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

An Improved Synthesis of Oxytocin[1]

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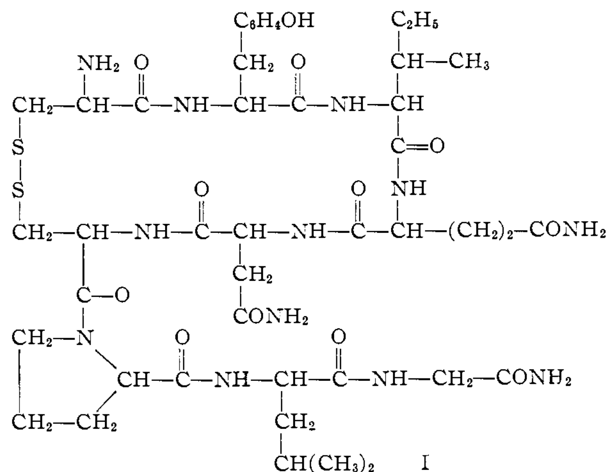
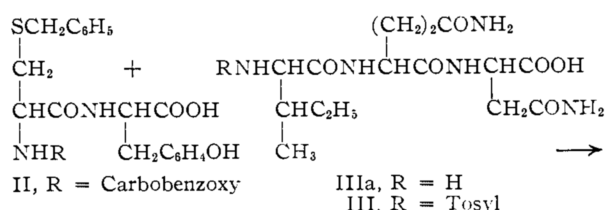
A new approach to synthetic oxytocin is described. By coupling S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine with L-isoleucyl-L-glutaminyl-L-asparagine and by the subsequent condensation of the resulting protected pentapeptide with S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, was prepared. After removal of the protective groups from the nonapeptide and oxidation to the disulfide, the biologically active material was isolated.

Oxytocin (I), the principal uterine-contracting hormone of the posterior pituitary gland, was isolated in highly purified form in this Laboratory,[2] and its structure was postulated[3-5] and proved by synthesis.[4] Since then, several other laboratories[6-9] have succeeded in the synthesis of similar nonpeptides differing from the one used here only in the protective groupings and in the intermediates used to make the nonapeptides. After removal of the protecting groups, the last step involving conversion by oxidation to the octapeptide, oxytocin, was carried out according to the method developed in this Laboratory.

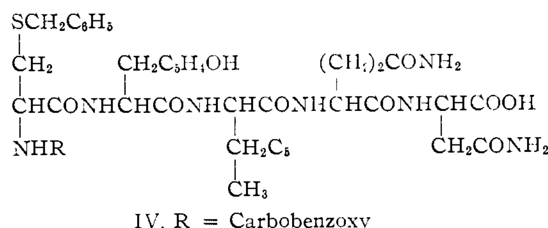
In the synthesis used earlier in this Laboratory,[4] the protected dipeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine (II)[10-13] was coupled with the heptapeptide amide L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-propyl-L-leucylglycinamide, which was, in turn, obtained from its tosyl derivative by removal of the protecting tosyl group and rebenzylation. This tosyl heptapeptide was prepared by coupling tosyl-L-isoleucyl-L-glutaminyl-L-asparagine (III)[14] with the tetrapeptide amide, S-benzyl-L-cysteinyl-L-propyl-L-leucylglycinamide (VIII).[4,15]

The synthesis reported in this paper uses the same dipeptide II, tripeptide III and tetrapeptide VIII derivatives, but in a method similar to that

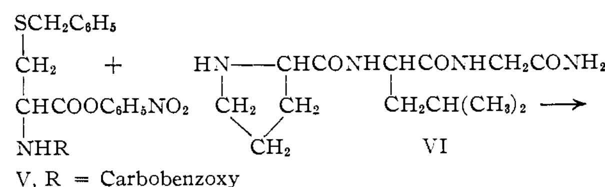




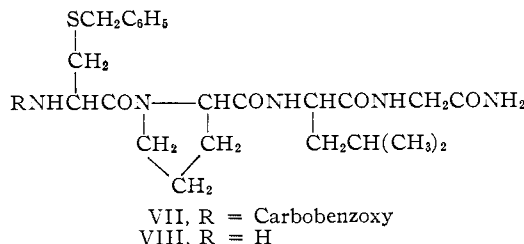
II, R = Carbobenzoxy IIIa, R = H; III, R = Tosyl



IV, R = Carbobenzoxy



V, R = Carbobenzoxy



VII, R = Carbobenzoxy
VIII, R = H

(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) A. H. Livermore and V. du Vigneaud, *J. Biol. Chem.*, **180**, 365 (1949); J. G. Pierce and V. du Vigneaud, *ibid.*, **186**, 77 (1950).

(3) V. du Vigneaud, C. Ressler and S. Trippett, *ibid.*, **205**, 949 (1953).

(4) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(5) H. Tuppy, *Biochim. Biophys. Acta*, **11**, 449 (1953); H. Tuppy and H. Michl, *Monatsh. Chem.*, **84**, 1011 (1953).

(6) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud and J. P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

(7) J. Rudinger, J. Honzl and M. Zaoral, *Coll. Czech. Chem., Communs.*, **21**, 202 (1956).

(8) M. Bodanszky, M. Szelke, E. Tomorkeny and E. Weisz, unpublished work, *cf.* L. Gyermek and Gy. Fekete, *Experientia*, **11**, 238 (1955).

(9) L. Velluz, G. Amiard, J. Bartos, B. Goffinet and R. Heymes, *Bull. soc. chim. France*, 1464 (1956).

(10) C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 417 (1944).

(11) C. W. Roberts and V. du Vigneaud, *J. Biol. Chem.*, **204**, 871 (1953).

(12) M. Bodanszky, *Nature*, **175**, 685 (1955); *Acta Chim. Hung.*, **10**, 335 (1957).

(13) B. Iselin, M. Feurer and R. Schwyzer, *Helv. Chim. Acta*, **38**, 1508 (1955).

(14) P. G. Katsoyannis and V. du Vigneaud, THIS JOURNAL, **76**, 3113 (1954).

(15) C. Ressler and V. du Vigneaud, *ibid.*, **76**, 3107 (1954).

IV + VIII ⟶

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SCH2C6H5         CH3            CONH2  SCH2C6H5
|                |              |      |
CH2              CHC2H5         CH2    CH2
|                |               \      \
CHCONHCHCONHCHCONHCHCONHCHCONHCHCON——CHCONHCHCONHCH2CONH2
|      |             |                 |     |      |
NHR    CH2C6H4OH     (CH2)2CONH2       CH2   CH2    CH2CH(CH3)2
                                          \ /
                                          CH2
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IX, R = Carbobenzoxy

1, Na, NH_3 ↓ 2, Air oxidation

I

used for synthesizing lysine and arginine vasopressins.[16,17] After removal of the tosyl group from III with sodium in liquid ammonia, L-isoleucyl-L-glutaminyl-L-asparagine (IIIa) was isolated as a crystalline monohydrate. Coupling of II and IIIa by the mixed anhydride procedure[18] with isobutylchloroformate gave the crystalline protected pentapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine (IV).

The preparation of VIII was improved by using the reaction between *p*-nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate (V)[19,20] and L-prolyl-L-leucylglycinamide (VI)[6,7,15] in dimethylformamide. The carbobenzoxy group was removed by treatment with HBr in acetic acid[21–23] and the HBr was eliminated from the resulting salt with the aid of the ion-exchange resin Amberlite IRA-400 in OH cycle. Yields in these steps were very satisfactory.

By combining IV with VIII by the mixed anhydride procedure,[18] the *o*-phenylenechlorophosphite method[24] or the dicyclohexylcarbodiimide method,[25] the protected nonapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IX)[4,6] was obtained.

Analysis of IX gave values which were close to the calculated ones and removal of the protecting groups gave a material with considerable oxytocic activity. From different batches of IX, 100-200 units of avian depressor activity could be obtained (from 1 mg. of the nonapeptide) and from purified samples as much as 260 units. Nevertheless, it was felt that in the case of such a non-crystalline high molecular weight substance, the synthesis cannot be regarded as complete at this stage because neither the satisfactory analytical values nor the biological activities can exclude the presence of impurities, especially diastereoisomers. On reduction, such impurities may lead to compounds with pharmacological properties similar to those of oxytocin, or to biologically inert or even to inhibitory substances. Therefore, the synthesis was completed not only by transformation of the protected nonpeptide into oxytocin but also by isolation of the oxytocin in highly purified form.

Dilute solutions of the reduction product of IX were aerated to form the disulfide compound.[26] After some preliminary purification, a solid with about 300 units of activity per mg. was obtained. Countercurrent distribution led to purified oxytocin showing avian depressor activity of approximately 500 units/mg.

(16) M. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward and V. du Vigneaud, THIS JOURNAL, **78**, 2905 (1956).

(17) P. G. Katsoyannis, D. T. Gish and V. du Vigneaud, *ibid.*, **79**, 4516 (1957).

(18) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **75**, 5556 (1953).

(19) M. Bodanszky, M. Szelke, E. Tomorkeny and E. Weisz, *Chem. and Ind.*, 1517 (1955); *Acta Chim. Hung.*, **11**, 179 (1957).

(20) B. Iselin, W. Rittel, P. Sieber and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

(21) D. Ben-Isbai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(22) G. W. Anderson, J. Blodinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952).

(23) R. A. Boissonnas and G. Preitner, *Helv. Chim. Acta*, **35**, 2240 (1952).

(24) G. W. Anderson, J. Blodinger, R. W. Young and A. D. Welcher, THIS JOURNAL, **74**, 5304 (1952); G. W. Anderson and R. W. Young, *ibid.*, **74**, 5307 (1952).

(25) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

Experimental[27,28]

p-Nitrophenyl S-Benzyl-N-carbobenzoxy-L-cysteinate (V).—Seven g. of S-benzyl-N-carbobenzoxy-L-cysteine[29] was dissolved in 22 ml. of tetrahydrofuran (freshly distilled from sodium); 4 g. of *p*-nitrophenol was added and then, while the mixture was cooled with ice-water, 4.2 g. of dicyclohexylcarbodiimide.[30] The solution was allowed to stand at room temperature for about 0.5 hr., after which time the precipitated urea derivative was filtered off and washed with 22 ml. of tetrahydrofuran. After removal of the solvent from the mother liquor and washings, the residue was triturated with 50 ml. of 0.2 *N* $KHCO_3$ solution, filtered and washed on the filter with 50 ml. of the same solution, 400 ml. of water and 100 ml. of 90% ethanol. Drying at room temperature over P_2O_5 *in vacuo* gave 8.2 g. (88%) of ester, m.p. 85–88°.

A sample recrystallized twice from absolute ethanol melted at 93–94°; $[\alpha]^{22}D$ −42° (*c* 1, dimethylformamide); $[\alpha]^{23}D$ −37° (*c* 1, methanol, dissolved with slight warming).

Anal. Calcd. for $C_{24}H_{22}O_6N_2S$: C, 61.79; H, 4.76; N, 6.01. Found: C, 61.96; H, 4.91; N, 5.91.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine (II).[10–13] **A.**—One hundred and forty g. of S-benzyl-N-carbobenzoxy-L-cysteine[29] and 80 g. of methyl L-tyrosinate[31] were dissolved in 200 ml. of dimethylformamide, and 94 g. of dicyclohexylcarbodiimide[25] was added at 0° in four portions. The following day, the reaction mixture was diluted with 650 ml. of ethyl acetate and filtered, and the urea derivative was washed with 500 ml. of ethyl acetate. The mother liquor and washings were pooled, extracted with 800 ml. of *N* HCl, 800 ml. of water, 3 × 300 ml. of 0.25% $KHCO_3$, 4 × 200 ml. of water, dried over $MgSO_4$ and evaporated to about 300 ml. After crystallization had been completed by the addition of 1250 ml. of hexane, the resulting methyl ester was filtered, washed with 500 ml. of hexane and air-dried. 214.5 g. (slightly above the theoretical yield) was obtained, m.p. 92–94°. A 20% aliquot of this ester was saponified by dissolving it in 160 ml. of methanol and adding 200 ml. of *N* NaOH with cooling. After 1 hr. at room temperature, the solution was filtered in the presence of charcoal, and, 40 minutes later, acidified with *N* HCl. The crystalline precipitate, which was washed with water and air-dried, weighed 75 g. The material was recrystallized from a hot mixture of 600 ml. of methanol and 300 ml. of

(26) When more concentrated solutions were subjected to oxidation, considerable amounts of solids were obtained which are almost insoluble in water and which are probably polymers of the disulfide I.

(27) The authors wish to thank Mr. Joseph Albert for carrying out the microanalyses reported herein.

(28) Capillary melting points were determined for all compounds and are corrected.

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

(30) D. F. Elliott and D. W. Russell, *ibid.*, **66**, 49P (1957).

(31) E. Fischer, *Ber.*, **41**, 855 (1908); *cf.* also (6).

water. The product was filtered, washed with 250 ml. of 80% and 100 ml. of 90% ethanol, dried first in air and finally over P_2O_5 *in vacuo*, with a yield of 32 g. (78% calculated on the basis of the protected cysteine); m.p. 200–202°, $[\alpha]^{23}D$ −14.8° (*c* 4.3, pyridine).

Anal. Calcd. for $C_{27}H_{28}O_6N_2S$: N, 5.51; neut. equiv., 508.6. Found: N, 5.54; neut. equiv., 502.

B.—11.7 g. of *p*-nitrophenyl S-benzyl-N-carbobenzoxy-L-cysteinate (V) and 5.5 g. of methyl tyrosinate were dissolved in 12.5 ml. of dimethylformamide. Three days later the mixture was diluted with ethyl acetate and treated as described in Section A. After saponification, etc., 9.9 g. (78%) of recrystallized protected dipeptide, m.p. 200–201°, $[\alpha]^{22}D$ −14.8° (*c* 4.56, pyridine), was obtained.

C.—30.7 g. of cyanomethyl S-benzyl-N-carbobenzoxy-L-cysteinate[13] and 17.5 g. of methyl tyrosinate were dissolved in 40 ml. of dimethylformamide, and 3 drops of acetic acid was added. After 5 days the reaction mixture was worked up in the manner already described. Seventy-eight per cent. of the theoretical yield and material, m.p. 201°, $[\alpha]^{22}D$ −14.4° (*c* 4.42, pyridine), was obtained.

L-Isoleucyl-L-glutaminyl-L-asparagine (IIIa).—Thirty-eight g. of the protected tripeptide, tosyl-L-isoleucyl-L-glutaminyl-L-asparagine,[32] was dissolved in about 3000 ml. of liquid ammonia and treated with sodium until a lasting blue color was obtained. Eleven g. of sodium was used. Addition of 30 ml. of acetic acid caused the precipitate to dissolve. The ammonia was allowed to evaporate, and the last traces were removed *in vacuo*. The contents of the flask were dissolved in 330 ml. of water and 160 ml. of acetic acid was added; the mixture was then filtered with charcoal and diluted with 3500 ml. of ethanol. The crystals which separated were filtered 2 days later, washed with 500 ml. of ethanol and dried *in vacuo* over P_2O_5 at room temperature. An additional 3 liters of ethanol was added to the filtrate, and a few days later a second crop of crystals was obtained, giving a total yield of 23 g. (81.5%). The substance has a poorly defined m.p. It sinters above 210° and decomposes between 220 and 235°.

Recrystallization was effected by dissolving the tripeptide (0.9 g.) in 5 ml. of water and adding 5 ml. of ethanol. 0.8 g. of purified tripeptide was obtained, which sintered at 225° and decomposed between 230 and 235°. The compound, dried over P_2O_5 at room temperature, contains one molecule of water of crystallization and has a rotation of $[\alpha]^{24}D$ −32.5° (*c* 1, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{15}H_{27}O_6N_5 \cdot H_2O$: C, 46.03; H, 7.47; N, 17.89; H_2O, 4.60. Found: C, 45.86; H, 7.39; N, 17.78; H_2O, 4.78.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine (IV).—16.8 g. of II[10–13] was suspended in 330 ml. of tetrahydrofuran (freshly distilled from sodium), and a clear solution was obtained on the addition of 5.5 ml. of triethylamine. After the addition of 4.5 g. of isobutylchloroformate at −40°, the mixture was allowed to warm to −10° with constant stirring. It was cooled again to −40°, a solution of 14.2 g. of Va and 5.5 ml. of triethylamine in 66 ml. water was added to the well-stirred mixture, and it was allowed to come to room temperature. After about 4.5 hr., 150 ml. of water and 75 ml. of *N* HCl were added, and the precipitate was filtered and washed with water. The yield was 25 g. (88%) of crude protected pentapeptide, which melted at 225–228° with decomposition. The product was boiled with 100 ml. of ethyl acetate, filtered off and washed with 400 ml. of ethyl acetate in four portions. 21.2 g. (74%), m.p. 236–238° with decomposition, was obtained. This pentapeptide derivative was dissolved in a mixture of tetrahydrofuran and water to give small needles on slow evaporation of part of the solvent. For analysis, 0.7 g. was washed with 100 ml. of boiling methanol, yielding 0.5 g., m.p. 245–247° with decomposition; $[\alpha]^{24}D$ −23.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{42}H_{53}O_{11}N_7S$: C, 58.39; H, 6.16; N, 11.35. Found: C, 58.47; H, 6.20; N, 11.17.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-leucylglycinamide (VII).—Ninety-four g. of *p*-nitrophenyl carbobenzoxy-S-benzyl-L-cysteinate and 58.6 g. of L-prolyl-L-leucylglycinamide[6,7,15] were dissolved in 100 ml. of dimethylformamide with some evolution of heat. With occasional stirring, a homogeneous solution was obtained in about 2.5 hr. By the next day, the reaction mixture had turned into a semi-solid mass of crystals. Two days later, 160 ml. of ethyl acetate was added and the mixture was well stirred to give a homogeneous suspension. After being allowed to stand in the refrigerator overnight, the crystals were filtered, washed with 500 ml. of ethyl acetate and dried *in vacuo* at room temperature. 100.5 g. (82%), m.p. 170–171.5° and $[\alpha]^{21}D$ −60.0° (*c* 2.0, dimethylformamide), was obtained.

Anal. Calcd. for $C_{31}H_{41}O_6N_5S$: C, 60.85; H, 6.75; N, 11.45. Found: C, 60.69; H, 6.86; N, 11.53.

Forty-five g. of this material was dissolved in 500 ml. of hot methanol and filtered with charcoal, and 300 ml. of water was added to the warm filtrate. The crystals obtained were separated the following day. 41.2 g. was recovered, m.p. 170–171.5°.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-propyl-L-leucylglycinamide (IX). A.—1.73 g. of protected pentapeptide IV and 1.1 g. of tetrapeptide amide VIII were dissolved in 10 ml. of dimethylformamide, and 0.83 g. of dicyclohexylcarbodiimide (100% excess) was added with stirring and cooling. The mixture was allowed to come to room temperature and to stand at this temperature for a few hours; the reaction mixture solidified. After storage overnight in the refrigerator, 1 ml. of acetic acid was added, followed by 90 ml. of water, which was added with cooling. The precipitate was filtered, washed with liberal amounts of water and dried at room temperature over P_2O_5 *in vacuo*, yielding 3.23 g. This substance was extracted with several portions of methanol (total 120 ml.) and gave 1.85 g. (70%) of white amorphous material, m.p. 230–238° dec.

After reduction with sodium in liquid ammonia, 1 mg. of this substance showed 120 units of avian depressor activity. When only the calculated amount of dicyclohexylcarbodiimide was used, a substance was obtained which after reduction contained only traces of biological activity (1 u./mg.). In another experiment, application of a 50% excess of the reagent resulted in material having 35 avian depressor u./mg. A 200% excess of the reagent increased the yield to 72%, 1 mg. of the product giving 140 units.

One gram of this material was dissolved in 15 ml. of dimethylformamide and filtered, and the filter was rinsed with 10 ml. of the same solvent. Two drops of acetic acid was added to the filtrate, followed by 200 ml. of distilled water with cooling and stirring. The precipitate was washed on the filter with water and dried over $CaCl_2$ at room temperature *in vacuo*. The dry solid, which was extracted in several portions with 100 ml. of hot methanol and then dried over P_2O_5 at room temperature *in vacuo*, weighed 0.50 g. and had m.p. 243–245° (decomposed at 247°) and $[\alpha]^{22}D$ −43° (*c* 2, dimethylformamide).

On reduction with sodium in liquid ammonia, 1 mg. of this purified protected nonapeptide gave 260 units of avian depressor activity.

Anal. Calcd. for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 58.98; H, 6.55; N, 12.70. Found: C, 58.6; H, 6.59; N, 12.55.

B.—17.26 g. of protected pentapeptide was dissolved in 200 ml. of dimethylformamide, 3 ml. of triethylamine and 150 ml. of tetrahydrofuran were added, the mixture was cooled to about −30°, and 2.8 g. of isobutylchloroformate was introduced. During 15 minutes, it was allowed to warm to −5°. It was recooled to −20° before 11 g. of tetrapeptide amide (sesquihydrate) was added. After being allowed to come to room temperature and after being stirred for another hour, the reaction mixture was poured into 3000 ml. of water and a few ml. of acetic acid was added to bring the *p*H to about 5. The precipitate was filtered off, washed with 1500 ml. of water and air-dried, yielding 23 g. (87%), m.p. 220–230° dec.

Upon extraction of 3 g. of this substance with 250 ml. of warm methanol, 0.65 g. of powder was left, m.p. 220–235° with decomposition. One mg. of this material, after reduction with sodium in liquid ammonia, had an avian depressor activity of 100 units.

C.—8.7 g. of protected pentapeptide and 5.5 g. of tetrapeptide amide were dissolved in 50 ml. of dimethylformamide, and 5.2 ml. of triethylamine and 3.75 g. of *o*-phenyl-

(32) This protected tripeptide was prepared by the method of P. G. Katsoyannis and V. du Vigneaud (see ref. 14). The tosyl-L-isoleucyl chloride used in this synthesis crystallized during the removal of $SOCl_2$. Recrystallized from hexane, m.p. 54–56°. *Anal.* Calcd. for $C_{13}H_{18}O_3NSCl$: N, 4.61; Cl, 11.67. Found: N, 4.73; Cl, 11.4.

enechlorophosphite were added at about $-10°$ after which the mixture was allowed to come to room temperature. On the following day, the solid reaction mixture was diluted with water (600 ml.) and 1 ml. of acetic acid was added to bring the *p*H to about 6. The precipitate was separated, washed well with water and dried in air; wt. 12.2 g. (92%), m.p. 223–227° dec. 6.1 g. was suspended in 150 ml. of methanol and stored at room temperature for 1 hr., when it was filtered, washed with a further 150 ml. of methanol and dried *in vacuo* at room temperature over P_2O_5. The yield was 4.8 g. (72%), m.p. 235–236°. The avian depressor activity of this product, after reduction with sodium in liquid ammonia, was approximately 200 u./mg.

Removal of the Protective Groups from the Nonapeptide Derivative IX and Preparation of Biologically Active Material.—1.34 g. of IX was dissolved in about 450 ml. of liquid ammonia and sodium was added in small portions until a blue color lasting for at least 15 minutes was obtained. 0.35 g. of sodium was required. Addition of 0.82 g. of ammonium chloride caused the white precipitate formed during the reaction to disappear. The ammonia was allowed to evaporate, the last 30–40 ml. being removed by evaporation from the frozen state *in vacuo*. Five hundred ml. of water was added to the residue, the *p*H of the solution was adjusted to 6.5 with acetic acid, and air was bubbled through until the nitroprusside sodium test for sulfhydryl groups disappeared (about 4 hr.). The solution was acidified to about *p*H 4 with acetic acid, filtered, evaporated *in vacuo* below room temperature to a small volume and then dried from the frozen state. The solid residue from two such reductions was extracted with 60 ml. of ethanol in several portions and filtered, and the solution was diluted with 500 ml. ethyl acetate. The precipitate thus obtained was filtered, washed with ethyl acetate and dried *in vacuo* over $CaCl_2$. It was extracted with pyridine in several portions, a total of 50 ml. being used. The filtered solution was diluted with 500 ml. of ethyl acetate, and the precipitated product was filtered, washed with ethyl acetate and dried over $CaCl_2$ *in vacuo*. One g. of amorphous powder was obtained with a total activity of approximately 300,000 units.

0.40 g. of this material was dissolved in 40 ml. of water saturated with butanol and placed in the first four tubes of a countercurrent distribution apparatus.[33] After 340 transfers in the solvent system 1:1 butanol–water,[34] the contents of Tubes 101–125 were pooled, evaporated *in vacuo* below room temperature to a small volume and lyophilized. The solid was dissolved in 12 ml. of ethanol and precipitated with 150 ml. of ethyl acetate, filtered, washed with ethyl acetate and dried *in vacuo* over P_2O_5 at room temperature. 0.10 g. of white powder was obtained with an avian depressor activity of approximately 500 u./mg.[35]

Anal. Calcd. for $C_{43}H_{66}N_{12}O_{12}S_2$: C, 51.27; H, 6.60; N, 16.69. Found: C, 51.51; H, 6.81; N, 16.36.

Acknowledgments.—The authors wish to thank Mr. Robert L. Tostevin and Miss Dade W. Tull for carrying out the bioassays and Mrs. Lorraine S. Abrash for technical assistance.

(33) L. C. Craig, *Anal. Chem.*, **22**, 1346 (1950).

(34) In subsequent experiments the solvent system butanol–ethanol–0.05% acetic acid (4:1:5) was used with advantage. The distribution coefficient of oxytocin in this system at 24° is about 0.43.

(35) Biological assay of oxytocic activity was made following "The Pharmacopeia of the United States of America," 14th revision, Mack Printing Co., Easton, Pa., 1950, p. 475.

NEW YORK, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL SCIENCES, SOUTHEAST LOUISIANA HOSPITAL]

Peroxidase Catalyzed Oxidations in Essentially Non-aqueous Media: The Oxidation of Phenothiazine and Other Compounds

BY D. J. CAVANAUGH[1]

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The peroxidase catalyzed oxidations of unsubstituted phenothiazine and several common donors for the reaction were carried out in water-poor media in which the primary solvent was propylene glycol. Demonstrable reactions occurred at water concentrations as low as 2 to 3% of the total volume. The reaction rates were dependent to a considerable extent upon the nature of the electron donor and were, therefore, not determined solely by such factors as hydration of the enzyme itself.

It has been shown that the phenothiazine derivative chlorpromazine serves very well as a donor in the horseradish peroxidase system.[2] It was desirable to determine the effect of substitution by comparison of the behavior of the parent compound with its derivatives in the peroxidase reaction. The low water solubility of phenothiazine prompted the examination of various organic solvents as media for the enzymic system. Propylene and ethylene glycol–buffer solutions supported the reactions even when the water volume was restricted to about 2% of the total volume of the reaction mixtures. The glycols had the further advantage that highly insoluble compounds such as phenothiazine could be retained in solution at fairly high concentrations after dilution of the glycol solution with water to give final water concentrations in excess of 20% of the total volume. These properties of the glycol solutions permitted study of the behavior of the enzyme over a wide range of water concentrations and the use of various organic reagents normally precluded by poor water solubility. This report is concerned with the oxidation of phenothiazine and several of the more common donors in buffered propylene glycol solutions.

Since the reactions were most conveniently followed by the polarographic determination of hydrogen peroxide, a brief addendum on peroxide polarography in glycol solutions has been included.

Experimental

Reagents.—All standard reagents were reagent grade or the best obtainable. Phenothiazine was purified by recrystallization from benzene after treatment of the solution with activated charcoal. Purpurogallin was synthesized by the peroxidase system, extracted into ether and recrystallized from alcohol. Stock solutions of autoxidizable compounds were stored in a freezer without deterioration on some occasions. Horseradish peroxidase and crystalline beef liver catalase were obtained commercially.[3] The enzyme stock solutions were stored frozen between experiments.

To determine qualitatively what the nature of the impurities in the enzyme were samples were studied by means

(1) Pharmacology Branch, Directorate of Medical Research, Army Chemical Center, Maryland.

(2) D. J. Cavanaugh, *Science*, **125**, 1040 (1957).

(3) Worthington Biochemicals, Inc., Freehold, New Jersey.