

# Evidence for Metastability in Stearoylsphingomyelin Bilayers<sup>†</sup>

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**ABSTRACT:** The physical properties of aqueous stearyl-sphingomyelin dispersions have been investigated by differential scanning calorimetry, fluorescence spectroscopy, and X-ray diffraction. These studies indicate that the thermodynamically most stable gel state of this sphingomyelin is more highly ordered than the corresponding form of other phospholipids. This gel undergoes a transition to a liquid-crystalline

conformation at 57 °C which is not rapidly reversible. Instead, the liquid-crystalline sphingomyelin supercools below 57 °C until a metastable gel is formed at 44 °C. The metastable gel slowly converts to the more highly ordered gel phase in an exothermic process at room temperature. The presence of various lipophilic substances enhances the stability of the metastable gel relative to the highly ordered form.

**A**lthough sphingomyelins comprise a significant fraction of the phospholipids in many tissues, their physical properties have only infrequently been investigated. Furthermore, most previous investigations utilized sphingomyelins from natural sources exhibiting heterogeneity in composition (Oldfield & Chapman, 1972; Shipley et al., 1974; Barenholz et al., 1976; Demel et al., 1977; Untracht & Shipley, 1977; Schmidt et al., 1977; Calhoun & Shipley, 1979a). To understand better the behavior of this class of phospholipids, physical studies were initiated with synthetic sphingomyelins which are homogeneous in fatty acid composition (Barenholz et al., 1976; Estep et al., 1979; Calhoun & Shipley, 1979a).

Previous experiments showed that DL-erythro-N-stearoylsphingosinephosphorylcholine (stearoylsphingomyelin)<sup>1</sup> displayed anomalous thermotropic behavior. For example, Barenholz et al. (1976) found a reproducible exotherm at 30 °C in differential scanning calorimeter thermograms for aqueous dispersions of this lipid, an effect not seen with other synthetic sphingomyelins. Further, the transition temperature and enthalpy change of the gel to liquid-crystalline phase transition were higher than the corresponding parameters of sphingomyelins containing shorter (palmitoyl) or longer (lignoceryl) fatty acid chains. In this paper we report the results of a systematic investigation of this anomalous thermotropic behavior. These results indicate that this lipid may exist in one or more metastable configurations at physiological temperatures and that the equilibrium gel conformation results in the formation of an unusually highly ordered phase.

## Experimental Procedures

**Materials.** DL-erythro-N-Stearoylsphingosinephosphorylcholine and DL-erythro-N-palmitoylsphingosinephosphorylcholine (palmitoylsphingomyelin) were supplied by Professor D. Shapiro (Weizmann Institute of Science, Rehovot, Israel) and Dr. C. F. Schmidt (Department of Biochemistry, University of Virginia, Charlottesville, VA). The lipid was purified by column or thin-layer chromatography on silicic acid and by washing in a biphasic solvent system containing chelating

Table I: Sphingomyelin Base Composition

sphingomyelin	mol % base	
	sphingosine	dihydro-sphingosine
N-palmitoylsphingosine-phosphorylcholine	86	14
N-stearoylsphingosine-phosphorylcholine		
57 °C endotherm preparation	85	15
44 °C endotherm preparation	81	19
44 °C endotherm-top <sup>a</sup>	78	22
44 °C endotherm-bottom <sup>a</sup>	85	15

<sup>a</sup> Samples resulting from separate purification of top and bottom halves of the major TLC spot from a preparation showing a 44 °C endotherm.

agents (Estep et al., 1978). The washing was followed by acetone precipitation or a recrystallization procedure adapted from that of Albon & Sturtevant (1978). To initiate recrystallization, we added 4 volumes of spectral grade hexane to 2.5% (w/v) of sphingomyelin in 7:1 (v/v) chloroform-methanol. The initially clear solution was allowed to evaporate under atmospheric pressure at room temperature for several days with the original volume being maintained by the periodic addition of hexane. The granular precipitate was then pelleted by centrifugation, the supernatant removed, and the residual solvent evaporated with a stream of nitrogen. The recrystallization was repeated and the lipid was dried at 40 °C over phosphorus pentoxide in vacuo. Sphingomyelin was stored in 7:1 (v/v) chloroform-methanol at -20 °C.

Previous analyses have shown that the stearyl-sphingomyelin contains more than 99.8% of the desired fatty acid (Barenholz et al., 1976). The amount of dihydrosphingosine base was assessed in several preparations by the methods of Gaver & Sweeley (1965) and Carter & Gaver (1967) except that a 1.9-m glass column packed with 3% OV-17, 100-120 mesh, Gas-Chrom Q support (Applied Science Laboratories, State College, PA) was used for gas-liquid chromatographic analysis. With a column temperature of 172 °C and a helium flow rate of 60 mL/min, the retention times for sphingosine and dihydrosphingosine (Sigma Chemical Co., St. Louis, MO) after silylation (SIL-PREP, Applied Science Laboratories) were approximately 21 and 24 min, respectively. The results of this

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<sup>1</sup> Abbreviations used: TLC, thin-layer chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene; stearyl-sphingomyelin, DL-erythro-N-stearoylsphingosinephosphorylcholine; palmitoylsphingomyelin, DL-erythro-N-palmitoylsphingosinephosphorylcholine.

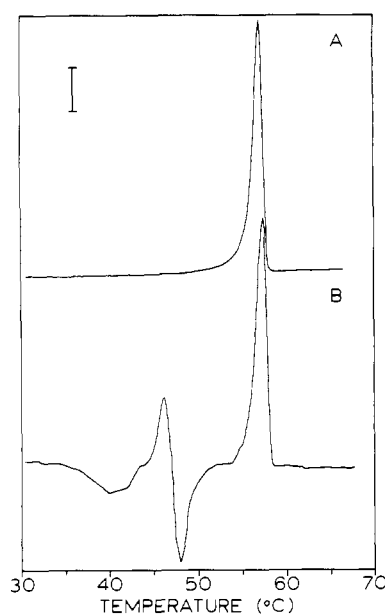


FIGURE 1: Calorimetric heating scans (scanning rate of 15 °C/h) of aqueous dispersions of stearyl sphingomyelin. Lipid concentration was approximately 10 mM. The vertical bar represents 2 kcal mol<sup>-1</sup>. See the text for details.

analysis are presented in Table I.

Stearyl sphingomyelin was also analyzed by thin-layer chromatography (TLC) on silica gel plates developed in 65:25:3.5:1.5 (v/v) chloroform-methanol-ammonia-water. Lipids were visualized by exposure to iodine vapor or phosphate stain (Vaskovsky & Kostetsky, 1968). The TLC chromatograms so obtained will be discussed under Results.

Cholesterol was purchased and purified as described previously (Estep et al., 1978). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was zone-refined Aldrich (Milwaukee, WI) Puriss grade.

**Methods.** The protocol for the preparation of aqueous lipid dispersions and the subsequent analysis by high-sensitivity scanning calorimetry was reported previously (Estep et al., 1978, 1979; Suurkuusk et al., 1976). In dispersions prepared for calorimetric analysis containing DPH, the precise concentration of fluorescent probe was assessed after completion of the experiment from absorbance of sample extracts dissolved in benzene by using an extinction coefficient of  $5 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup> at 380 nm. Several calorimetric scans were also performed with a Perkin-Elmer (Norwalk, CO) DSC-2 calorimeter as described by Calhoun & Shipley (1979a,b).

The temperature-dependent fluorescence anisotropy of DPH dissolved in sphingomyelin bilayers was measured and corrected for scattering as described by Lentz et al. (1976).

X-ray diffraction measurements on dispersions of sphingomyelin in excess aqueous 50 mM KCl were performed by using nickel-filtered Cu K $\alpha$  radiation (1.5418-Å wavelength) from an Elliot GX-6 rotating anode X-ray generator focused by cameras using either toroidal mirror (Elliot, 1965) or double mirror (Franks, 1958) optics. Samples were sealed in thin-walled capillaries (Charles Supper Co., Natick, MA) in a temperature-controlled sample holder. Diffraction patterns were recorded on Ilford Industrial G X-ray film, and the intensity distribution was derived by using a Joyce-Loebl Model 111-CS scanning microdensitometer.

## Results

In Figure 1 are displayed two increasing temperature scanning calorimeter thermograms of a sample of pure

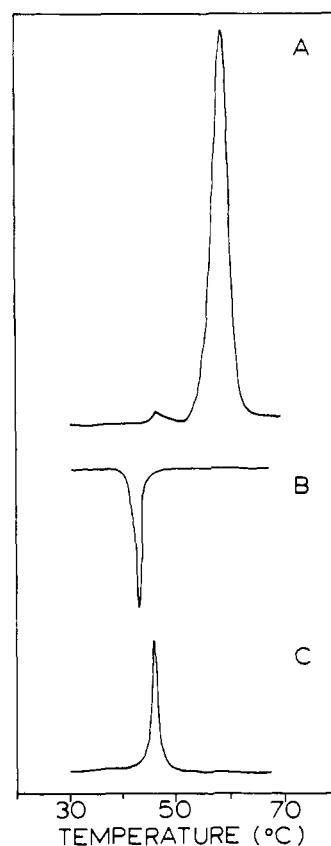


FIGURE 2: Successive calorimetric scans of an aqueous dispersion of stearyl sphingomyelin using the Perkin-Elmer instrument. The lipid concentration was approximately 50 wt % and the scanning rate 300 °C/h. (A) and (C) were recorded while heating the sample after holding at room temperature for greater than 2 h and less than 20 min, respectively. (B) was recorded while cooling the sample between scans (A) and (C).

stearyl sphingomyelin dispersed in excess 50 mM KCl. Thermogram 1A, typical for samples held at room temperature several hours prior to scan initiation, exhibits a single endotherm with an apparent heat capacity maximum at 57 °C and an associated enthalpy change of 20 kcal/mol. On several occasions, however, the 57 °C peak was preceded by an endotherm at 45 °C and one or more exotherms extending over the 35–50 °C temperature interval as shown in Figure 1B. Although it is difficult to reproduce such scans quantitatively, they tend to occur when heating is begun immediately after the sample has been cooled from temperatures above 57 °C. At times a small exotherm at 30 °C similar to that previously reported by Barenholz et al. (1976) was also observed. This behavior has not been observed with palmitoyl- or lignocerylsphingomyelin.

Several experiments were also performed with a Perkin-Elmer DSC-2 calorimeter which is capable of scanning in both cooling and heating modes. These scans were performed at a rate of 300 °C/h as compared to 15 °C/h with the high-sensitivity calorimeter. It is thus possible that the curves do not represent true heat capacity functions. Scans from one such sequence of experiments with stearyl sphingomyelin are shown in Figure 2. Scan 2A was obtained after the sample had been held at room temperature for several hours. A large 57 °C endotherm and a much smaller 46 °C endotherm are evident. A cooling scan of the same sample exhibited only an exotherm at about 44 °C. A subsequent heating scan, performed within minutes after cooling from above 57 °C, displayed only an endotherm at 46 °C. If the sample was maintained at 20 °C for several hours, the heating thermo-

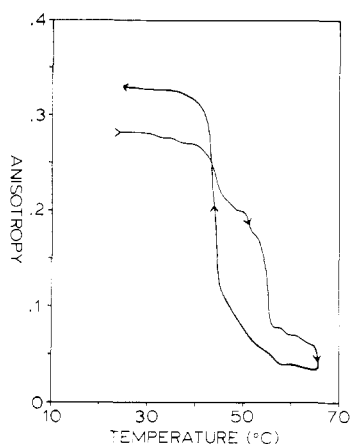


FIGURE 3: Fluorescence anisotropy of DPH dissolved in stearyl-sphingomyelin bilayers as a function of temperature. The lipid to dye mole ratio was approximately 1000:1. The lipid was incubated for more than 20 h before measurements were initiated. Arrowheads denote the direction of temperature change for each graph segment.

dynamics exhibited only the 57 °C endotherm.

Effects similar to those observed by calorimetry are seen by changes in the fluorescence anisotropy of DPH-labeled stearyl-sphingomyelin dispersions as shown in Figure 3. Samples incubated at room temperature for about 20 h exhibit two regions of rapid anisotropy change centered at 43 and 54 °C upon heating. Samples maintained at 65 °C for 90 min show only the transition at 43 °C upon cooling and when heated after being held at room temperature for several hours. While these results are not dependent upon scanning rate, it is necessary in some cases to hold dispersions at 20 °C for several days in order to observe the 54 °C transition. The difference in anisotropy below 40 °C for the heating and cooling runs depicted in Figure 3 is of unknown significance since it is not consistently observed.

The fluorescence experiments suggested that the presence of other compounds in the dispersion might stabilize the structure giving rise to the lower temperature endotherms. Dispersions of stearyl-sphingomyelin containing small amounts of cholesterol were also examined. The stearyl-sphingomyelin used in this experiment normally exhibited only a 57 °C endotherm on heating after being held at room temperature for several hours. The addition of small amounts of cholesterol results in the frequent appearance of endotherms in the 43–47 °C temperature interval as well as an exotherm in the 30–55 °C range.

In analogous studies with palmitoyl- and lignoceryl-sphingomyelin, the addition of small amounts of cholesterol or DPH caused modifications of the sphingolipid gel to liquid-crystalline phase transitions which were minor and insensitive to sample thermal history (Estep et al., 1979; B. R. Lentz, personal communication).

Although exact reproducibility of the thermotropic behavior of stearyl-sphingomyelin is difficult to obtain, several general conclusions can be drawn. First, the appearance of a 57 °C endotherm is more probable the longer a sample is held at 20 °C prior to scan initiation. Second, the enthalpy change corresponding to the 57 °C endotherm is 20 kcal/mol although it is always less when this peak is preceded by another endotherm in the 43–47 °C range. As shown in Figure 5 this enthalpy change is more than twice that observed for other phospholipids. Third, the total enthalpy change of the 43–47 °C endotherm never exceeds 7 kcal/mol. Finally the 57 °C endotherm is always seen in thermograms in which an exothermic process is detected. These observations also pertain

to samples containing a higher proportion of cholesterol. The thermotropic behavior of different synthetic preparations of stearyl-sphingomyelin varied and appeared to be dependent upon the presence of a contaminant in the preparation. One preparation exhibited a double endotherm at 44 °C which is similar to that seen in samples containing small amounts of DPH or cholesterol. No other endotherms were seen with this preparation even when the sample was held at room temperature for several days prior to heating. If, however, this sample was held at 4 °C for 2 months, the initial heating thermogram showed a 57 °C endotherm. Upon cooling and reheating, we found that only the low-temperature peaks were evident.

TLC analysis of this particular preparation indicated an impurity which was apparently a byproduct of the synthetic procedure. The identity of this impurity is unknown, although it migrates slightly faster than sphingomyelin on silica gel. By comparison, the sphingomyelin used for the experiments shown in Figures 1–4 contained only a trace of the faster moving material. This faster component, only observed under conditions of heavy loading (0.5 mg), comprised about 1% of total material as based on phosphate analysis. A similar faster moving component was also found in synthetic palmitoyl-sphingomyelin. It should be emphasized that the purity of material used in experiments shown in Figures 1–4 was at least as good as any commercially available synthetic phosphatidylcholine (B. R. Lentz, Y. Barenholz, and T. E. Thompson, unpublished results) and represents the highest purity currently attainable for synthetic sphingomyelins.

The various sphingomyelin preparations were also analyzed with respect to their long-chain base composition. The results are given in Table I. Only C<sub>18</sub>-sphingosine or C<sub>18</sub>-dihydro-sphingosine was found in these samples, with the dihydro-sphingosine content being higher in preparations which consistently exhibited the lower temperature transitions. However, no quantitative correlation between the occurrence of the high- and low-temperature endotherm and dihydro-sphingosine content was found.

On the basis of these results, a working hypothesis was formulated. This hypothesis assumes that the structural states achieved upon heating through the 43–47 °C transition and the 57 °C transition are identical. Since  $\Delta S = \Delta H/T$ ,  $\Delta S$  of the 43–47 °C transition is approximately one-third the  $\Delta S$  associated with the 57 °C transition. Assuming that the liquid-crystalline state obtained after either the low- or high-temperature transition is the same implies that the low-temperature state exhibiting the 43–47 °C transition is of higher entropy and thus more disordered than the state exhibiting the 57 °C transition.

X-ray diffraction patterns of fully hydrated sphingomyelin dispersions made from the two different synthetic preparations confirmed this hypothesis. The diffraction patterns obtained at 21 °C from samples giving rise exclusively to 43–47 °C or 57 °C endotherms are compared in Figure 4. Figure 4A shows the diffraction pattern from stearyl-sphingomyelin which exhibited only the lower temperature endotherms. The diffraction pattern obtained for the gel state shows a series of low-angle diffraction lines in the ratio 1:1/2:1/3:1/4... and a sharp wide-angle line at 4.2 (Å)<sup>-1</sup>. This is typical of a fully hydrated gel-phase phospholipid in a lamellar configuration with "hexagonal" chain packing. This pattern is quite similar to those reported previously for bovine brain sphingomyelin and palmitoyl-sphingomyelin (Shipley et al., 1974; Calhoun & Shipley, 1979b). The diffraction pattern arising from a stearyl-sphingomyelin sample which displayed only a 57 °C transition is shown in Figure 4B. Reflections consistent with

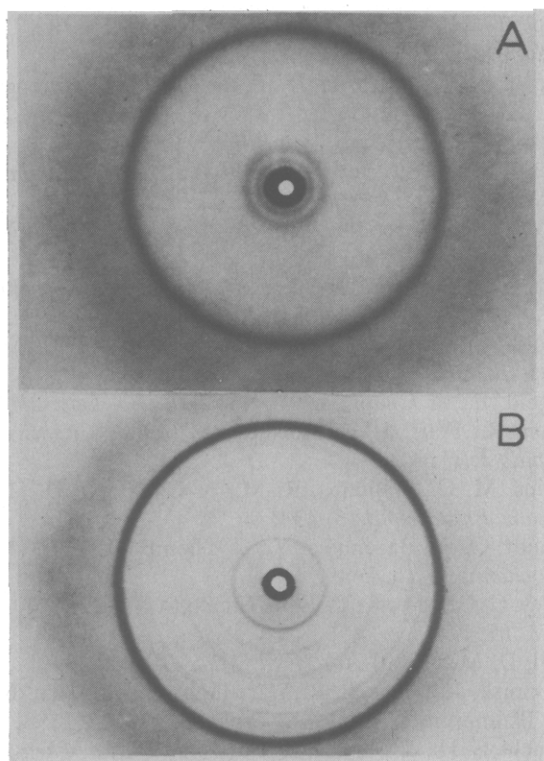


FIGURE 4: X-ray diffraction patterns at 21 °C of stearyl sphingomyelin dispersed in excess 50 mM KCl. Pattern A was from a preparation which normally exhibited endotherms only at 43–47 °C during heating while pattern B was from a preparation which displayed only 57 °C endotherms under the same conditions.

a lamellar phase, but with a repeat distance less than that shown in Figure 4A, are still observed. In the wide-angle region a number of sharp diffraction lines are observed, suggesting that the in-plane packing of the hydrocarbon chains is more ordered than that of the typical phospholipid gel phase. These X-ray diffraction data clearly show that there are distinct conformational differences between the samples. Interestingly, when a sample identical with that giving rise to the pattern shown in Figure 4A was held at 4 °C for 2 months, an X-ray diffraction pattern similar to that shown in Figure 4B was obtained, and subsequent calorimetric analysis of the sample showed a large 57 °C endotherm.

### Discussion

The changes in DPH fluorescence anisotropy and the enthalpy changes of the low- and high-temperature transition of stearyl sphingomyelin are consistent with the occurrence of a gel to liquid-crystalline phase transition. The data reported may be rationalized by the following hypothesis. The gel to liquid-crystalline phase transition of pure, fully hydrated stearyl sphingomyelin maintained in thermodynamic equilibrium occurs at 57 °C. Upon decreasing the temperature from 57 °C, the sphingomyelin supercools as a liquid-crystalline phase which undergoes a transition to a metastable gel phase at about 44 °C. Both the supercooled liquid-crystalline phase and the metastable gel phase are thermodynamically unstable relative to the equilibrium gel state at temperatures below 57 °C. However, kinetic barriers to the formation of the stable gel are large, and the presence of impurities impedes its formation. The association of the 44 °C endotherm with the existence of a metastable gel state is consistent with the correlation of exotherms below 44 °C with the appearance of the 57 °C transition, since heat evolution is indicative of the formation of a stable state from a kinetically trapped inter-

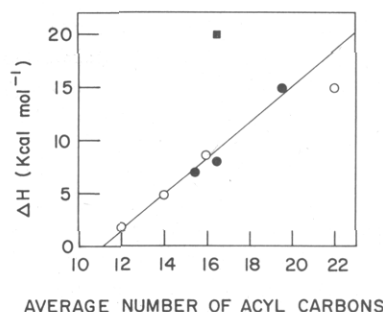


FIGURE 5: Enthalpy change during the gel to liquid-crystalline phase transition for (O) phosphatidylcholines and (●, ■) sphingomyelins having linear, saturated hydrocarbon chains vs. the average number of methylene carbons in the apolar chains of the molecules. The data for phosphatidylcholines are for species of identical chain length and are taken from Lentz et al. (1978) and Phillips et al. (1969). Sphingomyelin data are taken from Barenholz et al. (1976), Estep et al. (1979), and the present work.

mediate. Although, to our knowledge, no other naturally occurring phospholipid has shown such metastability, a number of biologically important triglycerides and cholesterol esters exhibit similar properties (Perron et al., 1969; Small, 1970; Loomis et al., 1979).

The apparent stabilization of the metastable gel structure by impurities is probably due to preferential accommodation of the impurities within the more disordered structure. Whether this results in a decrease in the Gibbs energy difference between the stable and metastable conformations and/or a change in the kinetics of interconversion is not known. This effect is not particularly specific since both DPH and cholesterol, as well as an unidentified substance detectable by TLC, promote its occurrence.

A question may be raised as to whether pure stearyl sphingomyelin exhibits metastability. All of the samples examined have shown this property to some extent, yet each sample contains a mixture of materials. No definitive answer to this question is possible until the nature of all substances present has been elucidated and the impurities have been removed. Nevertheless, even if pure stearyl sphingomyelin does not display metastability, the sensitivity of this lipid to the presence of other compounds is of interest. It is worth reemphasizing that dispersions of the closely related palmitoyl- and lignoceryl sphingomyelins containing comparable amounts of DPH, cholesterol, or endogenous impurities do not exhibit metastability.

According to the X-ray diffraction results, the stable gel conformation of the stearyl sphingomyelin dispersion is considerably more ordered than that of other phospholipids, including other sphingomyelins. This fact is underscored by a comparison of the enthalpy changes during the gel to liquid-crystalline phase transitions of various phospholipids. In Figure 5 these enthalpy changes are plotted as a function of the average hydrocarbon chain length for three synthetic sphingomyelins and several saturated phosphatidylcholines. The enthalpy changes for the saturated phosphatidylcholines are a nearly linear function of chain length. The enthalpy changes for palmitoyl- and lignoceryl sphingomyelin and the 43–47 °C transition of stearyl sphingomyelin form a similar series. However, the enthalpy change associated with the 57 °C transition of stearyl sphingomyelin is anomalously high. This result is consistent with the conclusion that the stable gel form is an unusually ordered structure.

It is not possible, with the data in hand, to establish unequivocally the molecular differences between the two gel forms. The principal difference may be due to variation in the degree

of hydration or the degree of hydrogen bonding between adjacent sphingomyelin molecules. In any case, changes in these or similar interactions during the gel-liquid-crystalline transitions must account for the differences in  $\Delta H$  and  $\Delta S$  for the two forms.

Brandts et al. (1978) have observed several irreversible transitions above 50 °C in human erythrocyte ghosts, at least some of which involve phospholipid. It is possible that some of these transitions may appear to be irreversible because they are transitions of the sort observed with stearyl-sphingomyelin. This possibility is strengthened by the fact that the content of C<sub>18</sub>-sphingolipids in this membrane is quite high.

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## Molecular Topography of the Phycocyanin Photoreceptor from *Chroomonas* Species†

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**ABSTRACT:** Molecular topology of a light-harvesting phycobiliprotein, phycocyanin from *Chroomonas* species of cryptomonad algae, was investigated by using spectroscopic techniques. The phycocyanin consists of four subunits of the form  $\alpha\alpha'\beta_2$ . The two small subunits,  $\alpha$  and  $\alpha'$ , cannot be differentiated by their molecular weights as determined by conventional methods such as sodium dodecyl sulfate gel electrophoresis and gel chromatography; the large subunit contains two chromophore molecules, one of which is phycocyanobilin. A dipole-dipole exciton coupling model was applied to interpret spectral properties of *Chroomonas* phycocyanin. Two main circular dichroic extrema, one with a strong negative band and the other with a positive band, are probably due to the result of exciton coupling of two phycocyanobilin molecules contained in the two  $\beta$ -subunit polypeptides. The visible absorption

spectrum of *Chroomonas* phycocyanin with two main peaks at 645 and 583 nm was analyzed to yield five spectral components with their maxima at 573, 592, 617, 636, and 652 nm. The 592- and 652-nm bands are the split bands of phycocyanobilins in the  $\beta$  subunit due to exciton coupling, while the other three bands correspond to three types of noninteracting chromophores, with the 573-nm band assigned to an unknown bilin chromophore in the  $\beta$  subunit, the 617-nm band assigned to phycocyanobilin in the  $\alpha$  subunit, and the 636-nm band assigned to the chromophore in the  $\alpha'$  subunit. On the basis of the proposed exciton coupling model and the fluorescence excitation polarization spectrum, a tentative molecular topography of *Chroomonas* phycocyanin is described in terms of spectroscopic parameters.

**O**n the basis of their color, there are two types of phycobilin photosynthetic accessory pigments. The red-colored phycocyanins absorb light in the 550–600-nm region, and the blue-colored phycocyanins, including allophycocyanin, show maximum absorption in the 600–650-nm region (O'Carra & O'hEocha, 1976; Bogorad, 1975; Glazer, 1976).

Of all the phycobiliproteins, the cryptomonad biliprotein has been known to display some properties distinctive from the rest. The most important distinctions are its location and aggregation in cells (Dodge, 1969; Gantt et al., 1971; Faust & Gantt, 1973). The biliproteins from blue-green and red

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