[CONTRIBUTION FROM DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY, CAMBRIDGE, MASS., AND THE DEPARTMENT OF BOTANY, COLUMBIA UNIVERSITY, NEW YORK, N. Y.]

The Asymmetric Incorporation of Isotopic Label in the Biogenesis of Hyoscyamine[1]

BY A. A. BOTHNER-BY,[2] R. S. SCHUTZ,[3] R. F. DAWSON AND M. L. SOLT

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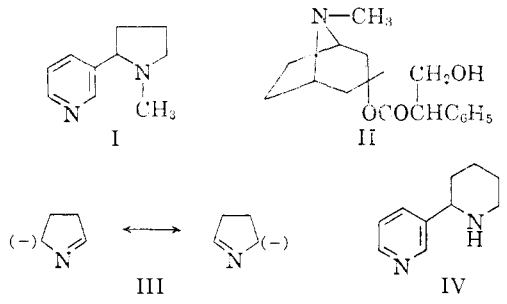
The distribution of label in hyoscyamine biosynthesized by roots of *Datura stramonium* from acetate-1-C^{14} was studied. One bridgehead carbon atom is labeled stereospecifically. A symmetrical precursor, such as occurs in the biosynthetic pathway for nicotine, thus does not occur in the pathway for hyoscyamine.

Introduction.—Considerable evidence[4-13] has accumulated concerning the biosynthetic incorporation of labeled precursors in the molecular skeletons of alkaloids. It is now possible to state, *e.g.*, that the pyrrolidine rings of nicotine (I) and of hyoscyamine (II) can arise from the ubiquitous amino acid metabolite, ornithine. Thus, Dewey, Byerrum and Ball,[4] Leete,[5] and Leete and Siegfried[6] have shown that when whole tobacco plants (*Nicotiana tabacum* L.) are grown on a medium containing ornithine-2-C^{14} they produce nicotine labeled at positions 2 and 5 of the pyrrolidine ring. In similar experiments using *Datura stramonium* L., Leete, Marion and Spenser[14] have demonstrated that ornithine-2-C^{14} is incorporated into hyoscyamine biosynthetically, the C^{14} appearing as bridgehead carbon. When the alkaloid was degraded they did not, however, ascertain whether incorporation of label was asymmetric, *i.e.*, whether all of the label appeared at one of the two diastereoisomeric bridgehead positions. In the case of nicotine it seems clear that a symmetrical intermediate must occur somewhere in the pathway leading from ornithine. Leete has pointed out that incorporation of the label into positions 2 and 5 of the pyrrolidine ring of nicotine might occur through the intervention of some such intermediate as III.

In the case of anabasine (IV), Leete has demonstrated that the piperidine ring may be formed biosynthetically from lysine. When lysine-2-C^{14} is administered to whole plants of *Nicotiana glauca* Grah. anabasine is formed in which all of the incorporated C^{14} appears in the piperidine ring carbon attached to the pyridine ring. In this case, therefore, no symmetrical intermediate analogous to III appears in the biosynthetic pathway.



I II III IV

It seemed desirable to learn whether such a symmetrical intermediate occurs in the biosynthesis of hyoscyamine by *Datura stramonium*.

Method.—Such a determination appeared feasible when based on the following approach. Hyoscyamine was to be prepared biosynthetically from acetate-1-C^{14} using cultures of excised *Datura stramonium* L. roots. We anticipated that C^{14} from this source would be incorporated into ornithine *via* established metabolic pathways of the Krebs cycle and amino acid interconversions according to the flow sheet outlined in Fig. 1. The ornithine would then be incorporated into hyoscyamine with possible stereospecific introduction of label at one bridgehead (Fig. 2). In order to detect asymmetric label, if it occurred, we proposed to degrade the alkaloid isolated from root cultures by the series of steps shown in Fig. 3. The crucial stage in this degradation was the resolution of the dimethylaminocycloheptadiene (α-methyltropidine). If it is supposed for the purpose of argument that label is introduced exclusively in position 5 of the hyoscyamine, then it can be deduced that one of the optical isomers of the α-methyltropidine should contain no activity in the carbon of the 7-membered ring attached to the nitrogen while the other optical isomer should contain all of the original bridgehead activity in this position.

The supposition that acetate-1-C^{14} could be incorporated into ornithine and that this could lead to the appearance of label in the expected positions has received support from the findings of Leete[15] and Griffith and Byerrum.[16] They observed just such a pattern of label in nicotine prepared biosynthetically from acetate-1-C^{14}.

For the purpose of preparing labeled hyoscyamine we supplied a total of 2.5 mc. (0.25 mg.) of

(1) Abstracted from a thesis presented by R. S. Schutz to Harvard University in partial fulfillment of the requirement for the degree of Doctor of Philosophy, April, 1959.

(2) Present address: Mellon Institute, 4400 Fifth Ave., Pittsburgh 13, Pa.

(3) Present address: Yeshiva University, Amsterdam Ave., and 186th St., New York 33, N. Y.

(4) J. L. Dewey, R. U. Byerrum and C. D. Ball, *Biochem. Biophys. Acta*, **18**, 141 (1955).

(5) E. Leete, *Chemistry & Industry*, 537 (1955).

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

(7) "Biochemie und Physiologie der Alkaloide," edited by K. Mothes, *Abhandl. Deutsch. Akad. Wissensch. (Berlin), Klasse für Chemie, Geologie u. Biologie*, **7**, 1956; publ. Akad. Verlag, Berlin, 1957.

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(9) E. Leete, *ibid.*, 977 (1958).

(10) G. Fodor, A. Romeike, G. Janszo and I. Koczor, *Tetrahedron Letters* **No. 7**, 19 (1959).

(11) D. Gröger, H. J. Wendt, K. Mothes and F. Weygand, *Z. Naturforsch.*, **14b**, 355 (1959).

(12) A. Romeike, *Naturwissensch.*, **16**, 492 (1959).

(13) R. F. Dawson, D. R. Christman, A. D'Adamo, M. L. Solt and A. P. Wolf, *J. Am. Chem. Soc.*, **82**, 2628 (1960).

(14) E. Leete, L. Marion and I. D. Spenser, *Can. J. Chem.*, **32**, 1116 (1954).

(15) E. Leete, *J. Am. Chem. Soc.*, **80**, 2162 (1958); *Chemistry & Industry*, 1477 (1958).

(16) T. Griffith and R. U. Byerrum, *Science*, **129**, 1485 (1959).

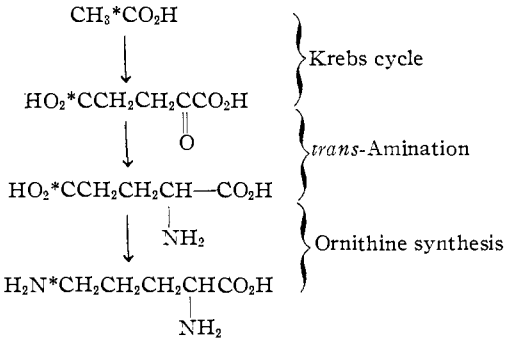

Fig. 1.

sodium acetate-1-C^{14} to approximately 300 microbially sterile cultures of the excised roots of *Datura stramonium* L. After a suitable incubation period the crude alkaloid was extracted from the root tissues and the spent culture fluids and added directly to inactive atropine carrier. The method of degradation as outlined in Fig. 3 was applied directly to this mixture.

The degradation products illustrated in this scheme, Fig. 3, were subjected to radioactivity analysis. The specific activities on a molar basis for two runs are given in Table I. In one case the degradation was carried out without resolution of the α-methyltropidine so that the specific activities represent the average activities of the carbons in corresponding positions on opposite sides of the mirror plane of symmetry of the tropane skeleton. In the second run the degradation was performed upon one pure diastereoisomer of α-methyltropidine so that the radioactivities represent single activities for one set of carbons on one side of the mirror plane of symmetry.

TABLE I

RADIOACTIVITY ASSAY OF PRODUCTS FROM THE DEGRADATION OF HYOSCYAMINE

Run	Compound	Specific activity[a] and probable error
I (racemic)	α-Methyltropidine[b]	48,500 ± 2500
	Cycloheptanone[c]	48,200 ± 2500
	Chlorobenzoic acid	4,600 ± 200
II (resolved)	α-Methyltropidine[d]	45,500 ± 2900
	Chlorobenzoic acid	150 ± 40

[a] Specific activity is reported as counts/minute/millimole. [b] As picrate. [c] As 2,4-dinitrophenylhydrazone. [d] As acid salt of dibenzoyltartaric acid.

Examination of the data as obtained reveals two interesting facts: (1) Since no significant amount of activity occurred in the chlorobenzoic acid obtained from the resolved diastereoisomer, it is apparent that all of the activity of the racemic material was concentrated at one of the two bridgehead positions; (2) there is considerable activity in positions other than the bridgehead.

The first fact means that under our conditions the biosynthetic pathway to hyoscyamine did not involve an intermediate in which the bridgehead carbons were symmetrically equivalent. It thus appears that the pathway of nicotine biosynthesis, in which such a symmetrical intermediate does occur, is the anomalous case.

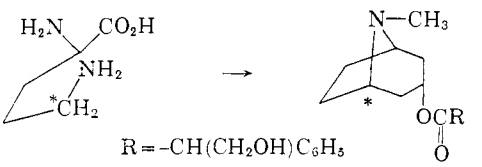

Fig. 2.

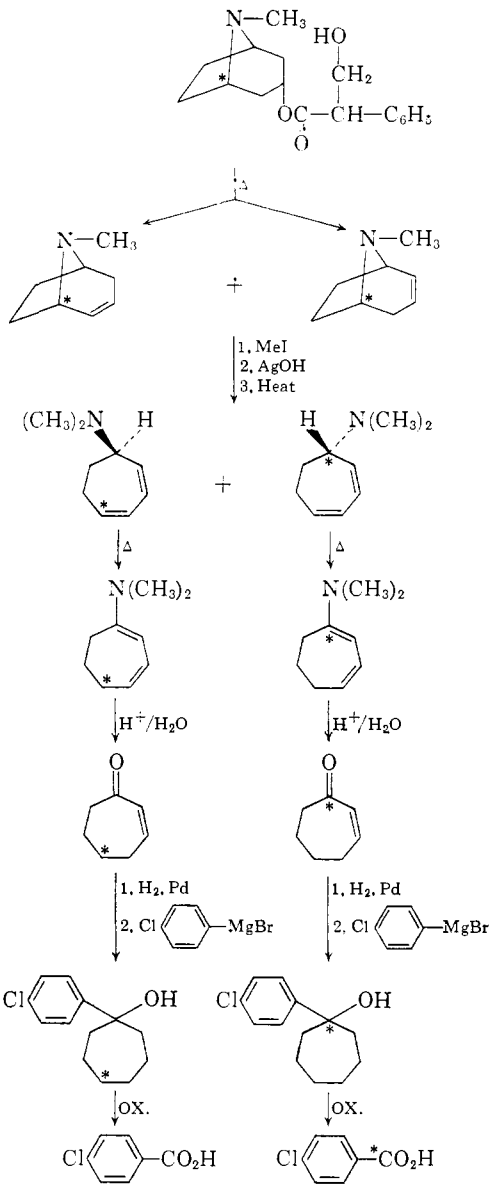

Fig. 3.

The second fact is compatible with the recent findings of Kaczkowski, Schütte and Mothes[17] on the distribution of label in hyoscyamine obtained by administration of acetate-1-C^{14} to *Datura metel*. They observed mainly incorporation into the C2–C3–C4 section of the tropane skeleton.

Leete[18] has recently performed experiments

(17) J. Kaczkowski, H. R. Schütte and K. Mothes, *Naturwiss.*, **47**, 304 (1960).

(18) E. Leete, private communication. We greatly appreciate Dr. Leete's kindness in informing us of his results prior to publication

similar to those reported above using ornithine-2-C^{14}, obtaining results in consonance with ours.

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Experimental

Growth of *Datura stramonium* and Administration of Acetate 1-C^{14}.—Seeds of *Datura stramonium* L., commonly known as Jimson weed, were obtained from Dr. D. M. Crooks of the U. S. Department of Agriculture at Beltsville, Md. The seeds were surface sterilized with mercuric bichloride in alcohol and germinated in sterile cultures. The roots which emerged from the seeds during germination were removed under sterile conditions with a scalpel and cultured thereafter in a solution of the following composition: (1) The major salt solution of P. R. White[19]; (2) to which was added 0.250 mg. of ferric sulfate plus 0.100 mg. of tartaric acid per liter; (3) 20 g. of recrystallized sucrose per liter; (4) vitamins as follows: thiamine hydrochloride 0.37 mg., nicotinic acid 0.123 mg., calcium pantothenate 0.47 mg., pyridoxine 0.21 mg., inositol 0.0216 mg. per liter.

The roots grew rapidly in this medium and were subcultured at frequent intervals by subdivision into many small segments, each segment containing two or three branch roots. In such a way the material can be subcultured indefinitely under entirely sterile conditions. Temperature of incubation was 28–30°.

A total of 294 cultures, each culture containing one root in 30 ml. of solution in a 125-ml. erlenmeyer flask, were given a total of 0.25 mg. of sodium acetate-1-C^{14} with a total activity of 2.5 mc. The sodium acetate was sterilized by filtration through a Pyrex UF disk and transferred aseptically into cultures. The roots were grown for about 5 days after each transfer before addition of the sodium acetate. The period of feeding was roughly speaking 9 and 10 days in all cases.

The roots were harvested as follows: The roots and the remaining culture solutions were poured into a Büchner funnel and the root mass washed with distilled water. The filtrates were reduced to low volume under reduced pressure. The roots were ground with sand in a mortar and extracted twice with water, once with dilute sodium hydroxide and finally with hot water. The extracts were filtered with Hyflo-Super-Cel, and the filtrates were combined with the concentrated residual culture solutions. The combined fluids were made alkaline with potassium hydroxide and continuously extracted in a liquid–liquid extractor with ether. The residues from the ether extraction were transferred quantitatively to a small screw-cap vial by batchwise transfer and evaporation of solvent.

Pyrolysis of Atropine and Preparation of Atropine Methiodide.—The pyrolysis was carried out in a sausage flask, the outer jacket of which was filled with concentrated sulfuric acid as a heat transfer medium. The receiver was provided with an outlet tube which was connected to a water aspirator, and was cooled with ice–salt mixture. The vacuum was maintained at 80–120 mm. by means of a needle valve. The total capacity of the inner vessel of the sausage flask was about 15 cc., but in practice the total charge was limited to about 5 g. of atropine and 2 cc. of mineral oil by a tendency to foam, particularly in the early stages. The use of mineral oil increased the yield considerably. Most of the distillate came over in the range 230–260°, but heating was generally continued to 280–300° to assure maximum yield. The product was then washed out of the receiver with ethyl acetate, dried over sodium sulfate, and treated with a considerable excess of methyl iodide. Formation of the methiodide was rapid and quickly completed. The filtration characteristics of the precipitate were superior if it was allowed to stand overnight in the refrigerator. The yield from 5 g. of atropine was generally about 3.8 g. of tropidine methiodide.

The radioactive alkaloid was diluted with 500 mg. of inactive carrier, pyrolyzed and the product quaternized by the above method, yielding 501 mg. of radioactive tropidine methiodide. The specific activity[20] of the methiodide was 2.5×10^6 counts per minute per millimole.

d,l-α-Methyltropidine.—The reaction was carried out by a procedure similar to that of Merling.[21] A solution of 2 g. of tropidine methiodide in 10 ml. of water was treated for 15 min. on the steam-bath with silver oxide freshly prepared from 7 g. of $AgNO_3$. The suspension was filtered directly into a distilling flask and distilled to near dryness. Additional water was added and distilled to carry over the last portions of product. The distillate was made strongly alkaline with concentrated sodium hydroxide solution and extracted with ether. Evaporation of the ether yielded α-methyltropidine; average yield 0.8–0.9 g.

The α-methyltropidine yielded a picrate, m.p. 149.5–150°. *Anal.* Calcd. for $C_{15}H_{18}N_4O_7$: C, 49.12; H, 4.95; N, 15.19. Found: C, 49.8; H, 4.96; N, 15.12.

Resolution of α-Methyltropidine.—d,l-α-Methyltropidine (0.40 ml.) and d-O,O-dibenzoyltartaric acid[22] (0.50 g.) were dissolved in 2 ml. of ethanol. After energetic and prolonged scratching a thick crystalline sludge formed. More ethanol was added and the suspension was filtered to yield 0.34 g. of salt, m.p. 131–136°. One recrystallization from ethyl acetate afforded 0.26 g., m.p. 131–131.5°. Further recrystallizations raised the m.p. to 132.8–133.0°. Prolonged heating of ethyl acetate solutions of the salt sometimes caused them to turn yellow, after which no salt could be recovered. This eventuality could be avoided by working rapidly, and by not concentrating the solutions prior to crystallization. The pure salt is a one-to-one salt. *Anal.* Calcd. for $C_{27}H_{29}O_5N$: C, 65.5; H, 5.9. Found: C, 65.2; H, 6.1.

(+)-α-Methyltropidine.—The resolved α-methyltropidine was liberated from the salt by solution in aqueous alkali, and extraction with ether; $[\alpha]^{25}D$ 141° ($CHCl_3$).

Cycloheptanone.—The conversion of α-methyltropidine to β-methyltropidine, cycloheptenone and cycloheptanone were usually performed without isolation of intermediates, in order to obtain maximum yields. In a typical run 0.85 ml. of α-methyltropidine was maintained at a temperature of 190–200° in an oil-bath for 45 min., cooled and treated with 3 ml. of 4:1 sulfuric acid. The cycloheptenone was immediately steam distilled. The distillation was continued well beyond the point at which clear distillate was obtained. To the aqueous emulsion of cycloheptenone thus obtained was added 10 mg. of 10% palladium-on-charcoal catalyst, and the suspension was hydrogenated at atmospheric pressure until no further hydrogen was taken up. The solution was filtered and extracted with several small portions of ether. The ethereal solution was dried and the ether fractionated off, yielding 0.12 ml. (30%) of cycloheptanone.

***p*-Chlorobenzoic Acid.**—In a typical run 100 mg. of cycloheptanone was treated with a 4-fold excess of *p*-chlorophenyl Grignard reagent in ether solution, 3 ml. of benzene was added, the ether distilled off, and the solution refluxed for 5 hours. Unreacted Grignard reagent was destroyed with excess aqueous ammonium chloride. The mixture was extracted with ether, the ethereal layer was filtered and evaporated, and the residue was steam distilled to remove volatile impurities. The residue was taken up in ether and extracted with aqueous bicarbonate to remove any *p*-chlorobenzoic acid formed by reaction of the Grignard reagent with adventitious carbon dioxide. 1-*p*-Chlorophenylcycloheptanol was obtained by evaporation as a gum (155 mg.) and was oxidized directly to *p*-chlorobenzoic acid, by refluxing for 6 hr. with 1.45 g. of potassium permanganate in 20 ml. of water. At the end of this period the solution was acidified, refluxed for 0.5 hr., and the remaining permanganate was destroyed with sodium bisulfite. The solution was acidified and extracted with ether. The ethereal layer was extracted with aqueous bicarbonate, the bicarbonate layer washed with ether, charcoaled, and acidified. The white precipitate was taken up in ether, recovered by evaporation and triply sublimed to yield 4–5 mg. of *p*-chlorobenzoic acid, m.p. 236–238°.

(19) P. R. White, *Am. J. Botany*, **25**, 348 (1938).

(20) Gas phase proportional counting.

(21) G. Merling, *Ann.*, **216**, 348 (1883).

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