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CELL GROWTH AND OUABAIN-SENSITIVE $^{86}Rb^+$ UPTAKE AND $(Na^+ + K^+)$-ATPase ACTIVITY IN 3T3 AND SV40 TRANSFORMED 3T3 FIBROBLASTS

H. K. KIMELBERG[a] and E. MAYHEW[b]

[a]*Division of Neurosurgery and Department of Biochemistry, Albany Medical College of Union University Albany, N.Y. 12208 and* [b]*Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, N.Y. 14263 (U.S.A.)*

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SUMMARY

The uptake of ouabain-sensitive $^{86}Rb^+$ uptake measured at 5 min and the uptake measured at 60 min was 4.5- and 2.7-fold greater respectively for SV40 transformed 3T3 cells compared to 3T3 cells during the late log phase of growth. This uptake, however, varied markedly with cell growth. Ouabain-sensitive $^{86}Rb^+$ uptake was found to be a sensitive indicator of protein synthesis as measured by total protein content. Cessation of cell growth as measured by total protein content was associated with a decline in ouabain-sensitive $^{86}Rb^+$ uptake in both cell types. This increased ouabain-sensitive cation transport was reflected in increased levels of $(Na^+ + K^+)$-ATPase activity for SV40 3T3 cells, which showed a 2.5-fold increase V but the same K_m as 3T3 cells.

These results are compared with the results of related work. Possible mechanisms for these effects are discussed and how changes in cation transport might be related to alterations in cell growth.

INTRODUCTION

There is considerable evidence that surface membranes play an important role in the control of cell growth, and are therefore likely to be involved in the loss of its regulation in malignant cells [1, 2]. One aspect of this involvement may well be through changes in membrane transport properties, which could affect cell growth by altering the intracellular availability of nutrients and metabolites [3]. Indeed, increased transport of sugars [4, 5] and amino acids [6, 7] has been found in several lines of transformed cells compared to their untransformed counterparts.

Another aspect of the way in which changes in the transport properties of surface membranes could control cell growth might be through alterations in the transport of substances which can act as effectors in controlling the rate of intracellular metabolic and synthetic reactions. There is some circumstantial evidence that intracellular levels of K^+ and possibly Na^+ affect cell growth [8–10], as

well as more extensively studied substances such as the cyclic nucleotides [11] and intracellular Ca^{2+} [12].

We and others have recently found that some transformed cells show increased $(Na^+ + K^+)$-ATPase activity [13–16] and increased ouabain-sensitive cation transport [14, 17], compared to their untransformed counterparts*. Both active cation transport [14, 18] and $(Na^+ + K^+)$-ATPase activity [16] have also been shown to vary with the rate of cell growth. This effect is also seen for the transport of sugars [19] and amino acids [6] perhaps reflecting similar growth-related changes in the properties of the surface membrane. It is also possible that effects of active cation transport on cell growth could be partly due to changes in Na^+-dependent amino acid transport [14, 18].

In this paper we report further studies on how ouabain-sensitive $^{86}Rb^+$ transport varies with the growth rate of 3T3 and SV40 transformed 3T3 cells in culture. Studies comparing the kinetics of the increased ouabain-sensitive $(Na^+ + K^+)$-ATPase activity in SV40 transformed 3T3 cells compared to untransformed 3T3 cells are also presented.

METHODS AND MATERIALS

Mouse BALB/c 3T3 and SV40 (simian virus 40) transformed 3T3 cells were cultured and measurements of $^{86}Rb^+$ uptake were performed in culture tubes as previously described [14]. In brief, cells were grown in 2 ml of Dulbecco's modified Eagle's medium (amino acids plus 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7\ H_2O$, 1.0 mM NaH_2PO_4, 5.6 mM glucose, 44 mM $NaHCO_3$, from GIBCO, Grand Island, N.Y.) in 15×150 mm test tubes on a roller drum (12 rev/h) at 37 °C in 5 % CO_2/95 % air atmosphere. The growth media was replaced with fresh media every 3 days. For measurement of $^{86}Rb^+$ uptake the growth medium was poured off and replaced with 2.2 ml of Dulbecco's phosphate buffered saline (136 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6\ H_2O$). Ouabain when added was in 0.2 ml of Dulbecco's phosphate buffered saline added to 2.0 ml of phosphate buffered saline, to give a final concentration of 1 mM ouabain. The tubes were preincubated in a shaking water bath for 15 min and then 5 μCi $^{86}Rb^+$, with a specific activity of 67 Ci/mol and representing $\approx$ 80000 cpm, was added. Uptake of $^{86}Rb^+$ was allowed to proceed, the tubes were centrifuged at 2000 rev./min in a refrigerated centrifuge and washed 3 times with cold phosphate buffered saline with the same centrifugation procedure. Total time for each wash was 1–2 min. Uptake of $^{86}Rb^+$ was then directly measured in a Packard automatic gamma counter. The uptake of $^{86}Rb^+$ was usually expressed in terms of μmol of K^+, treating $^{86}Rb^+$ as a tracer for K^+ [20]. The uptake was expressed per 10^6 cells with cell number determined from duplicate determinations on replicate tubes, or per mg total cell protein. Total cell protein and numbers of attached plus non-attached cells were determined as previously described [14].

ATPase determinations were made on cells collected by trypsinization or scraping from large culture vessels when they had reached growth stage just prior

* After this paper was submitted Banerjee and Bosman (ref. 41) also reported increased $^{86}Rb^+$ uptake in SV403T3 and murine virus transformed 3T3 cells compared to normal 3T3 cells.

to confluence. ATPase activities were not significantly different regardless of whether cells were scraped or trypsinized. The cells from different vessels were pooled, washed three times in 0.15 m NaCl and frozen at −70 °C for a maximum of 1 month before use. Before use the cells were thawed and diluted to a concentration of $\approx 10^7$ cells/ml in 1 mM histidine, pH 7.4. They were then sonicated for 1–2 minutes at 22 °C in a bath type sonicator (50 watts, Cole Parmer Inc.) which resulted in complete breakdown of the extra nuclear membranes but not the nucleus, as monitored by phase contrast microscopy. This procedure of sonicating in a bath type sonicator produced a homogeneous suspension and did not affect ATPase activities. It has been shown to efficiently disrupt several cell types without inactivating a number of enzyme activities [21]. Also, our ATPase activities are equal to or greater than those found for total homogenates in other studies where various homogenization techniques were used [14–16]. Aliquots of the suspension, usually equivalent to $\approx 10^6$ cells, were assayed for ATPase activity at pH 7.2 for 1 h as previously described [14, 22].

RESULTS

Time course of $^{86}Rb^+$ uptake by 3T3 and SV40 3T3 cells

A typical time course for total and ouabain-sensitive $^{86}Rb^+$ uptake is shown for 3T3 and SV40 3T3 cells in Fig. 1. These cells were harvested in the late log phase at

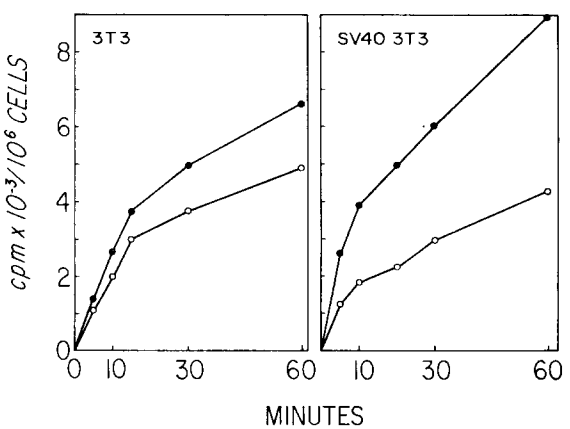

Fig. 1. Time course of total and ouabain-sensitive $^{86}Rb^+$ uptake by 3T3 and SV40 3T3 cells. Uptake of $^{86}Rb^+$ was determined for the indicated times as described in Materials and Methods. The cells were assayed at day 5 just before confluence. The cell densities per cm² during the growth cycle were:

Day	3T3	SV40 3T3
0	$2 \cdot 10^4$	$2 \cdot 10^4$
3	$8 \cdot 10^4$	$9 \cdot 10^4$
5	$1.1 \cdot 10^5$	$1.9 \cdot 10^5$
7	$1.3 \cdot 10^5$	$2.0 \cdot 10^5$

Each point for $^{86}Rb^+$ is the average of 3 determinations which did not differ by more than 10 %, and cell number is the average of 2 determinations on replicate tubes. (●), no ouabain; (○), +1 mM ouabain.

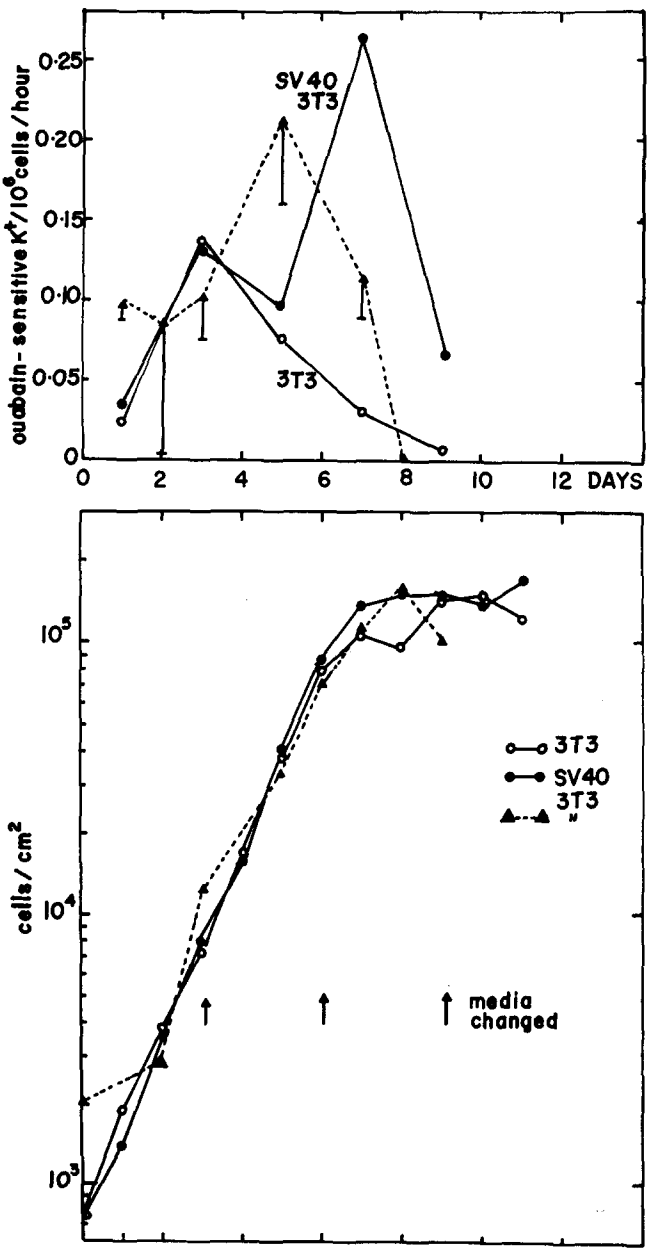

Fig. 2. Uptake of K^+ (measured as $^{86}Rb^+$) per 10^6 cells and cell growth of 3T3 and SV40 3T3 cells. Cells were grown and $^{86}Rb^+$ uptake measured as described in Materials and Methods. The arrows marked media changed refer to days of growth on which the media was poured off and replaced by fresh media. In the case of the experiments shown as continuous lines the uptake was measured in Dulbecco's modified Eagle's medium + 10 % serum instead of Dulbecco's phosphate balanced saline. Uptake shown is amount sensitive to 1 mM ouabain.

densities for the 3T3 and SV40 3T3 cells of 1.1 and $1.9 \cdot 10^5$ cells/cm^2, and a total protein content of 0.144 and 0.170 mg/10^6 cells respectively. These densities and the apparent stationary phase densities of 1.3 and $2.0 \cdot 10^5$ cells/cm^2 for 3T3 and SV40 3T3 cells (see legend to Figs. 1 and 2) are similar to those reported by Meisler [23] with frequent media changes. Our roller-drum culture conditions, which involves continuous movement of the cell cultures and media, might also result in higher cell densities due to increased nutrient availability. As can be seen the uptake of $^{86}Rb^+$ is approximately linear up to 15 min for the 3T3 cells, but non-linear even after 5 min

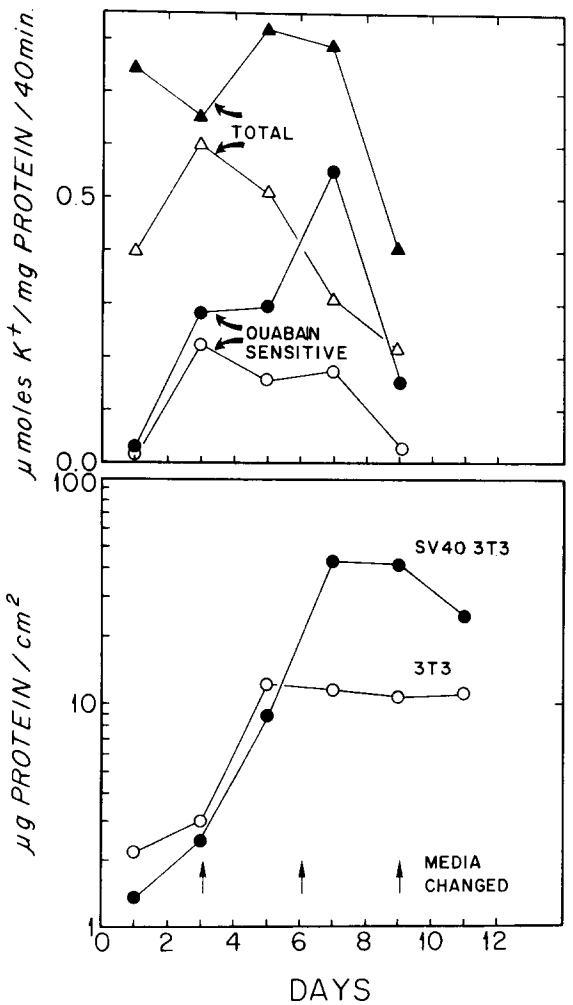

Fig. 3. K^+ uptake per total cell protein and cell growth. This figure represents the data from the experiments shown in Fig. 2 for the 3T3 and SV40 cells as continuous lines calculated as μmol K^+/mg protein and μg protein/cm^2. In this plot the total calculated uptake of K^+ from $^{86}Rb^+$ uptake is shown, as well as the amount inhibited by 1 mM ouabain. The uptake was measured in Dulbecco's modified Eagle's medium + 10 % serum. Protein determined on total cells as described in Materials and Methods. (●, ▲), SV40 3T3; (○, △), 3T3.

for the SV40 3T3 cells. 5 min was the earliest time for which we could make reliable measurements. The uptake sensitive to 1 mM ouabain was about 4.5-fold greater for SV40 3T3 cells compared to 3T3 cells at 5 min, while the total uptake was 1.9-fold greater. The ouabain-sensitive uptake of $^{86}Rb^+$ was 2.7-fold greater at 60 min for SV40 3T3 cells. The 1.18-fold higher protein content per cell for the SV40 3T3 cells would only slightly reduce the increased uptake for the transformed cells when expressed per mg of protein.

Effect of cell growth on $^{86}Rb^+$ uptake

Fig. 2 shows the ouabain-sensitive uptake of $^{86}Rb^+$, treated as a tracer for K^+ [20] and expressed as μmol $K^+/10^6$ cells per h for both 3T3 and SV40 3T3 cells. This was measured for the cells at different growth times for two separate growth curves for SV40 3T3 and one growth curve for 3T3 cells as shown. For the experiment shown as the dotted line the uptake was measured for 60 min, while for the continuous lines the uptake was measured for 40 min but recalculated for 60 min. Because of the deviation from linearity (Fig. 1) this will introduce a small error into the results. At cell densities $< 10^4$ cells/cm^2 both cell types showed comparable uptake. At cell densities greater than this, however, there was progressive decline in uptake for the 3T3 cells even though, in terms of cell number, they were still growing. In contrast, in both cases the SV40 3T3 cells showed a reproducible sharp increase in uptake at around 10^5 cells/cm^2 just before the stationary phase was reached. After this point the transformed cells showed a marked decline in ouabain-sensitive uptake.

The relationship between growth of 3T3 cells and ouabain-sensitive uptake of

TABLE I

INCREASE OF TOTAL CELL PROTEIN AND CELL NUMBER FOR 3T3 AND SV40 3T3 CELLS

The SV40 3T3 cells were seeded at $7.6 \cdot 10^2$ and the 3T3 cells were seeded at $7.5 \cdot 10^2$ cells/cm^2 on day 0 in culture tubes. Media was changed at days 3, 6 and 9 as indicated in Fig. 3. Cells were counted on each day and protein on every other day from duplicate tubes. The results shown are the average of these determinations. The growing area (12.8 cm^2) was estimated by adding a volume of dye equivalent to the volume of growth medium and measuring the area of the tube stained.

Day of growth	SV40 3T3			3T3		
	No. of cells per cm$^2 \times 10^4$	mg protein per 10^6 cells	μg protein per cm^2	No. of cells per cm$^2 \times 10^4$	mg protein per 10^6 cells	μg protein per cm^2
1	0.14	0.98	1.3	0.19	1.14	2.2
2	0.45			0.38		
3	0.79	0.31	2.4	0.72	0.41	3.0
4	1.53			1.66		
5	4.05	0.22	8.9	3.79	0.32	12.2
6	8.49			7.78		
7	13.46	0.32	42.9	10.51	0.12	11.7
8	14.65			9.65		
9	14.32	0.29	41.3	14.10	0.08	10.7
10	13.42			14.94		
11	16.67	0.15	24.5	12.00	0.09	11.1

$^{86}Rb^+$ became clearer when the data was expressed on the basis of total protein content. This is shown in Fig. 3 and is the data for both 3T3 and SV40 3T3 cells drawn as continuous lines in Fig. 2 recalculated on a protein basis. The uptake was measured for 40 min and is plotted for this time period. It can be seen that the decline in ouabain-sensitive $^{86}Rb^+$ uptake at day 5 for the 3T3 cells coincides with the day at which these cells show an abrupt cessation in the increase of total protein. In contrast, the similar point for the SV40 3T3 cells at day 7 is associated with the peak increase of $^{86}Rb^+$ uptake, which does, however, rapidly decline after this point. The maximum increased uptake for SV40 3T3 cells at day 7 compared to 3T3 cells is 3-fold when expressed per mg protein, which is somewhat less than the 10-fold difference found for the same time when expressed on the basis of cell number. The variability of cell protein with cell growth and number for this experiment is shown in Table I.

Kinetics of ATPase activity of 3T3 and SV40 3T3 cells

Cell homogenates from a growth period equivalent to that of the uptake experiments shown in Fig. 1 were used to study the kinetics of the ouabain-sensitive $(Na^+ + K^+)$-ATPase activity, shown in the lower panel in Fig. 4. The residual ouabain-insensitive Mg^{2+} ATPase activity is shown in the upper panel. These results indicate that the main difference in both ouabain-sensitive $(Na^+ + K^+)$-ATPase and ouabain-insensitive Mg^{2+} ATPase involves an increased V in both cases, whereas the K_m is essentially the same. The actual values obtained are shown in Table II. An increased V for SV40 3T3 cells but identical K_m was also found for the $(Na^+ + K^+)$-

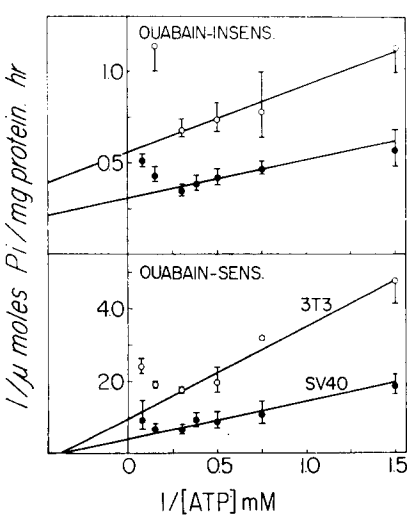

Fig. 4. Double reciprocal plots of ATPase activity of 3T3 and SV40 3T3 cells. The ATPase activity was measured with sonicated homogenates of whole cells harvested prior to confluence. The reaction was run for 60 min and performed as described in Materials and Methods. Each point is the average of 3–5 determinations ±S.E.M.

TABLE II

V AND K_m VALUES FOR ATPase ACTIVITY OF 3T3 AND SV40 3T3 FIBROBLASTS

Kinetic parameters from Fig. 4. All values were obtained directly from intercepts except the K_m values for the Mg^{2+} ATPase which were calculated from the slopes. Ouabain concentration was 1 mM.

Cells	V (μmol P_i/mg protein per h)		K_m (mM ATP)	
	Mg^{2+} ATPase (ouabain-insensitive)	(Na+K)-ATPase (ouabain-sensitive)	Mg^{2+} ATPase	(Na+K)-ATPase
3T3	1.79	1.05	0.66	2.70
SV40 3T3	3.23	2.50	0.68	2.70

ATPase activity when it was expressed on the basis of cell number [24]. For both $(Na^+ + K^+)$-ATPase and Mg^{2+} ATPase activity substrate inhibition occurs at ATP concentrations greater than 3.3 mM.

DISCUSSION

Cation transport and cell growth

The results reported in this paper and elsewhere [18] indicate a relationship between cell growth and ouabain-sensitive K^+ transport. Moreover, at cell densities between approx. 10^4 to 10^5 cells/cm^2 there is a markedly higher ouabain-sensitive uptake of $^{86}Rb^+$ for SV40 transformed 3T3 cells compared to the untransformed 3T3 cells. When these data are expressed on the basis of cell protein, ouabain-sensitive $^{86}Rb^+$ uptake appears to reflect the net protein increase. A peak of increased ouabain-sensitive uptake of $^{86}Rb^+$ for the SV40 3T3 cells and a subsequent sharp decline coincides with a cessation of growth as measured by total protein content. For 3T3 cells this sharp increase is not in $^{86}Rb^+$ uptake seen but only a gradual decline (see Fig. 3).

As briefly reviewed before [14], previous studies on several different systems have indicated that K^+ and Na^+ transport alters with changes in the rate of cell growth. Lubin [8] documented evidence that high intracellular K^+ levels are necessary for protein synthesis and growth in cultured mammalian cells, and Quastel and Kaplan [10] found that stimulation of lymphocytes by phytohemagglutin was accompanied by increased ouabain-sensitive K^+ uptake. Furthermore, fluctuations of Na^+ and K^+ levels during the cell cycle have been reported for mouse leukemic lymphoblasts [9]. It has also recently been shown that serum treatment of quiescent, confluent cultures of 3T3 cells resulting in stimulation of cell growth, was accompanied by a rapid increase in ouabain-sensitive $^{86}Rb^+$ uptake [18]. This is analogous to our findings of decreasing ouabain-sensitive $^{86}Rb^+$ uptake when 3T3 cells stopped growing (see Figs. 2 and 3). Mayhew and Levinson [25] found that ouabain reversibly inhibited growth in Ehrlich ascites cells. Recently this finding has been confirmed in mouse lymphoblasts, accompanied by decreased [^{3}H]leucine incorporation into protein [26]. In this latter study the authors attributed the effect to dissipation of the plasma membrane Na^+ gradient leading to inhibition of Na^+-dependent amino acid uptake.

$(Na^+ + K^+)$-ATPase and cell growth

Lelievre et al. [27] showed a 100-fold decline in $(Na^+ + K^+)$-ATPase activity with cessation of growth in a contact-inhibited mouse plasmocytoma line. We only found such a similar marked decline for both 3T3 and SV40 3T3 cells when the cells showed appreciable decreases in viability [14]. Elligsen et al. [16], using total homogenates of 3T3 and SV40 transformed 3T3 cells, found no noticeable differences in $(Na^+ + K^+)$-ATPase activity in both cell types at cell densities of $< 5.5 \cdot 10^4$ cells/cm^2. However, when the 3T3 cells stopped growing at $> 5.5 \cdot 10^4$ cells/cm^2 there was a 5–6-fold decrease in $(Na^+ + K^+)$-ATPase activity, leading to a 5–6-fold greater activity in SV40 3T3 cells at these higher densities since the transformed cells were still in exponential growth. The same study showed that when confluent 3T3 cells were stimulated to grow by adding fresh serum there was up to a 3-fold increase in $(Na^+ + K^+)$-ATPase activity. This is similar to the effect of serum on ouabain-sensitive $^{86}Rb^+$ uptake in 3T3 cells discussed above [18].

$(Na^+ + K^+)$-ATPase in normal and transformed cells

In contrast to our results [13, 14] and others [15, 16] of increased $(Na^+ + K^+)$-ATPase activity there have been a number of reports that $(Na^+ + K^+)$-ATPase activity is variable [28] or decreased [29–31] in transformed cells compared to normal cells. These differences may be related in part to use of homogenates or purified membrane fractions, and different cell types. In contrast, Kasarov and Friedman [15] and Elligsen et al. [16] have recently reported that total homogenates of several lines of transformed 3T3 cells show, at densities $> 5.5 \cdot 10^4$ cells/cm^2, a 4–6-fold increased $(Na^+ + K^+)$-ATPase activity compared to non-transformed 3T3 cells.

The increased $(Na^+ + K^+)$-ATPase of SV40 3T3 cells is associated with an increased V but the same K_m for ATP, which is in the range of the intracellular ATP concentrations of 2–3 mM reported for 3T3 cells [32]. Although such behavior is usually interpreted as indicating a change in the number of enzyme molecules present, it can also reflect an increased activity of the rate-limiting step for product formation. In a reconstituted system it has recently been shown that cholesterol, which decreases the fluidity of fluid phospholipid membranes, decreases the V of $(Na^+ + K^+)$-ATPase with no significant effect on the K_m for ATP [22]. Also, the increased sugar transport seen in transformed cells [4, 5] and the growth-dependent increased $^{86}Rb^+$ uptake in 3T3 cells [18] and transformed 3T3 cells [41] is also associated with an increased V but no effect on the K_m for the substrate. The similar kinetic behavior of the ouabain-insensitive ATPase is difficult to interpret since the assays were done on whole cell homogenates. Therefore, this activity would presumably involve the mitochondrial Mg^{2+}-dependent ATPase, as well as an unknown amount of Mg^{2+}-dependent ouabain-insensitive ATPase present in the plasma membrane.

Possible roles of altered cation transport and $(Na^+ + K^+)$-ATPase activity

Although there is evidence for a direct effect of intracellular K^+ levels on cell growth and macromolecular synthesis, this relationship is still circumstantial. There is some evidence of increased passive permeability to K^+ in transformed cells compared to normal cells [33], as well as in phytohemagglutin-stimulated lymphocytes [34]. This latter effect occurs in the same system in which stimulation by phytohemagglutin leads to increased ouabain-sensitive K^+ efflux, which would therefore appear to be needed to maintain constant intracellular K^+ levels.

Kasarov and Friedman [15] suggested that $(Na^+ + K^+)$-ATPase could control cell growth not only via K^+ changes, but indirectly by competing with adenylate cyclase for ATP. Both adenylate cyclase and $(Na^+ + K^+)$-ATPase are specifically localized in the plasma membrane and competition might involve changes in the steady-state level of a small local ATP pool, since studies on ATP levels indicate little variation in growing and density-inhibited 3T3 cells [32]. Relatively high K_m values for adenylate cyclase of 0.2 and 1.0 mM ATP have been found in normal and RSV-transformed chick embryo fibroblasts [35]. If adenylate cyclase in 3T3 and SV40 3T3 fibroblasts has K_m values for ATP in the same concentration range, then the intracellular ATP concentrations of 2–3 mM in 3T3 cells [32] and our values of 2.7 mM ATP for the K_m of the $(Na^+ + K^+)$-ATPase in 3T3 and SV40 3T3 cells are at least numerically consistent with such competition.

A recent paper [26], as already mentioned, has presented some evidence that ouabain inhibition of cell growth [25, 26] may be partly due to inhibition of Na^+-dependent uptake of amino acids due to dissipation of the Na^+ gradient across the plasma membrane. This possibility thus combines both the effect on cation transport and inhibition of the uptake of nutrient substance mechanisms referred to in the introduction.

If increased cation transport and $(Na^+ + K^+)$-ATPase activity involves an increased activity of a fixed number of sites it could be caused by increases in membrane lipid fluidity which has been shown to activate $(Na^+ + K^+)$-ATPase activity in a number of systems [24]. The temperature dependence of $(Na^+ + K^+)$-ATPase activity in 3T3 and SV403T3 cells at least suggests this possibility [14]. If this is correct, however, it would appear likely to involve a localized change in membrane lipid fluidity since recent studies comparing the total phospholipid composition and fatty acyl chain saturation of plasma membranes between BHK and polyoma virus transformed BHK cells have shown no significant differences [36]. In addition, direct measurements of membrane fluidity by a variety of physical methods have resulted in reports of increased [37, 38], similar [39] and decreased fluidity [40] of transformed cells compared to untransformed cells.

Irrespective of the exact reason for the changes in activity, this study and others discussed in this paper indicate that ouabain-sensitive ATPase-dependent cation transport is intimately involved as a cause or effect of changes in cell growth for 3T3 cells and is greater in transformed 3T3 fibroblasts for large portions of the growth curve. It is not clear how general this finding is with regards to transformed cells, although transformed baby hamster kidney cells [14] and malignant rat thymus cells [17] also showed increased $^{86}Rb^+$ and $^{42}K^+$ uptake.

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