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Regulation of Bilayer Stability in *Clostridium butyricum*: Studies on the Polymorphic Phase Behavior of the Ether Lipids†

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ABSTRACT: Three of the major phospholipids of the cell membrane of *Clostridium butyricum* are phosphatidylethanolamine (PE), plasmenylethanolamine (PlaE), and the glycerol acetal of plasmenylethanolamine. When cultured in the absence of biotin in media supplemented with a cis-unsaturated fatty acid, the cellular lipids become highly enriched with the fed fatty acid. Under these conditions, the ratio of the glycerol acetal of PlaE to the sum of PE plus PlaE increases markedly over that seen in cells containing mixtures of saturated and unsaturated fatty acids [Johnston, N. C., & Goldfine, H. (1985) *Biochim. Biophys. Acta 813*, 10–18]. We have studied the polymorphic phase behavior of the phospholipids from *C. butyricum* grown on oleic acid using differential scanning calorimetry, ^{31}P nuclear magnetic resonance, and X-ray diffraction. The mixed PE plus PlaE fraction undergoes a transition from the gel to liquid-crystalline state at −1.9 °C and a lamellar to reversed hexagonal (L → H) transition at or near 0 °C. The glycerol acetal of PlaE melts at 16.1 °C, and as predicted from lipid packing theory, the lamellar phase is stabilized, up to 50 °C. Addition of the oleate-enriched glycerol acetal of PlaE to dioleoylphosphatidylethanolamine, or the PE plus PlaE fraction from oleate-grown cells, stabilized the lamellar arrangement of the mixtures. A ratio of glycerol acetal of PlaE to total PE (PE plus PlaE) of 0.5, which is close to that found in cells grown on palmitic plus oleic acid, 0.6–0.7, did not produce a lamellar phase at 37 °C when the lipids enriched with oleic acid were tested, but a 1:1 mixture of these lipids was sufficient to produce the lamellar arrangement. In cells grown on oleic acid, the ratio is close to 2.0. It appears that these cells are capable of regulating the stability of the bilayer arrangement of the cell membrane by altering the ratio of the glycerol acetal of PlaE to the total PE fraction in response to changes in membrane lipid unsaturation.

Biological membranes contain mixtures of lipids which individually do and do not aggregate to form bilayers at physiological temperatures upon hydration. Examples of the former include phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, and diglycosyldiacylglycerols. Among the latter group are unsaturated species of phosphatidylethanolamine, plasmenylethanolamine, and monoglycosyldiacylglycerols [for general references, see Shipley (1973), Cullis and Hope (1985), and Lohner et al. (1984)]. To form bilayers, the mixtures of lipids in biological membranes appear to be carefully balanced to include appropriate proportions of lipids from the two groups

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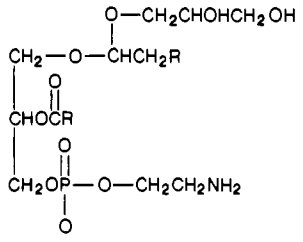
(Rilfors et al., 1984). The cellular regulation of the synthesis of lipids that functions to achieve these proportions is little understood at present.

Clostridium butyricum and *Clostridium beijerinckii* are auxotrophic for long-chain unsaturated fatty acids in the absence of biotin (Broquist & Snell, 1951). When grown under these conditions, the fed fatty acids are extensively incorporated into cellular phospholipids, resulting in membranes that contain over 95% unsaturated and cyclopropane chains (Khuller & Goldfine, 1975; Goldfine et al., 1977, 1981; Johnston & Goldfine, 1985). Growth of these organisms on mixtures of a saturated fatty acid, palmitic acid, and an unsaturated fatty acid, oleic acid, resulted in the synthesis of phospholipids that incorporated both types of chains. As the proportion of oleic acid in the medium was increased, the acyl chains became progressively more unsaturated, whereas the alk-1-enyl chains of the plasmalogens and the alkyl chains of their glycerol acetals were largely unsaturated at most ratios of the fed fatty acids (Johnston & Goldfine, 1985). In both organisms, enrichment of the phospholipid aliphatic chains with unsaturated fatty acids resulted in marked changes in the polar lipid composition.

Organisms of the *butyricum* group of clostridia contain unique glycerol acetals of their plasmalogens (I). In most



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of these species, the major plasmalogen is plasmenylethanolamine, but in *C. beijerinckii*, it is plasmenyl-*N*-methylethanolamine (Matsumoto et al., 1971; Johnston & Goldfine, 1983). As the degree of unsaturation of the phospholipids was increased, in the feeding experiments described above, the ratio of the glycerol acetal lipid to phosphatidylethanolamine plus plasmenylethanolamine increased from 0.7 to 2.0 in *C. butyricum* grown on various mixtures of palmitic and oleic acids (Johnston & Goldfine, 1985). The two other major membrane lipid classes are phosphatidylglycerol and cardiolipin. Approximately one-third of each is present in the form of plasmalogens (Goldfine et al., 1982). At high unsaturation, the proportion of phosphatidylglycerol decreased from 17% to 7% of total phospholipid, with little change in cardiolipin, which represented approximately 30% of phospholipid phosphorus. Similar changes were observed when *C. beijerinckii* was grown in the presence of biotin, resulting in mixed saturated and unsaturated chains, and then changed to growth on oleic acid in the absence of biotin (Khuller & Goldfine, 1975; Goldfine et al., 1977).

A simple shape concept has been used to predict the types of macroscopic structures that lipids will assume upon hydration (Cullis & de Kruijff, 1979; Israelachvili et al., 1980). According to this lipid packing theory, bilayers are formed by hydrated lipids that are approximately cylindrical in shape by virtue of a balance between the effective sizes of the polar head group and the volume occupied by the hydrocarbon chains. Lipids that are approximately conical in shape, with a small head group relative to the volume of the hydrocarbon chains, will tend to aggregate in the reversed hexagonal (H_{II}) or other nonlamellar phases, upon hydration. An increase in the effective polar head group area, as occurs in the conversion of phosphatidylethanolamine (PE)[1] to phosphatidylcholine or monoglycosyldiacylglycerol to diglycosyldiacylglycerol, serves to stabilize the bilayer packing arrangement. Examination of space-filling models of the glycerol acetal of PE plasmalogen (PlaE) led to the prediction that conversion of plasmenylethanolamine to its glycerol acetal would similarly serve to stabilize the bilayer packing arrangement of the membrane lipids of *C. butyricum* (Johnston & Goldfine, 1985; Goldfine, 1985). In this paper, we provide physical evidence concerning the phase behavior of the phospholipids of this organism which provides strong support for this hypothesis. We have examined the phase behavior of lipids from cells grown on oleic acid. The mixed phosphatidylethanolamine plus plasmenylethanolamine fraction and the glycerol acetal of PE plasmalogen have been examined separately and after mixing using differential scanning calorimetry (DSC), ^{31}P NMR, and X-ray diffraction. The ability of the glycerol acetal of PlaE to stabilize the bilayer packing arrangement of other unsaturated phosphatidylethanolamines has also been examined.

Materials and Methods

Materials

Vitamin-free casamino acids were obtained as a sterile solution from ICN-Nutritional Biochemicals (Cleveland, OH). Oleic and palmitic acids were obtained from Nu-chek Prep (Elysian, MN). Dioleoylphosphatidylethanolamine was obtained from Avanti (Birmingham, AL), and egg phosphatidylethanolamine was from Sigma (St. Louis, MO).

Methods

C. butyricum ATCC 19398 was grown anaerobically in 20-L carboys with casamino acids and fatty acids in the absence of biotin as described (Goldfine et al., 1977). Cells were concentrated with a Millipore Pellicon cassette system, followed by centrifugation at 8000*g* for 8 min. They were then washed twice with potassium phosphate buffer, 0.05 M, pH 7.2. All cultures were examined by phase-contrast microscopy for purity and the absence of spore formation, which occurs rarely if at all in cells grown in media devoid of biotin. Methods for lipid extraction with chloroform–methanol (2:1) have been described (Goldfine & Bloch, 1961).

The crude lipids were first separated into nonpolar and phospholipid fractions by acetone precipitation (Kates, 1972). Lipid purification was accomplished largely as described (Goldfine et al., 1981). The vinyl ether content was determined by the method of Gottfried and Rapport (1962), which was scaled down by a factor of 2.5. Lipid phosphorus was determined by the method of Bartlett (1959). Fatty acids were analyzed as their methyl esters, and aldehydes were analyzed essentially as described (Johnston & Goldfine, 1983).

^{31}P Nuclear Magnetic Resonance. Lipid (15–30 mg) was dried in 9-mm NMR tubes under nitrogen, and samples were then placed under high vacuum. They were dispersed in a D_2O Tris–acetate buffer containing EDTA as described (Cullis & de Kruyff, 1978). Spectra were recorded after 1–2-h equilibration on a Bruker WH/360 spectrometer operating at 145.7 MHz with broad-band proton decoupling. Accumulated free-induction decays were obtained from 1000 to 2000

[1] Abbreviations: DGDG, diglucosyldiacylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DSC, differential scanning calorimetry; MGDG, monoglucosyldiacylglycerol; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PlaE, plasmenylethanolamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

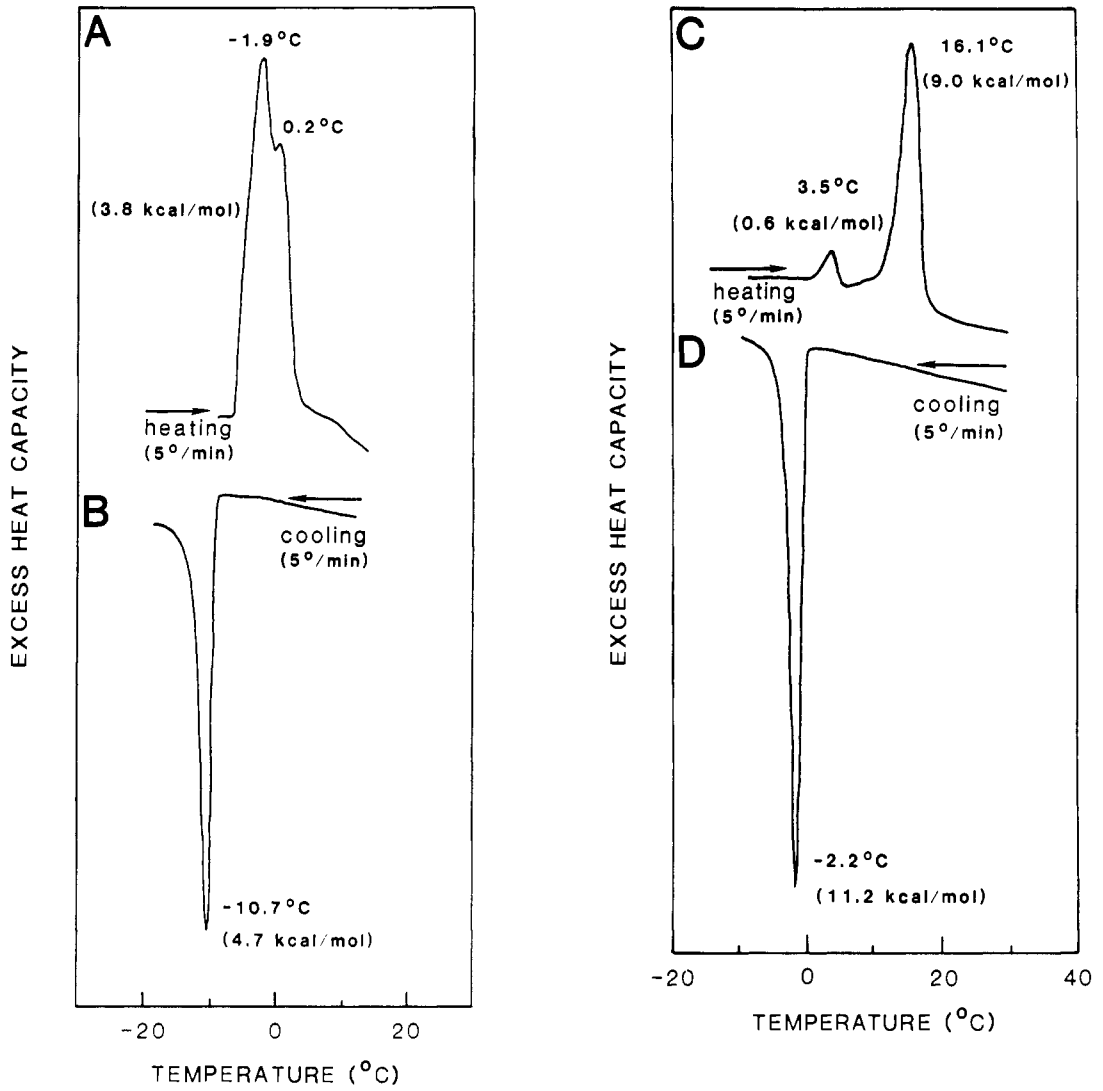

FIGURE 1: Differential scanning calorimetry of the phosphatidylethanolamine fraction from *C. butyricum* grown on oleic acid (preparation A, Table I) (A, heating; B, cooling), and glycerol acetal of plasmenylethanolamine from the same cells (preparation A, Table I) (C, heating; D, cooling). Heating/cooling rates = 5 °C/min.

transients by employing an interpulse time of 2.08 s.

Differential Scanning Calorimetry. DSC samples were prepared by weighing the lipids directly into stainless-steel pans and adding ~90 wt % water using a Hamilton syringe. The pans were hermetically sealed and transferred to a Perkin-Elmer DSC-2 scanning calorimeter (Norwalk, CT). Samples were heated and cooled at 5 °C/min. Transition temperatures were determined from the maximum of the excess heat capacity vs. temperature curves while the transition enthalpy was determined from the area under the peak, using gallium as a standard.

X-ray Diffraction. The oleate-enriched phospholipids from *C. butyricum* were weighed into constricted glass tubes and dissolved with chloroform. The chloroform was evaporated with nitrogen, and the samples were pumped overnight under high vacuum. Water was added gravimetrically to ~50% by weight, and the tubes were flame sealed and centrifuged through the constriction at room temperature (which is above the transition temperature for both lipids used in this study). Samples of hydrated lipids were then quickly transferred to quartz capillary tubes (internal diameter = 1 mm) (Charles Supper Co., Natick, MA), flame sealed, and transferred to a variable-temperature X-ray sample holder. X-ray diffraction patterns were recorded with photographic film using nickel-filtered Cu Kα X radiation from an Elliot GX-6 rotating anode generator (Elliot Automation, Borehamwood, England), which was collimated by double mirror optics or toroidal optics into a point source.

RESULTS

Phospholipids and Aliphatic Chain Compositions. The acyl and alkenyl chain compositions of the phospholipids from *C. butyricum* grown in biotin-free media with exogenous oleic acid are shown in Table I. As in previous work with this organism, growth under these conditions leads to very high enrichment with the fed fatty acid. In cells grown with oleic acid, from 92% to 98% of the acyl chains are either 18:1 or the 19-carbon cyclopropane fatty acid derived from 18:1. The cyclopropane content of the acyl chains is relatively low, especially in the glycerol acetal of PlaE. The alkenyl chains are even more highly enriched with the fed fatty acid than the acyl chains; however, in plasmenylethanolamine, there is a large but variable proportion of the 19-carbon cyclopropane chains.

Differential Scanning Calorimetry. Figure 1A,B shows differential scanning calorimetry (DSC) heating and cooling curves of the hydrated (84.5% water) phosphatidylethanolamine fraction of *C. butyricum* grown on oleic acid (preparation A, Table I). The curves were obtained after several heating and cooling cycles over the temperature range −15 → 17 °C. The heating curve shows an endotherm with a peak

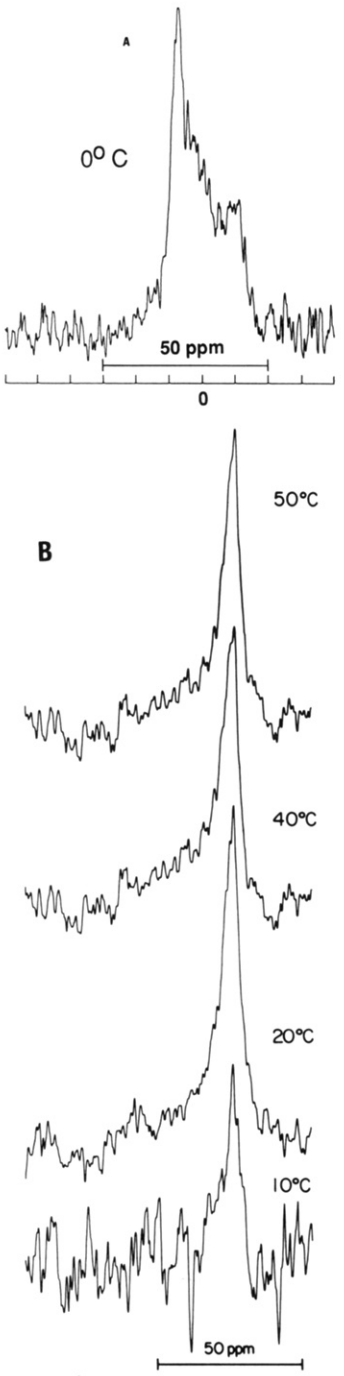

FIGURE 2: 145.7-MHz ^{31}P NMR spectra of aqueous dispersions of oleate-enriched (A) phosphatidylethanolamine fraction (46% plasmalogen; preparation C, Table I) and (B) glycerol acetal of plasmenylethanolamine (preparation A, Table I).

maximum at −1.9 °C and a shoulder at 0.2 °C, combined enthalpy ΔH = 3.8 kcal/mol. The corresponding exotherm exhibits supercooling, with a single transition observed at −10.7 °C, ΔH = 4.7 kcal/mol.

DSC heating and cooling curves of the hydrated (82.5% water) glycerol acetal of plasmenylethanolamine from cells grown on oleic acid (preparation A, Table I) are shown in Figure 1C,D. Two endotherms are observed on heating: a lower temperature and lower enthalpy transition at 3.5 °C (ΔH = 0.6 kcal/mol) and a higher temperature transition at 16.1 °C with a larger enthalpy of 9.0 kcal/mol. On cooling, the exotherm also exhibits supercooling, as shown previously (Goldfine et al., 1981), with only a single transition observed at −2.2 °C (ΔH = 11.2 kcal/mol).

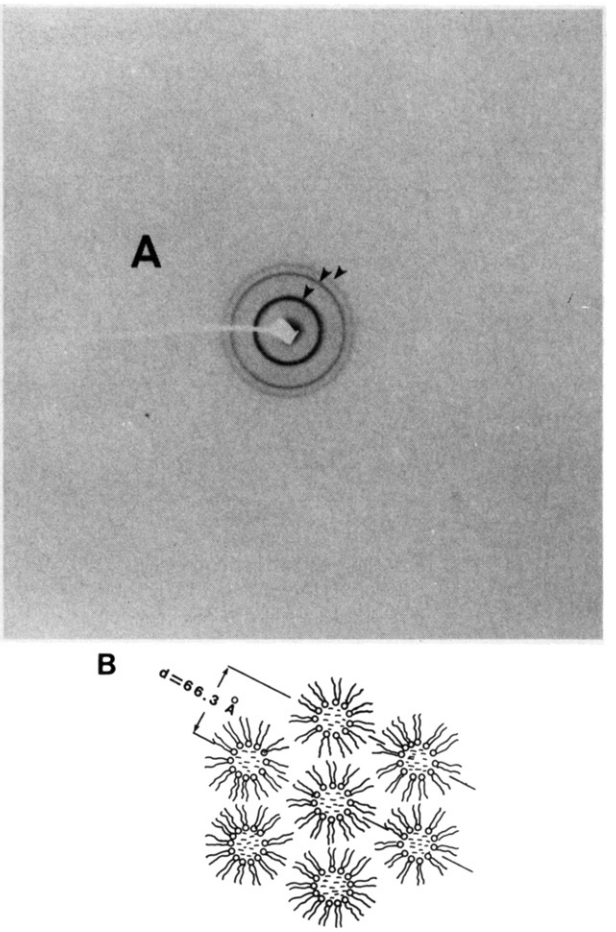

FIGURE 3: X-ray diffraction patterns and structure of the phosphatidylethanolamine fraction from *C. butyricum* grown on oleic acid (preparation A, Table I) at 24 °C and 50% hydration. (A) Diffraction pattern showing low-angle reflections; (B) structure of hexagonal II phase.

Aggregation States of the Pure Phospholipids. The aggregation states of the pure phospholipids were studied by ^{31}P NMR and low-angle X-ray diffraction of aqueous dispersions. ^{31}P NMR of oleate- and C_{19}-cyclopropane-enriched phosphatidylethanolamine indicates the presence of an inverted hexagonal phase at 0 °C along with a bilayer component (Figure 2A). Dioleoylphosphatidylethanolamine undergoes a lamellar to reversed hexagonal (L → H) phase transition between 5 and 10 °C (Gruner, 1985), and the presence of 46% plasmalogen would be expected to decrease the temperature at which this transition takes place (Boggs et al., 1981; Lohner et al., 1984). The ^{31}P NMR spectra of the glycerol acetal of plasmenylethanolamine from cells grown on oleate (Figure 2B) indicate that the lamellar phase of aqueous dispersions is stable at temperatures up to 50 °C.

X-ray diffraction of aqueous dispersions of the lipids obtained from cells grown on oleate confirms the results obtained with ^{31}P NMR. An X-ray diffraction pattern of hydrated (50 wt % water) oleate-enriched phosphatidylethanolamine (preparation A, Table I) at 24 °C is shown in Figure 3A. The low-angle region shows three reflections indexing in the ratio $1:(1/\sqrt{3}):(1/\sqrt{4})$, confirming the assignment of a hexagonal phase based on the ^{31}P NMR data shown in Figure 2A. The d_{10} reflection has a value of 66.3 Å corresponding to the separation of the planes that describe the packing of the rows

Table I: Phospholipid Acyl, Alk-1-enyl, and Alkyl Chain Composition (Weight Percent) of *C. butyricum* ATCC 19398 Grown on Oleic Acid[a]

	acyl chains						
	total phosphatidylethanolamine				glycerol acetal of plasmenylethanolamine		
		prepn B					
chain	prepn A	diacyl[b]	PlaE[c]	prepn C	prepn A	prepn B	prepn C
14:0	1.7	1.8	3.4	0.86		1.8	1.4
16:0	3.3	3.3	3.4	0.83	1.1	2.0	0.6
16:1				0.94		1.3	
18:0				0.72	0.9		
18:1	84.4	81.4	88.1	87.0	97.9	94.9	97.6
19:cy	8.8	13.6	4.1	12.9	tr[d]		
	alkenyl chains			alkyl chains		
	prepn A	prepn B PlaE	prepn C	prepn A	prepn B	prepn C
14:0		0.99	3.7			
16:0		tr			tr	
18:1	31.5	47.3	64.8	91.0	93.2	100
19:cy	68.5	51.7	31.3	8.9	6.8	

[a] The isolation of the lipids and analysis of the chains are described under Materials and Methods. The three preparations, A, B, and C, were from different cultures, and their use is described in the text and figure legends. The chains are described as follows: number of carbons:number of double bonds. 19:cy is the 19-carbon cyclopropane fatty acid or aldehyde. [b] In the analysis of preparation B, the diacylphosphatidylethanolamine acyl chains was analyzed separately from those of the plasmenylethanolamine. The total phosphatidylethanolamine fraction was treated with 90% acetic acid, and the resulting diacyl and lyso compounds were separated by silicic acid chromatography (Johnston & Goldfine, 1983). [c] Plasmenylethanolamine. [d] Trace.

of cylinders of the hexagonal phase (see Figure 3B). The axial separation of the lipid cylinders is calculated to be 76.6 Å. The wide-angle region (not shown) showed a single diffuse reflection at $1/4.6$ Å^{-1}, indicating the presence of melted hydrocarbon chains.

An X-ray diffraction pattern of hydrated (50 wt % water) oleate-enriched glycerol acetal of plasmenylethanolamine (preparation A, Table I) at 24 °C is shown in Figure 4A. The low-angle reflections in contrast to the low-angle reflections of oleate-enriched phosphatidylethanolamine (Figure 3A) indicate a lamellar structure. Four low-angle reflections can be observed in Figure 4A, with periodicities in the ratio $1:(1/2):(1/3):(1/4)$. This pattern is characteristic of a hydrated phospholipid bilayer structure of periodicity, glycerol acetal of plasmenylethanolamine bilayer plus intercalated water, d = 49.6 Å (see Figure 4B). On increasing the temperature to 50 °C, the lamellar phase is still observed (data not shown). The wide-angle region (not shown) at both 25 and 50 °C showed the diffuse $1/4.6$ Å^{-1} reflection characteristic of melted hydrocarbon chains. The X-ray diffraction results support the ^{31}P NMR data of Figure 2B which showed the existence of the lamellar phase of oleate-enriched glycerol acetal of plasmenylethanolamine up to 50 °C.

Model Studies on the Aggregation States of Mixtures of Phosphatidylethanolamine and the Glycerol Acetal of Plasmenylethanolamine. The amount of phosphatidylethanolamine from cells grown on oleic acid which was available for mixing experiments was limited. Therefore, the ability of unsaturated glycerol acetal lipid to stabilize the lamellar phase of natural and synthetic species of phosphatidylethanolamine was studied first. ^{31}P NMR spectra of aqueous dispersions of egg phosphatidylethanolamine showed that this lipid underwent a L → H phase transition which appeared to be almost complete at 20 °C. When oleate-enriched glycerol acetal of PlaE was added to egg phosphatidylethanolamine (PE:glycerol acetal ratio of 2:1), the lamellar phase was stabilized to at least 40 °C (data not shown). The ability of the oleate-enriched glycerol acetal derivative to stabilize the lamellar phase of dioleoylphosphatidylethanolamine (DOPE) was studied by ^{31}P NMR and X-ray diffraction. As noted above, this lipid undergoes a L → H transition at 5–10 °C, and ^{31}P NMR gave a typical H_{II} spectrum at 5 °C. Addition of 1 part oleate-enriched glycerol acetal of PlaE to 2 parts DOPE resulted in a lamellar spectrum up to 25 °C. At 46 °C, the spectrum showed the beginning of a lamellar to nonlamellar transition. A 1:1 mixture of the same two lipids produced a lamellar spectrum at 35 °C with little evidence of nonlamellar lipid at 46 °C (data not shown). Low-angle X-ray diffraction patterns

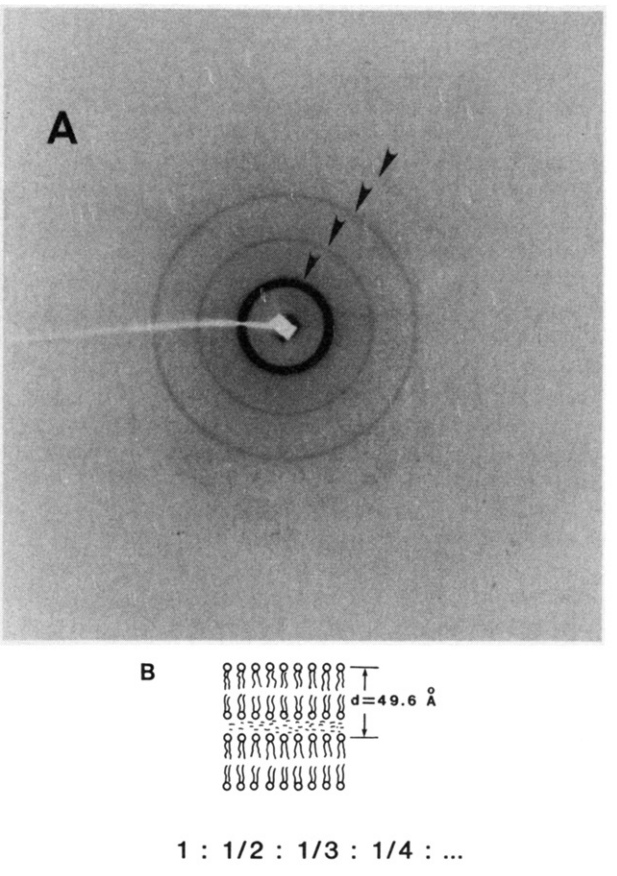

FIGURE 4: X-ray diffraction patterns and structure of glycerol acetal of plasmenylethanolamine from *C. butyricum* grown on oleic acid (preparation A, Table I) at 24 °C and 50% hydration. (A) Low-angle reflections; (B) structure of lamellar phase.

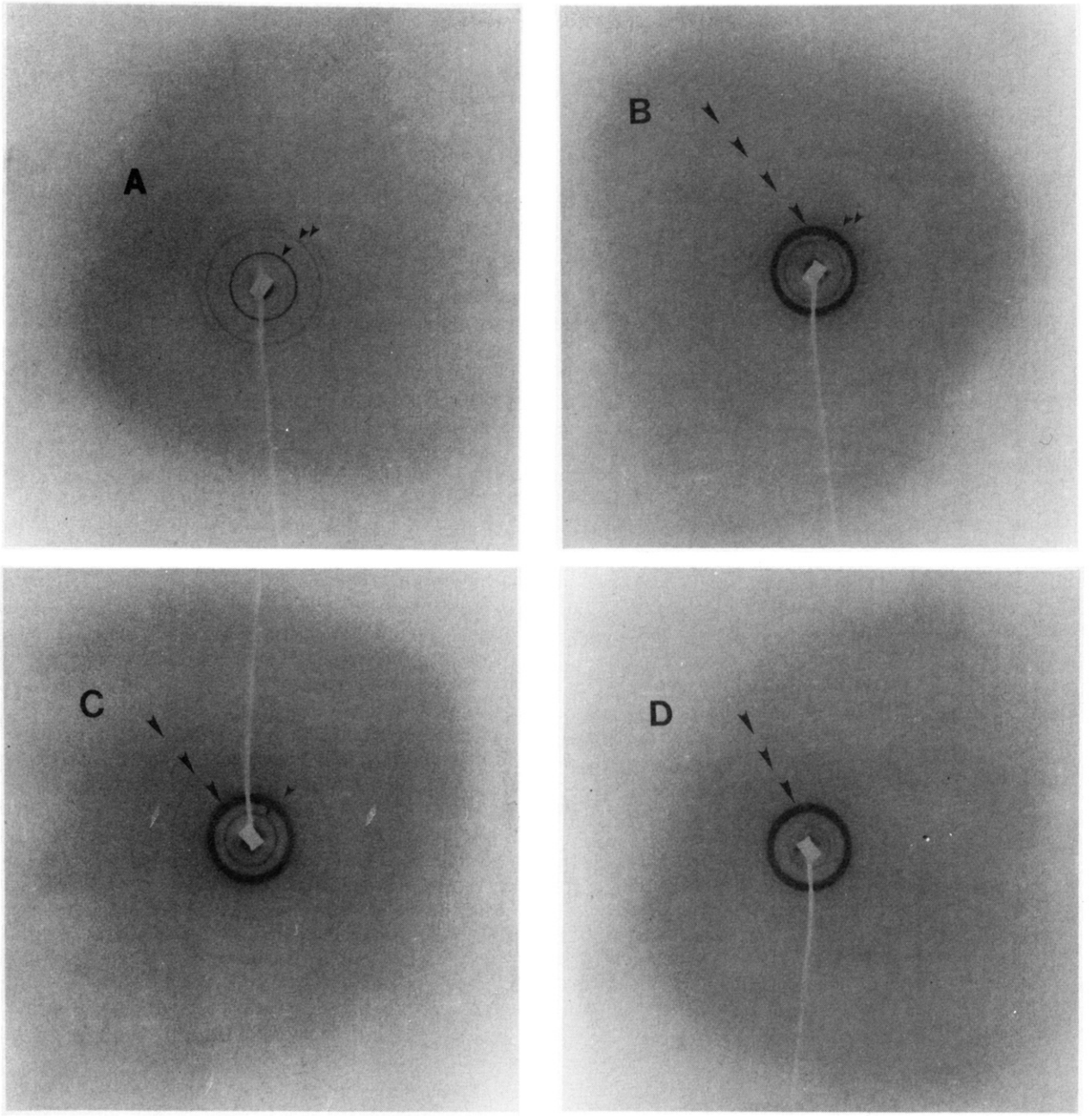

FIGURE 5: Low-angle X-ray diffraction patterns of dioleoylphosphatidylethanolamine (DOPE) with increasing percentages of gl cerol acetal of PlaE (preparation B, Table I) at 46 °C and 60% hydration: (A) 0%; (B) 33.4%; (C) 51%; (D) 75.3%.

of DOPE with increasing percentages of the oleate-enriched glycerol acetal PlaE at 46 °C and 60% hydration are shown in Figure 5A–D. Pure hydrated DOPE shows three low-angle reflections (Figure 5A) indexing in the ratio $1:(1/\sqrt{3}):(1/\sqrt{4})$ typical of a hexagonal phase with d = 61 Å, and an axial separation of the lipid cylinders of 70.4 Å. On addition of 33.4 mol % of oleate-enriched glycerol acetal of PlaE, several low-angle reflections are observed (Figure 5B) which can be indexed into two sets of reflections representing two coexisting phases. The first set of three reflections (small arrows) index in the ratio of $1:(1/\sqrt{3}):(1/\sqrt{4})$, corresponding to the hexagonal phase of DOPE, while the second set of four reflections (large arrows) index in the ratio $1:(1/2):(1/3):(1/4)$, indicating a lamellar organization of lipids and water with d = 50.8 Å. At 51% glycerol acetal of PlaE (Figure 5C), the lamellar reflections (large arrows; d = 51.3 Å) are dominant with only a very weak set of reflections (small arrows) corresponding to the hexagonal phase of DOPE. Further addition of glycerol acetal lipid to 75.3 mol % (Figure 5D) essentially abolishes the hexagonal reflections with primarily the lamellar reflections being observed (large arrows; d = 52.6 Å).

Aggregation States of Mixtures of Lipids from Cells Grown on Oleic Acid. The ^{31}P NMR spectrum of the phosphatidylethanolamine fraction from cells grown on oleic acid is shown in Figure 2A. This lipid contained mainly 18:1 and C_{19}-cyclopropane acyl and alkenyl chains and was 45.7% plasmenylethanolamine (Table I). The spectrum taken at 0 °C is that of a lipid in the reversed hexagonal phase with a small bilayer component. A mixture of the same PE fraction with oleate-enriched glycerol acetal of PlaE (2:1) produced a lamellar spectrum at 20 °C with a transition to a nonlamellar probably isotropic phase (Cullis & Hope, 1985) between 30 and 40 °C (Figure 6A). The isotropic component may arise from small vesicles. The transition did not appear to be complete at 40 °C. A 1:1 mixture of the two lipids yielded a spectrum characteristic of lamellar phase lipid at 35 and 40 °C, with some deterioration of the spectrum at 45 °C (Figure 6B). Low-angle X-ray diffraction of the 1:1 mixture at 46

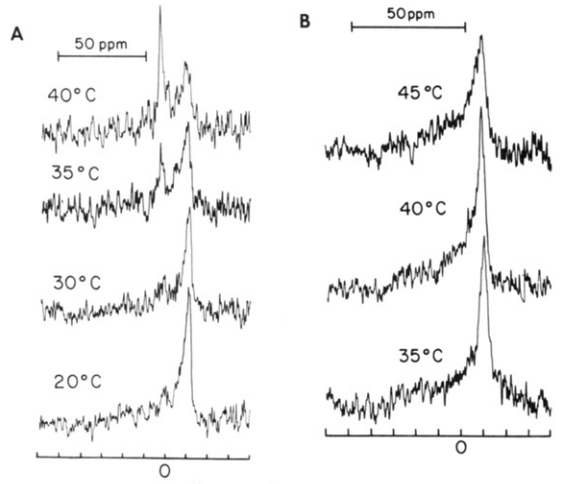

FIGURE 6: 145.7-MHz ^{31}P NMR spectra of aqueous dispersions of (A) phosphatidylethanolamine fraction (46% plasmalogen) plus glycerol acetal of plasmenylethanolamine (2:1 w/w). Both lipids were from *C. butyricum* grown on oleic acid (preparation C, Table I); (B) the same lipids (1:1 w/w).

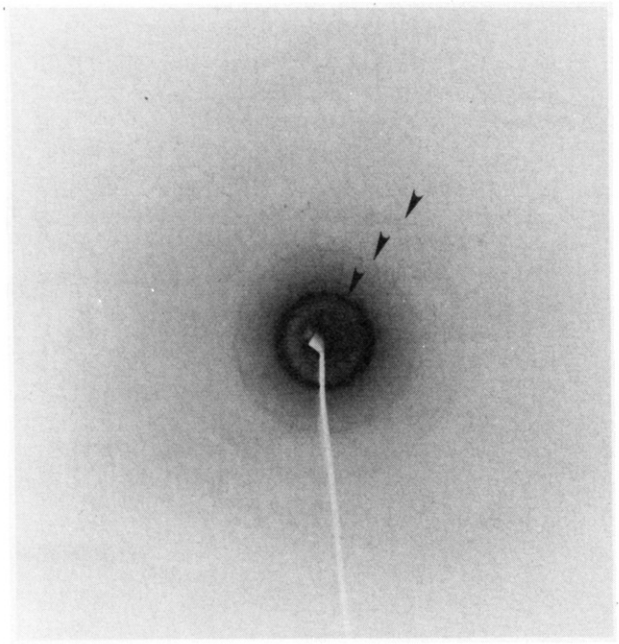

FIGURE 7: Low-angle X-ray diffraction pattern of a 1:1 (w/w) mixture of oleate-enriched *C. butyricum* glycerol acetal of PlaE–PE at 46 °C and 60% hydration.

°C (Figure 7) shows four reflections indexing in the ratio 1:(1/2):(1/3):(1/4), characteristic of a lamellar phase (d = 52.7 Å) as suggested by the ^{31}P NMR data (Figure 6B).

Discussion

The inability of unsaturated molecular species of phosphatidylethanolamine to form a lamellar phase has been known since the pioneering X-ray diffraction studies of Luzzati and his co-workers (Luzzati et al., 1966; Reiss-Husson, 1967). Recent work of Boggs et al. (1981) and Lohner et al. (1984) has shown that the plasmalogen form of phosphatidylethanolamine undergoes the L → H transition at lower temperatures than the diacyl form containing similar mixture of hydrocarbon chains. The potentiating effects of substitution of a cyclopropane fatty acid for an unsaturated fatty acid in a mixed chain phosphatidylethanolamine on the L → H transition have been studied by Perly et al. (1985). The major phospholipids of the cell membrane outer monolayer of *C. butyricum* are phosphatidylethanolamine, which is 50–75% plasmalogen, and the glycerol acetal of plasmenylethanolamine (Goldfine et al., 1982; Johnston & Goldfine, 1985). In cells grown on oleic (Johnston & Goldfine, 1985), *cis*-vaccenic, or linoleic acids (H. Goldfine, N. C. Johnston, and J. Rosenthal, unpublished results), the ratio of the glycerol acetal lipid to phosphatidylethanolamine plus plasmenylethanolamine increases markedly compared to cells grown on biotin or on mixtures of saturated and unsaturated fatty acids.

The studies presented here show the following: (1) The mixed phosphatidylethanolamine–plasmenylethanolamine fraction from cells grown on oleic acid cannot form bilayers at temperatures above 2 °C. (2) The glycerol acetal of plasmenylethanolamine enriched over 95% with cis-18:1 chains forms bilayers at temperatures well above the growth temperature of the cells. (3) A ratio of glycerol acetal of PlaE to phosphatidylethanolamine plus PlaE close to that found in cells grown with biotin or on palmitic plus oleic acids in the absence of biotin is inadequate to produce a lamellar phase at 37 °C when the lipid hydrocarbon chains are enriched with cis-18:1 plus C_{19}-cyclopropane, but (4) the ratio of glycerol acetal of PlaE to phosphatidylethanolamine plus PlaE found in oleate-grown cells (2.0) is more than sufficient to produce the lamellar arrangement.

The glycerol acetal of PlaE has a strongly hydrophilic group attached to the first carbon of the *sn*-1 chain. Presumably, its most stable arrangement in membranes would involve maximal contact of the hydroxyl groups with water. Space-filling models of this lipid (Goldfine & Johnston, 1980) and hydration studies (Goldfine et al., 1981) lead to the prediction that the additional glycerol residue will significantly increase the effective size of the polar head group, and this, according to lipid packing theory, should result in a markedly higher L → H phase transition temperature than phosphatidylethanolamine (Goldfine, 1985). This prediction has been confirmed in the present study. The addition of the glycerol acetal moiety increases the L → H phase transition temperature by over 45 °C.

The ability of the glycerol acetal of PlaE to stabilize the lamellar phase of natural and synthetic phosphatidylethanolamines was also tested. Our results show that addition of 33 wt % oleate-enriched glycerol acetal of PlaE to egg phosphatidylethanolamine results in a stable lamellar phase at physiological temperatures. A comparable study with soya phosphatidylethanolamine and egg phosphatidylcholine showed that addition of 50 mol % of the latter was required to stabilize the lamellar phase (Cullis & de Kruijff, 1978a). Soya phosphatidylethanolamine is richer in polyunsaturated fatty acids than egg phosphatidylethanolamine and undergoes a L → H transition below 0 °C. Our studies with DOPE and the oleate-enriched glycerol acetal of PlaE also mirror those of mixed PE–PC systems. Stabilization of the lamellar phase of DOPE at 37 and 46 °C was obtained by addition of ~50 wt % of the glycerol acetal of PlaE (see Figure 5). A similar result was obtained when DOPC was added to DOPE (Cullis et al., 1978). From these results, it appears that the addition of the glycerol moiety in acetal linkage to plasmenylethanolamine is approximately equivalent in its bilayer stabilizing effect to the addition of three methyl groups to the head group of phosphatidylethanolamine. However, it is important to consider other effects than increase in size of the polar head group. The effective area is also dependent upon charge and hydrogen bonding. Addition of three methyl groups to the polar head of phosphatidylethanolamine to form phosphatidylcholine decreases hydrogen bonding and thus increases bilayer stability (Boggs, 1984). While the conversion

of plasmenylethanolamine to its glycerol acetal should not significantly affect the charge, an increase in the ability to hydrogen bond is expected (Goldfine & Johnston, 1980), and this would tend to counteract the large increase in bulk of the polar head group.

This discussion does not take into account the influence of the other major lipids of *C. butyricum*, phosphatidylglycerol and cardiolipin, and the minor lipid classes, such as acyl phosphatidylglycerol (Koga & Goldfine, 1984), nor has there been any consideration of the membrane proteins. We have shown that the total lipids extracted from these cells give a typical lamellar phase ^{31}P NMR spectrum when dispersed in aqueous buffer (data not shown). Further, the predominant localization of the phosphatidylethanolamine, the major non-bilayer-forming lipid, and the glycerol acetal of PlaE in the membrane outer monolayer (Goldfine et al., 1982; Johnston & Goldfine, 1985) suggests that the interactions of these lipids are of greatest interest in studies on bilayer stabilization. The effects of proteins are less predictable. It is clear that individual membrane proteins can exert both bilayer stabilizing and destabilizing effects (Cullis & Hope, 1985). In *Escherichia coli*, it appears that the proteins of the inner membrane exert a bilayer stabilizing effect (Burnell et al., 1980). Future studies directed toward determining the influence of proteins on membrane organization in *C. butyricum* will obviously be important for a complete understanding of these phenomena.

Cellular Regulation of Bilayer Stability. The ability of cells to respond to changes in lipid hydrocarbon chain composition by altering the relative proportions of lipids that individually do and do not form bilayers has now been carefully documented in two species. The elegant work of Wieslander et al. (1980, 1981a,b) on *Acholeplasma laidlawii* [reviewed in Rilfors et al. (1984)] has shown that this organism adjusts the ratio of monoglucosyldiacylglycerol to diglucosyldiacylglycerol in response to changes in temperature, degree of unsaturation of the hydrocarbon chains, and the cholesterol content of the membranes. As these parameters are increased, the ratio of MGDG to DGDG decreases. Unsaturated species of MGDG are unable to form a lamellar phase at physiological temperatures and are stabilized by addition of unsaturated species of DGDG. MGDG and DGDG enriched in oleic acid cannot form bilayers if mixed in the proportions of these lipids found in cells grown on mixtures of palmitic and oleic acid; however, the lamellar phase is stabilized when mixed in the ratios found in cells grown on oleic acid alone. Indeed, comparison of the results of experiments with *A. laidlawii* and those obtained with *C. butyricum* shows a remarkable parallelism between the MGDG:DGDG and PE:glycerol acetal of PlaE ratios at comparable ratios of oleic to palmitic acid in the growth medium (Goldfine, 1985). These results suggest that a similar regulatory mechanism may be operative. At present, it is not known if control of membrane lipid ratios in these organisms is at the level of the gene or at the level of the membrane-bound biosynthetic enzymes themselves. In *Escherichia coli*, the ratio of anionic lipids to phosphatidylethanolamine is rigorously controlled. Marked changes in gene dose appear to have little influence on this ratio, suggesting that regulation is epigenetic (Raetz, 1982). One of us has proposed mechanisms by which the enzymes involved in phospholipid synthesis may be affected by the appearance of microdomains of non-lamellar lipids in the membrane (Goldfine, 1985). The formation of transient nonlamellar microdomains could enhance the access of water-soluble precursors to the enzymes involved in lipid interconversions. In addition, isotropic motion of the lipids may increase access of enzyme active sites to lipids that are predominantly located in the membrane outer monolayer. It may be significant that the lipids whose composition is regulated in *A. laidlawii* and *C. butyricum* appear to be largely located in the outer monolayer [see references in Goldfine (1985)].[2] The probable conversion of plasmenylethanolamine to the glycerol acetal lipid in clostridia is supported by the results of pulse–chase experiments with radioactive phosphorus (Koga & Goldfine, 1984).

Finally, Gruner (1985) has proposed that cells are capable of regulating the intrinsic radius of curvature of their membrane lipid aggregates. According to this hypothesis, membrane-bound enzymes homeostatically adjust the lipid composition to maintain a constancy of intrinsic curvature. It is further suggested that this value is adjusted so that the bilayer packing arrangement can be locally disturbed by membrane-active hydrophobic compounds or protein domains. The mechanism and control of lipid composition are obviously of considerable interest. Clearly, the *butyricum* group of clostridia provides interesting systems for the study of the control of membrane lipid composition.

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Inhibition of NADH–Ubiquinone Reductase Activity by *N,N'*-Dicyclohexylcarbodiimide and Correlation of This Inhibition with the Occurrence of Energy-Coupling Site 1 in Various Organisms†

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ABSTRACT: The NADH–ubiquinone reductase activity of the respiratory chains of several organisms was inhibited by the carboxyl-modifying reagent *N,N'*-dicyclohexylcarbodiimide (DCCD). This inhibition correlated with the presence of an energy-transducing site in this segment of the respiratory chain. Where the NADH–quinone reductase segment involved an energy-coupling site (e.g., in bovine heart and rat liver mitochondria, and in *Paracoccus denitrificans*, *Escherichia coli*, and *Thermus thermophilus* HB-8 membranes), DCCD acted as an inhibitor of ubiquinone reduction by NADH. By contrast, where energy-coupling site 1 was absent (e.g., in *Saccharomyces cerevisiae* mitochondria and *Bacillus subtilis* membranes), there was no inhibition of NADH–ubiquinone reductase activity by DCCD. In the bovine and *P. denitrificans* systems, DCCD inhibition was pseudo first order with respect to incubation time, and reaction order with respect to inhibitor concentration was close to unity, indicating that inhibition resulted from the binding of one inhibitor molecule per active unit of NADH–ubiquinone reductase. In the bovine NADH–ubiquinone reductase complex (complex I), [^{14}C]DCCD was preferentially incorporated into two subunits of molecular weight 49 000 and 29 000. The time course of labeling of the 29 000 molecular weight subunit with [^{14}C]DCCD paralleled the time course of inhibition of NADH–ubiquinone reductase activity.

Associated with the mitochondrial oxidative phosphorylation system are five energy-transducing enzymes: NADH–ubiquinone oxidoreductase (complex I), ubiquinol–cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV), ATP synthase (complex V), and nicotinamide nucleotide transhydrogenase (Hatefi, 1985; Earle & Fisher, 1980). Except for complex I, the others have been shown to be inhibited by the carboxyl modifying reagent *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] (Beechey et al., 1967; Casey et al., 1980; Beattie & Villalobo, 1982; Esposti et al., 1982; Phelps & Hatefi, 1981, 1984). In the case of the ATP synthase, the inhibition by DCCD is a universal feature and has been reported for the enzymes from chloroplasts and microorganisms as well (McCarty & Racker, 1967; Evans, 1970). DCCD

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Q, ubiquinone; Q_1, ubiquinone 1; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles; EDTA, ethylenediaminetetraacetic acid; FP, iron–sulfur flavoprotein fraction; IP, iron–sulfur protein fraction; HP, hydrophobic protein fraction; M_r, molecular weight estimated for relative mobility in SDS–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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