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Effects of Nystatin on Intracellular Contents and Membrane Transport of Alkali Cations, and Cell Volume in HeLa Cells

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Summary. Nystatin (50 μg/ml) had strong influence on the intracellular contents and membrane transports of monovalent ions and water in HeLa cells. The nystatin-induced changes in the intracellular ion content and cell volume were inhibited by sucrose, and Donnan and osmotic equilibria were attained. Using cells under conditions for these equilibria, the concentrations of intracellular impermeant solutes, their mean valence, the differences of their intra- and extracellular osmotic concentrations, and the circumferential tension of the cell membrane were determined. Stimulation by nystatin of the influx of one cation species, e.g. Rb, was inhibited by another cation species, e.g. Na. The stimulatory effect of nystatin on cation fluxes was reversible within 1 hr after ionophore addition, and after 1-hr treatment the intracellular contents of Na and K became proportional to their extracellular concentrations, provided that the sum of these concentrations was constant (300 mM). Similar proportionality was also observed in the presence of choline, provided that the choline concentration was less than those of the alkali cations. The implications of these results in relation to the osmotic properties of cultured cells, and the experimental regulation of alkali cations in the cells, are discussed.

Key Words nystatin · alkali cation · cation transport · cell volume · Donnan effect · HeLa cells

Introduction

Ionophores of monovalent cations, including neutral ionophores such as cyclic depsipeptides and macrocyclic tetralide nactins, carboxylic ionophores and polyene antibiotics, have been very useful in investigations of basic mechanisms of transport of ions through artificial lipid bilayer membranes and of the electrical properties of membranes (Mueller & Rudin, 1967; Pressman, 1968; Szabo et al., 1969; Cass et al., 1970; McLaughlin et al., 1972).

These ionophores have been valuable not only as models of carriers (e.g. depsipeptides and tetralide nactins) and of channels (e.g. polyene antibiotics) but also for stimulation of cation uptake and change in the intracellular contents of cations in red cells (Labelle, 1979; Haas et al., 1982) and other types of cells (Pietrzyk et al., 1978; Smith & Rozengurt, 1978; Mendoza et al., 1980). One example of application of the ion transport-stimulating effect of ionophore is the use of one of the polyene antibiotics, nystatin, in combination with sucrose to produce and investigate ionic and osmotic equilibria in red cells (Freedman & Hoffman, 1979). Another example is the development of a technique for controlling the intracellular levels of cations in red cells using nystatin (Cass & Dalmark, 1973). But no similar technique is available yet for nucleated cells, including cultured cells, since the intracellular environment of monovalent cations in these cells seems to be more complex than that of red cells (Smith & Adams, 1977).

In the present study, we demonstrated stronger influences of the channel-forming ionophore nystatin than of the carrier ionophores, valinomycin and monensin, on the fluxes and contents of cations in HeLa cells. Our results indicate that (1) nystatin is a useful tool for analysis of the dynamic and osmotic properties of the cell membrane and (2) the cation contents of the cells can be changed experimentally by dialyzing the cells in the presence of nystatin.

Materials and Methods

Cell Culture

HeLa S3 cells purchased from Flow Lab. were serially cultured in a modified Eagle's minimum essential medium (Miyamoto et al., 1976) supplemented with 10% (vol/vol) calf serum in glass culture flasks. HEPES was omitted from the culture medium. Cells in the exponential growth phase were dispersed with 0.5% trypsin and resuspended in the same culture medium at a density of 5 to 6 $\times$ 10^4 cells/ml. They were then inoculated into plastic culture dishes (60 mm diameter, Corning Glass Works) and incu-

bated in a CO_2 incubator at 37°C in a humid atmosphere of 5% CO_2 in air.

Cell Chilling

After 48-hr cultivation, the cultures were washed once with cold medium of the same composition as that used for incubation of the cells. This medium contained a fixed concentration of either Na or K or choline ions with or without nystatin. The other components of the medium were as reported previously (Ikehara et al., 1984), except that bicarbonate was omitted. Na medium and K medium were prepared as described previously (Ikehara et al., 1984), except that the concentrations of NaCl and KCl were varied. Choline medium contained 1 mM orthophosphoric acid instead of phosphate salt and was adjusted to pH 7.2 with 4 M choline; the stock solution of HEPES (1 M) was also adjusted to pH 7.2 with choline solution, and the concentrations of choline ions were adjusted appropriately with choline chloride. As the cells had to be incubated for 1 to 5 hr in an ice bath, HEPES was an essential requirement for preventing their detachment from the culture dishes.

Preparation of Ionophore Solutions

Appropriate amounts of nystatin were suspended in 15 ml of redistilled water and then solubilized by adding 0.5 ml of 0.5 N NaOH or KOH or 0.1 ml of 4 M choline. The concentrations of the antibiotic in stock solutions were adjusted with redistilled water to 100 times the final concentrations used for experiments. Valinomycin and monensin were dissolved at concentrations of 0.1 to 10 mg/ml in 99.5% ethanol, and mixed with an equal volume of 50% ethanol to give final concentrations of the ionophores in stock solutions of 0.05 to 5 mg/ml. These concentrations were 100 times the final concentrations used for experiments. The same amount of ethanol only was added to controls.

Assays

After incubations, the cells were washed six times in 15 sec as described elsewhere (Miyamoto et al., 1978) except that 0.15 M $LiNO_3$ was used instead of 0.15 M LiCl, and then 3.5 ml of cold redistilled water was added to each culture dish. The cells were scraped off the dishes with a silicone-rubber policeman. Even when the cells were incubated in hypertonic media, they were washed rapidly with this $LiNO_3$ solution, since we showed previously that the intracellular composition of alkali cations was not affected significantly by washing the cells in 15 sec with the normotonic $LiNO_3$ solution.

Then 2 ml of the resulting cell suspension was mixed with 2 ml of 30 mM $LiNO_3$. The mixture was kept overnight at room temperature to allow complete lysis of the cells and used for assay of alkali cations with Li^+ as an internal standard. The assay procedures were as reported previously (Ikehara et al., 1982) except that another type of flame-spectrophotometer (Model 170-30, Hitachi Ltd.) was used. Cl was determined by amperometry with NaCl as a standard. A 0.1 ml drop of 0.01 N CH_3COONa was added to 1 ml of the mixture and titration was carried out with 0.1 to 0.2 mM $AgNO_3$.

Another 1 ml of the cell suspension was mixed with 1 ml of 1 N NaOH and used for assay of protein by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

The diameter of the cells was measured microscopically. For this purpose, the cells were previously trypsinized. The cell volume of HeLa cells was reported not to be changed by trypsinization (Lamb & McCall, 1972). The cell volume was calculated from the cell diameter assuming that the cells were spheroids or ellipsoids. When the cells were greatly swollen, bubbles formed on their surface. The volume of a bubble was calculated from its diameter and height assuming that the bubble represented a portion of a sphere in shape.

The osmotic concentration of the medium was determined by the freezing point method with a Beckmann thermometer and sucrose as a standard.

Reagents and Miscellaneous Substances

Inorganic salts (specially pure grade) and Folin-phenol reagent were purchased from Wako Pure Chemical Co.; choline and choline chloride (specially pure grade) and calf serum were from Nakarai Chemicals; RbCl (superpure) was from Merck; nystatin, valinomycin, bovine serum albumin (fraction V) and HEPES were from Sigma Chemical Co.; monensin (sodium salt) was from Calbiochem-Boehring Corp.; trypsin was from Difco Lab. Concentrated solutions of vitamins (100×) and amino acids (50×) purchased from Flow Laboratories were used to prepare the modified minimum essential medium.

Results

Dose Response to Nystatin

On replacement of the normal medium at 0°C by isotonic medium containing 150 mM NaCl and various concentrations of nystatin without K, the intracellular Na (Na_i) content increased and the K (K_i) content decreased with increase in the nystatin concentration above 5 μg/ml (Fig. 1). In the absence of sucrose, the Na_i increased markedly and did not reach a plateau even with 200 μg/ml nystatin (Fig. 1*A*). This increase was accompanied by cell swelling, which increased with increase in the nystatin concentration. In contrast, Na_i reached a plateau with about 50 μg/ml nystatin in the presence of 40 mM sucrose and 130 mM Na (Fig. 1*B*), indicating inhibition of cell swelling by the osmotic effect of sucrose. On increase in the extracellular Na concentration to 300 mM, a similar but somewhat higher plateau of Na_i was observed in the presence of 40 mM sucrose at nystatin concentrations of more than 50 μg/ml (*data not shown*).

Time Course of Effects of Nystatin

The time-dependent changes in the intracellular contents of Na and Cl and in the cell volume after replacement of normal medium by hypertonic media containing nystatin were examined (Fig. 2). The contents of Na_i and Cl_i per mg cell protein, which

were proportional to the contents per cell, increased rapidly for 1 hr during incubation in media containing 300 mM NaCl, 50 μg/ml nystatin and various concentrations of sucrose (0 to 100 mM). The ions accumulated further at a lower rate for at least 5 hr in the absence of sucrose (Fig. 2*A* and *B*). In contrast, the ion contents remained at constant levels from 2 hr on in the presence of 30 to 40 mM sucrose and they rather decreased when 100 mM sucrose was added. In parallel with the increase in the ion contents, cell swelling occurred markedly in the absence of sucrose and less in the presence of 30 to 40 mM sucrose, whereas the cells tended to shrink slightly on addition of 100 mM sucrose (Fig. 2*E*). The cell swelling occurring with omission of sucrose was due to a Donnan effect of intracellular colloidal solutes, which caused a gradient of osmotic pressure across the cell membrane and consequent entrance of water into the cells. When 30 to 40 mM sucrose was added, the Donnan and osmotic equilibria could be established after certain increases in the cell volume. The intracellular concentrations of Na and Cl in the presence of 30 to 100 mM sucrose reached plateau levels after 2 hr (Fig. 2*C* and *D*), and differences in the levels at different sucrose concentrations were insignificant. In the absence of sucrose, the ion concentrations increased with time, but were not yet in equilibrium after 5 hr.

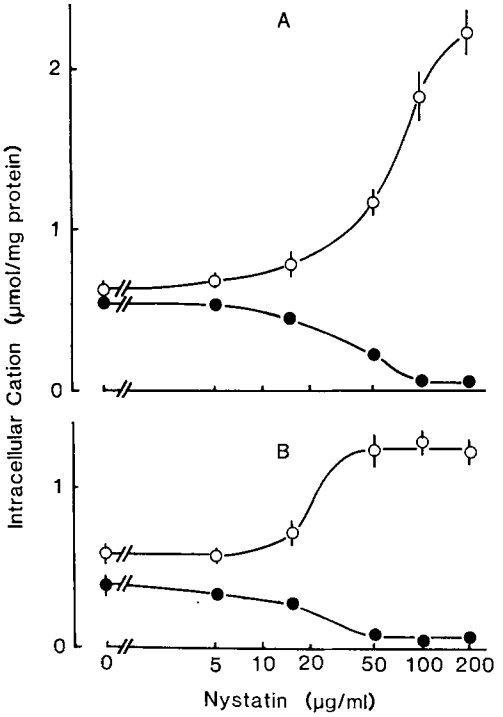

Fig. 1. Changes in the intracellular Na and K contents of HeLa cells as functions of the concentration of nystatin. The cells were chilled for 2 hr. (*A*) 150 mM Na without K or sucrose. (*B*) 130 mM Na with 40 mM sucrose and without K. ○, Na_i; ●, K_i

Effects of Sucrose Concentration

As sucrose restrained entry of monovalent ions into the cells in the presence of nystatin, we re-examined the effects of the sucrose concentration on the cell volume and the intracellular concentrations of the ions at 5 hr, i.e. in the plateau phase (Fig. 3). On addition of 15 to 100 mM sucrose to the hypertonic

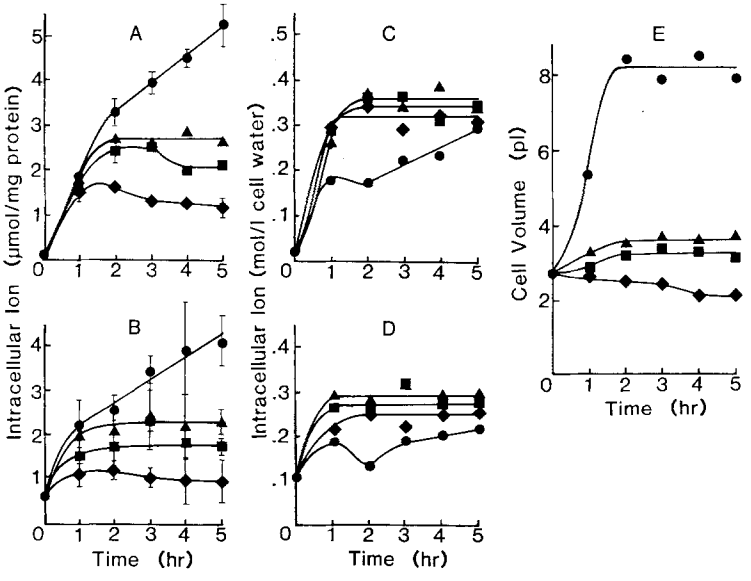

Fig. 2. Time-dependent changes in the intracellular Na and Cl contents and the cell volume of HeLa cells incubated in chilled media with 300 mM NaCl and various concentrations of sucrose in the presence of 50 μg/ml nystatin. (*A*) Na content per mg cell protein. (*B*) Cl content per mg cell protein. (*C*) Na concentration per liter cell water. (*D*) Cl concentration per liter cell water. (*E*) Cell volume. ●, no sucrose; ▲, 30 mM sucrose; ■, 40 mM sucrose; ◆, 100 mM sucrose. Points and bars in (*A*) and (*B*) are means ± SD for 4 to 8 samples. Cell volumes are means for 110 to 140 cells. Intracellular ion concentrations were calculated from the ion contents per mg protein assuming that the protein content of HeLa cells is 0.414 ng/cell and the volume of nonaqueous components corresponds to 17.6% of the normal cell volume

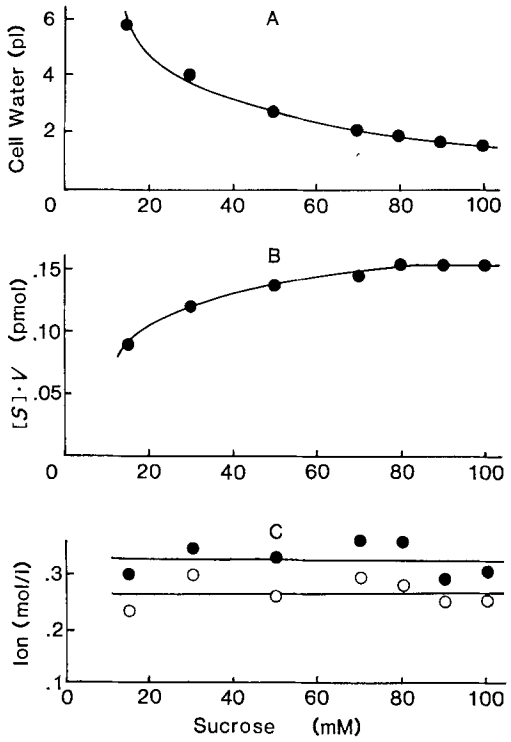

Fig. 3. Effects of the sucrose concentration in medium on the intracellular water content and the concentrations of Na and Cl in HeLa cells. The cells were chilled for 5 hr in medium with 300 mM NaCl and 50 μg/ml nystatin. (*A*) Effect on the volume of cell water. (*B*) Effect on the product of the sucrose concentration [*S*] and the volume of cell water *V*. (*C*) Effect on the intracellular concentrations of Na and Cl: ●, Na; ○, Cl. The regression curves and lines were obtained by the least-squares method. *See* legend to Fig. 2 for procedure for calculation of the intracellular ion concentration

medium containing nystatin, the volume of cell water again changed to various extents depending on the sucrose concentration (Fig. 3*A*). Figure 3*A* suggests that the cells retained the normal volume of cell water, 2.421 pl, in the presence of 61 mM sucrose. The concentrations of Na_i and Cl_i corresponding to $[m]_i$ and $[a]_i$ in the *Appendix* did not change significantly as functions of the sucrose concentration [*S*] (Fig. 3*C*). If [*S*] was sufficiently high, the circumferential tension of the cell membrane τ disappeared due to loss of cell water. Under such conditions, Eq. (17) of the *Appendix* is valid and [*S*] becomes equal to the concentration of cellular impermeant solutes [*P*]. Therefore, [*S*]*V* is equal to [*P*]*V*, i.e. the amount of the impermeant solutes per cell, where *V* represents the volume of cell water. This implies that [*S*]*V* remains unchanged when [*S*] is varied. Such a relation of [*S*]*V* *vs.* [*S*] is demonstrated in Fig. 3*B*, indicating that [*S*]*V* remains almost constant at 0.153 pmol, provided that [*S*] is

Table 1. Effect on the intracellular Na (Na_i) content of HeLa cells of 5-hr incubation in chilled hypertonic media containing 300 mM NaCl and different concentrations of sucrose and choline chloride in the presence of 50 μg/ml nystatin[a]

Solute		Osmotic concentration (mOsM)	Na_i (μmol/mg protein)	*P*
Sucrose (mM)	Choline (mM)			
0	70	564	3.15 ± 0.18	<0.01
40	40	565	2.08 ± 0.17	<0.01
100	0	558	1.14 ± 0.12	

[a] Values are means ± SD for the content of Na_i in 8 to 16 samples. The significances of differences between the two neighboring values were evaluated by Student's *t*-test.

higher than a critical concentration of about 84 mM. Figure 3*B* also shows that when [*S*] decreases below the critical concentration, [*S*]*V* decreases. The relation between [*S*] and [*S*]*V* can be described by the equation

$$[S]V = [P]V - (V/RT)2\tau/r,$$

which is derived from Eq. (15) and reveals that τ can be estimated by the difference between the height of the curve at any [*S*] and that at the plateau in Fig. 3*B*. Using the value for the amount of impermeant solutes and the data shown in Fig. 3*A* and *B*, we estimated [*P*], [*P*] − [*S*] and τ for cells with a normal cell volume as 63.2 and 2.2 mosmol/liter cell water and 0.022 N/m, respectively. '[*P*] − [*S*]' represents the difference in the osmotic concentrations across the cell membrane. Next, we estimated $[Na]_i - [Cl]_i$, i.e. $[m]_i - [a]_i$ in the *Appendix*, of cells incubated in the presence of 61 mM sucrose from the regression lines in Fig. 3*C* as 62.5 mmol/liter cell water. We have shown that the cells have a normal water content at this sucrose concentration. The osmotic concentrations of hypertonic media containing 300 mM NaCl and various concentrations of sucrose were determined previously to be 458, 487, 497 and 558 mOsM at sucrose concentrations of 0, 30, 40 and 100 mM, respectively. This result reveals that the extracellular concentrations of Na^+ and Cl^- ions and NaCl molecules, which correspond to $[M^+]_e$, $[A^-]_e$ and [*MA*] in the *Appendix*, remain constant at about 160, 160 and 140 mM, regardless of the sucrose concentration. By introducing these values into Eqs. (13), (14) and (16) we calculated the concentrations of Na^+ and Cl^- ions, the average valence of impermeable solutes, and the Donnan potential of the cells with a normal cell volume, as 191 and 129 mM, −0.99 and −5 mV, respectively.

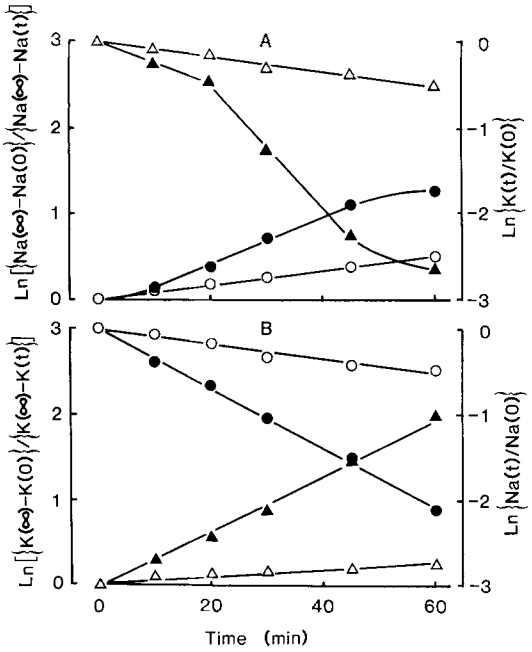

Fig. 4. Comparison of net fluxes of Na and K in chilled HeLa cells in the presence and absence of nystatin. The cells were preincubated for 3 hr in cold medium containing 300 mM Na or K without nystatin, and then the medium was changed at zero time to that containing 300 mM of the counter-cation with or without nystatin. Both media commonly contained 70 mM sucrose. (*A*) Na influx and K efflux. Na(0) = 0.103 μmol/mg protein; Na(∞) = 2.100 μmol/mg protein; K(0) = 1.574 μmol/mg protein. (*B*) Na efflux and K influx. Na(0) = 0.953 μmol/mg protein; K(0) = 0.369 μmol/mg protein; K(∞) = 2.100 μmol/mg protein. Na_i: ○, control; ●, with 50 μg/ml nystatin. K_i: △, control; ▲, with 50 μg/ml nystatin

Table 2. Stimulatory effect of nystatin on Na and K fluxes across the membrane of chilled HeLa cells[a]

Flux	Rate constant	
	Control (%/min)	Nystatin (%/min)
Na influx	0.53 ± 0.27	3.54 ± 0.62
Na efflux	0.64 ± 0.13	3.46 ± 0.79
K influx	0.57 ± 0.20	3.49 ± 0.71
K efflux	0.87 ± 0.25	7.54 ± 0.88

[a] Values are means ± SD for the rate constants in 4 to 5 samples. Rate constants were calculated as the slopes of linear parts of plots obtained by the procedure shown in Fig. 4. The parameters used in calculations were as follows: Na(0) = 1.093 ± 0.187 μmol/mg protein (197 ± 34 mM); Na(∞) = 2.100 ± 0.141 μmol/mg protein (394 ± 26 mM); K(0) = 1.474 ± 0.115 μmol/mg protein (266 ± 21 mM); K(∞) = 2.100 ± 0.100 μmol/mg protein (394 ± 19 mM).

Table 3. Effect of time of preincubation with 50 μg/ml nystatin on Rb influx into HeLa cells chilled for 3 hr in hypertonic medium containing 150 mM Na and K and 70 mM sucrose[a]

Preincubation time (min)	Rb influx (nmol/mg protein per min)
0	32.02 ± 1.76
10	54.35 ± 4.99
20	58.60 ± 5.36
30	68.40 ± 2.82
45	67.56 ± 4.05
60	67.48 ± 2.32

[a] Values are means ± SD for four samples. Rb influx was assayed for 5 min. The assay medium contained 150 mM Na, 100 mM K, 50 mM Rb and 70 mM sucrose in addition to nystatin.

Effect of Choline

In contrast to sucrose, choline ions did not fully inhibit accumulation of Na_i, suggesting that choline did not function effectively as an impermeable ion to counteract the Donnan effect (Table 1).

Cation Fluxes

After incubation of the cells for 3 hr in hypertonic medium containing 300 mM Na or 300 mM K with or without 50 μg/ml nystatin, the medium was replaced by medium containing the same concentration of the counter-cation. By this technique we could simultaneously determine the net influx of the cation into the cells and the net efflux of the counter-cation from the cells. The fluxes were apparently described by first-order reactions in relation to Na_i or K_i (Fig. 4). In the absence of nystatin, the curves had constant slopes for at least 60 min. In the presence of 50 μg/ml nystatin, the fluxes were significantly accelerated and the slopes became constant after a lag period of 10 to 20 min. The apparent rate constants of the fluxes were calculated by the least-squares method from the slopes of the linear parts of the curves. In the absence of nystatin, the influxes of the two cations were similar and K efflux was faster than the influxes of the two cations and Na efflux (Table 2). On addition of 50 μg/ml nystatin, the rate constants of Na influx, Na efflux and K influx increased about sixfold and that of K efflux increased about ninefold.

The flux rates seemed to become constant within 20 min after addition of nystatin, as calculated from the net changes in the intracellular cation contents. However, 30 min was required for unidirectional influx of one cation species, e.g. Rb, to reach a constant rate of more than double the rate determined without nystatin preincubation (Table 3). This lag time may be partly related to unstirred

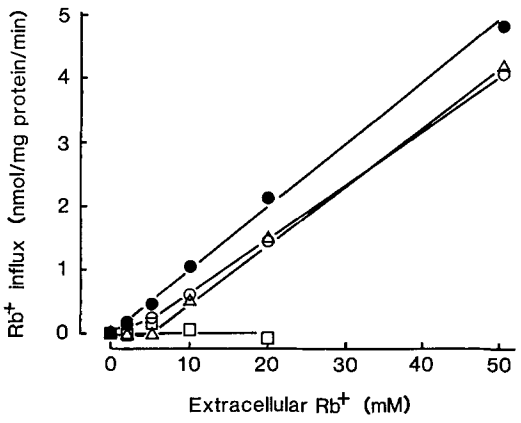

Fig. 5. Nystatin-stimulated part of Rb influx into HeLa cells in relation to the Rb concentration in medium containing various concentrations of Na. The cells were preincubated in chilled medium containing 150 mM Na, 150 mM K and 100 mM sucrose for 2 hr. Then, for assaying Rb influx, they were incubated for 10 min in chilled medium containing 150 mM K and various concentrations of Rb, Na and sucrose with or without 50 μg/ml nystatin. The nystatin-stimulated fraction of Rb influx was calculated by subtracting Rb influx in the absence of nystatin from that in the presence of nystatin. The sum of the concentrations of alkali cations plus half the sucrose concentration in the incubation medium was kept at 350 mM. ●, Na-free; ○, with 50 mM Na; △, with 100 mM Na; □, with 150 mM Na

layers, but is probably mainly the time necessary for nystatin molecules to become fully arranged in the membrane and act constantly.

Cation Influx in Relation to Concentration

The relation between the nystatin-stimulated fraction of influx of a cation species and its extracellular concentration, and the effect on this influx of another cation species were investigated (Fig. 5). Rb was chosen as the cation species, since low concentrations of this ion could be determined easily. The media used in this experiment all contained 150 mM K and various concentrations of sucrose of at least 50 mM to prevent cell swelling. In the absence of Na, nystatin-stimulated Rb influx was proportional to the Rb concentration up to 50 mM. When the Na concentration was increased to 50 to 100 mM, the linear curve was shifted to the right, which indicated that Na inhibited Rb influx at Rb concentrations of below 5 mM. On incubation with 150 mM Na, Rb influx at Rb concentrations below 20 mM was completely inhibited. These results imply that influx of one cation species through the channels formed by nystatin is inhibited by another cation species depending on the relative concentrations of the two cations. However, the results do not show whether the channels can distinguish one alkali cation from another.

Table 4. Reversibility of effect of 50 μg/ml nystatin on cation fluxes across the membrane of HeLa cells[a]

Preincubation time (hr)	Nystatin		Rate constant	
	Preincubation	Incubation	Na influx (%/min)	K efflux (%/min)
1	−	−	0.53	0.80
	+	−	0.76	0.94
	+	+	4.03	5.86
2	−	−	0.95	1.17
	+	−	3.60	4.01
	+	+	9.55	12.32
3	−	−	0.69	1.38
	+	−	10.26	3.35
	+	+	14.82	23.43

[a] The preincubation and incubation media contained 300 mM K or 300 mM Na and 70 mM sucrose. The symbols + and − indicate the presence and absence of nystatin in these media. The rate constants of cation fluxes were determined as shown in Fig. 4.

Reversibility of Nystatin Effect

Next we studied the maximum time of preincubation with nystatin after which either the antibiotic was hard to wash away or its stimulatory action on cation fluxes became irreversible, and hence, the increased permeability remained unchanged on removal of the antibiotic. Results showed that the rate constants of Na influx and K efflux returned to almost normal values when the preincubation time was limited to within 1 hr (Table 4); with longer preincubation times, the effects of nystatin on the fluxes of both cations became irreversible.

Effects of Nystatin on Na_i and K_i

The relations of the intracellular cation contents to the extracellular concentrations of the cations in the presence of nystatin and sucrose were investigated (Fig. 6). Although Na_i and probably also K_i were not in equilibrium after incubation for 1 hr (Fig. 2A), the incubation time was fixed at 1 hr in these experiments because the effect of nystatin was reversible within 1 hr (Table 4). When the sum of the Na and K concentrations in the medium was held at 300 mM but the concentrations of Na and K were changed, linear relations were observed between the contents of Na_i and K_i and the extracellular concentrations of these cations, and the slopes of the two linear

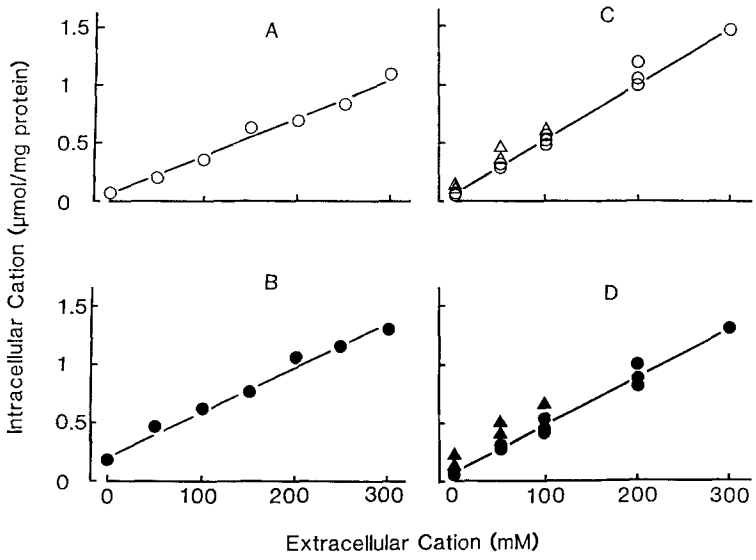

Fig. 6. Intracellular contents of Na and K in chilled HeLa cells in relation to the extracellular concentrations of the cations in the presence of 50 μg/ml nystatin and 70 mM sucrose. (*A*) and (*B*) The sum of the extracellular concentrations of Na and K was fixed at 300 mM. (*C*) and (*D*) The sum of the extracellular concentrations of Na, K and choline ions was kept at 300 mM. ○, Na_i and ●, K_i in the absence and presence of low concentrations of choline (≦150 mM) in the media. △, Na_i and ▲, K_i in the presence of high concentrations of choline (≧200 mM) in the media. The cells were incubated in these cold media for 1 hr

plots were not significantly different (Fig. 6*A* and *B*). Next, we used choline ions in addition to Na and K, keeping the sum of the extracellular concentrations of the three cations at 300 mM, but changing the concentrations of Na and K independently. Again, proportional relations of the contents of Na_i and K_i to their extracellular concentrations were observed (Fig. 6*C* and *D*). However, when the concentration of choline ions exceeded 200 mM, plots of Na_i and K_i deviated upward from linearity.

Effects of Other Ionophores

Valinomycin at concentrations of more than 0.5 μg/ml seemed to stimulate Na influx and K efflux, but its effect was very weak and was saturated at 5 μg/ml (*data not shown*). Monensin (0.5 to 50 μg/ml) had no influence on Na influx but slightly stimulated K efflux from HeLa cells (*data not shown*).

Discussion

The present study was undertaken to obtain basic data necessary for analyzing the osmotic properties of cultured cells and developing a suitable technique to regulate the intracellular levels of monovalent cations in the cells. The channel-forming ionophore, nystatin, was very useful for such studies.

Addition of nystatin induces swelling of red cells, which can be prevented by the presence of impermeant sucrose, resulting in rapid equilibration of Na and K (Freedman & Hoffman, 1979). Similar swelling of HeLa cells was observed upon addition of nystatin due to a Donnan effect of intracellular colloidal macromolecules with negative valences. This cell swelling was prevented to various extents by sucrose depending on its concentration in the medium. Although addition of 30 to 40 mM sucrose did not fully inhibit cell swelling, Donnan and osmotic equilibria were attained after certain increases in the cell volume. In contrast, the cells tended to shrink in the presence of 70 to 100 mM sucrose. The appropriate sucrose concentration for causing these equilibria without changing the cell volume was estimated to be about 60 mM. This is higher than the concentration of 33 mM reported for red cells incubated in isotonic medium containing nystatin (Freedman & Hoffman, 1979). The discrepancy is probably due to a difference in the intracellular conditions of impermeant solutes, especially colloidal anions, of the two types of cells. The intracellular concentration of impermeant solutes has been estimated to be about 20 mM in nystatin-treated red cells (Freedman & Hoffman, 1979), whereas it is about 60 mM in HeLa cells (*see* Results). Our results were obtained in the presence of nystatin. However, they obviously show that the cells maintain a normal volume by keeping the intracellular osmotic concentration about 2 mOsM higher than that of the medium. This difference in osmotic pressures would be counteracted and balanced by the circumferential tension of the cell membrane of about 0.02 N/m.

Experimental regulation of the intracellular levels of alkali cations is essential for analysis of the mechanisms of transport of cations across the cell membrane. So far, this has been achieved with red blood cells. When HeLa cells are chilled in medium with various concentrations of Na and K, the intra-

cellular contents of the cations change dependent on their external concentrations (Ikehara et al., 1984). However, the intracellular cation contents do not change in proportion to their external concentrations, and so cannot be regulated exactly by this chilling technique. Since dialysis of red cells in the presence of nystatin has been demonstrated to be a very good way of regulating the intracellular concentrations of monovalent ions (Cass & Dalmark, 1973), we tested this procedure on HeLa cells using three types of ionophores: valinomycin (a neutral ionophore), monensin (a carboxylic ionophore) and nystatin (a polyene antibiotic). We found that valinomycin and monensin had only slight effects, but that nystatin caused rapid and marked changes in fluxes and in the intracellular levels of alkali cations. The ineffectiveness of valinomycin and monensin was unexpected in contrast to reports of significant effects on cation permeability of valinomycin in Ehrlich ascites cells (Pietrzyk et al., 1978) and of monensin in various other cultured cells (Mendoza et al., 1980).

Addition of a derivative of ammonium, choline, enabled us to change the Na and K concentrations in the medium independently without great change in the osmotic pressure. Choline did not inhibit the nystatin-induced increase in the Na content of HeLa cells (Table 1), suggesting that choline ions can penetrate the cell membrane. This is not consistent with a report that with red cells, another derivative, tetramethyl ammonium, acts as an impermeant compound like sucrose (Joiner & Lauf, 1978). However, choline ions seemed to penetrate the membrane of HeLa cells less readily than alkali cations, because a temporary overshoot of the intracellular contents of the alkali cations was observed for 1 to 2 hr after medium change to hypertonic media containing a high concentration of choline ($\geqq$200 mM) and low concentration of alkali cation ($\leqq$100 mM), especially Na with later decrease to a steady level, probably due to slower entry of choline ions into the cells (*data not shown*). The temporary increase in the cation contents could result from a compensatory increase in loss of cell water and decrease in net cation efflux in response first to increase in the osmotic pressure of the medium due to the relatively impermeable solute and second to presumable hyperpolarization of the membrane. This increase in the intracellular cation contents was shown as an upward deviation from linearity in the plots in Fig. 6 and could be avoided by use of a more readily permeable derivative than choline.

Distinct cation selectivity of neutral and carboxylic ionophores, including valinomycin, nonactin-type carriers and monensin, has been observed in artificial systems (Pressman, 1968; Krasne & Eisenman, 1976), but low selectivity was reported with polyene antibiotics in lipid membranes (Andreoli & Monahan, 1968; Cass et al., 1970). Similarly, the effluxes of Na and K across the membranes of amphotericin B-treated red cells were reported not to be significantly different (Deuticke et al., 1973). No significant difference was found between the influxes of the two cations into nystatin-treated HeLa cells (Table 2), whereas K efflux was significantly greater than Na efflux. Since K efflux was slightly faster than K influx, even in control cells, there seemed to be a small difference in the native vectorial characters of the cell membrane in K transport. However, the difference was inadequate to explain the great difference between the two K fluxes in the presence of nystatin, which rather suggests asymmetric interaction of the ionophore molecules with membrane components on the two sides of the membrane. In all experiments, the cells were treated with nystatin at 0°C, which is the temperature at which K leakage from liposomes is maximally stimulated by the antibiotic (De Kruijff et al., 1974). In nystatin-treated frog skin, K efflux was not explained by K loss from the cells, suggesting that the efflux may be related in part to active transport (Nielsen, 1971). These results seem similar to those on HeLa cells, but the mechanisms of increased K efflux may be different, since the Na/K-pump activity was completely suppressed in chilled HeLa cells (Ikehara et al., 1982). The increases in permeability of red cells to monovalent ions induced by nystatin returned to normal values on removal of the antibiotic after treatment for 1 hr (Cass & Dalmark, 1973). The action of nystatin on HeLa cells was also reversible when the treatment time was limited to within 1 hr. On treatment for more than 2 hr, the cation fluxes increased and the effect of nystatin was hard to eliminate or became irreversible, indicating progressive change of the membrane.

Finally, we conclude that the dynamic and osmotic characters of the cell membrane of HeLa cells can be accurately analyzed and the contents of alkali cations in the cells are controlled by the technique of cell dialysis again nystatin.

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Appendix

Subscripts *i* and *e* refer to intra- and extracellular compartments separated by the cell membrane, which is permeable to monovalent ions M^+ and A^- and their neutral complex MA, but impermeable to an intracellular colloidal solute P and an extracellular neutral solute S (Fig. 7).

When the Donnan equilibrium is established for the diffusible solutes, we obtain the equations

$$[M^+]_i[A^-]_i = [M^+]_e[A^-]_e, \quad (1)$$

$$[MA]_i = [MA]_e = [MA], \quad (2)$$

where '[ ]' indicates the concentration of a solute. As the conditions for electroneutrality hold,

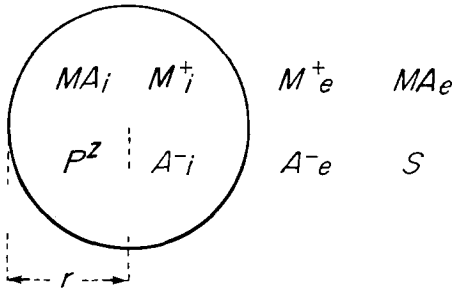

Fig. 7. Schematic presentation of distribution of permeable and impermeable solutes on both sides of the membrane of a spheroid cell

$$[M^+]_i - [A^-]_i + z[P] = 0, \quad (3)$$

$$[M^+]_e = [A^-]_e = C, \quad (4)$$

where z is the valence of P. Substitution of Eq. (4) into Eq. (1) gives

$$[M^+]_i[A^-]_i = C^2. \quad (5)$$

If the difference between the osmotic pressures of the two compartments is balanced by the circumferential tension τ of the cell membrane, then

$$[M^+]_i + [A^-]_i + [P] - ([M^+]_e + [A^-]_e + [S]) = (1/RT)2\tau/r, \quad (6)$$

where r is the radius of the spheroidal cell. Let

$$[m]_i = [M^+]_i + [MA]_i, \quad (7)$$

$$[a]_i = [A^-]_i + [MA]_i, \quad (8)$$

Then

$$[m]_i - [a]_i = [M^+]_i - [A]_i. \quad (9)$$

Equations (5) and (9) lead to

$$[M^+]_i^2 - ([m]_i - [a]_i) \cdot [M^+]_i - C^2 = 0.$$

Solution of this quadratic equation gives

$$[M^+]_i = [([m]_i - [a]_i) + \{([m]_i - [a]_i)^2 + 4C^2\}^{1/2}]/2. \quad (10)$$

Also

$$[A^-]_i = [-([m]_i - [a]_i) + \{([m]_i - [a]_i)^2 + 4C^2\}^{1/2}]/2. \quad (11)$$

By introduction of Eqs. (10) and (11) into Eq. (6), $[P]$ is written

$$[P] = 2C + [S] + (1/RT)2\tau/r - \{([m]_i - [a]_i)^2 + 4C^2\}^{1/2}. \quad (12)$$

Introduction of Eqs. (10), (11) and (12) into Eq. (3) gives

$$z = -([m]_i - [a]_i)/[2C + [S] + (1/RT)2\tau/r - \{([m]_i - [a]_i)^2 + 4C^2\}^{1/2}].$$

Assuming that $([m]_i - [a]_i)^2$ is negligibly smaller than $4C^2$, the above values can be approximated to

$$[M^+]_i \approx (2C + [m]_i - [a]_i)/2, \quad (13)$$

$$[A^-]_i \approx (2C - [m]_i + [a]_i/2, \quad (14)$$

$$[P] \approx [S] + (1/RT)2\tau/r, \quad (15)$$

$$z \approx -([m]_i - [a]_i)/\{[S] + (1/RT)2\tau/r\}. \quad (16)$$

When τ becomes zero in the presence of a sufficiently high concentration of sucrose, Eqs. (15) and (16) are simplified as

$$[P] \approx [S], \quad (17)$$

and

$$z \approx -([m]_i - [a]_i)/[S]. \quad (18)$$