effected (temperature kept to below 12°) and the solution then kept at 10° for 1 hr. After this time, the dropwise addition of 26 ml. of aqueous ammonia (sp. gr. 0.90) resulted in the separation of a white solid which, after 16 hr., was filtered over suction and washed successively with dilute ammonia and water. Recrystallization from dimethylformamide–water gave a mass of needles; yield 18.0 g. (40%), m.p. 223–224°.

Anal. Calcd. for $C_{23}H_{28}O_6N_4$: C, 60.5; H, 6.1; N, 12.3. Found: C, 60.4; H, 6.1; N, 12.3.

DL-Diaminopimelic Acid Diamide Diacetate.—The dicarbobenzoxylated diamide (21.5 g.) secured above was dissolved in 400 ml. of acetic acid and hydrogenated in the presence of palladium black catalyst. At the completion of hydrogenolysis, the catalyst was removed by filtration and the filtrate concentrated to dryness. After the addition of 25 ml. of water, the evaporation was repeated. The residual oil was used directly in the resolution step, below, without prior crystallization.

Enzymic Resolution.—The hydrogenolysis product, secured directly above, was dissolved in 300 ml. of water containing 1.15 g. of manganous acetate tetrahydrate, the *p*H adjusted to 6.5 with 2 *N* LiOH, 1.8 g. of lyophilized amidase powder[14] added, the *p*H adjusted to 8.0 with 2 *N* LiOH and the final volume brought to 470 ml., corresponding to 0.1 *M* in amide and 0.01 *M* in Mn^{++} concentration. The mixture was subsequently digested at 38° for 5 hr., at which point the manometric ninhydrin–CO_2 procedure revealed 50% hydrolysis of the substrate. Concentration of the digest to about 50 ml. was followed by its dialysis against 4 changes of water (about 900 ml. each) at 5°. The combined dialysates were concentrated to 50 ml. *in vacuo* and the concentrate (at *p*H 8.0) then run onto a column of Amberlite XE-64 (49 × 4 cm.) in the Li^+ form which had been equilibrated previously with lithium acetate (*p*H 6.5) and washed with water. Fractions were collected every 20 ml. and examined for ninhydrin-positive material. Tubes 19 to 31 contained L-diaminopimelic acid (R_f 0.57)[15] whereas tubes 19 and 20 contained, in addition, a small amount of a faster moving compound (R_f 0.63)[15] which, although not identified, was separable from the diamide (R_f 0.59),[15] the monoamide (R_f 0.53)[15] and lysine (R_f 0.55)[15] and which presumably could have arisen by autolysis of the enzyme. As no further ninhydrin positive material could be eluted with water, the eluent was changed to 1% acetic acid at tube 57. Emergence of ninhydrin-sensitive material began at tube 176 and was identified as the diamide (R_f 0.94).[23] This came off as an extended band (overlapping the end of the lithium) and was apparently uncontaminated with other detectable ninhydrin-positive compounds.

L-Diaminopimelic Acid.—The combined eluate of tubes 19–31 was concentrated to dryness, the residual material taken up in the minimal amount of hot water, treated with charcoal, filtered, adjusted to *p*H 6.5 with 2 *N* LiOH and 4 volumes of absolute ethanol subsequently added. A gelatinous precipitate formed which was filtered over suction and sucked dry to a white amorphous powder. Repetition of such precipitation, twice, finally yielded a product[24] which was ash and chloride free and which exhibited a single spot (R_f 0.57)[2] upon chromatographic examination; yield 3.5 g. (88%), $[\alpha]^{26}D$ +45.0° (1% in 1 *N* HCl).[25]

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.4; N, 14.7. Found: C, 44.1; H, 7.5; N, 14.5.

D-Diaminopimelic Acid.—The eluate from the column which contained the diamide was concentrated *in vacuo* to a sirup which, in turn, was dissolved in 1 l. of 3 *N* HCl and boiled for 6 hr. under reflux. Chromatographic analysis indicated complete hydrolysis at this point. The hydrolysate was concentrated to dryness and the residual material taken up in 1.5 *N* HCl and run onto a Dowex 50 column (40 × 4 cm.) which had been equilibrated previously with 1.5 *N* HCl. The acid strength of the eluent was increased to 2.5 *N* and the amino acid thereby eluted was uncontaminated by other ninhydrin-positive material or by lithium. After the combined eluate was evaporated to dryness, the D-form of the amino acid[24] was recovered and purified as given for the L-antipode, above; yield 2.9 g. (73%), $[\alpha]^{26}D$ −45.5° (1% in 1 *N* HCl).[25]

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.4; N, 14.7. Found: C, 44.1; H, 7.6; N, 14.6.

(23) A phenol–ammonia system on Whatman No. 1 paper was here employed.

(24) On drying *in vacuo* at 100° for 3–5 days, the compound lost 9% in weight, equivalent to one molecule of water of crystallization.

(25) Specific rotation of the monohydrate.

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids. VIII. Configuration of the Isomeric Octopines

BY NOBUO IZUMIYA, ROY WADE, MILTON WINITZ, M. CLYDE OTEY, SANFORD M. BIRNBAUM, ROBERT J. KOEGEL AND JESSE P. GREENSTEIN

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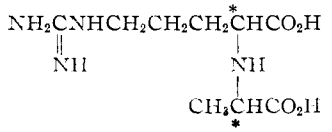
Preparation of the four stereoisomers of octopine was effected *via* a modification of previous methods involving the action of DL-α-bromopropionic acid, in alkaline solution, on L- or D-arginine. The epimeric mixture of (+)-octopine (natural form) and (+)-isoöctopine, secured with L-arginine, or (−)-octopine and (−)-isoöctopine, obtained when D-arginine was employed, was separated into the pure isomers through utilization of the picrate and flavianate derivatives. Optical rotation values of (+)-octopine and (+)-isoöctopine were $[\alpha]^{24}D$ +20.8° and +26.8° (2% in water), respectively, while their respective levorotatory antipodes exhibited values of −20.6 and −26.6° under the same conditions. (−)-Isoöctopine was alternatively prepared by the action of L-α-bromopropionic acid on D-arginine. Utilization of L- and DL-α-bromopropionyl-L-valine *in lieu* of α-bromopropionic acid in the above reactions resulted in the formation of the corresponding "octopinyl" peptides. Infrared spectra, apparent dissociation constants and optical rotatory dispersion data at 589, 578, 546, 435, 405 and 365 mμ, of the diastereomeric optical antipodes of octopine, are presented. From the latter data, calculations of the contribution (partial rotation) of the α- and α′-asymmetric centers to the observed rotation were effected and the empirical rule of Patterson and Brode then employed to assign tentatively a configuration to the alanine portion of each of the stereoisomeric octopines. Such assignment was confirmed unequivocally through the use of kinetic measurements which established that the reaction of L-α-bromopropionic acid with L-arginine, in alkaline solution, yielded (+)-octopine through a bimolecular substitution (SN2) mechanism.

Octopine was first isolated from octopus muscle by Morizawa,[1] in 1927, but it remained for Moore and Wilson[2] and for Akasi,[3] some ten years later, to establish independently the constitution of this compound as the guanidine derivative, α,α′-imino-(δ-guanidovaleric acid)-propionic acid



(1) K. Morizawa, *Acta Schol. Med. Univ. Imp. Kioto*, **9**, 285 (1927).

(2) E. Moore and D. W. Wilson, *J. Biol. Chem.*, **119**, 573, 585 (1937).

(3) S. Akasi, *J. Biochem. (Japan)*, **25**, 261, 281, 291 (1937).

This compound, wherein the asymmetric α- and α'-carbon atoms are joined to an imino nitrogen atom, may be visualized as having been derived from the condensation of one molecule of arginine and one of alanine, with the elimination of one molecule of ammonia. Since the compound contains two centers of asymmetry, the configuration of the natural isomer exists as one of four possible stereoisomeric forms. Although the noteworthy investigations of Akasi[3] have led to the assignment of an L-configuration to the arginine portion of the natural octopine molecule, early attempts[3,4] to assign unequivocally a configuration to the alanine portion have since been demonstrated as unsuccessful.[5,6] It is the determination of this latter configuration with which the present communication is primarily concerned. The confusion which has arisen with regard to the configuration of the alanine moiety has been ably reviewed by Herbst and Swart[6] and, more recently, by Neuberger.[7]

Results and Discussion

Preparation and Enzymic Behavior of the Isomeric Octopines.—Octopine was initially synthesized by Akasi,[3] in 1937, *via* the reaction of arginine with α-bromopropionic acid in alkaline solution. The natural isomer, which is dextrorotatory, was prepared from L-arginine and L-α-bromopropionic acid, whereas use of D-α-bromopropionic acid resulted in the formation of the diastereomeric (+)-isoöctopine. Utilization of DL-α-bromopropionic acid led to an epimeric mixture from which the former isomer could be separated as its insoluble picrate derivative. From the above, it followed that the arginine portion of the octopine molecule was of the L-configuration since none of the bonds radiating about its asymmetric α-carbon atom were broken during the reaction. The latter synthesis was repeated by Ackermann and Mohr[8] and improved by Herbst and Swart,[6] who isolated the (+)-isoöctopine as its sparingly soluble flavianate salt. Alternative syntheses have been described by Irvin and Wilson,[9] who employed the esters of arginine and α-bromopropionic acid, and by Knoop and Martius,[10] who prepared (+)-isoöctopine[11] by the platinum-catalyzed hydrogenation of an aqueous solution of L-arginine and pyruvic acid.[12]

For the preparation of the four isomeric octopines employed in the present investigation, the procedure of Herbst and Swart[6] appeared most promising. A solution of L-arginine·HCl, DL-α-bromopropionic acid and $Ba(OH)_2$, in the mole ratio of 1:1:4, was incubated at 37°, as directed by these investigators. In our hands, however, the disappearance of arginine, as measured by the manometric ninhydrin–CO_2 method, did not proceed to the reported[6] 80% over 72 hr., but rather ceased at only little more than half that value after about 12 hr. (Fig. 1). Since greater reac-

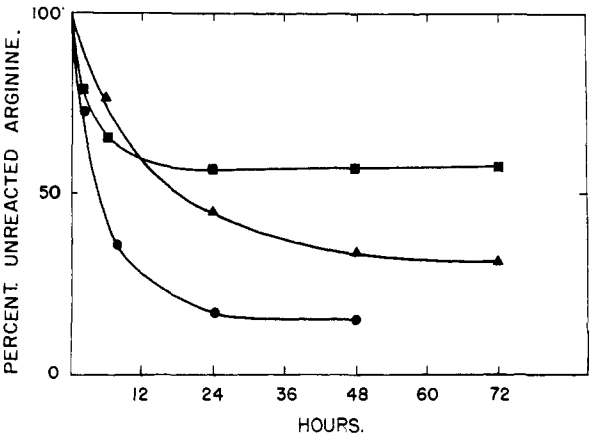


Fig. 1.—Disappearance of arginine in an alkaline solution of α-bromopropionic acid: ■, L-arginine:α-bromo acid:$Ba(OH)_2$ (1:1:4); ●, L-arginine:α-bromopropionic acid.$Ba(OH)_2$ (1:2:4); ▲, L-arginine:DL-α-bromopropionyl-L-valine:$Ba(OH)_2$ (1:2:4).

tivity was not only desirable on the basis of increased yield of product but imperative for effecting the kinetic measurements to be described below, study of other conditions was necessitated. It was found that a mole ratio of amino acid:α-halo acid:base of 1:2:4 led to an 85% disappearance of arginine in 48 hr. (Fig. 1). Under such conditions, the epimeric mixture of (+)-octopine and (+)-isoöctopine, secured with DL-α-bromopropionic acid and L-arginine, or (−)-octopine and (−)-isoöctopine, obtained when D-arginine[13] was employed, was separated into the pure isomers through utilization of the picrate[8] and flavianate[6] derivatives. The optical rotation values found for (+)-octopine and (+)-isoöctopine were $[\alpha]^{24}D$ +20.8° and +26.8° (2% in water), respectively, and were in agreement with previously reported values,[3,6] while their respective levorotatory antipodes exhibited values of −20.6 and −26.6° under identical conditions. (−)-Isoöctopine, alternatively prepared *via* the action of L-α-bromopropionic acid[14] on D-arginine, gave an $[\alpha]D$ of −26.8°.

An attempt to establish the configuration of the

(4) P. Karrer, R. Koenig and R. Legler, *Helv. Chim. Acta*, **24**, 127, 861 (1941).

(5) P. Karrer and R. Appenzeller, *ibid.*, **25**, 595 (1942).

(6) R. M. Herbst and E. A. Swart, *J. Org. Chem.*, **11**, 368 (1946).

(7) A. Neuberger, *Advances in Protein Chem.*, **4**, 297 (1948).

(8) D. Ackermann and M. Mohr, *Z. physiol. Chem.*, **250**, 249 (1937).

(9) J. L. Irvin and D. W. Wilson, *Proc. Soc. Exp. Biol. and Med.*, **36**, 398 (1937); *J. Biol. Chem.*, **127**, 555 (1939).

(10) F. Knoop and C. Martius, *Z. physiol. Chem.*, **258**, 238 (1939).

(11) The $[\alpha]D$ +22.3° (in water) of the isolated product, which was designated as (+)-octopine, was slightly higher than the +20.4° and +20.9° values previously ascribed to the natural isomer.[3,6] That this latter compound was, in actuality, pure (+)-isoöctopine, $[\alpha]D$ +25° (in water), was later indicated by Herbst and Swart,[6] who demonstrated its production by means of asymmetric hydrogenation.

(12) Attempts in this Laboratory to prepare "octopine," or its amide derivative, through the palladium-catalyzed hydrogenation of a mixture of α-keto-δ-guanidovaleric acid with L-alanine or DL-alaninamide·HBr, in aqueous solution, proved unsuccessful. Although the theoretical amount of hydrogen had been absorbed in 72–96 hr., no disappearance of alanine or its amide (an acid hydrolyzate of the hydrogenation mixture was here employed), as determined by the manometric ninhydrin–CO_2 method, could be ascertained. Somewhat greater success was encountered where ammonia was employed as the amine, the conversion of the keto acid to DL-arginine proceeding to some 19% of the theory in 72 hr. Failure of the former experiment conceivably could be attributed to a preferential reduction of α-keto-δ-guanidovaleric acid exclusively to arginic acid, whereas conversion to this compound is somewhat less when ammonia is employed.

(13) S. M. Birnbaum, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **60**, 496 (1956).

(14) E. Fischer and K. Raske, *Ber.*, **39**, 3981 (1906); S-C. J. Fu, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **76**, 6054 (1954).

octopines by the stereospecific action of L-amino acid oxidase (*Crotalus adamanteus*) and D-amino acid oxidase (hog kidney)[15,16] proved unsuccessful. The lack of susceptibility exhibited by these compounds, although not entirely unanticipated where the L-oxidase was employed, was nonetheless somewhat unexpected in the case of the D-oxidase, which readily oxidizes the imino acids, D-proline and the D-hydroxyprolines.[15] An effort was subsequently made to utilize the stereospecific hydrolytic action of aminopeptidase[16,17] on the "octopinyl"-L-valines, prepared by the action of L- or DL-α-bromopropionyl-L-valine on L-arginine in alkaline solution (Fig. 1). Complete lack of enzymic susceptibility again ensued. Similar attempts to utilize the hydrolytic action of hog kidney amidase[18] on the diamides of (+)-octopine and (+)-isoöctopine also proved unfruitful. Resort was thereupon made to the use of non-enzymatic methods, described below.

Rotatory Dispersion.—In an earlier paper of this series,[19] the optical rotatory dispersion of 42 L-amino acids was investigated at 589, 578, 546, 435, 405 and 365 mμ and described, for the most part, in terms of a two constant Drude equation.[20]

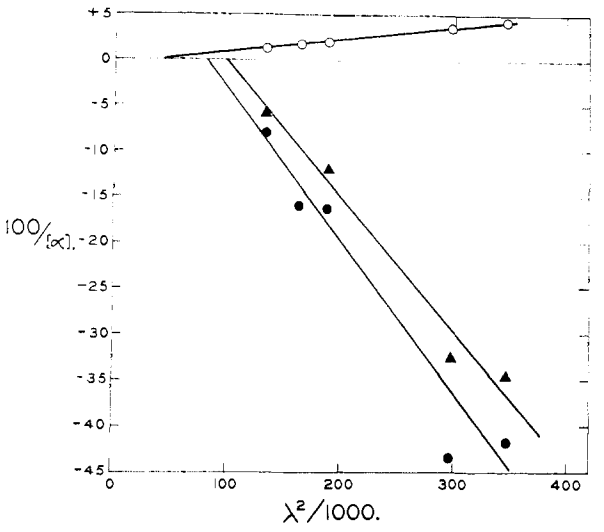


Fig. 2.—Rotatory dispersion of (+)-octopine, where ○ represents the dispersion of the arginine portion in both water and 5 *N* HCl, while that of the alanine portion is depicted by ● and ▲ in these same solvents.

That the rotatory dispersion of α-amino acids of a given optical configuration is possessed of certain characteristic regularities was previously established by the investigations of Patterson and Brode.[21] The empirical relation[22] between the rotatory behavior of α-amino acids and the magnitude of λ_0, which arose from these studies was later shown, in this Laboratory,[19] to have general applicability not only to those amino acids which contained a single α-center of asymmetry but, in modified form, to diasymmetric amino acids as well. Application of this relationship to the latter, more complex amino acids, could be made if the dispersion was based solely upon the contribution (partial rotation) of the α-asymmetric center to the total observed rotation of the molecule.[23] Such application is here extended to the isomers of octopine.

The rotatory dispersions of (+)-octopine and (+)-isoöctopine were determined, as 1% solutions in water and 5 *N* HCl, at 589, 578, 546, 435, 405 and 365 mμ with a photoelectric polarimeter of high precision. Partial rotations of each asymmetric center of the diastereomeric octopines were calculated from the experimental values at each wave length (Table I), as described previously.[19,23] A graphical plot of $100/\alpha$ *vs.* $\lambda^2/1000$ was employed to determine the character of the dispersion (Fig. 2), and the λ_0 for each asymmetric center calculated from the single term Drude equation. Such data revealed that the arginine portion of the (+)-octopine and the arginine and alanine portions of the (+)-isoöctopine molecules exhibited normal, positive dispersions and λ_0 values above 200, behavior characteristic of an L-configuration.[19-22] The alanine portion of the (+)-octopine molecule, however, showed a normal, negative dispersion with a λ_0 above 200, behavior attributable to a D-configuration.[24] On this basis, a configurational designation may be assigned to each of the four isomeric octopines (Table II). In accordance with the current rules of amino acid nomenclature, such assignment is based on the configuration of the smaller of the two amino acid radicals, *i.e.*, the alanine moiety, and, in addition, the prefix allo- is substituted for that of iso-.[25] As a consequence, natural (+)-octopine now becomes D-octopine.[26]

(15) J. P. Greenstein, *Advances in Protein Chem.*, **9**, 121 (1954); J. P. Greenstein, S. M. Birnbaum and M. C. Otey, THIS JOURNAL, **77**, 707 (1955).

(16) M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, *ibid.*, **78**, 2423 (1956).

(17) D. S. Robinson, S. M. Birnbaum and J. P. Greenstein, *J. Biol. Chem.*, **202**, 1 (1953).

(18) D. Hamer and J. P. Greenstein, *ibid.*, **193**, 81 (1951); *cf.* S. M. Birnbaum in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 397.

(19) M. C. Otey, J. P. Greenstein, M. Winitz and S. M. Birnbaum, THIS JOURNAL, **77**, 3112 (1955).

(20) The Drude equation expresses the relation between optical rotation and wave length as $\alpha = Ek_n/\lambda^2 - \lambda_n^2$, where α represents the rotation, *k* is a constant, λ is the wave length of the light employed for the measurements and λ_n is a series of wave lengths associated with the spectral absorption bands controlling the dispersion (*cf.* P. A. Levene and A. Rothen in H. Gilman's "Organic Chemistry," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1938, pp. 1779-1850). When a graphical plot of $1/\alpha$ against λ^2 in the visible region of the spectrum yields a straight line, the equation is expressed in its simplest or one-term form, $\alpha = k/\lambda^2 - \lambda_0^2$. If the resultant graph is not a straight line, additional terms generally must be added to express the dispersion linearly to at least a first approximation.

(21) J. W. Patterson and W. R. Brode, *Arch. Biochem.*, **2**, 247 (1943).

(22) According to such relationships, α-amino acids possess an L-configuration if, on the basis of the graphical use of the single term Drude equation, the following are met: (a) the dispersion is normal, positive, and the value of λ_0 is above 205 mμ; (b) the dispersion is normal, negative, and the value of λ_0 is below 140 mμ; and (c) the dispersion is anomalous (produced by partial rotations of opposite sign), and the sign of rotation changes from negative to positive with decreasing wave length.

(23) M. Winitz, J. P. Greenstein and S. M. Birnbaum, THIS JOURNAL, **77**, 716 (1955).

(24) The λ_0 values for the arginine portion of octopine and isooctopine were 220 and 232 for aqueous and 5 *N* HCl solutions, respectively, whereas the values of the alanine portion were 283 and 313 for these same solvents.

(25) *Cf.* H. B. Vickery, *J. Biol. Chem.*, **169**, 237 (1947).

(26) Some confusion has existed, in the past, with regard to the assignment of the configuration of the alanine portion when based upon rotational shifts induced by *p*H changes. Akasi[3] applied the Lutz-

TABLE I

CONTRIBUTION OF THE INDIVIDUAL ASYMMETRIC CARBON ATOMS OF THE OCTOPINES TO THE SPECIFIC ROTATION AT VARIOUS WAVE LENGTHS

λ, mμ	[α] In H_2O	[α] In 5 *N* HCl	[α] Arginine portion In H_2O	[α] Arginine portion In 5 *N* HCl	[α] Alanine portion In H_2O	[α] Alanine portion In 5 *N* HCl
			(+)-Octopine			
589	+20.9	+ 20.0	+23.3	+22.9	− 2.4	− 2.9
578	+22.8	+ 23.1	....	....	....	....
546	+25.4	+ 24.4	+27.7	+27.5	− 2.3	− 3.1
435	+43.5	+ 41.5	+49.6	+49.9	− 6.1	− 8.4
405	+53.4	+ 50.7	+59.6	....	− 6.2	....
365	+70.3	+ 66.7	+82.8	+83.9	−12.5	−17.2
			(+)-Isoöctopine			
589	+25.7	+ 25.7	+23.3	+22.9	+ 2.4	+ 2.9
546	+30.0	+ 30.6	+27.7	+27.5	+ 2.3	+ 3.1
435	+55.6	+ 58.3	+49.6	+49.9	+ 6.1	+ 8.4
405	+65.8	.....	+59.6	....	+ 6.2	....
365	+95.2	+101.0	+82.8	+83.9	+12.5	+17.2

TABLE II

CONFIGURATIONAL DESIGNATION AND NOMENCLATURE OF THE ISOMERIC OCTOPINES

Early designation	Radical configuration Arginine	Radical configuration Alanine	Present designation
(+)-Octopine (natural)	L	D	D-Octopine
(−)-Octopine	D	L	L-Octopine
(+)-Isoöctopine	L	L	L-Alloöctopine
(−)-Isoöctopine	D	D	D-Alloöctopine

Kinetic Measurements.—Reference has been made to the finding of Akasi[3] that (+)-octopine is formed in alkaline solutions of L-α-bromopropionic acid and L-arginine. In addition to the optical rotatory dispersion evidence cited above, consideration based on the probable course of the coupling reaction strongly implies that the α-halo acid should undergo a Walden inversion leading from an L-α-halo acid to a D-amino acid residue. Such implication arises from the finding of Fischer,[27] and of Abderhalden and Haase,[28] that inversion does occur upon the reaction of α-bromopropionic acid with either ammonia[27] or glycine.[28] In keeping with these findings, Karrer and Appenzeller[5] have demonstrated, still more recently, that optically inactive *meso*-α,α′-iminodipropionic acid is derived from the reaction of either L-alanine with L-α-bromopropionic acid or D-alanine with the D-α-halo acid. All of these reactions proceed *via* an SN2 mechanism.[29] By analogy, it would be anticipated that the reaction of arginine with α-bromopropionic acid should proceed through this same bimolecular mechanism with its implicit Walden inversion. If such fact could be substantiated by kinetic measurements, it would then provide unequivocal confirmation of the above configurational assignments to the isomeric octopines.

That the reaction of L-arginine with L-α-bromopropionic acid in alkaline solution is, indeed, of the second order was indicated by calculation of the first (k_1) and second (k_2) order rate constants, at various time intervals. Whereas the k_1 values decreased with time, the k_2 values remained constant, within the limits of experimental error. The rate of formation of octopine (dx/dt) was measured graphically from the slope of the concentration of unreacted arginine ($A - x$) *versus* time curve (Table III). The error accumulated during this calculation was of the order of 15% for the early values but fell to about 5% for the later ones. A plot of dx/dt *versus* $(A - x)(B - y)$, where $(B - y)$ equals unreacted α-bromopropionic acid, was linear within the limits of experimental error and thereby satisfied an equation of the form

$$dx/dt = k_2(A - x)(B - y)$$

required for an SN2 mechanism (Fig. 3). It should be noted that α-bromopropionic acid, in addition to reaction with arginine, undergoes an SN2 reaction with hydroxyl ions to an extent measured by the difference of $(B - y) - (A - x)$ at any given time.

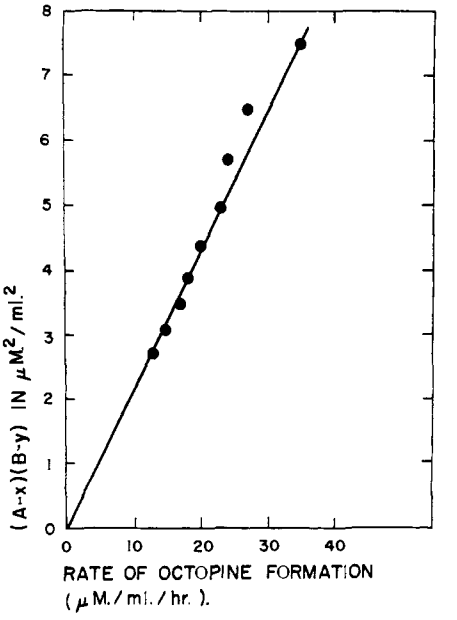


Fig. 3.—Dependence of rate of octopine formation upon product of reactant concentrations.

Apparent Dissociation Constants.—Irvin and Wilson[9] determined the constants of octopine to be pK_1' 1.36, pK_2' 2.40, pK_3' 8.76 and pK_4' (approximate estimate) as "somewhat less than 13." The titration curve of this substance was repeated, and the constants found at 25° were pK_1' 1.40, pK_2' 2.30, pK_3' 8.72 and pK_4' 11.34. The first three constants were in general accord with those noted by Irvin and Wilson. A similar titration curve for isooctopine yielded the values pK_1' 1.35, pK_2' 2.30, pK_3' 8.68 and pK_4' 11.25, which were essentially in agreement with those of the diastereoisomer.

Jirgensons rule (O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931); **65**, 784 (1932)) to the optical rotation curve at different *p*H values and suggested that the alanine portion of natural octopine had an L-configuration. This same technique, subsequently applied by Herbst and Swart,[9] led to different results and the suggestion that the alanine residue be given, rather, the provisional assignment of a D-configuration.

(27) E. Fischer, *Ber.*, **40**, 489 (1907).

(28) E. Abderhalden and E. Haase, *Z. physiol. Chem.*, **202**, 49 (1931).

(29) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

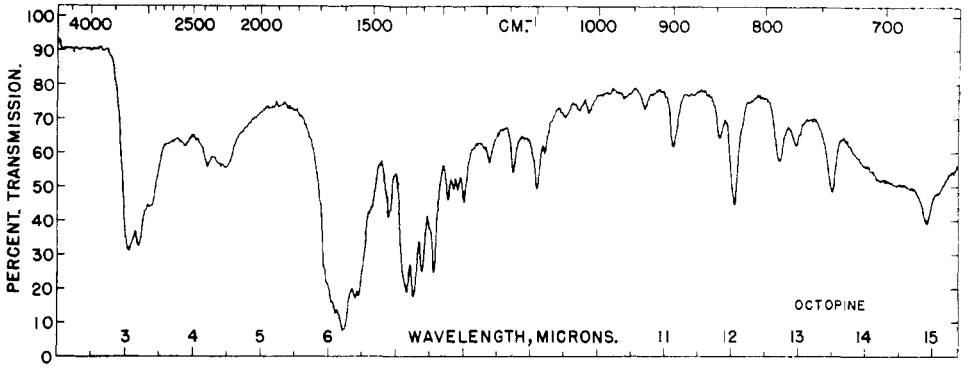


Fig. 4.—Infrared spectrum of (+)-octopine (D-octopine) in the solid state.

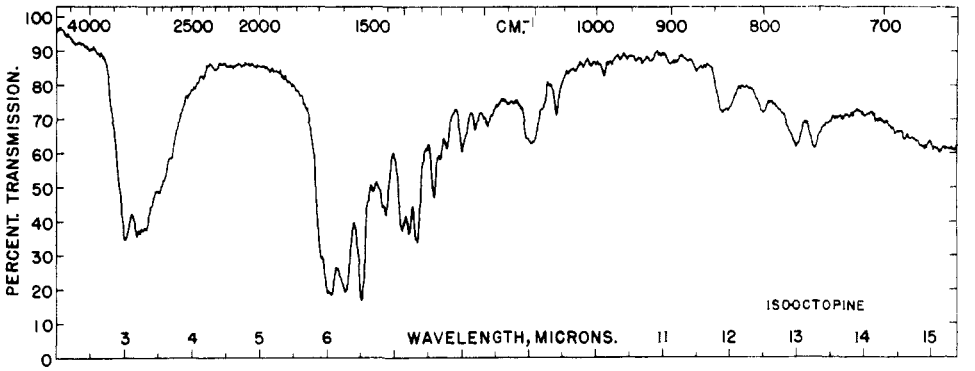


Fig. 5.—Infrared spectrum of (+)-isoöctopine (L-alloöctopine) in the solid state.

Infrared Spectra.—The solid state infrared spectra in the range of 3 to 15 μ were determined as described previously.[30] As noted in Figs. 4 and 5, the spectrum for either antipode of octopine is quite different from that of the isoöctopines. There are distinct bands in the 3.4 to 6.0 μ region present in the spectrum of the former compared with that of the latter compound. That the spectra of diastereoisomeric amino acids and peptides may be expected to differ is now an accepted fact.[30,31]

TABLE III

FORMATION OF (+)-OCTOPINE FROM L-ARGININE AND L-α-BROMOPROPIONIC ACID IN ALKALINE SOLUTION

Time, hr.	$(B - y)$[a]	$(A - x)$[b]	$\frac{(A-x)}{(B-y)} \times 10^{-4}$	dx/dt[c]
0.5	392	192	7.5	35
1.0	366	178	6.5	27
1.5	345	166	5.7	24
2.0	325	155	5.0	23
2.5	307	144	4.4	20
3.0	291	134	3.9	18
3.5	278	125	3.5	17
4.0	268	116	3.1	15
4.5	255	108	2.8	13

[a] Unreacted bromopropionate in μmoles/ml., where B equals initial concentration and y equals amount reacted. [b] Unreacted arginine in μmoles/ml., where A equals initial concentration and x equals amount reacted. [c] Rate of formation of octopine in μmoles/ml./hr.

(30) R. J. Koegel, J. P. Greenstein, M. Winitz, S. M. Birnbaum and R. A. McCallum, THIS JOURNAL, 77, 5708 (1955).

(31) E. Ellenbogen, *ibid.*, 78, 363 (1956); M. C. Otey and J. P. Greenstein, *Arch. Biochem. Biophys.*, 53, 501 (1954).

Experimental

I. Preparation of the Isomeric Octopines. (−)-Octopine.—Preparation of this compound was effected *via* a modification of the procedure of Akasi[3] and of Herbst and Swart.[6] As was discussed in the Results and Discussion Section, choice of the reactant ratios was dictated, in the main, by the rate of formation observed for octopine at various reactant concentrations.

A solution of 211 g. of D-arginine hydrochloride,[13] 306 g. of DL-α-bromopropionic acid and 632 g. of barium hydroxide octahydrate in 4 l. of water was incubated at 37° for 48 hr., after which time some 85% of the L-arginine had disappeared as measured by the manometric ninhydrin-CO_2 method. Removal of the barium ions was effected by the addition of 410 ml. of 10 N sulfuric acid to the digest, heating the mixture for 0.5 hr. on the steam-bath and filtering over suction. The pH of the filtrate was adjusted to 2.6–2.7 by the addition of the silver carbonate (about 280 g.), filtered and the filtrate concentrated to approximately 2.5 l. with a stream of air and concurrent warming on the steam-bath. To the concentrate was added 57 g. of flavianic acid and the resulting solution placed at 4° overnight. The precipitate of D-arginine flavianate was filtered off, washed with 0.02 N hydrochloric acid and the pooled filtrate and washings washed thrice with 1-butanol. Removal of trace amounts of 1-butanol in the aqueous layer was effected by concentration in a stream of air. About 100 g. of silver carbonate was added to the concentrate, the precipitate filtered over suction and the filtrate treated with hydrogen sulfide to remove residual silver ions. After removal of the precipitated silver sulfide, the filtrate was concentrated somewhat to remove residual hydrogen sulfide, subsequently adjusted to pH 6.5 by the addition of 6 N sodium hydroxide (about 60 ml.), filtered and the filtrate further concentrated to 3.3 l. This latter solution was heated nearly to boiling, 190 g. of picric acid dissolved therein and the reaction mixture allowed to stand at 4° overnight. The precipitated (−)-octopine picrate was collected, washed with cold water (*combined filtrate and washings saved for the recovery of* (−)-*isoöctopine*) and recrystallized from boiling water (about

7.5 l.). After drying for 48 hr. at 50°, the picrate melted at 229–230° dec.,[32] yield 186 g.

Decomposition of the picrate could be effected by suspending 186 g. in 2 l. of hot water and adding 2 l. of 11.5 *N* hydrochloric acid thereto. The mixture was heated over the steam-bath for 0.5 hr. with occasional stirring, cooled to 0°, filtered and the precipitate washed with cold 6 *N* hydrochloric acid. The combined filtrates were washed thrice with ether and the aqueous fraction evaporated to dryness *in vacuo*. Upon repeated additions of water to the residue, followed by evaporation, the excess hydrochloric acid was removed. The residue was taken up in water, silver carbonate (about 150 g.) added to remove chloride ion, and the precipitate removed by filtration. The filtrate, adjusted to *p*H 6.3–6.4 by the addition of triethylamine, was evaporated to dryness and the residue recrystallized twice from boiling water (600 ml.)–alcohol (400 ml.). After drying at 50° for 2 days, a 77-g. yield of (−)-octopine was obtained, m.p. 283–285°,[32] $[\alpha]^{24}D$ −20.6° (2% in water).

Anal. Calcd. for $C_9H_{18}O_4N_4$: C, 43.9; H, 7.4; N, 22.8. Found: C, 43.8; H, 7.3; N, 22.7.

(−)-Isoöctopine.—The combined filtrate and washings from (−)-octopine picrate were evaporated to 1.2 l., and 1.2 l. of 11.5 *N* hydrochloric acid added thereto. The solution was cooled to 0°, filtered and the filtrate extracted with ether. The aqueous fraction was evaporated to dryness *in vacuo* and the excess hydrochloric acid removed by repeated addition of water and evaporation. The residue, taken up in 1 l. of water, was treated with silver carbonate (about 130 g.) to remove chloride, filtered, the filtrate treated with hydrogen sulfide and the precipitated silver sulfide removed by filtration. The volume of the filtrate was adjusted to 1.3 l. by either evaporation or dilution, 147 g. of flavianic acid dissolved therein and the solution placed at 4°. Although the appearance of crystals of (−)-isooctopine flavianate did not commence until 7–9 days had elapsed, subsequent runs showed that this time could be reduced to several hours in the presence of seed crystals. The flavianate was filtered over suction and washed with ice-cold water. Since the solubility of the flavianate in water is appreciable at room temperature, it is recommended that these operations be carried out in the cold. Recrystallization was effected from 600 ml. of water, yield 160–180 g.

Decomposition of a solution of the above flavianate, in 700 ml. of hot water, was achieved by addition of a hot solution of 130 g. of barium hydroxide octahydrate, in 700 ml. of water. After cooling to 0°, filtering and washing the precipitate with dilute baryta solution, a stream of carbon dioxide gas was passed through the filtrate and the barium carbonate filtered off. Final traces of barium ion in the filtrate were removed by the careful dropwise addition of dilute sulfuric acid. Subsequent to filtration, the solution was concentrated to 120 ml. *in vacuo*. Addition of 360 ml. of absolute alcohol resulted in the precipitation of (−)-isooctopine as a gelatinous substance, which was converted into a white crystalline material upon warming. This was filtered over suction, recrystallized from hot water (150 ml.)–alcohol (450 ml.) and dried *in vacuo* over phosphorus pentoxide at 100° for 3 hr., yield 27.5 g., m.p. 263–264°,[32] $[\alpha]^{24}D$ −26.6° (2% in water).

Anal. Calcd. for $C_9H_{18}O_4N_4$: C, 43.9; H, 7.4; N, 22.8. Found: C, 43.6; H, 7.5; N, 22.5.

An alternative procedure for the production of (−)-isooctopine involved the reaction of 21.1 g. of D-arginine hydrochloride with 30.6 g. of L-bromopropionic acid[14] in alkaline solution in a manner comparable to that described above which involved use of the DL-α-halo acid. Incubation of the reaction mixture was allowed to proceed for 48 hr. at 37°, after which time 85% of the arginine disappeared. Isolation of the compound as the flavianate proceeded as above, 29.3 g. of flavianic acid being added to the neutralized solution (total volume of 230 ml.) for this purpose. Recrystallization and decomposition of the flavianate (yield 76 g.) subsequently yielded 12.0 g. of (−)-isoöctopine, m.p. 264–265°,[32] $[\alpha]^{24}D$ −26.8° (2% in water).

Anal. Found: C, 43.5; H, 7.6; N, 22.5.

(+)-Octopine.—This compound was prepared from L-arginine hydrochloride and DL-bromopropionic acid according to the same procedure employed for the levorotatory antipode described above; m.p. 283–284°,[32] $[\alpha]^{24}D$ +20.8° (2% in water). Values of $[\alpha]^{17}D$ +20.9° and $[\alpha]^{25}D$ +20° have been reported previously.[3,6]

(+)-Isoöctopine.—Isolation of this compound from the (+)-octopine picrate filtrate was achieved as described above for the levorotatory form, m.p. 264–265°,[32] $[\alpha]^{24}D$ +26.8° (2% in water). Previous values of $[\alpha]^{20}D$ +25.8° and +25° have been reported.[3,6]

II. Preparation of the "Octopinylvalines." L-α-Bromopropionyl-L-valine.—This compound was prepared from 58.5 g. of L-valine, 116 g. of L-α-bromopropionyl chloride[14] and 550 ml. of 2 *N* sodium hydroxide according to the usual Schotten–Baumann procedure. Acidification of the solution to congo red with 10 *N* hydrochloric acid led to the precipitation of beautiful crystals. Recrystallization was effected from 40% alcohol, yield 82%, m.p. 178°, $[\alpha]^{24}D$ −16.8° (in ethanol).

Anal. Calcd. for $C_8H_{14}O_3NBr$: C, 38.1; H, 5.6; N, 5.6. Found: C, 38.0; H, 5.6; N, 5.6.

DL-α-Bromopropionyl-L-valine.—This epimeric mixture was prepared as above with the exception that DL-α-bromopropionyl bromide was employed as the acylating agent, yield 81%, m.p. 168–173°, $[\alpha]^{24}D$ +2.3° (in ethanol). Since Fischer and Scheibler previously reported[33] a preparation of D-α-bromopropionyl-L-valine which exhibited an $[\alpha]^{20}D$ +20.9° (in alcohol), it follows from the above rotation of the L,L-form that the ratio of the L,L- to the D,L-stereoisomers in the epimeric mixture was 49:51.

Anal. Calcd. for $C_8H_{14}O_3NBr$: C, 38.1; H, 5.6; N, 5.6. Found: C, 38.3; H, 5.7; N, 5.6.

"D-Octopinyl"-L-valine (α,α'-Imino-(δ-guanidovaleric acid)-propionyl-L-valine.—A solution of 5.3 g. of L-arginine hydrochloride, 12.6 g. of L-α-bromopropionyl-L-valine and 15.9 g. of barium hydroxide octahydrate in 150 ml. of water was incubated at 37° for 72 hr., after which time some 69% of the arginine had disappeared. Treatment of the reaction mixture for removal of barium ions and unreacted arginine was effected as for (−)-octopine, as described above. After the 1-butanol treatment, paper chromatograms of the aqueous layer, which employed the 1-butanol–6 *N* HCl (7:3) or *n*-butanol–acetic acid–water (4:1:5) systems, indicated a single spot with an R_f value of 0.75 for the former and 0.46 for the latter system. These spots, developed *via* the Sakaguchi reaction as directed by Makizumi,[34] did not correspond to octopine (or isoöctopine) which showed respective R_f values of 0.35 and 0.12 for these same solvent systems. Values for L-arginine, under these same conditions, were 0.12 and 0.14, respectively. After the *p*H of the solution was adjusted to 6.5 with triethylamine, it was concentrated to a sirup *in vacuo*. Addition of alcohol resulted in the formation of an amorphous powder which was filtered off, washed with 90% alcohol and reprecipitated from water by the addition of 7–10 volumes of alcohol. The white, amorphous material was dried for 5 hr. *in vacuo* at 100°, yield 7.1 g., m.p. 180–184°, $[\alpha]^{24}D$ −3.1° (in water).

Anal. Calcd. for $C_{14}H_{27}O_5N_5$: C, 48.7; H, 7.9; N, 20.3. Found: C, 48.2; H, 8.1; N, 20.0.

In order to demonstrate the identity of the N-terminal octopine end of the above peptide, a sample was subjected to acid hydrolysis and the hydrolyzate subject to chromatographic analysis as above. Only a single spot, which corresponded to octopine, was observed. In addition, the hydrolyzate, upon treatment with picric acid, yielded D-octopine picrate. No precipitate of isoöctopine flavianate could be secured upon treatment with flavianic acid, however, a phenomenon demonstrated the relatively high stereochemical purity of the octopine residue.

"DL-Octopinyl"-L-valine.—An alkaline solution containing 25.2 g. of DL-α-bromopropionyl-L-valine and L-arginine was treated and the epimeric mixture of "D-octopinyl"-L-valine and "L-allooctopinyl"-L-valine isolated under conditions comparable to those employed with the L-α-bromoacyl

(32) Dependent upon the rate of heating and initial bath temperature, the picrates and flavianates of octopine and isoöctopine exhibited varying decomposition points. The sample to be checked was therefore simultaneously compared with an authentic sample of the compound, or its optical antipode, under conditions such that the latter would melt as follows: (+)-octopine, 283–284°; (+)-octopine picrate, 229–230°; (+)-isoöctopine, 264–265°; (+)-isoöctopine picrate, 210–212°.

(33) E. Fischer and H. Scheibler, *Ann.*, **363**, 136 (1908).

(34) S. Makizumi, *J. Chem. Soc.* (*Japan*), **73**, 737 (1952).

compound. The yield of the monohydrate was 15.5 g., $[\alpha]^{24}D$ −5.9° (in water). Chromatographic analysis, as described for the D,L-form above, revealed only a single spot.

Anal. Calcd. for $C_{14}H_{27}O_5N_5 \cdot H_2O$: C, 46.3; H, 8.1; N, 19.3. Found: C, 45.8; H, 8.2; N, 18.8.

In order to ascertain that the above compound was, in fact, an epimeric mixture, a sample of 7.27 g. was refluxed with 6 *N* hydrochloric acid for 24 hr. Paper chromatograms, as above, showed only a single spot corresponding to octopine (or isoöctopine). After removal of chloride ions with silver carbonate, the filtrate yielded 2.12 g. of D-octopine and 0.42 g. of L-alloöctopine, isolated *via* their respective picrate and flavianate derivatives. Although such data indeed indicated the presence of an epimeric mixture, the ratio of "D-octopinyl"- to "L-alloöctopinyl"-L-valine could not properly be determined from the final yields of D-octopine and L-alloöctopine subsequently secured, since large losses occur during the isolation of the flavianate of this latter compound.

III. **Rotatory Dispersion Measurements.**—The rotatory dispersion of (+)-octopine and (+)-isoöctopine was determined at 589, 578, 546, 435, 405 and 365 mμ, as 1% solutions in water and 5 *N* HCl, with a Rudolph photoelectric polarimeter. The procedure employed was similar to that described earlier.[19] Replicate readings were reproducible to ±0.003° (Table I).

IV. **Kinetic Measurements.**—Separate solutions of L-α-bromopropionic acid (632.4 mg.) in water (4.14 ml.) and of L-arginine·HCl (435.6 mg.) in 2 *N* sodium hydroxide (4.14 ml.) were allowed to equilibrate for 1 hr. in a water-bath at 39°. The solutions were then mixed and two 0.2-ml. samples removed immediately for Volhard titration and for Van Slyke ninhydrin-CO_2 determination. Similar sampling was effected at succeeding 30-minute intervals for 5.5 hr., by which time about 55% of the arginine had disappeared. Results were as shown in Table III.

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[CONTRIBUTION FROM THE DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

An Alanine-dependent, Ribonuclease-inhibited Conversion of AMP to ATP, and its Possible Relationship to Protein Synthesis

BY ROBERT W. HOLLEY[1]

RECEIVED AUGUST 3, 1956

An enzyme preparation from the soluble fraction of rat liver homogenate brings about conversion of radioactive AMP (adenosine 5′-monophosphate) into ATP (adenosine 5′-triphosphate) in the presence of L-alanine. Other amino acids, potassium acetate and sodium pyruvate have no effect. The L-alanine-dependent conversion of AMP to ATP is inhibited by ribonuclease.

There is considerable evidence that the first step in the synthesis of proteins from amino acids is an activation of the amino acid carboxyl groups by ATP.[2] The work of Hoagland[3] and Hoagland, Keller and Zamecnik[4] has suggested a detailed mechanism for this activation (eq. 1). These workers found that an enzyme preparation from the soluble fraction of rat liver homogenate brings about a rapid exchange between pyrophosphate and the terminal phosphates in ATP if amino acids are present. The amino acid activating enzymes could also be studied by demonstrating the formation of amino acid hydroxamic acids when the reaction was run in the presence of a high concentration of hydroxylamine. The activated amino acids have been formulated as enzyme-bound amino acyl-AMP derivatives[3,4]

$$\text{Enzyme} + \text{Amino Acid} + \text{ATP} \rightleftarrows \text{Enzyme–Amino Acyl-AMP} + \text{Pyrophosphate} \quad (1)$$

The assumption that this type of enzymatic activation of amino acids is the first step in protein synthesis rests primarily on the discovery of the enzymes in a fraction which is required for the *in vitro* incorporation of radioactive amino acids by rat liver microsomes.[3,4]

Strong support for the above formulation of the activation of amino acids has been furnished by DeMoss, Genuth and Novelli[5] who synthesized the L-leucyl derivative of AMP (thought to be the mixed anhydride of L-leucine and AMP) and showed that in the presence of amino acid activating enzyme obtained from *E. coli* this compound reacts with pyrophosphate to form ATP. Similar results were obtained by Berg and Newton[6] using a methionine activating enzyme obtained from yeast.

If it is assumed that amino acid activation of this type is the first step in protein synthesis, the question immediately arises as to the nature of the next step in protein synthesis. Presumably, the activated amino acid must react with something, or be transferred to something. As a working hypothesis, it was assumed that the activated amino acid reacts with some unknown material with liberation of AMP

$$\text{Enzyme–Amino Acyl-AMP} + \text{X} \rightleftarrows \text{Enzyme} + \text{Amino Acyl-X} + \text{AMP} \quad (2)$$

Such a reaction might be reversible and, if combined with the pyrophosphate exchange reaction (eq. 1), it might be detected by assaying for exchange of AMP into ATP.

A procedure for assay of AMP exchange was therefore devised. Crude enzyme preparations were incubated with radioactive AMP under conditions in which pyrophosphate was known to exchange with ATP. At the end of the incubation period, the ATP was precipitated as the barium salt and its radioactivity was determined.

The assay was first applied to extracts of spinach acetone powder, hog pancreas acetone powder and lyophilized *Lactobacillus plantarum* cells, extracts

(1) New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y. John Simon Guggenheim Memorial Foundation Fellow, 1955–1956.

(2) H. Borsook, *J. Cellular Comp. Physiol.*, **47**, Suppl. 1, 35 (1946).

(3) M. B. Hoagland, *Biochim. Biophys. Acta*, **16**, 288 (1955).

(4) M. B. Hoagland, E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.*, **218**, 345 (1956).

(5) J. A. DeMoss, S. M. Genuth and G. D. Novelli, *Proc. Natl. Acad. Sci.*, **42**, 325 (1956).

(6) P. Berg and G. Newton, *Federation Proc.*, **15**, 219 (1956).