STUDIES OF THE LIPID COMPOSITION OF THE EMBRYONIC CHICK LENS

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Thomas William Culp, Sr.

June, 1966

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ABSTRACT

The lipids of the embryonic chick lens were analyzed by a combination of chromatographic techniques. These techniques included silicic acid column chromatography, gas-liquid chromatography (GLC) and thin-layer adsorption chromatography (TLC).

The freshly extracted lenticular lipids were first separated into neutral and polar fractions using silicic acid column chromatography. These major lipid fractions were further separated into their respective lipid classes by gradient elution from silicic acid columns. The isolated lipid classes were quantitated, monitored by TLC and fatty acids determined by GLC.

Application of these chromatographic techniques separated the embryonic lenticular lipids into free and esterified cholesterol, glycerides, cephalin, lecithin and sphingomyelin. These lipid components were found to accumulate during lenticular development with the individual classes displaying characteristic accumulation patterns. The neutral lipids were found to exhibit an irregular distribution pattern which is interpreted to indicate a predominate metabolic role for these compounds. In contrast, the phospholipids showed a regular distribution pattern which indicated a structural role in the embryonic lens.

Fatty acids, as determined by GLC, were found to exhibit a highly diversified accumulation pattern with both saturated and unsaturated acids occurring in all stages of embryonic development. Individual fatty acids, including palmitic, oleic, stearic, were found to vary in their relative amounts depending on the developmental state of the embryo.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The earliest published report on lenticular lipids was made by Berzelius[1] in 1825. He and other early investigators had demonstrated for the first time the presence of fatty material in the lens. It was not, however, until the beginning of the twentieth century that the various classes of lenticular lipids were documented by Krause.[2]

The extremely slow progress exhibited by the early investigation of lenticular lipids has been due primarily to poorly refined analytical methods. However, in recent years the advent of more refined analytical tools as gas-chromatography, thin-layer adsorption chromatography now permit a more comprehensive and accurate analysis of the lipid classes.

In analyzing the bovine lens, Krause found the total lipid concentration to be minute consisting primarily of cholesterol, glycerides, and phospholipids. The phospholipids identified were lecithin, cephalin and sphingomyelin.

It is interesting to note that Pirie and Van Heyeningen[3] reported the concentration of lipid in the lens to be lower than other tissues of the eye. Cholesterol and phospholipids were indicated as the major lipids present.

In studying the fatty acids of the ox lens, Bartley et al.[4] found the lens to contain about the same proportion of unsaturated fatty acids as that found in the retina. The prevailing fatty acid was found to be oleic acid with slightly less palmitic acid reported. Only 7% of the fatty acids were polyenes with arachidonic acid comprising the bulk of this group.

Turchetto[5] reported similar results on the fatty acid composition of the crystalline lenses of dogs and rabbits. Utilizing gas-liquid chromatographic analysis, he found that both dogs and rabbits exhibited a similar fatty acid spectra which consisted of both saturated and unsaturated fatty acids. He reported palmitic to be the major saturated acid with oleic the principal unsaturated acid.

While the fatty acid composition of the total lipid of lenticular tissue has been investigated, little has been reported on the fatty acid composition of the individual lipid classes. Preliminary studies[6] have shown the fatty acid composition of the phospholipids of rabbit, bovine and human lenses. Again, the presence of both saturated and unsaturated acids was found with palmitic and oleic acids comprising the major portion of the fatty acids identified. Fatty acid analysis of the sphingomyelin fraction revealed unusually high concentration of the less common nervonic and lignoceric acids. These high molecular weight monoenes were also found in the lecithin and cephalin fractions of the rabbit, bovine and human lenses. Unusually high concentration occurred in the latter.

In a recent publication by Feldman et al.[7] a comparative study of the phospholipids of the bovine, rabbit and human lenses was carried out using silicic acid column chromatography. They were found to contain varying amounts of lecithin, cephalin and sphingomyelin. The phospholipids were shown to be the predominate lipid class of these lenses with quantities of the individual phospholipids varying with each species.

Similar methods were used to analyze the neutral lipids of the rabbit lens. The investigators[6] found the rabbit lens to contain

predominately cholesterol and glycerides. These results were consistent with the findings of Krause in an earlier study of the bovine lens.

Histochemical methods have been widely used in lipid chemistry and have been most applicable when the cytological location of lipids are desired. Wislocki[8] employing histochemical methods, demonstrated the presence of Sudan black positive material in the lens of the rhesus monkey. However, he made no attempt to localize the Sudophilic lipids in order to determine their cytoplasmic distribution. Hogan and Zimmerman[9] described a lipid membrane that was stainable with heavy metals which are found in younger fibers of the lens cortex but are absent in deeper cortex and nucleus regions. Dark[10] in a more comprehensive study using histochemical procedures, analyzed the distribution of lipids in general and of phospholipids specifically in the bovine lens. The total lipids, determined by Sudan black, were found in the cytoplasm of the anterior epithelium colored gray-blue with many discrete black granules. Also, the presence of Sudophilic substances were found in the cement material surrounding the fiber cells in which only slight staining of the cells occurred. A significant point noted by Dark was the presence of "lipid corpuscles" in the cement material. The phospholipids were localized by using Baker's acid hematin method. The cytoplasm of the anterior lens was stained a grayish color and contained closely packed blackish granules. The lens nucleus and the "lipid corpuscles" previously described were both stained an intense blue-blackish color.

In spite of the advances made in recent years in the elucidation of lenticular lipids, surprisingly little information is available in this

field. For example, various investigators have isolated and characterized the major lipid classes of the lens; however, no one has yet offered evidence to suggest a possible metabolic or structural role for these fatty compounds. Little is known of the structure of the various glycerides and phospholipids that are known to occur in the lens. The method and synthesis of these compounds also remains obscure. It would appear most likely that these lipids are synthesized de novo rather than transported from the surrounding lenticular environment. However, again no evidence is available to substantiate such a possibility.

The forthcoming presentation involves a study of lipids of the embryonic chick lens by the application of consecutive chromatographic techniques.

CHAPTER II

STATEMENT OF PROBLEM

A review of the literature has demonstrated the need for further work on lenticular lipids. By studying the classes of lipids and their dynamics in the embryonic lens, the investigator hopes to obtain information which will be of value in determining a role for these compounds in lenticular tissue. Also, a better understanding of the biochemistry of lenticular tissue is essential in determining the etiology of such lens anomalies as colobona, spherophalgia, cataracts, and other lenticular diseases.

The investigator's objective in doing this research was twofold:

1. To establish a growth pattern for the embryonic lens in order to determine the gross structural changes occurring with development;
2. To determine the classes of lipids that are found in the embryonic lens and the changes which these compounds undergo with development.

The author has chosen the chick embryo as an experimental animal. It is easily obtained with minimal expense, and requires little maintenance. Furthermore, the embryonic lens is an ideal tissue for analysis because it is derived embryonically from only one germ layer with no extrusion of cells occurring during growth. It has been compared by some investigators[3] to a pure bacterial culture in which all of the cells are the same size and age.

CHAPTER III

EXPERIMENTAL

Growth Studies - A study of the structural development of the embryonic lens was undertaken by (1) measuring the diameter and thickness of the lens to determine dimensional changes with age, and (2) determining the wet and dry weights of the developing lenticular tissue.

Fertile eggs from white Leghorn X New Hampshire Red chickens were incubated in a forced-draft incubator. Sampling of the embryonic lenses was begun on the eleventh day of incubation and was continued on alternate days until hatching. The embryo's age was determined by measuring the middle toe from its tip to the middle of the metatarsal joint and comparing this measurement with Lillie's Standard.[11] The lenses were excised by making an incision in the anterior region of the sclera and removing both lens and vitreous. The lens was then separated from the vitreous and blotted carefully on filter paper to remove extra-lenticular material. The measurement of the lenses was accomplished by placing the lens on the finger tip and taking dimensions with a Bausch and Lomb magnifying glass with a millimeter scale etched on the surface. The lenses were weighed immediately on a Mettler analytical balance to determine the wet weight and placed in a desiccator containing H_2SO_4 and allowed to stand for 48 hours under vacuum. The lenses were removed and weighed to determine the dry weight. A total of twenty freshly excised lenses were used for each age group in growth studies.

Lipid Extraction and Fractionation (See Figure 1 for scheme of analysis) - The extraction of lipids from the embryonic lenses was achieved

by using the procedure of Folch[12] which employs a 2:1 chloroform-methanol mixture. The number of lenses analyzed ranged from 755 for the 11th day embryonic lenses to 300 for the more mature ones (Table 2). The use of one hundred milligrams of wet tissue was found adequate for complete extraction of the freshly excised tissue. The total lipid, determined gravimetrically, was fractionated into neutral and polar fractions using silicic acid column chromatography as described in the procedure of Van Handel and Zilversmit.[13] Resolution was confirmed by thin-layer chromatography.

Further fractionation of the neutral lipids into two fractions was achieved by a modification of the procedure described by Horning et al.[14] A specially prepared glass column was packed with five grams of silicic acid (100-200 mesh) which was slurried in hexane. The first fraction, containing the hydrocarbons and steroid esters, was eluted off the column with a 20% benzene-hexane solution. The second fraction, containing the more polar compounds including glycerides, unesterified steroids, and free fatty acids, was eluted off with a 95% chloroform - methanol solution. The fractions were evaporated in vacuo and made up to volume in a volumetric flask. An aliquot of each fraction was then monitered by TLC to insure complete resolution of the desired components. The free and esterified cholesterol was determined by taking an aliquot of each fraction and determining cholesterol as described by Tschugaeff.[15] The glycerides were determined by taking an aliquot of the second fraction and preparing hydroxyamic acid derivatives as described in the ester-group determination by Antonis.[16]

The phospholipids were separated on a silicic acid (100 mesh) column by application of gradient elution with chloroform - methanol mixtures. They were fractionated into three fractions as described in the procedure of Newmann et al.[17] with the column effluent collected in graduated test tubes. The first fraction was eluted off the column with a 20% chloroform - methanol solution. It contained phosphatidyl ethanolamine and phosphatidyl serine. The second fraction, containing phosphatidyl choline, was eluted off with 38% chloroform - methanol, followed by the sphingomyelin fraction with 100% methanol. The total phosphorus present in each tube was determined by Fiske and Subbarow method to establish a breaking point between fractions. The tubes in each fraction were combined and weighed to determine the amount of each component present. The fractions were applied to TLC plates and developed to determine the degree of homogeneity obtained.

Monitoring by Thin-Layer Chromatography - The extreme rapidity and sensitivity of thin-layer adsorption chromatography has accounted for its ubiguitous application in scientific research. The use of silica gel plates was found by the author to be a valuable tool in monitoring silicic acid column effluents obtained by previously described methods.

An aliquot of each fraction was applied to the chromatoplate in quantities ranging from 25-100 micrograms. The plates were then developed in their respective solvents by ascending chromatography. The phospholipids were resolved by employing the following solvent systems: 65 parts chloroform, 25 methanol, 8 acetic acid and 4 water. The neutral lipids were separated with 2 parts chloroform to 1 part benzene solvent mixture. The visualization of the developed chromatoplate was carried out by spraying with a 0.2% alcoholic solution of 2^1, 7^1-dichlorofluorescein. The components were

then viewed under ultraviolet light and identified by comparing the Rf values with a known standard mixture.

Gas-Chromatographic Analysis - Methyl esters were prepared from the lipid fractions obtained by previously described methods. A maximum of twenty milligrams of lipid material was placed in a screw-top culture tube and solvent evaporated under a stream of nitrogen. A methylating reagent was prepared by making a 6% sulfuric acid methanol solution. One milliliter was added to each tube and loosely capped while warming at 60-70° C for twenty-four hours. The reaction was stopped by adding one milliliter of distilled water. The esters were then extracted by washing several time with hexane. The extract was added to a dry tube containing sodium sulfate and allowed to stand for thirty minutes. The extract was filtered and the hexane evaporated under a stream of nitrogen to obtain a 20% solution for gas-chromatographic analyses.

Methyl esters of the fatty acids were separated by using a Barber-Coleman Model 15 gas chromatograph equipped with an argon-ionization (triode) detector. Column packing material consisted of an inert phase (Gas Chrom P) coated with a stationary phase of 10% ECNSS-S. The twelve foot column was maintained at a temperature of 194° C (Temperature Programmed) and the flow rate of argon (carrier gas) was maintained at 30 psi. The triode detector was operated at a temperature of 225° C with a cell voltage of 750V.

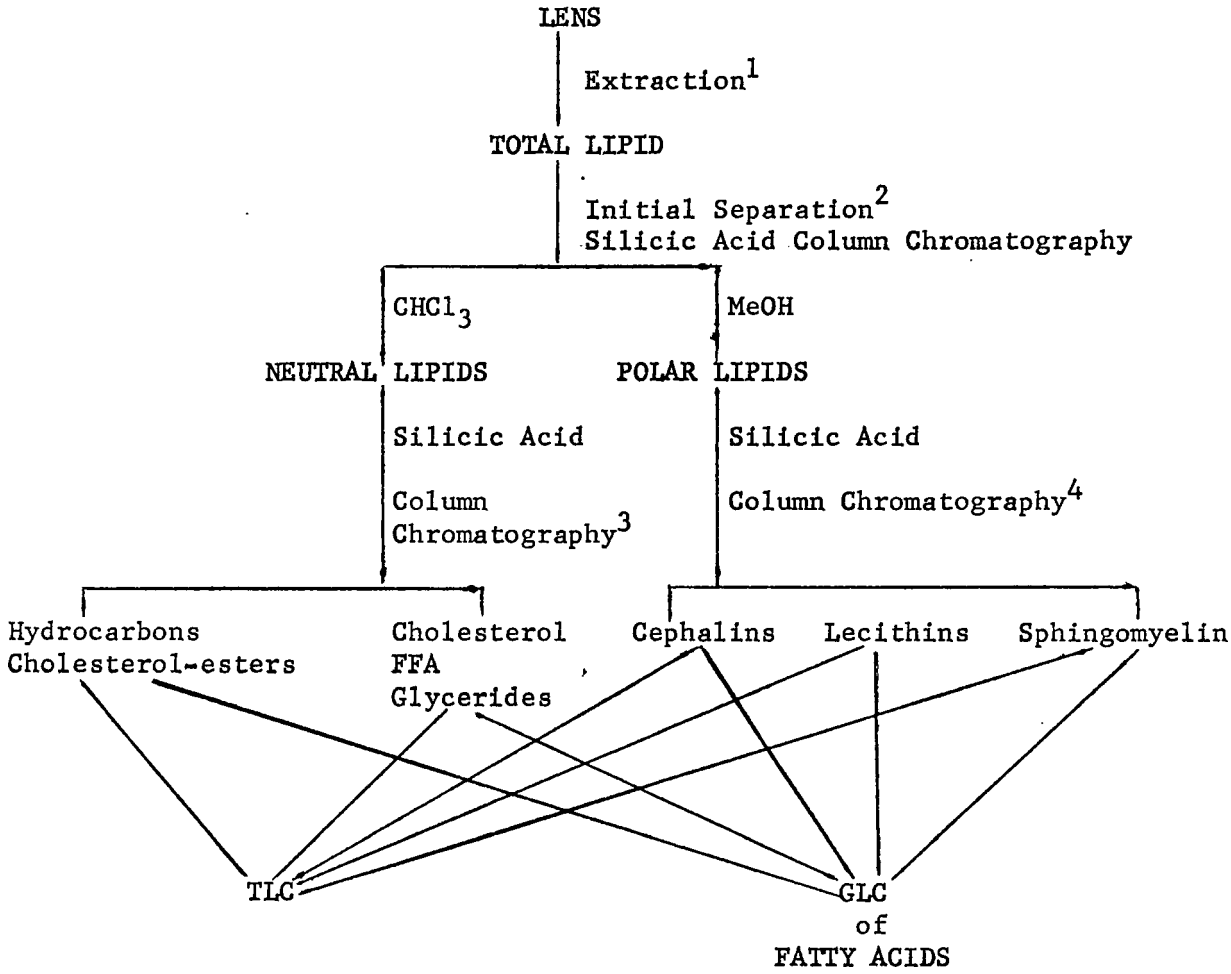
The methyl esters were injected into the column with a ten microliter Hamilton syringe. Peak heights and elution times were determined for each peak appearing on the chromatogram and fatty acids identified by comparing retention times (relative to methyl stearate) to those of a known standard

mixture (Table 1). A confirmation of the fatty acids identified was made by using a second chromatograph (Barber-Coleman 15) equipped with an 8% ethylene glycol adipate column and operated under conditions listed above.

The peak areas were measured by triangulation with quantitative response factors for the various methyl esters determined by a standard mixture.

FIGURE 1

SCHEME OF ANALYSIS OF THE EMBRYONIC CHICK LENS



1. Folch, J. Preparation of Lipid Extracts from Brain Tissue, J. Biol. Chem. 191: 833 (1951).

2. Van Handel, E. Micro-Method for the Direct Determination of Serum Triglycerides, J. Lab. Clin. Med., 50: 152 (1957).

3. Horning, M. G. Separation of Tissue Cholesterol Esters and Triglycerides by Silicic Acid Chromatography, J. Lipid Res. 1: 482 (1960).

4. Newman, H. A. Evidence for a Physiological Occurrence of Lysolecithin in Rat Plasma, J. Lipid Res. 2: 403-11 (1961).

TABLE 1

COMPARISON OF RELATIVE RETENTION TIMES
ON TWO STATIONARY PHASES

	RRT		
Carbon #	Diethyl Glycol Succinate[1] Standard	10% ECNSS-S[2] Standard	Diethyl Glycol Succinate Embryonic Lens*
C-8	0.06	0.06	-
C-9	0.08	0.08	0.09
C-10	0.11	0.11	0.12
C-11	0.14	0.14	0.15
C-12	0.19	0.19	0.18
C-13	0.25	0.25	0.22
C-14	0.33	0.32	0.32
C-14:1	0.39	0.40	0.39
C-15	0.44	0.44	0.43
C-16	0.58	0.57	0.56
C-16:1	0.67	0.68	0.65
C-17	0.76	0.76	0.72
C-18	1.00	1.00	1.00
C-18:1	1.17	1.14	1.13
C-18:2	1.48	1.44	1.40
C-18:3	1.98	1.87	1.84
C-19	1.31	1.32	1.34
C-20	1.73	1.75	1.75
C-20:1	2.01	2.02	1.99
C-20:4	3.46	3.19	3.26
C-20:5	4.53	4.13	4.43
C-21	2.27	2.42	2.38
C-22	2.98	3.09	2.96
C-23	3.95	4.04	3.99
C-24	5.10	5.44	5.34
C-24:1	6.17	6.05	6.10

*Averaged Values.

1. GLC conditions: 12 foot glass column packed with 8% ethylene glycol adipate; glow rate of argon (carrier gas) maintained at 30 psi; column temperature maintained isothermically at 194° C; triode detector operated at a temperature of 225° with cell voltage of 750V.

2. GLC conditions: 10% ECNSS-S stationary phase operated as above.

CHAPTER IV

RESULTS
GROWTH STUDIES

Growth Rate

A study of the growth of the embryonic lens was undertaken to express lipid changes as a function of growth. The growth rate, as determined by dimensional changes and tissue weights of the embryonic lens, showed a rapid increase in tissue size with age. A regression plot of the diameter and thickness of the embryonic lens demonstrated a linear increase with respect to age (Figure 2). Measurements of the early lens revealed a nearly spherical shape with the more characteristic lenticular shape appearing with age. Similar findings were reported by Hogan[9] for the fetal lens of the human. He reported an increase in the equatorial diameter after the seventh month of development. The dry weight of the developing avian tissue (indicative of cellular proliferation) demonstrated a constant increase with age. This amounted to a six fold increase from the eleventh through the twenty-first day (Figure 3). The moisture content revealed a similar regularity in the embryonic tissue through the seventeenth day, followed by a leveling off between the seventeenth and nineteenth day. A slight elevation in moisture occurred between the nineteenth and twenty-first day, just prior to hatching. This change in the moisture pattern of the developing lens seemed to parallel changes occurring in the lipid accumulation pattern exhibited by the embryonic lens (Figure 4). The erratic increase in both free and esterified cholesterol exhibited during this period indicated a possible metabolic relationship to the moisture changes occurring during this same period of development.

FIGURE 2

THICKNESS AND DIAMETER OF THE EMBRYONIC CHICK LENS

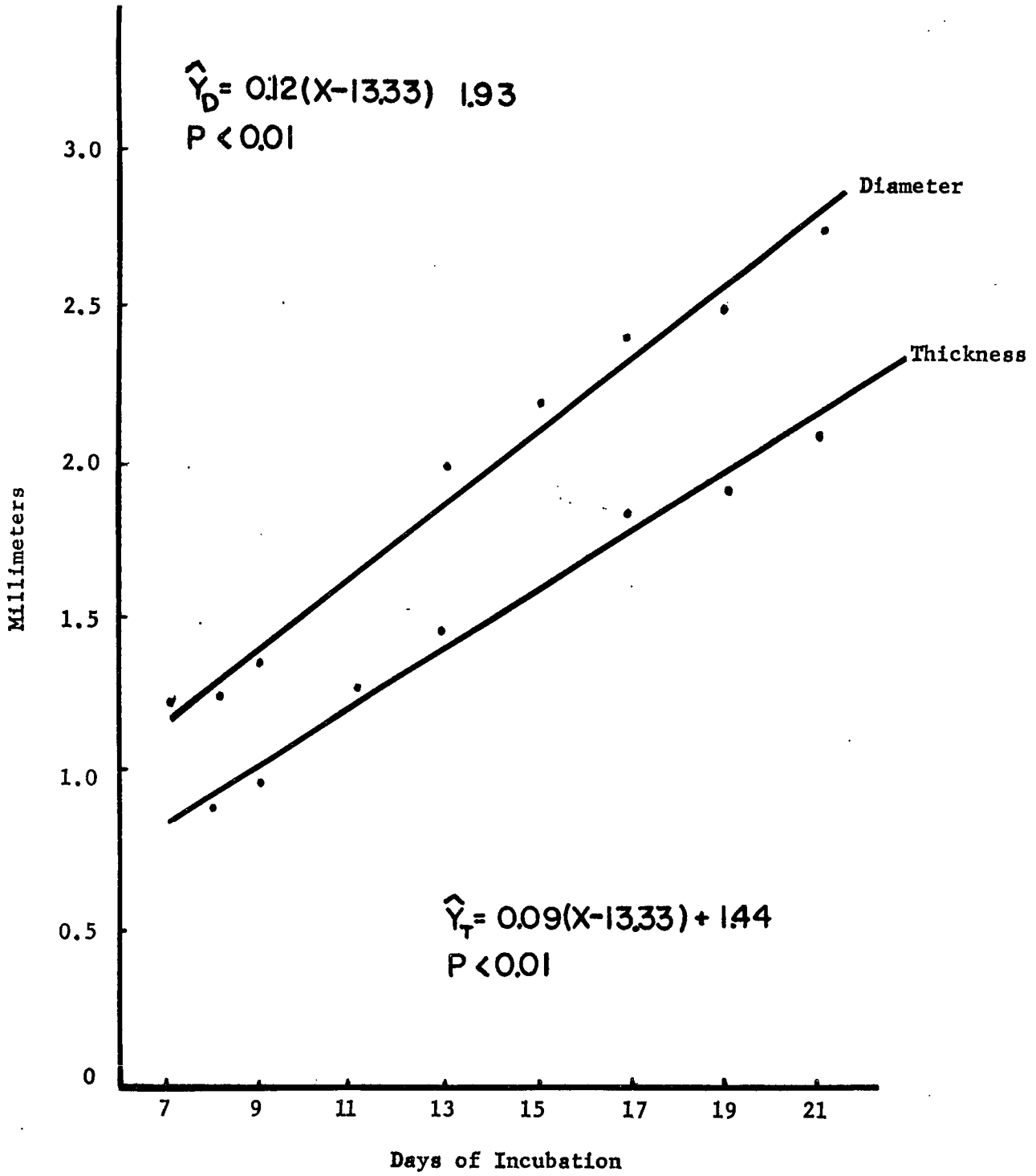


FIGURE 3

WET AND DRY WEIGHT OF THE EMBRYONIC CHICK LENS

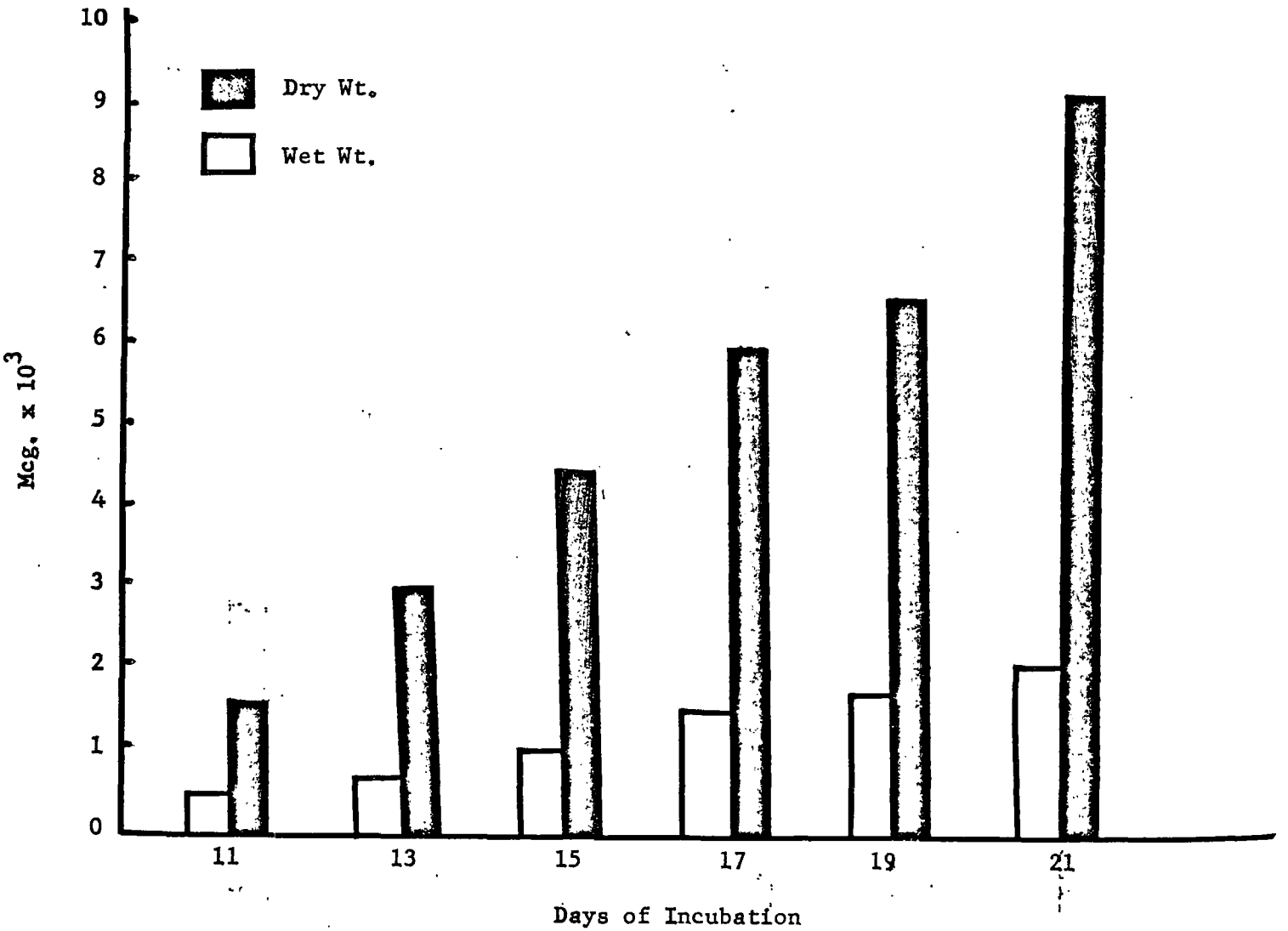
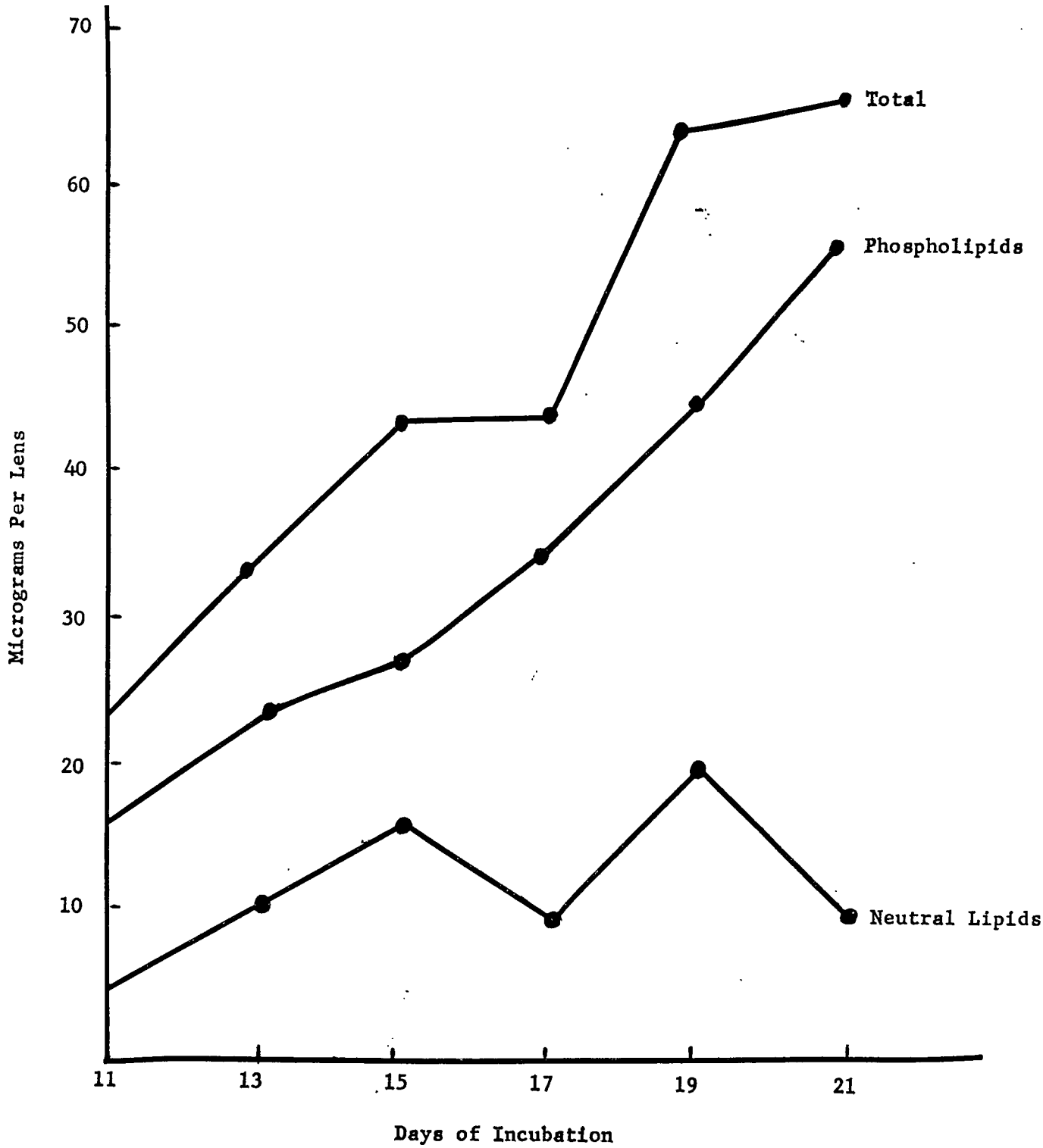


FIGURE 4

CROSS LIPID CONTENT OF THE DEVELOPING CHICK LENS



CHAPTER V

LIPID STUDIES

Lipid Distribution

A study of the individual lipid component of the embryonic lens was undertaken to aid in determining their role in the metabolic and structural development of the embryonic tissue. The accumulation pattern of the lipids of the developing lens showed a regular increase in some components while others illustrated irregular changes with age. These patterns implied both an increase due to cellular proliferation and growth, as well as a response to metabolic demands. Thus, to establish the basis for these exhibited accumulation patterns, the concentrations are presented in the following expressions of data:

1. Micrograms of lipid per lens -- in order to demonstrate the amount of lipid present at various stages of development and determine whether a deposition or depletion has occurred.
2. Micrograms of lipid per milligram wet weight of tissue -- to demonstrate the changes in the lipid present as related to growth of tissue.
3. Micrograms of lipid per milligram of dry weight of tissue -- to demonstrate the relationship of lipids to cellular proliferation.
4. Percent of each lipid component of total lipid -- to demonstrate the relative distribution of the different lipid components with respect to total lipid at various stages of development.

Gross Lipid Concentrations

The total lipid present in the chick lens showed a linear increase from the eleventh through the fifteenth day of development (Figure 4). Following this period of development, there was a leveling off which occurred between the fifteenth and seventeenth day. A sharp increase through the nineteenth day was followed by a slight elevation through the twenty-first day of development. This variable pattern exhibited by the total lipid was caused by changes which occurred in the neutral lipid components (Figure 4). On the seventeenth day, there was a three-fold reduction in the glycerides (Table 2). This accounted for the depletion shown by the total lipid pattern. However, a depletion is not observed in the total lipid between the seventeenth and nineteenth day of development due to increases in both free and esterified cholesterol (Figure 5). The phospholipids were the dominant lipid class of the developing lens, which accounted for approximately 70% of the total lipid (Table 2). This accumulation pattern of these phosphorus-containing compounds exhibited nearly a linear increase with age (Figure 4).

Free and Esterified Cholesterol

Cholesterol in the developing lenticular tissue was predominantly in the free form with only trace amounts of esterified cholesterol present. The free cholesterol increased gradually to the seventeenth day (Figure 5). A sudden rise occurred on the nineteenth day of development. This elevation expressed by free cholesterol was followed by a depletion on the twenty-first day. When considering free cholesterol as a function of

cellular growth and proliferation, a similar accumulation pattern was not observed. On the contrary, instead of an accumulation occurring, there was a gradual depletion of free cholesterol followed by an abrupt rise which again occurred on the nineteenth day of development (Tables 3 and 4). The presence of trace amounts of esterified cholesterol made it difficult to demonstrate changes which occurred in earlier stages of development. However, detectable amounts were determined on the nineteenth day of development (Tables 3 and 6). This was indicative of a similarity of accumulation patterns between free and esterified cholesterol. Fatty acid analysis at various stages of development showed esterified cholesterol to be present primarily as cholesteryl palmitate, cholesteryl oleate and cholesteryl palmitoleate (Table 8).

Glycerides

While the glycerides may be considered as one component, they generally exist in animal tissues as the mono-, di-, and, tri-fatty acid esters of glycerol. However, thin-layer chromatography has shown triglycerides to occur in the embryonic chick lens with only trace amounts of the mono and di-glycerides present. The glycerides exhibit a rather unique accumulation pattern in the embryonic lens. Consideration of the total glycerides present per lens indicates a gradual increase with the maximum developing on the fifteenth day (Table 3). Following this period of development there is a three-fold depletion which remained constant throughout the twenty-first day. When glycerides are viewed as a function of cell growth and proliferation, parallel patterns were observed. There

was a gradual accumulation with age to the fifteenth day of development. Again a sudden drop was observed which remained constant throughout the twenty-first day.

Phospholipids

On the basis of micrograms per lens, the cephalin fraction showed a gradual accumulation with age (Table 3). However, a reversal was found when micrograms of cephalin were viewed as related to cell growth and proliferation. On this basis, high concentrations of cephalin were observed during early developmental stages with a depletion appearing in latter stages.

The lecithin fraction exhibits a more irregular accumulation pattern. There is a gradual increase in micrograms per lens throughout the thirteenth day of development (Table 3). This increase is followed by a depletion on the fifteenth day. After this period, there is again a gradual increase which continues throughout the latter stages of development.

The sphingomyelin fraction demonstrated an accumulation pattern that was similar to the one exhibited by cephalin (Table 3). When considering micrograms per lens, there is a gradual increase with age. Both lecithin and sphingomyelin exhibited an irregular type of pattern when viewed in relation to growth and proliferation. Generally, amounts were high in early development with a gradual depletion occurring with age.

FIGURE 5

FREE AND ESTERIFIED CHOLESTEROL
OF THE EMBRYONIC CHICK LENS

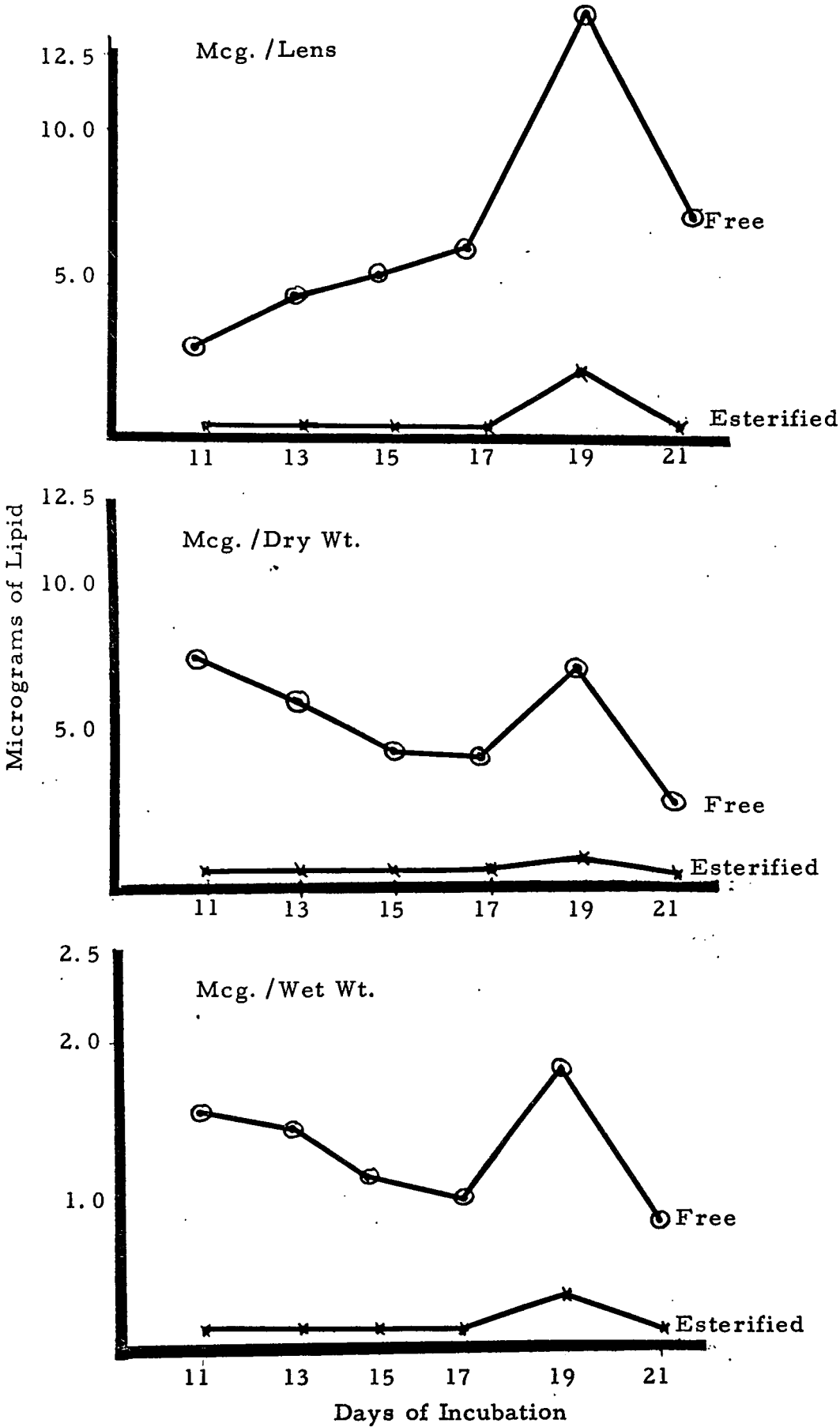


TABLE 2

PERCENTAGE OF NEUTRAL AND POLAR LIPIDS
IN THE EMBRYONIC CHICK LENS

Days of Incubation	Number of Lenses Analyzed	Total Lipid Mcg. Lipid/Lens	% Neutral	% Polar
11	755	22.2	15.4	84.6
13	400	33.6	32.3	67.7
15	363	41.7	35.6	64.4
17	300	42.6	34.5	65.5
19	300	63.6	33.6	66.4
21	300	65.6	27.1	72.9

TABLE 3

LIPIDS OF THE EMBRYONIC CHICK LENS

	Age in Days					
	11	13	15	17	19	21
	Mcg. Lipid/Lens					
Neutral Lipids						
Cholesterol Esters	-	-	-	-	2.0	-
Cholesterol	2.7	4.1	5.0	6.2	12.8	7.0
Glycerides	2.9	5.9	10.5	3.2	3.6	3.0
Total	5.6	10.0	15.5	9.4	18.4	10.0
Phospholipids						
Cephalins	7.9	11.0	14.6	16.0	19.0	26.0
Lecithins	6.0	8.8	5.6	8.5	17.6	19.0
Sphingomyelins	2.7	3.7	6.0	8.7	7.2	10.0
Total	16.6	23.5	26.2	33.2	43.8	55.0
Total Lipid	22.2	33.5	41.7	42.6	62.2	65.0

TABLE 4

LIPIDS OF THE EMBRYONIC CHICK LENS

	Age in Days					
	11	13	15	17	19	21
	Mcg. Lipid/mg. wet wt.					
Neutral Lipids						
Esterified Cholesterol	-	-	-	-	0.30	-
Free Cholesterol	1.54	1.39	1.15	1.03	1.95	0.787
Glycerides	1.67	1.98	2.40	0.53	0.55	0.335
Total	3.21	3.37	3.55	1.56	2.80	1.122
Polar Lipids						
Cephalins	4.54	3.72	3.34	2.67	2.89	2.87
Lecithins	3.45	2.97	1.29	1.42	2.67	2.03
Sphingomyelins	1.54	1.26	1.37	1.45	1.10	1.12
Total	9.53	7.95	6.00	5.54	6.66	6.02
Total Lipid	12.74	11.34	9.55	7.10	9.46	7.14

TABLE 5

LIPIDS OF THE EMBRYONIC CHICK LENS

	Age in Days					
	11	13	15	17	19	21
	Mcg. Lipid/mg. dry wt.					
Neutral Lipids						
Esterified Cholesterol	-	-	-	-	1.14	-
Free Cholesterol	7.58	6.03	4.72	4.26	7.31	3.35
Glycerides	8.21	8.60	9.90	2.19	2.08	1.44
Total	15.79	14.63	14.62	6.45	10.53	4.79
Polar Lipids						
Cephalins	22.36	16.12	13.75	11.04	10.85	12.38
Lecithins	16.98	12.86	5.31	5.86	10.05	8.72
Sphingomyelins	7.58	5.47	5.68	6.00	4.13	4.82
Total	46.92	34.45	24.74	22.90	25.03	25.92
Total Lipid	62.71	49.08	39.36	29.35	35.56	30.71

TABLE 6

LIPIDS OF THE EMBRYONIC CHICK LENS

	Age in Days					
	11	13	15	17	19	21
	% of Total Lipid					
Neutral Lipids						
Cholesterol Esters	-	-	-	-	3.21	-
Cholesterol	12.20	12.20	11.99	14.55	20.58	10.77
Glycerides	13.10	17.60	25.18	7.51	5.79	4.62
Total	25.60	29.80	37.20	22.10	29.60	15.40
Phospholipids						
Cephalins	35.59	32.84	35.01	37.56	30.55	40.00
Lecithins	27.03	26.27	13.43	19.95	28.29	29.23
Sphingomyelins	12.16	11.04	14.39	20.42	11.57	15.38
Total	74.40	70.20	62.80	77.90	70.40	84.60

CHAPTER VI

FATTY ACID STUDIES

Gross Consideration

The fatty acids composition, as determined by gas-liquid chromatography, consisted of a wide variety of fatty acids. Both saturated and unsaturated fatty acids were identified in all stages of embryonic development. Chain lengths of the saturated acids were found to range from dodecanoic acid to the higher molecular weight lignoceric acid. The unsaturated acids were primarily of the monoene type consisting mainly of oleic acid. The polyenes identified were associated primarily with the phospholipids which consisted generally of the C_{18} and C_{20} varieties.

The fatty acids of the total lipid were found to be composed of greater than 50% of the saturated acids (Table 7). Of this group, palmitic was present in highest concentration. It amounted to better than 40% throughout lenticular development. Oleic acid was the major unsaturated acid found. It comprised greater than 20%, and was followed by slightly lesser amounts of palmitoleic acid.

Cholesterol Ester

The fatty acids esterified to the cholesterol moiety were found to be mostly saturated (Table 8). Cholesteryl palmitate appeared in the highest concentrations, and was followed by cholesteryl stearate. The short chained fatty acids identified appeared only in the earlies stages of development. These included very small amounts of pelargonic and capric acids. In the latter stages of development (after the fifteenth day) a

few of the longer chain fatty acids were found. These included nonadecanoic, eicosanoic and heneicosanoic acids. Palmitoleic and oleic were the major monoenes identified with the cholesterol ester fraction. Small amounts of myristoleic and pentadecenoic acids also appeared irregularly throughout development. No polyenes were identified in the cholesterol ester fraction.

Glycerides

The accumulation pattern of the saturated fatty acids closely resembled the cholesterol ester in that both contained large amounts of palmitic acid in all the stages of development (Table 9). Also, small amounts of capric acid were found in the glycerides during early lenticular development. The longer chain alkane fatty acids consisted primarily of heneicosanoic acid with behenic and tricosanoic appearing only in early developmental stages.

The distinguishing difference between the cholesterol ester and glyceride fatty acids was the presence of the polyenes (i.e. linoleic and linolenic) found only in the glyceride molecule. The monoenes were found to consist basically of oleic acid with lesser amounts of the shorter chain palmitoleic acid.

Phospholipids

Palmitic, stearic and heneicosanoic acids were the major saturated fatty acids present in the cephalin fraction (Table 10). These acids amounted to well over 80% of the saturated acids and exhibited a rather uniform distribution throughout development.

A wide spectra of unsaturated fatty acids were found throughout lenticular development. Oleic acid comprised the bulk of this group. Lesser amounts of linoleic and the unsaturated eicosanoic acid also appeared irregularly during development. A similar fatty acid pattern was found to be exhibited for both lecithin and sphingomyelin (Tables 11 and 12). However, the sphingomyelin fraction was distinguished from the cephalin and lecithin fractions by large amounts of lignoceric and nervonic acids. These comprised as much as 16% of the total fatty acids on the twenty-first day of development (Table 12).

TABLE 7

FATTY ACID COMPOSITION OF THE TOTAL LIPID
OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 13	17	21
			Weight %	
Saturates				
C-12		-	0.89	-
14		-	3.90	7.40
15		-	1.34	0.80
16		40.39	40.13	46.41
18		7.89	6.46	5.78
20		-	9.48	-
21		4.49	4.57	3.05
	Total	52.7	66.7	63.7
Unsaturates				
C-16:1		10.2	8.58	7.52
18:1		36.5	22.4	26.6
18:2		0.59	2.23	1.20
20:1		-	-	0.19
	Total	47.3	33.3	36.3

GLC Conditions: (Refer to Table 1)

TABLE 8

FATTY ACID COMPOSITION OF THE CHOLESTEROL ESTER FRACTION OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 11	13	15	17	19	21
				Weight %			
Saturates							
C-9		1.7	-	2.7	-	-	-
10		2.1	-	3.2	0.4	-	-
11		-	3.0	-	-	-	-
13		-	-	-	-	-	-
14		4.6	5.7	5.3	4.9	6.1	8.8
15		6.7	11.9	-	4.8	6.0	5.5
16		12.0	6.9	17.1	33.8	17.4	31.0
17		6.8	2.7	5.3	-	-	5.2
18		7.3	3.5	13.7	7.5	10.3	8.2
19		3.9	-	-	3.8	8.1	9.2
20		5.1	-	-	2.5	-	-
21		-	-	-	4.9	12.4	9.8
	Total	50.1	33.8	47.2	62.4	60.3	77.7
Unsaturates							
C-14:1		6.2	9.2	6.3	2.3	5.4	-
15:1		8.4	6.9	-	5.4	-	3.3
16:1		15.1	8.06	13.5	10.0	12.2	8.7
18:1		6.7	4.1	9.8	13.8	16.1	7.0
	Total	41.43	33.0	36.07	33.0	39.6	18.9
Unidentified Components							
	Total	8.5	33.3	16.8	4.02	-	3.3

GLC Conditions: (Refer to Table 1)

TABLE 9

FATTY ACID COMPOSITION OF THE GLYCERIDE FRACTION OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 11	13	15	17	19	21
				Weight %			
Saturates							
C-10		1.2	2.1	4.5	-	-	-
11		1.3	-	-	-	-	-
12		-	-	-	0.91	1.0	0.5
13		-	-	-	-	-	-
14		2.1	2.2	2.7	4.8	4.6	4.1
15		1.4	1.3	1.3	2.5	2.7	2.4
16		24.8	17.8	22.2	30.3	33.3	23.8
17		-	0.8	0.9	1.5	1.3	1.3
18		11.0	12.9	12.3	14.1	9.5	15.3
19		-	-	2.3	-	1.8	1.8
21		4.5	9.3	14.5	1.7	4.7	5.5
22		-	1.4	-	-	-	1.2
23		6.2	2.8	-	-	-	-
24		-	-	-	-	3.02	2.2
	Total	52.5	49.8	60.8	55.9	61.8	58.1
Unsaturates							
C-16:1		3.2	2.2	1.1	6.4	5.3	4.8
18:1		21.5	18.5	7.1	25.3	15.4	17.6
18:2		5.8	5.2	-	1.7	-	-
18:3		-	5.1	2.4	-	8.5	6.2
	Total	30.6	30.9	10.6	34.7	29.2	28.7
Unidentified Components							
	Total	16.8	18.5	28.5	9.4	9.1	13.2

GLC Conditions: (Refer to Table 1)

TABLE 10

FATTY ACID COMPOSITION OF THE CEPHALIN FRACTION OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 11	13	15	17	19	21
				Weight %			
Saturates							
C-12		-	-	-	-	0.3	-
14		2.3	2.3	1.3	2.6	1.3	1.2
15		1.4	1.3	-	2.3	0.5	0.5
16		14.4	16.6	21.4	26.6	19.3	16.7
17		1.1	-	-	-	-	-
18		11.3	15.8	11.6	17.5	13.2	11.8
19		2.4	-	-	-	-	-
21		15.6	13.0	10.0	9.7	8.0	2.6
	Total	48.5	49.0	44.3	58.7	42.6	32.8
Unsaturates							
C-14:1		1.5	-	1.4	1.5	-	-
15:1		2.7	1.5	2.9	2.6	-	-
16:1		5.4	5.2	5.9	3.9	2.8	5.0
17:1		6.5	3.4	5.77	3.5	1.0	-
18:1		19.0	24.4	24.0	21.8	41.8	48.1
18:2		-	2.7	2.7	-	1.4	-
20:1		1.2	3.1	-	2.6	2.8	3.7
20:2		3.9	3.3	3.9	-	-	-
20:4		-	-	-	-	2.4	2.6
20:5		1.0	-	-	-	-	-
	Total	41.4	43.6	46.7	35.9	52.1	59.5
Unidentified Components							
	Total	10.1	7.4	9.0	5.3	5.7	7.6

GLC Conditions: (Refer to Table 1)

TABLE 11

FATTY ACID COMPOSITION OF THE LECITHIN FRACTION
OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 11	13	15	17	19	21
				Weight %			
Saturates							
C-12		0.3	-	-	-	-	-
13		0.3	-	-	-	-	-
14		3.2	4.2	2.1	1.6	3.3	3.0
15		1.0	0.7	1.1	-	-	-
16		34.2	29.0	51.2	54.9	36.8	33.6
17		0.8	-	-	-	-	1.0
18		4.2	7.2	6.2	6.2	4.9	4.8
19		-	2.0	1.9	-	-	-
20		-	1.3	-	-	-	-
21		3.5	7.9	9.4	12.9	-	2.5
22		-	2.6	-	-	-	-
	Total	47.5	54.9	71.9	75.6	45.0	44.9
Unsaturates							
C-14:1		0.6	-	1.1	-	-	-
15:1		0.6	0.6	1.4	1.1	-	-
16:1		8.1	3.6	2.9	3.0	8.1	9.2
17:1		0.84	0.93	1.98	3.38	-	-
18:1		37.1	27.8	18.0	11.2	41.6	37.8
18:2		1.2	1.6	-	2.1	0.9	-
20:1		0.7	0.8	-	-	0.9	1.4
20:3		-	1.5	-	-	-	-
20:4		2.4	-	-	-	2.0	3.1
	Total	51.5	36.8	25.4	20.78	53.4	51.5
Unidentified Components							
	Total	2.06	8.27	2.7	3.63	1.6	3.6

GLC Conditions: (Refer to Table 1)

TABLE 12

FATTY ACID COMPOSITION OF THE SPHINGOMYELIN FRACTION OF THE EMBRYONIC CHICK LENS

Carbon #		Days of Incubation 11	13	15	17	19	21
				Weight %			
Saturates							
C-14		3.3	3.4	4.6	2.8	4.5	3.5
15		1.7	-	-	-	1.7	-
16		41.6	26.9	46.1	47.8	62.0	45.6
18		3.5	2.5	3.2	3.5	7.0	4.0
19		-	-	3.9	-	-	-
20		-	-	-	-	2.0	1.4
21		4.2	1.9	11.3	8.3	-	-
22		2.7	3.5	2.3	-	7.0	4.8
24		-	5.6	3.0	-	-	5.9
	Total	57.0	43.8	74.4	62.4	84.2	65.2
Unsaturates							
C-14:1		-	1.1	-	-	-	-
15:1		2.8	2.8	1.1	2.1	-	-
16:1		5.2	6.0	2.3	4.5	4.9	4.1
17:1		6.16	9.32	1.99	4.57	-	-
18:1		13.6	10.5	10.6	14.79	10.9	14.2
18:2		-	-	-	3.5	-	-
20:2		4.9	5.8	1.6	3.7	-	-
20:3		3.8	3.8	-	-	-	-
24:1		-	5.4	5.6	-	-	16.5
	Total	36.16	44.7	23.19	33.17	15.8	34.8
Unidentified Components							
	Total	6.84	11.28	2.31	4.33	-	-

GLC Conditions: (Refer to Table 1)

CHAPTER VII

DISCUSSION

The existence of fatty material in lenticular tissue is well documented.[2, 3, 18] The introduction of gas-chromatography by James and Martin[18] in 1952, as well as other advanced lipid technology, has added impetuous to the study of lipids. However, the isolation and characterization of lipid material still remains a challenging problem.

As previously described, the phospholipids of the embryonic chick lens were separated employing silicic acid column chromatography. Here, especially, the separation into homogeneous components presented a formidable problem. Consequently, TLC (thin-layer adsorption chromatography) was employed to moniter the column effluents. This rapid and sensitive analytical tool proved not only valuable in developing methodology, but also provided a measure of the degree of homogeniety for the silicic acid fractions.

Cephalin was found to be the most abundant phospholipid occurring in the embryonic chick lens. These findings are consistent with earlier investigations on the rabbit and bovine lenses.[7] The human lens, however, seems to be an exception to this case. These same investigators reported sphingomyelin to be the major phospholipid of the human lens while cephalin was the minor phospholipid present.

The term "cephalin" is often used with hesitation by most lipid chemists because the name is misleading. It actually consists of two compounds: phosphatidyl serine and phosphatidyl ethanolamine. TLC analysis of the cephalin fraction has shown both these compounds to be

present in the embryonic chick lens; however, no attempt was made to quantitate these individual compounds because of the limited amount of lipid available for analysis.

The fatty acids (determined by gas-liquid chromatography) of cephalin were found to comprise a wide spectra of both saturated and unsaturated acids. Generally, the percent of saturated and unsaturated fatty acids was found to be of approximately equal distribution. Similar results were reported by Dittlse[19] in studies on the fatty acids of cephalin isolated from liver.

While lecithin (phosphatidyl choline) is generally the most prevalent phospholipid found in animal tissue, this is not true for lenticular tissue. This phospholipid fraction was monitered by TLC and found by this method to be homogeneous. However, some overlapping into the sphingomyelin fraction was observed.

The lecithin fraction was also found to contain a wide variety of both saturated and unsaturated fatty acids. The fatty acids, palmitic and oleic, comprised better than 60% of the total throughout development. An increased accumulation of palmitic acid with depletion of oleic was observed on the fifteenth throughout the seventeenth day of development. However, no explanation can be offered for this irregular fatty acid pattern.

Sphingomyelin was first discovered by Thudicum[20] in 1844. Since that time, it has been found to occur in numerous tissues, including lenticular. Krause[2] first demonstrated the presence of sphingomyelin in lenticular tissue while working with bovine lenses. This work has since been confirmed[7] and is now well established.

The author was the first to demonstrate the presence of sphingomyelin in the embryonic chick lens. This phospholipid was isolated by gradient elution on silicic acid columns as previously described. However, homogeneity as defined by TLC was not consistent with the other polor fractions. The sphingomyelin fraction was found to be contaminated with trace amounts of lecithin. Thus, these discrepancies were kept in mind during the evaluation of this data.

The distinguishing characteristic of the sphingomyelin fraction was found in its fatty acid composition. This fraction, unlike the other phospholipid fractions, was found to contain nervonic and lignoceric acids. Thannhauser and Boncoddo[21] first reported these unusually occurring fatty acids associated with sphingomyelin isolated from brain tissue. Similar results were reported by Feldman et al.[7] in a study of sphingomyelin isolated from bovine, rabbit and human lenses.

Free cholesterol is by far the most studied lipid occurring in lenticular tissue. The identification of cholesterol crystals in the cataratous lens and ease of quantitation have been the primary reasons.

The free form of cholesterol was found to be the major neutral lipid of the embryonic chick lens. This unesterified form of cholesterol was shown to comprise better than 10% of the total lenticular lipid (Table 6). Esterified cholesterol, consisting primarily of cholesteryl palmitate and cholesteryl oleate, appeared in very small quantities. Only on the nineteenth day of development were adequate amounts present for an accurate analytical determination. The values reported for cholesterol ester (Tables 3, 4, and 5) represents an average weight based on the total fatty acids identified with the cholesterol ester fraction.

The glycerides were also found to be a major constituent of the neutral lipids isolated from the embryonic chick lens. While the triglycerides were found to be most abundant, nevertheless the diglycerides and monoglycerides were also identified. Unlike the phospholipids, the glycerides were not present in sufficient amounts for accurate gravimetric analysis. Thus, an ester-group colorimetric determination was used. The values were adjusted by taking the average molecular weight of the glyceride fraction fatty acids.

Identified also with the neutral lipids of the embryonic lens was an unidentified compound (Plate 1). This compound was shown by TLC analysis to have an Rf value between that of free cholesterol and glyceride. It was found to occur in trace quantities in all stages of lenticular development.

Terroine[22] in 1944, first clearly differentiated between the two categories of lipids in vertebrates. He called these the éléments variable (which consist of the reserve fat) and the éléments constant (which represent the essential components of the protoplasm). The constant components were considered to be the phospholipids. These compounds, in the opinion of Terroine, remained relatively constant, regardless of physiologic variations. The variable components were considered the neutral lipids which exhibited changes in response to metabolic demands.

The two general classifications, as proposed by Terroine, are by no means exclusive. The lipids isolated from natural sources have been shown to have numerous functions. For instance, the animal glycerides generally serve as reserve energy for the organism and consequently would be classified as éléments variable. However, their role in membrane structure, lipoprotein

formation, etc., is well established. Thus, the classification proposed by Terroine is of general nature with obvious overlapping.

A similar distinction can be made for lenticular lipids as previously described by Terroine. The phospholipids seem to exhibit a constant increase with the development of the lens. Using Terroine's classification, these could be considered as élements constant. In contrast, the neutral lipids exhibit a rather irregular pattern with development. These can be considered as élements variable.

The phospholipids were found to comprise generally 70% of the total lipid found in the embryonic chick lens (Table 5). These compounds accumulated when considered on a basis of micrograms per lens but showed a depletion when expressed on a basis of cellular growth. To suggest the functional significance of these compounds in lenticular tissue would be premature, considering existing data. However, the role of phospholipids as structural elements is well established.[23,24] Thus, to include the possible participation of phospholipids in a structural role in the embryonic lens does not seem unreasonable.

The following seems to offer support to such a possibility:

1. The deposition of phospholipids in the embryonic lens was found to be constant as opposed to an irregular pattern exhibited by the neutral lipids.
2. Dark[10] has shown the lipids of the bovine lens to be particularly concentrated in the anterior of the lens. It is this region which contains the closely packed epithilial cells. These cells eventually differentiated into lens fiber cells comprising the core of the lens.

3. Van de Handel[13] has histochemically demonstrated the presence of lipid in the boundaries of the epithelial and fiber cells in the transitional layers near the nucleus.

These observations are by no means conclusive and definitely do not eliminate the other numerous functional possibilities for lenticular phospholipid. Dark[10] also demonstrated histochemically the presence of phospholipids in the cement matrix, cytoplasm and small amounts in the nuclear region of the lens. These findings further implicate the possible function of phospholipids as metabolic constituents.

Included in the adopted classification of elements variable is cholesterol, cholesterol esters, and glycerides. These lipids combined comprise approximately 30% of the total lipid found to occur in the embryonic chick lens. The participation of these lipids in a structural role is also well documented.[25] However, the irregular pattern exhibited by these compounds in the embryonic lens seems to imply a predominate metabolic role. This is particularly evident in the pronounced elevation in free and esterified cholesterol, just prior to hatching of the embryo (Figure 5). It seems possible that these irregular metabolic changes are preparation by the embryonic lens for its new visual role.

Another interesting observation is the depletion of the glycerides by the embryonic lens, just prior to steroid accumulation (Figure 8). If these steroids are synthesized de novo then it is possible that the fatty acids of the glycerides serve as a source for acetyl CoA. This possibility can be illustrated by the following scheme:

1. GLYCERIDES $\xrightarrow{\text{Lipase}}$ FATTY ACIDS + GLYCEROL

2. FATTY ACIDS $\xrightarrow{\text{B-Oxidation}}$ ACETYL CoA

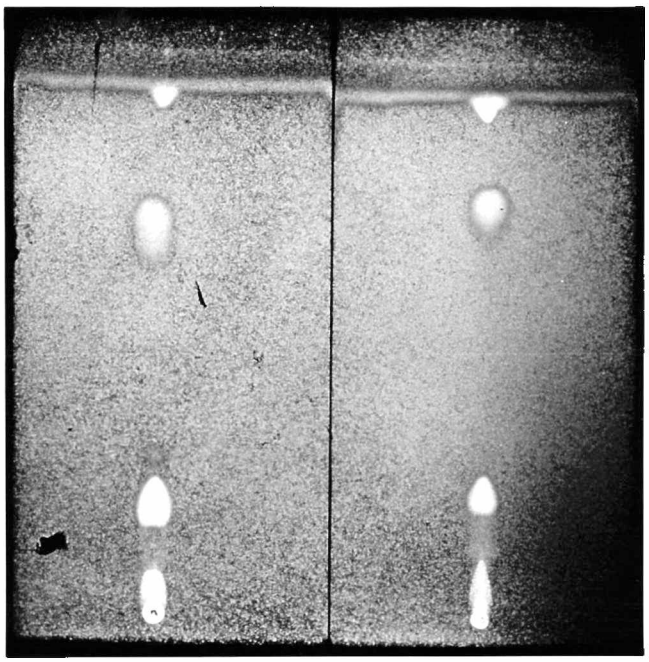
3. ACETYL CoA $\xrightarrow{\text{Sterol Synthesis}}$ CHOLESTEROL

4. CHOLESTEROL $\xrightarrow{\text{Fatty Acyl CoA}}$ ESTERIFIED CHOLESTEROL

PLATE 1

TLC CHROMATOGRAM OF THE 19 DAY EMBRYONIC CHICK LENS

Neutral Lipid Chromatogram



1
2
X
3
4
5

1
2
3
4
5

Embryonic Lens

Standard Mixture

Rf Values

Components	Standard		Embryonic Lens	
1	Cholesteryl Stearate	.96	Cholesterol Ester	.97
2	Tripalmitin	.76	Triglyceride	.73
X			Unidentified Compd.	.32
3	Free Cholesterol	.26	Free Cholesterol	.23
4	Mono and Diglycerides	.10	Partial Glycerides	.10
5	Phospholipids	---	Phospholipids	---

CHAPTER VIII

CONCLUSIONS

The findings of this study have shown a variety of lipids appearing throughout lenticular development. These compounds, when viewed over various stages of development, were found to exhibit characteristic accumulation patterns. In view of these exhibited patterns, the author has made several assumptions regarding possible functional roles for these embryonic lipids. These assumptions cannot, however, be substantiated with existing data.

The need for additional work in this field is obviously essential in determining the biochemical significance of this group of compounds in lenticular tissue. The data obtained from this investigation has raised a number of questions:

1. What is the origin of these lipids? Are they synthesized in the lens or transported from extra-lenticular surroundings? Or both?
2. What are the functional roles of these compounds in lenticular tissue?
3. What is the relationship, if any, of lipid metabolism to lenticular anamolies as cataracts, spherophakia, etc.?
4. Do the lipid classed have any specific relationship to cytological location?

CHAPTER IX

GENERAL SUMMARY

Studies of the structural growth and lipid distribution were undertaken on the embryonic chick lens. These studies began on the eleventh day of embryonic development and included alternate days until hatching of the embryo.

Structural growth of the embryonic lens, as determined by dimensions and wet and dry weights, exhibited increases throughout lenticular development.

Isolation and characterization of the lenticular lipids involved application of the following analytical tools:

1. Thin-layer adsorption chromatography - The sensitivity and rapidity of this analytical tool provided a preliminary exploration of the lenticular lipids. In addition, this technique was valuable in monitering silicic acid column effluents.
2. Silicic acid column chromatography - This method provided a means of separation of the lenticular lipids, by gradient elution, into individual lipid classes.
3. Gas-liquid chromatography - This highly sensitive analysis was used to determine the fatty acid composition of the lenticular tissue. Fatty acid analysis of the individual lipid classes provided valuable information regarding molecular structure.

The use of these methods for characterization of the lenticular lipids was not to provide a comprehensive analysis but rather a gross lipid investigation. These analyses revealed the embryonic lenticular tissue

to contain a variety of lipids including free and esterified cholesterol, glycerides, cephalin, lecithin, and sphingomyelin. These compounds were found to accumulate during lenticular development with the individual lipids displaying characteristic accumulation patterns.

Additional information was obtained by studying the fatty acid compositions of the individual lipids identified. These studies revealed the occurrence of a wide spectra of both saturated and unsaturated fatty acids with characteristic fatty acids identified with certain lipid classes. Generally, the fatty acids palmitic and oleic were the most abundant in this embryonic tissue.

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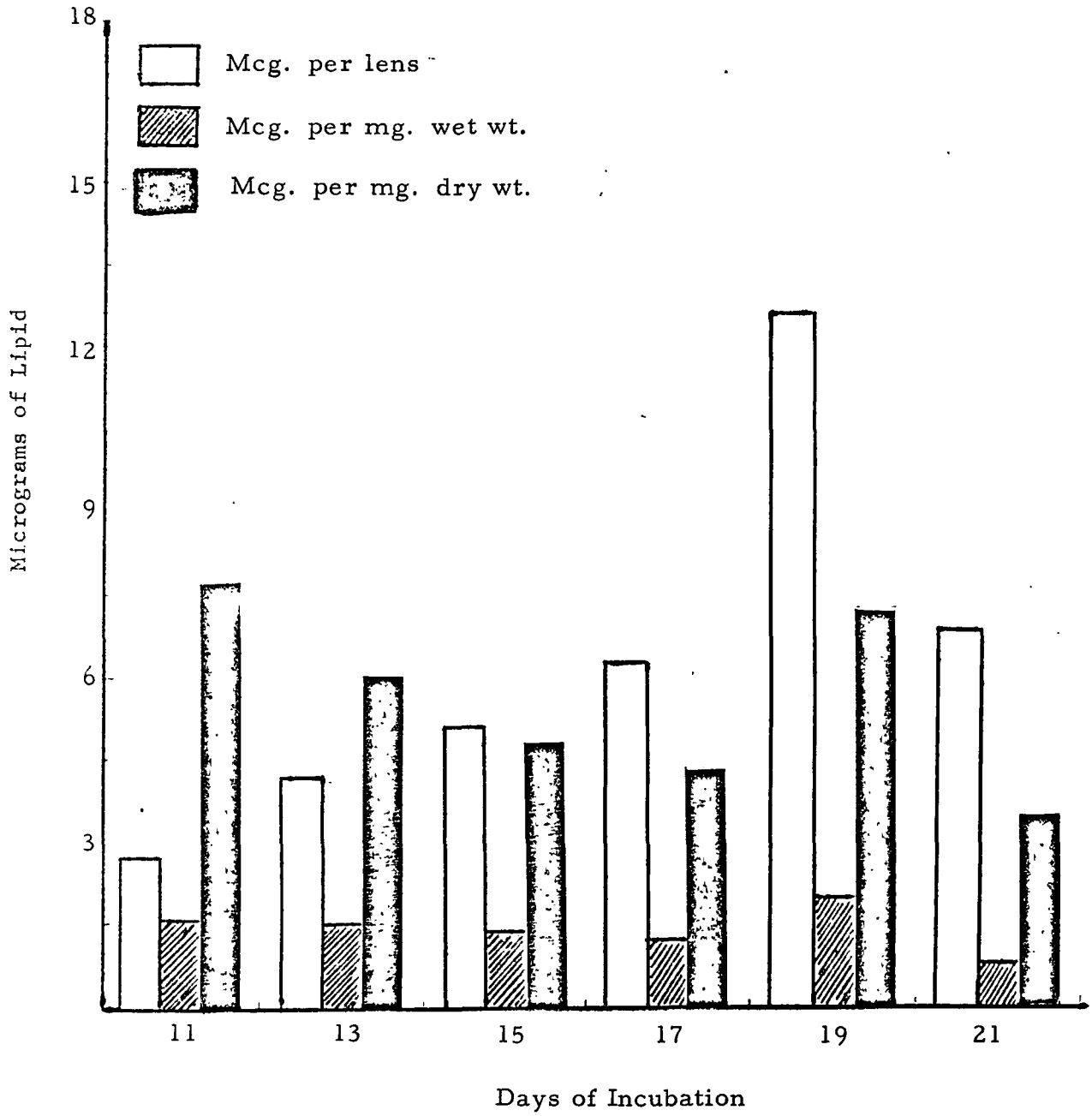
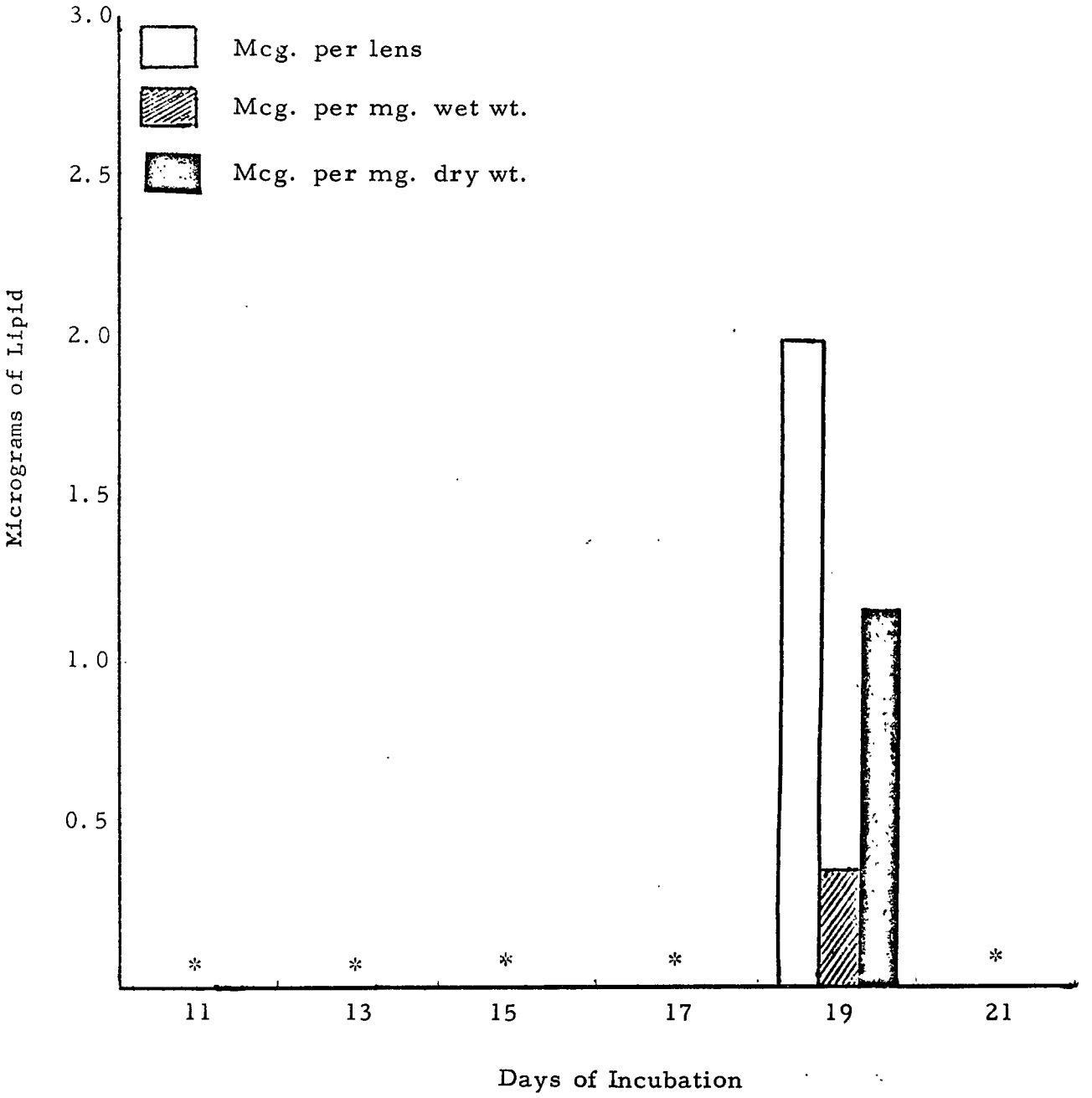
APPENDIX A

FREE CHOLESTEROL CONTENT OF THE EMBRYONIC CHICK LENS



APPENDIX B

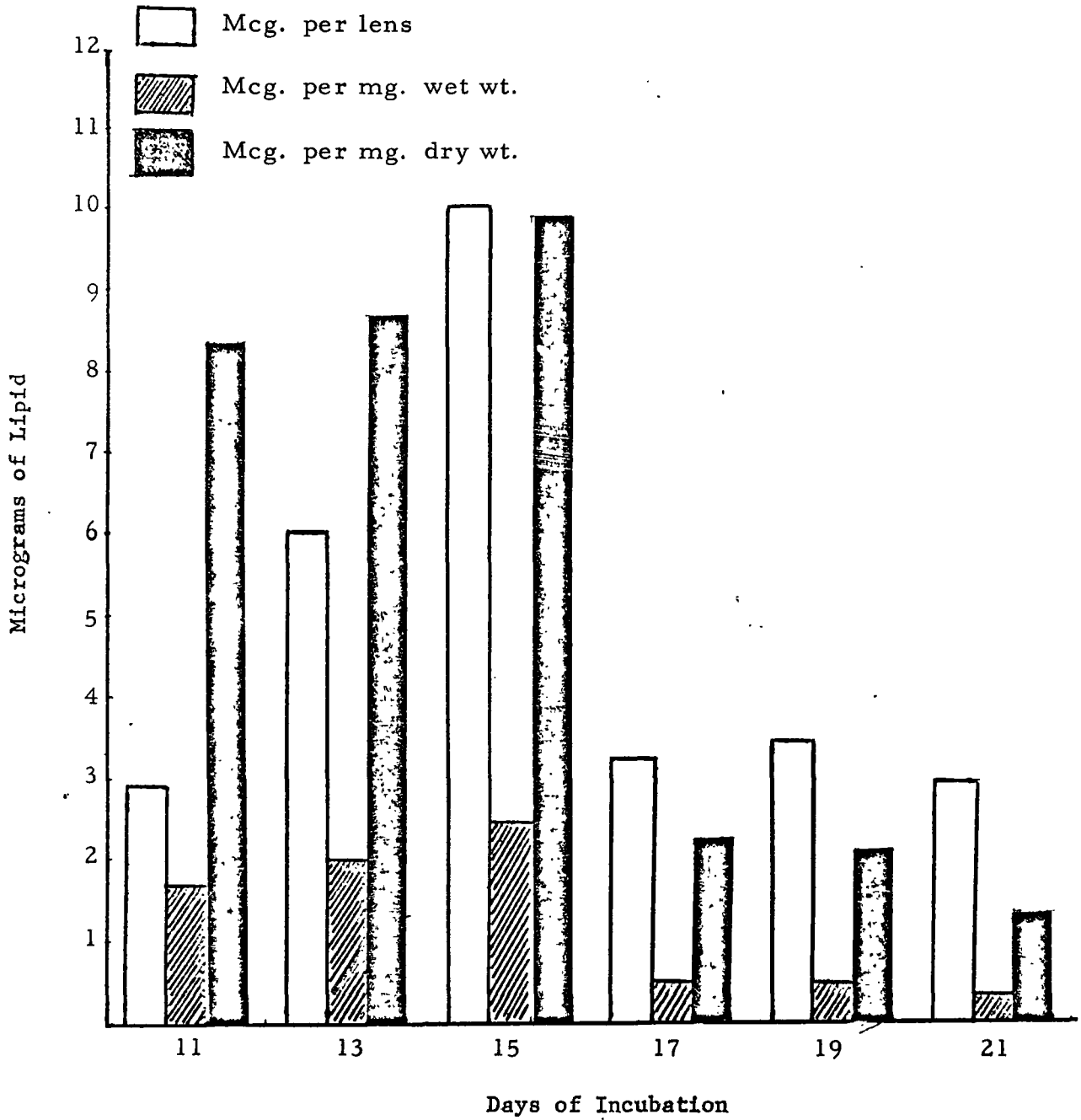
CHOLESTEROL ESTER CONTENT OF THE EMBRYONIC CHICK LENS



*Trace quantities.

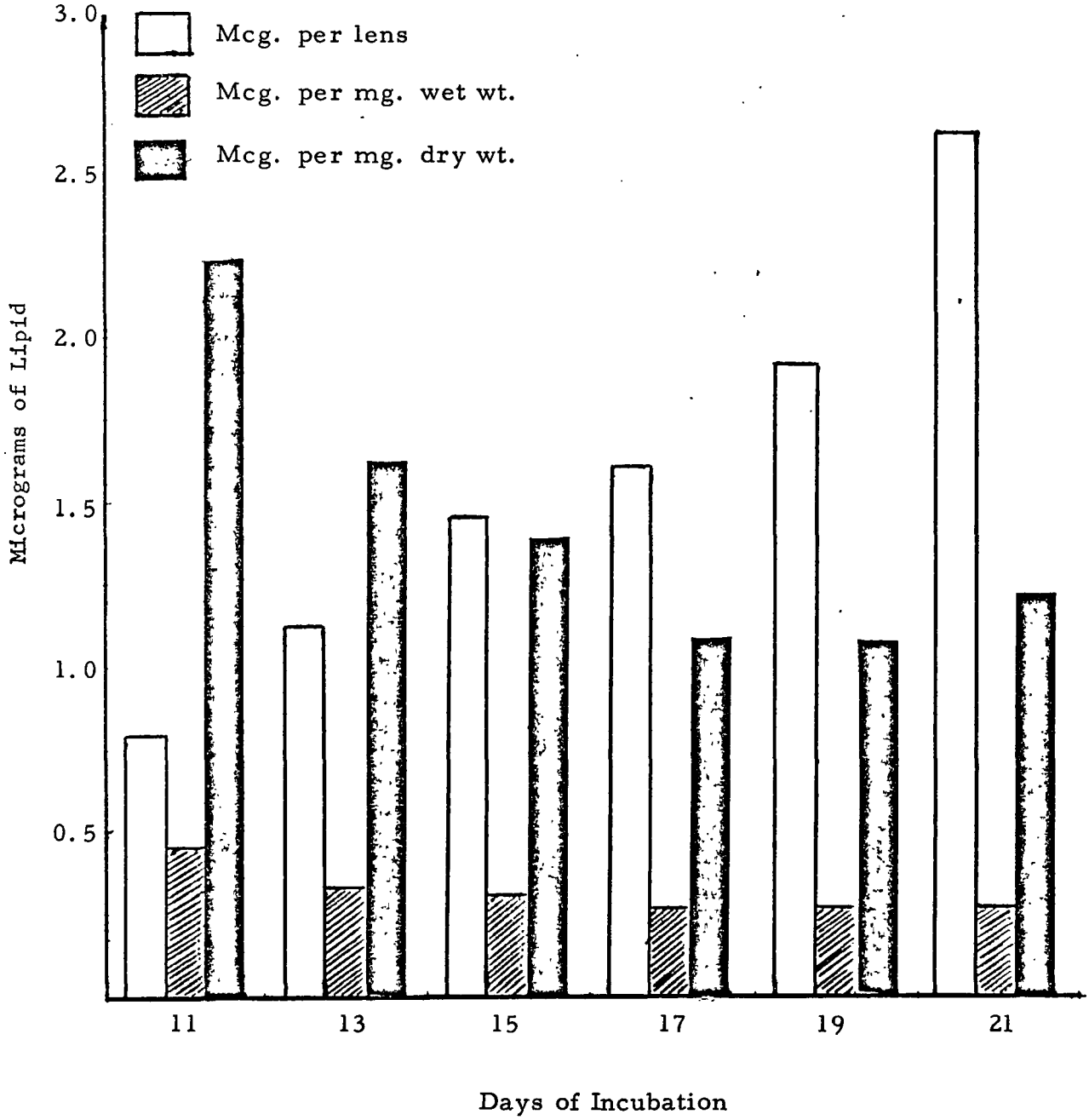
APPENDIX C

GLYCERIDE CONTENT OF THE EMBRYONIC CHICK LENS



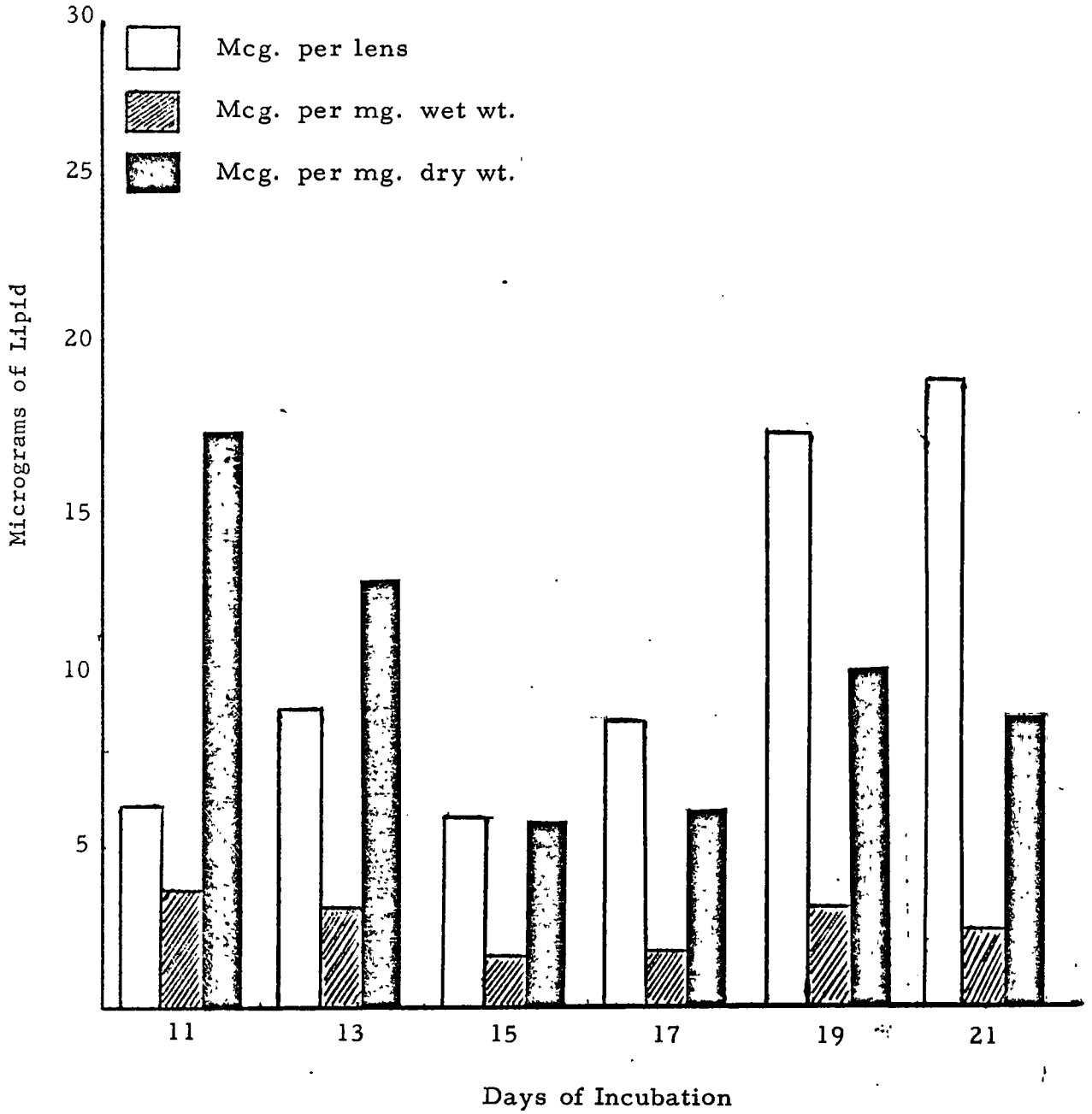
APPENDIX D

CEPHALIN CONTENT OF THE EMBRYONIC CHICK LENS



APPENDIX E

LECITHIN CONTENT OF THE
EMBRYONIC CHICK LENS



APPENDIX F

SPHINGOMYELIN CONTENT OF THE EMBRYONIC CHICK LENS