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Title Characterization of a Naturally Occurring Beta-methylcholine Phosphatide and Related Studies

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The major phospholipids of the larvae of the blowfly Phormia regina were investigated. A correlation was found to exist between phospholipid composition and the presence in the diet of certain nitrogen compounds. Two new phospholipids were isolated and characterized.

The phospholipids were separated by chromatography on silicic acid-Hyflo or by DEAE-cellulose columns after the lipids had been extracted with chloroform-methanol.

When the larvae were reared on an amino acid-containing diet and carnitine was used as a dietary choline substitute, the majority of the choline in the lecithin fraction was replaced by beta-methylcholine (1-dimethylaminoisopropyl alcohol). The beta-methylcholine was identified by melting points of the chloroplatinate and reineckate derivatives, by carbon

and hydrogen analyses of the chloroplatinate derivatives, and by comparative paper chromatography using several different solvent systems. Similar results were obtained when the dietary choline was replaced by authentic beta-methylcholine. The results demonstrate that carnitine is decarboxylated by the larvae of this insect.

If the dietary choline is replaced by either dimethylaminoethanol or 1-dimethylaminoisopropyl alcohol, these compounds appear as phospholipid components. The isolation, separation and identification of these phospholipid components is described. The incorporation of the dimethylaminoethanol was demonstrated by use of chain labeled dimethylaminoethanol.

When the larvae are reared on a casein-yeast diet, the choline phospholipids represent 20-25% of the total phospholipids; however, when the larvae are reared on a more defined diet and the dietary choline is replaced by carnitine, beta-methylcholine, dimethylaminoisopropyl alcohol or dimethylaminoethanol, the choline containing phospholipids are reduced to 2-3% of the total phospholipid. The lecithin content was the same when any one of the compounds was used as the dietary choline substitute. No radioactivity was detected in the phospholipid bound

choline when dimethylaminoethanol was added to the medium in place of choline.

These results show that in this larvae there is a relation between the presence of certain nitrogenous compounds in the diet and their appearance as phosphatide constituents. The larvae failed to grow if all of the above mentioned compounds were omitted from the diet. The larvae were unable to grow if diethylaminoisopropyl alcohol was used as the dietary choline substitute.

Ethanolamine-containing phospholipids were the predominant phospholipids in all instances investigated. Evidence is also presented for the existence of an ethanolamino-containing sphingolipid in this insect.

CHARACTERIZATION OF A NATURALLY OCCURRING BETA-METHYLCHOLINE PHOSPHATIDE AND RELATED STUDIES

by

LORAN LAMOINE BIEBER

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To Marion and Michael Bieber

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INTRODUCTION

Although the existence of phospholipids has been recognized since 1790 (96, p. 9) it is only recently that scientists have begun investigating the biological occurrence and chemistry of these compounds. Such studies were hindered because of the lack of adequate methods for the isolation and characterization of phospholipids and the fact that they often bind amino acids, salts and other material with the result that these latter materials are often included with the lipid components. Recent methods have aided in overcoming some of these difficulties, yet no single method appears to resolve completely a complex mixture of phospholipids.

The methods used for phospholipid separation and characterization are numerous. The most frequently used separation is chromatography on silicic acid-Hyflo Super Cel columns. The partial hydrolytic technique of Dawson (26), thin layer chromatography such as the method described by Lie and Nyc (64), DEAE-cellulose columns and chromatography using impregnated papers are all useful techniques which can be used to supplement one another. The partial hydrolytic procedure (24) recently improved by Dawson *et al.* (26)

may be very useful for more complete characterization of phospholipid mixtures. Dawson et al. (26) report the characterization of the base portion of phosphatides, and they are able to differentiate the amounts of ether, ester and plasmalogen of various phosphatides.

The chemistry of phospholipids and the methods of investigation have been reviewed by several persons. Hanahan's (44) book is probably the most thorough. Although the reviews of Marinetti (68), Rapport and Norton (79, p. 103-138), Heftman (45, p. 16R), and Jensen (58) are less comprehensive, they are more recent.

The earlier more complete characterizations of phospholipids were performed primarily on animal organs such as liver and brain. The major phosphatide generally contained choline while the bases, ethanolamine, serine, inositol and sphingosine were present in lesser amounts. However, as more organisms were investigated, it soon became apparent that the phospholipid composition varied greatly depending upon the source. Thus, Marr and Kaneshiro (59) have found that the phospholipids of Azotobacter agilis and Escherichia coli did not contain choline. No inositol has been found in the phospholipids of Staphylococcus aureus (70), Taenia taeniaformis, and Cysticerus faciolaris (75). Holobacterium cutirbrum contains 73% diphosphatidyl glycerol (88) and Anthopleura elegantissima contains sphingomyelin as the

principal phospholipid (6). Ethanolamine has been shown to be the major phospholipid base in several different systems (22, 59, 75, 88). Staphylococcus aureus phospholipids contain 66% phosphatidyl-glycerol (70).

In addition to the more common phospholipids, complex ones have been reported. Santiago-Calvo (87) suggested the existence of a phospholipid which contains four phosphorus atoms, two glycerol molecules and one inositol. Several workers have reported the presence of a triphosphoinositol phosphatide. Grado and Ballou (38) have investigated beef brain diphosphoinositide and found that it contained much triphophoinositide. A triphosphoinositide from ox brain has been characterized by Dawson and Dittmer (25) and Dittmer and Dawson (27, 28). Collins (21) has reported a phospholipid with a molecular weight of 2030. It contains two moles of glycerol-1-phosphate, one mole of ethanolamine, one mole of choline, one mole of sugar, one mole of a fatty alcohol, two moles of fatty acids and two unidentified nitrogen components.

Several phosphatides have been reported which contain nitrogen bases other than serine, ethanolamine, or choline. Phosphatidyl threonine has been isolated from tunny muscle (57), and a phosphatidyl inositomannoside has been characterized (26). Monomethylethanolamine and dimethylethanolamine have been shown to be phosphatide components in

Neurospora crassa (41, 42) and rat liver (14, 15). Carnitine has also (8, 76) been found to be a phospholipid component. This thesis adds two more components to the list, namely a β-methylcholine-containing phospholipid and a dimethylaminoisopropyl alcohol-containing phospholipid.

The non-phosphate linked portions of phospholipids have become increasingly diverse. The hydroxyl groups of glycerol have been shown to be free as in lyso-phosphatides, to be esterified with fatty acids, combined with aldehydes to form plasmalogens, joined in an ether linkage, or combined to each other by phosphate as in cardiolipin. The lengths of the carbon chains of the above residues can vary considerably.

From the above discussion it is evident that many different phospholipids have been found. Thus, it is not surprising that any single method of separation is not satisfactory by itself. Therefore, many investigators have characterized only the major phospholipids or have devoted their investigations to one or more particular phosphatides pertaining to their area of work. In mammalian systems several roles have been proposed for phospholipids. Some of the functions appear to be specific for particular phosphatides, while other roles may be of a more general nature. Their ubiquitous occurrence in membranes suggests a major

function in membrane structure. Sphingolipids are found in high concentration in nervous tissue and in the brain.

Phospholipids have been implicated in transport across membranes. Hokin and Hokin (51-54) have shown that phosphatidic acid and phosphatidyl inositol are involved in sodium ion transport. Phospholipids have been implicated in the electron transport system. If they are removed (30) electron transport activity is lost. The activity can be restored by adding back the phospholipids.

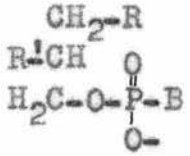
Certain phospholipids are intermediates in the metabolism of biological compounds. Bremer and Greenberg (14, 15, 16) demonstrated that phosphatidyl monomethylaminoethanol and phosphatidyl dimethylaminoethanol were intermediates in the biosynthesis of choline. The studies of Hall and Nyc (41, 42) and Artrom and Lofland (1) support this point of view. In another study (93, p. 374) the conversion of serine to choline is via phospholipid intermediates, since it is now thought that serine is decarboxylated while it is part of a phospholipid. Brockerhoff and Ballou (17) have proposed that monophosphoinositide is converted to triphosphoinositide without removal of the inositol from the original combination with glycerol. Thus diphosphoinositide would be an intermediate in the biosynthesis of triphosphoinositide. Benson (5) has reported that glycerolphosphoryl-glycerol is very rapidly metabolized

in actively photosynthesizing plants and has a high turnover number.

Hendler (46, 47) has evidence that amino acids and peptides are covalently bound to lipids. He suggests that lipid-amino acid complexes may represent stages of amino acid metabolism. Hunter and Goodsall (56, p. 34) concluded that phospholipid-amino acid complexes may be involved in the transfer of amino acids from the site of amino acid activation to the site of protein synthesis. Silberman and Gaby (89) concluded that a phospholipid-amino acid complex was present in Pseudomonas aeruginosa. They found a correlation between the utilization of amino acids and their uptake by the phospholipid complex of the cell. The phospholipids of heat killed cells did not take up labeled amino acids; neither did the phospholipids that had been extracted. McFarlane (71) has found amino acids covalently linked to glycerolphosphoryl-glycerol in certain bacteria. She identified glutamic and aspartic acids, alanine and lysine. Hill (48) concluded from his studies with certain bacteria that amino acids are intimately associated with phospholipids. Although the presence of some amino acids in phospholipid extracts may be artifacts as a result of the isolation procedure, the evidence shows a correlation between uptake of amino acids by phospholipids and amino acid metabolism.

The structures of the principal phosphatides described in this thesis are shown below.

The general structure of the glycerol-containing phospholipids is



When the phospholipid is in the diester form R and R' are fatty acid residues (-O-CO-aliphatic); when the phosphatide is a plasmalogen at least one of the R groups contains a vinyl ether (-O-C(H)=C(H)-aliphatic); if it is in the lyso form at least one of the R groups is an -OH. The structure of B can vary greatly.

Name of the Phospholipids	Structure of B
Lecithin	$-O-CH_2-CH_2-\overset{+}{N}-(CH_3)_3$
Phosphatidyl ethanolamine	$-O-CH_2-CH_2-NH_2$
Phosphatidyl serine	$-O-CH_2-CH\overset{+}{N}H_3-COO-$
Phosphatidyl threonine	$-O-CH(CH_3)-CH\overset{+}{N}H_3-COO-$
Phosphatidyl monomethylamino-ethanol	$-O-CH_2-CH_2-NH(CH_3)$
" dimethylaminoethanol	$-O-CH_2-CH_2-N(CH_3)_2$
" carnitine	$(CH_3)_3-\overset{+}{N}-CH_2-CH(OH)-CH_2-COO-$
" beta-methylcholine	$-O-CH(CH_3)-CH_2-\overset{+}{N}-(CH_3)_3$
" dimethylaminoiso-propanol	$-O-CH(CH_3)-CH_2-N(CH_3)_2$
" glycerol	$-CH_2-CHOH-CH_2OH$

Name of the phospholipid	Structure of B
Monophosphoinositide	-inositol
Diphosphoinositide	-inositol-PO_3H_2
Triphosphoinositides	-inositol-$(PO_3H_2)_2$
Phosphatidic acid	-H
Diphosphatidyl glycerol	-O-CH_2-CH_2OH-CH_2-O-phosphatidic acid
Phosphatidyl inositol-di-D-mannoside	-inositol-2(D-mannose)

Sphingomyelin has the following structure:

$$CH_3(CH_2)_{12}CH{=}CHCHOHCHCH_2OPO_2HOCH_2CH_2\overset{+}{N}(CH_3)_3$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad NHCO\text{-aliphatic}$$

Since a considerable portion of this thesis is related to studies with carnitine (β-hydroxy-γ-butyrobetaine), it is desirable to review also the limited number of biochemical investigations on this compound. Carnitine has been found in many sources (33), and although it is often present in large amounts, little is known of its biosynthesis and metabolism. The early biochemical investigations have been reviewed by Fraenkel (33, p. 74-118). Since 1957 the biosynthesis and metabolic fate of carnitine has been studied by several groups. Binon and Deltour (8) indicated that carnitine was a constituent of plasma lecithins, but Bender and Adams (3, 4) questioned its presence in plasma lipids. Fritz (35, 36) found a

stimulation of fatty acid oxidation by the addition of carnitine. Yue and Fritz (99) injected tritium labeled carnitine into dogs and found that the majority was excreted or deposited in the tissues. Of the latter, a small amount of activity was found in the lecithin fraction of phospholipids isolated from certain of the tissues. The nature of the activity in the lecithin fraction was not determined, but the authors stated that it may be part of some unidentified phospholipid. Wolf and Berger (97) in a rather extensive study were not able to find any good precursors of carnitine in the rat and concluded that the turnover of carnitine in the rat was slow. Mehlman and Wolf (76) have found a small amount of bound carnitine in the phospholipids of hens' eggs after they had been injected with chain labeled carnitine. There was a second labeled phospholipid present in the lecithin fraction, and these authors suggested that it might contain beta-methylcholine. Lindstedt and Lindstedt (66, 67) as well as Bremer (10, 11) have studied carnitine precursors in the rat. They reported that γ-butyrobetaine was a carnitine precursor, but neither γ-aminobutyric acid or its dimethyl analogue were converted to carnitine. Lindstedt and Lindstedt (65) have isolated a system <u>in vitro</u> which will convert γ-butyrobetaine to carnitine, and Bremer (12) obtained acetylation of carnitine with a mitochondrial

system. Lindstedt and Lindstedt (67) suggest that glycine-betaine could be an end product of carnitine metabolism. They found labeled betaine after feeding radioactive carnitine to P. pyocyaneus, an organism using carnitine as the sole energy source. They could not observe labeled betaine when the same techniques were used on rats. The investigations of Sakaguchi (86) indicate a relationship between betaine and carnitine, since they have shown that the betaine requirement of Pediococcus soyae can be replaced by carnitine.

Although many facets of insect biochemistry have been investigated, the phospholipids of these animals have rarely been studied. The amounts of the different classes of insect lipids have been determined, including the investigation by Sugiyama (92) on silkworm larvae, but the determination of the individual phosphatides has seldom been reported. Mitchell and Wren (99) have studied the phosphatides of Drosophila melanogaster. This study, although mainly qualitative, demonstrated that phosphatidyl ethanolamine was the predominant phospholipid in this organism. They were unable to detect any sphingomyelin, which is contrary to what had been found in other systems. Sridhara and Bhat (91) in a very brief note reported that phospholipids of the silkworm Bombyx mori contain 60% phosphatidyl choline but only 3.7% phosphatidyl

ethanolamine. Crone and Bridges (22) reported that the housefly Musca domestica contains 65% phosphatidyl ethanolamine as well as an ethanolamine-containing sphingolipid. As this thesis will describe the principal phospholipid of the egg, larvae and adult of the blowfly Phormia regina contained the base ethanolamine. No sphingomyelin has been detected in this organism.

This thesis is an extension of this research and is concerned with the isolation of two new phospholipids as well as extensive studies of alterations in phospholipid composition as a result of changes in dietary composition.

METHODS

Blowfly larvae were reared aseptically in 500 ml Erlenmeyer flasks. One diet consisted of casein, yeast extract, agar, cholesterol and Belar's salt solution as described by McGinnis et al. (72, p. 310). Another diet contained 3.4 g of the amino acid mixture, a 2.5 fold increase in the vitamin mixture described by McGinnis et al. (72, p. 313); in addition it contained 1.8 g agar, 160 mg RNA, 0.4 g Hammersten quality casein, 50 ml of double strength Belar's salt solution, 50 ml water and 100 mg cholesterol. It was necessary to mix the contents very well, and this was accomplished by use of a magnetic stirrer. The choline in the vitamin mixture was replaced by 25 mg of either carnitine chloride, β-methylcholine chloride, dimethylaminoethanol hydrochloride or dimethylaminoisopropanol hydrochloride. The above mixtures were thoroughly mixed, autoclaved for 20 minutes, cooled and inoculated with blowfly eggs which had been sterilized with 2000 ppm Zephiran chloride. In order to obtain sufficient amounts of larvae containing the β-methylcholine phosphatide, the amino acid mixture was replaced by 3.4 g of Hammersten quality casein. The larvae were tested for sterility, collected and stored in a deep freeze until needed.

The entire tissue was extracted by grinding successively in three aliquots of chloroform-methanol (2:1). Each grinding operation lasted about five minutes in a Waring Blendor. The material was allowed to stand in the solvent for approximately one and three hours after the second and third treatments. The organic phase was separated from the solid by centrifugation. No special precautions were taken to avoid oxidation. The total volume of solvent was at least nine times the weight of the wet tissue, and one-third of the total solvent was used for each extraction. The first chloroform-methanol extract contained an aqueous phase which was removed and washed twice with small amounts of chloroform. The chloroform washings were added to the combined organic extracts. The combined extracts were washed with 0.2 volume of 0.79% NaCl and then washed with 0.2 volume of a 50:50 mixture of 0.79% NaCl and methanol. When the methanol was eliminated in the second wash an interfacial fluff appeared. This fluff may have contained proteolipid as described by Folch *et al.* (32). Since the fluff was not obtained by using proper methanol concentrations, it was not investigated further.

The extract was evaporated to dryness under vacuum and low heat ($<50^{o}$), then dissolved as needed with chloroform. A small amount of chloroform insoluble material was obtained after evaporation. This material appeared to be

protein and probably came from proteolipid material such as the proteolipids described by Folch (32). The lipid extract was dissolved in chloroform and applied to silicic acid-Hyflo Super Cel columns as described by Hanahan et al. (43). The silicic acid had been dried at 105-110° C overnight. The columns were eluted with chloroform, then various ratios of chloroform-methanol and finally with methanol. The amount and composition of the various eluting solvents varied depending on the size of the column and the type of separation desired. The samples were collected with a drop counting fraction collector or a volume collector. Three hundred ten to four hundred drops per tube were collected.

Some of the phospholipid fractions were further purified and separated by rechromatographing on DEAE-cellulose columns similar to the method described by Rouser (84) and coworkers. The DEAE-cellulose was washed with methanol after the KOH and HCl treatments. The last wash was with chloroform, and the DEAE-cellulose poured into the column as a slurry in chloroform. The lipid material was applied to the column in a chloroform solution.

The lipid isolation procedure was tested for completeness of recovery of phospholipids. One hundred fourteen grams of larvae were extracted three times with chloroform-methanol as described above with a total of 900 ml of

solvent (chloroform-methanol 2:1). The residue was extracted a fourth time with chloroform-methanol by grinding for five minutes in a Waring Blendor and letting the mixture stand for 17.5 hours. After filtering the suspension, the total amount of phosphorus in the extract was found to be 8.8 μmoles, which was less than 1% of the phospholipid phosphorus obtained in the first three extractions. Therefore, extraction of phosphatides with this solvent pair appears to have been reasonably effective with three extraction operations, as routinely used. The aqueous phase obtained in the first chloroform-methanol extraction was evaporated to dryness and extracted three times with chloroform-methanol, 2:1, with the third extraction period being greater than 40 hours. The total phosphorus extracted amounted to 1.3 μmoles.

Loss of lipid phosphorus during the washing procedures was also tested. The extract obtained from the first three chloroform-methanol extractions was washed with 180 ml of 0.79% NaCl. This washing was evaporated to dryness and extracted three times with 2:1 chloroform-methanol. The third extraction was over 40 hours in duration. The amount of phosphorus in this wash was 0.1 μmole. One hundred ml of the organic phase were then extracted with 0.2 volume of a 50:50 mixture of methanol-0.79% NaCl. This aqueous layer contained 0.9 μmole of phosphorus, which was

equivalent to 4.5 μmoles of phosphorus (less than 0.5% of the total). The total phosphorus found in the various aqueous phases was less than 1% of the total phospholipid material extracted. The material lost in the aqueous phase was not investigated, since it represented such a small portion of the total.

Recovery of lipid phosphorus from the columns was tested by adding a known amount of lipid phosphorus to the column and measuring the total amount of phosphorus recovered from the column. Recovery from the silicic acid-Hyflo columns was from 94-98% of the phosphorus put on the column. The loss of lipid phosphorus was probably a result of incomplete transferring of the material and to errors in the method, rather than to actual material remaining on the column. Recovery of lipid phosphorus from the DEAE-cellulose columns varied from 50-100% depending on the nature of the phospholipid material applied to the column.

The position of the phospholipid peaks eluted from the columns was determined by phosphorus analyses according to the method of Bartlett (2).

The material which comprised the different fractions was pooled and the various analytical determinations were done on these fractions. Paper chromatography of the nitrogen bases was done on 6 N HCl hydrolysates of this

material. Some of the hydrolysates containing the nitrogenous components were applied to a Dowex 50 column (8 x 400 mm) and successively eluted with 0.5 N, 1.0 N and 1.5 N HCl similarly to the method of Christianson et al. (20). The resulting solutions were evaporated to dryness under vacuum and dissolved to 0.3 ml with distilled water. Aliquots were then taken for identification and determination of the nitrogenous components. Nitrogen was determined by the method of Lang (62). Inositol was detected by the method of Feigel and Gentil (29) and determined quantitatively by microbiological assay with Kleockera apiculata (ATCC 9774). The assay medium resembled closely that of Williams et al. (95). Amino acids were detected with ninhydrin and choline by Dragendorf's reagent. Hydrochlorides of ethanolamine, β-methylcholine, choline, dimethylaminoethanol, dimethylaminoisopropyl alcohol, monomethylaminoethanol and sphingosine were detected on paper chromatograms with iodine vapors. Ester determinations were done by the method of Snyder and Stephens (90). Plasmalogen was detected by a method similar to the one employed by Gray (39). Choline was determined by microbiological assay with Neurospora crassa, ATCC strain number 34486. The cultures were incubated five days at 30° C using Difco assay media. Beta-methylcholine was determined quantitatively by difference based on the total amount of

phosphorus of a purified lecithin fraction minus the amount of choline.

The lecithin fraction used for characterization of -methylcholine (Peak IV, Figure 4) was purified by rechromatographing on a silicic acid-Hyflo column. The column eluate containing the β-methylcholine phosphatide fraction was washed with 0.79% NaCl and evaporated to dryness under vacuum, and the lipid material was refluxed 24 hours in 6 N HCl, extracted with ether and evaporated to dryness using heat and reduced pressure. The residue was dissolved in water, streaked across Whatman #1 chromatography paper (18 x 11 inches) and developed with the solvent mixture, ethanol:conc. NH_4OH (95:5). After drying, one or more narrow strips were cut down from the chromatogram, and the position of the β-methylcholine chloride was located with iodine vapors and Dragendorf's reagent. The area corresponding to β-methylcholine chloride was removed from the chromatogram and eluted with water. The water eluate was evaporated to dryness, and the residue was dissolved in n-butanol, filtered and precipitated by addition of ether. The resulting precipitates were used for preparation of derivatives.

The chloride of methylcholine (1-trimethylamino isopropyl alcohol) was synthesized from 1-amino isopropyl alcohol and an excess of methyl iodide by a method similar

to the one Mazzetti and Lemmon (69) used for the synthesis of carnitine from 3-hydroxy-4-aminobutyric acid. The compound gave a positive Dragendorf test. The chloride was recrystallized from ethanol-ether. Later it was found that n-butanol-diethylether was a better solvent for recrystallization of the chloride.

Butanol solutions containing synthetic β-methylcholine chloride or the unknown chloride were filtered and an excess of chloroplatinic acid in n-butanol was added to the filtrate. The resulting precipitates were washed several times with n-butanol and finally with ether. The chloroplatinates were dissolved in a mixture of hot ethanol, with sufficient water to dissolve the precipitates, and filtered. Then hot n-butanol was added, and the solution was kept at room temperature until crystallization appeared to be complete. The crystals were collected, washed with ether and used for carbon-hydrogen analyses and the determination of decomposition points.

The reineckates were prepared by adding a 2% aqueous solution of reinecke salt to aqueous solutions of either known β-methylcholine chloride or the unknown chloride and allowed to stand overnight at 3° C. The precipitates were collected by centrifugation, washed twice with water, once with ethanol-ether, and decomposition points were determined. Carbon and hydrogen analyses were carried out by

the Galbrath Laboratories, Knoxville, Tennessee. Infrared analyses of the phosphatide in chloroform were performed in a Perkin-Elmer 21 double beam spectrophotometer, with the reference cell containing chloroform and the unknowns dissolved in chloroform.

Infrared analyses were done on some samples with a Beckmen IR5A spectrophotometer using micro cells. The samples were run in spectro grade CCl_4, and CCl_4 blanks were done with each determination.

The following solvent systems were used for paper chromatography: 95% ethyl alcohol: 28% NH_4OH (95:5), 1-butanol:methyl cellosolve: 20% acetic acid (40:15:20), Phenol:1-butanol:98-100% formic acid:H_2O (50:50:3:10) plus solid KCl with either washed Whatman #1 or papers impregnated with 1% KCl. Ethyl acetate:acetic acid:H_2O (3:1:3) upper phase, 1-butanol:ethyl alcohol:H_2O (9:1:10) in NH_3 vapors, n-propanol:NH_4OH conc:H_2O (60:30:10), t-butyl alcohol:H_2O:formic acid (16:4:1).

Larvae were grown on the amino acid diet when labeled dimethylaminoethanol was used. Radioactive dimethylaminoethanol hydrochloride was used in a concentration of 16 mg per flask and 2.0 microcuries of 1,2 C^{14} labeled material. The lipids were extracted and resolved as usual. The fractions obtained from silicic acid Hyflo chromatography which contained radioactivity were chromatographed on

DEAE-cellulose. Ten ml of the following solution was used for counting 0.5 ml of each unknown fraction: 120 g napthalene, 0.4 g POPOP, 8 g PPO, 200 ml methanol, 40 ml ethylene glycol, dissolved to 2.0 liters with para-dioxane. The samples were counted in a liquid scintillation counter.

The presence of sphingosine was investigated by detection with iodine vapors on paper chromatograms and by the quantitative modification of McKibbon's method (7, p. 533). Alkali-stable phospholipids (sphingolipids) were determined by adding normal NaOH to the material and stirring for an hour. The material was then left at 38° C for at least 19 hours. The NaOH solution was extracted several times with chloroform. The chloroform extract was washed with water and evaporated to dryness. Some of this material was rechromatographed on standard silicic acid-Hyflo columns.

Samples of 1-dimethylamino-2-propanol and 1-diethylamino-2-propanol were distilled, and the 61-63° C boiling fraction of the first and the 83-87° C boiling fraction of the latter were collected. They were treated with an excess of HCl and evaporated to dryness. These hydrochlorides were used in the diet and as standards for paper chromatography.

MATERIALS

Phosphatidyl ethanolamine, RNA, phosphatidyl serine, phosphatidyl inositol, myo-inositol, and serine were obtained from Nutritional Biochemicals Corp.; 1-amino-2-propanol and ethanolamine from Eastman Organic Corporation; choline chloride from Merck and Company; chloroform and methanol were Baker analyzed reagents; sphingomyelin from Sylvana Chemical Company; lecithin from British Drug Houses Ltd.; 1-diethylamino-2-propanol and 1-dimethylamino-2-propanol from Aldrich Chemical Company; 100 mesh silicic acid from Mallinckrodt Chemicals; Hyflo Super Cel from Johns-Mansville Products; methacholine chloride from Djac division of Borden Chemical Company; Kloeckera apiculata No. 9774 and Neurospora crassa No. 34486 from the American Type Culture Collection; choline assay media from Difco Laboratories; Baker analyzed hydrogen peroxide from J. T. Baker Chemical Company; DEAE-cellulose (Bio-Rad Cellex-D) from Bio-Rad Laboratories; and Dl-carnitine chloride was donated by International Minerals and Chemical Company and also by Fallek Products Incorporated.

RESULTS

Characterization of Normal Phospholipids

"Normal larvae" in this thesis will refer to larvae grown on the casein-yeast extract diet.

The results of the separation of different classes of phospholipids from larvae are shown in Figure 1. The total phosphorus in each fraction was determined by combining the eluates of each individual peak, diluting to a known volume, and then determining the total phosphorus content of the fraction. This was equivalent to the organic phosphorus in the fraction, since the inorganic phosphorus was negligible.

Table I shows the amounts of the principal phospholipid components present in the larvae. It can be seen that in most fractions the nitrogen content of the individual peaks was greater than the phosphorus content. Extensive washing failed to lower these N:P ratios significantly.

Peak I, the largest, is principally phosphatidyl ethanolamine; however, the positive aldehyde test indicates the presence of some phosphatidyl ethanolamine. Serine and threonine were also found in Peak I, indicating their presence as phospholipid components. Traces of other amino acids were detected in this peak. The front portion of the

Figure 1

Separation of different classes of phospholipids from 144 g of Phormia regina larvae. 310 drops per fraction were collected, and phosphorus determinations were carried out on every third fraction. C = chloroform and M = methanol. The column diameter was 26 mm, and 80 g silicic acid and 40 g Hyflo were used.

Figure 1

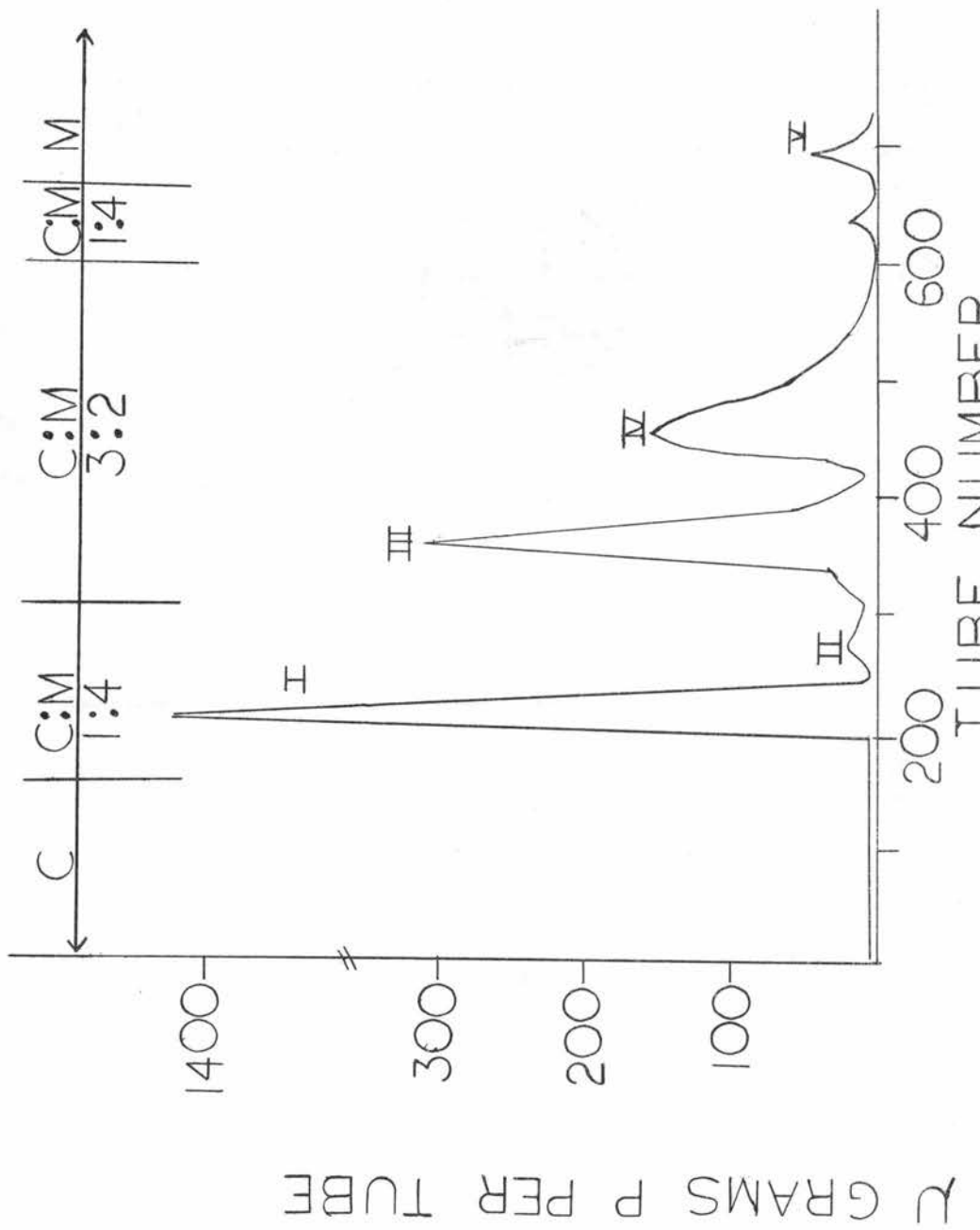

C
C:M 1:4
C:M 3:2
C:M 1:4
M
I
II
III
IV
V
100
200
300
400
200
400
600
TUBE NUMBER
γ GRAMS P PER TUBE

Table I

($CHCl_3$-MeOH extract)

Analysis of the Phospholipids of *Phormia regina* Larva**

Fraction No.	N/P Mole Ratio	% of total P in Fraction	Ester/P mole ratio	Ethanol-amine/P mole ratio	Ser./P mole ratio	Chol./P mole ratio	Inos./P mole ratio	% N Recovered
I.	1.01	58	2.28	0.92	0.03	--	--	96
II.	1.86	3.5	4.2	0.39	0.04	--	--	27*
III.	1.01	17	1.41	0.25	0.10	--	0.43	68*
IV.	1.00	20	2.04	--	--	0.92	--	92
V.	1.39	1.4	0.75 ?	0.20	0.10	0.92	--	108

*Black precipitate formed during hydrolysis

**Represents 144 g of larvae, wet weight

Figure 2

Separation of the material equivalent to Peak II of Figure 1. The column diameter was 16 mm; 30 g silicic acid and 15 g Hyflo were used. 400 drops per tube were collected, and phosphorus analysis was done on each fraction. C = chloroform and M = methanol.

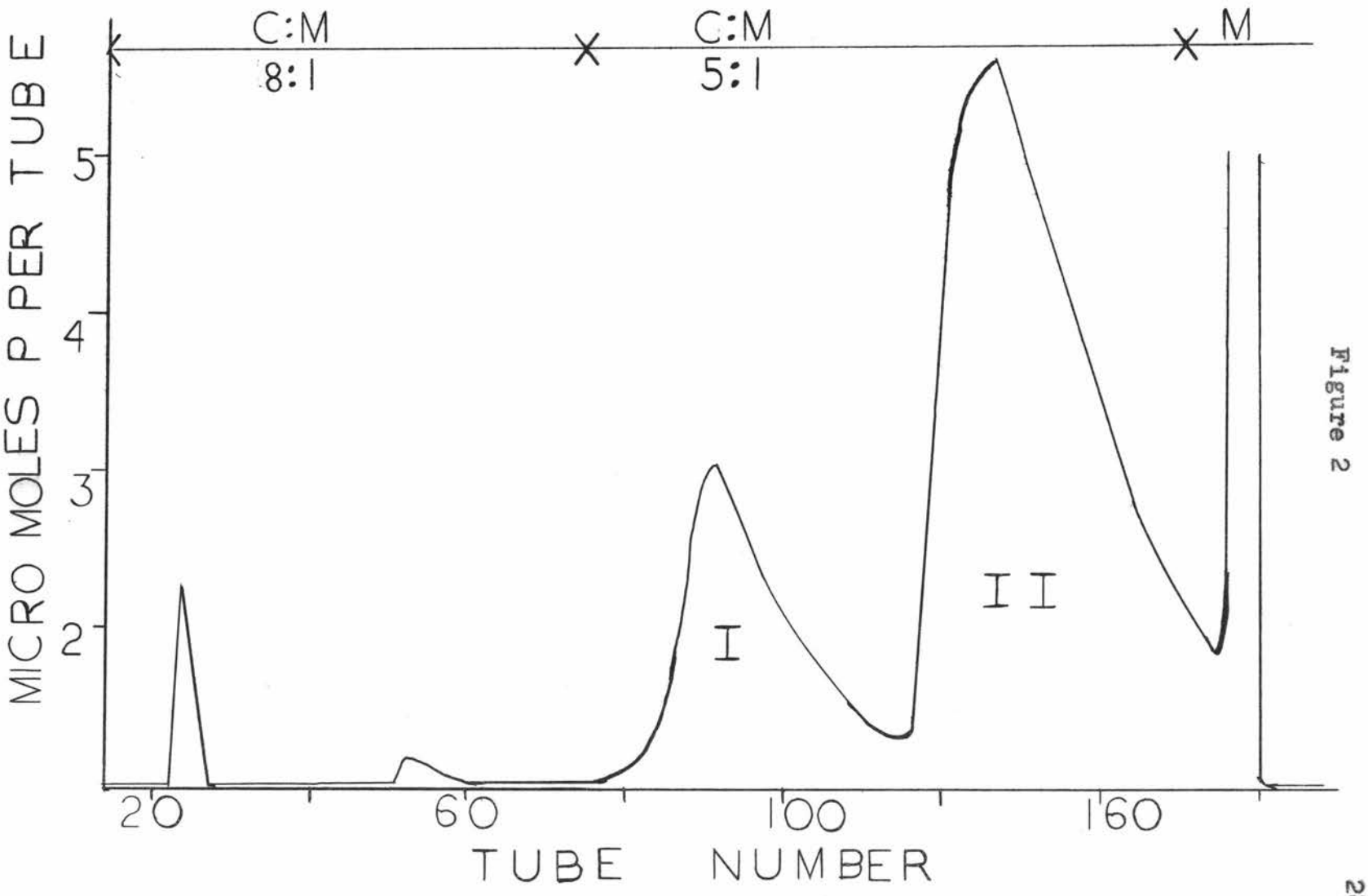
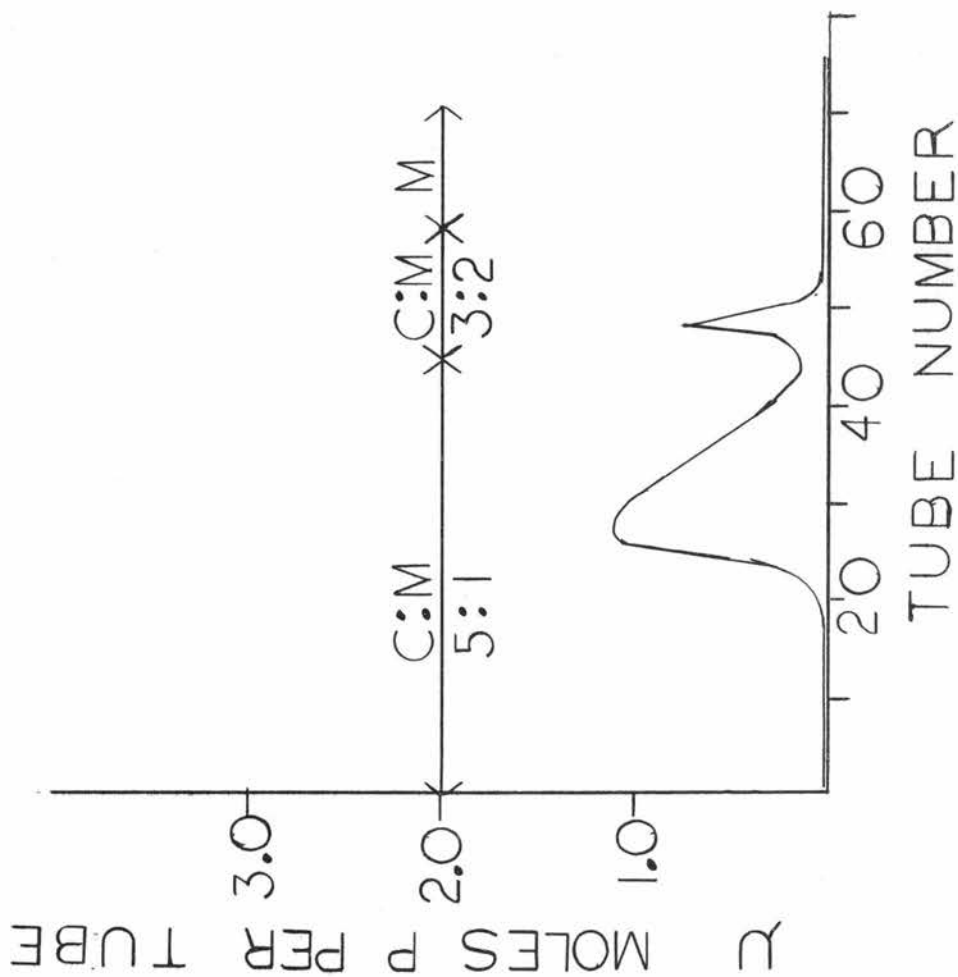

C:M
8:1
C:M
5:1
M
MICRO MOLES P PER TUBE
5
4
3
2
I
II
20
60
100
160
TUBE NUMBER

Figure 2

Figure 2A

Separation of the major peak from Figure 2 (Peak II) after it had been treated with 1 N NaOH for 20 hours at 38° C. Column diameter was 8 mm; used 8 g silicic acid; 4 g Hyflo. Phosphorus determinations were done on every tube.

Figure 2A



phosphatidyl ethanolamine peak (I) of the larvae was colored brown and had a high N:P ratio. This brown colored material, sometimes eluted as a shoulder, also appeared in elution patterns of the phospholipids isolated from larvae grown on the amino acid diet. The intensity of the color appeared to depend on the severity of heating during the evaporation of the solvent and may have been caused by oxidation of the fatty acids. The brown material generally contained more nitrogen than phosphorus. This would be in accord with the idea proposed by some investigators that oxidized phospholipids are more likely to include extraneous materials.

Peak II of Figure 1 contained high N:P ratios. It contained mainly ethanolamine but in addition some serine, threonine and other unidentified nitrogen components were detected. The sphingolipid material also occurred in this peak.

Figure 2 illustrates the re-chromatographing on silicic acid of a fraction equivalent to Peak II of Figure 4. This material represented 5.6% of the total lipid phosphorus. Figure 2 represents material isolated from larvae which had been reared on Hammersten's quality casein and a vitamin mixture in which the choline had been replaced by carnitine. Peak I of Figure 2 was shown to be only phosphatidyl ethanolamine by using Rouser's (83) hydrated

silicic acid technique. It had a nitrogen to phosphorus ratio of 1.0.

Peak II of Figure 2, representing approximately 3% of the total lipid phosphorus, contained ethanolamine as the major ninhydrin positive component. Sphingosine or a sphingosine-like material was detected with iodine vapors and in the chloroform soluble nitrogen isolated from a basic hydrolysate. The determination of base stable nitrogen gave 81, 43, and 31% of the material as base stable. The higher value had a N:P ratio less than 2.0, indicating incomplete hydrolysis of the non-sphingolipid material. The other two samples had N/P ratio of 1.97 and 2.0. Silicic acid-Hyflo chromatography of one of these basic hydrolysates (Figure 2A) gave one peak in the same position as Peak II of Figure 2. This demonstrated that the chloroform soluble-base treated material had the same silicic acid chromatographic properties prior and after hydrolysis. This material contained ethanolamine and sphingosine, indicating an ethanolamine-containing sphingolipid. However, traces of serine and threonine also could be detected in the acid hydrolysates of this material. The overall yield of this material was about 1.5% of the total lipid phosphorus. This value may be spuriously low since one does not know whether any of the material was hydrolyzed during the alkali treatment. Also, it is possible

that not all of the material in question was present in Peak II of the original column. Some of it may have overlapped into Peak III.

Peak III of Figure 1 contained inositol, serine, ethanolamine, threonine, and other ninhydrin positive materials. The ester values (Table I) indicated that much of the phospholipid was not in the diester form. The low ester value could be caused by lyso, ether, plasmalogen and sphingosine-containing compounds. The strong aldehyde test indicated the presence of a considerable amount of plasmalogen. The overall percentage of the total phospholipid represented by this peak was lower when the small columns were used. This may have been caused by the longer time of elution with the chloroform-methanol 4:1 or 5:1 when the small columns were used. Possibly more of it would be contained in Peak II. In addition it is known that plasmalogens are subject to slow hydrolysis by silicic acid. The serine and ethanolamine containing lyso-phosphatides might be found in fraction III. Peak IV of Figure 1 was predominantly phosphatidyl choline.

The last major fraction in Figure 1 contained choline, ethanolamine and certain amino acids. This material contained some lysolecithin. Infrared analysis of a peak equivalent to Peak V, Figure 1 gave a stronger -OH absorption band than the known diesters and also gave a weaker

ester absorption peak. This suggests that Peak V is at least partly in the lyso form. Some of the phospholipid material remained on the large columns for four or more days. The smaller columns were often resolved in less than a day, thus allowing less time for alteration of the phospholipids. Since certain plasmalogens are hydrolyzed on silicic columns, the appearance of Peak V on the large columns may be the result of degradation of these compounds.

Hydrolysates of the lecithin fractions, the chloroform:methanol 1:4 eluates and the methanol eluates did not give positive sphingosine tests, indicating the absence of sphingomyelin. The absence of sphingomyelin in this insect is further supported by the results obtained with larvae grown on the amino acid diet in which the dietary choline had been replaced by dimethylaminoisopropanol or dimethylaminoethanol. The choline-containing phospholipids represented less than 3% of the total phosphatides and no sphingosine could be detected in their acid hydrolysates. Thus if sphingomyelin is present at all, it appears to be in very small amounts.

The above discussion represents results obtained from normal larvae in which the phosphatides were separated on large silicic acid-Hyflo columns. The remainder of the discussion concerns results obtained from phospholipids

resolved on smaller silicic acid-Hyflo columns or on DEAE-cellulose. The only other large columns used were when large amounts of phospholipids were separated in order to obtain sufficient material for characterization of β-methylcholine.

Figure 3 is the elution pattern obtained by resolving a typical phospholipid extract from normal larvae on a DEAE-cellulose column. Only 84% of the phosphorus applied to the column was recovered. Peaks I and II of Figure 3 were combined and resolved on a standard silicic acid-Hyflo column. Peak III of Figure 3 was also chromatographed on a silicic acid column. It was somewhat surprising to find that 12% of Peak III was a choline-containing phospholipid. Rechromatographing the first three peaks on silicic acid made it possible to estimate the amount of ethanolamine phosphatide and choline phosphatide present. The overall choline content from the first three peaks represented 17-18% of the total lipid phosphorus. Hydrolysis and chromatography on paper of the lecithin fractions demonstrated choline was the predominant compound. Only traces of amino nitrogen were detected. This agreed well with the 19% value for lecithin obtained from the large silicic acid columns (Table I). The total ethanolamine-containing phospholipids obtained from the DEAE-cellulose separation represented 52% of the total phosphorus applied to the

Figure 3

Separation of the phospholipids isolated from 210 g of normal larvae. Used 25 g of DEAE-cellulose. The column diameter was 16 mm, and 400 drops per tube were collected. A phosphorus determination was carried out on the contents of every tube.

A = chloroform

B = chloroform-methanol (7:1)

C = chloroform-methanol (7:3)

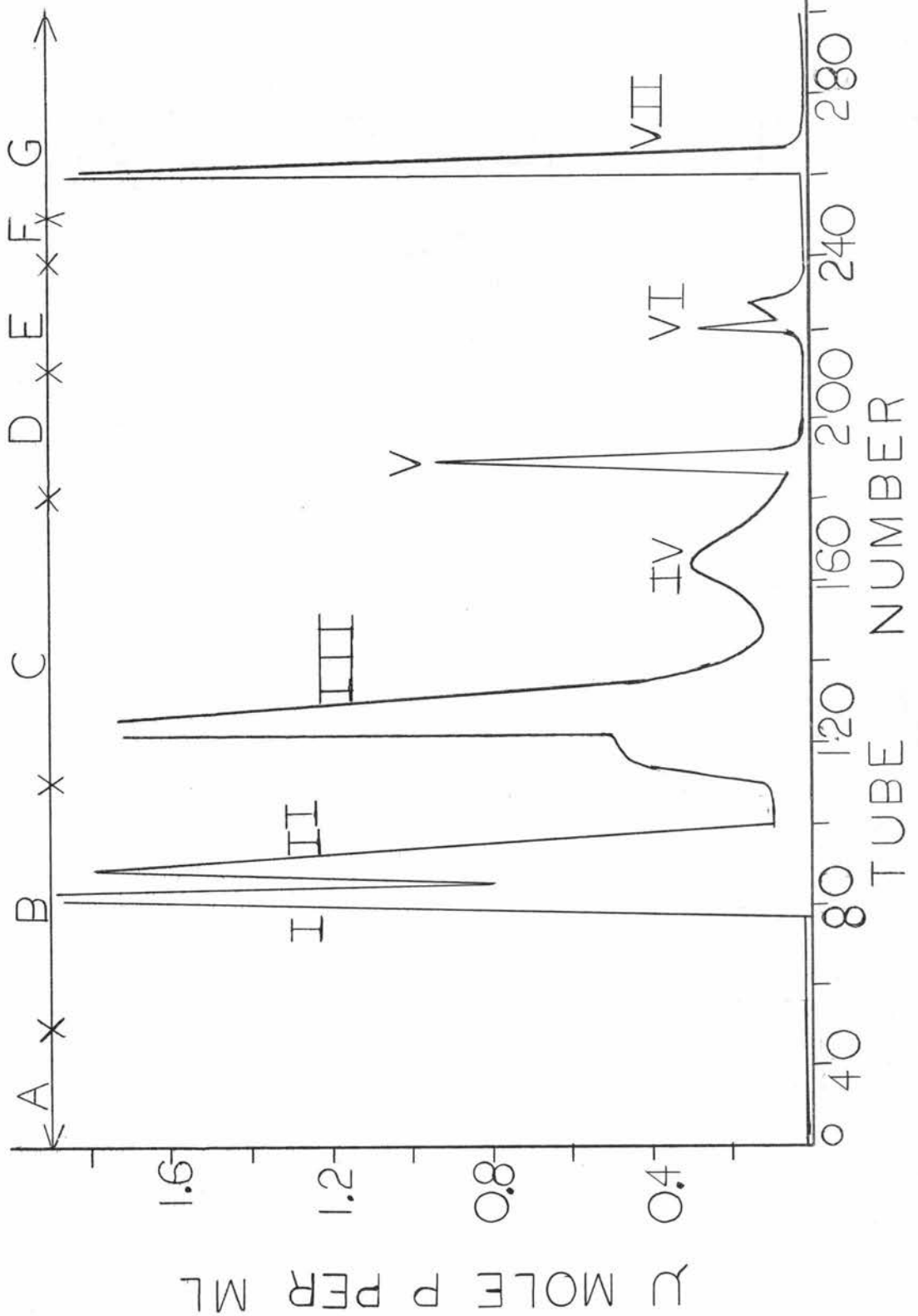
D = methanol

E = methanol-glacial acetic acid (1:1)

F = methanol

G = 9 volumes of $CHCl_3$-methanol (1:1) plus one volume conc NH_4OH

Figure 3


A
B
C
D
E
F
G
I
II
III
IV
V
VI
VII
0
40
80
120
160
200
240
280
TUBE NUMBER
0.4
0.8
1.2
1.6
μ MOLE P PER ML

column. This represented material eluted from the DEAE-cellulose column with chloroform-methanol (7:3), which according to Rouser (82) should be the ethanolamine-containing phosphatides and equivalent to the phosphatidyl ethanolamine isolated by silicic acid chromatography. The acid hydrolysates of these peaks contained some other amino nitrogen, but paper chromatography indicated predominantly ethanolamine. This does not include Peaks VI and VII of Figure 3. Peak VI should contain phosphatidyl serine and ganglioside material. If any phosphatidyl threonine had been present, it could be expected to be found in this fraction. This fraction represented less than 2% of the total phospholipid phosphorus and contained serine, threonine and other amino nitrogen. The last peak should contain inositol phosphatides and cardiolipin. It represented 13% of the total phospholipid. If this were all inositol phosphatide, it would be higher than the 7.3% obtained by the microbiological assay from the material obtained (Figure 1) from the large silicic acid columns. The microbiological assay gives a minimum estimate of the inositol present, since it is known that phosphatidyl inositol is very difficult to hydrolyze completely. The microbiological method will not differentiate diphospho- and triphosphoinositide from monophosphoinositide.

If cardiolipin were present, it would be expected in Peak I of Figure 1. It is possible that some cardiolipin may have been present in this peak, since 5 % of the material in this peak was uncharacterized. However, the variation in the method permits one to question the validity of such a small value.

The DEAE-cellulose separation of normal larvae phospholipids represented only one major separation; thus values given do not represent any average of multiple determinations. They were based on the amount of phosphorus present. However, the experiment tended to confirm the earlier results that ethanolamine phosphatides are the most abundant ones in the larvae when they are reared on the yeast extract-casein diet. The lecithin value was in good agreement, and the other phosphatide bases which were found in the silicic acid column eluates were found in the DEAE-cellulose eluates.

The material (equivalent to Peak III of Figure 4) from several columns, which was isolated from larvae reared on carnitine or β-methylcholine, was pooled and re-chromatographed over DEAE-cellulose. Only 51% of the phosphorus was recovered from the column, but several fractions were obtained. One contained choline, another contained β-methylcholine, another contained serine, threonine, and amino nitrogen. Three partly separated

fractions, eluted by the chloroform-methanol (7:3), contained ethanolamine. The major fraction contained inositol, and it represented 28% of the lipid phosphorus applied to the column. Assuming that this is entirely inositol phosphatide, it would represent 4-5% of the total lipid phosphorus as inositol phosphatide, which is close to the 7.3% obtained for normal larvae as shown in Table I. The number of fractions obtained from DEAE-cellulose demonstrates that the inositol containing peak obtained from silicic acid columns is a very heterogenous mixture.

Characterization of a Beta-Methylcholine Containing Phospholipid

The investigations cited previously demonstrated that larvae reared on casein and yeast extract had a lecithin content of about 20% of the total chloroform:methanol (2:1) extractable phospholipids. Since certain compounds could support larval growth when they replaced the dietary choline, it seemed advisable to see whether this change would be reflected in the phospholipid composition. Carnitine (3-hydroxy-4-trimethylaminobutyric acid) was selected as a substitute because little was known concerning its metabolic fate.

Sterile larvae were reared on the amino acid diet in which the dietary choline had been replaced by carnitine.

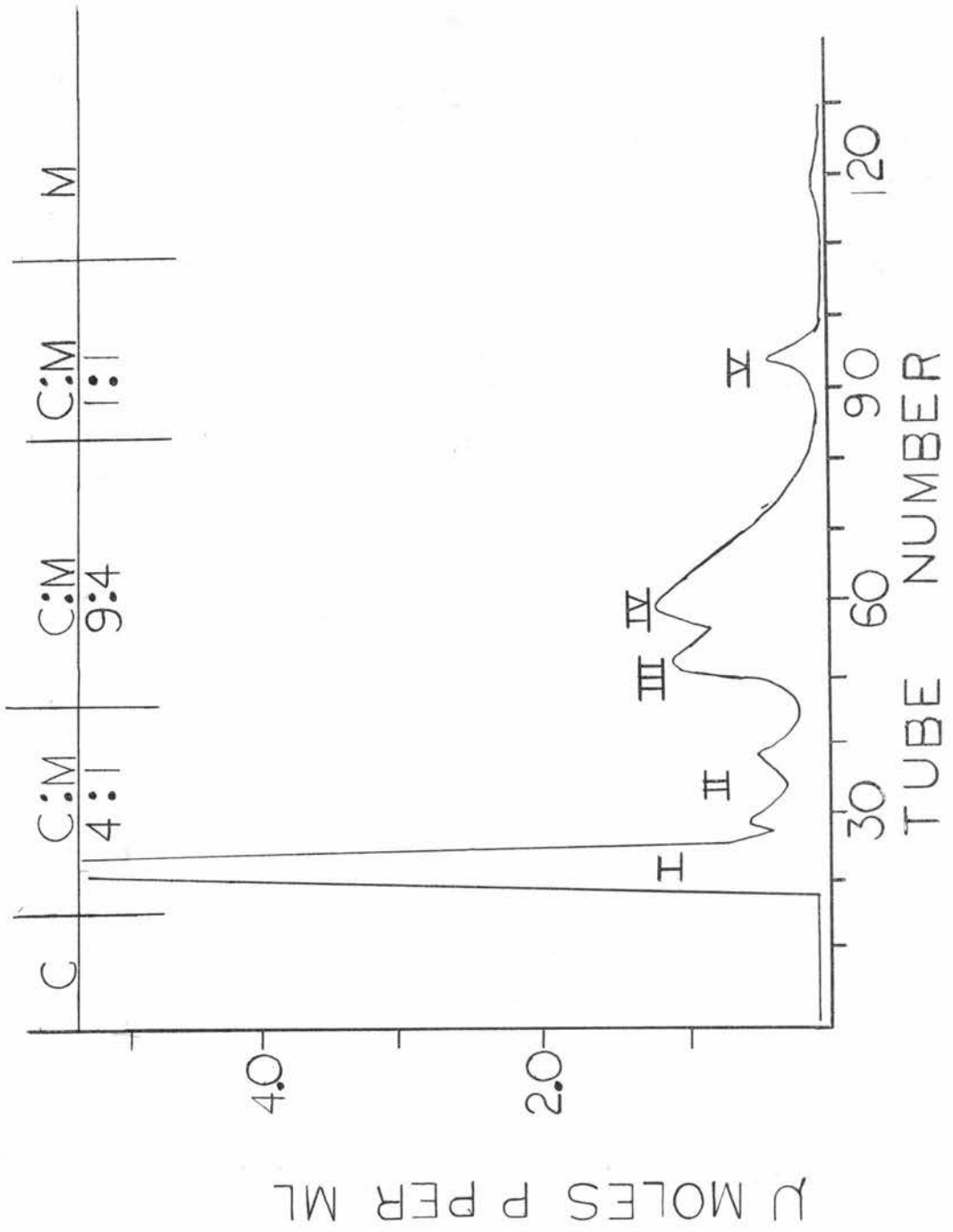
Tissue lipids were extracted and resolved as usual on silicic acid-Hyflo columns. The phospholipid profile appeared very similar to the profile reported earlier for the larvae grown on the normal diet; however, Peak IV appeared to come off the column slightly sooner than previously. The major Dragendorf-detectable substance in an HCl hydrolysate of the lecithin peak did not coincide with choline in the paper chromatographic solvent systems used. The compound was less sensitive to Dragendorf's reagent. The color was slower in developing, and it gave a slightly different color than choline. The color was more pronounced when the nitrogen bases were detected with iodine vapors prior to spraying with Dragendorf's reagent.

The compound had the same paper chromatographic properties as synthetic β-methylcholine chloride. The synthetic compound was also identical with a hydrochloric acid hydrolyzed sample of methacholine (acetyl-β-methylcholine). Phospholipid patterns, similar to those obtained when carnitine was used, were obtained with phospholipids isolated from larvae reared on a diet in which the choline had been replaced by β-methylcholine. Figure 4 is a typical elution pattern obtained from larvae reared on the amino acid diet using β-methylcholine or carnitine as the dietary choline substitute. Peak IV contained β-methylcholine and was further purified by running

Figure 4

Silicic acid-Hyflo separation of the phospholipids isolated from sterile larvae which had been reared on the amino acid-containing diet. The dietary choline had been replaced by β-methylcholine chloride. Collected 400 drops per fraction. Phosphorus determinations were done on every fraction. C = chloroform and M = methanol. 8 mm column diameter: 10 g silicic acid and 5 g Hyflo.

Figure 4


C
C:M
4:1
C:M
9:4
C:M
1:1
M
I
II
III
IV
V
30
60
90
120
TUBE NUMBER
2.0
4.0
μ MOLES P PER ML

over another silicic acid-Hyflo column as shown in Figure 5. Figure 5 represents the phosphorus pattern obtained by pooling the peaks (equivalent to Peak IV of Figure 4) from four different experiments in which the dietary choline had been replaced by carnitine.

The largest peak in Figure 5 was investigated. The major component of this fraction, detectable on paper chromatograms, was β-methylcholine. The ester/P ration was 2.0; N/P was 1.0. A microbiological assay indicated the presence of less than 1% choline; however, the small peak following the β-methylcholine-containing fraction contained both choline and β-methylcholine. The size of this fraction and its choline content depended on the point at which the solvent was changed in the original column.

In order to obtain sufficient material for adequate characterization of the β-methylcholine phosphatide, large amounts of larvae were reared on a diet in which the amino acids had been replaced by Hammersten quality casein. This had no noticeable effect on the phospholipid pattern, and β-methylcholine was still the major nitrogen component detectable in the lecithin fraction.

The β-methylcholine phosphatide is eluted from the silicic acid-Hyflo columns slightly more rapidly than the choline phosphatide, but complete separation of the two

Figure 5

Phosphorus profile of the chromatographing of the combination of four peaks equivalent to Peak IV of Figure 4. The largest peak, eluted by the chloroform-methanol (9:4), had a N/P ratio = 1.0; and choline/P = 0.01. Paper chromatography of the HCl hydrolysate indicated a large amount of β-methylcholine chloride and a trace of choline chloride. The larvae were grown on the amino acid diet with carnitine as the vitamin source. Phosphorus was estimated in every tube. Collected 400 drops per tube. C = chloroform; M = methanol. 8 g silicic acid; 4 g Hyflo and 8 mm diameter column.

Figure 5

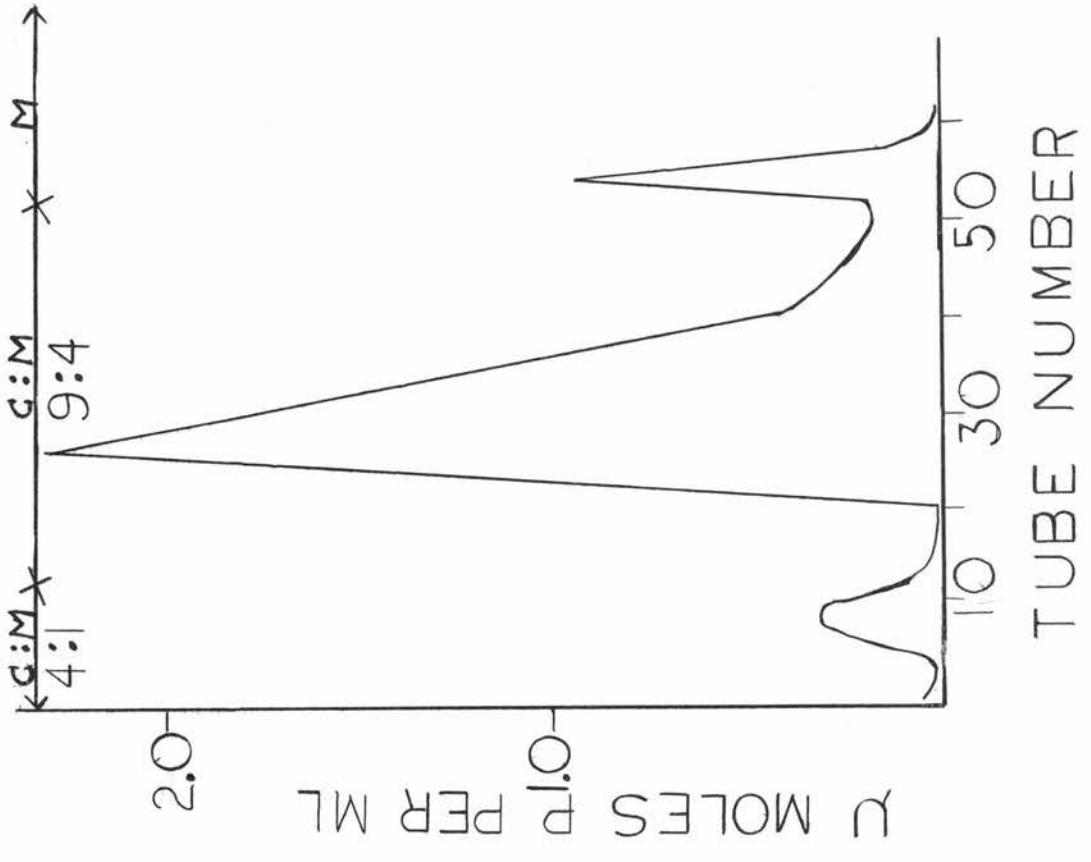


Table II

Composition of Phospholipid with Methylcholine

	Ratio
N/P	1.0-1.2
Ester/P	2.0-2.1
Choline	5% or less by microbiological assay

was not realized. A chloroform:methanol ratio of (9:4) gave better separation of the β-methylcholine and inositol-containing peaks than did (3:2) chloroform methanol. Figure 4 illustrates a typical elution pattern for larvae grown on β-methylcholine or carnitine; Peak IV contained 24% of the phospholipid phosphorus, with a choline/P ratio of 0.04; the remainder of the phosphatide contained mainly β-methylcholine with a small amount of material overlapping from Peak III. The major choline- and β-methylcholine-containing fractions (Peaks IV and V of Figure 4) represented from 21 to 27% of the lipid phosphorus when carnitine or methylcholine was used as a vitamin substitute for the dietary choline, whereas only 1-5% of the total lipid phosphorus eluted from the columns could be accounted for as choline.

Table II shows that the phosphatide in question is mainly, if not entirely, of the diglyceride type. The phospholipid pattern varied slightly from column to column. This was caused in part by the use of different amounts of eluting solvent and adsorbent. Occasionally a peak was obtained as a shoulder in front of Peak I.

No quantitative estimation of the individual phosphatide components was performed other than for choline and methylcholine. The nitrogen and inositol composition of the various phosphatide fractions obtained

Table III

Comparison of Melting Points of Derivatives of Synthetic Methylcholine and Naturally Occurring Unknown Nitrogenous Base

	Melting Point (°C)			
Salt	Synthetic	Unknown*	Mixed	Literature Value
Chloride	163-165	---	---	165° C
Reineckate	171-174d	170-173d	172-174d	---
Chloro-platinate	254-257	254-256	254-257	257° C

*Derivatives were made from the chloride isolated from larvae which had received carnitine as a replacement for the dietary choline.

from larvae reared on the defined diet was qualitatively the same as reported for normal larvae.

The growth of the larvae grown on the amino acid diet is slower than their growth on the normal diet; however, the growth rates appeared to be the same on the defined diet whether methylcholine or carnitine were used as the vitamin source. The rate of growth was slower when 1-dimethylamino-2-propanol or dimethylaminoethanol were used as the dietary choline substitute.

The growth of Neurospora crassa used for the choline assay was not affected by 1-50 micrograms of methylcholine as shown in Table V.

In order to substantiate that methylcholine was indeed a phospholipid component, a large amount of material equivalent to Peak IV was isolated, hydrolyzed and purified as described in the methods section. Chloroplatinate and reineckate derivatives of the unknown chloride and the and the synthetic β-methylcholine chloride were prepared. Table III shows the decomposition points obtained. The decomposition points of the synthetic and naturally derived chloroplatinates varied over a range of 2-3° but were similar, and the mixed decomposition points were not lower than those of the original starting materials. The same type of result was obtained with the reineckate derivatives. The melting point of the unknown chloride was not obtained,

since insufficient crystals were available. Difficulty was encountered in purifying the chloride, due to the extremely hygroscopic nature of this salt and also due to the presence of impurities probably arising during the elution of the chloride from the paper. The chloroplatinate and reineckate derivatives did not present these difficulties, since these derivatives were easily separated from the impurities present in the unknown chloride.

The carbon and hydrogen contents of the authentic and natural ("unknown") samples of the chloroplatinate derivatives were in good agreement as shown in Table IV. The values were the same as the calculated values for the chloroplatinate of β-methylcholine.

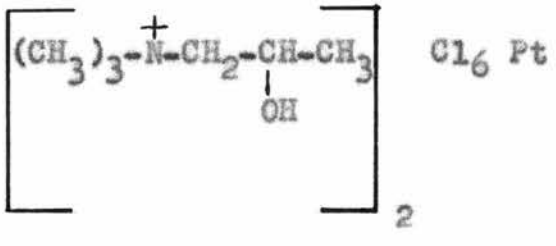
The infrared spectra of the phosphatide fractions in question are shown in Figure 6. The three fractions had the following major absorption peaks: 3325-3335 cm^{-1}, 2905 and 2835 cm^{-1} from CH stretching of methylene or methyl carbons, 1731-1736 cm^{-1} caused by the carbonyl of esters, 1460 cm^{-1}, 1373 cm^{-1} by CH_2-CH_3, 1258 cm^{-1} by P = O, 1158 cm^{-1} by P-O-aliphatic or ester, 1089-1064 cm^{-1} by P-O-aliphatic. These results are similar to the reported spectra of phospholipids (34, 40). The infrared data demonstrate that the lecithin fraction isolated from normal larvae is similar to the lecithin fraction isolated from larvae reared on the defined diet in which the choline was

Table IV

Elementary Composition of Synthetic and Unknown Methylcholine Derivatives

Compound	% C	% H
Syn. Chloroplatinate	22.02	4.83
*Unknown Chloroplatinate 1	21.79	4.76
2	22.37	5.07
Expected	22.36	4.97

Structure of chloroplatinate



*1 and 2 refer to chloroplatinate derivatives of the unknown chloride prepared by the same method but at different times.

The unknowns were obtained from phospholipids isolated from larvae which had been reared on a diet in which the dietary choline had been replaced by carnitine.

Table V

Effect of β-Methylcholine on the Microbiological Assay

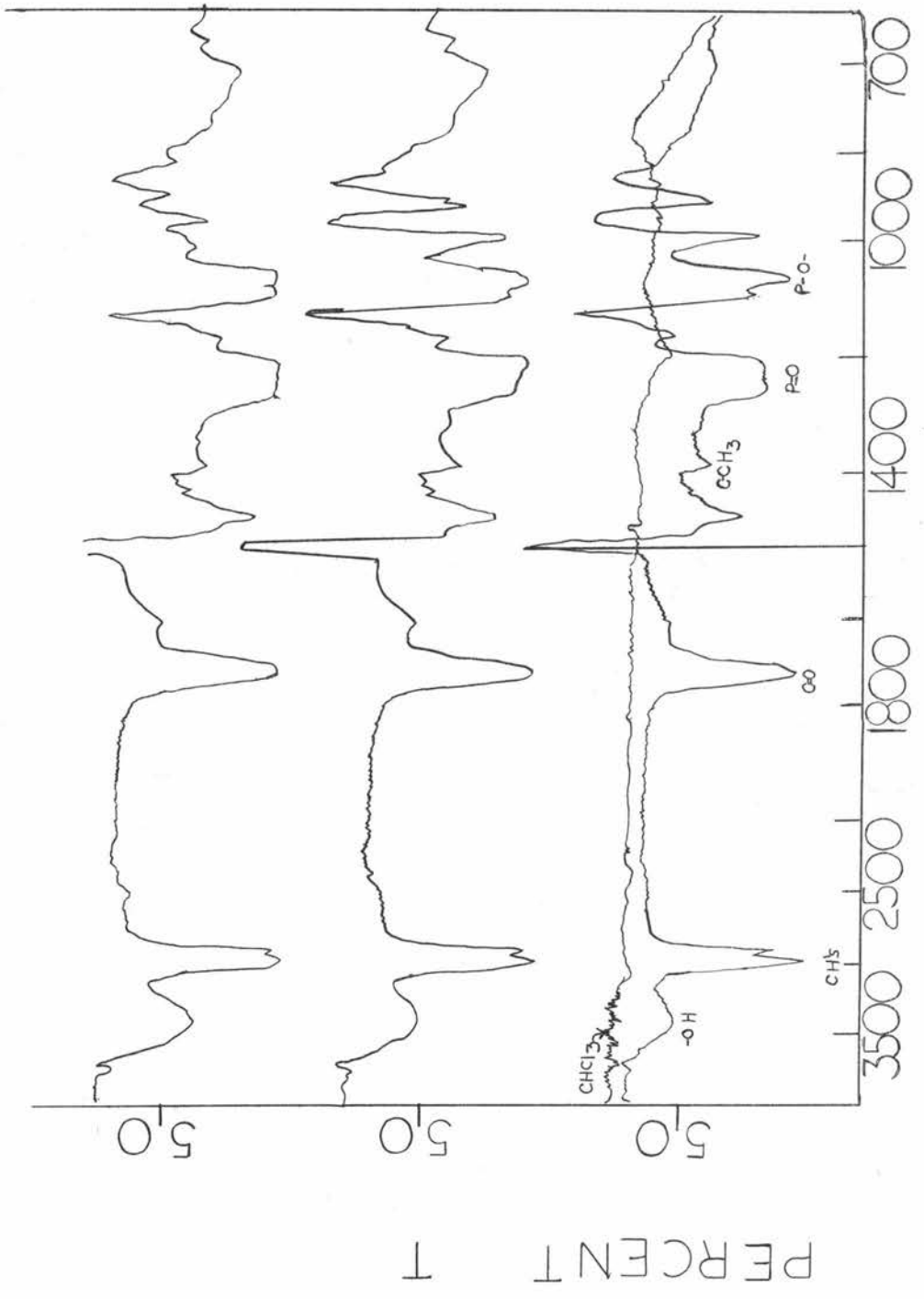
Choline Alone		Choline and Methylcholine		
Choline	Weight of mycelium	Choline	Methylcholine	Weight of mycelium
g	mg	g	g	mg
2.0	6.0	2.0	10.2	4.5
4.0	12.7	4.0	20.4	11.0
6.0	15.2	6.0	30.6	15.7
8.0	18.4	8.0	40.8	19.3
10.0	20.1	10.0	51.0	20.8
13.0	22.2	13.0	30.6	23.5
16.0	26.4	16.0	10.2	26.0
20.0	31.2	20.0	20.4	27.8
25.0	33.4	25.0	30.6	35.8
30.0	32.5	30.0	40.8	35.2
35.0	38.2	35.0	50.0	35.0
40.0	35.4	40.0	5.1	39.3
	291.7 mg			293.3

Neurospora crassa ATCC strain 34486; 5 day incubation at 30° C. Difco assay media.

Figure 6

Infrared spectra of the phospholipids equivalent to Peak IV of Figure 4. The top absorption spectrum is from normal larvae. The middle is the spectrum of the material from the major peak in Figure 5 and is phospholipid from larvae grown on the amino acid diet and carnitine. The bottom diagram is of the phospholipid from larvae grown on the amino acid diet and β-methylcholine. The fourth curve, with no appreciable absorption peaks, is $CHCl_3$ vs. $CHCl_3$.

Figure 6


PERCENT T
5.0
5.0
5.0
3500
2500
1800
1400
1000
700
FREQUENCY CM⁻¹
CHCl3
-OH
CH3
C=O
CCH3
P=O
P-O-

replaced by either carnitine or β-methylcholine. There are some differences in the "fingerprint" region which may be caused by unsaturated fatty acids. The weak peak at 1650 cm^{-1} may be produced by the presence of a small amount of plasmalogen. Gottfried and Rapport (37) reported that a strong band at 1650 is given by choline plasmalogen; however, the peak in the present samples is weak and may be caused by any one of several possible structures. The spectrum for the normal larval lecithin was obtained from the material whose analytical data is presented in Table I.

Studies With Dimethylaminoethanol as a Dietary Choline Substitute(1)

The effect of dietary DMAE on the phospholipid composition also was investigated. The larvae were reared on an amino acid diet in which the choline was replaced by DMAE. The phospholipids were extracted and resolved on silicic acid as described previously. Figure 7 is a typical elution pattern of such a determination. The pattern is different than the patterns shown in Figures 1 and 4. The total amounts of Peaks I and II have increased, and the lecithin peak is much smaller than in the previous

(1)The following abbreviations are used: DMAE = dimethylaminoethanol and DMAI = dimethylaminoisopropanol = 1-trimethylamino-2-propanol.

columns. From 70-80% of the total phospholipid was eluted by the first chloroform-methanol eluate (4:1 or 5:1).

The presence of dimethylaminoethanol in hydrochloric acid hydrolysates of Peaks I and II of Figure 7 was evident by paper chromatography. Since Peaks I and II were mixtures, they were further separated by resolution on DEAE-cellulose columns as shown in Figures 8 and 9. The first fraction, equivalent to Peak I of Figures 8 and 9, contained DMAE, and the other major peak contained predominantly ethanolamine. The ethanolamine peak sometimes came off in the chloroform-methanol 7:3 eluate and sometimes in the 10:1 or 7:1 chloroform-methanol eluate. This occurred in both the DMAE phosphatide studies and the DMAI phosphatide separations. The cause for this variation is not known. Table VI gives the values from two columns for this type of separation. The nitrogen values for some fractions were greater than anticipated. This may be due in part to contamination by DEAE-cellulose.

The fate of lipid DMAE was investigated by using 1-2C^{14} labeled DMAE. Figure 7 illustrates the phospholipid pattern and C^{14} pattern obtained by chromatography on silicic acid. Peaks I and II were further resolved by separation on DEAE-cellulose as shown in Figures 8 and 9. Figures 7-9 show that DMAE was incorporated into the lipid.

Figure 7

Phospholipids extracted from 66 g larvae which had been reared on a defined diet in which the dietary choline was replaced by DMAE (with addition of 20 curies of dimethylaminoethanol-1,2-C^{14}). 16 g silicic acid; 8 g Hyflo; 8 mm column diameter. Collected 400 drops per tube. Phosphorus was determined on each tube. The radioactivity was determined on every third tube starting with tube number 34. C = chloroform; M = methanol.

The dotted line is cpm per ml

The solid line represents micromoles P per ml

Figure 7

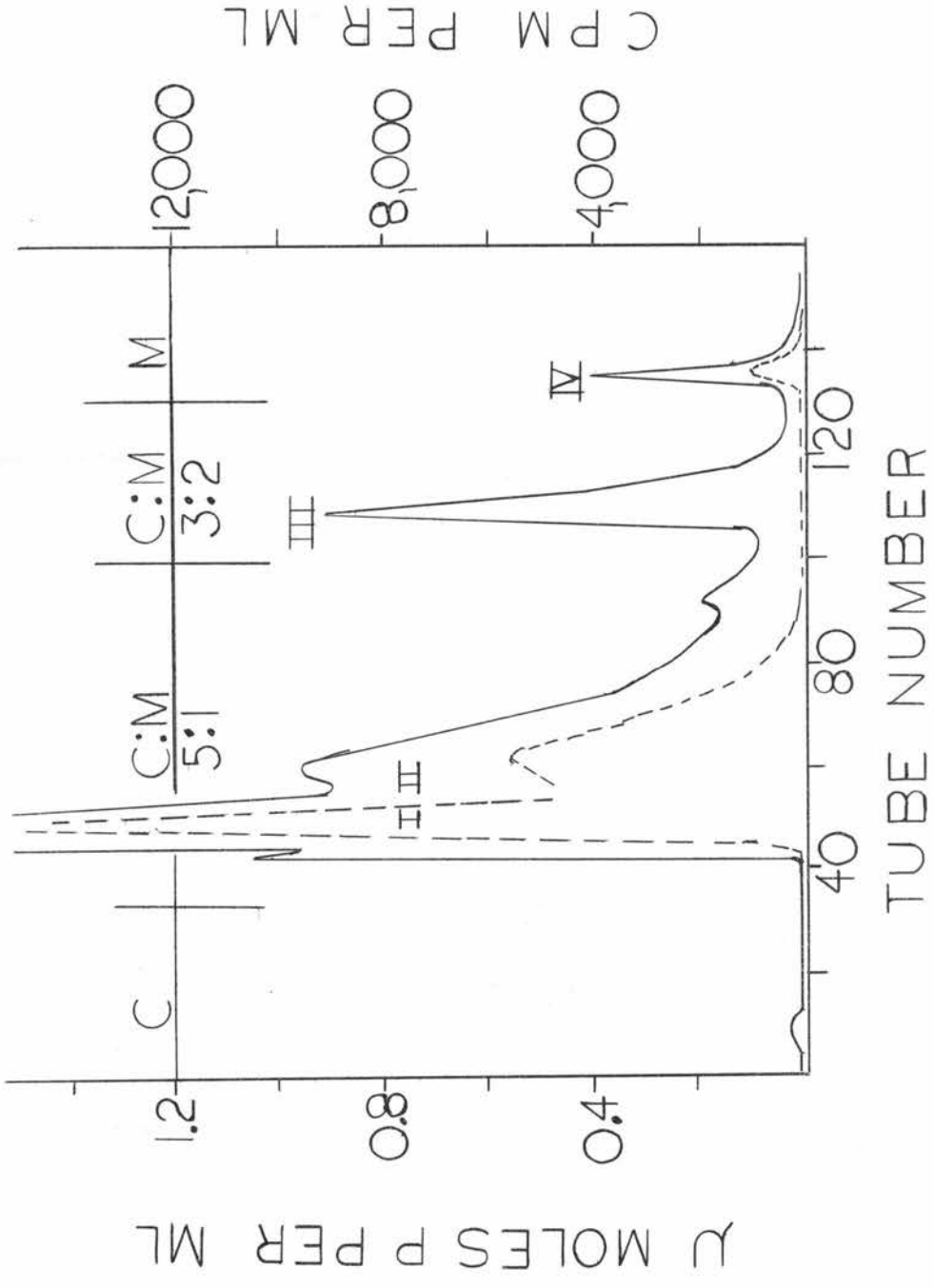

CPM PER ML
12,000
8,000
4,000
C:M
5:1
C:M
3:2
M
C
I
II
III
IV
40
80
120
TUBE NUMBER
1.2
0.8
0.4
μ MOLES P PER ML

Figure 8

Separation of the first peak (tubes 42-55) of Figure 7. 12 g DEAE-cellulose; 8 mm column diameter. C = chloroform; M = methanol. Collected 400 drops per tube. Radioactivity determinations and phosphorus analyses were carried out on alternate eluate samples.

- - - - cpm/ml

——— moles P/ml

Figure 8

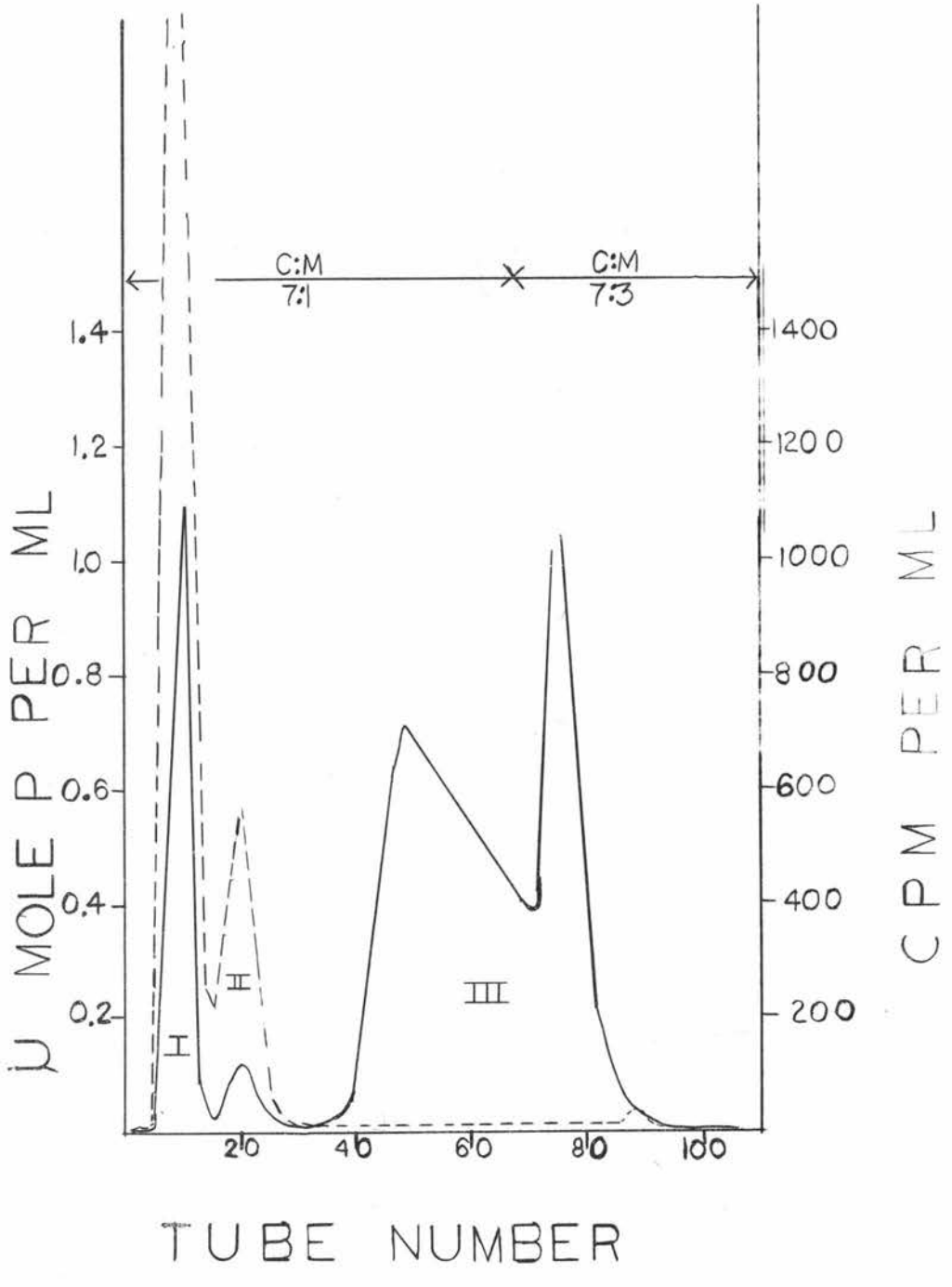

C:M
7:1
C:M
7:3
1.4
1.2
1.0
0.8
0.6
0.4
0.2
μ MOLE P PER ML
1400
1200
1000
800
600
400
200
CPM PER ML
I
II
III
20
40
60
80
100
TUBE NUMBER

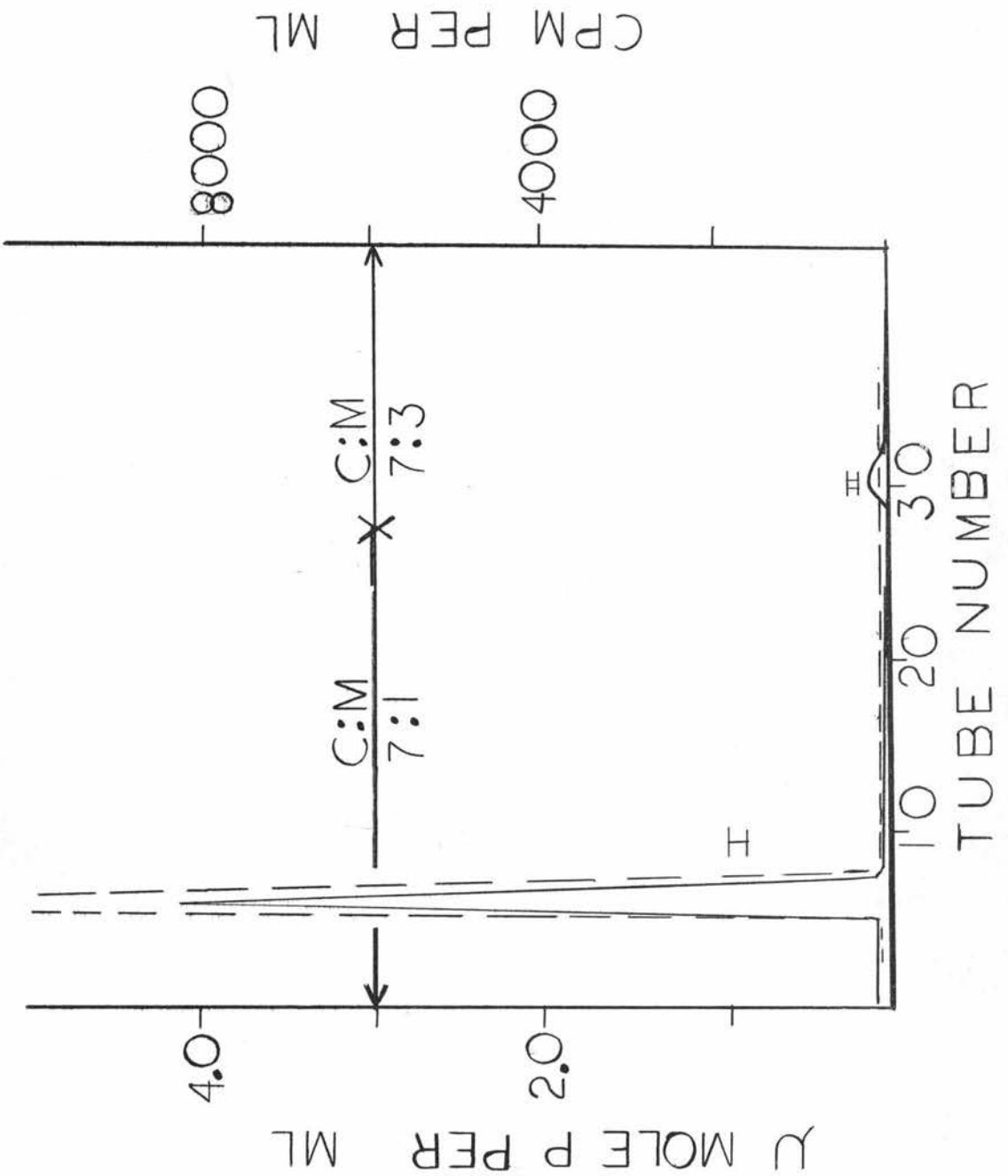
Figure 9

Separation on DEAE-cellulose of the second peak of Figure 7 (tubes 56-85). C = chloroform; M = methanol. Radioactivity and phosphorus determinations were carried out on sample eluate. Column, 8 mm diameter; 12-13 ml were collected per sample since a volumetric fraction collecter was used.

– – – – cpm/ml

——— moles P/ml

Figure 9


CPM PER ML
8000
4000
C:M
7:3
C:M
7:1
H
H
TUBE NUMBER
10
20
30
4.0
2.0
μ MOLE P PER ML

The amount of lecithin decreased from the 19% value in normal larvae to 2-3% of the total phospholipid when DMAE was used in the diet. An average of 2.4% was obtained from six individual columns (3.2, 1.9, 1.9, 2.6, 2.5, 2.6, and 1.4%). This represented at least six different batches of larvae. The 2.4% value represents the total phosphorus eluted in the lecithin fraction as well as the 1:4 eluate and the methanol eluate. Some of this material may not have been lecithin, but choline was the major compound detected in these fractions. Similar amounts of choline were found in the lecithin fraction when carnitine, β-methylcholine, and DMAI were used as dietary choline substitutes.

The labeled material in the lecithin fraction shown in Figure 7 was DMAE. Radioautograms, developed from paper chromatograms, demonstrated that the radioactive material was DMAE and not choline. Radioautography failed to reveal any choline in the radioactive DMAE standard. A small amount of radioactive monomethylaminoethanol was detected in the labeled DMAE standard when the solvent ethanol-conc. NH_4OH (95:5) was used. Dimethylaminoethanol cannot be detected when this solvent is used; consequently, traces of other compounds can be detected without interference from the DMAE. Apparently the hydrochloride of DMAE is removed by the NH_4OH, and the free base evaporates when the

Table VI

Analyses of the Fractions Containing Dimethylaminoethanol

Peaks Equivalent to Figure 8									Peaks equivalent to Figure 9			
Peak I			Peak II			Peak III			Peak I			Peak II
N/P	E/P	Compounds detected	N/P	E/P	Compounds detected	N/P	E/P	Compounds detected	N/P	E/P	Compounds detected	Compounds detected
1.1	2.0	DMAE	1.3	1.8	EA MMAE ?	1.1	2.08	EA Tr S & T	0.99	2.05	DMAE	Tr S & T EA
0.99	1.9	DMAE Tr S & T	1.03	1.6	EA MMAE ?	0.98	1.9	EA Tr S & T	1.04	1.9	DMAE	EA

S = serine; T = threonine; DMAE = dimethylaminoethanol; EA = ethanolamine; MMAE = monomethylaminoethanol; Tr = trace.

Percentage of phospholipid containing dimethylaminoethanol = Peak I of Figure 8 plus Peak I of Figure 9. The values for three different groups of larvae were 24%, 33% and 29%.

the chromatogram is dried.

As shown in Table VI, the phosphatidyl DMAE represented 24, 33 and 29 percent of the total lipid phosphorus as determined by separation of the silicic acid eluates with DEAE-cellulose. The 24% value may have been low, since a portion of this fraction was used for another determination prior to the determination of the total amount of phospholipid present.

The percentage of the inositol containing fraction (Peak III, Figures 1 and 4) varied from column to column. The highest value was 19% and the lowest value obtained was 6.2% of the total lipid phosphorus. This variation may not represent a change in the inositol content, since the amount of this fraction varied somewhat depending on when the solvent change was made. This peak is a mixture, and its increase or decrease could be caused by certain of the phosphatides coming off in different positions. Rathbone (80) has shown that similarly prepared phosphatidyl serine can be eluted from silicic acid columns in different positions.

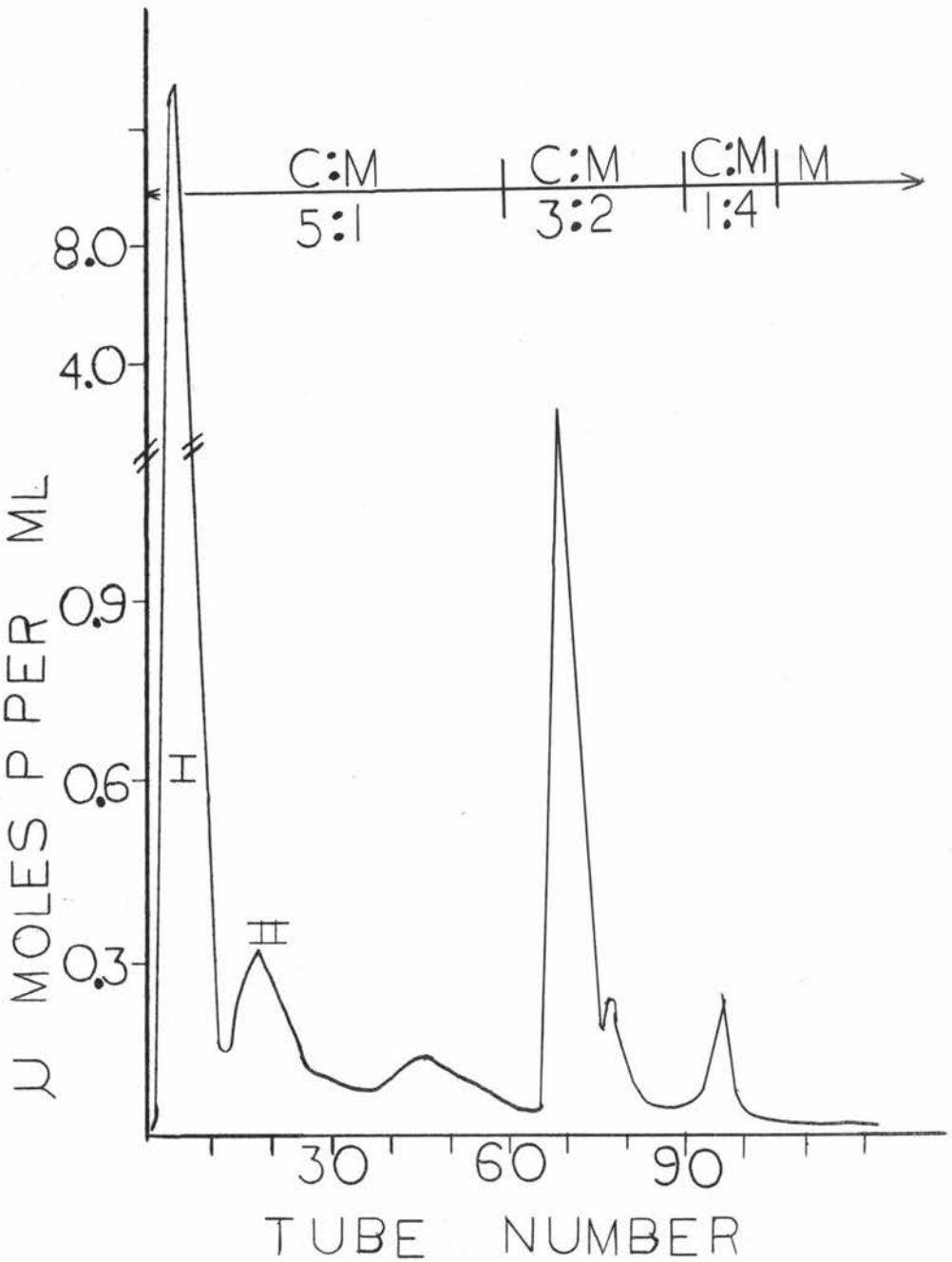
Studies Using Dimethylaminoisopropyl Alcohol as a Choline Substitute

Dimethylaminoisopropyl alcohol was used since it could be postulated as being a β-methylcholine precursor. The

Figure 10

Separation of the phospholipids isolated from 45 g of sterile larvae which had been reared on a diet containing dimethylaminoisopropanol. 16 g silicic; 8 g Hyflo; 8 mm diameter column. C = chloroform; M = methanol. Collected 400 drops per tube, and phosphorus was determined on every fraction. The neutral lipid was eluted from the column with chloroform prior to elution with C:M (5:1). A negligible amount of P was found in this pooled fraction.

Figure 10


C:M
5:1
C:M
3:2
C:M
1:4
M
8.0
4.0
0.9
0.6
0.3
μ MOLES P PER ML
I
II
30
60
90
TUBE NUMBER

same techniques were used as were used with DMAE. Diethylaminoisopropanol failed to support larval growth. Figure 10 illustrates the elution pattern obtained by chromatography of the lipid extract on silicic acid-Hyflo. Peaks I and II came off almost as one peak when a chloroform-methanol ratio of 4:1 was used as the eluting solvent. The two peaks appeared to be further apart when a 5:1 ratio was used. This also depended on the amount of material applied to the column, since less material would give sharper separations. The patterns were similar to those obtained with lipids isolated from larvae grown on the DMAE containing diet. Again the lecithin fraction was low with an average of 2.3% of the total lipid phosphorus from four columns (1.9, 1.9, 3.2 and 2.0%).

DEAE-cellulose separations were also performed on the individual fractions (Peaks I and II of Figure 10) as was done with the dimethylaminoethanol-containing material. The patterns were similar to the ones shown in Figures 8 and 9, and the first fraction contained DMAE. The DMAI-containing phosphatide represented a significant portion of the total phospholipids, but was less than the value found when DMAE was used as the dietary choline substitute. The total amount of lipid phosphorus represented by DMAI was an average of 17.6% from three individual runs. The individual values were 16, 17.4 and 19.4% of the total

lipid phosphorus. These values represent determinations on at least three different groups of larvae. The following N:P ratios for the DMAI phosphatide fractions were obtained: 1.0, 1.1, 1.1, 1.1, 1.6. These values were for material obtained from DEAE-cellulose columns and may have contained some extraneous DEAE-cellulose.

Infrared analyses were made on the DEAE-cellulose isolated DMAI and DMAE phosphatides described above. The fractions which were selected for infrared analyses had N/P ratios of 1.0-1.1, ester/P ratios of 1.9-2.0 and contained very little ninhydrin sensitive material. The following absorption peaks were obtained: 2.96 μ, 3.44 μ, 3.52 μ, 5.78 μ, 6.78 μ, 8.0-8.25 μ and 9.38 μ. These absorption maxima are typical for ester-containing phospholipids (34, 40).

DISCUSSION

Amino compounds other than serine, ethanolamine and threonine were present in varied amounts in the phospholipid hydrolysates, but their status as constituents of phospholipid molecules was not investigated. The possibility exists that they are artifacts, as the free amino acid concentration in insects is high (31, p. 63), and phospholipids have the property of rendering normally lipid insoluble compounds soluble in lipid solvents. Amino acids have been detected in phospholipid extracts (74, p. 328; 98, p. 2823; 78, p. 381; 89, p. 74; 48). Evidence has been presented by Hendler that amino acids and peptides are covalently linked to phospholipids. The significance of this information in insects cannot be evaluated with certainty at this time, but their involvement in amino acid metabolism is not ruled out.

The data in Table I demonstrates that ethanolamine-containing phospholipids are the major phosphatides in normal larvae. This was confirmed by the results obtained with DEAE-cellulose columns. Ethanolamine-containing phosphatides are also the major ones in the housefly, Musca domestica. The choline-containing phospholipids represented 18-19% of the total phospholipids. This is lower than the value obtained from many other sources. Although

examples of imperfect recovery of total nitrogen were encountered (believed due to either the small size of the material available for analysis or to difficulty with the determination in question); nonetheless, the measurements of the major fractions were generally satisfactory. The inositide and serine phosphatides were present in small amounts. The results indicate, but do not prove, the presence of a threonine-containing phospholipid. Every fraction which contained serine also contained threonine. A threonine phospholipid has been demonstrated in tunny muscle (57).

The failure to detect any sphingomyelin is surprising and would represent a departure from the pattern found in vertebrates. However, the presence in this insect of at least one non-choline containing sphingolipid may replace the sphingomyelin requirement. Crone and Bridges (22) did not find any sphingomyelin in the housefly, Musca domestica and Mitchell and Wren were unable to detect any sphingomyelin in Drosophila melanogaster. Both found other sphingolipids present, which were eluted from silicic acid columns in front of the lecithin fraction. Crone and Bridges have reported that their sphingolipid contains ethanolamine, which would be like the one tentatively identified earlier in this thesis.

The inositol-containing fraction isolated from silicic acid columns is very heterogeneous. It contains serine, threonine, ethanolamine, sometimes sphingosine as well as other ninhydrin sensitive materials. Carter et al. (19) have found similar results in the inositide fraction isolated from flax by silicic acid chromatography. Their material contained inositol, sphingolipid, phosphatidyl ethanolamine, phosphatidyl serine and glucosamine. They found calcium and magnesium present and have evidence to support the idea that these ions are involved in binding other components to the inositide.

The phospholipid investigations on larvae reared on a diet in which choline was replaced by carnitine demonstrates at least one step in the metabolism of carnitine in this insect. Several investigators have searched with little success for carnitine metabolites in aqueous extracts. Recently Mehlman has found β-methylcholine in the urine of pregnant rats after administration of labeled carnitine (private communication). The results demonstrate that carnitine causes the appearance of a β-methylcholine-containing phosphatide in the larvae of Phormia regina.

The results described in this thesis show that carnitine is decarboxylated in this insect. The expected product has been observed as a major replacement for choline in the phospholipids of carnitine fed larvae.

Whether carnitine is bound as a phosphatide in this insect is not known.

No carnitine was detected in the acid hydrolysates. However, carnitine is difficult to detect chemically and could have easily gone undetected. It has been found to be bound in hens' eggs after injection of the labeled compound. Very recently Mehlman (private communication) has found bound carnitine in the phospholipids isolated from chick embryo, chick liver, rat liver, rat embryo and red blood cells.

The lecithin fractions represented 21-27% of the total phospholipids when carnitine or β-methylcholine was the vitamin source, but only 1-5% of the phospholipid could be accounted for as phosphatidyl choline. The amounts of choline in the lecithin fraction were about the same when either carnitine, β-methylcholine, DMAE or DMAI were used as the dietary choline substitute. The small amount of radioactivity in the lecithin fraction, when chain labeled DMAE was used, was due to the presence of a small amount of DMAE and not the result of a synthesis of choline from DMAE. This is supported by the presence of a small amount of lipid bound choline when the other choline substitutes were used. The same amount of choline was present when DMAE was used instead of one of the other choline analogues. If there were a net synthesis of choline one could expect

more choline to be present when DMAE was used as the choline substitute. The average amount of the lecithin fraction was 2.3% of the phospholipid when DMAI was used, and six different batches of larvae reared on DMAE also gave an average of 2.4% of the phospholipid as lecithin, demonstrating very little difference in the amount of bound choline. It is interesting to speculate that this amount of choline may represent an irreducible minimum of an essential phosphatidyl choline.

DMAI phosphatide and DMAE phosphatide can replace most of the lecithin in this insect. The overall percentage of the DMAE phosphatide was greater than that of the DMAI-containing one.

The significance of this organism's ability to alter the phospholipid composition by alteration of one of the vitamins 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 which can be fulfilled by any one of several phospholipids. If this should be true, then the omission of one (containing choline) might not be disastrous to the organism; other bases might replace its phospholipid requirement. Whereas choline and β-methylcholine are similar in several respects, it is rather surprising to find a quaternary nitrogen being

replaced by a tertiary nitrogen, if the positive charge on the nitrogen is important. However, at physiological pHs the tertiary nitrogen would be protonated, and thus it would also have a positive charge. The normal larvae were reared on a casein-yeast extract diet and not on a natural meat diet. Hence we do not know the phospholipid composition of the wild larvae, but these animals represent an easily controlled system in which one can study the effect of certain planned changes. Since most meat has a high carnitine content, it may not be surprising that the larvae are able to readily metabolize carnitine.

The vitamin function of these compounds must also be considered. If they are omitted from the defined diet, the larvae will not grow. If the methyl groups of DMAI are replaced by ethyl groups, the larvae fail to grow. This could be a result of their role in methyl transfer or methyl donation. One can only speculate concerning any relation between the vitamin role and the phospholipid role of these compounds. Phospholipids are usually considered to be structural materials, whereas vitamins usually are not considered as being a part of structural material but are involved in certain metabolic activities such as functioning as cofactors in certain enzymatic reactions.

It will be interesting to see whether the phospholipid composition of some other systems depend on the intake or

availability of certain compounds. If so, it will be necessary to use strictly defined conditions in order to evaluate comparative studies.

SUMMARY

1. The phospholipids of Phormia regina larvae have been examined by chromatography on silicic acid.
2. The major constituents consist of ethanolamine- and choline-containing phospholipids. Ethanolamine phospholipids comprise 62% of the total phospholipid, and lipids of the lecithin type about 20%. In this respect the blowfly differs markedly from vertebrates. Serine and inositol phospholipids comprise a relatively small part of the total.
3. Sphingomyelin was not detected in any of the developmental stages under the conditions employed, but at least one sphingolipid was detected and was identified as an ethanolamine-containing sphingolipid. A method for its isolation is described.
4. When the dietary choline had been replaced by β-methylcholine or carnitine, larvae of the blowfly were shown to have a new phospholipid in which choline had been replaced by β-methylcholine. The β-methylcholine isolated from larval tissue was characterized by chromatography, carbon hydrogen analyses, and melting points of derivatives. This new base appears to arise from carnitine by decarboxylation in the larvae. The isolation and chromatography of the phosphatide

of β-methylcholine are described.

5. It was shown that dimethylaminoethanol can substitute for most of the choline of the phospholipids. A method for the separation of the dimethylaminoethanol phosphatide is described.
6. It was shown that dimethylaminoisopropyl alcohol can substitute for most of the phospholipid choline. A method for the isolation of the dimethylaminoisopropyl alcohol-containing phosphatide is described.
7. Dimethylaminoisopropyl alcohol and β-methylcholine can serve as vitamins in this insect.
8. The significance of these findings is discussed.

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