Opium Alkaloids IX: Detection of Coreximine in *Papaver somniferum* L. Based on Its Biosynthesis from Reticuline

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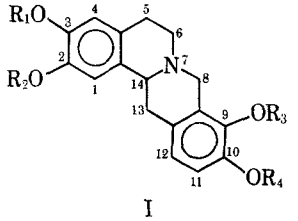
Abstract □ Protoberberines are biosynthesized in plants from reticuline in such a way that the *N*-methyl group of reticuline becomes the methylene group in the 8-position. It was demonstrated in this study that coreximine, a tetrahydro-ψ-berberine, is also derived from reticuline. (±)-Reticuline-(3-^{14}C) administered to intact opium poppies was incorporated into coreximine to an extent of 0.174%, and controlled degradation showed that the radioactivity was located at the C-6 position. Consequently, it could be concluded that the opium poppy is capable of converting reticuline to coreximine, and that coreximine, like scoulerine and isocorypalmine, is a normal member of the opium alkaloids. In the same way, it was shown that canadine, tetrahydropalmatine, stylopine, and berberine were not present in the plants in detectable amounts.

Keyphrases □ *Papaver somniferum*—coreximine detection □ Coreximine production from (±)-reticuline-(3-^{14}C)—*P. somniferum* □ IR spectrophotometry—identification □ NMR spectroscopy—identification □ GLC—identification □ Scintillometry—analysis

In 1962, Pfeifer and Teige (1) isolated a new opium alkaloid which they named somniferine (or base X), later shown to be identical with isocorypalmine (2) (I: $R_1 = R_3 = R_4 = CH_3$, $R_2 = H$). Scoulerine (I: $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$) was isolated from opium in 1965 (3) and has proved to be an important intermediate on the biosynthetic pathways leading to protopines, phthalide-isoquinolines, and benzophenanthridines (4, 5). In a patented process for chromatographic separation, Ose *et al.* (6) described the isolation of appreciable quantities of berberine (II: $R_1 + R_2 = CH_2$, $R_3 = R_4 = CH_3$) from opium poppies. This was disputed by Hakim *et al.* (7) who obtained negative tests for berberine in several varieties of opium poppy with paper chromatography and paper electrophoresis.

The protoberberines occur in a number of plant families. The most common substitution pattern is 2,3,9,10. More rarely are additional substituents found



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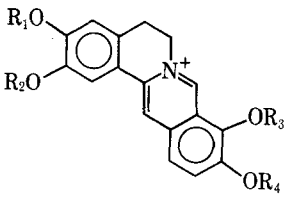
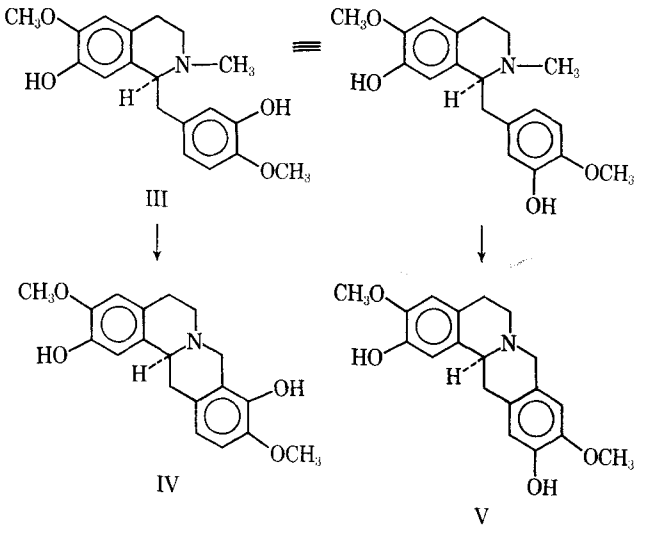


II

in positions 1, 5, or 13. Alkaloids having the same carbon skeleton but substituted in positions 2, 3, 10, and 11 are often referred to as tetrahydro-ψ-berberines (8).

The biosynthetic relationship of protoberberines to the benzylisoquinolines was recognized by Robinson (9) and demonstrated experimentally by Barton *et al.* (10), Battersby *et al.* (11), and Spenser and Gear (12). Based on feeding experiments performed with a great number of precursors and with different plants, it may be concluded that 2,3,9,10-substituted protoberberines (IV) are biosynthesized from (+)-reticuline (III) as illustrated in Scheme I.

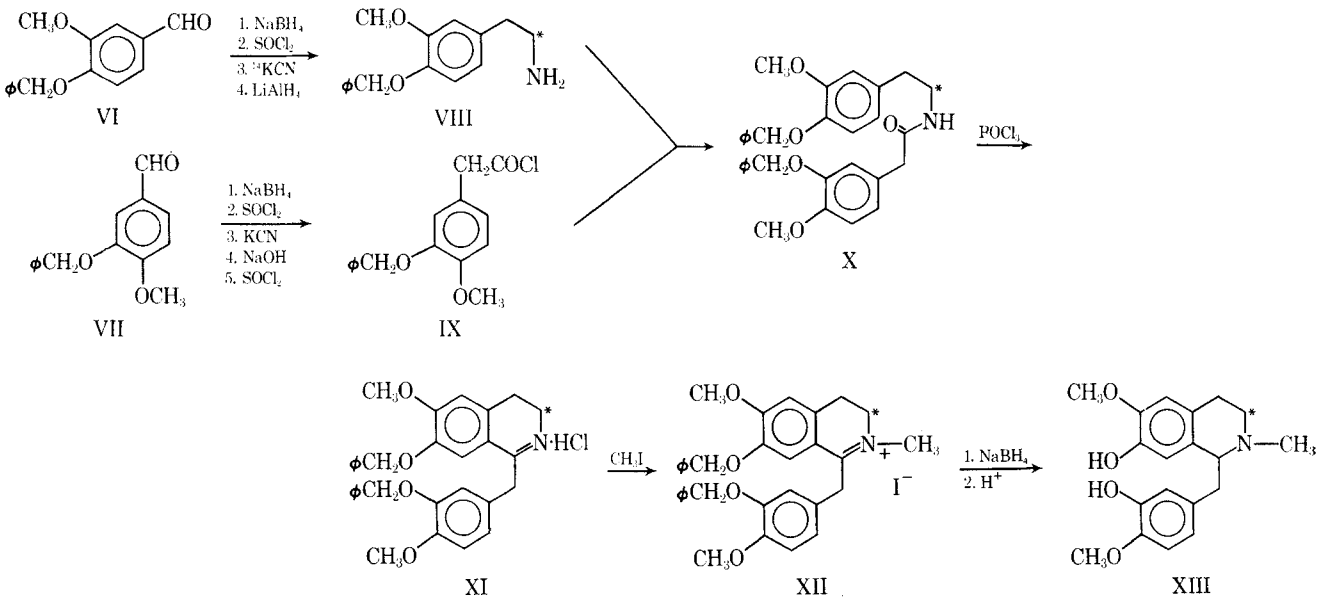


Scheme I—Proposed biosynthesis of protoberberines

The carbon atom in position 8, "berberine bridge," is derived from the *N*-methyl group of reticuline. The protoberberine (I and IV) is then oxidized spontaneously or enzymatically to the corresponding dehydro form (II). Several possible mechanisms have been proposed for the formation of the "berberine bridge" (10, 11).

From a mechanistic point of view, two products may be formed during the cyclization of reticuline. Coupling at the *ortho*-position to the hydroxyl group gives a tetrahydroberberine (IV), whereas coupling at the *para*-position gives a tetrahydro-ψ-berberine (V). In the living plant, this cyclization is probably under enzymatic control, and the product or products would depend on the presence of the required enzymes and on their specificity. Natural coreximine (V) is levorotatory and has the same absolute configuration as (−)-scoulerine (IV) and (+)-reticuline (III) (13, 14). It was, therefore, of interest to study the possibility that coreximine might be produced in the opium poppy since the potential precursor, (+)-reticuline, is known to be present (15–17). It was also decided to determine the presence or absence of related alkaloids, particularly in view of the conflicting observations regarding berberine.

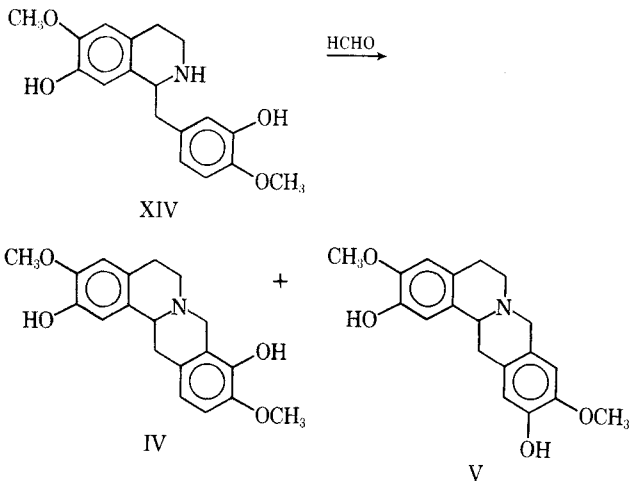
If these alkaloids exist in the opium poppy, the quantities would be too small to permit their actual isolation and characterization from a limited amount of plant material. The determination was, therefore, based on the hypothesis that they are biosynthesized in the plant by way of reticuline. Radioactively labeled (±)-reticuline was administered to opium poppies. Two weeks later the



Scheme II—Synthesis of (±)-reticuline-(3-^{14}C)

plants were harvested and extracted; the alkaloids, whose presence was to be determined, were added as "cold" carriers during the extraction (reverse isotope dilution). The alkaloids were then isolated and purified. Incorporation of the radioactive label in a position consistent with the biosynthetic pathways strongly suggested that the alkaloid is normally present in the plant. (±)-Reticuline-(3-^{14}C) and (±)-reticuline-(*N*-$^{14}CH_3$) were used as precursors. (±)-Reticuline-(3-^{14}C) was synthesized by the sequence of reactions illustrated in Scheme II (18).

(±)-Reticuline-(*N*-$^{14}CH_3$) was synthesized as described by Barton *et al.* (19). (±)-Coreximine for carrier dilution was prepared by condensation of (±)-norreticuline with formaldehyde (20) (Scheme III). This



Scheme III—Synthesis of (±)-scoulerine (IV) and (±)-coreximine (V)

gave a mixture of (±)-scoulerine and (±)-coreximine, which was readily separated by column chromatography on neutral aluminum oxide. (±)-Canadine was prepared by reduction of berberine sulfate with sodium borohydride (21). (±)-Stylopine was obtained by sodium borohydride reduction of coptisine which was isolated from coptis root.

RESULTS AND DISCUSSION

The conditions of the radioactive feedings and the results are given in Table I. The incorporation of radioactivity was calculated on the basis of the amount of "cold" carrier added.

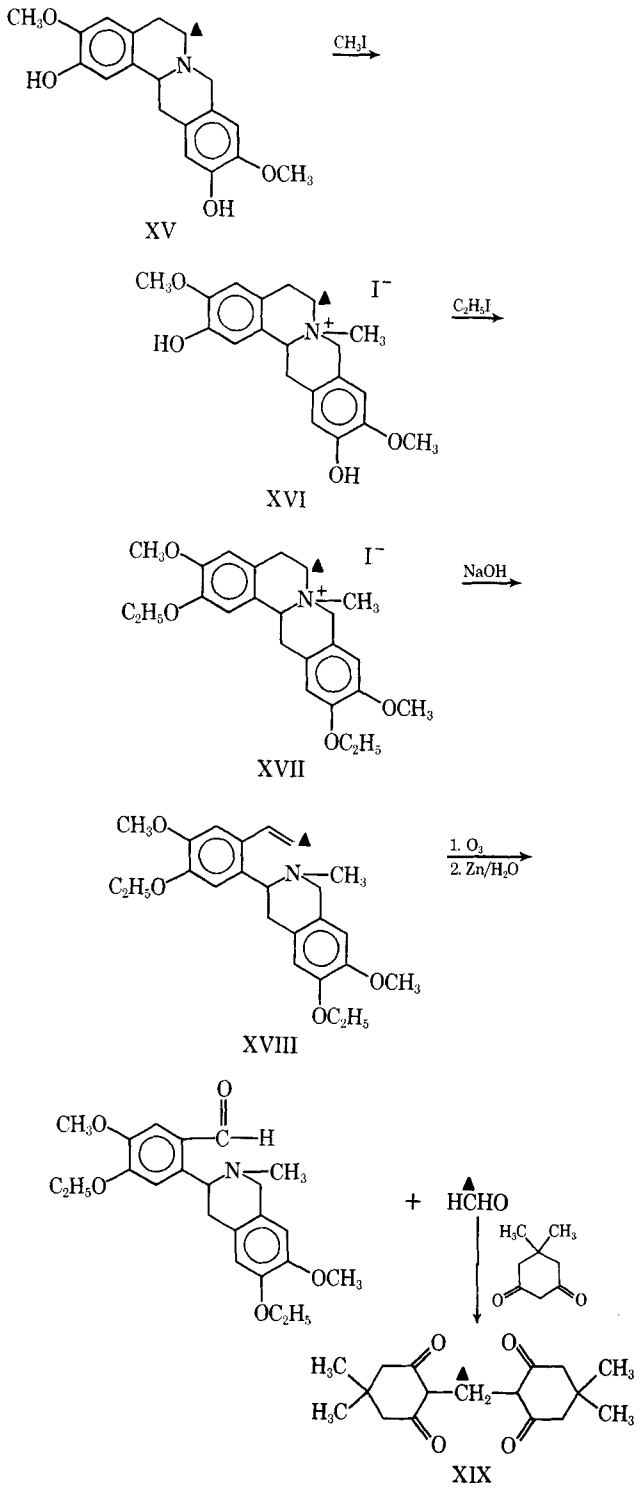
In view of the facts that (+)-reticuline is the proper precursor of the alkaloids studied, and that this substance is utilized by the plant for biosynthesis of a number of alkaloids, at least one of which occurs in large amounts (narcotine), an incorporation of 0.174% into coreximine is quite significant. It would not serve any useful purpose in this case to administer optically active reticuline, since this alkaloid is known to undergo rapid racemization in the plant (22). Radioactive coreximine was degraded to isolate the carbon atom in the 6-position, as illustrated in Scheme IV (23). The results showed that at least 90% of the radioactivity resided in this position. The slightly low specific activity of the dimedone derivative is a result of dilution by nonradioactive formaldehyde arising from the *N*-methyl group during the degradation process. A similar case was reported by Battersby *et al.* (24). Based on these results, one may conclude that the alkaloid coreximine is present in the opium poppy and that its biosynthesis proceeds along the same pathway as that reported for the protoberberines.

The incorporations of reticuline into canadine, tetrahydropalmatine, berberine, and stylopine were so small as to be negligible. Therefore, if these alkaloids were actually present in the varieties of opium poppies studied, the concentrations were too low to be detected. It is well known, however, that the alkaloid composition of *Papaver somniferum* varies greatly from one variety to another and that certain alkaloids may be virtually absent from some varieties while present in others.

EXPERIMENTAL

Equipment, Reagents, and Techniques—All melting points were determined with the Thomas-Hoover capillary melting-point apparatus and were uncorrected. The IR spectra were taken in potassium bromide with a Perkin-Elmer IR spectrometer, model 137. The NMR spectra were determined in deuterochloroform with the Varian Associates high-resolution spectrometer A 60-A, using tetramethylsilane as the internal standard. GLC analyses were carried out with a F&M biomedical gas chromatograph, model 400. Ozonolysis was performed on a Towers ozone apparatus, GE 150.

The radioactivity was determined by liquid scintillation counting with a Packard (model 3003) TriCarb liquid scintillation spectrometer. Two types of scintillation fluid were used: nonpolar (toluene type) and polar (dioxane type), depending on the solubility of the



Scheme IV—Controlled degradation of (±)-coreximine

substance. The sample, 0.5–2 mg., was weighed accurately on a Cahn gram electrobalance and dissolved in 0.1 ml. of methanol. Ten milliliters of scintillation fluid was added. The counting efficiency was calibrated with an internal standard of toluene-^{14}C. The efficiencies ranged from 80 to 84% for the nonpolar type and from 77 to 79% for the polar fluid. Berberine sulfate has an intense, yellow color and fluorescence, making a direct determination of its radioactivity difficult by scintillation counting. It was, therefore, reduced to (±)-canadine. About 0.1 mg. of sodium borohydride was added to the solution of berberine sulfate in methanol before addition of the scintillation fluid.

Cultivation of Plants and Administration of Labeled Precursors—The plants, *P. somniferum* L., Noordster and Indra varieties, were grown in flowerpots in a greenhouse, and the radioactive precursors were administered at the end of the flowering season as soon as the petals dropped. An aqueous solution of reticuline sulfate, containing the equivalent of about 3 mg./ml. of reticuline base, was injected into the top of the seed capsule as described by Battersby *et al.* (25). Each capsule received 0.3 ml. of the solution. The plants were allowed to grow normally for 1–2 weeks before they were harvested and stored in a freezer until they were required for extraction.

Four batches of plants were used. The first batch, consisting of 18 plants (Noordster variety), was fed a total of 19.9 mg. (80.31 μc.) of (±)-reticuline-(3-^{14}C); (±)-coreximine (210 mg.) and (±)-canadine (200 mg.) were used as "cold carriers." The second batch, 14 plants (Indra variety), was fed 36.6 mg. (144.2 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$); tetrahydropalmatine (250 mg.) was used as carrier. The third batch, 17 plants (Indra variety), was fed 34.1 mg. (134.3 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$), while berberine sulfate (250 mg.) was used for reverse isotope dilution. The fourth batch, consisting of 17 plants (Indra variety), was fed a total of 34.5 mg. (135.9 μc.) of (±)-reticuline-(*N*-$^{14}CH_3$); (±)-stylopine (250 mg.) was used as carrier.

Synthesis of Labeled Precursors—Several practice runs were always made with nonradioactive materials prior to the synthesis of the labeled compounds. The intermediates were characterized by TLC, NMR, IR spectroscopy, and melting points. The final products were compared with natural reticuline isolated from opium.

(±)-*Reticuline-(3-^{14}C)*—The synthesis did not differ in essential detail from that reported by Battersby *et al.* (18). The starting materials were isovanillin and vanillin. Benzylation with benzyl chloride under mildly basic condition yielded the corresponding *O*-benzyl ethers (VI and VII). The aldehyde groups of the *O*-benzyl ethers were reduced with sodium borohydride to the alcohols which, in turn, were converted to the chlorides with thionyl chloride. The 3-methoxy-4-benzyloxybenzyl chloride was reacted with potassium [^{14}C]-cyanide to give 3-methoxy-4-benzyloxyphenyl-[1-^{14}C]-acetonitrile. The nitrile was reduced with lithium aluminum hydride to the 3-methoxy-4-benzyloxyphen[1-^{14}C]ethylamine (VIII). The 3-benzyloxy-4-methoxybenzyl chloride was reacted with potassium cyanide to give 3-benzyloxy-4-methoxyphenyl acetonitrile. Hydrolysis with potassium hydroxide produced 3-benzyloxy-4-methoxyphenylacetic acid, which was converted to the acid chloride (IX) with thionyl chloride. Reaction of 3-methoxy-4-benzyloxyphen-[1-^{14}C]ethylamine (VIII) with 3-benzyloxy-4-methoxyphenylacetyl chloride (IX) under Schotten-Baumann conditions yielded *N*-(3-methoxy-4-benzyloxyphen[1-^{14}C]ethyl-3-benzyloxy-4-methoxyphenylacetamide (X).

1. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline hydrochloride (XI)—*N*-(3-Methoxy-4-benzyloxyphen[1-^{14}C]ethyl-3-benzyloxy-4-methoxyphenylacetamide (68 mg.) was dissolved in 5 ml. of dry toluene, and 0.5 ml. of freshly distilled phosphorus oxychloride was added. The solution was heated in an oil bath at 95–105° in a current of nitrogen, and the solution was concentrated in a rotary vacuum evaporator. The resulting yellow residue was dissolved in 2 ml. of 95% ethanol, and hydrogen chloride gas was bubbled through the solution for 2 min. The reaction mixture, after cooling, gave 52 mg. of yellow prisms, m.p. 202–203° [lit. (18) 201–203°].

2. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline methiodide (XII)—The dihydroisoquinoline hydrochloride (XI) (52 mg.), obtained in the preceding reaction, was suspended in 1.5 ml. of anhydrous methanol. A solution of 2 mg. of metallic sodium in 0.5 ml. of anhydrous methanol and 0.5 ml. of iodomethane were added, and the mixture was refluxed for 3 hr. under a stream of nitrogen. After 12 hr. in a refrigerator, the crystals were collected, giving 55 mg. of the methiodide, m.p. 195–196° [lit. (19) 197–198°].

3. (±)-Reticuline-(3-^{14}C) (XIII)—The crystals of 1-(3-benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydro[3-^{14}C]isoquinoline methiodide (XII) (55 mg.) were suspended in 2 ml. of methanol. The suspension was cooled in an ice bath, stirred with a magnetic stirrer, and 100 mg. of sodium borohydride was added in small portions. The reaction mixture was stirred for 14 hr. at room temperature. The solution was then evaporated to dryness, and 20 ml. of water and 1 ml. of 2 *N* sodium hydroxide were added. The mixture was extracted with chloroform (3 × 70 ml.) and the combined chloroform extracts were washed with water and evaporated to dryness to give *O,O*-dibenzylreticuline-(3-^{14}C), which was debenzylated without further purification.

Table I—Radioactive Feeding Experiments with *Papaver somniferum* L.

Precursor	Microcuries	Number of Plants	Year	Variety	Carrier Alkaloid	Incorporation, %	D g-radation, Activity in Isolated Fragment, %
(±)-Reticuline-(3-^{14}C)	80.31	18	1968	Noordster	(±)-Coreximine	0.174	90
(±)-Reticuline-(3-^{14}C)	80.31	18	1968	Noordster	(±)-Canadine	0.0011	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	144.2	14	1969	Indra	(+)-Tetrahydropalmatine	0.00085	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	134.3	17	1969	Indra	Berberine sulfate	0.00059	—
(±)-Reticuline-(*N*-$^{14}CH_3$)	135.9	17	1969	Indra	(±)-Stylopine	0.00058	—

The residue of *O,O*-dibenzylreticuline-(3-^{14}C) was dissolved in a mixture of 0.8 ml. of ethanol and 0.8 ml. of concentrated hydrochloric acid. The solution was heated in an oil bath at 130° for 1 hr., cooled, and evaporated to dryness. The residue was dissolved in 0.5 ml. of ethanol, and 10 ml. of ether was added to cause precipitation. The precipitate was collected, dissolved in a minimum amount of water, and washed twice with ether. The aqueous solution was basified with ammonium hydroxide to pH 8–9 and extracted with chloroform (3 × 10 ml.). The combined chloroform extracts were evaporated to dryness to give a residue of 30 mg. It was dissolved in ether, and a minimum amount of petroleum ether (b.p. 30–60°) was added to cause precipitation. This was repeated several times. TLC of the precipitate on silica gel with chloroform–methanol (9:1) and with ethanol–benzene (2:8) showed a single compound identical with natural reticuline isolated from opium. GLC analyses of the free base and the trimethylsilyl derivative, on columns containing *OV-1* and *OV-225* as stationary phases, gave only one peak having the same retention times as natural reticuline. The IR and NMR spectra of (±)-reticuline from the inactive runs were identical with those obtained with authentic reticuline. The (±)-reticuline-(3-^{14}C) had a specific radioactivity of 4.036 μc./mg. or 1.32 mc./mmole.

Synthesis of (±)-*Reticuline*-(N-$^{14}CH_3$)—This synthesis was essentially the same as described by Barton *et al.* (19). 3-Methoxy-4-benzyloxyphenethylamine was obtained by reaction of *O*-benzylvanillin with nitromethane, followed by reduction with lithium aluminum hydride.

1. 1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline methiodide-(*N*-$^{14}CH_3$)—1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline hydrochloride (490 mg.) was suspended in 25 ml. of ethyl acetate, and 25 ml. of a saturated solution of sodium bicarbonate was added. Nitrogen was bubbled through the solution until the solid was dissolved. The aqueous layer was extracted with ethyl acetate, and the combined ethyl acetate extracts were washed with water and evaporated to dryness. The residue was dissolved in 6 ml. of dry benzene and transferred to a reaction vessel 1 cm. in diameter and 10 cm. long. It was connected to a vacuum manifold system through which [^{14}C]-iodomethane (2 mc./141 mg.) was distilled into the benzene solution. The reaction vessel was sealed off and left at room temperature with occasional shaking. Yellow crystals appeared. After 4 days, the vessel was frozen in liquid nitrogen, and the seal was broken in a dry chamber. An excess of nonradioactive iodomethane was added, and the vessel was sealed again. After 2 days, the crystals were collected, m.p. 195–196° [lit. (19) 197–198°].

2. (±)-Reticuline-(*N*-$^{14}CH_3$)—The foregoing (*N*-methyl-^{14}C)-methiodide was reduced and debenzylated, and (±)-reticuline-(*N*-$^{14}CH_3$) was purified as described for the preparation of (±)-reticuline-(3-^{14}C); yield, 234 mg. of pure (±)-reticuline-(*N*-$^{14}CH_3$). It had a specific activity of 3.94 μc./mg. or 1.29 mc./mmole.

Alkaloids for Carrier Dilution—Tetrahydropalmatine and berberine sulfate were purchased[1].

Synthesis of Tetrahydroberberine[(±)-*canadine*] (*21*)—Berberine sulfate (500 mg.) was dissolved in 10 ml. of methanol, 500 mg. of sodium borohydride was added gradually, and the mixture was refluxed for 10 min. After addition of 50 ml. of water, the solution was extracted with chloroform (3 × 30 ml.), and the combined extracts were evaporated to dryness to give a residue of 400 mg. It was crystallized from 95% ethanol, m.p. 175–176° [lit. (21) 177–178°].

Synthesis of (±)-*Coreximine* (*20*)—

1. 1,2,3,4-Tetrahydro-7-hydroxy-1-(3-hydroxy-4-methoxybenzyl)-6-methoxyisoquinoline hydrochloride [(±)-norreticuline hydrochloride] (XIV)—1-(3-Benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3,4-dihydroisoquinoline hydrochloride (XI) (1.1 g.), prepared as described previously, was dissolved in 70 ml. of ethanol. The solution was shaken with hydrogen and 500 mg. of palladium (10%) on charcoal at room temperature and 1 atm. pressure. The uptake of hydrogen was complete in 3 hr. Removal of the catalyst and evaporation of the solvent left a residue which solidified on addition of water to give (±)-norreticuline hydrochloride monohydrate, 0.715 g., m.p. 165–166° [lit. (18) 165°]. The IR spectrum showed bands at 3250, 1500, 1450, 1300, 1270, 1240, 1120, 1030, and 860 cm.$^{-1}$.

2. 3,10-Dimethoxy-5,6,13,14-tetrahydro-8*H*-dibenzo(*a,g*)quinolizine-2,11-diol[(±)-coreximine] (20)—(±)-Norreticuline hydrochloride monohydrate (640 mg.) was dissolved in 4 ml. of ethanol. The solution was basified with concentrated ammonium hydroxide solution, and 100 ml. of water was added. It was then extracted with 3 × 40 ml. of chloroform. The combined chloroform extracts were evaporated to dryness to give a residue of 501 mg. The residue was dissolved in 24 ml. of methanol, and 5.5 ml. of 37% formaldehyde solution was added. The solution was refluxed for 1 hr. and the solvent evaporated, leaving a residue which consisted of a mixture of (±)-scoulerine and (±)-coreximine, as evidenced by TLC on aluminum oxide with chloroform–methanol (99:1). Authentic alkaloids were used for comparison of R_f values.

The residue was dissolved in a minimum amount of chloroform, transferred to a column of neutral aluminum oxide[2], and eluted with chloroform. The fractions containing coreximine were combined and evaporated to dryness. The residue (300 mg.) was crystallized from methanol, m.p. 237–240° [lit. (20) 238–239°].

(±)-*Stylopine*—Seventy grams of coptis root (botanical origin unknown) from Taiwan was ground, 100 ml. of 10% sulfuric acid was added, and the mixture was heated on a steam bath with frequent swirling for 20 min. After cooling to room temperature, the solution was made strongly alkaline with 80 ml. of 50% (w/v) sodium hydroxide solution and extracted with chloroform (4 × 200 ml.). The combined chloroform extracts were evaporated to dryness; the residue was dissolved in 50 ml. of methanol and reduced with sodium borohydride. After addition of 50 ml. of water, the solution was extracted with chloroform (3 × 100 ml.). Evaporation of the solvent gave a gum which was purified by column chromatography using neutral aluminum oxide and benzene. The fractions containing stylopine were combined and evaporated to dryness. The residue was crystallized from a mixture of chloroform and methanol to give 300 mg. of stylopine, m.p. 214–215° [lit. (26) 217°]. The mass spectrum[3] showed a fragmentation pattern characteristic of a protoberberine (27), with a molecular ion at *m/e* 323 and major fragments at *m/e* 148 and 174. Accurate mass determination of the molecular ion gave a mass of 323.114551 (calculated for $C_{19}H_{17}NO_4$, 323.115749).

Extraction, Separation, and Purification of Alkaloids—The plants were extracted by maceration with methanol in a high-speed

[1] Pierce Chemical Co.

[2] Woelm, activity IV.

[3] Associated Electronics Industries, model MS 902 high-resolution mass spectrometer.

blender[4], and the alkaloid carriers were added. The suspension was poured into a glass percolator and percolated with methanol until the extract gave negative tests for alkaloids. The extract was concentrated to a final volume of 1 l. in a rotary vacuum evaporator at 38° and shaken with about 150 ml. of ethyl acetate. The ethyl acetate layer was washed with 3 × 50 ml. of 0.5 *N* hydrochloric acid, and the washings were combined with the original aqueous solution (total alkaloids).

Canadine and Coreximine—The aqueous, acidic solution of total alkaloids from the plants to which (±)-canadine and (±)-coreximine had been added as cold carriers was extracted with several portions of chloroform. Evaporation of the chloroform gave a residue of weakly basic alkaloids. It was dissolved in chloroform (6 ml.) and transferred to a column of silica gel[5]; the alkaloids were eluted with a mixture of benzene and methanol (98:2). The elution of the alkaloids was monitored by micro-TLC. The fractions containing (±)-canadine were combined, evaporated to dryness, and rechromatographed on neutral alumina (50 g.) with benzene–chloroform (1:1). (±)-Canadine obtained in this way was crystallized from ethanol to constant radioactivity (10 d.p.m./mg.).

The aqueous solution remaining after extraction of the weakly basic alkaloids was basified to pH 8–9 with sodium bicarbonate and extracted with chloroform. The combined chloroform extracts were evaporated to dryness, and the residue was subjected to preparative TLC on silica gel with chloroform–methanol (9:1). The band corresponding to coreximine was extracted with methanol, and the extract was purified by chromatography on a column of neutral alumina (30 g.). Elution with chloroform gave coreximine, which was crystallized seven times from methanol to constant radioactivity (1480 d.p.m./mg.).

Tetrahydropalmatine—The weakly basic alkaloids, obtained as described from the batch of plants to which tetrahydropalmatine had been added as cold carrier, were chromatographed on a column of silica gel (60 g.). The elution was started with benzene, continued with a mixture of equal parts of ether and chloroform, and, finally, completed with chloroform–methanol (97:3). The fractions shown by micro-TLC to contain tetrahydropalmatine were combined and evaporated to dryness. The residue was crystallized three times to give constant radioactivity (11 d.p.m./mg.).

Stylopine—The aqueous, acidic solution of total alkaloid from the plants to which (±)-stylopine had been added as cold carrier was basified with ammonium hydroxide to pH 8–9 and extracted with chloroform (4 × 200 ml.). The chloroform extracts were evaporated to dryness, and the residue was chromatographed on a column of silica gel (70 g.) with chloroform. The fractions containing (±)-stylopine were combined, evaporated to dryness, and rechromatographed on neutral alumina (40 g.) with benzene. (±)-Stylopine obtained in this way was crystallized from a mixture of chloroform and methanol to constant radioactivity (7 d.p.m./mg.).

Berberine—The aqueous, acidic solution of total alkaloids obtained from the plants to which berberine had been added as cold carrier was extracted as described previously to remove the weakly basic alkaloids. The aqueous layer was then basified with ammonia and extracted several times with a mixture of chloroform–isopropyl alcohol (3:1). The extracts were combined and evaporated to dryness, and the residue was dissolved in 0.5 *N* hydrochloric acid. The solution was made strongly basic with sodium hydroxide and extracted repeatedly with chloroform. The combined chloroform extracts were washed with water and evaporated to dryness, yielding a residue of nonphenolic alkaloids. TLC showed it to contain berberine. It was chromatographed on a column of silica gel (65 g.), first with chloroform and then with chloroform–methanol (98:2). The fractions containing berberine were combined and concentrated to about 100 ml. Two milliliters of 10% sulfuric acid was added, and the solution was concentrated to a small volume (about 5 ml.) when yellow crystals of berberine bisulfate started to form. After 12 hr. in a refrigerator, the crystals were collected and recrystallized twice from ethanol to constant radioactivity (7 d.p.m./mg.).

Degradation of Radioactive Coreximine—Several practice runs were made with inactive material prior to the degradation of radioactive coreximine.

1. 2,11-Dihydroxy-3,10-dimethoxy-7-methyl-5,6,13,14-tetrahydro-8*H*-dibenzo(*a*,*g*)quinolizinium iodide [(±)-phellodendrine iodide] (XVI)—Coreximine (55 mg.) was dissolved in 5 ml. of methanol, and 3 ml. of iodomethane was added. The mixture was refluxed in a water bath. After 2 hr., the solution was evaporated to dryness. Addition of 1 ml. of warm methanol gave yellow crystals of (±)-phellodendrine iodide, 56 mg., m.p. > 300° [lit. (23) > 300°].

2. 2,11-Diethoxy-3,10-dimethoxy-7-methyl-5,6,13,14-tetrahydro-8*H*-dibenzo(*a*,*g*)quinolizinium iodide [(±)-*O*,*O*-diethylphellodendrine iodide] (XVII)—The crystals (±)-phellodendrine iodide (XVI) were dissolved in 4 ml. of 1 *N* alcoholic potassium hydroxide solution, and 4 ml. of iodoethane was added. The solution was refluxed in a water bath for 2 hr. Another 2 ml. of 1 *N* alcoholic potassium hydroxide and 2 ml. of iodoethane were added, and the reflux was continued for 2 hr. The resulting solution was evaporated to dryness to give a yellowish oily residue of *O*,*O*-diethylphellodendrine iodide (58 mg.).

3. *O*,*O*-Diethylphellodendrine methine (XVIII)—The residue (58 mg.) of *O*,*O*-diethylphellodendrine iodide was dissolved in 3 ml. of ethanol. Four grams of potassium hydroxide in 10 ml. of water was added. The solution was refluxed in an oil bath at 110–120° for 3 hr. and extracted with chloroform (5 × 20 ml.). The combined chloroform extracts were concentrated to dryness to give a residue of *O*,*O*-diethylphellodendrine methine (34 mg.), m.p. 116–117° [lit. (23) 116–117°]. The IR spectrum of the methine was identical with that reported by Tomita and Kunitomo (23).

4. Ozonolysis—The residue of *O*,*O*-diethylphellodendrine methine (XVIII) was dissolved in 5 ml. of ethyl acetate and cooled in a dry ice–chloroform bath; ozonized oxygen was passed through the solution. Finally, oxygen was passed through the resulting blue solution to expel excess ozone. The solution became colorless and was evaporated to dryness to give an oily residue. Zinc dust (0.2 g.), 20 ml. of water, and 10 mg. of silver nitrate were added. The mixture was refluxed in an oil bath for 30 min. Half of the water was then distilled at atmospheric pressure into a solution of 0.15 g. dimedone in 10 ml. of water and 4 ml. of ethanol. Water was added to the distilling flask and the distillation continued. The formaldehyde-dimedone, which separated over a period of 15 hr., was collected to give 16 mg. of crystals, m.p. 190–192°. It was recrystallized three times from 50% aqueous ethanol to a constant radioactivity of 1423 d.p.m./mg.

[4] Waring Products Co.
[5] Woelm, activity II.

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Determination of Time Course of *In Vivo* Pharmacological Effects from *In Vitro* Drug-Release Testing

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Abstract □ A mathematical approach is described which permits the computation of the temporal variation of pharmacological response intensity from the results of *in vitro* drug-release testing that linearly correlate or can otherwise be functionally related to *in vivo* drug availability. The development of optimized drug-release tests is suggested. Through the application of the described approach, such tests would permit the predictive estimation from *in vitro* data of the dependency of the pharmacological behavior of appropriate drugs on formulation factors. The dependency of the time course of pharmacological effects of a mydriatic drug on the drug-release characteristics of several types of dosage formulations is graphically shown.

Keyphrases □ Pharmacological response intensity correlation—*in vivo* drug availability □ Bioavailability time course determination—pharmacological data □ Drug-release tests, *in vitro*—optimization □ Absorption analysis, drug—theoretical considerations

It is well known that formulation factors can markedly influence the therapeutic activity and toxicity of pharmaceutical products. The advent of ever increasingly more effective and potent drugs emphasizes the imperative of concomitantly developing pharmaceutical dosage forms for optimal effectiveness, safety, and reliability. It is seldom practical to perform the exhaustive *in vivo* human testing of drug formulations that may be necessary to obtain a formulation possessing optimal drug-release characteristics and, therefore, the pharmacological response behavior that may be precisely desired. It is presently proposed that human testing of drug formulations for this purpose could be minimized if, instead of directly testing the many formulations themselves, *in vivo* studies are performed to establish "optimized *in vitro* drug-release tests" capable of predicting the *in vivo* bioavailability behavior of the drug as a function of formulation factors. Presently employed *in vitro* drug-release tests are generally inadequate for this purpose.

Morrison and Campbell (1), in their 1965 review article, concluded that: "It is apparent that *in-vitro* disintegration tests, as presently used, have certain inherent faults, and eventually must be modified or replaced by more critical tests of physiological availability." Aside from discrediting disintegration tests and replacing them by dissolution tests, apparently only limited progress has been reported since this time with regard to the modification and further development of *in vitro* tests that can reflect *in vivo* drug behavior.

It is often stated that the problem is quite complicated because a correlation of *in vivo* to *in vitro* release that is found with a particular test for a particular drug in a particular dosage form may not exist if another drug is substituted or the formulation altered. Few attempts have been reported to determine the limits to which this may be the case. The *in vitro* to *in vivo* drug availability correlations that have been found have always been after the fact. Quantitative correlations for a spectrum of dosage forms were well exemplified by the work of Cressman *et al.* (2) for one drug. The correlations, however, were of a single-point nature, *e.g.*, time for 50% of the drug to be released from the dosage form was correlated with the 50% release time *in vivo*.

In a previous report (3), a multiple-point quantitative *in vitro* to *in vivo* correlation procedure was recommended for providing criteria on which to gauge the adequacy of an *in vitro* drug-release test in reflecting *in vivo* drug availability. Also discussed was a quantitative approach to the development of *in vitro* drug-release tests, the expected limitations of such tests, and the manner in which the approach to the limits of applicability of a given test may be discerned. Levy and Hollister (4) and, more recently, Gibaldi and Weintraub (5), demonstrated that the required multiple-point linear correlations of amounts of drug released *in vitro* to the amounts of drug systemically absorbed *in vivo* (A_t) at corresponding times can be well accomplished for different dosage forms using the same dissolution test. The correlations can be further improved through suitably adjusting such test conditions