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A REVIEW ON THE USE OF BULK SPECIMEN X-RAY MICROANALYSIS IN CANCER RESEARCH

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Abstract

The freeze-fracture, freeze-drying (FFFD) method of biological bulk specimen preparation combined with quantitative X-ray microanalysis is suitable for the measurement of intracellular concentrations of biologically relevant elements in human biopsy or experimental animal materials. Especially useful information can be obtained regarding the intracellular Na^+/K^+ ratios being independent of the actual (and unknown) water content of the cytoplasm. The sustained increase of this ratio indicates a sustained depolarization of the cell membrane. These data are of importance from the point of view of the membrane hypothesis of mitogenesis (MHM). It has been revealed that the distribution histograms of the intracellular Na^+/K^+ ratio display a very significant broadening and an increase of the average values in human urogenital, thyroid and laryngeal tumors, as well as in experimentally induced cell proliferation models. Although MHM has been claimed to be invalid on the basis of some atomic absorption measurements of the intracellular monovalent ion concentrations as well as of some in vitro results obtained with amiloride, this review paper demonstrates that MHM may still be a valid hypothesis for the explanation of mitotic regulation.

KEY WORDS: Bulk specimen X-ray microanalysis, freeze-fracture freeze-drying method, urogenital cancer, thyroid cancer, laryngeal cancer, experimentally induced cell proliferation, intracellular Na^+/K^+ ratio, membrane hypothesis of mitogenesis.

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Introduction

The technique of X-ray microanalysis combined with electron microscopy has been developed during the late sixties and is since that time available for biological research. Although theoretical and practical problems of this analytical method are well known and have been described extensively in the literature (Beaman and Isasi, 1972, Hall et al. 1974, Russ, 1974, Panessa, 1974, Barbi, 1979, Chandler, 1979, Newbury, 1979, Marshall, 1980, Roomans, 1979, 1980, Gupta and Hall, 1981, 1982, 1984; Hall and Gupta, 1983, 1984, 1986; Hall, 1986, etc.), the biological applications of this method have still remained rather restricted. This fact may find its explanation in part in the need of relatively expensive instrumentation, but perhaps even more in the preparative difficulties in obtaining suitable biological specimens. In addition, although this method may reveal the elemental composition in rather small volumes, i.e., even in subcellular compartments, due to the complexity of the biological systems, the interpretation of such information is mostly difficult. For example, one can measure rather precisely the phosphorus content of the cytoplasm, however, this parameter does not tell us anything about the distribution of this element in various molecules, like RNA, nucleotides, proteins, phospholipids, etc. In other words, it is difficult to ask meaningful questions in biology and medicine to which X-ray microanalysis could offer the meaningful answers. Therefore, the majority of the X-ray microanalytical literature of the last 20 years treated more methodological problems than true biological ones.

During the mid-seventies, our laboratory developed a special technique called the freeze-fracture, freeze-drying (FFFD) bulk specimen preparation procedure (Zs.-Nagy et al. 1977). This method was designed basically for gerontological research where the main question to be answered was whether the intracellular monovalent ion contents of the postmitotic cells increase with age or not (Pieri et al. 1977). The bulk specimen method had also been extended to frozen-hydrated preparations allowing us to determine intracellular water and dry-mass contents (Zs.-Nagy et al. 1982). Advantages and

limitations of this method have extensively been described and critically reviewed by ourselves (Zs.-Nagy 1983, 1988, Lustyik and Zs.-Nagy 1985, 1988), and also commented on by others (Roomans 1981, Hall 1986, Hall and Gupta 1984, Gupta and Hall 1982, Elbers 1983, Roomans and Wroblewski 1985, Zierold 1986, Von Zglinicki et al. 1986). Therefore, this method can be considered as a known one and there is no need to repeat here the methodological details.

The aim of this review paper is to summarize the rationale of using the bulk specimen X-ray microanalytic method in cancer research based on the concept of Cone (1971) called by us (Zs.-Nagy et al. 1984) the membrane hypothesis of mitogenesis (MHM). The basic content of the MHM has recently been involved in the interpretation of the mitogenic response of fibroblasts (Rozengurt 1986). We started our work on human tumors during 1979-80 and a part of our early results was already mentioned in a review (Zs.-Nagy 1983). The present paper intends to give an overview of the more recent results and their interpretations.

Why use X-ray microanalysis for cancer research?

The rationale of using the bulk specimen X-ray microanalytic method for tumor tissue goes back to the hypothesis of Cone (1971) proposing that sustained depolarization of the cell membrane, i.e., an increase of the resting intracellular Na^+/K^+ ratio is the main regulatory factor in the control of cell division in both normal proliferative and cancerous tissues. The MHM was at the beginning supported mainly by indirect evidence (Orr et al. 1972, Cone and Tongier 1973, Cone and Cone 1976, Prescott 1976, Borgens et al. 1977, Rodan et al. 1978, Smith and Rozengurt 1978, Ihlenfeldt et al. 1981), and therefore, it gained only a limited attention for a long time.

More direct evidence was obtained through the experiments with amiloride (Glitzer and Steelman 1966, Baer et al. 1967). This is a specific Na^+-channel blocking drug (Baba et al. 1968, Bentley 1968, Salako and Smith 1970, Soudou and Hoshi 1977, Macfie et al. 1981); it was demonstrated that it can inhibit DNA synthesis in fertilized egg cells (Epel, 1977), cell proliferation in regenerating liver in vivo and also in cultured hepatocytes in vitro (Koch and Leffert 1979, Leffert and Koch 1979a, 1979b, Pieri et al. 1982, 1983a, 1984). Relevant data were found also on NIE-115 neuroblastoma cells (Moolenaar et al. 1981, Mummery et al. 1982), in fibroblasts (Villereal 1981, 1982) and in L-1210 murine leukemia cells (Pieri et al. 1983b). All these data showed that Na^+-influx is a necessary prerequisite for the initiation of cell division. More details can be found in the review of Boonstra et al. (1982).

Some contradictory opinions have also been published: experiments were performed showing that amiloride may have a nonspecific inhibitory effect on the cAMP-dependent protein kinases, i.e., it might influence the cellular functions not only as a Na^+-channel blocking agent (Holland et al. 1983). Amiloride inhibits protein kinase in an apparently competitive way, since the inhibition is overcome by increasing the ATP concentration, as shown in the experimental model of Holland et al. (1983). It turned out, however, that these latter data were not interpreted for the in vivo situation in terms of the available ATP/amiloride molar ratio (Zs.-Nagy et al. 1987), i.e., the arguments of Holland et al. (1983) are not strong enough to neglect the previously suggested interpretation of the effects of amiloride on the Na^+-channels. This problem will be considered in more detail in the discussion of this paper.

The situation was further complicated by some publications based on atomic absorption spectroscopic measurements of the intracellular sodium content of cultured cells washed up to seven times by sodium-free media, which were apparently contradicting the MHM (Sachs et al. 1974, Sanui and Rubin 1977, 1978, 1979, 1982, Moscatelli et al. 1979). However, it was shown later on that these contradictions were simply due to the fact that the washing procedure used for these cells removed more than 97 % of the intracellular sodium content (Zs.-Nagy et al. 1984).

As a consequence of the great need to learn more about the mechanisms of the mitotic regulation and to clarify the contradictory opinions mentioned above, during the late seventies there was an increasing need for a reliable measurement of the intracellular monovalent electrolyte contents during the cell cycle. X-ray microanalysis seemed to be the only possible choice. Since the MHM was found to be valid with this latter method in freeze-dried thick sections in various cell types of experimental animals (Smith et al. 1978, Cameron and Smith, 1980, Cameron et al. 1980), we decided to study human biopsy material by our technique. Cancer cells of human oral mucosa were also studied by X-ray microanalysis in sections (Wroblewski et al. 1983).

Criteria for using human biopsy materials

First of all, one had to establish certain criteria on the basis of which the monovalent electrolyte content (Na^+, K^+ and Cl^-) of the cells could be determined. These are in part ethical and in part technical criteria and can be summarized as follows.

1. Sampling is allowed from humans only, if the surgery is absolutely necessary in the interest of the patient.

2. Sampling must be carried out before any cytostatic treatment has been started.

3. Tissue sample can be removed without any mechanical or metabolical damage to it, i.e., special care must be taken by the operator to assure that the shortest possible time (less than 1 min) elapses between the cessation of blood circulation and the deep freezing of the sample.

4. The amount of the removed tissue must be sufficient for both the pathohistological

examination and the X-ray microanalysis, i.e., the diagnosis of the disease can be established.

5. Suitable control specimens can be obtained from the same type of tissue without any damage to the patient.

Preparative procedure and X-ray microanalysis

The biopsy materials were processed for energy-dispersive X-ray microanalysis by the freeze-fracture freeze-drying (FFFD) technique described in detail elsewhere (Zs.-Nagy et al. 1977, 1981, 1983, 1987, Zs.-Nagy 1983, 1988). Here we list only the main steps of the procedure and some practical information:

(a) Deep freezing of the sample in isopentane cooled to its melting point by liquid nitrogen. This procedure is carried out immediately after the removal of the tissue sample. The frozen samples can be maintained for a long time under liquid nitrogen without any risk.

(b) Fracture of the frozen samples by cooled scissors, still in the frozen state, in order to explore intact intracellular compartments. This step is usually realized immediately before the next procedure.

(c) Freeze-drying of the samples according to a special time and temperature schedule in order to exclude the possibility of the redistribution of the light elements between the intra- and extracellular spaces. After having reached room temperature, the FFFD specimens can be maintained in vacuum of about 10^{-3} Torr, in order to avoid any rehydration of them from the humidity present in air.

(d) X-ray microanalysis of the cell nuclei in the FFFD specimen (without using any coating layer) at 10 kV accelerating voltage in scanning electron microscope in secondary electron image mode. Our experiments were carried out mostly on a JEOL JSM 35C model equipped with an EDAX 711 type analyzer (EDAX System F). The incident beam current is kept at 45 μA, the effective beam current in the specimen amounted to about 1-2 pA when obtaining a count rate of 450 cps. The distance between the specimen and the X-ray detector is 36 mm, takeoff angle 28 degrees.

(e) Computer handling of the spectra resulting in the necessary information for the use of the mass fraction method of Hall et al. (1973) extended for bulk specimens (Zs.-Nagy et al. 1977; Zs.-Nagy 1983, 1988, 1989). This involves a background subtraction, calculation of the net peak integrals and the necessary "white count number" in the properly selected range of background. In possession of the peak-to-background ratios obtained under identical conditions on properly selected bulk standard crystals (Zs.-Nagy and Pieri 1976, Zs.-Nagy et al. 1977), this procedure gives relative elemental concentrations in the dry mass of the FFFD specimen.

(f) Calculation of the monovalent electrolyte concentrations for the intranuclear water. Usually for the tumor cells and their controls we estimate 25 % dry mass and 75 % water content of the cell nuclei. Final electrolyte concentrations are expressed as mEq/kg intracellular water.

Statistical evaluation of the data

For each analyzed cell nucleus elemental concentrations of Na^+, K^+ and Cl^- as well as the Na^+/K^+ molar ratio were calculated. Then individual averages were compared within the control and tumorous groups. As demonstrated for thyroid tumors (Zs.-Nagy et al. 1983), pooling together all the data within the same group gives a much more realistic standard deviation of the results than the averages of the individual means. As a matter of fact, the standard deviation of the individual means corresponds rather to the standard error of the mean within the sample. Therefore, the same method was used throughout. Elemental concentrations were compared between control and cancer groups by the Student's "t" test. Distribution histograms of the Na^+/K^+ ratio were prepared and compared with the Chi^2 test by a suitable computer program.

Some notes on the limitations of the method

It should be stressed that the FFFD bulk specimens are not suitable for high spatial resolution work (Zs.-Nagy 1983, 1988). Nevertheless, the main intracellular compartments like the nucleus and cytoplasm, as well as the extracellular space can easily be recognized in all tissue types studied. Usually the normal epithelial tissue can be recognized by the presence of a great number of intercellular processes, whereas the cancer tissue displays an irregular structure, the cells are of various size, the intercellular contacts are poor, and the whole image is characterized by a strong polymorphism (Zs.-Nagy et al. 1981, 1983, 1987).

The problem of necrotic cells in the tumor tissue

It is obviously of great importance to exclude from the measurement all the eventually occurring necrotic cells. Fortunately, these can be easily and safely identified even in the FFFD specimens on the basis of the following criteria:

(a) Their nucleus is not recognizable.

(b) There is no potassium ionic gradient in those cells as compared to the extracellular ionic composition. It should be stressed that even a serious decrease of the potassium gradient in itself does not indicate cell death, since viability tests may reveal the living state of such cells in cultures (Szállási et al. 1988).

(c) The necrotic cells have a high Ca^{2+} content, whereas Ca^{2+} is practically undetectable by the energy-dispersive X-ray microanalysis in most living cells.

(d) Due to the autolysis, the necrotic cells lose their nuclear and cytoplasmic P-content.

It should be stressed that necrotic cells occur very rarely in the tumors studied: they remain below 5 % of all cells because these tumors are removed in a relatively early stage.

A survey of the results obtained

Since from the point of view of the MHM the intracellular Na^+/K^+ ratio is the most relevant parameter, we summarize here the published data obtained by the bulk specimen X-ray microanalytic method (or its modification for cultured cells).

Urogenital cancers (Zs.-Nagy et al. 1981): Samples were taken from 10 patients suffering from invasive urogenital tumors. Pathohistology established 3 types of cancer: keratinizing (4 cases), transitional cell (4 cases) and hypernephroid carcinoma (2 cases). More than 250 cell nuclei were measured from each type of tumor. The results were compared to those obtained in intact human urothelium taken from patients without any malignant process. Proximal and distal tubular epithelial cells of rat kidney were also studied, since such cells could not be taken from healthy humans as controls for the hypernephroid cancers. The analyses revealed in all 3 types of cancers that the average intracellular sodium content increased more than three-fold, and the potassium content decreased 32, 16 and 13 %, respectively. The intracellular 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 rat kidney.

Thyroid tumors (Zs.-Nagy et al. 1983): Benign adenomas (5 cases) and differentiated (5 cases) as well as anaplastic carcinomas (6 cases) were selected for the studies. The tumor cells were compared to those obtained in apparently normal cells of the human thyroid epithelium removed for various reasons (7 cases). The number of normal cells analyzed was 349, whereas in the tumors listed above 408, 423 and 891 cells were measured, respectively. Due mostly to the increase of the Na^+ content, the distribution histograms of the Na^+/K^+ ratio show a considerable broadening toward the higher values, and the size of this shift correlates with the increasing malignancy of the tumors. The differences in the distribution histograms belonging to different tumors proved to be statistically significant. These alterations of the Na^+/K^+ ratio showed a correlation with the increasing aneuploidization of the thyroid cancer cells as measured by microfluorimetric methods (Lukács et al. 1983).

Laryngeal tumors (Zs.-Nagy et al. 1987): Laryngeal biopsy material was studied in 4 cases of benign character (135 cells) and in 15 cases of carcinoma planocellulare keratoides or non-keratoides (641 cells). The average intracellular Na^+/K^+ ratio was 0.13 ± 0.01 (S.E.M.) in the normal epithelial cells, with a regular Gaussian distribution. In case of carcinomas the average of the same ratio was 0.67 ± 0.03 and displayed an apparently non-normal, very broadened distribution histogram.

Hepatocytes in various conditions (Pieri et al. 1983a): Normal and regenerating liver as well as Morris hepatoma 3924A cells were compared as regards their monovalent ion contents and the Na^+/K^+ ratios. Na^+ and Cl^- contents were higher in both the nuclei and cytoplasm of regenerating liver and of the hepatoma cells, as compared to the normal hepatocytes. In addition, the hepatoma cells displayed higher Na^+ and Cl^- contents than the cells in regenerating liver. The potassium content of the cells was unaltered in all 3 models. The increased sodium content and the resulting increased Na^+/K^+ ratios are not due to a generalized increase of these parameters in all cells, but to the appearance of a new cell population in the regenerating liver and in the Morris hepatoma. These observations lended support to the MHM.

T_3-stimulated hepatocytes (Pieri et al. 1984): Hepatocytes can be stimulated to proliferate by a single pharmacological dose of triiodothyronine (T_3) resulting in an increased DNA synthesis and liver hyperplasia especially in female rats (Short et al. 1980). This model was used to study the correlation between the DNA synthesis and the Na^+/K^+ ratio during the first 22 hours subsequent to the injection of 0.2 mg T_3 per 100 g body weight to young female rats. These parameters increased gradually up to 22 hours, and amiloride treatment (20 mg/100 g body weight) inhibited both of them. This finding is consistent with the MHM.

Swiss/3T3 cells (Szállási et al. 1988): A slightly modified version of the bulk specimen X-ray microanalytic method was applied to cultured cells after enzymic (collagenase) harvesting. The intracellular Na^+/K^+ ratio increased abruptly to very high levels (1.5) already 5 min subsequent to the harvesting procedure, indicating a sustained depolarization of the cell membrane. This parameter decreased exponentially during the first 2 hours, reaching 0.11 and 0.06 by the end of the first an second hour, respectively. The total intracellular monovalent ion contents remained almost constant during this post-harvesting period. These alterations should be taken into consideration during various experimental designs when anchorage-dependent cell cultures for studies on the mitogenic stimulation are used.

General discussion

It is well established that the plasma membrane of living cells maintains an asymmetric ionic distribution between the extra- and intracellular space. Namely, in the resting, differentiated state of the cells the intracellular Na^+ content is low, whereas the K^+ content is high as compared to the extracellular space. This asymmetric distribution is due in part to the difference in the passive permeability of the cell membrane for these ions ($P_K:P_{Na}$ amounts to about 30 or more), and in part to a considerable energy expenditure through the Na^+-K^+-dependent ATPase (the "pump" enzyme) (Adelman 1971, Hope 1971). The resting membrane potential of the cells is about -40 to -100 mV, involving a low Na^+/K^+ ratio; even an extremely short collapse (for several msec) of this electric polarization during the regular passage of nerve impulses produces serious alterations in the

behavior of the cells, which is called the excited state. A sustained increase of the Na^+/K^+ ratio indicates a long-term depolarization of the cell membrane. The essential suggestion of Cone (1971) was that such a sustained depolarization of the cell membrane may be the main factor inducing the mitotic cycle in both normal and tumor cells.

In the above terms, it seems to be of importance that the human tumors studied so far display a very considerable increase of the average intracellular Na^+/K^+ ratio and a broadening of the distribution histogram of this parameter. Similar changes were observed in other models where cell proliferation was induced in various ways. This indicates that a great portion of the cells in these tissues may have depolarized cell membrane. Although the existence of such a phenomenon does not prove the causal interrelationship between the membrane depolarization and the initiation of the mitotic cycle, it must be emphasized that it deserves great interest. This is all the more true, since some of the methodological approaches to this problem which delivered negative evidence against the MHM (Sachs et al. 1974, Sanui and Rubin 1977, 1978, 1979, 1982, Moscatelli et al. 1979), proved to be incorrect (Zs.-Nagy et al. 1984).

Obviously, one has to ask the question how the increased Na^+ content of the tumor cells (i.e., the sustained depolarization of their cell membranes) can influence the mitotic activity of the cells. One can list several possibilities as follows. Ions can produce some nonspecific electrostatic interactions with the macromolecules, and can alter the conformation and solubility of the latter by some specific effects, too (Von Hippel and Schleich 1969). The charge distribution, the conformation and solubility of the proteins are directly related to enzyme activities, i.e., to functions of the living systems. The ionic effects on the nucleic acid-protein complexes can be interpreted even more directly on the basis of the so-called polyelectrolyte theory (Maurel and Douzou 1976, Douzou and Maurel 1977a, 1977b). It may also be relevant that some proteins are known to respond to depolarization of the cell membrane by a quick and reversible phosphorylation (Forn and Greengard 1978, Browning et al. 1979, Rozengurt 1986). Such processes may well be implicated in the mitotic regulation.

A question to be answered is the mechanism of the increase of the intracellular Na^+ content. Theoretical possibilities are:

(a) The cell membrane maintains its normal passive Na^+ permeability, however, the "pump" enzyme becomes insufficient, and therefore, the Na^+ content increases in the cell. An example of this possibility is offered by the case of the chemically induced carcinogenesis of the liver. In this process the disappearance of the "pump"-enzyme activity from the membrane was the very first, relevant and persisting observation (Emmelot and Scherer 1980).

(b) Na^+-permeability of the cell membrane increases in the resting cells, and although the "pump"-enzyme induction attempts to satisfy the increased requirement, the cells cannot maintain their homeostasis, in spite of the considerably increased pumping capacity. This second possibility has also been verified: the human thyroid papillary cancers display a 5-10-fold increase of the "pump"-enzyme activity (Mizukami et al. 1983), while the intracellular Na^+/K^+ ratio and the DNA-synthesis increased significantly (Lukács et al. 1983, Zs.-Nagy et al. 1983). Obviously the combination of these two possibilities may also occur in various models.

It is well known that Ca^{2+} ion is an important inductor of cell proliferation in numerous model systems (Epel 1980, Jaffe 1980, Whitfield et al. 1980, Rozengurt 1986). These observations gain a special importance, if we consider some recent data showing that most of the calcium effects on the mitosis are amiloride-sensitive, i.e., the target of the calcium effect may well be the Na^+ channel protein (Villereal 1981, 1982, Owen and Villereal 1982).

Lastly, the data of Holland et al. (1983) should be commented on. They described in vitro a non-specific inhibitory effect of amiloride on protein kinases and claimed that their results cast doubt on the role of the amiloride-sensitive Na^+/H^+ exchange system in the mitotic regulation. The inhibition of the protein kinases was shown using 0.1 mM of ATP and the same concentration of amiloride. They observed also that this inhibition of the cyclic AMP-dependent protein kinase was competitive with respect to ATP, i.e., the competition was relieved, if the ATP concentration was raised. Holland et al. (1983) concluded that due to the molecular structure of amiloride, it may inhibit protein kinases by competing at the ATP binding site.

These results seem to contradict rather strongly the amiloride-evidence in favour of the MHM. However, this contradiction is only apparent. Namely, the intracellular concentration of ATP in vivo is always much higher (in the range of 2-6 mM), whereas the concentration of amiloride especially in the in vivo experiments is much lower than in the model of Holland et al.(1983). For example, Koch and Leffert (1979) reported an ID_{50} = 0.02 mM amiloride for the inhibition of DNA synthesis of cultured hepatocytes, and ID_{50} = 25 mg/kg body weight for the same parameter of liver in vivo. Using this latter figure one can obtain molar concentrations of amiloride in vivo in the range of 0.01-0.02 mM, if one assumes an equal distribution of amiloride in the extra- and intracellular space, which is certainly an overestimation for the intracellular amiloride concentration. Therefore, one has to assume an ATP/amiloride molar ratio of at least 100 in the intracellular space. It means that the competitive inhibition of the protein kinases cannot be of great significance in vivo, especially if we consider that equal molar concentrations of ATP and amiloride caused a roughly equal binding of the two compounds to the protein kinases (see Table 2 of Holland et al. 1983). In light of this interpretation it seems to be obvious that the MHM cannot be disregarded on the basis of the arguments of Holland et al. (1983).

The results and considerations discussed above suggest that the MHM may still be a valid hypothesis for the mitotic regulation (see for details: Rozengurt 1986), and that the bulk specimen X-ray microanalysis represents a valid and solid approach to further studies in this field.

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Discussion with Reviewers

G.M. Roomans: The final stage of necrosis may be easy to identify, but how can one be sure about the intermediate stages of impaired energy metabolism that eventually result in necrosis? Wouldn't the inclusion of such cells in your sample give an increase in the Na/K ratio larger than that which can be attributed to MHM?
T. von Zglinicki: The morphological criteria applied exclude only the cells in late stages of necrosis. However, cellular lesion is not an all-or-none process but develops through many stages. An increased Na/K ratio even without an increase of Ca is not only indicative of rapidly dividing cells, but also of certain types of injury. The existence of a large number of malnourished, slowly dying cells could be the reason for the broadening of the Na/K distribution in thyroid and laryngeal tumors. In order to demonstrate an increased Na/K ratio in dividing, uninjured tumor cells, a cut-off at the lower side of the Na/K distribution histograms should be found. Was that the case?
Author: It is obviously possible that our data contain some cells which are on the way to necrosis. To the best of my knowledge, there is no method applicable for compact tissues under the X-ray microanalytic conditions to exclude such cells from consideration. Nevertheless, it seems to be of importance that in our experience such "dying" cells occur in healthy compact tissues very rarely. Energy shortage of the cells may be an important component contributing to the imbalance of the monovalent electrolytes, however, these cells are not obligatorily "dying" cells, as suggested by Dr. Von Zglinicki. We have shown on 3T3 fibroblasts that 99 % of the cells harvested by collagenase maintained their viability as revealed by trypan-blue uptake, whereas 95 % of them were able to exclude Erythrosin B as shown by X-ray microanalysis, meanwhile an extremely high Na/K ratio was found in them during the first 30 min subsequent to the harvesting (Szállási et al. 1988). In other words, the cells showing an increased Na/K ratio cannot be considered as "dying" or injured ones. Mitogenesis might be correlated with such a special state of the cells as suggested by the MHM. As regards the cut-off at the lower side of the Na/K distribution histograms, one cannot expect such a phenomenon, since the tumor tissue is never a pure clone of tumor cells, but contains numerous normal cells, too.

G.M. Roomans: According to the MHM, amiloride could be an effective drug in the treatment of cancer. Does this appear to be the case in practice?
Author: As far as I know, no systemic human studies on the antitumor activity of amiloride have been carried out so far. This is due, most probably, to the toxicity of this drug used practically only as a K-sparing diuretic in rather special cases. Since animal experiments showed unanimously that this drug blocks both the normal and tumorigenic mitogenesis both in vitro and in vivo, I would suggest to perform human trials under properly controlled conditions.

A. Warley: The author chooses to use the element phosphorous to sustain his argument that the subcellular information derived by X-ray microanalysis is not meaningful. Can the author really uphold this view if the diffusible elements are considered?
Author: As explained in this paper, the knowledge of overall cellular concentrations of elements belonging to various classes of molecules has a restricted value from a biochemical point of view. Apart from P one can mention numerous other elements like S, or (when using a window-less detector) C, N, O, etc. As regards the monovalent electrolytes, convincing NMR evidence is available showing that apart from some electrostatic interactions with the "fixed charges" of the macromolecules, the monovalents form one single class in the living cells (see for ref.: Zs.-Nagy et al. 1977).

A. Warley: The author concludes that the data of Holland et al. (1983) describing the inhibitory effects of amiloride on protein kinases does not reflect the in vivo situation. Would the author like to comment on the work of Lubin (1982) which indicates that amiloride is directly inhibitory for protein synthesis?
Author: I was present on the Symposium where Lubin delivered the lecture mentioned by the Reviewer. A detailed description of those experiments can be found in Lubin et al. (1982). It should be noted that the inhibitory effect of amiloride on the protein synthesis rate had already been observed much before Lubin, and this was explained by a decreased amino acid transport. There is no room here to comment on this problem in detail, but I can recommend a recently published extensive review (Grinstein et al. 1989) summarizing all relevant data on the effects of amiloride and its analogues. It is clear from the available data that the problem is very complex and numerous aspects of it are still unclear, nevertheless, the role of increased intracellular Na-content in the mitotic stimulation cannot be denied.

T. von Zglinicki: An increasing Na/K ratio is a rather weak indicator of decreasing membrane potential, resting not only on the assumptions involved in the derivation of the Goldman equation but also on the following ones: (i) The increase of the Na/K ratio is due to a decrease of K and/or there is a concomitant increase of Cl, and (ii) activity coefficients are essentially unchanged. How far are the results obtained from, e.g., thyroid tumors indicative for a decreased membrane potential? Are there more direct estimations of membrane potential available to support the MHM concept?
A. Warley: As I understand it, much of the work carried out on cell lines in culture indicates that there is an increase in intracellular (Na) as the cells enter the S phase of the cell cycle, the (Na) decreases as the cells progress

through the cell cycle. The author argues strongly that his results show a sustained depolarization of the cell membrane. Using the methods described can the author differentiate between the analysis of cells which are at a certain phase of the cell cycle, and cells which are showing a sustained increase in intracellular Na?.

Author: I disagree with Dr. Von Zglinicki in the first issue of his comment. Our recent (unpublished) results obtained on 3T3 cells by means of flow cytometry did prove that the cells showing an increased Na/K ratio after harvesting are really depolarized, and the decrease of this ratio is accompanied again by a polarization of the cell membrane. Although we do not have direct data on thyroid tumor cells, these results strongly suggest that similar situation may be assumed in other cell types, too. At present it is not possible to differentiate between the various phases of the cell cycle in the compact tissues, as Dr. Warley suggested above, but further studies on synchronized cell lines might result in progress in this field.

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