The last ethereal extract was taken to dryness and the solid sublimed at 150° and 3 mm. The acid was recrystallized from methanol, m.p. 233–235°.

Anal. Found: 11.5×10^6 d.p.m./mg. H. after dilution with carrier.

N'-Methylnicotinamide iodide prepared from this acid (diluted) was assayed. *Anal.* Found: 4.71×10^5 d.p.m./mg. H. The 2-pyridone from this iodide was also assayed. *Anal.* Calcd: 0.00 mμc./mg. H. Found: 302 d.p.m./mg. H. (0.06% of adjusted specific activity of iodide). The 6-pyridone was also obtained. *Anal.* Calcd: 5.29×10^5 d.p.m./mg. H. Found: 5.13×10^5 d.p.m./mg. H. (97% of the adjusted specific activity of the iodide).

Nicotinic Acid-6-*t*.—This acid was prepared in the same manner as the 2-t acid except that 6-bromo-3-picoline was the starting material.

Anal. Found: 5.54×10^6 d.p.m./mg. H. after dilution with carrier. The 6-pyridone prepared from this acid contained less than 4% of the adjusted activity of the acid.

Nicotinic Acid-5-*t*.—This acid could not be obtained in reasonable yield from 5-bromonicotinic acid by lithiation and treatment with tritium oxide. Consequently, 3-bromoquinoline was reacted with butyl lithium and finally with tritium oxide as described above. The tritiated quinoline was then oxidized with alkaline permanganate under reflux for 6 hr. The acid was isolated from the reaction mixture essentially as described above, m.p. 230° (subl.). The material was sublimed, recrystallized twice from ethanol and diluted with inactive nicotinic acid.

Anal. Found: 7.19×10^6 d.p.m./mg. H.

After a second dilution with carrier, this acid was converted to the corresponding 1-methylnicotinamide iodide. *Anal.* Found: 7.67×10^4 d.p.m./mg. H. The 2-pyridone was obtained in the usual manner. *Anal.* Calcd.: 8.62×10^4 d.p.m./mg. H. Found: 8.65×10^4 d.p.m./mg. H. (100.3% of the adjusted specific activity of the iodide). The 6-pyridone showed the following result. *Anal.* Calcd. 8.62×10^4 d.p.m./mg. H. Found: 8.38×10^4 d.p.m./mg. H (97.2% of the adjusted specific activity of the iodide).

6-Hydroxynicotinic Acid-N^{15}.[20]—In a solution of 80 ml. of ether and 20 ml. of methanol, 2 g. of coumalic acid was treated with an ethereal solution of diazomethane until the yellow color persisted, then the solvent removed under vacuum. The residual brownish solid was recrystallized from hot cyclohexane to give 1.08 g. of yellow needles of methyl coumalate, m.p. 68–70°.

A reaction tube containing 656 mg. of the ester was placed on a vacuum manifold, along with a "pants" vessel containing 600 mg. of 95.6% $N^{15}H_4NO_3$[21] in one arm and 4 ml. of 20% aqueous sodium hydroxide in the other. After evacuation of the system, the base was spilled onto the ammonium nitrate and all of the $N^{15}H_3$ and water frozen onto the ester with liquid nitrogen. The system was then taken to one atmosphere with nitrogen gas and the vessel removed and stirred overnight in a Dewar flask containing ice. Unused $N^{15}H_3$ was recaptured by distilling some of the aqueous portion into 3 ml. of 6 *N* hydrochloric acid on the vacuum manifold.

Then 5-ml. of 20% sodium hydroxide was added to the remainder of the solution and this refluxed for 5 minutes, using a cold-finger condenser, then allowed to cool. The solution was taken to *p*H 3 with 6 *N* hydrochloric acid and the 6-hydroxynicotinic acid prepitated after refrigeration. It was filtered, dissolved in a minimum amount of dilute aq. sodium bicarbonate and reprecipitated with 6 *N* hydrochloric acid, again after refrigeration. The material was centrifuged and then recrystallized twice from 5 ml. of 50% acetic acid to give 72.1 mg. of 6-hydroxynicotinic acid, m.p. 325°. This value is not in accord with the literature values of about 300°. However, commercial 6-hydroxynicotinic acid (Aldrich Chemical Co., Inc., 3747 North Booth St., Milwaukee, Wisc.) was also found to have a melting point of 323° and a mixed melting point showed no depression. The acid was assayed by a modification of Schoniger's method[22] for the simultaneous determination of carbon, hydrogen and nitrogen in which the nitrogen is trapped on charcoal rather than pumped away. It is purified by storage over hopcalite and potassium hydroxide pellets before being submitted to mass spectrometer analysis.

Anal. Atom % N^{15}, 101.7 ± 5%. Calcd. for $C_6H_5N^{15}O_3$, C, 51.43%; H, 3.60%; N, 10.71%. Found, C, 51.87%; H, 3.84%; N, 10.68%.

Nicotinic Acid-4-*d*.—The preparation of 3-picoline-1-oxide was carried out according to the directions of Boekelheide.[23] The 4-nitro-3-picoline-1-oxide was prepared by the method of Hertog,[24] to give material with m.p. 136–138°. Then 2 g. of this compound was treated with 10 ml. of acetyl chloride[25] in a reaction flask with a cold finger reflux condenser. There

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was copious evolution of nitric oxide, after which the solution was heated for 1.5 hr. at 50°. The cooled solution was treated with sufficient ice to hydrolyze the acetyl chloride, made basic slowly with solid sodium carbonate and extracted with 20 ml. portions of chloroform. This was dried overnight with potassium carbonate, filtered, taken to dryness and the residue recrystallized from acetone to give 1.7 g. of *4-chloro-3-picoline-1-oxide* (I), m.p. 120–122° d.

Anal. Calcd. for C_6H_6NOCl: C, 50.19; H, 4.21; N, 9.76, Cl, 24.70. Found: C, 50.39; H, 4.44;N, 9.71; Cl, 24.58%.

This material appears to be reasonably stable under ordinary conditions and shows only slight coloration after standing one year. An attempt to prepare the 4-bromo analog using acetyl bromide gave similar appearing white crystals when the chloroform was removed from the final extraction solution but these suddenly and spontaneously decomposed before they could be dissolved in acetone.

Attempts to prepare the deuterated picoline by direct reduction of I with zinc and D_2SO_4 were not successful. Therefore 4.168 g. of I were dissolved in 85 ml. of chloroform and 18 ml. of phosphorus trichloride added with the solution at 0°.[26] This was stirred for 45 minutes, then poured onto about 100 g. of ice. The solution was slowly made basic with 20% sodium hydroxide, then extracted with three 50 ml. portions of chloroform and the combined extracts dried over sodium sulfate. The solution was filtered, the sulfate washed with ether and the filtrate treated with dry hydrogen chloride; cloudiness appeared and then cleared again during this treatment. The solution was taken to dryness under vacuum and the residue was taken up in 95% ethanol and filtered to remove some insoluble yellow material. Ether was added to the warm solution until it started to grow turbid. Cooling overnight in a refrigerator produced 2.957 g. of *4-chloro-3-picoline HCl*, m.p. 165–170° (instantaneous, sealed capillary).

Anal. Calcd. for $C_6H_7NCl_2$: C, 43.93; H, 4.30; N, 8.54; Cl, 43.23. Found: C, 44.13; H, 4.48; N, 8.54; Cl, 43.25.

This material (1.39 g.) was dissolved in 25 ml. of 2 *N* D_2SO_4 in D_2O, 1.3 g. of zinc dust was added and the mixture heated at 100° for 2 hr.[27] After cooling, the solution was filtered and slowly made basic with potassium hydroxide pellets, an equal volume of water added and the solution (with suspended zinc hydroxide) extracted two days with ether in a continuous extractor. The ether solution was dried with Drierite, filtered, treated with dry hydrogen bromide and then taken to dryness. An oil was produced, which was dissolved in 150 ml. of water, taken to *p*H 6.5 and 3.57 g. of potassium permanganate added in small portions while the solution refluxed gently for 6 hr. The excess permanganate was destroyed with hydrogen peroxide and the basic solution was extracted overnight with ether. The manganese dioxide was then dissolved by the addition of conc. hydrochloric acid and the acid solution extracted with ether for 8 hr. Extraction for one day at *p*H 2.5–3 gave material which was transferred to a small vacuum sublimer with dimethylformamide and sublimed to give 263 mg. of nicotinic acid. This was recrystallized from 3 ml. of 95% ethanol before use.

The water from a dry combustion[18] of this compound was reduced over zinc at 650° in a sealed tube and the hydrogen submitted for mass spectrometer analysis.

Anal. Atom% D calcd., 20.0%; found, 19.7%, 20.1%.

The 1-methylnicotinamide iodide prepared from a diluted sample of this acid showed 0.49 atom% D. The 2- pyridone prepared from this material showed 0.54 atom% D, while the 6-pyridone contained 0.52 atom% D; the calculated value for these compounds, based on the iodide, is 0.55 atom% D.

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Potential Purine Antagonists. XXII. The Preparation and Reactions of Certain Derivatives of 2-Amino-6-purinethiol[1]

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A number of 6-alkylthio-2-aminopurines have been prepared by two routes: (1) *via* cyclization of certain 6-alkylthio-2,4,5-triaminopyrimidines with ethyl orthoformate and acetic anhydride, and (2) by alkylation of 2-amino-6-purinethiol. A new synthesis of 2-amino-6-purinethiol has been accomplished in which thiation and ring closure of 2,4-diamino-5-formylamino-6-hydroxypyrimidine is achieved in one step with phosphorus pentasulfide in pyridine. 2-Amino-8-methyl-6-purinethiol has been similarly prepared from 5-acetylamino-2,4-diamino-6-hydroxypyrimidine. The preparation of 2-amino-6-chloropurine is reported.

A study of the antitumor activity of various 6-alkylthiopurines[3–6] against Adenocarcinoma 755[7,8] has revealed that several 6-alkylthiopurines possess a better therapeutic index than does 6-purinethiol. 6-Ethylthiopurine (National Service Center No. 11588) and 6-*n*-propylthiopurine (National Service Center No. 11595) had been previously prepared[9] and submitted for antitumor screening. The antitumor activity of this series of compounds suggested the extension of synthetic work to include

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