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The incorporation of dimethylaminoethanol and dimethylamino*iso*propyl alcohol into *Phormia regina* phospholipids*

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SUMMARY

The effect of replacing dietary choline with dimethylaminoethanol and dimethylamino-*iso*propyl alcohol on the larval phospholipids of the blowfly *Phormia regina* was investigated. It was found that these compounds were incorporated into the phospholipids to the extent of approximately 30% of the total phospholipids when dimethylaminoethanol was used and approximately 18% when dimethylamino*iso*propyl alcohol was added to the diet. The separation of these phospholipids by silicic acid chromatography followed by chromatography on DEAE-cellulose is described. The amount of phospholipid-bound choline was greatly reduced when either of these two compounds was incorporated into the phosphatides. The possible significance of these findings is discussed.

Previous studies (1) showed choline to be a dietary requirement for the larvae of the blowfly, *Phormia regina*. However, choline is not "essential" since it can be replaced in the diet by the addition of one of several nitrogen compounds; namely, carnitine, γ-butyrobetaine, β-methylcholine, dimethylamino*iso*-propyl alcohol, or dimethylaminoethanol. Investigations of the phospholipid constituents of this organism (2) revealed that when choline was replaced by carnitine (β-hydroxy-γ-butyrobetaine), β-methylcholine (1-trimethylamino*iso*propyl alcohol), or γ-butyrobetaine, the nitrogen base found in the "lecithin" fraction was primarily β-methylcholine (3, 4).

Dimethylaminoethanol has been shown to be a component of phospholipids of *Neurospora crassa* (5, 6), and several investigators have shown that dimethylaminoethanol-containing phospholipids (7–11) are intermediates in the biosynthesis of lecithin from phosphatidyl ethanolamine. Ansell and Chojnacki (12, 13) have found that rat brain suspensions incorporate cytidine diphosphodimethylaminoethanol and cytidine diphosphomonomethylaminoethanol into phospholipids similar to the biosynthetic pathways described by Kennedy (14) for other phospholipids.

For these reasons and the obvious relations of dimethylaminoethanol to choline and dimethylamino-*iso*propyl alcohol to β-methylcholine, it seemed appropriate to investigate the larval phospholipids when *Phormia regina* is reared on a diet containing either dimethylaminoethanol or dimethylamino*iso*propyl alcohol.

METHODS

Larvae were reared on the amino acid diet described previously (14, 15) except that 25 mg of the hydrochlorides of dimethylaminoethanol or dimethylamino-*iso*propyl alcohol were substituted for choline. Commercial samples of dimethylaminoethanol and dimethylamino*iso*propyl alcohol were purified by distillation, (boiling ranges 61–63° and 83–87°, respectively). These fractions were treated with an excess of HCl and evaporated to dryness prior to their use in the diets or

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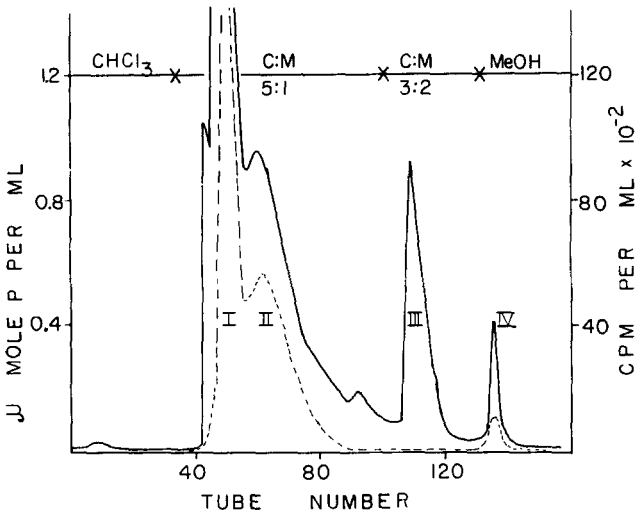

FIG. 1. Silicic acid-Hyflo Supercel chromatography of the phospholipids extracted from 66 g of larvae that had been reared on a defined diet in which the dietary choline was replaced by dimethylaminoethanol containing dimethylaminoethanol-1,2-C^{14}. The column diameter was 8 mm and consisted of 16 g silicic acid and 8 g Hyflo Supercel. C = chloroform; M = methanol. The dashed line represents cpm/ml and the solid line μmoles phosphorus/ml. Collected 400 drops (5.5–6.5 ml) per tube.

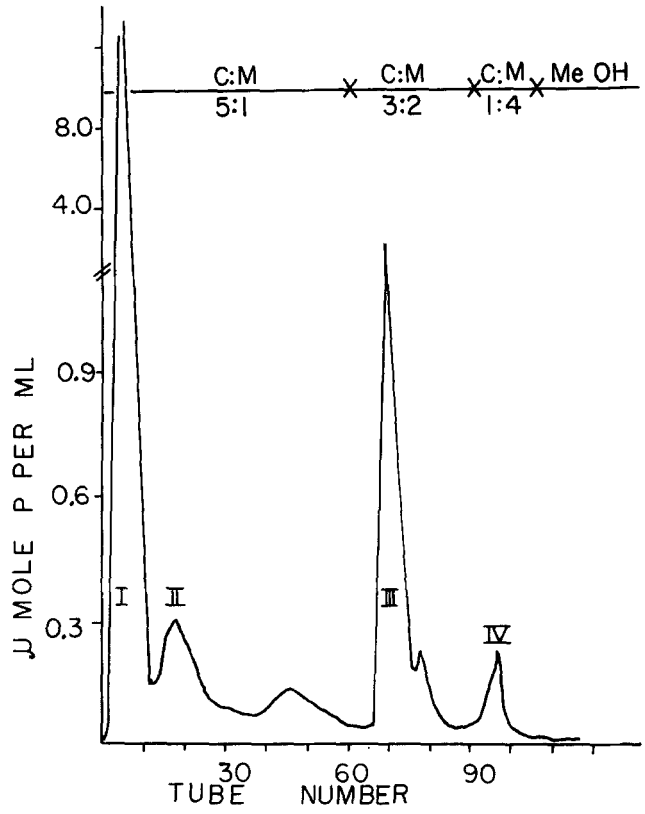

FIG. 2. Separation of the phospholipids isolated from 45 g of sterile larvae that had been reared on a diet containing dimethylamino*iso*propyl alcohol. Compare Fig. 1.

as standards for paper chromatography. Two microcuries (0.74 mg) of dimethylaminoethanol-1,2-C^{14} hydrobromide were mixed with 16 mg of unlabeled dimethylaminoethanol hydrochloride and added to each rearing flask where indicated. The procedures for extraction, purification, and resolution of the phospholipids on silicic acid columns (Figs. 1 and 2) have been described previously (2, 4). The total chloroform eluate from the silicic acid columns was often collected as one fraction. The phosphorus content of these neutral lipid fractions was always very low, and they were discarded. Since the lecithin content was very low and sphingomyelin was absent in this organism, the elution scheme was modified. The chloroform–methanol 1:4 was often eliminated and the amount of chloroform–methanol 3:2 was reduced. Under these conditions, the lecithin (Peak IV in Figs. 1 and 2) is eluted at the end of the chloroform–methanol 3:2 fraction or by the next eluting solvent.

One of the principal nitrogen constituents of Peaks I and II of Fig. 1 was dimethylaminoethanol, and that in Peaks I and II of Fig. 2 was dimethylamino*iso*propyl alcohol. Since these fractions also contained other nitrogen components, they were further purified by a second chromatography on DEAE-cellulose, exactly as described by Rouser et al. (16), except that the DEAE-cellulose was washed with methanol after the acid and base treatments. From 5 to 12 g of DEAE-cellulose was used per column (column diameter 8 mm). This procedure separated the new phospholipids (peak A, Figs. 3 and 4) from more acidic phospholipids, chiefly phosphatidyl ethanolamine.

Pooled fractions from the DEAE-cellulose column were hydrolyzed overnight in 6 N HCl, extracted with ether, and evaporated to dryness. The residues were dissolved in distilled water and chromatographed on a Dowex-50 ion exchange column (6-mm diam, height 30–35 cm), eluting with 0.5 and 1.0 N HCl. The individual fractions were evaporated to dryness, dissolved in distilled water, and made to a known volume. The tubes containing dimethylaminoethanol or dimethylamino*iso*propyl alcohol were located by paper chromatography using the methods described previously (2). The two most useful solvent systems for paper chromatography were 95% ethyl alcohol–conc NH_4OH (95 : 5) and phenol–*n*-butanol–98–100% formic acid–H_2O (50 : 30 : 3 : 10) plus KCl crystals. Whatman No. 1 paper impregnated with 1% KCl was used with the latter solvent system. Ester, nitrogen, and phosphorus determinations were made as described previously (2–4). Infrared analyses were performed with a Beckman IR5A spectrophotometer using micro cells. The samples were run in spectro grade CCl_4.

Radioactivity of the phospholipids eluted from the silicic acid and DEAE-cellulose columns was determined by adding 0.5-ml aliquots of the fractions to 10 ml of the following mixture: 120 g naphthalene, 0.4 g 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP), 8 g 2,5-diphenyloxazole (PPO), 200 ml methanol, 40 ml ethylene glycol, and sufficient *para*-dioxane to make 2.0 liters. The samples were counted in a scintillation counter.

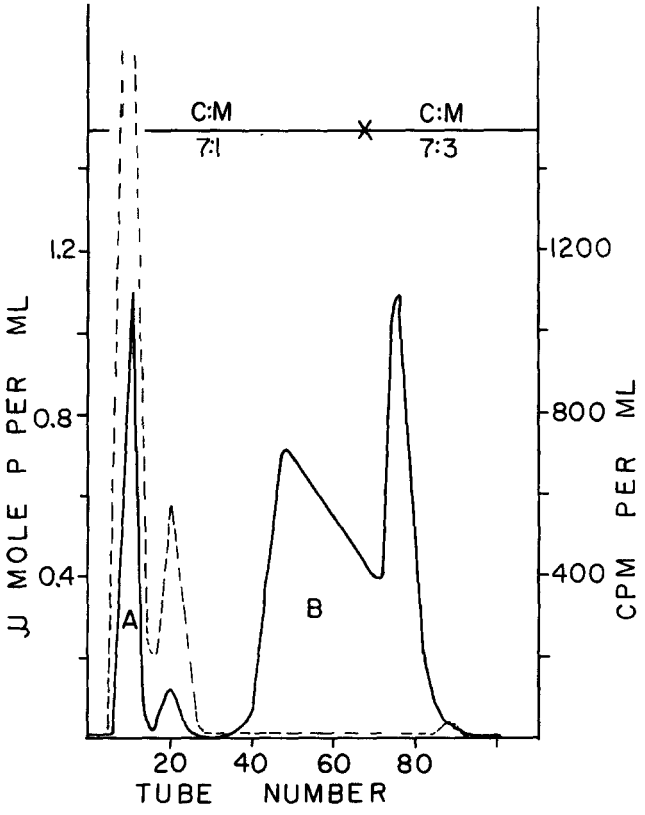

FIG. 3. Separation of Peak I of Fig. 1 (tubes 42–55) using 12 g DEAE-cellulose and an 8-mm diameter column. C = chloroform; M = methanol. The broken line represents cpm/ml and the solid line μmoles P/ml. Collected 5.5–6.5 ml (400 drops) per tube.

The recovery of lipid phosphorus from the columns represented by Figs. 3 and 4 varied from 88 to 101% of the phosphorus applied to the column, indicating that most, if not all, of the dimethylaminoethanol-, dimethylamino*iso*propyl alcohol-, and ethanolamine-containing phospholipids were recovered from the DEAE-cellulose. The eluates obtained represented only material eluted by chloroform–methanol 7:1 and 7:3 and would not include any of the more acidic phosphatides that may have been present in the first two peaks of Fig. 1.

RESULTS

In initial nutritional studies, it was found that larvae reared on a diet containing dimethylaminoethanol or dimethylamino*iso*propyl alcohol in place of choline or carnitine required 1–2 more days for maximum growth. If diethylamino*iso*propyl alcohol was used as the dietary substitute, the larvae failed to grow.

The pattern shown in Fig. 1 is typical for phospholipids isolated from larvae reared on a diet containing dimethylaminoethanol. Similar patterns were obtained when larvae were grown in the presence of dimethylamino*iso*propyl alcohol (Fig. 2). Figure 3 illustrates the resolution on DEAE-cellulose of Peak I of Fig. 1. Similar patterns were obtained from Peak I, Fig. 2 isolated from larvae reared in the presence of dimethylamino*iso*propyl alcohol. Acid hydrolysates of the fraction equivalent to Peak A of Fig. 3 contained dimethylaminoethanol or dimethylamino-*iso*propyl alcohol (demonstrated by paper chromatog-

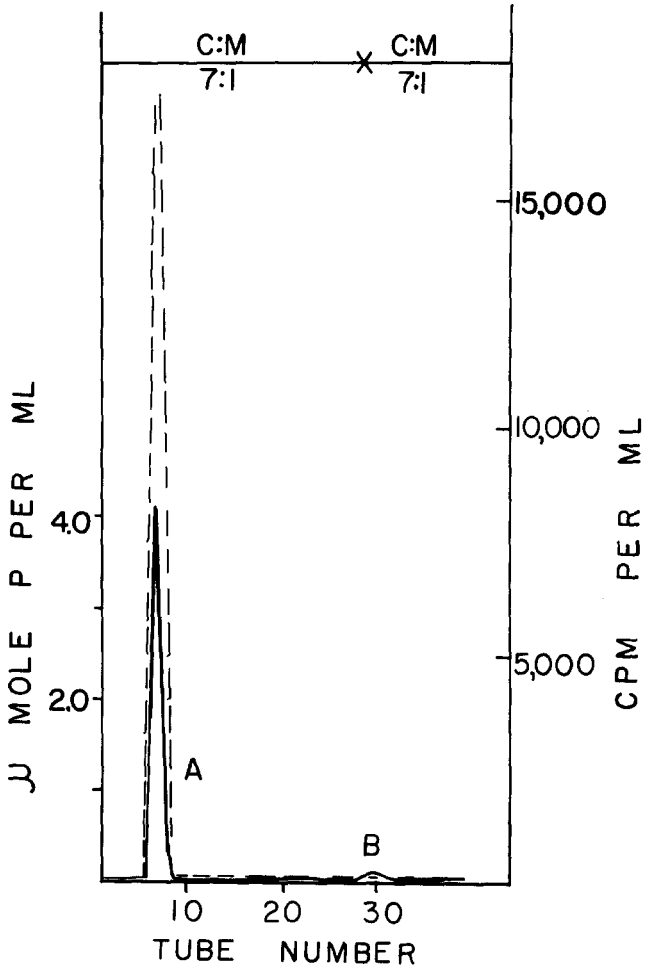

FIG. 4. Separation on DEAE-cellulose of Peak II of Fig. 1 (tubes 56–85). Collected approximately 13 ml per tube. Compare Fig. 3.

TABLE 1. ANALYSIS OF DIMETHYLAMINOETHANOL- AND DIMETHYLAMINOISOPROPYL ALCOHOL-CONTAINING FRACTIONS* OBTAINED FROM DEAE-CELLULOSE COLUMNS

Major Nitrogen Compound Detected in the Hydrolysate	Mole Percentage of the Total P Represented by this Material	DMIA/P Mole Ratio†	DMAE/P Mole Ratio†
Dimethylamino*iso*propyl alcohol	16.2	1.0	...
Dimethylamino*iso*propyl alcohol	19.4‡	0.9	...
Dimethylamino*iso*propyl alcohol	17.4	1.0	...
Dimethylaminoethanol	29	...	0.9
Dimethylaminoethanol	33	...	0.9

* Combined material corresponding to Peaks A of Figs. 3 and 4. The values were obtained from individual experiments.

† DMIA, dimethylamino*iso*propyl alcohol; DMAE, dimethylaminoethanol.

‡ Represents only the material equivalent to Peak A of Fig. 3. The total phosphatide percentage was 20.4% (Peak A of Fig. 3 + Peak A of Fig. 4).

raphy) depending on which was added to the diet, while acid hydrolysates of the other main fraction, Peak B, contained ethanolamine. Peak B of Fig. 3 was sometimes eluted with chloroform–methanol 7:1 and at other times with chloroform–methanol 7:3; however, in no instance did the fractions overlap. Figure 4 represents the rechromatography of Peak II of Fig. 1 on DEAE-cellulose. The first fraction (Peak A) contained dimethylaminoethanol or dimethylamino-*iso*propyl alcohol depending on which was added to the diet. The second fraction (Peak B) was an ethanolamine-containing phospholipid. Peaks A of Figs. 3 and 4 appeared to contain identical material, in spite of their being derived from two distinct peaks on silicic acid. Peak III contained inositol, lysophosphatidyl ethanolamine, and the major portion of plasmalogen as reported previously (2).

Known amounts of the phospholipids were hydrolyzed and the bases resolved on Dowex-50 columns. Nitrogen analysis of the eluted material gave N/P ratios of 0.9–1.0 (Table 1); very little, if any, amino nitrogen was detected.

The ester/P ratios of the dimethylaminoethanol- and dimethylamino*iso*propyl alcohol-containing fractions obtained from DEAE-cellulose fractionation were 1.9–2.1, demonstrating that the material was predominantly in the diester form. Infrared analysis gave the following absorption bands: 2.96 μ (NH or OH stretching), 3.44 and 3.52 μ (CH stretching), 5.78 μ (carbonyl of esters), 6.78 μ (CH_2–CH_3 stretching), 8.0–8.25 μ (P=O stretching), and 9.38 μ (P—O-aliphatic stretching). These absorption maxima are typical for ester-containing phospholipids (17, 18).

Dimethylamino*iso*propyl alcohol-containing material obtained from the DEAE-cellulose columns accounted for about 18% of the total lipid phosphorus; the corresponding figure for dimethylaminoethanol-containing phosphatide was approximately 30% (Table 1).

The amount of phosphatidyl choline found in the larvae decreased from 20% of the total phospholipid when they were reared in the presence of choline to 2–3% when dimethylaminoethanol or dimethylamino-*iso*propyl alcohol were substituted for this nitrogen base in the diet. An average of 2.4% (1.9–3.2%) was obtained from six individual experiments with dimethylaminoethanol and an average of 2.3% (1.9–3.2%) from four individual experiments with dimethylamino-*iso*propyl alcohol. These values represent the total phosphorus in the lecithin and any subsequent fractions eluted from the original silicic acid columns. Hydrolysis of this latter material indicated that choline was the major nitrogenous component although minor amounts of other nitrogen compounds were detected. Similar amounts of choline were found in the lecithin fraction when carnitine or β-methylcholine were used in the diet (15). The radioactive material in the lecithin fraction (Peak IV, Fig. 1) was dimethylaminoethanol, as determined by paper chromatography of the acid hydrolysates and subsequent radioautography. Radioautography failed to reveal any choline in the radioactive dimethylaminoethanol standard, although a trace of monomethylaminoethanol was detected. No explanation of the appearance of radioactivity in this fraction can be offered at this stage.

In all instances, the ethanolamine-containing phospholipids were the predominant ones, and the other fractions were qualitatively similar to what was reported previously (13).

DISCUSSION

It is apparent that dimethylaminoethanol and dimethylamino*iso*propyl alcohol are incorporated into the phospholipids of *Phormia regina* larvae when they are added to the diet in place of choline. Previously it was shown (4) that β-methylcholine (1-trimethylamino-2-hydroxypropane) was also incorporated into the larval phosphatides. In all three instances, these compounds replaced most of the phospholipid-bound choline. The amounts of this latter compound were approximately the same (2–3%) when either dimethyl-

aminoethanol, dimethylamino*iso*propyl alcohol, methylcholine, or carnitine were used as the dietary choline substitutes. In addition, synthesis of bound choline could not be detected when dimethylaminoethanol-C14 was added to the diet. It thus appears that the source of this small amount of phosphatidyl choline is most likely either the egg or dietary contamination. β-Methylcholine was not detected in the phospholipid hydrolysates when the larvae were reared in the presence of dimethylamino*iso*propyl alcohol, again indicating that methylation to the quaternary ammonium compound does not occur in the organism.

The significance of this organism's ability to alter the phospholipid composition when one of the dietary vitamins is changed cannot be fully evaluated at present. One of the major roles proposed for phospholipids is in membrane structure. It is possible that the phosphatides in question have a certain structural requirement that can be fulfilled by any one of several phospholipids. Although the replacement of choline by dimethylaminoethanol or dimethylamino*iso*propyl alcohol involves substitution of a tertiary nitrogen for a quaternary nitrogen, this may not be too surprising since at physiological pH the tertiary nitrogen could be protonated and thus carry a positive charge.

The reason for the consistent appearance of two partially separated dimethylaminoethanol- or dimethylamino*iso*propyl alcohol-containing phosphatides on the silicic acid columns is not known. The two peaks did not show this behavior when they were rechromatographed on DEAE-cellulose, but the resolving power of these columns is not great for nonacidic phospholipids. No significant difference in the ester-to-phosphorus ratio between the two fractions was found. The fatty acids were not investigated, hence their contribution to this behavior is not known. It is possible that these phospholipids exist partially in the nonprotonated and partially in the protonated form, since dimethylaminoethanol and dimethylamino*iso*propyl alcohol are weak bases, and the two forms may chromatograph differently on silicic acid columns (compare the complex chromatographic behavior of phosphatidyl serine, attributed by Marinetti, Erbland, and Stotz (19), Rathbone (20), and Rouser et al. (16) to the ion-exchange properties of silicic acid and Hy-flo Supercel in the presence of salts).

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