characteristics of the FPV in the squid retina, but it can account for three other observations as well. These are (i) Smith and Brown's (*9*) finding that the short-latency photovoltage of *Limulus* photoreceptors reverses sign when the recording microelectrode is thrust through the receptor cell membrane, (ii) Cone and Brown's report (*10*) that the vertebrate FPV disappears when rhodopsin in rods is disoriented by heat treatment, and (iii) our observation (*3*) that the squid FPV is similar in size and shape to that of vertebrates, even though the cell membranes are oriented and folded quite differently in the two types of retina. What physical process produces the membrane current? The short latency and simple kinetic form of the FPV (*3*) favor the idea that the plasma membranes of the outer segments themselves contain rhodopsin and that a photochemically induced change in its molecular charge distribution constitutes a change in the charge stored in the electrical capacitance of the membranes. For the biphasic FPV's described in our work, the charge displacements are such as to require the membrane's external surface to become relatively positive to its internal surface when rhodopsin is converted to acid metarhodopsin and relatively negative when the latter is reconverted to rhodopsin by the flash.

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Hexokinase Isoenzymes in Human Erythrocytes

Abstract. *The electrophoretic mobility of hexokinase from human erythrocytes and other tissues was studied with a new method that depends on the fluorescence of reduced nicotinamide-adenine dinucleotide phosphate for detecting enzyme activity on starch gel. The hexokinase of cord-blood erythrocytes has slightly different electrophoretic properties from that of adult red cells. Type I enzyme is split into type I_A and type I_F; the latter is more intense in cord blood; in hemolyzates of adult blood, the activity of the two bands is usually about equal. No type II enzyme was found in cord blood. The double type I band was present in red cells from adult rabbits.*

Four hexokinase isoenzymes found in rat tissues (*1–3*) have been numbered I, II, III, and IV in increasing order of electrophoretic mobility at *p*H 8.6 (*1*).

After analyzing the electrophoretic mobility of human erythrocyte hexokinase, Holmes *et al.* (*4*) concluded that normal adult erythrocytes contained only type I and type III enzymes. Red cells from newborns, infants, and adults with persistence of fetal hemoglobin contained an electrophoretically distinct hexokinase that moved somewhat faster than the type I enzyme detected in adult erythrocytes. This was identified as type II enzyme, although it moved more slowly than the type II enzyme of rat liver.

Exact localization of hexokinase when tetrazolium dyes are used in staining is difficult because of the relatively low activity of hexokinase in red cells and other tissues and because of nonspecific reduction of tetrazolium dyes by 2-mercaptoethanol incorporated in the gel to preserve hexokinase activity. In our study after electrophoresis, fluorescence of reduced nicotinamide-adenine dinucleotide phosphate (NADP) was used to reevaluate these findings. Apparently, the enzyme in red cells of newborns (*4*) does not coincide with type II enzyme of either rat liver or human tissues. Rather, comparison of the electrophoretic mobility of this enzyme with type II isoenzyme at different hydrogen-ion concentrations and in different human tissues indicates that it is a separate hexokinase isoenzyme, which we have designated I_F.

We studied human adult and cord-blood hemolyzates and extracts of other human, rat, and rabbit tissues, including leukocytes, liver, muscle, heart, kidney, and fat. Crude extracts were prepared in a 0.01*M* phosphate buffer, *p*H 7.0, containing 5 m*M* 2-mercaptoethanol, 5 m*M* neutralized sodium ethylenediaminetetraacetate (EDTA), and 10 m*M* glucose (*4*). Hemolyzates and leukolyzates were made from one volume of this medium per milliliter of packed, washed cells, frozen and thawed three times, and centrifuged at 30,900*g* for 30 minutes at 4°C. Tissue homogenates were prepared with 1 ml of medium per gram of fresh tissue and were centrifuged under the same conditions. Starch-gel electrophoresis was performed for 4 hours at 16 to 24 volts per centimeter in a horizontal system with a buffer containing 10 m*M* tris(hydroxymethyl)aminomethane (tris), 0.5 m*M* EDTA, 5 m*M* 2-mercaptoethanol; the *p*H was adjusted with HCl or with H_3PO_4 over the range 7.85 and 9.6. In the electrode compartment, the buffer, except for the 2-mercaptoethanol, was ten times more concentrated. Some experiments were made at *p*H 7.5 with 0.01*M* potassium phosphate buffer for the gel and 0.1*M* phosphate buffer in the electrode compartments. Stronger hexokinase bands were found when 1 m*M* glucose was incorporated in the gel, and this was done in most of our studies.

The position of hexokinase on the gel was ascertained by applying to the sliced gel a mixture comprised of 0.1*M* tris (*p*H 7.6), 5 m*M* adenosine triphosphate (ATP), 5 m*M* $MgCl_2$, 1.3 m*M* NADP, 0.4 unit of glucose-6-phosphate dehydrogenase per milliliter (*5*), and either 0.5 m*M* or 0.1*M* glucose at approximately 25°C. At intervals of 30 minutes, the gel was illuminated with ultraviolet light of long wavelength and photographed with a yellow filter.

Hexokinases I, II, and III from human, rat, and rabbit tissues have similar mobility (Figs. 1 and 2). Type III enzyme is strongly inhibited by high concentrations of glucose (0.1*M*), a useful property for identification of the band (*1, 3*). Conversely, type II is stabilized and better visualized in the presence of high concentrations of glucose. Enzyme from striated muscle and adipose tissue serve as reference standards for identifying enzyme bands in human erythrocytes.

In erythrocytes from normal human adults, a double type I band and a much weaker, single type III band are

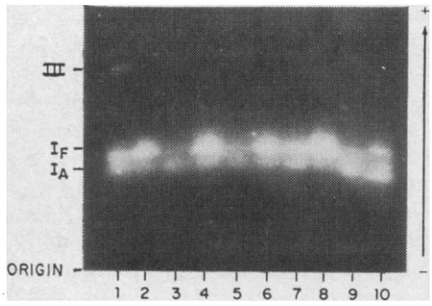

Fig. 1. Starch-gel electrophoresis of hemolyzate prepared from human adult blood and cord blood (picture taken after 1 hour). Tris-phosphate buffer, *p*H 8.0, and 0.5 m*M* glucose in the developing mixture were used. Channels *1, 3, 5, 7,* and *9* contain hemolyzates prepared from the blood of five adults. Channels *2, 4, 6,* and *8* contain hemolyzates prepared from the cord blood of four newborn infants. Channel *10* contains hemolyzate from adult rabbit blood. Hexokinase band III becomes visible only after prolonged staining.

found (Fig. 1). In most adults, the two type I bands have equal intensity. The slow band, comparable to the type I band encountered in other tissues (Fig. 2), is designated type I_A, and the fast component is designated I_F. The latter has not been found in other human adult tissues investigated (leukocytes, liver, adipose tissue, muscle, heart, and kidney) or in cord-blood leukocytes.

In human cord-blood hemolyzates, the double type I pattern is also present, but the I_F band is much more prominent (Fig. 1). A few normal adults

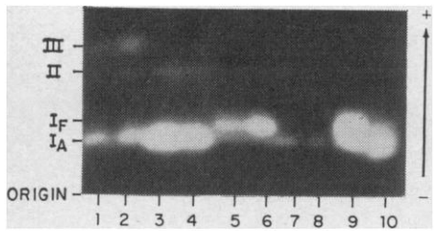

Fig. 2. Electrophoresis of hemolyzates of human blood and tissue extracts (picture taken after 1 hour). Concentrations of buffer and glucose were the same as in Fig. 1. Channels *1* and *2*, liver; *3* and *4*, muscle; *5*, adult hemolyzate ("fetal"-type pattern); *6*, cord hemolyzate; *7* and *8*, fat; *9*, hemoglobin-free fetal hemolyzate; *10*, hemoglobin-free adult hemolyzate. Hexokinase bands II and III become definite only after prolonged staining. The separation of bands I_F and I_A in hemoglobin-free hemolyzates (channels *9* and *10*) is incomplete because of the large amount of enzyme in these channels. When smaller amounts of enzyme were used, a clearly double-banded pattern appeared.

also show this "fetal" pattern. Type III hexokinase is not usually seen in cord-blood hemolyzates. When the electrophoresis is performed at *p*H 8.6, hemoglobin A masks band I_F, and hemoglobin F masks band I_A because of quenching by the large amount of hemoglobin present. An artifactual pattern is thus obtained, so that only band I_A is seen in adult red cells and only band I_F is seen in fetal red cells. Only at *p*H values such as *p*H 8.0, at which hemoglobin moves more slowly than hexokinase and therefore does not obscure the hexokinase pattern, does the double nature of red cell hexokinase type I become obvious. At *p*H 7.5, the duplication of type I apparently disappears, and any difference between cord-blood and adult hemolyzates vanishes. Erythrocyte extracts freed from hemoglobin by treatment with diethylaminoethyl cellulose (*6*) and concentrated by vacuum dialysis display the same electrophoretic pattern as crude hemolyzates. The fast-moving band I_F was compared with human type II enzyme for muscle, fat, and liver extracts. At any *p*H, the I_F band was always less anodic than band II. Although I_F tends to merge with I_A below *p*H 7.8, band II remains clearly distinguishable.

The duplication of type I enzyme in erythrocytes is not affected by the omission of 2-mercaptoethanol or of EDTA, incubation with 1 m*M* glutathione (oxidized), or electrophoresis in 2*M* urea. Glucose or phosphate had no effect. Omission of freezing and thawing in the preparation of the hemolyzates did not change the electrophoretic pattern.

We also considered the possibility that one of the bands represented 6-phosphogluconic dehydrogenase (6-PGD) acting upon 6-phosphogluconic acid generated in the glucose-6-phosphate dehydrogenase reaction. This was ruled out by the finding that partially purified hexokinase, which is free of 6-PGD activity and therefore gives no band when phosphogluconic acid is used as a substrate, still gives the typical hexokinase pattern with glucose and ATP as substrate.

In the absence of ATP, no band is obtained in erythrocytes, although in liver extracts occasionally there is a band moving toward the cathode. This band is present even when both ATP and glucose are omitted and is enhanced when NAD is substituted for NADP. It may be identical to the cathodic "low-K_m hexokinase" (*7*). Probably this band represents alcohol dehydrogenase (*8*).

A double type II band in rat liver has been described (*1, 9*), and a double band has been observed with type IV (glucokinase) in rat liver, kidney, and testis (*10*). This suggests that different hexokinase isoenzymes can exist in two or more metastable states like those of yeast hexokinase (*11*). Such duplicated bands may partly account for discrepancies in the number of hexokinase isoenzymes found in the red cells (*12*).

We confirm that the hexokinase pattern of human cord-blood erythrocytes differs slightly from that of adult erythrocytes. However, when the interfering effect of hemoglobin is removed, this difference is apparently not as great as it was originally thought to be. The hexokinase band which is enhanced in fetal cells is not type II hexokinase, but it represents the I_F isoenzyme which is also present in adults. The duplicated type I enzyme is not peculiar to the human species; it has also been found in adult rabbit erythrocytes (Fig. 1, channel *10*). These latter cells appear to have equally strong I_A and I_F bands and no type III band. Whether the "fast" type I band represents a unique erythrocyte isoenzyme or is due to special reactivity of type I hexokinase toward the environment in the red cell is unknown.

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