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Regulation of Ion Permeabilities of Isolated Rat Liver Cells by External Calcium Concentration and Temperature*

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Summary. Regulation of ion transport through the plasma membrane was studied on single cell suspensions of hepatocytes, obtained after perfusion of rat liver with collagenase/hyaluronidase solution. Steady-state intracellular K and Na contents were shown to be markedly dependent on external Ca concentration and temperature, the sum of both ion concentrations remaining nearly constant. In contrast, steady-state intracellular chloride content was found to be independent of external Ca concentration, but dependent on temperature. Using the constant field relations, the passive permeabilities P_K and P_{Cl} for potassium and chloride, respectively, were derived from the experimental data. At temperatures at and above 37 °C, with increasing external Ca concentration, P_K exhibits a sharp decrease at about 10^{-4} M. In contrast, P_{Cl} at 37 °C was found to be independent of Ca concentration within experimental error. Earth alkali ions other than Ca, show marked but different effects on P_K if compared at equal concentrations. Preincubation of the cells with cholesterol leads to a broadening of the dependence of P_K on external Ca concentration. The above results, as well as those on the dependence of P_K on external Ca concentration obtained by other authors, could be quantitatively described by a theoretical model of the plasma membrane proposed earlier. This model postulates regulatory binding sites, which cooperatively undergo a cation exchange of divalent cations by K^+ ions from the external medium, if the cation composition of the latter is altered.

Processes of ion transport through the plasma membrane of cells are interesting not only with regard to osmotic control but also in relation to regulation of basic metabolic functions of the cells. For instance, changes in ion permeabilities are observed as early steps in many hormone-induced biochemical responses: in noradrenaline action on liver (Haylett & Jenkinson, 1969), on brown fat cells (Horwitz, Horowitz & Smith, 1969), on

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toad skin (House, 1971); in glucagon action on liver (Friedmann, Somlyo & Somlyo, 1971; Friedmann & Dambach, 1973; Petersen, 1974), in adrenalin action on rat parotid slices (Selinger, Batzri, Eimerl & Schramm, 1973), etc. Similarly, inhibition or initiation of cell proliferation of fibroblasts have been shown to be closely associated with characteristic shifts in ion permeabilities of the plasmamembrane (Hülser & Frank, 1971; Orr, Yoshikawa-Fukada & Ebert, 1972; Cone & Tongier, 1974). In spite of the physiological relevance of ion transport through the plasma membrane, progress in the elucidation of its mechanisms has been very limited.

Major advances have recently been made with the introduction of phospholipid-bilayer membranes, demonstrating the principles of transit of ions through the hydrophobic core of membranes via carrier-like antibiotics such as valinomycin (cf. Läuger, 1972) or pore-forming substances such as gramicidin A (Hladky & Haydon, 1972; Bamberg & Läuger, 1973). However, bilayer studies have so far not contributed greatly to understanding the mechanisms of *regulation* of ion transport in nonexcitable cells. These regulation mechanisms have hitherto been investigated mostly using biological preparations, such as perfused organs or tissue slices. Most of these preparations, however, suffer from inadequate control of the nutritional, ionic, gaseous and hormonal environment of the cells. The evaluation of ion permeation characteristics is hampered further by the lack of knowledge of geometrical parameters of cell packing in the tissue such as intercellular spaces, exposed cell surface, etc., which specify the diffusion patterns of the permeating ion species in the tissue as well as the membrane permeabilities of the cells. For these and other reasons, single cell suspensions should preferably be used for the study of basic regulation mechanisms of ion transport through the plasma membrane.

We have studied some basic features of regulation of ion transport in single cell suspensions obtained from rat liver. This preparation has the following advantages: (*i*) rat liver has been well characterized by a multitude of biochemical investigations, so that any results on ion transport can be correlated meaningfully with other data and may provide for a basis of understanding the above-mentioned hormonal and other regulation mechanisms; (*ii*) in contrast to earlier attempts (Anderson, 1953; Jacob & Bhargava, 1962) there are now available reliable methods for isolation of viable single cell suspensions from this tissue (Schreiber & Schreiber, 1973); (*iii*) rat liver consists mostly of parenchymal cells, so that fairly homogeneous cell populations may be obtained (this paper).

We have studied the ion permeation characteristics of this preparation primarily in dependence on external calcium concentration and temperature

for the following reasons. Calcium seems to be intrinsically connected with the mechanism of regulation of cation transport, as suggested by numerous experimental studies. It has been shown for squid axon (Hodgkin & Keynes, 1957), frog skin (Curran, Herrera & Flanigan, 1963), kidney cortex (Kleinzeller, Knotkova & Nedvidkova, 1968), liver (Geyer, Sholtz & Bowie, 1955; Kalant & Hickie, 1968; Gilbert, 1972), and other tissue cells (Morril, Kaback & Robbins, 1964) that incubation in a calcium-free balanced salt solution leads to a decrease of the concentration gradients of alkali ions across the cell membrane. Thus, a general mechanism of regulation of alkali ion permeability by the external calcium concentration is suggested though not established quantitatively.

Another reason is the ubiquitous involvement of external calcium in hormone-induced cellular responses (Rasmussen, 1970), which is also suggested by recent work on the rat liver plasma membrane (Ray, Tomasi & Marinetti, 1967; Shlatz & Marinetti, 1972; Friedmann & Rasmussen, 1970).

Both these apparently general roles of calcium in relation to membrane phenomena might be closely related. Thus, it appears fruitful to characterize quantitatively first of all the effect of external calcium on ion permeabilities, and in a second step the alterations of these characteristics by hormone application.

Results on the first part of this program, using rat liver cells, are presented here, which show very steep characteristics of the dependence of potassium permeability on external calcium concentration. These findings are complementary to the recent observation by Mazet, Claret and Claret (1974) of a steep dependence of potassium permeability on external potassium concentration. These regulation characteristics are in quantitative agreement with a mechanism based on regulatory cation exchange in a membrane with cooperative subunit interaction, as proposed in earlier work (Adam, 1967, 1968, 1970 *a*).

Theoretical Relations

In the following, ionic permeabilities of the plasma membrane will be derived from the experimental data on ion permeation. Unfortunately, there is no unique way to define ionic permeabilities except by using a specific model of the membrane.

We shall use the Goldman-Hodgkin-Katz relations (GHK relations) for the passive ionic fluxes for the following reasons. Although the original derivation (Goldman, 1943; Hodgkin & Katz, 1949) used the constant

field assumption, which was critically discussed recently by Zelman (1968) and Zelman and Shih (1972), rederivations of the GHK relations by different authors (Patlak, 1960; Mullins & Noda, 1963; Barr, 1965; Simons 1973) have shown that they are valid under conditions more general than used initially. Fixed-charge models of the plasma membrane lead to relations very similar to the GHK relations (Adam, 1970*b*; Coster, 1973). Numerous applications to biological membranes have demonstrated the usefulness of these relations, in particular for description of ionic substitution experiments (cf. Williams, 1970).

The GHK relation for the passive flux Φ_L of ionic species L reads:

$$\Phi_L = P_L^* \frac{u}{e^{-u}-1}(C_{Li} - C_{Le}e^{-u}) \tag{1}$$

where

$$u = \frac{z_L F(\Psi_i - \Psi_e)}{RT}. \tag{2}$$

Here, the indices i and e denote the surfaces of the membrane phase directed to inside and outside of the cell, respectively; Ψ_i and Ψ_e are the corresponding electric potentials; C_{Li} and C_{Le} the corresponding concentrations of the ionic species L; P_L^* their permeability coefficient; z_L their valency (anions giving a negative sign for z_L); F the Faraday constant; R the gas constant.

In order to use the bulk concentrations L_i and L_e of the ionic species L in the inside and outside electrolyte solutions, respectively, in place of their concentrations C_{Li} and C_{Le} in the membrane, Hodgkin and Katz (1949) introduced into Eq. (1) the partition coefficient k_L being defined by:

$$C_{Li} = k_L L_i\,; \qquad C_{Le} = k_L L_e. \tag{3}$$

In case the membrane inner and outer surfaces are charged, the inner and outer electric surface potentials E_i and E_e, respectively, may be introduced yielding equations analogous to Eq. (3):

$$C_{Li} = k_L L_i e^{-z_L F E_i/RT}; \qquad C_{Le} = k_L L_e e^{-z_L F E_e/RT}. \tag{4}$$

Here E_i and E_e are taken relative to the electric potential of the internal and external electrolyte solution, respectively. For a complete description of ion transport through the plasma membrane, active transport has to be taken into account. Since the present data pertain to a steady state of ion concentrations in the intracellular compartment, we may use the following straightforward pump-leak model.

For any ionic species L of a steady-state intracellular concentration, there is a balance between passive flux Φ_L and active transport A_L:

$$\Phi_L = A_L. \tag{5}$$

From the studies on ouabain-sensitive Na/K-stimulated ATPase (cf. Harris, 1972), the active transport of Na and K may be assumed to be coupled:

$$A_{\mathrm{Na}} = -rA_{\mathrm{K}}, \tag{6}$$

where r is the active flux ratio which, for several tissues, is found to be in the range of $r \approx 1.5$ (Harris, 1972). Of course, r might depend on the intra- and extracellular ionic concentration. If $r \neq 1$, an electrogenic Na/K pump is implied.

From Eqs. (1) to (3), applied to Na and K, and Eqs. (5) and (6) a relation for the membrane potential $V_M = \Psi_i - \Psi_e$ is obtained (Mullins & Noda, 1963):

$$V_M = \frac{RT}{F} \ln \frac{rP_{\mathrm{K}}\mathrm{K}_e + P_{\mathrm{Na}}\mathrm{Na}_e}{rP_{\mathrm{K}}\mathrm{K}_i + P_{\mathrm{Na}}\mathrm{Na}_i}. \tag{7}$$

If surface potentials E_i and E_e are taken into account, Eq. (7) remains valid with:

$$V_M = E_e - E_i + (\Psi_i - \Psi_e). \tag{8}$$

In the derivation of Eq. (7) no statement was necessary as to the possible existence of an active Cl transport or whether or not Cl is in equilibrium across the membrane. As shown later in the discussion of the cellular ζ potentials the existence of surface potentials would lead to ion permeabilities differing only by a constant factor from those obtained without.

Thus, the analysis of the data will be based on Eqs. (1) to (3) yielding

$$P_{\mathrm{K}} = \Phi_{\mathrm{K}} \frac{\exp\left\{-\frac{FV_M}{RT}\right\} - 1}{\frac{FV_M}{RT}\left(\mathrm{K}_i - \mathrm{K}_e \exp\left\{-\frac{FV_M}{RT}\right\}\right)} \tag{9}$$

where

$$P_{\mathrm{K}} = P_{\mathrm{K}}^* k_L. \tag{10}$$

From the measurement of Φ_{K}, K_i, K_e and V_{M} the quantity P_{K} may be obtained. Since it proved rather difficult to obtain by direct measurement membrane potentials V_M of a precision comparable to the flux measurements, this quantity was determined indirectly from the alkali-ion con-

centrations via Eq. (7). Thus, for evaluation of P_K in dependence of the external parameters, such as Ca concentration, the experimental quantities Φ_K, K_i, K_e, Na_i and Na_e have been determined as described in the following.

Experimental Methods

Materials

Adult rats of strain Ch. B. B. THOM FW49 from Thomae, Biberach, BRD, weighing 180–240 g were used throughout. Collagenase (Type 1) and hyaluronidase (Type 1) were obtained from Sigma, St. Louis, Missouri, ^{42}K, ^{36}Cl and inulin-[^{14}C]-carboxylic acid from Amersham, UK. The nylon mesh of 80 μm pore size was purchased from Maierthaler KG, OHG, Köln, BRD. The standard basic salt medium (BS medium) used contained (mM): 118 NaCl, 2.5 Na_2HPO_4, 2.0 NaH_2PO_4, 6 KCl, 20 $NaHCO_3$, 50 D(+)-glucose and 0.14 phenol red, and was equilibrated with 5% CO_2 and 95% O_2 and maintained at pH 7.4. For a free Ca^{2+} concentration of 5×10^{-8} M, solutions containing 2×10^{-3} M EDTA and 1.2×10^{-3} $CaCl_2$ were used (Portzehl, Caldwell & Rüegg, 1964).

Preparation of Cell Suspensions by Recirculating Perfusion

The perfusion technique of Berry and Friend (1969) with modifications (Kolb, *unpublished*) was used as follows. The liver of anaesthetized animals (6.5 mg nembutal i.p./100 g living weight) was perfused through the portal vein with 50 ml BS medium followed by closed perfusion with back flow through the thoracic portion of the inferior vena cava of 150 ml BS medium containing 0.05% collagenase, 0.1% hyaluronidase and 10^{-5} M Ca. The closed perfusion was maintained at 34.5 °C under an atmosphere of 95% O_2 and 5% CO_2 for about 10–15 min using a flux of about 3.2 ml BS medium/g liver/min. The liver was carefully removed, cut into smaller pieces, transferred to a stoppered gassed vessel (95% O_2, 5% CO_2) containing 50 ml of perfusion medium and shaken for 3–5 min. Thereafter, the suspension was brought to a final volume of about 100 ml by adding BS medium containing 10^{-5} M Ca (washing medium). The suspension was transferred to four 50-ml centrifuge tubes and the cells were sedimented at $20 \times g$ for 1.5 min. After removing the supernatant, cells were resuspended in washing medium. This procedure was repeated twice. Finally, the cell pellet was resuspended in incubation medium as required for measurement. The dispersion and washing procedure was done at room temperature. Yields of 2.0 to 2.6×10^8 cells per gram wet weight of liver could be obtained consisting almost entirely of single parenchymal cells.

Respiration and Viability

About 1.8×10^6 cells were incubated in 3 ml of Krebs-Ringer's phosphate solution (Umbreit, Burris & Stauffer, 1972) at pH 7.4. Oxygen uptake was measured in a Warburg respirometer. The flasks, containing 0.15 ml of 10% KOH in their center wells, were incubated at 37 °C in an atmosphere of air. A constant respiration rate of 19.1 μl $O_2/10^6$ cells/hr could be measured for at least 4 hr of incubation at 37 °C. The presence of 1.2 mM Ca had no significant stimulatory or inhibitory effect on the respiration activity (Iype & Bhargava, 1965; Howard & Pesch, 1968; Berry & Friend, 1969). Hepatocytes after Na citrate perfusion of rat

liver showed upon addition of 1.2 mM Ca a decrease of respiration rate from 12.6 to about $0-1$ µl $O_2/10^6$ cells/hr.

It was found that 10^6 cells are equivalent to 1.96 ± 0.02 ($n=45$) mg of protein (mean $\pm$ standard error of mean, number of experiments in parentheses). Protein determination was done using the Biuret-method (Beisenherz *et al.*, 1953). The viability of liver cells was estimated by light microscopy counting the stained cells after 15-min incubation with 0.5% trypan blue. Cell counts were made with an improved Neubauer chamber; cell diameters were measured with an ocular micrometer of a Zeiss Universal microscope (C. Zeiss, Oberkochen, BRD). The percentage of cells stained by trypan blue treatment varies slightly from preparation to preparation; on the average we had 7% stained cells. Under light microscope the cells are round and have a well-defined outline. Electron-microscopic studies (Kolb, *unpublished*) showed that about 65% of the cells retain their structural integrity. No transformation of the parallel stacks of rough endoplasmic reticulum into isolated vesicles and no mitochondrial damage could be observed in this fraction. The remaining cells exhibit only some swelling of Golgi and cytoplasmic vacuoles.

Cell Electrolyte Contents

For determination of electrolyte contents, 3–10 ml aliquots of cell suspension ($1.5-8 \times 10^6$ cells) were taken and sedimented at $400 \times g$ for 2 min. After removing the supernatant carefully, the centrifuge tubes (no. 105/16.8 from Sarstedt, Numbrecht, BRD) were frozen in liquid nitrogen for 3–5 sec. The pellet could be removed from the tube as a frozen tablet and was filled into preweighed stoppered vessels. The water content and dry weight of the samples were obtained from the weights before and after drying in an air oven (90 °C) for about 48 hr. The electrolyte content was determined on extracts made by digesting each sample of pellet for 48 hr at room temperature with 0.1 HNO_3. This method permits complete recovery of the tissue sodium and potassium (Whittam, 1955). Chloride was determined by electrometric titration (Analytical Chloride Titrator, Aminco, USA); potassium and sodium with the flame photometer attachment for the Zeiss spectrophotometer PMQ 2. For K and Na analysis the tissue extract was diluted to give final K and Na concentrations of $1-2 \times 10^{-4}$ M. The electrolyte contents of liver cells are expressed as mmol/kg cell H_2O.

Intercellular Space

For calculation of cell water content the intercellular space of the pellet is needed. Cells were preincubated for 2 hr in BS medium which contained 1.2 mM Ca, 1.2 mM Mg and 2% inulin (Serva, Heidelberg, BRD) at 37 °C. After addition of 0.15 µCi inulin-[^{14}C]-carboxylic acid per ml suspension an aliquot was taken and sedimented at $400 \times g$ for 2 min. The pellet was weighed, 0.5 ml H_2O added and the suspension homogenized. Aliquots of 0.2 ml of homogenate were mixed with 10 ml Bray's solution (Bray, 1960) and counted in a Packard TriCarb 3375. The result was corrected for quenching. Binding and uptake of inulin-[^{14}C]-carboxylic acid was taken into account by sedimentation of an aliquot suspension through a layer of the medium containing 4% Ficoll (Pharmacia, Uppsala, Sweden). By this procedure cells could be qualitatively separated from the incubation medium. Standard calculations lead to a volume fraction of 0.692 ± 0.002 ($n=20$) for cellular volume of the pellet.

^{42}K and ^{36}Cl Influx

Cells were preincubated without label for 2 hr. Thereafter 0.1 µCi/ml suspension of ^{42}K or ^{36}Cl, respectively, were added. Aliquots of suspension were taken at different times

and sedimented 2 min at 400 × *g* through a layer of the same medium as used for preincubation but with 4% Ficoll added. For ^{42}K determination the γ-spectrometer attachment for the Packard TriCarb 3375 was used. The specific activity was corrected for radioactive decay. Measurements of ^{36}Cl were done in the same way as described for inulin-^{14}C determination. The ^{42}K and ^{36}Cl-influx were theoretically described by a simple two-compartment model using the equation

$$S_i(t) = S_{i0}(1 - \exp(-kt)). \tag{11}$$

Here, $S_i(t)$ is the specific activity at time t, and k the rate constant of influx. For k the following equation holds:

$$k = \frac{\Phi}{L_i} \frac{A}{V}, \tag{12}$$

where Φ denotes the influx, L_i the intracellular concentration of K or Cl, and A/V the cell surface per mg cells. Correction of k taking into account K or Cl efflux was done according to Keynes (1954). Using the latter correction, the rate constant k determined by Eq. (12) is augmented by a factor of about 1.05.

Dispersions with Cholesterol

Dispersions of cholesterol (recrystallized 3 times from methanol, m.p. = 148 °C) and BS medium containing 1 mg cholesterol per ml were prepared by ultrasonic treatment with a Branson sonifier for 10 min at level 8 and 10 A. Dispersion vessels were cooled with an ice/NaCl mixture and gassed with N_2.

Microcell Electrophoresis

The electrophoretic mobility, defined as migration velocity per electric field strength, was measured as described in Adam and Adam (1975). About 10^7 cells were suspended in 5 ml buffer at pH 7.2 and 37 °C containing: (a) 150 mM NaCl, 6 mM KCl, 1 g/liter D(+)-glucose, 10 mM triethanolamine hydrochloride, or (b) 140 mM NaCl, 6 mM KCl, 1 g/liter D(+)-glucose, 20 mM HEPES and measured in a thermostated rectangular cuvette of a Zeiss Zytopherometer. The results are determined as mean and standard error of mean from at least 20 determinations of migration velocity in each direction of the applied electric field strength.

Results

Steady-state K, Na and Cl Content

Isolated rat liver cells were incubated in BS medium containing 1.2 mM Ca at 37 °C and 0 °C. Fig. 1 presents the time course of K, Na and Cl content of liver cells. After the isolation procedure, which took place at 20–22 °C, the cells have lost about 60% of their K content which they are able to regain after a 90-min incubation at 37 °C. During this time K_i increases from 94.2 ± 1.8 (8) to 138.0 ± 2.2 (8) mmol/kg cell H_2O. At the

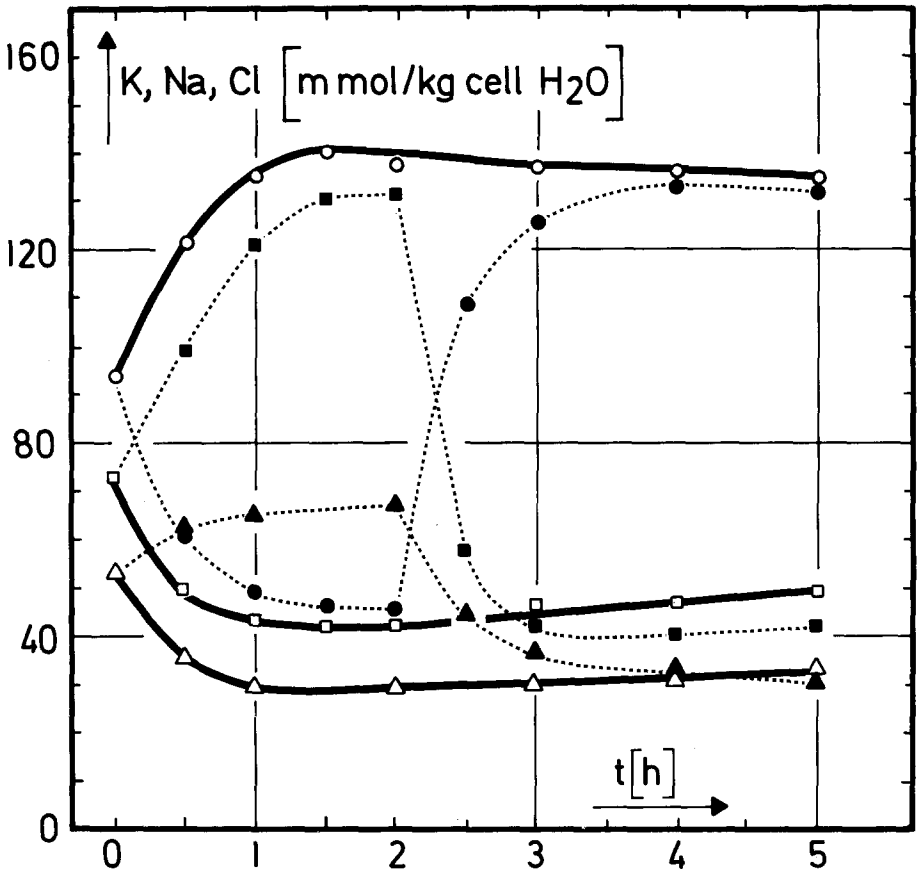

Fig. 1. K, Na and Cl content versus time at different incubation temperatures. (*a*) at 37 °C: ○, K; □, Na; △, Cl. (*b*) at 0 °C: ●, K; ■, Na; ▲, Cl starting from 21–22 °C at $t=0$ hr. After 2 hr of incubation in BS medium containing 1.2 mM Ca at 0 °C the medium was brought to 37 °C. The points are the mean ±SEM of eight separate experiments

same time Na_i falls from 73.1 ± 2.9 (8) to 42.1 ± 2.6 (8) mmol/kg cell H_2O and Cl_i from 52.8 ± 1.3 (8) to 29.7 ± 1.1 (8) mmol/kg cell H_2O.

After 90 min of incubation, the electrolyte content is nearly constant for another 150 min. We tested whether previous storage of the cells for about 2 hr at 0 °C before warming to 37 °C would lead to different and more stable electrolyte contents. This incubation procedure, which was used quite often by other authors (Flink, Hastings & Lowry, 1950; Judah & Ahmed, 1964; van Rossum, 1970) had no significant influence on the electrolyte contents of the cells at 37 °C (*see* Fig. 1). After about 4 hr of incubation at 37 °C, K_i starts to decrease and Na_i and Cl_i increase slightly. If Eagle's medium with 10% calf serum was used, the K content of 138.1 ± 1.8 (8) mmol/kg cell H_2O did not change for another 24 hr.

Effects of Calcium and Temperature on K, Na and Cl Content

In Fig. 2 the time course of cellular potassium is plotted for incubation at 37 °C in BS medium containing different Ca_e. As the Figure shows, cells incubated with 10^{-5} M or 5×10^{-8} M Ca_e, respectively, reach, after about 90 min, a stable K_i level which corresponds to 50–60% of the K content found in presence of 1.2×10^{-3} M Ca_e. A similar effect of calcium on K_i of isolated rat liver cells was observed by Howard, Lee and Pesch (1973). In order to verify the reversibility of the influence of calcium on K_i, Ca_e of the incubation medium was reduced from 1.2×10^{-3} M to 5×10^{-8} M by addition of 2×10^{-3} M EDTA and raised from 10^{-5} to

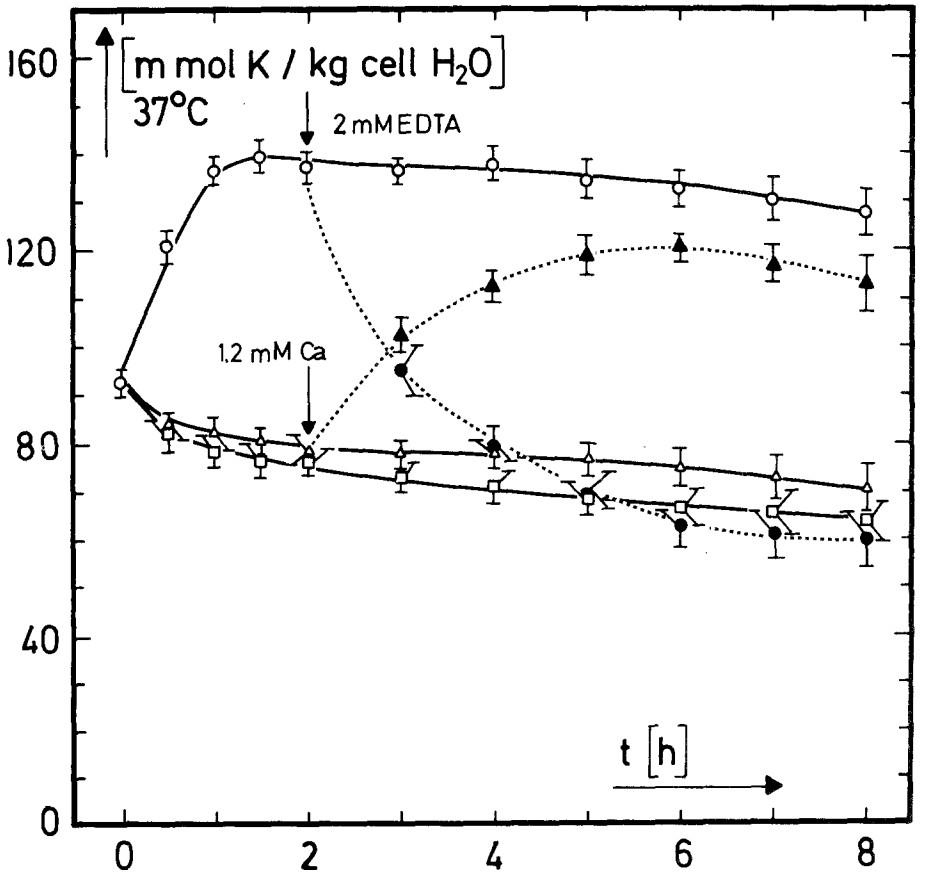

Fig. 2. K content versus time for different Ca_e: ○, 1.2 mM Ca_e; △, 0.01 mM Ca_e; □, 5×10^{-8} M Ca_e. After 2 hr of incubation Ca_e was lowered from 1.2×10^{-3} to 5×10^{-8} M by addition of 2 mM EDTA and raised from 1×10^{-5} to 1.2×10^{-3} M by addition of calcium, respectively, as indicated by the arrows. The points are the mean ± SEM of five separate experiments

1.2×10^{-3} M by addition of calcium. Lowering or raising Ca_e induced a nearly reversible change of K_i, as indicated by the dashed curves in Fig. 2. These experiments showed that net changes in electrolyte composition of liver cells, during incubation at different temperatures and Ca concentrations, are completed after at least 2 hr; therefore, we have used as standard incubation time 2–4 hr; thereafter the system was in a quasi stationary state and the effects of various factors on K, Na and Cl content could be measured.

First, the cellular K content was determined at different Ca_e and temperatures (*see* Fig. 3). For all temperatures, the cells gain their maximal K content in the range of 0.5–1.2 mM Ca_e. At temperatures higher than 25 °C a slight decrease in K_i is observed upon raising Ca_e from 1.2 to 2.4 mM. This effect may be due to inhibition of the Na/K-ATPase activity

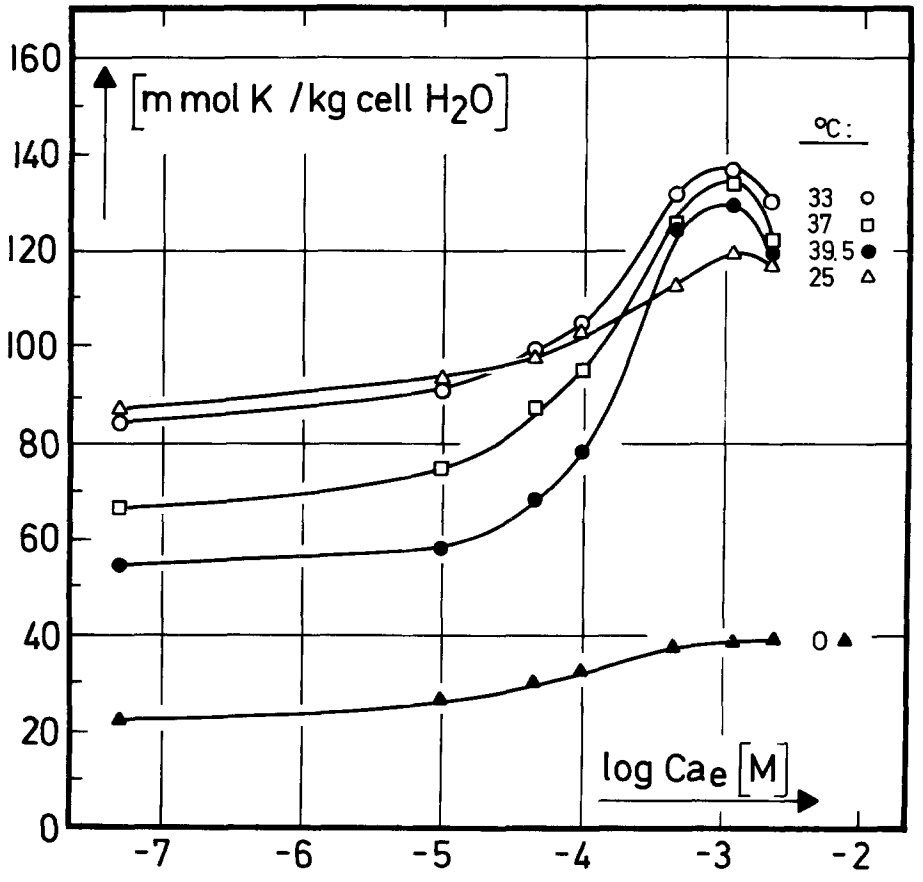

Fig. 3. K content versus external Ca concentration Ca_e. Curve parameter incubation temperature in °C: ●, 39.5; □, 37; ○, 33; △, 25; ▲, 0

above physiological external calcium concentration (Emmelot & Bos, 1966). The main effect of external calcium, however, is seen between 0.05 and 0.5 mM Ca_e. With increasing temperature K_i reacts more sensitively to changes of calcium in this concentration range. At 39.5 °C the decrease of K_i with decreasing Ca_e is maximal and amounts to about 60%. As can be seen, above 25 °C the presence of Ca exerts a larger effect than the incubation temperature on the retention of cellular potassium. Parallel to every measurement of K_i, we have determined intracellular Na content independently by flame spectrophotometry. At 37 °C and 1.2 mM Ca_e the sum of Na_i and K_i amounts to 175.9 ± 3.3 (14) mmol/kg cell H_2O. At lower temperatures and Ca_e this value is lowered by less than 8%. This means that Na_i under all incubation conditions behaves nearly inverse to K_i. Therefore, we have not reproduced here the data on Na_i explicitly as a function of Ca_e and temperature.

Fig. 4 shows the Cl content for different Ca_e and temperatures. Again an incubation time of 2–4 hr was used. At 1.2 mM Ca_e, a rise in temperature from 0 to 37 °C leads to a decrease of Cl_i from 79.8 ± 1.3 (8) to 32.2 ± 0.6 (8) mmol/kg cell H_2O. However, if the temperature is raised from 37 ° to 39.5 °C, Cl_i again increases by about 21%. The Cl content at all temperatures is only slightly dependent on Ca_e: the maximal change over 4–5 decades of Ca_e is less than 20%.

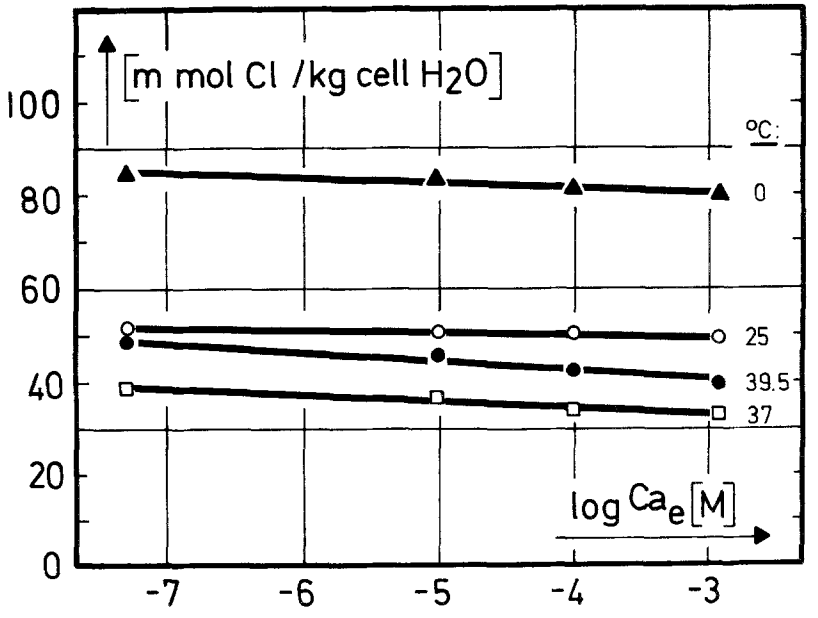

Fig. 4. Cl content versus external Ca concentration Ca_e. Curve parameter incubation temperature in °C: ●, 39.5; □, 37; ○, 25; ▲, 0

K Influx: Effect of Calcium and Temperature

The cells were preincubated for 2 hr in nonradioactive BS medium at different Ca_e and temperatures. Thereafter, ^{42}K was added to the cell suspension. Fig. 5 shows ^{42}K uptake in BS medium containing 1.2 mM Ca at 37 °C, averaged over four experiments. The curve drawn according to Eq. (11) agrees with the experimental values within the statistical error. This means that ^{42}K uptake by isolated liver cells can adequately be described by one exponential term. A rate constant of $k=0.0149\pm0.006$ (4) min^{-1} was calculated from a half logarithmic presentation of Fig. 5. With this Figure and a cell surface of 2.35 (44) cm^2/mg cell wet weight one derives by Eq. (12) a K influx of 9.3 ± 0.4 (4) pmol cm^{-2} s^{-1}. By this method the K influx was determined in dependence of Ca_e at different temperatures (*see* Fig. 6). Within the range of Ca_e and temperatures used, the ratio cell surface to mg cell wet weight differs by less than 10%. As Fig. 6 shows, K influx is independent of Ca_e within experimental error. The temperature dependence of K influx is qualitatively similar to that of Na/K-ATPase activity of rat liver (Bakkeren & Bonting, 1968).

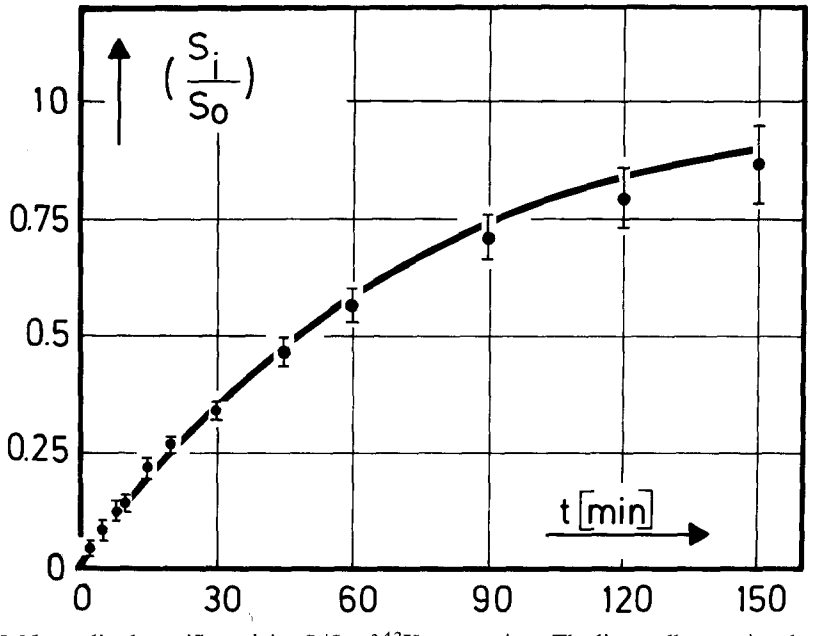

Fig. 5. Normalized specific activity S_i/S_0 of ^{42}K versus time. The liver cells were incubated in BS medium containing 1.2 mM Ca at 37 °C. Each point represents the mean ±SEM of four preparations. The solid line was calculated by Eq. (11) with a rate constant $k=0.0149\pm0.006(4)$ min^{-1} derived from a half-logarithmic presentation of $\left(1-\frac{S_i}{S_0}\right)$ versus time

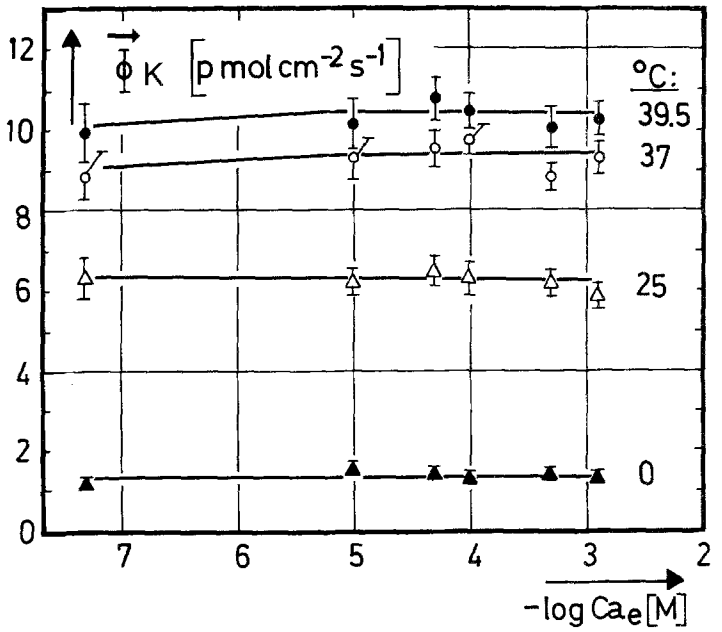

Fig. 6. K influx, Φ_K, versus external Ca concentration, Ca_e. Curve parameter incubation temperature in °C: ●, 39.5; ○, 37; △, 25; ▲, 0

K Content and K Influx in the Presence of Ouabain

Ouabain specifically inhibits the Na/K-ATPase activity and thereby lowers K_i. The Na/K-ATPase activity of liver cells is quite insensitive to ouabain concentrations up to 10^{-3} M (Beigelmann & Thomas, 1972). Similarly, Bakkeren and Bonting (1968) observed on Na/K-ATPase preparations in vitro that 10 mM ouabain is necessary to block its activity completely. We could confirm this observation by the following experiment. The cells were incubated at 37 °C in BS medium containing 1.2 mM Ca and different ouabain concentrations. K_i was found to decrease with increasing ouabain concentration. An asymptotic limit is attained at 10 mM ouabain where after 2 hr of incubation a K content of 15.4 ± 1.6 (6) mmol/kg cell H_2O was reached, which was nearly constant for another 2 hr. Under these conditions a K influx of 0.86 pmol cm^{-2} s^{-1} was measured.

Effect of Sr, Mg, Ba and La on K Content

If calcium in the incubation medium is exchanged by other divalent cations, in each case one observes a decrease of K_i with decreasing divalent

Table 1. Potassium content as a function of the extracellular concentration of different polyvalent cations in liver cells[a]

Medium	K_i (mmol/kg cell H_2O)			
(mM)	Mg	Sr	Ba	La
1.2	103.4±2.4	116.3±3.2	81.5±4.2	–
0.1	91.2±2.8	94.0±3.5	76.2±4.3	50.0±5.1
0.01	75.1±3.2	81.5±3.8	71.5±3.6	53.2±5.6

[a] Values are mean ±SEM of eight experiments in BS medium at 37 °C.

Table 2. Dependence of K_i content on Ca_e and Mg_e in liver cells[a]

Medium	K_i (mmol/kg cell H_2O)			
Mg_e(mM)	Ca_e(mM)			
	1.2	0.5	0.1	0.01
1.2	140.3±1.8	140.5±2.1	122.1±2.6	104.6±2.4
0.1	135.4±2.2	129.2±2.6	102.5±2.6	85.0±3.2

[a] Values are mean ±SEM of eight experiments in BS medium at 37 °C.

cation concentration. However, K_i maintained at 37 °C and at a fixed divalent cation concentration of 1.2 mM is lowered drastically in the sequence Ca > Sr > Mg > Ba (*see* Table 1). Addition of 0.1 mM Mg to BS medium containing different Ca_e does not produce any significant effect on K_i. However, by addition of 1.2 mM Mg the K_i is raised at low Ca_e; at 10^{-5} M Ca_e this rise amounts to about 23% (*see* Table 2). Lanthanum-chloride at concentrations above 0.01 mM apparently has a toxic effect; cells and cell fragments aggregate strongly and about 80–90% of these cells stain with trypan blue.

K Content as a Function of External Calcium in the Presence of Cholesterol

For these experiments the cells were incubated for about 3 hr in BS medium at different Ca_e. By addition of cholesterol (1 mg chol./ml BS medium) K_i increases (*see* Fig. 7). This increase is pronounced at Ca concentrations below 0.1 mM; here cholesterol increases K_i by 30% at an incubation temperature of 37° C and by 40% at 39.5 °C.

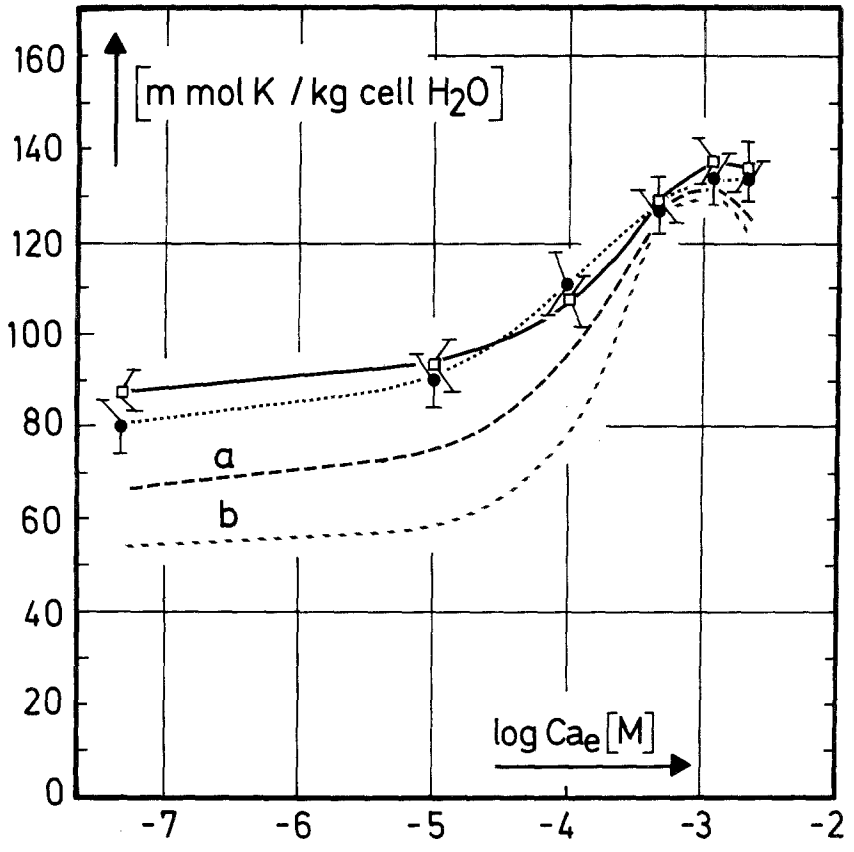

Fig. 7. K content versus external Ca concentration Ca_e in the presence of 1 mg cholesterol/ml BS medium at two temperatures: ●, 39.5 °C; □, 37 °C. Each point represents the mean ±SEM of five experiments. Curves *a* and *b* show the K control levels without addition of cholesterol at 37 and 39.5 °C

Calcium Dependence of Electrophoretic Mobility

Electrophoretic mobilities of rat liver cells, measured in two different buffers (20mM HEPES or 10 mM triethanolamine hydrochloride) coincided within experimental error. In Fig. 8 we have plotted therefore the averages from both runs, the error bounds being given by the larger of the standard errors of the individual measurement.

Within the precision of these measurements, electrophoretic mobility does not exhibit any change by altering Ca_e in the range 10^{-5} to 10^{-4} M, where K_i changes drastically. Only at $Ca_e = 5$ mM, i.e. a Ca concentration by a factor of about 50 higher than the range indicated above, is there observed a drop of electrophoretic mobility with increasing Ca_e.

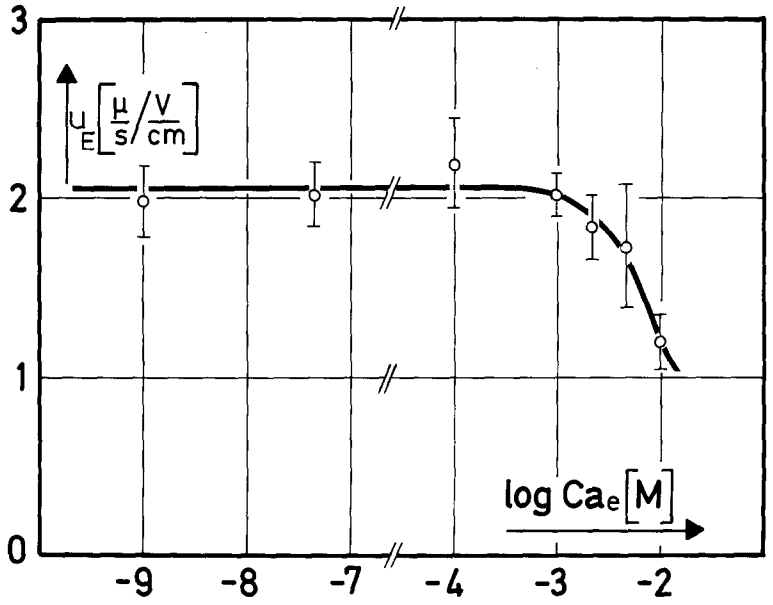

Fig. 8. Electrophoretic mobility u_E versus external Ca concentration; error bounds represent standard error of mean of about 40 determinations of migration velocity (*see text*)

Using the Smoluchowski relations $\zeta = u_E \cdot \eta/\varepsilon_0 \cdot \varepsilon$ and $\sigma = u_E \cdot \eta/l_D$ (Shaw, 1969), a zeta potential $\zeta = 20$ mV and a surface charge density $\sigma = 1.4 \times 10^{-6}$ coul cm^{-2} of the cells may be computed from $u_E = 2.0 \times 10^{-4}$ cm^2 V^{-1} s^{-1} as observed for $Ca_e < 10^{-4}$ M. Here the viscosity $\eta = 0.0069$ g s^{-1} cm^{-1} of the suspension medium at 37 °C, the dielectric constant $\varepsilon = 78$ and the Debye-length $l_D = 10^{-7}$ cm have been used, $\varepsilon_0 = 8.85 \times 10^{-14}$ coul V^{-1} cm^{-1} being the permittivity of vacuum.

Effect of Calcium on Membrane Potential and Passive Permeabilities for K and Cl

Electrophysiological measurements of the membrane potential of single rat liver cells are inherently difficult because of the lack of fixation of the cells for puncture by the microelectrode and have not yet been done with sufficient accuracy. Therefore we have calculated the membrane potential from the GHK relations by the following iteration procedure. The potassium influx is composed of passive and active components. In contrast, the potassium efflux may be considered purely passive and

according to Eq. (9) given by

$$\vec{\Phi}_K = P_K \frac{V_M F}{RT} \frac{K_i}{[1-\exp(-V_M F/RT)]}. \tag{13}$$

In the steady state of the pump-leak model, total influx and efflux are balanced. Our experiments have been designed to approximate steady state. Thus, the total K influx given in Fig. 6 may be taken as equal to the passive K efflux. The iteration procedure is started at the external Ca concentration of 1.2 mM by calculating the membrane potential and the K permeability according to Eqs. (7) and (9), respectively, using the measured values for K_e, K_i, Na_e, Na_i and $\vec{\Phi}_K$. In addition, the parameter $\alpha = P_{Na}/(r \cdot P_K)$ is needed in Eq. (7). From determinations of intracellular ion concentrations and membrane potential in physiological saline by different authors the following figures have been obtained: Williams, Withrow and Woodbury (1971), $\alpha = 0.13$ and $\alpha = 0.19$; Williams (1970) according to data from Wands, $\alpha = 0.25$; Schanne and Coraboeuf (1966), $\alpha = 0.30$; Claret and Mazet (1972), $\alpha = 0.32$; Claret, Claret and Mazet (1973), $\alpha = 0.30$; Mazet, Claret and Claret (1974), $\alpha = 0.31$. Evidently these numbers are within a fairly narrow range. Since P_K at 1.2 mM Ca_e is relatively insensitive to α, we may use in the following, $\alpha = 0.25 \pm 0.08$, and yet remain within the limits of precision $\Delta P_K/P_K \approx 10\%$ at 1.2 mM Ca_e set by experimental error.

For Ca concentrations below 1.2 mM, in the first iteration step V_m and P_K were calculated with $\alpha = 0.25$. With the figures P_K so obtained, α was corrected inversely proportional to P_K and the new value for α used in the second iteration step. After two iterations the third iteration gives a change of less than 3% in V_m and P_K, and thus yields the results given in the following figures. In this procedure $P_{Na}/(r \cdot P_K)$ at 1.2 mM Ca_e is assumed to be independent of temperature, and P_{Na}/r to be independent of Ca_e but change with temperature in proportion to P_K.

The membrane potential V_m resulting in this evaluation of the experimental data is given in Fig. 9 and turns out to be independent of Ca_e but strongly dependent on temperatures above 25 °C. For comparison, the equilibrium potentials E_{Cl} for chloride were computed from our data on intra- and extracellular Cl concentrations and found to agree approximately with the membrane potentials V_M (*see* Fig. 9), which is consistent with a purely passive distribution of chloride across the membrane.

In Fig. 10, P_K is plotted as a function of Ca_e and temperature, as resulting by the above procedure. There is a strong decrease of P_K with increasing

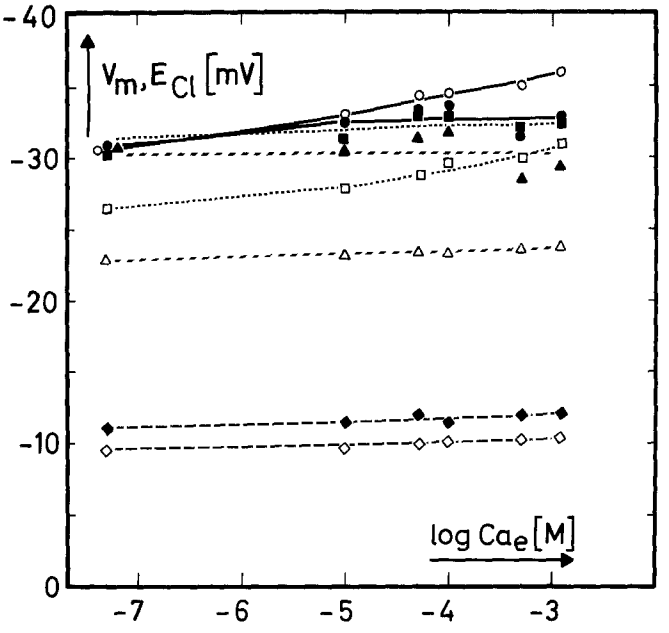

Fig. 9. Membrane potential V_m and chloride equilibrium potential E_{Cl} versus external Ca concentrations. Membrane potentials have been evaluated from experimental data using Eq. (7) at the following temperatures: ●, 39.5 °C; ■, 37 °C; ▲, 25 °C; ◆, 0 °C. Chloride equilibrium potentials were evaluated from the measured intra- and extracellular Cl contents using the Nernst equation at the following temperatures: ○, 39.5 °C; □, 37 °C; △, 25 °C; ◇, 0 °C

Ca_e at temperatures above 25 °C. This decrease occurs at about 10^{-4} M Ca_e and is maximal at 39.5 °C (Kolb & Adam, 1973, 1974).

If we used another α for evaluation of P_K, this would amount only to a parallel shift of the curve P_K *vs.* Ca_e along the ordinate axis. For instance, choosing $\alpha = 0.35$ as evaluated by Claret and Mazet (1972) from flux measurements on perfused rat liver at 37 °C, we would obtain P_K values only 0.23×10^{-7} cm/s lower than those given in Fig. 10.

Similarly, the relative shape of the curves P_K *vs.* Ca_e would not be changed, if allowance were made for an existence of surface potentials different on the inside and the outside face of the membrane. As an example we use $E_e = \zeta = -20$ mV as indicated by our electrophoresis data and choose $E_i = 0$ mV in order to provide for a fairly asymmetric situation. Here, Eq. (7) may be used again for evaluation of V_M. However, V_M in Eq. (13) is to be replaced by $(\Psi_i - \Psi_e) = V_M - (E_e - E_i)$ with $(E_e - E_i) = -20$ mV. Starting the iteration procedure at $Ca_e = 1.2$ mM and 37 °C, we obtain $(\Psi_i - \Psi_e) = -12.8$ mV yielding a P_K of about 35% lower than obtained

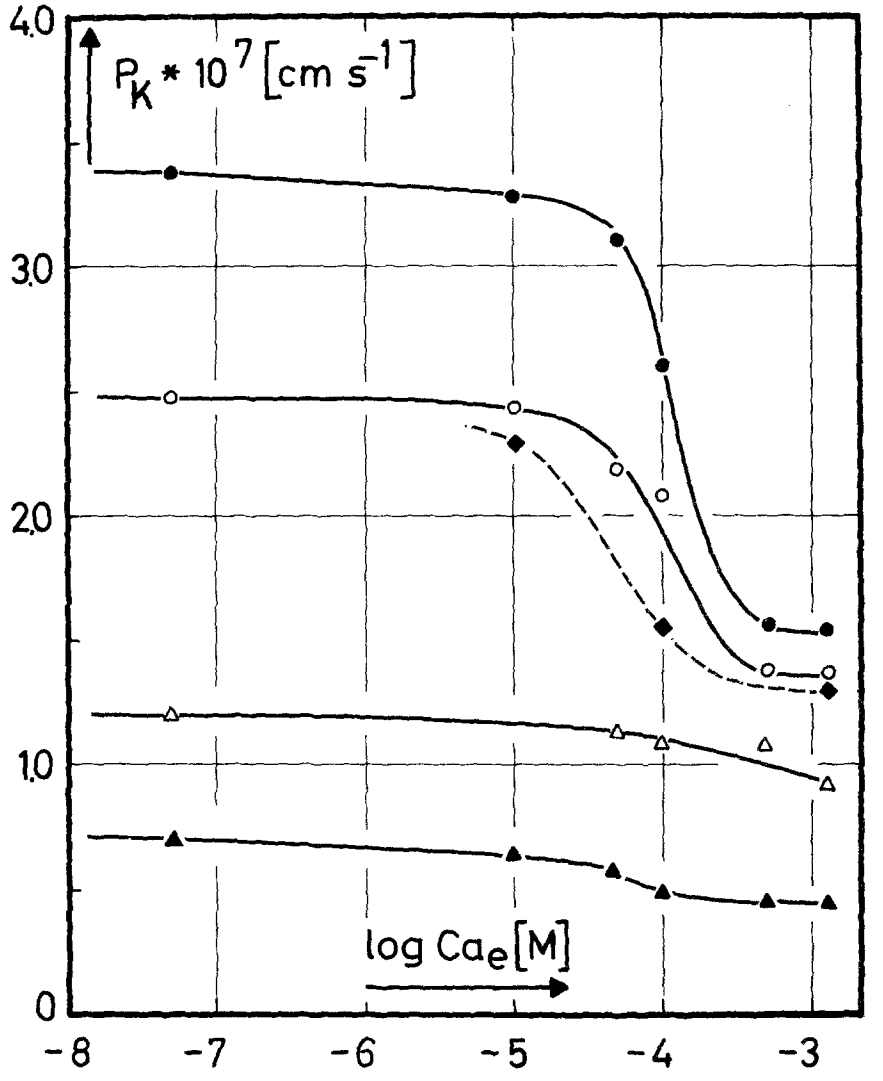

Fig. 10. K permeabilities P_K versus external Ca concentrations. The permeability values were evaluated from experimental data using Eq. (13). Curve parameter incubation temperature in °C: ●, 39.5; ○, 37; △, 25; ▲, 0. K permeabilities in the presence of 10 mM ouabain at 37 °C were calculated according to Eqs. (14) and (15) and are presented by ◆

without surface potentials. Performing the iteration for the other Ca_e values of interest we find that V_M (and thus $\Psi_i - \Psi_e$) is independent of Ca_e and leads to figures of P_K again by about 35% lower than without surface potentials. Thus, P_K for every Ca_e is changed by the same factor, but the relative shape of the dependence P_K on Ca_e remains unaffected.

As an independent check of the figures for P_K evaluated above and shown in Fig. 10, we have computed K permeabilities from cells at 37 °C, poisoned by 10 mM ouabain. As was mentioned before, cells treated with 10 mM ouabain are not in steady state of ionic concentrations. Therefore, we have to compute V_M from the complete GHK relation

$$V_M = \frac{RT}{F} \ln \frac{K_e + \alpha^* Na_e + \beta Cl_i}{K_i + \alpha^* Na_i + \beta Cl_e}. \tag{14}$$

Here $\beta = P_{Cl}/P_K$ and $\alpha^* = P_{Na}/P_K$, since any electrogenic contribution of the Na/K-ATPase is lost because of the inhibition yielding $r = 1$. Although the evaluation results in P_K practically independent of α for $0.2 \leqq \alpha \leqq 0.5$, for consistency we shall use $\alpha^* = 0.25 \cdot r = 0.375$ (where $r = 1.5$ was assumed for unpoisoned cells). The parameter β for unpoisoned cells will be determined below as $\beta = 1.45$. Starting with these figures, α^* and β, we compute P_K from influx measurements according to:

$$P_K = \frac{\vec{\Phi}_K (e^{-u} - 1)}{-u K_e e^{-u}} \tag{15}$$

where $u = FV_M/RT$.

The iteration procedure again converges rapidly and yields the figures of P_K for ouabain-treated cells as shown in Fig. 10. Evidently, these results coincide within experimental error with the corresponding K permeabilities of unpoisoned cells, thus confirming the previous evaluation. Membrane potentials for ouabain-treated cells evaluated by this procedure are given in Table 3 and again are in close agreement with chloride equilibrium potentials of these cells.

Chloride permeabilities P_{Cl} in dependence of Ca_e at 37 °C have been computed from membrane potentials of unpoisoned cells and from Cl influx

Table 3. Membrane potential V_m and equilibrium potential of chloride E_{Cl} in the presence of 10 mM ouabain in liver cells at 37 °C

Calcium (mM)	V_m (mV)	E_{Cl} (mV)
1.2	−4.5	−4.1
0.1	−3.6	−3.2
0.01	−5.0	−2.8

Table 4. Influence of the medium calcium concentration on the chloride influx Φ_{Cl} and the chloride permeability P_{Cl}

Calcium (mM)	Φ_{Cl} [a] (pmol cm^{-2} s^{-1})	P_{Cl} *10^7 (cm s^{-1})
1.2	11.1±0.4	1.98
0.5	10.5±0.4	1.96
0.01	12.8±0.6	2.29

[a] Values are the mean ±SEM of four experiments.

data shown in Table 4. Again the iteration procedure converges rapidly and within experimental error yields P_{Cl} independent of Ca_e (*see* Table 4). From $P_{Cl} = 1.98 \times 10^{-7}$ cm s^{-1} and $P_K = 1.36 \times 10^{-7}$ cm s^{-1} as determined above for unpoisoned cells at 37 °C, we obtain $\beta = P_{Cl}/P_K = 1.45$, which is numerically very similar to the figure $\beta = 1.6$ obtained by Claret and Mazet (1972). The absolute figures for P_K and P_{Cl} given above are larger by a factor of almost two than those obtained by Claret and Mazet (1972) from experiments on perfused rat liver. This difference is to be attributed in essence to our K^+ fluxes being about two times larger than those evaluated for perfused rat liver. We feel that this discrepancy stems from difficulties in determination of the effective cellular surface participating in K fluxes in perfused rat liver.

Discussion

First we shall discuss the temperature dependence of passive K permeability. As pointed out above, the ratio $\alpha = P_{Na}/(r \cdot P_K) = 0.25$ for unpoisoned cells at $Ca_e = 1.2$ mM and 37 °C is well substantiated from previous work of different authors. However, very little is known about this quantity at other temperatures. In the above evaluation of P_K, we have taken α at $Ca_e = 1.2$ mM as independent of temperature. Thus the temperature dependence of P_K at $Ca_e = 1.2$ mM shown in Fig. 10 is arbitrary. However, the quantity $\Delta P_K = P_K(Ca_e) - P_K(Ca_e = 1.2\,\text{mM})$ is a meaningful parameter for description of the temperature dependence of P_K at any calcium concentration different from 1.2 mM. According to the data of Fig. 10, ΔP_K at $Ca_e = 5 \times 10^{-8}$ M increases by a factor of 6.8 if temperature is raised from 0 ° to 39.5 °C. In contrast, the parameter $P_K(Ca_e = 5 \times 10^{-8}\,\text{M})/P_K(Ca_e = 1.2\,\text{mM})$ is increased by a factor of only about 1.3 by the same rise in temperature. A similar situation pertains to other $Ca_e \neq 1.2$ mM.

Thus, to a first approximation P_K may be split into a factor $A(Ca_e)$, which is only dependent on Ca_e, and a factor $B(T)$, which is only dependent on temperature, yielding $P_K = A(Ca_e) \cdot B(T)$. For fixed Ca_e, the temperature dependence $B(T)$, therefore, may be evaluated from that of ΔP_K as follows. Writing $B(T) = B_0 \exp\{-E_a/kT\}$, one obtains from the data of Fig. 10 for $Ca_e \leqq 0.1$ mM:

3.6 kcal/mol $\leqq E_a \leqq$ 10.9 kcal/mol for temperatures between 0° and 25 °C;

30.7 kcal/mol $\leqq E_a \leqq$ 50.2 kcal/mol for temperatures between 37° and 39.5 °C.

The activation energies E_a in the range 0° to 25 °C are of the order of those for diffusion coefficients of potassium ions in aqueous solutions: 2.8 kcal/mol $\leqq E_a \leqq$ 7.8 kcal/mol between 4° and 25 °C (Landolt-Börnstein, 1969).

For a tentative interpretation of the above figures, the membrane structures determining P_K below 25 °C may be considered as essentially frozen into a state with only a limiting number of K channels open, each of which allows transit of K^+ ions essentially as in free solution. At higher temperatures, structural rearrangements may take place in the membrane which lead to an opening up of more K^+ channels and, thus, to an increase in P_K. As suggested by the fairly large activation energies above 37 °C, these structural rearrangements in the membrane may involve cooperative interactions between membrane subunits. This interpretation is supported and further developed by the discussion of the Ca dependence of P_K.

The following causes might be invoked to interpret the dependence of P_K on Ca_e and will be discussed in this order: *i*) regulation of P_K via increased Ca_i due to Ca influxes depending on Ca_e; *ii*) regulation of P_K by electrostatic effects of divalent cations on the electric surface potential of the cells; *iii*) regulation of P_K by cooperative cation-exchange on regulatory subunits within the membrane.

i) Intracellular Ca contents have been found to be very low ($\lesssim 10^{-6}$ M) in different types of cells (Borle, 1973). Intracellular Ca homeostasis has been demonstrated to be mainly achieved by the action of mitochondria as a buffering and trapping compartment for Ca; transport of Ca across the plasma membrane was shown to be only a minor contribution to adjustment of Ca_i (Borle, 1973). Regulation of Ca_i by mitochondria appears to be particularly efficient in rat liver cells, where about 25% of the intracellular volume is occupied by mitochondria, displaying an enormous membrane surface area for binding and transport of Ca. Respiratory activity of isolated rat liver mitochondria of citrate-dispersed

(i.e. damaged) rat hepatocytes depends strongly on external Ca concentration. In contrast, respiratory activity of our enzymatically dispersed rat hepatocytes does not show any dependence on Ca_e between 10^{-5} and 1.2×10^{-3} M. In conclusion it appears very improbable that Ca_i in our preparation can be varied appreciably by Ca_e, which in turn argues against a regulation of P_K via Ca_i.

ii) From the above microcell electrophoresis data a ζ potential of -20 mV was evaluated for rat liver cells at 37 °C and $Ca_e < 5$ mM. McLaughlin, Szabo and Eisenman (1971) had proposed a mechanism whereby divalent cations exert their effect on ion permeability by changing the Gouy-Chapman potential of the electrolyte surface layer of the cells. However, according to our cell-electrophoresis data the ζ potential does not change in the region of Ca_e where drastic changes in P_K are observed. Furthermore, the electrostatic effect on the Gouy-Chapman potential should not depend on the particular species of divalent cations used, if equal concentrations are applied. However, as reported under Results and discussed in more detail later, Ca_e has by far the largest effect on P_K among the earth alkali ions tested; for instance Mg_e exhibits only about one-tenth of the activity of Ca_e. In addition, the steep dependence of P_K on Ca_e is not compatible with a purely electrostatic effect of Ca on the Gouy-Chapman boundary layer, as described by McLaughlin *et al.* (1971); thus this mechanism does not appear to account for the observed regulation characteristics of passive K transport in rat hepatocytes as was pointed out by Kolb and Adam (1973, 1974).

iii) Interaction of divalent cations with membrane structures determining K permeability might involve binding to regulatory sites. We have therefore applied the membrane model given earlier (Adam, 1967, 1968, 1970*a*) to a description of the present experimental data. This model of the plasma membrane proposed:

a) the existence of regulatory sites in the membrane, to which either a divalent cation or two monovalent cations may be bound, with the possibility of an exchange between both states of binding;

b) interactions between the regulatory sites rendering the above cation-exchange a cooperative process;

c) dependence of the cation permeability on the average binding state of the regulatory site. In particular, a one-to-one correspondence was proposed for an open cation-conducting "channel" with a regulatory site occupied by two alkali ions and for a closed "channel" with a site occupied by a divalent cation;

d) an asymmetric fixed-charge configuration of the two faces of the membrane.

In the following, we shall not use specifically postulate *d*) of the model; the corresponding theoretical parameters are, thus, not formulated explicitly. Postulate *c*) of the model also is formulated unnecessarily restrictively above, as a "channel" might be regulated by several regulatory sites. For sake of concreteness of the model, however, we shall use this postulate as stated above and, thus, assume P_K as proportional to the fraction n of regulatory sites occupied by two alkali ions. In addition, we assume that interaction of the regulatory site can occur only with cations from the outside electrolyte reservoir. The affinity of the regulatory site for Na_e is considered to be negligible, compared to that for K_e.

With these assumptions, the steady state of n is described by (Adam, 1968, 1970*a*):

$$\frac{n}{1-n} e^{\frac{wn}{kT}} = Q(T) \frac{K_e^2}{Ca_e + \gamma_Z \cdot Z_e}. \tag{16}$$

Here, the molecular field approximation for interaction between regulatory sites was used, the parameter $w<0$ describing positive cooperativity of the system. The quantity $Q(T)$ contains rate constants of the cation exchange and parameters concerning structural rearrangements in the membrane. Z_e is the concentration of a second divalent cation in addition to Ca_e in the extracellular medium. The parameter γ_Z describes the affinity of the regulatory site to divalent cation species Z relative to Ca and thus is a measure of the selectivity of the site.

According to postulate *c*) of the model we write

$$n = \frac{P_K - P_{K_0}}{P_{K_\infty} - P_{K_0}} \tag{17}$$

where in our case $P_{K_0} = P_K(Ca_e = 1.2\ \text{mM})$ and $P_{K_\infty} = P_K(Ca_e = 5 \times 10^{-8}\ \text{M})$ may be used.

In Fig. 10, we have plotted our experimental data on P_K at 37 °C, normalized according to Eq. (17), versus negative decadic logarithm of Ca_e. For comparison with theory, we have plotted also Eq. (16) using $K_e = 6$ mM, $Z_e = 0$, as given by the experimental conditions, and the theoretical parameters $w/kT = -3.2$ and $Q(T) = 0.47\ \text{M}^{-1}$. As is evident from Fig. 10, the experiments are well described by the theory. Any theoretical description using finite Ising-lattices (Hill & Chen, 1971) or a higher approximation for treatment of cooperative cation exchange on an infinite lattice (Adam, 1973) would give essentially the same result and is not attempted here in view of the lack of knowledge on details of

hepatocyte membrane structure. We may use Eq. (16) to describe the effect of different divalent cations shown in Tables 1 and 2.

From the above comparison of n as a function of Ca_e with theory, we may correlate $n=1/2$ at $\overline{Ca_e} \approx 10^{-4}$ M with $K_i \approx 100$ mM. According to Table 1, at $\overline{Mg_e} = 1.2$ mM this condition of $n=1/2$ is reached approximately with $K_i = 103.4$ mM. Here, and in the following, the bar indicates an abscissa for $n=1/2$. Thus, Eq. (16) with $n=1/2$ leads to $Q(T) \cdot K_e^2 \cdot e^{-w/2kT} = \overline{Ca_e} \approx \gamma_{Mg} \cdot \overline{Mg_e}$.

With the above figures, this relation yields $\gamma_{Mg} \approx 0.07$. This estimate may be checked by data given in Table 2, where $K_i = 104.6$ mM, i.e. $n \approx 1/2$, is reached at $\overline{Mg_e} = 1.2$ mM and $\overline{Ca_e} = 10^{-5}$ M, yielding $\gamma_{Mg} \approx 0.08$. For strontium an analogous estimate can be made with $K_i \approx 100$ mM for $Sr_e \approx 0.3$ to 0.5 mM, whence $\gamma_{Sr} \approx 0.2$ to 0.3. For Ba only an upper limit $\gamma_{Ba} < 0.07$ may be given. On the basis of the above evaluation of the effects of different divalent cations on P_K, the following sequence of affinity parameters of the regulatory site may be given: $\gamma_{Ca} > \gamma_{Sr} > \gamma_{Mg} > \gamma_{Ba}$.

Using Eq. (16), one may predict from our data on the dependence of P_K on Ca_e at fixed K_e the outcome of an experiment determining the dependence of P_K on K_e at fixed divalent cation concentrations. This latter type of experiment was done recently by Mazet *et al.* (1974), who used $Ca_e = 2.2$ mM and $Mg_e = 1.2$ mM. With these divalent cation concentrations and the theoretical parameters evaluated above, i.e. $w/kT = -3.2$, $Q(T) = 0.47\ M^{-1}$ and $\gamma_{Mg} = 0.07$, the theoretical curve n versus $2 \log K_e$, as shown in Fig. 11 may be computed (actually the abscissa $2 \log K_e$ is fixed by Eq. (16) relative to the abscissa $-\log Ca_e$). For a check by experimental results, we have normalized the data of Table 2 of Mazet *et al.* (1974) according to Eq. (17) with $P_{K_0} = P_K(K_e = 5.6$ mM) and $P_{K_\infty} = P_K(K_e = 112$ mM) and plotted the normalized data versus $2 \log K_e$ in Fig. 1. As is evident from Fig. 11, there is a remarkable agreement between the two sets of experimental data, indicating that both represent the same basic regulation mechanism.

The fairly large figure of $P_{K_\infty}(K_e = 112\ mM) = 9.1 \times 10^{-7}$ cm s^{-1} obtained by Mazet *et al.* (1974) is not incompatible with our $P_{K_\infty}(Ca_e = 5 \times 10^{-8}\ M) = 2.47 \times 10^{-7}$ cm s^{-1}, because the K conductance of an open "channel" may well be expected to depend on K_e. In Fig. 10, we have presented data showing that P_K at 37 °C for ouabain-treated and untreated cells coincide within experimental precision, although K_i in ouabain-treated cells is lower by a factor of about 10 than in untreated cells. This indicates that P_K does not depend on K_i, a feature which was anticipated already in Eq. (16).

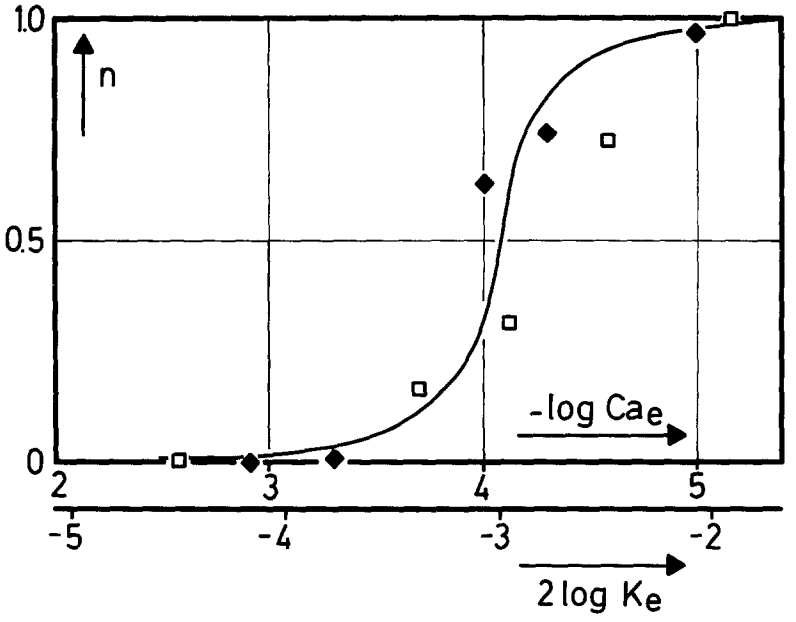

Fig. 11. Comparison of normalized K permeabilities as a function of external calcium and potassium at 37 °C, respectively. The normalized values n were calculated as described according to Eq. (17). ◆, normalized K permeability as a function of external Ca. Data were taken from Fig. 10. □, normalized K permeability as a function of K_e. Data were taken from Table 2 of Mazet *et al.* (1974). The theoretical curve has been drawn according to Eq. (16) with $w/kT = -3.2$

In conclusion, the mechanism of a cooperative cation exchange as formulated in Eq. (16), describes well the regulation of P_K by external concentration of divalent cations, as well as by external K concentration, and the absence of an effect of the internal K concentration.

There is available at present only slight evidence on the molecular nature of the regulatory site. From the theoretical description given above, one may surmise that negatively charged groups are an essential part of the regulatory cation-binding site. These groups may be located on membrane proteins or membrane lipids. Some evidence points to charged phospholipids in the lipid matrix of the plasma membrane as relevant molecular species involved in the regulation processes discussed above.

From work of Dod and Gray (1968) it is known that 12% of the phospholipids of rat liver plasma membranes have a net charge (6% phosphatidylserine and 6% phosphatidylinositol). Experiments on lamellar phospholipid model systems have shown that phase transitions (Träuble & Eibl, 1974) or phase separations (Ohnishi & Ito, 1973) may be induced by changing divalent cation content of the suspension media. Our results

shown in Fig. 7 show a broadening effect of preincubation of the cells with cholesterol on the Ca_e dependence of K permeation. This effect is consistent with a phospholipid nature of the regulatory site, as cholesterol in phospholipid model systems also has a broadening effect on thermal transitions (Chapman, 1973).

Further work is aimed, therefore, at a more detailed characterization of the molecular interactions in lamellar lipid systems (Adam, 1973) and of the physical state of the plasma membrane lipids as a prerequisite for molecular interpretation of regulation of cation transport in the multicomponent protein-lipid system of the plasma membrane.

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