Effect of Rb^+ Substituted for K^+ on HeLa Cells: Cellular Content and Membrane Transport of Monovalent Cations, and Cell Growth

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ABSTRACT. The usefulness of Rb^+ was investigated as an analog of K^+ in membrane transport and in supporting the growth of HeLa cells. On replacement of extracellular K^+ by Rb^+ , intracellular Rb^+ content increased linearly at 15 min. K^+ content declined in contrast with Rb^+ , but Na^+ content was unaffected. In Rb^+ -preloaded cells, Rb^+ and K^+ were exchanged after replacement of extracellular Rb^+ with K^+ . A kinetic analysis of intracellular Rb^+ content showed that most K^+ was exchangeable with Rb^+ , but a small part of K^+ was not. Both Rb^+ and K^+ influxes were equally inhibited by ouabain or furosemide. Rb^+ efflux was repressed by furosemide, whereas K^+ efflux was not influenced. When 90% of K^+ was replaced by Rb^+ in serum-free chemically defined medium, cell growth and protein synthesis were inhibited. After total replacement of extracellular K^+ by Rb^+ , cell growth ceased and cellular protein content decreased, while a small part of intracellular K^+ remained unexchanged. HeLa cells seemed to accumulate Rb^+ in preference to K^+ .

Rb^+ has been reported to behave more like K^+ than like the other alkali metal cations in membrane transport (1, 3, 5, 26, 38) or activating $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ (35, 41). In contrast, the selective uptake or accumulation of Rb^+ to K^+ has been reported for various organs and animal species (15, 18). This suggests that Rb^+ cannot be used as an analog for K^+ in every tissue. Selectivity appears to be minimal in erythrocytes, because Rb^+ has been shown to produce competitive inhibition of K^+ uptake (10) and to share the same path for active inward K^+ transport (17). Hence, Rb^+ has been a good tool with which to study the K^+ transport systems in erythrocytes (8, 9). However, some counter-evidence indicates that the relative affinities of Rb^+ and K^+ for the K^+ sites on the hypothetical carriers in erythrocytes are not equal (11, 17).

In the cultured mammalian cells Rb^+ has been used as a substitute for K^+ (6, 7, 13, 20, 28, 36), and as $^{86}\text{Rb}^+$ did not differ from $^{42}\text{K}^+$ in the inward transport system of mouse 3T3 cells, $^{86}\text{Rb}^+$ has proved to be a reliable substitute for K^+ in these cells (39). In cultured glioma and neuroblastoma cells, $^{86}\text{Rb}^+$ resembles $^{42}\text{K}^+$ in its ouabain-sensitive uptake (19). HeLa cells are reported not to discriminate $^{86}\text{Rb}^+$ from $^{42}\text{K}^+$ in membrane transport (12). K^+ leakage followed the same time course as $^{86}\text{Rb}^+$ leakage; however, the transport system seemed to require $^{86}\text{Rb}^+$ somewhat more than $^{42}\text{K}^+$. Also, data comparing the influx of the two cation species were not shown. In most of the reported experiments, tracer amounts of

$^{86}\text{Rb}^+$ were applied. The use of $^{86}\text{Rb}^+$ is based on evidence that the radioisotope is transported in the same way as K^+ across the cell membrane, and on the assumption that the isotope can be diluted evenly by intracellular K^+ as it is by extracellular K^+ . The assumption is reasonable, although it has not been tested by experiment. The intracellular K^+ content has not been determined simultaneously; it is assayed only when the intracellular $^{86}\text{Rb}^+$ concentration becomes high enough that a kinetic analysis can be made, or becomes saturated at which concentration the ratio of $^{86}\text{Rb}^+$ to K^+ is assumed to be equal inside and outside the cells. As measurement procedures are rather complex and a significant error occurs if the cells include a pool of unexchangeable K^+ , most investigators use a combination of other methods to assay the K^+ content, and they use replicate cultures. In the present study, 'non-radioactive' Rb^+ , totally or partly substituted for K^+ in the medium, was administered to the cells. Although 'non-radioactive' Rb^+ naturally contains radioisotopes, its radioactivity is so weak that its biological effects are negligible. The use of this Rb^+ has the following advantages and may have wide applicability. (1) Intracellular Rb^+ and K^+ contents can be assayed directly by flamephotometry, the results of which simultaneously determine the relationship between Rb^+ and K^+ transport in the same culture. (2) The maximal extent to which Rb^+ replaces K^+ is also determined by long incubation when necessary. This shows which cation species, Rb^+ or K^+ , is more selectively accumulated in cells. (3) Undesirable radiation effects on cells are avoided. However, data on the role of 'non-radioactive' Rb^+ applied in the same concentration range as K^+ , are not sufficient at the present time. Questions remain as to whether the inward and outward Rb^+ transport are identical with those of K^+ , and whether the cells grow in Rb^+ -substituted media.

Our intent in the present paper is to answer these questions and to show some simple techniques for the use of 'non-radioactive' Rb^+ as a tool to investigate membrane transport in cultured cells.

MATERIALS AND METHODS

Cell Cultures HeLa cells (strain S3) obtained from Flow Lab. were maintained in glass culture flasks ($40 \times 55 \times 150$ mm, Ikemoto Sci. Co.) containing 10 ml of modified minimum-essential medium (MEM) supplemented with 10% (v/v) calf serum (29). The modified MEM contained double amounts of glucose and amino acids, and triple amounts of vitamins and glutamine. Cells growing in the dense cultures were dispersed by 0.5% trypsin and inoculated at $6\text{--}7 \times 10^4$ cells/ml into Roux flasks containing 100 ml of the culture medium. They were then cultivated in a CO_2 -incubator in a humid atmosphere of 5% CO_2 and 95% air at 37°C . After three days, when the cultures were in the exponential growth phase, the cells were resuspended and seeded at 1.5×10^5 cells/ml in plastic culture dishes (60×15 mm, Falcon) containing 6 ml of the medium. These dishes were placed in the CO_2 -incubator for another 24 h.

Assays. Cultures were washed once and the culture medium was replaced with modified MEM containing 25 mM HEPES (pH 7.2). The culture dishes were then placed in the CO_2 -incubator for another 2–3 h, so that intracellular ionic conditions would come to equilibrium. The medium was again replaced with modified MEM that contained an equal concentration (5.4 mM) of RbCl in place of KCl , unless specifically stated. All procedures were carried out at 37°C . The initial rate of Rb^+ uptake was determined at 15 min (see Fig. 1), whereas K^+ uptake was measured with cells, preloaded with Rb^+ for 2–3 h, then incubated with K^+ for

15 min. When Rb⁺ or K⁺ uptake was over the medium was discarded. The culture dishes then were rinsed serially in five glass pots. Each pot contained 200 ml of ice-cold 0.15 M LiCl. After every four culture dishes, the highly stained contents of the first pot were discarded and a new pot of the same solution was placed at the last position. The time required to wash a dish in the five pots was about 15 sec. Six milliliters of ice-cold redistilled water was put in the dishes, then the cells were suspended with a silicon rubber policeman rotating at 150 cycles/min. Two and three milliliter samples of the suspension were placed in screw-capped test tubes (15 × 120 mm, Pyrex) containing 2 ml of 1 N NaOH and 3 ml of 30 mM LiCl, respectively. Therefore, a series of test tubes contained finally 0.5 N NaOH and the other 15 mM LiCl. These contents were left at room temperature for a day to lyse the cells completely. The mixtures containing LiCl were incubated in a boiling water bath for 10 min. This procedure facilitated extraction of the alkali metal cations as effectively as 1 N HNO₃ did. Finally, the extracts were frozen at -20°C. Before use, they were defrosted at room temperature and centrifuged at 3000 g for 10 min. The supernatant was used for flamephotometry.

Alkali metal cations were determined with a flame-apparatus (Model 139-0400, Hitachi Ltd.) with 15 mM LiCl as the internal standard. Whether Rb⁺, K⁺ or Na⁺ was chosen, measurement of the cation was not influenced by the other species. If the wavelength-dial of the apparatus was set to the wavelength for the line spectrum of the cation to be assayed, even twice the highest concentrations of the other cations never emitted light exceeding the background level.

Cellular protein dissolved in the 0.5 N NaOH was assayed by the method of Lowry et al. (25).

Cell Growth in the Chemically Defined Medium. Cells grown in modified MEM supplemented with calf serum for 24 h, were washed twice, then cultivated in the CO₂-incubator for 24 h with the chemically defined medium prescribed by Higuchi (16) and modified as described in the legend to Fig. 8. The KCl in the medium was replaced by RbCl in various concentrations and HEPES was omitted. Prior to and every 24 h after medium replacement, cells were sampled and dispersed in Dulbecco's phosphate buffered saline. Cell number was determined with a cell counter (Model CC-1002, Toa Med. Electron. Co.).

Chemicals and Others. Inorganic salts, glucose, ethanol and amino acids were purchased from Wako Pure Chem. Co.; RbCl (Suprapur) was from Merck; vitamins, miscellaneous materials, HEPES and ouabain were from Sigma Chem. Co.; furosemide was from Hoechst Japan Ltd.; trypsin (1:250) was from Difco Lab.; and calf serum was from the Res. Instit. of Microbial Diseases, Osaka Univ. For convenience, modified MEM was usually prepared with concentrated mixtures of amino acids (50 ×) and vitamins (100 ×) purchased from Flow Lab.

RESULTS

Transport of Rb⁺ in Relation to K⁺. After exchange of the medium containing 5.4 mM K⁺ with the medium containing 5.4 mM Rb⁺, the intracellular Rb⁺ content increased at a constant rate for the initial 15 min, then the rate dropped with time (Fig. 1). In contrast, the K⁺ content decreased in such a way that the sums of the Rb⁺ and K⁺ contents did not change with time up to 2 h, when the major portion of K⁺ had been replaced by Rb⁺. Similar time courses of ⁴²K⁺ uptake and leakage have been reported in other cells (14). Intracellular Na⁺ content was not effected by the medium exchange. Thus, Rb⁺ seemed to replace the equimolar amount of K⁺ without affecting Na⁺. The intracellular pool size of exchangeable K⁺, and the time required for Rb⁺ to replace half of the pool were estimated by the following equations. As Rb⁺ replaces the

Fig. 1. Time course of the cation contents in HeLa cells after replacement of the medium. Each point represents the mean of values determined for four samples. Medium containing 5.4 mM K^+ was replaced with medium containing 5.4 mM Rb^+ at time 0. ●, Rb^+ ; ○, K^+ ; □, sum of Rb^+ and K^+ ; and ▲, Na^+ .

equimolar amount of K^+ ,

$$dC(t)/dt = J_{Rb} \{1 - C(t)/C_p\}, \quad (1)$$

where $C(t)$ is the intracellular Rb^+ content at time t , J_{Rb} is the initial rate of Rb^+ uptake (17.5 nmol/mg of protein per min from Fig. 1), and C_p is the pool size of exchangeable K^+ . Therefore,

$$\ln \{C_p - C(t)\} = \ln C_p - (J_{Rb}/C_p)t, \quad (2)$$

$$t_{1/2} = C_p (\ln 2) / J_{Rb}, \quad (3)$$

where $t_{1/2}$ is the half saturation time of Rb^+ . By empirically changing the value for C_p we calculated whether there is a suitable value for C_p at which Eq. (2) holds. When the C_p is 890 nmol/mg of protein, the empirical value obtained from the left-hand and the theoretical value obtained from the right-hand of Eq. (2) fit well at any time (Fig. 2). This verifies the assumption that Rb^+ replaces an equimolar amount of K^+ . The $t_{1/2}$ was calculated as 35.3 min. Since the mean content of Rb^+ plus K^+ was 940 nmol/mg of protein, C_p should be about 95% of the total pool size of K^+ . Therefore, most of the intracellular K^+ is regarded as being distributed in a compartment, in which K^+ is exchangeable with extracellular Rb^+ , but a minor portion of K^+ is un-exchanged. Thus, there are at least two K^+ compartments in HeLa cells. This differs from results obtained by other investigators for HeLa cells (12) and for chick embryo fibroblasts (33). Their results were based on the method of kinetic analysis of the leakage of labeled cations and suggest a single K^+ compartment. In the Rb^+ -preloaded cells, the time courses of inward and outward Rb^+ transport were symmetric after replacement of the medium with one containing K^+ . These curves were parallel to the

Fig. 2. Time course of the Rb^+ -free fraction of the exchangeable K^+ pool after medium replacement. Points are empirical values obtained from the left-hand of Eq. (2). The straight line is the theoretical curve calculated from the right-hand of Eq. (2) in the RESULTS. See text on C_p and $C(t)$.

Fig. 3. Time course of intracellular cation contents after the normal medium was replaced with medium containing 5.4 mM Rb⁺: ●, Rb⁺; and ○, K⁺. Triangles indicate cation contents in Rb⁺-preloaded cells after the reverse medium exchange: ▲, Rb⁺; and △, K⁺.

time course curves of inward Rb⁺ and outward K⁺ transport in the control (Fig. 3). This shows that the transport of Rb⁺ is equal to the transport of K⁺ inward and outward across the plasma membrane. In the presence of extracellular 5.4 mM Rb⁺, ouabain inhibited Rb⁺ uptake at concentrations higher than 0.01 μ M (Fig. 4). The effect was strengthened as the ouabain concentration increased from 0.1 to 1 μ M, and was saturated at 10 μ M. This aspect of inhibition was similar to the same aspects reported for ⁸⁶Rb⁺ (12) and ⁴²K⁺ (4) uptake. A comparison of the inhibitory effects of 0.1–10 μ M ouabain on Rb⁺ and K⁺ uptakes revealed that the ouabain-sensitive K⁺ pump in HeLa cells cannot discriminate Rb⁺ from K⁺ because there was no significant discrepancy between the two dose-response curves. Furosemide slightly but definitely inhibited Rb⁺ uptake in HeLa cells (Fig. 5). The dose-response relation showed that the inhibitory effect took place at concentrations higher than 0.01 mM and that it was saturated above 0.2 mM. The effective concentration range was almost the same as the range reported for ⁸⁶Rb⁺ uptake in ascites cells (40). This was in sharp contrast to the fact that the inhibitory ouabain concentration was 100 times higher in ascites cells than in HeLa cells. Rb⁺ uptake was proportional to the concentration ratio of Rb⁺ to Rb⁺ plus K⁺ in the medium, in which the sums of the Rb⁺ and K⁺ concentrations were kept at either 0.54 or 5.4 mM (Fig. 6A). The linear relationship was valid even in the presence of 10 μ M ouabain and 0.9 mM furosemide (Fig. 6B). These results clearly show that Rb⁺ and K⁺ have equal affinities for the K⁺ binding sites on the hypothetical ouabain- or furosemide-sensitive carriers as interpreted from Eq. (7) of the APPENDIX. Simultaneous use of the two inhibitors induced an apparently synergistic action. This may be partly related to the decrease in the intracellular K⁺

Fig. 4. Ouabain-sensitive Rb⁺ and K⁺ uptake in relation to ouabain concentration in HeLa cells. Each point is the mean of the initial rates of cation uptake determined for four samples. The vertical bar is the S.D. of the mean. ○, Rb⁺ uptake after normal medium was replaced with medium containing 5.4 mM Rb⁺; and ●, K⁺ uptake into Rb⁺-preloaded cells after the reverse medium exchange.

Fig. 5. Rb⁺ uptake of HeLa cells in relation to the furosemide concentration. Each point is the mean of the initial rate of Rb⁺ uptake determined for four samples, and the vertical bar is the S.D. of the mean.

Fig. 6. Initial rates of cation transport in HeLa cells as functions of the Rb⁺ concentration in the medium, in which the sums of the Rb⁺ and K⁺ concentrations were maintained at constant values. Each point is the mean determined for four samples, and the vertical bar is the S.D. of the mean. A. Rb⁺ uptake and K⁺ loss. Circles, the sum of the extracellular Rb⁺ and K⁺ concentrations was 5.4 mM: ●, Rb⁺ uptake; ○, K⁺ loss. Triangles, sums were kept at 0.54 mM: ▲, Rb⁺ uptake; and △, K⁺ loss. B. Rb⁺ uptake in the presence of inhibitors. The sums of the extracellular Rb⁺ and K⁺ concentrations were 5.4 mM. ●, control; ○, 10 μM ouabain; △, 0.9 mM furosemide; and ▲, 10 μM ouabain plus 0.9 mM furosemide.

concentration caused by ouabain. Rb⁺ and K⁺ effluxes were almost unaffected in the presence of ouabain (data not shown). The K⁺ efflux was not influenced by furosemide (Fig. 7), which differs from the result reported for mouse 3T3 cells (39). Rb⁺ efflux was somewhat suppressed by furosemide. The analysis of the K⁺ leakage in our experiment was not accurate enough to be used to determine the K⁺ compartmentation.

Effects of Rb⁺ on Cell Growth and Cation Content. In a preliminary experiment, cells were grown in modified MEM with calf serum for 24 h, then they were incubated in the serum-free, chemically defined medium for another 72 h. The rate of cell growth in this medium did not differ significantly from the rate in modified MEM with calf serum. The sums of the extracellular concentrations of Rb⁺ and K⁺ were maintained at 5.4 mM. Cell growth was unaffected when half the extracellular K⁺ was replaced with Rb⁺ (Fig. 8). In addition, protein synthesis as judged from the protein content was not much inhibited (Fig. 9). The intracellular K⁺ content decreased to about half

Fig. 7. Time course of Rb⁺ and K⁺ contents in Rb⁺-preloaded HeLa cells after medium replacement. Cells were preincubated with 5.4 mM Rb⁺ for 40 min, then the medium was replaced with one containing 5.4 mM Rb⁺ or K⁺. $C(t)$ and C_0 are the cation contents at time t and 0. Closed symbols are for intracellular Rb⁺ in normal medium containing K⁺: ●, control; and ■, 1 mM furosemide. Open symbols are for intracellular K⁺ incubated in the Rb⁺-substituted medium: ○, control; and □, 1 mM furosemide.

that of the control value, but Rb⁺ was higher than K⁺ by 15% at 24 h (Fig. 10). The same tendency continued for another 48 h. Therefore, the sums of the Rb⁺ and K⁺ contents were higher than the K⁺ content of the control. Upon replacement of 90% of the extracellular K⁺ by Rb⁺, the rate of the cell growth slowed. Protein synthesis was also restrained to the same extent. The intracellular Rb⁺ content reached the level of the K⁺ content in the control, whereas the K⁺ content became extremely low. At 24 h the K⁺ content was still slightly higher than the intracellular K⁺ content observed when all the K⁺ in the medium was replaced by Rb⁺, but the amounts of the two contents became nearly equal with time. The variation in the Na⁺ content was insignificant. The total exchange of extracellular K⁺ with Rb⁺ halted cell growth, after a small increase, in 24 h, and finally the cell number decreased. Protein content was reduced at a constant rate. This suggests that protein synthesis is abolished. The intracellular Rb⁺ content reached that of the K⁺ of the control as stated above. Intracellular K⁺ decreased to 0.17–0.19 $\mu\text{mol/mg}$ of protein, then remained almost constant while protein was being decomposed. This K⁺ content may be very close to the pool size of unexchangeable K⁺ as already pointed out (Fig. 2). The intracellular Na⁺ content increased insignificantly with time.

Fig. 8. Growth of HeLa cells in chemically defined media containing various concentrations of Rb⁺ and K⁺. The culture medium was prepared as an enriched modification of Higuchi's medium (16). It contained twice the standard concentration of amino acids, and three times the concentration of vitamins and of glutamine. The sum of the Rb⁺ and K⁺ concentrations in the medium was kept at 5.4 mM. Each point is the mean determined for four samples: ●, 5.4 mM K⁺; ▲, 2.7 mM K⁺ and 2.7 mM Rb⁺; ■, 0.54 mM K⁺ and 4.86 mM Rb⁺; and ⊗, 5.4 mM Rb⁺.

Fig. 9. Protein content of HeLa cells grown in chemically defined media containing various concentrations of Rb^+ and K^+ : $\bullet$, 5.4 mM K^+ ; $\blacktriangle$, 2.7 mM K^+ and 2.7 mM Rb^+ ; $\blacksquare$, 0.54 mM K^+ and 4.86 mM Rb^+ ; and $\circ$, 5.4 mM Rb^+ .

Fig. 10. Monovalent cation contents in HeLa cells grown in chemically defined media containing various concentrations of Rb^+ and K^+ . The sum of the Rb^+ and K^+ concentrations in the medium was kept at 5.4 mM. Each point is the mean determined for four samples. A. Intracellular Na^+ . Symbols, see K^+ in B. B. Intracellular Rb^+ and K^+ . Symbols for K^+ : $\bullet$, 5.4 mM K^+ ; $\blacktriangle$, 2.7 mM K^+ and 2.7 mM Rb^+ ; $\blacksquare$, 0.54 mM K^+ and 4.86 mM Rb^+ ; and $\circ$, 5.4 mM Rb^+ . Symbols for Rb^+ : $\triangle$, 2.7 mM K^+ and 2.7 mM Rb^+ ; $\square$, 0.54 mM K^+ and 4.86 mM Rb^+ ; and $\times$, 5.4 mM Rb^+ .

DISCUSSION

$^{86}\text{Rb}^+$ has been considered to act in a manner similar to K^+ , although this is not true for every component of the K^+ transport system (39). There are data clearly indicating a discrepancy in the evaluation of $^{86}\text{Rb}^+$ for quantitative use as a substitute for $^{42}\text{K}^+$ in the same type of erythrocytes (10, 17). Whether or not Rb^+ substitutes for K^+ , most of the results reported on this point were obtained with tracer amounts of radioactive isotopes. Evidence on the usefulness of non-radioactive Rb^+ as an analog for K^+ is not yet conclusive. In the present study, extracellular K^+ was totally or partly replaced with Rb^+ , and the inward and outward transports of the cation across

the plasma membrane were investigated in HeLa cells. The conclusion that cells transport Rb⁺ almost equally as well as they do K⁺ was made from the following results. (1) Rb⁺ uptake and K⁺ loss in normal cells or the reverse in Rb⁺-preloaded cells followed symmetric time courses. (2) Rb⁺ and K⁺ bind to external K⁺ sites on the hypothetical ouabain-sensitive carriers with the same affinities. The similar result was obtained for the furosemide-sensitive carriers. (3) The Rb⁺ and K⁺ uptakes were almost equally sensitive to various concentrations of ouabain. (4) The Rb⁺ and K⁺ losses from the cells were not affected by ouabain. Only the Rb⁺ efflux was inhibited by furosemide, whereas the K⁺ efflux was insensitive to it. This evidence was obtained from experiments carried out for a short-term (in 2 h).

However, the aspect was different in experiments conducted for longer than 24 h. There was the tendency for intracellular K⁺ content to decrease with the time of culture in the control as has been reported for HeLa cells (42). The same tendency was observed for the sum of the Rb⁺ and K⁺ contents of Rb⁺-loaded cells, although the sum always exceeded the K⁺ content of the control. This indicates that cells accumulate Rb⁺ in preference to K⁺ as reported for other tissues (30, 34). Cells may accumulate Rb⁺ rather than K⁺ for the following reasons: the higher affinity of Rb⁺ for negative charges in cells, an integrated effect of imperceptible cation selectivity undetected in the short-term experiments, or enhanced cation selectivity in the transport system, which might occur during long-term incubation with Rb⁺.

A close relationship between extracellular K⁺ and protein synthesis has been pointed out for some tissues (2, 24). In cultured cells, growth and protein synthesis were suppressed as the K⁺ concentration in the medium decreased (21, 32). The growth of HeLa cells cultured in a medium supplemented with human serum was inhibited as the extracellular K⁺ concentration decreased (43). Ouabain is reported to reduce the rate of cell growth or protein synthesis by lowering either the K⁺ influx or its intracellular content (13, 20, 22, 27). Thus, protein synthesis and cell growth are regarded as dependent on the intracellular K⁺ concentration. In contrast, HeLa cells grown in serum-supplemented medium, in which all the K⁺ was replaced by Rb⁺, were only slightly inhibited. Rb⁺ could substitute for K⁺ to support cell growth (12). This result conflicts with the data reported in the present paper for a chemically defined medium. The reason for this discrepancy is not known, although serum might have a growth stimulating effect on cells grown in the Rb⁺-substituted medium. Protein synthesis and cell growth were significantly repressed when 90% of the extracellular K⁺ was exchanged for Rb⁺. The complete exchange of the K⁺ in the medium with Rb⁺ almost stopped growth within 24 h. However, Rb⁺ would not have any specific inhibitory effect because cell growth was not influenced even when Rb⁺ replaced half of the extracellular K⁺. An increase in the intracellular Na⁺ concentration to more than double that of the control did not affect the growth of L5178Y cells (37). This increase in Na⁺ was much higher than that observed in the present study. K⁺ is reported to be an essential requirement for the aminoacyl transfer reaction during protein synthesis (31), and Rb⁺ was far less effective than K⁺ in activating the reaction (23). Hence, the lack of cell growth and protein synthesis in the Rb⁺-substituted media should be due to the reduced intracellular K⁺ concentration. Neither a specific inhibitory effect of Rb⁺ nor a reduced Na⁺ gradient across the plasma membrane would cause the cessation of growth. This hypothesis is supported by the fact that even a small difference in the intracellular K⁺ content resulted in a significant difference in growth and in protein synthesis (compare the exchange of 90% and all of the extracellular K⁺

with Rb^+ in Figs. 8 and 9). Rb^+ cannot substitute for K^+ as a support for growth and protein synthesis in HeLa cells grown in a chemically defined medium.

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APPENDIX

Assume that Rb^+ and K^+ bind to the external K^+ sites and M^+ (a certain cation species) to the internal cation sites on a hypothetical membrane carrier, also that the exchange transport is carried out after all the sites have bound the cations. For typical cases:

(A) Binding to external sites is independent of binding to internal sites:

1) random binding to external sites,

$$\frac{J_{\max}}{J_{\text{Rb}}} = \left(1 + \frac{k_{\text{K}}}{k_{\text{Rb}}} \cdot \frac{[\text{K}]_o}{[\text{Rb}]_o}\right) \left(1 + \frac{1}{k_{\text{Rb}}[\text{Rb}]_o + k_{\text{K}}[\text{K}]_o}\right)^n; \quad (1)$$

2) simultaneous binding to external sites,

$$\frac{J_{\max}}{J_{\text{Rb}}} = \left(1 + \frac{k_{\text{K}}}{k_{\text{Rb}}} \cdot \frac{[\text{K}]_o}{[\text{Rb}]_o}\right) \left\{1 + \left(\frac{1}{k_{\text{Rb}}[\text{Rb}]_o + k_{\text{K}}[\text{K}]_o}\right)^n\right\}. \quad (2)$$

(B) Binding to external and internal sites take place simultaneously:

$$\frac{J_{\max}}{J_{\text{Rb}}} = \left(1 + \frac{k_{\text{K}}}{k_{\text{Rb}}} \cdot \frac{[\text{K}]_o}{[\text{Rb}]_o}\right) \left\{1 + \left(\frac{1}{k_{\text{Rb}}[\text{Rb}]_o + k_{\text{K}}[\text{K}]_o}\right)^n \left(\frac{1}{k_{\text{M}}[\text{M}]_i}\right)^m\right\}. \quad (3)$$

J_{Rb} is the Rb^+ influx; $J_{\max}$ the maximum Rb^+ influx; k_{Rb} , k_{K} and k_{M} the apparent dissociation constants of Rb^+ , K^+ and M^+ , respectively; n and m , the number of the external and internal sites on a carrier. As $[\text{Rb}]_o + [\text{K}]_o = C$, a constant concentration, Eqs. (1), (2) and (3) give the following equations:

$$\frac{J_{\max}}{cJ_{\text{Rb}}} = \left(1 + \frac{1}{k_{\text{Rb}}C}\right)^n, \quad (4)$$

$$\frac{J_{\max}}{cJ_{\text{Rb}}} = 1 + \left(\frac{1}{k_{\text{Rb}}C}\right)^n, \quad (5)$$

$$\frac{J_{\max}}{cJ_{\text{Rb}}} = 1 + \left(\frac{1}{k_{\text{Rb}}C}\right)^n \left(\frac{1}{k_{\text{M}}M_i}\right)^m, \quad (6)$$

where $cJ_{\text{Rb}} = J_{\text{Rb}}$ provided $[\text{Rb}]_o = C$, i.e. the medium contains only Rb^+ , and K^+ is omitted. If $k_{\text{Rb}} = k_{\text{K}}$, then Eqs. (1) and (4), (2) and (5), or (3) and (6) give

$$J_{\text{Rb}} = \frac{[\text{Rb}]_o}{C} \cdot cJ_{\text{Rb}} \quad \text{or} \quad \frac{[\text{Rb}]_o}{[\text{Rb}]_o + [\text{K}]_o} \cdot cJ_{\text{Rb}}. \quad (7)$$

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