Intermolecular interactions in lipid/carotenoid monolayers

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The collapse pressure at the air–water interface of monomolecular films of 1,2-distearoyl derivatives of phosphatidylcholine and digalactosyldiacylglycerol containing various proportions of the carotenoid astaxanthin was related to the composition of the monolayer. The results were analysed by using a regular-association approximation by which it is assumed that there is a stepwise formation of AB_i -type associations where A and B represent the diacyl lipid and astaxanthin respectively and $1 \leq i \leq 6$. This treatment provides an adequate description of the experimental data and permits calculation of equilibrium constants for the steps in complex-formation; each step is said to have the same equilibrium constant. The value for the collapse-surface-pressure increment associated with formation of AB_i complexes was also derived. Calculated values of equilibrium constants and collapse-surface-pressure increments are greater for phosphatidylcholine/astaxanthin mixed monolayers than for digalactosyldiacylglycerol/astaxanthin mixed monolayers. These differences are discussed in terms of intermolecular interactions between components in the two systems.

INTRODUCTION

The photosynthetic membrane of higher-plant chloroplasts and algae is organized so that light trapped to generate reducing equivalents is efficiently harnessed to produce metabolites required for growth and development. The carotenoids play an important primary role in the photosynthetic process, and they are organized in their arrangement within the membrane by special proteins (Matthews & Fenna, 1980) to form the light-harvesting chlorophyll–protein complexes. These complexes associate with the photochemical reaction centres in a manner that is regulated by a protein kinase-dependent process (Bennett, 1983) and is apparently stabilized by the membrane lipids. The dominant lipid of the membrane is monogalactosyldiacylglycerol (Douce *et al.*, 1973), a non-bilayer-forming lipid (Shipley *et al.*, 1973), which is believed to be required to package the membrane complexes into efficient photosynthetic units (Quinn & Williams, 1983).

To model the interaction of membrane lipids with components of the photosynthetic membrane we have examined the behaviour of monomolecular films consisting of mixtures of polar membrane lipids and carotenoids to determine whether there are specific interactions between these components. The behaviour of mixed monomolecular films of astaxanthin with digalactosyl-1,2-distearoylglycerol or 1,2-distearoyl phosphatidylcholine [1,2-distearoylglycerol(3)phosphocholine] have already been reported (Tomoaia-Cotişel *et al.*, 1984). Astaxanthin is a polar carotenoid found in some green algae and has an advantage over other less polar carotenoids in that it has a strong tendency to orient at an air–water interface. The earlier studies showed that the collapse pressure of the mixed monolayers was somewhat higher than might be expected if the two components exhibited ideal mixing. The increased stability of digalactosyldiacylglycerol/astaxanthin monolayers at high pressures was analysed assuming a regular

behaviour of the film and an apparent interaction parameter, ξ^M , was calculated that characterizes the attraction between the digalactosyldiacylglycerol and astaxanthin molecules of the monolayer (M). The numerical value for ξ^M was found to be -0.87 but the standard deviation of the experimental points on the theoretical curve for perfect behaviour was rather high ($\Delta = 1.106 \text{ mN} \cdot \text{m}^{-1}$). Mixed films of distearoyl phosphatidylcholine and astaxanthin were found not to conform to a regular solution approximation, and calculations of interaction parameters could not be applied. The present study is based on the assumption that the higher collapse pressure of mixed monolayers, as expected for perfect mixtures, is due to molecular associations between the molecules of the film. Accordingly, parameters can be derived that describe the nature of the molecular associations.

MATERIALS AND METHODS

The extraction, isolation and hydrogenation of digalactosyldiacylglycerol from leaves of 4–5-week-post-emergent bean plants (*Vicia faba* L. var. Express) to produce the 1,2-distearoyl derivative of the digalactosyldiacylglycerol have been described elsewhere (Sen *et al.*, 1981). 1,2-Distearoyl phosphatidylcholine was obtained from Sigma Chemical Co. and was used without further purification. Astaxanthin (all-*trans*-3,3'-dihydroxy-4,4'-dioxo- β -carotene) was obtained from Hoffmann–LaRoche in a chromatographically pure form. Details of the monolayer apparatus (Quinn & Esfahani, 1980) and methods for obtaining the surface pressure–area isotherms (Tomoaia-Cotişel *et al.*, 1983a, 1984) have been published elsewhere. The compression isotherms were recorded discontinuously by using a Wilhelmy method and at a temperature of 22°C . Monolayers were spread from a solvent mixture consisting of hexane/chloroform/ethanol (8:2:1, by vol.) at

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Fig. 1. Collapse pressures (π) of mixed monolayers as function of apparent molar fraction of lipid (x_A^m)

(a) Digalactosyldiacylglycerol/astaxanthin mixed monolayers; (b) distearoyl phosphatidylcholine/astaxanthin mixed monolayers. The broken curves were calculated by means of eqn. (4) by taking $x^M = x_A^m$; the continuous curves were calculated by means of eqn. (6') by using the numerical values given in Table 1.

an area per molecule of 1.3 nm^2 and allowed to equilibrate for 5 min before the commencement of compression. Collapse pressure was recorded when reduction in area of the monolayer did not result in a proportionate increase in surface pressure after equilibration of the film for 15 min.

RESULTS AND DISCUSSION

When mixed monomolecular films of astaxanthin with distearoyl derivatives of digalactosyldiacylglycerol or phosphatidylcholine are compressed to the point of collapse of the monolayer, the collapse pressure, π , is greater than predicted from the perfect behaviour of the components in the film. This is illustrated in Fig. 1, which shows the relationship between the collapse pressure of the pure components and mixed monolayers of astaxanthin (B) with digalactosyldiacylglycerol (Fig. 1a) and with phosphatidylcholine (Fig. 1b) as a function of the apparent molar fraction, x_A^m , of the diacyl lipids (A) in the monolayer. The composition of the film conforms to the expression:

$$x_A^m = \frac{n_A}{n_A + n_B} \quad (1)$$

where n_A and n_B represent the number of moles of distearoyl lipid and astaxanthin respectively spread at the interface.

At the collapse of an insoluble monolayer it is generally assumed that a metastable thermodynamic equilibrium is established between the monolayer (M) and the initially collapsed bulk phase (C). This implies that the chemical potentials of the components present in both phases are equivalent (Zsakó *et al.*, 1984). If we consider a binary mixture of two surfactants A and B, totally miscible both in monolayer (M) and in bulk phases (C) the chemical potentials of the components in the monolayer can be expressed as:

$$\mu_i^M = \mu_i^{\phi M} + kT \cdot \ln(f_i^M x_i^M) + \pi A_i' \quad (2)$$

and in the collapsed bulk phase as:

$$\mu_i^C = \mu_i^{\phi C} + kT \cdot \ln(f_i^C x_i^C) \quad (3)$$

where $\mu_i^{\phi M}$ and $\mu_i^{\phi C}$ are the standard chemical potentials of the component i in M and the chemical potential of the pure component i in C respectively, f_i^M and f_i^C are the activity coefficients of i in M and in C respectively, x_i^M and x_i^C are the corresponding molar fractions, π represents the surface pressure in the mixed monolayer, and A_i' the partial molecular area of i .

The chemical potentials, μ_i^M and μ_i^C , are assumed to be equal at the collapse of the monolayer. Furthermore, we may also assume that the bulk subphase liquid molecules are virtually squeezed out and excluded from the monolayer. In this case the relation $x_1^M + x_2^M = 1$ applies and it is evident that $x_1^C + x_2^C = 1$. The water molecules hydrating polar residues of the film molecules are, for this purpose, included as constituent components of the surfactant molecules. If surfactants A and B form perfect mixtures in both monolayer and bulk collapsed phase, then the activity coefficients in the respective phases are equal, $f_i^C = f_i^M = 1$. At the collapse of the monolayer, where $x_A^M = x_A^m$ and $x_B^M = 1 - x_A^m$, the following relationship can be obtained by combining eqns. (2) and (3):

$$(1 - x_A^m) \cdot \exp[(\pi - \pi_B)A_B/kT] + x_A^m \cdot \exp[(\pi - \pi_A)A_A/kT] = 1 \quad (4)$$

The values π , π_A and π_B represent the collapse pressure of the mixed monolayer and those of pure monolayers of A and B respectively, and A_A and A_B represent the mean molecular areas of A and B at the collapse of the pure monolayers. Numerical values of collapse characteristics were obtained from the experimental compression isotherms of the pure monolayers of distearoyl derivatives of digalactosyldiacylglycerol and phosphatidylcholine as well as astaxanthin and are presented in Table 1. By substituting the values given in Table 1 into eqn. (4), plots of the theoretical collapse pressure as a function of the composition, x_A^m , of the monolayer can be obtained and these are shown as broken lines in Fig. 1 for mixed monolayers of diacyl lipids and carotenoid by taking $x_A^m = x^M$. The marked positive deviation of mixed monolayers in the two systems from perfect behaviour suggests the existence of strong cohesive interactions between the lipids and the xanthophyll.

Application of the theory of regular solutions in accordance with the observed interactions shows that, if $\pi_B < \pi_A$, the maximum deviation from perfect behaviour would occur at $x_A^m > 0.5$. Reference to the curves in Fig. 1, however, indicates that for digalactosyldiacylglycerol/astaxanthin mixed monolayers (a) the point of maximum deviation is at $x_A^m \approx 0.5$ but that for phosphatidylcholine/astaxanthin monolayers (b) the maximum deviation occurs at $x_A^m < 0.5$. The deviations from perfect behaviour

Table 1. Surface characteristics of pure components, stability constants and surface-pressure increments of the regular associations and their minimum S.D. values

Component		Collapse pressure (mN·m ⁻¹)		Mean molecular area (nm ²)		K	$\Delta\pi$ (mN·m ⁻¹)	Δ (mN·m ⁻¹)
A	B	A	B	A	B			
Digalactosyldiacylglycerol	Astaxanthin	68	45	0.39	0.25	1.17	0.05	0.172
Distearoyl phosphatidylcholine	Astaxanthin	58	45	0.38	0.25	1.45	1.00	0.213

cannot therefore be explained in terms of the regular solution approximation.

An alternative approach is to consider the formation of molecular complexes or associations of the $A_m B_n$ type (Prigogine *et al.*, 1954). It is evident that the collapse pressure of monolayers containing only molecular species $A_m B_n$ must be greater than both π_A and π_B . Moreover, the composition of the monolayer at the point of maximum deviation from perfect behaviour will depend on both the values of m and n and the stability of the complex. It is clear in the case of phosphatidylcholine/astaxanthin mixed monolayers that molecular associations contain a predominance of carotenoid molecules over phospholipid molecules. If we assume the formation of AB_n -type molecular associations such that in the monolayer phase there is an equilibrium between molecular species A, B, AB, AB_2 , ..., AB_n , the reaction:

can be characterized in terms of equilibrium constants:

$$K_i = a_{AB_i} / (a_{AB_{i-1}} \cdot a_B)$$

where a_{AB_i} , $a_{AB_{i-1}}$ and a_B represent the activity of the corresponding molecular species in monolayer. In the perfect-solution approximation the equilibrium constant can be expressed:

$$K_i = x_{AB_i} / (x_{AB_{i-1}} \cdot x_B) \quad (5)$$

where x_i refers to the respective molar fraction in the monolayer phase. On the basis of the behaviour of pure monolayers of phospholipids (Tomoaia-Cotișel *et al.*, 1981) and xanthophylls (Chifu *et al.*, 1983, 1986) and the fact that the molecular area of the phospholipid is greater than that for the carotenoid at equivalent surface pressure, a maximum value of $n = 6$ appears to be reasonable for the AB_n complex, especially for phosphatidylcholine-astaxanthin. For the sake of comparison the same $n = 6$ value is presumed also for digalactosyldiacylglycerol-astaxanthin.

If we assume that both monolayer and bulk collapsed phases are perfect solutions of A, B and AB_i -type molecular complexes where $1 \leq i \leq n = 6$, the collapse pressure of the mixed monolayer will conform to a generalization of eqn. (4):

$$x_A \cdot \exp\left((\pi - \pi_A) \frac{A_A}{kT}\right) + x_B \cdot \exp\left((\pi - \pi_B) \frac{A_B}{kT}\right) + \sum_{i=1}^6 x_{AB_i} \cdot \exp\left((\pi - \pi_{AB_i}) \frac{A_{AB_i}}{kT}\right) = 1 \quad (6)$$

Eqn. (6) contains values for collapse pressures, π_{AB_i} and collapse molecular areas, A_{AB_i} , as well as the molar

fractions of all surfactant species. Calculation of the molar fractions requires knowledge of the equilibrium constants given by eqn. (5), none of which can be measured directly. This results in the generation of 18 parameters to be derived from the experimental π -versus- x_A^m curves (Fig. 1). To deal with this problem, we propose a simplified approach, referred to as a regular-molecular-association approximation. In this treatment the monolayer is assumed to be a perfect solution of regular associations, from which it follows that: (1) all values of K_i are the same, i.e. $K_i = K$ for $1 \leq i \leq 6$; (2) if B is associated with A or to AB_{i-1} , the same collapse pressure increment, $\Delta\pi$, will be obtained, i.e. $\pi_{AB_i} = \pi_A + i\Delta\pi$ by assuming $\pi_A > \pi_B$; (3) the molecular areas of the complex, AB_i , will be the sum of the molecular areas of the components, i.e. $A_{AB_i} = A_A + iA_B$.

On the basis of these assumptions, eqn. (6) can be expressed in an alternative form:

$$x_A \cdot \exp\left((\pi - \pi_A) \frac{A_A}{kT}\right) + x_B \cdot \exp\left((\pi - \pi_B) \frac{A_B}{kT}\right) + \sum_{i=1}^6 x_{AB_i} \cdot \exp\left((\pi - \pi_A - i\Delta\pi) \frac{(A_A + iA_B)}{kT}\right) = 1 \quad (6')$$

where the molar fractions x_{AB_i} can be calculated by using the relation:

$$x_{AB_i} = \frac{(1 - x_B)(x_B \cdot K)^i}{1 + \sum_{i=1}^6 (x_B \cdot K)^i} \quad (7)$$

The equilibrium molar fraction of B in monolayer, x_B , is obtained as the solution of a seventh-order equation, resulting from the expression of K and from the material balance equations:

$$x_A^m(1 - x_B) \cdot \sum_{i=1}^6 i(x_B \cdot K)^i = (1 - x_A^m - x_B)[1 + \sum_{i=1}^6 (x_B \cdot K)^i] \quad (8)$$

i.e. it can be calculated as a function of K and of the apparent molar fraction, x_A^m , initially spread, in the monolayer. The equilibrium molar fraction of A in monolayer, x_A , is obtained from:

$$x_A = 1 - x_B - \sum_{i=1}^6 x_{AB_i}$$

Derivation of K and $\Delta\pi$, the only two unknown parameters in eqns. (6'), (7) and (8) was obtained from the experimental data by using a double-minimization procedure as follows. Values of x_B at fixed values of K are calculated for all x_A^m monolayers for which the collapse pressure has been determined by using eqn. (8).

Fig. 2. Derivation of K and $\Delta\pi$ for regular associations in digalactosyldiacylglycerol/astaxanthin mixed monolayers

●, $\Delta\pi$; ○, Δ .

Fig. 3. Derivation of K and $\Delta\pi$ for regular associations in distearoyl phosphatidylcholine/astaxanthin mixed monolayers

●, $\Delta\pi$; ○, Δ .

The molar fractions of all other molecular species are obtained from eqn. (7), and eqn. (6') allows calculation of collapse pressures at designated $\Delta\pi$ values. By systematically varying $\Delta\pi$, a value of $\Delta\pi$ corresponding to the minimum standard deviation, Δ , of the experimental collapse-pressure values from the calculated values can be obtained. A plot of Δ at different values of K shows a minimum corresponding to the best values of K and $\Delta\pi$.

This procedure was applied to experimental data obtained from mixed monolayers of astaxanthin with

phosphatidylcholine or digalactosyldiacylglycerol, assuming the formation of AB_i -type regular molecular associations. The minimum standard deviations of the experimental points from the calculated values at designated values of K for digalactosyldiacylglycerol/astaxanthin mixed monolayers are presented in Fig. 2. It can be seen that the curve passes through a clear minimum when $K = 1.17$. Fig. 2 also contains a plot of the $\Delta\pi$ value corresponding to the minimum standard deviation as a function of K . It should be noted that, for low values of K , Δ increases with increasing $\Delta\pi$ values. $\Delta\pi$ values $> 1 \text{ mN}\cdot\text{m}^{-1}$ were not used because the collapse pressure of the 'pure' AB_6 cannot exceed the surface tension of water. Likewise, for high K values, Δ decreases with decreasing $\Delta\pi$, but negative values of $\Delta\pi$ were not used since we surmise that π_{AB_i} cannot be less than π_A . There is always a $\Delta\pi$ value ensuring the minimum of Δ at a point where a plot of Δ versus K reaches a minimum. Corresponding derivation of K and $\Delta\pi$ for phosphatidylcholine/astaxanthin mixed monolayers are shown in Fig. 3, and numerical values of the different parameters in the mixed films are given in Table 1. Note that $\Delta\pi$ values $> 3 \text{ mN}\cdot\text{m}^{-1}$ are possible because the collapse pressure of the phospholipid monolayer is lower.

The composition of mixed monolayers consisting of different apparent molar fractions of lipid can be calculated with a knowledge of K . Fig. 4 shows curves of the molar fraction of different molecular species x_i [$i: A, B, AB_i$ ($1 \leq i \leq 6$)] as a function of the apparent molar fraction of the lipid (x_A^m) in the digalactosyldiacylglycerol/astaxanthin system. Because at low x_A^m values the curves of AB_i -type complexes are compressed, the area of the Fig. 4(a) indicated by the broken lines is expanded in Fig. 4(b). Similar curves for phosphatidylcholine/astaxanthin mixed monolayers are shown in Figs. 5(a) and 5(b) respectively.

It can be seen from Figs. 4 and 5 that the molar fractions of the species A, B and AB of the diacyl lipid/carotenoid monolayers are relatively insensitive to values of K derived for the respective mixtures. Nevertheless, there is a rather significant modification of the molar fractions of the AB_i -type species, particularly at high values of i . This can be easily explained in terms of

the overall stability constant $\beta_i = \prod_{k=1}^i K_k$, which in our

approximation is equal to K^i . Thus in the region $0 < x_A^m < 0.5$ the number of complexes, especially of those with i values of 6, increases considerably, resulting in a shift of the maximum deviation from perfect behaviour at lower x_A^m values.

Evidence supporting the existence of regular molecular associations in mixed monolayers of diacyl lipids and astaxanthin is shown in Fig. 1, in which the continuous curves have been computed from eqn. (6') by using the numerical values given in Table 1. The excellent fit of these curves to the experimental data, such that the standard deviation does not exceed the experimental errors associated with measurement of the collapse pressure, indicates that a regular-association model provides an adequate description of the two systems.

The above analysis indicates that the AB_6 -type molecular associations are slightly more stable when astaxanthin complexes with phosphatidylcholine than with digalactosyldiacylglycerol. This could be due to formation of hydrogen bonds between the hydroxy

Fig. 4. Composition of the digalactosyldiacylglycerol/astaxanthin mixed monolayers as a function of x_A^m

Curves were calculated by means of eqn. (7) by using the K value given in Tables 1. The meaning of i is given by each curve.

Fig. 5. Composition of the distearoyl phosphatidylcholine/astaxanthin mixed monolayers as a function of x_A^m

Curves were calculated by means of eqn. (7) by using the K value given in Table 1. The meaning of i is given by each curve.

group of astaxanthin and the carbonyl oxygen atoms of the acyl ester bonds, which may be stronger in the case of the phospholipid because of an inductive effect of the negatively charged phosphate group. Alternatively, the oxygen atoms of the phosphate group itself may participate directly in hydrogen-bonding to the astaxanthin. The great difference between the collapse-pressure increment, $\Delta\pi$, due to molecular association can also be understood on the basis of a comparison of the monolayer properties of the pure diacyl lipids. The collapse pressure of pure digalactosyldiacylglycerol monolayers is considerably greater than that of monolayers of phosphatidylcholine, presumably because of hydrogen-bonding between the sugar residues of the galactolipid (Tomoaia-Cotișel *et al.*, 1983a,b). Consequently, complexing of astaxanthin to digalactosyl-

diacylglycerol will entail only a slight increase of collapse pressure. Intermolecular associations between phospholipid molecules, on the other hand, are weaker and provide scope for astaxanthin molecules to increase cohesion via the formation of molecular associations.

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