Determination of the electrophoretic mobility of chromosomes by free flow electrophoresis I. Morphology and stability

Isolated metaphase chromosomes of several fibroblastoid cell lines (Chinese hamster, Chinese hamster x human hybrid) were subjected to free flow electrophoresis (FFE) to study their electrophoretic mobility (EM). The morphology and stability of the chromosomes were unaffected by FFE as examined by cytogenetic methods and flow cytometry. The chromosomes of the complement all showed similar EM under most of the conditions applied. At neutral pH the EM of the chromosomes had the same sign as free DNA and about 2/3 of its magnitude. The variation of EM with buffer parameters such as ionic strength, valence of counterions, buffer capacity and dielectric constant of the solvent were investigated. Thermal denaturation increased the EM of the chromosomes by 20%. Partial denaturation might offer a possibility to separate or enrich large amounts of chromosomes by FFE.

1 Introduction

Free flow electrophoresis (FFE), as developed by Hannig [1], is commonly used to separate cells, cell organelles or macromolecules (for reviews see [2,3]). Under the influence of electric forces, such biological particles show a characteristic electrophoretic mobility (EM) depending on their charge density and the physicochemical conditions of the buffer used. According to different EM, particles can be separated electrophoretically. The carrier free system FFE has the following characteristics: (i) the electrophoresis buffer streams vertically through a separation chamber with a typical thickness of about 0.5 mm; (ii) the homogeneous electric field is arranged perpendicularly to the flow direction of the buffer; (iii) the sample is introduced to the chamber parallel to the streaming direction and flows continuously through the electric field (zone electrophoresis). A horizontal window in the lower part of the chamber may allow observation of the sample movement with a scanner that measures the extinction at the required wavelength or light scatter intensity.

Chromosomes consist of negatively charged DNA and a variety of differently charged proteins with atypical composition of about 30% DNA, 65% proteins and 5% RNA according to Nagl [4]. The protein composition of the metaphase chromosomes depends on the preparation method [5,6]. In this article the influence of FFE on the stability and morphology of chromosomes under a variety of FFE conditions was investigated. The influence of the different parameters determining EM was tested. To our knowledge this is the first time that chromosomes were successfully subjected to FFE.

2 Materials and methods

2.1 Isolation of chromosomes

All cell culture reagents were purchased from Serva (Heidelberg, FRG). Cells of the Chinese hamster cell line CHL and the Chinese hamster x human hybrid line ADA 13SC3 (kindly provided by P. Pearson, Leiden) were cultivated in Ham's F10 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (10000E/10000 µg/mL) in a humidified CO2 incubator (5%) at 37°C. Mitotic cells were collected overnight by a Colcemid-block (0.25 µg/mL medium) and harvested by shake-off. The mitotic cells from 10 culture flasks (175 cm² each) were pelleted by centrifugation (350 g; 15 min), chilled for 2 min at -20°C and transferred to 4°C. After 10 min the chromosome pellet was suspended in a hypotonic solution (10 mM Tris, 5 mM CaCl2, 10 mM NaCl, pH 7.5) and incubated for 15 min at room temperature. The hypotonic solution was removed by centrifugation and the chromosome pellet resuspended in 2 mL hexandiol buffer (0.75 M 1,6-hexanediol, 25 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 5 mM CaCl2, final pH adjusted to 3.2 with concentrated HAc), a modification of the hexylene glycol isolation buffer described by Stöhr and co-workers [7,8]. In the same buffer the mitotic cells were sonicated to obtain a suspension of dispersed chromosomes. The yield was between 1010-1011 chromosomes. This suspension was centrifuged at 400 g for at least 10 min. All reagents were of analytical grade.

2.2 FFE of chromosomes

The hexandiol buffer was removed and the...
chromosome pellet was resuspended in 5 mL Tris/HAc, pH 7.2, electrophoresis buffer containing different salts in different concentrations (see Section 3.) and 0.005 % Triton X-100. FFE was performed in an ElphorVap 21 (Bender & Hobein, Munich) in a chamber of 0.7 mm thickness and 250mm length, with an electrode distance of 100 mm and a field strength of 50-100 V/cm. The electrode buffer consisted of 50 mM Tris/HAc, adjusted to pH 7.2 ($\sigma = 2.4$ mS/cm), or 100 mM Tris/HAc, if the conductivity of the chamber buffer exceeded 1 mS/cm. For higher stability of the electrophoretic process, the electrode buffer was also rinsed along the membranes inside the separation chamber, reducing the effective width of the chamber to 80 mm. Unless stated otherwise, the temperature was adjusted to 5.1 °C. The sample flow rate was 1.7 mL/h, resulting in a 125 s field exposure time of the chromosomes. The minimum sample volume for one analytical run was 50 µL with a density not exceeding $5 \times 10^7$ chromosomes per mL, to avoid aggregation during electrophoresis. At the bottom of the separation chamber the absorbance at 280 nm was scanned up to 50 times parallel to the electric field, i.e. perpendicular to the streaming direction of the buffer. Scanning was performed with a high resolution scanning device constructed in our laboratory [9]. Seventy fractions were collected at intervals of 1.1 mm between the fractions. The scanner indicated the fractions containing the chromosomes and their EM was determined from the scanning profile. Since it was not possible to measure the effective field strength directly in the separation chamber, EM was determined indirectly as $EM = 175 \cdot d \cdot \sigma / t \cdot I$.

2.3 Microscopy and How cytometry

Chromosomes in the collected fractions were examined by microscopy and flow cytometry. Five µL of each fraction were dropped onto a microscopic slide and either stained by adding 5 µL of a 15 µM 4,6-diamidino-2-phenylindole (DAPI) solution in distilled water for examination by fluorescence microscopy (Orthoplan, Leitz) or air dried and G-banded [10].

In some experiments propidium iodide (PI) was added (7.5 mM in distilled water) to the electrophoresis buffer containing the chromosome suspension immediately after FFE to stabilize the chromosomes for flow cytometry (final concentration. 75 µM PI). For flow karyotyping [11,12,14] the chromosomes were stained with 5 µM DAPI. Flow cytometric measurements were performed on a FACS IV cell sorter (Beeton Dickinson) and an EPICS V cell sorter (Coulter Electronics). To excite the fluorochrome a krypton nm multiline) or an argon ion laser (EPICS V) set at 50 mW (351.1-363.8 nm multiline) was used. The measured flow histograms were smoothed slightly using a "k-nearest neighbor" procedure [13,14]. Background was corrected according to the $\chi^2$-method described by Dölle [13,15,16]. The peak positions and standard deviations were determined by an interactive procedure fitting Gaussians into each peak of the flow karyogram ([13,14] and Dölle et al., submitted).

3 Results

3.1 Morphology and stability of chromosomes after FFE

Isolated metaphase chromosomes were subjected to FFE in different buffers of low ionic strength, followed by analysis of the collected samples by microscopy and flow cytometry.

Fig. 1 shows a fluorescence micrograph of DAPI-
Figure 3. Flow cytometric measurements of Chinese hamster metaphase chromosomes (CHL) stained with 5 µM DAPI. Ordinate: Particle frequency, abscissa: logarithm of the DAPI fluorescence intensity. (a) Chromosomes isolated by the standard procedure [7,8] without FFE; only unfractionated chromosomes from a single culture flask were used and measured in hexandiol buffer, (b) Chromosomes of the dense sample before FFE after resuspending in the electrophoresis buffer and stabilizing with 75 µM PI. (c) The same chromosome sample as shown in (b) after FFE from a single fraction. Electrophoresis was performed as described in Fig. 1. After electrophoresis PI was added for stabilization to a final concentration of 75 µM.

stained chromosomes after FFE. The chromosomes exhibit a slight DAPI banding pattern and can be identified. G-banded chromosomes of the same also possible to subject chromosomes to a flow cytometer after FFE. In this technique a suspension containing the chromosomes is injected coaxially fraction are shown in Fig. 2. A G-banding pattern can be obtained from chromosomes after FFE. It is into the sheath fluid stream of a flow cell. By means of hydrodynamic focusing the chromosomes are confined within the core of the stream. For each chromosome the fluorescence emission is detected by photomultipliers during the few microseconds the particle needs to traverse the focal volume. The photomultiplier signal is logarithmically integrated, digitized and registered in a multichannel analyzer. Improved FFE separation of cells with flow cytometry was shown by Bauer and Kachel [29].

The chromosomes collected in different fractions after FFE were analyzed by flow cytometry (FACS IV). In Fig. 3 flow karyotypes of the Chinese hamster cell line (CHL) are shown. A typical flow karyotype of isolated chromosomes from mitotic cells of one culture flask only is given in Fig. 3 a. However, a dense chromosome suspension obtained from pooled mitotic cells of several flasks resulted in a lower resolution of the flow karyotype and peak broadening [18]. In Fig. 3b and 3c the flow karyotypes of a dense chromosome suspension before and after FFE, respectively, are shown. The two flow karyotypes are similar due to peak position. After electrophoresis (Fig. 3c) the typical peak pattern of the flow karyotype of the cell line (Fig. 3a and b) is still visible. The coincidence of the peak positions is almost complete (Table 1). Chromosomes were found in up to 5 adjacent fractions, depending on the FFE conditions, corresponding to a variability of the electrophoretic mobility of about 15%. The chromosomal composition was similar for all fractions, as verified by fluorescence microscopy (DAPI-staining). The flow cytometric analysis of adjacent fractions exhibits similar values in the fluorescence distributions of different fractions (Fig. 4c-f), indicating that differences in EM of the chromosomes are not correlated to DNA content or size of the chromosomes.

Fig. 4 shows flow histograms of the Chinese hamster x human hybrid cell line ADA 13 SC3, analyzed on an EPICS V sorter. A considerable reduction of background compared to the sample before FFE (Fig. 4b) was obtained. The "purification" can be shown after subtraction of a χ²-distribution for the background of the histogram. For this the ratio \( r = c/(t-c) \) is determined, where \( c \) is the number of chromosomal counts and \( t \) the total number of counts. The karyotype before FFE has a
The ionic strength of the carrier medium is defined as $I = \frac{1}{2}(c_1 z_1^2 + c_2 z_2^2)$, where $c_1$ and $c_2$ are the concentrations of the ions and $z_1$, $z_2$ the valences, respectively. Since the effect of the buffer alone was found to be linear, it can be neglected here. The EM behavior with increasing salt concentration was studied for both NaCl and CaCl$_2$; a "jump" near $I = 10$ mM was observed. A "jumping" tendency was observed also for trivalent cations such as spermidine and CO(NH$_3$)$_6$$^3+$. However, the transition point expected from the ionic strength to be in the range of 2 mM for trivalent cations occurred at much lower concentrations: about 120 $\mu$M for spermidine and about 10 $\mu$M for Co$^{3+}$ (if any). Addition of spermidine was correlated with a nonlinear change of conductance of the buffer, indicating an interaction between buffer ions and spermidine.

### 3.4 Effect of thermal denaturation on the EM and the stability of chromosomes

Thermal denaturation of the chromosomes prior to FFE was found to significantly alter the EM of the chromosomes (Fig. 8a-c and Table 3). The average EM of the thermally denatured chromosomes increased about 15% compared to native chromosomes. Mixing of native and denatured chromosome samples resulted in an average EM between the values of the pure native and the pure denatured fractions. The half width of the scanning profile of the mixed chromosome suspensions was increased about 20% after denaturation compared to chromosomes treated with acridine orange alone. Similar results were obtained by a combined treatment of chromosomes with acridine orange and thermal denaturation (Fig. 10). The EM was increased about 20% after denaturation compared to chromosomes treated with acridine orange alone. A mixture of both showed an electrophoretic separation of denatured and native chromosomes. The applied G-banding procedure resulted in a homogeneous staining of the chromosomes without any detectable banding pattern. The flow cytometric analysis (DAPI-staining) showed smearing of histograms, which made a clear correlation of peaks to chromosomes impossible. Similar results were obtained by a combined treatment of chromosomes with acridine orange and thermal denaturation (Fig. 10). The EM was increased about 20% after denaturation compared to chromosomes treated with acridine orange alone. A mixture of both showed an electrophoretic separation of denatured and native chromosomes.

### 4 Discussion

The EM of isolated metaphase chromosomes in suspension was determined by FFE in buffers of low ionic strength, making it possible to maintain a monodispersed chromosome suspension during the separation process. In other buffer systems e.g. hexandiol buffers of different pH values (see Section 2) or triethanolamine buffer, the chromosomes tended to aggregate or deteriorate in the electrophoresis chamber. Under appropriate conditions the morphology of the chromosomes was obviously unaffected by FFE. The chromosomes could still be identified by classical cytogenetic methods as shown in Figs. 1 and 2. The flow karyotypes in Figs. 3 and 4 show that it is possible to analyze the chromosomes in a flow cytometer after they had been subjected to FFE. The stability of the chromosomes required for flow cytometry was
Figure 4. Flow cytometric measurements of chromosomes of the Chinese hamster x human hybrid cell line ADA 13 SC3 stained with 5 µM DAPI. Ordinate: particle frequency; abscissa: logarithm of DAPI fluorescence intensity. (a) Flow karyotype of chromosomes isolated from one culture flask before FFE, measured in hexandiol buffer, (b) Flow karyotype of another dense sample of the same cell line from 10 culture flasks with rather high background before FFE but transferred to electrophoresis buffer before measurement, (c) to (f) Four adjacent fractions after FFE taken from the same suspension as in (b). FFE conditions as described in Fig. 1. In this experiment no PI was added before or after the run. Background was not corrected in the karyograms of (b) to (f) to show the loss of background during FFE. Corresponding structures of the flow karyograms are indicated by numbers.

Figure 5. Change of EM of chromosomes (CHL) with buffer capacity. Range of interest indicated by arrow (<—>); in this area a linear approximation is reasonable.

Figure 6. EM of chromosomes (CHL) with varying ethanol content of the FFE buffer.
increased by addition of PI after FFE, an intercalator known to stabilize chromosomes [21]. However, this was shown to be unnecessary if flow karyotyping is performed immediately after FFE (Fig. 4c-f). The flow karyotypes after FFE correspond quite well to the distributions obtained from freshly prepared chromosome suspensions (Figs. 3b, c and 4b, c, d and Table 1). The entire flow karyotype was still present in one fraction after FFE. Furthermore, the mean values of analogous peaks were almost identical before and after FFE.

Figure 7. Dependence of EM of chromosomes on ion concentration and valence. The concentrations are drawn on a logarithmic scale on the abscissa. (a) The chamber buffer consisted of 10 mM Tris/HAc pH 7.18, for NaCl (o) and 20 mM Tris/HAc, pH 7.18, for CaCl₂ (•). (b) Trivalent cations in a buffer containing 10 mM Tris/HAc, pH 7.18, and 1 mM NaCl in the case of spermidine (o) and no NaCl in the case of Co(NH₃)₆Cl₃ (•).

Figure 8. FFE-scanning profiles of Chinese hamster chromosomes showing the extinction at 280 nm on the ordinate and the fraction number on the abscissa. Electrophoresis was as described in Fig. 1. The cursor at fraction 48 represents the inlet point. (a) Untreated chromosomes; the cursor indicates the peak position at fraction No. 31.2. (b) An aliquot of the same sample after denaturation at 100 °C for 5 min; left cursor is at No. 28.7. (c) Two aliquots of the same sample as in (a) and (b) mixed immediately before electrophoresis.
Figure 9. Fluorescence micrograph of Chinese hamster chromosomes after thermal denaturation and FFE stained with 75 µM PI. The chromosomes were fixed on the microscopic slide by ethanol, after the thermal treatment the morphology of the chromosomes is well preserved. According to the centromeric index the chromosomes are (from left to right): No. X, 10, and probably No. 6 in a highly condensed state.

Since the same experimental setup of the flow cytometer (laser power, sheath pressure, photomultiplier potential, gain) was used for all measurements of one experiment, the following conclusions can be drawn: (i) No loss of DNA occurs during FFE within an experimental error of 2 %. (ii) All chromosome types have nearly the same EM and thus a similar charge density in spite of large differences in DNA sequence and morphology. The EM of native chromosomes was found to vary about 15 to 20 % (Fig. 8a; Table 2, first two columns). This indicates differences of chromosome structure in the suspensions investigated which were not correlated with differences in DNA sequence or morphology as visualized by cytogenetic methods. Similar results can be obtained with other chromosome isolation procedures [22, 23] as well as with buffers of higher ionic strength (unpublished data). In a variety of Tris/HAc buffers with neutral pH the concentration of ions as well as their valence changed the EM of all chromosome types in a similar way.

Table 1. Comparison of the peak positions of the flow karyotypes

<table>
<thead>
<tr>
<th>Peak number</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
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<tbody>
<tr>
<td></td>
<td>X0</td>
<td>σ</td>
<td>X0</td>
</tr>
<tr>
<td>1</td>
<td>168</td>
<td>3</td>
<td>168</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>2</td>
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</tr>
<tr>
<td>9</td>
<td>16</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 10. Scanning profiles of CHL chromosomes in FFE treated with 33 µM acridine orange for 2h before measurement. FFE conditions were as in Fig. 1 and 8. The cursor at fraction 46 represents the inlet point. (a) Chromosomes treated with acridine orange; the cursor indicates the peak position at fraction No. 32.5. (b) An aliquot of the same sample thermally denatured at 100 °C for 5 min, quenched on ice and subjected immediately afterwards to FFE; the cursor is at No. 29.6. (c) Two aliquots of the same sample as in (a) and (b) mixed 1:1 immediately before electrophoresis; the dashed cursors indicate the peak positions at fraction No. 32.9 and 28.0.

For all ions under investigation (Na⁺, Ca²⁺, Co³⁺, spermidine) a critical concentration exists at which a major change of EM of the chromosomes can be observed (Fig. 7). These data are summarized in Table 3. No comparable behavior of free DNA was found in equivalent experiments for NaCl and CaCl₂.
(data not shown). Condensation of DNA in solution, as well as in chromosomes in situ as recently described by Darzynkiewicz and Kapuscinski [24], should occur at much higher concentrations of the Co-complex (more than 10 mM compared to the transition observed at 10 μM) and not at all with NaCl or CaCl₂. For spermidine, however, our observations are in agreement with those of Ware and co-workers [25], who observed native DNA complexing with spermidine at a concentration of 100 μM (saturation value). From this it may be assumed that several kinds of binding of cations exist on chromosomes in suspension.

Table 2. Influence of denaturation on EM of chromosomes

<table>
<thead>
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<th>Native</th>
<th>Denatured</th>
<th>∆EM</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHL</td>
<td>1.64</td>
<td>1.89</td>
<td>10-15</td>
</tr>
<tr>
<td>CHL+AO</td>
<td>1.96</td>
<td>2.38</td>
<td>20-21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>Concentration</th>
<th>I</th>
<th>∆EM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 – x NaCl</td>
<td>8.5 ± 0.5</td>
<td>8.5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>20 – x CaCl₂</td>
<td>3.6 ± 0.5</td>
<td>10.5</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>10 – x Spermidine</td>
<td>0.11 ± 0.05</td>
<td>0.6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>10 – x (Co(NH₃)₆)Cl₃</td>
<td>0.01 ± 0.01</td>
<td>0.06</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Since we found the EM of native chromosomes to be similar for all chromosome types, specific treatment of one or a few chromosome types will be necessary for separation. Two such modifications are currently under investigation: (i) Thermal denaturation of isolated chromosomes resulted in a significantly altered EM (Figs. 8 and 10; Table 2). The EM of denatured chromosomes (peak position of the scanning profile) was 20 % higher than the EM of native chromosomes. This result is opposite to that obtained with DNA by Olivera and co-workers [26] and in our experiments (12 % lower than native DNA; data not shown). The notion of denaturing DNA in chromosomes in suspension by thermal treatment is supported by two observations: The DAPI fluorescence of chromosomes in suspension measured by flow cytometry decreases to about 1/7 of its intensity after thermal treatment compared to the flow karyotype before [14,31]; secondly, it was shown that it is possible to hybridize chromosomes in suspension after thermal treatment [27, 32]. From DNA it is well known that AT-rich sequences have a lower melting point than GC-rich sequences. Human chromosomes were found to differ in AT content by more than 20 % in certain regions [28]. Thus, partial thermal denaturation might offer a possibility to separate or enrich certain chromosomes or chromosome fragments by virtue of their different AT content. Currently the different effects of intercalating reagents on the EM and denaturation behavior of chromosomes are under investigation. First experiments with a combined treatment of chromosomes with the intercalator acridine orange and thermal denaturation (Fig. 10; Table 2) indicate that the effect of thermal denaturation on the EM of chromosomes can be increased in this way. (ii) Binding of antibodies was shown to lower EM of cells [33]. This effect can be shown with chromosomes as well [31,34]. If chromosome typespecific antibodies become available, chromosome separation with FFE may become possible. One step in this direction is an improved method of in situ hybridization of chromosomes in suspension.

This work is part of the thesis of F.F.B. and supported by the Deutsche Forschungsgemeinschaft (Cr60/6-1, 6-2). We thank Prof. Dr. K. Hannig for stimulating discussions and B. Hitzelberger for technical assistance.

Received November 28, 1988

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Electrophoresis 1989,10, 690-697


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