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György Lustyik
University of Minnesota

Imre Zs.-Nagy
University Medical School

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AGE DEPENDENT DEHYDRATION OF POSTMITOTIC CELLS AS MEASURED BY X-RAY MICROANALYSIS OF BULK SPECIMENS

György Lustyik* and Imre Zs.-Nagy

Fritz Verzár International Laboratory for Experimental Gerontology (VILEG)
Hungarian Section, University Medical School, H-4012 Debrecen, Hungary

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Abstract

In this paper we give a brief outline of our bulk specimen technique developed to measure intracellular water concentration in frozen-hydrated biological specimens by means of energy dispersive X-ray microanalysis. Fractured surface of the deep-frozen tissue samples is analyzed in an electron microscope (a specimen area of 15x11.5 μm is scanned) using 20 kV accelerating voltage and 1-5 pA effective beam current (measured in the specimen). Strong electric charging, which is the main problem associated with the low temperature X-ray microanalysis of frozen-hydrated specimens, is reduced by choosing optimum temperature range for the measurements (170-185 K) and by etching a thin surface layer on specimen surface. The main advantage of the method over other X-ray microanalytical techniques using sections and bulk specimens for water and dry-mass content determinations in cells (which are shortly reviewed) is the simple specimen preparation, the easy sample handling and the good stability of specimen during measurements. The main disadvantage is the poor spatial resolution as compared to the analysis of sections.

Measurements with our method provided meaningful results of the change in intracellular water contents in various postmitotic cells of rats dependent on age. The observed decline of the intracellular water contents results in increased ionic strength and slower diffusion in old cells than in young ones. These effects may be implicated in senescent deterioration of cell metabolism.

Keywords: Quantitative X-ray microanalysis, biological bulk specimens, frozen-hydrated specimens, cryopreparation, intracellular water content, cellular aging.

*Address for correspondence:
University of Minnesota, Department of Laboratory Medicine and Pathology, Box 198 Mayo,
Minneapolis, MN 55455, Phone No. (612)-626-2709

Introduction

Although the link between aging of a complex organism and aging of cells is not completely established, cellular aging may provide a relevant basis for the study of organismal aging. Highly differentiated somatic cells, as they age in vivo, exhibit impaired adaptive responses to a variety of stimuli.There is good reason to attribute the phenomena of cell aging in higher organisms to gradual, permanent loss of renewal due to modification in the expression of genetic information. Because no particular molecular mechanism of the cell has been established to be the cause of such modification, it is generally assumed that a large number of biochemical reactions in both nuclear and cytoplasmic steps of gene expression are involved.

Alteration of the physico-chemical state of the cytoplasm and the nucleoplasm are known to affect biochemical reactions and enzymatic processes. Therefore they may be implicated in senescent deterioration of cell function. Increased ionic strength and accompanying condensation of the cell matrix , which are predicted by the membrane hypothesis of aging (Zs.-Nagy 1978, 1979) are of special importance for the evaluation of age dependent slowing of the cell metabolism. The majority of observations concerning age-dependent compositional changes of cellular electrolytes came from X-ray microanalytical measurements (Pieri et al. 1977, Zs.-Nagy et al. 1979, Bertoni-Freddari et al. 1981). Recently we concluded from X-ray microanalytical measurements on rat brain cortical neurons and liver cells (Lustyik and Zs.-Nagy 1985, Bertoni-Freddari et al. 1981), cardiac myocytes and skeletal muscle cells (Lustyik 1986, Zglinicki and Lustyik 1986) that one of the consequences of aging in mammalian cells is a decline in intracellular water content of postmitotic cells. In this and in the accompanying paper (Zs.-Nagy I. 1987. The bulk specimen microanalysis of freeze-fractured, freeze-dried tissues in gerontological research. In this volume.) we describe X-ray microanalytical techniques and preparation methods appropriate for the measurement of electrolyte composition of tissue cells. The present study focuses on cellular water and dry-mass content measurements by energy dispersive X-ray microanalysis on deep-frozen, bulk biological samples and applications in aging studies.

Methods for water and dry-mass content measurements

Cryofixation of biological materials is routinely used for X-ray microanalytical work because it preserves the in vivo distribution of diffusible electrolytes. Excellent cryosectioning techniques have been developed in various laboratories to make both ultrathin and semi-thick sections (Barnard and Seveus 1978, Seveus, 1978, Federic, 1982, Gupta et al. 1978b, Roomans 1981, Roomans and Seveus, 1977, Roomans et al. 1982, Ross et al. 1981, Zierold 1980, 1982a, Wroblewski et al. 1985, Cameron and Smith, 1980), allowing X-ray microanalytical measurements either on freeze-dried or frozen hydrated samples. Intracellular water and dry-mass content measurements from cryosections are usually based on the Hall method (Hall, 1979, Hall and Gupta 1982, Hall et al. 1973) which needs two measurements, first in frozen hydrated then in freeze-dried specimens. The critical step of this technique is handling and transfer of deep-frozen cryosections (Zierold, 1982b, 1983) which has been successfully done only in a few laboratories.

Rick et al. (1978) and Dörge et al. (1978) suggested an alternate technique to measure dry-mass content (and indirectly water concentration) from freeze-dried cryosections. The method, which was further elaborated recently by Warner (1986), uses standards of known water content which have to be cut and freeze-dried together with the specimen. Recently Zierold described another method for measurement of dry-mass content in sections from relative dark-field intensity caused by a support film compared to the freeze-dried specimen (Zierold, 1986). The method overcomes most of the disadvantages of the previously used mass-thickness evaluations by Beer's law (Pozsgai and Barna, 1983, Siklós, 1983) and other derivations from backscattered and/or transmitted electron signals (Linders et al. 1982a,b, 1984, Linders and Hageman, 1983, Egerton, 1982, Reichelt et al. 1984).

Section technique and bulk specimen technique are frequently considered as two competing methods. We think, instead, of the two methods as complementary rather than mutually exclusive. It is often claimed that the large excitation volume restricts the spatial resolution in bulk specimens. It is true that spatial resolution is significantly better in sections than in bulk specimens. According to the range equations (Beaman and Isasi, 1972) the excited volume may be as large as 500 μm^3 in frozen-dried samples at 10 kV. However, this volume is less than 5 % of the hepatocyte or heart cell volume (Pieri et al. 1975, David et al. 1981) and even less compared to a neuron volume. Assuming 100 nm section thickness and a 2x2 um scanning area during analysis, the analyzed volume is only 0.4 μm^3. However, the analyst has to pay for this advantage with higher minimal detectable concentration of the analyzed element (assuming, e.g., 50 mM sodium concentration, the analyzed volume contains less than 5×10^{-16} g sodium in the above section) and with a risk for errors which are negligible in bulk specimen analysis (contribution of external X-ray sources to the collected spectra, local inhomogenities because of compression during section cutting, etc.). The same or similar differences are seen when frozen-hydrated cryosections and bulk specimens are compared. Both techniques have either strong or weak points, and they have their own fields of application. With the bulk specimen technique usually only questions associated with the average concentrations in whole cells, cytoplasm and nuclei can be addressed.

Since 1974 we have been using a bulk specimen technique for routine energy dispersive X-ray microanalytical work in our laboratory. The method was introduced by Zs.-Nagy et al. (1977) for measurement of electrolyte concentrations in freeze-dried specimens (Pieri et al. 1977, Zs.-Nagy et al. 1979). This method has been extended recently for determination of intracellular water and dry mass concentrations in frozen-hydrated bulk specimens of compact tissues (Zs.-Nagy et al. 1982, Lustyik and Zs.-Nagy 1981). The water concentration is determined from the increase of elemental concentrations due to the drying of fully hydrated samples, using the principle of the Hall method.

X-ray microanalysis of both frozen hydrated and freeze-dried bulk specimens is now an accepted method. Especially for freeze-dried specimens, quantitative methods are well developed (Zs.-Nagy et al. 1977, Roomans, 1981, Boekestein et al. 1980) but analysis of frozen-hydrated bulk samples also provides quantitative information (Marshall, 1980, 1982, Echlin et al. 1982, Echlin and Taylor, 1986). The main problem associated with X-ray microanalytical measurement of intracellular water content from deep-frozen specimens stems from the very low electric conductivity of ice at low temperature (Brombach, 1975). Although the conductivity increases to a certain extent above approximately 170 K, the risk of undesirable evaporation from the specimen also increases with increasing temperature. There are different ways to overcome difficulties arising from the extremely bad electric conductivity of biological samples at low temperature which results in strong charging under the electron beam in an electron microscope: (1) Because charging is less serious in thin samples, frozen hydrated sections have been used in few laboratories for low temperature X-ray microanalysis. (2) Space charge may be reduced by covering the specimen surface with a thin conductive layer of metals or carbon (Echlin and Taylor, 1986, Fuchs and Lindemann, 1975, Fuchs et al. 1978a,b, Marshall 1975, 1977, 1980). Beryllium was found to be one of the best coating materials because of its negligible interaction with the generated X-ray photons (Marshall and Carde, 1984, Marshall et al. 1985) but its use is limited because of its very high toxicity. (3) Etching of a superficial layer from the specimen surface was also reported to reduce specimen charging in deep-frozen specimens and improved both the contrast of the microscopic image and the quality of the generated X-ray spectra (Gupta et al. 1977, 1978a,b,c, Fuchs and Lindemann, 1975). Drying of a superficial layer of the specimen appeared to

be beneficial in our method of water content measurements.

Measurement of water concentration in bulk specimens

Principle of the applied method.

The mass fraction of water (W) in a frozen-hydrated sample can be calculated from the change of concentration of an evenly distributed element during drying of the sample:

$$W = 1 - \frac{C_w}{C_d} \qquad (1)$$

where C_w and C_d are concentrations in the wet and dry sample, respectively. C_w and C_d can be determined from peak to background ratios of the element (R_w and R_d) according to the continuum method of Hall (Hall et al. 1973, Hall and Gupta, 1986):

$$C_w = R_w G_w F \quad \text{and} \quad C_d = R_d G_d F \qquad (2a,b)$$

where F is a constant, independent of the sample, G represents the mean value $\overline{Z^2/A}$; Z and A are specimen atomic number and atomic weight. By definition: $G_w = (1-W)G_d + WG_{water}$. Since for soft biological tissues $G_d = 3.28$ for biological matrix (Hall et al. 1973) and $G_{water} = 3.666...$, we get:

$$W = \frac{R_d - R_w}{R_d + (G_{water}/G_d - 1)R_w} = \frac{R_d - R_w}{R_d + 0.1179\ R_w} \qquad (3)$$

It follows from Eq(3) that W can be determined without standards, by measuring peak to background ratios of an element first in fully hydrated, and then in the dried state.

Measurement of R_w and R_d:

Difficulties arise during measurement of R_w, and we have treated this question previously in detail (Zs.-Nagy et al. 1982, Lustyik and Zs.-Nagy, 1981, Zs.-Nagy, 1983, Lustyik and Zs.-Nagy, 1985). Because the electric conductivity of the low-temperature ice (below approximately 140 K) is very low, electrons from the beam of the electron microscope are trapped within the excited volume, building an intense negative space charge in bulk samples. The generated electric field may result in several kV of electric potential which reduces the effective accelerating voltage and restricts penetration of the exciting electrons. Because a large portion of the electrons is back-scattered from the surface, practically no secondary electron image can be observed and the generated X-ray spectra are seriously deteriorated. Although charging is less significant in thin frozen-hydrated sections than in bulk specimens, imaging is also not adequate in fully hydrated cryosections at low temperatures (Bulger et al. 1981, Echlin, 1978, 1979, Gupta and Hall, 1981, Gupta et al. 1978b, Saubermann et al. 1981a,b, Zierold, 1983).

Our method using frozen-hydrated bulk specimens is based on the observation that both excitability and microscopic image quality improves significantly when the specimen temperature exceeds approximately 165 K during warming up within the microscope column. This improvement may be attributed to the etching of the specimen surface and the increase of ice conductivity. This latter phenomenon is probable due to an increased proton mobility above 140-150 K (Franks, 1972).

Measurements are performed with an energy dispersive X-ray microanalyzer (EDAX F system) and a scanning electron microscope (JEOL JSM 35C) equipped with cold stages in the microscope column and airlock chamber (JEOL, Cryo Unit 1). Small, rod-shaped samples (diameter max. 1 mm) from the appropriate tissue are mounted on a specimen holder, frozen very quickly in cooled isopentane (113 K) and stored in liquid nitrogen until measured. Tissues should be fractured prior to the analysis within the airlock chamber at 140-150 K and introduced into the microscope column immediately. Samples are equilibrated with the cold stage of the microscope column for a few minutes (160-165 K) and measured during spontaneous warming (warming rate approximately 0.7 K/min) with 20 kV accelerating voltage. X-ray spectra are recorded continuously and peak to background ratios of potassium and phosphorous are calculated. At the begining, peak to background ratios show a continuous increase parallel with the improvement of the excitability of the specimen and the microscope image, then they are nearly constant within the temperature range of 170-185 K. Values in this stabilized interval can be considered realistic peak to background ratios. Above 185 K R_w displays a second increase due to drying in deeper layers of the specimen. A computer program has been written in order to select the constant values of the peak to background ratios and to avoid subjective error of selection.

Recently this method was further improved. A problem of spontaneous warming is that specimens spend only a relatively short time within the optimum temperature range allowing usually 10-12 R_w determinations on one sample. Recently we observed that peak to background ratios are constant for at least 2 h if specimen temperature is held between 175-180 K. Therefore, an electronic temperature stabilizer has been built into the microscope cold stage and measurements are performed at constant temperature of 175-180 K. This modification allows 60-100 consecutive measurements on the same sample. Peak to background ratios display only a very slow increase meanwhile. The intercept of the best fitting straight line through the R_w points versus time can be used to estimate the peak to background ratio of the wet sample.

R_d ratios are measured subsequent to drying of a 30-40 µm thick layer of the specimen at 210 K for 2-3 h. W is calculated from the Gaussian law of error propagation.

Problems associated with the measurements.

(1) One of the serious compromises of such measurements is the need for etching of the specimen surface. Freeze-drying is a very complex, incompletely described process, especially in structured biological samples. The rate of sublimation from ice can be described with the Knudsen equation based on the kinetic gas theory (see Umrath, 1983, Edelman, 1986). According to the calculation of Umrath (1983) 1 µm ice layer sublimates in about 15 min at 175 K (see also Fig.

Table 1. Intracellular water concentration (Mean +/- S.E.) in postmitotic cells of various tissues of rats dependent on age.

Cells	Age of rats	W (%)[a]	(b)	(c)
Brain cortical neurones	Young (1 month)	78.0 +/- 0.4		
	Adult (11 months)	76.3 +/- 0.6	N.S.	
	Old (25 months)	71.9 +/- 0.5	$p < 0.001$	$p < 0.001$
Liver	Young (1 month)	68.3 +/- 0.6		
	Adult (11 months)	66.5 +/- 0.3	N.S.	
	Old (25 months)	64.3 +/- 0.9	$p < 0.05$	N.S.
Heart cells	Young (1 month)	76.3 +/- 0.6		
	Adult (12 months)	72.0 +/- 1.0	$p < 0.05$	
	Old (24 months)	71.1 +/- 0.7	$p < 0.05$	N.S.
Skeletal muscle cells	Adult (13 months)	76.4 +/- 1.2	---	
	Old (30 months)	74.6 +/- 1.3	---	N.S.

[a] Data taken from Lustyik and Zs.-Nagy (1985), Lustyik (1986), Zglinicki and Lustyik, (1986).
[b] Level of significance of the difference from the young value.
[c] Level of significance of the difference from the adult value.
N.S. = not significant.

1. in Edelman, 1986). It is probable therefore, that large ice crystals completely evaporate from the surface of our frozen-hydrated samples during the initial warming phase leading to some underestimation of the mass fraction of the calculated cell water (vacuum pressure in the microscope column is 10^{-3} - 10^{-4} Pa).

Ice sublimation from the structured cellular network is significantly slower (Stephenson, 1953, Umrath, 1983) although it is difficult to estimate a realistic prolongation factor for the freeze-drying. Slower sublimation is due both to restricted diffusion in the dried shell of the biological materials (Malström, 1951, Stephenson, 1953, 1960) and to slow secondary drying (removal of the water molecules physically bound to biomolecules, MacKenzie 1965, 1981). Estimates of prolongation factors from the literature are scattered over a very wide range (Edelman, 1986, Umrath, 1983, Robbards and Sleytr, 1985). As discussed previously (Zs.-Nagy et al. 1982, Zs.-Nagy, 1983), assuming a prolongation factor of about 1000 results in quantitatively negligible dehydration of the structured cytoplasmic matrix. The calculation of Umrath (1983) predicts 13.7 nm/s etching rate from bulk ice at 183 K resulting in complete dehydration of less than 50 nm surface layer in 1 h under the above conditions. One can calculate from range equations (Beaman and Isasi, 1972) as well from measured and calculated penetration depths (Marshall, 1982, Marshall and Condron, 1985, Oates and Potts, 1985, Zs.-Nagy et al. 1982) that contribution of this dried layer to the generated X-ray spectra is less than 1% at 20 kV accelerating voltage.

The assumption that evaporation is slow in our samples is strongly supported by the fact that the peak to background ratio of potassium remains quite constant for at least 120 min at 175-180 K.

(2) Because electrons penetrate further into the sample, the analyzed volume is larger in dry samples then in the hydrated ones, therefore Eq(1) is valid only if the measured elements are uniformly distributed in both excited volumes. Although elements can be segregated in the cell to a certain extent, it seems to be an acceptable assumption that within the relatively large analysed volumes the average potassium and phosphorous concentrations are independent of the size of this volume.

(3) Overpenetration of the cells in the dry sample tends to cause an underestimation of the measured intracellular water content. This error does not significantly affect the measured W values in compact tissues where the extracellular space is very narrow and amounts only to a few % in the whole tissue volume. However, when using this technique one needs to avoid overpenetration of the analysed cells if the extracellular space is larger then 5-6 % in the whole tissue. Volume density (Weibel, 1969) of the extracellular space is usually higher in old tissues than in young tissue (David et al. 1981, Pieri et al. 1975).

Age-dependent decrease of the cell water

Our method appears to be useful for detecting decreasing water content during senescence in various postmitotic cells of CFY laboratory rats. In this section we give a brief outline of results obtained with our bulk specimen technique. Specimen preparation and further details of the employed measurements have been described previously (Zs.-Nagy et al. 1982, Zs.-Nagy, 1983, Lustyik and Zs.-Nagy, 1985).

Table 1 summarizes the average intracellular water concentrations of brain cortical neurons, hepatocytes, myocardial and skeletal muscle cells dependent on age. Because of poor spatial resolution of the analysis water content cannot be measured separately in cell compartments. However, it is possible to estimate the water content of nuclei in those cells where salt concentrations are determined in both the cytoplasm and the nuclei (Lustyik and Zs.-Nagy, 1985, Zs.-Nagy, 1983). Calculations may be performed assuming equal electrolyte concentrations in the two compartments (data not shown here).

It appears from the data of Table 1 that each cell type undergoes a considerable water loss during aging. The greatest decrease in water concentration (approximately 7.8 %) was observed in neurons.

It is noteworthy that the average tissue water content of the heart does not show significant change during aging. This is due to an increase of the volume density of the extracellular space in the myocardium of old animals (David et al. 1981) which compensates the water loss from the cardiac myocytes (Zglinicki and Lustyik, 1986). Similar changes can be observed in the liver of old rats.

Conclusions

Summarizing the discussed methods and results we can conclude that these measurements provide meaningful microanalytical results of the change in intracellular water content in rat postmitotic cells during aging. We do not claim that the suggested procedure is applicable for all biological materials without any modification; it has both advantages and disadvantages as compared with other published measurements. No doubt, the main disadvantage of the method over the section technique is the poor spatial resolution in the bulk specimens. It does not permit measurements in particular cell compartments. In contrast, its main attraction is the simple preparation, the easy sample handling and the relatively good stability of the specimens during the measurements. The effect of ice crystal damage (Zierold, 1984, Hall, 1986) and damage by the electron beam (Talmon et al. 1986, Glaeser and Taylor, 1978, Heide, 1982, Talmon, 1984) are also more significant in sections than in bulk specimens. Ice crystal formation can be avoided only with very high cooling rates (Bald, 1986, Franks, 1986) which can be achieved only within a thin surface layer of the tissue in bulk specimens (Haggis, 1986, Zglinicki et al. 1986).

Concerning the biological significance of the observed water loss from the postmitotic cells during aging, our results indicate alterations in the physico-chemical state of the cell matrix during aging. It should be noted that there are some uncertainties regarding the molecular characteristics of the aqueous state in biological systems. Numerous observations indicate that ions and other chemicals, interaction with macromolecular surfaces and cellular interfaces influence water properties (see for review Pethig, 1979), such as surface tension, local viscosity, electric and dielectric properties. Calorimetric, infrared, and NMR measurements (Kuntz and Kauzman, 1974) suggest that biomolecules bind water on their surface and that the "bound" water alters hydrodynamic properties of the biomolecules. Water molecules near the biomolecules rotate and translate more slowly than in the "bulk" water altering this way their infrared and Raman spectra and NMR relaxation times, and the associated water molecules have altered thermodynamic properties (Fung, 1986, Savage and Wlodawer, 1986). In living systems, recently Zimmerman et al. (1985), Merta et al. 1986, Cameron et al. (1985) described that cell water exists in different states which can be characterized with different NMR relaxation times. Edsall and McKenzie (1983) concluded that water is essentially bulk water in protein solutions, except the water adjacent to molecular surfaces carrying charge, internal water within folded peptide chains and interfaces between protein subunits. The nature of interaction between polyelectrolyte macromolecules and monovalent cations is also the subject of controversy (Burt, 1982, Gupta et al. 1984, Berendzen and Edzes, 1973, Cope, 1967).

However, we can conclude that there are at least two physico-chemical consequences of the observed decrease in water concentration:

(1) Increased dry-mass content in the cells suggests a higher cytoplasmic microviscosity in old cells than in young ones. Recently we measured a higher fluorescence emission anisotropy of fluorescein in the cytoplasm of hepatocytes isolated from old rats compared to young. These results can be due to both higher microviscosity surrounding the fluorescent probe (Cercek and Cercek, 1977) and to stronger interaction of the probe with the microtrabecular network of the cytoplasm (Bridgman and Reese, 1984, Wolosewick and Porter, 1979). Both effects may slow the cytoplasmic diffusion in the cells (Wojcieszin et al. 1984, Jacobson and Wojcieszin, 1981, Mastro and Keith, 1984, Gershon et al. 1985) as well the rate of various enzyme reactions (Gavish and Werber, 1979, Damjanovich and Somogyi, 1973, Somogyi and Damjanovich, 1977).

(2) On the other hand, our results indicate an increased wet weight concentration of electrolytes in old cells as compared to the young. According to the membrane hypothesis of aging (Zs.-Nagy, 1978, 1979, 1985) a decreased membrane permeability for potassium is responsible for increased ionic strength in the cytoplasm and nucleus. Interaction of ions with polyelectrolyte biomolecules provides a physical basis for effects of ions on enzymatic processes in cells (Douzou and Maurel, 1977, Record et al. 1985), which can contribute to the deterioration of the cell metabolism in senescence. Because free radicals may play a crucial role in cellular aging (Harman, 1982, Cutler, 1984, Zs.-Nagy and Floyd, 1984), the observed exponential decay of the superoxide dismutase activity (a key enzyme of the cellular defense mechanisms against oxidative damages; Fridovich, 1976) with increasing salt concentrations is an important example of such processes (Cudd and Fridovich, 1982, Semsei and Zs.-Nagy, 1984). Furthermore, observations of Record, Roe, and their research group (Record et al. 1976, 1978, Roe et al. 1984) suggest that this mechanism may explain the decrease of the mRNA

synthesis during senescence described in various cells and tissues (Semsei et al. 1982, Semsei and Richardson, 1986, Richardson and Semsei, 1987, Richardson et al. 1985, 1987). Roe and Record (1985) described for example that in an in vitro system the equilibrium constant of the open complex formation between Escherichia coli RNA polymerase and the λP_R promoter depends on the 20th power of the salt concentration, and a similar large salt effect was found for the RNA polymerase interaction with DNA (deHaseth et al. 1978, Lohman et al. 1980). One area where progress should be made in the future is testing of the importance of these effects in living, somatic cells.

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Discussion with Reviewers

A.T. Marshall: It has been shown by Marshall (1981) that etching of even a homogeneous sample results in an uneven removal of ice and a corresponding increase in variability of analytical data. Do you think this phenomenon would have an influence on your determination of Rw? The data from these experiments (i.e. Marshall, 1982) also suggest that etching proceeds at a somewhat faster rate than you calculate.
Authors: Etching certainly results in an uneven surface on frozen hydrated samples. We found variability of analytical data similar to your results (Marshall, 1981) when we applied a static electron beam or used line scanning. Scanning of a relatively large area with the electron beam reduced considerably the data scatter, most probably due to averaging over the local "tilting angles". However, we have less spatial resolution in our measurements than the 2-3 um resolution achievable in frozen hydrated samples with a static electron beam.

Etching rate can hardly be measured precisely. The conclusion that etching was negligible in our measurements was based on findings that: (1) Peak to background ratios of the measured elements (K and P) increased very slightly within 2 h, at 175-180 K. (2) Calculations based on the measured peak to background ratios resulted in realistic intracellular water concentrations. (3) Measurements on parallel samples were well reproducible. Nevertheless, etching rate depends on water content of the cells and on density of the structured cellular dry mass of the sample, and it may be faster in water-rich specimen than estimated in our measurements.

I. L. Cameron: Has your technique been validated using standard solutions of protein of known water and element content?
Authors: We have made such measurements. The problem with bulk specimens prepared from protein solutions of known composition is that they show considerably stronger charging than biological tissues. Because similar electrical behavior of the specimen and the standards is essential for the bulk specimen X-ray microanalysis, we cannot recommend the use of such standards for water content measurements in frozen hydrated bulk specimens.

I. L. Cameron: Which element was selected to ratio in wet and dry state in Eq.1? Does it make any difference if another element is selected for this purpose?
Authors: Peak to background ratios of potassium and phosphorous are generally measured simultaneously in both the wet and dry samples and cellular water concentrations are calculated parallel from the measurements. The difference between the results is usually less than 2-3 %, which is statistically not significant (see for further details: Zs.-Nagy et al. 1982, Lustyik and Zs.-Nagy, 1985).

B.L. Gupta: In the membrane hypothesis of aging it is not clear how does one dissociate the membrane permeability to potassium from the permeability to other ions such as Na, Cl etc. and the efficiency of Na/K pump. Do the authors also measure Na?
Authors: The membrane hypothesis of aging does not claim that membrane permeabilities to other ions than potassium are unchanged during cellular senescence. According to the hypothesis the ratio of permeability for potassium to sodium (P_K/P_{Na}) decreases in postmitotic cells during aging.

Sodium and chlorine concentrations were also measured in hepatocytes, brain cortical neurons, myocardial and skeletal muscle cells (Lustyik and Zs.-Nagy, 1985, Lustyik, 1986, Zglinicki and Lustyik, 1986). These concentrations showed a tendency to increase during aging, similar to potassium. However, the bulk of the increase of the ionic strength in the nucleus and the cytoplasm was caused by increase of cellular potassium content in those cells.

I. L. Cameron: It is stated in your introduction that aging in higher organisms can be attributed to a gradual-permanent loss of renewal. Granted that some cellular renewal systems have been shown to slow down with age, I know of no *in vivo* populations of mammalian cells which cease cellular renewal.
Authors: We do not think that aging leads to total cease of cell renewal in any population of vital mammalian cells. Cells die when the rate of any key metabolic process decreases below a critical level. Because turnover rate of some proteins can be as low as a few minutes (see for example: Schimke, 1975, Wiley, 1985), cell population can live only for a very short period of time without cellular renewal. This may be partly due to fast accumulation of damaged macromolecules. Slower accumulation can take place without stopping cell renewal, if the rate of macromolecular damage exceeds their elimination. It was shown, for example, than senescent cells have a decreased ability to degrade proteins, and this may account for accumulation of altered enzyme forms and reduction of protein turnover rate during cell aging (Rivett, 1986, Rothstein, 1983, Dice, 1982, Lavie et al. 1982).

B. L. Gupta: Superoxide dismutase is a zinc dependent enzyme. Similarly zinc-fingers (e.g. Transfer Factor IIIA) are important in gene-transcription. Other effects of zinc deficiency in health are also now well-recognised. Do the authors have any data on zinc?
Authors: We did not measure zinc routinely in our samples, although we are aware of the importance of this element in metabolic processes. Because zinc is present in very low concentration it is doubtful that intracellular zinc concentration could be determined quantitatively from bulk specimens.

On the other hand, zinc is only one element involved in the enzyme activity of superoxide dismutase (SOD). Most enzymes isolated from eukaryotic cells contain copper and zinc (cupro-zinc SOD). Mitochondrial SOD is manganese

dependent, and some bacterial enzymes contain manganese or iron (Fridovich, 1976).

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