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THE ADSORPTION OF LYSINE VASOPRESSIN AT IONIZED INTERFACES

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SUMMARY

It has been shown by surface potential measurements that lysine vasopressin and oxytocin may be bound by ionic surfaces to very varied extents. To dodecyl sulphate and phosphatidylserine monolayers the binding is very strong and is comparable to that for biological receptors such as those in toad bladder. For dioleyl phosphate and the carboxyl group of the polypeptide alamethicin, the binding is rather weaker while, for the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine, and for the erythrocyte surface, which contains two varieties of carboxylic acid group, no interaction seems to take place.

In no system does the lysyl amino group of the vasopressin appear necessary for adsorption and, in the dodecyl sulphate monolayers, the interaction is strong even when the ionization of the terminal α-amino group is suppressed.

INTRODUCTION

Vasopressin is a small polypeptide hormone whose main function in land vertebrates is to regulate water balance. It is thought to do this by increasing the permeability of certain epithelia to Na^+ and water [1], but the mechanism by which it is effective is not at present understood. However, whether the hormone is directly responsible for the permeability increase, or whether it achieves its effect through some intracellular intermediate, it seems that an interaction with the cell membrane must at some stage be involved. Vasopressin is a cation at neutral pH, owing to the presence of a basic amino acid, arginine or lysine, in its side chain. Thus, a factor which might contribute to its binding to a membrane is a charge interaction with acidic groups of lipid molecules or protein receptors.

Partly in an attempt to understand the contribution of proteins to membrane structure, the behaviour of well-characterized proteins at air-water and oil-water interfaces has been extensively studied [2]. Little comparable work has been carried out with small biological peptides. Snart and Sanyal [3] described the results of injecting vasopressin, oxytocin and angiotensin into low pressure stearic acid monolayers. Kafka and Pak [4] demonstrated that several polypeptide hormones, including vasopressin, could displace radioactively labelled calcium from monolayers of octade-

cyl phosphate. From neither of these pieces of work is it possible to draw quantitative conclusions concerning the nature or extent of binding of the peptides at the interface. The former authors did not take into account that vasopressin is soluble in the aqueous phase, and would not necessarily adsorb irreversibly. The latter work involved only a single high vasopressin concentration. The interaction of vasopression with black lipid films has been examined with a view to discovering whether or not it directly affects their water and ion permeability [5, 6]. The conclusions are not clear-cut [7], and it seems possible that the results depend strongly on the nature and state of the lipid, especially the presence of acidic components.

In the present study, the adsorption of vasopressin (and of the closely related substance oxytocin) by lipids and polypeptides at the air-water and oil-water interfaces has been examined by the measurement of interfacial potentials over a wide range of hormone concentrations in the aqueous phase. Both electrokinetic and compensation potential techniques have been used. The former had the advantage that the results could be interpreted, at least semi-quantitatively, in terms of surface ion densities, while the latter provided valuable qualitative information, especially with regard to uncharged forms of the interacting species.

From both types of experiment, it will be demonstrated that when negatively charged groups of lipids or other surfactants are present at an oil- or air-water interface, vasopressin adsorbs strongly and reversibly. It will also be demonstrated that, among the types of group examined, there is some specificity or selectivity in facilitating the adsorption of the peptide.

METHODS AND MATERIALS

Compensation potential measurements

Interfacial potential n easurements were made by means of a vibrating plate potentiometer similar to that described by Kinloch and McMullen [8]. A flat circular gold plate 2.5 cm in diameter was suspended less than 1 mm above the air-water interface and vibrated at approx. 70 Hz. The mean potential difference between the gold plate and interface was compensated by applying a direct current potential through a calomel electrode in the aqueous phase. The magnitude of this compensation potential was adjusted so as to reduce to zero the alternating current signal generated by vibration of the gold electrode.

To carry out an experiment, the compensation potential for the clean interface in the presence of the anionic surfactant was measured. When this had become constant, the gold plate was raised slightly and an aliquot of polypeptide added. The solution was stirred thoroughly by means of a magnetic stirrer, and the plate returned to a position the same distance from the interface. The difference between the new compensation potential and the initial value constituted the change in interfacial potential, ΔV. At low polypeptide concentrations, the solution had to be stirred several times over a period of 15 or more min before the new compensation potential became constant. Long diffusion times, consequent upon the low concentrations of polypeptide, proved to be a major disadvantage in the use of the technique, for the usual criterion of reliability of results is that there be no long drifts of potential with time. In practice, any drifts in potential which continued for more than 15 min were discounted. In a good experiment the compensation potential for the clean interface

was usually constant over this period of time (Fig. 2). The temperature for the compensation potential measurements was 20 ± 1 °C.

Electrophoretic mobility measurements

The electrokinetic measurements were made by means of a microelectrophoretic method using a closed cylindrical cell immersed in a water thermostat [9, 10]. The droplets of lipid selected for measurement were 2–5 μm in diameter and were large compared with the Debye-Hückel length ($1/\kappa$). The electrokinetic or zeta potential ζ could be calculated from the equation [11]

$$\zeta = \frac{4\pi\eta}{\varepsilon E} V_E \tag{1}$$

where V_E is the electrophoretic velocity, E is the applied field strength, η and ε are the viscosity and dielectric constant, respectively, of the solution in the electrical double layer adjacent to the surface and, in the present systems, were taken as equal to their values in the bulk [12]. In order that the observed velocity of the droplets should be equal to their electrophoretic velocity, the usual precaution was taken of focusing at the stationary level [9]. The measurement of the mobility of fresh human erythrocytes in 0.145 M NaCl (pH 7.4) provided a check on the accurate location of this level [10].

Most of the electrophoretic measurements were made on droplets of an emulsion of *n*-decane stabilized by the appropriate lipid or surfactant. The emulsions were formed by shaking about 0.05 ml of a mixture of decane plus the surfactant in 10 ml of 0.1 M NaCl buffered with acetate. To obtain reproducible results, the polypeptide was always added with a clean glass constriction micropipette after the emulsion had been formed. The suspension was then allowed to stand for about 30 min, being carefully inverted from time to time in order to 'stir' the suspension, before being transferred to the electrophoresis cell. For some experiments a vesicle suspension was formed by shaking only the lipid or surfactant with the aqueous phase. The stoichiometric lipid concentration in the decane suspension was usually about 10 mM. For dioleyl phosphate, it was necessary to use a higher concentration of 84 mM in order to obtain stable and homogeneous mobilities. The electrokinetic measurements were carried out at 25 ± 0.1 °C. For all experiments, the usual cleaning procedures were observed, using chromic acid for glassware, and rinsing well, first in singly and then in doubly distilled water. Aqueous solutions were made up using 'quartz distilled' water.

Materials

Egg phosphatidylcholine and bovine spinal cord phosphatidylserine were purchased from Lipid Products (Redhill, Surrey, U.K.). Both lipids were at least 99 % pure as determined by thin-layer chromatography using acid and alkaline solvent systems. Glyceryl monooleate was supplied by Nu Chek Prep (Minnesota, U.S.A.) and was more than 99 % pure. Synthetic dioleyl phosphate was a gift from Miss M. Yoshida (Department of Synthetic Chemistry, Tokyo Institute of Technology, Tokyo, Japan). Slight contamination by another phosphate-positive compound was indicated by thin-layer chromatography using a chloroform/acetone (80 : 20, v/v) solvent, but the material was not purified further. The sodium dodecyl sulphate was a pure speci-

men which had a critical micelle concentration in water of 8 mM, and showed no minimum in the vicinity of the critical micelle concentration and no ageing. Bacterial phosphatidylethanolamine was obtained from Koch-Light Ltd. The NaCl was Analar grade, which had been roasted at 700 °C to remove organic impurities. The *n*-alkanes were puriss grade from Koch-Light Ltd., and were further purified by passage through a column of aluminium oxide.

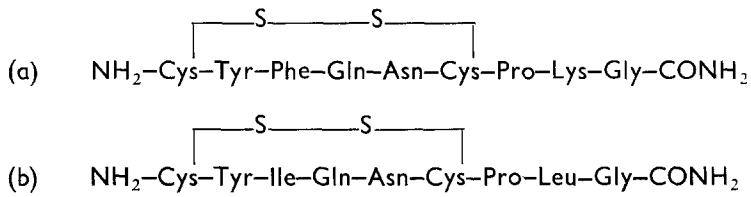


Fig. 1. a, lysine vasopressin; b, oxytocin.

Samples of synthetic lysine-8-vasopressin (Fig. 1) used were kindly supplied by Sandoz Ltd., (Basel and London) as aqueous solutions of 100 I.U./ml and 10 I.U./ml. These also contained known amounts of acetate buffer and chlorbutol preservative. Experiments were carried out with both samples, each giving similar results. The two stock solutions were distinct with respect to vasopressin concentration, chlorbutol concentration and buffer, and so it seems unlikely that the results were determined by anything other than the monomer vasopressin. Gel filtration on a superfine Sephadex G-25 column (0.1 M acetic acid/0.05 M ammonium acetate buffer, pH 4.33) of a vasopressin sample used in the experiments gave a single elution peak at about the correct molecular weight. The amino acid composition of this peak, and the initial concentration in the stock solution were checked by acid hydrolysis and estimation in an EEL autoanalyser. Vasopressin concentrations in stock solutions were routinely estimated from the measured absorbances at 225 and 275 nm using previously determined extinction coefficients at these wavelengths of 14000 and 1630 $l \cdot mol^{-1} \cdot cm^{-1}$, respectively. Synthetic oxytocin (Fig. 1) as syntocinon was also obtained from Sandoz Ltd. (London). Conversion factors from international units were 285 I.U./μmol for lysine-vasopressin and 450 I.U./μmol for oxytocin [1]. The alamethicin used was a sample obtained from Dr. J. Melling of the Microbiological Research Establishment, Porton, England, and was from the fraction '30' with an unblocked α-carboxyl.

RESULTS

Sodium dodecyl sulphate

The compensation potential measurements will be described first. The presence of chlorbutol in the vasopressin preparations had a small effect on the measured potentials (Fig. 2) and so, at the beginning of an experiment, an excess amount (0.54 mM) was added to the aqueous phase. For a clean air-0.1 M NaCl interface, the change in potential subsequent to the addition of vasopressin to the subphase to give a final concentration of $1.75 \cdot 10^{-7}$ M was less than 2 mV over a period of 20 min. When the anionic surfactant, sodium dodecyl sulphate was also present in the aqueous phase, however, large positive changes in potential were observed on addition of

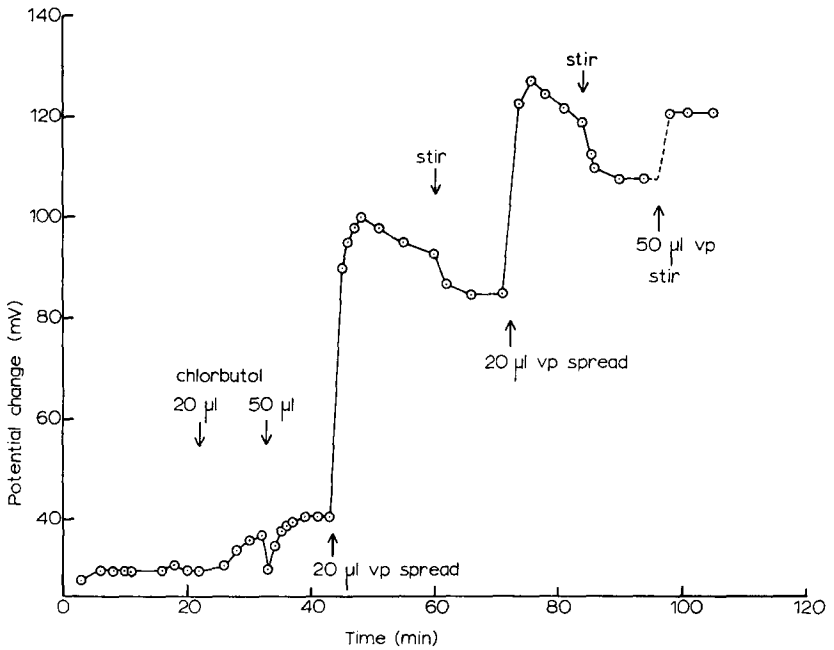


Fig. 2. Compensation potential changes at the air-aqueous solution interface. The aqueous phase consisted of 25 ml of 0.1 M NaCl plus $4 \cdot 10^{-5}$ M sodium dodecyl sulphate plus 0.002 M acetate, giving a pH of 4.8. The stock solution of chlorbutol was 5 mg/ml, and the stock vasopressin (vp) concentration was $3.5 \cdot 10^{-8}$ mol/ml (10 I.U./ml).

vasopressin. The results are summarized in Fig. 3 for three different surfactant concentrations, which corresponded to initial surface charge densities of one electronic charge per 250, 75 and 41 Å^2, respectively [13]. The similarity of the half saturation vasopressin concentrations for the three sodium dodecyl sulphate concentrations suggests that under the conditions of the experiment, there is no significant interaction in the bulk aqueous phase between vasopressin and sodium dodecyl sulphate. If the vasopressin was spread at the interface rather than injected into the bulk, the initial potential change was larger than normal, but decayed (more rapidly with stirring) to an equilibrium value (Fig. 2). This, taken with a negligible bulk interaction, argues for a reversible association at the interface.

Changes in the compensation potential $\Delta(\Delta V)$ may, in the present system be completely ascribed to changes in potential across the interfacial region, and contributions to it could arise both from changes in numbers or distribution of ions in the double layer and changes in number or orientation of dipoles in this region. This is normally expressed as [15]

$$\Delta(\Delta V) = 4\pi\mu\Delta N + \Delta\varphi_0 \qquad (2)$$

where ΔN is the change in the number of dipoles/unit area, μ is the normal component of each dipole moment and $\Delta\varphi_0$ is the change in the diffuse layer potential.

Although the interpretation of the data of Fig. 3 is complicated by the fact that, as the peptide adsorbs, there may also be additional adsorption of sodium

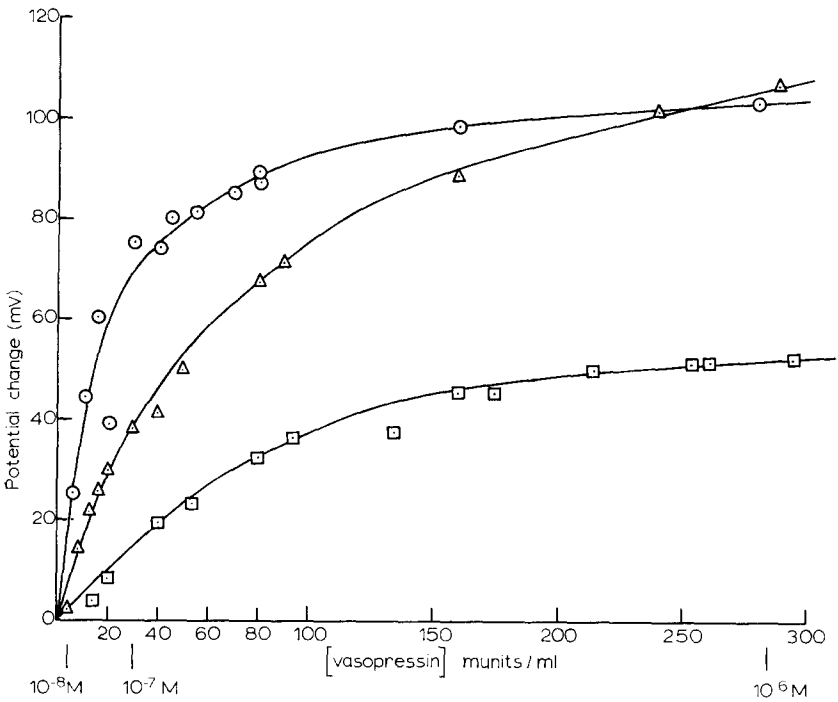
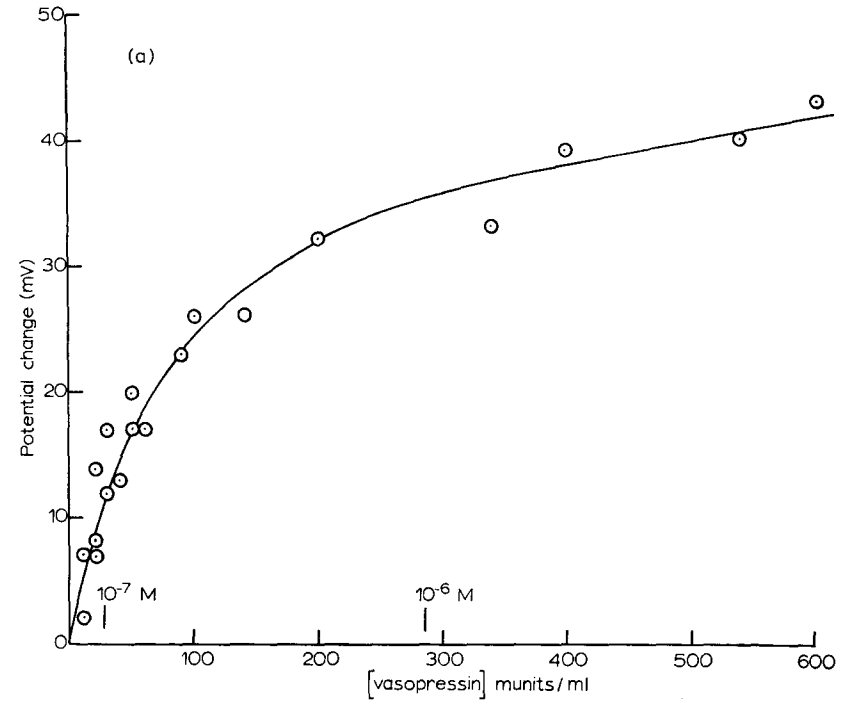


Fig. 3. Compensation potential changes at the air-aqueous solution interface for lysine vasopressin in three concentrations of sodium dodecyl sulphate. Aqueous phase: 0.1 M NaCl plus 0.002 M acetate, pH 4.8, plus sodium dodecyl sulphate: △, 10^{-5} M; ○, $4 \cdot 10^{-5}$ M; □, $4 \cdot 10^{-4}$ M.

dodecyl sulphate, it is easily shown that the potential changes observed cannot be explained solely in terms of the double layer potential and that a dipole contribution must be present. This makes a quantitative analysis of the results impossible. However, qualitatively it may be concluded that the concentration of vasopressin required for half saturation was about 10–20 munits/ml ($3.5 \cdot 10^{-8}$–$7 \cdot 10^{-8}$ M) and this agrees well with the electrophoresis results to be described later. Attempts to overcome some of the interpretive difficulties by using insoluble monolayers gave variable results and long time effects, probably owing to decreased efficiency of stirring in these systems. The large and reproducible compensation potential changes obtained using the sodium dodecyl sulphate do, however, allow a qualitative examination of the nature of the peptide association with the charged interface.

Lysine vasopressin contains three ionizable groups with pK values of 6.42 (α-terminal amino), 9.92 (tyrosine) and 10.36 (lysine ε-amino) [14]. The initial experiments described were all carried out at about pH 5 where nearly all of the vasopressin bears a double positive charge. At pH 8.8 the terminal α-amino group is suppressed. Under these conditions the surface potential changes are less by approx. 50 % but the strength of the adsorption is not greatly affected (Fig. 4). Similarly, oxytocin, which has a leucine residue instead of lysine, but which has an ionized terminal α-amino group at pH 4.9, adsorbs strongly (Fig. 4). Since neither the lysyl amino group, nor the terminal α-amino group seemed necessary for the adsorption it



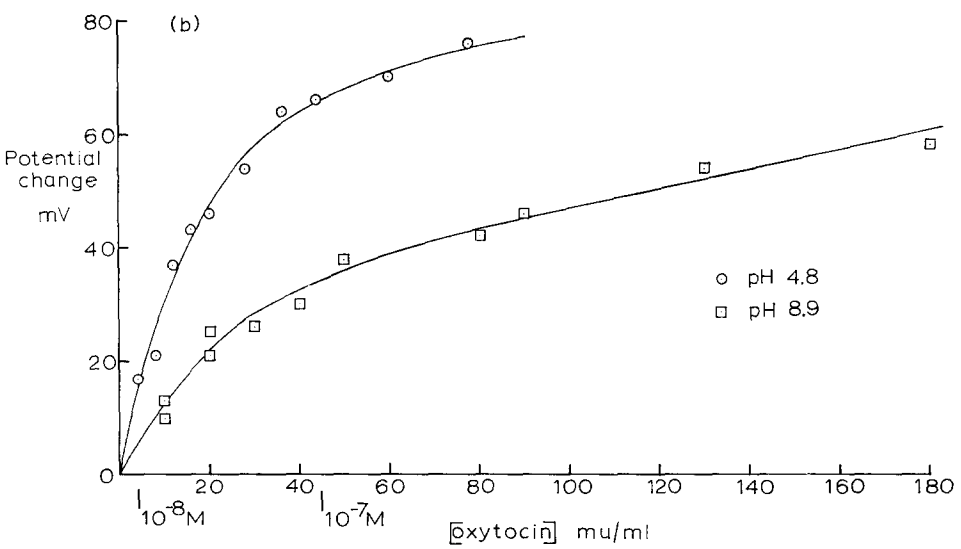


Fig. 4. Compensation potential changes as a function of hormone concentration. Aqueous phase 0.1 M NaCl plus $4 \cdot 10^{-5}$ M sodium dodecyl sulphate. (a) Lysine vasopressin, pH 8.8 (0.01 M sodium bicarbonate). (b) Oxytocin; ⊙, pH 4.8 (0.002 M acetate) and ⊡, pH 8.9 (0.01 M sodium bicarbonate).

was of interest to ascertain whether any positive charge at all was necessary. Compensation potential measurements are particularly valuable in this instance since the adsorption of un-ionized molecules can be followed through changes in dipole orientation at the interface (Eqn. 2). In fact, oxytocin, at pH 8.9, where it is uncharged, still appears to adsorb very strongly (Fig. 4). If, however, dodecyltrimethylammonium bromide (10^{-4} M) was substituted for the dodecyl sulphate, or if the surfactant were omitted altogether, no change in surface potential could be detected at either acid or alkaline pH values up to an aqueous concentration of about $9 \cdot 10^{-7}$ M. These results are consistent with the idea that neither a positive charge nor a lipophilic interaction is necessary for the binding of the oxytocin, but that a specific interaction occurs between the peptide and the sulphate head group.

The interaction of lysine vasopressin with sodium dodecyl sulphate was also studied by microelectrophoresis. Measurements were carried out on suspensions of droplets of glyceryl monooleate (6 mM) in decane, in 0.1 M NaCl containing $4 \cdot 10^{-5}$ M sodium dodecyl sulphate, at pH 4.9. This system has the advantages that the electrokinetic potential has been shown to be almost identical to the diffuse double layer potential (φ_0) [16] and that at $4 \cdot 10^{-5}$ M the sodium dodecyl sulphate adsorption is nearly maximal, so that additional adsorption in the presence of vasopressin should be small. Surface charge densities were calculated by substituting the zeta potential for φ_0 in the Gouy-Chapman equation [15]

$$\sigma = \frac{N_0}{F}\left(\frac{2\varepsilon RTc}{\pi}\right)^{\frac{1}{2}} \sinh \frac{F\varphi_0}{2RT} \tag{3}$$

where σ is the surface ion density in ions/cm^2, φ_0 is the diffuse layer potential, c is the electrolyte concentration in mol/cm^3, ε is the aqueous phase dielectric constant, N_0 is the Avogadro's number and F is the Faraday.

The control zeta potential (Table I) gave an area per sodium dodecyl sulphate molecule of 310 Å^2, which was similar to that of 330 Å^2 calculated from interfacial tension measurements [16]. The addition of vasopressin at concentrations comparable

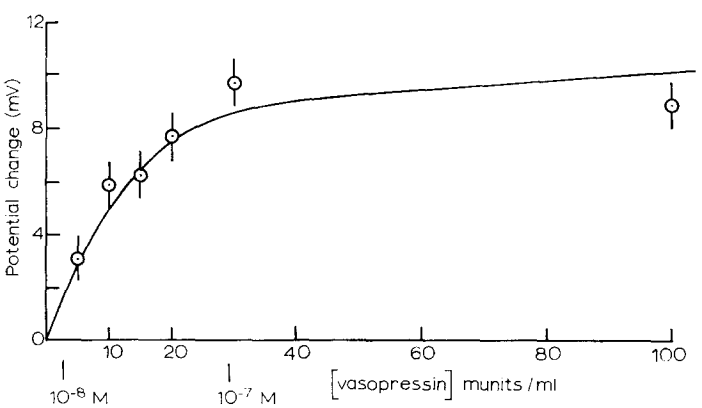


Fig. 5. Electrokinetic potential changes in the presence of vasopressin for an emulsion of glyceryl monooleate/decane, in 0.1 M NaCl plus $4 \cdot 10^{-5}$ M sodium dodecyl sulphate plus 0.002 M acetate, pH 4.4.

to those used in the compensation potential measurements reduced the zeta potential, the maximum change observed being about 10 mV (Fig. 5). The electrokinetic measurements give a half saturation of the potential at a vasopressin concentration ($\simeq 3 \cdot 10^{-8}$ M) which is in good agreement with that obtained from the compensation potential measurements.

At high concentrations, the adsorption could also be detected in this system by interfacial tension measurements using the drop volume technique, and this provided another means of establishing the reversibility of the peptide adsorption. A drop of a glyceryl monooleate-decane solution was suspended from the tip of an Agla syringe in an aqueous solution containing the sodium dodecyl sulphate and vasopressin, and about 5 min was allowed for the adsorption to reach equilibrium. The drop was then carefully and slowly retracted. Had the adsorption been irreversible, retraction would have produced a compression of the adsorbed monolayer, a lowering of the tension and detachment of the drop, but this did not occur. It could be argued that the vasopressin-sodium dodecyl sulphate complex had desorbed, but this requires postulating a bulk interaction between the sodium dodecyl sulphate and vasopressin, which is contrary to the inferences of the previous section.

Egg phosphatidylcholine

A major component of cell membranes is the zwitterionic lipid, phosphatidylcholine, which contains both phosphate and trimethylammonium groups. The presence of the phosphate in the head group and the ubiquity of the lipid made it of interest to see whether any adsorption of vasopressin could be detected in this system. Control suspensions of egg phosphatidylcholine/decane droplets in 0.1 M NaCl (pH 5.6) had a scarcely detectable mobility, and the zeta potential was $< \pm 2.0$ mV. In the presence of 5 units/ml ($1.8 \cdot 10^{-5}$ M) vasopressin at pH 4.9, no difference from the control potential could be found. The limitations on the accuracy of the mobility measurements in this system are related to focusing at the stationary level and the small number of droplets. This accuracy can be improved by measuring the mobility as a function of the distance, x, across the tube.

TABLE I

CONTROL ELECTROKINETIC POTENTIALS

All emulsions made in 0.1 M NaCl plus 0.002 M acetate, pH 4.9, except *0.02 M NaCl plus 0.004 M acetate, pH 5.4.

System	Potential (mV) (mean ± S.E.)
Glyceryl monooleate/decane $+4 \cdot 10^{-5}$ M sodium dodecyl sulphate	-58.6 ± 0.5
Phosphatidylserine/decane	-56.0 ± 0.6
Phosphatidylserine/phosphatidylcholine (1 : 1)/decane	-45.3 ± 0.3
Phosphatidylserine vesicles	-59.6 ± 0.6
Dioleyl phosphate/decane	-76.2 ± 0.9
Dioleyl phosphate vesicles	-75.7 ± 2.2
Alamethicin/decane* ($1.5 \cdot 10^{-4}$ M + 10 % (v/v) ethanol)	-61.0 ± 0.8

The application of a potential difference across the ends of a capillary tube leads to a movement of the fluid near the tube wall towards the cathode owing to the negative charge on the surface of the glass. In a closed cell, this electroosmotic component is balanced by a return flow of the fluid along the axis of the tube. It has been shown that the liquid flow velocity in a closed cylindrical tube of radius R is given by [17]

$$V = V_s \left(\frac{2r^2}{R^2} - 1\right) \tag{4}$$

where V_s is the velocity close to the surface of the tube and r is the distance from the axis of the tube. The observed velocity, V_0, of a droplet is the sum of the electrophoretic velocity, V_E, and the liquid flow velocity, i.e.

$$V_0 = V_E - V_s + V_s \left(\frac{2r^2}{R^2}\right) \tag{5}$$

V_0 is thus a linear function of r^2. From Eqn. 4 it may be seen that the stationary level is at a distance $r = \frac{1}{2}R\sqrt{2}$ from the axis of the tube.

Fig. 6 shows the results of measuring the velocity of phosphatidylcholine vesicles as a function of the distance x from the front wall of the tube. The control zeta potential was $\leqslant +1$ mV in 0.024 M salt (pH 5.6) and this corresponded to a mean charge density of less than one electronic charge per $4 \cdot 10^4$ Å^2. Addition of 5 units/ml

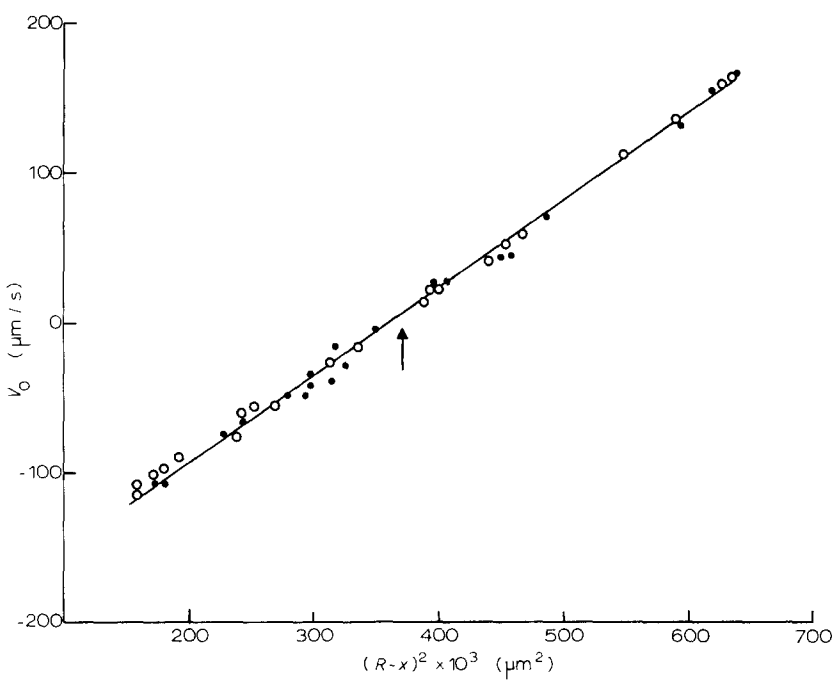


Fig. 6. The velocity of phosphatidylcholine/decane droplets as a function of the distance from the axis of the electrophoresis tube. Aqueous phase; ○, 0.024 M NaCl, pH 4.9; ●, 0.024 M NaCl, pH 4.9, plus $1.8 \cdot 10^{-5}$ M lysine vasopressin. The arrow indicates the position of the stationary level.

($1.75 \cdot 10^{-5}$ M) vasopressin to the vesicle suspension, or formation of the suspension in the vasopressin solution, had no effect on this control potential. It may be noted that since vasopressin has no effect on the slope, there can be negligible adsorption on to the glass surface of the capillary tube. Electrophoresis experiments also showed that, at pH 8.9, where the terminal α-amino group is suppressed, vasopressin (at 2.5 μM) does not adsorb detectably on to phosphatidylcholine liposomes.

Support for, and extension of, the above observations was obtained from compensation potential measurements on monolayers of phosphatidylcholine (and phosphatidylethanolamine) spread at the air-aqueous solution interface. Egg phosphatidylcholine in chloroform/methanol solution was added to the interface until further additions produced no further permanent change in the measured potential. The final potential relative to the original air-solution potential was 468 ± 7 mV (air phase positive) over eight experiments. Injection of lysine vasopressin into the aqueous solution, to give a final concentration of 2.5 μM, did not change the compensation potential by more than 5 mV at either pH 4.9 or 8.9. Similarly, injection of oxytocin, to give 0.18 μM, produced less than 5 mV change in potential at these pH values. Such potential changes as were observed were not considered significant since, in these systems, the time which must be allowed to elapse after the addition of the hormone in order to ensure that equilibrium had been reached, is relatively long (approx. 15 min) and small drifts in the reference potential could have occurred. Comparable experiments with phosphatidylethanolamine and vasopressin gave similarly negative results.

Phosphatidylserine

The strong interaction of vasopressin with sulphate groups, noted earlier, could be of interest biologically since such groups occur in the sulphatide lipids. But the more common types of anionic group encountered in proteins and lipids are carboxyl and phosphate ions. Phosphatidylserine contains both these ions and its interaction with vasopressin and oxytocin has been studied using electrokinetic and compensation potential methods.

The control zeta potentials for phosphatidylserine/decane droplets and phosphatidylserine vesicles were similar, but low compared with that expected from the Gouy-Chapman theory assuming the area per lipid head group in the vesicle to be about 60 Å^2. Dilution of the phosphatidylserine with phosphatidylcholine caused the zeta potential to fall (Table I). The control values are in good agreement with those obtained by Bangham and Papahadjopoulos [18, 19]. The low potentials may be related to the complex nature of the phosphatidylserine head group. The effect of vasopressin on the zeta potentials of phosphatidylserine/decane and phosphatidylserine/phosphatidylcholine (1 : 1, w/w)/decane droplets in a medium of pH 4.9 are shown in Fig. 7. Despite the uncertainty about contributions to the control potentials, the half saturation concentrations in the two systems are similar, being about 20 munits/ml ($7 \cdot 10^{-8}$ M). At pH 8.9 and 2.5 μM vasopressin, no potential change was found.

The reversibility of the vasopressin interaction with phosphatidylserine was examined in two ways. In two experiments, a phosphatidylserine/decane emulsion containing $8.75 \cdot 10^{-7}$ M vasopressin at pH 4.9 was diluted with 0.1 M NaCl to give a vasopressin concentration of $7 \cdot 10^{-8}$ M. The zeta potential of the undiluted solution was -44.5 mV, and of the diluted sample was -50.4 mV, thus indicating reversi-

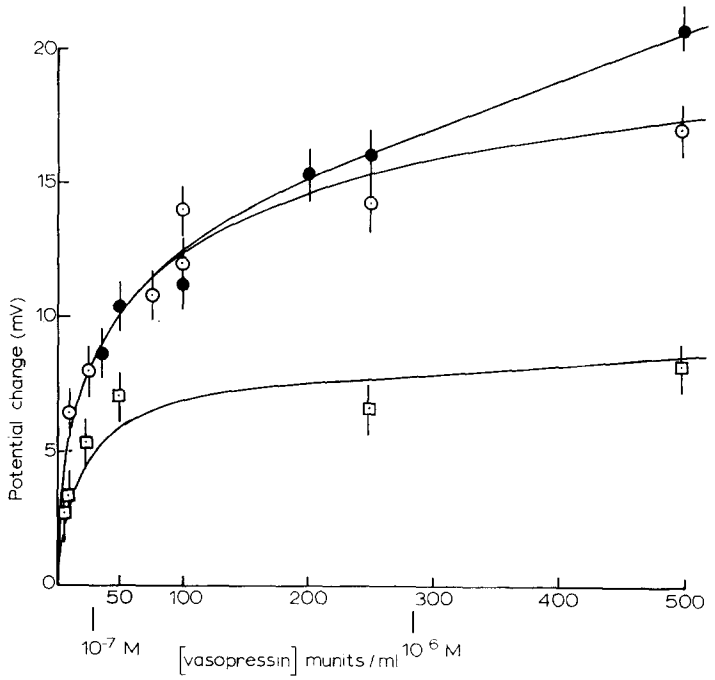


Fig. 7. Electrokinetic potential changes in the presence of vasopressin of emulsions of ○, phosphatidylserine/decane; ⊡, phosphatidylserine and phosphatidylcholine (1 : 1, w/w)/decane and ●, dioleyl phosphate/decane. Aqueous phase: 0.1 M NaCl plus 0.002 M acetate, pH 4.9.

bility, but there were very few drops in the diluted suspension, and some heterogeneity was always observed on dilution. The reversibility was also examined with the compensation potential apparatus. A ΔV of 377 mV was measured on spreading excess phosphatidylserine from ethanol onto a clean air-0.1 M NaCl (pH 4.9) interface. A further ΔV change of +27 mV was observed on adding vasopressin to give 2.8 μM, and this was reduced by about 20 mV on diluting the subphase 25-fold and stirring. A further addition of vasopressin returned the potential to its original value. The conclusion from both types of experiments is that the vasopressin interaction with the charged phosphatidylserine interface is a reversible one.

Oxytocin at 0.18 μM and pH 4.9 produced a change in compensation potential of the phosphatidylserine monolayers of approx. 110 mV. At pH 8.9, on the other hand, neither lysine vasopressin (at 2.5 μM) nor oxytocin (at 0.18 μM) had any significant effect. However, as with the phosphatidylcholine systems, long equilibration times could have masked changes of less than 5 mV. From arguments given in Discussion it is concluded that when the ionization of the terminal α-amino group of both vasopressin and oxytocin is suppressed the interaction with phosphatidylserine is greatly reduced.

Dioleyl phosphate

The adsorption of vasopressin at an interface containing only phosphate groups was examined using the synthetic surfactant, dioleyl phosphate. The zeta potentials measured for dioleyl phosphate/decane drops were reduced on addition of vasopressin (Fig. 7) with a half saturation concentration of about $2.6 \cdot 10^{-7}$ M.

Peptide carboxyl

Several compounds were tested in an attempt to obtain an interface with a pure peptide carboxyl. The one chosen for study was the polypeptide antibiotic alamethicin, since it had the advantage that it could be added to the hydrocarbon only, and adsorbed irreversibly at the decane-water interface. Adsorption of the alamethicin at a glyceryl monooleate/decane-water interface has, however, been found to be reversible, having been studied by interfacial tension measurements [20]. It was possible, therefore, using the latter system, to estimate the extent to which the zeta potential reflected the true surface charge density. The titration of the alamethicin was first examined in this system and was found to behave as a single acidic group, being completely ionized above pH 6.0 (Fig. 8). At an aqueous concentration of 10^{-5} M, the alamethicin adsorption should have saturated [20], and the area per molecule inferred from the zeta potential for the plateau, using the Gouy-Chapman relation was 890 Å^2. The limiting area per molecule obtained from interfacial tension measurements was 600 Å^2 (pH 5.6). Zeta potentials of droplets of decane plus alamethicin ($1.5 \cdot 10^{-4}$ M +10 % (v/w) ethanol) were also measured in 0.1 M NaCl and the values obtained

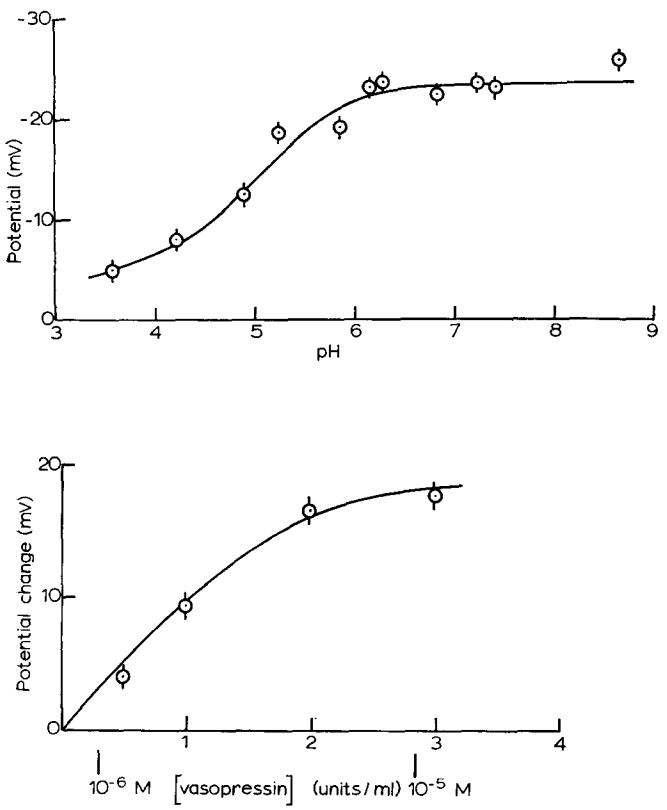


Fig. 8. Top: Electrokinetic potentials as a function of pH for an emulsion of glyceryl monooleate/decane, in 0.1 M NaCl plus 10^{-5} M alamethicin. Bottom: Electrokinetic potential changes in the presence of vasopressin for an emulsion of alamethicin/decane, in 0.02 M NaCl plus 0.004 M acetate, pH 5.4.

were −33.0 (pH 5.3) and −49.6 mV (pH 7.3); the latter potential gave an area per molecule of about 400 Å^2. In a study of the adsorption of alamethicin at air-0.1 M NaCl and decane-0.1 M NaCl interfaces, Haydon and Gordon (unpublished) found a limiting area per molecule of 300–500 Å^2 which is in agreement with that inferred from the zeta potential. They also concluded that the alamethicin adsorption was not reversible, contrary to the situation at the glyceryl monooleate/decane-water interface.

The zeta potential measurements for studying the effect of vasopressin were carried out on suspensions made from decane plus alamethicin. No detectable change was observed at pH 7.3, but an effect was seen at pH 5.4 on lowering the ionic strength (Fig. 8). The half saturation was 3.4 μM which is considerably higher than that seen with the other charged surfactants studied.

In order to discover whether this low affinity for the peptide carboxyl occurred in another system, the adsorption of vasopressin to human erythrocytes was examined. The negative charge on the erythrocyte surface is mainly due to carboxyls, these being partly sialic acid residues and partly protein carboxyls [21]. In two experiments, no change in the erythrocyte zeta potential was found in the presence of 10^{-5} M vasopressin (pH 5.2). Thus, the affinity of vasopressin for both carboxyl systems seemed less than for either phosphate or sulphate.

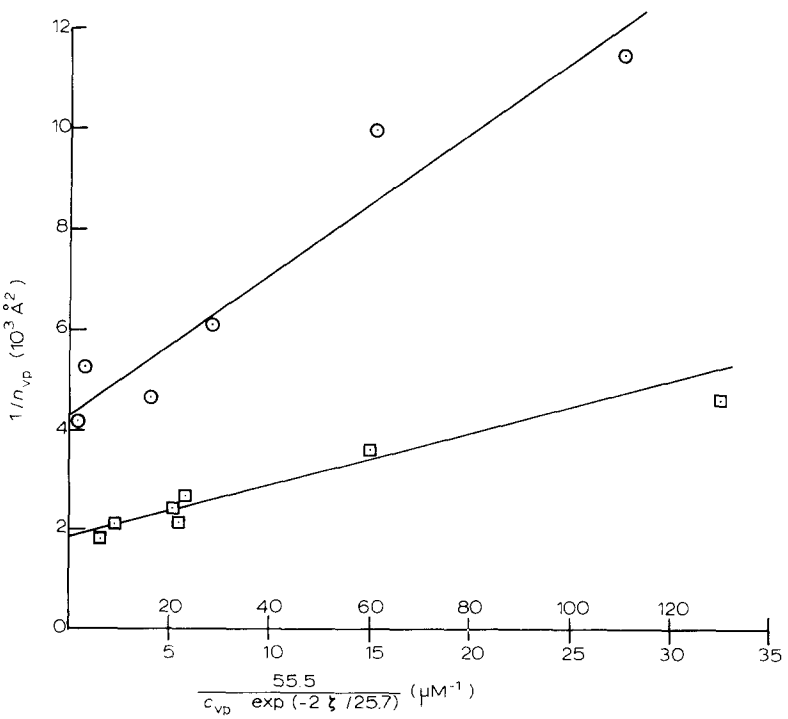


Fig. 9. Vasopressin binding (from Eq. 8) for phosphatidylserine systems; □, phosphatidylserine; ○, phosphatidylserine/phosphatidylcholine (1 : 1).

The free energies of the interactions

A simple treatment of the electrophoretic results was attempted by use of the Stern adsorption isotherm [22, 15] which has previously been applied successfully to the binding of cations by the erythrocyte membrane [23, 24]. In this treatment the number of ions, n, adsorbed per unit area is given by

$$n = \frac{\bar{x}Ne^{-\Delta\bar{\mu}^{\ominus}/RT}}{1+\bar{x}e^{-\Delta\bar{\mu}^{\ominus}/RT}} \tag{6}$$

where N is the number of binding sites available and $\bar{x}$ is the mol fraction of adsorbing species in the bulk phase. The standard electrochemical potential of adsorption per mol, $\Delta\bar{\mu}^{\ominus}$, is

$$\Delta\bar{\mu}^{\ominus} = \Delta\mu^{\ominus} + zF\varphi_0 \tag{7}$$

where $\Delta\mu^{\ominus}$ is the standard chemical potential of adsorption and φ_0 is the surface potential. Thus, for a charged molecule, the adsorption is determined both by affinity for binding sites and by the diffuse double layer potential φ_0. For vasopressin, in the systems to be analysed, $z = 2$, and on substitution and inversion, Eqn. 6 becomes

$$\frac{1}{n_{vp}} = \frac{1}{\bar{x}NKe^{-2F\varphi_0/RT}} + \frac{1}{N} \tag{8}$$

where $K = e^{-\Delta\mu^{\ominus}/RT}$. Hence, a plot of $1/n_{vp}$ against $1/\bar{x}e^{-2F\varphi_0/RT}$ should be linear, with

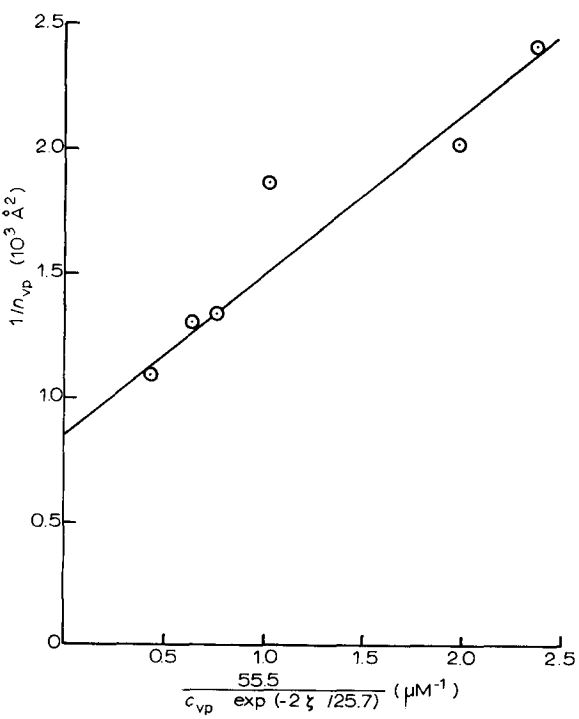


Fig. 10. Vasopressin binding (from Eq. 8) for the dioleyl phosphate system.

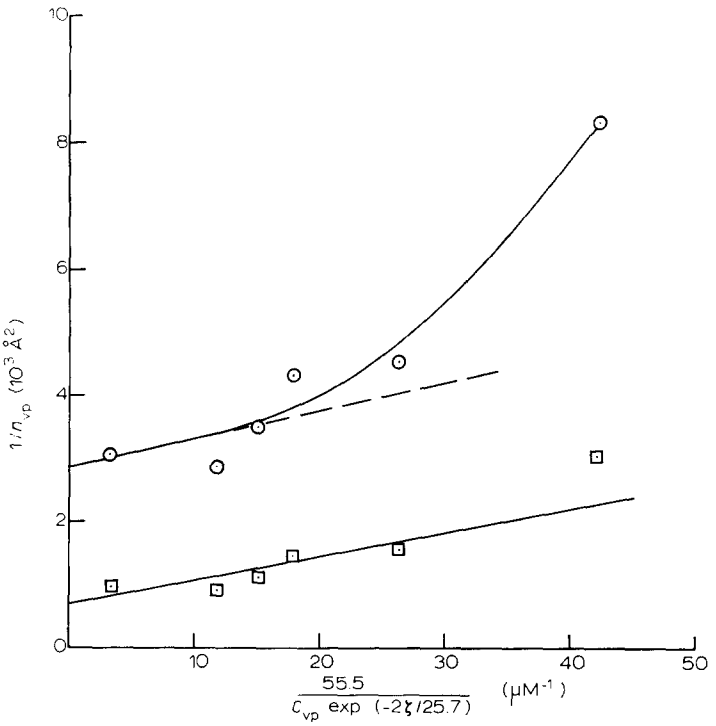


Fig. 11. Vasopressin binding (from Eqn. 8) for the glyceryl monooleate/decane-sodium dodecyl sulphate system. ○, uncorrected and □, corrected for additional adsorption of sodium dodecyl sulphate using Eqn. 9.

TABLE II

BINDING PARAMETERS FOR VASOPRESSIN OBTAINED FROM THE STERN ISOTHERMS FOR VARIOUS CHARGED GROUPS

System	$\frac{1}{N}$ (Å^2)	K	$\Delta\mu^{\ominus}$ (kcals/mol)	$C_{\frac{1}{2}}^{S}$ (M)*	$C_{\frac{1}{2}}^{B}$ (M)*
Phosphatidylserine/decane	1850	$1.8 \cdot 10^7$	9.9	$2.9 \cdot 10^{-6}$	$7.2 \cdot 10^{-8}$
Phosphatidylserine/phosphatidylcholine (1 : 1)/decane	4300	$6.1 \cdot 10^7$	10.6	$9.1 \cdot 10^{-7}$	$3.4 \cdot 10^{-8}$
Dioleyl phosphate/decane	850	$1.3 \cdot 10^6$	8.3	$4.3 \cdot 10^{-5}$	$3.5 \cdot 10^{-7}$
Glyceryl monooleate/decane-sodium dodecyl sulphate	2900	$6.6 \cdot 10^7$	10.7	$8.4 \cdot 10^{-7}$	$1.2 \cdot 10^{-8}$
Glyceryl monooleate/decane-sodium dodecyl sulphate**	700	$1.0 \cdot 10^7$	9.9	$2.92 \cdot 10^{-6}$	$4.4 \cdot 10^{-8}$
Alamethicin/decane		$< 2.8 \cdot 10^5$	< 7.4	$> 2 \cdot 10^{-4}$	$> 3.5 \cdot 10^{-6}$

* $C_{\frac{1}{2}}^{S} = \frac{55.5}{K}$, $C_{\frac{1}{2}}^{B} = \frac{55.5}{Ke^{-2\zeta/25.7}}$

** With the correction for additional adsorption of sodium dodecyl sulphate.

a slope of $1/NK$ and intercept on the ordinate axis of $1/N$. For the purpose of the analysis the zeta potential was taken as an approximation to φ_0 [12, 16, 25] and n_{vp} was determined from the potential using the Gouy-Chapman equation 3.

It may be seen from Figs. 9 and 10 that good linear inverse plots were obtained for the phosphatidylserine and dioleyl phosphate systems. The best fits were obtained by least squares regression analysis, and the parameters obtained are listed in Table II. The regression coefficients for all three systems were between 0.95 and 0.98.

Some attempt was made to correct for the additional adsorption of sodium dodecyl sulphate in the glyceryl monooleate/decane-sodium dodecyl sulphate system. This was done by assuming that the increased surface excess of the sodium dodecyl sulphate ($\Gamma - \Gamma_0$) was determined solely by the potential change, i.e.

$$\Gamma = \Gamma_0 \, e^{-F\Delta\zeta/RT} \tag{9}$$

although this neglects any specific interaction between vasopressin and sodium dodecyl sulphate. The slope of the curve at the intercept with the ordinate was used to determine K, since the number of binding sites, N, was not constant. The value of K deduced was not significantly altered by the correction for the additional sodium dodecyl sulphate, although the inferred vasopressin adsorption was increased considerably.

An analysis of the alamethicin data was not possible, the fit being poor. One possible explanation of this could be that, since the measurements were made near the pK of the alamethicin, a change in ionization of the carboxyl contributed to the measured potential in the presence of vasopressin. An attempt to analyse the compensation potential data in terms of Eqn. 8 gave meaningless results, even after correction for the additional sodium dodecyl sulphate adsorption. The reason for this is almost certainly that, as mentioned earlier in this section, the compensation potential did not correspond to the change in φ_0, but that the major part of it arose from dipole reorientation.

The fit for those systems that could be analysed was surprisingly good considering the assumptions that were required. The limiting area per molecule of vasopressin ($1/N$) was always greater than would be expected from complete surface coverage on charge neutralization, and it is likely that the adsorption was underestimated. The half saturation concentrations are only to be considered accurate to an order of magnitude, but the standard adsorption free energy, $\Delta\mu^{\ominus}$, is relatively insensitive to small errors.

The strength of binding of vasopressin by the various compounds may be arranged in the following order: sodium dodecyl sulphate (sulphate), phosphatidylserine (phosphate+carboxyl) > dioleyl phosphate (phosphate) > alamethicin (carboxyl). It would be unwise, however, to conclude that this represented a general order of binding strength for the groups concerned. It is known, for example, that the two types of carboxyl on the erythrocyte surface exhibit very different affinities for methylene blue [21].

DISCUSSION

It has been demonstrated that, at pH 4.9, where all amino groups are ionized, both lysine vasopressin and oxytocin adsorb strongly at interfaces containing dodecyl

sulphate ions. At pH 8.9, where the vasopressin has only its lysyl group ionized, and where oxytocin is un-ionized, the adsorption of both substances is still strong. The similarity of the latter two sets of results (Fig. 4) suggests that the lysyl amino group plays little part in the vasopressin adsorption, and the fact that the un-ionized oxytocin adsorbs at all suggests that lipophilic or other non-Coulombic interactions contribute substantially to the strength of the adsorption. As no evidence for any adsorption of either hormone was found for an air-water interface in absence of sodium dodecyl sulphate, or when a quaternary ammonium surfactant was present, it seems that lipophilic interactions can be ruled out. The obvious remaining interaction involves hydrogen bonds. The standard free energy change of approx. 10 kcal/mol (Table II) would correspond to the formation of at least 2–3 such bonds per hormone molecule.

Both vasopressin and oxytocin, at pH 4.9, were shown to adsorb strongly on to phosphatidylserine interfaces also. However, unlike the sodium dodecyl sulphate system, no zeta or compensation potential changes could be detected for either vasopressin or oxytocin at pH 8.9. It can be argued persuasively, though not rigorously, from these results that an interaction with phosphatidylserine may occur only when the terminal α-amino groups of the hormones are ionized. Thus, an absence of any change in the compensation potential on adding hormone does not necessarily indicate absence of adsorption because (a) an un-ionized molecule, such as oxytocin at pH 8.9, may produce no change in the surface dipole moment μ (Eqn. 2), and (b) an ionized molecule, such as lysine vasopressin at pH 8.9, may yield values of $\Delta\varphi_0$ and $4\pi n\mu$ which are equal and opposite. But both these eventualities seem rather unlikely in the present systems. The fact that for phosphatidylserine at pH 4.9 the compensation potential changes are larger than the electrokinetic potential changes, suggests that any dipole contribution must be positive. There is, therefore, no indication that for vasopressin at pH 8.9 the $\Delta\varphi_0$ and $4\pi n\mu$ terms would cancel, as required in (b) above. Furthermore, the suggestion of a positive dipole contribution for the singly ionized vasopressin, together with the evidence that this molecule does not adsorb on to phosphatidylserine, makes it seem unlikely that oxytocin at pH 8.9 adsorbs on to phosphatidylserine, phosphatidylcholine or phosphatidylethanolamine.

Although, for phosphatidylserine, H bonding does not seem to play a part (at least, in absence of the ionized α-amino group) the interaction is still very strong (Table II) and clearly involves substantial non-Coulombic effects. In common with the sodium dodecyl sulphate system is the finding that the lysyl amino group again plays no essential rôle.

In contrast to the sodium dodecyl sulphate and phosphatidylserine, the interaction of vasopressin with dioleyl phosphate at pH 4.9 is somewhat weaker. For phosphatidylcholine, no interaction was detectable for either vasopressin or oxytocin at either pH 4.9 or 8.9. Thus, in spite of an anionic group and possibilities for hydrogen bonding, phosphatidylcholine appears totally unreactive. As some interaction was found with dioleyl phosphate, it would seem that the presence of the trimethylammonium group or, in the case of phosphatidylethanolamine, the amino group, must have a completely inhibiting effect.

Despite the inaccuracies in the present adsorption data, it can be concluded that the interaction of the peptide with the various charged groups is very strong, and the half saturation concentrations $c_{\frac{1}{2}}^{B}$ are comparable to those observed for many biological receptors. For example, the data may be compared with the binding to the

carrier protein neurophysin, in the posterior pituitary, and to receptors in epithelia. In the latter case, binding is reflected only indirectly by the magnitude of the observed increase in water permeability. It should be noted that in the biological situation, experiments have usually been carried out at an alkaline pH, where the ionization of the terminal amino group will have been supressed.

The binding of lysine vasopressin to bovine neurophysin has been studied by equilibrium dialysis and the affinity constants obtained were about $2 \cdot 10^4$ (pH 7.18) [26] and about 10^5 (pH 5.8) [27]. The interaction is reversible and little selectivity between oxytocin and vasopressin is exhibited. It has been suggested that ionic or hydrogen bonding between the essential terminal amino group of the peptide and a carboxyl on the carrier protein provides a basis for complex formation (it is of interest in this connection that in none of the present experiments was the lysyl amino group found to be essential), although the hydrophobic residues at positions 2 and 3 are also important, and may be involved in non-polar interactions. The major qualitative features of the hormone-neurophysin interaction can be reproduced in model peptides containing only the first three residues [28]. The binding constants found for the biological system are of the same order of magnitude as that determined here for the interaction between lysine vasopressin and the alamethicin carboxyl.

The binding of lysine vasopressin to toad bladder epithelial cells, as assayed by the water permeability response, has an affinity constant of about 10^7 (pH 7.75). In the same system, oxytocin has an affinity constant of about 10^8 [29], but both peptides are less effective than the endogenous hormone arginine vasotocin. The biological receptors would be expected to have other important properties to determine selectivity, and this is supported by the variety of minor modifications in peptide structure which diminish activity [29]. The findings in the present work do suggest, however, that a negative charge on the receptor could contribute to the peptide's interaction. In both the phosphatidylserine and dodecyl sulphate systems examined in the present study, half saturation concentrations are much smaller than for neurophysin and are comparable to those given for the toad bladder. All three types of negative group which have been examined here are likely to occur in the epithelial cell membrane. Although no detailed lipid analysis could be found for the toad bladder, a considerable amount is known about the lipids of the mammalian kidney, where it has been shown that sulphatides constitute a significant component [30]. This is of special interest in view of the strong interaction found between vasopressin and sulphate.

It is not necessarily implied that there is any parallel between the hormone receptors in neurophysin and toad bladder. The mammalian antidiuretic hormone receptors may also be quite separate to both in that they have evolved a further specificity discriminating strongly in favour of the vasopressins. Replacement of the lysine in position 8 by any other basic residue (e.g. arginine, ornithine, diaminobutyric acid) retains the mammalian antidiuretic activity, but replacement with a neutral amino acid considerably diminishes the activity [1].

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REFERENCES

1 Handbook of Experimental Pharmacology (Berde, B., ed.), (1968) Vol. XXIII, Neurohypophysial Hormones and similar Polypeptides, Springer-Verlag, Heidelberg
2 Miller, I. R. and Bach, D. (1973) Surf. Colloid Sci. 6, 185–260
3 Snart, R. S. and Sanyal, N. N. (1968) Biochem. J. 108, 369–373
4 Kafka, M. S. and Pak, C. Y. C. (1969) Biochim. Biophys. Acta 193, 117–123
5 Graziani, Y. and Livne, A. (1971) Biochem. Biophys. Res. Commun. 45, 321–326
6 Fettiplace, R., Haydon, D. A. and Knowles, C. D. (1971) J. Physiol. Lond. 221, 18–20P
7 Fettiplace, R. (1974) Ph. D. Dissertation, University of Cambridge
8 Kinloch, C. D. and McMullen, A. I. (1959) J. Sci. Instrum. 36, 347–349
9 Bangham, A. D., Flemans, R., Heard, D. H. and Seaman, G. V. F. (1958) Nature 182, 642–644
10 Seaman, G. V. F. and Heard, D. A. (1960) J. Gen. Physiol. 44, 251–268
11 Overbeek, J. Th.-G. (1952) in Colloid Science (Kruyt, H. R., ed.), Vol. 1, p. 207, Elsevier, Amsterdam
12 Haydon, D. A. (1964) in Recent Progress in Surface Science (Danielli, J. F., Pankhurst, K. G. A. and Riddiford, A. C., eds.), Vol. 1, pp. 134–145, Academic Press, New York
13 Matijevic, E. and Pethica, B. A. (1958) Trans. Farad. Soc. 54, 1382–1389
14 Campbell, B. J., Sun-Chu, F. and Hubbard, S. (1963) Biochemistry 2, 764–769
15 Aveyard, R. and Haydon, D. A. (1973) An Introduction to the Principles of Surface Chemistry, Cambridge University Press
16 Haydon, D. A. and Myers, V. B. (1973) Biochim. Biophys. Acta 307, 429–443
17 Overbeek, J. Th.-G. (1952) in Colloid Science (Kruyt, H. R., ed.), Vol. 1, p. 218, Elsevier, Amsterdam
18 Bangham, A. D. and Papahadjopoulos, D. (1966) Biochim. Biophys. Acta 126, 181–184
19 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240–254
20 Gordon, L. G. M. and Haydon, D. A. (1975) Phil. Trans. R. Soc. Lond. Ser. B. 270, 433–447
21 Haydon, D. A. and Seaman, G. V. F. (1967) Arch. Biochem. Biophys. 122, 126–135
22 Stern, O. (1924) Z. Electrochem. 30, 508–516
23 Haydon, D. A. and Seaman, G. V. F. (1962) Proc. R. Soc. Ser. B. 156, 533–549
24 Seaman, G. V. F., Vassar, P. S. and Kendall, M. J. (1969) Arch. Biochem. Biophys. 135, 356–362
25 Carroll, B. J. and Haydon, D. A. (1975) J. Chem. Soc. Faraday Trans. I, 71, 361–377
26 Breslow, E. and Walter, R. (1972) Mol. Pharmacol. 8, 75–81
27 Breslow, E. and Abrash, L. (1966) Proc. Natl. Acad. Sci. U.S. 56, 640–646
28 Breslow, E., Aanning, L., Abrash, L. and Shmir, M. (1971) J. Biol. Chem. 246, 5179–5188
29 Rasmussen, H., Schwartz, I. L., Young, R. and Marc-Aurele, J. (1963) J. Gen. Physiol. 46, 1171–1189
30 Karlsson, K. A., Samuelsson, B. E. and Steen, G. O. (1973) Biochim. Biophys. Acta 316, 317–335