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CHLOROPHYLL a INTERACTIONS WITH CHLOROPLAST LIPIDS *in vitro*

Terry Trosper and Kenneth Sauer

February 26, 1968

Chlorophyll *a* Interactions with Chloroplast Lipids *in Vitro*[*]

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SUMMARY

Purified chloroplast glycolipids--galactosyldiglycerides and sulfoquinovodiglyceride--form relatively strong complexes with chlorophyll *a*, as measured by their ability to dissociate chlorophyll dimers in carbon tetrachloride solution. The chloroplast lipids form stable monolayers at a water-nitrogen interface, with maximum packed areas of 39, 44 and 73 $Å^2$-molecule^{-1} for sulfolipid, monogalactolipid and digalactolipid, respectively. Mixed monolayers of chlorophyll *a* with sulfolipid or monogalactolipid exhibit compression behavior characteristic of ideal two-dimensional solutions.

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INTRODUCTION

That chlorophyll a is located in chloroplasts of green plant cells was recognized in the last century.[1] More recent work indicates that the pigment is contained entirely in the lamellae of these organelles.[2,3] The specific molecular environments of chlorophylls in vivo are, however, largely unknown. Results of studies by Emerson's group on complementary effects of different wavelengths of activating light in inducing chloroplast reactions provided clear evidence that the pigments in higher plants and algae are present in more than one form.[4] This has since been confirmed by a wealth of evidence from absorption, fluorescence, optical rotation and photochemical activation spectra, from differential extractions and enzyme susceptibilities, etc.[5] Part of the chlorophyll appears to be in an aggregated state and, in part, it appears to be associated with amphiphilic surface-active structural lipids.

Accordingly, we have studied the interactions of chlorophyll a with chloroplast glycolipids, mono- and digalactosyl diglyceride and sulfoquinovodiglyceride, in three-dimensional solutions and in monolayers at a nitrogen-aqueous interface. Chlorophyll a tends to dimerize in carbon tetrachloride solution,[6,7] the extent of dimerization depending on the pigment concentration and the presence of polar solvents or Lewis bases.[6-8] The spectral properties of both monomer and dimer in this solvent have been reported in detail.[7] By observing changes in absorption of mixed solutions, we are able to demonstrate that the structural lipids interact strongly with chlorophyll a in carbon tetrachloride.

Pressure-area isotherms of mixed monolayers of pigment and lipid are used to determine miscibility of the two components. Favorable comparison with theoretical curves for ideal mixing implies that complexes with new

spatial requirements are not formed. Fluorescence properties of the mixed films[9] aid in the interpretation of the results.

EXPERIMENTAL

Materials

Chlorophyll a was isolated from spinach chloroplasts by the method of Anderson and Calvin[10] and rechromatographed on sugar, if necessary, as previously described.[9]

The spreading solvent benzene (J. T. Baker or Baker and Adamson, reagent grade) was distilled from sodium hydride. Chloroform, methanol, and acetic acid were distilled immediately prior to use. Reagent grade carbon tetrachloride was taken directly from freshly opened bottles. Nitrogen was bubbled through all solvent systems before they were put in contact with the lipids.

The chloroplast structural lipids were isolated and purified by a combination of column and thin layer chromatographic procedures similar to those previously described by Rosenberg, et al.[11] The entire preparation was carried out under nitrogen gas. Once-washed spinach chloroplasts obtained according to the method of Park and Pon[12] were extracted with chloroform:methanol, 2:1 (v/v), until the residue was pinkish- or yellowish- brown. We isolated sulfolipid from the combined extracts following the method of O'Brien and Benson.[13] However, column chromatography proved unsatisfactory for separating the pigments completely from the two galactolipids. Thus these lipids were purified by thin layer chromatography of the column eluates.

Plates coated with Silica Gel G,[14] and activated 20 minutes at 110°C just prior to use, were streaked with eluate which had been concentrated by evaporation. Monogalactolipid was recovered from the last third of the chloroform:methanol, 9:1 (v/v) eluate from the first (Florisil) column, and digalactolipid from the chloroform:methanol, 2:1 (v/v) eluate from the second (DEAE) column. One plate was spotted to be used for detection of the lipid bands. We developed the plates in solvent systems suggested by Nichols.[15] Monogalactolipid separated satisfactorily in chloroform:methanol, 9:1 (v/v), whereas chloroform: methanol, 9:2 (v/v) proved to be a better solvent for the more polar digalactolipid. The spotted plate, after drying, was sprayed with 50% H_2SO_4 and charred at 180°C for 15 minutes to locate the lipids. Then the bands corresponding to the galactolipid on the other streaked plates were scraped off and the lipid eluted with chloroform:methanol, 9:1 (v/v). After evaporation to dryness the lipid residue was resuspended to a concentration of 1 mg/ml in benzene.

We checked the purity of the isolated lipids by thin layer chromatography using chloroform:methanol:acetic acid:water, 85:15:12:1 (v/v)[15] as developing solvent, and by the anthrone sugar test following hydrolysis. Despite rechromatography on thin layer, we were unable to free digalactolipid completely from chlorophyll-like contaminants, which remained at a relative concentration of approximately 0.01 mole percent. Therefore, we did not use this lipid for three-dimensional solution studies.

Methods

Anthrone sugar test. Anthrone reagent gives a green color with galactose, the maximum absorption of the rather broad band occurring at 625 nm.[15] The product formed in the presence of sulfoquinovose, however, absorbs further in the blue, having a maximum at 592 nm. The anthrone reaction is very sensitive to the conditions of the test. Because we wished to detect small quantities of sugar, we chose the following conditions based on reported procedures.[15-17] They proved sufficiently sensitive and fairly reproducible.

A stock anthrone solution, 10 mg/ml of concentrated H_2SO_4, was aged for four hours in the dark, and then stored in the refrigerator. No stock solution was kept more than two days. 200 to 500 microgram aliquots of glycolipids and 50 to 250 microgram galactose standards were hydrolyzed in 2 ml reagent grade H_3PO_4 (85%) for fifteen minutes at 90 to 95°C, and then cooled 5 minutes in ice. Then 5 ml of freshly prepared anthrone reagent (1 ml anthrone stock solution in 24 ml H_2SO_4/H_2O, 2:1) was added and the solution stirred vigorously. The mixture was heated for 12 minutes at 90 to 95°C, and then cooled in ice in the dark for 30 minutes to allow full color development. The optical density of the solutions from 520 to 700 nm was then recorded on a Cary 14 spectrophotometer, using the control solution (2 ml H_3PO_4 + 5 ml anthrone reagent heated as were the samples) in the reference compartment. Galactose standards gave an optical density ratio at the two wavelengths of interest, 592/625, of 0.75 under these test conditions. Owing to the presence of other lipid hydrolysis products, this ratio was slightly higher for the galactolipids. Sulfolipid hydrolysis products produced a ratio of 1.3 (see Fig. 1). Ratios of

the order of unity, which were obtained from lipid samples not purified by thin layer chromatography, indicated cross-contamination of sulfo- and galactolipids. The sugar concentrations of the samples were calculated from the absorbances of the known galactose standards. From these data we determined the original amount of lipid in the samples and used this as a further criterion of purity. Molecular weights computed from the structures given by Benson[18] were used in these calculations.

Absorption difference spectroscopy. Before dissolution in carbon tetrachloride, a weighed amount of dried chlorophyll a was stored overnight in the dark in a nitrogen box through which gas circulated slowly. The stock solution, 1.6×10^{-4} M, was kept in the dark under nitrogen.

Sample solutions of pigment and monogalactolipid were prepared by resuspending an aliquot of lipid, which had been evaporated to dryness, in a measured amount of stock chlorophyll solution. The sulfolipid, however, was not sufficiently soluble in the carbon tetrachloride solution to permit use of this procedure. Instead, a stock solution of sulfolipid in carbon tetrachloride was prepared and aliquots of this added to a measured amount of the stock chlorophyll a solution. This method proved satisfactory, but we could not use such large excesses of the lipid as were attainable in the experiments with the galactolipid.

Monolayer studies. Compression characteristics of pure and mixed films were observed with the monolayer fluorometer described elsewhere.[9] For measurements of pressure-area behavior and monolayer stability, the spreading solution was deposited on the subphase from a micropipette after the surface had been swept clean, the barrier positioned, and the apparatus covered and flushed with nitrogen gas. After the lapse of a

few minutes for complete evaporation of the solvent and formation of the lipid monolayer, the torsion balance was zeroed and the film compressed by movement of the barrier at a constant rate. The torsion balance was adjusted to null position and the surface pressure read every 30 seconds. Compression was stopped when the barrier reached the end of the trough, or when film collapse was indicated by a leveling off or decrease in surface pressure. If we stopped compression before collapse, the stability of the monolayer at the final pressure could be observed. In all cases, the films were re-expanded and the zero-point compression checked to ascertain that the lipid did not leak past the float or barrier during the experiment. Spreading solutions were prepared by mixing aliquots of known concentration of chlorophyll a and lipid solutions in benzene.

RESULTS

Absorption Difference Studies

Upon addition of lipid to chlorophyll a solutions in carbon tetrachloride, the absorbance showed a decrease centered at 682 nm and a concomitant increase at 663 nm. Absorptions at these wavelengths are associated with the dimer and monomer-lipid complex, respectively, and the difference spectra (Fig. 2) indicate that the concentration of monomer complex is increased at the expense of dimer upon addition of lipid. Fig. 3 shows the relative absorbance change at 682 nm, $\Delta A/A_{ref}$, where A_{ref} is the absorbance of the pigment solution without lipid, as a function of the relative amount of lipid added. The solid curve is obtained theoretically, assuming an equilibrium constant for one-to-one complex formation of 8×10^3 liters/mole. We

computed this constant, as well as the dimerization constant and the extinction coefficient of the complex at 682 nm, from the spectral data for the galactolipid system, the known total pigment and lipid concentrations, and the monomer and dimer extinction coefficients. The agreement between theory and experiment justifies the assumption that a one-to-one complex is the only new species formed. Calculations yielded $\epsilon_c = 2.3 \pm 0.07 \times 10^4$ l-mole^{-1}-cm^{-1}, $K_c = 8 \pm 2 \times 10^3$ l-mole^{-1}, based on $K_d = 4.4 \pm 1.1 \times 10^4$ l-mole^{-1} for galactolipid complexing. Chlorophyll a sulfolipid systems appear to behave similarly. We were unable to obtain sufficient data at high sulfolipid concentrations to treat these data quantitatively in the same fashion as for the galactolipids.

Monolayer Studies

The chloroplast lipids formed stable compressible monolayers of the liquid-expanded type[19] on 10^{-3}M phosphate buffer, pH 7.6, in a nitrogen atmosphere. The pressure-area curves and collapse points (Fig. 4) of freshly prepared materials were reproducible, and gave maximum packed areas of 39, 44, and 73 Å^2-molecule^{-1} $\pm$ 10% at a surface pressure of 12 dyne-cm^{-1} for sulfolipid, monogalactolipid, and digalactolipid, respectively. These values are reasonable provided one hexose moiety of the molecules extends into the aqueous subphase, thereby reducing the surface area required.

As the lipids are slightly water-soluble, we checked the stability of the monolayers with time at pressures between 10 and 13 dynes/cm. Fig. 5 is a plot of the surface pressure of a sulfolipid film maintained at constant area. The pressure fell slightly at first and then remained constant for several minutes. Monogalactolipid films at constant area maintained constant pressures in this range for over 20 minutes.

Mixed monolayers of chlorophyll a and sulfolipid or monogalactolipid, at low mole fractions of pigment, behaved essentially as pure lipid films. Pressure-area data of mixed monolayers containing larger amounts of chlorophyll a are shown in Fig. 6a and b. The dashed curves in these figures indicate theoretical compression behavior for an ideal two-dimensional solution of the two components, calculated from

$$A(\pi) = \Sigma_i x_i a_i(\pi) ,$$

where x_i is the mole fraction and $a_i(\pi)$ the area per molecule of species i at surface pressure π. The data fit the theoretical predictions within spreading error of approximately 5%. Monolayers of completely immiscible components would also obey this equation. However, such films would collapse at the lowest collapse pressure of a component, rather than reproducibly at a pressure intermediate to those of the pure components,[20] as we observed in our systems.

Mixed monolayers of monogalactolipid and chlorophyll a were stable with time at pressures of 15 dynes-cm^{-1} and below, at all concentration ratios investigated. However, when the chlorophyll a mole fraction exceeded about 0.04 in sulfolipid films, the monolayers were not reproducibly stable with time at pressures above approximately 10 dynes-cm^{-1}.

DISCUSSION

We note that our dimerization constant for chlorophyll a in carbon tetrachloride, $4.4 \pm 1.1 \times 10^4$ l-mole^{-1} is higher than the previously reported value, $1.0 \pm 0.4 \times 10^4$ l-mole^{-1}.[7] The discrepancy may be due to our storing the dried pigment under streaming dry nitrogen gas and using fresh solvent also flushed with nitrogen. This procedure may have removed some complexing water molecules otherwise present, and thus enhanced dimer formation.

The relative strengths of pigment-pigment and pigment-lipid interactions may be compared by considering the relative free energies of interaction. On this basis the chlorophyll-lipid interactions are weaker than those between chlorophyll molecules in the dimer. Sauer and Ku found that ethanol is a complexing agent of similar strength to that of the plant lipids, while the chlorophyll a-water complex is somewhat weaker.[8]

We conclude that galactolipid, and probably also sulfolipid, form strong complexes with chlorophyll a, and will compete effectively with water for the pigment. Thus, in the presence of excess lipid, chlorophyll a complexes will be formed at the expense of chlorophyll a aggregation, even in an environment containing water molecules.

The results of the monolayer studies are consistent with this interpretation. The apparently ideal compression behavior of the mixed films suggests that the pigment is dispersed in the lipid in a two-dimensional solution. The increase in chlorophyll a fluorescence yield and polarization as pigment concentration was decreased in the monolayers[9] further supports this hypothesis.

The instability of sulfolipid films containing more than 0.05 area fraction chlorophyll a is inconsistent with our other results and the conclusions just drawn. We also noted a residual fluorescence polarization in this system.[9] The anomalous behavior might be indicative of a phase change in the system above a given mole fraction of pigment, such as formation of lipid-pigment complexes which, although

they occupy the same surface area as the individual molecules, are either water soluble or unstable. The polarization measurements indicate that chlorophyll may be partially oriented in such a configuration. The state of pigment aggregation and presence of one or several species cannot be ascertained from the available data. Aggregated pigment would contribute little to depolarization, because its fluorescence yield is considerably lower than that of monomers. However, the ability of sulfolipid to break up chlorophyll a dimers in solution suggests that the presence of pigment aggregates may be thermodynamically unfavorable.

The extension of these results to biological material is somewhat tenuous, because even the liquid-gas interface environment of the monolayers is a poor approximation to chloroplast lamellar surfaces. Also, the presence of many other molecular species may affect the chlorophyll-chlorophyll and chlorophyll-lipid interactions studied here. In addition, we must consider experimental evidence that the pigment is present in several degrees of orientation and states of aggregation in the plant.[5,21-23]

The evident random dispersion of chlorophyll by monogalactolipid in monolayers, and the random breakup of pigment dimers in solution by this lipid, allow us to suggest that the bulk fraction of randomly oriented pigment in chloroplast lamellae may be associated with this lipid. Similarly, the aggregated, oriented forms of chlorophyll a *in vivo* are probably not in such an environment. The sulfolipid results are more ambiguous, but they might be interpreted as indicative of a specific complexing and partial orientation of localized high concentrations of pigment by this surface active lipid. We note reports of the occurrence of sulfolipid in conjunction with chlorophyll a appearance and disappearance and fluorescence polarization changes during greening,[25] which also suggest that the lipid is involved in pigment organization *in vivo*.

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FIGURE LEGENDS

Fig. 1. Anthrone color reaction with galactose (———), hydrolyzed chloroplast galactolipid (-------) and hydrolyzed sulfolipid (— — —). Relative absorbances have not been normalized to unit weight of sugar.

Fig. 2. Absorption difference spectra. Reference solution, 1.6×10^{-4} M chlorophyll a in carbon tetrachloride; sample solution, same pigment concentration with varying amounts of monogalactolipid. a. Lipid/chlorophyll = 8.2. b. Lipid/chlorophyll = 5.2. c. Lipid/chlorophyll = 3.1. d. Lipid/chlorophyll = 2.0. e. Baseline.

Fig. 3. Relative change in absorbance at 682 nm as a function of the lipid/chlorophyll a ratio in solutions of carbon tetrachloride. Experimental data for monogalactolipid (o) and sulfolipid (▲). Theoretical curve calculated for formation of a 1:1 complex with equilibrium constant $K_c = 8 \times 10^3$ liters/mole.

Fig. 4. Pressure-area characteristics of monolayers of chloroplast glycolipids. mGdG = monogalactodiglyceride; dGdG = digalactodiglyceride. Results are shown for four or more samples of each lipid. The linear extrapolations to zero pressure yield the maximum areas per molecule occupied by a uniform monolayer.

FIGURE LEGENDS (continued)

Fig. 5. Time dependence of the surface pressure of a chloroplast sulfolipid monolayer under nitrogen maintained at constant area, 33 $Å^2$ per molecule after compression at a rate of 4 $Å^2$-molecule^{-1}-min^{-1}.

Fig. 6. Surface pressure-area curves for mixed monolayers of chlorophyll a with isolated chloroplast lipids. (a) Monogalactodiglyceride. (b) Sulfolipid. Mole fractions of chlorophyll as indicated. Results are shown for three samples in each case. The curves shown are the theoretical pressure-area behaviors expected for ideal two-dimensional solutions.

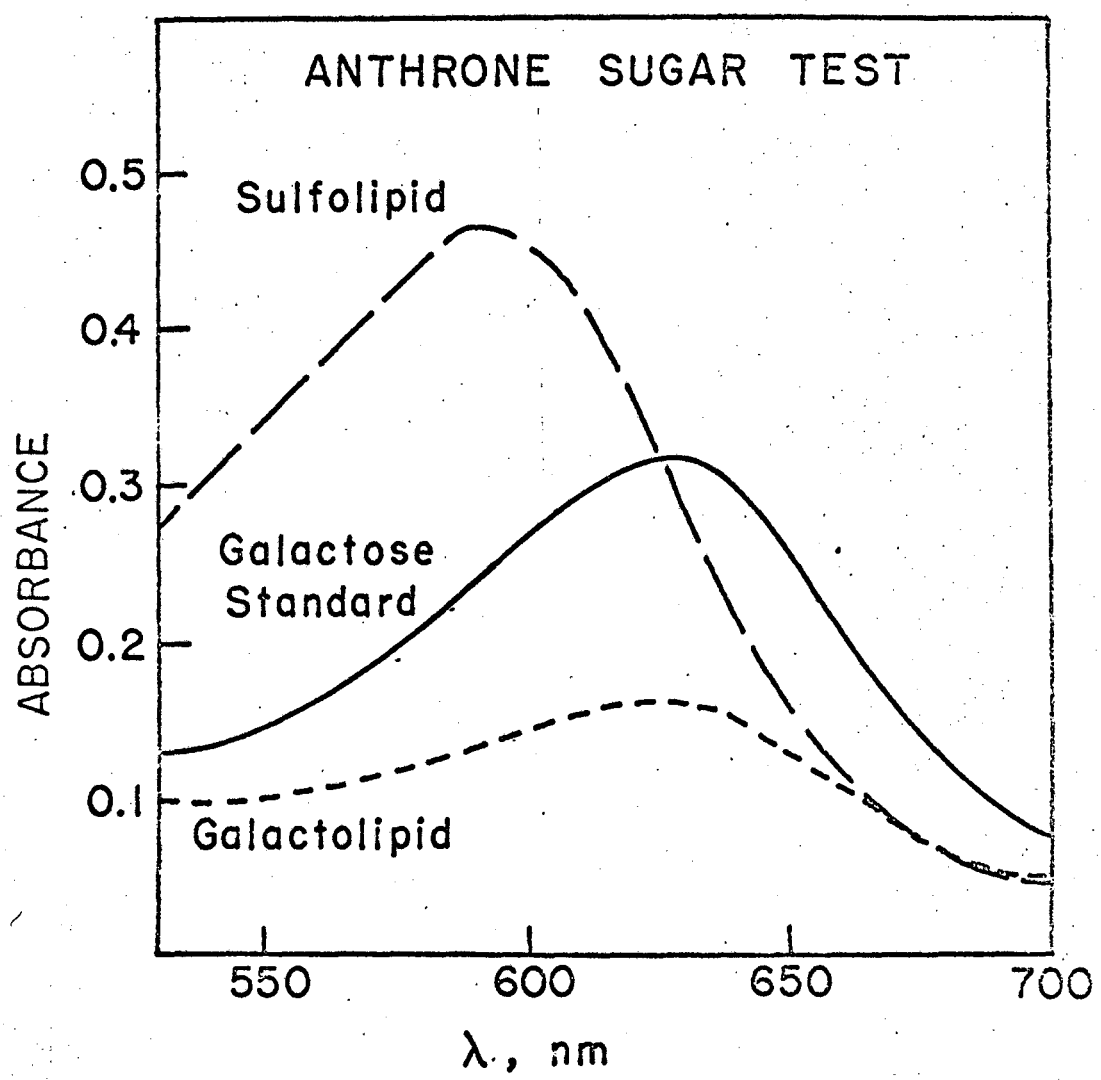

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Fig. 1

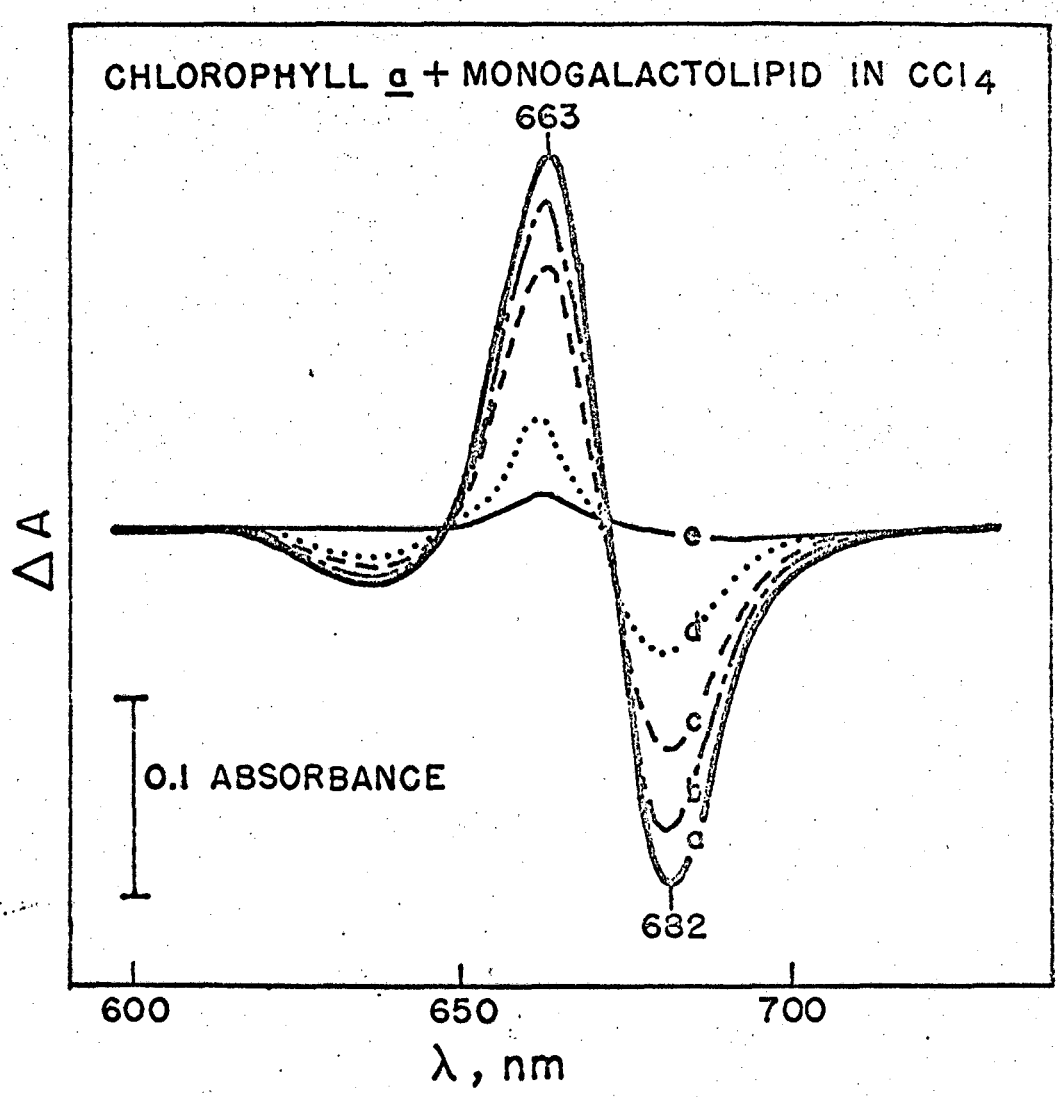

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Fig. 2

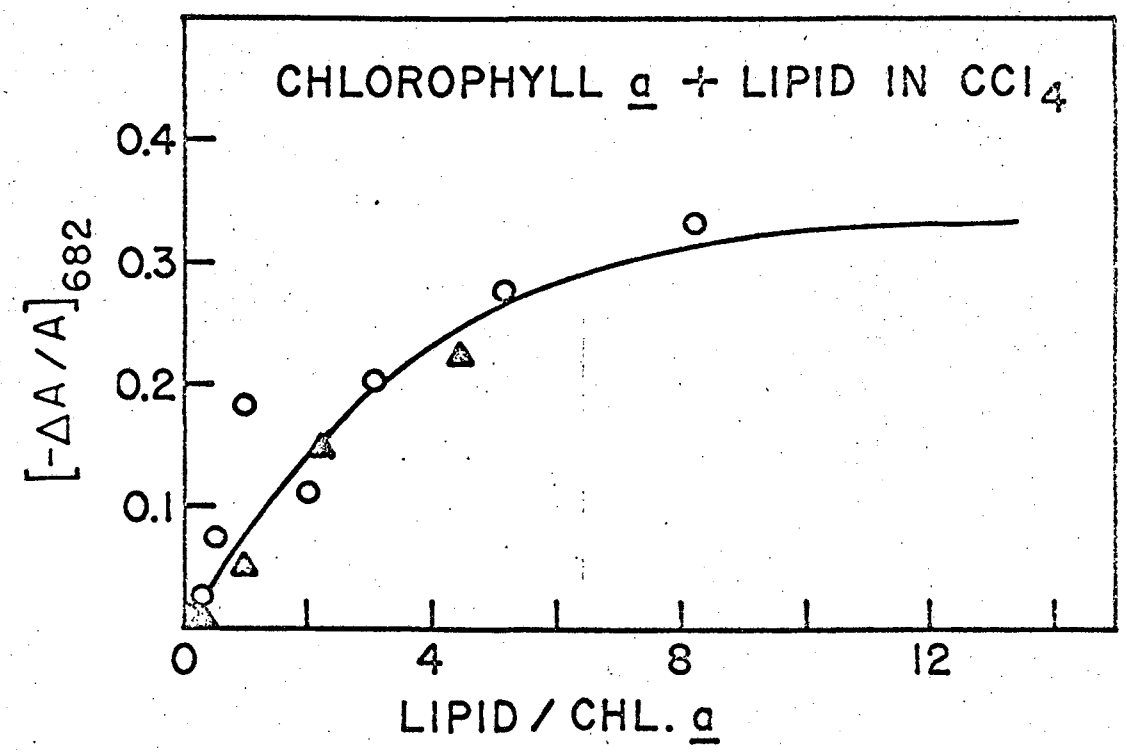

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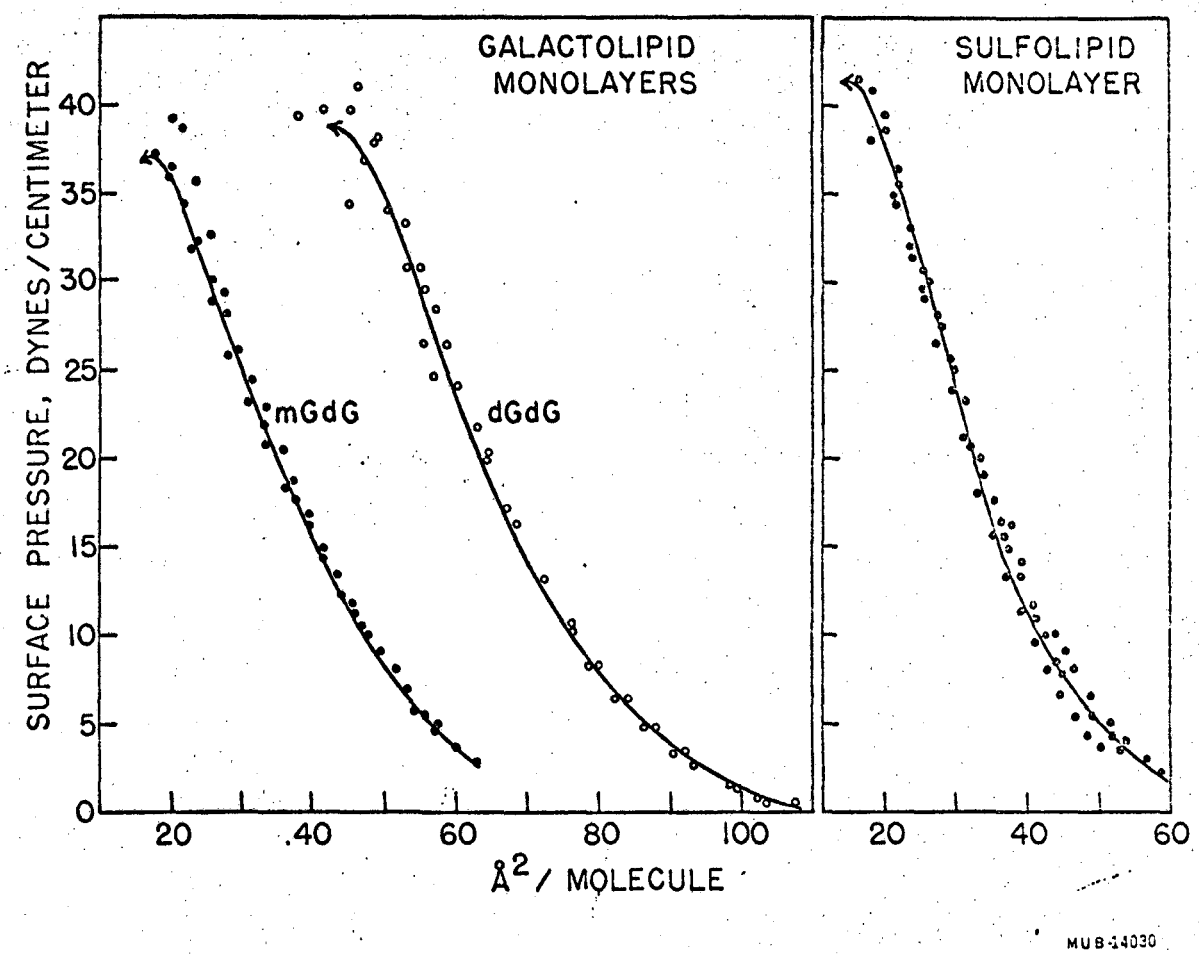
Fig. 3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SULFOLIPID
MONOLAYER
mGdG
dGdG
SURFACE PRESSURE, DYNES/CENTIMETER
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10
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Fig. 4

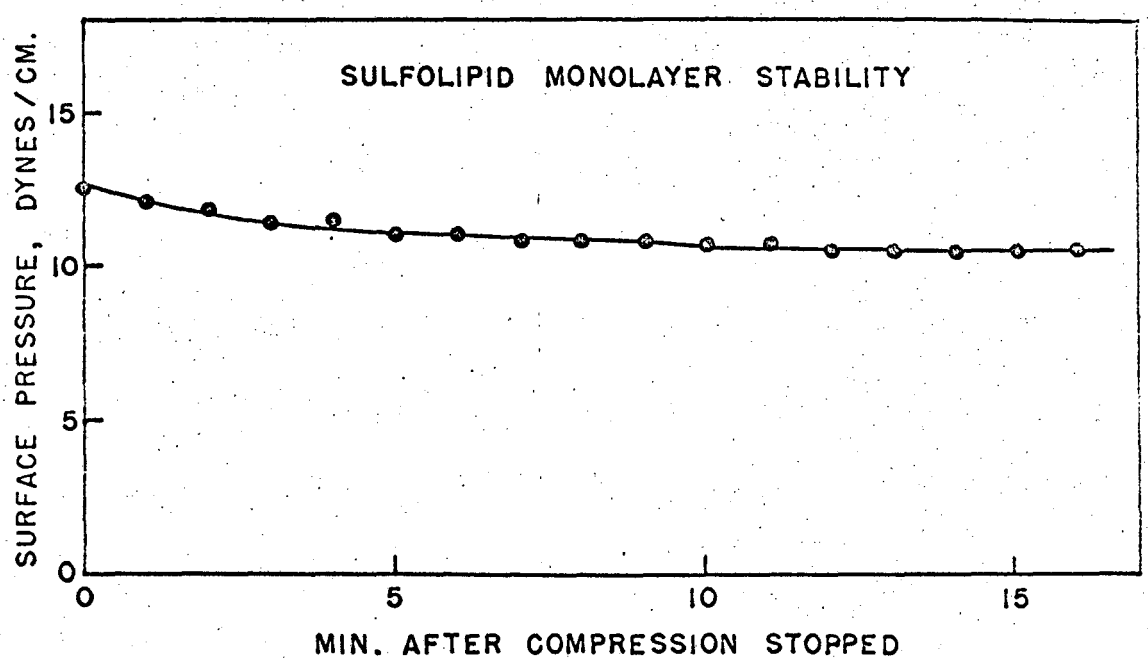

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Fig. 5

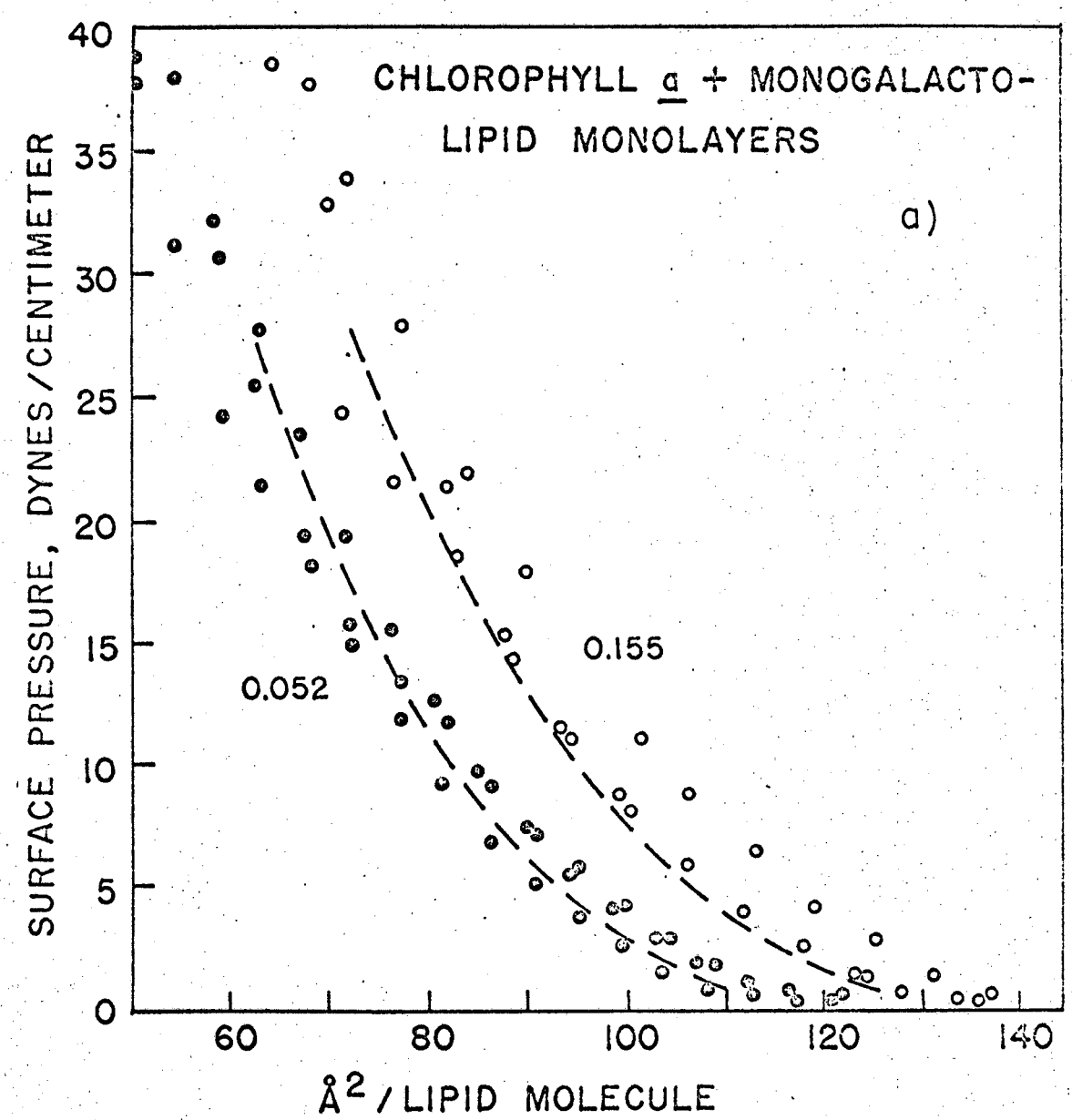

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Fig. 6a

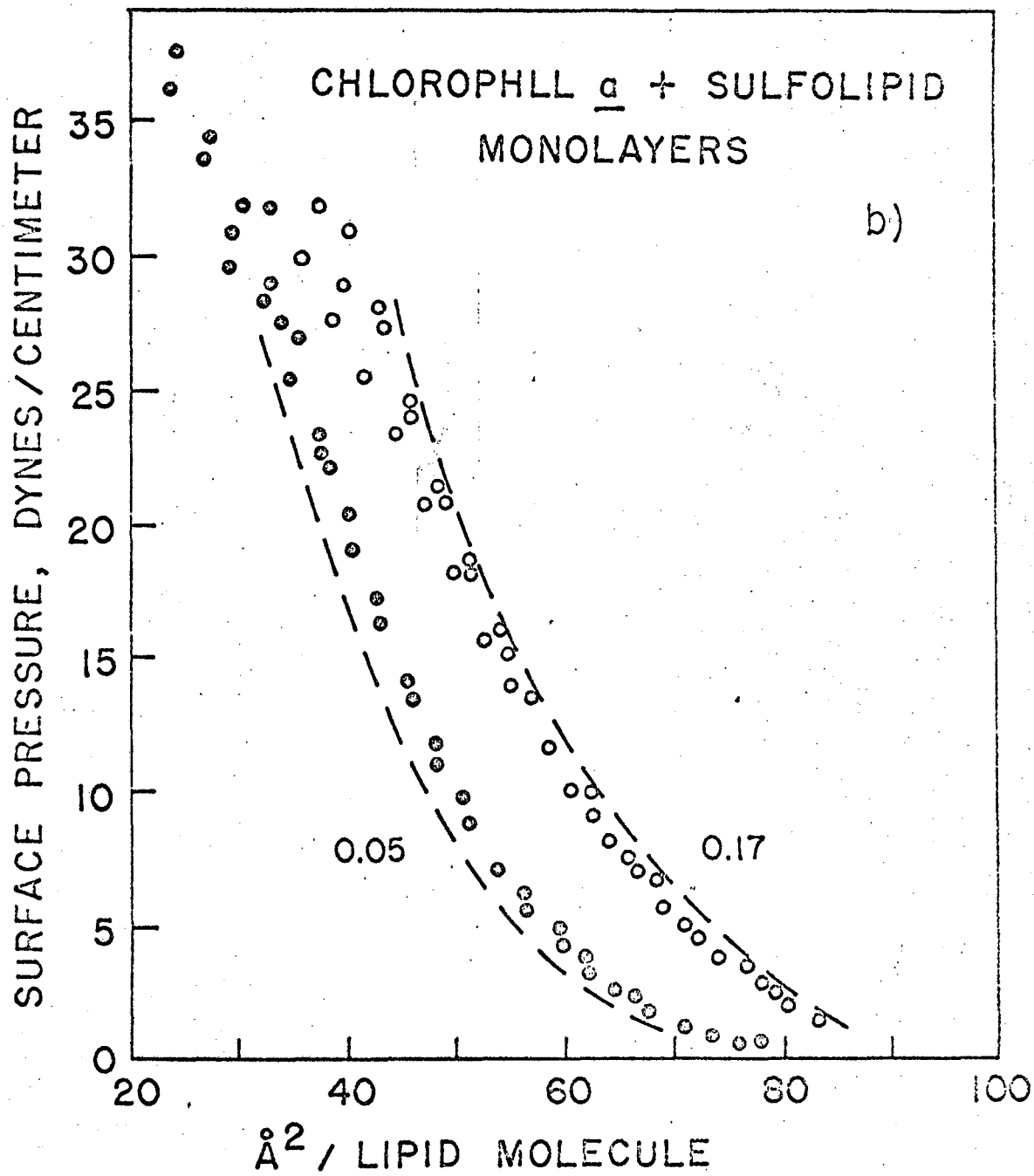

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Fig. 6b

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