DIFFUSION RATES OF CELL SURFACE ANTIGENS OF MOUSE-HUMAN HETEROKARYONS

II. Effect of Membrane Potential on Lateral Diffusion

MICHAEL EDIDIN and TAIYIN WEI

From the Biology Department, The Johns Hopkins University, Baltimore, Maryland 21218

ABSTRACT

The rate of appearance, in a population of mouse-human heterokaryons, of cells with intermixed mouse and human surface antigens may be used to estimate the rate of lateral diffusion of the antigens in a single cell. Most heterokaryons appear to restrict diffusion of their surface antigens. These restrictions are altered by exposing either heterokaryons or their parent cells to conditions that change cell surface membrane potential. Media containing unphysiological concentrations of potassium ion, drugs, affecting the Na^+, K^+ ATPase, or a channel-forming antibiotic, gramicidin, all affect lateral mobility of cell surface antigens in a manner consistent with a common effect on membrane potential.

KEY WORDS diffusion · membrane potential · heterokaryons · cell surface · surface antigens

A newly formed population of mouse-human heterokaryons rapidly changes from one in which so-called segregate heterokaryons, bearing separate areas of mouse and human antigens on their surfaces, predominate to one in which the bulk of the heterokaryons have fully intermixed surface antigens (7). Analysis of the rate of this change in the population leads to the conclusion that individual heterokaryons allow different rates of diffusion of their surface antigens; 85–90% of the heterokaryons appeared to restrict free diffusion of their surface antigens to some extent (5). This restriction is not affected by Colcemid or cytochalasin B treatment of heterokaryons and of the parent cells fused to form them.

Estimates of the mean diffusion constants for the H-2 and HLA antigens of heterokaryons are in good agreement with estimates obtained by using another technique, fluorescence photobleaching for diffusion of labeled proteins of mouse and rat cells, suggesting that the Sendai virus used to form heterokaryons does not itself greatly alter the cell membrane, so as to give falsely high or low estimates of diffusion in heterokaryons. However, the maximum rates reported for diffusion in heterokaryons are 2- to 10-fold higher than those found in photobleaching. After reports that Sendai virus treatment altered permeability and caused depolarization of cell membranes (17), we attempted to exacerbate this effect by incubation of parent cells and heterokaryons in media that might be expected to mimic or counteract the depolarization caused by the virus. We find that incubation of cells in medium of altered potassium ion content, expected to depolarize or hyperpolarize the cell membrane, drastically alters the mobility of surface antigens of heterokaryons. Other treatments of cells, with the drugs ouabain, gramicidin, and diphenylhydantoin, also affect membrane antigen diffusion rates.

The data are consistent with an effect of membrane potential upon the restricted diffusion of cell surface antigens in heterokaryons.

MATERIALS AND METHODS

Cell lines, antisera, salt solutions, and fusion procedures are described in the preceding paper (5). "High potassium" Hanks' solution (high K^+ medium) was made up to contain 2.5, 5, or 10 times the standard (5.6 mM) potassium ion concentration (14, 28, or 56 mM K^+), with KCl. "Low potassium" Hanks' solution (low K^+ medium) was 0.56 mM or 0.06 mM in KCl. Equivalent amounts of NaCl were removed from or added to a high K^+ or low K^+ medium to maintain osmotic balance. Ouabain was prepared as a 3×10^{-3} M stock in *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES)-Hanks' solution. It was diluted into Hanks' solution before use. Gramicidin D (Sigma Chemical Co., St. Louis, Mo.) was prepared as a 3×10^{-4} M stock in 95% ethanol. 5,5'-diphenylhydantoin (Sigma Chemical Co.) was prepared as a 10^{-2} M solution in dimethylsulfoxide (DMSO).

Unless otherwise noted in the section Results, parent cells were pretreated with media of altered potassium ion concentration or with drugs for 30 min, in ice, before fusion.

RESULTS

Exposure of parent cells to medium containing concentrations of K^+ ion other than 5.6 mM drastically altered the mobility of the surface antigens in heterokaryons derived from the treated cells. In Fig. 1 are plotted pooled data from seven control experiments, showing the range of antigen segregate heterokaryons remaining at various times after initiating fusion, together with similar plots for cells pretreated with either high K^+ or low K^+ medium. It will be seen that high potassium medium decreased the fraction of segregates in the initial sample, made 3 min after initiating fusion, while it scarcely affected the rate at which interdiffusion of surface antigens proceeded in the remaining segregates. Low potassium medium, on the other hand, had no effect on the fraction of segregates appearing in the initial population sample, but effectively blocked further loss of segregates from the heterokaryon population.

The low potassium medium had to be applied before initiating fusion, and in the cold in order to be effective. Fig. 2 compares the time history of heterokaryons either made from parent cells treated with low K^+ medium (Fig. 2*a*) or treated, immediately after their formation, with low K^+ medium (Fig. 2*b*). Table I summarizes the times and temperatures at which altered potassium ion

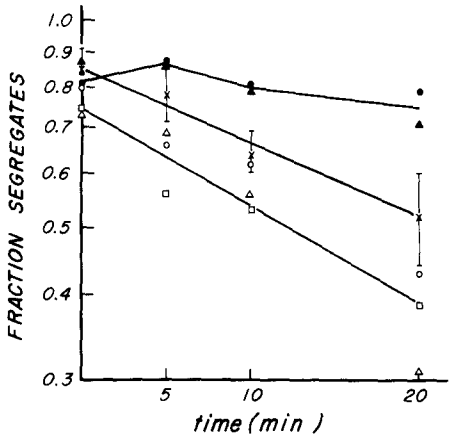

FIGURE 1 Effect of treatment of heterokaryon parent cells with "high potassium" or "low potassium" medium before fusion. (X) Mean of seven control experiments. The bars indicate the maximum range of values found in the seven experiments. Open symbols: parent cells pretreated with high potassium medium. (△) 14 mM K^+; (□) 28 mM K^+; and (○) 56 mM K^+. Closed symbols: parent cells treated with low potassium medium. (●) 0.56 mM K^+; and (▲) 0.06 mM K^+.

Hanks' solution had to be applied to effect alterations in the antigen diffusion rates of heterokaryons. In it, we consider the experiment in three time periods: prefusion, fusion, and postfusion. Treatment in the prefusion period is treatment of heterokaryon parent cells, in suspension, before addition of any Sendai virus. Treatment during fusion was carried out for 13 min only, 10 min at 0°C and 3 min at 37°C, in the presence of concentrated virus. For postfusion treatment, medium containing drugs or altered concentrations of potassium ion was added upon dilution of the concentrated cell/virus suspension after 3 min at 37°C; newly formed heterokaryons were maintained in the medium for 20–40 min thereafter.

Only prefusion treatment, of parent cells rather than heterokaryons, had an effect on apparent diffusion rate of surface antigens.

The effects of treatment with Hanks' solution of altered potassium ion content could be approximated by treatment of heterokaryons with drugs either acting on the sodium-potassium ATPase or forming membrane channels for Na^+ and K^+. Treatment of parent cells with 3 mM ouabain for 1 h before fusion resulted in cell populations in which 55% of heterokaryons had intermixed their surface antigens at a rate approaching that expected for free diffusion, while the rates of diffusion in the remaining segregate heterokaryons

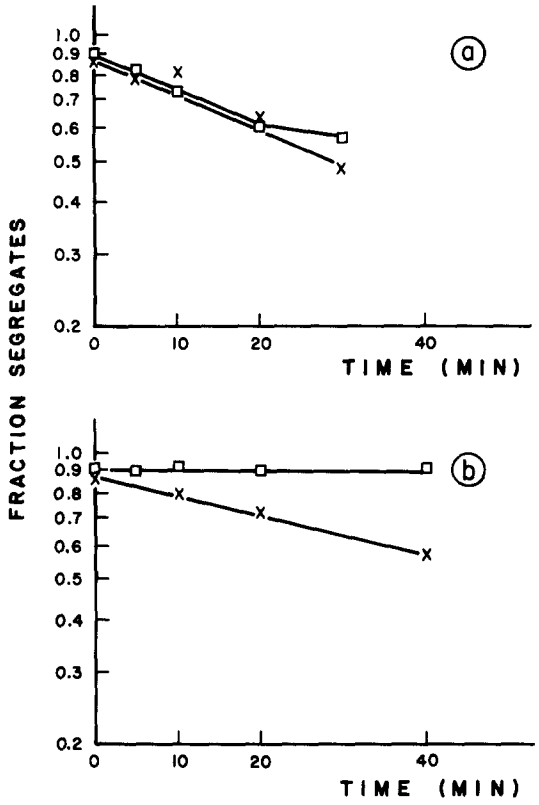

FIGURE 2 Effect of low potassium treatment, at different times during the course of a fusion experiment, on lateral diffusion of heterokaryon antigens. (*a*) Parent cells exposed to low potassium medium, in ice, for 30 min before fusion. (X) Control, untreated cells; and (□) cells in low K^+ medium. (*b*) Heterokaryons exposed to low potassium medium from the beginning of fusion until 40 min after the initial population sample was taken. (X) Control, untreated; and (□) cells in low K^+ medium.

TABLE I

Effect of Hanks' Solution with Altered Potassium Ion Content on Heterokaryon Surface Antigen Lateral Diffusion

Medium	Time of application	Temperature	Effect on diffusion
		°C	
Low K^+	Prefusion	0	Inhibits
Low K^+	Prefusion	37	None
High K^+	Prefusion	0	Enhances
High K^+	Prefusion	37	Enhances
Low K^+	Fusion or postfusion	37	None
High K^+	Fusion or postfusion	37	None

seemed varied to the same extent as in control populations (Fig. 3*a*). Lower concentrations of ouabain had either no effect (at 10^{-3} mM) or an intermediate effect (at 1 mM) on the population (Fig. 3*b* and *c*).

5,5′-diphenylhydantoin, when applied to parent cells for 1 h at 37°C before fusion, gave a result approximating that after treatment with low potassium medium. In the experiment shown here (Fig. 4), approx. 20% of heterokaryons allowed interdiffusion of their surface antigens, and all of these were observed in the first sample taken after initiating fusion.

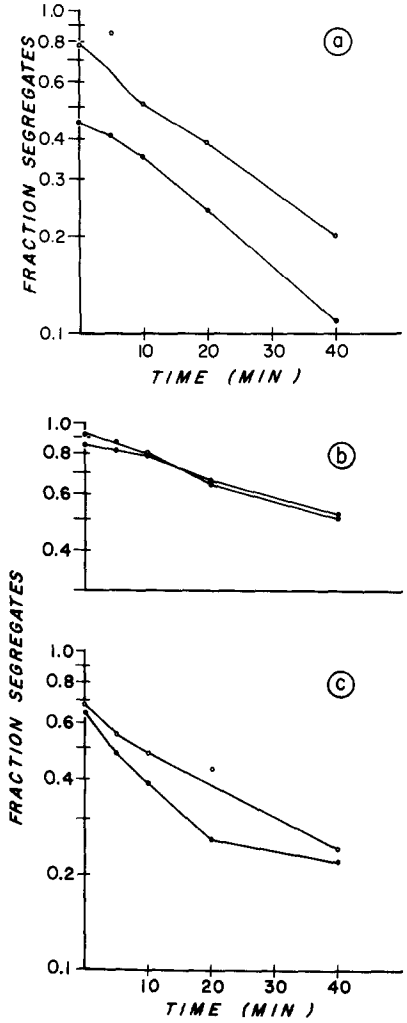

FIGURE 3 Effect of ouabain treatment of parent cells on heterokaryon antigen diffusion rates. (*a*) 3 mM ouabain for 1 h at 37°C before fusion. (○) control; and (●) 3 mM ouabain. (*b*) 10^{-3} mM ouabain, otherwise as Fig. 3*a*. (*c*) 1 mM ouabain, otherwise as Fig. 3*a* and *b*.

Gramicidin D treatment of parent cells before fusion resulted in a majority of the heterokaryon population's allowing free diffusion of surface antigens (only 45% segregates remaining in the initial population sample taken 3 min after initiating fusion). Again, the remaining segregate heterokaryons were apparently little affected by gramicidin treatment (Fig. 5).

Diameters of single cells and heterokaryons from standard fusions, fusions of high potassium Hanks'-treated, ouabain-treated, or 5,5'-diphenylhydantoin-treated parent cells were measured with a filar micrometer and compared to the diameters and calculated volumes of parent single cells. Sendai virus itself causes a 40% increase in single cell volume immediately after initiating fusion. Single cells from 40-min samples were slightly less swollen, with volumes about 30% greater than those of control single cells not treated with Sendai virus. None of the treatments that modified the antigen mobility altered the cell volumes significantly with respect to the volumes observed for single Sendai virus-treated cells. The volume of the limited number of dikaryons measured (8–10 for each treatment) was within 10% of twice the volume of single virus-treated cells. Again, no difference was found between treated groups.

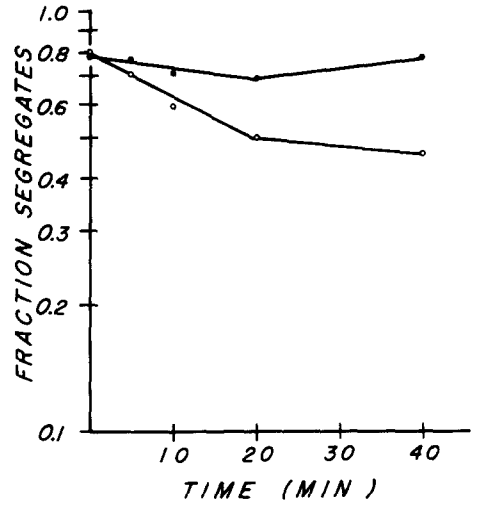

FIGURE 4 Effect of 5,5'-diphenylhydantoin treatment of parent cells on heterokaryon antigen diffusion rates. (●) 5×10^{-4} M DPH for 1 h at 37°C before fusion; and (○) control.

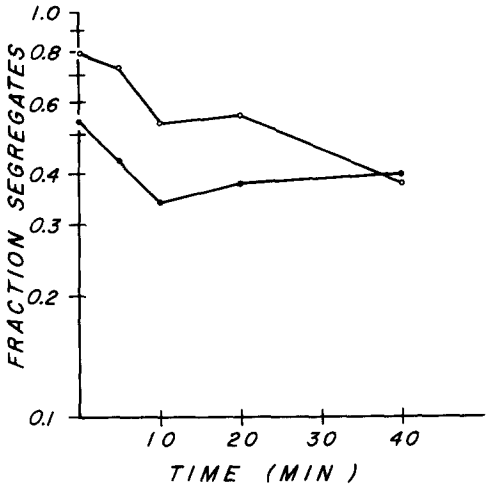

FIGURE 5 Gramicidin D treatment of parent cells at room temperature. (●) 3×10^{-6} M gramicidin for 10 min at room temperature; and (○) control.

DISCUSSION

Membrane potentials of nonexcitable cells are generally not so high as tnose in nerve and muscle cells (26). However, it appears that potentials in nonexcitable cells change in response to changes in external potassium ion concentration in the direction predicted from studies on nerve and muscle (8, 11). In the experiments described here, treatments expected to alter membrane potential result in alterations in the lateral diffusion rates of membrane antigens of heterokaryons. Increasing the external K^+ concentration increases the proportion of heterokaryons whose surface antigens diffuse at or near the maximum rate predicted, apparently by releasing the constraints on mobility of antigens in the subpopulation of heterokaryons which, in control, partly restrict diffusion of their surface antigens. On the other hand, reducing the external K^+ concentration fails to affect the cells allowing maximal diffusion of surface antigens, but increases restrictions on diffusion in all other heterokaryons.

Drugs affecting the cell surface (Na^+, K^+)-ATPase, the sodium pump, altering the internal concentrations of Na^+ and K^+ and thus altering membrane potential have effects in the directions predictable from the results obtained with K^+-rich or -poor medium. Ouabain, an inhibitor of the sodium pump (9, 23), when applied at the high concentrations required to inhibit the mouse pump (14), has an effect, like that of high K^+ medium, of increasing the rates of lateral diffusion in all heterokaryons, with a substantial fraction allowing free diffusion. We would expect that ouabain acts to reduce internal levels of cell potas-

sium, and to increase internal Na^+, again reducing membrane potential relative to the medium (14).

Because of the low affinity of ouabain for the mouse Na^+, K^+-ATPase, especially when applied, as in the present experiments, in medium containing potassium, concentrations used were 1–3 × 10^3 greater than are effective on human cells (4). Hence, it might be argued that the ouabain effect seen on heterokaryons is not due to an effect on the ATPase-pump, but is a consequence of the nonspecific binding that occurs at high concentrations of the drug (2). At least some evidence against this is provided by the action of the drug 5,5′-diphenylhydantoin. This drug is thought to act by stimulating the Na^+, K^+-ATPase (15, 27); hence, it ought to increase internal potassium ion concentrations and slightly raise cell membrane potential. Indeed, its effect is similar to that of low K^+ medium, inhibiting diffusion of surface antigens in most heterokaryons in a population. Thus, the two drugs that have opposite effects on the Na^+, K^+-ATPase have opposite effects on antigen diffusion rates, and this antithesis follows that predicted from the effects of altered external K^+ concentration on antigen diffusion.

A third approach to altering ionic composition and membrane potential of our cells has been treatment with the antibiotic gramicidin. This compound forms sodium- and potassium-conducting channels through artificial and natural membranes (10, 20). It has been shown to depolarize cell and membrane preparations (19). Treatment with gramicidin D effected a greatly increased fraction of cells with a high diffusion rate for surface antigens at the expense of those heterokaryons allowing intermediate rates of antigen diffusion. The remaining heterokaryons appeared unaffected by the drug over the time of observation.

Thus, three different approaches to altering transmembrane potentials, alteration of potassium ion concentration in the medium but not in the cells, alteration of potassium ion content of cells by interfering with the Na^+, K^+-ATPase, and alteration of the passive permeability of the membrane to potassium, all affect the rate of lateral diffusion of surface antigens in heterokaryons.

Heterokaryon populations appear to include a subpopulation, usually 10–15% of the total heterokaryons, whose surface antigens interdiffuse with a rate, $D \simeq 1\text{–}4 \times 10^{-9}$ cm^2 s^{-1}, which is that predicted for free diffusion (5). The remainder of the heterokaryons place some restrictions on antigen diffusion, resulting in apparent diffusion constants 10–30 times slower than the maximum rate. Indeed, still greater constraints may be imposed in some cells; we do not follow the heterokaryon population for more than 40 min after initiating fusion. Changes in membrane potential appear to affect only those cells which restrict diffusion to some extent, though we cannot accurately estimate changes in antigen diffusion constants of cells in the first, 3-min, sample from the population. Depolarizing treatments enhance the diffusion of surface antigens in cells that otherwise restrict surface lateral diffusion. This is seen as an increase in the fraction of all heterokaryons whose antigens have intermixed in the 3 min required to initiate fusion between mouse and human parent cells, and, in most instances, further loss of segregates from the treated population parallels the loss from the control population. Hyperpolarizing treatments, on the other hand, fail to alter the fraction of heterokaryons with freely interdiffusion surface antigens; this fraction in the first sample taken is the same as in the controls. Hyperpolarizing treatments do affect all cells with any degree of restriction on antigen diffusion, converting them all to cells in which interdiffusion appears, on the time and spatial scale of our measurement, to be completely restricted. Thus, changes in membrane potential appear to convert a partial restriction on, or anchor of, integral membrane proteins to one of two extreme states, fully unlocked or fully locked. While all measurements of surface antigen diffusion are made in heterokaryons, the membrane potential effects on restriction of mobility appear to take place in the parent cells used for fusion. Only pretreatment of parent cells is effective in causing the changes described here. Incubation of forming and newly formed heterokaryons in high or low K^+ medium does not alter the diffusion rates of their surface antigens (Table I). Whatever the locking and unlocking process, it seems to require some time to be effective. This observation is consonant with Okada's observation of the brief time-course of Sendai-virus-induced depolarization of fused cells (17). This transient depolarization is not enough to free the surface antigens from constraints on their diffusion; this is evident both from the data presented here on the effect of low K^+ medium, and from previous experiments indicating that other cultured cells, notably 2° mouse fibroblasts and human WI-38 cells, form heterokaryons whose H-2 and HLA antigens diffuse at least sixfold slower than those of transformed cells (6).

The data on timing of high K^+ and low K^+ treatments also suggest that restrictions to mobility may be applied or removed without intervening cellular metabolism. The effect of high K^+ medium is similar whether applied at 0°C or 37°C, while low K^+ is effective only when applied at 0°C.

Whatever the mechanisms, if potential-dependent alterations in membrane protein diffusion are widespread they could be the basis for several effects reported for non-nervous system cells. In particular, extended exposure to depolarizing conditions has been reported to induce mitosis in otherwise quiescent cells (3) and to enhance [^{3}H]thymidine uptake and growth of normally dividing cells (16), though other data indicate that at extremes such culture conditions may interfere with cell proliferation (18, 25). A rapid flux of potassium and rubidium ions has been observed immediately after treatment of lymphocytes and tumor cells with mitogenic lectins (1, 12, 21, 24) and after mitotic stimulation of quiescent 3T3 cells by serum (22); recently, membrane depolarization has been implicated in the block to polyspermy in sea urchins (13). It may be that all of these systems involve some membrane proteins whose mobility in the plane of the membrane is required to trigger or maintain the series of events leading to mitosis, or to the early surface alterations in sea urchin development. However, such speculation must be tempered by the fact that altered mobilities may be secondary consequences of changes in membrane potential, and we must look elsewhere to establish a chain of causation from altered potential to cell division or other biological effects.

This paper was supported by a contract CB-43922 and a grant AM11202 from the National Institutes of Health. This is contribution 920 from the Department of Biology, The Johns Hopkins University.

Received for publication 10 December 1976, and in revised form 19 May 1977.

REFERENCES

1. AVERDUNK, R. 1976. Early changes of "leak flux" and the cation content of lymphocytes by concanavalin A. *Biochem. Biophys. Res. Commun.* **70:**101–109.
2. BAKER, P. F., and J. S. WILLIS. 1972. Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol. (Lond.).* **224:**441–462.
3. CONE, C. D., JR., and C. M. CONE. 1976. Induction of mitosis in mature neurons in central nervous system by sustained depolarization. *Science (Wash. D. C.).* **192:**155–157.
4. DAHL, JUNE L., and L. E. HOKIN. 1974. The sodium-potassium adenosine triphosphatase. *Annu. Rev. Biochem.* **43:**327–356.
5. EDIDIN, M., and T.-Y. WEI. 1976. Diffusion rates of cell surface antigens of mouse-human heterokaryons. I. Analysis of the population. *J. Cell Biol.* **75:**475–482.
6. EDIDIN, M., and A. WEISS. 1974. Restriction of antigen mobility in the plasma membranes of some cultured fibroblasts. *In* Control of Proliferation in Animal Cells. (B. Clarkson and R. Baserga, editors.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 213–219.
7. FRYE, L. D., and M. EDIDIN. 1970. The rapid intermixing of all surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7:**319.
8. GOLDMAN, D. E. 1943. Potential, impedance and rectification in membranes. *J. Gen. Physiol.* **27:**37–60.
9. GLYNN, I. M. 1974. The action of cardiac glycosides on ion movements. *Pharmacol. Rev.* **16:**381–407.
10. HARRIS, E. J., and B. C. PRESSMAN. 1967. Obligate cation exchanges in red cells. *Nature (Lond.).* **216:**918–920.
11. HODGKIN, A. L., and B. KATZ. 1949. The effect of sodium on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.).* **108:**37–77.
12. INOUÉ, M., K. UTSUMI, and S. SENO. 1975. Effect of concanavalin A and its derivative on the potassium compartmentation of Ehrlich ascites tumor cells. *Nature (Lond.).* **255:**556–557.
13. JAFFE, LAURINDA A. 1976. Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature (Lond.).* **261:**68–71.
14. LAMB, J. F., and M. G. A. MACKINNON. 1971. Effect of ouabain and metabolic inhibitors on the Na and K movements and nucleotide contents of L-cells. *J. Physiol. (Lond.).* **213:**665–682.
15. LEWIN, E., and V. BLECK. 1971. The effect of diphenyl hydantoin administration on sodium-potassium-activated ATPase in cortex. *Neurology.* **21:**647–651.
16. MCDONALD, T. F., H. SACHS, C. W. ORR, and J. D. EBERT. 1972. External potassium and baby hamster kidney cells: intracellular ions, ATP, growth, DNA synthesis, and membrane potential. *Dev. Biol.* **28:**290–303.
17. OKADA, Y., J. KOSEKI, J. KIM, Y. MAEDA, T. HASHIMOTO, Y. KANNO, and Y. MATSUI. 1975. Modification of cell membranes with viral envelopes during fusion of cells with HVJ (Sendai Virus). I. Interaction between cell membranes and virus in the early stage. *Exp. Cell Res.* **93:**368–378.
18. ORR, C. W., M. YOSHIKAWA-FUKADA, and J. D. EBERT. 1972. Potassium: effect on DNA synthesis

and multiplication of baby hamster kidney cells. *Proc. Natl. Acad. Sci. U. S. A.* **69:**243–247.

19. PODLESKI, T., and J.-P. CHANGEAUX. 1969. Effects associated with permeability changes caused by Gramicidin A in electroplax membrane. *Nature (Lond.).* **22:**541–545.
20. PRESSMAN, B. C. 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* **45:**501–530.
21. QUASTEL, M. K., and J. F. KAPLAN. 1970. Early stimulation of potassium uptake in lymphocytes treated with PHA. *Exp. Cell Res.* **53:**230.
22. ROZENGURT, E., and L. A. HEPPEL. 1975. Serum rapidly stimulates ouabain-sensitive $^{86}Rb^{+}$ influx in quiescent 3T3 cells. *Proc. Natl. Acad. Sci. U. S. A.* **72:**4492–4495.
23. SCHATZMAN, H. J. 1953. Herzglykoside als Hemmstoffe für den activen Kalium und Natriumtransport durch Erythrocytenmembran. *Helv. Physiol. Pharmacol. Acta* **11:**105–115.
24. SEGAL, G. B., B. R. GORDON, M. A. LICHTMAN, M. M. HOLLANDER and M. R. KLEMPERER. 1976. Exodus of $^{42}K^{+}$ and $^{86}Rb^{+}$ from rat thymic and human blood lymphocytes exposed to phytohemagglutinin. *J. Cell Physiol.* **87:**337–344.
25. STAMBROOK, P. J., H. G. SACHS, and J. D. EBERT. 1975. The affect of potassium on the cell membrane potential and the passage of synchronized cells through the cell cycle. *J. Cell Physiol.* **85:**283–292.
26. WILLIAMS, J. A. 1970. Origin of transmembrane potentials in nonexcitable cells. *J. Theor. Biol.* **28:**287–296.
27. WOODBURY, D. M. 1955. Effect of diphenylhydantoin on electrolytes and radiosodium turnover in brain and other tissues of normal, hyponatremic and postical rats. *J. Pharmacol. Exp. Ther.* **115:**74.