Glutamine Isomers of Oxytocin and Deamino-oxytocin: Synthesis and Pharmacological Properties of [4-N^4-Methyl-L-asparagine]-oxytocin and [1-β-Mercaptopropionic Acid, 4-N^4-Methyl-L-asparagine]-oxytocin

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SUMMARY

Analogues of oxytocin and deamino-oxytocin ([1-β-mercaptopropionic acid]-oxytocin) with an N^4-methyl-L-asparagine residue in place of the glutamine residue in position 4 have been prepared and tested for their biological activities. [4-N^4-Methyl-L-asparagine]-oxytocin was found to possess 119 ± 5 units/mg of avian vasodepressor activity, 41 ± 2 units/mg of rat oxytocic activity, less than 0.5 unit/mg of rat pressor activity, and approximately 0.045 unit/mg of rat antidiuretic activity. The hydro-osmotic activity of the analogue was 2.23 ± 0.40% of that of crystalline deamino-oxytocin upon assay in the toad bladder system. The corresponding activity values for [1-β-mercaptopropionic acid,4-N^4-methyl-L-asparagine]-oxytocin were 186 ± 4 units/mg, 59 ± 2 units/mg, less than 0.4 unit/mg, approximately 0.012 unit/mg, and 1.17 ± 0.11%, respectively. Most significantly, the replacement of the *N*-terminal amino group in [4-N^4-methyl-L-asparagine]-oxytocin by hydrogen elicited not only a quantitative but even a qualitative alteration in the hydro-osmotic activity.

INTRODUCTION

Considerable information on the relation between the structure of neurohypophysial hormones and their biological activities has been gathered during the last two decades. One point of focus in these structure-activity studies is the functional role of the glutamine residue in position 4 of oxytocin. In this context two analogues were synthesized, [4-glutamic acid]-oxytocin (1) and [4-ornithine]-oxytocin (2), both retaining as a first approximation the length of the side chain of the glutamine residue present in the natural hormone. While the latter retained a high degree of oxytocin-like biological activities, the former exhibited an extremely low level of these activities.

In a continued effort to explore the steric and electronic role of the glutamine residue in oxytocin, we decided to prepare and evaluate pharmacologically [4-N^4-methyl-L-asparagine]-oxytocin, an analogue bearing an amino acid residue isomeric with the glutamine residue, and [1-β-mercaptopropionic acid,4-N^4-methyl-L-asparagine]-oxytocin, in which a further change has been made by replacing the half-cystine residue in position 1 by a β-mercaptopropionic acid residue.

The polypeptide intermediates of [4-N^4-

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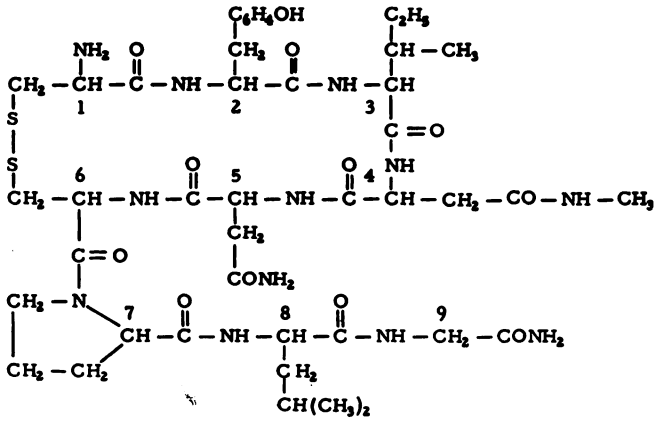

FIG. 1. *Structure of [4-N^4-methyl-L-asparagine]-oxytocin*
Numbers indicate the positions of the individual amino acid residues.

methyl-L-asparagine]-oxytocin (Fig. 1) and its deamino analogue were prepared by the *p*-nitrophenyl ester method of peptide synthesis (3). The *p*-nitrophenyl N^2-carbobenzoxy-N^4-methyl-L-asparaginate was obtained from N^2-carbobenzoxy-N^4-methyl-L-asparagine, which in turn was isolated after saponification of N^2-carbobenzoxy-N^4-methyl-L-asparagine benzyl ester[1] prepared earlier in these laboratories (4). The key intermediates were then deprotected and oxidized to yield [4-N^4-methyl-L-asparagine]-oxytocin and its deamino analogue, respectively, by methods employed in the synthesis of oxytocin (5) and deamino-oxytocin (6). The purified analogues, isolated after partition chromatography on Sephadex G-25 (7), behaved as single, homogeneous compounds when tested by paper electrophoresis and paper and thin layer chromatography in several solvent systems; furthermore, they gave the expected values upon elementary analysis and upon analysis for ninhydrin-reactive components following acid hydrolysis.

[4-N^4-Methyl-L-asparagine]-oxytocin and its deamino analogue were tested for their biological activities in the avian vasodepressor, rat oxytocic, rat pressor, and rat antidiuretic assay systems, and their mean potencies along with standard errors are given in Table 1. These data might suggest that whether the glutamine residue in oxytocin is replaced by an N^4-methyl-L-asparagine residue or by an ornithine residue, the biological effects encountered are comparable. Both of these oxytocin analogues retained a high potency as compared with [4-glutamic acid]-oxytocin (1). It was recently speculated (12) that the drastic loss of activity for the latter compound was due to the introduction of a negative charge at position 4 of the oxytocin molecule, thus profoundly changing the distribution properties of this analogue.

Both [4-N^4-methyl-L-asparagine]-oxytocin and its deamino analogue were also tested for hydro-osmotic activity on the isolated toad bladder according to the method of Eggena *et al.* (8) and were found to have a potency of 2.23% ± 0.40 and 1.17% ± 0.11, respectively, relative to crystalline deamino-oxytocin. When [4-N^4-methyl-L-asparagine]-oxytocin and oxytocin were compared for their ability to evoke a maximal response, a measure of intrinsic hormonal activity (13), both compounds were found to be comparable (viz. 97.8% ± 7.02). However, under identical experimental conditions [i.e., at the pH optimum (8.4) for toad bladder reactivity to neurohypophysial hormones (14)], [deamino-4-N^4-methyl-L-asparagine]-oxytocin evoked only 70.9% ± 2.6 of the maximal response (Fig. 2).

[1] This compound was previously referred to as N^2-carbobenzoxy-*N*-methyl-L-asparagine benzyl ester.

TABLE 1

Biological activities of oxytocin and deamino analogues modified in position 4

Compound	Vasodepressor (fowl)	Oxytocic (rat)	Pressor (rat)	Antidiuretic (rat)	Water flux (toad bladder)[a]
			USP units/mg		
Oxytocin[b]	507 ± 23	546 ± 18[c]	3.1 ± 0.1	2.7 ± 0.2	182 ± 9[d]
[1-β-Mercaptopropionic acid]-oxytocin[e]	975 ± 24	803 ± 36	1.44 ± 0.06	19 ± 1	100[d]
[4-N^4-Methyl-L-asparagine]-oxytocin	119 ± 5	41 ± 2	<0.5	~0.045	2.23 ± 0.40
[1-β-Mercaptopropionic acid,4-N^4-methyl-L-asparagine]-oxytocin	186 ± 4	59 ± 2	<0.4	~0.012	1.17 ± 0.11
[4-Ornithine]-oxytocin[f]	163 ± 5	58 ± 1.6	<0.1	~0.029	1.60 ± 0.10
[4-Glutamic acid]-oxytocin[g]	~0.5	~1.5	—[h]	—	

[a] As a percentage of crystalline deamino-oxytocin on a molar basis (see ref. 8).
[b] Values reported by Chan and du Vigneaud (9).
[c] Value reported by Chan, O'Connell, and S. R. Pomeroy (10).
[d] See ref. 8.
[e] Values reported by Ferrier, Jarvis, and du Vigneaud (11).
[f] See ref. 2.
[g] See ref. 1.
[h] Negligible values are signified by dashes.

On the basis of earlier experience it is not surprising that the replacement of the primary amino group by a hydrogen atom in a neurohypophysial peptide results in an enhancement of the avian vasodepressor and rat oxytocic activities and in a diminution of the rat pressor and toad bladder hydro-osmotic activities. But to our knowledge a qualitative alteration in hormonal activity has not previously been encountered in association with such a replacement. A detailed investigation of this finding is under way.

EXPERIMENTAL PROCEDURE[2]

N^2-Carbobenzoxy-N^4-methyl-L-asparagine. N^2-Carbobenzoxy-N^4-methyl-L-asparagine benzyl ester (4) (3.7 g) in 30 ml of dioxane was saponified with 10.5 ml of 1 N NaOH for 3 hr. The mixture was acidified, and the product was extracted into ethyl acetate–ether (1:1). The organic solution was washed with 1 N hydrochloric acid followed by water, and the product was then extracted into 1 N sodium bicarbonate. The bicarbonate solution was washed with ethyl acetate–ether, acidified, and extracted with ethyl acetate. The organic solution was separated, washed with 1 N hydrochloric acid, water, and saturated sodium chloride, and then dried over anhydrous magnesium sulfate. The solvent was removed under vacuum; the product, crystallized from 95% ethanol, weighed 1.1 g; m.p. 142–144°.

$C_{13}H_{16}N_2O_5$

Calculated:	C 55.7,	H 5.76,	N 10.0
Found:	C 55.8,	H 5.90,	N 9.95

p-Nitrophenyl N^2-Carbobenzoxy-N^4-methyl-L-asparaginate. N^2-Carbobenzoxy-N^4-methyl-L-asparagine (11 g) was dissolved in 50 ml of dimethylformamide, and 4.54 g of *p*-nitrophenol were added. The mixture was cooled in an ice bath, and 6.1 g of dicyclohexylcarbodiimide, dissolved in 10 ml of dimethylformamide, were added. After 3 hr of stirring at 0°, the solution was stirred for an additional 2 hr at room temperature. Then acetic acid (0.3 ml) was added, the mixture was cooled to 0°, and the precipitated dicyclohexylurea was filtered off and quickly washed with 20 ml of ice-cold dimethylformamide. Upon the addition of 70 ml of water, the product crystallized and was recrystallized from methanol; m.p. 155–156°; $[\alpha]_D^{24}$ −28.7° (*c*, 2, in dimethylformamide).

$C_{19}H_{19}N_3O_7$

Calculated:	C 56.9,	H 4.77,	N 10.5
Found:	C 57.1,	H 4.95,	N 10.2

N^2-Carbobenzoxy-N^4-methyl-L-asparaginyl-L-asparaginyl-S-benzyl-L-cysteinyl-

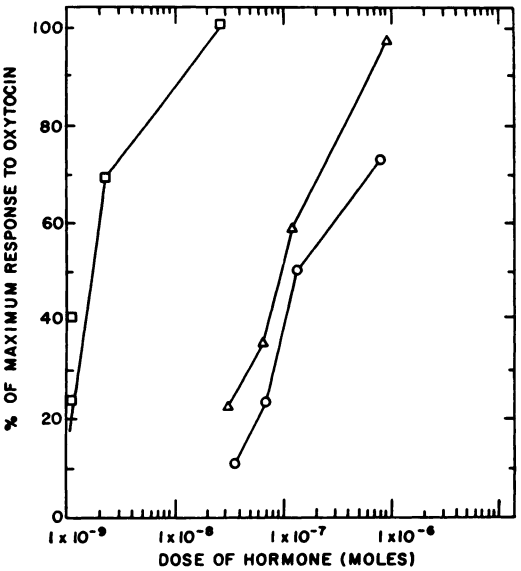

FIG. 2. *Cumulative log dose-response curves for oxytocin* (□——□), *[4-N^4-methyl-L-asparagine]-oxytocin* (△——△), *and [1-β-mercaptopropionyl-4-N^4-methyl-L-asparagine]-oxytocin* (○——○) *at pH 8.4 according to the procedure of Eggena et al. (8)*

Oxytocin served as a standard for control hemibladders. Each analogue was tested in contralateral experimental hemibladders against the standard at various response levels.

[2] All melting points were determined with a Thomas-Hoover capillary melting point apparatus and are corrected. Optical rotations were determined with a Carl Zeiss photoelectric precision polarimeter set at 0.005°. The samples for elementary analysis were dried for 12 hr at 100° over P_2O_5 in a vacuum and analyzed by Galbraith Laboratories, Knoxville, Tenn. Peptide hydrolysates were chromatographed on a Beckman/Spinco model 120C amino acid analyzer, using Beckman custom research resin PA-28.

L-prolyl-L-leucylglycinamide. Carbobenzoxy-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (3) (1.8 g) was decarbobenzoxylated by treatment with 20 ml of 4 N hydrobromic acid in glacial acetic acid for 30 min. The salt was precipitated with 400 ml of ether and washed by decantation with four 400-ml portions of ether. The salt was then isolated by filtration and dissolved in 150 ml of methanol. The solution was passed through a Rexyn RG1 column (Fisher, hydroxide form), which then was washed with 100 ml of methanol. After evaporation of the solvent and washings, the free pentapeptide was dissolved in 3 ml of dimethylformamide and allowed to react with *p*-nitrophenyl N^2-carbobenzoxy-N^4-methyl-L-asparaginate. The next day ethyl acetate was added, and the solid material was filtered off and washed with ethyl acetate and ethanol; yield, 1.8 g; m.p. 256–258° (with decomposition); $[\alpha]_D^{24}$ −68.3° (*c*, 1, in 97% formic acid).

$C_{40}H_{56}N_9O_{10}S$

Calculated:	C 56.3,	H 6.49,	N 14.8
Found:	C 56.1,	H 6.63,	N 14.6

Carbobenzoxy-L-isoleucyl-N^4-methyl-L-asparaginyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protected hexapeptide (1.7 g) was decarbobenzoxylated with hydrobromic acid in acetic acid and freed of hydrogen bromide by the method already described. The free hexapeptide obtained from the methanol solution was dissolved in 4 ml of dimethylformamide and treated with 0.85 g of *p*-nitrophenyl carbobenzoxy-L-isoleucinate. After 2 days the product was isolated as described in the preceding section; yield, 1.5 g; m.p. 261–263° (with decomposition); $[\alpha]_D^{24}$ −73.1° (*c*, 1, in 97% formic acid).

$C_{46}H_{66}N_{10}O_{11}S$

Calculated:	C 57.1,	H 6.88,	N 14.5
Found:	C 56.9,	H 7.11,	N 15.1

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-N^4-methyl-L-asparaginyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The heptapeptide (1.4 g) was decarbobenzoxylated and then freed of hydrogen bromide in the usual manner, and the free heptapeptide was dissolved in 4 ml of dimethylformamide and treated with 0.84 g of *p*-nitrophenyl *N*-carbobenzoxy-*O*-benzyl-L-tyrosinate. Isolation in the manner already described yielded 1.41 g of octapeptide; m.p. 262–264°; $[\alpha]_D^{24}$ −53.2° (*c*, 1, in 97% formic acid).

$C_{62}H_{81}N_{11}O_{13}S$

Calculated:	C 61.0,	H 6.69,	N 12.6
Found:	C 61.0,	H 6.90,	N 12.8

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-N^4-methyl-L-asparaginyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protected octapeptide (0.70 g) was suspended in 2,2,2-trifluoroethanol (10 ml) and treated for 10 min with anhydrous hydrogen bromide at 0°. After being stirred at room temperature for 20 min more, the solution was concentrated to dryness under vacuum and the residue was triturated with dry ether. The hydrobromide salt of the nonapeptide was dried and then dissolved in 5 ml of dimethylformamide. The solution was cooled in an ice bath while the pH was adjusted to 8 with triethylamine. The precipitated triethylamine hydrobromide was filtered off, and the free peptide was coupled with 0.30 g of *p*-nitrophenyl *N*-carbobenzoxy-*S*-benzyl-L-cysteinate. After 2 days the product was isolated in the usual manner to give 0.32 g of solid material; m.p. 246–248°; $[\alpha]_D^{24}$ −58.4° (*c*, 1, in 97% formic acid).

$C_{65}H_{86}N_{12}O_{14}S_2$

Calculated:	C 59.0,	H 6.55,	N 12.7
Found:	C 58.8,	H 6.53,	N 12.9

[4-N^4-Methyl-L-asparagine]-oxytocin. The protected nonapeptide (257 mg) was dissolved in 250 ml of anhydrous liquid ammonia at its boiling point and then reduced with sodium until a blue color remained for 30 sec. The ammonia was then removed under vacuum from the frozen state. The residue was dissolved in 500 ml of water containing 0.20 ml of trifluoroacetic

acid; the pH was adjusted to 8.2 by addition of 2 N NH_4OH, and the resulting solution was treated with an excess of 0.01 N potassium ferricyanide. After 30 min the pH was adjusted to 6.5 with dilute trifluoroacetic acid, and the ferrocyanide and excess ferricyanide ions were removed by treatment of the solution with AG3-X4 resin (Bio-Rad) in the chloride form. The solution obtained after removal of the resin was lyophilized. The resulting powder was taken up in 5 ml of the upper phase of the solvent system 1-butanol–benzene–pyridine–0.1% acetic acid (6:2:1:9) and purified by partition chromatography on Sephadex G-25. Folin-Lowry color determinations (15) revealed a major peak ($R_f = 0.39$), which was isolated by pooling of the fractions. Twice the volume of water was added, and the resulting solution was concentrated under vacuum to a small volume and lyophilized to yield 58 mg of a white powder; $[\alpha]_D^{23}$ −12.0° (*c*, 0.5, in 1 N acetic acid). A sample of this material migrated as a single component when subjected to paper electrophoresis in 6% acetic acid, chromatography on Whatman No. 1 paper in the upper phase of the solvent system 1-butanol–acetic acid–water (4:1:5), and thin layer chromatography on Silica Gel G in the following solvent systems: 1-butanol–acetic acid–water (4:1:5, upper phase), 1-butanol–pyridine–acetic acid–water (3:2:0.6:1.2), and 1-butanol–acetic acid–water (7:1:2). A sample of the lyophilized powder was submitted for elementary analysis without further drying.

$C_{43}H_{66}N_{12}O_{12}S_2 \cdot 2H_2O$

Calculated:	C 49.5,	H 6.76,	N 16.1
Found:	C 49.8,	H 6.35,	N 15.9

Another sample was hydrolyzed under vacuum in 6 N HCl at 110° for 21 hr and analyzed for ninhydrin-positive material according to the procedure of Spackman, Stein, and Moore (16). The following molar ratios were obtained, glycine being taken as 1.0: aspartic acid, 1.9; proline, 1.00; glycine, 1.00; cystine, 0.9; isoleucine, 0.9; leucine, 1.0; tyrosine, 0.8; ammonia, 2.1; and methylamine, 0.8.

*S-Benzyl-β-mercaptopropionyl-*L*-tyrosyl-*L*-isoleucyl-N^4-methyl-*L*-asparaginyl-*L*-asparaginyl-S-benzyl-*L*-cysteinyl-*L*-prolyl-*L*-leucylglycinamide.* The protected octapeptide amide (0.70 g) was deprotected with hydrobromic acid and converted to the free peptide with triethylamine as previously described. The free octapeptide was then coupled with *p*-nitrophenyl *S*-benzyl-β-mercaptopropionate (200 mg) in 5 ml of dimethylformamide. After 2 days the product was isolated as described above; yield, 0.506 g; m.p. 243–247°; $[\alpha]_D^{24}$ −56.6° (*c*, 1, in 97% formic acid).

$C_{57}H_{79}N_{11}O_{12}S_2$

Calculated:	C 58.2,	H 6.78,	N 13.1
Found:	C 57.6,	H 6.76,	N 13.1

*[1-β-Mercaptopropionic acid,4-N^4-methyl-*L*-asparagine]-oxytocin.* The preceding nonapeptide (400 mg) was reduced, oxidized, deionized, and lyophilized in the manner described for [4-N^4-methyl-asparagine]-oxytocin. The resulting powder was taken up in 5 ml of the upper phase of the solvent system 1-butanol–benzene–water, containing 3.5% acetic acid and 1.5% pyridine (1:1:2), and purified by partition chromatography on Sephadex G-25. Folin-Lowry color determinations revealed a major peak ($R_f = 0.41$), which was isolated as described previously and lyophilized to yield 113 mg of a white powder; $[\alpha]_D^{23}$ −64.9° (*c*, 0.5, in 1 N acetic acid). The analogue migrated as a single component when subjected to thin layer chromatography on Silica Gel G in the three solvent systems described previously for [4-N^4-methyl-L-asparagine]-oxytocin. The lyophilized powder was submitted for elementary analysis without further drying.

$C_{43}H_{65}N_{11}O_{12}S_2 \cdot 3H_2O$

Calculated:	C 49.4,	H 6.84,	N 14.7
Found:	C 49.4,	H 6.50,	N 14.3

A sample, hydrolyzed in 6 N HCl under vacuum at 110° for 21 hr, showed upon amino acid analysis the following molar ratios relative to glycine as 1.0: aspartic acid, 1.9; proline, 1.0; glycine, 1.0; isoleucine, 0.9; leucine, 1.0; tyrosine, 0.9; cys-

tine, 0.2; mixed disulfide of cysteine and β-mercaptopropionic acid, 0.4; ammonia, 1.8; and methylamine, 1.0.

Resolution of ammonia and methylamine during amino acid analysis. The analyses were carried out on a 19 × 0.9 cm column, employed for the resolution of basic amino acids and amines. Temperature was maintained from the start to the end of the chromatogram at 55°; the flow rate of the buffer (0.38 N sodium citrate buffer, pH 4.2) was set at 50 ml/hr.

Methods of biological assay. Avian vasodepressor activities were measured in conscious chickens by the method of Munsick, Sawyer, and van Dyke (17). Assays of oxytocic activity were performed on isolated uteri from rats in natural estrus by the procedure of Holton (18) as modified by Munsick (19), with the use of magnesium-free van Dyke-Hastings solution as bathing fluid. Rat pressor assays were carried out on male rats as described in the United States Pharmacopoeia (20). Assays for antidiuretic activity were performed on male rats according to the method of Jeffers, Livezey, and Austin (21) as modified by Sawyer (22).

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REFERENCES

1. I. Photaki and V. du Vigneaud, *in* "Proceedings of the European Peptide Symposium" (L. Zervas, ed.), p. 235. Pergamon Press, London, 1965; *J. Amer. Chem. Soc.* **87**, 908 (1965).
2. R. T. Havran, I. L. Schwartz and R. Walter, *J. Amer. Chem. Soc.* In press.
3. M. Bodanszky, *Nature* **175**, 685 (1955); M. Bodanszky and V. du Vigneaud, *Nature* **183**, 1326 (1959).
4. R. Walter, I. L. Schwartz, L. J. Trauth, M. C. Berman and D. H. Schlesinger, *Can. J. Chem.* **44**, 2348 (1966).
5. M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.* **81**, 5688 (1959).
6. V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope and R. D. Kimbrough, Jr., *J. Biol. Chem.* **235**, PC64 (1960); D. B. Hope, V. V. S. Murti and V. du Vigneaud, *J. Biol. Chem.* **237**, 1563 (1962).
7. D. Yamashiro, *Nature* **201**, 76 (1964); D. Yamashiro, D. Gillessen and V. du Vigneaud, *J. Amer. Chem. Soc.* **88**, 1310 (1966).
8. P. Eggena, I. L. Schwartz and R. Walter, *J. Gen. Physiol.* **52**, 463 (1968).
9. W. Y. Chan and V. du Vigneaud, *Endocrinology* **71**, 977 (1962).
10. W. Y. Chan, M. O'Connell and S. R. Pomeroy, *Endocrinology* **72**, 279 (1963).
11. B. M. Ferrier, D. Jarvis and V. du Vigneaud, *J. Biol. Chem.* **240**, 4264 (1965).
12. J. Rudinger, *Proc. 3rd Int. Congr. Endocrinol., Mexico City* (June 1968).
13. E. J. Ariëns, *Molecular Pharmacology*, Vol. I. Academic Press, New York, 1964.
14. P. F. Gulyassy and I. S. Edelman, *Biochim. Biophys. Acta* **102**, 185 (1965).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.* **30**, 1190 (1958).
17. R. A. Munsick, W. H. Sawyer and H. B. van Dyke, *Endocrinology* **66**, 860 (1960).
18. P. Holton, *Brit. J. Pharmacol.* **3**, 328 (1948).
19. R. A. Munsick, *Endocrinology* **66**, 451 (1960).
20. "The Pharmacopoeia of the United States of America," 17th rev., p. 749. Mack Publishing Co., Easton, Pa., 1965.
21. W. A. Jeffers, M. M. Livezey and J. H. Austin, *Proc. Soc. Exp. Biol. Med.* **50**, 184 (1942).
22. W. H. Sawyer, *Endocrinology* **63**, 694 (1958).