Intracellular Na^+:K^+ Ratios in Human Cancer Cells as Revealed by Energy Dispersive X-Ray Microanalysis

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ABSTRACT Intranuclear sodium, potassium, and chloride contents were measured by energy-dispersive x-ray microanalysis in freeze-fractured, freeze-dried, bulk-tumor samples taken from 10 patients suffering from invasive urogenital cancers. Human biopsies were carried out during the first diagnostic interventions before any cytostatic treatment had been applied. Pathohistological diagnosis established the malignancy in each case. The cancers were classified in three types: keratinizing, transitional cell, and hypernephroid carcinoma. More than 250 cell nuclei were measured from each type of cancer. The results were compared with those obtained in intact human urothelium taken from patients having no malignant processes. Proximal and distal tubular epithelial cell nuclei representing the origin of human hypernephroid cancer were also measured in rat kidney because corresponding healthy human material cannot be obtained. The analyses revealed, in all three types of cancer cells, that the average intranuclear sodium content increased more than three-fold, the potassium content decreased 32, 16, and 13%, respectively; meanwhile the chloride content increased, but to a lesser extent than did the sodium. The intranuclear Na^+:K^+ ratios were more than five-fold higher in the cancer cells on the average, and their distribution histograms were much broader than in the normal human urothelium and in the tubular cell nuclei of the rat kidney. The results obtained fit well with the theory of Cone, C. D., Jr. 1971. *J. Theor. Biol.* 30: 151–181 according to which the sustained depolarization of the cell membrane may be of mitogenic effect.

The theory of Cone (7) proposes that sustained depolarization of cell membrane (i.e., an increase in the resting-state intracellular Na^+:K^+ ratio) is involved in the regulation and control of cell divisions during both normal and cancerous growth of tissues. Although several experimental approaches have been attempted to test this assumption, both in vivo and in vitro (3, 8, 9, 21, 24), the evidence obtained has been mostly circumstantial and indirect. Some authors have even refuted the basic concept. Sachs et al. (22) are of the opinion that, although the membrane potential of isolated cells of Chinese hamster lung fluctuates during the cell cycle, the intracellular sodium content plays no causal role as a regulator of mitotic activity.

The application of x-ray microanalytic methods for biology opened the way for direct measurements of intracellular elemental concentrations. Data have been published (5, 6, 25) on different cell types from experimental animals: (*a*) transformed cells (hepatomas and mammary adenocarcinomas); (*b*) normal counterparts of the transformed cells; (*c*) normal, rapidly dividing cells; and (*d*) normal, slowly dividing cells. All the results obtained support the existence of a correlation between the high sodium content and the proliferative capacity of the cells (5, 6, 25). Although these data still do not prove the causal role of high intracellular sodium content in the regulation of cell proliferation, they gain a certain significance in the light of some further experimental results. Namely, observations have been published during the last decade (see, for details, 12) demonstrating that one of the first relevant alterations of hepatocytes during chemically induced carcinogenesis is the focal disappearance of the membrane-bound ATPase activity (i.e., the deficiency of the "sodium pump") and, furthermore, that this enzyme remains undetectable even in the cancer cells of the later stages. Koch and Leffert (17) have shown in vitro in hepatocyte cultures, and in vivo, in regenerating liver, that sodium influx is necessary to initiate cell proliferation, because a specific sodium-influx inhibitor (Amiloride) is able to block the hepatocyte proliferation.

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From the above results, it seemed to us worthwhile to collect data from human cancers to reveal the actual monovalent electrolyte composition of the nuclei of malignant (i.e., invasively growing) cells. By means of an energy-dispersive, x-ray microanalytic method, elaborated for biological bulk specimens (29), investigations were started on some kinds of human cancers where the sampling criteria for a safe quantitative microanalysis could be realized. The studies described in this paper revealed a positive correlation between the high intracellular sodium content and the Na^+:K^+ ratio and malignant cell proliferation as well.

MATERIALS AND METHODS

Sampling Criteria

Human tumors can be used for x-ray microanalysis of the monovalent electrolytes (Na^+, K^+, Cl^-), if the following criteria are met: (*a*) Sampling must be carried out before any cytostatic treatment has been started. The best method is to take samples during the first diagnostic intervention and use the material obtained if the pathohistological analysis defines the nature of the tumor. (*b*) The material removed from the patients must be mechanically and metabolically intact. Materials from surgical interventions during which the removal of the tissue has been preceded by blood vessel ligatures for a long period of time are surely damaged by hypoxia or anoxia, and they must be excluded from the microanalysis. (*c*) Samples should be taken only in cases where the intervention is absolutely necessary in the interest of the patient.

These criteria could be met in some of the human urogenital tumor cases listed below.

Materials

10 cases of human malignant tumors were included in our studies. The ages of patients were between 37 and 77 yr: three of the patients were women. The clinical diagnosis was urinary bladder papilloma in six cases, two of which were recidivist tumors. Two patients suffered from penis cancer and two others had hypernephroid cancer.

The removal of material from the bladder papillomas was performed during the first cystoscopy when material was also taken for pathohistology. Samples from the other cases were obtained during the therapeutic interventions (nephrectomy and amputation of penis) as quickly as possible before the tissue samples could have been damaged by hypoxia. The diagnosis for malignant tumors was established in each case by pathohistological analysis and also by considering the clinical picture. The malignant nature of both cases of hypernephroid cancer was supported by the relatively rapid growth of the tumor and, in one case the invasive character of the tumor: this tumor was ~20 cm in diameter and was so tightly infiltrating the adjacent tissues that nephrectomy could not be performed. The patient became cachectic later on and died. In the other case of hypernephroid cancer, a tumor ~9 cm in size was found in the removed left kidney.

The results obtained on the cancer cells are compared with those obtained on apparently intact human urinary bladder epithelium taken from three patients. Cystoscopy was performed in these cases for diagnostic purposes because of some urological complaints. Samples were used as controls only if the pathohistological analysis excluded the presence of any malignant tumors.

The removed tissue pieces were prepared for x-ray microanalysis by means of the freeze-fracture, freeze-drying (FFFD) technique described in detail elsewhere (29). Here we repeat only the main steps of the preparative procedure: (*a*) The small tissue pieces (about 1 × 1 × 2 mm in size) were deep-frozen in isopentane and cooled by liquid nitrogen immediately after removal to immobilize the monovalent electrolytes. The length of time between the tissue excision and the freezing was usually ~15–20 s and never exceeded 30 s of both the control and tumor samples. (*b*) The frozen tissue pieces were fractured by scissors perpendicular to the longer axis in the isopentane at its melting point (−160°C) to explore the internal compartments of intact cells inside the tissue block. (*c*) The tissue pieces were freeze-dried in a JEOL JEE-4X vacuum evaporator equipped with a JEOL EE-ACE freeze-drying attachment (JEOL U.S.A., Analytical Instruments Div., Cranford, N.J.). Specimen temperature was kept between −80° and −100°C for ~4–5 h, then warmed to room temperature within ~2–3 h. The vacuum was of the order of 10^{-6} Torr during the whole procedure, and the temperature of the cold finger of the attachment was maintained with liquid nitrogen. The dried pieces were preserved in a vacuum at room temperature until the measurement was performed. (*d*) The surface of breaking, in the frozen state, can be recognized macroscopically because of its white color. Other surfaces of cutting at room temperature, before freezing, are never white. The dried specimens were examined, without any coating layer, in a JEOL JSM 35C scanning electron microscope at 10 kV accelerating voltage in secondary electron image mode. In this type of specimen, one cannot expect a high spatial resolution; nevertheless, it is possible to recognize the main tissue components such as cells and extracellular space, fibrous ground substance, etc. One could also see the cell nuclei if the plane of breaking passed through them. The electrostatic charging is minimal; at least it is not able to displace the electron beam as shown by the in-focus pictures taken with ~1-min exposure time (Figs. 2 and 3). (*e*) The x-ray spectra were recorded by means of an EDAX System F consisting of a usual energy-dispersive x-ray detector with Be-window (Edax International Inc., Prairie View, Ill.), a 711 type multichannel analyzer and a Data General NOVA 3 computer of 32 Kbyte memory size (Data General Corp., Westboro, Mass.). The breaking plane of the FFFD specimen was oriented in the horizontal plane controlled by the light microscope. The takeoff angle of the detector was 28° and the distance between it and the specimen was 36 mm. Cell groups suitable for microanalysis were selected by using low magnifications; then the nucleus to be analyzed was brought into the center of the screen, and the magnification was increased until the analyzed microarea remained within the nucleus. Usually a magnification of ×50,000 was sufficient for this purpose. Considering the technical parameters of the microscope used, the analyzed area of a nucleus was ~10 μm^2. The penetration depth of 10 kV electrons is maximally ~4 μm in this type of specimen; however, ~74% of the x-ray photons arrive from the upper half of this distance as shown earlier (29). Each spectrum was recorded for 40 s, in the range of 0–8 keV, applying an output count rate of ~4–500 cps, and using a slow scanning speed. During the analyses, an effective beam current of 2–3 pA could be measured between the earth and the specimen. After completion of the analysis, the magnification was lowered and the surface of the nucleus was inspected again. (*f*) The recorded spectra were handled first by using the program package of EDAX called FDEM. Each spectrum was smoothed once, and the background subtraction was carried out. The peak list was printed together with the integral of counts between 4 and 6 keV energies where no significant peaks occur in the biological specimens. The mass fraction method of Hall et al. (14) was used for quantitative analysis, extended for biological bulk specimens by applying a special selection of standards and avoiding the necessity of ZAF correction (28, 29). Calculation of peak to background ratios, preparation of histograms, etc. were performed by means of special utility programs written by us in BASIC for the NOVA 3 computer (Data General Corp.).

The tissues were examined histologically after freeze-drying as well. For this purpose, some of the dried tissue pieces were embedded in Araldite, starting from a 1:1 mixture of propylenoxide and Araldite. Sections of 1- or 2-μm thickness were cut and studied after either staining with a methylene-blue-azur combination in the light microscope or without staining in the phase-contrast microscope.

RESULTS

According to the pathohistological analysis, the tumor samples in our studies proved to be cancers of invasive character and could be classified in three main groups: (*a*) Squamous cell carcinoma. Two of the urinary bladder papillomas as well as the two penis cancers belong to this group. The extent of keratinization was higher in the latter two cases than in the former ones; nevertheless the individual comparison of the data obtained allowed us to pool these results into one class. (*b*) Transitional cell carcinoma. Four urinary bladder papillomas belong to this group. No keratinization was found in these samples. (*c*) Adenocarcinoma of the kidney of clear cell type. The two cases of hypernephroid carcinoma belong to this group.

The typical morphological features of these three groups could be clearly recognized in the tissue samples even after freeze-drying. Fig. 1 demonstrates the histological pictures of a normal urothelium and the three types of cancer, taken from freeze-dried and araldite-embedded material by means of light or phase-contrast microscopy. The same characteristic cell types were also clearly recognizable in the FFFD specimen in the secondary electron image (Figs. 2 and 3). One could select the proper zones using lower magnifications and then perform the x-ray microanalysis within the cell nucleus of proper size. The smallest nuclei were omitted from the analysis because in such cases, the fracture plane must have passed the nucleus far from its equatorial zone, therefore, the probability of overpen-

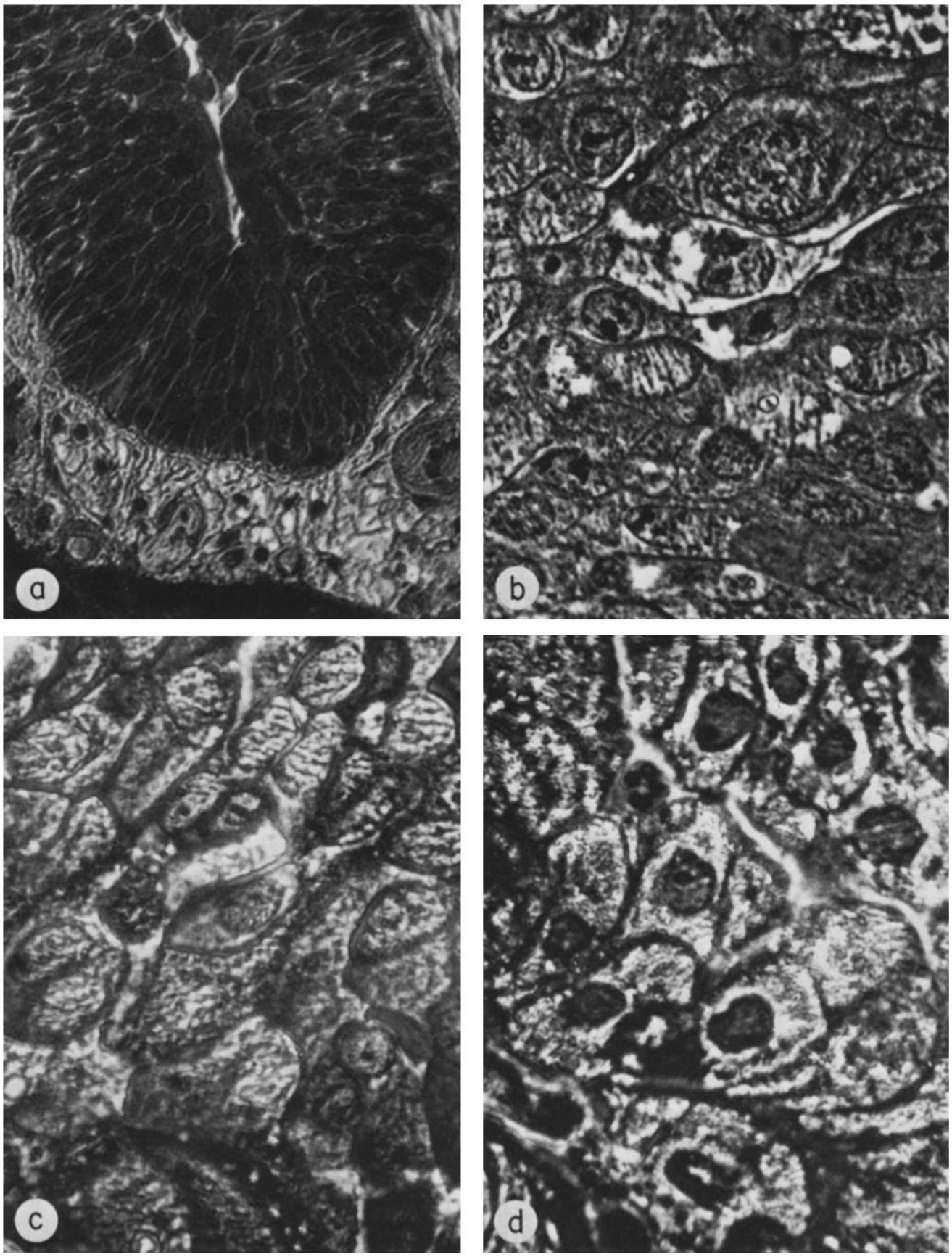

FIGURE 1 Light microscopic images of the normal urothelium (a) and types I–III cancers (*b–d*), respectively (as seen in sections of freeze dried samples after Araldite embedding). (*a*) Stained section, × 400. (*b–d*) Phase-contrast micrographs, × 1,000.

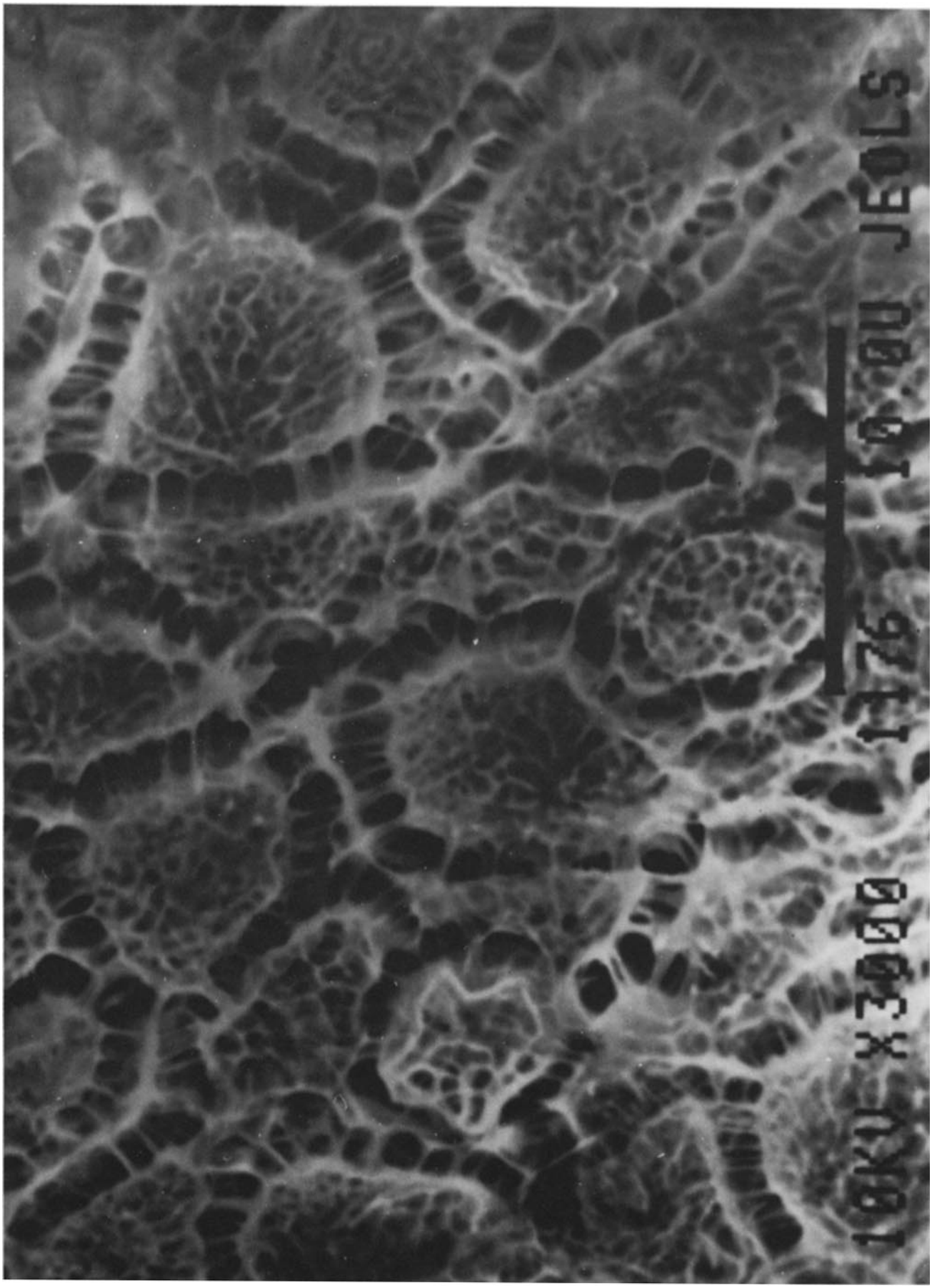

FIGURE 2 Secondary electron image of the normal urothelium in FFFD specimen. The breaking plane passed nearly parallel to the surface and explored the cells near the basal layer. The bar, 10 μm. × 6,800.

etration by the electron beam increases in such samples. Because the cytoplasmic region of many cancer cells was very narrow and also of irregular shape, and, to prevent eventual errors due to overpenetration by the electron beam into the extracellular space, the cytoplasmic monovalent ion contents have not been analyzed. It would have been possible to increase the magnification or to use point-form beam for the analysis to assure the analyzed area within the cytoplasm on the fractured plane, however, one cannot be sure as regards the shape of the cell in the whole of 4 μm from where the x-ray photons arrive.

It is obviously very important to consider only living tumor cells when performing the analyses. Although the tumor samples were always taken from the regions where no apparent macroscopic signs of necrosis could be observed, some necrotic cells were present in our samples. However, on the basis of the following criteria, one can distinguish the necrotic cells from the living ones. (*a*) The morphology of the necrotic cells is altered, and their nucleus is hardly recognizable. (*b*) The necrotic cells are not able to maintain the monovalent gradients against the extracellular space. Therefore, they contain similar concentrations of sodium, potassium, and chloride ions as do the extracellular fibrous regions. It is even more interesting that the equivalent Cl^- content of the necrotic cells is roughly equal to the sum of sodium and potassium, which is never seen in living cells. (*c*) The necrotic cells contain high amounts of Ca^{2+}, whereas the living ones do not. (*d*) The P-contents of the living cell nuclei in the tumors are high, even somewhat higher than in the normal living control cells, whereas the necrotic cell nuclei contain very low concentrations of P, because their components are being autolysed.

It should be noted that necrotic cells, as defined by the above criteria, occurred almost exclusively in the hypernephroid cancer, whereas they were found very rarely in the other two types of tumors studied. Therefore, it could safely be assumed that our results indeed concerned living tumor cells. Necrotic cells were never considered in our analyses.

Fig. 4 demonstrates some typical x-ray spectra recorded from normal and cancer cell nuclei. Fig. 4 *a* is the spectrum of a normal cell nucleus in the bladder epithelium. It is clear from the spectrum that the sodium content is low (at an energy of 1.04 keV), and that the potassium content is rather high (at 3.31 keV). Fig. 4 *b* shows a spectrum recorded in the nucleus of a cancer cell of type II. The increase in sodium and the decrease in potassium content is apparent even without quantitation. Quite similar spectra were recorded in all three types of cancers. The calculated concentrations for the three main monovalents are summarized in Table I.

Table I gives the mass fraction values for the monovalents, as calculated in the biological dry mass. To convert these concentrations into values regarding the intranuclear water as well, one has to know the dry mass content of the nuclei. It seems to be safe to put this value at 25%. Using this hypothetical figure involving 75% water content of the nuclei, one can obtain the monovalent concentrations in millimolar units, also given in Table I. It should be pointed out that any eventual error in this hypothetic water content may influence the absolute concentrations of the monovalents, but has no effect on the ratios calculated from the concentrations obtained in the dry mass of the nuclei. Therefore, we calculated the Na^+:K^+ ratio for each nucleus, and the results are given in histograms (Fig. 5).

It is evident from the histograms of Fig. 5 that the intracellular Na^+:K^+ ratios of the normal epithelial cells fall in a rather narrow range; their average is 0.11. However, the cancer cells display a quite different distribution of this parameter. All the malignant tumors studied have a much higher average value for the Na^+:K^+ ratio: it increased to 666, 508, and 508% of the control value in the three groups of cancer cells, respectively. The data of Table I show that this increase is due mostly to the net increase of the intranuclear sodium content (358, 348, and 317% of the control value in the three types of cancer cells, respectively), whereas the decrease of potassium content in the cancer cell nuclei effected the ratio to a lesser extent (the decreases were 32, 16, and 13%, respectively).

It should be noted that the values of the Na^+:K^+ ratio found in the malignant tumors and falling in the range of the normal epithelium (i.e., below 0.3) were always found in nuclei belonging to cells that showed some morphological signs of normal differentiation. For instance, in the bladder cancers such cells were to be found in more or less uniformly stratified epithelial cell groups often having a recognizable basement membrane. Furthermore, they were surrounded by an intercellular space through which some intercellular processes could be seen, quite resembling the normal epithelium shown in Fig. 2. In the hypernephroid cancers, one can often find regular tubular arrangements of the cells: the Na^+:K^+ ratios were always below 0.3 in the nuclei of such cells. On the other hand, the cancer cells displaying the tightly packed, irregular appearance with nuclear polymorphism, like those shown in Fig. 3, or the true clear cells of the kidney tumors, always contained a very high amount of sodium and, as a consequence, displayed a very high Na^+:K^+ ratio. Our measurements were carried out randomly; we measured all the living cell nuclei that appeared in the field of view on the FFFD specimen and were of suitable size. If one takes out from the histograms of malignant tumors the values of the Na^+:K^+ ratio belonging to the normal range, the averages for cancer cells will still be higher.

DISCUSSION

Apart from blood cells, human materials are not used very frequently for experimental purposes by cell biologists. In the given case, we decided to analyze the intracellular monovalent concentrations in human tumor cells for the following reasons: (*a*) The importance of testing the validity of Cone's hypothesis (7) on naturally occurring malignant human tumors is obvious. A positive correlation between the increase in intranuclear sodium content and the invasive character of the tumor may considerably strengthen the evidence obtained so far in different animal cells and experimental tumors (5, 6, 25). (*b*) The malignancy of the tumors selected for our studies could be established unequivocally by the pathologist on the basis of the invasive character of the cancers. According to the criteria of grading tumors defined by Koss (18), the bladder cancers studied were of grades II and III. (*c*) The technique of removal of the tissue samples by either cystoscopic or surgical intervention could assure that <30 s elapsed between the interruption of living condition and the freezing of the samples. It should be noted that, even in cases of experimental animals (e.g., rats), the removal of specific parts of the brain, etc., requires somewhat more time. Brain cortex samples can be removed from a rat within 1 min after the animal is killed. Nevertheless, redistribution artifacts between the extra- and intracellular space were not observed within the period of several minutes, until the brain cortex did not become completely anoxic.[1]

[1] I. Zs.-Nagy. Unpublished observations.

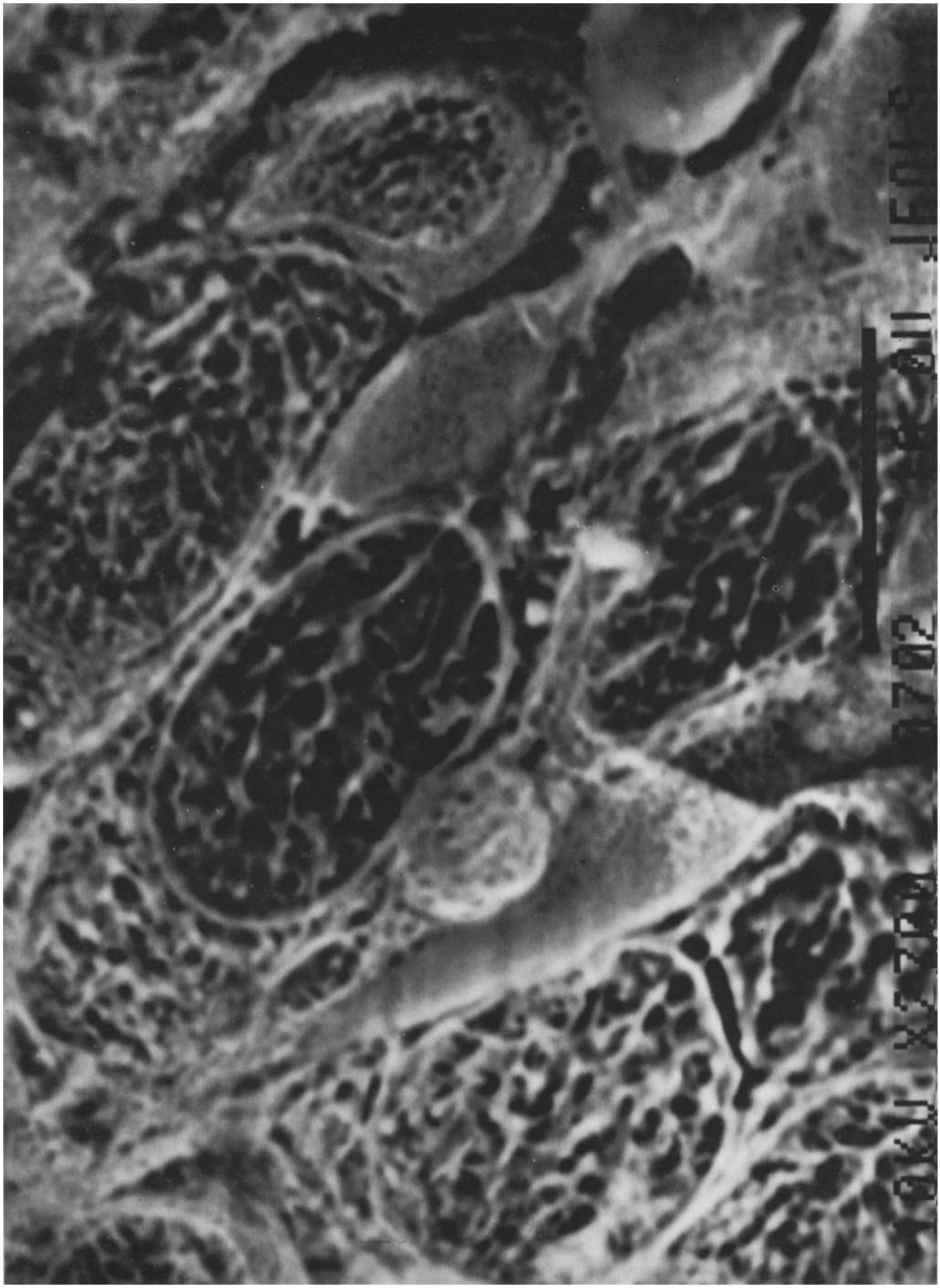

FIGURE 3 Secondary electron image of cell groups of a type II cancer (FFFD specimen). Note the nuclear polymorphism and the irregular intercellular contacts. The printed bar, 10 μm. × 6,000.

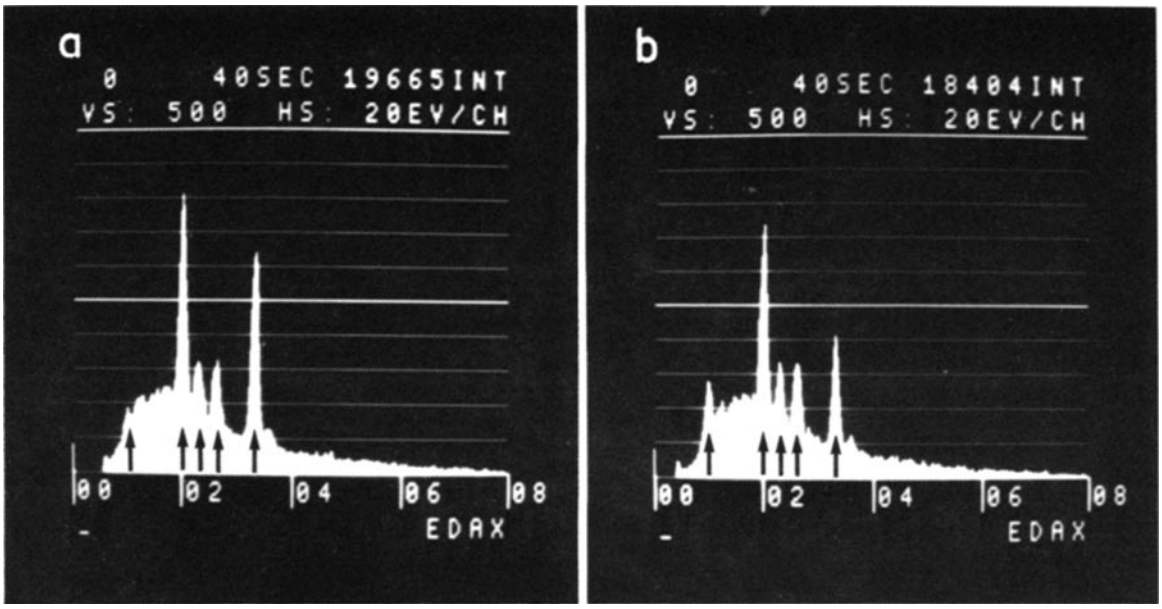

FIGURE 4 Typical x-ray spectra from normal (*a*) and cancerous (*b*) cell nuclei. The peaks marked by black arrows are from left to right: Na^+, P, S, Cl^-, K^+. Note the marked differences in the Na^+ and K^+ peaks. Na^+:K^+ molar ratios are 0.14 and 0.92 for *a* and *b*, respectively.

TABLE I

Monovalent Electrolytes in Cell Nuclei of Normal Epithelial and Cancerous Cells

Cell type (number of nuclei)	Dry mass*			Intranuclear water*			Total monovalents
	Na^+	Cl^-	K^+	Na^+	Cl^-	K^+	
	%			meq/kg			meq/kg water
Normal cells (388)	0.1617 ± 0.0073	0.4976 ± 0.0130	2.5046 ± 0.0247	23.4 ± 1.0	46.7 ± 1.2	214.1 ± 2.1	284.2
Type I cancer (255)	0.5783 ± 0.0238	0.7873 ± 0.0189	1.7016 ± 0.0384	83.8 ± 3.4	73.9 ± 1.8	145.4 ± 3.3	303.1
Type II cancer (287)	0.5632 ± 0.0345	0.8856 ± 0.0332	2.0947 ± 0.0718	81.6 ± 5.0	83.2 ± 3.1	179.0 ± 6.1	343.8
Type III cancer (272)	0.5122 ± 0.0233	0.7309 ± 0.0203	2.1820 ± 0.0426	74.3 ± 3.4	68.6 ± 1.9	186.5 ± 3.6	329.4

The milliequivalent per kilogram water values were calculated assuming 75% intranuclear water content by weight. All the monovalent concentrations of the cancer cells differ significantly ($P \ll 0.001$) from the respective normal values.
* ± SEM

Furthermore, evidence has been published recently (23) showing that the artificial electrolyte gradients prepared at microscopic dimensions in gelatin blocks are not destroyed by redistribution, even when analyzed in frozen-hydrated sections at relatively high temperatures (−30°C). Because our material was kept at a temperature < −80°C during freeze-drying, we can safely assume that no significant postmortem redistribution of the monovalents could take place during the preparative procedure.

The energy-dispersive, x-ray microanalytic method used in our studies is, in principle, similar to that applied by others on sectioned material (5, 6, 25). However, our method of preparation is much quicker and allows us to measure a greater number of cells. In addition, we calculated the concentration in the intracellular water. Nevertheless, the results obtained by our method are completely comparable to those of the authors cited above.

The bulk specimen analysis we used also has a certain risk. Namely, we can see the cell nucleus in the plane of breaking, but we cannot be sure that the actual shape of each nucleus is spheric, i.e., we do not know whether the nucleus reaches, in depth, the necessary distances for avoiding overpenetration by the electron beam. This possibility, however, cannot influence seriously the results obtained, for the following reasons: (*a*) Most of the nuclei are indeed of spheric or ovoid shape; if we measure a great number of broken nuclei of sufficient size, in most of the cases 10 kV electrons will not overpenetrate the nucleus. (*b*) In those few cases where overpenetration may occur due to the irregular shape of the nucleus, some of the x-ray photons may arrive at the detector from the cytoplasmic regions. This, however, can never result in a higher positive monovalent concentration in the nucleus because due to the lower water content of the cytoplasm as compared with the nucleus, one always obtains somewhat lower Na^+ and K^+ concentrations in the dry mass of the cytoplasm (2, 20).

We are of the opinion that x-ray microanalysis, as applied in our studies, is a useful and reliable method for looking for correlations between the actual monovalent ion composition of the nuclei and the proliferative capacity of the cells.

A further problem arises when interpreting our results. Namely, human control tissue could be obtained only from the urinary bladder epithelium. This can be considered as a proper control only for types I and II bladder cancers. According to present knowledge, all renal adenocarcinomas, including the hypernephroid cancer, arise from the epithelium of the renal tubules (26). More precisely, there is histologic, ultrastructural,

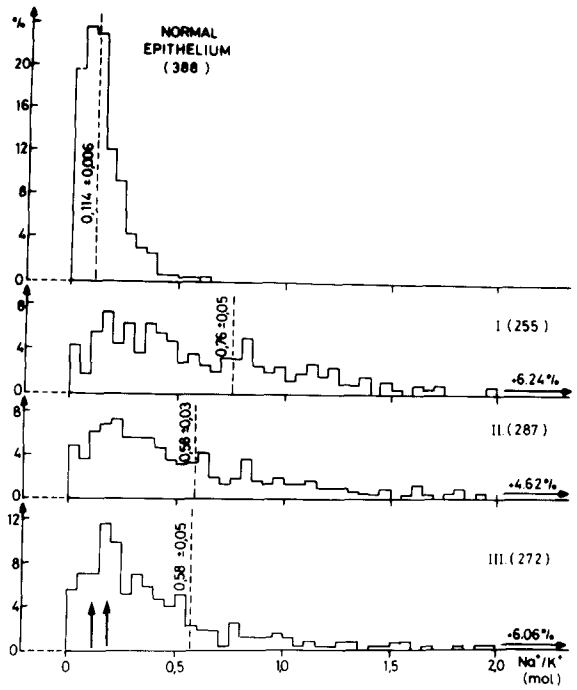

FIGURE 5 Distributions of the Na^+:K^+ molar ratios in the normal and cancerous cell nuclei. The number of measured nuclei per group is given in parentheses. Percentage values at the right side of histograms indicate cell nuclei having Na^+:K^+ molar ratios >2.0. Averages ± SEM are given for each histogram. Arrows in the histogram of hypernephroid cancer (type III) at the values 0.165 and 0.103 indicate the averages of Na^+:K^+ ratios observed in the proximal and distal tubular epithelial cell nuclei of rat kidney, respectively. For more details, see Discussion.

and immunologic evidence indicating that hypernephroid cancer originates in the proximal tubular epithelium (1). However, it is not possible to obtain sufficiently fresh, healthy, human kidney material for control purposes. According to our knowledge, no x-ray microanalytic data are available regarding the intranuclear monovalent contents of the proximal tubular epithelium of mammals. Bulger et al. (4) have published data on the elemental content of cells in the rat renal papillary tip, based on x-ray analysis of frozen-hydrated sections; however, they analyzed only the collecting duct cells, the papillary epithelial cells, and the interstitium. These cell types are not comparable to the proximal tubular epithelium, because there is a very special osmotic load on the cells at the papillary region that is absent in the proximal tubular epithelium.

To obtain data regarding the monovalent electrolyte contents of the nuclei of the proximal tubular epithelium, we have started a series of analyses on rat kidney by using the same method of x-ray microanalysis as applied to the tumor tissues. A complete description of the results will be published elsewhere. Here we intend to mention only the following important facts: (*a*) The proximal and distal tubular epithelia can be distinguished with absolute certainty even in the FFFD specimen in secondary electron image on the basis of morphological features. (*b*) The Na^+:K^+ molar ratios are 0.165 ± 0.011 and 0.103 ± 0.011 in the proximal and distal tubular epithelial cell nuclei (averages ± SEM of 50 and 53 cells, respectively). Because these molar ratios are very near to that obtained in the normal human bladder epithelium (Fig. 5), we believe that one can compare, without any great risk, the results obtained in hypernephroid cancer to those obtained in the bladder epithelial cells.

The present investigations demonstrate unanimously that the high proliferating capacity of invasively growing cancer cells is accompanied by a significantly increased intranuclear Na^+:K^+ ratio due mostly to an increased Na^+ content. Because the nuclear membrane does not represent any barrier for the light elements such as Na^+ and K^+, similar ratios must also occur in the cytoplasm. This finding fits well with the theory of Cone (7).

Koch and Leffert (17) have demonstrated convincingly the causal role of sodium influx in the initiation of cell proliferation. Although the mechanism of the possible effect of the intracellular sodium on the regulation of cell division is far from clear, it seems to be worth-while to point out some data that may be of importance. Namely, ions are able to influence macromolecules in aqueous solutions in essentially two different ways: first, the ions can produce some nonspecific electrostatic interactions and, second, they can alter by specific effects the conformation and solubility of macromolecules (16). The charging properties, the conformation and solubility characteristics of the biological macromolecules are directly related to the functions of living systems; therefore, one can believe that the effects of ions are of great importance, in spite of the fact that such effects on the transition temperature for a polymer chain aggregation process are still not clearly understood (15). The so-called polyelectrolyte theory (10, 11, 19) offers a more precise explanation for the ionic effects on the nucleic acid-enzyme complexes (for details, see 27).

It must be emphasized, however, that the absolute values of the Na^+ content or even the Na^+:K^+ ratio alone cannot be regarded as the regulatory factor, even if the theory of Cone (7) is true. The transmembrane potential of a given cell type is determined by the intra- and extracellular ion concentrations as well as by the permeability ratios of the cell membrane for those ions. Therefore, the Na^+ contents and also the Na^+:K^+ ratios may well be different in two types of cells, while their membrane potential is identical, or vice versa. As a matter of fact, the nondividing nerve cells of the brain cortex of healthy rats display two to three times higher Na^+:K^+ ratios than the hepatocytes of the same animals (2, 20). This means that each type of differentiated cell has a characteristic ionic composition that determines the resting potential of the membrane together with the actual ionic permeability of its own cell membrane. Transient depolarizations of the membrane represent the excited state of the cell, whereas alterations resulting in a sustained depolarization are mitogenic effects (8, 9, 17).

It is still an open question as to how the sustained depolarization of the cell membrane is able to trigger the mitotic process. One has to suppose the existence of directly potential-dependent mechanisms in this regulation. The existence of such possibilities is suggested by the results of Forn and Greengard (13). They have shown that the reversible phosphorylation (i.e., the "activation") of a specific neuronal protein in rat cerebral cortex is regulated by cyclic nucleotides and also by depolarizing agents. A high level of phosphorylation of this neuroprotein was achieved by a 10-fold increase in the extracellular potassium concentration or by the addition of veratridine to the brain slices. Both treatments give identical results, i.e. the membrane becomes depolarized, and this depolarization influences the protein in spite of the fact that Na^+ influx occurs only during the veratridine experiment. Although the functional significance of this neuroprotein has not been clarified,

it represents an example of proteins being directly sensitive to the actual level of the membrane potential.

In light of these thoughts, we regard the considerable increase in the Na^+:K^+ ratios in many tumor cells, as compared with their controls, as an indication of a sustained depolarization of their cell membrane. We are of the opinion that this may indicate a causal relationship with the high mitotic activity of the cancer cells, as predicted by the hypothesis of Cone (7).

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NOTE ADDED IN PROOF: After completion of this manuscript, the following information has become available: experiments on serum-stimulated growth of mouse N1E-115 neuroblastoma cells fully support the role of increased Na^+-influx in the regulation of the cell cycle. For details, see the following reference: Moolénaar, W. H., C. L. Mummery, P. T. van der Saag, and S. W. de Laat. 1981. Rapid ionic events and the initiation of growth in serum-stimulated neuroblastoma cells. *Cell.* 23:789–798.

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