**A NEW METHOD FOR GEOMETRICAL DEMONSTRATION
OF LDH ISOENZYMES
—HISTOCHEMICAL ELECTROPHORESIS—**

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This paper describes a new method for the geometrical demonstration of lactate dehydrogenase (LDH) isoenzymes in tissues and organs (histochemical electrophoresis). The principle of this method can be found in the following points. (i) The tissues obtained from various organs were frozen, and cut in 8 μ slices with a cryostat. The sections were placed on a piece of filterpaper and dried immediately under a fan. (ii) As a result, the enzyme molecules of the tissue were resorbed in the surface layer of the filter paper. (iii) Then, the filter paper is treated by standard electrophoresis. The data obtained with this new method were compared to those of tissue extracts of the heart, liver and kidney of dogs. This new method makes it possible to investigate the distribution of isoenzymes in tissue as a whole. Therefore, it may enable us to clarify the geometrical distribution of LDH isoenzymes in various organs.

Recently, the analysis of serum enzyme patterns, particularly LDH isoenzyme, has been used in the clinical diagnosis of various diseases (1, 3, 5, 6, 7). Electrophoresis is the most common method used in clinical and experimental studies, including the analysis of tissue extracts (2, 4, 5).

This paper describes a new method which combines the methods of histochemistry and electrophoresis. This new method is able to clarify the geometrical distribution of the enzyme in the total sections. The authors tentatively designate this method "histochemical electrophoresis".

METHODS

(A) The electrophoretic pattern of LDH isoenzymes in "histochemical electrophoresis" is detected by a modification of agar-agarose gel electrophoresis (6).

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The migration bands were designated H4, H3M1, H2M2, H1M3, and M4, seriatim from the anode to the cathode. For this experiment, five mongrel dogs weighing about 10 kg were used. The experimental dogs were given an intramuscular injection of ketamin hydrochloride solution (10 mg/kg). Operation was performed under intermittent positive pressure respiration, using pure oxygen in a Takaoka respirator.

All tissues obtained from the various organs were frozen at -10°C , and cut in $8\ \mu$ sections with a cryostat. The sections were placed on a piece of filterpaper and dried immediately under a fan. These filterpapers were used for the "histochemical electrophoresis" as follows.

1) *Buffer stock solution.*

a) Sodium veronalate-HCl buffer: pH 8.4, $\mu=0.04$, sodium veronalate 8.5 g, hydrochloric acid (N) about 11.5 ml, aq. dist. ad. 1,000 ml.

b) Tris (hydroxymethyl) aminomethane-HCl buffer: pH 7.4, tris (hydroxymethyl) aminomethane 7.2 g, hydrochloric acid (N) about 50 ml, aq. dist. ad. 1,000 ml.

2) *Preparation of agar-agarose gel plates.*

The agar-agarose powder (Difco special agar-noble, 3.5 g, and Behringer agarose, 0.5 g) was added to 500 ml of veronalate-HCl buffer solution and heated until the solution in a boiling water bath became completely clear. The clear warm 200 ml of agar-agarose solution was poured into a 25/25/5 cm glass box until it covered the bottom entirely. When a perfectly horizontal surface was obtained and the agar-agarose is solidified, a 20/20 cm glass plate was placed carefully on the surface, then covered with another 300 ml agar-agarose gel solution.

3) *Application of the tissue sample.*

The glass plate covered with the agar-agarose layer was cut and three to five pieces of the prepared filterpapers were placed 1 cm to the left of the plate center, and left this way for 20 to 30 minutes. Then the filterpapers were removed with their tissues from the agar-agarose layer.

FIG. 1. Histochemical electrophoresis box.

4) *Electrophoretic run.*

The glass plate (20/20 cm) was placed in the "histochemical electrophoresis" box and the buffer vessels filled with the sodium veronalate-HCl buffer. (Fig. 1).

Electrophoresis should be carried out under a constant current of 120 mA for three and a half hours in a cold room (0°C).

5) *Staining.*

The agar-agarose glass plate was incubated in the dark for one hour at 37°C using the following staining solution. (This solution should be prepared just before use.)

Tris-HCl buffer pH 7.4, 0.05 M,	168 ml
KCN, 0.06 M	15 ml
Sodium lactate, 2 M,	12 ml
Nicotine amide adenine dinucleotide (NAD),	120 mg
Nitroblue tetrazolium (NBT),	90 mg
Phenazine methosulphate (PMS),	15 ml

6) *Fixation.*

The agar-agarose plate was fixed for 20 min in approx. 300 ml of the solution containing water, methanol, and acetic acid in a ratio of 14 : 5 : 1 by volume, respectively.

7) *Designation.*

The staining bands are designated H4, H3M1, H2M2, H1M3, and M4, seriatim from the anode to the cathode.

(B) Other samples of various tissues were homogenized at 0°C. The homogenized solution was centrifuged at 4,000 r.p.m. for 15 minutes, the supernatants were used for the determination of LDH isoenzymes as a control. These LDH isoenzymes were detected by agar-agarose gel electrophoresis (6), and the zones were designated LD5, LD4, LD3, LD2, and LD1 from the anode to the cathode.

RESULTS

The following data were obtained with this new method, "histochemical electrophoresis". Figures 2 and 3 show the electrophoretic patterns of cryostat sections from the kidney (No. 1, 2, and 7), the heart (No. 3, 4, 5, 8, and 10), and the liver (No. 6, and 9). Number 1 is the pattern of a section from the coronal section of the apical portion of the kidney, No. 2 is that of half of a coronal section of the apical portion of the kidney, and No. 7 is that of a coronal section of the middle segment of the kidney. As seen in No. 1, 2, and 7, in the renal cortex area, H4 and H1M3 were found in moderate stainability, H3M1 and H2M2 in the least stainability, and M4 in the greatest stainability; thus, $M4 > H4 > H1M3 > H3M1 \geq H2M2$. In the area of the renal medulla H4 was found in moderate stainability, H3M1 and H2M2 in a lower staining degree, and H1M3 and H4 in the lowest staining reaction; $H4 > H3M1 \geq H2M2 > H1M3 \geq M4$.

In Fig. 4, the LDH isoenzyme pattern of homogenized renal cortex reveals

FIG. 2. Patterns of histological electrophoresis of LDH isoenzymes in thin tissue sections of kidney (No. 1 and 2), and heart (No. 3, 4 and 5).

the gradual decrease in stainability of the bands in the following order: LD1 > LD2 > LD3 > LD5 > LD4, while that of homogenized renal medulla shows the following order: LD3 > LD4 > LD2 > LD1 > LD5.

The histochemical electrophoretic patterns of No. 3, 4, and 5 show sections of the heart. Number 3 shows a horizontal section of the heart at the apical portion, No. 4 the posterior wall of the left ventricle, No. 5 and 8 the anterior wall of the

FIG. 3. Patterns of histological electrophoresis of LDH isoenzymes in thin tissue sections of liver (No. 6 and 9), kidney (No. 7), and anterior wall (No. 8), and left ventricle (No. 10) of the heart.

FIG. 4. LDH isoenzyme patterns in tissue extracts (kidney, liver, and heart).

left ventricle, and No. 10 the middle portion of the left ventricle. As seen in No. 3, 4, 5, 8 and 10, the electrophoretic patterns of the heart show large amounts of H₄ and H₃M₁. These patterns are like those of LDH isoenzymes in the heart extract, as shown in Fig. 4.

Numbers 6 and 9 are the histochemical electrophoretic patterns of the liver sections; showing a significant amount of M₄, some H₁M₃, and a slight amount of H₂M₂ and H₃M₁. These histochemical electrophoretic patterns are like those of LDH isoenzymes in the liver extract as shown in Fig. 4.

DISCUSSION

Enzymatic analysis has been performed on serum and tissue extracts mainly by biochemical methods. For staining enzymes, histochemical methods are most commonly used for the tissue sections. The principle of this method is to fix on filterpaper the enzymes isolated on the cut surfaces of the tissue.

Histochemical electrophoresis obtained from this new method was compared with that of tissue extracts from the heart, liver and kidney of dogs. The findings of the heart and liver were in good agreement, but those of the kidney showed some discrepancies. In the renal cortex, the most concentrated pattern was in the anode side migrations (M₄ and H₁M₃) for both methods, but in the cathode side band (H₄) the histochemical electrophorogram showed a more stainable pattern than that of the routine electrophoresis. In the renal medulla, the least stained zone was in the anode side band for both methods, but the most concentrated zone was found in the middle band in the routine electrophoresis and in the cathode side band with the histochemical electrophorogram. The reason for these discrepan-

cies requires further study.

The new method, histochemical electrophoresis, as designated by the authors, may enable clarification of the precise distribution of LDH isoenzymes in the cryostat sections of various organs. The procedure of the technique is simple and the findings distinct, thus it is valuable for laboratory tests in medical use and biological studies as well.

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