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Retention of ^{45}Ca in Rats and Lambs Associated with the Onset of Nutritional Muscular Dystrophy

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Abstract

Nutritional myopathy has been induced in both rats and lambs by feeding diets low in selenium. The distribution of ^{45}Ca, administered as $^{45}CaCl_2$, has been examined firstly by autoradiography, and secondly by measuring the excretion of ^{45}Ca in urine and faeces. Autoradiographs of skeletal muscle from unsupplemented animals showed radioactivity over discrete muscle fibres at a stage when no abnormalities were apparent using conventional staining techniques. Similar retention of ^{45}Ca was found in some of the tubules in the kidneys of selenium-deficient rats.

Total excretion in urine and faeces of lambs, examined for 48 h after intravenous administration of $^{45}CaCl_2$, showed that in normal animals 18·6% of the dose was excreted, whereas in dystrophic lambs 12·0% was lost. The difference was significant at the 2% level.

The respiratory rates of isolated skeletal muscle mitochondria, measured polarographically in the presence of glutamate and pyruvate as substrates, were low for dystrophic rats. Respiratory control indices were 1·0 for the same preparations but for supplemented rats they were all above 1·0. The differences in respiratory rates were significant at the 1% level.

The major conclusion drawn from the results of these experiments is that one of the first effects of selenium deficiency which can be visualized is the abnormal retention of calcium by individual muscle fibres.

Introduction

The deposition of calcium in dystrophic muscle has been a recognized feature of white muscle disease, the selenium-deficiency syndrome of ruminants, for many years (Oldfield *et al.* 1958). Later work failed to show an increase in plasma calcium in ewes, lambs or calves affected by the disease (Schubert *et al.* 1961). The effectiveness of selenium and vitamin E in bringing about a remission in gross calcification was noted in this work, but no study was made of the significance of calcium deposition in the early stages of the disease. Later reports from New Zealand (Andrews *et al.* 1968) and Australia (Gardiner 1969) refer to calcification and recognize it as a feature in the overall pathology.

Work published recently from this laboratory (Godwin *et al.* 1974) showed that calcium retention occurred early in the development of experimental white muscle disease. Associated with this calcium retention was a reduction in respiratory rate of mitochondria isolated from dystrophic muscle when incubated in the presence of certain substrates.

The selenium-deficient syndrome in rats is associated with hepatic necrosis (Schwarz 1958). Recently Rotruck *et al.* (1972), by adding 0·3% DL-methionine to these necrogenic diets, prevented liver necrosis without removing other manifestations of selenium deficiency. In such animals typical dystrophic lesions occur in muscle.

This paper reports the retention of ^{45}Ca in both rats and lambs in dystrophic muscle following administration of $^{45}CaCl_2$, using autoradiography. In addition to the autoradiographic data obtained, ^{45}Ca excretion via urine and faeces has been measured in normal and dystrophic lambs.

Materials and Methods

(a) Experimental Arrangements

Two experiments were run, one with rats and the other with lambs. In the former, 122 weanling rats from the specific pathogen-free colony established in this laboratory (Godwin *et al.* 1964) were distributed over the following four dietary treatments:

(1) basal diet, 38 animals;
(2) basal diet plus 0·5 ppm selenium as Na_2SeO_3, 38 animals;
(3) basal diet plus 50 i.u. vitamin E (Duphaphral E, N.V. Philips-Duphar, Amsterdam) per kilogram, 38 animals;
(4) basal diet plus selenium and vitamin E as in (2) and (3), 8 animals.

The basal diet was identical to that used by Rotruck *et. al* (1972).

Animals were killed at 0, 1, 2, 3, 4, 5, 6, 8, and 10 weeks after being introduced to the diets.

In the second experiment normal Merino lambs were weaned onto dystrophogenic hay in the manner described elsewhere (Godwin 1972; Godwin *et al.* 1974). Some were given no selenium supplement and were allowed to develop white muscle disease, others were supplied with a selenium supplement either as an oral drench or as an intraruminal heavy pellet (Godwin *et al.* 1974). A third but smaller group was given a selenium supplement after developing white muscle disease and was designated the recovery group (Kuchel and Godwin, unpublished data).

(b) Experimental Procedures

(i) *Autoradiography*

Rats were injected intraperitoneally with ^{45}Ca as $^{45}CaCl_2$ (200 μCi/100 g body weight) 1 h before slaughter. ($^{45}CaCl_2$ was obtained from Radioamersham, Bucks., England.) After slaughter of the rats, samples of gastrocnemius muscle, kidney and liver were plunged into an ethanol and dry ice mixture. Sections were cut using an H-R Slee Cryostat and were mounted and processed using the technique of Appelton (1964). Ilford Nuclear Emulsion was diluted 1 : 2 with cold glass-distilled water. Sections were stained after development (usually 3–4 days) with haematoxylin and eosin.

Lambs used for autoradiography were cannulated and the isotope introduced into an external jugular vein. Lambs weighing 25 kg were given 1 mCi ^{45}Ca as $^{45}CaCl_2$ in normal saline. The animals were slaughtered 3 h after injection. The following tissues were sampled and plunged into an ethanol and dry ice mixture: semitendinosus, biceps femoris and adductor muscles, left ventricle and liver.

(ii) *Excretion rate of ^{45}Ca*

In all, 10 lambs were examined for determination of the excretion rate of ^{45}Ca following the intravenous administration of $^{45}CaCl_2$ in normal saline. Animals received 3–4 μCi ^{45}Ca per kilogram body weight. After administration of the isotope, the animals were housed in metabolism cages for 48 h; urine was collected separately and immediately acidified with HNO_3; faeces were collected and weighed.

Urine was added directly to scintillation fluid for measurement of radioactivity. Faeces were dried at 90°C and small weighed amounts were combusted in a muffle furnace at 800°C for 1 h before dissolving in 6N HCl.

The composition of the scintillation fluid was 4·0 g 2,5-diphenyloxazole, 100 mg dimethyl-1,4-*bis*(4-methyl-5-phenyloxazal-2-yl)benzene dissolved in 1 litre toluene, 840 ml Triton X-100 and finally 184 ml water. Counts were corrected for quenching and for decay rate.

Of the 10 lambs used, four were unsupplemented with either selenium or vitamin E and six were supplemented to prevent the onset of white muscle disease. Two received 100 mg α-tocopherol acetate (vitamin E) weekly, two received 3 mg selenium as sodium selenite orally twice weekly and two received an intraruminal heavy selenium pellet. Two from the unsupplemented group were subsequently allowed to recover by supplementation and ^{45}Ca excretion rates were measured before and after recovery.

(iii) *Mitochondrial characteristics*

Mitochondria were isolated from pooled gastrocnemius and soleus muscles from rats, in order to measure their respiratory rates and respiratory control indices. The techniques used have been described elsewhere (Godwin *et al.* 1974).

Respiratory properties of the mitochondria were examined in the presence of glutamate, pyruvate and malate, and DL-palmitylcarnitine. The final concentrations of the substrates were 5·4 mM except for DL-palmitylcarnitine which was 0·4 mM.

Examination of the density of the mitochondrial suspensions was carried out using phase-contrast microscopy. Janus green was used intravitally to test the viability of the preparations.

(*c*) *Additional Assessments Made*

Assessment of the dystrophic condition of lambs was based on measurement of whole blood selenium using the method of Watkinson (1966) and on creatine kinase levels in plasma which were measured at weekly intervals using the Boehringer CPK Activated UV system. During the recovery phase following selenium supplementation to dystrophic animals, measurements were made daily.

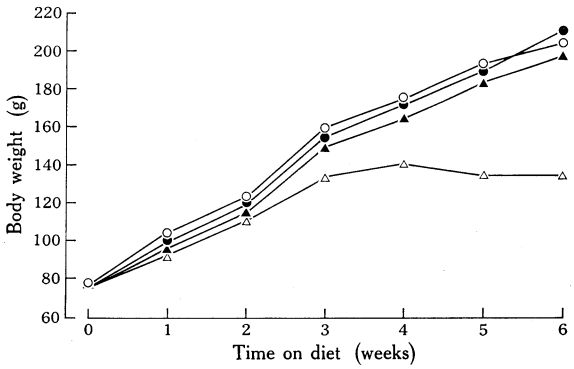

Fig. 1. Weekly average body weights for groups of eight rats on the four diets. △ Group 1. ● Group 2. ▲ Group 3. ○ Group 4. Note the fall-off in body weight increase after the third week in group 1.

Results

Autoradiographs prepared from rat skeletal muscle demonstrated localization of ^{45}Ca within discrete fibres scattered throughout the section; this was observed only in the unsupplemented group of rats. The earliest stage at which isotope localization could be detected was at the third week. At this stage no abnomalities could be detected in histological preparations of skeletal muscle, and very little difference in weight gain had occurred in the unsupplemented group (Fig. 1). Autoradiographs of unsupplemented and supplemented rats are shown in Figs 2*a* and 2*b*. From these observations the general conclusion was drawn that ^{45}Ca localization is well established by the sixth week. At this stage, dystrophic degeneration was already present in skeletal muscle (Fig. 3).

Creatine kinase levels in plasma, measured at the sixth and seventh weeks, showed higher averages in the unsupplemented group of rats but the differences did not reach a significant level. In the four groups (basal diet, basal diet plus selenium, basal diet plus vitamin E, and basal diet plus selenium and vitamin E) the values for the sixth week were 28,16, 18 and 12 units/100 ml plasma respectively and those for the seventh week were 41, 20, 9 and 28 units/100 ml plasma respectively.

Autoradiographs of kidney sections from the same rats showed similar ^{45}Ca localization in tubules of the collecting ducts (see Fig. 4).

Measurement of the respiratory properties of the mitochondria isolated from skeletal muscle of rats slaughtered at 6 weeks showed abnormal rates of respiration and low respiratory control indices. Table 1 shows respiratory rates of skeletal muscle

mitochondria isolated from gastrocnemius and soleus muscles of rats fed the dystrophogenic diets with and without vitamin E and selenium supplementation. The values show a significant lowering of the respiratory rate in the unsupplemented animals when glutamate and pyruvate were added to the *in vitro* system as substrates. Respiratory control indices for the same preparations are also shown in Table 1.

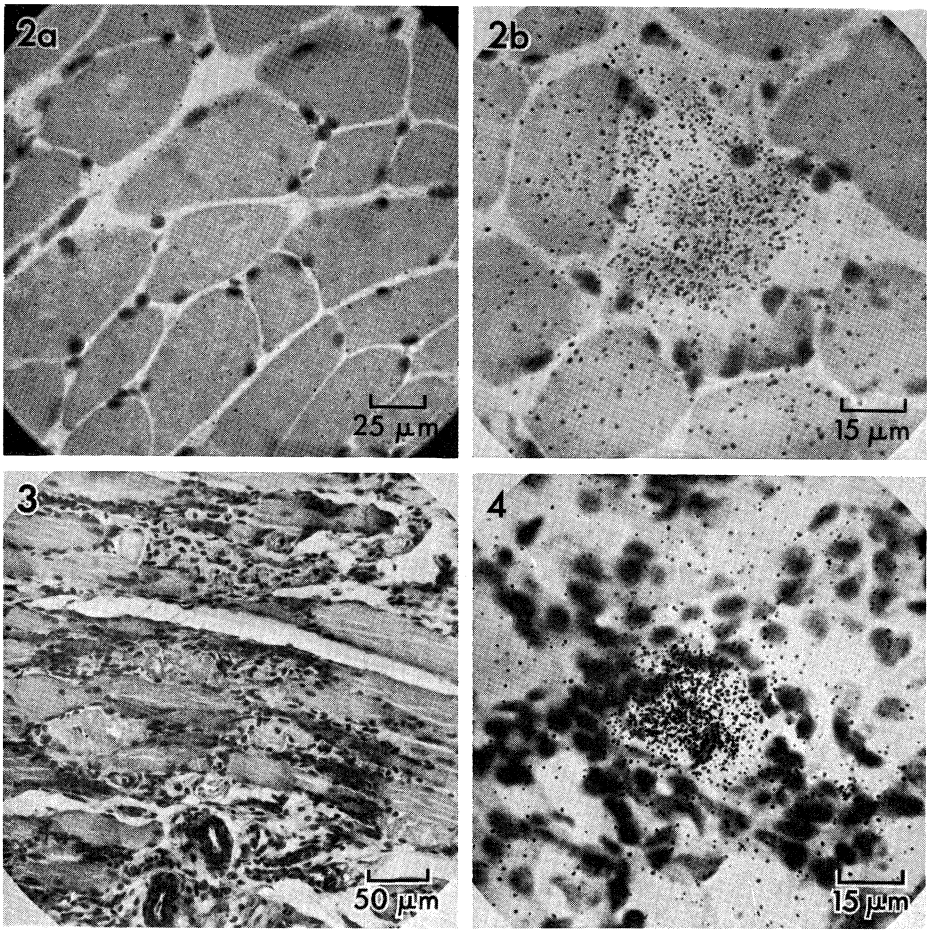

Fig. 2. Transverse sections of gastrocnemius muscle of rats showing ^{45}Ca localization over a single muscle fibre in the selenium-unsupplemented animal (*b*) compared with mild overall activity in the normal animal (*a*).

Fig. 3. Longitudinal section of rat gastrocnemius muscle showing typical degenerative lesion found in rats fed a low-selenium intake supplemented with 0·3% methionine to prevent hepatic necrosis.

Fig. 4. Section through rat kidney showing ^{45}Ca localization over kidney tubule in dystrophic animal.

Calcium excretion rate, obtained by measuring the total percentage loss of ^{45}Ca via the faeces and urine over a 48-h period following intravenous injection, was found to be low in dystrophic lambs. The values were $12 \cdot 00 \pm 2 \cdot 15\%$ for the unsupplemented group and $18 \cdot 60 \pm 2 \cdot 15\%$ for the selenium-supplemented group ($t = 3 \cdot 05$, $P < 0 \cdot 01$).

Two of the lambs that were allowed to recover from white muscle disease were used for measurement of ^{45}Ca excretion before and after recovery. The values are shown in the following tabulation.

Lamb No.	^{45}Ca excretion initially (%)	^{45}Ca excretion after recovery (%)
073	12·32	26·74
541	6·50	20·13

These values suggest that the retention of calcium is reversible when there is remission of the dystrophic condition.

Table 1. Respiration rates and respiratory control indices of skeletal muscle mitochondria from rats in the four dietary treatment groups

Rates measured in presence of ADP and different substrates by polarographic technique are expressed as μatoms oxygen per minute per milligram protein (shown in italics). The volume of the reaction vessel was 2 ml, temperature was 30°C and the final concentrations in the medium were: 0·225M sucrose, 10 mM phosphate, 5 mM $MgCl_2$, 20 mM KCl, 20 mM tris–HCl, pH adjusted to 7·4. Statistical analysis is based on a logarithmic transformation. Each value is the mean ± standard error for six animals. Respiratory control indices (= rate of respiration in absence of ADP ÷ rate of respiration in presence of ADP) are given in parentheses

Treatment group	Substrates		
	Glutamate	Pyruvate	DL-Palmitylcarnitine
1	*13·0* 1·22±0·13 (1·0)	*42·0* 1·56±0·13 (1·0)	*19·7* 1·24±0·13 (1·0)
2	*76·7* 1·83±0·13 (2·9)	*106·3* 2·00±0·13 (4·0)	*56·3* 1·66±0·13 (1·6)
3	*68·4* 1·78±0·14 (3·7)	*106·4* 1·99±0·14 (3·4)	*67·8* 1·57±0·14 (1·9)
4	*89·8* 1·83±0·14 (3·4)	*122·8* 2·07±0·14 (3·2)	*30·6* 1·32±0·14 (1·1)
P[A]	<0·01	<0·01	n.s.

[A] Group 1 compared with groups 2, 3 and 4.

Autoradiographs prepared from a supplemented and an unsupplemented lamb, from cryostat sections of semitendinosus, biceps femoris and adductor muscles, showed a pattern of localization of activity similar to that described earlier in the paper for rats (see Figs 5*a*, 5*b* and 5*c*). Again, the dystrophic nature of the muscle was confirmed by histological examination (Fig. 6).

Discussion

The importance of calcium in the physiology of muscle has been recognized since the classical studies of Ringer (1883). Recent reviews by Ashley (1971) and Simkiss

(1974) cover specific ways in which calcium is known to be involved in cellular function. One way, for example, is the intracellular regulation of calcium ion concentration by the mitochondria which, according to Lehninger (1970), is so important in the energy economy of the cell that it may take primacy over oxidative phosphorylation. Therefore, when there is a disturbance in normal calcium distribution or concentration within muscle it is likely to be of importance and may underlie the degenerative processes occurring in dystrophic muscle.

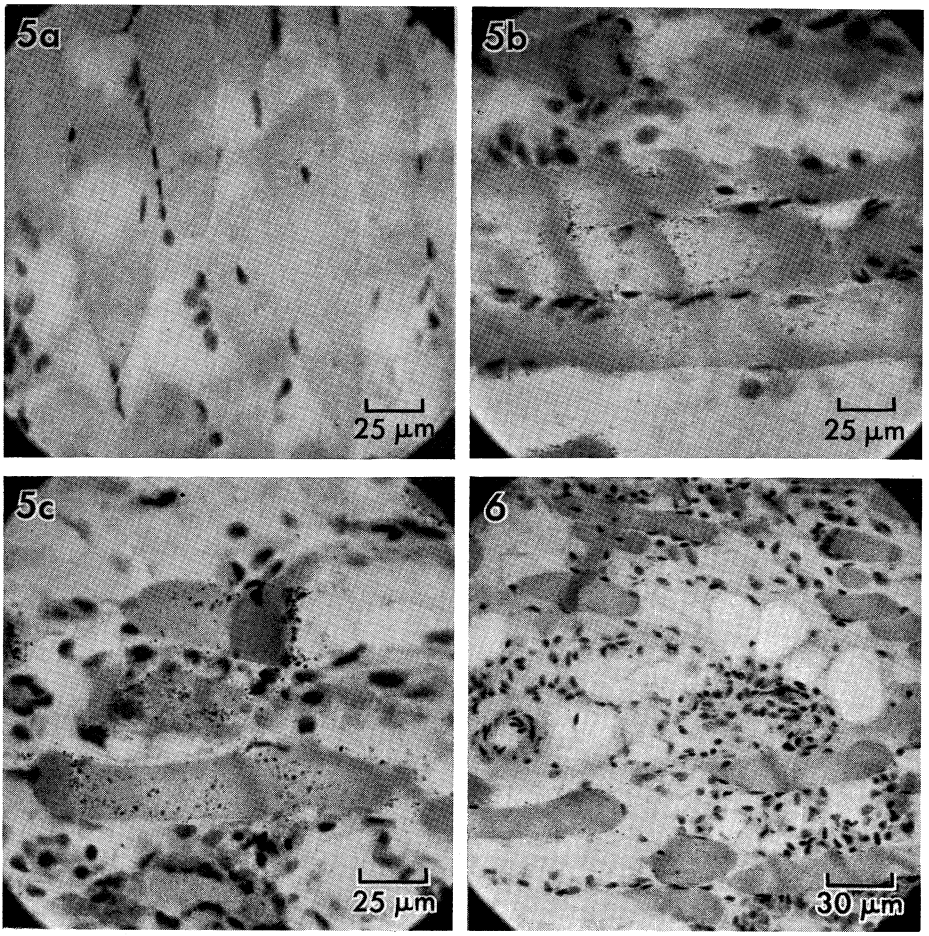

Fig. 5. Longitudinal section through semitendinosus muscles of lambs showing ^{45}Ca localization over muscle fibres of selenium deficient lambs (*b,c*) compared with virtually no retention in the selenium-supplemented lambs (*a*).

Fig. 6. Semitendinosus muscle from lamb showing dystrophic lesion of the type associated with ^{45}Ca retention in certain discrete fibres during the early phases of the myopathy.

The autoradiographs show that at a very early stage of the dystrophic process, before abnormalities are present as judged by conventional histological techniques, there is retention by scattered, discrete muscle fibres of ^{45}Ca following the administration of $^{45}CaCl_2$. The observation fits the later histopathological picture of normal

and abnormal fibres sometimes lying side by side. Although these observations allow the assumption to be made, it is presumably reasonable to suppose that those fibres showing the presence of radioactivity are those that would later show degenerative breakdown.

Recently, working with hereditary muscular dystrophy in hamsters, Mezon *et al.* (1974) separated out distinct populations of mitochondria having high calcium levels by means of a sucrose density-gradient centrifugation technique. They postulated that the reason for the reduced rate of respiration in mitochondria isolated from dystrophic muscle, which they had observed earlier (Wrogemann *et al.* 1970), was loss of calcium from certain organelles and its uptake by others, thus rendering all the mitochondria defective. In the later publication ruthenium red was used to prevent calcium exchange during the isolation of the mitochondria, a devise used by these same authors previously (Thakar *et al.* 1973).

The observations reported in this paper are important, therefore, for a number of reasons. They lend further support for the essential similarity of the hereditary and nutritional types of muscular dystrophy (Godwin 1972; Godwin *et al.* 1974). They show that the earliest abnormality during the onset of selenium deficiency may be with respect to an ion, the redistribution of which would be expected to cause profound changes in muscle integrity. They also fully support the view of Mezon *et al.* (1974) that it is now possible to reconcile the selective picture of histopathological damage in muscle with a generalized defect in isolated mitochondria.

Other features of the autoradiographic data are of interest. There was little evidence of ^{45}Ca retention in heart muscle and it may be significant that in our earlier study (Godwin *et al.* 1974) no abnormalities occurred in heart muscle mitochondrial respiratory activity even at a time when white muscle disease was well advanced. Kidney damage has been recognized as a feature of the pathology of selenium deficiency in rats (Schwarz 1961), and the autoradiographs of the kidney show that ^{45}Ca retention is an early feature of the subsequent tubular damage. No retention of ^{45}Ca was apparent in autoradiographs of livers from the dystrophic group.

To our knowledge no observations have been made of the respiratory activity of mitochondria isolated from dystrophic rat muscle. Whanger (1973) examined muscle slices from normal and myopathic lambs and found reduced amounts of glutamate converted to carbon dioxide by slices from the selenium-deficient lambs. Three substrates were examined: glutamate, pyruvate and succinate. In the case of the latter two, slightly more of the radioactivity from labelled substrates was incorporated into protein by normal slices than by muscle slices from dystrophic lambs. Our observations on respiratory rates in skeletal muscle mitochondria show a reduced rate of respiration in the case of both glutamate and pyruvate. Similar work in lambs (Godwin *et al.* 1974) has shown a reduced respiratory rate in the presence of DL-palmitylcarnitine, but this was not the case with the rat. Our conclusions support those of Whanger, therefore, that selenium may have a role to play in glutamate metabolism. The indication from Whanger's tissue slice work that pyruvate may be affected seems confirmed by our positive results in the rat.

The low respiratory control indices obtained with each of the three substrates further point to mitochondrial dysfunction. The significance of this was discussed in the earlier lamb work (Godwin *et al.* 1974). The respiratory control index is a rigorous test of normal mitochondrial function (Lehninger 1964).

The difference obtained between the supplemented and unsupplemented groups of lambs, with respect to the excretion of ^{45}Ca over a 48-h period, suggests a more rapid turnover of calcium in the soft tissues of the normal lamb. This may reflect an overall greater turnover of calcium, including that in the bony structures, but the muscle autoradiographs allow conclusions to be drawn about only that one tissue.

Acknowledgment

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