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Tittel: Effect of submaximal isometric exercise on total Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} contents of muscle.

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Sammendrag:

Forandringer i musklenes innhold av Ca^{2+}, Mg^{2+}, Na^{+} og K^{+} under repetert statisk arbeid ble undersøkt. Arbeidet besto av repeterte kontraksjoner på 30% av maksimal kraft med begge knestrekkerne inntil utmattelse (73 ± 36 min). Hver kontraksjon ble holdt i 6 s med 4 s hvile i mellom. Før, under og etter arbeid ble muskelbiopsier tatt for senere analyse av elektrolyttene.
Resultatene viser en initial økning i Na^{+} og Ca^{2+}, og et fall i K^{+} og Mg^{2+}. Deretter ble konsentrasjonen av Ca^{2+}, K^{+} og Mg^{2+} normalisert, mens Na^{+} konsentrasjonen steg videre. Forandringene var små, og det er uklart hvilken betydning de har for utvikling av tretthet og eventuell muskelskade.

Stikkord:
skjelett muskel
statisk arbeid
muskeltretthet
elektrolyttbalanse
natrium, kalsium, kalium, magnesium

Key words:
skeletal muscle
exercise
isometric contractions
muscle fatigue
electrolyte balance
sodium, calcium, potassium, magnesium

Effect of submaximal isometric exercise on total Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} contents of muscle.

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to my two Jans

Preface

The investigation here reported was carried out at the National Institute of Occupational Health, department of work physiology, Oslo, Norway, in the period from january to august 1991. The investigation was part of a program, studying fatigue in low intensity intermittent isometric exercise. Tutor in Oslo was Dr. MD. O. M. Sejersted, tutor in Amsterdam was Prof. dr. A. J. Sargeant.

I would like to thank both tutors and all other persons who have helped me complete this study. Special thanks to Dr. MD. Ole M. Sejersted for being an inspirating tutor; Prof. dr. A. J. Sargeant and drs. H. G. Westra for reviewing the almost final version; Per-Kristian Lunde, for guiding me on the lab work; Dr. Nina K. Vøllestad for reviewing several versions of the report; Elisabet Børsheim, Per Holmstad and Petter Gramvik for stimulating company in "Studentrommet". I would also like to thank Stichting Het Vrije Universiteits- fonds, Stichting Dr Hendrik Muller's Vaderlandsch Fonds and Stichting Bekker-La Bastide Fonds for financial contributions for my stay in Oslo.

Amsterdam, January 8, 1992 Esther Verburg

Summary

Calcium, magnesium, potassium and sodium were measured in biopsies taken before and during repeated submaximal contractions.

8 healthy male human subjects performed two-legged isometric contractions for 6 seconds at 30% maximal voluntary contraction (MVC) with a rest of 4 seconds in between, until exhaustion (72,5 ± 35,5 min). Exhaustion was defined as the point when the subjects were unable to maintain the target force for the required 6 seconds. Throughout the exercise period and at exhaustion, muscle biopsies were taken from the vastus lateralis of both legs. Control biopsies in rest were taken before the beginning of exercise. After taking the biopsies these were directly frozen in liquid nitrogen. Before drying at 90°C the frozen muscle biopsies were dissected free from blood, fat and connective tissue. Dry weight was determined. The muscle biopsies were digested by heating in 65% HNO_3 for 3 hours at 60-70°C. Samples from the digest were taken for analysis of Ca, Mg, K, and Na concentration. Ca and Mg were measured with Inductively Coupled Plasma Spectrophotometry (ICPS) and K and Na with Flame Atomic Emission Spectrophotometry.

Testing of the method showed high precision and accuracy. Repeated measurements on human muscle samples gave a variation coefficient that ranged from 5,7% for Mg, 7,9% for K, 12,2% for Ca to 14,4% for Na. High correlations were found when sample dry weight was related to sample electrolyte concentration. The Y-intercepts of the regression lines did not differ significantly from zero.

Mean resting concentrations (sd) were: 3,161 (0,358) mmol Ca/kg dry wt, 36,83 (3,78) mmol Mg/kg dry wt, 382,8 (42,8) mmol K/kg dry wt, 130,1 (64.1) mmol Na/kg dry wt. After 15 minutes of exercise, the electrolyte concentrations reached a peak (in the case of calcium and sodium) or nadir (magnesium and potassium), the significant mean differences (se) being +15,96% (6,11%) for Ca, +65,03% (12,99%) for Na, -4,73% (1,67%) for Mg and -11,36% (3,95%) for K. During the following 15 minutes of exercise, the concentrations returned to approximately resting values in the cases of Ca, Mg and K. Sodium content either increased further or fell back to the resting value. After 30 minutes of exercise there was a tendency for a small uptake of Calcium and small losses of Mg and K. The changes found were, however, not significant, although the exhaustion values of calcium were significantly higher than resting. Sodium concentrations increased significantly towards exhaustion.

It is excluded that the temporal changes in electrolyte concentration are caused by artifacts or by systematic measurement errors. The ratio figures show that random errors also could be excluded. Variation coefficients and reproducability found when testing the method support this. The changes cannot have be caused by a time-order of measurement error, since samples were measured in random order. It is concluded that the changes should therefore be physiologically explained.

The potassium loss, although not significant, compares favorably with the changes due to exercise found in blood concentration in other studies. The time-pattern found for the changes in intracellular electrolyte concentration has however not been reported before. It is speculated that the pattern represents a disturbance of the electrolyte balance of the cell, and its back-regulation.

It is concluded that exercise seems to cause temporal changes in the muscle concentration of all the electrolytes measured, but that more research is needed before any conclusions can be drawn on the role of these four electrolytes in the development of fatigue. The results do not reject the hypothesis of a role for calcium or any of the other electrolytes in the development of fatigue.

Contents

Chapter 1

Introduction

In search for the causes of fatigue, several studies have led to the finding that exercise causes a loss of potassium from the muscle (Clausen and Everts, 1988b [8],Sjøgaard, 1988 [23], Sahlin and Broberg, 1989 [20] and Vøllestad et al., 1991 [30]). It has been suggested that this disturbance of the potassium balance may play a role in the mechanism of fatigue (Sjøgaard,1988 [23], Vøllestad and Sejersted, 1988 [28], Vøllestad et al., 1991 [30],Clausen and Everts, 1988b [8]).

Vøllestad et al., 1988 ([28]) found in earlier research on intermittent low intensity isometric exercise, a linear decrease in maximum voluntary contraction (MVC) force, reaching about 50% of control at exhaustion. Only negligible changes were found in lactic acid, creatine phosphate and glycogen in the exercising muscle, excluding them as causes of the gradual developing fatigue in this type of exercise. In a follow-up study, with the same type of exercise, they found a continuous loss of potassium from the exercising muscle, when measuring in the blood plasma (Vøllestad et al., 1991 [30]). In a review, Vøllestad and Sejersted, 1988 ([28]), also proposed a role for calcium in the mechanism of fatigue.

In the present investigation, the effect of exercise on the concentration of four electrolytes (calcium, magnesium, potassium and sodium) in working muscle is studied. Measurements were made on biopsies from the vastus lateralis, taken before and during intermittent isometric leg extensions at 30% maximum voluntary contraction (MVC) force. The biopsies were taken during experiments in 1984 and 1985, and had only been used partly for other analyses.

Before measuring on the exercise-biopsies, a method for measuring the four electrolytes in biopsies was developed, and thoroughly tested.

Chapter 2

Methods

Calcium, magnesium, potassium and sodium were measured in biopsies originating from experiments carried out with humans in 1984 and 1985. These biopsies had been stored in a refrigerator at -80°C.

The above mentioned experiments had the following protocol: 8 healthy male human subjects made two-legged isometric quadriceps contractions at 30% maximal voluntary contraction (MVC) for 6 seconds with 4 seconds of rest between until exhaustion (72,5 ± 35,5 min). Exhaustion was defined as the point when the subjects were unable to maintain the target force for the required 6 seconds. The force of the knee extensors was measured with a strain gauge connected to the ankles. The legs had to generate equal force simultaneously. The subjects were asked to make a brief maximal contraction after 11, 21, and 35 min and about 5 min before exhaustion. Throughout the exercise period and at exhaustion, muscle biopsies were taken from the vastus lateralis of both legs. Control, resting biopsies were taken before the start of exercise. Directly after taking, the muscle biopsies were frozen in liquid nitrogen. Part of the biopsies were to be analyzed for fibre type composition. These were quickly rolled on a filter paper with NaCl to remove most of the blood before being frozen in liquid nitrogen. For the present investigation, the remainders of all the biopsies taken in those experiments were used. These remainders had been stored at -80°C, and were now taken out.

The frozen muscle biopsies were dissected free from visible blood, fat and connective tissue, then weighed. After attaching the biopsies to preweighed platinum hooks, they were placed in an oven to dry overnight at 90°C. The dry muscle biopsies were weighed and dry weight was calculated. The muscle biopsies were digested by heating in 250 μl 65% HNO_3 for 3 hours at 60 - 70°C. The digest was then diluted with deionized and distilled

water to 4000μl and taken for analysis of Ca and Mg concentration with Inductively Coupled Plasma Spectrophotometry (ICPS). From the remaining of these 4000μl dilutions, 250μl was taken and diluted to 2000μl with 1% Cs solution. This was taken for analysis of Potassium concentration with Flame Atomic Emission Spectrophotometry (FAES). A further 500μl was taken from the 4000μl dilution, diluted to 2000μl with 1% Cs solution, and taken for measurement of Sodium with FAES.

Before commencing analysis of the experimental biopsies, the method for preparation and analysis was tested on standardized bovine liver powder, pig muscle, rat muscle and human muscle, to see if the method was reliable enough. Seven different techniques for preparation and analysis of muscle biopsies were found in the literature. Two of them were chosen to be further examined, because of their simplicity and (relative) inexpensiveness, and because of the availability of the necessary analysis instruments (both in Dørup, et al., 1988 [10]). The two methods use the same analysis techniques (the actual measuring of the concentration in the sample), but different preparation techniques. Both preparation techniques are so called "wet ashing" techniques, one using 65% HNO_3 for the digestion of the muscle samples, the other using 30% H_2O_2 for this purpose. An advantage of this last technique is, that concentrated acids do not need to be used. This makes this method suitable for analysis by instruments that do not allow the use of these acids, for example analysis with ion-selective electrodes. Presently the analysis of the prepared samples was performed by atomic emission spectrophotometry (AES). In view of the small amounts of calcium and magnesium expected to be found in a muscle biopsy, Inductively Coupled Plasma Spectrophotometry (ICPS) was considered the best instrument for analysis of these electrolytes. Amounts of potassium and sodium expected in a muscle biopsy allowed the use of the cheaper Flame Atomic Emission Spetrophotometry (FAES) technique.

Initially, before digesting, the biopsies were dried and fat was extracted, according to the method described by Sjøgren et al. (1987) [24]. This was done to avoid errors due to possible electrolyte content of fat. The following preparation technique was established:

1. Frozen muscle biopsies were dissected free from visible fat, connective tissue, connective tissue and blood in a refrigerating chamber (-30 to -25°C).
2. The dissected muscle samples were weighed on a balance that was placed in the same refrigerating chamber.

3. The weighed muscle samples were attached to preweighed platinum hooks and dried over night at 90°C.
4. The dry muscle samples were again weighed (still attached to their hooks and using the same balance, but now at room temperature). They were then placed in 3,0 ml petroleum ether for 3 hours in disposable polypropylene tubes, to extract fat.
5. The fat-extracted samples were dried for 3 hours at 90°C, then weighed to obtain fat free dry weight.
6. The biopsies were digested in either 250μl 65% HNO_3, or 1,0 ml 30% H_2O_2. No times for digesting were available from the literature, so to begin with, the tubes were placed in room temperature until the biopsies were visibly digested.
7. The H_2O_2 digest was evaporated at 90°C overnight, and then resuspended in 4000 μl 5% TCA. The resuspension was analyzed for calcium and magnesium by Inductively Coupled Plasma Spectroscopy (ICPS).
8. The HNO_3 digest was diluted with distilled and deionized water to 4000 μl before analyzing calcium and magnesium content by ICPS.
9. From both the HNO_3-dilution and the H_2O_2-resuspension, 250 μl was further diluted to 2000 μl with distilled and deionized water, containing 1% Cs solution. The dilutions were analyzed for potassium (K) content by Flame Atomic Emission Spectroscopy (FAES).
10. From both the HNO_3-dilution and the H_2O_2-resuspension, 500 μl was diluted to 2000 μl with distilled and deionized water, containing 1% Cs solution. The dilutions were analyzed for sodium (Na) content by FAES.

If not written otherwise these were the instruments and chemicals that were used (in order of use):

- balance: Cahn 27 automatic electrobalance.
- platinum hooks: weighed between 36 and 50 mg.
- petroleum ether: petroleum spirit, pronalys AR, b.pt. 40°-60°C, May and Baker Ltd, Dugenham, England.
- HNO_3: at first KEBO Lab AB, Oslo (Puriss); later Chem Scan AS, Elverum Norway (Scan Pure).
- H_2O_2: Merck, Darmstadt BRD (zur analyse ISO);
- TCA: Trichloressigsure, Merck, Darmstadt BRD (pro analysi) in destilled and deionized water.
- Inductively Coupled Plasma Spectrophotometer: Per kin El mer ICP-5500. Wavelenght for calcium: 393,37 nm; magnesium: 279,55 nm. Two

standards were used for calibration, 0,500 ppm and 1,000 ppm of both Ca and Mg, the blank contained only distilled and deionized water.

- Cs solution: Spectrascan element standard for atomic spectroscopy (Cs, 1000 ppm), Teknolab AS, Drøbak Norway.
- Flame Atomic Emission Spectrophotometer: Perkin Elmer 5000, flame air-acetylene. Wavelength for potassium: 766,5 nm; two standards: 1,000 ppm and 2,000 ppm and a blank containing 0,5% HNO_3 or 0,65% TCA were used for calibration. Wavelength for sodium: 589,0 nm; two standards: 0,500 ppm and 1,000 ppm and a blank containing 1% HNO_3 or 1,25% TCA.
- Calibration standards were made by diluting element standards: Spectrascan element standards for atomic spectroscopy (Ca, Mg, K, and Na, each solution contained 1000 ppm), Technolab AS, Drøbak, Norway.

The methods with HNO_3 and H_2O_2 were tested and further developed.

2.1 Testing the methods with standard bovine liver powder

The main purpose of this phase was to see if the methods worked when following the description given above and to see if the methods gave accurate results. Dried and powdered Bovine Liver, with known Ca, Mg, K and Na content, was used (Analytical Standards AB, Kungsbacka Sweden (Pl 2366, S-43400)). Small amounts of the powder (2 to 7 mg) were weighed (Mettler AT 250 Fact balance) and put into teflon tubes. In these tubes the powder was prepared and analyzed as described above from point 6. From the weight of the powder and the measured concentration in the sample, the concentration of each electrolyte was calculated. This was compared with the concentration given by the producers of the powder. Unfortunately, no description of their analysis method or the accuracy of the concentration was given.

2.2 Testing the methods with pig muscle

The main purpose of this phase was to find out if the different techniques (the two digesting techniques and the extraction of fat) resulted in the same values for electrolyte contents. Pig muscle was used (a muscle from the jaw which was low in tendon content and which seemed very homogenous in fibre type composition). The muscle was dissected from a pig that had been killed

only just before, and frozen immediately in liquid nitrogen. From this large piece of muscle (approximately 50 g), 24 samples, weighing about 15 mg, were dissected out. The samples were free of visible blood, fat and tendon. The 24 samples were divided into 4 groups:

1. H_2O_2 method with fat extraction;
2. H_2O_2 method without fat extraction;
3. HNO_3 method with fat extraction;
4. HNO_3 method without fat extraction.

The groups were matched so that mean wet weights were the same. The samples were prepared according to the methods discribed earlier. Group means were then compared with each other, using students t tests for comparing means (two sided, $\alpha = 0{,}05$).

Taking into account the results from the first two stages, (see chapter 3) it was decided to change the preparation method before further testing, in that the digestion was only done with HNO_3 and no longer with H_2O_2. In addition, fat extraction was left out. (Summary of the method as finally used can be seen in the appendix.)

2.3 Testing the method with rat muscle

The main purpose of this third stage was to estimate the accuracy and size of error of the method. A second purpose was to see if the estimated electrolyte content of the smaller biopsies (down to 5 mg wet weight) differed from that of the larger biopsies. Rat vastus lateralis muscle was used. Just after the rats had been killed, the muscle was dissected out and frozen immediately in liquid nitrogen. From the large piece of muscle, fifteen small samples, free from visible fat, connective tissue and blood, were dissected out. The weights were 5, 10, 15, 25 and 50 mg, three of each weight. The muscle samples were then prepared for analysis according to the HNO_3 method but without fat extraction, as described above.

2.4 Testing the method with human muscle

The main purpose of this phase was to estimate accuracy and errors, and reproducibility, but now for human muscle. Another purpose was to see if weight of the samples was of any influence on the estimation of electrolyte contents. A third purpose was to find out whether the biopsy needles could cause pollution of calcium, magnesium, potassium or sodium. Two samples

(each weighing approximately 10 g) were cut from m. Vastus Lateralis of a 63 year old male patient undergoing bone-surgery (an old fracture in his femur had to be corrected), but who was otherwise healthy. One sample was frozen immediately after removal in liquid nitrogen. The second sample was packed in aluminium foil, put on ice and transported to the institute (this took approximately half an hour). Here 10 biopsies were taken with a biopsy-needle from the unfrozen sample and then frozen in liquid nitrogen. From the first, frozen sample were cut 15 smaller pieces, with varying weight (ranging from 2 to 20 mg wet weight). All biopsies and pieces were then prepared according to the method mentioned above. For the calculation of the inter assay variation, 5 other samples were cut from the frozen piece, all weighing approximately 10 mg. With each batch of biopsies that was prepared and analyzed (1 batch each week), one of those 5 samples was prepared and analyzed. After analysis of all 5 samples, means and standard deviations of the concentrations of electrolytes (expressed in mmol/kg dry weight) were calculated, to estimate reproduceability of the preparation and analysis technique.

Chapter 3

Results of testing the method

3.1 Testing the methods with standard bovine liver powder

Both methods worked as digesting techniques. The digesting of the biopsies took, however, 48 hours at 75°C with the H_2O_2 method, while the biopsies were digested within a day in HNO_3, after being heated at 75°C for 2 hours.

expected concentration in mmol/kg powder	Ca	Mg	K	Na
mean measured conc.	2,850	24,21	219,2	110,01
sd	0,165	1,05	20,1	34,60
variation coefficient	5,8%	4,3%	9,2%	31,4%
n	11	11	11	11
concentration given by producers	2,994	24,69	254,7	105,67
student t-test $H_0 : \mu$ = given value				
t value	-2,902	-1,50	-5,9	0,42
p-value	0,01<p<0,02	0,1<p<0,2	p<0,01	p>0,5

Table 3.1: Mean measured electrolyte concentration of the powder, with the HNO_3-method. Results of statistical testing of the mean measured concentration against the concentration given by the producers are also shown.

Tables 3.1 and 3.2 show the results from the electrolyte analysis, and

the statistical calculations. The mean measured concentration was tested against the concentration given by the producers of the powder, with a student-t test, for testing a mean. H_0: mean measured electrolyte concentration = concentration given by the producers, $\alpha = 0{,}05$, two-sided testing. The differences between given and measured electrolytes were in some cases significant. It can be seen in tables 3.1 and 3.2 that the HNO_3 method tended to systematically underestimate the electrolyte concentrations, and that the H_2O_2-method tended to overestimate.

Concentration of in mmol/kg powder	Ca	Mg	K	Na
mean measured conc.	3,158	25,17	278,4	130,01
sd	0,411	1,90	21,0	22,82
sd in % of mean	13,0%	7,5%	7,5%	17,6%
n	12	12	6	6
concentration given by producer	2,994	24,87	254,7	105,70
student t-test $H_0 : \mu$ =given value				
t-value	1,381	0,883	2,758*	2,610*
p-value	0,1<p<0,2	0,2<p<0,5	0,02<p<0,05	0,02<p<0,05

* significant, $\alpha = 0{,}05$

Table 3.2: Mean measured electrolyte concentrations of the powder, with the H_2O_2-method. Results of statistical testing of the mean measured concentration against the concentration given by the producers are also shown.

3.2 Testing the methods with pig muscle

24 samples were cut from the piece of jaw muscle. These were devided into four groups, with matched wet weight. The samples in group 1 and 2 were digested using the H_2O_2-method, but fat was extracted only from the samples in group 1. The samples in group 3 and 4 were digested using the HNO_3-method, but fat was extracted only from the samples in group 3. The mean

weights of the samples for each group are given in table 3.3.

group	wet weight		dry weight		fat free dry weight	
	mg	(sd)	mg	(sd)	mg	(sd)
1	15,03	(0,09)	2,85	(0,09)	2,80	(0,10)
2	15,04	(0,09)	2,82	(0,08)	-	
3	15,03	(0,08)	2,86	(0,14)	2,81	(0,14)
4	15,03	(0,09)	2,72	(0,04)	-	

Table 3.3: **Mean sample wet weight, dry weight and fat free dry weight for each group of the pig muscle samples.**

Mean water content of the samples was 81,3% (sd 0,8%) of the wet weight. Mean amount of fat extracted from the samples was 1,8% of the dry weight, or 0,34% of the wet weight.

This time H_2O_2 could not digest the whole muscle sample, not even after 48 hours at 90°C (with closed tubes). Fresh H_2O_2 was tried later, but gave a similar result. Since it could not be made sure that there were no electrolytes left in the undigested parts of the muscle, the method was considered less applicable than the HNO_3 extraction method and further development was discontinued.

group	Ca	Mg	K	Na
	mmol/kg wet weight (standard deviation)			
3	0,865	9,03	89,1	21,8
	(0,045)	(0,45)	(7,8)	(1,9)
4	0,796	8,14	75,6	18,8
	(0,016)	(0,47)	(5,7)	(1,2)
difference	0,069*	0,89*	13,5*	3,0*
p-value	p<0,01	0,01<p<0,02	0,01<p<0,02	0,01<p<0,02

* = significant

Table 3.4: **Pig muscle. Results of the electrolyte analysis on the samples of group 3 and 4. Concentrations are given in mmol/kg wet weight. Results of statistical testing of the difference between the means of both groups are also given.**

Results of the electrolyte analysis of the samples treated according to the HNO_3-method are shown in table 3.4 and 3.5. Table 3.4 gives the results in mmol/kg wet weight, table 3.5 in mmol/kg dry weight. Both tables also give the results of statistical testing of the differences between the means of group 3 and group 4. The test used is a students t-test for the difference between two means, $\alpha = 0{,}05$.

group	Ca	Mg	K	Na
	mmol/kg dry weight (standard deviation)			
3	4,48	46,7	461,7	113,0
	(0,14)	(1,1)	(26,0)	(6,9)
4	4,40	45,0	417,6	103,7
	(0,05)	(2,0)	(28,6)	(5,9)
difference	0,08	1,9	44,1*	9,3*
p-value	0,2<p<0,5	0,05<p<0,1	0,02<p<0,05	0,02<p<0,05

* = significant

Table 3.5: Pig muscle. Results of the electrolyte analysis on the samples of groups 3 and 4. Concentrations are given in mmol/kg dry weight. Results of statistical testing of the group means are also given.

	Ca	Mg	K	Na
mean concentration	1,391	12,35	449,4	87,64
sd	0,081	0,22	16,3	7,58
sem	0,021	0,06	4,2	1,96
variation coefficient	5,8%	1,8%	3,6%	8,6%
n	15	15	15	15

Table 3.6: Mean electrolyte concentrations (in mmol/kg dry weight) of rat vastus muscle.

The difference between group 3 (HNO_3-digestion and fat extraction) and group 4 (HNO_3-digestion without fat extraction) was significant for all electrolytes, when concentrations were expressed in mmol/kg wet weight, but only significant for potassium and sodium when comparing concentrations expressed in mmol/kg dry weight. The mean concentrations of electrolytes

	Ca	Mg	K	Na
R-squared	0,9962	0,9999	0,9987	0,9918
Y-intercept	-0,588	1,233	-32,93	-0,984
X coefficient	5,681	48,87	456,0	87,56
se of Y-estimate	1,427	2,27	67,6	32,42
n	15	15	15	15
Statistical testing of the Y-intercept estimate				
se of Y-intercept	0,613	0,97	29,1	13,93
t-test t	-0,959	1,267	-1,134	-0,071
degrees of freedom	13	13	13	13
p-value	0,2<p<0,5	0,2<p<0,5	0,2<p<0,5	p>0,5

Table 3.7: Results of linear regression analysis (dry weight (mg) vs biopsy content (μmol)) and statistical testing, rat vastus muscle.

were always higher for group 3.

3.3 Testing the method with rat muscle

The measured concentration in each sample in mmol/kg dry weight is shown in table 3.6. Mean concentration of calcium was 1,391 mmol/kg dry wt, of magnesium 12,35 mmol/kg dry wt, of potassium 449,4 mmol/kg dry wt, and sodium 87,64 mmol/kg dry wt. Standard deviations were not large and the variation coefficients ranged from 1,8% for magnesium, 3,6% for potassium, 5,8% for calcium to 8,6% for sodium.

Results of the linear regression analysis can be seen in table 3.7. Linear regression resulted in very high R^2 values, 0,9962 for Calcium, 0,9999 for Magnesium, 0,9987 for Potassium, and 0,9918 for Sodium. Statistical testing of the Y-intercepts showed that these were not significantly different from zero.

3.4 Testing the method with human muscle

Table 3.8 shows the mean electrolyte concentrations (in mmol/kg dry wt) of 13 samples (taken from the immediately frozen piece). Means were 4,175,

36,60, 413,8 and 74,8 mmol/kg dry weight (respectively for calcium, magnesium potassium and sodium). Variation coefficients varied from 5,9% for magnesium, 7,9% for potassium, 12,5% for calcium to 14,4% for sodium.

	Ca	Mg	K	Na
mean concentration	4,175	36,60	413,8	74,8
sd	0,522	2,17	32,7	10,8
sem	0,145	0,60	9,1	3,6
variation coefficient	12,5%	5,9%	7,9%	14,4%
n	13	13	13	9

Table 3.8: **Mean electrolyte concentrations (in mmol/kg dry weight) of human muscle (vastus lateralis).**

	Ca	Mg	K	Na
R-squared	0,9935	0,9983	0,9984	0,9902
Y-intercept	0,520	2,00	-16,7	-45,84
X-coefficient	3,626	34,39	437,1	98,19
se of Y-estimate	0,377	1,81	22,8	12,02
n	13	13	13	9
statistical testing of the Y-intercept estimate				
se of Y-intercept	0,185	0,89	11,2	8,22
degrees of freedom	11	11	11	7
t-test t	2,804*	0,249*	-1,484	-5,575*
p-value	0,01<p<0,02	0,04<p<0,05	0,1<p<0,2	p<0,01
* significant, $\alpha = 0{,}05$				

Table 3.9: **Results of linear regression analysis (sample dry weight (mg) vs sample electrolyte content (μmol)) and statistical testing, human vastus lateralis muscle.**

Linear regression analysis of the dry weight (in mg) of the samples against the sample electrolyte content (in μmol) resulted in very straight lines. A plot of dry weight against content with the calculated regression line can be seen in figure 3.1. A to D. Results of the linear regression analysis can be seen in table 3.9.

Again very high R^2 values were obtained, with a value for calcium of 0,9935, for magnesium 0,9983, for potassium 0,9984 and for potassium 0,9902. Statistical testing of the Y-intercept (with a student t-test and α = 0,05) showed that this time the difference from zero was significant (two-sided) for calcium, magnesium and for sodium. Calcium and magnesium were overestimated, sodium was underestimated.

In figure 3.2. A to D, the sample dry weight is plotted against the residual error (regression estimate - measured value). No significant relations between these two were found.

	Ca	Mg	K	Na
mean concentration (mmol/kg dry weight)				
biopsy-needle samples	4,225	32,26	383,3	148,3
sd	0,327	2,28	30,8	13,0
non-needle samples	4,175	36,60	413,8	74,8
sd	0,522	2,17	32,7	10,8
difference	0,050	-4,34*	-30,5*	73,5*
p-value	0,5<p<0,8	p<0,002	0,02<p<0,05	p<0,002

* significant, α = 0,05

Table 3.10: Results of statistical testing of the difference between 10 biopsy-needle samples and 13 non-needle samples.

	Ca	Mg	K	Na
mean concentration	3,532	35,35	421,1	86,93
sd	0,131	1,84	28,6	15,14
se	0,059	0,82	12,8	6,77
variation coefficient	3,7%	5,2%	6,8%	17,4%

Table 3.11: Mean concentrations and standard deviations of 5 samples that were analyzed in five different weeks. Mean weight of the samples was 2,41 mg dry weight (10,06 mg wet weight).

Mean concentrations (in mmol/kg dry wt) of 10 biopsy-needle samples were: 4,225 (0,327); 32,26 (2,28); 383,3 (30,8) and 148,3 (13,0) mmol/kg dry wt (sd) for respectively calcium, magnesium, potassium and sodium. These

were not significantly different from the earlier mentioned 13 samples taken from the immediately frozen piece of muscle for calcium ($0,5<p<0,8$). Significant differences were obtained for the other 3 electrolytes (Mg: $p<0,002$, K: $0,02<p<0,05$, Na: $p<0,002$). The statistical test used here was a students t-test for the comparison of two group means, $\alpha = 0,05$, two-sided testing. The results are summarized in table 3.10.
Table 3.11. shows the results of the reproducibility testing. Mean concentrations are given of five samples, prepared and analyzed in five different weeks. Standard deviations and variation coefficients are also given.

3.5 Discussion of the method

The (significant) differences between given and measured values for standardized bovine liver powder, may possibly be caused by:

(i) a very unstable balance, which made it very difficult to measure the weight of the powder in each tube. This balance was not used after the powder-stage.

(ii) pipetting errors causing the standards, used for calibration of the spectrophotometers, to be incorrect. Calibration standards were dilutions from a standard analytical solution, that was very concentrated compared with the final dilution (the standard analytical contained 1000ppm of the electrolyte and the dilutions 0,5 to 2 ppm). As a consequence, small pipetting errors of about 2% may have caused a large error in the concentrations of the electrolyte in the standards, even when diluting in two steps.

In later stages, parallel calibration standards were made, and tested against other standards, to pick out the best (with smallest chance on calibration errors) pair of calibration standards.

When testing the method with pig muscle, it was found that the H_2O_2 used in this study, was not able to digest the whole sample (weighing approximately 15 mg wet weight). Since it could not be made sure that there were no electrolytes left in the remainders, and since these remainders would complicate the preparation technique (at least centrifugion would be necessary), the method was considered less applicable and further development was discontinued.

When testing the effect of fat extraction, the difference between group 3 (with fat extraction) and group 4 (no fat extracted), was always significant when comparing mean concentrations in mmol/kg wet weight, and only insignificant in the case of calcium and magnesium when comparing in

mmol/kg dry weight. It may therefore be concluded from the results that fat extraction causes the calcium and the other electrolyte concentrations to be higher. Since the fat content of the dissected samples were very low (at least in pig), amounting to 1,7% of the dry weight and 0,3% of the wet weight, and since these amounts of fat were not expected to give rise to significant errors in the estimation of biopsy-electrolyte content, fat extraction was left out from the method.

Testing the method with rat muscle showed that, at least for Ca, Mg and K, the method, as established after the first two stages with powder and pig muscle, is able to accurately measure the concentration of electrolytes in samples ranging in weight from 5 to 50 mg wet weight. Standard deviations were not large and correlation coefficients were high, indicating high precision. Y-intercepts that were not significantly different from zero, indicating that the method is also accurate. Sodium shows rather large variation. Since these results, obtained from rat muscle, may not apply to human muscle, the stage with human muscle was also included.

For Ca, Mg and K high correlation coefficients and the low standard deviations were found for human, indicating that the precision of the method is again high, although the R-squared values were somewhat lower and the variation coefficients somewhat higher, than for rat vastus muscle.

Testing of the Y-intercept revealed that it was not significantly different from zero in the case of calcium, but significant in the case of the other three electrolytes. The cause of this significance probably lies in calibration errors. These errors can cause the concentration of an electrolyte to be both under- or overestimated. The direction of the error will be the same for the whole batch, but may be different for each electrolyte measured, since calibration standards contained dilutions of four different standard element solutions. The Y- intercept for calcium in rat muscle is the opposite to those found for human muscle, supporting the above suggestion. The variation coefficient of Sodium is again rather large. In addition, the Y-intercept is very large.

It was also tested whether small samples (weighing less than 5 mg wet weight) would give accurate results. The results showed, as indicated by the lack of a relation between the sample weight and the residual error, that the method was able to precisely measure the concentration, even when sample weight was less than 5 mg wet weight. Measurements on five samples all weighing approximately 10 mg wet weight, in five different weeks, revealed that the method was also highly reproducible for Ca, Mg and K. Standard deviations were very small, even though the measurements were done in five

different weeks. Again sodium results show a large variation.

The biopsy-needles, similar to and similarly treated as those that were used in the experiments of 1984 and 1985, did not contaminate the biopsies with calcium. Significancy was, however, obtained when comparing the magnesium, potassium and sodium concentrations of samples taken with a needle and samples not taken with a needle and concentrations were higher for the needle-group. Only in the case of sodium the difference was extremely large. It is not possible to say whether these contaminations were caused by the needles. Differences in treatment directly after the collecting of the muscle tissue (one piece was frozen immidiately in liquid nitrogen, while the other piece remained unfrozen for a little more than half an hour), may have played a role here. If the needles used in 1984 and 1985 would have caused contamination of the biopsies, this would result in an overestimation of the concentration in the biopsies. This would, however, not influence the magnitude or the direction of changes.

In conclusion, the method can be considered reliable to measure the concentration of calcium, magnesium and potassium in biopsies from human muscle. The method is both precise and accurate. As for sodium, this is not the case. Especially in the testing with human muscle, sodium has shown large variations in all samples. It can be concluded that the sodium concentration in biopsies is difficult to measure with this method. Results of the exercise experiments should therefore be interpreted carefully with regard to sodium.

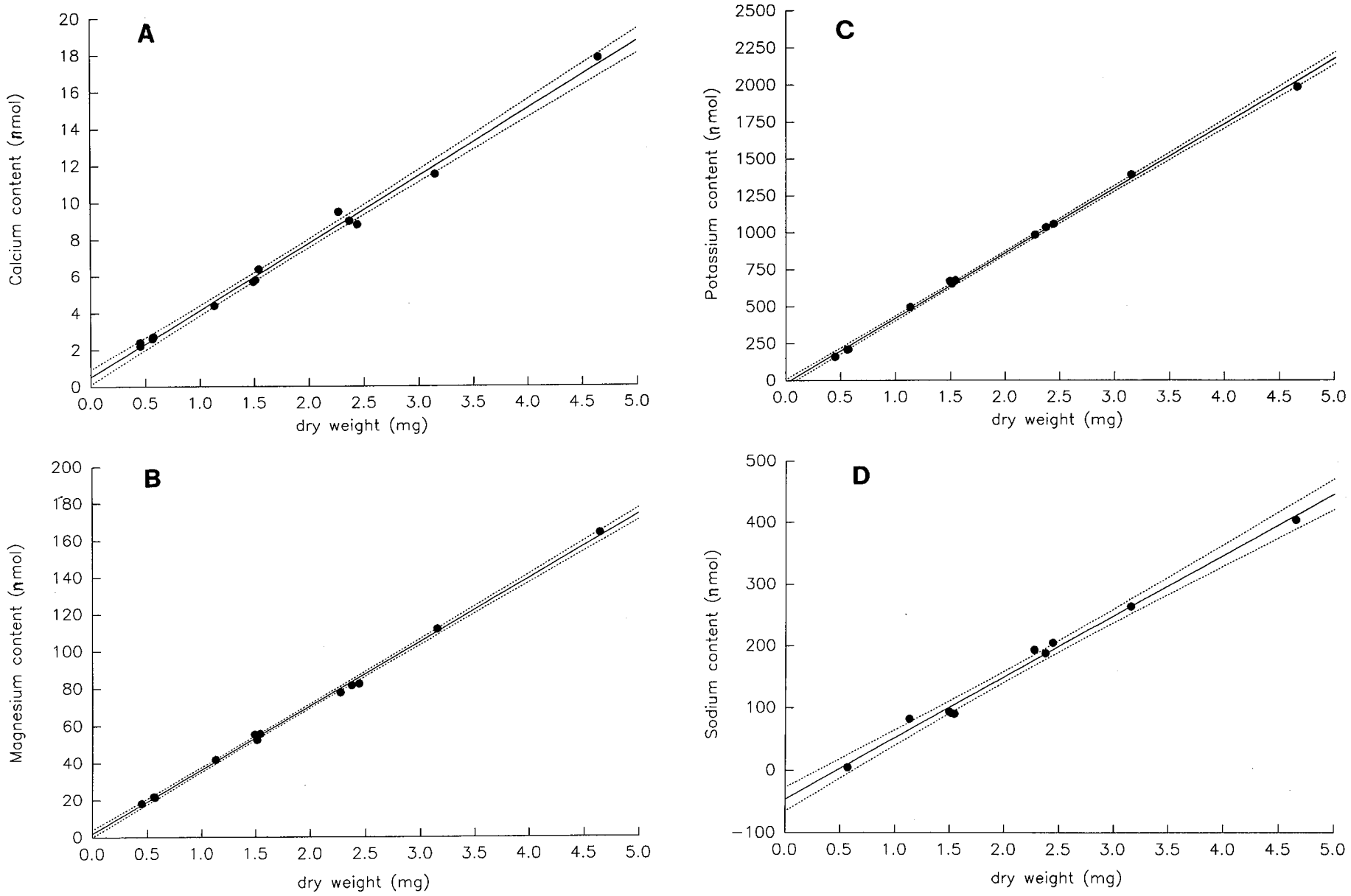

Figure 3.1: Calcium(A), Magnesium(B), Potassium(C) and Sodium(D) content plotted against Sample dry weight. The solid line represents the linear regression relation and the dashed line the 95% confidence interval. R= 0.9968 for Calcium, R= 0.9992 for magnesium, R= 0.9992 for Potassium and R= 0.9951 for Sodium.

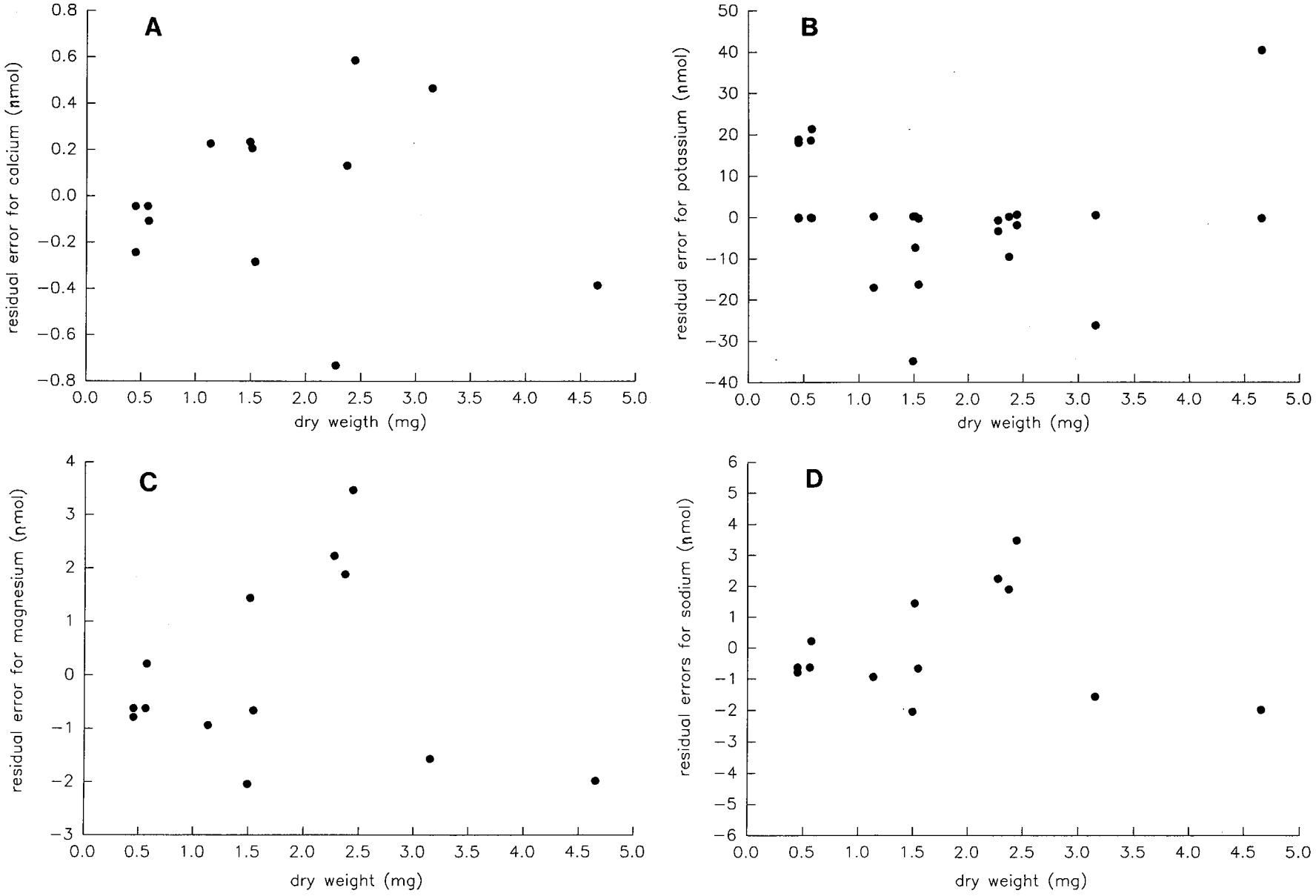

Figure 3.2: Residual errors (regression Y-estimate - experimental value for Calcium(A), Magenesium(B), Potassium(C) and Sodium(D) plotted against sample dry weight

Chapter 4

Results of the exercise experiments

Because of the long storage period of the biopsies, water evaporated from the biopsies. Results are therefore only expressed per kilogram dry weight.

Table 4.1 gives a summary on the subjects.

subject	age (years)	weight (kg)	height (cm)	time to exhaustion (min.)
1	21	79	182	45
2	23	80	188	36
3	25	67	173	60
4	31	72	176	80
5	23	88	196	67
6	23	81	186	91
7	30	66	180	105
8	25	79	184	(103)
mean	25,1	76,5	183	73
sd	3,6	7,5	7	25

Table 4.1: Subject summary. Age, weight, height, and time to exhaustion are shown. Subject 8 never reached exhaustion, but exercise was discontinued after 103 minutes

Table 4.2 to 4.5 show "raw" data used in all calculations. Means are shown when parallel biopsies (two or more biopsies from the same point in time) were available. From persons 3 and 5 no exhaustion biopsies were available. Subject 8 never reached exhaustion, but exercise was discontinued after 103 minutes.

time (min)	1	2	3	4	5	6	7	8	time (min)
0	2.971	3.688	3.461	3.084	3.044	3.223	3.321	2.495	0
5	3.313	4.379	2.984		3.305				5
15	3.941	4.667	3.016	3.195	3.913		3.735	3.013	15
30		3.637	2.974	3.430	3.537		2.766	2.844	30
36		4.492							36
45	3.370						3.856	3.065	45
55						3.531			55
60							3.579	3.304	60
80				3.331					80
91						3.642			91
92							3.288		92
101							3.718		101
103								2.715	103
105							3.911		105
exhaustion	3.370	4.492		3.331		3.642	3.911		exhaustion

Table 4.2: Concentration-data for calcium for all subjects (identified by numbers 1 to 8). Concentration is given in mmol/kg dry weight, means are given when parallel biopsies were available. From subjects 3 and 5 no exhaustion biopsies were available, subject 8 never reached exhaustion.

time (min)	1	2	3	4	5	6	7	8	time (min)
0	43.47	38.80	37.83	36.48	36.86	37.51	31.10	32.60	0
5	42.46	40.56	39.24		37.63				5
15	42.75	38.41	35.31	36.52	35.35		27.11	30.43	15
30		40.17	39.03	36.18	41.04		29.67	33.88	30
36		42.60							36
45	41.80					38.54	30.92	33.65	45
55						37.60			55
60							32.27	36.21	60
80				36.33					80
91						38.60			91
92							30.34		92
101							25.82		101
103								31.98	103
105							29.82		105
exhaustion	41.80	42.60		36.33		38.60	29.82		exhaustion

Table 4.3: Concentration-data for magnesium for all subjects (identified by numbers 1 to 8). Concentration is given in mmol/kg dry weight, means are given when parallel biopsies were available. From subjects 3 and 5 no exhaustion biopsies were available, subject 8 never reached exhaustion.

time (min)	1	2	3	4	5	6	7	8	time (min)
0	454.2	374.3	431.7	393.3	362.1	377.2	340.1	329.3	0
5	447.5	424.6	417.2		377.9				5
15	429.8	389.5	379.3	354.7	334.4		244.9	261.0	15
30		337.8	414.0	364.5	387.5		330.1	363.3	30
36		429.7							36
45	406.7					375.2	319.9	350.1	45
55						353.8			55
60							375.5	403.0	60
80				372.9					80
91						360.7			91
92							305.8		92
101							219.9		101
103								315.9	103
105							317.2		105
exhaustion	406.7	429.7		372.9		360.7	317.2		exhaustion

Table 4.4: Concentration-data for potassium for all subjects (identified by numbers 1 to 8). Concentration is given in mmol/kg dry weight, means are given when parallel biopsies were available. From subjects 3 and 5 no exhaustion biopsies were available, subject 8 never reached exhaustion.

time (min)	1	2	3	4	5	6	7	8	time (min)
0	74.9	146.6	172.9	107.7	240.0	171.0	81.2	46.8	0
5	107.5	187.4	144.0		317.4		144.7		5
15	156.6	167.4	228.5	182.8	367.0		69.0	93.2	15
30		238.3	130.9	245.6	121.6			117.6	30
36		217.6							36
45	123.1					116.3	129.2	140.8	45
55						135.4			55
60							99.2	123.4	60
80				289.5					80
91						208.6			91
92							142.1		92
101							282.5		101
103								189.7	103
105							145.9		105
exhaustion	123.1	217.6		289.5		208.6	145.9		exhaustion

Table 4.5: Concentration-data for sodium for all subjects (identified by numbers 1 to 8). Concentration is given in mmol/kg dry weight, means are given when parallel biopsies were available. From subjects 3 and 5 no exhaustion biopsies were available, subject 8 never reached exhaustion.

In figure 4.1 A to D the mean electrolyte concentrations (mmol/kg dry weight) are plotted against time (min.). It can be seen that almost all subjects show a tendency for a peak (or nadir in the case of magnesium

and potassium) at approximately 15 minutes. After this peak (or nadir) the electrolyte content decreases (increases) again, to a level approximating resting at 30 minutes. Towards exhaustion, no clear changes in one direction can be seen, although there may be a tendency for calcium and sodium to be higher, and magnesium and potassium to be lower at exhaustion.

An ANOVA two factor variance analysis revealed both a significant subject effect and a time effect on all four measured electrolyte concentrations of the muscle.

Figures 4.2 A to D show the individual plots of electrolyte concentrations against time. In order to exclude subject effects, concentrations are expressed in percentage of the resting value. The patterns already apparent in figures 4.1 A to D are even more explicit when looking at the individual changes. Statistical testing of the values found for 15 and 30 minutes against resting values and 15 minutes against 30 minutes, with a Wilcoxon matched pairs signed rank test ($\alpha = 0.05$), showed that the changes from 0 to 15 minutes were significant, but that the changes from 15 to 30 minutes were just non-significant. The concentration of electrolytes at 30 min were not significantly different from resting. The differences between the electrolyte concentrations at exhaustion and before exercise were also tested. This gave two- sided significant results for calcium and sodium, one-sided significance for magnesium but potassium concentration did not differ significantly from resting at exhaustion. Results of testing and p-values can be seen in table 4.6.

Figure 4.3 A to C show the ratios of calcium over potassium (A), calcium over magnesium (B) and potassium over magnesium (C), plotted against time. It can be seen that approximately the same time-pattern of changes as in the concentration curves reappears in the first two ratio curves. The curve of the ratio of potassium over magnesium is in most cases almost flat.

Difference tested:	0 - 15 min.	15 - 30 min.	0 - 30 min.	0 - exhaustion
1. Calcium				
mean difference	+15,96%	-11,64%	+1,53%	+13,8%
sd	16,16%	14,68%	14,49%	5,3%
se	6,11%	5,99%	5,91%	2,2%
n	7	6	6	6
T+	25	3	10	21
T-	3	18	11	0
p one-sided	0,039*	0,078	0,500	0,016*
p two-sided	0,078	0,156	1,000	0,032*
2. Magnesium				
mean difference	-4,73%	+7,95%	+2,75%	+ 0,41%
sd	4,43%	5,60%	5,33%	5,26%
se	1,67%	2,28%	2,17%	2,15%
n	7	6	6	6
T+	1	20	15	9
T-	27	1	6	12
p one-sided	0,016*	0,031*	0,219	0,042*
p two-sided	0,032*	0,062	0,438	0,084
3. Potassium				
mean difference	-11,36%	+11,21%	-1,13%	-2,68%
sd	10,45%	16,20%	8,02%	8,88%
se	3,95%	6,62%	3,28%	3,62%
n	7	6	6	6
T+	1	18	9	6
T-	27	3	12	16
p one-sided	0,016*	0,078	0,422	0,219
p two-sided	0,032*	0,156	0,844	0,438
4. Sodium				
mean difference	+65,03%	-15,55%	+58,62%	+114,70%
sd	34,38%	76,71%	71,75%	105,70%
se	12,99%	31,27%	29,29%	43,16%
n	7	6	6	6
T+	28	7	15	21
T-	0	14	6	0
p one-sided	0,008*	0,281	0,219	0,016*
p two-sided	0,016*	0,562	0,438	0,032*

*=significant

Table 4.6: Results of statistical testing. Test: Wilcoxon Matched Pairs Signed Rank test. Tested are differences in time of electrolyte concentrations. $\alpha = 0{,}05$.

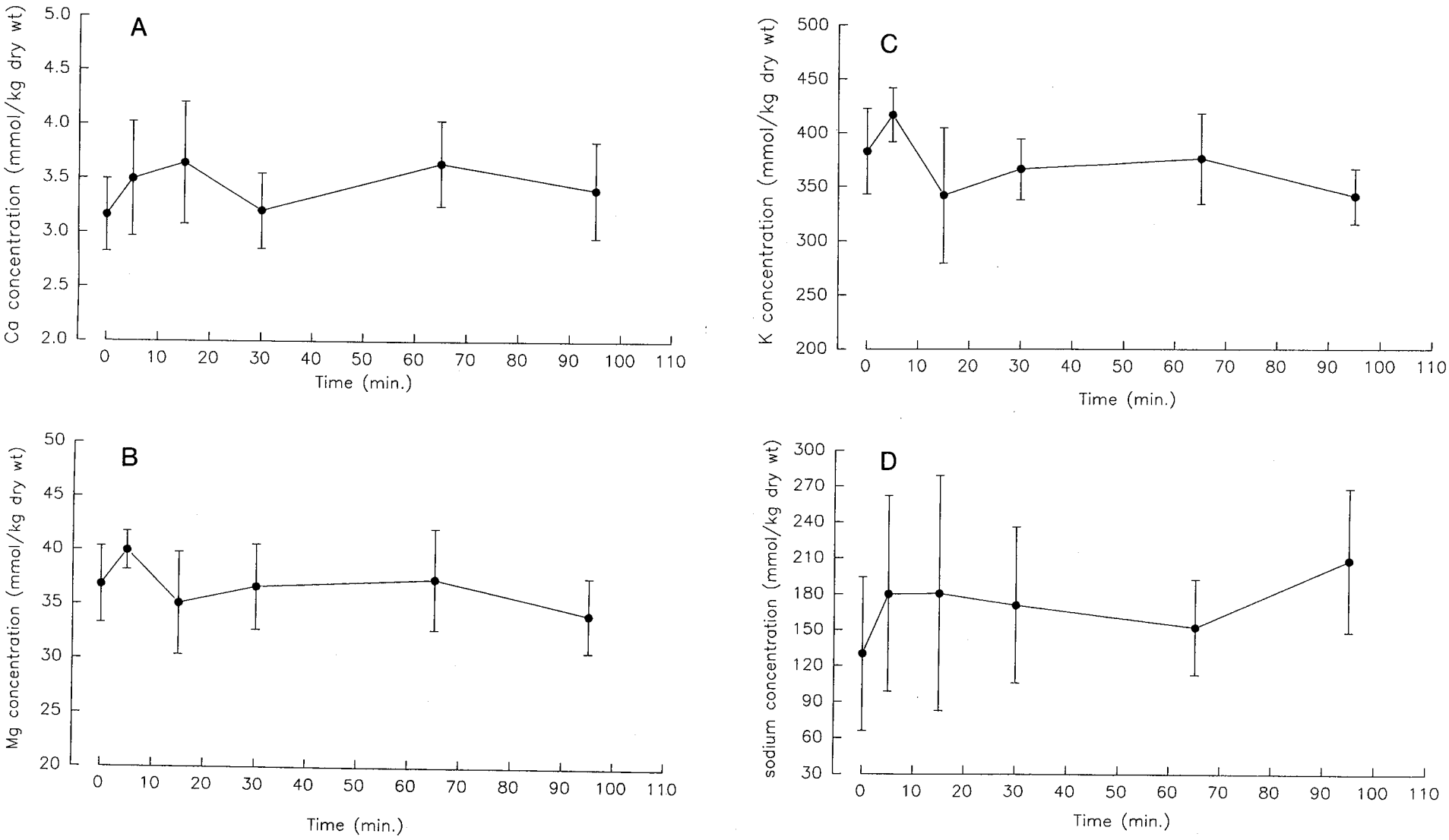

Figure 4.1: Mean calcium (A), magnesium (B), potassium (C) and sodium (D) comcentrations plotted against time. Means were taken at 0, 5, 15 and 30 minutes; a mean was taken for exhaustion biopsies of subjects 1 en 2 and non-exhaustion biopsies of the other subjects between 31 and 104 minutes, plotted at 65 minutes of the other subjects. Another mean was taken for the exhaustion biopsies of subjects 4, 6 and 7 and the last biopsy of subject 8, plotted at 95 minutes. The vertical bars represent standard deviations.

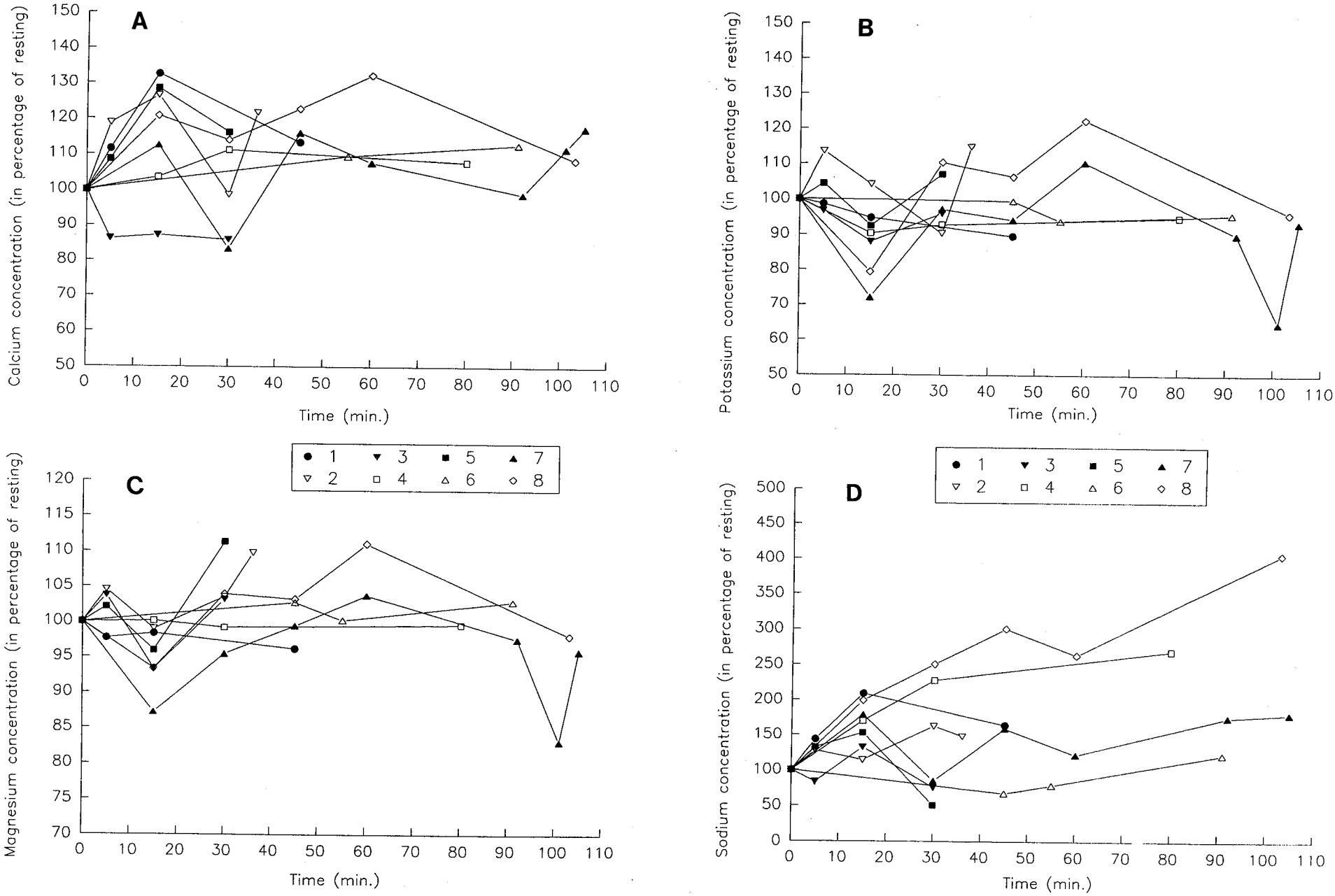

Figure 4.2: Calcium (A), magnesium (B), potassium (C) and sodium (C) concentration in percentage of resting plotted against time

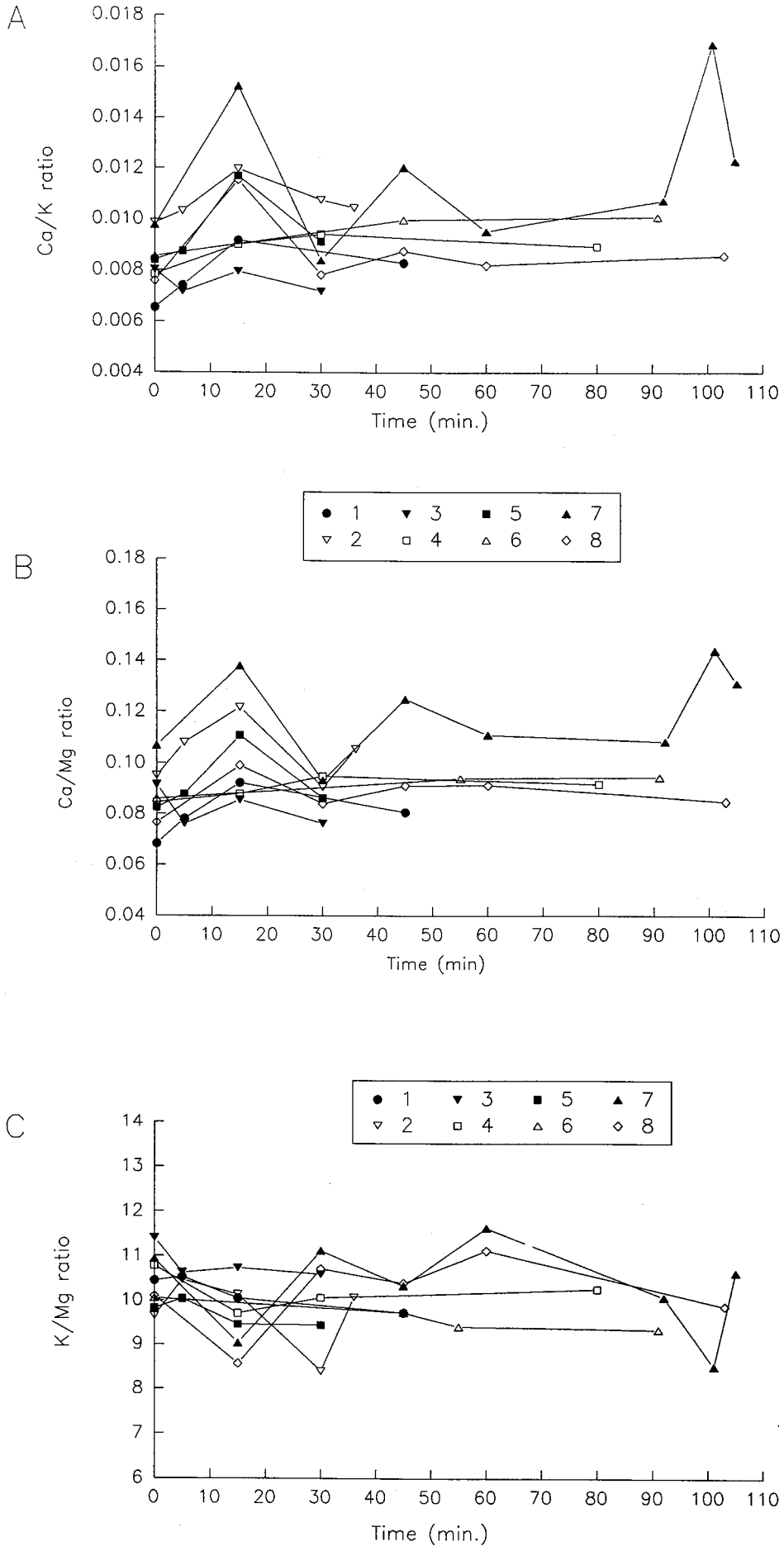

Figure 4.3: Ratios of calcium over potassium (A), calcium over magnesium (B) and potassium over magnesium (C) plotted against time. Data for all biopsies are shown.

Chapter 5

Discussion

All measured electrolytes in human muscle biopsies taken before and during exercise, showed a significant concentration variation over time. ANOVA two factor variance analysis revealed both a subject effect and a time effect. When looking at the relative change in electrolyte concentration over time, there seems to be a common pattern for all subjects, independent of how long they could exercise before exhaustion. This pattern consisted of a peak (for calcium) or a nadir (for magnesium and potassium) at 15 minutes, followed by a return to resting level at 30 minutes. After this, no further significant changes were seen towards exhaustion. For calcium this means that, until 15 minutes of exercise the muscle takes up calcium, but releases it again after 15 minutes. At 30 minutes the concentration of calcium is approximately back to resting levels, and does not change after this. For magnesium and potassium the pattern of change is exactly the opposite. The first 15 minutes of exercise, the muscle looses magnesium and potassium, after which the amount lost is taken up again by the muscle, reaching resting levels at 30 minutes. After 30 minutes no further changes occur. Sodium follows a pattern similar to that of calcium the first 15 minutes. The initial rise is followed either by a further rise, or by a fall back to resting levels in the period between 15 and 30 minutes of exercise. At exhaustion, all subjects from which exhaustion biopsies were available, show a higher muscle sodium concentration compared to resting.

Post hoc statistical testing of the differences between 0 and 15 minutes, 15 and 30 minutes, 0 and 30 minutes and 0 and exhaustion, gave significant differences between 0 and 15 minutes for all four electrolytes ($p \leq 0{,}039$), with the exception that the difference for calcium was only significant when tested one-sided. Significance was also obtained when testing the difference between 0 and exhaustion, but only for calcium and sodium ($p = 0{,}032$ in

both cases). The test used was the Wilcoxon matched pairs signed rank test, with $\alpha = 0{,}05$ and testing two sided.

The low variation coefficients, the high reproducability and the recovery of standards when testing the method with muscle samples from three different species, show that the method is a reliable tool for measuring calcium, magnesium and potassium in muscle biopsies. Systematic errors due to calibration, would not be able to cause such a reoccurring pattern, and it is very unlikely that the same errors were made for every batch of samples analyzed (biopsies from two subjects were analyzed in each batch, and different calibration standards were used). When looking at the ratio-figures, showing the ratio of calcium over potassium and calcium over magnesium, it can be seen that these curves show the same temporal change as the concentration curves. Pipetting errors could cause errors in either direction for the different samples, but would be expected to have the same effect (the same direction) on all the electrolytes measured on one sample. That this is not the case can be seen in the ratio-figures. Here the same temporal pattern as in the concentration curves reappears, showing that calcium and magnesium and calcium and potassium move in exactly opposite directions. The curve of the ratio of potassium over magnesium is in most cases approximately flat. Each point in these figures represents a ratio of two values that were obtained from the same sample. Errors dependent on the order of analysis are unlikely, as the biopsies were not analyzed in the time-order of their taking. It is thus unlikely that the present variations in time of the concentration of the exercise biopsies have been caused by errors in the measurements of the electrolyte concentrations.

Since the measurements are apparently correct, then the question remains whether they represent what they should represent, i.e. the (total) intracellular and interstitial concentration in the muscle. Contaminations with other tissues than muscle and contamination with blood could cause the measured electrolyte concentration to not be representative for muscle. However, the biopsies were dissected carefully free from blood and non-muscular tissues, and only small amounts (if any) could have remained in the biopsy. Since blood plasma concentration of at least potassium does not change much, compared to the amount in the cell (about 1 mM change in blood plasma in this type of exercise (Vøllestad et al. 1991 [30]) compared to a total intracellular concentration of about 150 mM (Sejersted, 1992 [22])), it would be very unlikely that small amounts of blood remaining in the biopsy could cause the changes over time found in the present investigation. Thus it can be concluded that the measured temporal changes probably reflect

changes in the muscle parenchymal electrolyte concentrations.

The concentrations in biopsies represent both intracellular and interstitial concentration. If one assumes that the interstitium is in constant equilibrium with the blood (which is very likely since the blood capillary membranes are higly permeable to small ions), then the changes in biopsy- concentration should represent changes in intracellular concentration. Different fiber type distribution in biopsies can give different intracellular electrolyte concentrations, due to differences in electrolyte concentration between fast-twitch and slow-twitch fibers. In the present experiment, however, there was not found any significant relation between the electrolyte content and the fiber type distribution of the biopsy. The changes over time in muscle electrolyte concentration could therefore not have been caused by different fibre type distributions in the biopsies.

In conclusion, it seems that exercise causes temporal changes in electrolyte concentration of the muscle cells, and that calcium, potassium and magnesium are closely related to each other. When calcium shifts in one direction over the cellular membrane, potassium seems to move in exactly the oppposite direction. The same is true for magnesium compared to calcium. Potassium and magnesium seem to move in the same direction. As for sodium, considering the difficulties met in measuring this ion, conclusions cannot be drawn as sharp. Sodium seems also to change over time of exercise, but in a somewhat different way than the other three measured electrolytes. Instead of a peak or nadir at 15 minutes of exercise, the sodium concentration seems to rise continuously towards exhaustion.

A study of the literature on the subject of exercise and electrolyte content of muscle did not reveal any earlier research reporting the same pattern of change in muscle electrolyte concentration with exercise. Comparable total electrolyte concentrations in human muscle were, however, found earlier by Dørup et al.(1988) [10], Ericsson (1984) [11], Jackson et al.(1985) [15], Sjøgren et al.(1987) [24], Sylvén et al.(1991) [27], Lunde and Jebens (personal communication).

Vøllestad et al.(1991) [29] reported a continuous loss of K^+ from the muscle, when measuring blood plasma content of the femoral vein and artery together with blood flow during similar exercise as in the present investigation. Although their blood-data do not reveal a similar pattern as presently found in muscle, the accumulated loss of K^+ is comparable to the (insignificant) loss found presently. Changes in electrolyte concentrations in exercising human skeletal muscle were reported by Sjøgaard (1988) [23], measuring on biopsies taken before and at 30 and 60 minutes of exercise. This revealed

a (insignificant) continuous loss of K^+ from and a continuous uptake of Na^+ in the muscle amounting to a total of 11 and 14 mmol/kg dry wt respectively at exhaustion. The exercise was however different from that done presently, namely a continuous isometric contraction at 5% MVC. Sahlin and Broberg (1989) [20] also report a loss of a comparable amount of K^+ from the muscle due to 60% VO2max dynamic exercise, measured in blood plasma. All conclude that this change in muscle K^+ concentration may play a role in the mechanism of fatigue. Vøllestad and Sejersted (1988) [28] propose a role for calcium. The results of the present investigation reveal that there are temporal changes in the concentrations of the four electrolytes. More research is needed before any conclusions can be drawn on their role in the development of fatigue. The results do not reject the hypothesis that K^+ or calcium or the two other electrolytes play a role in fatigue. Fatigue may even be caused by an overall disturbance of the electrolyte balance.

5.1 Speculations

In the following paragraph speculations are made in an attempt to explain the time-pattern found for the changes in intracellular electrolyte concentration. The speculations are based on reports of research on the effects of exercise, fatigue or stimulation on the cell. Changes in muscle intracellular electrolyte concentration can only occur when the ions are transported over the sarcolemma via transport-proteins, since the sarcolemma is otherwise impermeable to ions. There are three types of transport proteins in the membrane: channels (the direction of transport depends on the electrochemical gradient), transporters (f.e. exchangers) and pumps (which are primarily active transporters) (Stryer, 1988 [26]). Transport of K^+ and Na^+ is known to happen through channels that open when the muscle is stimulated. At each action potential some nmol of K^+ slips out of the cell and about the same amount of Na^+ slips into the cell (Clausen and Everts, 1988a [7]). In order to recreate the membrane potential, K^+ and Na^+ have to be pumped back. One known mechanism in muscle cells is the Na,K ATPase, which pumps 2 K^+ in and 3 Na^+ out for each ATP hydrolyzed (Skou, 1965 [25]; reviewed in Sejersted, 1992 [22]). Other mechanisms for active transport of K^+ and Na^+ are not yet found in skeletal muscle cells. Some controversy exists on the existence of a Na^+/Ca^{2+} exchanger, which is found in many other tissues and which plays a major role in the ion balance of the heart muscle cell. Gilbert and Meissner (1982) [13] found Sodium- Calcium exchange in

rabbit sarcolemmal vesicles, but their vesicle population may have been contaminated with plasmalemma from other tissues than skeletal muscle. The existence of a $Na^+/K^+/Cl$–cotransporter in skeletal muscle, which is for example found in heart myocytes (Liu et al.1989 [17]), is also questionable, since there are no reports of such a transporter in skeletal muscle.

Known calcium transport sites are the dihydropyridine-sensitive Ca^{2+}-channel (Barhanin et al.1989 [1], Beam et al.1989 [2], Carafoli, 1987 [5], Curtis and Catterall, 1984 [9]). It is however not known whether this channel transmits a large "leak" of calcium into the cell. Active transport of calcium out of the cell happens via the Calcium-ATPase, which can pump 1 Ca^{2+} per ATP hydrolyzed (Carafoli, 1987) [5], and if existing, also via the Na^+/Ca^{2+} exchanger, which exchanges 1 Ca^{2+} against 3 Na^+. One article, written by Kirley (1988) [16], reports the existence of a Mg^{2+}-ATPase in rabbit skeletal muscle transverse tubule. This ATPase is stimulated by millimolar concentrations of Ca^{2+} or Mg^{2+}, but not by micromolar concentrations of calcium. The physiological role of this ATPase is however not known. It is speculated that this ATPase may be important in removing cytosolic Ca^{2+} when its concentration exceeds 10^{-7}M. No reports are found on magnesium transport across the sarcolemma. The above mentioned transport systems are reviewed in figure 5.1, showing a schematic picture of a muscle cell and its environment.

Reports on the rate of transport of the Na^+/K^+-ATPase pump are manyfold. Most studies have been done on rat muscle. Clausen and Everts (1988b) [8] calculated a theoretical maximum pump rate for humans of 4000 nmol K^+/g wet muscle/min, assuming a maximum ATP turnover rate of 8000/min for this pump, 2 ions of potassium pumped per hydrolyzed ATP and a pump density of 0,25/g wet muscle. Equivalent Na^+-maximum pump rate would be 6000 nmol Na^+/g wet wt/min. Under optimal conditions they obtained an experimental value for the rate of K^+ pumping that corresponded to 90% of this theoretical maximum. No rate values of the Ca^{2+}-ATPase of the sarcolemma are reported, but data on rate of calcium influx or efflux are given in Gilbert and Meissner (1982) [13] and in Everts and Clausen (1986) [12]. Gilbert and Meissner report an optimum efflux rate of 4,6 to 6 nmol/mg protein/min, which corresponds to 0,133 to 0,174 nmol/g muscle/min with a yield of 29 mg protein/kg muscle. Optimum influx rate reported was 18,7 - 21,1 nmol/mg protein/min, which corresponds to 0,542 - 0,612 nmol/g muscle/min. These figures were measured in rabbit white muscle isolated sarcolemmal vesicles. Everts and Clausen report much higher Ca^{2+}-uptake rates, based on tracer studies. The values reported were

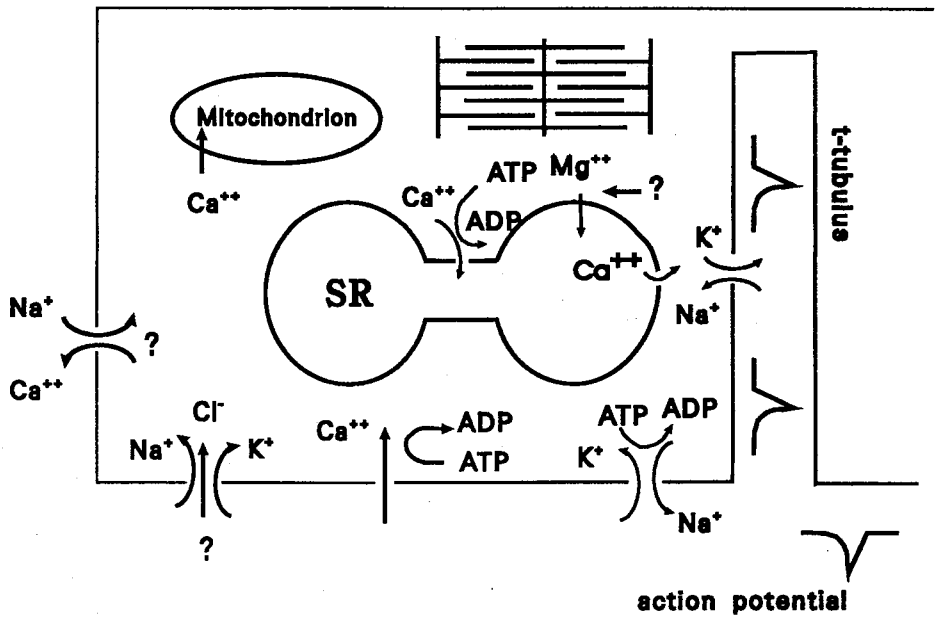

Figure 5.1: Schematic picture of the transportsystems in the muscle cell

measured in rat soleus and amount to 5,5 nmol/g wet wt/min. Compared to this value, it is interesting to see that the rate of calcium uptake in the muscle during the first 15 minutes of exercise in the present experiments was about 5,3 nmol/g wet wt/min, if assuming a mean increase in Ca^{2+} concentration of 0,4 mmol/kg dry wt in 15 minutes (see figure 4.1 A), and a muscle water content of 80%.

When looking at potassium losses and sodium uptake the first 15 minutes of exercise in the present study, mean rates can be calculated amounting to 550 nmol K^+ lost/g wet wt/min and 750 nmol Na^+ increase/g wet wt/min. Vøllestad and Bigland- Ritchie (1991) [29] report firing rates of 11,5 Hz in the first 5 min of similar exercise as presently used, increasing to 16 Hz after 30-35 min. If a loss of 7 nmol K^+/action potential/g wet muscle (as reported by Clausen and Everts, 1988b [8]) is assumed then the muscle would loose 11,5×60×7=4830 nmol/g wet wt/min in the first 5 minutes of exercise, increasing to 16×60×7=6720 nmol/g wet wt/min after 30-35 minutes, if no pumps or other transport mechanisms would be active. Even when assuming 100% activity, the ATPases would not be able to pump back the amount of K^+ lost with each action potential, and the muscle would loose 830 nmol/kg

wet wt/min, when using the above mentioned figures. This value is higher than the presently found rate of loss of 550 nmol/g wet wt/min. Similar calculations for sodium, would give an influx of Na^+ of 4692 nmol Na^+/g wet wt/min increasing to 6528 nmol Na^+/g wet wt/min, if assuming an influx of 6,8 nmol/action potential/g wet wt, and if no pumps or other transport mechanisms would be active. In this case however, the Na,K-ATPase alone should be able to prevent the influx of Na^+ due to action potentials. A pump rate of approximately 65% could explain the influx rate found for the first 15 minutes of exercise. Sejersted (1992) [22] calculated theoretical steady state intracellular Na^+ concentrations that would be reached at certain stimulation frequencies. Stimulating a muscle at 11,5Hz would give a rise in steady state level of intracellular Na^+ concentration of approximately 11 mM. Assuming a (unchanging) muscular water content of 80% and a muscle density of 1 kg/l, this value would correspond to 55 mmol/kg dry wt. In the first five minutes of the present exercise, intracellular Na^+ concentration increased on average 50 mmol/kg dry wt. He used, however a slightly lower influx rate per action potential for Na^+ than Clausen and Everts: 3-5 nmol/g wet wt/action potential in stead of 7 nmol/g wet wt/action potential. Calculating with Sejersted's Na^+ influx values (and assuming that the K^+ efflux is of the same magnitude), the picture of the capacity of the muscle to keep the ionic balance is somewhat less pessimistic than above. With a K^+ efflux of 3-5 nmol/g wet wt/a.p., the K^+ lost if no transport mechanisms were active, would amount to 2070-3450 nmol/g wet wt/min in the first five minutes of exercise when the muscle is stimulated at 11,5 Hz. Na,K-ATPase pumps, activated at 52-85% of their maximum (assuming a maximum pump rate of 4000 nmol/g wet wt/min), would be able to prevent this loss of K^+. Since the maximum pump rate for sodium is apparently larger than for potassium, the pumps would be able to maintain intracellular sodium even at a lower percentage of the maximal rate. No other transport mechanism for Na^+ than the Na,K-ATPase is known in the skeletal muscle cell, that transport Na^+ out of the cell (under physiological conditions). The other two transport mechanisms mentioned, involving sodium, most probably transport sodium into the cell. It may thus be speculated that the Na,K-ATPase pump rate is well below 85% of its maximum, and that K^+, and probably also Na^+, is transported into the cell via other mechanism. In this context it is interesting to note that Liu et al.(1989) [17] report an interaction of Na/K/Cl-cotransport with the Na^+,K^+-ATPase pump in cultured chick cardiac myosites. The existence of such other transport systems would also make it easier to explain the influx of sodium and the back-regulation

of the electrolyte balance after 15 minutes of exercise.

Why the muscle takes up calcium the first 15 minutes of exercise and subsequently releases it after 15 minutes, can also only be speculated. Byrd et al.(1989) [4] report that calcium uptake rates into the SR decrease after 20 minutes of treadmill run in deep fibres of rat gastrocnemius and vastus muscles. Gollnick et al.(1991)[14] report a decrease in calcium uptake by the SR in humans after high-intensity exercise. Melzer et al.(1986) [18] report a decay in the rate of removal of cytosolic free calcium following release from SR, after fatiguing stimulation, in single frog skeletal muscle and suggest that this decrease may be due to decreased pump activity, but also due to increased saturation of the slow-calcium buffering proteins in the cytosol, parvalbumin. It can then be speculated that during the first 15 minutes of exercise, the rate of the Ca uptake in the SR is large enough to take back all of the calcium released from the terminal cisternea during activation. This rate may even be so large and the pump rate of the Ca pumps in the sarcolemma so low, that the calcium that leaked into the muscle due to action potentials, is also pumped into the SR. After 15 minutes, the rate of uptake into the SR may be decreased, and the rate of transport of calcium out of the cell (not necessarily only by the Ca^{2+}ATPase pumps of the sarcolemma) may be increased, so that the leaked calcium and an extra amount are pumped out of the cell, and leading to a net loss of calcium from the cell until 30 minutes of exercise.

The reason for the changes in magnesium concentration in the muscle are hard to explain, since only very little is known about transport of magnesium across membranes. For the cell it is important to keep the cytosolic free magnesium unchanged, so that if there should be some imbalance, possibly caused by the imbalance of the other electrolytes, this would most likely be backregulated.

The time course of the imbalance and its back regulation may be much shorter for the individual fibers, than the 30 minute time course found for whole muscle. Vøllestad and Bigland-Ritchie found that in this kind of exercise, about 50% of the fibres (most of them slow twitch) are activated at the start of exercise, and that increasingly more fibres are recruited towards exhaustion (Vøllestad, personal communication). If then the individual fibers have the same imbalance and back-regulation pattern, but the time course of it is much shorter, the accumulated pattern for the entire muscle may have a longer time-course.

The above section is of course very speculative. Not much data is available on the actual amount of ions pumped or released through chan-

nels, and the effect of exercise on the pump and release rates. The little that is known comes often from experiments with animals or entirely different tissues, which may give results that are not at all applicable for humans and the present type of exercise. It is obvious that more research is needed. Experiments with single fibers or even with whole muscles, in which cytosolic and extracellular (in the surrounding media) calcium and other ions can be measured, should be able to verify or reject some of the above speculations. Further experiments with muscle biopsies from exercising humans are already planned. A series of measurements on the electrolyte content of biopsies from isometric intermittent exercise at 45% MVC are planned for september and october. After this a new series of experiments is planned, with intermittent isometric exercise at decreasing force level in the 6 s activity period, hoping to exclude recruitment of new fibres. Electrolyte content will again be measured in biopsies. The results of these measurements may be able to answer some questions about the role of fibre recruitment in these changes in muscle electrolyte concentration.

Chapter 6

Conclusions

Exercise seems to cause transient changes in muscle electrolyte concentration, that are dependent on the duration of exercise, but not on how long a person can exercise until exhaustion. The electrolytes measured presently, calcium, magnesium and potassium seem to be closely related, in that changes occur in all the electrolyte concentrations at the same point in time of exercise. The muscle tends to take up calcium and loose potassium and magnesium the first 15 minutes of exercise. In the case of calcium, magnesium and potassium, this uptake or loss seems to be corrected again by the muscle, and concentrations are back to approximately resting levels at 30 minutes of exercise. After 30 minutes a slow calcium uptake and a slow magnesium and potassium release may be possible, but the changes presently found were not significant, although exhaustion values were significantly higher than resting values for calcium. As for sodium, there is no such clear pattern. Individual differences are large. Considering the methodological problems with measuring the sodium concentration, no sharp conclusions can be drawn as to the influence of exercise on the muscle concentration of this ion.

The reasons for the apparent imbalances and backregulations are unclear, and more research is needed to confirm or reject the speculations made. The results do not reject the hypothesis of a role in fatigue for potassium or calcium, as proposed earlier by Clausen and Everts (1988b) [8], Sahlin and Broberg (1989) [20], Sjøgaard (1990) [23] and Vøllestad et al.(1991) [30], or for the other two electrolytes measured.

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Appendix

Summary of the method as used for the exercise biopsies.

1. Frozen muscle biopsies were dissected free from visible fat, connective tissue and blood in a refrigerating chamber (-30 to - 25C)
2. The dissected biopsies were weighed on a balance placed in the same refrigerating chamber.
3. The weighed biopsies were attached to preweighed platinum hooks and dried overnight at 90C.
4. The dry muscle biopsies were again weighed (still attached to their hooks and using the same balance, but now at room temperature).
5. The biopsies were then placed in teflon tubes, and 250 μl 65% HNO_3 was added. The tubes were closed tightly and placed in an oven at 60 to 70 C, for 2 to 3 hours. This ensured the biopsies to be fully digested.
6. After cooling down, the HNO_3-digest was diluted with 3750μl distilled and deionized water to 4000μl. These samples were analyzed for calcium and magnesium contents with ICPS.
7. Of the remaining of the 4000μl dilutions, 250μl was taken and diluted with 1% Cs standard solution to 2000μl. This was analyzed for potassium (K) content with FAES.
8. Also of the remaining of the 4000μl dilutions, 500μl was diluted with 1% Cs standard solution to 2000μl. This was analyzed for sodium (Na) content with FAES.

If not stated otherwise these were the instruments and chemicals that were used (in order of use):

- balance: Cahn 27 automatic electrobalance.
- platinum hooks: weighed between 36 and 50 mg.
- HNO_3: Chem Scan AS, Elverum Norway (Scan Pure).
- Inductively Coupled Plasma Spectrophotometer: Perkin Elmer ICP-5500. Wavelenght for calcium: 393,37 nm; magnesium: 279,55 nm. Two standards were used for calibration, 0,500 ppm and 1,000 ppm of both Ca and Mg, the blank contained only distilled and deionized water.
- Cs solution: Spectrascan element standard for atomic spectroscopy (Cs, 1000 ppm), Teknolab AS, Drøbak Norway.

- Flame Atomic Emission Spectrophotometer: Perkin Elmer 5000, flame air-acetylene. Wavelength for potassium: 766,5 nm; two standards: 1,000 ppm and 2,000 ppm and a blank containing 0,5% HNO_3 were used for calibration. Wavelength for sodium: 589,0 nm; two standards: 0,500 ppm and 1,000 ppm and a blank containing 1% HNO_3.
- Calibration standards were made by diluting element standards: spectrascan element standards for atomic spectroscopy (Ca, Mg, K, and Na, each solution contained 1000 ppm), Technolab AS, Drøbak, Norway.