Biochem. J. (1967) 102, 87

A Method for the Degradation of Radioactive Nicotinic Acid

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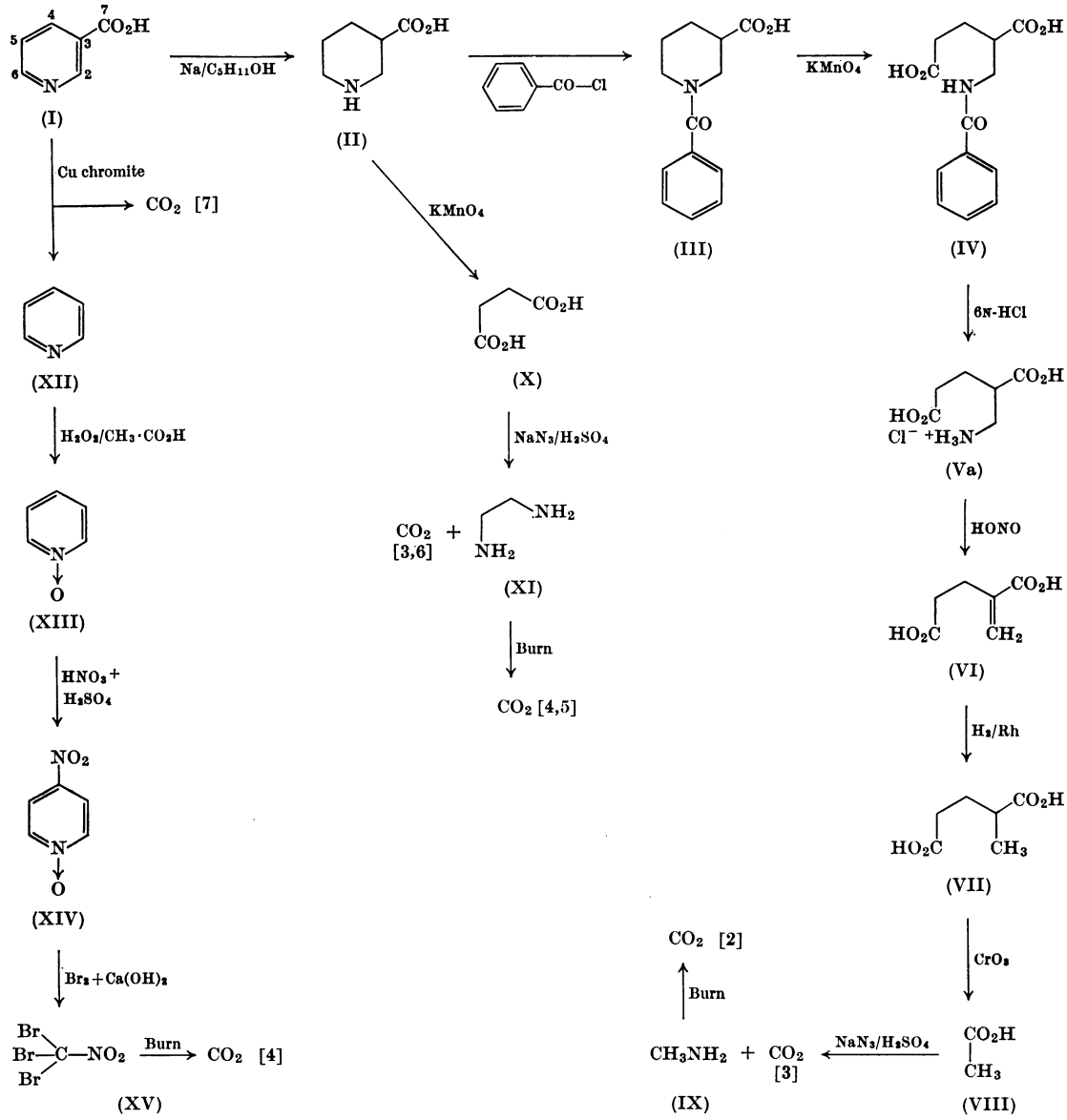
(*Received* 8 *June* 1966)

A chemical degradation scheme is reported, which permits the measurement of the radioactivity of each carbon atom of nicotinic acid. Nicotinic acid is decarboxylated by heating with copper chromite to give carbon dioxide (C-7) and pyridine. The pyridine is converted into 4-nitropyridine 1-oxide, which is heated with aqueous calcium hypobromite to give tribromonitromethane. Combustion of the latter gives carbon dioxide derived from C-4 of the nicotinic acid. Nicotinic acid is also reduced to nipecotic acid, which is oxidized to succinic acid by acidic potassium permanganate. Stepwise degradation of the succinic acid by standard procedures gives two samples of carbon dioxide, which correspond to C-3, C-6 and C-4, C-5 of the nicotinic acid. Benzoylation of the nipecotic acid, followed by oxidation with permanganate at pH 7, gives 5-amino-4-carboxyvaleric acid; this is converted into 2-methyleneglutaric acid by the action of nitrous acid. Hydrogenation of the 2-methyleneglutaric acid over rhodium in methanol gives 2-methylglutaric acid, which is oxidized with dilute chromic acid to acetic acid. Stepwise degradation of the acetic acid by standard procedures gives two samples of carbon dioxide, which correspond to C-2 and C-3 of the nicotinic acid. Thus the radioactivities of C-2, C-3, C-4 and C-7 are determined directly and those of C-5 and C-6 by difference. The method was shown to be isotopically valid for [2,3,7-^{14}C]-, [4,6-$^{14}C_2$]- and [5-^{14}C]-nicotinic acid.

For further studies on the biosynthesis of nicotinic acid (Scott & Hussey, 1965) a method was required for the determination of radioactivity in each carbon atom of the nicotinic acid molecule. The problem of devising such a degradation pathway lies in opening the heterocyclic ring to give a high yield of a known product. Decarboxylation has been used in several studies (Albertson & Moat, 1965; Mothes, Gross, Schütte & Mothes, 1961; Scott & Hussey, 1965), but it is not possible to distinguish between C-2 and C-6 or between C-3 and C-5 of the symmetrical pyridine. Direct oxidative attack on the unsaturated pyridine ring system gives low yields of products; for instance, the oxidation of nicotinamide methiodide with alkaline ferricyanide gives only 5% of the corresponding pyrid-2-one (Dawson, Christman, D'Adamo, Solt & Wolf, 1960). It was therefore decided to reduce nicotinic acid and to study the oxidative cleavage of the resulting hexahydronicotinic acid (nipecotic acid).

The degradation scheme for nicotinic acid (I) is shown in Scheme 1. Under varied conditions of pH and temperature, nipecotic acid (II) was oxidized by potassium permanganate to give succinic acid (X) and unchanged nipecotic acid; the conversion into succinic acid was virtually complete when excess of acidic permanganate was used. Benzoylnipecotic acid (III), however, was oxidized by permanganate to the benzoyl derivative of an unknown amino acid (IV). This amino acid was shown to be 5-amino-4-carboxyvaleric acid (Vb) by comparison with the authentic compound, which was synthesized by the catalytic hydrogenation of disodium 1-cyanoglutarate (XVII). 5-Amino-4-carboxyvaleric acid hydrochloride (Va) is converted by nitrous acid into methyleneglutaric acid (VI). Since rhodium was reported to catalyse a rapid and specific hydrogenation of conjugated systems (Breitner, Roginski & Rylander, 1959), it was tested as a catalyst in the hydrogenation of 2-methyleneglutaric acid. In methanolic solution, at room temperature and atmospheric pressure, the hydrogenation of 2-methyleneglutaric acid was complete after 30min. in the presence of one-quarter of its weight of catalyst (5% rhodium on charcoal).

As expected for a lone methyl group in an aliphatic compound, 2-methylglutaric acid (VII) gives a 100% yield of acetic acid when oxidized by dilute chromic acid in the Kuhn & Roth (1933) procedure for the determination of methyl groups.



Scheme 1. Degradation of nicotinic acid.

The acetic acid was distilled and collected by the method of Ma & Breyer (1960). The radiochemical degradations of succinic acid and acetic acid are well tried and established in the literature (Phares, 1951; Strassman & Weinhouse, 1953).

Nicotinic acid (I) is decarboxylated efficiently by heating with copper chromite, provided that certain precautions are taken. Sublimation and decarboxylation of nicotinic acid both commence at 235°; even with a very slow gas flow rate, it is possible to drive nicotinic acid through several inches of heated copper chromite supported on asbestos, with only slight decarboxylation. Consequently, the nicotinic acid was intimately mixed with five times its weight of copper chromite catalyst and the mixture suspended in silicone oil. Decarboxylation was complete after heating this mixture for 1 hr.

The nitration of pyridine (XII) is notoriously difficult, but the nitration of pyridine 1-oxide

(XIII) proceeds smoothly (Ochiai, 1953). Tribromonitromethane (bromopicrin) (XV) is formed from 4-nitropyridine 1-oxide (XIV), as from any nitrated aromatic compound, by heating with calcium hypobromite.

MATERIALS AND METHODS

Chemicals. These were obtained from the following suppliers: 5% rhodium on charcoal from Koch–Light Laboratories Ltd., Colnbrook, Bucks.; Dowex 50 from Howe and Co. Ltd., London, W. 11; silicone fluid MS550 from Hopkin and Williams Ltd., Chadwell Heath, Essex; NE213 liquid scintillator and methanolic 1·0M-Hyamine hydroxide from Nuclear Enterprises Ltd., Sighthill, Edinburgh; all radioactive chemicals were from The Radiochemical Centre, Amersham, Bucks.

Compounds not commercially available were prepared as follows. 2-Methyleneglutaric acid was prepared by bringing malonic ester into reaction with formaldehyde in the presence of piperidine, followed by acid hydrolysis of the ester (Buchman, Reims & Schlatter, 1942). Copper chromite was prepared according to Lazier & Arnold (1943). [5-^{14}C]Nicotinic acid and [4,6-$^{14}C_2$]nicotinic acid were prepared by treating appropriately labelled glycerol with aniline in conc. H_2SO_4. The resulting quinoline was oxidized to quinolinic acid, which was decarboxylated to nicotinic acid (Pastan, Tsai & Stadtman, 1964). [7,3,2-$^{14}C_3$]-Nicotinic acid, synthesized by the same method from [U-^{14}C]aniline, was kindly provided by Mr J. P. Glynn.

Other materials were purchased from British Drug Houses Ltd., Poole, Dorset.

Infrared-absorption spectra. Samples were mulled with Nujol and placed between polished disks of NaCl. The spectra were recorded with a Unicam SP.200 spectrophotometer.

Elemental analyses. These were performed by G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford.

Melting points. These were measured with a Gallenkamp electric melting-point apparatus (design 889339) and were uncorrected.

Measurement of radioactivity. Carbon dioxide was produced by totally burning samples with the combustion fluid of Van Slyke & Folch (1940) or by specific decarboxylation reactions (see below). The apparatus was flushed with CO_2-free N_2, and the effluent gases were passed through 5ml. of 5% (w/v) $KMnO_4$ in N-H_2SO_4 and then 5ml. of carbonate-free 2N-NaOH. The absorbed CO_2 was released by acid, measured manometrically, then transferred quantitatively to methanolic 1·0M-Hyamine hydroxide (3ml.) by means of the apparatus described below. Carbonated Hyamine hydroxide solution (0·5ml.) was added to NE213 liquid scintillator (4ml.) and the mixture was counted in an IDL Tritomat 6020 scintillation counter (Isotope Developments Ltd., Beenham, Berks.). The counting efficiency was 70%. Samples were counted in triplicate.

Apparatus for the measurement and reabsorption of carbon dioxide (Fig. 1)

Construction. Each manometer arm (50cm.) was made from a soda-glass micro-burette, in which 5cm. represented 1ml. A scale was mounted behind the manometer as an aid to equalizing the mercury levels. Volumes were read from the original graduations of the micro-burette. The rest of the apparatus was made from Pyrex. The upper horizontal tube was internal diameter 1cm.; other internal tube diameters were 7mm. and 3mm. and they are drawn to scale in Fig. 1. Except for the thick-walled rubber tubing of the manometer, all other rubber tubing was internal diameter 4mm. and wall thickness 2mm. The ground-glass joint at tap 2 to flask A was B14; all other glass joints were B12. Flask A had a capacity approx. 30ml. and bulb B approx. 20ml. Bulb C was a syringe bulb (capacity 50ml.). The rear entry valve of C was removed and replaced by an inlet tube, the valve was remounted between the inlet tube and a T-tube, and the joint sealed with a collar of thick rubber tubing. The side arm of the T-tube carried a small rubber balloon (D), which acted as a pressure reservoir during the compression stage of each pumping operation. The bubbler (E) was based on the design of Phares (1951); the volume was 3ml. at the liquid levels shown.

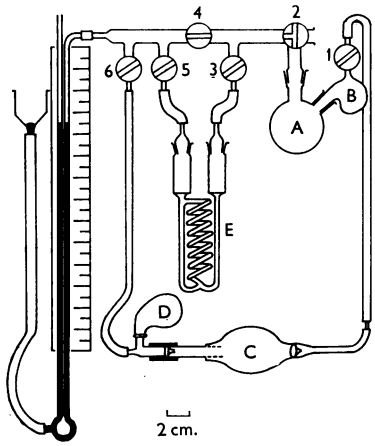


Fig. 1. Apparatus for the measurement and reabsorption of CO_2. Details are given in the Materials and Methods section.

Operation. Carbonated 2N-NaOH solution was transferred to flask A and a small polythene-covered bar magnet was added. Side bulb B contained 10N-H_2SO_4 (3ml.). Taps 1, 3, 5 and 6 were closed, tap 4 was opened and tap 2 was turned so that flask A and the manometer were connected to the atmosphere. After equilibrium at room temperature, the level of mercury in the manometer was recorded and tap 2 was turned so that the apparatus was a closed system and flask A was connected, via the upper horizontal tube, with the manometer. Bulb B was turned to tip the acid into flask A, and the solutions were thoroughly mixed by magnetic stirring. To dissipate the heat produced in mixing the acid and base, a beaker containing 2l. of water at room temperature was raised until flask A was completely immersed. After 10min. the mercury levels were adjusted until equal and the new level was recorded. The volume of CO_2 evolved was calculated from the change in manometer levels and corrected to normal temperature and pressure.

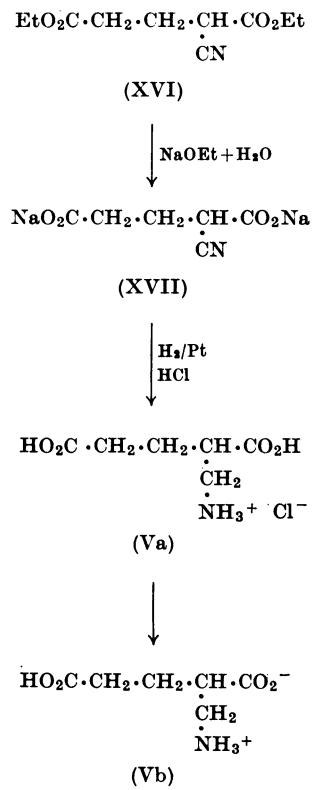
Tap 4 was then closed; taps 1, 3, 5 and 6 were opened and the total gas of the system was then continually circulated through the bubbler, which contained methanolic 1·0M-Hyamine hydroxide (3ml.), by means of the syringe bulb. There are small dead spaces on each side of tap 4, but these were efficiently flushed. The blank space from the top of the mercury to the exit to tap 6 was also efficiently flushed, probably because the mercury continually oscillates during pumping. In the author's apparatus, fifty compressions of the syringe bulb were sufficient to ensure the complete absorption of 3ml. of $^{14}CO_2$ by the Hyamine hydroxide solution.

EXPERIMENTAL

Reduction of nicotinic acid (I) *to nipecotic acid* (II). The method of Clemo, Ormston & Ramage (1931) was adapted for this conversion. Nicotinic acid (50mg.) and dry amyl alcohol (3ml.) were heated under reflux and finely divided sodium (170mg.) was added over a period of 30min. The reaction mixture was heated for a further 30min., cooled and extracted with water (2×5ml.). The aqueous extract was acidified with conc. HCl and evaporated to dryness under reduced pressure. Nipecotic acid hydrochloride was extracted from the residue by heating with absolute ethanol (10ml.); the mixture was cooled to 0° and filtered. After this extraction had been done three times, the ethanol extracts were combined and taken to dryness by distillation. Recrystallization of the residue from chloroform–acetone gave 50mg. (yield 75%) of nipecotic acid hydrochloride, which started to sublime with decomposition at 240° (Found: C, 43·6; H, 7·4; N, 8·4; Cl, 21·4; Calc. for $C_6H_{12}NO_2Cl$: C, 43·63; H, 7·27; N, 8·48; Cl, 21·2%).

Preparation of benzoylnipecotic acid (III). Equimolar proportions of nipecotic acid and benzoylchloride were stirred vigorously in alkaline aqueous solution. The mixture was kept alkaline to phenolphthalein by the continual addition of 5N-NaOH and at 0° by immersion of the reaction vessel in ice–water. After the completion of the reaction, which was marked by the disappearance of the benzoylchloride and the cessation of acid production, the mixture was acidified to Congo red by the addition of excess of conc. HCl. Benzoylnipecotic acid was precipitated in essentially 100% yield; it was removed by filtration, washed with water and recrystallized from aqueous ethanol, m.p. 187–188° (Found: C, 66·8; H, 6·6; N, 5·9; Calc. for $C_{13}H_{15}NO_3$: C, 66·95; H, 6·43; N, 6·00%).

Synthesis of 5-amino-4-carboxyvaleric acid (Vb) *and its hydrochloride* (Va) (*Scheme* 2). Ethyl-γ-carbethoxy-α-cyanobutyrate (XVI) (prepared by the method of Koelsch, 1943) (1g.) was added to a solution of sodium in aqueous ethanol, which was prepared by dissolving sodium (2g.) in absolute ethanol (20ml.) and adding water (2ml.). The precipitated disodium 1-cyanoglutarate (XVII) was collected by filtration, washed with alcohol and then with diethyl ether and dried over P_2O_5. The yield was 1g., i.e. essentially theoretical; this was all dissolved in water (50ml.) and hydrogenated in the presence of PtO_2 (200mg.) for 48hr. at 20° and atmospheric pressure. After removal of the catalyst by filtration, the solution was evaporated to dryness under reduced pressure and the residue dissolved in water (10ml.) and transferred to a column (length 20cm., diam. 1cm.) of Dowex 50 (H^+ form). The column was eluted with 1·5N-HCl (60ml.); 3ml. fractions were collected



Scheme 2. Synthesis of 5-amino-4-carboxyvaleric acid.

and analysed by paper chromatography in the system butan-1-ol–acetic acid–water (4:1:1, by vol.). Fractions 9–16, which contained ninhydrin-positive material, were evaporated to dryness under reduced pressure and the residue was recrystallized from aqueous acetone: 63mg. (yield 64%) of the amino acid hydrochloride was obtained; this was an hygroscopic, white solid, which darkened and decomposed above 200°. The hydrochloride (60mg.) was converted into the free amino acid by boiling with pyridine (2ml.). Excess of pyridine was distilled and the pyridine hydrochloride removed by sublimation. The residue was recrystallized twice from water, to give a white crystalline solid, which darkened and decomposed at about 200°, depending on the rate of heating (Found: C, 44·7; H, 7·0; N, 8·3. $C_6H_{11}NO_4$ requires C, 44·7; H, 6·6; N, 8·7%). The 2,4-dinitrophenyl derivative of the amino acid, prepared by the method of Levy & Chung (1955) and recrystallized from aqueous ethanol, had m.p. 187° (Found: C, 43·8; H, 4·2; N, 12·3. $C_{12}H_{13}N_3O_8$ requires C, 44·0; H, 3·9; N, 12·8%).

The infrared spectrum (Fig. 2) of the amino acid hydrochloride was rather diffuse, with little fine structure, due to the many polar forms of the hydrochloride of an acidic amino acid. Nevertheless, the band at 1220cm.$^{-1}$ can be assigned to the C–O vibration and that at 1720cm.$^{-1}$ to the C=O stretch; these bands are present in all amino acid hydrochlorides. The infrared spectrum of the free amino acid (Fig. 2) shows a strong C–O band at 1230cm.$^{-1}$, typical

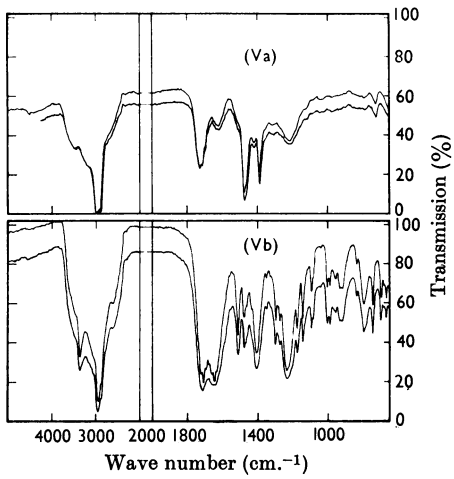


Fig. 2. Infrared spectra of 5-amino-4-carboxyvaleric acid (Vb) and its hydrochloride (Va). Upper trace: material from the hydrogenation of disodium 1-cyanoglutarate (XVII); lower trace: material from the oxidation of benzoylnipecotic acid (III).

of dicarboxylic amino acid. A strong band at 1710cm.$^{-1}$ indicates the C=O stretch of the un-ionized carboxyl group of a dicarboxylic amino acid. Deformation of the NH_3^+ group gives rise to two bands: the stronger lies between 1650 and 1640cm.$^{-1}$ and the weaker band appears at 1510cm.$^{-1}$.

Preparation of 5-amino-4-carboxyvaleric acid (Vb) *and its hydrochloride* (Va) *from benzoylnipecotic acid* (III). An aqueous solution (10ml.) containing benzoylnipecotic acid (100mg.) and $KMnO_4$ (100mg.), pH adjusted to 7 with NaOH, was stirred for 24hr. at 20°. The mixture was boiled briefly to coagulate the hydrated MnO_2, which was removed by filtration. The filtrate was taken to dryness under reduced pressure and the residue heated under reflux for 18hr. with 6N-HCl (5ml.) to hydrolyse the benzoylated product. Excess of HCl was removed by repeatedly evaporating the mixture to dryness under reduced pressure with the addition of extra water. The residue was dissolved in 1ml. of water and placed on a column (length 20cm., diam. 1cm.) of Dowex 50 (H^+ form). The column was eluted with 60ml. of 1·5N-HCl; 3ml. fractions were collected and analysed by paper chromatography in the system butan-1-ol–acetic acid–water (4:1:1, by vol.). Fractions 2 and 3 contained small amounts of succinic acid. Fractions 9–16, which contained the main ninhydrin-positive material, were evaporated to dryness under reduced pressure, purified further by paper chromatography (butan-1-ol–acetic acid–water, 4:1:1, by vol.) and eluted again from Dowex 50. The resulting amino acid hydrochloride was converted into the free amino acid with pyridine as described above. It was identical with 5-amino-4-carboxyvaleric acid: the infrared spectra were identical (Fig. 2), co-chromatography with authentic 5-amino-4-carboxyvaleric acid gave a single ninhydrin-positive spot (R_F0·25) in the system butan-1-ol–acetic acid–water (4:1:1, by vol.), and treatment with nitrous acid, as subsequently described, gave the same yield of 2-methyleneglutaric acid (VI) in each case.

Oxidation of nipecotic acid (II) *to succinic acid* (X). Nipecotic acid hydrochloride (20mg.) in 2N-H_2SO_4 (5ml.) was titrated with 2% $KMnO_4$, with the reaction mixture kept at 100°, until the red colour of the permanganate persisted for 1min. Excess of permanganate was destroyed by the addition of a drop of dilute formic acid and the reaction mixture placed on a column (length 10cm., diam. 1cm.) of Dowex 50 (H^+ form). The column was eluted with water, the first 10ml. of eluate evaporated under reduced pressure, and the residue sublimed under reduced pressure to give 9mg. of succinic anhydride (75% yield). This was dissolved in water (5ml.), titrated to pH7 with 0·02N-NaOH and the solution evaporated to give sodium succinate. The infrared spectrum of the product was identical with that of authentic disodium succinate. Paper chromatography of the sublimed succinic anhydride in the system ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (20:1:4, by vol.) showed the presence of only one organic acid, corresponding to succinic acid at R_F0·3.

Conversion of nicotinic acid (I) *into 5-amino-4-carboxyvaleric acid hydrochloride* (Va) *and succinic acid* (X). When radioactive nicotinic acid was degraded, these conversions were performed as follows. Nicotinic acid (50mg.) was heated under reflux in dry amyl alcohol (3ml.) and finely divided sodium (170mg.) was added over a period of 30min. Heating was continued for a further 30min., water (10ml.) was added and the reaction mixture distilled under reduced pressure. Distillation was repeated with the further addition of water until the residue was in aqueous solution (5ml.) and free from amyl alcohol.

Benzoylchloride (18mg.) was added to a portion of the alkaline, aqueous solution of nipecotic acid (3ml.) and stirred vigorously for 1hr. at 0°. The total reaction mixture was adjusted to pH7 with 2N-H_2SO_4 and the volume was adjusted to 5ml. with water. Potassium permanganate (55mg.) was added and the mixture stirred at room temperature for 24hr. A portion (5ml.) of 12N-HCl was added and the mixture heated under reflux for 18hr. The solution was evaporated to dryness under reduced pressure and 5-amino-4-carboxyvaleric acid hydrochloride was purified from the residue by chromatography on Dowex 50 as described above.

The remaining 2ml. of alkaline aqueous nipecotic acid solution was acidified with 2N-H_2SO_4, titrated with 2% $KMnO_4$ at 100° and the succinic acid isolated and purified as described above. The succinic acid was degraded by the method of Strassman & Weinhouse (1953).

Oxidation of 5-amino-4-carboxyvaleric acid hydrochloride (Va) *to 2-methyleneglutaric acid* (VI). 5-Amino-4-carboxyvaleric acid hydrochloride (11·6mg.) was dissolved in water (50ml.), 1N-H_2SO_4 (2ml.) and 2% $NaNO_2$ (15ml.) were added and the mixture was heated at 100° for 15min. Excess of nitrous acid was destroyed by the addition of 10% urea (10ml.). The reaction mixture was then extracted three times with 50ml. of diethyl ether. The pooled ether extracts were dried over anhydrous Na_2SO_4, filtered, and blown to dryness in a stream of N_2. Recrystallization of the residue from water gave 6mg. (66% yield) of 2-methyleneglutaric acid, m.p. 128–130° (Found: C, 50·5; H, 5·9; Calc. for $C_6H_8O_4$: C, 50; H, 5·6%). The infrared spectrum of the product was identical with that of authentic 2-methyleneglutaric acid.

Reduction of 2-methyleneglutaric acid (VI) *to 2-methylglutaric acid* (VII). 2-Methyleneglutaric acid (10mg.) and catalyst (2mg. of 5% rhodium on charcoal) in methanol (3ml.) were hydrogenated at room temperature and atmospheric pressure until the theoretical amount of hydrogen was consumed. The catalyst was removed by filtration. Distillation of the filtrate gave a residue of practically pure 2-methylglutaric acid in essentially 100% yield, which crystallized on standing overnight, m.p. 77–79° (Found: C, 49·3; H, 6·9; Calc. for $C_6H_{10}O_4$: C, 49·31; H, 6·85%). The infrared spectrum was identical with that of authentic 2-methylglutaric acid.

Decarboxylation of nicotinic acid (I). An intimate mixture of nicotinic acid (20mg.) with copper chromite (100mg.) was suspended in silicone oil (MS550) (1ml.) and placed in the apparatus shown in Fig. 3. The apparatus was flushed with dry CO_2-free N_2, while the tube containing the decarboxylation mixture was heated to 235° in a bath of silicone oil. The effluent gas was passed first through a 30ml. pear-shaped, double-necked flask cooled in ice, as shown in Fig. 3, to collect the pyridine (XII), and then through a bubbler containing carbonate-free 2N-NaOH (3ml.) to collect the CO_2. Decarboxylation was complete after 1hr. at 235°. Some of the pyridine, however, remained condensed in the delivery tube. This was washed into the receiver by adding acetic acid (the solvent for the next conversion—see below) through the small entry port in the delivery tube.

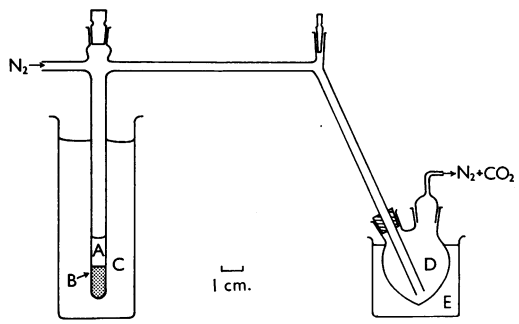


Fig. 3. Apparatus for the decarboxylation of nicotinic acid. A, Silicone oil; B, mixture of nicotinic acid and copper chromite; C, bath of silicone oil, which is heated to 240°; D, receiver for the pyridine; E, water at 0°.

Conversion of pyridine (XII) *into 4-nitropyridine 1-oxide* (XIV). The method of Ochiai (1953) was adapted as follows: after the decarboxylation of nicotinic acid (20mg.), the solution of pyridine in acetic acid (approx. 12mg. in 3ml.) was heated for 3hr. at 80° with aqueous 35% H_2O_2 (0·5ml.). A further 0·5ml. of 35% H_2O_2 was added and the mixture maintained at 80° for an additional 3hr. The mixture was concentrated under reduced pressure, diluted with 2N-HCl (5ml.) and concentrated again to give approx. 17mg. of white, solid pyridine 1-oxide hydrochloride. Nitric acid (sp.gr. 1·48) (15mg.) and H_2SO_4 (sp.gr. 1·84) (0·05ml.) were added to the pyridine 1-oxide hydrochloride and the solution was heated at 130° for 3½hr. The nitration mixture was diluted with ice-cold 10% Na_2CO_3 soln. (3ml.) and the yellow 4-nitropyridine 1-oxide was extracted with cyclohexanol (2×3ml.). The solution in cyclohexanol was evaporated to dryness under reduced pressure and the residue was dissolved in water (0·2ml.). This solution of crude 4-nitropyridine 1-oxide was distributed over a 5in. starting line on an 8in.-square thin-layer plate of deactivated silicic acid (0·5mm. thick). After development of the plate with $CHCl_3$ for a distance of 5in. from the starting line, the band of 4-nitropyridine 1-oxide was detected by its opacity to ultraviolet light (R_F about 0·3).

Formation of tribromonitromethane (XV) *from 4-nitropyridine 1-oxide* (XIV). This is essentially the method used by Bolas & Groves (1870) for the formation of tribromonitromethane ('bromopicrin') from picric acid. The 4-nitropyridine 1-oxide and its carrier silicic acid were scraped from the above thin-layer chromatogram and this mixture was used directly. A mixture containing $Ca(OH)_2$ (2·5g.), water (10ml.) and bromine (1ml.) was prepared and cooled to 0°. The silicic acid, which contained the 4-nitropyridine 1-oxide, was added, steam was passed and the mixture heated to 100°. The first 4ml. of distillate contained the oily tribromonitromethane (approx. 20mg.),

Table 1. *Distribution of radioactivity in test samples of nicotinic acid*

Nicotinic acid, 300mg. in each case, was degraded with the method of determination of radioactivity, described in the text.

	Radioactivity (above background) (counts/min./m-mole of C)		
True labelling pattern (according to synthesis) of the nicotinic acid ...	[5-^{14}C]-	[4,6-$^{14}C_2$]-	[2,3,7-$^{14}C_3$]-
Origin of CO_2			
C-2	0·0	0·0	436·0
C-3	0·0	0·0	440·0
C-4	0·0	237·0	0·0
C-3,6	0·0	115·0	219·0
C-4,5	105·0	121·0	0·0
C-5 (by difference)	210·0	2·5	0·0
C-6 (by difference)	0·0	230·0	−2·0
C-7	0·0	0·0	441·0
Total activity of the nicotinic acid (measured directly)	35·0	80·0	214·0
Total activity of the nicotinic acid (summated from the degradation results)	35·0	77·8	219·5

which was washed twice with water by centrifugation, then burned to CO_2.

RESULTS AND DISCUSSION

Table 1 shows the results from the degradation of [5-^{14}C]-, [4,6-$^{14}C_2$]- and [2,3,7-$^{14}C_3$]-nicotinic acid. There was no cross-contamination with radioactivity between different carbon atoms during the degradation procedure. Succinic acid (X) from the oxidation of nipecotic acid (II) therefore contains only carbon atoms C-3 to C-6. Other mechanisms for the conversion, which could give rise to succinic acid containing carbon atoms C-2 to C-5, are thus ruled out. Sampling and counting techniques are therefore the chief sources of error, which is approximately $\pm 5\%$ for the individual carbon atoms, and $\pm 3\%$ for the calculated total activity of the nicotinic acid. The small value for C-5 represents an apparent cross-contamination of 3%, but this is mathematical in origin, since C-5 is determined by difference; C-6 even has a small negative value for the same reason.

When the carbon dioxide was precipitated and counted as barium carbonate (Scott & Hussey, 1965) the error between individual carbon atoms was 15%. The accuracy of the present method therefore depends upon the use of liquid-scintillation counting.

I thank Mr C. Skelton, who made all the glass apparatus, and Carreras Ltd., who bought all the radioactive materials.

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