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Lipid Activation of Undecaprenyl Pyrophosphate Synthetase from *Lactobacillus plantarum*†

Charles M. Allen, Jr.,* and Janine D. Muth

ABSTRACT: *Lactobacillus plantarum* undecaprenyl pyrophosphate synthetase is a soluble enzyme which has an in vitro requirement for detergent or phospholipid for activity. It is activated by the anionic detergents deoxycholate, dodecyl and cetyl sulfate, as well as Triton X series detergents. Brij 35, 56, and 96 and cetyltrimethylammonium bromide were ineffective in activating the enzyme. *L. plantarum*, *Escherichia coli*, and bovine cardiolipin, egg phosphatidic acid, and oleate are all good activators of the enzyme in the absence of detergent. *L. plantarum* phosphatidylglycerol and lysylphosphatidylglycerol, several lecithins, dipalmitylphosphatidic acid, phosphatidylserine, and the C_6–C_{18} saturated fatty acids (except C_{16}) are all ineffective over a wide concentration range. However, in the presence of 0.1% Triton X-100, dipalmitylphosphatidic acid, phosphatidylserine, and C_6–C_{18} saturated fatty acids exhibit a concentration-dependent stimulatory effect, with the C_{12} and C_{14} fatty acids being most effective. Mixtures of C_{10} or C_{14} fatty acids with Brij 35 demonstrated enzyme activation similar to the fatty acids–Triton X-100 mixtures. Using mixtures of Brij 35 and C_{14} fatty acid, it was observed that a fixed molar ratio of fatty acid to detergent (2:1) was required for optimal activity. Temperature dependence of egg phosphatidic acid activation of the enzyme showed a marked increase in enzyme activity from 30 to 40 °C, while both cardiolipin and Triton X-100 exhibit a decrease in stimulatory activity in this temperature range. These results suggest that an optimal surface charge and fluid lipid bilayer are required for enzyme activity.

Long chain polyprenyl phosphates have been shown to play a central role as glycosyl carriers in the biosynthesis of bacterial cell wall polysaccharides (Hemming, 1974) and have been strongly implicated in a similar role in the biosynthesis of oligosaccharide precursors of glycoproteins (Lennarz, 1975). All the enzymes reported to date which metabolize these polyprenyl phosphates have been described as being membrane associated and activated by a variety of lipids and detergents. Prenyl transferases which synthesize undecaprenyl pyrophosphate, a glycosyl carrier in bacterial systems, have been described in *S. newington* (Christenson et al., 1969), *M. lysodeikticus* (Kurokawa et al., 1971), and *L. plantarum* (Keenan and Allen, 1974a). The enzyme from *L. plantarum* has been partially purified and demonstrated to be stimulated by several detergents and cardiolipin (Keenan and Allen, 1974a,b; Allen et al., 1976). We report here a study of the effectiveness of a variety of natural and synthetic phospholipids, fatty acids, and detergents to stimulate undecaprenyl pyrophosphate synthetase.

Experimental Procedure

Materials

L. plantarum (ATCC 8014) was obtained from the American Type Culture Collection. Sorbents for chromatography were silica gel G (E. Merck), silicic acid (Bio-Sil HA, Bio-Rad Laboratories), and silica gel G without gypsum, on plastic sheets from Brinkmann Instruments. Amberlite XAD-2 was obtained from Mallinckrodt Chemical Works. Farnesyl py-

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TABLE I: Fatty Acid Content of Phospholipids.

Lipids	Source	Major fatty acid components (percent of total)						
		14:0	16:0	16:1	18:0	18:1	18:2	Cyclopropane
Cardiolipin	Bovine (Sigma)[a]		2.8	4.6		12.6	79.4	
Cardiolipin	*E. coli* (Supelco)[b]		32.5		1	38.3		28.4
Cardiolipin	*L. plantarum*[a]	3.2	22.7	10.3	Tr	33.3		30.6 (C_{19})
Phosphatidylglycerol	*L. plantarum*[a]	4.8	26.6	9.6	Tr	29.1		28.3 (C_{19})
Whole cell	*L. plantarum*[c]	0.7	6.7	13.9	1.3	26.4		48.1 (C_{19})

[a] Data obtained by analysis as described in Methods. [b] Data obtained from manufacturer. [c] Literature values (Thorne and Kodicek, 1962).

rophosphate (36% trans, trans and 45% cis, trans) and Δ^3-[1-^{14}C]isopentenyl pyrophosphate (0.45 μCi/μmol) were the preparations described earlier (Keenan and Allen, 1974a). *E. coli* cardiolipin (CL)[1] and phosphatidylglycerol (PG) were purchased from Supelco Inc. Bovine heart CL, egg lecithin (PC), egg phosphatidic acid (PA), dipalmityl-PA, and phosphatidylserine (PS) were obtained from Sigma Chemical Co. Dilauryl-PC was obtained from Analabs, Inc. Fatty acids, detergents, and other reagents were of reagent grade purity.

Methods

Thin-Layer Chromatography. TLC of phospholipids was carried out on freshly prepared silica gel G glass plates activated for 20 min at 80 °C immediately before use. Solvents for TLC of the phospholipids were chloroform–methanol–water (65:25:4, v/v) (solvent I), chloroform–methanol–7.4 N ammonium hydroxide (17:7:1, v/v) (solvent II), diisobutyl ketone–acetic acid–water (80:50:7, v/v) (solvent III), and chloroform–methanol–1 M NH_4OH (65:25:4, v/v) (solvent IV).

Phospholipid Preparation. Total cell lipid was prepared from whole *L. plantarum* cells or a 48 000*g* Triton X-100 extracted cell pellet (Keenan and Allen, 1974a) by the method of Bligh and Dyer (1959). The chloroform–methanol extract was clarified by filtration through Whatman PS 1 paper and the filtrate taken to dryness at room temperature. Phospholipids were precipitated from a chloroform solution (0.8 mL) by the addition of 10 volumes of acetone (8 mL). The phospholipids were repeatedly precipitated from chloroform solution to remove contaminating neutral lipid. This phospholipid preparation (maximum of 30 mg) was suspended in chloroform and applied to a silicic acid column (2 g) packed in chloroform. The phospholipids were eluted from the column by sequential addition of 15-mL aliquots of chloroform–methanol in the following proportions: 100:0, 98:2, 90:10, 85:15, 75:25, 60:40, 50:50, 0:100. CL eluted in fraction 3 (90:10), PG in fraction 4 (85:15), and lysyl-PG in fraction 6 (60:40).

CL was further purified by thick-layer silica gel G chromatography in solvent I, R_f 0.8. Its TLC mobility was the same as bovine heart CL in several solvent systems giving R_f values of 0.93, 0.84, and 0.65 in solvents I, II, and III, respectively.

PG and lysyl-PG were rechromatographed on silicic acid columns as described above. PG was identical with *E. coli* PG giving R_f values of 0.46 and 0.71, respectively, by TLC in solvents III and IV. It had an R_f value of 0.63 in solvent I.

The R_f's of lysyl-PG on TLC in solvents II and III were 0.61 and 0.45, respectively. Because of its lability in neutral and mildly basic solutions, highest yields were obtained when slightly acid buffer (0.1 M acetate, pH 4.7) was used in washing whole cells and extracting whole cell lipids (Houtsmuller and van Deenen, 1965).

The phospholipids were assayed by the dichromate oxidation method of Amenta (1964) using standard curves determined with bovine CL. Analyses of the fatty acid methyl esters obtained from *L. plantarum* CL and PG were determined by isothermal (173 °C) GLC on a 6-ft 10% DEGS/Gas Chrom Q column. These results and the analyses of the fatty acids obtained from other phospholipids, obtained commercially, are listed in Table I.

Enzyme Preparation. Undecaprenyl pyrophosphate synthetase was prepared from cell-free extracts of *L. plantarum* obtained by lysozyme or French press treatment. The enzyme was purified chromatographically in Triton X-100 containing buffers through the hydroxylapatite step as previously described (Allen et al., 1976). Before testing the enzyme for lipid stimulation, the hydroxylapatite enzyme was depleted of Triton X-100 by treatment with Amberlite XAD-2 at 100–150 mg of resin per mg of Triton X-100 for 10–20 min as previously described (Keenan and Allen, 1974b). This procedure left 0.1 to 0.2% Triton X-100 remaining. Dilution of enzyme in the assay mixtures gave levels of Triton X-100 which were not effective in stimulating the enzyme activity.

Enzyme Assay. The standard assay solution for measuring lipid activation contained in a final volume of 0.5 mL, 0.2 M Tris buffer, pH 7.5, 50 μM farnesyl pyrophosphate, 60 μM Δ^3-[1-^{14}C]isopentenyl pyrophosphate (30 000 dpm), 0.2 mM $MgCl_2$, and detergent-depleted hydroxylapatite purified enzyme. Depending on the experiment, detergent, sonicated phospholipid, or fatty acid was also included. The reaction mixtures were incubated for 30 min at 40 °C unless otherwise indicated and product formation was determined following acid hydrolysis as previously described (Keenan and Allen, 1974a).

An organic solution of each phospholipid to be tested was brought to dryness with a nitrogen stream, suspended in a solution containing 0.2 M Tris buffer, pH 7.5, 0.2 mM $MgCl_2$, and 50 μM farnesyl pyrophosphate, flushed with nitrogen and dispersed immediately prior to assay by sonication in a plastic test tube for 5 min at 0 °C using a Biosonic II sonicator at 150 W. Aliquots were then transferred to the assay tubes. Farnesyl pyrophosphate was included in the sonication mixture for technical reasons, i.e., to maintain small and constant enzyme reaction volumes. In the case of all fatty acids, except C_6, C_8, and C_{10} which did not require sonication, the same procedure was followed except that farnesyl pyrophosphate was omitted

[1] Abbreviations used: CL, cardiolipin; PG, phosphatidylglycerol; PC, lecithin; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane.

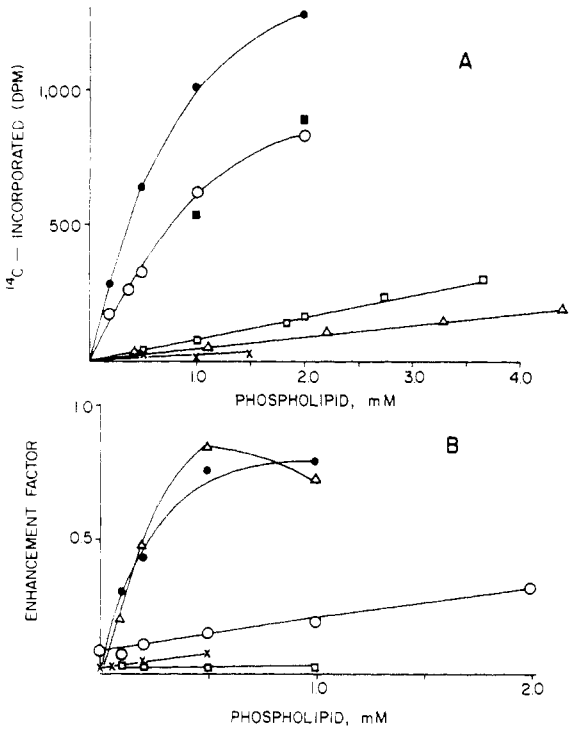

FIGURE 1: Activation of synthetase by phospholipids in the absence of Triton X-100. Reactions were carried out with sonicated phospholipid at 37 °C as described in Methods. (A) Product formed is shown for varying concentrations of *L. plantarum* CL (○), *L. plantarum* PG (□), *L. plantarum* whole phospholipid extract (△), *L. plantarum* lysyl PG (X), *E. coli* CL (■), and bovine CL (●). (B) The enhancement factor for enzyme stimulation is shown for varying concentrations of dipalmityl-PA (○), egg PA (△), bacterial PS (X), dilauryl-PC (□), and bovine CL (●). An enhancement factor of 1.0 indicates the same extent of stimulation as obtained with 0.5% Triton X-100 alone.

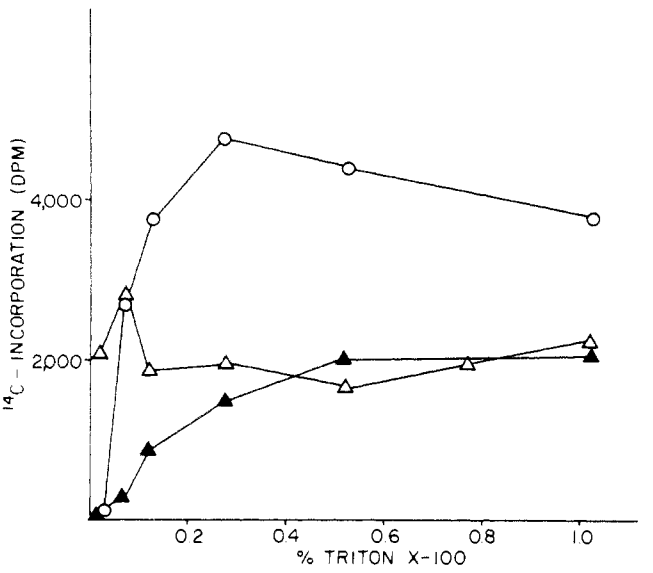

FIGURE 2: Effect of Triton X-100 on enzyme stimulation by phosphatidic acid. Reactions were carried out as described in Methods in the presence of varying concentrations of Triton X-100. The enhancement factor for enzyme stimulation is shown for assay mixtures containing Triton X-100 alone (▲), 0.5 mM dipalmityl-PA (○), and 0.5 mM egg PA (△).

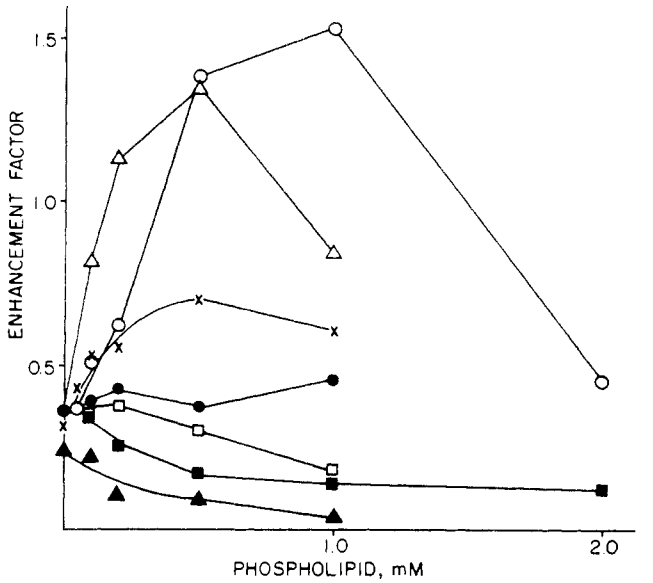

FIGURE 3: Activation of synthetase by phospholipids in the presence of 0.1% Triton X-100. Reactions were carried out as described in Methods in the presence of 0.1% Triton X-100 and varying concentrations of sonicated phospholipid. The enhancement factor is shown for bovine CL (●), dipalmityl-PA (○), egg PA (△), bacterial PS (X), dilauryl-PC (□), egg PC (■), and bacterial PE (▲).

from the sonication mixtures. Control experiments showed that palmitic acid sonicated in the presence or absence of farnesyl pyrophosphate gave the same stimulatory behavior. Similarly sonicated or unsonicated CL gave the same stimulatory response.

Results

Detergent-depleted undecaprenyl pyrophosphate synthetase had previously been demonstrated to be inactive in the absence of detergent but was reactivated by various detergents and bovine CL. The results reported here describe a more complete investigation of the effect of lipids on enzymic activity.

Activation by Phospholipid. L. plantarum phospholipids were isolated and purified from cultures harvested in late log phase. Three of the major phospholipid components, CL, PG, and lysyl-PG, were tested for their ability to stimulate detergent-depleted undecaprenyl pyrophosphate synthetase. Figure 1A demonstrates that, of the individual *L. plantarum* phospholipids tested, CL is the only one which exhibited good activity. The major negatively charged phospholipid, PG, having the same fatty acid content, showed little activity. The whole phospholipid extract and the positively charged lysyl-PG also demonstrated little activity. *E. coli* CL which had a similar fatty acid content to *L. plantarum* CL, Table I, showed the same activity as *L. plantarum* CL. Bovine CL with approximately 80% $C_{18:2}$ fatty acid showed consistently higher activity at all concentrations tested.

The effectiveness of other phospholipids is presented in Figure 1B and Table II, as an enhancement factor representing the ratio of extent of product formed in the presence of phospholipid to the extent of product formed in the presence of 0.5% Triton X-100. The zwitterionic species dilauryl-PC (Figure 1B), egg PC, dimyristyl- and bacterial PE (data not shown) and the negatively charged phospholipids bacterial PS, and dipalmityl-PA showed little or no activity. However, egg PA was as effective as bovine CL.

When dipalmityl-PA was tested in the presence of varying concentrations of Triton X-100, however, this phospholipid was found to enhance the activity of the enzyme beyond that observed with Triton X-100 alone at each Triton X-100 concentration tested (Figure 2). Mixtures of egg PA and Triton X-100 at concentrations below 0.3% Triton X-100 showed

TABLE II: Lipid Activation of Undecaprenyl Pyrophosphate Synthetase.

Lipid	Minus Triton X-100		Plus 0.1% (1.6 mM) Triton X-100	
	Enhancement factor[a]	mM concn for ½ max effect	Enhancement factor[b]	mM concn for ½ max effect
Phospholipids				
Egg phosphatidic acid	0.8	0.35	1.0	0.10
Dipalmitylphosphatidic acid	0.2	*c*	1.2	0.32
Bovine cardiolipin	0.8	0.30	0.1	
Phosphatidylserine	0.05	*d*	0.4	0.10
Fatty acids				
Oleic ($C_{18:1}$)	0.9	0.96	1.3	0.54
Stearic (C_{18})	0.07	*c*	0.8	0.28
Palmitic (C_{16})	0.80	1.30	1.4	0.24
Myristic (C_{14})	0.20	0.26	2.1	0.21
Lauric (C_{12})	0.10	0.85	2.3	0.30
Capric (C_{10})	0.04	*e*	1.0	1.20
Caprylic (C_8)	0	*e*	0.2	2.0
Caproic (C_6)	0	*e*		
Detergents				
Deoxycholate	1.0	0.33	2.3	0.85
Dodecyl sulfate	0.3	0.075	2.4	0.15
Cetyl sulfate	0.9	0.040	2.8	0.28
Cetyltrimethylammonium bromide	0	*c*	0.3	0.25
Brij 35, 56, 96[g]	0	*f*		

[a] Ratio of extent of product formed in the presence of lipid to the extent of product formed in 0.5% Triton X-100 determined for each experiment. [b] The extent of product formed in a control containing 0.1% Triton was subtracted before factor was calculated. [c-f] Highest concentration tested was 10, 0.5, 5 mM and 20 mM, respectively. [g] HLB numbers are 16.9, 12.9, and 12.4, respectively (Umbreit and Strominger, 1973).

stimulatory activity, but at higher Triton X-100 concentrations the phospholipid was ineffective.

Most of the phospholipids were then tested in the presence of a fixed Triton X-100 concentration (0.1%) (Figure 3 and Table II). Egg PA showed a stimulatory effect similar to that observed in the absence of Triton X-100. Furthermore, both dipalmityl-PA and PS showed enhancement effects not observed in the absence of Triton X-100. CL showed little stimulatory effect in the presence of Triton X-100. PC and PE, however, demonstrated an inhibitory effect on the enzyme partially stimulated by Triton X-100. Whole *L. plantarum* phospholipid extract also showed a marked inhibitory effect over the same concentration range tested for the other lipids (data not shown).

Control experiments performed with CL and enzyme prepared in the absence of Triton showed that residual Triton X-100, remaining in enzyme solutions after treatment with the nonionic resin, was not necessary for the stimulatory effect seen with lipids tested in the absence of Triton supplementation.

Purified membrane fractions from *L. plantarum* exhibited no enzyme activity. Sonication or treatment with Triton X-100 did not activate any synthetase activity in these membrane fractions. Furthermore, the membranes were not effective as a substitute for detergent or purified phospholipids in activating the detergent-depleted hydroxylapatite purified enzyme. Sonication or suspension of these membranes in Triton X-100 also did not potentiate the ability of these membranes to activate the enzyme.

Activation by Fatty Acids. The effect of simpler amphipathic lipids was also examined using various fatty acids. The C_6-C_{18} saturated fatty acids, with the exception of palmitic acid, demonstrated little ability to stimulate activity (Figure 4A). The unsaturated fatty acid, oleate, however, showed good stimulatory activity at a concentration 20 times lower than palmitate. However, when these same fatty acids were tested in the presence of 0.1% Triton X-100, there was a general stimulatory effect seen when the carbon chain was greater than 10 (Figure 4B, Table II). The relative enhancement effect above the level of 0.1% Triton X-100 alone demonstrated optimal stimulatory activity for C_{12} and C_{14} with decreasing activity for longer or shorter chain fatty acids (Figure 5A).

A comparison of the concentration of fatty acid required to give ½ maximum enhancement effect in the presence of 0.1% Triton X-100 as a function of the number of carbons in the fatty acid is illustrated in Figure 5B and Table II. The saturated fatty acids with 12–18 carbons were each ½ maximally effective at approximately 0.25 mM concentration. Decreasing carbon number, i.e., C_{10} and C_8, required increasing fatty acid concentration to give the maximum effect. Oleic acid had a ½ maximum stimulatory effect at a concentration, 0.54 mM, which is greater than the corresponding C_{18} saturated fatty acid.

Enzyme stimulation by fatty acids in the presence of a detergent, which was ineffective alone in stimulating enzyme activity, was tested using Brij 35 and C_{10} and C_{14} fatty acids. In 0.1% Brij 35, each fatty acid (Figures 6A and 6B) showed an optimal concentration for maximum stimulation as demonstrated with Triton X-100. However, the concentrations for peak activities for myristic acid (C_{14}) and capric acid (C_{10}) were 1.5 and 10 mM, respectively, compared with 0.5 and 2 mM when 0.1% Triton X-100 was used. Therefore, a higher concentration of fatty acid was required for optimal activity when the shorter chain fatty acid was tested with either detergent.

The effect of varying C_{10} or C_{14} fatty acid concentrations at different fixed detergent concentrations is also illustrated

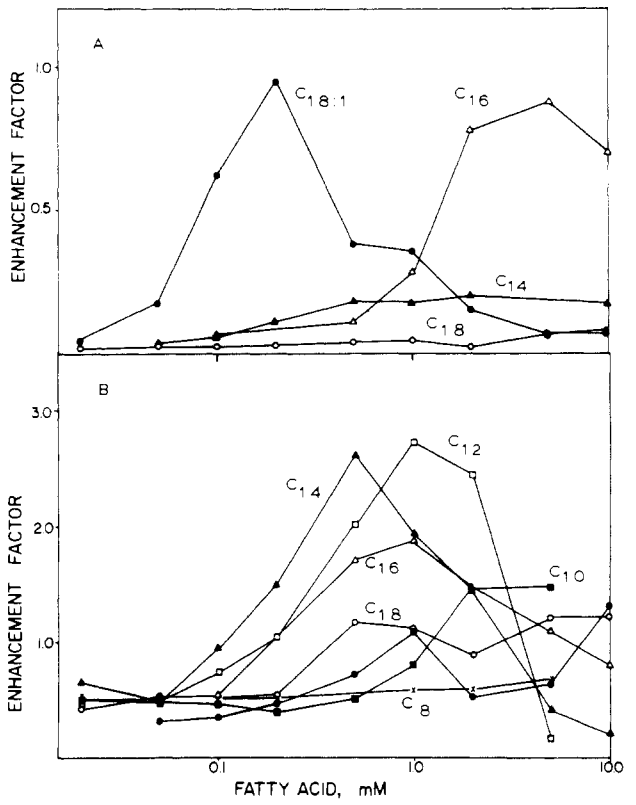

FIGURE 4: Activation of synthetase by fatty acids. Reactions were carried out as described in Methods with varying concentrations of fatty acids in the absence (A) or presence (B) of 0.1% Triton X-100. The enhancement factors are shown for varying concentrations of fatty acids: C_8 (X), C_{10} (■), C_{12} (□), C_{14} (▲), C_{16} (△), C_{18} (○), and $C_{18:1}$ (●). In the absence of Triton, the C_6, C_8, C_{10}, and C_{12} fatty acids show little or no stimulatory activity.

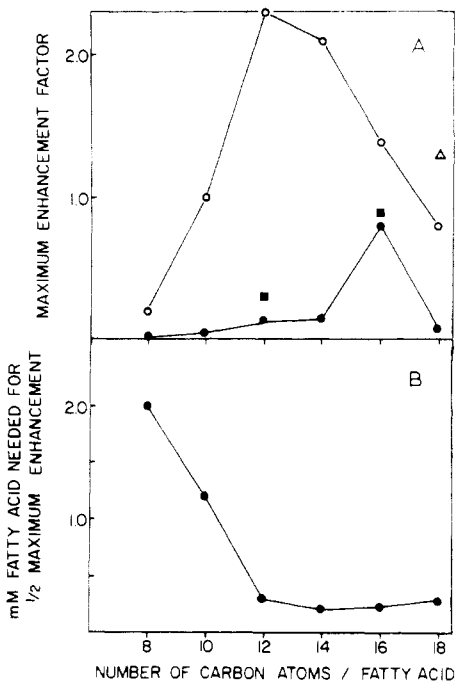

FIGURE 5: The extent and efficiency of activation of synthetase as a function of fatty acid chain length. (A) The maximum enhancement factor observed for each fatty acid, tested in the presence of 0.1% Triton X-100 (see Figure 4B), was plotted as a function of the number of carbons in the fatty acid chain, after subtraction of the stimulatory effect of 0.1% Triton X-100 alone. Open and closed symbols represent effects in the presence and absence of Triton X-100, respectively. Closed squares represent effect with corresponding alkyl sulfates. Open triangle (△) represents enhancement due to oleic acid ($C_{18:1}$). (B) The mM concentration of fatty acid necessary to give the ½ maximum enhancement effect in the presence of 0.1% Triton X-100 is plotted here as a function of the number of carbons in the fatty acid chain.

in Figures 6A and 6B. In each case the maximum amount of product formed increased with an increase in detergent concentration. This is seen more clearly in Figure 7 for myristic acid where the product formation is plotted for varying Brij concentrations at different fixed fatty acid concentration. The concentration of Brij necessary to achieve optimal activity at each myristic acid concentration studied also increased with increased fatty acid concentration. A plot of the C_{14} fatty acid concentration vs. detergent concentration necessary to give optimal activity gave a straight line indicating that there was a fixed molar fatty acid to detergent ratio (2:1) required for optimal activity. In the case of C_{10} fatty acid (Figure 6B), increasing Brij concentration did not affect the optimal fatty acid concentration necessary for enzyme stimulation.

Activation by Detergents. The alkyl sulfates, dodecyl and cetyl sulfate, showed a stimulatory effect similar to that seen with the corresponding chain length fatty acids, although at considerably lower concentrations (Figure 5A and Table II), and over a very narrow concentration range. Cetyltrimethylammonium bromide (concentration range 0.02–10 mM) and Brij 35, 56, and 96 (concentration range 0.02–20 mM) showed no stimulatory effect. Cetyltrimethylammonium bromide exhibited only a small enhancement effect in the presence of 0.1% Triton X-100. Dodecyl and cetyl sulfate were also tested individually with 0.1% Triton and showed an enhancement effect much like the corresponding chain length fatty acids with remarkably similar concentrations for the ½ maximal effect.

Temperature Dependence of Phospholipid Activation. Studies on the temperature dependence of phospholipid activation were made using 0.5% Triton X-100, 0.5 mM egg PA, and 0.6 mM CL with the hydroxylapatite purified enzyme (Figure 8). There is a marked increase in enzymic activity over the temperature range of 30–40 °C when egg PA was used. In the presence of either CL or Triton X-100, the enzyme activity decreased over this same temperature range.

Discussion

Many membrane-associated enzymes have been described which are inactive in the absence of lipids but are stimulated by the addition of detergents and phospholipids. β-Hydroxybutyrate dehydrogenase is activated by the zwitterionic phospholipid PC (Gazzotti et al., 1975), whereas the anionic phospholipid CL is ineffective (Fleischer et al., 1966). On the other hand, $(Na^+ + K^+)$ATPase is activated by the anionic phospholipids PG and PS (Kimelberg and Papahadjopoulos, 1972). Undecaprenol kinase is stimulated by a variety of both anionic and zwitterionic phospholipids (Sandermann, 1974; Gennis and Strominger, 1976). A number of detergents and phospholipids stimulate the activity of undecaprenyl pyrophosphate synthetase. The mechanism by which these agents affect enzyme activity is not clear, but the lipophilic nature of these agents suggests some type of interaction of this soluble enzyme with a lipophilic surface.

Triton X- series detergents were generally found to activate undecaprenyl pyrophosphate synthetase, whereas other nonionic detergents were ineffective (Keenan and Allen, 1974a). In view of reports (Umbreit and Strominger, 1973; Gennis and Strominger, 1976) that some membrane-associated enzymes

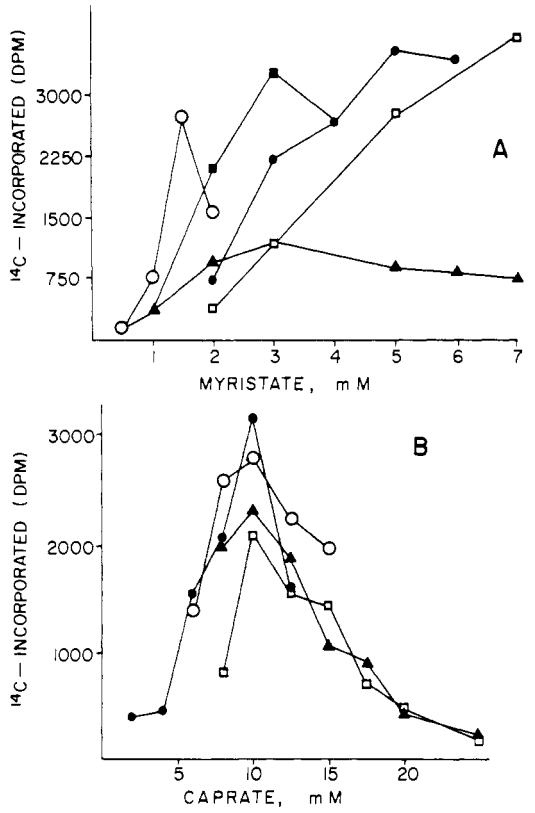

FIGURE 6: Activation of synthetase by myristate and caprate in the presence of Brij 35. Reactions were carried out as described in Methods with varying concentrations of fatty acid and Brij 35. (A) ^{14}C-labeled product formed at varying myristate (C_{14}) concentrations at different concentrations of Brij 35: 0% (▲); 0.1%, 0.81 mM (○); 0.15%, 1.22 mM (■); 0.3, 2.44 mM (●); and 0.4%, 3.26 mM (□). (B) ^{14}C-labeled product formed at varying caprate (C_{10}) concentrations at different concentrations of Brij 35: 0.03%, 0.24 mM (□); 0.06%, 0.49 mM (▲); 0.1%, 0.81 mM (○); and 0.2%, 1.63 mM (●).

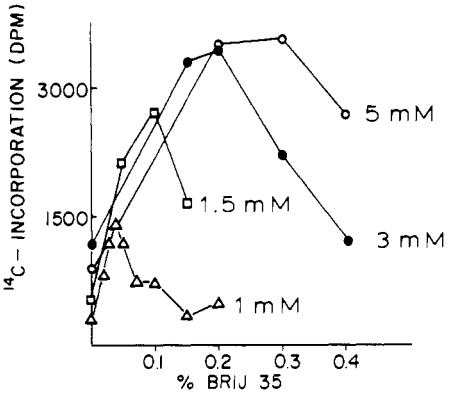

FIGURE 7: Dependence of enzymic activity on myristate/Brij ratio. Reactions were carried out as described in Methods and Figure 6. The ^{14}C-labeled product formed at varying concentrations of Brij 35 is shown at different concentrations of myristate: 1 mM (△), 1.5 mM (□), 3 mM (●), and 5 mM (○).

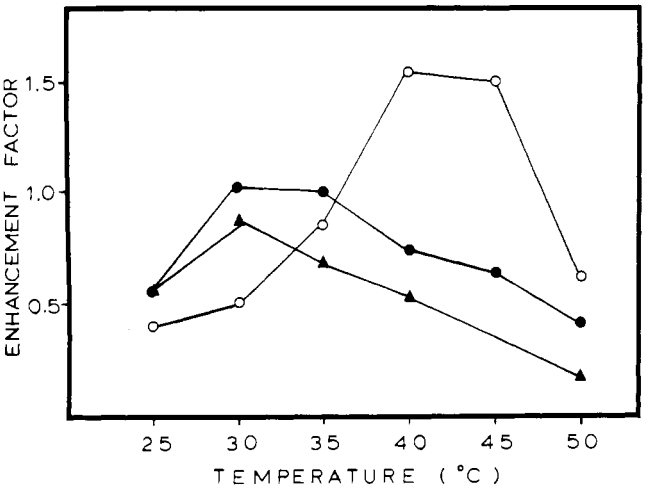

FIGURE 8: Temperature dependence of phospholipid activation. Reactions were carried out as described in Methods using 0.5 mM sonicated egg PA (○), 0.6 mM unsonicated CL (▲) or 0.5% Triton X-100 (●) at the indicated temperatures. The enhancement factor representing enzyme activity is presented for each condition relative to the enhancement factor of 0.5% Triton X-100 at 35 °C, which was taken as 1.0.

are stimulated by detergents having HLB (hydrophobic-lipophilic balance) values over a very limited range, this possibility was examined in the case of this synthetase. Whereas the Triton X- series detergents whose HLB numbers fall in the range 10.5 to 14.5 seem to be particularly active in stimulating the synthetase, other detergents, Brij 96 and Brij 56, with HLB numbers in this region are not effective. The use of the HLB number alone to predict the potential of a detergent for activating an enzyme is therefore not applicable in this case.

The marked concentration dependent stimulation of enzyme activity shown earlier with deoxycholate (Allen et al., 1976) was also seen here with the negatively charged detergents dodecyl sulfate and cetyl sulfate as well as long chain fatty acid. This emphasizes the importance of carefully examining the concentration dependence of each effector before drawing conclusions on their effectiveness.

An earlier description of possible natural phospholipid effectors showed that bovine CL activated the enzyme, whereas *E. coli* PG and zwitterionic phospholipids were ineffective in stimulating enzyme activity (Keenan and Allen, 1974b). We reported here that *E. coli* CL and egg PA were also effective activators.

It was also of importance, however, to test the phospholipids found naturally in *L. plantarum* and to compare their activity with those phospholipids already tested. CL, which represented about 12% of the total *L. plantarum* phospholipid, exhibited good activity. Its activity was only about 60% of that of bovine CL but essentially the same as *E. coli* CL. The major phospholipid isolated from *L. plantarum*, PG, exhibited little stimulatory activity even though it has a net negative charge and the same fatty acid content as CL. The whole lipid extract and the positively charged lysyl-PG were inactive. It appeared, therefore, that CL was the only phospholipid in *L. plantarum* capable of stimulating the enzyme.

In an attempt to examine the properties of the phospholipid that were necessary for activation, experiments were carried out to determine if the ineffective lipids were effective in the presence of detergents. The lack of stimulation by dipalmityl-PA in the absence of detergent seemed to indicate a lack of fluidity in the phospholipid bilayer or micelle since egg PA containing unsaturated fatty acid residues was effective. A mixture of 0.1% Triton X-100 and dipalmityl-PA dramatically stimulated enzymic activity above that expected with detergent alone. This is consistent with an increase in the fluidity of the phospholipid aggregate if it is assumed that the detergent inserted into the phospholipid bilayer or the phospholipid was dispersed into mixed micelles with the detergent.

The stimulatory effect was, however, dependent on the

concentration of phospholipid used. A decrease in stimulation of activity at concentrations of phospholipid higher or lower than the optimal effective concentration suggested that an optimal phospholipid to detergent ratio was required for maximum activity. This optimal ratio might correspond to an optimal surface charge density on the lipid bilayer.

Similar but less dramatic effects were seen with PS. The active phospholipids CL and egg PA both exhibited the lack of stimulatory activity at high Triton X-100 concentration. This effect is difficult to explain but may indicate a low level of association of phospholipid with detergent due to the presence of unsaturated fatty acid moieties in the phospholipids and/or the preference of the enzyme for the active detergent micelles rather than the phospholipid bilayer.

If surface charge density was important in determining the activation of the enzyme, then simple fatty acids should also activate the enzyme. The observation that fatty acids C_6–C_{14} and C_{18} gave no activity in the absence of detergent is explainable on the basis of the lack of micellular structure for C_6–C_{12} at the concentrations tested (Shinoda, 1963) and a lack of fluidity in the lipid bilayer in the case of the longer chain fatty acids C_{14} and C_{18} at the temperature tested. The micelles of the unsaturated fatty acid, oleate, are apparently fluid enough to permit good stimulation. The reason C_{16} fatty acid is effective is not clear. Mixtures of C_{12} or C_{14} saturated fatty acids and Triton X-100 demonstrated a dramatic increase in enzymic activity with a concentration-dependent effect similar to that described for PA.

Similar findings using fatty acid–detergent mixtures have been made by Gennis and Strominger (1976) with the membrane associated C_{55}-isoprenoid alcohol phosphokinase from *S. aureus* and Cunningham and Hager (1971), with the soluble enzyme pyruvate oxidase from *E. coli*. Others have shown that the incorporation of anionic lipids into synthetic PC micelles greatly enhanced PC breakdown by phospholipase A (van Deenen, et al., 1963; Slotboom et al., 1976).

The hypothesis that a specific surface density of negative charge was optimal for enzyme stimulation was tested further in experiments using mixtures of C_{10} and C_{14} fatty acid with the detergent Brij 35, which had no stimulatory effect alone. The use of Brij 35 made it possible to vary the detergent concentration without increasing enzymic activity due to detergent alone. The fatty acid concentration dependent stimulation of enzyme in the presence of Brij 35 was similar to that observed with Triton X-100.

The observed dependence of enzymic activity on the concentration of fatty acid or phospholipid and detergent may be explained by variations in the physical structures of the resulting lipid–detergent complexes. Esfahani et al. (1972) have presented evidence that Brij 36T disrupted the lipid interactions in elaidate membranes resulting in a marked stimulation of succinic-ubiquinone reductase activity. Also, Warner and Dennis (1975) explained kinetic behavior of PS decarboxylase with mixtures of phospholipid and Triton X-100 on the basis of changes in the phospholipid to detergent ratios in the mixed micelles.

An explanation of the results observed with the undecaprenyl pyrophosphate synthetase, using fatty acids as the stimulating agents, is discussed below. Myristic acid (C_{14}) at concentrations greater than about 10 mM, in the absence of detergent, is found in extended micelles or bilayers. The addition of detergent would result in the insertion of detergent into the bilayer diluting out the surface charge density. If a specific surface charge density was necessary for optimal enzyme interaction and activity, then low enzyme activity at high fatty acid to detergent ratios would indicate the charge density was too high for optimal activity. The results of experiments with C_{14} fatty acid and either Brij 35 or Triton X-100 are consistent with this hypothesis. Addition of detergent to mixtures having a high fatty acid to detergent ratio would have decreased the charge density until an optimal charge density and optimal activity were reached. Further addition of detergent would have resulted in a dilution of the negative surface charge below the optimal level. Decreased enzyme interaction with this surface would have resulted in decreasing activity. Adjusting the C_{14} fatty acid to Brij ratio in this manner resulted in changes in enzymic activity predicted by the model. These same effects would be observed if the myristic acid was transfered to a detergent micelle instead of detergent inserting into the lipid bilayer.

At higher concentrations of fatty acid, corresponding higher concentrations of detergent would have been necessary to give the appropriate fatty acid to detergent ratio necessary for optimal enzymic activity. This would have resulted in a constant ratio of fatty acid to detergent for optimal stimulatory activity independent of fatty acid concentration used. This effect was seen in the case of myristic acid and Brij 35.

The maximum synthetase activity observed in experiments, where detergent concentration was fixed and fatty acid was varied, demonstrated an increase in extent of product formed as the fixed detergent concentration was increased. This is explained by an increase in the number of optimally charged micelles, or total optimally charged surface area, with increasing concentrations of detergent. At low detergent concentrations, the amount of appropriately charged surface would be rate limiting but enzymic activity leveled off at higher detergent concentrations where a step other than enzymic–lipid interactions could be rate limiting.

The stimulation of activity with mixtures of Brij 35 or Triton X-100 and the shorter chain fatty acids requires further explanation. In contrast to the results seen with fatty acids larger than C_{12}, when shorter chain fatty acids were used, it was necessary to use increasingly higher concentrations of fatty acid to obtain ½ maximal activity as the fatty acid chain length was decreased (Figure 5B).

The shorter chain fatty acids have relatively high cmc values and should not be found as micelles at the concentrations tested. In detergent solutions, increasing the concentration of free fatty acid will result in an increase in the fatty acid to detergent ratio in the mixed micelles formed. The number of fatty acids of a given chain length absorbed by the detergent, however, is dependent on the partitioning of the fatty acid between the aqueous phase and the hydrophobic portion of the detergent micelle. The position of this equilibrium would be expected to be dependent on the chain length of the fatty acid being used. Therefore, for shorter chain fatty acids, a higher concentration of fatty acid will be required to obtain the optimal fatty acid to detergent ratio necessary for optimal enzymic activity. Similar effects have been demonstrated by Romstead and Cordes (1968) studying the micellular catalyzed hydrolysis of *p*-nitrophenyl fatty acid esters using cetyltrimethylammonium bromide, and by Sullivan et al. (1974) studying the chain length dependency of alkanol stimulated transport of *o*-nitrophenyl galactoside, by lactose permease in *E. coli*.

With the C_{10} fatty acid–Brij 35 mixture, in contrast to the results obtained with the C_{14} fatty acid, optimal synthetase activity was observed for the same fatty acid concentration at all detergent concentrations tested. Nevertheless, an increase in detergent concentration at the optimal fatty acid concen-

tration still resulted in a corresponding increase in enzymic activity.

These observations are explained by a combination of effects: (1) a small association constant for absorption of C_{10} fatty acid by the Brij micelles, and (2) the relatively low concentration of Brij being used resulting in only a small fraction of the total fatty acid being used to form active mixed micelles. Increasing the Brij concentration would have resulted in a minor change in free fatty acid concentration so that the number of fatty acid molecules absorbed onto each Brij micelle would have been determined only by the fatty acid concentration.

The temperature studies support the suggestion that lipid bilayer fluidity is important for enzyme activity. The marked increase in enzymic activity from 30 to 40 °C, using egg PA, occurs in a temperature range where a phase transition might be expected. The disodium salt of egg PA has a phase transition at about 20 °C (Ranck et al., 1974). However, the transition state temperature, under the conditions of our experiments, should be higher than 20 °C because of the presence of Mg^{2+} and the fact that the phospholipid would not be completely ionized at pH 7.4 (Trauble and Eibl, 1974). The temperature effect is not a simple kinetic effect on the enzyme as seen with the controls, with Triton X-100 and CL which exhibited a decrease in stimulatory activity in this temperature range. The apparent increase in enzymic activity with greater lipid fluidity may be explained by either increased association of the enzyme with the lipid hyrophobic core or increased solubilization of the reaction product, undecaprenyl pyrophosphate.

These results indicate that undecaprenyl pyrophosphate synthetase requires an optimal negative surface charge and fluid lipid bilayer for in vitro activity. We would suggest that similar requirements are needed in the in vivo situation and are provided by interaction of this soluble enzyme with the intercellular membrane surface. The water soluble substrates, farnesyl and Δ^3-isopentenyl pyrophosphate, would be polymerized to the more hydrophobic product, undecaprenyl pyrophosphate at the membrane surface. The product would then enter the lipid phase of the membrane for subsequent utilization in its well-described role as carbohydrate carrier.

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