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Polarographic Studies on Natural Peptides. II. Polarography of Oxytocin, Lysine- and Arginine-Vasopressin in Co(III) Ammonia–Ammonium Chloride Solutions[1a]

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The polarographic behavior of the natural peptides, oxytocin, lysine- and arginine-vasopressin in cobaltic ammonia–ammonium chloride solutions has been studied and the relationship to the so-called "protein wave" indicated. Parameters studied with respect to their effect on the polarographic pattern were peptide concentration, pressure on the dropping mercury electrode, variations in ammonia concentration, the effect of peptide concentration on the half-wave potential of cobaltic ion, the influence of the cobaltic ion concentration and the effect of *p*H on the wave height.

Since Brdicka discovered a polarographic pattern with protein which consists of characteristic waves in a solution of 0.1 *N* ammonia and 0.1 *M* ammonium chloride–hexammine cobaltic chloride or cobaltous chloride, much has been reported on the behavior of various proteins in this type of solution. In general, the protein wave has been studied most in ammoniacal trivalent cobalt solutions since the polarographic waves are better defined and larger in this media. As indicated in the preceding paper,[2] we have chosen to study the peptide hormones from the posterior pituitary gland in an effort to understand better the pattern obtained during polarography of "deproteinized" serum filtrate. This is of interest since the pattern shows characteristic elevations in approximately 90% of cancer patients. In the previous paper[2] we reported the behavior of oxytocin, lysine- and arginine-vasopressin in ammonia–ammonium chloride solutions containing cobaltous ion. The present paper is a parallel study of these same substances in similar solutions containing cobaltic ion.

Experimental

Experimental methods are the same as in the preceding paper[2] except cobaltic hexammine chloride was used to make up the cobalt solutions in place of cobaltous chloride.

Results and Discussion

1. Origin of the Polarographic Reduction Waves of the Octapeptide Hormones.—Figure 1A shows the usual pattern of the "protein double wave" from the filtrate reaction[3] of serum. Although Jurka[4] described a third wave following the so-called "protein double wave," she did not report the behavior of this third wave in detail. Millar[5] also observed a third wave with insulin and bovine plasma albumin. In our laboratory we have studied this third wave using "deproteinized" serum filtrates from normal and cancer patients. As indicated in Fig. 1A the sensitivity of the galvanometer must be decreased four-fold in order to observe this wave. From Fig. 1 it is apparent that lysine-vasopressin (Fig. 1C) and arginine-vasopressin (Fig. 1D) give patterns very similar to that of the "deproteinized" serum filtrate, the only difference being the resolution of a third and fourth wave instead of a single wave in this area of the pattern. The pattern of oxytocin (Fig. 1B), although differing from the general character of the other patterns, is of particular interest to polarography since the so-called "minimum effect" (i_m in Fig. 1) returns to the level of the cobalt diffusion current. A similar minimum effect has also been obtained with acetylated lysine-vasopressin (see below). From the peak potentials indicated in Fig. 1, it can be seen that there is a progressive shift toward a more negative potential as one progresses from oxytocin (Fig. 1B) to lysine-vasopressin (Fig. 1C) and arginine-vasopressin (Fig. 1D). This shift shows some correlation with the isoelectric point for these compounds, the more basic peptide (arginine-vasopressin) having the more negative peak potential for all of the component waves in the pattern. This pattern is also very much like that which Muller obtained with an ultrafiltrate of pooled, untreated plasma.[6] The peak potentials of "deproteinized" serum filtrate show values intermediate between those of lysine-vasopressin and arginine-vasopressin. Balle-Helears[7] and Sasai[8] are of the opinion that the first wave of serum filtrate may depend on polysaccharide in mucoprotein. Although a contribution from this source cannot be eliminated from consideration in the case of the "deproteinized" serum filtrate, the simple disulfide-containing peptides, arginine- and lysine-vasopressin, give a pattern with all the essential characteristics without the involvement of any carbohydrate moiety.

Muller[6a] described a catalytic wave from the ultrafiltrate of serum in a trivalent cobalt–ammonia solution. He suggested that the first and second waves depend on the protein composition, the first wave being independent of a sulfhydryl or a disulfide, but dependent upon the arginine or lysine residues of the protein or polypeptides. In a later report[6b] it was shown that asparagine also gives a wave in the same range as wave I. From Fig. 1B, it can be seen that oxytocin, containing no lysine or arginine, shows this same wave. Therefore, Muller's explanation, to apply here, must involve the asparagine portion of oxytocin (see section 4).

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(2) H. Sunahara, D. N. Ward and A. C. Griffin, THIS JOURNAL, **82**, 6017, (1960).

(3) I. M. Kolthoff and J. J. Lingane, "Polarography," Vol. II, 2nd. Ed., Interscience Publishers, Inc., New York, N. Y., 1952, p. 866.

(4) E. Jurka, *Collection Czechoslov. Chem. Commun.*, **11**, 243 (1939).

(5) G. J. Millar, *Biochem. J.*, **53**, 385 (1953).

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(7) E. Balle-Helears, *Bruxelles-medical.*, **36**, 1 (1956).

(8) T. Sasai, *Rev. Polarography*, **5**, 26 (1957) (in Japanese).

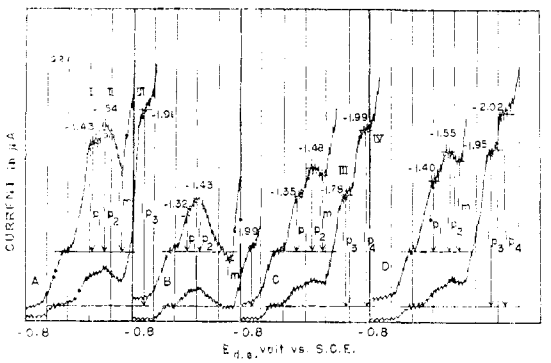

Fig. 1.—Polarograms of serum filtrate (A), oxytocin (B), lysine-vasopressin (C), arginine-vasopressin (D): A, serum filtrate, Sen = 1/50 and 1/200; B, oxytocin, 5.0 μg./ml., Sen = 1/50 and 1/200; C, lysine-vasopressin, 5.0 μg./ml., Sen = 1/50 and 1/200; D, arginine-vasopressin, 5.0 μg./ml., Sen = 1/50 and 1/200. Supporting electrolyte, hexammine cobalt(III) chloride, 1.1 m*M*/l., NH_4Cl, 0.1 *M* and NH_3, 0.1 *M* containing 2 drops of Triton × 100 0.1% solution per 5 ml. of sample volume.

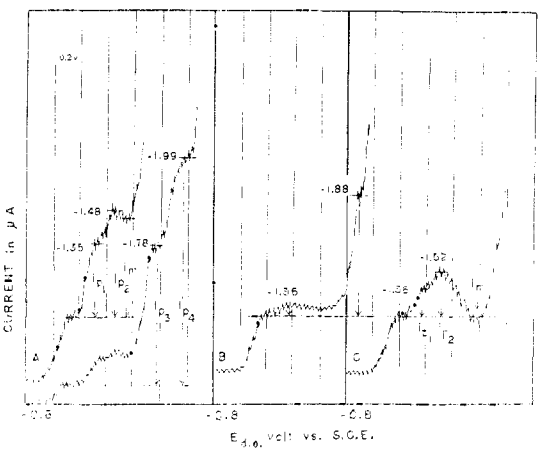

Fig. 2.—Polarograms of lysine-vasopressin (A), S,S'-bis-(benzyl)-lysine-vasopressin (B) and bis-(acetyl)-lysine-vasopressin (C): A, lysine-vasopressin, 5.0 μg./ml., Sen = 1/50 and 1/200; B, S,S'-bis-(benzyl)-lysine-vasopressin, 20 μg./ml., Sen = 1/50; C, bis-(acetyl)-lysine-vasopressin, 13 μg./ml., Sen = 1/50. Supporting electrolyte, hexammine, cobalt(III) chloride, 1.1 m*M*/l., NH_4Cl, 0.1 *M* and NH_3, 0.1 *M* containing 2 drops of Triton × 100 0.1% solution per 5 ml. of sample volume.

In order to learn more concerning the source of the polarographic pattern obtained with the peptides in Fig. 1, behavior of S,S'-bis-(benzyl)-lysine-vasopressin and bis-(acetyl)-lysine-vasopressin was investigated. As shown in Fig. 2, S,S'-bis-(benzyl)-lysine-vasopressin showed no first or second wave at 5.0 μg/ml., but in higher concentration (20 μg./ml., Fig. 2B), a small wave at the peak potential of the first wave of lysine-vasopressin appeared. From this it may be concluded that wave II (numbered as in Fig. 1) depends upon the presence of a disulfide bond in the molecule. Wave I also seems to be indirectly dependent upon the presence of the disulfide bond since the wave height was considerably decreased without it. In place of waves III and IV the benzylated vasopressin showed a single wave (−1.88 V, Fig. 2B)

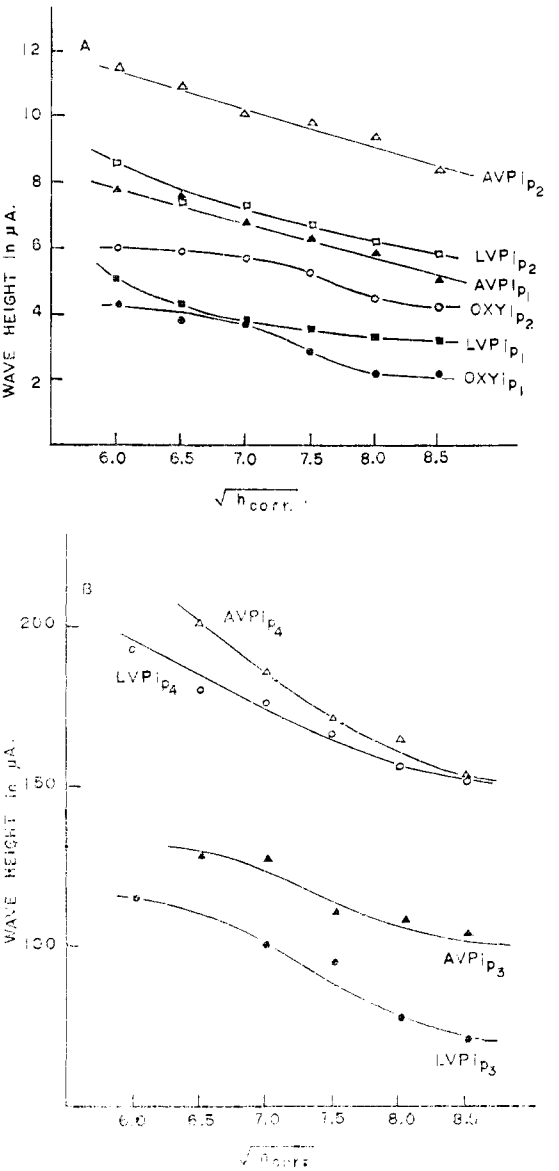

Fig. 3.—The relationship of wave height to effective pressure on the dropping mercury: (A) OXY i_{p1} and OXY i_{p2}, waves I and II of oxytocin; AVP i_{p1} and AVP i_{p2}, waves I and II of arginine-vasopressin; LVP i_{p1} and LVPi_{p2}, waves I and II of lysine-vasopressin. (B) AVP i_{p2} and AVP i_{p4}, waves III and IV of arginine-vasopressin; LVP i_{p3} and LVP i_{p4}, waves III and IV of lysine-vasopressin. Concentration of peptides: oxytocin, 13.9 μg./ml.; arginine-vasopressin, 5.0 μg./ml.; lysine-vasopressin, 5.0 μg./ml. Supporting electrolyte: hexammine cobalt(III) chloride, 1.1 m*M*/*l*, NH_4Cl, 0.1 *M* and NH_3, 0.1 *M* containing 2 drops of Triton × 100 0.1% solution per 5 ml. of sample volume.

intermediate between the peak potential of waves III and IV of the original lysine-vasopressin (Fig. 2A). In Fig. 2C, bis-(acetyl)-lysine-vasopressin showed waves I and II, but wave III and IV disappeared. Thus it may be concluded that waves III and IV of the original vasopressin depend upon the free amino groups. The minimum effect (i_m, Fig. 2C) which returns completely to the level of the cobalt limiting current has already been noted as similar to that of oxytocin. The data

for oxytocin and acetylated vasopressin thus suggest that the "minimum effect" was influenced by the relative number of basic groups in the molecule.

2. The Relationship of Wave Height to Peptide Concentration.—For this study the concentration of the ammonia, ammonium chloride and hexammine cobaltic chloride was kept constant and the peptide concentration varied. The data fit the Langmuir adsorption isotherm; *i.e.*, the plot of C/H against C was a straight line. Adsorption coefficients of wave I and wave II of oxytocin were calculated as 52.9 and 100.7, respectively. Wave III was sufficiently ill-defined at the various concentrations studied that an accurate plot of C/H against C could not be made. The plot of C/H against C for arginine- and lysine-vasopressin was a straight line for all peaks. The adsorption coefficients of arginine-vasopressin were calculated: 115 (wave I), 277 (wave II), 10000 (wave III) and 18314 (wave IV). For lysine-vasopressin the calculation gave: 386 (wave I), 740 (wave II), 12000 (wave III) and 28570 (wave IV).

Thus, as in the previous paper,[2] an adsorption phenomenon is clearly indicated as being involved in the reduction of the peptides at the dropping mercury electrode.

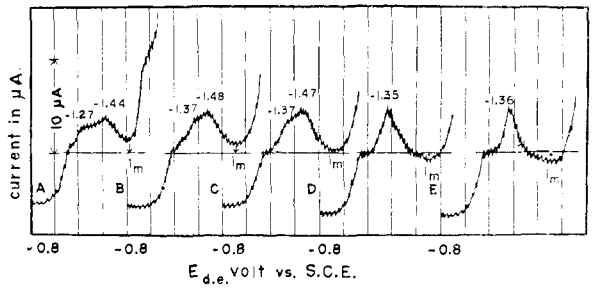

Fig. 4.—The effect of alteration of the concentration of ammonia on the oxytocin catalytic wave: A, 0 (pH 7.85); B, 0.05 M (pH 8.75); C, 0.1 M (pH 9.0); D, 0.04 M (pH 9.6); E, 0.7 M (pH 9.88). Oxytocin: 5.0 μg./ml. in hexammine cobalt(III) chloride, 1.1 mM/l, NH_4Cl, 0.1 M and NH_3 added as indicated; 2 drops of Triton × 100 0.1% solution per 5 ml. of sample volume.

3. Relationship of Wave Height to Pressure on the Dropping Mercury Electrode.—The effect of increasing pressure on the dropping mercury electrode during polarography in cobaltic-ammonia ammonium chloride solutions was investigated as in the preceding paper.[2] In general, the results were essentially the same in either cobaltic or cobaltous solutions; namely, increasing pressure gave a decrease in wave height. However, as can be seen in Fig. 3 the curves for oxytocin (i_{p1} and i_{p2} Fig. 3A) and vasopressin (i_{p3}, Fig. 3B) showed an inflection point between mercury pressures (corrected) of 49 and 64 cm. which was not observed with the cobaltous solutions. A plot of wave height against the surface area of the mercury drop (as obtained by the equation $A = kt^{2/3}$) also reflected this same inflection point. It is not clear why an inflection point should be observed under these conditions for the particular waves involved. Apart from this phenomenon the general character of behavior was that which would be expected for

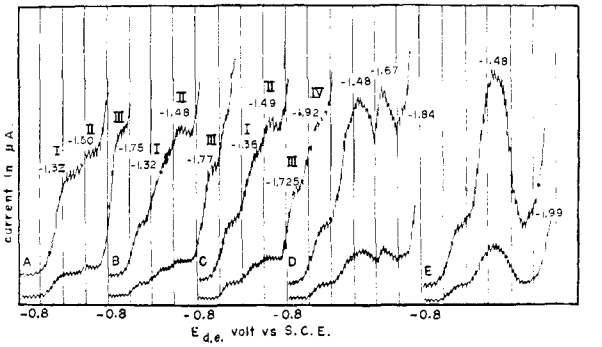

Fig. 5.—The effect of alteration of the concentration of ammonia on the lysine-vasopressin catalytic wave: A, 0 (pH 7.35); B, 0.05 M (pH 8.8); C, 0.1 M (pH 9.13); D, 0.4 M (pH 9.68); E, 0.7 M (pH 9.98); F, 1.0 M (pH 10.1). Lysine-vasopressin: 5.0 μg./ml. in hexammine cobalt(III) chloride, 1.1 mM/l, NH_4Cl, 0.1 M and NH_3 added as indicated; 2 drops of Triton × 100 0.1% solution per 5 ml. of sample volume.

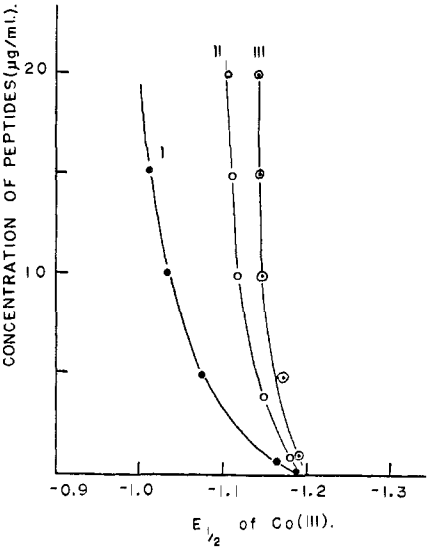

Fig. 6.—The relationship between half-wave potential of Co(III) and peptide concentration: I, lysine-vasopressin; II, S,S′-bis-(benzyl)-lysine-vasopressin; III, bis-(acetyl)-lysine-vasopressin. Supporting electrolyte: $Co(NH_3)_6Cl_3$, 1.1 mM/l., NH_4Cl, 0.1 M and NH_3, 0.1 M containing 2 drops of Triton × 100 0.1% per 5 ml. of sample volume.

reduction involving an adsorption at the mercury drop, *i.e.*, increased surface area gave an increased wave height and increased mercury pressure gave a decreased wave height, all of which may be taken as further evidence for the conclusion reached in section 2.

4. Influence of Ammonia Concentration.—The concentration of ammonium chloride or ammonia is an important factor in the production of the protein double wave polarographic pattern in trivalent cobalt systems as Hata and Matsushita[9] have shown. Figure 4 shows the change in the polarographic pattern of oxytocin with increasing ammonia concentration from 0 to 1.0 N and a constant concentration of 0.1 M ammonium chloride

(9) T. Hata and S. Matsushita, "Mem. Research Inst. Food Sci.," Kyoto Univ. No. 7, 33 (1951) (in English).

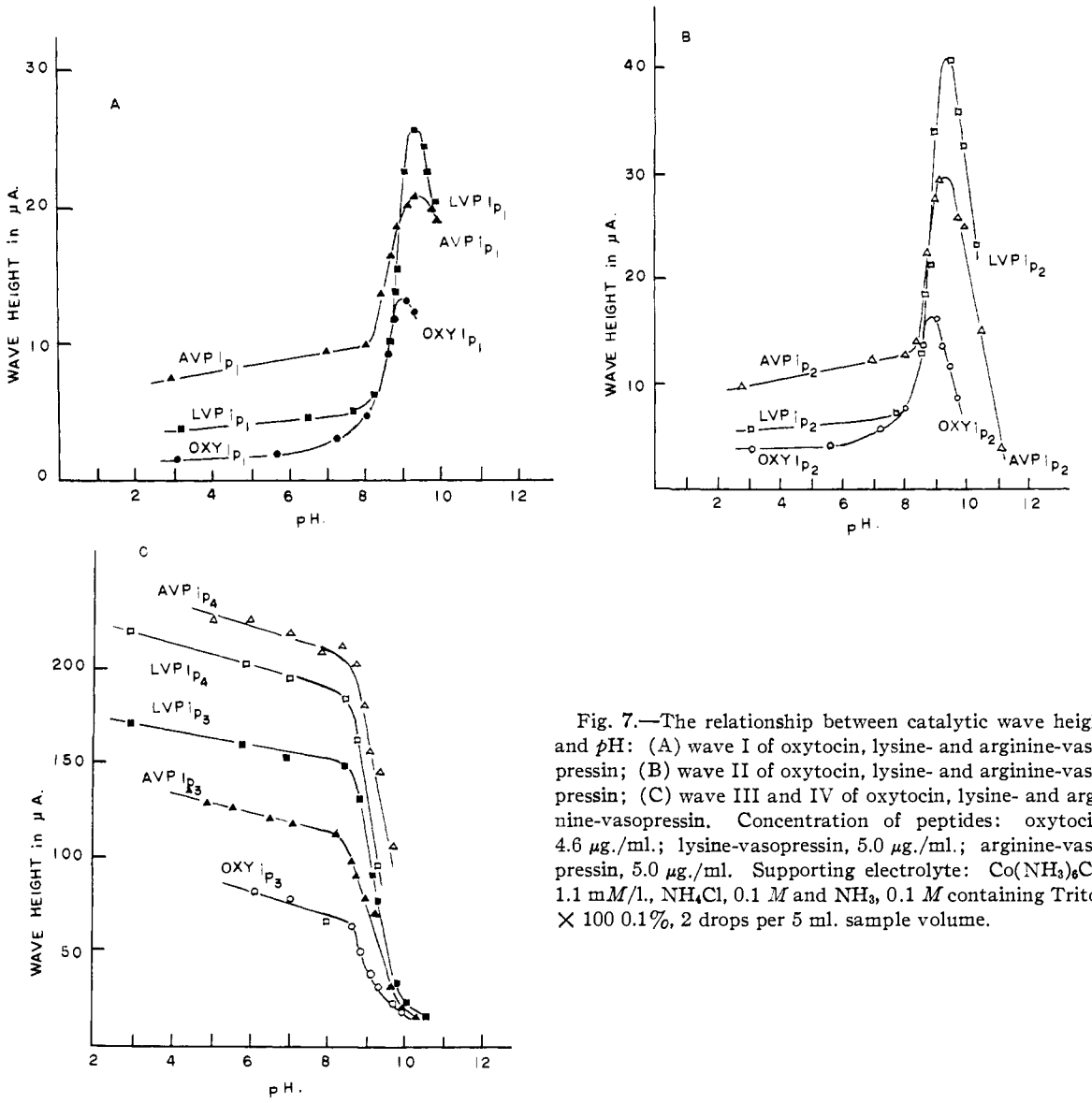

Fig. 7.—The relationship between catalytic wave height and *p*H: (A) wave I of oxytocin, lysine- and arginine-vasopressin; (B) wave II of oxytocin, lysine- and arginine-vasopressin; (C) wave III and IV of oxytocin, lysine- and arginine-vasopressin. Concentration of peptides: oxytocin, 4.6 μg./ml.; lysine-vasopressin, 5.0 μg./ml.; arginine-vasopressin, 5.0 μg./ml. Supporting electrolyte: $Co(NH_3)_6Cl_3$, 1.1 m*M*/l., NH_4Cl, 0.1 *M* and NH_3, 0.1 *M* containing Triton X 100 0.1%, 2 drops per 5 ml. sample volume.

and 1 m*M* hexammine cobalt chloride. The general character of the pattern was quite different from that observed in cobaltous systems, where waves I and II were seen to merge with increasing ammonia concentration. Between 0.1 and 0.4 *N* (Fig. 4C and D) wave II abruptly disappeared and wave I remained as a single sharp peak. This difference may be explained by the difference in *p*H of the various solutions (see legend, Fig. 4), as is shown in section 7. As noted in section 1 the "minimum effect" was greater in those compounds without charged amino groups. The more pronounced minimum effect observed in progressing from Fig. 4A to 4E correlated well with increasing *p*H and thus decreasing charge on the amino groups. It may also be noted that wave III for oxytocin disappeared with increasing ammonia concentration.

Increasing the ammonia concentration caused a slight shift in the peak potential of wave I to a more negative value for both oxytocin and vasopressin; the other waves were not shifted.

Using an ammonia concentration of 0.72 *N*, Muller[6a] reported a pattern similar to that of Fig. 4E for the polarogram of an ultrafiltrate of plasma. From subsequent studies[6b] Muller concluded that wave I (Fig. 4D and E) may be produced by arginine, lysine, ornithine, asparagine or 2,4-diaminobutyric acid. We have confirmed the production of such a pattern by the compounds mentioned and have also shown that glutamine, cystine and a number of other amino acids do not give this pattern. From this, by elimination of other possibilities, it may be concluded that the asparagine residue in oxytocin must be responsible for this wave. There is also chemical evidence that an asparagine residue in a peptide chain possesses an unusual susceptibility to reduction.[10]

The effect of varying ammonia concentration on the wave pattern for lysine-vasopressin is shown in Fig. 5. The behavior of arginine-vasopressin was the same for the ammonia concentrations studied. In general the catalytic reduction currents

(10) C. Ressler, THIS JOURNAL, 78, 5956 (1956).

obtained with vasopressin were two to three times as great as those obtained with an equivalent concentration of oxytocin. Since wave II became very pronounced with increased ammonia concentration (in marked contrast to the case observed with oxytocin), it was difficult to draw a conclusion as to the effect on wave I. From the diagrams obtained with the galvanometer at a lower sensitivity (lower curves, Fig. 5) wave I still persisted although virtually obscured by wave II. Wave II itself showed a remarkable increase with increasing concentration of ammonia. It is suggested that the molecule of vasopressin shows a much greater tendency to form a reducible ammonia cobalt complex than oxytocin. Apart from this suggestion we have no explanation for the marked difference in the behavior of wave II of oxytocin and vasopressin with respect to ammonia concentration. Waves III and IV (Fig. 5) showed a progressive decrease with increasing ammonia concentration which was probably a function of the degree of ionization of the basic groups, or alternatively, a competition of the basic groups and ammonia for complex formation with the cobalt.

5. The Effect of Peptide Concentration on the Cobaltic Ion Half-wave Potential.—The effect of peptide concentration on the cobaltic ion half-wave potential was studied for the peptides lysine-vasopressin; S,S′-bis-(benzyl)-lysine-vasopressin; and acetylated vasopressin. The results are summarized in Fig. 6. The effect was essentially the same as that obtained with cobaltous ion,[2] thus the same reasoning may apply to the relationships of cobaltic-ammonia-peptide complexes for these three peptides, *i.e.*, vasopressin forms a stronger complex than acetylated vasopressin which in turn forms a stronger complex than the benzylated vasopressin.

6. Relationship of Wave Height to the Cobaltic Ion Concentration.—The relationship of wave height to cobalt concentration for the protein double wave was studied by Jurka[4] and Tropp, *et al.*,[11] who found that the data fit a parabolic function.

The relation between wave height and the concentration of trivalent cobalt was studied over the range 10^{-4}, to 10^{-2} M trivalent cobalt in 0.1 N ammonia and 0.1 M ammonium chloride. At the highest concentration the current at the various peaks was too great for accurate measurements. As previous investigators have found, the wave height followed a parabolic type of response for all the waves as a function of cobaltic ion concentration, except for wave I of all three peptides where the relationship was linear.

The peak potential of wave I and II was shifted to a more negative potential as the cobaltic ion concentration was increased—a difference of 0.26 volts going from a Co^{111} concentration of 1×10^{-3} to 7×10^{-3} M. The shift was linear over the concentration range studied.

7. Influence of *p*H on the Peptide Catalytic Waves.—Millar[12] studied the effect of *p*H on wave I and II for a number of proteins. All proteins studied gave a maximum wave height at a *p*H near 9. The peptides studied here showed the same behavior, as can be seen in Fig. 7A and B. Calculated either on a molar or concentration basis, lysine-vasopressin gave the highest peak current in the area of the maximum but at *p*H values below eight arginine-vasopressin showed considerably higher peak current values. Oxytocin always gave peak current values inferior to either of the vasopressins.

Wave III, in Millar's studies,[12] showed a gradual decline with increasing *p*H until at approximately *p*H 9 there was a precipitous drop and the wave was virtually eliminated at *p*H about 10. Again the peptides show this same behavior for wave III and also for wave IV in the case of the vasopressin samples, as shown in Fig. 7C. The data suggests that ionized basic groups are necessary for the production of waves III and IV and that the waves decrease rapidly as the *p*H goes from about 8.5 to 10.0, the range in which the basic groups would be expected to lose their charge.

(11) C. Tropp, L. Juhling and F. Geiger, *Z. physiol. Chem.*, **262**, 225 (1959).

(12) G. J. Millar, *Biochem. J.*, **53**, 393 (1953).