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COMPARISON OF PURIFIED LACTATE DEHYDROGENASES FROM NORMAL RAT LIVER AND MORRIS HEPATOMAS IN RATS AND IN CULTURE

R. J. CARLOTTI[a], G. F. GARNETT[a], W. T. HSIEH[a*], A. A. SMUCKER[a**], C. S. VESTLING[a] and H. P. MORRIS[b]

[a]*Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242, and* [b]*Department of Biochemistry, Howard University, Washington, D.C. 20001 (U.S.A.)*

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SUMMARY

Lactate dehydrogenase (EC 1.1.1.27) from Morris rat hepatomas grown in vivo and from Morris rat Hepatoma 7288c grown in cell culture has been maximally purified for comparison with lactate dehydrogenase from normal rat liver. A rabbit antiserum directed against normal rat liver lactate dehydrogenase has been produced to allow immunochemical characterization. Other studies included heat inactivation, oxamate inhibition, ethanol sensitivity, starch gel electrophoresis, and polyacrylamide gel electrophoresis. By each criterion the comparisons revealed enzyme identity.

INTRODUCTION

Research in this laboratory has focused on a complete characterization of the enzyme, lactate dehydrogenase (EC 1.1.1.27) from normal rat liver (Gibson et al. [1] and Davisson et al. [2]). The purpose has been to investigate the properties and function of the highly purified enzyme in sufficient detail so that the "same" enzyme could be isolated from rat hepatomas of differing characteristics and studied in a similar fashion. Although many enzymes from tumors have been assayed or purified to some extent, the paucity of studies on highly purified enzymes has prompted a partial characterization of purified lactate dehydrogenase, a key metabolic dehydrogenase, from two Morris hepatomas and one in cell culture for comparison with normal rat liver lactate dehydrogenase.

Our selection includes the Morris Hepatomas 7793 (intermediate growth rate) and 7777 (fast growing) cultivated in vivo. No. 7793 is classified as a well-differentiated trabecular carcinoma of minimal metabolic deviation compared to normal rat liver. No. 7777 is considered to be a poorly differentiated hepatoma which should be ex-

Abbreviation: HTC cells, Hepatoma Tissue Culture cells.

* Permanent address: Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan, China.

** Permanent address: Department of Chemistry, Goshen College, Goshen, Ind. 46526, U.S.A.

pected to exhibit greater metabolic deviation. In principle, the feature of minimal metabolic deviation should enable the investigator to detect subtle changes which may accompany conversion from a normal to a cancerous state [3]. Recently, we have also acquired from Dr Daryl K. Granner of the Department of Internal Medicine, Morris Hepatoma 7288c grown in vitro, hereafter referred to as HTC cells (Hepatoma Tissue Culture). This hepatoma is classified as a "less well-differentiated trabecular carcinoma of intermediate growth rate, not a minimal deviation type" [4].

MATERIALS AND METHODS

The transplantable Morris hepatomas (7777 and 7793) were grown in the rear legs of young Buffalo strain male rats (150–200 g body weight). No. 7777 required about one month for development; No. 7793 required about four months. The tumors were excised, freed from adhering tissue, frozen on solid CO_2 and stored at -20 °C.

The HTC cell culture was derived from ascites tumor cells which were originally obtained from the solid Morris Hepatoma 7288c [5]. The cells were grown to a maximum density of 10^6 cells/ml, harvested, and frozen.

Lactate dehydrogenase from rat liver or Morris hepatoma

The procedure described by Hsieh and Vestling [6] was used with a few minor modifications. Solutions were concentrated with the aid of Amicon ultrafilters*. Rechromatography on carboxymethyl cellulose was employed when needed.

Development of rabbit antiserum against normal rat liver lactate dehydrogenase

An antibody was prepared against normal rat liver lactate dehydrogenase by injecting five rabbits with maximally pure normal rat liver enzyme according to the methods described by Williams and Chase [7]. Each rabbit was injected subcutaneously with 1 ml of a solution containing 3 mg of normal rat liver lactate dehydrogenase dissolved in 140 mM NaCl and emulsified with 1 ml of Freund's complete adjuvant. Six weeks after immunization a 1-mg booster of normal rat liver lactate dehydrogenase was administered similarly. On the fourth and fifth day after boosting, each rabbit yielded 50 ml of whole blood. After clotting the serum was decanted, centrifuged and stored at -20 °C in sealed tubes.

Absorption of raw antiserum

During preliminary immunodiffusion, a small amount of impurity in the "pure" normal liver lactate dehydrogenase was indicated by a faint extra line of precipitin. This is due to the rabbit's biological amplification of trace antigenic contaminants. This antibody against the impurity was removed from the antiserum by absorption with crude rat liver homogenate (1 g normal rat liver per 10 ml of 500 mM NaCl) according to the methods described by Raffel [8].

A modification of the Ouchterlony technique [9] was employed using a 1%

* Complete information is available from Scientific Systems Division, Amicon Corporation, 21 Hartwell Ave., Lexington, Mass. 02173, U.S.A.

solution of Agar-Noble (Difco Laboratories) in barbital–acetate buffer, pH 8.6, 0.1 ionic strength (Hartman–Leddon Co., Philadelphia). The center well was filled to capacity with undiluted antiserum, while pure enzyme approximating 1 mg/ml in 100 mM potassium phosphate, 100 mM $(NH_4)_2$ SO_4, 1 mM 2-mercaptoethanol, pH 7.6, buffer occupied the peripheral wells. Incubation at room temperature proceeded overnight.

Immunoelectrophoresis

With the agar previously described, immunoelectrophoresis was performed on diffusion plates with a central trough for antiserum flanked by a well on either side for the antigen according to the method of Osserman [10]. The current was maintained for 1 h at 4 mA per slide in parallel on a Gelman electrophoresis apparatus. Both the anode and cathode sides of the trough contained barbital–acetate buffer, pH 8.6, 0.1 ionic strength. After the electrophoresis was completed, the agar troughs were opened and filled with 0.1 ml of the antiserum. Diffusion proceeded overnight at room temperature.

Enzyme inhibition by anti-normal rat liver lactate dehydrogenase

Inhibition studies of lactate dehydrogenase in the presence of increasing amounts of antiserum directed against the normal rat liver enzyme were carried out according to a modification of the method of Chang and Bollum [11]. In each experiment a control without added antiserum was included as well as a control containing an aliquot of serum from a non-immunized rabbit. Samples were incubated at 37 °C for 1 h in a constant temperature water bath. Remaining enzyme activity was measured at 340 nm in the Cary 15 recording spectrophotometer. A cuvette with a 1-cm cell light path contained 110 mM lithium lactate, 30 mM sodium veronal buffer, pH 8.6, 0.2 mM NAD, 7.65 mM NaCl, and 2.75 mM $NaHCO_3$, and 50 μl of sample.

A somewhat different procedure was used in comparing HTC lactate dehydrogenase with normal enzyme. It was found that antiserum from individual rabbits showed appreciable and varying amounts both of enzyme activity and of non-specific NAD reductase (reduction of NAD in the absence of any added substrate). Both activities were eliminated without any apparent effect on antibody activity by heating the serum rapidly to 70 °C, holding for 10 min, then cooling as rapidly as possible on ice. A gelatinous precipitate was removed by centrifugation at 23 000 $\times$ g for 1 h at 0–3 °C.

Stability and heat inactivation

The concentration and quantity of enzyme used was 7.5 μg in 1 ml of 100 mM potassium phosphate, 100 mM $(NH_4)_2$ SO_4, 1 mM 2-mercaptoethanol, pH 7.6, buffer. The mixture was held for 1 h at the following temperatures: 5, 25, 37, 60, and 65 °C. Other identical samples prepared as above were held at 25 °C for 24 and 48 h. After the appropriate incubation period, all samples were assayed for remaining enzyme activity.

Enzyme inhibition by oxamate

Enzyme from normal rat liver, intermediate growth rate Morris Hepatoma 7793, and fast-growing Morris Hepatoma 7777 were compared by calculating the

percent activity remaining after addition of inhibitor. A solution of pure enzyme (approx. 6 μg in 1 ml of 500 mM NaCl and 18 mM $NaHCO_3$) was incubated with various concentrations of oxamate as inhibitor. After 5 min at 25 °C, the remaining activity was measured.

Comparison of enzyme by starch gel electrophoresis

Starch gel electrophoresis was performed according to the modified method described by Mann and Vestling [12] with the use of 200 mM lithium lactate as the substrate for 100 μg of enzyme. Also a current of 200 mA instead of 30 mA was applied.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed with 8% gels by a modification of the procedure of Davis [13]. Samples were prepared for application by diluting maximally pure lactate dehydrogenase with 40% sucrose, or by dissolving solid sucrose in the enzyme solution to make a final 40% concentration of sucrose. 20 μl of sucrose–enzyme solution containing either 0.5 or 5.0 μg enzyme were applied to the gels. 10 μl of a bovine serum albumin solution (5 mg per ml) in 40% sucrose were added, followed by a layer of cathode buffer at the top of the tube [14].

The tubes were then placed in the grommets of the electrophoresis unit. The cathode chamber contained the buffer with 5 drops of 0.05% bromophenol blue per liter of buffer. Electrophoresis was carried out at 2.0 mA per gel, 300–450 V. The visible migration of the major albumin–bromophenol blue band was noted, and the time taken for this band to reach the 35-mm mark was recorded for each gel. For each gel, the total electrophoresis time was twice the time taken for the bovine serum albumin–bromophenol blue band to reach the 35-mm mark, which usually was about 2 h.

Staining was done via two different procedures. Protein staining and destaining was done with Coomassie blue according to Weber and Osborn [15]. Activity staining was done by 10–30 min incubation of the gels in 108 mM lithium lactate, 1.56 mM NAD, 10.8 mM NaCl, 540 μM $MgCl_2$, 135 mM Tris, 320 μM nitroblue tetrazolium chloride, 86 μM phenazine methosulfate, pH 8.6. Protein-stained gels contained approx. 5.0 μg of lactate dehydrogenase, and activity-stained gels contained 0.5 μg of lactate dehydrogenase.

Sodium dodecylsulfate–polycrylamide gel electrophoresis

This procedure is according to Weber and Osborn [15]. The protein standards and their molecular weights were sperm whale myoglobin (17 800), chymotrypsinogen (25 000), and ovalbumin (45 000), all purchased from Mann Research Laboratories. A 25-μg sample of protein in 100 mM potassium phosphate, 100 mM $(NH_4)_2\ SO_4$, 1 mM 2-mercaptoethanol, pH 7.6, was applied to each tube.

RESULTS

A comparison of the purification of lactate dehydrogenase from four sources

There are considerable differences in the extraction of enzyme from normal rat

liver from hepatomas (Table I). The units* are those recommended in the "Report of the Commission on Enzymes of the International Union of Biochemistry (Pergamon Press, 1961)".

The specific activities in the initial extraction step vary as shown in Table I. The low specific activity encountered after Steps B and C in extractions from the slower growing hepatoma necessitated an additional step in the extraction. The specific activities in the range of 91–97 are indicative of highly purified enzyme.

TABLE I

A COMPARISON OF LACTATE DEHYDROGENASE PURIFICATION FROM NORMAL RAT LIVER, MORRIS HEPATOMAS 7793, 7777, AND HTC CELLS

Tissue	Normal rat liver		Morris Hepatoma 7793		Morris Hepatoma 7777		HTC	
Initial units/g tissue	81		32		96		83	
Procedure*	Specific activity (units/ml/ $A_{280\ nm}$)	Recovery (%)	Specific activity	Recovery (%)	Specific activity	Recovery (%)	Specific activity	Recovery (%)
Step A	1.2	100	0.69	100	0.80	100	3.6	100
Step B	1.7	74	1.1	83	1.1	84	8.5	92
Step C	3.9	63	2.6	74	5.7	37		
Step C'**			15	66				
Step D2	47	53	53	35	86	25	51	54
Step E2	110	50	92	24	97	24	97	51

* Step A, 20% ethanol, 500 mM NaCl extraction. Step B, 0.3 g $(NH_4)_2SO_4$/ml. Step C, 5–30% ethanol and dialysis vs 20 mM potassium phosphate, 1 mM β-mercaptoethanol (pH 6.0).

** Step C', single step non-gradient elution with 50 mM potassium phosphate, 1 mM β-mercaptoethanol (pH 6.0). This addition to the procedure is only necessary in the case of Morris Hepatoma 7793 due to the relatively low specific activity after Step C. A successful continuation of the fractionation can thus be achieved. Step D2, CM-cellulose gradient elution with 20 mM → 75 mM potassium phosphate, 1 mM β-mercaptoethanol (pH 6.0). Step E2, DEAE-cellulose.

Heat inactivation of lactate dehydrogenase

There were not significant differences in the stability of either hepatoma enzyme when compared to normal rat liver enzyme. In all cases incubation at 65 °C for 1 h completely inactivated the lactate dehydrogenase.

Oxamate inhibition

No differences were seen in the oxamate inhibition of enzyme from either normal rat liver or the Morris hepatomas.

* The lactate dehydrogenase activity units are those described by Hsieh and Vestling [12]. Lithium L-lactate was used as substrate at one-half the concentration of sodium DL-lactate formerly used.

Immunodiffusion (Ouchterlony technique)

With absorbed anti-normal rat liver enzyme the reaction of immunological identity is evident (Fig. 1a, top), and the picture in this specific case is typical of each comparison. The line of precipitin reaction is continuous at any intersection of zones. These results provide evidence for enzyme identity.

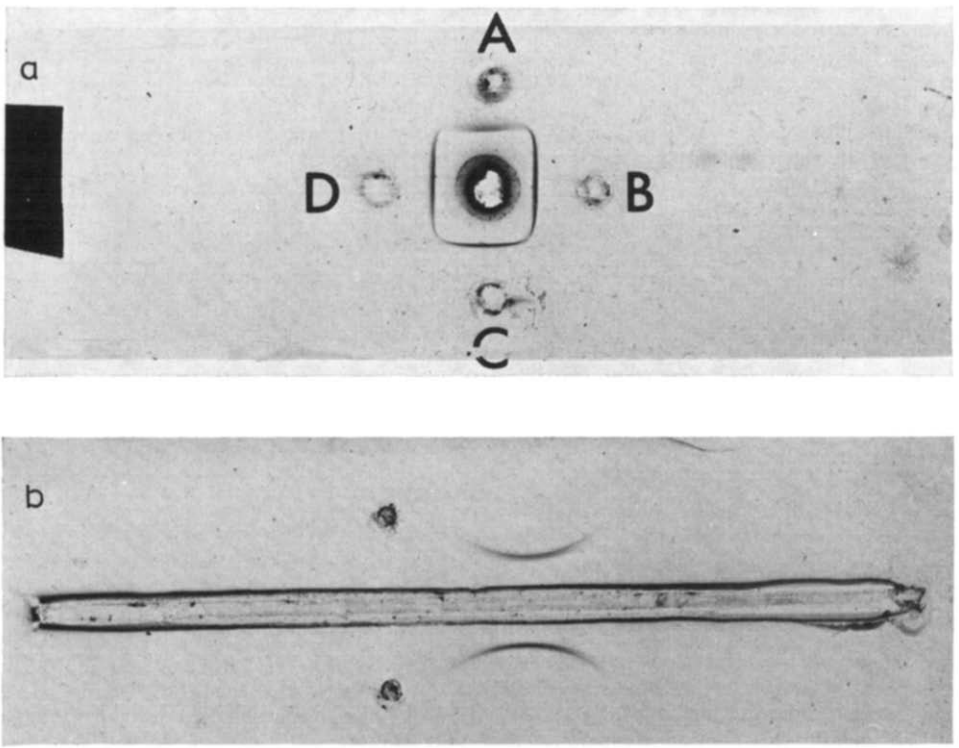

Fig. 1. (a, top) Immunodiffusion showing the reactions of identity with 50 μg amounts of normal liver lactate dehydrogenase (Wells A and D), Morris Hepatoma 7793 lactate dehydrogenase (Well B), and Morris Hepatoma 7777 lactate dehydrogenase (Well C) as detected by 50 μl of anti-normal lactate dehydrogenase serum (Center well) which had been treated with rat liver homogenate to absorb traces of antibody against antigen impurities (see text). (b, bottom) Immunoelectrophoresis showing the comparison between 50-μg quantities of normal liver lactate dehydrogenase (upper) and Morris Hepatoma 7777 lactate dehydrogenase (lower) following electrophoresis for 1 h at 4 mA, pH 8.6, and immunodiffusion for 16 h at 25 °C. Anti-normal lactate dehydrogenase serum which had been treated with rat liver homogenate to absorb traces of antibody against antigen impurities (see text) was placed in the center trough.

Immunoelectrophoresis

The immunoelectrophoresis studies support the conclusions drawn above from immunodiffusion experiments. The results of a typical experiment are shown in Fig. 1b, bottom. In every case the precipitin patterns demonstrate identity for the enzyme involved.

Enzyme inhibition by anti-normal rat liver lactate dehydrogenase

The nearly identical patterns of enzyme inhibition are apparent (Figs 2a and 2b). Enzyme from normal rat liver, intermediate growth rate Morris hepatoma, and fast-growing Morris hepatoma are all inhibited to the same extent at each dilution of antiserum (Fig. 2a). A similar comparison holds for normal enzyme and HTC enzyme (Fig. 2b). The highly sensitive reaction between antibody and antigen and the measurement of catalytic function reveal no structural differences between normal and hepatoma lactate dehydrogenase.

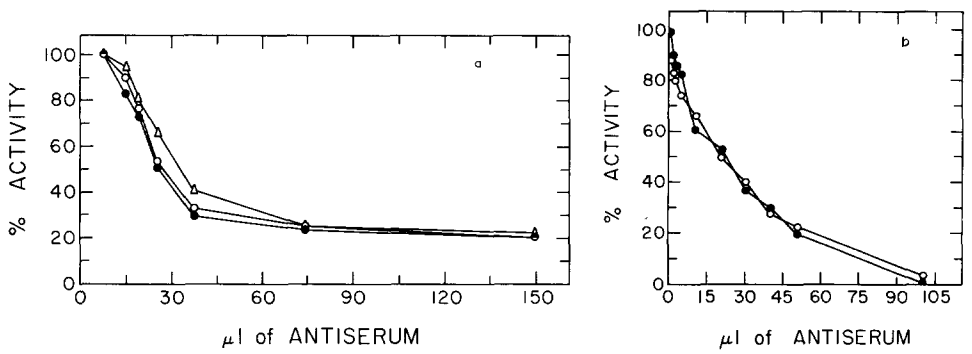

Fig. 2. (a) Activity remaining after inhibition by rabbit antiserum directed against normal rat liver lactate dehydrogenase. Systems containing 30 μg of normal enzyme (○—○), Morris Hepatoma 7777 enzyme (△—△), and Morris Hepatoma 7793 enzyme (●—●) were incubated for 1 h at 37 °C in the presence of appropriately diluted antiserum before assaying. The solvent was 100 mM potassium phosphate, 100 mM $(NH_4)_2SO_4$, 1 mM β-mercaptoethanol, pH 7.6. The antiserum was not heat treated to remove small amounts of non-specific NAD reductase. (b) Activity remaining after inhibition by heat-treated rabbit antiserum directed against normal rat liver lactate dehydrogenase. Systems containing 0.1 μg of normal (○—○) or HTC lactate dehydrogenase (●—●) were incubated for 40 min at 37 °C in the presence of appropriately diluted antiserum before assaying. The solvent was 500 mM NaCl and 18 mM $NaHCO_3$.

Starch gel electrophoresis

Enzyme from normal rat liver, intermediate growth rate Morris hepatoma, and fast-growing Morris hepatoma each move with identical mobility and display a single spot which migrates to the cathode under the conditions employed.

Polyacrylamide gel electrophoresis

The enzyme from the normal rat liver, intermediate growth rate Morris hepatoma, fast-growing Morris hepatoma, and HTC cells migrated with the same mobility. When the gels were stained for catalytic activity or protein content, the patterns were identical. In Fig. 3 there is presented a representative result with activity staining. Similar results involving protein and activity staining were found with all combinations of normal and hepatoma lactate dehydrogenase.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

The monomers of highly purified enzyme from each source also migrated with identical mobilities. When plotted on semi-log paper with the mobilities versus

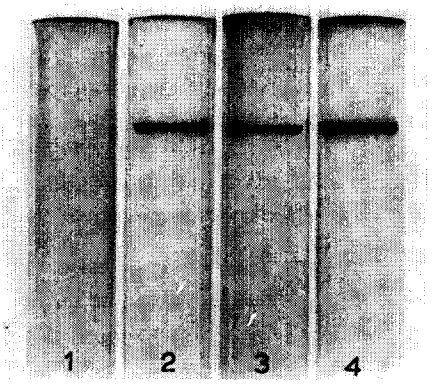

Fig. 3. Polyacrylamide gel electrophoresis of normal liver lactate dehydrogenase, HTC lactate dehydrogenase, and a mixture of the two. Zones detected by activity staining. See text for details. Tubes numbered from the left: 1, control with no applied enzyme; 2, normal enzyme; 3, HTC enzyme; 4, normal enzyme plus HTC enzyme.

molecular weights of known protein standards, the enzyme from normal rat liver, intermediate growth rate Morris hepatoma, and fast-growing Morris hepatoma shows the same molecular weight (about 35 000) in each case.

DISCUSSION

Lactate dehydrogenase has been maximally purified from intermediate growth rate Morris Hepatoma 7793, fast-growing Morris Hepatoma 7777, and from HTC cells. Results from sodium dodecylsulfate–polyacrylamide gel electrophoresis reveal a monomer from each tumor source of identical mobility when compared to that from normal rat liver enzyme and to that from pure M_4 type rabbit muscle enzyme. Indeed, this report appears to be the first involving the characterization of highly purified lactate dehydrogenase from a tumor source, whether cultivated in vivo or in vitro.

These experiments strongly indicate identity among the lactate dehydrogenases studied. This conclusion is supported by the similar results obtained from heat inactivation, oxamate inhibition, immunodiffusion and immunoelectrophoresis, enzyme inhibition by antibody directed against normal rat liver enzyme, starch gel electrophoresis, polyacrylamide gel electrophoresis, and sodium dodecylsulfate–polyacrylamide gel electrophoresis. Starch gel electrophoresis of lactate dehydrogenase isoenzymes from other tumors also reveals a similar pattern of near identity to normal rat liver [16].

The only difference among the three lactate dehydrogenases appears during purification (Table I). The initial extractable enzyme units and specific activity in the first step are clearly lowest in the intermediate growth rate Morris Hepatoma 7793. The final specific activity of enzyme from the fast-growing Morris Hepatoma 7777 and of HTC cells compares very closely with that of enzyme from normal rat liver.

Some correlations have been made regarding tumor growth rate and the ribonucleotide reductase activity of Morris hepatomas [17]. The initial specific activity of ribonucleotide reductase from fast-growing tumors was always higher than that

from more slowly growing tumors. Similar findings also hold true for lactate dehydrogenase [18, 19].

There is a trend in the pattern of enzyme development during carcinogenesis toward reversion to the prenatal form as reported by Weinhouse and co-workers [20, 21]. Their investigations show that some rat hepatoma isozymes of phosphorylase, kinetically and immunologically different from normal rat liver, are identical to those of normal fetal rat liver. Studies by Farron et al. [22] using zone electrophoresis of lactate dehydrogenase extracts reveal that Morris Hepatoma 7800 does not display the small amounts of isoenzyme-4 shown by adult and fetal liver.

The conclusion from the results in the present paper is that the demonstrated identity of the pure lactate dehydrogenases from normal rat liver and from three Morris hepatomas indicates that the original carcinogenic event(s) did not involve alterations in gene expression related to the biosynthesis of lactate dehydrogenase. This conclusion is of course limited to those comparisons which have been made. The possibility of other more subtle changes exists and is being explored.

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