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Cryptoxanthin, echinenone and hydroxyechinenone films at air-water interface

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ABSTRACT

The surface pressure-area (π-A) isotherms, as well as cycles of compression and recompression experiments with cryptoxanthin, echinenone and hydroxyechinenone at the air-water interface were presented. Molecular areas of these one-head carotenoids differ from those of their two-head analogs zeaxanthin, canthaxanthin and astaxanthin. Essential differences were observed at surface pressures (lower than 20 mN m^{-1}) in the pairs cryptoxanthin-zeaxantin and echinenone-canthaxanthin. In contrast, molecular areas of hydroxyechinenone and astaxanthin are essentially different at surface pressures (higher than 10 mN m^{-1}). Data were interpreted in terms of carotenoid crystallization at the air-water interface.

1. INTRODUCTION

Carotenoids are the essential pigments in biological systems. Their functional role in the photosynthetic process has been thoroughly studied [1–5]. Their presence in the photosynthetic apparatus [6] is associated with two essential physiological functions. First, they act as accessory pigments, harvesting light energy and transferring it to chlorophylls [7]. Second, they are known to protect the photosynthetic apparatus, particularly chlorophyll molecules, against harmful photosensitized destruction [8]. These functions are due to their large integral absorption coefficients in the visible region and efficient singlet-singlet energy transfer to chlorophylls.

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The Langmuir film technique provides a well defined and a biomimetic model to study plant pigments [9–15] including chlorophyll-carotenoid interactions to understand their role *in vivo*. As a rule, amphiphilic molecules investigated in monolayers at air-water interface have their hydrophilic groups situated at one end of the molecule. In this respect, certain carotenoids [12, 16, 17] and other substances [18-21] constitute exceptional surfactants. It can be expected that some of the specific properties of the carotenoid films are the result of the presence of two hydrophilic groups at opposite ends of the molecules. The main objective of this paper was to elucidate the role of the feature of amphiphilic carotenoid molecules by examining the films of cryptoxanthin, echinenone and hydroxyechinenone (Fig. 1a and b). These molecules have hydrophilic groups only at one end and are one-head analogs of zeaxanthin, canthaxanthin and astaxanthin, which have polar groups at both ends of the molecules.

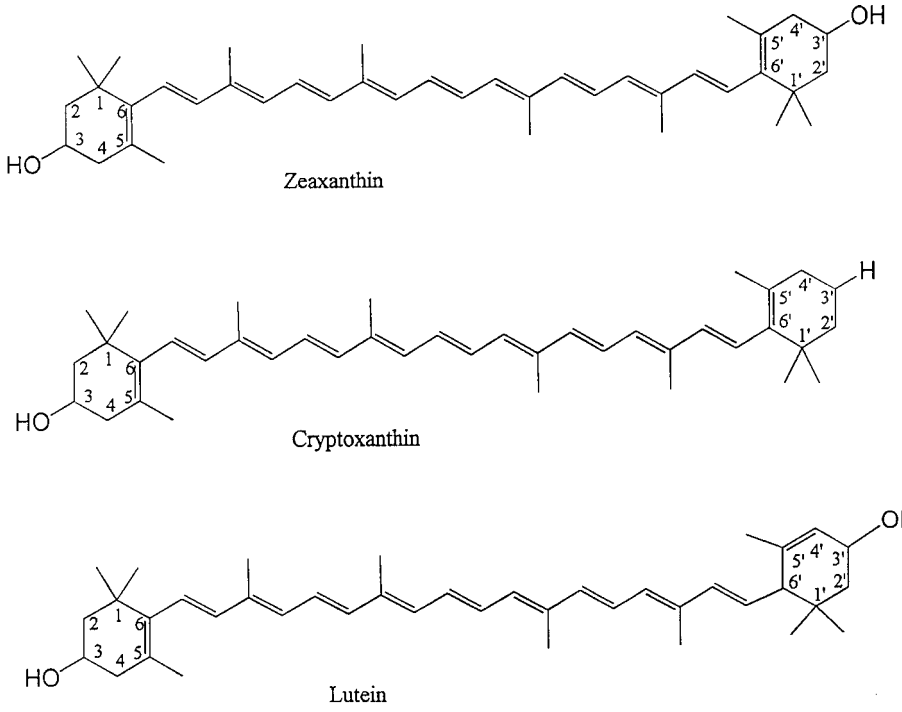

Fig. 1a. Molecular structures of zeaxanthin, cryptoxanthin and lutein

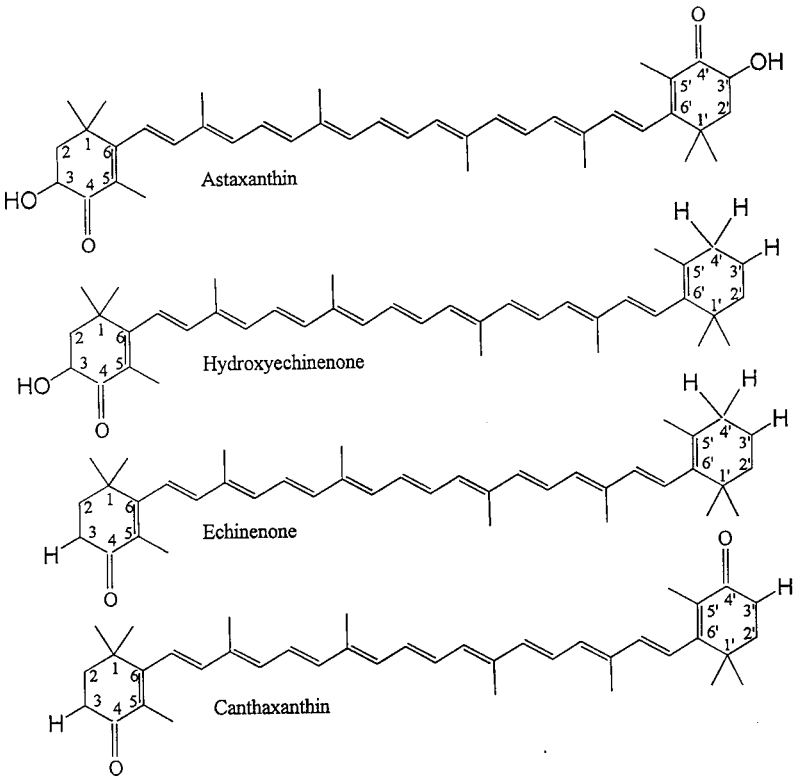

Fig. 1b. Molecular structures of astaxanthin, hydroxyechinenone, echinenone and canthaxanthin

2. MATERIALS AND METHODS

(3R)-cryptoxanthin, echinenone and racemic hydroxyechinenone were provided by Hoffmann-la Roche and Co. (Basel) and were used without further purification. Cares were taken to prevent the chemical degradation of carotenoids. Purity tests were conducted on activated silica gel plates using benzene-ethyl acetate-methanol (15/4/1, v/v/v) for echinenone and hydroxyechinenone, and methylene chloride-ethyl acetate (4/1, v/v) for cryptoxanthin as migration solvents. Hydroxyechinenone produced a single spot on Thin-Layer Chromatography plates. Besides the main spot on TLC plates of echinenone, two additional weaker spots were observed. Cryptoxanthin produced one spot with a tail behind it. The TLC patterns, as well as absorption spec-

tra for all three carotenoids were the same before and after the experiments.

All the surface pressure-area isotherms of one-head carotenoids presented in this paper were obtained on a lab-built Langmuir trough controlled by a computer.

For deposition on the surface of the subphase, echinenone was dissolved in benzene and hydroxyechinenone was dissolved in the benzene-ethanol (9/1, v/v) mixture. For cryptoxanthin both solvent systems were used. In all cases, a 10^{-3} M phosphate buffer, pH 7.0, was used as a subphase. Water has a resistivity of 18 MΩ cm and a surface tension of 72 mN m^{-1}. All the experiments at the air-water interface were performed at 20±1°C.

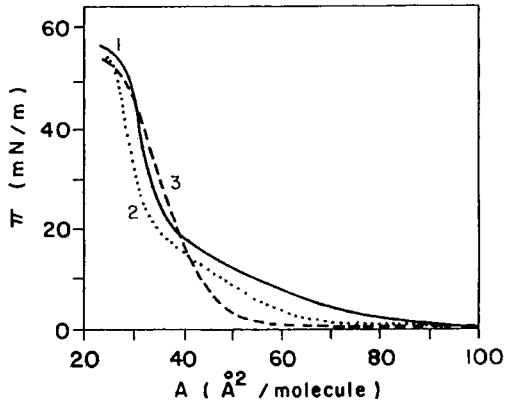

Fig. 2. π-A isotherms of cryptoxanthin and zeaxanthin: 1 (—) cryptoxanthin spread at different initial molecular area in the range 130–250 $Å^2$ $molecule^{-1}$, spreading solvent: benzene-ethanol (9:1, v/v) and rates of compression: 4 to 8 $Å^2$ $molecule^{-1}min^{-1}$. 2 (•••) cryptoxanthin spread at an initial molecular area of 600 $Å^2$ $molecule^{-1}$, spreading solvent: benzene and rate of compression 36 $Å^2$ $molecule^{-1}min^{-1}$. 3 (----) zeaxanthin spread at an initial molecular area of 130 $Å^2$ $molecule^{-1}$, spreading solvent: benzene-ethanol (9:1, v/v) and rate of compression 4 $Å^2$ $molecule^{-1}min^{-1}$

3. RESULTS

The surface pressure-area (π-A) isotherms of cryptoxanthin, echinenone and hydroxyechinenone films at the air-water interface are reproducible and show less dependence on the initial molecular area than those of analogous carotenoids having polar groups at the both ends of their molecules. However, the films of these compounds are unstable except at zero surface pressure.

In the case of cryptoxanthin film, we have measured the π-A isotherms using two different spreading solvents and varying the rates of compression. Curves 1 and 2 in Figure 2 show a similar shape, but

a difference of 5–10 $Å^2$ $molecule^{-1}$ is observed. Both curves were reproducible for the same fresh solution spread at air-water interface, i.e. three fresh solutions were used to determine the π-A isotherms and three isotherms were obtained for each fresh solution (9 isotherms are reproducible within ±2 $Å^2$ $molecule^{-1}$). The films behave as a liquid expanded (LE) layer at low surface pressures (0–20 mN m^{-1}) and as a liquid condensed (LC) layer at higher surface pressures (20–55 mN m^{-1}). The 5–10 $Å^2$ $molecule^{-1}$ difference between curve 1 and 2 is due to the rate of compression since the use of pure benzene in experimental conditions of curve 1 (benzene instead of benzene-ethanol) presents the same π-A isotherm in the limit of the experimental error (±2 $Å^2$ $molecule^{-1}$). The π-A isotherm of zeaxanthin (Fig. 2, curve 3) presents the same phases as cryptoxanthin, i.e. liquid expanded (LE) film (0–18 mN·m^{-1}) and liquid condensed (LC) film (18–55 mN m^{-1}), but the range of molecular areas for the phases are different: curve 3, 40–70 LE, 30–40 LC; curve 2, 35–80 LE, 26–35 LC; curve 1, 40–100 LE, 30–40 LC. The two OH groups of the zeaxanthin situated at the extremity of the conjugated chain provide stronger attraction to water compare to one OH group for cryptoxanthin. The two hydrophilic moieties of the zeaxanthin stabilize the film at air-water interface. In the case of cryptoxanthin, we should consider an important interaction between conjugated chains that explains the liquid expanded phase starting at large molecular area.

The π-A isotherms of echinenone films are more easily reproducible than those of canthaxanthin. The dispersion of molecular area amounts to 5 $Å^2$ $molecule^{-1}$ at low surface pressure and is equal to 2 $Å^2$ $molecule^{-1}$ at high surface pressure. Figure 3, curve 1 presents π-A isotherm of echinenone film. The π-A isotherm of canthaxanthin film (curve 2, Fig. 3) was recorded with the same compression rate (8 $Å^2$ $molecule^{-1}min^{-1}$) as curve 1, in Figure 3. In the plateau portion of the canthaxanthin π-A isotherm, the echinenone film shows zero surface pressure. The differences between the two curves become negligible in their steep regions.

In contrast to the two previous pairs of analogs, hydroxyechinenone and astaxanthin surface pressure-area isotherm show completely different surface properties at air-water interface. For surface pressures higher than that of the astaxanthin plateau, hydroxyechinenone has a molecular area twice that of astaxanthin film. In Figure 4, the effect of initial molecular area on the π-A isotherm of hydroxyechinenone films is also shown. Within the experimental error, there is not much difference except at surface pressure higher than 30 N m^{-1}.

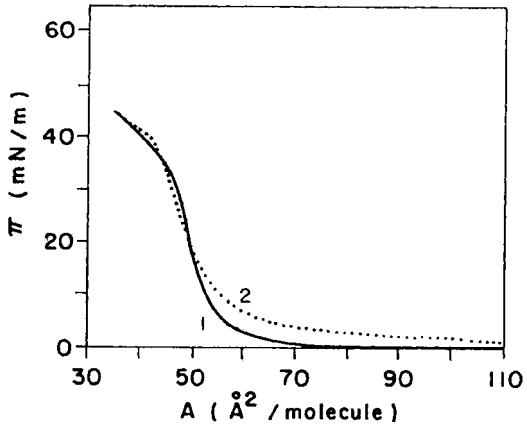

Fig. 3. π-A isotherms of echinenone and canthaxanthin films from benzene solution. In both films initial molecular area is equal to 130 Å^2 molecule^{-1}, rate of compression 8 Å^2/molecule min^{-1}; 1 (—) echinenone, 2 (•••) canthaxanthin

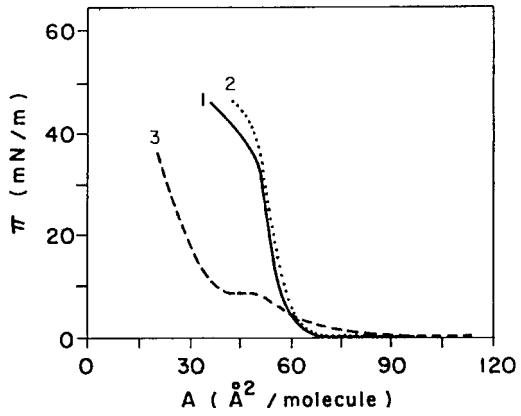

Fig. 4. π-A isotherms of hydroxyechinenone and astaxanthin films. 1 (—) hydroxyechinenone, initial molecular area 130 Å^2 molecule^{-1}. 2 (•••) hydroxyechinenone, initial molecular area 200 Å^2 molecule^{-1}. 3 (----) astaxanthin, initial molecular area 130 Å^2 molecule^{-1}. In all cases the rate of compression was 8 Å^2 molecule^{-1}min^{-1}

The cryptoxanthin films observed under white reflected light with naked eye seem to be perfectly uniform at low surface pressures. Some heterogeneities appear in the middle of steep portion of π-A isotherm (Fig. 2, curve 1). In the case of echinenone and hydroxyechinenone films, blue clouds with irregular black holes become visible at zero surface pressure, at the molecular area approximately 160 Å^2 molecule^{-1}. Under compression, the black holes become smaller and disappear

when the surface pressure starts to increase. The films are uniform as long as the molecular area remains larger than 50 Å^2 molecule^{-1} approximately. At a molecular area of this order, fracture of the film can be observed in the vicinity of the compressing barrier. No details were noticed in the one-head carotenoid film spread in a white trough under a microscope. But in a dark field, colored spots can be seen in echinenone films even at a molecular area as large as 900 Å^2 molecule^{-1}.

Visible heterogeneities in the echinenone monolayers suggested that a kind of crystallization took place at the interface. In order to investigate this process we performed recompression experiments. For the first time, a monolayer was compressed from the initial molecular area of 130 Å^2 molecule^{-1} to a certain final molecular area A_f. The monolayer rested in compressed state for a few minutes. Next, the movable barrier was returned to its initial position what made the surface pressure fall down to zero. The monolayer stayed at zero surface pressure for 15 minutes and was compressed again.

Let us assume that the monolayer under consideration consists of the compressible part with molecular area A_L and non-compressible (crystallized) structures with molecular area A_C. Now, the mean molecular area can be expressed as:

$$A_1 = c_1 A_C + (1 - c_1) A_L \tag{1}$$

and

$$A_2 = c_2 A_C + (1 - c_2) A_L \tag{2}$$

where $c_{1,2}$ are molar fractions of the non-compressible structures. The inferior indices 1 and 2 refer to the first and the second compression. The elimination of the surface pressure dependent value of A_L from (1) and (2) results in the equation relating A_2 to A_1:

$$A_2 = cA_C + (1 - c)A_1 \ , \tag{3}$$

where $c = (c_2 - c_1)/(1 - c_1)$.

Equation (3) gives a linear relation between A_2 and A_1. We fitted straight lines to the portions of the experimental curves $A_2(A_1)$ corresponding to surface pressure intervals 0–11 mN m^{-1} and 12–30 mN m^{-1}. The parameters of those fitted straight lines allowed us to determine c and A_C. At final molecular area of the first compression not lower than 40 Å^2 molecule^{-1} we found that A_C in cryptoxanthin monolayers be-

longs to the interval 36.7–44.5 Å^2 molecule^{-1}. In the case of echinenone monolayers (Fig. 5) we obtained $A_C \cong 53–57$ Å^2 molecule^{-1} at $A_f \in$ (47–60 Å^2 molecule^{-1}). Hydroxyechinenone monolayers gave exactly the same π-A curves during the both compressions.

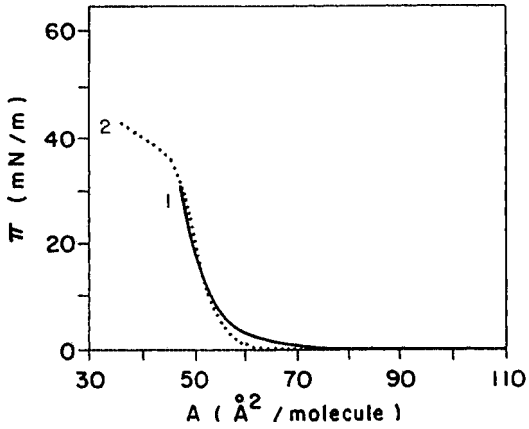

Figure 5. π-A isotherms of echinenone film recorded during the compression (—) and recompression (•••) of the film. Initial molecular area 130 Å^2 molecule^{-1}, rate of compression 8 Å^2 molecule^{-1}min^{-1}

4. DISCUSSION

Let us start our discussion with an interpretation of the surface properties of the cryptoxanthin films. At low surface pressures (less than 20 mN m^{-1}), we have a liquid expanded film. Cryptoxanthin molecules interact with water only at one end (Fig. 1a), and all possible orientations of molecules are allowed. If the accessible area is large enough, they have a rather flat orientation for geometrical reasons. Under compression they are reoriented to form wider angles with the plane of the film. However, apart from shape of π-A isotherm, we have to explain the instability of the surface pressure and the formation of non-compressible structures demonstrated by compression-decompression cycle experiments. These two properties of cryptoxanthin films can be explained by the existence of domains, apart from separate molecules,

with the structure presented schematically in Figure 6. Such domains can be formed due to the action of dispersion forces. Dispersion interactions consist in the induced dipole-dipole interactions [22], and their energy depends strongly on the distance between interacting molecules and on the relative orientation of molecules. Due to the long chain of conjugated bonds, carotenoid molecules have a pronounced anisotropy of polarizability with maximal value in the direction of their long axis.

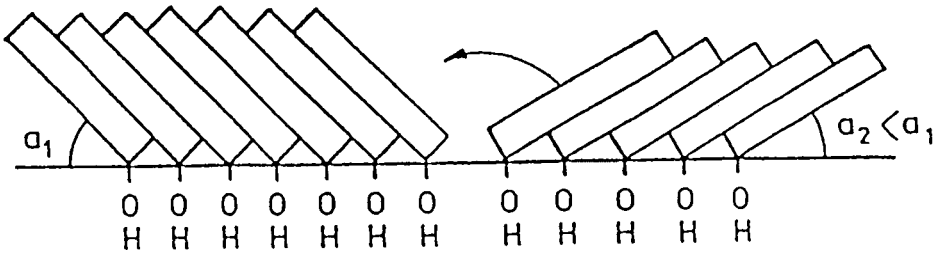

Fig. 6. Hypothetical structure of domains in cryptoxanthin film. Molecules are represented by rigid rods. The arrow marks a possible mechanism of relaxation

Reorientation of molecules, which results in wider angles between the long axis of molecules and the plane of the film, leads to shorter distances between molecules and makes more probable induction of a dipole moment in the molecule due to fluctuations of charge distribution in its neighbors. Both factors make the energy of dispersion interactions bigger at a more vertical orientation and the free energy of the film is lowered. In such a domain, no molecule can be reoriented independently from its neighbors. The only way for rapid decrease of the molecular area, for example under compression, is a simultaneous reorientation of the whole domain. In spite of the relative weakness of the dispersion forces, such reorientation could require some work, if the domain is large enough, and an increase of surface pressure is observed. The reorientation of cryptoxanthin molecules can be attained also by succeeding reorientation of separate molecules at the border of the domain, as marked by the arrow in Figure 6. We propose such mechanism of relaxation for the decrease of surface pressure at constant total molecular area of the film. Domains formed during the first compression of the film are stable enough to be detected during recompression as non-compressible aggregates. The presence of two ionone rings with bigger diameter than that of the polyene chain implies that at certain angles between molecules and the plane of the film, further reorientation to more vertical position should be coupled with separation of the molecules or with the fracture of the film. As a result, we observed a rapid increase of surface pressure or steep part of the π-A isotherm (compression from 37 to 29 $Å^2$ $molecule^{-1}$ in curve 1, Fig. 2).

We believe that the same mechanism of relaxation is valid in the case of echinenone and hydroxyechinenone films. The domains in surface films of these two carotenoids are supposed to have the same structure as those of cryptoxanthin. The non-compressible aggregates on the echinenone monolayers have molecular areas in the range 53-57 $Å^2$ $molecule^{-1}$ which is equal to mean molecular area in the steepest portion of isotherms in Figure 5, where the both isotherms almost coincide. The same molecular area of the non-compressible aggregates were obtained at lower surface pressures, where the isotherms of the first and second compression are noticeably different. Fairly constant molecular area of echinenone non-compressible aggregates is a consequence of the special properties of the keto-oxygen atom in position 4, which constitutes the hydrophilic group in echinenone molecule. This oxygen atom and the subphase can form two hydrogen bonds with well-defined orientation. The bonds should be coplanar with the ring and directed at an angle of 120° with respect to the double bond $C^4 = 0$. As a result of this, reorientation of echinenone molecule to more vertical position can strain or break one of the hydrogen bonds of echinenone molecule with water and increase the free energy of the domain. On the other hand, such reorientation lowers the dispersion contribution to the free energy of the domain. These two factors create a local minimum of free energy, which determines unequivocally the angle between the long axes of the molecules and the plane of the film. The energy minimum is too shallow to prevent relaxation in compressed film, but is deep enough to preserve domains with well defined molecular area at zero surface pressure between two succeeding compressions.

In comparison with echinenone, hydroxyechinenone molecule has one additional hydrophilic group, hydroxyl in position 3, which can form three hydrogen bonds with subphase. These bonds form defined angles with the bond $C^3 - 0$, but their orientation with respect to the plane of the ring is not strictly defined, because the hydroxyl can rotate around the single bond $C^3 - 0$. It seems to us that the local minimum of free energy created by the opposite effects of hydrogen bonds and dispersion forces on the orientation of molecules is much wider in hydroxyechinenone films than in the films of echinenone.

In the case of hydroxyechinenone, the strain or breaking the hydrogen bond of the keto-oxygen atom can be compensated by relaxing or forming an additional hydrogen bond with hydroxyl. Thus, the minimum of free energy in hydroxyechinenone films is too wide to determine the unique orientation of the molecules. On the other hand, its influence at zero surface pressure is strong enough to destroy the domains, which might be created by dispersion forces (that is, to cancel all the changes in the film structure which took place during the first com-

pression). Consequently, recompression of the film gives the same π-A isotherm as the first compression.

How does this picture change in the presence of one or more hydrophilic groups at the opposite end of the molecules? Interactions between hydrophilic groups at the upper surface of monolayer stabilize the structure of domains and transform them into solid crystals. On the basis of our microscopic observations, we can say that such crystals in astaxanthin and canthaxanthin films are multilayered. In spite of the possibility that the image of the network in zeaxanthin and lutein films can be created by monolayered domains with a different orientation in the plane of the film, the formation of solid crystals in these films is also highly probable because of their ability to deflect Wilhelmy's plate from vertical position even at very low surface pressure. Astaxanthin and canthaxanthin films acquire such a property only after compression to smaller molecular area than those corresponding to the plateau that is after crystallization. Moreover, comparison of molecular areas of lutein with the orientation of molecules in lutein film deposited on the quartz slide confirms the crystallization hypothesis [15].

The presence of hydrophilic groups at the opposite ends of carotenoid molecules creates a potential barrier for crystallization. Formation of domains like those in Figure 6 or crystals by carotenoid molecules, which are bound to water at both ends, should be preceded by detaching one end of the molecule from the subphase. It means that some work should be performed. This work can be performed by compression only. The fact that astaxanthin molecule can form more hydrogen bonds with water than canthaxanthin molecule is a reason for higher surface pressure of plateau in astaxanthin films than in the films of canthaxanthin.

Crystallization of zeaxanthin and lutein in films at zero surface pressure can be explained by the fact that ionone rings are not coplanar with one another, or with the polyene chain [23, 24]. Both molecules have their hydroxyls in positions 3 and 3' at the very ends. So, it is possible, and we assume it that zeaxanthin and lutein molecules have such configuration at the interface that makes highly improbable their interaction with water at both ends simultaneously. Oxygen atoms in positions 4 and 4' in a canthaxanthin molecule can simultaneously interact with water at any angle between the planes of 5, 6 and 5', 6' double bonds (Fig. 1b).

5. CONCLUSION

Considering our data on carotenoid films, we conclude that the investigated carotenoids, which have hydrophilic groups at both ends of

their molecules crystallize at the air-water interface at very low surface pressure. Most probably, interfacial films of cryptoxanthin and echinenone are also crystallized when compressed, because molecular areas in these films have similar values to that in zeaxanthin and canthaxanthin films (see Figs. 2 and 3). We can not exclude the existence of a monolayer form of a film in the case of hydroxyechinenone based on the results of the compression and decompression cycle experiments and also, because of evidently larger molecular areas in hydroxyechinenone films than those in the films of astaxanthin, compressed with the same rate and starting from the same molecular area (Fig. 4). It is also possible that a cryptoxanthin film forms a true monolayer at low surface pressure.

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