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DIFFUSIBLE SODIUM, POTASSIUM, MAGNESIUM, CALCIUM AND PHOSPHORUS IN FROG SKELETAL MUSCLE

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SUMMARY

1. A microvolumetric analytical method has been developed to measure the endogenous concentrations of diffusible elements in muscle cells.

2. Single twitch fibres from frog muscle were skinned under oil and 0·2 nl drops of isosmotic sucrose solution, held in the tips of specially constructed pipettes, were placed in contact with the skinned fibres. After 0–10 min, the microdrops were removed and analysed with a wave-length dispersive X-ray spectrometer.

3. The uptake of Na, K, Mg, and P into the microdrops was well fitted by a single exponential function, while the uptake of Ca was better represented by the sum of two exponential functions. All elements analysed except Ca reached diffusional equilibrium within 5 min of placing the microdrop on the fibre, while Ca was still not equilibrated at 10 min.

4. For freshly isolated muscle fibres, diffusible element concentrations in the microdrops at equilibrium were (in mM, mean $\pm$S.D.): Na, $7{\cdot}6 \pm 7{\cdot}2$; K, 82 ± 36; Mg, $5{\cdot}8 \pm 3{\cdot}0$; P, 51 ± 19. Diffusible Ca concentration (at 10 min elapsed sampling time) was $0{\cdot}7 \pm 0{\cdot}4$ mM.

5. Results from experiments in which microdrops were equilibrated with skinned fibres pre-soaked in an artificial (Ca-free) solution support the notion that the exogenous solutes can replace the endogenous diffusible contents of a skinned fibre by soaking the skinned fibre in a relatively large volume of the artificial solution.

6. Total Na, K, Mg, Ca, and P content of whole muscle was measured by electron probe analysis of muscle extracts. In freshly isolated muscle, whole muscle element content was (in mmol/kg wet weight, mean $\pm$S.D.): Na, 21 ± 8; K, 120 ± 26; Mg, $9{\cdot}7 \pm 2{\cdot}6$; Ca, $2{\cdot}2 \pm 0{\cdot}5$; P, 76 ± 18.

7. Extracellular fluid volumes of freshly isolated whole muscles were estimated by compartmental analysis of Na efflux. Extracellular element concentrations were measured by electron probe analysis of frog plasma. Using the extracellular fluid volume and concentration estimates, extracellular contributions were subtracted from measurements of the element content of whole muscle to yield estimates of total intracellular element concentration (in mmol/l myoplasmic water).

8. Based on the values for the intracellular total and diffusible element concentrations, the diffusible/total content fraction in freshly isolated muscle is estimated to be: Na, 0·38; K, 0·48; Mg, 0·42; Ca, 0·22; and P, 0·47.

INTRODUCTION

Na, K, Mg, Ca and P are important constituents of all living cells. In muscle, it is widely recognized that diffusible ionic forms of these elements play direct roles in generating the transmembrane resting and action potentials (Na^+ and K^+) and in generating and regulating muscle force and shortening (Mg^{2+}, $MgATP^{2-}$, and Ca^{2+}). The skinned muscle fibre (Natori, 1954) is a particularly useful preparation for investigating direct effects of these diffusible ions on contractile and metabolic processes, since removal or disruption of the sarcolemma permits direct access to the interior of the cell (see review by Stephenson, 1981).

It is generally assumed that the fluid contents of a skinned fibre can be replaced with an artificial solution by soaking the skinned fibre in a relatively large volume of the solution. This suggests the hypothesis that freely diffusible solutes can equilibrate between the extracellular and intracellular solvent phases in skinned fibres. The present experiments were carried out to test this hypothesis, as well as to gain some information about the actual concentrations of the endogenous solutes themselves.

It is rarely possible to distinguish the concentration of a freely diffusible solute from that of its bound forms inside a cell; in most cases, only total concentrations of a solute can be measured. For example, Ingram, Ingram & Hogben (1972), Somlyo, Shuman & Somlyo (1977) and Somlyo, Gonzales-Serratos, Shuman, McClellan & Somlyo (1981) recently used electron probe methods to examine the intracellular distributions of elements in frog muscle cryosections, but, because the spatial resolution could not reach the molecular level, the element contents measured included both diffusible and bound forms. For Na, Somlyo *et al.* (1981) found a relatively uniform 38 ± 23 mmol/kg dry fibre mass over a 20–300 μm^2 range of myoplasmic areas. (Means ± 1 S.D. given throughout text unless otherwise indicated.) This average corresponds to 11 ± 7 mmol Na/kg fibre water (assuming 0·30 kg dry fibre/kg fibre water: Somlyo *et al.* 1981), similar to that ($9{\cdot}2 \pm 1{\cdot}0$ mmol Na/kg fibre water) obtained by Ingram *et al.* (1972). Other element concentrations obtained by Somlyo *et al.* (1981) correspond to 129 ± 29 mmol K/kg, 12 ± 4 mmol Mg/kg, $2{\cdot}6 \pm 1{\cdot}4$ mmol Ca/kg and 82 ± 18 mmol P/kg fibre water. The concentrations of the diffusible forms of these elements, however, are likely to be less than these values.

Recently, Horowitz, Paine, Tluczek & Reynout (1979) developed an ingenious method of measuring the concentrations of freely diffusible solutes in frog oocytes. In their method, a droplet of gelatin is injected into a cell. The gel droplet excludes the cytoplasm, but diffusible solutes enter, reaching diffusional equilibrium with it. The method of Horowitz *et al.* (1979) requires that the volume of the injected droplet be comparatively small to minimize dilution of the cell fluid and serious disruption of the intracellular structures. Yet the droplet must be large enough to allow manipulation and analysis of the droplet using conventional microanalytical methods. Horowitz *et al.* (1979) used 50–100 nl droplets whose dimensions relative to the much larger oocyte easily satisfied these volume criteria. In contrast, muscle cells from vertebrates (such as frogs) are comparatively tiny, and they possess an organized myofilament lattice that will not accommodate 50–100 nl gel droplets without introducing serious dilution and disruption artifacts.

In this paper we describe a sampling and spectrometric method which, though similar in some respects to the method of Horowitz *et al.* (1979), allows the concentration of diffusible elements to be measured directly in vertebrate muscle cells. A brief description of this method and some preliminary results have been published (Maughan & Lechene, 1980; Maughan, 1982; Maughan, 1983). In this method, a relaxed muscle fibre is placed under oil and stripped of its sarcolemma, as in the technique of Natori (1954), and a comparatively small droplet of isosmotic sucrose solution placed *on* the membrane-free surface of the stripped fibre rather than *in* it. After equilibration with the cell fluid, the microdrop is removed, freeze-dried, and, along with calibration standards, analysed with an X-ray spectrometer to yield direct estimates of the concentrations of freely diffusible elements.

TABLE 1. Composition of Ringer solutions. All solutions at pH 7·2 (20 °C). The $CaCl_2$ level in propionate (prop.) containing solution D was increased to keep the Ca^{2+} activity equivalent to that of the propionate-free solution A. Isotonic cobalt-Ringer solution was made by adding 10 mM-$CoCl_2$ and by subtracting 17 mM-NaCl or Na propionate. Ferritin-containing Ringer solution was made by adding 10 g calcium-free horse spleen ferritin (ICN Pharmaceuticals, Plainview, NY, U.S.A.) to 100 ml of solution A. All chemicals, unless otherwise noted in text, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

	Concentrations (mM)							
Solution	KCl	NaCl	$CaCl_2$	$TrisCl_2$	HEPES	Na prop.	Na H_2PO_4	Na_2 HPO_4
A	2·5	109	1·80	10	—	—	—	—
B	2·5	119	1·80	—	3·0	—	—	—
C	2·5	115	1·80	—	—	—	0·85	2·15
D	2·5	—	2·88	10	—	108	—	—

METHODS

Preparation

Muscle fibres were obtained from *Rana pipiens* (Hazen's Frog Farm, Alburg, VT, U.S.A.) and *Rana temporaria* (Charles Sullivan Co., Nashville, TN, U.S.A.). The frogs were kept in fresh-water pens (20 °C) and sacrificed by decapitation within approximately 1 week of delivery. Semitendinosus muscles were isolated and adherent connective tissues, fat plaques and small blood vessels removed. In some experiments, the anterior branch of the tibialis muscle from *R. temporaria* was also isolated.

Two preliminary procedures for obtaining isolated skinned fibres were used: in the first procedure, muscles were isolated and then incubated for 10–90 min in solution A (Table 1) containing 1–3 μg tetrodotoxin/ml, or in solutions B–D (Table 1). Each muscle (referred to throughout as an 'incubated muscle') was removed from the incubating solution, blotted gently with filter paper, and transferred to a glass bottom dish (8 cm diameter, 1 cm deep) half-filled with water-saturated mineral oil (Alrich Chemical Co., Milwaukee, WI, U.S.A.). In the second procedure, freshly isolated muscles were blotted and directly placed under the water-saturated mineral oil. Thus these muscles (referred to throughout as 'freshly isolated muscles') were never exposed to any artificial solution before being immersed in oil. The temperature of the oil and fibre were maintained at 20 °C with a thermoregulating Peltier device (Cambion, Cambridge, MA, U.S.A.).

Under oil, a bundle of 20–30 surface fibres was clipped from the dorsal head of a muscle. A single 1 cm long fibre segment was then isolated by progressively subdividing the bundle. The single fibre segment was manually stripped of its sarcolemma (skinned) by clasping one end with forceps and peeling back the sarcolemma with a sharp needle (Natori, 1954). This exposed the underlying

myofibrils and intracellular fluid. The sarcolemma was discarded along with adhering myofibrils and extracellular fluid. The skinned fibre segment was then straightened and trimmed to 0·5 cm.

Some fibre segments were obtained from whole fibres rather than cut fibre bundles. Whole fibres were isolated (tendon to tendon) in solution B (Table 1) containing 20 μg *d*-tubocurarine/ml. Following the dissection, one tendon was attached to an adjustable mechanical ground and the other tendon was attached to a strain gauge (Kulite, Ridgefield, NJ, U.S.A.). The viability of the fibre was tested with an electrical stimulus and the isometric twitch and tetanus force were recorded. Usable fibres sustained 2 s tetani of at least 0·3 N/mm^2 (striation spacing 2·2 μm) and relaxed completely following stimulation. The fibre was then transferred to the oil-filled dish in a Ringer-filled 'transfer boat'. Nearly all of the Ringer solution clinging to the fibre was stripped off by pulling the fibre into the oil. The fibre was then skinned and trimmed as described above.

Each muscle segment was photographed through an inverted compound microscope (with 0·1, 0·25, and 0·65 numerical aperture lenses: Olympus Corp., New Hyde Park, NY, U.S.A.) using a 35 mm camera. Print enlargements were made, and fibre segment lengths and widths measured directly from the fibre image against a calibrated grid. Mean striation spacing was 2·1–2·3 μm, estimated by averaging the length of ten sarcomeres at five places in the region sampled.

Water-saturated mineral oil was produced by shaking one part distilled/de-ionized water and two parts mineral oil for 2 h, centrifuging the mixture (1000 *g* for 15 min), and drawing the water-saturated oil off the top.

Equilibration of 0·2 nl microdrops with fibre fluid

Fig. 1 (top panels) illustrates the method of equilibrating small droplets with a skinned muscle fibre. The microdrop was formed as follows: a specially constructed glass pipette was inserted into the oil and a small amount of oil aspirated into the pipette. The pipette was transferred to a drop of 0·25 M-sucrose (+5 μM-EGTA) solution adjacent to the fibre, and a microdrop aspirated into an elongated cavity at the tip of the pipette (Fig. 1*A*). The glass pipette was linked via air-filled tubing to a syringe used to draw and expel the oil and microdrop fluid.

Microdrop volumes ranged from 0·1 to 0·3 nl (about 0·1–1 % of the volume of the skinned fibre). A microdrop in the tip of a pipette was transferred to the fibre and the microdrop placed in contact with and held against the surface of the skinned fibre (Fig. 1*B*). Contact was confirmed by eye using an inverted microscope. The 5 μM-EGTA in the microdrop chelated trace Ca^{2+} which would have otherwise activated the fibre upon contact.

Surface tension between the water and oil phases in the pipette constriction stabilized the microdrop. However, we noted in preliminary experiments that a microdrop containing $\leqslant$0·25 M-sucrose tended to be drawn immediately into the fibre unless suction was applied to the pipette, whereas microdrops containing $\sim$ 0·25 M-sucrose tended to be stable. This result suggests that the osmolarity of frog myoplasm is $\sim$ 0·27 osmol/l, assuming an osmotic coefficient of 1·08 for the 0·25 M-sucrose solution in osmotic equilibrium with it (Weast, 1973).

After 0·5–10 min of contact with the fibre, the pipette was removed and the microdrop drawn up into the shank of the pipette (Fig. 1*C*) between oil droplets, thereby isolating the microdrop between oil seals and preventing evaporation. The procedure was then repeated, using other pipettes and droplets, and different contact times. Finally, a sample of sucrose/EGTA solution was aspirated into a pipette to establish 0 min contact concentrations (background levels). After sampling, the pipettes with their microdrops were immediately frozen and stored at −70 °C.

Electron probe analysis of microdrops

Fig. 1 (bottom panels) illustrates the method of analysing microdrops according to the X-ray spectrometric method of Lechene & Warner (1979). Details of the method are presented in Bonventre, Blouch & Lechene (1980). Pipettes with their frozen microdrops were shipped (frozen with dry ice) to the National Laboratory of Biotechnology Resource in Electron Probe Microanalysis (NBREM: Harvard Medical School, Boston, MA, U.S.A.). At NBREM the microdrops were thawed, expelled under water-saturated oil from the pipettes (Fig. 1*D*), and 1–3 ultramicrodrops aspirated into a carefully calibrated 20–30 pl volumetric pipette (also shown in Fig. 1*D*). The volumetric pipette was then transferred to a beryllium block, and the ultramicrodrops expelled under water-saturated oil. Five calibration ultramicrodrops (containing various known concentrations of the elements selected for analysis) were deposited alongside the sample ultramicrodrops (Fig. 1*E*) using the same volumeteric pipette (re-cleaned).

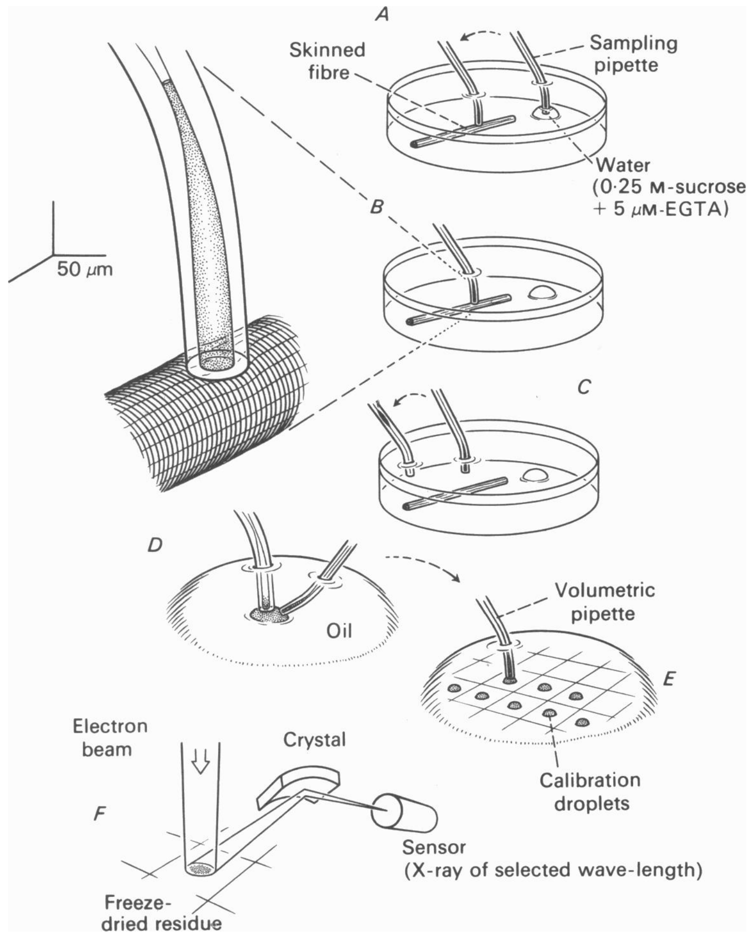

Fig. 1. Protocol for sampling and analysing muscle fibre fluid using microdrop equilibration and X-ray spectroscopy. *A*, technique of placing the microdrop on the surface of a skinned muscle fibre segment. The microdrop is formed in the 0·1–0·3 nl chamber in the tip of the sampling pipette. Both microdrop and fibre are under oil. *B*, equilibration. The microdrop, still contained in the end of the pipette tip, is allowed to equilibrate with the fibre fluid for 0–10 min. The enlargement (to scale) illustrates the microdrop and fibre in contact. *C*, removing the microdrop. The microdrop is aspirated into the shank of the pipette between oil droplets, frozen, and stored at −70 °C. *D*, ejection of thawed microdrop from sampling pipette and aspiration of 2–3 aliquots of the microdrop by volumetric pipette (composite illustration). Both procedures performed on an oil-covered beryllium platform. *E*, calibration. The aliquots are ejected onto another oil-covered beryllium platform beside five ultramicrodrops (of identical volume) from calibration solutions. (The same volumetric pipette is used to deposit the sample aliquots and calibration droplets.) The highly polished beryllium platforms have scratch grids which are used to identify the location of each sample. *F*, analysis. The oil is removed by washing with *meta*-xylene, and the fluid samples and calibration droplets are frozen and freeze-dried. The platform with its freeze-dried residues is placed in a scanning electron microscope equipped with a wave-length dispersive X-ray spectrometer. Each residue is exposed to the electron beam for 10 s, and the number of photons counted by a photon detector. The wave-length of the characteristic X-ray is selected by moving an appropriate diffraction crystal into the path of some of the X-ray photons emerging from the bombarded residue. The number of photons emerging is proportional to the amount of each element in the residue and, therefore, the concentration of the element in the microdrop; the proportionality constant is determined by the calibration solutions. The sampling was conducted at the University of Vermont School of Medicine, the X-ray analysis at the National Laboratory of Biotechnology Resource in Electron Probe Microanalysis at Harvard Medical School.

The oil on the block covering the ultramicrodrops was removed by washing with *meta*-xylene and the samples immediately frozen in isopentane at −150 °C. The ultramicrodrops were freeze-dried under vacuum (10^{-5} Torr, −70 °C), resulting in a thin uniform crystal residue 20–40 μm in diameter. The entire residue was exposed to the beam of a Cameca MS 46 microprobe (Cameca, Courbevoie, Paris) and the number of X-ray photons of a characteristic wave-length emitted from the residue recorded and counted for 10 s by an X-ray detector (Fig. 1*F*). X-ray emissions were selected using tuned potassium acid phthalate and pentaerythritol diffracting crystals. Typical electron beam parameters were: accelerating potential 20 keV, current 200 nA, diameter 50 μm.

Different spectrometer settings were used to examine Na, K, Mg, Ca, P, Cl, S, Zn, Co, and Fe. Preliminary tests of microdrops equilibrated with freshly isolated fibres showed no detectable signals from Zn, Co and Fe over background counts. X-ray counts from the other elements (Na through to S) were taken from each ultramicrodroplet residue (of the several from each microdrop), and the counts averaged to yield a count rate for each microdrop. Linear regression lines relating count rate to concentration in the standard solutions were used to determine element concentrations in the samples. Linear regression coefficients were typically 0·999 (see Bonventre *et al.* 1980, for representative examples of the calibration curves). The sensitivity of the analysis was about 0·1 mM (typical background level).

Preliminary tests suggested that some Cl and S vaporizes during the analysis. Loss of both these elements results from drying or ashing Cl- and S-containing material under conditions where little or no loss of Na, K, Mg, Ca and P occurs (see Results). Thus, some Cl and S may be vaporized by the electron beam during the electron probe analysis (Borland, Biggers & Lechene, 1977), the extent of Cl and S loss depending on a balance between the escape of volatile compounds of these elements into the vacuum and retention of these elements by other materials which burn and coat the sample. Because of the uncertainties associated with Cl and S measurements, we omitted them from the present analysis.

Construction of 0·2 nl sample pipettes

The pipettes used to hold the microdrops were constructed from borosilicate glass capillary tubing (Kimble Division, Owen-Illinois, Inc., Toledo, OH, U.S.A.). The tubing was heated and drawn with a De Fonbrune microforge (Sage Instruments, Cambridge, MA, U.S.A.) to form a thin, hollow segment used to store single or sequential (serial) liquid samples. Near the tip, a constriction was produced with the microforge to form a 0·1–0·3 nl elongated cavity used to hold the microdrop (Fig. 1*B*: inset). Microdrop dimensions were: diameter at pipette tip 34·7 ± 4·8 μm, length 315·5 ± 51·7 μm, volume 191·1 ± 63·1 pl ($n = 38$). Microdrop volumes were measured in each pipette by drawing a representative droplet into the cylindrical shank of the pipette, photographing it, and calculating the cylindrial volume from dimensions taken from the photograph.

The pipettes were cleaned in acetone and chloroform, and then siliconized to reduce wetting of samples to the walls of the pipette. Siliconization involved hydrating the cleaned pipettes in a humidifying chamber overnight, flushing the pipettes with a 7 % v/v solution of silicon-containing Surfacil (Pierce Chemical Co., Rockford, IL, U.S.A.) in chloroform, and rinsing the pipettes in toluene. Between uses, the pipettes were recleaned and stored in a dust-free sealed box.

Elemental analysis of whole muscle and blood plasma

To obtain a measure of what fraction of the total intracellular element content is diffusible, fluid extracts of freshly isolated *R. pipiens* semitendinosus muscle were analysed. Muscles were blotted, weighed, and dried overnight at 100 °C. The dried muscles were reweighed and ashed overnight in platinum crucibles at 600 °C. The ash from each muscle was dissolved in a volume of 0·1 M-ammonium citrate corresponding to the wet weight of the muscle minus the dry weight. The extracts were then transferred to Pyrex vials and shipped to NBREM for analysis.

Plasma concentrations (required for the extracellular corrections) were obtained as follows: immediately after sacrifice, 20–80 μl of blood was drawn from the ventricle of the exposed heart into a heparinized syringe. The blood was transferred to a plastic vial, centrifuged, and the plasma removed. Ice cold ethanol (four times the measured volume of the supernatant) was added to precipitate proteins. The mixture was shaken in a vortex mixer and centrifuged (3000 *g* for 1 min). The plasma supernatant was transferred to a Pyrex drying tube and spun overnight in a vacuum evaporator. Distilled water (equal to the volume of plasma supernatant minus the volume of precipitated proteins and trapped plasma) was added to the residue. The extract was transferred to Pyrex vials and shipped to NBREM for analysis.

RESULTS

Validation of method

Dessication of fibre in oil. Skinned fibres under oil slowly dessicate, even in water-saturated oil (Maughan & Godt, 1979). To examine the rate of water extraction from a skinned fibre under the present conditions, widths were carefully measured at various times at the same location on a fibre. The rate at which the widths decreased ranged from 0 to 0·044 %/min. The corresponding rate of volume decrease was 0–0·088 %/min; i.e. as much as 5 % over the 60 min duration of a typical experiment. Since the solute in the fibre fluid becomes concentrated as water is taken up by the oil (Lechene & Warner, 1979), the elements may concentrate by as much as 6 % during a typical hour-long experiment (assuming an 88 % fluid content: Baylor, Chandler & Marshall, 1982). Additional concentration of the solute may occur in the microdrop during the 10–30 min sampling period at 20 °C (Lechene & Warner, 1979; see also Discussion).

Absence of freezing and storage artifacts. Two tests indicated that solvent and solute concentrations remained constant during storage of the frozen microdrops. (a) Microdrop volumes were $1{\cdot}01 \pm 0{\cdot}06$ (range: 0·89–1·09; $n = 5$) of their initial volumes after 12 days of storage. (b) Electron probe analysis of microdrops of a test solution, first aspirated into the pipettes from a vial of the solution and then stored frozen for up to 12 days yielded element concentrations which were $1{\cdot}02 \pm 0{\cdot}1$ (range: 0·88–1·14; $n = 12$) of the concentrations obtained from a direct (electron probe) analysis of the fresh test solution. Results (a) and (b) together suggest that neither freezing nor storage *per se* affected the results.

Test uptake curves. In a control experiment that more closely approximated the actual test conditions, microdrops were placed in contact with 10 μl drops of test solution for 0–10 min. The uptake of Na, K, Mg, Ca and P into the microdrops (Fig. 2) was well fitted by a single exponential curve (continuous lines). The exponential time constants, indicated by τ under each curve, were derived by a non-linear least squares parameter estimation routine. Final equilibration values were reached within 2–5 min.

The extrapolated equilibrium concentrations and the mean of the 5 and 10 min values were, as a whole, $1{\cdot}10 \pm 0{\cdot}12$ (range: 0·97–1·26; $n = 12$) of the actual concentrations within the test solution. Assuming all elements concentrate equally, the average over-estimate was therefore ~ 10 % in this set of experiments. Other experiments suggest an average over-estimate of ~ 6 % (see legend of Table 2, rows 3 and 4).

The uptake of each element into the microdrops (Fig. 2) can, as a first approximation, be modelled as a simple diffusion process in one dimension (Crank, 1956). The composite diffusion constant (D) of the element is related to the exponential time constant of the uptake curve (τ) and the length of the microdrop in the pipette tip (L) through the expression (Maughan, 1983)

$$D = 4\, L^2 \tau^{-1} \pi^{-2}. \qquad (1)$$

Values for D are listed alongside τ in Fig. 2. As a whole, the diffusion constants are consistent with values one would expect from aqueous salt solutions of similar composition and temperature. For example, D_{Na} (~ $1{\cdot}0 \times 10^{-5}$ cm^2/s) is roughly comparable to that of Na^+ in 0·15 M-NaCl solution ($1{\cdot}47 \times 10^{-5}$ cm^2/s at 20 °C: Wang, 1952). Likewise, D_K (~ $1{\cdot}1 \times 10^{-5}$ cm^2/s) is roughly

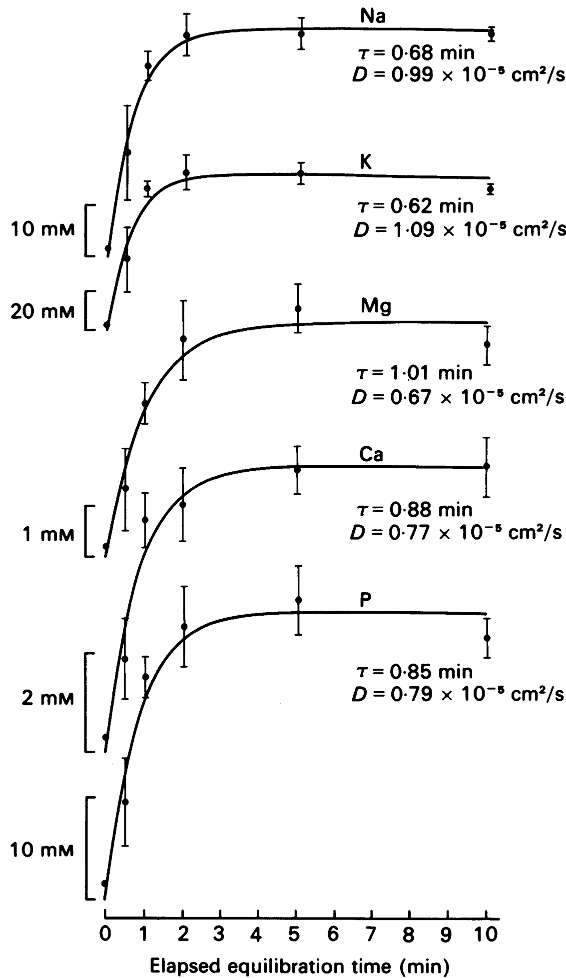

Fig. 2. Uptake of Na, K, Mg, Ca, and P into microdrops in contact with a test solution containing 64·3 mM-KCl, 15 mM-Na_2 creatine phosphate, 5 mM-$CaSO_4$, 4·75 mM-$MgCl_2$, 5 mM-EGTA, 3·15 mM-Na_2ATP, 20 mM-imidazole, 10·9 mM-KOH (pH 7·0; 20 °C). Average concentrations ±1 S.E. of mean. The element concentration (ordinate) was measured by X-ray spectroscopic analysis of the microdrops. The time axis (abscissa) indicates the duration which the microdrop remained on the test solution droplets. The exponential time constants (τ) and associated composite diffusion constants (D) are given alongside each curve. Temperature, 20 °C.

comparable to that of K^+ in 0·15 M-KCl solution ($2·17 \times 10^{-5}$ cm²/s at 20 °C: Friedman & Kennedy, 1955).

Element concentrations in droplets equilibrated with skinned fibres from incubated muscles

Microdrops were placed in contact with skinned fibre segments under oil. The appearance of most fibre segments remained unchanged during the sampling period, indicating that the fibres were relaxed and stable. Occasionally, however, a fibre

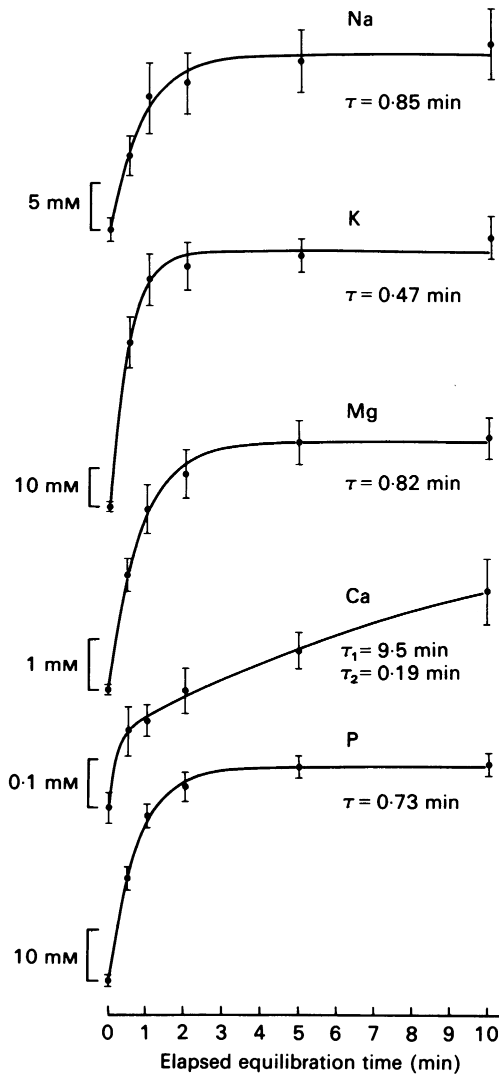

Fig. 3. Uptake of Na, K, Mg, Ca and P into microdrops in contact with frog muscle fluid. Each graph illustrates the time course of uptake of the element into the microdrop from the skinned fibre myoplasm (average concentrations ± 1 S.E.). Note that Ca has not reached equilibrium at 10 min. Exponential time constants, τ, are given alongside each curve. Pooled results from semitendinosus muscles from *R. pipiens* (twelve fibres), semitendinosus muscles from *R. temporaria* (seven fibres), and anterior tibialis muscles from *R. temporaria* (five fibres). Microdrop volume: fibre volume ratio had a range of 0·001–0·01. Temperature, 20 °C.

segment would contract at the cut ends and eventually collapse to a fat short segment; results from 'collapsed' fibres were omitted.

Fig. 3 shows the time course of Na, K, Mg, Ca and P equilibration between fibre segments and microdrops. The mean values were obtained from *R. pipiens* and *R. temporaria* muscles incubated in various solutions. All elements except Ca

equilibrated within 5 min of placing the microdrop on the fibre, while Ca apparently required > 10 min to equilibrate. Likewise, the uptake of all elements except Ca was well fitted by a single exponential function (τ given alongside each curve). The uptake of Ca was better represented by the sum of two exponential functions. The rates of Na, K, P and Mg uptake into the microdrops were not significantly different ($P > 0{\cdot}05$, Student's unpaired t test) than from the drop of test solution, whereas the rate of Ca uptake was considerably slower.

TABLE 2. Concentrations (means ±S.D) of diffusible elements in 0·2 nl microdrops equilibrated for 10 min with skinned fibres from *R. pipiens* semitendinosus muscles. Row 1: results from fibres taken from muscles incubated in solution D (Table 1). Row 2: results from fibres taken from freshly isolated muscles. Row 3: results from fibres of row 2 after incubation in test solution (20 mM-Na_2ATP, 15 mM-$MgCl_2$, 10 mM-KCl, 50 mM-NaCl, 20 mM-imidazole, 5 mM-EGTA, 32 mM-KOH; pH 7·0). Row 4: results from direct electron probe analysis of test solution. (Separate analyses of the test solution by flame photometry yielded [Na] 83·7±3·1 mM and [K] 47·3±5·0 mM ($n = 3$).) All concentrations, except those of Ca, are close to equilibrium values. The equilibrium [Ca], extrapolated from the double exponential fit to the uptake time course (Fig. 3), is estimated to be ~ 1 mM

			Concentrations (mM)				
No.	Condition	n	Na	K	Mg	Ca	P
1	Fibre (incubated)	5	27·2±15·5	90·1±26·8	5·8±1·7	0·6±0·3	59·4±12·9
2	Fibre (freshly isolated)	11	7·6±7·2	82·0±35·9	5·8±3·0	0·7±0·4	51·0±19·2
3	Fibre+test solution	11	83·0±12·4	47·9±7·0	14·4±2·2	0·7±0·3	57·8±7·7
4	Test solution	20	84·4±10·4	41·9±3·9	13·8±1·4	< 0·2	53·4±4·4

The average concentrations depicted in Fig. 3 are pooled values from different species, muscle types, and incubation media. Included in the standard errors are variations due to differences in the species, muscle type and incubation media used, as well as fibre-to-fibre and frog-to-frog variation. The time constants, however, are factors of diffusion and therefore depend on factors such as the length and temperature of the microdrop and not on factors which affect concentration.

Selected results (Table 2, row 1) from *R. pipiens* semitendinosus muscles *incubated* for 30–60 min in solution D (Table 1) indicate that average concentrations of diffusive elements range from < 1 mM (Ca) to 90 mM (K). Except for Ca, the concentrations listed are equilibrium values (exponential time constants < 1 min: Fig. 3).

Preliminary results suggested that neither the length nor the volume of the microdrop influenced concentrations significantly over the narrow range of microdrop lengths (about 0·2–0·4 mm) and volumes (about 0·1–0·3 nl) used. Further, neither the location of a pipette on a given fibre nor repetitive sampling at one location appeared to influence the results. Preliminary results from isolated whole single fibres were also similar ($P > 0{\cdot}05$) to corresponding results from fibres cut from the whole muscle, suggesting that the results do not necessarily depend on the particular way a fibre was dissected.

Element concentrations in droplets equilibrated with skinned fibres from freshly isolated muscles

Results from microdrops equilibrated with skinned fibres obtained from *freshly isolated* muscles (Table 2, row 2) produced estimates of average diffusible concen-

trations ranging from < 1 mM (Ca) to 82 mM (K). As in row 1, the concentrations listed are equilibrium values (exponential time constants < 1 min: Fig. 3), except for Ca.

Elemental analysis of droplets equilibrated with skinned fibres that were soaked in a solution of known composition

After each skinned fibre in the previous set of experiments was sampled, the fibre was transferred to a large (adjacent) drop of test solution (composition given in Table 2). The composition of the test solution was such that the elemental concentrations differed appreciably from expected *in vivo* values. After incubating the fibre for 30 min in the test solution, a fibre was stripped of excess (extracellular) fluid by carefully moving the skinned fibre out of the drop, through the oil bath, and along the glass bottom of the dish. A droplet was then equilibrated with the fibre for 10 min. Except for a lateral swelling of the fibre (Maughan & Godt, 1979), the appearance of the fibre remained unchanged.

Element concentrations in microdrops equilibrated with the skinned fibres pre-incubated for 30 min in the test solution were not significantly different ($P > 0.1$) from the concentrations of the test solution (Table 2, compare rows 3 and 4). Calcium was not present in the test solution, thus its presence in the microdrop indicates that during the previous half-hour incubation period all of the endogenous Ca did not diffuse out of the fibre.

Absence of extracellular fluid contamination

Cobalt, which is present in muscle cells in trace amounts, was used to verify the absence of appreciable extracellular fluid contamination of the microdroplets. Results from muscles incubated for 1–2 h in Ringer (solutions A and D: Table 1) containing 10 mM-$CoCl_2$ were compared with results from muscles incubated in similar solutions which were free of Co (*R. pipiens* semitendinosus muscles). No detectable signal from Co (over background counts) was found in the microdrops from either group. Analogous results were obtained from experiments using (iron-containing) ferritin as an extracellular marker. These results support the assumption that direct contamination of the fibre fluid by extracellular fluid was negligible.

Elemental analysis of whole muscle and plasma samples

Table 3 summarizes the results of an electron probe analysis of extracts of whole muscle. Average total concentrations ranged from 2·9 mmol/kg muscle water (Ca) to 157 mmol/kg muscle water (K). Chlorine and sulphur were undetectable (< 0·1 mM); compounds of these elements were probably vaporized as the muscles were dried or ashed, as would be expected under conditions of intense radiation (see Methods).

To estimate intracellular *in vivo* element concentrations ($[x]_i$), extracellular contributions ($[x]_o$) were subtracted from total concentrations ($[x]$):

$$[x]_i = [x]\,(r+1) - r[x]_o, \tag{2}$$

where r is the ratio of extracellular to intracellular fluid volume. Given nominal values for r (0·10, as determined from the Na efflux experiment described below), $[x]$ (Table 3, row 3), and $[x]_o$ (plasma values, given below), eqn. (2) yields values for the total intracellular concentration of each element listed in Table 3 (row 4).

Element concentrations in the extracellular (interstitial) fluid were assumed to be close to frog plasma values. Although one would expect the inorganic ion concentrations in the interstitial fluid to differ somewhat from those of plasma fluid (Manery, 1954), the expected differences are small and were therefore neglected.

TABLE 3. Concentrations of elements in fluid extracts of whole frog muscles after centrifugation. Row 1: results from ashed muscles (citric acid added to residue, neutralized with ammonium hydroxide). Row 2: results of row 1 re-expressed as mmol/kg of dry muscle mass. Row 3: results of row 1 re-expressed as mmol/kg wet mass minus dry mass. Row 4: results of row 3 after subtracting extracellular components (*see Methods). Na and K results from flame photometry of pooled muscle extracts were similar to the means listed below

No	Concentration (in units of):	Na	K	Mg	Ca	P
1	mmol/kg wet mass	20·5 ± 8·1	120·3 ± 26·2	9·7 ± 2·6	2·2 ± 0·5	76·1 ± 17·6
2	mmol/kg dry mass	89·5 ± 35·4	525·3 ± 114·4	42·4 ± 11·4	9·6 ± 2·2	332·3 ± 76·9
3	mmol/kg wet minus dry mass	27·5 ± 9·1	156·8 ± 34·1	12·6 ± 3·4	2·9 ± 0·6	99·1 ± 22·9
4	mmol/kg wet minus dry mass*	19·9	172·1	13·9	3·2	108·9

The electron probe analysis of de-proteinated plasma samples yielded: [Na] 104·3 ± 18·4 mM, [K] 3·5 ± 1·5 mM, and [P] 1·2 ± 0·5 mM ($n = 14$). [Mg] and [Ca] were < 0·2 mM. Flame photometric analysis of Na and K in pooled samples from the same lot yielded 97·3 ± 14·6 mM-Na and 3·2 ± 0·9 mM-K ($n = 6$), comparable to the results from the electron probe analysis.

Removal of extracellular fluid from muscle tissue

Extracellular fluid was removed before each of the above experiments on freshly isolated muscles by centrifuging the muscles on top of moist filter paper for 4 min at 1000 *g* (4 °C), according to the method of Ling & Walton (1975). The average wet mass prior to centrifugation was 139·3 ± 37·5 mg *versus* 126·6 ± 34·4 mg following centrifugation. Average weight loss as a result of centrifugation was 9·1 ± 2·5 %, comparable to the 9·4 %, reported by Ling & Walton (1975). From a parallel set of Na efflux experiments modelled after those of Neville & White (1979), we confirmed that virtually all of the spun-off fluid was extracellular. In uncentrifuged muscles, the extracellular fluid volume was 14·2 ml/100 g whole muscle, comparable to the 12 ml/100 g reported by Neville & White (1979) for freshly isolated muscle from *R. pipiens*. Centrifugation reduced the extracellular fluid volume by about half (to 7·7 ± 4·0 ml/100 g). In the uncentrifuged muscles, the mean ratio of extracellular water volume to intracellular water volume was 0·21 ± 0·08, while that for centrifuged muscles was 0·10 ± 0·06.

DISCUSSION

Diffusive elements in muscle

The major advantage of the microdrop–electron probe analytical method introduced here is that one can measure an otherwise elusive parameter: the intracellular concentration of diffusible elements. The 0·2 nl liquid droplets are considerably smaller than single vertebrate muscle cells (thus avoiding dilution

effects), and X-ray spectroscopy of the droplets allows all elements of the periodic table after boron in atomic weight to be analysed with a sensitivity of ~ 0·1 mM and a resolution of ~ 2 fmol. The concentrations measured are distinct from measures of ionic activity, such as those from ion-selective electrode recordings, and measures of total concentration, such as those from flame photometry of liquid extracts. Our results suggest that cellular fluid of freshly isolated frog muscle fibres in contact with a microdrop can be viewed as an ~ 0·27 osmol/l mixed salt and protein solution, with estimates of mean *in vivo* concentrations for Na, K, Mg, Ca and P given in Table 2 (row 2). Incubation of a freshly isolated muscle in artificial solutions can change some intracellular concentrations appreciably. For example, skinned fibres from muscles incubated for 30–60 min in a special isotonic low-chloride solution (D: Table 1) contained 3·6 times more diffusible Na than fibres from freshly isolated muscles (Table 2, row 1 *versus* row 2).

Concentration values for diffusible elements in skinned fibres which had themselves been incubated in an artificial Ca-free solution were found to be comparable to values obtained from direct analysis of the artificial solution. The concentrations of most of the salts in the solution bathing the skinned fibre were intentionally set outside the expected range of physiological values. This was done in order to demonstrate clearly that at least some of the diffusible solutes reach diffusional equilibrium. Thus the results of this experiment support the hypothesis that the diffusible contents of a skinned fibre can be essentially replaced by an artificial solution by merely soaking the skinned fibre in a relatively large volume of the solution.

A limitation of the equilibrated microdrop method is that each element concentration measured is a *total* concentration which includes all molecular forms of the diffusive element. Thus individual species, such as K^+, Na^+, Mg^{2+}, Ca^{2+}, and free phosphate (or compounds of these species, including $MgATP^{2-}$ and creatine phosphate) cannot be specifically identified. Nevertheless, a concentration limit can be established for each species (Table 2), and in some cases actual concentrations can be estimated by assuming reasonable values for the concentrations and affinity constants of the primary moieties.

For example, diffusible Na and K are probably almost entirely uncomplexed because of their low binding affinity to most other diffusible species (Sillen & Martell, 1971). Thus average diffusible [Na^+] and [K^+] is ~ 8 and 82 mM, respectively. Diffusible Mg, on the other hand, is mostly complexed (Maughan, 1983); it binds primarily to ATP^{2-} (about 4·8–5·0 mM of the 5·8 mM average), parvalbumin (0·2–0·4 mM) and creatine phosphate (0·4–0·6 mM). The present results are consistent with estimates of 0·2–0·6 mM for free [Mg^{2+}] which have been reported for frog muscle (Gupta & Moore, 1980; Baylor, Chandler & Marshall, 1982; Maughan, 1983; Gupta, Gupta, Yushok & Rose, 1983; Garfinkel & Garfinkel, 1984). On the other hand, the present results are not consistent with other estimates of free [Mg^{2+}] which have also been reported (4·4 mM: Cohen & Burt, 1977; 3·3 mM: Hess, Metzger & Weingart, 1982; 1·2–6 mM: Baylor, Chandler & Marshall, 1982; 3·8 mM: Lopez, Alamo, Caputo, Vergara & DiPolo, 1984; 3–4 mM: Close & Lannergren, 1984). We have as yet no clear explanation for these differences.

The Ca data present a somewhat different picture. The exceptionally slow diffusion of Ca into the microdrops suggests one, or a combination of two possibilities: either

the diffusible Ca is bound to macromolecules of low mobility, or Ca is itself diffusion-limited within the fibre, possibly by chemical interaction with intracellular structures (Kushmerick & Podolsky, 1969). In any case, free Ca^{2+} (52 nM: Weingart & Hess, 1984) is only a tiny fraction of all the diffusible Ca in relaxed frog muscle.

Much of the diffusible Ca is probably complexed with parvalbumin, a soluble 11 kDa protein whose concentration in frog muscle is 0·3–0·4 mM (Gosselin-Rey & Gerday, 1977; Gillis, Piront & Gosselin-Rey, 1979). Parvalbumin has two high affinity binding sites for Ca^{2+} (dissociation constant ~ 10 nM: Johnson, Charlton & Potter, 1979) which also bind Mg^{2+} competitively, though not so strongly (dissociation constant ~ 0·1 mM: Potter, Robertson, Collins & Johnson, 1980). Thus, if the free [Mg^{2+}] in relaxed frog muscle is as low as 0·2–0·6 mM (see references above), then up to three-quarters of the sites on diffusible parvalbumin are occupied by Ca^{2+}. If, on the other hand, free [Mg^{2+}] is as high as 3–4 mM (see other references above), then less than one-quarter of the sites are occupied by Ca^{2+}.

Because parvalbumin diffuses much more slowly than free Ca^{2+}, it is tempting to speculate that the slow component of the Ca uptake curve (Fig. 3) represents the Ca-parvalbumin complex. If so, then the slow longitudinal diffusion of Ca reported previously in relaxed muscle fibres (Kushmerick & Podolsky, 1969) may reflect not so much an electrostatic restraint of free Ca^{2+} by fixed charged structures within the cell, as Kushmerick & Podolsky (1969) suggest, as a slow diffusion of (Ca-loaded) endogenous parvalbumin.

Diffusible P, like Mg and Ca, is almost entirely complexed, consisting mainly of creatine phosphate, adenosine tri-, di-, and monophosophate, and free phosphates. The average concentration of diffusible P (51 mM) is consistent with the combined averages of the free phosphate compounds as measured by nuclear magnetic resonance (32·7 mmol P/kg whole muscle: Dawson, Gadian & Wilkie, 1980, or 56 mmol P/kg myoplasmic water, assuming (1/0·58) kg whole muscle/kg myoplasmic water: Baylor, Chandler & Marshall, 1983).

Any estimate of *in vivo* diffusible element concentrations must take into account the extent to which ions redistribute during the preparatory procedures. Conveniently, stripping the sarcolemma from the fibre under oil removes residual fluid. Furthermore, the transverse tubular lumen occupy < 1 % of the water volume of a fibre (Peachey, 1965), so this source of extracellular contamination can probably be neglected (Maughan, 1983). Thus, direct contamination of the microdrop by extracellular fluid is unlikely, as the absence of extracellular markers Co and Fe confirmed.

More likely, redistribution of ions across the sarcolemma could result from reduced metabolic ion pump activity after the frog is sacrificed. The amount redistributed would depend on how quickly, i.e. to what extent, metabolic activity declines once homoeostasis is disrupted. Further redistribution of permeant species could occur upon depolarization of the membrane when the sarcolemma is cut or stripped. The impact of these procedures is difficult to assess, although the number of ions transferred is probably negligible compared to the total number available, even after a prolonged depolarization of the membrane (Woodbury, 1982).

Another, perhaps more serious source of solute redistribution is likely to occur as a result of fibre and droplet dehydration, as mentioned previously (Results). In particular, the validation experiments indicate that the diffusible element concentrations in the microdroplets are slightly higher than expected from direct analysis of a test solution. The data of Table 2 interpreted in this way suggest that solutes concentrate by a factor of ~ 6 %, assuming that the elements concentrate equally and that the dispersions in values reflect measurement error. This is close to the amount calculated previously from fibre shrinkage under oil.

Restricted elements in freshly isolated muscle

The total element content of a cell can be divided operationally into freely diffusible and restricted fractions (Dick & McLaughlin, 1969):

$$[x]_i = [x]_d + [x]_r, \tag{3}$$

where i, d, and r refer to total, diffusible and restricted concentrations respectively. Let $[x]_i$ be the concentration obtained from the analysis of the muscle extracts after subtracting the estimated extracellular component (cf. Methods) and $[x]_d$ the equilibrium concentration obtained from the analysis of the microdroplet. Thus, values for $[x]_d/[x]_i$, derived from eqn. (3) are: Na, 0·38; K, 0·48; Mg, 0·42; Ca, 0·22 and P, 0·47.

Presumably, microdrop element equilibrium concentrations represent diffusible concentrations in the extra-myofibrillar fluid as well as in most of the intra-myofibrillar fluid. This assumption is based on the premise that, at the assumed intracellular ionic strength of 0·14–0·16 M (Gordon, Godt, Donaldson & Harris, 1973), most contiguous aqueous regions within the cell are relatively far (i.e. many Debye lengths) from the fixed charges on the surfaces of the filaments and membranes, and are therefore at very nearly the same potential. (For a 0·14–0·16 M-salt solution, the Debye length is about 1 nm.) Therefore, in the absence of significant potential gradients, ion concentrations (and thus element concentrations) are similar.

Appreciable ion concentration differences, however, have been postulated between certain inner regions of the myofibrils and the rest of the myoplasm (see Stephenson, Wendt & Forrest, 1981, and references therein). These differences are most marked in the immediate vicinity of fixed charges (Elliott, 1973) and in regions neighbouring chemical reactions (Godt & Baumgarten, 1984). In particular, a certain number of Na^+, K^+, Mg^{2+} and Ca^{2+} ions are restricted in the sense they are counterions which balance the net negative charge ($\sim$ 57 mequiv/l: Maughan & Godt, 1980) on the actin- and myosin-containing filaments. However, the total amount of restricted Na^+, K^+, Mg^{2+}, and Ca^{2+} ($<$ 57 mM, taking into account the divalent cations) is not sufficient to account for all the restricted Na, K, Mg and Ca ($>$ 170 mM, i.e. the difference between the sum of the total and the sum of the diffusible element concentrations).

The concentrations of intracellular Na, K, Mg, Ca, and P obtained by Ingram *et al.* (1972) and Somlyo *et al.* (1977, 1981) lie between the present mean values for diffusible intracellular concentration (Table 2) and the estimates of total intracellular concentration (Table 3). This finding is consistent with both diffusible and restricted elements being included in the electron probe analysis of muscle tissue sections. However, the standard deviations about the means listed in Table 2 for freshly isolated or pre-incubated fibres (rows 1 and 2) are quite large – being larger, for example, than those given by Somlyo *et al.* (1981) – so that in some cases the numerical results appear to be hardly better than order-of-magnitude. However, the standard deviations listed for fibres pre-bathed in a test solution or from the test solution alone (rows 3 and 4) are comparatively small, which suggests that the spread of the results from freshly isolated or pre-incubated fibres is mainly derived from fibre-to-fibre variation rather than from uncertainties of sampling or X-ray analysis.

It is of interest to compare the diffusible/total element content of Na^+ and K^+ with activity coefficients derived from ion-selective micro-electrode measurements. For example, Na^+ activity coefficients of 0·20 (Lev, 1964) –0·27 (Lee & Armstrong, 1974), and K^+ activity coefficients of 0·66–0·69 (Lee & Armstrong, 1974) have been reported, compared with the diffusible/total values of 0·38 and 0·48 for Na^+ and K^+ respectively

in the present study. Both sets of measurements imply substantial ion binding or sequestration. The sarcoplasmic reticulum and mitochondria could potentially accommodate substantial amounts of Na^+ and K^+, but the electron probe results of Somlyo *et al.* (1981) indicate that the Na and K contents of the longitudinal sarcoplasmic reticulum, terminal cisternae, and mitochondria are not appreciably different than those of the bulk cytoplasm. More likely, substantial amounts of Na^+ and K^+ are directly absorbed to fixed cellular proteins and lipids. Free carboxyl groups of the aspartic and glutamic acid residues are present in ample amounts (Yates & Greaser, 1983), and are therefore likely binding sites at neutral (intracellular) pH. However, distribution isotherms for each element, of the kind reported by Horowitz & Miller (1984), are needed to determine unambiguously the size of the bound pools.

The rather substantial amount of restricted P (57·9 mM) is likely that bound to or incorporated into fixed contractile and cytoskeletal proteins and membrane lipids. For example, actomyosin binds up to 7 mmol P/kg whole muscle (Barany & Barany, 1973), or about 12 mmol P/kg myoplasmic water (assuming 1/0·58 kg whole muscle/kg myoplasmic water: Baylor *et al.* 1983). Phospholipids chelate 60 mmol P/kg dry mass (estimated by M. Barany and quoted in Somlyo *et al.* 1977), or about 20 mmol P/kg myoplasmic water (assuming 0·34 kg dry mass/kg myoplasmic water: Somlyo *et al.* 1981). Estimates of intracellular total P content and, therefore, comparison of diffusible and total P is further complicated by the fact that some phosphorus in whole muscle is bound to surface membranes and connective tissue.

A rather substantial amount of restricted Mg (about 8 mM) and Ca (about 2·6 mM) is, like Na, K, and P, probably adsorbed to intracellular proteins and lipids. In addition, about 1·1 mM-Ca, or about half of the restricted Ca, is sequestered in the sarcoplasmic reticulum of resting frog muscle. (Somlyo *et al.* (1981) obtained 117 ± 48 mmol Ca/kg dry terminal cisternae (t.c.), which corresponds to $48 \times 0.39 \times 0.035 \times (1/0.58) = 1.1$ mmol Ca/kg myoplasmic water, assuming 0·39 kg t.c. dry mass/kg t.c. wet mass, 0·035 l t.c./l fibre, (1/0·58) kg fibre/kg myoplasm, and 1·0 kg/l for both t.c. and fibre.)

Charge and osmotic balances

Of the major cations in the microdrop, Na^+ and K^+ together contribute about +90 mequiv/l, while Mg^{2+} (0·2 mM), Ca^{2+} (0·1 μM) and other species of Na, K, Mg and Ca probably contribute < 1 mequiv/l. To satisfy charge neutrality, the +90 mequiv/l in the droplet must, at equilibrium, be balanced by −90 mequiv/l. This negative charge comes primarily from the P-containing anions, including creatine phosphate (about 40 mM, or ~ 80 mequiv/l, calculated from the data of Dawson *et al.* 1980) and $MgATP^{2-}$ (about 5 mM, or ~ 10 mequiv/l, *ibid*). Chloride also contributes to the anion complement of the microdrop, although the contribution is likely to be small (< 3 mM: Macchia & Baumgarten, 1979). Various nucleotides, phosphates, and amino acids together probably contribute < 5 mequiv/l (Conway, 1957).

The osmolarity of the microdrop in equilibrium with the intracellular fluid was estimated to be ~ 0·27 osmol/l by the simple visual test described previously (see Methods). We estimate that the total particle concentration of the species listed above is only 0·15 osmol/l, so that a considerable amount of osmotically active solute remains to be accounted for. Carnosine, lactate, bicarbonate, and other organic

substances contribute to the osmolarity of the microdrop, but, because the electron probe cannot distinguish these compounds, their concentrations will have to be measured by other methods.

Relevance to design of a bathing medium for skinned fibres

While the average diffusible concentrations of Na, K, Mg, Ca and P can be measured, their concentrations within restricted intracellular domains can only be inferred. Nevertheless, the experimentally relevant concentrations are those in the solutions bathing skinned fibres. We suggest that an appropriate reference solution for skinned fibre experiments is the contents of a microdrop in complete equilibrium with the myoplasm of a freshly isolated fibre at rest.

To illustrate this point, consider Mg and Ca, whose ionic forms play key roles in the generation and regulation of contraction. Because of the influence of the electrostatic field associated with the net negative fixed charge on the myofilaments, the concentration of these divalent cations near the filaments may be as much as twenty-five times greater than the concentration in the bulk fluid (Godt, 1981). Thus, if one wants to study the relationship between force and intracellular free [Ca^{2+}] at various intracellular free [Mg^{2+}] in skinned fibres, the concentrations which directly influence contraction will be those concentrations at the Ca^{2+} and Mg^{2+} binding sites on the contractile and regulatory proteins. However, the relevant concentrations are not those near the filaments, but rather those in the bulk intracellular fluid far (i.e. many Debye lengths) from the filaments, i.e. those of the experimental solutions. If the intracellular fixed charge density in the skinned fibre preparation remains close to that *in vivo* and if the structural integrity of the fixed proteins remains intact, then the resulting force–[Ca^{2+}]–[Mg^{2+}] relationships will also be close to those *in vivo*.

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