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INTERSTITIAL SPACE OF MOUSE SKELETAL MUSCLE

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SUMMARY

1. A new preparation of mouse skeletal muscle, prepared from pectoral muscles, is described.

2. The sorbitol space of this muscle, both *in vivo* and *in vitro*, has been measured with dynamic loading of the muscle *in vitro* as an experimental variable.

3. The Na^+ and K^+ contents of the muscle have been determined and the apparent intracellular concentration for these ions calculated both *in vivo* and after incubation *in vitro*.

4. Histological studies on the incubated muscle have been made so as to permit comparison of the changes in the chemical measurements with changes in the ultrastructure of the muscle.

5. The results of these experiments show that there is an increase in the apparent extracellular space of the muscle following incubation. This increase is constant, and independent of the load, with the important exception that unloaded muscles do not reach an equilibrium during the period of incubation and have a much greater apparent extracellular space.

6. Intracellular Na^+ and K^+ concentrations are consistent with the sorbitol being restricted to an extracellular phase in the loaded muscle; but the evidence implies that sorbitol in the unloaded muscle penetrates into a space from which Na^+ is excluded.

7. The total water content of the muscle per unit weight is unchanged by incubation, indicating that the apparent change in sorbitol space is in the ratio of intracellular space to extracellular space rather than by addition of water to the extracellular space.

The significance of these results is discussed with reference to the use of such preparations for *in vitro* studies.

INTRODUCTION

In this report we describe a new preparation of skeletal muscle designed for use in *in vitro* studies of muscle function. We also present data on the changes of

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intracellular Na^+ and K^+ and the volume of the interstitial space (i.s.) of muscle in this preparation as they occur during incubation. We have investigated the changes in these parameters as a function of time and the amount of load placed on the muscle. The ultrastructure of the muscle after incubation has been studied in the electron microscope.

Our purpose in making these observations was to determine whether the muscle in this preparation reached and maintained a reproducible steady state during incubation under varying conditions and, if so, how the electrolyte levels and i.s. volume in this steady state differed from those *in vivo*.

Maintaining the correct intracellular–extracellular concentration gradients for ions is fundamental to normal cell function. We chose to measure Na^+ and K^+ as an index of this function. The interstitial space is of importance because *in vitro* this channel is the only pathway by which the cells in the deeper portions of the preparations can exchange substrates and excretory products with the incubation medium. Since the passage of solutes through the i.s. is diffusion-limited, changes in its volume will alter rates of exchange between muscle and incubation medium and hence modify metabolic functioning of the muscle. Additionally, as is described below, a knowledge of the i.s. volume is necessary for the calculation of intracellular electrolyte concentrations.

Measurement of Na^+ and K^+ is a simple flame photometric procedure, but measurement of the i.s. is more complex. Although an anatomic compartment corresponding to the i.s. can be described, the space is best defined (Aukland & Nicolaysen, 1981) as 'the equilibrium distribution space of extracellular tracers minus plasma volume'. In *in vitro* systems the plasma volume is ignored.

Most methods for assay of interstitial space depend upon one of two types of measurement. In efflux studies, such as those analysed by Neville & Mathias (1979), the kinetics of the loss of a chosen substance such as Na^+, Cl^-, xylose, or sucrose from an incubated portion of tissue are studied. The results are resolved into a series of fast and slow efflux rates, and the interstitial space is considered to be that from which the most rapid efflux has occurred. Influx studies, conversely, utilize large (inulin, dextran) or small (sorbitol, sucrose, CoEDTA, SO_4^{2-}) molecules or ions which are considered to be strictly excluded from the intracellular space. By measuring the concentration of such substances in plasma or incubation medium and in the tissue after an equilibrium has been established, the interstitial space can be calculated. Choice of the correct tracer is important. It has generally been concluded (Poole-Wilson & Cameron, 1975; Barclay, Hamley & Houghton, 1959) that the 'inulin space' is an inadequate measure of the total extracellular space. Poole-Wilson & Cameron (1975) showed that, in skeletal muscle, the extracellular space calculated from inulin was considerably less than that calculated from sucrose or CoEDTA. Ions may be inadequate for measurements because of their charges. Recently, Macchia, Page & Polimeni (1979) showed that the 'sulphate space' is considerably smaller in skeletal muscle than the 'sucrose space'. These authors consider the difference to be due to an 'excluded ion' effect.

Uncharged small molecules seem to give results which are directly comparable and Brading & Jones (1969) showed that sorbitol is distributed in a manner very similar to that of CoEDTA or sucrose.

For these studies we chose to use the equilibrium distribution of carrier-free [^{3}H]sorbitol by an influx method as our measure of interstitial space.

We designed the muscle preparation in order to have a simple *in vitro* system in which we could study the changes in whole muscle which occurred as a response to its experimental manipulation. The preparation, therefore, had to meet certain criteria:

(1) the preparation should be thin enough to prevent the diffusion of substrates and oxygen from being a limiting factor;

(2) it should be large enough to enable multiple assays to be done on the same piece of tissue after incubation;

(3) the technique of preparation should be simple so that reproducible preparations can be made in a short time (less than 4 min): this permits the simultaneous incubation of several muscles.

We (Zacks & Sheff, 1965) had previously used portions of the mouse pectoral muscle in incubation studies and we decided to develop our preparation from this muscle since it fulfills the first three criteria. Two further criteria were:

(1) that the muscle should show minimal ultrastructural changes after incubation;

(2) that leakage from the muscle, as judged by loss of protein into the incubation medium, should also be minimal.

Our preliminary experiments with this preparation have been reported in abstract form (Sheff, Brodsky & Zacks, 1979). In that work we found that glucose at 150 mg/dl., insulin (5 i.u./ml.) and weight loading of 1 g per muscle gave us a preparation which retained its morphological integrity during incubation for 60 min in Krebs–Henseleit bicarbonate Ringer solution. We have used these conditions as the basic incubation system in these experiments.

Despite the extensive pathology literature on the correlation of ultrastructural changes with muscle dysfunction, few studies of muscle function *in vitro* contain any information on the direct effects of the incubation on the morphology of the muscle. Similarly, stretch and tenotomy have been used as experimental variables in *in vivo* studies on muscle, but, with the exception of the studies of Hider, Fern & London (1971), little attention has been paid to loading of the muscle in *in vitro* studies. We have addressed these questions in this paper since we believe that they are important in comparing the results of *in vitro* studies with those from *in vivo* studies.

METHODS

Animals

A breeding colony of white mice, originally Swiss-Webster stock, obtained from Charles River Farms has been kept for several generations in our Animal Facility. Animals from 25 to 65 days of age from this stock were used in these experiments. Originally, we had intended to consider the age of the animal as a variable in this study, but early measurements indicated that there was no significant age differential in the results.

Reagents

All inorganic reagents and glucose were obtained as the highest grade available from Fisher Scientific Company, Fairlawn, NJ 00410, U.S.A. [^{3}H]Sorbitol (1 mc/ml.) was obtained from the New England Nuclear Corporation, Boston, MA, U.S.A., dissolved in 90 % ethanol. This was diluted with sterile 0·9 % saline before use. Protosol, a tissue solubilizer, and Biofluor, a scintillation 'cocktail', were also obtained from New England Nuclear, Boston, MA, U.S.A. Insulin was obtained from Sigma, St. Louis, MO, U.S.A.

Equipment

The muscle incubation bath which was built for use with the preparation will be described below.

Radioactive counting was done in a Packard 460 CD liquid scintillation beta counter. This instrument has the capacity to automatically generate and store quench correction curves so that all count rates were corrected directly to disintegrations/min.

Electrolyte measurements (Na^+ and K^+) were made using an IL146 Flame Photometer using standards which had been prepared for the assay of serum and urine electrolytes.

Spectrophotometry was carried out in a Beckman Model 25 double-beam recording spectrophotometer.

Micro (100 μl.) haematocrits were measured using an International microcapillary centrifuge system.

In vivo measurements

We are using the definition: i.s. equals extracellular space minus plasma volume. Therefore, in order to find the interstitial space *in vivo* it was necessary to find the volume of the plasma in the muscle samples. We determined the plasma volume from the haemoglobin content of the muscle and the haematocrit of the circulating blood at the same time as we measured the total extracellular space with [^{3}H]sorbitol.

Mice were injected with 5 μc of [^{3}H]sorbitol into the peritoneal cavity. At chosen intervals they were anaesthetized with chloroform, the chest wall was opened and a sample of blood drawn from the right ventricle. The entire pectoral muscle group from one side of the animal was then removed at its insertion and origin and quickly weighed. The muscle was homogenized in 1·5 ml. of distilled water and a clear supernatant obtained by centrifugation at 20,000 *g* for 15 min. This supernatant was aerated in a 1 ml. microcuvette and an absorption spectrum from 600 to 400 nm was obtained in a Beckman Model 25 spectrophotometer. The absorptions at the maxima of 577 and 545 nm for oxyhaemoglobin were measured and compared with the absorptions for these maxima which were obtained for a 1:200 dilution for the heparinized whole blood drawn from the right ventricle of the same animal and haemolysed in distilled water. From this data, the residual whole blood in the muscle following its removal from the animal was calculated.

The haematocrit of the whole blood was determined in an International Microhaematocrit system and from this and the whole blood measurement the plasma volume in the muscle was determined.

In some cases 0·5 ml. of the homogenate was taken before centrifugation and 0·5 ml. of 0·1 N-HCl containing 15 m-equiv. LiCl/l. was added. After 72 hr in the cold the precipitate was removed by centrifugation and the Na^+ and K^+ concentrations determined by flame photometry.

The radioactivity of the muscle was determined as follows: 0·5 ml. of the muscle supernatant was dried to constant weight at 105 °C. 200 μl. of distilled water was added to the dried residue and it was allowed to rehydrate. 1 ml. of Protosol (New England Nuclear) was added to the residue to dissolve it, and following its dissolution 14·5 ml. of Biofluor, a commercial scintillation 'cocktail' by New England Nuclear containing POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) and PPO (2,5-diphenyloxazole) in toluene, was added. Following the addition of the Biofluor, 0·35 ml of 2N-HCl were added to each sample to provide the final sample for counting. This system gives a clear homogenous solution with a minimum of quenching.

Tritiated sorbitol in the blood was assayed by diluting 30 μl. of heparinized blood with 1·5 ml. of 0·9% NaCl and centrifuging to remove the red cells. 100 μl. of the supernatant was heated for 1 hr with 1 ml. of Protosol and prepared for scintillation counting as described above for tissue. From this data and haematocrit the specific activity of tritiated sorbitol in the plasma (disintegrations/min. μl.) was calculated. The net counts (disintegrations/min. mg of tissue) were obtained by subtracting the counts in the residual blood from the total measured counts in the tissue.

Then:

$$\textit{In vivo}\ \text{i.s.} = \frac{\text{Total counts} - \text{plasma counts}}{\text{Plasma specific activity (disintegrations/min.}\ \mu\text{l.)}}$$

In vitro measurements

The *in vitro* incubation apparatus is shown in Fig. 1. A rectangular chamber, internal dimensions 1 × 7 × 9 cm length, was made from $\frac{1}{8}$ in. thick Lucite. The chamber was divided into two sections by a piece of Lucite extending the length of the chamber 1 cm from the bottom. This internal wall was pierced with $\frac{1}{16}$ in. holes at approximately 0·5 cm intervals and by a 2 mm i.d. O_2–CO_2 inlet

vertical glass tube which extends above the top of the chamber as shown. When the chamber is filled with incubation medium, little passes into the lower portion and the O_2–CO_2 mixture which is used both for mixing and for oxygenation rises through the perforations. A removable bar sits in a bracket (not shown) and is notched so that hooks may be hung from it. The whole chamber is provided with lugs (not shown) which enable it to be supported in a 37 ° water bath. Provision is also made for the humidification of the gas supply by bubbling it through a trap containing 0·9 % NaCl at 37 °C. 40 ml. of incubation medium provides satisfactory volume for studies.

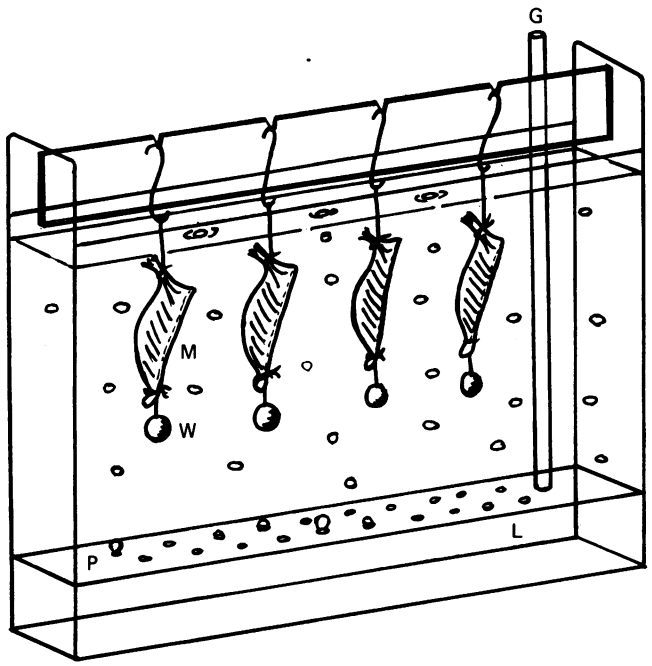


Fig. 1. Incubation apparatus. The incubation bath is 7 × 9 × 1 cm. Muscles (M) hang in the incubation medium and are lightly extended by weights (W). Humidified 95 % O_2–5 % CO_2, enters via the tube G. The gas enters a water-free compartment (L), and bubbles up through a perforated plate (P), both stirring and oxygenating the medium.

Tissues for incubation were prepared from mice in the following manner. The mice were anaesthetized with chloroform and the skin rapidly removed from the chest. By cutting across the diaphragm and up the side of the chest wall, the chest could be reflected so that the heart was accessible. The ascending vena cava was cut just prior to its entry into the right auricle and the animal was perfused with ice cold 0·9 % NaCl by injecting the saline via a 27 gauge needle into the tip of the left ventricle. A needle of this size provided sufficient resistance so that tissue damage did not occur due to too great a pressure. The animal was perfused with approximately 5 ml. of saline in this manner in 30–60 sec. The pectoral muscles were raised as a group by a ligature tied just proximal to their severed insertions and the sternum was then divided in the mid line so that the origins of the muscles were not damaged. The rib cage was then cut through approximately $\frac{1}{8}$ in. from the centre line of the sternum, lateral to the origin of the muscles, and the muscles attached to the sternum at one end and held by the ligature at the other were removed in their entirety from the animal. An appropriately chosen weight of 1–4 g (modified from a fishing weight) was attached to the xiphoid by means of a second ligature. The free end of the ligature around the distal end of the muscles was then looped around one end of a light aluminium hook, the other end of the hook passing over a notch in the supporting bar in the incubation bath. By this means, the muscle hung freely in the incubation medium, extended by its load.

The incubation medium for these experiments was a Krebs–Henseleit bicarbonate Ringer solution (Umbreit *et al.* 1959) which was fortified with 150 mg glucose/dl., all essential amino acids

at 0·2 mM and 5 i.u. insulin/ml. Preliminary studies have indicated that these conditions gave a satisfactory preservation of the muscle structure during 1 hr incubation. The bath was both stirred and oxygenated with 95% O_2–5% CO_2 which had been pre-humidified as mentioned above. The incubation chamber sat in a water bath at 37·5 °C.

Tissues were incubated in the bath for chosen periods of time, and on removal the muscle was cut away below the upper ligature and from its insertion on the sternum. The portions of the muscle thus obtained were rapidly and lightly blotted, weighed wet, and then dried overnight to constant weight at 105 °C. The dried muscles were cooled and reweighed and then either rehydrated with 0·2 ml. of distilled water for 24 hr for tritium assay, or incubated directly in 0·1 N-HCl (1·3 ml.) for 72 hr for Na^+ and K^+ assays. Following rehydration they were dissolved in Protosol and prepared for scintillation counting in the same manner as that used for *in vivo* measurements. For measurements of interstitial space, [^{3}H]sorbitol was added to the bath to a concentration of 10 μc per 40 ml.

In a few experiments, a similar but larger chamber, containing 500 ml. of incubation medium with 100 μc of tritiated sorbitol was employed. In each case 20 μl. portions of the incubation mixture were counted in the same system to determine the specific activity (disintegrations/min. μl.) of the tracer. The ratio of the specific activity of the muscle (disintegrations/min. 100 mg wet weight) to the specific activity of the medium gives the number of microlitres of interstitial fluid per 100 milligrams wet weight of tissue.

In order to determine electrolyte contents, some samples were extracted for 72 hr in 0·1 *N*-HCl after being dried. At the end of the extraction Li^+ was added as LiCl to a concentration equal to that in a standard with 15 m-equiv. Li^+/l., prepared for flame photometry, which contained 100 m-equiv. Na^+/l. and 100 m-equiv. K^+/l. These solutions were then assayed by a standard technique on an IL 146 flame photometer and the Na^+ and K^+ contents of the tissues calculated.

The intracellular concentrations of Na^+ and K^+ were calculated from the equation:

$$\text{Intracellular concentration} = \frac{C_T V_T - C_E V_E}{V_T - V_E}.$$

C_T is the total concentration of the ion in m-equiv./l. total tissue water, and V_T is the total tissue water in microlitres (wet weight − dry weight). V_E is the i.s. in microlitres and C_E the concentration of the ion in the incubation medium (or plasma) with which the i.s. is equilibrated. Plasma Na^+ and K^+ were measured by standard methods. Plasma Na^+ was 149 m-equiv./l. (S.D. ± 1·8). Measurements of K^+ concentration showed a much wider variation. The mean was 6·7 m-equiv./l. with a range of 5·2–8·4 m-equiv./l. (S.D. = 1·3). The elevated values were probably due to the slight haemolysis which was visible in some samples. However, trial calculations showed that changes of as much as 2·5 m-equiv./l. in the external concentration of K^+ resulted in changes of only 0·8 m-equiv/l. in the calculated intracellular K^+ concentration. Values for non-haemolysed plasma were similar to the 5·4 m-equiv./l. found by Burns & DeLannoy (1966). Because of the relative insensitivity of the calculated intracellular concentration of K^+ to changes in the plasma K^+ we have used a standard plasma concentration of 5·4 m-equiv./l. in our calculations.

RESULTS

In preliminary experiments, we found [^{3}H]sorbitol to be a more satisfactory tracer for i.s. measurements in the mouse than radio-labelled inulin. The results that we obtained with its use by the methods described above are shown in Tables 1 and 2.

The results for *in vivo* experiments are the first group in Table 1. These results show that there is little change in the apparent i.s. between samples taken 60 min and 120 min after the intraperitoneal injection of [^{3}H]sorbitol. We have used the result for 120 min (i.s. = 14·4% of wet weight of the tissue) as our estimate of the equilibrium interstitial space of the mouse pectoral muscle *in vivo*.

The second group of results in Table 1 refer to measurements obtained with the *in vitro* incubation system. In the first two sets, the apparent i.s. was measured at time intervals from 10–60 min after the tissue had been placed in an incubation

medium containing [^{3}H]sorbitol. These results compare the rate of change of apparent i.s. in unloaded muscle and muscle loaded with 1 g. At all sample times, the unloaded muscle had a greater apparent i.s. than the muscle loaded with 1 g. Further, the loaded muscle appeared to reach an equilibrium value for the i.s. by 40 min, but the unloaded muscle did not appear to reach a steady state. In view of these results, further experiments were performed in which loads of 2 and 4 mg were applied to the muscle

TABLE 1. Apparent interstitial and intracellular spaces calculated from the distribution of sorbitol and measurements of dry weight by methods described in the text

Experimental conditions	Incubation time (min)	Apparent interstitial space (μl./100 mg wet weight)	Apparent intracellular space (μl./100 mg wet weight)
In vivo			
Five animals each time period	20	5·1 S.D. = 0·8	—
	60	12·4 S.D. = 4·0	—
	120	14·4 S.D. = 2·2	62·4
In vitro		(Range)	
No load, three animals each time period	10	20·2 (18·4–24·9)	
	20	33·7 (27·1–38·8)	
	40	45·1 (40·8–48·0)	
	60	52·0 (44·3–60·8)	27·4
1 g load, three animals each time period	10	15·3 (13·9–17·1)	
	20	22·9 (20·8–24·5)	
	40	33·1 (30·5–33·3)	
	60	31·3 (28·4–39·0)	46·9
2 g load, eight animals	60	29·7 S.D. = 5·9	43·7
4 g load, eight animals	60	33·2 S.D. = 3·4	46·8

and the apparent i.s. was measured at 60 min. All measurements for loaded incubated muscles give closely comparable values when measured at this time. Therefore, we have used a mean value of 31 % of wet weight as the standard interstitial space for loaded muscle *in vitro* after 60 min of incubation. Na^+ and K^+ were measured as described above.

The values for the i.s. from Table 1 were used in calculations of corrected intracellular concentrations for Na^+ and K^+. The results are shown in Table 2. The numbers in parenthesis after each type of preparation represent the number of animals used for that assay. For *in vivo* and *in vitro* experiments extracellular Na^+ and K^+ concentrations were known; the former by flame photometry of the serum samples from the mice, the latter by calculation from the known concentrations of the incubation medium. This data plus measurements of wet and dry weight and volume of the i.s. were substituted in the equation given in the Methods and the intracellular concentrations of Na^+ and K^+ calculated.

The amount of Na^+ found in the controls was somewhat greater than that which has been reported for rat muscles such as the quadriceps by Poole-Wilson & Cameron (1975), but, since there is such a wide variation in the literature regarding intracellular

Na^+ concentration, this result falls within the range that has been reported for other tissues. The K^+ concentrations of the controls are similar to those found by other authors.

As with the apparent i.s. there is a considerable difference between the results obtained for loaded and unloaded muscles. All of the loaded muscles show a slight, statistically not significant, increase in the amount of intracellular sodium and a larger, statistically significant ($P < 0{\cdot}01$) decrease in the concentration of intracellular K^+. There is an apparent decrease in the sum of these two monovalent ions of

TABLE 2. Calculated values for intracellular Na^+ and K^+ and percentage dry weight of mouse muscle

		m-equiv./l. cell water		
Experimental conditions	n	Na^+	K^+	Dry weight as % wet weight
In vivo control	17	37·4(7·8)	163·2(14·5)	23·2(1·55)
In vitro, no load	12	21·7*	177(34·8)	20·6(1·36)
1 g load	15	44·8(16·7)	132(13·8)	21·1(0·9)
2 g load	8	50·9(33·1–62)†	132(114–150)†	23·5(1·2)
4 g load	8	41·4(32·8–50·7)†	135(121–148)†	23·1(1·0)

Numbers in parentheses are ± S.D.

* Some animals in this group had calculated Na^+ concentrations of less than 0; see text.

† Four animals in each group, numbers in parenthesis are range.

approximately 20 m-equiv./l. By contrast, results for muscles which had not been subject to load showed a much greater variance of calculated concentrations as indicated in the table. In particular, the apparent Na^+ content was considerably diminished. No statistical parameters could be calculated for the intracellular Na^+ in the unloaded *in vitro* preparation since, in some cases, the calculations gave a negative value for the concentration of Na^+. We interpret this unusual result as indicating that under these conditions sorbitol has penetrated into a space in the muscle from which Na^+ is wholly or partly excluded.

In summary, the results for intracellular electrolyte concentrations are similar to those for distribution of sorbitol in the i.s. That is, there are relatively few differences between the results obtained from animals with varying load placed on them during incubation, but a marked difference between these and the unloaded muscles. There is an apparent loss of K^+ from the muscle during the period of incubation of approximately 20 % and a small (up to 10–15 %) increase in the apparent intracellular Na^+ concentration.

The last column of Table 2 summarizes results obtained from the dry weight as percentage of wet weight. These results show that there is virtually no change in this ratio for those muscles which were loaded with 2 and 4 g as compared with the *in vivo* controls. Muscles which were incubated with no load or 1 g show a slight, but significant ($P < 0{\cdot}005$) decrease in the percentage dry weight, though with greater variance, and again there is a statistically significant difference ($P < 0{\cdot}005$) between these tissues and the *in vivo* controls in this quantity.

For histological studies the muscles were fixed *in situ*. As an *in vivo* control a mouse

was prepared as described and perfused with saline. Before removing the muscle, the animal was perfused with a further 10 ml. of 2·5 % glutaraldehyde and 2 % paraformaldehyde in 0·2 M, pH 7·4 cacodylate buffer. The muscle was removed after fixation. The incubated muscles were fixed while still suspended by replacing the incubation medium with the same fixative, in which they were allowed to remain for 1 hr. Tissues for light microscopy were then transferred to 10 % formalin. They were then processed by standard techniques and haemotoxylin and eosin stained sections obtained. Tissue for electron microscopy was post-fixed in osmium tetroxide, embedded in Epon, and thin sections for electron microscopy obtained by standard techniques. The sections were examined at various levels of magnification in both the light and electron microscope.

Sorbitol measurements indicated that the i.s. has doubled in volume *in vitro* as compared with its size *in vivo*. However, we were unable to find consistent changes in the histology of the muscles in the light microscope which would have provided a morphological parallel to the sorbitol measurements. Portions of the tissue showed some splitting between the myofibres, particularly in the unloaded muscles. However, in no case did the whole muscle show changes which could be interpreted as a generalized increase in the extracellular space.

Our electron microscopic studies concentrated on the intracellular structure of the muscles. The Plate shows representative sections of muscle, all at the same magnification. *A* is control *in vivo* muscle; *B* is unloaded incubated muscle; *C* is muscle incubated with a 1 g load; and *D* is incubated muscle with a 4 g load. Muscle with a 2 g load was similar to that with a 1 g load in all respects. We paid particular attention to the sarcomere length; the structure of the mitochondria; the width of the intermyofibrillar spaces; and the appearance of the sarcoplasmic reticulum.

Sarcomere length was measured on twenty-five randomly chosen sarcomeres in low power electron micrographs of each muscle sample. Only the muscle incubated with a load of 4 g, with a sarcomere length of 2·22 μm (S.D. = 0·15), showed a statistically significant change from the control sarcomere length of 1·77 μm (S.D. = 0·11).

Although there were no other consistent quantifiable differences between the various muscle samples, other qualitative differences were found. The mitochondria in all samples were well preserved. However, mitochondria in muscle from a 4 g load incubation showed the presence of dense granules. These are known to appear when muscle is subjected to any of a number of metabolic insults (Weiss, 1955; Zacks & Sheff, 1970).

The intermyofibrillar spaces in unloaded and 1 and 2 g loaded muscles also appeared to be enlarged as compared with those in control and 4 g loaded muscles. When the width of these spaces was measured in moderate power (31,000 ×) electron micrographs the spaces in the control muscle never exceeded 0·2 μm in width. On the other hand at least 20 % of the spaces in all the incubated tissues with the exception of those with a 4 g load were between 0·2 and 0·3 μm in width.

As we discuss below, the sarcoplasmic reticulum is important as a Na^+ containing space and alterations in its size can change the apparent intracellular Na^+ concentration. Examination of the micrographs leaves the impression that this component is more pronounced in the incubated specimens (again with the exception of that

loaded with 4 g) than it is in the controls. However, its appearance may have been enhanced by the increase in width of the intermyofibrillary space where it is most easily seen. We could not measure any differences in the width of the tubules of the reticulum between controls and incubated specimens.

Our general conclusion from the histological studies is that there are no significant consistent differences in structure between the control muscle and all of the experimental samples, except for the muscle loaded with 4 g. With the exception of the hyperextension and mitochondrial dense bodies found in the 4 g loaded muscle none of the changes seen in the other incubated muscles are indicative of pathological alterations. Further, the changes seen in the microscope do not correlate with the changes in interstitial space measured with [^{3}H]sorbitol. The differences between the i.s. in the control, the unstretched muscle and the loaded muscles have no morphologic parallel. The i.s. of the 4 g muscle, as measured with [^{3}H]sorbitol is very similar to that found for the other weight loaded muscles while its morphology shows significant differences. In general, the incubated samples show no evidence of having undergone degradation as a result of incubation.

DISCUSSION

As was stated in the introduction we undertook this study in order to determine how this new preparation behaved *in vitro* with respect to its i.s. and electrolyte content. We have looked for morpological changes in order to see whether the muscle underwent pathological alterations during incubation. Our intent is to use these observations as a basis on which to assess the degree to which the muscle achieves a steady state during incubations, and how that steady state differs from that found *in vivo*.

The results we found for the size of the i.s. *in vivo* are similar to those reported in the literature for rats, rabbits, and frogs. Poole-Wilson & Cameron (1975) measured the i.s. in rabbit quadriceps and found a volume of 9–10 % of the wet weight of the tissue. Results for the rat gastrocnemius (by influx studies) were given by Macchia *et al.* (1979). They found that, for sucrose but not for sulphate, the apparent extracellular space was approximately 15 % of the wet weight of the tissue. Rogus & Zierler (1973) measured efflux rates from rat extensor digitorum longus *in vitro* using sucrose and found a total sucrose space of approximately 20 % of wet weight. These authors also used inulin and, in long-term studies, found an inulin space of 13 % of the wet weight of the muscle. Our data showing that the sorbitol space of mouse skeletal muscle *in vivo* is 14·4 % of wet weight of the tissue is thus in good accord with previously published data for other mammals.

A space of similar size has been found for frog sartorius muscle by a variety of methods, from the early studies with inulin, sodium and magnesium (Boyle, Conway, Kane & O'Reilly, 1941) to recent data (Neville & White, 1979) in which the kinetics of efflux of a variety of small molecular and ionic loads were measured.

The increase in the apparent extracellular space found after incubation is also consistent with the literature. Neville & White (1979), in reviewing the literature and results of their own experiments, found an increase of approximately 50 % in the extracellular space of frog sartorius muscle studied *in vitro* as compared with that

of the freshly excised muscle. We can find no comparable *in vitro/in vivo* studies using the same tracer in both systems in mammalian muscle, but the sucrose space of 20 % reported for rat muscle by Rogus & Zierler (1973) is larger than the *in vivo* space reported for the same muscle by Macchia *et al.* (1979) and Poole-Wilson & Cameron (1975) as cited above.

The origin of this increased i.s. is important in assessing the comparative functioning of the muscle *in vivo* and *in vitro*. The lack of change of the dry weight to wet weight ratio places a constraint upon the source of the increased i.s. Although we have reported small decreases in this ratio for unloaded muscle and that incubated with a 1 g weight, the change, though statistically significant, amounts to an increase in water content of less than 5 μl./100 mg wet weight of muscle. This is far less than the increase in i.s. Thus, the increase in i.s. must either be at the expense of another compartment into which sorbitol penetrates *in vitro* but not *in vivo*, or is due to a portion of the extracellular space being less available to the tracer *in vivo*. Since we calculate the intracellular space by subtracting the measured i.s. from the total water content of the muscle either of these possibilities would result in an apparent decrease in the intracellular space.

The two possible non-extracellular compartments into which sorbitol might enter during incubation are the sarcoplasm and the sarcoplasmic reticulum (s.r.). The s.r. is totally within the muscle cell, but is a morphologically distinct space. Our electrolyte measurements can be used to assess the possible entry of sorbitol into these spaces.

Our results for K^+, which are similar to those found by other workers, are not informative in this respect. However, our results for the intracellular concentration of Na^+ is higher than that found by other workers, particularly those (cited below) who measured Na^+ by efflux methods. We believe that this difference is due to the fact that our method will include the s.r. as part of the intracellular space unless the sorbitol penetrates into it, whereas efflux methods separate the Na^+ in the s.r. from that in the sarcoplasm by measuring their differing efflux rates. Harris (1963) concluded that much of the 'intracellular' Na^+ was in the s.r. and not in the sarcoplasm, and this conclusion has been supported by Birks & Davey (1969), Rogus & Zierler (1973), and Neville & White (1979). Rogus and Zierler estimated that 94–98 % of measured 'intracellular' Na^+ was in the s.r., and that the volume of the s.r. was 12·4 μl./100 mg wet weight of muscle in the rat. Further, their efflux studies indicated that the Na^+ in the s.r. was in rapid equilibrium with the Na^+ in the extracellular fluid. If this applies to mouse muscle, then we can estimate the volume of the s.r. in the muscles by taking it to be the volume which would be occupied by 96 % of the intracellular Na^+ if it were at the same concentration (143 m-equiv./l.) as it is in the incubation medium for *in vitro* studies or at plasma concentration (149 m-equiv./l.) for *in vivo* studies. This calculation yields the following volumes in μl./100 mg wet weight for the s.r.: *in vivo*, 15·1 μl.; incubated muscles: with 1 g, 14·1 μl.; with 2 g, 14·9 μl.; with 4 g, 13·0 μl.

These estimates are consistent with the results of Rogus & Zierler (1973) and also show little change between muscle *in vivo* and *in vitro*. Thus, although the volume of the s.r. is similar to the increase in the i.s. found *in vitro* it is unlikely to be the source of that increase. If sorbitol had penetrated into the s.r. *in vitro* but not *in vivo*

both the calculated s.r. space and the apparent intracellular concentration of Na^+ would be greatly diminished. These considerations also apply to a possible intracellular penetration of sorbitol *in vitro*. If sorbitol penetrates into the sarcoplasm of a muscle which is still capable of excluding Na^+ by active mechanisms, then our measurements of intracellular sodium would be decreased. Further, the calculated value for intracellular K^+ would be increased. We believe this to be the case for muscles incubated without load, and take it as an indication of abnormal membrane function in those samples. The results for the other incubated muscles are not consistent with this possibility. If the incubated muscles became 'leaky' so that the sarcoplasm could equilibrate with the incubation medium, then we would expect to find a large increase in the apparent interstitial space, but the calculated electrolyte concentrations in the reduced apparent intracellular space would not be significantly reduced. However, such a system would not reach an equilibrium value for the i.s., whereas in our system all of the loaded incubated muscles reached such an equilibrium relatively rapidly.

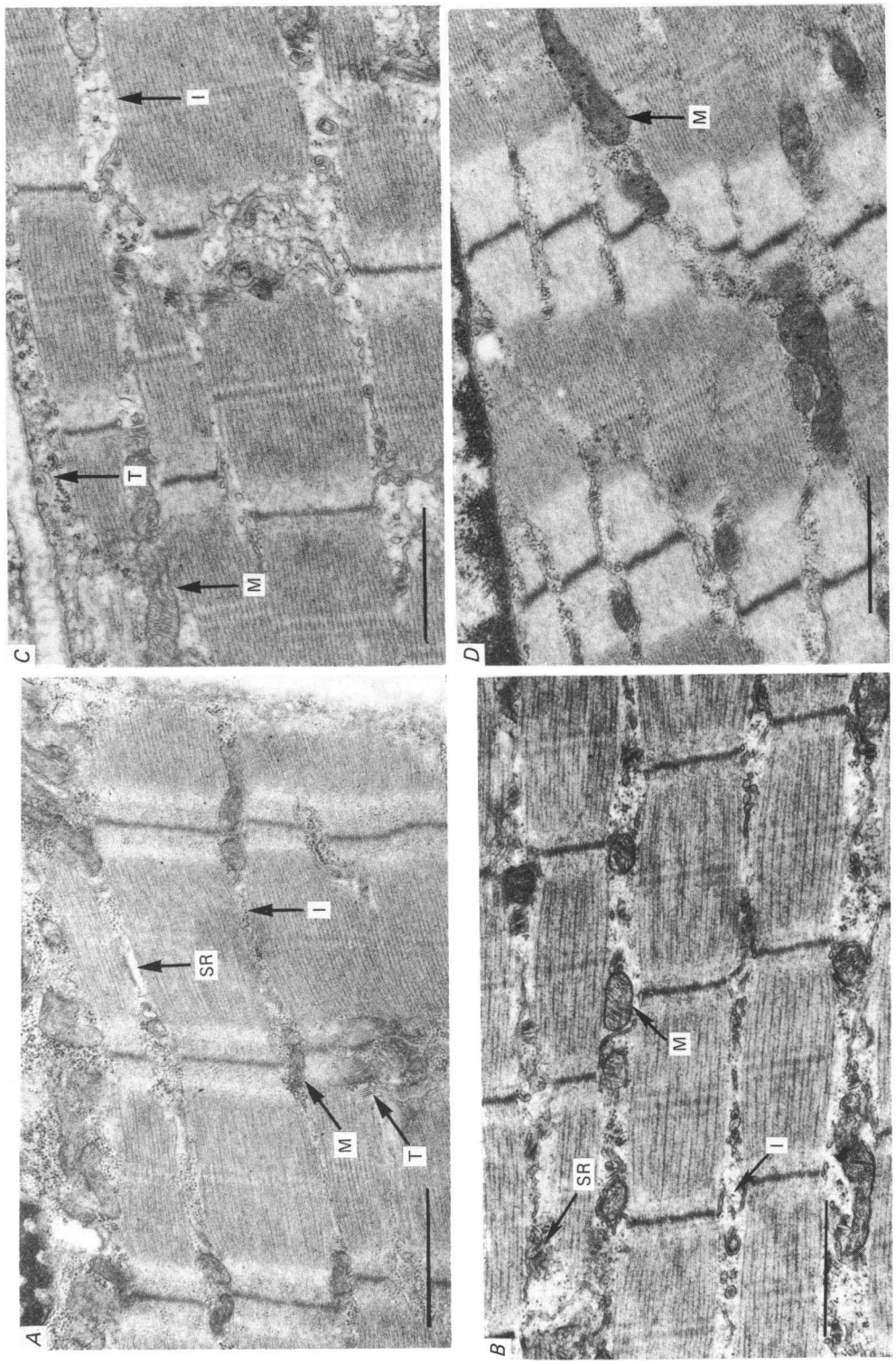
We therefore conclude that our electrolyte and i.s. measurements indicate that during incubation the sorbitol can enter into a portion of the extracellular space which is not readily available to it *in vivo*. We suggest that this arises from the anatomic structure of our preparation. *In vivo* sorbitol reaches the i.s. by diffusion through capillary walls. The myofibres are richly supplied with such capillaries while the connective tissue is much less well-vascularized. The complexity of the connective tissue space has been reviewed by Aukland & Nicolaysen (1981) and it presents many barriers to diffusion. Thus, *in vivo*, a perivascular i.s. may be readily available for rapid diffusion of the sorbitol while diffusion to the relatively avascular portions of the connective tissue is very slow. If the two diffusion rates are sufficiently different an apparent equilibrium space will be measured which corresponds to the readily available i.s. *in vivo*.

Conversely, *in vitro*, both surfaces of the thin sheet of muscle are completely exposed to the incubation medium. Thus, there is an easier diffusion path for the sorbitol into the connective tissue space. In addition the complex glycoproteins on the surfaces of the connective tissue matrix may have a different degree of hydration in the *in vitro* system and thus provide less of a barrier to diffusion.

Our experiments cannot answer the question as to which is the 'true' interstitial space, and hence the 'true' intracellular space. However, they are sufficient to answer the question asked at the beginning of the study.

The results indicate that a satisfactory preparation of mouse skeletal muscle can be made as described. The ultrastructure of the muscle is not significantly altered during incubation. Providing that a sufficient, but not too great a load, is placed on the muscle during incubation it maintains electrolyte levels which are comparable with those *in vivo*, particularly when the contribution of the s.r. is taken into account. Muscles with 1 or 2 g loads come to a satisfactory equilibrium with the incubation medium and do not appear to permit inert tracers such as sorbitol into the sarcoplasm. Conversely, muscles incubated without a load appear to make unsatisfactory preparations, and in particular appear to undergo changes in cell permeability. Loads of 4 g per muscle result in hyperextension of the muscle accompanied by electron microscopic evidence of metabolic insult.

This study points up the necessity of specifying the conditions of incubation very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closely and the importance of measuring the effects of incubation *per se* if valid results are to be obtained from *in vitro* studies of muscle function.

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EXPLANATION OF PLATE

The plate shows electron micrographs of tissues prepared as described in the text. *A*, the control tissue; *B*, the tissue incubated without load; *C*, the tissue after incubation with a 1 g load; *D*, the tissue after incubation with a 4 g load. The final magnification is 31,000 × and the bar in the corner of each micrograph is 1 μm in all cases. M, mitochondria, I, intermyofibrillar spaces; SR, sarcoplasmic reticulum; T, transverse tubular system.