QUANTITATIVE LOCAL X-RAY SPECTRAL MICROANALYSIS OF SINGLE ERYTHROCYTES

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The use of x-ray spectroscopy to study the inorganic composition of cell suspensions is limited mainly by imperfections in the method of preparing the specimens. The choice of backing for microanalysis must take into account the need for its high electrical and thermal conductivity and also minimal background radiation; the method of preparation of the specimens, moreover, must ensure preservation of the ionic composition of the cells and the possibility of independent analysis of single cells. Local x-ray microanalysis with dispersion by wavelength was first used to study the content of elements in erythrocytes during maturation by Kirk et al. [4]. A defect of this method is the impossibility of recording all elements simultaneously. This drawback is eliminated by the use of x-ray power spectroscopy for these purposes.

The aim of this investigation was to devise a quantitative method of determining the content of inorganic elements in isolated cells by local x-ray spectral microanalysis with power dispersion.

EXPERIMENTAL METHOD

Freshly isolated erythrocytes from healthy human peripheral blood were used. To separate plasma from erythrocytes the blood was centrifuged 3 times in 0.1 M mannitol, buffer with 20 mM Tris, and titrated with acetic acid to pH 7.4 at 0-2°C. Films were then made on electron-microscopic grids with a large hole ("blinds") covered with Parlodion, and dried in air. The site of analysis was located in a scanning electron microscope in secondary electrons. Spraying with metals and carbon was not used. X-ray microanalysis was carried out on a "KEVEX" 5,100 spectrometer (USA), based on a "Hitachi" S-500 electron microscope (Japan), with the aid of a special carbon adapter, so that cells could be analyzed on an ultrathin backing, making virtually no contribution to background radiation, and with a consequent increase in the signal to noise ratio. The area of scanning was chosen so that the cell was completely covered.

For quantitative analysis of the experimental data, calibration curves plotted with the aid of standards were used. To prepare the standard solutions a 20% solution of bovine serum albumin was used, with the addition of 5% glycerol as cryoprotector, and known quantities of the sales NaCl and KH_2PO_4 were dissolved. Next, small drops of standard solutions were frozen and cut on a freezing microtome at −25°C. Sections 5 μ thick were mounted on the "blinds" and dried in the frozen state. Sections of the standards were analyzed within concentration range of 40-800 meq/kg dry weight at 5 points. The spectra thus obtained were analyzed with allowance for Hall's assumption [3], that the dry weight of the specimen is proportional to the background in the region of energies with no characteristic peaks, and which is chosen a priori (4.50-5.00 keV). The mass fractions for each element were calculated by the following equation:

$$A_x = (I - I_x) I_b,$$

where A_x is the mass fraction of element x, I the complete integral of the peak of element x, I_x the integral of the background beneath the peak of elemeent x, and I_b the integral within the energy range 4.50-5.00 keV.

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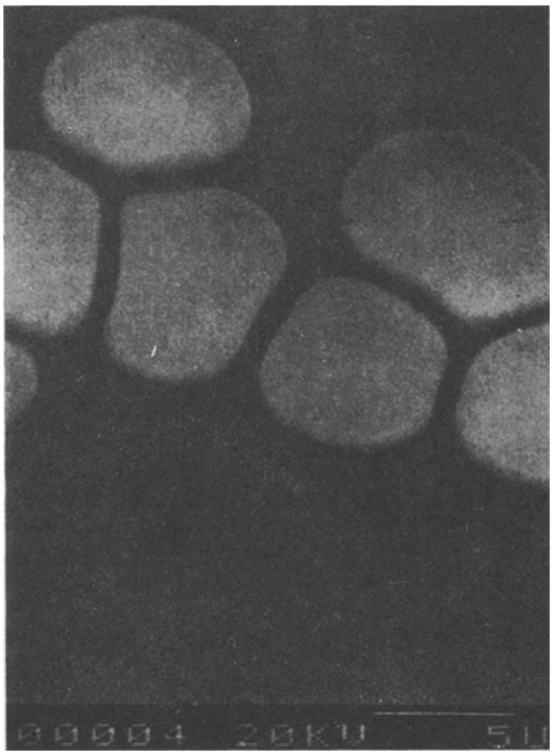

Fig. 1. General view of human peripheral blood erythrocytes in 0.1 M mannitol solution, prepared for x-ray microanalysis. Scanning electron microscopy, preparation not sprayed. Magnification, 2800×.

The calibration curves thus obtained were analyzed by the method of least squares, and coefficients of correlation and linear regression equations were calculated.

EXPERIMENTAL RESULTS

By x-ray power spectroscopy it was possible to study the content of the elements sodium, potassium, phosphorus, and chlorine in single erythrocytes. The intensity of the characteristic lines for these elements was sufficient for semiquantitative analysis of the signals. The density of the suspension was chosen so that single cells did not overlap each other, and they could be analyzed independently (Fig. 1). For each cell chosen for analysis, simultaneous recording of the elements was thus possible. After mathematical analysis of the results obtained with standard solutions, coefficients were caluclated for linear regression equations, whose appearance was as follows.

$$C_{Na} = 4399.73\, A_{Na} + 78.48,$$
$$C_{Cl} = 275.02\, A_{Cl} + 7.37,$$
$$C_{P} = 586.36\, A_{P} - 37.74,$$
$$C_{K} = 329.01\, A_{K} - 67.83,$$

where C denotes the concentration of the corresponding element (in meq/kg dry weight), and A the mass fraction of the corresponding element.

The calibration curves had the appearance shown in Fig. 2. To study loss of the elements during washing of the erythrocytes to remove the external medium (plasma) they were incubated in a solution of 0.1 M mannitol at 0-2°C. It was found that if the erythrocytes were allowed to stand for 3 h in the cold no loss of the elements sodium, potassium, chlorine, and phosphorus took place (Table 1). Comparison of the concentrations of elements obtained in these investigations with the results of other studies [1] led to the conclusion that the elements are well preserved in erythrocytes during washing and air-drying of the films. However, a considerable and significant decrease in the chlorine content in these experiments must be

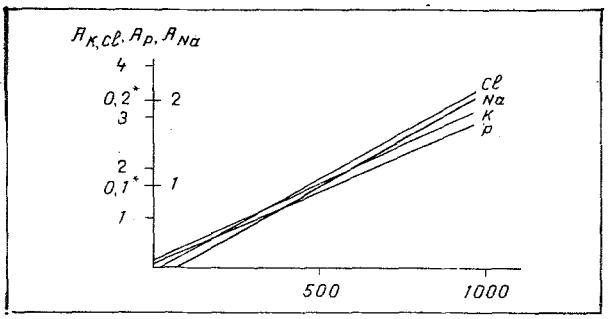

Fig. 2. Calibration curves for elements sodium, phosphorus, chlorine, and potassium (coefficients of correlation 0.91, 0.97, 0.92, and 0.99, respectively). Abscissa, concentration of elements (in meq/kg dry weight of specimen); ordinate, mass fractions of elements.

TABLE 1. Content of Elements in Healthy Human Peripheral Blood Erythrocytes (in meq/kg dry weight)

Elements	Control erythrocytes	Experiments		
		to study action of 10^{-7} M ouabain for 15 min	after standing for 3 h at 0-2°C	of fixation with 2.5% glutaraldehyde
Sodium	236,84±17,60	188,47±8,80*	206,07±83,53	Not determined
Chlorine	43,12±3,58	32,12±1,93*	34,87±1,38	» »
Potassium	428,98±20,73	27,58±8,23**	382,91±10,53	» »
Phosphorus	97,12±4,28	102,99±11,14	97,12±6,45	» »

Legend. *P < 0.05, **P < 0.001.

mentioned. This was evidently due to the very high rate turnover of chlorine ions in erythrocytes [2].

To modify the internal elementary composition of the erythrocytes, treatment with valinomycin, a specific potassium ionophore [5], and fixation with 2.5% glutaraldehyde solution in 0.1 M phosphate buffer was used. In these experiments incubation of erythrocytes in Krebs' physiological saline in the presence of 10^{-7} M valinomycin at 37°C for 15 min led to the outflow of potassium ions from the erythrocytes (the potassium level under these circumstances was 6.4% of its initial value), and the erythrocytes themselves underwent conversion into echinocytes. Analysis of erythrocytes fixed for 2 h showed that the characteristic peaks of the elements sodium, phosphorus, chlorine, and potassium were not differentiated from the background below them (Table 1).

The results are evidence that power-dispersion x-ray microanalysis can be successfully used to determine the content of inorganic elements and ions in isolated cells. The method described above may be suitable for research under expedition and clinical conditions and it permits different subpopulations of cells to be studied without preliminary separation, which under certain circumstances (for example, if one particular subpopulation is extremely small or it cannot be separated by centrifugation), may prove to be of decisive importance during the choice of a method of investigation.

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