Interaction of Polypeptide Hormones with Lipid Monolayers

By R. S. SNART AND N. N. SANYAL
Department of Zoology, University of Sheffield

(Received 15 January 1968)

1. The interaction between lipid monolayers spread on the surface of water and oxytocin, [8-arginine]-vasotocin and [1-asparagine-5-valine]-angiotensin II in the subphase was investigated in a Langmuir surface trough by studying the changes in pressure produced on injection of various quantities of the polypeptide solution under the film. 2. The effect of 2M- and 4M-urea on the character of the adsorption is reported. 3. Structures for the adsorbed films formed in this way are suggested. 4. If the lipid monolayer is taken as a suitable model of cell membranes, then it may be supposed that the effect of such structures forming in cell membranes would be to provide effective 'pores' to facilitate the movement of water and other small molecules across the membrane.

The cell membrane has been supposed by Danielli & Davson (1943) to consist of a bimolecular layer of lipid with an adsorbed monolayer of surface-denatured protein on each side of the lipid with a further layer of adsorbed native protein. Model structures of such membranes have been achieved by an injection technique as used by Schulman & Rideal (1937). Qualitative studies on injecting protein solution beneath unimolecular films of lipid were carried out by Schulman (1945), Doty & Schulman (1949) and Matalon & Schulman (1949), all of whom found a significant increase in surface pressure at constant film area. Eley & Hedge (1956) showed quantitatively that the adsorption of protein on lipid monolayers was determined by adsorption of one lipid molecule at each peptide link in the protein, forming a surface-denatured adsorbed layer of protein; further increases of pressure were observed that they suggest indicate adsorption of a second native layer of protein. Confirmation of the adsorption corresponding to the first adsorbed layer of protein has been obtained with cholesterol and bovine plasma albumin (Snart, 1965), but no further increase in pressure occurred after this first adsorbed layer was formed. A preliminary report of the adsorption of [8-arginine]-vasotocin and oxytocin on lipid monolayers has been communicated (Sanyal & Snart, 1967).

The molecular interactions of steroid hormones with lipid membranes have attracted attention (Willmer, 1961; Snart, 1964; Taylor & Haydon, 1965), but little is known about the interaction of polypeptide hormones with artificial membranes, though some direct effects on cell membranes have been reported (Berde & Cerletti, 1958).

The present work suggests a possible mechanism of action at lipid membrane sites.

METHODS

The interaction between lipid monolayers spread on the surface of water and oxytocin, [8-arginine]-vasotocin and [1-asparagine-5-valine]-angiotensin II in the subphase was investigated in a Langmuir surface trough by studying the changes in pressure produced on injection of various quantities of the polypeptide solution under the film. This

CyS-Tyr-Ile-Gln-Asn-CyS-Pro-Leu-GlyNH₂

Oxytocin

CyS-Tyr-Ile-Gln-Asn-CyS-Pro-Arg-GlyNH₂

[8-Arginine]-vasotocin

Asn-Arg-Val-Tyr-Val-His-Pro-Phe

[1-Asparagine-5-valine]-angiotensin II

technique is similar to that used for the study of the interaction between protein in the subphase and lipid monolayers.

To avoid contamination, the paraffin wax used to coat the trough was freed from surface-active impurities by heating with hot N-NaOH followed by hot N-HCl. After thorough washing the wax was recrystallized twice from light petroleum (b.p. 40–60°). The water was redistilled with alkaline KMnO₄. Nitrogen was passed over the covered trough during the experiments. Samples of cholesterol (British Drug Houses Ltd., Poole, Dorset), stearic acid (British Drug Houses Ltd.) and lauric acid (Professor A. S. C. Lawrence, Sheffield) were all recrystallized to constant melting point from light petroleum (b.p. 40–60°), which itself was AnalaR (British Drug

Houses Ltd.) redistilled. The samples of oxytocin (Ferring A.-B., Malmö, Sweden), [8-arginine]-vasotocin (Dr B. Berde, Sandoz Ltd., Basle, Switzerland) and [1-asparagine-5-valine]-angiotensin II (Hypertensin; Ciba Ltd., Basle, Switzerland) were used as supplied.

Solutions were made of cholesterol in benzene, stearic acid and lauric acid in light petroleum (b.p. 40–60°), oxytocin (16mg./100ml.) in water, [8-arginine]-vasotocin (10mg./100ml.) in water and [1-asparagine-5-valine]-angiotensin II (24mg./100ml.) in water.

By using an Agla microsyringe 12×10^{16} molecules of lipid were injected on to the water surface, at the natural pH of 6.7, in the Langmuir surface trough. The initial pressure of the film was adjusted to 10 dynes/cm. for cholesterol and 2 dynes/cm. for fatty acids. Trouble was experienced with lauric acid at this pressure, but after considerable effort these films were held at 2 dynes/cm. The polypeptide solution was injected through the lipid monolayer and the pressure of the film increased, reaching a maximum value within 50 min. Sweeping the surface on the non-lipid side of the mica floating barrier showed the absence of leakage or surface adsorption of injected polypeptide past the barrier. The same results were obtained whatever the site of injection of the polypeptide. This increase in pressure was measured by a horizontal torsion wire that was sensitive to 0.02 dyne/cm. and used to obtain isotherms for adsorption of polypeptide on the lipid monolayers. The effect of 2M- and 4M-urea (AnalaR; British Drug Houses Ltd.), which was found to be free from surface-active impurities, on the adsorption was studied.

RESULTS

The increase in pressure of a stearic acid monolayer after the injection of oxytocin solution beneath the film is shown (Fig. 1). The pressure increases to a maximum value after 50 min. This time-interval was required for all equilibrium adsorption measurements. No polypeptide surface film forms in the absence of the lipid monolayer. The isotherms obtained by plotting this pressure increase (at constant area) against the amount of polypeptide in the substrate are similar for both oxytocin and

Fig. 1. Variation of surface-pressure increases $\Delta\pi$ with time after the injection of oxytocin solution (containing 45 mμmoles of oxytocin) beneath a monomolecular layer of stearic acid (12×10^{16} molecules) at a surface of water.

Fig. 2. Increase of surface pressure $\Delta\pi$ with amount of oxytocin (mμmoles) injected through a monomolecular layer of cholesterol (12×10^{16} molecules) at a surface of water.

Table 1. Analysis of the adsorption isotherms obtained for the adsorption of polypeptide hormones on lipid monolayers

Values for the lipid/polypeptide ratio corresponding to inflexion points on the adsorption isotherms are given along with the film pressure.

	First break: lipid/ hormone ratio	Pressure (dynes/cm.)	Second break: lipid/ hormone ratio	Pressure (dynes/cm.)
(A) Cholesterol/oxytocin (starting pressure 10 dynes/cm., area $42 \text{ Å}^2/\text{mol.}$)				
(1)	21.0	15.3	6.0	20.8
(2)	18.8	15.3	6.3	20.1
(3)	24.0	13.5	6.4	17.7
(B) Stearic acid/oxytocin (starting pressure 2 dynes/cm., area $24 \text{ Å}^2/\text{mol.}$)				
(1)	20.2	6.9	6.1	11.8
(2)	20.2	7.3	6.0	12.2
(3)	22.7	4.9	5.8	8.3
(C) Lauric acid/oxytocin (starting pressure 2 dynes/cm., area $24 \text{ Å}^2/\text{mol.}$)				
(1)	19.6	7.5	6.1	10.6
(2)	20.9	6.8	6.2	11.9
(D) Cholesterol/[Arg ⁸]-vasotocin (starting pressure 10 dynes/cm., area $42 \text{ Å}^2/\text{mol.}$)				
(1)	22.0	16.8	6.5	20.4
(E) Stearic acid/[Arg ⁸]-vasotocin (starting pressure 2 dynes/cm., area $24 \text{ Å}^2/\text{mol.}$)				
(1)	23.0	7.8	6.5	11.8
(F) Lauric acid/[Arg ⁸]-vasotocin (starting pressure 2 dynes/cm., area $24 \text{ Å}^2/\text{mol.}$)				
(1)	20.6	7.9	6.3	12.1
(G) Cholesterol/[Asn ¹ -Val ⁵]-angiotensin II (starting pressure 10 dynes/cm., area $42 \text{ Å}^2/\text{mol.}$)				
(1)	7.1	20.9	—	—
(H) Stearic acid/[Asn ¹ -Val ⁵]-angiotensin II (starting pressure 2 dynes/cm., area $24 \text{ Å}^2/\text{mol.}$)				
(1)	7.1	8.2	—	—

[8-arginine]-vasotocin adsorbing at the surface of a cholesterol or fatty acid monolayer (Fig. 2). The adsorption isotherms are linear with respect to the amount of polypeptide injected, which suggests that before saturation all the polypeptide is adsorbed on the lipid monolayer. The isotherms show two marked discontinuities, which are believed to represent stable surface structures. The lipid/polypeptide ratios were determined at each inflexion point, and the results are tabulated in Table 1, along with the pressure changes. Lauric acid films behaved well during the adsorption experiments.

[1-Asparagine-5-valine]-angiotensin II adsorbs on both cholesterol and fatty acid monolayers, giving rise to an isotherm with only one discontinuity (Fig. 3). In all other respects the adsorption is similar to that of the other polypeptides. The lipid/polypeptide ratios and the final pressures are shown in Table 1.

The effect of 2M- or 4M-urea (pH 6.8–6.9) was to increase $\Delta\pi$, giving rise to adsorption isotherms for

Fig. 3. Increase of surface pressure $\Delta\pi$ with amount of [1-asparagine-5-valine]-angiotensin II (mμmoles) injected through a monomolecular layer of cholesterol (12×10^{16} molecules) at a surface of water.

Fig. 4. Adsorption isotherm of oxytocin on a monomolecular layer of stearic acid (12×10^{16} molecules) on 2M-urea subphase.

both oxytocin and [1-asparagine-5-valine]-angiotensin II (Fig. 4) adsorbing on a stearic acid monolayer, which resembles Langmuir type I monomolecular isotherms. They were therefore analysed by using an equation of the form:

$$\Delta\pi = ap/(1 + bp)$$

where $\Delta\pi$ represents the increase in pressure at constant area, p represents the total amount of polypeptide injected and a and b are constants characteristic of the adsorption. We plotted (Fig. 5) the amount of polypeptide injected per unit pressure increase against the amount of polypeptide injected to obtain values for the adsorption constants. Evaluation of these constants for the systems studied are shown in Table 2, along with the lipid/polypeptide ratios obtained at the point of saturation and the final pressure of the films. The full significance of this type of adsorption is not understood.

DISCUSSION

The final and intermediate structure proposed for the adsorbed films of oxytocin and [8-arginine]-vasotocin on lipid monolayers are shown in Fig. 6. These are based on the idea (Eley & Hedge, 1956) that the polar groups of the lipid associate with peptide links in proteins. A space-filling model (Fig. 7) of the polypeptide chain shows that the proline group helps to give a six-peptide ring structure. The 6:1 lipid/polypeptide ratio is believed to represent a ring of six lipid molecules adsorbed at each peptide link associated with the ring of the hormone. The latter ring is one of the essential structural requirements for physiological

Fig. 5. Adsorption of oxytocin on a stearic acid monolayer in the presence of 2M-urea: a plot of the amount of polypeptide injected (mμmoles) per unit pressure increase (dynes/cm.) against the amount injected (mμmoles).

Table 2. *Analysis of the adsorption isotherms obtained for oxytocin and [1-asparagine-5-valine]-angiotensin II on lipid monolayers, with a urea subphase*

The results were analysed in terms of the equation:

$$\Delta\pi = ap/(1 + bp)$$

where $\Delta\pi$ represents the increase in pressure of constant area, p represents the total polypeptide injection and a and b are constants characteristic of the adsorption.

	a	b	Lipid/ hormone ratio	Final pressure (dynes/cm.)
Oxytocin/stearic acid (2M-urea)	3.05×10^9	2.09×10^8	8.9:1	14.2
[Asn ¹ -Val ⁵]-angiotensin II/stearic acid (4M-urea)	2.72×10^9	1.54×10^8	6.8:1	16.5
[Asn ¹ -Val ⁵]-angiotensin II/stearic acid (2M-urea)	3.05×10^9	2.16×10^8	7.2:1	14.2
[Asn ¹ -Val ⁵]-angiotensin II/cholesterol (2M-urea)	2.91×10^9	2.10×10^8	6.8:1	21.8

Fig. 6. Proposed structures for the surface films resulting from the adsorption of oxytocin/[8-arginine]-vasotocin at the surface of lipid monolayers. Structure A corresponds to a 6:1 lipid/polypeptide ratio and structure B corresponds to a 21:1 lipid/polypeptide ratio.

activity (Berde & Cerletti, 1958). The 21:1 lipid/polypeptide ratio requires stabilization by adsorption of three peptide groups in the tail part of the hormone. The tail unit of the hormone could help to stabilize our structure B by sweeping out the space between each ring structure such that particular orientations may be represented by the shaded region in Fig. 6. This would require, not only that the eight peptide links in the polypeptide

should be adsorbed on lipid molecules, but also that the terminal amide group should do so.

If these lipid monolayers may be taken as suitable basic models of cell membranes, it may be supposed that the effect of such structures forming in cell membranes would be to provide effective 'pores' to facilitate the movement of water and other small molecules. Such biological effects of the octapeptide hormones have been demonstrated (Koefoed-Johnsen & Ussing, 1953; Leaf & Dempsey, 1960) and are consistent with those expected if 'pore' size had been changed. For the polypeptide hormones to act in this manner they would have to displace cellular protein from the polar groups of the membrane lipids. As the work done in expansion of the lipid film on adsorption of these hormones is similar to that observed with the lipid-protein interaction (Snart, 1965), this could occur by competitive interaction. However, it is considered more reasonable to suggest that the disulphide bridge could help such a displacement interaction, by attaching itself to cellular protein. There is evidence to suggest (Rasmussen, Schwartz, Schoessler & Hochster, 1960) that the disulphide bonds are important in the binding of the hormone to thiol groups of a membrane protein.

In more recent work it has been proposed that adenosine 3',5'-(cyclic)-phosphate acts as an intracellular intermediate in the effect of the neurohypophyseal hormones and has been shown to mimic their activity (Handler, Butcher, Sutherland & Orloff, 1965; Orloff & Handler, 1962, 1964). It is possible to accept an involvement of adenosine 3',5'-(cyclic)-phosphate in the hormonal effect of the neurohypophyseal hormones while realizing that the interaction of the hormone with lipid membranes

Fig. 7. Photograph of space-filling atomic model of basic octapeptide structure, placed on the bench as it may be considered to lie at the lipid interface. The hexagonal ring of six peptide units can be traced by the oxygen atoms, which are supposed, along with the nitrogen atoms, to associate with the polar groups of the lipid. It is possible to swing the tail unit out of this plane by bending about the proline group.

would explain the principal mechanism of action of these hormones.

The adsorption of [1-asparagine-5-valine]-angiotension II on cholesterol and stearic acid monolayers was carried out as a means of establishing the nature of the adsorption, [1-asparagine-5-valine]-angiotension II being a linear polypeptide. The result confirms that one lipid molecule adsorbs at each peptide link in the polypeptide, giving a 7:1 lipid/polypeptide ratio.

The increase in pressure observed on a urea subphase is in contrast with the corresponding decrease reported (Eley & Hedge, 1956) in the lipoprotein system. With oxytocin there is no further sign of the intermediate structure, and the final structure now corresponds to a 9:1 lipid/polypeptide ratio. This ratio tends to confirm the idea, not only that the eight peptide links in the polypeptide adsorb on lipid, but that the terminal amide group also does so. The amide groups on the asparagine and glutamine side chains are probably not of a suitable length to allow the amide group to assume a suitable configuration on the surface.

It was hoped to distinguish, if possible, between different hormones by using an urea subphase. However, the results shown in Table 2 suggest that no significant difference exists in strength of adsorption. Any biological selection therefore cannot involve specific lipid membrane sites, and selective inhibitors, such as the inhibitor of oxytocin found in toad bladder (Karlin & Overweg, 1965),

would be needed as a means of obtaining specificity in responsive tissues.

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