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Lipids of *Salmonella typhimurium* and *Escherichia coli*: Structure and Metabolism

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The nature and quantity of the phospholipids of *Salmonella typhimurium* and *Escherichia coli* K-12 have been examined. The main classes of phospholipids, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin have been completely characterized. Four minor compounds have been detected: phosphatidylserine, phosphatidic acid, and two partially characterized lipids. The phospholipid composition of the two organisms is quite similar, the only difference is the absence of one of the minor components and a decreased level of all components in *E. coli*. A study of the turnover of the phosphate in the phospholipids demonstrated no turnover in phosphatidylethanolamine, a slow turnover in phosphatidylglycerol, and a slow turnover in cardiolipin with, possibly, a transfer of phosphate from phosphatidylglycerol to cardiolipin. The amino acid phenylalanine is shown to become incorporated intact into lipidic compounds which have been partially characterized. Methods for the isolation and separation of lipids have been examined for their utility with these bacteria.

Salmonella typhimurium and *Escherichia coli* K-12 have been studied extensively in terms of genetics and biochemical genetics. However, a biochemical genetic approach, which has been so successful in other areas of metabolism, has rarely been applied to the study of lipids or of the role of lipids in active transport. Part of the difficulty has been the scarcity of biochemical data available on the lipids of these gram-negative bacteria (24).

The present paper describes the lipids of *S. typhimurium* and *E. coli* and the methods for their characterization. The identification, quantities, and phosphorus turnover of the major classes of phospholipids of *S. typhimurium* are presented. It is hoped that these results will be of use as a basis for future biochemical genetics studies.

In connection with a study of active transport of phenylalanine in *S. typhimurium*, labeled phenylalanine is shown to be incorporated into lipidic compounds. It is not clear whether these lipoamino acids are involved in active transport or whether they participate in some other metabolic function.

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MATERIALS AND METHODS

Growth of bacteria. *S. typhimurium*, wild type (strain LT-2), and *E. coli*, K-12 W3101 (gal$^-$), were grown at 37 C in medium E, which is 0.074 M in phosphate (44), with glucose (0.4%) as carbon source, in a New Brunswick rotary shaker. The optical density of the cultures and the dry-weight contents were assayed as described (2).

Extraction of lipids. The procedure was as described by Bligh and Dyer (3) for fish. This method was compared with other extracting and washing procedures reported in the literature (7, 14, 22) and was found more convenient than any of them. It is much less time-consuming and gives a better yield of extracted lipid (either because of better extraction or because of decreased loss by degradation). It is easily applied to samples of any volume and density. The method consists of an extraction of the lipids in a monophasic system in which methanol, chloroform, and water (i.e., bacterial culture) are in the proportions 2:1:0.8 (v/v). If a very dense suspension of cells is used, a correction must be made (1 g of wet weight of cells contains 0.8 ml of water). The lipids are separated from the water-soluble material by diluting the extraction mixture with 1 volume of chloroform followed by 1 volume of water. After centrifugation, the chloroform layer is removed completely by gently inserting a pipette through the water-methanol phase and through the pellet which forms at

the interphase. The extractions were performed at 0 C for 10 min. Chloroform layers so obtained were evaporated to dryness in a rotary evaporator at 10 C or less. The dry lipid was dissolved in a small volume of chloroform and stored at −20 C under nitrogen.

This procedure extracted 95% of the lipid and had no preferential extraction properties. This was demonstrated on a radioactive preparation by adding enough chloroform to the residue (pellet plus water-methanol layer) to create a monophasic system, then heating the tube at 55 C for 10 min. The second chloroform layer so obtained contained only 5% of the total lipid extracted by both extractions and all lipids appeared to be present in the same proportions. Lowering the *p*H of the water phase to about *p*H 4.0 during extraction, made no difference on the pattern of lipids extracted.

Thin-layer chromatography of lipids. The extracted lipids were separated by thin-layer chromatography (TLC) on commercially available plates (Mann) coated with Silica Gel G (containing $CaSO_4$ binder). The plates were developed in acetone shortly before use and then dried. No heat activation was used. The chromatographic tanks were lined with filter paper which was thoroughly impregnated with the freshly made solvent immediately before starting development. Approximately 1 mg of lipid could be applied and chromatographed satisfactorily. Development was in the following solvents, either mono- or two-dimensionally. (When a two-dimensional system was used, the plates were allowed to dry at room temperature for about 30 min, after development in the first direction, with a blast of cold air over the surface.) Solvent 1. Chloroform-methanol-water (65:25:4, v/v; reference 45). Solvent 2. Chloroform-methanol-7 N ammonia (60:35:5, v/v; reference 36). Solvent 3. Chloroform-methanol-concentrated ammonia (70:21:1, v/v; reference 4). Solvent 4. Chloroform-methanol-glacial acetic acid (65:25:8, v/v; reference 36). The R_F of the major lipids in each of the above systems is shown in Table 1 and their formulas in Fig. 1.

TABLE 1. *R_F values of lipids in thin-layer chromatography*

Lipid	Solvent system			
	1	2	3	4
Phosphatidylethanolamine	0.40	0.38	0.14	0.35
Phosphatidylglycerol	0.32, 0.50[a]	0.52	0.20	0.56
Cardiolipin	0.68	0.55	0.24	0.95
Phosphatidylserine	—	0.15	—	0.17
PG-X	0.59	0.75	0.48	0.90
Phosphatidic acid	—	0.08	—	0.58
Y	0.44	0.68	0.39	0.78
Lipo-amino acid A	0.45	0.73	0.32	0.67
Lipo-amino acid B	0.80	0.93	0.74	0.41

[a] Phosphatidylglycerol gives two spots in this solvent (presumably two different salt forms), unless previously run in an acidic or basic system (as in Fig. 4).

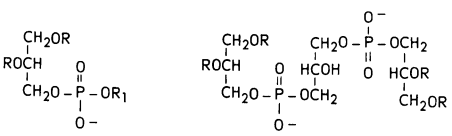

FIG. 1. *Formulas of phospholipids.*

Lipids were unspecifically visualized with iodine vapors.

Amino groups were identified with a ninhydrin spray (1 g of ninhydrin in 600 ml of absolute ethyl alcohol, 200 ml of acetic acid, 80 ml of γ-collidine. This solution can be stored for a few months in a freezer).

The presence of organic phosphate was detected with the phosphate spray reagent described by Dittmer and Lester (12).

The following sequence could be used on the same plate: iodine vapors, ninhydrin, phosphate reagent.

The presence of vicinal glycols was detected by periodate oxidation followed by the acetylacetone reagent described by Schwartz (40). This reagent was found more satisfactory than a periodate-Schiff spray (34) if the plates were examined not later than a few hours from the time of spraying. At later times, lipid classes with no vicinal glycols also gave a positive reaction. This spray was also used for the identification of the hydrolysis products on paper chromatograms.

A permanent record of the stained chromatograms was obtained by the blueprint method of Eisenberg (13), which gives a mirror image. For publication, in order to give a true copy of the original TLC, a mirror image of the blueprint has been reproduced.

For elution and further analysis of lipids visualized with iodine vapors, the regions of interest were scraped off the plate with a spatula and the silicic acid placed in a Pasteur pipette obstructed with glass wool. Lipids were eluted slowly from the small columns thus obtained, with 2 ml of chloroform-methanol (1:1, v/v), followed by 2 ml of methanol.

Autoradiograms were obtained by placing Kodak X-ray film (Blue Sensitive, single coated) over the TLC plate. About 3 mμc of ^{14}C (i.e., 2,000 counts/min in a gas flow counter) can be easily detected by overnight exposure. Quantitative estimation of radioactivity was obtained by scraping the zone of interest off the plate with a spatula, placing the powder in Bray's solution (5), and counting it in a scintillation counter.

Column chromatography. Column separation was performed at 0 C on activated silicic acid (Unisil, 200 to 325 mesh, Clarkson Chemical Co., Williamsport, Pa.) by use of a continuous gradient of methanol in chloroform. The silicic acid was washed in methanol, followed by chloroform with which the column was equilibrated. The apparatus used was the column described by Kredich and Ames (27). Only

Teflon tubing was used; no grease was employed in any portion of the column. The gradient was created by placing chloroform in a tightly stoppered mixing vessel and methanol-chloroform (1:1, v/v) in the reservoir vessel and simply connecting them at the bottom. The volume of fluid in the mixing vessel remains constant; therefore, the gradient obtained has a convex shape as indicated by the equation presented by Cherkin, Martinez, and Dunn (8). The proportion of methanol in chloroform can be adjusted, if desired, so as to make use of the first portion of the curve only, which is approximately linear. Also, the concentration of methanol in chloroform at which a certain lipid begins to be eluted can be calculated from that equation. The columns used usually had a ratio of diameter to length of approximately 50. The flow rate of eluant varied with the size of the column; it was adjusted to approximately 2.0 ml per min in a 100-cm long column. The fractions were analyzed by TLC.

Preparation of Ba salt of cardiolipin. Following the procedure of Pangborn (37), the acetone-insoluble lipids were dissolved at room temperature in methanol-chloroform (9:1, v/v), and an aqueous solution of 20% $BaCl_2$ was added to give a 2% final concentration of $BaCl_2$. The precipitate formed was collected by centrifugation at room temperature, redissolved in methanol-chloroform (9:1, v/v), and precipitated a second time with $BaCl_2$. The Ba salt of cardiolipin was placed on a column of Dowex 50, K^+ form, which had been washed exhaustively with methanol and then chloroform, and was eluted with methanol-chloroform (9:1). It was assumed that all the cardiolipin eluted from the column was in the K salt form. Only a qualitative assay was done for the presence of Ba in the HCl eluate of the Dowex 50 after passage of the Ba salt of cardiolipin. The behavior of the isolated cardiolipin on TLC was unaltered when compared with that of cardiolipin in crude lipid extracts.

Analytical methods. Total phosphate was assayed by the method of Ames (1).

The presence of vicinal glycols in intact lipids was assayed by periodate oxidation and assay of the formaldehyde liberated. The periodate oxidation was performed as follows: the lipid (about 0.01 to 0.1 μmole) was dissolved in 50 μliters of chloroform, and 25 μliters of a periodic acid solution (0.02 M in chloroform-95% acetic acid, 1:1, v/v) was added. The mixture was kept at room temperature for 5 min. The formaldehyde formed was assayed as follows (26): 5 μliters of 1.0 M Na arsenite and 25 μliters of 1 N H_2SO_4 were added; after 5 min at room temperature, 1.0 ml of chromotropic acid was added and the tube was heated at 100 C for 10 min. The color, which is stable for at least 5 hr, was read at 570 mμ. Glycerol, which liberates 2 μmoles of formaldehyde per μmole, was used as standard.

Vicinal glycols appearing after mild alkaline hydrolysis (10) were detected as follows. The lipid (approximately 0.02 μmole) was hydrolyzed by dissolving it in 20 μliters of chloroform and 25 μliters of ethyl alcohol; 5 μliters of 0.5 N NaOH in 95% ethyl alcohol was then added, and the mixture was kept at room temperature for 5 min. The vicinal glycols thus formed were assayed by adding to the above mixture 100 μliters of 0.2 M Na acetate buffer (*p*H 4.8) and then 10 μliters of 0.05 M Na periodate. After 10 min at room temperature, the formaldehyde formed was detected by the chromotropic acid assay as described for unhydrolyzed lipids. Ethyl alcohol is used instead of methanol during the hydrolysis, because methanol interferes with the chromotropic acid assay.

For structure identification of the lipids, mild alkaline hydrolysis was performed as described above. The volumes of reagents were increased as necessary. After 10 min at room temperature, 1.0 ml of water was added for each 0.5 ml of mixture. Carrier deacylated lipids were added when necessary. The mixture was then neutralized by filtering it on a column made from a Pasteur pipette stoppered with glass wool and containing about 50 mg of Dowex 50 W-X8 (5 meq/g), in the acid form, for each 500 μliters of mixture. The water layer was separated, a drop of 1 N ammonia was added to it, and it was lyophilized. The water-soluble products thus obtained were analyzed by paper chromatography.

Paper chromatography was performed on Whatman no. 1 paper and was by the ascending technique. The solvents commonly used were: (A) isopropanol-ammonia-water (7:1:2, v/v/v; reference 38); (B) methanol-98% formic acid-water (80:13:7, v/v/v; reference 10); (C) butanol-propionic acid-water (71:36:50, v/v/v; reference 35); and (D) phenol saturated with water-acetic acid-ethyl alcohol (10:1:1.2, v/v/v; reference 10).

Most water-soluble products were identified by paper chromatography in at least two of the above solvents. Visualization was with ninhydrin or with periodate oxidation and the acetylacetone spray (40) for vicinal glycols (which was found preferable to the available sprays for the identification of organic phosphate). Autoradiography was used for chromatograms of labeled products.

Table 2 shows the R_F values obtained for the water-soluble hydrolysis products in each of the solvent systems. System A was preferred when possible, because system B caused some breakdown of glycerolphosphorylglycerol and di(glycerolphosphoryl)glycerol. The acid lability of these compounds has been mentioned in the literature (6).

TABLE 2. *R_F values of the hydrolysis products obtained by mild alkaline hydrolysis of phospholipids*

Hydrolysis product	Solvent system			
	A	B	C	D
Glycerolphosphorylethanolamine	0.44	0.46	0.23	0.63
Glycerolphosphorylserine	—	—	—	0.24
Glycerolphosphorylglycerol	0.44	0.76	0.10	0.29
Di(glycerolphosphoryl)glycerol	0.27	0.70	0.10	0.29
α-Glycerophosphate	0.14	0.72	0.16	0.31
Deacylated lipid Y	0.57	—	0.24	—

The dinitrophenyl derivative of lipo-amino acid A was obtained as described by Macfarlane (31).

Chemicals. Glycerolphosphorylethanolamine, glycerolphosphorylserine, and di(glycerolphosphoryl)-glycerol were obtained by mild alkaline hydrolysis of commercial phosphatidylethanolamine, phosphatidylserine, and cardiolipin, respectively. Glycerolphosphorylglycerol was kindly supplied by E. P. Kennedy. DL, α-Glycerolphosphate and phosphatidylserine were from Mann Fine Chemicals, Inc., New York, N. Y. Phosphatidylethanolamine (dipalmitate) and snake venom (*Crotalus terrificus terrificus*) were from Sigma Chemical Co., St. Louis, Mo. Beef heart cardiolipin was from Nutritional Biochemicals Corp., Cleveland, Ohio. Pure phosphatidic acid was generously supplied by D. B. Berkowitz. Pancreatic lipase was from Worthington Biochemical Corp., Freehold, N.J. Palmitoylphenylalanine was synthesized as described by Hendler (16). ^{14}C-L-serine (120 mc/mmole) was obtained from Schwarz Laboratories, Inc., Mt. Vernon, N.Y. Carrier-free ^{32}P-orthophosphate was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Lipid components of Salmonella typhimurium. The total lipid extracted by the Bligh-Dyer procedure amounts to 6.3% of the dry weight of the bacteria. The neutral lipids are separated from the phospholipids by precipitating the phospholipids with cold acetone. Each fraction contained less than 5% of the other fraction. The acetone-soluble lipids represent 10.4% of the total lipids (or 0.66% of the dry weight of the bacteria). The phospholipids are 89.6% of the total lipids (or 5.7% of the dry weight of the bacteria).

Neutral lipids. The concentrated chloroform solution of the neutral lipid has a bright orange color produced by material tentatively identified as coenzyme Q. The material responsible for the color was partially purified by silicic acid chromatography. Its identification as coenzyme Q was done according to Lester et al. (29). The spectrum of the orange material dissolved in ethyl alcohol was analyzed before and after reduction with a crystal of $NaBH_4$. The characteristic maximum at 275 mμ disappeared after reduction with $NaBH_4$, and a new maximum appeared at 290 mμ, which had an intensity 3.5 times lower than that at 275 mμ (Fig. 2). The concentration of coenzyme Q was calculated by assuming $\Delta E^{1\%}_{1\,cm}$ at 275 mμ (oxidized-reduced) = 166 (average for the values given in reference 29). The content of coenzyme Q was found to be 0.05% of the dry weight of the cell or 7% of the neutral lipid. No further research into the nature of the neutral lipids was done.

Phospholipids. The phospholipids were separated and identified by TLC, hydrolysis to water-soluble products, color reactions, and partial purification by silicic acid column chromatography. The content of each class of lipids was calculated after ^{32}P incorporation into growing bacteria and separation of the lipids by TLC, and is reported in the following section.

Figures 3 and 4 show representative two-dimensional separations of the crude lipid extract. The neutral lipids (spot NL) run with the front in both solvents.

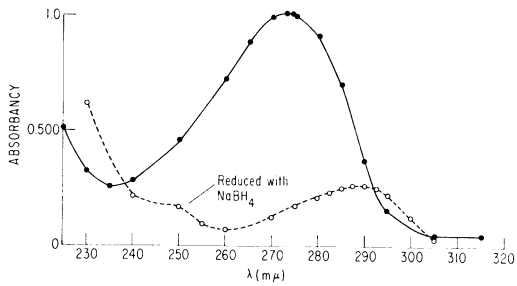

FIG. 2. *Spectrum of coenzyme Q from Salmonella typhimurium before and after reduction. The neutral lipid, in chloroform, was placed on a silicic acid column prepared in chloroform. An orange-yellow band was completely eluted upon washing the column with chloroform. The fractions containing the orange material eluted with the chloroform washing were pooled, the solvent was completely evaporated, and the residue was dissolved in ethyl alcohol. Reduction was with a crystal of $NaBH_4$ as described in reference 29.*

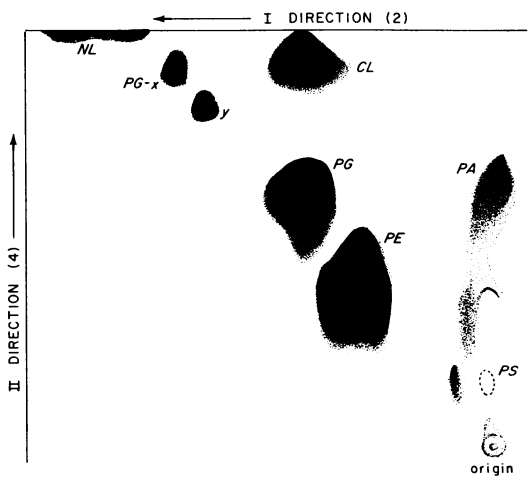

FIG. 3. *Two-dimensional thin-layer chromatogram of total lipids from Salmonella typhimurium. Development was first in the horizontal direction in solvent 2 (chloroform-methanol-7 N ammonia, 60:35:5, v/v), then in the vertical direction in solvent 4 (chloroform-methanol-acetic acid, 65:25:8, v/v). Approximately 1 mg of lipid was applied at the origin. For description of each spot see text. The spot named NL is the neutral lipid.*

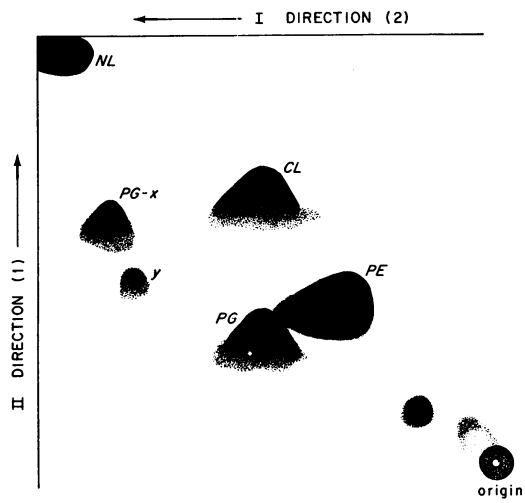

FIG. 4. *Two-dimensional thin-layer chromatogram of total lipids from Salmonella typhimurium. Development was first in the horizontal direction in solvent 2 (chloroform-methanol-7 N ammonia, 60:35:5, v/v), then in the vertical direction in solvent 1 (chloroform-methanol-water, 65:25:4, v/v). See also legend to Fig. 3.*

Spot PE was identified as phosphatidylethanolamine by the following criteria: (i) identity of chromatographic behavior in TLC with synthetic phosphatidylethanolamine and purified commercial phosphatidylethanolamine from various sources, in several chromatographic systems; (ii) reactivity with ninhydrin; (iii) presence of phosphate; (iv) the labeled compound purified by TLC yields glycerolphosphorylethanolamine upon mild alkaline hydrolysis; and (v) it incorporates label as ethanolamine when the bacteria are exposed to ^{14}C-serine.

Spot PG was identified as phosphatidylglycerol by the following criteria: (i) presence of phosphate; (ii) a positive reaction on TLC with the acetylacetone spray reagent, indicating presence of vicinal glycols in the unhydrolyzed lipid; (iii) upon periodate oxidation, the intact purified lipid yields one mole of formaldehyde per mole of phosphate; and (iv) the labeled compound purified by TLC yields glycerolphosphorylglycerol upon mild alkaline hydrolysis.

Spot CL was identified as cardiolipin (diphosphatidylglycerol) on the basis of the following criteria. (i) The R_F is the same as that of commercial beef heart cardiolipin in several solvent systems, and is the same as that reported by several authors. (ii) It forms a precipitate with barium (37). By use of this property, it was partially purified by precipitation as Ba salt and chromatography on silicic acid. (iii) The purified lipid yields one mole of formaldehyde upon periodate oxidation per mole of phosphate, after mild alkaline hydrolysis. (iv) The labeled compound purified by TLC yields di(glycerolphosphoryl)glycerol upon mild alkaline hydrolysis.

Spot PA, which is less than 1% of the total phospholipid, was identified as phosphatidic acid because it liberates α-glycerolphosphate upon mild alkaline hydrolysis and has the same chromatographic properties as pure phosphatidic acid in TLC solvent systems 2 and 4. In the TLC of Fig. 3, phosphatidic acid may overlap with some other iodine-stainable material, because the amount of phosphatidic acid present as assayed by the concentration of ^{32}P (*see below*) is probably too small to be visible with iodine.

Spots PG-X and Y have not been completely identified and are being investigated further. They both contain phosphate as indicated by the phosphate spray and by ^{32}P incorporation. They have the following characteristics.

Spot PG-X, upon mild alkaline hydrolysis, liberates glycerolphosphorylglycerol. It behaves like an acidic lipid on TLC and it is retained on diethylaminoethyl cellulose columns (17) after removal of the nonionic and zwitterion lipids with nonionic solvents. PG-X runs differently from PG in all the chromatographic systems used. This fact, together with the different turnover properties, distinguished PG from PG-X.

Spot Y did not give any of the common hydrolysis products.

The spot marked PS in Fig. 3 is the position of phosphatidylserine, identified by TLC of ^{32}P-labeled lipids. The amount present was too small to be visualized with iodine. Its identification was based on the following criteria. (i) It contains phosphate (by ^{32}P incorporation). (ii) The labeled compound, purified by TLC, yields glycerolphosphorylserine upon mild alkaline hydrolysis. (iii) It incorporates ^{14}C label from uniformly labeled ^{14}C-serine very rapidly, behaving like an early intermediate—the amount of ^{14}C-serine incorporation is already at its maximum at 30 sec of exposure to ^{14}C-serine; the label is completely "chased" by a second exposure to a 100-fold excess of unlabeled serine. (iv) Its chromatographic behavior is similar to that of commercial phosphatidylserine and that described in the literature.

Amounts of phospholipids present. To assay the content of each phospholipid class in bacteria growing exponentially, the whole culture was labeled with ^{32}P and the lipids were extracted. The labeled lipids were separated on TLC by a two-dimensional system (usually solvent 2 in the first direction and solvent 4 in the second direc-

tion), and visualized with iodine and autoradiography (Fig. 5). The regions of the chromatograms which were radioactive were scraped off and counted. From the specific activity of the ^{32}P (usually about 5×10^5 counts per min per μmole as calculated from the concentration of phosphate in the medium, i.e., 0.074 M) and the dry weight of the bacteria, the concentrations of the phospholipids were calculated. A certain variability was encountered, perhaps resulting from the stage of growth of the bacteria, even though all experiments were done in logarithmic phase. Table 3 shows representative values. A total of 73 μmoles of phospholipid was present in 1 g (dry weight). Most of the label appeared in phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

Phospholipids of E. coli. The pattern of phospholipids of *E. coli* K-12 (W3101) was investigated and found to be qualitatively the same as that of *S. typhimurium* except for the absence of spot PG-X. This was demonstrated both by iodine visualization of a TLC plate (as in Fig. 3, on which the *E. coli* lipids were exactly superimposable) and by a ^{32}P-incorporation experiment (Fig. 6). The quantitative assay of the ^{32}P-labeled phospholipids is presented in Table 3. The total amount of phospholipid was less than in *Salmonella* (i.e., 3.4% of the dry weight), but the percentage of each lipid was very much the same, except for a slight increase in the percentage of cardiolipin and of spot Y and the absence of spot PG-X. These values are qualitatively similar to those published for a strain of *E. coli* B (30). That *E. coli* and *S. typhimurium* have similar patterns is to be expected, since they have been demonstrated to be quite similar in numerous respects.

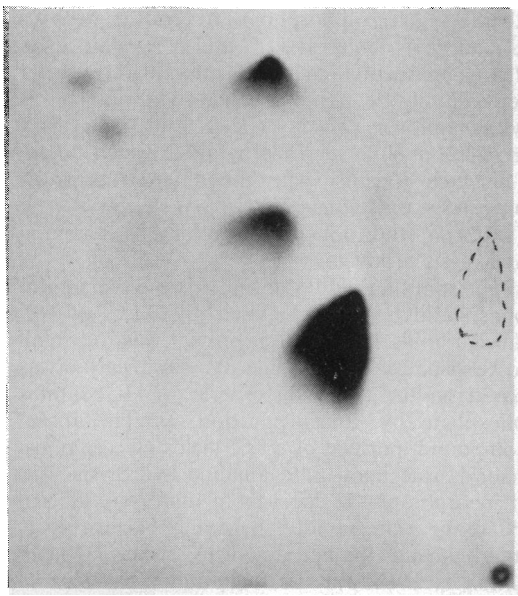

FIG. 5. *Autoradiogram of ^{32}P-labeled lipids from Salmonella typhimurium. The two-dimensional thin-layer chromatogram, with which this autoradiogram should be compared, is shown in Fig. 3. The dashed line defines the region where phosphatidic acid runs; the counts present in this compound could be detected in the scintillation counter, but were not sufficient to give a strong spot on the X-ray film. In this experiment, the lipids were extracted from 600 μg (dry weight) of bacteria which had been growing exponentially for about 4 hr in a medium containing ^{32}P-orthophosphate (5×10^5 counts per min per μmole). During the extraction, 20 mg (dry weight) of cold bacteria was added as carrier.*

Turnover of phospholipids of S. typhimurium. The question arose as to whether any of the lipids detected had a high turnover rate, indicative of an involvement in some active metabolic function rather than a structural one. Kanfer and Kennedy already presented data on this point (22), but since no mention was made of the cardiolipin component, it was decided to look into the turnover problem again. The following experiment was therefore performed. Cells labeled with ^{32}P were centrifuged and resuspended in unlabeled medium, and the growth continued. Samples were extracted at various times and analyzed qualitatively and quantitatively by two-dimensional TLC. Figure 7 shows the results of such an experiment.

There was no turnover of the phosphate in phosphatidylethanolamine and in cardiolipin. A rather slow turnover appeared in phosphatidylglycerol; i.e., approximately 50% of the label was lost in 60 min (about one generation time). The cardiolipin showed a peculiar behavior—it had an initial decrease, followed by a slight increase, which was probably due to incorporation of the products of turnover of phosphatidylglycerol. (This point will be discussed later.) Preliminary results indicated no turnover of PG-X and phosphatidic acid and a slow turnover of Y.

No experiments were done on the turnover of phosphate in phosphatidylserine, though it is expected to occur in accordance with its involvement in the biosynthesis of phosphatidylethanolamine (23) and with the demonstrated turnover of the serine moiety (*see above*).

Amounts of phospholipids present in S. typhimurium cells grown in low-phosphate medium. The low-phosphate medium used in these experiments was the same as the regular medium, except that instead of the $Na(NH_4)HPO_4$, NH_4Cl was added at a final concentration of 0.012 M, and the final

TABLE 3. *Phospholipid content of Salmonella typhimurium in high- and low-phosphate medium, and of Escherichia coli K-12*[a]

Lipid	*S. typhimurium*				*E. coli* K-12	
	High-phosphate medium		Low-phosphate medium			
	μmoles/g (dry weight)	Per cent of total phospholipid	μmoles/g (dry weight)	Per cent of total phospholipid	μmoles/g (dry weight)	Per cent of total phospholipid
Phosphatidylethanolamine	55	75	37	78	33	69
Phosphatidylglycerol	13	18	8.7	18	9	19
Cardiolipin[b]	3.3	4.5	1.5	3.2	3.1	6.5
Phosphatidylserine[c]	0.16	0.2	—[d]		—	
Y	1.6	2.2	0.35	0.7	2.9	6
PG-X	0.5	0.7	—		Undetectable	Undetectable
Phosphatidic acid	0.16	0.2	—		—	

[a] Extraction and assay procedures are described in the text.
[b] The cardiolipin concentration is calculated on the basis of two molecules of P per molecule of cardiolipin.
[c] The phosphatidylserine concentration was determined by ^{14}C-serine incorporation.
[d] Not assayed.

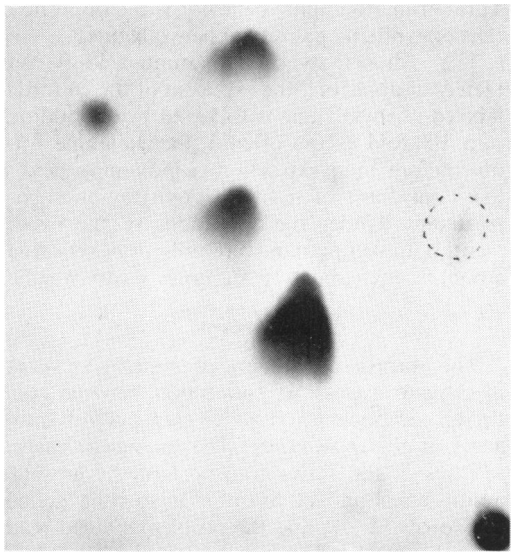

FIG. 6. *Autoradiogram of ^{32}P-labeled lipids from Escherichia coli K-12. Experimental details are the same as those described in legend to Fig. 5.*

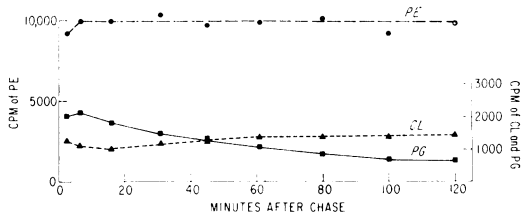

FIG. 7. *Turnover rates of ^{32}P in phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin in Salmonella typhimurium. Bacteria had been growing exponentially for 4 hr in the presence of ^{32}P-orthophosphate (about 5 × 10^5 counts per min per μmole). At time zero, a sample was centrifuged, the pellet resuspended in the same volume of warm unlabeled medium, and samples extracted (with the addition of 20 mg, dry weight, of cold carrier bacteria) at the times indicated. A culture containing no ^{32}P received the same treatment and was used for following the growth curve (which was normal) of the resuspended bacteria. The earliest sample in the figure represents the lipids from 260 μg of labeled bacteria.*

concentration of K_2HPO_4 was 0.006 M. The *p*H was adjusted to 7.0 with NaOH. The bacteria were grown and utilized for experiments before any *p*H change in the medium was detectable. The bacteria grew as well in this medium as in the high-phosphate medium. The bacteria were labeled and analyzed during growth in the low-phosphate medium exactly as described for the regular medium. The values obtained for the amounts of phospholipids under such conditions are shown in Table 3.

The total amount of phospholipid was considerably reduced (from 72.9 to 47.5 μmoles/g, dry weight) under conditions of growth in a low-phosphate medium, even though the proportions of each phospholipid were not greatly changed.

Incorporation of phenylalanine into lipids of S. typhimurium. Exponentially growing cells were exposed to 10^{-5} M L-phenylalanine-^{14}C for 1 min, and then the lipids were extracted and examined by TLC and autoradiography. Two radioactive

spots were detected (Fig. 8). One (lipo-amino acid A) runs just ahead of phosphatidylethanolamine in the neutral system 1 and has an R_F of 0.45. The other (lipo-amino acid B) runs with an R_F of 0.8 in the same system. Neither of these compounds appeared if the ^{14}C-phenylalanine was added immediately after the extracting solvents.

Lipo-amino acid A liberates phenylalanine upon 6 N HCl hydrolysis at 110 C for 12 hr. It reacts with dinitrofluorobenzene, yielding a dinitrophenyl-derivative of the intact lipid, from which dinitrophenyl-phenylalanine is obtained by

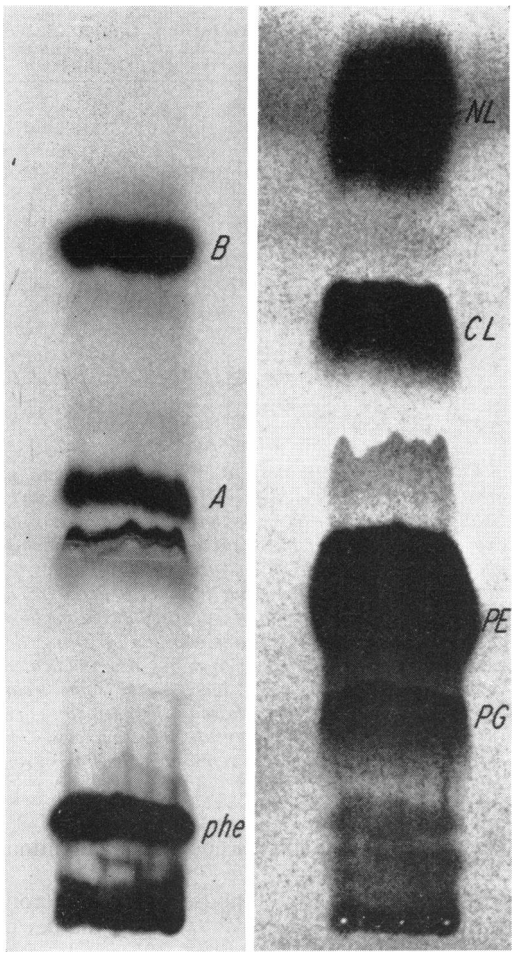

FIG. 8. *Autoradiogram (left) of the thin-layer chromatogram (right) of total lipids from Salmonella typhimurium after exposure to ^{14}C-phenylalanine. Cells were incubated at 37 C with 10^{-5} M ^{14}C-phenylalanine (200 μc/μmole), for 1 min immediately before lipid extraction. Development was in solvent 1 (chloroform-methanol-water, 65:25:4, v/v). A and B refer to lipo-amino acid A and lipo-amino acid B, respectively.*

6 N HCl hydrolysis. It is completely labile to mild alkaline hydrolysis and is degraded by snake venom and by pancreatic lipase. Upon treatment with aqueous alkali (i.e., shaking 1.0 ml of a chloroform solution with 1.0 ml of 1 N ammonia at room temperature for 30 min), it liberates about half of the radioactivity as free phenylalanine. A compound such as the phenylalanine ester of phosphatidylglycerol (31) would have these characteristics.

Lipo-amino acid B liberates phenylalanine upon 6 N HCl hydrolysis at 110 C for 12 hr. It contains no phosphate. It is degraded by pancreatic lipase, but not by snake venom. It has an R_F of 0.5 in TLC developed in acetone. It is dissimilar from the compound (palmitoylphenylalanine) described by Fukui and Axelrod (15) for the following reasons. In contrast to palmitoylphenylalanine, it is alkali-labile (60 min at room temperature in ethanolic 0.5 N NaOH). It has an R_F of 0.93 in the basic TLC system 2 and an R_F of 0.41 in the acidic TLC system 4. This chromatographic behavior is the opposite of that of synthetic palmitoylphenylalanine.

The radioactivity of lipo-amino acid B completely disappears if the exposure of the culture to labeled phenylalanine is followed by the addition of a 100-fold excess of cold phenylalanine for 1 min before lipid extraction. Lipo-amino acid A loses only part of its radioactivity upon such a treatment. Under the conditions of the experiment, both compounds were present in very small amounts, each about 10 mμmoles/g (dry weight).

DISCUSSION

The purpose of the work presented here was to investigate a possible connection between some lipidic component and active transport of amino acids in *S. typhimurium*. The biological characteristics of the active transport of the aromatic amino acids and of histidine have been defined previously (2, 41) and the problem seemed ready for a biochemical approach. The lipids were the object of attention because the membrane delimiting the cell, and presumably conferring to it its characteristic of impermeability, is supposed to be constituted largely of lipid. In *E. coli*, approximately 70% of the cell lipid is present in the purified membranes (19). It therefore seemed reasonable that a water-soluble compound, like an amino acid, might form a covalent bond with a particular lipid, becoming lipid-soluble itself. Several types of covalent compounds between amino acids and lipids have been found (reviewed in 33), though in no case is it clear that they are involved in transport. Particularly interesting is the demonstration of the involvement

of t-ribonucleic acid in the biosynthesis of amino acid esters of phosphatidylglycerol (28).

In the course of investigating phenylalanine transport, the formation of several phenylalanine-containing lipids was observed. As part of the study of these compounds, a detailed study of the lipids of *S. typhimurium* was undertaken.

Because of the lability of these phenylalanine-lipid compounds, a method of extraction was sought which was neutral, rapid, and effective at 0 C. The rapid and simple Bligh-Dyer extraction technique has been found to be superior to other methods. The extraction is at neutrality and at 0 C, conditions which are unlikely to cause any breakdown of lipid. A total of seven phospholipids could be extracted by this method. The main classes of compounds were identified, after complete separation on a single, two-dimensional, TLC. The results agree with the preliminary data on the major lipid classes of *S. typhimurium* and *S. newington* presented by Macfarlane (32) and Dankert et al. (9), respectively.

Phosphatidylethanolamine is the most abundant component; it represents 75% of the total phospholipid and has no significant rate of turnover. Kanfer and Kennedy (22) had already shown similar results for the phosphatidylethanolamine of *E. coli* B. Its main function might be structural, but this does not exclude its involvement in other functions, such as its participation in the lipopolysaccharide biosynthesis (39).

The next most abundant component is phosphatidylglycerol, which constitutes 18% of the total phospholipid of *S. typhimurium*. Though its route of biosynthesis has been identified (25; Y. Chang and E. B. Kennedy, Federation Proc. **26**:277, 1967), nothing is known about its function. It has a fairly slow turnover rate; it takes more than one generation (48 min) for half the phosphatidylglycerol to turn over. These data agree with those presented on the turnover of phosphatidylglycerol in *E. coli* B by Kanfer and Kennedy (22). The significance of the phosphatidylglycerol turnover will be discussed together with the turnover of cardiolipin.

Cardiolipin represents approximately 5% of the total phospholipid. In the turnover study (Fig. 7), an initial decrease in the amount of label is followed by an increased incorporation of counts into cardiolipin. This might be explained if the label lost from phosphatidylglycerol were incorporated into cardiolipin without going through a pool of inorganic phosphate, or of other compounds which can be diluted with the unlabeled phosphate of the medium. Preliminary evidence concerning the biosynthetic pathway of cardiolipin (42) demonstrates, in fact, that the intact molecule of phosphatidylglycerol gets converted, in vitro, to cardiolipin by addition of a new phosphatidic acid moiety. However, in vivo, the recovery of the counts lost from phosphatidylglycerol into cardiolipin is not complete, since cardiolipin itself seems to be turning over. Also, phosphatidylglycerol might be turning over independently from its possible conversion to cardiolipin, and, as it is continuously synthesized during the chase experiment, its specific activity is continuously decreasing.

The four minor phospholipids detected represent approximately 3% of the total phospholipid. One of these, phosphatidic acid, was identified as phosphatidic acid because it released α-glycerophosphate upon mild alkaline hydrolysis, and because of its chromatographic behavior in several TLC systems. However, it did not turn over as expected for a key metabolic intermediate in the biosynthesis of all phospholipids. This point is under further investigation. Spot PG-X is probably phosphatidylglycerol carrying some additional component which increases its chromatographic mobility in all solvents tried. This compound did not turn over. Spot Y does not give any of the common hydrolysis products and will have to be investigated further. It is very unlikely that spot Y is the polyisoprenoid lipid involved in the biosynthesis of the *Salmonella* O-antigen (46) and of the cell wall peptidoglycan of *Micrococcus lysodeikticus* (18), because their mobilities on TLC are very different (11). It is possible that the polysioprenoid compound (which is more polar) is not extracted under the conditions used, or that it is present in undetectable amounts, or, finally, that it is present mostly as the nonphosphorylated derivative (which would be part of the "neutral fat" class).

The metabolic intermediate phosphatidylserine was, as expected (23), present in very small amounts, and its serine moiety turned over rapidly. The label from this portion was incorporated as ethanolamine into the phosphatidylethanolamine molecule.

I examined the lipids of *E. coli* K-12 because the data available in the literature (21, 30; J. H. Law, Bacteriol. Proc., p. 129, 1961) have been collected on strains of *E. coli* other than K-12. This is the strain utilized in bacterial genetics experiments and it seemed pertinent to explore its lipid composition at a moment when the field of lipid research is ripe for genetic inquiry.

As expected, *E. coli* and *S. typhimurium* have very similar lipid composition. The differences might be due to the slow growth rate of *E. coli* K-12 in minimal medium (a doubling time of 48 min). In fact, growth conditions can affect lipid composition (at least quantitatively).

The lipids of *E. coli* K-12 are similar to those

of a strain of *E. coli* B investigated by Lubochinsky et al. (30). Qualitative identification of the phospholipids of another strain of *E. coli* has been presented by Kaneshiro and Marr (21). Recently, a thorough paper has appeared (20) on the composition and turnover of the phospholipids of *E. coli* B. The data agree very well with those presented here for *S. typhimurium* and *E. coli* K-12.

The data presented by Kennedy and his coworkers (22, 43) in connection with their work on the biosynthesis and turnover of lipids are quite fragmentary and were not obtained on the individually purified lipid classes; the presence of cardiolipin and some of the minor components was not mentioned. As cardiolipin is degraded fairly easily under acid conditions, it is conceivable that some of it (or of phosphatidylglycerol) was degraded to phosphatidic acid under the conditions they used for extraction. However, a recent report (42) by the same group indicates that a pathway for the biosynthesis of cardiolipin exists in *E. coli*.

Of interest is the experiment in which *S. typhimurium* is grown in a low-phosphate medium. This medium has a 10-fold decrease in the amount of phosphate and it does not significantly affect the bacterial growth rate; however, it causes a significant decrease in the amount of each phospholipid present. This indicates that the concentration of phosphate in the medium somehow regulates the synthesis of phospholipids. This possibility should be kept in mind when doing studies of ^{32}P labeling under conditions of very low phosphate in the medium. The pattern of labeling might represent a heavily altered composition of the lipidic constituents.

Preliminary experiments have demonstrated the incorporation of the amino acid phenylalanine into lipidic material. A complete identification of the two compounds formed has not yet been possible because of the very small amount of material available. However, neither is a compound of the type isolated by Fukui and Axelrod (palmitoylphenylalanine, 15). The properties of one of them suggest that it might be an ester of phosphatidylglycerol (31), but more evidence is necessary to confirm this point.

The function of these two compounds is uncertain at present. Because they occur in such small amounts, are formed very rapidly, and because lipo-amino acid B turns over very fast, either or both compounds might be involved in the active transport of phenylalanine into the cell. As pointed out above, a combination between amino acids and lipids could fulfill the role of an intermediate in active transport. A definite demonstration of such an involvement will come only from the detailed biochemical analysis of the synthesis and breakdown of these compounds.

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