N O T I C E

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Kidney Cell Electrophoresis

Mid-Term Progress Report

Contract NAS 9-15584

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January 1980

TASK I. Development and testing of electrophoresis solutions.

The testing of buffers and media continued, using additional new human kidney cell explants obtained from Microbiological Associates, Inc. These were designated HFK-6 through HFK-9. The earlier source, Grand Island Biological Company, is no longer supplying human kidney cells. During this 6-month reporting period a problem requiring additional research arose. Fetal calf serum became scarce then essentially unavailable. Alternatives tested for the growth of HFK-7 and HFK-8 cells included Calf serum, newborn calf serum, donor calf serum, "bobby" calf serum, and bovine (adult) serum. No qualitative differences were observed between cultures of these cells grown in fetal and newborn calf sera at 10% concentration in Eagle's BME. If anything, newborn serum was better (EXP 1064). A more quantitative evaluation was made using our colony-forming human cell line T-1E, which shares phenotypic properties with HeLa cells. It was found (EXP 1069) that plating efficiencies were not significantly different whether fetal, newborn, calf, or bovine sera were used at 10% concentration and that growth curves in fetal and newborn sera were indistinguishable. Experimentation therefore continued using newborn calf serum on a routine basis.

Comparisons between PBG and A-1 buffers continued at various stages of cell preparation and electrophoresis. The formulae for these solutions were given in the previous progress report. Emphasis was placed on the effect of spending 4 hr at 4°C in the two buffers containing DMSO or glycerol on the cells' ability to attach and spread in culture and to form confluent monolayers. The regular culture medium "BME-10" was used as control standard. The effects of 5% DMSO or glycerol were compared in complete medium, and it was found (EXP 1036) that without freezing DMSO produced denser cultures than glycerol in 72 hr, and the cultures in the two solutions were comparable after 96 hr. Supernatants contained cells that later attached in both cases, and cells from both treatments ultimately produced confluent sheets. When 5% glycerol was used in A-1 or PBG buffers (EXP 1037) for storage at 4°C for 4 hr it was found that A-1 plus glycerol led to slightly higher viability than PBG plus glycerol, as shown in Table 1. It is concluded that, if cells are not to be frozen, A-1 buffer is slightly better. However, see below under "freezing" task.

Another step in the procedure was examined, namely the use of EDTA or scraping in place of trypsinizing to prepare cells for electrophoresis. Although neither procedure was as gentle as our usual trypsinization it was clear that incubation of monolayers in 0.37% EDTA in Puck's Saline A (PSA), which produced 48% attached and flat cells in 24 hr was superior to scraping cells in PSA and no EDTA, which produced less than 20% cells attached and spread in 24 hr (EXP 1062). Due to an earlier decision to try to avoid trypsinization in future electrophoresis runs, cell dispersal for electrophoresis is now being done with 0.37% EDTA in PSA. Monolayers are incubated 10 min in this solution, and suspending fluid is added to dilute the EDTA.

TASK II. Optimization of freezing and thawing.

It was previously reported that freezing and thawing of HFK cell suspensions resulted in large numbers of non-adherent but apparently viable cells. These cells were subjected to further study, and no evidence could be found that they are capable of proliferation (EXP 1024). Attempts to evaluate their DNA content by laser flow cytofluorometry failed when it was found that the 4N HCl used in Feulgen hydrolysis destroyed these cells (EXP 1027, 1039). It is tentatively concluded that these are dead cells whose unattached carcasses do not readily lyse or otherwise disappear from cultures. Cells with this peculiar behavior have not been found in large numbers in recent explants.

Glycerol and DMSO (5%) were compared as freezing agents in complete medium (EXP 1036). Cells frozen 18 days in liquid nitrogen attached more quickly after thawing in glycerol than in DMSO. On the second and third days, however, the cultures were indistinguishable. When 5% glycerol was used in A-1 or PSB and cells were frozen 2 days, the results shown in Table 2 (EXP 1037) were obtained, and it was concluded that PSB is superior to A-1 when cells are frozen in glycerol, despite the above finding that cells in A-1 buffer survive better when freezing is not part of the procedure.

The effect of incubation time after thawing and before adding medium was investigated. By allowing cells to attach 1 hr and removing medium and replating the supernatant while changing medium on the attached cells, it was possible to conclude that 75% of the cells (HFK-6) attach in the first 2 hr after thawing and that they tolerate the residual DMSO (diluted to about 0.8%) for this 2-hr period (EXP 1083).

In a combined 3-day freezing test of glycerol vs. DMSO and A-1 vs. PBG it was found, as indicated in Table 3 (EXP 1060) that combining glycerol and DMSO was very bad for the cells, otherwise glycerol and DMSO were about the same in A-1 buffer, but in this particular test glycerol appeared to be better than DMSO in PBG; however, the fraction of cells that also spread in 24 hr was actually greater in DMSO--28% vs. 13%. Cells spread more slowly yet (10-12% in 24 hr) in the A-1 buffer solutions.

The results of freezing and thawing experiments to date slightly favor the use of 5% DMSO in PBG, but it is also evident that 5% glycerol in A-1 is really not very different. It seems that 50-60% viability (in terms of cell attachment in culture) is to be expected under several of the conditions tested. The present data do not give very strong reasons for making recommendations of any particular combination of buffer and freezing agent.

TASK III. Procedures for evaluation of separated kidney cells.

The two principal analytical procedures applied to separated fractions of kidney cells after density gradient electrophoresis in the laboratory are Coulter counting (including volume distribution analysis) and cell culture. A 16-channel log-volume distribution is obtained on every fraction counted. A 100-channel linear distribution will be acquired on every fraction counted as the quality of the separations merits in the near future. Cell culturing procedures in the early phases of the project were plagued with contamination, but this is no longer the case, since a sterilization filter is now used at the column inlet so that all solutions (except cells) that enter the column are sterile regardless of their history. This addition has substantially increased the success rate of post-separation cell culture analysis. Several separated fractions have been grown and are about to be submitted to urokinase assay (EXP 1076, 1077).

A final choice of urokinase assay procedure has been made. The reaction volume has been greatly reduced, and the acid concentration has been adjusted to minimize interference and temperature dependence while maximizing the extinction coefficient of the colored product. Aliquots of test solution are brought to 1.86 ml by the addition of 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0. To this is added 0.14 ml of 0.0152 M CBZ-gly-gly-arg-4-methoxy-2-naphthylamide, which has been dissolved in 50% ethanol, 0.05 M tris buffer. This is incubated at 37°C for 20-80 min, after which the sample tubes are placed in an ice-water bath and 0.2 ml of 1.0 M HCl is added and mixed well. Then 0.4 ml of fast blue B diazonium salt, 2 mg/ml is added, and after a 20 min reaction period optical density is read at 520 nm. This procedure makes it possible to test small volumes from culture supernatants with a sensitivity range that is reasonable for cells that are actively producing urokinase. The fluorometric version of this assay is much more sensitive; however, it was found to be somewhat erratic in our hands, and cells that are truly of interest should produce enough UK for the colorimetric version of the assay. This artificial substrate is also an excellent substrate for trypsin, and the exact extent to which trypsin interferes with the assay has been studied; the data are currently undergoing analysis.

The thrombolytic urokinase assay and the erythropoietin assay received only limited attention at the primary contract site. Some clot-lysis tests will be done at the University of Rochester, and erythropoietic assays are in a state of rapid flux, but it appears that the more recent techniques will be even more amenable to flow cytophotometric assay.

Rebuilding of the flow cytophotometer for light-scattering viability assay and for erythropoietin studies is now nearly complete. A theoretical study was conducted for the optimization of optics, which can now be changed freely, owing to the acquisition of a high-quality optical table. However, progress in this area is still behind schedule.

TASK IV. Electrophoretic mobility characterization of kidney cells.

By combining the results of this work period with those presented in previous progress reports it becomes clear that different primary cultures produce different electrophoretic mobility profiles. Some additional examples are presented here. Cell strain HFK-7 produced copious quantities of non-adherent cells, and the electrophoretic migration plot of Fig. 1 indicates quite clearly that the non-adherent cells belonged to a completely different mobility class from the fibroblasts, which dominated this cell strain at the 6th passage, when this separation was performed. The morphology of the cells in the fractions was determined by phase-contrast microscopy of the plated cultures. As was found to be the case with other HFK strains, this cell strain was much more heterogeneous at earlier passages. Fig. 2 shows the migration plot (EXP 1028) of a separation experiment with strain HFK-7 at passage #2, which is the earliest possible culture passage available to us for study. In this experiment alternating fractions were plated and counted so that quantitative counts (including volume distributions) and evaluations in culture could be obtained on adjacent fractions. This is the procedure followed in about 75% of the HFK separation experiments. The density gradient electrophoretic distribution profile, Fig. 3, confirms that these cells are very broadly distributed with respect to electophoretic mobility. Although 60 pairs of fractions were collected over a 12-cm migration distance in this experiment, electrophoretic heterogeneity was not manifested as a number of sharp peaks but as a broad distribution. Both cases have been seen in past experiments, but sharp peaks are more likely to appear when smaller fraction are collected.

Additional recent experiments have emphasized the significance of electrophoretic mobility distributions obtained by the density gradient method. To determine whether distributions such as the one shown in Fig. 3 represent true mobility distributions or distributions distorted by the method, an old experiment was repeated in which mixed fixed erythrocytes of rat, chicken, and rabbit were subjected to density gradient electrophoresis and to free-zone electrophoresis in which the buffer resembled the solution at the mid-point of the electrophoresis column. The free-zone electrophoresis was carried out at the University of Uppsala. In both experiments chicken cells were chosen to dominate the distribution by being added in the highest concentration. The profiles in Fig. 4 and Fig. 5 are in reasonable agreement that the coefficient of variation (CV) is about 7% in both cases. Since the cells were from the same suspension it is concluded that significant distortions of mobility profile do not occur in density-gradient electrophoresis. Therefore, profiles such as Fig. 3 and those presented in earlier reports are meaningful representations of electrophoretic mobility distributions in human kidney cell populations.

The necessary experimental work has been completed for the estimation of standard electrophoretic mobilities by density gradient electrophoresis. To insure that a proper standard of comparison is being used, a sample of formaldehyde-vapor-fixed human RBC's was obtained from the laboratory of G. V. F. Seaman. Their electrophoretic mobility distribution was determined by our method of microscopic analytical electrophoresis (Fig. 6) and found to agree very well with published findings under standard conditions-- 0.145 M NaCl and $NaHCO_3$ buffer. It is concluded from this result that our microscopic analytic electrophoresis data can be used as a baseline for obtaining standard mobilities. Our rat RBC mobility data, shown vs. ionic strength in Fig. 7, can be used in conjunction with the migration plot of Fig. 8 (EXP 1029) to indicate that, roughly, a migration rate of 1 cm/hr under the conditions of this experiment corresponds to 1 um-cm/V-sec at physiological ionic strength and standard viscosity and temperature. Otherwise standard mobilities can be determined in individual experiments by using the applied current, the column diameter, and the conductivity, which for rather unclear reasons, has a slight dependence upon Ficoll concentration, as Fig. 9 indicates.

The development of laser-Doppler electrophoresis has encountered several successes in recent months. A procedure for chamber coating with methylcellulose to eliminate electroosmosis has been developed and tested by microscopic electrophoresis. Two water-cooled chambers have been built for the laboratory. The complete system, now mounted on a high-quality optical table, has been tested at known rates of fluid flow, and Doppler shift frequencies have been recorded with very little noise and excellent fidelity, as indicated in the test power functions of Fig. 10.

A few recent density gradient experiments have been performed using a 22 cm long column and longer electrophoresis times. This experimental arrangement leads to higher final resolution. Cultures have been started from fractions from such runs, and urokinase testing on these is about to begin.

Table 1. Effect of medium composition on the viability of cells stored at 4°C for 4 hr.

MEDIUM	PER CENT CELLS ATTACHED AFTER 24 HR	48 HR	72 HR	96 HR
"BME-10"	95	100	Confluent	Confluent
A-1 + 5% glycerol	76	77	77	Confluent
PBG + 5% glycerol	68	63	70	81

Table 2. Effect of medium composition on the viability of cells frozen in liquid nitrogen for 2 days.

MEDIUM	PER CENT CELLS ATTACHED AFTER 24 HR	48 HR	72 HR	96 HR
A-1 + 5% glycerol	29	19	20	23
PBG + 5% glycerol	39	45	33	53

Table 3. Effect of medium composition on the attachment of cells frozen in liquid nitrogen for 3 days and examined 24 hr after thawing.

BUFFER	% GLYCEROL	% DMSO	% CELLS ATTACHED
A-1	5	0	57
"	5	5	0
"	0	5	57
"	0	10	0
PBG	5	0	72
"	5	5	0
"	0	5	49
"	0	10	48

Figure 1. Distance migrated in the density gradient column as a function of time of electrophoresis of human kidney HFK-7 cells. The lower envelope describes the migration of a narrow band consisting of round, non-adherent cells, and the upper envelope describes the migration of a broadly distributed band of cells consisting mainly of fibroblasts. The culture was in its 6th passage.

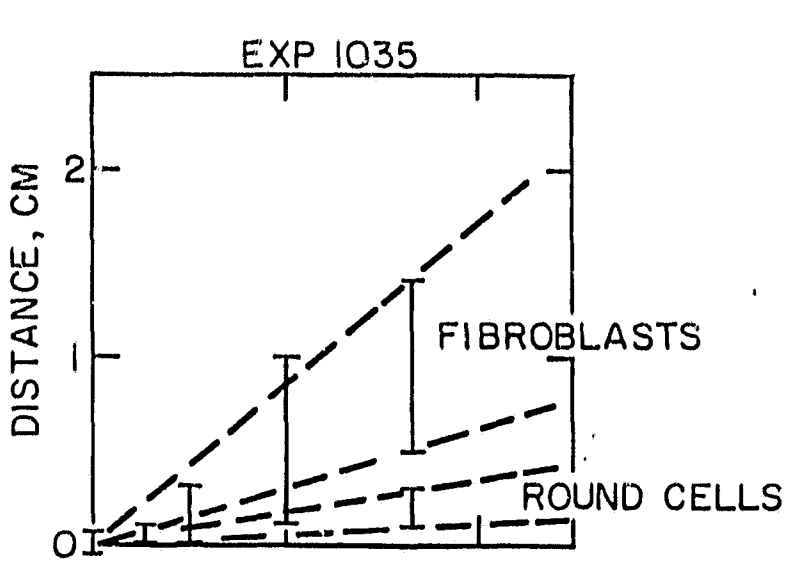

Figure 2. Electrophoretic migration of HFK-7 cells at the 2nd passage, as in Fig. 1. The uppermost envelope describes the migration of an extremely diffuse band of cells having a mixture of morphologies. The electrophoretic heterogeneity of this early-passage culture is reflected in the distribution shown in Fig. 3, in which the fraction numbers correspond to the numbers on the right axis of this migration plot.

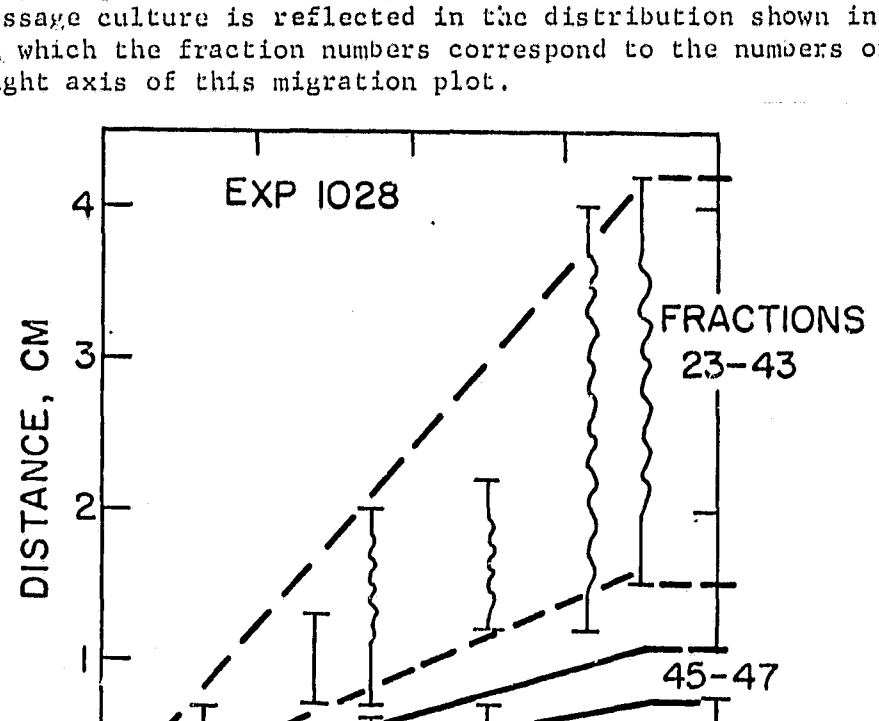

Figure 3. Electrophoretic profile of HFK-7, 2nd passage, cells subjected to density gradient electrophoresis for 3.75 hr and harvested in 1 ml fractions. Fraction numbers correspond to numbers in Fig. 2. Electrophoresis was upward, from right to left on the plot.

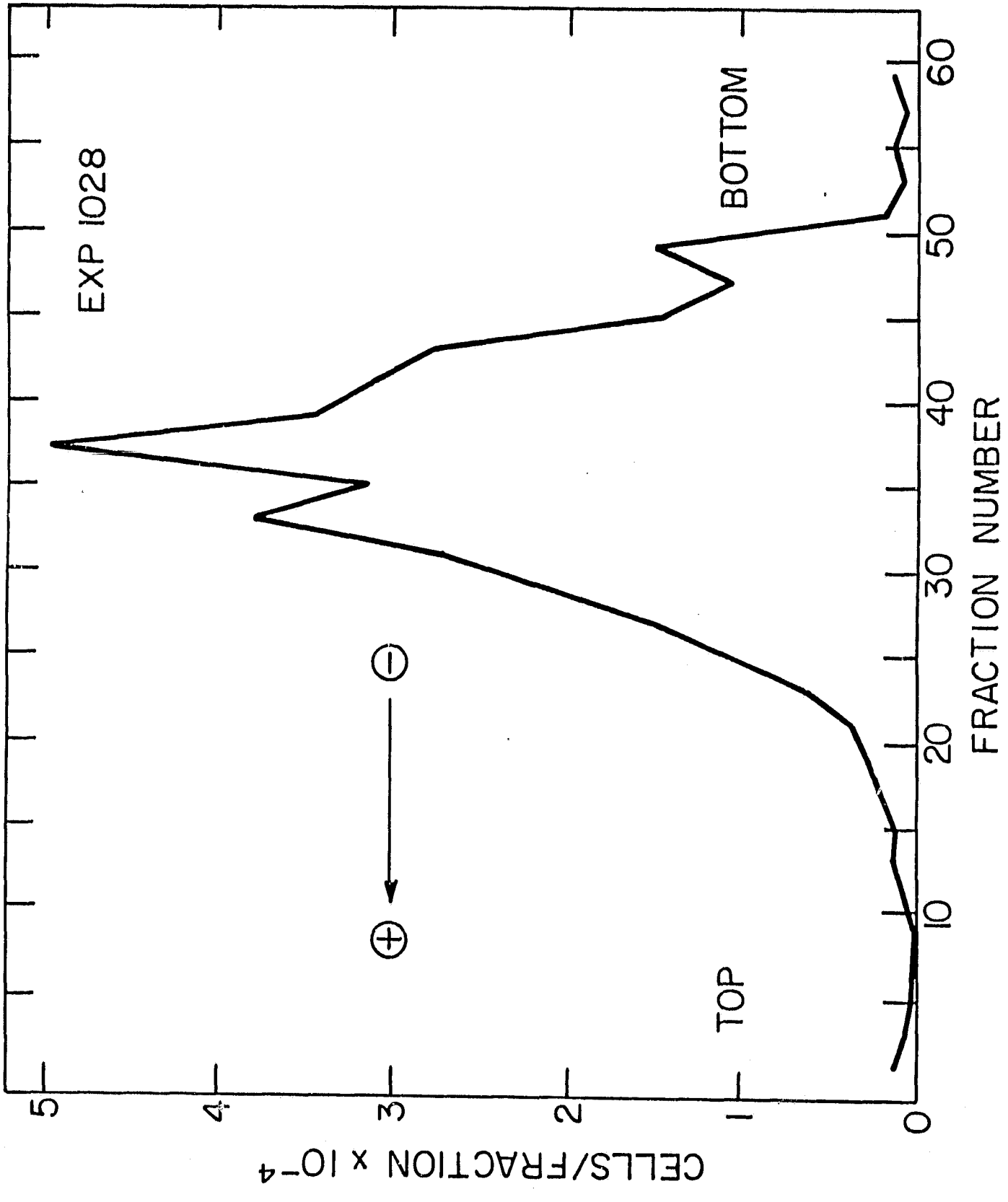

Figure 4. Electrophoretic profile of glutaraldehyde-fixed chick erythrocytes subjected to density gradient electrophoresis for 2 hr to provide an indication of migration velocity (Fig. 8) and electrophoretic mobility distribution of a standard particle.

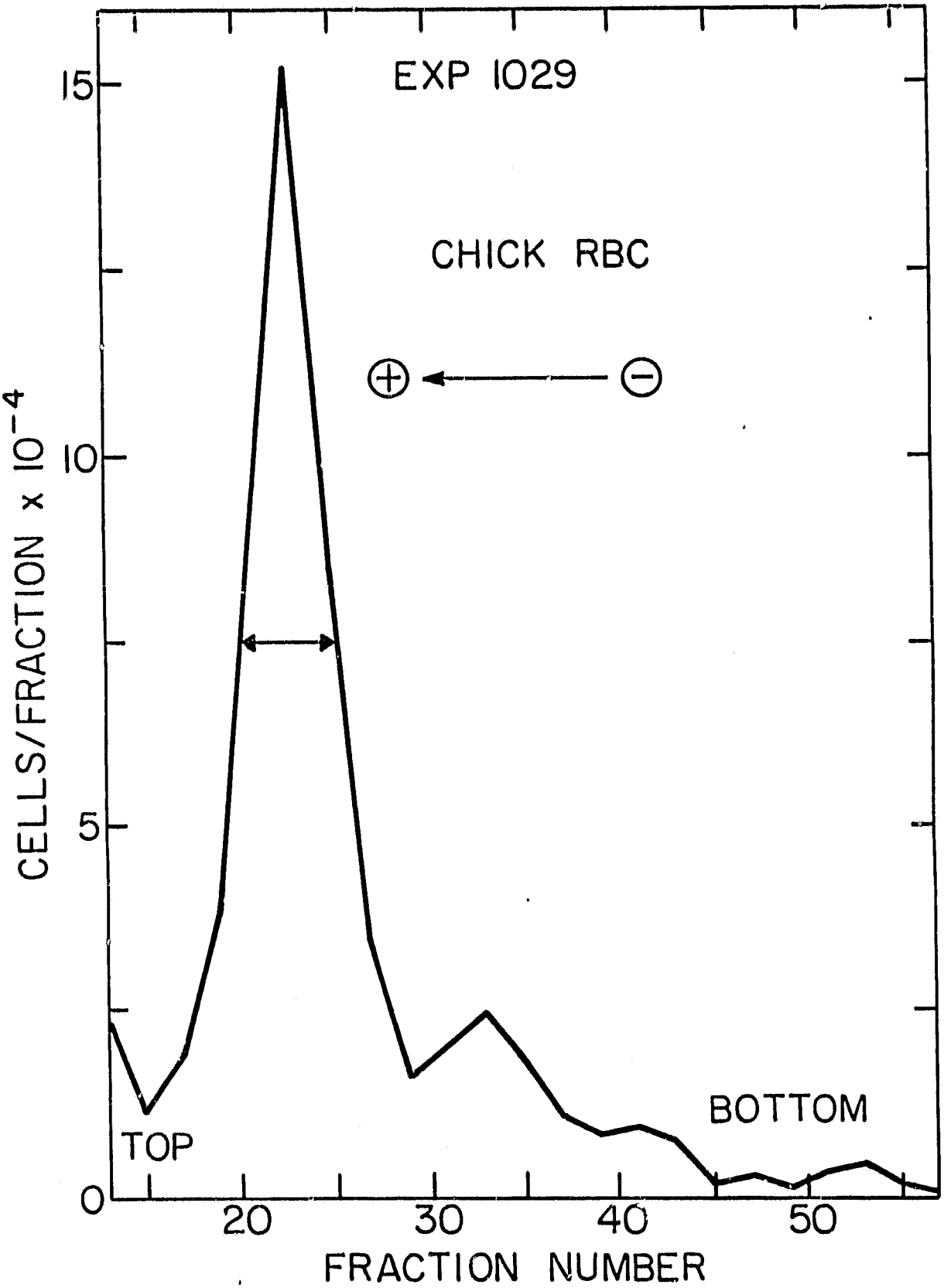

Figure 9. Electrophoretic profile of glutaraldehyde-fixed chick erythrocytes subjected to free-zone electrophoresis for 63 min to provide a true reference mobility distribution with which to compare density gradient electrophoretic distributions.

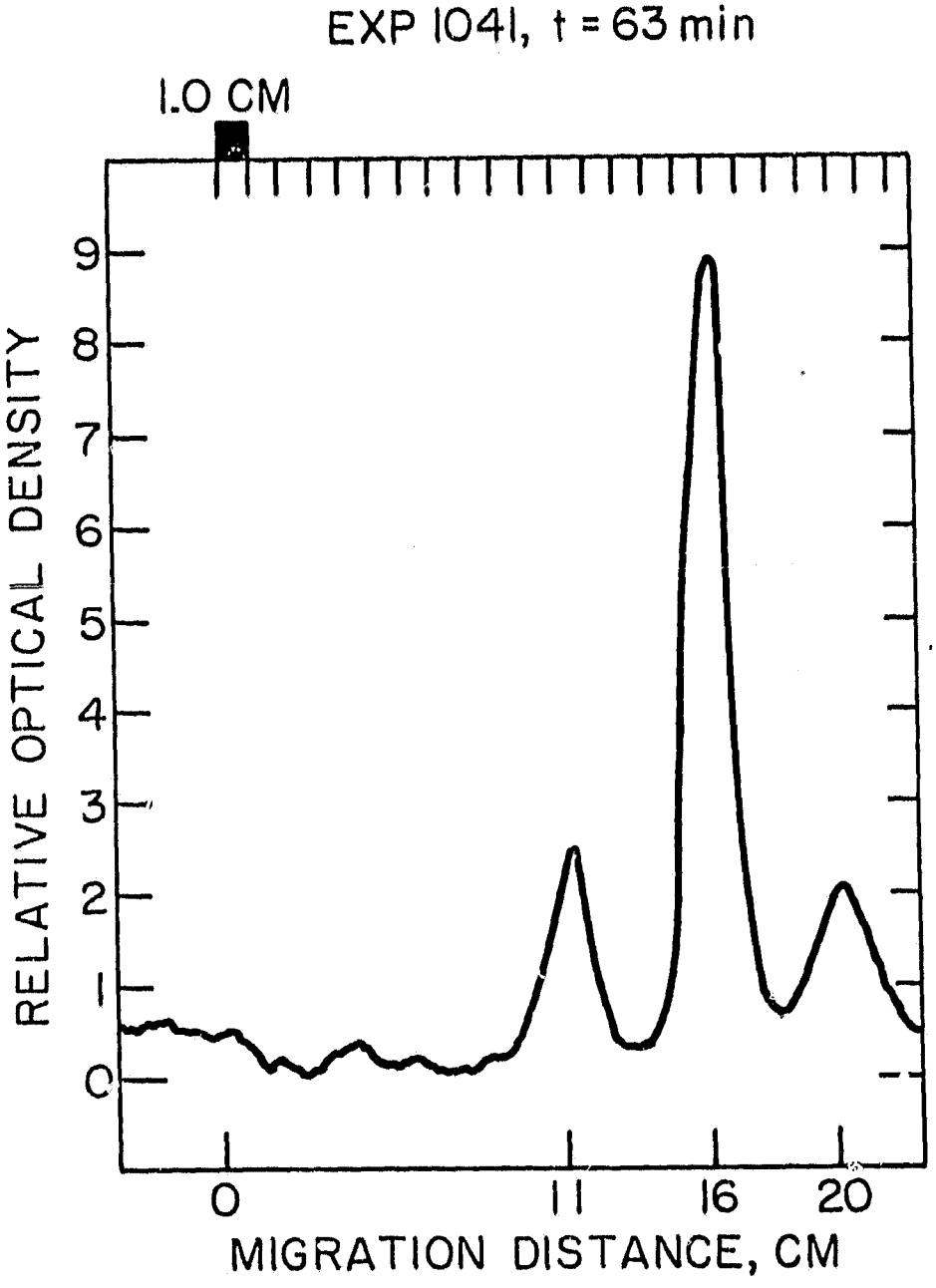

Figure 6. Electrophoretic mobility distribution of formaldehyde-fixed human erythrocytes measured microscopically with a Zeiss Cytopherometer to provide a reference value for a standard particle for comparison with other laboratories and with test and experimental particles used in the present research.

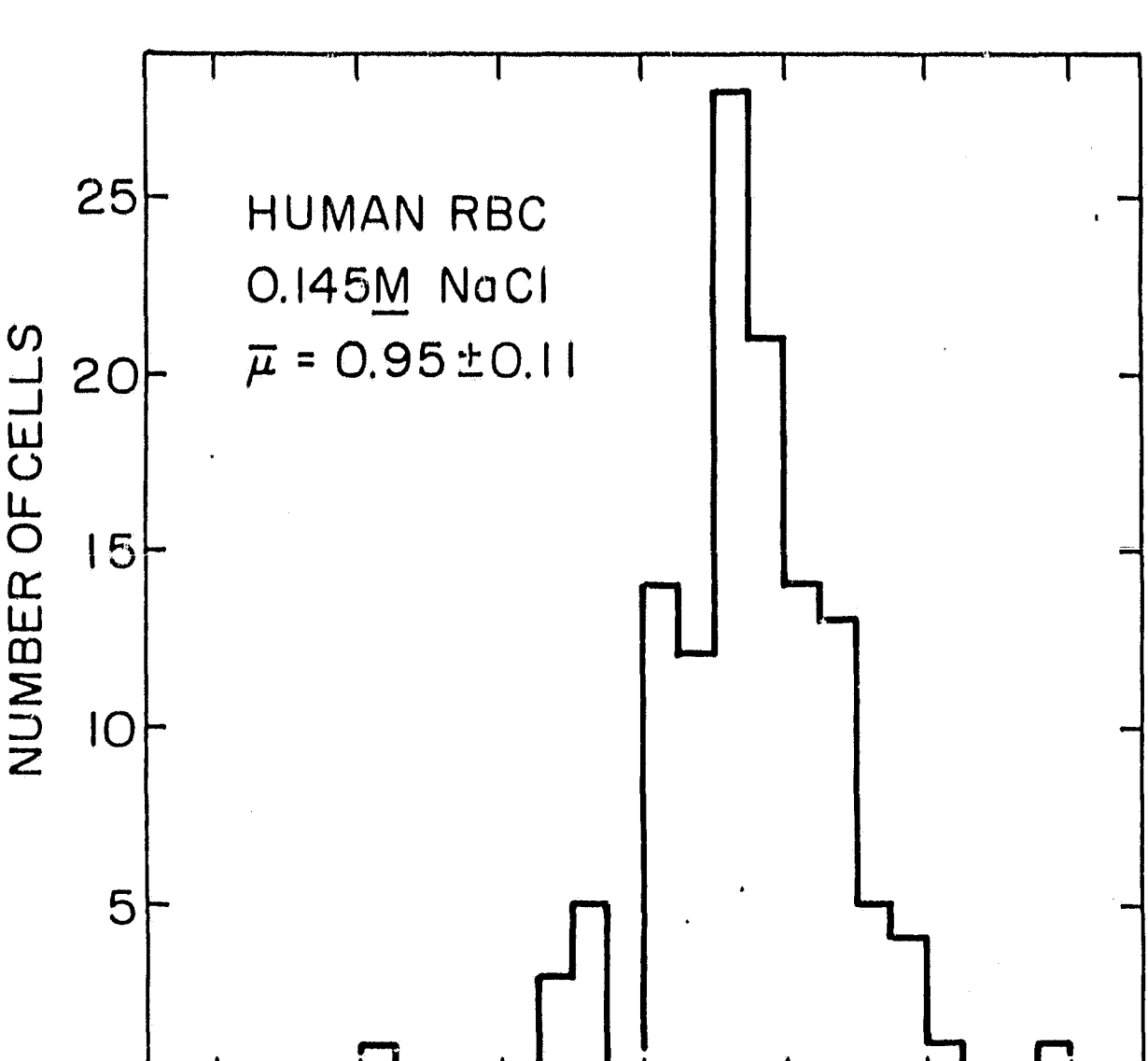

Figure 7. Electrophoretic mobility of glutaraldehyde-fixed rat erythrocytes as a function of ionic strength, as determined by microscopic analytical electrophoresis.

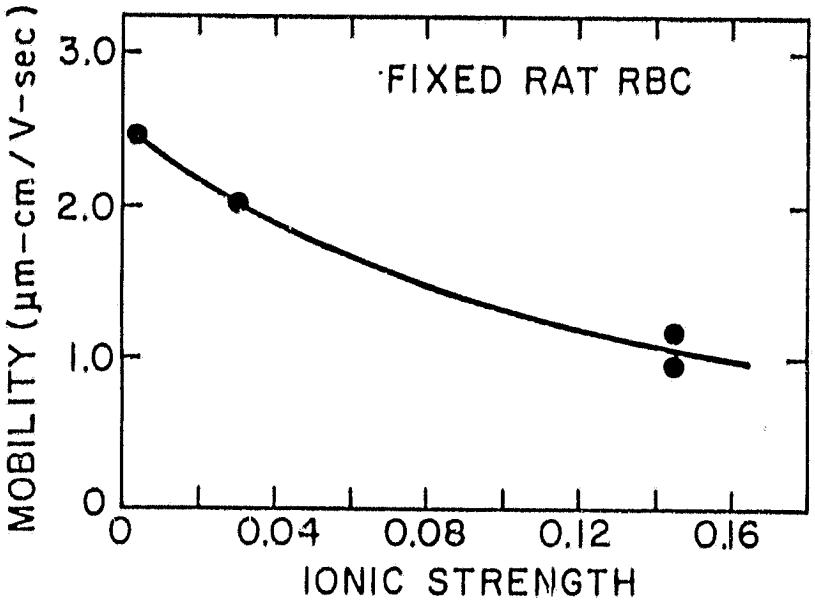

Figure 8. Electrophoretic migration of fixed chick erythrocytes. The slope of the straight line divided by the field strength is the "Ficoll gradient mobility" of the cells, an empirical measurement of their electrophoretic mobility which can be standardized by comparison with analytical electrophoresis under standard conditions of temperature, viscosity, and ionic strength.

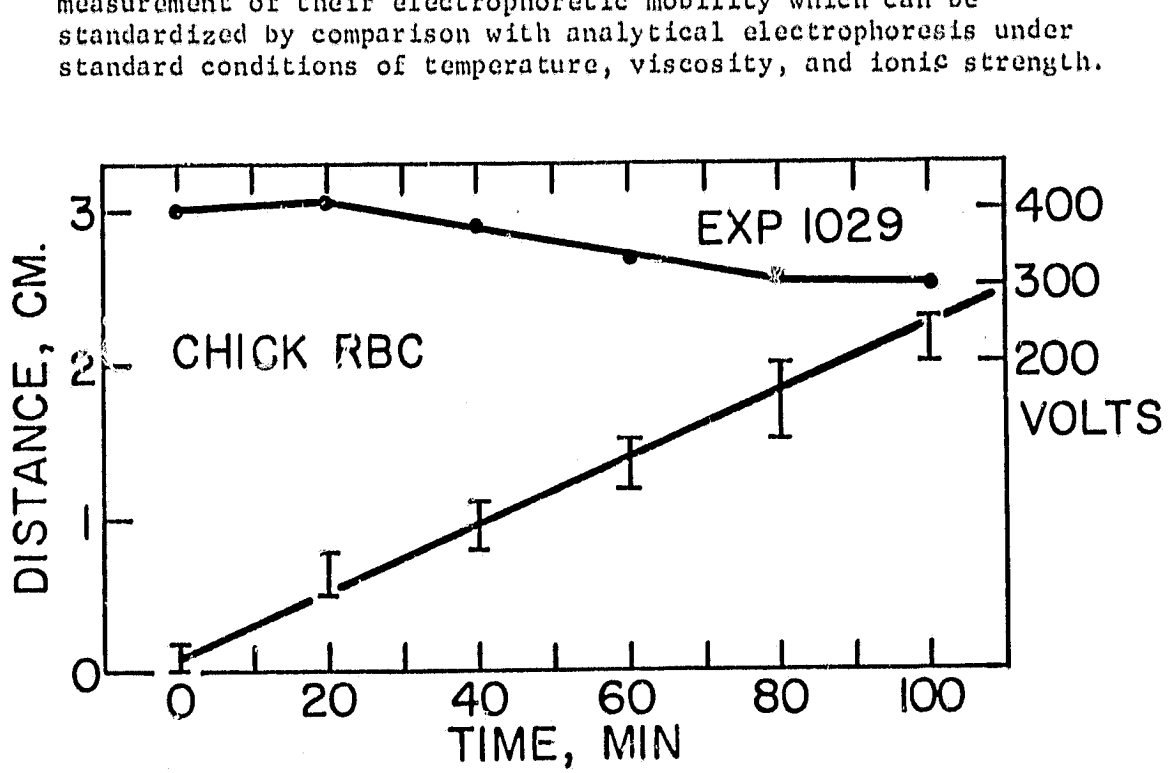

Figure 9. Conductivity of Ficoll-sucrose solution mixtures used in density gradient column electrophoresis. The apparent conductivity falls with increasing Ficoll concentration.

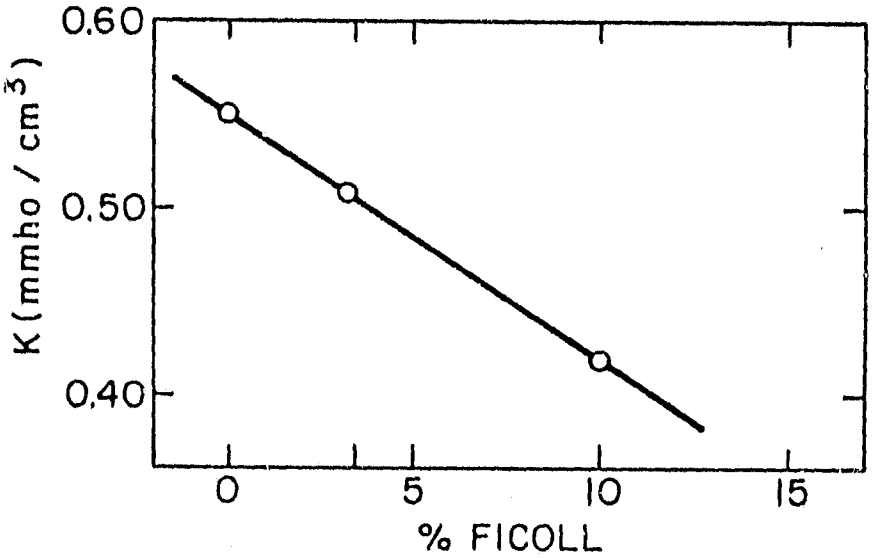

Figure 10. Doppler shift frequency distribution measured in laser-Doppler electrophoresis system. In this test liquid was pumped through the electrophoresis light-scattering chamber at a known, low velocity. The main peak is correctly positioned at 96Hz, and its expected side bands occur and 66 and 124 Hz. In addition to the CRT graphics display shown here, a print-out of the relative power in each channel serves as the primary data set. The estimated coefficient of variation of the main peak is 2.3%.