Lipid Changes in the Chick Hatching Muscle[1] (34564)

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A key role for the musculus complexus (hatching muscle) in the hatching process has been suggested for the chick, as well as other avian species, due to the sudden increase in muscle size just prior to the time of breaking the egg shell (pipping). Chemical and enzymatic characteristics of the hatching muscle in the chick have been investigated by George and Iype (1), Smail (2), Brooks and Ungar (3), and Ramachandran *et al.* (4). The earlier observations on the imbibition of water and the loss of glycogen in this muscle prior to hatching are now well documented. Values of lipid content in the developing bird are known to vary widely in tissues, particularly embryonic tissue (5). The changes in lipid content of the skeletal muscles may have special significance during hatching, but the contribution of the lipids has not been studied adequately. Specific analytical procedures employing gas-liquid chromatography and a densitometric assay on thin-layer plates were developed to examine in detail lipid changes occurring in the m. complexus prior to and during the period of hatching of the chick.

Material and Methods. White leghorn eggs (Ghostley Poultry Farm, Coon Rapids, Minnesota) were used for the experiments. The eggs were transferred from the farm to the incubator (Humidaire Co., New Madison, Ohio) in the laboratory on day 14 of incubation. The complexus and shank muscle (gastrocnemius) were removed daily from the developing chicks from day 17 of incubation to hatching. The m. complexus is a paired muscle situated at the back of the skull between the cervical vertebrae and the parietal bones. The gastrocnemius muscle was used as a skeletal muscle control. No attempt was made to determine the sex of the embryos.

All solvents used in these experiments were ACS reagent grade. Chloroform and methanol were redistilled. *N*-Pentane was 99 mole % minimum (Phillips Petroleum Co. Special Products Division, Bartlesville, Oklahoma).

Extraction of lipids. Total lipid extracts were prepared from pools of tissues by the method of Folch *et al.* (6) with 20 vol of chloroform–methanol (2:1) in the homogenizer. Nonlipid was removed from extracts by partition according to the method of Folch *et al.* (6) using 0.017% magnesium chloride in the first partition. The lipid extract was dried under nitrogen and then *in vacuo* at 30° until constant weight was obtained. The weighed lipid was stored in *n*-pentane at −15°.

Analysis of lipids. Duplicate determinations were made on pools of extracts of 4–8 birds in four separate experiments. Fatty acid composition of the total lipids were determined by gas-liquid chromatography (GLC) of methyl esters prepared by the direct methylation method of Morrison and Smith (7). Boron trifluoride-methanol (14%) reagent (Applied Science Laboratories, Inc. State College, Pa.) was added under nitrogen in the proportion of 1 ml of reagent/4–16 mg of lipid. At the end of 90-min boiling, benzene and methanol were added to give the proportions of 35% BF_3-methanol reagent/30% benzene/35% methanol to methylate sterol esters, and the methanolysate was boiled for another 30 min. The *n*-pentane extract of the esters was stored at −15°.

The methyl esters were analyzed in a

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Perkin-Elmer 881 gas chromatograph with flame ionization detector. The column was 6 ft × ¼ in. o.d. packed with 20% diethylene glycol succinate polyester (Supelco, Inc. Bellefonte, Pa.) on 100/120 Chromosorb W (AW-DMCS, Applied Science Laboratories, State College, Pa.). The injector temperature was 265°, the detector 220° and the column was operated at 190°. The nitrogen carrier gas was at a flow rate of 33 ml/min. Attenuation was at ×20. Good separations could be carried out at 165° column temperature but at approximately twice the retention time. An internal standard of lauryl methyl ester was used after it was determined that it was present only in trace amounts in the muscle extracts. The standard served as an adequate check on the reproducibility of the injections.

The peaks in the chromatograms were identified by reference to standard samples of methyl esters (Nutritional Biochemicals Corporation, Cleveland, Ohio) and qualitative mixtures (Applied Sciences Laboratories, State College, Pa.). Quantification was made on the basis of the direct proportionalities of the peak areas measured by triangulation. All determinations were done in duplicate. The carbon numbers of methyl esters were verified by a nonpolar column packed with 20% Apiezon L. (Metropolitan-Vicker Electrical Co. Ltd. Distributed by Shell Co.) on 100/120 Chromosorb W (HP AW-DMCS, Johns-Manville).

Individual lipid fractions were separated by thin-layer chromatography. The cholesteryl esters, triglycerides, free fatty acids, and cholesterol of the neutral lipids were quantitated by densitometry using sulfuric acid charring similar to the method of Blank *et al.* (8). The polar lipid fraction was determined by difference. The thin-layer plate contained silica gel G (Brinkman Instruments Inc., L. I., N. Y.) and the solvent system used was hexane–diethyl ether–formic acid (80:20:1, v/v/v). After a 20-min period for development, the chromatoplate was sprayed with 50% (v/v) aqueous sulfuric acid and then heated at 180° in an oven (Precision Thelco, model 18). The optimum heating time was 30 min for cholesteryl esters and cholesterol and 110 minutes for triglycerides and free fatty acids. A standard curve was run with each lipid fraction for quantitation. All determinations were done in duplicate. The densitometry was carried out with an Aminco-Bowman spectrophotofluorometer using a thin-layer chromatogram scanner (American Instrument Company, Inc., Silver Spring, Maryland). The individual peaks of the chromatograms were cut out and weighed. Pure cholesteryl palmitate, cholesterol, tripalmitin, and palmitic acid (Lipids Preparation Laboratory of the Hormel Institute, Austin, Minnesota) were used as standards for reference and quantitation.

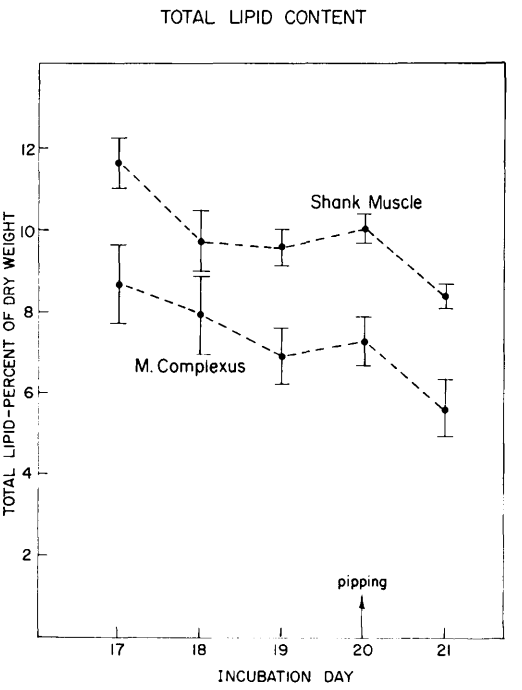

FIG. 1. Total lipid content of m. complexus and shank muscle of the developing chick. Each vertical line represents the standard deviation of the mean value of four experiments.

Results. The total lipid of the m. complexus determined on a dry weight basis decreased an average of 40% between day 17 of incubation and hatching (day 21). The total lipid of the shank muscle decreased an average of 25% during the same period. Figure 1 demonstrates the quantitative changes of total lipids in four separate experiments in both

TABLE I. Fatty Acid Composition of Total Lipids of Developing Chick Muscles Determined as Methyl Esters on GLC.[a]

	Musculus complexus						Shank muscle					
Day: / Fatty acid	17	18	19	20	21	Ratio day 21/17	17	18	18	20	21	Ratio day 21/17
C_{16}	2.27	1.93	1.64	1.80	1.73	0.63	3.04	2.52	2.90	2.56	2.19	0.72
C_{18}	1.58	1.27	1.16	1.19	0.88	0.56	2.01	1.61	1.83	1.52	1.73	0.71
$C_{18=1}$	1.57	1.64	1.70	1.16	1.15	0.73	2.53	1.99	2.70	2.46	2.52	0.99
$C_{18=2}$	1.15	1.07	1.07	0.92	0.77	0.67	1.82	1.67	1.96	1.75	1.32	0.73
$C_{20=4}$	0.81	0.72	0.63	0.51	0.56	0.69	1.36	1.09	1.29	0.90	0.53	0.39
Total	7.38	6.63	5.90	5.58	4.79	0.65	10.76	8.85	10.68	9.19	7.99	0.74

[a] (mg/100 mg of tissue dry wt; pools of 4–8 chick muscles/group, av of 2 expts.); trace amounts of C_{12}, C_{14}, and $C_{16=1}$ fatty acids are not listed.

m. complexus and shank (gastrocnemius) muscle of the developing chick during the hatching period. The shank muscle is richer in total lipid. However, there was no difference in the composition of fatty acids of the total lipids between m. complexus and shank muscle as shown by the results of GLC analysis in two experiments in Table I. Results of densitometric quantitation on TLC of fractionated neutral lipids in two experiments are presented in Table II. Between day 17 of incubation and hatching, the decrease of triglycerides was 41% in m. complexus and 11% in shank muscle. There were no apparent differences in other lipid fractions. Cholesteryl esters in both m. complexus and shank muscle decreased 34%. Cholesterol decreased 46% in m. complexus and 43% in shank muscle. There was no change in free fatty acids during the same period. Polar lipids (calculated by difference) decreased 40% in m. complexus and 31% in shank muscle. The R_f values which were identical for the zones of the standards and for the muscle extracts were 0.89 for cholesteryl esters, 0.59 for triglycerides, 0.31 for free fatty acids, and 0.14 for cholesterol. The polar lipids remained at the origin. The H_2SO_4 charred zones on TLC of a mixture of cholesteryl ester, methyl ester, triglyceride free fatty acid, cholesterol, and polar lipid are shown in Fig. 2. No attempt was made at this time to assay for the individual polar lipids.

Discussion. Attention has been given for some time to the participation of the m. complexus in the hatching of the chick (1, 2, 9). As a result of the rapid increase in size of the

TABLE II. Lipid Class Analysis of Developing Muscle of Chick Embryos by Densitometric Measurement of Sulfuric Acid Charred Zones on TLC Plates.[a]

	Musculus complexus					Shank muscle				
Day:	17	18	19	20	21	17	18	18	20	21
Total lipids	8.15	9.73	7.45	7.58	4.86	11.40	10.95	9.35	10.40	7.78
Neutral lipids (av of 2 determinations)										
Cholesteryl esters	0.58	1.12	0.78	0.45	0.38	0.87	0.78	0.86	0.64	0.57
Triglycerides	0.84	1.64	0.62	0.72	0.49	0.71	0.69	0.62	0.60	0.63
Free fatty acids	0.09	0.07	0.08	0.08	0.09	0.09	0.19	0.05	0.08	0.06
Cholesterol	0.69	0.62	0.61	0.74	0.37	1.22	1.11	0.97	0.96	0.69
Polar lipids[b]	5.95	6.28	5.36	5.69	3.53	8.51	8.18	6.85	7.42	5.83

[a] (mg/100 mg of tissue dry wt; pools of 4–8 chicks/group, av of 2 expts.).

[b] Calculated by difference.

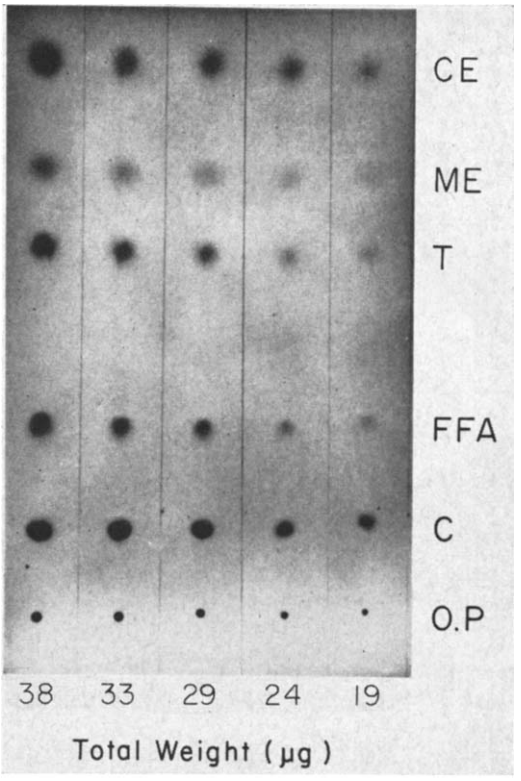

FIG. 2. Thin-layer chromatogram of a standard lipid mixture (The Hormel Institute, Austin, Minnesota) and phosphatidyl ethanolamine (Superco, State College, Pa.). The mixture contains (equal weight %) CE, cholesteryl oleate; ME, methyl oleate, T, triolein, FFA, oleic acid; C, cholesterol; P, phosphatidyl ethanolamine. O was origin. Solvent system was hexane–diethyl ether–formic acid (80:21:1, v/v/v) and the thin-layer plate was silica gel G. The plate was developed for 20 min. 50% (v/v) aqueous sulfuric acid was used for spraying. Heating temperature was 180°.

hatching muscle due to water imbibition just prior to the pipping of the egg shell, this muscle has been assigned an important role in the hatching process and has been suggested to be under endocrine control (3). The extent of water uptake by the chick muscles was reported by Smail (2) and Brooks and Ungar (3). After a survey of a number of enzyme activities Ramachandran *et al.* (4) using the gastrocnemius muscle for comparison, reported that those biochemical changes which were unique for the m. complexus during the hatching period included : (a) water imbibition up to hatching day, (b) a decrease in glycogen content and an increase in some glycolytic enzymes, and (c) an increase in adenosinetriphosphatase and acid phosphatase activity. The glycogen stores in the hatching muscle are depleted during the hatching period. The decrease of triglyceride as well as glycogen stores in the m. complexus during the period just prior to pipping and hatching is consistent with the view that the muscle is undergoing contraction, which with the increased swelling of the muscle to act as a cushion, provides the necessary force for the breaking of shell.

At the time of pipping, the absorption of the yolk sac by the chick embryo has not been completed and sufficient food material remains in the yolk as a potential source of energy to more than sustain the chick during and immediately after the hatching process. The changes in lipid content of the hatching muscle especially and in the skeletal muscle at pipping, therefore, may reflect the state of activity of these muscles specifically rather than the general nutritional state of the bird. The size of the hatching muscle is barely discernible at day 16 and increases from 50 mg on day 17 to more than 600 mg on day 21. A large proportion of this sudden weight increase is the result of water imbibition, which reaches a maximum at the time of pipping and the water content and muscle size decreases rapidly over the next 3 days after hatching.

The values for lipid content obtained in this study agree generally with classical values for the bird and in the specific instances with those of Bloor (5) who reported as dry weight values in bird skeletal muscle, 4.31% phospholipid and 0.31% cholesterol. One previous study on the role of lipids in the hatching muscle was made by George and Iype (1) who reported no change in the lipase activity during the hatching process. In the present study as shown Fig. 1, total lipids in both m. complexus and shank muscle decreased at the same rate from day 17 of incubation to hatching day. The total lipid content was lower initially and the percent loss in the m. complexus was at least 50%

greater. The decrease of 41% of triglycerides in the hatching muscle compared to an 11% decrease of triglycerides in shank muscle (Table II) substantiated the view that triglycerides were being utilized in the m. complexus as a source of energy for muscle contraction. In this regard, the initial increase in concentration of the triglyceride fraction in the m. complexus may be significant. The decrease in the cholesterol, cholesteryl ester, and polar lipid fractions was similar in m. complexus and shank muscle between days 17 and 21 of incubation.

The direct densitometric or fluorometric measurement of components of mixtures separated on thin-layer plates offers the possibility of a rapid and sensitive assay procedure applicable to lipids, steroids, and similar substances (10). For the lipid fractions described in this study, the individual components, free fatty acids, triglycerides, cholesterol, and cholesterol esters were assayed separately, since optimum conditions necessary for the proper development of the charred sulfuric acid-treated zones varied with each component. The free fatty acids were difficult to char and to obtain accurate measurements in the free form or as methyl esters, however, this is not critical since the GLC procedure for the measurement of the fatty acid methyl esters still remains the method of choice. Reproducibility in the densitometric measurement on TLC plates of cholesterol, cholesterol esters, and triglycerides can be achieved by strict adherence to uniformity in the preparation, spotting, and spraying procedures and in the development of the charred zones. For the triglyceride fraction, for example, linear plots were obtained between 1 to 6 μg of tripalmitin standards and estimates of duplicate assays of extracts agreed within 5%. The variation of triglyceride values for each day listed in Table II was within 15%. The use of the densitometric procedure on TLC plates for the measurement of these lipid fractions should be considered as a practical procedure for routine use.

Summary. GLC analyses of the hatching muscle of the developing fetal chick revealed that both hatching muscle and shank muscle had similar compositions of total fatty acids, although shank muscle was richer by 50% in total lipids. There was a loss of total lipids during the hatching period in both muscles the relative loss being greater in hatching muscle. Analysis of lipid constituents on TLC using a densitometric assay procedure demonstrated the larger decrease specifically of triglycerides in the hatching muscle during this period. The participation of lipids in the contraction of the hatching muscle of the chick embryo at the time of pipping is suggested.

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