METABOLISM OF PHOSPHOLIPIDES BY BACTERIAL ENZYMES*

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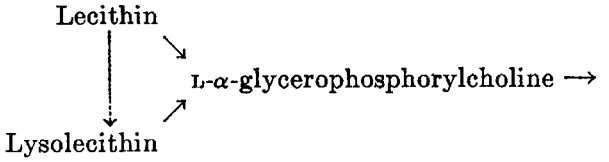
The hydrolysis of lecithin and cephalin at each of the four ester linkages by enzymes derived from plant, animal, and microbial sources has been described in numerous reports and reviewed recently by Zeller (2). However, relatively little work has been done on the systematic metabolic breakdown of the complex phospholipide molecule by a single cell type. Schmidt *et al.* (3) have demonstrated that the degradation of lecithin by pancreas and other tissues leads to the accumulation of glycerophosphorylcholine, but the mechanism of this conversion, as well as the nature of the further metabolism of this product, remains to be clarified.

We have studied the metabolism of phospholipides by a strain of *Serratia plymuthicum* isolated from soil by the enrichment culture technique. The results derived from the use of resting cell suspensions and partially purified enzyme preparations obtained from these cells indicate the following sequence of reactions:[1]



L-α-glycerophosphate + choline

The formation of lysolecithin as an intermediate in the conversion of lecithin to GPC could not be established, owing to the excessive lysolecithinase activity of the lecithinase fractions.

Cephalin appeared to be metabolized by a comparable mechanism involving glycerophosphorylethanolamine as an intermediate.

* A preliminary report of this work was presented (1).

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[1] The following abbreviations are used throughout the paper: GPC, glycerophosphorylcholine; GPE, glycerophosphorylethanolamine; GP, glycerophosphoric acid.

Methods

Materials—Egg yolk lecithin, used for enzyme studies, was purified according to Pangborn (4) and on analysis gave the following:[2]

Found.	C 66.09, H 8.10, N 1.86, P 4.18, N:P 0.99
Pangborn.	" 1.94, " 4.21, " 1.01

The choline content was determined directly from the lecithin-iodine complex (see below), and as free choline after release by acid (1 N HCl, 1 hour at 100°) or by enzymatic hydrolysis. In a sample containing 0.8 mg. of lecithin, 0.75 μM was determined as a lecithin-iodine complex, and 0.73 and 0.75 μM of choline were determined after acid and enzymatic hydrolyses, respectively. These values, which represent only about 75 per cent of the theoretical, indicate that some impurity of inorganic nature or a compound similar to choline was present. Where lecithin has been used in these investigations, the determinations have depended solely on the estimation of choline content. Lecithin emulsions were obtained by placing a distilled water suspension in a Raytheon sonic oscillator, model 9 KC, for 10 to 15 minutes at approximately 20°. *Crude egg lecithin* (used as a growth substrate), *crude animal lecithin*, and *cephalin* were products of the Nutritional Biochemicals Corporation. *Lysolecithin* was isolated from rice (5) or prepared by enzymatic hydrolysis of egg lecithin (6).

Sphingomyelin, *cardiolipin*, and *dipalmitoleyl-L-α-lecithin* were kindly supplied by Dr. H. E. Carter, Dr. H. D. Piersma, and Dr. D. J. Hanahan, respectively. L-*α-Dimyristoyllecithin* and L-*α-dimyristoylcephalin* were obtained through the kindness of Dr. E. Baer.

GPC was generously furnished by Dr. G. Schmidt, or prepared by his method (7). The choline content of the Schmidt preparation was 100 per cent of theory; that of our own was only 72 per cent, but the elementary analysis was approximately correct. The former sample was always used

$C_8H_{22}NPO_7 \cdot 2CdCl_2$.	Calculated.	C 14.97, H 3.46, N 2.18, P 4.83
	Found.	" 15.06, " 3.33, " 2.21, " 4.50

in crucial experiments. *GPE* was a gift from Dr. E. Baer.

The barium salt of DL-α-GP (8) was converted to the potassium salt by treatment with Dowex 50 (K^+) resin. The organic P content was 83 per cent of theory, and the periodate consumption was 90 per cent of theory. *Diphenyl phosphate* was a product of The Dow Chemical Company.

Particulate *GP dehydrogenase* was prepared from rabbit muscle according to Green (9). This preparation did not oxidize either β-GP or D-α-GP.

[2] Microanalyses in this paper were performed by the Microanalytical Laboratory of the National Institutes of Health under the supervision of Dr. W. C. Alford.

Determinations—Choline was determined routinely by the method of Appleton *et al.* (10). This method depends on the formation of an iodine complex which is insoluble in water but which in ethylene dichloride yields a solution with strong light absorption in the ultraviolet region. The sample was treated with an equal volume of cold 3 per cent perchloric acid and centrifuged in the cold. Choline-containing substances, such as lecithin and lysolecithin, are found in this precipitate. 0.5 ml. of the supernatant fluid, containing 0.05 to 0.5 μM of free choline, was treated with 0.2 ml. of iodine reagent (12.5 gm. of KI and 9.8 gm. of I_2 dissolved in water to 250 ml.) and kept in an ice bath for 15 minutes. After centrifugation, the supernatant fluid was removed by aspiration, the precipitate dissolved in ethylene dichloride (reagent grade) to a volume of 10 ml., and the optical density determined at 365 mμ in a Beckman model DU spectrophotometer. The molar extinction coefficient, referred to choline, is approximately 2.7×10^4.

This method for choline determination was also applied to the measurement of *lecithin* and *lysolecithin*. The sample, at neutral pH, was treated directly with iodine reagent. The insoluble lecithin-iodine complex was collected by centrifugation, dissolved in ethylene dichloride, and measured in the spectrophotometer. The lysolecithin-iodine complex, which remains soluble at neutral pH, may be precipitated by acidifying the sclution. The extinction coefficients appeared to be the same as that of the choline complex; the value obtained was corrected when necessary for free choline, determined as described above on an aliquot sample.

Choline was also determined in a few instances by the reineckate method of Glick (11), which gave satisfactory agreement with the procedure described above.

Lysolecithin was also determined by the red blood cell hemolysis test of Bernheimer (12). Quantitative results were obtained only when the amount of lysolecithin was adjusted within the narrow range of 0.15 to 0.25 μM; similar results have been reported by Collier (13). The hemolysis test applied to egg and rice lysolecithin preparations gave results in good agreement with those from the choline analyses.

GPC, which is not determined in the choline estimation by the iodine method, was estimated in the perchloric acid supernatant fluid as free choline which appeared after acid (100° in 1 N HCl for 20 minutes (14)) or enzymatic hydrolysis (see below). Both methods of hydrolysis released equivalent amounts of choline.

Ethanolamine was determined by an unpublished method of Axelrod and Brodie.[3] To 2.0 ml. of sample (pH 7.0 to 8.0) containing 0.25 to 1.0 μM

[3] Personal communication. We should like to thank Dr. B. B. Brodie and Dr. J. Axelrod for making available to us their unpublished methods.

of ethanolamine were added 0.5 ml. of 5 per cent $NaHCO_3$ solution and 0.1 ml. of dinitrofluorobenzene solution (0.1 ml. of dinitrofluorobenzene diluted to 2.0 ml. with 95 per cent ethanol). The mixture was incubated at about 80° for 1 hour in stoppered test-tubes. After the solution was cooled, 8 ml. of chloroform were added, and the mixture was vigorously shaken for 10 minutes in a mechanical shaker and centrifuged. The upper layer was removed by aspiration, and 5 ml. of the chloroform layer were transferred to a glass-stoppered container, to which were added 30 ml. of petroleum ether (b.p. 30–60°) and 4 ml. of 6 N HCl. The mixture was shaken manually for several minutes, and the optical density of the bottom aqueous layer was determined at 420 mμ in the spectrophotometer. 1.0 μM of ethanolamine gave a density reading of approximately 1.0 in a cell of 1 cm. light path.

Protein and *phosphorus* were determined by the methods of Lowry *et al.* (15) and Fiske and Subbarow (16), respectively.

Periodate titrations were performed by Jackson's method (17) modified to a micro level. *α-GP* was determined according to Burmaster (18).

Isolation and Properties of Organism—The organism was isolated from soil by the enrichment culture technique on a medium containing 0.1 per cent GPC, 0.1 per cent Difco yeast extract, 0.15 per cent K_2HPO_4, 0.05 per cent KH_2PO_4, and 0.02 per cent $MgSO_4 \cdot 7H_2O$ in distilled water. Details of the isolation technique were previously described (19). Four strains were isolated; one which produced red pigment and liberated free choline when grown in a lecithin-containing medium was used throughout this study.

The organism is a small aerobic rod, gram-negative, non-motile, and non-acid fast. Under aerobic conditions, it produces a bright red pigment which is soluble in alcohol but only slightly soluble in water. It does not decompose alkylamines; it produces a small but definite amount of gas when grown on dextrose and forms acetylmethylcarbinol. From these observations, the isolated organism has been tentatively classified as a strain belonging to *S. plymuthicum* according to Bergey's manual (20).

Cultivation of Organism and Preparation of Crude Enzyme Fractions—Cells were grown in the above medium with the addition of lecithin (0.2 per cent) but lacking GPC. The lecithin was dissolved in ether, sterilized separately by filtration, and poured into the hot medium. Ether was removed by mechanical shaking while the whole medium was hot. The cells were cultivated in 20 liter glass carboys containing 10 liters of the medium at about 26° for 16 hours with constant, reciprocal shaking. The cells were harvested by centrifugation in a Sharples supercentrifuge, washed once with a 0.5 per cent NaCl-0.5 per cent KCl solution, and frozen. At −10°, cells retained their activity for at least 6 months. The yield of cells was

approximately 4 gm. (wet weight) per liter of medium in a large scale culture and 6 to 10 gm. per liter in smaller scale cultures.

Cell-free extracts were prepared by the method of McIlwain (21). Washed cells were ground in a mortar with 4 times their weight of alumina (Alcoa A-301) at 0° for 5 minutes. The cell paste was extracted with 10 parts of glycylglycine buffer (pH 9.0, 0.02 M); the whole slurry was centrifuged at 0° at about 6000 × *g* for 15 minutes. The supernatant fluid was a slightly turbid suspension of cell fragments and contained a negligible number of intact cells. This preparation, referred to as "bacterial homogenate," contained 3.5 to 4.0 mg. of protein per ml.

The homogenate was centrifuged in a Spinco centrifuge model L (rotor No. 40) at 110,000 × *g* for 1 hour. The clear upper layer, representing about 90 per cent of the fluid, was removed with a syringe; the turbid fluid layer overlaying the residue was discarded. The residue was washed once with a volume of glycylglycine buffer equal to that of the original homogenate by centrifuging at 110,000 × *g* for 1 hour, and resuspended in the same volume of buffer. The clear supernatant fluid and the washed residue, referred to as Fractions S and R, contained approximately 2.2 and 1.3 mg. of protein per ml., respectively.

Assay of Lecithinase—The assay was based on the rate of choline liberation from purified egg lecithin in the presence of Fraction R or S as a source of lysolecithinase (see below) and an added excess of purified GPC diesterase (see below). The test system contained 1 μM of lecithin, 10 μM of potassium phosphate buffer (pH 7.0), and 0.1 ml. of crude Fraction S (when Fraction R was assayed), or 0.1 ml. of crude Fraction R (when Fraction S was assayed), 0.05 ml. of purified GPC diesterase (25 units), and water to a total volume of 0.4 ml. Incubation was carried out at 25° for 1 hour. Under these conditions the rate of reaction was proportional to the enzyme concentration.

Assay of Lysolecithinase—The assay was based upon the rate of choline liberation from lysolecithin in the presence of an added excess of purified GPC diesterase. The test system contained 1 μM of lysolecithin, 10 μM of phosphate buffer (pH 7.0), 0.05 ml. of purified GPC diesterase (25 units), lysolecithinase, and water to a total volume of 0.4 ml. The incubation was carried out at 25° for 30 minutes.

Assay of GPC Diesterase—The test system contained 0.2 ml. of GPC (0.02 M, pH 7.0), 0.1 ml. of glycylglycine buffer (0.2 M, pH 8.85), and 0.1 ml. of enzyme. After 10 minutes at room temperature (25–27°), the reaction was stopped by the addition of 0.4 ml. of perchloric acid (6 per cent) and free choline was determined in a 0.2 ml. aliquot. A unit of enzyme was defined as the amount hydrolyzing 1 μM of GPC during a 1 hour interval under these conditions, and the specific activity was defined as units

per mg. of protein. With about 1 unit of enzyme in this test system, the reaction was linear for more than 1 hour. With a crude, cell-free extract, proportionality between rate and the amount of enzyme was obtained between 0 and 4 units, whereas with purified enzyme preparations proportionality was observed between 0 and 10 units; this suggests the presence of an inhibitor in crude extracts. As will be shown under "Results," several metal ions were found to be inhibitory; the inhibition at high enzyme concentration could, therefore, be due to metal impurities in the crude enzyme preparations.

TABLE I

Adaptive Nature of Organism

The incubation mixtures (at 26°) contained 1 μM of each substrate, 20 μM of each buffer, and about 0.15 mg. of protein in a total volume of 0.4 ml. The pH and buffers were as follows: lecithin (acetate, pH 5.7), lysolecithin (acetate, pH 6.3), GPC (glycylglycine, pH 8.9), choline (phosphate, pH 7.0). Lecithin and choline were incubated for 1 hour; lysolecithin and GPC were incubated for 10 minutes. The values represent the micromoles of substrate decomposed calculated for a 10 minute incubation period and 1 mg. of protein.

Substrates	Cell suspension		Cell homogenate	
	Lecithin medium	Broth medium	Lecithin medium	Broth medium
Lecithin	0.005	0.002	0.033	0.003
Lysolecithin	0.310	0.047	0.515	0.038
GPC	0.105	0.077	0.176	0.034
Choline	0.000	0.000	0.000	0.000

Results

Adaptive Nature of Organism—The activities toward lecithin, lysolecithin, GPC, and choline of cells grown on a lecithin-containing medium and on ordinary broth were compared with both resting cell suspensions and broken cell preparations. As shown in Table I, the values were 5 to 14 times greater with extracts from lecithin-grown cells; similar results were also observed with the resting cell suspensions. Lysolecithinase activity was by far the most potent of the activities tested. Choline was observed to accumulate in lecithin-containing culture media, and no choline-removing activity was detectable with any of the preparations. The failure to utilize choline explains an observed requirement for yeast extract as a source of nitrogen and perhaps other nutrients as well.

Phosphorylcholine was not converted to choline by the intact or broken cell preparations; this suggests that this compound is not an intermediate in the formation of choline from lecithin.

Metabolism of Lecithin and Lysolecithin

Lecithin Breakdown—When purified egg lecithin was incubated with the bacterial homogenate, choline was liberated at a rapid rate, but no significant amounts of lysolecithin or GPC accumulated. With Fraction R or Fraction S alone, the removal of lecithin and the appearance of chcline were either absent or very slow, but, with Fractions R and S combined, as rapid a disappearance of lecithin and formation of choline were observed as with the original homogenate (Fig. 1).

Heating of the bacterial homogenate (at 97° for 5 minutes at pH 9.0) did not significantly reduce its ability to remove lecithin, but decreased the

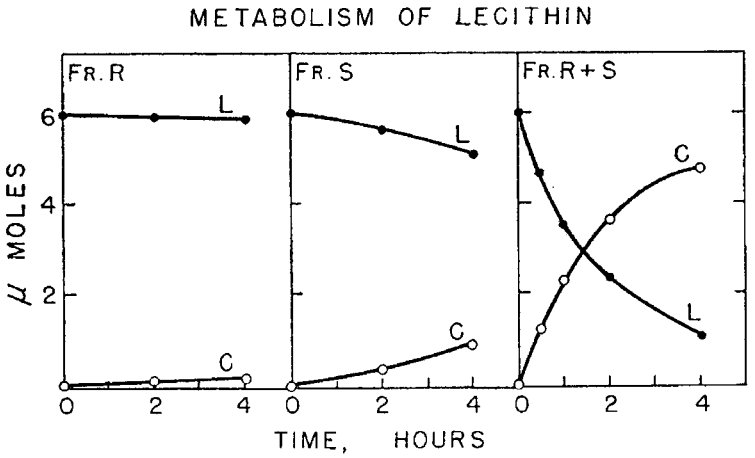

FIG. 1. Lecithin metabolism. The incubation mixtures contained 0.6 ml. of lecithin (0.01 M), 0.3 ml. of 0.5 M acetate buffer, pH 5.6, and 0.1 ml. of Fraction S (1.3 mg. of protein) or 0.1 ml. of Fraction R (0.7 mg. of protein), as indicated, in a final volume of 2.4 ml. The incubation was at 26°. *L* = lecithin; *C* = choline. No lysolecithin or GPC was detected throughout the incubation period.

liberation of free choline and led to the accumulation of GPC (Table II). The presence of Mg^{++}, which inhibits the breakdown of GPC (see below), further depressed the formation of free choline by the heated preparation (Table II).

Attempts to demonstrate the formation of a lysolecithin as an intermediate in the conversion of lecithin to GPC did not succeed, presumably as a result of the relatively high activity of lysolecithinase. Unlike a lysolecithinase of animal origin (22), which was rather heat-labile as compared with the accompanying lecithinase, the bacterial enzymes were both heat-stable. Heating of the bacterial homogenate at 100° for 10 minutes at pH 7.0 caused only a 50 to 80 per cent inactivation of both lysolecithinase and lecithinase.

Lysolecithin Breakdown—When lysolecithin was incubated with either Fraction S or Fractions R and S, a rapid release of free choline was observed

without any appreciable accumulation of GPC (Fig. 2). Incubation with Fraction R alone, however, resulted in only a slight liberation of free choline, although the rate of lysolecithin removal was undiminished. GPC accumulated instead of choline to an extent approximating the lysolecithin that had disappeared (Fig. 2). As in the case of lecithin breakdown, heat-

TABLE II

Accumulation of GPC As Intermediate in Lecithin Breakdown

The incubation mixtures contained 1 μM of lecithin, 25 μM of acetate buffer (pH 5.7), and enzyme (0.6 mg. of protein) in a total volume of 0.4 ml. The incubation was for 2 hours at 25°.

Substrate	Enzyme preparation		
	Homogenate	Heated homogenate	Heated homogenate + 0.04 M Mg^{++}
	μM	μM	μM
Choline	0.575	0.128	0.032
GPC	0.000	0.457	0.506

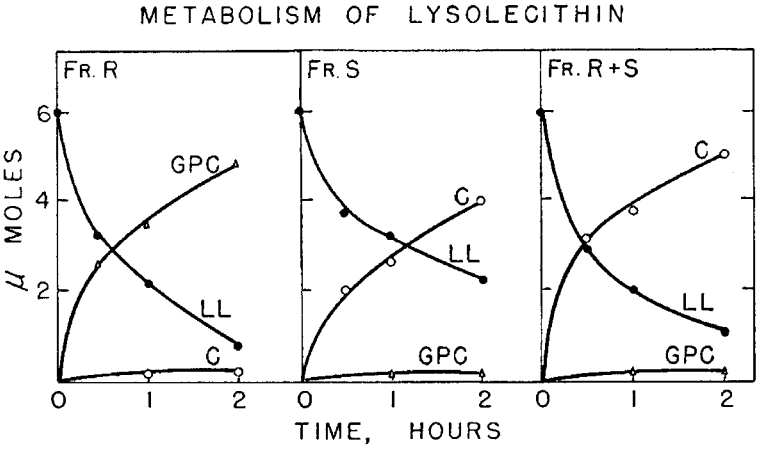

FIG. 2. Lysolecithin metabolism. The conditions were the same as those described in Fig. 1, except that 0.6 ml. of lysolecithin (0.01 M) was used instead of lecithin. *LL* = lysolecithin; *C* = choline.

ing of Fraction S (at 100° for 5 minutes) also limited the reaction to the formation of GPC. Thus with 0.1 ml. of *unheated* Fraction S (incubated with 1 μM of lysolecithin and 5 μM of glycylglycine buffer, pH 8.5, in a volume of 0.4 ml. for 2 hours at 26°), 0.442 and 0.018 μM of choline and GPC respectively, were formed; with *heated* Fraction S, 0.025 and 0.461 μM of choline and GPC, respectively, were produced.

Specificity of Reaction—Since both Fractions R and S were required to initiate lecithin degradation, but either Fraction R or S alone degraded

lysolecithin, it might be assumed that the liberation of an unsaturated fatty acid from lecithin was more complicated than the release of a saturated fatty acid. However, this assumption is not supported by the observation that dipalmitoleyllecithin, which has two unsaturated fatty acids, was readily attacked by either Fraction R or S alone; when both fractions were combined, the activity was merely additive (Table III). Dimyristoyllecithin was attacked by Fraction S but only slightly by Fraction R.

Crude animal lecithin was metabolized either by Fraction S or R, although at a somewhat slower rate than was egg lecithin, and the combined activities of Fractions S and R were almost additive.

TABLE III

Specificity of Lecithinase

The reaction mixtures contained 1 μM of each substrate, 5 μM of acetate buffer (pH 5.7), and 0.15 ml. of Fraction S or 0.15 ml. of Fraction R, as indicated, in a total volume of 0.4 ml. The incubation was carried out at 26° for 2 hours. The values represent the sum of free and acid-labile choline or ethanolamine and are expressed as micromoles.

Substrate	Enzyme preparation		
	Fraction R	Fraction S	Fractions S + R
Lecithin (egg)	0.021	0.120	0.582
Dipalmitoleyllecithin	0.502	0.296	0.760
Dimyristoyllecithin	0.001	0.104	0.139
Lecithin (animal)	0.275	0.168	0.390
Cephalin "			0.037
Dimyristoylcephalin			0.176
Sphingomyelin			0.000

Ethanolamine was shown to be liberated from cephalin, although at a much slower rate when crude animal cephalin was used as a substrate. However, when dimyristoyllecithin and dimyristoylcephalin were compared, the amounts of choline and ethanolamine released were of about the same order of magnitude.

Under the test conditions no free choline was released from phosphorylcholine and sphingomyelin. Attempts to adapt this organism by growing it in the presence of sphingomyelin were unsuccessful; the growth was poor and there was no liberation of free choline into the medium.

Cardiolipin was not metabolized by the whole bacterial homogenate. Cleavage of the diester linkages would have released phosphate monoester groups which, in the presence of added monoesterase (from human semen),[4]

[4] Human semen was fractionated with ammonium sulfate, and a fraction collected between 0.5 and 0.8 saturation was used after dialysis.

should have resulted in the appearance of inorganic phosphate, but none was detectable.

Purification and Metal Requirements of Lecithinase—Fraction R was made soluble by butanol treatment according to Morton (23). To 5 ml. of the chilled suspension was added 0.5 ml. of *n*-butanol with constant mechanical stirring. After 5 minutes at 0°, the mixture was centrifuged in a Servall angle centrifuge (SS-1) at 13,000 r.p.m. for 30 minutes. Almost 100 per cent of the activity remained in the supernatant fluid if Ca^{++} was added to the incubation mixture; prior to butanol treatment, the activity was completely sedimented under these conditions. The supernatant fluid was dialyzed against 0.01 M K_2HPO_4 at 3° for 4 to 6 hours (until the odor of butanol was no longer detectable). Excessive dialysis led to inactiva-

TABLE IV

Effect of Ca^{++} on Lecithinase

The assay was carried out as described under "Methods" except that GPC diesterase was omitted. The protein content of Fraction R before butanol treatment was 1.7 mg. per ml.; after butanol treatment the preparation contained 0.62 mg. per ml., and after dialysis 0.52 mg. per ml.

Steps	Choline liberation,* μM	
	No Ca^{++}	0.01 M Ca^{++}
Fraction R	0.083	0.138
Butanol treatment	0.044	0.142
Dialysis	0.003	0.095

* The values represent the sum of free and acid-labile choline.

tion of the enzyme. This preparation contained 80 to 100 per cent of the original activity and only 25 to 30 per cent of the protein. (Additional purification (2- to 4-fold) was obtained by fractionation between ammonium sulfate saturations of 30 and 50 per cent; but this latter step was not used routinely because yields of less than 30 per cent were obtained when the removal of butanol in the previous step was incomplete.) An almost obligatory requirement for Ca^{++} was observed at this stage (Table IV). The saturation level was reached with 0.01 M Ca^{++}, and inhibitory effects were observed at concentrations above 0.02 M. Mg^{++} and Mn^{++} had no demonstrable effect.

Fraction S was purified by adsorption on calcium phosphate gel and elution with 0.5 M dipotassium phosphate. After such treatment, the enzyme was observed to lose activity by passage through a Dowex 50 (K^+) column, while treatment with Dowex 1 (formate) resulted in little loss of activity, although most of the protein was removed.

On the basis of these observations, various metal ions were tested. Fe^{++} was found to be the most effective in substituting for Fraction S (Table V), while Fe^{+++} was inactive. The saturation level of Fe^{++} was at about 0.01 M. Unlike the action of untreated Fraction S, which (in the presence of Fraction R) can lead to the complete degradation of the lecithin substrate, the action of either gel-treated Fraction S or Fe^{++} (in the presence of Fraction R) ceased after only 20 to 30 per cent of the substrate was removed, although the rate of the reaction is comparable to or even greater than the rate with Fraction S.

TABLE V

Effect of Various Metal Ions on Lecithinase Action of Fraction R

The test system contained 0.5 μM of lecithin, 25 μM of glycylglycine buffer (pH 7.4), 5 μM of $CaCl_2$, 0.1 ml. of Fraction R, and 5 μM of metal ions, as indicated, in a total volume of 0.5 ml. The incubation was carried out for 1 hour at 38°. With 0.1 ml. of Fraction S, 0.096 μM of choline was liberated under the same conditions.

Salt	Choline liberated,* μM	Salt	Choline liberated,* μM
None	0.024	$Fe(NH_4)_2(SO_4)_2$	0.140
$MgCl_2$	0.043	$FeCl_2$	0.145
$MnCl_2$	0.048	$FeCl_3$	0.017
$CuCl_2$	0.078	$CoCl_2$	0.046
$FeNH_4(SO_4)_2$	0.032	$ZnCl_2$	0.036

* The values represent the sum of free and acid-labile choline.

Metabolism of GPC

Purification of GPC Diesterase—To each 100 ml. of the crude cell-free extract (homogenate) were added 31.5 gm. of $(NH_4)_2SO_4$. After 30 minutes at 0°, the precipitate was centrifuged and 30 gm. of $(NH_4)_2SO_4$ were added to the supernatant fluid. The precipitate was collected by centrifugation and dissolved in 50 ml. of glycylglycine buffer (0.02 M, pH 9.0) (Ammonium Sulfate I, Table VI). A 2-fold increase in activity at this stage was probably due to the removal of metallic inhibitors in the crude extract.

To each 50 ml. of Ammonium Sulfate I were added 18.2 gm. of $(NH_4)_2SO_4$. The precipitate was centrifuged and to the supernatant fluid were added 6.5 gm. of $(NH_4)_2SO_4$. The precipitate, collected by centrifugation, was dissolved in 10 ml. of glycylglycine buffer (0.04 M, pH 9.0) (Ammonium Sulfate II, Table VI).

To 9 ml. of Ammonium Sulfate II in a 0° bath were added 1 ml. of 1 M sodium acetate and then 6.5 ml. of acetone (−10°) dropwise with mechanical stirring. After standing for 5 minutes, the precipitate was removed by

centrifugation, and 4 ml. of acetone were added to the supernatant fluid. After 5 minutes at 0°, the precipitate was collected by centrifugation and dissolved in 4 ml. of 0.01 M K_2HPO_4 (acetone, Table VI).

Stability of Enzyme—The purified preparations were stored at −10° for at least 6 months without any appreciable loss of activity. Heating the

TABLE VI

Purification of GPC Diesterase

Fraction	Total activity	Specific activity
	units	*units per mg. protein*
Crude extract	4,500	24
Ammonium Sulfate I	10,200	437
" " II	5,000	1135
Acetone	3,850	5600

TABLE VII

Balance Study of GPC Hydrolysis

The incubation mixture contained 100 μM of GPC, 100 μM of glycylglycine buffer (pH 8.85), and 0.12 mg. of enzyme protein (1100 units per mg. of protein) in 2.0 ml. of water. The incubation was at 26° for 2 hours. The values are expressed in micromoles.

Hrs.	Choline		Periodate titration	α-Glycerophosphate*	L-α-Glycerophosphate†
	Iodine complex	Reineckate			
0	0	0	97.0	93.6	0
2	101	93	97.0	94.0	105
Δ	+101	+93	0.0	+0.4	+105

* Burmaster method which involves the estimation of acid-labile phosphate (1 hour in 1 N HCl at 100°) after periodate oxidation.

† Assay with L-α-glycerophosphate dehydrogenase.

enzyme preparations at pH 9.0 for 5 minutes at 50°, 75°, or 100° destroyed 15, 95, or 100 per cent of the activity, respectively.

Products of GPC Breakdown—The enzymatic breakdown of GPC resulted in the production of L-α-glycerophosphate and choline in equimolar amounts equivalent to the GPC metabolized (Table VII). Choline determined by two different methods yielded similar results. The values obtained by periodate titration and Burmaster's method, in which α-GP and GPC cannot be differentiated, showed no change. Specific enzymatic assay of L-α-GP indicated reasonable agreement with the theoretical value.

It is significant that the enzymatic hydrolysis of lecithin, unlike alkaline hydrolysis, yields only the α form of GP.

Substrate Specificity of GPC Diesterase—GPE was the only substrate besides GPC among a group of choline-containing phosphate diesters which was split by the enzyme preparation at a rate comparable with the splitting of GPC. Under test conditions, which resulted in the release of 1 μM of choline from GPC in 60 minutes, there was no detectable choline release from purified egg lecithin, lysolecithin, animal lecithin, sphingomyelin,

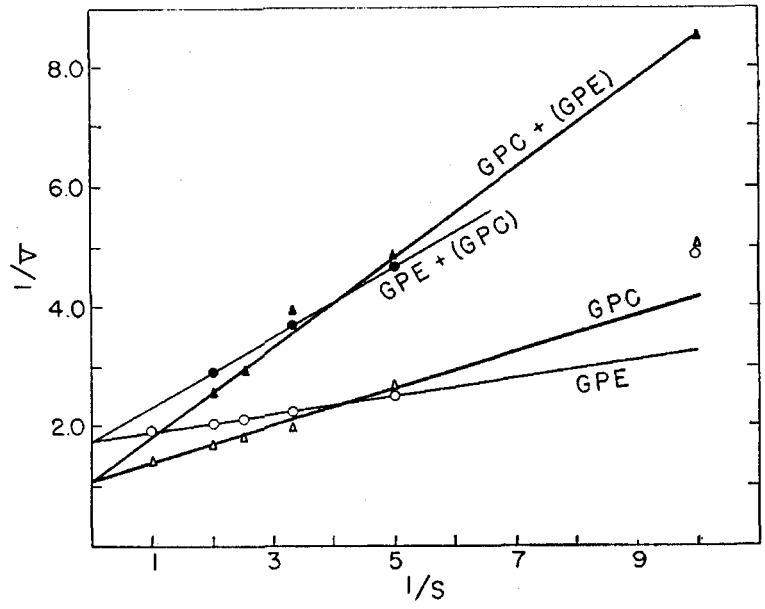

FIG. 3. Influence of substrate concentration (S) on rate of reaction ($\overline{V}$), with and without competitive inhibitors. The incubation mixtures contained 0.1 ml. of 0.2 M glycylglycine buffer, pH 8.9, 0.1 ml. of GPC diesterase (ammonium sulfate Fraction II, 12.4 γ of protein), 2 μM of inhibitors and substrates, as indicated, in a total volume of 0.4 ml. The incubation was carried out at 26° for 10 minutes.

dipalmitoleyllecithin, or dimyristoyllecithin in a 120 minute incubation period.

Non-choline-containing phosphate diesters, except GPE, did not appear to be attacked. Diphenyl phosphate released neither phenol nor phosphate monoester groups (determined by subsequent treatment with human semen phosphatase) in the presence or absence of Mg^{++} (0.01 M) at pH 8.9. Diphenyl phosphate (0.005 M) inhibited GPC splitting by 15 per cent. Splitting of yeast ribonucleic acid and of polynucleotides resistant to ribonuclease action was tested by the method of Kunitz (24).[5] Less than 1 per cent of the rate of GPC splitting was found with either substrate in the presence or absence of Mg^{++} (0.03 M).

[5] These tests were carried out in collaboration with Dr. L. A. Heppel.

A slight contamination with monoesterase activity was present in the purified preparations. Only trace amounts of inorganic phosphate were released from α-GP (at the rate of 0.2 per cent of the rate of GPC splitting). Adenosine-5-phosphate was hydrolyzed at about 3 per cent of the rate of GPC splitting; however, this activity, in contrast to that of GPC diesterase (see below), was not inhibited by Mg^{++} (0.01 M) but rather increased 2-fold.

Influence of Substrate Concentration; Identity of GPC and GPE Diesterase —Lineweaver-Burk plots (25) of the hydrolysis rates at varying GPC and GPE concentrations are shown in Fig. 3. From these curves Michaelis

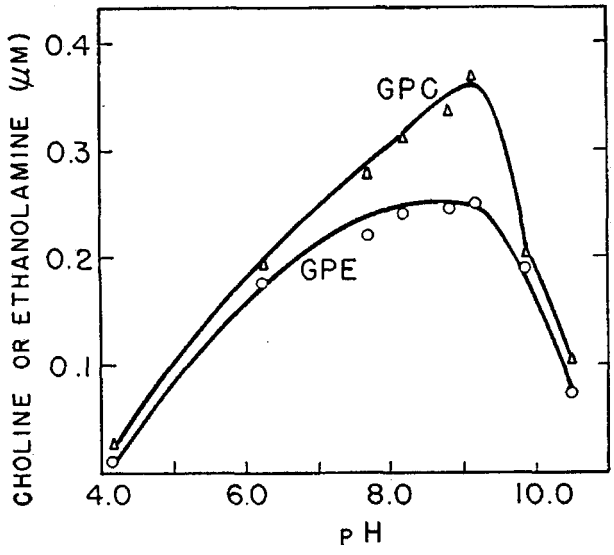

FIG. 4. Rate of enzymatic hydrolysis of GPC and GPE as a function of pH. The standard assay conditions were employed with 0.05 ml. of ammonium sulfate Fraction II containing 6.22 γ of protein. The buffers employed (0.05 M) were acetate (pH 4.2), phosphate (pH 6.2), glycylglycine (pH 7.7, 8.2, 8.9) and glycine (pH 9.2, 9.8, 10.5).

constants of 1.2×10^{-3} and 2.5×10^{-3} M were calculated for GPE and GPC, respectively. GPE and GPC were observed to be competitive inhibitors of GPC and GPE splitting, respectively. The dissociation constants of the inhibitor-enzyme complex[6] were calculated to be 2.4×10^{-3} M for GPE and 1.8×10^{-3} M for GPC. These results are taken as an indication that a single enzyme is responsible for the hydrolysis of GPC and GPE.

Influence of pH and Metal Ions—Maximal activity for the splitting of

[6] $K_I = (K_S(I/K_S + S))/((v - v_I)v_I)$. K_S, dissociation constant of enzyme-substrate complex; K_I, dissociation constant of enzyme-inhibitor complex; S, concentration of substrate; I, concentration of inhibitor; v, velocity; v_I, velocity in the presence of inhibitor.

both GPC and GPE was observed in the range pH 8 to 9 (Fig. 4). At a concentration of 0.001 M, Mg^{++} and Mn^{++} inhibited GPC splitting by more than 90 per cent and Zn^{++} inhibited the rate by about 60 per cent. Ethylenediaminetetraacetate (0.0001 M) inhibited GPC splitting by 91 per cent, but this inhibition could not be overcome by any metals tested nor did prolonged dialysis against water reduce the activity of the enzyme.

DISCUSSION

These results demonstrate that GPC is an intermediate in the release of choline from lecithin and lysolecithin. While proof is lacking that lysolecithin is the intermediate in the conversion of lecithin to GPC, this is strongly suggested by the 14-fold greater lysolecithinase activity of lecithin-grown cells than of broth-grown cells. The very high content of lysolecithinase activity in these extracts relative to their lecithinase content is adequate to explain the failure to accumulate lysolecithin. Monsour and Colmer (26) recently reported the formation of choline from egg yolk by a number of strains belonging to the genus *Serratia*. They attributed the reaction to a lecithinase C activity by which, as demonstrated with certain plant tissues (27), choline is split directly from lecithin to yield phosphatidic acid as the other product. Our study of lecithin metabolism in *S. plymuthicum* suggests another interpretation of Monsour and Colmer's findings.

From an investigation of the mechanism of the lecithinase action of pancreas preparations, Shapiro (28) concluded that the primary reaction was a transfer rather than hydrolysis of a fatty acid ester linkage. While the over-all result observed in our bacterial system is the hydrolysis of both fatty acid ester bonds, it has not been excluded that a transfer reaction involving an unstable intermediate is involved. Further purification of the bacterial lecithinase is needed to resolve this question.

It has generally been assumed that the action of lecithinase A is to split only the ester linkage with the unsaturated fatty acid (2). From the present investigation, it is apparent that a fully saturated lecithin (dimyristoyllecithin) and an unsaturated one (dipalmitoleyllecithin) are both attacked at comparable rates. Although it cannot be inferred from these results that a single enzyme is responsible for these several activities, it is nevertheless clear that such activities do exist. With the availability of these highly purified and well characterized lecithins, it should become possible to establish whether the α- or β-fatty acid ester linkage is the primary and exclusive site of attack.

Belief in the existence of naturally occurring β-lecithin (which yields β-glycerophosphate on chemical hydrolysis) has been virtually abandoned as the result of the work of a number of investigators. Particularly note-

worthy are the recent contributions of Baer and Kates (29) demonstrating that the synthetic α-lecithins also yield β-glycerophosphate under the conditions employed for the hydrolysis of naturally occurring lecithin. The present work adds further confirmation by specifically identifying L-α-glycerophosphate as the exclusive product of the bacterial metabolism of egg lecithin.

SUMMARY

1. The metabolism of certain phospholipides has been studied with enzyme preparations from a strain of *Serratia plymuthicum*. This organism was isolated by enrichment culture on a medium containing glycerophosphorylcholine (GPC) and was routinely grown on a lecithin-yeast extract medium.

2. Extracts from cells cultured on lecithin released free choline from lecithin, lysolecithin, and GPC at a rate 5 or more times greater than that obtained with extracts from broth-grown cells.

3. Heat-treated extracts split lecithin and lysolecithin with the accumulation of GPC. Fractionation of the extract by high speed centrifugation yielded residue (R) and supernatant (S) fractions, both of which were essential for the splitting of lecithin. Fraction R was made soluble by butanol treatment, partially purified, and shown to require Ca^{++}. Fraction S was replaced by Fe^{++} to a limited extent. Lysolecithinase activity was present in both Fractions R and S. The action of these enzyme preparations on saturated and unsaturated lecithin, cephalin, and other related compounds has been studied.

4. An enzyme hydrolyzing GPC to L-α-glycerophosphate and choline, named GPC diesterase, has been partially purified. Kinetic data indicate that glycerophosphorylethanolamine (GPE) is also a substrate for this enzyme.

5. These results suggest the following pathway for the bacterial metabolism of lecithin (or cephalin): Lecithin and lysolecithin are converted to GPC, which is then split into L-α-GP plus choline. Cephalin is metabolized to L-α-GP and ethanolamine by way of GPE.

BIBLIOGRAPHY

1. Hayaishi, O., *Federation Proc.*, **12**, 216 (1953).
2. Zeller, E. A., in Sumner, J. B., and Myrbäck, K., The enzymes, **1**, pt. 2, 986 (1951).
3. Schmidt, G., Hecht, L., Fallot, P., Greenbaum, L., and Thannhauser, S. J., *J. Biol. Chem.*, **197**, 601 (1952).
4. Pangborn, M. C., *J. Biol. Chem.*, **188**, 471 (1951).
5. Iwata, M., *Biochem. Z.*, **224**, 430 (1930).
6. Fairbairn, D., *J. Biol. Chem.*, **157**, 633 (1945).
7. Schmidt, G., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 523 (1945).

8. Bailly, M. C., *Compt. rend. Acad.*, **206**, 1902 (1938).
9. Green, D. E., *Biochem. J.*, **30**, 629 (1936).
10. Appleton, H. D., Levy, B. B., Steele, J. M., and Brodie, B. B., *Federation Proc.*, **9**, 146 (1950).
11. Glick, D., *J. Biol. Chem.*, **156**, 643 (1944).
12. Bernheimer, A. W., *J. Gen. Physiol.*, **30**, 337 (1947).
13. Collier, H. B., *J. Gen. Physiol.*, **35**, 617 (1952).
14. Schmidt, G., Hecht, L., Strickler, N., and Thannhauser, S. J., *Federation Proc.*, **8**, 249 (1949).
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
16. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
17. Jackson, E. L., in Adams, R., Organic reactions, New York, **2**, 341 (1944).
18. Burmaster, C. F., *J. Biol. Chem.*, **164**, 233 (1946).
19. Hayaishi, O., and Kornberg, A., *J. Biol. Chem.*, **197**, 717 (1952).
20. Breed, R. S., Murray, E. G. D., and Hitchens, P., Bergey's manual of determinative bacteriology, Baltimore, 6th edition (1948).
21. McIlwain, H., *J. Gen. Microbiol.*, **2**, 288 (1948).
22. Noguchi, S., *J. Biochem.*, Japan, **36**, 113 (1944).
23. Morton, R. K., *Nature*, **166**, 1092 (1950).
24. Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).
25. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
26. Monsour, V., and Colmer, A. R., *J. Bact.*, **63**, 597 (1952).
27. Hanahan, D. J., and Chaikoff, I. L., *J. Biol. Chem.*, **169**, 699 (1947); **172**, 191 (1948).
28. Shapiro, B., *Nature*, **169**, 29 (1952).
29. Baer, E., and Kates, M., *J. Biol. Chem.*, **175**, 79 (1948); **185**, 615 (1950).