for example cytochrome c, which bind strongly to co-factors such as haem.

Finally, the third group comprises a different class of proteins which have not been crystallised without co-factors. Analyses of their properties in solution show that they do not fold in a well defined way, and tend to form a random coil at relatively low temperatures. Proteins in this group, for example troponin-C (rabbit) and ribosomal proteins, have a large proportion (≃ 25%) of charged amino acid residues. There is a paucity of information in the literature on the relationship between the hydrophobic to charged amino acid residues in proteins and their surface activity.

The investigations of Papahadjopoulos and his associates on the effect of various proteins on the transition temperature (T_c) of lipids however, permitted them to identify three broad types of interaction as follows:

(i) Surface adsorption, for example, as shown by ribonuclease and polylysine;

(ii) Surface adsorption followed by partial penetration and deformation of the lipid bilayer as, for example, manifested by the basic myelin protein A_1, and also by cytochrome c and haemoglobin; and finally,

(iii) Complete penetration of the lipid bilayer by the protein, as observed in myelin proteolipid, apoprotein and gramicidin (Papahadjopoulos et al., 1975). The ratios of the hydrophobic to charged amino acid residues in some of these proteins and in VSG are listed in Table 5.

Table 5 The ratio of hydrophobic to charged amino acid residues (H/C) for various proteins.

Protein	H/C	Reference
Ribonuclease	2.4	Williams (1979)
Mellitin	3	Knoppel (1979)
Variant surface glycoprotein	3	Cross (1975)
β-Casein		
K-casein		
apo A-I	1.2	Osborne (1977)
apo A-II	1.7	Osborne (1977)
Lysozyme	2.2	Williams (1979)
Cytochrome c	1.5	Williams (1979)
Protein A_1 of myelin membrane	1.5	Williams (1979)

The term 'protein hydrophobicity' is used on the basis of amino acid composition and surface activity properties (see Section 5.4). The ratio (H/C) was calculated on the basis of amino acid composition divided into three main classes:

(i) Hydrophobic: leucine, valine, isoleucine, alanine, proline, methionine, cysteine, phenilalnine, tyrosine, tryptophan and threonine.

(ii) Charged: lysine, arginine, glutamic acid and aspartic acid.

(iii) Intermediate: glycine, serine, asparagine, glutamine and histidine.

From the above discussion it is clear that this ratio does not contain sufficient information to assess the overall 'hydrophobicity' of the protein. For example, VSG and mellitin have identical values for this ratio but their behaviour at the air-water interface is totally different. Most investigations have concentrated on the interaction of plasma lipoproteins with phospholipids. Segrest et al. (1974) have shown that the ability of apolipoprotein to interact and form complexes with phospholipids could not readily be explained on the basis of their amino acid composition, nor from direct inspection of their primary structures. These authors used evidence from space-filling models of apoproteins of known sequence, and have suggested the existence of amphipathic helices in these proteins. Where the distribution of charged amino acids with a type of steric hindrance to charge could partially explain the occurrence of ion pairs in restricted regions of the amphipathic helix. Then the possibility of interaction with oppositely charged groups in phospholipids becomes greatly increased (Morrisett et al., 1977).

The complete amino acid sequence for VSG has not been determined. So far, there are partial sequences for the carboxyl terminus (Johnson & Cross, 1979), and an indirect sequence of the whole molecule from cDNA sequences (Cross et al., 1980). Once the complete sequence is known it may be possible to construct a space-filling model in order to show the distribution of the charged amino acid residues and search for possible binding sites.

5.5.5 Quantifying the interaction of VSG with lipids

The quantification of the VSG-lipid interaction was considered in Section 5.4. The modified Gibbs adsorption isotherm analysis was applied

to the interaction of VSG with various phospholipids. Since the adsorption of VSG occurred at an interface containing a lipid monolayer, it was important to quantify the formation of the first layer of VSG beneath the lipid film. The method suggested by Eley & Hedge (1956) permitted the calculation of the slope $^{d\pi}/_{d\log_c}$ at the point where formation of the first layer is completed. Thus the surface excess of VSG calculated for various monolayers takes into account only the first layer of VSG deposited beneath the lipid film (Table 1(a)). Comparison of the number of amino acid residues penetrating lipid monolayers calculated from the Gibbs adsorption equation and other calculations, shows markedly differing values in some cases. The values showing the best correlation are those obtained using the equation due to Smaby & Brockman (1978) for calculations involving a miscible system. It is clear from these calculations that the bulk of VSG molecule inserted into lipid monolayers is extremely small, bearing in mind that the total surface area of this protein molecule unfolded at the interface is estimated to be 8220$\mathring{A}^2$. It could be assumed, therefore, that these calculated values of amino acid residues represent a part of the molecule which is attached to the lipid surface. Under these conditions the adsorbed protein molecule is not completely unfolded and therefore different from the structure of a spread protein monolayer. The above assumptions have been indirectly substantiated by the findings of Morrisett (Morrisett et al., 1977) that the α-helical content of apo C-III is increased in the presence of phospholipids. Even in the absence of a lipid monolayer it appears that various polypeptides still retain some of their secondary structure (Mitchell et al., 1970; Malcolm, 1973). Also

application of the Gibbs adsorption equation to various proteins shows that the area occupied per protein molecule is smaller than that expected for an unfolded protein, thus implying that there is no relationship between values for the area occupied and the molecular size of the protein (see review by MacRitchie, p. 303, 1977). In other words, the molecular area of BSA estimated from the π-A isotherms of spread monolayers of protein is about 10,000$\mathring{A}^2$, and in contrast to this value, the area of BSA adsorbed showed an average value of 265$\mathring{A}^2$, corresponding to seventeen amino acid units.

The low values for penetrating amino acid units obtained from other calculations such as the geometric model, coefficient of lateral compressibility, and relative increase in area, could be due to over-estimation of the amount of VSG which had mixed with the lipid monolayer. It is thus not possible to arrive at a definite conclusion as to which treatment provides the best approximation for the number of penetrating amino acid units. The result from the geometric model may be excluded, however, on the grounds that it has not taken the lateral pressure of the film into account, and therefore assumes that the available surface area is entirely occupied by the molecules of VSG. This is well illustrated in Fig. 5.12 where it is seen that the total surface pressure at a VSG:phospholipid molar ratio of 1:1 did not reach values higher than 10 mN/m.

CHAPTER 6

INFLUENCE OF PH, IONIC STRENGTH, AND DETERGENT ON THE ANTIGEN-LIPID INTERACTION IN MONO- AND HETERO-MOLECULAR FILMS

CHAPTER 6

INFLUENCE OF PH, IONIC STRENGTH, AND DETERGENT ON THE ANTIGEN-LIPID INTERACTION IN MONO- AND HETERO-MOLECULAR FILMS

Study of the interaction of VSG with monomolecular films has proved highly informative. The ease of obtaining physicochemical values such as compressibility coefficient, critical surface pressure, and adsorption at the lipid-water interface has shown the monolayer model to be suitable for these studies.

6.1 Mixed monolayers

The use of heteromolecular films as a model is of importance because they can be made to approximate natural systems. The study of insoluble monolayer mixtures can be carried out in a similar way to that for a monolayer containing a single molecular species. The characteristics of the mixed film, however, do not necessarily correspond to an average of the characteristics of the individual components as discussed below. Despite the considerable complexity of working with a system consisting of an insoluble mixed lipid monolayer and an added water-soluble component, the study is potentially attractive.

At this stage, it is pertinent to discuss the theoretical and practical ways of evaluating the miscibility properties of heteromolecular lipid films. If the components are immiscible they will obey the equation

$$A_{1,2} = N_1A_1 + N_2A_2 \qquad (6.1)$$

where $A_{1,2}$ is the average molecular area units, N_1 and N_2 are the mole fractions of the components, A_1 and A_2 are the molecular areas

for their respective monomolecular films at the same surface pressure. Surface potential should also obey a similar equation

$$\Delta v_{1,2} = N_1 \Delta v_1 + \Delta v_2 N_2 \qquad (6.2)$$

The application of the thermodynamics of mixing at the interface analogous to that applied to gases and liquids provides other arguments. Thus from the Gibbs free energy equation (5.4), with the intensive variables held constant [Temperature (T), surface tension (γ) and pressure (p)] we obtain,

$$G^s = \sum_i \mu_i n_i \qquad (6.3)$$

the superscript s denotes surface property.

μ_i is in equilibrium with the bulk phase and can be written $\mu_i = RT \ln n_i$ and the equation (6.3) becomes

$$G^s = \sum_i n_i RT \ln n_i \qquad (6.4)$$

For two components at the liquid surface the free energy of mixing of the components 1 and 2 at the interface is

$$\Delta G_{mix} = nRT (x_i \ln x_1 + x_2 \ln x_2) \qquad (6.5)$$

n is the total amount of components at the interface ($n = n_1 + n_2$), and x_1 and x_2 are the mole fraction for each component.

Since $\left(\frac{\partial G}{\partial T}\right)_{p,n,\gamma} = -s$

The entropy of mixing is

$$\Delta s_{mix} = -(\partial G_{mix} / \partial T)_{p,n_1,n_2} = - nR(n_1 \ln x_1 + x_2 \ln x_2) \qquad (6.6)$$

mix is the abbreviation for mixing

Since ln x is negative G_{mix} is negative and the entropy of mixing is a positive quantity. It is in ideal mixtures where the 1-1, 1-2 and 2-2 interactions are not zero but they are all virtually the same and using

$$\Delta G_{mix} = \Delta H_{mix} - T\Delta S_{mix} \qquad (6.7)$$

at constant temperature the enthalpy of mixing is $\Delta H_{mix} = 0$ (constant p and T). 1 denotes the component n_1 and 2 denotes the component n_2. In real mixtures in which the 1-1, 1-2 and 2-2 interactions are different, an enthalpy change is involved ($\Delta H_{mix} = 0$) in the disruption of 1-1 and 2-2 interactions and their replacement by 1-2 interactions. If the interactions 1-1 and 2-2 are more favourable than the 1-2 the enthalpy of mixing is positive ($\Delta H_{mix} > 0$) and its contribution to ΔG_{mix} may overcome the favourable entropy term and the components will be immiscible.

Any deviation of mixed monolayer behaviour from equations (6.1) and (6.2) provides evidence for miscibility in the film. At low surface pressures, however, some mixed films have been found to be miscible whilst they remain immiscible at high surface pressure (Gaines, 1966, p. 281).

Therefore, the use of the surface phase rule† for detecting miscibility in heteromolecular monolayers at the equilibrium spreading

†The following definition of the surface phase rule for the monolayer system is analogous to Gibb's phase rule (Gaines, 1966).

$$F = C^B + C^S - P^B - q + 3 \qquad (6.8)$$

where F is number of degrees of freedom, C^B number of components of the system, P^B number of bulk phases, C^S number of components confined to the surface and q number of surface phases in equilibrium with one another. Surface tension, temperature and external pressures are system variables.

pressure has proved to be of extreme importance. It is very well illustrated in the study of condensed heteromolecular lipid monolayers carried out by Gershfeld (1974). The two components DPPC and CHL (crystals) were added to water, the mixture was stirred vigorously, the surface tension and surface composition were monitored periodically until no change in both parameters was detected. The use of radio-labelled CHL showed that at equilibrium CHL remained at the same surface concentration as for a pure saturated solution. This was taken to indicate that DPPC had been completely excluded from the surface and, therefore, both components (DPPC and CHL) were immiscible in surface film. This result contradicts that found in the spread film experiment, where solutions of CHL and DPPC at fixed molar ratios in a volatile organic solvent were spread on an aqueous surface followed by the determination of the π-A isotherm. The latter indicated that π was a function of the mean average area (van Deenen et al., 1962; Müller-Laudau et al., 1979). Thus from the spread film experiment it seems that the two lipid components are miscible at all film pressures. Gershfeld (1974) explains this discrepancy in equilibrium terms: the spread film is assumed to be a non-equilibrium system in view of the possible occurrence of supercompression and the high surface viscosity in condensed films. In equilibrium spreading pressure experiments a real equilibrium is probably reached and then, the compound with the highest equilibrium spreading pressure will preferentially adsorb to the surface when the components are immiscible. Details of the Gibbs phase rule are not given here because equilibrium spreading pressure experiments were not attempted since spread film experiments were found to be more appropriate for the present study. The main objective in using

mixed lipid films was to find out whether VSG could compete with phospholipids for sterols to form a 'mixed' VSG-sterol-phospholipid film, which could be a feature on the cell surfaces.

6.1.1 Mixed monolayers of phosphatidylcholine and phosphatidylethanolamine

Monolayers of EYPC and PE in a 1:1 molar ratio spread at the air-water interface showed a π-A isotherm typical of an expanded lipid film. Precautions taken during the compression and expansion were as described for monomolecular films. Specially preformed films for studying the VSG interaction were never compressed to the collapse point. Comparison of the displacement of the π-A isotherm in the presence of VSG, both in the mixed film and in the monomolecular lipid film of each component respectively, did not show significant differences within experimental error. This is illustrated by the graph inset (Fig. 6.1) where the $\Delta\pi_{VSG}$ values have been found to be equivalent to those observed for PE and EYPC monomolecular monolayers.

It should be noted here that in these calculations no attempts were made to calculate δ as a function of amount of VSG adsorbed at the lipid surface. In order to follow the behaviour of the VSG-lipid interaction the VSG-lipid molar ratio of 1:6 was kept constant. Changes in surface potential, in general follow the pattern observed in experiments with monomolecular films of phospholipids. At very low surface pressures of lipid, the presence of VSG raised the $\Delta\delta$ by about 50 mV compared with that found for lipids alone; and this difference diminished simultaneously with the increase of surface pressure. This suggests that VSG mixes with lipid molecules at low surface pressure, but is squeezed out at high surface pressure.

Fig. 6.1

Mixed monolayer of egg yolk phosphatidyl choline-phosphatidyl ethanolamine spread at the air-water interface. The lipids were mixed previously before adding to the interface.

(O) equimolar mixture of EYPC and PE, 6 nmol each.

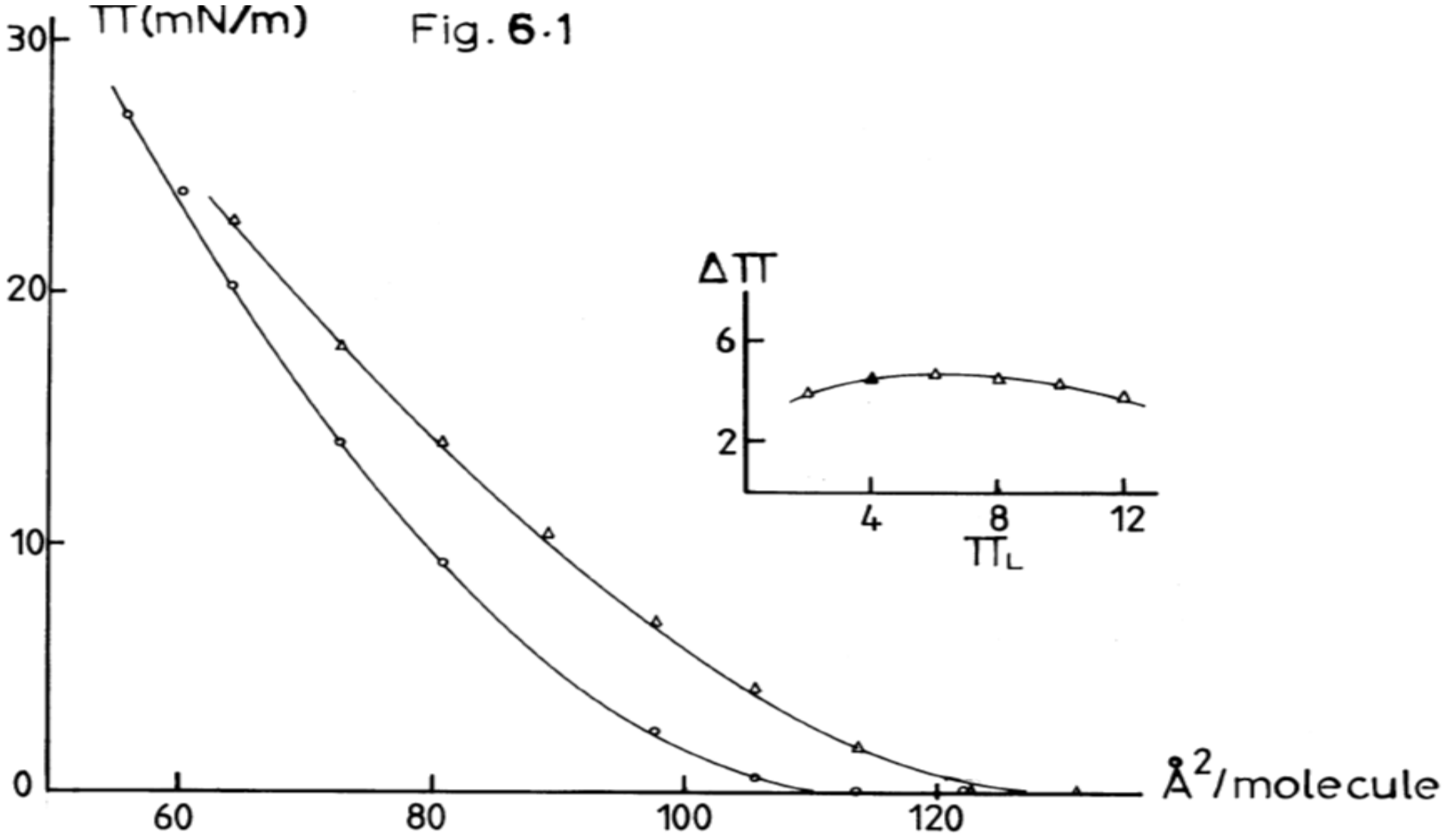
(Δ) VSG was added by touching the lipid surface at near zero surface pressure for a VSG:phospholipid mol ratio of 1:6, and the force-area curve was determined after 1 hr.

Figure inset.

$\Delta\pi$ is the difference between the surface pressure from the phospholipid-VSG monolayer and the phospholipid monolayer as determined from force-area curves (see equation 5.1). The critical surface pressure is taken at the inflexion point where $\Delta\pi$ start decreasing.

Subphase pH 6.8.

Fig. 6.1



6.1.2 Mixed monolayers of phosphatidylethanolamine, sphingomyelin and cholesterol

Interaction of VSG_{151} with monolayers containing PE, SPH and CHL were carried out as described above. Fig. 6.2 shows that increasing the ratio of cholesterol caused less expansion of the π-A isotherm. Increasing the CHL:phospholipid mole ratio from 1:5 to 1:3, however, did not cause a significant increase in Δπ values when an equivalent amount of VSG was added (Fig. 6.2, inset). Changes in Δπ for CHL-phospholipid ratio of 1:1 gave higher values than those above. Discontinuity of surface pressure changes can also be deduced from the plot of Δπ versus lipid surface pressure (πL). The point where Δπ starts to decline ($\Delta\pi_{critical}$) indicates the total surface pressure (Δπ + πL) at which VSG is expelled. When this value of $\Delta\pi_{critical}$ is transferred to the π-A isotherm it then becomes possible to identify the lipid surface area at which the exclusion process occurs.

The critical surface pressure was found to be dependent on initial available area and initial surface pressure. This is illustrated in Fig. 6.3(a), where the addition of VSG at 72$\mathring{A}^2$, instead of 92$\mathring{A}^2$ and zero surface pressure (see Fig. 6.2), drastically reduced the displacement of the π-A isotherm. A similar result was obtained when CHL was replaced by ERG for the same lipid composition and VSG concentration (Fig. 6.3b).

Although increasing the VSG concentration produced a displacement in the π-A isotherm, the $\pi_{critical}$ values showed no significant changes as evident from the plots of the Δπ versus πL (Fig. 6.3 inset). Thus at the VSG-lipid mole ratio of 1:4.5 the following $\pi_{critical}$ values were obtained: 14 mNm^{-1} for CHL:SPH:PE (1:1:1), and 11 mNm^{-1} for

Fig. 6.2

Interaction of VSG_{151} with phospholipid-cholesterol monolayers. The phospholipid:cholesterol mol ratio is indicated in the figure. 2 nmol of VSG was added to the CHL:SPH:PE (1:1:1 mol ratio) monolayer for a VSG:phospholipid mol ratio of 1:6. Similarly 2.5 nmol of VSG was added to the CHL:SPH:PE (2:1:1 mol ratio) monolayer for the VSG:phospholipid ratio 1:6.

Figure inset.

$\Delta\pi$ for the interaction of VSG with each lipid monolayer was determined as described in Fig. 6.1.

(▲) CHL:SPH:PE 2:1:1

(△) CHL:SPH:PE 1:1:1

(□) CHL:SPH:PE 1:2:2

Subphase pH 6.8.

Force-area curves of (o) the lipid mixed film and in presence of (△) VSG. Surface potentials for (o, ●) lipid films and (□) in presence of VSG.

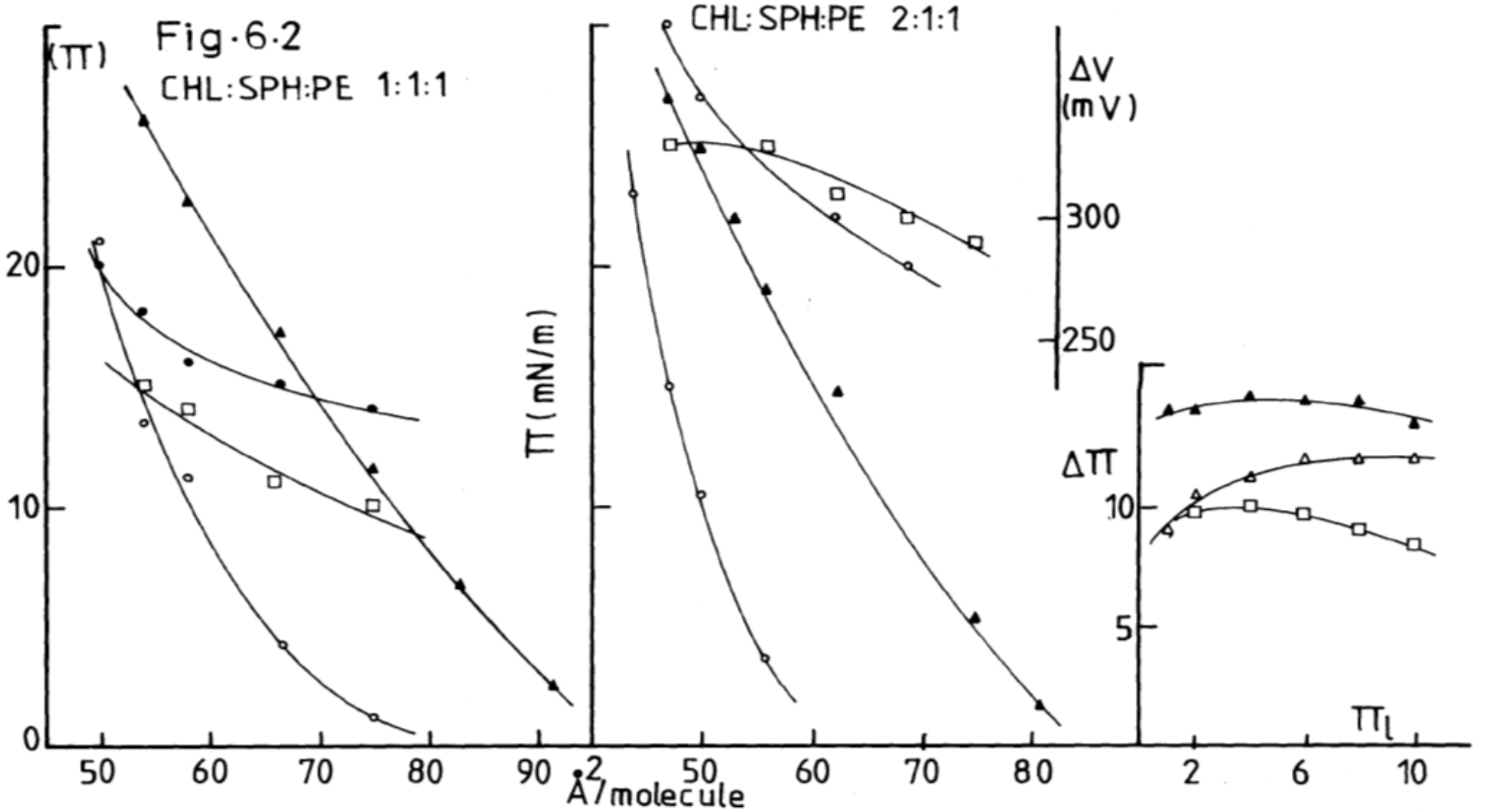

Fig·6·2
(π)
CHL:SPH:PE 1:1:1
CHL:SPH:PE 2:1:1
π (mN/m)
ΔV (mV)
300
250
Δπ
20
10
0
5
50
60
70
80
90
2
6
10
Å²/molecule
πl

Fig. 6.3

(a) Interaction of VSG_{151} with mixed films of CHL:PE:SPH 1:1:1 mol ratio. 4 nmol of VSG was injected beneath the lipid film for a VSG:phospholipid mol ratio 1:4.5 and the force-area curve was measured after 1 hr. The procedure used in these experiments differs from that described in Fig. 6.2, in that VSG was added to the lipid monolayer at an initial lipid surface area of 72 $Å^2$ instead of 92 $Å^2$. The initial surface pressure (zero surface pressure) was identical for both procedures.

(b) As in (a), except that cholesterol was replaced by ergosterol.

Subphase pH 6.8.

FIG. 6.3.a

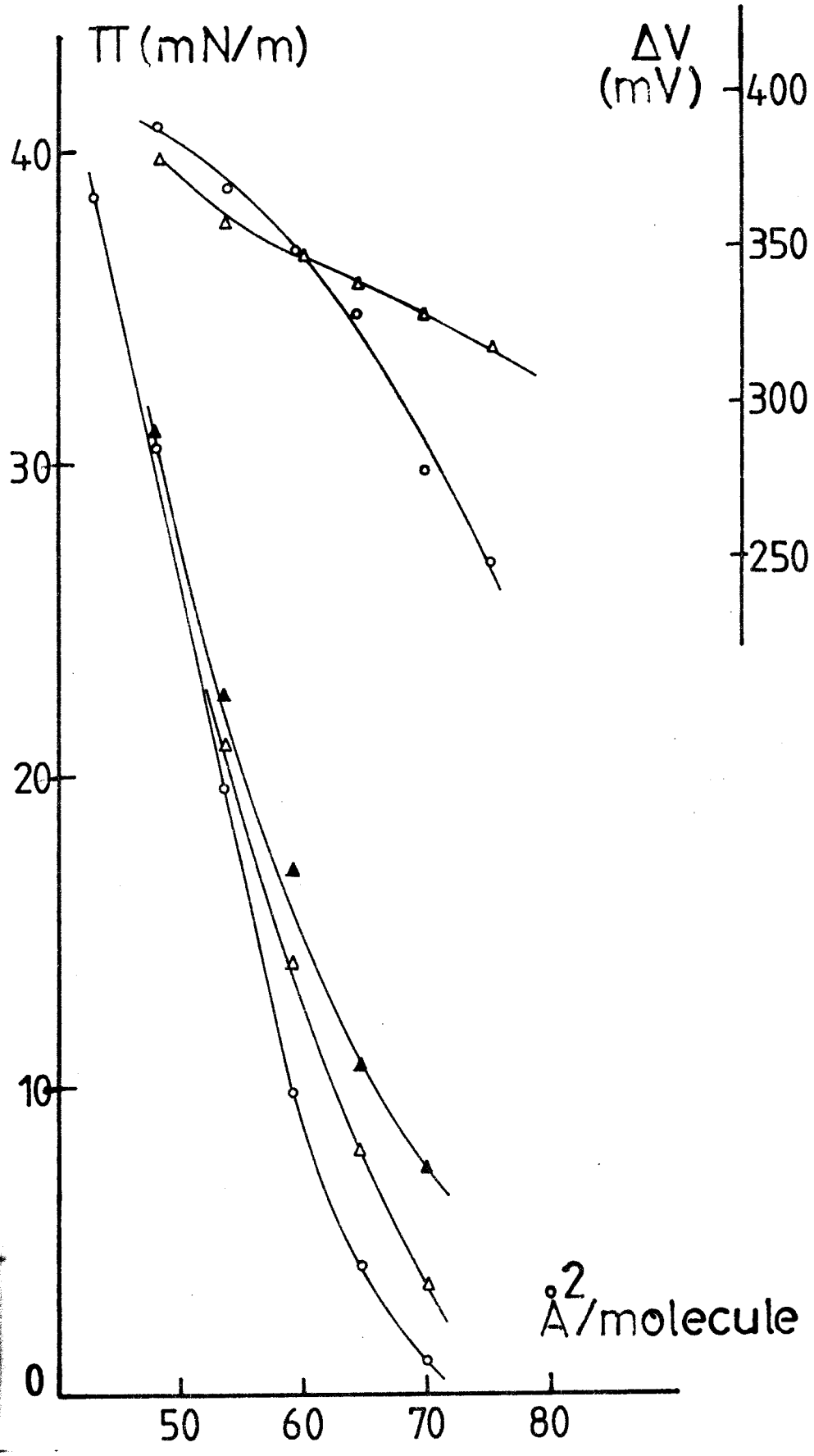
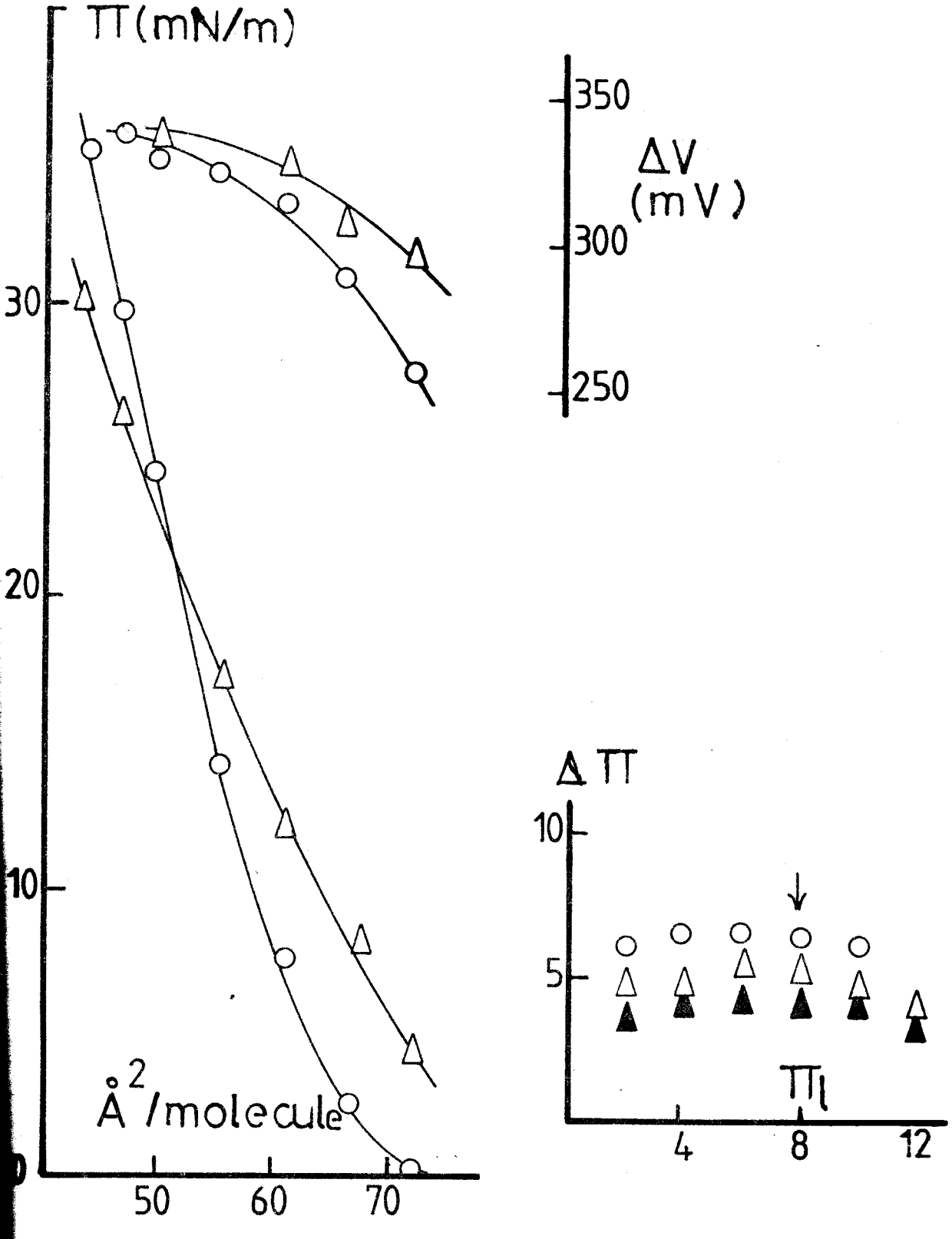


Fig. 6.3. b.


Π (mN/m)
30
20
10
0
50
60
70
Å² /molecule
350
300
250
ΔV
(mV)
ΔΠ
10
5
4
8
12
Π l

ERG:SPH:PE; whereas at the increased VSG concentration of 1:2:3 VSG-lipid mole ratio, the identical $\pi_{critical}$ value of 12 mNm^{-1} obtained for the above respective systems falls within the range of the earlier values at reduced VSG concentration. Changes in surface potential in the presence of VSG maintained the typical pattern described above (Fig. 6.2). One possible interpretation of these results is that VSG adsorbs to the available surface area (AA) until a limiting surface pressure is reached. However, it appears that an equilibrium between adsorbed VSG and that in the subphase may have to be considered, since increasing the VSG concentration may lead to further displacement of the π-A isotherm.

6.1.3 Effects of pH

The effects of pH on the compressibility of VSG inserted into lipid monolayers were studied. In these experiments the antigen VSG-221 was injected beneath the lipid monolayer. The initial surface pressure was raised to 4 mNm^{-1} and the π-A isotherm obtained. This is compared with the estimations from similar experiments performed at near to zero surface pressure and reduced available surface area 75$\AA^2$ per average of lipid molecules containing cholesterol and 85 to 90$\AA^2$ in absence of cholesterol (see Fig. 6.4). The figure illustrates the compressibility characteristics in the presence and absence of CHL with the subphase at pH 7. It is worth noting from these results that this antigen appears to be slightly more active than VSG-151.

$\Delta\pi_{critical}$ in presence of CHL (CHL:PC:PE:SPH 1:1:1:1) as calculated from the figure inset was 18 ± 1 mNm^{-1}; and the calculated equivalent lipid surface area was 58 ± 2$\AA^2$. In the absence of CHL these values were 13* ± 1 mNm^{-1} and 69 ± 2$\AA^2$ respectively.

* this figure is the average value of three experiments.

When VSG-221 (isoelectric point 6.5 ± 0.1) was injected beneath pre-formed phospholipid monolayers with the subphase at pH 5.5, a remarkable difference in the above values was observed in the presence and absence of CHL (Fig. 6.5). In the presence of CHL the $\Delta\pi_{critical}$ value was about 17* ± 1 mNm^{-1} for an area of 65 ± 2$\mathring{A}^2$ whereas the calculated value in the absence of CHL was 10 ± 2 mNm^{-1} for an area of 77 ± 2$\mathring{A}^2$.

Studies with VSG-151 under identical conditions yielded qualitatively similar results. Quantitatively, however, there was a difference in $\Delta\pi_{critical}$ values at pH 5.5 and pH 7.0 in the presence of CHL. Thus $\Delta\pi_{critical}$ from pH 5.5 to 5.8 was 22 ± 1 mNm^{-1} at a surface area of 61 ± 2$\mathring{A}^2$ (average values of three experimens each) whilst from pH 6.0 to pH 7.0 the estimated values were 15 ± 1 mNm^{-1} and 56 ± 3$\mathring{A}^2$ respectively (average values of 5 experiments). In the absence of CHL the changes in $\Delta\pi_{critical}$ in the pH range (pH 5.5 to pH 7.0) were negligible the average calculated value being 12 ± 2 mNm^{-1}.

When the mixture of phospholipids (PE-SPH-EYPC) was replaced by EYPC, while maintaining the same ratio of CHL, similar displacements of the π-A isotherm as those shown in Fig. 6.4 and 6.5 were observed at pH 5.5. In the absence of CHL, however, at any point between pH 5.5 and pH 8.0 $\Delta\pi_{critical}$ was estimated to be 17 ± 1 mNm^{-1} (Fig. 6.5, inset). Experiments carried out in the pH range 5.4 to 8.0, showed that the displacement of the π-A curve, due to the low pH of the subphase was maintained until pH 5.8 above which this became insignificant (not shown).

*this figure is the average of three experiments.

Fig. 6.4

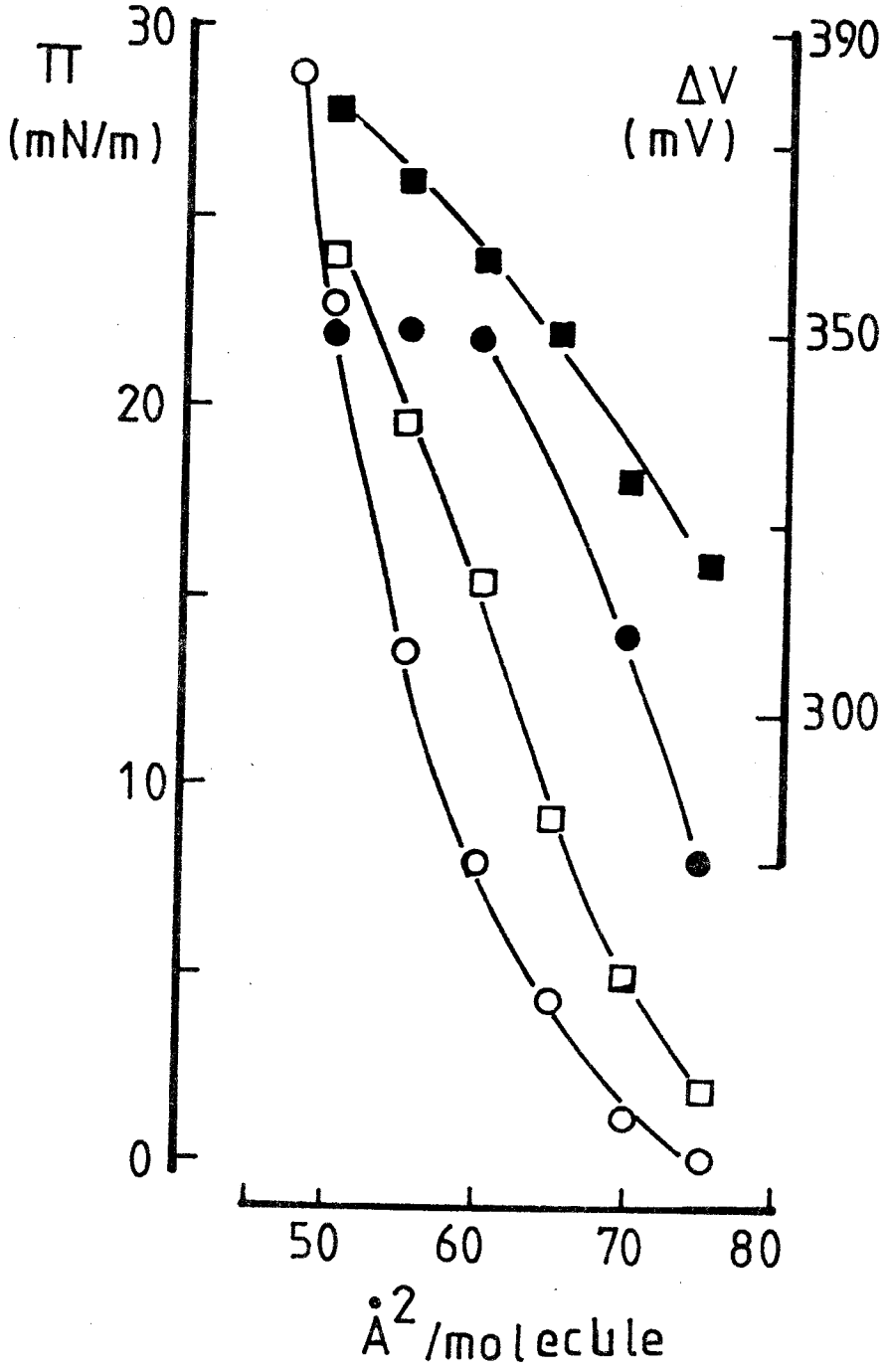
Interaction of VSG_{221} with mixed films of phospholipid–**cholesterol.** 3 nmol of VSG was injected beneath a lipid **film of** PC:SPH:PE:cholesterol 1:1:1:1, for a VSG: **phospholipid** mol ratio of 1:4.5. Force-area curve was deter-**mined** as described in Fig. 6.3.

Subphase pH 7.

Force-area curves for (o) mixed lipid films and in (□) presence of VSG.

Surface potentials for (●) the mixed lipid film and after added (■) VSG.

Fjg. 6.4



6.1.4 Effect of ionic strength

The effects of change in ionic strength were studied either by forming lipid monolayers on a subphase of fixed ionic strength, or indirectly by exchanging the subphase of a pre-formed monolayer with salt concentrations of varying ionic strength. The π-A curves of these monolayers were determined in the presence and absence of VSG-151. The components of the lipid system were PE-EYPC-CHL in a 1:1:1 molar ratio on a subphase maintained at ionic strengths of 0.01, 0.079, 0.145 and 0.5 M with NaCl and KCl respectively (Fig. 6.5).

6.2 Effect of the detergent sodium cholate on the phospholipid-antigen interaction

Sodium cholate, a negatively charged detergent, has been shown to cause the disaggregation of the dimeric forms of VSG-121 and VSG-221 thus allowing the formation of monomers (Auffret et al., 1981). In the presence of sodium cholate (1%, w/v) the cross-linking of VSG by dimethylsuberimidate was not observed. It has also been shown that in aqueous buffer proteins having many regions with hydrophobic residues (i.e. structural proteins) form complexes in the absence of detergent by having their hydrophobic sites bound to each other (Knoppel et al., 1979). These proteins also have a higher affinity for charged detergents than do water soluble proteins (Robinson & Tanford, 1975). The fact that VSG-121 and VSG-221 respectively form cross-links has led to the assumption that in solution each exists as an equilibrium mixture in which the dimeric form predominates (Auffret et al., 1981). Hence it can be postulated that the low interaction between VSG and phospholipids is a consequence of the presence of VSG monomers which are the only molecular forms able to adsorb to the lipid

Fig. 6.5

(a) VSG_{221} was added to a lipid film of PE:PC:SPH:cholesterol 1:1:1:1 and force-area curves determined. The mol ratio of VSG:phospholipid was 1:4.5, the initial surface pressure and initial lipid surface area was as described in Fig. 6.3.

(b) Procedure was as in (a), except that cholesterol was omitted in the lipid film.

Subphase pH 5.5.

Figure inset.

Changes in surface pressure ($\Delta\pi$) were determined from the force area curves (see legend to Fig. 6.1).

(O) Phospholipids:cholesterol 1:1

(Δ) Phospholipids only.

Similar curves were obtained for VSG:phospholipid mol ratio of 1:6.

Force-area curves for (o) mixed lipid films and in (□ , △) presence of VSG. Surface potentials are indicated in filled symbols.

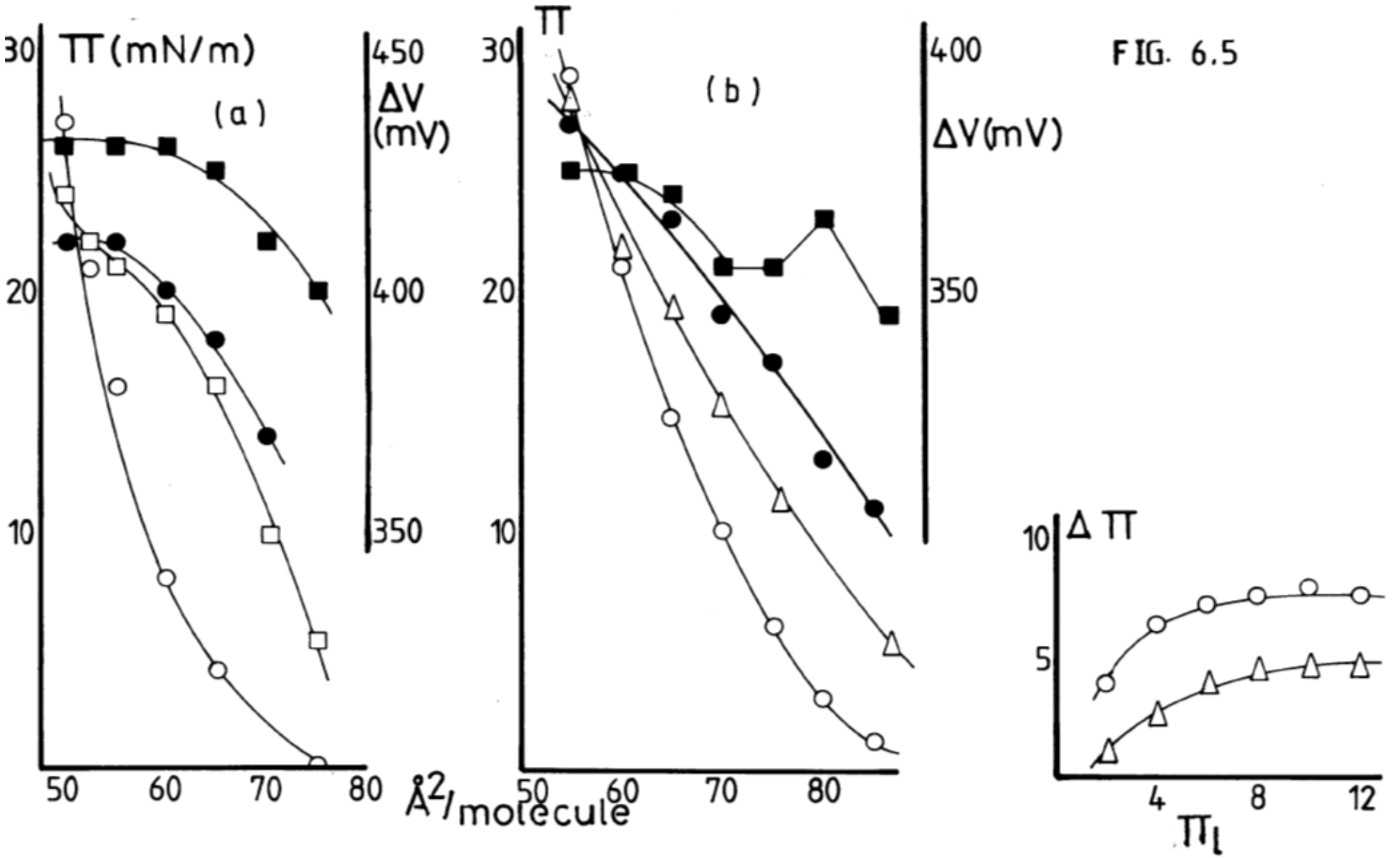

30
Π (mN/m)
(a)
450
ΔV
(mV)
20
400
10
350
50
60
70
80
Å²/molecule
Π
(b)
ΔV(mV)
Δ Π
5
4
8
12
Π_l

FIG. 6.5

monolayers. To verify this hypothesis, VSG was pre-incubated with sodium cholate and the mixture added to the monolayer. The displacement of the π-A curve was much larger than that observed for the same ratio of EYPC/VSG151 in the absence of cholate (Fig. 6.6). The ΔV-A curves also behaved similarly. Exchanging the subphase with fresh buffer led to significant reduction in these isotherms, and infact, after such treatment the π-A curves attained uniform stable values. In comparison with the isotherms in Fig. 5.2, this was equivalent to increasing the VSG-PC ratio to 2:3. Not surprisingly cholate, a surface active detergent, capable of decreasing the water surface tension, has been found to interact with EYPC (Fig. 6.7). The detergent could however, be removed from the phospholipid monolayer by extensive washing, monitored by the reduction of radioactive cholate. A similar result was obtained with SPH, a phospholipid which failed to interact with VSG even at very low surface pressure; see Section (5.1.7). The removal of cholate from SPH monolayers however, required a much larger volume of buffer than for EYPC. Thus it is apparent from the π-A isotherm (Fig. 6.8) that even after washing with 300 ml of buffer some cholate still remained inserted into the SPH monolayer. It was only after a volume of 500 ml had been used that the π-A isotherm was brought to the values observed in the experiments with SPH alone. When the same amount of VSG151 was mixed with various concentrations of cholate and then added to the SPH monolayer, however, the $\Delta\pi_{VSG}$ was found to be dependent on the original cholate concentration (Fig. 6.9). This suggests that in the presence of VSG cholate is not completely removed from SPH film even after a large volume of buffer has been exchanged. Evidence to support this co-operative effect is

Fig. 6.6

Effect of incubating VSG with sodium cholate and then added to a phospholipid monolayer. 4 nmol of VSG in 0.5% sodium cholate was injected beneath the phosphatidyl choline monolayer (14 nmol) and after 1 hr of incubation (△) surface pressure and surface potential were measured. Then, the monolayer was washed extensively with 200 ml of fresh buffer and (□) force-area curves and surface potential were determined.

Subphase pH 6.8.

Fig. 6-6

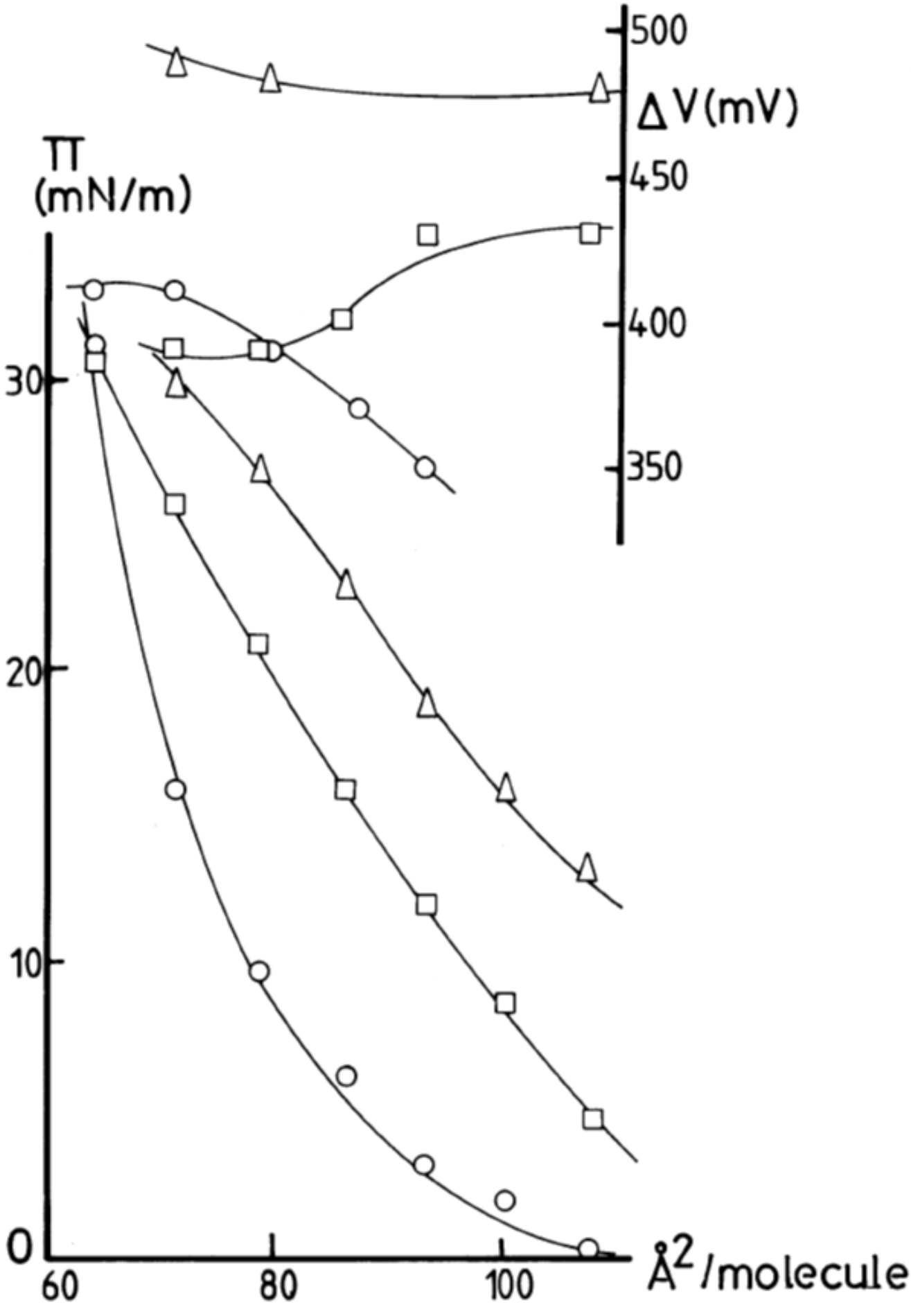


Fig. 6.7

1 mg of sodium cholate dissolved in the subphase buffer was injected beneath the phosphatidyl choline monolayer. After 1 hr of incubation the force-area curve was determined. The monolayer was washed with ∿ 300 ml of fresh buffer at approximately 20 mNm^{-1} and subsequently the force-area curve was measured.

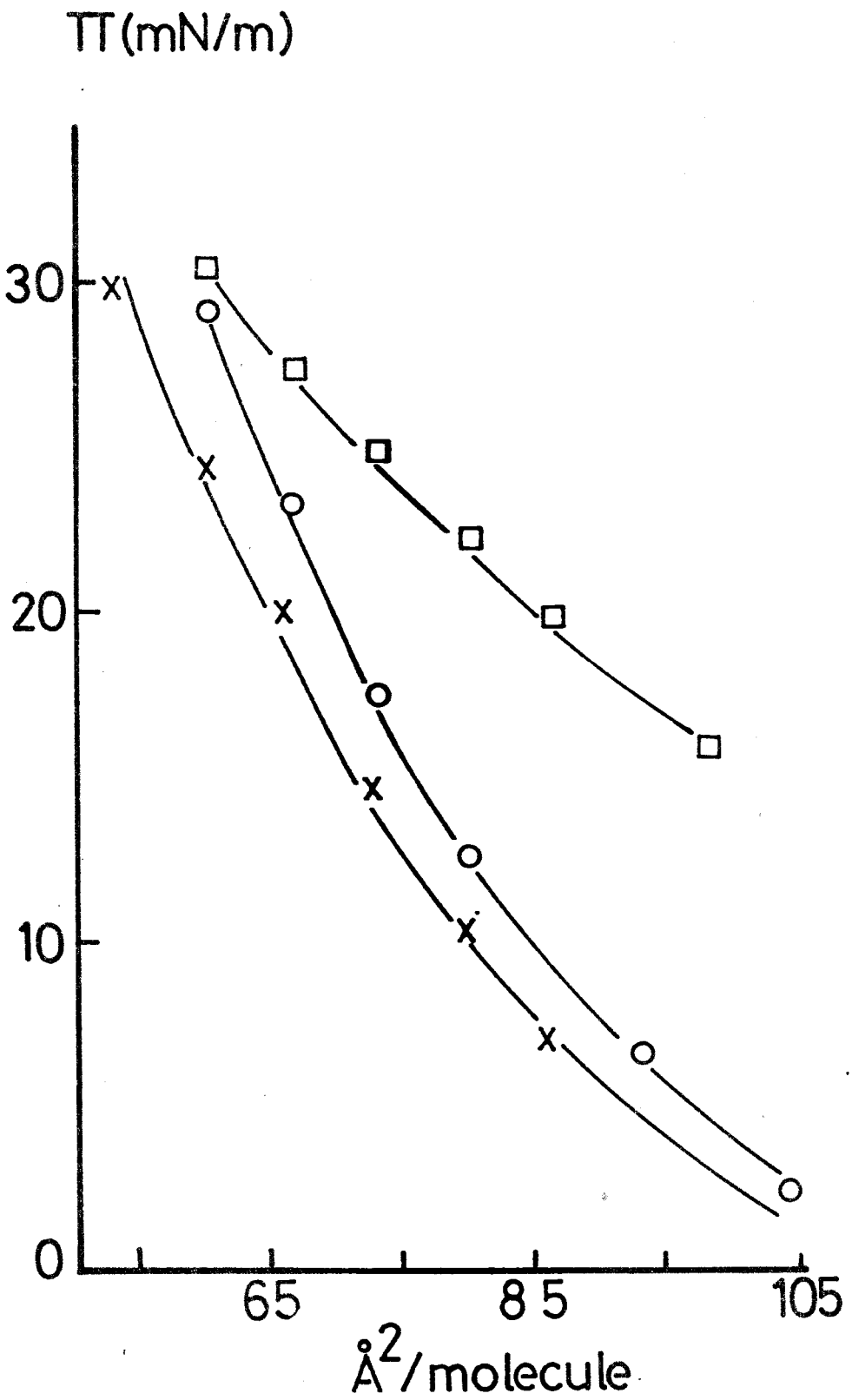
(O) egg yolk phosphatidyl choline

(□) In presence of sodium cholate

(X) After washing

Subphase pH 6.8.

Fig. 6.7



illustrated in Fig. 6.10 where it is shown that neither the π-A nor Δν-A isotherm was affected after such extensive washing. Under the experimental conditions used the concentration of VSG_{151} was doubled (8 nmole) while that of sodium cholate was maintained at the equivalent of 7.25 μM in the bulk phase. Other experimental conditions are as shown in Fig. 6.9.

Two main differences are evident from the results of the above experiments. First, the π-A and Δν-A isotherms of the pre-washed monolayers remained very stable and reproducible; whereas the π-A isotherm of the monolayers containing double the concentration of cholate was less stable. Secondly, extensive washing of both monolayers resulted in very stable π-A and Δν-A isotherms. The order of magnitude of the differences noted above are apparent from Figs. 6.9 and 6.10. These comparative effects were observed in the pH range 7.4 to 8.0. No experiments were attempted below the isoelectric point of VSG_{151}.

6.2.1 Effect of sodium cholate on the cholesterol-antigen interaction

A similar effect to that already noted for SPH and EYPC was observed when cholesterol monolayers were treated with cholate. The π-A isotherm was however, less displaced than for the corresponding SPH and EYPC results (Fig. 6.11). Extensive washing caused a shift in the π-A isotherm to that found in the absence of cholate. The corresponding changes in Δν-A isotherm seemed to support this observation. When VSG was incubated with equivalent amounts of cholate, and then added to CHL films such large changes in surface pressure as those observed in EYPC- or SPH-cholate isotherms were not detected. Furthermore, removal of cholate by washing from the CHL-VSG-cholate monolayers

Fig. 6.8

Effect of sodium cholate in sphingomyelin monolayers. To a preformed sphingomyelin monolayer (18 nmol) 1 mg of sodium cholate dissolved in the subphase buffer was added. Determination of surface pressure and surface potential was carried out as described in Fig. 6.7.

Sphingomyelin alone: (○) surface pressure and (●) surface potential.
Sphingomyelin plus cholate: (□) surface pressure and (■) surface potential.
Sphingomyelin-cholate after washing: (△) surface pressure and (▲) surface potential.

Subphase pH 6.8.

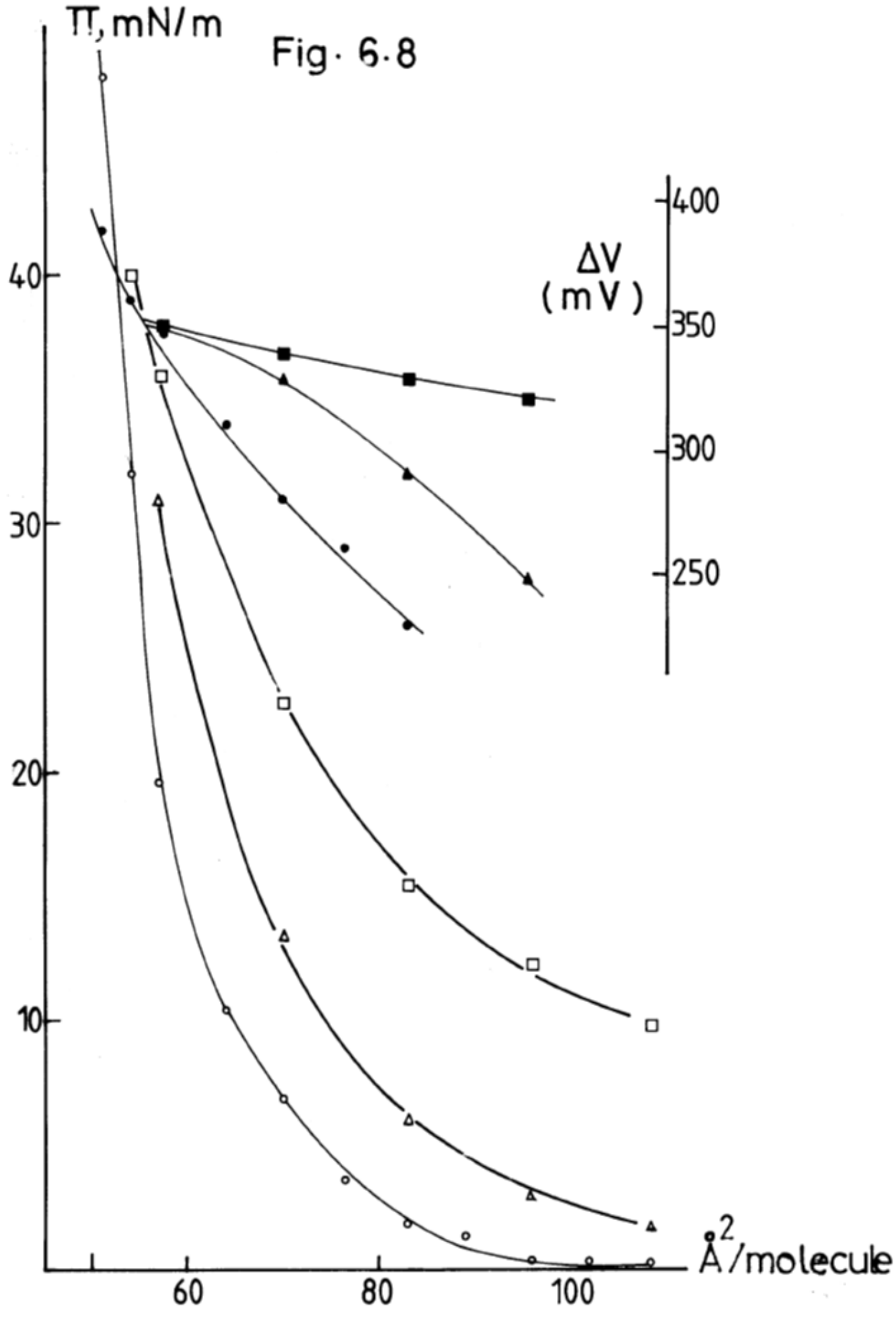

Π, mN/m
Fig. 6.8
ΔV
(mV)
400
350
300
250
40
30
20
10
60
80
100
Å²/molecule

Fig. 6.9

Effect of pre-incubating VSG with sodium cholate before adding to the sphingomyelin monolayer. 4 nmol of VSG was pre-incubated with 1 mg of sodium cholate in aqueous buffer and then injected beneath a sphingomyelin monolayer (17 nmol). Determination of surface pressure, surface potential and other procedures are as described in Fig. 6.8 and 6.7.

	surface pressure	surface potential
Sphingomyelin alone:	(0)	(0)
Sphingomyelin plus cholate-VSG:	(Δ)	(Δ)
After washing:	(□)	(■)

Figure inset.

Similar experiments were carried out as described above except that the concentration of VSG and sodium cholate were changed. $\Delta\pi$ is estimated from force-area curves after washing extensively the monolayer.

(□) 4 nmol VSG in 0.5% (w/v) sodium cholate

(Δ) 4 nmol VSG in 0.25% (w/v) sodium cholate

(0) 8 nmol VSG in 0.25% (w/v) sodium cholate.

Fig. 6.9

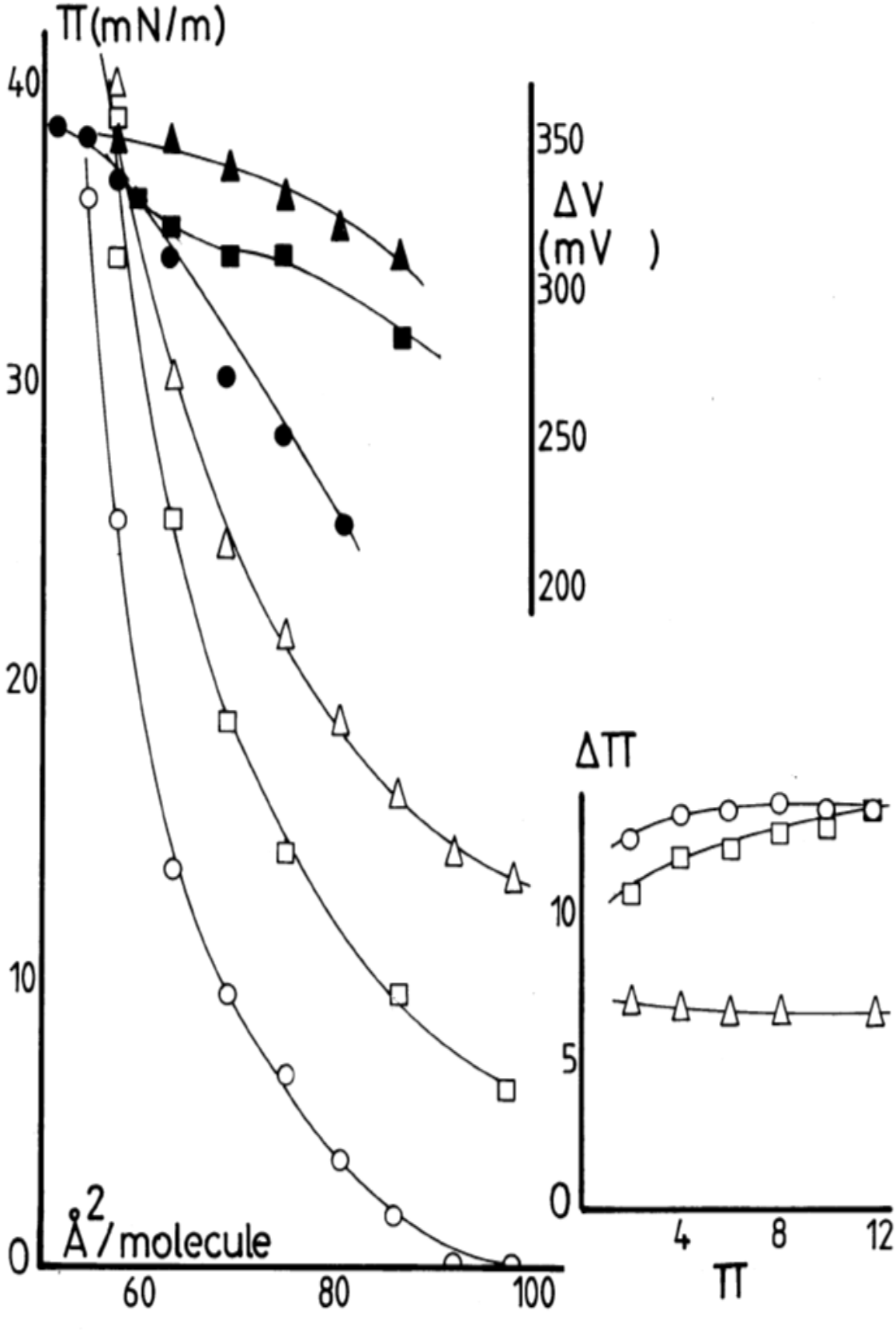

Π(mN/m)
40
30
20
10
0
Å²/molecule
60
80
100
350
ΔV
(mV)
300
250
200
ΔΠ
10
5
0
4
8
12
Π

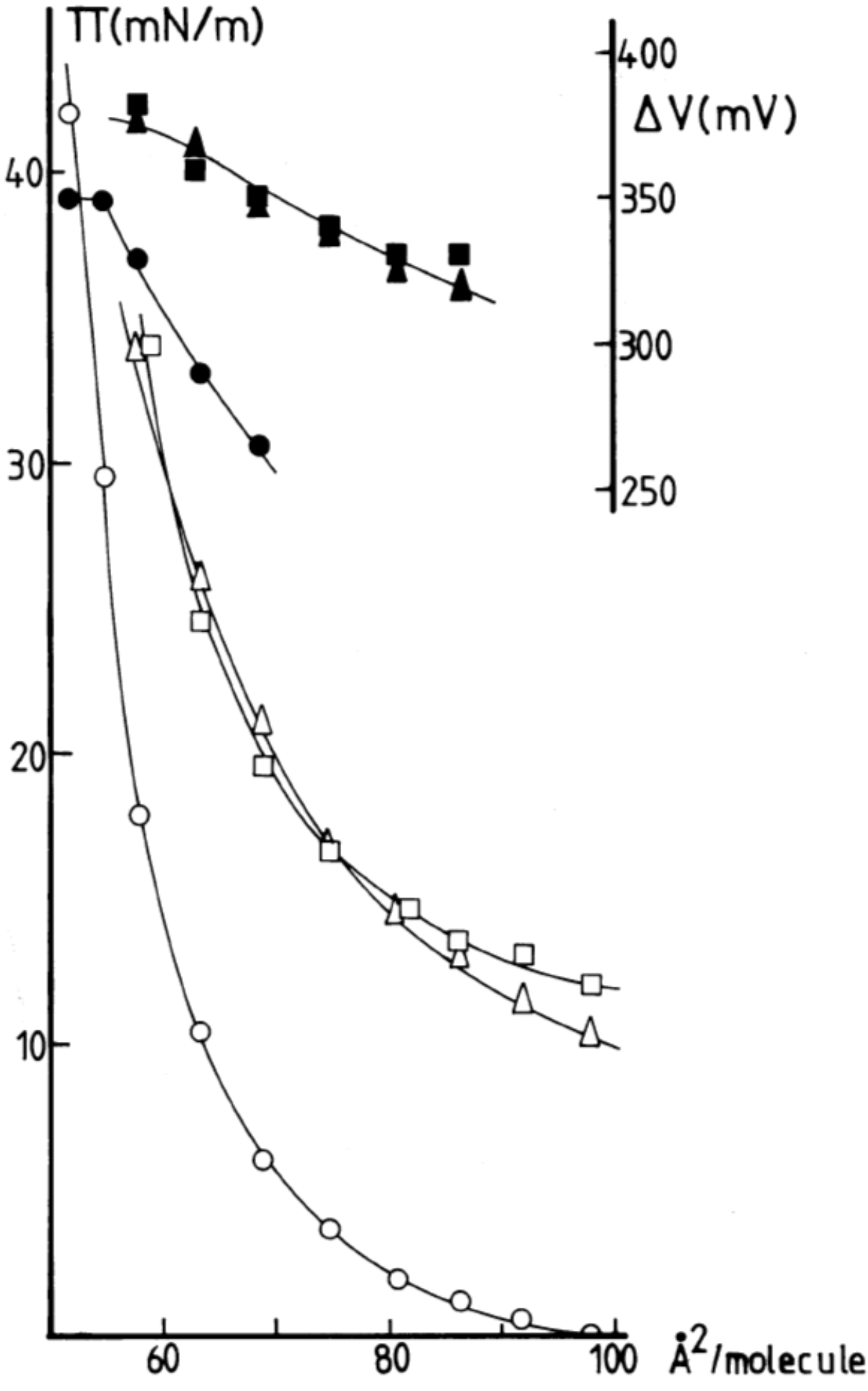
Fig. 6.10

Interaction of VSG dissolved in a sodium cholate solution with sphingomyelin monolayers. Determinations of surface pressure, surface potential and removal of the detergent were carried out as described in Fig. 6.9. 8 nmol of VSG in a solution of sodium cholate of 0.25 (w/v)% or 0.58 µmoles was added to a sphingomyelin monolayer (18 nmol).

	surface pressure	surface potential
Sphingomyelin alone:	(○)	(●)
Sphingomyelin-VSG-cholate:	(□)	(■)
After washing:	(△)	(▲)

Subphase pH 6.8.

Fig. 6.10



did not significantly alter the final π-A isotherm from that obtained in the absence of cholate (Fig. 6.11). That the changes in Δν-A isotherm may be taken as evidence for the ejection of cholate from lipid monolayers appears to be substantiated by the results of experiments using radiolabelled [^{14}C]-cholate as discussed in the following section.

6.2.2 Use of the radiotracer [^{14}C]-cholate

The experiments described above indicate that VSG mixed with cholate is readily inserted into phospholipid monolayers at an enhanced rate compared to the interaction occurring in the absence of cholate. The presence of cholate, however, appears to be unnecessary for the interaction of VSG with cholesterol. These observations were further investigated using radiolabelled [^{14}C]-cholate (specific activity 52.6 Ci/mole). 4 μCi of radiolabelled [^{14}C]-cholate was injected beneath monolayers composed of CHL and EYPC respectively, with an initial surface pressure of 5 ± 0.5 mNm^{-1}. Changes in Δπ values and radioactivity were then recorded continuously for 1 hr. From the results (Fig. 6.12) some observations can be made on the bahaviour of the two systems (i.e. CHL and EYPC): (a) the EYPC system became stabilised after 20 minutes, changes in Δπ after this period remained uniform. The average radioactivity after 20 minutes was 200 ± 50 cps; (b) in contrast, changes in Δπ for the cholesterol monolayers were variable for the first 30 minutes but stabilised thereafter. The mean radioactivity was 20 ± 10 cps.

Under similar experimental conditions, VSG was injected beneath monolayer films of cholesterol and EYPC, which had been pre-incubated with radiolabelled [^{14}C]-cholate. The results (Fig. 6.12) were obtained

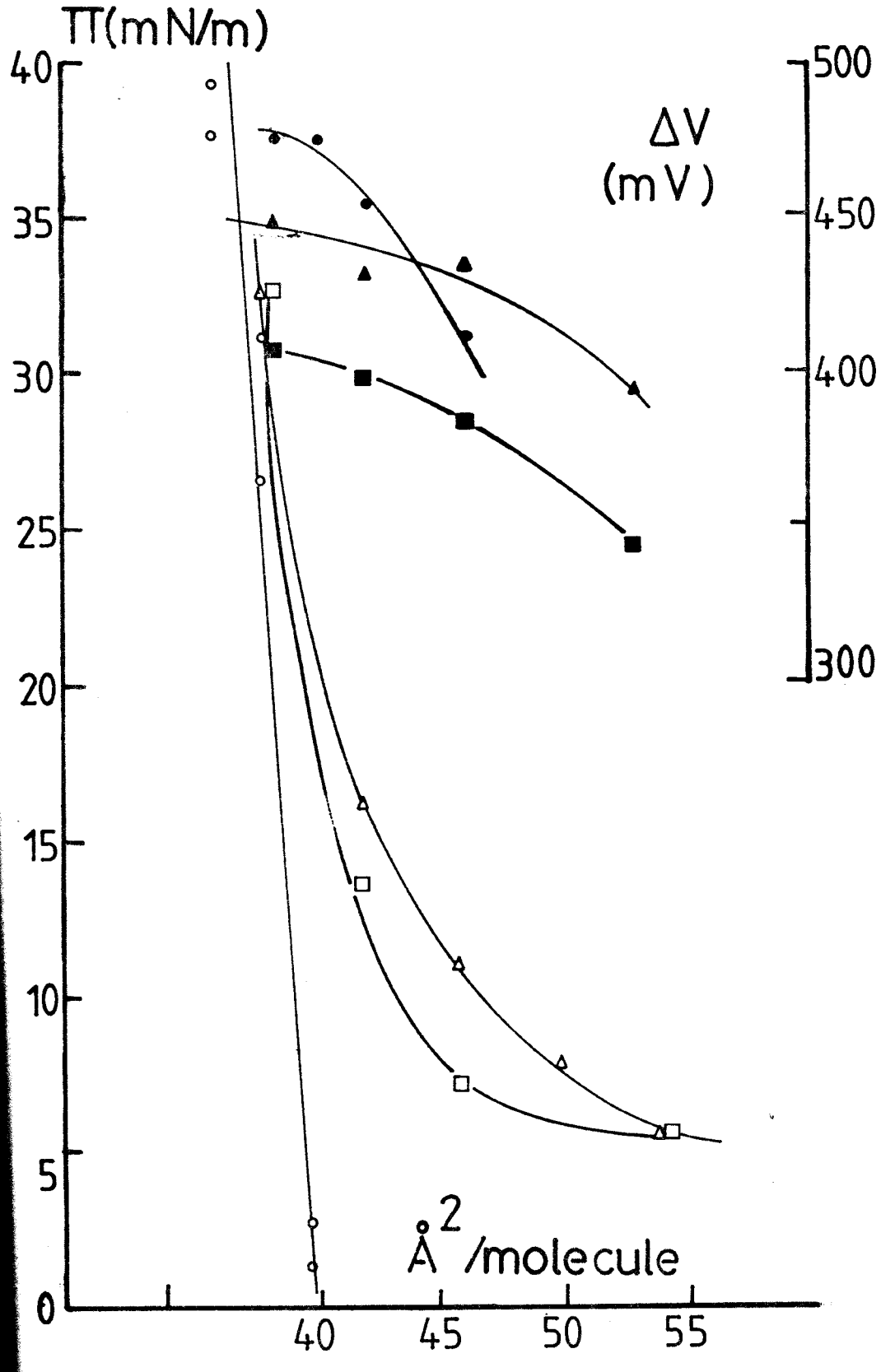
Fig. 6.11

Interaction of VSG previously dissolved in sodium cholate with cholesterol monolayers. The procedures of incubation, and washing of the monolayer are essentially the same as described in Fig. 6.7. 4 nmol of VSG in 0.5% sodium cholate (1.1 μmol) was injected beneath a cholesterol monolayer (25 nmol), and after 1 hr surface pressure and surface potential were determined.

	Surface pressure	Surface potential
Cholesterol alone	(O)	(●)
Cholesterol-VSG-cholate	(△)	(▲)
After washing	(□)	(■)

Subphase pH 6.8.

Fig. 6.11


TT(mN/m)
40
35
30
25
20
15
10
5
0
ΔV
(mV)
500
450
400
300
Å 2 /molecule
40
45
50
55

with initial conditions of 130 cps radioactivity (76 nmoles [^{14}C]-cholate), and 5 mNm^{-1} surface pressure before the injection of VSG. VSG showed a slow rate of penetration into monolayers; and a slight reduction in surface radioactivity was also observed. In cholesterol monolayers an initial surface pressure of 5 mNm^{-1} had the effect of reducing the increase of surface radioactivity. It was also evident that the rate of penetration by VSG was significantly higher than that observed for EYPC films; but as is shown below, this was still far less than that observed when the experiments were performed in the absence of cholate.

Control experiments showed that a similar concentration of [^{14}C]-cholate attained a surface equilibrium (140 ± 10 cps) after 30 minutes. Changes in surface area did not affect this value. Equally, there were no appreciable changes in surface tension, a maximum value of 2 mNm^{-1} being observed. The addition of VSG to the system led to a reduction in surface radioactivity (90 ± 10 cps), but caused no observable change in the surface tension. Decreasing the surface area, however, caused an increase in the surface pressure and a reduction in surface radioactivity. Presumably the presence of chemical groups such as polar groups and hydrocarbon chains in the lipids at the air-water interface provides stabilising conditions for cholate to remain strongly adsorbed.

Force-area curves and radioactivity curves for cholesterol and EYPC in the presence of cholate, and cholate plus VSG respectively, are shown in Fig. 6.13. It is worth pointing out the following from these results: (a) at large surface area or low surface pressure cholate is inserted into EYPC monolayers but an increase in surface pressure caused it to be progressively ejected. This process was very significant with surface pressures above 20 mNm^{-1}. The addition of VSG_{151} at low

surface pressure caused a reduction in surface radioactivity. Washing with a trough volume (100 ml) of non-radioactive buffer, similarly caused a drastic reduction in surface radioactivity. Since the π-A curve remained unaltered, however, it can be concluded that VSG was not the monolayer component being removed in that process; (b) in contrast, the interaction of cholate with cholesterol was markedly different from its interaction with EYPC. Even at a surface pressure lower than that observed for EYPC alone, the surface radioactivity of a cholesterol monolayer was about three times less than that recorded for EYPC. In addition, increasing the surface pressure to about 20 mNm^{-1} caused a drop in the surface radioactivity to a level equivalent to that observed after washing the monolayer. When the cholesterol monolayer was removed the surface radioactivity increased to values of 95 ± 10 cps. The addition of VSG to a cholesterol monolayer in the presence of [^{14}C]-cholate, however, did not appear to have any marked effect on surface radioactivity.

6.2.3 Incubation of VSG and [^{14}C]-cholate before adding it to a lipid monolayer

The experiments described above demonstrated the effects of 0.76 μM [^{14}C]-cholate on the interaction of VSG with lipids. To enhance this interaction, however, VSG was pre-incubated with radiolabelled [^{14}C]-cholate (0.67 mM) for 15 minutes at room temperature, and the mixture then added beneath the lipid film (Fig.6.14). When the pre-incubated VSG-cholate mixture was added to a cholesterol monolayer, the behaviour of the system was markedly different from that of a system composed of cholate and cholesterol only. There was a high initial surface radioactivity for the first 10 minutes followed by a steady decline until

Fig. 6.12

Interaction of [^{14}C]cholate with phosphatidyl choline monolayers and cholesterol monolayers was determined using the film penetration technique. 77 nmoles of [^{14}C]cholate was injected beneath the lipid film at an initial surface pressure of 5 mNm^{-1}. Changes in surface pressure and surface radioactivity were recorded for 1 hr. Subsequently 7.5 nmol of VSG was injected beneath this film and the procedure was as above. The subphase was continuously stirred.

(a) Phosphatidyl choline monolayer:

	surface pressure	surface radioactivity
EYPC-[^{14}C]sodium cholate:	(○)	(●)
After added VSG	(△)	(▲)

(b) Cholesterol monolayer

Cholesterol-[^{14}C]cholate:	(△)	(▲)
After added VSG:	(□)	(■)

Subphase pH 6.8.

Fig. 6.12.

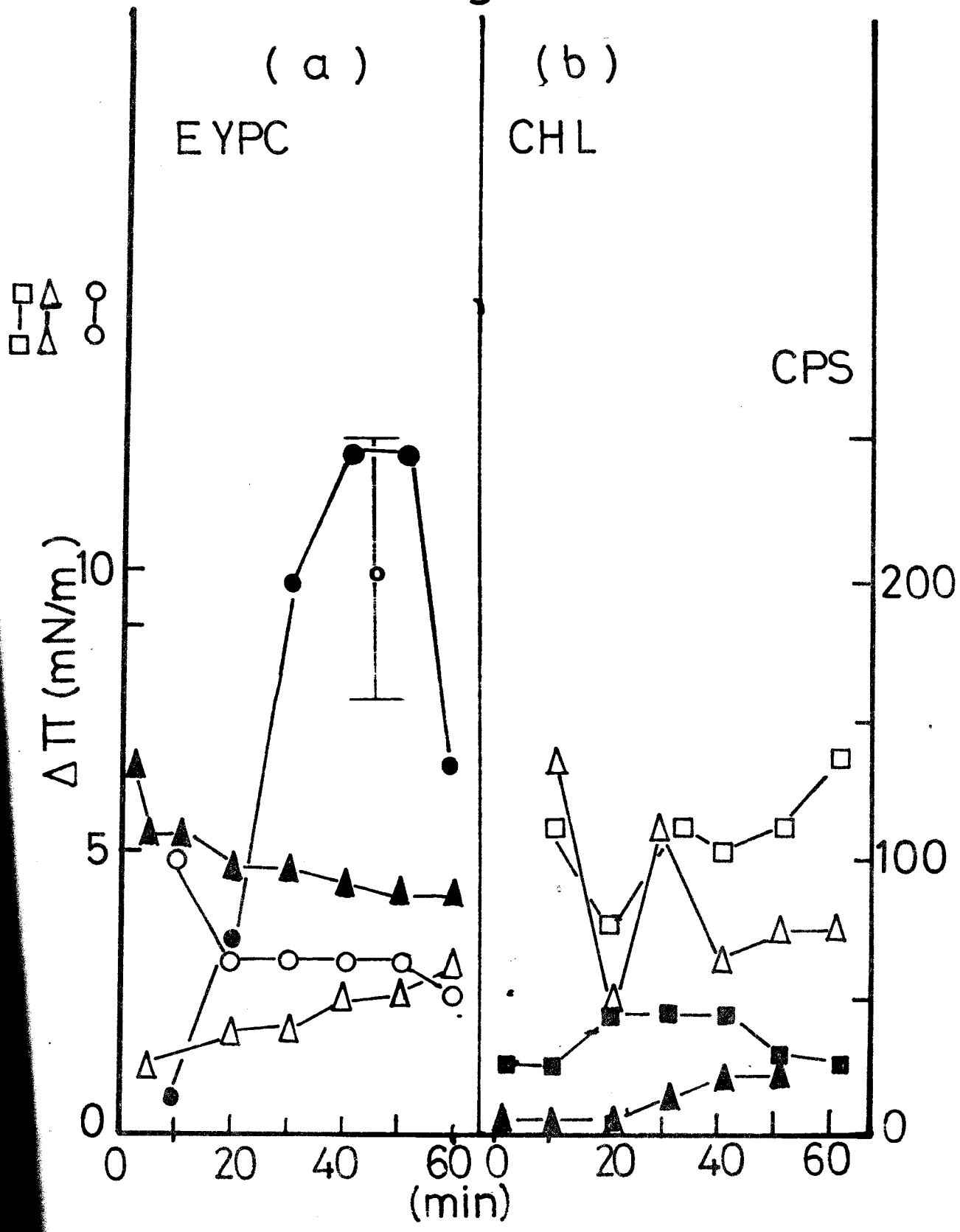


Fig. 6.13

Interaction of [^{14}C]cholate with phosphatidyl choline monolayers and cholesterol monolayers was estimated using force-area curves. 77 nmoles of [^{14}C]cholate was added to a lipid film at near to zero surface pressure and after 1 hr force-area curves were determined. VSG was added to this lipid-[^{14}C]cholate monolayer and force-area curves were measured after 1 hr.

(a) Phosphatidyl choline (14 nmol)

	Surface pressure	Surface radioactivity
Phosphatidyl choline:	(○)	
Phosphatidyl choline-[^{14}C]cholate:	(□)	(●)
After added VSG:	(△)	(▲)

(b) Cholesterol (30 nmol)

Cholesterol:	(○)	
Cholesterol-[^{14}C]cholate:	(△)	(●)
In presence of VSG:	(□)	(■)

Subphase pH 6.8.

Fig. 6.13.a

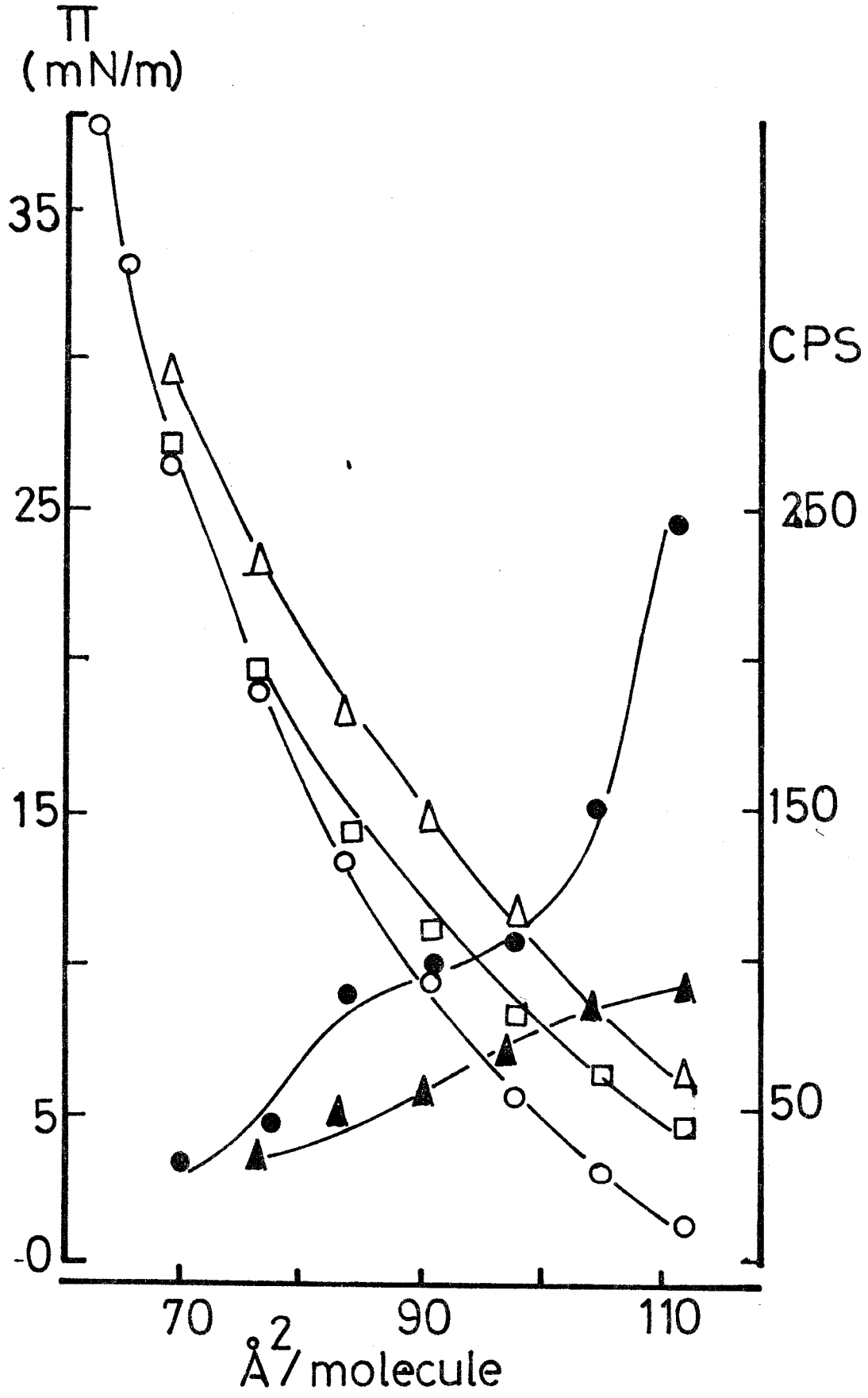
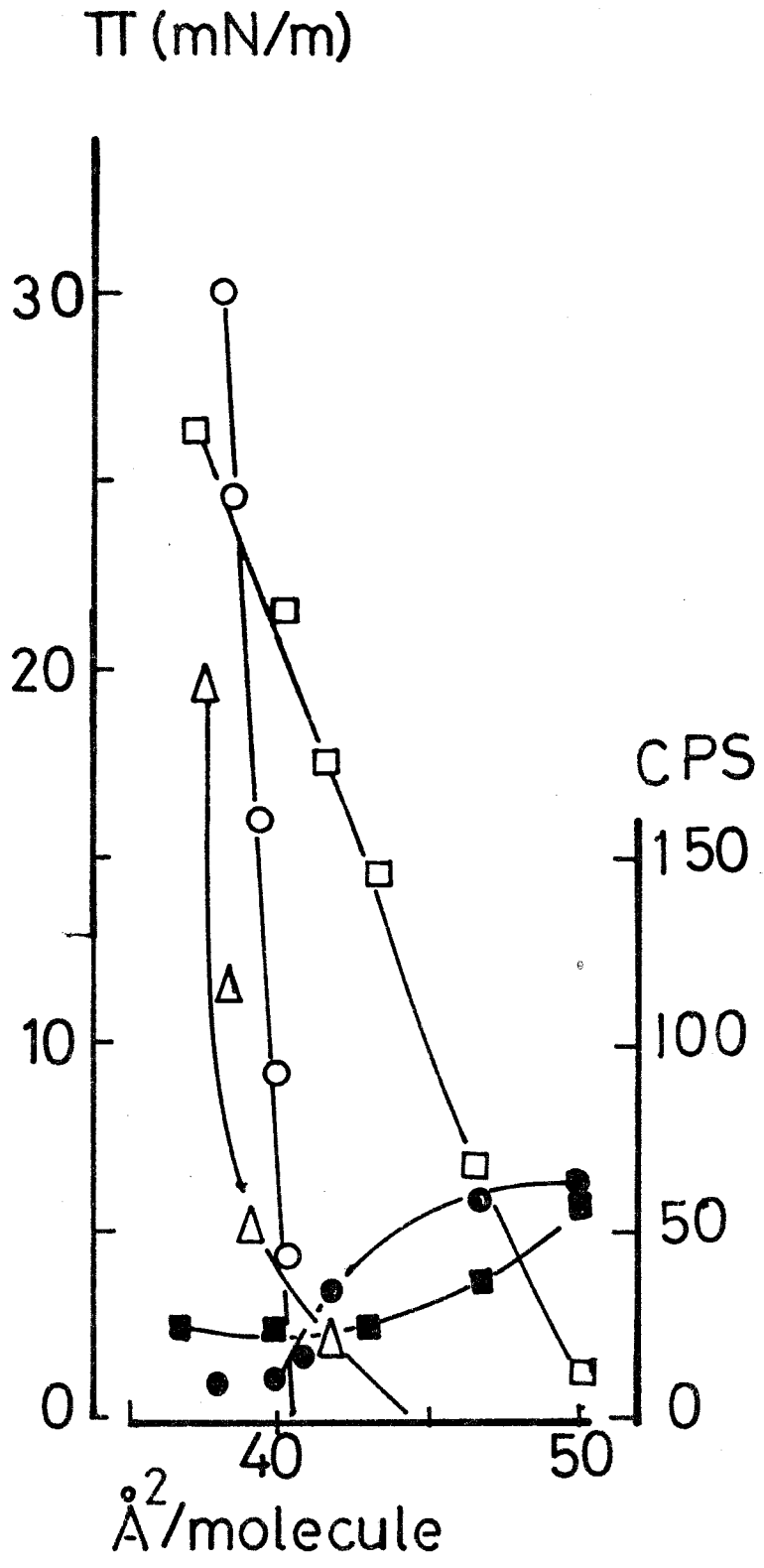


Fig. 6.13.b



the counting rate dropped to background levels (50 ± 10 cps). Changes in surface pressure parallel those observed in the penetration of cholesterol monolayers by VSG (Fig. 6.16a).

Corresponding experiments with EYPC monolayers revealed that when the total surface pressure ($\Delta\pi + \pi_{initial}$) had a value of about 10 mNm^{-1}, radiolabelled cholate began to be squeezed out of the monolayer. The total surface pressure after 60 minutes incubation however, was much lower than that observed in experiments using non-radioactive ("cold") cholate (Fig. 6.6). When the same concentration of VSG (4 nmoles) and a higher concentration of radiolabelled cholate (1.58 mM) were treated as above a similar value of total surface pressure (9 ± 1 mNm^{-1}) was recorded. The surface radioactivity however, increased to 170 ± 20 cps as against a value of 70 ± 10 cps recorded for cholesterol monolayers under similar conditions. The radioactivity of the bulk phase increased to 40 ± 10 cps in contrast to a value of 10 cps obtained when the experiments were performed in the presence of 76 nmoles [^{14}C]-cholate. The force-area curves and radioactivity-area plots of the results from the above experiments are shown in Fig. 6.15.

These results show the relation of surface radioactivity between monolayers of cholesterol and phosphatidylcholine after adding a mixture of [^{14}C]-cholate and VSG. Surface radioactivity values for EYPC films are clearly higher than those determined for cholesterol film. The surface radioactivity was compared within the range of 4 to 20 mN/m. These results taken together with those illustrated in Fig. 6.13 can be interpreted to suggest that cholate binds to VSG; this would explain the relatively high incorporation of cholate into cholesterol monolayers. The plot of radioactivity versus π_{total} (Fig. 6.15

Fig. 6.14

Interaction of lipid monolayers with VSG mixed previously with radio-labelled cholate was measured using the film penetration technique. The difference between these experiments and those described in Fig. 6.12 is the order of addition of VSG and the radioactive detergent. In these experiments VSG and [^{14}C]cholate were previously incubated and then added to the lipid film. The experimental conditions were as described in Fig. 6.12.

Cholesterol (30 nmoles)	surface pressure	surface radioactivity
4 nmoles of VSG-76 nmoles of [^{14}C]cholate	(O)	(□)
phosphatidyl choline (15 nmoles)		
4 nmoles of VSG-76 nmoles of [^{14}C]cholate	(●)	(Δ)

Subphase pH 6.8.

Legend for Fig. 6.15.a on page 200

FIG. 6.14

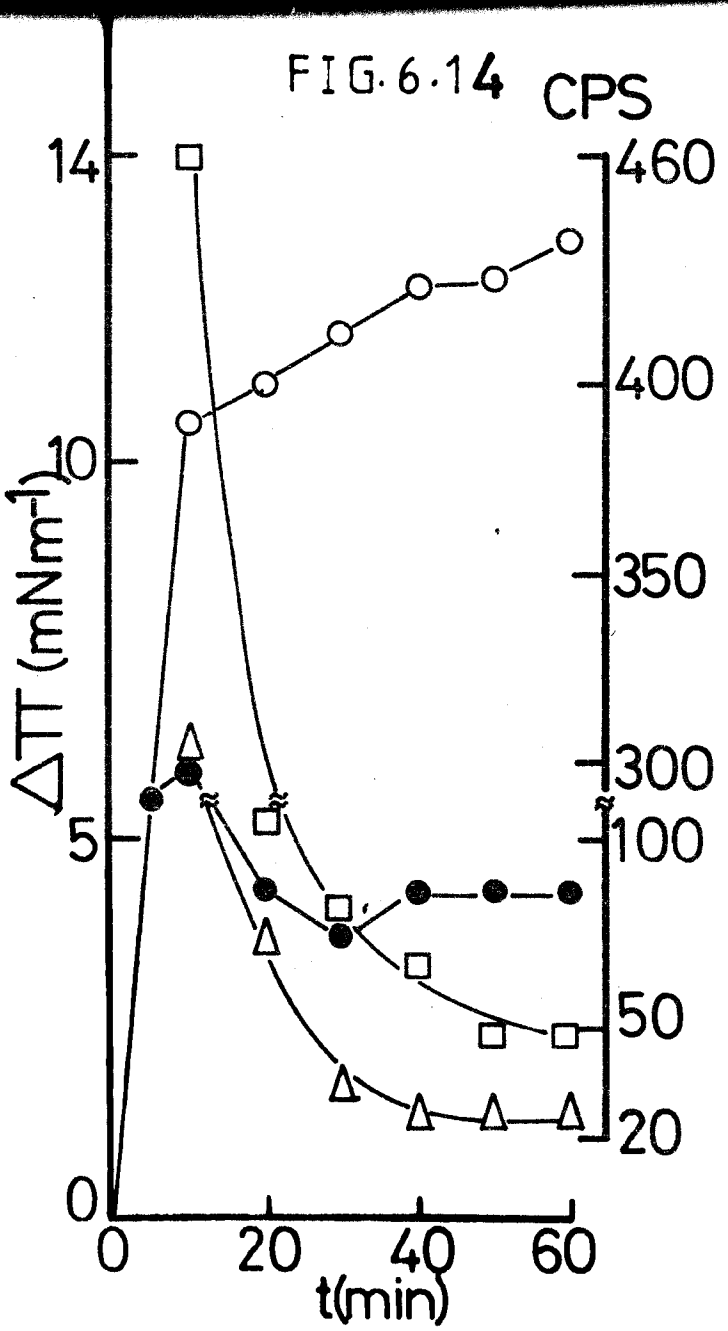
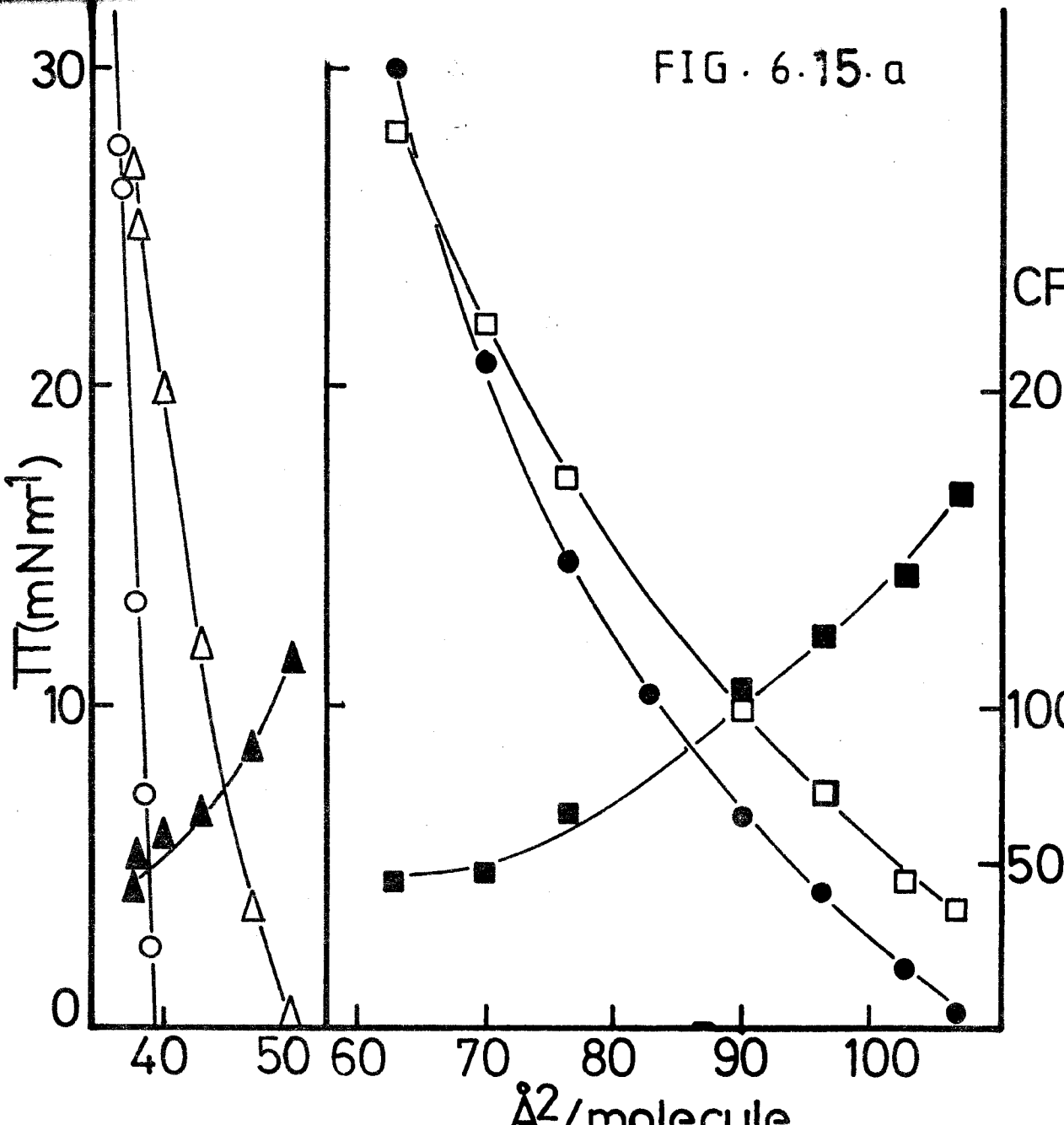


FIG. 6.15.a



equally suggests that even at nearly zero surface pressure [^{14}C]-cholate could partially penetrate the cholesterol monolayer.

6.3 Penetration of lipid monolayer by VSG at constant surface area

Studies of the interaction of VSG with lipids at constant surface area and an initial surface pressure of 5 mNm^{-1} were carried out in a small circular trough. Other experimental details were as described in Fig. 6.16. The magnitude of the lipid-VSG interaction was dependent on the lipid composition rather than on its physical state.

Antigen was injected beneath the lipid film, and changes in $\Delta\pi$ were then recorded for 60 minutes. Figure 6.16a shows a plot of $\Delta\pi$ versus time for both cholesterol and DPPC monolayers. In the cholesterol system $\Delta\pi$ increases very rapidly during the first 20 minutes and then tends towards an asymptote. Phospholipids on the other hand, behaved differently and showed a pattern characteristic of that for DPPC in which $\Delta\pi$ increases slowly to reach a value approximately one third of that obtained for cholesterol (Fig. 6.16a). These $\Delta\pi$ values for DPPC remained at 1.8 $\pm$ 0.8 mNm^{-1} even when the concentration of VSG was raised by four times the amount used in cholesterol monolayers.

The $\Delta\pi$ values observed for phospholipids were independent of the thermotropic transition temperature (Tc) for these lipids. Hence at the experimental (room) temperature the values of $\Delta\pi$ obtained for DMPC (Tc 24°C), DSPC (Tc 54°C), DPPC (Tc 41°C) were indistinguishable from those for DLPC (Tc -1.8°C) EYPC (Tc -15°C) and SPC (Tc <20°C). At room temperature DSPC and DPPC form condensed monolayers in comparison with DMPC, DLPC, EYPC and SPC which form expanded films. On interaction with VSG both groups of lipids show an increase in $\Delta\pi$ values whose average is 1.5 $\pm$ 1.1 mNm^{-1} (Fig. 6.16b).

Fig. 6.15a

Interaction of VSG (previously mixed with [^{14}C]cholate) and lipid monolayers estimated from force-area curves. 4 nmol of VSG was incubated with 76 nmol of radio-labelled cholate and injected into the subphase of the lipid film either with cholesterol or phosphatidyl choline at zero surface pressure. After 1 hr the force-area isotherm and surface radioactivity were determined.

Cholesterol (30 nmol)	surface pressure	surface radio-activity
VSG-[^{14}C]cholate	(Δ)	(Δ)
Phosphatidyl choline (15 nmol)		
VSG-[^{14}C]cholate	(□)	(■)

Subphase pH 6.8.

(on page 198).

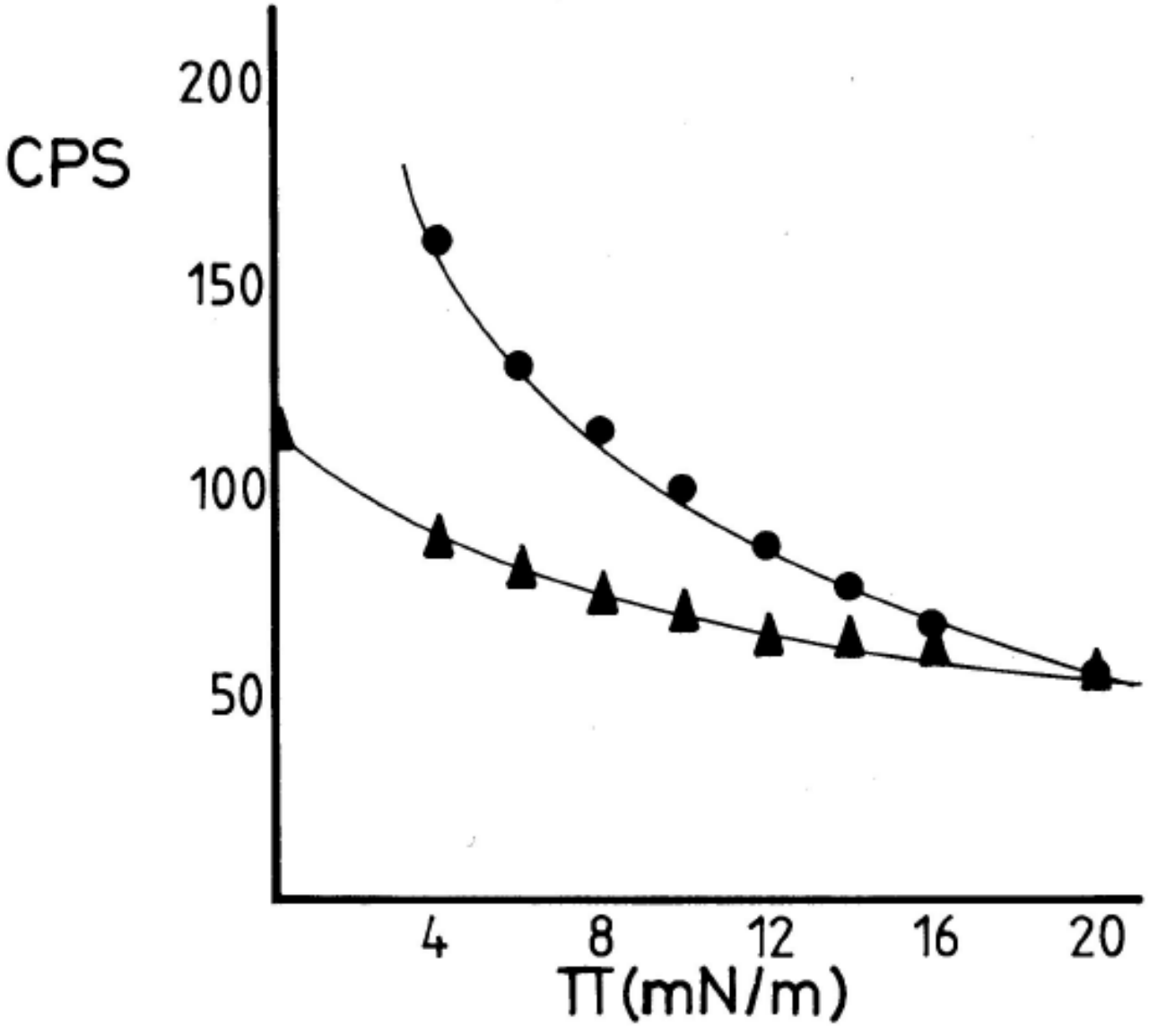
Fig. 6.15b

Relation amongst surface radioactivity, surface pressure and lipid class for [^{14}C]cholate mixed with VSG. The values for surface radio-activity and surface pressure were taken from the results described in Fig. 6.15a.

Phosphatidyl choline (0)

Cholesterol (Δ).

Fig. 6.15b



A further characterisation of the interaction of VSG with cholesterol and EYPC was attempted using penetration experiments. These were performed at the initial surface pressure of 5 mNm^{-1} at which EYPC and other short chain phosphatidylcholine have been reported to form monolayers existing in the liquid expanded, rather than the gaseous state (Phillips et al., 1975a). Various subphase concentrations of VSG 151 were obtained by injecting the appropriate amount of VSG beneath the lipid monolayer film. The resulting $\Delta\pi$ was then recorded for 60 minutes.

A plot of $\Delta\pi$ versus VSG concentration reveals an apparent saturation effect, in which the surface pressure tends towards an asymptote with increasing amounts of antigen. It can be seen that in cholesterol monolayers, relatively small amounts of VSG are able to produce significant changes in the surface pressure, but in phospholipid monolayers this effect was less apparent (Fig. 6.17a). From these results, various ranges of VSG concentration were established. These caused different changes of surface pressure according to the lipid class. Generally, it was observed that a large increase in $\Delta\pi$ was always associated with the cholesterol monolayers.

Direct assessment of the extent of the interaction was made using the Capacity for Film Penetration (CFP) method of Pethica (Pethica, 1955). This author first employed the technique to quantify the penetration of lipid films by detergents. Later, Camejo applied it to the interaction of HDL-apoprotein in lipid film monolayers (Camejo et al., 1968). In this type of analysis, the relationship between the molar ratio of antigen and $\Delta\pi_{eq}$ may be transformed into a linear form using the Langmuir equation:

$$1/\Delta\pi_{eq} = 1/\Delta\pi_{max} (1 + K/C) \qquad (6.3$$

Fig. 6.16

Penetration of lipid monolayers by VSG.

0.5 nmol of VSG was injected beneath lipid films at constant surface area and at an initial surface pressure of 5 mN/m. The rate of penetration measured in changes of surface pressure was recorded continuously for 1 hr.

(a)

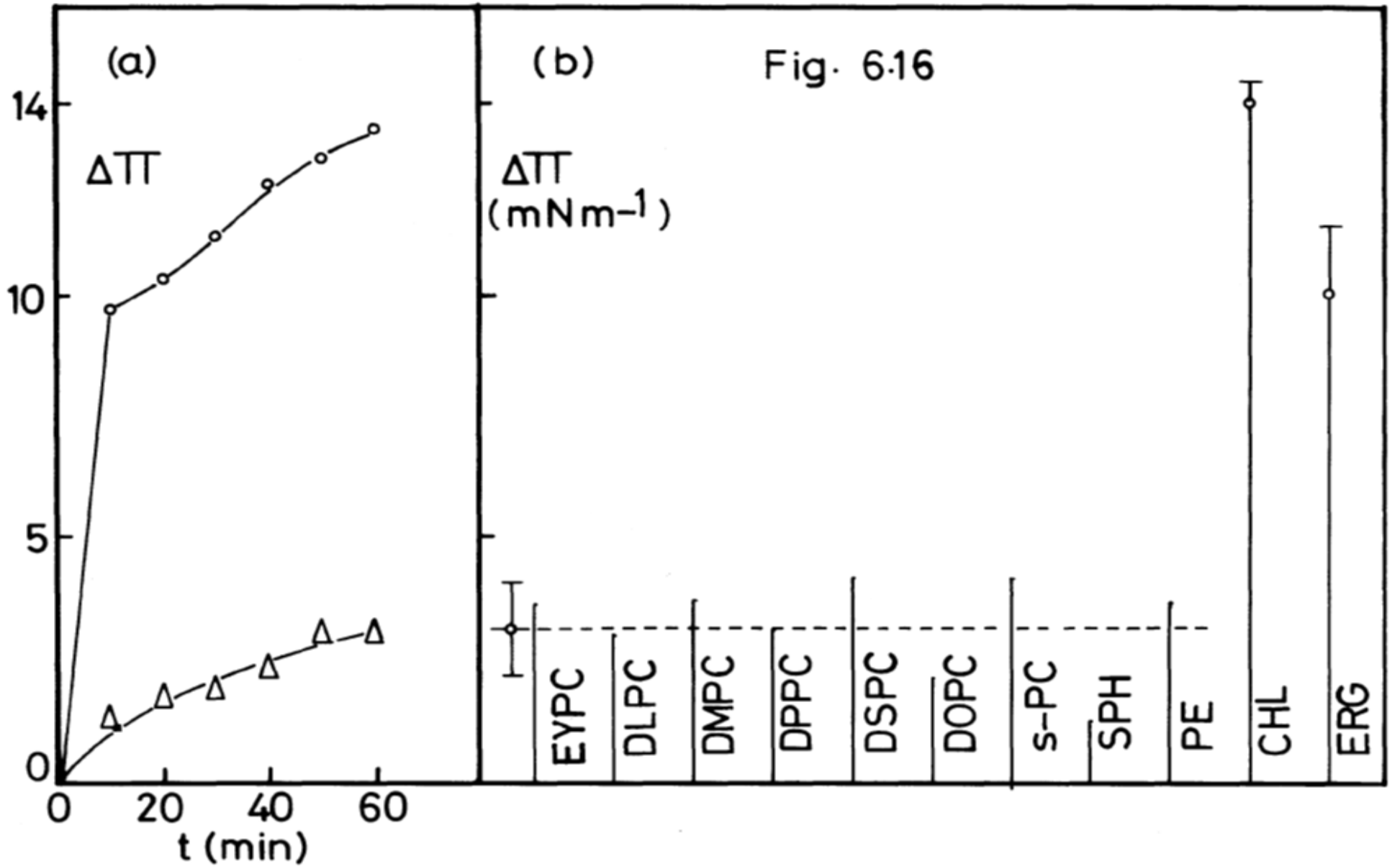
Cholesterol (O)

Dipalmitoyl phosphatidyl choline (Δ).

(b)

The rate of penetration for the lipids indicated in this figure was taken at 60 min. The procedure was as described in (a).

The subphase at pH 6.8 was stirred continuously.


(a)
(b)
Fig. 6.16
14
10
5
0
ΔΠ
ΔΠ
(mNm⁻¹)
0
20
40
60
t (min)
EYPC
DLPC
DMPC
DPPC
DSPC
DOPC
s-PC
SPH
PE
CHL
ERG

where $\Delta\pi_{max}$ is the maximum increase in surface pressure, C is the concentration of protein, i.e. antigen, in the bulk phase, and $\Delta\pi_{eq}$ is the value measured for each antigen concentration when equilibrium is reached. The plot of $^{1}/\Delta\pi_{eq}$ versus $^{1}/C$ for cholesterol and EYPC are shown in Fig. 6.17b). The slope of the straight line was determined by linear regression analyses. The reciprocal of the slope obtainec ($\Delta\pi max/_{K}$) was used as a measure of the capacity for film penetration (CFP). The values of CFP for VSG 151 estimated for cholesterol and EYPC monolayers were 1000 ± 43 and 42 ± 28 mNm^{-1} $mmol^{-1}$l, respectively.

6.3.1 Penetration by VSG of mixed film of phospholipid-cholesterol with excess of cholesterol

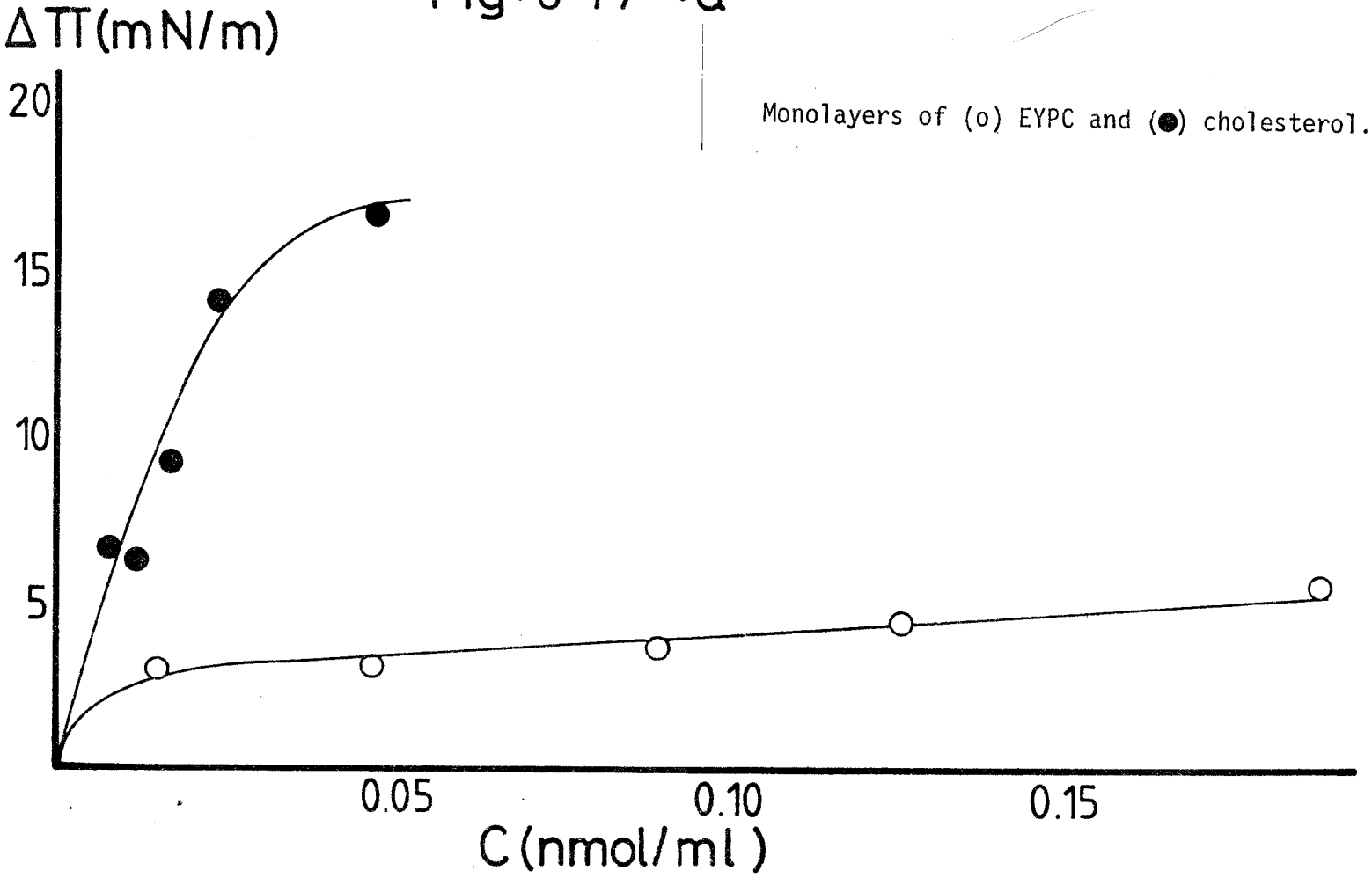
The mechanism proposed for Lateral Phase Separation in lipid monolayers has suggested the cholesterol-phospholipid interaction to be dependent on the ratio of phospholipid to cholesterol in the system. It has been postulated that the formation of cholesterol-phospholipid complexes of fixed stoichiometry would lead to one of the components forming a separate phase (Tajima & Gersnfeld, 1978). The isolation, from human erythrocyte membranes, of band 3 proteins having a high affinity for cholesterol (Klappauf & Schubert, 1977) provided an opportunity to test this hypothesis. Band 3 proteins penetrated mixed monolayers of EYPC-cholesterol and the rate of penetration was enhanced as the concentration of excess cholesterol was increased. These results have lent support to the existence of 2:1 and 1:2 stoichiometrics of cholesterol-EYPC (Schubert & Marie, 1982). These data do not argue against the formation of other complexes (i.e. 1:1) which have been reported by other authors (Gershfeld, 1978; Cadenhead & Müller Landau, 1979).

Fig. 6.17

(a) Monolayers of egg yolk phosphatidyl choline and cholesterol were spread at the air-water interface. Various concentrations of VSG were injected beneath these monolayers at constant surface area and at an initial surface pressure of 5 mN/m. Changes in surface pressure were recorded for 1 hr and are the values plotted. Subphase pH 6.8.

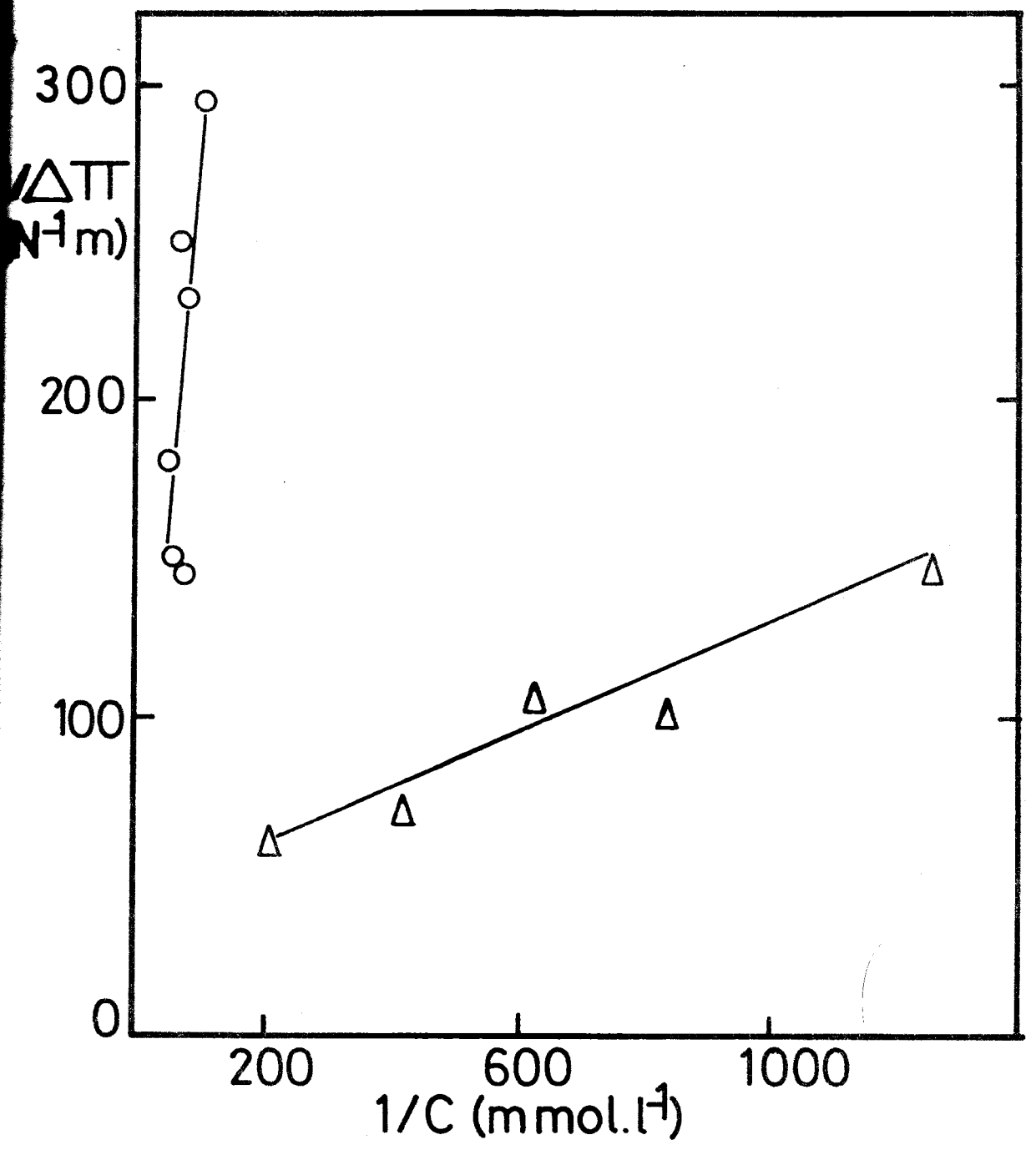
(b) The reciprocal of the $\Delta\pi$ values are plotted against the reciprocal of the VSG concentrations, the values were taken from (a).

Fig. 6.17 .a



Monolayers of (o) EYPC and (●) cholesterol.

Fig. 6.17.b



In the present studies, film penetration experiments were developed to test if VSG would bind cholesterol through a mechanism of Lateral Phase Separation as described above. The results are summarised in Fig. 6.18. A fixed concentration of VSG_{151} was used. After 30 minutes incubation it was observed that almost 85% penetration had occurred (Fig. 6.16a). The positive experiment control consisted of the penetration of pure films of cholesterol by identical quantities of VSG (Fig. 6.16).

It is evident from these results that VSG interacts with cholesterol in a mixed monolayer composed of cholesterol-DPPC, and that such an interaction is pH-independent. When the DPPC in the system was replaced with EYPC, however, the results indicate that the rate of penetration is not affected by changing the ratio of EYPC to cholesterol. In EYPC monolayers VSG_{151} produced an increase in $\Delta\pi$ values of the order of 4.4 mNm^{-1}; whereas in mixed monolayers composed of EYPC and various molar ratios of cholesterol (EYPC:CHL, 1:1; 1:2; 1:3) the $\Delta\pi$ values (4.4 ± 0.8 mN/m) were significantly identical.

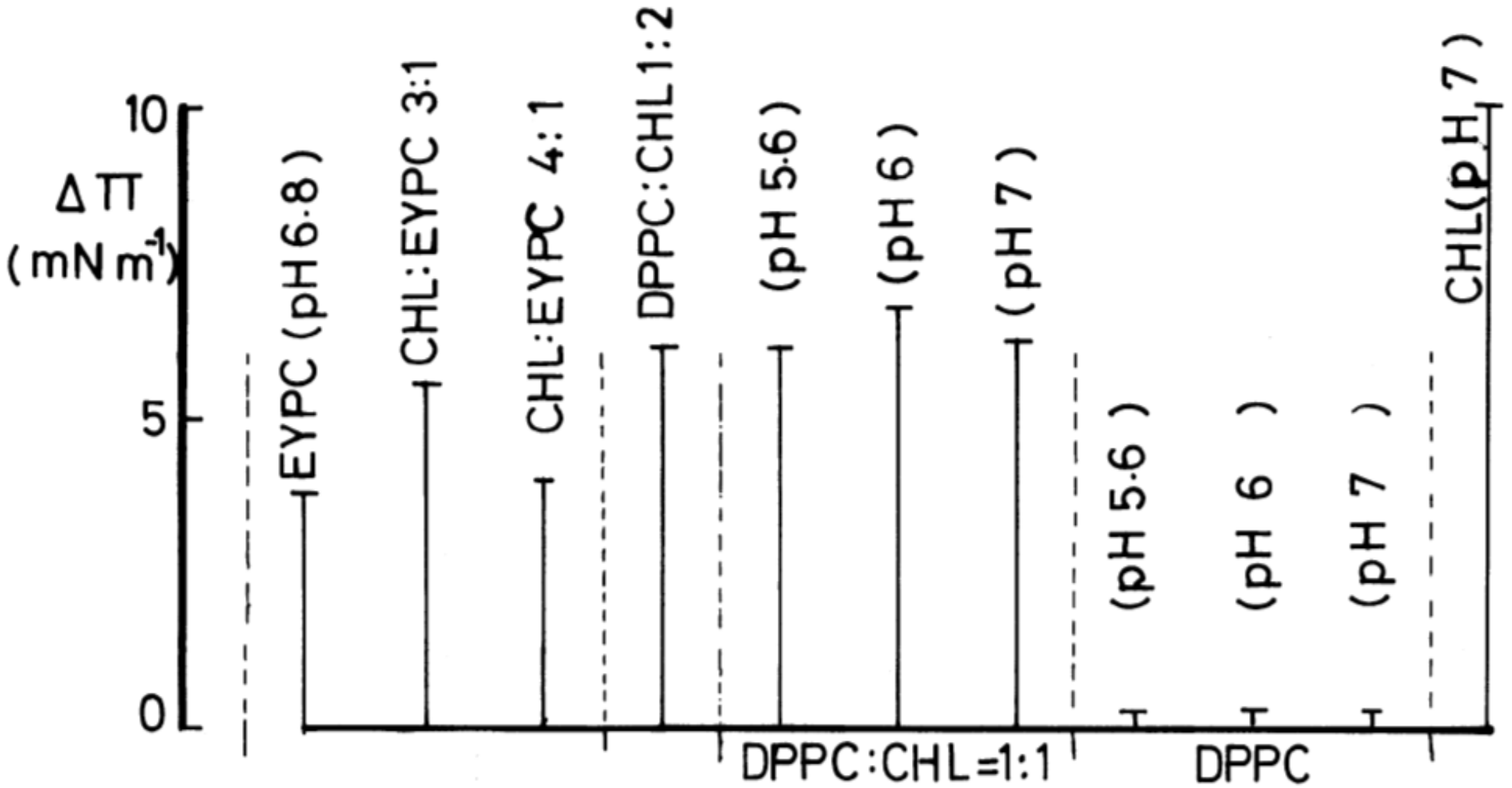
6.4 Effect of electrolytes on the interaction between VSG and phospholipids

Calcium ions (Ca^{2+}) bind strongly to negatively charged lipids (Hauser & Phillips, 1979). The binding to uncharged phospholipids, however, is negligible unless an acidic phospholipid is present (Quinn & Dawson, 1972). This binding to monolayers has been correlated with an increase in surface potential (ΔV), and surface radioactivity respectively (Hauser & Dawson, 1967; Colacicco & Basu, 1978). It has also been shown that protein conformations can be altered through interaction with inorganic cations (e.g. Na^+, K^+, Mg^{2+}, Ca^{2+}), small organic

Fig. 6.18

Film penetration experiments were carried out with mixed film of DPPC/cholesterol and EYPC/cholesterol in the small circular trough. A concentration of VSG (64 pmol) was injected beneath these lipid films at an initial surface pressure of 5 mN m^{-1}, other conditions were as described in Fig. 6.17. The values of lipid composition are in mol ratio, and the pH values are indicated in brackets.

Fig·6·18


10
5
0
ΔΠ
(mN m⁻¹)
EYPC (pH 6·8)
CHL:EYPC 3:1
CHL:EYPC 4:1
DPPC:CHL 1:2
(pH 5·6)
(pH 6)
(pH 7)
(pH 5·6)
(pH 6)
(pH 7)
CHL(pH 7)
DPPC:CHL=1:1
DPPC

cations, or interaction with anions (see Williams, 1979). Proteins with a high content of charged amino acid residues are particularly susceptible to this type of interaction. Hence Concanavalin A folds in the absence of calcium ions; whereas pervalbumins (from fish muscles) trofonin C bind metal ions to external protein loops (Williams, 1979).

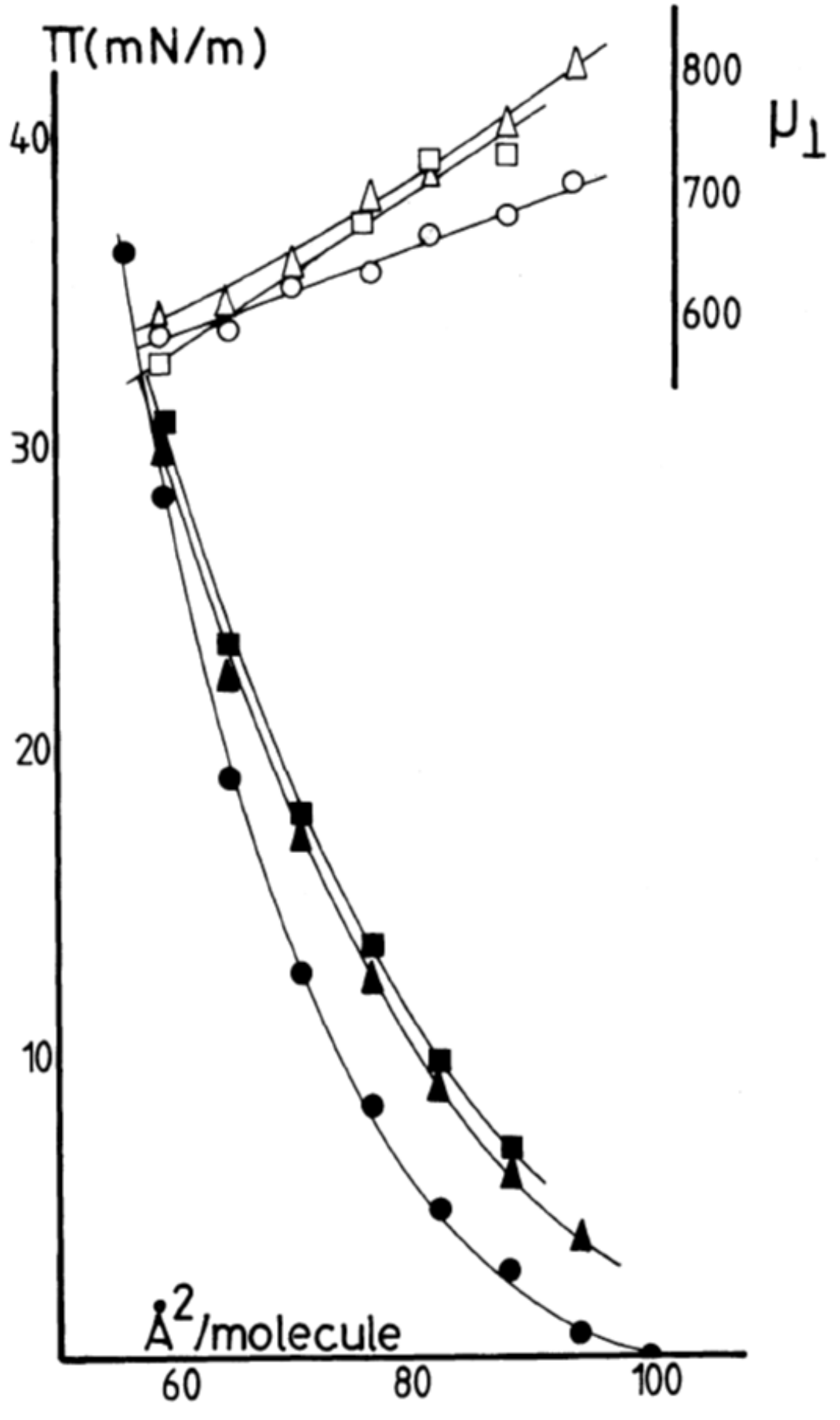
The effects of electrolytes on the interaction between phosphatidylethanolamine monolayers and VSG_{151} were studied by assessing any changes in π-A and ΔV-A curves both in the presence and absence of these ions. Similar concentrations (145 mM) of KCl and NaCl respectively were used, whereas the concentration of calcium ions (as $CaCl_2$) varied between 0.01 to 2 mM.

The results from these experiments show that the presence of electrolytes such as K^+, Ca^{2+} and Cl^- ions did not produce any significant changes in either the π-A or ΔV-A curves. These observations are in agreement with the results discussed earlier (Chapter 5, Fig. 5.6). It is also evident that the presence of Ca^{2+} ions did not significantly alter the π-A curves in PE monolayer alone, or during its interaction with VSG. Experiments on the effects of ions on compressibility were carried out in the pH range 5.6 to 7 in the presence of KCl. Again, no changes in the displacement of π-A curves were observed. Similarly, curves of surface dipole moment (μ) versus area from these experiments failed to show any significant differences in the presence and absence of calcium ions (Fig. 6.19).

6.5 Quantitative estimation of VSG bound to lipid monolayers

The studies described above have shown that VSG interacts with lipid monolayers and that the nature of this interaction depends on the lipid composition of the system. Quantitative estimations of the number

Fig. 6.19



of VSG molecules inserted into lipid monolayers have already been described in Chapter 5. These calculations, however, have been based on such physical parameters as surface pressure, surface area per lipid molecule, and the concentration of VSG in the Subphase respectively. These are indirect methods of estimation and the figures obtained can only be considered to be approximate values.

The initial step in the process of penetration of lipid monolayers by proteins is the adsorption of the latter to the water-lipid interface (Papahadjopoulos, 1975). Changes in the surface potential of the monolayer could therefore, be used to follow this process. However, a quantitative evaluation using this parameter is not easily accomplished (Section 5.4.6). Therefore, the application of other methods to estimate quantitatively the amount of VSG adsorbed to lipid monolayers is necessary.

6.5.1 Direct estimation of adsorbed VSG

Protein determination was employed to estimate the amount of VSG adsorbed by lipid monolayers. The modified Lowry protein determination method was adopted (Colacicco, 1969). Mixed monolayers of EYPC:PE: Cholesterol (1:1:2) were prepared and the antigen added beneath the lipid monolayer with the bulk phase maintained at pH 6.8. After one hour, samples (2 mls) containing the monolayer were collected as previously described (Section 4.10.3) and the protein content determined.

The results from these studies were, however, not satisfactory and showed significant variations in the estimated amount of VSG adsorbed even for the same samples under identical conditions. The values of adsorbed protein ranged from 0 to 50% (w/w), as a percentage of that originally added to the monolayers.

These discrepancies probably arose because the amount of protein falls below the range of sensitivity (2 µg) detectable by this method. For similar reasons this method could not be used to follow the reduction in protein concentration in the bulk solution (Calacicco, 1969; Phillips & Sparks, 1980).

6.5.2 Indirect estimation of adsorbed VSG

Experiments in which radio-labelled [^{125}I] VSG was added into the subphase solution of a lipid monolayer, and radioactivity of the solution was determined against time of incubation were carried out. Results from these experiments showed a significant non-specific adsorption to the trough surface. Changes in surface pressure of the lipid monolayer were higher than those expected for a very low concentration of VSG (10 pg ml^{-1}). Therefore the interference of BSA in the binding of VSG to the lipid film is inferred. BSA is added to the labelled VSG solution to protect it against non-specific adsorption and structural damage, it is discussed below.

These results suggest that the introduction of even small quantities of new components into the system produce significant alterations. Thus it appeared that more promising results might be obtained by the use of a radioimmunoassay technique (RIA) since this would permit the analysis of components present in very low concentrations (nanogram quantities) within the system. Reproducibility in this highly sensitive technique, however, has been known to be subject to the methodology employed.

The 'coated tube' method was adopted in these studies. Details have been given in the Materials and Methods Section. Under the experimental conditions used a minimum concentration of 78 ng/ml could be measured. The quantitative approach employed using the RIA technique,

was to compare the amount of each sample to produce 50% inhibition of antibody binding with the amount of unlabelled VSG needed to achieve 50% of inhibition of binding of [^{125}I] VSG to specific antibody in a standard RIA (Fig. 6.20). Thus the sample was diluted several times and the dilution points plotted, thereby generating a sample curve. The standard concentration which produced 50% inhibition of the tracer binding was multiplied by the dilution factor of the sample which gave a similar value. The values thus obtained (micrograms/ml) were assumed to represent the concentration of VSG in the subphase after the incubation time. This plot of the VSG concentration versus incubation time in the presence and absence of cholesterol in the monolayer is shown in Fig. 6.21. It is evident from these results that the adsorption of VSG to cholesterol monolayers cannot be differentiated from its non-specific adsorption to both the teflon surface and the air-water interface.

6.5.3 Adsorption of radio-labelled [^{125}I]-VSG to lipid monolayers: direct estimation

The radio-labelling of the antigen used in these experiments was performed as previously described (Section 4), with the modification that BSA was not added to the final radiolabelled solution. The physical properties of labelled [^{125}I] VSG in absence of BSA has to be examined whereas BSA in solution of radiolabelled proteins has demonstrated the following functions:

(a) to stabilise the concentration of the protein solution since at the low concentrations involved losses due to nonspecific adsorption to container walls become substantial;

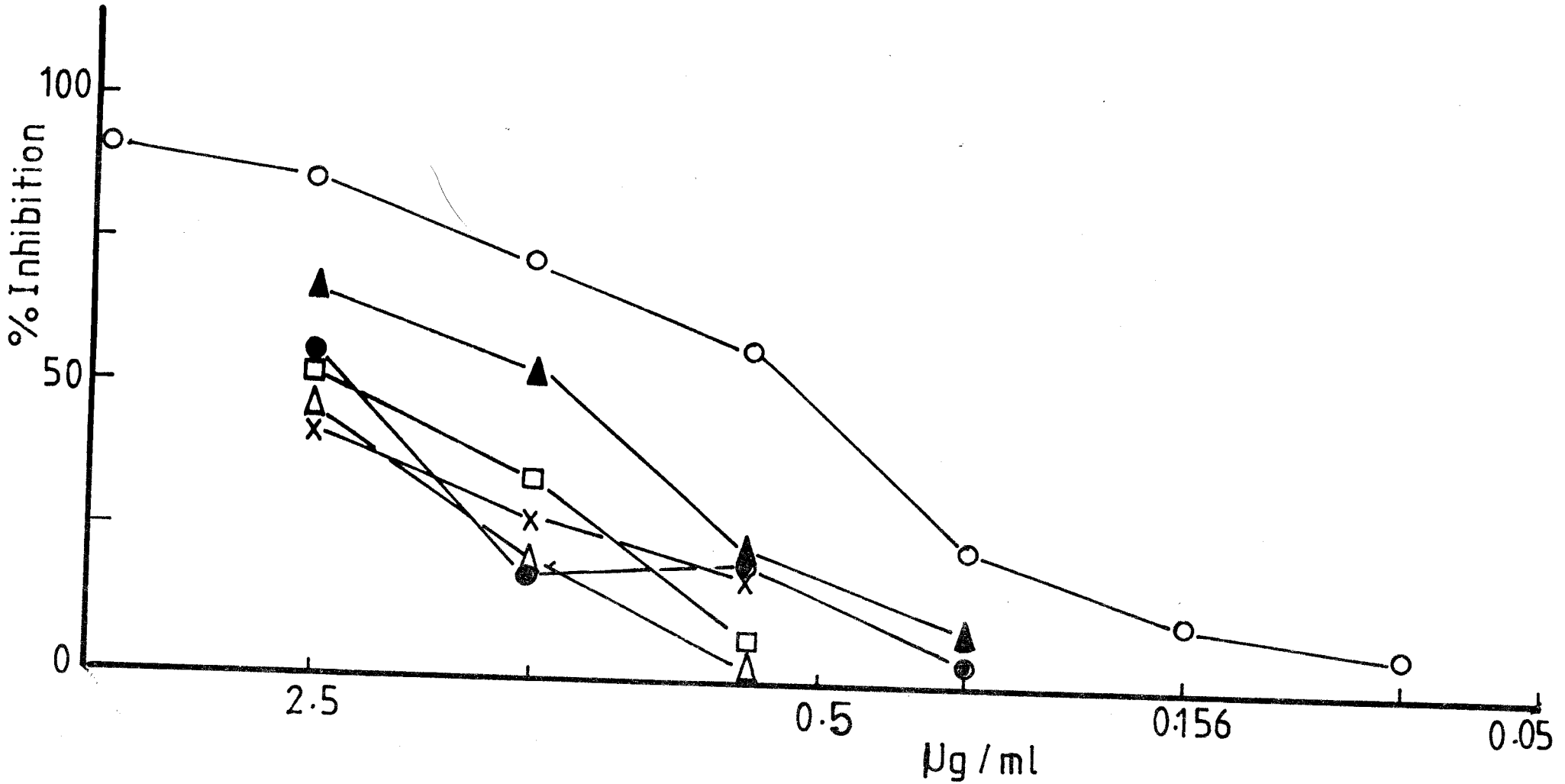
(b) to protect the proteins from denaturation resulting from unfolding at the air-water interface; and finally,

Fig. 6.20

Estimation of non-specific adsorption of VSG to the teflon trough and probably at the air water interface. 500 µg (8 nmol) of VSG was added to 100 ml of aqueous buffer contained in the large trough and samples were withdrawn at (O) 40 min, (△) 60 min, (□) 80 min, (X) 100 min and (Δ) 120 min. Each sample was diluted serially and placed in wells of a microtitre plate already coated with the specific antibody (Section 4.3.4). Finally 10 µl of radioactive antigen [^{125}I]VSG$_{151}$ with an approximate activity of 10,000 cpm was added and after 2 hrs, the plate was washed with buffer to remove free antigen. The radioactivity of each sample was measured and compared with the standard values for further calculations.

(o) Standard curve.

Fig. 6.20



(c) as a diluent to reduce the radiation emitted from labelled proteins (Chard, 1978, Chapter 3; Cooper, 1977, Chapter 8).

The labelled antigen in the absence of BSA is liable to all the losses mentioned above. The antigen was checked for possible damage due to iodination, first by observing its specific binding to antibody linked to Sepharose 4-B; and secondly, by counting the radioactivity present in a TCA precipitate as described in the Materials and Methods section. The problem due to nonspecific adsorption to plastic tubes was resolved by collecting samples into glass containers. Non-radio-labelled VSG was added to give a final concentration of 100 µg/ml. Although this raised the total VSG concentration (non-labelled plus labelled VSG), the small volume of the solution (15 to 30 µl) added beneath lipid films contained insufficient VSG to saturate even a cholesterol monolayer.

Analysis of the specific binding of [^{125}I]-VSG to antibody fixed to Sepharose 4-B showed the binding to be 55-70%. This corresponds to the results obtained when VSG containing BSA in solution was similarly analysed. Non-specific binding was corrected for by using antibody against mice erythrocytes fixed to Sepharose 4-B. [^{125}I] VSG was incubated with the non-specific antibody linked to Sepharose 4-B, for 16 hours at 4°C. The suspension was centrifuged and the radioactivity of VSG bound to Sepharose 4-B determined (see Section 4.4). The values thus obtained were within the range of 4 to 15% binding. This percentage of binding is the resultant value after dividing the radioactivity of the pellet by the initial total radioactivity per sample. A more detailed analysis of specific binding is shown in Fig. 6.22 from which it is evident that the binding of radio-labelled antigen to specific antibody is inhibited

Fig. 6.21

Changes of the concentration of VSG in the presence or absence of a cholesterol monolayer. These values were calculated from radioimmunoassay determinations.

2.5 µg/ml initial concentration of VSG.

(●) cholesterol monolayer

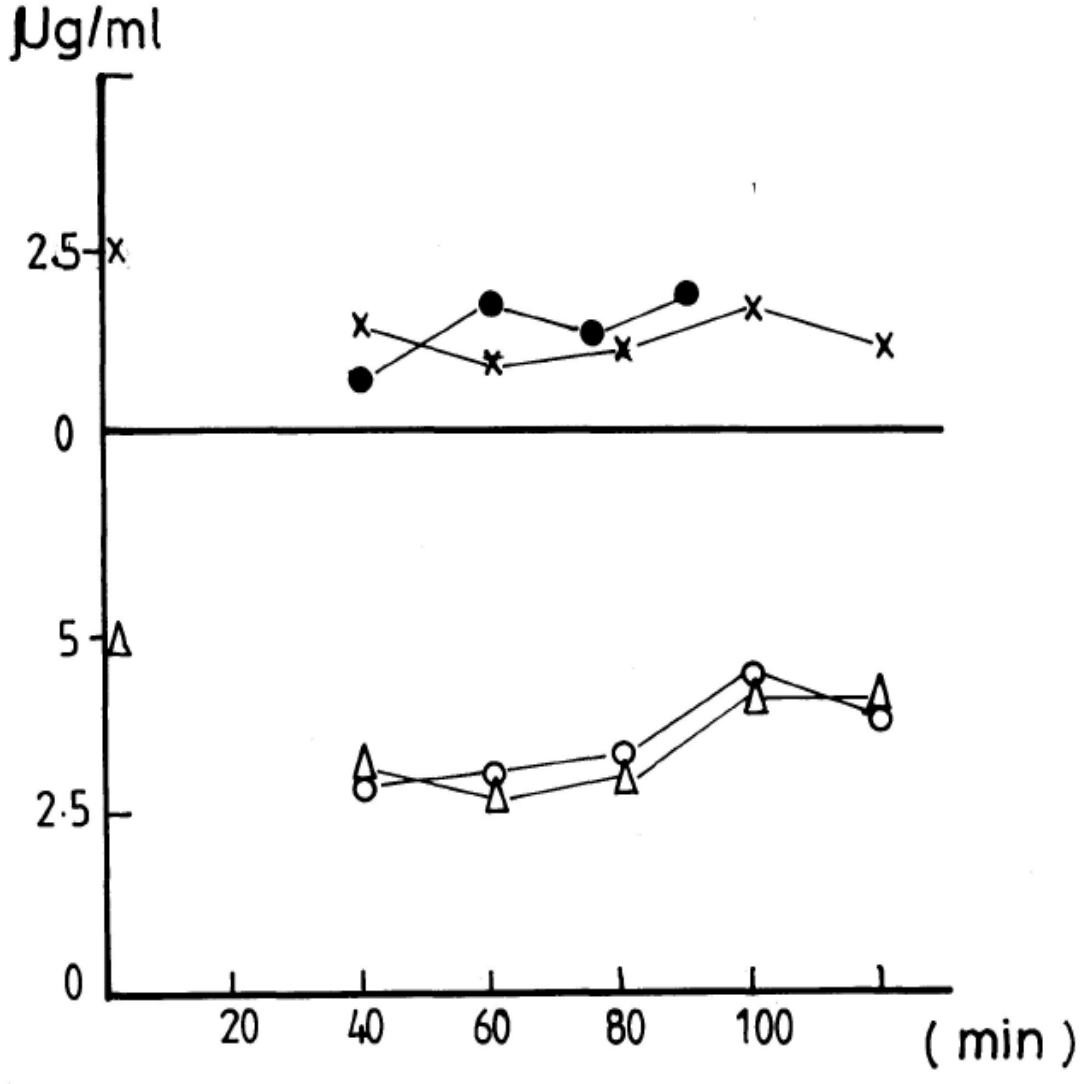
(X) no lipic monolayer

5 µg/ml initial concentration of VSG

(○) cholesterol monolayer

(△) no lipid film.

Fig. 6.21



by increasing the concentration of non-radiolabelled VSG; thus suggesting that both forms of the antigen are competing for the same binding site. The structural integrity of the antigen molecule was checked using polyacrylamide gels and the molecular weight so determined was in agreement with that of native antigen.

It is concluded from the above results that the antigenic determinant site of the VSG molecule is not affected by the incorporation of iodine.

Under the labelling conditions used the incorporation of ^{125}Iodine was limited to 0.22 iodine atoms per molecule of VSG (302 Ci/mol). Another reason for limiting the amount of ^{125}I incorporated into the protein was to avoid the undesirable "decay catastrophe" effect due to the disruption of a molecule bearing an iodine atom by a neighbouring decaying iodine atom (Chard, Ch. 3, 1978). If the incorporation was reduced to about 0.01 iodine atom per molecule of VSG (19.7 Ci/mol), the radioactivity of molecules adsorbed to the lipid monolayers could not be distinguished from that of the bulk solution. Radiolabelled VSG prepared and stored in the absence of BSA must be used within one week of preparation.

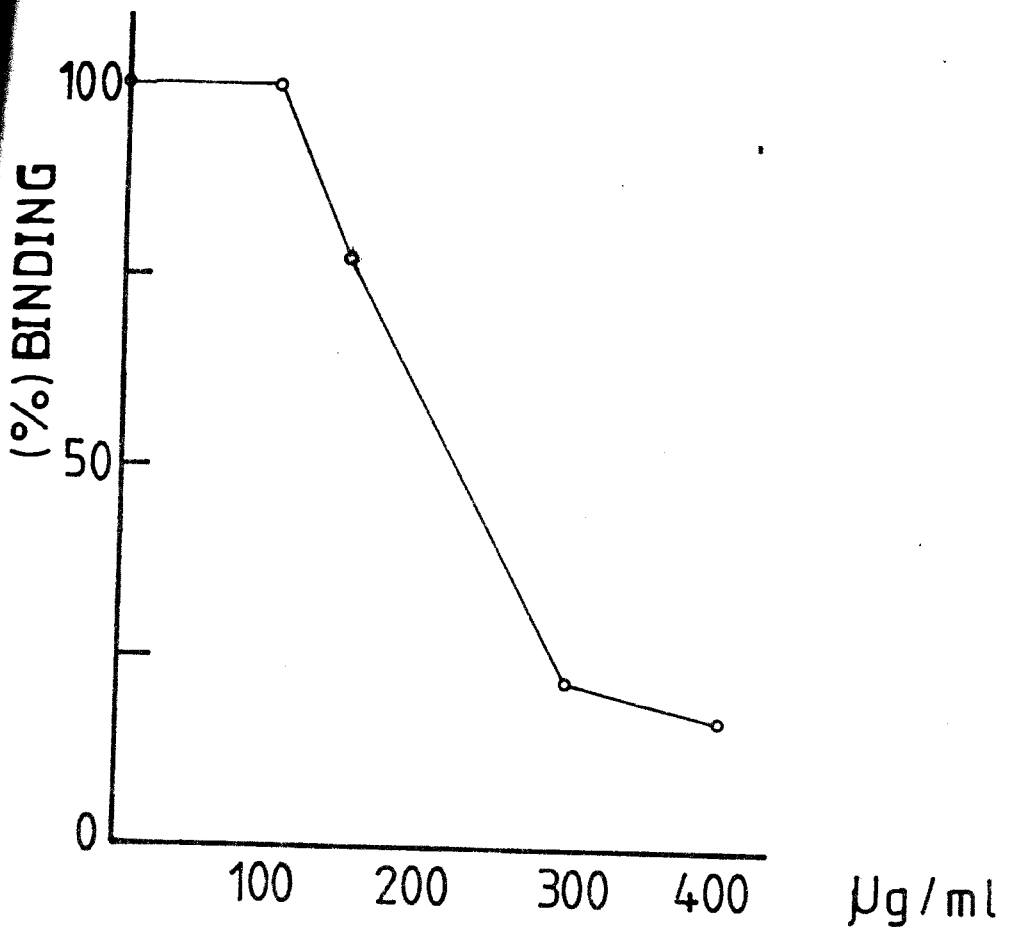
The adsorption of [^{125}I]-VSG$_{151}$ to lipid monolayers was studied using a diffusion method. A volume of 15 µl of radio-labelled VSG containing a total of 4.5×10^6 CPM was injected beneath a pre-formed lipid monolayer while stirring the subphase. The mixture was then incubated at room temperature for exactly one hour. The lipid monolayer was collected by aspiration through a glass capillary connected to a graduated 5 ml-container. The tip of the glass capillary was held at one end of the trough just touching the water surface whilst the monolayer was compressed and aspirated (see Section 4.10.3).

Fig. 6.22

Specific binding of VSG_{151} to specific antibody linked to Sepharose 4-B. Various concentrations of non radiolabelled VSG and 5 µl of radioactive ^{125}I-VSG (approx. 14 x 10^3 CPM) were incubated with 10 µl of the antibody-Sepharose 4B for a final volume of 50 µl.

Specific Binding of ^{125}I-VSG_{151} to specific antibody linked to Sepharose 4-B. Various concentrations of non-radiolabelled VSG and a fixed amount of radioactive ^{125}I-VSG (approximately 14 x 10^3 CPM) were incubated with 10 µl of the specific antibody linked to Sepharose 4-B, final volume 50 µl. After 2 hrs of incubation at 30°C the samples were centrifuged and washed with buffer. The pellet radioactivity was counted and divided by the initial radioactivity.

Fig. 6-22



Similar samples from the subphase were simultaneously collected. The latter served as a basis for the correction for the free labelled antigen that is unattached to the lipid film. It was found necessary to exchange 100 ml of the radioactive solution with fresh non-radioactive buffer since the high content of radioactivity in the bulk solution rendered an accurate determination of radioactivity incorporated into the monolayer extremely difficult. By this procedure the amount of radioactivity in the bulk solution was reduced by 80%.

Studies of the adsorption of [^{125}I]-VSG to lipid monolayers comprising three classes of lipids were undertaken. The lipids chosen are sphingomyelin, cholesterol and dimyristoyl phosphatidylcholine. The results are shown in Table 1.

The percentage of adsorption for the same lipid monolayer was determined to within a 10% error margin.

Adsorption of [^{125}I]-VSG to cholesterol monolayers, as calculated either by percentage of adsorbed radioactivity or by net radioactivity are not significantly different from those of DMPC. Similar studies with mixed monolayers composed of DMPC and cholesterol gave values that were practically identical to the above. The observed increase in surface pressure following the addition of radiolabelled VSG to cholesterol monolayers indicates adsorption and penetration by VSG.

Studies similar to those described above showed that for two mixed monolayers, one composed of cholesterol and Filipin, and the other of cholesterol and cytochrome c, the values of adsorption were 2 and 3 times higher than those obtained for cholesterol alone. These results suggest that there is a positive cooperative effect due to the non-specific interaction of VSG with the components of the two systems. The process of VSG adsorption to these mixed monolayers is complex. It is not possible

Table 6.1 Adsorption of VSG to lipd monolayers

Lipids	Additives	Moles ratio (lipid/additive)	% Adsorption (Radioactivity CPM)	Δπ (nM/m)
Dmpc	-		0.6[b]	0
DMPC	cholesterol	1:1	0.4[b]	-0.5
Cholesterol	cytochrome c	15:1	1.2[b]	+3
Cholesterol	filipin	3:2	0.76 ± 0.1	-2
Cholesterol	-	-	0.4 ± 0.04	+5.5
SPH	-	-	2.1[b]	-1
SPH[a]	-	-	0.16[b]	-1.3

Percentage adsorption was calculated as the radioactivity collected from the monolayer divided by the initial total radioactivity added. The radioactivity of the monolayer was corrected for background by substracting the value obtained for the subphase without a monolayer film. Cholesterol, sphingomyelin (SPH) and dimyristoyl phosphatidyl choline (DMPC) monolayers were formed at the air-water interface with 24, 12 and 12 nmole respectively. Mixed monolayers were formed by spreading the previously mixed lipid solution. Mixed DMPC-cholesterol films were formed at equimolar ratios (12 nmole each). To form mixed cholesterol-filipin films cholesterol (24 nmole) was first spread, and subsequently filipin (16 nmole) was added beneath it. Mixed cytochrome c-cholesterol monolayers were formed by adding cytochrome c (16 nmole) beneath the pre-formed cholesterol monolayer (24 nmole) and allowed to equilibrate for 1 hour. To each lipid monolayer a fixed volume of 15 µl of a [^{125}I] VSG solution containing 40 ng/µl of VSG with a total radioactivity of 4.5 x 10^6 CPM was added. The radio-iodination method used produced an incorporation of 0.22 molecules iodine per molecule of VSG. Other conditions are described in the text. The change in surface pressure (Δπ) is the difference between the final surface pressure and the initial pressure (5 nM/m^{-1}). The radioactive buffer subphase was exchanged with 100 ml of non-radioactive buffer (pH 6.8) and then the monolayer was collected by aspiration (Section 6.5.3).

a Monolayer collected without exchanging the subphase.

b Mean square deviation was not determined because of the small number of determinations (n = 3).

to deduce from the present data whether the high values of adsorption of VSG occur by cooperative interaction with cytochrome c. Cytochrome c interacts with phospholipids by a combination of electrostatic and hydrophobic forces. Initially it binds the polar head groups and subsequently penetrates partially the lipid bilayer (Papahadjopoulos, 1977). Then, the hydrophilic part of the molecule must be exposed to the aqueous medium and may interact with charged groups of VSG. Other types of non-specific interactions however, can occur where the surface area of the cholesterol monolayer was expanded approximately 2 times by cytochrome c to keep the surface pressure constant. Although judging by the changes in surface pressure VSG penetrates this mixed film composed of cholesterol and cytochrome c. Positive cooperativity as observed above has been described for other systems (Ketis & Grant, 1982; Eytan, 1982). Thus the binding of Concanavalin A to liposomes containing a Concanavalin A receptor is enhanced when the reconstituted liposomes were previously coated with BSA (Ketis & Grant, 1982).

On the other hand results for sphingomyelin monolayers show these to have the highest values of adsorption for VSG. This process was however, not preceded by insertion of VSG in the lipid film. In Section 5.5 it was discussed that sphingomyelin by forming hydrogen bonds with VSG could obstruct the penetration of the lipid monolayer. Is that process inducing any orientation of VSG molecules as to produce a positive cooperativity between VSG molecules?

The answer to this question cannot be provided with only these results. It would be necessary to study if sphingomyelin mixed with phosphatidylcholine or cholesterol can affect the adsorption of such amounts of VSG. Studies of binding of VSG to liposomes containing sphingomyelin could contribute to clarify these results.

6.6 Discussion

The presence of VSG in monolayers of mixed phospholipids displaced the lipid π-A isotherm in a similar way to those for monolayer of each component of the mixture. Changes in critical surface pressure ($\Delta\pi_{critical}$) can be interpreted as partial penetration of these mixed films of VSG.

$\Delta\pi_{critical}$ values were dependent on the initial surface pressure, and for 5 mN/m it was reduced to values near to zero. Below 5 mN/m $\Delta\pi_{critical}$ showed maximum values. The presence of cholesterol in these mixtures (PE/EYPC/SPH) at neutral pH gave results similar to those for the phospholipids (Fig. 6.2). From the previous sections there is seen to be a certain degree of chemical recognition at the interface, as well as an energetic barrier that VSG has to surmount, thus phospholipid monolayers even at near zero surface pressure restrict the insertion of VSG into the interface. Expanding the available surface area of the monolayer brought about an increase in the $\Delta\pi_{critical}$ values (Fig. 6.4a). $\Delta\pi_{critical}$ values were different for monolayers of mixed phospholipids (PE/EYPC/SHP) at pHs ranging from 5.4 to 5.8 only when cholesterol was present. $\Delta\pi_{critical}$ values above pH 6.0 were similar for mixed phospholipids and in presence of cholesterol. Interestingly, substituting the mixture of phospholipids by egg yolk phosphatidyl choline abolished this difference in $\Delta\pi_{critical}$ at different pH values regardless of the presence of cholesterol, although the π-A isotherms were displaced significantly at pH values below 5.8 and in presence of cholesterol. This result may be interpreted that the mixture of PE/EYPC/SPH does not form stable monolayers. It has been demonstrated that spingomyelin and EYPC do not form homogenous ideal mixtures below 20°C (Untracht &

Shipley, 1977). In an early section it was demonstrated that VSG does not interact with a spingomyelin monolayer (Section 5.1). Therefore it may be that VSG penetrates certain areas of the monolayer rendering it unestable. At pH 5.4 VSG could have a conformation which is more likely to interact with cholesterol in the phospholipid cholesterol film. Similar results in film penetration experiments, have been obtained for expectrin. This molecule showed a larger rate of penetration at pH 3.5 than at neutral pH (Juliano et al., 1971).

The weak interaction between VSG and phospholipid monolayers appears to be independent of electrostatic interactions, whereas changes in the concentrations of KCl and NaCl up to 145 mM, or $CaCl_2$ up to 2 mM, did not alter the π-A or ΔV-A isotherms significantly (Section 6.4). These ionic concentrations has been found to disrupt cytochrome c - phospholipid complexes (Ivanetich et al., 1974). Hydrogen bonding in combination with Van der Waals forces may cause this VSG-phospholipid interaction. Phosphatidyl ethanolamine (PE) was mainly used in these experiments. PE has been found to bind Ca^{2+} weakly at pHs ranging from 4 to 9; above pH 10 PE binds Ca^{2+} strongly having a maximum at 30°C (Rojas & Tobias, 1965; Seimiya et al., 1976). These experiments, however, did not show either displacement of VSG or bridging of VSG to PE as observed with other proteins (Juliano et al., 1971). Interestingly experiments carried out with the whole parasite showed that the surface coat was not released by Ca^{2+} unless a calcium ionophore was included; however, a clear relationship between the releasing mechanism of VSG and the rôle of Ca^{2+} plus ionophore was not given (Bowles & Voorheis, 1982).

The use of the detergent sodium cholate increased the insertion of VSG into spingomyelin and phospholipid monolayers. Thus the dissociation

into monomers appears to be necessary for film penetration. The removal of [^{14}C]cholate was more easily accomplished in EYPC films than in spingomyelin monolayers (Section 6.2). The method used to remove cholate from monolayer is analogous to detergent dialysis, except that the subphase is exchanged continuously. A possible sequence of events for the process of inserting protein molecules using this method is shown (Fig. 6.23). One conclusion from the results detailed in Section 6.2 is that cholate binds preferentially to spingomyelin rather than to phosphatidyl choline. Is this interaction related to the structural similarities between cholesterol and cholate? It has been found that cholesterol bonds preferentially to spingomyelin in a phosphatidyl choline-spingomyelin mixture (Demel et al., 1977; Van Dijck et al., 1977)

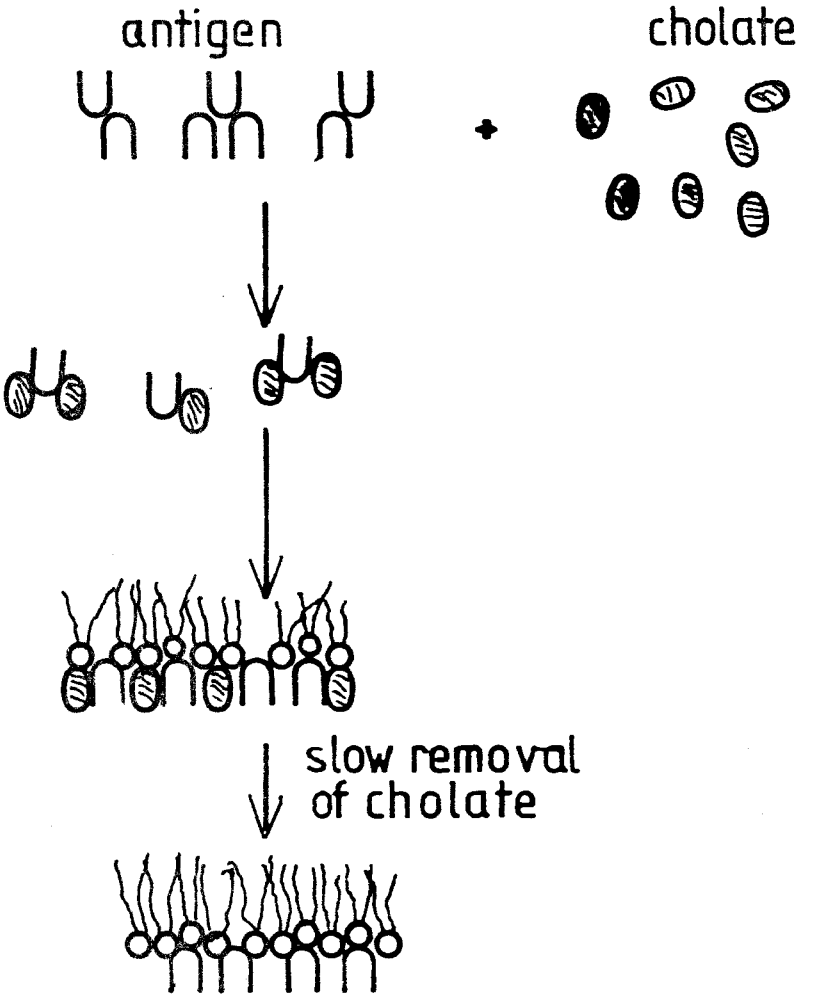
Film penetration experiments using VSG uncubated with [^{14}C]cholate show a rapid increase in radioactivity in cholesterol monolayers. This effect was not detected when radiolabelled cholate was injected beneath the cholesterol film (Section 6.2.3). These results can be interpreted as the reversible binding of VSG to cholate and during the penetration process cholate becomes detached from the molecule of VSG. Furthermore, the difference between EYPC and cholesterol films in decreasing surface radioactivity can be interpreted in terms of cholesterol binding to VSG at a similar site as cholate (Fig. 6.16b). Therefore cholate may be displaced by a combination of specific binding competition and a surface pressure higher than the equilibrium surface pressure. For EYPC films cholate is displaced by the effect of high surface pressure only.

Direct assessment of the interaction between antigen and lipid was made using the capacity for film penetration (CFP) method of Pethica (1955). This method appears to be the most appropriated for comparing

Fig. 6.23

Insertion of VSG dissociated by detergent in phospholipid monolayers. A schematic representation of possible events occurring during the insertion of VSG dissociated with sodium cholate. Subsequently the detergent is removed by exchanging the lipid monolayer subphase with fresh buffer.

Fig. 6.23



penetration in different films (Section 6.3). An alternative method is the relative penetration capacity. The plot of $\Delta\pi$ versus initial surface pressure gives a straight line, and extrapolating to $\Delta\pi = 0$ provides the relative penetration capacity. This is interpreted as the surface pressure at which proteins are excluded from the lipid monolayer (Fig. 6.24). For cholesterol films this value was approximately 37 mNm^{-1} which is 8 mNm^{-1} higher than that estimated by $\Delta\pi_{critical}$ (Table 5.4).

Applying the CFP method to estimate the cholesterol film penetration by band 3-protein at an initial surface pressure of 10 mNm^{-1} and at various protein concentrations gave a value of 5×10^6 mNm^{-1} $mmol^{-1}l$. Band 3-protein is an integral membrane protein of human erythrocyte with a molecular weight of 1×10^5 (Tyler J.M., 1979). The experimental values to do this calculation were taken from Klappauf & Schubert (1977). This CFP value is two order of magnitude higher than that calculated for VSG (5×10^3, see Table 7.2). This strong interaction may be due to a major affinity of band 3-protein by cholesterol in comparison with VSG. It has been found that band 3-protein exhibits a preferential order of interaction with lipids which resembles that observed for VSG. This order of affinity decreases as follows: cholesterol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline and spingomyelin (Klappauf & Schubert, 1977; Schubert & Marie, 1982).

VSG readily penetrates mixed films of DPPC-cholesterol, whereas in the absence of cholesterol the penetration does not occur (Section 6.3.1). The rate of penetration by VSG was not, however, proportional to the cholesterol concentration. This can be interpreted as cholesterol increasing the surface area available in the monolayer for VSG. This agrees with the relationship between surface pressure and area per

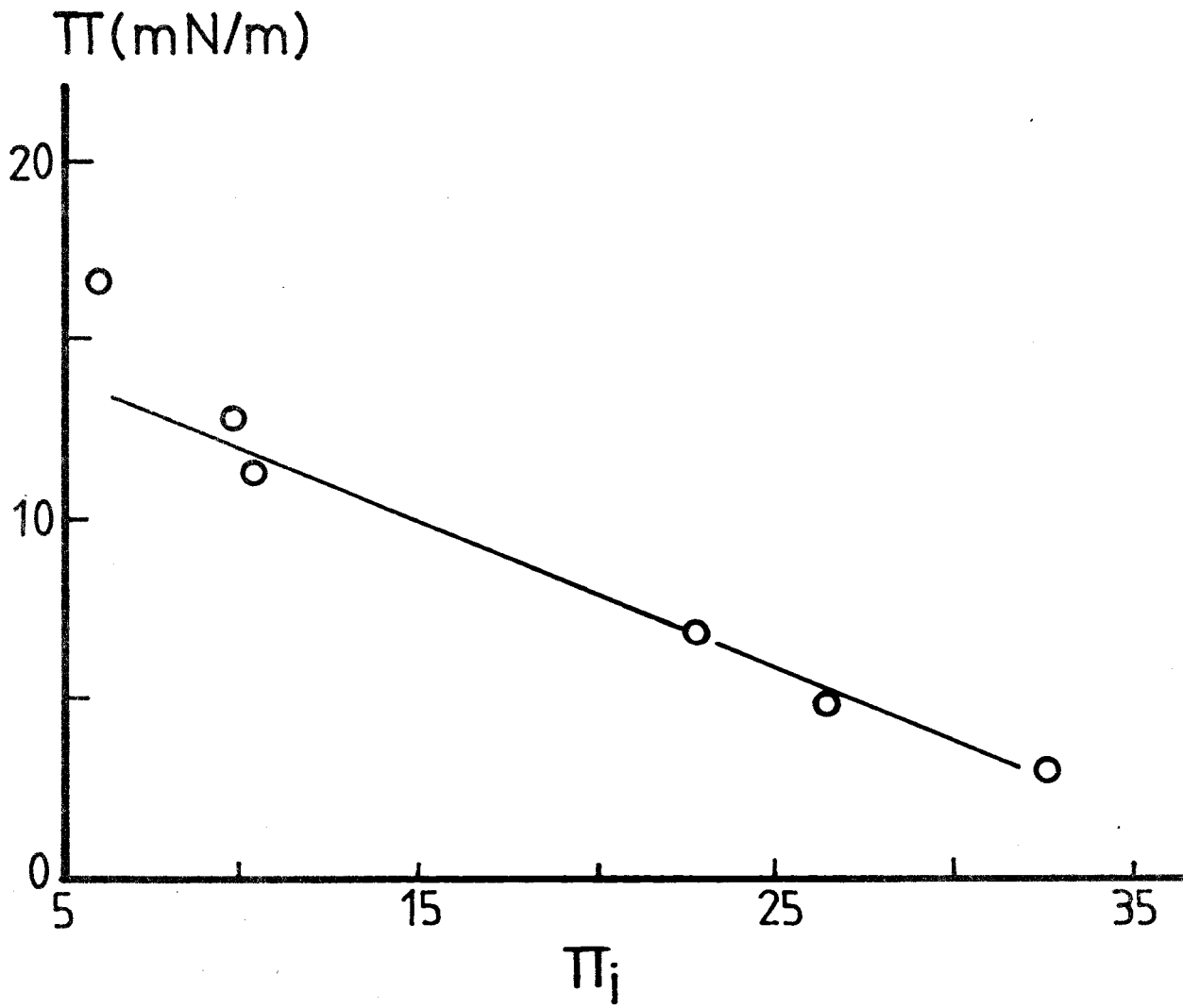
molecule deduced from the π-A isotherms for DPPC and EYPC monolayers. Thus at the same surface pressure the area per molecule of EYPC is larger than that for DPPC (Section 5.1). The existence of lateral phase separation in mixed DPPC-cholesterol films is not yet completely clear (Gershfeld & Pagano, 1972; Lundberg, 1982). The finding that the penetration rate by VSG does not increase in relation to the proportion of cholesterol suggests various possibilities: (i) cholesterol in excess is distributed at random; (ii) the formation of clusters of different size are distributed at random - the existence of cholesterol-poor, or cholesterol-rich clusters has been suggested using differential scanning calorimetry (Snyder & Freyre, 1980); and (iii) VSG binds phospholipid polar groups with an orientation which inhibits the binding by other molecules of VSG.

Fig. 6.24

Penetration of cholesterol monolayers at different initial surface pressures by VSG. 0.63 nmol of VSG was injected beneath cholesterol monolayers at various initial surface pressures. $\Delta\pi$ values were taken as the difference between the stable final surface pressure and the initial surface pressure.

Subphase pH 6.8.

Fig. 6.24



CHAPTER 7

STUDY OF THE AFFINITY OF VSG FOR CHOLESTEROL USING CHOLESTEROL DERIVATIVES AND POLYENE ANTIBIOTICS, AND THE INTERACTION OF VSG WITH LIPID BILAYERS

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STUDY OF THE AFFINITY OF VSG FOR CHOLESTEROL USING CHOLESTEROL DERIVATIVES AND POLYENE ANTIBIOTICS, AND THE INTERACTION OF VSG WITH LIPID BILAYERS

7.1 Interaction of protein with sterols: brief commentary

In the two preceding chapters monolayer experiments showed that VSG has a marked affinity for cholesterol and ergosterol. Members of Trypanosoma brucei contain a high proportion of sterols ranging from 21 to 35% of total lipids (Venkatesan & Ormerod, 1976). The bloodstr form contains cholesterol which is obtained from the host. Ergostero is the main component in the culture form and is synthesized by the parasites (Dixon et al., 1972; Venkatesan & Ormerod, 1976; Beach et a 1979). Cholesterol is present in most plasma membranes of eucaryotic cells and in T.brucei the mole ratio of cholesterol to phospholipids 3:2 (Voorheis, 1979). It is unlikely therefore that the occurrence of such strong interaction between VSG and cholesterol molecules found in the monolayer model will have no biological significance. This become more significant when experiments are considered which show that cholesterol brings about lateral phase separation of phospholipids (Gershfeld, 1978; Van Dyck et al., 1976). Furthermore the affinity of some membrane proteins for specific lipids suggests structural recogni- tion between protein and lipids, i.e. apoprotein A-I has a high affinit for DMPC. Similarly, the myelin basic protein A1 has affinity for cerebroside sulphate; and the band 3 of erythrocyte for cholesterol (Pownall et al., 1979; London et al., 1974; Klappauf & Schubert, 1977). Thus it seemed important to investigate whether the interaction between VSG and cholesterol is directed by specific recognition of the whole or

part of the structure of the cholesterol molecule. This study was carried out using the monolayer system and various cholesterol derivatives.

7.2 Surface properties of sterol derivatives

7.2.1 β-Sitosterol

β-sitosterol is a sterol commonly found in plants. Its molecular structure is similar to cholesterol but with the modification of possessing an ethyl group in position 24 of the diphatic chain. Figure 7.1 shows the structure of β-sitosterol and other sterol compounds. The force-area curve of β-sitosterol at the air-water interface exhibits the general properties of a condensed monolayer. The limiting surface area was of 37Å^2 per molecule, a value identical to that determined for cholesterol (Fig. 7.2). Force-area curves of this compound were significantly displaced by small quantities of VSG. Thus the presence of 0.06 mol fraction of VSG could displace the π-A isotherm by a similar amount to that for cholesterol (Fig. 5.8a).

7.2.2 Lanosterol

Lanosterol is an animal sterol obtained from the wool fat of sheep. It possesses a double bond in position C-8,9 and some additional methyl groups attached on carbons of the steroid nucleus, thus distinguishing it from cholesterol and ergosterol. The force-area curve at the air-water interface shows the characteristic of a condensed film. The addition of VSG caused a displacement of the π-A and ΔV-A curves respectively (Fig. 7.2).

7.2.3 The synthetic sterol derivatives of amino-cholestene and amino-cholestane

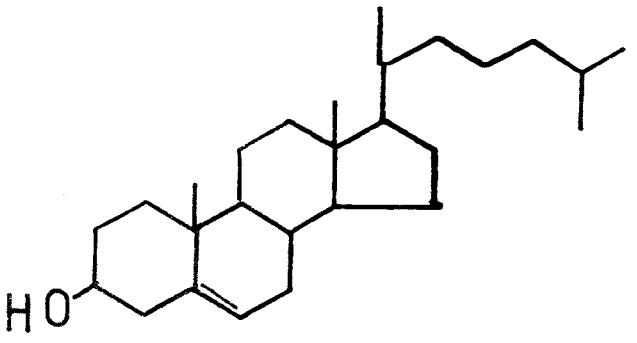
The force-area curves of amino-cholestene and amino-cholestane appear

Fig. 7.1

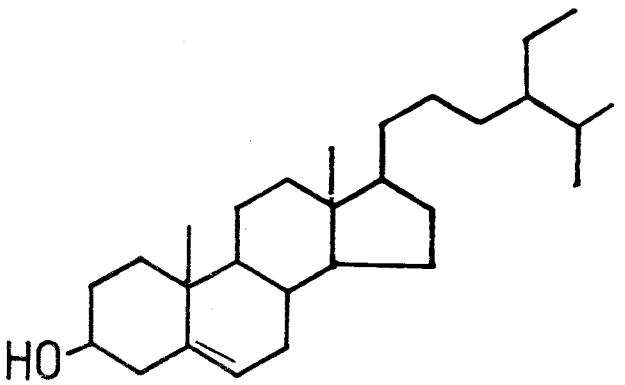
Structure of some common sterols.

Fig. 7.1

Cholesterol



Sitosterol



Ergosterol

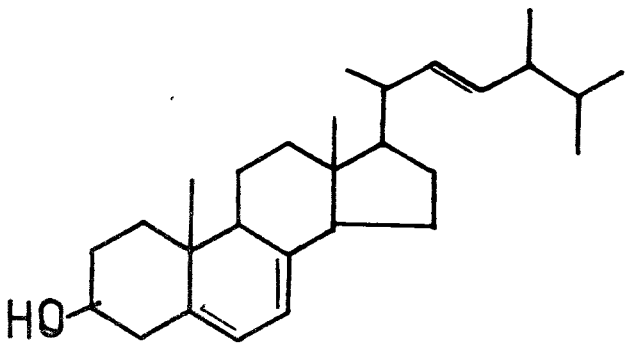
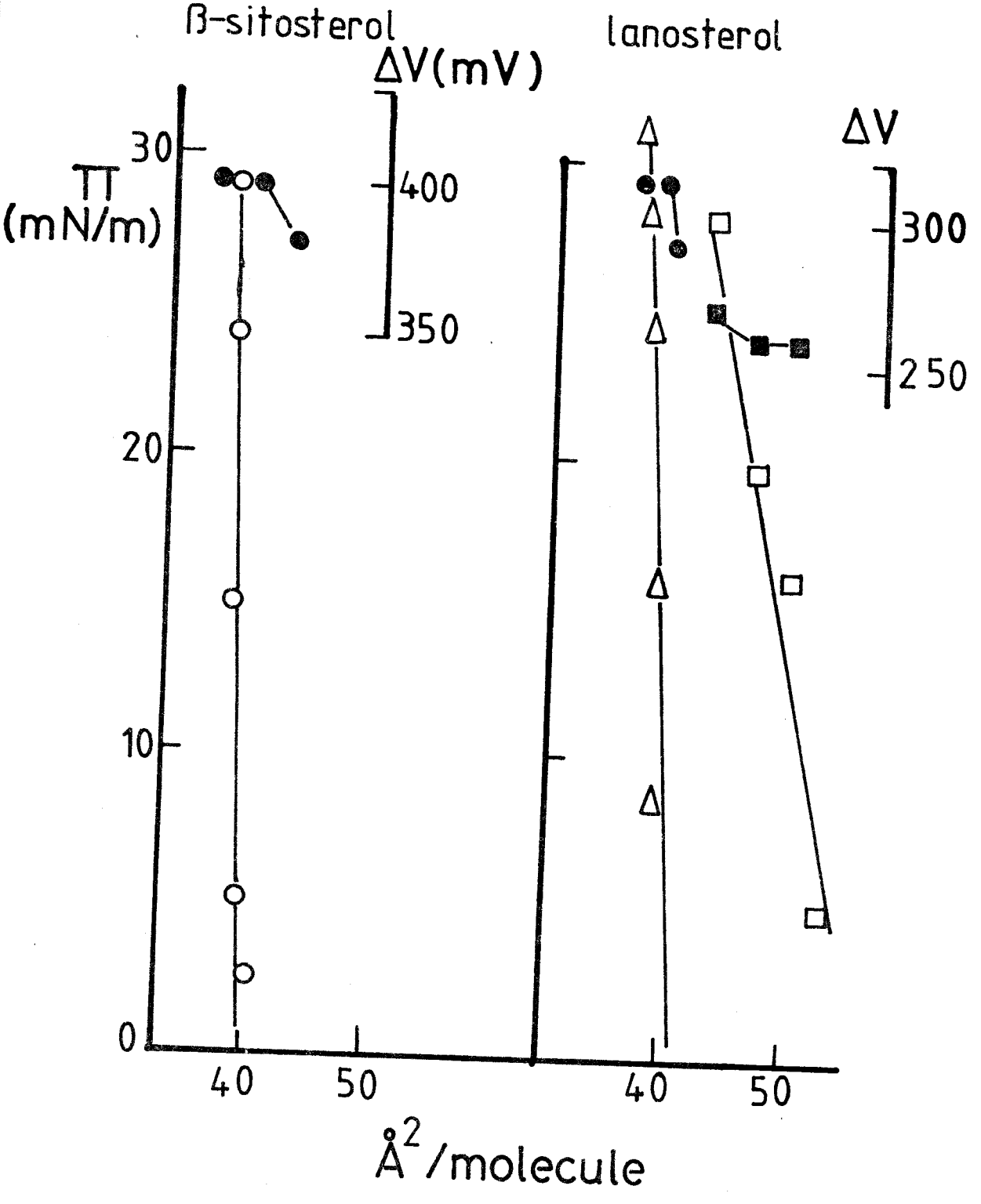


Fig. 7.2

β-sitosterol monolayer (30 nmol) spread at the air-water interface: (○) surface pressure and (●) surface potential. Lanosterol monolayer (30 nmol): (△) surface pressure and (▲) surface potential. 3.2 nmol of VSG injected beneath the lanosterol film (30 nmol): (□) surface pressure and (■) surface potential.

Subphase pH 7.2.

Fig. 7.2


β-sitosterol
lanosterol
ΔV(mV)
ΔV
400
350
300
250
π
(mN/m)
30
20
10
0
40
50
40
50
Å²/molecule

to be less condensed than those of β-sitosterol or lanosterol. Thus the part of the π-A curve which can be compressed from nearly zero surface pressure to approximately 25 mN/m for amino cholestene corresponds to the less condensed phase, whilst above this surface pressure the film becomes completely condensed.

Amino cholestane shows a short range of surface pressure (up to 12 mN/m) for the compressible part of the π-A curve. Studies of phase transitions for these compounds were not attempted.

The addition of 0.04, 0.08 and 0.1 mol fraction of VSG induce a considerable displacement of the π-A isotherms for both amino-cholestene and amino-cholestane. The surface potential in the presence of 0.1 mol fraction of VSG was stable at large area per molecule of amino-sterol, and was increased when the film was compressed up to a surface pessure of 30 mN/m (Fig. 7.3).

In comparison with the values of ΔV for the amino-sterols the surface potential changes in the presence of VSG were relatively higher over the range of compression shown above. This differs from the ΔV at surface pressures above 20 mN/m when cholesterol is the film forming molecule (see section 5.2.1). The interacting part of VSG may have the same dipole orientation as that of the amino-sterol and this is reflected by an increase in the surface potential (see Section 5.4.6).

7.2.4 Acetamido-cholestene and acetamido-cholestane

These sterol derivatives show force-area curves with a short range of surface pressure (up to ~ 13 mN/m) and these π-A curves resemble those described for amino-cholestane (Fig. 7.4). The addition of 0.1 mol fraction of VSG induces a prompt displacement of the π-A curve. The critical surface pressure for acetamido-, and amino-sterol derivatives

Fig. 7.3

(a) Amino cholestene monolayer (23 nmol) spread at the air water interface: (○) surface pressure and (●) surface potential. 1.1 nmol of VSG was injected beneath the amino cholestene monolayer (23 nmol) at near to zero surface pressure and force-area curves were determined after 1 hr, (□) surface pressure and (▲) surface potential.

(b) Acetamido cholestene monolayer (27 nmol) formed at the air-water interface: (○) surface pressure and (●) surface potential. 2.5 nmol of VSG was injected beneath the monolayer, the procedure was as in (a).
(□) surface pressure and (▲) surface potential.

Fig. 7.3

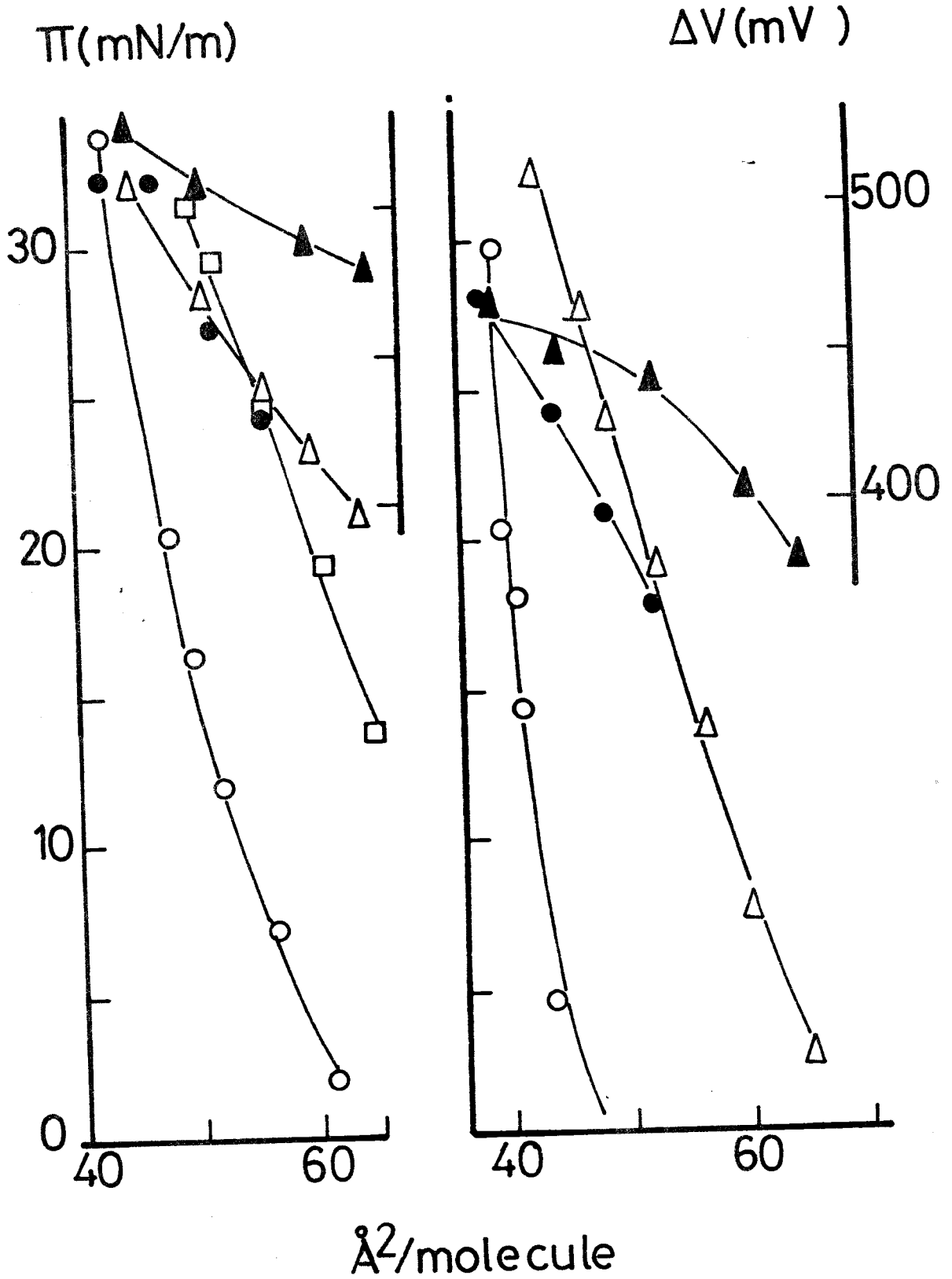


Fig. 7.4

(a) Acetamido cholestane monolayers (27 nmol) formed at the air-water interface: (O) surface pressure and (●) surface potential. 2.7 nmol of VSG was injected under this film. Other conditions are described in Fig. 7.3. (Δ) force-area curve, (▲) surface potential.

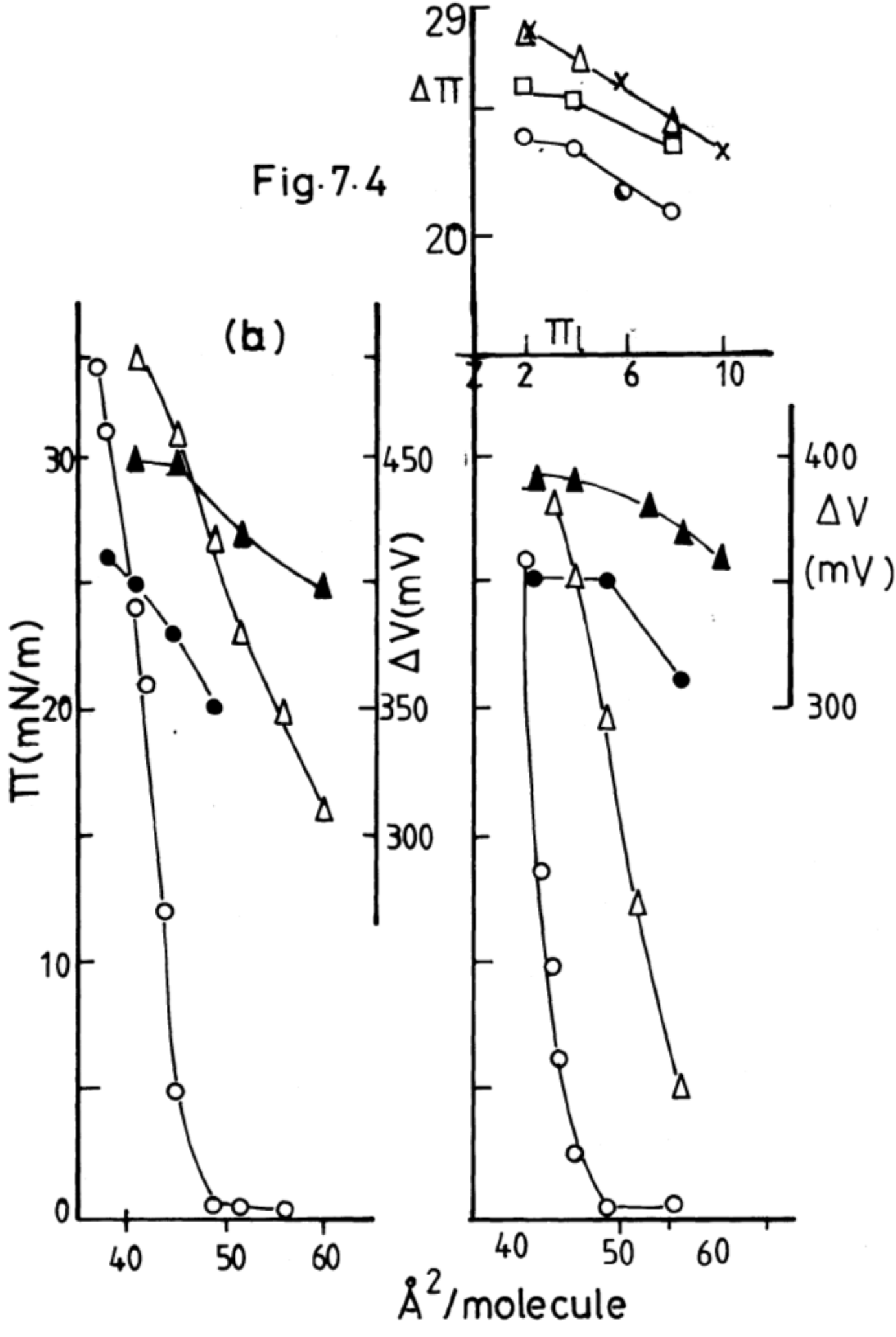
(b) Amino cholestane monolayer (27 nmol) spread at the air-water interface: (O) surface pressure and (●) surface potential. 2.7 nmol of VSG was added beneath the amino cholestane monolayer and (Δ) surface pressure and (▲) surface potential were determined.

Figure inset

Comparative plots of $\Delta\pi$ versus lipid surface pressure amongst sterol derivatives. $\Delta\pi$ was determined from force-area curves of lipid monolayers containing 0.1 mol fraction of VSG. (O) acetamido cholestane, (□) amino cholestane, (Δ) acetamido cholestene, (X) amino cholestene.

Subphase pH 7.2.

Fig. 7.4


29
ΔΠ
20
Π
2
6
10
(b)
30
20
10
0
Π(mN/m)
450
350
300
ΔV(mV)
400
ΔV
(mV)
300
40
50
60
Å²/molecule

shows no significant difference with a value of 28 ± 2 mN/m (inset of Fig. 7.4).

7.2.5 Cholesteryl phosphoryl choline

The force area curve of cholesteryl phosphoryl choline at the air-water interface is more expanded than those shown above. Addition of 0.14 mol fraction of VSG did not, however, provide a large displacement of the π-A curve. Changes in surface potential in comparison with pure films of cholesteryl phosphoryl choline showed a slight increase at large area per molecule, and a reduction in ΔV for areas less than 70$\mathring{A}^2$ per molecule (Fig. 7.5). Interestingly these changes in surface potential are different from those shown for the interaction of VSG with amino- and acetamido-sterols (see Fig. 7.3 and 7.4). It is possible that the interacting part of VSG cannot contribute to the dipole moment by a restriction imposed through the polar groups of phosphoryl choline. Thus the contributing dipole cannot be parallel with that of the sterol, and its angle θ with respect to the interfacial director cannot be zero.

$$\mu_{l_{VSG}} = \mu \cos \theta \qquad \text{(Section 5.4.6).}$$

The surface potential ΔV is influenced by the nature of the permanent dipoles, the presence of ionogenic groups, and the ionic double layer (equation 5.20). The contribution from these components to ΔV can be expressed using equation (5.20) as

$$\Delta V = 4\pi n_c \mu_{l,c} + 4\pi n_{VSG} \mu_{l,VSG} + \psi_o \qquad (7.1)$$

c refers each of the parameters to cholesteryl phosphorylcholine, other nomenclatures have been given (section 5.4.6).

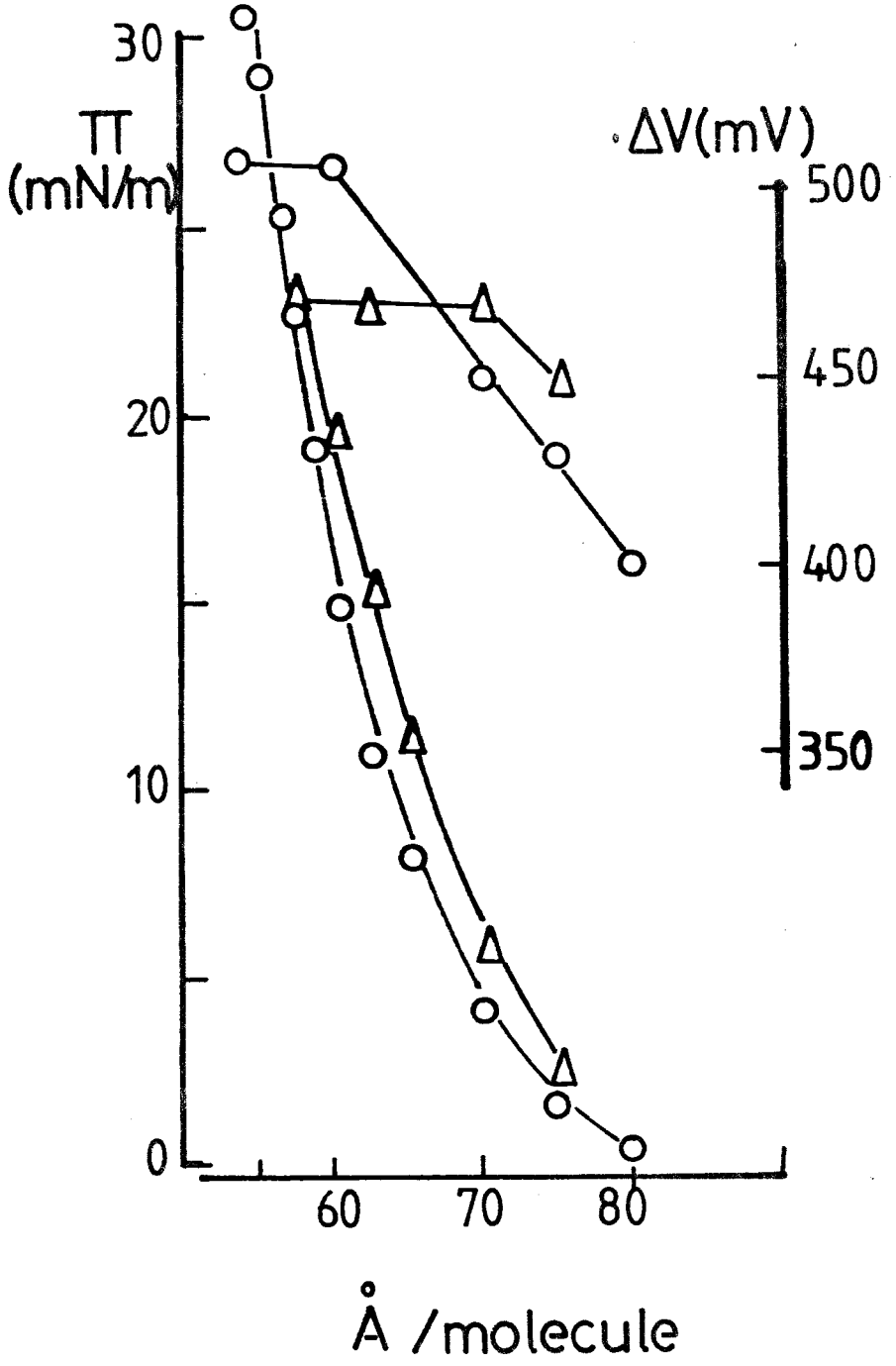
The ionic double layer potential (ψ_o) is assumed to be zero due to electrical neutrality of the film. It may be inferrred also from

Fig. 7.5

Cholesteryl phosphatidyl choline monolayers (20 nmol) formed at the air-water interface: (O) force-area curves and (O) surface potential curves.

3.3 nmol of VSG was injected under this lipid film and (Δ) surface pressure curves and (Δ) surface potentials were determined after 1 hr.

Fig. 7.5



equation (7.1) that at high surface pressure if VSG is ejected from the monolayer its contribution to ΔV is zero, and therefore ΔV is only determined by the film-forming molecules.

The high surface pressure part of the π versus A isotherm is linear for most of the sterol derivatives described above. It is possible to extrapolate to π equal to zero and so obtain a limiting area per molecule at zero surface pressure (A_0) (Gaines pp. 186, 1966; Aveyard & Haydon pp 89, 1973). The values of A_0 for various sterol derivatives are shown (Table 7.1). The variability of A_0 for amino-cholestene and cholestanyl phosphate could be explained on the basis that the condensed state is obtained at high surface pressure. These compounds show a $\delta\pi/\delta A$ lower than those for the other sterol derivatives. Since the inflection point separating the π-A isotherms of the condensed and non-condensed states is not clearly demarcated, the error in extrapolating this short linear part of the isotherm may, therefore, be appreciably higher (Fig. 7.5).

7.2.6 Cholesteryl phosphate, cholestanyl phosphate and cholesterol acetate

These sterol derivatives form monolayers with varying stabilities at the air-water interface. Cholesteryl phosphate and cholestanyl phosphate monolayers are stable at a surface pressure no higher than 30 mNm^{-1}, whilst the limiting pressure for cholesterol acetate is 15 mNm^{-1}. In fact compressing these films above these surface pressures followed by expansion and recompression, results in a shift of the π-A curves to low values of surface area. This indicates that some film forming molecules are presumably solubilized at high pressures. Studies of equilibrium at low pressure, however, were not attempted. The force-

Table 7.1 Limiting area (Ao) for sterol derivatives.

Sterol derivative	($\AA^2$/Ao molecule)
Cholesteryl acetate	40 ± 1
Amino cholestene	54 ± 5
Amino cholestane	45 ± 2
Acetamido cholestene	42 ± 3
Acetamido cholestane	44 ± 2
Cholesteryl phosphate	39 ± 3
Cholestanyl phosphate	41 ± 5
Cholesteryl phosphoryl choline	65 ± 2

(Subphase: 10 mM Tris-HCl, 156 mM KCl, pH 7.2).

area and surface potential curves of these compounds are shown (Fig. 7.6). The shape of the π-A isotherm for cholesterol acetate shows the characteristics of a condensed film. Its collapse pressure of 15 ± 1 mN/m is 2.5 times smaller than that of cholesterol (Kwong et al., 1971). The sterol phosphate derivatives form less condensed films as shown by their respective π-A isotherms. Their interaction with variant surface glycoproteins was investigated using the technique of film penetration described in the following section 7.3.

7.3 Structure-activity relationships for the interaction of cholesterol with VSG. Film penetration studies

The determination of the critical surface pressure from the force-area curves for the interaction between VSG and the amino- and acetamido-sterols showed no differences among the sterol derivatives used. Therefore a study measuring the capability for penetration of these films by VSG would provide more information on their specific interactions. These experiments were carried out in the small circular trough described earlier (Materials & Methods, section).

The rate of penetration plotted as $\Delta\pi$ versus time shows that the interaction of VSG with these compounds is dependent on the initial surface pressure and on the concentration of VSG (Fig. 7.7a, b and c). On the other hand, sterols with phosphate and amide substituent groups required higher amounts of VSG to yield significant values of $\Delta\pi$. In comparison, amino- and hydroxy-substituted sterols need about 20 times less VSG to obtain the same $\Delta\pi$ value (Fig. 7.8). For some sterol derivatives an initial surface pressure of 10 mN/m reduced the rate of penetration by VSG considerably. In addition obtaining the plateau of the curve $\Delta\pi$ versus time, took longer than 2 hours. For most of the sterol derivatives higher $\Delta\pi$ values, and a clearly defined equilibrium

Fig. 7.6

(a) Cholesteryl phosphate monolayers (27 nmol) spread at the air-water interface: (○) force-area curve and (●) surface potential.

Cholestanyl phosphate film (27 nmol).

(△) force-area curve and (▲) surface potential.

(b) Cholesterol acetate (30 nmol) at the air-water interface: (○) surface pressure and (●) surface potential.

Subphase pH 7.2.

Fig. 7.6

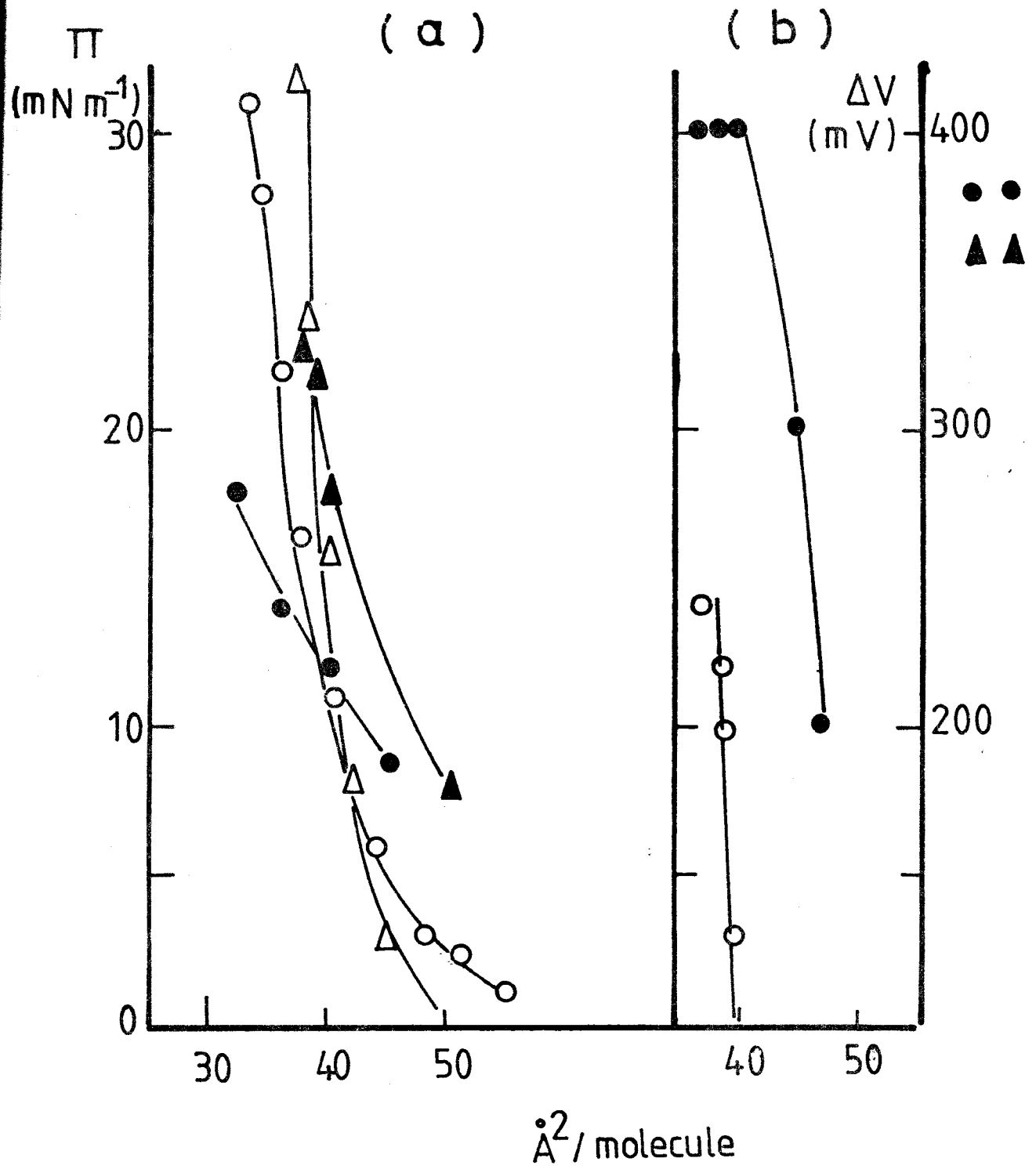


Fig. 7.7

Film penetration experiments. The sterol derivative monolayers were formed at the air-water interface at constant surface area. VSG was injected beneath the lipid film and changes in surface pressure determined.

(c)

Aminocholestene:

	VSG:lipid mol ratio	initial surface pressure
(○)	1:6.5	11
(△)	1:6.5	14
(□)	1:13	14

(a)

Cholestanol:

(○)	1:10	10
(△)	1:20	10
(□)	1:5	10
(▲)	1:15	10

(b)

Amino cholestane

(○)	1:5	11
(●)	1:12.5	11
(△)	1:12.5	11

(d) Penetration rate determined at 60 min.

VSG:lipid mol ratio 1:10

Initial surface pressure: 10 mN/m

(⊓) amino cholestane

(▨) amino cholestene

(▩) cholestanol

(☰) β-sitosterol

Subphase pH 7.2.