A STUDY OF THE APPLICABILITY OF THE ELECTRON MICROPROBE TO A QUANTITATIVE ANALYSIS OF K AND Na IN SINGLE HUMAN RED BLOOD CELLS

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1. Introduction

Conventional analytical procedures only permit the determination of average concentrations of cell constituents in many cells. In contrast, the electron microprobe allows one to measure the amounts of the various elements present in individual cells. However, the application of the microprobe encounters the difficulty that during the preparation of the cells for analysis uncontrolled losses of diffusible cell constituents such as K and Na may take place [1, 2]. In the present paper an attempt was made to design a technique which minimizes such losses. A standardization procedure was applied to assess the success of the preparation procedure. This involved the preparation of cells of varying K and Na contents and a comparison of the averages of the K and Na *X*-ray intensities as measured in many single cells by means of the electron microprobe with the average K and Na contents of the population as determined by flame photometry.

2. Materials and methods

The specimens for microprobe analysis were prepared as follows: Human red cells were loaded with various concentrations of K and Na by incubation in the presence of either p-chloromercuribenzene sulfonate (PCMBS) [3] or nystatin [4] in isotonic media containing varying proportions of KCl and NaCl, the sum ($K^+ + Na^+$) being maintained at 150 mequiv. To avoid colloid osmotic hemolysis, 20 mM sucrose was present. After treatment the cells were first washed 3–4 times in their respective incubation media without nystatin or PCMBS and then 3 times in 150 mM LiCl solution containing 20 mM sucrose. After PCMBS treatment, during the first wash 0.1 mM cystein was present. Determinations of Na and K were done by flame photometry. The results are expressed in mM/kg of the cell weight existing prior to the loading procedure.

For the preparation of specimens for microprobe analysis celloidin films were made from a celloidin solution in isoamyl acetate (0.05% w/v). A clean slide was dipped into the solution, pulled out, and air-dried at room temperature over night. During drying the slide was tilted to an angle of about 30°. Subsequently, the film covered slide was gently moved through the surface of a dilute suspension of cells in the LiCl medium (about .1% w/v). Upon immersion of the slide, the film became detached and floated on the surface. Thereafter a beryllium block with a polished face uppermost (8 × 6 × 6 mm) was introduced underneath the film and lifted through the surface. This led to sandwiching the cells between the polished surface of the block and the celloidin film. The preparation was quickly frozen by contact with dry ice and dried under vacuum at −40°C. After drying, the temperature was raised to a little above room temperature, and the vacuum was released. After freeze-drying, the celloidin film no longer formed a coherent layer on top of the cells. In many places the film was disrupted and the cells were

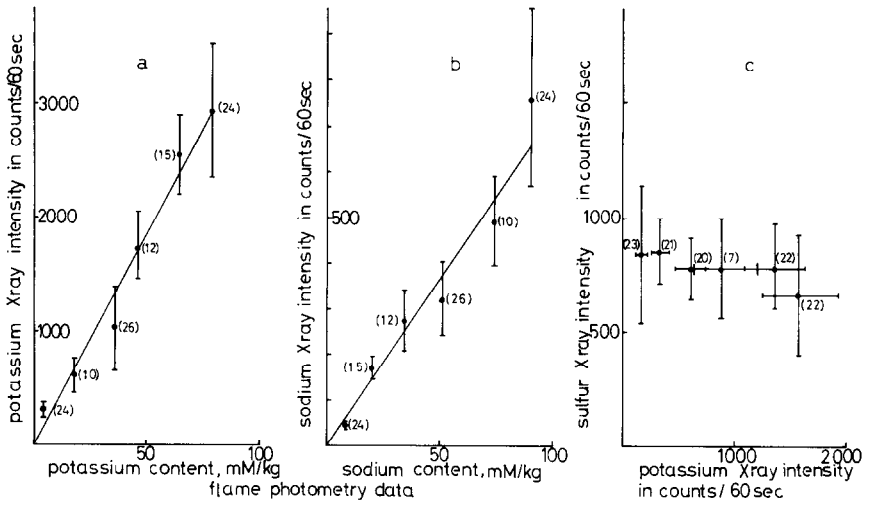

Fig. 1a, 1b, 1c. Averages of K and Na *X*-ray intensities from individual cells plotted against average contents of K or Na determined by flame photometry. The intracellular K and Na concentrations were varied as described in the text. In this and in other figures standard deviations of *X*-ray intensities are represented by bars and the numbers in brackets refer to the numbers of cells investigated. The straight lines are calculated by the method of least squares. In fig. 1c. Ordinate and abscissa represent *X*-ray intensities of *N*a and K respectively, as obtained by averaging the signals from individual cells. K was measured with the crystal PET 4 whose sensitivity is one half the sensitivity of the crystal PET 3 which had been used for measuring Na.

exposed. Among the exposed cells, those chosen for microprobe analysis were not in contact with each other and were of normal shape. Cells which rested underneath the still continuous layer of celloidin showed no detectable sodium signal and, for this reason, were disregarded.

The electron microprobe analyzer used was a Cameca MS 46. Two elements could be measured simultaneously: S and K by means of 2 pentaerythritol crystals (PET 3 and PET 4, respectively) and Na and K by means of a potassium acid phtalate crystal (KAP) and the PET 3. The accelerating voltage was 12 kV and the current 120 nA. The diameter of the beam was slightly larger than that of the red cells (8–12 μ). The use of beryllium as a support for the cell preparations ensured a low background; since its conductivity for heat and electrons is high, no coating with carbon was necessary. Background was measured on the exposed beryllium close to the cells. The readings on any single cell were independent of time.

3. Results

Fig. 1a and 1b shows that there exist linear relationships between K or Na contents as measured by flame photometry and the mean values of net counting rates from individual cells as obtained in the electron microprobe. In the same collection of cells, there were no significant variations of the sulfur contents (fig. 1c). This indicated that there exists no relationship between the experimentally induced variations of the Na and K contents and the total protein contents of the cells.

Each standard deviation in fig. 1a and 1b represents an overall error which comprises statistical variations of counting rates, uncontrolled changes of cation composition due to the manipulations during specimen preparation, and biological variations. In the Na determinations, the counting rates were low and at the lowest concentrations the statistical counting error was of the same magnitude as the total error. However, at the higher Na concentrations, the total error exceeded the statistical counting error by a factor of 3. More obvious were the results with K: the total error exceeded the counting error at least 3 times at the lowest K contents and up to 10 times at the highest K contents. This indicated that sample preparation or biological variations or both significantly contributed to the observed scatter.

Biological variations could consist in variations of cell volume. Electron microprobe data as represented in fig. 1a and b refer to quantities which are proportional to amounts of K or Na per cell. The equilibration procedure in nystatin- or PCMBS-containing media leads to the adjustment of the intracellular concentrations. Hence cells with equal concentrations of K or Na but different volumes will give different *X*-ray intensities. This source of variability can be eliminated as follows: using the slopes of the curves in fig. 1a and 1b, the net intensities can be related to amounts of K and Na inside each single cell. If one assumes that during exposure to nystatin or PCMBS the cells are at osmotic equilibrium, then for each cell the sum (K + Na) should be roughly proportional to its water content; the molar fractions Na/(Na + K) or K/(Na + K) (the latter is identical to 1 − Na/(K + Na)) as calculated from the electron microprobe data should be identical with the molar fractions in the corresponding incubation media. Fig. 2 shows that the data points recalculated by this procedure are in fact close to the identity line, and that the statistical variations are very much smaller than in fig. 1a and b. The slight systematic deviation of these data points from the identity line suggest that after equilibration at zero Na concentration in the medium the cells still retain some Na.

The data points represent averages of determinations in many cells. To demonstrate the behavior of the individual cells, we plotted for each cell the estimate of the molar fraction Na/(Na + K) against the corresponding sum (Na + K). Fig. 3 shows that relatively large variations of (Na + K) are accompanied by much smaller variations of Na/(Na + K). The findings in this figure as well as those in fig. 2 strongly suggest that the observed variations between the individual cells do not primarily represent artifacts but are largely of biological origin.

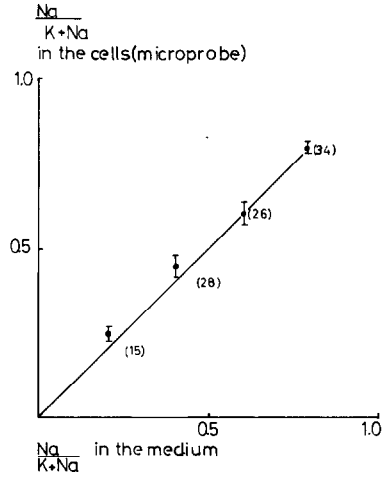

Fig. 2. Molar ratios Na/(K + Na) deduced from electron microprobe data as function of the molar ratios in the incubation media with which the cells were equilibrated prior to analysis. The straight line represents identity.

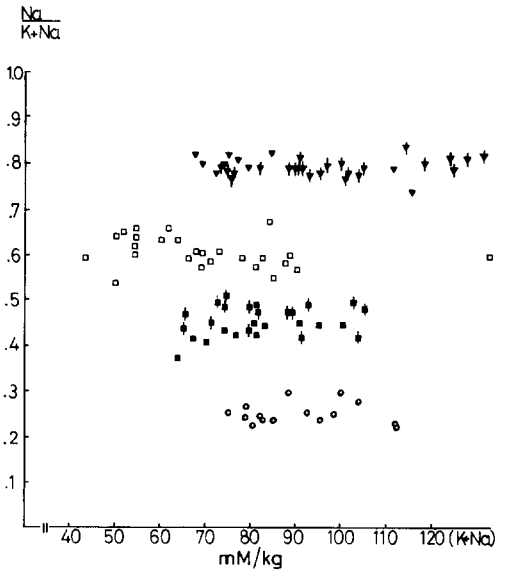

Fig. 3. Determinations of $\frac{Na}{K + Na}$ in individual cells plotted against the corresponding values of (K + Na). The molar ratios were formed after converting the *X*-ray intensities into quantities which are proportional to the Na and K content, respectively. The conversion was achieved by means of standard curves such as those in fig. 1a and 1b. The different symbols refer to the various incubation media used to vary the intracellular K and Na contents (see fig. 2). Each pair of symbols ▼ and ▼ or ♦ and ■ refers to experiments done under identical conditions at different times. Data points represented by symbols without a bar are from the experiment depicted in fig. 1.

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