The isolation and characterization of phospholipids containing mono- and dimethylethanolamine from *Neurospora crassa**

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SUMMARY

Mono- and dimethylethanolamine containing phospholipids have been isolated from a choline-requiring mutant strain of *Neurospora crassa*. These phospholipids have been chemically degraded, and shown to be the phosphatidyl esters of mono- and dimethylethanolamine. The implications of the accumulation of these compounds by the mutant of *Neurospora* are discussed.

We have previously reported the isolation of MMEA[1] and DMEA from the lipid hydrolyzate of *Neurospora crassa*, strain 47904 (1). The chromatographic behavior of these metabolites indicated that they were the phosphatidyl derivatives of MMEA and DMEA. The isolation, from 47904, of the corresponding free amines and also the phosphate esters of these two bases (2) suggests two alternate pathways for the synthesis of phosphatidyl-MMEA, phosphatidyl-DMEA, and lecithin: (*a*) Kennedy's scheme (3) via the cytidine diphosphate base, and (*b*) a direct methylation of cephalin. This latter proposal is supported by the work of Bremer and Greenberg (4, 5), and that of Artom and Lofland (6, 7).

This paper is concerned with the separation and chemical characterization of the phospholipids from "wild-type" *Neurospora*, strain 1A, and from the mutant strain, 47904. A quantitative estimate of the amounts of MMEA, DMEA, and choline liberated on hydrolysis of the phospholipids from strain 47904 has been published previously (1).

EXPERIMENTAL

Cultures. *Neurospora crassa*, strain 47904, previously described by Horowitz *et al.* (8, 9) synthesizes MMEA and DMEA (10), but is unable to convert these compounds to choline at the normal rate, due to a mutation at a genetic locus concerned with the synthesis of choline. This mutant has also been shown to accumulate both MMEA-P and DMEA-P (2). Large quantities of these four compounds accumulate in the mycelium of a 47904 culture grown under forced aeration.

The "wild-type" *Neurospora*, strain 1A, by comparison, accumulates large quantities of choline and phosphorylcholine, no MMEA or MMEA-P, and trace amounts of DMEA and DMEA-P.

The quantitative estimation of the free bases, MMEA, DMEA, and choline, as well as the bases liberated from phosphate esters after hydrolysis, is determined with another choline-requiring mutant, *Neurospora crassa*, strain 34486 (11).

Growth and Extraction of Microorganisms. In each experiment a 6-liter culture of *Neurospora crassa*, strain 47904 or 1A, is grown for 6 or 10 days at 25° under forced aeration on minimal medium (12). The mycelium is homogenized with distilled water in a Waring Blendor and filtered. Trace amounts of lipids are recovered from the filtrate by extraction with chloroform before the filtrate is discarded. In earlier studies (1), the total lipids were removed from the homogenized mold by extraction with ether and alcohol; however, in later experiments low temperature chloroform extractions

[1] The following abbreviations are used: MMEA, monomethylethanolamine; DMEA, dimethylethanolamine; MMEA-P, phosphorylmonomethylethanolamine; DMEA-P, phosphoryldimethylethanolamine; GPM, glycerophosphorylmonomethylethanolamine; GPD, glycerophosphoryldimethylethanolamine; GPC, glycerophosphorylcholine.

according to Wren and Mitchell (13) were employed. The total lipid recovered from either strain 1A or 47904 corresponds to 10 ± 1% of the dry weight of the mycelium. Essentially the same results are obtained from studies on phospholipids obtained by both extraction methods. These lipid extracts are washed free of nonlipid contaminants by the method of Folch et al. (14). Since the phospholipids in peaks A and B (Fig. 1) are readily oxidized, all procedures concerned with these materials were designed to minimize exposure of these lipids to air.

Silicic Acid. The chromatographic separation of total lipids on a silicic-acid column is based on a solvent system previously employed for blood lipids by Mead and Fillerup (15), while the separation of the phospholipids on silicic acid is carried out according to the method of Wren and Mitchell (13).

Silicic acid (Baker analyzed) is prepared essentially according to the method described by Hirsch and Ahrens (16), except that it is used without being ground in an Abbé mill. Fifty grams of the washed and dried silicic acid is dusted into a column (2.8 cm diameter) provided with a sintered glass plug at its lower end, which is covered with a piece of thick filter paper. The silicic acid is packed to a column height of about 11.8 cm by applying suction to the lower end of the column and tapping the sides of the glass. A thick filter paper disk is pressed firmly on the top of the silicic acid to minimize distortion of the upper surface during elution.

The silicic-acid column described above is washed with 100-ml aliquots of methanol, acetone, and diethyl ether, respectively. The sample is put on the column in diethyl ether, and the nonpolar lipids are removed by passing through 500 ml of ether. The phospholipids are eluted from the column with 500 ml of methanol. The phospholipids recovered from either strain 1A or 47904 represent about 3.5% to 4% of the dry weight of the mycelium. A comparison of the phospholipids from these two strains of *Neurospora*, based on the methylated ethanolamine content, was made in a previous publication (1).

For the separation of phospholipids, a silicic-acid column packed identically to that described above is washed with 400 ml of chloroform. The methanol eluate from the previously described silicic-acid column is evaporated to dryness and the lipid residue applied to the column in 5% methanol in chloroform (v/v). The column is developed with increasing percentages of methanol in chloroform at a rate of flow set at 14 to 16 ml/hour. Samples are collected at half-hour intervals. The test tubes in which the samples are collected are covered with funnels during collection to prevent evaporation. For phosphorus analyses, aliquots of each tube are taken to dryness and determined according to the method of Bartlett (17). Samples are prepared for the methylated ethanolamine analysis of each eluate fraction by taking aliquots to dryness and subsequently hydrolyzing each residue by autoclaving
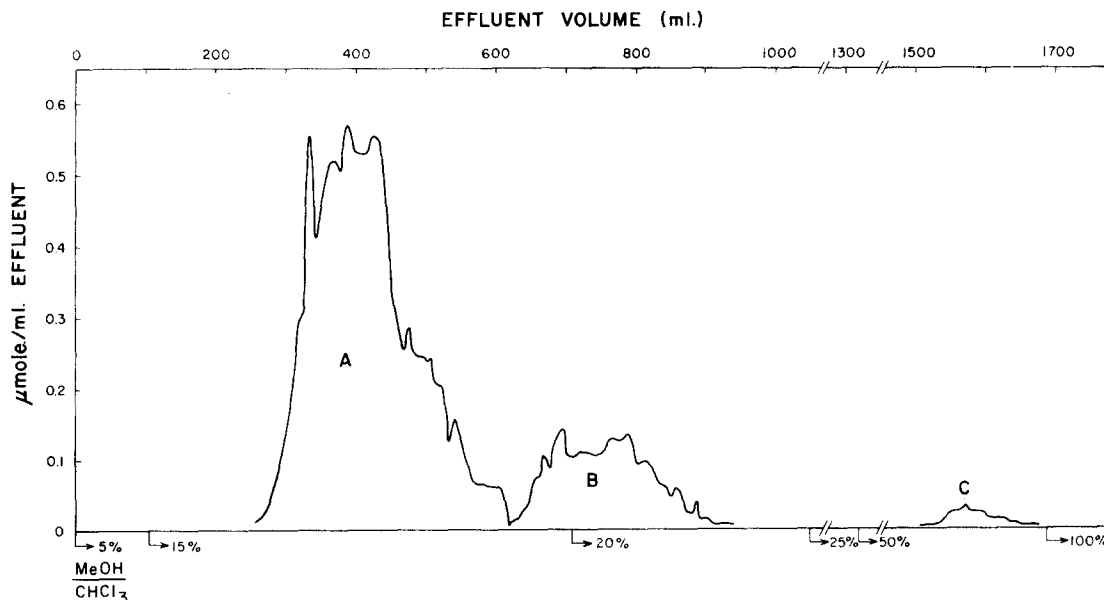

FIG. 1. Silicic-acid chromatography of phospholipids extracted from *Neurospora crassa*, strain 47904. The column was charged with 675 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced. The values on the ordinate are expressed as micromoles of MMEA (peak *A*), DMEA (peak *B*), and choline (peak *C*) per milliliter of eluate.

for 6 hours with 3% sulfuric acid at 250°F. The cooled hydrolyzates are extracted with chloroform to remove any lipid contaminants. The MMEA, DMEA, and choline in the aqueous phase are assayed as previously described (11). Figures 1 and 2 show the results of a typical separation. The results are expressed as micromoles of MMEA, DMEA, or choline per milliliter of eluate. Phospholipids from both 6- and 10-day-old cultures gave essentially the same pattern.

Ion-Exchange Resins. The separation of free MMEA, DMEA, and choline on a Dowex 50 cation-exchange column (15 × 450 mm, hydrogen form) has been described previously (10). The choline chloride used in the present studies was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Monomethylethanolamine, obtained from Eli Lilly and Company, Indianapolis, Indiana, was redistilled in a 28-inch Vigreaux fractionating column, and the fraction boiling at 155°–155.5° was used in these studies. DMEA (Eastman-Kodak Company, Rochester, New York) was redistilled in a similar column. A fraction boiling at 58°–60° at 50-mm pressure was employed as an authentic reference sample.

Both MMEA-P and DMEA-P are products obtained by the chemical degradation of the phospholipids found in *Neurospora*, strain 47904. These esters are identified by comparing their chromatographic behavior with authentic samples on both a Dowex 50 cation-exchange column (15 × 450 mm, hydrogen form) (2) as well as on a Dowex 1 × 4 anion-exchange column (15 × 450
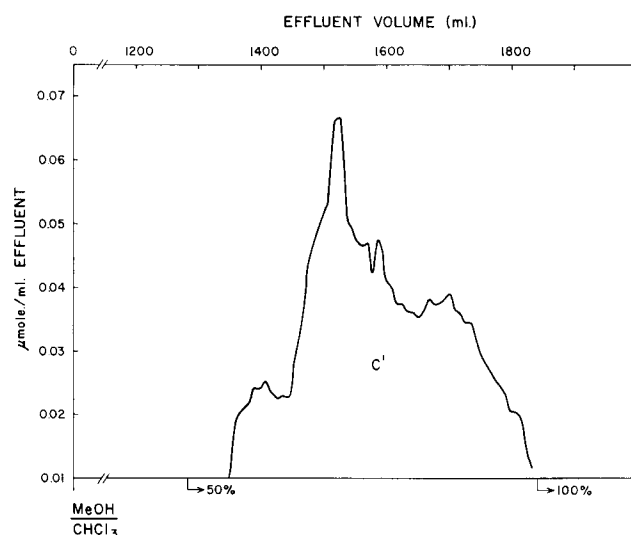

FIG. 2. Silicic-acid chromatography of phospholipids extracted from *Neurospora crassa*, strain 1A. The column was charged with 101 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced. Solvent changes between 0 and 1200 ml were the same as in Figure 1. The values on the ordinate are expressed as micromoles of choline per milliliter of eluate.

mm, acetate form). Upon elution of the above anion-exchange column with 200 ml of water, followed by 0.1 N acetic acid, authentic MMEA-P and DMEA-P both emerge from such a column with concentration peaks, as determined by bioassay, at 295 to 305 ml of total eluate. Chromatography on the Dowex 50 cation-exchange column with 0.3 N HCl as the eluting solvent gives individual peaks for MMEA-P and DMEA-P with maxima at 340 ml and 407 ml, respectively. These values differ slightly from the previously published values for MMEA-P and DMEA-P (2). The Dowex 50 resin used in these experiments was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey. The authentic MMEA-P and DMEA-P employed in the present studies were those described in a previous publication (2).

Degradation of Phosphatidyl MMEA and DMEA. The phospholipids contained in peaks *A* and *B* (Fig. 1) are subjected to mild alkaline hydrolysis according to the method of Dawson (18). The neutralized hydrolyzate is partitioned between chloroform-methanol and water by the method of Folch *et al.* (14). The organic phase contains the fatty acids which result from the hydrolysis of the phospholipids. These are recovered and stored under nitrogen for analysis by gas-liquid chromatography. The aqueous phase contains the glycerophosphoryl esters of the two methylated ethanolamines. The glycerol moiety is removed from these products of hydrolysis to yield the phosphate esters of MMEA and DMEA by the method of Fleury *et al.* (19) as follows: A slight excess of periodate is allowed to react with the phosphate diesters for 30 minutes. The periodate and iodate ions present at the completion of the oxidation are removed as the insoluble barium salts by the addition of barium carbonate. The filtrate is taken to dryness at 25° and dissolved in a small volume of 1 N sodium acetate buffer at pH 4. A stoichiometric amount of phenylhydrazine is added, based on the amount of periodate previously consumed by the sample. The latter values are obtained on small aliquots of the periodate reaction mixture by back titration of unused periodate. The buffered reaction mixture is kept at 37° for 1 hour, after which it is extracted several times with 5-ml volumes of ether. The aqueous phase contains the desired phosphate esters of MMEA and DMEA, which are then characterized by comparing their behavior on ion-exchange resin columns with authentic materials.

RESULTS

Identification of MMEA, DMEA, and Choline. MMEA, DMEA, and choline were obtained with C[14]

in-their methyl groups from the completely hydrolyzed phospholipids of either culture 1A or 47904, which had been grown on isotopically labeled formate-C^{14}. The isolation of these amines on a Dowex 50 column with 1.5 N HCl as the eluting solvent has been described in previous communications (10, 20). Each of the isolated labeled amines was isotopically diluted with authentic unlabeled material, and the resulting mixture rechromatographed on a second Dowex 50 column. In each case the biological activity, as measured by bioassay with *Neurospora crassa*, strain 34486, is superimposed upon the radioactivity in the chromatographic patterns, which are plotted for eluate fractions. Further confirmation of the identity of the isolated three amines was obtained by comparing the growth response of *Neurospora crassa*, strain 34486, to each isolated substance, with simultaneous assays on authentic material. In each case the growth response of the organism to increasing aliquots of the isolated amines in question corresponds to the growth curves obtained on authentic samples. The crystalline picrate of MMEA (m.p. 148°–150°) was isolated from the phospholipid hydrolyzate of two combined 6-liter cultures of strain 47904 grown for 10 days. This sample does not exhibit a melting point depression when mixed with an authentic sample (m.p. 148°–149.5°).

Each of the fractions *A*, *B*, *C*, and *C'* indicated in Figures 1 and 2 were hydrolyzed according to the method of Horowitz and Beadle (11). The hydrolyzates were chromatographed on Dowex 50 columns to determine the distribution of the methylated ethanolamines contained within each fraction, the eluates being assayed by means of *Neurospora crassa*, strain 34486. These experiments have shown that fraction *A* contains only MMEA, fraction *B* contains only DMEA, and fractions *C* and *C'* both yield only choline. Hence there is a clear separation of phospholipids based on amine content as well as chromatographic behavior. Since peaks *A* and *B* (Fig. 1) almost completely replace the lecithin found in the normal strain (Fig. 2), further studies on the chemical characterization of these materials were based on the conjecture that these peaks contained the phosphatidyl esters of MMEA and DMEA. In the work discussed in the following section, GPM was studied more extensively than GPD since much larger quantities of the former were available.

Characterization of MMEA-P, DMEA-P, and GPC. When known chemical degradative methods previously used for studying the chemistry of lecithin and cephalin are applied to the two unknown phospholipid fractions *A* and *B* (Fig. 1), the products obtained are those normally expected from a phosphatidyl derivative.

Mild alkaline hydrolysis of all of the phospholipid fractions (18) yields fatty acids which are readily extractable from an aqueous phase with chloroform. The aqueous phases from the hydrolyzates, remaining after the release and extraction of fatty acids with organic solvent, contained the biologically active amines in ester forms, presumably as the glycerophosphoryl bases. Since the expected water-soluble hydrolysis product of fractions *C* and *C'* is a known and available substance previously described in the literature, its identity is readily confirmed by comparing its chromatographic behavior on a column of powdered cellulose (15 × 450 mm) with an authentic sample. The eluting solvent in this study is a mixture consisting of eight parts of 99% ethyl alcohol and one part of 0.1 N acetic acid-pyridine buffer, pH 4.5.[2] The elution peak of the unknown sample is between 243 to 254 ml of the eluting solvent, and is indistinguishable from the behavior of authentic GPC. Since the expected corresponding hydrolysis products from peaks *A* and *B*, GPM and GPD, respectively, are chemically unknown, the identification of these materials by direct comparison with authentic materials is impossible at this time. An unequivocal identification of these degradation intermediates by classic chemical approaches will be attempted when adequate amounts of pure materials are available. Work directed toward this goal has been slow.

Before proceeding further with the degradation of these materials, a preliminary experiment was performed on GPM to determine whether it is oxidized by periodate, and whether its chromatographic behavior on ion-exchange resins resembles that of GPC, which has a similar dipolar-ion structure. A phosphate diester such as GPC passes readily through anion-exchange resins without expressing an appreciable anionic character. The substance believed to be GPM also exhibits this predicted behavior and passes through chromatographic columns of Dowex 1 × 4 at the solvent front when distilled water is employed as the eluting solvent. An aliquot of GPM used up 10% more than the theoretical amount of periodate according to the method of Voris *et al.* (21).

The biologically active substances resulting from the mild alkaline hydrolysis of fractions *A* and *B*, presumably GPM and GPD, were degraded to MMEA-P and DMEA-P according to the method of Fleury *et al.* (19), by a procedure previously applied to the degradation of authentic GPC[3] and glycerophosphorylinositol (22). Glycolaldehydephosphorylcholine, obtained by

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[2] B. J. Crocken and J. F. Nyc. Unpublished results.
[3] M. O. Hall and J. F. Nyc. Unpublished results.

treating GPC with periodate, spontaneously decomposes to yield phosphorylcholine when allowed to react with phenylhydrazine at 37°. By an analogous procedure, Brown *et al.* (22) obtained phosphorylinositol from glycerophosphorylinositol. The MMEA and DMEA phosphate esters, which were obtained by this degradation method, were characterized by comparing their behavior with authentic samples on both the Dowex 1 anion- and the Dowex 50 cation-exchange columns described above. The elution pattern of these phosphate esters was indistinguishable from that of the authentic controls in these chromatographic studies.

Characterization of Phospholipids. When a hydrolyzed sample of total phospholipid from strain 47904 is subjected to paper chromatography according to Bremer and Greenberg (4), it is found to contain MMEA, DMEA, choline, ethanolamine, serine, and two unidentified ninhydrin-staining spots. An inositol test was positive (23). The total phospholipid contains only 0.05 mmole of plasmalogen per gram as determined by the potassium tri-iodide method of Rapport and Alonzo (24).

About one-third of the total phospholipid from strain 47904 is recovered as peak *A* (Fig. 1). A typical sample contains 0.7 mmole of MMEA and 1.2 mmoles of phosphorus (17) per gram of material. The low iodine uptake of 0.014 mmole per gram of this lipid indicates that about 1% of the phosphorus in the sample is accounted for by plasmalogens. When a hydrolyzate of peak *A* is chromatographed on paper (4), the ninhydrin-staining contaminants are ethanolamine (estimated at 0.05 mmole per gram of lipid on the basis of the ninhydrin color reaction) and traces of serine. The sample does not contain detectable amounts of inositol. A surprising observation is the fact that about a half of the MMEA-containing material in crude phospholipid extracts of strain 47904 is not eluted from a silicic-acid column by 50% methanol-chloroform. The phosphatidyl-MMEA in peak *A* can be rechromatographed on a silicic-acid column without appreciable loss of material. It is not known at present whether the unidentified MMEA-containing material is an artifact resulting from the isolation procedure.

To further purify the phosphatidyl-MMEA in peak *A*, the tubes comprising this fraction were combined, evaporated to dryness, and the resulting residue was rechromatographed on silicic acid under conditions identical to those employed for the experiment illustrated in Figure 1. As can be seen in Figure 3, a separation into two phosphorus-containing peaks, A^1 and A^2, is accomplished. Peak A^1, which contains all of the MMEA, gives a molar ratio of MMEA to phosphorus of 0.92. There is no measurable loss of
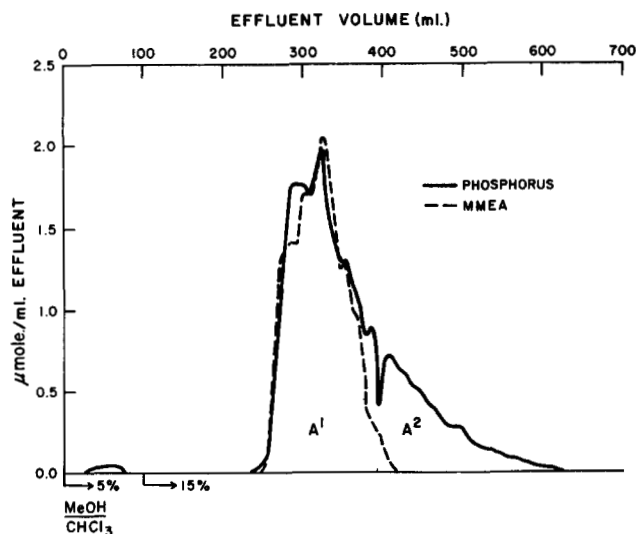

FIG. 3. Rechromatography of peak *A* on silicic acid. The column was charged with 227 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced.

the MMEA moiety during the rechromatography of peak *A*, and the total recovery of phosphorus in the fractions corresponding to peaks A^1 and A^2 is 92% of the amount originally contained in the sample subjected to rechromatography. Studies employing paper chromatography revealed that most of the ethanolamine and all of the serine, which were previously present in hydrolyzates of peak *A*, are now in peak A^2. This fraction contains 26% of the total phosphorus recovered after the rechromatography of peak *A*.

One gram of the total lipid from strain 47904 yields 150 mg of phospholipid in the eluate fraction corresponding to peak *B* (Fig. 1). Essentially all of the DMEA-containing lipid in the total lipid fraction is recovered in the material contained in peak *B*. A typical sample contains 0.7 mmole of DMEA and 1.3 mmoles of phosphorus per gram of material. When a hydrolyzate of the lipid in peak *B* is chromatographed on paper, the only ninhydrin-staining materials which are observed are ethanolamine and serine. Estimates based on such studies indicate that the phosphatidyl derivatives of ethanolamine and serine are the major contaminants of phosphatidyl-DMEA. Inositol-containing phospholipids are not found in this fraction. The iodine uptake of 0.02 mmole per gram of lipid shows that the plasmalogens represent only a small fraction of the lipids in peak *B*. Further purification of phosphatidyl-DMEA has not been effected at this time.

DISCUSSION

The isolation of phospholipids containing MMEA

and DMEA from a *Neurospora* mutant grown only on sugar, salts, and biotin establishes these substances as naturally occurring products of cell metabolism (1). These phospholipids have now been identified as the phosphatidyl esters of the corresponding methylated ethanolamines. The natural occurrence of these lipids suggests that they play a metabolic role as precursors of lecithin, and thus may be obligatory intermediates in the *de novo* synthesis of a choline moiety. The studies with *Neurospora* parallel studies with mammalian tissues in two other laboratories. Bremer and Greenberg (4, 5) have shown that rat liver microsomes incubated with (Me-C[14])-adenosylmethionine were able to incorporate the label into phospholipids from which radioactive MMEA and DMEA could be isolated. In these studies it was noted that the maximum incorporation of radioactivity into cytidinediphosphocholine occurred 1 to 2 hours later than that in the phospholipid choline. More recently, Artom and Lofland (6, 7) have incubated enzymatically prepared phosphatidyl-DMEA-C[14] with a rat liver homogenate, and found that unless adenosylmethionine is added to the incubation mixture, no label is found in lecithin. No isotope dilution effect is noted when unlabeled DMEA is added to the incubation flasks. This indicates that DMEA is not split off the phospholipid moiety before methylation, but rather that the methylation occurs on the intact phospholipid. The above workers (4 to 7) believe that the methylation of phosphatidyl ethanolamine or phosphatidyl-DMEA represents an important pathway in the biosynthesis of lecithin.

Since the same mutant of *Neurospora* in which these MMEA- and DMEA-containing phospholipids are found also accumulates free MMEA, DMEA (10), and the phosphate esters of these two bases (2), an important consideration arises concerning the precursor-product relationship of these bases and base esters to the corresponding phosphatidyl derivatives. Mycelial extracts of 6- and 10-day-old *Neurospora* 47904 cultures both contain essentially the same concentration of the phosphatidyl esters of MMEA, DMEA, and choline. In contrast to this, earlier work by Horowitz (9) showed that free MMEA did not appear in the medium of a liquid culture of this organism until after the sixth day of growth—at which time appreciable autolysis can occur. He used *Neurospora*, strain 34486, to quantitate the amount of base in the media of cultures of strain 47904. Because strain 34486 responds to MMEA, DMEA, and choline, he would have detected the presence of any one of these bases in his time-accumulation studies. Since the free methylated ethanolamines appear only in the media of aging 47904

cultures, they may be, to some extent, autolysis products formed by the degradation of the corresponding phospholipids. Artom *et al.* (7, 25) have shown that in rat liver slices DMEA can be incorporated into a phospholipid, presumably by a pathway analogous to that proposed by Kennedy (3) for the biosynthesis of phosphatidyl ethanolamine and lecithin. The proposed degradation of MMEA- and DMEA-containing phospholipids in autolyzing cultures of *Neurospora* may represent a reversal of Kennedy's synthetic pathway. The DMEA-containing phospholipid prepared by Artom and Lofland (7) was eluted from a silicic-acid column by a 4 to 1 chloroform-methanol eluant. This corresponds well with the behavior of the phosphatidyl-DMEA in peak *B* (Fig. 1), which was obtained from *Neurospora*.

It is well established that serine can give rise to ethanolamine by decarboxylation. In view of the experiments reported here, and those by Bremer and Greenberg (4, 5) and Artom and Lofland (6, 7), it is interesting to speculate that a phosphatidyl serine decarboxylase might exist. This could catalyze the decarboxylation of phosphatidyl serine to phosphatidyl ethanolamine, which would then be the initial methyl acceptor for lecithin synthesis. It is noteworthy in this connection that Hübscher *et al.* (26) have shown that the biosynthetic pathway of phosphatidyl serine differs from that of phosphatidyl choline or phosphatidyl ethanolamine.

Preliminary experiments have shown a large difference in the fatty acid composition of the various phospholipids from strains 1A and 47904. Further purification of the phospholipids from peaks *A*, *B*, *C*, and *C'* is necessary before any conclusions can be drawn from these results. Work directed toward this goal is in progress in this laboratory.

The single-gene mutant of *Neurospora*, strain 47904, lacking choline, differs phenotypically from the normal "wild-type" strain 1A only in its reduced rate of growth (8), greater aminopterin resistance (27), and colonial form of growth on liquid medium. Despite the fact that the mutant strain 47904 lacks most of the lecithin normally found in other strains, it exhibits a normal vegetative and sexual life cycle (20).

Of interest is the observation (28) that the *tubercle bacillus*, like strain 47904, also lacks lecithin. It is not known at this time whether this organism has substituted MMEA- and DMEA-containing phospholipids for the lecithin which it lacks.

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