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A Fluorescent Aminolipid from a Green Photosynthetic Bacterium†

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ABSTRACT: Kenyon and Gray [Kenyon, C. N., & Gray, A. M. (1974) *J. Bacteriol. 120*, 131–138] first reported the presence of phosphate-free aminolipids in green sulfur bacteria, and we now present a preliminary chemical characterization of the aminolipid isolated from *Chlorobium limicola* f. *thiosulfatophilum*. A major component of our membrane preparations, this lipid contains no phosphorus, glycerol, sugar, ornithine, or lysine. Ultraviolet absorption and fluorescence spectra indicate that the amino moiety of the lipid is an aromatic heterocyclic compound. Infrared spectra indicate that the lipid is a secondary or tertiary amide, and gas chromatographic analysis of the hydrolyzed lipid shows that for each 1100 g of lipid, 1 mol of myristic acid ($C_{14:0}$) is linked in an amide bond. Acid hydrolysis of the lipid yields two fluorescent substances, A (ninhydrin positive) and B (negative), in addition to myristic acid. Proton nuclear magnetic resonance (NMR) studies indicate that substance A contains a butyl group attached to a conjugated ring carbon, two equivalent ethyl groups attached to one or two nitrogen atoms, and two downfield protons (8.4 ppm), perhaps attached to a ring carbon adjacent to a ring nitrogen as in adenine. Substance B also appears to contain a butyl group (not attached to a conjugated ring) as well as two vinyl protons (7.4 and 7.7 ppm) across a ring double bond. *Chlorobium* aminolipid appears to be a new type of lipid, enriched in our membrane preparations from green sulfur bacteria.

In green sulfur bacteria (Chlorobiaceae) roughly half the inner surface of the cytoplasmic membrane is covered with green patches composed of chlorosomes (40 × 70 nm to 100 × 260 nm) and associated base plates (Staehelin et al., 1980; Olson, 1980). Photosynthetic reaction centers inside the membrane are concentrated under the patches. The chlorosomes contain bacteriochlorophyll c, d, or e, which traps the incoming light and transfers the energy to the bacteriochlorophyll a in the reaction centers via the bacteriochlorophyll a in the base plate (Olson, 1980). The reaction center complexes are hydrophobic protein complexes embedded in the membrane, but the base plate consists of hydrophilic bacteriochlorophyll a–proteins arranged in a two-dimensional crystal between the membrane and the chlorosome. These proteins are presumably bound to the membrane by hydrogen bonds, because they can be removed from membranes and/or reaction center complexes by the use of guanidine hydrochloride or arginine hydrochloride but not by the use of high salt or the nonionic detergent Triton X-100 (Olson, 1980). However, the nature of the linking groups on the inside surface of the

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0006-2960/83/0422-1819$01.50/0

membrane has not been determined.

In the course of characterizing a membrane fraction enriched in reaction centers, we found an unusual amino-containing lipid as a major component. Kenyon & Gray (1974) had earlier reported the presence of one or more unidentified aminolipids in five strains of green sulfur bacteria, and very recently, Knudsen et al. (1982) found a similar aminolipid (1–3 μg/mg of dry cell) in three strains of *Chlorobium limicola* f. *thiosulfatophilum*. Since these aminolipids have not been found outside the green sulfur bacteria, they may be required for the unique structure of the photosynthetic apparatus in these organisms.

In this paper we show that *Chlorobium* aminolipid (from *C. limicola* f. *thiosulfatophilum*) is a major component of our membrane preparations and that the aminolipid is unrelated to the known ornithine- and lysine-containing lipids of other bacteria and yeasts (Kawanami et al., 1968; Prome et al., 1969; Kawanami, 1971; Knoche & Shively, 1972; Wilkinson, 1972; Kenyon, 1978). Furthermore, *Chlorobium* aminolipid contains one or two fluorescent chromophores that appear to be aromatic heterocyclic compounds. These observations establish *Chlorobium* aminolipid as a new type of lipid.

Materials and Methods

Organism. Chlorobium limicola f. *thiosulfatophilum* 6230 (strain Tassajara) was grown autotrophically with and without acetate as described previously (Olson et al., 1973).

Unit-Membrane Vesicles. These were prepared from fresh or frozen cells as described previously (Knaff et al., 1979; Olson & Thornber, 1979). Some preparations were treated with 2 M NaCl and others with 2 M guanidine hydrochloride.

Extraction Procedure. Lipids were extracted from pelleted membrane preparations with chloroform–methanol–water (1:2:0.8 v/v) by a modification of the Bligh–Dyer procedure as used for bacteria and cell fractions (Kates, 1972). The isolated lipids were dissolved in chloroform for TLC.[1]

Thin-Layer Chromatography. One-dimensional TLC was performed in chloroform–methanol–ammonia (13:5:1 v/v) (solvent I), chloroform–methanol–acetone–acetic acid (10:2:4:1) (solvent II), chloroform–methanol–water (65:25:4) (solvent III), or chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1) (solvent IV) (Kates, 1972) with the following plates: precoated TLC plates (silica gel 60, EM Laboratories) and Prekotes (Adsorbsil 5, Applied Science Laboratories). Spots were located by spraying with water or exposing to iodine vapor. After removal of the water or iodine, the plates were sprayed with ninhydrin for aminolipids, followed by molybdate (Vaskovsky & Kostetsky, 1968) for phospholipids. Glycolipids (and other lipids with vicinal OH groups) were visualized on a separate plate with Schiff's reagent (Shaw, 1968). Sometimes glycolipids were visualized with α-naphthol (Kates, 1972).

Purification of Chlorobium Aminolipid. For isolation and purification of larger quantities of lipid, fresh or frozen cell paste was suspended in water, and methanol–chloroform was added to give a solvent ratio of methanol–chloroform–water of 2:1:0.8. After several hours of extraction at ~20 °C 1 volume each of chloroform and water was added to bring the solvent ratio to 1:1:0.9 and to cause phase separation. The methanol–water layer was discarded, and the chloroform layer was filtered to give an extract of lipids and pigments. The chloroform was removed in vacuo and the residue dissolved in a minimum volume of chloroform. This residue was fractionated by acetone precipitation at 0 °C (Kates, 1972). A mixture of aminolipid, phospholipids, and about 25% of pigments was precipitated by this treatment. The supernatant, containing MGDG and about 75% of pigments, was discarded. The precipitate was dissolved in 90% methanol–water and partitioned with an equal volume of hexane. Carotenoid, chlorophyll, and phospholipids moved into the hexane layer, while aminolipid and 50% of chlorophyll remained in the methanol–water layer. This layer was evaporated to dryness in vacuo and redissolved in hexane–2-propanol–water (6:8:1.1) (solvent V) for HPLC (Guerts van Kessel et al., 1977). Isocratic HPLC was carried out on a silica gel (EMSG 60, 5-μm) column (1.0 × 25 cm) with absorbance detection at 206 nm. Solvent V was pumped at about 3.5 mL/min at pressures between 50 and 100 atm (800 and 1600 psi). The aminolipid was eluted from the column about 10 min after injection and was further purified by one-dimensional TLC with solvent III. From 50 g of wet cell paste the yield of purified aminolipid was approximately 1 mg by dry weight.

Infrared Absorption Spectra. A sample in solution was placed dropwise on a AgCl window (50-mm diameter, 1.5-mm thickness) so as to form a circle about 5 mm in diameter. The dried sample on the window was then placed in the beam of a FT–IR spectrometer (Nicolet Model 7199B) and the spectrum recorded at a resolution of 1 or 4 cm^{-1} from 400 to 4000 cm^{-1}.

Ultraviolet Spectra. Absorption spectra were recorded from 200 to 400 nm on Cary 14R and 118 spectrophotometers. Absorbance values were recorded at 1-nm intervals with the Cary 118 spectrophotometer. At each wavelength a fourth-order polynomial was fitted to the seven data points for λ, λ ± 1 nm, λ ± 2 nm, and λ ± 3 nm by the method of least squares (Savitsky & Golay, 1964). The zero- and second-derivative values for each wavelength were calculated from the fitted polynomials. Fluorescence excitation and emission spectra were measured on a Perkin-Elmer spectrophotofluorometer MPF-4.

Fatty Acid Analysis. Aliquots of aminolipid (9.3 μg) were treated with 10% (w/v) BCl_3 in methanol (Campisi & Scandella, 1980) and incubated at 70 °C for various time intervals. The fatty acid methyl esters thus formed were assayed with a Hewlett-Packard GC, Model 5830A, equipped with a silicone column (3% SE-30) operated at 225 °C. The methyl ester of $C_{20:0}$ fatty acid was used as an internal standard.

Nuclear Magnetic Resonance Spectra. Proton NMR spectra were obtained with a Bruker WH-360 spectrometer. 4,4-Dimethyl-4-silapentane-1-sulfonate and tetramethylsilane served as standards in D_2O and organic solvents, respectively.

Results

Membrane Preparation Lipids. After membrane preparations (Olson & Thornber, 1979; Olson, 1980) were extracted and analyzed by TLC (see Figure 1), the main lipids were found to be MGDG (L1), aminolipid (L3), PE (L2), PG (L5), and two other phospholipids, possibly PI (L4) and DPG (L6). (Membrane preparations treated with 2 M NaCl and 2 M guanidine hydrochloride gave essentially the same results.) From the size and staining behavior of the TLC spots, it was estimated that the aminolipid was a major component of the total lipid pool. Membrane preparations appeared to contain the same lipids found in whole cells but in different proportions.

[1] Abbreviations: DGDG, digalactosyl diglyceride; DPG, diphosphatidylglycerol; FT–IR, Fourier transform–infrared; GC, gas chromatography; HPLC, high-performance liquid chromatography; MGDG, monogalactosyl diglyceride; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SL, sulfolipid; TLC, thin-layer chromatography.

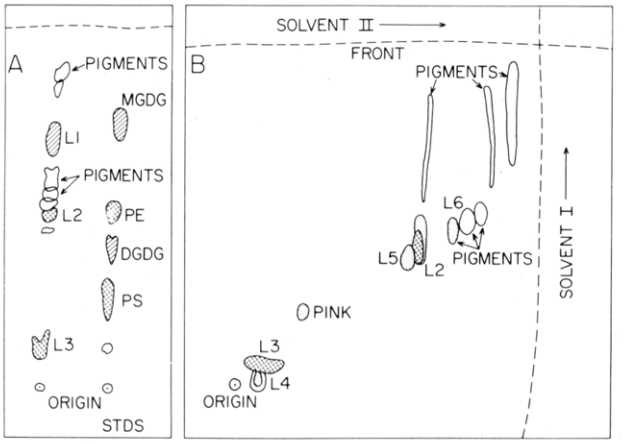

FIGURE 1: Thin-layer chromatography of lipids extracted from unit-membrane vesicles on silica (Adsorbsil 5). (A) One-dimensional chromatogram of lipid extract from vesicles treated with 2 M guanidine hydrochloride. Spots were sprayed with ninhydrin (crosshatched = positive) and Schiff's reagent (hatched = positive) on separate plates. (B) Two-dimensional chromatogram of lipid extract from vesicles treated with 2 M NaCl. Spots were sprayed with ninhydrin followed by molybdate (stippled = positive). Solvents are defined under Materials and Methods.

Table I: R_f Values[a] × 100 of β-Aminolipid (L3), PS, and PE in Three Solvent Systems

	solvent		
lipid	I	III	IV
L3	3	16	23
PS	8	17	56
PE	32	42	53–56

[a] Detected by sulfuric acid charring on EM 60 TLC plates.

The proportion of PE varied in various lipid extracts from whole cells, and in some cases high levels of PE were correlated with the presence of colorless, motile organisms (not *C. limicola*) seen in the cultures under the microscope. In the absence of these organisms, the levels of PE were very low, but never zero.

Chlorobium Aminolipid. This lipid was separated from most of the other lipids by several steps of solvent extraction and partitioning followed by HPLC (see Materials and Methods). A small amount of PE was removed by preparative TLC in solvent III. Purity was assayed from the infrared absorption spectrum; the usual contaminant (if any) was PE. Contaminated samples were rechromatographed by TLC in solvent III. The purified aminolipid migrated as a single spot in each of the solvents I, III, and IV. The R_f values are listed in Table I. After TLC in solvents I and/or II, this lipid (3–10 μg) gave a single ninhydrin-positive spot (purple color) and negative reactions with molybdate and Schiff's reagent (see Figure 1). With α-naphthol the lipid initially stained pink and then turned yellow. The absorption spectrum [E_{220} = 1.96 ● 0.06 $(g/L)^{-1}$ cm^{-1}] and the second derivative dissolved in solvent V indicated absorption bands at 225 and 231 nm. The fluorescence emission spectrum (Figure 2) showed a maximum at 340 nm with excitation maxima at 232, 288, and ~325 nm. There was no excitation maximum at 225 nm. When the native α-form was incubated in 0.1 mM HCl in methanol at 20 °C for 17 h, it was converted irreversibly to the β-form, which appeared to have a lower fluorescence yield in solvent V than the α-form. The infrared and NMR characteristics of both forms are summarized in Tables II and III. In neither form was there an infrared band in the vicinity of 1730 cm^{-1}, showing that no ester bonds are present. Strong bands

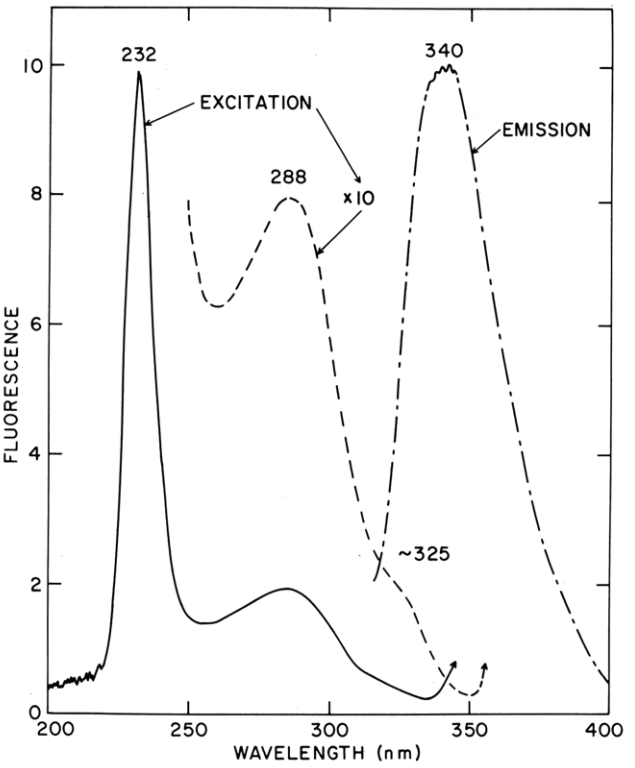

FIGURE 2: Fluorescence of α-aminolipid in solvent V. Excitation spectrum for λ_{em} = 350 or 370 nm. Emission spectrum for λ_{ex} = 232 nm.

Table II: Trough Positions in Infrared Spectra of α- and β-Aminolipid

α spectrum		β spectrum	
trough	$\bar{\nu}$ (cm^{-1})	trough	$\bar{\nu}$ (cm^{-1})
		α	3414 ± 2
a	3287 ± 5	β	3301 ± 1
			3190 ± 5
		δ	3046 ± 4
b	2951 ± 2	b	2951 ± 2
c	2921 ± 3	c	2921 ± 3
d	2852 ± 3	d	2852 ± 3
e	1641 ± 1	ε	1637 ± 1
f	1610 ± 6	ζ	1620 ± 1
g	1520 ± 2	η	1528 ± 1
h	1466 ± 0	θ	1465 ± 1
i	1401 ± 3	ι	1396 ± 1
j	1293 ± 2	κ	1293 ± 2
		λ	1262 ± 1
k	1206 ± 1	μ	1214 ± 2
		ν	1178 ± 0
l	1120 ± 15		
m	1068 ± 4	ξ	1078 ± 1
n	1035 ± 1	ο	1026 ± 1
o	888 ± 1	π	914 ± 1
p	830	ρ	840 ± 1
		σ	818 ± 0
q	721 ± 0	τ	720 ± 0
r	670	υ	671 ± 2
		φ	593 ± 1

around 1630 cm^{-1} (Figure 3) indicated an amide linkage.

Fatty Acid Analysis. After hydrolysis and esterification in BCl_3–methanol, a single fatty acid methyl ester peak was observed upon GLC (see Materials and Methods). This peak was identified as methyl myristate ($C_{14:0}$) by its retention time relative to several standards. The amount of methyl myristate released increased with the incubation time in BCl_3–methanol up to a maximum of 11 h. Under these hydrolysis conditions ester-linked fatty acids are usually released from lipids within

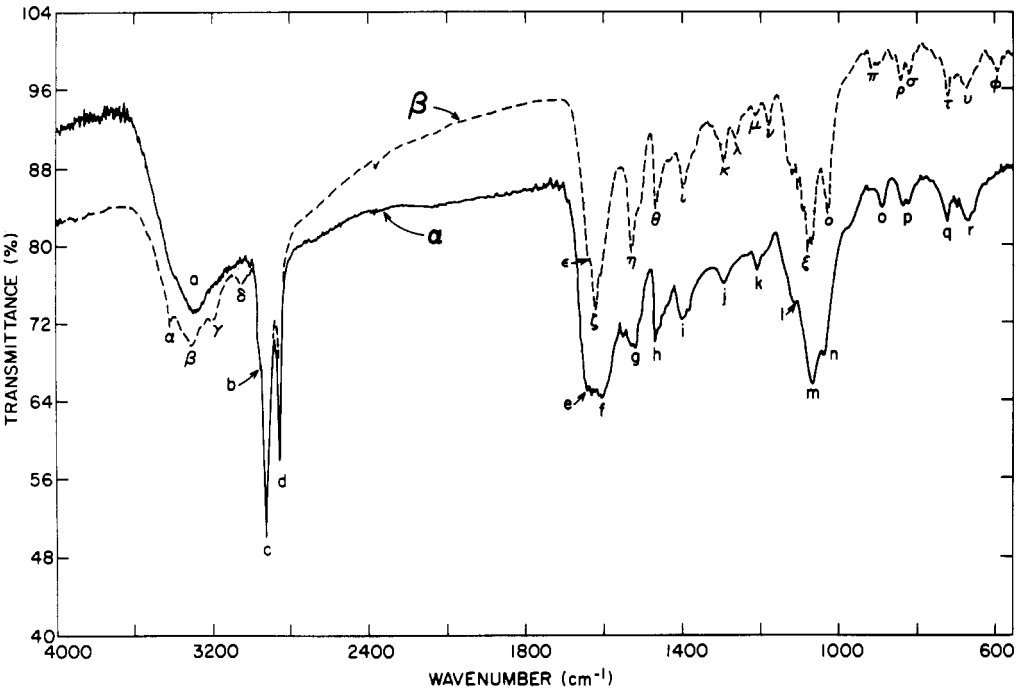

FIGURE 3: Infrared spectra of α- and β-forms of aminolipid. Form β may be obtained by treating form α with weak acid. See text for details. The wavenumbers corresponding to the various absorption bands are listed in Table I.

30 min (C. Scandella, unpublished results). The release of fatty acid followed pseudo-first-order kinetics, so that

$$\ln (L_0 - P) = \ln L_0 - kt$$

where L_0 is the initial amount of fatty acid bound to aminolipid and P is the amount of fatty acid released at time t. Several values of L_0 between 1.89 and 1.98 μg were chosen, and the correlation coefficient $r = \sum xy/(\sum x^2 + \sum y^2)$ [where $x = t$ and $y = \ln (L_0 - P)$] was calculated for each value of L_0. The maximum correlation coefficient value of −0.9983 corresponded to $L_0 = 1.91$ μg. Since the dry weight of the aminolipid sample was 9.3 μg, the minimum molecular weight of the lipid is (9.3 μg of lipid/1.91 μg of $C_{14:0}$) × 228 μg of $C_{14:0}$ mol^{-1} = 1100 g of lipid (mol of $C_{14:0}$)$^{-1}$.

Acid Hydrolysis. Aliquots of lipid (0.1–0.2 mg each) were hydrolyzed at 110 °C in 6 N HCl for 5, 22, and 96 h and the products partitioned between methanol–water and chloroform before analysis by TLC in solvent III (Figure 4). No differences were noted among the three times of hydrolysis. The methanol–water phase contained two substances that migrated 4.5 cm (A) and 5.5 cm (B) from the origin. A was ninhydrin positive; B was ninhydrin negative. The chloroform phase contained a small amount of A in addition to a substance C that migrated 12 cm from the origin. Substance C comigrated with a $C_{14:0}$ fatty acid standard on the plate. The proton NMR spectra (Table III) of substance C and authentic $C_{14:0}$ were identical; thus, substance C was identified as myristic acid.

Amino Acid Analysis. The products of acid hydrolysis were examined on the amino acid analyzer, and the ninhydrin-positive substance A was found to have a retention time of 145 min. Of the 16 amino compounds tested[2] only γ-amino-*n*-butyric acid (144 min) fell in this range. On the analyzer the ninhydrin reaction with A yielded a product for which $A_{440}/A_{570} \approx 1.8$ instead of 0.2, the value for most amino acids other than proline and hydroxyproline. Treatment of A with KOH or NH_4OH (pH ~13) converted it irreversibly to a new form A′ with a retention time of 149 min and a "normal" A_{440}/A_{570} ratio. No known amino acid was found among the hydrolysis products of the lipid.

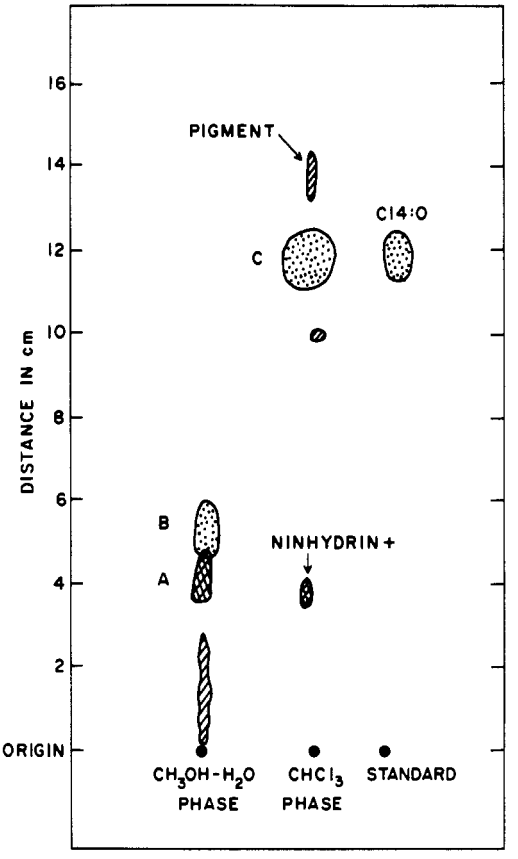

FIGURE 4: Thin-layer chromatography of aminolipid acid hydrolysate (6 N HCl, 110 °C, 22 h) on silica (EM 60) in solvent III.

[2] The amino compounds tested (retention time in minutes in parentheses) are tyrosine (122), phenylalanine (126), glucosamine (132–), galactosamine (133+), γ-amino-β-hydroxybutyric acid (139), β-alanine (140–), β-aminoisobutyric acid (140+), δ-aminolevulinic acid (~141), mannosamine (141), γ-amino-*n*-butyric acid (144), hydroxylysine (148), β-amino-*n*-butyric acid (151), δ-amino-*n*-valeric acid (151), ornithine (153), ethanolamine (154), and ammonia (158).

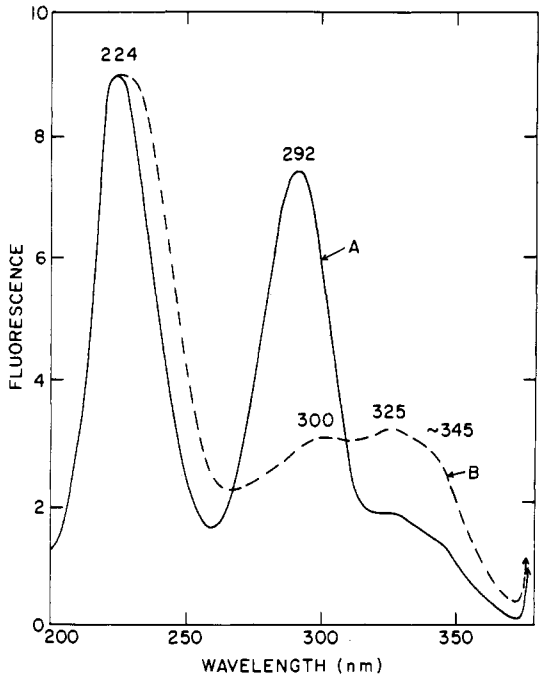

FIGURE 5: Fluorescence excitation spectra for components A and B dissolved in water. pH ~5.2 for A and 7.6 for B. λ_{em} = 390 nm.

Fluorescence of Substance A. Dissolved in solvent V, substance A emitted at ~347 nm with excitation maxima at 237, 288, and ~326 nm. The resemblances to the fluorescence characteristics of the native lipid (Figure 2) suggested that A contained the fluorescent moiety of the lipid. When A was dissolved in water (pH ~5), its fluorescence emission peak shifted to 390–400 nm, and the excitation spectrum (Figure 5) exhibited shoulders at ~345 nm not evident in solvent V. When an aqueous solution of A was treated with KOH (pH ~11), the fluorescence emission peak shifted down to 360 nm, and the fluorescence yield increased about 3×. The excitation spectrum for this new form A′ is shown in Figure 6. When the pH was lowered to 7.4 with HCl, the emission peak shifted back to ~390 nm, and the yield dropped again to its former level. The excitation spectrum also changed to that for the intermediate form of A′ (Figure 6). As the pH was lowered to 3.3, the excitation spectrum changed to that for the acid form of A′. The acid form of A′ was clearly not the same as the acid form of A (pH 5.2), since the excitation peak at 332 nm for A′ (Figure 6) did not coincide with either peak at 325 or 345 nm for A (Figure 5).

By plotting fluorescence excitation and absorbance changes as a function of pH, we determined two proton dissociation constants for A′ as shown in Figure 7. From ΔA_{285}, we determined a pK_1 of ~5.6, and from ΔA_{300} and ΔA_{240} we determined a pK_2 of ~9.6. From ΔEX_{290} (reference pH 3.3) and ΔEX_{250}, we found pK_1 values of 5.5 and 5.6, and from ΔEX_{250} (reference pH 11.4) and ΔEX_{317} we found a pK_2 of ~9.

Fluorescence of Substance B. Dissolved in solvent V, substance B had fluorescence characteristics similar to those of substance A. When B was dissolved in water (pH ~8), its emission peak also shifted to 400 nm, and its excitation spectrum (Figure 5) showed a much diminished peak at 300 nm compared to the spectrum of B in solvent V. When B in water was treated with KOH (pH ~11), the fluorescence emission shifted only slightly from 400 to 410 nm, and the fluorescence yield stayed about the same. The excitation spectrum for this new form B′ is shown in Figure 8. When the pH was lowered to 3.0, the emission shifted back to 400

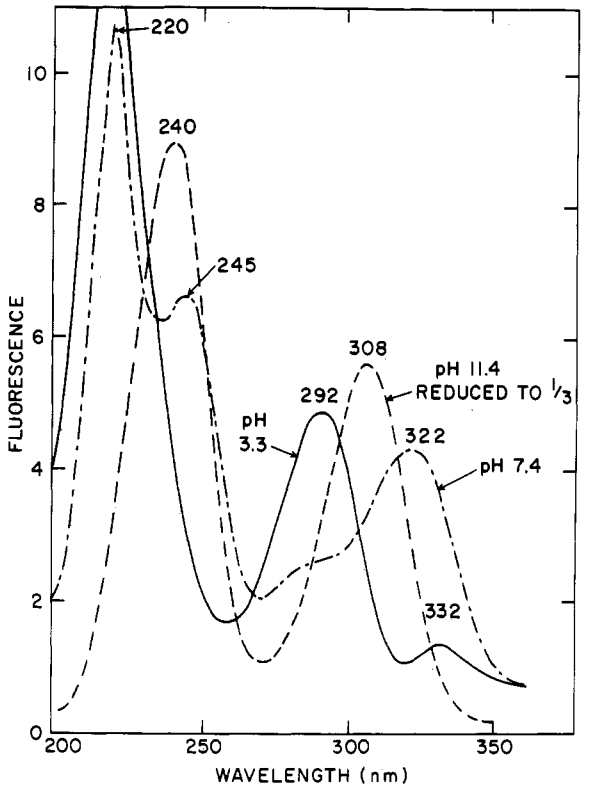

FIGURE 6: Fluorescence excitation spectra for A′ in water. λ_{em} = 360 (pH 11.4) or 380 nm (pH 3.3 and 7.4).

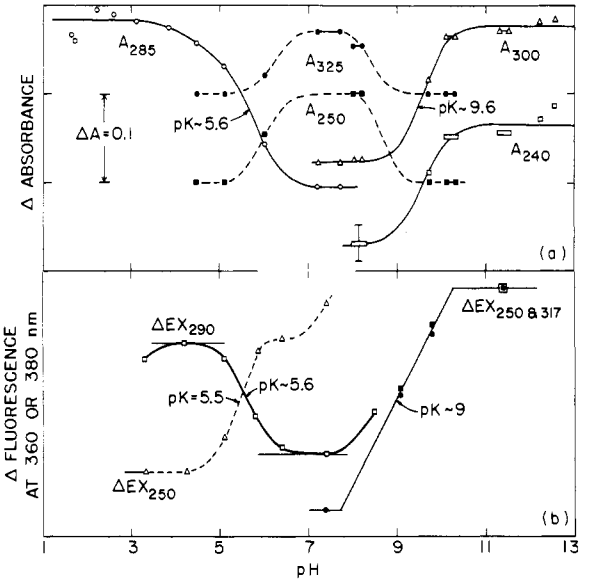

FIGURE 7: (a) Absorbance changes (ΔA) and (b) fluorescence excitation changes (ΔEX) in A′ with changing pH.

nm, and the excitation spectrum changed to the intermediate form (Figure 8). As the pH dropped to 2.0, the excitation spectrum for the acid form (Figure 8) appeared. The intermediate form of B′ was quite similar to B at pH 7.6 (Figure 5) in excitation spectrum, although B′ showed a peak at 265 nm not found in the spectrum of B.

We determined dissociation constants for B′ by plotting fluorescence excitation changes as a function of pH as shown in Figure 9. From ΔEX_{300} (reference pH 2), we found 2.5 < pK_1 < 3.0, and from ΔEX_{350} and ΔEX_{270} (reference pH 12.0) we found pK_2 = 4.6.

Infrared Spectra of A and B. Most spectra for both A and B showed one broad band ($\Delta\bar{\nu}$ = 80–160 cm^{-1}) in the region

Table III: Proton Chemical Shifts (NMR) for α- and β-Aminolipid and Products of Acid Hydrolysis

A in D_2O			A′ in 0.1 M KOH in D_2O			A′ in 0.1 M KCl + H_2O in D_2O	
ppm	multiplicity	spins	ppm	multiplicity	spins	ppm	multiplicity
0.91[a]	3	2-3	0.87[a]	3	3	+	?
			1.03[a]	3	5		
1.26[a]	3	12	1.31[a]	4-5	4	1.27[a]	3
1.97	1	36[b]	1.90	1	20[b]	2.08	1
			2.38	1-2	2-3		
			2.54[a]				
				4-5	5		
			2.56				
3.05[a]	2	5-6				3.05	2
4.8[c]		many	4.8[c]		many	4.8[c]	
8.43	1	2-3	8.44	1		8.23	1

B in D_2O			B′ in 0.1 M KOH in D_2O			B′ in 0.1 M KCl + H_2O in D_2O	
ppm	multiplicity	spins	ppm	multiplicity	spins	ppm	multiplicity
0.80[a]	3	4	0.80	br	4	0.80	?
1.19[a], 1.21	2-3	8	1.19	br	9	1.19, 1.21	?
1.90	1	4	1.90	1	3	2.08	1
4.8[c]		many	4.8[c]		many	4.8[c]	
7.38[a]	2	1	7.36[a]	2		7.39[a]	2
7.71[a]	2	1	7.70[a]	2		7.71[a]	2

C (fatty acid) in $CDCl_3$			α-lipid in $CDCl_3$–CD_3OD (2:1)			β-lipid (β-form) in $CDCl_3$–CD_3OD (2:1)		
ppm	multiplicity	spins	ppm	multiplicity	spins	ppm	multiplicity	spins
			0.70	1	1.5	0.72	1	3
0.88[a]	3	3.0	0.88[a]	3	3	0.90	3	3
1.26	1	21	1.26	1		1.28	1	
1.31	1		1.30	1	23	1.3	br	28
			1.34	1		1.36	1	
			1.51	br	3	1.5	br	2
1.59[c]			1.60	br	3	1.64	br	2
			1.70[a]	1 or 3	2	1.73	1	1
			2.02	br	4	2.02	br	1
2.36[a]	3	2.2	2.21[a]	3	3	2.24	br	1
			3.36[d]			3.36[d]		
			3.68[e]	2	50[c]	3.68[e]	2	120[c]
			4.3[d]			4.3[d]		
			5.34	br	1.2	5.34	1	
			6.86	br	3-4	6.86	?	
			7.44[f]			7.4[f]		

[a] $J \sim 8$ Hz. [b] Protons exchange with solvent. [c] H_2O. [d] CH_3OH. [e] $J = 2.9$ Hz. [f] $CHCl_3$.

1630–1740 cm^{-1} and another very broad band ($\Delta\bar{\nu}$ = 600–800 cm^{-1}) at about 3430–3440 cm^{-1} (A) or 3120–3430 cm^{-1} (B). Details of these spectra were poorly reproduced from sample to sample.

Proton NMR Spectra of A and B. These data are summarized in Table III. The NMR spectrum for A′ was the same as that for A except that the 8.43-ppm resonance in A was shifted to 8.23 ppm in A′. The spectra for B and B′ were the same. The spectra for A and B were distinctly different except for corresponding peaks at 0.8–0.9, 1.2–1.3, and 1.9–2.0 ppm.

Discussion

The lipid composition of the membrane preparations used in this study is compared to results for whole cells in Table IV. *Chlorobium* aminolipid, which represents about 2–3% of the total lipid in whole cells (Knudsen et al., 1982), appears to be significantly enriched in our membrane preparations. The trivial name "*Chlorobium* aminolipid" is based on the color reaction with ninhydrin, since only free amino groups are known to react with ninhydrin to give the characteristic purple color. As previously observed by Kenyon & Gray (1974), *Chlorobium* aminolipid contains no phosphate.

Table IV: Lipid Composition of Membranes and Whole Cells of *C. limicola* f. *thiosulfatophilum*

	whole cells		
lipid	Kenyon & Gray (1974) and Kenyon (1978)	Knudsen et al. (1982)	membranes (this work)
MGDG	+	+++	+
glycolipid (lipid 2)[a]	–	++	–
DPG (cardiolipid)[a]	+	+	+
PE	–	+	+
PG	+	+	+
L4	+	–	+
aminolipid (lipid 1)[a]	+	+	+
glycolipid II	+	+	–
SL	+	+	–
lyso-PE	–	tr	–

[a] Names in parentheses are those used by Knudsen et al. (1982).

Contrary to the report of Knudsen et al. (1982), we found no evidence for carbohydrate in this lipid. The yellow color upon

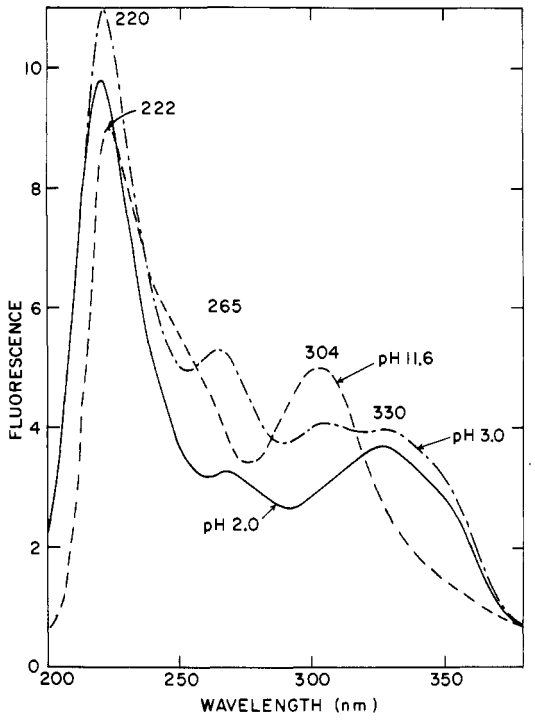

FIGURE 8: Fluorescence excitation spectra for B′ in water at pH 2.0, 3.0, and 11.6. λ_{em} = 400 nm.

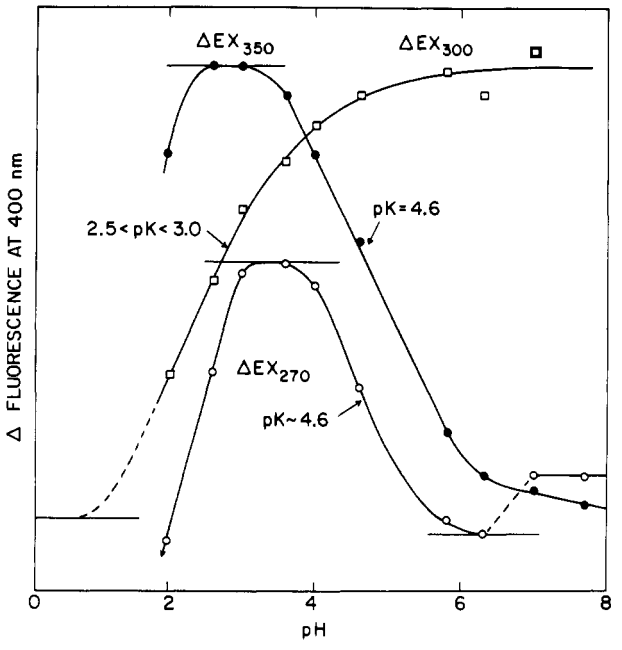

FIGURE 9: Fluorescence excitation changes (ΔEX) in B′ with changing pH.

staining with α-naphthol indicated only a polar lipid. The negative reaction with Schiff's reagent further demonstrated the absence of vicinal hydroxyl groups.

The ultraviolet absorption and fluorescence data indicated that the fluorescence of the aminolipid (Figure 2) was due to one or two fluorescent components related to the products of acid hydrolysis, A and B. These components, as well as A and B, appeared to be aromatic heterocyclic structures such as flavins, indoles, purines, pyrimidines, and pteridines. Since the infrared spectra of A and B showed a strong C=O stretching band, we looked for aromatic ring structures containing a carbonyl group. Also, since A was ninhydrin positive, we expected an amino group attached to the ring. Flavins were

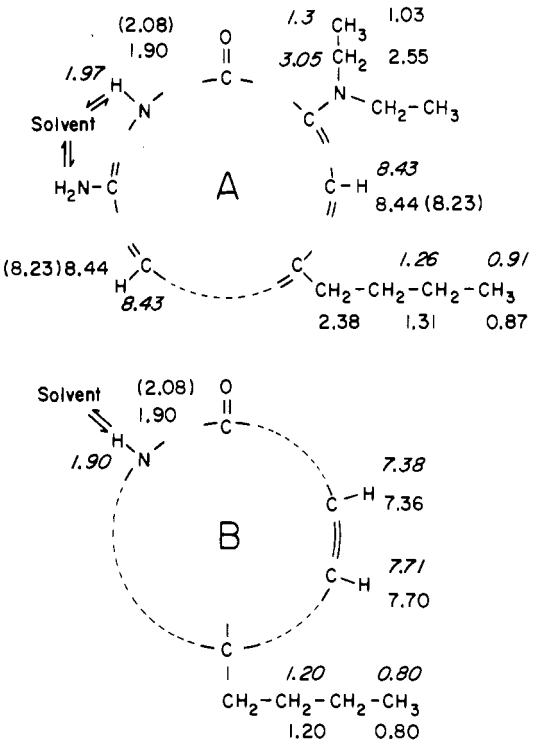

FIGURE 10: Interpretation of infrared and proton NMR spectra for A, A′, B, and B′. Numbers indicate chemical shifts in ppm. Those numbers in italics are for A and B before addition of KOH; those numbers in roman script are for A′ and B′ in 0.1 M KOH; those numbers in parentheses are for A′ and B′ in 0.1 M KCl + H_2O.

eliminated from consideration because they absorb and fluoresce in the visible range. Indoles, purines, and pyrimidines were ruled out because their absorption spectra lack bands in the region 325–345 nm. The 2-amino-4-oxo-7,8-dihydropteridines (7,8-dihydropterins) with absorption bands at ~230, ~280, and 320–330 nm at pH 7 (Nagai, 1968; Blakley, 1969) most closely matched the spectral characteristics of the intact aminolipid and compounds A and B dissolved in solvent V. The 7-hydroxypterins (isoxanthopterins) at pH 1 had bands that matched almost as well with bands at ~210, ~290, and ~340 nm (Blakely, 1969). (At pH 1 a 7-hydroxypterin is a 7-oxopterin with a 7,8 single bond as in 7,8-dihydropterin.) Comparison of the fluorescence excitation spectra for A in solvent IV and A′ in water (Figure 6) suggests that A exists in the acid form (pH 3.3) in solvent V and in the intact lipid.

Although comparisons can be made between certain pterin spectra (Fukushima & Akino, 1968; Nagai, 1968; Blakley, 1969) and the ultraviolet spectra of A′ and B′, the weight of the evidence is against A′ and B′ being pterins. The effect of pH on the spectra of A′ and B′ differs from its effect on the spectra of known pterins. In the latter case, protonation of the C4–N3 amide system at low pH causes the longest wavelength maximum to move 30–50 nm to shorter wavelengths because of the loss of a double bond in the pyrimidine ring (R. L. Blakely, personal communication). For both A′ and B′, lowering the pH causes the longest wavelength maximum (absorbance or fluorescence excitation) to move to longer wavelengths. This behavior is not easily reconciled with the pterin ring structure. Therefore, we conclude that substances A′ and B′ probably are not pterins.

The assignments for the chemical shifts listed in Table III for A, A′, B, and B′ are given in Figure 10. The assignments are most straightforward for A′ in 0.1 M KOH: Two ethyl groups (1.03 and 2.55 ppm) appear to be attached to one or

two nitrogen atoms from the close match to the corresponding values for 2-(ethylamino)ethanol, compound 296 ($C_{12}H_{17}N_3$), procaine, and 3-(diethylamino)-2,2-dimethylpropionaldehyde (Bhacca et al., 1962, 1963). The butyl group (0.87, 1.31, and 2.38 ppm) appears to be attached to a conjugated ring carbon. The shifts (0.87 and 1.31 ppm) assigned to the $CH_3(CH_2)_2$– protons in the butyl chain are typical of aliphatic chains [e.g., *n*-octane and 1-decanol (Bhacca et al., 1962)], and it seemed reasonable to assign the 2.38-ppm shift to the $-CH_2-$ protons connecting the chain to the ring carbon as in 2-methyl-3-*n*-amylpyrrole (Bhacca et al., 1962). Two protons (8.44 ppm) appear to be attached to conjugated ring carbons adjacent to ring nitrogens as in adenine. This chemical shift is also typical of vinyl protons in pterins (Fukushima & Akino, 1968; Dieffenbacher & von Philipsborn, 1969; Poe, 1973).

For A, the two ethyl groups (1.3 and 3.05 ppm) bonded to nitrogen(s) resemble the examples given in the literature for *N*-ethyl-*N*-phenyl-2-aminoethanol and 3-amino-4-methoxybenzenesulfonic acid diethylamide (Bhacca et al., 1963) in which the N atoms are more electron withdrawing than in the case of A′ in 0.1 M KOH. The chemical shift assignments for the $CH_3(CH_2)_2$– protons (0.90 and 1.26 ppm) in the butyl chain remain about the same as those in A′ in KOH except for the absence of a chemical shift to assign to the $-CH_2-$ protons connecting the chain to the ring. The chemical shifts at 1.9–2.1 ppm in both A and B are assigned to protons on ring nitrogens as in *N*-methylpiperazine (Bhacca et al., 1962). The protons exchange with the solvent (D_2O/H_2O), and in the case of A, the number of spins is much larger than the number of A molecules.

The assignments for B were ambiguous except for the 7.38- and 7.71-ppm doublets (J = 8.1 Hz), which were assigned to vinyl protons across a ring double bond. For corresponding vinyl protons in 8-methylpterin dissolved in trifluoroacetic acid, the chemical shifts are also doublets (J = 3.5 Hz) at 8.78 and 8.85 ppm (Dieffenbacher & von Philipsborn, 1969). The other chemical shifts (0.80 and 1.20 ppm) were assigned to a butyl chain apparently connected to a carbon atom not part of a conjugated ring as in compound 493 ($C_7H_{12}O_3$) (Bhacca et al., 1963).

The lack of reproducibility in the infrared spectra of both A and B suggested that these compounds may be sensitive to light and/or oxygen. The very broad bands observed may have been caused by polymerization. The irreversible conversion to A to A′ under alkaline conditions may be due to a ring rearrangement that permits the amino group in A′ to react freely with ninhydrin.

The infrared spectrum of the intact aminolipid in either form (α or β) shows no band in the vicinity of 1730 cm^{-1}; there are no ester bonds in the molecule. This means that the lipid cannot contain glycerol bound in a glyceride backbone. According to Kates (1972), the absorption bands in the 3300-cm^{-1} region are assigned to NH stretch modes of primary and/or secondary amino groups (free or bonded), while the sharp bands at 2951, 2921, and 2852 cm^{-1} are assigned to C–H stretching modes (probably in the $-CH_3$ and $-CH_2$ groups of the $C_{14:0}$ fatty acid). The bands at about 1640 cm^{-1} are assigned in part to C=O stretch modes in secondary or tertiary amides, while bands between 1620 and 1520 cm^{-1} are assigned in part to NH deformation modes in primary and/or secondary amino groups. The strong bands in the region 1120–1020 cm^{-1} are assigned to the C–O stretch mode of hydroxyl groups and/or alkyl ethers.

The long time (11 h) required to liberate the $C_{14:0}$ fatty acid from aminolipid with BCl_3 indicates an amide linkage of the fatty acid to the rest of the lipid as in ceramide and sphingomyelin. We assume one fatty acid per lipid molecule and thereby estimate the minimum molecular weight of the lipid to be about 1100.

In the NMR studies of the intact lipid it was straightforward to assign the $C_{14:0}$ resonances, 0.88, 1.26, and 1.31 ppm, but the 2.36-ppm resonance had disappeared. At present, we are unable to interpret the remaining chemical shifts in the NMR spectra of the lipid. We are also unable to detect the presence of substances A and/or B in the intact lipid by NMR. Since the $C_{14:0}$ fatty acid spectrum is so clearly present, it may be that there are several fatty acid molecules per fluorescent chromophore in the lipid. If this is correct, there must be additional nonfluorescent components also in the lipid. The absorption band at 225 nm indicates that a nonfluorescent chromophore is present as well as fluorescent chromophore(s) absorbing at 232 nm.

A crucial question in this study is whether our membrane preparations were significantly contaminated with chlorosome material or other lipid-containing cell components. Previous studies (Olson & Thornber, 1979; Olson, 1980) showed that our membrane preparations contained closed unit-membrane vesicles (30–100 nm in diameter) with a mean diameter of about 60 nm, as well as open membrane fragments. No intact chlorosomes were visible. These membrane preparations contained tightly bound reaction-center complexes, cytochromes, carotenoid, bacteriopheophytin c (Olson, 1981), a newly discovered porphyrin P665 (Swarthoff et al., 1982), and possibly small and variable amounts of monomeric bacteriochlorophyll c (674 nm). In the preparations extracted for lipids, the ratio of aggregated bacteriochlorophyll c (744 nm) to bacteriochlorophyll a (810 nm) was estimated to be about 1:7; the ratio of bacteriopheophytin c and P665 (674 nm) to bacteriochlorophyll a was estimated to be about 3:5. It is, therefore, possible that *Chlorobium* aminolipid might be concentrated in the chlorosomes and might have been a "contaminant" in our membrane preparations. It seems more probable, however, that the aminolipid is concentrated in the membranes derived from the cytoplasmic membrane, but a further investigation of the lipid composition of purified chlorosomes (Schmidt, 1980) must be carried out to establish where the aminolipid is concentrated.

Acknowledgments

We appreciated the help of Nicholas Alonzo with the amino acid analyses and the help of H. W. Siegelman with the HPLC apparatus. Donald Lawler recorded the NMR spectra in Alan McLaughlin's laboratory, and Stanley Seltzer (Brookhaven National Laboratory Chemistry Department) helped us interpret the data. Jeff Elhai (Stony Brook) performed the GLC analysis.

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Metabolism of 4-Pentenoic Acid and Inhibition of Thiolase by Metabolites of 4-Pentenoic Acid†

Horst Schulz

ABSTRACT: The metabolism of 4-pentenoic acid, a hypoglycemic agent and inhibitor of fatty acid oxidation, has been studied in rat heart mitochondria. Confirmed was the conversion of 4-pentenoic acid to 2,4-pentadienoyl coenzyme A (CoA), which either is directly degraded via β-oxidation or is first reduced in a NADPH-dependent reaction before it is further degraded by β-oxidation. At pH 6.9, the NADPH-dependent reduction of 2,4-pentadienoyl-CoA proceeds 10 times faster than its degradation by β-oxidation. At pH 7.8, this ratio is only 2 to 1. The direct β-oxidation of 2,4-pentadienoyl-CoA leads to the formation of 3-keto-4-pentenoyl-CoA, which is highly reactive and spontaneously converts to another 3-ketoacyl-CoA derivative (compound X). 3-Keto-4-pentenoyl-CoA is a poor substrate of 3-ketoacyl-CoA thiolase (EC 2.3.1.16) whereas compound X is not measurably acted upon by this enzyme. The effects of several metabolites of 4-pentenoic acid on the activity of 3-ketoacyl-CoA thiolase were studied. 2,4-Pentadienoyl-CoA is a weak inhibitor of this enzyme that is protected against the inhibition by acetoacetyl-CoA. The most effective inhibitor of 3-ketoacyl-CoA thiolase was found to be 3-keto-4-pentenoyl-CoA, which inhibits the enzyme in both a reversible and irreversible manner. The reversible inhibition is possibly a consequence of the inhibitor being a poor substrate of 3-ketoacyl-CoA thiolase. It is concluded that 4-pentenoic acid is metabolized in mitochondria by two pathways. The minor yields 3-keto-4-pentenoyl-CoA, which acts both as a reversible and as a irreversible inhibitor of 3-ketoacyl-CoA thiolase and consequently of fatty acid oxidation.

4-Pentenoic acid is a hypoglycemic agent and an inhibitor of fatty aicd oxidation [for a recent review, see Billington et al. (1978a,b)]. Although the inhibition of fatty acid oxidation by 4-pentenoic acid has been well documented (Senior et al., 1968; Brendel et al., 1969; Williamson et al., 1970), the molecular basis of this inhibition has not been fully elucidated. Bressler et al. (1969) have concluded from their studies that metabolites of 4-pentenoic acid, which are slowly or not at all metabolized, sequester coenzyme A (CoA) and carnitine and thereby inhibit fatty acid oxidation. In contrast, Sherratt and co-workers (Billington et al., 1978a) have proposed that one or several metabolites of 4-pentenoic acid inhibit at least one of the enzymes of β-oxidation in a reversible fashion. Although these authors have observed the inhibition of isolated acetoacetyl-CoA thiolase (EC 2.3.1.9) by 2,4-pentadienoyl-CoA (Holland et al., 1973), they have been unable to detect the inactivation of the same enzyme in mitochondria preincubated with 4-pentenoic acid (Billington et al., 1978a). However, Fong & Schulz (1978) have demonstrated the inactivation of both 3-ketoacyl-CoA thiolase (EC 2.3.1.16) and acetoacetyl-CoA thiolase when coupled rat heart mitochondria were incubated with 4-pentenoic acid. Preliminary evidence pointed to 3-keto-4-pentenoyl-CoA as the inhibitory metabolite of 4-pentenoic acid (Schulz & Fong, 1981). However, a definite

† From the Department of Chemistry, City College of the City University of New York, New York, New York 10031. *Received September 23, 1982.* This work was supported in part by Grant HL 18089 of the National Heart, Lung, and Blood Institute and by a City University of New York Faculty Research Award.

0006-2960/83/0422-1827$01.50/0