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CAROTENOIDS AS RAMAN-ACTIVE PROBES OF ERYTHROCYTE MEMBRANE STRUCTURE

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SUMMARY

1. Erythrocyte ghosts exhibit resonance-enhanced Raman bands at 1530 cm^{-1} and 1165 cm^{-1} attributable to ν(–C=C–) and ν(=C–C=), respectively, of the conjugated polyene chains in carotenoids. In lipid extract of ghosts, these resonance-enhanced bands lie at 1527 and 1158 cm^{-1}. The spectra indicate the presence of membrane-bound β-carotene.

2. The resonance-enhanced Raman spectrum of β-carotene in lecithin liposomes is identical to that obtained with hexane or chloroform solutions.

3. Increasing proportions of cholesterol in cholesterol-lecithin liposomes up to a cholesterol: phospholipid molar ratio of 0.8–0.9 drastically decreases the intensity of both resonance-enhanced bands.

4. In ghosts the carotenoid bands respond to membrane perturbations. Trypsinization, lysolecithin treatment and reduction of pH increase the intensities of the 1530 and 1165 cm^{-1} bands. In contrast, a decrease in the intensity of both bands follows equilibration of ghosts for 15 min at approx. 50 °C or addition of (0.1 %) sodium dodecyl sulfate.

5. We suggest that perturbants known to change lipid-protein interactions in erythrocyte membranes modify the microenvironment and/or configuration of the membrane-bound carotenoid.

INTRODUCTION

We have recently described the Raman spectra of hemoglobin-free ghosts isolated from freshly drawn human blood [1]. A prominent feature of these spectra is the presence of two sharp, intense bands at 1530 and 1165 cm^{-1}. These bands were documented to arise from the –C=C– and =C–C= stretching vibrations of a conjugated polyene system, apparently carotenoid in character [1]. The intensity of the bands was shown to be resonance enhanced due to vibronic coupling [1].

The degree to which the intensity of a Raman scattering band can be enhanced by vibronic coupling can approach 10^6 [2] and, at a given Raman excitation wavelength, depends markedly upon the spectral overlap between electronic absorption

spectrum of the polyenes and the Raman excitation band, as well as the intensity of the electronic transition [3–5]. Since both of these qualities can vary with the configuration and environment of carotenoids, their resonance Raman spectra can be expected to reflect some features of their microenvironment, i.e. carotenoids, as well as other conjugated polyenes might act as sensitive, natural Raman-active "probes" of membrane structure.

We have now obtained further evidence indicating that the carotenoid responsible for the 1530 cm^{-1} bands and 1165 cm^{-1} bands is β-carotene and have accordingly compared the resonance-Raman spectra of β-carotene in several solvents, and in lecithin liposomes plus and minus cholesterol, with the resonance-Raman spectra of native and structurally perturbed [6–8] erythrocyte ghosts. Our data document the usefulness of carotenoids as Raman-active membrane probes, support previous conclusions [8] about erythrocyte membrane structure and provide new insight into the state of cholesterol in erythrocyte ghosts.

EXPERIMENTAL

Chemicals. Egg lecithin (unsaturated) and lysolecithin was obtained from Lipid Products (South Nutfield, Great Britain). β-Carotene form Nutritional Biochemicals (Cleveland, Ohio) and cholesterol, sodium dodecyl sulfate and crystalline trypsin from Sigma (St. Loius, Mo., U. S. A.). All other reagents were of analytical grade.

Preparation of liposomes. Liposomes were prepared in essence as described previously [8]. Lecithin with or without cholesterol in chloroform solution was combined with β-carotene, also in chloroform, to give a carotene: lectithin molar ratio of 10^4. After removal of the chloroform at 4 °C by a flow of N_2, 0.1 M NaCl was added and the resulting suspension sonicated for 10 min at 4 °C using a Sonic Dismembranator, Quigley-Rochester (N.Y.), power step 10. All of these operations were carried out in the dark. The final concentration of β-carotene in the liposome suspensions was 10^{-5} M and the lecithin level 0.1 M.

Erythrocyte ghosts. Erythrocyte ghosts were isolated from freshly drawn human blood exactly as in ref. 8. Treatment with trypsin with or without lysolecithin was exactly as in ref. 8. Protein was measured fluorimetrically as in ref. 8. Lipid extracts were prepared as in ref. 1.

Raman spectroscopy. Solutions of β-carotene, liposomes, erythrocyte ghosts or lipid extracts were transferred to 0.9–1 mm internal diameter Kimex capillaries. After sealing, the sample capillaries were placed in a Harney-Miller cell for temperature control. The temperature was controlled by a flow of N_2 regulated by a telethermometer. Temperature control was checked by melting point measurements on standard lipids in the laser beam.

Raman spectra were recorded by a Ramalog 4 Raman spectrometer (Spex Industries, Metuchen, N.J., U.S.A.) interfaced to an Interdata (Model 70) computer, An Ar^+ laser (Spectra Physics model 164), tuned at 488 nm, 200 mW power at the sample, was used as an excitation source for ghosts and 60–70 mW for liposomes. Raman scattering at right angles to the laser beam was detected by a thermoelectrically cooled photomultiplier (RCA 31034) and recorded in terms of photons/s. The "dark" counts of the photo cell were < 100 counts/s. Raman scattering from the

samples gave counts in the order of 10^5/s in the case of liposomes and 10^4 in the case of ghosts. Scanning was done through the computer (loaded with VIE8D Ramacomp Computer Program, Spex Industries). We used the following specifications for the ghost spectra. Maximum time and minimum time at each data point = 3 and 1 s, respectively; photon counts = 10^4/s maximum, 100/s minimum; scanning step = 1 wave number. For liposomes, the maximum and minimum times were 0.5 s each, the maximum and minimum counts 10^5/s and 10^2/s, respectively, and the scanning step = 1 wave number. The program does not scan the spectrometer at a rate linear with respect to time. Rather, scans are incremental between data points and no counts are recorded when the spectrometer is moving between data points. The photon counts were stored in the computer memory during scanning (2–4 scans). The averaged stored spectra were then plotted on the Ramalog recorder. The ghost spectra were obtained at 18 °C. The liposome spectra were routinely recorded at 12 °C for long-term stability. There is no difference in the positions or relative intensities of the resonance-enhanced β-carotene bands between 12 and 18 °C.

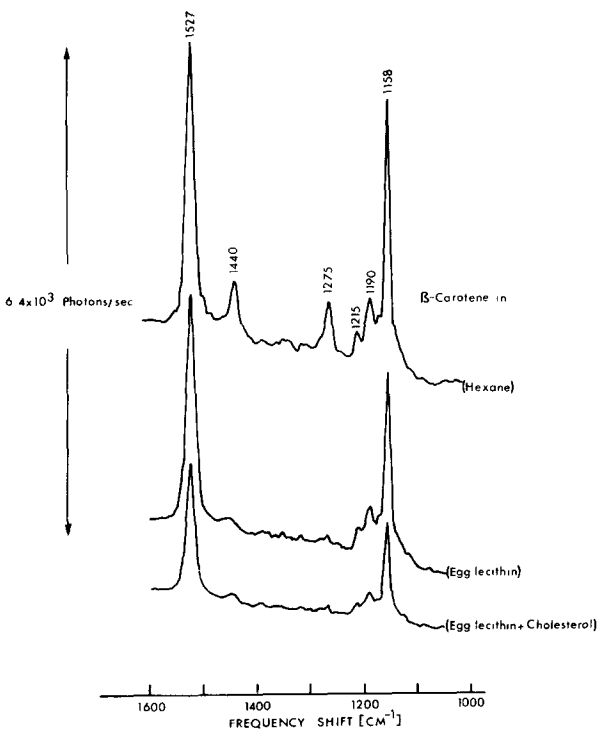

Fig. 1. Resonance-enhanced Raman spectra of β-carotene in *n*-hexane and egg lecithin liposomes with or without cholesterol. Excitation wave length, 488 nm; power, 70 mW; resolutions, approx. 3 cm^{-1}. Raman intensities are in photons/s. (Full scale = $6.4 \cdot 10^3$).

RESULTS

β-Carotene in organic solvents

As reported by Rimai and associates [9], β-carotene in hexane solution exhibits Raman scattering bands at 1527, 1215, 1190 and 1158 cm^{-1} (Fig. 1). The peaks at 1527 and 1158 cm^{-1} are resonance enhanced and have been assigned to the double-bond stretching mode ν(–C=C–) and single-bond stretching mode ν(=C–C=), respectively, of the conjugated double-bond system. Resonance enhancement is prominent because the electronic absorption band of the conjugated double-bond system (λ_{max} = 476 nm) markedly overlaps the wavelength of the exciting light (488 nm).

The CH deformation band at 1440 cm^{-1} was used as an internal reference to evaluate the variation of the 1527 cm^{-1} band with β-carotene concentration. For this we integrated the scattering intensity between 1510 and 1560 cm^{-1} for the 1527 cm^{-1} band, and between 1430 and 1470 cm^{-1} for the 1440 cm^{-1} band. We find that the log of the ratio of the integrated intensities (I_{1527}/I_{1440}) increases linearly with β-carotene concentration. The relative intensities of the 1527 and 1158 cm^{-1} bands do not change with β-carotene concentration. The intensities of both bands stay constant during 20 min irradiation.

We have evaluated the intensity variation of the 1527 and 1158 cm^{-1} bands with solvent composition by using varying proportions of chloroform and methanol. The results, illustrated in Fig. 2, show that the scattering intensity (relative to the intensity of the CH deformation band) decreases with increasing dielectric constant. The relative intensities of the 1527 and 1158 cm^{-1} bands stay constant at different proportions of chloroform and methanol.

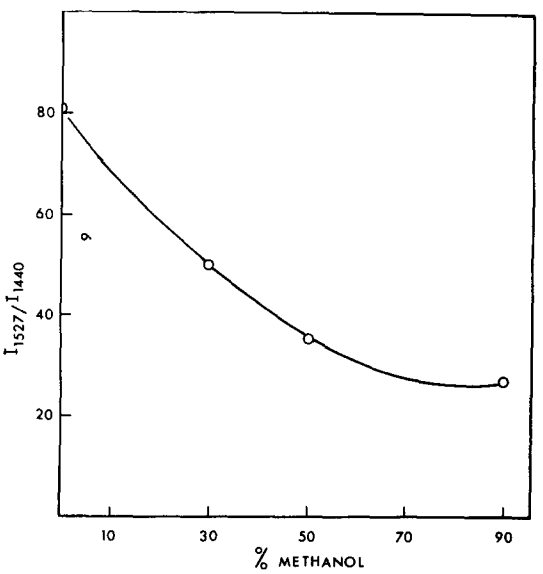

Fig. 2. The ratio of the intensity integrals between 1510–1560 cm^{-1} (I_{1527}) and 1430–1470 cm^{-1} (I_{1440}) as a function of the proportion of methanol (% methanol) in chloroform. The points marked in the curve are an average value of at least three recordings.

β-Carotene in egg lecithin liposomes

The resonance-Raman spectrum of β-carotene in egg lecithin liposomes is shown in Fig. 1. The peak positions of the resonance-enhanced bands, 1527 and 1158 cm^{-1}, are identical to the position in hexane.

β-Carotene and lecithin-cholesterol liposomes

Incorporation of increasing proportions of cholesterol into the liposomes progressively decreases the intensities of the 1527 and 1158 cm^{-1} β-carotene bands. This is illustrated in Fig. 3, which gives the ratio of the integrated intensity of the 1527 cm^{-1} band to the integrated intensity of the CH deformation band near 1450 cm^{-1}. Since the CH deformation band of lecithin with or without cholesterol is less intense than that of hexane, we used integration intervals of 1500 to 1560 cm^{-1} for the 1527 cm^{-1} band and 1430 to 1470 cm^{-1} for the CH deformation band. The relative intensity of the 1527 cm^{1-} band decreases linearly up to a cholesterol: phospholipid molar ratio of 0.8–0.9, leveling off at 7 % of the value without cholesterol. The relative intensities of the 1527 and 1158 cm^{-1} bands remained unchanged.

This suggested the possibility that β-carotene is extruded into the aqueous phase in the presence of cholesterol and is thereupon oxidated [10]. We therefore prepared aqueous cholesterol: β-carotene codispersions using molar ratios corresponding to those in the liposomes, using the same suspension method as for liposomes. The intensity of the 1527 cm^{-1} band (relative to the CH deformation band was equivalent to that found with cholesterol: lecithin liposomes at cholesterol: phospholipid molar ratios > 0.8.

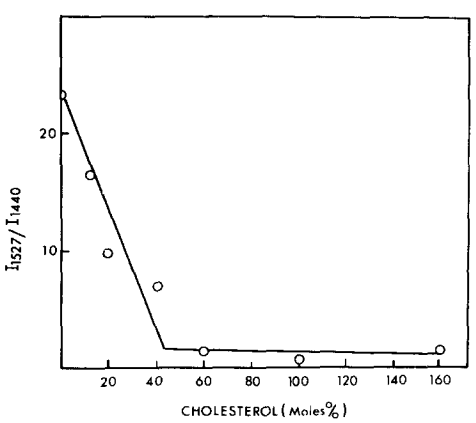

Fig. 3. The ratio of the intensity integrals between 1500–1560 cm^{-1} (I_{1527}) and 1430–1470 cm^{-1} (I_{1440}) as a function of cholesterol proportion in egg lecithin-β-carotene-cholesterol liposomes.

Erythrocyte ghosts

Erythrocyte ghosts prepared from freshly drawn human blood exhibit sharp peaks at 1530 and 1165 cm^{-1} (Fig. 4). As we have already demonstrated [1], these are resonance-enhanced bands arising from membrane-associated carotenoid molecules. The intensity ratio 1530 cm^{-1}/1165 cm^{-1} is 1.0, compared with 1.2–1.3 for β-carotene in hexane or lecithin liposomes (Table I). In lipid extracts of ghosts, redissolved in chloroform, the resonance-enhanced bands lie at 1527 and 1158 cm^{-1} (Table I).

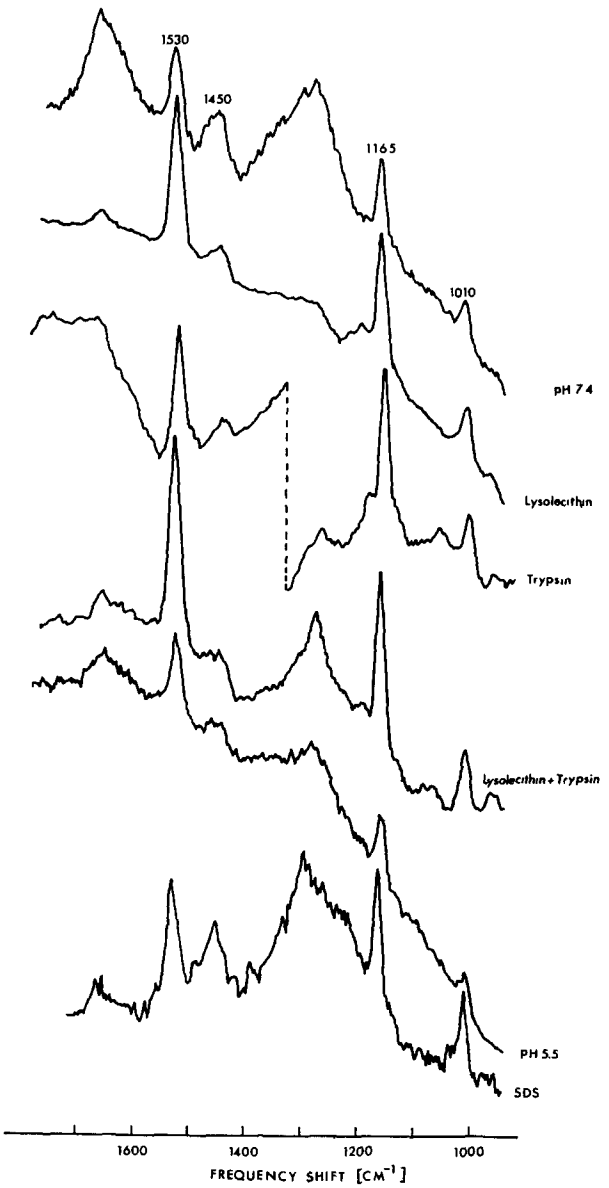

Fig. 4. Raman spectra of native erythrocyte (pH 7.4) and membranes perturbed as marked. Excitation wave length, 488 nm; power, 200 mW; resolution, approx. 6 cm^{-1}. Dotted vertical line in trypsin plus ghost spectrum indicates change in the background for purpose of presentation done in the replotting.

We have previously shown by spin-label techniques that the organization of erythrocyte membrane lipids can be altered by lowering pH, as well as by trypsinization and lysolecithin treatment [8]. These manipulations, as well as heat treatment and exposure to sodium dodecyl sulfate also alter many features of the Raman spectrum

TABLE I

RELATIVE INTENSITIES OF THE RESONANCE-ENHANCED ν(–C=C–) and ν(=C–C=) BANDS IN ERYTHROCYTE GHOSTS, LECITHIN LIPOSOMES AND ORGANIC SOLVENTS, AND VARIATION OF THEIR INTENSITY IN ERYTHROCYTE GHOSTS UNDER DIFFERENT CONDITIONS

ν(–C=C–) and ν(=C–C=) are at 1527 and 1158 cm^{-1}, respectively, with β-carotene in chloroform or hexane solution, liposomal suspensions and ghost-lipid extracts in chloroform, and at 1530 and 1165 cm^{-1}, respectively in ghosts.

System	Intensity ratios (at listed frequencies (cm^{-1}))			
	1530 : 1010	1530 : 1450	1165 : 1010	1530 : 1165
Ghost				
control (pH 7.4)	2.3	1.8	2.5	1.0
pH 5.5	3.5	4.6	3.0	1.3
plus lysolecithin	3.6	6.4	3.0	1.3
trypsinized	3.0	8.0	4.0	0.8
trypsin plus lysolecithin	3.6	7.0	3.1	1.1
plus sodium dodecyl sulfate	1.2	1.5	1.5	0.9
50 °C	1.2	1.3	1.3	1.0
Ghost-lipid extract	–	–	-	1.4
Egg lecithin plus β carotene			–	1.2
β-Carotene (organic solvents)	–	–	–	1.3

of the membranes, including the carotenoid bands but do not influence the intense scattering peak at 1010 cm^{-1} due to tryptophan and phenylalanine. The responses of the 1530 and 1165 cm^{-1} bands to these perturbations is thus conveniently monitored by use of the 1010 cm^{-1} band as an internal reference.

As shown in Fig. 4 and Table I, reduction of pH from 7.4 to 5.5, increases the relative intensities of the 1530 and 1165 cm^{-1} bands from 2.3 and 2.5 to 3.5 and 3.0, respectively. Similarly, addition of lysolecithin (100 μg/mg ghost protein) markedly increase the relative intensities of the 1530 and 1165 cm^{-1} bands (Fig. 4, Table I). Equivalent trends are found using the intensity ratio 1530 cm^{-1}/1450 cm^{-1}.

In contrast, equilibration of the ghosts for 15 min at approx. 50 °C and addition of sodium dodecyl sulfate (10 μg/mg ghost protein) drastically decrease the intensities of the two carotenoid bands (Fig. 4, Table I).

DISCUSSION

We have previously presented evidence that the 1530 and 1165 cm^{-1} Raman bands of erythrocyte ghosts can be assigned to resonance-enhanced ν(–C=C–) and ν(=C–C=) stretching vibrations arising from minute amounts of conjugated polyene, probably carotenoid in character [1]. Similar resonance-enhanced Raman bands have been reported for frog sciatic nerve [11].

We suspect that the carotenoid involved is β-carotene, rather than vitamin A for the following reasons: (a) Lipid extracts of erythrocyte ghosts exhibit ν(–C=C–) and ν(=C–C=) bands at 1527 and 1158 cm^{-1}, respectively, the frequencies found with β-carotene [9]. (b) In ghosts, their lipid extracts and β-carotene [9] the intensity ratio $I(\nu(\text{–C=C–})/I(\nu(\text{=C–C=}))$ lies near unity, whereas it is approx. 2.5 in vitamin

A and its derivatives, due to the low intensity of the ν(=C–C=) band [3–5]. (c) While the ν(–C=C–) band of protein-bound vitamin A (or vitamin A derivatives) lies near the frequency found in β-carotene or ghosts (1530 cm^{-1}) the intensity ratio $I(\nu(\text{–C=C–}))/I(\nu(\text{=C–C=}))$ as increases further, e.g. to $\simeq 10$ in rhodopsin [12] and to $\simeq 4.5$ in bacteriorhodopsin [13]; this is due to the still lower relative intensity of the ν(=C–C=). (d) While the position of the ν(=C–C=) band in the case of free vitamin A and its analogues corresponds to that found in ghosts (1161–1170 cm^{-1}; [3–5]), its position is very variable in rhodopsin (1153 cm^{-1}; [12]) or bacteriorhodopsin (1168–1172 cm^{-1}; [13]) and its relative intensity is always much less than observed in ghosts. (e) Depending on conditions the ν(=C–C=) peaks of β-carotene can shift to frequencies slightly higher than 1158 cm^{-1}.

For these reasons, we have compared the resonance-Raman spectra of β-carotene codispersed with egg lecithin containing from 0 to 50 mol % cholesterol. As the cholesterol: phospholipid ratio increases, the intensity of both resonance-enhanced carotenoid bands diminishes to the same level observed with aqueous codispersions of β-carotene (10^{-5} M) and cholesterol. We suspect therefore that liposomes containing equimolar proportion of cholesterol and lecithin cannot accomodate β-carotene, and the carotenoid is in such cases restrained to the aqueous phase and there oxidized. However, the cholesterol: phospholipid ratio in ghosts is near unity, and the carotenoid is not extruded therefrom during the repeated washing required for ghost preparation. Moreover, the carotenoid resonance bands intensify after several membrane perturbations, indicating that their intensity in native membranes actually underestimates the carotenoid present. We, therefore, suggest that cholesterol and β-carotene do not share the same locations in the ghost membranes.

We can interpret the decreased intensity of the ν(–C=C–) and ν(=C–C=) bands after exposure to sodium dodecyl sulfate, or after heating at 50 °C, to gross disruption of membrane structure and consequent oxidation of the conjugated double-bond system. The enhancement of the ν(–C=C=) bands by pH reduction, trypsinization and lysolecithin treatment in contrast, suggest a change in electronic absorption secondary to manipulations known to reduce the constraints on lipid mobility that exist in native membranes [6–8]. This could be due to a change in microenvironment and/or polyene chain configuration [14–16]. Recent data [17] show that the intensity of the carotenoid bands also change reversibley with a thermotropic lipid state transition occurring in erythrocyte membranes at approx. 19 °C.

Concerning the origin of the membrane-bound β-carotene, one should consider serum high density lipoprotein since recent data [18] indicate that this is an important component of erythrocyte membranes and since nearly 80 % of serum β-carotene is associated with high density liproprotein [19].

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