Separation of Lactate Dehydrogenase Isozymes by Paper Electrophoresis* (33526)

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Many investigators have used electrophoresis to separate different molecules that bear the same enzymatic activity, often referred to as isozymes. Agar (1), starch (2), or polyacrylamide (3) are used as supports, rather than paper, because although amnipulation of the former is more difficult, the usual paper strip gives bad resolution. This limitation is especially evident when the problem is to separate isozymes obtained from tissue extracts.

Previous studies have shown that separation improves if paper is impregnated with dilute solutions of salt or buffers (4), but this procedure is not sufficient for the quantitative determination of all the different enzymatic fractions. If proteins are added to homogenates of organs before electrophoretic migration is attempted, however the separation improves, and the activity of lactate dehydrogenase isozymes increases. Different heterogeneous proteins such as human blood serum and gelatin have been tried, as well as pure proteins such as bovine and human serum albumin, hemoglobin, or gamma globulin.

A mixture of albumin and gamma globulin incorporated into different homogenates allows a perfect electrophoretic separation of the five LDH isozymes. The method described in the present report is not only simple and economic but also has good precision and could be easily introduced in any routine laboratory. It can be also be applied, with suitably modified incubation media, to the detection of other dehydrogenase isozymes, e.g., alcohol, glutamate, and malate.

Homogenized extracts of human, rabbit, and rat tissue were prepared according to Kaplan (5) except that 0.9% (w/v) sodium chloride was added instead of sucrose. Ratio of salt solution to wet tissue was 5:1 (v/w). The amount of extract used in electrophoresis depends on its activity and is usually between 10 and 20 μl.

Electrophoresis was carried out at room temperature on Whatman 3 MM paper strip (30 $\times$ 4 cm), using Michaelis Veronal buffer of ionic strength (u) 0.075, pH 8.2, in the electrophoretic vessels and a 50–60% dilution of the same buffer as paper impregnating solution (4). At 300 V separation time was 5 hr. Detection was made by the tetrazolium method (6).

The use of human serum to improve the separation of isozymes from tissue extracts was tested, because the available information indicates good resolution of the serum isozymes by paper electrophoresis (7), and because serum itself can be considered as a polyextract, containing enzymes from the different organs of the body. Although serum is both useful and economical, however it must not be used in amounts higher than 5 μl, otherwise the isozymogram of the extract might be contaminated with LDH from the serum itself.

The conditions which were studied improve the separation of the lactate dehydrogenase from the extracts. Impregnating the paper with dilute buffer allows the separation of two and sometimes even three active fractions. If gelatin or gamma globulin is incorporated in the extracts three active fractions are usually obtained. Bovine and human serum albumin all clearly separate the five expected isoyzmes. Figure 1 shows that gamma globulin can only separate those isozymes (M type) that migrate more slowly than it does.

Similarly to what happens in the separa-

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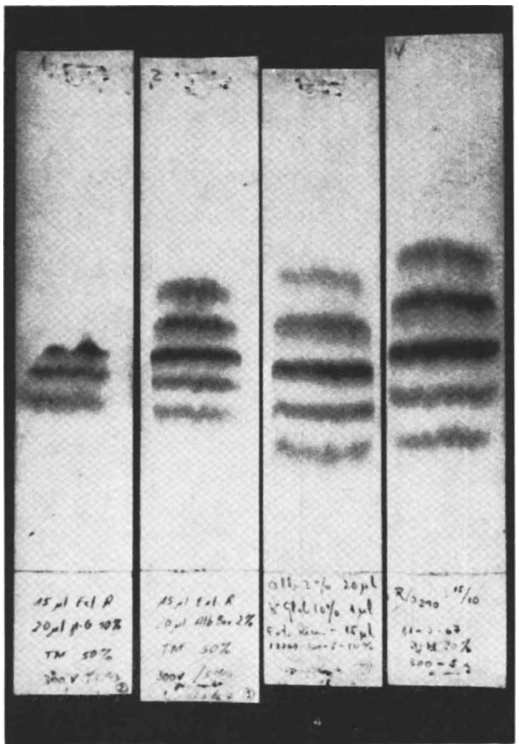

FIG. 1. All strips carry 15 μl of rat kidney homogenate, plus, from left to right: (first strip) 20 μl of 10% gamma globulin; (second strip) 20 μl of 2% bovine serum albumin; (third strip) 20 μl of 2% serum albumin and 1 μl of 10% gamma globulin; (fourth strip) 10 μl of human normal serum.

tion of serum proteins (8) it is believed that when serum albumin is added to tissue extracts, electrophoresis of the isozymes present is not carried out on the supporting paper itself, but rather on a coat of albumin that has gradually impregnated the strip during migration. In the particular case of paper electrophoresis it is extremely important that added protein migrate faster than isozymes fractions. The function of the amount of protein added to the homogenates is evident: increasing the albumin from 0.1 to 0.4 mg improves the resolution of the isozymes and the appearance of strongly colored fractions shows that enzymatic activity also increases. The detection of albumin shows that this protein always migrates further on the paper strip than the enzymatic proteins.

Human albumin and gamma globulin were mixed in a proportion similar to that of normal serum (4 parts and 1 part) and used together in the amount of 500–700 μg as separating agents in order to take advantage of the separatory capacity of albumin and at the same time increase the protein concentration in the migratory field with gamma globulin. Moreover it has the added advantage that it does not contain isozymes that might interfere. Figure 1 compares the effect of the mixture to that of serum. Results obtained are quite satisfactory, because there is an increment over the "full distance" (distance measured in millimeters between the front of the first isozymes and the rear of the last one) (4). The separation of isozymes is good and the compact fractions allow a clear densitometric diagram.

When the electrophoretic separation of the strip bearing extract alone is compared to that with the added protein, it can be observed that the extract proteins alone are not enough for good resolution of the isozymes. On the other hand, the incorporation of the mixture of serum albumin and gamma globulin to the different human homogenates in the same ratio as a "synthetic human serum" allows a definite separation of the five isozymes with their characteristic pattern, see Fig. 2.

A reasonable supposition is that proteins, when incorporated to the extracts, protect enzymatic fractions from loss of activity due to the general conditions of electrophoresis: electric field, temperature, time, and so on (9). The enzyme's conservation under improved conditions in the normally adverse electrophoretic environment might result in an apparent increase in formazan production. In order to eliminate this possibility, varying amounts of gelatin, agar, starch, serum albumin, gamma globulin, and other substances were added in turn, to equal amounts of LDH in test tubes. The values in duplicate of reduced nitro-blue-tetrazolium obtained, detected spectrophotometrically, were different in each case, but always much higher than those obtained with the same amount of enzyme but no added macromolecules (10).

Results in Table I suggest that, under the

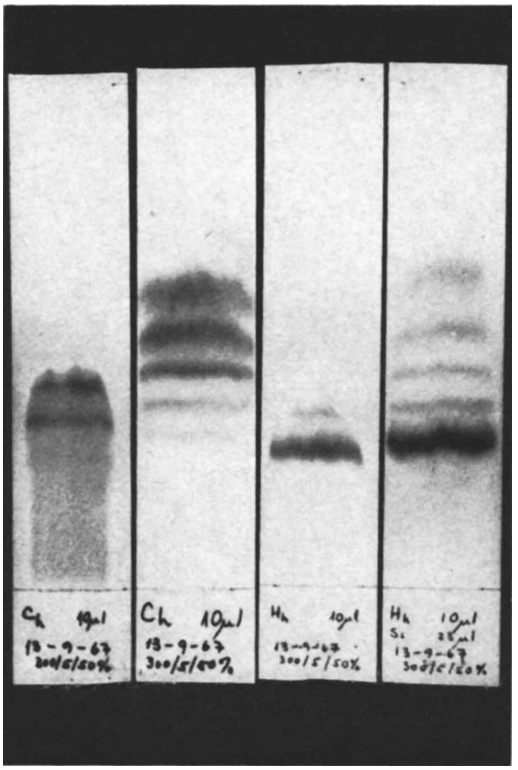

FIG. 2. From left to right: (first strip) 10 μl of human heart homogenate; (second strip) 10 μl of the same homogenate plus 700 μg of the mixture human serum albumin and gamma globulin; (third strip) 10 μl of human liver homogenate; (fourth strip) 10 μl of the same extract plus 700 μg of the same mixture.

conditions of this experiment, the presence of certain molecules clearly induces an increase in the activity of the dehydrogenase, and the electrophoretic deterioration of the enzyme, if it exists, seems to be slight.

Summary. If proteins are added to homogenates of organs before paper electrophoresis, better separation and increased activity of the LDH isozymes is obtained. This increase was confirmed by the spectrophotometric determination of the formazan produced. A mixture of human albumin and gamma globulin in the normal serum ratio (4:1) gives a good electrophoretic resolution of the five isozymes.

TABLE I. Formazan Produced by LDH and in the Presence of Macromolecules.

Macromolecules added[a] (μg)	ΔOD $\times$ 10^3/min at 540 mμ
LDH[b]	20
+ agar (100)	37
+ starch (100)	40
+ serum albumin (100)	46
+ gelatin (100)	50
+ gamma globulin (100)	60

[a] The macromolecules added to the system without LDH gave negative results.

[b] LDH type III crystalline from beef heart, Sigma Chem. Co., St. Louis, Missouri (enzymatic activity corresponding to 1 μg of protein).

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