ELECTROPHORESIS OF LACTIC DEHYDROGENASE IN BORATE-BUFFERED STARCH-GEL

M. C. BLANCHAER

Department of Biochemistry, St. Boniface General Hospital, St. Boniface, Manitoba

and

Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

INTRODUCTION

It has been suggested[1-4] that examination of the serum lactic dehydrogenase (LDH) isoenzyme pattern may be helpful in identifying tissues affected by disease. Cellular damage to tissues such as myocardium or liver results in characteristic abnormalities in the relative proportions of the 5 LDH isoenzymes demonstrable in serum by electrophoresis in barbiturate-buffered agar[2,4], starch-grain[1] and starch-gel[3] media. Unfortunately, as might be predicted from tissue LDH isoenzyme patterns[3], serum isoenzyme patterns are not entirely tissue-specific[4], particularly when there is a possibility of more than one tissue contributing to the serum LDH.

The ability of electrophoresis in borate-buffered starch-gel[5] to detect many more serum protein fractions than other electrophoretic methods suggested that this procedure might also resolve serum LDH into isoenzyme patterns of greater tissue specificity. Some support for this was found in the results of Markert and Møller[6], who applied this method to a study of LDH isoenzymes in animal tissues.

METHODS

Serum and tissue homogenates were subjected to electrophoresis in horizontal starch-gels as described by Smithies[5], except for minor modifications indicated below. Human tissues obtained within eight hours after death were homogenized ice-cold in 10 volumes of the 0·022 mM borate buffer, pH 8·6, used to prepare the starch-gels, and centrifuged at 6500*g* for 10 min at 0° to remove debris. Serum was recentrifuged and stored at −20° until used. The slits for the insertion of samples were cast in the gel as described in Smithies' vertical electrophoresis method[7], but were increased in width from 0·75 mm to 2·0 mm. The serum or homogenate was made into a slurry with washed starch-grains and run into the slits, which were then covered with a thin waterproof plastic film (" Saran Wrap ", Dow Chemical Company), and a current of constant amperage sufficient to produce an average drop of 11 volts/cm in the gel was applied for 6 h at 4°. The gel was slit longitudinally, and to each cut surface was applied a sheet of Whatman No. 1 filter paper sprayed with a solution containing DPNH (90 mg/100 ml) and sodium

pyruvate (1·0 g/100 ml) in barbiturate buffer, pH 8·4, $\mu = 0{\cdot}05$. After moist incubation at 37° for 25 min, the paper was stripped off and the gels inspected under ultra-violet light (4-watt Sylvania fluorescent lamp F4T5/BLB) and photographed through a medium green filter on Kodak Panatomic-X film. An exposure of 3 min at f/2·8 with the ultra-violet lamp 15 cm above the gel, followed by high-contrast development, reproduced the LDH bands detected visually, and occasionally revealed faint bands not seen by the eye. The gels were notched to identify the position of the LDH bands and then stained for protein with amido-black. The filter paper sheets that had been stripped from the gels and dried also showed the LDH bands under ultra-violet light.

Variations of the above technique were found to offer no advantages, nor did they yield inferior results. These variations included vertical electrophoresis[7], applying the sample directly to the slit without making a starch-grain slurry or replacing the latter with filter paper or blotting paper, prolonging or shortening the electrophoresis time at 4°, and using wider or narrower sample slits.

Some of the samples were also subjected to an agar electrophoresis procedure[2,4] in which the positions of the individual LDH isoenzymes relative to the major serum proteins are approximately the same as those reported by previous workers using barbiturate-buffered starch-grain[1], starch-gel[3] and continuous paper electrophoresis[8]. In these experiments, individual LDH bands seen on the starch-gels were excised, inserted into a slit in an agar-gel, and subjected to a second electrophoresis[4], usually together with the original sample applied directly to another slit in the same agar slab.

The *total* serum lactic dehydrogenase was determined with a commercial kit (Sigma Chemical Company, St. Louis, Mo., U.S.A.). The upper limit of normal values was 400 Berger–Broida (BB) units/ml.

RESULTS

The LDH isoenzymes on the starch-gel appeared under ultra-violet light as dark bands that varied in intensity and width but not in position relative to the protein fractions. Omission of the pyruvate from the substrate mixture resulted in the absence of LDH bands. A total of eight LDH isoenzymes was seen in 226 gels obtained with 46 sera and 31 tissue homo-

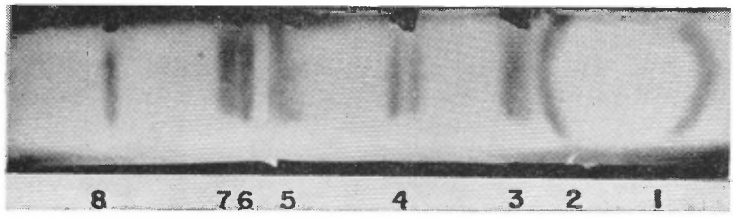

Figure 1. Ultra-violet photograph of a borate-buffered starch-gel after electrophoresis, showing the lactic dehydrogenase isoenzymes in the serum of a patient with acute myeloblastic leukaemia

genates, but in only one sample, the serum from a patient with acute myeloblastic leukaemia, were all eight bands found simultaneously (*Figure 1*).

Location of LDH isoenzymes on starch-gel

Figure 2 shows the position of the LDH isoenzymes on starch-gel and on agar in relation to the serum protein fractions in these media. The positions of the isoenzyme bands excised from starch-gel and run a second time on agar are shown by arrows labelled with the number of samples examined.

Although the positions of the isoenzymes in starch were constant relative to serum protein fractions, the activity of the individual LDH bands bore no relationship to the intensity of the staining of the associated protein bands, but depended rather on the enzyme source. Band 3 occurred in all

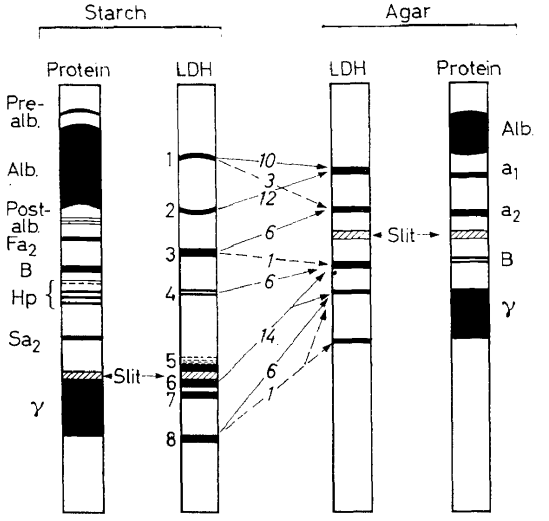

Figure 2. Serum and tissue lactic dehydrogenase isoenzymes detected by electrophoresis in borate-buffered starch-gel and barbiturate-buffered agar. The isoenzymes are labelled 1–8; the *italicized figures* on the arrows refer to the number of samples in which an excised starch-gel isoenzyme band appeared on agar in the indicated position after a second electrophoresis

samples, followed in order of frequency by bands 2, 4, 6, 8, 5, 1 and 7. Band 4 was almost always resolved into a doublet which occupied the same position as cholinesterase, aromatic esterase and acid phosphatase in borate-buffered starch-gel[9]. Band 5 was an ill-defined smear which did not appear consistently even in simultaneous replicate runs of a single sample on the same gel, which suggested it might be an artefact produced by delayed entry of enzyme into the gel. Band 6 was frequently quite broad, but an additional band 7 was resolved only in one kidney and one liver homogenate and in the sera of 7 patients. In 3 of the latter, band 7 was detected repeatedly, but in the remaining 4 it could not be consistently separated from band 6.

Samples showing band 8 invariably produced two γ-globulin LDH bands when the original enzyme sample was applied directly to a slit in agar and

subjected to electrophoresis. However, band 8 from the same samples excised from starch-gels and re-run on agar showed a second γ-globulin band of LDH activity in only 1 of 7 samples. This discrepancy probably reflects the lability of LDH isoenzymes of low mobility[10].

LDH isoenzymes in tissue homogenates

Homogenates of different tissues were subjected to simultaneous electrophoresis on a single gel in order to compare the position and activity of the isoenzyme bands (*Figure 3*). The relative activities of the bands were confirmed in a number of samples by electrophoresis of a series of dilutions of the original 10 per cent homogenate in borate buffer. Increasing dilution resulted in the progressive disappearance of bands until, in the highest dilution, only the isoenzyme with the highest activity was detectable.

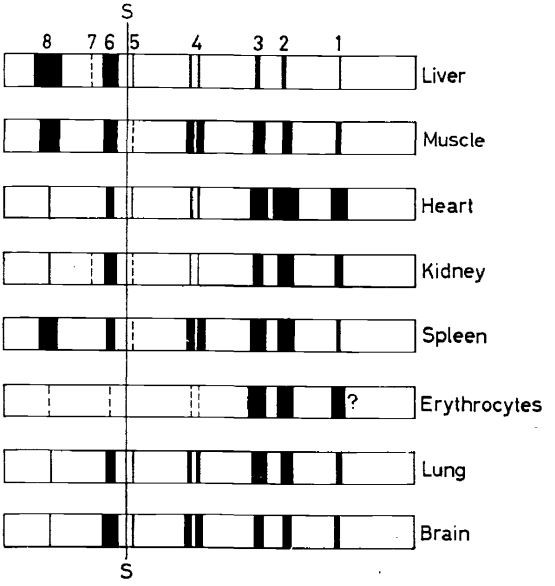

Figure 3. Lactic dehydrogenase isoenzymes in tissue homogenates

In a total of 63 electrophoretic studies of liver, muscle, kidney, brain, heart, spleen, and lung homogenates, never more than eight isoenzymes were detected in any one sample and rarely fewer than five. Results on erythrocyte homogenates were equivocal because haemoglobin partially obscured the position of some of the LDH bands.

With most electrophoretic methods[1-4], liver and muscle homogenates give similar isoenzyme patterns, *i.e.* high activity of the isoenzymes migrating with the "γ-globulins". In the present method, as in the barbiturate-buffered starch-gel procedure[3,10], LDH activity was proportionally greater in the "γ-globulin" region in liver than in skeletal muscle, while the latter tissue had somewhat greater activity in bands 1–4. Muscle consistently yielded six bands, in contrast to total of one and five isoenzymes reported[10,3] with barbiturate-buffered starch-gel. In heart, kidney and

erythrocytes, bands 1, 2 and 3, associated with the α-globulins, were very active but band 4 was relatively weak in these three tissues. Minor differences included very low activity in bands 5–8 of erythrocytes and the presence in kidney of a more intense band 6 than that seen in heart and erythrocytes.

Spleen homogenates showed patterns similar to those of skeletal muscle, but had relatively higher activity in bands 1–4. Bands 1–4, 6 and 8 were present in lung and brain homogenates, with the greatest activity in bands 3, 4 and 6.

It would appear that the present method is able to resolve more isoenzyme bands and to detect lower concentrations of the individual isoenzymes in tissue homogenates with greater consistency than other methods. Nevertheless, the results suggest that the increased sensitivity and resolving power of borate-buffered starch-gel offers no great advantage in differentiating between tissues which also yield similar isoenzyme patterns in barbiturate-buffered media.

LDH isoenzymes in serum

The majority of normal subjects showed LDH bands 3 and 6 in their sera, but very occasionally bands 2, 8 and 1 were also found, in that order of frequency (*Figure 4*).

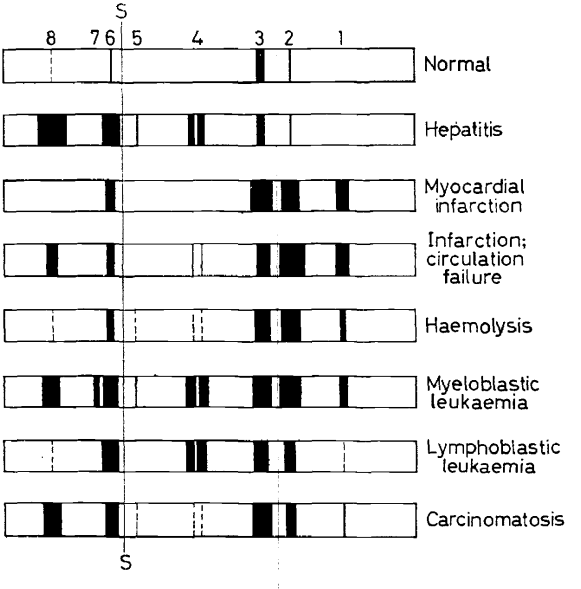

Figure 4. Lactic dehydrogenase isoenzymes in serum

Strikingly abnormal findings were seen in myocardial infarction. These findings are similar to those reported in barbiturate-buffered starch-grain[1], starch-gel[3,10] and agar[3,4], *viz.* intense activity of the two isoenzymes in the α-globulin region. However, in borate-buffered starch-gel, three bands were enhanced after infarction, *i.e.* 3, 2 and 1 in that order of intensity. In

the 14 cases of myocardial infarction examined, strong band 2 and 3 activity persisted after the total serum LDH had receded to nearly normal levels as the subjects recovered. Band 4 was faint or absent in all. Band 1 was usually detectable as long as the total serum LDH remained above normal.

Although band 1 was found more consistently in myocardial infarction than in other conditions, it was also detected in 2 patients with acute myeloblastic leukaemia having serum LDH levels of 1400 BB units/ml and in 1 subject with acute intravascular haemolysis. The latter case also had intense bands 2 and 3, making this serum indistinguishable from the " characteristic " pattern of myocardial infarction.

The sera of the 2 cases of acute myeloblastic leukaemia examined contained all of the isoenzymes described above except for band 7 which was present in only one of these subjects. Band 6 was broad and intense and, as mentioned above, band 1 was strong. It is of interest that all of these isoenzymes were detectable even when, as in one of these subjects, the total serum LDH level (460 BB units/ml) was only slightly elevated. A single case of chronic myelogenous leukaemia, not shown in *Figure* 4, was examined before and after treatment. Initially, his serum LDH fell within the upper limits of the normal range, but bands 2, 3, 5 and 6 were abnormally intense. After myleran treatment, the serum LDH level dropped to 200 BB units/ml and, of the three bands seen previously, band 3 was the only one that remained detectable. However, a fairly intense band 8 appeared during treatment, suggestive of a continuing subclinical manifestation of the disease and/or toxic effects of treatment. Two additional acute leukaemic subjects with the monocytic and lymphoblastic types of the disease had strong band 6 activity like the 2 myeloblastic cases discussed above (*Figure 4*). However, unlike the myeloblastic cases, bands 1 and 8 were very faint.

Early carcinoma without metastases did not produce an abnormal LDH isoenzyme pattern. However, in advanced carcinoma with metastases and elevated serum LDH levels, abnormal isoenzyme patterns were always seen. Bands 6 and 8 were usually present and band 4 barely detectable.

The enhanced activity in bands 6 and 8 in carcinoma patients with metastases was also seen in subjects with viral and toxic hepatitis and with cirrhosis, but bands 2 and 3 were weaker than in the carcinoma patients. It is of interest that band 8 often appeared in cholangiolytic hepatitis, simple obstructive jaundice of a few weeks' duration, congestive heart failure, and in patients who, after myocardial infarction, suffered engorgement of the liver as a result of circulatory failure. In the latter group bands 1–3 appeared within 24 h of the infarction, to be followed by the gradual appearance of bands 8 and often 6 also. It appears likely that the secondary appearance of these bands reflects liver involvement, but muscle and spleen cannot be excluded since they also have strong bands 6 and 8 (*Figure 3*).

Summary

1. The lactic dehydrogenase (LDH) isoenzyme patterns of human serum and tissue homogenates were examined with a modification of the Smithies' borate-buffered starch-gel technique.

2. A total of eight LDH isoenzymes was detected, in contrast to a maximum of five reported in barbiturate-buffered electrophoretic media.

3. The present method appears to be no more tissue-specific than other procedures in identifying the source of an elevated serum LDH when two or more diseased tissues with similar isoenzyme patterns were suspected of contributing to the serum LDH.
4. Probably because of the relative large volume of serum used, the present method is able to detect abnormal isoenzyme patterns in the absence of a markedly elevated total serum LDH levels in some patients with leukaemia, myocardial infarction, congestive heart failure, carcinomatosis and obstructive jaundice.

This work was supported by grants from the Muscular Dystrophy Association of Canada and the National Cancer Institute of Canada.

References

[1] E. S. Elliot and A. G. Bearn. *J. Clin. Invest.*, **37**, 672 (1958)
[2] R. J. Wieme. *Clin. Chim. Acta*, **4**, 46 (1959)
[3] F. Wróblewski, C. Ross and K. Gregory. *New Engl. J. Med.*, **263**, 531 (1960)
[4] M. C. Blanchaer. *Clin. Chim. Acta*, **6**, 272 (1961)
[5] O. Smithies. *Biochem. J.*, **61**, 629 (1955)
[6] C. L. Markert and F. Møller. *Proc. Natl. Acad. Sci. U.S.*, **45**, 753 (1959)
[7] O. Smithies. *Biochem. J.*, **71**, 585 (1959)
[8] B. R. Hill. *Ann. N. Y. Acad. Sci.*, **75**, 304 (1958)
[9] C. A. Dubbs, C. Vivonia and J. M. Hilburn. *Science*, **131**, 1529 (1960)
[10] P. G. W. Plagemann, K. F. Gregory and F. Wróblewski. *J. Biol. Chem.*, **235**, 2282 (1960)