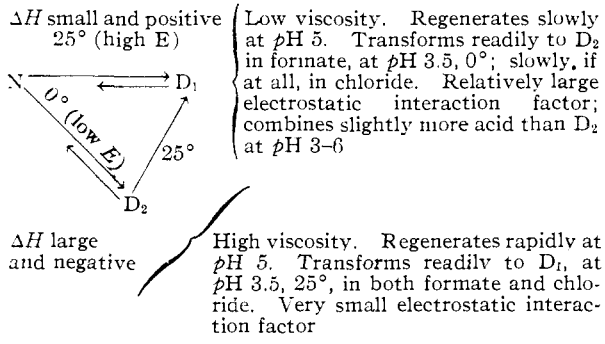
present in this temperature range, then the reversibility of the reaction $D_1 \rightleftarrows D_2$ in formate should permit determination of the equilibrium between these forms and, from the temperature dependence of the equilibrium constant, the heat of transformation may be calculated. For example, if the transformation is 90% complete in each direction at each of the temperatures in Fig. 7, a value of ΔH of about 30,000 cal. would be deduced. The heat absorbed in denaturation itself for at least one of these temperatures cannot be less than half of ΔH for the $D_1 \rightarrow D_2$ transformation and may be greater by an indeterminate amount. However, the protonation of 22 unmasked imidazole groups, which is incident to denaturation by acid, will appear (by its attendant heat evolution) to reduce the true ΔH of denaturation by about 140,000 cal. when ΔH is measured in other ways. The values estimated earlier[16] from the *p*H displacement of the equilibrium by changes in temperature have been shown to be of doubtful significance because of the complex regeneration reaction[3] which involves the transient formation of an intermediate.

Effect of *p*H on Viscosity on Denatured Protein.—Since substituting formate ion for other buffer anions in kinetic experiments on denaturation has the effect of lowering by a constant amount, the *p*H, at which the same rates are obtained, it appeared possible that the peculiar effects of formate ion on viscosity might be manifested in other acids at a high *p*H, *i.e.* about 3.8–3.9. The effect of *p*H on viscosity of denatured protein at 25° at a single ionic strength was therefore investigated.

The results obtained at three *p*H values with protein denatured at a single *p*H are shown in Fig. 8. It is clear that the *p*H has a marked effect on intrinsic viscosity. The Huggins' constant is not affected. However, in HCl ηi at *p*H 3.75 is not the same as in formate at the lower *p*H—and its relation to concentration bears no resemblance to that prevailing in formate. The effect of formate on viscosity is thus highly characteristic and specific. The *p*H of denaturation is without effect on the viscosity, although the viscosity depends on the *p*H at which it is determined.

Recapitulation.—All of the evidence presented in this paper which indicates that ferrihemoglobin denatured by acid at 0° differs from the denatured protein formed at 25°—and which thus constitutes distinguishing properties of the two products—may be summarized in an expanded form of the diagram used earlier in this paper.



Acknowledgment.—Generous provision by Prof. G. Scatchard of the Department of Chemistry, Massachusetts Institute of Technology, of facilities and of numerous opportunities for helpful discussion, is gratefully acknowledged.

(16) E. M. Zaiser and J. Steinhardt, THIS JOURNAL, **76**, 2866 (1954).

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

Pantethine Analogs. The Condensation of Pantothenic Acid with Selenocystamine, with Bis-(β-aminoethyl) Sulfide and with 1,2-Dithia-5-azepane (a New Ring System)[1,2]

BY WOLFGANG H. H. GÜNTHER[3] AND HENRY G. MAUTNER

RECEIVED NOVEMBER 13, 1959

As part of a project concerned with the development of potential coenzyme A antagonists, the following compounds were synthesized: selenopantethine, bis-(β-pantothenoylaminoethyl) sulfide and N-pantothenoyl-1,2-dithia-5-azepane. These compounds were prepared by the condensation of pantothenic acid with selenocystamine, bis (β-aminoethyl) sulfide and 1,2-dithia-5-azepane, respectively. The last compound represents a new heterocyclic system.

In the design of antimetabolites with potential carcinostatic activity, little emphasis has been placed on possible antagonists of coenzyme A or compounds related to it. Yet the literature contains several reports which suggest that tumor tissue might be more sensitive to the action of antagonists of coenzyme A than normal tissue. Thus, the capacity of tumors to utilize acetate and to oxidize fatty acids appears to be lower than that of non-neoplastic tissue,[4] rat tumors are reported to be deficient in coenzyme A and in pantothenic acid-content, as compared to normal tissues,[5] and in at least some neoplasms the coenzyme A content parallels certain synthetic capacities of the tumor.[6] Reports that the utilization of acetate is the first cell function, in a bacterial system, to

(1) Part of this material was presented before the Biological Chemistry Section at the American Chemical Society Meeting, Atlantic City, N. J., September 1959, 76-C.

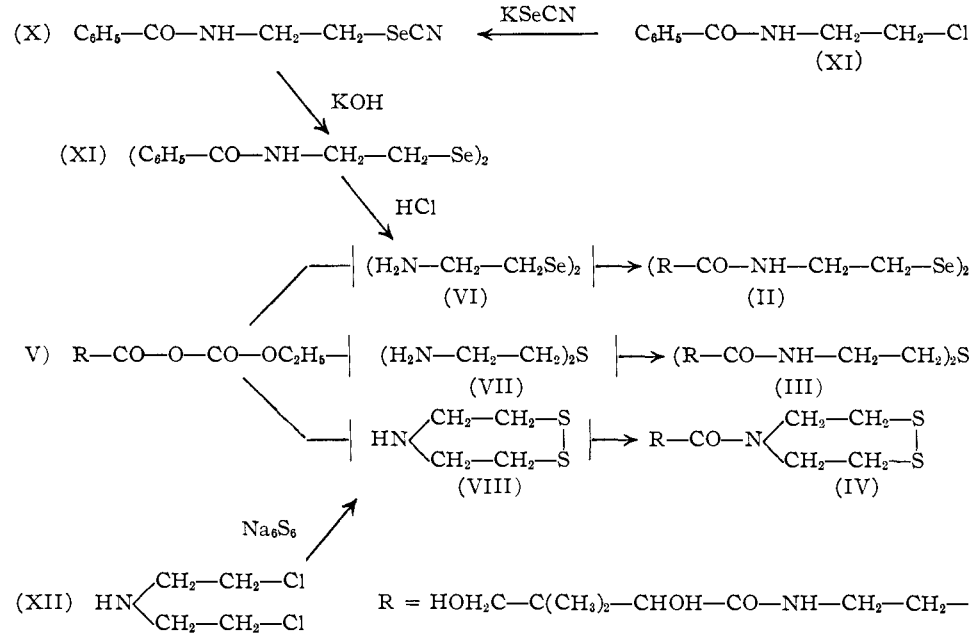
(2) This work was supported in part by a grant (CY-3937) from the National Institutes of Health, Public Health Service. We are indebted to the Wellcome Trust, London, Great Britain, for a generous travel grant to W. H. H. Günther.

(3) James Hudson Brown Memorial Post-doctoral Fellow, 1958–1959.

(4) J. P. Greenstein, "Biochemistry of Cancer," Academic Press, Inc., New York, N. Y., 1954, p. 374.

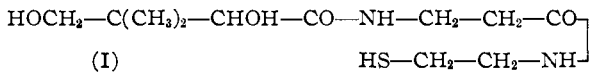
(5) H. Higgins, *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **75**, 462 (1950).

(6) P. Emmelot and L. Bosch, *Brit. J. Cancer*, **9**, 327 (1955).



be depressed by 6-mercaptopurine,[7] a clinically useful antileukemic agent, and that the antimitotic activity of this compound can be reversed by coenzyme A,[8] again suggested the desirability of synthesizing possible antimetabolites of coenzyme A.

It was felt that in such a project attention should first be directed towards the preparation of analogs of pantetheine I. Pantetheine represents the



(I)

major portion of coenzyme A and can be converted to 4′-phosphopantetheine and to coenzyme A by either synthetic[9,10] or biological[11] methods. Presumably it will be possible to convert pantetheine analogs to coenzyme A analogs by similar means.

Since thio-acyl derivatives serve as key intermediates in the metabolic functions of coenzyme A, [12,13] with the acyl residues being attached to the single sulfur atom of the molecule, it seemed that efforts in designing analogs should be centered at the terminal β-mercaptoethylamino group. Since sulfur and selenium compounds are essentially isosteric and the reactivities of thio-acyl and seleno-acyl compounds appear to be rather similar,[14] replacement of the sulfur atom in pantetheine by selenium seemed of interest. In recent years several selenium analogs of biologically active sulfur compounds have been synthesized[15–19]; some of these showed considerable activity in microbial and tumor systems.[20–23] Selenocystine was reported to have clinically useful effects in the treatment of leukemia.[24] The finding that the turnover-rate of L-cystine is very high in the leukocytes of acute leukemia,[25] making them unusually sensitive to the action of cystine antagonists,[25] suggests the possibility that the synthesis of coenzyme A from cystine may be inefficient in leukemic cells and added interest to the question whether selenopantetheine (II), and eventually seleno-coenzyme A, would replace or antagonize the natural coenzyme.

Two other compounds which seemed worth synthesizing were bis-(β-pantothenoylaminoethyl) sulfide (III) and N-pantothenoyl-1,2-dithia-5-azepane (IV).

While pantetheine and its disulfide form, pantethine, are presumably readily interconvertible in biological systems, it was hoped that III might fit receptor sites for pantethine without being capable of facile reduction. On the other hand, reduction of IV would lead to the formation of a bifunctional pantetheine.

The synthetic scheme used in the preparation of the above compounds is summarized in Table I.

The pantethine analogs were prepared by the amide synthesis of Boissonas,[26] condensing the mixed carbonic ester-anhydride of pantothenic acid (V)[27] with selenocystamine (VI), bis-(β-aminoethyl) sulfide (VII) and 1,2-dithia-5-azepane (VIII), respectively.

(7) E. T. Bolton and H. G. Mandel, *J. Biol. Chem.*, **227**, 833 (1957).
(8) J. J. Biesele, *Ann. N. Y. Acad. Sci.*, **60**, 228 (1954).
(9) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).
(10) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **81**, 1265 (1959).
(11) G. D. Novelli in "Methods of Enzymology," Vol. II, Academic Press, Inc., New York, N.Y., 1955, p. 623.
(12) F. Lynen, *Harvey Lectures*, **48**, 210 (1954).
(13) F. Lynen, *Ann. Rev. Biochem.*, **24**, 653 (1955).
(14) H. G. Mautner and W. H. H. Günther, unpublished data.
(15) H. G. Mautner and W. D. Kumler, THIS JOURNAL, **78**, 97 (1956).
(16) H. G. Mautner, *ibid.*, **78**, 5292 (1956).
(17) H. G. Mautner and E. M. Clayton, *ibid.*, **81**, 6270 (1959).
(18) F. W. Campbell, H. G. Walker and G. M. Coppinger, *Chem. Revs.*, **50**, 279 (1952).
(19) G. Bergson, *Acta Chem. Scand.*, **11**, 1607 (1957).
(20) H. G. Mautner, *et al.*, *Antibiotics Chemotherapy*, **6**, 51 (1956).
(21) H. G. Mautner, *Biochem. Pharmacol.*, **1**, 169 (1959).
(22) H. G. Mautner and J. J. Jaffe, *Cancer Research*, **18**, 294 (1958).
(23) A. S. Weisberger and L. G. Suhrland, *Blood*, **11**, 11 (1956).
(24) A. S. Weisberger and L. G. Suhrland, *ibid.*, **11**, 19 (1956).
(25) A. S. Weisberger, L. G. Suhrland and J. Seifter, *ibid.*, **11**, 1 (1956).
(26) R. A. Boissonas, *Helv. Chim. Acta*, **34**, 874 (1951).
(27) R. Schwyzer, *ibid.*, **35**, 1903 (1952).

Selenocystamine (VI) was prepared in high overall yield by an adaptation of an early synthesis of Coblentz.[28] β-Chloroethylbenzamide (IX) was made from ethylene imine and then converted to β-selenocyanoethylbenzamide (X). Condensation of two molecules of X yielded N,N-dibenzoylselenocystamine (XI), which could be hydrolyzed to selenocystamine by means of concentrated hydrochloric acid.

Bis-(β-aminoethyl) sulfide was prepared by the reaction of ethylene imine with hydrogen sulfide.[29]

1,2-Dithia-5-azepane(VIII) appears to represent a new heterocyclic system. The hydrochloride of this substance was prepared by the reaction of bis (β-chloroethyl) amine (XII) with sodium disulfide in aqueous solution at 0°, followed by steam distillation of the product, acidification of the distillate with hydrochloric acid and evaporation to dryness. The yield of VIII was substantially improved when a catalytic amount of potassium cyanide was added during the steam distillation. Presumably this caused the initially formed polymeric disulfides to dissociate into sulfhydryl and thiocyanate fragments, recombination of which yielded the volatile cyclic monomer and regenerated the cyanide.

Free VIII was obtained by solvent extraction from alkaline solution. The base is an oil with an odor resembling that of piperidine; it polymerizes very readily and, as yet, is available in analytical purity only in the form of the hydrochloride.

The pantethine analogs II, III and IV were purified by means of column chromatography on activated alumina. Further synthetic work with these and related compounds is now in progress.

Selenopantethine is capable of replacing pantethine completely, on a mole for mole basis, in *Lactobacillus helveticus*,[30] an organism requiring preformed pantethine and presumably capable of converting it to coenzyme A. No growth occurred when selenopantethine was replaced by pantothenic acid and selenocystamine or pantothenic acid and cysteine. It may be suggested that selenopantethine is being converted to selenocoenzyme A which is capable of carrying out the metabolic functions of the thio analog. This finding of a biologically active selenium compound is interesting in view of recent reports of selenium containing dietary factors[31,32] and of claims that selenium is an essential trace element.

Further work with selenopantethine and the other analogs described here is now in progress and will be reported elsewhere.

Acknowledgments.—We are indebted to Miss Elaine Perry and to Mrs. Joyce Briggs for their valuable technical assistance during the course of this investigation.

(28) V. Coblentz, *Ber.*, **24**, 2131 (1891).

(29) S. Gabriel and G. Eschenbach, *ibid.*, **30**, 2497 (1897).

(30) H. G. Mautner and W. H. H. Günther, *Biochim. Biophys. Acta*, **36**, 561 (1959).

(31) K. Schwarz and C. M. Foltz, THIS JOURNAL, **79**, 3292 (1957).

(32) E. L. Patterson, R. Milstrey and E. L. R. Stokstad, *Proc. Soc. Exptl. Biol. Med.*, **95**, 617 (1957).

Experimental

β-Chloroethylbenzamide (IX).—This compound was made from ethylene imine (Chemirad Corp., Port Washington, N. Y.) by the method of Bestian.[33]

β-Selenocyanoethylbenzamide (X).—β-Chloroethylbenzamide (18.3 g.) and potassium selenocyanate (14.5 g.) were dissolved in absolute ethyl alcohol (250 ml.). The solution was heated under reflux with stirring for 18 hr. in a system protected from atmospheric moisture. The hot solution was filtered and water added until a slight turbidity persisted. β-Selenocyanoethylbenzamide (21.0 g., 84% of theory) crystallized on cooling. After recrystallization from dilute alcohol, the substance melted at 89–90°.[34]

Anal.[35] Calcd. for $C_{10}H_{10}N_2OSe$: C, 47.66; H, 3.93; N, 11.27. Found: C, 47.44; H, 3.98; N, 11.07.

N,N'-Dibenzoylselenocystamine (XI).—β-Selenocyanoethylbenzamide (14.4 g.) was dissolved in ethanol (30 ml.) and a solution of potassium hydroxide (10 g.) in 80% ethanol (70 ml.) was then added with shaking. The clear solution was heated for a few minutes on a steam-bath and then allowed to cool to yield crystalline N,N'-dibenzoylselenocystamine (11.1 g., 85% of theory) as pale yellow needles; m.p. 144–145° (dec.) after recrystallization from ethanol.

Anal. Calcd. for $C_{18}H_{20}N_2O_2Se_2$: C, 47.59; H, 4.44; N, 6.17. Found: C, 47.74; H, 4.56; N, 6.26.

Selenocystamine Dihydrochloride.—N,N'-Dibenzoylselenocystamine (10 g.) was dissolved in a mixture of ethanol (30 ml.) and concentrated hydrochloric acid (30 ml.) and the solution heated under reflux for 36 hr. Water (50 ml.) was then added, the solution cooled in ice and filtered from the precipitate of benzoic acid. The filtrate was evaporated to dryness under reduced pressure and the residual selenocystamine dihydrochloride (5.3 g. = 75% of theory) was recrystallized from 95% ethyl alcohol. Yellow platelets, m.p. 177–179° (decomp.); m.p. lit.,[28] 178°.

Selenocystamine (VI).—Selenocystamine dihydrochloride (7.0 g.) was dissolved in water (25 ml.) and sodium hydroxide (2.0 g.) was added. The clear yellow solution was extracted continuously with ether until the aqueous phase was colorless. The ethereal solution was dried over solid potassium hydroxide and the solvent removed under reduced pressure. Free selenocystamine (4.9 g.) was obtained as a yellow oil which was used for the next step without further purification.

N,N'-Dipantothenoyl-selenocystamine (Selenopantethine) (II).—Calcium pantothenate (10.0 g.) was dissolved in water (25 ml.) and triethylamine (10 ml.) was added. The calcium was then precipitated by the addition of a solution of oxalic acid (1.87 g.) in water (25 ml.). The precipitate was separated by centrifugation, washed with small amounts of water, and the combined supernatant layers were evaporated under vacuum. The residual oil was dried by azeotropic distillation under reduced pressure with ethanol and benzene and then dried *in vacuo* over phosphorus pentoxide until the weight remained constant.

The dry, oily triethylamine salt (12.5 g.) was dissolved in dry dimethylformamide (50 ml.) and cooled to −5° in a 250-ml. flask fitted with stirrer, dropping funnel and calcium chloride tube. A solution of ethyl chloroformate (4.1 g.) in ethyl acetate (25 ml.) was added dropwise to form the mixed ester anhydride V. Stirring and cooling were continued for 30 minutes before selenocystamine (4.9 g.) in ethyl acetate (25 ml.) and triethylamine (10 ml.) were introduced through the dropping funnel.

The reaction mixture was stirred for 1 hr. at room temperature, then freed from the precipitated salts. The solvents were removed under reduced pressure (oil pump) at a temperature not exceeding 50°. The light brown, oily residue (16.2 g.) was dissolved in methanol (20 ml.), and acetone was then added until a slight cloudiness persisted (approx. 110 ml.). This solution was then passed through a column of activated alumina (110 g.; "Woelm" neutral alumina, Brockmann grade *III*; column diameter 34 mm.), and eluted with acetone containing 15% of methanol. The first fraction of 150 ml. was discarded, the next fraction (250 ml.) was evaporated to dryness under reduced pressure

(33) H. Bestian, *Ann.*, **566**, 210 (1950).

(34) All m.p.'s are uncorrected.

(35) Microanalyses were performed at the Schwarzkopf Laboratories, Woodside, N. Y.

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]

BY HENRY RAPOPORT, FRANK R. STERMITZ AND DON R. BAKER

RECEIVED NOVEMBER 19, 1959

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).