material (R_f 0.02), the presence of a major (XL, R_f 0.35) and a minor (XLI, R_f 0.39) spot. Separation of this mixture on one chromatoplate (20 × 20 cm) using 50% benzene–hexane provided: (1) 3 mg of a very polar contaminant, which remained unchanged in its R_f value upon further oxidation with Jones' reagent;[14] (2) 6 mg of chromatographically homogeneous 14α-ketone XL; (3) 2.8 mg of a mixture of the 14α-ketone XL with slightly more mobile material, presumably the 14β-ketone XLI. Recrystallization of 5α-ergostan-15-one (XL) from a small amount of methanol afforded an analytical specimen which exhibited mp 143–144°; λ_{max}^{Nujol} 5.73 μ. The optical rotatory dispersion data are presented in Table II.

Anal. Calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.08; mol wt, 400. Found: C, 83.70; H, 11.81; mol wt (mass spectroscopy), 400.

A 5-mg sample of 5α-ergostan-15-one (XL) was epimerized in 2.5% methanolic potassium hydroxide solution as previously described (see preparation of XXXVII). For the rotatory dispersion data of the crude residue, see Table II.

An insufficient amount of 15-keto steroid coupled with the observed similarity[51] in R_f values between the 14α epimer XL and the 14β epimer XLI precluded isolation of pure 5α,14β-ergostan-15-one (XLI) sufficient for analysis. Base-catalyzed epimerization of 5α,14β-ergostan-15-one (XLI), containing a little of its 14α epimer (XL), gave a semicrystalline residue identical on thin-layer chromatographic comparsion with that obtained from an identical epimerization reaction with pure 5α-ergostan-15-one (XL); this comparison further supports the fact that the more mobile spot (tlc) obtained during equilibration of 5α-ergostan-15-one (XL) in basic medium is, indeed, 5α,14β-ergostan-15-one (XLI).

A difference in molecular amplitude between −64 and −124° for unknown 5α,14β-ergostan-15-one (XLI) only corresponds to a variation of 22–18%, respectively, for the amount of 14β epimer XLI present in the equilibrium mixture. These values for the equilibrium composition are very close to that determined (see Table II) for 20-methyl-5α-pregnan-15-one (XXXVIII). The value (20% 14β) expressed in Table II assumes an amplitude of −95° for the unknown 5α,14β-ergostan-15-one (XLI).

(51) Closer here than for the other C-14 epimeric 17β-alkyl-5α-androstan-15-ones (XXXIV–XXXIX) described in this paper.

The Biosynthesis of Nicotine in *Nicotiana glutinosa* from Carbon-14 Dioxide. Labeling Pattern in the Pyrrolidine Rings[1,2]

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Abstract: A systematic degradation has been developed which permits isolation of each carbon atom of the pyrrolidine ring of nicotine. The independent synthesis of specifically labeled intermediates obtained during this sequence and their degradation have confirmed the integrity of the entire process. This degradation has been applied to numerous samples of nicotine obtained from short-term $^{14}CO_2$ biosynthesis with *N. glutinosa*, and in each experiment the pyrrolidine ring showed an unsymmetrical labeling pattern, a condition contrary to the accepted symmetrical intermediate hypothesis of pyrrolidine ring formation. Ornithine feeding experiments, from which the symmetrical theory had evolved, were applied to *N. glutinosa* and results were identical with those in other species. These experiments establish a greatly different labeling pattern in the pyrrolidine ring from CO_2 than from preformed precursors such as ornithine.

The formation of the pyrrolidine ring of nicotine in various *Nicotiana* species has been studied extensively since the initial observation that ornithine-2-^{14}C was incorporated only into the pyrrolidine ring, and that carbon-14 was found exclusively, and equally, at positions 2′ and 5′, leading to the proposal of a symmetrical pyrroline intermediate.[4] Glutamic acid was found to act as a precursor of the pyrrolidine ring in a similar manner, but to a lesser extent.[5] These data were interpreted to mean that ornithine is a close or immediate precursor of the pyrrolidine ring of nicotine and, furthermore, that the relationship between glutamate, derived by the tricarboxylic acid cycle, and ornithine is very close *via* the intermediacy of glutamic semialdehyde.[6,7] This interpretation led to development of the generally accepted[8] glutamate symmetrical intermediate hypothesis for pyrrolidine ring biosynthesis.

Support for this hypothesis was obtained by feeding various labeled tricarboxylic acid cycle intermediates to intact plants or root cultures of *Nicotiana*,[7,9] and results were generally consistent with the predicted pyrrolidine ring labeling pattern. Several exceptions to the calculated labeling pattern were noted,[10] the most significant contradiction being found in the pyrrolidine ring derived from short-term administration of acetate-2-^{14}C.[7] Only under longer term, more equilibrating conditions did this precursor provide a labeling pattern in agreement with that predicted by the hypothe-

(1) Preliminary results of this work have been reported: A. A. Liebman, F. Morsingh, and H. Rapoport, *J. Am. Chem. Soc.*, **87**, 4399 (1965).

(2) Sponsored in part by the United States Atomic Energy Commission and Grant MH 12797 from the National Institutes of Health, U. S. Public Health Service.

(3) Public Health Service Postdoctoral Research Fellow of the National Institute of General Medical Sciences.

(4) E. Leete, *Chem. Ind.* (London), 537 (1955); L. J. Dewey, R. U. Byerrum, and C. D. Ball, *Biochim. Biophys. Acta*, **18**, 141 (1955).

(5) B. L. Lamberts and R. U. Byerrum, *J. Biol. Chem.*, **233**, 939 (1958).

(6) E. Leete, *J. Am. Chem. Soc.*, **78**, 3520 (1956).

(7) P. L. Wu, T. Griffith, and R. U. Byerrum, *J. Biol. Chem.*, **237**, 887 (1962).

(8) (a) A. R. Battersby, *Quart. Rev.* (London), **15**, 259 (1961); (b) K. Mothes and H. R. Schutte, *Angew. Chem.*, **75**, 265 (1963); (c) E. Ramstad and S. Agurell, *Ann. Rev. Plant Physiol.*, **15**, 143 (1964); (d) E. Leete, *Science*, 147, 1000 (1965).

(9) D. R. Christman and R. F. Dawson, *Biochemistry*, **2**, 182 (1963).

(10) For a review of the labeling pattern produced in the pyrrolidine ring of nicotine after administration of various carbon-14 precursors see W. L. Alworth, A. A. Liebman, and H. Rapoport, *J. Am. Chem. Soc.*, **86**, 3375 (1964).

sis,[11] and the possibility of alternate pathways for glutamate or pyrrolidine ring biosyntheses in *Nicotiana* was raised. In addition, the possible difficulties in interpreting data obtained from long-term precursor feeding experiments[8d,11] has been noted.

Further difficulties with the glutamate symmetrical intermediate hypothesis were encountered and some revision of the original postulate was made after the finding that ornithine-α-^{15}N contributed no ^{15}N to nicotine in contrast to a reasonable incorporation into the pyrrolidine ring from ornithine-δ-^{15}N when administered to sterile root cultures of *N. tabacum*.[12] However, when α-N-methylornithine-N-$^{14}CH_3$ and δ-N-methylornithine-N-$^{14}CH_3$ were taken up by rooted leaves and excised root cultures of *N. rustica*,[13] the radioactivity in the N-methyl group of nicotine from the α-N-methyl group was four times that from the δ compound. This difference seems too large to explain on the basis of transmethylation reactions, and therefore these data are in direct contrast with those obtained from feeding ornithine-^{15}N (α and δ).

We have previously reported[14] initial results on nicotine biosynthesis obtained from relatively short-term exposure of intact *Nicotiana glutinosa* to $^{14}CO_2$. Of significance in these experiments was the finding of a very small percentage of label incorporated into C-2′ of the pyrrolidine ring, a condition contrary to the glutamate symmetrical intermediate hypothesis, assuming known glutamate biosyntheses. Accordingly, nicotine obtained from $^{14}CO_2$ biosynthesis was further degraded[10] in an effort to test this theory of pyrrolidine ring formation. Degradative methods were limited to isolation of C-2′ and C-5′, and essentially equal label was demonstrated at these positions.[15] However, the difference between ^{14}C incorporation in C-2′ plus C-5′ and C-3′ plus C-4′ ruled out the possibility of the pyrrolidine ring being formed from glutamate directly *via* the tricarboxylic acid cycle, and an alternate pathway was proposed.

To fully evaluate the glutamate symmetrical intermediate hypothesis, a complete degradation of the pyrrolidine ring of nicotine, obtained from $^{14}CO_2$ biosynthesis, was required. Existing degradations for the isolation of C-2′ and C-5′[16] are not adaptable to further unambiguous determination of either C-3′ or C-4′. The original degradation of nicotine[17] *via* dibromocotinine has recently been utilized in assessing incorporated tracer at C-3′ and C-4′ after administration of acetate-2-^{14}C in intact *N. rustica*;[11] however, this degradation is based on the incorrect structure of dibromocotinine.[18] In view of this, the low yields, and the fact that C-3′ and C-4′ values could only be assigned by difference, an efficacious method of pyrrolidine ring degradation was still lacking. Such a method has now been developed and is presented below.

(11) P. L. Wu and R. U. Byerrum, *Biochemistry*, **4**, 1628 (1965).
(12) E. Leete, E. G. Gros, and T. J. Gilbertson, *Tetrahedron Letters*, 587 (1964).
(13) H. B. Schröter and D. Neumann, *ibid.*, 1279 (1966).
(14) W. L. Alworth, R. C. DeSelms, and H. Rapoport, *J. Am. Chem. Soc.*, **86**, 1608 (1964).
(15) The Schmidt procedure used for the isolation of C-5′ has been reexamined and found to be inadequate in that exclusion of sulfur dioxide in the collected gases was incomplete. Therefore, the values reported for C-5′[10] are lower than the true values by an indeterminable amount.
(16) Pyrrolidine ring degradations have been reviewed in ref 10.
(17) A. Pinner, *Ber.*, **26**, 292 (1893).
(18) A. M. Duffield, H. Budzikiewicz, and C. Djerassi, *J. Am. Chem. Soc.*, **87**, 2926 (1965).

Degradative Procedures

Since the pyrrolidine ring was shown to incorporate essentially equal amounts of ^{14}C at C-2′ and C-5′ after $^{14}CO_2$ biosynthesis,[10] determination of activity at either C-3′ or C-4′ would be sufficient to test a symmetrical intermediate precursor because the total activity at C-3′ and C-4′ could be found by difference. In effect, determination of C-2′ would also provide the value for incorporation at C-5′, and a direct analysis of C-3′ would enable assignment of the C-4′ value by difference. The degradation path for determination of C-2′ and C-3′ is shown in Scheme I.

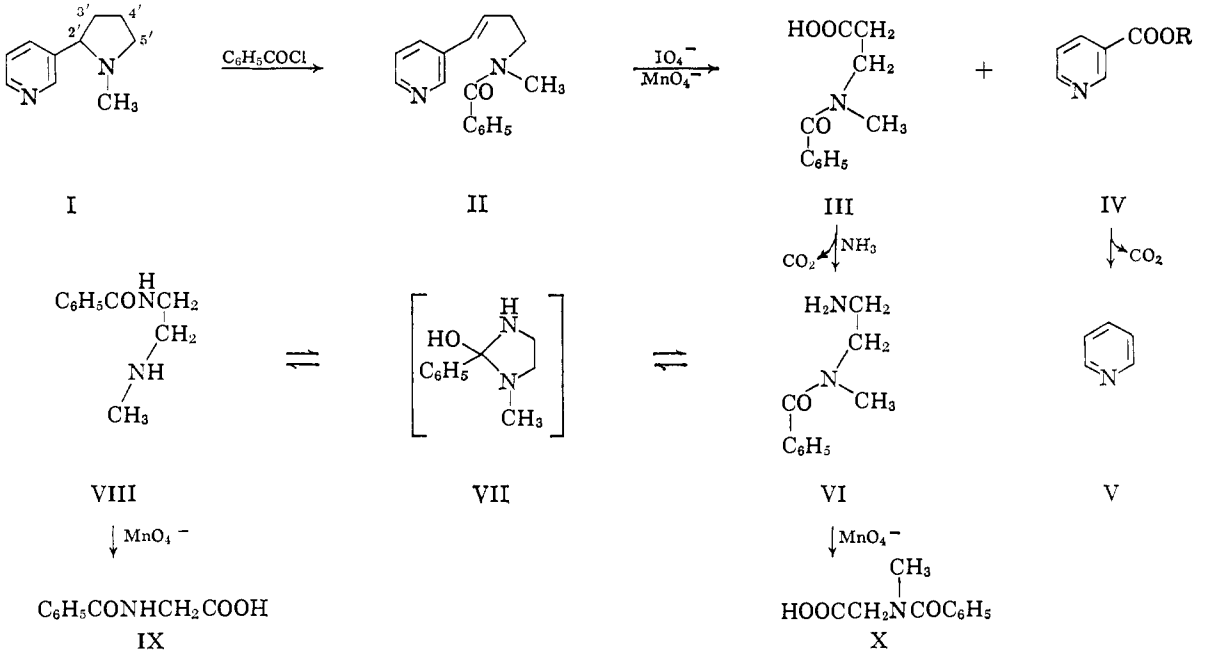
The conversion of nicotine to N-benzoylmetanicotine (II)[19,20] was slightly modified, and improved yields of the crystalline product II were obtained. N-Benzoylmetanicotine, in dilute aqueous solution, was cleaved by periodate–permanganate oxidation[21] and the N-benzoyl-N-methyl-β-alanine (III) was recovered from the acidified solution by continuous extraction with methylene chloride. Nicotinic acid (IV) was isolated as the methyl ester and converted to pyridine (V) and carbon dioxide in the usual manner.[10] Decarboxylation of the β-alanine derivative III was accomplished *via* the Schmidt reaction. Since III is an N-benzoyl amino acid, and the Schmidt reaction is carried out in concentrated sulfuric acid, the possibility existed that the evolved CO_2 might be diluted as a result of hydrolysis and subsequent decarboxylation of benzoic acid. We tested this possibility by preparing N-benzoyl-N-methyl-β-alanine from inactive nicotine and benzoyl chloride-7-^{14}C. In addition, β-alanine-1-^{14}C[22] was converted to N-methyl-β-alanine-1-^{14}C by way of the tosylate,[23] which after hydrolysis and benzoylation[24] provided N-benzoyl-N-methyl-β-alanine-1-^{14}C.[25] Each of the labeled compounds was treated with hydrazoic acid in sulfuric acid, and the evolved CO_2 was collected as barium carbonate and analyzed for radioactivity. Results of these experiments are summarized in Table I and clearly demonstrate that no significant contamination occurs during the Schmidt reaction. Therefore, the carbon dioxide obtained corresponds only to C-3′ of nicotine.

Table I. Schmidt Degradations of Specifically Labeled N-Benzoyl-N-methyl-β-alanines

Compound	Specific activity, dpm/mmole	% of total
N-Benzoyl-7-^{14}C-N-methyl-β-alanine	185,000	100
$BaCO_3$	900	0.5
N-Benzoyl-N-methyl-alanine-1-^{14}C	522,000	100
$BaCO_3$	539,000	105

(19) A. Pinner, *Ber.*, **27**, 1053 (1894).
(20) E. Spath and G. Bobenberger, *ibid.*, **77**, 362 (1944).
(21) R. U. Lemieux and E. von Rudloff, *Can. J. Chem.*, **33**, 1701 (1955).
(22) Purchased from New England Nuclear Corp.
(23) J. P. Greenstein and M. Winitz, "Chemistry of Amino Acids," Vol. 3, John Wiley and Sons, Inc., New York, N. Y., 1961, p 2756.
(24) J. L. O'Brien and C. Niemann, *J. Am. Chem. Soc.*, **79**, 80 (1957).
(25) The synthesis of this degradation product and the isolation of nicotinic acid fromthe periodate–per manganate oxidation established beyond question the structure of N-benzoylnicotine.

Scheme I. Degradation of Nicotine to Obtain C-2′ and C-3′



The residual solution from the Schmidt reaction on N-benzoyl-N-methyl-β-alanine was alkalinized and extracted to yield an amine fraction. Thin layer chromatography revealed several compounds to be present in this oil, and purification consisted only of molecular distillation; infrared analysis established the presence of amide and amino groups. The distilled material was treated with alkaline permanganate[26] and the acidic oxidation products were isolated. After hydrolysis, paper chromatography established the presence of glycine and sarcosine along with small amounts of methylamine and dimethylamine. The presence of glycine, resulting from hydrolysis of hippuric acid (IX), is readily accountable by invoking the intermediate cyclol VII to explain the migration of the benzoyl group. In subsequent experiments, the acidic fraction resulting from permanganate oxidation was applied to silica gel and separated into its various components by continuous elution with chloroform.[27] By this process, the major components, hippuric acid and benzoylsarcosine, were isolated from the reaction mixture in a ratio of 9:1. Hippuric acid may be conveniently degraded by hydrolysis, followed by conversion to dimethylglycine,[28] which on oxidation with lead tetraacetate yields the carboxyl as carbon dioxide and the methylene as formaldehyde.

By this procedure (III → IX) the maximum yield of hippuric acid was only 3%, and therefore 40 or 50 mmoles of nicotine would be required to complete the degradation. The poor steps in the sequence are the Schmidt reaction and subsequent oxidation. Accordingly, these reactions were examined in detail, using N-benzoyl-N-methyl-β-alanine-2-^{14}C,[29] and results demonstrated that both reactions led to several side products. Polymeric material was obtained during the Schmidt reaction, and some overoxidation resulted from the permanganate treatment since benzamide and N-methylbenzamide could be isolated. In spite of considerable effort, these reactions could not be improved sufficiently to allow further degradation. Only pyridine ring, C-2′, C-3′, and C-4′ plus C-5′ as hippuric acid (and in low yield) may be obtained by this series of reactions; accordingly, alternate degradation paths were sought.

Since N-benzoyl-N-methyl-β-alanine (III) was a readily obtained degradation product containing C-3′, -4′, -5′, and N-methyl of the pyrrolidine moiety of nicotine, it remained the focus of renewed degradative efforts.[30] The logical sequence still appeared to be removal of the carboxyl as carbon dioxide or some other unambiguous carbon unit and retention of the residual carbons as a glycine derivative.

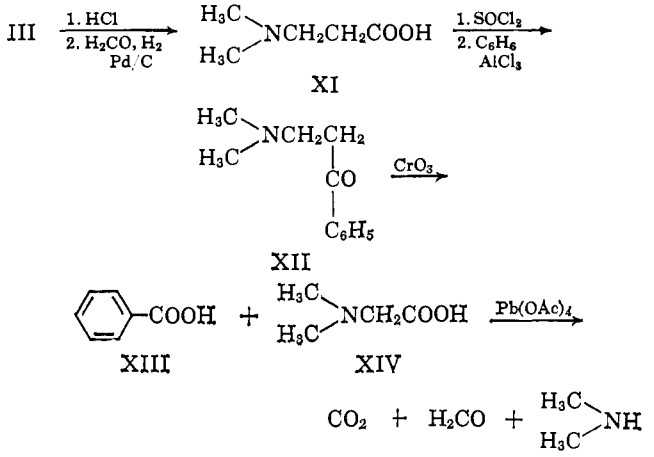
After a number of abortive attempts,[31] a highly convenient degradation was developed, as delineated in Scheme II. N,N-Dimethyl-β-alanine (XI) was prepared from the parent N-benzoyl-N-methyl-β-alanine by hydrolysis and reductive methylation.[28] Treatment of XI with thionyl chloride yielded the corresponding acid chloride, which when subjected to the Friedel–Crafts reaction in benzene afforded β-dimethylaminopropiophenone (XII). This product was identical with that obtained by the Mannich reaction of acetophenone, formaldehyde, and dimethylamine.[32]

Oxidation of this amino ketone XII was accomplished smoothly with chromium trioxide in acetic acid–trifluoroacetic acid. After isolation of benzoic acid (XIII), the chromium salts were conveniently

(26) E. F. Phares, *Arch. Biochem. Biophys.*, 33, 173 (1951).
(27) H. Rapoport and J. Bordner, *J. Org. Chem.*, 29, 2727 (1964).
(28) R. E. Bowman and H. H. Stroud, *J. Chem. Soc.*, 1342 (1950).
(29) Prepared from β-alanine-2-^{14}C which was purchased from Nuclear Research Chemicals, Inc.

(30) A degradation scheme for N-methyl-β-alanine has recently been presented [K. S. Yang, R. K. Gholson, and G. R. Waller, *J. Am. Chem. Soc.*, 87, 4184 (1965)] although no yields are given. It involves exhaustive methylation, elimination, and hydrogenation to propionic acid followed by two Schmidt reactions and two permanganate oxidations to complete the degradation. The multiplicity of steps and the nature of the intermediates (all liquids) made this procedure unattractive for repeated and small-scale analyses.
(31) Alternate approaches involved the preparation of N-benzoyl-N-methyl-β-aminopropiophenone (XV), 3-dimethylamino-1,1-diphenylpropanol (XVI), and 3-dimethylamino-1,1-diphenylpropene (XVII), but none of these could be developed into a consummate degradation. Their preparation is described in the Experimental Section.
(32) C. E. Maxwell, *Org. Syn.*, 23, 30 (1943).

Scheme II. Degradation of N-Benzoyl-N-methyl-β-alanine



precipitated by addition of bisulfite and alkali, and ion-exchange chromatography of the filtrate yielded only dimethylglycine (XIV) hydrochloride[33] which was purified by sublimation.

Glycine has previously been degraded (as the N-tosyl derivative) by Kolbe electrolysis,[34,35a] and dimethylglycine has also been treated in this manner.[35b] We found more convenient lead tetraacetate degradation[36] which provided each of the carbon atoms of dimethylglycine in good yields and in an unambiguous manner. The carboxyl was obtained as barium carbonate, the methylene as formaldehyde dimedone, and the N-methyl group as N,N-dimethyl-*p*-bromobenzenesulfonamide. The reaction sequence and yields in this degradation of the pyrrolidine ring of nicotine are summarized in Table II.

Before applying the total procedure to plant material, it was necessary to ensure that the integrity of each carbon of the pyrrolidine ring would be maintained throughout each step of the degradation. Since glycine-2-^{14}C was readily available, the corresponding N,N-dimethyl compound was prepared and was subjected to the conditions of the lead tetraacetate oxidation. As expected, the resulting formaldehyde dimedone derivative contained more than 99% of the initial radioactivity and the barium carbonate less than 1%.

To test the propiophenone preparation and its oxidation to benzoic acid and dimethylglycine, two specifically labeled propiophenones were prepared. N-Benzoyl-N-methyl-β-alanine-1-^{14}C was converted to β-dimethylaminopropiophenone (XII) labeled specifically at the carbonyl position, and Mannich reaction[32] between acetophenone, formaldehyde-^{14}C,[37] and dimethylamine hydrochloride gave β-dimethylaminopropiophenone-β-^{14}C. Chromic acid oxidation of these amino ketones and lead tetraacetate oxidation of the

(33) Initially, dimethylglycine was eluted from a basic ion-exchange column with acetic acid. Repeated evaporation of the eluate led to a greatly diminished yield of this volatile amino acid [L. A. A. Sluyterman and H. J. Veenendaal, *Rec. Trav. Chim.*, 70, 1049 (1951)].

(34) R. P. Linstead, B. R. Shepard, and B. C. L. Weedon, *J. Chem. Soc.*, 2854 (1951).

(35) (a) A. R. Battersby, H. M. Fales, and W. C. Wildman, *J. Am. Chem. Soc.*, 83, 4098 (1961); (b) W. C. Wildman, H. M. Fales, and A. R. Battersby, *ibid.*, 84, 681 (1962).

(36) The lead tetraacetate procedure used for these degradations was partially developed in these laboratories by Dr. I. Murakoshi and is based on the degradation of acylglycines [O. Sus and S. Rosenberger, *Ann.*, 564, 137 (1949)].

(37) Purchased from California Corporation for Biochemical Research.

Table II. Reaction Sequence and Yields in the Degradation of the Pyrrolidine Ring of Nicotine

Compound	→ Product(s)	Yield, %
Nicotine (I)	N-Benzoylmetanicotine (II)	70
N-Benzoylmetanicotine (II)	N-Benzoyl-N-methyl-β-alanine (III)	83
	Methyl nicotinate (IV, R = CH_3)	60
Methyl nicotinate (IV, R = CH_3)	Barium carbonate (C-2′)	50
	Pyridine (V)	60
N-Benzoyl-N-methyl-β-alanine (III)	N,N-Dimethyl-β-alanine (XI)	90
N,N-Dimethyl-β-alanine (XI)	β-Dimethylaminopropiophenone (XII)	95
β-Dimethylaminopropiophenone (XII)	Benzoic acid (C-3′) (XIII)	48
	Dimethylglycine (XIV) hydrochloride	30
Dimethylglycine (XIV) hydrochloride	Barium carbonate (C-4′)	50
	Formaldehyde dimedone (C-5′)	48
	N,N-Dimethyl-*p*-bromobenzenesulfonamide (N-CH_3)	24

Table III. Degradation of Specifically Labeled β-Dimethylaminopropiophenones

Compound	Specific activity, dpm/mmole	% of total
β-Dimethylaminopropiophenone-carbonyl-^{14}C	4,080	100
Benzoic acid	4,114	100
β-Dimethylaminopropiophenone-β-^{14}C	57,672	100
Benzoic acid	0	0
Barium carbonate (C-4′)	560	0.9[a]
Formaldehyde dimedone (C-5′)	57,154	99.1

[a] The small percentage of activity found in the carbon dioxide is probably the result of slight further oxidation of formaldehyde.

dimethylglycine resulting from the β-labeled compound provided the results in Table III, clearly establishing the integrity of each carbon through the degradation path. The form in which each carbon is counted is summarized in Figure 1.

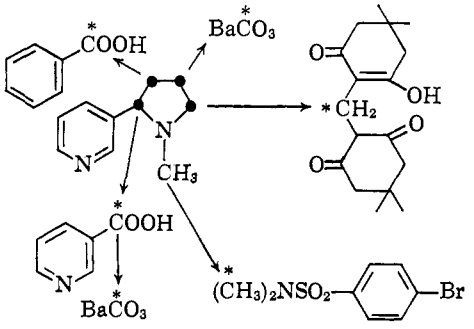


Figure 1. Isolation of the various carbon atoms of the pyrrolidine ring of nicotine.

Results and Discussion

A number of $^{14}CO_2$ biosyntheses were performed in order to test the hypothesis for pyrrolidine ring formation. Early experiments (aerial XII and root XIII) provided nicotine which was degraded by Scheme I

Table IV. Percentage of Total Nicotine Activity Found in Various Degradation Products after $^{14}CO_2$ Biosynthesis

Sample	Source	Specific activity, dpm/mmole[a]	Nicotine activity, %
Nicotine	XII aerial (70 days)	27,652	100
Methyl nicotinate		22,672	82.0
Pyridine		21,973	79.5
$BaCO_3$ (C-2′)		553	2.0
N-Benzoyl-N-methyl-β-alanine		4,654	16.8
$BaCO_3$ (C-3′)		473	1.7
N-CH_3		722[b]	2.6
C-4′ + C-5′ (by difference)			13.1[c]
Nicotine	XIII root (85 days)	50,198	100
Methyl nicotinate		39,134	78.0
Pyridine		38,050	75.8
$BaCO_3$ (C-2′)		1,398	2.8
N-Benzoyl-N-methyl-β-alanine		10,410	20.7
$BaCO_3$ (C-3′)		1,538	3.1
N-CH_3		2,540[b]	5.0
Hippuric acid (C-4′ + C-5′)		6,785	13.5
Nicotine	XIII aerial (85 days)	35,519	100
Methyl nicotinate		28,715	80.8
Pyridine (by difference)		28,200	79.4
$BaCO_3$ (C-2′)		496	1.4
N-Benzoyl-N-methyl-β-alanine		6,640	18.6
Benzoic acid (C-3′)		992	2.8
$BaCO_3$ (C-4′)		1,444	4.1
Formaldehyde dimedone (C-5′)		1,509	4.3
N,N-Dimethyl-*p*-bromobenzenesulfonamide		2,326	6.6
Nicotine	XIV root (53 days)	37,677	100
Methyl nicotinate		28,416	75.4
Pyridine		27,529	73.0
$BaCO_3$ (C-2′)		488	1.3
N-Benzoyl-N-methyl-β-alanine		9,396	24.9
Benzoic acid (C-3′)		2,130	5.6
$BaCO_3$ (C-4′)		2,249	5.9
Formaldehyde dimedone (C-5′)		2,098	5.6
N,N-Dimethyl-*p*-bromobenzenesulfonamide		2,945	7.8
Nicotine	XV seedling (6 days)	53,774	100
Methyl nicotinate		41,342	76.9
Pyridine		39,303	73.1
$BaCO_3$ (C-2′)		1,766	3.3
N-Benzoyl-N-methyl-β-alanine		11,967	22.2
Benzoic acid (C-3′)		2,903	5.4
$BaCO_3$ (C-4′)		2,617	4.9
Formaldehyde dimedone (C-5′)		2,798	5.2
N,N-Dimethyl-*p*-bromobenzenesulfonamide		3,522	6.5

[a] All scintillation counting was performed with a Nuclear-Chicago 720 series liquid scintillation instrument as previously described.[14] [b] N-Methyl determinations for experiments XII and root XIII were performed with samples of methyl iodide obtained by the Herzig–Meyer reaction as previously described.[14] [c] Average of 12.5, N-benzoyl-N-methyl-β-alanine − [(C-3′) + (N-CH_3)], and 13.7, nicotine − [methyl nicotinate + (C-3′) + (N-CH_3)].

and the data, therefore, are not complete for each individual carbon atom. However, the nicotine samples obtained in subsequent experiments (aerial XIII, root XIV, and seedling XV) were completely degraded by Scheme II. The results of these degradations are listed in Table IV.

In each of these experiments, biosynthesis was allowed to proceed for 6 hr and the CO_2 level in the exposure chamber[14] was maintained at the normal atmospheric concentration by the addition of $^{12}CO_2$ as needed. Each experiment utilized 10 mcuries of $^{14}CO_2$ per plant, generated in one pulse from barium carbonate whose carbon was over 90% ^{14}C. (Experiment XV, 6-day-old seedlings, utilized 10 mcuries of $^{14}CO_2$ for 30 g of fresh weight of plant material.) The factors that differed substantially in each experiment were the age and growth conditions of the plants. Experiment XII was performed with 70-day-old *N. glutinosa* grown hydroponically in a rudimentary greenhouse; subsequent experiments utilized plants grown in a considerably more efficient greenhouse. The chronological age of the plants used in exposure XIII was 85 days; however, they appeared to be considerably more advanced than plants of comparable age grown under the previous conditions. For exposure XIV, 53-day-old plants were chosen. *N. glutinosa* seedlings (exposure XV) were obtained by germinating 300 mg of seeds on a disk of Whatman seed-test paper, for 2 days in the dark. The seedlings were then maintained for 96 hr on alternating light and dark cycles of 12 hr each, on half-strength, nutrient solution.[14] After this time, the fresh weight of each disk was from 4–5 g and six of these disks were used for the exposure. Differences among these plants are summarized in Table V.

If these growth differences have an effect on the metabolism of the biological system, differences in the formation of a metabolite, such as nicotine, are not unexpected. However, from each experiment (Table IV), it is clear that the pyrrolidine ring labeling pattern can-

Table V. Comparison of *N. glutinosa* Used for Exposures XII–XV

Exposure	Plants, no.	Age, days	Height, cm	Leaves, no.	Length, Roots, cm	—Mass, g, per plant— Aerial	Roots
XII	4	70	30	10–11	10–15	45–50	8–10
XIII	5	85	45	12–14	25–30	70–75	24
XIV	3	53	15–20	8–10	20–25	30–35	15
XV		6	0.4–0.5	.....	.....	30 (total)	

not be symmetrical. For example, in expt XII, the total of C-2′ (2%) and C-3′ (1.7%) is 3.7% whereas C-4′ + C-5′ (by difference) is 13.1%. A similar representation is found in expt XIII (root). Here, C-2′ + C-3′ is 5.9% of the total and C-4′ + C-5′ (by direct determination) is 13.5%. The aerial nicotine obtained from this experiment shows 4.2% at C-2′ + C-3′ and 8.4% at C-4′ + C-5′. Again, from exposure XIV, the total at C-2′ + C-3′ is 6.9% and is 11.5% at C-4′ + C-5′. The labeling pattern resulting from *glutinosa* seedlings also shows this unsymmetrical feature since 8.7% of the total was incorporated at C-2′ + C-3′ and 10.1% at C-4′ + C-5′. Striking is the consistently low incorporation at C-2′ (1.3–3.3%).

From these data, considerable variation is seen in N-methyl incorporation and also in C-3′ incorporation. From expt XIV, the incorporation of ^{14}C into C-3′ is over three times the quantity found in expt XII, yet these samples were derived from plants closely related in size. However, the age of the plants differed considerably and, in addition, XII represents an aerial sample whereas XIV was obtained from roots. Evidence has been presented[14] for some independent synthesis of root and aerial nicotine. In samples to which the total degradation (Scheme II) was applied, an interesting similarity of incorporated label is seen at C-4′ and at C-5′.

The lack of symmetry in the pyrrolidine ring found in these experiments is a contradiction to the glutamate symmetrical intermediate hypothesis. A possible explanation is a species difference since experiments from which the symmetry postulate evolved were carried out with species other than *glutinosa*. This possibility was tested by hydroponically feeding ornithine-2-^{14}C[37] (50 μcuries, 26 mcuries/mmole) to four mature *N. glutinosa* plants.[14,38] A modification of the usual technique[39] was employed in that biosynthesis was allowed to proceed for only 6 hr, since data obtained from $^{14}CO_2$ exposures[14] showed that *de novo* nicotine synthesis occurs rapidly in *glutinosa*. With other species, the fact that ornithine does incorporate into nicotine is indisputable, and we felt that a reconciliation with $^{14}CO_2$ data might be obtained with a reasonably short-term biosynthesis. Also, biosynthesis as long as several days provides ample time for complete equilibration of various precursor pools, and any potential nonequilibrium differences will not be observed.

(38) It is of interest to note that the previously unreported administration of ornithine-5-^{14}C also appears to give symmetrical incorporation into the pyrrolidine ring. Although the total incorporation was considerably less than with ornithine-2-^{14}C, the two primary degradation products, N-benzoyl-N-methyl-β-alanine and methyl nicotinate, each contained approximately half of the incorporated label.

(39) E. Leete and K. J. Siegfried, *J. Am. Chem. Soc.*, 79, 4529 (1957).

Since a wealth of data has been gathered on ornithine incorporation into the pyrrolidine ring, the determination of the activity at C-2′ was the primary concern of this experiment. Uptake of the tracer was rapid and essentially quantitative. The incorporation into nicotine was 1.3% of the total ornithine activity administered, which is considerably higher than incorporation after a 7-day biosynthesis using intact *N. tabacum*.[39] The plants were separated into aerial and root portions and the nicotine obtained was degraded to provide the results listed in Table VI.

Table VI. Percentage of Total Nicotine Activity Found in Various Degradation Products after Ornithine-2-^{14}C Biosynthesis

Sample	Specific activity, dpm/mmole	Nicotine activity, %
Nicotine (aerial)	25,125	100
Methyl nicotinate	12,763	50.8
Pyridine	94	0.4
$BaCO_3$ (C-2′)	12,675	50.6
N-Benzoyl-N-methyl-β-alanine	13,122	52.3
Nicotine (root)	44,866	100
Methyl nicotinate	21,425	47.8
N-Benzoyl-N-methyl-β-alanine	23,238	51.8

From these results, we can only conclude that the pathway from exogenous ornithine to the pyrrolidine ring of nicotine is the same in all *Nicotiana* species tested. In addition, this is further evidence that ornithine does incorporate into the pyrrolidine ring *via* a symmetrical intermediate; but in view of the results from $^{14}CO_2$ biosynthesis, the relationship between ornithine incorporation and the true biosynthesis of the pyrrolidine ring is undefined.

Conclusions

The most significant conclusion that may be drawn from these results is that the labeling pattern of the pyrrolidine ring formed from $^{14}CO_2$ exposure is greatly different from that produced from precursor feedings. Since the $^{14}CO_2$ exposures represent normal growth conditions, it became conceivable that the labeling pattern *via* a symmetrical intermediate produced from precursor feedings might result from a minor or aberrant pathway. Examples of such biosynthetic pathways have been demonstrated.[40]

(40) The formation of L-ascorbic acid in strawberries from exogenous glucuronic acid derivatives was shown [F. A. Loewus, *Phytochemistry*, 2, 109 (1963)] to be greatly different than from glucose. Furthermore, the conversion of glucose to ascorbic acid in this system did not proceed by a pathway involving glucuronic acid, thus effectively demonstrating two independent biosyntheses of a metabolite. Similar phenomena

Previously, the symmetry feature observed in the pyrrolidine ring required nicotine biosynthesis to occur *via* bond formation between an intact pyrroline, derived from ornithine and the pyridine moiety.[30,41] The present experiments, by removing the requirement of a symmetrical intermediate for the pyrrolidine ring, also remove the requirement of the four-carbon unit combining intact. Thus a variety of possibilities must now be considered for the elaboration of nicotine from a pyridine moiety.

Significant differences are found in the labeling pattern of the pyrrolidine ring of nicotine derived from each exposure. This is not surprising, considering the variations among the plants used in these experiments. The most consistent data are the low level of incorporation at C-2′ which varies from 1.3 to 3.3%. The N-methyl incorporation shows a great amount of variation which is not unexpected. Excepting the aerial nicotine from expt XIII, the total incorporation of C-4′ + C-5′ is quite similar, *viz.* 13%. Furthermore, in each of the degradations where individual values were assigned, these two positions were almost equal in label. The combination of similar incorporation and the fact that in all cases C-4′ + C-5′ represent, 60–70% of the pyrrolidine ring total (N-methyl incorporation not being considered) may indicate an involvement of glycolic acid which has been found[42] to be equally labeled and to contain much of the early photosynthetically fixed carbon in tobacco leaves. Also, since 6-hr exposures were used, and the carbon dioxide concentration was maintained by adding $^{12}CO_2$, we do not know if low activity (at C-2′) means slow incorporation of ^{14}C, or rapid incorporation of ^{14}C followed by equally rapid incorporation of ^{12}C.

To eliminate the interpretive difficulties of the present work, future experiments will involve short-term steady-state[43] $^{14}CO_2$ exposures of *glutinosa* seedlings. We have found that a well-controlled environment (including temperature, humidity, and lighting) produces highly uniform growth of these seedlings. Ten grams of such seedlings (fresh weight derived from 600 mg of seeds, 6 days after seeding) incorporated 18,000 dpm into nicotine after 18 min of steady-state exposure at a $^{14}CO_2$ concentration of 0.04%. Thus a series of relatively short-term exposures under controlled, reproducible conditions is now feasible.

have been noted in alkaloid biosynthesis, *e.g.*, hyoscyamine formation in *Datura*, into which ornithine is unsymmetrically incorporated [E. Leete, *J. Am. Chem. Soc.*, 84, 55, (1962)] compared with symmetrical incorporation of putrescine;[8b] see also E. Leete and M. C. L. Louden, *Chem. Ind.* (London), 1725 (1963). Also, when codeine methyl ether is fed to *P. somniferum*, it is converted to codeine, but codeine methyl ether is not the normal precursor of codeine (G. Blaschke, this laboratory).

In the area of *Nicotina* alkaloid biosynthesis, the conversion of cadaverine to anabasine by a pea seed enzyme extract was shown [K. Mothes, H. R. Schutte, H. Simon, and F. Weygand, *Z. Naturforsch.*, 14b, 49 (1959)] to proceed in an entirely different manner than in *N. glauca* [E. Leete, *J. Am. Chem. Soc.*, 80, 4393 (1958)]. In addition, *N. glauca* incorporates cadaverine into the piperidine ring in a symmetrical fashion in contrast to a unsymmetrical incorporation of lysine.[6]

These examples all illustrate the fact that exogeneously supplied, related precursors can be incorporated into a metabolite *via* different biosynthetic paths and, therefore, their position in the actual biosynthetic sequence is tenuous.

(41) R. F. Dawson, D. R. Christman, A. D'Adamo, M. L. Solt, and A. P. Wolf, *ibid.*, 82, 2628 (1960).

(42) I. Zelitch, *J. Biol. Chem.*, 234, 3077 (1959); 240, 1869 (1965).

(43) See J. A. Bassham and M. Calvin, "The Path of Carbon in Photosynthesis," Prentice-Hall, Inc., Englewood Cliffs, N. J., 1957, for pertinent references.

Experimental Section[44]

Biosynthesis. *Nicotiana glutinosa* plants used for exposures XII–XIV were grown hydroponically and exposed to $^{14}CO_2$, and the nicotine was extracted and purified as previously described in detail.[10,14] The nicotine specific activity was determined by the liquid scintillation technique;[10] conversion to N-benzoylmetanicotine provided a solid derivative which showed essentially the same specific activity. Seedlings used for exposure XV were grown from 300 mg of seeds per dish. The seeds were allowed to germinate in the dark for 2 days on moist Whatman seed-test paper. After this time, the water was replaced by half-strength nutrient solution, and the seedlings were allowed to grow at constant temperature and humidity for alternating 12-hr periods of light and dark. Alkaloid isolation was the same as from mature plants.

N-Benzoylmetanicotine (II). The procedure of Späth and Bobengerger[20] was modified as follows. To nicotine (I), 5 mmoles (810 mg), was added a solution of 5.25 mmoles (740 mg) of benzoyl chloride in 5 ml of anhydrous xylene, and the mixture was magnetically stirred and heated at 145°, under nitrogen, for 20 hr. It was cooled to 100°, 25 ml of 10% hydrochloric acid was added, and the mixture was stirred for 0.5 hr, allowing the temperature to drop to 60°. The two phases were separated, and the aqueous layer was washed with 25 ml of benzene. The pH of the aqueous solution was adjusted to 11, and the resulting mixture was extracted with four 50-ml portions of methylene chloride. The total organic extract was shaken with two 30-ml portions of 2 *N* acetic acid to remove unreacted nicotine, followed by a 1 *N* sodium hydroxide wash. The solution was then dried, filtered, and evaporated to a crystalline residue of 976 mg (73%). Thin layer chromatography (silica gel, methanol elution) showed only one spot (red, R_f 0.78) when the plate was sprayed with 5% ethanolic *p*-aminobenzoic acid and exposed to cyanogen bromide vapor. Recrystallization was effected in greater than 90% recovery from cyclohexane or methylcyclohexane to yield white needles, mp 78–79° (lit.[21] mp 82°), of N-benzoylmetanicotine (II).

Anal. Calcd for $C_{17}H_{18}N_2O$: C, 76.7; H, 6.8; N, 10.5. Found: C, 76.8; H, 6.7; N, 10.4.

Oxidation of N-Benzoylmetanicotine (II). A. N-Benzoyl-N-methyl-β-alanine (III). N-benzoylmetanicotine (II), 10 mmoles (2.07 g), was dissolved in 4 l. of water containing 30 mmoles of anhydrous potassium carbonate, 80 mmoles of sodium metaperiodate, and 1.3 mmoles of potassium permanganate.[21] The mixture was stirred magnetically at room temperature until homogeneity resulted, and after a total reaction time of 20 hr, sufficient sodium bisulfite was added to reduce all of the iodine present to iodide. The acidic solution was evaporated at 50°, *in vacuo*, to a volume of 200 ml, which was then extracted continuously with methylene chloride for 24 hr. The extract was dried, filtered, and evaporated to a residual oil which was dissolved in 1 *N* sodium hydroxide solution (50 ml). Extraction with ether or methylene chloride removed any neutral impurities and, after reacidification, continuous extraction yielded 1.72 g (83%) of N-benzoyl-N-methyl-β-alanine (III). Recrystallization (80% recovery) from benzene–cyclohexane (3:1) gave colorless needles of mp 67–70°.

Anal. Calcd for $C_{11}H_{13}NO_3$: C, 63.7; H, 6.3; N, 6.7. Found: C, 63.5; H, 6.2; N, 6.7.

B. Nicotinic Acid (IV). The residual aqueous solution was then made alkaline to pH 9, extracted with methylene chloride to remove any unreacted starting material, and evaporated to dryness. Treatment as previously described[10] yielded methyl nicotinate (60%).

Decarboxylation of Methyl Nicotinate. The previously described technique[10] was modified in that the temperature used to effect decarboxylation of nicotinic acid was increased to 450°.

Decarboxylation of N-Benzoyl-N-methyl-β-alanine. A solution of 207 mg (1 mmole) of the acid III in 5 ml of concentrated sulfuric acid was swept with a slow stream of nitrogen, which then bubbled through 10 ml of a 5% solution of potassium permanganate[26] into a gas washing apparatus containing 6 ml of carbonate-free 0.1 *N* sodium hydroxide. With magnetic stirring, 1 ml of 1.03 *M* hydrazoic acid solution (in chloroform) was added at room temperature, and the decarboxylation vessel was heated to 70° over the course of 1 hr. This process was repeated three times providing a

(44) Each of the commercially available radiochemicals used in this work was subjected to paper chromatography and radioautography to verify the purity before use. Ultramicroanalyses were performed by the Microchemical Laboratory, Department of Chemistry, University of California, Berkeley.

total reaction time of 3 hr and 300 mole % excess of hydrazoic acid. The addition of saturated barium chloride solution to the alkali precipitated the evolved carbon dioxide as barium carbonate, which after thorough washing and drying at 80° (10 μ) weighed 78 mg (40%). A blank experiment, performed under exactly the same conditions, yielded less than 0.5 mg of barium carbonate.

The residual sulfuric acid–chloroform mixture was cooled to 0° and added with stirring to 30 ml of ice cold 6 *N* sodium hydroxide at a rate such that the internal temperature did not exceed 15°. After the addition was completed, 100 ml of water was added to provide homogeneity, and the solution was adjusted to pH 10 by the addition of phosphoric acid and then extracted continuously with methylene chloride for 48 hr. The extract was dried, filtered, and evaporated *in vacuo* to a residue of 47 mg of viscous oil. Short-path distillation [70° (10 μ)] resulted in a colorless oil showing infrared absorption at 3550, 3400 (amino), and 1640 cm^{-1} (amido). The partially purified oil was dissolved in 3 ml of 5% aqueous potassium permanganate and 0.5 ml of 0.5 *N* sodium hydroxide. The solution was heated at 95° for 20 min[26] and cooled, and excess permanganate was destroyed with 95% ethanol. The mixture was filtered through Celite, the precipitate was thoroughly extracted with boiling water, and the combined filtrates were acidified to pH 1 and extracted continuously with methylene chloride for 18 hr. The extract was dried, filtered, and evaporated, leaving a residue of 43 mg. A small portion was hydrolyzed with 10% hydrochloric acid, and paper chromatography of the hydrolysate (Whatman No. 4, descending, phenol–water, 4:1, ninhydrin detection) established the presence of glycine (R_f 0.42) and sarcosine (R_f 0.68). The remainder was applied to 50 g of silica gel and eluted with chloroform for 16 hr,[27] which effectively removed benzoylsarcosine (less than 1 mg). Elution was continued with methanol, and the first 200 ml of eluate was evaporated to a solid residue. This material was dissolved in 10 ml of pH 9 phosphate buffer and was extracted with four 20-ml portions of chloroform to remove benzamide. The aqueous solution was acidified and extracted with chloroform continuously for 2 hr. After drying, the extract was evaporated to a residue of 6 mg (3%) of hippuric acid which was recrystallized from petroleum ether (30–60°)–absolute ethanol to give material, mp 184°. Paper chromatography of a hydrolyzed sample showed only glycine to be present.

N-Benzoyl-N-methyl-β-alanine-2-^{14}C. β-Alanine-2-^{14}C,[29,44] 100 μcuries, 0.54 mcuries/mmole, was diluted with inactive β-alanine to a total of 20 mmoles which was converted to the tosylate in 62% yield in the described manner.[23] To 920 mg of N-tosyl-β-alanine-2-^{14}C so obtained was added 1.410 g of inactive material, making a total of 10 mmoles. The compound was dissolved in 12 ml of 3 *N* sodium hydroxide and heated with 1.633 g (11.5 mmoles) of methyl iodide in a sealed tube at 67° for 1.5 hr as described.[23] Isolation of the product in the usual manner followed by crystallization from water yielded material which softened at 71–74° and melted at 91–98°. The literature records two melting points for N-methyl-N-tosyl-β-alanine, 73–74°[45] and 110–111°.[46] To ensure complete reaction, our product was retreated under the methylation conditions and after crystallization from 10% ethanol showed mp 110–111°.

Anal. Calcd for $C_{11}H_{15}NO_4S$: C, 51.3; H, 5.9; N, 5.5. Found: C, 51.2; H, 5.8; N, 5.7; specific activity, 4.8 × 10^6 dpm/mmole.

N-Methyl-N-tosyl-β-alanine-2-^{14}C (1.2 g, 4.7 mmoles) was heated with 3.75 ml of concentrated hydrochloric acid in a sealed tube at 100° for 22 hr. The tube was then cooled, and the contents were removed with the aid of 15 ml of water rinse. The pH of the solution was adjusted to 5.5; the solution was then applied to 100 ml of AG 50W-X4, 200–400 mesh, H^+ ion-exchange resin. The column was washed with water until the eluate was neutral, and the product was obtained by elution with 1.5 *N* ammonium hydroxide, being detected in the eluate with ninhydrin. The solution, containing ammonium N-methyl-β-alanate, was evaporated at reduced pressure to a residue of 453 mg. This product was dissolved in 3.8 ml of water containing sodium hydroxide (424 mg, 10.6 mmoles), and the solution was cooled in ice–water. Benzoyl chloride (747 mg, 5.3 mmoles) was added dropwise over a 20-min period, and the isolation of the product was carried out as described.[24] N-Benzoyl-N-methyl-β-alanine-2-^{14}C was obtained in over-all yield of 50% from the N-methl-N-tosyl compound; specific activity, 4.7 × 10^6 dpm/mmole.

N-Benzoyl-N-methyl-β-alanine-1-^{14}C. β-Alanine-1-^{14}C,[22] by exactly the same process, was converted to N-benzoyl-N-methyl-β-alanine-1-^{14}C; specific activity, 522,000 dpm/mmole.

N-Benzoyl-N-methyl-β-aminopropiophenone (XV). N-Benzoyl-N-methyl-β-alanine (763 mg, 3.7 mmoles) was mixed with 1.1 ml of thionyl chloride under a nitrogen atmosphere. The reaction mixture was heated on the steam bath for 15 min, after which time it was allowed to remain at room temperature for 16 hr. Excess thionyl chloride was removed at reduced pressure, and the crude acid chloride, dissolved in 5 ml of benzene, was injected into a nitrogen-filled reaction flask containing aluminum chloride (2.0 g, 15.1 mmoles) and 7 ml of benzene. Stirring was continued at ice–water temperature for 2 hr, after which time the reaction mixture was poured into a cooled aqueous solution of phosphoric acid (2 ml of concentrated phosphoric acid and 23 ml of water). The acidic solution was extracted (four 30-ml portions) with chloroform, and the chloroform solution was washed with saturated aqueous bicarbonate (two 30-ml portions). Two chloroform extractions (20 ml each) of the bicarbonate solution followed, and the combined chloroform extracts were dried, filtered, and evaporated to yield 891 mg of a clear yellow oil. Distillation [150° (1 μ)] resulted in 736 mg (79%) of pure oily ketone XV.

Anal. Calcd for $C_{17}H_{17}NO_2$: C, 76.4; H, 6.4; N, 5.2. Found: C, 76.0; H, 6.1; N, 5.2.

A 2,4-dinitrophenylhydrazone was prepared and, after recrystallization from ethanol–acetone, melted at 222–224°.

Anal. Calcd for $C_{23}H_{21}N_5O_5$: C, 61.7; H, 4.7; N, 15.7. Found: C, 61.3; H, 4.3; N, 15.8.

β-Dimethylaminopropiophenone (XII). N-Benzoyl-N-methyl-β-alanine (III) (1.87 g, 9.0 mmoles) was mixed with 30 ml of 10% hydrochloric acid, and the reaction mixture was heated at reflux for 20 hr. Cooling resulted in crystallization of benzoic acid. This was filtered, and the aqueous solution was extracted with ether to remove any remaining benzoic acid. The acidic aqueous solution was evaporated to dryness, leaving crystalline N-methyl-β-alanine hydrochloride which was dissolved in 45 ml of water, and to this was added 3 g of 36% formaldehyde and 1.35 g of 10% palladium on carbon. The mixture was hydrogenated for 18 hr as described[28] and yielded 1.25 g (91%) of crystalline N,N-dimethylamino-β-alanine (XI) hydrochloride. A 461-mg (3 mmoles) sample of XI was stirred under a nitrogen atmosphere with 2 ml of thionyl chloride. The reaction mixture was heated to about 50°, and heating and stirring were continued until all the salt was dissolved or for 0.5 hr maximum. The crude reaction mixture was evaporated to dryness, and to the residue was added 2.0 g (15 mmoles) of aluminum chloride. Benzene (20 ml) was injected into the reaction flask, and the mixture was allowed to stir at room temperature for 2 hr. The product was isolated by pouring the reaction mixture into ice–water, adjusting the pH to 10, and extracting with methylene chloride. Evaporation of the dried methylene chloride solution resulted in 90–100% yields of amino ketone XII which exhibited identical properties by tlc [silica gel, methanol–chloroform (1:1) elution, iodine detection] with authentic material prepared by the Mannich reaction of acetophenone, formaldehyde, and dimethylamine.[32]

β-Dimethylaminopropiophenone-β-^{14}C. During the preparation of the Mannich ketone,[32] a trace of aqueous formaldehyde-^{14}C was added to the solution. The amino ketone hydrochloride that was isolated was recrystallized from 2-propanol to a constant specific activity of 57,672 dpm/mmole.

3-Dimethylamino-1,1-diphenylpropanol (XVI). The amino ketone XII (1 mmole) was dissolved in 10 ml of anhydrous ether, and the solution was stirred at room temperature while 1.5 ml (*ca.* 3.25 mmoles) of an etheral solution of phenyllithium was added. After 1 hr, water was carefully added, and the resulting two layers were separated, the aqueous layer being extracted several additional times with methylene chloride. The combined ether and methylene chloride solutions were dried over potassium carbonate and filtered, and the filtrate was evaporated to give 181 mg (62%) of a crystalline product, mp 107–120°. Recrystallization from hot ethanol yielded the pure propanol XVI, mp 165°.

Anal. Calcd for $C_{17}H_{21}NO$: C, 80.0; H, 8.3; N, 5.5. Found: C, 80.0; H, 8.2; N, 5.6.

3-Dimethylamino-1,1-diphenylpropene (XVII). The propanol XVI (500 mg, 1.95 mmoles) was mixed with 1.5 ml of acetic anhydride (*ca.* 12 mmoles), and the mixture was heated to reflux. After 5 hr, the excess acetic anhydride was removed at reduced pressure, and the residue was made alkaline and extracted several times with chloroform (total volume of 75 ml). The combined ex-

(45) G. S. Misra and R. S. Asthana, *J. Prakt. Chem.*, **4**, 270 (1957).
(46) E. Späth, P. P. Wibaut, and F. Kesztler, *Ber.*, **71**, 100 (1938).

tracts were dried, filtered, and evaporated to yield 400 mg of an oil; λ_{max}^{EtOH} 250 mμ; homogeneous by tlc.

Chromic Acid Oxidation of β-Dimethylaminopropiophenone (XII). The amino ketone XII (5.0 mmoles) was mixed with 10 ml of trifluoroacetic acid, and to the solution was slowly added over a period of 1 hr a mixture prepared from chromium trioxide (2.5 g, 25 mmoles), water (4 ml), and glacial acetic acid (40 ml). After the addition was complete, the reaction mixture was heated at 50° with stirring for 3 hr. The mixture was evaporated at reduced pressure and 50°, leaving a residue which was dissolved in 100 ml of 5% sulfuric acid and then was extracted with five 25-ml portions of ether. The combined extracts were filtered and evaporated, and the addition of bisulfite to an alkaline solution of the residue precipitated chromium salts. After filtration, acidification followed by extraction into ether yielded benzoic acid which was purified by sublimation (290 mg, 48%).

The original aqueous solution was treated with 4 g of sodium bisulfite, adjusted to pH 10, and filtered, and the filtrate was washed with methylene chloride. The aqueous solution was then adjusted to pH 6 and applied to 175 ml of AG 50W-X8, 200–400 mesh, hydrogen form, cation-exchange resin. The column was washed with water until the eluate was neutral and then with 1.5 *N* ammonium hydroxide. The first 100 ml of basic eluate was evaporated at 50° and reduced pressure until the pH of the solution was about 8. The solution was then applied to 100 ml of AG 1-X8, 200–400 mesh, hydroxide form, anion-exchange resin. Again, the column was washed with water until the eluate was neutral and then with 2 *N* hydrochloric acid. The first 100 ml of acidic eluate was evaporated to a semisolid residue, water was added, and the process repeated until all of the excess hydrochloric acid was removed, leaving a crystalline residue of N,N-dimethylglycine (XIV) hydrochloride, 200 mg, 30%. This product was purified by sublimation [115° (10 μ)] to yield material, mp 182–183°.

Anal. Calcd for $C_4H_{10}ClNO_2$: C, 34.4; H, 7.2; N, 10.0. Found: C, 34.7; H, 7.4; N, 10.0.

Lead Tetraacetate Oxidation of Dimethylglycine (XIV) Hydrochloride. Dimethylglycine hydrochloride (100 mg, 0.72 mmole) was mixed with 12 ml of benzene, and lead tetraacetate (450 mg, 1.0 mmole) was added to this heterogeneous mixture. While maintaining a sweep of dry nitrogen through the apparatus and with stirring, the reaction vessel was heated to 55°. The liberated carbon dioxide was swept into a gas washing vessel containing 8 ml of 0.1 *N* carbonate-free sodium hydroxide. Gas evolution was complete after 2 hr, and the carbon dioxide was obtained in the usual manner as barium carbonate, 60 mg, 40%. A parallel reaction, omitting the dimethylglycine, gave less than 1 mg of barium carbonate.

The gas washing vessel was removed and replaced by a distillation take-off, whose tip was immersed in a solution (6 ml) prepared from 5.6 g of 5,5-dimethyl-1,3-cyclohexanedione dissolved in 100 ml of 50% ethanol. Water (5 ml) was added to the benzene residue, and while maintaining a slow nitrogen sweep, the mixture was heated to 150–160°. When almost dry, an additional 10 ml of water was added and distillation was continued. This process was repeated twice more, and the total distillate was then evaporated at 50° and reduced pressure after standing at room temperature for 24 hr. The residue was recrystallized several times from 95% ethanol to yield pure formaldehyde dimedone derivative, 100 mg, 48%, mp 192°.

The oxidation vessel was now equipped with a dropping funnel containing 15 ml of 20% sodium hydroxide solution. The distillation take-off was replaced with a gas washing vessel containing 10 ml of 5% hydrochloric acid. While maintaining the nitrogen stream, the alkali was added to the residue in the oxidation vessel which was then heated to 70°. After 2 hr, the acidic wash solution was removed and evaporated at reduced pressure to a residue of 20 mg (34%) of dimethylamine hydrochloride.

The amine salt, 16.2 mg (0.22 mmole), dissolved in 100 μl of water, was added to a solution of *p*-bromobenzenesulfonyl chloride (54 mg, 0.22 mmole) in 80 μl of *p*-dioxane, and 280 μl of 10% aqueous potassium hydroxide was added. The mixture was then shaken for 30 min, an additional 280 μl of the potassium hydroxide solution was added, and the mixture was warmed in a water bath at 55–60° for 2 hr with occasional shaking. The solid was removed, washed with water until the washings were no longer alkaline, dried *in vacuo* at room temperature, and then sublimed [70° (10 μ)] to yield N,N-dimethyl-*p*-bromobenzenesulfonamide, 38 mg, 72%, mp 90–92° (lit.[47] mp 94°).

(47) C. S. Marvel and F. E. Smith, *J. Am. Chem. Soc.*, **45**, 2696 (1923)

Microbiological Hydroxylation of Monocyclic Alcohols

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Abstract: As part of a broad study of the microbiological oxygenation of simple monocyclic systems, cyclododecanol exposed to *Sporotrichum sulfurescens* gave a mixture of dioxygenated products that was oxidized to cyclododecane-1,5-dione, cyclododecane-1,6-dione, and cyclododecane-1,7-dione. The relative yields of the isomeric diones can be rationalized on conformational grounds and suggest a hypothetical enzyme–substrate model in which oxygenation occurs at a methylene group about 5.5 A from the electron-rich substituent of the substrate. Oxygenations of cyclotridecanol to cyclotridecane-1,7-dione and of cyclotetradecanol to cyclotetradecane-1,6-dione were also observed. Cyclohexanol oxygenation *per se* was not seen, but the more lipophilic N-phenylcarbamate was oxygenated to the 4-hydroxy derivative.

The oxygenation of an unactivated methylene group is a reaction at which microorganisms are still more adept than organic chemists, in spite of many advances made in recent years by the macroorganisms. This difference has been widely exploited during the past 15 years to oxygenate microbially a variety of steroids,[1a] and somewhat less widely to oxygenate terpenes,[1b] hydrocarbons,[1c] alkaloids,[1d] and other substances.[1e]

(1) As leading references, see: (a) E. Vischer and A. Wettstein, *Advan. Enzymol.*, **20**, 237 (1958); S. H. Eppstein, P. D. Meister, H. C. Murray, and D. H. Peterson, "Vitamins and Hormones," Vol. XIV, Academic Press Inc., New York, N. Y., 1956, pp 359–432; O. Hanc, "Global Impacts of Applied Microbiology," M. P. Starr, Ed., John Wiley and Sons, Inc., New York, N. Y., 1963, p 420; (b) P. K. Bhattacharyya, B. R. Prema, B. D. Kulkarni, and S. K. Pradhan, *Nature*, **187**, 689 (1960); P. J. Chapman, G. Meerman, I. C. Gunsalus, R. Srinivasan, and K. L. Rinehart, Jr., *J. Am. Chem. Soc.*, **88**, 618 (1966); (c) G. W. Fuhs, *Arch. Mikrobiol.*, **39**, 374 (1961); (d) E. Meyers and S. C. Pan, *J. Bacteriol.*, **81**, 504 (1961); G. D. Griffith, R. U. Byerrum, and W. A. Wood, *Proc. Soc. Exptl. Biol. Med.*, **108**, 162 (1961); (e) D. J. Siehr, *J. Am. Chem. Soc.*, **83**, 2401 (1961); V. Prelog and H. E. Smith, *Helv. Chim. Acta*, **42**, 2624 (1959).