A COMPARISON OF ION SHIFTS WITH ADENOSINE TRIPHOSPHATE AND CREATINE PHOSPHATE LEVELS IN MUSCLE

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SUMMARY

ATP and CrP have been estimated in toad sartorius muscle by a modification of existing techniques which has enabled large numbers of analyses to be performed.

Levels of these compounds *in vivo* and after soaking the sartorius in normal Ringer are compared.

The effect of high Ringer levels of K^+, Rb^+, Cs^+ and Li^+ on the ATP and CrP content have been correlated with ion shifts in the same muscle.

The effect of a small number of metabolic inhibitors on the ATP and CrP level has been correlated with the Na^+ and K^+ content. The findings have been found consistent with the hypothesis of a 3-phase system for muscle, whereby a constant feed in of metabolic energy will not be required to maintain the ionic gradients.

INTRODUCTION

The maintenance of the normal ionic pattern of the muscle cell involves a separation of Na^+ and K^+, and results in large concn. gradients of these ions across the cell membrane. Since studies with radioactive tracers have shown that there is a constant exchange of all the ions of the system across the membrane this ionic partition cannot result from the exclusion by the membrane of certain of the ionic species. Numerous theories have been propounded to explain the mechanism of ionic discrimination, (for bibliography see SIMON *et al.*[1]). Broadly speaking there are 2 main schools of thought. Firstly, there is a widely held opinion that the steady state ionic concns. are maintained by the active outward transport of the excluded constituents, which may or may not be linked with an active inward movement of accumulated constituents. It is obvious that this type of system would require the constant feed in of metabolically derived energy for the postulated ionic movements.

The second type of mechanism proposed has resulted from an attempt to reduce the energy requirements of the system. It has been postulated that the accumulation of K^+ results from some type of adsorption onto the cell structure. This view has been put forward separately by LING[2], HARRIS[3,4], and TROSHIN[5], and is also held by the authors[1,6,7]. We have proposed that muscle should be regarded as a 3-phase system, there being an extracellular phase and 2 intracellular phases, the free intracellular phase and the ordered phase. The ability of the cell to differentiate between similar ionic species depends upon the ability of the ordered phase to either accumulate or

References p. 495.

exclude these ions. Excluded ions will, therefore, be confined to the free intracellular phase, where it is assumed they will be in what approximates to a diffusion equilibrium with the external medium. Deviations in the behaviour of the excluded ions from that expected in this initial hypothesis have been discussed elsewhere[6,7]. It suffices to say here that such a system would obviate or reduce the continuous feed in of energy necessary for the "pump" hypothesis.

An evaluation of the metabolic status of the muscle during the course of various ion shifts could be expected to throw light on the mechanism of ionic discrimination. In this and a subsequent paper[8] are recorded expts. in which we have attempted to correlate the ATP and creatine phosphate level of the cell, and the oxygen uptake and aerobic glycolysis with the ion movements.

METHODS

The sartorius muscle of the toad, *Bufo marinus*, was used throughout this study. The treatment of the muscles was essentially similar to that reported previously[1]. Paired muscles were always taken, one of which was used as the control. Sufficiently large numbers were used to permit a statistical evaluation of the results. Particular care was taken in blotting the muscles, in order to avoid contraction, as this was found to reduce the resting creatine phosphate level of the tissue.

At the conclusion of each expt. the muscles were frozen in liquid air, and homogenized with 2-ml aliquots of a 5 % solution of trichloracetic acid in a chilled pyrex homogenizer. The homogenate was centrifuged at 2° and the supernatant fluid was divided into 3 portions and analysed as follows: (a) 1 portion was analysed for ATP and ADP without further treatment. (b) A second portion was diluted 1:5 and analysed for cations. (c) 1 ml of the 1 in 5 dilution was neutralized with 0.4 *N* NaOH to pH 7.0 in the cold, and made up to 5 ml with iced distilled water. This fraction was analysed for creatine and creatine phosphate.

Analysis of ATP and ADP

50-μl aliquots of each extract were spotted onto Whatman No. 1 chromatography paper. A blank was run for each pair of muscles. The chromatograms were developed for 6 h by the technique of KREBS AND HEMS[9], using isopropyl ether–90 % formic acid (3:2). After drying the paper was cut 1.5 inches above the starting line. The chromatogram was then run overnight in the reverse direction, using *iso*-butyric acid–1 *N* ammonia–0.1 *M* EDTA (100:60:1.6). The paper was dried and photographed in u.v. light to localize the ATP, ADP and (when present) AMP. The method was similar to that suggested by MARKHAM AND SMITH[10].

The paper was then cut and the adenosine phosphates eluted with 6 ml of water. The absorption due to adenosine was estimated in a Beckman model DU spectrophotometer at 260 μm[11], using a photomultiplier attachment to increase the sensitivity. Standard ATP samples were run with each determination. This method is described more fully in another communication[12].

Cation analysis

The diluted extract was analysed for Na^+, K^+ and Li^+, Cs^+ and Rb^+ (when present) using a Beckman flame spectrophotometer. We noted marked interference

References p. 495.

when estimating K^+, Cs^+ and Rb^+ in the same sample. These errors were corrected with the aid of an interference curve in a manner similar to that described by LUBIN AND SCHNEIDER[13].

Creatine and creatine phosphate analysis

The third fraction was analysed for creatine and creatine phosphate by the method of ENNOR AND ROSENBERG[14] with the following modification. Instead of adding the diacetyl and α-naphthol separately these solutions were mixed together in bulk, and 3 ml of the mixture was added for colour development. This modification in no way affected the reproducibility of the results or the full recovery of added creatine.

The muscle extract was tested for the presence of guanidine and glycocyamine which are known to interfere in the diacetyl reaction. This was done using an ascending paper chromatographic technique, with 95 % ethanol as solvent[15]. The spots were detected with a diacetyl spray which was made by adding 2 ml of an alkaline 1 % solution of α naphthol to 1 ml of a 5 % solution of diaceytl and diluting the resulting mixture to 10 ml[15]. This reagent was more sensitive than the alkaline ferricyanide-nitroprusside reagent suggested by SMITH[16].

The following R_F values were obtained: Toad-muscle extract 0.06, muscle extract plus 0.04 μM creatine 0.06, 0.04 μM creatine in 5 % trichloracetic acid 0.05, 0.04 μM guanidine plus toad extract 0.49 and 0.06, 0.04 μM glycocyamine and muscle extract 0.56, 0.06. Thus both these interfering substances would appear to be absent.

Solutions

The Ringer solutions used were modifications of those described previously[17] and will be detailed in the text. Aureomycin was added to the Ringer for all expts. of more than 4 h duration to prevent bacterial contamination.

Results in vivo

The toads were killed by pithing, the muscles were rapidly excised and frozen in liquid air. They were then analysed as described in the METHODS section. The ATP content ∓ S.E. was 4.7 ∓ 0.3 mmole/kg (18 observations). This was the figure obtained for winter toads (see below). The assay of creatine phosphate gave most erratic results which could be related to the activity of the toad during pithing. Various methods of sedation were tried, but no constant levels were obtained.

Muscles soaked in Ringer

ATP, CrP and Cr levels: It is well known[1] that when excised muscles are held in Ringer solution there is a movement of Na^+ into the cell, and a loss of K^+. These ionic movements along the concn. gradient are prevented by soaking the tissue in homologous plasma[6], but it is not known whether this stabilizing effect of plasma is due to a direct effect on the cell metabolism. The ATP and ADP level of muscles *in vivo* was compared with that of companion muscles soaked in Ringer for 4 h. There was no significant change in ATP or ADP concn., the mean ATP level was 5.4 mmoles/kg in both series of muscles. Creatine phosphate levels tended to be stabilized during the 4 h soaking. Since there was a large seasonal variation in the total creatine

References p. 495.

level of the muscle, we have compared in all expts. the ratio of creatine phosphate to creatine (Cr P/Cr) rather than the absolute amounts.

The authors have shown previously[18] that the inorganic phosphate level of the cell is not altered by soaking in Ringer.

Seasonal variation: When considering the level of high-energy phosphates in soaked muscles obtained over a 7-month period we noted that both ATP and Cr P/Cr tended to be highest in the winter months. The variation which we observed is shown in Table I.

TABLE I

VARIATION OF HIGH-ENERGY PHOSPHATES WITH SEASON IN 1958 IN MUSCLES SOAKED 4 h IN NORMAL RINGER

	ATP mmoles/kg ± *S.E.*	$\frac{CrP}{Cr}$ ± *S.E.*
May	5.3 ± 0.3 (16)	0.76 ± 0.04 (16)
June	5.7 ± 0.2 (8)	1.16 ± 0.10 (8)
July	6.1 ± 0.4 (9)	1.31 ± 0.06 (6)
August	7.5 ± 0.6 (4)	1.49 ± 0.07 (4)
September	5.4 ± 1.0 (3)	0.99 ± 0.04 (4)
October	6.2 ± 0.4 (12)	1.59 ± 0.09 (11)
November	6.1 ± 0.6 (4)	0.99 ± 0.06 (4)

The effect of high KCl Ringer: Increases in the external level of K^+ have been shown to increase the membrane conductance[19], to increase the respiration[8], to increase the resting heat production[20], and to increase the flux rates of Na^+ and K^+[4,21]. The resting potential is decreased by high external K^+[23], the decrease being proportional to the log of the external K^+ level. We have attempted to ascertain whether an alternation in the external K^+ level is reflected in an altered amount of high-energy phosphate compounds in the muscle.

The high-energy phosphate and ionic content of muscles soaked in normal Ringer for 4 h was compared with that of muscles soaked in Ringer which contained increasing amounts of KCl. The Na^+ content of the Ringer was kept constant. Groups of 4 pairs of muscles were used in each expt.

The results are shown in Tables II and III.

(a) The high K^+ Ringer has not caused a significant alteration in the Na^+ level of the cell. The losses shown in this table are not significant. (b) The K^+ content of the muscle was significantly increased at all levels of external K^+. Intracellular levels have not been calculated, as we did not estimate the extracellular space in these muscles, but the values obtained using a group mean of 20 % for the extracellular space were of the same order as those reported previously[1]. (c) High K^+ Ringer caused a significant decrease in ATP level at 20 mmoles/l external K^+, both when compared with controls in normal Ringer, and with muscles in 10 mmoles/l KCl. There was no significant change at other concentrations of K^+. (d) Similarly, the ratio Cr P/Cr was significantly lowered only at the 20 mmoles/l K^+ level, both when compared with controls in normal Ringer, and with controls in 10 mmoles/l KCl. (e) There was no significant vol. change in any muscles. (f) Muscles in high KCl Ringer failed to respond to electrical stimulation. All muscles contracted when first placed in these solutions, but the contracture passed off after approximately 10 min in K^+ levels of

References p. 495.

TABLE II

THE EFFECT OF HIGH POTASSIUM RINGER ON ATP AND CrP IN MUSCLE

	10 mmoles/l		*20 mmoles/l*		*40 mmoles/l*		*100 mmoles/l*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treate*
ATP, mmoles/kg.	4.9	4.8	7.5	7.1*	3.9	4.7	3.7	4.0
ADP, mmoles/kg	2.6	2.4	1.8	1.7	2.4	2.8	2.7	2.5
ATP/ADP	1.9	2.0	4.2	4.2	2.0	1.4	1.4	1.6
CrP, mmoles/kg	11.3	11.0	11.6	6.9	3.8	2.7	8.7	9.2
Cr, mmoles/kg	6.5	8.2	6.9	16.1*	15.4	17.4	8.1	10.5
CrP/Cr	1.7	1.3	1.7	0.3*	0.3	0.2	1.1	0.9
Na, mmoles/kg	40.6	40.6	51.2	48.8	50.1	38.5**	51.0	44.2
K, mmoles/kg	71.7	76.4**	68.4	80.9**	70.6	96.3**	70.4	137.5**

* Significant.
** Highly Significant.

TABLE III

THE EFFECT OF VARIATION IN POTASSIUM LEVEL ON ATP AND CP IN TOAD MUSCLE

	10 mmoles/l vs. 20 mmoles/l		*20 mmoles/l vs. 40 mmoles/l*	
	Treated "10"	*Treated "20"*	*Treated "20"*	*Treated "40"*
ATP, mmoles/kg	7.0	4.0*	7.2	6.3
ADP, mmoles/kg	1.4	1.1	1.4	1.1
ATP/ADP	5.1	5.6	5.1	5.6
CrP, mmoles/kg	10.2	4.3	2.9	8.3
Cr, mmoles/kg	8.0	15.7	18.1	9.9
CrP/Cr	1.3	0.3*	0.2	0.9
Na, mmoles/kg	52.3	49.4	46.2	52.0
K, mmoles/kg	77.0	80.4	81.6	92.3*

* Significant.

TABLE IV

THE EFFECT OF RUBIDIUM RINGER ON ATP AND CrP IN TOAD MUSCLE

	20 mmoles/l		*40 mmoles/l*		*100 mmoles/l*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ATP, mmoles/kg	5.5	5.0	5.0	4.9	5.3	5.1
ADP, mmoles/kg	3.0	3.5	3.4	3.4	3.5	3.2
ATP/ADP	1.8	1.5	1.4	1.4	1.5	1.6
CrP, mmoles/kg	10.2	6.8	8.1	6.5	9.6	8.6
Cr, mmoles/kg	12.0	17.0	10.9	15.0	9.6	12.6
CrP/Cr	0.9	0.4*	0.7	0.4	1.0	0.7
Na, mmoles/kg	47.7	39.8**	57.4	47.8	48.3	42.9*
K, mmoles/kg	72.8	65.6	71.4	56.8*	90.0	50.2*
Rb, mmoles/kg	—	7.9	—	47.4	—	78.6

* Significant.
** Very significant.

References p. 495.

40 mmoles/l and under. With levels of 100 mmoles/l K^+ the muscles tended to remain bunched over the 4-h period.

The effect of high RbCl Ringer: Rb^+ and Cs^+ are known to depolarize muscle cells in a manner similar to that produced by K^+, but to a lesser extent[13]. LUBIN AND SCHNEIDER[13] have demonstrated that these ions substitute for K^+ in frog muscle, and this has been bound to hold for a wide variety of other tissues. A full report on the effect of Rb^+ and Cs^+ on the ionic content of toad muscle will be published from this laboratory at a later date.

The high-energy phosphate and ionic content of muscles soaked in normal Ringer was compared with that of muscles soaked in Ringer to which was added various levels of RbCl. No K^+ was included in these solutions. The experimental conditions were the same as those used in the previous section.

The results are shown in Table IV.

(a) The high Rb Ringer has produced a drop in the Na^+ content of the muscle at each level tested. As the Na^+ level of control and test Ringers was the same this represents a significant decrease in the Na^+ space of the muscle. The term "space" in this context refers to the total ionic concns. divided by the Ringer level of the same ion, and is an index of the amount of muscle water available to the ion in question. (b) There was a loss of K^+ at all levels of Rb^+, but this loss was not significant at the 20 mmoles/l Rb^+ level. Rb^+ entered the muscle to replace the lost K^+. (c) There was no significant change in the ATP content of the muscle at any level of Rb^+ tested. (d) There was a significant lowering of the ratio Cr P/Cr only at the 20 mmoles/l Rb^+ level. (e) As was found in the high KCl Ringer expts. the muscles were unexcitable in the test Ringer. There was no significant volume change. The muscles all contracted when placed in the Rb Ringer, but the contracture passed off after approximately 15 min.

The effect of high CsCl Ringer: A similar series of expts. was carried out using Ringer to which had been added varying levels of CsCl. No K^+ was included in these solutions. Again groups of 4 pairs of muscles were used in each expt. 2 expts. were performed at the 20 mmoles/l CsCl level, one of 4-h duration, the second of 18-h

TABLE V

THE EFFECT OF CAESIUM RINGER ON ATP AND CrP IN TOAD MUSCLE

	4 h		*18-h soaking*					
	20 mmoles/l		*20 mmoles/l*		*40 mmoles/l*		*100 mmoles/l*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ATP, mmoles/kg	5.4	4.5	4.6	4.3	6.8	6.6	4.5	4.2
ADP, mmoles/kg	2.5	2.3	1.6	1.5	1.2	1.0	1.3	1.0
ATP/ADP	2.2	2.0	2.9	2.9	5.7	6.6	3.5	4.2
CrP, mmoles/kg	8.9	5.9	7.3	3.0	5.7	4.5	5.7	4.5
Cr, mmoles/kg	8.7	13.1	10.5	13.5	12.8	17.3	12.8	13.3
CrP/Cr	1.0	0.5**	0.7	0.2*	0.5	0.3*	0.9	0.3*
Na, mmoles/kg	43.4	46.0	63.0	71.8	58.5	60.4	60.7	74.6
K, mmoles/kg	71.5	68.5	71.1	50.0	62.5	58.7	59.4	48.6
Cs, mmoles/kg	—	8.51	—	18.4	—	28.6	—	64.2

* Significant.
** Very Significant.

References p. 495.

duration. At other levels of Cs^+ tested the muscles were soaked for 18 h, since the rate of incorporation of Cs^+ into the cell was found to be slower than that for Rb^+. The results of these expts. are shown in Table V. (a) There was a slight but non-significant increase in the Na^+ content of the muscles at all levels of CsCl. (b) The Cs Ringer has caused an irregular loss of K^+ from the cell, which was only significant at the 20 mmoles/l level in muscles soaked for 18-h. (c) The entry of Cs^+ into the muscle at the 20 mmoles/l level was greater after 18 h soaking than at 4 h. At 40 and 100 mmoles/l CsCl the amount of Cs^+ in the muscle was less than was found with Rb^+, although the muscles were soaked for 12 h longer in the Cs^+ expt. (d) The high CsCl Ringer did not cause a significant change in the ATP level of the muscle at any level. (e) There was a significant decrease in the ratio of Cr P/Cr at all levels of Cs^+ tested. (f) The muscles were unexcitable in the Cs Ringer. There was no significant volume change. The contracture found on immersing them in the Cs^+ solution passed off after approximately 15 min.

The effect of high Li^+ Ringer: It has been shown elsewhere[6] that Li^+ may be regarded as a partial substitute for cellular Na^+. It was found to be without effect on K^+ and Cl^- levels, but caused an increase in the amount of "bound" Na^+ in the cell. We, have, therefore, attempted to ascertain whether this effect could be correlated with any change in the level of high-energy phosphate compounds in the muscle. 2 expts. were carried out in which muscles were soaked for 4 h and 18 h in Ringer in which 50 mmoles/l Li^+ was substituted. Companion muscles were soaked in normal Ringer, and 4 pairs of muscles were used in each expt. The results are set out in Table VI. (a) The interaction between Li^+ and Na^+ was similar to that reported

TABLE VI

THE EFFECT OF LITHIUM RINGER ON ATP AND CrP IN MUSCLE

50 mmoles/l Li, 80 mmoles/l Na.

	4 h		*18 h*	
	Control	*Treated*	*Control*	*Treated*
ATP, mmoles/kg	5.8	5.1	4.8	4.9
ADP, mmoles/kg	0.8	0.9	1.9	2.1
ATP/ADP	7.2	5.7	2.5	2.4
CrP, mmoles/kg	11.8	10.6	6.4	5.0
Cr, mmoles/kg	7.3	7.0	12.2	13.7
CrP/Cr	1.7	1.6	0.44	0.49
Na, mmoles/kg	51.3	31.2*	71.6	30.1**
K, mmoles/kg	72.4	65.1	68.5	56.6
Li, mmoles/kg	—	14.2	—	20.4

* Significant.
** Very Significant.

previously[6]. That is, the Na^+ space exceeded the Li^+ space at 4 h soaking, but they were similar in magnitude at 18 h. (b) There was a slight but insignificant loss of K^+ at both soaking times. (c) There was no change in the level of ATP or CrP at either times of soaking, compared with companion muscles soaked in normal Ringer.

Thus there is a slightly different pattern of results for each of the cations tested. K^+ and Rb^+ are similar in that they effected the level of high-energy phosphate only

References p. 495.

at the 20 mmoles/l level, while Cs^+ produced a lowering of the CrP level under al conditions, K^+ and Rb^+ tended to decrease the amount of muscle water available to Na^+, or to decrease the Na^+ space, while Cs^+ caused an entry of Na^+. It would seem likely that the loss of K^+ found in the Rb^+ expts. has resulted from an exchange between the 2 ions, whilst the irregular K^+ losses found in the Cs^+ expts. were due to a toxic effect of this ion. Thus the slow rate of entry of Cs^+ into the cell may result from the fact that Cs^+ does not appear able to substitute for K^+ to any great extent.

It would appear from the results of these expts. that profound changes in the ionic pattern of the soaking medium may result in very small changes in the level of ATP and CrP in the cell. Thus the changes in ionic pattern brought about by the foreign ions cannot be ascribed to any gross alteration in the metabolic status of the cell, but must be related to finer control mechanisms.

The effect of metabolic inhibitors

There have been many reports in the literature on the effect of metabolic inhibitors on the ionic pattern of a particular tissue. It is noteworthy, however, that very little attempt has been made to correlate the effect of the inhibitor on both the metabolic cycle and the ions. It is usually noted that (say) dinitrophenol causes K^+ loss from the cell, and its effect on oxidative phosphorylation is inferred from other studies. We have attempted such a correlation with a small series of metabolic inhibitors, and although the results must be considered preliminary there have been several interesting findings.

Iodoacetic Acid: The ATP and CrP and ionic content of muscles soaked in normal Ringer was compared with that of companion muscles soaked in Ringer to which was added 2 mmoles/l iodoacetic acid. All muscles contracted on immersion in the test Ringer, but the contracture passed off in 2 muscles, while the other two twitched continuously throughout the 4-h soaking period. The reason for this difference is not known, but the twitching has entirely altered the pattern of results obtained, and we have thought it worthwhile to list the data in full, rather than give an average value. The figs. set out in Table VII provide an excellent example of the value of individual correlations as opposed to the statistical treatment of grouped results.

TABLE VII

THE EFFECT OF IODOACETIC ACID ON ATP AND CRP IN MUSCLE

Treated muscles in toads 1 and 2 twiched spontaneously throughout the expt. Treated muscles in toads 3 and 4 were quiescent.

	1		*2*		*3*		*4*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ATP, mmoles/kg	6.3	3.0	5.5	3.0	4.8	5.1	5.2	4.8
ADP, mmoles/kg	3.0	3.3	2.3	2.4	3.4	3.2	3.5	3.0
ATP/ADP	2.1	0.9	2.4	1.3	1.4	1.6	1.5	1.6
CrP, mmoles/kg	12.7	0.9	12.4	—	10.1	9.7	12.0	8.6
Cr, mmoles/kg	10.6	13.0	7.8	13.0	9.1	12.2	10.9	12.0
CrP/Cr	1.20	0.07	1.59	—	1.11	0.80	1.10	0.72
Na, mmoles/kg	57.2	76.0	53.2	77.4	62.6	53.8	52.3	51.8
K, mmoles/kg	78.8	48.6	74.5	44.7	66.4	72.7	73.0	65.8
Volume change %	−2	+8	−2	+6	+5	−2	−4	−5

References p. 495.

The quiescent muscles showed slight losses of CrP, and virtually no change in ATP levels, while the Na^+ and K^+ content was unaltered. The muscles which underwent spontaneous contractions showed marked lowering of the ATP and CrP levels, and there was a movement of Na^+ and K^+ along the respective concentration gradients.

2,4 Dinitrophenol, methylene blue, and sodium azide: Similar expts. to that with iodoacetic acid were carried out with these three inhibitors. The levels at which the inhibitors were added to normal Ringer were as follows: 2,4 dinitrophenol, 2 mmoles/l; methylene blue 0.5 mmoles/l; and sodium azide, 5 mmoles/l. The results of these expts. are set out in Table VIII. (a) All the inhibitors brought about a gain

TABLE VIII

THE EFFECT OF METABOLIC INHIBITOR ON HIGH-ENERGY PHOSPHATES AND IONIC LEVELS

	2 mmoles/l DNP		*0.5 mmoles/l Methylene Blue*		*5.0 mmoles/l NaN_3*	
	Control	*Treated*	*Control*	*Treated*	*Control*	*Treated*
ATP, mmoles/kg	6.3	5.1*	6.5	3.8**	6.8	4.4*
ADP, mmoles/kg	2.8	3.8	2.8	3.5	2.4	2.5
ATP/ADP	2.3	1.4	2.4	1.1	2.8	1.8
CrP, mmoles/kg	9.3	2.0	11.9	1.0	11.2	1.6
Cr, mmoles/kg	7.9	17.0	8.7	18.7	7.5	21.2
CrP/Cr	1.3	0.1**	1.4	0.1**	1.5	0.1**
Na, mmoles/kg	49.6	55.5	62.3	65.7	51.7	64.4*
K, mmoles/kg	74.7	57.1*	75.1	58.4**	70.3	39.3*

* Significant
** Very Significant.

of Na^+ by the muscles, but this gain was only significant in the azide expt. (b) There was a significant loss of K^+ from the muscle in all expts. The loss of K^+ would appear to be proportionately greater than the Na^+ gain. (c) There was a significant loss of both ATP and CrP in all expts. (d) The muscles showed a significant increase in volume only in dinitrophenol.

Thus it would appear that gross interference with the metabolic cycle causing a decrease in the high-energy phosphate compounds leads to a movement of both Na^+ and K^+ with the concn. gradient. Toad muscle is somewhat resistant to the action of these inhibitors, since high levels were needed to bring about demonstrable effects. It is intended to pursue this investigation using a technique to measure the rate of turnover of ATP. It is likely that lower concns. of inhibitors would effect the turnover rate than are necessary to cause actual depletion.

The expt. with iodoacetic acid is of interest since only the muscle showing spontaneous activity had lowered levels of ATP an CrP, and showed a loss of K^+ and gain of Na^+. Since one must assume a similar interference with metabolism in both groups of muscles it would appear that ionic movement along the concn. gradient did not occur until the high energy stores had been depleted by activity. This recalls an expt. quoted by LING[2], who found that iodoacetic acid did not cause K^+ loss when muscles were held at 0°, although similar concns. caused marked losses at room temperature.

References p. 495.

DISCUSSION

One of the main difficulties inherent in the concept of active transport when applied to the steady state ionic maintenance of the single cell has been the large energy requirement of the system. There have been numerous estimates of the proportion of the resting metabolism necessary to operate the Na^+ pump. The values arrived at have shown great variation, depending upon the assumptions made by each author, but it would appear to be a large proportion. USSING[23] has put forward the concept of exchange diffusion, whereby a carrier mediated movement of Na^+ across the membrane could take place without the feed in of energy. It could be said that we have enlarged this hypothesis to include all of the constituents in the free intracellular phase. Thus USSING has assumed that the Na^+ exchange due to thermal movement takes place across the membrane. We postulate that there is an intracellular region (the free intracellular phase) which is freely accessible to Na^+, and it is the rate of movement of the ion in and out of this region which is measured in tracer expts. If the ability of the ordered phase to exclude certain ions is assumed to reside in the maintenance of the structural integrity of this phase, then it is clear that little or no metabolic energy need be directly channelled for the transport of ions.

It is not possible to say on the evidence presented in this paper whether such a channelling has occurred or not. It appears suggestive that it has not. To test this point beyond doubt would require, as DAVIES[24] has pointed out, "...Measuring the quantitative relations between the number of ions moved in a given time and the amount of oxygen or substrate used, or say ATP broken down, in the same time. This gives a lower limit to the ratio of ions moved to substrate utilized and can be used to test any hypothesis of the mechanism of active transport." The practical difficulties in making this evaluation are considerable, but it appears the only way of reaching a definite conclusion. This correlation has been made for frog skin[25,26] and the conclusion was reached that less than 1 mole of oxygen was used to transport 1 mole of Na^+. There is, however, no doubt that the net movement of Na^+ across this skin is in fact active transport, and is an energy dependent process of the same nature as secretion.

BARTLEY *et al.*[27] have demonstrated very high rates of exchange of intramitochondrial ions, even at 0°, and have assumed that the rate of exchange is not associated with the expenditure of energy. CALDWELL AND KEYNES[28] have estimated that 4 phosphate bonds must be broken down for each Na^+ extruded in cephalopod axons.

The experiments of KEYNES AND MAISEL[29] on frog muscle will be discussed in a subsequent paper[8].

A parallel has been demonstrated by FLECKENSTEIN *et al.*[30] between ATP turnover and oxygen uptake, but not between muscular contraction and ATP turnover. In this respect it is of interest that we were unable to correlate the contracture of the muscle in K^+, Rb^+ or Cs^+ with changes in the ATP and CrP levels. (See ref.[30]). It is known that high K^+ solutions increase both the oxygen uptake and the rate of movement of K^+ both into and out of the cell. It remains to be seen whether one can correlate these 2 effects of K^+ with each other, and with a change in ATP turnover.

We have noted the curious finding that K^+ (and to a lesser extent Rb^+) lowers the content of high-energy phosphate compounds only at a level of 20 mmoles/l.

This effect is paralleled by both the oxygen uptake and the resting heat produc-

References p. 495.

tion. It resembles to some extent the uncoupling of oxidative phosphorylation by dinitrophenol, but we are at a loss to explain why it should occur only at one level of K^+.

The expts. with metabolic inhibitors indicate a relation between ATP loss and K^+ loss, but until the rates of loss are compared one cannot infer a cause and effect relationship.

Although the the findings in this paper must be regarded as preliminary, they appear to be consistent with the 3-phase theory[1]. Thus the exchange between Na^+ and Li^+ in the free intracellular phase has not altered either the ATP and CrP level or produced a significant K^+ loss. Rb^+ appears to substitute for K^+ in the ordered phase without altering the metabolic pattern, while Cs^+ is somewhat toxic. The metabolic inhibitors cause movement of Na^+ and K^+ along their concentration gradients only after the high-energy phosphate stores have been depleted.

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