TABLE V

RELATIVE AMOUNTS OF 2′- AND 3′-ISOMERS FORMED IN HYDROLYSIS AND METHANOLYSIS OF MONOESTERS OF NUCLEOTIDES

	Hydrolysis[b]			Methanolysis[b]	
Product[a]	RNA	Monomethyl esters	2′:3′-Cyclic esters	Yeast RNA	Calf liver RNA
C-2′-P[46]	43	46	45	43	43
C-3′-P	57	54	55	57	57
A-2′-P	43	46	..	31	33
A-3′-P	57	54	..	69	67
U-2′-P	42	40	40	38	38
U-3′-P	58	60	60	62	62
G-2′-P	44	47	45	31	35
G-3′-P	56	53	55	69	65

[a] The hydrolysis products are the nucleotides as such. The methanolysis products are the monomethyl esters of the nucleotides. [b] See footnotes *a* and *b*, Table I.

each of the methyl ester peaks increased by several per cent. while each of the peaks corresponding to the cyclic esters decreased by a corresponding amount. The same effect was observed with the calf liver RNA (peaks 3, 6, 7, 8, 10 and 11, Fig. 2).

Since the base-catalyzed hydrolysis of monoesters of 2′- or 3′-nucleotides involves 2′:3′-cyclic phosphates as intermediates,[17,23,40] the ratio of 2′- to 3′-isomers formed on hydrolysis of a monoester of a given nucleotide should be independent of the particular ester which is being hydrolyzed. The data given in Table V illustrate this point for hydroxide ion-catalyzed hydrolysis (0.5 *N* NaOH, 30–37°, 17–20 hr.) of RNA, monomethyl esters of nucleoside-2′(3′)-phosphoric acids and nucleoside-2′:3′-phosphoric acids. The ratios of 2′- to 3′-isomers also are given in this table for the monomethyl esters of the nucleotides which are formed in the methanolysis of RNA (65°, 1 *N* methoxide ion). These ratios vary significantly from the corresponding ratios in hydrolysis. This is not surprising. The isomer ratio obtained in hydrolysis is determined by the relative rates of breaking the C_2' and C_3' P–O bonds in a 2′:3′-cyclic phosphate. On the contrary, the isomer ratios of the methyl esters obtained in methanolysis reflect not a rate phenomenon, but the relative thermodynamic stabilities of the two isomers in a given pair.

Preliminary experiments carried out recently[55] indicate that methoxide ion-catalyzed methanolysis is applicable to the determination and identification of RNA end-groups of type II in whole turnip yellow mosaic virus nucleoprotein. It will be of interest to determine the fate of the protein in this degradation.

(55) These experiments were done by R. Markham, M. W. Rees and D. Lipkin (Senior Visiting Fellow, Office for European Economic Coöperation, 1960) in the laboratories of the Virus Research Unit, Agricultural Research Council, Cambridge, England.

[CONTRIBUTION FROM DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NEW YORK]

The Synthesis of 1-(Hemi-homocystine)-oxytocin and A Study of Some of its Pharmacological Properties[1]

BY DEREK JARVIS,[2] MIKLOS BODANSZKY AND VINCENT DU VIGNEAUD

RECEIVED JUNE 19, 1961

1-(Hemi-homocystine)-oxytocin, an analog of oxytocin in which the half-cystine residue of the hormone which bears the free amino group is replaced by a half-homocystine residue, has been synthesized and tested for some of the pharmacological properties characteristic of oxytocin. This analog, which possesses a 21-membered disulfide ring in contrast to oxytocin, which contains a 20-membered ring, did not exhibit avian depressor or rat pressor activity. It possessed a very slight but definite oxytocic activity (0.75 unit per mg.). The mixed disulfide of L-cysteine and L-homocysteine has been synthesized, and its chromatographic behavior in the Beckman–Spinco amino acid analyzer has been shown to be the same as that of the mixed disulfide produced by acid hydrolysis of 1-(hemi-homocystine)-oxytocin.

The complete elucidation of the structures of oxytocin[3] and the vasopressins[4] demonstrated for the first time the occurrence of a twenty-membered disulfide ring in nature. Subsequently, a cyclic disulfide of the same size was found in insulin.[5] The question arises as to whether the size of the cyclic moiety is critical for the possession of biological activity. An indication that this might be true in the case of oxytocin was afforded by the pharmacological behavior of synthetic 4-isoglutamine oxytocin[6] which was found to be completely devoid of oxytocic and avian depressor activities. This substitution of isoglutamine for glutamine in oxytocin leads to an increase in the size of the disulfide ring from twenty members, as in oxytocin, to twenty-two members. However, concomitant with this, the carboxamide grouping, which in oxytocin is separated from the ring by two methylene units, is attached directly to the ring in the isomeric octapeptide. 5-Isoasparagine oxytocin,[7] an analog of oxytocin in which isoasparagine replaces asparagine, was also inactive. In this instance the cyclic moiety has

(1) This work was supported in part by a grant from the National Heart Institute, U. S. Public Health Service, Grant No. H-1675.

(2) N.A.T.O. Research Fellow.

(3) V. du Vigneaud, C. Ressler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954); H. Tuppy, *Biochim. et Biophys. Acta*, **11**, 449 (1953); H. Tuppy and H. Michl, *Monatsh.*, **84**, 1011 (1953).

(4) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, *J. Am. Chem. Soc.*, **75**, 4880 (1953); E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **206**, 353 (1954); V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 4751 (1954); R. Acher and J. Chauvet, *Biochim. et. Biophys. Acta*, **12**, 487 (1953).

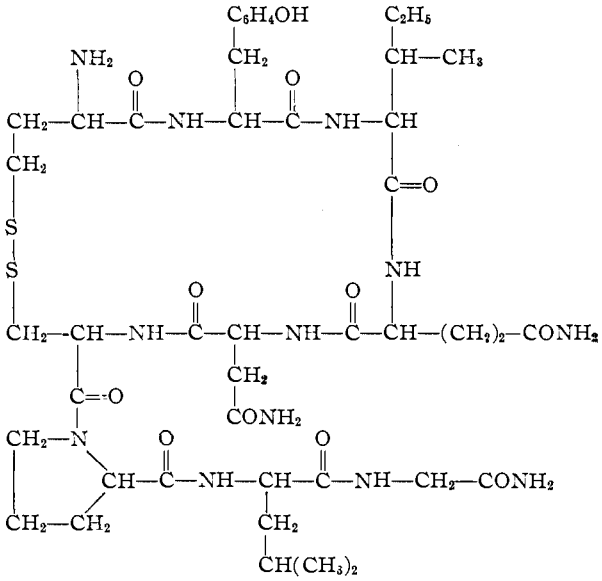
(5) F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **58**, vi (1954); A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *ibid.*, **60**, 541 (1955).

(6) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957).

(7) W. B. Lutz, C. Ressler, D. E. Nettleton, Jr., and V. du Vigneaud, *ibid.*, **81**, 167 (1959).

been enlarged by one methylene unit to give a twenty-one membered ring and the carboxamide grouping of the substituted residue is attached directly to the ring. That the proximity of the carboxamide grouping to the ring may be of significance to the biological activity of oxytocin is indicated by the fact that 5-glutamine-oxytocin[8] was practically without activity.[9] This analog, in which the asparagine residue is replaced by a glutamine residue, differs from oxytocin only in the increased separation, by one methylene unit, of the carboxamide grouping in the 5 position from the ring. An analog of oxytocin in which the disulfide ring was enlarged to twenty-three members has also been synthesized. In this case, the insertion of an additional tyrosine residue between the tyrosine and isoleucine residues of oxytocin resulted in the disappearance of the pharmacological properties characteristic of oxytocin.[10]

As a further contribution to the study of the effect of the size of the disulfide ring of oxytocin upon its biological properties, we have synthesized 1-(hemi-homocystine)-oxytocin (I), an analog of oxytocin which differs from oxytocin only in the replacement of the half-cystine residue in the 1-position by a half-homocystine residue. Such a substitution leads to a twenty-one membered disulfide ring, larger by one methylene unit than that in oxytocin. It should be pointed out, however, that this change makes the free amino group one methylene unit more remote from the disulfide bond.



1-(Hemi-homocystine)-oxytocin (I)

The important intermediate for the synthesis of 1-(hemi-homocystine)-oxytocin was the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II). Our first approach to the synthesis of the nonapeptide II was through the condensation of S-benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine (III) with S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide by the *o*-phenylene chlorophosphite method of Anderson and his co-workers as described for the protected nonapeptide corresponding to oxytocin.[11] However, the oxytocin analog prepared from the nonapeptide did not give a satisfactory analysis. It is of interest that the product obtained was devoid of avian depressor and rat pressor activities. The preparation of two intermediates used in this synthesis, S-benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosine and the pentapeptide III, for which satisfactory analytical data were obtained, is described in the Experimental part. The synthesis of 1-(hemi-homocystine)-oxytocin was subsequently accomplished by the application of the nitrophenyl ester method which was recently used for the synthesis of oxytocin.[12] A distinctive feature of this procedure is that amino acid residues are incorporated singly into the peptide chain from the amino terminal end by means of their protected nitrophenyl esters. Thus, for the synthesis of the protected nonapeptide II, the same octapeptide derivative, O-benzyl-N-carbobenzoxy-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV) was used as for the synthesis of oxytocin. O-Benzyl and N-carbobenzoxy protecting groups were removed from the octapeptide intermediate IV and the free base was allowed to react with S-benzyl-N-carbobenzoxy-L-homocysteine *p*-nitrophenyl ester to give the required nonapeptide intermediate II. All of the protecting groups were removed from the latter compound by means of sodium in liquid ammonia, as in the original synthesis of oxytocin. The dithiol so obtained was oxidized in neutral solution by titration with a solution of potassium ferricyanide.[13] The oxidation product was subjected to countercurrent distribution in the system butanol–ethanol–0.05% acetic acid (4:1:5). One major component was found and this was isolated by concentration and lyophilization. The compound has a partition coefficient (K) of approximately 0.41 in this system, whereas the partition coefficient of oxytocin in the same system is approximately 0.43.[11]

1-(Hemi-homocystine)-oxytocin was tested for avian depressor,[14] rat uterine-contracting[15] and rat pressor[16] activities; in each test the U.S.P. Standard posterior lobe powder served as reference. Within the limits of sensitivity of the

(8) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956).

(9) B. Berde, W. Doepfner and H. Konzett, *Brit. J. Pharmacol.*, **12**, 209 (1957).

(10) St. Guttmann, P.-A. Jaquenoud, R. A. Boissonnas, H. Konzett and B. Berde, *Naturwissenschaften*, **44**, 632 (1957).

(11) M. Bondanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).

(12) M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(13) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope and R. D. Kimbrough, Jr., *J. Biol. Chem.*, **235**, PC 64 (1960).

(14) The avian depressor assay was carried out by the method of Coon [*Arch. Intern. Pharmacodynamie*, **62**, 79 (1939)], as modified by Munsick, Sawyer and van Dyke [*Endocrinology*, **66**, 860 (1960)].

(15) The uterine-contracting activity was determined according to the method of Holton [*Brit. J. Pharmacol.*, **8**, 328 (1948)], as modified by Munsick [*Endocrinology*, **66**, 451 (1960)].

(16) "The Pharmacopeia of the United States of America," 16th Revision, Mack Printing Co., Easton, Pa., 1960, p. 546.

methods employed, the analog did not appear to possess any avian depressor or rat pressor activity. A sample of the reduced nonapeptide taken before oxidation also showed no avian depressor activity. 1-(Hemi-homocystine)-oxytocin did exhibit a slight oxytocic activity (0.75 unit per mg.), less than one five hundredth of that possessed by oxytocin. Thus, either the size of the ring or the relation of the free amino group to the disulfide bond or both of these factors are exceedingly important to the biological activity of oxytocin. Further work is in progress to evaluate the influence of each of these factors.

It may be recalled that isoglutamine oxytocin and the analog with a tyrosine residue inserted between the tyrosine and isoleucine residues of oxytocin showed some inhibitory activity. The former compound[17] inhibits the pressor action of vasopressin on the blood pressure of the rat, and the latter[10,18] inhibits the action of oxytocin on the isolated uterus of the rat and the rabbit and in the avian depressor assay. A preliminary investigation showed that 1-(hemi-homocystine)-oxytocin inhibits the action of oxytocin in the avian depressor assay but only to a very slight degree.

An amino acid analysis of an acid hydrolysate of the hormone analog was carried out according to the method of Spackman, Stein and Moore[19] in a Beckman–Spinco amino acid analyzer. Initially the usual 50° system was employed.[20] No new peak appeared on the chromatogram representing the mixed disulfide formed between cysteine and homocysteine, but the isoleucine peak was abnormally high, representing a ratio of 1.7 instead of 1.0. Hence, it was tentatively assumed that the peaks corresponding to isoleucine and the expected mixed disulfide had coincided. Homocystine and cystine were also present, in amounts accounting for approximately one-half of the disulfide-containing material. Both had presumably arisen from an acid-catalyzed disulfide interchange reaction which the mixed disulfide had undergone during hydrolysis. The other amino acids and ammonia appeared in the expected ratios. Cysteine and homocysteine were oxidized together in equal molar portions and the oxidation product, which was expected to consist of a mixture of cystine, homocystine and the mixed disulfide, was analyzed in the 50° system. Three peaks were observed, those of cystine and homocystine, and a third peak which appeared at a position identical with that of the abnormally high isoleucine peak obtained by analysis of an acid hydrolysate of the analog. This afforded strong evidence for the coincidence of the isoleucine and mixed disulfide peaks in the analysis of the acid hydrolysate. Known amounts of leucine and isoleucine were added to the oxidation mixture and samples of the total mixture were analyzed in the 30–50° system, whereupon partial resolution of the composite isoleucine-mixed disulfide peak occurred, and in the 30° system, where the mixed disulfide peak merged completely with that of leucine. Separation of isoleucine, the mixed disulfide and leucine into three distinct peaks was achieved in the 30–40° system. However, the precise location of the mixed disulfide peak between the isoleucine and leucine peaks is very sensitive to the ionic strength, as well as the *p*H, of the buffer.[21]

The mixed disulfide of L-cysteine and L-homocysteine[22] was prepared by oxidizing the two thiols together and isolated by the chromatographic procedure of Hirs, Moore and Stein[23] from the reaction mixture in analytically pure form. The synthetic material and that obtained by hydrolysis of the analog behaved identically upon chromatographic analysis in the amino acid analyzer. Schöberl aud Gräfje[24] have prepared this mixed disulfide in which the half-homocystine residue is racemic by treating the thiolsulfinate of DL-homocystine with L-cysteine.

Having ascertained the advantage of the 30–40° system, an acid hydrolysate of 1-(hemi-homocystine)-oxytocin was analyzed in this system and the expected amino acid composition was found.

Experimental[25]

S-Benzyl-N-carbobenzoxy-L-homocysteine.—This compound was prepared from S-benzyl-L-homocysteine according to the procedure used for the preparation of S-benzyl-N-carbobenzoxy-L-cysteine from S-benzyl-L-cysteine.[26] Two recrystallizations from benzene–petroleum ether (b.p. 30–75°) gave 67.5 g. (85%), m.p. 86.5–87.5°, $[\alpha]^{20}D$ −14.4° (*c* 1.11, ethanol); $[\alpha]^{20}D$ −1.5° (*c* 1.03, ethyl acetate); lit.[27] m.p. 85–88°; $[\alpha]^{25}D$ −11.7° (*c* 1.5, ethanol).

Anal. Calcd. for $C_{19}H_{21}O_4NS$: C, 63.5; H, 5.89; N, 3.90. Found: C, 63.7; H, 5.84; N, 3.93.

S-Benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosine.—S-Benzyl-N-carbobenzoxy-L-homocysteine (7.2 g.) was dissolved in dimethylformamide (20 ml.), and methyl L-tyrosinate (4 g.) was added, the solution was cooled to 0° and dicyclohexylcarbodiimide (4.2 g.) was added. The mixture was allowed to stand overnight at room temperature. Ethyl acetate (100 ml.) was added, and the N,N′-dicyclohexylurea was filtered off and washed with ethyl acetate. The filtrate was then washed successively with water, 1 *N* HCl, water, 1% $KHCO_3$ solution, and water again, and then dried with $MgSO_4$. The solvent was removed *in vacuo* and the residual syrup was diluted with ethyl acetate (25 ml.) and then with petroleum ether, b.p. 60–70° (200 ml.). The product separated as an oil which soon crystallized, m.p. 130–131°. This crude ester (8 g.) was dissolved in methanol (60 ml.) and 1 *N* NaOH (40 ml.) was added. During the addition the mixture was stirred and cooled with water. After 1.5 hr. the solution was filtered to remove small amounts of impurities. The filter was rinsed with 60%

(17) C. Ressler and J. R. Rachele, *Proc. Soc. Exptl. Biol. Med.*, **98**, 170 (1958).

(18) H. Konzett, *Helv. Physiol. Acta*, **15**, 419 (1957).

(19) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(20) Subsequently it became necessary to modify the conditions of chromatography in order to resolve isoleucine, leucine and the mixed disulfide of cysteine and homocysteine. Temperature was the variable and in each case the temperature change and the buffer change were introduced at the same effluent volume. The change in the influent buffer was made at 315 ml. and observed at 405 ml.

(21) Attention has previously been called to the sensitivity of cystine to the *p*H of the buffer during column chromatography by Moore and Stein [*J. Biol. Chem.*, **192**, 663 (1951)].

(22) This mixed disulfide has also been prepared by Frimpter (*J. Biol. Chem.*, **236**, PC 51 (1961)) in connection with his identification of this disulfide in cystinuric urine.

(23) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

(24) A. Schöberl and H. Gräfje, *Ann.*, **617**, 71 (1958).

(25) Capillary melting points were determined for all compounds and are corrected. Optical rotations were measured in 2 dm. tubes, the estimated error being ±0.01°.

(26) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).

(27) E. C. Herrick and C. W. Todd, *Chem. Abs.*, **50**, 4214g (1956).

ethanol (25 ml.), and the filtrate was then acidified with 5 *N* HCl (8.5 ml.) before the addition of 50 ml. of water. An oil separated which readily crystallized. After being washed with water and dried, the product weighed 7.5 g. (72%), m.p. 138–140°, $[\alpha]^{23}D$ +16° (*c* 4, pyridine).

Anal. Calcd. for $C_{28}H_{30}O_6N_2S$: C, 64.3; H, 5.79; N, 5.36. Found: C, 64.1; H, 5.93; N, 5.41.

S-Benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine (III).—The same procedure was used as for the preparation of the analogous protected pentapeptide containing a cysteinyl residue.[11] The protected dipeptide (2.1 g.) and L-isoleucyl-L-glutaminyl-L-asparagine (1.9 g.) were coupled by the isobutyl-chlorocarbonate method to give 3.1 g. of protected pentapeptide, m.p. 230–233° (sinters at 215°), $[\alpha]^{25}D$ −18° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{48}H_{55}O_{11}N_7S$: C, 58.8; H, 6.31; N, 11.2. Found: C, 58.9; H, 6.32; N, 11.0.

S-Benzyl-N-carbobenzoxy-L-homocysteine *p*-Nitrophenyl Ester.—S-Benzyl-N-carbobenzoxy-L-homocysteine (10.8 g.) and *p*-nitrophenol (5.5 g.) were dissolved in tetrahydrofuran (80 ml.), and dicyclohexylcarbodiimide (6.2 g.) was added to the solution at 0°. The reaction mixture was allowed to stand for 3 hr. at 0° and overnight at room temperature. The N,N′-dicyclohexylurea was filtered off and washed with tetrahydrofuran. The filtrate was evaporated *in vacuo* to a viscous oil which solidified on being triturated with hexane. Recrystallization from ethanol (60 ml.) gave 10.3 g., m.p. 72.5–74.5°. A sample recrystallized from the same solvent for analysis melted at 74.5–75.5°, $[\alpha]^{20.5}D$ −35.8° (*c* 1.23, methanol).

Anal. Calcd. for $C_{25}H_{24}O_6N_2S$: C, 62.5; H, 5.00; N, 5.83. Found: C, 62.5; H, 5.12; N, 5.78.

S-Benzyl-N-carbobenzoxy-L-homocysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II).—Hydrogen bromide (4 *N*) in glacial acetic acid (25 ml.) was added to a solution of O-benzyl-N-carbobenzoxy-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[12] (3.5 g.) in glacial acetic acid (35 ml.) at room temperature. After 2 hr., ether (400 ml.) was added to the solution. When the precipitated hydrobromide had settled, the supernatant liquor was decanted and the precipitate was washed with ether (3 × 100 ml.), each washing being followed by decantation. After being dried *in vacuo* over potassium hydroxide pellets for 30 minutes, the hydrobromide was dissolved in dimethylformamide (45 ml.), the solution was cooled to room temperature and triethylamine (3.5 ml.) was added, followed by S-benzyl-N-carbobenzoxy-L-homocysteine *p*-nitrophenyl ester (1.7 g.). The reaction mixture was stirred for about 40 hr. The mixture was evaporated to dryness in a rotary evaporator at a temperature below 20° and the residue was shaken well with ethyl acetate (250 ml.). The precipitate was filtered off, washed with ethyl acetate (100 ml.), ethanol (150 ml.) and ethyl acetate (100 ml.) and dried *in vacuo* over calcium chloride. This material, m.p. 223–226°, was dissolved in dimethylformamide (50 ml.), the solution was filtered and the filter was washed with approximately 5 ml. of the same solvent. The filtrate was swirled gently as it was diluted with 0.2 *N* acetic acid (400 ml.) and the suspension obtained was allowed to stand overnight at 5°. The precipitate was filtered off, washed with 0.1 *N* acetic acid (200 ml.) and water (200 ml.) and dried *in vacuo* over $CaCl_2$ at room temperature; yield, 2.9 g., m.p. 231–233° (dec.), softening at about 229°. The material was reprecipitated a second time in the same way. The melting point was unchanged; $[\alpha]^{20}D$ −47.9° (*c* 1.33, dimethylformamide).

For analysis, a sample (115 mg.) was suspended in pyridine (3 ml.), and the suspension was heated in a water-bath. Water was added dropwise to give a clear solution and the addition continued until a precipitate was about to form. The hot solution was filtered and the filtrate was allowed to cool to room temperature. The precipitate obtained was filtered off, washed with water (25 ml.) and dried *in vacuo* over P_2O_5 at 100° for 40 hr.; yield, 105 mg., m.p. 232–234° (dec.), softening about 231°. A second precipitation by the same means left the melting point unchanged; $[\alpha]^{20}D$ −48.5° (*c* 1.02, dimethylformamide). The material was dried for 48 hr. to constant weight and analyzed.

Anal. Calcd. for $C_{66}H_{88}O_{14}N_{12}S_2$: C, 59.3; H, 6.63; N, 12.6. Found: C, 58.7; H, 6.71; N, 12.3.

1-(Hemi-homocystine)-oxytocin (I).—The procedure for the reduction of two 250-mg. batches of compound II was essentially the same as that described for the synthesis of oxytocin. The reduced material was aerated in aqueous solution at a concentration of 1 mg. per ml. at *p*H 6.8 for approximately 2.5 hr. Oxidation was completed by means of a solution of recrystallized potassium ferricyanide (0.011 *N*), which was added dropwise from a burette to the swirled solution of peptide. The oxidant acted as its own indicator, the solution becoming pale green when oxidation was complete. Ferrocyanide and ferricyanide ions were removed by pouring the oxidized solutions onto a column of AG 3-X4, 100–200 mesh in the chloride form. The *p*H of the effluents was adjusted to 7 with ammonia, and the solutions were shown to be free from ferrocyanide. The colorless solutions also gave a negative reaction to nitroprusside and a positive reaction to cyanide-nitroprusside. After being allowed to stand overnight at 5°, the solutions were combined and concentrated to about 50 ml. in a rotary evaporator at a temperature below 20°.

The concentrate was introduced into the first six tubes of a 200-tube countercurrent distribution apparatus and distributed in the system butanol–ethanol–0.05% acetic acid (4:1:5) for 550 transfers. Determinations of the Folin–Lowry[28] color values after 190 and 388 transfers both indicated a major peak with a distribution coefficient of approximately 0.4 accompanied by a more slowly moving minor peak having a distribution coefficient of approximately 0.2. Throughout the distribution emulsions were encountered which necessitated a settling time of 20 minutes. At the end of the distribution and at the two interim determinations of the Folin–Lowry color values, the emulsions were located in those tubes which contained the major peak. The distribution pattern remained the same after 550 transfers, the main peak appearing in tube numbers 141–179 with a maximum in tube number 160. The good agreement between the distribution curve obtained by plotting the Folin–Lowry color measurements and the theoretical curve calculated for the *K* value 0.41 was taken to indicate a high degree of purity of the material. The total contents of tube numbers 141–179 were pooled and concentrated to about 120 ml. in a rotary evaporator at a temperature below 20°. The concentrate was lyophilized to give 110 mg. of a fluffy white powder. For elemental analysis a sample of the material was dried *in vacuo* over P_2O_5 at 100° for 7.25 hr. and a loss in weight of 6% was observed.

Anal. Calcd. for $C_{44}H_{63}O_{12}N_{12}S_2$: C, 51.8; H, 6.72; N, 16.5. Calcd. for $C_{44}H_{63}O_{12}N_{12}S_2 \cdot C_2H_4O_2$: C, 51.1; H, 6.71; N, 15.5. Found: C, 50.6; H, 6.88; N, 15.7 (corrected for 0.47% ash). $[\alpha]^{24}D$ −5.9° (*c* 0.51, 1 *N* acetic acid).

A sample was hydrolyzed in 6 *N* HCl *in vacuo* at 110° for 17 hr. and analyzed in the 30–40° system of the Beckman–Spinco amino acid analyzer. The following molar ratios were obtained, isoleucine being taken as 1.0; aspartic acid (1.1), glutamic acid (1.0), proline (1.1), glycine (0.9), leucine (1.0), tyrosine (0.9), cystine (0.2), homocystine (0.2), mixed disulfide of cysteine and homocysteine (0.6) and ammonia (3.1).

Mixed Disulfide [1(L),7(L)-Diamino-4,5-dithiaheptane-1,7-dicarboxylic Acid].—L-Cystine (2.4 g.) and S-benzyl-L-homocysteine (4.5 .g) were reduced together by means of sodium in liquid ammonia (200 ml.). Glacial acetic acid (0.1 ml.) was added to the reduction mixture, ammonia was removed by evaporation and lyophilization, and the residue was dissolved in water (60 ml.). The *p*H of the aqueous solution was adjusted to 8 with dilute ammonia and the solution was extracted with ether (25 ml.) and oxidized by aeration, a small amount of ferric chloride being added as catalyst. The *p*H of the suspension was adjusted to 6 with acetic acid and the precipitate filtered off and extracted with boiling water (2 × 200 ml.). The aqueous extracts were combined and allowed to cool to 5°. The precipitate which formed was filtered off and dried *in vacuo* over P_2O_5.

The required material was isolated from the precipitate by chromatography on a column of Dowex 50W-X2, 200–400 mesh. The separation was carried out at about 28° and the procedure followed was based upon, and similar to, that de-

(28) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. *J. Biol. Chem.*, **193**, 265 (1951).

scribed by Hirs, Moore and Stein.[23] A portion (0.7 g.) of the precipitate was suspended in 0.2 *M* ammonium formate buffer (*p*H 3.11), and concentrated hydrochloric acid was added until the precipitate dissolved. The solution was poured on to a column of resin (5 × 33 cm.) which had been prepared and allowed to equilibrate with 0.2 *M* ammonium formate buffer (*p*H 3.11). The sample was allowed to sink into the resin under atmospheric pressure and rinsed in with 20 ml. of buffer. The buffer reservoir was reconnected to the column which was mounted over a fraction collector. The rate of flow of buffer through the column was adjusted to 45–50 ml. per hour and the effluent was collected in 23 ml. fractions.

Alternate fractions were analyzed qualitatively for disulfide-containing material by means of the cyanide–nitroprusside test.[29] After the elution of the first peak in tube numbers 69–77, the buffer was changed to 0.2 *M* ammonium formate (*p*H 3.28). Tube numbers 150–210 comprised a second peak. At approximately tube 260 the buffer was changed to 0.2 *M* ammonium formate (*p*H 3.40) and at approximately tube 310, to 0.2 *M* ammonium formate (*p*H 4.10). A third peak was contained in tube numbers 350–370. The contents of tube numbers 150–210 were pooled, evaporated in a rotary evaporator to approximately 50 ml., diluted with about 100 ml. of water and evaporated again, to about 20 ml., to remove excess free acid originally present. The *p*H of the suspension was adjusted to 6 by the addition of 2 *N* ammonium hydroxide and the suspension was evaporated to dryness in a rotary evaporator at a temperature below 30°. The white residue was resuspended in water (100 ml.) and the suspension was filtered. The precipitate was washed with water and recrystallized from boiling water (200 ml.) to give 190 mg. of prismatic needles which began to decompose when heated above 250°. Analysis in the amino acid analyzer showed the compound to be chromatographically pure; $[\alpha]^{22}D$ −63.2° (*c* 0.89, 1 *N* HCl).

Anal. Calcd. for $C_7H_{14}O_4N_2S_2$: C, 33.1; H, 5.55; N, 11.0; S, 25.2. Found: C, 33.3; H, 5.55; N, 11.1; S, 25.2.

(29) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

NOTE ADDED IN PROOF.—Dr. David Yphantis of The Rockefeller Institute, using short column equilibrium centrifugation, has found that 1-(hemi-homocystine)-oxytocin behaves as a monomer at pH 5.6 in 0.15 *M* ammonium acetate. We wish to thank him for this information.

Acknowledgments.—The authors wish to thank Mr. Joseph Albert for the microanalyses reported herein, Mrs. Lorraine S. Abrash for the amino acid analyses and Miss Lenore McAteer and Miss Shirley R. Pomeroy for the biological assays.

[CONTRIBUTION NO. 173 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER, COLORADO]

Effect of Binding of Ions and Other Small Molecules on Protein Structure. IX. The Binding of Acetic Acid to Insulin at *p*H 4[1]

BY JOHN R. CANN

RECEIVED MAY 29, 1961

The electrophoretic behavior of insulin in acetate buffers at *p*H 4.0 is interpretable over a seven-fold range of acetic acid concentration in terms of the binding of a single mole of undissociated acid to the protein. The effect of acid binding on the electrophoretic behavior of proteins is discussed in the context of conformational changes with concomitant changes in the *pK*'s of several carboxyl groups on the proteins.

Introduction

The various peaks in the electrophoretic patterns of a variety of proteins in acidic media containing acetate buffer (NaAc–HAc) or other carboxylic acid buffers constitute a single reaction boundary modified in some instances by mild convective disturbances.[2–5] In the case of serum albumin the reaction boundaries arise as a result of interaction of the protein with undissociated buffer acid and, in some but not all rising patterns, possess a fine structure due to the superimposed isomerization reaction. (At a sufficiently high concentration of acetate buffer, the pattern is a reaction boundary attributable entirely to the isomerization reaction.) The reaction boundaries of ovalbumin and bovine γ-pseudoglobulin arise solely from interaction of these proteins with undissociated buffer acid. Equilibrium constants for the binding of undissociated buffer acids by bovine serum albumin (BSA) and ovalbumin have been computed[2] from electrophoretic patterns using the concepts of the theory of weak-electrolyte moving boundaries. These investigations have now been extended to insulin whose electrophoretic behavior at *p*H 4 is interpretable over a sevenfold range of acetic acid concentration in terms of the binding of a single mole of undissociated acid to the protein.

Experimental

Electrophoresis was carried out at field strengths of 3 to 8 volts $cm.^{-1}$ in the standard 11 cc. Tiselius cell with the Spinco model H electrophoresis-diffusion instrument. Schlieren patterns were recorded photographically with the cylindrical lens optical system. Following electrophoretic separation of the peaks in a reaction boundary, portions of solution were removed from various positions in the rising limb of the Tiselius cell for conductance measurements. The crystalline zinc insulin was kindly supplied by the Eli Lilly Company. Samples of the protein were dissolved in $10^{-3}M$ HCl and the resulting solutions dialyzed for 3 hr. against $10^{-2}M$ HCl followed by 24 hr. against buffer with three changes of dialysate. Within experimental error, the *p*H and resistance of the dialyzed protein solution were the same as those of the buffer.

Results

Representative electrophoretic patterns of insulin in a series of *p*H 4.0 media containing varying concentrations of NaAc–HAc, the ionic strength being maintained constant at 0.02 *M* with NaCl, are presented in Fig. 1. It will be noted that the

(1) Supported in part by research grant No. E-1482 (C9) from the National Institute of Allergy and Infectious Diseaes of the National Institutes of Health, United States Public Health Service, and in part by the Damon Runyon Fund and the American Cancer Society. The author wishes to thank Mr. Stanley Fisher for his technical assistance.

(2) J. R. Cann, *J. Biol. Chem.*, **235**, 2810 (1960).

(3) J. R. Cann and R. A. Phelps, *J. Am. Chem. Soc.*, **79**, 4672 (1957).

(4) J. R. Cann, *J. Phys. Chem.*, **63**, 210 (1959).

(5) J. R. Cann and R. A. Phelps, *J. Am. Chem. Soc.*, **81**, 4378 (1959).