Electrolyte Metabolism in HeLa Cells

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ABSTRACT Methods have been developed to study cellular Na, K, and Cl concentrations in HeLa cells. Cell [Na] and [K] are functions of the age of the culture. As the culture grows [K], expressed in mmols/liter cell H_2O, rises from an initial value of 121 to a peak of 206 at about 4 days, and thereafter falls until it has almost returned to the initial value by the 9th day. [Na] falls as [K] rises, but there is no fixed relationship between the cellular concentrations of the two cations. There is, however, a correlation between generation time and cellular [K]. Measurements of net K uptake and net Na extrusion were carried out during 1 hour incubation at 37°C of low K cells. Both net K uptake and net Na extrusion took place against chemical concentration gradients, so that at least one transport system must be active; if the Cl distribution is passive both net K uptake and net Na extrusion are active. Studies with inhibitors of respiration and glycolysis lead to the conclusion that respiration is not required for these net transports, which appear to derive their energy from glycolytic sources.

The present study is concerned with the electrolyte metabolism of the HeLa cell, a cell type obtained from an epidermoid carcinoma of the cervix in 1951 (1) and since then maintained in culture. The cells obtained from such cultures are relatively uniform and are suitable for quantitative experimental work. The electrolyte concentrations and water content in HeLa cells have been shown to vary with the age of the culture. Furthermore the role of metabolism in maintaining ionic concentrations has been investigated and it has been found that glycolysis is an important source of energy for Na and K transport.

EXPERIMENTAL METHODS

Cell Culture

The original stock of HeLa cells used in these experiments was obtained from Microbiological Associates. These cells were grown in monolayers on a flat glass surface (area, 44 cm^2) in milk dilution bottles (Corning No. 1367) using the growth medium described by Puck and others (2); its composition is shown in Table I. All stock solutions were freshly prepared at monthly intervals. Cells were subcultured weekly by

trypsinizing full grown cultures in 0.05 per cent trypsin solution (2) until most of the cells were freed from the surface (3 to 5 minutes). The trypsin was then inactivated by dilution with the nutrient medium. New bottles were adjusted to a final cell concentration of 10^5 cells per ml (10^6 cells per bottle). The culture medium in each bottle was changed daily, a treatment found necessary in order to obtain reproducible growth rates.

All solutions were sterilized by filtration under pressure through a millipore filter at weekly intervals whether the solutions were freshly prepared or not. Thus, bacterial contamination was virtually eliminated. The cultures were subdivided and their

TABLE I

COMPOSITION OF GROWTH MEDIUM*, ‡

Substance	Concentration
	mM
NaCl	88.4
$NaHCO_3$	23.5
Na_2HPO_4	0.36
KCl	2.70
KH_2PO_4	0.25
$MgSO_4$	0.64
$CaCl_2$	0.24
Glucose	0.72
L-Amino acids	1.10
Vitamins	0.01
Total Na (determined analytically)	152.4±0.2 (9)
Total K (determined analytically)	5.5±0.1 (13)
Total Cl (determined analytically)	129±5 (10)
Osmolarity of medium (mOsM)	293±0.3 (5)
pH	7.4

* In addition, the medium contained 20 per cent human serum, 10 per cent horse serum, 0.3 mM hypoxanthine, 1.4 μg/ml phenol red, and 296 μg/ml streptomycin and 335 units/ml of penicillin.

‡ Errors in this and subsequent tables are standard errors. The number of experiments is given in parentheses.

medium changed at room temperature inside a bacteriological hood previously sterilized by ultraviolet light. Cells about to be used for experiments were trypsinized in the 37°C room in which the cultures normally grew.

The cultures were tested for the presence of pleuropneumonia-like organisms by plating out old medium onto ascites fluid agar plates. When this type of contamination was suspected, oxytetracycline 2.5 μg/ml was added to the medium during one or two subcultures (3).

The growth of a culture (comprising 20 to 30 bottles of cells) was measured by periodic cell counts. Cells from 2 to 5 bottles (depending on the density of the population) were trypsinized until the cell sheet was completely free of the glass surface. This took 10 to 12 minutes, after which the cells were suspended in fresh medium in a

10 ml volumetric flask. Cell counts were made in a hemacytometer of 0.1 mm depth with bright line, double improved Neubauer ruling. Approximately 900 cells were counted for each determination, resulting in a standard deviation of about 3 per cent (4) and the results were expressed in terms of the number of cells per bottle.

Measurement of Electrolyte Concentration

In order to measure intracellular electrolyte concentrations, cells from 2 to 5 bottles were trypsinized and suspended in nutrient medium to form a suspension of about 1 per cent cell volume per total volume. The suspension was transferred to a 50 ml Erlenmeyer flask and incubated in a water bath at 37°C with sufficient shaking to keep the cells in suspension. This incubation period was found necessary to bring cells into a quasi-steady state with respect to intracellular ion concentrations. After 1 hour the suspension was centrifuged in specially designed centrifuge tubes of 3 ml capacity. These tubes, similar to those previously described in detail (5, Fig. 1), consist of an upper reservoir and a lower tube of capillary glass with a precision made bore having a diameter of 1.02 mm and a volume of 43.3 μl. After these tubes had been filled with cell suspension, they were centrifuged at 4°C for 20 minutes at 10,000 $\times$ *g* in a specially constructed horizontal rotor. After centrifugation, a portion of the resulting column of closely packed cells was extruded onto a tared aluminum disc and weighed immediately on a quartz helix balance (capacity 19 mg). A correction for evaporation was made as previously described (5). The pellet was then dried overnight at 100°C and reweighed to obtain the dry weight, which ranged from 1 to 2 mg.

The pellets of dried cells were ashed with 50 to 100 μl concentrated distilled nitric acid in vycor glass tubes over a micro-Bunsen burner. The residue was taken up in 5 ml distilled water and the K and Na contents determined on the Beckman flame photometer modified by Solomon and Caton (6). Cellular ion concentrations were calculated after correcting for the trapped medium in the pellets. The latter was measured using I^{131}-albumin and was found to be 100 $\pm$ 3 μl/gm wet weight (SE, 16 determinations) when the cells were suspended in complete growth medium. The standard deviation in a series of 10 replicate pellets was 2 per cent for [K] and 6 per cent for [Na]. For Cl measurements, dried cell pellets were suspended in 1 ml of 1 per cent $NaNO_3$ or KNO_3 and shaken overnight on a mechanical shaker. The Cl content was then determined potentiometrically by the method of Ramsay, Brown, and Croghan (7) as modified by Whittembury *et al.* (8). The standard deviation of the intracellular [Cl] in a series of 10 replicate pellets was 5 per cent.

In order to determine whether the cells were injured from the trypsinization prior to the experiments, freshly trypsinized cells were treated with lissamine green. Holmberg (9) has shown that cells of 4 culture types became permeable to lissamine green after injuries which resulted in an inhibition of cell respiration and certain enzyme activities. Using this method we have demonstrated that treatment with trypsin does not injure cells sufficiently to make them permeable to lissamine green since 94 to 95 per cent of growing cells from cultures of various ages were found to be impermeable to the dye.

RESULTS AND DISCUSSION

Growth of Culture

The cell count per bottle as a function of the age of the culture is plotted in Fig. 1. The 3 cultures shown are typical of 17 such experiments. After a variable lag period, the cells increased in number almost exponentially until there were about 10^7 cells per bottle. At this point the entire lower surface of the bottle was covered with a single layer of cells; during the ensuing 2 to 5

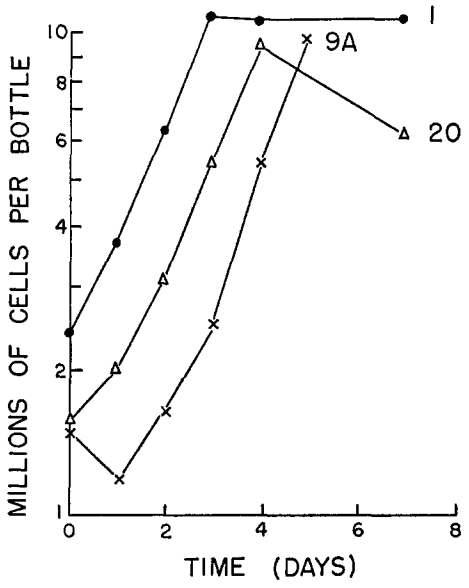

FIGURE 1. Growth curve of 3 HeLa cell cultures, expressed in terms of cell numbers.

days the cells declined in number, often by only a few per cent but sometimes by as much as 35 per cent.

The time required for the cells to double in number (the generation time) seemed to decrease during the growth period of the culture as shown in Fig. 2. On the 1st day of subculture the cells either remained at their original number or else divided with a generation time of 30 to 40 hours. During the following days the generation time fell progressively to a value of 25 to 27 hours, at which time there were 0.9 to 1.2 $\times$ 10^7 cells per bottle.

Electrolyte Concentration

The ratio of dry to wet weight of packed HeLa cells obtained from cultures of all ages was 18.5 $\pm$ 0.8 per cent (SE, 36 determinations). The solid content of the cells is calculated to be 20.6 per cent of the wet weight after correction for trapped medium.

The cellular K and Na concentrations in HeLa cells, measured after 1 hour's incubation at 37°C, were found to depend on the age of the culture. These results are shown in Fig. 3 which presents the average results for 4 experiments for Na and 5 for K. The K concentration rose steadily from an initial value of 121 ± 5 mmols/liter cell H_2O to a maximum concentration of 207 ± 12 mmols/liter cell H_2O. In the next 6 days the concentration decreased until it returned to about its original value. The Na concentration, also shown in Fig. 3, started at 90 ± 26 mmols/liter cell H_2O and then fell to 32 mmols/liter cell H_2O and remained near that level for several days

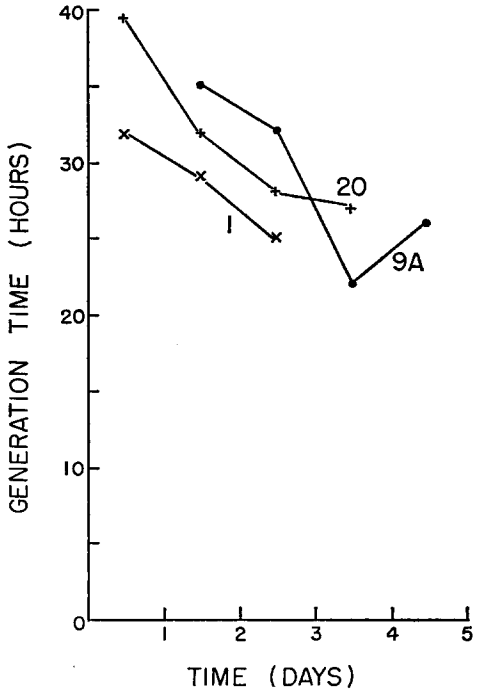

FIGURE 2. Change in generation time with growth of culture in 3 representative cultures.

before rising slowly. Table II shows that the sum of the intracellular concentrations of Na, K, and Cl rose synchronously with K concentration.

These results indicate that HeLa cells grown in monolayers exhibit progressive changes in K, Na, and Cl concentration during the course of growth of the culture. Since the nutrient medium bathing the cells was replaced every day throughout the period of growth, nutrients were equally available at every stage and the accumulation of toxic products was avoided. It does not therefore appear likely that the cycle of K concentration may be attributed to changes in the medium. An examination of Figs. 1 and 3 indicates that the rise in cell K concentration coincides with the increase in cell growth rate. The subsequent decline in cell K concentration coincides with the cessation

of cell growth, and the usual decline in cell number. In Fig. 4 the intracellular K concentration is plotted as a function of the generation time of the culture from which the cells were obtained. The regression line is statistically significant ($p < 0.01$) which suggests that cell K concentration and cell division are somehow related.

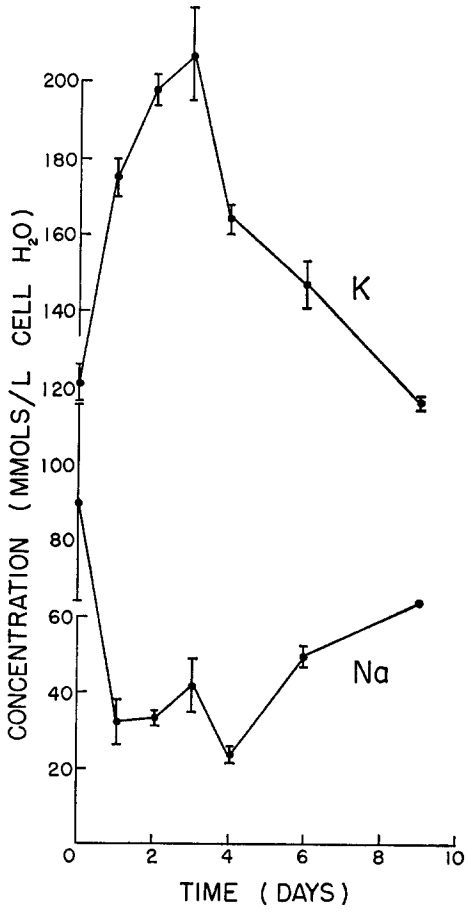

FIGURE 3. Na and K concentrations of HeLa cell cultures throughout growth phase. Each point represents the mean of 5 experiments for K and 4 for Na. Errors are standard errors.

In addition, changes in chemical composition of the cells during growth of the culture are indicated by the absolute increase in cation concentration. Table II shows that growth is accompanied by only small changes in Cl concentration. Therefore electroneutrality can be preserved only by the accumulation of impermeant anions if we assume that the cellular HCO_3 concentration remains constant. The data in Table II may be used to calculate the cellular anion concentration and valency, based on the restraints of electroneutrality and isosmolarity and the assumption that there are no significant cellular cations other than K and Na. This calculation leads to a cellular

TABLE II

K, Na, AND Cl CONCENTRATIONS IN HeLa CULTURES*

Age of culture	K	Na	Cl	Total	Dry weight / Wet weight
days		*mmols/l cell H_2O*			*per cent*
0	130	55	49	234	20.9±0.2
1	171	18	40	229	20.7±0.6
2	192	29	34	255	20.0±0.2
3	226	30	40	296	20.4±0.4
4	170	32	43	245	20.0±0.3
7	130	45	55	230	

* Result of one experiment, typical of 2 in which Cl was measured simultaneously with Na and K.

concentration of 75 mOsM for the sum of indiffusible anions and HCO_3 (assuming a mean osmotic coefficient of 0.93) with an average valency of −1.8 meq charge/mOsM at the beginning of the experiment. After 3 days

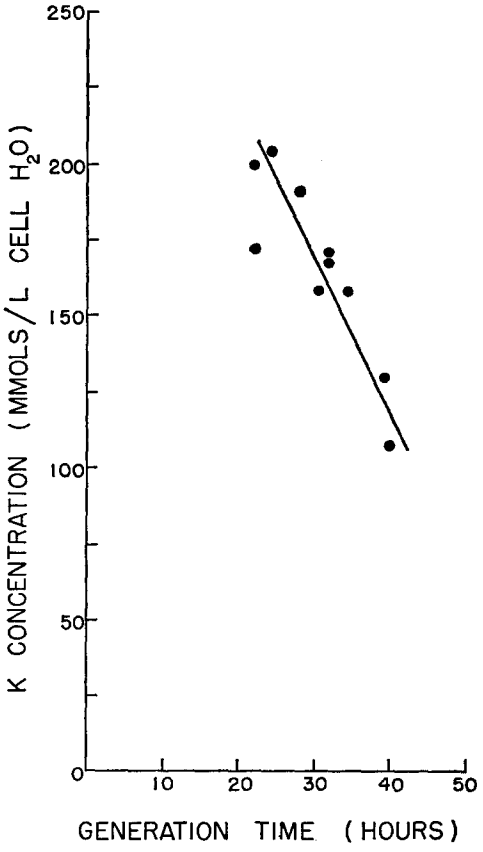

FIGURE 4. Change in cell K concentration with generation time. The line has been drawn by the method of least squares and corresponds to the equation: [K] = 325 − 5.1 t, in units of mmols K/liter cell H_2O. The correlation is significant ($p < 0.01$).

the concentration of indiffusible anions plus HCO_3 has shrunk to 18 mOsM. The deficit in anions is then 12 meq of negative charges per milliosmol which would be consistent with a coalescence of the initial indiffusible anions to produce large polyvalent anions, as for example nucleic acids. Salzman's (10) data on RNA and DNA in HeLa cells are expressed in amount per cell and are not directly comparable with the results of our calculations which are given in mmols/liter cell H_2O.

The figures in Table II may be used to calculate the ratio of net K uptake to net Na extrusion. These ratios vary from about 1:1 to 34:1 indicating that net K uptake is not linked in any definite ratio to net Na extrusion. Since net K and Na movements are directed oppositely, each against its concentration gradient, at least one flux must be active. If we assume the Cl distribution to be passive, we may calculate that the interior of the growing cell is 33 mv negative to the medium. If this conclusion is valid, then both K influx and Na efflux would be active processes, taking place against their respective electrochemical potential gradients.

Experiments were carried out to determine the effect of medium K concentration in the range of 1 to 45 mM on cell K concentration. Freshly trypsinized 2 day old cells were suspended in a salt solution, isosmolar with the growth medium, containing in mM: KH_2PO_4, 0.44; Na_2HPO_4, 0.34; $NaHCO_3$, 4.2; $MgSO_4$, 0.84; $CaCl_2$, 0.13; glucose, 2.0; and phenol red, 0.02 gm/liter, with sufficient NaCl and KCl to bring the sum of Na and K concentrations to 140 mM. The suspension was incubated for 1 hour at 37°C. At the beginning and end of that time aliquots of the suspension were centrifuged in cytocrit tubes and cell Na and K determined according to the methods described earlier. Only those 4 experiments were accepted in which the control sample after incubation in the medium given in Table I had an average K concentration of 153 mmols/liter cell H_2O. Fig. 5 shows that cell K concentration varies with extracellular K concentration at low concentrations, but at extracellular K concentrations greater than 20 mM the cell K concentration approaches its maximum and is almost independent of the extracellular K concentration. The external K concentration that gives half the maximal cell K concentration was found to be 1.3 mM. The Na concentration falls as the K concentration rises, though not in a 1:1 ratio, which is consistent with the absence of a direct link between net Na and K movements.

Effects of Metabolism on Electrolyte Concentrations

The effects of glucose and of various metabolic inhibitors were studied by measuring net K uptake and Na extrusion in cells which came from bottles kept undisturbed for at least 12 hours at 37°C. After the cells were trypsinized and harvested cell K and Na concentrations were measured immediately after resuspension in growth medium and again after 1 hour's incubation at

37°C under differing conditions. Since the first measurement was taken in advance of the usual 1 hour incubation period, these cells were characterized by a lowered K concentration. Table III shows that such K-depleted cells

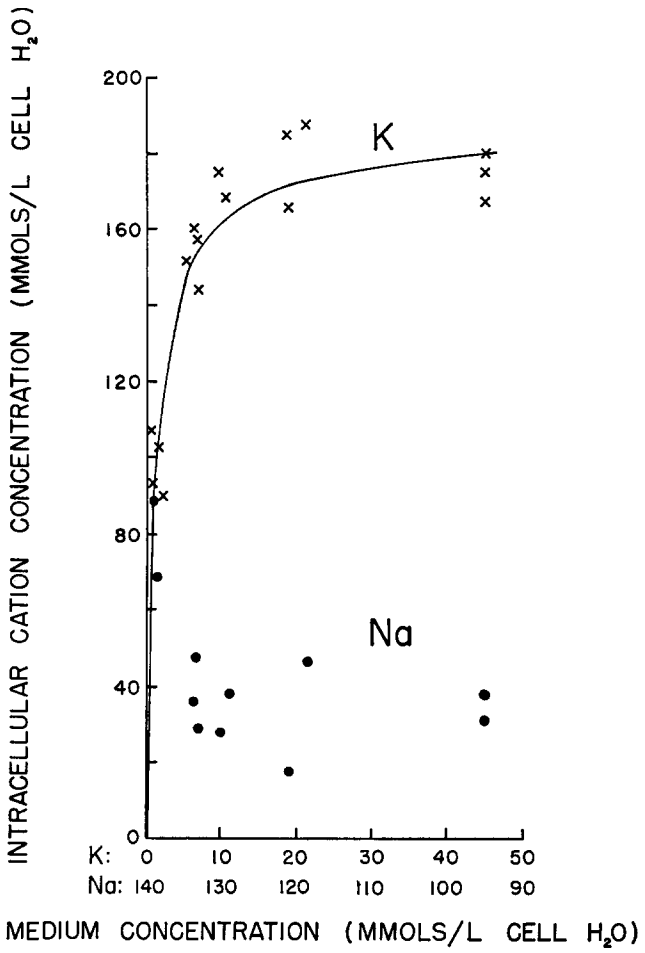

FIGURE 5. Relationship between K and Na concentrations in the medium and cell K and Na concentration. Total [Na] + [K] in medium = 140 mM. The curve for K has been fitted to the data by the use of least squares according to the method of Lineweaver and Burk (11).

were normally characterized by cell K concentrations in the range of 120 to 141 mmols K/liter cell H_2O. When suspended in growth medium they responded by rapid accumulation of K to levels of about 160 mmols/liter cell H_2O; at the same time Na was extruded. The cellular concentrations of both Na and K came to a quasi-steady state within 1 hour. The top section of Table III shows that the addition of glucose after a preliminary starvation

period of 1 hour had no effect on K accumulation or Na depletion. The cells were washed 3 times with a glucose-free solution before incubation started; no traces of glucose were therefore carried over into the incubating medium. The cells were next starved for 24 hours, care being taken to dialyze the serum used in the medium until it was free of glucose. These starved cells were incubated for 1 hour and the results were compared with those obtained with

TABLE III

EFFECT OF GLUCOSE ON INTRACELLULAR K AND Na CONCENTRATIONS

	K	Na
	mmols/l cell H_2O	
1 hr. starvation (3 experiments)		
Before incubation	128±9	82±17
After 1 hr. incubation with glucose	150±3	42±5
After 1 hr. incubation without glucose	153±5	34±2
24 hr. starvation (3 experiments)		
Starved cells		
Before incubation	121±2	64±14
After 1 hr. incubation with glucose	173±3	29±3
After 1 hr. incubation without glucose	158±2	23±0.2
Control cells (unstarved)		
Before incubation	141±20	57±15
After 1 hr. incubation with glucose	165±0.3	40±8

TABLE IV

EFFECT OF TEMPERATURE ON INTRACELLULAR K AND Na CONCENTRATIONS

	K	Na
	mmols/l cell H_2O	
Before incubation	130±3	78±1
After 1 hr. at 37°C	161±1	28±2
After 1 hr. at 4°C	106±9	89±22

unstarved cells. Deprivation of glucose had little effect on the ability of HeLa cells to accumulate K and extrude Na. HeLa cells grown under conditions similar to ours are known to contain large stores of glycogen (12); these, together with other reserves, may well serve as the source of energy for ion transport.

Table IV shows the results of 2 experiments in which K-depleted cells were incubated in growth medium for 1 hour at 4°C. The normal gain of K and loss of Na observed at 37°C were reversed at 4°C so that the cells underwent a further loss of K and gain of Na. Since the net cation movements involved

are oppositely directed, this observation cannot be attributed solely to a change in the transmembrane potential difference at lower temperatures. Instead, these results support our previous suggestion that active transport processes which apparently depend upon an intact metabolic energy source are responsible for maintenance of the intracellular concentrations of either Na or K or both.

In an effort to discover whether the energy source for Na and K transport was respiration or glycolysis, cells were treated with a number of metabolic

TABLE V
EFFECT OF GLYCOLYTIC INHIBITORS ON K AND Na CONCENTRATIONS

	K		
		After incubation	
	Before incubation	Control	+Inhibitor
	mmols/l cell H_2O	*mmols/l cell H_2O*	
Iodoacetate, 1 mM	120±9	162±2	137±5
NaF, 5 mM	108±3	160±1	155±0
NaF, 30 mM	142	167	117
Na oxamate, 38 mM	97±6	165±5	113±10
	Na		
		After incubation	
	Before incubation	Control	+Inhibitor
	mmols/l cell H_2O	*mmols/l cell H_2O*	
Iodoacetate, 1 mM	66±8	21±2	44±2
NaF, 5 mM	73±1	24±2	48±0
NaF, 30 mM	50	18	103
Na oxamate, 38 mM	99±6	33±3	66±16

inhibitors. There was little significant effect on net K uptake and Na extrusion when the cells were treated for 1 hour with the following compounds which inhibit respiration or its coupling to oxidative phosphorylation: 2,4-dinitrophenol (10^{-4} M), KCN (10^{-3} M), antimycin A (1 μg/ml), and NaN_3 (8×10^{-4} M). Two experiments were performed with antimycin A and 3 with the other inhibitors. Similarly, there was no significant effect on net K uptake and Na extrusion in 3 experiments in which the cells were incubated under an atmosphere of N_2. It can be concluded that cation transport is not solely dependent on aerobic metabolism, at least during short term experiments.

In contrast to the results with respiratory inhibitors, glycolytic inhibitors exerted a pronounced effect on K accumulation and Na extrusion. Table V

shows that Na iodoacetate, fluoride, and oxamate (exposure time 1 hour) reduced both K uptake and Na extrusion. The action of oxamate is particularly revealing; this compound, which inhibits glycolysis through lactic dehydrogenase, has been found to inhibit growth and glycolysis in parallel manner in the HeLa cell (12); these inhibitions are first noticed at a Na oxamate concentration of 40 mM. In the present experiments inhibition of cation movements was observed with 38 mM oxamate. The inhibition was not due to the increase in osmolarity of the medium since additions of 38 mM sodium chloride or pyruvate (an analogue of oxamate) were without significant effect on K uptake. The inhibitory effect of oxamate was not reversed by simultaneous addition of pyruvate, in contrast to the findings with the lactic dehydrogenase (13), and the effect observed by Papaconstantinou and Colowick (14) on growth and glycolysis. It should be noted, however, that these latter effects were measured after periods of some days (40 to 200 hours) whereas the experiments reported here lasted only 1 hour.

These results, which indicate that HeLa cells derive their energy for net ion transport largely from glycolysis, can be compared to those on ascites tumor cells by Maizels and others (15), who showed glycolysis to be an important source of energy for Na transport, though respiration also contributed to the process. The present experiments strongly suggest that glycolysis is essential to the maintenance of the normal Na and K concentrations in HeLa cells.

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