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

The Biosynthesis of Opium Alkaloids. Alkaloid Interconversions in *Papaver somniferum* and *P. orientale*[1]

BY FRANK R. STERMITZ AND HENRY RAPOPORT

RECEIVED APRIL 15, 1961

The observation has been made that among hydrophenanthrene alkaloids in Papaveraceae (a) thebaine is the most widely occurring and (b) thebaine is the most rapidly formed, and with the highest specific activity, when $^{14}CO_2$ is fed. The primary role of thebaine has been further corroborated by feeding experiments with *P. somniferum* using radioactive thebaine, codeine and morphine, in which it is found that thebaine is converted to codeine and morphine, codeine is converted to morphine, and no other conversions take place within this group. Also, in *P. orientale*, thebaine appears to be converted to oripavine. This establishes thebaine as the precursor of the other hydrophenanthrene alkaloids and O-demethylation as an important biosynthetic pathway. Comparison of specific activities of fed and recovered thebaine indicates a rapid synthesis and transformation and an important role in the plant's economy for this alkaloid. Various biogenetic proposals are reviewed in the light of these findings.

Introduction

Although many experiments on radioactive amino acid incorporation into alkaloids have been conducted in the past decade,[2] little attention has been directed toward establishing interrelationships among the various alkaloids themselves.[3] In a previous report,[4] we presented the first experimental evidence for an interconversion series in the biosynthesis of the opium alkaloids, and now we wish to present evidence which, together with the previous work,[4] constitutes proof of the proposed interrelationships.[5]

Comparative rates of $^{14}CO_2$ incorporation indicated that, in *Papaver somniferum*, thebaine (Ia) is the first hydrophenanthrene alkaloid formed and is converted by successive O-demethylations to codeine (IIa) and finally to morphine (IIb). This proposal was also supported by the fact that morphine, codeine and oripavine (Ib) are found in a number of species of Papaveraceae, but only together with thebaine, while the converse is not true. These relationships are shown in Table I.[6]

TABLE I

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. strigosum Schur.[d]	−	−	−	+
P. intermedium O. ktze.[d]	−	−	−	+

[a] 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. Yunusov and A. P. Orekhov, *Ber.*, 68B, 2158 (1935). [c] R. A. Konovalova and U. V. Kiselev, *Zhur. Obshchei Chim.*, 18, 855 (1948). [d] F. Santavy, M. Maturova, A. Nemeckova, H. B. Schröter, H. Potesilova and Vl. Preininger, *Planta Med.*, 8, 167 (1960).

Using the randomly-labeled morphine, codeine and thebaine readily available from $^{14}CO_2$ biosyntheses,[4] we have investigated this interrelationship in the present work by feeding the three alkaloids to plants of *P. somniferum* and determining the amount of radioactivity incorporated into other members of the series. In addition, large doses of non-active thebaine were fed to the plants to determine whether any gross physical changes could be observed. Finally, radioactive thebaine was fed to a plant of *P. orientale* to see whether conversion to other alkaloids occurred in this species as well.

Methods

Plant Growth.—The plants were grown in pots containing vermiculite, and feeding was accomplished through use of a nutrient solution as previously described.[4] Plants used in the present work were grown exclusively in the greenhouse. Varieties of *P. somniferum* L. which were used were "U.S. D.A. No. M92" and "U.S.D.A. No. M73" from seeds grown at Mesa, Arizona, in 1956[7a] and "paeoniflorum fl. pl.".[7b] The M92 variety was of Afghanistan origin and gives plants containing morphine, codeine, thebaine and narcotine as the main alkaloids. The "paeoniflorum" was of unknown origin and the main alkaloids isolated from derived plants were found to be morphine, codeine, thebaine and papaverine. The *P. orientale* L. seed was a "mixed" commercial variety. This species, unlike *P. somniferum* L., can be clonally propagated and strain varieties maintained from a single seed.[8]

Alkaloid Feeding Techniques.—The alkaloid was dissolved in a minimum amount of 0.1 *M* potassium dihydrogen phosphate, from which solution plant feeding was accomplished in two ways. In one method, a slit one-half inch long was cut diagonally upward into the plant stem. The edge of a one ml. beaker containing the alkaloidal solution was then pressed into the slit so that the upper flap of plant stem extended to the bottom of the beaker. An air blower directed toward the plant leaves helped to cause rapid uptake of the one ml. of solution, usually in from 2 to 4 hr. This method was used in the large dose, non-active thebaine feedings and in one radioactive thebaine feeding. Root absorption of the alkaloids was the more general method for bringing about alkaloid uptake. The plant was removed from the vermiculite, keeping the root mass intact, and the excess clinging vermiculite washed from the root hairs. Thorough removal of the vermiculite is advisable since some alkaloid adsorption by the vermiculite itself was found to occur. The plant was then usually allowed

(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) For a listing of some recent reviews, see footnote 2 in ref. 4.

(3) See, however, (a) H. Birecka and T. Sebyla, *Bull. acad. polon. sci., Ser. sci. biol.*, 8, 183 (1960), and previous papers; (b) A. Romeike, *Naturwissenschaften*, 47, 64 (1960); (c) T. C. Tso and R. N. Jeffrey, *Arch. Biochem. Biophys.*, 80, 46 (1959), and previous papers.

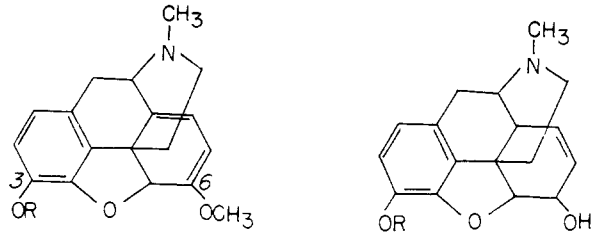
(4) H. Rapoport, F. R. Stermitz and D. R. Baker, *J. Am. Chem. Soc.* 82, 2765 (1960).

(5) A preliminary report of a portion of this work has appeared: F. R. Stermitz and H. Rapoport, *Nature*, 189, 310 (1961).

(6) The botanical classification of *P. paeoniflorum*, Hort., a species included in a previous table,[4] has since been questioned (H. G. Boit, private communication.) In addition, morphine already may have been removed from the total bases prior to the work reported [H. G. Boit and H. Ehmke, *Naturwissenschaften*, 45, 315 (1959)].

(7) The seed was kindly provided by: (a) Dr. Norris W. Gilbert, U.S.D.A. Crops Research Division, Mesa, Arizona, and (b) Prof. H. G. Boit, Humboldt University, Berlin.

(8) R. F. Dawson and C. James, *Lloydia*, 19, 59 (1956).



Ia, thebaine, R=CH_3
Ib, oripavine, R=H

IIa, codeine, R=CH_3
IIb, morphine, R=H

Fig. 1.

to stand with roots immersed in a nutrient solution for 10–24 hr. prior to transfer to the radioactive alkaloid solution. Since the root mass was quite large, the original solution was diluted to 20–50 ml. with distilled water so that total immersion of the roots was possible. The root feeding was most convenient with plants which had been maintained in the rosette stage, an 80 g. plant absorbing about 25 ml. of solution overnight. For the stem incision feedings, more mature plants with elongated stems were used.

In the root feeding experiments, a very rapid disappearance of activity from solution was noted immediately after introduction of the plants. An appreciable activity drop (10–20%) took place by the end of five minutes, and this usually reached 50% by the end of 1 to 2 hr., although an insignificant drop in solution level had occurred. Subsequent loss of activity corresponded exactly with the amount of solution absorbed. It was found that the initial rapid activity loss was due to root hair adsorption of the alkaloid since most of the activity could be recovered by a single washing of the roots with 0.1 *M* phosphoric acid. At the end of each feeding, the solution or residue remaining was analyzed by paper chromatography and radioautography to determine if any decomposition had occurred to give radioactive fragments. Of the six feedings reported below, one did show some evidence of decomposition and this experiment was discontinued. None of the others gave any such indication and no activity was found in the solution residues which did not correspond exactly with the alkaloid fed.

Alkaloid Detection and Isolation.—The alkaloids of *P. somniferum* and *P. orientale* were isolated and purified as previously described.[4] Again, fresh frozen plant material was used except in the case of the plants which were fed large doses of inactive thebaine. Alkaloid isolation from these plants and from the controls was accomplished on air dried material. The previous methods of paper chromatography which were developed for *P. somniferum*[4] also gave good separation of the *P. orientale* alkaloids with the exception of thebaine and isothebaine. Here the use of unbuffered paper and 1% tartaric acid solution as solvent, described by Dawson and James,[8] gave good separation.

Radioactivity Measurements.—Counting of discrete samples to give the values listed in Results was with a Packard Automatic "tri-Carb" liquid scintillation spectrometer. The values reported (disintegrations per minute) are corrected for the efficiency of the counter, which was about 50%, using a toluene–ethanol–dioxane mixture as solvent. It was often convenient in preliminary work to count active spots directly on paper chromatograms. This was done with a modified Geiger–Mueller tube having a gold-spattered thin Mylar window.[9]

Results

Radioactive Alkaloid Preparation.—Five plants of *P. somniferum*, U.S.D.A. No. M92, were placed in the large chamber previously described,[4] were allowed to absorb a total of 70 millicuries of $^{14}CO_2$ over the course of two days and were killed on the third day. The weights and specific activities of the pure alkaloids isolated from the fresh plants are listed in Table II.

(9) J. A. Bassham and M. Kirk, *Biochim. et Biophys. Acta*, **43**, 447 (1960).

TABLE II

70 MILLICURIES, 3 DAY, $^{14}CO_2$ BIOSYNTHESIS

Alkaloid	Amount isolated, mg.	Specific acitivity, d.p.m./μmole
Morphine	11.0	2.00×10^6
Codeine	1.09	8.65×10^6
Narcotine	1.06	5.66×10^6
Thebaine	0.39	1.80×10^7

Alkaloid Feeding Experiments in P. somniferum.—In Table III are listed the results of some feeding experiments using radioactive substrates. It is apparent from these that excellent incorporation of radioactivity has been accomplished from thebaine to codeine and morphine and from codeine to morphine. Since the alkaloids which were fed were from a $^{14}CO_2$ biosynthesis, considerable activity was in the methyl groups on oxygen and nitrogen.[4] It was necessary therefore to demethylate the isolated alkaloids to insure that good incorporation of the ring structures had occurred. This was done in the manner previously described[4] and the results are given in Table IV. It can be seen that the codeine and morphine isolated retain large amounts of activity in the ring structure and, indeed, the ratio of methyl group to ring specific activity has changed only slightly from what it was in the fed thebaine. The slight decrease in the ratio, which becomes somewhat larger in comparing the morphine reisolated with the morphine fed, probably is due to transmethylation, as discussed below.

Thebaine (non-radioactive) was fed to two plants of *P. somniferum*, U.S.D.A. No. M73, by the stem slit method. One plant was 80 cm., another 70 cm. in height at the beginning of the experiment. Two controls of identical height and age were maintained together with the test plants. Thebaine (8 mg. at each feeding) was fed to the two test plants on the first, third, sixth, eighth and twelfth day and the plants were harvested on the fifteenth day. Each successive feeding was given by making a new incision at an internode higher than the previous cut. Each of the plants was just beginning to bud at the beginning of the experiment. During the fifteen days, each plant, tests and controls, grew about 30 cm., blossomed and formed a normal capsule. No physical difference was observable between the controls and thebaine-fed plants during the growth period. After being removed from the pots, the plants were air dried in the dark. Alkaloid analyses then were made with the results of one test and control listed in Table V.

The second plant and control were analyzed only approximately by paper chromatography, but again the test plant showed a large increase in codeine. The amounts of morphine were about the same in test and control.

***P. orientale* Radioactive Alkaloid Feeding.**—Over the course of 41 hr. a rosette-stage plant of *P. orientale* was allowed to absorb 0.56 mg. of $^{14}CO_2$ biosynthetically-labeled thebaine by root uptake. The total activity was 762,000 d.p.m. At the end of this time, the plant was frozen and the usual[4] alkaloid separation carried out. A total of 385,000 d.p.m. was extracted from the butanol–benzene

TABLE III

^{14}C-LABELED ALKALOID FEEDING EXPERIMENTS

Plant no.	Alkaloid fed	Isolated	Amount (mg.)	Total act. (d.p.m.)	Specific act. (d.p.m./μmole)	Growth time (hr.)
1	Morphine	.....	0.089[a]	621,000[a]	2,000,000	40
		Morphine	1.53	337,000	62,000	
		Codeine	0.088	873[b]	...	
		Thebaine	.021	None	...	
2	Codeine	.....	.12[a]	3,500,000[a]	8,650,000	40
		Morphine	1.03	355,000	98,300	
		Codeine	0.28	402,000	425,000	
		Thebaine	.042	None	...	
3	Thebaine	.....	.040	2,290,000[a]	18,000,000	40
		Morphine	.87	77,000	19,200	
		Codeine	.19	66,000	105,000	
		Thebaine	.026	29,900	321,000	
4	Thebaine	.....	.085[a,c]	5,040,000[a]	18,000,000	24
		Roots	...	2,470,000[d]	...	
		Plant	...	618,000	...	
5[e]	Thebaine	.....	.46	841,000	570,000	30
		Morphine	1.83	19,000	2,980	
		Codeine	0.24	29,100	36,500	
		Thebaine	.16	81,000	162,000	
		Papaverine	.30	None	...	

[a] The values listed for amount and total activity adsorbed are based on loss of activity from solution. This gives a value perhaps 20–40% high, since a large degree of root adsorption without further transportation occurs. See Methods and Plant 4, this Table. The roots were not included in the analysis of the first three plants. [b] Testing of the fed morphine by isotopic dilution with inactive codeine indicated a slight contamination with active codeine (less than 0.1%). The amount would not quite account for the total of 873 d.p.m. [c] Some decomposition of the fed thebaine had occurred in the feeding beaker (see Methods). [d] About half the activity was adsorbed alkaloid and the remainder obtained by the usual alkaloid extraction. The latter was mostly thebaine, but other (non-alkaloidal) activity was also present. [e] This plant was the "paeoniflorum" variety and the feeding was accomplished through a stem slit.

TABLE IV

COMPARISONS OF RING SKELETON AND METHYL GROUP ACTIVITIES

Compound	Specific activities (d.p.m./μmole × 10^{-3})					Methyl/Ring ratios		
	Total	O^6-methyl	O^3-methyl	N-methyl	Ring	O^6-methyl	O^3-methyl	N-methyl
Thebaine fed[a]	18,000	7610	2390	2660	5440	1.4	0.44	0.49
Codeine isolated	105	..	20.8	27.6	56.4	..	0.37	.49
Morphine isolated	19.2	..	....	5.3	13.9	..	..	.38
Morphine fed[b]	2,000	..	....	680	1320	..	..	.51
Morphine isolated	62.8	..	....	17.3	45.5	..	..	.38

[a] Plant 3, Table III. [b] Plant 1, Table III.

TABLE V

LARGE DOSE THEBAINE-FEEDING EXPERIMENTS

Plant	Amount thebaine fed, mg.	Dry weight, g.	Morphine, mg.	Amount codeine isolated, mg.	Thebaine, mg.
Control	None	17.5	20.4	5.0	1.3
Test	40	16.0	16.4	12.3	15.9

solution into 0.5 *M* sulfuric acid while a total of 240,000 d.p.m. of probably non-basic material was present in various other washings and residues. The solution from which the plant had been fed was analyzed and found to contain thebaine and isothebaine, the latter being inactive. Successive increments of potassium hydroxide solution were added to the 0.5 *M* sulfuric acid solution and extractions with chloroform were carried out with the results

*p*H	Activity (d.p.m.) extracted	Alkaloids
6	270,000	Thebaine and isothebaine
9	32,000	5 spots (3 major)
14	7,000	None

The thebaine and isothebaine from the *p*H 6 extraction were separated on paper,[8] and all the activity was found to coincide with thebaine. As determined by radioautography of the paper strip of the *p*H 9 extraction, alkaloid spots at R_F 0.76 and 0.66 (both major components) were inactive while spots at R_F 0.52 (major) and 0.04 (trace) were active. Some activity was also present at R_F 0.60 where there was no corresponding alkaloid spot. The smallest of the major components, occurring at R_F 0.48, appeared to have some activity, although the activity mark of the R_F 0.52 spot slightly overlapped this area. In this chromatographic system, (2:1:1 *n*-propyl alcohol:water:ether and 0.2 *M* potassium dihydrogen phosphate buffered paper), thebaine and isothebaine run together at about R_F 0.70. The alkaloids at R_F 0.48 and 0.52 have been found to occur also in plants which were not fed thebaine but have not as yet been separated other than on paper. Their behavior on solvent extraction from buffer solution (not extracted at *p*H 6, difficultly extracted at *p*H 11, more easily at *p*H 9), corresponds to that of morphine as does the R_F value (morphine = 0.50) and spot color. This is the behavior one would expect of oripavine (Ib), a previously reported alkaloid of *P. orientale* (see Table I). The ultraviolet spectrum of the mixture

of the two has the typical ($\lambda_{max.}$ 286, $\lambda_{min.}$ 263) shape of the morphine-type alkaloids. Positive identification of either as oripavine is awaiting comparison with an authentic sample.

Discussion

Alkaloid Interconversions.—The combined results of Tables III and IV show a clear thebaine to codeine to morphine conversion. Based on ring skeleton activities derived from Table IV, a 7.5 and 5.0% incorporation of total activity from thebaine to morphine and codeine, respectively, was achieved in plant 3. Because of the alkaloid adsorption by the root hairs, as discussed in Methods, these per cent. incorporation values are minimal and more realistic values would be perhaps 1.5–2 times higher. The per cent. incorporation of specific activity is somewhat lower, due to a number of factors. The dilution of active alkaloids with the corresponding compounds already present in the plant is important in some cases. This dilution factor is high with regard to morphine since the amounts fed were small compared to the total amount of morphine in the plant. This is not the case with respect to codeine and thebaine since here the amounts absorbed were at least of the same order of magnitude as the amounts of inactive alkaloids already present. However, a large drop in specific activity still was noted in comparing the specific activities of the fed thebaine and codeine with the activities of the same alkaloids isolated after 24 to 40 hr. It was thought that this possibly might be due to a rapid exchange of methyl groups, in which a labeled methyl on the alkaloid oxygen or nitrogen atom is replaced by an inactive one from the plant metabolic pool as depicted below. Here the X, Y and R groups are

$$>C^*\text{-}OC^*H_3 + X\text{-}Y \longrightarrow C^*\text{-}OX + Y\text{-}C^*H_3$$

$$>C^*\text{-}OX + RCH_3 \longrightarrow C^*\text{-}OCH_3 + R\text{-}X$$

unspecified but could represent either enzyme surfaces or discrete compounds. Since the methyl groups are much more active than the ring carbons, the effect would be a considerable lowering of the specific activity of the alkaloid without, necessarily, converting it to a new alkaloid. However, the results of Table IV indicate only a small change in the ratios of methyl group to ring carbon activities, these change being only slightly outside experimental error with the exception of the morphine N-methyl data. The most likely explanation for the specific activity drop seems to be that a relatively rapid synthesis and transformation of codeine and, especially, thebaine is taking place in the plant. As a rough estimate, the amount of thebaine synthesized by the plant in one or two days is of the same general magnitude as the amount present at any given time. This is supported by the previous evidence[4] that incorporation of labeled carbon from $^{14}CO_2$ into the ring structure of these alkaloids can occur in less than two hours. The exact lower limit has not as yet been determined.

The proved interconversions above clearly establish O-demethylation of alkaloids as a metabolic pathway. No significant reversibility of any of the steps was encountered. The high yields indicate that the thebaine to codeine to morphine transformation must be the major pathway and establish the primacy of thebaine among the hydrophenanthrene opium alkaloids.[10] Additional conversions (other than the reversal of these steps) are of course not excluded. Another example of thebaine metabolic activity is provided by its conversion to two alkaloids in *P. orientale*. One of these is very likely oripavine, again a demethylated thebaine derivative. One previously-held theory[11] depicted the fully methylated alkaloids as end products, the plant being desirous of deactivating –OH and –NH groups. This is certainly not true in the present case. However, since some intermediate must previously have been methylated prior to thebaine formation, the idea of detoxification *via* methylation could conceivably still hold true at an early stage. It will be interesting to determine whether the original methylation has taken place on an alkaloid or alkaloid-type intermediate or on some earlier precursor.

Of some interest are the results of the feeding of large doses of inactive thebaine to plants of *P. somniferum*. During the growth period studied, large amounts of thebaine had no effect whatsoever on the physically observable plant characteristics. Analysis of the air-dried plants showed a large increase in codeine content, in keeping with the proven pathway, but no increase in morphine content. However, the process of air-drying can have profound effects on alkaloid content, as was shown in the extensive and thorough work of Miram and Pfeifer,[12] and it is perhaps futile at this time to speculate on the lack of an increase in morphine.

Biogenesis and Biosynthesis of the Morphine Alkaloids.[13]—It seems appropriate at this time to comment on the relationship of the present work to other biosynthesis experiments and to biogenetic theory in general. Most theories on the formation of morphine-type alkaloids are variations of a scheme derived from suggestions of Winterstein and Trier[14] and Gulland and Robinson.[15] The former workers were the first to suggest that phenylalanine could serve as the precursor of both a β-phenylethylamine derivative and a C_6–C_2 fragment which could then combine to give the skeleton of the benzylisoquinoline alkaloids, equation 1. Gulland and Robinson then proposed an intramolecular aromatic coupling reaction of the benzylisoquinoline to furnish the hydrophenanthrene skeleton, equation 2. These and particularly subsequent theories[16] also included attempts to

(10) G. Kleinschmidt [*Pharmazie*, **15**, 663 (1960)] also has observed the demethylation of radioactive codeine to morphine (in 7% conversion) by isolated leaves. However, he considers this a secondary transformation and draws no biosynthetic implications.

(11) *E.g.*, H. B. Schröter, *Encycl. of Plant Physiol.*, **8**, 844 (1958); R. Miram and S. Pfeifer, *Naturwissenschaften*, **45**, 573 (1958).

(12) R. Miram and S. Pfeifer, *Sci. Pharm.*, **27**, 34 (1959); **28**, 15 (1960).

(13) We have used the term biosynthesis to refer to actual experimental work with plants; hypothetical schemes or *in vitro* experiments have been termed biogenesis.

(14) E. Winterstein and G. Trier, "Die Alkaloide," Borntraeger Press, Berlin, 1910, p. 307.

(15) J. M. Gulland and R. Robinson, *Mem. Proc. Manchester Lit. and Phil. Soc.*, **69**, 79 (1925).

(16) (a) R. Robinson and S. Sugasawa, *J. Chem. Soc.*, 3163 (1931); (b) R. Robinson, "The Structural Relations of Natural Products,"

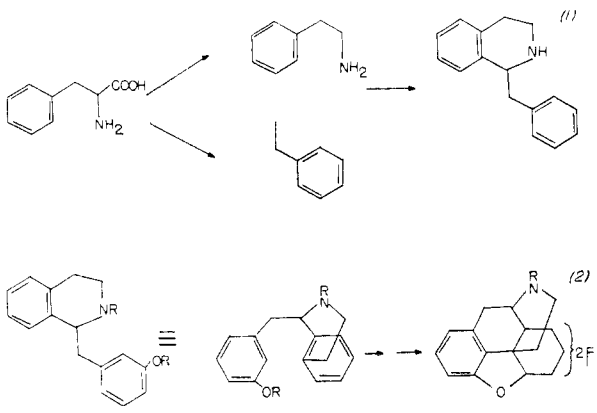


Fig. 2.

establish more detailed mechanisms from a consideration of the relative placement of the oxygen functions, emphatically pointing out[16b] or stating with qualification[16f] that the particular stage at which methylation takes place was unknown and not pertinent to the basic scheme.

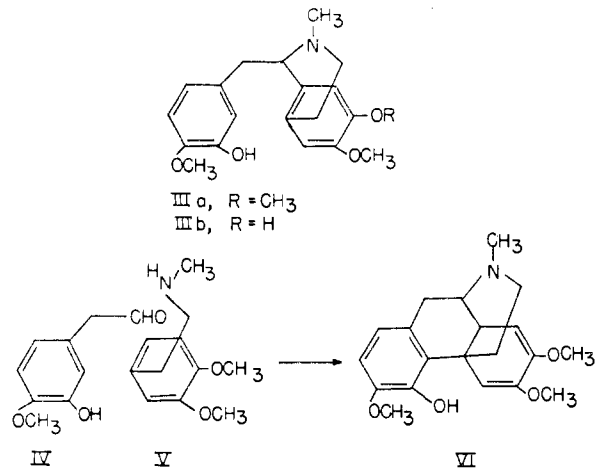
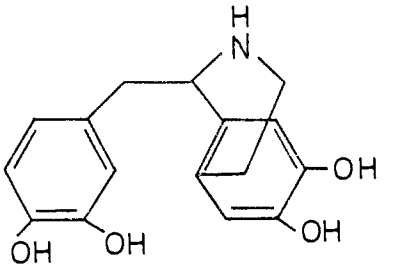


Fig. 3.

The present results provide experimental data as a basis for consideration of these various alternatives. They exclude any scheme which requires the primary formation of morphinone enol[16c] and those[16] which provide for the independent or concurrent formation of morphine, or codeine without the intervention of thebaine. Still tenable are direct approaches to thebaine, *e.g.*, through laudanine (IIIa)[16d] through IIIb[16f,g] and through the condensation of IV and V and VI[16g] and thence to thebaine.

Leete[17] and Kleinschmidt and Mothes[18] have presented papers describing the details of their biosynthesis experiments in the morphine alkaloid field, and Battersby and co-workers, in a series of preliminary communications,[19] also have indicated the course and first results of their work. Of these, the most pertinent to the present results are those[19e] in which specifically-labeled norlaudanosoline (VII) was fed to mature *P. somniferum*



VII

Fig. 4.

plants and radioactive papaverine, thebaine, codeine and morphine were isolated from the plants fourteen days later.[20] However, the emphasized[19e] close structural correspondence of norlaudanosoline and morphine is deceptive since norlaudanosoline apparently can function as a true morphine precursor only by being methylated to another intermediate, which then undergoes ring closure leading to thebaine and finally morphine. If norlaudanosoline can be established definitely[21] as one of the normal biosynthesis intermediates, it would appear that O- and N-methylation must occur almost as the next step. Our results establish that the methylation must occur prior to hydrophenanthrene alkaloid formation, but no limitation has previously been set as to how early in the pathway it might take place.

Three groups[17-19] fed labeled tyrosine to plants of *P. somniferum* and obtained radioactive morphine,[17,19a] narcotoline[18a] and papaverine,[19c] labeled in accordance with the basic biogenetic scheme (equations 1 and 2) and also radioactive thebaine[19f] and codeine[19f] (position of label unknown). The radioactive yields were low[17,18a] (0.017% incorporation of total activity and 0.23% incorporation of specific activity,[17a] for example) or not reported.[19a,c,f] In spite of the fact that many explanations can be devised for low incorporation

Clarendon Press, Oxford, 1955, p. 84; (c) C. Schöpf, *Naturwissenschaften*, **39**, 21 (1952); (d) K. W. Bentley, *Experientia*, **12**, 251 (1956); (e) T. Cohen, *Chem. & Ind. (London)*, 1391 (1956); (f) D. H. R. Barton and T. Cohen, "Festschrift Arthur Stoll," Birkhauser, Basle, 1957, p. 123; (g) G. Stork in "The Alkaloids," Vol. VI, edited by R. H. F. Manske, Academic Press, Inc., New York, N. Y., 1960, p. 242.

(17) E. Leete, *J. Am. Chem. Soc.*, **81**, 3948 (1959).

(18) (a) G. Kleinschmidt and K. Mothes, *Z. Naturforsch.*, **14b**, 52 (1959); (b) G. Kleinschmidt and K. Mothes, *Arch. Pharm.*, **15**, 948 (1960).

(19) (a) A. R. Battersby and B. J. T. Harper, *Chem. & Ind. (London)*, 364 (1958); (b) *ibid.*, 365 (1958); (c) *Proc. Chem. Soc.*, 152 (1959); (d) A. R. Battersby, R. Binks and D. J. LeCount, *ibid.*, 287 (1960); (e) A. R. Battersby and R. Binks, *ibid.*, 360 (1906); (f) A. R. Battersby and B. J. T. Harper, *Tetrahedron Letters*, No. 27, 21 (1960).

(20) The statement[19e] that norlaudanosoline was the most efficient morphine precursor yet found was made prior to the publication[5,10] of the demethylation results. The exact efficiency of norlaudanosoline was not reported and is not calculable from the data presented.

(21) The indication that norlaudanosoline activity was incorporated into all the hydrophenanthrene alkaloids and papaverine as well makes it quite likely that this compound, at the very least, does have an entrance into the normal biosynthetic pattern. However, the remote possibility that norlaudanosoline is a substance completely foreign to *P. somniferum* cannot be excluded. The compound as yet has not been isolated as a natural product. Any number of metabolic pathways might hold true for norlaudanosoline as a foreign substance, including a direct ring closure to give morphine without first passing through thebaine. The capacity of plants to perform chemistry unrelated to their normal processes has been little studied.

in plant systems (diversion of free amino acids to many things other than alkaloids, high pool dilution, failure to reach synthesis site, failure to penetrate cells, etc.), low incorporation values should not be ignored or deemed irrelevant.

The rapid alkaloid biosynthesis from carbon dioxide demonstrated here and in previous work[4] indicates that tyrosine, if it is a main precursor, might be expected to give better incorporation yields than were reported.[17,18a] The incorporation which was achieved is more in line with what might be expected from a minor precursor or even through an aberrant synthesis. This possibility is also raised by the interesting results of Kleinschmidt and Mothes,[18b] who showed a higher incorporation into the alkaloids of *P. somniferum* from glucose than from tyrosine, although generally accepted metabolic pathways[22] indicate that tyrosine is formed from glucose. Since the results[18b] held not only for excised leaves, but also for the isolated opium sap, the questions of differing absorption rates, ability to reach synthesis sites or to penetrate cells, etc., do not seem to be pertinent. At the same time, the observation[19f] that tyrosine incorporation appears to initiate at thebaine and thence proceed to codeine and morphine is consistent with the present results.

Concurrent investigations in these Laboratories are being directed toward establishing alkaloid intermediates in the pathway prior to thebaine, clarifying the earlier biosynthesis steps and studying the possible relationships of biosynthesis to alkaloid function.

(22) B. D. Davis, *Symposium on Microbial Metabolism Report*, VI, International Congress of Microbiology, Rome, 1953, p. 23, and subsequent work.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS]

Studies on Synthetic Polypeptide Antigens. III. The Synthesis and Physico-Chemical Properties of a Group of Linear-Chain Antigens[1]

BY EDWARD FRIEDMAN, THOMAS J. GILL III[2] AND PAUL DOTY

RECEIVED APRIL 3, 1961

This paper describes the synthesis and physico-chemical properties of a group of synthetic polypeptides of immunological interest containing different combinations and proportions of L-glutamic acid, L-lysine, L-tyrosine and L-phenylalanine. They were in the molecular weight range of 50,000 to 100,000 and contained 15–20% helix when studied in 0.15 *M* saline-phosphate at *p*H 7.6, a solvent which closely resembled physiological conditions. The sedimentation constants and intrinsic viscosities closely fitted empirical equations relating them to the molecular weights of the polymers.

Introduction

The preceding paper in this series[3] described the immunological properties of a group of linear-chain synthetic polypeptides. This paper will describe the synthesis and physico-chemical properties of these polypeptides.

Materials

N-Carboxy-anhydrides (NCA).—The NCA's used in the kinetic studies and the polymerizations were obtained from the Pilot Chemical Company, Watertown, Massachusetts.

Solvents.—Reagent grade benzene was purified by refluxing it over calcium hydride and distilling it immediately before use. Reagent grade methanol was purified by adding a sufficient amount of sodium to react with any water in it and shortly thereafter distilling it.

Sodium Methoxide.—Sodium metal was dissolved in methanol and the solution added to three times its volume of benzene. The normality was 0.0516 *N* as determined by titration against benzoic acid.

Barium Hydroxide.—Solid barium hydroxide was dissolved in water containing 5 ml. of 1-butanol per liter. The solution contained 0.015 mole of barium hydroxide per 1000 g. of solution as determined by titration against potassium acid phthalate and also by conductivity measurements. The latter were made by flushing a known quantity of carbon dioxide, evolved by adding standardized potassium permanganate to a solution of sodium oxalate in 1 *N* sulfuric acid, into the barium hydroxide solution and measuring the decrease in its conductivity.[4]

Buffers.—The saline-phosphate buffers were made up to 0.11 *M* in sodium chloride and 0.04 *M* in phosphate. The ratio of monohydrogen phosphate to dihydrogen phosphate was adjusted to give the desired *p*H.[5] All of the measurements, except where indicated, were made in this buffer at *p*H 7.6 which closely resembled physiological conditions. The carbonate buffer was 0.15 *M* and *p*H 10.0.[5]

Experimental

Kinetics.—The kinetics of co-polymerization of the N-carboxyanhydrides of γ-benzyl-L-glutamate, ε-carbobenzoxy-L-lysine and O-carbobenzoxy-L-tyrosine at 25° were studied in order to determine the conditions for the synthesis of the polymers. The amino acids were mixed in a mole ratio of 3:2:1 to give a 1% solution in benzene, and sodium methoxide was used as the initiator at an anhydride:initiator ratio of 400. The conditions were found by Idelson and Blout to give high molecular weight polymers.[6] The rate of polymerization was measured by the carbon dioxide evolution method of Doty and Lundberg[4]: the reaction was followed by flushing the carbon dioxide evolved through a standardized solution of barium hydroxide and measuring the decrease in its conductivity. The kinetics data are plotted in Fig. 1 where the reaction is seen to deviate from simple first-order kinetics at 36 minutes, at which time it is 90% complete. The rate constant for the initial linear portion of the curve is 13.2 l./mole sec.

Polymer Preparation.—Polypeptides 8, 9, 10 and 12 were prepared by the method described above. The reactions were stopped when they were 90% complete, because of the deviation from simple first-order kinetics, by bubbling anhydrous HBr through the solution for 1.5 hr. and flushing out the excess with pre-purified nitrogen. This procedure removed the protecting groups from all of the amino acid residues and precipitated the polymer. The precipitated

(1) Work supported by a grant from the National Science Foundation (G-7487).

(2) Junior Fellow of the Society of Fellows, Harvard University.

(3) T. J. Gill III and P. Doty, *J. Biol. Chem.*, in press.

(4) R. D. Lundberg and P. Doty, *J. Am. Chem. Soc.*, **79**, 3961 (1957).

(5) "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 143, 146.

(6) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 2387 (1958).