Abstract. Electrophoretic patterns of enzymes in single vertebrate erythrocytes may be visualized with the use of capillary tube polyacrylamide columns and stains which amplify the enzymatic activity. The following points have thereby been demonstrated:

1. Peripheral blood of metamorphosing bullfrogs (Rana catesbeiana) contains two molecular forms of hemoglobin: embryonic (tadpole) and adult (frog). Only one of these forms is found in each erythrocyte.

2. Human umbilical cord blood contains fetal and adult hemoglobins; both forms may be found in individual erythrocytes, in varying proportions.

3. Both lactate dehydrogenase isozymes found in whole blood of bullfrogs and humans are found in individual erythrocytes, but the proportions differ from cell to cell.

Introduction. Several vertebrate species undergo a change as they develop in the predominant hemoglobin they synthesize, from an embryonic to an adult form. The two hemoglobin forms contain different polypeptides, hence developmental hemoglobin changes represent specific gene regulation. To understand the mechanism of regulation, it is necessary to know whether separate cells synthesize embryonic and adult hemoglobins or whether a single cell can synthesize both.

Many vertebrate enzymes have been found to be composed of multiple molecular forms, often derived from multiple genes for the enzyme subunits. Lactate dehydrogenase (LDH), for example, is a tetramer composed of two different subunits, A and B, encoded by nonallelic genes; the subunits tetramerize nonrandomly or randomly to produce one of five basic molecular forms, called isozymes. It has not yet been ascertained whether such enzyme multiplicity results from gene regulation at the level of the individual cell or the cell population or clone, i.e., whether each cell contains all molecular forms or only contributes a single component to the isozyme complement of the tissue.

Methods and Materials. Larval and adult stages of Rana catesbeiana were purchased from Lemberger Co., Oshkosh, Wis., and also caught in ponds in the New Haven vicinity. Erythrocytes were collected into heparinized amphibian-Ringer's solution at 4°C, by cardiac puncture of adults and by cutting the tip off the ventricle in tadpoles; this blood was washed free of plasma three times in cold Ringer's solution. Fresh heparinized blood from human umbilical cord was obtained by the courtesy of the Yale-New Haven Hospital Obstetrics Department, and was washed three times in phos-
phosphate-buffered saline at 4°C. A portion of each sample was lysed with 2 volumes of distilled water plus 0.1 volume of toluene and electrophoresed on standard disc acrylamide gels which were stained from hemoglobin, lactate dehydrogenase, and glucose 6-phosphate dehydrogenase to exclude genetic variants.

For single-cell studies, three samples from genetically similar individuals were pooled and single cells were collected by micromanipulation. Glass micropipettes with tips about 30–40 μm in diameter were fitted to a rack and pinion type micromanipulator. The erythrocytes were diluted to about 5000/ml in Ringer’s buffer and one drop was placed on a glass slide. The cells were collected individually under a light microscope at 100 power. When a cell was sucked into the micropipette tip (Fig. 1), it was lysed by the addition of two volumes of water and brief agitation (Fig. 2). The lysate was stored in a capillary test tube at 4°C until electrophoresed.

![Fig. 1](image1.jpg)
![Fig. 2](image2.jpg)
![Fig. 3](image3.jpg)
![Fig. 4](image4.jpg)

**Fig. 1.**—An erythrocyte of *Rana catesbeiana* is isolated in a micropipette.

**Fig. 2.**—Addition of 2 vol of water, and brief agitation by sucking in and out, lysed the cell.

**Fig. 3.**—Polyacrylamide gel electrophoresis of hemolysates from *Rana catesbeiana* (8 cm gels).

**Fig. 4.**—Electrophoresis in capillary columns of single erythrocytes from metamorphosing *Rana catesbeiana*. Benzidine stain.

Polyacrylamide electrophoresis was performed in 0.4–0.45 mm diameter glass capillaries by a method adapted slightly from that published. The tubes, filled with small pore gel by capillarity and sealed at one end with hemotocrit putty, were stood vertically in a stand punched out of a small cardboard box. About 1/2 cm of solution was removed with a micropipette, and water was layered on top. In general the method was precisely that used for larger gels except that micropipettes were used for layering, and proportionally twice the recommended volume of spacer gel was used. The columns were
run in a scaled-down standard disc electrophoresis apparatus at \( \frac{1}{4} \) mA per tube, constant current, at room temperature, for approximately 5 min, or until the tracking dye was \( \frac{1}{4} \) cm from the end. At this time, the entire apparatus was immersed in an ice-water bath and each gel removed as rapidly as possible by forcing it out with propylene glycol from a syringe with a no. 30 needle.

For the location of hemoglobin, Coomassie blue was sufficiently sensitive, but the benzidine stain was preferred. For lactate dehydrogenase and glucose 6-phosphate dehydrogenase, standard stains were used but were concentrated 20 times. Substrate and coenzyme controls were included for all experiments by the omission of these materials from the stains. All stains were warmed to 37°C before gels were added. Gels were incubated in the stains until clear banding appeared (3-8 hr for all but the benzidine stain which took 2-3 min). Densitometry was performed on a Joyce-Loebl microdensitometer with the use of a 0.5 mm lucite carrier.

Another method of staining was with fluorescein-conjugated antibody to the proteins. The gels were fixed in 95% ethanol for 2 hr, washed in phosphate-buffered saline, incubated at 37°C for 1 hr with the fluorescein-conjugated antibody, washed thoroughly, and examined at once in a Zeiss fluorescent microscope at about 360 nm. The methods of antibody induction in rabbits and preparation of antibody-fluorescein conjugates have been published.

Results. The enzymes from single cells could be visualized reproducibly.

1. **Bullfrog hemoglobin**: Three *Rana catesbeiana* were bled at metamorphic climax (hind limb/tail ratios of 1.0-2.6). Both tadpole and frog-type hemoglobins were present at this stage (Fig. 3). Two hundred gels were run. Half were stained for hemoglobin and half for protein. Only a single area of hemoglobin was obtained for each erythrocyte (Fig. 4). As judged by the distance from the tracking dye, 28% of the cells contained hemoglobin with the mobility of the adult form, while 72% contained the tadpole form. No gels were seen with two bands. Twenty-five erythrocytes taken from the tails of three pre-metamorphic tadpoles and 25 taken from three adults displayed single hemoglobin bands with the mobility of embryonic and adult hemoglobins, respectively. Although with larger samples many minor bands are observed at both hemoglobin peaks, with single cells chiefly the major bands were observed. Confirmation of the identity of adult hemoglobin in twelve gels was obtained with fluorescein-conjugated monospecific antibody to frog hemoglobin.

2. **Human hemoglobin**: Two hundred gels were run with umbilical cord erythrocytes from three samples and stained for hemoglobin with benzidine reagent. 46% displayed varying proportions of two different bands (Fig. 5) while the remainder had only one. Nine out of twelve gels with only one band stained with fluorescein-conjugated monospecific antibody to human fetal hemoglobin F.

3. **Lactate dehydrogenase and glucose 6-phosphate dehydrogenase**: Each of 24 bullfrog and 24 human cord erythrocytes contained the same lactate dehydrogenase isozyme complements as did whole hemolysates (Fig. 6); however the proportions were not always the same. In one female frog were three isozymes of glucose 6-phosphate dehydrogenase. Twenty-four gels were analyzed. Some gels contained only one of these isozymes while others contained the other two isozymes; the situation suggested sex-linkage and X-chromosome inactivation but more heterozygous individuals must be studied to confirm this.
**Discussion.** The developmental change in *Rana catesbeiana* hemoglobin must be accomplished by the appearance at metamorphic climax of a new clone of erythrocyte precursors that synthesize adult hemoglobin. A similar mechanism appears to hold for mouse hemoglobins. In contrast, the change in human hemoglobins during embryonic development seems to depend upon a gradual individualistic shift in synthesis in precursor cells from a predominance of fetal, to a predominance of adult, hemoglobin. Since the human hemoglobins differ by one polypeptide chain, and the frog hemoglobins by at least one, the hemoglobins reflect changes in specific gene activity. The differences in localization of the genetic regulatory mechanism controlling the ontogenetic hemoglobin switches are basic to further work on the molecular basis for such switches. For the medical scientist, therapeutic control of the fetal-adult hemoglobin switch could offer relief from the symptomatology of such congenital hemolytic anemias as sickle cell disease.

The appreciation that multiple molecular forms of lactate dehydrogenase are present within each cell is basic to understanding the origin of isozymes. It is interesting that even though each cell was capable of producing both LDH isozymes present in the whole tissue, considerable heterogeneity was apparent in the proportions of these isozymes. In the case of the frog, the two isozymes are A4 and B4, products of two different genes, so that differences in their proportions in different cells would reflect heterogeneity in gene activity in a relatively homogeneous cell type. Although it was not possible to stain for hemoglobin and LDH in the same cell, there was no reason to suspect a correla-
tion between tadpole or adult hemoglobin synthesis and LDH subunit synthesis, for there was no difference in isozyme pattern between tadpoles and adults.

Another conclusion derived from the LDH data is that the controls upon subunit association, which prevent the A and B subunits from tetramerizing randomly in frogs as they do in mammals, are not due to isolation of the cells synthesizing the two subunits; both are synthesized in the same cell but cannot associate. This has previously been shown for fish LDH as well.3

This technique allows one to analyze the activity of genes in single cells. Clones in culture do not yield the same kind of information since, as the LDH data have shown, apparently uniform cell populations may display heterogeneity in gene activity. By electrophoresis of enzymes from single cells it is possible to quantify X-inactivation, compare activities of alleles in different cells, compare cells from different stages in a maturation sequence as in erythropoiesis and spermatogenesis, and study genetic individuality within homogeneous cell populations. At present, radioactive amino acid uptake into the erythrocytes prior to electrophoresis is being developed as a means to quantify the activities of single genes more precisely.

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Abbreviations: LDH, lactate dehydrogenase.

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† Present address: Department of Medical Genetics, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104.