The Biosynthesis of Opium Alkaloids. Reticuline as the Benzyltetrahydroisoquinoline Precursor of Thebaine in Biosynthesis with Carbon-14 Dioxide*

Robert O. Martin,† Mitchum E. Warren,‡ and Henry Rapoport

ABSTRACT: The natural occurrence of the diphenolic benzyltetrahydroisoquinoline alkaloid, reticuline, was established in fresh budding plants and seedlings of *Papaver somniferum* L. Exposure of such plants to $^{14}CO_2$ for 1–3 hr was followed by determination of the radioactivity incorporated: (a) into reticuline and thebaine (I) and (b) into the *N*- and *O*-methyl groups for both alkaloids. These results confirm those from feeding experiments, and establish beyond question the role of reticuline as the true biosynthetic benzyltetrahydroisoquinoline precursor of the hydrophenanthrene alkaloids. The high rate of incorporation of radioactivity into the total alkaloids as well as into thebaine and reticuline both in seedlings and in mature plants should place beyond doubt the intimate involvement of these alkaloids in the economy of the plant. Our findings constitute positive evidence for the operation of the same biosynthetic relationships (*i.e.*, CO_2 → reticuline → thebaine → codeine → morphine) in seedlings as in mature plants. Simple chambers were developed for the exposure of single mature plants and seedlings to $^{14}CO_2$. The potential of gas chromatography with simultaneous mass and activity measurement, used in conjugation with carbon-14 dioxide feeding of plants in order to detect small amounts of active metabolites and to compare specific activities of related compounds, is clearly illustrated.

Earlier work in this laboratory (Rapoport *et al.*, 1960; Stermitz and Rapoport, 1961) established the rapid *de novo* synthesis of thebaine (I) (Chart I) from $^{14}CO_2$ and its primacy in the hydrophenanthrene alkaloid series. These reports together with similar studies on the biosynthesis of nicotine alkaloids (Alworth *et al.*, 1964; Liebman *et al.*, 1965, 1967) provide adequate demonstration of the value of the method which involves growing plants in the presence of $^{14}CO_2$ and determining the relative rates and amounts of radioactivity incorporated into different molecules or different portions of the same molecule.

Barton and Cohen (1957) earlier suggested the diphenolic alkaloid, reticuline (IIa), as a likely precursor of thebaine (I). Recent evidence (Barton *et al.*, 1965; Battersby *et al.*, 1965b), based on feeding experiments, showed that (−)- and (+)-reticuline could serve as precursors of thebaine. If reticuline is a true precursor of thebaine, it should be found with a specific activity greater than that of thebaine after short-term exposures of plants to $^{14}CO_2$ (Zilversmit *et al.*, 1943). The maximum ratios of the specific activities should exceed the inverse mole ratios of the two alkaloids in the plant, this difference becoming greater with shorter exposure times (Rapoport *et al.*, 1960).

To study the incorporation of $^{14}CO_2$ into the various opium alkaloids in order to determine possible precursors of thebaine, as well as interrelationships of all these alkaloids, work with single plants exposed for short periods (1–3 hr) to relatively high activities of $^{14}CO_2$ (3–10 mc) appeared attractive. A combination gas–liquid partition chromatography (glpc)–flow-counting system was clearly indicated as the best method to give qualitative separations as well as quantitative data for the microgram amounts of alkaloids to be encountered together with a measurement of their radioactivities. Successful glpc of a number of opium alkaloids has been reported (Lloyd *et al.*, 1960; Brochmann-Hanssen and Baerheim-Svendsen, 1962; Yamaguchi *et al.*, 1962) and continuous-flow counting of ^{3}H and ^{14}C is well established (Wolfgang and Rowland, 1958; Cacace, 1961).

Methods

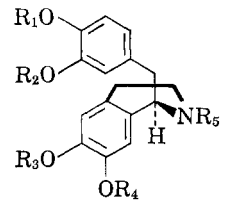
Alkaloid Standards and Reagents. (+)-Reticuline perchlorate was synthesized by a modification of the procedure of Gopinath and Viswanathan (1959). Norlaudanosine (IIf) was prepared by electrolytic reduction of papaverine (Corrodi and Hardegger, 1956); laudanosine (IIe) was synthesized by methylation of the nor compound. Laudanosoline (IId) was prepared by sealed-tube acid hydrolysis of laudanosine (Pymen and Reynolds, 1910).

* From the Lawrence Radiation Laboratory and the Department of Chemistry, University of California, Berkeley, California. *Received April 19, 1967.* Sponsored in part by the U. S. Atomic Energy Commission and the National Institutes of Health, U. S. Public Health Service.

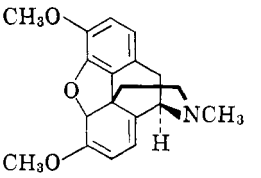
† Present address: Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada.

‡ U. S. Public Health Service postdoctoral fellow.

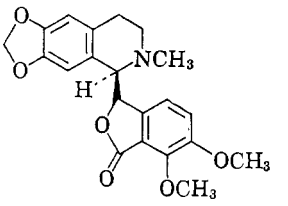
CHART I



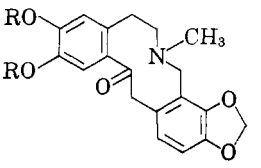
H	CH_3	
IIa, $R_{2,4}$	$R_{1,3,5}$	(−)-reticuline
b, R_1	$R_{2,3,4,5}$	(−)-laudanine
c, R_4	$R_{1,2,3,5}$	(−)-codamine
d, $R_{1,2,3,4}$	R_5	(−)-laudanosoline
e,	$R_{1,2,3,4,5}$	(−)-laudanosine
f, R_5	$R_{1,2,3,4}$	(+)-norlaudanosine
g, $R_{1,2,3,4,5}$		(+)-norlaudanosoline



thebaine (I)



1-(*R*)-(−)-narcotine (III)



IVa, R = CH_3 cryptopine

b, $\frac{R}{R}$ = $>CH_2$ protopine

The free base forms of these alkaloids were easily generated from their salts in an inert atmosphere[1] by passage of an aqueous solution of the salt, diluted with ethanol–chloroform, through a small column of Dowex 1 (OH^-). Barium carbonate-^{14}C having a specific activity of 127 μc/mg was obtained from Oak Ridge National Laboratories. Methylene chloride and benzene used for extractions were redistilled reagent grade.

Plant Growth. All plants were *Papaver somniferum* L., U. S. Department of Agriculture variety M92a, started from seeds as previously described (Rapoport *et al.*, 1960) except that all growth was in a greenhouse with supplementary lighting provided from 6 AM to 6 PM by G. E. Power Groove lights located 70 cm above the pots (about 1000 ft-candles). This variety of *P. somniferum* L. contains morphine, codeine, thebaine, and narcotine as the main alkaloids. Seedlings for exposure to $^{14}CO_2$ were grown on moistened filter paper in disposable plastic culture dishes (150 × 13 mm). The filter paper was kept moistened with nutrient solution by a paper wick passing through a slot cut in the bottom of the dish. Seeds were sterilized for 1 min in a 3% solution of hydrogen peroxide, rinsed, and dried by blotting. Germination was allowed to take place in the dark at 30° for the first 2 days, then on the bench top at 25°. With the dishes covered with inverted, slightly raised dishes of the same size, healthy seedlings free of contamination by fungi could be maintained for 2 weeks.

Biosynthesis. For exposure of single, mature plants to $^{14}CO_2$, a chamber and associated equipment similar to that previously described (Rapoport *et al.*, 1960) was used with the exception that the chamber was a tall cylinder 3 ft × 9 in., and the original $^{14}CO_2$ loop was replaced by direct generation from $Ba^{14}CO_3$.

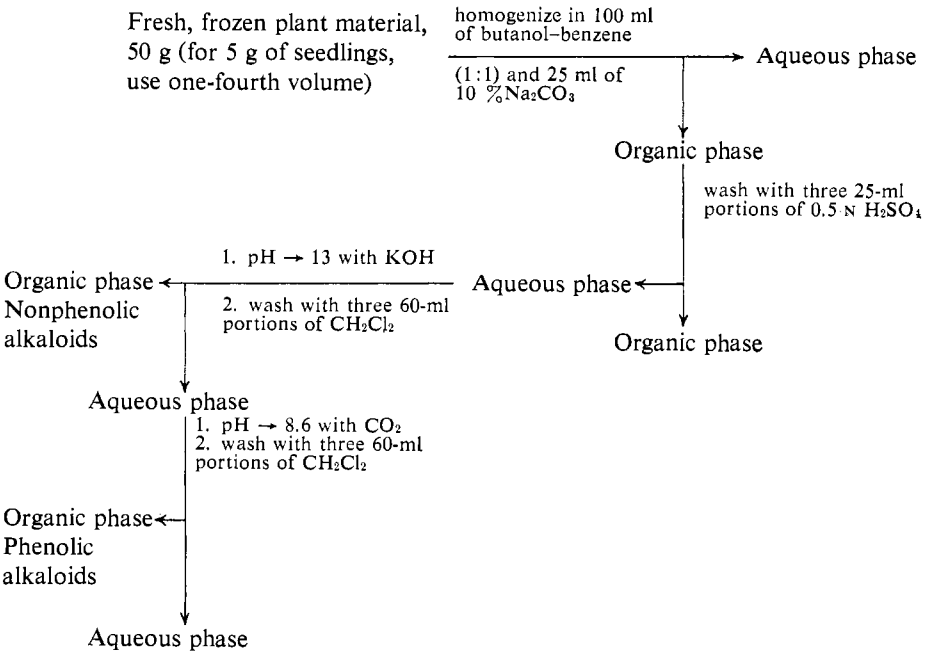
For studies with seedlings, the chamber consisted of an 8-in. diameter desiccator bottom with a greased, flat rubber gasket on the top rim. A circle of 0.5-in. thick lucite, provided with hose fittings for $^{14}CO_2$ circulation, weighted by lead bricks, served as a lid. Lighting was by means of two 500-w photo-flood bulbs placed at a distance of 1 ft from opposite sides of the chamber. Excess heat was dissipated by interposition of two water-cooled infrared filters between the chamber and the lights. This arrangement proved satisfactory for exposing three dishes of seedlings (about 5 g) for periods of up to 3 hr.

Alkaloid Isolation. A mature plant, after removal from the chamber, was cut into small pieces and dropped into liquid nitrogen. The frozen material was then ground for a few seconds in a Waring Blendor, under a stream of nitrogen.[2] The sodium carbonate solution, 0.5 g of sodium bisulfite, butanol–benzene, and any carrier alkaloid desired were then added and the extraction was carried through Scheme I.

[1] All operations were carried out in a polyethylene glove bag (I²R, Inc., Chettenham, Pa.), inflated under slow nitrogen flush.

[2] All subsequent operations were carried out in vessels flushed with nitrogen.

SCHEME I



Seedlings were washed into a sintered-glass funnel, sucked damp dry, and quickly frozen with liquid nitrogen. These frozen seedlings were ground in a glass Ten-Broeck homogenizer three times with 10% sodium carbonate and butanol–benzene, the organic layer being separated by centrifugation each time.

Where it was anticipated that analysis of these fractions would not be carried out immediately, they were converted to their HCl salts by addition of a small amount of 0.5 M HCl in methanol to the extract after removal of the methylene chloride. All fractions were dried *in vacuo* and stored under nitrogen at 5°.

Glpc Proportional Counting Apparatus. The principle technique enabling us to carry out multiple analysis on single mature plants or a few grams of seedlings was that of glpc combined with continuous combustion and flow counting. Our method differed from the system reported by Cacace (1961) in that (a) the column temperature was programmed and (b) microgram amounts of material were to be detected and counted. The total column effluent was passed through a triode, argon ionization mass detector (Teranishi *et al.*, 1960), followed by combustion over CuO to CO_2 and H_2O, drying, mixing with propane, and counting in a flow-through proportional counter (Cacace, 1961). A dual pen recorder simultaneously plotted the output of a counting ratemeter and the ion current from the mass detector, giving radioactivity and mass in differential type curves. The useful mass range was 0.1–20 μg and minimum activities of about 100 cpm were detectable in any one peak.

Glpc columns for resolution of alkaloid mixtures were prepared by filling precoiled 6 ft × 0.25 in. o.d. aluminum tubes *in vacuo* with the coated stationary phase. As a nonpolar stationary phase, Anakrom ABS (80–100-mesh) was double coated by the filtration technique, first with 0.5% of Versamide 900 as a tail reducer (Kabot and Ettre, 1964), then with 2% of a methylvinylsilicone (UC-W-96). Columns, filled with this material, were conditioned for 15 hr at 250°. The other stationary phase was the same support as above, single coated with 1% of a cyanosilicone (XE-60). Columns of this material were conditioned overnight at 240°.

For the analysis of alkaloid mixtures the injector was operated at 230–240° and the column oven started at 170–180°, programmed to increase at a rate of 2–3°/min to a maximum of 290 (for the W-96 column) or 250° (in the case of the XE-60 one). The detector was operated at 260–280°. Nonphenolic alkaloids were injected as their free bases dissolved in methylene chloride.

Phenolic alkaloids were chromatographed as their TMS[3] ethers prepared by the rapid method described by Sweeley *et al.* (1963) for sugars. Reaction appeared to be complete in 30 min at room temperature as judged from constant peak heights by glpc and ultraviolet spectra in the case of morphine and reticuline collected from the glpc effluent.

Collection of individual alkaloids from the glpc column effluent was easily accomplished by inserting an L-shaped, 2.5-in. length of 1/8-in. o.d. Pyrex tubing filled with glass beads into the detector exit, using Teflon Swagelok ferrules. Nonphenolic or phenolic alkaloids as TMS ethers were trapped in this way.

[3] Abbreviation used: TMS, trimethylsilyl.

Specific Activity Determination of Various Alkaloids. With the high voltage adjusted so that the counter was operating in the center of its plateau region, it was standardized as follows. Several 2-μl injections of hexane-^{14}C of known activity (A) (1650 and 440 dpm/μl) were made. The counts registered after 1.5 min by the proportional counter on a scaler were noted and the average background for a similar period was substracted to give the net counts (N) registered per injection of standard. The dynamic counter efficiencies (N/A) calculated from these data were in the range of 30–40%.

Standardization of the mass detector[4] was accomplished by injection of 2–4 μl of serially diluted samples of pure alkaloids or mixtures of known composition. As the detector linearity was limited and response depended on the structure of the individual alkaloid, it was desirable to prepare, by dilution, standards that were as close as possible in concentration to that of the plant extracts. The two standardizations described above allowed the activity and mass of an unknown sample of opium alkaloids to be determined.

Other Chromatographic Methods. A. IMPREGNATED PAPER. Squares (8 in.) of alumina-impregnated paper (Schleicher & Schuell 1967), developed ascending in two dimensions with diethyl ether (peroxide free) followed by chloroform (dried over $MgSO_4$), completely resolved up to 50 μg of each of the alkaloids shown in the formula diagram in less than 6 hr. Iodoplatinic acid reagent (Mumer and Machebouef, 1949) was used to locate the alkaloids.

B. PAPER. Whatman No. 3MM paper developed in an ascending manner with butanol–acetic acid–water (63:10:27) (Tomita and Kikkawa, 1956) resolved (as hydrochlorides) reticuline (IIa) (R_F 0.7) from the two major interfering alkaloids in the phenolic fraction of mature plants, namely morphine (R_F 0.4) and narcotine (III) (R_F 0.8).[5] The monophenolic base laudanine (IIb) was unresolved from reticuline. Borate-impregnated paper (Swain, 1953) was used with this solvent to check for the presence of any *o*-diphenolic alkaloids.

C. THIN LAYER. Phenolic as well as nonphenolic alkaloids (spotted as HCl salts) were resolved using layers of silica gel (Woelm, binderless). Ascending development (under nitrogen) in chloroform–diethylamine (9.5:0.5) (Waldi *et al.*, 1961) for 2 hr completely resolved morphine (R_F 0.03), reticuline (R_F 0.12), laudanine (R_F 0.30), and narcotine (R_F 0.78). For purification of reticuline for optical activity determinations the phenolic fraction (free base) was chromatographed on large plates using benzene–ethanol (80:20) for development. Radioautograms were made using Eastman duplitized no-screen X-ray film.

Determination of Total Methyl Group Activities. Reticuline (as the perchlorate salt) and thebaine obtained from a 5-day seedling $^{14}CO_2$ experiment were diluted with carrier and recrystallized to constant specific activities. About 15 mg of each alkaloid was subjected to a standard Herzig-Meyer and Zeisel determination. The total methyl iodide generated from each alkaloid was collected in 0.5 ml of dry toluene cooled in a Dry Ice–acetone trap. An excess of triethylamine (5% solution in absolute ethanol) was added and the mixture was allowed to stand at room temperature for 24 hr. Evaporation of the solvent *in vacuo*, crystallization of the residue two times from 1.5 ml of dry isopropyl alcohol, and drying at 50°/0.01 mm for 10 hr provided pure methyltriethylammonium iodide, mp 297° dec. Weighed samples of the original alkaloids and of the methyltriethylammonium iodide were counted using a liquid scintillation counter.

Results

Alkaloid Extraction. Prior to making a search for possible precursors of thebaine, the stability and recoveries from the extraction scheme of a series of alkaloids thought likely to be encountered was tested. The results are shown in Table I.

Glpc of Opium Alkaloids. The following nonphenolic alkaloids were completely resolved from one another on a UC-W-96 column in 35 min (temperature program

TABLE I: Per Cent of Alkaloids Recovered from Extraction.[a]

Alkaloid	Fraction: pH 13 Nonphenolics	pH 8.6 Phenolics	pH 11.8[b] Mixed Alkaloids
Codeine	90	0	90
Cryptopine	*c*	—	33
Laudanine	0	90	50
Laudanosine	90	0	90
Laudanosoline	0	0	0
Morphine	0	80	1
Narcotine	1	90	50
Narceine	—	—	10[d]
Norlaudanosine	—	—	90
Papaverine	90	0	90
Protopine	—	—	25
Reticuline	0	90	50
Thebaine	90	0	90

[a] Recoveries determined by glpc. In the case of reticuline, thebaine, and morphine, this was also checked by ultraviolet spectra. [b] This fraction was obtained by adjusting the pH to 11.8 instead of 13 before washing with methylene chloride. [c] Not determined. [d] Determined by ultraviolet spectra only which showed an altered product.

[4] We are indebted to Dr. Teranishi of the Western Regional Laboratory, U. S. Department of Agriculture, Albany, Calif., for assistance in building a modification of this detector.

[5] Although narcotine is considered a nonphenolic alkaloid, it appears in the phenolic fraction due to the presence of a lactone ring which opens during the pH 13 extraction.

from 180 to 250°, argon flow, 60 cc/min): codeine, thebaine, laudanosine, papaverine, cryptopine, and narcotine in order of increasing retention time. The pairs, laudanosine–norlaudanosine and protopine–cryptopine, were only partly resolved.

The resolution of the four phenolic alkaloids morphine, laudanosoline, reticuline, and laudanine in order of increasing retention times was complete on an XE-60 column in 16 min (temperature programmed from 170 to 235°; argon flow, 60 cc/min). Laudanosine was eluted shortly after (18 min) laudanine.[6]

A neopentyl glycol succinate column showed promise of effecting a similar resolution. Low percentages of liquid phase were important with both polar columns to ensure elution of all the alkaloids before the maximum temperature permissible for these phases (250°) was reached.

Collection of 10–20-μg samples of codeine, morphine-TMS, and reticuline-TMS ethers after glpc gave compounds with unchanged paper chromatographic mobilities as well as ultraviolet spectra (including alkali shifts for morphine and reticuline). The latter data indicated better than 90% recovery for morphine and reticuline from glpc.

The Natural Occurrence of Reticuline in P. somniferum L. Prior to searching for radioactive reticuline, establishment of its natural occurrence was desirable. About 500 g of fresh whole plant material, 1 week prior to blossom, was extracted according to the scheme. Paper chromatography of the phenolic fraction in butanol–acetic acid–water revealed an alkaloid in the expected reticuline region of the chromatogram, between two larger spots of morphine and narcotine. Chromatography of a large portion of the fraction on heavier paper, elution of the reticuline area, and rechromatography showed the alkaloid to be either reticuline or laudanine.[7] The absence of *o*-diphenolic material was shown by chromatography on borate-impregnated paper. Glpc analysis of the TMS ethers of the eluted alkaloid spot on the nonpolar column again indicated the major component to be either reticuline or laudanine plus a small amount of morphine. Finally, by use of the slightly polar XE-60 column, identity of reticuline was established. Co-injection of the unknown with a nearly equal amount of reticuline standard gave a single peak. No laudanine was detected but two small peaks were observed between the morphine and reticuline. These may possibly have been a tri- and tetraphenolic alkaloid, though neither appeared to be identical with the laudanosoline standard. Thin layer chromatography (tlc) and ultraviolet spectra confirmed the identity of reticuline. Approximately 2 mg of reticuline was isolated compared with about 20 mg of thebaine.

Optical Activity of Reticuline from Fresh Plant Material. The phenolic alkaloids from 35 *fresh* capsules (mature plants, 2–4 weeks after petal fall) were purified twice by preparative tlc (benzene–ethanol solvent). Chromatographically pure reticuline (0.84 mg) was obtained which in 95% ethanol gave an $[\alpha]_D$ of −97.5° or a molecular rotation $[\Phi]_D$ of −317° (*c* 0.04) [reported $[\Phi]_D$ +376° (ethanol) by Gopinath *et al.* (1959) and $[\Phi_D]$ +344 (*c* 1.5, ethanol) by Arndt and Baarschers (1963) for the antipodal material from other plant species].

The phenolic alkaloids from eight fresh mature, decapsulated plants yielded no detectible reticuline by the above procedure. The capusles from these plants yielded 0.36 mg of pure reticuline; $[\alpha]_D$ −110° and $[\Phi]_D$ −342° (*c* 0.02, ethanol).

Comparison of Reticuline and Thebaine Specific Activities from a Budding Plant (Biosynthesis I). A poppy plant, 1 day prior to blossoming and weighing 50 g, was exposed for 1 hr to 3 mc of $^{14}CO_2$. The plant was immediately frozen, 1.2 mg of reticuline perchlorate was added,[8] and the alkaloids were extracted. Paper chromatography of the phenolic fraction freed the reticuline from the bulk of morphine and narcotine. Autoradiography of two parallel chromatograms, one on borate paper, showed more than 90% of the radioactivity to be in the reticuline area, with no detectible activity in the morphine or narcotine. Glpc analysis of the eluted reticuline confirmed this and showed thebaine to be the major active compound in the nonphenolic fraction. From the glpc data, the specific activities of the undiluted alkaloids were calculated and compared, together with the total activities of each fraction; these are given in Table II.

Alumina paper chromatography of the nonphenolics followed by autoradiography confirmed that thebaine was the major radioactive alkaloid. Tlc and autoradiography showed reticuline to be the only active alkaloid in the area eluted from the first mentioned paper chromatogram. Elution of thebaine and reticuline areas after tlc and determination of their specific activities by ultraviolet and scintillation counting gave values in good agreement with the glpc data.

Alkaloid Spectrum of Seedlings and $^{14}CO_2$ Incorporation into Thebaine in Seedlings and Blossoming Plants.

[6] The tetramethoxy alkaloid, laudanosine, was injected to see if it had the predicted retention time, based on the assumption that the more TMS substituents and the fewer methyl groups there were on the alkaloid, the lower a retention time it should have. The observed order of elution seems to bear this out, as well as to indicate that the TMS group is less polar than the methyl group. Also, the fact that reticuline and the TMS esters of reticuline and laudanine showed identical retention times on the nonpolar column indicates the TMS groups have little effect on volatility in spite of an increase in molecular weight of nearly 50% in the case of reticuline. Tetra-*O*-TMS-laudanosoline has over twice the molecular weight of the parent phenol.

[7] There are three other possible monophenolic compounds isomeric with laudanine. Only codamine has been reported in opium. Also there are five other isomers of reticuline, two of which would be *o*-diphenols. None were detected during these studies.

[8] The amount of added carrier in all cases was kept small (no more than ten times the estimated natural concentration in contrast to much larger amounts usually added in such studies), in order not to overload the chromatographic sytems used. This permits autoradiography, a most sensitive test of radiochemical purity, to be used to its fullest advantage.

TABLE II: Reticuline–Thebaine Relationships in Biosynthesis with $^{14}CO_2$.

Biosynthesis No.	I	II	III
Plant age (days)	120	5	5
$^{14}CO_2$ absorbed (mc)	3.0	2.4	3.8
Duration of exposure (hr)	1.0	2.5	2.75
Total alkaloid activity (dpm/mc per hr of exposure per g of plant)	1.5×10^4	7.0×10^4	5.4×10^4
Total thebaine dpm/reticuline dpm	3:1	4:1	6:1
Thebaine specific activity (dpm/μmole)	4×10^5	4×10^5	9×10^4
Reticuline specific activity[a] (dpm/μmole)	1×10^7	8×10^6	5×10^6
Thebaine concentration in plant (μmoles/g)	0.012	0.1	0.1
Reticuline concentration in plant (μmoles/g)	0.0006	0.01	0.01
Carrier reticuline added (μmoles)	2.7	3	0
Thebaine:reticuline molar ratios[a]	20:1	10:1	10:1

[a] Calculations are based on the maximum reticuline concentration.

TABLE III: Specific Concentration of Several Alkaloids in Plants of *P. somniferum L.* at Different Ages.

	Specific Concn in μg/g of Plant (fresh wt)				
Age (days)	Codeine	Thebaine	Reticuline	Protopine + Cryptopine	Narcotine
91[a]	44	3.7	*b*	0.6	150
91[c]	35	2.0	0.2	0.5	140
60	125	15	—	1	83
51	80	0.6	—	1.6	53
8	6	31	—	1	4
7	1.5	75	—	<1	<1
5	<4	125	<6	<6	<6
3	—	1	—	—	—

[a] Plant on day after blossom opened. [b] No attempt was made in these cases to detect reticuline. [c] Plant 1 day prior to blossoming.

The findings of Pfeifer and Heydenreich (1961) that thebaine is the first alkaloid detectable in *P. somniferum* together with earlier experiments in this laboratory (Rapoport *et al.*, 1960; Stermitz and Rapoport, 1961) led us to investigate the possibilities of using seedlings for alkaloid biosynthesis studies. Germinating seedlings were analyzed for total alkaloids after 3, 5, 7, and 8 days by glpc. The results are tabulated in Table III together with results from older plants for comparison. In no case, using 3–5 g of seedlings, was reticuline detectable. With a predetermined limit of sensitivity, the maximum concentration of reticuline was calculated, and from this value a minimum thebaine:reticuline ratio (see Table II). A most promising aspect of the seedling results was the very simple alkaloid spectrum, especially the absence of codeine, morphine, and narcotine, the major interfering alkaloids in older plants.

As a preliminary to determination in seedlings of reticuline:thebaine specific activity ratios, 5- and 7-day-old seedlings were exposed for 2.5 hr to 3 mc of $^{14}CO_2$. A comparison of the thebaine specific activities with those from two earlier biosyntheses with older plants is made in Table IV together with the thebaine concentration in plants of different ages.

Several interesting comparisons are possible. First, the rate of incorporation of radioactivity into the total alkaloids and thebaine is five and eight times greater, respectively, with 5-day than with 7-day seedlings, the latter incorporating at rates near to those of mature plants. Second, the incorporation of activity into the

TABLE IV: Thebaine Concentration and $^{14}CO_2$ Fixation into Alkaloids as a Function of Plant Age.

Plant age (days)	3	5	7	8	30	50	90	110
Thebaine concentration (μg/g of fresh plant wt)	1	125	75	31	1.6	0.6	3	4
Thebaine specific activity ($\times 10^{-2}$ dpm/μmole per mc per hr)	—	11.9	1.5	—	—	—	1.0	1.1
Total alkaloid activity ($\times 10^{-4}$ dpm/mc per hr)	—	14.7	2.7	—	—	—	5	—

total alkaloids and thebaine parallels the percentage of thebaine concentration in the plants as a function of age. Third, the thebaine accounts for only about 10–20% of the total alkaloid activity.

Biosynthesis Using Seedlings. The results of exposure of 5-day seedlings to $^{14}CO_2$ are given in Table II. Except for biosynthesis III, carrier reticuline was added just before beginning the extraction of each batch of plants. The phenolic fraction was analyzed by glpc without any prior purification by paper chromatography. In the case of biosynthesis I, nonphenolics were checked by alumina paper chromatography (and tlc for biosynthesis II) and autoradiography which established thebaine as the major, if not the only, radioactive alkaloid in all cases. Tlc of the phenolics from II showed reticuline contained more than 80% of the activity of this fraction.

Comparison of the Total N- and O-Methyl Group Specific Activity with that of Total Activity for Thebaine and Reticuline. Results of the total demethylation of diluted samples of thebaine and reticuline, both from a 3-hr biosynthesis using 5-day seedlings, showed that the methyl groups accounted for 17% of the total activity in reticuline and 20% in thebaine.

Discussion

A comparison of the undiluted specific activities of reticuline and thebaine from short-term $^{14}CO_2$ biosynthesis shows that of reticuline to be greater than that of thebaine. The specific activity ratios of reticuline to thebaine are 20:1 and 55:1 for 5-day seedling and 25:1 for the budding plant, respectively, thereby exceeding the thebaine:reticuline molar ratios of 10:1 and 20:1 for the two age groups, respectively. Thus a condition necessary to confirm reticuline as the benzyltetrahydroisoquinoline precursor has been met. These molar ratios are also consistent with the role of reticuline as an active biosynthetic intermediate, *i.e.*, one which is rapidly turned over and therefor edoes not accumulate to any large extent.

Further support for the view that all the carbons of reticuline are incorporated directly into thebaine is given by the fact that the fraction of activity in the *O*- and *N*-methyl groups is substantially the same for thebaine (20%) and reticuline (17%) from a 5-day seedling experiment.

Even if the mass of reticuline present was so low as to escape detection by glpc, one might expect to see a radioactivity peak for reticuline of the same magnitude as for thebaine, unless the rate of reticuline turnover is different from that of thebaine, because the reticuline:thebaine specific activity ratio must be at least equal to their inverse mass ratio.[9] Indeed, analysis of the alkaloids from 5-day seedlings exposed to $^{14}CO_2$ as above, but with no carrier added, showed a large activity, but no mass, peak for reticuline. Thebaine showed similar activity but with a substantial mass peak. Since the ratio of the *total* activities of thebaine:reticuline was from 3:1 to 6:1, and both compounds are known to be recovered to the same extent, it is clear that the rate of turnover of reticuline is more rapid than that of thebaine.

The latter conclusion raises the question of the chirality of naturally occurring reticuline, since only the (*R*)-(−) isomer should lead to thebaine[10] [assuming no inversion during the conversion; see, however, Battersby *et al.* (1965b) for the incorporation of both (−)- and (+)-reticuline presumably through the common intermediate, 1,2-dehydroreticuline], and there is very little accumulation of reticuline. It was suggested in our earlier communication (Martin *et al.*, 1964) that (*S*)-(+)-reticuline may prove to be the precursor of 1-(*R*)-(−)-narcotine (III), the major nonphenanthrene alkaloid in our variety of poppy, which has been shown (Ohta *et al.*, 1963; Battersby and Spencer, 1964) to have the same steric configuration as (*S*)-(+)-reticuline. In our blossoming plants, narcotine is present in quite substantial proportions, the reticuline:thebaine:codeine:morphine:narcotine ratios being about 1:20:60:300:250. It is also noteworthy that narcotine appears in seedlings about the same time the maximum thebaine concentration is found but at about $^1/_{20}$ the concentration. Narcotine from a 3-hr biosynthesis with 5-day seedlings was moderately radioactive (autoradiograms).

Brochmann-Hanssen and Nielson (1965) reported the occurrence of optically active reticuline in crude opium of unidentified origin, with a predominance of the (+) isomer over the (−) by a ratio of 3:2. Battersby *et al.* (1965a) reported a ratio of 6:1 for (+)- over (−)-

[9] This is true assuming that (a) the *de novo* synthesis of reticuline and thebaine is small during the 1–3-hr exposure to $^{14}CO_2$ compared to existing pools of these alkaloids, and (b) the activity incorporated into both alkaloids during the period of $^{14}CO_2$ exposure is an average value.

[10] Of interest is the fact that reticuline isolated by Gopinath and Viswanathan (1959) from *Anona reticulata* and by Arndt and Baarschers (1963) from *Phylica rogersii* in both cases was the almost pure (*S*)-(+) enantiomorph.

reticuline, respectively, isolated by carrier dilution from opium poppies (var. noordster) of unspecified age, and racemic reticuline from our 5-day seedlings.

Our isolation of the almost pure (−) isomer from *fresh* mature capsules is in striking contrast to the above results. This would seem to indicate the hazards of making deductions about biosynthetic interrelations of alkaloids from such optical activity data alone since: (a) such material as crude opium is of undefined origin with respect to plant species or age and might involve changes which occur during drying of the latex to yield crude opium; (b) the alkaloid spectrum of the *fresh* plant material is seldom known or reported; and (3) this alkaloid spectrum apparently varies considerably from plant to plant and over the course of a day as well.

These points suggest the value of obtaining data about a whole spectrum of alkaloids isolated from fresh single plants. Even then such data must be evaluated with caution, since the relative pool sizes determined by isolation of the actual alkaloids is a reflection of both the turnover rate and rate of synthesis for a particular compound in question. For example, two compounds or isomers in a plant may accumulate in only very small amounts; in one case the small pool size may be the result of a very slow rate of synthesis; in the other it may be due to a rapid synthesis and turnover. Similar considerations are necessary in interpreting observation concerning optical activity of such isolated compounds. On the other hand, kinetic data from short-term $^{14}CO_2$ biosynthesis appear to be the most reliable basis for determining the dynamic interrelations among a large group of alkaloids, such as is present in *P. somniferum*.

The early appearance of high concentrations of thebaine in seedlings and the high rate of incorporation of radioactivity into thebaine and reticuline should place beyond doubt the intimate involvement of these alkaloids in the economy of the plant.[11] Our findings also constitute positive evidence for the operation of the same biosynthetic relationships (*i.e.*, CO_2 → reticuline → thebaine → codeine → morphine) in seedlings as in mature plants.[12,13]

Several advantages were derived from working with seedlings. First, a supply of practically uniform plant material could be grown in less than 1 week. Second, the alkaloid spectrum was greatly simplified (absence of the major interfering alkaloids morphine and narcotine in any significant amounts). Third, the rates and efficiency of $^{14}CO_2$ incorporation into alkaloids of seedlings were as good or better than with older plants, and small amounts of highly radioactive seedlings could be extracted rapidly and easily.

[11] Fairbairn and Wassel (1964) and Fairbairn and Paterson (1966) present additional evidence for active metabolic roles for the morphine-type alkaloids, as suggested by the refeeding experiments of Stermitz and Rapoport (1961).

[12] Massicot (1961) suggested that different metabolic pathways for the morphine-type alkaloids exist in seedlings compared with mature plants.

[13] Neubauer (1964) presented negative though complimentary evidence to support this view.

References

Alworth, W. L., DeSelms, R. C., and, Rapoport, H. (1964), *J. Am. Chem. Soc. 86*, 1608.

Arndt, R. R., and Baarschers, W. H. (1963), *J. Chem. Soc.*, 2244.

Barton, D. H. R., and Cohen, T. (1957), Festschrift Arthur Stoll, Basle, Birkhauser, p 117.

Barton, D. H. R., Kirby, G. W., Steglich, W., Thomas, G. M., Battersby, A. R., Dobson, T. A., and Ramuz, H. (1965), *J. Chem. Soc.*, 2423.

Battersby, A. R., Evans, G. W., Martin, R. O., Warren, Jr., M. E., and Rapoport, H. (1965a), *Tetrahedron Letters*, 1275.

Battersby, A. R., Faulkes, D. M., and Binks, R. (1965b), *J. Chem. Soc.*, 3323.

Battersby, A. R., and Spencer, H. (1964), *Tetrahedron Letters*, 11.

Brochmann-Hanssen, E., and Baerheim-Svendsen, A. (1962), *J. Pharm. Sci. 51*, 1095.

Brochmann-Hanssen, E., and Nielsen, E. (1965), *Tetrahedron Letters*, 1271.

Cacace, F. (1961), *Nucleonics 19*, 45.

Corrodi, H., and Hardegger, E. (1956), *Helv. Chim. Acta 39*, 889.

Fairbairn, J. W., and Paterson, A. (1966), *Nature 210*, 1163.

Fairbairn, J. W., and Wassel, G. (1964), *Phytochemistry 3*, 253.

Gopinath, K. W., Govindachari, T. R., Pai, B. R., and Viswanathan, N. (1959), *Ber. 92*, 776.

Gopinath, K. W., and Viswanathan, T. W. (1959), *Ber. 92*, 1657.

Kabot, F. J., and Ettre, L. S. (1964), *J. Gas Chromatog. 2*, 21.

Liebman, A. A., Morsingh, F., and Rapoport, H. (1965), *J. Am. Chem. Soc. 87*, 4399.

Liebman, A. A., Mundy, B. P., and Rapoport, H. (1967), *J. Am. Chem. Soc. 89*, 664.

Lloyd, H. A., Fales, H. M., Highet, P. F., Vanden-Heuvel, W. J. A., and Wildman, W. C. (1960), *J. Am. Chem. Soc. 82*, 3791.

Martin, R. O., Warren, M. E., Jr., and Rapoport, H. (1964), *J. Am. Chem. Soc. 86*, 4726.

Massicot, J. (1961), *Ann. Pharm. Franc. 19*, 44.

Mumer, R., and Machebouef, M. (1949), *Bull. Soc. Chim. Biol. 31*, 144.

Neubauer, D. (1964), *Planta Med. 12*, 43.

Ohta, M., Tani, H., Morazume, S., Kodaira, S., and Duritama, K. (1963), *Tetrahedron Letters*, 1857.

Pfeifer, S., and Heydenreich, K. (1961), *Naturwissenschaften 48*, 222.

Pymen, F. L., and Reynolds, C. (1910), *J. Chem. Soc. 97*, 1320.

Rapoport, H., Stermitz, F. R., and Baker, D. R. (1960), *J. Am. Chem. Soc. 82*, 2865.

Stermitz, F. R., and Rapoport, H. (1961), *J. Am. Chem. Soc. 83*, 4045.

Swain, T. (1953), *Biochem. J. 53*, 200.

Sweeley, C. C., Bently, R., Makita, M., and Wells, W. W. (1963), *J. Am. Chem. Soc. 85*, 2497.

Teranishi, R., Nimmo, C. C., and Corse, J. (1960), *Anal. Chem. 32*, 896.
Tomita, M., and Kikkawa, K. (1956), *Chem. Pharm. Bull. (Tokyo) 4*, 230.
Waldi, D., Schnackerz, K., and Munter, F. (1961), *J. Chromatog. 6*, 61.
Wolfgang, R., and Rowland, F. S. (1958), *Anal. Chem. 30*, 903.
Yamaguchi, S., Isao, S., Okuda, S., and Tsuda, K. (1962), *Chem. Pharm. Bull. (Tokyo) 10*, 755.
Zilversmit, D. P., Ententman, C., and Fishler, M. C. (1943), *J. Gen. Physiol. 26*, 325.

Chemical, Physical, and Biological Properties of a Lipopolysaccharide from *Escherichia coli* K-235*

Floyd C. McIntire, H. William Sievert, Grant H. Barlow, Richard A. Finley, and Agnes Y. Lee

ABSTRACT: Studies on a lipopolysaccharide (LPS) from the cells of *Escherichia coli* K-235 were focused upon the relationship of toxicity, pyrogenicity, and antibody neutralization to the state of aggregation, molecular charge, and lipid content. Disaggregation to a unit of 400,000 mol wt was achieved by three different procedures: (1) removal of esterified fatty acids by alkaline hydroxylaminolysis, (2) the introduction of approximately 200 succinyl groups/molecule, and (3) dissolution with an equal weight of sodium dodecyl sulfate (SDS). Succinylation markedly increased the anionic character of LPS. Both succinylation and the removal of lipid gave high yields of completely water-soluble products which showed surprisingly little evidence of heterogeneity. A high degree of molecular asymmetry is indicated by very low $s_{20,w}$ values in relation to light-scattering figures for molecular weights. Disaggregation by SDS did not decrease pyrogenicity in the rabbit. Compared to LPS dissolved with SDS, the succinyl derivative showed no great loss of pyrogenicity and toxicity, but there was a marked loss of ability to neutralize antibody to LPS. The removal of lipid resulted in a very great loss of pyrogenicity and toxicity, but only slight loss of antibody-neutralizing ability.

Lipopolysaccharides[1] from Gram-negative bacteria are usually obtained as aggregates with particle weights of millions when prepared by the phenol–water extraction (Westphal and Jann, 1965; Schramm *et al.*, 1952) which allows a minimum of opportunity for the cleavage of covalent bonds. The high particle weight of these preparations and their low solubility in water have made homogeneity studies difficult (Nowotny *et al.*, 1966; Beer *et al.*, 1966), and thus there is a question as to whether the many interesting biological properties belong to one or several different molecules in the same preparation.

In most investigations in the past a reduction in particle size of LPS preparations has been achieved only by procedures which would break covalent bonds (Neter *et al.*, 1956; Nowotny, 1963; Tripody and Nowotny, 1966; Ribi *et al.*, 1962; Haskins *et al.*, 1961; Johnson and Nowotny, 1964). In these studies one cannot tell whether changes in physical state and biological properties resulted from disaggregation (intermolecular) or degradation (intramolecular). Recently evidence has been presented for the reversible dissociation and inactivation of LPS preparations by the use of SDS (Oroszlan and Mora, 1963) and NaD (Rudbach *et al.*, 1966). While the dissociation with either SDS or NaD indicates the importance of hydrophobic bonding in the aggregation of LPS molecules, there is no suggestion as to the involvement of other types of intermolecular forces. The molecular size has not been defined; usually only sedimentation coefficients have been reported without other data necessary for meaningful calculations, and light scattering has been employed either inadequately or not at all.

Our investigations of the relationships among the physical, chemical, and biological properties of an *Escherichia coli* LPS are presented here, with emphasis on homogeneity, the factors responsible for molecular aggregation, and the relationship of aggregation to toxicity.

* From the Molecular Biology Department, Research Division, Abbott Laboratories, North Chicago, Illinois. *Received February 15, 1967.*

[1] Abbreviations used: LPS, lipopolysaccharide; PS, polysaccharide obtained after removal of lipid from LPS; SDS, sodium dodecyl sulfate; NaD, sodium deoxycholate; KDO, 2-keto-3-deoxyoctonic acid; MPD-3, minimal pyrogenic dose, 3 hr.

Materials and Methods

Preparation of the Lipopolysaccharide. E. coli K-235