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CHEMICAL MODIFICATION BY TRINITROBENZENESULFONATE OF A LIPID AND PROTEINS OF INTRACYTOPLASMIC MEMBRANES ISOLATED FROM *CHROMATIUM VINOSUM* AND *AZOTOBACTER VINELANDII*

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SUMMARY

1. The structure of intracytoplasmic membranes of a photosynthetic bacterium *Chromatium vinosum* and a nitrogen-fixing bacterium *Azotobacter vinelandii* was studied by chemical modification of amino groups of phosphatidylethanolamine and proteins with trinitrobenzenesulfonate.

2. Almost all the constituents of intracytoplasmic membranes of *C. vinosum* were solubilized in a mixture of chloroform, methanol and trichloroacetic acid. One-third of proteins in the intracytoplasmic membranes of *C. vinosum* was found solubilized in a mixture of chloroform and methanol. By using a column chromatography with Sephadex LH-20 in organic solvents, the unmodified as well as the trinitrophenylated proteins and also the trinitrophenylated phosphatidylethanolamine were separated from the other colored substances.

3. In the chemical modification of the intracytoplasmic membrane preparations, 30 % of phosphatidylethanolamine and 15 % of protein amino groups in *C. vinosum* and 45 % of phosphatidylethanolamine and 20 % of protein amino groups in *A. vinelandii* were estimated to be exposed to the aqueous phase. In the single-layered liposomes composed of phosphatidylethanolamine and phosphatidylglycerol with a ratio of 2 : 1, 40 % of phosphatidylethanolamine were estimated to be exposed to the aqueous phase.

INTRODUCTION

Intracytoplasmic membranes are called chromatophores when isolated from photosynthetic bacteria and are the site of primary processes of photosynthesis. Observation with the electron microscope of some photosynthetic bacteria revealed that they are spherical vesicles of single membrane of diameter from 500 to 1000 Å

Abbreviation: N_3Ph, trinitrophenylated

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[1–7]. In a photosynthetic sulfur bacterium, *Chromatium vinosum*, the intracytoplasmic membranes can be isolated in a highly purified form by centrifugation in a sucrose density gradient. This preparation is called light particles according to Cusanovich and Kamen [7].

A well developed intracytoplasmic membrane system was also found in a nitrogen-fixing bacterium, *Azotobacter vinelandii* [8, 9]. Observation with the electron microscope seems to show spherical vesicles having a diameter from 500 to 1000 Å [8]. The intracytoplasmic membrane preparation from this bacterium seems to consist also of closed vesicles as indicated by studies on the proton translocation [10].

A chemical reagent, 2,4,6-trinitrobenzenesulfonate reacts with the primary amino groups to form trinitrophenylated amino groups [11]. This reagent is suitable for the investigation of the structure of biological membranes, since it is soluble in an aqueous solution and penetrates the biological membranes very slowly [12], and reacts with the amino groups in proteins and phosphatidylethanolamine at neutral pH. The trinitrophenylated amino groups can be identified and quantified by their characteristic absorption bands at about 340 and 410 nm [11].

It is estimated in this study that lipid content of the intracytoplasmic membranes of *C. vinosum* is about 60 % of the dry weight(see Results). Phospholipids which occupy 93 % of the lipid fraction are composed of 55 % phosphatidylethanolamine, 40 % phosphatidylglycerol and 5 % cardiolipin [13]. In the case of the intracytoplasmic membrane of *A. vinelandii*, the lipid content was reported to be about one-fifth of dry weight. 93 % of the lipid fraction was due to phospholipids which were composed of 64 % phosphatidylethanolamine and 36 % other unidentified ninhydrin-negative phospholipids [14].

In order to analyze the trinitrophenylated (N_3Ph) membranes, it is desirable to isolate N_3Ph-proteins and N_3Ph-phosphatidylethanolamine from other colored substances. Acidic mixtures of organic solvents were used to solubilize biological membranes of various origins [15–20]. Gel filtration chromatography on Sephadex LH-20 separates the membrane proteins from the other membrane components in the acidic organic solvent systems [15, 17].

In this study, we found that almost all of the proteins in the intracytoplasmic membranes of *C. vinosum* was solubilized in a mixture of chloroform/methanol/trichloroacetic acid, and that one-third of the membrane proteins was soluble in a mixture of chloroform/methanol. Using these organic solvent mixtures, we investigated the trinitrophenylation of intracytoplasmic membranes of *C. vinosum* and *A. vinelandii* and also of liposomes.

METHODS AND MATERIALS

C. vinosum was cultured at 30 °C for 2 days under an anaerobic condition in an inorganic medium of Bartsch [21] under illumination with incandescent lamp light, 8000 lux. *A. vinelandii* (ATCC 9046) was grown at 30 °C in the nitrogen-free medium of Burk [22] as described previously [23].

Intracytoplasmic membranes of *C. vinosum* and *A. vinelandii* were prepared in almost the same manner. The harvested cells were suspended in 300 mM sucrose containing 50 mM phosphate buffer, pH 7.8, and were ruptured through a French

pressure cell (Ohtake) at a pressure of 200 Kg/cm^2 in *C. vinosum* and 300 Kg/cm^2 in *A. vinelandii*. A crude membrane fraction which sedimented between $25000 \times g$ for 15 min and $100000 \times g$ for 60 min was purified by a centrifugation at $60000 \times g$ for 2 h on a linear density gradient of sucrose from 15 to 50 % (w/v) in 50 mM phosphate buffer, pH 7.8. A main colored band appearing at 20–25 % in *C. vinosum* or at 20–30 % in *A. vinelandii* of sucrose concentration included a purified preparation of the intracytoplasmic membranes which is called light particles in the case of *C. vinosum* [7]. They were collected by centrifugation at $100000 \times g$ for 1 h. All the above preparative procedures were performed at 0–4 °C.

Liposomes were formed from the mixture of phosphatidylethanolamine and phosphatidylglycerol with a molecular ratio of 2 : 1 according to Huang [24] except that phosphate buffer, pH 7.7, was used in place of Tris-acetate buffer. Phosphatidylethanolamine and phosphatidylglycerol were extracted from cells of *C. vinosum* with a mixture of chloroform/methanol (7 : 3, v/v) and were purified by the column chromatography of silicic acid and the thin-layer chromatography of silica gel. The liposomes were separated by the gel filtration through a Sepharose 2B column to the single-layered and the multi-layered liposomes.

Light particles of *C. vinosum* were solubilized using a mixture of chloroform/methanol/trichloroacetic acid (50 : 50: 1, v/v/w) or extracted by a mixture of chloroform/methanol (7 : 3, v/v) (see Results).

The protein fraction which is soluble in the mixture of chloroform and methanol will be called chloroform/methanol-soluble. The fraction which is insoluble in this mixture but soluble in the chloroform/methanol/trichloroacetic acid mixture will be called chloroform/methanol-insoluble. The protein fraction which is solubilized directly in the mixture of chloroform/methanol/trichloroacetic acid will be called chloroform/methanol/trichloroacetic acid-soluble. The sum of the chloroform/methanol-soluble and the chloroform/methanol-insoluble fractions produces the chloroform/methanol/trichloroacetic acid-soluble fraction. The protein fractions solubilized in the above-mentioned solvent mixtures were separated from the other components of membrane by gel filtration chromatography with Sephadex LH-20. The trinitrophenylation of phosphatidylethanolamine and proteins did not alter their solubility to the solvent mixtures.

In the case of the intracytoplasmic membranes of *A. vinelandii*, lipids were extracted with a mixture of chloroform/methanol (7 : 3, v/v). A complete solubilization of the components of intracytoplasmic membrane was performed by using aqueous solution of 2 % sodium dodecyl sulfate containing 200 mM sodium phosphate buffer, pH 6.0.

For the chemical modification, the light particles of *C. vinosum* and the intracytoplasmic membrane preparations of *A. vinelandii* were suspended in 300 mM sucrose containing 50 mM phosphate buffer, pH 7.6. The liposomes were suspended in 100 mM NaCl containing 20 mM phosphate buffer, pH 7.7. The concentration of phosphatidylethanolamine of these membranes were, at most, 2 mM in these suspensions. Sodium trinitrobenzenesulfonate was solved in 300 mM sucrose containing 50 mM phosphate buffer, pH 7.6 in the modification of the membranes of *C. vinosum* and *A. vinelandii* or in 100 mM NaCl and 20 mM phosphate buffer, pH 7.7 in the modification of the liposomes. The trinitrophenylation was initiated by mixing 4 ml of the membrane suspension and the same volume of the freshly dissolved sodium

trinitrobenzenesulfonate solution. The final concentration of trinitrobenzenesulfonate was 100, 50 or 10 mM, and was much higher than that of phosphatidylethanolamine. The reaction mixture was kept in the dark at 20 °C. At a designed time, an aliquot of the reaction mixture was passed through a column of Sephadex G-25 previously equilibrated with 200 mM phosphate buffer, pH 6.0, to terminate the reaction and isolate the trinitrophenylated membranes from trinitrobenzenesulfonate. The membranes were collected by a centrifugation at 100000 × *g* for 60 min. In the observation with the electron microscope, the trinitrophenylation did not alter the shape and the size of the light particles.

N_3Ph-Phosphatidylethanolamine was isolated by column chromatography with Sephadex LH-20 as shown in the results. The amount of N_3Ph-phosphatidylethanolamine was determined in the N_3Ph-phosphatidylethanolamine fractions of the chromatography by measuring the absorbance at 335 nm and 407 nm which was due to the absorption of N_3Ph-amino groups. The total amount of modified and unmodified phosphatidylethanolamine was determined after the unmodified phosphatidylethanolamine in the chloroform/methanol extract was all chemically modified to N_3Ph-phosphatidylethanolamine. The trinitrophenylation of phosphatidylethanolamine in the extract was performed as follows: 1 ml of the chloroform/methanol (7 : 3, v/v) extract of membrane was mixed with 1.1 ml of methanol containing 10 mM sodium trinitrobenzenesulfonate and 0.56 ml of 5 mM phosphate buffer, pH 7.8, under which condition the reaction mixture was a monophasic solution as described by Bligh and Dyer [25]. The reaction mixture was kept at 30 °C in the dark. After 6 h of reaction time N_3Ph-phosphatidylethanolamine was separated by Sephadex LH-20 column chromatography and its amount was determined spectroscopically. The half reaction time of the trinitrophenylation of phosphatidylethanolamine in this method was about 6 min. At 6 h, no unmodified phosphatidylethanolamine was detected in the color development with iodine vapor or ninhydrin on the thin-layer chromatography with silica gel.

In the light particles of *C. vinosum* the degree of trinitrophenylation of the membrane proteins was determined using a spectroscopic method after the protein fraction was separated from other colored substances. The estimation was based on the assumption that the absorption spectrum for N_3Ph-amino groups was identical with that of N_3Ph-phosphatidylethanolamine and that the absorbance at 280 nm due to the aromatic amino acid residues of proteins was not affected by the trinitrophenylation of amino groups. The extent of trinitrophenylation was calculated by using the ratio of the absorbance at 335 nm due to the N_3Ph-amino groups to the absorbance at 280 nm due to the proteins but after correcting the mutual overlapping of the absorption bands at these wavelengths. The degree of trinitrophenylation of protein fractions was defined as the amount of N_3Ph-amino groups divided by the sum of the N_3Ph-amino groups and the unmodified but reactive amino groups. The trinitrophenylation of all of the reactive amino groups of proteins was performed by treating the light particles with 100 mM trinitrobenzenesulfonate at pH 8.7 at 35 °C for 48 h. Amino acid analysis of the proteins indicated that more than 75 % of lysine was trinitrophenylated in the procedure. Since the hydrolysis treatment of proteins reconverts a large part of N_3Ph-amino acid to the original amino acids [11], this result implies that almost all of the ε-amino groups of lysine must be trinitrophenylated in the modification procedure.

In the intracytoplasmic membrane preparations of *A. vinelandii*, a large part of proteins was insoluble in the mixtures of organic solvents. Thus, the membrane was solubilized in 2 % sodium dodecyl sulfate solution containing 200 mM phosphate buffer, pH 6.0. The amount of N_3Ph-amino groups of proteins was calculated by subtracting from the total amount of N_3Ph-amino groups the amount of N_3Ph-phosphatidylethanolamine which was determined in the organic solvent systems.

Polyacrylamide gel electrophoresis was performed according to Hoober [26]. Determination of phosphorus was according to Bartlett [27]. Thin-layer chromatography of lipids was performed using Wako gel B-5 with a developing solvent chloroform/methanol/water (70 : 30 : 5, v/v/v). Sodium trinitrobenzenesulfonate was obtained from Tokyo Kasei Co. Sephadex LH-20 and G-25 and Sepharose 2B were from Pharmacia.

RESULTS

Solubilization and fractionation of membrane proteins of light particles of C. vinosum

When the light particles of *C. vinosum* were mixed with 10 volumes of a mixture of chloroform/methanol/trichloroacetic acid (50 : 50 : 1, v/v/w) and agitated for a few minutes, almost all the membrane constituents were solubilized. After a centrifugation at 20000 × *g* for 10 min, only a very small amount of precipitate was formed. Judging from the dry weight determination more than 95 % of the membrane components were found solubilized in this solvent mixture.

The proteins thus solubilized (the chloroform/methanol/trichloroacetic acid-soluble proteins) were separated by the gel filtration chromatography of Sephadex LH-20 from the other components of low molecular weights, such as phospholipids, carotenoids and bacteriopheophytin *a*. Fig. 1 shows an elution pattern of the chloro-

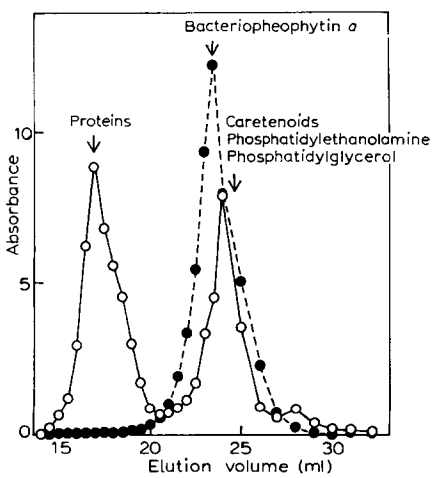

Fig. 1. Separation of chloroform/methanol/trichloroacetic acid-soluble protein fraction in the light particles of *C. vinosum* from pigments and lipids by column chromatography with Sephadex LH-20. The extract in the chloroform/methanol/trichloroacetic acid mixture (50 : 50 : 1, v/v/w) was eluted by the same mixture. Column size, 1.7 × 23 cm. ○, absorbance at 280 nm; ●, absorbance at 755 nm. The absorbance of each fraction was measured after dilution.

form/methanol/trichloroacetic acid extract of light particles of *C. vinosum* from the Sephadex LH-20 column. Elution of carotenoids, phosphatidylethanolamine and phosphatidylglycerol was detected by applying the eluted fractions to the thin-layer chromatography of silica gel.

The trinitrophenylated light particles were also solubilized in the mixture of chloroform/methanol/trichloroacetic acid. The N_3Ph-protein fraction was also separated from the other substances by column chromatography with Sephadex LH-20. The trinitrophenylation of amino groups of proteins did not alter the behavior of proteins in the chromatography with Sephadex LH-20.

A mixture of chloroform/methanol (7 : 3, v/v) solubilized all of the lipids and a part of proteins (chloroform/methanol-soluble proteins) in the light particles of *C. vinosum*. The chloroform/methanol-soluble protein fraction was also separated from the other components in the column chromatography with Sephadex LH-20 by using a mixture of chloroform/methanol in a ratio of 1 : 1 (v/v) as an eluent.

The mixture of organic solvents of chloroform/methanol/trichloroacetic acid (50 : 50 : 1, v/v/w) was found to solubilize proteins in the membrane preparations of chloroplasts, mitochondria and inner membranes of *Escherichia coli*. The mixture without trichloroacetic acid, however, extracted only a very small portion of proteins in these membrane preparations. This behavior is in contrast to that observed with intracytoplasmic membranes of the photosynthetic bacteria.

Properties of protein fractions

Fig. 2 shows the results of polyacrylamide gel electrophoresis of the protein fractions in the presence of sodium dodecyl sulfate and urea. In the chloroform/methanol-soluble and the chloroform/methanol-insoluble fractions of *C. vinosum*, no common bands were found (Figs. 2B and 2C). This indicates that the extraction with the chloroform/methanol mixture completely fractionates the membrane proteins. The electrophoresis in 16 % polyacrylamide gel of the chloroform/methanol-soluble fraction suggested that the chloroform/methanol-soluble fraction contained three kinds of polypeptide chains (Fig. 2D). The apparent molecular weights of these polypeptide chains were estimated to be 12 000, 11 500 and 9500 by using hemoglobin and chymotrypsin as marker polypeptide chains.

The chloroform/methanol-soluble proteins are likely to correspond to the band 15 protein of *Rhodopseudomonas spheroides* described by Fraker and Kaplan [28] and by Takemoto and Lascelles [29] and to the 9000 dalton protein described by Clayton and Clayton [30]. According to these authors [28–30], this protein contains bacteriochlorophyll *a*.

The amino acid composition of the chloroform/methanol-soluble and the chloroform/methanol-insoluble protein fractions of *C. vinosum* is shown in Table I. The chloroform/methanol-soluble fraction was rich in the hydrophobic amino acid residues. The polarity index defined by Capaldi and Vanderkooi [31] for the chloroform/methanol-soluble fraction was as low as 30.3 while it was 42.1 in the chloroform/methanol-insoluble fraction.

In the absorption spectra of chloroform/methanol-soluble fraction of *C. vinosum*, no appreciable absorption bands were observed in the region of wavelengths 300–900 nm. In contrast, chloroform/methanol/trichloroacetic acid-soluble and chloroform/methanol-insoluble fractions of this bacterium showed a clear band at

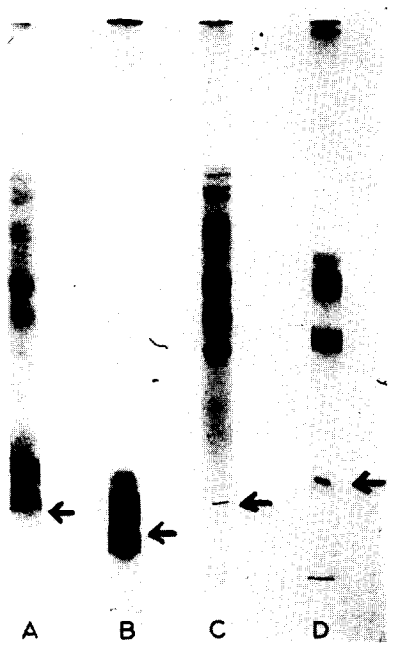

Fig. 2. Polyacrylamide gel electrophoresis of protein fractions of *C. vinosum* in the presence of sodium dodecyl sulfate and urea. Proteins were separated from lipids and pigments in the column chromatography with Sephadex LH-20 and then, after being solubilized in sodium dodecyl sulfate and urea, were subjected to the electrophoresis. Concentration of polyacrylamide was 8 % in A, B, and C, and 16 % in D. Arrows indicate the position of the tracking dye. A, chloroform/methanol/trichloroacetic acid-soluble fraction. B and D, chloroform/methanol-soluble fraction. C, chloroform/methanol-insoluble fraction.

397 nm due to heme groups of cytochromes. These findings indicate that the heme proteins are soluble only in the acidic solvent mixture. The absorption spectrum of N_3Ph-proteins in each fraction was a sum of the spectra of the unmodified proteins and the N_3Ph-amino groups with peaks at 335 and 407 nm.

The proportion of the chloroform/methanol-soluble and chloroform/methanol-insoluble protein fractions to the chloroform/methanol/trichloroacetic acid-soluble one is estimated by comparing the absorbance at 280 nm and 397 nm in the absorption spectra of these fractions. The calculation is based on the findings that the absorption of heme groups at 397 nm appears only in the chloroform/methanol/trichloroacetic acid-soluble and the chloroform/methanol-insoluble fractions and that the absorption at 280 nm is due to tryptophane and tyrosine. From the contents of these amino acids (Table I) and the ratio of molar extinction coefficients (about 3.5 according to Bailey et al. [33]), it is estimated that the chloroform/methanol-soluble protein fraction amounts to one-third of the chloroform/methanol/trichloroacetic acid-soluble protein fraction in *C. vinosum*.

The chloroform/methanol-soluble proteins were found to be soluble also in a mixture of acetone/methanol (7: 2, v/v). Cusanovich [34] had reported that the protein content of the light particles of *C. vinosum* would be 26–28 % dry weight. However, this value was calculated on an assumption that the mixture of acetone/

TABLE I

AMINO ACID COMPOSITION OF CHLOROFORM/METHANOL-SOLUBLE AND CHLOROFORM/METHANOL-INSOLUBLE PROTEIN FRACTIONS OF *C. VINOSUM*

The protein fractions were separated from the other components by the column chromatography, and the proteins were hydrolysed in 6 M HCl at 110 °C for 24 h.

Amino acid	Mol %	
	Chloroform/methanol-soluble	Chloroform/methanol-insoluble
Lys	4.1	4.6
His	3.5	2.1
Arg	1.6	5.1
Asx	5.9	10.1
Glx	8.3	10.1
Thr	5.1	5.5
Ser	4.0	5.4
Pro	5.7	6.4
Gly	5.8	8.6
Ala	12.8	12.5
Cys/2	—*	—*
Val	8.0	5.3
Met	3.1	1.5
Ile	6.0	5.3
Leu	12.8	8.6
Tyr	1.5	2.8
Phe	7.3	4.3
Trp**	4.6	1.9
Polarity	30.3	42.1

* Not detected

** Determined according to Goodwin and Morton [32].

methanol would have extracted the lipids but left all of the proteins unsolved. After correcting for the solubilization of chloroform/methanol-soluble protein fraction in this solvent mixture, it is estimated that the protein fraction amounts to about 40 % dry weight of the intracytoplasmic membranes of *C. vinosum*.

Isolation of N_3Ph-phosphatidylethanolamine

Fig. 3 shows the elution pattern of the extract with the chloroform/methanol mixture (7 : 3, v/v) of trinitrophenylated light particles of *C. vinosum* in the Sephadex LH-20 column chromatography developed by a mixture of chloroform/methanol (2 : 1, v/v). N_3Ph-phosphatidylethanolamine was separated in this mixture of solvents as a yellow-colored substance from the other colored materials, such as N_3Ph-proteins, bacteriochlorophyll and carotenoids. The recovery of N_3Ph-phosphatidylethanolamine in this method was 98–99 %. This value was larger than that obtained from the thin-layer plate of silica gel i.e. 96 % in our results.

Molar extinction coefficients of N_3Ph-phosphatidylethanolamine at 335 and 407 nm in the mixture of chloroform/methanol (2 : 1, v/v) were estimated to be 12400 and $4900 \cdot M^{-1} \cdot cm^{-1}$, respectively, on the basis of phosphorus content in N_3Ph-phosphatidylethanolamine.

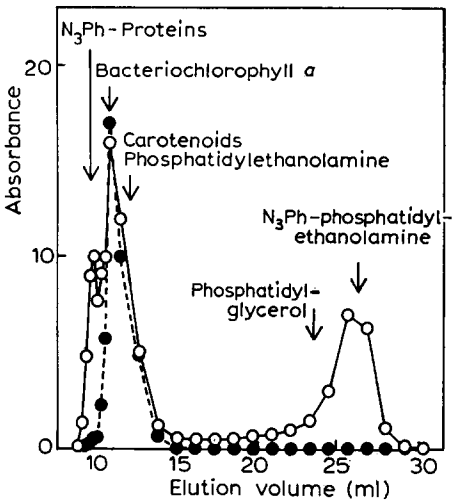

Fig. 3. Elution pattern of the column chromatography with Sephadex LH-20 eluted by chloroform/methanol mixture (2:1, v/v). The sample was extracted with chloroform/methanol (7.3 v/v) from the light particles of *C. vinosum* after trinitrophenylation at 100 mM trinitrobenzenesulfonate for 1 h. Column size 1.5×15 cm. ○, absorbance at 335 nm; ●, absorbance at 775 nm. Absorbance was measured after dilution.

Trinitrophenylation of light particles of C. vinosum

The absorption spectrum of the light particles changed upon trinitrophenylation. The difference spectrum due to the modification in the region of 250 to 500 nm was very similar to the spectrum of N_3Ph-phosphatidylethanolamine. This finding indicates that the change in absorption in this wavelength region should be due to the trinitrophenylation of amino groups in phosphatidylethanolamine and/or proteins. Absorption changes also appeared in the region of 800 to 900 nm. The absorption band at 885 nm decreased, while the bands at 800 and 850 nm increased. These changes in bacteriochlorophyll *a* absorption may have been produced by an alteration of membrane characteristics of light particles upon the trinitrophenylation of amino groups.

Fig. 4 shows a time course of trinitrophenylation of light particles, which was measured by an increase in absorbance at 425 nm divided by the absorbance at 590 nm. In this case the absorption at 590 nm of bacteriochlorophyll *a* was assumed to be unaltered upon modification. It is clear in Fig. 4 that the time course consisted of at least two components with different reaction rates. The fast component was complete within 10 min, but the slower components within hours. These findings suggest that a part of amino groups of phosphatidylethanolamine and/or proteins in the membrane was readily attacked by trinitrobenzenesulfonate solved in the surrounding aqueous phase and the other part was not.

Time courses of trinitrophenylation of the light particles were compared in the presence and absence of 300 mM sucrose. No essential difference was found in the time courses.

In order to investigate the feature of chemical modification of the intracytoplasmic membranes in detail, the trinitrophenylation was determined in phos-

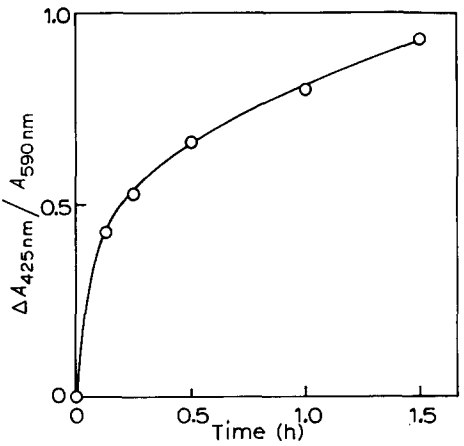

Fig. 4. Time course of trinitrophenylation of light particles of *C. vinosum* in100 mM trinitrobenzenesulfonate. Trinitrophenylation was presented by an increase in the absorbance at 425 nm divided by the absorbance at 590 nm.

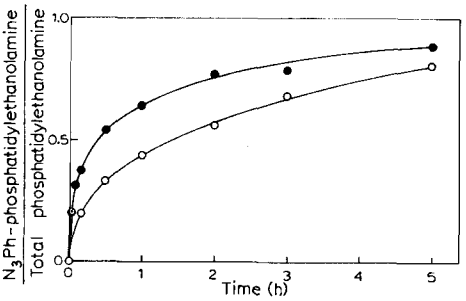

Fig. 5. Trinitrophenylation of phosphatidylethanolamine in the light particles of *C. vinosum*. Time courses of trinitrophenylation of phosphatidylethanolamine in 100 mM, (●) and 10 mM, (○) trinitrobenzenesulfonate.

TABLE II

HALF-REACTION TIME IN MIN OF TRINITROPHENYLATION OF PHOSPHATIDYLETHANOLAMINE IN THE FAST-, SLOWLY- AND VERY SLOWLY-REACTING COMPONENTS IN THE INTRACYTOPLASMIC MEMBRANE PREPARATIONS OF *C. VINOSUM* AND *A. VINELANDII*

	10 mM Trinitrobenzenesulfonate			100 mM Trinitrobenzenesulfonate		
	Fast	Slow	Very slow	Fast	Slow	Very slow
C. vinosum	13	150	Not measured	1.5	17	180
A. vinelandii	12	200	—	2.0	23	—

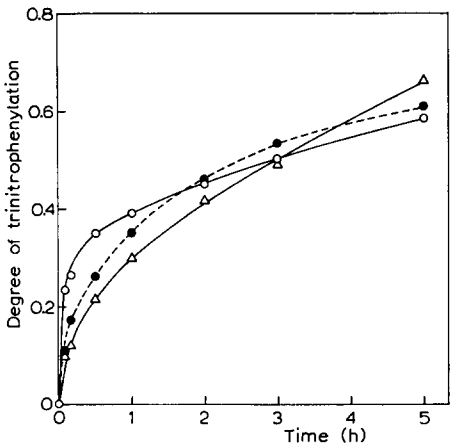

Fig. 6. Time courses of trinitrophenylation of the three protein fractions of the light particles of *C. vinosum* in 100 mM trinitrobenzenesulfonate. ○, chloroform/methanol-soluble fraction; △, chloroform/methanol-insoluble fraction; ●, chloroform/methanol/trichloroacetic acid-soluble fraction.

phatidylethanolamine and proteins by separating these components in the chromatography with Sephadex LH-20 in the organic solvent mixtures. Fig. 5 shows time courses of trinitrophenylation of phosphatidylethanolamine in the light particles of *C. vinosum*. The course was composed of more than one component with different rates of modification. Since trinitrobenzenesulfonate existed in the reaction mixture in far molar excess, pseudo-first-order kinetics could be assumed. The relationship between the logarithm of the amount of unmodified phosphatidylethanolamine versus the reaction time indicated that three components of phosphatidylethanolamine with different reaction rates seemed to exist. In Table II are summarized the half reaction times of the fast, slowly and very slowly reacting components. It is noted that the reaction rate was more than 10-times higher in the fast reacting component than in the other slower components. It was estimated in the semi-logarithmic plot that the fast reacting part of phosphatidylethanolamine amounted to about 30 % of the total phosphatidylethanolamine. Since trinitrobenzenesulfonate penetrates the membrane only very slowly [12], it is assumed that this amount of phosphatidylethanolamine is exposed to the water phase on the outer surface of the light particles.

Fig. 6 represents the time course of trinitrophenylation of the chloroform/methanol-soluble, the chloroform/methanol-insoluble and the chloroform/methanol/trichloroacetic acid-soluble protein fractions. Also in the amino groups of the proteins, there were fast and slowly reacting components. In the chloroform/methanol-soluble fraction, the fast reacting amino groups amounted to 22 % of the total amino groups. In the chloroform/methanol-insoluble fraction, the fast reacting amino groups were about 7 %. In the chloroform/methanol/trichloroacetic acid-soluble fraction, the fast reacting amino groups were 13 %. It can be inferred that one part of the amino group of membrane proteins is exposed to the aqueous phase and the other part is hidden.

Trinitrophenylation of intracytoplasmic membrane preparation of A. vinelandii

Fig. 7 presents time courses of trinitrophenylation of phosphatidylethanol-

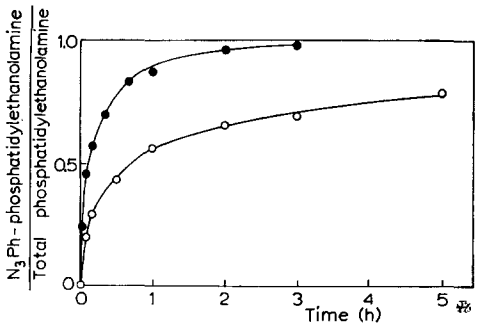

Fig. 7. Trinitrophenylation of phosphatidylethanolamine in the intracytoplasmic membrane preparations of *A. vinelandii*. Time courses of trinitrophenylation of phosphatidylethanolamine in 100 mM, (●) and 10 mM, (○) trinitrobenzenesulfonate.

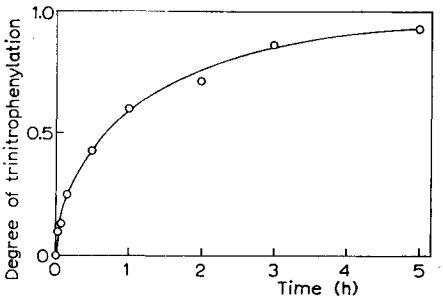

Fig. 8. Time course of trinitrophenylation of proteins in the intracytoplasmic membrane preparations of *A. vinelandii* in 100 mM trinitrobenzenesulfonate.

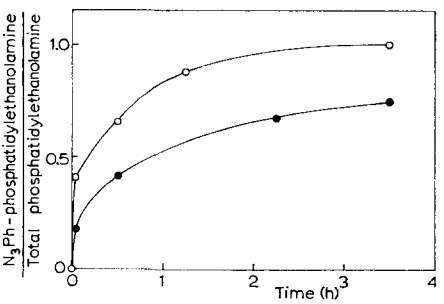

Fig. 9. Trinitrophenylation of single- and multi-layered liposomes. Time course of trinitrophenylation of phosphatidylethanolamine in 50 mM trinitrobenzenesulfonate. ○, single-layered liposomes; ●, multi-layered liposomes.

amine in the intracytoplasmic membrane preparation of *A. vinelandii*. There were more than one component with different reaction rates. The relationship between logarithm of the amount of unmodified phosphatidylethanolamine versus the reaction time indicated the existence of two components of phosphatidylethanolamine with different reaction rates. The third very slow component, which existed in the light particles of *C. vinosum*, seemed lacking in this membrane preparation. It was noted that the half-reaction times of fast and slowly reacting components were about the same as those in the membranes of *C. vinosum*. The fast reacting component of phosphatidylethanolamine in the membrane of *A. vinelandii* was estimated to be about 45 %.

Fig. 8 shows the time course of trinitrophenylation of the proteins in the membranes of *A. vinelandii*. Also in this membrane, the fast and slowly reacting components were observed. The fast reacting amino groups amounted to about 15 %.

Trinitrophenylation of liposomes

Fig. 9 shows time courses of trinitrophenylation of phosphatidylethanolamine in the single and the multi-layered liposomes that were composed of phosphatidylethanolamine and phosphatidylglycerol with a ratio of 2 : 1. The time course consisted of the fast and slowly reacting components. In the single-layered liposomes, the fast reacting component of phosphatidylethanolamine amounted to 40 % of the total, while in the multi-layered liposomes it amounted to 20 %. It is also noted that there seems to be only one slow component present in the single-layered liposomes, while more than one slow component seems to exist in the multi-layered liposomes. The fact that the trinitrophenylation in the multi-layered liposomes is slower than in the single-layered ones suggests that trinitrobenzenesulfonate penetrates only very slowly into the liposome membranes as suggested by Gordesky et al. [12].

Distribution of amino groups in the intracytoplasmic membrane vesicles of C. vinosum and A. vinelandii and in the liposomes

What proportions of amino groups of lipids and proteins are exposed to and

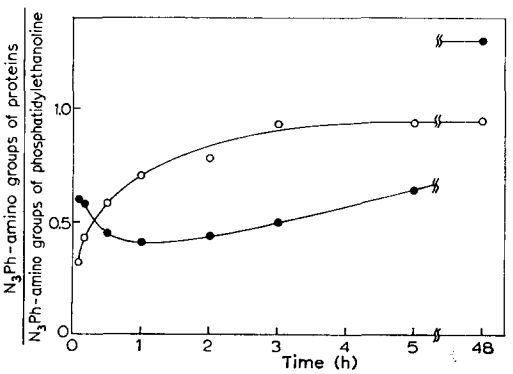

Fig. 10. Time course of the ratio of the amounts of N_3Ph-amino groups in proteins to phosphatidylethanolamine in the intracytoplasmic membrane preparations of *C. vinosum*, (●); and *A. vinelandii*, (○). The membrane preparations of *C. vinosum* and *A. vinelandii* were trinitrophenylated in 100 mM trinitrobenzenesulfonate.

TABLE III

RELATIVE AMOUNTS OF EXPOSED AND HIDDEN AMINO GROUPS OF PHOSPHATIDYLETHANOLAMINE AND PROTEINS IN THE INTRACYTOPLASMIC MEMBRANE PREPARATIONS OF *C. VINOSUM* AND *A. VINELANDII* AND IN THE SINGLE- AND THE MULTI-LAYERED LIPOSOMES

The ratio of the amounts of exposed to hidden phosphatidylethanolamine is estimated in Figs. 5, 7, and 9. The ratio of the amounts of exposed and total amino groups of phosphatidylethanolamine to those of proteins were estimated in Fig. 10. The amount of hidden amino groups of proteins are obtained by subtracting the amount of the exposed amino groups from the total. Relative values are presented by normalizing the values for the exposed phosphatidylethanolamine to unity.

Materials	Exposed	Hidden	Total
C. vinosum			
Phosphatidylethanolamine	1.0	2.0	3.0
Proteins	0.6	3.4	4.0
A. vinelandii			
Phosphatidylethanolamine	1.0	1.2	2.2
Proteins	0.3	1.7	2.0
Single-layered liposomes			
Phosphatidylethanolamine	1.0	1.5	2.5
Multi-layered liposomes			
Phosphatidylethanolamine	1.0	5.0	6.0

hidden from the aqueous phase at the outer surface of membrane vesicles can be estimated on the assumption that the fast reacting components of amino groups are exposed to the aqueous phase. Fig. 10 shows time courses of the ratio of the amounts of N_3Ph-amino groups of proteins to those of N_3Ph-phosphatidylethanolamine. After reacting for 5 min, the fast reacting component of amino groups ($\tau_{\frac{1}{2}} \approx$ 2 min) must have been almost completely modified, while almost no reaction occurred in the slow components ($\tau_{\frac{1}{2}} \gtrsim 20$ min). Therefore, the ratio of exposed amino groups of proteins to phosphatidylethanolamine was obtained in this region of time. It was 0.6 in the light particles of *C. vinosum* and 0.3 in the membrane preparation of *A. vinelandii*. The ratio of total amounts of amino groups of proteins to phosphatidylethanolamine was obtained after the complete modification of membrane preparations. It was 1.3 in the light particles of *C. vinosum* and 0.9 in the membrane preparation of *A. vinelandii*.

Table III summarizes the distribution of the exposed and the hidden amino groups in phosphatidylethanolamine and proteins in the intracytoplasmic membrane preparations and the liposomes. It is noted that in both of the biological membranes, more amino groups are hidden in the proteins than in phosphatidylethanolamine.

DISCUSSION

The experimental results on the trinitrophenylation of the intracytoplasmic membrane preparations of *C. vinosum* and *A. vinelandii* are interpreted in terms of the closed vesicular structure of the membranes. According to the studies of Gordesky et al. [12] and Litman [35, 36], trinitrobenzenesulfonate does not (or only does very

slowly) penetrate the biological and artificial membranes. In the intracytoplasmic membrane preparation of *A. vinelandii* and in the single- and the multi-layered liposomes, phosphatidylethanolamine was modified by trinitrobenzenesulfonate in the fast and slow phases. In addition to these phases, a very slow phase appeared in the light particles of *C. vinosum*. It is supposed that the fast reacting component of phosphatidylethanolamine is exposed to the aqueous phase at the outer surface of the vesicular structure of membranes. The rate of this modification is limited directly by the reaction between the amino groups and trinitrobenzenesulfonate. The slowly reacting component of phosphatidylethanolamine is probably exposed at the inner surface of the vesicular structure. The reaction must be limited by the process of trinitrobenzenesulfonate penetrating the lipid bilayer of membranes. It is difficult to propose the situation of the very slowly reacting component of phosphatidylethanolamine in the light particles of *C. vinosum*.

The amino groups of membrane proteins are trinitrophenylated in at least two phases. The half time of the fast phase seemed to be the same as that in phosphatidylethanolamine. The slow phases, however, may be composed of more than one minor phase. Four situations of amino groups are considered in the membrane proteins. In the first case, the amino groups are exposed to the aqueous phase on the outer surface of the membrane. In the second case, the groups are exposed but on the inner surface of the membrane. In the third case, the amino groups are buried in the inside of the membrane or covered by another protein molecule. In the fourth case, the groups are buried in the folding of peptide chains of proteins. The first case corresponds to the fast reacting component of amino groups and the other cases to the slowly reacting components.

In the results on the amino acid analysis, the chloroform/methanol-soluble and the chloroform/methanol-insoluble proteins of intracytoplasmic membranes of *C. vinosum* were rich in the hydrophobic amino acid residues. The chloroform/methanol-soluble proteins are supposed to be "integral" proteins of the membrane [37], since they showed a very low polarity index, 30.3. 22 % of amino groups of the chloroform/methanol-soluble protein fraction and 7 % of those of chloroform/methanol-insoluble protein fraction were fast modified by trinitrobenzenesulfonate (Fig. 6). These findings may suggest that most of the chloroform/methanol-soluble proteins are on the outer surface of the membrane, and that most of the chloroform/methanol-insoluble proteins are located on the inner surface or buried in the membrane matrix.

In the single-layered liposomes composed of phosphatidylethanolamine and phosphatidylglycerol, the proportion of phosphatidylethanolamine located on the outer surface was 40 %. If the lipid molecules were symmetrically distributed on both sides of the membrane vesicles with a diameter 250 Å [24], this value would be calculated to be 58 %. However, this discrepancy is similar to the finding by Michaelson et al. [38] in the liposomes composed of phosphatidylcholine and phosphatidylglycerol. This mixture may have almost the same electric properties as that of phosphatidylethanolamine and phosphatidylglycerol. Israelachvili [39] proposed a model in which the electrostatic repulsion of negative charges of the head group of phosphatidylglycerol decreases the population of phosphatidylglycerol on the inner surface and increases that on the outer surface. This will result in a decrease in the population of neutrally charged lipids such as phosphatidylcholine and phosphatidylethanol-

amine on the outer surface. In the intracytoplasmic membranes of *C. vinosum* and *A. vinelandii*, such electrostatic forces might produce an influence on the distribution of lipids and proteins on the inner and the outer surfaces, although no experimental results on this problem have been reported for biological membranes.

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