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IONIC TRANSPORT AND MEMBRANE POTENTIAL OF RAT LIVER CELLS IN NORMAL AND LOW-CHLORIDE SOLUTIONS

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SUMMARY

1. The ouabain-sensitive component of Na efflux and K influx amount to 58 and 72% respectively. Taking into account also (*a*) the diffusional passive fluxes of Na (5%) and K (13%), as estimated by Ussing's equation and (*b*) the ouabain-insensitive Na-Na exchange (28%), 85% (K) and 90% (Na) of the measured total fluxes can be accounted for.

2. Na efflux is diminished when K is partially or totally removed from the medium. This effect is reversible, indicating probably activation of the Na pump by external K.

3. The coupling ratio of Na and K ouabain-sensitive fluxes is equal to 1·58, suggesting that three Na ions are removed from and two K ions are carried into the cell in one cycle of the pump. Hence, in liver cell membranes, the Na pump must be electrogenic.

4. A tenfold decrease in $[Cl]_o$ by substitution with an impermeant anion results in a membrane hyperpolarization and a decrease in $[Cl]_i$. Cl loss from the liver is compensated by an equivalent loss of intracellular K to preserve electroneutrality.

5. The measurement of passive fluxes indicates that Cl removal from the perfusing solutions increases P_K but does not alter P_{Na}.

6. Addition of ouabain brings about a depolarization which is three times greater in low-Cl solutions (21·9 mV) than in normal-Cl solutions (6·8 mV).

7. It is concluded that hyperpolarization which develops when Cl ions are removed can be accounted for entirely by (*a*) the increase in P_K, (*b*) the increase of the contribution of the electrogenic pump to membrane potential.

INTRODUCTION

It has been established (Leaf, 1956; McLean, 1960; Elshove & Van Rossum, 1963; Van Rossum, 1966*a*, *b*) that the greatest part of net Na and K uphill transport and ^{24}Na efflux in rat liver slices depends on

external temperature, tissue respiration and it is inhibited by cardiac glycosides. These movements of Na were also suppressed by K removal from incubation media and activated by an increase of external K. It was concluded that Na and K transport in liver slices against their concentration gradients was the result of an active Na extrusion coupled to K uptake which may be linked to the presence of a Na-K ATPase in liver plasma membrane (Emmelot, Bos, Benedetti & Rumke, 1964). To account for the net ion transfer between intra and extracellular compartments, Elshove & Van Rossum (1963) suggested a sequence of events on the basis of an electroneutral mechanism exchanging Na for K.

However, recent observations from electrophysiological studies are in favour of an electrogenic exchange of Na and K. Williams, Withrow & Woodbury (1971*a*, *b*) demonstrated that nephrectomy or injection of ouabain (10 mg kg^{-1}) induced an hyperkalaemia associated to an hyperpolarization in rat liver cells *in situ*. In isolated and perfused liver such a mechanism accounts also adequately for the discrepancy between measured and calculated potential from the passive Na and K fluxes (Claret & Mazet, 1972). The observed difference allowed an estimate of the coupling ratio, i.e. the number of Na carried out per K carried inwards in one cycle of the pump, at 1·64. Furthermore, it was suggested that part of the hyperpolarization which develops in the perfused liver when foreign anions are substituted for Cl is the result of a change in the contribution of the electrogenic pump to membrane potential.

In the present study, the ionic membrane conductance and the coupling Na/K ratio were tentatively calculated from passive and ouabain-sensitive fluxes, in order to estimate the current produced and the potential further generated by the pump across the membrane. This figure was compared to actually measured potential drop during ouabain inhibition in normal and low-Cl solutions. Preliminary accounts of these results have appeared elsewhere (Claret & Mazet, 1971).

METHODS

The normal physiological solutions used in these experiments contained (mM): Na, 130; K, 5·6; Ca, 2·8; Mg, 1·0; Cl, 127; HCO_3, 12; HPO_4, 2·0 and glucose, 1 g. The low-Cl solutions were obtained by replacement of NaCl with equimolar quantities of Na benzene sulphonate, making $[Cl]_o$ equal to 13 mM. All the solutions were maintained at 38° C and pH 7·4 and were equilibrated with 97 % O_2 and 3 % CO_2.

The analytical procedures (total ion content, extracellular space and total water), the methods used to calculate fluxes and make the necessary corrections, and the techniques for recording membrane potential have been described previously (Claret & Mazet, 1972).

Radioactive tracers were supplied by C.E.A. (Saclay), for ^{42}K and ^{24}Na, and Radiochemical Centre (Amersham), for [^{14}C]inulin and [^{35}S] benzene sulphonate. The activity

of samples containing ^{24}Na or ^{42}K was counted directly in an autogamma spectrometer (Nuclear), and those containing ^{14}C or ^{35}S in a liquid Scintillation Spectrometer (Intertechnique).

The effects of ouabain (10^{-3} M) or K (0 and 15 mM) on Na extrusion were tested during 10 min. The rate constant k of Na efflux was determined by the ratio of activity collected in the effluent (counts min^{-2}) over the tracer amount remaining in the tissue (counts min^{-1}). For K concentrations of 1, 2 and 5·6 mM the rate constant was obtained directly from the slow component of ^{24}Na loss in experimental washout solutions. To determine the ouabain sensitive K flux, the uptake of labelled ^{42}K appearing in tissue within 5 min was first measured. Then the inhibitor was added into the loading solution, and the uptake of ^{42}K was determined again between the 10th and 15th min. To test the effect of ouabain on passive Na influx, the liver was first perfused with the inhibitor. After 5 min, ^{24}Na was added into the solution, then at the 8th min ^{24}Na was removed from the perfusing fluid. The uptake was obtained by integrating the efflux curve.

In a set of low-Cl experiments, net K movement was determined. It is equal to the difference in K concentration between perfusing fluid and effluent multiplied by the flow rate (μmole.min^{-1}). [K] were measured by flame photometry (Eppendorf).

The numerical values are given as mean values ± S.D. of an observation.

RESULTS

Effects of ouabain on Na efflux

It has been reported in a previous paper (Claret & Mazet, 1972) that a small fraction of the Na efflux was made up by a Na-Na exchange process, insensitive to ouabain but essentially dependent on external Na concentration. Its average value in rat liver was 3·3 p-mole cm^{-2} sec^{-1}, i.e. about 28 % of the total Na flux. To test whether the remaining Na efflux could be totally accounted for by an active extrusion, ouabain was added into the perfusing fluid.

After loading with ^{24}Na, the loss of the tracer was followed up during 16–20 min to determine the control efflux. At this time, addition of ouabain (10^{-3} M) results in a decline of the rate constant of Na efflux, which falls to about one half of the control value in 4–6 min. At the same time, the perfusion flow-rate also decreases (see *Effects of ouabain and K on flow rate*, page 95). These effects were progressively reversed with the removal of the inhibitor. However, in some experiments Na extrusion did not reappear with ouabain removal and the ^{24}Na efflux-curve flattened further with time.

Quantitative results can be obtained from the experiments of this type using the equations of Hodgkin & Keynes (1955). The analysis of ten experiments gave a maximal inhibition after 5 min: ouabain decreased Na efflux from 12·6 to 5·35 p-mole cm^{-2} sec^{-1}, that is a reduction of 57·6 %. The mean value of ouabain-sensitive efflux amounts to 7·25 ± 1·9 p-mole cm^{-2} sec^{-1}. If ouabain perfusion was maintained, a moderate increase of Na efflux was frequently observed. This slow increase of Na loss in the

presence of ouabain may be accounted for by an increase in intracellular Na occurring as a result of the inhibition of the Na extrusion. This effect in turn may enhance the ouabain-insensitive Na exchange or rise a residual fraction of the pump remaining unblocked by ouabain (Lamb & MacKinnon, 1971).

Effects of K on Na efflux

When the liver was perfused with a K-free solution for 10 min the mean value of Na extrusion, estimated from three experiments, decreased from 11·3 to 6·1 p-mole cm^{-2} sec^{-1}, i.e. the K-dependent Na efflux amounts to 46·5% of total Na efflux. As in the ouabain experiments the flow rate decreased. The Na efflux of perfused rat liver in K-free or ouabain 10^{-3} M solutions displays a similar pattern to that of liver slices (Van Rossum, 1966*a*, *b*). Moreover, the mean inhibition in K-free solutions is slightly lower than that obtained with ouabain. This observation may indicate the persistence of an active but reduced extrusion of Na in K-free solutions.

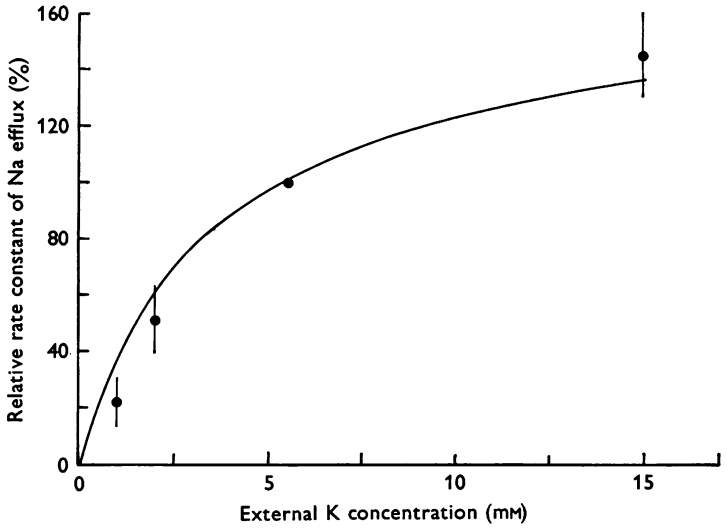

Fig. 1. Dependence of the Na efflux on external [K]. Ordinate: increment in relative rate constant for loss of ^{24}Na above the rate constant measured in K-free solutions. Abscissa: external [K] in mM. The curve is established according to the equation of a rectangular hyperbola:

$$B = B_{max}\,[K]_o/(K_m + [K]_o)$$

where B is the relative rate constant of Na efflux, B_{max} is the maximal rate constant. The constant K_m corresponds to the value of $[K]_o$ at which the binding sites are half-filled with potassium. B_{max} was taken as 160% and K_m as 3·0 mM. At low-Cl concentration, experimental points seem to deviate from the theoretical curve.

The effect of other K concentrations on Na efflux was tested by varying $[K]_o$ from 0 to 15 mM. Fig. 1 shows that the increase of Na efflux generated by external K is maximal above 15 mM-K and seems to deviate at low-K concentrations from a theoretical rectangular hyperbola. This inflexion point below 3 mM-K is consistent with the presence of a multisite carrier within the membrane, the activation sites of which are reversibly linked to more than one K ion. Incidentally, if in liver perfused with low-K solutions, there is a net leak of K from the cells resulting in a local accumulation of K against the membrane, the actual K concentration might be greater than the nominal $[K]_o$ so making the results more likely to be sigmoid (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969).

Effect of ouabain on K influx

If the coupling ratio is higher than one, the ouabain-sensitive K influx must be smaller than the ouabain-sensitive Na efflux. To test this hypothesis further, K influx was measured in livers perfused without and then with ouabain. The mean of nine control experiments for ^{42}K uptake was $1{\cdot}07 \pm 0{\cdot}08$ m-mole kg^{-1} min^{-1}, a value which yields an influx of 6·4 p-mole cm^{-2} sec^{-1}. In the presence of ouabain, K uptake decreased to $0{\cdot}28 \pm 0{\cdot}17$ m-mole kg^{-1} min^{-1}. The calculated K influx after correction for the post-ouabain depolarization (see below) was 1·83 p-mole cm^{-2} sec^{-1}. Thus, the ouabain inhibited component of K influx amounts to $4{\cdot}6 \pm 1{\cdot}8$ p-mole cm^{-2} sec^{-1}, i.e. about 72% of the total influx.

Although experimental determinations on Na and K fluxes were not performed in the same livers, the mean values of ouabain-sensitive fluxes were significantly different ($P < 0{\cdot}01$). The coupling ratio r, thus calculated, was $7{\cdot}25/4{\cdot}60 = 1{\cdot}58$. This figure is close to that estimated from membrane potential measurements. It suggests that three Na ions are rejected from and two K ions are pumped into the hepatic cell in one cycle of the pump, and unless either ion is moved with Na or K, that an electrogenic process may effectively modify the diffusion potential predicted by constant-field equations.

Effects of ouabain on passive fluxes

The effect of ouabain on Na influx was obtained by integrating the last exponential of the efflux curve in the presence of ouabain. The average value of five experiments for Na uptake was $1{\cdot}80 \pm 0{\cdot}7$ m-mole kg^{-1} min^{-1} after correction for ouabain depolarization (see below). The Na influx was estimated at 14·8 p-mole cm^{-2} sec^{-1}. This value is not significantly different from that obtained in the absence of ouabain (13·4 p-mole cm^{-2} sec^{-1}; Claret & Mazet, 1972). If ouabain has an effect on Na influx, it is trivial compared to its effect on Na efflux.

It was not possible to determine in the same way the effect of ouabain on K efflux. The inhibitor brought about a large and progressive decrease in flow rate. As a result ^{42}K accumulated probably in the interstitium, making rate constant determinations highly questionable.

Effects of perfusion with low-Cl solutions

In a previous study it was reported that removal of external Cl results in a slow hyperpolarization (Claret & Mazet, 1972). It was suggested that the observed hyperpolarization may be due to (*a*) an increase in P_K and (*b*) the removal of short-circuiting effect of Cl ions on potential generated

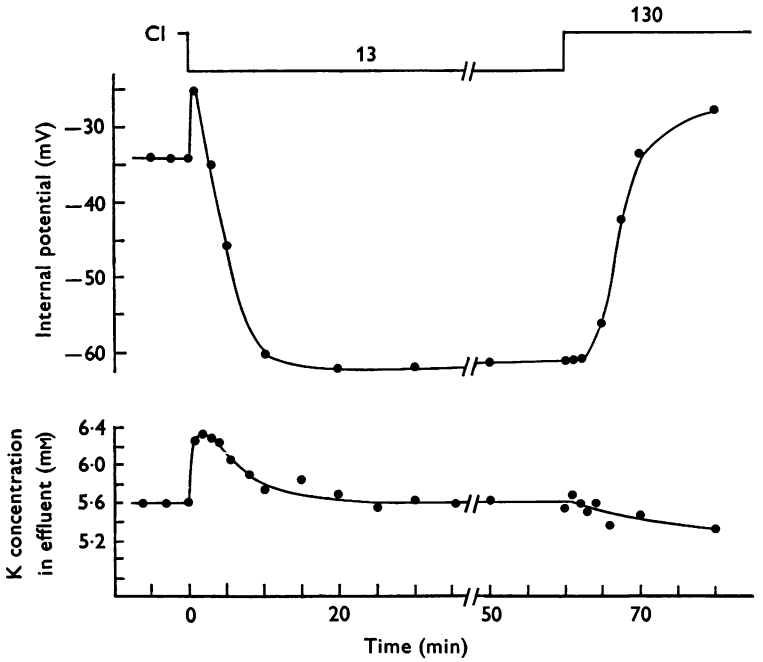

Fig. 2. Effect of tenfold $[Cl]_o$ decrease on membrane potential and on [K] in effluent in a typical experiment. Ordinate, upper curve: membrane potential (mV); lower curve, [K] in effluent (mM). Abscissa: time in minutes. Note the absence of symmetry of the effects of removal and admission of external Cl on E_m and $[K]_o$.

by the electrogenic pump. Fig. 2 shows the changes in membrane potential (E_m) and the simultaneous net K movements, when benzene sulphonate was substituted for Cl. The tenfold decrease in $[Cl]_o$ results in an immediate depolarization corresponding to a shift of E_{Cl}. Then, a sudden outflux of K occurs, followed by a slow hyperpolarization which reaches a maximum and stable value in 15–20 min. The loss of K from the liver decreases

exponentially with time. When returning to normal medium, E_m does not change for a few minutes and no opposite movement of K occurs. Then, the amplitude of E_m decreases and K movement is reversed, i.e. K enters the cell. Table 1 shows that the mean absolute value of E_m increases from 33·7 mV (Cl solutions) to 63·8 mV (benzene sulphonate solutions).

TABLE 1. Total and extracellular water (ml. kg^{-1} wet wt.), total ion content (K_T, Na_T, Cl_T, m-mole kg^{-1} wet wt.) and membrane potential (mV) of rat livers perfused during 60 min in chloride or benzene sulphonate solutions. The number of observations is given in brackets. Intracellular ion concentrations (m-mole $l.^{-1}$ of cells), are calculated as mentioned in Methods

	Cl solutions	Benzene sulphonate solutions
Total water	722 ± 27 (8)	704 ± 20 (11)
Extracellular water	257 ± 32 (8)	243 ± 33 (10)
K_T	73·5 ± 3·2 (12)	76·0 ± 4·0 (8)
Na_T	44·5 ± 5·2 (13)	47·9 ± 4·6 (8)
Cl_T	48·0 ± 4·5 (9)	3·3 ± 1·7 (12)
E_m	−33·7 ± 1·7 (21)	−63·8 ± 3·2 (27)
$[K]_i$	104	107
$[Na]_i$	17·1	23·0
$[Cl]_i$	22·1	0·3

In order to determine the origin of the observed hyperpolarization, intracellular concentrations and fluxes of K and Na were determined and the effect of ouabain on membrane potential was compared in livers perfused with Cl and benzene sulphonate solutions.

Permeability to benzene sulphonate. Before measuring intracellular ionic concentrations, it was necessary to test whether the anionic conductance was suppressed when benzene sulphonate was substituted for Cl, i.e. whether benzene sulphonate anions enter the cell compartment or not. The content of [^{35}S]benzene sulphonate was measured in tissue as function of time. The kinetic of its distribution is similar to that of [^{14}C]inulin. As demonstrated by Casteels (1971) in smooth muscle, the total liver concentration of [^{35}S]benzene sulphonate increases rapidly to 32·2 ± 2·2 m-mole kg^{-1} wet wt., then very slowly after 10–20 min. After 60 min, the concentration of anion was only 35·4 ± 5·3 m-mole kg^{-1}. This result suggests that the membrane permeability to benzene sulphonate anions, P_{BS}, is small compared to that of other ions.

Water and ionic contents. Table 1 shows water and ionic concentrations measured after 60 min of equilibration in Cl and benzene sulphonate solutions. While the total water is decreased, extracellular water, measured on the basis of [^{14}C]inulin space is not significantly different in livers perfused with benzene sulphonate solutions and controls. Assuming that the density of liver was equal to 1·05 g.ml^{-1} and the volume/surface ratio

to 2·5 μm (Claret & Mazet, 1972), the surface of liver cells has the same value in both solutions, i.e. 2·8 cm^2 mg^{-1}.

During benzene sulphonate substitution, the intracellular cationic concentrations are increased whereas $[Cl]_i$ decreases considerably, suggesting that Cl leaves the intracellular compartment. Since $[Cl]_i$ in control experiments was 22·1 m-mole $l.^{-1}$ of cells and the mean liver weight was 7·7 g, it may be estimated that Cl loss amounts to 115 μmole per liver. If benzene sulphonate is relatively impermeant, i.e. if no exchange Cl benzene sulphonate occurs through the membrane, it is likely that Cl leaves the cell with K to maintain electroneutrality. This may explain the sudden outflux of K that occurs with a mean rate constant of 0·16 min^{-1}, as calculated from seven experiments. The integration of K-leakage curve allows an estimate of the total amount of K lost at 106 μmole (i.e. 17·5 % of cell K content per liver of 7·7 g). The identity of the cationic and anionic net losses suggests that Cl leaves the cell as KCl and therefore confirms that membrane permeability to benzene sulphonate is relatively low. To preserve osmotic equilibrium this movement must be accompanied by water increasing slightly $[K]_i$ and to a greater extent $[Na]_i$. Consistent with this hypothesis is the observation that water content in livers perfused with low-Cl solutions is lower than that measured in livers perfused with normal-Cl solutions.

Na and K fluxes

The amplitude of hyperpolarization remains unchanged if the perfusion with low-Cl solutions is maintained. After 3 hr, the mean value of membrane potential is $-60·2$ mV. This observation suggests that intracellular ionic concentrations are in steady state, i.e. the influx is equal to the efflux for any ion. Na uptake determined by integrating the efflux curve in livers, averaged to $2·32 \pm 0·87$ m-mole kg^{-1}, a value which corresponds to a corrected Na influx of 17·1 p-mole cm^{-2} sec^{-1}. The mean rate constant of Na efflux was $0·170\ min^{-1} \pm 0·18$ ($n = 9$) which is not different from that obtained in the presence of Cl. From this figure and the internal Na (Table 1), the Na efflux amounts to 16·3 p-mole cm^{-2} sec^{-1}. Assuming no change in the amplitude of ouabain-insensitive Na for Na exchange in low-Cl media, i.e. 3·3 p-mole cm^{-2} sec^{-1}, passive flux along its electrochemical gradient may be estimated at 13·8 p-mole cm^{-2} sec^{-1}. The permeability coefficient, P_{Na}, calculated from passive influx, $[Na]_o$ and membrane potential amounts to $4·1 \times 10^{-8}$ cm sec^{-1}. This figure does not differ from that obtained for P_{Na} in livers perfused with normal solutions ($P_{Na} = 4·0 \times 10^{-8}$ cm sec^{-1}; Claret & Mazet, 1972).

K uptake was estimated from the activity of ^{42}K present in the tissue after 5 min of loading, corrected for the outflux of ^{42}K at that time. The

mean of K uptake was $1{\cdot}04 \pm 0{\cdot}08$ m-mole kg^{-1} min^{-1} ($n = 9$). The rate constant for ^{42}K loss was not altered over a period of 2 hr. Its mean value was $1{\cdot}01 \pm 0{\cdot}25$ hr^{-1} ($n = 4$). After corrections, the efflux was estimated at 7·7 p-mole cm^{-2} sec^{-1}. The membrane permeability calculated from this value, $[K]_i$ and membrane potential was 20×10^{-8} cm sec^{-1}. This figure is 2·6 times greater than that obtained in livers perfused with physiological Ringer solutions (Claret & Mazet, 1972) which is in agreement with previous data suggesting that E_m becomes progressively sensitive to $[K]_o$ in low-Cl solutions.

Despite the drastic changes in K permeability the unidirectional fluxes for each ionic species are identical, indicating that the tissue is effectively in steady state.

Effects of ouabain on E_m in normal and low-Cl solutions

If the potential generated by the Na-K pump is greater in low-Cl solutions, the addition of ouabain should result in a greater depolarization. The data confirm this interpretation. The presence of 10^{-3} M ouabain alters rapidly the membrane potential in livers perfused with normal-Cl as well as with low-Cl media. Six to ten minutes after addition of inhibitor, the absolute value of E_m was decreased from $35{\cdot}0 \pm 2{\cdot}3$ mV ($n = 13$) to $28{\cdot}2 \pm 3{\cdot}3$ mV ($n = 12$) and $63{\cdot}4 \pm 3{\cdot}0$ ($n = 20$) to $21{\cdot}5 \pm 5{\cdot}7$ mV ($n = 20$) in normal and low-Cl solutions respectively. Although E_m largely fluctuated from sample to sample in benzene sulphonate ouabain solutions, the amplitude of the depolarization induced by ouabain was significantly greater (21·9 mV) than that observed in the presence of Cl (6·8 mV). A small depolarization was also reported in rat liver slices (Biegelman & Thomas, 1972) and in dog liver perfused with blood (Lambotte, 1970) with cardiac glycosides. However, in the latter, it was detected only after 20 min of perfusion with ouabain.

Effects of ouabain and K on flow rate

The data reported in this study demonstrates that hepatic flow rate declines progressively when the liver is perfused with a K-free or ouabain-added medium. On the average, flow rate decreases from 23·3 to 17·2 ml. min^{-1} in 10 min when K was removed and from 21·1 to 16·0 ml. min^{-1} when ouabain was added. Boyer (1971) also reported an ouabain-induced decrease in flow rate in perfused rat liver. If the solution is switched back to Ringer, the flow rate increases slowly to its control value, but prolongation of the perfusion with the test solution cuts down drastically hepatic flow rate. Its mean value, estimated in three experiments, after 25 min of ouabain perfusion was 3·48 ml. min^{-1}. On the contrary, the addition of ouabain in benzene sulphonate solutions does not alter hepatic

flow rate. Ten minutes after addition of the drug in low-Cl solutions, it was as high as 98% of the control value.

Since the magnitude of flow rate is drastically reduced under ouabain perfusion or by K removal, it is tempting to speculate that its changes reflect a variation of Na-K pump as postulated by Boyer (1971). Unfavourable metabolic conditions lead extracellular electrolytes, chiefly Na and Cl, and water to accumulate in the hepatic cell (Leaf, 1956; Heckman & Parsons, 1959; Elshove & Van Rossum, 1963). It is likely that the observed changes in flow rate result from cellular swelling and partial occlusion of hepatic capillaries. This hypothesis is supported by the fact that ouabain does not change hepatic flow rate, when added in low-Cl media. If the membrane is truly impermeant to benzene sulphonate anions, the net influx of Na occurring as a result of the pump inhibition is limited to a passive one-to-one Na-K exchange. Thus, intracellular Na concentration increases but the net ionic content remains unchanged, and no cellular swelling occurs.

DISCUSSION

The existence of a common coupled mechanism for Na-K transport in rat liver cells has been demonstrated by the dependence of the net uphill movement of Na and ^{24}Na efflux on external K concentration, which occurs when cold incubated slices are subsequently rewarmed at 38° C (Elshove & Van Rossum, 1963; Van Rossum, 1966*a*, *b*). It was also reported that K removal from external solutions is less effective than cardiac glycosides to obtain inhibition of Na extrusion. Na efflux from perfused liver displays a similar pattern. As shown in Fig. 2, its magnitude decreases in low-K solutions and increases in high-K solutions, an observation indicating activation of the Na pump by external K. Also the average inhibition of Na efflux obtained by K-free solutions amounts to 46·5% as opposed to 57·8% obtained with ouabain.

In liver slices incubated in K-free solutions, ouabain diminishes slightly the efflux of Na but it does not change tissue Na content (Van Rossum, 1966*b*). It was, accordingly, suggested that a small part of the active Na extrusion was independent of K inwards transport. This hypothesis, though not tested directly here, could account for our data. The small difference between ouabain-sensitive Na efflux and Na efflux affected by K removal (about 12%) could be ascribed to ouabain-sensitive Na-Na exchange. It seems unlikely that liver cell membranes exhibit an ouabain-sensitive Na for Na exchange in 5·6 mM-K solutions, since the addition of the inhibitor does not alter Na influx. However, K removal may induce this process which is not operative in normal conditions, as already shown in red cells (Garrahan & Glynn, 1967), skeletal muscle (Keynes & Stein-

hardt, 1968) or partly poisoned giant axons (Baker *et al.* 1969). An alternative explanation is that the small inhibition of Na extrusion in K-free media is accounted for by residual activity of the Na-K pump, as a result of incomplete removal of K from the interstitium. Indeed, the inhibition of the pump in K-free media results in a decrease in flow rate and net K cellular leakage. Both effects may promote a local accumulation of K in the vicinity of the membrane and hence allow the pump to operate at a reduced rate (Baker *et al.* 1969). This hypothesis is supported by the theoretically estimated value of $[K]_0$ in the microenvironment against the cell membrane. Using the mathematical model developed by Claret & Mazet (1972) for rat liver, the actual K concentration against the pumping sites, when the liver is perfused with K-free solutions, may be estimated at about 0·3–0·6 mM.

A survey of the data reported here allows an estimate of the different components of Na and K fluxes in normal conditions. Na efflux is made up from at least three components, namely (1) ouabain-sensitive (58 % of the total Na efflux); (2) ouabain-insensitive (28 % of the total efflux); (3) passive movement of Na predicted from Ussing's equation (5 % of the total Na efflux). This leaves some 10 % unaccountable. K influx is made of two parts, i.e. (1) ouabain-sensitive (72 % of the total K influx) and (2) passive movement of K predicted from Ussing's equation (13 % of the total K influx). It seems likely that the unexplained parts of Na efflux and K influx are active fluxes, resistant to the concentration of ouabain used here. This hypothesis is supported by the fact that the ouabain-sensitive fluxes account for 83 and 85 % of the expected Na and K fluxes respectively from a complete inhibition of the pump. The similarity of these figures suggests effectively that ouabain does not block completely the Na-K exchanging transport mechanism (Lamb & McKinnon, 1971).

The ratio of the ouabain-sensitive fluxes of Na and K is 1·58 suggesting that three Na are carried out while two K are carried inwards, in one cycle of the pump. Taking into account this ratio and the actual Na efflux, the net cationic flux produced by the pump is found to be 3·1 p-mole cm^{-2} sec^{-1}. This figure multiplied by the Faraday allows an estimate of the pump current at 0·30 μA cm^{-2}. Since membrane conductance of liver cells is 52 μmho cm^{-2} (Claret & Mazet, 1972), the potential generated by the active cationic transport amounts to 5·8 mV. This value, obtained indirectly, is in good agreement with the depolarization actually measured when ouabain was added (6·8 mV).

When the liver is perfused with a solution in which benzene sulphonate was substituted for Cl, chloride leaves the cellular compartment as KCl. The loss of KCl is followed by the amount of water required to preserve cellular tonicity. However, this movement of water results in an increased

concentration of intracellular indiffusible particles. When the liver is again perfused with normal Ringer ($[Cl]_o$ = 130 mM), no change in K and Cl net fluxes is observed for a few minutes (E_m also remains stable). This observation is consistent with a decrease in chloride permeability in low-Cl media as was suggested in a previous paper (Claret & Coraboeuf, 1970). Inwards diffusion of Cl would slow down if P_{Cl} is decreased, despite a favourable driving force. After a few minutes, K and Cl movements reverse, suggesting that there is a progressive increase in P_{Cl}. The fact that benzene sulphonate solutions reduce Cl permeability and increase K permeability could be explained by an interaction between anions and membrane lipoproteins, as already reported for foreign anions (Wieth, 1970; Barker & Levitan, 1971; Casteels, 1971).

The origin of the hyperpolarization which develops as a result of Cl removal is a complex subject. Since G_{Cl} accounts for 58% of G_m in liver cells (32 μmho cm^{-2} sec^{-1}) it would be expected that the replacement of Cl by an impermeant anion increases the membrane resistance and consequently leads to greater hyperpolarization by any electrogenic pump. This, associated to the increase in potassium permeability induced by low-Cl solutions, might explain the observed hyperpolarization. Using the Mullins & Noda's equation (1963) which takes into account ionic permeabilities and pump activity dependent on membrane conductance, it is possible to predict the actual membrane potential (E'_m). Since P_{BS} is negligibly small, K and Na ions share the whole membrane current, and the unidirectional fluxes are close to each other, the theoretical membrane potential is given by

$$E'_m = -RT/F \ln \frac{r[K]_i + \alpha[Na]_i}{r[K]_o + \alpha[Na]_o},$$

where α is the relative K/Na permeability, i.e. 0·20 ($3{\cdot}9 \times 10^{-8}$ cm sec^{-1}/ 20×10^{-8} cm sec^{-1}) and r the coupling-ratio of the pump (3/2). Using the values of intracellular ionic concentrations, as determined above, the predicted potential at 38° C is $-53{\cdot}0$ mV. This value is significantly higher than the membrane potential actually measured ($E_m = -63{\cdot}8$ mV), an observation indicating that the 2·6-fold increase of P_K in low-Cl solution and the removal of the short-circuiting effect on electrogenic Na-K pump are not sufficient to account for the total hyperpolarization.

The difference between the theoretical and measured potential might be due to an overestimation of the ratio of Na/K permeability. The main error in calculating α would originate from the estimate of the Na diffusion flux. Indeed, the magnitude of this flux is obtained by subtracting from the total Na influx the ouabain-insensitive Na-Na exchange flux which was assumed not to be altered by Cl removal. If Na-Na exchange across

the membrane was enhanced by the increase of $[Na]_i$ (from 17·1 to 23·0 m-mole $l.^{-1}$ of cells), the diffusional Na influx might have been overestimated. However, this mechanism could account for the measured value of E_m only if P_{Na} and, consequently, diffusional Na influx were halved and if the magnitude of the Na-Na process was increased by a factor of three. Such an increase is unlikely, since it has been demonstrated that the ouabain-insensitive Na-Na exchange is nearly saturated at relatively low internal sodium concentration (Keynes & Steinhardt, 1968).

Another hypothesis to account for the difference between calculated and measured potential is that the coupling ratio r was underestimated in low-Cl media, i.e. liver cells are endowed with a flexibly coupled Na-K pump. Consistent with this hypothesis are the effects of ouabain on membrane potential. If the inhibitory action of the drug is confined to Na extrusion, the greater depolarization observed in low-Cl media (22 mV versus 6·8 mV in normal-Cl media) implies that the pump shares a greater part in the build-up of the overall membrane potential, when chloride is removed from the medium. Fitting the data obtained with low-Cl solutions and the observed membrane potential of -64 mV into the Mullins & Noda's equation yields a coupling ratio, r, of 2·65. This figure suggests that under these conditions five Na ions are extruded from and two K ions are pumped into the cell in every cycle of the pump. Such an increase in r ratio is in agreement with the fact that unidirectional fluxes for each ionic species remain identical despite the change in membrane potential. If this hypothesis is correct, the observed 30 mV hyperpolarization in low-Cl media could be attributed to the sum of three effects: (*a*) an increase in P_K induced by foreign anion, (*b*) a decrease in membrane conductance consecutive to the removal of Cl short-circuiting effect and (*c*) an enhancement of the pump current, resulting from a change in coupling ratio. Further experiments are needed to elicit the mechanism of the pump in benzene sulphonate solutions.

The data reported in this study brings experimental evidence in favour of the hypothesis advanced earlier (Williams *et al.* 1971*a*, *b*; Claret & Mazet, 1972) of an electrogenic Na-K transport in rat liver cells. The pump extrudes three intracellular Na ions while carrying inwards two extracellular K ions in physiological solutions but it seems likely that Cl removal may indirectly increase the Na/K coupling ratio to 5/2. Therefore it might be concluded that the Na pump of liver cells is probably a labile system with a rather variable degree of direct coupling between Na and K fluxes, depending on the conditions prevailing at a given moment, as it has been reported in other tissues (Mullins & Noda, 1963; Keynes & Rybova, 1963; Adrian & Slayman, 1966; Mullins & Brinley, 1969).

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