PHOSPHOLIPID PATTERNS IN THE DEVELOPING CHICK EMBRYO

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1962

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Date thesis is presented October 16, 1961

Typed by Lilah N. Potter

ACKNOWLEDGMENT

The author expresses his gratitude to Dr. Robert W. Newburgh and Dr. Vernon H. Cheldelin for their guidance and valuable criticism during these formative years.

Thanks are extended to the Science Research Institute for providing the facilities and technical assistance necessary for the completion of this study.

My thanks to my wife for her encouragement and to my fellow students for many helpful suggestions and discussions.

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INTRODUCTION

The phospholipids are a heterogeneous class of compounds. They have been shown to be complex esters which contain a nitrogenous base or a sugar, an alcohol, and long chain fatty acids. They may be generally divided into three groups, namely the phosphoglycerides, the phosphoinositides and the sphingomyelins. The phosphoglycerides are diacylesters of a nitrogenous base derivative of L-α-glycerophosphate. Included in the phosphoglycerides are lecithin (phosphatidyl choline), phosphatidyl ethanolamine, phosphatidyl serine, and the plasmalogens (acetal phospholipids). The lysophosphoglycerides may be included here also. They are the monoacyl counterparts of lecithin, phosphatidyl ethanolamine and phosphatidyl serine. The phosphoinositides consist of monophosphoinositides, which are diacylesters of glycerylphosphorylinositol, and the diphosphoinositides whose structure is still uncertain. Complex inositides have been isolated but appear to occur mainly in plant tissue. The sphingomyelins are a group of compounds in which a long chain nitrogenous base, sphingosine or some closely related compound, is esterified with phosphorylcholine and bound to a fatty acid by an amide linkage.

Several other phospholipids which are much less abundant than the foregoing should be mentioned. These include the phosphatidic acids (diacylesters of L-α-glycerophosphate), the polyglycerolphosphatides and cardiolipin. The lysophosphoglycerides also could be included with these minor phospholipids since they are not nearly as abundant as are the phosphoglycerides. The structure and properties of the phospholipids are discussed in the treatises by Deuel (8, p. 405-474) and Hanahan (10, p. 1-177).

As early as 1793 Fourcroy (49, p. 9) showed the existence of complex fatty compounds in tissues. Vauquelin (49, p. 9) was the first investigator to prove the presence of phosphorus as a constituent of fatty material. Since the discovery of the phospholipids as constituents of tissue many investigators have contributed to the literature on the chemistry and occurrence of these compounds as is evident in the reviews by Wittcoff (49) and Deuel (8, p. 405-472).

It was soon evident that the phospholipids were present in all the tissues of the animal body. The total amount of phospholipid present as well as the relative amount of each individual phospholipid present may vary considerably with the tissue. The proportion of the total lipid present as phospholipid also varies with the

tissue. This ubiquity suggests that the phospholipids may well be concerned with certain functions performed in or by all tissue. There is now good evidence that the phosphatides are intimately concerned with cell structure and with metabolic processes involved in certain structural entities within the cell. It has been suggested that the phosphatides are involved in the processes of secretion and protein synthesis. Other important roles of the phosphatides are fat transport and blood coagulation. The role of the phosphatides in nervous function as components of the myelin sheath is still uncertain. Reviews dealing with the metabolism and functions of the phosphatides have been compiled by Wittcoff (49), Dawson (7, p. 188-229), Kennedy (18, p. 119-148), and Rossiter and Strickland (38, p. 69-127).

Egg yolk phospholipids have been studied extensively. Gobley (49, p. 9 and 320) first isolated phosphatides from eggs in 1846. Koch and Woods (19) reported that cephalin was the major constituent of egg yolk phospholipids. In later studied by Nottbohm and Mayer (33), Kaucher et al. (17, p. 203-215) and Tsuji et al. (44), it was reported that lecithin was the major component of the phospholipid fraction in the yolk. The most complete analysis of the egg yolk phospholipids is that of

Rhodes and Lea (37). By the use of alumina and silicic acid columns the phospholipids were separated and purified. Their results show the egg yolk phospholipids to contain phosphatidyl choline (73.0%), lysophosphatidyl choline (5.8%), sphingomyelin (2.5%), phosphatidyl ethanolamine (15.0%), lysophosphatidyl ethanolamine (2.1%), and inositol phosphatides (0.6%). The plasmalogen content was low (0.9%) and 0.2% amino acid containing phospholipid was reported.

In 1909 Plimmer and Scott (35) studied the changes in lipid phosphorus during the development of the chick embryo. They found a decrease in the phosphatide content of the residual egg yolk, particularly after the sixteenth day of incubation. At the same time the lipid phosphorus of the embryo was found to increase. A large increase in inorganic phosphorus was observed and it was suggested that the phospholipids were being degraded to supply inorganic phosphorus for bone ossification. Masia and Fukutomi (26) obtained similar results in their study of the egg yolk and embryo phospholipids. Cahn and Bonot (6, p. 399-479) reported the phospholipid content of the hen's egg to decrease fifty percent during the development of the embryo. At the same time the embryo phospholipid content was found to increase with age. Needham, citing the work of Cahn (31, p. 1208-1209),

states that the total amount of lecithin phosphorus rose regularly with age. On the basis of wet weight the lipid phosphorus increased up to the fifteenth day and then remained constant. On the basis of dry weight the lipid phosphorus increased until the tenth day of incubation and then proceeded to fall. Sereno et al. (42) showed the lecithin content of the egg yolk to decrease during development with a concomitant increase in the developing embryo. At the same time the total lecithin content in the system was found to decrease. Jost and Sorg (16) demonstrated a parallelism between the fall of phospholipid phosphorus and total fatty acid content in the egg yolk during incubation. It was suggested that the phospholipids were being degraded in the yolk, but after the splitting off and oxidation of their fatty acids they were resynthesized in the embryo from fatty acids supplied from the neutral lipids. Kugler (20) stated that phospholipid metabolism in the chick embryo reached its maximum between the fifteenth and eighteenth days of incubation. This conclusion was based on the observation that the yolk decreased to the greatest extent during this time and the increase in embryo phospholipids was the greatest during the same period. In a later study Kugler (21) observed that the lecithin to cephalin ratio remained three to one throughout

development. Tsuji et al. (44), in contrast to the work of Kugler (21), found considerable variation in the ratio of choline containing to non-choline containing phospholipids in the developing chick embryo. The percentage of tissue concentration of total lipid phosphorus and lipid choline was found to remain relatively constant in the residual yolk but the percentage of these substances in the embryo decreased continually during incubation. The decrease in lipid phosphorus and lipid choline in the yolk was greatest between eighteen and twenty-one days incubation. The decrease in yolk weight and the increase in embryo weight were greatest during the same period of time. The increment of lipid phosphorus in the embryo was observed to be at a maximum somewhere between twelve and eighteen days of incubation. Szepsenwol et al. (43) studied the phospholipids in several individual tissues of the developing chick embryo. Lipid phosphorus was found to increase in all tissues studied from the eighth day of incubation. The greatest increase occurred in the brain. The lipid phosphorus decreased before hatching in all the tissues studied with the exception of the brain, where it was found to continue to increase.

The question of whether the phospholipids of the chick are synthesized in the embryo or transferred intact

from the yolk to the embryo has been studied by Hevesy *et al.* (13). Eggs were injected with radioactive phosphate and incubated for various lengths of time up to eighteen days. The phospholipids extracted from the yolk showed negligible radioactivity while those extracted from the embryo had incorporated a considerable amount of the isotope. Thus they reasoned that since the embryo lipids showed a higher specific activity than the yolk, the embryo must be capable of phospholipid synthesis. By the use of hexosephosphate, labelled with radioactive phosphate, it was shown that the embryo phospholipids also became labelled upon incubation while the yolk phospholipids did not. It was proposed that the yolk phospholipids are hydrolyzed to yield inorganic phosphate which is in turn transported to the embryo for the synthesis of the necessary phosphate compounds. Branson *et al.* (3), using eggs injected with radioactive phosphorus, came to the conclusion that the embryo first uses the inorganic phosphorus present in the egg yolk for synthetic purposes. Upon the depletion of the supply of inorganic phosphorus the organic phosphorus compounds of the yolk are hydrolyzed by a phosphatase activated or released by the embryo and the inorganic phosphate released is utilized by the embryo for synthetic purposes. These results do not exclude the possibility of *de novo*

synthesis of some or a particular phospholipid together with direct transfer of other yolk phospholipids to the embryo.

It is evident from a survey of the literature that considerable work has been done on phosphorus compounds in the developing chick embryo, including some on the phospholipids. Very little work on the separation of the individual phospholipids during the development of the chick has been attempted. In view of the recent advances in the methods of separation and identification of the phospholipids and the many suggested roles of the phospholipids in metabolism and function, a study of the phospholipids appearing during embryonic development seemed to warrant consideration.

MATERIALS AND METHODS

Materials

Commercial preparations of the following compounds were used: Choline chloride from Merck and Co., chloroform and methanol from J. T. Baker and Co., sphingomyelin from Sylvana Chemical Co., lecithin from British Drug Houses Ltd., phosphatidyl serine, phosphatidyl ethanolamine, inositol phosphatide, myo-inositol and serine from Nutritional Biochemicals Corp., ethanolamine from Eastman Organic Chemicals, 100 mesh silicic acid from Mallinckrodt Chemicals, Hyflo Super-Cel from Johns-Mansville Products, Kloeckera apiculata No. 9774 and Neurospora crassa No. 34486 from American Type Culture Collection, choline assay medium from Difco Laboratories, deoxyribose nucleic acid (DNA) from Krischell Laboratories, Inc.

Methods

Fertile eggs from White Leghorn chickens were obtained from Hanson's Hatchery, Corvallis, Oregon. The eggs were incubated at 100-101°F for the required time in electric incubators.

Unincubated eggs were used to prepare egg yolk lipids. Embryo lipids were extracted from embryos of

eggs incubated four, six, twelve, fourteen and eighteen days. The embryos were removed from the eggs, rinsed in saline to remove any adhering yolk and membranes, then dropped into a solution of two parts of chloroform to one part of methanol. The number of individuals used in preparation of the various lipid samples and the approximate wet weight of the samples are shown in Table I. The wet weight of the material was estimated by weighing the solvent before and after addition of the embryos.

The extraction of the lipids from the egg yolk and embryos was carried out by homogenizing the wet material in a Waring blendor with a solution of two parts of chloroform to one part methanol. The total volume of solvent used in the extraction was ten times the weight of the wet tissue. The extraction was carried out in two steps, approximately one-half of the solvent being used in the first extraction with an extraction time of fifteen minutes. After centrifugation, the precipitate was homogenized in the Waring blendor with the second portion of the solvent. This extraction was of thirty minutes duration. After centrifugation, the supernatant liquid was removed and combined with that from the first extraction. The precipitate was saved for protein and DNA determination. The combined extracts were washed essentially by the method of Folch *et al.* (9, p. 499-500)

TABLE I

Material	No. Embryos Used	Wet Weight (g)
Unincubated egg yolk	3	40
Four day embryo	500	43
Six day embryo	100	40
Twelve day embryo	12	68
Fourteen day embryo	6	66
Eighteen day embryo	3	66

using 0.88% KCl. A volume of the wash solution equal to 0.2 of the volume of the lipid extract was thoroughly mixed with the lipid extract. After centrifugation, the aqueous layer was removed. The second washing was repeated three times using a solution of chloroform, methanol and 0.74% KCl (3:48:47). This was done by layering an aliquot of the solution over the chloroform layer without disturbing the surface of the liquid. The aqueous layer was removed by siphoning. In order to check the completeness of the lipid extraction, the residue remaining after the usual extraction procedure was allowed to stand in ten times its volume of chloroform for twelve hours. After filtration, the supernatant fraction was made to a known volume and aliquots were taken for phosphorus determinations. In a similar manner, the aqueous washings were dried and extracted with chloroform. This was done in order to ascertain the amount of lipid phosphorus lost in the washing procedure. From the results obtained both methods were considered to be adequate.

The washed lipid extract was evaporated to dryness in a rotary vacuum evaporator. The dried lipid sample was dissolved in 50 ml of chloroform and an aliquot was removed for total lipid phosphorus determination. The lipid solution was again evaporated to dryness in the

vacuum evaporator and dissolved in a small amount of chloroform. This solution was then applied to a silicic acid column. The lipid sample was usually applied to the column within four hours from the start of its preparation.

Ten grams of dried silicic acid and 5 g of Hyflo Super-Cel were successively washed and sucked dry on a sintered glass funnel with methanol and diethyl ether. The mixture was then made into a slurry with chloroform and poured into a glass column plugged with glass wool. The silicic acid column, 1.4 cm x 28 cm, was washed overnight by allowing chloroform to flow through it. The appropriate amount of lipid solution was applied to the column (0.6-1.0 mg lipid phosphorus per g of silicic acid). The neutral lipids were eluted with chloroform and the phospholipids with increasing concentrations of methanol in chloroform, using nitrogen pressure to maintain a flow rate of 1.0-1.5 ml per minute. The elution scheme used is shown in Table II. Fractions of 340 drops were collected with an automatic drop counting fraction collector. Usually between 160 and 170 tubes were collected from each column. The volume per tube ranged from approximately 5 ml with the chloroform to 8 ml with methanol.

The positions of the phospholipid peaks were

TABLE II

Elution Scheme

Chloroform		200 ml
Chloroform:methanol	9:1	180 ml
Chloroform:methanol	4:1	200 ml
Chloroform:methanol	3:2	250 ml
Chloroform:methanol	1:4	100 ml
methanol		100 ml

determined by phosphorus analysis (method of Bartlett (2)) on an aliquot of the eluate from each tube. The tubes containing the individual peaks were combined and diluted to a known volume. These pooled samples were used for subsequent qualitative and quantitative investigations of the nature of the phospholipids.

Phosphorus determinations on the pooled samples also were carried out by the method of Bartlett (2). The micro-Kjeldahl nitrogen determination described by Lang (22) was used for all nitrogen determinations.

Samples from each phospholipid peak were dried and hydrolyzed with 6N hydrochloric acid overnight at 100-110°C in sealed tubes. The hydrolysates were washed twice with petroleum ether to remove fatty acids. After drying in a vacuum desiccator, the hydrolysates were dissolved in distilled water and used for paper chromatography.

The lipid hydrolysates were chromatographed on Whatman No. 1 chromatography paper that had been washed successively with dilute hydrochloric acid and distilled water. Ascending one-dimensional chromatography was used with two separate solvent systems, ethanol-ammonia as described by Artom *et al.* (1) and butanone-methylcellosolve-acetic acid-water as described by Magee (23).

After drying, the papers were sprayed with an ethanolic solution of ninhydrin for amino acids and other amine compounds. Dragendorf's reagent was used for detection of choline. Inositol was detected by a general carbohydrate test with ethanolic silver nitrate and by a specific test for inositol as described by Nagai (30). Sphingosine was detected by dipping the papers in a solution of sodium fluorescein as described by Saito (41).

Qualitative paper chromatography of the unhydrolyzed phospholipid peaks was carried out on Whatman No. 1 chromatography paper treated with formaldehyde, acetic acid and ammonium thiocyanate similar to the procedure described by Hörhammer et al. (14). This consisted of thoroughly wetting the papers, rolled in a cylinder, with a solution of formaldehyde, acetic acid and ammonium thiocyanate (100:6.5:0.2, v/v/w). The soaked papers were autoclaved for eight hours at 121°C and 15 pounds pressure. The water washed and air dried impregnated papers were spotted with solutions of the individual phospholipid peaks and with solutions of commercial samples of lecithin, sphingomyelin, phosphatidyl inositol, phosphatidyl serine and phosphatidyl ethanolamine. The solvent system consisted of the upper phase of the system butanol-acetic acid-water (4:1:5) mixed with diethyl ether in the ratio of four parts upper phase to

one part of diethyl ether (v/v). The chromatograms were run in a cold laboratory at 0-4°C by the ascending technique. After drying, the papers were sprayed with a solution of ninhydrin in acetone to detect amine containing compounds or dipped in a 0.25% aqueous solution of malachite green to detect lipids in general.

Attempts at quantitative paper chromatography of hydrolysates of the peaks containing serine and ethanolamine by the method of Magee (23) were unsuccessful. The failure was considered to be due to the use of insufficient amounts of the phosphatides, since known samples of serine and ethanolamine carried through the same procedure gave adequate standard curves.

Quantitative choline determinations on the peaks shown to contain choline were carried out using a Neurospora crassa mutant, ATCC No. 34486, as described by Horowitz and Beadle (15). The cultures were grown on sterile Bacto choline assay medium containing the proper aliquots of hydrolysates from peaks V and VI. Recrystallized choline chloride, equivalent to 0.2 to 40 μg of choline, was used as a standard. The cultures were allowed to grow for five days at room temperature. After autoclaving for 10 minutes, the spores were collected, dried under a heat lamp and weighed. Choline concentration was directly related to spore weight. Table III

TABLE III

Typical Standard Curve for Choline

μg choline	mg *N. crassa* spores
-	2.3
1	6.4
2	8.6
4	14.
8	22.
10	27.
15	33.
20	38.
25	40.
30	37.
40	41.

shows the results of a typical standard curve.

Kloeckera apiculata, ATCC No. 9774, was used for quantitative estimation of inositol in the phospholipid peaks shown to contain inositol by paper chromatography. The assay medium resembled closely that described by Williams et al. (48). The cultures were grown at 30°C and read turbidimetrically in a Coleman Jr. Spectrophotometer at 625 m about every 24 hours up to 120 hours. A standard curve was run with each determination using myo-inositol as the standard. Results of a typical standard curve are shown in Table IV.

For quantitative assay of sphingosine, a modification of the McKibbin and Taylor procedure (28) was used. Aliquots of the peaks to be analyzed for sphingosine were dried and hydrolyzed in test tubes with 4 ml of saturated barium hydroxide, using a sand bath to maintain a temperature of approximately 100°C. After 5 hours 0.4 ml of concentrated hydrochloric acid was added to each sample and the hydrolysis continued for an additional 2 hours. The hydrolysates were extracted with three portions of chloroform, the first consisted of 5 ml and the final two of 3 ml each. The combined extracts were dried and dissolved in a known volume. Aliquots were used for total nitrogen determination by the micro-Kjeldahl method (22).

TABLE IV

Typical Standard Curve for Inositol

μg inositol	O.D. at 625 mμ at 120 hours
0.2	0.01
0.4	.017
0.7	.025
1.0	.031
1.5	.043
2.0	.051
3.0	.068
5.0	.11
8.0	.18
10.	.19
20.	.21
30.	.22
40.	.21

Qualitative identification of sphingosine in the extracts was established with fluorescein as described previously (41).

Separation of phosphatidyl serine from phosphatidyl ethanolamine in peaks II and III was achieved by a modification of the method of Rouser (40). Five grams of dried silicic acid were successively washed and sucked dry on a sintered glass funnel with methanol and then chloroform. After slurring the mixture in a solution of four parts of chloroform to one part of methanol, it was poured into a glass wool plugged column of 1.0 cm diameter. When the column neared dryness 20 ml of a solution of chloroform, methanol and concentrated ammonium hydroxide in the ratio of 80:20:1 was passed through the column. Nitrogen pressure was used to maintain a flow rate of 0.5-0.7 ml per minute. The lipid sample, in a solution of chloroform and methanol in the ratio of four parts chloroform to one part methanol, was applied to the column when it neared dryness. The elution was carried out with 150 ml of a solution of chloroform and methanol in the ratio of four parts chloroform to one part methanol, followed by 50 ml of methanol. Approximately 35 tubes were collected from each column. Phospholipid peaks were located by phosphorus analysis as described previously (2).

Protein and DNA determinations were carried out on the residual material remaining after lipid extraction. The residue was dried, weighed and pulverized with a mortar and pestle. A portion (200 mg) was taken and homogenized with cold 5% trichloroacetic acid in a Dounce homogenizer. After centrifugation the precipitate was extracted again with 5% trichloroacetic acid for 15 minutes in an ice bath. The precipitate obtained by centrifugation was extracted with 95% ethanol and centrifuged. The precipitate was extracted twice with 5 ml portions of 5% trichloroacetic acid for 15 minutes at 90°C. The combined extracts were used for DNA determination by the diphenylamine reaction as described by Burton (5). The precipitate remaining after the final extraction was used for total protein nitrogen determination by the micro-Kjeldahl method described earlier.

RESULTS

Results are given in Figures 1-6 for the separation of the lipids extracted from unincubated egg yolk and chick embryos from eggs incubated for four, six, twelve, fourteen and eighteen days. Considerable difference is observed between the profile of the egg yolk and those from the embryos. On the other hand, the profiles of the embryo lipid separations at different ages remain quite similar except for changes in the relative amounts of lipid phosphorus contained in the individual peaks. Table V shows the percent of the total lipid phosphorus in the individual peaks. The different values were obtained in separate experiments. Table VI lists the major nitrogen constituents and other components identified by paper chromatography and spot tests. The range of the nitrogen/phosphorus values obtained for each peak are also shown.

Comparison of the lipid constituents of yolk with those of the embryo shows that the most striking change observed is the emergence of serine containing phospholipids. In addition, it can be seen that two new phospholipid peaks appear in the embryo, namely I and III. It is thought that peak III of the egg yolk is a solvent change artifact due to tailing from peak II. The small

FIGURE I.

EGG YOLK LIPIDS

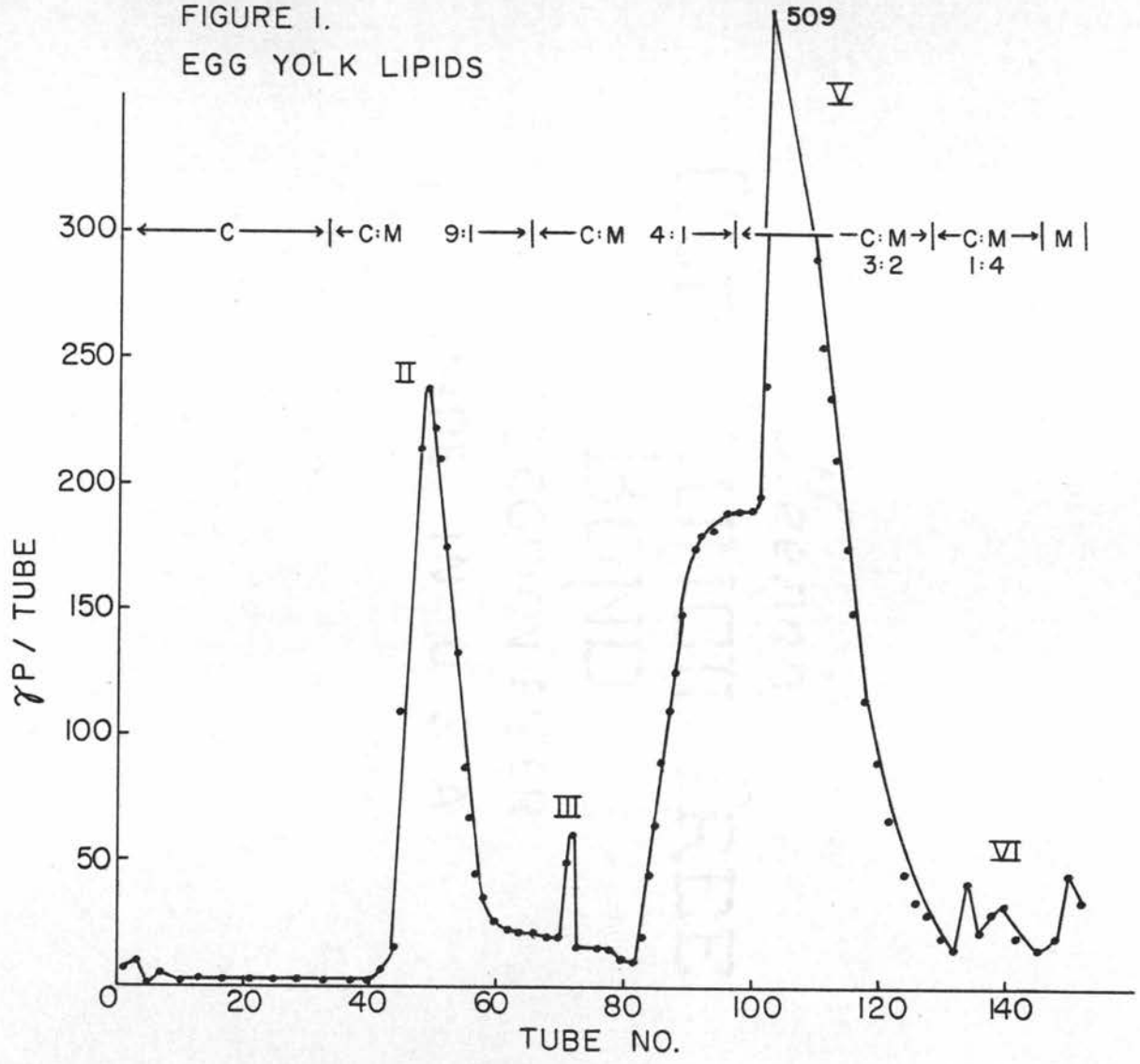


FIGURE 2

4 DAY EMBRYO LIPIDS

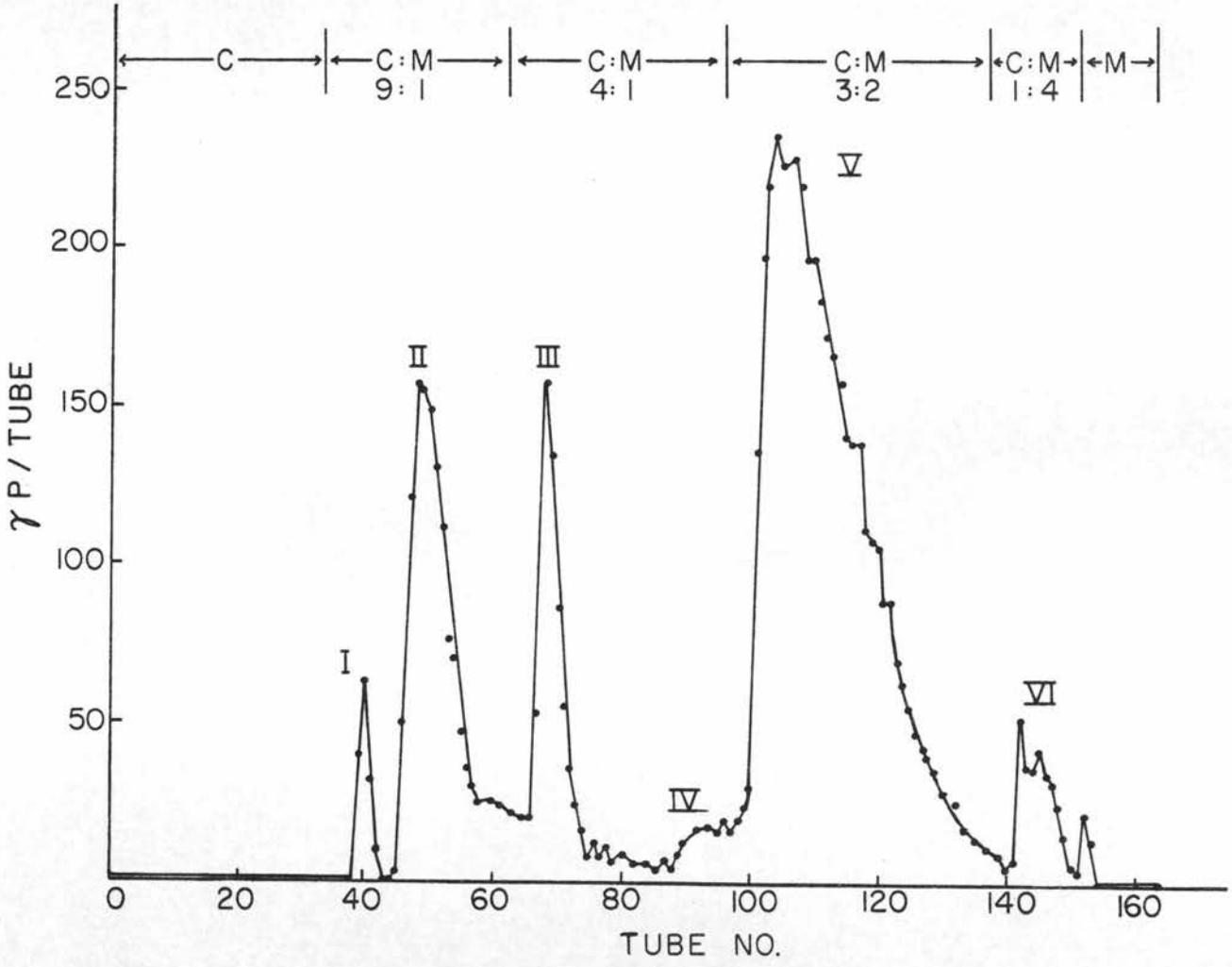


FIGURE 3
6 DAY EMBRYO LIPIDS

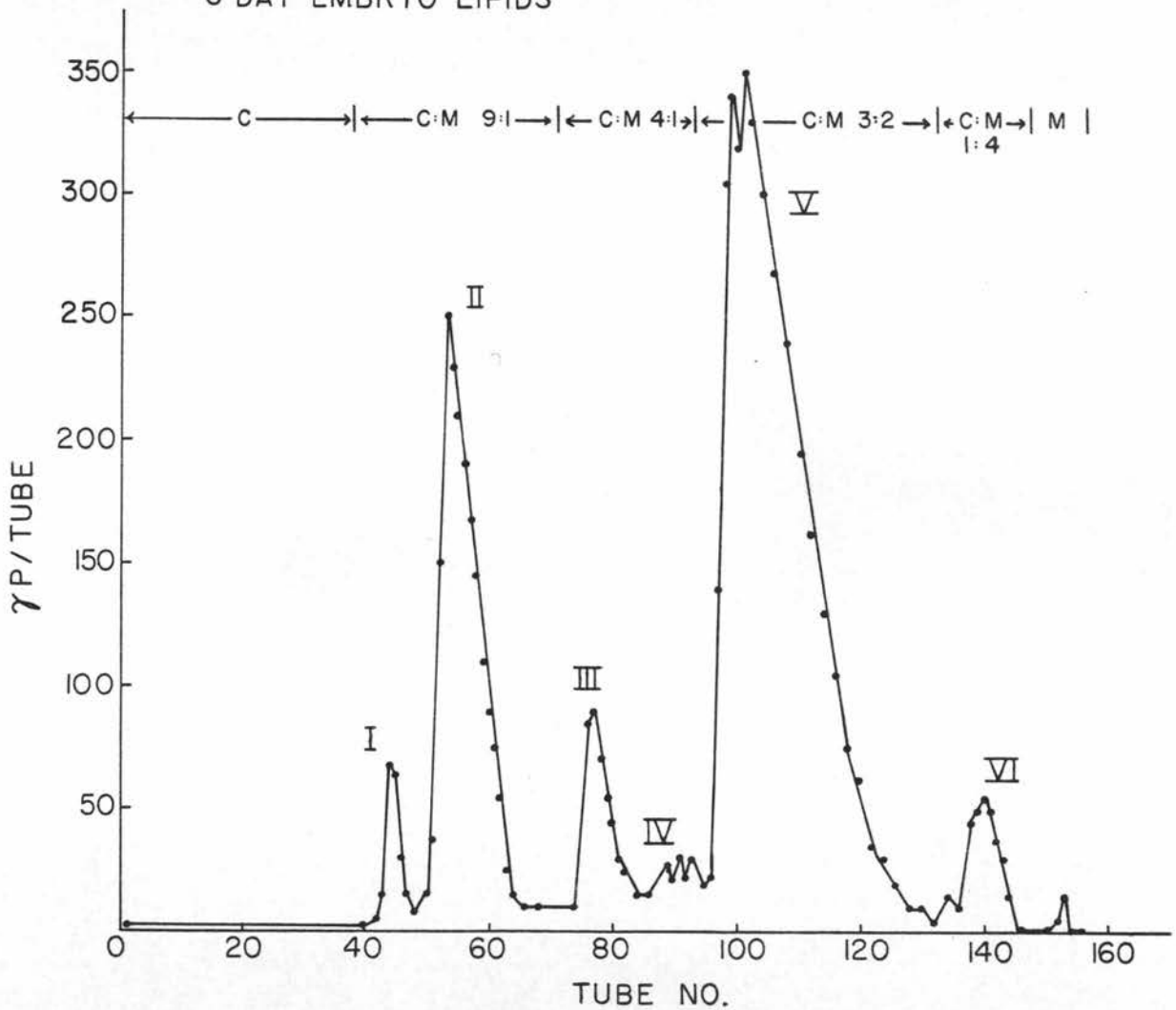


FIGURE 4
12 DAY EMBRYO LIPIDS

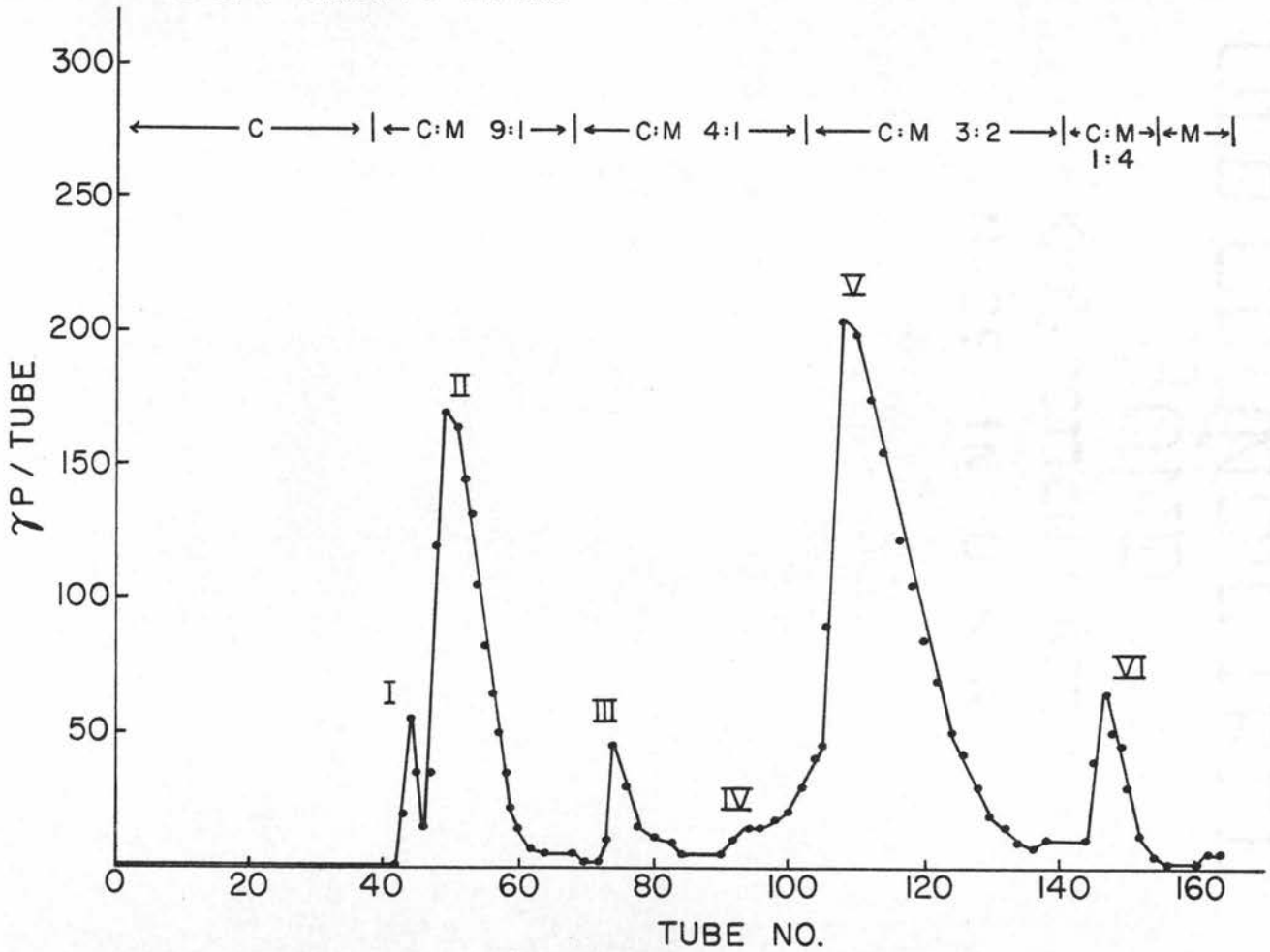


FIGURE 5
14 DAY EMBRYO LIPIDS

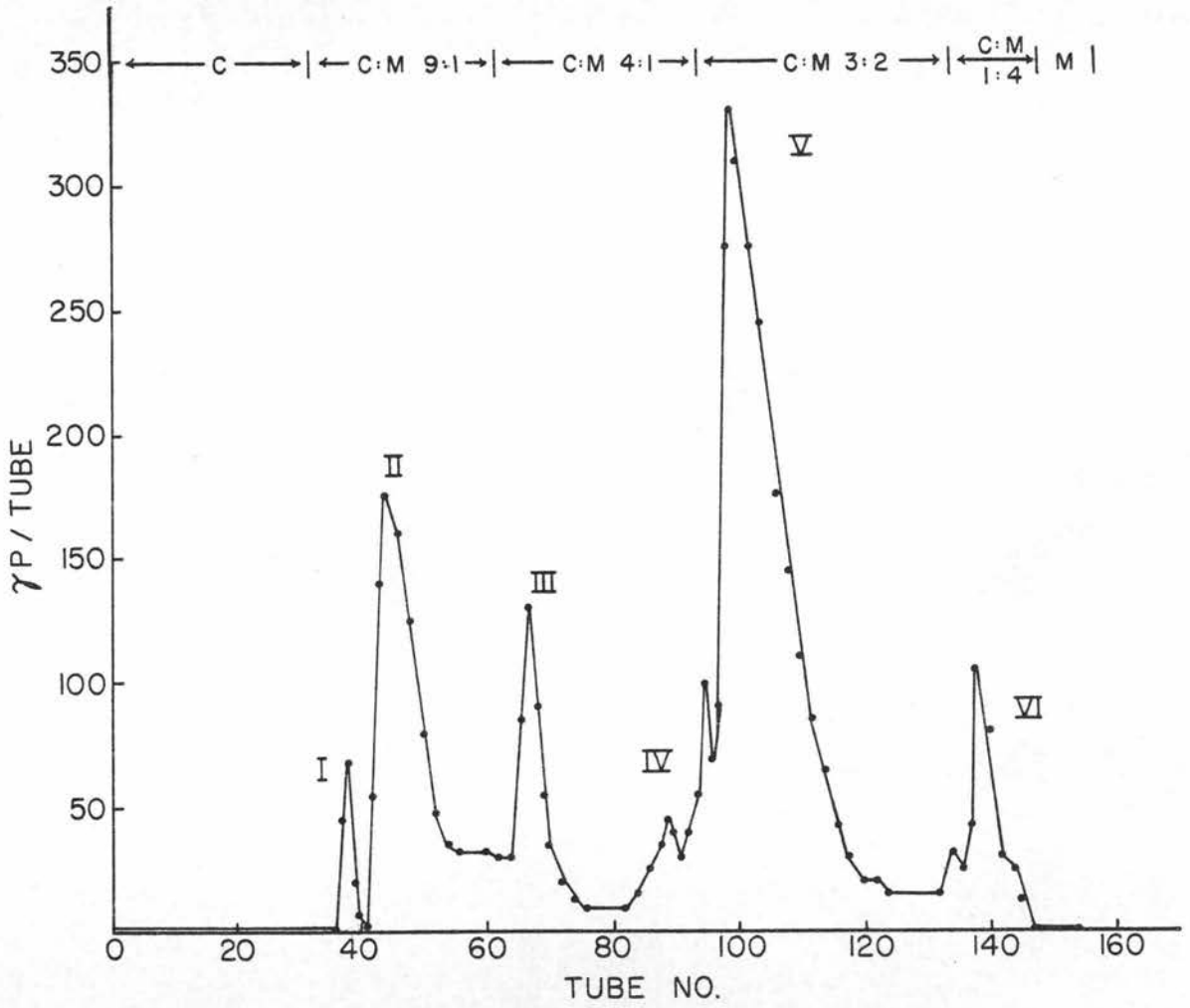


FIGURE 6

18 DAY EMBRYO LIPIDS

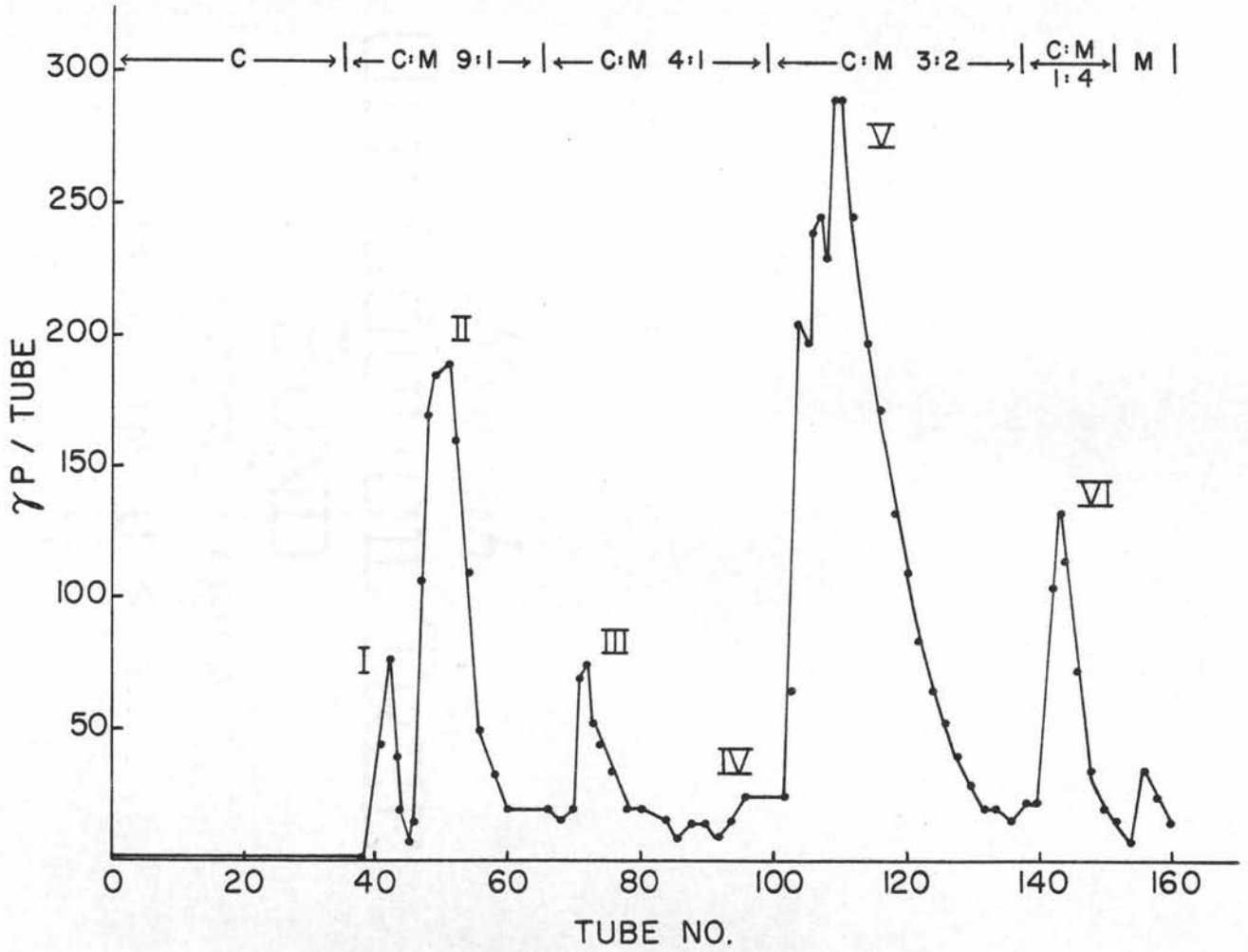


TABLE V

Percent Lipid Phosphorus
in Individual Phospholipid Peaks
Eluted from Silicic Acid Columns

	Peak Number					
	I	II	III	IV	V	VI
	%	%	%	%	%	%
Egg		19.	3.9		72.	5.3
4 day embryo	2.8	19.	10.	3.2	58.	6.2
6 day embryo	4.0	21.	10.	6.5	52.	8.9
	2.4	23.	6.5	3.9	58.	6.5
	2.9	23.	11.	4.3	55.	4.5
12 day embryo	2.9	26.	4.5	2.1	56.	9.1
	3.1	25.	5.6	2.3	54.	10.
	3.3	23.	7.6	4.7	51.	11.
14 day embryo	3.2	25.	5.0	5.8	50.	11.
	2.4	25.	5.3	2.8	53.	11.
	2.4	24.	8.2	5.0	50.	11.
	2.7	22.	8.1	4.8	50.	12.
18 day embryo	2.2	19.	8.6	7.3	51.	12.
	3.4	22.	5.6	10.	45.	14.
	2.8	21.	7.6	3.7	50.	14.
	2.9	18.	11.	4.1	51.	13.

TABLE VI

Ratios of Nitrogen to Phosphorus
and Principal Constituents
of Eluted Phospholipid Peaks

	Peak No.	N/P	Major Constituents
Egg	I		
	II	1.1	Ethanolamine
	III	1.1	Ethanolamine
	IV		
	V	1.1	Choline Inositol
	VI	1.6-2.1	Choline Sphingosine
4 day embryo	I	0.9	Serine
	II	1.0-1.2	Ethanolamine Serine
	III	1.1-1.5	Ethanolamine Serine
	IV	0.9-1.0	Ethanolamine Serine Inositol
	V	1.0-1.1	Choline Inositol
	VI	1.5-1.9	Choline Sphingosine
6 day embryo	I	1.0-1.2	Serine
	II	1.0-1.1	Ethanolamine Serine
	III	1.1-1.3	Ethanolamine Serine
	IV	0.8-1.0	Ethanolamine Serine Inositol
	V	1.0-1.1	Choline Inositol
	VI	1.9-2.1	Choline Sphingosine
12 day embryo	I	0.7-1.0	Serine
	II	1.1-1.3	Ethanolamine Serine

Continued on next page

TABLE VI - Cont.

	Peak No.	N/P	Major Constituents
	III	1.0-1.2	Ethanolamine Serine
	IV	0.7-0.9	Ethanolamine Serine Inositol
	V	1.0-1.1	Choline Inositol
	VI	1.5-2.0	Choline Sphingosine
14 day embryo	I	1.0-1.5	Serine
	II	1.0-1.1	Ethanolamine Serine
	III	1.0-1.1	Ethanolamine Serine
	IV	0.7-0.9	Ethanolamine Serine Inositol
	V	0.9-1.2	Choline Inositol
	VI	1.5-2.1	Choline Sphingosine
18 day embryo	I	1.2-2.3	Serine
	II	1.0-1.3	Ethanolamine Serine
	III	1.1-2.5	Ethanolamine Serine
	IV	0.8-1.3	Ethanolamine Serine
	V	1.0-1.5	Choline Inositol
	VI	1.9-2.0	Choline Sphingosine

shoulder labelled IV in the embryo profiles is not present in the egg yolk profile.

Peaks II and III of the egg yolk have ethanolamine as the sole constituent. Peak V contains choline and a small amount of inositol lipid while peak VI has choline and sphingosine as the identified lipid components.

The only constituent identified in acid hydrolysates of peak I of the embryos is serine. Peaks II and III of all ages of the embryo studied have been shown to contain serine and ethanolamine phospholipids. The small shoulder just preceeding the major phospholipid peak is a mixture of serine, ethanolamine and inositol phosphatides. The major peak consists mainly of choline phospholipids with a small amount of inositol phospholipid and peak VI has been shown to contain choline and sphingosine as the constituents of the phospholipids. The high nitrogen/phosphorus values obtained on this peak would suggest that the sphingolipids are predominant.

In an effort to estimate the amount of serine and ethanolamine phospholipids present in peaks II and III of the embryos, they were separated further. Typical separation curves for peaks II and III are shown in Figures 7 and 8, respectively. Chromatography of the eluted lipid peaks on impregnated paper revealed single spots with ninhydrin and with malachite green, IIa and

FIGURE 7

SEPARATION OF PEAK II USING CHLOROFORM:METHANOL, 4:1, AND METHANOL

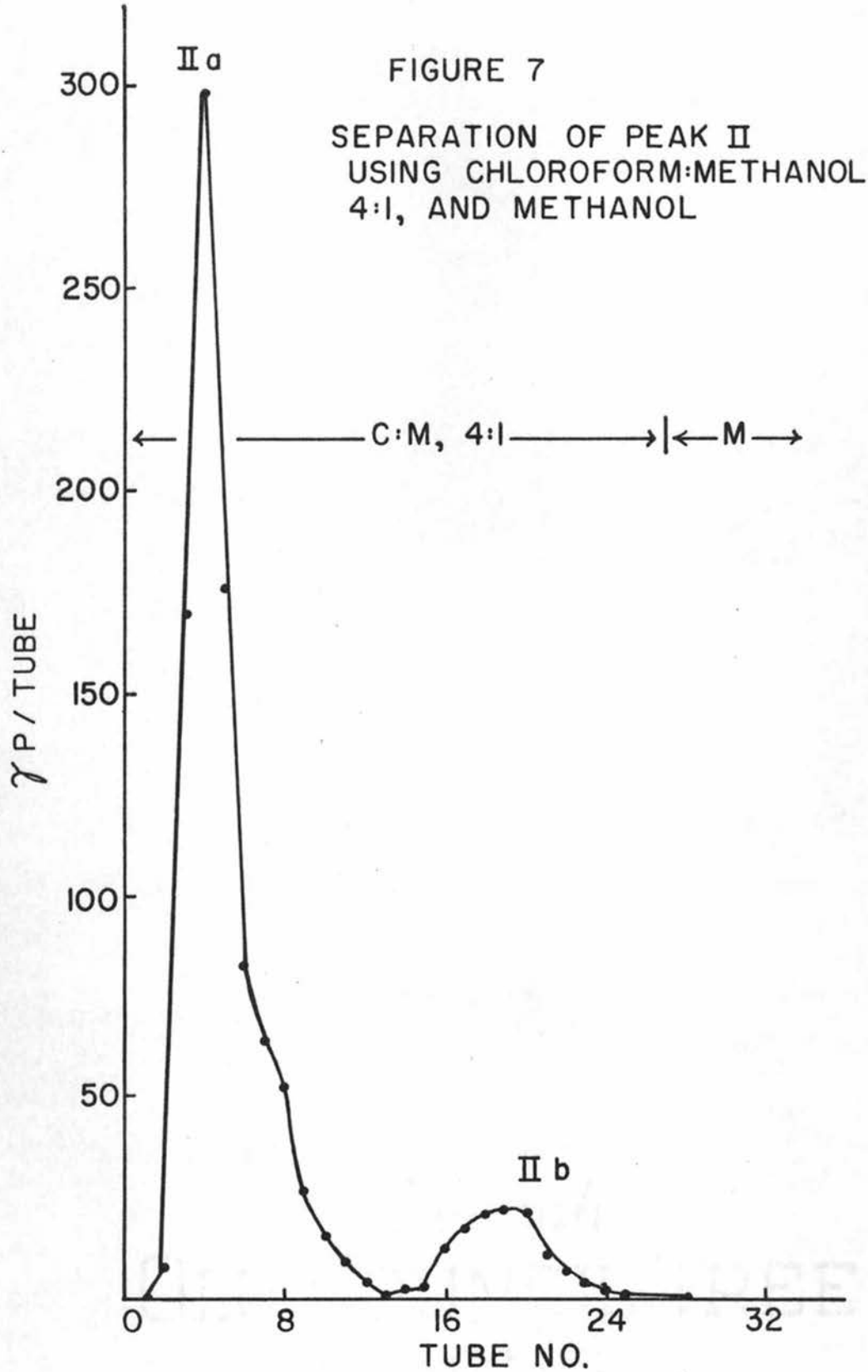
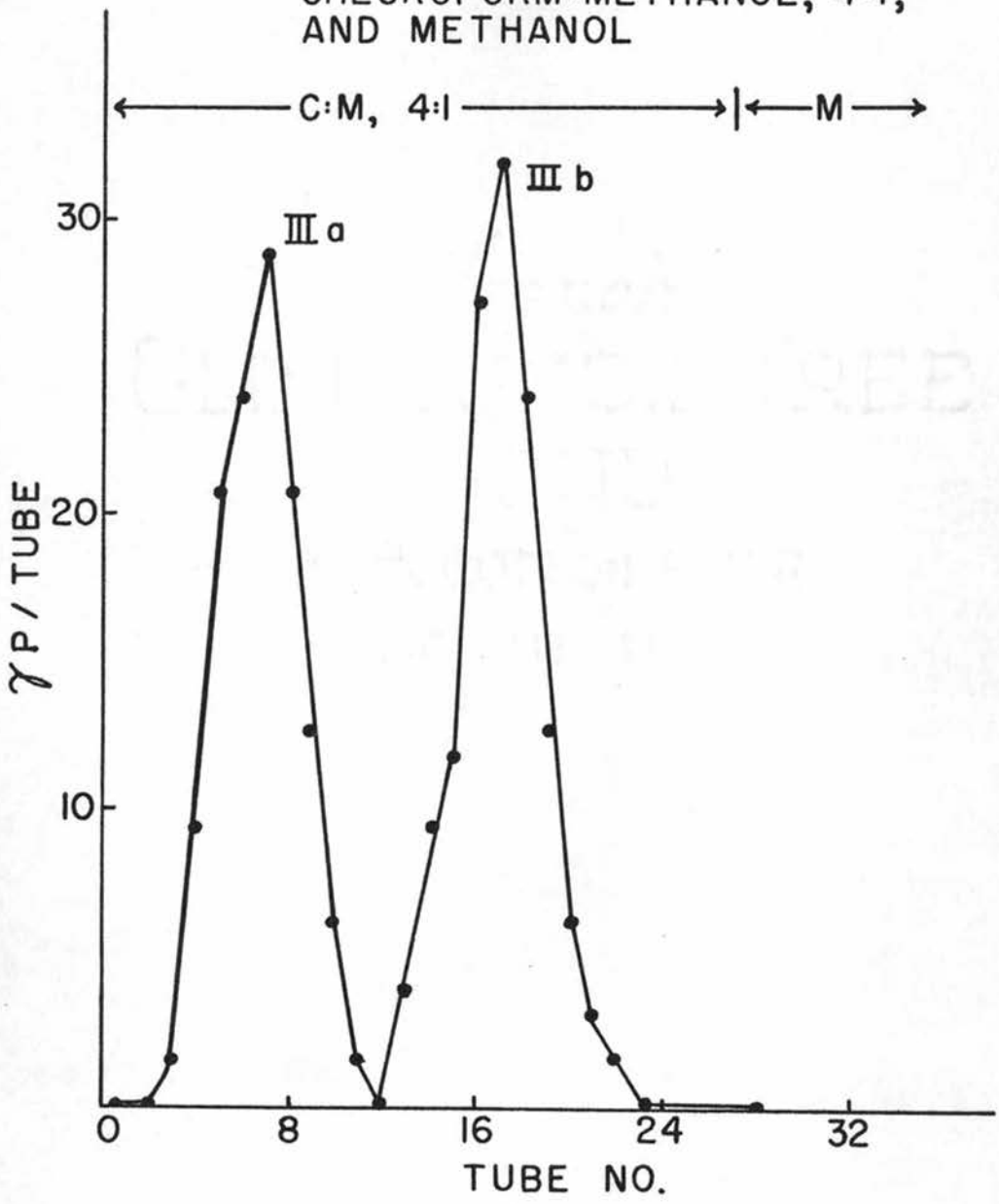


FIGURE 8
SEPARATION OF PEAK III USING CHLOROFORM:METHANOL, 4:1, AND METHANOL



IIIa containing phosphatidyl ethanolamine and IIb and IIIb containing the serine phosphatide. Paper chromatography of the acid hydrolysates of the separated peaks showed the presence of mainly ethanolamine in peaks IIa and IIIa, with very minor amounts of serine and unidentified ninhydrin reactive compounds. One of the unidentified compounds was shown to contain phosphate by spraying with the solution described by Burrows et al. (4). Peaks IIb and IIIb were shown to contain serine and minor quantities of other ninhydrin reactive components.

Table VII gives the percent of the total lipid phosphorus present as phosphatidyl serine and phosphatidyl ethanolamine in peaks II and III. Calculations are based on the assumption that peaks IIa and IIIb have ethanolamine as the sole phosphatide component and peaks IIb and IIIb have phosphatidyl serine as the only phospholipid component.

Table VIII gives the data on estimation of inositol present in peaks IV and V. The calculations are based on the assumption of a molar ratio of unity of inositol/phosphorus in the inositol phosphatides present.

Table IX gives the quantitative data obtained from analysis of peaks V and VI for choline and peaks VI for sphingosine. The values for choline/phosphorus are

TABLE VII

Percent of Total Lipid Phosphorus Present as Serine and Ethanolamine Phospholipids

	Peak No.	Constituent	Total Lipid P
			%
4 day embryo	IIa	Ethanolamine	18.
	IIIa		6.6
	IIa + IIIa		24
	IIb	Serine	1.5
	IIIb		4.0
	IIb + IIIb		5.5
6 day embryo	IIa	Ethanolamine	22.
	IIIa		6.5
	IIa + IIIa		28.
	IIb	Serine	1.2
	IIIb		4.2
	IIb + IIIb		5.4
12 day embryo	IIa	Ethanolamine	20.
	IIIa		2.7
	IIa 2 IIIa		23.
	IIb	Serine	4.4
	IIIb		2.9
	IIb + IIIb		7.3
14 day embryo	IIa	Ethanolamine	19.
	IIIa		4.2
	IIa + IIIa		23.
	IIb	Serine	3.1
	IIIb		3.9
	IIb + IIIb		7.0
18 day embryo	IIa	Ethanolamine	14.
	IIIa		3.8
	IIa + IIIa		18.
	IIb	Serine	6.9
	IIIb		3.7
	IIb + IIIb		11.

TABLE VIII

Estimation of Percent of Total Lipid Phosphorus Present as Phosphoinositides

	Peak No.	Phosphorus	Total Lipid P
		%	%
4 day embryo	IV	29.	0.9
	V	5.5	3.2
6 day embryo	IV	27.	1.2
	V	7.0	3.7
12 day embryo	IV	30.	1.3
	V	3.9	1.9
14 day embryo	IV	15.	0.7
	V	5.2	2.6
18 day embryo	IV	12.	0.5
	V	8.0	4.1

TABLE IX

Estimation of Ratios
of Choline to Lipid Phosphorus
and Sphingosine to Lipid Phosphorus

	Peak No.	Choline/P Acid Hydrolysis	Choline/P Basic Hydrolysis	Sphingosine N/P
Egg	V		1.0	
	VI		0.9	1.0
4 day embryo	V	0.8	0.8	
	VI	0.5	0.6	0.9
6 day embryo	V	0.9	1.7	
	VI	0.3	0.6	1.3
12 day embryo	V	0.9	0.8	
	VI	0.2	0.4	0.8
14 day embryo	V	0.8	1.3	
	VI	0.2	0.4	0.9
18 day embryo	V	0.8	0.7	
	VI	0.2	0.6	1.0

usually lower than unity, which is in part due to the presence of inositides in peak V. Table X gives choline/phosphorus ratios calculated using molar phosphorus values for peak V from which the molar amounts of inositol present in peak V have been subtracted, assuming inositol/phosphorus ratio of unity.

Table XI shows the change in lipid phosphorus during the development of the chick embryo when compared to DNA, protein nitrogen and fat-free dry weight. The ratio of lipid phosphorus to DNA appears to remain relatively constant in all the ages of the embryo studied. The ratios of lipid phosphorus to protein nitrogen and fat-free dry weight, on the other hand, decrease steadily throughout development, as does the ratio of DNA to protein nitrogen. The differences in the two separate determinations are in part due to the fact that in the first set of values the extraction of DNA was only carried out once, while in the second determination the extraction was repeated a second time. All values of lipid phosphorus on the six day embryo in the second set of values are too low due to loss of some of the lipid during preparation of the sample.

Table XII summarizes the data on percent of total lipid phosphorus present in each individual phospholipid.

TABLE X

Estimation of Ratio of Choline to Phosphorus
using Total Phosphorus Values
Corrected for Inositol in Peak V

	μM Choline	Total μM P - μM Inositol	Choline/P
4 day embryo	89	107	0.85
6 day embryo	84	89	0.94
12 day embryo	117	122	0.98
14 day embryo	112	128	0.89
18 day embryo	81	97	0.85

TABLE XI

Estimation of Lipid Phosphorus Present
on the Basis of DNA, Protein Nitrogen and Dry Weight

	mg Lipid P / mg DNA	mg Lipid P / mg Protein N*	mg Lipid P / g Dry Wt.+	mg DNA / mg Protein N*
Egg	3.2	0.18		0.06
6 day embryo	0.12	0.064	6.5	0.53
12 day embryo	0.13	0.059	5.0	0.44
14 day embryo	0.13	0.046	4.3	0.35
18 day embryo	0.17	0.035	2.5	0.20
4 day embryo	0.10	0.057	5.6	0.55
6 day embryo	0.095	0.042	4.7	0.45
12 day embryo	0.11	0.045	4.8	0.41
14 day embryo	0.11	0.032	3.7	0.30
18 day embryo	0.11	0.019	2.5	0.17

*Fat-free Kjeldahl nitrogen
+Fat-free Dry weight

TABLE XII

Average Percent of Eluted Lipid Phosphorus Present as Individual Phospholipids

Peak No.	Constituents	Percent Lipid Phosphorus					
			Embryo Age (in days)				
		Egg	4	6	12	14	18
I	Serine Phospholipids		2.8	3.1	3.1	2.7	2.8
II	Serine Phospholipids		1.5	1.2	4.4	3.1	6.9
	Ethanolamine Phospholipids	19.	18.	21.	20.	19.	14.
III	Serine Phospholipids		4.0	4.2	2.9	3.9	3.7
	Ethanolamine Phospholipids	3.9	6.4	6.5	2.7	4.2	3.9
IV	Inositol Phospholipids		0.9	1.2	1.3	0.7	0.5
	Serine Phospholipids						
	Ethanolamine Phospholipids						
V	Choline Phospholipids	72.	58.	55.	53.	51.	49.
	Inositol Phospholipids		3.2	3.7	1.9	2.6	4.1
VI	Sphingomyelin	5.3	6.2	6.6	10.	11.	13.

DISCUSSION

The egg is the source of nourishment for the embryonic development of all animals except the mammals. It is known to contain considerable amounts of lipid material including phospholipid. The phospholipids of the chicken egg yolk have been separated, purified, and identified as previously indicated. Very little information on the separation and identification of the individual phospholipids of the developing chick embryo is available. The chick embryo would seem to be an ideal source of material for such a study because of the ease of identifying the stages of development and the relative abundance of material. The major drawback to its use in the study of phospholipids is the number of embryos required to obtain a sufficient amount of material at the early incubation stages. The phospholipids are noted for being easily autooxidized and being subject to autolysis during extraction and purification (10, p. 13-15; 40, p. 113-114). Considering these facts, dissection of the many embryos required for such a study at the early stages could become a limiting factor, since relatively long periods of time may elapse between the beginning of the dissection and the application of the lipid to the column. It is conceivable that such lengths of time

might result in extensive alteration of the extracted lipids and thus introduce certain artifacts.

Table V shows the results of the individual separations of egg yolk and chick embryo phospholipids. It can be seen that there is some variation between individual separations of the phospholipids from the same embryonic age. Considering the methods and biological material used, the agreement is remarkably good. The variation may be due to several factors: the silicic acid columns used were poured from unused silicic acid. Although the silicic acid may be from the same lot, it is difficult to obtain columns with identical properties. Other differences such as temperature variation on a given day and differences in temperature from day to day certainly have an effect on the elution of the phospholipids. Uncertainty in localizing the exact tube to begin a particular fraction also may affect the percentage of lipid phosphorus found in each peak, particularly peaks III and IV, where the separation was not very precise.

Not listed in Table VI as major constituents, but usually present in small amounts, were certain unidentified ninhydrin reactive compounds. At least one of these coincided with a phosphate spot and thus may have been serine or ethanolamine phosphate. The possibility cannot

be excluded that the remainder of these unidentified compounds may have been components of the phospholipids. It seems more likely that they were artifacts produced by extraction, probably amino acids rendered soluble by the lipid solvents as described by Dawson (7, p. 192). Contamination of lipid extracts is a common occurrence and is not limited to amino acids but may also include sugars, sterols, urea and many inorganic compounds. Although nitrogen contaminents are present as evident from paper chromatography, most of the N/P ratios are remarkably near unity as shown in Table VI, indicating a relatively small amount of contamination by non-lipoidal nitrogen.

The separation of lysophosphoglycerides from phosphoglycerides was not achieved in this study. Comparison of the results of the egg yolk lipid separation with the results of Rhodes and Lea (37) may still be of interest, if one considers the total ethanolamine phospholipid and the total choline phospholipid as a basis for comparison. Table XII shows 23% of the total lipid phosphorus as ethanolamine and 77% as choline, if peaks II and III are considered as ethanolamine and peaks V and VI as choline containing phospholipids. These values compare favorably with the respective literature values of 17.1% and 82.3%. The variation observed may be due to differences in the

breed of chicken used or the diet of the hens from which the eggs were obtained.

It can be seen from Figures 2-6 that the phospholipid profiles remain similar throughout the period of development studied. The major changes taking place appear to be in the relative amounts of lipid phosphorus located in each individual peak as shown in Table XII. It is interesting to note that the total percentage of the lipid phosphorus present in peaks I-IV and peaks V-VI remains essentially constant throughout the period studied.

The only constituent identified in peak I of the embryo lipids is serine. Similar results were obtained by Hanahan et al.(11, p. 693) in a study of the rat liver phosphatides. In view of the wide range of nitrogen to phosphorus ratios obtained for peak I and the evidence of nitrogen contaminants in the lipid extracts, the possibility exists that this peak may contain some nitrogen contaminants such as amino acids. Chromatography of the nitrogen constituents after hydrolysis indicated that the principal nitrogen compound was serine but small amounts of other amino acids were encountered. Since this peak constitutes 4% or less of the total phospholipid this also means that the nitrogen determinations

are subject to a greater error than occurs with larger peaks. Thus values of N/P ratios of 0.7 to 1.5 reasonably may be considered 1.0.

Peaks II and III have been shown to contain serine phospholipids and ethanolamine phospholipids in varying amounts. The reason for the unsuccessful separation of these two types of phospholipids is unknown but this seems to be a common phenomenon in silicic acid chromatography (40). Further separation of these compounds as shown in Figures 7 and 8 has permitted calculation of the percent phosphatidyl serine and phosphatidyl ethanolamine present in peaks II and III as shown in Table VII. The assumption was made that all the phosphorus eluted in the first peak was ethanolamine and that in the second peak was serine. There appears to be an increase in the phosphatidyl serine between fourteen and eighteen days of incubation as shown in Table XII. Correlation of this change with morphological or functional change in the embryo at a corresponding time seems unwarranted because of the lack of information on the distribution and function of phosphatidyl serine in the chick embryo.

Inositol has been shown to be present in peak IV to the extent shown in Table VIII, assuming a ratio of unity for inositol to phosphorus for all the inositides

present. The microorganism used for quantitative assay of inositol has a lower growth response to inositol phosphate than to inositol (10, p. 110). The relative proportion of inositol and inositol phosphate in the hydrolysates was not determined due to the small amounts of total phospholipid occurring in this peak. It seems evident that the inositides represent only a small percent of the total phospholipids and little change in concentration occurs during development. Serine and ethanolamine also were shown to be present in peak IV. Hanahan (11, p. 693-695) has shown the presence of serine and ethanolamine in the inositide fractions of phospholipids from beef liver, rat liver and yeast.

As is evident from Figures 2-6, peak V is the major phospholipid peak throughout development, representing approximately one-half of the total lipid phosphorus. The major constituent is lecithin but a significant amount of inositol containing lipid is also present as shown in Table VIII. It can be seen from Table IX that the ratio of choline to phosphorus is less than unity. The presence of inositol is in part responsible for these low ratios. Table X shows a recalculation of the ratios, using corrected lipid phosphorus values obtained by subtracting the molar values of inositol from the total molar phosphorus values. Such

calculations result in a ratio of choline to phosphorus nearer unity. It appears that the relative lecithin content of the embryo decreases slightly during development.

Peak VI is probably the most interesting since it shows the most significant change. The presence of sphingosine and choline and the ratios of nitrogen to phosphorus approaching two indicate this peak consists mainly of sphingomyelins. The most striking change in relative concentrations of the phospholipids is found in this peak. The percentage of lipid phosphorus as sphingomyelin at least doubles between the fourth and the eighteenth days of incubation. The possible significance of this change will be discussed.

Although the situations are not the same and the techniques used were different, it is interesting to compare the changes in relative proportions of lecithin, cephalin and sphingomyelin in the developing chick with those obtained from the growing rat by Williams et al. (46, p. 466). Studying rats at birth, fifteen days, forty-five days and seventy days of age, they showed the percent lipid phosphorus as lecithin to change from 74% at birth to 33% at forty-five days and 37% at seventy days of age. Cephalin was found to be 20%, 59% and 50%, respectively, while sphingomyelin increased from 6% to

13%. Table XII shows lecithin to decrease from 58% to 49% between four and eighteen days incubation while sphingomyelin increased from 6% to 13%. If one considers peaks I-IV as cephalin, very little change is noted in the cephalin fraction. In a subsequent study Williams et al. (47) studied the changes in the relative amounts of lecithin, cephalin and sphingomyelin in several individual rat tissues at fifteen, forty-five and seventy days of age. In general there appeared to be an increase in the relative percentage of cephalin and a decrease in the lecithin with increasing age in all the tissues studied. Sphingomyelin appeared to vary independently, increasing in some tissues, decreasing in some and remaining relatively constant in others. Such results illustrate the futility of attempting to correlate changes in gross phospholipid composition with specific morphological or functional changes.

Nevertheless, the doubling of the relative sphingomyelin content between four and eighteen days of incubation seems to be significant enough to warrant some consideration. It has been suggested that brain formation during development takes place in four rather distinct periods (27, p. 179-180). The first period is one of rapid cell division in which the cell number of the brain nearly reaches the adult level, even though

the weight is only a fraction of the adult weight. The second period is considered to be characterized by growth in size of the individual cells. In the third period of formation, the overall growth rate decreases, but some new processes commence, the most prominent of these in its effect on activity and composition, is myelin sheath formation. In the fourth period the overall growth proceeds slowly to the adult level while myelination remains quite active. The period of active myelination and development of well defined electrocorticograms in the chick embryo is between ten and nineteen days of incubation (12, p. 39). A marked increase in the sphingomyelin content in the chick embryo brain between the thirteenth and sixteenth days of incubation is reported by Mandel et al. (24). In the phospholipids of the whole chick embryo, an increase in the sphingomyelin content from 6% to 13% between four and eighteen days incubation is seen in Table XII. This period of incubation nearly spans the active myelination period of the chick embryo and the increase in sphingomyelin during this time may well be due mainly to the phenomenon of myelination. The evidence presented is only suggestive and it should be recognized that equating quantitative changes in the sphingomyelin content with myelin sheath formation suffers, not only from lack of information on the lipid

composition of the sheath, but also rests on the unproven assumption that an increase in sphingomyelin represents myelin sheath lipid simply because it increases at the same time the myelin sheath appears. Nevertheless, it seems reasonable to suggest a correlation between this morphological and biochemical change.

Consideration of the data presented in Table XI shows the ratio of lipid phosphorus to deoxynucleic acid (DNA) to remain relatively constant throughout the period of development studied. Since DNA can be used as an indication of the cell number, this could be taken as an indication of a constant amount of lipid phosphorus per cell, regardless of cell type. Such a conclusion would seem to be untenable in view of the suggestions made previously about the formation of the brain. For example, if the brain has nearly the adult number of cells during the first stage of development, 0-10 days incubation, its DNA content would of necessity remain relatively constant during the remainder of its development and growth. On the other hand, Mandel et al. (24) have shown a large increase in the phospholipid content of the chick brain between twelve and sixteen days of incubation, both in quantity and in concentration as percent of dry weight. From this example it would be obvious that the lipid content of a brain cell does not remain

constant during development, but increases. A more likely suggestion is that the relatively constant ratio of lipid phosphorus to DNA is a coincident happening during differentiation, that is, individual tissues vary in their relative phospholipid content, some increasing while others are decreasing. The net effect of such changes in the individual tissues results in a constant ratio of lipid phosphorus to DNA.

As one would expect, the DNA to nitrogen ratios decrease throughout development. Such a change results from continued growth of the cells which were produced during the early stages of rapid proliferation and the general tendency for the mitotic index to decline during development (25). The continued growth results in increased nitrogen accumulation while the declining mitotic index results in a decreasing rate of DNA accumulation.

The DNA results are in general agreement with those of Wang et al. (45), who showed a decline in the ratio of DNA to fat-free dry weight in the chick embryo during development. Such a change is observed in the data presented in Table XI. Novikoff and Potter (34) and Reddy et al. (36) have studied the changes in DNA during the development of the chick embryo. The results of these workers do not appear to agree but Marrian et al. (25) states that this is due to an error in a

caption in the publication of Novikoff and Potter (34). Recalculation of the data shows it to be in agreement with that of Reddy et al. (36). Thus the DNA content on a dry weight basis is shown to increase until about fourteen days of incubation and then to decline. It is difficult to compare the dry weight data mentioned above with the fat-free dry weight data presented in Table XI.

This investigation has shown that certain changes in the phospholipids occur during the development of the unincubated egg into an embryo and further changes, namely in the relative concentrations of the individual phospholipids, occur within the embryo as development progresses. The change noted between egg and embryo is suggestive of phospholipid synthesis by the embryo. The presence of such a synthetic system in the embryonic tissue as well as phospholipids with the same constituents as those found in adult tissues emphasizes the unity of biochemistry, while diversity is evidenced by the changes in the relative concentration changes in the phospholipids during development and the variation between individual tissues as noted in the introduction and discussion.

SUMMARY

The phospholipids of unincubated egg yolks and embryos from four, six, twelve, fourteen and eighteen days of incubation have been separated on columns of silicic acid and Hyflo Super Cel. The major phospholipid components of the peaks obtained have been identified by hydrolysis and paper chromatographic techniques. Quantitative estimations of ethanolamine, serine, choline, inositol, and sphingosine containing phospholipids were made.

Phospholipids of the egg yolk were found to contain ethanolamine, inositol, choline, and sphingosine. The embryo phospholipids were found to have the same constituents as the egg yolk with the addition of serine phospholipids.

The lipid profiles of the egg yolk were found to differ considerably from those obtained from the embryos. Only three peaks were evident in the egg yolk, while six separate fractions were obtained in the separations of all embryonic ages studied. The pattern of the phospholipids in the embryos remained similar throughout the period of development studied. Changes in the relative amounts of lipid phosphorus in the individual peaks were observed, particularily in the sphingomyelin containing

peak. The sphingomyelins were found to increase from 6% of the total lipid phosphorus at four days incubation to 13% at eighteen days of incubation. It has been suggested that this change may be a reflection of active myelination in the nervous system of the developing chick embryo. The lecithin content was found to decrease from 58% to 49% during the same period of time.

On the basis of DNA the lipid phosphorus content was found to remain relatively constant between four and eighteen days of incubation. Lipid phosphorus content based on fat-free Kjeldahl nitrogen content and fat-free dry weight were both found to decrease throughout the developmental period studied.

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