Effect of enzymic (collagenase) harvesting on the intracellular Na^+/K^+ ratio of Swiss/3T3 cells as revealed by X-ray microanalysis

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Summary

Swiss/3T3 cell cultures were harvested with 0·05% collagenase and after centrifugation the pellet was prepared by the freeze-fracture/freeze-drying (FFFD) method for bulk-specimen X-ray microanalysis. Time-dependent variations in the intracellular monovalent elemental concentrations (Na^+, K^+ and Cl^-) as well as of the Na^+/K^+ ratio were followed for 120 min subsequent to harvesting. The quantitative measurements revealed a very considerable increase in the intracellular Na^+ and Cl^- accompanied by a decrease in the K^+ concentration as soon as 5 min after harvesting. The Na^+/K^+ ratio had increased by this time to about 1·5 on average. These changes indicate a sustained depolarization of the cell membrane. During the first 60 min this depolarization tended to normalize as demonstrated by an exponential decrease in the intracellular Na^+ and Cl^- and an increase in the K^+ content involving a decrease in the Na^+/K^+ ratio. The total intracellular monovalent ion concentration remained almost constant during this post-harvesting period. These results suggest that harvesting represents a serious depolarizing stimulus to the cells, the consequences of which are restored only after 1–2 h. These alterations should be taken into consideration during various experimental designs when using anchorage-dependent cell cultures.

Key words: enzymic harvesting of cultured cells, collagenase, intracellular Na^+/K^+ ratio, X-ray microanalysis of cultured cells, Swiss/3T3 cells, sustained depolarization of the cell membrane.

Introduction

Anchorage-dependent cell cultures are widely used in various research fields like cell physiology, microbiology, cancer research, etc. These cultured cells have to be harvested either mechanically or chemically during each subculturing procedure and before the preparation of cells for the given experiments. This procedure involves a serious alteration of the cells and, although some authors have suggested that these alterations should be considered, little is known about the effect of various harvesting procedures on the overall status of the cells.

The most commonly used harvesting procedure is scraping cells off the plates, which causes a heavy mechanical trauma to the cells. As a matter of fact, in murine neuronal-enriched cultures free fatty acids and diglycerides are liberated from the cell membrane, which shows a partial inhibition of Na^+/K^+-dependent ATPase activity and an increased production of malondialdehyde (Demediuk *et al.* 1985). Scraping has been shown to increase the uptake of macromolecules like labelled dextrans, ovalbumin or immunoglobulins as well as various dyes like Lucifer Yellow ('scrape-loading', for details see McNeil *et al.* 1984; El-Fouly *et al.* 1987) into cultured cells. In monolayer-cultured endothelial cells and monkey kidney cells, treatment with collagenase or trypsin as well as mechanical harvesting damaged the integrity of the membrane lipids as revealed by measuring the liberation of isotopically labelled membrane compounds (Kirkpatrick *et al.* 1985). The enzymic procedures proved to be less traumatic than the mechanical ones in these experiments.

During the recent years we have been particularly interested in the role the intracellular monovalent ions may play in the mitotic regulation and cancerous growth of cells (Zs.-Nagy *et al.* 1981, 1983, 1987). When we started to extend our experiments to cloned

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cell lines, we noticed that the intracellular monovalent ion concentrations displayed unusually wide scattering in numerous cell preparations, which were identical in all aspects except that different time intervals had elapsed between harvesting and deep-freezing of the samples. Since this problem seems to be of great importance not only from the point of view of the experiments originally designed for studying the correlation between the intracellular monovalent ion concentrations and the mitotic activity, but also from a general cell-biological aspect, we decided to perform systematic measurements of the intracellular monovalent ion concentrations from 5–120 min subsequent to harvesting by collagenase digestion.

Materials and methods

Cloned Swiss/3T3 mouse cell line (Paterson Laboratories, Manchester, UK) was used. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories) supplemented with 10 % foetal calf serum (HUMAN Serobacteriological Institute, Budapest) and grown in a humidified incubator at 37 °C with 5 % CO_2 in an air/gas phase.

Exponentially growing cells were harvested by incubating in 0·05 % collagenase IA (SIGMA) dissolved in Hanks' solution for 2–3 min at 37 °C. The cells were separated from the bottom of Petri dishes by gentle shaking and then the same volume of DMEM was added.

The harvested cells were stored in suspension for 5–120 min in a humidified incubator at 37 °C with 5 % CO_2 in air/gas phase; samples were prepared at 5, 15, 30, 45 , 60 and 120 min.

Cell suspensions were centrifuged at 600 *g* for 2–3 min and 5 μl of 1 % gum arabic solution (in DMEM) was added to the pellet. This was necessary to ensure the necessary mechanical stability of the pellets during the later preparative steps. The pellets obtained this way were not compact in appearance, they were similar to a dense liquid. This dense liquid was quench-frozen by dropping it in isopentane cooled by liquid nitrogen, and prepared for energy-dispersive X-ray microanalysis by means of the freeze-fracture/freeze-drying (FFFD) technique described in detail elsewhere (Zs.-Nagy *et al.* 1977; Zs.-Nagy, 1983, 1988). The FFFD samples were further treated like the compact tissues.

The main steps of the X-ray microanalysis were the following: (1) the FFFD bulk specimens were used for X-ray microanalysis of the cells without using any coating layer, at 10 kV accelerating voltage in a JEOL JSM-35C scanning electron microscope equipped with an EDAX System F. The incident beam current was kept at 45 μA, the effective beam current in the specimen amounted to about 1–2 pA when obtaining a rate of 350–450 cts s^{-1}. The distance between the specimen and the X-ray detector was 36 mm, and the takeoff angle 28°. Forty cells were measured from each sample. Analysis time for each spectrum was 40 s. (2) Computer elaboration of the spectra according to the mass fraction method of Hall *et al.* (1973) extended for bulk specimens (Zs.-Nagy & Pieri, 1976; Zs.-Nagy *et al.* 1977; Zs.-Nagy, 1983, 1988). (3) Calculation of the monovalent electrolyte concentrations for the dry mass of the cells and also for the estimated intracellular water content.

Elemental concentrations of Na^+, K^+ and Cl^-, as well as the Na^+/K^+ molar ratio were calculated for each cell. Elemental distribution histograms were prepared for each sample and compared with those of identical post-harvest incubation time by using the χ^2 test. Since no significant differences could be detected within the groups, data belonging to each identical post-harvest time were pooled together. During the final elaboration of our data, a distribution histogram of the Na^+/K^+ molar ratio was prepared for each group and compared by the χ^2 test, using a suitable computer program.

It is obviously of great importance to exclude from the measurements all the cells having lost their viability during the preparation procedure. The viability of the cells was measured in portions of each sample before freezing by means of the exclusion criteria of the vital dye Erythrosin B in the light microscope. Fortunately, this dye contains four iodine atoms, which can easily be detected also in the X-ray spectra (L-line gives a peak at the energy of 3940 eV, excited sufficiently by 10 kV incident beam), i.e. the viability test could also be used for the FFFD specimens. This principle has already been used by others (Walker *et al.* 1984).

Results

The FFFD bulk specimen X-ray microanalytical method results in average intracellular elemental concentrations, provided the exciting electron beam is not overpenetrating the cell volume (Zs.-Nagy, 1983, 1988). Therefore, one cannot expect any high spatial resolution from this type of investigation. Nevertheless, the quality of the image is sufficient for recognition of the cell borders and the cells can be distinguished satisfactorily from the extracellular space (Fig. 1). The cells are usually not broken in the FFFD specimen; however, the breaking plane goes through the specimen so that practically no extracellular substance remains attached to the surface of the cells bombarded by the incident electron beam. This statement is supported by the fact that an iodine peak (derived mostly from the extracellular space) appears very rarely in the X-ray spectrum.

The morphology of the bulk specimens showed a characteristic time-dependent change. In samples frozen within a short time (5–30 min) subsequent to harvesting, the cells are homogeneously distributed, whereas in specimens obtained after a longer time (1–2 h) groups of 10–15 aggregated cells were frequently seen. These small cell groups were fairly well separated from each other.

The size of the harvested 3T3 cells is sufficiently large for meeting the criterion that (when using 10 kV accelerating voltage) the exciting beam should remain within the cell volume. This was fully confirmed by the

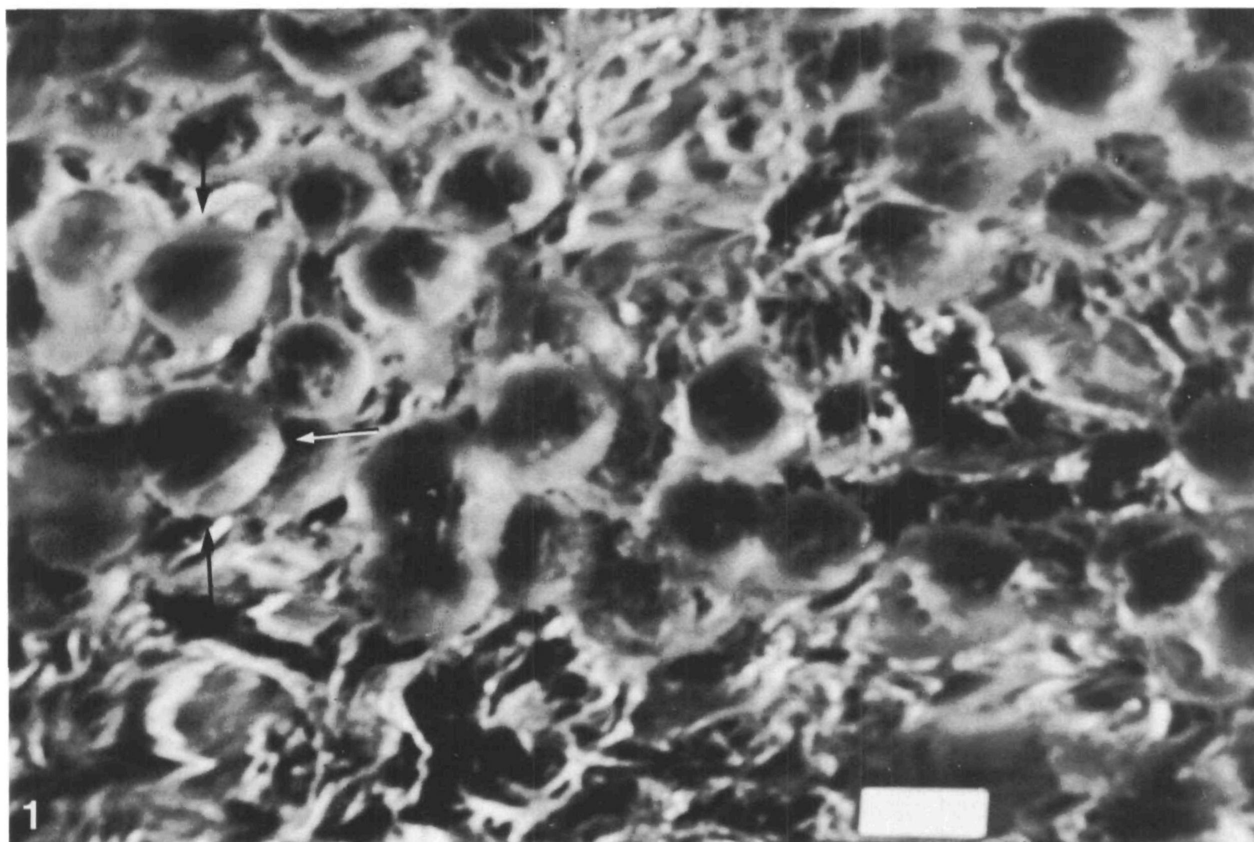

Fig. 1. Low-power scanning electron micrograph of an FFFD specimen of the 3T3 cells. Arrows indicate clearly recognizable spherical cells. Note the relatively negligible electrostatic charge of the secondary electron image. X-ray microanalysis was performed on such specimens as follows: cells were selected randomly at low magnification, then the latter was increased up to ×35 000 to ×45 000 so that an area of about 0·6 μm×1·0 μm was scanned above the central part of the cells. Bar, 10 μm.

X-ray microanalytical variant of the erythrosin-exclusion test. We obtained a very high (about 99 %) viability of 3T3 cells by light microscopy, i.e. about 1 % of the cells contained erythrosine, whereas a clearly recognizable iodine peak occurred in the FFFD samples of the same cells somewhat more frequently (in about 5 % of the cells). This difference is due to the overpenetration of certain cells by the exciting beam, since iodine is present everywhere in the extracellular space. Our observations indicate that the probability of overpenetrating occasionally flattened or otherwise irregularly shaped cells is very low (about 4 %). The use of erythrosin B as a marker of electron beam overpenetration seems to be a considerable advancement in the X-ray microanalysis of cultured cells.

Cell samples could not be prepared later than 2 h after harvesting, because the cells adhere to the wall of the centrifuge tube. Therefore, six time intervals were chosen, as indicated in the methodological description, up to 120 min; three samples were prepared and analysed at each sampling time, resulting in a total of 120 measurements per group.

Table 1 shows the mean values of the elemental concentrations of monovalent ion ratios per sampling time calculated as percentages of the dry mass. It is noteworthy that Na^+ and Cl^- contents are very high, while K^+ content is unusually low, in the 5 min sample. These values show opposite changes in the later samples: Na^+ and Cl^- contents decrease very considerably during the first 30 min and keep decreasing thereafter, meanwhile the K^+ content increases during the same time. The sum of the three monovalent concentrations displays only moderate and non-significant alterations (Table 1).

Table 2 shows the concentrations of the monovalent electrolytes calculated for the intracellular water and the Na^+/K^+ ratio. Since the intracellular water content has not been determined, we used an assumed value (75 %, w/w). It should be stressed that although differences between the actual and the assumed water contents may have an influence on the absolute values of the elemental concentrations of Table 2, they cannot abolish the very strong time-dependent changes of these concentrations. On the other hand, it should be emphasized that the Na^+/K^+ ratio is not dependent on

Table 1. *Monovalent electrolyte concentrations in 3T3 fibroblasts after various time intervals subsequent to harvesting by collagenase digestion*

Sampling time (min)	Na^+	Cl^-	K^+	Total
5	0·406 ± 0·017	1·637 ± 0·059	0·726 ± 0·039	2·769
15	0·238 ± 0·017	1·262 ± 0·071	1·388 ± 0·036	2·888
30	0·178 ± 0·013	1·129 ± 0·049	1·695 ± 0·047	3·002
45	0·135 ± 0·011	1·054 ± 0·049	1·910 ± 0·035	3·099
60	0·112 ± 0·008	0·932 ± 0·035	1·953 ± 0·030	2·997
120	0·068 ± 0·007	0·736 ± 0·033	2·065 ± 0·028	2·869

Values are average percentages of the dry mass measured in 120 cells per group ± S.E.M.

Table 2. *Monovalent electrolyte concentrations in 3T3 fibroblasts after various time intervals subsequent to harvesting by collagenase digestion*

Sampling time (min)	Na^+	Cl^-	K^+	Na^+/K^+	Total
5	58·8 ± 2·4	153·6 ± 5·5	61·7 ± 3·3	1·443 ± 0·107	274·1
15	34·6 ± 2·4	118·4 ± 6·7	118·6 ± 3·1	0·351 ± 0·031	271·6
30	25·8 ± 1·8	105·9 ± 4·7	144·7 ± 4·0	0·226 ± 0·025	276·4
45	19·6 ± 1·7	98·9 ± 4·6	163·2 ± 3·0	0·136 ± 0·013	281·7
60	16·3 ± 1·2	87·4 ± 3·3	166·8 ± 2·6	0·107 ± 0·009	270·5
120	9·9 ± 1·0	69·1 ± 3·1	176·3 ± 2·4	0·059 ± 0·006	255·3

Average values in mEq kg^{-1} intracellular water measured in 120 cells per group ± S.E.M. (estimated intracellular water content: 75 % w/w).

the actual water content, i.e. the values given in Table 2 are valid molar ratios at any level of hydration and characterize the resting membrane potential of the cells.

Fig. 2 shows the distribution histograms of the Na^+/K^+ molar ratios. These data reveal that harvesting causes a considerable and rather long-lasting depolarization of the cell membrane, due most probably to the usual ionic mechanisms (i.e. influx of Na^+ and efflux of K^+). Since the viability tests (and even eventual further culturing experiments) show that the cells remain practically fully viable, one can assume that the reconstitution of the intracellular monovalent ion composition takes place by the known cell membrane functions, i.e. by active pumping out of Na^+ against uptake of K^+.

Discussion

It has been postulated that sustained depolarization of the cell membrane involving intracellular monovalent ionic changes may have a regulatory role in mitogenesis (Cone, 1971). This problem and relevant experiments and measurements have been extensively reviewed (Rozengurt & Mendoza, 1986; Geering, 1986; Villereal, 1986). One of the most direct and relevant methods for following intracellular monovalent ionic changes is quantitative electron-probe X-ray microanalysis (Gupta & Hall, 1981; Hall & Gupta, 1984; Zs.-Nagy, 1983, 1988).

Owing to a great number of technical difficulties related to the cryosectioning method, the FFFD bulk-specimen X-ray microanalysis (Zs.-Nagy *et al.* 1977; Zs.-Nagy, 1983) seems to be one of the most practical approaches for measuring average intracellular elemental concentrations in a great number of cells within a reasonably short time. This method, with some small technical improvements (like the addition of gum arabic to the cell suspension before freezing as well as the use of X-ray microanalytic erythrosin B test), is sufficiently reliable also for cultured cells behaving quite similarly to bulk specimens prepared from compact tissues.

Several authors have mentioned the observation that very marked intracellular monovalent ion changes occurred when cells were isolated from each other (Quistorff *et al.* 1973; Barnabei *et al.* 1974; Kendall *et al.* 1985; Warley, 1986) or from a Petri dish (Norrie *et al.* 1982; Warley *et al.* 1983*a*,*b*). Unfortunately, nobody has studied this problem systematically, although the available data suggest that isolating cells from, e.g., Petri dishes or from each other causes a reversible increase of the intracellular Na^+/K^+ ratio. Our measurements revealed that the phenomena mentioned above most probably relates to a sustained

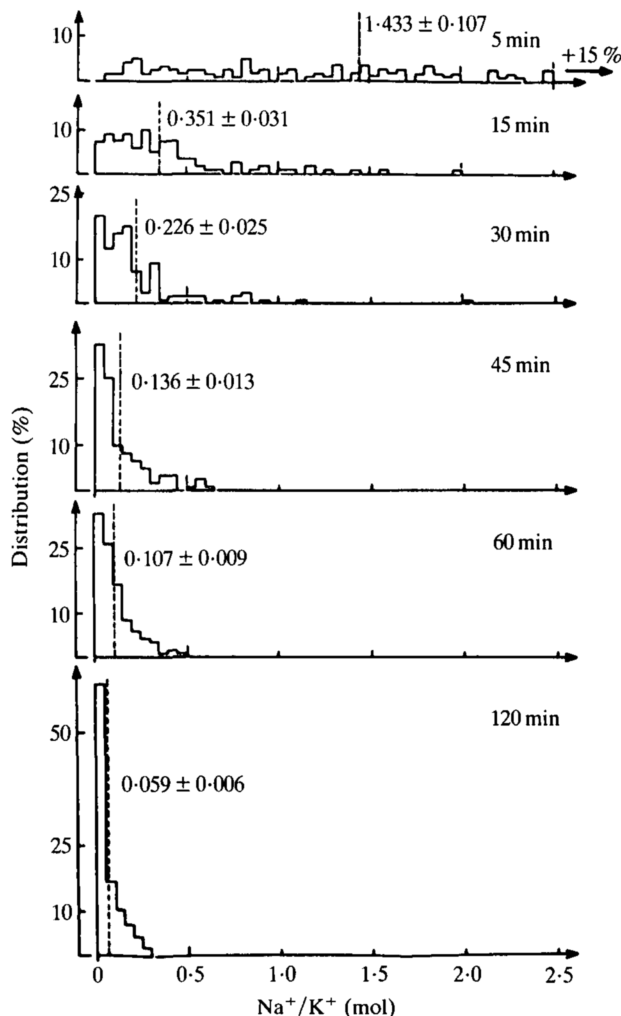

Fig. 2. Distribution histograms of the Na^+/K^+ molar ratios at various sampling times as indicated. Perpendicular broken lines indicate the average value (±S.E.M.) of 120 cells measured in three samples at each time. The histogram scale is 0·05 units per class. The arrow on the right of the 5 min histogram indicates that 15 % of the values were even greater than 2·5.

depolarization process in each type of cell studied, which was provoked by the harvesting procedure, and which is restored during the first 2 h. Such a change in the membrane status of the harvested cells must be taken into consideration in all types of experimental approaches where ion compositions or membrane functions, as well as other phenomena depending on them, are being studied.

Although we do not know much about the processes that occur during the first 5 min after harvesting, one can list some data that may be relevant to the mechanism of the rapid Na^+ and Cl^- influx, and K^+ efflux, caused by harvesting. For example, an important step during the initial processes may be a decrease in the activity of Na^+,K^+-ATPase, the structural configuration of which is altered by damage to membrane phospholipids with a consequent decline in enzymic activity, as shown by Demediuk *et al.* (1985).

According to Binggeli & Weinstein (1986), sodium channels and gap junctions may be involved in growth initiation and inhibition. Though no direct evidence is available, on the basis of this theory one can suppose that during the adhesion of cells to Petri dishes a determined distribution of the sodium channels comes into being, which is altered by harvesting so that the cell membrane becomes considerably more permeable to sodium (i.e. it becomes depolarized) than during the normal resting state. Although considerable differences can be assumed between the effects of enzymic and mechanical harvesting procedures, the phenomena related to 'scrape-loading' (McNeil *et al.* 1984; El-Fouly *et al.* 1987) deserve further, special interest.

Apart from their methodological significance, the time-dependent changes in the intracellular monovalent ion contents after harvesting may be of importance from a general cell-biological point of view. For example, further studies may use this phenomenon as a model for (1) obtaining detailed information about the mechanisms producing sustained depolarization of the cell membrane, and (2) analysing the regulatory role of the cell membrane in metabolism and mitotic activity.

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