*Anal.* Calcd. for $C_{23}H_{33}O_8N_5$: C, 54.4; H, 6.58; N, 13.8. Found: C, 54.3; H, 6.66; N, 13.4.

**Ethyl Tosyl-L-isoleucylglycinate.**—A solution of tosyl-L-isoleucyl chloride (prepared from 2.9 g. of tosyl-L-isoleucine and phosphorus pentachloride) in 15 ml. of anhydrous ether was added slowly to a suspension of 2.5 g. of ethyl glycinate hydrochloride and 3.5 ml. of triethylamine in 50 ml. of anhydrous ether and the mixture was allowed to stand at room temperature for 12 hours. The white precipitate was filtered off and washed with ether. After trituration with water to remove the triethylamine hydrochloride, 3.34 g. of product, m.p. 159–160°, was obtained; yield 89%. Another 0.25 g., m.p. 152–154°, was afforded by extraction of the ether filtrate successively with water, dilute HCl, dilute aqueous $KHCO_3$ and water, followed by removal of the ether *in vacuo*. Recrystallization of the second crop from ethanol raised the m.p. to 158–160°. For analysis, the tosyl dipeptide ester was recrystallized twice from ethanol and then melted at 160–161°.

*Anal.* Calcd. for $C_{17}H_{26}O_5N_2S$: C, 55.1; H, 7.07; N, 7.56. Found: C, 55.1; H, 7.30; N, 7.55.

**Tosyl-L-isoleucylglycine.**—The tosyl dipeptide ester (1.6 g.) was dissolved in 9 ml. of ethanol and 5 ml. of 1 *N* NaOH was added. The mixture was warmed on a water-bath (70°) for 1 minute, allowed to stand at room temperature for 2 hours, acidified to congo red paper with HCl and then concentrated *in vacuo* to dryness. The residue was dissolved in ethyl acetate plus a few drops of water and this solution was extracted twice with dilute aqueous $KHCO_3$. Acidification of the aqueous phase to congo red paper precipitated a crystalline product which was filtered off and washed with water; wt. 1 g. (71%), m.p. 185°. The m.p. remained unchanged after precipitation of the tosyl dipeptide from dilute aqueous $KHCO_3$ with HCl; $[\alpha]^{21}D$ −26.7° (*c* 1.1, 0.5 *N* $KHCO_3$).

*Anal.* Calcd. for $C_{15}H_{22}O_5N_2S$: C, 52.6; H, 6.47; N, 8.18. Found: C, 52.3; H, 6.63; N, 8.20.

Unchanged ethyl ester could be recovered by concentration of the ethyl acetate phase *in vacuo*.

**Methyl Tosyl-L-isoleucyl-L-leucinate.**—Tosyl-L-isoleucyl chloride (from 4.2 g. of tosyl-L-isoleucine and phosphorus pentachloride) was dissolved in 20 ml. of anhydrous ether and added to a suspension of 3.6 g. of methyl L-leucinate hydrochloride and 5 ml. of triethylamine in approximately 75 ml. of anhydrous ether. After 12 hours the precipitate was filtered off, washed with ether and triturated with water; wt. 4.6 g., m.p. 145–146°. The ether filtrate was shaken successively with water, dilute HCl, dilute aqueous $KHCO_3$ and water. Removal of the ether *in vacuo* and recrystallization of the residue from ethanol gave an additional 0.5 g. of product, m.p. 146–147°; over-all yield 85%. After two recrystallizations from ethanol, the m.p. was 147–148°.

*Anal.* Calcd. for $C_{20}H_{32}O_5N_2S$: C, 58.2; H, 7.82; N, 6.79. Found: C, 58.2; H, 7.84; N, 6.87.

**Tosyl-L-isoleucyl-L-leucine.**—This tosyl dipeptide was prepared by saponification of 2 g. of methyl tosyl-L-isoleucyl-L-leucinate (dissolved in 20 ml. of ethanol) with 5.5 ml. of 1 *N* NaOH according to the procedure described for the preparation of tosyl-L-isoleucylglycine; wt. 1 g., m.p. 152–154°. The ethyl acetate phase containing unreacted ester was concentrated *in vacuo* and the residual oil was dissolved in 8 ml. of ethanol and saponified in the same manner using 2 ml. of 1 *N* NaOH. The second crop (0.3 g.) melted at 151–153°. For analysis, a portion was recrystallized from a mixture of ethyl acetate and petroleum ether and dried; m.p. 163–165°; $[\alpha]^{21}D$ −24.7° (*c* 1.1, 0.5 *N* $KHCO_3$).

*Anal.* Calcd. for $C_{19}H_{30}O_5N_2S$: C, 57.3; H, 7.58. Found: C, 57.2; H, 7.73.

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

NEW YORK, N. Y.

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

# The Synthesis of Oxytocin[1]

BY VINCENT DU VIGNEAUD,[2] CHARLOTTE RESSLER, JOHN M. SWAN,[3] CARLETON W. ROBERTS AND PANAYOTIS G. KATSOYANNIS[4]

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A cyclic octapeptide amide (I) having the hormonal activity of oxytocin has been synthesized through the condensation of N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine and the heptapeptide amide L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IVa) to yield the protected nonapeptide amide VI followed by reduction with sodium in liquid ammonia and oxidation of the resulting sulfhydryl nonapeptide. IVa was prepared by the condensation of S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide with tosyl-L-isoleucyl-L-glutaminyl-L-asparagine followed by removal of the tosyl group from the condensation product. The biologically active synthetic material thus obtained has been purified by countercurrent distribution and compared with natural oxytocin as to potency, specific rotation, partition coefficients, amino acid composition, electrophoretic mobility, infrared pattern, molecular weight, enzymatic and acid inactivation and chromatography on the resin IRC-50. The synthetic material and natural oxytocin were also compared with respect to milk ejection and induction of labor in the human as well as rat uterus contraction *in vitro*. The crystalline flavianates prepared from the synthetic material and from natural oxytocin were found to have the same crystalline form, melting point and mixed melting point. All of these comparisons afforded convincing evidence of the identity of the synthetic product with natural oxytocin. This synthesis thus constitutes the first synthesis of a polypeptide hormone.

Oxytocin, the principal uterine-contracting and milk-ejecting hormone of the posterior pituitary gland,[5] was obtained from the latter in this Laboratory in highly purified form,[6–8] and isolated as a crystalline flavianate.[8] The purification was effected by application of countercurrent distribution[9,10] to posterior pituitary material which

(1) A preliminary report of this work has appeared [V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953)].

(2) Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this study. Acknowledgment is also made to Parke, Davis and Company, Armour and Company, and Eli Lilly and Company for placing at our disposal posterior pituitary material used as starting material for preparations of the purified oxytocin.

(3) Fulbright Scholar on Smith–Mundt grant-in-aid, on leave from Wool Textile Research Laboratory, C.S.I.R.O., Australia.

(4) Fellow of State Scholarships Foundation of Greece.

(5) The uterine-contracting activity of pituitary extracts was reported by H. H. Dale in 1906 [*J. Physiol.*, **34**, 163], and the milk-ejecting activity by I. Ott and J. C. Scott in 1910 [*Proc. Soc. Exp. Biol. Med.*, **8**, 48]. For a discussion of numerous chemical and biological investigations of the posterior pituitary gland including the early work on the subject, reference might be made to the review by H. Waring and F. W. Landgrebe ["The Hormones," Vol. 2, G. Pincus and K. V. Thimann, Ed., Academic Press, Inc., New York, N. Y., 1950, pp. 427–514].

(6) A. H. Livermore and V. du Vigneaud, *J. Biol. Chem.*, **180**, 365 (1949).

(7) J. G. Pierce and V. du Vigneaud, *ibid.*, **182**, 359; **186**, 77 (1950).

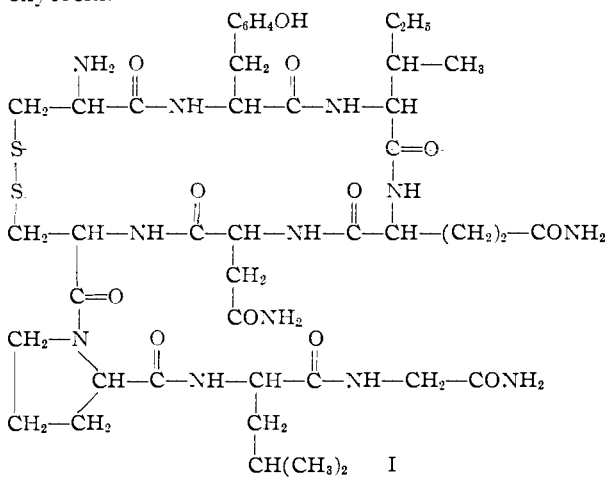
(8) J. G. Pierce, S. Gordon and V. du Vigneaud, *ibid.*, **199**, 929 (1952).

(9) L. C. Craig, *ibid.*, **155**, 519 (1944).

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

had received preliminary purification according to the procedure of Kamm and co-workers.[11] Amino acid analysis by the starch column method of Moore and Stein[12] showed that hydrolysates of the highly purified material contained leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine and cystine in equimolar ratios to each other and ammonia in a molar ratio of 3 to any one amino acid.[7]

The active principle appeared to be a polypeptide of molecular weight approximately 1000.[7,13] Evidence was obtained through oxidation with performic acid[14] and desulfurization with Raney nickel[15] that the polypeptide was some type of cyclic structure involving the disulfide linkage. Further studies including determination of terminal groups,[13,16-18] degradation with bromine water[19,13] and determination of sequence of amino acids by Edman degradation and by partial hydrolysis with acid,[18] along with the assumption that glutamine and asparagine residues were present rather than their isomers, allowed structure I to be postulated for oxytocin.[18,20]



I

It may be pointed out that this octapeptide amide is made up of a cystine-containing cyclic pentapeptide amide linked to a tripeptide amide. One-half of the cystine residue possesses a free amino group and is joined to the rest of the cyclic portion of the molecule through its carboxyl group, while the other half of the cystine residue is connected through its amino group to the rest of the cyclic portion of the molecule and through its carboxyl group to the tripeptide prolylleucylglycinamide. Synthetic proof of this structure was obviously desirable.

In this connection it may be recalled that in early experiments[21] with less purified oxytocin preparations, it was found that oxytocin could be reduced and reoxidized without appreciable inactivation. Treatment of the reduced active material with benzyl chloride resulted in inactivation. Early attempts to regenerate active material from the benzylated oxytocin by debenzylation with sodium in liquid ammonia were not successful. A reinvestigation of this problem recently[22] with highly purified oxytocin preparations led to the isolation of the biologically inactive S,S'-dibenzyl derivative of oxytocin, which on treatment with sodium in liquid ammonia according to the procedure of Sifferd and du Vigneaud for cleaving benzyl thioethers[23] gave rise to biologically active material. A study of the latter led to the conclusion[22] that the hormone had been regenerated from its benzylated derivative.

In terms of structure I, the reduction of oxytocin would represent the opening of the ring at the disulfide linkage to give a disulfhydryl open chain nonapeptide. Benzylation would then effect the conversion of the sulfhydryl form to a dibenzyl nonapeptide, with two residues of benzylcysteine taking the place of one of cystine in the original molecule. Debenzylation would convert the dibenzyl nonapeptide back to the sulfhydryl form from which the ring would be regenerated through the formation of the disulfide by aeration.

With the successful regeneration of oxytocin from the dibenzyl derivative of natural oxytocin, a way was opened to an approach to the synthesis of oxytocin on the basis that structure I represents that of oxytocin. Thus, if the nonapeptide N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI) could be synthesized, the removal of the carbobenzoxy and benzyl protecting groups with sodium in liquid ammonia should give the reduced form of oxytocin and the latter upon oxidation should yield oxytocin.

It may be noted that this synthetic approach utilizes the type of procedure for the synthesis of cysteine- and cystine-containing peptides introduced by du Vigneaud and Miller[24] in a synthesis of glutathione. In the latter synthesis N-carbobenzoxy-γ-glutamyl-S-benzylcysteinylglycine was prepared as the key intermediate in which the carbo-

(11) O. Kamm, T. B. Aldrich, I. W. Grote, L. W. Rowe and E. P. Bugbee, THIS JOURNAL, **50**, 573 (1928).

(12) S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 53 (1949).

(13) C. Ressler, S. Trippett and V. du Vigneaud, *ibid.*, **204**, 86 (1953).

(14) J. M. Mueller, J. G. Pierce, H. Davoll and V. du Vigneaud, *ibid.*, **191**, 309 (1951).

(15) R. A. Turner, J. G. Pierce and V. du Vigneaud, *ibid.*, **193**, 359 (1951).

(16) H. Davoll, R. A. Turner, J. G. Pierce and V. du Vigneaud, *ibid.*, **193**, 363 (1951).

(17) H. G. Kunkel, S. P. Taylor, Jr., and V. du Vigneaud, *ibid.*, **200**, 559 (1953).

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

(19) J. M. Mueller, J. G. Pierce and V. du Vigneaud, *ibid.*, **204**, 857 (1953).

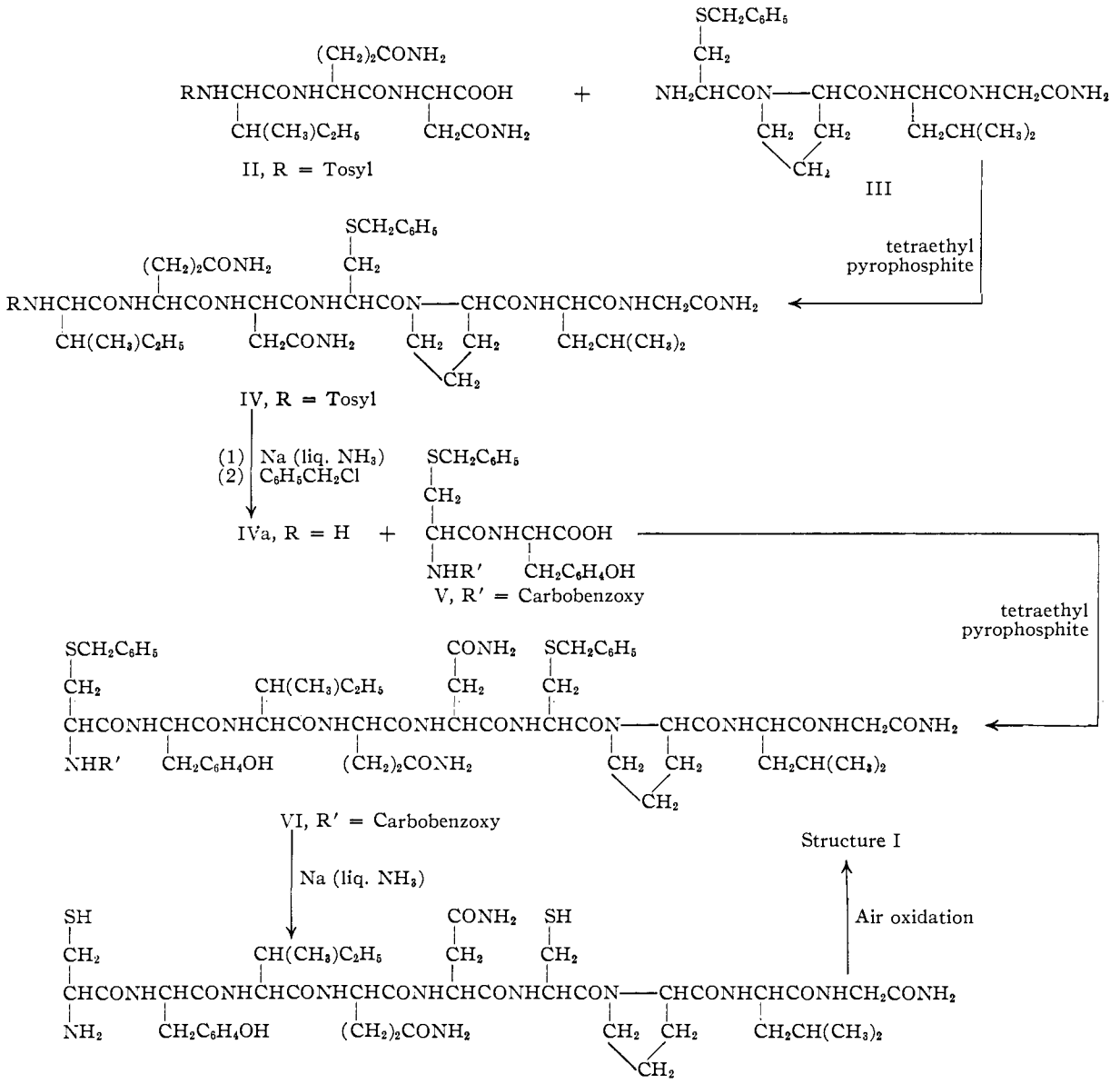
(20) After the proposal of this structure and the degradative studies involved had been submitted to *J. Biol. Chem.* (ref. 18) and after the preliminary report of the synthetic proof of the structure had been submitted to THIS JOURNAL (ref. 1), an identical proposal for the structure of oxytocin by H. Tuppy appeared. This proposal was based on the data from this Laboratory on composition, molecular weight, terminal groups and on the cyclic structure involving the disulfide linkage, together with his independent studies on the sequence of amino acids involving partial hydrolysis with acid and with an enzyme [H. Tuppy, *Biochim. et Biophys. Acta*, **11**, 449 (1953); H. Tuppy and H. Michl, *Monatsh. Chem.*, **84**, 1011 (1953)]. It is extremely interesting that the interpretation of the data and the assumptions made were quite parallel in both laboratories.

(21) R. R. Sealock and V. du Vigneaud, *J. Pharmacol. Exp. Therap.*, **54**, 433 (1935).

(22) S. Gordon and V. du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, **84**, 723 (1953).

(23) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(24) V. du Vigneaud and G. L. Miller, *ibid.*, **116**, 469 (1936).




benzoxy group was used for protection of the amino group[25] and the benzyl group for protection of the sulfhydryl group. The protective groups were finally removed to yield glutathione by cleavage with sodium in liquid ammonia.[23,24]

The preparation of the desired intermediate (VI) for oxytocin was accomplished by coupling N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine[26,27] (V) with the heptapeptide amide, L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IVa), prepared in turn from the coupling product of tosyl-L-isoleucyl-L-glutaminyl-L-asparagine[28] (II) with S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (III). The synthesis of the tetrapeptide amide III,[29] the synthesis of L-glutaminyl-L-asparagine[30] and the synthesis of the tosyl tripeptide II[31] are described separately.

Condensation of II with III was effected by the use of tetraethyl pyrophosphite according to the general procedure of Anderson, Blodinger and Welcher[32] for peptide synthesis. The "amide" procedure[32] was used with several modifications. The protected heptapeptide amide IV was obtained in approximately 40% yield as a white amorphous solid.[33] Removal of the tosyl[34] group from IV was effected with sodium in liquid ammonia, by which the benzyl group was also removed. The resulting reduced compound was then benzylated

(25) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932).

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

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

(28) Tosyl is used to designate the *p*-toluenesulfonyl grouping.

(29) C. Ressler and V. du Vigneaud, THIS JOURNAL, **76**, 3107 (1954).

(30) J. M. Swan and V. du Vigneaud, *ibid.*, **76**, 3110 (1954).

(31) P. G. Katsoyannis and V. du Vigneaud, *ibid.*, **76**, 3113 (1954).

(32) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(33) This protected heptapeptide amide has recently been obtained from glacial acetic acid as micro-needles, m.p. 236.5–237.5°.

(34) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

with benzyl chloride[35] in the same medium to yield the heptapeptide amide IVa. The latter was condensed with N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine (V) in the presence of tetraethyl pyrophosphite to yield the desired nonapeptide derivative N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI) in approximately 30% yield.

VI was then treated with sodium in liquid ammonia, by which both the benzyl and carbobenzoxy groups were removed. The reduced material obtained after evaporation of the ammonia was oxidized by aeration in dilute aqueous solution at *p*H 6.5 and tested for biological activity.

The reaction product was assayed by the rat uterine strip method[36,37] and by the chicken vasodepressor method of Coon,[38] which utilizes the property of oxytocin of lowering the blood pressure of the fowl and has been adopted by the United States Pharmacopeia as the method of assay for oxytocin.[39] Based on the assumption that the synthetic material has a potency equivalent to that of the purified natural material,[8] the yield of biologically active material, as determined by the two types of assay, ranged in several runs from 20 to 30% of the calculated amount from the protected nonapeptide VI. The yield approximated closely the yield of oxytocic activity obtainable under similar conditions from the S,S'-dibenzyl derivative of natural oxytocin.[22]

For purification, the crude active material was distributed by countercurrent distribution in the system 0.05% acetic acid–*sec*-butyl alcohol for 300 transfers. Analysis of the distribution indicated that approximately 90% of the biologically active material was present in a single peak having a partition coefficient ($K$ 0.35) in agreement with that of natural oxytocin. The material in the peak tube was not distinguishable in potency from one of our best preparations of natural oxytocin[8] when the two were assayed repeatedly against one another. The contents of 16 tubes representing the bulk of the material in the peak were combined, concentrated and lyophilized. Bioassay of the latter using the Coon method indicated that its potency was close to but somewhat less than that of the material in the peak tube.

The synthesis of the biologically active material has been repeated from the beginning on a somewhat larger scale and the results originally obtained were confirmed. At the time of the first synthetic run the tetrapeptide amide and the protected tripeptide were available only as white amorphous solids. In the more recent run it was possible to use these two intermediates in crystalline form and furthermore the countercurrent distribution was carried through approximately 1500 transfers.

The potency of the final synthetic product was compared with that of the purified natural oxytocin[40] by assaying one against the other with the synthetic material and the natural oxytocin serving interchangeably as the standard in the assay. The detailed procedure of assay as outlined in the United States Pharmacopeia[39] was rigorously followed. The potencies of the synthetic product are expressed as percentages of the potency of natural oxytocin with the corresponding standard error. In four U.S.P. assays in which the synthetic material was used as the standard, it was found to have 96% (s.e. 7), 94% (s.e. 12), 86% (s.e. 7) and 98% (s.e. 3) of the potency of the natural oxytocin. In three U.S.P. assays in which the natural oxytocin was used as the standard, the synthetic material was found to have 90% (s.e. 9), 83% (s.e. 11) and 89% (s.e. 10) of the potency of the natural oxytocin. As an over-all test of our assay procedure, a series of three U.S.P. assays were run in which natural oxytocin was assayed against itself. In each of the latter three assays a solution of natural oxytocin was divided into two parts, one of which was used as the standard and the other as the "unknown." Values of 91% (s.e. 17), 90% (s.e. 7) and 96% (s.e. 4) were obtained for the "unknown." It is apparent from the assay results that the potency of the synthetic product is close to that of the natural material.

The synthetic material was found to be as effective in the induction of labor in the human as was the purified natural oxytocin.[41] When the synthetic compound and natural oxytocin were tested for milk-ejecting activity in the human, as has been reported,[42] they were found indistinguishable in effectiveness. Approximately 1 $\gamma$ of either the synthetic or natural material given intravenously to patients induced milk ejection in 20–30 seconds.

The synthetic material possessed the specific rotation $[\alpha]^{21.5}D$ $-26.1 \pm 1.0°$ ($c$ 0.53, water) compared to $[\alpha]^{22}D$ $-26.2°$ ($c$ 0.53, water) for natural oxytocin.[22] In addition to having, under the same conditions, a partition coefficient in agreement with that of natural oxytocin in the system used for purification (0.05% acetic acid–*s*-butyl alcohol) the synthetic substance had the same partition coefficient ($K = 1.24$) as natural oxytocin[8] in a second solvent system (0.01 $M$ ammonia–*s*-butyl alcohol).[6] Determination of the amino acid composition gave the expected values. At two different hydrogen ion concentrations the synthetic and natural material exhibited the same electrophoretic mobility on paper.[43] No differences were detected in the infrared patterns.[44] The synthetic

(35) V. du Vigneaud, L. F. Andrieth and H. S. Loring, THIS JOURNAL, **52**, 4500 (1930).

(36) J. H. Burn, *Quart. J. Pharm. Pharmacol.*, **4**, 517 (1931).

(37) J. H. Burn, D. J. Finney and L. G. Goodwin, "Biological Standardization," 2nd Edition, Oxford University Press, 1950, p. 180.

(38) J. M. Coon, *Arch. intern. pharmacodynamie*, **62**, 79 (1939).

(39) "The Pharmacopeia of the United States of America," fourteenth revision, Mack Printing Co., Easton, Pa., 1950, p. 475.

(40) The purified natural oxytocin when assayed against the U.S.P. Standard Powder showed an activity of approximately 450–500 U.S.P. units/mg. (ref. 8). The assignment of an exact unitage is of course extremely difficult due to the nature of the bioassay and of the standard involved.

(41) The uterine-contracting activity in the human was tested through the kindness of Professor R. Gordon Douglas, Dr. Kenneth Nickerson and Professor Roy W. Bonsnes of the Department of Obstetrics and Gynecology.

(42) K. Nickerson, R. W. Bonsnes, R. G. Douglas, P. Condliffe and V. du Vigneaud, *Am. J. Obstet. Gynecol.*, in press.

(43) Electrophoretic analysis was performed by Dr. Sterling P. Taylor, Jr.

(44) The infrared spectra were obtained by Mr. Robert J. Brotherton.

material formed an active flavianate which had the same crystalline form (fine silky needles) and agreed in melting point with the flavianate obtained from natural oxytocin.

On treatment with bromine water the synthetic material has been found to undergo the cleavage into two fragments encountered with natural oxytocin. Both give rise on treatment with bromine water followed by chromatography on paper in phenol to two ninhydrin-positive spots, $R_f$ approximately 0.3 and 0.85, which have been identified as β-sulfoalanyldibromotyrosine and a sulfonic acid heptapeptide, respectively.[19,13]

A preliminary study on the molecular weight of the synthetic material gave a value in the expected range.[45] As in the case with natural oxytocin,[46] the synthetic material was inactivated on treatment with dilute acid at 90° or with chymotrypsin at 25°. Both the synthetic and natural products chromatographed as a single substance on the resin Amberlite IRC-50.[47]

These comparisons of the physical, chemical and biological properties of the synthetic product with those of the purified natural oxytocin justify in our estimation the conclusion that the synthetic octapeptide amide is oxytocin and that structure I represents that of the hormone.

## Experimental[48]

**Tosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV).**—To a solution of 1.19 g. of S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide[29] (III) in 5 ml. of anhydrous diethyl phosphite in a 50-ml. r.b. flask fitted with a stirrer and protected from moisture was added 1.2 ml. of tetraethyl pyrophosphite[32] and the clear solution was heated in an oil-bath at 85° for several minutes; 1.31 g. of finely divided tosyl-L-isoleucyl-L-glutaminyl-L-asparagine[31] (II) was then added in three portions within 10 minutes. After the last addition a further 0.8-ml. portion of tetraethyl pyrophosphite and 6 ml. of diethyl phosphite were added. The gelatinous mixture was stirred vigorously and heated at the bath temperature of 85–90° for 50 minutes. At the end of this time the precipitate was separated by centrifugation and washed with 2 ml. of diethyl phosphite. Addition of 10 ml. of ice-water converted the gelatinous precipitate to a white solid which was readily separated by filtration. The solid was allowed to stand for 5 minutes in 5 ml. of 1% sodium bicarbonate solution. It was then filtered off and washed with water; wt. 1.37 g., m.p. 219.5–223° dec. The dried solid was washed twice with warm absolute methanol; wt. of the residue 1.01 g. (41%), m.p. 234–236° dec. In successive runs, varying amounts of product of different degrees of purity were obtained initially. However, after the purification with methanol, the yields approximated that of the run described.

For analysis, the compound was dissolved in warm aqueous methanol, centrifuged and allowed to precipitate slowly; m.p. 236.5–237.5°.

*Anal.* Calcd. for $C_{45}H_{66}O_{11}N_{10}S_2$: C, 54.8; H, 6.74; N, 14.2. Found: C, 54.4; H, 6.85; N, 13.8.

Material ranging in m.p. from 234–236° to 236.5–237.5° was used for further synthetic work.

**Preparation of Protected Nonapeptide Amide VI Followed by Reduction and Oxidation.**—A solution of 1.53 g. of tosyl heptapeptide amide IV in 35 ml. of liquid ammonia in a 3-necked r.b. flask protected by a drying tube and fitted with a stirrer was held at −40° and approximately 409 mg. of sodium was added in small portions until the end-point, as indicated by a deep blue color throughout the solution lasting at least 30 seconds, was reached. A white precipitate appearing near the end-point was kept dispersed throughout the solution by vigorous stirring and 0.214 ml. of freshly distilled benzyl chloride was added. After 40 minutes an additional portion of 0.16 ml. of benzyl chloride was added. After a total of 1–1.5 hours, 0.94 g. of ammonium chloride was added, when the precipitate dissolved, and the clear solution was allowed to evaporate spontaneously. The dry solid residue was collected and extracted several times with ether. The white solid was suspended in 4 ml. of ice-water, filtered, washed on the filter with small volumes of ice-water and dried *in vacuo*. The L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IVa) thus obtained weighed 0.94 g. and was used directly for coupling with N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine.

To a suspension of 0.4 g. of the heptapeptide amide IVa in 2 ml. of diethyl phosphite in a test-tube protected from moisture was added 0.5 ml. of tetraethyl pyrophosphite. The mixture was heated with stirring in a bath at 85° for several minutes. A solution of 0.4 g. of N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine (V) in 2 ml. of diethyl phosphite was then added in two portions to the gelatinous mass, and after 10 minutes an additional 0.3 ml. of tetraethyl pyrophosphite was added. The mixture was then stirred and heated in a bath at 85° for an additional 45 minutes. At the end of this period the contents were cooled and poured into 30 ml. of ice-water. The white precipitate which resulted was separated by filtration and washed several times with 3% sodium bicarbonate solution at 40° and then several times with water at 40°. It was then filtered and dried; wt. 0.43 g. The N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI) was extracted with small volumes of warm acetone until the extracts remained colorless, and was then washed several times with warm absolute methanol; wt. 211 mg. (33%), m.p. 224–225° dec. with softening at 215°. This material was converted to biologically active material by reduction in liquid ammonia.

The reduction procedure was essentially that described by Gordon and du Vigneaud[22] for the regeneration of oxytocin from its S,S'-dibenzyl derivative. Eighty mg. of the protected nonapeptide amide VI was dissolved in 10 ml. of anhydrous liquid ammonia in a 100-ml. r.b. flask fitted for magnetic stirring and connected to an evacuation system. The reaction was carried out at the boiling point of the solution. Reduction was accomplished by dipping a fine glass capillary containing sodium below the surface of the solution until a blue color appeared throughout. Fifteen mg. of ammonium chloride was then added, the flask immediately evacuated and the contents swirled until frozen. The ammonia was removed by lyophilization. The white fluffy residue was then added portionwise to 400 ml. of $CO_2$-free water with vigorous stirring. The *p*H was adjusted to 6.5 with 1% acetic acid and the solution aerated with $CO_2$-free air for 2 hours. Four ml. of 5% acetic acid was added. For isolation purposes, this procedure was repeated several times and the solutions were combined at this point, concentrated to a small volume in a rotary evaporator[49] at a temperature below 30° and lyophilized. The product from 360 mg. of the crude protected nonapeptide VI was assayed by the Coon method[38] and found to possess an activity of approximately 29,000 units.

**Isolation of Synthetic Biologically Active Material.**—The material was placed in the first 6 tubes of the all-glass countercurrent distribution apparatus[10] and distributed in the system 0.05% acetic acid–*s*-butyl alcohol. By following the weight distribution and absorption at 275 mμ, it was apparent that after 200 transfers considerable separation into at least four components had been effected. Assay for biological activity[38] indicated that the activity was concentrated in a single peak, having the partition coefficient $K = 0.35$. Within the deviations expected experimentally, this value agrees with the partition coefficient ($K = 0.37$)[22] obtained for oxytocin regenerated from the S,S'-dibenzyl derivative and with that obtained earlier for

(45) Preliminary sedimentation studies on the molecular weight of natural oxytocin and the synthetic material were kindly made by Dr. Howard K. Schachman and Dr. W. F. Harrington of the University of California.

(46) S. P. Taylor, Jr., and V. du Vigneaud, unpublished data.

(47) S. P. Taylor, Jr., *Proc. Soc. Exp. Biol. Med.*, **85**, 226 (1954).

(48) Capillary melting points were determined and are corrected.

(49) L. C. Craig, J. C. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

natural oxytocin ($K$ = 0.41, 0.39).[8] The active material was also found to have the same partition coefficient as natural oxytocin ($K$ = 1.24)[8] in a second solvent system (0.01 $M$ ammonia–$s$-butyl alcohol).[6] The distribution between 0.05% acetic acid and $s$-butyl alcohol was continued for another 100 transfers. The material from a peak tube (tube 71) was assayed against a sample of one of our most highly purified preparations of natural oxytocin and was found equal in potency with the latter. The material (55 mg.) obtained by concentration of the combined contents of tubes 69–81 followed by lyophilization, however, was somewhat lower in potency.

In the second run the material resulting from the treatment of 890 mg. of VI had an activity of approximately 81,000 units. It was placed in the first 8 tubes of the countercurrent distribution apparatus and distributed for 176 transfers. The contents of tubes 42–74 were then subjected to further countercurrent distribution for an additional 1250 transfers using fresh solvent. The distributions of weight and ultraviolet absorption at 275 mμ were followed. Biological assay indicated that approximately 90% of the starting activity was present within one peak (tubes 90–155). The contents of tubes 105–132 were concentrated to a small volume in a rotary evaporator[49] and finally lyophilized to dryness. Seventy-five mg. of a white fluffy solid was obtained from this fraction and 65 mg. from the combined side fractions.

**Comparison of Synthetic and Natural Oxytocin.**—The potency of the lyophilized synthetic material, obtained as described in the preceding section from tubes 105–132, was compared with that of the purified natural oxytocin by assay according to the Coon method[38] following the procedure outlined in the United States Pharmacopeia.[39] For this comparison seven U.S.P. assays were carried out. The responses in blood pressure obtained in one of these assays are given in Table I.

TABLE I

ASSAY OF SYNTHETIC AND NATURAL OXYTOCIN[a]

| Time | Sample | Dose, ml. | Response[b] Drop in blood pressure, mm. |
|---|---|---|---|
| 2:30 | S | 0.15 | 17 |
| 2:35 | N | .15 | 15 |
| 2:40 | S | .15 | 12 |
| 2:45 | N | .18 | 15 |
| 2:50 | S | .20 | 13.5 |
| 2:55 | N | .16 | 10 |
| 3:00 | S | .25 | 13 |
| 3:05 | N | .25 | 14 |
| 3:10 | S | .30 | 13.5 |
| 3:15 | N | .24 | 11 |
| 3:20 | S | .30 | 11 |
| 3:25 | N | .36 | 15.5 |
| 3:30 | S | .40 | 12.5 |
| 3:35 | N | .40 | 17 |
| 3:40 | S | .40 | 12 |
| 3:45 | N | .48 | 18 |
| 3:50 | S | .50 | 14 |
| 3:55 | N | .40 | 13.5 |
| 4:00 | S | .50 | 11.5 |

[a] Solution N of natural oxytocin (0.203 mg./ml.) at dilution 1:500 is assayed against solution S of synthetic oxytocin as the standard (0.209 mg./ml.) at dilution 1:500. [b] The values show the response of the standard and the unknown at the various dose levels. One will note the falling off in sensitivity of the bird during the course of the assay, which is taken into account in the calculation of the comparative potencies of the standard and unknown. The potency and the standard error are calculated as described in the Pharmacopeia.[39] In this assay the potency of the synthetic material was found to be 96% of the potency of the natural oxytocin with a standard error of 7.

The results of the assays, which are discussed in the first section of this paper, indicated that the potency of the synthetic material was close to that of natural oxytocin. It may be pointed out that some degree of racemization may have occurred during the course of the various steps in the synthesis. Furthermore, a small amount of diastereoisomeric forms may have arisen during the various procedures involved in the isolation of the synthetic product and of the natural product as well. Small amounts of diastereoisomers could conceivably be present in either of these products even after the extensive purification. It may be noted that on concentration of dilute acetic acid solutions of natural oxytocin followed by lyophilization some degree of inactivation is occasionally encountered in this Laboratory.

The synthetic material possessed the specific rotation $[\alpha]^{21.5}D$ −26.1 ± 1.0° ($c$ 0.53, water) compared to $[\alpha]^{22}D$ −26.2° ($c$ 0.53, water) for natural oxytocin.[22]

An active crystalline flavianate was prepared from the synthetic material by the procedure previously described[8] for the preparation of the natural oxytocin flavianate. To 5.2 mg. of the synthetic material dissolved in 0.26 ml. of water was added 0.15 ml. of 5% flavianic acid solution and the solution was seeded with a few crystals of natural oxytocin flavianate and allowed to stand in the refrigerator. After 3 days the crystals were separated by centrifugation, washed in this manner with 0.25% flavianic acid followed by cold water and then dried by lyophilization. The flavianate of the synthetic material had the same crystalline form (fine silky needles) as the flavianate prepared from natural oxytocin and had a m.p. of 182–187°, compared with 181–185.5° for the natural oxytocin flavianate. Admixture of the flavianates from the synthetic and natural material did not cause a depression in m.p. Both these values are somewhat lower than that previously reported for the flavianate of natural oxytocin.[8] However, the m.p. was found to depend on the rate of heating.

Amino acid analysis by the starch column chromatographic method[12] of the synthetic material after hydrolysis showed the expected composition,[7] expressed as molar ratios: leucine 1.00, isoleucine 1.00, tyrosine 0.83, proline 0.92, glutamic acid 0.91, aspartic acid 0.93, glycine 0.98, cystine 0.87 and ammonia 3.04. The infrared spectra of the synthetic and natural substances were identical, showing three main broad absorption bands at 3.10, 6.05 and 6.58 μ.

Preliminary studies[45] on the molecular weight of the natural oxytocin and the synthetic material using the synthetic boundary ultracentrifuge cell of Pickels, Harrington and Schachman[50] showed that the sedimentation coefficient for both materials was about the same and equal to about 0.34. These results suggested that the molecular weight of the material in solutions at 0.3 μ at $p$H 4.5 is in the neighborhood of 1000. This value is in the same range as that previously reported for the molecular weight of natural oxytocin by the thermoelectric osmometer method.[7,13] All the results were in accord with what would be expected of the cyclic polypeptide represented by structure I which has a calculated molecular weight of 1007.

Two experiments were run in which the synthetic and natural oxytocin were compared by electrophoretic analysis on filter paper.[43] The procedure used was that described previously for natural oxytocin.[17] Fifty γ of the synthetic material and a similar amount of natural oxytocin were applied separately to a strip of Whatman No. 3 MM filter paper and 300 v. was applied for 2 hours. Glycine buffer at $p$H 9.7 was used in one experiment and acetate buffer at $p$H 4.5 in the other. The material was stained with the bromo phenol blue–mercuric chloride reagent. In both cases, the synthetic material exhibited the same mobility as natural oxytocin.[17]

When either 0.8 mg. of the synthetic material or a mixture of 0.34 mg. of the synthetic material and 0.37 mg. of natural oxytocin was chromatographed on Amberlite IRC-50, no indication of the presence of more than one component was obtained.[47]

Perhaps the least understood reaction encountered in the degradation studies on oxytocin was the cleavage of the molecule into two fragments when performic acid–oxidized oxytocin was treated with bromine water.[19,13] The smaller fragment was identified as β-sulfoalanyldibromotyrosine and the larger fragment as a sulfonic acid heptapeptide in which the amino group of the isoleucine residue was free.[13] It appeared likely on the basis of further degradative work[18,51]

(50) E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci. U.S.*, **38**, 943 (1952).

(51) C. Ressler and V. du Vigneaud, unpublished data.

that these two fragments were adjacent and were connected through a simple peptide linkage involving the carboxyl group of tyrosine and the amino group of isoleucine, and the structure for oxytocin was postulated in accordance with this view.[18] However, inasmuch as such a degradation had not, so far as was known, been previously encountered in the chemistry of polypeptides and proteins, it was desirable to apply the degradative procedure to the synthetic compound. The early experiments[19] in this Laboratory on the cleavage of oxytocin itself with bromine water had given low yields of the fragments. Rather than apply the reaction to performic acid-oxidized synthetic material, the bromine water cleavage directly on oxytocin was reinvestigated. It has been found that cleavage in good yield can be obtained at $-10°$ with oxytocin under the conditions used in the case of the performic acid-oxidized oxytocin. A comparison of this reaction with the synthetic and natural compounds was made. Both give rise on treatment with bromine water followed by chromatography on paper in phenol to the two ninhydrin-positive spots, $R_f$ 0.3 and 0.85, which have been identified as β-sulfoalanyldibromotyrosine and a sulfonic acid heptapeptide, respectively.[18,19]

The comparisons of the biological activities of the synthetic and natural oxytocin including the rat uterus contraction *in vitro*, the induction of labor in the human, the avian vasodepressor activity and the milk-ejecting activity in the human have been presented and discussed in the first section of this paper.

NEW YORK, N. Y.

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

# Erythromycin. I. Properties and Degradation Studies[1]

BY EDWIN H. FLYNN, MAX V. SIGAL, JR., PAUL F. WILEY AND KOERT GERZON

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The physical and chemical properties of erythromycin, a new and useful antibiotic, have been studied. Degradative studies have led to partial structures for erythromycin and some of its derivatives and degradation products.

Erythromycin[2] is a therapeutically useful, wide-range antibiotic produced by a strain of *Streptomyces erythreus*.[3] A brief description of some of its properties has been given.[3] It is a crystalline, colorless compound which is slightly soluble in water but dissolves easily in most of the common organic solvents. Crystals are obtained readily from aqueous acetone, aqueous alcohol or chloroform. Other solvents have been employed but those mentioned are the most useful. Crystals can be obtained from aqueous solution, but in this solvent solubility varies inversely with temperature in the ordinary temperature range. As might be expected from this temperature effect, the resulting crystals are hydrated. The hydrated crystals lose water on drying at 56° under reduced pressure. When dried in this manner, the crystals are hygroscopic and if exposed to moisture, regain the weight which was lost on drying. When erythromycin is allowed to crystallize from aqueous acetone, unstable solvated crystals are obtained which lose crystallinity on drying over phosphorus pentoxide. When crystallized from either solvent (see Experimental) the compound melts at 135–140°, and, if a slow rate of heating is continued, it partially resolidifies. It then melts at 190–193°. Solvated crystals are also obtained from other organic solvents. A detailed report on the crystallography of erythromycin is being published.[4]

Erythromycin is a base exhibiting a $pK'_a$ of 8.6 when titrated in 66% dimethylformamide. It dissolves readily in dilute aqueous acids and forms crystalline salts with mineral acids. It is optically active with a specific rotation of $-73.5°$ in methanol (corrected to an anhydrous basis). The compound absorbs weakly in the ultraviolet; the single broad band has a maximum at about 278 mμ, ε 27. Its infrared absorption (Fig. 1) in chloroform solution is characterized by intense bands in the 8.5–10.0 μ region. These bands are probably due to the presence of C–O–C groupings. In the 5.5–6.0 μ region two maxima are observed. The first, at 5.78 μ, is comparable in intensity and position to the carbonyl band observed for a normal ester or six-membered lactone. The second band is at 5.91 μ and has an intensity which is about half that of the 5.78 μ band. A maximum at 2.84 μ indicates the presence of hydroxyl groupings in the molecule.

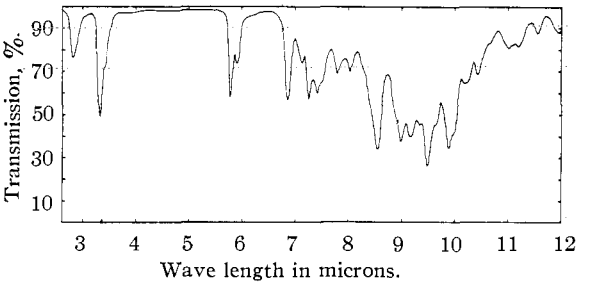


Fig. 1.—Infrared spectrum of erythromycin: 5.5% in $CHCl_3$, cell path, 0.093 mm.

The molecular weight of erythromycin was estimated by two independent methods. The free base, two salts and two other derivatives were subjected to X-ray crystallographic analysis.[5] Five independent molecular weight values were calculated. The average value derived for the molecular weight of the free base was 736. Maximum deviation from the average was 6 units. This method has a possible maximum error of ±2%, although it is felt that the results are more precise

(1) Presented before the Division of Organic Chemistry at the National Meeting of the American Chemical Society at Chicago, Illinois, September 6–11, 1953. Subsequent to the presentation of this paper, an article appeared which described some experiments similar to those recorded here. See R. B. Hasbrouck and F. C. Garven, *Antibiotics and Chemotherapy*, **3**, 1040 (1953).

(2) The Eli Lilly and Company trade-mark for the antibiotic erythromycin is "Ilotycin."

(3) J. M. McGuire, R. L. Bunch, R. C. Andersen, H. E. Boaz, E. H. Flynn, H. M. Powell and J. W. Smith, *Antibiotics and Chemotherapy*, **2**, 281 (1952).

(4) S. F. Kern and H. A. Rose, in preparation.

(5) A detailed report of the method used will be published by H. A. Rose and S. F. Kern.