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INTRACELLULAR POTASSIUM AND CELL GROWTH IN MOUSE FIBROBLASTS TRANSFORMED BY SIMIAN VIRUS

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SUMMARY

The effects of cell density on total K influx, ethacrynate-sensitive K influx, and the intracellular concentrations of sodium and potassium ions were studied in Simian Virus 40 transformed mouse fibroblasts (designated as SV3T3 cells). This study shows that total K influx increases with cell population density. The ethacrynate-sensitive K influx increases with increased cell density up to about 1.3×10^5 cells cm^{-2} and then decreases with a further increases in cell number. The level of $[K]_i$ increases with cell density while the $[Na]_i$ decreases up to about 1.25×10^5 cells cm^{-2} and then increases with further increase in cell number. In accordance with the above findings, the K^+ leakiness decreases in confluent cultures. The high level of K is correlated with the cell's continued ability to grow in a very dense culture.

INTRODUCTION

The intracellular level of K has an important physiological role in the normal functioning of a cell. Scholnick *et al.* (1) has shown that reduction of K^+ uptake depresses glycolysis in ascites tumour cells. In addition, Ledbetter and Lubin (2) found that when the intracellular K^+ concentration of cultured human fibroblast cell is below 60-80% of control level, a further reduction in the concentration of K^+ results in a proportionate reduction in the rate of protein and DNA synthesis and, consequently, the rate of cell growth.

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In this study the levels of intracellular K were determined in SV3T3 cells at various cell population densities to determine the possible relationship between cell growth and intracellular K concentration. Evidence of the factors involved in regulating the intracellular level of K at the various cell population densities was obtained from transport studies by using Rubidium as an isotopic tracer, assay of the enzyme Na KATPase, and direct measurement of the intracellular ion contents.

MATERIALS AND METHODS

Cell Culture

SV3T3 cells and materials required for cell growth, such as Dulbecco's modification of Eagles minimal essential medium, newborn calf serum and trypsin, were obtained from Flow Laboratories. Continuous cultures of the cells were maintained in the laboratory in Roux bottles at 37℃ in a medium supplemented with 10% serum. For experimental purposes the cells were grown in 5 cm petri dishes.

Intracellular sodium and potassium measurement

The cells were washed four times with ice-cold Calcium-sorbitol solution to remove the extracellular sodium and potassium ions. The washing was completed in forty seconds. From the washed cells, sodium and potassium ions were extracted for two hours in 3 mls of deionized water. The extracted sodium and potassium ions were measured on an EEL (model A) flame photometer. The measured sodium and potassium values were expressed as mmole/l of intracellular water. Cell number and volume were obtained by using the Coulter Counter from parallel plates of cells that were similarly washed with ice-cold Calcium-sorbitol solution in forty seconds.

Influx measurement

Because of a longer half life ^{86}Rb was used instead of ^{42}K as an isotopic tracer for the K influx since it has been shown to be translocated across the cell membrane by a similar mechanism to K in cultured cells (3). ^{86}Rb influx is, therefore, referred to as K^+ influx in this work.

K-influx was measured by incubating the cells at external K^+ of 5 mmole/l in ^{86}Rb-Krebs for ten minutes at 37℃. Incubation was terminated by aspirating the radiolabelled Krebs solution, and the extracellular isotope was removed by rapidly rinsing the plates of cells with ice-cold Krebs solutions. The washed cells were trypsinized in 0.25% trypsin, and the cell number and volume were determined by using the Coulter Counter. The ^{86}Rb content of the samples was counted by detecting Cerenkov radiation in a liquid scintillation spectrophotometer.

Influx is defined here as the entry of ion (radiolabelled) for a short period of time compared to the $t_{1/2}$ of exchange. It can be expressed by the following formula

$$J_{in} = [C]_i/t$$

Where J_{in} is the influx in mmole/l. and $[C]_i$ the intracellular concentration of C (mmole/l) at the time t (min).

Values for influx per suface area were calculated from the following formula derived from Lamb *et al* (4)

$$J_{in} = [C]_i/t.\ V/A$$

Where J_{in} is the influx in p moles/cm^2 sec., V and A are the mean cell volume (u^3), and surface area (u^2), respectively. Values of V and A were obtained in each experiment from the Coulter readings.

Enzyme assay:

($Na^+ + K^+$) - ATPase (E.C. 3.6.1.4) was assayed by estimating the inorganic phosphate released from ATP by the enzyme. The assay was carried out as follows:

1 ml. buffer (40 mM triethanolamine with 0.1 mM EDTA at pH 7.6) + 0.2 mls of sample + 0.2 mls of (1 mM $MgCl_2$ + 1 mM ATP), at pH 7.4 were incubated for 30 mins. at 37℃ in two sets, one in the presence, the other in the absence of, 0.2 ml of (60 mM NaCl + 10 mM KCl).

To measure the non-enzymic hydrolysis of ATP and the sample phosphate content, blanks were run at the same time. The total ATPase activity was obtained in assays carried out in the presence of Na^+, K^+ and Mg^{2+}. The basal enzyme activity, i. e., the activity due to Mg^{2+} - ATPase, was obtained in assays carried out in the absence of Na^+ and K^+. The difference between the total ATPase activity and the Mg^{2+} ATPase activity gives an estimate of the ($Na^+ + K^+$) -ATPase activity.

RESULTS

K^+ influx and NaKATPase activity

Fig. 1 shows that total K^+ influx increases with cell population density. On comparing low and high density cultures, we found that the total influx increases by 2.1 fold when expressed as per litre cell water (Fig. 1a) and by 3. 1. fold when expressed as per cell surface area (Fig. 1b). The larger changes seen in influx values when expressed as per cell surface were mainly due to the reduction in cell size with increasing cell population density (Fig. 2). Though the results clearly show an increase in K^+ influx, it was necessary to express them as both per litre cell water and per cell surface area. Expressing it per litre cell water would eliminate the effects due to variations in cell size at different densities. On the other hand, expressing it per surface area would accommodate the area available for exchange, would show the availability of the cell's membrane to the medium surrounding it, and would indicate that transport is a membrane, albeit 'surface' phenomena.

The active component of the total influx of K^+ was considered to be sensitive to ethacrynate, shown to inhibit partially purified NaKATPase preparation from L cells (5). Ouabain could not be used since the SV3T3 cells are not sensitive to the drug (6).

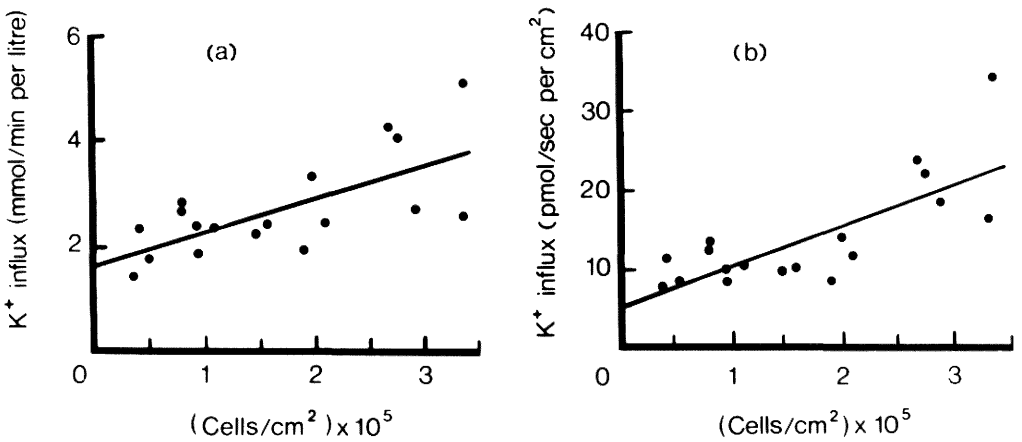

Fig. 1. Effect of cell density on K^+ influx into SV3T3 cells. Cells were grown to various densities in 5 cm dish. Influx was measured as described in the methods and expressed as per litre cell water in a) and as per surface area of cell in b). ^{86}Rb was used as a tracer for K^+. Influx was measured for 10 mins in labelled Krebs at external $[K^+]$ of 5 mmole/l. Cells and experimental solutions were maintained at 37℃ throughout the experiment. Each point represents the mean of 4 observations. Line was established by the method of least squares. The increase in K^+ influx with increase in cell density is significant in both a) and b) ($r=0.6758$, $P < 0.01$ and $r=0.7601$, $P < 0.01$ respectively).

Preliminary experiments show that the maximal degree of inhibition by ethacrynate was at 10^{-3}M, and this concentration was used to determine the active component of K influx.

The ethacrynate-sensitive K influx was found to increase with an increase in cell density up to about 1.3×10^5 cells cm^{-2} and then to decrease with a further increase in cell number (Fig. 3). The ethacrynate-sensitive K^+ influx constitutes between 61% to 5% of the total influx over the density range studied.

Fig. 4 shows the effects of cell density on the NaKATPase activity which closely follows the ethacrynate-sensitive component of K transport. The low ethacrynate-sensitive K^+ influx and the low enzyme activity of a sparse culture were further examined to see if the density effect reflects the ethacrynate-sensitive K^+ influx and the enzyme activity of dense cultures when the experimental cells are grown form a dense culture source in a roux bottle. For these purposes the experiments were also conducted on cells taken from a sparse culture source. Similar results were obtained.

Intracellular ion contents

Fig. 5 shows that the $[Na]_i$ decreases with an increase in cell number up to about 1.25×10^5 cells cm^{-2}; with a further increase in cell number the $[Na]_i$, however, increases. Although the $[Na]_i$ changes with cell density are consistent with the trend found in the ethacynate-sensitive K influx and the results of the NaKATPase activity, changes in $[Na]_i$ have been shown to have no effect on the diuretic-sensitive K influx in HeLa cells (7; 8). In addition, changes in $[Na]_i$ have also been shown not to affect a serum-

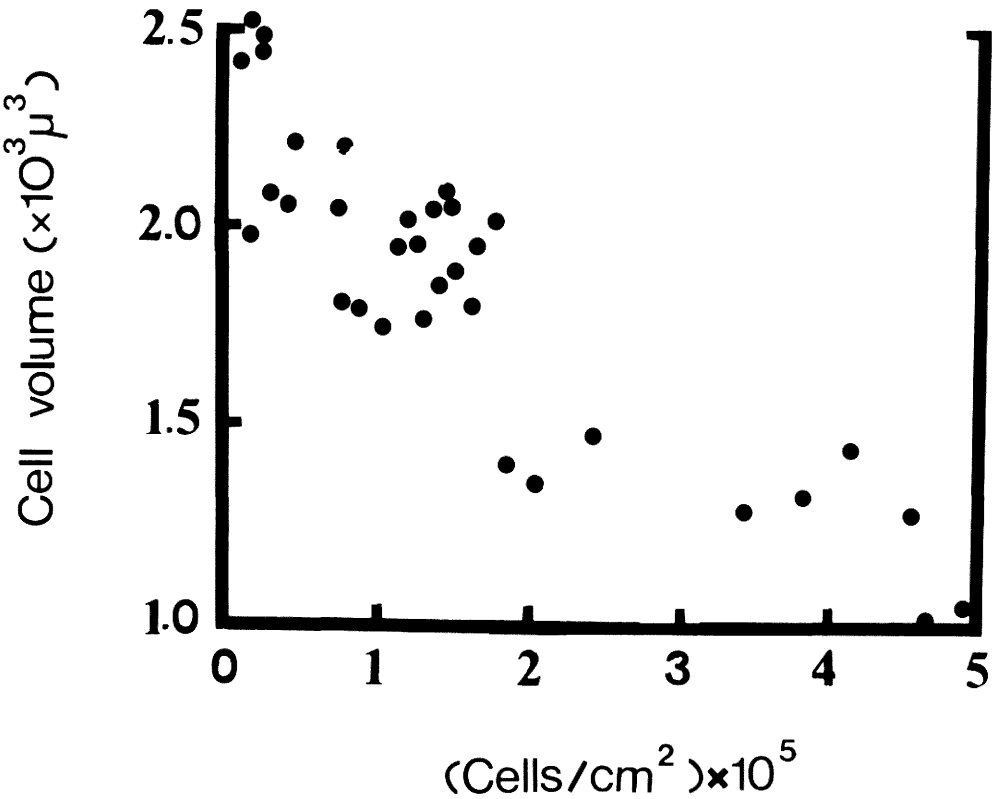

Fig. 2. Effect of cell density on the volume of SV3T3 cells grown in Dulbeccos' medium + 10% newborn calf serum. Cells were grown to various densities and trypsinised, and an aliquot of the sample was taken for cell number and volume determination by using the Coulter Counter. Each point is the mean of 2-4 observations. Line was established by the method of least squares. There is significant correlation between cell volume and density ($r = -0.8548$, $P < 0.01$).

stimulated increase in Na-K pump activity in 3T3 and HeLa cells (9).

The $[K^+]_i$, though it appears to remain constant up to about 1×10^5 cells cm^{-2}, increases 2 fold at high cell density, and this action contradicts the low ethacrynate-sensitive K influx at high cell density and also the low level of NaKATPase activity. The $[K]_i$ at high cell density suggests an increase in the ethacrynate-insensitive K influx and/or a decrease in the leakiness of K^+ at high cell density. This possibility was investigated.

An investigation on K^+ leakiness in SV3T3 cells

The cells were grown to sparse and confluent cultures and at the end of the growth period one set of cells at both densities was blocked for 10 minutes with 10^{-3}M ethacrynate in zero K^+ Krebs; control cells were not treated with ethacrynate. The cells were then incubated in fresh media in sufficient time to allow the cells to recover their

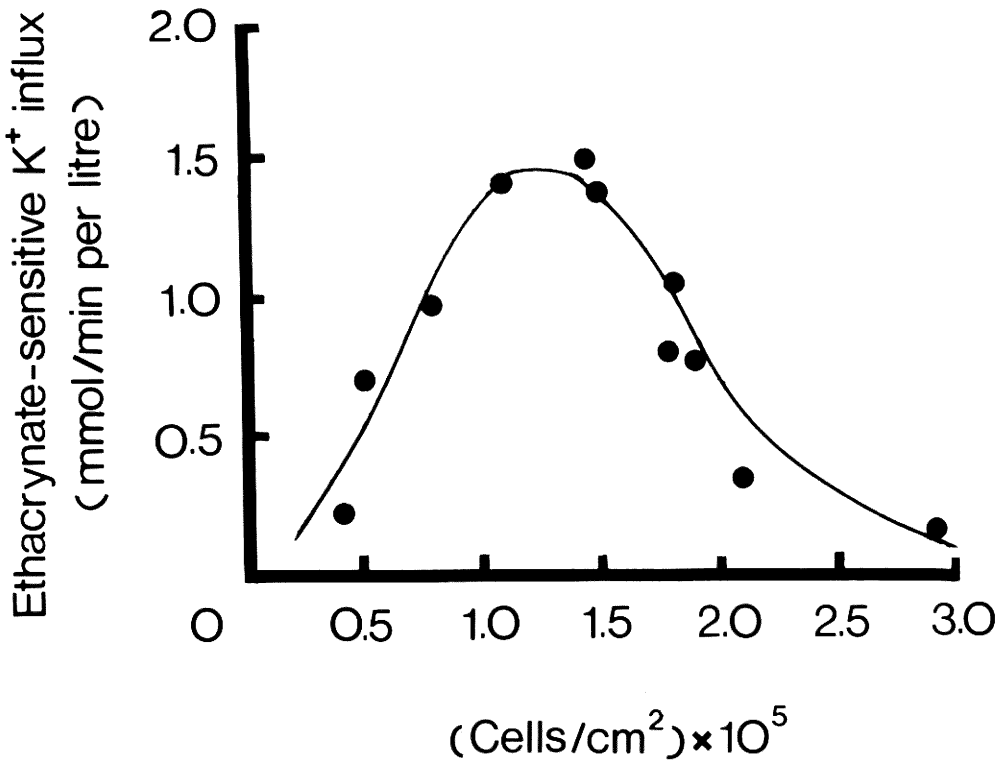

Fig. 3. Effect of cell density on ethacrynate-sensitive K^+ influx in SV3T3 cells. Cells were grown to the various densities, and the influx was measured for 10 mins. in labelled Krebs at external [K^+] of 5 mmole/l in the presence or absence of ethacrynate (10^{-3} M). Each point is the mean of 4 observations. The increase in ethacrynate-sensitive K^+ influx is followed by a decrease with an increasing cell density.

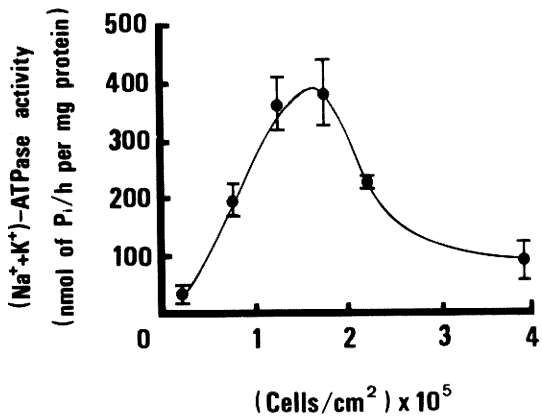

Fig. 4. Effect of cell density on the (Na^+ + K^+)-ATPase activity in SV3T3 cells. Cells were grown to the various densities in plates. (Na^+ + K^+)-ATPase activity was measured in a broken cell preparation and expressed as nmole Pi/10^6 cells. hr. Each point is the mean of 5 observations; bars are standard error of the mean.

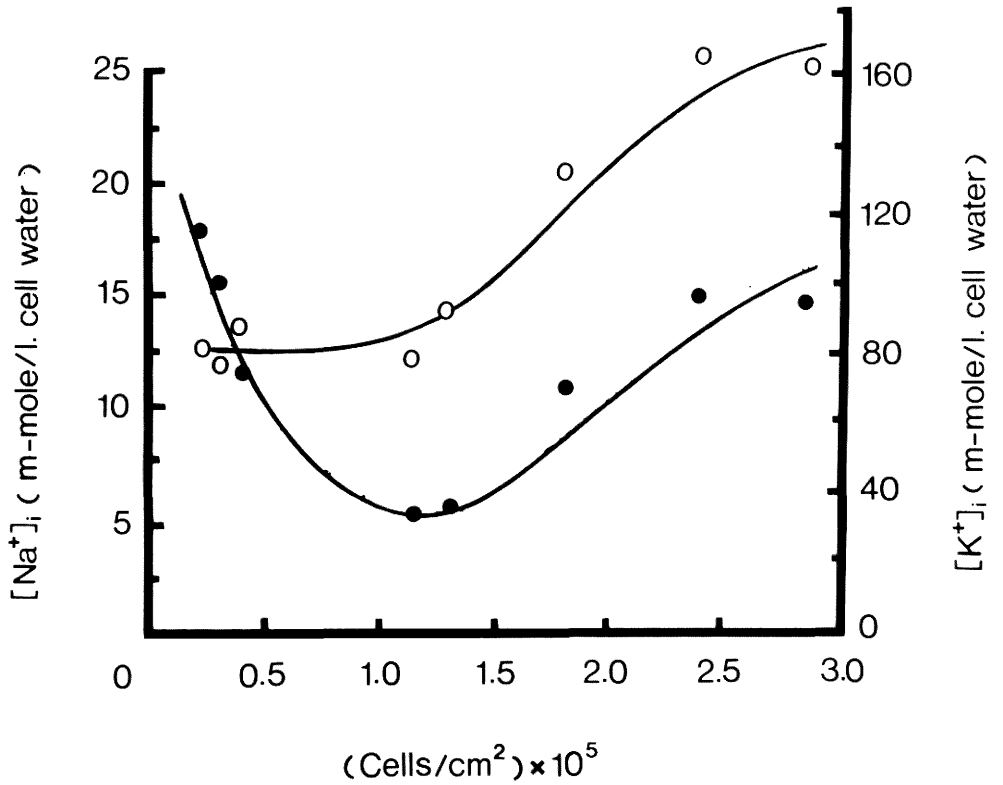

Fig. 5. Effect of cell density on the $[K^+]_i$ and $[Na]_i$ in SV3T3 cells. The intracellular ions were measured as described in the methods. Each point represents the mean value obtained from 6 determinations. The lines were established by eye.

steady state. At the end of the incubation period, the intracellular K^+ was estimated. The intracellular concentration of K in the ethacrynate-treated cell would represent the steady state level of $[K^+]_i$ due to ethacrynate-insensitive K^+ influx. This level of $[K^+]_i$ when expressed as a percent of $[K^+]_i$ in control cells represents the percent of K remaining in the cell, and this result is taken as a measure of K^+ leakiness. Fig. 6 shows that the percent of K remaining increases by 2.6 fold from 33% in sparse cultures to 86% in confluent cultures, suggesting a decrease in K^+ leakiness as the cells approach confluency. This finding is in reasonable agreement with the 2-fold increase in $[K^+]_i$ at high cell density.

Discussion

Mouse cells transformed by Simian Virus 40 are characterized by their reduced sensitivity to contact inhibition (10) and their ability to grow in multilayers as opposed to the monolayer growth of the normal cell (11). Furthermore, transformed cells can grow in the absence of anchorage to glass or plastic (12). These properties of the cells coupled

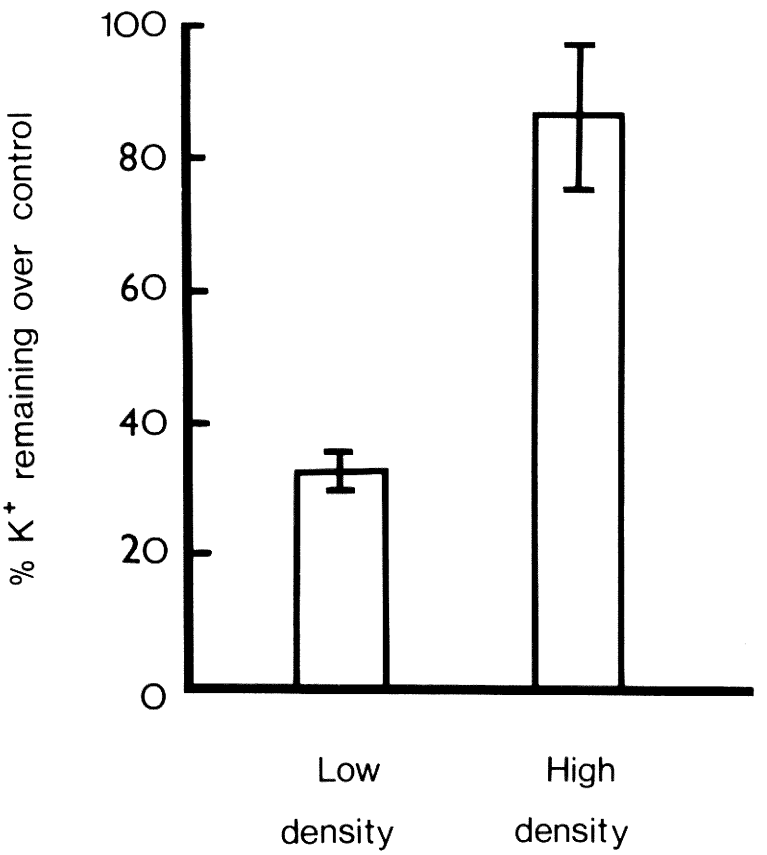

Fig. 6. Effect of cell density on the leakiness of K^+ in SV3T3 cells. Cells were grown in 5 cm plates to low and high densities, the final densities being 58, 867 cells/sq. cm. and 251, 305 cells/sq. cm. respectively. At zero time cells were washed twice in warm K-free Krebs followed by incubation for 10 mins. at 37℃ in 3 mls. of K-free Krebs containing 10^{-3}M ethacrynate. Control plates of cells were similarly incubated, but in K-free Krebs without the addition of drugs. At the end of the incubation period the solution was removed and the cells washed in three changes of Krebs containing 5 mmole K^+. Fresh media (3 mls.) were then added to each plate, the media were then equilibrated with 95% air 5% CO_2, and the cells were incubated at 37℃ for one hour. At the end of the incubation period the cells were processed for intra-cellular ion determinations as described in the methods. The percent of K^+ remaining in cells blocked by ethacrynate over that of control cells is taken as a measure of K^+ leakiness. The percent of K^+ remaining over control at low density was 33.34 ± 2.23 (5)%, and that at high density was 85.99±10.44 (8)%. The number in parenthesis indicates the number of observations, and the error is S. E.

with their capacity for extensive mutilayer growth enable them to attain saturation densities of about 25-fold higher as compared to the normal line. The reduced volume with increasing cell density observed in this study would mean an icrease in surface area/volume ratio of the cells, and this finding would mean an increase in the availability of the cell's membrane for the transport of nutrients. This is consistent with the total increase in K^+ influx with the increase in cell numbers. The component of K^+ influx that

increases is possibly sensitive to diuretics as hypertonic cell shrinkage (induced by sorbitol) in SV3T3 cells stimulates bumetanide-sensitive K^+ influx, thus suggesting that cellular shrinkage activates the diuretic sensitive cotransport system (13). The diuretic sensitive (ethacrynate) cotransport system in SV3T3 cells possibly constitutes the ouabain insensitive component of K^+ influx as in HeLa cells Aiton *et al* (14) have shown that it constitutes about 35% of ouabain insensitive K^+ influx, but because of the insensitivity of the mouse cells to ouabain we could not ascertain the proportion of passive K^+ influx (ouabain-insensitive) that is mediated by ethacrynic acid.

There is a well-established body of evidence showing that inhibition of growth in 3T3 cells is associated with a reduction in the intracellular level of K (15; 16). The role of K in the growth control of transformed cells in not well established although transport of nutrients essential for growth is increased in the transformed cells (17). This finding has been correlated with their escape from density dependent inhibition of growth. The present work demonstrates that the intracellular level of K and its transport in SV3T3 cells increase with cell density, and this activity is due mainly to the decrease in K^+ leakiness in dense culture and to an increase in the ethacrynate-insensitive K influx with cell density.

The intracellular level of K has a crucial role in protein synthesis. Inhibition of protein synthesis following reduction of K^+ levels has been found in bacteria (18) and several mammalian cell lines (1; 2). In K depleted human fibroblast, however, this inhibition was shown not to be limited by the intracellular ATP content and by the rate of transport of amino acids (2). Since a high level of K is not inhibitory for macromolecular synthesis and cell growth, such observed levels of K in a dense culture of transformed cells is conducive to the continued survival of these cells in a crowded environment, a condition limited to the normal parental line. Transformed cells apprently adapt and servive in a crowded environment by increasing the availability of K for macromolecular synthesis. It is likely that the high metabolic rate and metastasizing nature of malignant cells may also be due to increase availability of K in these cells.

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