NASA CR-
160353

# KIDNEY CELL ELECTROPHORESIS

Progress Report

Contract NAS 9-15584



Paul Todd

403 Althouse Laboratory

The Pennsylvania State University

University Park, Pennsylvania 16802

June 1979

TASK I. Development and testing of electrophoresis solutions.

The testing of buffers continued in stages. In addition to the testing of laboratory electrophoresis buffers reported previously, space-rated electrophoresis buffer (for example "A-1") and freezing solutions have been tested. The results, some of which are summarized in Table 1, indicate that the previously used A-1 buffer (1) may be quite suitable for zero-gravity electrophoresis but is not so suitable as a solution in which to freeze cells. Contrary to expectation the addition of ficoll to DMSO seemed to have a harmful effect on the cells at 4° C, our usual electrophoresis temperature. In actual freezing and thawing experiments A-1 buffer and PBG (2) were equivalent and resulted in viability slightly under 60%. Since PBG was less toxic than A-1 in the presence of DMSO (they are equivalent without DMSO), the freezing procedure (3) itself appears to be responsible for some of the cell lethality.

The composition of PBG (2) is given in Table 2, and the composition of A-1 buffer (1), as formulated in our laboratory, is given in Table 3. The cells used in viability testing were obtained from Grand Island Biological Co., Grand Island, New York, and a total of six explants were obtained for study through the course of the project. They were designated HFK and HFK-1 through HFK-5. The data of Table 1, for example, were obtained using HFK-5. Testing of solutions was performed by trypsinizing the cells in 0.05% trypsin, 0.5 mM EDTA, and centrifuging and resuspending at a concentration of $10^5$ cells/ml in test solution for the designated time and treatment conditions. The composition of the trypsinizing solution is given in Table 3.

In early stages of viability testing cells were treated in monolayer culture. An interesting result was found in that a constant fraction of the cells was non-adherent and continued to multiply in suspension. Since non-adherent cells were scored as "dead" in these experiments, any change of medium led to "increased" viability by removing non-adherent cells, dead or alive. This observation is a corollary of our previoulsy-reported results which indicated the existence of an electrophoretically-separable non-adherent cell population capable of continued proliferation in complete culture medium, which is Eagle's basal medium (BME) (4). Under continued subcultivation these cells do not attach. This phenomenon introduces ambiguity into the viability measurement and the figures quoted in Table 1 are probably somewhat low.

The solutions used in the Table 1 viability study cover a wide range of ionic strengths, and complete medium, PBG, and A-1 buffers all provide about 70% viability with ionic strengths of approximately 0.16, 0.013, and 0.0097 M, respectively. Ionic strength tolerance is therefore not a limiting factor in human kidney cell electrophoresis.

Complete medium at 4° C is rather toxic to HFK-5 cells (Table 1). This is not surprising, since cells of human kidney epithelioid line T-1 have also been found susceptible to low temperature exposure in complete medium, and similar tests on HFK-4 explant cells also resulted in less than 60% survival. Storage and processing of cells at low temperature is definitely more successful when most of the ingredients of culture medium are omitted. It is possible that the complete nutrient mixture causes unbalanced cell metabolism at low temperature or the high ionic strength of complete medium is harmful at low temperature. The toxicity

of medium at low temperature should not be considered a serious practical problem, since none of the plans for kidney cell electrophoresis in the laboratory or in space include the maintenance of cells in complete medium or high-ionic-strength solutions at low temperature.

Concerning the ultimate choice of buffer for electrophoresis and freezing, it appears that a slightly improved version of either PBG-sucrose or A-1 would be suitable. They are similar in composition (Table 2 and Table 3) and toxicity (Table 1). Both use phosphate as buffering ion, supply glucose in excess of 1%, have ionic strength around 0.010 M, provide sodium, potassium, and chloride ion, do not become toxic when DMSO is added, and have similar osmolarities. PBG-sucrose is 264 mosM, and A-1 is 248 mosM (5). Their major difference lies in their divalent cation content. PBG-sucrose provides $Mg^{++}$ at 0.5 mM, whereas A-1 removes divalent cations using $Na_2EDTA$ at 0.3 mM, a similar concentration. The viability data to date, therefore, provide no evidence for a role of divalent cations in the retention of viability at 4° C during electrophoresis or freezing.

TASK II. Optimization of freezing and thawing.

It was found that, in complete medium with 10% DMSO, the customary rate-freezing procedure (3) resulted in cell killing that was cell concentration dependent. About 5-10 x $10^6$ cells/ml are recommended for good viability through a freeze-thaw cycle in complete medium. It was found, however, that in the presence of 10% DMSO both electrophoresis buffers (Table 1) produced much higher viability than that found by freezing in complete medium (sometimes less than 10%). It will be possible to test whether serum or ionic strength is responsible for the large difference between complete medium and the buffers as a freezing solution.

The freezing procedure we use (3) requires that cells spend some time at 4° C, and the observation (Table 1) that cells are less viable in complete medium or ficoll plus DMSO at 4° C could be relevant to the freezing problem. It is surprising that macromolecules that presumably stay outside the cell would have such an effect. It was originally expected that macromolecules in solution might have a cryoprotective role and that their addition might permit reductions in DMSO concentrations. This hypothesis has now been dismissed, at least as it applies to experiments with kidney cells. It has also become apparent that adding ficoll to space electrophoresis buffer would not serve a useful purpose.

TASK III. Procedures for evaluation of separated kidney cells.

Development of procedures for culturing cells was reported in the mid-term progress report, and repeated electrophoresis experiments continue to indicate that cells from nearly all electrophoretic fractions form monolayer cultures when the ficoll-gradient fractions are diluted directly into complete medium, BME + 10% fetal bovine serum. From the supernatant medium on the cultures an aliquot of medium was removed periodically and assayed for urokinase or frozen for later

assay. Cultured fractions were observed daily by phase-contrast microscopy, so that viability and morphology could be recorded. As will be noted below, an unexpected cell type emerged, and this would have been missed without microscopic evaluation of living cultures from all of the fractions. Cells which failed to attach multiplied in suspension, and this represented a particular electrophoretic fraction. Even these cells apppear to grow well in BME + 10% fetal bovine serum. Since the ability to attach and spread is not a reasonable viability criterion for these cells, the trypan blue method of staining for dead cells was used. In our procedure this consists of adding 0.1 volume of 0.4% trypan blue in Hanks' balance salts solution to cells suspended in completed medium. After a freeze-thaw cycle in complete medium this category of cells is about 40% viable by this criterion.

A survey of proteolytic assay methods for urokinase activity was performed. The esterolytic method, described in the previous report, was further evaluated with a modified substrate and found to be unsuitable. As noted before, the esterolytic activity of urokinase upon ALME or AGLME substrates is somewhat difficult to assay under non-rigorous conditions. This stems from the level of intereference by other enzymes, and care must be taken in the interpretation of ALME esterase activity when complex biological fluids are being assayed as other enzymes such as plasmin, trypsin, or thrombin may be present. These three can also hydrolyze the ALME substrate (6). Trypsin has been present in reaction mixtures because it was used in the preparation of cell cultures prior to assay. The extent of interference is not known at this time, but it is thought that it could be great. EDTA has been observed to give a false positive color reaction and has produced high blank values. Now, it has been found (7) that trypsin inhibitors, such as SBTI and mingin (urinary trypsin inhibitor), can completely abolish trypsin activity while leaving the urokinase quite unaffected. This addition may have merit for this assay as well as for the 4-methoxy-2-NA procedure (below). Kinetic parameters of three synthetic substrates are given in Table 5. Allowing $V_{max}$ to be an indicator of enzyme sensitivity or specificity, it is seen that the ALME and AGLME are superior to the 4-MeO-2-NA moiety. However, a trade-off is enacted by using the latter as substrate at this time due to its reproducibility and simplicity. Nevertheless, standardization curves for the esterolytic assay are shown on Figure 1. Variation is not too great for the urokinase dilutions. However, in order to obtain these results, the assay of White and Barlow had to be modified and, in general, required too much time for assay. Urokinase standardizations for both ALME and AGLME were worked out and the results parallel those found by various investigators (8-11).

The enzymatic hydrolysis of the synthetic esters ALME and AGLME was carried out by the previously published method of White and Barlow (11). In the past, this method has been known to be laborious and the variation in the assay large. The reactions of the method are shown diagrammatically as follows:

$$\text{ALME (or AGLME)} \xrightarrow{\text{Urokinase}} \text{MeOH} \xrightarrow{\text{Oxidation}} \text{Methanal (HCHO)}$$

$$\text{Methanal} + \text{Chromotropic Acid} \xrightarrow[\text{heat}]{} \text{Colorimetric Complex (purple)}$$

Chromotropic Acid = 4,5-dihydroxy-2,7,naphthalenedisulfonic acid disodium dissolved in 10 ml distilled $H_2O$ and then mixed with 90 ml of 67% sulfuric acid.

The colorimetric product concentration is determined by measuring absorbance at 580 nm.

Some testing of cell-culture supernatants was done with this method, and 7th passage cultures of HFK-1 cells showed about 200 CTA units/ml medium and 7th-passage cultures of HFK cells showed none. This finding of such low activity is consistent with the notion that the percent differentiated, secreting cellsdecreases with increasing passage number. Furthermore, it appeared that HFK cells were mostly fibroblasts at this passage level.

Heavy emphasis was placed on the use of naphthylamide derivatives of oligopeptide substrates of plasminogen activator. Colorimetric and fluorometric tests for the liberated 4-methoxy-2-naphthylamine were explored and applied to culture human kidney cell supernatant media. The synthetic substrate is similar to the amino acid sequence at the cleavage site in plasminogen and the 4-methoxy-2-naphthylamine moiety, when hydroxylzed via urokinase from its peptides, lends itself well to colorimetric analysis when coupled to diazonium salts such as Fast Blue B. The hydrolysis of this synthetic substrate was carried out by the previously published method of Huseby *et al.* (12) with corresponding results appearing in Figure 2, which shows standard curves. The reactions are shown diagrammatically as follows:

$$\text{CBZ-gly-gly-arg-4-MeO-2NA} \xrightarrow{\text{Urokinase}} \text{4-MeO-2NA (to be coupled)}$$

$$\text{4-MeO-2NA} + \text{Fast Blue B} \xrightarrow[\text{5 min.}]{} \text{colorimetric product}$$

The colorimetric product that is produced lends itself to visible spectrophotometry. The sample chamber of the spectrophotometer was cooled to 10° C using a water-bath circulation pump as an aide to make reaction times uniform. Absorption was read at 520 nm.

Abbreviations used are:

CBZ-gly-gly-arg-4-MeO-2-NA = Nα-Benzyloxycarbonyl-gly-gly-arg-4 methoxy-2-naphthylamide · HCl

4-MeO-2-NA = 4-methoxy-2-napthylamine

Fast Blue B = 4,4'-diamino-3,3'-dimethoxy-biphenyl

The following detailed procedure for the routine use of this method for assays of supernants was worked out and applied. The materials needed include:

| | |
|---|---|
| Tris buffer | 0.05 M, pH 8.0 |
| HCl | 1.0 M |
| Fast Blue B dye | 0.1 mg/ml (to be made fresh for each assay) |

For the assay of urokinase from cell cultures, the following standard conditions are used:

> Tris buffer 0.05 M, pH 8.0, 10 ml containing 1.2260 mg substrate per ml; distilled water, 1.0 ml; 0.5 ml of trypsin-free tissue culture fluid to be assayed are mixed well in a small test tube (dispo Culture). Final concentration of substrate is $8 \times 10^{-4}$ M.

Important: Substrate used is CBZ-gly-gly-arg-4-MeO-2-NA (HCl) - it is soluble in the Tris buffer only if an amount of EtOH is present in solution. This amount should not exceed the volume of Tris buffer. This rationale stems from the fact that the urokinase will remain active and unblocked by the EtOH when introduced into the Tris/EtOH/substrate solution. Therefore, add the weighed solid to 1/2 the volume of Tris that would normally make up the desired concentration, then add EtOH gradually until the solid dissolves. Be patient and use a hot water bath as an aid to complete the solution process. The amount of added EtOH should not exceed the amount of Tris. In summary, add solid to 1/2 volume Tris buffer, dissolve with minimal EtOH, then fill to the mark with Tris buffer. Do this in a fume hood.

For low concentrations of urokinase (0 to 30 CTA units/ml), a standard incubation time of 120 minutes is used. For higher concentrations (30 to 100 CTA units/ml), incubation times of 30 to 60 minutes are used. Incubation temperature is 37° C. After appropriate incubation times, the reaction is stopped by the addition of 0.1 ml of 1.0 M HCl. To each aliquot is then added 1.0 ml of Fast Blue B dye @ 1.0 mg/ml. This dye solution must be made up only prior to each assay as it decomposes rapidly. The colored coupling product is then allowed to develop for 5 minutes and is read at 520 nm. The blank used is complete except for the addition of the cell culture supernatant fluid -- volume should be made up with appropriate fresh tissue culture media. The values obtained for absorbance are taken to the appropriate standard curve and the corresponding value of urokinase CTA units/ml is noted. Results obtained using this method are described later.

The fluorescent assay method holds considerable promise, due to its sensitivity, so it was subjected to preliminary testing.

This direct assay also uses N-carbobenzoxy-glycine-glycine-L-arginine-4-methoxy-2-napthylamide (CBZ-Gly-Gly-Arg-4-MeO-2NA). Hydrolysis of the amide bond releases fluorescent 4-MeO-2NA. This assay is quantitative, easy to perform, and more sensitive than the AGLME assay. The method here allows the accurate and reproducible determination of very low amounts of plasminogen activators (13).

Fluorescence measurements were made using an American Instrument Co. SPF-125 Spectrofluorometer (Thanks to Dr. Roy H. Hammerstedt, Department of Biochemistry and Biophysics) equipped with a 150-watt xenon light source. The corrected excitation and emission maxima for 4-MeO-2NA were at 342 and 426 nm, respectively. Because continuous excitation resulted in a slow decrease in the fluorescence signal, the exictation shutter was kept closed except during the brief periods of data acquisition. Fluorescence intensity of 4-MeO-2NA in 0.05 M Tris-HCl was directly proportional to concentration over the range $5 \times 10^{-9}$ to $5 \times 10^{-7}$ M and was independent of pH between 7.0 and 9.5.

For the direct assay of urokinase, 20 µl of 0.1 M CBZ-Gly-Gly-Arg-4-MeO-2NA in dimethylformamide (DMF) was added to 1.96 ml of 0.05 M Tris-HCl, pH 8.75, followed by a 20-µl aliquot of urokinase stock solution, 25 to 2500 CTA/ml. The fluoresence intensity at 426 nm was measured every minute for 10 minutes. The rate of the linear increase of fluorescence is determined and converted to nanomoles of 4-MeO-2NA/ml/min. All fluorescence measurements were made at 22¼ C.

The kinetics of the urokinase-catalyzed hydrolysis of CBZ-Gly-Gly-Arg-4-MeO-2NA are illustrated in Figure 3. The enzymatic production of 4-MeO-2NA is linear for at least 15 minutes. Bigbee et al., found, by using a working concentration of urokinase of 1 to 5 I.U./ml, that urokinase concentrations as high as 100 I.U./ml could be determined using the same assay conditions. The practical lower limit of sensitivity with their fluorometer was about 0.5 I.U./ml of enzyme. They found that this assay is 50-fold more sensitive than the ester-substrate assay with AGLME or the colorimetric amide-substra' assays with N-α-acetyl-L-lysine-p-nitroanilide or with CBZ-Gly-Gly-Arg-4-MeO-2NA. This direct assay is at least an order of magnitude more sensitive than the standard clot-lysis assay (14) and is as sensitive as the fibrinolytic method described by Johnson et al. (9). The assay is very rapid, is sensitive over a wide range of urokinase concentrations, and can be performed at room temperature.

Preparations were made for examining a sensitive radiochemical esterolytic assay for plasminogen activator. Specificity is assumed by using plasminogen as the urokinase substrate and plasmin, the product, as esterolytic agent. The reactions involved in this indirect method are as follows (16):

$$\text{Plasminogen} \xrightarrow{\text{Urokinase}} \text{Plasmin}$$

$$\text{TAME } (^3\text{H}) \xrightarrow{\text{Plasmin}} (^3\text{H})\text{-MeOH}$$

To be counted by liquid scintillation

TAME: N-α-p-tosyl-L-arginyl-methyl ester

The assays are performed in tubes placed in liquid scintillation counting vials. At the end of the experiment, the generated $(^3\text{H})$-MeOH

is extracted into the liquid scintillation cocktail and counted. Unhydrolyzed substrate largely remains in the aqueous phase and contributes only a small fraction of the counts. The facile separation of $^{3}H$-labeled MeOH from the ester substrate allows for the simple and highly sensitive assay for urokinase. This method gives good results in excellent agreement with the classical fibrin plate assay. It stresses simplicity and high specificity. This indirect method can quantitate 0.01 CTA units of urokinase activity and does not show the interferences found with the other two methods. Recipes and purchase sources for this procedure have been prepared, but it has not yet been tested in the laboratory.

Further development of fibrin plate assay methods was aimed at being able to distinguish urokinase-producing cells from non-producers by microscopic examination of collected fractions following electrophoresis. The method of Marsh and Gaffney (17) was adapted to use in tissue-culture dishes.

Marsh and Gaffney recommended the use of 10 ml 0.1% fibrinogen (fbgn), 2 casein units plasminogen (psgn), and 0.5 ml thrombin (tmbn, 10 NIH units/ml) in a 9.0 cm petri dish. We decided to try 2 ml fbgn in 35 mm petri plates. Solutions of 0.1% to 0.8% fbgn were prepared and 2 ml aliquots pipetted into 35 mm petri plates. Thrombin was then added to each plate, the mixture swirled briefly, and the plates allowed to set until clots were firm. All clots began to form almost immediately upon addition of tmbn and were firm by 5 mins after tmbn addition. After the clots were firm, the plates were placed into the incubator at 37°C. The plates were observed at three hours and showed no change (lysis). At 12 hours all clots were completely lysed. The first set of experiments was repeated using a 1 ml aliquot of fbgn since the first clots were somewhat thick. The clot formation and lysis times were essentially the same; however, subsequent runs were made using 1.5 ml fbgn and clots appeared more uniformly thick.

The next step was to determine the effect of plasminogen on the lifetime of the clot. A stock solution prepared with 0.5 mg psgn dissolved in 10 ml phosphate buffer (pH 7.5) contained 6.45 Worthington units psgn/ml and was used in all subsequent runs. Various amounts of psgn were added to plates. Using 1.5 ml 0.3% and 0.6% fbgn, we prepared plates with 1 ml, 0.5 ml, 0.25 ml, and 0.16 ml psgn and allowed clots to form as usual (no change in clotting time). The plates were placed in the 37°C incubator and observed at various times. After 3 hours incubation the 0.3% fbgn plates with 1 and 0.5 ml psgn had begun to liquify. At 12 hours all 0.3% fbgn plates and 0.6% fbgn plates with 1 ml psgn were liquified and at 18.75 hours all plates except 0.6% fbgn with 0.16 ml psgn showed some degree of liquification. With these results, we decided to use the smaller amounts of psgn in experiments. Up to this point we had prepared a fbgn/psgn mixture in the plate and then added tmbn. In the experimental case we felt that a fbgn/psgn mixture on top of cells would possibly allow some psgn to be activated before tmbn was added and the clot formed and so opted to try a tmbn/psgn mixture added to fbgn in the culture plate. When the clots were prepared we noted the time and observed a 30-45 second delay in clot formation time for the tmbn/psgn mixture.

The clot staining method was also tested. Mixtures of hematoxylin stain (no glacial acetic acid) and Harris hematoxylin stain were prepared and an attempt made to stain the clots. Staining proved to be an inadequate method by which to read lysis zones. The stain does not spread evenly over the clot surface (hence, uneven staining) and such a large amount was needed that excess would have to be washed off. (Washing the clot has a tendency to destroy the clot). In view of these results we recommend reading lysis zones by means of an overhead projector as described by Marsh and Gaffney. The clot shows definite fibers running through it which gives it a cludy white appearance.

The full procedure was tested. Two clots were made in a petri plate and 20 samples of urokinase (UK) added to the center of the clot. One UK sample was a standard dilution of UK and the other a supernatant from a 2nd-passage culture of HFK-3 cells. The procedure included pouring off the media, washing twice with a small amount of Hanks' Balanced Salt Solution, then applying the clot components. Residual Hanks' colored the clots a pale peach and greatly increased to time needed for clot formation (0.6% fbgn clot increased from 5 to 10 mins). Conclusions could not be drawn from this experiment, and it is anticipated that a slightly modified version of this procedure will be suitable for the detection of urokinase-producing cells and/or colonies.

Preliminary testing of the fibrin slide method of Kwaan and Astrup was also carried out. This relatively simple assay involves two steps: the formation of an artificial clot and then the addition of an enzyme (UROKINASE) to dissolve the clot. The actual dissolving away of the clot is detected by the appearance of holes (lysis zones) in the stained clot. The following is a brief outline of the procedure:

1. On ordinary precleaned microscope slides, an area of 2.5 x 4.0 cm was delineated with waterproof ink.
2. 10 µl of thrombin solution was spread evenly over this area and allowed to dry quickly at room temperature.
3. Then, 60 µl of fibrinogen solution was applied and spread quickly with a glass rod, evenly distributing the solution by gentle tilting.
4. Slides were placed on a horizontal glass plate in a moist chamber at room temperature for 1 hour to complete clot formation.
5. Cell suspensions were usually prepared as a smear on the glass slide, either by simple drying or after brief fixation in 50% methanol and then covered. Cells from electrophoretic fractions were collected by Cytospin.
6. The slides were then incubated for appropiate lengths of time in a moist chamber at 37°C.
7. The incubated slides were fixed in 10% formaldehyde solution for varying periods of time.
8. After staining with Harris' Alum Hematoxylin without acetic acid, the slides were mounted in glycerine jelly.
9. For comparison, solutions of urokinase (or tissue activator) were applied with a glass capillary (diameter 1.5 mm) delivering approximately 5 µl by gently touching the fibrin film.

In five experiments using this method and variants of it, the background (spontaneous clot lysis zones) was found unacceptably high. According to Kwaan and Astrup, "to achieve a detailed and discrete localization of active sites, the fibrinogen must be rich in plasminogen, low in inhibitor content with clottability above 85-90%, containing no spontaneous fibrinolytic activity (including such caused by bacterial contamination), be free of citrate and be soluble at high concentration (about 1%) without sedimentation. We have found no commercial preparations which fulfill all of these criteria, in particular, they are low in plasminogen and therefore low in sensitivity to activators". Further testing of this method has been abandoned.

A procedure for the in vitro assay of erythropoietin (ESF) was adopted and learned, but not yet applied to the assay of cultured kidney cell supernatants. The erythrocytic colony formation (CFU-E) method of McLeod et. al. (19) was learned in the laboratory of Dr. E. L. Alpen of Lawrence Berkeley Laboratory. In this method erythropoietic stem cells of mouse femoral bone marrow are stimulated by ESF to differentiate into erythroid cells that multiply into microcolonies (4-16 cells) and incorporate iron-59. After femurs are removed by as clean surgery as possible, the procedure is as follows:

1. Flush each femur with 2.0 ml of cold collection medium ("supplemented medium"), and pool the suspensions on ice.
2. Disperse the cells gently with a 23 then a 27 gauge needle.
3. Count nucleated cells/ml. Hemacytometer and Coulter counter with ZAP give the same result, so either method can be used.
4. Add the suspensions to plasma culture medium at $4.5 \times 10^5$ cells/ml.
5. Add human urinary ESF standard or test sample to give final concentration in the vicinity of 0.25 U/ml.
6. Mix 0.8 ml of suspension with 0.2 ml bovine plasma for clot formation. Keep plasma at room temperature just prior to addition. Mix well.
7. Plate 0.15 ml of this mixture in the bottom of each of the central 8 wells of a 24-well tissue-culture plate. The button that forms when this is done should be allowed to clot in the absence of vibrations. The remaining wells of the micro-culture plate should be filled with 1.0 ml sterile distilled $H_2O$ for moisture retention in the covered plate.
8. Leave at room temperature until the clots have formed.
9. Incubate at 37°C in 4% $CO_2$ incubator, pH 7.14 for 30 hr.
10. Add 0.15 ml of $^{59}FeCl_3$ solution. This radioiron is provided by New England Nuclear Corp. with specific activity around 20 mCi/mg Fe in buffered sodium citrate. It should be diluted in Earle's Balanced Salt Solution.
11. Incubate cultures 18 hr with the isotope.
12. Remove medium from the wells.
13. Fix the buttons with 5% glutaraldehyde in 0.01 M phosphate buffer pH 7.0-7.2 for 6 min.
14. Rinse in several changes of distilled $H_2O$ over 2.5 - 3.0 hr period.

15. Lift clots and transfer them to acid cleaned slides.
16. Dry slides in air at room temperature.
17. Dip slides in Kodak NTB2 nuclear track emulsion at 45-50°C.
18. Expose radioautographs for 5 days in darkness at 4°C.
19. Develop radioautographs in D-170 developer.
20. Stain with Giemsa.
21. Count the total number of radioactively labeled colonies per clot, where a colony is a group of 8 or more cells. Use 160-200X magnification.

The important solutions for this procedure are:

1. Citrated bovine plasma, stored at -20°C, obtainable from Grand Island Biological Co. (GIBCO).
2. Reconstituted beef embryo extract dissolved in "supplemented medium", also from GIBCO.
3. "Supplemented Medium"--Eagle's MEM with Hanks' Balanced Salt Solution as base plus Eagle's non-essential amino acids and pyruvate at the standard culture-medium concentrations, 50-100 U or mcg penicillin and streptomycin/ml.
4. Heat inactivated (60°C, 30 min) fetal calf serum, stored at -20°C.
5. ESF solution, dissolved at 10 U/ml in "supplemented medium", not filtered, stored at -20°C.
6. L-asparagine, 2 mg/ml in "supplemented medium", filter sterilized and stored at -20°C.
7. Culture medium NCTC-109 plus antibiotics, stored at 4°C, single strength.
8. Bovine serum albumin Fraction V, 10% stock solution made by dissolving at 4°C for 2.5 hr., obtained from Sigma Chemical Co.

These solutions are combined for the execution of the steps in the above procedure as described by McLeod et. al. (19). An alternative to this procedure involves omitting the radioactive label and radioautography and staining for hemoglobin in the traditional manner with benzidine derivatives.

TASK IV. Electrophoretic Mobility Characteristics of Kidney Cells

With continued acquisition of fresh explants of human kidney cells, a serial strain numbering system was adopted. The first explant was called HFK, the second HFK-1, etc. Total consistency was not expected among different explants, but some regular patterns emerged. Cultures that were low in fibroblasts were the most interesting, and all of these also developed populations of viable, non-adherent cells, which could be distinguished and enumerated in cultures by phase microscopy. In early electrophoretic separation experiments it was considered desirable to culture trypsinized cells in suspension for 6 hr before electrophoresis. This procedure normally permits surface glycoprotein regeneration in continuous cell lines, but it killed all but the non-adherent cells in early-passage human kidney cell suspensions. All electrophoresis experiments are now done using freshly-trypsinized cells.

Early-passage cells of strain HFK-1 were subjected to density gradient electrophoresis after mixing with fixed rat erythrocytes (RBC). Figure 4 shows the resulting electrophoretic profile. It indicates that the electrophoretic mobility of the modal HFK-1 cells is about 70% that of the fixed RBC's in the Ficoll gradient (in which the origin was around fraction 35), and the non-adherent cells had about 60% the mobility of RBC's. Non-adherent ("round") cells were fewer in number than attaching cells, and their average mobility was lower. This particular population was not studied for its urokinase activity.

Human kidney HFK-3 cells at the first passage were separated by density gradient electrophoresis, and fractions were grown in culture in 5 ml complete medium in 60 mm tissue culture dishes for two weeks. After the supernatant was sampled for urokinase activity it was sampled for non-adherent cells. The monolayers were trypsinized and also counted. It appeared from the cell-count profile of Figure 5 that fractions containing the high numbers of attached cells also contained high numbers of unattached cells. Coulter volume spectrometry revealed that non-adherent cells were smaller than attached cells by as much as a factor of 4 in volume (Figure 6). The fractions highest in cells, 21-23 in this experiment, did not coincide with maximum urokinase production shown in Figure 7, which describes the same experiment (plotted as empty circles). It therefore appears that urokinase producing cells were a rather small proportion of this particular explant and that UK activity was high, on a per cell basis, in the lower-mobility fractions, namely fractions 25-32.

Similar separation experiments were performed at passage 1 and passage 2 of strain HFK-3, and as Figure 7 indicates, specific cell fractions were found to produce UK in both cases. Unfortunately, the applied electric fields were not the same in the two experiments, so the fractions cannot be directly related. In both cases, UK producing cells appeared to be at least bimodally distributed among the collected fractions. It is possible that there will be more than one electrophoretic population of cells producing UK in cultures in general. The cell population described by the data of Figure 8 behaved similarly. There were two electrophoretic peaks of urokinase producing cells and evidence that there were two populations whose UK production increased between day 12 and day 13 in culture after electrophoretic separation. In these experiments UK activity was assayed on aliquots of supernatant culture medium using the colorimetric 4-methoxy-2-naphthylamide method (Figure 2). Activity units per viable cell in separated fractions have not yet been determined.

In summary, electrophoretic separations have produced populations of viable adherent and non-adherent cells and cell fractions capable of producing differing amounts of UK upon subsequent cultivation. There may be more than one elctrophoretic cell subpopulation capable of UK production.

## REFERENCES


1. Allen, R. E. , Barlow, G. H., Bier, M., Bigazzi, P. E., Knox, R. J., Micale, F. J., Seaman, G. V. F., Vanderhoff, J. W., Van Oss, C. J., Patterson, W. J., Scott, F. E., Rhodes, P. H., Nerren, B. H., and Harwell, R. J., Electrophoresis technology experiment MA-011. In Apollo-Soyuz Test Project Summary Science Report Vol. I, NASA-SP-412, NASA, Washington, D C., 1977, pp. 307-334.

2. Boltz, R. C. Jr., Todd, P. Streibel, M. J., and Louie, N. K., Preparative electrophoresis of living cells in a stationary ficoll gradient. Prep. Biochem. 3, 383-401 (1973).

3. Schroy, C. B. and Todd, P., Simple method for freezing and thawing cultured cells. Tissue Culture Assoc. Manual 2, 390-310 (1976).

4. Eagle, H., Propagation in fluid medium of a human epidermoid carcinoma, strain KB. Proc. Soc. Exp. Biol. Med. 89, 362-364 (1955).

5. Knox, R. J., Analytical study of electrophoretic characterization of kidney cells. Final Report, Contract NAS8-32162, to George C. Marshall Space Flight Center. August 1978.

6. Celander, D. R., and Guest, M. M., The biochemistry and physiology of urokinase. American Journal of Cardiology. 6, 409-421, 1960.

7. Sobel, G. W., Hohler, S. R., Jones, N. W., Dowdy, A. B., and Guest, M. M., Urokinase: An activator of Plasma profibrinolysin extracted from urine. American Journal of Physiology. 171, 768, 1952.

8. Sherry, S., Alkjaersig, N., and Fletcher, A., Assay of Urokinase Preparations with the Synthetic Substrate Acetyl-L-Lysine Methyl Ester. Journal of Laboratory and Clinical Medicine. 64, 145-152, 1964.

9. Walton, P. L., Biochim. Biophys. Acta. 132, 104-114, 1967.

10. Johnson, A. J., Kline, D. L., and Alkjaersig, N., Assay Methods and Standard Preparations for Plasmin, Plasminogen and Urokinase in Purified Systems, Washington, D. C., International Committee on Haemostasis and Thrombosis, N.H.I. Standardization, 1967.

11. White, W. F., and Barlow, G. H., Urinary Plasminogen Activator (Urokinase). Methods in Enzymology. 19, 665, Academic Press, 1970.

12. Huseby, P. M., et al., Studies of Tissue Culture Plasminogen Activator, Part II. Thrombosis Research. 10, 680-84, 1977.

13. Bigbee, W. L., et al., Sensitive Fluorescence Assays for Urokinase using Synthetic Peptide 4-Methoxy-2-Napthylamide Substrates. Anal. Biochemistry, submitted 12/13/77 and accepted, 1-20.

14. See Ref. 9

15. Johnson, A. J., et al., Thromb. Diath. Haemorrh. 21, 259-272, 1969.

16. Imanari, T., et al., Sensitive radiochemical esterolytic assays for urokinase. Clinica Chimica Acta. 71, 267-276, 1976.

17. Marsh, N. A., and Gaffney, P. J., The rapid fibrin plate - a method for plasminogen activator assay. Thrombos. Haemostas. (Stutg.) 38, 545-551 (1977).

18. Kwaan, H. C. and Astrup, T., 17, Demonstration of Cellular Fibrinolytic Activity by the Histochemical Fibrin Slide Technique. Laboratory Investigation. No. 2, pages 140-5, 1967.

19. McLeod, D. L., Shreeve, M. M., and Axelrad, A. A.: Improved plasma culture system for production of erythrocytic colonies in vitro: Quantitative assay method for CFU-E. Blood 44, 517-534 (1974).

Table 1. Viability of suspended cells undergoing various treatments in electrophoresis buffers and freezing solutions. Viability criterion was attachment and spreading in culture within 24 hours after plating. PBG = phosphate-buffered glucose, recipe in Table 2.

| SOLUTION | TREATMENT | | % VIABLE |
|---|---|---|---|
| Complete medium | Room temp. | 3h | 71 |
| Complete medium | 4°C | 3h | 58 |
| PBG + 6.8% sucrose | 4°C | 3h | 68 |
| PBG + 6.8% sucrose + 10% DMSO | 4°C | 3h | 75 |
| PBG + 6.8% sucrose + 5% DMSO + 5% glycerol | 4°C | 3h | 62 |
| PBG + 5% sucrose + 10% ficoll | 4°C | 3h | 69 |
| PBG + 5.9% sucrose + 5.0% ficoll | 4°C | 3h | 61 |
| PBG + 5.9% sucrose + 5.0% ficoll + 10% DMSO | 4°C | 3h | 49 |
| A-1 buffer | 4°C | 3h | 70 |
| A-1 buffer + 10% DMSO | 4°C | 3h | 66 |
| A-1 buffer + 10% DMSO | Freeze-thaw cycle | | 58 |
| PBG + 6.8% sucrose + 10% DMSO | Freeze-thaw cycle | | 57 |

Table 2. Composition of phosphate-buffered glucose (PBG) and PBG-sucrose, which is PBG with 6.8% sucrose to give isosmolarity.

| COMPONENT | MW | g/l | M |
|---|---|---|---|
| KCL | 74.56 | 0.20 | 0.00268 |
| $MgCl_2 \cdot 6H_2O$ | 203.33 | 0.10 | 0.00049 |
| $Na_2HPO_4$ | 141.96 | 1.15 | 0.00810 |
| $KH_2PO_4$ | 139.09 | 0.20 | 0.00144 |
| Glucose | 180.16 | 10.00 | 0.05551 |

Table 3. Composition of zero-g electrophoresis buffer used in viability tests (A-1).

| COMPONENT | MW | g/ℓ | M |
|---|---|---|---|
| NaCℓ | 58.45 | 0.380 | 0.00642 |
| $Na_2HPO_4$ | 94.98 | 0.167 | 0.00176 |
| $KH_2PO_4$ | 136.09 | 0.050 | 0.000367 |
| $Na_2EDTA$ | 372.24 | 0.125 | 0.000336 |
| Glucose | 180.16 | 40.0 | 0.222 |
| Glycerol | 92.10 | 47.34 | 0.514 |

Table 4. Composition of trypsinizing solution used before viability studies and electrophoresis.

| COMPONENT | g/ℓ |
|---|---|
| NaCℓ | 8.00 |
| KCℓ | 0.40 |
| $NaHCO_3$ | 0.35 |
| $Na_2HPO_4 \cdot 7H_2O$ | 0.09 |
| $KH_2PO_4$ | 0.06 |
| $Na_2EDTA$ | 0.20 |
| Glucose | 1.00 |
| Phenol red | 0.01 |
| Trypsin, bovine pancreatic 1:250 | 0.50 |

Table 5. Comparison of kinetic parameters of three synthetic substrates of urokinase.

| SUBSTRATE | $K_m$ * | $V_{max}$ ** |
|---|---|---|
| CBZ-gly-gly-arg-4-MeO-2-NA | $2.6 \times 10^{-3}$ | $3.29 \times 10^{-5}$ |
| Acetyl-lysine methyl ester | $7.7 \times 10^{-4}$ | $1.00 \times 10^{-3}$ |
| Acetylglycl-lysine methyl ester | $5.9 \times 10^{-4}$ | $2.50 \times 10^{-3}$ |

*molarity

**µmole substrate/CTA unit/minute

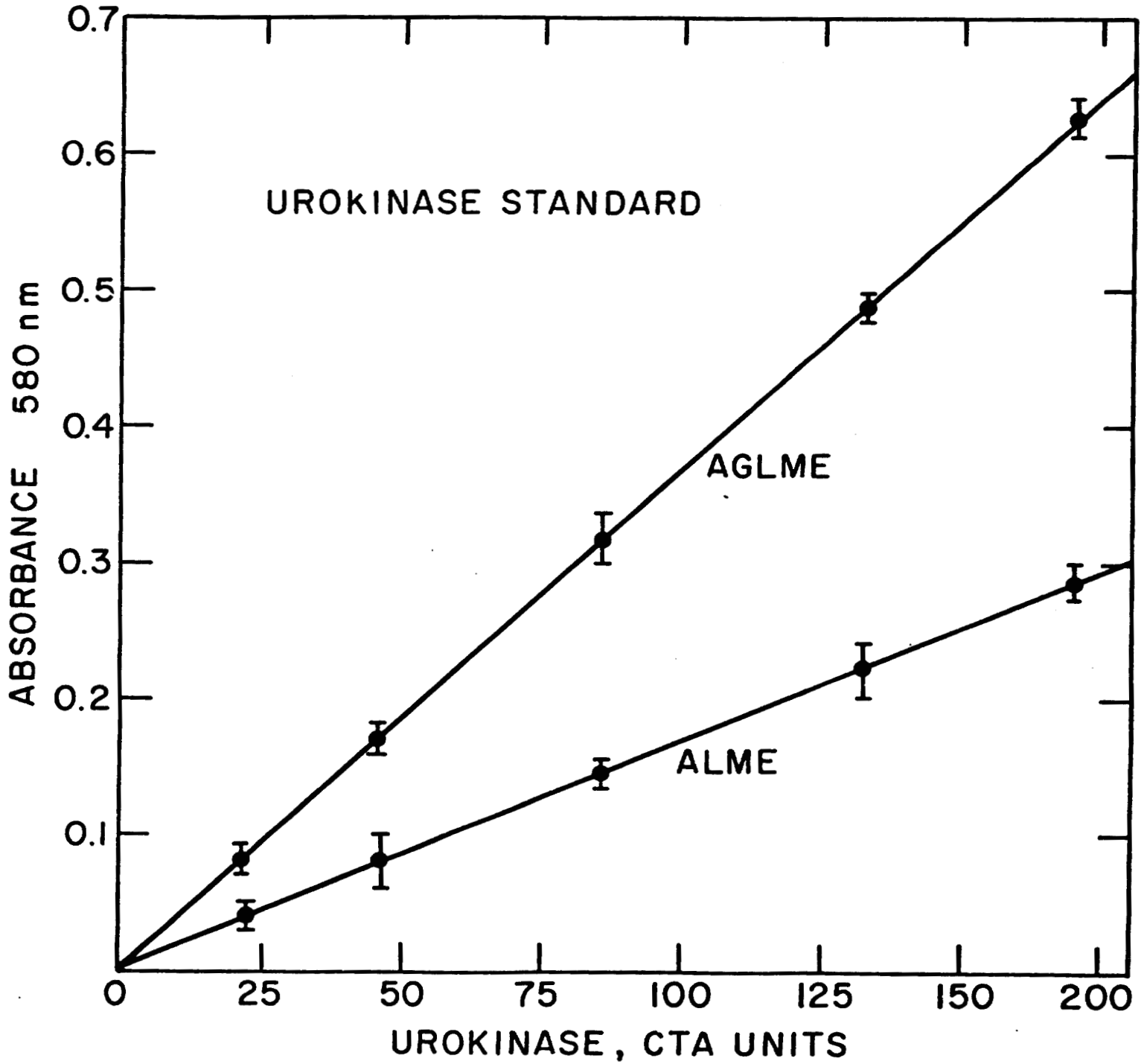



Figure 1. Spectrophotometric standardization curves for the esterolytic determination of urokinase activity using ALME or AGLME as substrate.

Figure 2. Spectrophotometric calibration curve for the proteolytic determination of urokinase activity using the synthetic 4-methoxy-2-naphthylamide substrate and Fast Blue B dye.

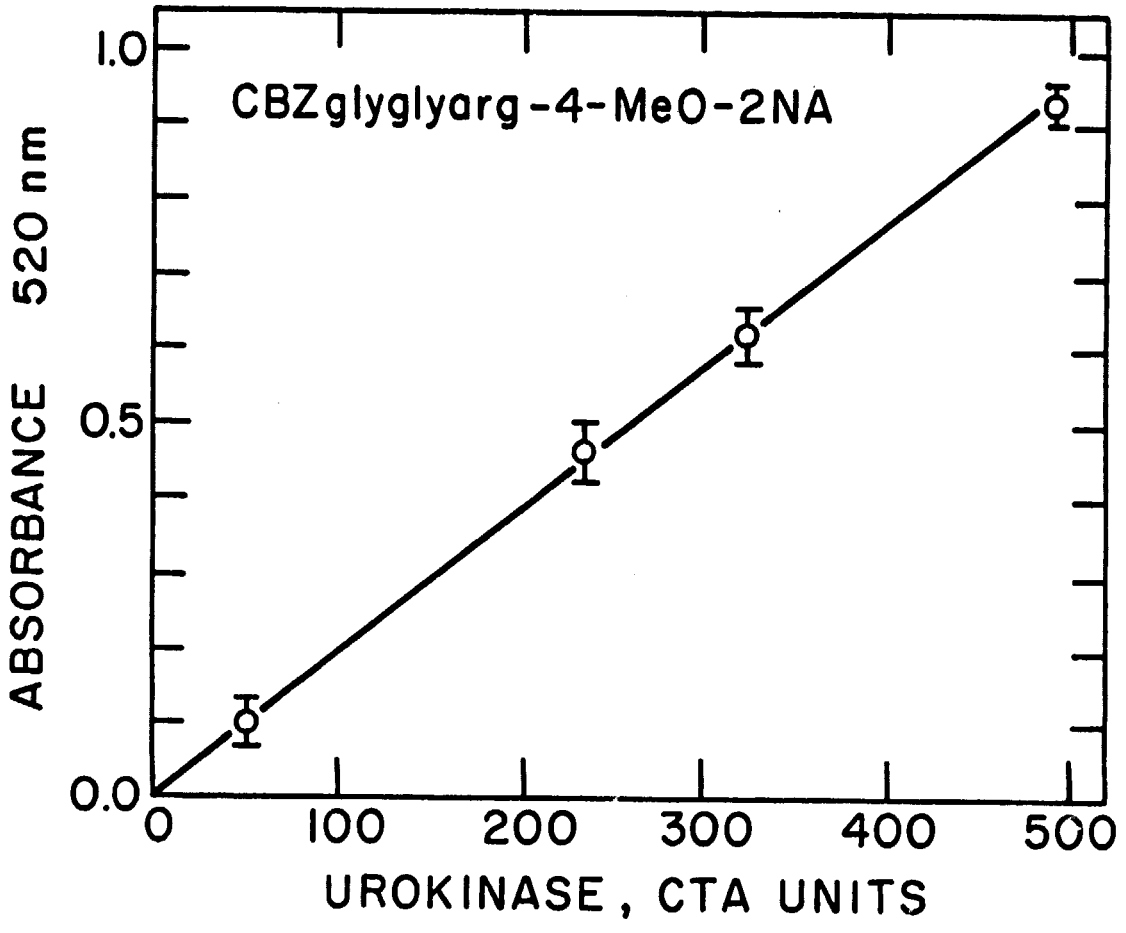


Figure 3. Reaction kinetics of the urokinase-catalyzed hydrolysis of synthetic 4-methoxy-2-naphthylamide substrate as determined by spectrofluorometry.

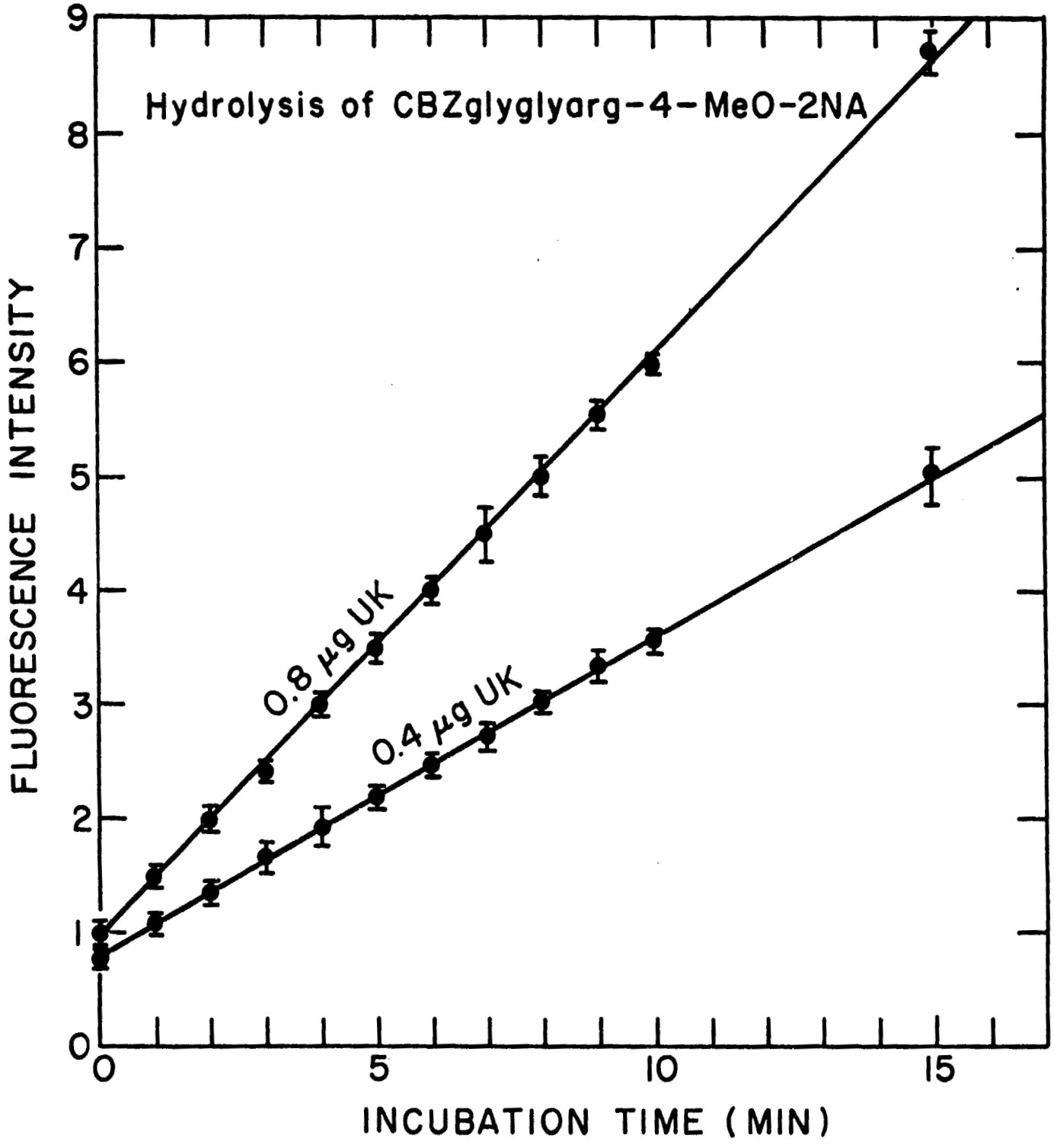


Figure 4. Density gradient electrophoretic profile of early-passage HFK-1 cells co-electrophoresed with fixed rat red blood cells as markers. The mobility of the kidney cells in the peak fraction is estimated to be 70% of that of the RBC's. Fractions were collected directly into culture vessels, and the number of cells per 16X field was counted 24 hr later.

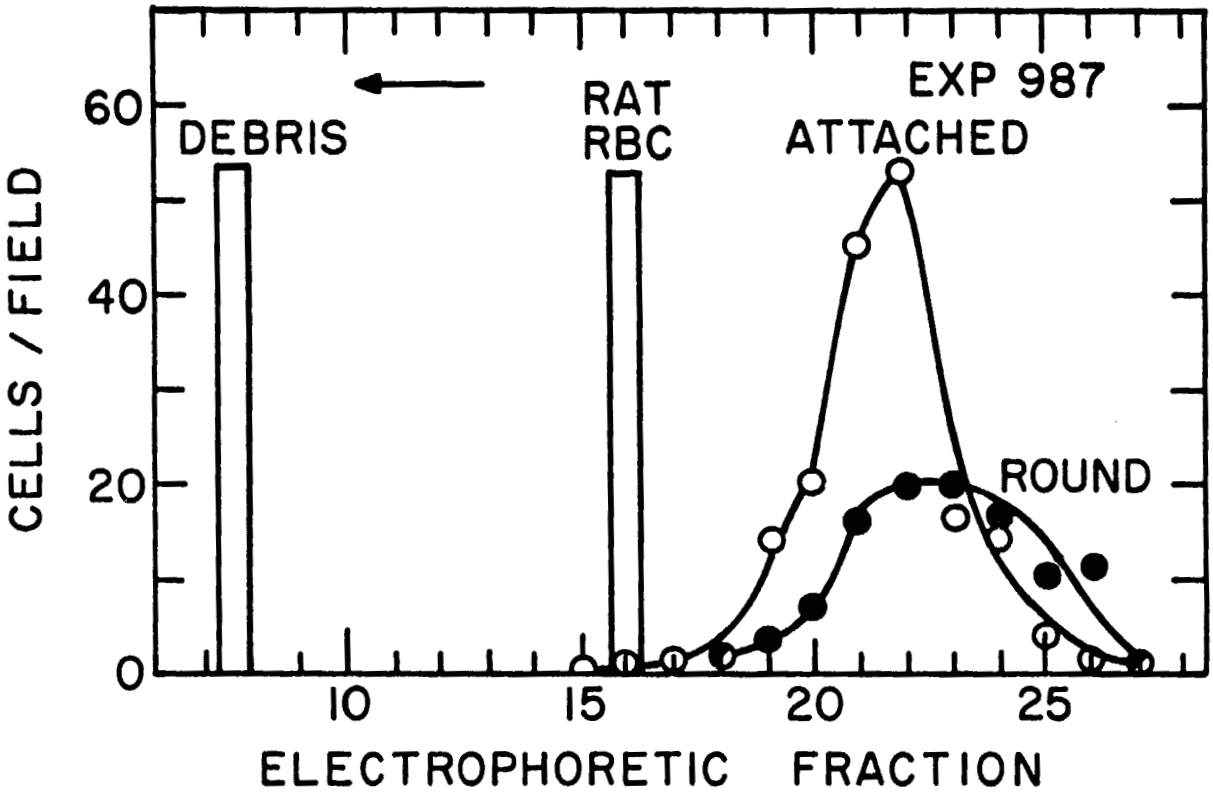


Figure 5. Cell counts in cultures two weeks after collection of density-gradient electrophoretic fractions of HFK-3 cells. Suspended cells were non-adherent and were determined by Coulter counting of the supernatant medium in each culture. Attached cells were determined by trypsinizing each monolayer and Coulter counting. Volume distributions were obtained for both components of each cultured fraction. Electrophoretic migration was toward the left.

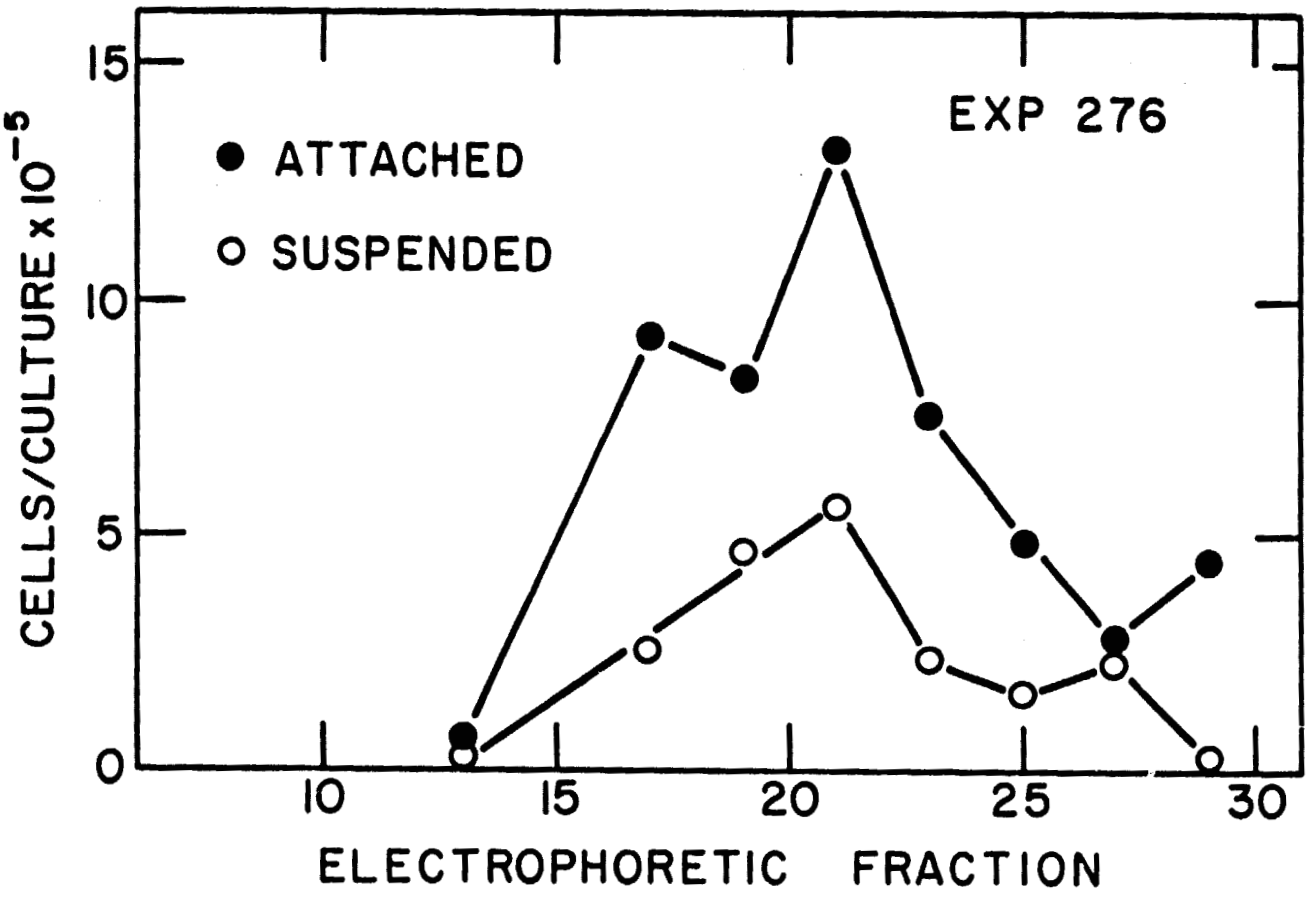
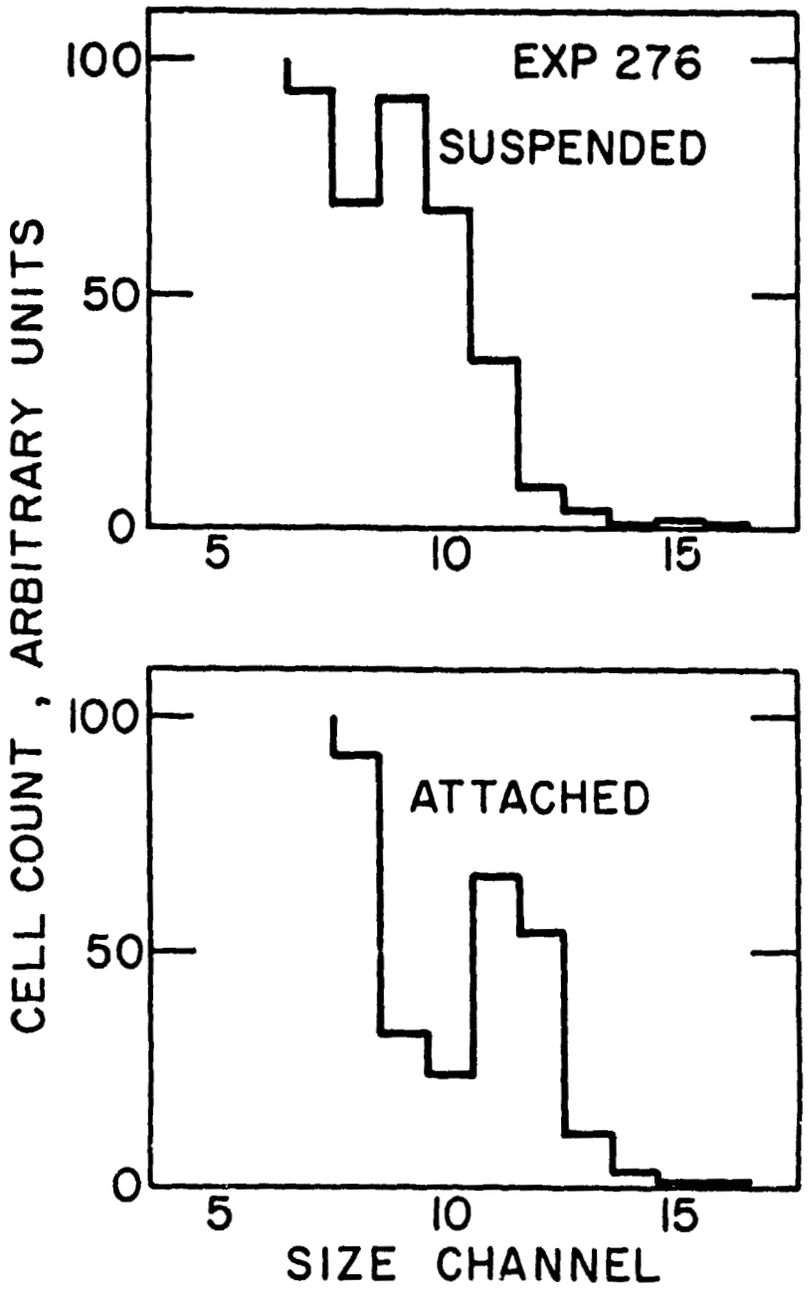


Figure 6. Coulter log-volume distributions of one fraction of cells from profile shown in Figure 5. The ordinate is proportional to cells per log-volume interval, and the abscissa is proportional to the logarithm of cell volume; each channel represents 2X the volume of the preceding channel, so that suspended cells have about ½ the volume of attached cells.



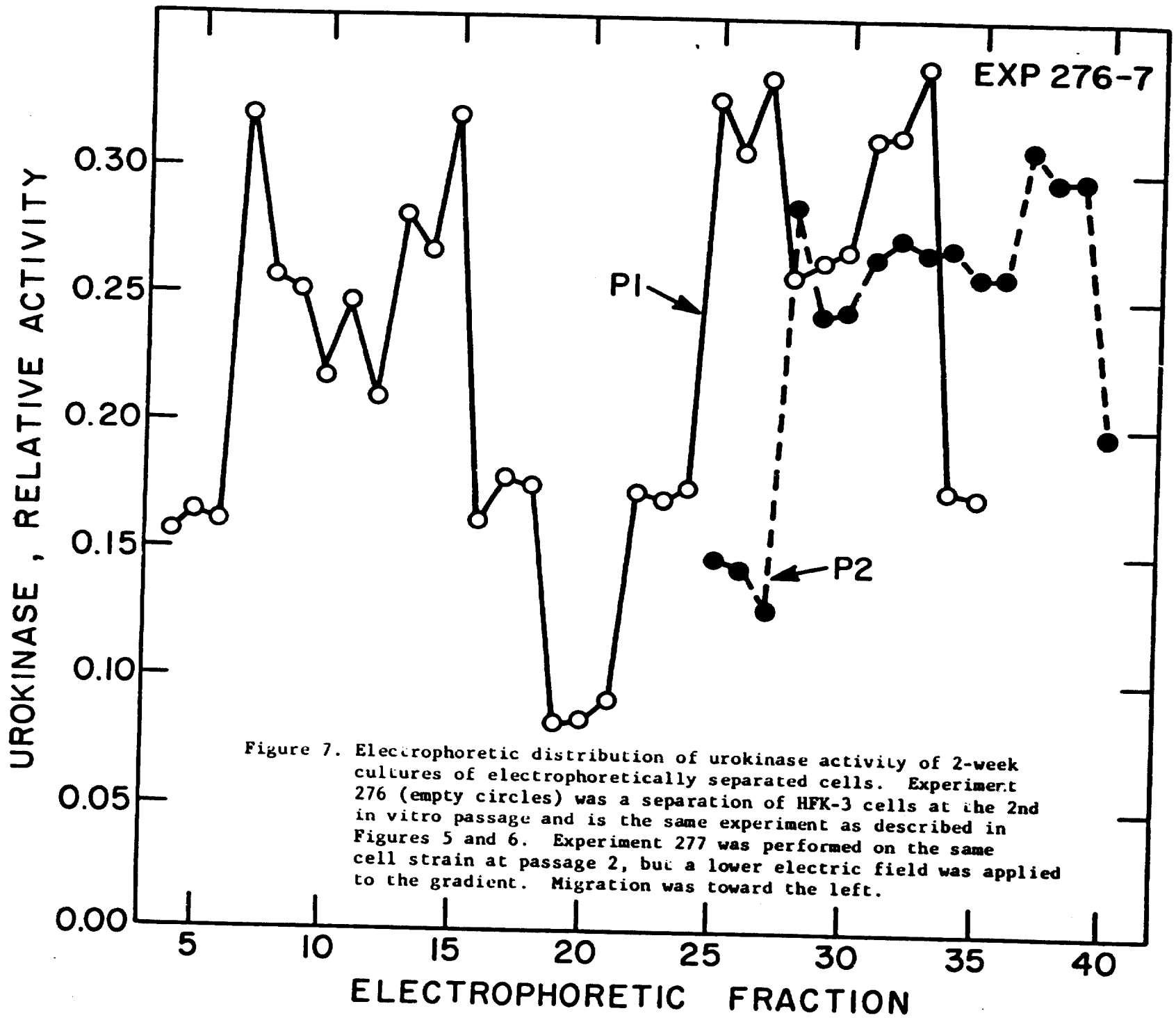



Figure 7. Electrophoretic distribution of urokinase activity of 2-week cultures of electrophoretically separated cells. Experiment 276 (empty circles) was a separation of HFK-3 cells at the 2nd in vitro passage and is the same experiment as described in Figures 5 and 6. Experiment 277 was performed on the same cell strain at passage 2, but a lower electric field was applied to the gradient. Migration was toward the left.

Figure 8. Urokinase activity of supernatants of cultures of cells separated by density gradient electrophoresis. The same cultures were sampled on successive days, and increased production was found in high and low, but not intermediate, mobility cells.