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The Biogenesis of the Alkaloids of *Colchicum*. I. The Incorporation of Phenylalanine into Colchicine[1]

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The administration of DL-phenylalanine-3-C^{14} to sprouting *Colchicum byzantinum* corms led to the formation of radioactive colchicine. Systematic degradation of the alkaloid showed that all the activity was located on one carbon strongly suggesting that phenylalanine is one of the precursors of colchicine. The results are discussed with respect to previously suggested biogenetic schemes for colchicine.

The curative properties of *Colchicum* were known to the ancient civilizations of Greece, Egypt and India. Colchicine is the most important active principle found in the various species of *Colchicum* and this alkaloid has been the subject of extensive investigations by pharmacologists, biologists and chemists.[2] Chemical investigations of colchicine have recently culminated in its total synthesis.[3] This article is an account of preliminary tracer work designed to elucidate its biogenesis in the plant.

There have been several schemes proposed for the biogenesis of colchicine, and these will be briefly discussed since they have served as working hypotheses for our tracer studies. Anet[4] suggested that colchicine may be biogenetically related to the flavones (III), the tropolone ring of colchicine being formed by the ring enlargement of the catechol ring by the insertion of a one carbon fragment.[5] It has recently been established that phenylalanine is a precursor of the 3,4-dihydroxyphenylpropane moiety found in many flavonoid compounds, the other aromatic ring being derived from three acetate units.[6] Thus if the Anet-Robinson scheme were correct the administration of phenylalanine-3-C^{14} (I) to *Colchicum* should yield colchicine (IV) labeled at the position indicated in Fig. 1. Belleau[7] suggested that colchicine is formed by the oxidative coupling of two molecules of 3,4,5-trihydroxyphenylpyruvic acid (V) to yield compound VI. The quinonoid ring of this compound then undergoes fission and cyclization of the resultant fragments shown diagrammatically in Fig. 2 results in the formation of the carbon skeleton of colchicine. Phenylalanine-3-C^{14} which

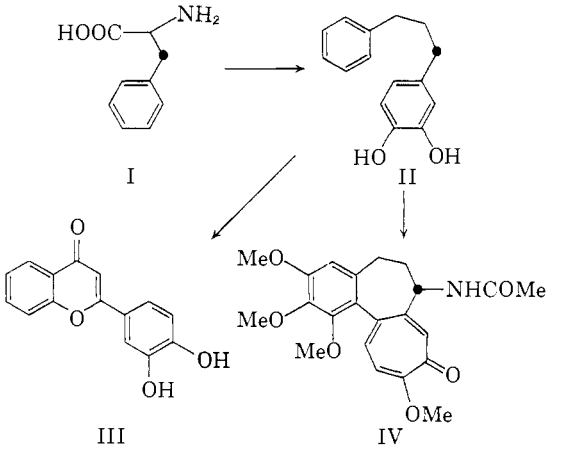

Fig. 1.—Anet–Robinson scheme for colchicine biogenesis.

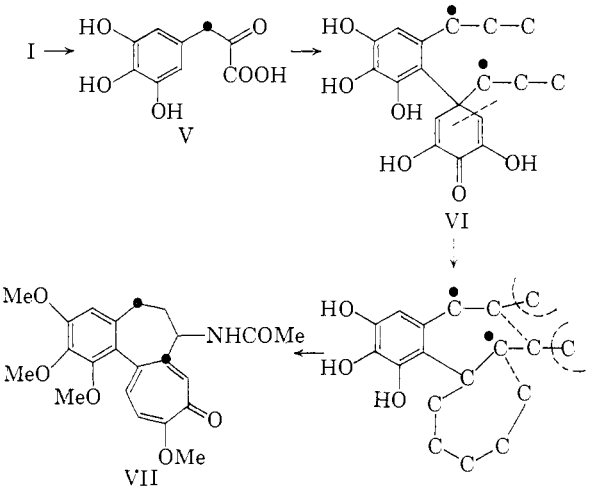

Fig. 2.—Belleau's scheme for colchicine biogenesis.

is a plausible precursor of V would thus be expected to yield colchicine labeled as shown in formula VII. Wenkert[8] proposed that colchicine is formed by the condensation of the protonated Schiff base VIII and 5-hydroxytropolone (IX). He suggested that compound VIII is formed from prephenic acid. However, it is considered equally plausible that VIII could arise from phenylalanine. If this were the case colchicine derived from I would be labeled as shown in formula X. Wenkert suggested that the tropolone IX was formed from shikimic acid. Phenylalanine is a metabolite of

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(2) *cf.* "Colchicine," by O. J. Eigsti and P. Dustin, The Iowa State College Press, Ames, Iowa, 1955. J. W. Cook and J. D. Loudon in "The Alkaloids," Vol. II, 1952, p. 261, and W. C. Wildman in "The Alkaloids," Vol. VI, Ed. by R. H. F. Manske, Academic Press, Inc., New York, N. Y., 1960, p. 247.

(3) J. Schreiber, W. Leimgruber, M. Pesaro, P. Schudel and A. Eschenmoser, *Angew. Chem.*, **71**, 637 (1959); E. E. van Tamelen, T. A. Spencer, D. S. Allen and R. L. Orvis, THIS JOURNAL, **81**, 6341 (1959).

(4) F. A. L. Anet's suggestion is recorded in the presidential address of Sir Robert Robinson to the Royal Society, *Nature*, **166**, 924 (1950).

(5) This proposal for the mode of formation of some naturally occurring tropolones was first elaborated by Robinson in his presidential address.[4]

(6) T. A. Geissman and T. Swain, *Chem. and Ind. (London)*, 984 (1957); H. Grisebach, *Z. naturforsch.*, **12b**, 227, 597 (1957); *ibid.*, **13b**, 335 (1958); H. Reznik and R. Urban, *Naturwissenschaften*, **44**, 592 (1957); E. W. Underhill, J. E. Watkin and A. C. Neish, *Can. J. Biochem. Physiol.*, **35**, 219, 229 (1957).

(7) B. Belleau, *Experientia*, **9**, 178 (1953).

(8) E. Wenkert, *ibid.*, **15**, 165 (1959).

shikimic acid,[9] but it seems unlikely that this biochemical transformation is reversible and we would not expect phenylalanine-3-C^{14} to yield colchicine labeled in the tropolone ring if this hypothesis is correct.

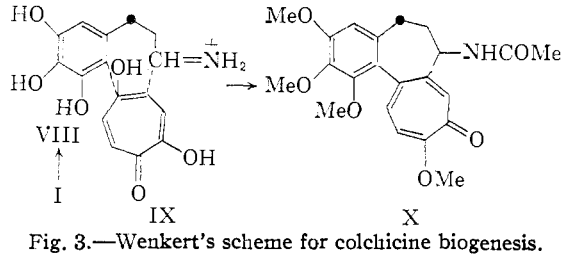

Fig. 3.—Wenkert's scheme for colchicine biogenesis.

Since all these biogenetic schemes involved phenylalanine or compounds closely related to it, it seemed logical to administer radioactive phenylalanine to *Colchicum*. Geiling's group obtained radioactive colchicine by allowing *C. autumnale* plants to grow in an atmosphere containing radioactive carbon dioxide.[10] It was our original intention to use this species in our experiments since it has received the most extensive investigation. However, our horticultural supplier was unaware of our scientific endeavors and provided us with *C. byzantinum*, a species native to Greece and Asia Minor, which has larger corms and produces more spectacular flowers. This species, like *autumnale*, belongs to the subgenus *Eucolchicum* and flowers in September. Fortunately this species proved to be a good source of colchicine. Phenylalanine-3-C^{14} was administered to the sprouting corms by a wick arrangement, details being recorded in the Experimental section. After one month the colchicine was isolated and purified until it had a constant specific activity. A further check on its radiochemical purity was obtained by acid hydrolysis to colchiceine[11] which had the same specific activity as the colchicine. The radioactive colchicine was oxidized with alkaline potassium ferricyanide to yield 3,4,5-trimethoxyphthalic anhydride using the same conditions as reported by Haworth for the oxidation of chebulinic acid.[12] This oxidizing agent gave better yields of the anhydride than alkaline potassium permanganate which had previously been used to oxidize colchicine.[13] The anhydride was converted to 3,4,5-trimethoxybenzoic acid by refluxing with concentrated hydrochloric acid.[14] This acid was decarboxylated by heating in quinoline in the presence of copper chromite catalyst, the evolved carbon dioxide being absorbed in barium hydroxide to yield barium carbonate. This barium carbonate and all the other intermediates in this degradative sequence had essentially the same specific activity as the colchicine (*cf.* Table I) indicating that all the activity in the radioactive colchicine was located on the carbon indicated by a heavy dot in formula X, Fig. 3.

This decisive result is thus compatible with Wenkert's hypothesis but does not agree with the schemes of Belleau and Anet as shown in Figs. 1 and 2. However, the possible relationship of colchicine to the biogenesis of flavonoid compounds is attractive and our results are consistent with a modified Anet-Robinson scheme which is illustrated in Fig. 4. In this scheme ring A of

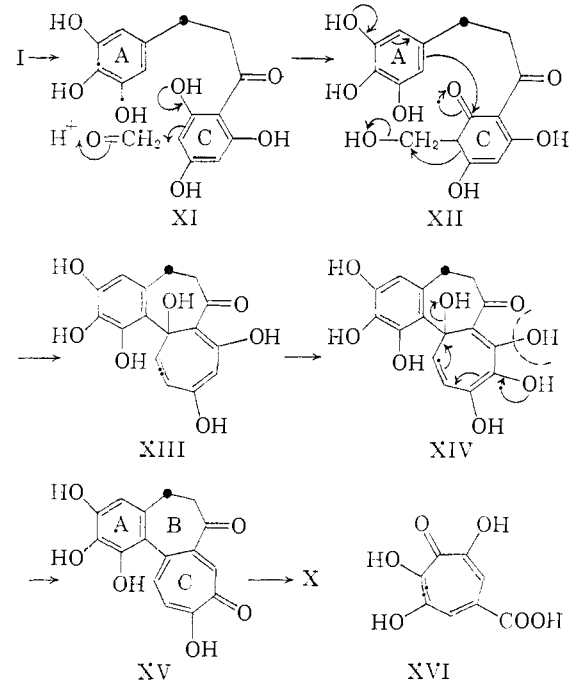

Fig. 4.—Modified Anet-Robinson scheme.

colchicine corresponds to the 3,4-dihydroxyphenyl substituent of the flavones. The acetate derived ring C in the intermediate XI reacts with formaldehyde or its biological equivalent to yield compound XII. Enlargement of ring C (perhaps facilitated by prior phosphorylation of the primary alcohol group) as indicated in formula XII, followed by condensation with ring A yields XIII which has the required carbon skeleton of colchicine. Nuclear hydroxylation of the potential tropolone ring yields XIV, which on elimination of water and loss of a hydroxy group affords compound XV. Final steps are uneventful, involving methylation of the four hydroxy groups, reductive amination of the carbonyl group in ring B and acetylation of the resultant amino group. It has been recently shown[15] that six of the carbons of the tropolone ring of the mold metabolite puberulic acid (XVI) are derived from acetic acid. This significant result offers considerable support to our hypothesis for the origin of the tropolone ring of colchicine. We have fed acetate-C^{14} to *Colchicum* and our results will be reported in a later publication.

(9) *cf.* B. D. Davis in "Symposium on Amino Acid Metabolism," Ed. by W. M. McElroy and H. B. Glass, The Johns Hopkins Press, Baltimore, Maryland, 1955, p. 799.

(10) E. J. Walaszek, F. E. Kelsey and E. M. K. Geiling, *Science*, **116**, 225 (1952).

(11) S. Zeisel, *Monatsh.*, **7**, 557 (1886).

(12) R. D. Haworth and L. B. de Silva, *J. Chem. Soc.*, 3511 (1951).

(13) A. Windaus, *Sits. ber. Heidelberg. Akad. Wiss.*, 1 (1910).

(14) R. L. Alimchandani and A. N. Meldrum, *J. Chem. Soc.*, **117**, 964 (1920). There is a typographical error in this paper, the carboxyl group in structures V and VI being in the wrong position.

(15) J. H. Richards and L. D. Ferretti, *Biochem. Biophys. Research Communs.*, No. 2, 107 (1960).

Experimental[16]

Administration of DL-Phenylalanine-3-C^{14} to *Colchicum byzantinum*-Ker-Gawl and Isolation of the Colchicine.—It was not possible to inject the corms directly with a hypodermic syringe because of their hardness. A cotton thread was inserted into the surface layer (penetrating about 5 mm.) of a sprouting corm by means of a fine needle. The two ends of the thread were placed in a 5 ml. beaker containing a solution of the tracer. Ten corms were fed in this way with DL-phenylalanine-3-C^{14} [17] (17.4 mg. in 10 ml. of sterile distilled water, total activity–5.67×10^8 c.p.m.[18]). Each plant thus received 1 ml. of solution which was absorbed in a few hours. Water was added daily to the corms by addition to the beakers in which the ends of the cotton threads were placed. The sprouting corms produced pale purple flowers and remained healthy. After one month the corms and flowers were comminuted in a Waring Blendor with methanol. The mixture was filtered and the residue washed with more methanol to bring the total volume to 2.5 l. The air-dried marc weighed 260 g. The methanol solution was evaporated to dryness *in vacuo* and the residue dissolved in water (400 ml.), filtered and extracted with petroleum ether (b.p. 40–60°) (2×200 ml.). The aqueous portion was made alkaline with sodium bicarbonate, filtered from a small amount of solid and then extracted with chloroform (7×200 ml.). The chloroform extract, dried over sodium sulfate, was evaporated *in vacuo* and the residue dissolved in benzene (15 ml.) and chromatographed on 60 g. of Woelm alumina (activity I).[19] The column was first eluted with 1:1 benzene–chloroform (100 ml.) and then with chloroform (500 ml.). The chloroform eluate was evaporated, redissolved in chloroform (15 ml.) and then diluted with petroleum ether (200 ml.) when a greyish white solid separated. Solution of this material in a small amount of chloroform followed by precipitation with petroleum ether was repeated three times yielding pure colchicine (235 mg.) which had an infrared spectrum identical with authentic colchicine.[20] The radioactive colchicine obtained from the plant was diluted with inactive material and rechromatographed on alumina and crystallized from ethyl acetate until it had a constant activity. The incorporation of tracer into the colchicine was 0.06%.

Degradation of the Radioactive Colchicine. Colchicine.[11]—Colchicine (50 mg.) was dissolved in water (5 ml.) containing 0.03 ml. of concentrated hydrochloric acid and refluxed gently for 2 hr. The solution was filtered hot and on cooling pale yellow needles of colchiceine hydrate separated (29 mg.), m.p. 151–157°. Analysis on material dried at 60° indicated a composition corresponding to the hemihydrate in agreement with Zeisel.[11]

Anal. Calcd. for $C_{21}H_{23}O_6N \cdot 0.5H_2O$: C, 63.98; H, 6.14; N, 3.55. Found: C, 64.20; H, 6.28; N, 3.58.

3,4,5-Trimethoxyphthalic Anhydride.—Colchicine (400 mg.) was added to a solution of potassium hydroxide (17.5 g.) and potassium ferricyanide (100 g.) in 400 ml. of water and heated on a steam-bath with stirring for 12 hr. At this time more potassium hydroxide (17.5 g.) and potassium ferricyanide (100 g.) were added. Similar additions of alkali and ferricyanide were made after 36 hr., and after 60 hr. the mixture was cooled and the yellow crystals of potassium ferrocyanide filtered off and washed with a little cold water. The filtrate was acidified with 50% sulfuric acid and the solution (400 ml.) extracted with ether in a continuous extractor for 24 hr. The ether extract was evaporated without drying and the residue sublimed (160–180°, 0.1 mm.). The pale yellow sublimate (76 mg., m.p. 144–145°) was crystallized from a mixture of benzene and petroleum ether to yield colorless plates of 3,4,5-trimethoxyphthalic anhydride (51 mg.), m.p. 146–147°.

Anal. Calcd. for $C_{11}H_{10}O_6$: C, 55.46; H, 4.23. Found: C, 55.68; H, 4.45.

3,4,5-Trimethoxybenzoic Acid.—3,4,5-Trimethoxyphthalic anhydride (41 mg.) was refluxed for 12 hr. with concentrated hydrochloric acid (1 ml.). The solution was filtered hot and on cooling colorless needles of 3,4,5-trimethoxybenzoic acid (19 mg.) separated, m.p. 166–167°, identical with authentic material (mixed m.p., infrared spectrum).

Decarboxylation of 3,4,5-Trimethoxybenzoic Acid.—The trimethoxybenzoic acid (15 mg.) was refluxed with copper chromite catalyst (10 mg.) in quinoline (2 ml.) for 1 hr. in a current of carbon dioxide free nitrogen. The exit gases were bubbled through barium hydroxide solution yielding barium carbonate (11 mg.).

The activities of the carrier free colchicine and its degradation products are recorded in Table I.

TABLE I
All activities are in c.p.m./m*M*

	Specific act., $\times 10^{-6}$
Colchicine	5.4
Colchiceine hemihydrate	5.4
3,4,5-Trimethoxyphthalic anhydride	5.2
3,4,5-Trimethoxybenzoic acid	5.1
Barium carbonate	5.0

(16) Melting points are corrected. Analyses were carried out by Mrs. Olga Hamerston and her assistants at the University of Minnesota.

(17) Purchased from Research Specialties Co., Richmond, California.

(18) Counts were carried out in a Nuclear-Chicago model D-47 Q gas flow counter using a "Micromil window." Determinations were carried out on samples of finite thickness, making corrections for efficiency and self absorption.

(19) Purchased from Alupharm Chemicals, New Orleans, La.

(20) The authors thank Dr. E. C. Kornfeld of the Eli Lilly Co. for a generous sample of colchicine. The alkaloid was also purchased from Nutritional Biochemicals Co., Cleveland, Ohio, and purified by chromatography on alumina (*cf.* J. N. Ashley and J. O. Harris, *J. Chem. Soc.*, 677 (1944)).