and dried to constant weight over phosphorus pentoxide to yield essentially pure selenopantethine (4.0 g., 35% of theory) as a highly hygroscopic, yellow glass. Further elution of the column yielded only dark brown oils of uncertain composition.

Analytically pure selenopantethine was obtained as an extremely hygroscopic yellow glass by a second chromatography on alumina (15 g.), using the same solvent system.

Anal. Calcd. for $C_{22}H_{42}N_4O_8Se_2$: C, 40.72; H, 6.53; N, 8.65; Se, 24.36. Found: C, 40.84; H, 6.46; N, 8.43; Se, 24.02.

The equivalent weight of selenopantethine was determined by means of an iodine–sodium thiosulfate titration[36] and found to be 630.7 (theoretical value 648.3).

1,2-Dithia-5-azepane Hydrochloride (VIII).—Sodium sulfide nonahydrate (48 g.) and powdered sulfur (6.4 g.) were heated together until a clear red melt of sodium disulfide was obtained. After cooling to room temperature a solution of sodium carbonate (30 g.) in the minimum amount of water was added, together with chipped ice, to bring the total volume to 800 ml. This solution was then mixed with a solution of β,β-dichlorodiethylamine hydrochloride (30 g.) in water (50 ml.) and ice (150 g.). The resulting mixture was kept at about 0° for 48 hr. and then steam-distilled.

When the *p*H of the distillate dropped from an initial value of 9 to about 8 a solution of potassium cyanide (0.5 g.) in 6 *N* sodium hydroxide (100 ml.) was added and the distillation continued until about 6 l. of distillate had passed over. These were acidified with 6 *N* hydrochloric acid and evaporated to dryness under reduced pressure. The residue was dried azeotropically by distillation with benzene and ethyl alcohol and crystallized from absolute alcohol. Colorless prisms (16.4 g., 55% of theory) m.p. 178° resolidified, then decomposed at 230°. $C_4H_{10}ClNS_2$ requires 20.65% Cl; found 20.38% Cl.

The 3,5-dinitrobenzoate was prepared by reaction of the hydrochloride and 3,5-dinitrobenzoyl chloride in pyridine at room temperature and purification of the product by filtration over neutral activated alumina ("Woelm," act. grade I) in benzene solution. Colorless needles m.p. 152–154°.

Anal. Calcd. for $C_{11}H_{11}N_3O_8S_2$: C, 40.09; H, 3.38; N, 12.76; S, 19.48. Found: C, 39.96; H, 3.30; N, 12.74; S, 19.43.

(36) Houben-Weyl, "Methoden der Organischen Chemie," Vol. IX, 4th Ed., Georg Thieme Verlag, Stuttgart, Germany, p. 1103.

N-Pantothenoyl-1,2-dithia-5-azepane (IV).—The mixed carbonic ester-anhydride of pantothenic acid was prepared from calcium pantothenate as described previously in the synthetis of selenopantethine. At −5° a suspension of 1,2-dithia-5-azepane hydrochloride (7.9 g.) in dimethylformamide (25 ml.) and triethylamine (10 ml.) was added dropwise with stirring. With continued stirring, the solution was allowed to warm to room temperature over a period of about 1 hr., filtered from precipitated salts, and evaporated at reduced pressure (oil pump) to yield a tan-colored oil (14.1 g.).

Five grams of this residue were chromatographed on activated alumina (100 g., "Woelm," Brockmann grade III, column diameter 34 mm.) using acetone containing 10% methanol as solvent. The first 200 ml. of eluate (1.5 g. dark brown oily residue) were discarded, the next 2000 ml. yielded IV (2.15 g. = 43% of theory) as a colorless, highly hygroscopic glass.

Anal. Calcd. for $C_{13}H_{24}N_2O_4S_2$: C, 46.42; H, 7.15; N, 8.38; S, 19.04. Found: C, 46.50; H, 7.28; N, 8.05; S, 18.90.

Bis (β-Pantothenoyl-aminoethyl) Sulfide (III).—Bis (β-aminoethyl)-sulfide (VII; 2.5 g.), dissolved in ethyl acetate (20 ml.) and triethylamine (5 ml.), was dropped slowly into a stirred solution of the mixed ester-anhydride V, and the reaction product was worked up as described in the preceding experiment. An amber oil (11.7 g.) was obtained.

This residue was dissolved in methanol (30 ml.) and acetone (180 ml.) was added until the solution became permanently turbid. Chromatography on activated alumina ("Woelm," Brockmann grade III, 110 g. in column of 34 mm. diameter) and elution with acetone containing 15% methanol yielded a first solvent-containing fraction (320 ml.; 2.29 g. oily residue) which was discarded; the second fraction (1500 ml., 4.65 g. colorless glass = 44% of theory) was regarded as impure sulfide III. To obtain a product of analytical purity this fraction was dissolved in methanol (15 ml.), acetone (300 ml.) was added and the solution chromatographed on alumina as above. Elution with acetone containing 5% methanol (500 ml.) yielded a fraction (500 ml. = 0.23 g. residue), which was discarded, further elution with acetone containing 15% methanol (2000 ml.) yielded pure sulfide III (3.48 g. = 33% of theory).

Anal. Calcd. for $C_{22}H_{42}O_8N_4S$: C, 50.55; H, 8.10; N, 10.72; S, 6.13. Found: C, 50.56; H, 8.27; N, 10.45; S, 5.87.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND LAWRENCE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

The Biosynthesis of Opium Alkaloids. I. The Interrelationship among Morphine, Codeine and Thebaine[1]

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Plants of *Papaver somniferum* L. were grown in the presence of $C^{14}O_2$ for two hour, six hour and eight day periods, after which morphine, codeine and thebaine were isolated. The incorporation of radioactivity into each of these alkaloids was determined for (1) the intact compounds, (2) the various O- and N-methyl groups and (3) the ring skeleton. Differences found in the ring skeleton labeling are best accommodated by a scheme in which thebaine is the precursor of the other morphine alkaloids. Morphine appears to be a storage product formed from codeine by demethylation. In addition, these short periods of biosynthesis have allowed the development of a simplified biosynthesis chamber adaptable for research purposes and yet capable of producing appreciable quantities of alkaloids of high specific activity.

Introduction

Investigations and speculations on the biosynthetic mechanisms of alkaloid formation in the plant have increased markedly in the last decade. The number of reviews which have recently appeared[2] indicate the extent of present interest in this problem and are sufficient to provide a background of current ideas on alkaloid biogenesis. The reviews of Marion,[2c] Poisson[2d] and Mothes[2e,f] describe, in addition, the methods which have been

(1) The work described in this paper was sponsored in part by the United States Atomic Energy Commission and Grant B-570 from the National Institute of Neurological Diseases and Blindness, Public Health Service.

(2) See for example (a) R. Robinson, "The Structural Relations of Natural Products," Clarendon Press, Oxford, England, 1955; (b) R. B. Woodward, *Angew. Chem.*, **68**, 13 (1956); (c) L. Marion, *Bull. soc. chim. France*, 109 (1958); (d) J. Poisson, *Ann. biol.*, **34**, 395 (1958); (e) K. Mothes, *Pharmazie*, **14**, 121 (1959); (f) K. Mothes, *ibid.*, **14**, 177 (1959); (g) E. Wenkert, *Experientia*, **15**, 165 (1959).

used to obtain experimental evidence relating to the mechanism of alkaloid formation.

One of the first methods used was the synthesis of alkaloids *in vitro* by bringing together possible highly reactive precursors under pseudo-physiological conditions.[2e] Although often leading to the synthesis of the alkaloid, this method is open to the criticism that such conditions do not parallel those found in the plant. A number of techniques involve the detection of some change in alkaloid formation resulting from a given applied environmental change.[2d] Thus, one may observe an increase or change in alkaloid content after feeding a certain presumed precursor, omitting certain factors from a nutrient, or even changing light or temperature conditions. In certain cases, grafting experiments using scion and stock from different plants, one containing a given alkaloid and another not having such a compound, have yielded results particularly interesting in regard to possible sites of synthesis. However, an environmental change probably has an effect on the entire plant and thus may be effecting the alkaloid-forming process only remotely. Although these experiments and those involving *in vitro* syntheses are incapable of proving a biosynthetic pathway or even the involvement of any given compound as a precursor, they have certainly suggested directions for further research, and may be valuable as supporting evidence.

The chief method used today in investigating alkaloid biosynthesis is the feeding of radioactively-labeled compounds which, usually from a consideration of structural relationships, are thought to be likely precursors of the alkaloid.[2d,f] However, this method, when not carefully undertaken or interpreted has a number of shortcomings which are sometimes overlooked or ignored. Ideally, a true precursor should satisfy the criteria of specificity and yield. Specificity is necessary in order to eliminate the possibility of degradation to simpler molecules followed by resynthesis. Even in this case, specificity might result, and in its most exacting form, specificity would require a multiply-labeled precursor and a pattern of labeling, both as to position and ratio of activity, in the alkaloid identical with that in the precursor. Unfortunately, these standards of specificity have rarely been met.

The problem of yield is a particularly difficult one with plants and the percentage incorporation of specific activity (where reported) seems usually to be extremely low. Many of the studies result in a percent incorporation of specific activity below 0.1% and sometimes below 0.01%, far less than would be anticipated merely by pool-size dilution. It is certainly possible that such low radioactivity yields may reflect a transport or similar physiological phenomenon, but isolated cases[3] have indicated that values of 2 to 3% can be obtained. Low incorporation also may mean that the plant is only forming a small amount of its alkaloid from the compound fed or that the compound is an extremely distant precursor. Such possibilities would be interesting in themselves but would yield relatively little information as regards the total biosynthetic scheme.

As an alternative to the above procedures, we have undertaken the study of alkaloid biosynthesis using radioactive carbon dioxide. A number of workers[4] have previously prepared radioactive alkaloids by plant growth in the presence of $C^{14}O_2$ but their objective has been mostly preparative and not mechanistic. McIntosh, Kelsey and Geiling[4a] first used this technique with the opium poppy and it was developed so that eventually morphine was isolated having an activity of about 0.23×10^6 disintegrations per minute per micromole (d.p.m./μmole).[4b] In the same manner Evertsbusch and Geiling[4c] from *Atropa belladonna* obtained atropine having 0.20 d.p.m./μmole and Peets and co-workers[4d] isolated from *Rauwolfia serpentina* reserpine having 0.55×10^6 d.p.m./μmole. Kuzin and Merenova[4e] also investigated the biosynthesis of morphine alkaloids using $C^{14}O_2$ but obtained alkaloids of lower specific activity. In general, the plants were grown in an enclosed chamber for periods of from 20 to 40 days in an atmosphere which contained appreciable amounts of $C^{14}O_2$ over the entire time. It was assumed that this method would produce randomly labeled alkaloids and no chemical degradations of the materials obtained were reported. Aronoff,[4f] in attempting to use this technique mechanistically, grew excised tobacco leaves in $C^{14}O_2$ for 3 hours. After continuing normal growth for 48 hours, anabasine was isolated. A partial degradation indicated that the alkaloid was uniformly labeled.

The present work was initiated to obtain C^{14} labeled morphine for human and plant metabolism studies as well as to study the mechanism of biosynthesis of the opium alkaloids. Although using $C^{14}O_2$ introduces much complexity into the biosynthetic pathway study, it offers possibilities in learning the detailed mechanism that other precursor studies do not. The method in general involves growing plants in the presence of $C^{14}O_2$ and observing the relative rates and amounts of radioactivity incorporated into different molecules or different portions of the same molecule.[5]

Methods

Plant Growth.—Except as noted otherwise, the plants used in this work were from seed of *Papaver somniferum* L., var. *alba*, U.S.D.A. No. 40, grown at Somerton, Arizona in 1950.[6] A complete description of the cultivation of such poppies has been given by Mika.[4b] In our work, the seeds were germinated in 10 inch pots containing vermiculite and were thinned to one or two plants per pot for continued growth. The nutrient solution of Hoagland and Broyer,[7]

(3) (a) E. Leete, L. Marion and I. D. Spenser, *Nature*, **174**, 650 (1954); (b) D. Gröger, H. J. Wendt, K. Mothes and F. Weygand, *Z. Naturforsch.*, **14b**, 355 (1959); (c) H. R. Schütte and E. Nowacki, *Naturwissenschaften*, **46**, 493 (1959).

(4) (a) B. J. McIntosh, F. E. Kelsey and E. M. K. Geiling, *J. Am. Pharm. Assoc.*, **39**, 512 (1950); (b) E. S. Mika, *Bot. Gaz.*, **116**, 323 (1955); (c) V. Evertsbusch and E. M. K. Geiling, *Arch. intern. pharmacodynamie*, **105**, 175 (1956); (d) E. A. Peets, A. R. Schulert, J. Skok and W. Charney, *J. Am. Pharm. Assoc.*, **47**, 280 (1958); (e) A. M. Kuzin and V. I. Merenova, *Biokhimiya*, **19**, 616 (1954); (f) S. Aronoff, *Plant Physiol.*, **31**, 355 (1956).

(5) See for example, J. A. Bassham and M. Calvin, "The Path of Carbon in Photosynthesis," Prentice-Hall, Englewood Cliffs, New Jersey, 1957.

(6) The seed was kindly provided by Dr. Norris W. Gilbert, U.S.-D.A. Crops Research Division, Mesa, Arizona.

(7) D. R. Hoagland and T. C. Broyer, *Plant Physiol.*, **11**, 471 (1936).

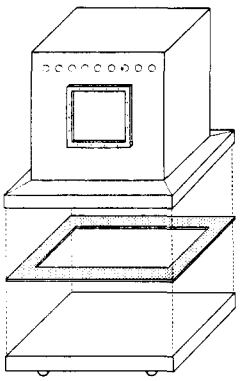


Fig. 1.—Large biosynthesis chamber.

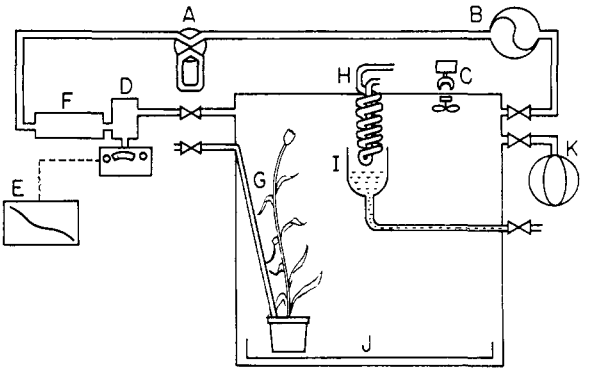


Fig. 2.—Diagram of biosynthesis chamber in operation.

with the substitution of an iron–Versenol solution for iron tartrate, was used to water and feed the plants. Extreme variation in plant size and maturation date was noted if both seed germination and plant growth took place exclusively in the greenhouse. To obtain a standard poppy, seeds were germinated in the dark, and controlled lighting was used for bud induction as a supplement to normal greenhouse growth. In the summer, plants were maintained on short days for the first 30 to 50 days and then placed in the greenhouse where normal bud induction took place. In the winter, the process was reversed.

Biosyntheses.—For multi-plant biosyntheses, a chamber 30 × 30 × 30 inches and containing a ten inch square opening for an access port (Fig. 1) was constructed of $^3/_8$ inch Lucite. The sides were screwed together and sealed with chloroform. A door was constructed of Lucite to fit over the access port, and this was bolted to the chamber during a run. Between the door and chamber was placed a rubber gasket which was well greased with stopcock grease. A false bottom for the chamber was constructed of wood to which rollers were attached. To the bottom edges of the chamber was affixed a two inch wide flange of one and one-half inch Lucite. A large rubber gasket, well greased, was placed on top of the wooden bottom, and the chamber proper was tightly clamped on with wood clamps.

In Fig. 2 is diagrammed the chamber in operation. In a typical experiment, $C^{14}O_2$ is generated from $BaC^{14}O_3$ with sulfuric acid in a vacuum line in the usual manner and collected in a carbon dioxide feed loop which is then inserted in the system at point A (loop shown closed to the system). A diaphragm air pump (B) circulates air and pumps the $C^{14}O_2$ into the chamber where it is mixed rapidly by means of a magnetic powered fan (C). Air leaving the chamber is monitored at D by an ionization chamber and vibrating reed electrometer connected to a recorder (E). The carbon dioxide content is then measured at F by an infrared analyzer. A chamber this size can accommodate about nine ten-inch pots. One such pot and the manner in which nutrient is fed is indicated in the diagram as G. Nine $^1/_8$ inch valves were threaded through the chamber just above the access port. By means of a short length of flexible tubing, a glass tube was affixed to the valve on the inside and the tube was extended to the surface of the vermiculite in each pot. In order to control the humidity, a one-half inch stainless steel coil (H) through which refrigerated water can circulate was attached to the top of the chamber. The condensate is caught in a trap (I) suspended from the coil and the water can be removed while the chamber is in operation. A large stainless steel pan (J) was placed on the bottom of the chamber to simplify cleaning. On either side of the chamber was placed a bank of nine 46 inch "daylight" fluorescent lights. With aluminum foil placed behind the lights as a reflector and the lights placed about ten inches from the chamber, a light intensity of about 1000 f.c. at the center of the chamber was usually obtained. In order to dissipate pressure changes in the chamber due to changes in the air temperature, a vinyl balloon (K) was attached to the system.

This chamber is a modification of that described by Kuzin and Merenova.[4e] While not as elegant as the "Argonne" chamber described by Scully and co-workers,[8] it is much simpler to construct, and more flexible as a research instrument. As the results below indicate, quite high activities may be obtained using this chamber, and in many cases an elaborate system[8] and long growth periods are not necessary.

For single plant, short term biosyntheses, a small chamber was constructed by cutting a large cylindrical battery jar and using it inverted on a glass plate. The chamber measured nine inches in diameter and was 18 inches high. Again, a cooling coil was affixed to the inside of the top of the chamber. This chamber could be interchanged with the large one so as to utilize the same monitoring system.

Each of the biosyntheses herein reported was begun with plants two to five days prior to the blossoming stage. Because of day length variation during the growth season, the plants were not always of the same chronological age. The $C^{14}O_2$ was generated with concentrated sulfuric acid from $BaC^{14}O_3$ having a specific activity of approximately 120 μc/mg. This was accomplished in a vaucum line and the $C^{14}O_2$ was trapped by cooling a loop in liquid nitrogen. The loop was transferred to point "A" in Fig. 2 and the contents were pumped into the chamber containing the plants. In using the large chamber, the carbon dioxide concentration was kept between 0.02 and 0.4% throughout the period of biosynthesis by adding small portions of Dry Ice as needed through a hole in the top of the chamber. The hole was ordinarily kept sealed with a stopper. With four 20 inch plants in the large chamber, the relative humidity was generally 80 to 85% and the temperature 18 to 24°. Because of the volume of the large chamber, the amount of carbon dioxide added to the atmosphere when introducing the $C^{14}O_2$ was small compared to the amount already present. When using the small chamber, however, the initial carbon dioxide concentration would reach 1%. If the biosynthesis period using the small chamber was sufficiently long so that this concentration would drop below normal (approximately 0.03%), the inlet tube to the chamber was opened and air was pulled through the system. The same technique was used if it was necessary to stop the biosynthesis before the activity in the chamber had dropped to a safe level. In each of the experiments (with the one exception noted below) the only introduction of $C^{14}O_2$ took place at the beginning of the run. In the large chamber containing four 20 inch plants, about 70% of the radioactivity was taken up in three hours and slightly over 90% in 24 hours. With a 12 inch plant in the small chamber, 90% of the introduced activity was usually taken up by the plant in about three hours.

Alkaloid Isolation.—At the end of a typical biosynthesis, the poppy plant (20 g. wet weight) was cut in small pieces, dropped into liquid nitrogen in a large mortar, and ground to a powder. Aqueous sodium bicarbonate, 25 ml. of a 10% solution, was added and then 100 ml. of a 1:1 mixture of butanol and benzene.[9] The resultant mixture was shaken vigorously for a few minutes to cause an emulsion, and this was then allowed to stand at room temperature for 40 hours.[10] The mixture was filtered, washed with a portion of butanol–benzene, and the layers were separated. The organic phase was extracted three times with 45 ml. of 0.5 *N* sul-

(8) N. J. Scully, W. Chaney, G. Kostal, R. Watanabe, J. Skok and J. W. Glattfield, "Biosynthesis in Carbon-14 Labeled Plants," U. N. Conference on Peaceful Uses of Atomic Energy, Geneva, Switzerland, August 1955.

(9) L. B. Achor and E. M. K. Geiling, *Anal. Chem.*, **26**, 1061 (1954).

(10) E. Leete, THIS JOURNAL, **81**, 3948 (1959).

furic acid, and the *p*H of the acidic solution was adjusted to 11.8 by the addition of potassium hydroxide. This was extracted three times with 100-ml. portions of methylene chloride. The *p*H of the aqueous solution was adjusted to 8.6 by the addition of phosphoric acid, and the solution was then extracted continuously for 16 hours with chloroform.

The residue from evaporation of the chloroform was dissolved in 5 ml. of 0.1 *M* phosphoric acid and was washed twice with equal volumes of methylene chloride. After having been adjusted to *p*H 8.6, the aqueous phase was extracted eight times with equal volumes of chloroform. Evaporation of the chloroform yielded a residue of morphine. The morphine at this point was usually sufficiently pure so that it could be quantitatively analyzed by observing the absorption at 287 mμ (ε = 1,460, 95% ethanol). Small amounts of radioactive impurities were often present and for final purification cellulose powder chromatography was employed (see below).

The methylene chloride extract of the *p*H 11.8 aqueous phase was dried and evaporated to dryness. The residue was dissolved in 5 ml. of 0.1 *M* phosphoric acid and was washed three times with equal portions of methylene chloride. This extract yielded a residue which, when chromatographed on paper, showed the presence of an alkaloid or alkaloids having the same R_F value as papaverine and narcotine. The *p*H of the aqueous layer was adjusted to 11.8 with potassium hydroxide solution and was extracted with three equal volumes of methylene chloride. After the methylene chloride solution was evaporated, a residue was left which contained codeine and thebaine. These were separated by alumina chromatography (see below). From the alumina chromatography codeine was obtained which was free from radioactive impurities or impurities which absorbed in the ultraviolet. The quantity of codeine was determined from the absorption at 287 mμ (ε = 1,550 in 95% ethanol). The thebaine was usually sufficiently pure for ultraviolet analysis at 285 mμ (ε = 7,680 in 95% ethanol), but often required a further purification by cellulose powder chromatography to free it from radioactive impurities.

Many of the literature procedures for the quantitative estimation of morphine alkaloids in micro amounts involve determinations in the presence of other interfering substances for which allowance is made or a blank constructed. As described above, the analyses here were conducted on pure samples or samples containing only traces of non-interfering materials.

Chromatographic Techniques.—For the purification of the plant alkaloids and degradation products, both cellulose powder and alumina column chromatography were employed. A Standard Grade Whatman cellulose powder was used which was buffered by stirring a slurry of the powder in 0.2 *M* potassium dihydrogen phosphate for a few minutes in a Waring blender. The material was filtered, dried thoroughly, and repowdered in the blender. A solvent system was developed which would separate most of the mixtures encountered and consisted of a 2:1:1 by volume mixture of *n*-propyl alcohol, ether and water. The cellulose was applied to the column (10 × 350 mm.) in a slurry of the solvent mixture and packed by applying an air pressure of 10 lb./sq.in. on top of the column. In order to obtain even distribution on the column, the alkaloid was usually dissolved in a minimum amount of a suitable solvent and evaporated onto a thick filter paper disk which was then placed on top of the column.

For the separation of codeine and thebaine and the purification of some codeine derivatives, alumina chromatography was used.[11] For this, Reagent Grade Merck alumina was employed. It was basic, a water slurry having a *p*H of about 10. The column (10 × 350 mm.) was prepared in benzene in the usual manner and the alkaloid mixture was applied by dissolving the residue in a minimum of benzene. As eluent, a mixture of 88.5% benzene, 10% chloroform, and 1.5% isopropyl alcohol was used. This eluted thebaine fairly rapidly, but codeine-type compounds were held somewhat more tightly and spread into many fractions. However, if about 1% methanol was added to the eluent after thebaine had passed through, the codeine could be concentrated into three or four fractions.

The propanol–ether–water solvent system was also used for most of the paper strip chromatography. A buffered paper was prepared by dipping sheets of Whatman No. 4 in 0.2 *M* potassium dihydrogen phosphate and allowing them to drip dry. Using the descending technique, the R_F values observed were: morphine = 0.50, codeine = 0.60, thebaine = 0.75, papaverine and narcotine = 0.90. An iodoplatinic acid reagent spray[12] was used to detect the spots. Morphine gives a blue-violet spot, codeine and thebaine a violet one, and papaverine and narcotine red-violet spots.

An additional system was necessary to separate morphine from normorphine and codeine from norcodeine. This was composed of *t*-butyl alcohol, methyl ethyl ketone, diethylamine and water (175:215:10:100), and when employed on the buffered paper gave the following R_F values: morphine = 0.85, normorphine = 0.60, codeine = 0.90, norcodeine = 0.75. The iodoplatinic acid reagent was less satisfactory in this case, gave dark spots for each alkaloid and was not as sensitive as with other solvent systems.

No prior equilibration period was needed with either system and excellent separation was obtained in 2 to 4 hours.

Radioactivity Measurements.—Aliquots were removed from the alkaloid solution used for the ultraviolet analysis and spread in a uniform, "infinitely thin" film on aluminum plates. These were counted in a Nuclear Chicago Q gas flow counter (Model 47) using a "Micromil" window. Counter efficiency was about 40%. If direct comparison was to be made between a number of compounds or a series resulting from a degradation, such samples were counted successively at a given time.

To check the radioactive purity, an aliquot was spotted on buffered paper, a strip was developed and analyzed to determine if the only radioactivity present coincided with the alkaloid spot. This was done either by radioautography on the low activity compounds or by use of a Nuclear Chicago Model C-100A "ActigraphII" for counting and automatically recording activity along the entire strip. Where necessary, a second check was made by running another strip on the same material, but employing a different solvent system.

Degradations.—In order to determine the amount of activity which entered the basic carbon skeleton of each alkaloid the N-methyl and O-methyl groups were removed from morphine, codeine and thebaine according to the scheme diagrammed in Chart 1.

The procedure of von Braun[13] was used to remove the N-methyl group from codeine and morphine, and the pyridine hydrochloride–ether cleavage[14] was used to remove the O^3-methyl group from codeine. These reactions could not be used directly on thebaine so thebaine was first converted to dihydrocodeine. Pyridine hydrochloride cleavage of the dihydrocodeine removed the O^3-methyl group and again the von Braun reaction was used to remove the N-methyl substituent. The conversion of thebaine to dihydrocodeine resulted in removal of the O^6-methyl group. This conversion was based on a series of reactions similar to that employed by Conroy,[15] to convert thebaine to neopine. A number of the reaction conditions and reagents had to be modified. While the exact procedures of Conroy usually gave good results on a gram scale, adaptation of these to milligram quantities gave somewhat variable results. The neopine was obtained crude and was hydrogenated directly to dihydrocodeine, which was the first intermediate in the thebaine degradation purified for radioactive counting. Quantitative analysis of the degradation products was accomplished by observing the characteristic peaks at 285–287 mμ having the following molar extinction coefficients: normorphine, 1510; norcodeine, 1490; dihydrocodeine, 1600; dihydromorphine, 1440; dihydronorcodeine, 1590.

In the description of the degradation steps below, those reactions which were used on a number of similar compounds, *e.g.*, the von Braun reaction on morphine, codeine, or dihydrocodeine, will only be delineated once. Degradations were usually performed on diluted material. Sufficiently active morphine and codeine were always obtained so that a number of determinations could be made. This was also true of thebaine in the long term runs. However, only one degradation of thebaine was possible with material from each short term run.

(11) C. C. McElheny, G. DeLaMater and R. D. Rands, *Anal. Chem.*, **26**, 819 (1954).

(12) R. Mosser and M. Machebouef, *Bull. soc. chim. biol.*, **31**, 1144 (1949).

(13) J. von Braun, *Ber.*, **47**, 2312 (1914).

(14) H. Rapoport, C. H. Lovell and B. M. Tolbert, THIS JOURNAL, **73**, 5900 (1951).

(15) H. Conroy, *ibid.*, **77**, 5960 (1955).

Morphine Degradation.—Morphine, 0.49 mg. having 54,200 d.p.m./μmole, was heated at reflux under nitrogen with 0.2 ml. of acetic anhydride for 16 hours. The anhydride was removed *in vacuo* and the residue was dissolved in 2 ml. of benzene. This was washed twice with 0.5 *M* sodium carbonate and twice with water. The benzene solution was dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was dissolved in 1 ml. of chloroform and this solution was added to a solution of 2.5 mg. of cyanogen bromide in 1 ml. of chloroform which was cooled in ice water. The resulting solution was allowed to stand for 30 minutes in ice and then 30 minutes at room temperature. It was then heated at reflux for 2.5 hours. At the end of this time, the sclution was evaporated to dryness and two 1-ml. portions of chloroform were added and each evaporated. The residue was heated for five minutes at 100° with 0.3 ml. of concentrated hydrochloric acid after which 3 ml. of water was added and the solution was heated at reflux for three hours. It was then evaporated to dryness and the residue was dissolved in 1 ml. of a methanol–water mixture. This solution was evaporated onto a thick filter paper disk about 10 mm. in diameter and placed on top of a column of 0.2 *M* KH_2PO_4-buffered cellulose powder. Elution was with the propanol–ether–water solvent mixture. About 0.8 ml. fractions were collected and normorphine, 0.046 mg. having 50,200 d.p.m./μmole, was present in fractions 14 and 15.

Codeine Degradation.—Codeine, 7.0 mg. having 50,200 d.p.m./μmole, was placed in a 5 mm. tube and 30 mg. of anhydrous pyridine hydrochloride was added. The tube was evacuated immediately and sealed. It was then heated for seven minutes at 210 to 220°. The tube was cooled, opened, and the solid was dissolved in 2 ml. of 0.1 *M* phosphoric acid. This was extracted twice with methylene chloride and the pH of the aqueous solution was adjusted to 5. This was then extracted five times with two-ml. portions of methylene chloride, readjusting the *p*H to 5 after each extraction. The *p*H of the aqueous solution was then adjusted to 11.8 and extracted twice with methylene chloride. The *p*H of the aqueous layer was once more adjusted, this time to 8.6 and extracted six times with 2-ml. portions of chloroform. Evaporation of the chloroform solution to dryness gave a residue showing the presence of 0.95 mg. of morphine having 40,100 d.p.m./μmole. This material was usually pure morphine free from any radioactive contaminants. When not pure, it could be purified by repeating the extraction scheme or better by cellulose powder chromatography as with the normorphine.

Thebaine Degradation.—Thebaine, 3.27 mg. having 16,700 d.p.m./μmole, was dissolved in 1 ml. of carbon tetrachloride and 2.5 mg. of crystalline flakes of N-bromosuccinimide was added. The mixture was stirred at room temperature for 16 hours. It was then filtered and the filtrate was evaporated to dryness. The residue was dissolved in 1 ml. of dry ether and 0.2 ml. of a saturated solution of lithium aluminum hydride in ether was added. This solution was stirred under nitrogen for two hours and then 0.5 ml. of ethyl acetate was added, followed by 2 ml. of 1 *N* hydrochloric acid. The layers were separated and the acidic solution was washed twice with equal volumes of ether. It was then made basic by the addition of a solution of potassium hydroxide and potassium sodium tartrate and extracted four times with 3 ml. portions of chloroform. The chloroform solution was evaporated to dryness. A small portion of the residue was chromatographed on a buffered paper strip. The spray reagent showed the presence of a large spot coinciding with neopine and three additional minor spots. The residue was dissolved in 1 ml. of absolute ethanol, about 1 mg. of platinum oxide catalyst was added, and the mixture was hydrogenated under atmospheric pressure for two hours. Filtration and evaporation of the filtrate to dryness left a residue which was chromatographed on alumina as described above. About 2.5-ml. fractions were collected. Unknown alkaloidal material was found in fractions 17 to 19. After fraction 21 was collected, about 1% methanol was added to the eluent and dihydrocodeine was eluted in fractions 26 to 30. A total of 0.45 mg. of pure dihydrocodeine having, 5,010 d.p.m./μmole was obtained. Of this, half was cleaved with pyridine hydrochloride to yield 0.052 mg. of dihydromorphine having 3,840 d.p.m./μmole and half was treated by the von Braun procedure to give 0.034 mg. of dihydronorcodeine having 4,620 d.p.m./μmole.

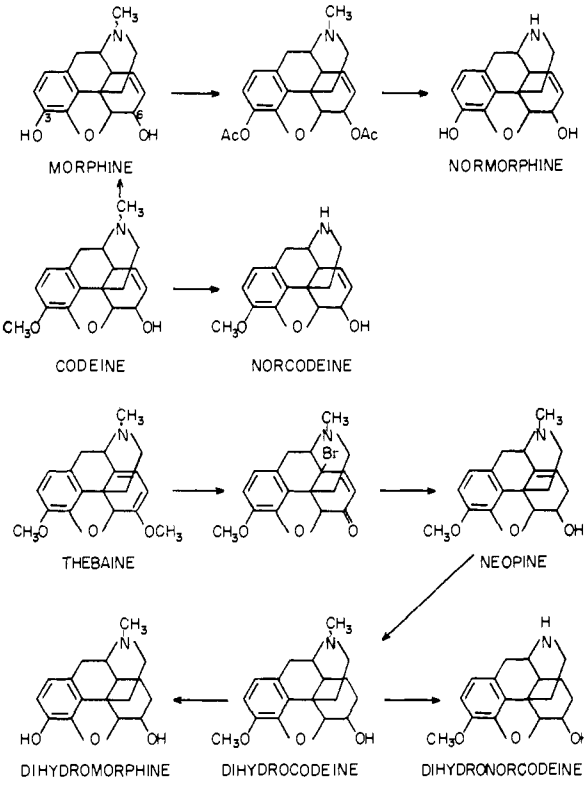


Chart 1.—Demethylation of morphine alkaloids.

Results

In Table I are listed the results of ten biosyntheses. Although the first six runs were exploratory in nature, the partial results are in agreement with the more complete and carefully controlled experiments 7 to 10. Discussion will, in general, refer to the latter, "standardized" runs. In experiments 7 and 8, plants which were 3.5 months old were used while those of experiments 9 and 10 were 2.5 months old. All were at the same physiological stage at the beginning of each experiment: 2 to 5 days before blossoming. Since the emphasis in the runs described was on obtaining radioactively pure alkaloids, narrow fractions were often taken in chromatographies and hence the quantities reported in Table I do not reflect accurately the relative amounts of each alkaloid actually present in the plant. Separate quantitative analyses of plants identical to those of runs 9 and 10 gave a morphine:codeine:thebaine ratio of 15:3:1. As it was difficult to achieve both chronological and physiological age correspondence for each experiment, it was arbitrarily decided to standardize the state of maturity in this initial work. Since differing activities of $C^{14}O_2$ and different plant weights were used in each run, vertical comparison of absolute specific activities between the various runs cannot be made. The results will be discussed in terms of comparisons of specific activity ratios among the three morphine-type alkaloids as found in each experiment.

From Table I it can be seen that the specific activities, with the exception of runs 4 and 8, are in the order thebaine>codeine>morphine. To determine if these ratios hold for the carbon ring

TABLE I

BIOSYNTHESIS RESULTS AND TOTAL ACTIVITIES OBTAINED

Run, no.[a,b]	$C^{14}O_2$, (mC.) absorbed	Time under $C^{14}O_2$	Time under normal air	Wet wt. plants, g.	Morphine Mg.	Morphine 10^3d.p.m./μmole	Codeine Mg.	Codeine 10^3d.p.m./μmole	Thebaine Mg.	Thebaine 10^3d.p.m./μmole
1	0.40	3 hr.	3 hr.	43.9	..	0.38	..	3.5	..	27.4
2	1.06	2 hr.	...	36.9	..	>2.6	..	6.3	..	23.4
3	1.07	6 hr.	10 days	34.7	..	108	..	127	..	190
4	1.00	6 hr.	5 days	34.1	..	128	..	211	..	209
5	20.0	5 hr.	1 hr.	6.1	..	50	..	1440	..	7000
6	36.9	8 days	...[d]	170	37.1	1030	3.0	1450	Not isolated	
7	23.5[c]	8 days	...[d]	190	46.4	505	5.2	572	3.4	667
8	39.2[d]	8 days	...[d]	143	28.9	1530	4.7	1430	2.8	806
9	21.5[e]	1.8 hr.	0.3 hr.	21	1.3	10.6	0.33	197	0.07	2810
10	20.1[f]	3 hr.	3 hr.	8	0.75	202	0.18	4040	0.004	16500

[a] Runs 1 to 6 were preliminary trials using different plant varieties at varying growth stages. [b] In all runs except No. 6, the $C^{14}O_2$ was introduced only at the beginning. In Run 6, 24.5 mC. was introduced in the chamber at the beginning and 12.4 mC. added in the course of six days. [c] 90+% of activity was absorbed in the first 24 hours. [d] Although CO_2 was added to the chamber, the plants were allowed to reabsorb their expired $C^{14}O_2$. [e] 50% of activity was absorbed in 0.5 hours, 95% in 1.8 hours. [f] 97% of activity was absorbed in three hours.

skeleton or are simply a reflection of the number of N-methyl and O-methyl groups, these methyl groups were removed from the alkaloids in a number of cases. The activities remaining in the ring carbons are listed in Table II. In Table III is given a complete breakdown of the amount of activity present in each of the methyl groups.

TABLE II

SPECIFIC ACTIVITY IN RING CARBONS

Run no.	Time	Nuclear specific activity (10^3 d.p.m./μmole) Morphine	Codeine	Thebaine
6	8 days	690	..	..
7	8 days	463	346	200
8	8 days	1230	..	..
9	2 hr.	4.8	77	300[a]
10	6 hr.	166	1920	2780

[a] Minimum estimation. See discussion in "Results."

In the 2 hour run (No. 9) insufficient dihydrocodeine was isolated from thebaine and it was not possible to complete the degradation. The value given for the N-methyl group in Table III represents a maximum estimated from the results of the other runs so that a minimum value for the ring skeleton results. Actually the N-methyl activity might be expected to correspond more with Run 5 which showed a much lower value in comparison with the O^3- and O^6-methyls.

TABLE III

COMPARISON OF SPECIFIC ACTIVITIES (10^3 D.P.M./μMOLE) OF PERIPHERAL METHYL GROUPS AND RING CARBONS

Run no.	Compound	Total	N-Methyl	O^3-Methyl	O^6-Methyl	Ring carbons
9	Morphine	10.6	5.8[a]	.....	....	4.8
(2 hr.)	Codeine	197	48.2[b]	51.8[c]	....	77
	Thebaine	2810	340[d]	343[e]	1830[f]	300[d]
10	Morphine	202	36[a]	.....	....	166
(6 hr.)	Codeine	4040	1240[b]	876[c]	....	1920
	Thebaine	16500	398[g]	2020[e]	11500[f]	2580
7	Morphine	505	42[a]	.....	....	463
(8 days)	Codeine	575	140[a]	89[c]	....	346
	Thebaine	667	166[g]	132[e]	169[f]	200

[a] Normorphine counted. [b] Norcodeine counted. [c] Morphine counted. [d] Maximum estimation for N-methyl giving minimum estimation for ring carbons. See discussion in "Results". [e] Dihydromorphine counted. [f] Dihydrocodeine counted. [g] Dihydronorcodeine counted.

Discussion

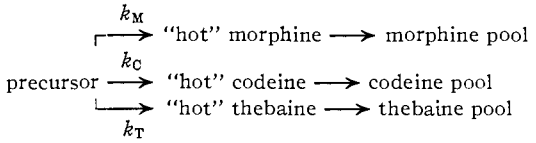
From the results of runs 6 to 8 listed in Table I it is seen that morphine of high specific activity can be obtained using the biosynthetic modifications introduced here. From run 8, morphine was obtained which had more than six times the specific activity previously reported.[4b] Strict comparison with all the biosyntheses reported in the literature[4] is, however, not possible since the total amount of $C^{14}O_2$ fed and total amount of alkaloids isolated were not always reported. However, the method of introducing the $C^{14}O_2$ at one time and conducting the biosynthesis for eight days instead of using growing periods of 20 to 40 days and maintaining a reasonable $C^{14}O_2$ level over the entire time allows for a considerable saving in time and operational maintenance. Indeed, the eight day period was an arbitrary choice for the initial experiments, and analysis of the results of all the runs (see below) indicates that the morphine may have reached its highest specific activity considerably prior to eight days. This possibility is now being investigated. If the alkaloid desired is metabolically stable, the total activity will continue to increase with time, and the point at which the biosynthesis is stopped will probably depend upon individual needs. The specific activity of the morphine from run 8 was sufficiently high so that it could be diluted, demethylated and remethylated to give morphine labeled exclusively in the ring carbons, and this was subsequently used for studies on the metabolic fate in humans.[16]

From the data in Table I it is seen that the specific activities are, with the exception of runs 4 and 8, in the order thebaine>codeine>morphine. Since this is also the order of the number of N- and O-methyl groups present in the alkaloids, it was possible that most of the radioactivity in the molecules was present in the relatively labile methyl groups. The removal of these groups gave the results listed in Tables II and III and shows that in the two and six hour runs the same order of specific activities obtains even with respect to the main carbon skeleton. However, the difference be-

(16) Part of a study of morphine metabolism under investigation in this Laboratory.

tween the alkaloids has diminished considerably and Table III shows that the labile methyl groups do indeed become labeled much faster than the gross ring structure. The removal of the methyl groups from the alkaloids of run 7, an eight day run, results in a reversal of the order of specific activities when comparing the ring nucleii. This brings the results of runs 4 and 8 into agreement with the others.

In respect to the short term runs, it seems very likely that only a small amount of alkaloid is synthesized during the course of the experiment as compared with the total amount of each alkaloid already present in the plant. Since the total plant material is analyzed, a large dilution of each "hot" alkaloid must occur. If one assumes that morphine, codeine and thebaine are synthesized at approximately the same rate, then the observed order of specific activities would be thebaine> codeine>morphine since the morphine formed would undergo the greatest dilution with material present in the plant and thebaine the least. This relationship may be pictured in Scheme I where $k_M = k_C = k_T$ and "precursor" represents a structure close to that of the three alkaloids.



Scheme I

In evaluating such a scheme it is necessary to know the exact mole ratio of the three alkaloids in the plant and the results obtained for plants identical to those used in the short term runs give a morphine:codeine:thebaine ratio of 15:3:1. If Scheme I with $k_M = k_C = k_T$ obtains, then the maximum difference in specific activities (caused by dilution) would be represented by the morphine: codeine:thebaine ratio of 1:5:15. The observed ratio for the six hour run is 1:12:17 and for the two hour biosynthesis is 1:16:60. These ratios differ significantly enough from those calculated for simple dilution that this mechanism seems to be ruled out.

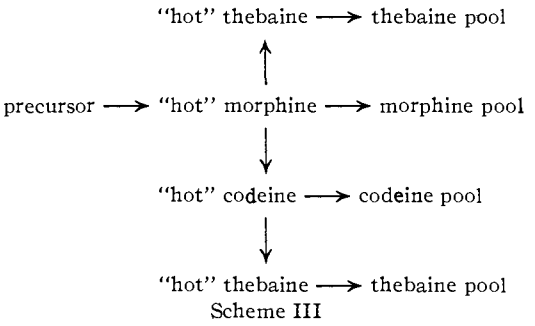
Since the actual ratio of morphine to codeine to thebaine in the plant is 15:3:1, one might consider that the rates of alkaloid formation are in the same ratio. This would be depicted by Scheme I where $k_M:k_C:k_T = 15:3:1$. However, this possibility can be eliminated since it would result in alkaloids having approximately the same specific activity, and this is not observed.

Schröter[17] has pictured biological methylation as a process of deactivation in which the active hydrogens of the –OH, $-NH_2$ and >NH are methylated and the resultant compounds more or less relegated to storage unless demethylated again. If such were the case with regard to the morphine-type alkaloids, then Scheme II might represent the relationship among these alkaloids.

precursor → morphine → codeine → thebaine

Scheme II

However, this would result in a ratio of specific activities opposite to that observed. A somewhat different relationship which still represents methylated compounds as being secondarily formed is expressed in Scheme III. In this representation



Scheme III

codeine and thebaine are formed from morphine only at the site of morphine synthesis itself and not from the general morphine pool in the plant. As depicted, thebaine could also come from codeine in the same manner. If such a relationship holds, all the "hot" alkaloids in the most favorable case might have approximately the same specific activities and would be diluted either by passing into the inactive pool or by being mixed with the pool alkaloids in the isolation procedure. However, again the maximum divergence of specific activities possible would be that represented by the dilution factors and this does not correspond to the experimental facts as discussed in regard to Scheme I. In addition, such a scheme seems to favor the idea that the poppy plant possesses one particular site or at least a number of relatively isolated sites for alkaloid synthesis. To date most of the evidence is negative with regard to finding a specific synthesis site and indeed, Kleinschmidt and Mothes[18] have presented evidence that alkaloid synthesis can take place in the latex or milky sap which is distributed throughout the plant.

Another possible relationship among the alkaloids is that depicted in Scheme IV and is actually

precursor M → morphine
precursor C → codeine
precursor T → thebaine

Scheme IV

a statement of the possibility that there is no relation among them in regard to their manner of formation. The data as presented here could neither support nor eliminate such a possibility but it is rejected on the grounds that some correspondence undoubtedly exists in the modes of formation of such complex but structurally similar substances.

The most obvious and simplest relationship which would be in accord with and explain the data obtained is that of Scheme V. The specific activity

precursor → thebaine → codeine → morphine

Scheme V

ratios found in the two and six hour runs and an examination of the nuclear specific activity ratios in the alkaloids from the eight day run tend to

(17) H. B. Schröter, *Pharmazie*, **10**, 141 (1955).

(18) G. Kleinschmidt and K. Mothes, *Z. Naturforsch.*, **14b**, 52 (1959).

support the hypothesis of Scheme V. The eight day run can be interpreted as a reverse of the short term runs. Since $C^{14}O_2$ was introduced in one dose at the beginning of the experiment, one can imagine that the alkaloids have reached a maximum level of specific activity at a certain time, perhaps 24 to 48 hours later, and that the introduction of unlabeled carbon dioxide thereafter has resulted in a lowering of the specific activity. If $C^{14}O_2$ entered thebaine first, followed by codeine and morphine, thebaine would also pass through the peak activity and diminish in activity first. The nuclear specific activity data of run 7 in Table II supports this idea. The six hour run involved the absorption of $C^{14}O_2$ for three hours followed by ordinary air for three hours and hence it seems possible that the peak differences in alkaloid specific activity had already been reached and the reverse trend had set in. This would explain the relatively low thebaine specific activity from this run.

If such a relationship as Scheme V is indeed the correct one, it has some interesting corollaries. If thebaine and codeine are formed more rapidly than morphine one might expect that the quantity of these alkaloids would exceed that of morphine. Since this is not the case, it follows that codeine and thebaine must also be metabolized faster than morphine. In much of the early literature alkaloids have been considered only as storage or end products, while more recent studies with labeled alkaloids have led some authors to believe that they are more intimately connected with metabolism. Our results certainly indicate that codeine and thebaine are active in some way in the plant's metabolism. It would be of interest to determine whether this metabolism consists merely in the conversion to morphine or in other directions as well. Whether or not morphine is also metabolically active cannot be directly deduced from the specific activity data. However, Miriam and Pfeifer[19] showed that over a period of four weeks of poppy plant growth the percentage of morphine increased steadily while the percentages of codeine and thebaine remained essentially constant. This would be in accordance with the hypothesis that thebaine and codeine are active intermediates in the plant, more or less in equilibrium with other metabolites, while morphine is a storage product which undergoes little change at least in the growth stages investigated. The authors[19] did not comment on the morphine, codeine, thebaine relationships just discussed and indeed used data from the same table on narcotoline and narcotine relationships to come to the conclusion that narcotoline is methylated to narcotine in accord with the Schröter hypothesis. The data presented[19] are insufficient to justify such a conclusion. In another publication, Pfeifer[20] adopted the opposite viewpoint and considered it probable that the O-demethyl alkaloids are the end-products of biosynthesis.

(19) R. Miriam and S. Pfeifer, *Naturwissenschaften*, **45**, 573 (1958).

Also in accord with Scheme V is the observation that morphine has been found to occur only together with codeine and thebaine while the reverse is not true.

These relationships are seen in Table IV. In all cases where O^3- or O^6-demethylated octahydrophenanthrene alkaloids have been found, they are accompanied by thebaine. Two species have the O^3, O^6-demethylated alkaloid (morphine) and the O^6-demethylated alkaloid (codeine). One has only the O^6-demethylated alkaloid, while two others have only the O^3-demethylated alkaloid (oripavine). These data are neatly explained by Scheme V which proposes the primacy of thebaine.

TABLE IV

OPIUM ALKALOIDS IN PAPAVERACEAE

Species	Morphine	Codeine	Oripavine	Thebaine
P. somniferum L.	+	+	−	+
P. setigerum D.C.[a]	+	+	−	+
P. orientale L.[b]	−	−	+	+
P. bracteatum Lindl.[c]	−	−	+	+
P. paeoniflorum, Hort.[d]	−	+	−	+

[a] H. Asahima, T. Kawatani, M. Duo and S. Fujita, *Bull. Narcotics, U. N. Dept. Social Affairs*, **7**, No. 2, 20 (1957). [b] R. A. Konovalova, C. Yunosov and A. P. Orekhov, *Ber.*, **68B**, 2158 (1935). [c] R. A. Konovalova and U. V. Kiselev, *Zhur. Obschei Chim.*, **18**, 855 (1948). [d] H. G. Boit and H. Ehmke, *Naturwissenschaften*, **45**, 315 (1958).

In regard to the amounts of activity present in each methyl group as indicated in Table III, a number of comments can be made. It is seen that these substituents must indeed have higher specific activities than most, or perhaps all of the ring carbons, even in the eight day biosyntheses. The experiments were not specifically designed to investigate the methylation process and such techniques as using an air flush or varying times in runs 4 and 5 would tend to obscure the exact meaning of the values obtained. In addition, the possibilities that transmethylations with various methylating agents are taking place would render any interpretation of the methyl activities extremely tenuous. One value, however, is of sufficient interest to merit specific notice and this is the very high specific activity noted in the O^6-methyl group of thebaine. This would indicate that a different methylating agent was used for that part of the thebaine molecule as compared to the other positions or at least a different manner of methylation.

Enough evidence seems to have been found to make a relationship similar to Scheme V the most likely and further investigation is being directed towards establishing its details. In addition, a search for earlier precursors is being conducted through the use of $C^{14}O_2$ exposure times shorter than two hours.

(20) S. Pfeifer, *Bull. Narcotics, U. N. Dept. Social Affairs*, **10**, No. 3 (1958).