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# THE MOLECULAR DIMENSIONS OF PORCINE NEUROPHYSIN AND SOME THERMODYNAMIC PARAMETERS OF THE REACTION WITH LYSINE VASOPRESSIN

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SUMMARY

1. The molecular weight of porcine neurophysin was estimated by molecular sieve chromatography and by analytical ultracentrifugation and was found to be in the order of 13,000.

2. Internal evidence for the homogeneity of the preparation of neurophysin with respect to molecular weight was obtained in the ultracentrifugation experiments.

3. The frictional ratio of neurophysin was 1·1 which suggests that the molecular form of the protein approximates to a sphere.

4. The molecular weight and frictional ratio were not affected by temperature change (10–34° C) or by twofold change in protein concentration.

5. The binding of [$^{14}$C]lysine vasopressin to porcine neurophysin was studied at 0, 27 and at 45° C, and double reciprocal plots of the binding were shown to be curvilinear at 27 and at 45° C and rectilinear at 0° C.

6. Concordant estimates for maximum binding capacity were obtained by extrapolations from the data at 27 and 45° C by applying two independent empirical methods of approximation; these agreed with the estimate obtained by extrapolation of the straight line, fitting data obtained at 0° C, being approximately 1 mole lysine vasopressin per 13,000 g protein.

7. The association constant and thermodynamic parameters of the reaction were estimated for near saturation conditions. The reaction is entropy driven.

8. The binding of lysine vasopressin was found to be dependent on protein concentration. No dependence of oxytocin binding on protein concentration was apparent.

## INTRODUCTION

The peptide hormones of the neurohypophysis can be bound by neurophysin, a protein also found in neurohypophysial extracts. It has been shown that in the presence of L-cystine, an inhibitor of the reaction between lysine vasopressin (LVP) and porcine neurophysin, there are non-linear reflexions of the double reciprocal plots (Thomas & Ginsburg, 1966) which, it is suggested, indicate that the amino acid acts as an allosteric inhibitor. If such were the case, deviation from linearity should occur at low free ligand concentrations even in the absence of inhibitor. One of the aims of the present investigation was to study the binding reaction at free hormone concentrations lower than those which could be conveniently measured by biological assay. This was made possible by the availability of LVP labelled with [$^{14}$C]phenylalanine (Thomas, Havranek & Rudinger, 1967).

Studies of the molecular dimensions of the protein in the presence and in the absence of LVP and the investigation of the effect of neurophysin concentration on binding are described. The bearing of these results on the applicability of models proposed for allosteric reaction (the tautomeric model of Monod, Wyman & Changeux, 1965, and the more general model involving a polymerizing system proposed by Nichol, Jackson & Winzor, 1967) is discussed.

The formation of complexes between the peptide hormones and neurophysin involves ionic interaction between the ionized free $\alpha$ amino of the hemicystine in the hormone and the free carboxyl groups in the protein (Stouffer, Hope & du Vigneaud, 1963; Ginsburg & Ireland, 1963, 1964). However, it has been suggested that the specificity of the binding reaction is conferred by the formation of secondary hydrophobic bonds (Ginsburg & Ireland, 1964; Breslow & Abrash, 1966). In the present paper, estimates of thermodynamic parameters of the binding reaction are given and discussed in relation to the nature of the binding reaction.

## METHODS

Porcine neurophysin was prepared by the method of Ginsburg, Jayasena & Thomas (1966).

2-Phenylalanine [U-$^{14}$C]-8-lysine vasopressin prepared by Thomas *et al.* (1967), was used in most of the binding experiments; the potency of the preparation was approx. 250 i.u. (rat pressor activity) per $\mu$-mole and the specific activity was 7 $\mu$ Ci per $\mu$-mole. Unlabelled LVP used in ultracentrifugation experiments was either prepared synthetically (parallel to the synthesis of the radioactive vasopressin) or was material purified from porcine neurohypophysis, kindly donated by the National Institutes of Health, Bethesda, Maryland. Synthetic oxytocin (Syntocinon Sandoz Ltd.) was used. Where necessary hormones provided in solution were concentrated by freeze drying before use.

The following buffers were used: 0·05 M sodium phosphate, pH 5·8, prepared by dissolving 0·26 g $Na_2HPO_4$ and 7·627 g $NaH_2PO_4$ in distilled water to a final volume of 1 l., and adding L-lysine to 1 mM; 0·1 M sodium acetate, pH 5·5 and 0·1 M pyridine acetic acid, pH 5·8, prepared by mixing appropriate amounts of sodium hydroxide and pyridine with acetic acid and diluting to 0·1 M acetate; modified Munsick solution, pH 5·8, containing 1 mM L-lysine (Ginsburg *et al.* 1966).

*Markers.* The following substances were used as markers in experiments involving molecular sieve chromatography: blue Dextran 2000, mol. wt. $2 \times 10^6$ (Pharmacia Ltd.); cytochrome *c*, type 2, horse heart, mol. wt. $1{\cdot}24 \times 10^4$ (Koch Light Laboratories, Ltd.); dinitrophenolarginine, mol. wt. 330, prepared after the method described by Fraenkel-Conrat, Harris & Levy (1954).

In a few experiments, cyanocobalamin (mol. wt. $1{\cdot}35 \times 10^3$, a gift from Dr D. R. Ferguson) was used as a marker.

*Estimation of protein.* Protein in solution was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein on thin layer plates was visualized by printing on to filter paper and staining by the method of Zahn & Rexroth (1955).

*Estimation of hormone.* (i) Biological assay. LVP was assayed by the method of Dekanski (1952) and oxytocin by the method of Holton (1948). (ii) Measurement of radioactivity. The activity of radioactive LVP was measured on an automatic gas flow detector (Nuclear Chicago, Model D 47). Aliquots of 2·0 ml. of the solution to be measured were layered on to clean aluminium planchets (31 mm in diameter) and dried. Before layering the planchets were coated with 0·1 ml. of a 1 % solution of Cetavalon and dried. Samples of LVP of known activity were used for comparison.

*Molecular sieve chromatography.* Columns of Sephadex G 50 and G 75 and thin layer plate of Sephadex G 50, G 75 and G 100 (Pharmacia Ltd.) were used.

*Analytical ultracentrifugation.* A Beckman Model E analytical ultracentrifuge was used. (1) Molecular weights were determined by the approach to equilibrium method, using a synthetic boundary cell for the determination of protein concentration. (2) Diffusion coefficients were calculated from the rate of spread of the peak in the synthetic boundary cell. (3) The sedimentation coefficient was estimated from ultracentrifugation runs at 20·2° C and at a speed of 59780 rev/min. For the calculation of molecular weight, diffusion and sedimentation coefficient, the methods described by Schachmann (1957) were used.

The amino acid composition of porcine neurophysin (determined by Dr B. J. Pickering, using an EEL automatic amino acid analyser) was used to calculate the partial specific volume of neurophysin according to the method described by Schachmann (1957) and the average hydrophobicity as described by Bigelow (1967).

*Measurement of hormone binding.* The film dialysis technique of Craig, King & Stracher (1957) as modified by Ginsburg & Ireland (1964) was used. In experiments involving the use of radioactive LVP, 0·05 M phosphate, pH 5·8 containing 1 mM-L-lysine was used as the buffer. When biological assays were involved, calcium-free modified Munsick solution, pH 5·8 containing 1 mM L-lysine was used. The presence of L-lysine inhibits the uptake of LVP by the Visking membrane (Ginsburg *et al.* 1966). Buffered solutions containing various concentrations of hormone (0·2–20 μM-LVP or 2·9–12 μM oxytocin) and protein (64 or 250 μg/ml.) were allowed to equilibrate to the experimental temperature and 0·49 ml. of each solution was placed in the inner compartment of a dialysis cell. The inner compartment solution is separated from the outer compartment which contains 10 ml. buffer solution, by a membrane of $\frac{18}{32}$ in. (1·48 cm) Visking tubing. Preliminary experiments in the absence of protein showed that the concentration of LVP in the outer compartment reached 25, 50 and 78 % of the equilibrium concentration after dialysis for 28, 17·5, and 30 min at 0, 27 and 45° C respectively, and that the concentration of oxytocin reached 50 % of the equilibrium concentration after 17·0 min at 27° C. The solutions containing protein and hormone were dialysed for these times and the concentration in the outer compartment determined. The

hormone concentration that would have obtained in the outer compartment at equilibrium was calculated to give the free hormone concentration ($A$). From that value and from the known initial contents of the inner compartment, the bound hormone ($r$) was obtained.

## RESULTS

### *The molecular dimensions of porcine neurophysin*

*Molecular sieve chromatography.* The results of experiments by thin layer molecular sieve chromatography of neurophysin and various marker substances of known molecular weight on Sephadex of various grades are shown in Table 1. These indicate that the molecular weight of porcine

TABLE 1. $R_{\text{blue dextran}}$ values of neurophysin and marker substances in thin layer molecular sieve chromatography on Sephadex G 50, Sephadex G 75 and Sephadex G 100. In all cases 0·05 M phosphate buffer, pH 5·8, was used

| | | 0–4° C | | | 45–50° C | | |
|---|---|---|---|---|---|---|---|
| Substance | Mol. wt. | G 50 | G 75 | G 100 | G 50 | G 75 | G 100 |
| 2:4 Dinitrophenyl arginine | 330 | < 5 | < 5 | < 5 | 46 | 34 | 34 |
| Cyanocobalamin | $1{\cdot}34 \times 10^3$ | — | — | — | — | — | 36 |
| Cytochrome *c*, type 2, horse heart | $12{\cdot}4 \times 10^3$ | 53 | 49 | 60 | 75 | 70 | 51 |
| Porcine neurophysin | — | 55 | 49 | 51 | 73 | 60 | 57 |

neurophysin is in the same order as that of cytochrome *c*. An estimate of molecular weight, calculated on the basis that log mol. wt. is proportional to distance travelled on the plate gave a mean value of $1{\cdot}2 \times 10^4$ for neurophysin. The experiments were carried out under two conditions of temperature; in an oven (45–50° C) and in a cold room (0–4° C) and the estimates of molecular weight were not affected significantly by alteration of temperature. Although neurophysin appeared to travel as a single component, this cannot be taken as evidence of homogeneity by molecular weight because the method used to visualize protein (elution on to filter paper followed by staining) tends to spread the spots.

The patterns of elution of porcine neurophysin from columns ($19{\cdot}5 \times 1{\cdot}5$ cm) of Sephadex G 50 and G 75 (Table 2) confirm that the molecular weight of neurophysin is approximately equal to that of cytochrome *c*, and once again alteration in temperature conditions (cold room 0–4° C, room temperature 18–20° C) had no effect on the position of neurophysin relative to the markers. Using the relationship between log mol. wt. and elution volume described by Andrews (1964) the molecular weight of porcine neurophysin was calculated to be $1{\cdot}3 \times 10^4$ from experiments at 0–4° C and $1{\cdot}2 \times 10^4$ from experiments at 18–22° C.

A direct comparison with the elution pattern on Sephadex G 75 (data taken from Ginsburg & Ireland, 1965), confirms that the molecular weight of the porcine protein is considerably less than that of the bovine product.

In a separate experiment, porcine neurophysin was applied to the self-same G 75 column used by Ginsburg & Ireland (1965) equilibrated with the same buffer, and the elution patterns were compared (Table 2). Using the 90,000 and 25,000 molecular weight proteins of Ginsburg & Ireland (1965) as markers the estimate for the molecular weight of porcine neurophysin is $1{\cdot}4 \times 10^4$.

*Analytical ultracentrifugation.* Approach to equilibrium experiments were performed under the various conditions indicated in Table 3 and molecular weights were calculated from measurements made both at the meniscus and at the bottom of the ultracentrifuge cell. In runs carried

TABLE 2. Elution volumes of porcine neurophysin and markers eluted from column of Sephadex G 50 and G 75

| | | 0–4° C | | 18–20° C | | |
|---|---|---|---|---|---|---|
| Substance | Mol. wt. | G 50* | G 75* | G 50* | G 75* | G 75† |
| 2:4-Dinitriphenyl arginine | 330 | 19·5 | 22 | 20 | 21·5 | — |
| Cytochrome *c*, type 2, horse heart | $12{\cdot}4 \times 10^3$ | 10 | 13 | 8 | 12 | — |
| Blue dextran | $2 \times 10^6$ | 7 | 6 | 6 | 6 | — |
| Bovine neurophysin‡ | $2{\cdot}5 \times 10^4$ | — | — | — | — | 15‡ |
| Bovine neurohypophysial‡ protein | $9{\cdot}0 \times 10^4$ | | — | — | — | 9‡ |
| Porcine neurophysin | — | 10·5 | 12 | 8·5 | 12 | 18 |

* = experiments using 0·05 M phosphate buffer, pH 5·8 and column $19{\cdot}5 \times 1{\cdot}5$ cm.
† = experiments using 0·1 M pyridine acetate buffer pH 5·8 and column $27 \times 1{\cdot}1$ cm.
‡ = data from Ginsburg & Ireland (1965).

out at rotor speeds of 12,590, 15,220 and 17,980 rev/min, marked discrepancies occurred between values of molecular weights calculated from measurements made at the meniscus ($M_m$) and those calculated from measurements made at the bottom of the cell ($M_b$). Inspection of the photographic plates showed that while extrapolation of the curves of $dc/dx$ (where $c$ is the concentration of the protein and $x$ the distance from the axis of rotation) to the boundary between the silicone oil and the aqueous phase at the bottom of the cell was easy, giving reproducible results, extrapolation of $dc/dx$ to the meniscus was difficult, several extrapolations from the same photograph producing as many different values. As a result estimates of $M_m$ varied widely from 15,000–35,000 while values for $M_b$ were relatively consistent averaging about 13,000; invariably $M_m > M_b$, values of $M_m/M_b$ ranging from 1·3 to 2·3. In an attempt to obtain consistent estimates of $M_m$, a run was carried out at a higher speed (24,630 rev/min). Reproducible extrapolations were obtained from both the meniscus and from the bottom of the cell, giving values of $M_m$ = 13,600, $M_b$ = 13,760. These estimates agree with $M_b$ values obtained at lower

centrifugal speeds but are not consistent with the low speed $M_m$ values, confirming the impression that the latter are unreliable and should be disregarded. During the course of the run at 24,630 rev/min, the values of $c_b/c_m$ (where $c_b$ is the concentration of protein at the bottom of the cell and $c_m$ is the concentration of protein at the meniscus) increased gradually to 2·3 with the duration of the centrifugation but estimates of $M_m$ and $M_b$ were not dependent on the period of centrifugation. Doubling the initial

TABLE 3. Estimates of molecular weight, diffusion coefficient and frictional ratio of neurophysin under different conditions of concentration, temperature and presence of ligand in the analytical ultracentrifuge

| Experimental conditions | | | | | |
|---|---|---|---|---|---|
| Cell contents | Neurophysin* mean concentration | Temperature (° C) | Mol. wt. | $D_{tw}$ cm²/sec × 10⁶ | $f/f_0$ |
| Neurophysin alone | 495 | 10 | 12,400 | 0·90 | 1·12† |
| Neurophysin alone | 412 | 20 | 12,400 | 1·29 | 1·07† |
| Neurophysin alone | 827 | 20 | 13,000 | 1·20 | 1·15† |
| Neurophysin alone | 437 | 34 | 13,200 | 1·89 | 1·05† |
| Neurophysin + oxytocin or LVP (2:1 molar excess of hormone) | 439 | 10 | 12,200 | 0·92 | 1·10† |
| Neurophysin + oxytocin or LVP (2:1 molar excess of hormone) | 618 | 20 | 12,200 | 1·38 | 1·00† |
| Neurophysin + oxytocin or LVP (2:1 molar excess of hormone) | 550 | 34 | 13,300 | 1·74 | 1·14† |
| Neurophysin + LVP (20:1 molar excess of hormone) | 720 | 20 | 14,700 | 1·34 | 1·00‡ |

* = protein concentrations are expressed in arbitrary units of refractive index and lie within the range 5–10 mg/ml.; refractive index is temperature dependent and concentrations at different temperatures should not be compared.

† = based on mol. wt. 13,000.

‡ = based on mol. wt. 14,700.

concentration of neurophysin did not affect the estimate of molecular weight (Table 3). These results indicate the homogeneity of the protein by molecular weight.

*The effect of temperature and of the addition of hormone on the molecular weight of porcine neurophysin.* The estimates of molecular weight of porcine neurophysin from approach to equilibrium analytical ultracentrifugation were unaffected by temperature over a range of 24° (Table 3) or by the presence of relatively low concentrations of hormone (one molecule of oxytocin or two molecules of LVP per molecule of neurophysin). The presence of a large excess of hormone (20 molecules of LVP per molecule of neurophysin) increased the estimate of molecular weight to 14,700, a value consistent with the binding of one or two molecules of LVP (mol. wt. 1056) per molecule of neurophysin.

*The diffusion coefficient and frictional ratio of porcine neurophysin.* Diffusion coefficients ($D$) were obtained by centrifugation in synthetic boundary cells at 34,000 rev/min. The coefficients were calculated from the slope of the line relating $(A/H_{max})^2$ to time ($A$ = area of the peak; $H_{max}$ = the maximum height of the peak). The linearity of the relationship confirmed the homogeneity of the protein with respect to molecular weight. The radius of an anhydrous sphere ($r_o$) of molecular weight, 13,000 is 15·38 Å and this value was used in the calculation of frictional ratio ($f/f_o$) under various conditions. These values (Table 3) were not affected consistently by temperature, protein concentration or by the presence of hormone. The mean value of $f/f_o$ (1·1) was used to obtain a final estimate of $D_{20,w}$ of $1{\cdot}244 \times 10^{-6}$ cm$^2$ sec$^{-1}$.

*The sedimentation coefficient of porcine neurophysin.* A solution of porcine neurophysin (5 mg/ml. in 0·1 M sodium acetate, pH 5·5) was centrifuged at 59,780 rev/min at 20·2° C for 100 min. A sedimentation coefficient of $s_{20,w}$ = 2·22 S was obtained. When, after centrifugation for 20–100 min, the peak had broadened sufficiently to allow accurate measurement of area, it was found that $(A/H_{max})^2$/time remained constant, indicating homogeneity. Substitution of the estimate of $D_{20,w} = 1{\cdot}244 \times 10^{-6}$ cm$^2$ sec$^{-1}$ into the equation

$$M = (\mathrm{RTs})/D(1-v\rho)$$

where $s$ is the sedimentation coefficient, $v$ is the partial specific volume of solute, $\rho$ is the viscosity of the solvent, gives a value of 15,000 for the molecular weight of porcine neurophysin. This value is approximate since neither $D_{20,w}$ nor $s_{20,w}$ was extrapolated to zero concentration before application of the Svedberg equation.

*The partial specific volume and average hydrophobicity of porcine neurophysin.* From the amino acid composition of porcine neurophysin (Table 4) and using values for the partial specific volumes of amino acid residues (Schachmann, 1957) the partial specific volume of porcine neurophysin was calculated as 0·709 c.c./g. Also from the amino acid composition and following the calculations described by Bigelow (1967) the average hydrophobicity and frequency of charged side chains at pH 6 were estimated as 850 cal/residue and 0·258 respectively.

### *LVP binding by neurophysin*

*Experiments at different protein concentrations and over an extended range of ligand concentration.* The estimated binding at 0, 27 and 45° C of [$^{14}$C]LVP by neurophysin measured by film dialysis experiments is shown in Fig. 1 in which log (moles LVP bound per 13,000 g protein) is plotted against log (free hormone concentration), i.e. log $r$ is plotted against log $A$. The same

data appear in Fig. 2 in which $r^{-1}$ is plotted against $A^{-1}$. Values of $A$ in the present experiments extended over a fifty-fold range ($5 \times 10^{-9}$ M–$2{\cdot}5 \times 10^{-7}$ M) compared with a tenfold range ($7 \times 10^{-8}$ M–$7 \times 10^{-7}$ M) in earlier experiments (Ginsburg *et al.* 1966; Thomas & Ginsburg, 1966). In contrast to results obtained at 27° C over the narrower range of $A$ in which the reciprocal

TABLE 4. The amino acid composition of porcine neurophysin. These analyses were kindly performed by Dr B. Pickering, using an EEL amino acid analyser. They have been corrected for recovery and for molecular weight. Residues are given to the nearest integer

| Amino acid | Residue | Amino acid | Residue |
|---|---|---|---|
| Tryptophan | Not estimated | Glycine | 19 |
| Lysine | 6 | Alanine | 11 |
| Histidine | 1 | Cystine ($\frac{1}{2}$) | 14* |
| Ammonia | 11 | Valine | 6 |
| Arginine | 6 | Methionine | 1 |
| Aspartic acid | 11 | Isoleucine | 3 |
| Threonine | 4 | Leucine | 9 |
| Serine | 7 | Tyrosine | 2 |
| Glutamic acid | 19 | Phenylalanine | 4 |
| Proline | 10 | | |

* not determined as acid—not accurate.

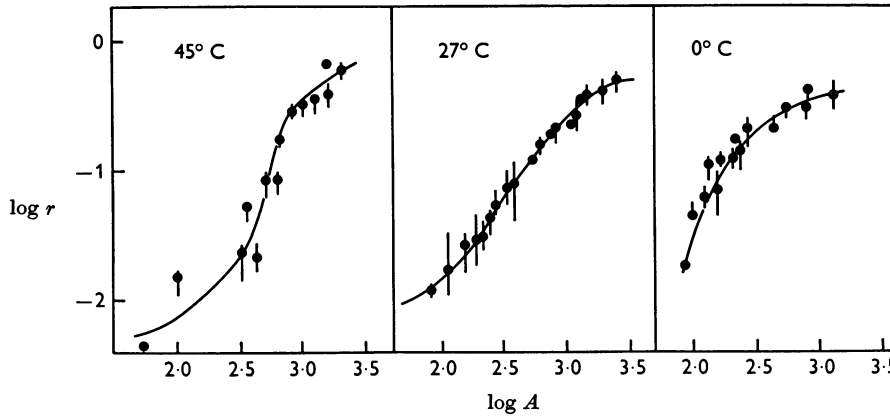


Fig. 1. The binding of [$^{14}$C]LVP to porcine neurophysin (64 $\mu$g/ml.) at 0, 27 and 45° C in 0·05 M phosphate buffer, pH 5·8. Ordinate log $r$ where $r$ is m-moles hormone bound by 13,000 mg of protein. Abscissa log $A$ where $A$ is free hormone concentration, $\mu$-moles per litre. Most of the points give the means of three determinations and the vertical lines indicate the range of values for log $r$.

plots appeared to be linear, the plots in Fig. 2 for experiments at 45 and 27° C are concave towards the $r^{-1}$ axis while at 0° C the points may be fitted by a straight line $r^{-1} = 1{\cdot}116 + 0{\cdot}154(A^{-1})$.

For the purpose of extrapolating the curves to the $r^{-1}$ axis (the intercept gives the maximum binding capacity of the protein or, if the preparation is homogeneous, the number of binding sites) two types of approximation

were used. In the first of these, only values obtained at relatively high concentrations of free hormone ($> 8{\cdot}75 \times 10^{-8}$ M at 45° C, $> 5{\cdot}65 \times 10^{-8}$ M at 27° C) were considered, and straight lines were fitted to these points. These lines are described by the equations:

$$\text{at } 27° \text{ C}, \quad r^{-1} = 0{\cdot}713 + 0{\cdot}371\ (A^{-1}); \quad \text{and}$$

$$\text{at } 45° \text{ C}, \quad r^{-1} = 0{\cdot}763 + 0{\cdot}255\ (A^{-1}).$$

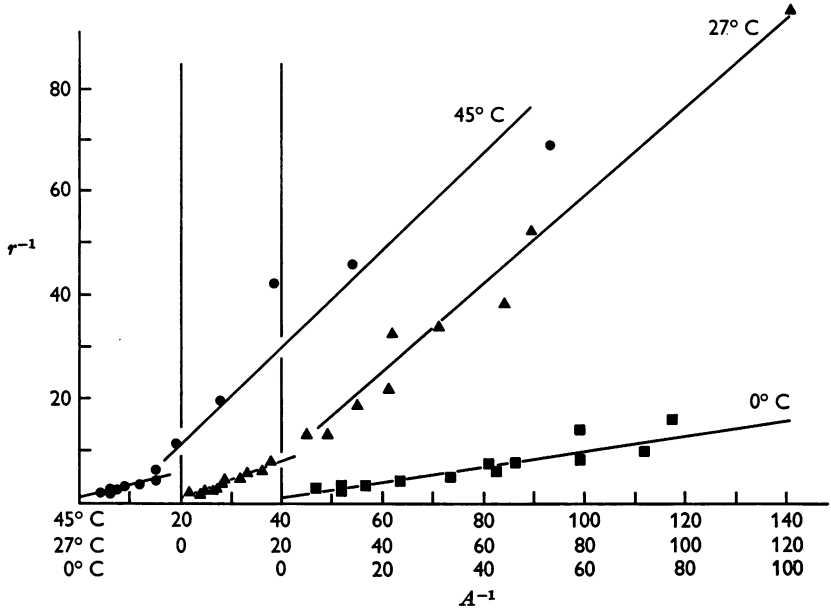


Fig. 2. The binding of [$^{14}$C]LVP to porcine neurophysin. The data given in Fig. 1 are here expressed in the form of double reciprocal plots. Ordinates $r^{-1}$, abscissa $A^{-1}$. For definitions of $r$ and $A$ see legend to Fig. 1.

Such extrapolations are likely to provide an underestimate of the intercept. At relatively low free hormone concentration (at 27° C, $< 5{\cdot}65 \times 10^{-8}$ M; at 45° C, $< 8{\cdot}75 \times 10^{-8}$ M) the slopes of the lines were 0·66 and 1·25 at 27 and 45° C respectively, and each of these is significantly different ($P < 0{\cdot}001$) from the corresponding value in the equation above.

In the second method of approximation, advantage was taken of the fact that when the values obtained in experiments at 27 and 45° C were plotted as $\log r^{-1}$ against $\log A^{-1}$, the points were fitted by the straight lines (Fig. 3) described by the following equations:

$$\text{at } 27° \text{ C}, \quad \log r^{-1} = 1{\cdot}183 \log (A^{-1}) - 0{\cdot}535; \quad \text{and}$$

$$\text{at } 45° \text{ C}, \quad \log r^{-1} = 1{\cdot}392 \log (A^{-1}) - 0{\cdot}769.$$

$r^{-1}$ was therefore plotted against $A^{-(1{\cdot}183)}$ for experiments at 27° C and

against $A^{-(1\cdot392)}$ for experiments at 45° C (Fig. 4) and the points were fitted by lines described by the equations

$$\text{at } 27° \text{ C}, \quad r^{-1} = 0\cdot956 + 0\cdot270\ (A^{-1\cdot183}); \quad \text{and}$$

$$\text{at } 45° \text{ C}, \quad r^{-1} = 1\cdot307 + 0\cdot159\ (A^{-1\cdot392}).$$

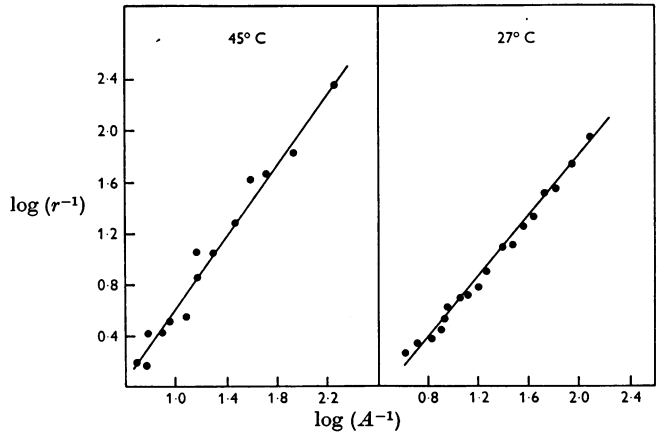


Fig. 3. The binding of [$^{14}$C]LVP to porcine neurophysin. The data obtained at 27 and 45° C are here expressed as log reciprocal plots. Ordinate log ($r^{-1}$), abscissa log $A^{-1}$. For definition of $r$ and $A$ see legend to Fig. 1.

Such extrapolations are likely to provide an overestimate of intercept.

An additional method was used to determine the value of the intercept at 0°. Barber, Welch & Mackay (1967) have shown that for a transport system obeying Michaelis–Menten kinetics

$$\log(v^{-1}) - \log(1 + K_m/c) = \log(V_{max}^{-1}) + (K_m - K_o)/2\cdot303(c + K_o),$$

where $v$ is the initial rate of transport at substrate concentration $c$, $V_{max}$ is the maximum initial rate of transport at very high substrate concentration, $K_m$ is the Michaelis constant for the system and $K_0$ is an estimate of Michaelis constant. $\text{Log}(v^{-1}) - \log(1 + K_0/c)$ is plotted $(c + K_0^{-1})$ and a line of zero slope intercepting the $\log(v^{-1}) - \log(1 + K_0/c)$ axis at log $(V_{max}^{-1})$ is obtained when $K_m = K_0$. The equation describing the binding of ligand to a protein possessing $n$ equivalent identical binding sites,

$$r = nk^{-1}A/(1 + k^{-1}A),$$

where $k$ is the microscopic dissociation constant, is analogous in form to the Michaelis–Menten equation and $\log(r^{-1}) - \log(1 + K_0/A)$ was plotted against $(A + K_0)^{-1}$. ($K_0$ was an estimated value for the dissociation constant of the reaction). A straight line of zero slope (Fig. 5) was obtained when $K_0 = 0\cdot118$, and the intercept of this line corresponded to a maximum binding capacity of 1 mole LVP per 15,500 g protein.

Estimates of maximum binding capacity made in these various ways, and under different conditions of temperature are shown in Table 5. There is good agreement between the estimates obtained by the different calculations, and the maximum binding capacity does not vary with tempera-

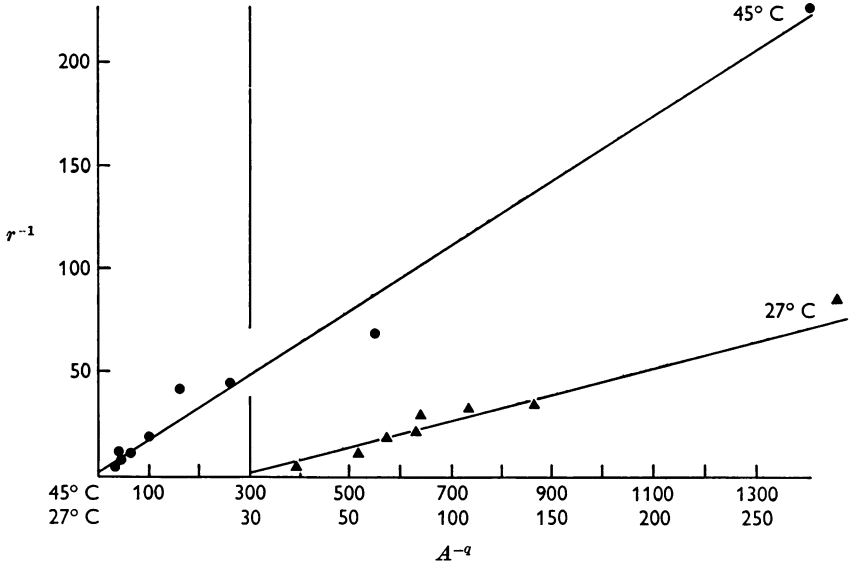


Fig. 4. The binding of [$^{14}$C]LVP to porcine neurophysin. The data obtained at 27 and 45° C are expressed in a modified double reciprocal plot. Ordinate $r^{-1}$, abscissa $A^{-q}$ where $q$ is 1·183 at 27° C and 1·392 at 45° C (see text for explanation). Some points near the origin have been omitted for clarity, but all points were used in calculating lines of best fit.

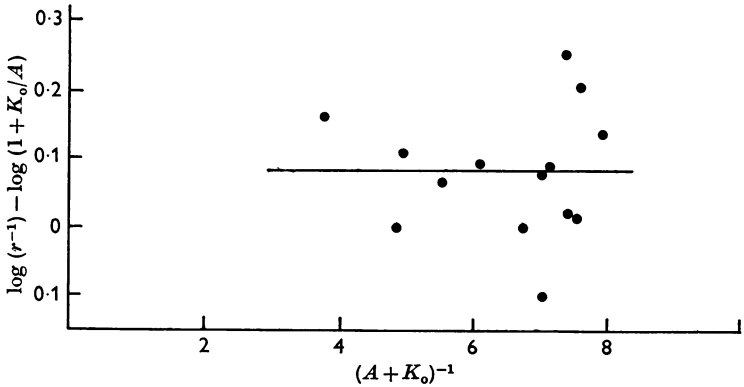


Fig. 5. The binding of [$^{14}$C]LVP to porcine neurophysin at 0° C. The data are expressed in a manner analogous to that of Barber *et al.* (1967). Ordinate, log $(r^{-1}) - \log(1 + K_o/A)$, abscissa $(A + K_o)^{-1}$ where $K_o$ is an estimate of the dissociation constant of the reaction (0·118 $\mu$M in this case). For definition of $r$ and $A$ see legend to Fig. 1.

ture. The mean for all these values taken together is one mole of LVP per 12,500 g protein.

*Thermodynamic parameters of the reaction.* Estimation of the thermodynamic parameters of the binding reaction is complicated by the fact that whatever model is used to describe allosteric reactions, more than one association constant must be considered. An approximation was therefore used. As saturation is approached an allosteric reaction approximates to a non-allosteric one; $k_{max}$ is defined as the association constant calculated by extrapolation to saturation from data obtained at relatively high ligand concentrations interpreted according to the classical model for non-allosteric reactions. This is assumed to approximate to the greater of the intrinsic association constants defined in either the model of Monod *et al.* (1965) or in the model of Nichol *et al.* (1967). $k_{max}$ was estimated in two ways. In the first, the values of the intercept and slope of the reciprocal plot calculated from data obtained at 0° C and from data obtained at high free ligand concentrations at 27 and 45° C were used to calculate $k_{max}$ in accordance with the equation

$$k_{max} = (r_{max}\,\text{slope})^{-1}.$$

Values of $k_{max}$ obtained in this way are shown in Table 5.

The second method involved the use of the Hill plot (Wyman, 1963). Hill plots at 0, 27 and 45° C (Fig. 5) were obtained by calculating the fractional saturation ($y$) on the basis of a maximum binding capacity of one mole LVP per 12,500 g neurophysin. $\text{Log}(y/(1-y))$ was plotted against $\log A$ and the points were fitted by lines described by the following equations:

$$\text{at } 0°\text{ C}, \quad \log(y/(1-y)) = 0{\cdot}83 + 1{\cdot}05 \log A,$$

$$\text{at } 27°\text{ C}, \quad \log(y/(1-y)) = 0{\cdot}93 + 1{\cdot}42 \log A,$$

$$\text{at } 45°\text{ C}, \quad \log(y/(1-y)) = 1{\cdot}22 + 1{\cdot}66 \log A.$$

The Hill plot can be used to distinguish allosteric and non-allosteric reactions. In the latter case only, are the points fitted by a straight line of unit slope which intercepts the $\log(y/(1-y))$ axis at $\log k$. With an allosteric reaction the curve is asymptotic to a line of unit slope at extreme values of $\log A$ and the asymptote at the higher values of $\log A$ will intercept the $\log(y/(1-y))$ axis at $\log k_{max}$. The lines of unit slope passing through the points at high values of $\log A$ (Fig. 6) will therefore intercept the $\log(y/(1-y))$ axis at a position equal to or less than $\log k_{max}$. Values of $k_{max}$ obtained in this way are shown in Table 5. There is no consistent variation of $k_{max}$ with temperature within the range 0–45° C, indicating a low enthalpy of reaction. The agreement between the values at the various temperatures obtained from the Hill plots is close and the difference

between the value at 0° C and those at 27 and 45° C obtained from the reciprocal plots is probably exaggerated as in the latter two cases a straight line is used to approximate to a curve.

The equation:

$$\ln k_{max} = -\Delta H/RT + c$$

TABLE 5. Estimates of maximum binding capacity and association constant of the neurophysin-LVP reaction and derived values for free energy, enthalpy and entropy of reaction

| Operating temperatures (° C) | $(r_{max})^{-1}$ g protein per mole ligand | | | $k_{max}$, $\mu M^{-1}$ | | | $-\Delta G$ | $\Delta H$ | $\Delta S$ |
|---|---|---|---|---|---|---|---|---|---|
| | *b* | *d* | *c* | *a* | *b* | *c* | (kcal. mole$^{-1}$) | | cal. deg.$^{-1}$ mol.$^{-1}$ |
| 0 | 15,000 | — | 15,600 | 5·6 | 7·3 | 8·5 | 8·5 | 0 | 31·2 |
| 27 | 9,300 | 12,500 | — | 4·5 | 2·8 | — | 9·5 | 0 | 31·6 |
| 45 | 10,000 | 18,600 | — | 6·3 | 4·4 | — | 9·9 | 0 | 31·2 |

*a* = estimated from Hill plots.

*b* = estimated from the double reciprocal plots. In estimating $k_{max}$ the intercept was corrected to the reciprocal of 1·04 and the slope of the line adjusted accordingly.

*c* = estimated in a manner analogous to that of Barber *et al.* 1967.

*d* = estimated from extrapolation of plots shown in Fig. 4.

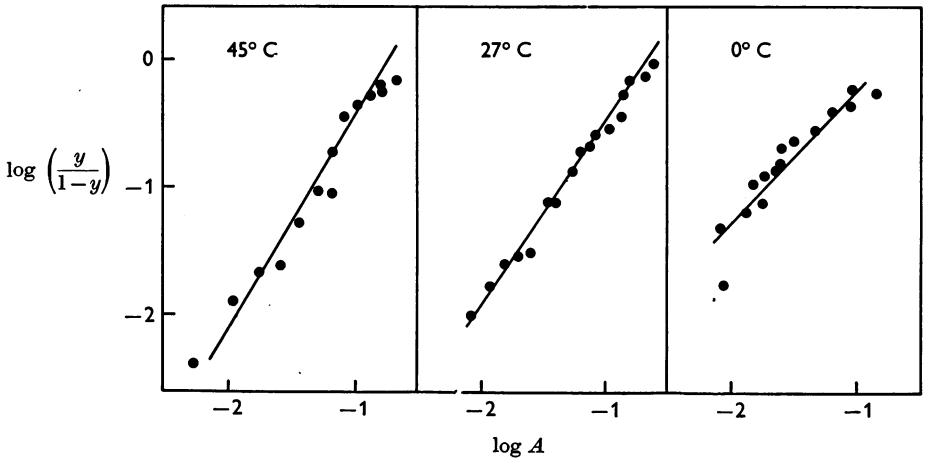


Fig. 6. The binding of [$^{14}$C]LVP to porcine neurophysin at 0, 27 and at 45° C expressed in the form of Hill plots. Ordinate log $(y/(1-y))$, abscissa log $A$, where $y$ is the fractional saturation. For definition of $A$ see legend to Fig. 1.

was used to calculate the enthalpy of reaction both from the Hill plot and from the reciprocal plot. In each case data are available for three different temperatures, permitting the solution of three pairs of simultaneous equations, giving, altogether, six separate estimates of enthalpy. These estimates are:

from the Hill plots, +4·15, +0·96 and −1·25 (mean = +1·29) kcal. mole$^{-1}$;

and from the reciprocal plots, +5·7, −2·1 and −6·0 (mean = −0·8) kcal.mole$^{-1}$
giving an overall mean value of +0·2 kcal.mole$^{-1}$ which is not significantly different from zero. In estimating the free energy and entropy of reaction, it will be assumed that enthalpy = 0.

The free energy of reaction was calculated from the average value of $k_{max}$ in accordance with the equation $\Delta G = -RT \ln k_{max}$ and the entropy in accordance with the equation $\Delta G = \Delta H - T\Delta S$. These estimates are shown in Table 5.

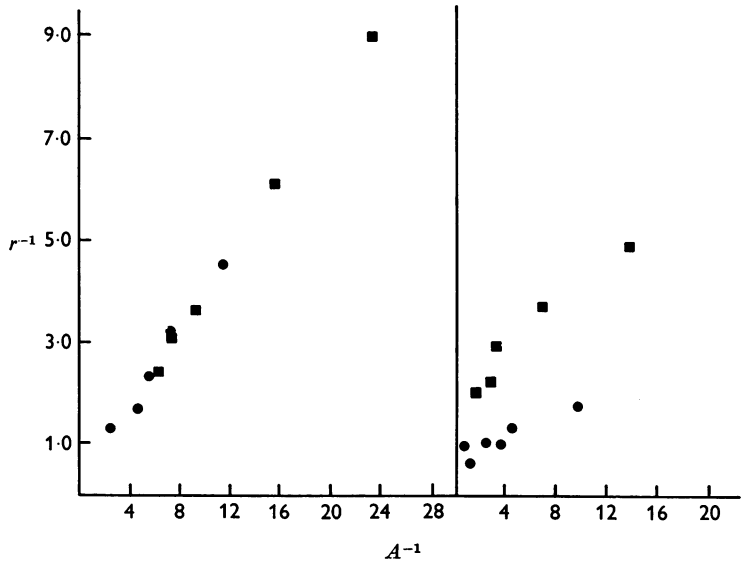


Fig. 7. The binding of LVP (right) and oxytocin (left) to porcine neurophysin at 27° C in modified Munsick solution pH 5·8. Ordinate $r^{-1}$, abscissa $A^{-1}$. Hormone concentrations were estimated by biological assay. For definition of $r$ and $A$ see legend to Fig. 1. ● Protein concentration = 64 $\mu$g/ml. ■ Protein concentration = 250 $\mu$g/ml.

The binding of arginine vasopressin (AVP) and oxytocin to bovine neurophysin under different conditions of protein concentration has been studied by Ginsburg & Ireland (1965). These experiments showed that the binding of AVP was markedly reduced at higher protein concentration, particularly at low values of $A$; the influence of protein concentration on the binding of oxytocin was not as marked as with AVP and was of doubtful significance. Similar experiments have now been carried out with LVP, oxytocin and porcine neurophysin and reciprocal plots of the results obtained as shown in Fig. 7. The points obtained from experiments with oxytocin clearly are fitted by the same line whether the protein concen-

tration was 64 or 250 $\mu$g/ml., while in experiments with LVP for any given value of $A$ the value of $r$ is always less at the higher protein concentration.

## DISCUSSION

We have assumed previously (Ginsburg *et al.* 1966) that molecular weight of neurophysin from porcine sources was in the same range as that of bovine neurophysin (25,000 approximately, Ginsburg & Ireland, 1965; 19,000–20,000, Hollenberg & Hope, 1967). The experiments described in this present paper suggest that the molecular weight of the protein from porcine neurohypophyses is, in fact, much smaller, possibly in the order of 13,000.

In the approach to equilibrium experiments at 24,630 rev/min, the agreement of the estimates of molecular weight from both the upper and lower boundaries is an index of the homogeneity of the preparation with respect to molecular weight. Furthermore, other methods for determining molecular weight (sedimentation and diffusion coefficients; molecular sieve chromatography) give values consistent with those obtained by the approach to equilibrium method. The linear relationship between $(A/H_{max})$ with time is a further indication of homogeneity. The estimate 13,000 is thus unlikely to be grossly in error due to heterogeneity, but might be a slight overestimate because our estimate of the partial specific volume of the protein (obtained from the partial specific volume of the constituent amino acid residues) did not take into account the compactness probably conferred on the molecule by the large number of disulphide bonds.

In earlier work (Ginsburg *et al.* 1966) we assumed that the formation of complex between LVP and porcine neurophysin was described by the classical model for non-allosteric reactions given by Klotz (1953). Maximum binding capacity was estimated by extrapolation of straight lines fitting points in the double reciprocal plots to the $r^{-1}$ axis. It was later shown that (Thomas & Ginsburg, 1966) in the presence of L-cystine, curves concave to the $r^{-1}$ axis fitted the experimental points better than did straight lines. It was predicted that such a phenomenon would become overt at low free ligand concentrations even in the absence of L-cystine and the experiments carried out at 27 and 45° C covering an extended range of free ligand concentration, described in this paper, substantiate that prediction. This implies that the double reciprocal plots in fact intercept the $r^{-1}$ axis higher than the straight lines fitted to the experimental points and thus the values for maximum binding capacity were overestimates.

In estimating maximum binding capacity, it is assumed that maximum binding is realized when the free ligand concentration is infinite (i.e. when $A^{-1} = 0$). The extrapolation of the curves in the double reciprocal plots

to the $r^{-1}$ axis was carried out making use of two different empirical approximations, one of which is intended to overestimate and the other to underestimate the value of the intercept. These approximations gave estimates of intercept in good agreement with one another and with the value calculated on the classical model from data obtained at 0° C, under which condition the reciprocal plot is rectilinear. The mean value obtained in these ways is therefore not likely to be grossly in error and corresponds to a maximum binding capacity of one mole of LVP per 12,500 g neurophysin.

This estimate is consistent with the increase in molecular weight of neurophysin observed in analytical ultracentrifugation of the protein in the presence of a large excess of LVP and it is in close agreement (on a weight scale) with the maximum binding capacity of bovine neurophysin for oxytocin (2 mole hormone per 25,000 g protein) estimated by Breslow & Abrash (1966). As predicted, it contrasts with earlier estimates of maximum binding capacity of porcine neurophysin (4 moles LVP or 14 moles oxytocin per 25,000 g protein (Ginsburg *et al.* 1966) and indeed of bovine neurophysin (4 mole LVP or 7 g moles oxytocin per 25,000 g protein (Ginsburg & Ireland, 1964). The close agreement with the estimate of Breslow & Abrash (1966) (who also assumed the linearity of the double reciprocal plot) may be explained as the experiments of these authors were carried out at much higher free ligand concentrations. Assuming that the lines of best fit approximate to tangents to a curve concave to the $r^{-1}$ axis, a line drawn from data obtained at high values of $A$ will intercept the $r^{-1}$ axis at a higher point (and consequently give a lower estimate of maximum binding capacity) than will a line drawn from data obtained at lower values of $A$. It is likely that the discrepancy between estimates of the maximum binding capacity of the proteins for oxytocin and for arginine and lysine vasopressin may be resolved in a similar way, since the experiments were carried out using vasopressin in higher concentration than oxytocin.

If the preparation is homogeneous, the maximum binding capacity of 1 mole LVP per 12,500 g protein implies that each molecule of porcine neurophysin has only one site capable of binding hormone. The representation of allosteric effects by the 'plausible model' of Monod *et al.* (1965) requires at least two binding sites per protomer. This model can be regarded as a special case of the more general system of Nichol *et al.* (1967) in which it is postulated that protein monomer co-exists with a single higher polymer; both may bind a low molecular weight ligand and the number of binding sites on each molecular form may be any integral value (including zero). By appropriate choice of constants, the model of Nichol *et al.* would yield curves similar to those obtained experimentally

in the LVP-neurophysin system, e.g. double reciprocal plots concave to the $r^{-1}$ axis. It should be noted that if neurophysin is a polymerizing system, the molecular weight estimate, 13,000, represents an average molecular weight of monomer and polymer.

Other evidence supporting application of the model of Nichol *et al.* to the LVP-porcine neurophysin system is given by experiments in which the conditions of protein concentration were altered. The equilibrium between polymer and monomer will be concentration-dependent and since the number of sites and/or the affinities of the sites for ligand will depend on molecular form, so binding also will be dependent on protein concentration. In fact, with the LVP-porcine neurophysin and the AVP-bovine neurophysin systems the binding is reduced at higher protein concentration and this suggests that the number of binding sites and/or their affinities for ligand are lower in the polymeric form than in the monomer. However, the dependence of oxytocin binding on protein concentration is either not demonstrable (with porcine neurophysin) or much less prominent (with bovine neurophysin) and this difference between oxytocin and the vasopressin peptides is a subject of work in hand.

Change in temperature is likely to affect separately association constants governing binding of ligand to protein, and polymerization. The degree of curvilinearity of the double reciprocal plots (measured from slopes of the log reciprocal plots) increases with temperature, implying an increase in the relative concentration of polymer (i.e. the association constant $L = (n\text{-mer})/(\text{monomer})^n$ increases with temperature); positive enthalpy is consistent with the involvement of hydrophobic bonds in formation of polymer. We were unable to demonstrate a temperature dependent change in average molecular weight, though in the approach to equilibrium experiments there was a trend to increase of molecular weight with increasing temperature, which was, however, within the limits of experimental error; similarly, the ultracentrifugation experiments provided no evidence for depolymerization of the protein in the presence of high concentrations of LVP. However, if the abundance of polymer is low, it might be impossible to detect changes in average molecular weight due to depolymerization.

The average hydrophobicity and frictional ratio of neurophysin are consistent with stability of the protein in globular form (Bigelow, 1967) but the hydrophobicity is less than that of any of the known polymerizing systems quoted by Bigelow (1967).

Owing to the curvilinearity of the reciprocal plots at 27 and 45° C the association constant of the ligand-protein reaction at these temperatures had to be estimated by approximation. Although the agreement between values obtained in different ways and at different temperatures was only moderate, the results showed that there was no consistent change with

temperature. The average value for the enthalpy of reaction was thus approximately zero. The thermodynamic parameters of formation of hydrophobic bonds are opposite in size to, though smaller in magnitude than those of aqueous solutions of hydrocarbons (Scheraga, 1963) and consequently owe their negative free energy of formation to an increase in entropy. Since the enthalpy of reaction in the formation of hydrophobic bonds is either a low negative value or positive, the zero estimate obtained is compatible with the suggestion of Ginsburg & Ireland (1964) and Breslow & Abrash (1966), that the specificity of the reaction is conferred by secondary hydrophobic bonds. This conclusion can be reinforced because the free energy realized by the reaction (in agreement with the results obtained by Breslow & Abrash, 1966) is much greater than that of an ionic bond between the $\alpha$ amino group on the hormone and free carboxyl groups on the protein.

*Note added in proof*

T. C. Wuu and M. Saffran (IIIrd International Congress of Endocrinology, July, 1968) have reported the isolation of a polypeptide from porcine neurohypophyses capable of binding LVP and oxytocin at pH 5·8, and mol.wt. 9,170, calculated from amino acid analysis. If this value represents the molecular weight of the monomer, there is no inconsistency with our finding that the *weight average* molecular weight of the system is of the order 13,000.

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