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Potassium: Effect on DNA Synthesis and Multiplication of Baby-Hamster Kidney Cells

(cell cycle/membrane potential/synchronization/transformation)

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ABSTRACT **The relations between DNA synthesis, cell multiplication, and external potassium concentration have been investigated in cultured baby-hamster kidney cells. When the potassium concentration was raised from 8 mM to 114 mM by equimolar replacement of sodium, DNA synthesis and cell multiplication were almost completely inhibited. This inhibition was reversible even after 72 hr of incubation in medium with a high concentration of potassium. There is a consistent difference between the cultured cells and polyoma virus-transformed cells in response to high-potassium medium, a higher-potassium concentration being required to inhibit multiplication of polyoma virus-transformed cells to the same extent as that of the nontransformed cells.**

The regulation of the processes of cell growth and division remain incompletely understood. A number of procedures are currently available that will impose at least some degree of synchronous growth upon a population of cells. The methods fall into two major categories: those where a metabolic poison is used and those that utilize physical manipulations of the cells. In the former category, most work has emphasized the inhibition of DNA synthesis by a disruption of nucleotide metabolism, e.g., thymidine blockade. However, such techniques do not always lend themselves to studies of the initiation and control of nucleic acid and protein synthesis (1). In the second category, cell synchrony can be brought about by physically dislodging and collecting mitotic cells. For a short period these cells then progress synchronously through the cell cycle. The method has the drawback of being time-consuming, particularly if large numbers of cells are required.

Cone (2) described the reversible inhibition of multiplication of Chinese-hamster ovary (CHO) cells by reducing the Na^+/K^+ ratio. This article is the first in a series that will describe the changes that occur in baby-hamster kidney (BHK) cells cultured in media in which sodium has been replaced by potassium. A major effect of increasing the potassium concentration is the inhibition of DNA synthesis.

MATERIALS AND METHODS

Cell Lines. Stocks of BHK 21 cells (line C 13-404) and the polyoma-transformed variant PyBHK H3 were obtained originally from Dr. H. V. Aposhian. These cell lines are incubated at 37.5°C on Falcon plastic petri plates in Eagle's minimal essential medium supplemented with 5% tryptose phosphate broth and 10% calf serum (MEM5 10) in an atmosphere containing 5% CO_2 in air. Calf serum (Lot No. A 3073G) was obtained from Grand Island Biological Co.

Defined Medium. Ham's F 12 medium, supplemented with 2× and 1× amino-acid and vitamin concentrations, respectively, and containing 10% calf serum, is referred to subsequently as normal medium. A medium in which each Na^+ salt has been replaced with its K^+ equivalent, but which in all other respects is identical to normal medium, is referred to as 100% K^+ medium. The concentrations of Na^+ and K^+, together with the respective tonicities of both normal and 100% K^+ medium, are shown in Table 1.

In the experiments reported below, cells have been maintained in media containing different concentrations of K^+. These were prepared by mixing normal medium with 100% K^+ medium in different proportions. Thus, if seven volumes of 100% K^+ medium are mixed with three volumes of normal medium, a medium containing 114 mM K^+ and 60 mM Na^+ is obtained; such a medium is referred to as 114 mM K^+ medium.

Conditioned Medium was prepared by incubation of logarithmically growing cells in either normal or 114 mM K^+ medium for 36 hr. The medium was then decanted and centrifuged at 1000 × *g* to remove debris before re-use.

Determination of Cell Density. Cells were removed from the plate with 0.25% trypsin (Trypsin, 1:250, Nutritional Biochemicals) and the density was determined by counting of an appropriate dilution in a hemocytometer.

Chemical Analysis. DNA, RNA, and protein have been analyzed by Fleck and Munro's (3) modification of the Schmidt and Thannhauser procedure (4). Briefly, the method is as follows: Cells are washed three times with Hanks' balanced salt solution and the plates are put on ice. 5% ice-cold perchloric acid is added, and 10 min later the cells are scraped off the plates. The acid-insoluble material is obtained by centrifugation. RNA is extracted from the pellet with 0.3 N

TABLE 1. *Na^+ and K^+ content of normal and high-K^+ media*

	Normal (mM)	100% K^+ (mM)
Na^+ (mM)	158	14.5
K^+ (mM)	8	150
	(324 milliosmoles/liter)	(318 milliosmoles/liter)

Abbreviations: BHK cells, baby-hamster kidney cells; PyBHK cells, polyoma virus-transformed baby-hamster kidney cells.

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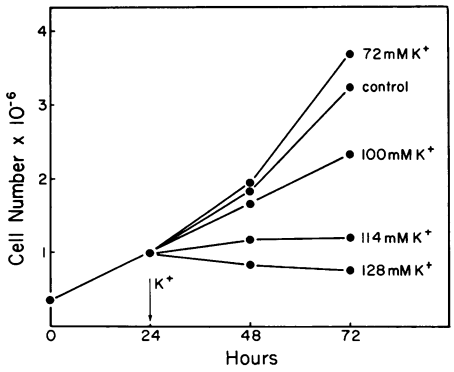

FIG. 1. The multiplication of BHK cells seeded at 3×10^5 cells/plate as a function of external K^+ concentration.

NaOH at 37°C for 45 min. The mixture is acidified with 10% perchloric acid and centrifuged. The resulting supernatant is analyzed spectrophotometrically for RNA ($A_{260} = 1 = 34$ μg of RNA). DNA is extracted from the pellet with 5% perchloric acid at 80°C for 30 min, and its concentration is determined on the basis of $A_{260} = 1 = 32$ μg of DNA. The ratio 280/260 nm was always within the range 0.825 ± 0.025, which shows little, if any, protein contamination. The pellet remaining after DNA extraction is dissolved in 1 N NaOH at 37°C for 60 min, and protein is determined by the procedure of Lowry *et al.* (5).

Radioactivity. [^{3}H]Thymidine (16.4 Ci/mmol) and [^{14}C]-uridine (0.05 Ci/mmol) were obtained from Schwarz Bio-Research Inc.

Pulse-Labeling Experiments. A measure of the rate of DNA and RNA synthesis was obtained by incubation of the cells with [^{3}H]thymidine and [^{14}C]uridine, respectively. They were then washed three times with glucose-free Hanks' solution containing 2 mM thymidine, followed by one wash with cold glucose-free Hanks' medium containing 1 mM sodium azide. The cells were transferred to ice, chilled, and 5% trichloroacetic acid containing 100 mM sodium pyrophosphate was added. 10 min later, the cells were scraped off the plates. The acid-insoluble material was collected on Whatman GF/C filters and washed four times with cold 5% trichloroacetic acid containing 100 mM sodium pyrophosphate. The filters were dried and counted in toluene flour in a Packard liquid scintillation counter.

RESULTS

The effect of modification of K^+ concentration on cell growth

BHK cells were seeded on 60 × 15 mm plates at a density of 3×10^5 cells/plate in MEM5 10 medium. After 24 hr, the medium was changed to either normal or K^+ medium, the latter ranging in concentration from 72 mM K^+ to 128 mM K^+. The cell density was determined (two plates/point) at 24-hr intervals. The findings are presented in Fig. 1. At 72 mM K^+, the rate of cell growth exceeds that of the control (8 mM K^+); above 72 mM K^+, cell growth is progressively reduced. At 114 mM K^+, the cell density remains about constant, whereas at 128 mM K^+ cells soon round up, detach from the plate, and die. Microscopically, the cells maintained at 114 mM K^+ appear healthy; however, it can be seen (Fig. 2) that their morphology is altered. The cells are flattened and present markedly fewer extended processes. These experiments have been repeated with PyBHK cells. The findings are shown in Fig. 3. The data are similar to those presented in Fig. 1, i.e., high extracellular K^+ concentrations inhibit cell growth. However, it has been repeatedly observed that there is a

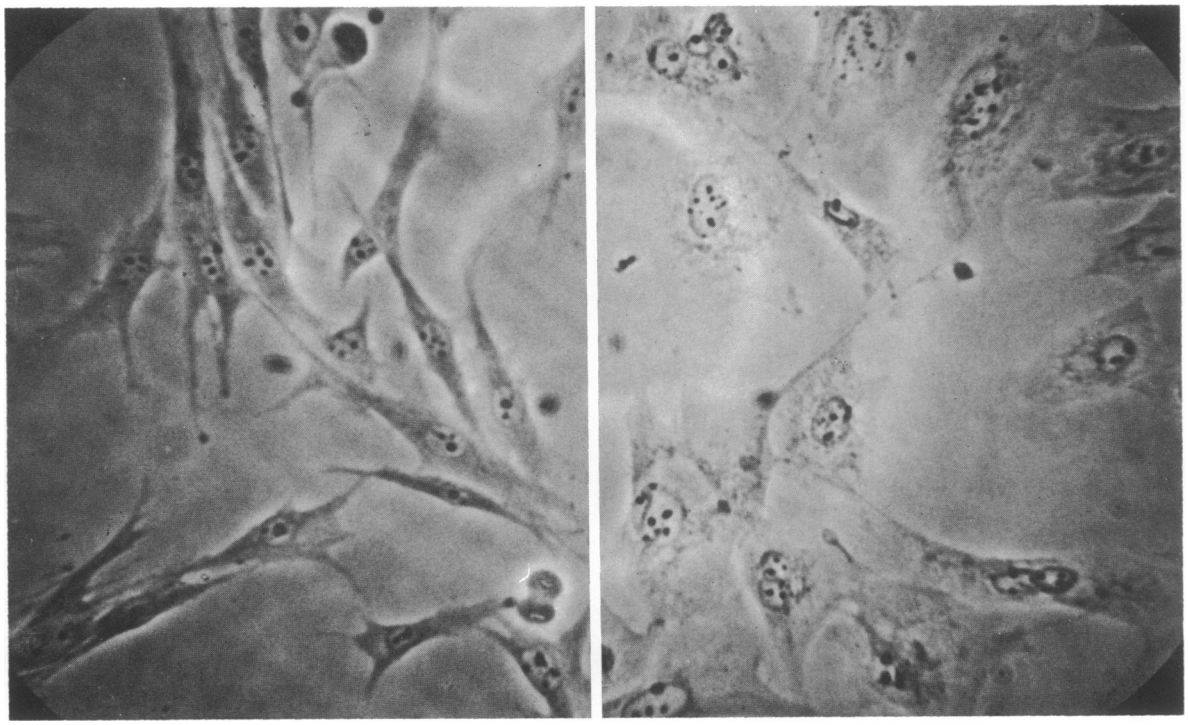

FIG. 2. (*Left*) Phase-contrast photomicrograph of BHK cells in normal medium (8 mM K^+), ×300. (*Right*) Phase-contrast photomicrograph of BHK cells maintained in 114 mM K^+ medium, ×300.

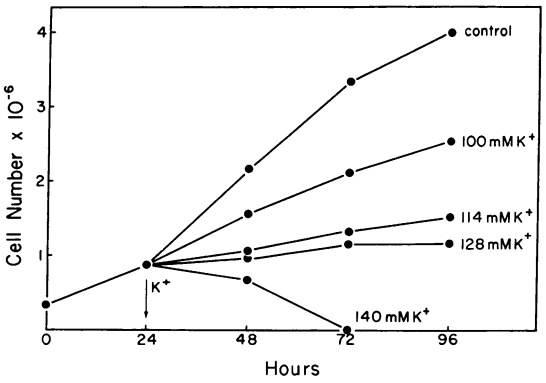

FIG. 3. The multiplication of PyBHK cells seeded at 3×10^5 cells/plate as a function of external K^+ concentration.

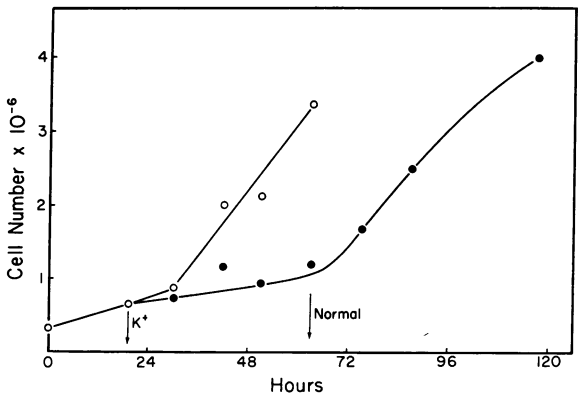

FIG. 4. The recovery of multiplication of BHK cells after 45 hr in 114 mM K^+ medium. *Arrows* indicate time of introduction and removal of high-K^+ medium.

consistent difference between BHK and PyBHK cells in response to K^+ medium; a higher K^+ concentration is required to inhibit growth of PyBHK cells to the same degree as BHK cells. For example, 114 mM K^+ medium allows for significant growth of PyBHK cells, whereas this concentration of K^+ almost completely inhibits growth of BHK cells.

From these results it was clear that by appropriately modifying the K^+/Na^+ ratio in the growth medium, cell division in both BHK and PyBHK cells can be inhibited. These observations prompted us to investigate the character of this inhibition more closely. The remainder of this paper describes an attempt to answer two questions: Is the inhibition of cell division by high concentrations of K^+ reversible? Is it possible to detect any biochemical changes that might elucidate the biochemical basis of the phenomenon?

The recovery of cells maintained in high K^+

The design of the experiments described in Figs. 1 and 3 was used again, BHK cells being seeded at 3×10^5 cells per plate in MEM5 10. After 20 hr, the medium was changed to either normal medium or to 114 mM K^+ medium. The cell density was determined (two plates/point) at 12 hr intervals for a period of 48 hr. Cell growth was minimal in 114 mM K^+ medium and exponential in normal medium (Fig. 4). At 58 hr, the K^+ medium was replaced with normal medium and cell density was measured during the recovery period. It is clear from Fig. 4 that the inhibition of cell division by K^+ medium is reversible.

Chemical analyses of cells maintained in high K^+ medium

BHK cells were plated at 7×10^5 cells per plate in MEM5 10 in 100 × 20 mm dishes. 24 hr later, the medium was changed to either 114 mM K^+ or to normal medium. Chemical analyses were made on cells from duplicate plates 24, 48, and 72 hr later. At 72 hr, fresh normal medium was added to the remaining plates and these cells were analyzed 24 hr later. The data are presented in Table 2. It is clear that as cell multiplication slows due to high extracellular K^+ concentration (114 mM), so does the synthesis of DNA, RNA, and protein, and that the synthesis of these molecules resumes when normal medium is supplied. However, when adjusted to the number of cells present, the values are all higher in the cells exposed to 114 mM K^+. There are at least two possible interpretations of these results: (*a*) synthesis of these macromolecules occurs slowly in the absence of cell division; or, (*b*) a degree of synchrony has been imposed upon these cells so that their chemical composition differs from that of cells in a randomly growing population.

DNA and RNA synthesis by BHK cells in 114 mM K^+ medium

The effect of 114 mM K^+ medium on the biosynthesis of DNA and RNA was examined to determine how rapidly the K^+ blockade was initiated. Cells were pulsed with [^{3}H]thymidine and [^{14}C]uridine at different times after the initiation of a high-K^+ regimen. The experimental design was as follows:

TABLE 2. *Chemistry of BHK cells maintained in 114 mM K^+ medium*

Treatment	Hours	Cell density ×10⁶	Total DNA (μg)	DNA per 10⁶ cells (μg)	DNA K^+/ normal	Total RNA (μg)	RNA per 10⁶ cells (μg)	RNA K^+/ normal	Total protein (μg)	Protein per 10⁶ cells (μg)	Protein K^+/ normal
K^+ medium	22	0.75	8.5	12	1.9	35.3	47.1	1.67	125	159	1.46
Normal medium		2.8	17.6	6.3		82	29.3		305	109	
K^+ medium	46	1.23	10.4	8.5	1.1	34	29.2	1.49	162	129	1.65
Normal medium		6.0	47.2	7.7		119	19.6		473	78	
K^+ medium	70	2.05	19.5	9.5	1.35	66	32.2	1.62	238	116	1.27
Normal medium		7.8	54.5	7.0		155	19.8		722	92	
Recovery											
K^+-treated cells	24	6.0	27	4.5	0.6	91	15	0.68	371	62	0.67
Normal cells		9.2	72	7.8		206	22.4		845	92	

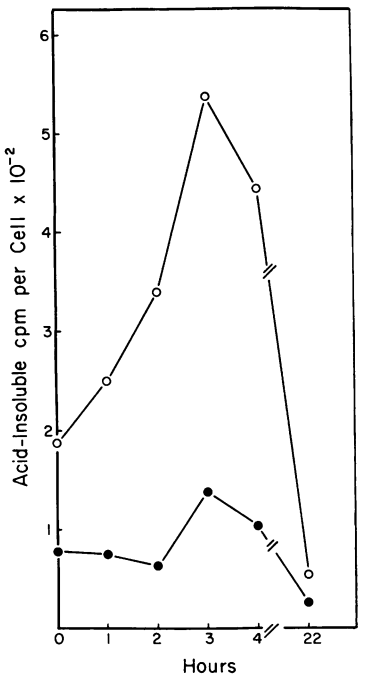

FIG. 5. The effect of conditioned 114 mM K^+ medium on the rate of DNA synthesis in BHK cells. O—O, conditioned normal medium; ●—●, conditioned high-K^+ medium.

exponentially growing BHK cells were fed with either conditioned normal medium or conditioned 114 mM K^+ medium. Conditioned medium was used in these experiments because it was found at the outset that fresh medium temporarily halted BHK cell growth and the synthesis of all macromolecules. At the times indicated in Figs. 5 and 6, 5 μCi/ml of [^{3}H]thymidine or 0.5 μCi/ml of [^{14}C]uridine were added to duplicate plates in either normal or 114 mM K^+ medium. Zero time, therefore, denotes both the time of medium change and the first radioactive pulse. The cells were pulsed for 15 min and the acid-insoluble radioactivity was measured. Several observations emerge from the data presented in Fig. 5. DNA synthesis in K^+ medium is immediately reduced to an extent about half that of the control. Subsequently, the low amount of DNA synthesis in K^+ medium is maintained, whereas during the next 4-hr DNA synthesis on a per-cell basis in normal medium increases 2- to 3-fold. By 22 hr, the rate of DNA synthesis in the control cells is also greatly reduced. Quite clearly, the reduced rate of DNA synthesis is not due to the conditioned medium becoming depleted or inhibitory, since fresh conditioned medium given at 22.75 hr does not alter the rate of DNA synthesis. It might be anticipated that DNA synthesis should remain constant on a per-cell basis in a logarithmically growing population of cells. The data shown in Fig. 5 are repeatable and occur whether fresh or conditioned medium is used. We have no explanation for this phenomenon. It does not appear to be a typical "negative pleiotropic response" (6), nor is it readily explained as either a transport effect (7), or as a clock mechanism based on cyclic changes in pH of the medium (8).

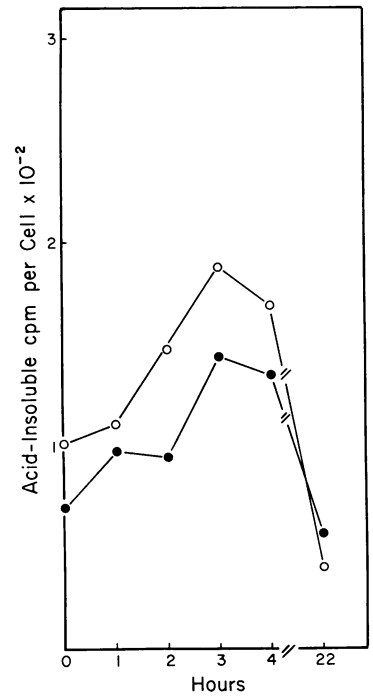

FIG. 6. The effect of conditioned 114 mM K^+ medium on the rate of RNA synthesis in BHK cells. O—O, conditioned normal medium; ●—●, conditioned high-K^+ medium.

Incubation in 114 mM K^+ medium causes a small, but significant, reduction in RNA synthesis during the first 4 hr of incubation (Fig. 6); by 22 hr, the difference between normal and 114 mM K^+ medium is insignificant.

The synchronization of BHK cell growth by K^+ blockade

The immediate and reversible effect of high concentrations of K^+ on cell multiplication suggested the use of K^+ as a means of initiating synchronous cell growth. To explore this possibility, BHK cells were maintained in 114 mM K^+ medium for 24 hr. At this time, the K^+ medium was replaced with normal medium, and each successive hour DNA synthesis was measured as described in the previous experiment. Duplicate plates were used to determine cell density, DNA synthesis, and the number of mitotic figures. Mitotic figures were counted in five separate fields on each plate. The data are shown in Fig. 7. The major feature of Fig. 7 is the presence of two synchronous waves of DNA synthesis. The initial wave, which represents about 15% of the larger wave, occurs immediately upon release from K^+ blockade and is followed by a peak of

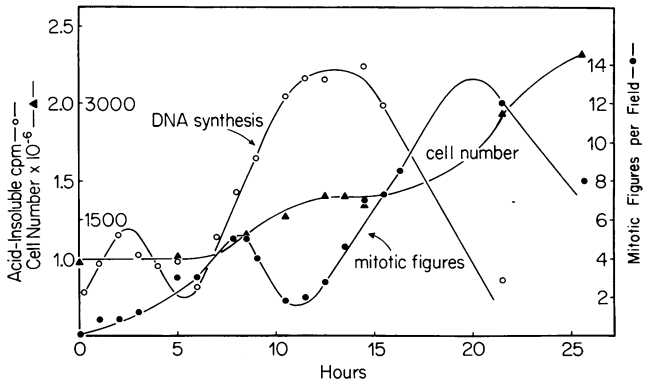

FIG. 7. DNA synthesis, cell number, and mitotic figures in BHK cells maintained in normal medium after 24 hr in 114 mM K^+ medium. O—O, DNA synthesis; ▲—▲, cell number; ●—●, mitotic figures.

mitotic activity. Little, if any, increase in cell density occurs during this period. It can be concluded that a group of cells (about 15% of the population) were blocked either in late-G1 or early-S phase. The major wave of DNA synthesis peaks about 13 hr after release from K^+ medium. It, too, is followed by a synchronous burst of mitotic activity.

The period between the peaks of DNA synthesis and mitotic activity is 5–6 hr. Consequently, it can be concluded that cells require 5–6 hr to progress from S to M phase in the BHK cell cycle. Since the doubling time of these cells is 18 hr, the length of the G1 phase is calculated to be about 12 hr. The principal blockade created by incubation in 114 mM K^+ medium must occur about the middle of the G1 period. This conjecture is confirmed by the data of Fig. 7 that show that peak DNA synthesis for the majority of cells released from K^+ blockade occurs 13 hr after release.

DISCUSSION

In this report we have shown that high concentrations of extracellular K^+ can prevent the multiplication of both BHK and PyBHK cells. It has also been shown that the concentration of extracellular K^+ ordinarily used in the culture of BHK cells is suboptimal. At concentrations between 36 and 72 mM K^+, the rate of BHK cell multiplication is enhanced. These observations are novel for BHK cells, but similar data have been presented for LM-strain mouse fibroblasts (9). The inhibition of growth is reversible, since it has been shown that replacement of a high-K^+ medium with normal medium results in an immediate return to exponential growth. Highly contact-inhibited 3T3 and simian virus 40-transformed 3T3 cells are also reversibly inhibited by high K^+ (Yoshikawa-Fukada, M, unpublished observations).

We have already remarked on one surprising feature observed in cells maintained in 114 mM K^+, namely that, on a per-cell basis, they contain higher concentrations of DNA, RNA, and protein. As suggested above, this could be the consequence of a slow synthesis of macromolecules without concomitant cell division. There are, of course, complex and variable relations between phases of the cell cycle and enzyme activity, so that a synthesis of protein without cell division, or even without DNA replication, is not completely unexpected (1). A second possible explanation, namely that cells may be clustered at a point in the cycle where, in comparison to a randomly growing population, their content of macromolecules is elevated, is substantiated by the finding that cells released from K^+ blockade passed through a cell cycle synchronously. The majority of cells are held in mid-G_1 phase, while a smaller fraction appears to be blocked at the G_1–S border.

The pulse-labeling experiments indicated a very marked effect of K^+ on DNA synthesis. Upon addition of high concentrations of extracellular K^+, the rate of DNA synthesis is immediately reduced; upon release from K^+ blockade, DNA synthesis resumes.

RNA synthesis is only moderately affected by 114 mM K^+. However, release to normal medium resulted in an increased rate synthesis of RNA (10). An analysis of the polysome profile from cells in normal and 114 mM K^+ medium shows that after 24 hr in 114 mM K^+ medium, the content of polysomes in the cell is reduced; upon release from the K^+ medium, the polysomes rapidly reappear (10).

These observations raise a number of critical questions: Does K^+ flow into the cell and thus rapidly disrupt the metabolic machinery? Is the cessation of cell growth in high-K^+ medium due to a reduction in cellular ATP?

The question of K^+ entry into the cell has been investigated both by electrophysiological methods and, more directly, by chemical analysis of intracellular K^+ and Na^+ concentrations in 114-mM K^+ medium as a function of time (5 min and 24 hr). Concurrently the concentrations of ATP were determined (McDonald, T. F., Sachs, H. G., Orr, C. W. & Ebert, J. D., unpublished).

At 5 min of exposure to 114 mM K^+, there is no change in intracellular concentration of K, yet DNA synthesis is dramatically reduced. A small shift in intracellular concentration of K is observed at 24 hr, but it could hardly account for the differences in growth and DNA synthesis observed.

Nor is there any change from the normal in the ATP content of BHK cells incubated for 24 hr in 114 mM K^+ medium. Similarly, 3T3 and simian virus 40-3T3 cells incubated in high-K^+ medium have a normal ATP content (Yoshikawa-Fukada, M., unpublished).

The effect of K^+ on DNA replicase activity has also been examined (11). Although the replicases of BHK and PyBHK appear to differ when a number of criteria are examined, they do not differ significantly in their susceptibility to K^+. It appears unlikely that the inhibition of DNA synthesis by K^+ is mediated through an inhibition of DNA replicase activity. We are left with the question of whether the changes observed in DNA synthesis and cell multiplication due to the external K^+ concentration are mediated through resultant changes in the membrane potential. This aspect will be treated in subsequent papers.

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