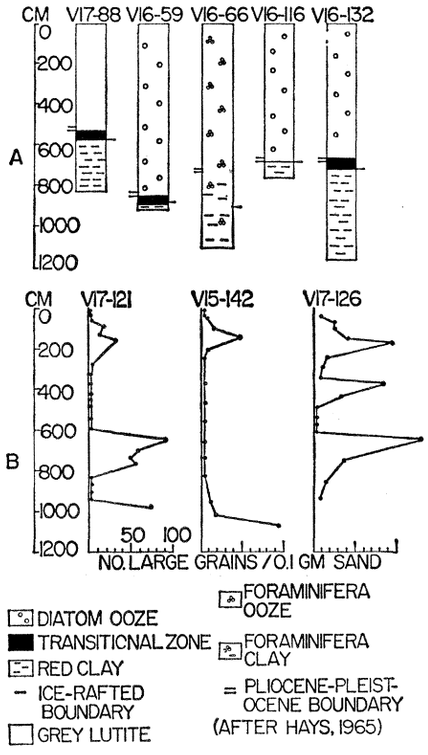

Fig. 2. *A*, Lithology of five cores from the deep-sea floor off Antarctica showing the position of the Pliocene-Pleistocene boundary, based on studies of radiolarians (*4*) and on the first appearance of ice-rafted grains. *B*, Variation of the number of large ice-rafted grains with depth in three cores from the oceanic rise in the Argentine Basin.

et al. (*7*) show that the uppermost brownish-gray zone was probably deposited during the Holocene and that the cores penetrate two to three major cold climatic periods in the Pleistocene, with a probable sedimentation rate of 2 to 5 cm per 1000 years. The zones containing assemblages associated by Groot *et al.* with glacial stages correspond very closely to the zones containing ice-rafted grains shown in Fig. 2*B*.

The results suggest that the distribution of easily recognizable, ice-rafted grains in deep-sea cores from areas around 40° to 50°S latitude can be effectively used to subdivide the Pleistocene into glacial and interglacial climatic phases, as was shown for comparable latitudes in the North Atlantic (*9*), and that the initiation of ice-rafting as evidenced in deep-sea sediments in high temperate latitudes probably is a good indicator of the initiation of the Pleistocene glaciation. Cores from south of the Polar Front contain ice-rafted material continuously after first appearance, indicating continuity of glacial conditions on Antarctica. This indicator of glacial conditions provides an independent check on the climatic interpretation of fossil fauna and flora, as demonstrated in the North Atlantic, where excellent correlation has been established by us (*10*) between zones assigned to glacial stages on the faunal evidence of Ericson *et al.* (*11*) and zones containing high concentrations of ice-rafted particles.

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12. We thank Thayer Willis and Anthony Cok, who made preliminary examinations of some of the Antarctic cores. Important support for the study was provided by a Ford Foundation grant and by a Fulbright travel grant for J.R.C. supported by ONR and NSF. Lamont Geological Observatory contribution No. 862.

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Electrophoresis of Hemoglobin in Single Erythrocytes

Abstract. *A technique for electrophoretic analysis of the hemoglobin mixtures from single erythrocytes has been developed. Hemoglobin mixtures were separated into their constituents, A_2, A, C, S, and J, at least qualitatively.*

Attempts have been made to isolate and fractionate genetically different hemoglobin mixtures from single cells. By the use of microspectrophotometric techniques, hemoglobin F can be separated from hemoglobin A, and these two fractions can be accurately determined (*1*). Unfortunately other hemoglobins, including A_2, S, C, and J, which are present in single cells in normal or pathologic conditions cannot be detected by these methods. The problem of measuring the products of single-gene activity in single cells has become more important as a way of answering questions related to genetic expressions in various phases of cell differentiation and of solving problems concerning the mechanism of the transformation from hemoglobin F to hemoglobin A during the passage from fetal to adult life. Since the heterogeneity of cell preparations so far obtainable forbids this analysis on a large scale, the analysis on single cells is mandatory.

Since electrophoresis is widely used for fractionation of various hemoglobin fractions, mainly because of differences in electric charge, the customary techniques of electrophoresis have been adapted for use with very small (picogram, 10^{-12} g) quantities in order to analyze hemoglobin mixtures. An electrophoresis method for picogram quantities has been developed by Edström (*2*) for the analysis of RNA–DNA bases of single cells. Our attempts to use Edström's technique for the hemoglobin separation, however, failed because of difficulty in placing single cells on the cellulose fiber, which he used as a supporting medium, and because hemoglobin A_2 was undistinguishable from hemoglobin A in those few occasions where an electrophoretic run was obtained. We now describe an apparatus and results for electrophoretic analysis of the hemoglobin from single erythrocytes with polyacrylamide gel as a supporting medium. The degree of separation of various hemoglobin fractions compares favorably with the separation obtained by polyacrylamide electrophoresis on a large scale.

The electrophoresis was conducted under direct microscopic observation in a special chamber. This chamber was made from a normal glass slide with glass tubes (2.5 cm in length, 0.9 cm inside diameter, 1.2 cm outside diameter) glued on its surface, 5.5 cm apart. There was at the base of each tube a semicircular opening which faced the middle of the slide. The tubes were filled to one-fourth of their volume with 1 percent agarose (Calbiochem) solution in a buffer (0.03*M*, *p*H 8.5) composed of glycine (ammonia free) and HCl-tris. The agarose was connected through the semi-circular openings with a layer (1.5 mm thick) of 1 percent agarose solution, in the same buffer, which covers the slide surface between the tubes. After complete gelation of the agarose a slot (0.7 cm wide) was cut perpendicularly

to the long axis of the agarose layer (Fig. 1). These agarose angles are used as buffer bridges to provide electrical connections with the extremities of the acrylamide fiber. This fiber is represented by the track remaining in the middle of a collodion-coated coverslip (22 by 22 mm) after a thin stainless steel wire (2 cm long, 15 μ in diameter) is pulled across a drop (10 mm^3) of acrylamide-erythrocyte suspension placed in the middle of one edge of the coverslip. Another drop (10 mm^3) of the same suspension is placed at the extremity of the track on the opposite edge of the coverslip. The acrylamide-erythrocyte suspension was prepared from a solution containing 20 percent acrylamide monomer (Eastman); 1 percent *N, N'* methylenebisacrylamide (Eastman), 10 percent glycerol, 2 ml of a 10 percent aqueous *N, N, N',N'*-tetramethylethylenediamine (Eastman), and 2 ml of a 10 percent aqueous solution of ammonium persulfate, in glycine-tris buffer (0.05*M*, *p*H 8.9). To 1 ml of this solution, 0.4 ml of 50 percent aqueous solution of polyethylenimine (Borden) and 0.01 ml of a washed suspension of erythrocytes in saline, containing about 5×10^5 cells/mm^3, were added. After vigorous stirring, the acrylamide-erythrocyte suspension was ready for the production of the fiber. With some experience fibers can be prepared which, in the middle portion, are 10 μ thick and 100 μ wide. The erythrocytes are distributed at random in the fiber, and the number of cells per unit length of the fiber should be limited to avoid the overlapping of the hemoglobin fractions from various erythrocytes during the electrophoretic run. A concentration of 5 to 10 red blood cells in a 200-micron section of the fiber is adequate.

The use of a discontinuous buffer system (glycine-HCl-tris and glycine-tris) was suggested by the experiments on disc electrophoresis because concentration of various fractions during the electrophoresis is enhanced by discontinuity in voltage and *p*H gradient, resulting at the boundary zone between trailing ion (glycine) and leading ion (chloride ion) with respective mobilities of -15 and -37 mobility units (1 mobility unit is equal to 10^{-5} cm^2 $volt^{-1}$ sec^{-1}) due to the "Kohlrausch phenomenon" (5). A nonpolar substance, polyethylenimine, which increases the viscosity of the liquid phase in the gel pores is added to limit the diffusion of the hemoglobin. Since it is impossible to measure the viscosity directly on the fiber, the amount of polyethylenimine added depends on the type of separation desired and must be estimated empirically.

After the fiber was pulled, the coverslip was inverted (fiber downwards) over the slot on the microscope slide. The two acrylamide drops were placed in contact with the agarose bridges by a slight pressure on the coverslip. The whole electrophoretic chamber was placed in an air-tight plastic chamber and filled with carbon monoxide, which had been bubbled through a saturated solution of magnesium acetate at 20°C. The fiber rapidly reached equilibrium in an atmosphere of CO (65 percent relative humidity). After 5 minutes, the time required for the acrylamide to gel completely, the fiber was covered with liquid paraffin delivered from a pipette that had previously been inserted between the slide and coverslip. The paraffin prevents evaporation and, as a nonpolar convecting medium, dissipates heat which may develop during electrophoresis.

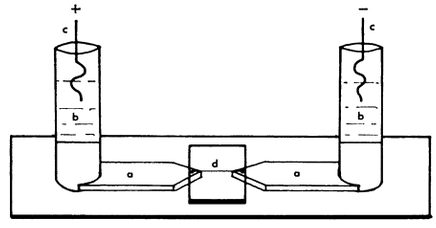

Fig. 1. Electrophoretic chamber. (*a*) Agarose bridges; (*b*) buffer reservoirs; (*c*) platinum wire electrodes; (*d*) polyacrylamide fiber on collodion-coated coverslip.

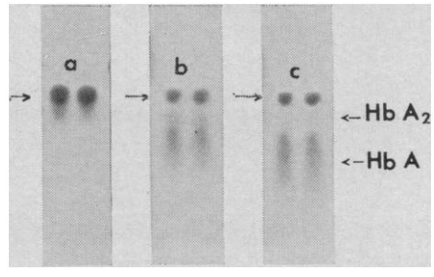

Fig. 2. Electrophoresis of the hemoglobin of two erythrocytes from a normal adult subject: *a*, *b*, and *c* are pictures taken at 1, 3, and 4.5 minutes after the current was turned on. The conditions are: 1000 v/cm; glycine-HCl-tris discontinuous buffer; *p*H 8.5, 0.03*M*; relative humidity of the fiber, 65 percent. The picture was taken at 418 mμ ($\times$ 600). Unattached arrows indicate starting zone.

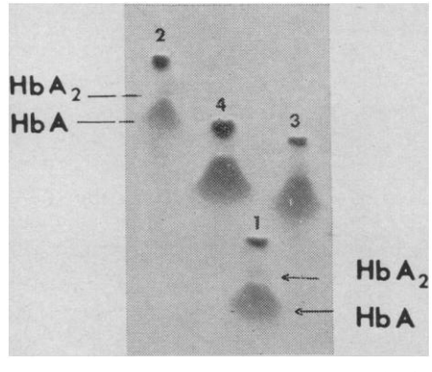

Fig. 3. Electrophoresis of the hemoglobin of four erythrocytes from a normal adult subject, 45 minutes after starting the current. Relative humidity of the fiber: 44 percent; other conditions same as in Fig. 2 ($\times$ 600).

The electrophoretic chamber was then removed from the air-tight chamber, and a suitable portion of the acrylamide fiber with the embedded erythrocytes was selected by microscopic observation ("Zeiss WL" microscope illuminated by a "Bausch and Lomb" monochromator. Light was supplied by a quartz-iodine lamp). The wavelength of 418 mμ was used since it coincides with the absorption maximum of carboxyhemoglobin (CO Hb) in the Soret region. The two tubes of the electrophoretic chamber were filled with glycine HCl-tris buffer (0.03*M*, *p*H 8.5), and two platinum wire electrodes were inserted. Current (d-c) at 800 volts was supplied from a regulated power supply, furnishing about 1.5 to 2 μa.

Figure 2 shows in sequence the beginning of the run, and the separation of hemoglobin from the erythrocytes of two normal adults. One minute after the current was applied, hemoglobin started to move from the cells (Fig. 2*a*). Three minutes later almost all the hemoglobin migrated from the cell (Fig. 2*b*), and at 4.5 minutes the separation of the hemoglobin in two fractions was evident (Fig. 2*c*). The stroma of the erythrocytes could be seen at the starting zone, particularly if the microscope condenser was lowered in order to obtain a phase-contrast effect. Sometimes the stroma disappeared shortly after the beginning of the migration, probably because of complete lysis (Figs. 4*a*, 5*a*, 5*b*). Calculation of the migration ratio for the small fraction in respect to the main fraction (arbitrarily set equal to 1) gave a value of 0.50 to 0.55. These values are comparable to the values of Nakamichi and Raymond (*4*) for the hemoglobin A_2 in an acrylamide-gel electrophoresis. Thus the fraction with slower mobility represents hemoglobin

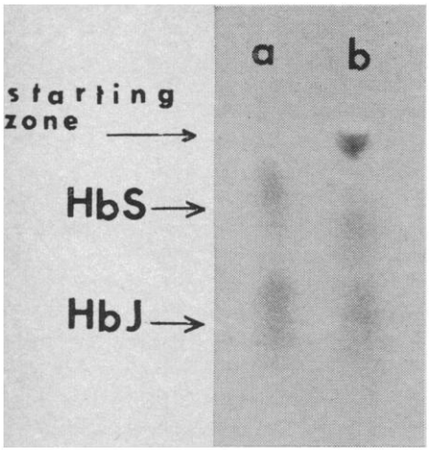

Fig. 4. Electrophoresis of the hemoglobin of two erythrocytes from a patient with hemoglobin S and J disease. The starting zone for cell *a* is indicated by the arrow. The conditions are the same as in Fig. 2. The picture was taken 5 minutes after the start of the current (× 600).

A_2 and the major fraction is constituted by hemoglobin A.

Electrophoresis for longer than 5 minutes was of no advantage since the image of the hemoglobin A_2 fraction fades, and identification becomes very difficult.

Figure 3 shows the electrophoretic pattern of four erythrocytes from a normal adult person. The separation into two fractions of the hemoglobin became less evident from cells No. 1 and No. 2 to cells No. 3 and No. 4. The degree of separation was parallel to the increasing concentration of the major hemoglobin component (hemoglobin A), which was highest in cell No. 4. It has not been possible to prove whether cell No. 4 totally lacked

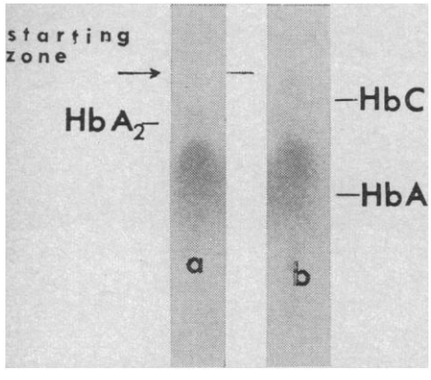

Fig. 5. Electrophoretic pattern of the hemoglobin of a normal adult subject (*a*), and of a patient with hemoglobin C disease (*b*) on two different fibers. The starting zone for both cells is marked by the arrow. The pictures were taken 4.5 minutes after starting the current (× 1000).

the minor component or if its absence was due to a stronger trailing effect of the major hemoglobin A fraction. To obtain an answer would require longer runs to separate hemoglobin A completely from hemoglobin A_2. A similar observation on the decrease in separation between two fractions of close electrophoretic mobilities when one was present in very high concentration with respect to the other has been made by Robinson *et al.* (*5*) who used agar-gel electrophoresis.

The result shown in Fig. 3 was obtained with acrylamide fibers equilibrated at 44 percent relative humidity with CO that was passed through a saturated solution of potassium carbonate. The decreased water content of this fiber in respect to the fiber in Fig. 2 resulted in a reduction of diffusion of the fractions, which appears better defined.

Figure 4 shows the separation of hemoglobin components from single cells of a hemoglobin S patient and another component with an electrophoretic mobility higher than hemoglobin A on paper electrophoresis, which probably is represented by hemoglobin J. Electrophoresis of hemoglobin from two cells of this patient shows (Fig. 4) two clearly distinctive components in roughly similar concentrations. Their relative mobilities of 0.07 and 1.3, in respect to hemoglobin A, are suggestive for hemoglobin S and hemoglobin J, respectively. In Fig. 4 the stroma of cell *a* is undetectable; and the starting zone, which is different for the two cells, can be established only from the position of the cell before the current was applied.

Figure 5 shows the electrophoretic patterns of hemoglobin from two erythrocytes on two different fibers. The concentration of hemoglobin C in this patient was 6 percent as evaluated by ordinary paper electrophoresis. In both cases the erythrocytic stroma was not detectable, and the starting zone was established by the same criteria as for cell 4*a*. The comparison of the electrophoretic patterns of cells *a* and *b* shows in both cells the presence of a major component (hemoglobin A) with similar electrophoretic mobility, and a minor component, which in cell *b* has a slower mobility than in cell *a*. This fraction is identified with hemoglobin C.

The results show the feasibility of microelectrophoretic analysis for the separation of various fractions of protein mixtures from single-cell cytoplasm. The hemoglobin from single erythrocytes is a model for investigation of the limits of the resolution of this technique because the high absorption of the heme in the Soret region permits a direct detection of the fractions. Attempts to stain the hemoglobin fractions with amidoblack or similar substances before or after fixation with acetic acid, heat, or ethanol were totally unsuccessful. The high absorption of acrylamide in the ultraviolet limits the possibility of using their absorption at 280 mμ. Interference microscopy might be applied to solve the problem of detection and quantitation of other proteins or compounds which do not absorb in the visible range provided the acrylamide gel fiber is optically homogeneous. The possibility of isolating hemoglobin A, A_2, S, C and hemoglobin J from single cells, as shown by our results, opens the field of analysis of γ-, β-, and δ-hemoglobin chains in single cells on a quantitative basis, and of studying genetic activity of erythroblasts where the synthesis of various polypeptide chains takes place. Although no precise figures are available, electrophoresis on acrylamide has a much greater sensitivity for very small quantities than other types of electrophoresis. Since the average amount of hemoglobin in single cells is 30×10^{-12} g, and the average hemoglobin A_2 content is 2.5 percent of total hemoglobin, as little as 7×10^{-13} g of hemoglobin may be detected with this technique.

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