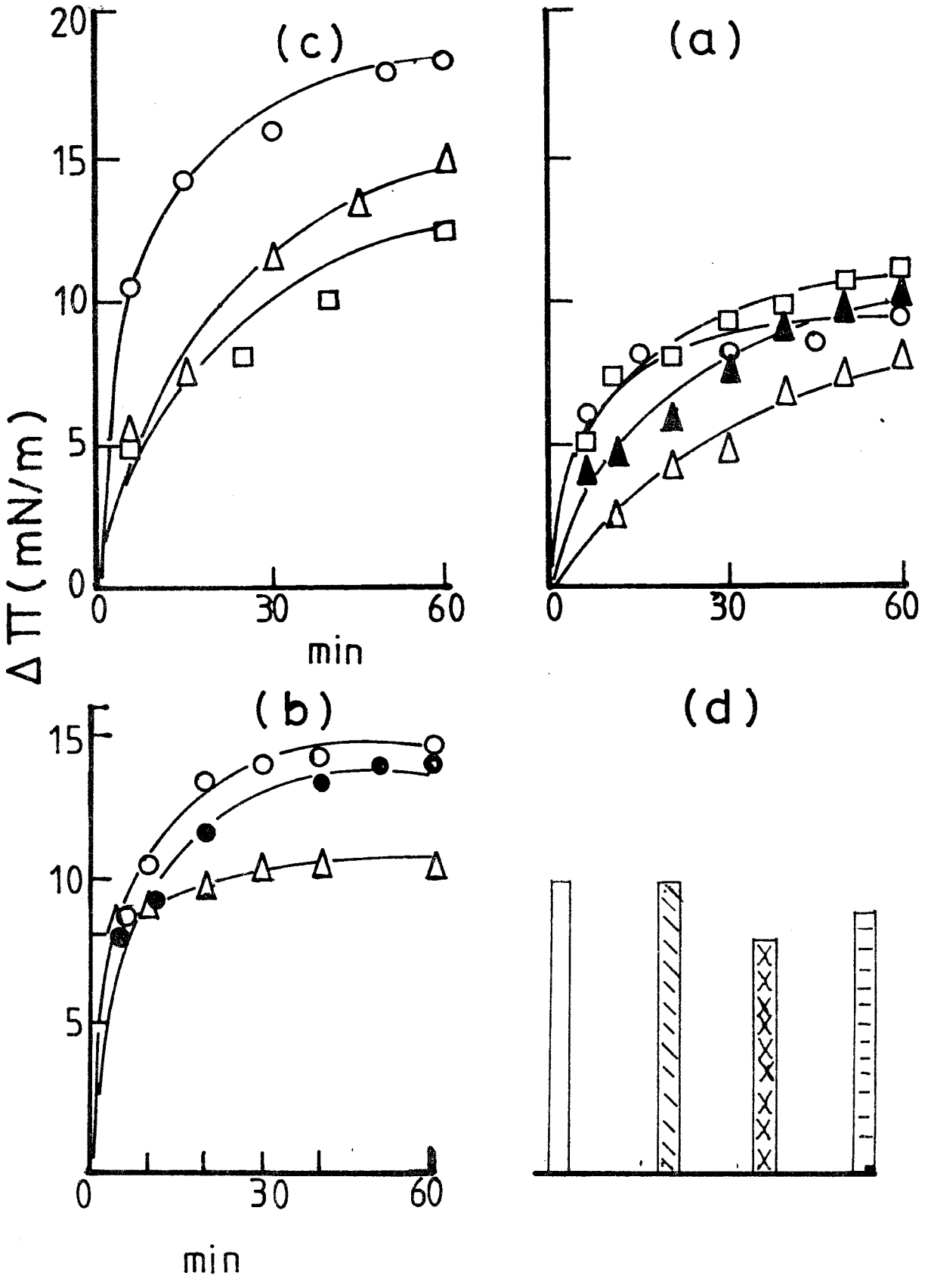
Fig. 7.7



were obtained at surface pressures below 10 mN/m. Figure 7.8 shows the interaction between sterol derivative films and various concentrations of VSG. In particular it shows the weak interaction of acetamido sterols and cholesteryl phosphoryl choline with VSG.

Although, cholestanol, the amino-sterols and β-sitosterol can interact strongly with VSG even at an initial pressure of 10 mN/m, as Fig. 7.7 d shows, over 1 hour of incubation, when a concentration higher than 0.014 mN of VSG was used the equilibration time was much greater than 1 hour. Thus an initial surface pressure of 5 mN/m was considered the most suitable for studying the interaction between sterol derivatives and VSG. Although, the interaction of these sterol derivatives with VSG can be classified qualitatively by their rate of penetration by VSG, it is desirable to do this quantitatively. To this end, application of the "Capacity for Film Penetration (CFP)" originally devised and defined by Pethica (1954) was used (Section 6.3).

It is important to state the experimental conditions for the subphase of the sterol monolayers was continuously stirred throughout the experiment. The stirring caused minor fluctuations in the π values ($\pi i = 5 \pm 0.5$ mN/m) but significant variations in the average surface pressure were not observed. This observation was used as a criterion for the stability of the lipid films and to check the absence of contaminants.

The addition of VSG to sterol derivatives at this initial surface pressure led to an increase in surface pressure ($\Delta\pi$) measured at equilibrium. This $\Delta\pi$ is dependent on the concentration of VSG, and the plot of $\Delta\pi$ versus antigen concentration shows apparent saturation of the lipid films. Furthermore the plateau of these curves is different for the various sterol derivatives. Thus the amino-sterols, cholesterol

Fig. 7.8

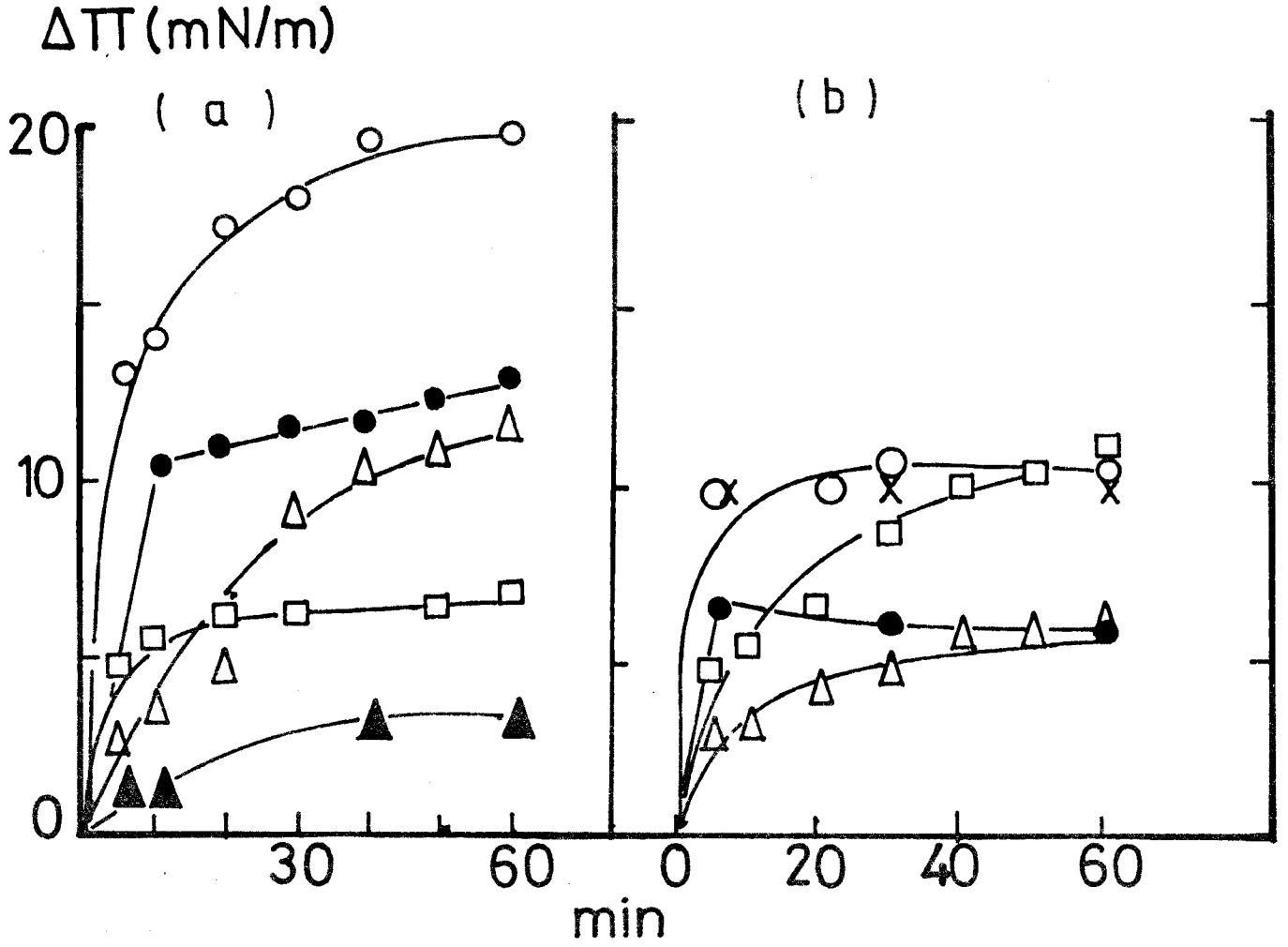
Rate of penetration of VSG for various sterol monolayers at constant surface area and at an initial surface pressure of 5 mN/m.

(a)	VSG (µg/ml)
(O) amino cholestene	1.6
(●) amino cholestene	0.6
(Δ) cholestanol	0.1
(□) acetamido cholestene	3.1
(▲) cholesteryl phosphoryl choline	3

(b)	
(O) Lanosterol	1.5
(▲) cholesteryl ammonium	0.6
(X) cholesterol acetate	1.5
(●) cholesteryl phosphate	3.1
(□) cholesteryl ammonium	1.2

Subphase pH 7.2.

Fig. 7.8



and cholestanol show higher $\Delta\pi$ values than acetamido-sterols and phospho-sterols (Fig. 7.9). The plot of the reciprocal of $\Delta\pi$ versus the reciprocal of the concentration of VSG results in a straight line with a slope which is calculated by linear regression (Fig. 7.10). The reciprocal of this slope is defined as the Capacity for Film Penetration (CFP) and permits a quantitative estimate of the interaction of VSG with lipid films. Table 7.2 shows the values of CFP for the penetration of each sterol derivative by VSG. According to these values the interaction of VSG with sterol derivatives follows a general order:

amino sterols = cholestanol > cholesterol = β-sitosterol > acetamido sterols = cholesterol acetate > cholesteryl phosphate.

7.4 Polyene antibiotics

The polyene antibiotics, filipin and amphotericin B, were used as putative competitors in the binding of cholesterol by VSG (Fig. 7.11). These compounds have been found to be highly specific for sterols with a 3-β-hydroxyl group, planar steroid nucleus and apolar chain at position C-17 (Readio & Bittman, 1982; Blau et al., 1982). Furthermore, they are able to interact with sterols present in biological membranes forming polyene-sterol complexes leading to the disruption of membrane function and ultimately to leakage of cellular constituents (Norman et al., 1976). Comparing the effect of filipin and amphotericin B on cholesterol- and ergosterol-containing membranes by measuring the permeability of intracellular markers, i.e. potassium, relative binding of filipin and amphotericin B, or by inducing polyene sensitivity in cells, led to the conclusion that amphotericin B binds more tightly to ergosterol and filipin to cholesterol than when the order is interchanged (Bittman, 1974; Gale, 1974; Schiffman & Klein, 1977; Readio &

Fig. 7.9

Increasing the concentration of VSG in the monolayer subphase raises the surface pressure until an apparent saturation occurs.

(□) amino cholestene

(●) cholestanol

(x) cholesterol

(Δ) cholesteryl phosphate

(▲) acetamido cholestene

Initial surface pressure 5 mN/m, subphase pH 7.2.

Fig. 7.10

The plot of the reciprocal of $\Delta\pi$ versus the reciprocal of the concentration of VSG results in a straight line.

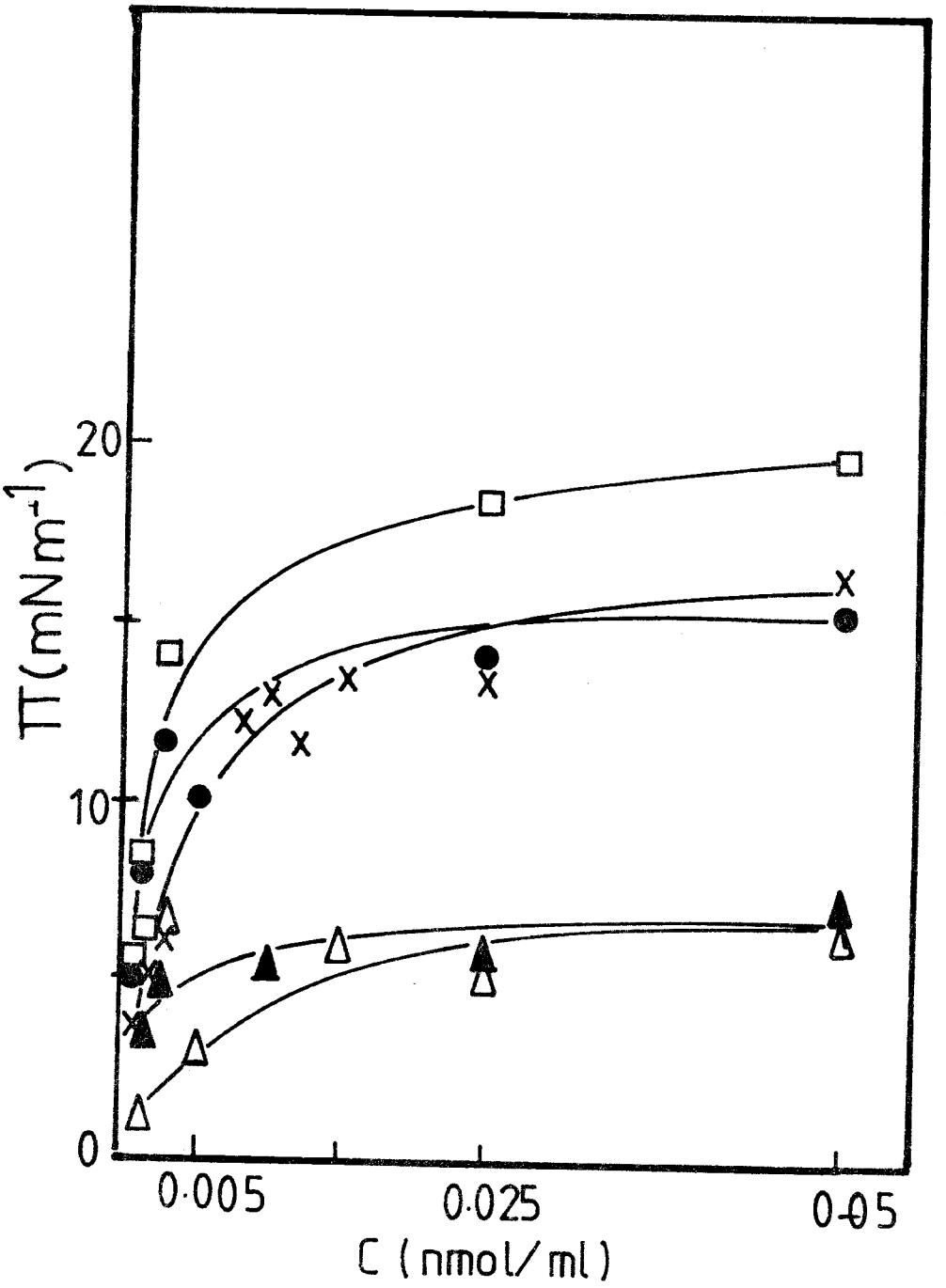
(■) amino cholestane

(X) cholesterol

(□) β-sitosterol

(Δ) acetamido cholestane

Fig. 7.9



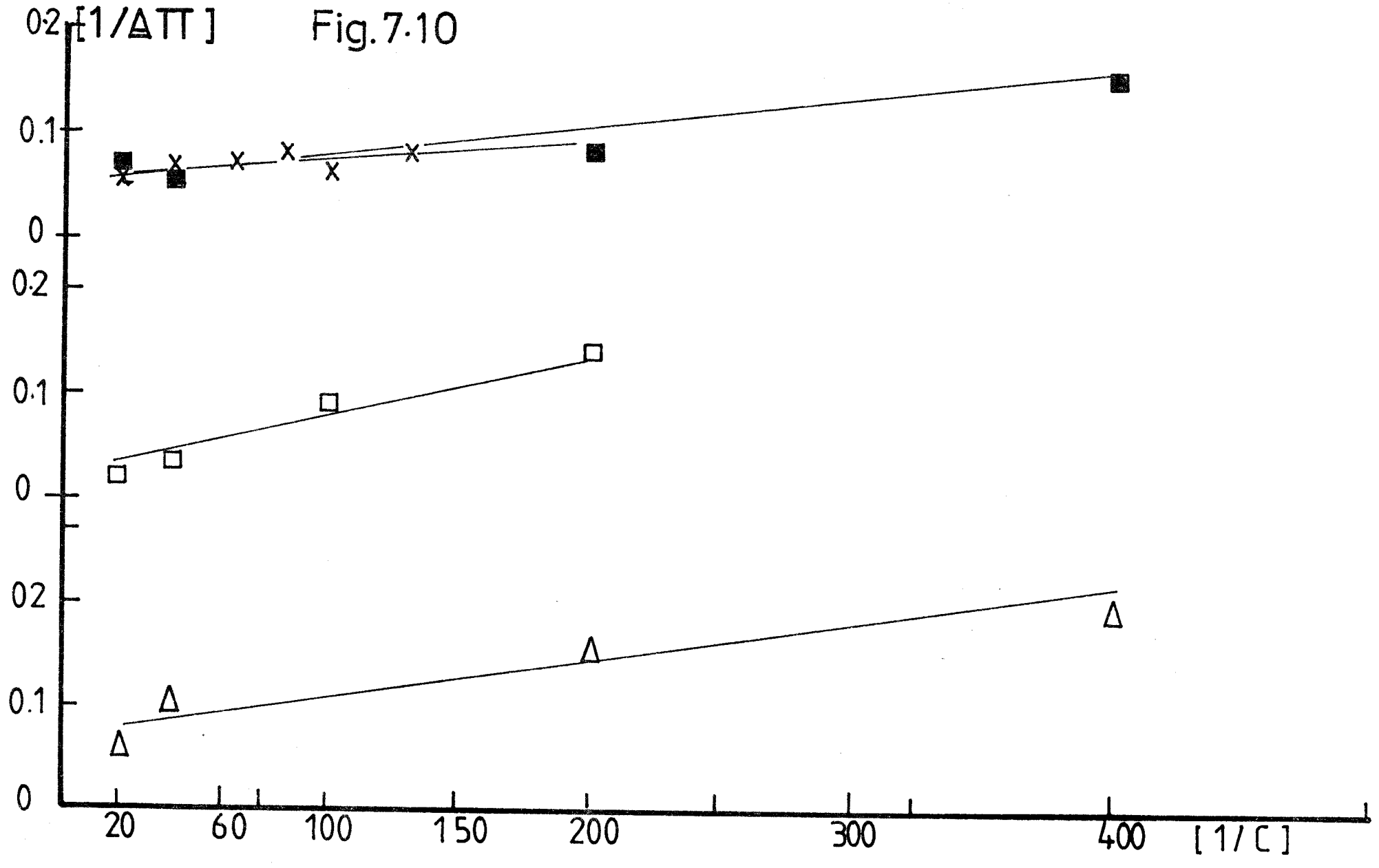


Fig. 7·10

Table 7.2 Capacity for film penetration (CFP) by VSG_{221} in monolayers made with derivatives of cholesterol

Derivative	CFP:mNm^{-1} $mmol^{-1}l$
Cholesterol	5542 ± 170
Aminocholestene	18854 ± 38
Acetamido-cholestene	2882 ± 28
Cholesteryl phosphate	1038 ± 103
Cholesteryl acetate	2094 ± 36
Cholestanol	18542 ± 88
Aminocholestane	19932 ± 62
Acetamido-cholestane	3227 ± 58
β-sitosterol	4841 ± 41

The capacity for film penetration (CFP) was estimated as the reciproca of the slope for the straight line plot of $1/\Delta\pi$ versus $1/C$ according to Camejo (1968) and Pethica (1953).

Bittman, 1982).

The mode of action of polyene antibiotics differs from that of digitonin in that they do not remove sterols from the plasma membrane but form polyene-sterol complexes visualized as 'pits' in sterol-containing membranes (Lampen et al., 1962; De Kruiff & Demel, 1974).

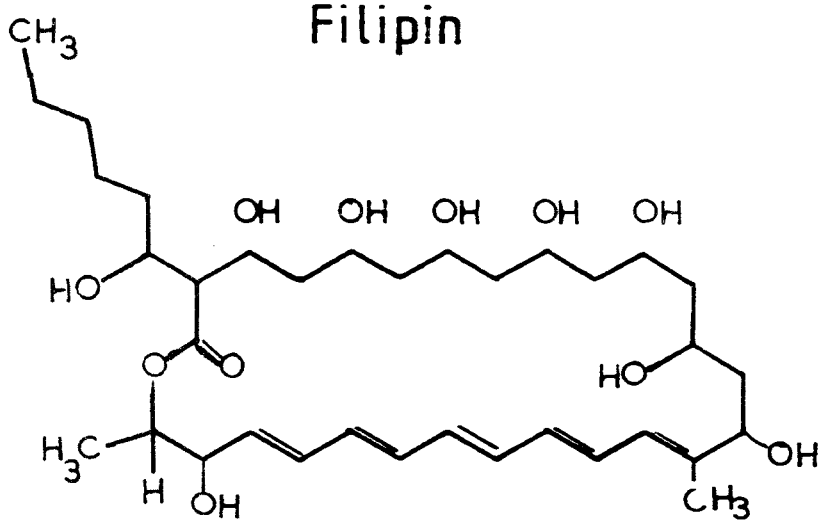
7.4.1 Amphotericin B

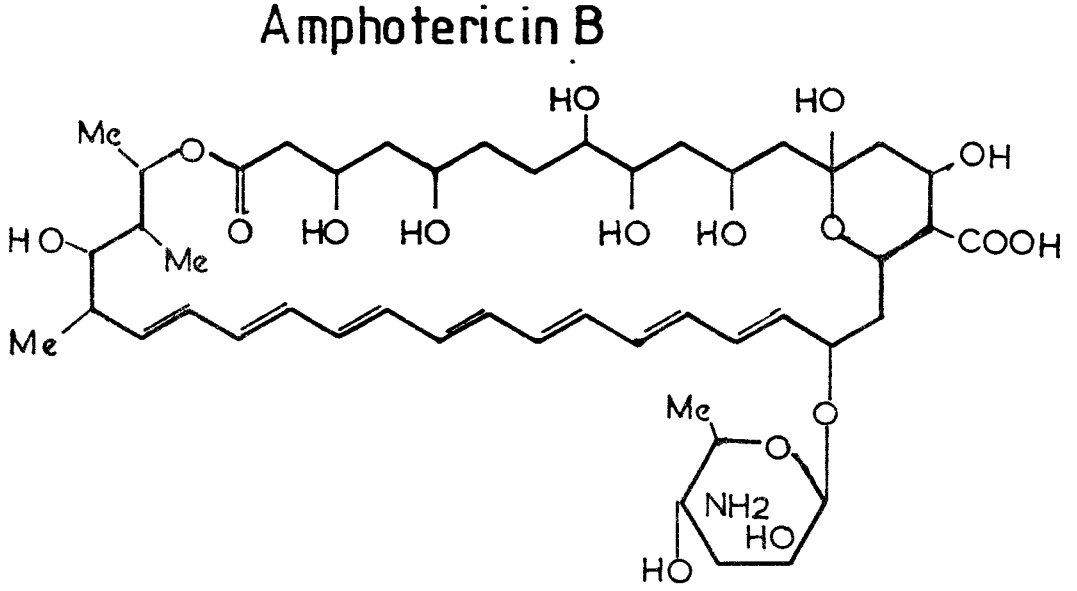
Amphotericin B added, at a mol ratio of filipin to ergosterol of 5:1, to an equimolar lipid film of EYPC: ergosterol led to a significant displacement of the force area curve (Fig. 7.12). Surface potential values determined at areas per molecule larger than 60Å^2 were found to be significantly higher for lipids alone than in presence of amphotericin B.

The addition of VSG to films containing EYPC-ergosterol-amphotericin B induced a displacement of the π-A curves. Interchanging the order of addition of VSG and amphotericin B to the mixed lipid film resulted in similar displacements. It can be assumed therefore that the simultaneous adsorption of amphotericin B and VSG do not disrupt the lipid monolayer. The magnitude of the $\Delta\pi$ values determined from the force-area curves, however, are different according to the order of addition to the mixed lipid film already containing one of them (Fig. 7.12d). Nevertheless the inflexion point ($\Delta\pi_{critical}$) of the curve of $\Delta\pi$ versus π lipid was found to be similar (18 ± 1 mN/m) for the lipid film containing both amphotericin B and VSG and was independent of the order of addition. Since $\Delta\pi_{critical}$ for the lipid film containing VSG (18 ± 1 mN/m) is very close to that containing amphotericin B (17 ± 1 mN/m), then both VSG and amphotericin B are probably expelled simultaneously from the monolayer. This latter assumption is supported by the fact that radioactive cholesterol is not removed when either amphotericin B or VSG is

Fig 7.11

Structure of Filipin and Amphotericin B.





added separately or when both are added together to the lipid monolayer (Fig. 7.13). Furthermore, VSG and amphotericin B added to a film of SPH:PE:cholesterol (1:1:2, mol ratio) at a mol ratio of VSG to cholesterol 1:4 and amphotericin B to cholesterol 2:1, showed values of $\Delta\pi_{critical}$ as indicated above. The substitution of ergosterol for cholesterol produced no apparent modification on the interaction of VSG and amphotericin B with the lipid monolayer.

The rate of penetration of lipid monolayers by VSG, with molecular composition as above including amphotericin B, at an initial surface pressure of 5 ± 1 mN/m, yielded similar values (4 ± 0.5 mN/m) to that measured for the lipid film without amphotericin B (Fig. 7.12a). No further conclusions can, however, be drawn from the latter result since the rate of penetration of phospholipids without cholesterol or ergosterol by VSG gave similar values (see Section 6.3).

7.4.2 Filipin

Filipin was added to a lipid film at 1-2 mN/m and after 30 min the π-A isotherm was determined. In contrast to amphotericin B, less filipin was necessary to produce a significant displacement of the π-A isotherm. Fig. 7.14a shows the effect of adding filipin for an equimolar ratio of EYPC:cholesterol:filipin.

The presence of VSG in equimolar ratio of EYPC and cholesterol does not inhibit the displacement of the π-A isotherm by filipin (Fig. 7.14b). The addition of such a large amount of VSG to the lipid film produced very large displacements of the π-A isotherm. Thus the $\Delta\pi$ was measured as the difference between the values of surface pressure after adding the last component minus the value for the previous π-A isotherm (inset of Fig. 7.15). If cholesterol is omitted in the lipid film the

Fig. 7.12

(a) Amphotericin B (Amph-B) was injected beneath a pre-formed monolayer of ergosterol:phosphatidyl choline (6:1:1 mol ratio). The monolayer was equilibrated for 1 hr before determining the (Δ) force-area curve. Then 3 nmol of VSG was injected and after 1 hr the (□) force area curve determined.

(b) As in (a), except that the order of addition was reversed.

(c) Δπ was determined from the force-area curves. Critical surface pressure is determined from the plot of Δπ versus lipid surface pressure.

VSG or AMPH-B were first added according to the following order:

(■) ERG/EYPC/VSG/AMPH-B

(□) ERG/EYPC/AMPH-B

(▲) ERG/EYPC/VSG

(Δ) ERG/EYPC/AMPH-B/VSG

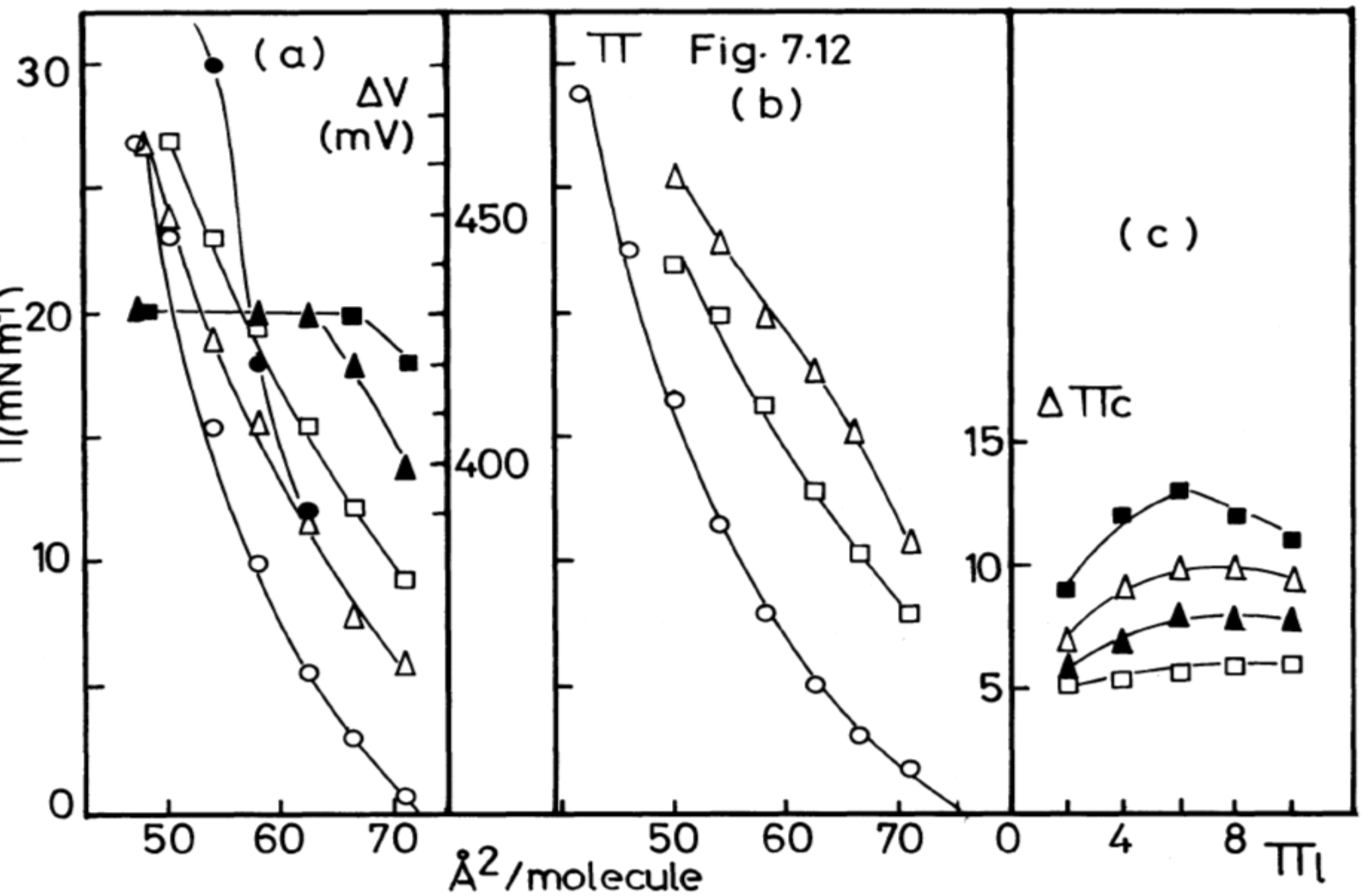


Fig. 7.12
(a)
ΔV
(mV)
450
400
(b)
Π
(c)
ΔΠc
30
20
10
0
15
10
5
50
60
70
0
4
8
Π(mNm⁻¹)
Å²/molecule
Πl

Fig. 7.13

The lipid monolayer composed of phosphatidyl choline: cholesterol:[^{14}C]cholesterol 1:1:1 mol ratio was spread at the air water interface. VSG and filipin were injected under this lipid monolayer and surface pressure and surface radioactivity determined:

(○) EYPC:[^{14}C]cholesterol

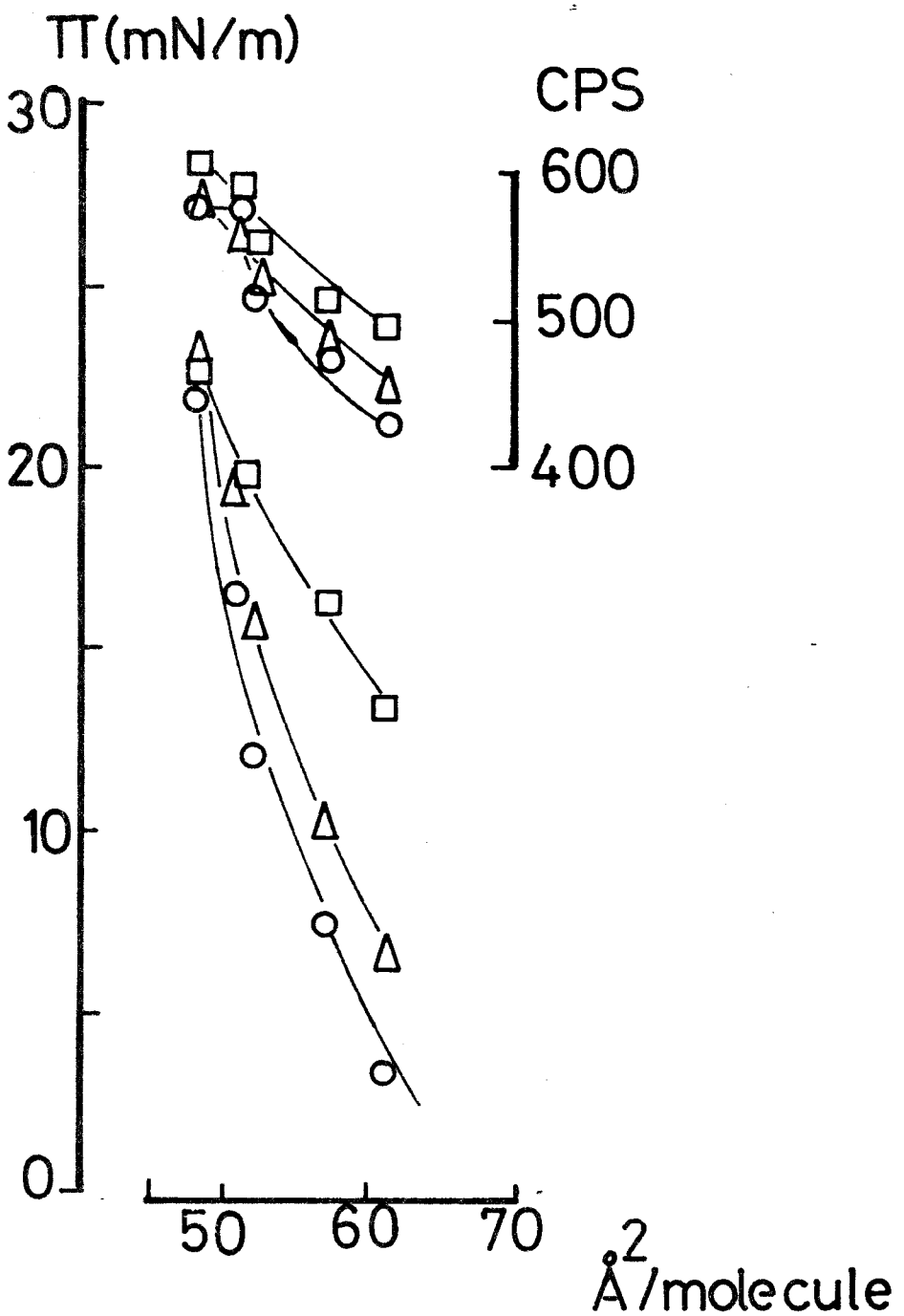
(△) 3.3 nmol of VSG

(□) 10 nmol of filipin

Other conditions as in Fig. 7.14.

Subphase pH 7.2.

Fig. 7.13



π-A isotherm is slightly displaced when filipin is added and this leads to reduction in $\Delta\pi$ values (Fig. 7.15).

Although these results suggest that VSG reduces the interaction of filipin with cholesterol, the presence of phospholipid may further decrease that interaction. In fact when the mole ratio of phospholipid to cholesterol is higher than to one penetration of the film by filipin is reduced (Norman, 1976). Thereafter, monolayers of cholesterol were used in studying the interaction with filipin and VSG. Fig. 7.16 shows the π-A isotherms corresponding to the addition of filipin or VSG to monolayers of cholesterol-VSG or cholesterol-filipin respectively.

It is not possible to measure the respective $\Delta\pi$ from these π-A isotherms because of the shape of the curves. Thus it seemed more appropriate to measure the relative increase in area (RIA). RIA is the relative increase in area per molecule of cholesterol as the result of adding another surface component at the same surface pressure (see Section 5.4.4). The results of RIA for filipin added to a pre-formed cholesterol-VSG monolayer and for VSG added to a filipin-cholesterol film were too variable to discern any competition between VSG and filipin for cholesterol.

The addition of filipin to cholesterol monolayers reduces the surface potential to values of 280 ± 40 mV. This effect could be explained assuming that filipin interacts with the hydroxyl (-OH) group and with the sterol nucleus of cholesterol thus preventing the cholesterol molecule from normal alignment with respect to the place of the monolayer. As explained above this would diminish the value of the surface dipole moment. It is worthwhile noting that ΔV is reduced even when filipin is added to a film formed by cholesterol and VSG (Fig. 7.16).

The apparent competition between filipin and VSG for cholesterol was further investigated using the technique of film penetration. These

Fig. 7.14

Filipin or VSG was injected beneath a pre-formed monolayer of EYPC/cholesterol for an equimolar ratio. Force-area curves were determined after 1 hr. The order of addition of VSG and filipin to the lipid monolayer is as follows:

(0) cholesterol/EYPC

(□) cholesterol/EPYC/VSG

(▲) cholesterol/EYPC/filipin

(△) cholesterol/EYPC/VSG/filipin.

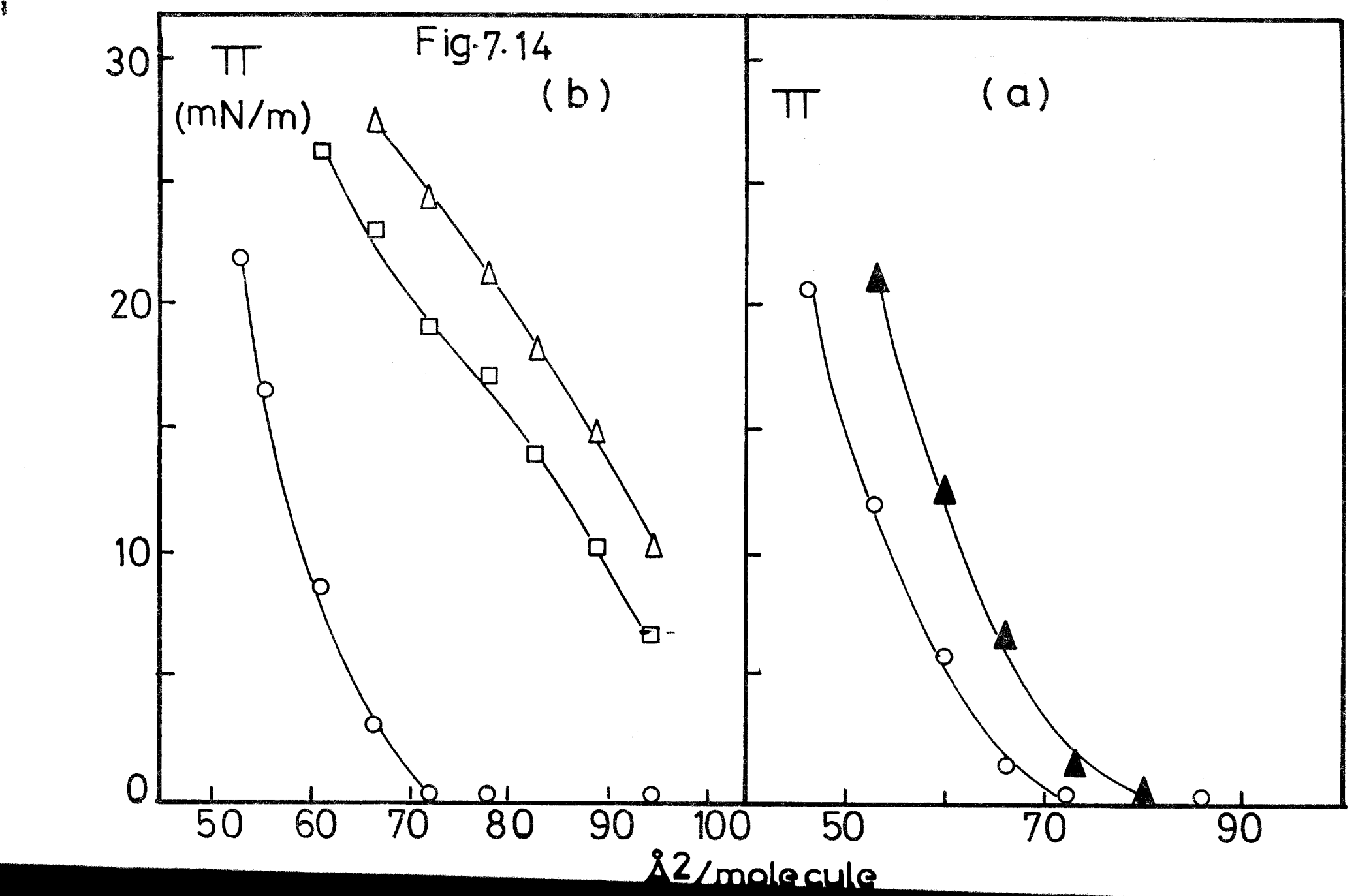

Fig·7·14
(b)
(a)
π
(mN/m)
30
20
10
0
50
60
70
80
90
100
50
70
90
Å2/molecule

Fig. 7.15

VSG was injected beneath a preformed phosphatidyl choline monolayer and after 1 hr the force-area curve was determined. Then Filipin was injected at an initial surface pressure of 2 ± 1 mN/m and the force-area curve determined after 1 hr.

(O) EYPC (4 nmol)

(Δ) EYPC plus 8 nmol of VSG

(□) After added 8 nmol of filipin

Subphase pH 7.2.

Figure inset

Filipin was added to a pre-formed lipid monolayer for a final composition:

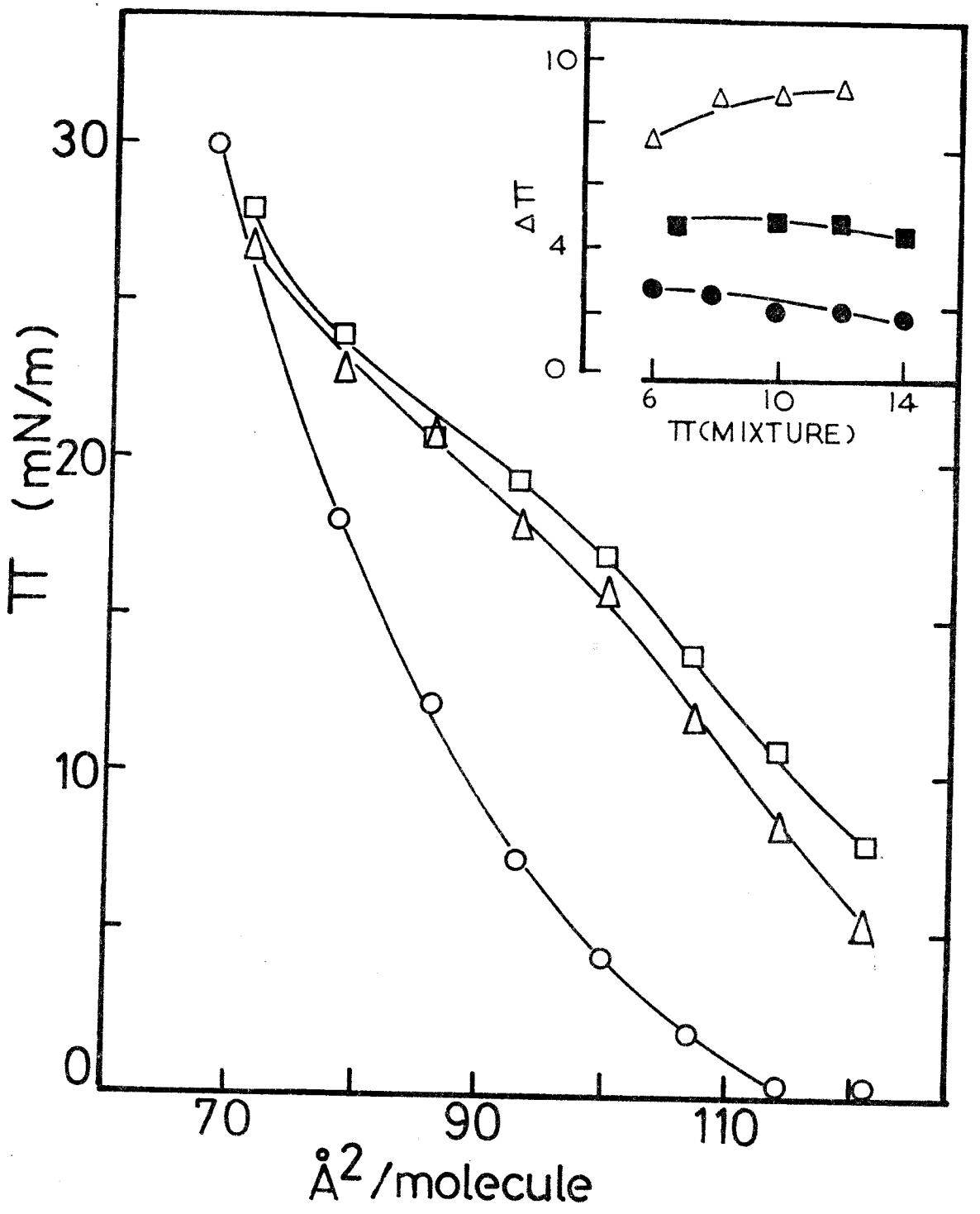
(Δ) cholesterol:EYPC:filipin 1:1:1

(■) cholesterol:EYPC:VSG:filipin 1:1:1:1

(●) EYPC:VSG:filipin 1:8:1:1

$\Delta\pi$ was determined from the force-area curves and plotted versus the surface pressure of the mixed film.

Fig. 7.15



experiments were aimed at comparing the adsorption of filipin and VSG to a cholesterol monolayer, in particular under conditions in which these compounds have to surmount an energy barrier (i.e. high surface pressure) to penetrate the monolayer.

Briefly, cholesterol was spread at the air-water interface and the surface pressure adjusted to 14 $\pm$ 1 mN/m. The subphase at pH 7.2 was continuously stirred during the experiment.

Figure 7.17 shows the rate of penetration by filipin or VSG injected separately beneath a film of cholesterol; and the rate when both filipin and VSG were added together varying the order of addition. Injecting filipin into the cholesterol film at a mol ratio of 1:2 produced a rapid increase in surface pressure which reached equilibrium in approximately 40 minutes. The value of 39 mN/m obtained at equilibrium approaches the average value of 42.6 mN/m which is the collapse pressure for cholesterol (Demel et al., 1968; Muller & Cadenhead, 1979; Cadenhead et al., 1982). The effect is interpreted as a strong interaction between filipin and cholesterol since the collapse pressure for filipin monolayers have an average value of 15 mN/m (Demel et al., 1968; Norman et al., 1976).

The rate of penetration of a cholesterol film by VSG (mol ratio of VSG to cholesterol 1:10) shows large values over the first 30 minutes after which these values become less pronounced and the plot $\Delta\pi$ versus time tends towards an asymptote.

The same Fig. 7.17 shows the rate of penetration of a cholesterol-filipin film and a cholesterol-VSG film by VSG and filipin respectively at the initial surface pressure as above. The values of the rate of penetration of VSG is reduced to approximately 40% of the control value. Furthermore when these pre-formed cholesterol-filipin films were prepared at an equimolar ratio the rate of penetration was reduced

by 90%. Even when the initial surface pressure was reduced to 8 ± 1 mN/m, the $\Delta\pi$ value at 60 minutes was only 2 ± 1 mN/m. Thus the penetration values of $\Delta\pi$ higher than 5 mN/m for a filipin:cholesterol mol ratio of 2:1 can be interpreted as resulting from penetration of the available cholesterol molecules by VSG.

The stoichiometry of filipin:cholesterol has been determined as 1:1 from fluorescence measurements and from differential scanning calorimetry (Norman et al., 1976; Schroeder et al., 1973). The rate of penetration of pre-formed VSG-cholesterol films by filipin follows a rather different profile in comparison with those described above. The high values of increasing pressure for the first 10 minutes are a clear indication of the accessibility of cholesterol to filipin regardless of the presence of VSG. After 10 minutes the rate of penetration starts decreasing and the $\Delta\pi$ values are reduced continuously during the next 60 minutes. This may indicate that disruption of the monolayer has occurred. Hence the possibility that VSG is being expelled from the monolayer, and therefore disrupting the film, is considered reasonable because the higher surface pressure reached after adding filipin exceeds the critical surface pressure for the cholesterol-VSG film (Section 5.4.5).

7.5 Studies of protein-cholesterol binding by the method of gel filtration

Hummel & Dreyer (1962) described a quantitative method using gel filtration for the study of the binding of cytidine 2'-phosphate by ribonuclease. This method is analogous in principle to equilibrium dialysis and is briefly as follows. A column of Sephadex is equilibrated with a solution containing a substance of low molecular weight (A Then a sample of this solution containing a protein (P) is added to this

Fig. 7.16

Interaction of VSG and filipin with cholesterol monolayers. Force-area curves were determined after 1 hr.

(a) cholesterol (20 nmol): (O) surface pressure and (●) surface potential. 2.4 nmol of VSG added to the cholesterol film: (Δ) surface pressure and (▲) surface potential. 10 nmol of filipin added to the cholesterol-VSG film: (□) surface pressure and (■) surface potential.

(b) as in (a) except that the order of addition of filipin and VSG was reversed.

Subphase pH 7.2.

Fig. 7.16

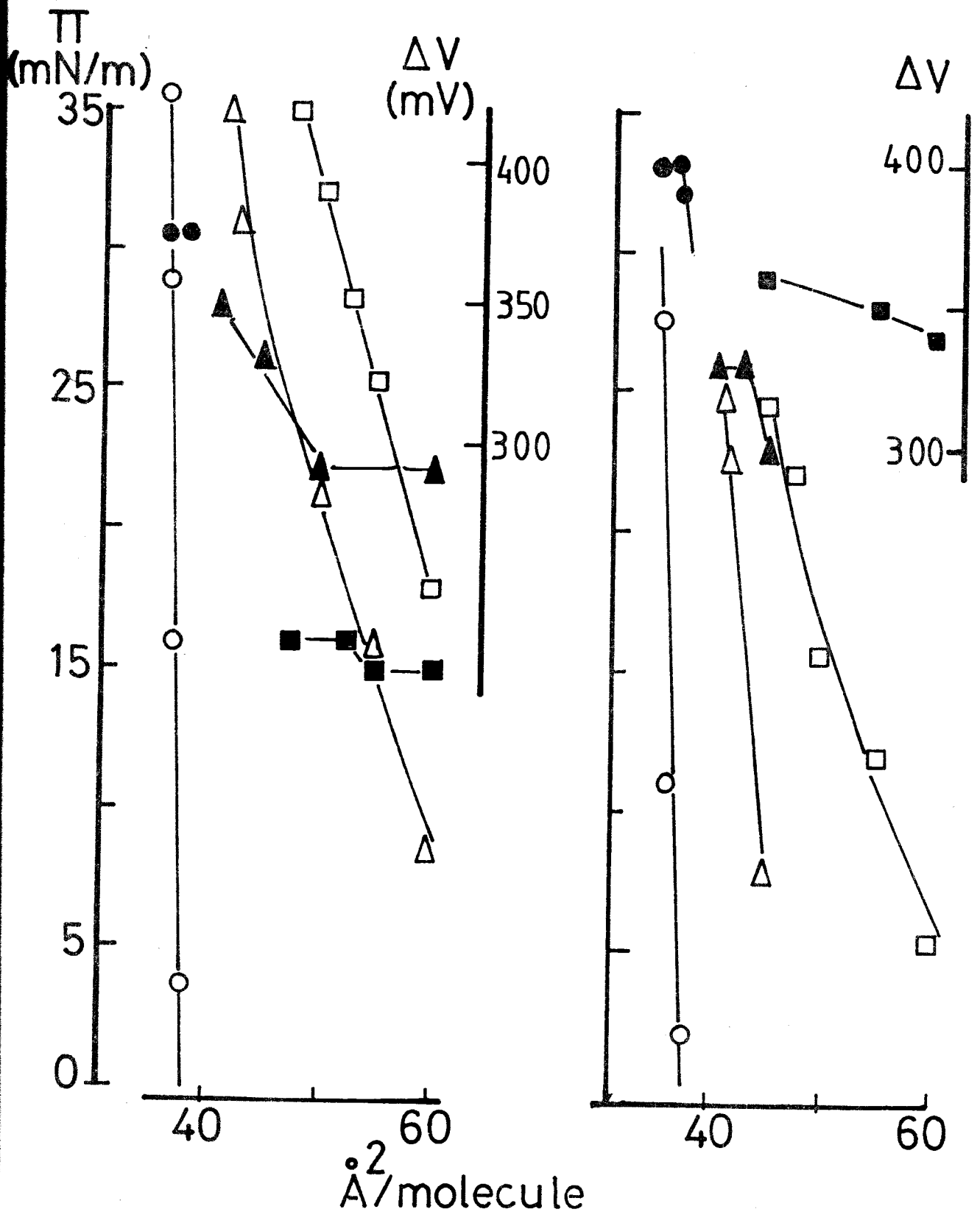

π
(mN/m)
35
25
15
5
0
ΔV
(mV)
400
350
300
ΔV
400
300
40
60
40
60
Å²/molecule

Fig. 7.17

Cholesterol-VSG monolayers or cholesterol-filipin monolayers were formed at the air-water interface. Then VSG or filipin was injected beneath the appropriate mixed film at an initial surface pressure of 14 ± 1 mNm and the rate of penetration measured at constant surface area.

(O) cholesterol:VSG:Filipin 20:2.4:10 mol ratio

(Δ) cholesterol:VSG 10:1 mol ratio

(●) cholesterol:filipin 20:10 mol ratio

(□) cholesterol:filipin:VSG 20:10:2.4 mol ratio

Subphase pH 7.2.

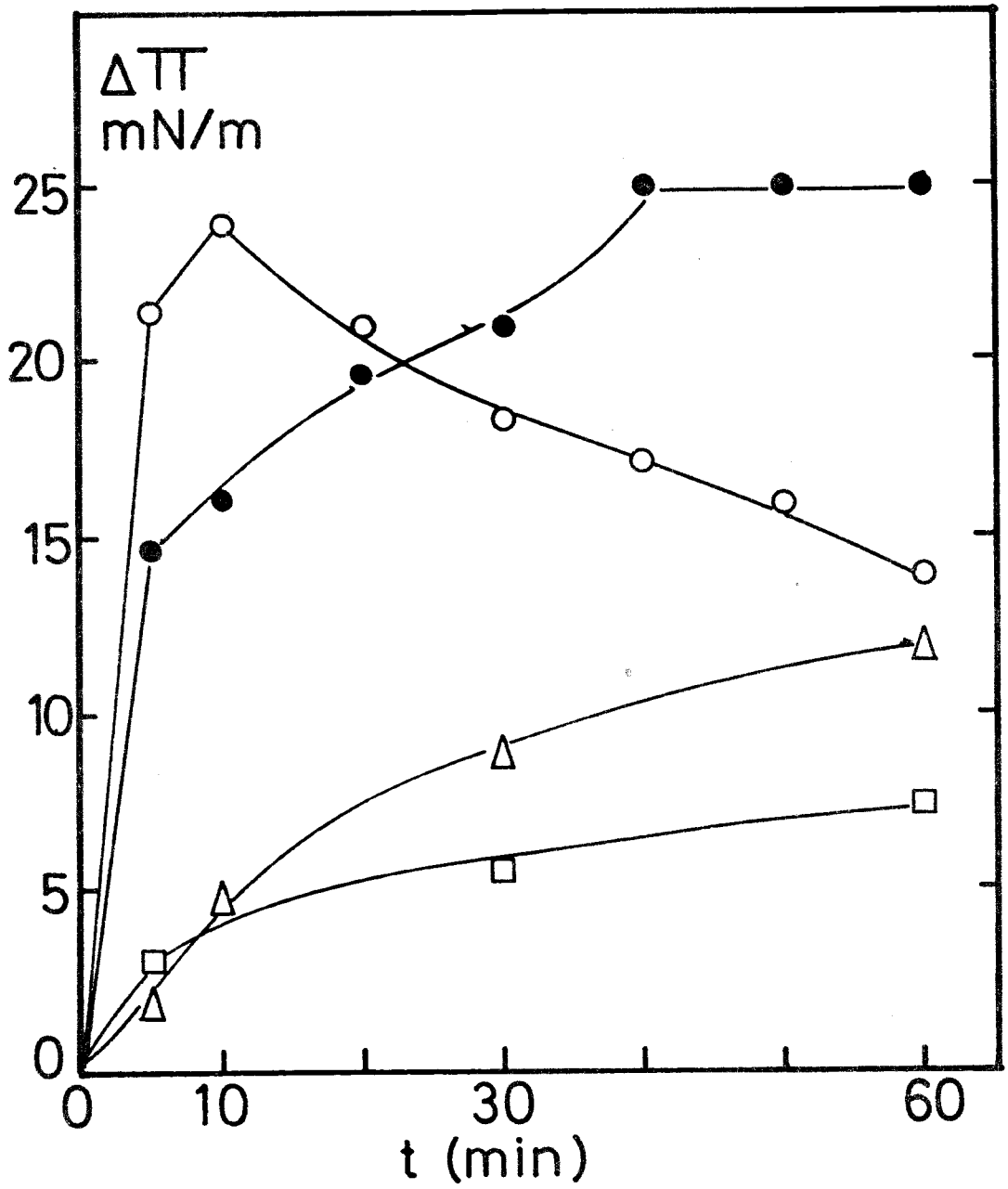


Fig. 7.17

column. The protein (P) is known to bind reversibly to molecules of the substance (A). The column is thereafter eluted with a solution containing the substance A; the concentration of A is measured in fractions of the eluant solution. The peak corresponding to the P-A complex emerges at the excluded volume of the column and the concentration of A rises above the equilibrium level (the base line). Then at some point after the protein-peak the concentration of A in the eluat is decreased below the base line level forming a trough. The amount of free A removed from the solution, as indicated by this trough is equal to the excess of A found in the protein peak. The formation of this trough is also a criterion of equilibrium of the substance A in the chromatographic column.

This method was adopted in these studies. The protein is the antigen, VSG, and the substance of low molecular weight is cholesterol. The antigen is excluded in the void volume of the various Sephadex type columns (Section 4.11) whereas, cholesterol is retarded. Cholesterol is a compound poorly soluble in water with a critical micelle concentration of 20-30 mN (Haberland & Reynolds, 1973). Use of radiolabelled [^{3}H]cholesterol, however, makes detection and estimation of its concentration easy. Details of the procedure for equilibration of the Sephadex column with the radioactive buffer (RB) and the collection of fractions have been given (Section 4.11). The sample volume containing the antigenic glycoprotein was applied after a stable baseline had been obtained. The high radioactive zone was located in the void volume of the column (Fig. 7.18a). The presence of the antigen in this peak was verified by continuous monitoring at 280 nm or when very low concentrations of VSG were applied, samples from each peak fraction were tested with specific antibody using the immunodiffusion method

(Section 4.5). A zone of decreased radioactivity was not however, observed in these experiments. This indicates that cholesterol is not in a proper equilibrium with the column matrix or that the cholesterol solution is a mixture of monomers and aggregates of cholesterol. Increasing the concentrations of VSG up to 200 µg did not enhance the binding of labelled cholesterol. The ratio of cholesterol to VSG in the protein peak however, was affected with this latter condition. Thus, it was found to contain about 4 times less cholesterol per mol of VSG than for quantities of VSG higher than 100 µg was added. Then for 200 µg VSG a saturation of the system occurs (Fig. 7.18). On the other hand increasing the radioactive concentration up to 3 nM and use of VSG under 100 µg (1.6 nmol) showed no significant alteration in the ratio of cholesterol to VSG as shown below.

In section 6.1.3 it has been shown that mixed films containing cholesterol and VSG are more stable at pH values below 6. Thus, the estimation of binding of cholesterol to VSG was carried out at pH 5.6 and pH 7.2 respectively. Representative elution diagrams at these pH values are shown in Figs. 7.18a and b.

The average value for the ratio of cholesterol to VSG contained in the protein-cholesterol peak was calculated. The cholesterol bound to VSG was estimated as follows:

$$\text{nmoles of cholesterol bound} = \frac{\sum_i (\Delta Rix\ Vi)}{118.96 \times 10^6} \qquad (7.2)$$

Ri is the difference between the radioactivity (DPM) of fraction i of the peak and that of the base line, Vi is the volume of fraction i, and 118.96×10^6 DPM $nmol^{-1}$ is the specific activity of [^{3}H]cholesterol.

Thus the values of the mol ratio of cholesterol to VSG using the amount of cholesterol calculated with the equation (7.2) are the

Fig. 7.18

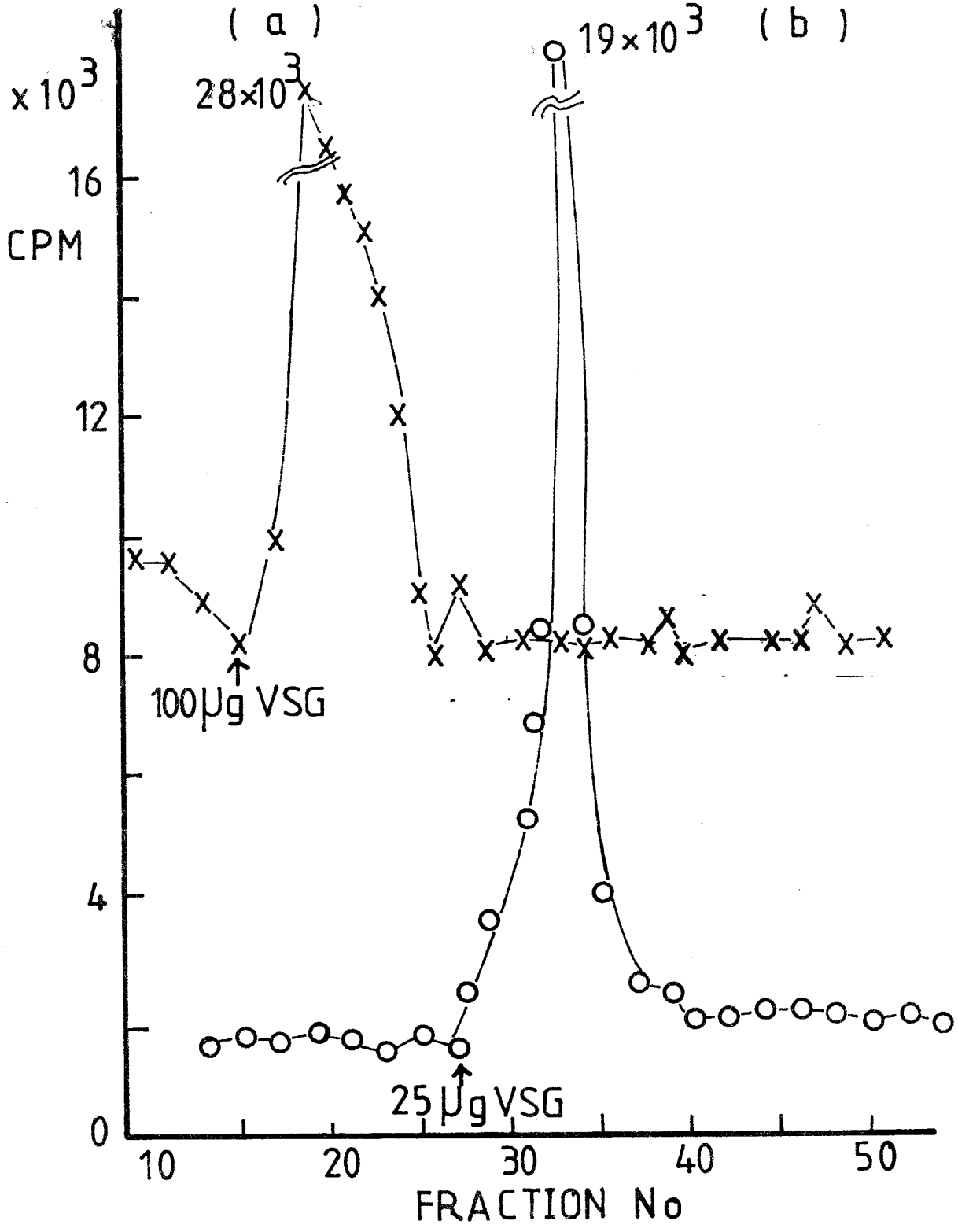
Study of the cholesterol-VSG interaction in aqueous solution. The Sephadex column was equilibrated with a radioactive buffer of [^{3}H]cholesterol (specific activity 54 C/mmol) and after established the base line VSG was added. The position where VSG was added is indicated in the graph.

(a) 25 µg of VSG was added and 0.2 ml fractions were collected for counting of radioactivity. Details are shown in the text and in Section 4.11.

(b) As in (a), except that 100 µg of VSG_{221} was used.

Buffer: 10 mµ Tris-HCl, 145 mM KCl, 0.02% azide, pH 7.2.

Fig. 7.18


(a)
(b)
x 10 3
28×10 3
19×10 3
16
CPM
12
8
4
0
100µg VSG
25µg VSG
10
20
30
40
50
FRACTION No

following:

pH	number of experiments	Cholesterol:VSG
5.6	3	1:95 ± (34)
7.2	4	1:103 ± (27)

The value in bracket is the root mean square deviation.

The ratio of cholesterol to VSG determined by this method was not affected by the pH of the solution. The ratio of cholesterol bound per molecule of VSG then presents a weak interaction; however, a definite conclusion cannot be given with these results alone. The significance to be placed on this data will be examined in the discussion of this Chapter.

7.6 Interaction of antigen with lipid bilayers

This study was aimed at determining whether VSG could adsorb to liposomes when both are mixed together in an aqueous solution. The self-association process of peptides or proteins into higher structures is driven by hydrophobic, electrostatic and hydrogen bonding among the amino acid residues in them (Frieden, 1971). For example, in the specific interaction of peptides with proteins at least four types of bonding may be envisaged: (1) electrostatic interaction between oppositely charged ions; (2) interaction between apolar side-chain groups; (3) hydrogen bonding either involving side-chain groups such as those of tyrosine and glutamic acid (Fairclough & Fruton, 1966).

There is evidence to support the view that the same specific mechanisms are involved in the readsorption of extrinsic proteins on the plasma membrane (Kagawa, 1974). Reconstitution of membrane proteins into liposomes using a variety of procedures has been reported (Rosseneu, 1977; Eytan, 1982). These can be grouped into the following three main categories.

(i) Detergent solubilization. The isolated membrane proteins are dissolved together with phospholipids in cholate and subsequently the detergent is removed by dialysis overnight (Kagawa, 1973). After removal of the detergent closed proteo-liposomes are formed.

(ii) Sonication. A lipid dispersion and the protein mixture are ultra-sonically irradiated to form the proteo-liposome complexes. Although this procedure is suitable for the reconstitution of some proteins, it is detrimental to most others (Scanu, 1966; Carroll & Racker, 1977). Brief sonication, however, can be made efficient if liposomes are initially formed by sonic irradiation of lipids alone, the protein subsequently added and the protein-liposome mixture further sonicated (Rosseneu, 1977).

(iii) Direct incorporation. Membrane proteins are added to liposomes already formed by sonic irradiation and incubated while being mechanically shaken. The hydrophobic moiety of amphipatic proteins, such as cytochrome b_5, is easily inserted into liposomes using this method (Enoch, 1977).

The procedure adopted in these studies was that of direct incorporation. Since this study was aimed at investigating whether VSG could bind to liposomes by specific lipid affinity rather than inducing it by detergents or ultrasonic irradiation, these methods were not used.

The details regarding unilamellar liposome preparation, liposome-VSG incubation, centrifugation in a gradient of sucrose (16% to 40%) and collection of the samples are given in Section 4.14. Fig. 7.19 shows the values of absorbance (280 nm) and gradient density plotted against the number of fractions collected. Gradient density was calculated after measuring the refractive index of each fraction. VSG alone run in this sucrose gradient shows a peak at approximately 28% of sucrose (w/v) in comparison to 26 ± 1% when VSG was previously

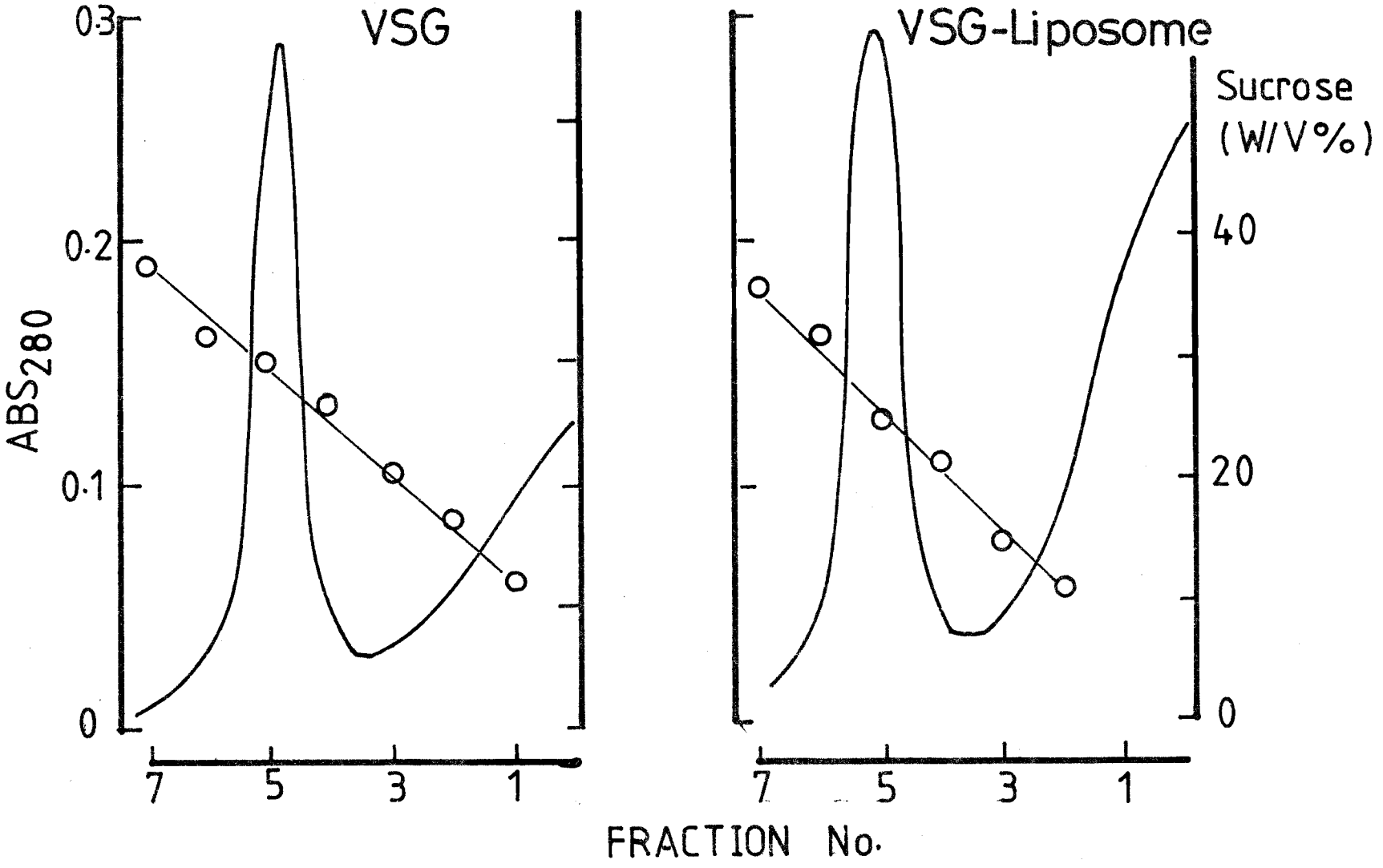
Fig. 7.19

Studies of liposome-VSG interaction using 16% to 40% sucrose gradients centrifuged at 170,000 x g_{av} for 20 hrs at 5°C.

(a) VSG (0.5 mg) run alone in the sucrose gradient.

(b) VSG-liposomes run in a similar sucrose gradient at the same time as (a).

Fig. 7.19


VSG
VSG-Liposome
0.3
0.2
0.1
0
ABS_{280}
Sucrose
(W/V%)
40
20
0
7
5
3
1
FRACTION No.

incubated with phospholipid liposomes. Similar results are obtained for a phospholipid:cholesterol mol ratio of 1:4:1 and 1:1. The absorbance of this peak is not affected by incubation with liposomes. Therefore, it is inferred that only very small amounts of VSG are retained in the supernatant fractions 1 and 2 where most of the liposomes can be located (Morriset et al., 1973).

Thus it seems that there is no reconstitution of VSG with these lipids or that interaction is weak and may be disturbed by the high concentration of sucrose (Scanu & Granda, 1966). Quantification of the protein and lipid contents at this stage were not attempted.

7.7 Effects of VSG on the physical properties of lipid mixtures

7.7.1 The model of lipid-protein interaction at the temperature of phase transition

Studies on the mechanism of the interaction of proteins and lipids have shown that the temperature of the phase transition for mixture of lipids is of importance for the recombination of the protein and lipid (Pownall et al., 1978). The possible significance of phase boundary in mediating the penetration of molecules through the lipid membrane arose from experiments of passive transport of sodium (Papahadjopoulos et al., 1973). A maximum of transport of Na^+ was observed around the phase transition temperature of phospholipid vesicles, where the boundaries between gel and liquid crystalline phases predominate (Kanehisa & Tsong, 1978). In this phase transition interval phospholipid molecules are found in clusters which undergo rapid fluctuations between solid and fluid phases (Freire & Biltonen, 1978). Swaney (1980) have proposed a model where the insertion of proteins into phospholipid bilayer occur at pocket excess volume which accompany equilibrium fluctuation of phase states.

The model system which has contributed most to these studies is the recombination of apoprotein A-I (apo A-I), the major protein constituent of human high-density lipoprotein, with pure phospholipids and with binary mixtures. The finding that the high rate of association of apo A-I with DMPC occurred only in the vicinity of the gel to liquid-crystalline transition of this phospholipid (24^{o}C), suggested that protein insertion is facilitated by volume changes which occur at the melting temperature (Pownall et al., 1978). The reactivity of apo A-I with phospholipids decreases as the length of the acyl chains increases (Swaney & Chang, 1980; Pownall et al., 1978). Thus the recombination of apo A-I with DPPC is very poor even at the transition temperature; however, binary mixtures of DPPC and DMPC result again in a high recombination at the transition temperature. Furthermore, as the mole fraction of DPPC in the binary mixture increases the rate of recombination with apo A-I decreases. Similarly apo A-I shows a poor recombination with DSPC unless DMPC is incorporated in the lipid dispersion.

The temperature-programming technique has been the method most used in these studies. The process of recombination between protein and lipid dispersions can be followed by measuring the clearance of turbidity as the temperature in increased. The clearing of turbidity is due to the formation of small protein-lipid complexes (Trauble et al., 1974; Behof et al., 1978). The reliability of the method above has been demonstrated by correlating the transition temperature from heating curves for the mixture of DMPC and DPPC with phase diagram data derived from the calorimetric data (Swaney & Chang, 1980).

7.7.2 Studies of recombination of phospholipid mixtures with VSG

The studies described in section 7.7.1 were adopted and applied to the system of VSG and phospholipid mixtures, and phospholipid-cholesterol

mixtures (for description of the method see Section 4.13). Figure 7.20 shows the curves of absorbance (325 nm) of a binary mixture of DSPC/DMPC (0.28 mol fraction DSPC) when the temperature is linearly increased at a rate of $0.5^{o}C\ min^{-1}$. The transition temperature calculated from this graph is $26.8^{o}C$, a value very close ro that determined by Swaney (1980). The same figure shows the curve obtained when VSG is added to this binary mixture. The mol ratio of phospholipid to VSG was 57:1 and the temperature and absorbance of the samples were continuously monitored throughout the entire heating programme. It can be concluded from these results that VSG failed to recombine with the mixture of DSPC/DMPC when when the VSG to phospholipid concentration was increased to a mol ratio of 1:8 and 1:30.

The proportion of DSPC to DMPC was reduced to 0.1 mol fraction DSPC and its phase transition temperature obtained from the plot of absorbance versus temperature was $25.6 \pm 0.5^{o}C$ (n = 5). Figure 7.20 shows the curves for the mixture of phospholipids alone and in the presence of VSG; the inflexion point however, is slightly altered for the lipid suspension containing VSG and the new value is $28.5^{o}C$ (n = 3) instead of $25.5^{o}C$. In the absence of DSPC however, the addition of VSG to a lipid suspension of DMPC:cholesterol:VSG failed to increase the transition temperature. Increasing the mol ratio of VSG to phospholipid from 1:8 to 1:4 and 1:3.5 did not promote either clearing of turbidity or increase of the phase transition temperature (Fig. 7.21).

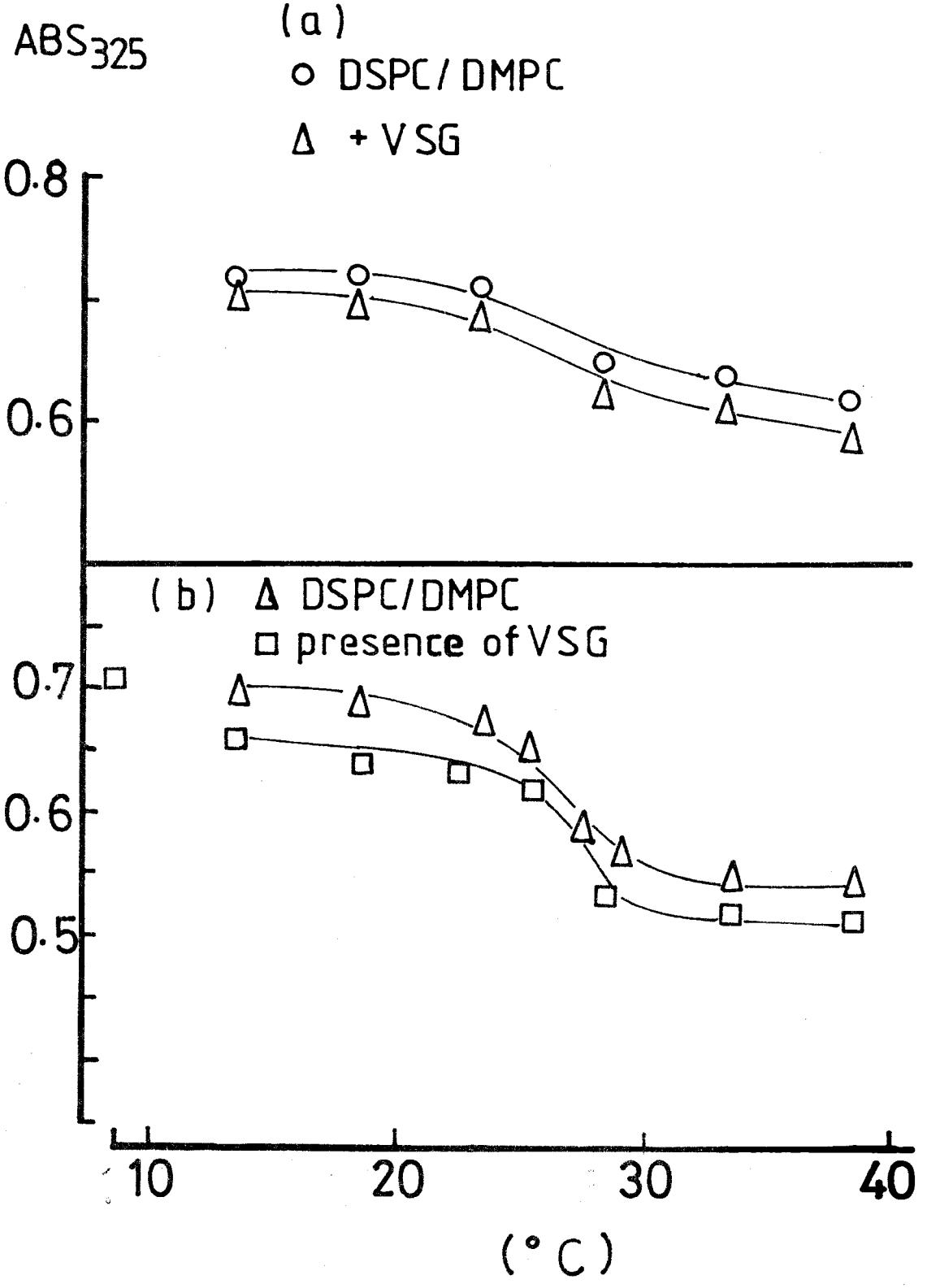
The increase of the transition temperature observed for the mixture phospholipid-cholesterol-VSG is difficult to explain from the available data. This method can only detect recombination if the interaction of the protein can disrupt the multilamellar liposomes to form small liposomes (Pownall et al., 1978). On the other hand an increase in the

Fig. 7.20

(a) Representative curves of absorbance versus temperature of a binary mixture of DSPC/DMPC, 0.28 mol fraction DSPC. The VSG/phospholipid mol ratio is 1:57. The decrease in absorbance was continuously recorded.

(b) The proportion of DSPC to DMPC was reduced to 0.1 mol fraction DSPC, and VSG was added to a 1:8 VSG:phospholipid mol ratio.

Fig. 7.20


ABS325
(a)
○ DSPC/DMPC
Δ + VSG
0.8
0.6
(b) Δ DSPC/DMPC
□ presence of VSG
0.7
0.6
0.5
10
20
30
40
(°C)

transition temperature has been observed when proteins instead of distorting the packing of the aliphatic chains contribute to stabilizing the crystallization process (Epand & Sturtevant, 1982). For example, the polypeptide hormone glucagon causes an increase in the transition temperature of DMPC to 26°C. The crystallization of phospholipids can be prolonged by multivalent cations, therefore increasing the transition temperature. For proteins which cause a similar effect it may be suggested that the interaction is restricted to the polar groups of phospholipids and the fatty acid chains are not involved (Bach & Miller, 1976). In contrast a decrease in the phase transition has been found with proteins that partially penetrate the bilayer causing a distortion of the packing of the fatty acid chains and so preventing crystallization as found with the myelin basic protein (Chapman & Urbina, 1974; Boggo & Moscarello, 1978). The effect of these proteins on the phase transition was estimated using differential scanning calorimetry. This technique applied to the VSG-liposome interaction may clarify the experiments obtained using absorption and temperature-programming. At the end of these experiments samples from the mixtures of VSG-phospholipid and VSG-phospholipid-cholesterol were centrifuged at 12000 x g_{av} for 1 minute at room temperature and washed twice with buffer pH 7.2. The pellets and supernatants were analyzed by polyacrylamide gel electrophoresis (Section 4.2.2). The pellets sample were either applied directly onto gels or were extracted with 80% acetone for 2 hours at 4°C, centrifuged at 4000 x g_{av} for 10 minutes and finally resuspended in 100 µl of buffer. Figure 7.21 shows the bands of VSG for the pellet samples and for the supernatants. Most of the antigen remains in the supernatant and very little is bound to the liposomes. Thus the presence of cholesterol seems not to make any difference to the binding of VSG. This result is confirmed by quantitative analysis using the technique of radioimmuno-

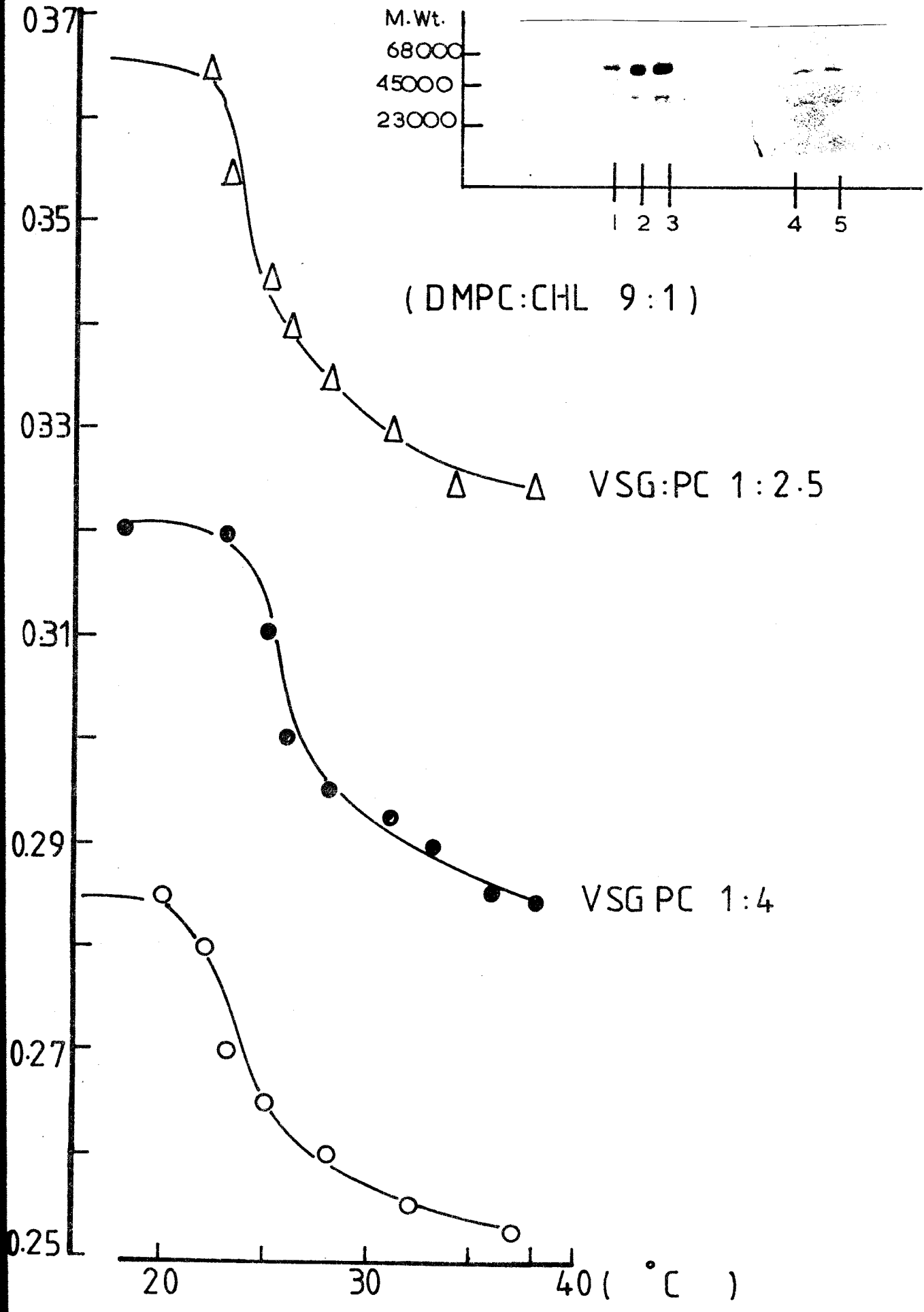
Fig. 7.21

Multilamellar liposomes with a lipid composition DMPC: cholesterol of 9:1 were incubated with VSG at 1:2.5 VSG: phospholipid mol ratio. Absorbance was recorded continuously throughout the entire heating programme.

Figure inset

1. VSG alone
2. Supernatant from phosphatidyl choline liposomes
3. Supernatant from phosphatidyl choline cholesterol liposomes
4. Pellet from phosphatidyl choline liposomes-VSG
5. Pellet from phosphatidyl choline/cholesterol/VSG liposomes.

Fig. 7.21


ABS325
0.37
0.35
0.33
0.31
0.29
0.27
0.25
M.Wt.
68000
45000
23000
1 2 3 4 5
(DMPC:CHL 9:1)
VSG:PC 1:2.5
VSG PC 1:4
20
30
40 (°C)

assay (Section 4.3.4). The number of molecules of VSG bound to multilamellar liposomes is very little and the mol ratio of phospholipid per VSG was of approximately 4500.

Experiments at a fixed temperature were carried out in order to establish if the clearing of turbidity was a time-dependent process. Liposomes of the following composition: DSPC/DMPC (1:9 mol ratio) and DSPC/DMPC/cholesterol (1:9:1 mol ratio) were equilibrated at 31°C in buffer pH 7.4. After 10 minutes VSG was added to each liposome preparation at a ratio of phospholipid to VSG of 14:1 and changes in absorbance (325 nm) were monitored for 4 hours. The initial value of absorbance did not change significantly during this period of incubation. In conclusion, no recombination occurs at long period of incubation above the transition temperature of the mixture of phospholipids. These results show that under conditions of direct recombination VSG is unable to produce clearing of turbidity at the phase transition temperature. Then, it is inferred that there is no disruption of the bilayer; hence the formation of small protein-lipid complexes cannot be detected. It can be concluded that VSG does not interact with the acyl chains of phospholipids and therefore no recombination occurs according to the definition given above. Figure 7.22a shows the results of increasing the ratio of VSG to phospholipid (1:2 mol ratio). Again, no clearing of turbidity is observed.

Cholesterol present in mixture of phospholipids tends to stabilize the interaction with VSG (Section 6.1.2). Swaney (1980) found that the mixture of DMPC/DSPC (1:1 weight ratio) does not recombine with apo A-I unless cholesterol is added in low proportions (11% w/w). Studies of the recombination of VSG with a dispersion of mixed phospholipids containing cholesterol were carried out. From the plot of absorbance

versus temperature the inflexion point was determined at 25.5°C. Although this value was obtained for a mixture containing phospholipid-cholesterol (DMPC:DSPC:cholesterol 9:1:3) it corresponds to the phase transition temperature of the binary mixture DSPC/DMPC as stated above. The fact that the phase transition for the phospholipid mixture is still detectable even in presence of cholesterol is explained on the basis that the proportion of cholesterol used is below 0.5 mol fraction above which phase transition is totally undetectable (Mabrey et al., 1978 Demel et al., 1977). Fig. 7.22b shows the curves of absorbance against temperature for the phospholipid-cholesterol mixture alone and with VSG (VSG:phospholipid 1:8 mol ratio). VSG fails to clear the turbidity of this lipid suspension.

7.7.3 Binding studies of VSG to liposomes

The binding of VSG to liposomes appears to be of a weak type according to the results above. Therefore it was pertinent to characterize further the binding of VSG to liposomes. With a weak binding it is important to isolate the liposome-VSG complex from the free antigen within a very short time to avoid dissociation of the complex. An appropriate method is centrifuging the system in equilibrium followed by analysis of antigen in the supernatant and in the pellet (see Alvin et al., 1980). This invariably necessitates the use of multilamellar liposomes. The application of this method to the system of VSG-liposome suspensions, however, was not very successful probably because non-specific adsorption of VSG to plastic container produced a high background in absence of phospholipids.

Thereafter the samples of liposomes-VSG were incubated in buffer pH 7.4 of 10 mM Tris-HCl, 145 mM KCl, 1 mM EDTA and 0.02% azide, at room temperature further with continuous mechanical mixing on a rotating

Fig. 7.22

(a) A binary mixture of DMPC:DSPC (0.1 mol fraction DSPC) was incubated with VSG for a VSG/phospholipid mol ratio of 1:2. Other conditions are as described in Fig. 7.20.

(b) Cholesterol was added to the phospholipid mixture: DMPC:DSPC:cholesterol 9:1:3 mol ratio. VSG was added to this liposome suspension for a mol ratio of 1:8 VSG/phospholipid mol ratio.

Fig. 7.22(a)

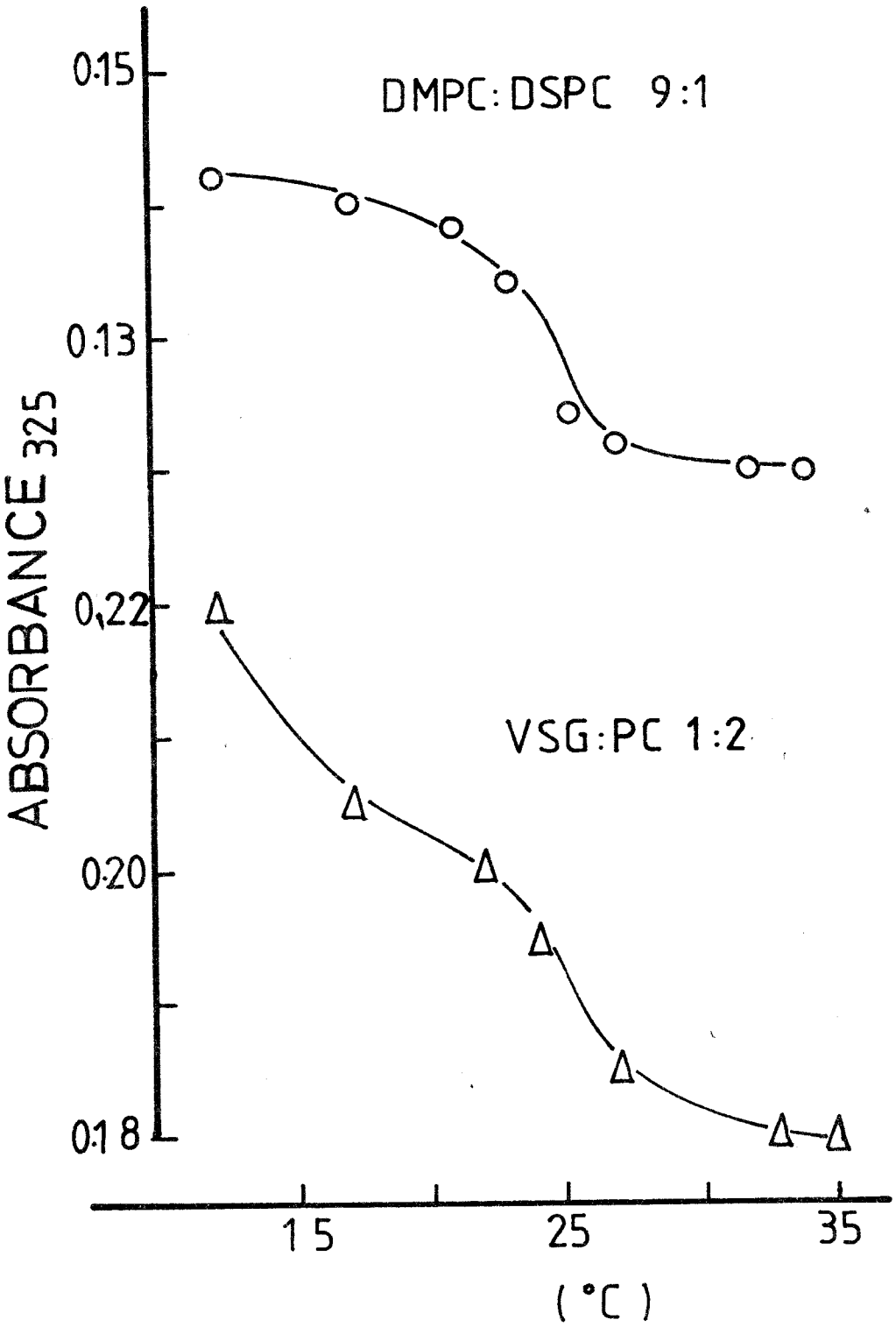

0·15
0·13
0,22
0·20
0·18
ABSORBANCE 325
DMPC:DSPC 9:1
VSG:PC 1:2
15
25
35
(°C)

Fig. 7.22.b

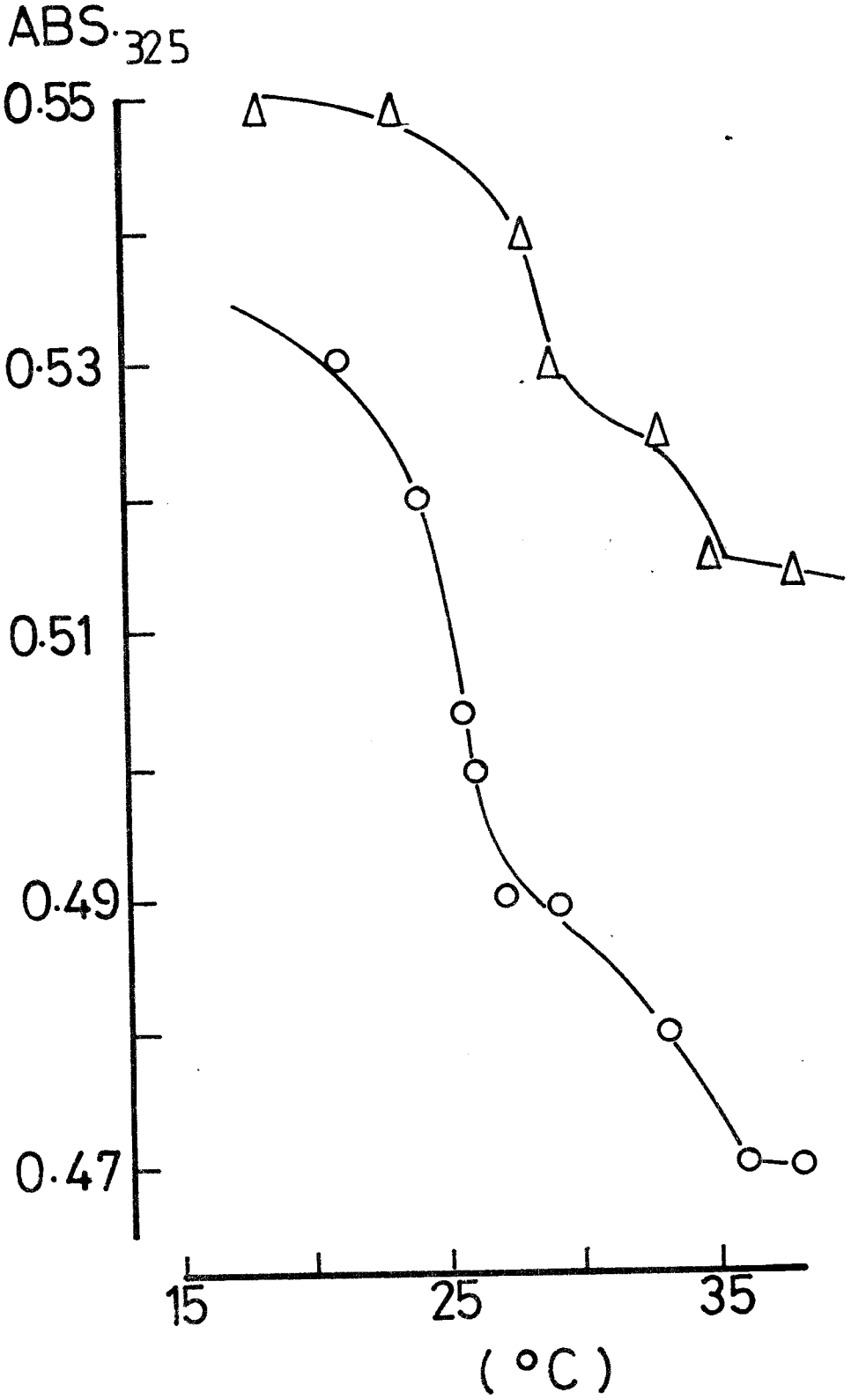
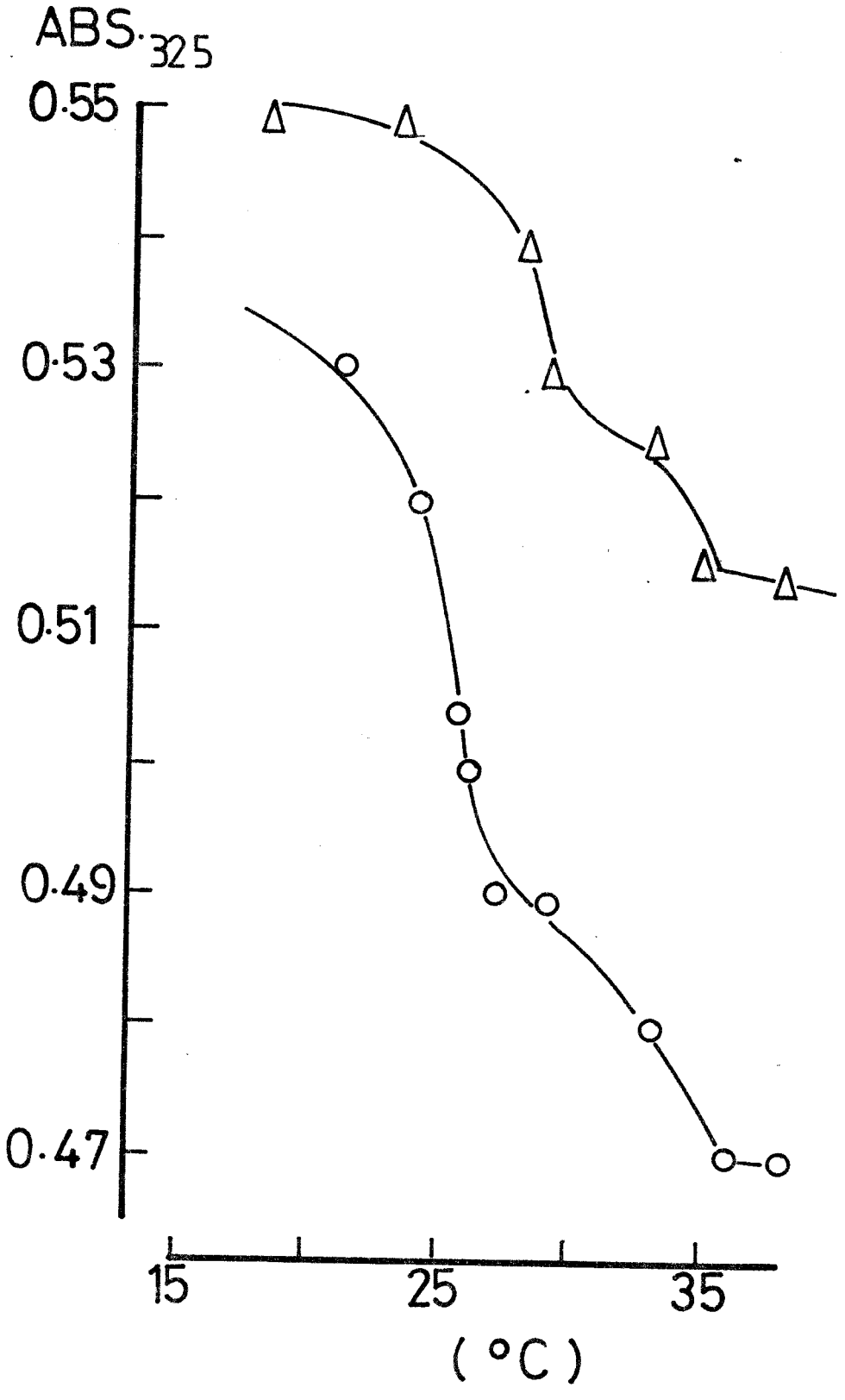


Fig. 7.22.b



well for 1 hour. At the end of this incubation the samples were transferred to new 1 ml-plastic tubes, centrifuged at 12000 x g_{av} for 5 minutes and the pellet counted in a γ-ray scintillation spectrometer. Table 7.3 shows the results of incubating a fixed amount of phospholipids and phospholipid-cholesterol with radiolabelled antigen [^{125}I]-VSG. These results suggest that liposomes of phospholipid-cholesterol bind less antigen than those of phospholipids. VSG, however, binds to only a very small amount of phospholipids. The results obtained after adding non-labelled VSG to these suspensions suggest that this binding is non-specific.

The method adopted was that of rapid ultrafiltration previously used to estimate the binding of amphiphilic peptides to phospholipid-cholesterol liposomes (Fukushima et al., 1981). Unilamellar liposomes were prepared using the method of injecting 1 ml of lipids in pure ethanol into 50 ml of buffer pH 7.4. The vesicle suspension was concentrated by ultrafiltration in a membrane XM 300 with a cutoff of mol wt 3×10^5 (see Section 4.8). This suspension of concentrated liposomes was purified by passing it through a column of Sepharose 4-B. Thus two populations of liposomes EYPC and EYPC/cholesterol were prepared and thei phospholipid concentration determined. Various concentrations of these liposome solutions were incubated with 100 µg per ml of VSG with shaking at 37°C for 1 hour. Thereafter each sample was passed through the membrane XM 300 and the content of protein in the supernatant and in the liposome pellets retained in the membrane, were determined by the method of Lowry et al. (1951). The amount of antigen bound to liposomes was calculated as the difference between the concentration of VSG in the solution without liposomes and that in the liposomes after passing through the membrane. This procedure showed less variation than when the

Table 7.3 Binding of VSG to phospholipid liposomes

I. Egg yolk phosphatidylcholine

Unlabelled VSG		[^{125}I] VSG bound
Concentration µg/ml	Total amount nmol	µmol VSG bound/mol phospholipid
0	0	0.1
142	1.6	0.1 (0.04)
286	3.1	0.05 (0.01)
576	6.4	0.07 (0.02)

II. Egg yolk phosphatidylcholine cholesterol (1:1 mol ratio)

Concentration µg/ml	Total amount nmol	µmol VSG bound/mol phospholipid
0	0	0.08
142	1.6	0.09 (0.02)
286	3.1	0.08 (0.01)
572	6.4	0.05 (0.01)

The total amount of phospholipid per sample (1.5 µmol) was the same for both EYPC and EYPC-cholesterol dispersions. Samples of the lipid dispersions and antigen were incubated in 0.7 ml of buffer at pH 7.4 with continuous stirring at room temperature for 1 hour. After centrifugation, the sample pellets were counted on a γ-ray scintillation spectrometer. Specific activity of the iodinated [^{125}I]VSG was 302 Ci/mmol. The value in brackets after the amount bound is the root mean squared deviation.

liposomes retained on the membrane were resuspended and the content of its protein measured.

The amount of protein bound to liposomes is relatively small and is not dependent on phospholipid concentration. Thus it was found that 0.46 µmol per ml of phospholipid binds approximately 0.48 nmol of VSG per ml; however, a substantial increase of the phospholipid concentration did not increase the binding of VSG. This can be interpreted as a possible saturation of VSG in solution, probably the presence of dimers of VSG reduce the efficiency of binding as demonstrated in other systems (Morriset et al., 1977; Auffret & Turner, 1981). Furthermore, liposomes of phospholipid-cholesterol (0.2 mol fraction of cholesterol) bind less antigen than those of phospholipids. For example 0.76 µmol per ml of phospholipids (phospholipid-cholesterol liposomes) binds approximately 0.08 nmol per ml of VSG.

The variations observed in the quantification of antigen bound per phospholipid molecule makes it difficult to estimate the binding constant of VSG. These results however confirm first the weak interaction of VSG with phospholipids. Consequently, VSG does not reconstitute in liposomes at least in these experimental conditions. Is this weak interaction of VSG with phospholipid liposomes related to the finding of low interaction of these components in the monolayer system? It is discussed in the last section of this Chapter.

7.8 Discussion

The preferential interaction of VSG with cholesterol may be due to specific structural recognition. It was shown in section 6.3 that the rate of film penetration by VSG was approximately four times higher for cholesterol than for DPPC and DSPC monolayers. At room temperature

all these compounds form condensed monolayers as evidenced by their π-A isotherms. Further investigation to establish whether VSG recognizes a special group of cholesterol or the whole molecule was carried out and is described in Section 7.3. In these studies cholesterol derivatives were used. When the 3β-hydroxyl group (3β-OH) of cholesterol (cholest-5-en-3β-ol) was substituted by acetate, acetamido, phosphoryl choline or phosphate groups, the film penetration of VSG was inhibited. On the other hand substituting the 3β-OH group with an amino group seemed to enhance film penetration. Similar results were also found when the substituent was the ammonium group ($-N^{+}(CH_3)_3I^{-}$) (not shown in Table 7.2). Small differences in the acyl group of cholesterol did not alter the interaction with VSG; β-sitosterol (5 cholest-5-en-24-ethyl-3β-ol) gave a similar capacity for film penetration (CFP) value to cholesterol. Complete saturation of the steroid ring appears, however, to enhance film penetration. Cholestanol (5α-cholestan-3β-ol) showed a significantly higher CFP value in comparison with cholesterol. The cholestanol derivatives derived showed similar CFP values to those from cholesterol (Table 7.2). These results are interpreted to mean that the hydrophilic part of the sterol molecule determines the degree of penetration by VSG. Positively charged substituents, or hydroxyl groups in the 3β position of the cholesterol molecule possibly favour the adsorption of and penetration by VSG by either electrostatic or hydrogen bonding interaction. On the other hand substitution by negatively charged groups or neutral groups may not favour adsorption of VSG to monolayers, and this therefore results in a relatively low rate of penetration (Section 7.3). On the basis of these results it can be concluded that slight modification of the hydrophobic side of cholesterol does not effect the sterol-VSG interaction. Major modification of the

hydrophobic side of the steroid molecule, such as ergosterol (cholest-5,7,22-trien,24 methyl, 3β-ol) and lanosterol (cholest-8,24-dien-4,4 14α trimethyl-3β-ol), appears to reduce the rate of film penetration by VSG; however, this interaction was not determined quantitatively. For the Folch-Lees protein, a protein purified from myelin membrane (bovine spinal cords) it has been found that its strong interaction with cholesterol is diminished if cholesterol-like molecules with modifications in the hydrophobic chain are used to form the film (London et al., 1974).

Studies of the affinity of VSG for cholesterol were also undertaken using polyene antibiotics of known affinity for cholesterol. The relative interaction and competition between VSG and filipin for cholesterol was better discerned using the technique of film penetration. When filipin was injected beneath a pre-formed VSG-cholesterol monolayer an apparent ejection of VSG molecules was produced. The high initial surface pressure reached during the film penetration by filipin can explain in part the ejection of VSG from the monolayer. The critical surface pressure at which VSG is ejected from a cholesterol monolayer was found to be approximately 29 mNm^{-1} (Table 5.4).

The rate of penetration of filipin-cholesterol monolayers by VSG decreased as the proportion of filipin was increased. This suggests that filipin competes for cholesterol with VSG. An equimolar ratio of filipin to cholesterol inhibited the penetration by VSG by approximately 90% (Section 7.4). These results can be interpreted as filipin bonding to the 3β-OH group of cholesterol making it inaccessible to VSG. This is in agreement with the results shown in Section 7.3 where cholesterol derivatives containing acetate, acetamido or cholesteryl phosphoryl choline substituents inhibited monolayer penetration by VSG. The significance of VSG penetrating filipin-cholesterol monolayers, with filipin

at below an equimolar ratio, can be explained by considering the equimolar stoichiometry of the filipin-cholesterol interaction (Norman et al., 1972; Shroeder et al., 1973). VSG may thus adsorb and penetrate small patches of free (i.e. uncomplexed) cholesterol. The use of filipin to study the affinity of a protein for cholesterol has not been previously reported. There is indirect evidence that the formation of the filipin-cholesterol complex can be inhibited by viral membrane proteins (retrovirus); this has been detected by electron microscopy (Feltkamp & van der Waerden, 1982).

The molar ratio of cholesterol to VSG ($\sim$ 1:100) in aqueous solution was determined by the method of Hummel & Dreyer (1962). This value may be an approximation to the true ratio of the number of cholesterol molecules bound to VSG; but one of the equilibrium conditions is not satisfactorily met (Section 7.5). The increase in the concentration of VSG up to 200 µg for a baseline of 1 nM of radiolabelled [^{3}H]cholesterol resulted in no increase of the binding of cholesterol to VSG. One possible explanation is that cholesterol in aqueous solution was below its critical micelle concentration (25 to 40 nM, Haberland & Reynolds, 1973), and under these experimental conditions was a mixture of monomers and aggregates. Although the cholesterol solution was passed continuously through the Sephadex column, the equilibrium between the outside and the inside of the gel matrix may have been slow enough so as to increase the monomer concentration inside of the gel and cause the formation of dimers. There is also evidence suggesting that proteins may become aggregated during their passage through a gel filtration column, presumably by an undefined interaction with the gel matrix (Maddy, 1972).

Evidence against the attainment of the equilibrium condition is as follows: Once the baseline for radio-labelled [^{3}H]cholesterol (1 nM)

was established, a volume of 250 µl containing ∿ 3 [^{3}H]cholesterol solution was added. The result was a radioactive peak with a width larger than those shown in Section 7.5. The presence of this peak is interpreted as cholesterol being unable to attain equilibrium rapidly enough on the Sephadex column. In dialysis experiments it has been found that it takes 20 hours to reach 95% of the equilibrium values for cholesterol present in aqueous dispersion as monomers (Haberland & Reynolds, 1973). These authors also showed that cholesterol micelles have heterogenous molecular dimensions and that micelle formation is a gradual process and does not show a critical phenomenon over a narrow range of concentration as observed with detergents (see Tanford, 1980, Chapter 7). If this process occurs in the gel matrix then the chance of VSG interacting with cholesterol would be significantly reduced.

The determination of the relationship of VSG bound to uni-lamellar liposomes is described in Section 7.7.3. Assuming VSG bound to the liposome surface (see below) the value of 0.48 nmol of VSG per 0.46 µmol of phospholipid can be derived to 45028Å^2 per molecule of VSG. The value of 2830 cm^2 per µmol of phospholipid was used in this calculation and corresponds to the surface area for unilamellar liposomes determined by the uranyl titration method (Klein, 1972; and references contained therein). This value of area per molecule of VSG is equivalent to a mol ratio of VSG to phospholipid of 1:750, and as demonstrated in Chapter 5 VSG when present at this ratio does not affect the force-area curves of phosphatidyl choline monolayers. The correlation between lipid monolayers and liposomes is, however, difficult to establish since the properties of each system are different. For example penetration of lipid monolayers by VSG is surface pressure dependent (Section 7.3) and whereas this factor can be controlled in monolayers it cannot be considered adequately in liposomes.

The determination of surface pressure in liposomes is a difficult problem and values of 5 mNm^{-1} (Phillips & Chapman, 1968) and 30 mNm^{-1} (Demel et al., 1975) have been estimated indirectly using the area per molecule of phospholipid measured by X-ray diffraction. A value of zero surface pressure has been estimated theoretically using the basic thermodynamics of interface for small liposomes (Tanford, 1979; White, 1980). If the surface pressure is above 5 mN/m, VSG cannot penetrate, and therefore probably it binds only by electrostatic interactions. This seems to be a real possibility where in addition to the low binding of VSG, there is also the results obtained from programming temperature experiments which indicate that VSG cannot interact with the phospholipid acyl chains (Section 7.7.1).

7.9 Concluding remarks

The lipid monolayer is an excellent model for determining interfacial properties. Its application to various physiological systems has contributed to a better understanding of the relationship between structure, activity and interfacial forces. For example certain lipolytic enzymes are denatured and inactivated at low surface pressure; however, an increase in activity proportional to the increase in the surface pressure is observed (see Verger & Pattus, 1982). The monolayer model has shown that the water soluble form of VSG exhibits behaviour very close to that of an extrinsic protein (Singer & Nicolson, 1972). VSG partially penetrates phospholipid monolayers at low surface pressures. Force area curves show that after re-compression and expansion of these monolayers the antigen remains adsorbed. Is this a feature of the mechanism occurring on the parasite surface? Very little is known about the turnover of VSG in the surface coat. It seems to be a rapid and probably continuous active mechanism. Taylor & Cross (1977) have found that radioactive [^{35}S] L-methionine is incorporated into the surface coat in approximately 30

minutes, and McConnell *et al.* (1981) have shown that VSG can be synthesized *in vitro* in less than 14 minutes.

VSG showed a preferential order of interaction with lipids as follows: cholesterol≥ergosterol>phospholipids>sphingomyelin. Mixed monolayers of cholesterol and phospholipid, however, inhibited the strong interaction of VSG with cholesterol. Experiments of film penetration showed that unsaturated phosphatidyl cholines inhibited the penetration by VSG even at a mol ratio of cholesterol to phosphatidyl choline of 4:1 (Fig. 6.19).

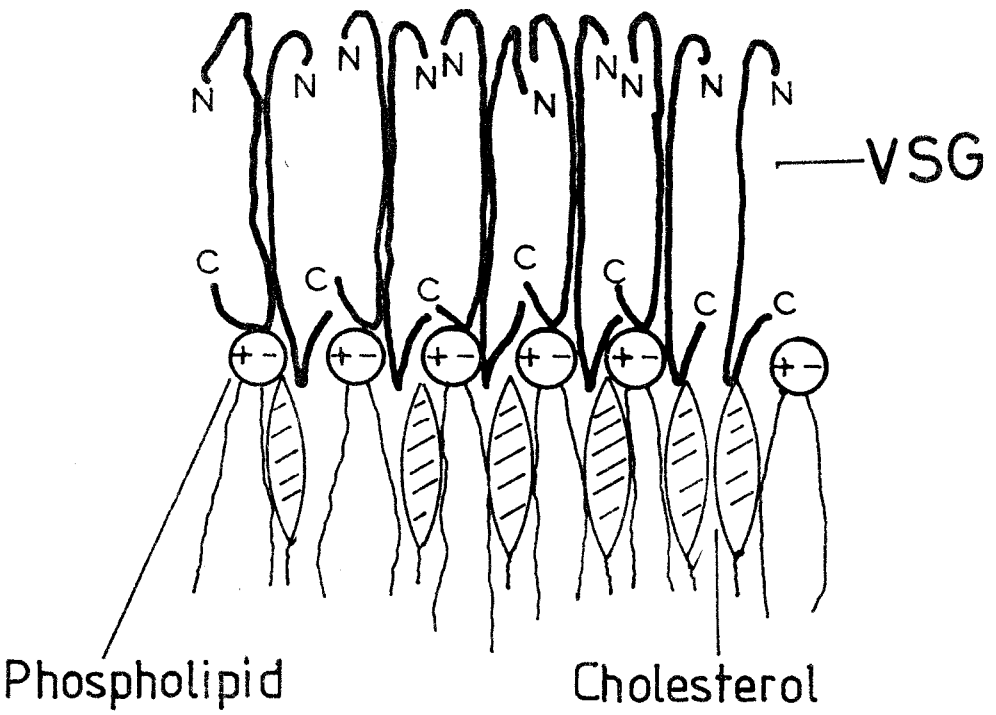
The use of cholesterol derivatives has contributed to establishing the importance of the 3β-OH group of cholesterol in the interaction with VSG. Results with cholesterol derivatives suggest that positive charges exposed on the surface membrane bind VSG with an orientation favouring film penetration. Negative charges may bind VSG but with an inappropriate orientation. From liposome experiments it can be concluded that electrostatic interaction of VSG with the polar groups of phospholipids is not enough as to keep it adsorbed to the liposome surface. It is suggested that a net positive charge, or an integral protein may be present in the parasite membrane surface. The cooperative interaction of protein with protein at the membrane surface has been discussed in Section 6.5.3. The presence of a membrane form of VSG (mf-VSG) in the surface coat has been reported (de Almeida & Turner, 1983) and its significance in the arrangement of VSG at the parasite surface is discussed in Chapter 2.

Preliminary studies of this antigen (kindly donated by Dr M.J. Turner), using the monolayer system, show that 0.032 nmole per ml of mf-VSG in monolayers of DPPC:cholesterol (1:1 mol ratio) gives changes in surface pressure of 8 ± 1 mN/m, a value slightly higher than those observed for water soluble VSG (Fig. 6.19). A possible arrangement of VSG in the surface membrane is shown in Fig. 7.23.

Fig. 7.23

Schematic representation of the possible interaction between VSG molecules and membrane lipids. This model considers the occurrence of heterogenous mixtures of VSGs bound to the lipid surface. Some VSG containing hydrophobic regions may partially penetrate the outside monolayer. Probably some could also bind free cholesterol present in the cholesterol-rich domains. Other VSGs could bind to already attached VSG, thus forming dimers.

Fig.7.23



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