INTRACELLULAR DISTRIBUTION OF CALCIUM IN DEVELOPING BREAST MUSCLE OF NORMAL AND DYSTROPHIC CHICKENS

ETHEL COSMOS, Ph.D.

From the Division of Cell Biology, Institute for Muscle Disease, Inc., New York

ABSTRACT

To follow the intracellular distribution of calcium in the breast muscles of developing chickens, Ca^{45} was injected into the albumen of predeveloped eggs. Since the embryos were grown in a radioactive medium, a complete exchange of the isotope for its non-radioactive counterpart in muscles was accomplished. Subcellular particulates of the muscle cells were separated by the method of differential centrifugation. Analysis of the separated fractions showed that in the muscles of the 13-day embryo, when the nuclear-myofibrillar ratio is high, 65 per cent of the muscle calcium is in the nuclei. With the increased synthesis of myofibrils, the nuclear-myofibrillar ratio decreases with a concomitant fall in radioactivity. Thus, calcium was not associated with the developing myofibrils. At the time of hatching, when myofibrils perform physiological work, the highest level of calcium is in the mitochondria. This suggests that the mitochondria play a key role in the physiological activities of calcium in the cell. The microsomal fraction reaches a maximal level of calcium when the adult composition of muscle is attained. Results of investigations on dystrophic muscles show changes in the calcium distribution of the fractions as early as the 3rd week of embryonic development, which are interpreted to indicate an alteration in the protein metabolism of the cell, or an early destruction of muscle tissue. Further, alterations in the calcium content of fractions which seem to regulate the movements of this ion in the cell are discussed. A new technique for homogenizing tissues from embryos of different ages is presented.

INTRODUCTION

The turnover of muscle calcium is facilitated by muscular activity; this has been demonstrated in frog muscle both *in vivo* and *in vitro*. *In vitro*, the turnover of calcium after periodic contraction is incomplete; *in vivo*, total exchange is realized only after several hours of activity (1). Having established the fact that movement of calcium is aided by muscular activity, we can formulate the question: Under physiological conditions, what intracellular components of the muscle cell participate in the binding and releasing of calcium?

Several approaches to this problem have been attempted, one of which was radioautography. With frog muscle, radioautographs were realized only in experiments *in vitro* in which tissues were soaked in radioactive media for long periods (2). These preparations recorded the presence of radioactivity in sarcoplasmic structures, perhaps the endoplasmic reticulum, but gave no indication of the sites of a more tightly bound calcium not exchangeable under these *in vitro* conditions (1).

A second approach involved the separation of individual muscle fractions by the method of differential centrifugation (3). Since we were concerned primarily with the localization and not the uptake of calcium by the various cell fractions,

chicken embryos were grown in a medium made radioactive as a result of the injection of $Ca^{45}Cl_2$[1] into the albumen of predeveloped eggs. This technique assured a complete labeling of all calcium-containing structures with the radioactive isotope.

The age period selected for these studies on chicken muscle was from 13 days *in ovo* through 1 month *ex ovo*, since in this period development of muscle ranges from muscle cells containing few myofibrillar structures (day 13) to muscle that has reached its adult composition (1 month) (4).

The studies were extended to the dystrophic chicken whose breast muscle loses the ability to contract (5). Further, the fact that dystrophy in the chicken is hereditary and that symptoms are manifest early was a challenge to investigate possible alterations in the calcium metabolism in the embryo and in pre-adult periods. Before this investigation, information was available only on adult dystrophic animals (5).

MATERIALS AND METHODS

Fertilized eggs from normal and dystrophic chickens were obtained from the University of Connecticut, Storrs, Connecticut,[2] and eggs from normal chickens from Shamrock Farms, New Jersey. Between 24 and 48 hours after incubation, the eggs were injected with 60 to 120 μc of $Ca^{45}Cl_2$ in a maximum volume of 0.1 cc of the radioactive solution per egg.

General Procedures

All procedures were performed at 4°C. Animals were decapitated and bled; the breast muscle was rapidly excised and placed in precooled (0°C) homogenizing fluid. Final dilutions of all samples were 10:1 w/v (*ex ovo* tissues) or 6:1 w/w (*in ovo* tissues). Before homogenization, tissues were finely chopped with scissors, then gently homogenized in a power-driven glass tissue-grinder fitted with a radially serrated teflon pestle machined to a clearance of 0.005 to 0.007 inch. The temperature of the homogenate was always kept below 3°C by alternating the homogenization periods of 50 seconds with rest periods of 30 seconds. Total grinding time was regulated by microscopic examination for each homogenate.

Because of the scope of the period of development which was selected, it was necessary to experiment with buffer media and homogenizing techniques suitable to the age of the experimental tissue. Our aim was to select media which provided the energy requirements of the cell particulates and which gave preparations of relaxed myofibrils. Further, the method of homogenization had to be controlled carefully since severe grinding of tissues destroys myofibrils and mitochondria, and smaller particles of each portion so destroyed contaminate successive residues. A binocular Zeiss Photomicroscope equipped with phase contrast lenses (magnification, 2000) was used to examine the preparations.

Preparation and Analysis of Adult Muscle

HOMOGENIZING FLUID: The solution selected primarily to satisfy the energy requirements of the cell contained the following substances: Imidazole, 0.01 M; KCl, 0.1 M; $MgCl_2$, 0.005 M; K-oxalate, 0.001 M; K_2HPO , 0.005 M; MgATP, 0.002 M; PEP, 0.01 M; PEP Kinase, 0.05 mg/ml. The energy systems were added to the solutions immediately before homogenization.[3]

DIFFERENTIAL CENTRIFUGATION: A Spinco Model L ultracentrifuge was used to fractionate the muscle particulates. *Fraction I*, centrifuged at 2700 RPM in a 30 Rotor for 15 minutes (R_{max}-856 *g*), consisted primarily of myofibrils, nuclei, and connective tissue; *Fraction II*, the mitochondrial residue, was centrifuged at 8500 RPM in the 30 Rotor for 20 minutes, (R_{max}-8481 *g*); *Fraction III*, designated as the microsomal fraction, contained all small particles centrifuged at 36,000 RPM for 60 minutes in the 40 Rotor (R_{max}-117,363 *g*); *Fraction IV*, was the supernatant fluid from the high-speed centrifugation.

RADIOACTIVE DETERMINATIONS: Residues were quantitatively transferred to quartz crucibles, dried at 100°C overnight for dry weight determinations (DW), and then incinerated at 550°C, or wet-ashed with HNO_3. Appropriate dilutions of the dry or wet-ashed residues were made to give aliquots for radioactive counting of less than 1.0 mg per planchet. A low background (approximately 1.5 to 1.8 CPM) gas flow windowed counter (Nuclear Chicago) was used to determine the radioactivity in each fraction. Specific activities (SA) were determined as CPM mg DW. To correct for decay values of the isotope and for radioactive dilution effects *ex ovo* due to calcium intake in the diet, excretory losses, and bone growth, the SA of the tissue was divided by the SA of the serum expressed as CPM mg serum. Final results are

[1] $Ca^{45}Cl_2$ was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[2] I am indebted to Dr. Walter Landauer for a generous supply of eggs.

[3] Abbreviations used: ATP, Disodium salt of adenosine-5′-triphosphate (Calbiochem); PEP, Crystalline tricyclohexylammonium salt of 2-phosphoenolpyruvic acid (Boehringer and Soehne, Germany); PEP kinase, rabbit muscle pyruvate kinase (Boehringer and Soehne, Germany). The MgATP was made by mixing equimolar solutions of $MgCl_2$ and ATP.

expressed as SA of each fraction in CPM mg DW divided by the CPM mg serum.

Preparation and Analysis of Embryo Muscle

HOMOGENIZING FLUID (HF): Microscopic examination showed that solutions tested for the *ex ovo* breast tissue were unsuitable for the embryonic tissues. Mitochondria swelled to many times their original size and resembled "crescent"-type structures (6); nuclei, usually oval-shaped in normal preparations, were fully rounded structures with an outer membrane swelling to the same size as the nucleus; myofibrils became gelatinous or supercontracted. These microscopic observations indicated that the solutions used for adult tissues were hypotonic to the subcellular structures of the embryo tissue. In addition, a reverse ionic distribution is characteristic of *in ovo* muscle (7). The problem was not merely one of solution tonicity since homogenization of the tissue in 0.44 to 0.88 M sucrose obviated the swelling of the subcellular particulates, but successive washings of each fraction with fresh buffer solution resulted in an increase in the amount of radioactivity released rather than the decrease to be expected with isotope losses. This increased loss of Ca^{45} with successive washings implied a dilution of substances needed for the fractions to retain their radioactivity. One alternative was to use the animal's own tissue fluid for homogenization. Non-radioactive breast muscles of embryos of similar ages were homogenized in 0.44 M sucrose at a 10:1 dilution (w/w) and centrifuged at 50,000 RPM for 90 minutes in a Spinco 50 Rotor (R_{max}-198,425 *g*). The resulting supernatant was used undiluted as a homogenizing fluid (*HF*) for the tissues of the radioactive embryos in a final dilution of 6:1 (w/w). Approximately 1 gm of breast muscle was obtained by pooling muscles of embryos of similar weight. One breast yields close to 0.35 gm of muscle. Each tissue sample was examined under a dissecting microscope to remove visible fat, blood vessels, and contaminating feathers.

DIFFERENTIAL CENTRIFUGATION: *Fraction I* was centrifuged at 2700 RPM in a 30 Rotor for 15 minutes. Owing to the density of the medium, *Fraction II* was centrifuged at 18,000 RPM (8) for 16 minutes (R_{max}-25,718 *g*), and *Fraction III* at 50,000 RPM for 53 minutes (Spinco 50 Rotor was used for each). After one complete centrifugation series, a 2-ml aliquot of the supernatant (*Fraction IV*) was used to wash the residue of *Fraction I*. The resulting supernatant was used as a wash of *Fraction II*, and the supernatant then obtained was used as a wash of *Fraction III*. The final supernatant was analyzed separately and designated as the supernatant wash. Radioactivity did not increase in the wash solutions.

NITROGEN ANALYSIS AND RADIOACTIVE DETERMINATIONS: Owing to the rapid changes which take place in the ratio of extracellular to cellular phase of embryonic tissue, SA is expressed as CPM mg nitrogen (N) rather than as CPM mg dry weight. Residues were dissolved in 0.05 N NaOH (9), brought to volume, and aliquots taken for radioactive plating and for nitrogen analysis by a modification of the Kjeldahl procedure of McKenzie and Wallace (10). The total muscle nitrogen per gm wet weight tissue was approximately 9 to 15 mg for embryos of 13 to 21 days. The amount of N contributed by the homogenizing fluid (*HF*) was approximately 0.4 mg/ml of fluid. To correct for the added N in the *HF* medium and for the relatively large volume of supernatant fluid occluded in the low-speed fraction, the following relationship was used:

$$[(V_h - DW) - V_s]\, N/\text{ml} = T_n$$

where:

V_h = Volume of homogenate sample.
DW = Dry weight volume of muscle sample (Table I).
V_s = Volume of supernatant obtained experimentally (*Fraction IV*).
N/ml = Nitrogen content in mg/ml of V_s.
T_n = Total nitrogen content of fluid occluded in *Fraction I*.

This amount (T_n) and its equivalent CPM were subtracted from corresponding values of *Fraction I* and added to *Fraction IV*. To check the calculated value of recoverable supernatant, homogenates were centrifuged at approximately 200,000 *g* for 2 to 3 hours. The resulting volume of supernatant agreed to within 2 per cent of the calculated value. Further, the amount of nitrogen added by the *HF* was subtracted from the supernatant fraction. Thus, the total nitrogen of *Fraction IV* was calculated with the following relationship:

$$(T_s + T_n) - T_{hf} = T_{ns}$$

where:

T_s = Total supernatant nitrogen recovered experimentally (*Fraction IV*).
T_n = Total nitrogen content of the occluded fluid subtracted from *Fraction I*.
T_{hf} = Total nitrogen content of the added homogenizing fluid (*HF*).
T_{ns} = Total calculated nitrogen of the supernatant (*Fraction IV*).

This calculated value (T_{ns}) was used to determine the SA of *Fraction IV*. Calculations to correct for fluid occluded in the pellets of *Fractions II* and *III* were unnecessary since differences in the N content

of these fractions isolated in 0.44 M sucrose and the *HF* medium were slight, especially after a dry recentrifugation of each pellet.

To correct for isotope decay in the *in ovo* tissues, the SA of the muscle fraction was divided by the SA of the heart.[4] For this analysis, hearts were cleaned of aortas, bisected, and drained on glass by wiping the opened heart across the glass surface. These were analyzed for counts and total nitrogen as described above. The SA in embryo fractions then is expressed as CPM mg N of the fraction divided by the CPM mg N of the pooled hearts.

Finally, precautions were taken to keep experimental equipment free of calcium. Glassware was soaked in citric acid and HCl. Only glass-distilled water was used for rinsing and for making solutions.

RESULTS AND DISCUSSION

Since these experiments were carried out over a period of active development and growth in the chicken, a knowledge of the muscle structure at the various stages of development was helpful for the interpretation of the experimental findings. Routine histological preparations (done by Miss Yolanda Pagan) were made of each experimental tissue. Examination of these preparations (Fig. 1) and homogenate samples showed that in a 13-day embryo the muscle is composed mainly of nuclei, mitochondria, microsomes, and the precursors of myofibrils, myoblasts, and myotubles. Biochemical data also indicate the presence of a high nuclear-myofibrillar ratio at this stage of development (11). During the last 7 days of embryonic development, the high nuclear-myofibrillar ratio is rapidly reversed, owing to the increased synthesis of both connective tissue and myofibrils. Further, the bulk of myofibrils formed during this period up to 30 days after hatching serve to *dilute* other muscle components.

Fig. 2 correlates the age in days and weight in grams of normal animals. Owing to the variability in weights of embryos sacrificed on a specific day after incubation, the days of incubation could not be used as a criterion of age (4). The straight line is drawn through points obtained from one batch of normal eggs and was the basis for determining the exact age of experimental embryos. Each point of the line is an average of 6 embryos of similar weight. The scatter points were not used in drawing the line, but they are included to emphasize the variability in weights of embryos sacrificed on the same day. The large increase in weights from approximately 19.5 days to hatching (21 days) results from the absorption of the yolk sac. Before the animals were weighed, all embryonic membranes were removed, the yolk sac cut, and the embryos were blotted on wet filter paper.

Fig. 3 shows the distribution of calcium among the various subcellular fractions in the breast muscle of normal chickens from 13 days *in ovo* to hatching (21 days). In the 13-day embryo, the low-speed fraction contains almost 65 per cent of the total calcium of the muscle; and by 30 days

TABLE I

Dry Weights of Developing Breast Muscle of Normal and Dystrophic Chicken Embryos

Age of embryo	Dry wt as per cent wet wt	
	Normal	Dystrophic
days		
12	7.5 (7.1–8.0)	7.4 (7.1–7.8)
13	8.2 (7.9–8.6)	8.0 (7.5–8.5)
14	8.4 (8.3–8.7)	8.9 (8.2–9.4)
15	8.9 (8.3–9.7)	9.6 (8.8–11.3)
16	10.6 (9.6–12.3)	10.0 (9.4–10.6)
17	11.5 (10.2–13.3)	11.2 (10.6–12.3)
18	12.4 (11.1–13.4)	11.8 (10.9–13.8)
19	12.7 (12.0–13.5)	12.0 (11.1–12.9)
20	12.2 (9.8–14.4)	13.2 (12.2–14.5)
21	12.1 (10.2–13.9)	13.9 (12.9–15.7)

Samples were tightly folded in tin foil at 4°C, weighed (wet weight), dried under an infrared lamp, and allowed to remain in a vacuum oven at 100°C overnight before dry weights were determined. The range of dry weight is indicated under each average. A total of 107 dry weight determinations was done for normal breast tissue, and 97 for dystrophic tissue.

[4] Serum samples obtained from bleeding decapitated embryos were contaminated with the radioactivity of extraembryonic fluids. More tedious methods of removing blood from the animals would not permit rapid excision of the breast tissue. As a result, heart tissue was used to correct for isotope decay in the embryos.

ex ovo the value has dropped to approximately 10 per cent of the total. During this entire period, synthesis of myofibrils continues. Since there is no concomitant increase in the SA, these data suggest that this calcium is not associated with the contractile structures.

The high SA seen in in *Fraction I* of the 13-day embryo can probably be attributed to calcium in the nuclei since these structures make up the greater portion of the low-speed fraction at this period. Were there no other structures taking up calcium in this fraction, the curve would show a continual decline corresponding to a dilution of the nuclear material per unit weight of sample. However, in the period between days 14 and 19, we note a sharp increase and then a decrease in SA. This would have to be associated with a fraction showing a marked increase in development during this short period, but which, after hatching, showed a decrease in terms of percentage of the total nitrogen of the muscle. The myofibrils are an unlikely repository of the calcium since they continue to be synthesized after birth (12, 13). However, analyses of connective tissue in *Fraction I* indicate a sharp increase from amounts of less than 2 per cent of the total nitrogen at day 14 to about 16 per cent at day 19. In the adult, connective tissue nitrogen comprises less than 5 per cent of the total nitrogen. These data agree with values previously published. Further, from day 19 to day 21 there is an actual drop in the percentage of connective tissue as related to total protein (4). Connective tissue is fully differentiated at hatching.

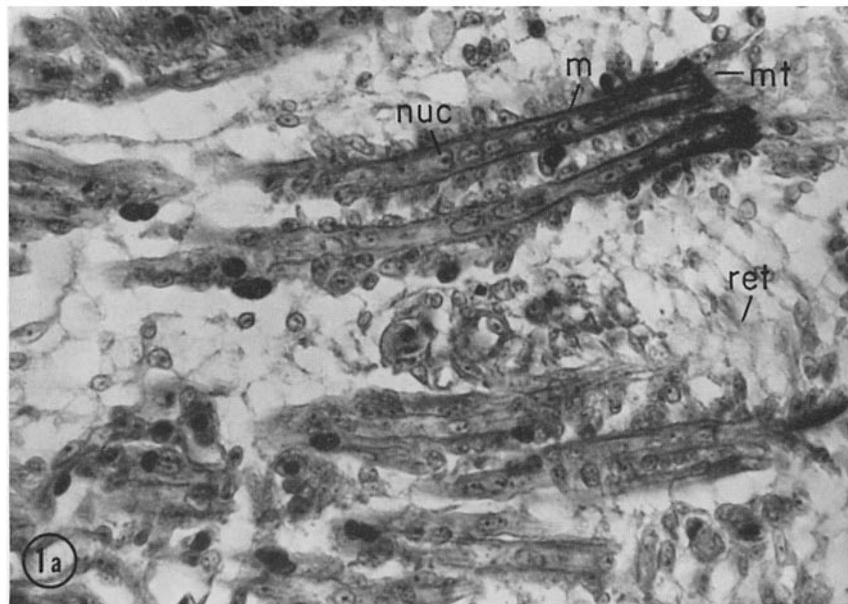
Unlike it does in the low-speed fraction, the SA of the mitochondria and microsomes increases progressively during development. The change in SA of mitochondria from about 30 per cent of the total at day 13 to a maximum at hatching indicates a key role of mitochondria in the physiological activities of calcium. The gradual increase in the SA of the microsomal fraction may be correlated with the development of the sarcotubular system concomitant with that of the myofibrils. The role of calcium of this system, then, is only maximal when the adult composition is reached. The increase in both mitochondrial and microsomal SA parallels an increase in the energy systems of muscle tissue during this period of development (12, 14, 15). The fact that energy systems are at a low level and that oxygen is in relatively poor supply may account for the low SA seen during embryonic development of both the mitochondrial and microsomal fractions.

The soluble substances of the supernatant contain less than 0.5 per cent of the total SA in all ages studied.

In Fig. 4 the distribution of calcium in the breast muscle of the dystrophic chicken is shown. As in the normal animal, the highest percentage of calcium is in *Fraction I*, at a time when the nuclear-myofibrillar ratio is high. Since up to day 18 the SA of *Fraction I* of dystrophic muscle drops less than 10 per cent of its day 14 value, in contrast to a drop of approximately 50 per cent from day 13 to 15 in the normal tissue, we concluded that the nuclear-myofibrillar ratio continues high as a result of increased synthesis of nuclei or of decreased synthesis of myofibrils. The additional peak of increased SA between days 14 and 19 in the normal animal is noted also in the dystrophic tissue, but is of a different magnitude.

Observations made during the period around hatching may be difficult to interpret because of the many rapid changes that occur in water shifts, nitrogen content (4), and body weight (Fig. 2). Thus, before hatching, these parameters suddenly increase, and then after hatching they decrease. The decrease in SA noted during this period reflects the rapid growth of myofibrils with its diluting of the calcium-associated structures, nuclei and connective tissue.

In Fig. 5 the intracellular distribution of calcium in the muscle of normal chickens is compared with that of dystrophic chickens. The 14-day embryo was selected for comparison since it is at this stage of development that differences between normal and dystrophic animals are first apparent in these experiments. Animals younger than 13 or 14 days were not studied, because of the difficulties in obtaining samples of breast muscle. At this stage of development, the high water content (Table I) prevents proper manipulation of the muscle tissue. In both the embryonic and adult tissues, the SA of *Fraction I* is higher in the dystrophic muscle and the SA of *Fractions II* and *III* is lower in the abnormal muscle. *Fraction IV* is not included since the SA of this fraction was similar in both series. The distribution in the 14-day embryo represents the calcium localization in non-functioning muscles. In the adult muscle, which performs physiological work, the calcium distribution is reversed. The large increases in



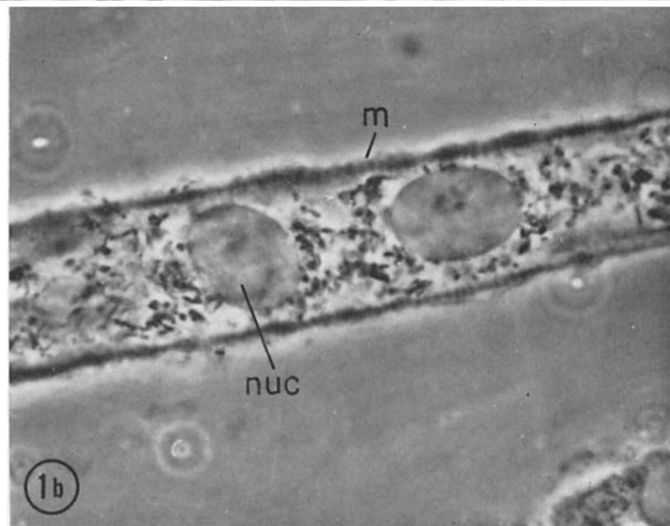


FIGURE 1 *a* Photomicrograph of the breast muscle of a 13-day chick embryo to show the characteristic high nuclear-myofibrillar ratio. The tissue was fixed in formal-calcium, cut at 5 μ, and stained with hematoxylin and eosin. Magnification, 509. Myofibrils, *m*; nuclei, *nuc*; myotubules, *mt*; reticulum, *ret*.
FIGURE 1 *b* Photomicrograph to show an enlargement of a myotubule taken from a freshly homogenized breast muscle of a 13-day chick embryo. Characteristic of the myotubule are the centrally localed nuclei, young myofibrils limited to the periphery of the tubule, and cellular inclusions seen as dark bodies inside the tubule (mainly mitochondria). As the myofibrils continue to be synthesized, filling the tubule, the nuclear-myofibrillar ratio decreases. Magnification, 2035 (phase contrast). Myofibrils, *m*; nucleus, *nuc*.

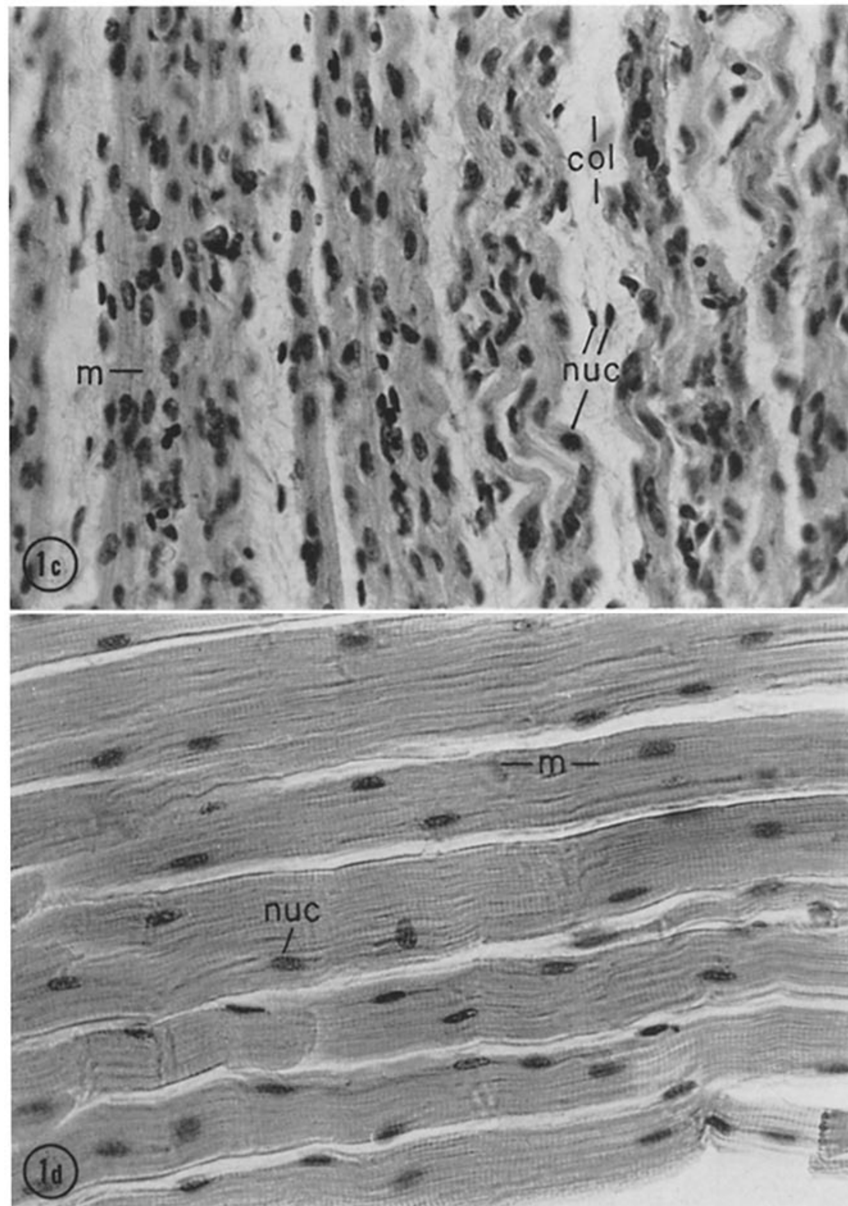


FIGURE 1 *c* Photomicrograph of a 19-day chick embryo breast muscle fixed, stained, and cut as described in Fig. 1 *a*. The number of myofibrils has greatly increased as has the amount of collagenous connective tissue between fibers. Magnification, 509. Myofibrils, *m*; collagenous connective tissue, *col*; nuclei, *nuc*.

FIGURE 1 *d* Photomicrograph of the breast muscle of a 1-month-old adult chicken fixed, stained, and cut as described in Fig. 1 *a*. In the adult muscle the nuclear-myofibrillar ratio is low (*cf*. Figs. 1 *a* and *c*). The muscle is more compact due to the increase in size of muscle fibers and the decrease in spacing between fibers. The dry weight of this muscle is 24 per cent of the wet weight as compared to 8 per cent in the 13-day embryo (Table I). Magnification, 509. Myofibrils, *m*; nuclei, *nuc*.

SA of *Fractions II* and *III* emphasize their importance in regulating the movements of calcium in the cell.

The role of calcium in both the contraction and relaxation of muscle fibers has been empasized. The emphasis to date has been placed upon myofibrils and microsomes and upon the formulation preliminary observations, made on both normal and dystrophic tissue, emphasize the importance of mitochondria in both the releasing and binding of calcium *during* or *after* contraction and relaxation. One might postulate that the sarcoplasmic reticulum (microsomal fraction) may be the mediator for transporting calcium from the cell

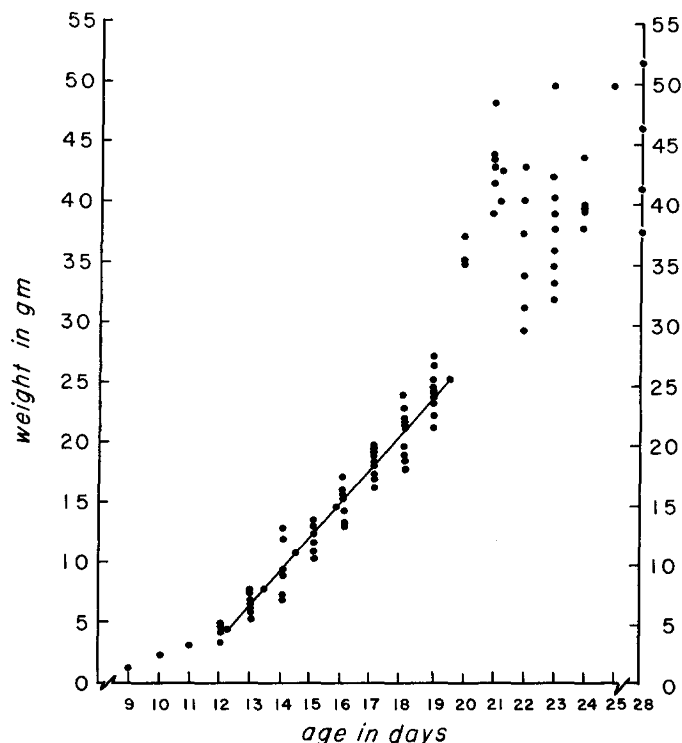


FIGURE 2 Age correlated to weight. The straight line is drawn through points obtained from one batch of normal eggs. Each point is an average of 6 embryos of similar weight. The scatter points indicate the variability in weights of embryos sacrificed on a specific day after incubation and the inaccuracy of using the days of incubation as a criterion of age. Note the large increase in weight from day 19.5 to hatching (21 days), followed by much variability after hatching.

that free calcium in the muscle leads to contraction of myofibrils (16, 17) and that removal of calcium either by chelating agents (18) or physiologically by microsomes gives relaxation (19). However, in our preparations, we have shown that mitochondria in the presence of all other components of the cell demonstrate a greater affinity for calcium than do other subcellular structures. Further, experiments not presented here indicate an increase in myofibrillar calcium and a decrease in mitochondrial calcium in preparations of supercontracted myofibrils. These membrane to the cell interior, but the mitochondria may regulate calcium movement within the cell during activity. Both structures seem to be dependent on some form of energy for the uptake and retention of calcium *in vitro* (20–22); both of them show an increase in calcium content during the period of rapid myofibrillar synthesis, except that a maximal mitochondrial level is attained at hatching when myofibrils are ready to perform physiological work.

In dystrophic muscle which progressively loses its normal function, an upset in the distribution of

calcium was observed as early as the last week of embryonic development. If the high SA of *Fraction I* in the embryo is due to an increased synthesis of nuclei, it would imply an alteration in the cell's protein metabolism at any early period. Examination of histological sections of *ex ovo* dystrophic muscle shows early increases in number of nuclei identified as muscle and phagocytic cell nuclei (eosinophilic, basophilic, mononuclear cells). The invasion of cells seems to be associated with myofibrillar destruction. The increased calcium in the embryo may be associated with the nuclei of invading cells; if so, it would imply a destruction of tissue during the early formation of contractile proteins.

Connective tissue structures, however, also show an affinity for calcium (Fig. 3). Preliminary experiments done in this laboratory indicate that connective tissue removed from *Fraction I* (*i.e.*, tissue insoluble in 0.05 N NaOH) (9) can precipitate 90 per cent of the radioactivity of the fraction. Further, increases in the amount of connective tissue are seen early in the dystrophic tissue (by 6 weeks *ex ovo*). Connective tissue high in calcium could serve to alter the calcium medium for membranes of muscle cells and thus affect both membrane permeability and excitability (23, 24).

The foregoing experiments represent initial efforts to investigate the distribution of calcium in the subcellular structures of developing and adult muscle as it exists in the living cell. Media were selected for the suspension of the cell fractions which would provide environmental conditions and energy requirements of the living cell, since solutions facilitating the uptake of exogenous calcium by isolated subcellular fractions *in vitro* (20–22) are also instrumental in retention of endogenous calcium by cell structures (21).

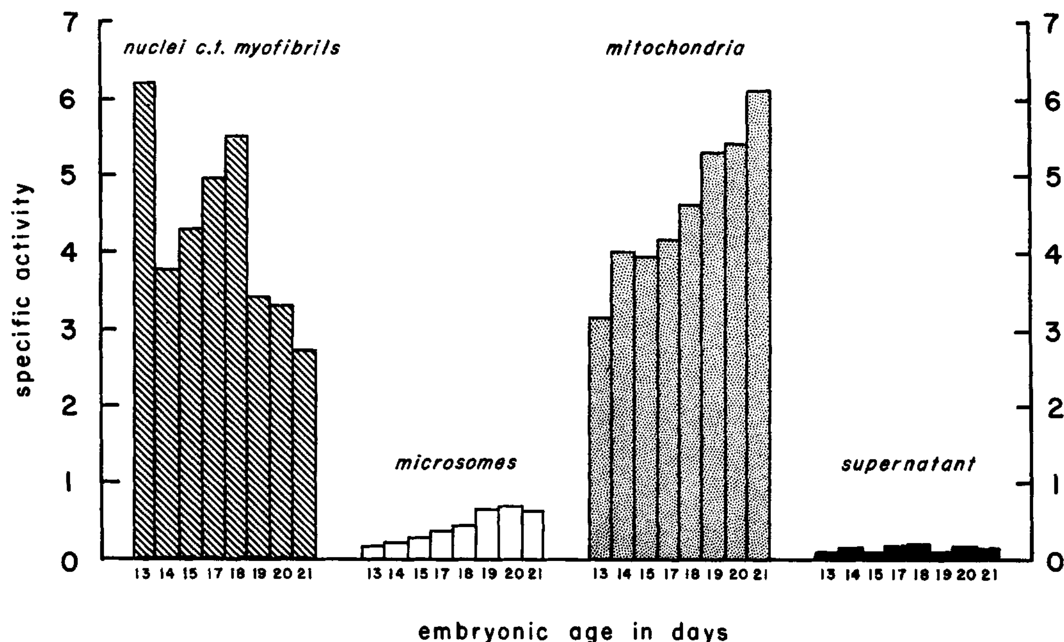


FIGURE 3 The intracellular distribution of calcium among the subcellular fractions of muscles from normal embryos. Each bar represents a pooling of 4 to 8 embryos, depending on the age. Specific activity is expressed as CPM mg N of the fraction divided by the CPM mg N of the pooled hearts. Kjeldahl nitrogen analysis on each fraction was done in duplicate or triplicate; CPM were determined on sample weights from 0.1 to 1 mg for each fraction. The high SA of a 13-day embryo of *Fraction I* (nuclei, connective tissue, myofibrils) is associated with nuclei; the second peak between day 14 and 19, with connective tissue proliferation; and the low SA from day 19 to 21, with increased myofibrillar synthesis serving to dilute the other structures containing calcium. The increases in the SA of the mitochondrial and microsomal fractions (*Fractions II* and *III*) are associated with increased energy systems and functioning myofibrils (see text). The supernatant (*Fraction IV*) contained less than 0.5 per cent of the total muscle calcium in experiments of all the age groups studied.

The use of oxalate in the incubating media of sarcoplasmic reticulum fragments (19, 22) results in increases in the uptake of calcium by these structures either by facilitating transport or by precipitating calcium in the tubules (22). Oxalate added to the medium in the present study would presumably enter the microsomal fragments and precipitate the calcium *in situ*.

Slater and Cleland (25) have reported on investigations indicating that during homogenization the sarcosomes (mitochondria) of rat heart muscle bind all the calcium in the tissue, even when only 30 per cent of the total sarcosomes are released into the medium. They concluded that *in vivo* all the calcium is found in the sarcoplasm or extracellular spaces, and that it is bound by the sarcosomes only *in vitro*. The fact that studies *in vitro* show that 70 per cent of the calcium in heart ventricles is rapidly exchanged for Ca^{45} in the bathing medium compared to 20 per cent in skeletal muscle under similar conditions (26) indicates that calcium in the heart is more loosely bound. In none of the experiments reported in the present paper was all of the tissue calcium in the mitochondria (see Figs. 3 to 5), but, instead, the calcium was distributed among the cell fractions in amounts depending upon the developmental stage or physiological activity of the muscle. Further differences between dystrophic and normal tissue were also observed.

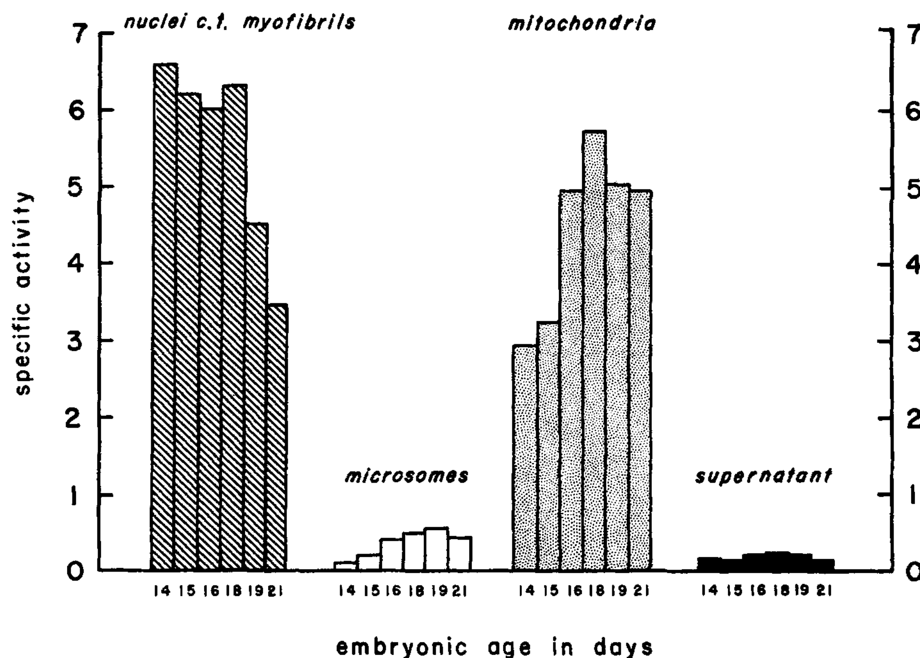


FIGURE 4 The intracellular distribution of calcium in the breast muscle of dystrophic chicken embryos. Each bar represents a pooling of 4 to 8 embryos, depending on the age. Specific activity is expressed as CPM mg N of the fraction divided by the CPM mg N of the pooled hearts. Kjeldahl nitrogen analysis on each fraction was done in duplicate or triplicate; CPM were determined on sample weights from 0.1 to 1 mg for each fraction. For discussion, see text.

Since activities of the cell are greatly retarded at low temperatures, homogenization followed by the isolation of fractions was done at approximately 0°C. Studies of calcium uptake by isolated kidney mitochondria incubated *in vitro* in media which seem to afford optimum conditions for the uptake of calcium indicate that at 0°C the level of calcium in the mitochondria remains relatively constant. When the incubation temperature was raised from 0° to 30°C, the mitochondrial calcium increased about fourfold (21). These experiments emphasize that at low temperatures the mobility of calcium, as well as metabolic activities of the cell, is reduced.

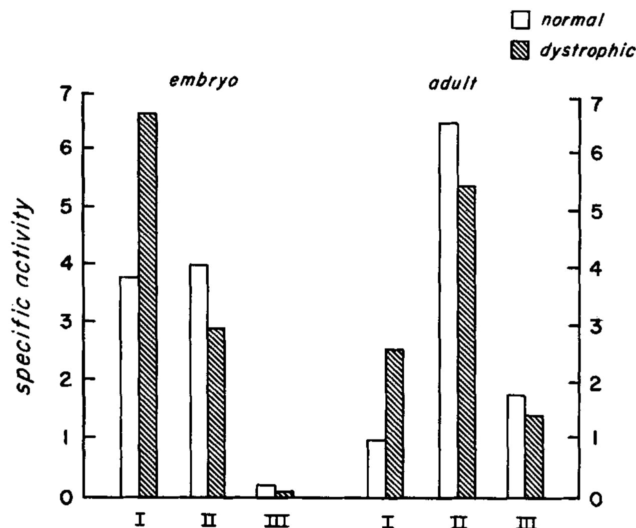


FIGURE 5 The figure contrasts the intracellular distribution of calcium in breast muscles of normal chickens with that of dystrophic chickens for the embryonic and adult animals. Specific activity (SA) is expressed as CPM mg N in the embryo, and CPM mg DW in the adult. Owing to the decreased radioactivity of adult tissue, samples to be analyzed for radioactivity were ashed at 550°C. The shaded bars refer to the dystrophic animals, and the plain bars, to the normal animals. The 14-day embryos were selected for comparison since it is at this stage that differences between normal and dystrophic tissues are first apparent. The adult series represents the SA of tissues from experiments of 26- to 34-day animals (5 to 6 experiments, normal and dystrophic), since a leveling off of SA was seen during this time. Roman numerals refer to the fractions: nuclei, connective tissue, myofibrils constitute *Fraction I*; mitochondria, *Fraction II*; microsomes, *Fraction III*. The graph emphasizes the intracellular distribution of calcium in a muscle which is not performing physiological work (14-day embryo) and in one which is functional as an adult tissue. In the adult, the SA of the mitochondria (*Fraction II*) is more than 3 times greater than the SA of the microsomes (*Fraction III*). In the dystrophic animal, the SA of the mitochondria and the microsomes is lower than corresponding values in the normal tissue, and the SA of *Fraction I* is higher.

Reports of part of this work have been given elsewhere (27, 28).

The author thanks Dr. Anthony Soldo for his discussions and criticisms and Miss R. Brown and Mr. E. Durham for technical assistance.

This work was supported by a grant from Muscular Dystrophy Associations of America, Inc.

Received for publication, January 15, 1964.

REFERENCES

1. COSMOS, E., Factors influencing movement of calcium in vertebrate striated muscle, *Am. J. Physiol.*, 1958, **195**, 705.
2. COSMOS, E., Autoradiography of Ca^{45} in ashed sections of frog skeletal muscle, *Anal. Biochem.*, 1962, **3**, 90.
3. ANDERSON, N. G., Techniques for the mass isolation of cellular components, *in* Physical Techniques in Biological Research, (G. Oster and A. W. Pollister, editors), New York, Academic Press, Inc., 1956, **3**, 299.
4. ROBINSON, D. S., Changes in the protein composition of chick muscle during development, *Biochem. J.*, 1952, **52**, 621.
5. ASMUNDSON, V. S., and JULIAN, L. M., Inherited muscle abnormality in the domestic fowl, *J. Heredity*, 1956, **47**, 248.
6. HARMAN, J. W., and FEIGELSON, M., Studies on mitochondria. V. The relationship of structure and oxidative phosphorylation in mitochondria of heart muscle, *Exp. Cell Research*, 1952, **3**, 509.
7. DICKERSON, J. W. I., and WIDDOWSON, E. M., Chemical changes in skeletal muscle during development, *Biochem. J.*, 1960, **74**, 247.
8. WITTER, R. F., WATSON, M. L., and COTTONE, M. A., Morphology and ATP-ase of isolated

mitochondria, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 127.

9. Lilienthal, J. L., Jr., Zierler, K. L., Folk, B. P., Buka, R., and Riley, M. J., A reference base and system for analysis of muscle constituents, *J. Biol. Chem.*, 1950, **182**, 501.
10. McKenzie, H. A., and Wallace, H. S., The Kjeldahl determination of nitrogen: A critical study of digestion conditions—temperature, catalyst and oxidizing agent, *Australian J. Chem.*, 1954, **7**, 55.
11. Robinson, D. S., Changes in the nucleoprotein content of chick muscle during development, *Biochem. J.*, 1952, **52**, 628.
12. Herrmann, H., Studies of muscle development, *Ann. New York Acad. Sc.*, 1952, **55**, 99.
13. Dickerson, J. W. T., The effect of growth on the composition of avian muscle, *Biochem. J.*, 1960, **75**, 33.
14. Csapo, A., and Herrmann, H., Quantitative changes in contractile proteins of chick skeletal muscle during and after embryonic development, *Am. J. Physiol.*, 1951, **165**, 701.
15. Robinson, D. S., A study of the adenosinetriphosphatase activity of developing chick muscle, *Biochem. J.*, 1952, **52**, 633.
16. Heilbrunn, L. V., and Wiercinski, F. J., The action of various cations on muscle protoplasm, *J. Cell. and Comp. Physiol.*, 1947, **29**, 15.
17. Niedergerke, R., Local muscular shortening by intracellularly applied calcium, *J. Physiol.*, 1955, **128**, 12P.
18. Bozler, E., Relaxation in extracted muscle fibers, *J. Gen. Physiol.*, 1954, **38**, 149.
19. Weber, A., Herz, R., and Reiss, I., On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum, *J. Gen. Physiol.*, 1963, **46**, 679.
20. Ebashi, S., and Lipmann, F., Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle, *J. Cell Biol.*, 1962, **14**, 389.
21. Vasington, F. D., and Murphy, J. V., Calcium uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation, *J. Biol. Chem.*, 1962, **237**, 2670.
22. Hasselbach, W., and Makinose, M., Die Calciumpumpe der "Erschlaffungsgrana" des Muskels und ihre Abhangigkeit von der ATP-Spaltung, *Biochem. Z.*, 1961, **333**, 518.
23. Carleton, B. H., Blair, H. A., and Latchford, W. B., Effects of the chlorides of potassium, calcium, and sodium on the α excitability of muscle, *J. Cell. and Comp. Physiol.*, 1938, **12**, 223.
24. Paul, D. H., The effects of calcium and magnesium on mammalian muscle fibres, *J. Physiol.*, 1960, **151**, 566.
25. Slater, E. C., and Cleland, K. W., The effect of calcium on the respiratory and phosphorylative activities of heart-muscle sarcosomes, *Biochem. J.*, 1953, **55**, 566.
26. Henrotte, J. G., Cosmos, E., and Fenn, W. O., Calcium exchange in isolated turtle ventricle, *Am. J. Physiol.*, 1960, **199**, 779.
27. Cosmos, E., Meetings of the Society of General Physiologists, Woods Hole, Massachusetts, 1963.
28. Cosmos, E., Second Conference on Muscular Dystrophies, Seton Hall, Jersey City, 1963.