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CANTHAXANTHIN – THE STRONG MODIFIER OF THE LIPID MEMBRANE PROPERTIES

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Canthaxanthin (β,β-carotene 4, 4' dione) is a pigment widely used as a food and cosmetics colorant. Although considered safe, canthaxanthin may produce some undesirable effects on human health caused mainly by the formation of crystals in the *macula lutea* membranes of the retina of an eye. Experiments show that canthaxanthin toxicity towards the lipid membranes can be the result of its molecular interactions with the lipid molecules. All the results of experiments done on model systems such as monolayers of pure canthaxanthin as well as mixtures of canthaxanthin and lipids, oriented bilayers or liposomes indicate a very strong effect of canthaxanthin on the physical properties of lipid membranes. As compared to other xanthophylls the striking difference is that the effects of canthaxanthin at a molecular level are observed at much lower concentration of the pigment in the lipid phase (as low as 0.05 mol% with respect to lipid). Analysis of the molecular interactions of canthaxanthin showed a molecular mechanisms such as: strong van der Waals interactions between the canthaxanthin molecule and the acyl chains of lipids introducing an ordering effect of canthaxanthin on the lipid membranes, restrictions to the segmental molecular motion of lipid molecules, modifications of the surface of the lipid membranes, effect on the membrane thermotropic properties such as forming new thermotropic phases and finally interactions based on formation of the hydrogen bonds both between the keto groups of canthaxanthin and ester carbonyl group of lipid as well as between the keto groups of canthaxanthin and the lipid acyl chain directly or with the mediation of the water molecules.

INTRODUCTION

Canthaxanthin (β,β-carotene 4, 4' dione) is a naturally occurring pigment which appears as an intermediate during the synthesis of astaxanthin. It has been first isolated from the edible mushroom, *Cantharellus cinnabarinus* (Haxo 1950). This pigment is reported to be synthesized *de novo* by algae and plants and by a certain bacteria or fungi in addition to primary carotenoids (Czygan 1968). In the case of animals and human it can be also formed from other carotenoids ingested with food (Britton, Liaaen-Jensen et al. 2007).

Canthaxanthin was first synthesized from β-carotene (Petracek and Zechmeister 1956a) followed by complete synthesis by Isler et al. (Isler, Montavon et al. 1956) and by Isler and Schudel (Isler and Schudel 1963).

Over the last decade canthaxanthin has been of wide interest as a source of added colour of food and beverages as well as tanning creams and pills (Gupta 1985; Lober 1985). The market research show that an attractive-looking product and the guaranty of a healthy and long life is the main element affecting the customers choice (Baker and Gunther 2004). The experiments on a mammary tumours show that canthaxanthin can act as a strong antioxidant; it's anti-tumour and radical quenching action has been proved (Chew, Park et al. 1999). The anti-cancer effectivity of canthaxanthin examined on cells in culture has been reported lower than lutein but higher that zeaxanthin (Kozuki, Miura et al. 2000; Palozza, Maggiano et al. 1998; Gradelet, Astorg et al. 1997).

Several alternative hypotheses have been proposed to explain the anti-tumour effects of this carotenoid, including its ability to act as an antioxidant; for reviews see: (Burton and Ingold 1984; Palozza and Krinsky 1992a; Palozza and Krinsky 1992b; Krinsky 1993), to potentiate immune responses (Bendich and Shapiro 1986), to enhance gap junctional communication directly (Zhang, Cooney et al. 1992) or through the formation of 4-oxo-retinoic acid (Hanusch et al. 1995). The presence of canthaxanthin in the diet rich in ascorbic acid, β-carotene and vitamin E resulted in remarkable decrease in the free radical level in the blood, liver, kidneys or heart tissues, what was an indicator of the synergistic effect of this pigment (Chen and Tappel 1995). Despite the positive effects registered, there is a growing body of publications describing the undesirable effects on human health following the usage of canthaxanthin such as: canthaxanthin retinopathy (McGuinnes 1985; Daicker 1987; Weber, Michaelis et al. 1987; White 1988; Arden 1989; Bopp, el-Hifnawi et al. 1989), retinal dystrophy

(Hennekes 1986) or aplastic anaemia (Bluhm, Branch et al. 1990).

The canthaxanthin role in the lipid membranes has not been yet explained, although it is known that this pigment does not have the provitamine A activity and it does not play the role in the process of photosynthesis as other xanthophylls (Gupta 1985).

Many experiments done on animal (Weber, Michaelis et al. 1987; Goralczyk, Barker et al. 2000) as well as on humans (Macdonald, Holti et al. 1984; McGuinnes 1985; Daicker 1987; Weber, Michaelis et al. 1987; White 1988; Arden 1989; Bopp, el-Hifnawi et al. 1989) show that after the usage of canthaxanthin even in small quantities (as coloured food or cosmetics) the molecular aggregates of canthaxanthin can be formed and deposited in the tissues especially in the *macula lutea* of an eye. The effect is stronger when accompanied with the high blood pressure or diabetics, even in the case of relatively young patients.

Interestingly, even in the case of a complete healthy patient the pigment can be found in the membranes of the *macula lutea* when used with food or cosmetics during the long period.

It has been proposed that the toxicity of the pigment towards the lipid membranes is the result of forming crystalline aggregates even at very small concentration of canthaxanthin (Sujak, Gabrielska et al. 2005). One can not exclude the hypothesis that about the toxicity of canthaxanthin may decide also its interactions with lipid membrane proteins.

The effect of canthaxanthin on membranes formed with different lipids containing canthaxanthin was studied by means of several techniques including: electronic absorption spectroscopy, linear dichroism, X-ray diffractometry, DSC, ^{1}H-NMR spectroscopy and FTIR spectroscopy. It appears that canthaxanthin present in the lipid membranes at relatively low concentration (below 1 mol% with respect to lipid) modifies significantly physical properties of the membranes. All the results demonstrate a very strong modifying effect of canthaxanthin with respect to the dynamic and structural properties of lipid membranes.

STRUCTURE AND SOME PHYSICAL PROPERTIES OF CANTHAXANTHIN

Chemical structure of canthaxanthin

Canthaxanthin chemically is a tetraterpene (Petracek and Zechmeister 1956b; Britton 1995) having the conjugated double bond system which constitutes a rigid, rod-like skeleton of the molecule, which can play a key role in the function of canthaxanthin and its interactions within the lipid membrane. The molecule is ended with cyclic ionone ring. It belongs to xanthophylls, class of carotenoids pigments containing oxygen as it has a keto groups in the position 4 and 4' (see Fig. 1.). Due to a dark-orange tincture of canthaxanthin it has been widely used as a natural colorant.

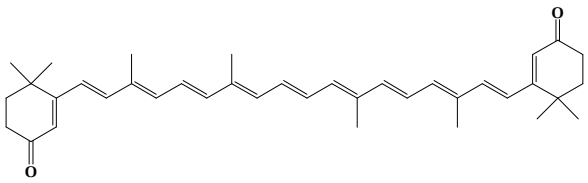

Fig. 1. Chemical structure of canthaxanthin

Canthaxanthin solubility

Canthaxanthin dissolves almost in all known organic solvents. It reveals minor solubility in water, especially at small molar concentrations. At high concentrations in water canthaxanthin forms molecular crystals which are eye-visible and tend to settle on the bottom of the vessels (Sujak 2007, not published observation).

Carotenoids are soluble in lipids and the solubility depends on the lipid profile; the degree of solubility depends on their lipophilicity and polarity (Page and Davies 2006). The experiments show that the solubility of canthaxanthin strongly depends on the length of the hydrophobic core of the lipid, dimension of the polar head zone as well as on the presence of the esther carbonyl groups (Sujak, Strzalka et al. 2007). It has been generally concluded that canthaxanthin demonstrates the highest solubility in lipids having dimension of the hydrophobic core comparable to the distance between keto groups of canthaxanthin. It has also been concluded that canthaxanthin is more miscible with phosphocholines than with phosphoethanolamines. The miscibility of canthaxanthin with lipids having esther carbonyl groups is comparable with phosphocholines at a molar concentration higher than 0.5 mol% in respect to lipid. At smaller molar concentration miscibility of canthaxanthin is bigger for lipids containing esther carbonyl groups (Sujak, Strzalka et al. 2007).

Specific molecular area occupied by canthaxanthin molecule

The specific molecular area of canthaxanthin, measured with aid of the monomolecular layer technique from the monolayer formed at the air-water interface, amounts 68±4 Å^2 (Sujak, Gagos et al. 2007). The result indicates that the surface occupied by a canthaxanthin molecule is bigger than in the case of other carotenoid pigments such as lutein and zeaxanthin (~42 ±4 Å^2) (Sujak 2000; Sujak and Gruszecki 2000). Although the isotherm of compression is similar to other xanthophyll - lutein, canthaxanthin occupies bigger molecular area. The experiments show that the molecular surface occupied by canthaxanthin depends on the sub-phase (54 Å^2, on the phosphate buffer pH 8 (Diarra, Hotchandani

et al. 1986), 60 $Å^2$, phosphate buffer pH 7 (Sielewiesiuk 1988)) as well as on the rate of monolayer compression (Sielewiesiuk 1988; Sielewiesiuk, Veeranjaneyulu et al. 2002). This is the indication of an exceptional behaviour of this molecule in the environment in which formation of the hydrogen bonds is possible. The distance between canthaxanthin keto groups amounts ~27 Å (2.7 nm), therefore the surface occupied by a horizontal oriented canthaxanthin amounts ~250 $Å^2$.

Electronic absorption spectra of canthaxanthin in organic solvents

The conjugated bond system of the polyene is responsible for the pigment absorption properties. The spectra in the region between 400 nm and 550 nm correspond to the electronic transition between the ground energy level ($1A_{g^-}$) and the S_2 ($1B_{u^+}$) state (Britton 1995; Gruszecki and Strzalka 2005). Similarly to the other carotenoid pigments substituted with keto groups at the 4 and/or 4' position, such as astaxanthin, the absorption spectra of canthaxanthin recorded at room temperature do not display vibronic substructure typical of other polar carotenoids (Britton, Liaaen-Jensen et al. 2004). The dipole transition moment of the $1A_{g^-} \rightarrow 1B_{u^+}$ transition of canthaxanthin was determined on the basis of integration of the absorption spectrum recorded in ethanol as 15.3 Debye (Sujak, Gabrielska et al. 2005).

Although similarly to other carotenoids the maximum of absorption shifts towards longer or shorter wavelengths in different organic solvents according to the polarizability term of the solvent (expressed as: $(n^2-1)/(n^2+2)$, where n is the refraction index of the solvent) (Andersson, Gilbro et al. 1991), the behaviour of canthaxanthin is exceptional. The dependence of the position of absorption maximum on a wavenumber scale on the dielectric properties of the chromophore environment is not only linear as in the case of other xanthophylls but forms two proximally parallel lines depending on the fact whether or not organic solvents applied are able to form hydrogen bonds with canthaxanthin keto groups either directly or indirectly via water molecules. Generally the linear dependence for the organic solvents able to form hydrogen bonds is shifted towards lower energies (longer wavelengths) as compared to other solvents (Sujak, Gabrielska et al. 2005).

Electronic absorption spectra of canthaxanthin in DPPC membranes

Canthaxanthin incorporated into model lipid membranes, liposomes formed with DPPC, displays electronic absorption spectra very different from those recorded in organic solvents (Sujak, Gabrielska et al. 2005). Interestingly, this is not dependent on the physical state of lipid. Both the main phase transition of DPPC ($P_{\beta'} \rightarrow L_{\alpha}$, at ~41°C) as well as the phase pre-transition ($L_{\beta'} \rightarrow P_{\beta'}$ at ~35°C) almost do not affect the molecular organization of canthaxanthin or at least this can not be seen by the electronic absorption technique (Sujak, Gabrielska et al. 2005). Such a behavior is opposite to the very strong effect observed in the case of other xanthophyll pigments such as lutein and zeaxanthin (Sujak, Okulski et al. 2000). The main spectral band observed at 492 nm represents the main absorption band of canthaxanthin. On the incorporation of canthaxanthin to the DPPC membranes the band broadens which corresponds well with the dependence of the maximum position on the dielectric properties of canthaxanthin environment described in the previous paragraph (Andersson, Gilbro et al. 1991) in terms of forming or not the hydrogen bonds between the keto groups of the pigment and the esther carbonyl groups of DPPC molecules at two opposite borders of the hydrophobic core of the bilayer. It was additionally explained in terms of possible distortions in the canthaxanthin molecular geometry upon binding to the lipid bilayer such as torsional deformations about the single C-C bonds (Krawczyk and Olszowka 2001). Interestingly, depending on the pigment concentration the long wavelength band appears at 560 nm in most cases except the samples with very low concentration of canthaxanthin. On the one hand this band can be assigned to the 0-0 vibronic transition of canthaxanthin as visible on the low temperature as well as on Stark spectra (Krawczyk and Olszowka 2001). On the other hand, for the high concentrations the possibility is that it represents molecular aggregates in which the chromophore axes are tilted with respect to the axis connecting the centers of molecules or J-type aggregates (Kasha 1963; Hochstrasser and Kasha 1964; Kasha 1965). The measurements of the canthaxanthin orientation show the differences depending on the carotenoids concentration.

LOCALIZATION AND ORIENTATION OF CANTHAXANTHIN IN LIPID MEMBRANES

Canthaxanthin localization and orientation in the lipid membranes

Polar carotenoid pigments such as canthaxanthin are long enough to span the membrane bilayer in such a way that their hydrophilic groups are anchored at two opposite polar zones of the membrane. In general, hydrocarbons locate into the inner, hydrophobic part of the membrane (Gruszecki and Strzalka 2005). A non-polar polyene chain of canthaxanthin incorporated into the lipid membranes is located similarly to unsaturated acyl chains or free polyunsaturated fatty acids, but owing to the fact that the polyene chain is rigid, different structural and dynamic effects can be expected (Sujak, Gabrielska et al. 2005). The localization and orientation

of canthaxanthin within the lipid membrane is determined by the localization of the non-polar polyene chain on the one hand, and the localization of the hydrophilic groups which remain in contact with the polar head groups of the lipid bilayer, on the other hand. The binding of canthaxanthin to the DPPC lipid bilayer has been found to be associated with the formation of hydrogen bonds, via water bridges, between the keto groups of the carotenoid located at the 4 and 4' positions and the ester carbonyl groups of the lipid molecules at two opposite borders of the hydrophobic core of the bilayer (Sujak, Gabrielska et al. 2005).

The dimension of the hydrophobic core of DPPC is comparable with canthaxanthin length, which implies the vertical orientation with respect to the plane of the membrane (Sujak, Gabrielska et al. 2005). Unlikely other xanthophylls the orientation angles of canthaxanthin in the lipid phase depend on the actual concentration of the pigment in respect to the lipid. The mean angle between the dipole transition moment and the axis normal to the plane of the DPPC membrane was determined as 20^o- at 0.5 mol% and 47^o- at 2 mol% canthaxanthin (Sujak, Gabrielska et al. 2005). This suggests that molecular structures can be formed, characterized by chromophores tilted with respect to the axis normal to the plane of the membrane. The angle of 20^o suggests roughly the vertical orientation of the axis connecting opposite polar groups of the xanthophyll, taking into consideration the angle between the molecular polarization axis and the axis connecting the keto groups at the 4 and 4' positions. The angle of 47^o implies that similarly to other xanthophyll pigment – lutein, canthaxanthin incorporated into lipid membranes can be distributed among two pools: one spanning the lipid bilayer roughly perpendicularly to the surface of the membrane and one parallel to the membrane, localized in the head group region. The population of the horizontal fraction increases with the increase in the concentration of the pigment in the lipid phase. The results are consistent with the data on the orientation of canthaxanthin in the single monomolecular layer.

Canthaxanthin orientation in single or mixed mono-layers

FTIR linear dichroism method was applied to determine orientation of canthaxanthin in mono-component monolayers. The orientation angles between the dipole transitions of C=C as well as C=O vibrations determined as $\alpha_{c=c} = 65^o$ and $\alpha_{c=o} = 70^o$ indicate that the monomolecular layer is organized in such a way that the pigment chromophores and even axes connecting the opposite polar groups are tilted with respect to the normal to the plane of the membrane (Sujak, Gagos et al. 2007).

The band corresponding to the C=C vibrations was applied to a linear dichroism analysis of orientation of canthaxanthin in the two-component DPPC-cantha-xanthin monomolecular layers as this band is not present in the pure lipid but exclusively in the carotenoids spectrum. The results indicated that the angle between the normal to the plane of the monolayer and the axis defined by C=C bonds amounted 29^o at 0.5 mol% and 48^o at 5 mol% of canthaxanthin, respectively (Sujak, Gagos et al. 2007), which stays in agreement with the data reported for bilayers, discussed in the previous paragraph.

MOLECULAR MECHANISMS RESPONSIBLE FOR THE CANTHAXANTHIN ACTION WITHIN THE MEMBRANES

All the results of the experiments show the very strong effect of canthaxanthin on the lipid membranes. Based on the data a few mechanisms of canthaxanthin interaction with the lipid membranes can be listed.

Process of aggregation

Forming of aggregates of canthaxanthin in the *macula lutea* membranes has been reported as observed with an aid of ophthalmoscope (McGuinnes 1985; Hennekes 1986; Daicker 1987; White 1988; Arden 1989; Bopp, el-Hifnawi et al. 1989; Harnois 1989).

In the case of most carotenoids being strong hydrophobic molecules the process of aggregation takes place in the environment of the hydrated organic solvents. In the case of other xanthophylls such as lutein or zeaxanthin the spectral shifts towards shorter wavelengths are observed indicative of forming a H-type aggregate called often a "card-pack" aggregate (Gruszecki 1990; Gruszecki 1999; Gruszecki and Strzalka 2005; Wang, Du et al. 2005). The process of aggregation of canthaxanthin is not easy to observe by means of the UV-Vis absorption technique.

The only published spectrum of aggregated cantha-xanthin comes from the paper by Salares and Young (Salares, Young et al. 1977). The authors published the spectrum of canthaxanthin in which the shift of the maximum of absorption towards shorter wavelengths was observed. They observed similar effect for other keto-carotenoids such as astaxanthin and echnenone. Intere-stingly, in spite of using comparable molar concentra-tions of canthaxanthin (1×10^{-5}M) the repetition of expe-riment didn't bring similar results (Sujak 2007- not pub-lished). Unfortunately the doubts can be raised as the methods of purification of the pigments have changed since the 80s. In our case (Sujak 2007- not published) addition of water to an ethanol solution of canthaxanthin resulted in a gradual decrease of the main absorption maxima accompanied with the precipitation of the pig-ment and its settling down on the bottom of the measu-rement cuvette. The low intensity

long wavelength maximum of absorption can be observed similarly to this reported for canthaxanthin dissolved in DPPC at ca. 600 nm (Sujak, Gabrielska et al. 2005). This bathochro-mic shift of the main absorption maximum can be an indication of the presence of the J-type aggregates or aggregates in which the chromophore axes are tilted with respect to the axis connecting the centres of mole-cules (Kasha 1963; Hochstrasser and Kasha 1964; Kasha 1965) on one hand or the 0-0 vibronic transition of canthaxanthin; as described above. At the concentrations close to 10^{-4}M the shift of the absorption maximum towards shorter wavelengths is observed (at 375 nm; Sujak 2007 – in preparation) indicating the presence of the H-type molecular structures (Kasha 1963; Hochstrasser and Kasha 1964; Kasha 1965; Hager 1970; Gruszecki 1990). This demonstrates that different kind of aggregates can be formed, depending on the canthaxanthin concentration.

The formation of aggregated molecular structures of canthaxanthin in hydrated organic solvents as well as in the lipid membrane can be predicted on the basis of information that it can form hydrogen bonds.

Unfortunately the process of canthaxanthin aggregation in the lipid membranes can not be monitored straightforward by the observation of the shifts of the main absorption spectra. The temperature profiles show that the molecular organization of canthaxanthin unlikely other xanthophylls is not dependent on the physical state of lipid (for both the main phase transition of DPPC at ~41^{o}C as well as the phase pre-transition at ~35^{o}C) (Sujak, Gabrielska et al. 2005).

For other xanthophyll pigment (lutein and zeaxanthin, violaxanthin) the same process of formation of the aggregated forms was observed in lipid membranes (Yamamoto and Bangham 1978; Mendelsohn and van Holten 1979; Gruszecki 1990; Sujak, Okulski et al. 2000), see also (Gruszecki 1999) for review. The aggregation level of these xanthophylls depended strongly on both the concentration of the pigment and the fluidity of the lipid phase. The temperature-dependent reorganisation of xanthophylls molecular structures was clearly observed from the measured temperature profiles (Sujak 2000; Sujak, Okulski et al. 2000). It has been shown that in the temperature region corresponding to the main phase transition monomerisation took place. On the basis of the measurements it has been concluded that the molecular forms such as dimers, trimers or tetramers are present. In the case of canthaxanthin such a conclusion could not be drawn based on the observations of the absorption spectra.

However the conclusions about the process of canthaxanthin aggregation can be based on the experiments done on model membranes containing canthaxanthin with the aid of other techniques.

Ordering effect of canthaxanthin on the lipid membranes

The ordering effect of canthaxanthin with respect to alkyl lipid chains is most probably based upon the hydrophobic van der Waals interactions with rigid carotenoid molecule containing conjugated double bond system (for carotenoids in general, see: Gruszecki 1999; Gruszecki and Strzalka 2005).

The thickness of the hydrophobic core of the canthaxanthin-modified membranes formed with DPPC, calculated from the diffractometrically-determined periodicity parameter of the lipid multibilayer shows that it is larger than the distance between the keto groups of canthaxanthin (Sujak, Gabrielska et al. 2005). The reported canthaxanthin dimension between the canthaxanthin keto groups amounts ~2.7 nm (Bart and MacGillavry 1968), while the experimentally determined hydrophobic core thickness of DPPC amounts 3.2 nm at 40^{o}C (Sujak, Gabrielska et al. 2005). Such a result is diagnostic for a strong carotenoids-lipid interaction, where the alkyl chains are forced to adopt extended conformation. The measured growth in thickness of the hydrophobic core of canthaxanthin-supplemented DPPC gives information that this can be the result of the ordering effect of the pigment towards the acyl chains of the lipid (Sujak, Mazurek et al. 2002; Sujak, Gabrielska et al. 2005).

The analysis of the FTIR absorbance spectra on monolayers containing canthaxanthin in the region responsible for the scissoring vibrations of the CH_2 groups in the head group region, the methylene waging progression as well as of C-H stretching vibrations of methyl and methylene groups indicate a condensing effect of canthaxanthin with respect to the lipids (Sujak, Gagos et al. 2007). The elimination of the *end-gauche* and *double-gauche* conformations of lipid alkyl chains has been observed (Sujak, Gagos et al. 2007). The new low-wavenumber component corresponding to an ordered lipid phase has been observed upon incorporation of canthaxanthin. The results of the ^{1}H-NMR show that canthaxanthin exerts restrictions to the segmental molecular motion of lipid molecules both in the headgroup region and in the hydrophobic core of the bilayer (Sujak, Gabrielska et al. 2005).

It has been concluded on the basis of the experiments on the canthaxanthin-lipid monolayers as well as on canthaxanthin-containing membranes that canthaxanthin promotes extended conformation of alkyl lipid chains. The measurements of the isotherms of compression show the effect of removal of the semi-plateau in the DPPC monolayer containing between 0.2 and 1 mol% of canthaxanthin, even at relatively low surface pressure (Sujak, Gagos et al. 2007). It has been concluded that this represents the molecular interactions of alkyl lipid chains and the rigid polyene chains of canthaxanthin,

leading to the ordering of the hydrocarbon lipid chains and promoting vertical orientation of a certain fraction of canthaxanthin.

The ordering effect of canthaxanthin with respect to DPPC membranes emerges also from the analysis of the size distribution of the canthaxanthin-pigmented liposomes. The size distribution profiles show that the incorporation of canthaxanthin results in the appearance of the new pool of liposomes, which dimensions are shifted towards higher diameter values. Additionally canthaxanthin affects the physical properties of the liposomes so that vesicles tend to aggregate.

The strong van der Waals interaction of canthaxanthin and alkyl chains of lipid were also concluded on the basis of the FTIR spectra of DPPC multibilayers containing canthaxanthin (2 mol%; Sujak, Gabrielska et al. 2005). In particular the position of the band corresponding to the scissoring vibrations of the CH_2 groups of alkyl chains (1470 cm^{-1}) was shifted towards lower wavenumbers upon incorporation of canthaxanthin into the membranes. Additionally this band became narrower which indicated an ordering effect of canthaxanthin with respect to the hydrocarbon membrane core.

The FTIR measurements on single DPPC monomolecular layer containing canthaxanthin (0.5 and 5mol%) revealed the existence of the band at 2839 cm^{-1} representing an ordered lipid phase correlating with the band at 2960 cm^{-1} representing the antisymetric stretching vibrations giving further support for the strong ordering effect of canthaxanthin on the lipid phase (Sujak, Gagos et al. 2007).

Modifications of the surface of the lipid membranes

The analysis of the unilamellar DPPC and EYPC liposome size distribution profiles shows that canthaxanthin affects the physical properties of the liposomes so that vesicles tend to aggregate (Sujak, Gabrielska et al. 2005). The effect was strongly pronounced in the rigid state of liposome membranes. All the liposomes were prepared at the temperature of 50^{o}C and analyzed at 20^{o}C (below the main phase transition of DPPC) and 50^{o}C (above the main phase transition of DPPC). A liposome aggregation was not observed in the fluid state of the lipid membranes both in the EYPC liposome suspension under the room temperature as well as in the DPPC liposomes incubated at 50^{o}C. It has been concluded that the molecular mechanisms directly involved in the liposome aggregation were related to the surface properties of lipid membranes (Sujak, Gabriels-ka et al. 2005). Such a conclusion is not surprising considering that the incorporation of canthaxanthin pigment exerts interactions within the headgroup region. On the other hand the question aroused why such behaviour is strongly pronounced in the rigid phase of lipid and not in the lipid fluid state. It has been sugges-ted that the molecular motions of lipid molecules in the fluid phase of the membrane and the membrane surface deformations prevent vesicle aggregation.

The ^{1}H-NMR experiments additionally confirmed that the presence of cantha-xanthin at the concentrations of ca 1 mol% caused the DPPC vesicle aggregation (Sujak, Gabrielska et al. 2005).

The FTIR measurements on the DPPC multibilayers containing canthaxanthin showed that the band at 1068 cm^{-1} assigned to C-O-C-P-C stretching vibrations was very sensitive to the pigment presence (Sujak, Gabrielska et al. 2005). Upon binding of canthaxanthin this band became shifted to lower wavenumbers by almost 20 cm^{-1}, which suggested strong interaction in this region being close to the surface of the membrane as well as the location at least a certain fraction of the pigment in the polar headgroup region. Interestingly, the choline group $N^+(CH_3)_3$ was almost insensitive to the presence of canthaxanthin, remaining virtually at the same position (968 cm^{-1}). The analysis of the position of the spectral bands corresponding to the symmetric and antisymme-tric stretching vibrations of the PO_2^- (at 1099 and 1053 cm^{-1}, respectively) shifted towards lower wavenumbers by 10 and 30 cm^{-1}, respectively, indicate the immobi-lization of this fragment of the lipid molecule, probably due to the hydrogen bond formation (Sujak, Gabrielska et al. 2005).

The analysis of the FTIR spectra of single monomolecular layer shows that incorporation of canthaxanthin is associated with the shift of the center of the band assigned to PO_2^- antisymmetric stretching vibration towards lower wavenumbers (Sujak, Gagos et al. 2007). Similarly to experiments with multibilayers the band representing vibrations of the choline group was insensitive to the presence of canthaxanthin in the monolayer. In general, the spectral analysis indicated the possibility of hydrogen bonding to the phosphate groups.

Changing of the membrane thermotropic properties

The effect of canthaxanthin on the thermotropic properties of lipid membranes formed with different lipids has been measured and discussed in detail in (Sujak, Strzalka et al. 2007) . Generally the DSC peaks were less intensive and broadened upon addition of canthaxanthin and the midpoint temperature of the phosphatidylocholines tended to decrease. Such an effect was similar to the behaviour of the most of the polar carotenoids which shift the main phase transition temperature of the lipids towards lower values which is also concentration–dependent. Carotenoids influence also the lipid pretransition by shifting the mid-point temperature towards lower values (for review, see (Gruszecki 2004; Gruszecki and Strzalka 2005)). The only difference between polar carotenoids and canthaxanthin was that the greatest changes in the characteristic thermal parameters occurred at canthaxanthin concentration as low as 0.05 mol% (Sujak, Strzalka et al. 2007).

It has been generally concluded that carotenoids fluidise the membrane in its gel phase and rigidify the membrane in its liquid crystalline phase (Subczynski et al., 1992; Jezowska et al., 1994; Strzalka and Gruszecki, 1994; Gabrielska and Gruszecki, 1996; Castelli et al., 1999; Suwalsky et al., 2002; Gruszecki and Strzalka, 2005; Jemiola-Rzeminska et al., 2005). The effects from polar carotenoids have been found to be much greater than for nonpolar pigments. Polar carotenoids have been found to considerably decrease cooperativity and the molar heat capacity of the main phase transition; a two-fold decrease in molar heat capacity required ~1 mol% of polar carotenoid, while a similar effect was obtained only by using ca. 10 mol% of non-polar pigments (Kostecka-Gugala et al., 2003; Gruszecki and Strzalka, 2005).

The strongest influence of canthaxanthin on the main phase transition and pre-transition has been observed for the lipid having the thinnest hydrophobic region DMPC (C-14) as compared with DPPC (C-16) or DSPC (C-18) (Sujak, Strzalka et al. 2007).

In the case of DMPC the total disappearance of the pre-transition peak has been observed which provided an information about the fluidisation of the $L_{\beta'}$ phase. The influence of canthaxanthin depends strongly on the hydrophobic core dimension. In the case of DPPC the hydrophobic zone dimension is comparable to the canthaxanthin molecule length while the hydrophobic core of DSPC is bigger than its size (Small 1986).

Component analysis for the lipids differing in the thickness of the hydrophobic cores indicated a distinct cooperativity change, which most probably colligated with the formation of new thermotropic phases. In the case of DMPC the low- and high-temperature phases were observed, while DPPC revealed low-temperature and DSPC high-temperature component. Lowering the temperature of the mid-point temperature indicates that canthaxanthin enters the hydrocarbon core of the lipid. The results obtained from the analysis clearly indicate fluidising of the $P_{\beta'}$ phase of DPPC as reported previously for other xanthophylls (Kolev and Kafalieva 1986; Kostecka-Gugala, Latowski et al. 2003) and rigidifying effect in the case of DSPC (Sujak, Strzalka et al. 2007).

The effect of canthaxanthin has been almost negligible in the case of phosphatidylethanolamines. The absence of the ester carbonyl group results in different thermotropic behavior, especially for low canthaxanthin concentrations. The phosphoetyloamines are tightly packed as compared to phosphocholines so it is of a great possibility that canthaxanthin is more miscible with phosphatidylcholines. Although the effect of canthaxanthin has not been as pronounced as in the case of phosphocholines a rapid decrease in the cooperativity of the lipids was noticed at the canthaxanthin concentrations between 0.2 and 0.5 mol% (Sujak, Strzalka et al. 2007).

The existence of esther carbonyl groups (DPPC vs DHPC) resulted in narrowing the pre-transition component especially for the canthaxanthin concentration as small as 0.05 mol%, which suggests an ordering effect of canthaxanthin. Addition of canthaxanthin results in the shift of the main transition peak position towards higher temperatures (~3 ^{o}C at 0.1 mol % of canthaxanthin in DHPC) which also accounts for the ordering effect of canthaxanthin (Sujak, Strzalka et al. 2007).

Interactions via formation of the hydrogen bonds

The possibility of forming of the hydrogen bonds via the canthaxanthin keto groups results straight from the canthaxanthin structure (Petracek and Zechmeister 1956b). The analysis of the position of the maksimum of absorption spectra of this pigment versus the polarizability term of the solvent indicated different behaviour of the pigment depending on the fact whether or not organic solvents applied are able to form hydrogen bonds with canthaxanthin keto groups either directly or indirectly via water molecules (Sujak, Gabrielska et al. 2005).

The analysis of the FTIR spectra of DPPC membranes containing 2 mol% of canthaxanthin in the region corresponding to the carbonyl group vibrations (band at ca 1657 cm^{-1} in the KBr environment (Bernhard and Grosjean 1995; Sujak 2007 - in preparation) indicated the appearance of the low intensity band at 1659 cm^{-1} assigned to the keto groups of canthaxanthin incorporated into the membrane. The main band corresponding to the ester carbonyl group stretching vibrations of lipids that centers at 1736 cm^{-1} is also affected as indicated by the appearance of the spectral component with the maximum at 1712 cm^{-1} accompanied with the disappearance of the band at 1751 cm^{-1} (Sujak, Gabrielska et al. 2005). Such a shift in the components of the main C=O stretching spectral band indicated that DPPC molecules can interact with canthaxanthin via the lipid ester carbonyl groups (Sujak, Gabrielska et al. 2005).

The spectrum of the single monomolecular canthaxanthin layer shows features characteristic of the pigment in the hydrated form. The band centered at 3191 cm^{-1}, characteristic of O-H stretching vibrations of water and the band with the maximum at 1661 cm^{-1}, representing the antisymmetric stretching vibrations of two keto groups located at the 4 and 4' positions of the pigment molecule, hydrogen bonded to water molecules. The absorption band with the maximum at 1736 cm^{-1} represents the antisymmetric stretching vibrations of the keto groups that are not involved in the molecular interactions via hydrogen bonding (Sujak, Gagos et al. 2007).

The presence of water bonded to canthaxanthin has been concluded on the basis of the band corresponding to the stretching O-H vibrations. The analysis of FTIR spectra showed a very interesting effect of appearance of the low intensity bands with the maximum at 2727 cm^{-1}

and 2528 cm^{-1} correlated with the hydration of the pigment. The position of the mentioned bands does not change even at the substitution of the H_2O vapours with the D_2O. This indicates that they may correspond to C-H stretching vibration of the canthaxanthin polyene, shifted towards lower frequencies resulted from creation of the hydrogen bonds upon incorporation of the water molecules (Sujak-personal communication).

CONCLUSIONS

Canthaxanthin toxicity towards the lipid membranes can be the results of its molecular interactions with the lipid membranes. All the results of experiments done on model systems such as monolayers of pure canthaxanthin as well as mixtures of canthaxanthin and lipids, oriented bilayers or liposomes indicate a very strong effect of canthaxanthin on the physical properties of lipid membranes. As compared to other xanthophylls such as lutein or zeaxanthin the effects of canthaxanthin at a molecular level are observed at much lower concentration of the pigment in the lipid phase (as low as 0.05 mol% with respect to lipid). The action of canthaxanthin in the membranes is realised by the molecular mechanisms such as: strong van der Waals interaction between polyene chain of canthaxanthin and the lipid chains, modifications of the lipid properties in the polar head zone, introduction of a new thermotropic phases upon incorporation of canthaxanthin as well as forming of the hydrogen bonds between canthaxanthin keto groups and the C=O group of lipid or hydrogen bonds between the polyene chain and water. This last mechanism can have a crucial significance both in forming of the molecular aggregates of canthaxanthin as well as in the trans-membrane transport of the water.

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