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Consequences of the Inhibition of Cardiolipin Metabolism in *Haemophilus parainfluenzae*

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Examination of phospholipid metabolism in *Haemophilus parainfluenzae* with inhibitors of various cellular functions indicated that macromolecular synthesis and lipid metabolism can be dissociated at least for a short time. Two classes of inhibitors have relatively specific effects on cardiolipin (CL) metabolism. Pentachlorophenol and *p*-hydroxymercuribenzoate blocked CL synthesis but allowed CL hydrolysis to phosphatidic acid and phosphatidyl glycerol (PG); 3,3',4,5'-tetrachlorosalicylanilide (TCS) and carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP) blocked CL hydrolysis with the stoichiometric accumulation of CL. It appeared as if TCS and *m*-CCCP inhibited a vital activity coupled with the hydrolysis of CL by the highly active, CL-specific phospholipase D found in this organism. Because TCS and *m*-CCCP are thought to act by destroying the proton gradient thereby interrupting energy-dependent transport, it is possible that a highly active portion of the cellular CL could be coupled to some phase of this process.

In *Haemophilus parainfluenzae*, it appears as if a portion of the minor phospholipid, cardiolipin (CL), has a very rapid metabolism (19). A CL-specific phospholipase D in *H. parainfluenzae* hydrolyzes CL to equimolar amounts of phosphatidic acid (PA) and phosphatidyl glycerol (PG) and is present in the cells at an activity 100 times greater than the activities of phospholipases a_2 and *c* in the organism (14, 15). The CL-specific phospholipase D activity is equal to that of the phosphatidyl serine decarboxylase, the enzyme responsible for the synthesis of phosphatidyl ethanolamine (PE) which comprises 77 to 80% of the phospholipid (15). This proved surprising, as CL accounts for only 3% of the cellular phospholipid in exponentially growing cells (20). The CL-specific phospholipase D of *H. parainfluenzae* requires Mg^{2+} (K_m about 1.3 mM) and is inhibited in the presence of ethylenediaminetetraacetic acid (EDTA) (14). Addition of EDTA to growing cells results in accumulation of CL with a loss of PG (14, 19), suggesting that this enzyme is involved in CL hydrolysis in vivo.

In the present study, the inhibitors of proton conduction 3,3',4,5'-tetrachlorosalicylanilide (TCS) and carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-CCCP) have been shown to block the hydrolysis of CL in vivo with a corresponding slowing of the growth rate. TCS and *m*-CCCP had no effect on isolated CL-specific phospholipase D itself and so appear to inhibit some process coupled with rapid CL metabolism.

MATERIALS AND METHODS

Materials. Oligomycin, L-chloramphenicol, gramicidin D, *N*-ethylmaleimide (NEM), 2 *N*-heptyl-4-hydroxyquinoline N-oxide (HOQNO), *p*-hydroxymercuribenzoate (*p*HMB), 2,4-dinitrofluorobenzene, and *N*,*N*'-dicyclohexyl-carbodiimide (DCCD) were purchased from Sigma Chemical Co., St. Louis, Mo. Cycloserine, valinomycin, rifampin, bacitracin, polymyxin B sulfate, L-cysteine, and *m*-CCCP were supplied by Calbiochem, Los Angeles, Calif. Pentachlorophenol (PCP), glutathione, and TCS were purchased from Eastman Kodak Co., Rochester, N.Y. Puromycin and β-2-thienylserine were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Tetracycline was purchased from E. R. Squibb & Sons, New York, N.Y. Kanamycin sulfate was purchased from Bristol Laboratories, Division of Bristol-Myers Co., Syracuse, N.Y. Antimycin A-K-35203 was purchased from Ayerst Laboratories Inc., New York, N.Y. 2,4-Dinitrophenol was purchased from the Amend Drug and Chemical Co., Inc., New York, N.Y. 2,4-Dibromophenol was purchased from the Chemicals Procurement Laboratories Inc., New York, N.Y. 4,7-Diphenyl-1,10-phenanthroline (Bathophenanthroline) was purchased from G. Frederick Smith Chemical Co., Columbus, Ohio. Thenoyltrifluoroacetone was purchased from the Fisher Scientific Co., Fair Lawn, N.J. Dithiothreitol was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Penicillin G was purchased from Pfizer Laboratories, Div. Chas. Pfizer and Co., New York, N.Y. Monoactin was a gift from W. Keller-

Schierlein. Nigericin (X-464) was donated by R. L. Harned. Tetramethyldipicrylamine (TMPA) was supplied from Naval Dental Research Institute. Rutamycin was gift from the Lilly Laboratories, Eli Lilly & Co., Indianapolis, Inc. Other reagents were as described previously (14–22).

Growth of H. parainfluenzae. Cells were grown and harvested as described previously (16). Growth was measured as the absorbance at 750 nm in 13-mm round test tubes. An absorbance of 0.50 corresponded to a dry wt of 0.28 mg per ml (16).

Screening procedures for inhibitors of CL metabolism. Cultures of *H. parainfluenzae* were grown with 1 mCi $H_3{}^{32}PO_4$ per 300 ml for three to four doublings during the early exponential growth phase. The cells were collected by centrifugation at 37 C and resuspended in 60 ml of prewarmed medium containing no ^{32}P. Inhibitors were dissolved in 95% (v/v) ethyl alcohol, and 0.3 ml of the solution was added to 25 ml of prewarmed medium in 250-ml Erlenmeyer flasks. Portions (5 ml) of the bacterial suspension were added to the inhibitor medium mixture, and the cells were incubated for 1 hr at 37 C. After 1 hr, 20 ml of cells and medium were added to 75 ml of chloroform:methanol [1:2 (v/v)]; the mixture was shaken and extracted for 1 hr. Then 25 ml of chloroform and 25 ml of water were added, and mixing was repeated. After standing overnight, the chloroform layer was recovered; the total ^{32}P was determined, and the phospholipids were deacylated by mild alkaline methanolysis (21). The glycerol phosphate esters derived from the lipids, glycerol phosphorylglycerol (GPG) from PG, α-glycerol phosphate (GP) from PA, glycerol phosphorylethanolamine (GPE) from PE, and diglycerylphosphorylglycerol (GPGPG) from CL, were separated chromatographically on acid-washed aminoethylcellulose paper (23, 25). The esters were located by autoradiography, and the ^{32}P was determined by scintillation spectrometry (14, 23).

Phospholipase D assay. The phospholipase D, prepared from *H. parainfluenzae* by sonic treatment in 50 mM tris(hydroxymethyl)aminomethane buffer (Tris) (*p*H 7.5) containing 0.5% (v/v) Triton X-100 and 10 mM $MgCl_2$, was recovered in the supernatant portion after centrifugation at 31,000 × *g* for 30 min (14, 15). ^{14}C-labeled CL was prepared from *Escherichia coli* and suspended in Triton X-100 before addition to the enzyme (15). The products of CL hydrolysis were analyzed chromatographically as described above. Protein was determined colorimetrically (12).

RESULTS

Effects of inhibitors on growth and CL metabolism. The study of CL metabolism in exponentially growing cells has proved very difficult because of the large PG pools (19). Consequently, CL metabolism was examined in the presence of various inhibitors (Table 1). In these experiments, *H. parainfluenzae* was grown with $H_3{}^{32}PO_4$ for several generations and then transferred to nonradioactive medium containing the inhibitors. After 1 hr with the inhibitors, the entire sample (cells plus medium) was extracted and the lipids were analyzed. By analyzing the entire sample, complications which have been observed in *H. parainfluenzae* (16–18) resulting from the loss of membrane fragments were eliminated. During exponential growth the cells lost about 20% of the ^{32}P from the total lipids in 1 hr. This is largely from PE which comprises 76 to 80% of the total phospholipid. In the period of growth in the absence of inhibitors, the CL lost about 30% of its ^{32}P, and the PG lost 60% of its ^{32}P. These rates of loss represented the minimum rate of loss of ^{32}P from the lipid as any recycling of ^{32}P would result in an apparent rate of loss slower than the true rate of loss (22).

The membrane adenosine triphosphatase inhibitor DCCD (5) inhibited growth, speeded up the loss of ^{32}P from CL, and slowed ^{32}P loss from PG and PE. The enzyme inhibitors NEM, dinitrofluorobenzene, and arsenite inhibited growth and loss of ^{32}P from CL and PE and were without effect on the loss of ^{32}P from PG. Arsenate slowed the growth and loss of ^{32}P from all the lipids. The most marked effects on CL metabolism occurred with PCP and *p*HMB, which stopped growth, stimulated loss of ^{32}P from CL which apparently accumulated quantitatively in PG, and stopped the loss of ^{32}P from PE. The inhibitors TCS and *m*-CCCP stopped growth and loss of ^{32}P from PE and caused an accumulation of ^{32}P in CL that balanced the loss from PG. The inhibitor TMPA, which has effects similar to TCS (3, 13, 24), was similar except that it stimulated loss of ^{32}PG as well as the accumulation of ^{32}P into CL without affecting the metabolism of PE.

These experiments were repeated with *E. coli* K-12, which lost 30% of the ^{32}P from CL and PE and 74% of the ^{32}P from PG in 1 hr in an experiment like that described in Table 1. The inhibitors EDTA, arsenite, arsenate, NEM, and dinitrofluorobenzene showed exactly the same responses as in *H. parainfluenzae*. With *m*-CCCP and TCS, there was accumulation of ^{32}P from CL, rather than a rapid loss, and loss of ^{32}P from PG and PE not detected in *H. parainfluenzae*.

Effect of m-CCCP and PCP on CL metabolism. The inhibitors *m*-CCCP and PCP appeared to stop growth and turnover of PG and PE. *m*-CCCP appeared to inhibit CL catabolism. Pentachlorophenol appeared to inhibit CL synthesis (Table 1). The kinetics of these processes were examined by growing *H. parainfluenzae* with $H_3{}^{32}PO_4$ for several divisions and then transferring the culture into medium containing the inhibitor but no ^{32}P. When the culture was incubated with 10^{-4} M *m*-CCCP, there was an immediate loss of ^{32}P from PG which was exactly balanced by the gain of ^{32}P in the CL (Fig. 1a). The ^{32}P in the total lipid extract, the PE, and the

TABLE 1. *Effect of inhibitors on growth, proportions of cardiolipin (GPGPG), phosphatidyl glycerol (GPG), and total phospholipid in exponentially growing Haemophilus parainfluenzae*[a]

Inhibitor	Final concn[b]	Relative growth[c]	Per cent ^{32}P recovered in		
			GPGPG[d]	GPG[e]	Total[f]
Control (zero time)[g]		—	4.95	16.8	100
Control		100	3.40	6.57	78.8
Tetracycline	100 μg/ml	70.4	4.26	2.07	78.3
Chloramphenicol	1 mM	39.5	2.68	0.89	71.4
Puromycin	400 μg/ml	37.3	3.74	8.79	70.9
Kanamycin	300 μg/ml	39.5	1.62	6.21	60.3
Rifampin	100 μg/ml	4.8	4.46	7.23	77.8
Cycloserine	1 μg/ml	—	1.69	7.25	63.1
Penicillin G	1,000 units/ml	lysis	1.38	6.10	57.0
Bacitracin	1 mg/ml	100	2.86	8.82	64.9
Polymixin	600 μg/ml	0.0	1.95	11.2	68.2
Gramicidin D	1 mg/ml	—	3.26	9.15	67.8
Valinomycin	22 μg/ml	100	3.43	5.63	71.1
Monactin	100 μg/ml	100	2.78	4.90	75.3
Nigericin	100 μg/ml	64.3	4.45	6.85	90.1
Oligomycin	10 μg/ml	63.6	2.84	4.56	63.0
Rutamycin	0.5 mM	—	2.32	3.88	55.0
Antimycin A	0.2 mM	100	4.07	4.93	65.7
HOQNO[h]	0.2 mM	63.6	4.76	5.57	73.5
Thenoyltrifluoroacetone	0.2 mM	21.2	5.33	5.92	84.6
KCN	1 mM	78.3	4.06	4.29	77.6
Bathophenanthroline	0.5 mM	87.7	4.54	5.77	73.5
Dinitrophenol	1.0 mM	63.6	5.42	6.56	80.3
Dibromophenol	0.2 mM	24.2	5.44	12.7	86.1
DCCD	0.2 mM	30.3	2.89	15.8	75.4
NEM	0.1 mM	9.2	5.45	6.44	87.2
*p*HMB	1 mM	0.0	0.76	23.8	100
Pentachlorophenol	1 mM	0.0	0.27	18.6	100
Dinitrofluorobenzene	1 mM	5.7	5.00	6.49	88.8
Arsenate	1 mM	10.0	6.08	8.39	87.0
Arsenite	1 mM	18.9	7.89	6.33	83.7
TCS	0.01 mM	0.0	10.7	10.3	101
m-CCCP	0.1 mM	0.0	10.8	11.2	103
TMPA	0.1 mM	—	10.2	4.37	100

[a] Exponentially growing *H. parainfluenzae* were grown with 1 mCi $H_3{}^{32}PO_4$ per 300 ml for three to four doublings. The cells were harvested by centrifugation at a bacterial density of about 0.14 mg (dry wt) per ml and resuspended in prewarmed medium containing no ^{32}P and the inhibitor dissolved in ethyl alcohol. The lipids were extracted without centrifugation of the cells and deacylated, and the proportions of ^{32}P in the lipid extract and in the glycerol phosphate esters were determined (23). Unless otherwise indicated, all growth data and ^{32}P proportions were determined after 1 hr of growth in medium without ^{32}P (chase period).

[b] Inhibitors were dissolved in 95% (v/v) ethyl alcohol and added to the medium. The final concentration of ethyl alcohol was 0.5% (v/v).

[c] Change in cell density measured as absorbance at 750 nm in 13-mm diameter round tubes during 1 hr of growth after transfer into the nonradioactive medium (chase period). The control culture (containing 0.5% ethyl alcohol) increased in density 2.4-fold and was taken as 100%. Increases in density during the chase period in the presence of inhibitors (such as clumping of the cells) precluded measurement of the bacterial density (indicated as –).

[d] Proportion of the total ^{32}P found in GPGPG, the ester derived from cardiolipin.

[e] Proportion of the total ^{32}P found in GPG, the ester derived from phosphatidyl glycerol.

[f] Proportion of the total ^{32}P found in the lipid extract as compared to the zero time control.

[g] Determinations made just after transfer to nonradioactive medium.

[h] Abbreviations: 2 *N*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO); *N*,*N*′-dicyclohexyl-carbodiimide (DCCD); *N*-ethylmaleimide (NEM); *p*-hydroxymercuribenzoate (*p*HMB); 3,3′,4,5′-tetrachlorosalicylanilide (TCS); carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-CCCP); tetramethyldipicrylamine (TMPA).

PA did not change at all. These data suggest that CL is formed from PG without PA metabolism and that *m*-CCCP inhibits the rapid degradation of CL. When the culture was incubated with 10^{-3} M PCP, there was a loss of ^{32}P from CL (96% of the CL in 3 min, or 4.8% of the total lipid ^{32}P) that was balanced by an increase in the PG of 2.4% of the total lipid ^{32}P and an increase in

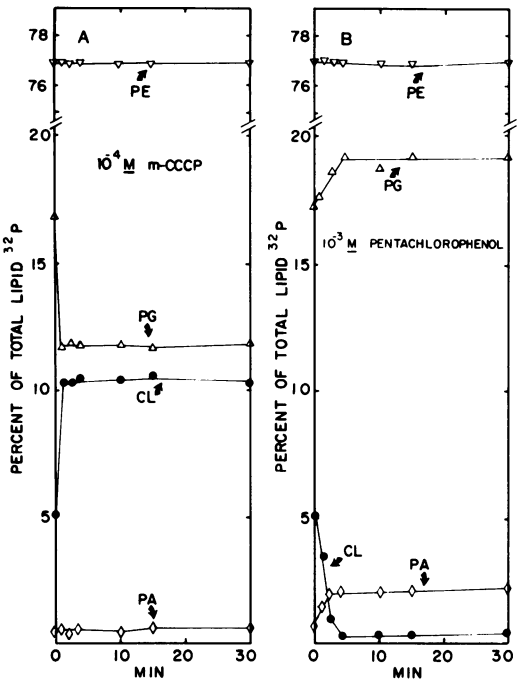

FIG. 1. *Effect of (A) carbonyl cyanide m-chlorophenyl hydrazone (m-CCCP) and (B) pentachlorophenol on the metabolism of cardiolipin (CL) in H. parainfluenzae. Cells were grown with $H_3{}^{32}PO_4$ for three to four divisions, resuspended in medium in the presence of the inhibitor but in the absence of ^{32}P, and sampled as in Table 1. The ^{32}P in the phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and phosphatidic acid (PA) was determined after chromatographic separation of the glycerol phosphate esters derived from the lipids by deacylation (20). The numbers represent the per cent of the total ^{32}P in the lipid extract in each component. With both inhibitors, the total ^{32}P in the lipid extract did not change throughout the experiment.*

PA of 1.7% of the total lipid ^{32}P (Fig. 1b). Half of the ^{32}P lost from the CL was recovered in PG, and 38% of the remaining ^{32}P lost from CL was recovered in the PA, suggesting that the catabolism of CL involves the CL-specific phospholipase D which yields PA and PG as hydrolysis products. PCP blocks the synthesis of CL without affecting its degradation.

Relationship between growth and inhibition of PG and CL metabolism. The inhibition of CL synthesis by *p*HMB and PCP, measured as the accumulation of ^{32}P into PG, and the inhibition of CL hydrolysis by *m*-CCCP and TCS, as measured by the accumulation of ^{32}P in CL, correlated directly with the inhibition of growth (Fig. 2). The inhibitory effects of *p*HMB, PCP, and *m*-CCCP could be detected within 1 min after their addition to the culture, but the accumulation of ^{32}P into CL in the presence of TCS required 5 min to be detected (Fig. 3).

In these experiments, the cells were grown with $H_3{}^{32}PO_4$ and then transferred to medium containing the inhibitors but no ^{32}P, as in the experiments illustrated in Table 1. The bacterial density and the lipids were analyzed after 1 hr of incubation with the inhibitor and in the absence of ^{32}P. From the data in Table 1, it appears that other inhibitors such as chloromycetin, kanamycin, oligomycin, rutamycin, polymixin, DCCD, PCP, and *p*HMB both slow the growth rate and enhance the rate of CL catabolism. The inhibitors tetracycline, puromycin, rifampin, thenoyltrifluoroactone, dinitrophenol, dibromophenol, NEM, dinitrofluorobenzene, arsenite, arsenate, and TMPA, like *m*-CCCP and TCS, slow the growth rate and inhibit the catabolism of CL.

Effects of inhibitors on the activity of the CL-specific phospholipase D. The inhibitors TCS, *m*-CCCP, TMPA, arsenite, arsenate, and NEM inhibit the hydrolysis of CL in growing *H. parainfluenzae* (Table 1) but have no effect on the hydrolysis of CL by the CL-specific phospholipase D in cell-free preparations from *H. parainfluenzae* (Table 2). The inhibitors *p*HMB and PCP, which inhibit the synthesis of CL, also do not affect the activity of phospholipase D in vitro. DCCD, which inhibits CL synthesis and the turnover of PG and PE, inhibited the activity of the phospholipase D in vitro.

Effect of cysteine on m-CCCP inhibition of CL hydrolysis. The inhibition of CL turnover and growth was completely abolished in the presence of a 100-fold molar excess of cysteine (Table 3). Other thiol compounds were not effective in relieving the inhibition of CL hydrolysis by *m*-CCCP. Cysteine at 10 mM had no affect on the inhibition of growth or lipid metabolism with 0.01 mM TCS although TCS and *m*-CCCP seemed to have very similar affects on lipid metabolism in *H. parainfluenzae*. Inhibitors which interact with thiols like arsenite or with amines like dinitrofluorobenzene blocked CL catabolism to a lesser extent than TCS or *m*-CCCP (Table 1). The sulfhydryl inhibitor NEM had no detectable affect on CL catabolism; *p*HMB blocked synthesis of CL but had little effect on turnover. The CL that accumulates accounts for the PG that disappears. These inhibitors, however, do not affect the activity of the CL-specific phospholipase D in vitro, suggesting that the vital cellular activity blocked by these agents is coupled to the rapid CL metabolism demonstrated in this organism.

DISCUSSION

It is clear from the data in Table 1 that inhibition of synthesis of protein, nucleic acids, or cell

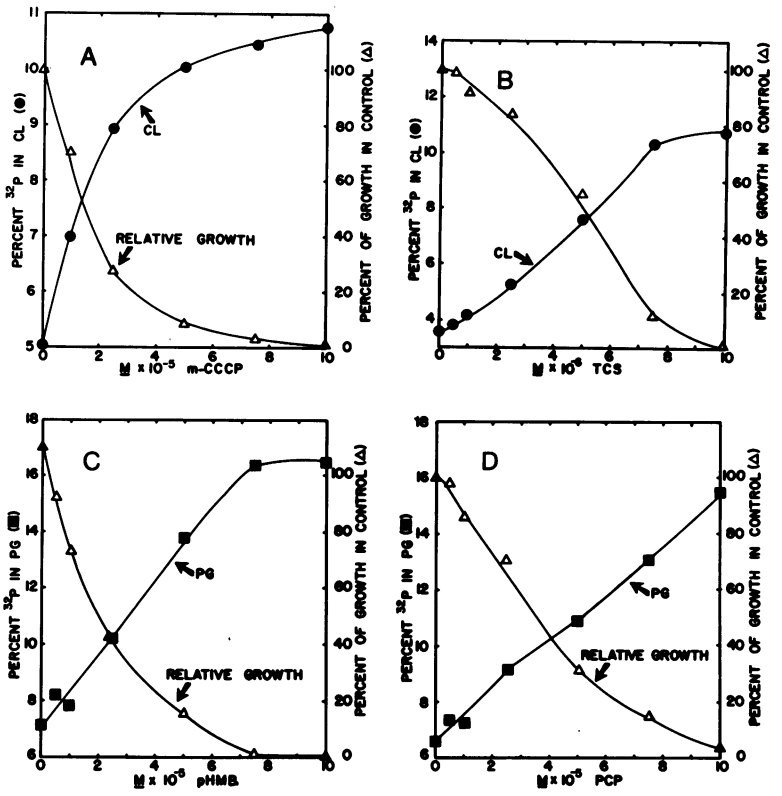

FIG. 2. *Effect of (A) carbonyl cyanide m-chlorophenyl hydrazone (m-CCCP), (B) 3,3',4,5'-tetrachlorosalicylanilide (TCS), (C) N,N'-dicyclohexylcarbodiimide (DCCD), and (D) pentachlorophenol (PCP) on growth, phosphatidyl glycerol (PG), and cardiolipin (CL) metabolism in H. parainfluenzae. Cells were grown with $H_3{}^{32}PO_4$, centrifuged, resuspended in medium containing the inhibitor in the absence of ^{32}P as in Table 1. After 1 hr, the bacterial density and the proportions of ^{32}P on the CL or PG were measured. The bacterial density was measured as the absorbance at 750 nm and is plotted (right hand axis) as the per cent of the growth in the control culture (△). The CL or PG was measured as in Table 1 and is expressed as the per cent of the ^{32}P in the total lipid that was recovered in CL or PG (left hand axis) after 1 hr (● or ■).*

wall need not immediately affect the metabolism of CL, PG, or PE of *H. parainfluenzae*. Similar data for *E. coli* have been published (1). The finding that there is very rapid metabolism of a portion of the minor lipid CL and that a highly specific phospholipase D is present in *H. parainfluenzae* with surprisingly high activity (15) makes the metabolism of CL especially interesting to study. Again from the data in Table 1, inhibitors of protein synthesis and translation had various effects on CL metabolism. Inhibitors of cell wall formation or electron transport function generally speeded up the metabolism of CL, and uncouplers of oxidative phosphorylation together with sulfhydryl and amino group enzyme inhibitors slowed the loss of ^{32}P from CL.

Two classes of inhibitors had very characteristic effects on CL metabolism. Both classes stopped growth and lipid metabolism except for CL synthesis or catabolism. PCP and *p*HMB blocked CL synthesis, but hydrolysis continued yielding PG and PA from CL (Fig. 1). The PA liberated from CL was further hydrolyzed so that less than half of the ^{32}P lost from CL was recovered in PA. (The other half of the ^{32}P lost from CL was recovered in PG.) The loss of ^{32}P from PA was much more rapid at lower inhibitor concentrations. Purification of the CL-specific phospholipase D from *H. parainfluenzae* eliminated the PA hydrolyzing activity (15). The other class of inhibitors, *m*-CCCP and TCS, blocked the hydrolysis of CL with little effect on CL synthesis, so that CL accumulated at the expense of PG (Fig. 1). This work strengthens the supposition that PG is the precursor of CL as has been shown in a strain of *Staphylococcus aureus* (Short and White, *submitted for publication*) and in *Micrococcus lysodeikticus* (A. J. DeSiervo and M. R. J. Salton, Bacteriol. Proc., p. 69, 1970).

The effects of these two classes of inhibitors greatly strengthen the claim that the highly ac-

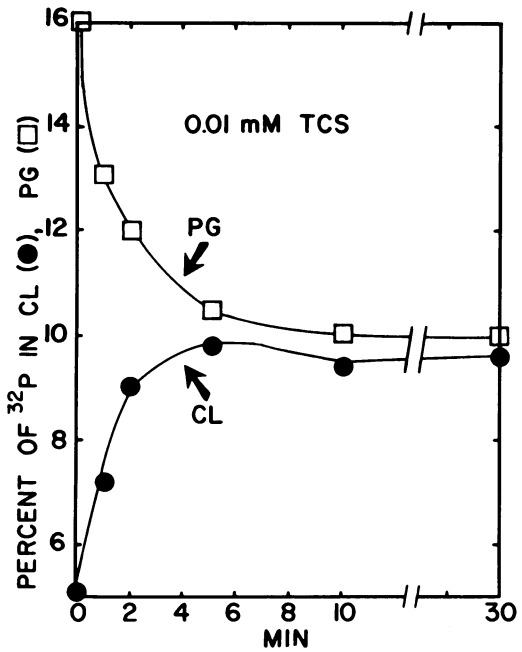

FIG. 3. *Time-course of inhibition of CL hydrolysis by 3,3',4,5'-tetrachlorosalicylanilide (TCS). The per cent of the lipid ^{32}P in cardiolipin (●) and phosphatidyl glycerol (□) was determined as in Fig. 2.*

tive, CL-specific phospholipase D in *H. parainfluenzae* is involved in CL hydrolysis in vivo. EDTA inhibits the activity of the enzyme in vitro and in vivo and causes the accumulation of CL and the loss of PG (14, 15). With PCP and *p*HMB, which do not affect the activity of the enzyme in vitro (Table 2) but which block synthesis of CL, the metabolism of PG, and almost all the metabolism of PA, the expected products of CL hydrolysis accumulate in vitro (Fig. 1).

Inhibition of CL synthesis (*p*HMB and PCP) or of CL hydrolysis (*m*-CCCP or TCS) inhibited the growth of *H. parainfluenzae* in proportion with the effect on CL metabolism (Fig. 2). This suggested that CL synthesis and hydrolysis were coupled to vital processes in the cells. The fact that none of these inhibitors affect the activity of the CL-specific phospholipase D in vitro (Table 2) suggested that the activities inhibited by these agents are coupled to the hydrolysis of CL by phospholipase D. The coupling appears to involve at least two activities, as the inhibition of phospholipase D activity by *m*-CCCP can be relieved by excess cysteine (Table 3) and occurred within 1 min after its addition. Excess cysteine had no effect on the inhibition of CL hydrolysis by TCS, and TCS required 5 min before CL hydrolysis was blocked (Fig. 3).

What vital activity could be coupled to the rapid metabolism of CL? Both of the inhibitors

TABLE 2. *Effect of inhibitors on the cardiolipin-specific phospholipase D from Haemophilus parainfluenzae in vitro*[a]

Inhibitor[b]	Final concn (mM)	Relative activity[c] (%)
Control		100
TCS	1	111
m-CCCP	1	120
TMPA	1	83.8
Arsenite	1	101
Arsenate	10	84.3
NEM	1	97.9
Pentachlorophenol	1	103
*p*HMB	1	96.8
DCCD	1	0
DCCD	0.5	1.89
DCCD	0.1	81.7

[a] CL-specific phospholipase D was prepared from *H. parainfluenzae* which was ruptured sonically in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.5) containing 0.5% (v/v) Triton X-100 and 10 mM $MgCl_2$. The preparation was then centrifuged at 31,000 × *g* for 30 min, and the supernatant fluid was utilized for the experiment. The analysis mixture contained the cell-free phospholipase D preparation (0.14 mg of protein), 60 nmoles of CL, labeled with ^{14}C (280 counts per min per nmole) and isolated from *E. coli*, in a final volume of 1 ml.

[b] Inhibitors were dissolved in ethyl alcohol and added to the enzyme mixture; final ethyl alcohol concentration with inhibitors or control was 2.5% (v/v). See Table 1 for abbreviations.

[c] The enzyme mixture was incubated for 15 min at 37 C resulting in the hydrolysis of 41% of the CL. The lipids were extracted, separated chromatographically on silica gel-impregnated paper, located by autoradiography, and the ^{32}P in the CL, PA, and PG was determined (14). The data are given as the per cent of CL hydrolysis in the control tube.

TCS and *m*-CCCP are inhibitors of oxidative phosphorylation (3, 9, 23) and appear to act by destroying the impermeability of the bacterial membrane to protons in *Streptococcus faecalis* (4, 8). Destruction of the proton gradient stops the energy-dependent transport of phosphate, potassium, and amino acids in this organism (4, 6–8). It has been shown that the diglyceride moieties at the ends of the CL molecule in *H. parainfluenzae* do not lose ^{14}C during pulse-chase experiments during exponential growth. During exponential growth, ^{14}C is lost from the middle glycerol, and the two phosphates lose ^{32}P at different rates (22). It would appear that repeated synthesis and hydrolysis of CL could be vital in changing the structure of molecules coupled to CL in the membrane. The fact that part of the CL molecule remains in situ as the middle section is hydrolyzed and resynthesized from a

TABLE 3. *Effect of inhibition of cardiolipin catabolism by m-CCCP and thiol-containing compounds*[a]

Inhibitor + protector	Relative growth	Per cent of ^{32}P recovered in		
		GPGPG	GPG	Total
Control (zero time)	—	4.95	16.8	100
Control	100	3.40	6	78.8
m-CCCP (0.1 mM)	0.0	11.6	12.0	100
m-CCCP (0.1 mM) + dithiothreitol	11.6	10.9	8.57	90.4
m-CCCP (0.1 mM) + cysteine	100	5.32	8.41	77.1
m-CCCP (0.1 mM) + glutathione	6.9	11.3	8.62	99.1
m-CCCP (0.1 mM) + thienylserine	2.3	11.9	9.46	95.9

[a] Experiments were performed and analyzed as in Table 1 except that the thiols at 10 mM were added with the 0.1 mM carbonyl cyanide *m*-chlorophenyl hydrazone (*m*-CCCP) at the start of the chase period.

portion of the PG pool could be related to stretching on CL hydrolysis and contraction on CL synthesis. It is hoped that vesicles similar to those utilized so elegantly by Kaback (10, 11) can be stimulated by D-lactate or its equivalent in *H. parainfluenzae* and that the involvement of the rapid CL metabolism in transport processes can be detected.

ACKNOWLEDGMENT

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