Biochimica et Biophysica Acta, 691 (1982) 193–200
Elsevier Biomedical Press

BBA 71344

A SERIAL STUDY OF MUSCLE MICROSOMES DURING THE EARLY GROWTH OF GENETICALLY DYSTROPHIC CHICKENS

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(Received March 8th, 1982)

Key words: Muscular dystrophy; Microsome; Ca^{2+} uptake; ATPase; Protein distribution; Electron microscopy; (Chicken breast muscle)

Two lines of genetically involved and control chickens were compared with regard to the onset of muscle dystrophy during the early stages of growth ex ovo. Definite structural and functional involvement of pectoralis muscle developed within the first 4–5 weeks. In parallel experiments, microsomal membranes were obtained weekly from pectoralis muscle during the first 14 weeks ex ovo. The microsomes were studied with respect to ultrastructural features, protein composition, Ca^{2+} uptake and ATPase activity. Microsomal preparations obtained from all newborn chickens contain two types of vesicles: one type reveals an asymmetric distribution and 'high density' of particles on freeze-fracture faces which is characteristic of sarcoplasmic reticulum (SR) membrane; the other type reveals a symmetric distribution and 'low density' of particles. The yield of 'low density' microsomes from muscle of normal birds is very much reduced as the chicks grow from 1 to 4–5 weeks ex ovo. On the contrary, it remains high in chicks developing muscle dystrophy. Ca^{2+} uptake and coupled ATPase activity are found to be of nearly identical specific activity in control and genetically involved newborn chicks. The specific activity of the control birds, however, increases as the chicks grow from 1 to 4–5 weeks of age, while the specific activity of the dystrophic birds remains low. Such a difference appears to be related to the relative representation of sarcoplasmic reticulum and 'low density' vesicles in the microsomal preparations. It is concluded that failure to obtain a normal differentiation of muscle cell membranes is a basic defect noted in the early growth of genetically involved chickens. This defect appears along with the earliest signs of the dystrophic process.

Introduction

Alterations of sarcotubular membranes in the breast muscle of genetically dystrophic chickens have been shown by microscopic and biochemical studies on whole muscle and isolated microsomes [1–10]. These studies, however, were performed in 6–8-week-old chicks, at an age when the dystrophic process is fully established. Therefore, it is not apparent as yet what is the role of sarcotubular membrane alterations in the pathogenesis of the dystrophic process and muscle functional impairment. We have thus carried out a serial study to monitor the establishment of membrane alterations within the timetable of muscle involvement. These studies were rendered possible by the availability of control and dystrophic chickens differing only with respect to a single gene that determines the dystrophic disease [11,12].

Material and Methods

Line 412 (control) and 413 (dystrophic) chickens were obtained from the University of California Davis, Department of Avian Sciences. Birds at the age of 1 to 14 weeks were used at one week

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Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*, *N*'-tetra-acetic acid.

intervals, at which time their gross muscle function was checked. Chickens were placed in a supine position and the number of times that they were able to right themselves out of ten trials (FN) was established. Following killing, the breast muscle was dissected, weighed and the appearance of dystrophic and control muscles was compared.

Microsomes were prepared from breast muscle according to the procedure described earlier [5,9]. Special attention was paid to keep the pH between 6.8 and 7.0 during the homogenization step, and to separate the microsomal fraction from floating or pelleted contaminants during the differential centrifugation. This was found to be very important especially after the centrifugation which followed the 40 min incubation with 0.6 M KCl, when a soft as well as a hard pellet were present at the bottom of the centrifuge tubes. Such a selective transfer of the supernatant microsomal fraction reduced the yield but greatly improved purity and reproducibility of the preparations. The final microsomal fraction was suspended in 10 mM Mops (pH 7.0) and 30 % sucrose, and assayed within one day from isolation.

Ca^{2+} uptake was measured at 25°C by isotopic distribution and filtration methods [13]. The reaction mixture contained 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$ and 0.2 mg protein/ml. The reaction was initiated by addition of 2 mM ATP and interrupted by filtration at serial times. The residual calcium in the filtrate was determined by scintillation counting. ATPase activity was measured at 37°C in a reaction mixture containing 0.1 mM $CaCl_2$, 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 0.1 mM EGTA and 0.0125 mg protein/ml.

Steady-state levels of phosphoenzyme intermediate were measured at 4°C. The reaction mixture contained 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.95 mM $CaCl_2$, 1 mM EGTA and 1.0 mg protein/ml. The reaction was started with 0.1 mM [γ-^{32}P]ATP and stopped after 10 s with acid. Pellets were washed four times, dissolved as described earlier [14], and aliquots were taken for scintillation counting and protein assay.

Rapid kinetic experiments on phosphoenzyme formation and P_i production in the transient state at 25°C, were carried out with the aid of a Dionex multimixing apparatus, as described previously [14]. The reaction mixture contained 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.25 mg protein/ml and 0.1 mM ATP. The reaction was quenched in 3.5% trichloroacetic acid and 0.1 mM cold P_i.

Electrophoretic protein patterns of microsomes prepared from breast muscle of chicken at different developmental stages were obtained by polyacrylamide gel electrophoresis in the presence of SDS, according to the method described by Weber and Osborn [15].

Freeze-fracture replicas were stained as described previously [5], and examined with a Philips 200 electron microscope.

Results

Development of the disease

A preliminary objective of our studies was to establish a time sequence for development of functional signs of muscle dystrophy in the particular line of chickens used for our biochemical and ultrastructural observations. To this effect, the most practical evaluation of breast muscle involvement is the 'exhaustion score' test. This test consists of placing birds in the supine position ten times, and counting the number of times that they are able to right themselves. The validity of this test was established in large populations of dystrophic chickens exhibiting a score of nearly zero,

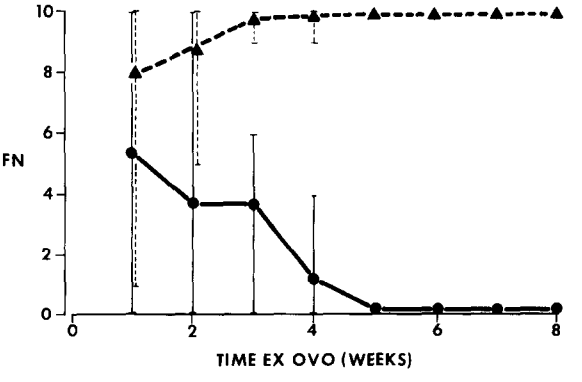

Fig. 1. Righting ability of developing chickens (FN = number of times that the chickens are able to right themselves up out of ten). Line 413 dystrophic (●———●) chickens were compared to line 412 control (▲———▲) chickens. 15–30 birds were tested per each point.

whereas a score of ten was obtained in normal controls [12,16].

We submitted all birds used in our studies to the 'exhaustion score' test, including 15–30 birds for each week up to 14 weeks and for each line of genetically involved (line 413) and normal (line 412) chicks. We found that the righting ability was in all chicks less than optimal (6–8/10) during the first week ex ovo, and deteriorated progressively in the dystrophic line to reach 0/10 after 5 weeks ex ovo, whereas it improved in the normal line to reach 10/10 after 3 weeks ex ovo (Fig. 1).

Involvement of the pectoralis muscle during the first 4–5 weeks was further revealed by macroscopic enlargement of the muscle. Furthermore, ultrastructural observations on fixed and sectioned samples showed a progressive appearance of alterations in the sarcotubular system and myofibrillar disorganization.

It became clear that a meaningful role of intracellular membrane alterations with respect to the pathogenesis of the dystrophic process must be established within the first 4–5 weeks ex ovo.

Yield of microsomal protein

In comparative protein determinations we found that the highest yield of microsomal proteins was obtained from 1-week-old chicks, while a sharp reduction of yield was observed as the birds grew during the first four weeks. We attribute such a reduction mostly to development of myofibrillar protein and increase in the ratio of myofibrillar to membrane proteins. The yield underwent a slower reduction with age after the first four weeks and up to fourteen weeks. At all ages the yield of microsomal protein was somewhat higher from dystrophic than from control birds.

Protein disposition in microsomal membranes

Microsomal preparations of sarcoplasmic reticulum membrane obtained from adult skeletal muscle possess an extraordinary specificity in their protein composition inasmuch as 70–80% of the total protein is accounted for by a component migrating as a 100000 mol. wt. polypeptide chain in SDS gels [17]. This component corresponds to the Ca^{2+}-dependent ATPase first purified by MacLennan [18,19], and its typical disposition in the membrane is revealed by particles appearing on the concave (cytoplasmic) freeze-fracture faces of sarcoplasmic reticulum microsomes with a density higher than $2000/\mu m^2$ [20–22]. Due to the large number of particles visible on the concave faces (while the convex faces are almost totally deprived of particles), we refer to these vesicles as having a 'high density and asymmetrical disposition of particles'. Since there are no particles on the convex faces, the total density of particles per membrane area corresponds to that observed on the concave faces. Such a disposition of particles is typical of the sarcoplasmic reticulum membrane as visualized in muscle fibers [5,23].

When we proceeded to characterize the pattern of particle distribution on freeze-fracture faces of microsomal membranes obtained from chicken pectoralis muscle as a function of development, we found that soon after hatching (1–2 weeks) the microsomes of both genetically involved and control chicks included a large population of vesicles with particle density lower than $1500/\mu m^2$ (Figs. 2 and 3). In these vesicles, an approximately equal particle density was found on both concave and convex freeze-fracture faces. We refer to these vesicles as having a 'low density and symmetrical disposition of particles'. In 'symmetrical' vesicles the total density of particles in the membrane was the sum of the densities found on the concave and convex faces.

It is clear from Figs. 2 and 3, that during the first four weeks of growth after hatching, the fraction of vesicles with a 'low density and symmetrical disposition of particles' was substantially reduced in favor of the fraction of vesicles with a 'high density and asymmetrical disposition of particles'. However, this change occurred to a much lesser extent in the dystrophic birds (Fig. 3). The electron microscopic pictures revealed the obvious difference between high-density and low-density particle distributions on concave and convex freeze-fracture of control, as compared to dystrophic sarcoplasmic reticulum vesicles. Quantitative determinations were better revealed by statistical evaluation in Fig. 3. and in Table I as shown below.

It should be noted that independent of age and dystrophic involvement, electrophoretic analysis of solubilized microsomal proteins revealed nearly identical patterns, with the 100000 mol. wt. ATPase

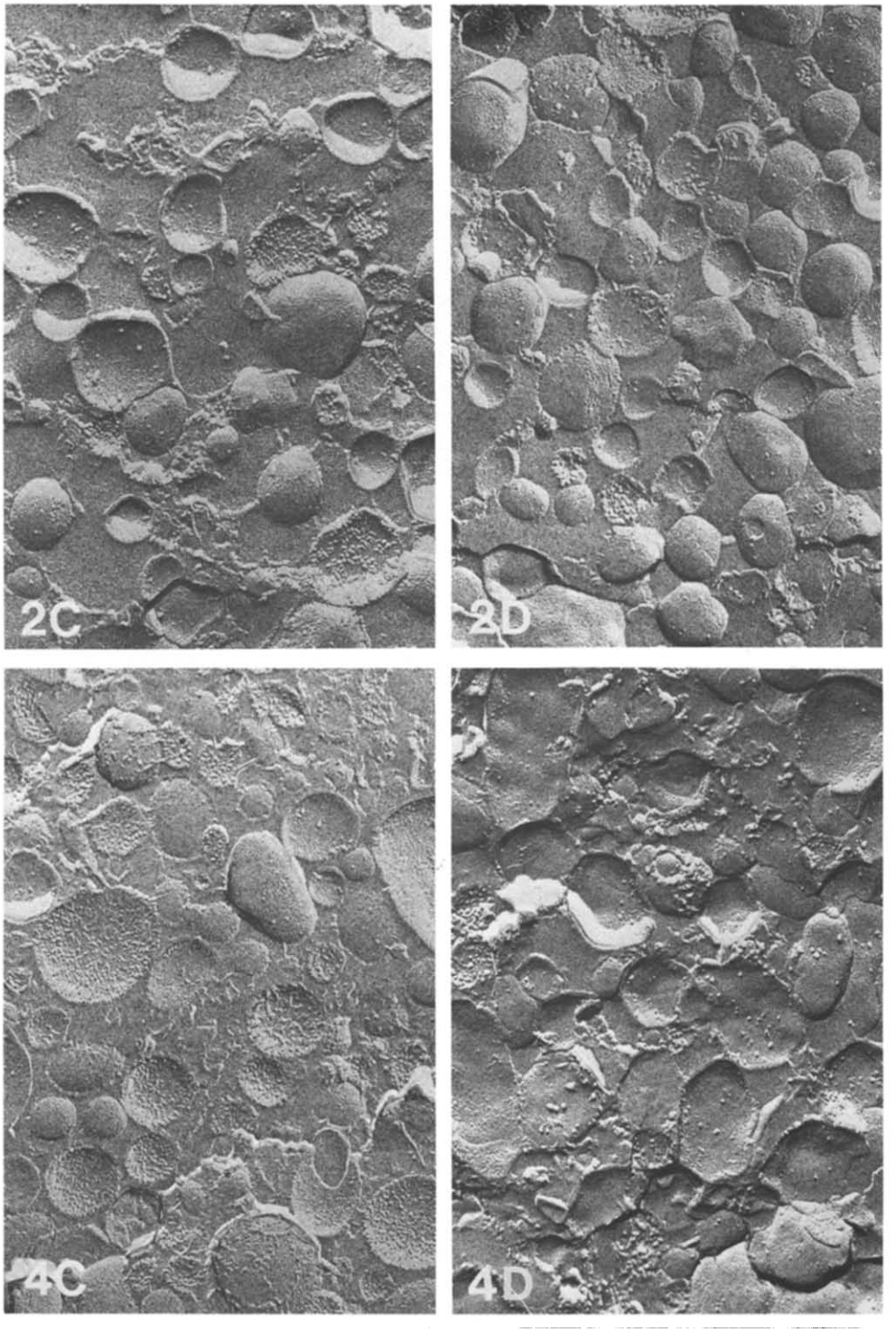

Fig. 2. Freeze-fracture replicas of microsomes from dystrophic (D) and control (C) chicken breast muscle at age 2 and 4 weeks, respectively. All micrographs shown at ×80000.

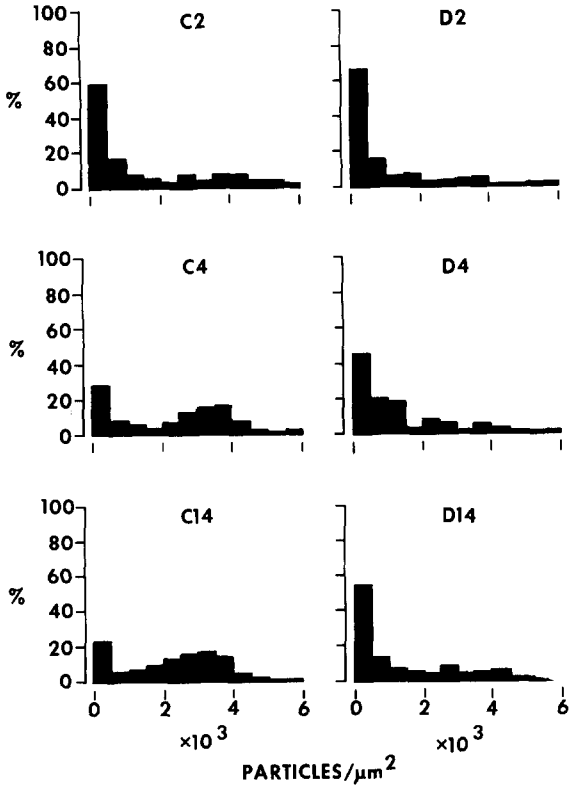

Fig. 3. Distribution of concave fracture faces observed in sarcoplasmic reticulum preparations from 2-4- and 14-week-old dystrophic (D) and control (C) chickens muscle. Particle densities were estimated from approx. 1500 fracture faces.

TABLE I

MICROSOMAL PROTEIN YIELD FOR DYSTROPHIC AND CONTROL CHICKEN MUSCLE

Microsomal yield, in reference to wet weight of muscle, is based on protein determinations (Folin method) on microsomal preparations, and on the distribution of protein particles in low and high density vesicles (see text). LS, 'low density' embryonic membranes; HA, sarcoplasmic reticulum membranes. D, dystrophic; C, control.

Age (weeks of ex ovo)	Yield of microsomal protein (mg/g muscle)	
	LS	HA
2 D	0.67±0.05	0.34±0.03
C	0.41±0.04	0.31±0.03
4 D	0.24±0.03	0.19±0.03
C	0.04±0.01	0.25±0.03
14 D	0.14±0.01	0.25±0.02
C	0.02±0.01	0.15±0.04

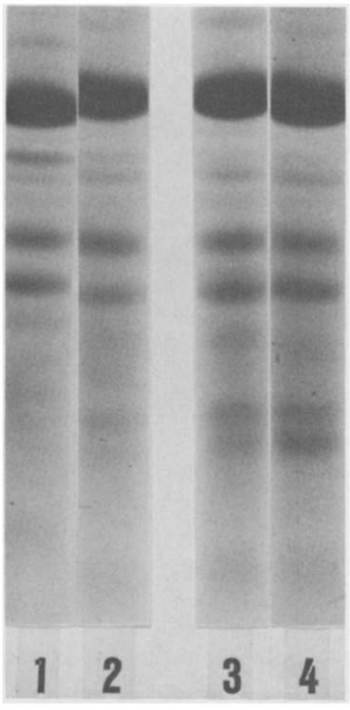

Fig. 4. SDS-polyacrylamide gel electrophoresis patterns of sarcoplasmic reticulum isolated from chicken breast muscle. 1 and 2: dystrophic; 3 and 4: control; 1 and 3: 2-week-old; 2 and 4: 4-week-old chickens.

band accounting for more than 80% of the total protein (Fig. 4). Therefore, it was apparent that this enzyme was the main protein component in the membrane fractions obtained in our studies, independent of pattern of particles disposition in freeze fracture faces.

The specific yields (Y) of protein associated with membranes exhibiting a low-density symmetric pattern (LS) or a high-density asymmetric pattern (HA) can by approximated by:

$$Y_{LS} = \frac{N_{LS}}{N_{tot}} \cdot Y_{tot},$$

and

$$Y_{HA} = \frac{N_{HA}}{N_{tot}} \cdot Y_{tot},$$

where Y_{tot} is the total yield obtained by protein determinations on the microsomal preparations, and N is the number of particles per unit mem-

brane area obtained by multiplying density (D) by the fraction of vesicles (F) exhibiting a specific pattern, and assuming that the average area of vesicles in various fractions is approximately equal. In this case:

$$N_{LS} = \sum_{D_1}^{D_{1500}} D \cdot F \cdot 2$$

(particles present on both concave and convex faces),

$$N_{HA} = \sum_{D_{1500}}^{D\max} D \cdot F$$

(particles present only on concave faces)

where D_1, D_{1500}, and D_{max} indicate the densities of particles on the fracture faces. D_1 to D_{1500} and D_{1500} to D_{max} ($D_{max} = 6000$ particles/μm^2) refer to LS or HA membranes, respectively.

Although such estimates of specific yield include gross approximations, they still reveal obvious features of the enzyme distribution in the two systems, which are useful in the interpretation of functional findings. It is clear (Table I) that the yield of protein associated with LS (low density) membrane is approx. 60% of the total microsomal protein in preparations obtained from 2-week-old chicks both in control and dystrophic lines. The yield of protein associated with LS membrane then falls rapidly with age in the control line reaching a value of approx. 15% of the total microsomal protein in 4-week-old chicks. On the contrary, a value of 55% is still found in dystrophic chicks of the same age.

Calcium uptake and ATPase activity

Examples of ATP dependent calcium uptake by microsomes obtained from chicken pectoralis muscle are given in Fig. 5. Generally, the maximal level of calcium uptake (in the absence of oxalate), was approximately the same in control and dystrophic microsomes obtained from 1-week-old chicks. However, when the microsomes were obtained from chicks aged 4 weeks or older, the specific activity of the control microsomes increased approximately 30%. This change is in agreement with the relative increase in yield of vesicles with dense and asymmetric disposition of

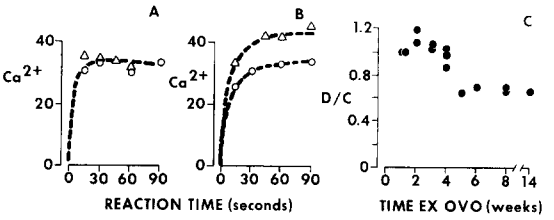

Fig. 5. (A) and (B). Ca^{2+} uptake by microsomes obtained from 1 week (A) and 5 week (B) old chickens, expressed in nmol per mg protein. Ca^{2+} uptake was initiated by addition of ATP in the presence of $^{45}CaCl_2$ and the reaction was stopped by filtration. The filtrate was collected for scintillation counting. The reaction mixture contained: 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 0.05 mM $CaCl_2$ and 0.2 mg protein/ml. (C) Ratios of maximal Ca^{2+} uptake levels in dystrophic (D) and control (C) chickens.

particles (Fig. 3 and Table I), e.g. vesicles of sarcoplasmic reticulum membrane properties. A clear difference between the calcium uptake capacity of control and dystrophic microsomes becomes apparent in preparations obtained from 3–4-week-old chicks (Fig. 5C).

It is known that ATP utilization for calcium uptake by sarcoplasmic reticulum vesicles, includes formation of a phosphorylated enzyme intermediate by transfer of the ATP terminal phosphate onto the enzyme protein, followed by hydrolytic cleavage of the intermediate and release of inorganic phosphate. Examples of phosphoenzyme

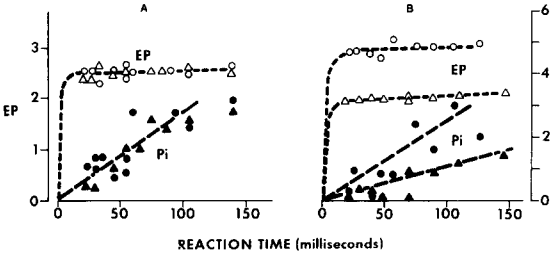

Fig. 6. Initial velocity of phosphoenzyme formation and P_i production. Phosphoenzyme intermediate (EP) formation (open symbols) and P_i production (closed symbols) were followed by starting the reaction with [$\gamma - ^{32}$P]ATP. The reaction mixture contained: 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM $CaCl_2$, and 0.25 mg protein/ml. The reaction was quenched with acid. Microsomes from dystrophic (△, ▲) and control (○, ●) muscle of 1 (A) and 5(B) week old chickens were used. The phosphoenzyme formed and P_i produced were expressed in nmol per mg protein.

levels and velocities of P_i release by chicken microsomes are shown in Fig. 6. Here again, we found that these parameters were nearly identical in control or dystrophic microsomes obtained from 1-week-old chicks. However, the specific activity of control microsomes increased as the chicks developed to ages of 4 weeks and older. At this stage, the phosphoenzyme levels of control microsomes were approx. 30–40% higher than those of dystrophic microsomes. The velocity of P_i production was proportionally higher indicating that, in fact, the enzyme turnover was identical. Similar relationships were observed when phosphoenzyme levels were measured following 15-s incubation in ice with saturating ATP (Table II). It is apparent then, that the lower activity of microsomes obtained from newborn (control and dystrophic) chicks, and dystrophic chicks of all ages is due to the presence of protein that does not participate in the reaction.

In addition to the Ca^{2+}-dependent ATPase, the chicken muscle microsomes contained a rather high Ca^{2+}-independent ATPase activity. The maximal velocity of this enzyme was approx. 1 μmol/mg protein per min (37°C), and did not change significantly with age or in relation to dystrophic involvement. ATP utilization by this mechanism was not accompanied by accumulation of measurable levels of phosphorylated enzyme intermediate.

TABLE II

STEADY-STATE LEVELS OF PHOSPHOENZYME

Phosphoenzyme intermediate (EP) levels were measured at 4°C. Reaction was started with [γ-^{32}P]ATP and after 10 s stopped by acid. The reaction mixture contained: 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM ATP, 0.95 mM $CaCl_2$, 1 mM EGTA and 1.0 mg protein/ml. The level of EP formed when $CaCl_2$ was omitted did not exceed 2%. Values are means ± S.E. (n = 3–4 at each age).

Age (week ex ovo)	nmol EP per mg protein	
	Dystrophic	Control
2	3.10±0.30	2.85±0.10
3	2.90±0.25	2.97±0.25
4	2.45±0.30	3.25±0.30
5–14	2.00±0.20	2.83±0.35

Discussion

Previous reports on abnormalities of sarcotubular membranes in the breast muscle of dystrophic chickens, have raised the question of whether such abnormalities are the primary cause of muscle dysfunction in the genetic disease. We have attempted to answer this question by monitoring membrane alterations and impairment of muscle function during early development. Chickens are a convenient model for this purpose, inasmuch as the functional development of sarcoplasmic reticulum membrane [24,25] as well the establishment of the dystrophic process (Fig. 1) are not fully completed until several days after hatching.

A distinctive feature of microsomal preparations obtained from chicken breast muscle (when compared to rabbit skeletal muscle), is the presence of a large number of vesicles with 'low density and symmetrical disposition' of particles, in addition to vesicles displaying structural and functional characteristics of sarcoplasmic reticulum membrane. The 'low density' vesicles were separated [5] from the sarcoplasmic reticulum vesicles, and tentatively identified with T-tubule membranes on the basis of their appearance in freeze-fracture preparations. However, their main protein component displays an electrophoretic mobility identical to that of sarcoplasmic reticulum ATPase (Fig. 4). Furthermore, no significant (Na^+K^+)-ATPase activity is associated with the 'low density' vesicles. On the contrary, heterogeneous electrophoretic patterns and significant $(Na^+ + K^+)$-ATPase acitivity have been attributed to tubular preparations by independent studies [26–28]. At any rate, considering that the yield of 'low density' vesicles is maximal soon after hatching (approx. 60% of the total protein yield), and is very much reduced (approx. 10% of the total microsomal protein) within 4–5 weeks after hatching, it is reasonable to attribute an embryonic character to these membranes. With respect to the dystrophic disease, a significant finding is that the yield of 'low density' microsomes is higher in genetically involved chicks in the early postnatal development, and is not reduced in parallel with the control birds in later stages of development. Even 14 weeks after hatching, the yield of 'low density' microsomes corresponds to approx. 50% of the

total microsomal protein from dystrophic birds, while it is reduced to approx. 15% in the controls. It is noteworthy that differences of protein yield become very large when expressed in terms of membrane area, owing to the much lower density of protein assembly in the embryonic, as compared to the sarcoplasmic reticulum membranes.

Studies of calcium transport, enzyme phosphorylation and ATPase activity indicate that, in general, the specific activity of microsomes obtained from chickens is somewhat lower than that of rabbit or lobster muscle microsomes which are most often used for this type of studies. The specific activity of the chicken microsomes is even lower in the early stages of development and in the dystrophic chickens. In all cases, the lower specific activity can be accounted for by the presence of the 'low density' microsomes, assuming that their protein is inactive.

In dystrophic birds the specific activity of sarcoplasmic reticulum vesicles remains approximately the same from the 1st to the 14th week following hatching, while muscle performance deteriorates dramatically within that period of time. Therefore, alterations of calcium uptake by sarcoplasmic reticulum are not the primary cause of muscle dysfunction. On the other hand, a feature that differentiates control and genetically involved chicks during the early development is the failure of the genetically involved chicks to undergo a comparable reduction in the yield of 'low density' or 'embryonic' membranes from their muscle cells. This difference develops in parallel with the manifestation of muscle dysfunction. It is possible that a part of these 'low density' membranes derives from swollen and deranged T-tubulus structures, and the muscle functional impairment may be partly ascribed to a defect in excitation-contraction coupling. More generally however, our studies indicate a basic defect in membrane development. This defect may interfere with cellular homeostasis, permitting development of degenerative phenomena, and breakdown of the fiber [29,30].

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