

## Morphological changes in skeletal muscles in vitamin E-deficient and refed rabbits\*

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1. Nutritional muscular dystrophy was induced in young rabbits by giving them a vitamin E-deficient diet. Dystrophic animals were rehabilitated by the addition of vitamin E to the diet. Controls were fed on a supplemented diet containing 50 mg DL- $\alpha$ -tocopheryl acetate/kg.

2. Muscle weight and fibre diameter were determined in seven skeletal muscles, from the fore-limb (biceps brachii), the trunk (pectoralis descendens), the hind-limb (gastrocnemius, semitendinosus, soleus, plantaris and tibialis cranialis). Muscle weights, except for those of the soleus and semitendinosus, were significantly reduced in vitamin E-deficient animals when compared with the control animals. A monophasic fibre diameter distribution was obtained for all muscles from all the groups studied. Distribution curves for vitamin E-deficient muscles showed a consistent decrease in fibre diameter. The extent of change in distribution varied among different muscles. On refeeding, the redistribution curve returned to the normal pattern in the biceps brachii, semitendinosus, gastrocnemius, plantaris, tibialis cranialis and pectoralis descendens but not in the soleus.

3. No significant changes in bone length or weight were detected in the humerus, femur and tibia in vitamin E-deficient rabbits compared to the control animals.

4. Transverse and longitudinal sections of the seven muscles were studied by light microscopy. In the vitamin E-deficient animals, typical hyaline degeneration was observed with much infiltration of collagen and lymphocytes. The extent of degeneration varied among muscles, the gastrocnemius being most affected and the semitendinosus least affected. Muscles in the rabbits refed vitamin E showed that the infiltration with collagen and lymphocytes had almost disappeared.

Nutritional muscular dystrophy (NMD) is similar to hereditary muscular dystrophy (HMD) in that both myopathies involve skeletal muscle wasting, creatinuria and increased activity of numerous blood enzymes (Schapira & Dreyfus, 1963). However, no damage to the nerve or motor end-plate occurs in NMD (Roger, Pappenheimer & Goettsch, 1931; Chor & Dolkart, 1939). Lesions in skeletal muscles constitute a universal observation in all laboratory animals that have been subjected to vitamin E depletion (Mason, 1973). Loss of body-weight and skeletal muscle weight were observed in NMD in rabbits fed on a vitamin E-deficient diet, the loss of structural components ultimately causing a loss of muscle function (Telford, 1971). Ultrastructural damage occurs in skeletal muscles in animals with NMD (Howes, Price & Blumberg, 1964; Van Vleet, Hall & Simon, 1967, 1968; Sweeny, Buchanan-Smith, de Mille, Pettit & Moran, 1972; Olson, 1974). No studies have been reported on changes in the distribution of the diameter of the muscle fibres induced by the atrophy produced in NMD. In contrast, the atrophy of skeletal muscles in HMD produces a biphasic distribution of fibre diameters. This distribution indicates that some muscle fibres hypertrophy to compensate for the degradation of others (Rowe & Goldspink, 1969; Shafiq, Gorycki & Milhorat, 1969).

This study was undertaken first to determine if the skeletal muscle atrophy of NMD produced distribution curves for muscle fibre diameter similar to those obtained for HMD. Secondly, the effect of vitamin E deficiency on various skeletal muscles throughout the body was measured to indicate if the nutritional stress was similar in all muscles. A survey

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of the literature indicated that many studies on NMD have been performed on one muscle only, the muscle often being unnamed. Thirdly, subjective studies using the light microscope showed that the addition of vitamin E arrested the degenerative process in hamster muscles (West & Mason, 1955, 1958). The present study was designed therefore to determine the changes in muscle fibre size distribution during vitamin E therapy of vitamin E-deficient rabbits. The rabbit was chosen as the experimental animal because its muscle can be depleted to an exceptionally low level of tocopherol in a short period. Rabbit hind-leg muscle had about one-third the tocopherol content found in rat muscle (Green, Diplock, Bunyan & Edwin, 1961).

#### EXPERIMENTAL

##### *Animals*

Eighteen white male weanling New Zealand rabbits (Oak Crest Rabbitry, Edina, Minnesota 55436, U.S.A.), average weight 1.2 kg, were divided into four groups each of four or five animals for the following dietary treatments. Group 1 was fed on a modification of the vitamin E-deficient diet of Draper, Bergan, Chiu, Csallany & Boaro (1964) (Table 1) for 24 d. Group 2 was given a control diet made by supplementing the vitamin E-deficient diet with 50 mg DL- $\alpha$ -tocopheryl acetate/kg for 24 d. This diet did not contain folic acid, biotin or cyanocobalamin. These vitamins are synthesized in the intestinal tract of the rabbit (National Academy of Sciences-National Research Council, 1966). Group 3 was fed on the vitamin E-deficient diet for 28 d; then each rabbit was given an oral dose of 50 mg DL- $\alpha$ -tocopheryl acetate and fed on the control diet for a further 22 d. Group 4 was fed on the control diet for 28 d.

Rabbits were caged individually in raised metal cages (360  $\times$  400  $\times$  600 mm) which were located in an environmental-control room with temperature maintained at  $21 \pm 2^\circ$  and alternate 12 h of light and darkness. Food and water were supplied *ad lib*.

All rabbits were killed by an overdose of sodium pentobarbital administered intraperitoneally. They were maintained at  $20^\circ$  for 4 h before dissection to ensure that rigor mortis had developed. Initial studies revealed that 4 h was adequate time for muscle pH to drop from 7.2 to an ultimate value of 6.2.

##### *Skeletal muscle and bone sampling*

Muscles of the fore-limb (biceps brachii), the trunk (pectoralis descendens) and hind-limb (semitendinosus, gastrocnemius, soleus, plantaris and tibialis cranialis) were dissected intact from origin to insertion, freed of adhering fat and connective tissue and weighed. The entire muscle was fixed in neutral formalin (100 ml/l). All muscles were named according to Barone (1973).

Three bones, the humerus, tibia and femur, were dissected from each rabbit and trimmed of attached muscle and tendon. Bone length and weight were determined. These bones were chosen as indirect indicators of muscle length.

##### *Fibre diameter determination*

Individual muscles were fixed for at least 7 d to ensure total penetration of neutral formalin (100 ml/l). Fibre diameter was determined by the method of Hegarty & Naudé (1970). This method produces no structural damage in dystrophic muscle fibres (Landing, Dixon & Wells, 1974). A sample of approximately 25 mg was cut from the middle of the muscle. Fibres were separated in 0.7 ml Ringer-Locke solution using a Polytron-20 blade homogenizer (Kinematica GmbH, Lucerne, Switzerland). Sampling sites were kept as near constant as possible for each type of muscle to minimize the effects of variation within the muscles. Measurements of diameter were made by projection of images of the isolated

Table 1. Composition of vitamin E-deficient diet given to weanling rabbits

Ingredients (/kg)	
Vitamin-free casein*	200 g
Glucose*	204 g
Maize starch†	400 g
Stripped lard*	70 g
Cod-liver oil‡	30 g
Salt mix 4164‡§	40 g
Vitamin B-complex mix‡	5 g
Cellulose*	50 g
Choline chloride*	1 g
Retinyl palmitate*	500 mg
Ergocalciferol*	2 mg
DL- $\alpha$ -tocopheryl acetate*¶	—

\* INC Biochemicals, Cleveland, Ohio.

† Nugert Distributors Inc., Stockton, California.

‡ Teklad Test Diet, Madison, Wisconsin.

§ Supplied (g/kg diet): calcium (as carbonate and dibasic phosphate) 5.93, chloride (as sodium chloride) 2.62, copper (as sulphate) 0.0018, iodine (as potassium iodide) 0.0012, iron (as ferric citrate) 0.11, magnesium (as carbonate) 0.41, manganese (as sulphate) 0.018, phosphorus (as dibasic calcium and potassium phosphate) 3.11, potassium (as citrate and dibasic phosphate) 4.81, sodium (as chloride) 1.70, sulphur (as cupric and manganese sulphate) 0.011, zinc (as carbonate) 0.01.

|| Supplied (g/kg diet): thiamin 0.25, riboflavin 0.25, pyridoxine 0.25, calcium pantothenate 2, nicotinic acid 1, *p*-aminobenzoic acid 5, *myo*-inositol 10.

¶ Vitamin E-deficient diet was analysed for tocopherol content using the method of Chow, Draper & Csallany (1969). No tocopherol was detected.

fibres on white paper at a magnification of about  $\times 450$  (Large Universal Research Microscope; Zeiss, Oberkochen, West Germany). Fibres (100) were measured at random from each sample.

#### Histology

Skeletal muscles, fixed in neutral formalin (100 ml/l), were embedded in paraffin wax. Cross and longitudinal sections (six  $\mu\text{m}$  thick) were cut and stained by Masson's Trichrome (Lee, 1968).

#### Statistical methods

The significance of the difference between mean values was determined by Student's *t* test.

### RESULTS

#### Body-weight changes

The four groups of rabbits on different dietary treatments showed an initial weight gain (Fig. 1). The vitamin E-deficient groups (groups 2 and 4) had a very sharp decrease in body-weight at 15–22 d. This sudden weight loss was accompanied by a significant decrease in food intake. An oral dose of 50 mg DL- $\alpha$ -tocopheryl acetate was given to the animals in group 3 after 28 d on the vitamin E-deficient diet. Food intake was restored to normal after the 2nd day of administering vitamin E, resulting in a rapid gain in body-weight similar to the control animals. The growth curves of the vitamin E-deficient rabbits resembled those produced by MacKenzie & McCollum (1940) although the diet used in the present study was more purified (Draper *et al.* 1964).

#### Muscle weight

Muscles from the vitamin E-deficient animals appeared to be pale and exudative with scattered yellow fatty streaks, as reported by Van Vleet *et al.* (1968). In general, muscles

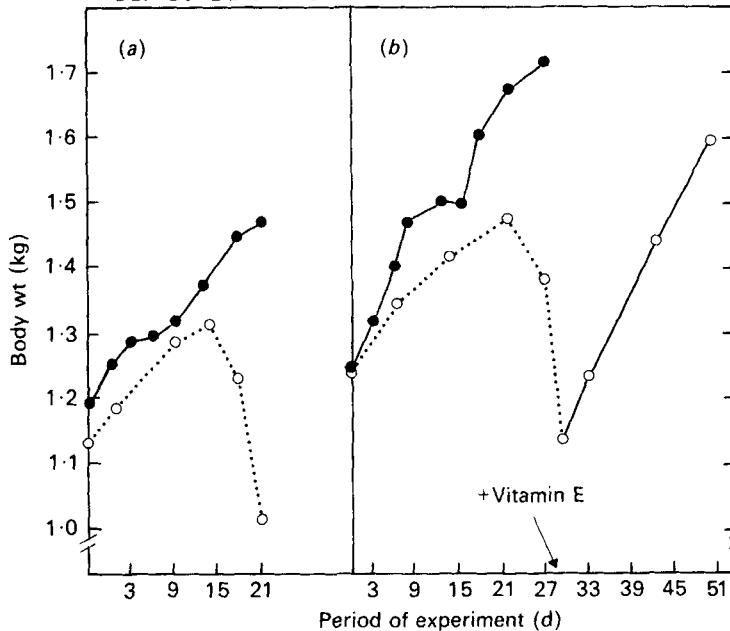


Fig. 1. Growth curves of groups of rabbits (a): (○), vitamin E-deficient (group 1); (●) vitamin E-supplemented (group 2); (b): (○), vitamin E-refed (group 3); (●), vitamin E-supplemented (group 4). Each line represents a mean of four or five animals. For details of groups and treatments, see p. 362.

of vitamin E-deficient rabbits weighed less than the corresponding control muscles and were also a smaller proportion of body-weight (Table 2). The weights of the soleus and semitendinosus muscles showed no significant differences between vitamin E-deficient rabbits and their controls. Weights less than those of the control rabbits were obtained for the biceps brachii, gastrocnemius, plantaris and tibialis cranialis. However, in the rabbits that were re-fed with vitamin E for 22 d, the weights of the biceps brachii and tibialis cranialis were not significantly less than in the controls, but the gastrocnemius and plantaris muscles weighed less than in the controls (group 4). This observation suggests an incomplete regeneration in the gastrocnemius and plantaris muscles.

#### *Bone length and weight*

The three bones that were measured served as an indirect indicator of the length of the muscles attached to the bones. Bone lengths and weights are given in Table 3. There were no significant differences between the vitamin E-deficient rabbits and the appropriate controls. This indicates that muscle length is relatively constant in all groups and that any changes in muscle weight must be due to change in the transverse dimension of the muscle.

#### *Fibre diameter and distribution*

The mean fibre diameter of seven skeletal muscles from the vitamin E-deficient and control rabbits are presented in Table 4. The fibre diameters of muscles from vitamin E-deficient animals were significantly smaller than the control animals in the biceps brachii, gastrocnemius, soleus, plantaris and tibialis cranialis. The diameter of the fibres in the semitendinosus and pectoralis descendens muscles were not significantly different from the controls. After 22 d of refeeding vitamin E in the diet the mean fibre diameter returned to a value comparable to that of the control animals with the exception of the gastrocnemius and soleus, which were still significantly smaller.

Table 2. Muscle-weight relative to body-weight (mg/g) of six muscles from vitamin E-deficient and sufficient rabbits

Muscle ... Group no.	Treatment†	Biceps brachii		Semitendinosus		Gastrocnemius		Soleus		Plantaris		Tibialis cranialis	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Vitamin E-deficient 24 d	0.71***	0.03	3.22	0.19	2.27***	0.23	0.57	0.05	1.04***	0.06	0.77*	0.08
2	Control, 24 d	1.03	0.03	3.45	0.13	3.73	0.14	0.56	0.03	1.50	0.04	0.98	0.05
3	Vitamin E-deficient refed	0.82	0.01	3.18	0.09	2.38**	0.10	0.55	0.01	1.14**	0.03	0.89	0.04
4	Control, 28 d	0.90	0.04	2.93	0.10	3.67	0.23	0.52	0.00	1.42	0.04	1.02	0.05

Mean values significantly lower than the corresponding control value: \*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.001$ .  
 † For details, see p. 362.

Table 3. Bone length and bone weight of vitamin E-deficient and sufficient rabbits

Group no.	Treatment†	Bone length (mm)						Bone wt (g)					
		Humerus		Femur		Tibia		Humerus		Femur		Tibia	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Vitamin E-deficient 24 d	59.4	0.80	76.5	1.20	85.0	1.60	2.82	0.13	5.57	0.20	4.44	0.20
2	Control, 24 d	57.2	1.00	74.7	0.60	82.7	1.00	2.77	0.16	6.09	0.27	4.96	0.21
3	Vitamin E-deficient refed	62.5	0.40	80.4	0.70	89.7	0.80	3.63	0.02	7.40	0.25	5.58	0.08
4	Control, 28 d	59.9	1.20	76.9	1.50	86.4	1.50	3.51	0.25	7.39	0.45	6.03	0.39

† For details, see p. 362.

Table 4. Mean fibre diameter ( $\mu\text{m}$ ) of various muscles from vitamin E-deficient and sufficient rabbits  
(Mean values with their standard errors)

Group no.	Treatment†	Biceps brachii		Semitendinosus		Gastrocnemius		Soleus		Plantaris		Tibialis cranialis		Pectoralis descendens	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	Vitamin E-deficient 24 d	40.53**	0.73	43.03	2.51	41.52**	2.42	50.54***	1.32	34.14***	1.48	38.55*	1.80	39.12	1.74
2	Control, 24 d	51.11	1.95	48.17	2.73	52.43	1.18	62.67	1.73	46.53	1.78	46.67	2.06	44.03	1.89
3	Vitamin E-deficient refed	44.19	2.18	45.43	0.74	47.62*	0.58	55.17*	1.00	44.32	1.11	39.18	0.82	40.63	0.91
4	Control, 28 d	49.95	2.57	46.26	3.00	50.41	0.50	61.80	2.11	49.61	2.12	43.05	2.65	46.33	2.55

Mean value significantly lower than the corresponding control value: \*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.001$ .

† For details, see p. 362.

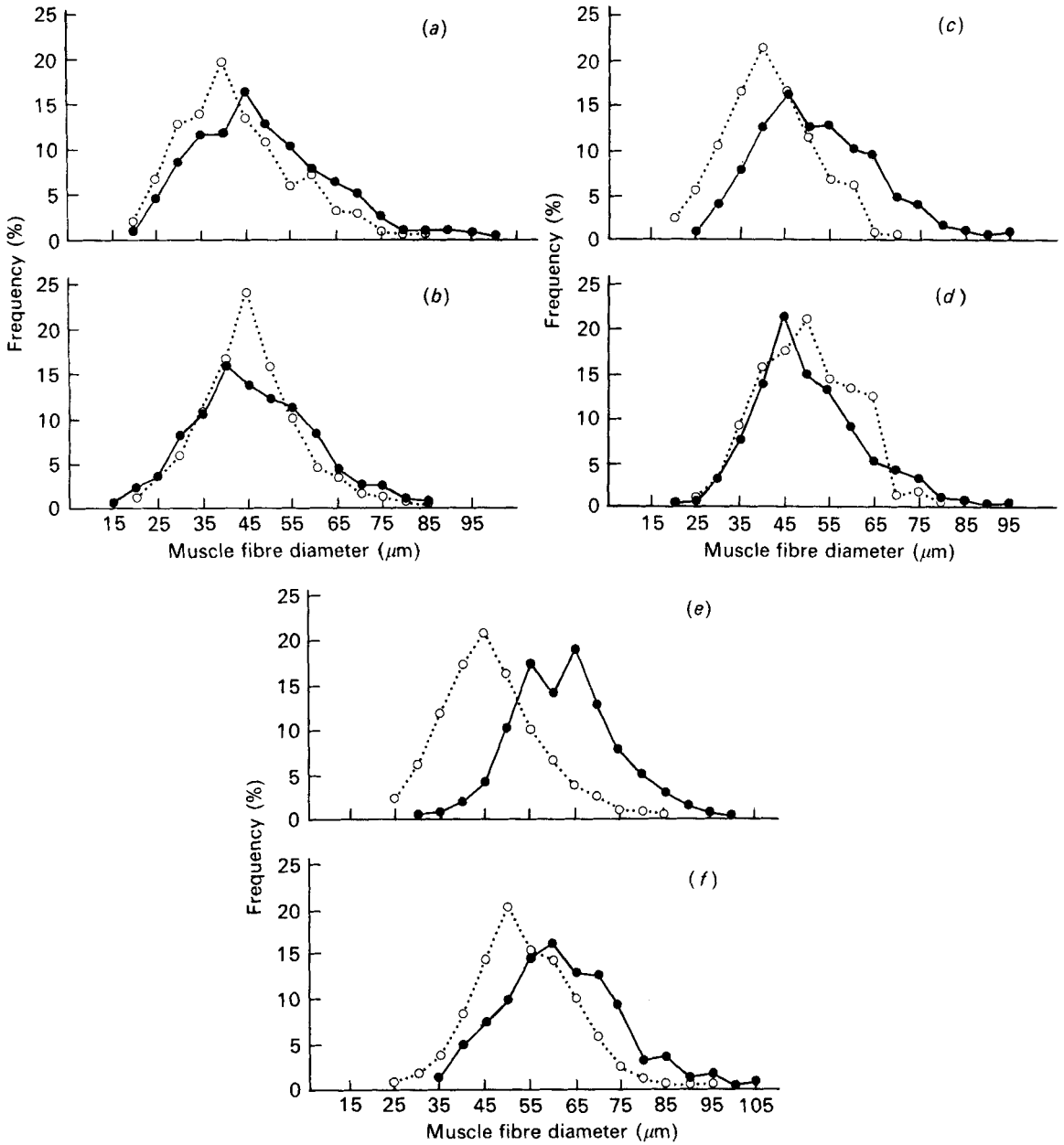


Fig. 2. Muscle fibre diameter distribution curves from rabbits: (○), vitamin E-deficient (group 1); (●), vitamin E-supplemented (group 2); (a) semitendinosus, (c) gastrocnemius, (e) soleus; (○), vitamin E-refed (group 3); (●), vitamin E-supplemented (group 4): (b) semitendinosus, (d) gastrocnemius, (f) soleus.

Fibre diameter distribution curves for the semitendinosus, gastrocnemius and soleus muscles are shown in Fig. 2a-f. In general, all muscles showed a monophasic distribution. The distribution of the fibre diameter of vitamin E-deficient rabbits showed a consistent decrease in every muscle studied. The extent of the decrease was greatest in the soleus and



the gastrocnemius and least in the semitendinosus. Upon refeeding the vitamin E-deficient animals, the distribution pattern of the semitendinosus and gastrocnemius muscles returned to that of the control animals. The exception was the soleus muscle, suggesting incomplete regeneration during the 22 d refeeding period.

#### *Light microscopy*

No structural changes were observed in the skeletal muscles from the rabbits fed on the vitamin E-supplemented diet (Plate 1*a* and Plate 2*a*). Histopathologic changes were observed in all seven muscles from the vitamin E-deficient rabbits (Plate 1*b, c*, and Plate 2*b, c*). Unpublished observations in this laboratory (A. C. Chan) indicated that no histopathologic lesions occur in muscles from rabbits that were pair-fed the control diet. This result indicates that all morphologic changes in the muscles were due to a dietary deficiency of vitamin E; the reduced food intake during the latter portion of the feeding period was not the cause of the abnormal morphologic changes in the muscles. The myopathy was characterized by widespread contraction clot formation, coagulation necrosis of muscle fibre segments, and extensive invasion of macrophages and mononuclear cells suggesting a mild inflammatory reaction (Plate 1*b, c* and Plate 2*b, c*). Involved segments showed loss of striations, transformation of myofibrils and sarcoplasm into a homogeneous coagulum and irregular distribution of nuclei. Substantial infiltration of collagen and fat replacing the degenerating fibres was common. The extent of degeneration was not the same in all muscles studied. The semitendinosus showed a mild degeneration with almost no collagen or fat (Plate 2*c*). Spontaneous regeneration was also observed in areas of extensive necrosis. Vitamin E therapy to the deficient rabbits removed almost completely the collagen infiltration, and the cross-striation pattern was returned to normal (Plate 1*d*). However, the removal of collagen was not complete in some sections of the muscles (Plate 2*d*).

#### DISCUSSION

It has been established that a deficiency of vitamin E in the diet produces NMD in the rabbit. The present study confirms this observation, but the extent of the atrophy varied between different skeletal muscles. The criteria for the extent of atrophy were changes in muscle weight, muscle fibre diameter and subjective evaluation of light micrographs. The reasons for the variable response by the muscles to the vitamin E deficiency may include the many differences in biochemical and physiological properties between skeletal muscles in adequately nourished animals (Close, 1972). Secondly, since young growing animals were used, each skeletal muscle was at a different phase of development. Skeletal muscles do have different relative growth patterns in animals consuming an adequate diet (Butterfield & Berg, 1966). Furthermore, the pioneering work of Hammond (1932) showed that the normal development of the skeleton and musculature is more advanced in the head and fore-limbs than in the hind-limb during the early development of mammals. Skeletal muscles from the fore- and hind-limbs were chosen in the present study because if the stage of development was significant in NMD, the cephalic muscles would be degenerated more than the muscles located caudally. The location of the skeletal muscles in the body was not a factor in the development of NMD in either the present study or that of Telford (1971). Differences in weight and in fibre diameter between different skeletal muscles have been observed also when adult mice were given a reduced nutrient intake (Rowe, 1968). Therefore, the concept of differential responses by skeletal muscles to a variety of nutritional stresses has been documented. However, the mechanisms involved may be different for different nutritional stresses. The atrophy in skeletal muscles from vitamin E-deficient



animals was accompanied by increased proteolytic activity (Koszalka, Mason & Krol, 1961; Weinstock, 1966; Noguchi, Takano & Kandatsu, 1972).

A significant decrease in body-weight of the rabbits fed on the vitamin E-deficient diet did not affect the length of the three bones measured. This indicates that the observed changes in the weight of the muscles and in the diameter of the muscle fibres may have occurred only in the transverse dimension of the muscle.

Addition of vitamin E to the diet of vitamin E-deficient animals causes improved reproduction (Draper *et al.* 1964) and decreased erythrocyte haemolysis (Draper & Csallany, 1969) in rats, and repair of testicular tissue in the hamster (Mason & Mauer, 1975). No detailed studies have been undertaken to determine if skeletal muscles undergoing NMD can be repaired by vitamin E except for the subjective evaluations of West & Mason (1955, 1958) on hamster muscles. The results of the present study indicate that the extensive atrophy of the muscles produced a decrease in muscle fibre diameter. This resulted in a uniform shift downwards in the monophasic distribution curve for muscle fibre diameter. However, the shape of the curve was similar to that obtained for the control animals. Atrophy in the muscles due to HMD produced a biphasic distribution for fibre diameter (Rowe & Goldspink, 1969; Shafiq *et al.* 1969). The small phase was due to degenerating muscle fibres, and the large phase was due to fibre hypertrophy to compensate for the loss of muscle fibres (Rowe & Goldspink, 1969; Shafiq *et al.* 1969). Goldspink (1972), in a review of the literature on postembryonic growth and differentiation of striated muscle, indicated that the number of muscle fibres is genetically fixed in utero or shortly after birth. If these fibres are lost due to atrophy, then they are not replaced because hyperplasia has ceased. Examination of the light micrographs in the present study indicated that hyperplasia had occurred when vitamin E was added to the diet. However, care must be exercised in the interpretation of fibre loss in vitamin E deficiency. Reference to Plate 2*c* illustrates that if a transverse section were to be cut at one portion of this sample a muscle fibre would be observed. If the transverse section was cut in another portion of the sample it would be concluded that the fibre had atrophied.

Ultrastructural studies showed extensive damage to the mitochondria and myofibrillar proteins in vitamin E-deficient animals (Howes *et al.* 1964; Van Vleet *et al.* 1967, 1968; Sweeny *et al.* 1972; Olson, 1974). However, in the present study the addition of vitamin E to the animal restored the normal appearance of the muscle. Connective tissue still remained in some muscles, and the damage was not fully repaired in some of the muscles during the 22 d rehabilitation. However, the conclusion was that vitamin E therapy restored the normal appearance of the muscle fibres. The mechanism whereby this was achieved is unclear. It has been documented that there is increased protein synthesis in skeletal muscles from nutritionally-dystrophic animals (Nichoalds, Diehl & Fitch, 1967; Simard & Srivastava, 1974). Ashmore & Doerr (1971) have presented an interesting speculation suggesting that fibre splitting may be a normal reaction in skeletal muscles subjected to severe metabolic stress. This splitting would facilitate the exchange of metabolites by increasing the available surface area. The implications of this suggestion in the rehabilitation of muscles undergoing NMD have not been studied.

The observations in the present study demonstrate that the damage to the contractile proteins have been repaired by vitamin E therapy. It cannot be inferred from this study that full mechanical efficiency has been restored to the skeletal muscles.

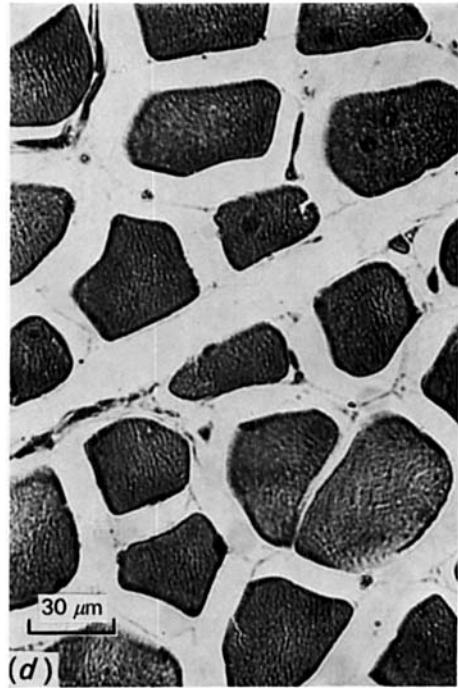
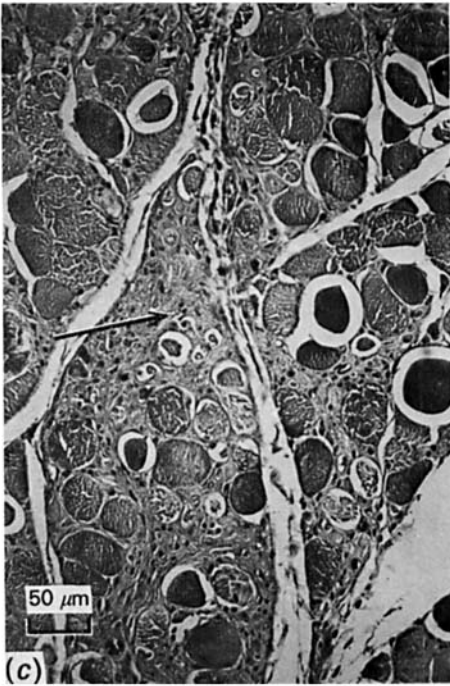
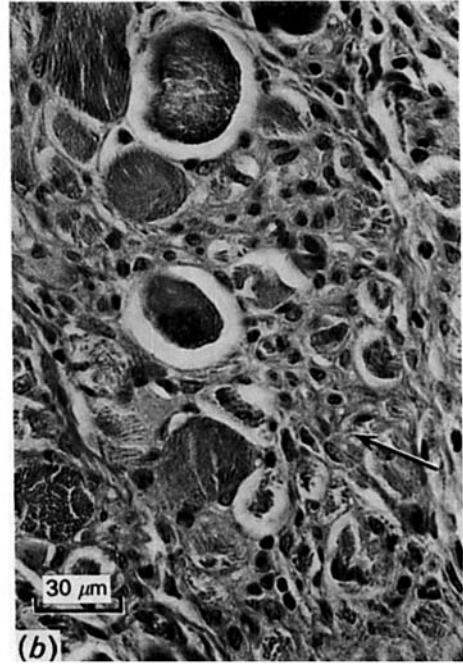
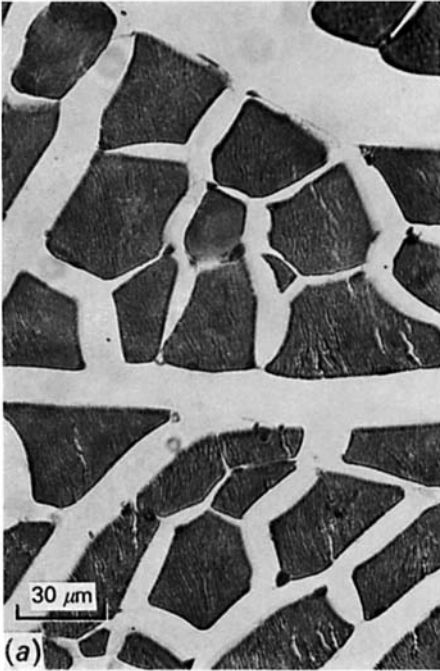
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## EXPLANATION OF PLATES

Plate 1. Transverse sections of rabbit muscles (a) vitamin E-supplemented rabbit, semitendinosus muscle; (b), (c) vitamin E-deficient rabbit, gastrocnemius muscle; (d) rabbit rehabilitated from vitamin E deficiency, semitendinosus muscle. Many of the muscle fibres in (b) and (c) have atrophied and have been replaced by connective tissue (†).

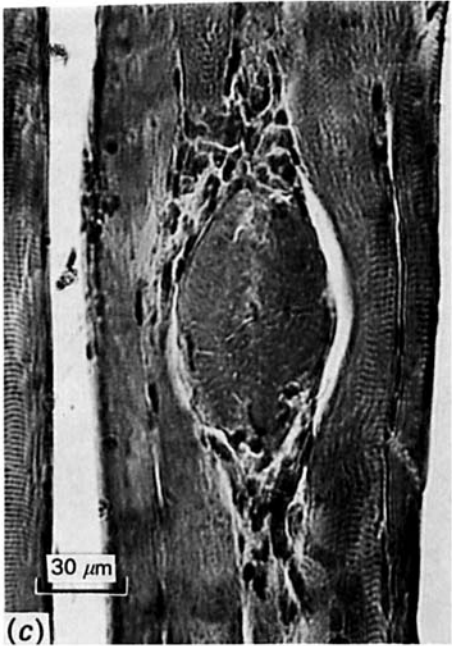
Plate 2. Longitudinal sections of rabbit muscles (a) vitamin E-supplemented rabbit, semitendinosus muscle; vitamin E-deficient rabbit, (b) plantaris muscle and (c) semitendinosus muscle. Note the extent of atrophy of the muscle fibres in (b) and (c). Rabbit rehabilitated from vitamin E deficiency, semitendinosus muscle. Note that some connective tissue (†) remained in (d); the muscle fibres were normal.



A. C. CHAN AND P. V. J. HEGARTY

(Facing p. 370)





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