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THE ROLE OF LACTATE IN THE ACTIVE EXCRETION OF SODIUM BY FROG MUSCLE

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The active excretion of Na in quantity from isolated Na-rich frog muscle was first demonstrated by Desmedt (1953) and by Carey, Conway & Kernan (1959). The latter workers made frog sartorii Na-rich (about 59 m-equiv/kg) by immersing the muscles during the night in cold K-free Ringer–Conway fluid (Boyle & Conway, 1941) containing 120 mM-Na. These muscles when reimmersed at room temperature (18° C) for 2 hr in a similar Ringer's fluid with Na reduced to 104 mM and with the addition of 10 mM-K excreted in the average about 18 m-equiv Na/kg. If the Na concentration of the reimmersion fluid was 120 mM, there was in the average little or no excretion of Na. They also found that sartorii immersed during the night in cold K-free Ringer's solution with 104 mM-Na accumulated an average of 47 m-equiv Na/kg, and that these muscles when reimmersed in the same fluid with the addition of 10 mM-K also showed little or no average Na excretion. The above procedures will be referred to in the following text as 120, 0/104, 10; 120, 0/120, 10; and 104, 0/104, 10; respectively.

To explain the need to reduce the Na concentration of the recovery fluid and to increase the K concentration above the normal plasma level of 2·5 mM to bring about Na excretion the concept of the critical energy barrier was introduced by Conway (1960). The energy barrier takes into account the osmotic and electrical gradients against which the Na must be extruded and is expressed by the relation,

$$dG/dn = RT.\ln([Na]_o/[Na]_i) + E_mF, \quad (1)$$

where dG/dn is the free energy change per equivalent of Na extruded, E_m the mean membrane potential of the muscle fibres (outside minus inside) measured in the recovery fluid, and $[Na]_o$ and $[Na]_i$ the sodium concentrations in the recovery fluid and fibre water, respectively. It has been shown (Conway, Kernan & Zadunaisky, 1961) that above a critical energy barrier of approximately 2 cal/m-equiv Na the muscles would not excrete Na under the experimental conditions described. If the membrane

potential and corresponding electrical energy gradient were reduced by increasing the external K concentration, and/or if the external [Na] was reduced below a certain critical value, thereby decreasing the osmotic component of the energy barrier, extrusion of Na would take place.

The critical energy barrier above which muscles were unable to excrete Na may be related to the maximum energy available from metabolism for the process of active transport. The level of the energy barrier as calculated (Conway *et al.* 1961) for rat and frog muscles *in vivo* was about 4 cal/m-equiv Na. The difference between this value and the 2 cal/m-equiv Na found with isolated muscle may be due to a decline in available energy caused by deterioration of the muscle, or by the absence from the medium of an essential substance normally present in frog plasma.

With this in mind, particular note was taken of the findings of Manery, Gourley & Fisher (1956), Smillie & Manery (1960) and Zierler (1959) that addition of insulin alone or with lactate to Ringer's fluid in which isolated frog or rat muscles were immersed prevented loss of K from the muscles, and in some cases led to an uptake by the muscles of as much as 8 m-equiv K/kg, as well as stimulating oxygen consumption. Earlier, Gourley & Fisher (1954) reported the stimulation of lactate oxidation in isolated muscles by insulin.

In the present communication experiments are described in which lactate and insulin, separately and together, were added to the recovery fluid in various amounts to produce Na excretion under conditions already described in which excretion of Na would not otherwise take place. Assuming that the energy for Na transport here comes primarily from the oxidation of lactate, the extrusion of Na was examined under conditions where electron transfer via lactate dehydrogenase was inhibited. It has been shown by Vallee (1960) that *o*-phenanthroline will inhibit electron transfer by lactate dehydrogenase by chelating the Zn atoms which bind the enzyme to diphosphopyridine nucleotide (DPN). The inhibition has been reversed by addition of Zn salt to the medium. The effect of this inhibitor alone and in the presence of Zn on Na excretion is here examined in the presence of lactate. The effect of iodoacetate and of ouabain on Na transport was also investigated.

Pyruvate, the end product of the oxidation of lactate by lactate dehydrogenase, might be expected to decrease the rate of lactate oxidation if added to the recovery fluid (Plagemann, Gregory & Wróblewski 1960). If the energy for active transport comes from the oxidation of lactate, pyruvate or an inhibitor of lactate dehydrogenase should inhibit the excretion of Na from Na-rich muscles if added to the recovery fluid.

Evidence arising from membrane potential measurements is also presented to indicate that during the long Na excretion observed from loaded

muscles the accompanying K uptake is a passive process. New values for the critical energy barrier in the presence of insulin and lactate are presented.

METHODS

Isolated companion sartorii of *Rana temporaria* were used in all experiments described here. With some variations, the treatment of the muscles was as already described (Carey *et al.* 1959). The muscles in all cases were first made Na-rich by immersion during the night in cold K-free Ringer–Conway fluid (Boyle & Conway, 1941) containing either 104 or 120 mM-Na. They were reimmersed always in the same Ringer's fluid as was used for the loading, but with the addition of 2·5 mM-K, as well as lactate, insulin or both. Insulin alone was used at concentrations of 30, 50 and 100 u./l., DL- and L-lactate alone at 5 mM. Insulin and lactate together were used at concentrations of 30 u./l. and 1 mM, respectively. After analysis of the muscles for Na, the amount of Na excreted was estimated by a comparison of sets of companion muscles: one set was analysed after the first immersion, the other set reimmersed for 3 hr in the recovery fluid and then analysed.

Inhibitors. When the effect of inhibitors on Na excretion was being examined, both sets of Na-rich muscles were reimmersed in recovery fluid, but to one set the inhibitor was added. The inhibitors used included 2 mM iodoacetate, 1 mM *o*-phenanthroline, 10^{-6} M ouabain and 1 mM pyruvate. The latter was used in the presence of insulin. When reversal of *o*-phenanthroline inhibition by Zn was investigated both sets of companion muscles were reimmersed in recovery fluid with the inhibitor present, but to one set 1 mM-$ZnSO_4$ was also added.

Chemical methods. For analysis the muscles were blotted on moist filter paper, weighed and digested in boiling concentrated HNO_3. When oxidation was complete the acid was evaporated and the residue dissolved in 10 ml. of de-ionized water. This was then analysed for Na and K by means of the Beckmann flame photometer. Where the concentration of K or Na in the muscle fibre water was required this was calculated, allowance being made for the extracellular space as measured by inulin.

Potential measurements. The membrane potentials of the muscles in recovery fluid were measured by the micro-electrode technique (Graham & Gerard, 1946), the arrangement already described (Conway *et al.* 1961) being used. For each muscle used the membrane potential of about 20 fibres surface was measured.

When comparison was being made between the observed resting potential and the calculated K equilibrium potential, the former was measured immediately on reimmersion in recovery fluid, and the latter obtained by analysis of the muscles and use of the Nernst equation, $E_K = RT/F.\ln([K]_i/[K]_o)$, where $[K]_o$ and $[K]_i$ were the potassium concentrations in the reimmersion fluid and muscle fibre water, respectively. Here the 120, 0/120, 2·5 procedure was used with insulin 30 u./l. and 1 mM lactate in the recovery fluid. With the companion muscles the effect of addition of 1 mM *o*-phenanthroline to the recovery fluid on the membrane potential of the muscle was examined. The potential was measured immediately before and after the addition of the inhibitor, and the muscles were analysed and E_K calculated as described above.

Sets of 20 muscles were used in all potential measurements described and mean values calculated.

When calculating the values of the critical energy barrier in the presence of insulin and lactate by equation 1, the potentials were measured after 3 hr reimmersion in recovery fluid at room temperature (procedures 120, 0/120, 2·5 and 104, 0/104, 2·5) with insulin 100 u./l., with 5 mM lactate or with insulin 30 u./l. and 1 mM lactate present. The muscles were immediately analysed for Na to provide a value for $[Na]_i$ to be used in calculating the osmotic component of the critical energy barrier (eqn. 1).

RESULTS

As the companion muscles of each frog were treated differently in the individual experiments, a mean was arrived at for muscles under a particular set of conditions irrespective of the treatment which the companion muscles received. For example, of the muscles used to obtain a mean level of Na in muscle after reimmersion in Ringer's fluid containing lactate and insulin (Table 2), some of the companion muscles were analysed after the first immersion, others were used to measure inhibition by reimmersion in Ringer's fluid with lactate, insulin and inhibitor present.

TABLE 1. Sodium excretion from Na-rich muscles on reimmersion in recovery fluid with addition of insulin and lactate

Conditions	Mean Na content (±S.E.) of muscles before and after 3 hr reimmersion (m-equiv/kg wet wt. of muscle)
120, 0/120, 2·5 procedure	
After first immersion	59·4 ± 2·4 (21)
On reimmersion with	
insulin (30 u./l.)	48·7 ± 2·4 (11)
5 mM DL-lactate	49·6 ± 3·3 (14)
5 mM L-lactate	42·2 ± 1·5 (36)
insulin (50 u./l.)	37·4 ± 1·8 (14)
insulin (30 u./l.) + DL-lactate (1 mM)	38·7 ± 1·0 (35)
104, 0/104, 2·5 procedure	
After first immersion	46·9 ± 1·2 (31)
On reimmersion with	
insulin (50 u./l.)	37·5 ± 1·5 (36)
insulin (100 u./l.)	25·1 ± 1·0 (18)
insulin (30 u./l.) + 1 mM DL-lactate	36·2 ± 1·8 (23)

Number of experiments indicated in brackets.

The effect of adding insulin and lactate to the recovery fluid on the Na excretion from Na-rich muscles under the conditions 120, 0/120, 2·5 and 104, 0/104, 2·5 is shown in Table 1. It will be noted that L-lactate produced a greater stimulation of Na excretion than DL-lactate. Greater excretion also occurs when insulin and lactate are used together. The most striking result in Table 1 is perhaps the amount of Na excreted and the low level of Na reached when insulin 100 u./l. was added to the recovery fluid in the 104, 0/104, 2·5 procedure. Here the concentration in the muscles fell from a mean of 46·9 to 25·1 m-equiv/kg, a loss of 21·8 m-equiv. The final level reached was very close to the median value of 23·9 m-equiv/kg reported by Conway (1957) for freshly dissected frog muscle.

The effect of the inhibitors ouabain, iodoacetate and *o*-phenanthroline on Na excretion in the 120, 0/120, 2·5 procedure is shown in Table 2. In the presence of insulin and lactate, the Na level of the muscles fell from about

58 to about 31 m-equiv/kg, a loss of 27 mM-Na during the 3 hr reimmersion (Table 2). The cardiac glycoside ouabain at the low concentration of 10^{-6} M inhibited this excretion by about 55%. Iodoacetate, the powerful inhibitor of anaerobic metabolism, cut off completely the Na excretion by the muscles. *o*-Phenanthroline, the chelating agent which Vallee (1960) reported to block electron transfer by lactate dehydrogenase by combining with the Zn of the molecule, inhibited Na transport by about 85%. If, however, Zn salt was added to the recovery fluid and muscles before addition of inhibitor the Na transport was not appreciably changed. In this case the final level of Na in the muscles was 38·8 compared with 37·4 m-equiv/kg for the control. The lower level of muscle Na reported in Table 2 compared with Table 1 in presence of insulin and lactate may be due to seasonal variation in the frogs, as the experiments were carried out at different times of the year.

TABLE 2. Effect of ouabain, iodoacetate and *o*-phenanthroline on Na excretion in the 120, 0/120, 2·5 procedure with lactate and insulin present in recovery fluid

	Mean Na content (±S.E.) of muscles after reimmersion (m-equiv/kg wet wt. of muscle)			
Conditions	Control	Inhibited	Inhibitor used	(mM)
(*A*)	32·6±1·4	46·5±1·8 (11)	Ouabain	10^{-3}
(*A*)	30·6±2·5	59·8±3·6 (14)	Iodoacetate	2
(*B*)	37·4±2·6	54·8±2·6 (34)	*o*-Phenanthroline	1
Reversal of inhibition by Zn		38·8±2·8 (16)	*o*-Phenanthroline	1
			+$ZnSO_4$	1

Mean Na content after first immersion = 57·9±4·2 m-equiv/kg wet wt. Number of experiments indicated in brackets. Additions to recovery fluid: (*A*) 1 mM DL-lactate +insulin 30 u./l.; (*B*) 5 mM L-lactate.

The effect of replacing lactate with pyruvate in the recovery fluid on the amount of Na excreted (120, 0/120, 2·5) was also examined. In the presence of insulin 50 u./l. the mean Na concentration in the muscles fell from 56·8 to 40·5 m-equiv, a loss of 16·3 m-equiv/kg. With addition of 1 mM lactate to this recovery fluid a final Na level of 32·7 m-equiv/kg was reached, indicating a loss of 24·1 mM-Na. On addition of 1 mM pyruvate instead of lactate to the recovery fluid in the presence of insulin the final Na concentration of the muscles was 50·3 m-equiv/kg after the 3 hr reimmersion, indicating a loss of only 6·5 mM-Na, and a 60% inhibition of Na excretion from the muscles.

Membrane potentials of Na-rich sartorii were measured during Na excretion, at the beginning of the reimmersion period with 1 mM lactate and insulin 30 u./l. (120, 0/120, 2·5). About 5 min was allowed to pass before potentials were measured, so as to allow K_o to diffuse evenly through the

muscles. Observations were then made over a period of about 15 min. The mean value found was 97·6 ± 0·3 mV. The calculated K equilibrium potential for the same muscles was 91·0 ± 0·8 mV. This difference of 6·6 mV between observed and calculated potentials has a high level of significance ($P < 0·01$). When Na transport was inhibited by addition of *o*-phenanthroline to a set of companion muscles in recovery fluid, the potential fell from about 97 mV to a mean value of 93·4 ± 0·8 mV within minutes of addition of inhibitor. It then differed from the K equilibrium potential as calculated for this set of muscles by only 2·4 mV. The difference between the observed and calculated potentials was then not very significant ($P = 0·05$). The difference between the observed and calculated potentials here is interpreted as due to the activity of the Na pump, because the inhibition of the active transport abolishes this difference and also for reasons which will be discussed later.

The critical energy barrier was calculated by eqn. 1, as described in Methods for muscle in recovery fluid with insulin 100 u./l., 5 mM L-lactate or with both insulin 30 u./l. and 1 mM DL-lactate present. The values obtained were 3·74 cal/m-equiv Na with insulin 100 u./l., 2·99 cal/m-equiv Na with 5 mM L-lactate, and 3·05 cal/m-equiv Na with insulin 30 u./l. + 1 mM DL-lactate.

DISCUSSION

It is evident that addition of insulin or lactate to the Ringer's fluid in which the Na-rich sartorii were reimmersed markedly stimulated the excretion of Na. In their presence the sartorii were able to excrete Na in quantity under conditions where previously there was no excretion. The more marked stimulation produced by L-lactate compared with the same concentration of DL-lactate is probably due to the greater affinity shown by lactate dehydrogenase for the former. Plagemann *et al.* (1960) found that lactate dehydrogenase isolated from rabbit muscle oxidized L-lactate 20 times more rapidly than DL-lactate in the presence of DNP. The rate of oxidation of mixtures of the two isomers was roughly proportional to the amount of the L-lactate present.

The greater effectiveness of insulin over lactate in stimulating Na transport may be due to the fact that insulin seems to act on the membrane (Gourley, 1957), from which it may be lost during the long period of soaking in K-free Ringer's fluid and during the reimmersion in recovery fluid when it is absent from the medium. When insulin alone is present in the recovery fluid, on the other hand, there is probably already present in the muscle fibres a large store of lactate, due particularly to the muscle contraction which takes place during the killing of the frogs, and also in some cases during the immersion in K-free Ringer's fluid.

Taking into consideration the finding of Gourley & Fisher (1954) that

insulin increases the rate of lactate oxidation by isolated frog muscle, it is suggested here that the extra energy supply needed to bring about Na excretion and to raise the total energy gradient against which this ion can be moved, comes from the oxidation of lactate by lactate dehydrogenase. If lactate oxidation is an essential step in supplying the energy, it might be expected that a specific inhibitor of lactate dehydrogenase, namely *o*-phenanthroline, would be a strong inhibitor of Na excretion in the presence of lactate. That this is indeed the case is evident from Table 2. Iodoacetate, a strong inhibitor of anaerobic metabolism, and the cardiac glycoside ouabain also produced marked inhibition of Na excretion.

Reversal of *o*-phenanthroline inhibition of Na transport by the addition of Zn, which parallels the reversal of inhibition of electron transfer observed by Vallee (1960), is further evidence (Table 2) of the involvement of lactate dehydrogenase in Na transport.

The inhibitory effect of pyruvate on Na transport found here is also of interest in this respect. Severin (1959) has shown that addition of pyruvate to tissues such as amphibian muscle with relatively low rates of respiration decreases the oxygen consumption, the pyruvate apparently competing with oxygen as acceptor with formation of lactate. It is not surprising, then, that the addition of pyruvate in the presence of insulin produced 60% inhibition of Na excretion, whereas addition of the same concentration of lactate caused a 48% increase over that occurring with insulin 50 u./l. alone in the recovery fluid.

The higher values of the critical energy barrier found following addition of lactate or insulin to the recovery fluid are interpreted here as due to the stimulation of oxidative metabolism and the associated electron transfer involved. The highest value found, 3·7 cal/m-equiv Na in the presence of insulin 100 u./l., is associated with practically complete removal of the Na accumulated by the muscle fibres during the immersion overnight in cold K-free Ringer's fluid. This value is very close to the calculated level *in vivo* of 4 cal/m-equiv Na (Conway *et al.* 1961) and is far greater than the value of 2 cal/m-equiv Na found (Conway *et al.* 1961) with isolated muscles in Ringer–Conway fluid without insulin or lactate present. These substances would normally be present in plasma, where they may also play a part in the process of active transport of Na *in vivo*.

Membrane potential and the Na pump. Measurement of the membrane potential of sartorii during active transport should give some indication as to whether the Na ion, the K ion or both are actively transported. The fact that at the beginning of the reimmersion period, when active transport was at a maximum, the mean potential observed was significantly greater than the K equilibrium potential calculated for the same muscles suggests that this difference may be due to the active process. This is also indicated by

the fact that the addition of an inhibitor to the recovery fluid reduces this difference to a level no longer very significant. Zierler's (1959) finding that insulin increased the membrane potential of excised rat muscle by about 5 mV has been interpreted by him as responsible for the net K influx which he also observed. The mechanism proposed by him for the increased potential was a potential difference generated between the two surfaces of the membrane by metabolic processes.

The fact that the interior of the muscle fibres is negatively charged with respect to the exterior suggests that net Na exit will have the effect here of increasing the membrane potential, while net K entrance will have the opposite effect. If then active excretion of Na by an active process is responsible for the increased potential, the K entrance should take place passively to restore conditions of electrical neutrality within the muscle fibres. It is assumed that Cl like K will move passively to reach a Donnan equilibrium when Na excretion is complete. It has already been found (Kernan, 1961) that the membrane potential observed at the end of the 3 hr reimmersion period agrees well with the K equilibrium potential calculated at the same time, which observation is consistent with this view.

SUMMARY

1. Frog sartorii made Na-rich by immersion during the night in cold K-free Ringer–Conway fluid containing 120 or 104 mM-Na, and re-immersed in the same fluid at room temperature with addition of 2·5 or 10 mM-K, could not excrete Na. When insulin or lactate alone or together were added to the reimmersion fluid up to 27 m-equiv Na/kg muscle was excreted over a 3 hr period.

2. *o*-Phenanthroline, an inhibitor of lactate dehydrogenase, and also ouabain and iodoacetate inhibited the Na excretion. In presence of insulin in the recovery fluid addition of 1 mM pyruvate inhibited Na excretion by 60%, the same concentration of lactate stimulated Na excretion by 48%.

3. These results are interpreted as evidence that the energy for active transport here comes from the oxidation of lactate by lactate dehydrogenase.

4. These substances increase the electrochemical gradient against which Na can be extruded by the Na-rich muscles as measured by the critical energy barrier.

5. The mean membrane potential measured during Na excretion is significantly greater than the K equilibrium potential calculated for the same set of muscles. The observed and calculated potentials are in better agreement following addition of the inhibitor of Na transport *o*-phenanthroline, to the reimmersion fluid.

6. The increased potential here is interpreted as due to the activity of the Na pump, the K entering freely to restore electrical neutrality within the fibres.

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