Critical comparison of lactate dehydrogenase isoenzyme separation by isoelectric focusing and disc electrophoresis in polyacrylamide microgels

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Critical comparison of lactate dehydrogenase isoenzyme separation by isoelectric focusing and disc electrophoresis in polyacrylamide microgels

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(Paper received June 13, 1978)

Dedicated to Dr. Ernst Neuhoff on his 80th birthday

Summary: It is shown that upon fractionation of lactate dehydrogenase isoenzymes by isoelectric focusing in microgels, the most anodic LDH 1 is largely inactive, and the cathodic fractions are predominant in the isoenzyme pattern, even of those organs like heart and brain, which are reported to contain mainly LDH II. LDH II and LDH III. Upon focusing of isolated rat LDH I and LDH V, an inactivation of the anodic isoenzyme of 86% relative to LDH V was observed. This inactivation is due to the acid denaturation of the enzyme protein at its isoelectric point as shown by incubation experiments with pure isoenzymes in test tubes. Excellent resolution of the isoenzymes can also be achieved with a newly developed micro-disc-electrophoretic technique. The isoenzyme patterns compare favourably with the results reported in the literature, although in this case the most cathodic isoenzyme LDH V is partially inactivated.

Enzyme: lactate dehydrogenase, L-lactate:NAD-oxidoreductase (EC 1.1.1.27), with isoenzymes LDH 1-5.
Triton X-100 increases the colour intensity of the bands two- to four-fold after enzyme staining with tetrazolium blue. This effect is independent of the fractionation technique used. The detergent does not influence the enzymatic reaction but promotes the formazan development, thus acting as an unspecific amplifier which further increases the sensitivity of enzyme detection in microgels.

The present results show that a relevant interpretation of isoenzyme patterns is only possible if the principle hazards of the separation technique applied are thoroughly considered.

**Key words:** Lactate dehydrogenase isoenzymes, microgel electrophoresis, isoelectric focusing.

Isoenzyme patterns of various tissues are determined for the most part by electrophoresis. In addition to starch gel electrophoresis which was widely used earlier (see references [1,2]), acrylamide gel electrophoresis, according to Ornstein [3] and Davis [4], and more recently, isoelectric focussing in polyacrylamide gels have been the principal methods used. In principle, isoelectric focussing is the ideal separation method for isoenzymes since it separates only according to differences in the isoelectric points of the molecules, and indeed, isoenzymes have different isoelectric points although they often have almost identical molecular weights.

With the introduction of micro gel electrophoresis, an accurate enzyme activity determination directly in the gel has also become possible [5,6]. Isoelectric focussing in microgel [7,8], therefore, appeared to be the optimal method for the study of the lactate dehydrogenase isoenzyme distributions. Very reproducible separations and enzyme-kinetic measurements can be made by micro-isoelectric focussing. However, in comparison with results obtained by other methods, it becomes apparent that the anodic bands are always very faintly stained in isoelectric focussing. This is especially true for organs such as the heart and brain which are said to
contain mainly LDH 1 and LDH 2. This problem led us to systematically compare the isoenzyme determination by isoelectric focussing and by disc electrophoresis in microgels since isoelectric focussing is used more often for the study of isoenzyme patterns.

Methods

Extracts of various rat organs were fractionated. Homogenates, 10-25% (w/v), of the organs in 10 mM phosphate buffer, pH 7.5, were centrifuged for 1 hr at 100,000 x g, and the clear supernatant was used for the fractionation after suitable dilution with glycerol (final concentration of 25% for isoelectric focussing) or sucrose-NAD solution (final concentration of 35% sucrose-1 mM NAD for disc electrophoresis).

Isoelectric focussing was carried out according to the method of Bispink and Neuhoff[8] in 7% acrylamide gels with 4% Ampholine, pH 3.5-10, or 4% Servalyte, pH 2-11, as the ampholyte. At the anode, 0.1% acetic acid, and at the cathode, 370 mM tris, pH 11.2, were used as ion carriers since this achieved a better resolution of the cathodic fractions than with the originally specified system with hydrogen carbonate as the catholyte.

The fractionation was terminated after 30 min at constant 100 V.

The fractionation of isoenzymes in 20% polyacrylamide microgels described earlier[6] is less suitable since in this system, the isoenzyme bands are so close to each other that optical determination is affected. Therefore, for disc electrophoresis, the method of Diez and Lumbrano[9,10] was used on the micro scale. With an unmodified gel mixture (T = 5.5%, C = 2.6%, tris/C1 gel buffer, pH 8.9), 13 mM tris/glycine, pH 8.4, was

\[ T = g \text{ acrylamide/100 ml; } C = g \text{ bis(acryloylamido)-methane/100 g acrylamide} \]
used as the electrode buffer. Electrophoresis lasts only 10-12 min with a constant voltage of 120 V. Triton gels were prepared by adding 0.05% Triton X-100 to the respective gel mixtures.

For the identification of the isoenzymes, the gels were incubated following fractionation for 10-15 min in the tetrazolium system used by Quentin and Neuhoff[7] and then differentiated in 7.5% acetic acid. Refractionation experiments were carried out using the method of Neuhoff and Schill[11].

Crudely purified isoenzyme fractions were obtained by ion-exchange chromatography of rat heart extracts on DEAE Sephadex as described earlier[12]. The enzymatically active column fractions were combined, concentrated, and dialyzed against 10 mM phosphate buffer, pH 7.5. Suitable dilutions of these fractions with glycerol or sucrose were used for the electrophoretic separation, as described for organ extracts.

The influence of Ampholine of different pH ranges on the lactate dehydrogenase activity was tested on pure isoenzymes of pig (Boehringer, Mannheim). Isoenzymes were incubated with Ampholine solutions (pH 4.2, 8.1, 8.6) and the enzyme activity measured colorimetrically by the tetrazolium method instead of by the usual optical test in order to make the conditions during the enzymatic reaction as similar as possible to those in the gel. Additional enzyme activity measurements were made using the optical test according to Gay et al[13].

To study the influence of Triton X-100 on the colour reaction, the same tetrazolium mixture was used in the cuvette as for the enzyme staining in the gel, and the change of the extinction followed at 520 nm.
Results

Fig. 1 shows gels from disc electrophoretic separations and isoelectric focussing of similar extracts from the rat heart, cortex and cerebellum. After isoelectric focussing (Fig. 1 right), isoenzymes LDH 3, LDH 4 and LDH 5 predominate in all organs, while after electrophoretic separation, mainly the anodic fractions LDH 1 and LDH 2 are produced but LDH 5 is scarcely detectable.

Fig. 1. Disc electrophoresis (left) and isoelectric focussing (right) of lactate dehydrogenase isoenzymes from heart (a), cortex (b), and cerebellum (c) of the rat in microgels. The gels are arranged so that the cathode always lies above. In disc electrophoresis, the samples are applied at the cathodic end of the gel, whereas in isoelectric focussing, at the anodic end. For disc electrophoresis, half (heart) or two-thirds (brain) of the sample which were focussed was applied. All gels were incubated for 6 min in the tetrazolium system.
In Table 1 the relative distributions of isoenzymes after densitometric determination of the gels from Fig. 1 are compared. The quantitative determination confirms the predominance of the cathodic fractions after focussing, while after electrophoresis, the patterns correspond largely to the proportions given in the literature\(^1,2\). For comparison, the distribution of the rat heart isoenzymes as determined by column chromatography is also shown in Table 1. Disc electrophoresis and column chromatography produce concurring results.

Table 1. Relative distribution of lactate dehydrogenase isoenzymes in heart, cortex and cerebellum of the rat after isoelectric focussing and disc electrophoresis in micro gels and in the rat heart after column chromatography on DEAE Sephadex. Data in \% of the total enzyme activity.

<table>
<thead>
<tr>
<th></th>
<th>LDH1</th>
<th>LDH 2</th>
<th>LDH 3</th>
<th>LDH 4</th>
<th>LDH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herz</td>
<td>8.0</td>
<td>10.6</td>
<td>32.0</td>
<td>10.3</td>
<td>39.0</td>
</tr>
<tr>
<td>Cortex</td>
<td>7.6</td>
<td>3.5</td>
<td>26.4</td>
<td>30.9</td>
<td>31.6</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>6.8</td>
<td>-</td>
<td>23.2</td>
<td>36.3</td>
<td>33.7</td>
</tr>
<tr>
<td>Herz</td>
<td>23.9</td>
<td>37.1</td>
<td>-</td>
<td>10.0</td>
<td>7.7</td>
</tr>
<tr>
<td>Cortex</td>
<td>20.7</td>
<td>19.6</td>
<td>27.1</td>
<td>24.8</td>
<td>-</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>41.6</td>
<td>23.3</td>
<td>19.7</td>
<td>15.3</td>
<td>-</td>
</tr>
<tr>
<td>Herz</td>
<td>39.3</td>
<td>36.9</td>
<td>16.7</td>
<td>6.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Herz = heart; Cortex = cortex; Cerebellum = cerebellum; Isoelektrofokussierung = isoelectric focussing; Diskelektrophorese = disc electrophoresis; Chromatographie = chromatography

Results of the fractionation of isolated rat isoenzymes are shown in Table 2. Activities were first measured by the optical test, and fractions LDH 1, LDH 2 and LDH 5 mixed in proportions given in the
Table 2. Relative activity distributions after mixing and fractionation of isolated lactate dehydrogenase isoenzymes by isoelectric focussing and disc electrophoresis.

<table>
<thead>
<tr>
<th></th>
<th>LDH 1</th>
<th>LDH 2</th>
<th>LDH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Küvette</td>
<td>2.7</td>
<td>0.42</td>
<td>1</td>
</tr>
<tr>
<td>Isoelektrofokussierung</td>
<td>0.41 (84.5%)</td>
<td>0.20 (52.4%)</td>
<td>1</td>
</tr>
<tr>
<td>Küvette</td>
<td>0.24</td>
<td>0.46 (52.4%)</td>
<td>3.0</td>
</tr>
<tr>
<td>Diskelektrophorese</td>
<td>0.20 (52.4%)</td>
<td>1.23 (59%)</td>
<td></td>
</tr>
</tbody>
</table>

Küvette = cuvette; Isoelektrofokussierung = isoelectric focussing; Diskelektrophorese = disc electrophoresis

table for isoelectric focussing and electrophoresis. After fractionation of these mixtures, the stained gels were determined by densitometry. Obviously, during isoelectric focussing, LDH 1 is mostly inactivated, about 84.5%, based on LDH-5 activity. In contrast to focussing, LDH 5 is inactivated about 59%, based on LDH-1 activity, during disc electrophoresis. The absolute activities in the cuvette and in the gel obviously cannot be compared. Therefore, the values are regarded only as relatively rough approximations of the actual activity changes since the mixing proportions are based on isoenzymes measured separately in the cuvette. However, mixing as such can indeed change the activities. It was shown, e.g., that in the gel, bands were always stained more intensely after fractionation of a certain mixing of isoenzymes than after fractionation of the individual isoenzymes. This protective effect of the homologous protein, which has already been known for a long time \cite{14}, could explain the somewhat contradictory behaviour of LDH 2 which is indeed inactivated in isoelectric focussing, as expected; however, after disc electrophoresis, it even shows an activity increase.
Fig. 2. Influence of Ampholine of different pH ranges on the activity of pure lactate dehydrogenase isoenzymes of pig (Boehringer). LDH 1, 0.5 µg, (180 mU) and 0.5 µg of LDH 5 (225 mU) were incubated for 30 min at room temperature in 100 µl of Ampholine (4%) or buffer solution which contained 15% glycerol. Ten (10) µl of these mixtures were added to 190 µl of the usual tetrazolium solution. The reaction was stopped at various times with 100 µl of 7.5% acetic acid, and after suitable dilution, the extinction was measured at 520 nm. For the increase of the optical density as a measure of the enzyme activity, the following regressions and correlation coefficients (r) in n experiments were obtained.

**LDH 1**
- Tris(Cl), pH 8.1  
  \[ Y = (0.036 + 0.0012)X + 0.133 + 0.0077; r = 0.982 \text{ (n = 8)} \]
- Tris/Essigsäure, pH 8.1  
  \[ Y = (0.038 + 0.0009)X + 0.118 + 0.0061; r = 0.998 \text{ (n = 2)} \]
- Ampholine 3.5, pH 4.2  
  \[ Y = (0.057 + 0.0014)X + 0.136 + 0.0087; r = 0.972 \text{ (n = 6)} \]
- Essigsäure Acetat, pH 4.2  
  \[ Y = (0.047 + 0.0011)X + 0.118 + 0.0074; r = 0.981 \text{ (n = 5)} \]
- Ampholine 7, pH 8.2  
  \[ Y = (0.041 + 0.0019)X + 0.167 + 0.0128; r = 0.986 \text{ (n = 5)} \]

**LDH 5**
- Tris(Cl), pH 8.1  
  \[ Y = (0.044 + 0.0007)X + 0.1 + 0.0038; r = 1 \text{ (n = 1)} \]
- Ampholine 7, pH 8.6  
  \[ Y = (0.044 + 0.0014)X + 0.113 + 0.0076; r = 0.995 \text{ (n = 2)} \]

Tris/Cl = tris/Cl; Tris/Essigsäure = tris/acetic acid; Ampholine = Ampholine; Essigsäure Acetat = acetic acetate

Inkubationszeit [min] = incubation time [min]
In incubation experiments with pure isoenzymes of pig (Fig. 2), whether Ampholine itself or simply the acid pH value leads to the inactivation of LDH 1 was tested. Incubation in 10 mM tris/HCl, pH 8.1, the same medium, therefore, in which enzyme is stained in the gel, was used as the control. All test mixtures contained 15% glycerol in addition.

Incubation of LDH 1 in 4% Ampholine in water, pH range 3.5-5 (measured pH in the mixture, 4.2), led to a distinct inactivation of the enzyme. The enzyme was still somewhat more strongly inactivated by an acetic acid buffer without Ampholine at pH 4.2, while in a tris/acetic acid buffer, pH 8.1, the activity was unchanged. On the other hand, Ampholine of pH range 7-9 (measured pH, 8.2) led to a small increase of the activity.

Ampholine of pH range 7-10 (measured pH in the mixture, 8.6) had no influence on the activity of LDH 5.

By re-electrophoresis of the individual bands of disc electrophoresis and of focussing, the homogeneity of the bands was tested following the respective fractionations. The positions of bands 1-4 after fractionation of the total extract and after refractionation were in agreement. Only LDH 5 was not detected since it does not migrate in the gradients in this system, as electrophoresis of the total extract showed.

Fig. 3 shows the isoenzyme distributions in different rat organs after disc electrophoresis and isoelectric focussing in gels with or without 0.05% Triton X-100. In this figure, the predominance of the cathodic fractions after focussing and the anodic isoenzymes after disc electrophoretic separation is evident once again. However, independent of the separation methods, the colour of the bands in Triton gels is two to
Fig. 3. Influence of Triton X-100 on activity and isoenzyme distribution after enzyme fractionation in microgels with different organs.
As measure of the enzyme activity, the areas of peaks were converted into arbitrary units after densitometric determination of the coloured gels.
Since in each case the same amounts of sample were used for the fractionation, with and without Triton, and gels were incubated for the same time in the tetrazolium mixture, the activities, with and without Triton, are directly comparable. The relative portions of the individual isoenzymes are given on the columns in % of the total activity. Black columns: with Triton X-100 (0.05%); blank columns: without Triton X-100.
Muskel = muscle; Isoelektrifokussierg. = isoelectric focussing; Elektrophorese = electrophoresis; Herz = heart; Cortex = cortex; Aktivität = activity
four times as intense as in Triton-free gels. The separation itself is essentially not influenced by the detergent, the bands simply become somewhat broader and less sharp (Fig. 4).

Fig. 4. Influence of Triton X-100 on the activity of the lactate dehydrogenase isoenzymes from heart (a), cortex (b) and skeletal muscle (c, adductors) of rat after disc electrophoretic fractionation in micro-gels. Amounts of sample and incubation times were the same for gels with (+) and without (-) 0.05% Triton.

During incubation, a relatively intense background colour which strongly affects the determination is formed in gels with higher Triton concentrations (0.1-0.5%); with the concentration of 0.05% Triton X-100 chosen here, however, this does not occur. The increase of the optical
density during the tetrazolium staining was linear in Triton-containing gels so that in this case as well, the enzyme activity can be measured quantitatively in the gel. This agrees with previous results in the isoelectric-focussing gel[8].

In the usual optical test (reverse reaction lactate $\rightarrow$ pyruvate), Triton X-100 did not influence the reduction of the coenzyme and did not have a direct effect on the enzymatic reaction. However, if the colour reaction was carried out in the cuvette with the same tetrazolium mixture as in the gel, the increase of the optical density at 520 nm in the system with Triton was about five times as high as that without Triton. However, the maximal effect was achieved only if Triton was added before the addition of the enzyme or of the substrate. But if the detergent was added to the total system, the effect was smaller, the later the addition was made after the start of the enzymatic reaction.

Discussion

A comparison of the lactate dehydrogenase isoenzyme patterns of different rat organs after isoelectric focussing and disc electrophoresis clearly shows high activity losses of the anodic isoenzyme fractions in the isoelectric-focussing gel. Quantitative experiments with isolated isoenzymes confirm that a considerable inactivation of LDH 1 occurs during isoelectric focussing. The relative activity loss of LDH 1 of 86.3%, of course, gives only a rough check since activities in the cuvette and gel cannot be directly compared. Besides, the mixing proportion of the isoenzymes measured individually in the cuvette was taken as a basis for the calculation; in dilute solutions, mixing alone of different isoenzymes changes their
Weller et al have indicated the lability of anodic isoenzymes after isoelectric focussing\(^{[15,16]}\). These authors focussed in sucrose gradients and traced activity losses to protein precipitation often observed in the acid range of the pH gradient. According to Dale (cited by Latner\(^{[17]}\)), LDH 1 is inactivated very easily by anodic oxidation. Without investigating their observation further, Klose and Spiegelmann\(^{[18]}\) also mention that the mouse heart extract shows remarkably little LDH-1 activity after isoelectric focussing. In their comparison of LDH fractionation by electrophoresis and isoelectric focussing, Chamoles and Karcher\(^{[19]}\) point out in particular the numerous secondary bands which can appear in isoelectric focussing. Bispink and Neuhoff\(^{[18]}\) have shown that this effect depends on the conditions of the separation. However, the densitogram of a focussing of human heart extract by Chamoles and Karcher\(^{[20]}\) shows very low activity in the region of the anode in comparison with the cathodic fractions; no importance is attributed to this finding.

According to the results given here, this activity loss of LDH 1 is obviously due to acid denaturation of the enzyme protein. The direct contact of the sample with the acid ion carrier leads to a partial inactivation of the enzyme which is largely prevented by coating the sample with glycerol\(^{[6]}\). But during fractionation, LDH 1 is always found in the acid range of the pH gradient.

Incubation experiments in combination with a direct cuvette test have shown that LDH 5 does not influence in the region of its isoelectric point. On the other hand, LDH 1 is distinctly inactivated by incubation at pH 4.2. This is not due to a specific effect of Ampholine since acetic
acid buffer at pH 4.2 had the same effect. As the incubation in tris/acetate buffer at pH 8.1 shows, the acetate ions themselves also do not cause inactivation. Inactivation is thus attributed to the acid milieu alone. The activity loss at the low pH value in incubation experiments was substantially less than the inhibition of the LDH-1 bands in isoelectric focussing. However, it should be borne in mind that conditions in the incubation mixture are not identical with those in the gel where the enzyme is concentrated very heavily.

The results presented here appear to contradict an earlier study\[7\] in which the lactate dehydrogenase patterns of various rabbit organs in the isoelectric-focussing microgel did not show the predominance of cathodic fractions observed in the rat. In the rabbit heart, e.g., only LDH 1 was found. In tests using isoelectric focussing modified according to Bispink\[8\], we also found predominantly LDH 1 in the rabbit heart and an additional, very weak LDH-2 band which was apparently not detected in the earlier method with its lower sensitivity. We were also able to confirm the patterns found earlier in other rabbit organs with small differences. On the one hand, this shows the large differences in the isoenzyme pattern of the same organs from different species. But, on the other hand, the variable stability of lactate dehydrogenase isoenzymes of different species at low pH values should be considered.

The conditions of isoelectric focussing of lactate dehydrogenase isoenzymes can be chosen so that the five classical isoenzymes of mammal tissues are detected as homogeneous bands. However, organ patterns are distorted in that anodic isoenzymes are largely inactivated in the isoelectric-focussing gel, and accordingly, the relative portion of the
cathodic fractions is too high. This error, attributable to the method itself, compelled us to take great care in interpreting the isoenzyme patterns ascertained by isoelectric focusing of the organ extracts with regard to their physiological importance.

A micro disc electrophoretic method by which the lactate dehydrogenase isoenzymes can be satisfactorily separated is presented here for the first time. The reliable and very rapid fractionation of the five isoenzyme bands gives organ patterns which correspond well with the literature data and agree with results from ion-exchange chromatography (Table 1). Besides, its detection sensitivity is higher than that in the case of focusing. As with other electrophoretic methods[10,21-23], the main difficulty here is with LDH 5 which migrates only very slowly toward the anode in the basic buffer system due to its high isoelectric point[16]. Relative to LDH 1, the activity of LDH 5 is so greatly reduced after electrophoresis that the cathodic band is often no longer detectable, although focusing shows that the extract contains active LDH 5. In macro gel electrophoresis, Diez and Lumbrano were able to solve this problem through polymerization of a 5.5% "coating gel" on the sample[10]. We have not yet tested this possibility; in any case, such an additional step would mean a real disadvantage for routine applications. Our experiments confirm that the sucrose concentration in the sample is decisive for the quality of the separation[10]. For micro disc electrophoresis, 35% sucrose is optimal. Albumin addition to the sample or even to the gel and buffer, as recommended by various authors[21-23], did not have any effect on the LDH-5 activity. Even by micro disc electrophoresis, therefore, the "true" isoenzyme pattern cannot be determined. However, agreement with the results of other
separation methods shows that the disc electrophoretic patterns correspond to the proportions in vivo far more than the isoelectric-focussing patterns. For the determination of organ patterns, therefore, disc electrophoresis is preferred, while for special studies, e.g., testing the purity of anodic isoenzymes, isoelectric focussing will often be more advantageous.

The influence of Triton X-100 on the intensity of the colour of the bands, described here for the first time, is independent of the actual separation methods. The promoting effect of colloids such as albumin, agar or agarose on formazan formation has been long known \cite{24,25}. A facilitated electron transfer from the reduced coenzyme to the tetrazolium salt has been suggested as the mechanism. This could also occur with Triton since the analysis of the reaction in the cuvette shows that the detergent does not act in the enzymatic but in the indicator reaction. Consequently, a non-specific strengthening mechanism exists and the relative portions of isoenzymes are almost equal in Triton and Triton-free gels. With initially very high activity of a certain band, it was shown that the strengthening effect is smaller than with bands with lower activity. This leads to pronounced shifts of the relative portions in favour of the anodic fractions, especially with muscle extracts with a predominant LDH-5 portion. In this case, the colour of the active bands probably reaches a saturation value very rapidly, while the extinction of the less active bands continues to increase linearly. Shifts in the pattern also occur in the same way in Triton-free gels if the activity differences of the individual isoenzymes are very pronounced or the amounts of sample applied are too large.

The findings presented here show that excellent fractionations of lactate dehydrogenase isoenzymes can be achieved on the micro scale, not
only by isoelectric focussing but also by disc electrophoresis. But at the same time, it is also demonstrated that isoenzyme patterns can be interpreted only if the principal possibilities of error of the separation methods are known. That similar erroneous interpretations are possible after separation of other isoenzymes is not excluded, and therefore, a critical analysis of the separation methods used must precede the interpretation of the patterns in each case.

References

2. The isoenzymes of lactate dehydrogenase.

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