ELECTROPHORETIC SEPARATION OF HEXOKINASE ISOENZYMES IN AGAR GEL

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The writers suggest a method of electrophoretic separation of the hexokinase isoenzymes in agar gel. This method is much less laborious than other known methods of separating these isoenzymes and it requires a smaller quantity of expensive reagents.

Of the existing methods of separation of isoenzymes, because of its simplicity and convenience, electrophoresis in agar gel has become widely used in the laboratory. However, so far as the separation of the isoenzymes of hexokinase (2.7.1.1), a key enzyme of glycolysis, is concerned there is a detailed account of electrophoresis in starch gel [4] but no information on electrophoresis in agar. Nevertheless, electrophoretic separation of hexokinase isoenzymes in starch gel is relatively laborious, and quantitative determination of the stained isoenzymes is difficult because of the opacity of the gel.

The proposed method of determining hexokinase isoenzymes is based on the method of electrophoretic separation of lactate dehydrogenase isoenzymes in agar gel suggested by Yurkov and Alatyrtsev [1], and the staining method of Katzen and Schimke [4]. The most effective conditions for electrophoretic separation were chosen and optimum proportions of the substrate mixture for the color reaction were found.

The complete analysis is made up of the following principal stages: the preparatory treatment of the tissues, preparation of the agar gel, electrophoresis, and staining the isoenzymes.

Preparation of the Tissues for Electrophoresis. The animals were killed by decapitation. The tissues for testing were quickly removed, washed free from blood with cold tris-HCl buffer, pH 7.4, containing 5 mM Na_2EDTA and 5 mM 2-mercaptoethanol (ME). The samples were homogenized in two volumes of the same buffer for 1 min. The homogenate was centrifuged at 4°C for 1 h at 30,000 g. Erythrocytes, separated from plasma, were twice washed with three volumes 0.9% NaCl solution with 0.01 M glucose. Hemolysis was carried out by addition of an equal volume of a mixture of bidistilled water and ether in the ratio of 3:1 to the erythrocytes [5]. The bidistilled water contained 5 mM Na_2EDTA, 5 mM ME, and 0.01 M glucose. The hemolysate was centrifuged at 4°C for 20 min at 30,000 g.

Electrophoresis. The supernatants were applied in a volume of 0.05 ml by a micropipet to a groove in the agar one-third of the distance from the edge of the side, as the origin. A 1.5% agar gel was used instead of the ordinary 1% gel. With this concentration of agar, the separation of the isoenzymes was clearer. The gel was made up in 0.021 M tris-HCl buffer, pH 8.4, containing 0.02 M boric acid, 0.001 M Na_2EDTA, 5 mM ME, and 0.01 M glucose. The glucose was added to the gel to stabilize the hexokinase activity. The electrode buffer had the following composition: 0.21 M tris-HCl buffer, pH 8.0, 0.15 M boric acid, 0.47 M Na_2EDTA, 5 mM ME. By using, instead of Na-barbital buffer, a tris-HCl buffer of the same pH, as Brewer [3] showed, the formation of a layer of hemoglobin above the hexokinase isoenzymes is prevented. Electrophoresis was carried out for 3.5 h at 4°C with a voltage of 90 V and current 30 mA.

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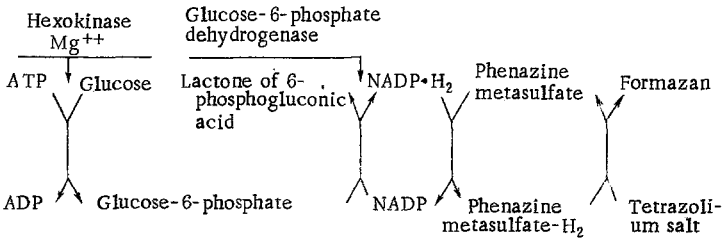

Fig. 1. Scheme of reactions used to determine hexokinase by Katzen and Schimke's method.

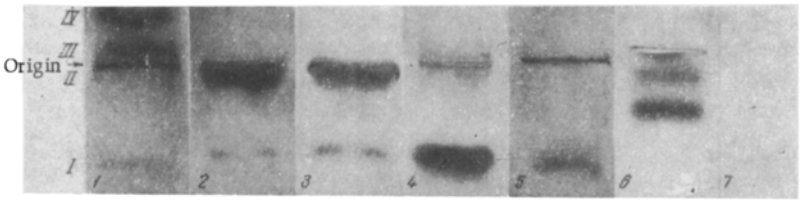

Fig. 2. Distribution of hexokinase isoenzymes in rat tissues and human erythrocytes: 1) liver; 2) muscle; 3) heart; 4) kidney; 5) brain; 6) erythrocytes; 7) control (staining without ATP, the same in all tissues); I-IV) types of hexokinase isoenzymes.

Staining. The slides with agar gel were placed in a bath containing the substrate mixture with the following components: 0.1 M tris-HCl buffer, pH 7.4, 0.5 mM NADP, 10 mM $MgCl_2$, 5 mM ATP, 2 mM NaCN, 20 mg glucose-6-phosphate dehydrogenase to 60 ml of solution, 0.13 mM phenazine metasulfate, and 0.47 mM nitro-BT. The NADP concentration was reduced to one-tenth of that in the usual formula [4], which did not affect the intensity of staining but which made the method very much cheaper. Remembering that the optimum for catalytic activity of hexokinase occurs when the ratio Mg^{++}:ATP = 2:1 [2], the $MgCl_2$ concentration was doubled. No glucose was added to the staining mixture, because the glucose added to the gel was sufficient to reveal all types of isoenzymes. All components of the substrate mixture were added immediately before incubation. Incubation at 37°C continued for 1.5 h.

The principle of determination of the hexokinase isoenzymes is based on their role in transferring the third phosphate group of ATP to the C6 atom of the hexose. The glucose-6-phosphate thus formed is oxidized with the aid of glucose-6-phosphate dehydrogenase. The NADP is thereby converted into its reduced form. The phenazine metasulfate added to the reaction mixture, accepting hydrogen from NADP · H_2, transfers it to the dye nitro-BT which, on reduction, forms insoluble formazan salts (Fig. 1). These salts are adsorbed wherever the hexokinase isoenzymes are found.

Four types of hexokinase isoenzymes were detected in rat tissues. Types I and II migrate toward the cathode, and III and IV toward the anode. In the character of distribution of their isoenzymes, the tissues differed significantly from each other. Four types of isoenzymes, for example, were found in the liver, types I and II (mainly II) in muscle, types I and II in about equal intensity in the heart, and types I and II (mainly I) in the kidney. Two factions were found in human and rat erythrocytes (Fig. 2). Comparison with the results of electrophoresis in starch gel [1] showed agreement as regards both the order of arrangement of the types of hexokinase isoenzymes from cathode to anode and in the distribution of types in the rat tissues.

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