Hereditary Muscular Hypertrophy in the Bovine
I. Histological and Biochemical Characterization* (34257)

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Muscle hypertrophy (double muscling) is an inherited disorder of skeletal muscle growth and development in domestic cattle. The condition is widespread among herds of several European countries (1) and apparently first appeared in the United States in the early 1930's (2).

Although the inheritance of the character has not been firmly established, most studies indicate it to be the result of a single gene pair with the phenotype of the heterozygote being variably intermediate between the alternative homozygous conditions (3). The phenotype of the homozygous mutant is best described as a generalized hypertrophy of the skeletal musculature. Although other organ systems appear to be affected also, these effects may be secondary to the muscular involvement. Death of calves and of cows is common at parturition due to dystocia, this problem being most evident in the extreme phenotypes. Most homozygous animals are born with some evidence of hypertrophy. Immediately after birth, gross hypertrophy is rapid and progressive for several weeks, but then declines in rate until mature body weight is reached (3).

Muscle from these animals has considerably less intramuscular fat than normal muscle, and the hypertrophied animal produces considerably more meat protein on a body weight basis (4–6). In light of the voluminous evidence that an excess of saturated animal fats in the diet is in part responsible for a variety of cardiovascular diseases, animals manifesting this condition may serve as an important dietary protein source with a lower percentage of animal fat than is presently available in normal beef.

Although this mutant has been recognized for many years, few definitive studies have been done at the cellular level; thus, little is known about the biochemistry or physiology of the trait. Available reports do suggest however that the trait does have several characteristics which would make it desirable as an experimental model for studies in muscle growth and development.

In the present studies, animals from a herd of "double-muscled" animals established for genetic studies were used to define some of these histological and biochemical parameters with respect to normal, heterozygous and homozygous hypertrophied animals.

Materials and Methods. Biopsies were performed on three 9-months-old calves from each of the normal, heterozygous and homozygous genotypes. The semitendinosus and triceps brachii were biopsied on each side of each animal. One piece was subjected to histological and histochemical examination while the other was analyzed for protein, DNA, and RNA concentration. At 12 months of age, one animal from each genotype was sacrificed, and the musculature was grossly examined.

One part of the tissue removed for histological examination was fixed in 10% neutral formalin and the other was immediately frozen in a dry ice–acetone mixture. The formalin-fixed pieces were cross-sectioned and stained with H. & E. The frozen sections were cross-sectioned in a cryostat at —20° and incubated for determination of succinic dehydrogenase activity according to the method of Pearse (7).

The other biopsy sample was homogenized in 19 vol of 0.14 *N* KCl and aliquots were

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withdrawn for protein determinations by the method of Lowry *et al.* (8) after incubation with 0.3 *N* KOH at 37° for 30 min. RNA and DNA were determined by the method of Munro and Fleck (9).

Blood samples were withdrawn from 12 homozygous hypertrophied animals and from 13 normal animals at approximately 1 year of age. The plasma was collected by centrifugation and assayed for creatine phosphokinase (CPK) activity. Preliminary experiments showed no significant difference in activity in plasma when compared to serum. The enzyme was assayed colorimetrically according to the following scheme (Sigma):

$$\text{ADP} + \text{phosphocreatine} \xrightarrow{\text{CPK}} \text{ATP} + \text{creatine}, \quad (1)$$

$$\text{Creatine} + \alpha\text{-naphthol} + \text{diacetyl} \longrightarrow \text{color complex}. \quad (2)$$

Extinction was read at 520 mμ and results were expressed as millimicromoles of creatine phosphorylated per minute at 25°.

Results and Discussion. A. Histological. Examination of the H. & E. sections disclosed no apparent differences between the muscle fibers of the hypertrophied and normal animals. In the hypertrophied condition, no signs of structural abnormalities were seen. All fibers were polygonal in shape and included a wide range of diameters. There appeared to be no abnormally large or small fiber populations. However, since the cross-sectional area of the fibers was not accurately determined for the purpose of plotting a population distribution, it is possible that there was a shift in mean fiber size within the normal range. Nuclei were peripherally located. There was no indication of increased numbers of nuclei in the hypertrophied fibers as seen in cross-section nor did there appear to be any difference in nuclear morphology.

Fat was essentially absent between the fiber bundles of the hypertrophied muscle whereas there was considerable deposition between the bundles of the fibers from normal animals. No differences in connective tissue content were noted. From the biopsies it was not possible to determine if hyperplasia is responsible for the generalized hypertrophy. However, this histological study does allow us to conclude that no degenerative changes of the hypertrophied muscle are evident up to 12 months of age. This is in agreement with the report of Kidwell *et al.* (10). Increased numbers of smaller fibers which might have indicated a continuing formation of new fibers were not seen. The only alteration observed was the difference in fat deposition between the fiber bundles referred to above.

B. Histochemical. Most, if not all muscles, of cattle and other higher animals contain varying distributions of fiber types, classified according to their type of metabolism (11). Many systems have been devised to classify fibers with different degrees of activities of various metabolic pathways, but generally, two basic fiber types are recognized. Red fibers (type 1) have a relatively higher capacity for aerobic metabolism than white fibers (type 2), higher concentrations of myoglobin, an increased vascular supply, and a lower capacity for glycolysis (12). They have slower contraction speeds but are capable of more sustained contractile activity (11), presumably due to the greater capacity to produce ATP via the oxidative pathway. Importantly here, red fibers, on the average have a considerably smaller cross-sectional area than do white fibers (13).

Much recent evidence, gathered from cross-innervation and re-innervation studies (14) shows that the basic type of metabolism of the muscle fiber is set by its motor neuron. If white (type 2) and red (type 1) fibers are cross-innervated by their respective neurons, the types of metabolism are reversed. The type of metabolism which each fiber has can be determined histochemically by localization of any of several enzymes (12). The most commonly used is succinic dehydrogenase, since it is tightly bound to the mitochondrion and provides an accurate estimation of the capacity for oxidative metabolism (15). The triceps in the normal beef animal contains a predominance of fibers with higher oxidative enzyme activities (red fibers) whereas the semitendinosus contains a majority of fibers with higher glycolytic activities (white fibers). In the histochemical comparison of hypertrophied muscle with normal muscle, it was obvious that all hypertrophied muscles contained far fewer red

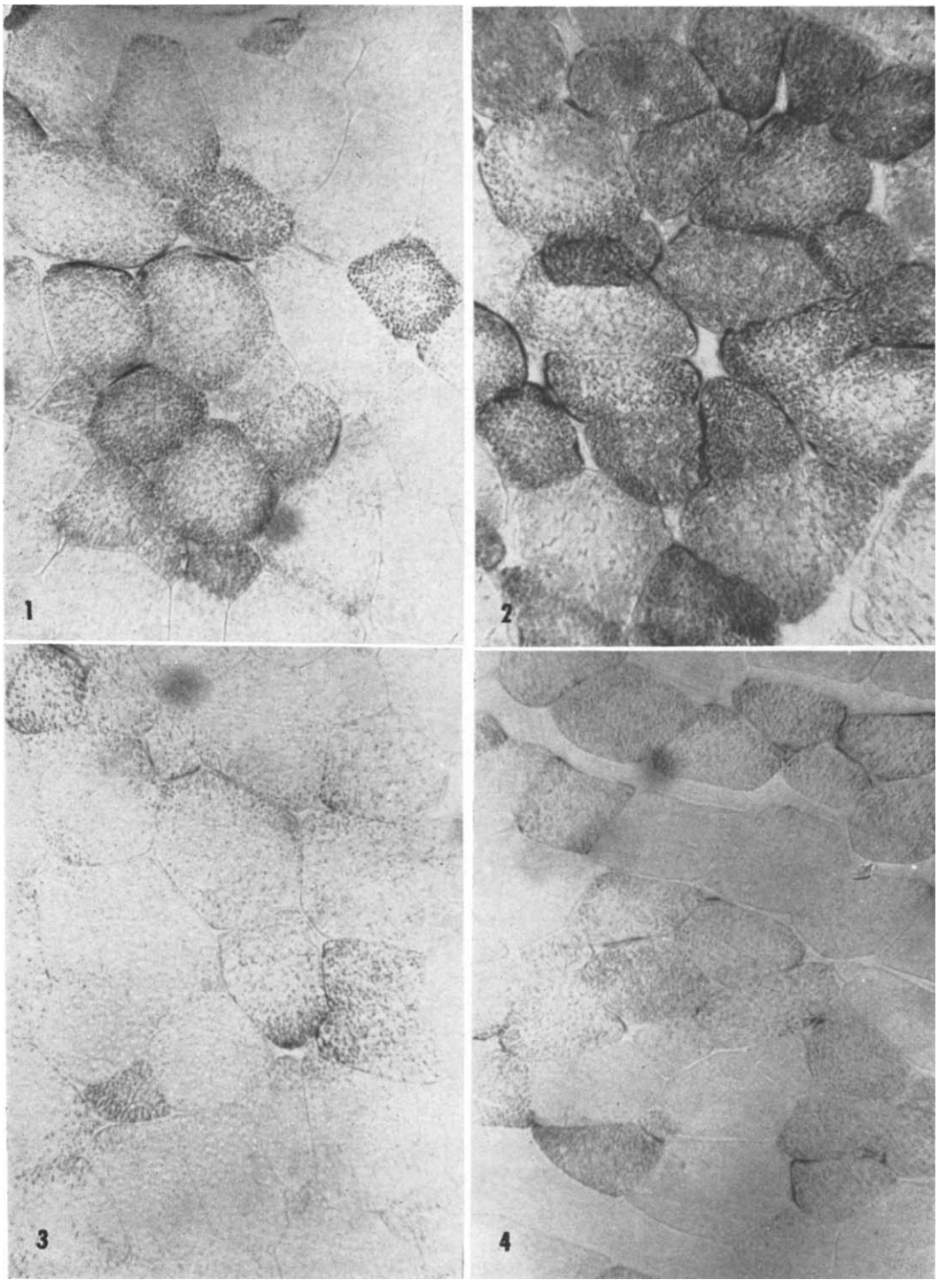

FIG. 1. Cross-section normal semitendinosus incubated for succinic dehydrogenase activity; ×400.

FIG. 2. Cross-section normal triceps incubated for succinic dehydrogenase activity; ×400.

FIG. 3. Cross-section hypertrophied semitendinosus incubated for succinic dehydrogenase activity; ×400.

FIG. 4. Cross-section hypertrophied triceps incubated for succinic dehydrogenase activity; ×400.

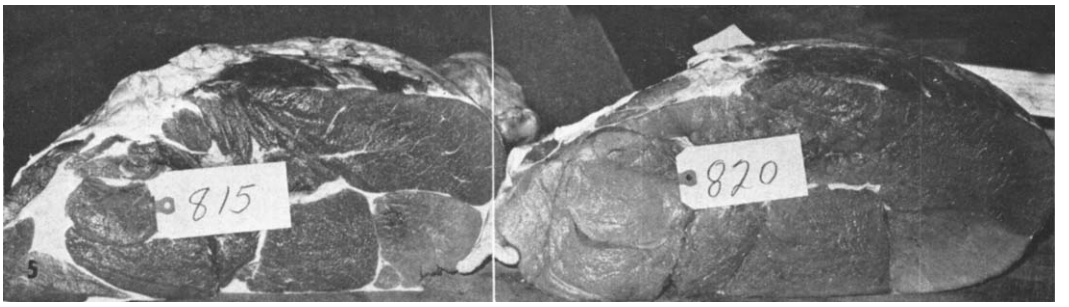

FIG. 5. Cross-cut through entire right hind leg. The normal leg (no. 815) shows considerable extra-, inter-, and intramuscular fat. All muscles in the hypertrophied leg (no. 820) are considerably lighter in color than the corresponding muscles of the normal leg.

fibers than did corresponding normal muscles (Figs. 1–4). In addition, red fibers in hypertrophied sections contained fewer mitochondria than the equivalent fibers in the normal sections. This was true of each muscle biopsied. This alteration again became obvious when the animals were slaughtered. All muscles of the hypertrophied animal were seen to be lighter in color than the corresponding muscles of the normal animal (Fig. 5). As a result of these observations, we suggest the cause of the generalized hypertrophy to be the result of an increase in proportion of glycolytic-type fibers in each muscle.

C. Biochemical. It has been demonstrated by a number of workers that the mean amount of DNA per nucleus of somatic cells is constant for individual species. DNA has been used, therefore, as a reference substance against which to measure changes in other cytoplasmic constituents and also as a measure of the number of nuclei present in a given organ and tissue. Total wet weight of the muscle divided by the total DNA content provides an index of cytoplasm/nucleus, since the DNA content per nucleus is constant at 2.5×10^{-9} mg (16). The "cytoplasm per nucleus" provides an index of the cell numbers and cell size and, when measured during growth, an index of the rate of cellular hyperplasia and hypertrophy. A difference in the cytoplasm per nucleus in multinucleated muscle cells could mean either differentially hypertrophied fibers, with a constant nuclei number or a differential rate of nuclei formation during growth. Moss (16) postulated that in chickens the cytoplasm per nucleus was constant throughout posthatching growth, while Robinson (17) showed in pigs, and Winnick and Noble (18) in rats, an increasing ratio of cytoplasm per nucleus during early growth indicating a relatively greater rise in cytoplasmic constituents relative to DNA as growth proceeded.

Table I presents the DNA, RNA, and protein concentrations of biopsies from 3 animals of each genotype. One animal of each genotype was sacrificed and the muscle weights were obtained (Table I). From these data total DNA, RNA, and protein values were calculated for the whole muscle. The concentrations of DNA, RNA, and protein appear to be the same in each of the 3 genotypes. Muscle weights, and the total DNA, RNA, and protein values were distinctly different. The consistency of the cytoplasm:DNA ratio, or the protein:DNA ratio imply that in development the muscles from the 3 genotypes are similar and that the hypertrophied muscles are not due to an increase in the cytoplasm per nucleus. The hypertrophied muscles contain more nuclei and more cytoplasm with the ratio remaining constant. Histochemical observation has indicated that there are more glycolytic-type fibers in the hypertrophic muscle.

Biochemical or mechanical injury to muscle tissue results in liberation of intracellular enzymes into the general circulation (19, 20). Because of its high specific activity in muscle as compared to other tissues, an increase in CPK in the serum has been used as a sensitive aid in the diagnosis of muscular

TABLE I. Nucleic Acid and Protein Values in Bovine Semitendinosus Muscle.

	Nonhypertrophied homozygous	Heterozygous	Hypertrophied homozygous
DNA (mg/100 g of wet tissue)[a]	25.4	26.4	27.0
RNA (mg/100 g of wet tissue)[a]	85.0	80.0	87.0
Protein (g/100 g of wet tissue)[a]	20.4	20.9	19.7
Muscle weight (g) corrected[b]	1078	1312	1534
Total DNA (mg)	272	346	414
Total RNA (mg)	916	1050	1334
Total protein (g)	220	274	341
Ratio weight:DNA (g/mg)	3.96	3.80	3.70
Ratio protein:DNA (g/mg)	0.81	0.79	0.82

[a] Each value represents the mean of 3 animals. The values obtained for the three genotypes were not significantly different.

[b] Value for 1 animal per group corrected for body weight.

dystrophy and other types of muscle disease (19, 21, 22).

No significant difference was found between serum CPK activities in the normal and hypertrophied animals used in this study (Table II). These results are consistent with those seen in the histological study, and with those of Kidwell *et al.* (10), indicating that muscular degeneration is not present in this syndrome, at least during the normal growth period. On the other hand, we have seen that in a nutritionally induced muscular dystrophy in calves, CPK activities increase 10–40 times those values reported here, with significant increases being apparent before degeneration is detected histologically (unpublished results). Despite the fact that activities were not significantly different in this study, there was a tendency for the values of the hypertrophied animals to be increased over those of normal animals. This is possibly due to the increased muscle mass of these animals relative to other body components. It could also be due to the fact that white muscle fibers have higher CPK activity than red muscle fibers (23).

The following observations are in accord with the hypothesis that this hypertrophied muscle condition is caused by a disproportionate number of white fibers developing during myogenesis. (i) There is no interfiber accumulation of other tissue or cell types which could account for the gross hypertrophy. In fact there is considerably less fat than in normal muscle (Fig. 5); (ii) whereas red fibers metabolize fatty acids as an energy source (12), white fibers are primarily glycolytic. Teleologically then, hypertrophied muscle should have considerably less demand for intramuscular fat. (iii) A population of abnormally large fibers was not seen, nor was there any evidence of pseudohypertrophy; (iv) there were no signs of degeneration and resulting cellular infiltration; (v) there was no change in DNA, RNA, protein:DNA or RNA:DNA ratios; (vi) newborn hypertrophied calves were observed to be less active than normal calves. This is possibly due to lower capacity to produce high energy intermediates; (vii) hypertrophied animals showed a reduced tendency to bleed at the time of injury, whereas the normal muscle bled profusely. This observation might be explained on the basis that white muscle has a lower capillary:fiber ratio than red muscle and a lower rate of blood flow (24). In addition, preliminary results of a

TABLE II. Serum Creatine Phosphokinase Activities in the Normal and Hereditary Hypertrophied Bovine.

Genotype	No. of animals	Mean[a]	Range[a]
Normal	13	11.7	0–41
Hypertrophy	12	16.8	0–34

[a] Values represent the number of millimicromoles of creatine phosphorylated/min at 25°; means are not significantly different.

histochemical localization of capillaries indicated that the vascular supply to hypertrophied muscle is lower than it is in normal muscle. Since the basic type of metabolism of a fiber is set by its innervation, it would seem that the disproportionate number of white-type fibers is determined during early myogenesis. As mentioned above, most of the mutant animals are hypertrophied at birth.

Normal neonatal muscle in the chick is more oxidative in nature, with glycolytic-type fibers developing subsequently (25). However, in the normal bovine, histochemical localization of succinic dehydrogenase indicated that the skeletal muscle acquires an increasing capacity for oxidative metabolism with age. This is further indicated by observing the "paleness" of muscle from young calves (veal) versus the "redness" of muscle from older animals (beef). In the chick, hereditary muscular dystrophy results in continued production of oxidative enzymes in neonatal muscle (25), therefore, seeming to maintain the embryonic state. Since the normal development of bovine skeletal muscle seems to be in opposite direction to that of the chicken, the increase in glycolytic-type muscle fibers may in this case also reflect a maintenance of the embryonic state. It is possible then that the processes of hereditary dystrophy and hereditary hypertrophy may be concerned with the same regulatory mechanisms.

The results of this study suggest that this mutant could well serve as an experimental animal for investigation of muscle growth and development, and perhaps for the investigation of neural control mechanisms. The processes of domestication and selection for large muscles in meat-producing animals and birds may be important mechanisms whereby genetic modifiers can accumulate to support a generalized increase in anaerobic-type muscle. In addition to the physiological importance of the muscle fiber types to the animal, they are important to the meat consumer. The differences in protein distribution must have an effect on the quality of the meat as well as its postmortem behavior. White-type muscle, because of its high glycolytic capacity, would be more susceptible to physical and biochemical stress than red-type muscle. Thus in continually selecting for large muscled animals, we may be inadvertently selecting for muscle which has different biochemical and physical characteristics.

Summary. Muscles from three normal calves and three calves with hereditary muscular hypertrophy were biopsied at nine months of age and assayed histochemically for succinic dehydrogenase activity, and histologically for evidence of muscular degeneration. In addition, the biopsy samples were analyzed for total DNA, RNA, and protein. Blood samples from 13 normal and 12 hypertrophied animals were taken at 12 months of age and assayed for creatine phosphokinase activity. Histologically, no degenerative changes were detected in hypertrophied animals. No significant differences were found in concentrations of DNA, RNA, protein, or in serum creatine phosphokinase activity between normal and hypertrophied animals. There was a significant decrease in succinic dehydrogenase activity of the hypertrophied muscle. There were fewer reacting fibers, and the activity was less in those fibers from hypertrophied muscle which did react for succinic dehydrogenase. It is concluded that degeneration is not a part of this syndrome, and that bovine hereditary muscle hypertrophy is associated with a disproportionate number of glycolytic-type fibers. Since these are, on the average, larger than oxidative-type fibers, this observation could explain the gross hypertrophy of the skeletal musculature in this mutant.

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