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Regulation of Passive Potassium Transport of Normal and Transformed 3T3 Mouse Cell Cultures by External Calcium Concentration and Temperature †

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Summary. Regulation of passive potassium ion transport by the external calcium concentration and temperature was studied on cell cultures of 3T3 mouse cells and their DNA-virus transformed derivatives. Upon lowering of external calcium concentration, passive potassium efflux generally exhibits a sharp increase at about 0.1 mM. The fraction of calcium-regulated potassium efflux is largely independent of temperature in the cases of the transformed cells, but shows a sharp increase for 3T3 cells upon increasing temperature above 32 °C. In the same range of temperature, the 3T3 cells exhibit the phenomenon of high-temperature inactivation of the residual potassium efflux at 1 mM external calcium. At comparable cellular growth densities, the transformed cell lines do not show high-temperature inactivation of "residual" potassium efflux. These results are consistent with the notion of a decisive role of the internal K^+ concentration in the cell-density dependent regulation of cell proliferation. In particular, the growth-inhibiting effect of lowering the external Ca^{2+} concentrations is considered as largely due to a rise of passive K^+ efflux and a subsequent decrease of internal K^+ concentration. The experimental data on the Ca^{2+} dependence of passive K^+ flux are quantitatively described by a theoretical model based on the constant field relations including negative surface charges on the external face of the membrane, which cooperatively bind Ca^{2+} ions and may concomitantly undergo a lateral redistribution. The present evidence is consistent with acidic phospholipids as representing these negative surface charges.

Key words: K^+-transport, Ca^{2+}-effects, 3T3 cells, cell proliferation

Primary processes in the chain of events leading to stimulation or inhibition of cell proliferation by external agents or cellular growth density are occurring in the plasma membrane. Since the pioneering study of Lubin (1967) a large number of investigations have provided ample evidence that transport of monovalent cations plays a major role in regulation of cell proliferation. The cells studied most completely in this regard are mouse 3T3 cells. Sutherland (1972) has formulated four criteria, which in a first survey have to be met as a necessary condition if a substance is to be considered a second messenger in humoral regulation. These criteria of Sutherland are a convenient basis for a discussion of a possible role of the intracellular K^+ concentration as a second messenger in growth regulation. In fact, putting together the results on active K^+ transport (Elligsen, Thompson, Frey & Kruuv, 1974; Kasarov & Friedman, 1974; Rosengurt & Heppel, 1975; Kimelberg & Mayhew, 1975, 1976; Banerjee & Bosmann, 1976; Bourne & Rosengurt, 1976; Spaggiare, Wallach & Tupper, 1976; Tupper, Zorgniotti & Mills, 1977), as well as on passive K^+ transport and on intracellular K^+ content of normal and transformed cells (Cone & Tongier, 1974; Rosengurt & Heppel, 1975; Pollack & Fisher, 1976; Ernst & Adam, 1978, 1979; Adam, Ernst & Seher, 1979; Ledbetter & Lubin, 1979), evidence may be presented complying with all four criteria of Sutherland, if formulated for the intracellular K^+ concentration as a second messenger with regard to cell-density dependent growth regulation (for a discussion of much of the evidence *see* Adam et al., 1979). Apparently, this conclusion cannot be extended to the situation of growth inhibition by serum deficiency (Tupper & Zografos, 1978).

† This work is dedicated to the memory of Max Delbrück (deceased March 10, 1981), in whose laboratory in 1966 the earlier version of the present theoretical model was developed by one of the authors.

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A possible role of intracellular K^+ concentration and/or K^+ transport in growth regulation is also indicated by results on a large number of other cell types: sarcoma 180 cells (Lubin, 1967), lymphocytes (Kaplan, 1977), Girardi cells (Lamb & McCall, 1972), baby hamster kidney (BHK) cells (McDonald, Sachs, Orr & Ebert, 1972), L-cells (Quissel & Suttie, 1973), mouse lymphoblasts (Shank & Smith, 1976), and human fibroblasts (Ledbetter & Lubin, 1977, 1979).

More recent work has pointed to a specific role of Na^+ transport in hormone- or serum-dependent growth stimulation of quiescent 3T3 and other cells (Smith & Rosengurt, 1978a, b; Adam et al., 1979; Koch & Leffert, 1979; Rosengurt, Legg & Pettican, 1979).

On the other hand, the external Ca^{2+} concentration has been shown to affect specifically the growth behavior of 3T3 cells (Balk, 1971; Dulbecco & Elkington, 1975; Boynton & Whitfield, 1976a, b; Rubin, Terasaki & Sanui, 1978, 1979; van der Bosch, Sommer, Maier & Rahmig, 1979; Paul & Ristow, 1979) and other cell types (Whitfield, Rixon, Perris & Youdale, 1969; Balk, Whitfield, Youdale & Braun, 1973; Frank, 1973; Rixon & Whitfield, 1976; Swierenga, MacManus & Whitfield, 1976; Whitfield, MacManus, Rixon, Boynton, Youdale & Swierenga, 1976; Boynton, Whitfield, Isaacs & Tremblay, 1977; Rubin et al., 1978; Balk, Polimeni, Hoon, LeStourgeon & Mitchell, 1979; Hazelton, Mitchell & Tupper, 1979). Furthermore, the content, distribution, transport and exchange of Ca^{2+} in normal and transformed 3T3 mouse cells shows specific features which are consistent with a membrane-related function of surface-membrane localized Ca^{2+} in cell-density dependent regulation of growth (Tupper & Zorgniotti, 1977; Tupper, Del Rosso, Hazelton & Zorgniotti, 1978; Vannucci, Del Rosso, Cella, Urbano & Chiarugi, 1978; Hazelton & Tupper, 1979; Sanui & Rubin, 1979).

A possible link between the effects of external Ca^{2+} concentration and K^+ transport on cell proliferation is suggested by experimental results indicating that external Ca^{2+} ions are intrinsically connected with the mechanism of regulation of (passive) monovalent cation transport. It has been shown for squid axon (Hodgkin & Keynes, 1957), frog skin (Curran, Herrera & Flanigan, 1963), liver cells and salivary gland cells (Loewenstein, 1967), kidney cortex (Kleinzeller, Knotkova & Nedvidkova, 1968), liver (Geyer, Sholtz & Bowie, 1955; Kalant & Hickie, 1968; Gilbert, 1972; Kolb & Adam, 1976), 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 and/or membrane potential. For isolated rat-liver cells the dependence of passive K^+ permeability on external Ca^{2+} concentration and temperature has been investigated in considerable detail (Kolb & Adam, 1976), exhibiting a fairly sharp decrease with increasing external Ca^{2+} concentration at about 10^{-4} M if temperature is at or above 37 °C.

As the dependence of cell proliferation on external Ca^{2+} concentration and on K^+ transport has been studied most closely for 3T3 cells and their transformed derivatives, it appeared most fruitful to study the relation between external Ca^{2+} concentration and passive K^+ transport on normal and transformed 3T3 cells in a detail comparable to the study on liver cells (Kolb & Adam, 1976). Such an analysis as shown in the following does not only give valuable insights into the mechanism(s) of Ca^{2+}-dependent growth regulation of normal cells, but also is of considerable interest with regard to different Ca^{2+}-dependent growth characteristics for transformed cells (van der Bosch et al., 1979). In particular, the validity and possible generalization of the proposal of Gilbert (1972) of a higher affinity of receptor sites in the membrane of transformed cells compared to normal as determining their different dependence of cellular K^+ content on external Ca^{2+} concentration could be checked and answered negatively.

Furthermore, this investigation contributes to elucidation of the basic mechanism(s) of regulation of passive K^+ transport by external Ca^{2+} concentration in mammalian cells, as it yields very steep Ca^{2+} characteristics as in the case of liver cells (Kolb & Adam, 1976), which suggest a regulation mechanism involving a cooperative binding of Ca^{2+} in the membrane and concomitant lateral redistribution of its (negatively) charged constituents.

Materials and Methods

Cell Preparations

Stocks of Swiss 3T3, Polyoma-3T3 (PY-3T3), and SV40-3T3 (line 101) cells were kindly supplied by Prof. M.M. Burger, Basel. Cells were maintained antibiotic-free at 37 °C on 95 mm plastic tissue-culture plates (Greiner, Nuertingen) in Dulbecco's modification of Eagle medium supplemented with 10% heat-inactivated newborn-calf serum in a moist atmosphere containing 10% carbon dioxide. Media and supplements were obtained from Flow Laboratories, Bonn.

Cells were seeded at a density of $1–3 \times 10^3$ cm^{-2} and the stem cultures passaged every 4 days. Growth medium was changed three times weekly. Measurements were made only 24 hr at least after a change of growth medium. Final cell densities of the cell lines used were $2–3 \times 10^5$ cm^{-2} for SV40-3T3, $1–2 \times 10^5$ cm^{-2} for PY-3T3, and $2–4 \times 10^4$ cm^{-2} for 3T3 cells.

Measurement of Cell Surface Area

Cell surface areas were determined essentially as described by Seher and Adam (1978). Briefly, micrographs of cells still attached to the petri plate but with the growth medium replaced by tris-buf-

fered Earle's solution (in mM: 116 NaCl, 5.4 KCl, 0.9 $NaH_2PO_4 \cdot H_2O$, 5.1 glucose, 0.14 phenol red, 20 Tris-HCl, pH 7.4) were taken at different cell densities using a Zeiss microscope IM35 with phase contrast optics at a total magnification of 800×. Outline areas of the cells were determined with a mechanical planimeter and calibrated using micrographs of the grid of a Neubauer hemocytometer. The cell-outline areas of 10–40 micrographs of different cells were determined for each independent preparation (petri plate). Cell-surface area was evaluated as twice the outline area. As checked by determination of cell heights using the micrometer of fine focus adjustment, while observing cells grown on glass microscope coverslips under differential interference contrast (Nomarski optics), contributions to cell-surface area arising from the vertical dimensions of the cells amount to only 5–15% (Seher & Adam, 1978; and *in preparation*) and can be neglected for the purposes of the present paper, in particular as the cell-surface areas turned out to be independent of Ca^{2+} concentration and cell growth density.

Measurement of Passive Potassium Efflux

In order to minimally perturb the physiological state of the cells growing on the culture substrate and in view of its considerable expediency for the large number of flux determinations involved, potassium transport was measured with ion-specific electrodes, recording K^+ activity directly above the cells during incubation with a modified Ringer's solution containing 0.1 mM K^+ (Adam et al., 1979). Low external potassium concentration in the incubation medium served two purposes: *i*) to provide for an electrochemical gradient of potassium across the cell membrane leading to net K^+ efflux, and *ii*) to practically eliminate active potassium influx. Inactivation of active K^+ influx at external K^+ concentration of about 0.1 mM has previously been demonstrated for the same cell types (Rosengurt & Heppel, 1975).

For an efflux experiment, growth medium was removed from the petri plate, and the cell sheet rinsed twice with 5 ml of modified Ringer's solution (MRS) used for measurement (144 mM NaCl, 0.1 mM KCl, 10 mM triethanolamine, $CaCl_2$ concentration adjusted as required, pH 7.4). Thereafter, the cell sheet, remaining on the petri plate, was incubated with 10 ml MRS and the potassium efflux from the cells recorded continuously with an ion-specific electrode (Philips model IS 561 K, Philips Elektronik-Industrie, Hamburg), containing a sensing membrane dotted with valinomycin. The reference electrode (double junction, model 90-02, Orion Research, Cambridge, Mass.) was connected to the measuring solution by a reference salt bridge (model 563853 from Beckmann Instruments, Muenchen) filled with 10% CsCl solution, thereby preventing any leakage of potassium from the reference electrode to the incubation solution. The electrodes were connected to a millivoltmeter with high input impedance (model E500 from Metrohm, Herisau, Switzerland; or model 640 from Knick, W. Berlin) and the observed potential difference recorded with a potentiometer recorder (model Servogor RE571, Goerz-Electro, Wien). The petri plate was thermostated during incubation in an aluminum block, the whole assembly including electrodes being agitated at about 15 cycles/min and maximum inclination of two degrees on a gyratory moving table (model Reax 3 from Heidolph, Kelheim).

Potassium efflux ϕ_K in pmol cm^{-2} sec^{-1} was evaluated from the initial slope (dE/dt) of recorded difference E of the electrical potential between potassium electrode and reference electrode versus time t using the relation (Cammann, 1973):

$$\phi_K = \frac{2.303}{SAN} V K_{eo} \left(\frac{dE}{dt}\right)_{t\ 0} \quad (1)$$

where $V = 10$ ml is the extracellular volume, $K_{eo} = 0.1$ mM is the initial K^+ concentration of the incubation solution, A is the cell-surface area (determined as described above), N is the number of cells per plate and was determined by electrical counting (model *ZF*, Coulter Electronics, Krefeld) after release of the cells from the plate in Ca-free MRS with 0.25% trypsin, this procedure following each experiment. The response characteristics S of the electrode assembly may be expressed in $mV/\log(K_{e1}/K_{eo})$. This quantity S was determined by calibration at $K_{eo} = 0.1$ mM and $K_{e1} = 0.5$ or 1.0 mM in MRS under the same conditions before and after each experimental recording, and at 25 °C was in the range of 52 to 59 mV. The response of the electrode assembly proved to be very reproducible and fast (response time ≈1–2 min). As was obvious from the calibration runs for determination of S, any efflux of Cs^+ from the salt bridge turned out to be negligible as, in fact, could be shown even for a salt bridge filled with KCl.

By incubation for various intervals in the absence of the potassium electrode and comparison with the result of continuous presence of the electrode, we have ascertained that any effect on the results of leakage of valinomycin from the potassium electrode can be excluded.

As an additional corroboration to earlier results on inactivation of active K^+ uptake of 3T3 cells (Rosengurt & Heppel, 1975) and other cells (Garrahan & Glynn, 1967; Ducouret-Prigent, Lelievre, Paraf & Kepes, 1975) under the conditions of the present experiments ($K_e \approx 0.1$ mM), we have measured K^+-effluxes after preincubation of 3T3 or SV40-3T3 cells for 10 or 30 min at 37 °C in Ringer's solutions containing 5–150 mM K^+ with or without 1–3 mM ouabain, and within an experimental error of 10–15% for individual measurements could not detect any difference in the subsequent K^+ efflux into MRS with or without 1–3 mM ouabain, respectively.

Temperatures were accurate within ±0.5 °C, as determined by Pt100 resistance thermometer.

Further details on the method of K^+-efflux experiments with ion-specific electrodes are given by Adam et al. (1979).

K^+-efflux measurements for each temperature were all done from one seeding on one day, each Ca^{2+} concentration being measured in triplicate, i.e. by three independent recordings from a separate cell preparation (petri dish) each. Results on efflux measurements are given as the arithmetic mean ± standard error of the mean.

Results

Growth Parameters and Cell Surface Area

As shown in our earlier work (Adam et al., 1979), passive K^+ effluxes of 3T3 and SV40-3T3 cells depend markedly on cell growth density. By a specified growth procedure, we have, therefore, adjusted cell densities to prescribed figures as closely as practicable. For 3T3 cells, we used a cell density of $(3.5 \pm 0.4$ SD$) \times 10^4$ cm^{-2}, for PY-3T3 cells of $(8.2 \pm 1.1$ SD$) \times 10^4$ cm^{-2}, and for SV40-3T3 cells of $(5.2 \pm 0.7$ SD$) \times 10^4$ cm^{-2}. The figure for 3T3 cells is near saturation density of the cell monolayer, and for the transformed cells at least approximate a monolayer covering the culture plate.

Cell surface areas determined according to the procedure outlined under Materials and Methods are given in Table 1. It is found that the cell-surface area of PY-3T3 cells is less than half of the cell-surface area of 3T3 cells. This confirms for another transformed derivative of 3T3 cells a finding made earlier for SV40-3T3 cells (Seher & Adam, 1978).

Table 1. Cell growth densities ± standard deviation and average cell surface areas ± standard error of the mean (number of independent preparations in parentheses) for the cell lines investigated

Cell line	3T3	PY-3T3	SV40-3T3
Average cell density			
$N/10^4$ cm^{-2} ± SD	3.5 ±0.4	8.2 ±1.1	5.2 ±0.7
Cell surface area			
$A/10^{-5}$ cm^2 ± SEM	4.53±0.14 (43)[a]	1.92±0.07 (9)	1.68±0.06 (9)[a]

[a] Taken from Seher and Adam (1978).

Table 2. Arrhenius parameters describing temperature dependence of basic passive K^+ efflux ϕ_K^1 (at $Ca_e = 1$ mM) and of the extent $\Delta\phi_K^0$ of Ca^{2+}-dependent passive K^+ efflux, using Eqs. (2) and (5), respectively

Cell line	$\log \frac{A_\phi^1}{\text{pmol cm}^{-2}\text{ sec}^{-1}}$	$\frac{E_\phi^1}{\text{kJ mol}^{-1}}$	$\log \frac{A_{\Delta\phi}^0}{\text{pmol cm}^{-2}\text{ sec}^{-1}}$	$\frac{E_{\Delta\phi}^0}{\text{kJ mol}^{-1}}$
3T3	10.7	59	13.8	77
PY-3T3	9.4	49	9.1	48
SV40-3T3	9.4	51	12.4	69

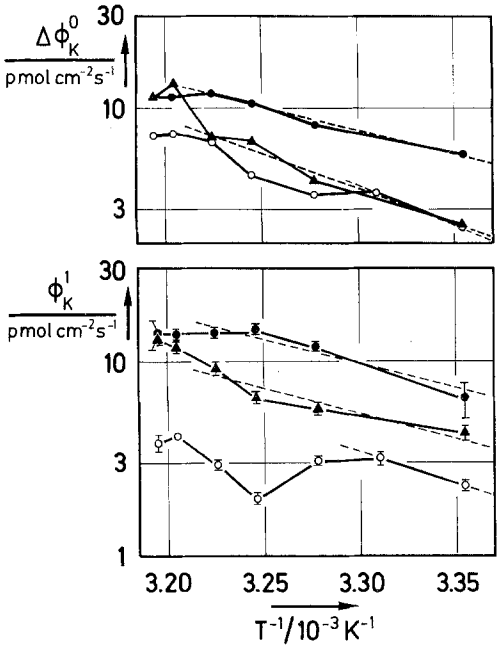

Fig. 1. Components of K^+ efflux versus reciprocal temperature for 3T3 (○), PY-3T3 (●), and SV40-3T3 cells (▲). *Upper part:* Extent $\Delta\phi_K^0$ of K^+ efflux which is regulated by external Ca^{2+} concentration. This quantity $\Delta\phi_K^0$ was obtained from the fit of Eqs. (3) and (4) to the data given in Fig. 2–4. *Lower part:* Basic K^+ efflux ϕ_K^1 at 1 mM external Ca^{2+} concentration (error bars indicate standard error of the mean from three independent experiments). Broken curves indicate fit of the Arrhenius relations Eqs. (2) and (5) for $T < 39$ °C (transformed cell lines) and $T < 32$ °C (3T3 cells); parameters of the fit are given in Table 2

Temperature Dependence of Passive K^+ Flux at $Ca_e = 1$ mM

The dependence on temperature of passive K^+ fluxes at an external Ca^{2+} concentration of $Ca_e = 1$ mM is given in Fig. 1, lower part. As is evident from this Figure (broken lines), the logarithm of K^+ efflux of the transformed cell lines at temperatures $T \leqq 37$ °C within experimental error depends linearily on reciprocal temperature. An activation energy of about $E_\phi^1 \doteq 50$ kJ mol^{-1} and a pre-exponential factor of about $\log (A_\phi^1/\text{pmol cm}^{-2}\text{ sec}^{-1}) = 9.4$ has been evaluated for the transformed cell lines at $T \leqq 37$ °C (*see* Table 2) using the Arrhenius relation:

$$\phi_K^1 = A_\phi^1 \exp\{-E_\phi^1/RT\}. \quad (2)$$

At temperatures $T \geqq 39$ °C there are deviations from this relation for transformed cells which appear to transcend experimental error but nevertheless are fairly small.

In contrast, 3T3 cells exhibit an N-shaped curve in its dependence of $\log \phi_K^1$ on $1/T$, which only at $T \leqq 29$ °C yields a slope $E_\phi^1 = 59$ kJ mol^{-1} comparable to that of transformed cells (Table 2). The N-shaped temperature characteristic for 3T3 cells was referred to in our earlier report as "high-temperature inactivation" of passive K^+ efflux (Ernst & Adam, 1978). This interesting phenomenon could be shown to be even more pronounced at lower cell growth densities, and at low cell growth densities it has been found to apply for SV40-3T3 cells also (Adam et al., 1979). High-temperature inactivation of passive fluxes is a characteristic opposite to that of low-temperature inactivation, as observed for active K^+ influx of 3T3 and SV40-3T3 cells (Kimelberg & Mayhew, 1975), for (Na+K)-activated ATPase (Priestland & Whittam, 1972; Kimelberg & Papahadjopoulos, 1972, 1974; Grisham & Barnett, 1973; Kimelberg, 1977) or for sugar transport into bacterial cells (Schairer & Over-

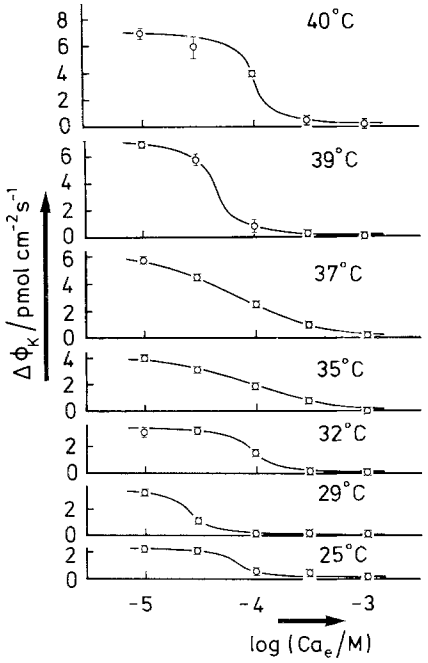

Fig. 2. Ca^{2+}-regulated component $\Delta\phi_K = \phi_K - \phi_K^1$ of K^+ efflux ϕ_K versus external Ca^{2+} concentration (on logarithmic scale) for 3T3 cells at different temperatures. Error bars indicate standard error of the mean from three independent experiments. Curves are computed from Eqs. (3) and (4) using figures w/kT, Q and $\Delta\phi_K^0$ as shown in Fig. 5

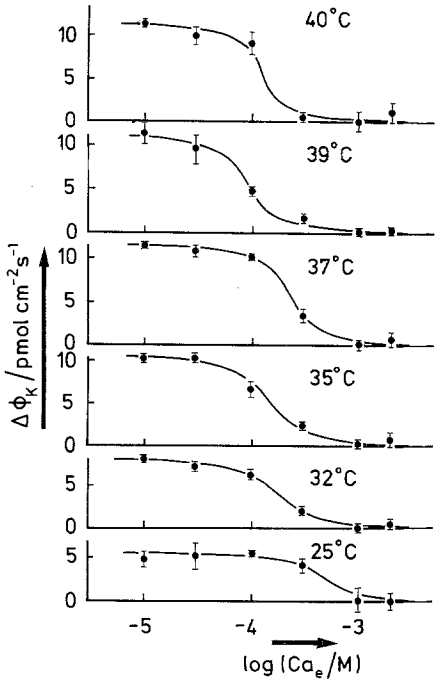

Fig. 3. Ca^{2+}-regulated component $\Delta\phi_K = \phi_K - \phi_K^1$ of K^+ efflux ϕ_K versus external Ca^{2+} concentration (on logarithmic scale) for PY-3T3 cells at different temperatures. Error bars indicate standard error of the mean from three independent experiments. Curves are computed from Eqs. (3) and (4) using figures w/kT, Q and $\Delta\phi_K^0$ as shown in Fig. 5

ath, 1969; Overath, Schairer & Stoffel, 1970; Wilson, Rose & Fox, 1970; Esfahani, Limbrick, Knutton, Oka & Wakil, 1972; Träuble & Overrath, 1973; Linden, Wright, McConnell & Fox, 1973; Thilo, Träuble & Overath, 1977).

Ca^{2+}-Dependence of Passive K^+ Flux at Different Temperatures

At fixed temperature, passive K^+ efflux of all cell lines studied here decreases monotonously if the external Ca^{2+} concentration Ca_e is raised, and eventually reaches a lower plateau at or near $Ca_e = 1$ mM. In order to arrive at a clear presentation of the considerable number of experimental data on the dependence of passive K^+ efflux on Ca_e and T, we have plotted in Figs. 2 through 4 the difference $\Delta\phi_K = \phi_K - \phi_K^\infty$ of the Ca_e-dependent K^+ efflux ϕ_K and its lower plateau ϕ_K^∞, which in most cases is given by ϕ_K^1, i.e. by the K^+ efflux at $Ca_e = 1$ mM, as shown in Fig. 1. Only for the figures $\Delta\phi_K$ of SV40-3T3 cells at and below 35 °C, as given in Fig. 4, we have used ϕ_K^∞ slightly lower than ϕ_K^1, as that characterizes the lower plateau more closely (*see* Fig. 4, lower curves).

The general characteristics of regulation of passive K^+ transport of all the cell lines investigated here

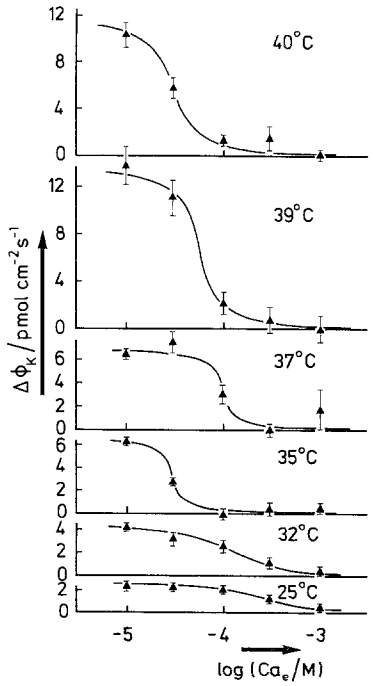

Fig. 4. Ca^{2+}-regulated component $\Delta\phi_K = \phi_K - \phi_K^\infty$ of K^+ efflux ϕ_K versus external Ca^{2+} concentration for SV40-3T3 cells at different temperatures. Error bars indicate standard error of the mean from three independent experiments. Curves are computed from Eqs. (3) and (4) using figures w/kT, Q, and $\Delta\phi_K^0$ as given in Fig. 5

resemble each other: a lower plateau at about $Ca_e \geqq 1$ mM, a more or less steep rise at about $Ca_e = 0.1$ mM to a higher plateau, which is attained at about $Ca_e = 0.1$ mM to a higher plateau, which is attained at about $Ca_e = 10^{-5}$ M. With rising temperature, the difference between low and high Ca^{2+} plateaus of K^+ flux increases markedly. All these features are very similar, albeit not the same in every quantitative detail, as observed for isolated rat liver cells (Kolb & Adam, 1976).

Any quantitative discussion of the considerable amount of data contained in Figs. 2–4 requires some data reduction. The fairly steep characteristics of dependence of ϕ_K on Ca_e (Figs. 2–4), similar to those observed for isolated liver cells (Kolb & Adam, 1976), strongly suggest a cooperative change of the physical state of the membrane upon variation of external Ca^{2+} concentration. We shall, therefore, use for quantitative description a theoretical formulation given previously (Adam, 1967, 1968, 1973), which is based on a cooperative cation-binding in a two-dimensional lattice of subunits in the plasma membrane. At the present stage of our argument, this theoretical description is intended only as a phenomenological compilation of the data, allowing for convenient extrapolation or interpolation. In the Appendix we have given a detailed molecular model describing the Ca^{2+}-dependent regulation process of passive K^+ transport as due to cooperative binding of Ca^{2+} to pairs of negative charges. Application of this specific model to the experimental data, derivation of the molecular parameters of the model and their comparison with independent experimental results will be deferred to the Discussion section. Here, we need only the basic relations. The Ca^{2+}-dependent passive K^+ flux may be approximated as proportional to the fraction n of negatively charged binding sites on the cell surface, which are not bound by Ca^{2+} ions (*cf.* Eqs. (A8) and A13)):

$$\Delta\phi_K = \Delta\phi_K^0 \cdot n \tag{3}$$

where $\Delta\phi_K = \phi_K - \phi_K^\infty$, as before, and $\Delta\phi_K^0 = \phi_K^0 - \phi_K^\infty$ corresponds to the passive K^+ flux attained asymptotically at low external Ca^{2+} concentrations.

The steady state of the two-dimensional cooperative assembly of Ca^{2+}-binding sites on the external face of the plasma membrane in the molecular field approximation is given by (*cf.* Eq. (A9)):

$$\frac{n}{1-n}\exp\left\{\frac{wn}{kT}\right\} = \frac{Q}{Ca_e} \tag{4}$$

where $1-n$ is the fraction of sites occupied by Ca^{2+} ions, whereas n is the fraction of sites not bound by Ca^{2+} ions. Further $w \leqq 0$ is the cooperativity parameter, Q is a parameter depending on temperature and on monovalent cation concentrations, but not on the external Ca^{2+} concentration Ca_e.

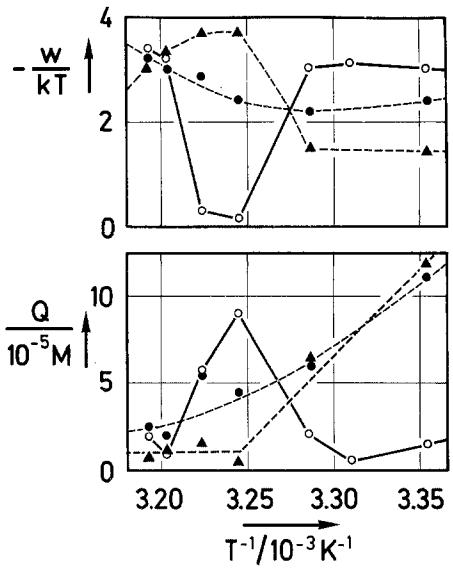

Fig. 5. Parameters-wk/T and Q versus reciprocal temperature for 3T3 (○), PY-3T3 (●), and SV40-3T3 cells (▲). These parameters are evaluated according to Eqs. (3) and (4) of the text from the data given in Fig. 2–4

Choosing a set of the three parameters $\Delta\phi_K^0$, w and Q for each temperature investigated, the curves given in Figs. 2–4 were calculated. As is evident from these Figures, an excellent fit is obtained by the relations (3) and (4), describing the experimental data fully within their statistical error bounds. The parameters $\Delta\phi_K^0$, w and Q, chosen for each temperature are given in Fig. 1, upper part, Fig. 5, upper part, and Fig. 5, lower part, respectively.

The meaning of the parameter $\Delta\phi_K^0$ is most obvious; it is the maximum extent of regulation of ϕ_K by external Ca^{2+} concentration. As is evident from Figs. 2–4, $\Delta\phi_K^0$ is well approximated by $\Delta\phi_K$ at $Ca_e = 10^{-5}$ M.

For the transformed cell lines, the temperature dependence of $\Delta\phi_K^0$ at $T \leqq 37$ °C within experimental error is described by an Arrhenius relation

$$\Delta\phi_K^0 = A_{\Delta\phi}^0 \exp\{-E_{\Delta\phi}^0/RT\} \tag{5}$$

(*see* Fig. 1, upper part, broken lines). The activation energies $E_{\Delta\phi}^0$ and pre-exponential factors $A_{\Delta\phi}^0$ thus derived are given in Table 2. At temperatures $T \geqq 39$ °C, there are deviations from this relation, which are beyond experimental error and are parallel to those of ϕ_K^1 from relation (2) used for the temperature dependence of ϕ_K^1. Similar to the situation encountered with regard to ϕ_K^1 of 3T3 cells, the temperature dependence of $\Delta\phi_K^0$ of 3T3 cells cannot be approximated

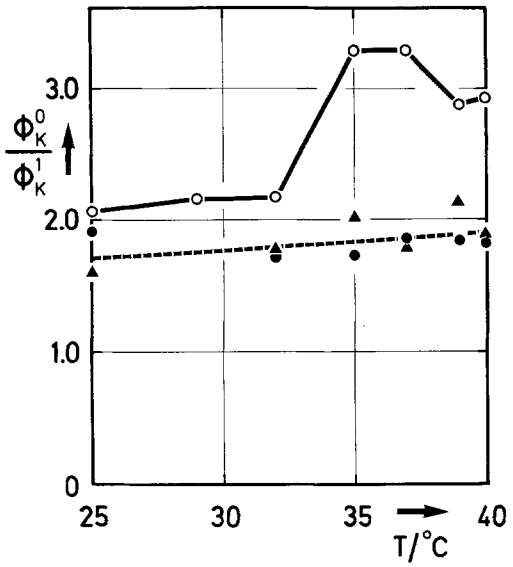

Fig. 6. Ratio of K^+ efflux ϕ_K^0, extrapolated to very low external Ca^{2+} concentration, to K^+ efflux ϕ_K^1 at 1 mM external Ca^{2+} concentration versus temperature for 3T3 (○) PY-3T3 (●), and SV40-3T3 cells (▲)

by a single activation energy $E^0_{\Delta\phi}$, but shows indications of high-temperature inactivation. Only at temperatures $T \leqq 29$ °C, the temperature dependence of $\Delta\phi_K^0$ of 3T3 cells yields an activation energy $E^0_{\Delta\phi}$ comparable to that applicable to SV40-3T3 cells over a much larger temperature range (*see* Table 2).

If we plot ϕ_K^0/ϕ_K^1 versus temperature, any temperature dependences common to the Ca^{2+}-dependent K^+ efflux $\Delta\phi_K^0$ and to the basic K^+ efflux ϕ_K^1 will cancel each other. Furthermore, such a reduced plot should cancel terms dependent on cell surface area and/or intracellular K^+ content and therefore facilitates comparison of different cell lines. As Fig. 6 shows, the flux ratio ϕ_K^0/ϕ_K^1 of transformed cells indeed turns out to be independent of temperature within experimental error. Interestingly, the flux ratio of ϕ_K^0/ϕ_K^1 of 3T3 cells exhibits a drastic rise by about 50% occurring between 32 °C and 35 °C (*see* Fig. 6), i.e. at the temperatures of high-temperature inactivation of ϕ_K^1 (Fig. 1).

Considering the temperature dependences of the parameter $-w/kT$ and Q, one observes that large figures of $-w/kT$ generally correspond to low figures of Q, and vice versa (*see* Fig. 5). An interesting feature, which clearly is beyond the uncertainty of the experiments and their evaluation, concerns the temperature dependence of $-w/kT$ and Q of 3T3 cells. At precisely the temperature range of high-temperature inactivation of ϕ_K^1 and of the drastic step-up of ϕ_K^0/ϕ_K^1, the "cooperativity parameter" $-w/kT$ drops to nearly zero, whereas Q exhibits a maximum. In contrast, the transformed cell lines show an essentially monotonous dependence of $-w/kT$ and Q on temperature.

Table 3. Affinities q of Ca^{2+} binding to membrane surface sites regulating passive K^+ transport at different temperatures evaluated from the data given in Fig. 5 using Eq. (6)

q (mM)	T (°C)					
	25	32	35	37	39	40
3T3	≈0.07	0.094	0.097	0.067	0.045	0.101
PY-3T3	≈0.3	0.180	0.149	0.229	0.090	0.124
SV40-3T3	≈0.2	0.130	0.030	0.099	0.055	0.030

As shown in the Appendix, an intrinsic affinity q of the cation-binding sites on the cell surface for Ca^{2+} may be derived from Q by elimination of the dependence on the cooperativity parameter $-w/kT$, using:

$$Q = q\, e^{w/2kT}. \tag{6}$$

The affinity q is simply characterized by the external Ca^{2+} concentration yielding half maximal saturation of the sites. Table 3 represents the figures for q which, according to Eq. (6), are computed from the figures on $-w/kT$ and Q given in Fig. 5. As is evident from Table 3, the strong dependence of Q on temperature (which is opposite to that of $-w/kT$) does not appear for q. Here, the figures of q for $T = 25$ °C should be disregarded, as they are subject to considerable error due to the smallness of the corresponding fluxes ϕ_K. The averages of q for $T > 25$ °C calculated from Table 3 for each of the cell lines are: $q_{3T3} = (0.081 \pm 0.024$ SD) mM; $q_{PY\text{-}3T3} = (0.154 \pm 0.053$ SD) mM; and $q_{SV40\text{-}3T3} = (0.069 \pm 0.044$ SD) mM.

According to these figures, normal and transformed cell lines cannot be distinguished with regard to their affinities q for Ca^{2+} binding to ϕ_K-regulating surface sites.

Discussion and Theoretical Model

Significance of the Parameters $\Delta\phi_K^0$, w/kT, and Q

Since the primary experimental data were evaluated and will be discussed in terms of the parameters $\Delta\phi_K^0$, w/kT, and Q, we wish to discuss first the significance of this data reduction. The experimental procedure was such as to perform the measurements for one temperature on one day using parallel preparations for each of the 5 to 6 Ca^{2+} concentrations done in triplicate. Thus, measurements at different temperatures were made on different days, i.e. in principle on different preparations which, however, were matched as far as practicable with regard to growth

procedure and cell density. Nevertheless, the variation between preparations used for different temperatures may be expected to be the major source of error. In comparison, the error resulting from the fit of relations (3) and (4) to the experimental results may be assessed more easily, although a simple regression analysis does not appear possible. The theoretical curves were fitted by trial and error taking into account the experimental error of the primary data. The uncertainty of the final figures chosen for $\Delta\phi_K^0$, w/kT, and Q may be estimated by this procedure as being mostly within $\pm 10\%$, in few cases within $\pm 20\%$. Only for the temperatures showing small fluxes $\Delta\phi_K^0$, i.e. for 25 °C and to a lesser extent for 32 °C, is the error in the choice of these parameters considerably larger, in particular with regard to parameter Q. As is evident from the earlier sections and also from the following discussion of the results in terms of the theoretical model, there are striking correlations between different experiments and different aspects of the experiments, which are beyond these error bounds of the experiments and their evaluation, indicating that the data on $\Delta\phi_K^0$, w/kT, and Q may form a useful basis for discussion of the experimental results.

Correlation of the Effects of Ca_e or T on Passive K^+ Fluxes with Those on Cell Proliferation

It has been observed consistently that normal cells do not proliferate at low external Ca^{2+} concentrations, but that transformed cells have a much lower Ca^{2+} requirement allowing for proliferation at very low levels of $Ca_e \leqq 10^{-4}$ M (references given in the Introduction). As to possible mechanisms of Ca-bound regulation of proliferation, some authors have suggested specific interactions between Ca and the metabolism of cyclic AMP including some redistribution of intracellular Ca pools (Boynton & Whitfield, 1976*a*, *b*; Boynton et al., 1977). More recently, fairly detailed evidence has been put forward attributing to calcium only an indirect role in growth control, whereas magnesium was concluded to be involved in processes more proximal to the intracellular events of cell replication (McKeehan & Ham, 1978; Rubin et al., 1978, 1979; Bowen-Pope, Vidair, Sanui & Rubin, 1979).

In addition to this intracellular role of Mg in regulation of proliferation, detailed evidence has been adduced showing that the intracellular K^+ concentration fulfills all criteria which according to the statements given by Sutherland (1972) may be required for a second messenger in the chain of events leading to stimulation or inhibition of cell division (*see* Introduction). Accordingly, an increase of K^+ efflux induced by an external perturbation (such as lowering

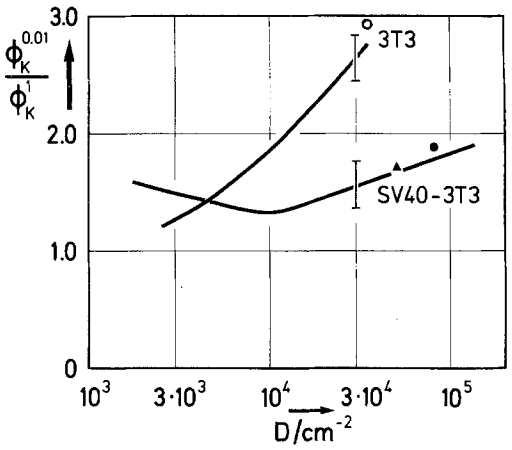

Fig. 7. Ratio of K^+ efflux $\phi_K^{0.01}$ at 10^{-5} M external Ca^{2+} concentration to K^+ efflux ϕ_K^i at 1 mM external Ca^{2+} concentration versus cellular growth density D in cm^{-2} for 3T3 (○), PY-3T3 (●), and SV40-3T3 cells (▲) (*present work*). Full curves are the results taken from Adam et al. (1979). Error bars indicate standard error of the mean at cell density 3×10^4 cm^{-2}. Temperature in these experiments was (37 ± 0.5) °C

of external Ca^{2+} concentration or raising temperature from 25 to 39 °C) should lower the intracellular K^+ concentration and thus negatively affect cellular proliferation, provided the effect on passive K^+ efflux is not compensated by any effect on active K^+ influx. These predictions may be checked using the results presented above, together with some data obtained in parallel studies (Adam et al., 1979; van der Bosch et al., 1979) employing the same stem cultures and growth conditions as in the present work. In Fig. 7, we have plotted the ratio of passive K^+ effluxes at very low ($Ca_e^{2+} \leqq 10^{-5}$ M) and at physiological ($Ca_e \geqq 10^{-3}$ M) external Ca^{2+} concentrations for different cell densities of normal and transformed 3T3 cells. This efflux ratio describes the maximum extent of stimulation of passive K^+ efflux with lowering of Ca_e. As is evident, the extent of Ca^{2+}-dependent regulation of K^+ efflux of 3T3 cells increases with cell density almost by a factor of three, indicating a strong effect of Ca_e on the balance of internal K^+ concentration at saturation densities. This effect is entirely consistent with the results of the parallel study (van der Bosch et al., 1979) showing a substantial depression of 3T3 cell proliferation at $Ca_e \approx 4\times10^{-5}$ M and its almost entire suppression at $Ca_e \approx 10^{-5}$ M. This range of Ca^{2+} concentrations affecting cell growth coincides fully with that affecting K^+ efflux (*see* Fig. 2, 37 °C). In contrast, the extent of Ca^{2+}-dependent regulation of K^+ efflux of transformed cells does not change very much with cell density and at cell densities of saturation of normal cells is substantially smaller than that of normal cells (*see* Fig. 7). Again this finding is consistent with the proposed effect of the balance of internal K^+ concentration on cell proliferation,

since the parallel growth study (van der Bosch et al., 1979) demonstrated almost negligible effects on SV40-3T3 cell proliferation of external Ca^{2+} concentrations in the range of 10^{-5} M $\leqq Ca_e \leqq 2.5 \times 10^{-3}$ M. These results are consistent with an indirect (viz. membrane-related) role of the external Ca^{2+} concentration in regulation of cell division of normal cells as proposed by Rubin et al. (1978, 1979) and in addition give some insight into the specific perturbations with regard to the pertinent regulation characteristics of transformed cells. Our results are inconsistent with the interpretation suggested by Gilbert (1972) of the dependence of intracellular K^+ content of different cell types on external Ca^{2+} concentration. It was found that with lowering of Ca_e the K^+ content of normal and transformed cells exhibited drastic decrease, which for transformed cells occurred at much lower figures of Ca_e than for hepatocytes (Gilbert, 1972). His results on hepatocytes have been largely confirmed by later work (Kolb & Adam, 1976). Interpretation of these differences between normal and transformed cells as resulting from a higher affinity of the plasma membrane of transformed cells for binding of Ca (Gilbert, 1972) does not appear conclusive as the intracellular K^+ content results from a complex balance of passive efflux and active uptake of K^+ ions and thus requires some detailed analysis. As the figures of Table 3 indicate clearly, the steep rises of K^+ efflux in fact occur at figures of Ca_e which are very similar for normal and transformed cells.

A further check on the relation between balance of intracellular K^+ concentration and cellular proliferation is provided by our data on temperature dependence of K^+ efflux of 3T3 and SV40-3T3 cells (Fig. 1, lower) at physiological Ca_e. For both cell lines the K^+ efflux increases by a factor of about two with increase of temperature from 35 to 39 °C. Since active K^+ uptake is affected much less ($\approx$35%) by this rise of temperature (Kimelberg & Mayhew, 1975), intracellular K^+ concentration may be expected to be substantially lower at 39° as compared to 35 °C. As this inbalance of intracellular K^+ concentration relates to saturation density of 3T3 cells and to about confluence of SV40-3T3 cells, it is to be expected that according to the role of intracellular K^+ as a second messenger in growth regulation the cell density-dependent inhibition of proliferation for both cell lines at 39 °C is more effective than at 35 °C. This prediction is fully confirmed by the parallel growth study (van der Bosch et al., 1979).

Thus, the results discussed above give detailed additional evidence supporting suggestions on intracellular K^+ content as a second messenger in growth regulation based on earlier data (Lubin, 1967; Pollack & Fisher, 1976; Ledbetter & Lubin, 1977, 1979; Adam et al., 1979; Ernst & Adam, 1979). Further information on the mode of action of intracellular K^+ concentration in growth control appears to stem from observations of protein synthesis in sarcoma 180 cells (Lubin, 1967) and human fibroblasts (Ledbetter & Lubin, 1977) which is severely inhibited if the intracellular K^+ concentration drops below 60–80% of normal, where RNA synthesis is hardly affected. Analogous conclusions could be drawn for 3T3, 3T6 and SV40-3T3 cells, the proliferation of which is strongly inhibited by low external K^+ concentration (Pollack & Fisher, 1976). Studies on cell-free systems (Schreier & Staehelin, 1973) are in detailed agreement with these results on cellular systems.

The sensitivity of growth of both normal and transformed cells to lowered intracellular potassium according to Pollack and Fisher (1976) and also following from the temperature dependence of growth and K^+ efflux of 3T3 and SV40-3T3 cells (*present work;* Adam et al., 1979; van der Bosch et al., 1979) is of considerable interest. It points to a specific perturbation of regulation of K^+ transport in transformed cells by growth density and/or external Ca^{2+} concentration.

Clearly, the role of K^+ as an intracellular regulator of protein synthesis and eventually of cell proliferation does not exclude an equally important and/or complementary role of intracellular Mg^{2+} in protein synthesis and growth control (Rubin et al., 1978, 1979; Schreier & Staehelin, 1973).

Furthermore, the present data and their interpretation do not exclude that Ca^{2+} ions fulfill important and specific functions within the cells. For instance, recent work has provided evidence for 3T3 cells on a calmodulin-mediated intracellular function of Ca^{2+}, in particular associated with the mitotic apparatus and/or assembly-disassembly of microtubuli (Marcum, Dedman, Brinkley & Means, 1978; Means & Dedman, 1980). In analogy to other systems (Roufogalis, 1980), even a more proximal role of the Ca^{2+}/calmodulin system in the primary chain of events of growth regulation may be surmised (Means & Dedman, 1980).

Thus, although the present data add evidence to the suggested role of intracellular K^+ content as a second messenger, the relative importance and the specific interrelations of the anorganic cations K^+, Mg^+, and Ca^{2+} and the cyclic nucleotides as intracellular effectors (second messengers) remain to be elucidated.

Application of the Theoretical Model of Ca^{2+}-Dependent Regulation of ϕ_K

In order to apply the theoretical relations derived in the Appendix for interpretation of the present ex-

Table 4. Average area per Ca^{2+}-binding site on the external membrane surface (*see text*)

Cell preparation		Average area per Ca^{2+}-binding site in 10^{-17} m^2				
Cell type	Growth density	Theoretical model[a]		Direct measurement[b]	Cell electrophoresis[c]	
		25–32 °C	35–37 °C		< 20 °C	> 30 °C
SV40-3T3	0.3	–	2.2	–	–	–
SV40-3T3	0.4–0.8	–	–	1.4	–	–
SV40-3T3	5	1.4–1.7	1.4–1.7	–	1.85	1.85
SV40-3T3	18–24	–	–	1.25	–	–
3T3	0.3	–	4.1	–	–	–
3T3	0.4–0.8	–	–	4.1	–	–
3T3	1.2–1.8	–	–	0.4	–	–
3T3	3.5	1.1–1.4	0.6–0.8	–	2.6	1.85

[a] Theoretical model of cooperative Ca^{2+} binding (*present work*).
[b] Computed from experimental data on surface-bound Ca^{2+} (Tupper & Zorgniotti, 1977) and on surface area per cell (Seher & Adam, 1978).
[c] Derived from microcell electrophoretic data on surface charge density (Adam & Adam, 1975).

perimental results, we observe that electrophysiological measurements on the membrane potential of parallel cultures of 3T3 and SV40-3T3 cells did not show any difference between 25 and 37 °C (Adam et al., 1979). Furthermore, the membrane potential between $Ca_e = 10^{-5}$ M and $Ca_e = 10^{-3}$ M changed only a little and for the cell densities used here can be represented by $V_M = -13.5 \pm 6.0$ mV for 3T3 cells and by $V_M = -18.5 \pm 6.0$ mV for SV40-3T3 cells (Adam et al., 1979).

According to the model given in the Appendix, the external Ca^{2+} concentration regulates ϕ_K through binding to the external membrane surface and thus modifying the external surface potential E_e. The internal surface potential E_i and the intrinsic membrane permeability P_K for K^+ are assumed to be negligibly dependent on Ca_e^{2+}. At any given temperature T the ratio of ϕ_K^0/ϕ_K^∞ according to Eqs. (A2) and (A3) depends only on the reduced potential differences u^0 and u^∞ between inner and outer face of the membrane:

$$\phi_K^0/\phi_K^\infty = u^0 (1 - e^{-u}) / u^\infty (1 - e^{-u}). \quad (7)$$

Here, u^0 and u^∞ correspond to very small and very high external Ca^{2+} concentrations, respectively.

According to the experimental results on ϕ_K^0/ϕ_K^1 shown in Fig. 6, the ratio ϕ_K^0/ϕ_K^∞ for transformed cells within experimental error is independent of temperature. Using Eqs. (7) and (A2), this result suggests that the external surface potential E_e^0 at low Ca_e is independent of temperature. Thus, the temperature dependences of $\phi_K^\infty \approx \phi_K^1$ and $\Delta\phi_K^0$ according to Eqs. (A2) and (A3) are essentially given by C and should coincide. As is evident from Fig. 1 and from the activation parameters given in Table 2 and also from Fig. 6, for transformed cells this prediction is borne out within experimental error. Using reasonable figures of $|E_i| \leqq 50$ mV, it is seen that the temperature dependence of ϕ_K^1 or $\Delta\phi_K^0$ within the precision of a few percent is given by that of P_K.

For a more detailed comparison of the model with experiments on transformed cells, we use $T = 37$ °C, $V_M = -18.5$ mV (Adam et al., 1979) and the temperature average $\phi_K^0/\phi_K^1 = 1.84 \pm 0.15$ SD (*see* Fig. 6). Application of Eqs. (A2), (A3), (A6) and (A7) yields E_e^0 largely independent of the figure E_i chosen. If E_i varies between 0 and -30 mV, E_e^0 is obtained between -32 and -27 mV, respectively. Using Eq. (A13) with $E_e^0 = -30$ mV, $l_D \approx 10^{-7}$ cm, $\varepsilon_0 = 8.85 \times 10^{-14}$ CV^{-1} cm^{-1} and $\varepsilon = 80$, an average surface area per Ca^{2+}-binding site, i.e. for a pair of negative charges, of 1.51×10^{-17} m^2 is predicted from K^+-efflux data on transformed cells. In Table 4, this figure is compared with the results of two different types of experiments taken from the literature.

i) The amount of surface-bound Ca was determined by Tupper and Zorgniotti (1977). For SV40-3T3 cells with a cell volume of 1521 ± 86 μm^3 they found the surface-bound fraction of cell Ca as 150 pmol/μl cell volume. Using these numbers and the surface area per cell (Table 1), we obtain an average cell-surface area per Ca^{2+}-binding site of 1.25×10^{-17} m^2.

Somewhat larger amounts of "surface-bound Ca^{2+}" were determined by Sanui and Rubin (1979) using a procedure different from that of Tupper and Zorgniotti (1977). Since in the procedure of Sanui and Rubin (1979) the cells were washed with isotonic nonelectrolyte solution before release of Ca^{2+} from the surface by acidification, it may be expected that the released Ca^{2+} is derived not only from Ca^{2+} bound directly to the negatively charged groups in the surface, but also from Ca^{2+} of the Gouy-Chapman interfacial layer and, therefore, should be larger than that determined by the method of Tupper and Zorgniotti (1977)

which, after washing with an electrolyte solution, corresponds more closely to actually surface-bound Ca^{2+} (in the "Stern-layer").

ii) Cell surface charge density σ_o at very low Ca_e was determined directly using micro-cell electrophoresis yielding for SV40-3T3 cells $\sigma_0 \times l_D = -1.72 \times 10^{-13}$ C cm^{-1} independent of temperature between 25 and 40 °C (Adam & Adam, 1975). With a Debye length $l_D = 10^{-7}$ cm, we obtain an average area for a pair of surface charges of 1.85×10^{-17} m^2.

Obviously, the three independent estimates for the average area per Ca^{2+}-binding site on the cell surface agree well with each other, providing support for the validity of the theoretical model.

For a more detailed application of the theoretical model, the surface potential E_i at the inner face of the membrane for simplicity is taken as zero. This conforms at least qualitatively to the theoretical expectation that generally the density of negatively charged entities should be smaller on the inner face of the membrane than on the outer face (McLaughlin & Harary, 1974). Then Eqs. (3), (4), (A13) and (A14) are applicable and w/kT and Q may be evaluated as given in Fig. 5. Using in addition $E_e^0 = -30$ mV, as derived before, we obtain from Eq. (A14): $w' - w = 5.79$ kJ mol^{-1}. As $-w/kT$ of transformed cells is in the range between 1.5 and 3.7 if temperature varies between 25 and 37 °C, the range of the "intrinsic" cooperativity parameter $-w'/kT$ is between 3.7 and 6.0. The "intrinsic" cooperativity w' is thus attenuated by the contribution of "negative" cooperativity resulting from the electrochemical activity of Ca^{2+}.

The parameter w' is defined as:

$$w' = w_{00} + w_{11} - 2w_{01} \tag{8}$$

where w_{11} is the interaction energy of an empty binding site in a lattice/environment of empty binding sites, whereas w_{00} and w_{01} are the interaction energies of a binding site occupied with Ca^{2+} in a lattice/environment of occupied and empty binding sites, respectively (Guggenheim 1952; Adam, 1970). This formulation appears appropriate here as it includes interactions of non-nearest neighbors, partly resulting from electrostatic energy terms between the charged binding sites and between the sites and bound Ca^{2+}.

Turning to application of the theoretical model to 3T3 cells, we observe that, in contrast to the situation for transformed cells, the extent of the Ca^{2+}-dependent process of regulation of ϕ_K varies greatly with temperature (*see* Fig. 1, upper, and Fig. 6). This temperature- dependent variation of the state of the plasma membrane of 3T3 cells is reflected also by all other parameters evaluated above: high-temperature inactivation of ϕ_K^1, minimum and maximum of $-w/kT$ and Q, respectively. It is, therefore, appropriate to separately apply the model to different temperature ranges.

At $T = (28 \pm 3.5)$ °C we have $\phi_K^0/\phi_K^1 = 2.13 \pm 0.06$ SD (Fig. 6). Using further $V_M = -13.5$ mV (Adam et al., 1979) and applying Eqs. (A2), (A3), (A6) and (A7), the quantity E_e^0 turns out to be largely independent of the figure of E_i chosen. If E_i varies between 0 and -30 mC, E_e^0 is found between -42 and -32 mV, respectively. Using again Eq. (A13), the average membrane areas per Ca^{2+}-binding site turn out to be between 1.10×10^{-17} m^2 and 1.41×10^{-17} m^2, respectively.

At $T = (36 \pm 1)$ °C we have $\phi_K^0/\phi_K^1 = 3.28 \pm 0.01$ SD (Fig. 6). Here the same procedure yields E_e^0 varying between -74 and -58 mV, if E_i is chosen between 0 and -30 mV, respectively. As an estimate for the average membrane area per Ca^{2+}-binding site, we thus obtain figures between 0.61×10^{-17} m^2 and 0.78×10^{-17} m^2, respectively. This density of Ca^{2+}-binding sites for 3T3 cells is 2 to 2.5 times that obtained before for transformed 3T3 cells. For comparison, we may use data on the amount of surface-bound Ca^{2+} in 3T3 cells, which for a cell volume of 3749 ± 269 nm^3 bind 485 pmol Ca^{2+}/µl cell volume to their surface (Tupper & Zorgniotti, 1977). Using these figures and a cell-surface area of 3T3 cells as given in Table 1, we obtain an average area per Ca^{2+}-binding site of 0.42×10^{-17} m^2. This independent method thus yields a density of Ca^{2+}-binding sites in the surface of 3T3 cells three times higher than that of SV40-3T3 cells, again being in reasonable agreement with the prediction of our model.

It is of considerable interest with respect to the membrane-bound mechanisms of cell-density dependent regulation of cell proliferation that the dependence on temperature of the relative extent ϕ_K^0/ϕ_K^1 of Ca^{2+}-regulated passive K^+ efflux (Fig. 6) is largely paralleled by its dependence on cell-growth density (Fig. 7) as computed from our earlier results on parallel cultures (Adam et al., 1979). Actually, in Fig. 7 the ratio $\phi_K^{0.01}/\phi_K^1$ is plotted versus cell density, where $\phi_K^{0.01}$ is the passive K^+ efflux at $Ca_e = 10^{-5}$ M, which, however, according to Figs. 2–4 in good approximation may be taken as ϕ_K^0. The striking parallelity for normal and transformed cells of the temperature dependence and the cell-density dependence of ϕ_K^0/ϕ_K^1 strongly supports the inference drawn earlier (Adam et al., 1979) that with regard to the physical state of the plasma membrane increasing cell density is equivalent to increasing temperature, i.e. may imply increasing "fluidization". Using $V_M = 13.5$ mV, $E_i = -10$ mV, $l_D = 10^{-7}$ cm, $\varepsilon = 80$, $\varepsilon_0 = 8.85 \times 10^{-12}$ CV^{-1} m^{-1} in Eqs. (A2), (A3), (A6) and (A7) as before, we wish to apply the theory to decribe the dependence of E_e^0 on cell density of 3T3 cells. At the cell densities

3×10^3 cm^{-2} and 3×10^4 cm^{-2}, the data from Fig. 7 are $\phi_K^{0.01}/\phi_K^1 \approx 1.25$ and 2.90, giving $E_e^0 = -11$ and -58 mV, respectively. Using again Eq. (A13), we obtain average areas per Ca^{2+}-binding site of 4.12×10^{-17} m^2 and of 0.80×10^{-17} m^2, respectively (*see* Table 4). The figures may be compared with those derived from the data of Tupper and Zorgniotti (1977) on the dependence of cell density of surface-bound Ca^{2+} determined directly. At cell densities of exponential growth ($4–8 \times 10^3$ cm^{-2}), these authors determined the amount of surface-bound Ca^{2+} as 65 pmol/μl cell volume, which at these cell densities was given as 2718 ± 65 μm^3. Using in addition the (density-independent) cell surface area of 3T3 cells (Table 1), we obtain an average area per Ca^{2+}-binding site of 4.08×10^{-17} m^2 at low cell density, whereas at saturation density the data of Tupper and Zorgniotti (1977) yield an average area per Ca^{2+}-binding site of 0.42×10^{-17} m^2, as discussed before (*see* Table 4). As is obvious from these data on the dependence of the average membrane area per Ca^{2+}-binding site on cell density of 3T3 cells, there is a very good agreement between the direct measurements of Tupper and Zorgniotti (1977) and the predictions from our model on Ca^{2+}-dependent regulation of ϕ_K. For 3T3 cells near saturation density, however, the area per pair of negative surface charges as derived from microcell electrophoresis data at $T > 25$ °C (Adam & Adam, 1975) is 1.85×10^{-17} m^2, and thus differs considerably from the results obtained by the other methods at similar growth densities (Table 4). It is of considerable interest, though, that upon lowering the temperature the surface charge density determined microelectrophoretically decreases by a factor of 1.4, which resembles the decrease with temperature of σ_0 for 3T3 cells by a factor of about 1.8, as is evident from the figures presented above (*cf.* Table 4). A similar temperature dependence is absent in the case of SV40-3T3 both for the microcell electrophoretic data (Adam & Adam, 1975) and for the data derived above from application of the theoretical model to the experimental results shown in Fig. 6. Furthermore, the independence of surface-bound Ca^{2+} of cell growth density of SV40-3T3 cells (Tupper & Zorgniotti, 1977) is in a striking agreement with E_e^0 of SV40-3T3 cells being largely independent of cell growth density as follows from the insignificant dependence of $\phi_K^{0.01}/\phi_K^1$ on cell density (Fig. 7).

For a more detailed application of the theoretical model to experimental data on ϕ_K of 3T3 cells, it is convenient to specify the surface potential at the inner face of the membrane, and we shall use for 3T3 cells $E_i = -10$ mV. This choice conforms at least qualitatively to the theoretical expectation for equilibrium distribution of negatively charged entities between inner and outer face of the membrane (MacLaughlin & Harary, 1974) and does not appear to be unduly restrictive as it was shown above that the parameter E_e^0 is very insensitive for E_i. With this choice, Eqs. (3), (4), (A13) and (A14) may be applied to derive w/kT and Q as shown in Fig. 5. Using this linear approximation in the case $T = (28.5 \pm 3.5)$ °C with $\phi_K^0/\phi_K^1 = 2.13$, we obtain from Eq. (A17) $E_e^0 = -32$ mV in reasonable agreement with the figures derived above using Eqs. (6) and (7) directly. Applying Eq. (A14) with $-w/kT = 3$ (Fig. 5), we may thus evaluate the "intrinsic" cooperativity parameter as $w' = (13.7$ kJ mol^{-1}, which is in the range found for transformed cells. This intrinsic cooperativity parameter reflects molecular interactions of vacant and occupied Ca^{2+}-binding sites, as discussed before, and may, therefore, be assumed to be independent of the state of the membrane, e.g. independent of lipid composition and/or temperature. Since at $T = (36 \pm 1)$ °C a surface potential $E_e^0 = -68$ mV is derived by the procedure given above, the cooperativity parameter $-w/kT$ may be calculated using Eq. (A14) and is found to be $-w/kT = 0.22$. This is in striking quantitative agreement with the drastic decrease of $-w/kT$ for 3T3 cells between 32 and 35 °C, as given in Fig. 5, upper. Even the increase of $-w/kT$ between 37 and 39 °C can be accounted for at least qualitatively: at $T = (39.5 \pm 0.5)$ °C we have $\phi_K^0/\phi_K^1 = 2.9$, which according to Eq. (A17) yields $E_e^0 = -57$ mV and according to Eq. (A14) with $w' = -13.7$ kJ mol^{-1} results in $w/kT = -1.04$.

The application of the theoretical model of Ca^{2+}-dependent regulation of ϕ_K to the present experimental data, thus gives a very reasonable and detailed description and furthermore yields a considerable number of correlations between different aspects of the experimental data providing for some trenchant checks on internal consistence of the model.

Molecular Constituents of the Cell Membrane with Regard to Regulation of ϕ_K

According to our model, Ca^{2+} binding was assumed to affect passive K^+ transport via a change of external surface potential but not via a change of the intrinsic permeabilities P_K of the entities effecting the actual transit of the K^+ ions through the membranes. Therefore, the temperature dependence of passive K^+ transport in the case of transformed cells could be attributed in the essence to that of P_K. It is of interest to compare the Arrhenius parameters describing this temperature dependence (Table 2) with those of pore-forming antibiotics in artificial lipid bilayer membranes. The activation energy of the single-channel conductance for Cs^+ transport through monazomycin

channels in a diphytanoyllecithin bilayer was found to be about 42 kJ mol^{-1} (Bamber & Janko, 1976), whereas that of Na^+ ions through the gramicidin channel in dioleoyllecithin bilayer membrane was found to be 31 kJ mol^{-1} (Bamberg & Läuger, 1974). These figures compare well with those of E^1_ϕ and $E^0_{\Delta\phi}$ between 48 and 69 kJ mol^{-1} for transformed cells (Table 2). For 3T3 cells, activation energies of passive K^+ efflux of this order of magnitude are observed only at temperatures below 32 °C.

At higher temperature, simultaneous changes with temperature of the parameters ϕ^1_K, $\Delta\phi^0_K$, ϕ^0_K/ϕ^1_K, and w/kT are superimposed on this simple Arrhenius behavior, confirming and extending the earlier observations of a process of "high-temperature inactivation" (Ernst & Adam, 1978). This process has been interpreted as due to K^+ transport molecules being more effective and/or residing preferentially in the cholesterol-rich and/or quasi-crystalline regions of a heterogeneous plasma membrane, which upon rising temperature above 32 °C diminish greatly by redistribution of cholesterol between laterally coexisting phases in the membrane (Ernst & Adam, 1978; Adam et al., 1979). This interpretation is consistent with detailed experimental results on analogous phenomena of high-temperature inactivation observed for channel-forming antibiotics in artificial lipid bilayer membranes (De Kruijff, Gerritsen, Oerlemans, van Dijck, Demel & van Deenen, 1974; Boheim, Hanke & Eibl, 1980). Such a change with temperature of the physical state of the plasma membrane of 3T3 cells at fixed external Ca^{2+} concentration, e.g. lateral redistribution of chlolesterol and/or K^+-transport molecules between different coexisting phases, may well result in a higher fraction of K^+-transport molecules participating in the Ca^{2+}-induced change of external surface potential and/or experiencing a higher surface-charge density in the liquid crystalline regions of the membrane, as is consistent with our data on variation of E^0_e with temperature.

The following indirect evidence points to (pairs of) negatively charged phospholipids as the molecular entities binding Ca^{2+} ions and thereby governing the external surface charge density near the passive K^+-transport mediating structures in the membrane.

i) Phosphatidyl serine (PS) and phosphatidyl inositol (PI) are the predominant negatively charged phospholipids in the plasma membrane, amounting to a molar fraction of 0.09 of the total plasma-membrane lipids of SV40-3T3 cells (Perkins & Scott, 1978). Since the cross-sectional area of cholesterol is roughly that of a phospholipid, which approximately may be given as 0.7 nm^2, the average membrane-surface area per pair of negatively charged lipids is $2 \times 0.7\ nm^2/0.09 = 1.55 \cdot 10^{-17}\ m^2$, which compares well with the figures given in Table 4 for the average area per Ca^{2+}-binding site of transformed cells.

However, this agreement between density of Ca^{2+}-binding sites and density of (pairs of) negatively charged phospholipid molecules does not extend to the case of 3T3 cells at saturation density (Perkins & Scott, 1978). This discrepancy might result from the prevalence in the cell membrane of 3T3 cells at high growth densities of di- and triphospho-inositides (*cf.* Hendricksen & Reinertsen, 1971; Michell, 1975).

ii) If negatively charged phospholipids are the pertinent Ca^{2+}-binding sites of the model, the interaction terms contributing to w' should partly result from selective interactions of the hydrocarbon chains of negatively charged lipids and those of amphiphilic phospholipids and/or cholesterol. A striking indication of such nonelectrostatic contributions to the negative figures of w' is the observation of a great preponderance of saturated hydrocarbon chains $C_{18:0}$ for phosphatidyl serine and phosphatidyl inositol of normal and SV40-transformed 3T3 cells as compared to the amphiphilic phospholipids having mostly short ($C_{16:0}$) or unsaturated (18:1) hydrocarbon chains (Perkins & Scott, 1978).

Virtually the same observation was made for plasma membranes of hepatocytes and different hepatoma (Van Hoeven, Emmelot, Krol & Oomen-Meulemans, 1975). Furthermore, an analogous observation was made for phosphatidyl inositol in the plasma membrane of BHK cells, where separate information on hydrocarbon-chain saturation of phosphatidyl serine is not given (Micklem, Abra, Knutton, Graham & Pasternak, 1976).

This apparently general feature of predominant saturation of the fatty-acid chains of the negatively charged lipids should favor their lateral aggregation once the repulsive interaction of the net negative charge of the lipids is compensated by Ca^{2+} binding, the latter thus being rendered positively cooperative ($w' < w \leqq 0$). These arguments seem to apply *a fortiori* to interactions of negatively charged phospholipids with the predominant lipid in the plasma membrane: cholesterol.

In the case of high Ca_e the complexes of negatively charged lipids and Ca^{2+} may be expected to separate from the cholesterol-containing membrane areas. In any case, studies on binary mixtures of acidic and neutral phospholipids do not appear to give representative models of Ca^{2+}-induced phase separations in mammalian plasma membranes, if such a differentiation of saturation of the corresponding fatty-acid chains and/or the effect of cholesterol is not taken into account.

iii) Multiple studies on lipid model systems have given ample evidence for the existence of Ca^{2+}-induced phase transitions in suspensions of negatively charged phospholipids (Papahadjopoulos, 1968; Träuble & Eibl, 1974; Hause, Darke & Finer, 1976) and, more pertinent for the present discussion, of Ca^{2+}-induced phase separations in mixtures of acidic and neutral phospholipids (Ohnishi & Ito, 1973, 1974; Papahadjopoulos, Poste, Schaeffer & Vail, 1974; Jacobson & Papahadjopoulos, 1975; Papahadjopoulos, Vail, Newton, Nir, Jacobson, Poste & Lazo, 1977; van Dijck, de Kruijff, Verkleij, van Deenen & de Gier, 1978). According to this latter group of investigators,

"the Ca^{2+}-bound negatively charged lipid forms rigid domains embedded in the otherwise liquid crystalline lipid" (Lee, 1977). The quantitative relations for cooperative Ca^{2+}-binding given in the Appendix are consistent with such a lateral phase separation of negatively charged phospholipids. There are some strikingly parallel features observed for these binary lipid model systems and the Ca^{2+}-dependent regulation process for passive K^+ transport: 1) The mol fraction of solid phase phosphatidyl serine in the presence of excess Ca^{2+} is rather insensitive to temperature (Ito, Ohnishi, Ishinaga & Kito, 1975), which corresponds well with ϕ_K^0/ϕ_K^1 being largely independent of temperature in the case of transformed cells (Fig. 6); 2) the affinity of binding of Ca^{2+} to the negatively charged lipids is considerably higher (about $10\times$) than that of Mg^{2+} (Ohnishi & Ito, 1974; Newton, Pangborn, Nir & Papahadjopoulos, 1978); which corresponds to the effectivity of Ca^{2+} for regulation of passive K^+ transport in hepatocytes being about 14 times that of Mg^{2+} (Kolb & Adam, 1976).

iv) An interaction of Ca^{2+} ions with neuraminic acids in the outer cell membrane as pertinent with regard to Ca^{2+} regulation of ϕ_K appears very improbable from the following experiments. Passive K^+ transport in 3T3 and SV40-3T3 cells was not affected by treatment with neuraminidase (Adam et al., 1979) at concentrations that modify cell-surface charge (Adam & Adam, 1975). In full consistence with the conclusions from the present work, cell growth could also not be affected by application of neuraminidase to the growth medium (Adam, *unpublished results*).

The theoretical analysis presented above is, therefore, entirely consistent with a cooperative binding of Ca^{2+} ions to, and a concomitant lateral molecular redistribution of, negatively charged lipids in the liquid crystalline phase of transformed 3T3 cells, the amount of this liquid crystalline phase in the plasma membrane being independent of temperature, which appears a reasonable assumption at least for $T \leqq 37\,°C$. In the case of 3T3 cells, the situation is more complicated, since a temperature-dependent change in the outer cell membrane appears superimposed on the Ca^{2+}-dependent regulation process. Clearly, further evidence is needed in order to be able to exclude the possibility of regulation of ϕ_K by interaction of Ca^{2+} with nonphospholipid molecular entities in the plasma membrane, such as membrane proteins or glycolipids.

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Appendix

Theoretical Formulation of a Molecular Model for Regulation of ϕ_K

Regulation of passive K^+ transport by external Ca^{2+} concentration has been described as governed by cooperative binding of Ca^{2+} to negatively charged sites of the plasma membrane (Adam, 1967, 1968, 1970; Kolb & Adam, 1976). In our previous formulation of the model, however, the dependence of the passive K^+ flux on the (binding) state of the membrane was not derived from a specific model, but simply taken as a linear relation.

In order to be more specific on this aspect, passive K^+ efflux ϕ_K is described by the constant field equation allowing for surface potentials E_e and E_i at the outer and inner faces of the plasma membrane, respectively (Kolb & Adam, 1976; Adam, Läuger & Stark, 1977):

$$\phi_K = P_K \frac{u}{1-e^{-u}}\left(K_i e^{-\frac{FE_i}{RT}} - K_e e^{-\frac{FE_e}{RT}} e^{-u}\right). \tag{A1}$$

Here, P_K is the intrinsic membrane permeability, K_i and K_e are external and internal (bulk) concentrations of K^+, respectively. The reduced difference u of electrical potential between inner and outer face of the membrane is given by

$$u = \frac{F}{RT}(V_M + E_i - E_e), \tag{A2}$$

where V_m is the electric membrane potential difference determined by the electric potentials in the bulk electrolyte phases.

Since in our experiments $K_e \ll K_i$ and $-V_M \lessapprox 30\,mV$ (Adam et al., 1979), we may use $K_e \exp\{-FV_M/RT\}/K_i \ll 1$, and therefore obtain:

$$\phi_K = C\frac{u}{1-e^{-u}}, \tag{A3}$$

where

$$C = P_K K_i \exp\left\{-\frac{FE_i}{RT}\right\}. \tag{A4}$$

If $|u| \ll 1$, the series expansion of e^{-u} in Eq. (A3) yields:

$$\phi_K \approx C\left(1+\frac{u}{2}\right). \tag{A5}$$

In the following, we shall assume that P_K and E_i do not depend on the external Ca^{2+} concentration. Since according to our experimental procedure K_i is independent of Ca_e, and therefore the same applies to C, any dependence of ϕ_K on Ca_e derives from u, in particular through E_e. For small surface potentials, we may use the approximation

$$E_e = \frac{\sigma l_D}{\varepsilon \varepsilon_o} \tag{A6}$$

where σ = cell surface charge density (at the sites of passive K^+ transport), l_D = Debye length, ε = relative dielectric constant and ε_o = permittivity of vacuum.

The dependence of σ on Ca_e will be described by the following minimal model (a more detailed theoretical formulation re-

moving most of the restrictions of the present model will be published elsewhere).

The surface charge σ is considered to consist of negatively charged groups which (after lateral redistribution) cooperatively bind Ca^{2+} ions. In the most simple case, one Ca^{2+} ion is bound by a pair of negative charges (viz. two negatively charged phospholipids), which is referred to as a "Ca^{2+}-binding site". The intrinsic binding affinity of alkali ions to the negatively charged groups is considered to be negligible (Eisenberg, Gresalfi, Riccio & McLaughlin, 1979). Furthermore, 1:1 binding of Ca^{2+} per charged group is expected to occur only after saturation of the 2:1 binding and will be neglected in the following. If n and $1-n$ are the fractions of vacant sites and sites occupied by a Ca^{2+} ion, respectively, the cell surface charge density σ may be written:

$$\sigma = \sigma_0 n \tag{A7}$$

where σ_0 is the surface charge density without any binding of Ca^{2+} to the sites.

Using Eqs. (A2) and (A3)–(A7), we have:

$$\phi_K = C\left[1+\frac{F}{2RT}\left(V_M + E_i - \frac{\sigma_0 l_D}{\varepsilon \varepsilon_0} n\right)\right]. \tag{A8}$$

Cooperative binding of Ca^{2+} ions to a two-dimensional assembly of binding sites has been formulated in the molecular field approximation (Adam, 1967, 1968, 1970; Kolb & Adam, 1976) as:

$$\frac{n}{1-n} e^{\frac{w'}{kT}n} = \frac{Q}{\tilde{C}a_e} \tag{A9}$$

where $\tilde{C}a_e$ is the electrochemical activity of Ca^{2+} ions near the binding sites on the membrane surface:

$$\tilde{C}a_e = Ca_e \exp\left\{-\frac{2FE_e}{RT}\right\} \tag{A10}$$

and $w' \leqq 0$ the cooperativity parameter of Ca^+ binding whereas Q describes the affinity of the binding sites for Ca^{2+}.

Clearly, the formulation of Eq. (A9) gives only a crude approximation of the statistical problem in the case of laterally redistributing negatively charged membrane molecules (viz. acidic phospholipids) in an excess of neutral membrane constituents (viz. amphiphilic phospholids and cholesterol).

The result of a more detailed derivation of the partition function in the molecular field approximation for a two-dimensional system of three species of membrane subunits (neutral, vacant negatively charged and bound negatively charged, i.e. pairwise occupied by a Ca^{2+} ion) can be approximated as (Adam, *to be published elsewhere*):

$$\frac{v^2}{1-v} e^{\frac{v'v}{kT}} = \frac{Q'}{\tilde{C}a_e}. \tag{A11}$$

Here, v is the vacant fraction of negatively charged subunits, whereas $v' \leqq 0$ and Q' are parameters depending on the ratio of negatively charged sites to neutral sites and on interaction parameters. Although Eq. (A11) is not symmetrical if v is plotted *vs.* $\log Ca_e$, its deviations from Eq. (A9) are inconsequential for the present purposes, so that the discussion of the present data will be based on Eq. (A9). Observing that E_e depends on n by Eqs. (A6) and (A7), we may write:

$$E_0 = E_e^0 n \tag{A12}$$

$$E_e^0 = \frac{\sigma_0 l_D}{\varepsilon_0 \varepsilon} \tag{A13}$$

and thus recast Eqs. (A8) and (A10) into Eq. (4), where

$$w = w' - 2e_0 E_e^0 \tag{A14}$$

where e_0 is the elementary charge.

According to Eq. (A9), n approaches 0 or 1 if Ca_e is very large or very small, respectively. If the membrane potential V_M is independent of Ca_e, we obtain from Eq. (A8):

$$\phi_K^\infty = C\left[1+\frac{F}{2RT}(V_M + E_i)\right] \tag{A15}$$

$$\phi_K^0 = C\left[1+\frac{F}{2RT}(V_M + E_i - E_e^0)\right] \tag{A16}$$

$$\phi_K^0/\phi_K^\infty = \left[1+\frac{F}{2RT}(V_M + E_i - E_e^0)\right] \Big/ \left[1+\frac{F}{2RT}(V_M + E_i)\right]. \tag{A17}$$

Furthermore, Eqs. (A8) and (A13) yield Eq. (3) if:

$$\Delta\phi_K^0 = -C\frac{FE_e^0}{2RT}. \tag{A18}$$

Lastly, we wish to evaluate the temperature dependence of the parameter Q. Eq. (3) may be written in a more symmetrical form. Defining an intrinsic affinity q of the negatively charged binding sites for Ca^{2+} by

$$Q = q e^{w/2kT} \tag{A19}$$

we obtain

$$\frac{n}{1-n} e^{-\frac{w}{2kT}(1-2n)} = \frac{q}{Ca_e}. \tag{A20}$$

Here, q indicates the external Ca^{2+} concentration for half-maximal saturation of the cation-binding sites.

References

Adam, G. 1967. Nervenerregung als kooperativer Kationenaustausch in einem zweidimensionalen Gitter. *Ber. Bunsenges. Phys. Chem.* **71**:829–831

Adam, G. 1968. Ionenstrom nach einem depolarisierenden Sprung im Membranpotential. *Z. Naturforsch.* **23b**:181–197

Adam, G. 1970. Theory of nerve excitation as a cooperative cation exchange in a two-dimensional lattice. *In:* Physical Principles of Biological Membranes. F. Snell, J. Wolken, G. Iversen, and J. Lam, editors. pp. 35–64. Gordon and Breach Science Publishers, New York

Adam, G. 1973. Cooperative transitions in biological membranes. *In:* Synergetics. H. Haken, editor. pp. 220–231. B.G. Teubner, Stuttgart

Adam, G., Adam, G. 1975. Cell surface charge and regulation of cell division of 3T3 cells and transformed derivatives. *Exp. Cell Res.* **93**:71–78

Adam, G., Ernst, M., Seher, J.-P. 1979. Regulation of passive membrane permeability for potassium ions by cell density of 3T3 and SV40-3T3 cells. *Exp. Cell Res.* **120**:127–139

Adam, G., Läuger, P., Stark, G. 1977. Physikalische Chemie und Biophysik. p. 311ff. Springer Verlag, Berlin-Heidelberg-New York

Balk, S.D. 1971. Stimulation of the proliferation of chicken fibroblasts by folic acid or a serum factor in a plasma containing medium. *Proc. Natl. Acad. Sci. USA* **68**:1689–1692

Balk, S.D., Polimeni, P.I., Hoon, B.S., LeStourgeon, D.N., Mitchell, R.S. 1979. Proliferation of Rous sarcoma virus-infected,

but not of normal, chicken fibroblasts in a medium of reduced calcium and magnesium concentration. *Proc. Natl. Acad. Sci USA* **76**:3913–3916

Balk, S.D., Whitfield, J.F., Youdale, T., Braun, A.C. 1973. Roles of calcium, serum, plasma, and folic acid in the control of proliferation of normal and Rous sarcoma virus-infected chicken fibroblasts. *Proc. Natl. Acad. Sci. USA* **70**:675–679

Bamberg, E., Janko, K. 1976. Single channel conductance at lipid bilayer membranes in presence of monazomycin. *Biochim. Biophys. Acta* **426**:447–450

Bamberg, E., Läuger, P. 1974. Temperature-dependent properties of gramicidin A channels. *Biochim. Biophys. Acta* **367**:127

Banerjee, S.P., Bosmann, H.B. 1976. Rubidium transport and ouabain binding in normal and virally transformed mouse fibroblasts. *Exp. Cell Res.* **100**:153–158

Boheim, G., Hanke, W., Eibl, H.-J. 1980. Lipid phase transition in planar bilayer membrane and its effect on carrier and pore mediated ion transport. *Proc. Natl. Acad. Sci. USA* **77**:3403–3407

van der Bosch, J., Sommer, I., Maier, H., Rahmig, W. 1979. Density-dependent growth adaptation kinetics in 3T3 cell populations following sudden $[Ca^{2+}]$ and temperature changes. A comparison with SV40-3T3 cells. *Z. Naturforsch.* **34c**:279–283

Bourne, H.R., Rosengurt, E. 1976. An 18,000 molecular weight polypeptide induces early events and stimulates DNA synthesis in cultured cells. *Proc. Natl. Acad. Sci. USA* **73**:4555–4559

Bowen-Pope, D.F., Vidair, C., Sanui, H., Rubin, A.H. 1979. Separate roles for calcium and magnesium in their synergistic effect on uridine uptake by cultured cells: Significance of growth control. *Proc. Natl. Acad. Sci. USA* **76**:1308–1312

Boynton, A.L., Whitfield, J.F. 1976*a*. Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells. *Proc. Natl. Acad. Sci. USA* **73**:1651–1654

Boynton, A.L., Whitfield, J.F. 1976*b*. The different actions of normal and supranormal calcium concentrations on the proliferation of BALB/c 3T3 mouse cells. *In Vitro* **12**:479–484

Boynton, A.L., Whitfield, J.F., Isaacs, R.J., Tremblay, R. 1977. The control of human WI-38 cell proliferation by extracellular calcium and its elimination by SV-40 virus-induced proliferative transformation. *J. Cell. Physiol.* **92**:241–248

Cammann, K. 1973. Das Arbeiten mit ionenselektiven Elektroden. p. 134. Springer-Verlag, Berlin

Cone, C.D., Jr., Tongier, M., Jr. 1974. Contact inhibition of division: Involvement of the electrical transmembrane potential. *J. Cell. Physiol.* **82**:373–386

Curran, P.F., Herrera, F.C., Flanigan, W.J. 1963. The effect of Ca and antidiuretic hormone on Na transport across frog skin. II. Sites and mechanism of action. *J. Gen. Physiol.* **46**:1011–1027

Dijck, P.W.M van, Kruijff, B. de, Verkleij, A.J., Deenen, L.L.M. van, Gier, J. de 1978. Comparative studies on the effects of pH and Ca^{2+} on bilayers of various negatively charged phospholipids and their mixtures with phosphatidyl-choline. *Biochim. Biophys. Acta* **512**:84–96

Ducouret-Prigent, B., Lelievre, L., Paraf, A., Kepes, A. 1975. Relationship between intracellular K^+ concentrations and K^+ fluxes in growing and contact-inhibited cells. *Biochim. Biophys. Acta* **401**:119–127

Dulbecco, R., Elkington, J. 1975. Induction of growth in resting fibroblastic cell cultures by Ca^{++}. *Proc. Natl. Acad. Sci. USA* **72**:1584–1588

Eisenberg, M., Gresalfi, T., Riccio, T., McLaughlin, S. 1979. The adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry* **18**:5213–5223

Elligsen, J.D., Thompson, J.E., Frey, H.E., Kruuv, J. 1976. Correlation of $(Na^+\text{-}K^+)$ATPase activity with growth of normal and transformed cells. *Exp. Cell Res.* **87**:233–240

Ernst, M., Adam, G. 1978. High-temperature inactivation of passive potassium transport in electrically non-excitable cells. *Z. Naturforsch.* **33c**:937–940

Ernst, M., Adam, G. 1979. Dependence of intracellular alkali-ion concentrations of 3T3 and SV40-3T3 cells on growth density. *Cytobiologie* **18**:450–459

Esfahani, M., Limbrick, A.R., Knutton, S., Oka, T., Wakil, S.J. 1972. The molecular organization of lipids in the membrane of *E. coli*: Phase transitions. *Proc. Natl. Acad. Sci. USA* **68**:3180

Frank, W. 1973. Stimulation of embryonic rat cells in culture by calf serum: VI. Calcium and potassium ions as cofactors. *Z. Naturforsch.* **28c**:322–328

Garrahan, P.J., Glynn, I.M. 1967. The behaviour of the sodium pump in red cells in the absence of external potassium. *J. Physiol. (London)* **192**:159–174

Geyer, R.P., Sholtz, K.J., Bowie, E.J. 1955. Influence of calcium on potassium concentration in rat liver *in vitro*. *Am. J. Physiol.* **182**:487–492

Gilbert, I.G.F. 1972. The effect of divalent cations on the ionic permeability of cell membranes in normal and tumour tissues. *Eur. J. Cancer* **8**:99–105

Grisham, C.M., Barnett, R.E. 1973. The role of lipid-phase transition in the regulation of the (sodium + potassium) adenosine triphosphatase. *Biochemistry* **12**:2635–2637

Guggenheim, E.A. 1952. Mixtures. p 29ff. Clarendon Press, Oxford

Hauser, H., Darke, A., Phillips, M.C. 1976. Ion binding to phospholipids. Interaction of calcium with phosphatidyl serine. *Eur. J. Biochem.* **62**:335–344

Hazelton, B., Mitchell, B., Tupper, J.T. 1979. Calcium, magnesium, and growth control in the WI-38 human fibroblast cell. *J. Cell Biol.* **83**:487–498

Hazelton, B.J., Tupper, J.T. 1979. Calcium transport and exchange in mouse 3T3 and SV40-3T3 cells. *J. Cell Biol.* **81**:538–543

Hendricksen, H.S., Reinertsen, J.L. 1971. Phosphoinositide interconversion: A model for control of Na^+ and K^+ permeability in the nerve axon membrane. *Biochem. Biophys. Res. Commun.* **44**:1258–1264

Hodgkin, A.L., Keynes, R.D. 1957. Movements of labelled calcium in squid giant axons. *J. Physiol. (London)* **138**:253–281

Hoeven, R.P. van, Emmelot, P., Krol, J.H., Oomen-Meulemans, E.P.M. 1975. Studies on plasma membranes. XXII. Fatty acid profiles of lipid classes in plasma membranes of rat and mouse livers and hepatomas. *Biochim. Biophys. Acta* **380**:1–11

Ito, T., Ohnishi, S., Ishinaga, M., Kito, M. 1975. Synthesis of a new phosphatidylserine spin-label and calcium-induced lateral phase separation in phosphatidylserine-phosphatidylcholine membranes. *Biochemistry* **14**:3064–3069

Jacobson, K., Papahadjopoulos, D. 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentration of bivalent cations. *Biochemistry* **14**:152–161

Kalant, H., Hickie, R.A. 1968. Effects of divalent cations on K^+ transport in liver and morris hepatoma 5123 tc. *Cancer Res.* **28**:2086–2091

Kaplan, J.G. 1977. The role of cation flux in triggering and maintaining the stimulated state in lymphocytes. *In:* Regulatory Mechanisms of Lymphocyte Activation. D.O. Lucas, editor. p. 51ff. Academic Press, New York

Kasarov, L.B., Friedman, H. 1974. Enhanced $Na^+\text{-}K^+$-activated adenosine triphosphate activity in transformed fibroblasts. *Cancer Res.* **34**:1862–1865

Kimelberg, H.K. 1977. The influence of membrane fluidity on the activity of membrane-bound enzymes. *In:* Dynamic Aspects

of Cell Surface Organization. G. Poste and G.L. Nicolson, editors. pp. 205–293. Elsevier, Amsterdam

Kimelberg, H.K., Mayhew, E. 1975. Increased ouabain-sensitive $^{86}Rb^+$ uptake and sodium and potassium ion-activated adenosine triphosphatase activity in transformed cell lines. *J. Biol. Chem.* **250**:100–104

Kimelberg, H.K., Mayhew, E. 1976.Cell growth and ouabain-sensitive $^{86}Rb^+$ uptake and $(Na^+\text{-}K^+)$-ATPase activity in 3T3 and SV40 transformed 3T3 fibroblasts. *Biochim. Biophys. Acta* **455**:865–875

Kimelberg, H.K., Papahadjopoulos, D. 1972. Phospholipid requirements for $(Na^+\text{-}K^+)$-ATPase activity: head-group specificity and fatty acid fluidity. *Biochim. Biophys. Acta* **282**:277–292

Kimelberg, H.K., Papahadjopoulos, D. 1974. Effects of phospholipid acyl chain fluidity, phase transition and cholesterol on $(Na^+\text{-}K^+)$-stimulated adenosine triphosphatase. *J. Biol. Chem.* **249**:1071–1080

Kleinzeller, A., Knotková, A., Nedvidkova, J. 1968. The effect of calcium ions on the steady-state ionic distributions in kidney cortex cells. *J. Gen. Physiol.* **51**:326s–334s

Koch, K.S., Leffert, H.L. 1979. Increased sodium ion influx is necessary to initiate rat hepatocyte proliferation. *Cell* **18**:153–163

Kolb, H.A., Adam, G. 1976. Regulation of ion permeabilities of isolated rat liver cells by external calcium concentration and temperature. *J. Membrane Biol.* **26**:121–151

Kruijff, B. de, Gerritsen, W.J., Oerlemans, A., Dijck, P.W.M. van, Demel, R.A., Deenen, L.L.M. van 1974. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. II. Temperature dependence. *Biochim. Biophys. Acta* **339**:44–56

Lamb, J.F., McCall, P. 1972. Effect of prolonged ouabain treatment on Na, K, Cl, and Ca concentration and fluxes in cultured human cells. *J. Physiol. (London)* **225**:599–617

Ledbetter, M.L.S., Lubin, M. 1977. Control of protein synthesis in human fibroblasts by intracellular potassium. *Exp. Cell Res.* **105**:223–236

Ledbetter, M.L.S., Lubin, M. 1979. Transfer of potassium. A new measure of cell-cell coupling. *J. Cell Biol.* **80**:150–165

Lee, A.G. 1977. Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. *Biochim. Biophys. Acta* **472**:285–344

Linden, C.D., Wright, K.L., McConnell, H.M., Fox, C.F. 1973. Lateral phase separations in membrane lipids and the mechanism of sugar transport in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **70**:2271–2275

Loewenstein, W.R. 1967. Cell surface membranes in close contact. Role of calcium and magnesium ions. *J. Colloid Interface Sci.* **25**:34–46

Lubin, M. 1967. Intracellular potassium and macromolecular synthesis in mammalian cells. *Nature (London)* **213**:451–453

Marcum, J.M., Dedman, J.R., Brinkley, B.R., Means, A.R. 1978. Control of microtubule assembly-disassembly by calcium-dependent regulator protein. *Proc. Natl. Acad. Sci. USA* **75**:3771–3775

McDonald, T.F., Sachs, H.G., Orr, C.W., Ebert, J.D. 1972. Multiple effects of ouabain on BHK cells. *Exp. Cell Res.* **74**:201–206

McKeehan, W.L., Ham, R.G. 1978. Calcium and magnesium ions and the regulation of multiplication in normal and transformed cells. *Nature (London)* **275**:756–758

McLaughlin, S., Harary, H. 1974. Phospholipid flip-flop and the distribution of surface charges in excitable membranes. *Biophys. J.* **14**:200–209

Means, A.R, Dedman, J.R. 1980. Calmodulin – An intracellular calcium receptor. *Nature (London)* **285**:73–77

Michell, R.H. 1975. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta* **415**:81–147

Micklem, K.J., Abra, R.M., Knutton, S., Graham, J.M., Pasternak, C.A. 1976. The fluidity of normal and virus-transformed cell plasma membrane. *Biochem. J.* **154**:561–566

Morril, G.A., Kaback, H.R., Robbins, E. 1964. Effect of calcium on intracellular sodium and potassium concentrations in plant and animal cells. *Nature (London)* **204**:641–462

Newton, C., Pangborn, W., Nir, S., Papahadjopoulos, D. 1978. Specificity of Ca^{2+} and Mg^{2+} binding to phosphatidylserine vesicles and resultant phase changes of bilayer membrane structure. *Biochim. Biophys. Acta* **506**:281–287

Ohnishi, S., Ito, T. 1973. Clustering of lecithin molecules in phosphatidylserine membranes induced by Ca^{++}-binding to phosphatidylserine. *Biochem. Biophys. Res. Commun.* **51**:132–138

Ohnishi, S., Ito, T. 1974. Calcium-induced phase separations in phosphatidylserine-phosphatidylcholine membranes. *Biochemistry* **13**:881–887

Overath, P., Schairer, H.U., Stoffel, W. 1970. Correlation of *in vivo* and *in vitro* phase transitions of membrane lipids in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **67**:606–612

Papahadjopoulos, D. 1968. Surface properties of acidic phospholipids: Interaction of monolayers and hydrated liquid crystals with uni- and bi-valent metal ions. *Biochim. Biophys. Acta* **163**:240–254

Papahadjopoulos, D., Poste, G., Schaeffer, B.E., Vail, W.J. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta* **352**:10–28

Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G., Lazo, R. 1977. Studies on membrane fusion. III. The role of calcium-induced phase changes. *Biochim. Biophys. Acta* **465**:579–598

Paul, D., Ristow, H.J. 1979. Cell cycle control by Ca^{++}-ions in mouse 3T3 cells and in transformed 3T3 cells. *J. Cell. Physiol.* **98**:31–40

Perkins, R.G., Scott, R.E. 1978. Differences in the phospholipid, cholesterol, and fatty acyl composition of 3T3 and SV3T3 plasma membranes. *Lipids* **13**:653–657

Pollack, M., Fisher, H.W. 1976. Dissociation of ribonucleic acid and protein synthesis in mammalian cells deprived of potassium. *Arch. Biochem. Biophys.* **172**:188–190

Priestland, R.N., Whittam, R. 1972. The temperature dependence of activation by phosphatidylserine of the sodium pump adenosine triphosphatase. *J. Physiol. (London)* **220**:353–361

Quissel, D.O., Suttie, J.W. 1973. Effect of fluoride and other metabolic inhibitors on intracellular sodium and potassium concentrations in L cells. *J. Cell. Physiol.* **82**:59–64

Rixon, R.H., Whitfield, J.F. 1976. The control of liver regeneration by parathyroid hormone and calcium. *J. Cell. Physiol.* **87**:147–156

Rosengurt, E., Heppel, L.A. 1975. Serum rapidly stimulates ouabain-sensitive $^{86}Rb^+$ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* **72**:4492–4495

Rosengurt, E., Legg, A., Pettican, P. 1979. Vasopressin stimulation of mouse 3T3 cell growth. *Proc. Natl. Acad. Sci. USA* **76**:1284–1287

Roufogalis, B.D. 1980. Calmodulin: Its role in synaptic transmission. *Trends Neuro-Sci.* **Oct.**:238–241

Rubin, A.H., Terasaki, M., Sanui, H. 1978. Magnesium reverses inhibitory effects of calcium deprivation on coordinate response of 3T3 cells to serum. *Proc. Natl. Acad. Sci. USA* **75**:4379–4383

Rubin, A.H., Terasaki, M., Sanui, H. 1979. Major intracellular cations and growth control: Correspondence among magnesium content, protein synthesis, and the onset of DNA synthesis in Balb/c 3T3 cells. *Proc. Natl. Acad. Sci. USA* **76**:3917–3921

Sanui, H., Rubin, A.H. 1979. Measurement of total, intracellular

and surface bound cations in animal cells grown in culture. *J. Cell. Physiol.* **100**:215–226

Schairer, H.U., Overath, P. 1969. Lipids containing trans-unsuturated fatty acids change the temperature characteristic of thiomethylgalactoside accumulation in *Escherichia coli*. *J. Mol. Biol.* **44**:209–214

Schreier, M.H., Stähelin, T. 1973. Initiation of mammalian protein synthesis: The importance of ribosome and initiation factor quality for the efficiency of *in vitro* systems. *J. Mol. Biol* **73**:329–349

Seher, J.P., Adam, G. 1978. Dependence of cellular surface area on growth density of 3T3 and SV40-3T3 cells. *Z. Naturforsch.* **33c**:739–743

Shank, B.B., Smith, N.E. 1976. Regulation of cellular growth by sodium pump activity. *J. Cell. Physiol.* **87**:377–388

Smith, J.B., Rosengurt, E. 1978*a*. Lithium transport by fibroblastic mouse cells: Characterization and stimulation by serum and growth factors in quiescent culture. *J. Cell. Physiol.* **97**:441–450

Smith, J.B., Rosengurt, E. 1978*b*. Serum stimulates the Na^+, K^+ pump in quiescent fibroblasts by increasing Na^+ entry. *Proc. Natl. Acad. Sci. USA* **75**:5560–5564

Spaggiare, S., Wallach, M.H., Tupper, J.T. 1976. Potassium transport in normal and transformed mouse 3T3 cells. *J. Cell. Physiol.* **89**:403–416

Sutherland, E.W. 1972. Studies on the mechanism of hormone action. *Science* **177**:401–408

Swierenga, S.H.H., MacManus, J.P., Whitfield, J.F. 1976. Regulation by calcium of the proliferation of heart cells from young adult rats. *In Vitro* **12**:31–36

Thilo, L., Träuble, H., Overath, P. 1977. Mechanistic interpretation of the influence of lipid phase transitions on transport functions. *Biochemistry* **16**:1283–1290

Träuble, H., Eibl, H. 1974. Electrostatic effects on lipid phase transitions. Membrane structure and ionic environment. *Proc. Natl. Acad. Sci. USA* **71**:214–219

Träuble, H., Overath, P. 1973. The structure of *Escherichia coli* membranes studied by fluorescence measurement of lipid phase transitions. *Biochim. Biophys. Acta* **307**:491–512

Tupper, J.T., Del Rosso, M., Hazelton, B., Zorgniotti, F. 1978. Serum-stimulated changes in calcium transport and distribution in mouse 3T3 cells and their modification by dibutyryl cyclic AMP. *J. Cell. Physiol.* **95**:71–84

Tupper, J.T., Zografos, L. 1978. Effect of imposed serum deprivation on growth of the mouse 3T3 cell. *Biochem. J.* **174**:1063–1065

Tupper, J.T., Zorgniotti, F. 1977. Calcium content and distribution as a function of growth and transformation in the mouse 3T3 cell. *J. Cell Biol.* **75**:12–22

Tupper, J.T., Zorgniotti, F., Mills, B. 1977. Potassium transport and content during G_1 and S phase following serum stimulation of 3T3 cells. *J. Cell. Physiol.* **91**:429–440

Vannucci, S., Del Rosso, M., Cella, C., Urbano, P., Chiarugi, V. 1978. Surface glycosaminoglycans and calcium distribution in 3T3 cells. *Biochem. J.* **170**:185–187

Whitfield, J.F., MacManus, J.P., Rixon, R.H., Boynton, A.L., Youdale, T., Swierenga, S. 1976. The positive control of cell proliferation by the interplay of calcium ions and cyclic nucleotides. A review. *In Vitro* **12**:1–18

Whitfield, J.F., Rixon, R.H., Perris, A.D., Youdale, T. 1969. Stimulation by calcium of the entry of thymic lymphocytes into the deoxyribonucleic acid-synthetic (S) phase of the cell cycle. *Exp. Cell Res.* **57**:8–12

Wilson, G., Rose, S.P., Fox, C.F. 1970. The effect of membrane lipid unsaturation on glycoside transport. *Biochem. Biophys. Res. Commun.* **38**:617–623

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