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Reactions of Cupric Ion with Lysine Vasopressin and Acetyllysine Vasopressin*

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Lysine vasopressin was purified by ion exchange chromatography and gel filtration to provide a peptide of adequate purity for quantitative physical chemical studies. The three *pK*'s of the peptide were determined by spectrophotometric and potentiometric titrations. Spectral analyses of cupric ion–lysine vasopressin solutions revealed that a 1:1 complex is formed with an absorption maximum at 525 mμ. Similar measurements with copper–acetyllysine vasopressin solutions demonstrate no evidence of complex formation of the type exhibited by copper–lysine vasopressin. Titration of the 1:1 copper–lysine vasopressin and spectral analyses provide data to support the hypothesis that the complex forms by interaction of the cupric ion at the free terminal α-amino acid group of the peptide followed by subsequent loss of three hydrogen atoms from neighboring peptide bonds. A tetradentate structure consistent with this interpretation is presented. The apparent molecular extinction coefficient, stability constant, and free energy of formation of the complex have been estimated. The use of solutions of copper–lysine vasopressin under conditions expected to maintain maximum complex formation resulted in no significant alteration of the effect of the hormone in the toad bladder assay.

There is considerable evidence indicating that cupric ions react with peptides in solution by interaction at the free terminal amino end with subsequent release of protons from the peptide nitrogen atoms. Dobbie and Kermack (1955a) described the formation of the 1:1 copper-glycylglycine complex as a reaction of cupric ion at the *N*-terminal amino group of the peptide followed by chelation at the peptide nitrogen atom to produce the loss of a peptide proton and subsequent loss of a proton from a coordinated water molecule. This interpretation of the reaction was supported by work of Datta and Rabin (1956) and by studies of Koltun *et al.* (1960). When the 1:1 copper-triglycine complex was titrated by Dobbie and Kermack (1955b), they observed that three equivalents of alkali per copper atom were taken up in the *p*H range 4–8. During the addition of the first two equivalents of alkali the color of the solution deepened from a pale greenish blue to a very dark blue, but thereafter the color of the solution changed through violet to a deep purple, while the third equivalent of alkali was added. This corresponded to shift in the wavelength of the maximum absorption of the solution from about 650 mμ to about 550 mμ. They suggested that this color change is produced by the formation of a tridentate complex in which the copper is attached to the free terminal amino group and the nitrogen atoms at two peptide bonds.

Martin *et al.* (1960) reported that tetraglycine reacts in a 1:1 molar mixture with cupric ions to release three protons in addition to the proton from the terminal amino group, and their data indicated that ionization of peptide hydrogens had occurred. Koltun *et al.* (1963) have also studied the reaction of tetraglycine with cupric ions, and these workers found that reaction of 1 mole of cupric ion with 1 mole of tetraglycine and 4 moles of base produced a complex with a wavelength maximum of 520 mμ and extinction coefficient of 145 $mole^{-1}$ cm^{-1}. The uptake of 4 moles of base by this complex occurred approximately between *p*H 4 and 10.5, and the dissociating protons were identified as those of the terminal α-amino group and three peptide bond nitrogens.

Campbell *et al.* (1960) described the spectral characteristics of the 1:1 copper–lysine vasopressin complex and the 1:1 copper-oxytocin complex, and Breslow (1961) reported data concerning the reaction of cupric ion with oxytocin and its derivatives. The work of Breslow suggested that cupric ion reacted with oxytocin to complex with the terminal α-amino group and to produce subsequent ionization of protons from neighboring peptide bonds. The evidence presented in this paper indicates that the cupric ion reacts with lysine-vasopressin to from a 1:1 complex by interaction at the terminal α-amino group with concomitant release of three protons from peptide nitrogen atoms.

Experimental

Purification of Lysine Vasopressin (LVP).[1]—An acetone powder of whole hog pituitary glands from which corticotropin had been removed by treatment with oxycellulose was kindly supplied by Dr. J. B. Lesh of Armour Pharmaceutical Company. The preparation of this powder has been described previously (Porath and Schally, 1962). The material was subjected to chromatography on carboxymethylcellulose and rechromatographed on the same exchanger using the techniques described by Schally *et al.* (1960a) and Schally *et al.* (1960b). The lysine vasopressin was then purified by Sephadex gel filtration in a manner similar to that of Porath and Schally (1962). A column, 3.2 × 68 cm, was packed with G-25 Sepha-

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[1] Abbreviation used in this paper: LVP, lysine vasopressin.

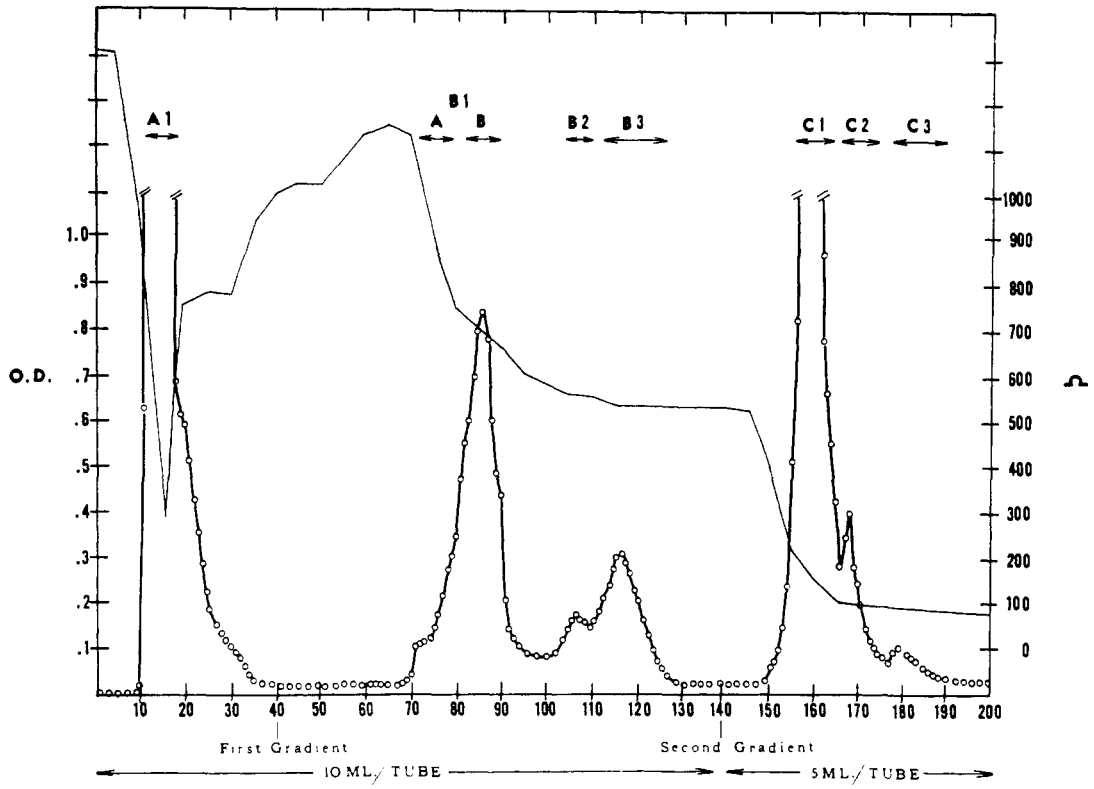

FIG. 1.—Purification of lysine vasopressin on carboxymethylcellulose. The elution curve was obtained from the following experiment: 1.3 g of the acetone powder of whole hog pituitary dissolved in a 0.01 M NH_4OAc buffer at *p*H 4.5 was applied on a carboxymethylcellulose column, 1.9 × 50 cm. Elution with the starting buffer was continued until tube 40. The first gradient to 0.02 M NH_4OAc at *p*H 6.0 was started at tube 40. The second gradient to 0.2 M NH_4OAc at *p*H 7.0 was started at tube 140. The flow rate was 4 ml/min. Lysine vasopressin was found to be concentrated in fraction C_1.

dex, medium grade, according to the procedure of Flodin (1961). A solution of 102 mg of the carboxymethylcellulose-purified LVP in 5 ml of water was added to the column. After washing with 200 ml of water, the column was eluted with 0.005 M ammonium acetate. The flow rate of the column was 4 ml/min. The elution curve was obtained by measuring the optical density at 280 mμ and the resistance of the effluent. The material in the tubes of the major peak were collected and lyophilized.

Analyses of Lysine Vasopressin.—The moisture content of LVP was determined by heating at 105° in a vacuum oven for 16 hours. Amino acid composition of purified LVP was carried out using the Spinco amino acid analyzer and the method of Moore *et al.* (1958). Paper chromatography was performed using as solvent *n*-butanol–glacial acetic acid–water in the ratio of 4:1:5. Paper electrophoresis of the product employed the E-C Apparatus Co. Model 1331, and the following buffer systems: sodium acetate–acetic acid, *p*H 4–6; sodium phosphate, *p*H 7; tris(hydroxymethyl) aminomethane-HCl, *p*H 8–9; glycine-NaOH, *p*H 10–12. Stains employed were bromophenol blue (Durrum, 1950) and 0.2% ninhydrin in acetone. Drs. Guillemin and Schally supplied the U.S.P. rat pressor assay (Guillemin *et al.* 1962). A sample of LVP supplied by Drs. Guillemin and Schally was employed as a comparison in certain analyses. This sample had been purified by carboxymethylcellulose chromatography and countercurrent distribution as described by Schally *et al.* (1960b).

Spectral Analyses.—The spectra of various LVP-copper complexes were determined by analysis of appropriate solutions in the Carl Zeiss PMQ II spectrophotometer. The length of the light path through the solution was 1 cm, and the temperature was controlled by means of a temperature-control jacket through which water was circulated from a Brinkmann-Haake Ultra Thermostat Type F.

Potentiometric Titrations.—These titrations were carried out using a Radiometer Automatic Titrator Type TTT1 with a Radiometer Titrigraph Type SBR2 and the Type SBU1 syringe burette. The electrodes employed were the Model G222B semimicro glass electrode and Type K4312 calomel reference electrode. Temperature was controlled by using a jacketed titration vessel through which water passed from a Brinkmann-Haake Ultra Thermostat Type F. The vessel was fitted with a rubber bung with appropriate holes for electrodes and nitrogen delivery and exhaust. All titrations were performed with nitrogen bubbling through the reaction mixture. Stirring was also facilitated by means of a micromagnetic stirring bar. In a typical titration a 1:1 mixture of 5.00×10^{-4} M LVP and 5.00×10^{-4} M $CuCl_2 \cdot 2H_2O$ in 0.16 M KCl were titrated with 0.0099 M NaOH or 0.0110 N HCl.

Spectrophotometric Titrations.—Solutions of 5.00×10^{-4} M LVP in 0.16 M KCl were titrated with 0.0099 M NaOH as previously described. Samples were withdrawn from the titration vessel at appropriate *p*H values, and the optical density measured at 2425 A, 2900 A, and 2950 A immediately in the sgectrophotometer.

Preparation of Acetyllysine Vasopressin.—Bis-acetyllysine vasopressin was prepared according to method described by Sunahara *et al.* (1960). The product was purified by chromatography on carboxymethylcellulose equilibrated with 0.01 m NH_4OAc buffer. The acylation reaction mixture was added directly to the column and elution continued with the starting

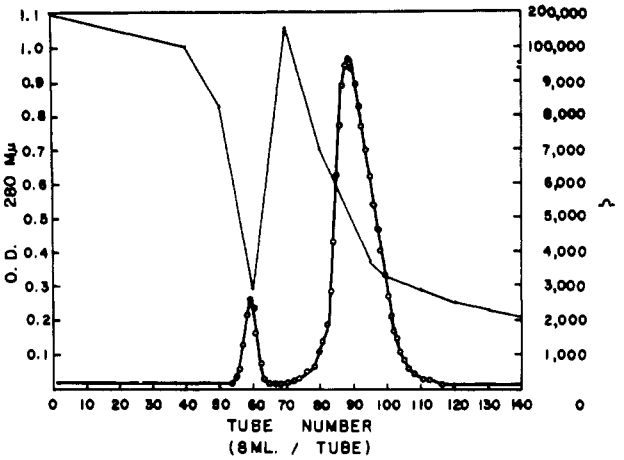

FIG. 2.—Purification of lysine vasopressin on Sephadex G-25. The elution curve was obtained from the following experiments: 102 mg of material purified by carboxymethylcellulose chromatography in 5 ml of water was applied on a Sephadex G-25 column, 3.2 × 68 cm. Elution with water was continued until tube 25. Stepwise elution with 0.005 M NH_4OAc at *p*H 6.0 was then applied. The line —O—O—O represents optical density, OD, and the line, ———, represents resistance in ohms, Ω. The flow rate was 4 ml/min. Purified lysine vasopressin was obtained from the major peak.

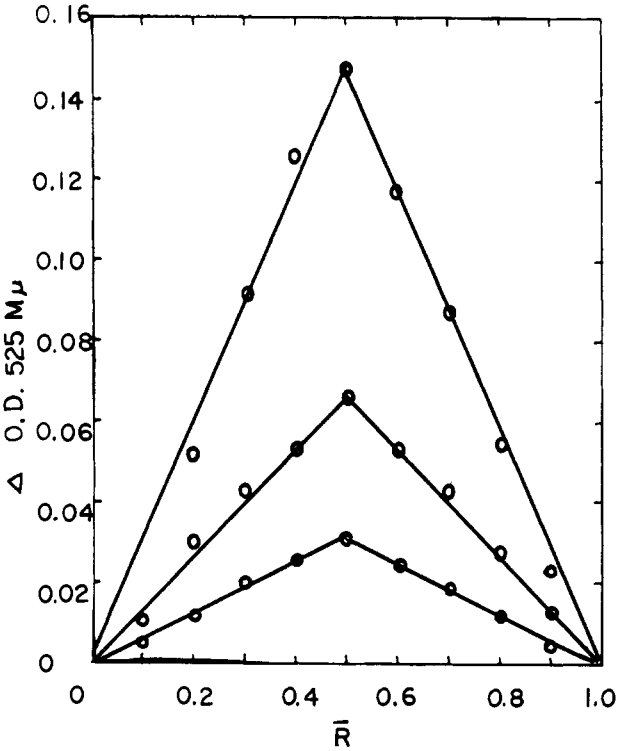

FIG. 3.—Continuous variation plots for the cupric ion–lysine vasopressin complex at *p*H 8.00. The total concentrations of copper plus vasopressin were 2.00×10^{-3} M for the upper curve, 1.00×10^{-3} for the middle curve, and 5.00×10^{-4} for the lower curve. $\bar{R}$ is the mole fraction of Cu^{++} and at $\bar{R}$ values above 0.7 turbidity was observed due to the formation of cupric hydroxide.

buffer. After the acetylated product passed through the column, the unreacted LVP and other products were obtained by gradient elution to 0.05 M NH_4OAc at *p*H 6.8. The acetylated product was further purified by gel filtration on Sephadex G-25 as described above. The final product exhibited only one dissociable group in the *p*H range 3–11, and this group was identified as the tyrosine phenoxyl group by means of spectrophotometric and potentiometric titration.

Toad Bladder Assay.—The method employed was similar to that described by Bentley (1958). Bladders were dissected from the toad *Bufo marinus*, and one lobe of the bladder was used as a control for the other. The bladders containing a 1:6 dilution of Ringer's solution at *p*H 7.7 were equilibrated in a bath of 100 ml of Ringer's solution at *p*H 7.7, and the average water loss was established by weighing the bladders at appropriate times. A 0.10-ml aliquot of a 2.00×10^{-5} M LVP solution was introduced to the bath on the serosal side of the bladder, and the initial response was measured to establish that the bladders were responsive to the hormone. The bladders were then washed, the bathing solutions changed, and the average water loss again established. A 1:1, 10:1, or 100:1 copper-LVP aliquot of the same concentration (2.00×10^{-5} M) was then added and the response determined. Again the hormone was washed out, the solutions changed, and equilibrium established. Finally another 0.1-ml aliquot of 2.00×10^{-5} M LVP was added to determine if the bladders were still responsive.

RESULTS

Purification of LVP.—The fractionation of LVP by carboxymethylcellulose chromatography is shown in Figure 1. Fraction C_1, was pooled, lyophilized, and rechromatographed on carboxymethylcellulose. The material was then purified by means of gel filtration on Sephadex G-25 as shown on Figure 2. The major peak was pooled and lyophilized three times to remove ammonium acetate.

Analyses of LVP.—The moisture content of various preparations ranged from 3 to 7%. Amino acid analyses gave the constituent eight amino acids in a 1:1 mole ratio, and calculation of the amino acid content demonstrated that the final product was over 98% pure. This range of purity was the same as that obtained for the sample that had been provided by Drs. Schally and Guillemin and that had been prepared by carboxymethylcellulose chromatography and countercurrent distribution. The final product from gel filtration migrated as one spot in paper chromatographic analysis. The peptide was shown to be homogeneous by paper electrophoresis at 6 *p*H values from 6 to 12. The U.S.P. rat pressor assay of a typical product was 296 units/mg.

Spectral Analyses.—Examination of the spectrum of lysine vasopressin in the presence of cupric ion solutions demonstrated the presence of a copper–lysine vasopressin complex that absorbed maximally at 525 mμ. The composition of the complex at *p*H 8.00 was determined by Job's method of continuous variations (Job, 1936) and the analysis is presented in Figure 3. The total concentrations of copper plus vasopressin for the three curves are 2.00×10^{-3} M, 1.00×10^{-3} M, and 5.00×10^{-4} M. At R values above 0.7 turbidity was observed due to the formation of cupric hydroxide and these solutions were clarified by centrifugation. The alteration of the spectrum of the 1:1 Cu-LVP complex as the *p*H of the solution increases is shown in Figure 4. The chromatographic purification of acetyllysine vasopressin is described in Figure 5, and a comparison of the spectrum of a 1:1 mixture of Cu-acetyl-LVP with that of the 1:1 Cu-LVP complex is given in Figure 4.

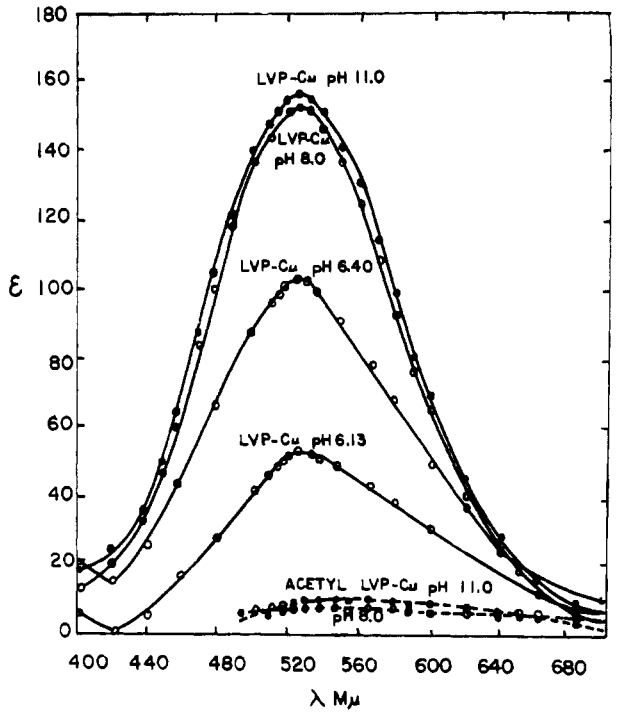

FIG. 4.—Spectra of 1:1 cupric ion–lysine vasopressin and 1:1 cupric ion–acetyllysine vasopressin at various *p*H's. The concentrations of the 1:1 mixtures of metal-peptide were 5.00 × 10^{-4} M with respect to $CuCl_2 \cdot 2H_2O$, acetyl-LVP, and LVP. The *p*H was adjusted by additions of 0.0099 M NaOH.

Determination of pK's LVP.—The pK_1 (α-NH_2) of the peptide was evaluated by the method of Li *et al.* (1954) from titrimetric data reported in Table I. The pK_2 (phenoxyl) of the peptide was evaluated by the method of Paiva and Paiva (1962) from spectrophotometric titration data presented in Table II. The value of pK_3 (ϵ-NH_2) was estimated from data obtained by potentiometric titration using the value of

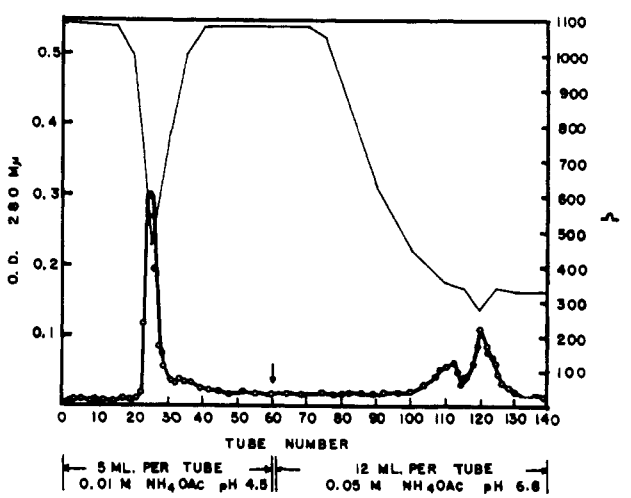

FIG. 5.—Chromatographic purification of acetyllysine vasopressin. The elution curve was obtained from the following experiment: The acylation mixture from 31.45 mg of LVP was diluted to 5.0 ml with 0.01 M NH_4OAc at *p*H 4.5 and applied on a carboxymethylcellulose column, 2.2 × 50 cm, that had been equilibrated with the same buffer. Elution with starting buffer was continued until tube 60. Stepwise elution with 0.05 M NH_4OAc at *p*H 6.8 was started at tube 60. The flow rate was 2.5 ml/min. Bis-acetyllysine vasopressin was obtained from the first peak.

TABLE I

DISSOCIATION CONSTANT OF α-TERMINAL AMINO GROUP (pK_1)[a,b]

n[c]	*p*H	$\frac{n-2}{3-n}$	*p*K
2.85	5.73	5.67	6.48
2.75	5.95	3.00	6.42
2.65	6.20	1.86	6.47
2.50	6.40	1.00	6.40
2.40	6.55	0.67	6.42
2.30	6.70	0.43	6.35
		Av.	6.42

[a] Calculated by method of Li *et al.* (1954). [b] In aqueous solutions at 25.0°. [c] n = moles of protons bound per mole peptide.

TABLE II

TYROSINE DISSOCIATION CONSTANT OF LVP[b,a]

Wavelength (A) *p*H		8.69	9.29	9.80	10.28	Average
2425	ε	1510	2862	5095	7423	
	*p*K	9.93	9.87	9.90	9.91	9.90
2900	ε	424	667	1086	1514	
	*p*K	9.92	9.91	9.91	9.95	9.92
2950	ε	233	502	994	1464	
	*p*K	9.93	9.91	9.90	9.94	9.92

[a] Calculated by method of Paiva and Paiva (1962). [b] In aqueous solutions at 25.0°.

TABLE III

ESTIMATED DISSOCIATION CONSTANT OF ε-AMINO GROUP (pK_3)[a,b]

n[c]	*p*H	$\frac{n}{1-n}$	$\frac{1+(H^+)(2-n)}{K_2(1-n)}$	*p*K
0.70	10.47	2.34	2.22	10.49
0.60	10.52	1.50	1.88	10.42
0.50	10.55	1.00	1.71	10.32
0.40	10.59	0.67	1.56	10.21
			Av.	10.36

[a] Calculated by method of Li *et al.* (1954). [b] In aqueous solutions at 25.0°. [c] n = moles of protons bound per mole peptide.

pK_2 reported in Table II. This estimation was made using the equation of Li *et al.* (1954), and the results are presented in Table III.

Potentiometric Titration of LVP and 1:1 Cu-LVP.—Titrations of LVP and the 1:1 Cu-LVP complex are presented in Figure 6. These curves were constructed according to the method described by Kenchington (1960), and they have been corrected for a small amount of ammonium acetate that was not released upon lyophilization.

Stability Constant and Extinction Coefficient of the 1:1 Cu-LVP Complex.—The molecular extinction coefficient and the stability constant of the 1:1 Cu-LVP complex were evaluated from spectrophotometric data according to the method described by Turner and Anderson (1949). The results are presented in Table IV.

Toad Bladder Experiments.—The effect of adding a 1:1 Cu-LVP mixture to the toad bladder assay system is shown in Figure 7.

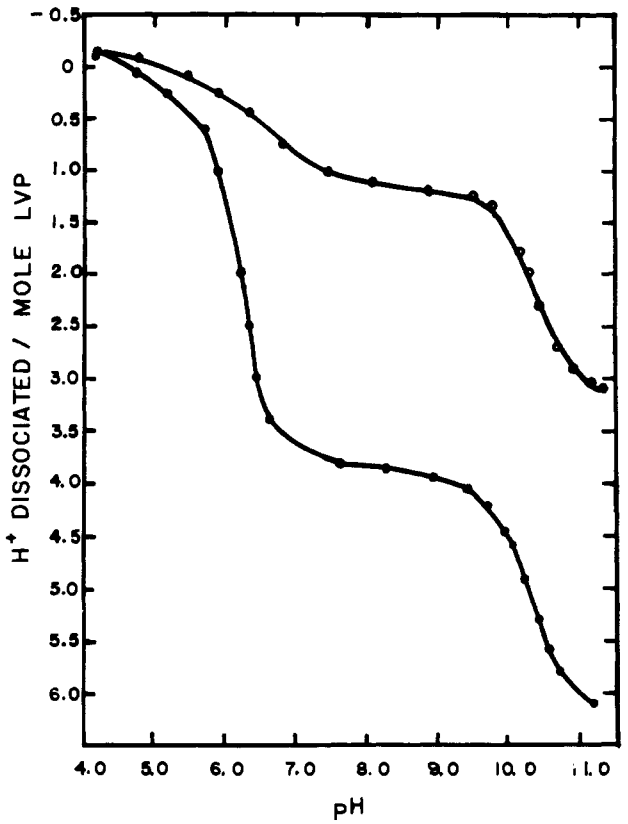

FIG. 6.—Titrations of lysine vasopressin and 1:1 cupric ion–lysine vasopressin complex. Upper curve: A solution of 5.00×10^{-4} M LVP in 0.16 M KCl titrated with 0.0110 M HCl and 0.0099 M NaOH at 25.0°. Lower curve: A solution of 5.00×10^{-4} M LVP and 5.00×10^{-4} M $CuCl_2 \cdot 2H_2O$ in 0.16 M KCl titrated with 0.0099 M NaOH and 0.0110 M HCl at 25.0°.

DISCUSSION

The analyses of the final LVP product demonstrated that adequate purity had been achieved to provide significant quantitative information in the subsequent studies. The method of continuous variations (Figure 3) applied at 525 mμ indicates that the mole ratio of copper to lysine vasopressin in the complex is 1:1. The spectral analyses of the 1:1 Cu-LVP complex at various *p*H's (Figure 4) shows that the absorption of the complex increases appreciably up to *p*H 8. The absorption curves above *p*H 8 are practically superimposable. This suggests that complex formation is essentially complete, and that this occurs before the disassociations of the phenoxyl and ε-amino groups are appreciable. Previous reports that oxytocin forms a copper complex with the same spectral characteristics as Cu-LVP indicate further that the ε-amino group is not involved in complex formation (Breslow, 1961; Campbell *et al.*, 1960). Analyses of the spectrum of a 1:1 mixture of Cu-acetyl-LVP (Figure 4) at *p*H 8 and *p*H 11 demonstrate no evidence of complex formation of the type exhibited by Cu-LVP. This result was confirmed by potentiometric titration of the Cu-acetyl-LVP solution. In this titration precipitation of cupric hydroxide occurred at *p*H values above 6. These data indicate that blocking the terminal α-amino group of the peptide results in a product that does not form the 1:1 complex.

TABLE IV

APPARENT ASSOCIATION CONSTANT OF CU-LVP[a]

Total (LVP)[b] × 10^{-3}	Total (Cu^{++}) × 10^{-3}	(Cu-LVP) × 10^{-3}	OD	ε[c]	K_1 × 10^5	log K_1
0.900	4.10	0.898	0.125	139	1.40	5.14
0.965	0.965	0.898	0.125	139	2.00	5.30
1.09	3.92	1.08	0.150	139	1.91	5.27
1.15	1.15	1.08	0.150	139	2.41	5.38
1.27	3.74	1.26	0.175	139	1.70	5.23
1.34	1.34	1.26	0.175	139	2.36	5.37
1.46	3.55	1.45	0.200	138	2.31	5.36
1.53	1.53	1.45	0.200	138	2.72	5.43
					Av.	5.31

[a] Calculated by method of Turner and Anderson (1949). [b] ( ) = molar concentration. [c] ε = molecular extinction coefficient of 1:1 Cu-LVP complex at 525 mμ.

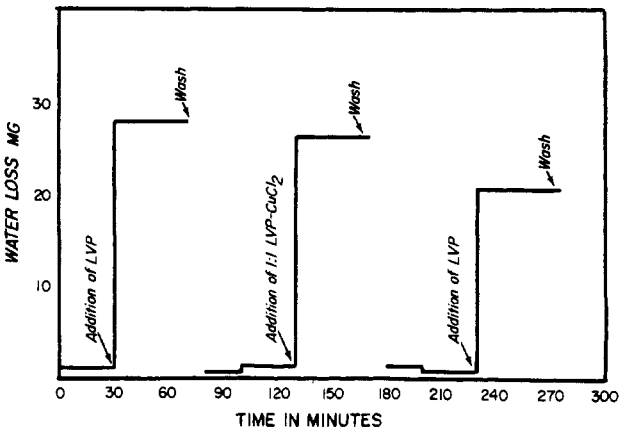

FIG. 7.—Effect of 1:1 cupric ion–lysine vasopressin on water transport. The addition of 0.1 ml of a 2.00×10^{-5} M 1:1 mixture of Cu-LVP, was substituted for the same concentration of LVP in the same assay. The *p*H in each case was 7.7.

The titrations shown in Figure 6 indicate that during the formation of the Cu-LVP complex three protons are dissociated in the *p*H range 4–8 in addition to the proton normally dissociated from the terminal α-amino group at *p*H 6.42. The *pK*'s of the phenoxyl group (9.92) and the ε-amino group (10.4), are unaltered, confirming the hypothesis that these dissociations are not involved in complex formation. The apparent *pK*'s of the protons that are dissociated during complex formation indicate that these protons are ionized from the peptide nitrogen atoms rather than from co-ordinated water molecules in the complex. Dobbie and Kermack (1955a) and Datta and Rabin (1956) presented evidence to demonstrate that dissociation of a proton from a coordinated water molecule in the 1:1 copper-glycylglycine complex occurs a about *p*H 9.6. The last apparent *pK* of dissociation caused by Cu-LVP complex formation occurs at *p*H 6.8. The spectral alteration described by Dobbie and Kermack (1955b) for the formation of the 1:1 copper-triglycine complex, and the spectral characteristics of the 1:1 copper-tetraglycine complex (Koltun *et al.*, 1963) are similar to those reported in this paper for 1:1 Cu-LVP–complex formation. The results described suggest that the 1:1 Cu-LVP complex forms by interaction of the cupric ion at the free terminal α-amino group of the peptide followed by subsequent loss of three hydrogen atoms from neighboring peptide bonds. A tetradentate structure consistent with this interpretation is presented in Figure 8. The molecular extinction coefficient and stability constant were reported in Table IV. From the apparent dissociation constant, one may calculate the free energy of forma-

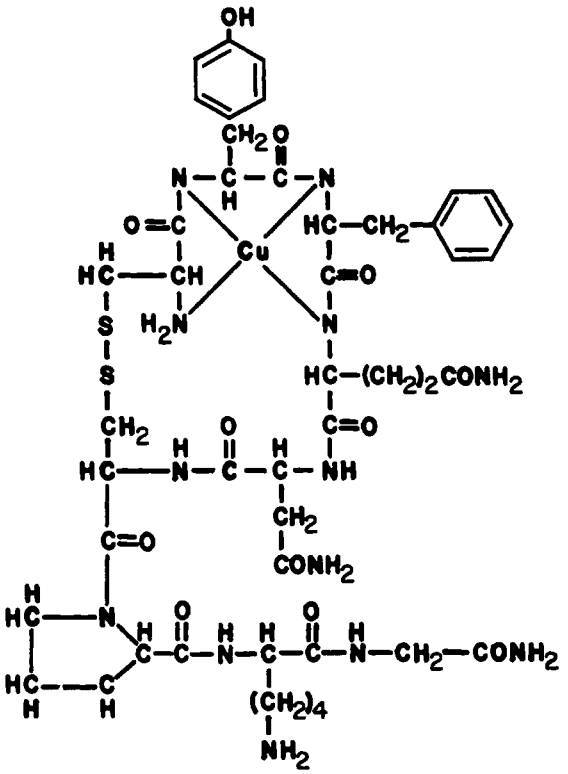

FIG. 8.— Proposed structure of cupric ion–lysine vasopressin complex.

tion: $\Delta F = -RT \ln K$. This gives a value of −7200 cal/mole for the free energy of formation of the complex. These apparent values are based on the assumption that there is no other appreciably absorbing species present in the solution under these conditions. Measurement of the absorption spectra of $CuCl_2 \cdot 2H_2O$ and LVP in separate experiments demonstrated that this is a reasonable assumption for these free components at 525 mμ. However, the absence of absorption from other complex species under these conditions will have to be more rigorously established by independent methods.

The use of solutions of Cu-LVP under conditions expected to maintain maximum complex formation resulted in no significant alteration of the effect of the hormone in the toad bladder assay (Figure 7). No appreciable alteration in hormonal activity was observed in solutions of 1:1, 10:1, or 100:1 copper-hormone composition. Thus under the conditions of the assay there seems to be no evidence of copper-induced hydrolysis of the disulfide bond such as that reported by Klotz and Campbell (1962) for the model compound, 2,2'-(hydroxy-6-sulfonaphthyl-1-azo)diphenyl disulfide. It seems likely that nonspecific binding of copper to other ligands in the assay system provides enough free hormone to elicit the normal response. It is also possible that the binding of hormone to copper is not strong enough to compete with binding of LVP at the specific site of hormone interaction on the bladder.

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