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MODULATION OF LACTATE DEHYDROGENASE ACTIVITY BY ENZYME-PROTEIN INTERACTION

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Some lactate dehydrogenase modulator proteins have been isolated from the lactate dehydrogenase-free crude mitochondrial fraction of rabbit muscle, beef liver and chicken liver. It was shown that beef and chicken liver mitochondrial extracts exhibited activatory capacity in contrast to the inhibitory capacity of rabbit muscle mitochondrial extracts. All modulators can be precipitated by 80% ammonium sulphate saturation and show high anodic electrophoretic mobility and heat stability. Modulators have higher affinity for alkaline pI lactate dehydrogenase isoenzymes, independent of whether the M and H subunits are predominant. The inhibitor and the activator molecules compete for lactate dehydrogenase since their modulatory capacity was nullified when similar relative amounts were used. This study shows the existence of analogous proteins with an acidic pI in the different mitochondrial fractions which modify lactate dehydrogenase activity.

KEY WORDS: Lactate dehydrogenase, mitochondrial fraction. protein modulators, inhibitor, activator

INTRODUCTION

The most extensive model of structural organization of metabolism in the cytosol has been proposed by Ureta[1] in assuming individual complexes formed by the enzymes contributing to a metabolic route. The requirement to integrate identical, or at least similar, catalytic properties into different enzyme complexes, while granting selectivity in protein-protein interactions, is regarded as the reason for the evolution of different isoenzymes. In fact, the kinetic and regulatory properties of isoenzymes are often similar, and the most prominent diversity between them lies in the difference of their electric charge exhibited at physiological pH values.

Nevertheless, the glycolytic enzyme molecules in the cellular milieu are interacting not only with other soluble enzyme molecules, but also with membranous structures such as mitochondria[2–4] and the contractile filaments[5–7]. The soluble and bound forms are kinetically distinct.[8–12] This event has been extensively reported for lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) from many sources associated with different particulate subcellular structures and, in general, binding causes an important decrease of enzyme activity.[13–18] The binding capacity of lactate dehydrogenase to the mitochondrial fraction depends on the net charge of the isoenzyme, since, the binding process does not occur with H-type isoenzymes with an acid pI or with M-type isoenzymes with pI near 6, but binding and the concomitant enzyme inhibition are oberved with M or H-type isoenzymes with pI near 7 or higher.[18,19] Thus, the modulation of lactate dehydrogenase activity by enzyme-membranous particles or enzyme-contractile filaments interactions may be very important in

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explaining the physiological significance of the localization and compartimentation of this enzyme in relation to its isoenzymatic composition.

In previous studies performed in our laboratory, a lactate dehydrogenase inhibitor protein was isolated from rabbit skeletal muscle crude mitochondrial fraction and its molecular characterization carried out.[20,21] The author suggested that this inhibitor protein might be part of the mitochondrial fraction binding site for lactate dehydrogenase. In this paper, two lactate dehydrogenase activator proteins were isolated from beef and chicken liver mitochondrial fractions, using the same methodology as for inhibitor isolation and some of their characteristics were studied. We have conducted a comparison between their modulatory effect on different sources of lactate dehydrogenase isoenzymes. All modulators have higher affinity for alkaline pI lactate dehydrogenase isoenzymes showing that this modulation of lactate dehydrogenase activity by enzyme-protein interactions is also isoenzyme dependent.

MATERIALS AND METHODS

Materials

Lactate dehydrogenase substrates were freshly prepared solutions in 5 mM sodium phosphate buffer (pH 6) of sodium pyruvate and β-nicotinamide adenine dinucleotide disodium salt (NADH) (Boehringer, Mannheim, F.R.G.). NADH was prepared immediately before use to avoid the formation of specific enzyme inhibitors. All other chemicals used were analytical reagent grade (Merck or Boehringer).

Isolation of Mitochondrial Fractions and Cytosoluble Lactate Dehydrogenase

Homogenates from beef or chicken liver and rabbit skeletal muscle were obtained by trituration of fresh tissues with cold 0.25 M sucrose solution, pH 6, (1:8, w/v) in a Potter-Elvejhem homogenizer refrigerated with an ice-water bath using 10 up-down strokes at 700 rpm of a loose fitting teflon pestle, 95–115 μm clearance (B. Braun-Melsungen, type 853202). Beef and chicken liver crude mitochondrial fractions were obtained from their homogenates by differential centrifugation as described previously.[18] Lluis' method[16] was used to prepare rabbit muscle crude mitochondrial fraction. Different sources of cytosoluble lactate dehydrogenase were obtained from the post-mitochondrial supernatants by centrifugation at 105.000 × g for 90 min at 2°C (Beckman ultracentrifuge L5-75).

Isolation of Modulators

Each crude mitochondrial fraction (1 g) free from overtly assayable lactate dehydrogenase activity (crude mitochondrial fraction washed three times with 0.15 M NaCl. pH 6), obtained as described previously, was homogenized with 30 ml of 0.15 M NaCl solution (pH 6) and disrupted by sonication at 0°C in a 150 Vibrasons (Kerry Ultrasonics, KT-150) at 15 μm wave amplitude for 120s (30s fractions). The suspensions were centrifuged at 70.100 × g for 60 min at 2°C. The supernatants showed lactate dehydrogenase activity (0.07, 2.64 and 3.05 U/ml for beef liver, chicken liver and rabbit muscle mitochondrial extracts respectively). This activity was removed by heating each sample in boiling water for 10 min before checking for the presence of the modulators.

Enzyme Activity

The lactate dehydrogenase activity was determined by observing the linear absorbance changes at 340 nm for periods less than 2 min with a PYE-Unicam SP 1700 recording spectrophotometer in a 1 cm light-path cell. The reaction was initiated by the addition of cold soluble enzyme solution to cells containing the optimum substrate concentrations for each enzyme source (pyruvate 0.4 mM, 0.8 mM and 0.3 mM for beef liver, rabbit skeletal muscle and chicken liver respectively; in all cases 0.1 mM NADH concentration was used), in 5 mM sodium phosphate buffer pH 6. The enzyme unit (U) is the amount of enzyme causing the disappearance of 1 μmol NADH/min.

The modulatory capacity of extracts isolated from the crude mitochondrial fractions was calculated as a percentage inhibition or activation of a sample of cytosoluble lactate dehydrogenase whose activity was determined (as described above) in the absence and presence of the amount of modulator stated in each assay.

Electrophoresis

Discontinuous polyacrylamide disc gel electrophoresis (running gel T = 6.5%, C = 2.6% and stacking gel T = 2.9%, C = 25%) was carried out as described previously,[20] using 0.37 M Tris-HCl buffer, pH 8.9, as anode solution and Tris-glycine (0.65 M Tris and 1.9 M glycine) buffer, pH 8.5, as cathode solution. The process was run at room temperature for 30 min at 2 mA/gel. Coomassie brilliant blue (0.02%) in water-acetic acid-ethanol (200:16:50) was used for staining (3–8 h at room temperature). Destaining was allowed by successive washings with the same reagent without Coomassie.

Isoelectric focusing (IEF)

The lactate dehydrogenase isoenzymes were separated by agarose gel IEF using 1.0 mm thick agarose gel plates containing 6.5% Pharmalyte (Pharmacia Fine Chemicals) in the pH range 3–10. The electrode solutions used were 1.0 M NaOH and 0.05 M H_2SO_4. The gel plates were prepared as outlined in the Pharmacia Fine Chemicals instruction pamphlet 52-1536-01. The samples containing 0.1–0.2 lactate dehydrogenase units were applied onto filter-paper strips placed near the anodic end and were separated with the power limit set at 15 W and the voltage limit set at 1500 volts. The sample application strips were removed after 45 min and the separation continued for a further 45 min. Immediately after each run the plates were placed in a bath of lactate dehydrogenase reagent.[22] When development of the purple colour took place plates were then fixed in a 7% acetic acid solution and dried as indicated in the above-mentioned pamphlet.

RESULTS AND DISCUSSION

Characterization of Lactate Dehydrogenase Modulators

It has been reported that rabbit muscle inhibitor is extremely heat-stable.[20] This protein characteristic was comparatively investigated for the three modulator

TABLE I
Heat stability of mitochondrial lactate dehydrogenase modulators.

Source of protein Modulator	% remaining capacity of modulator at given time of heat-treatment		
	10 min	30 min	60 min
Rabbit muscle	100	96.7	93.5
Beef liver	100	65.7	55.9
Chicken liver	100	79.8	74.0

10 ml of mitochondrial extracts were heated in a boiling-water bath for the given times. After extensively dialysis against 5 mM sodium phosphate buffer (pH 6) the modulatory capacity of 0.2 ml was determined, as described under enzyme activity in the methods, using 6.2×10^{-3} U of rabbit muscle cytosoluble lactate dehydrogenase. Percentage remaining capacity was referred to the lactate dehydrogenase inhibition (rabbit muscle) or activation (beef or chicken liver) due to modulator after 5 minutes of heating treatment.

preparations. The results obtained are shown in Table I. Beef and chicken liver lactate dehydrogenase modulators also showed high heat stability. This characteristic was used for its purification. Thus, mitochondrial extracts were heated in boiling water for 10 min, cooled rapidly in a ice-water bath, centrifuged at 25.500 × g for 30 min at 2°C and dialysed for 24 h against 5 mM sodium phosphate buffer (pH 6) to remove the NaCl.

Figure 1 shows the electrophoretic behaviour of the heated modulators-containing

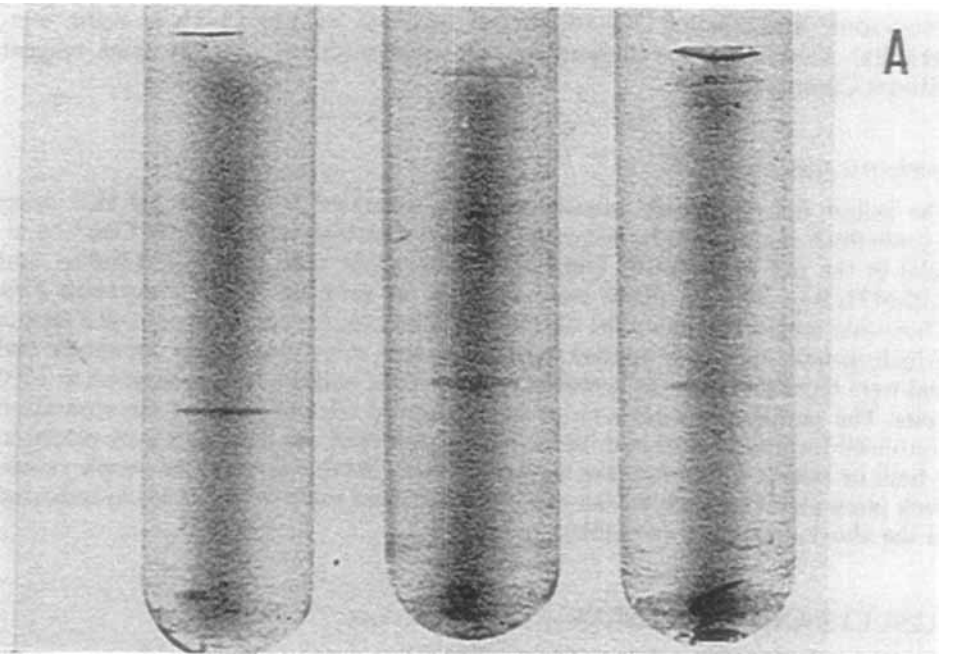
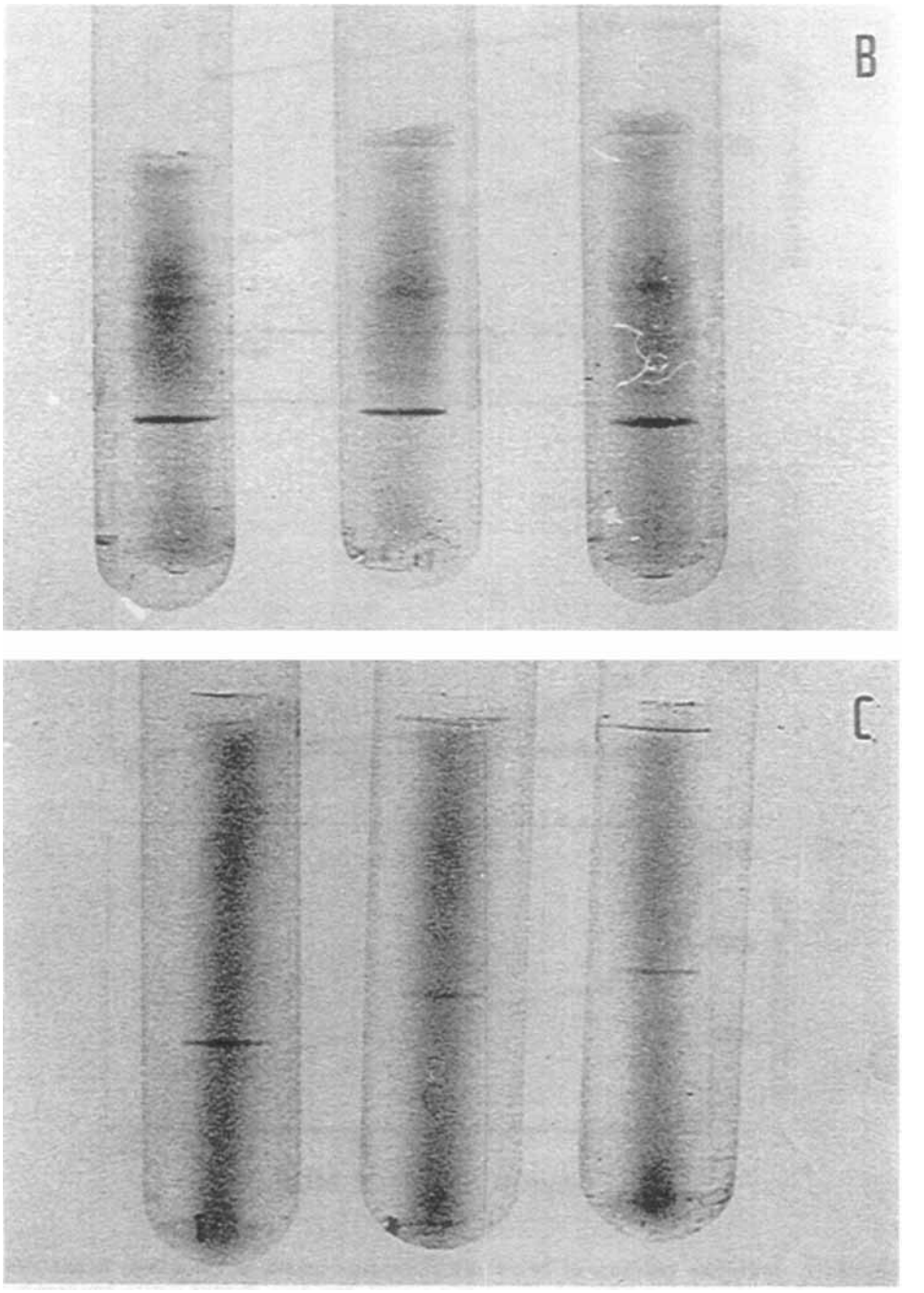


FIGURE 1 Electrophoretic behaviour of lactate dehydrogenase modulators. Discontinuous polyacrylamide disc gel electrophoresis was carried out as described in the text. Applied samples were 300 μl of the purified lactate dehydrogenase modulators from: (A) rabbit muscle (A_{280} = 0.320). (B) beef liver (A_{280} = 0.530) and (C) chicken liver (A_{280} = 0.610). Top to bottom direction means anodic mobility. Left to right: extracts heated in a boiling-water bath 10, 30 and 60 min.



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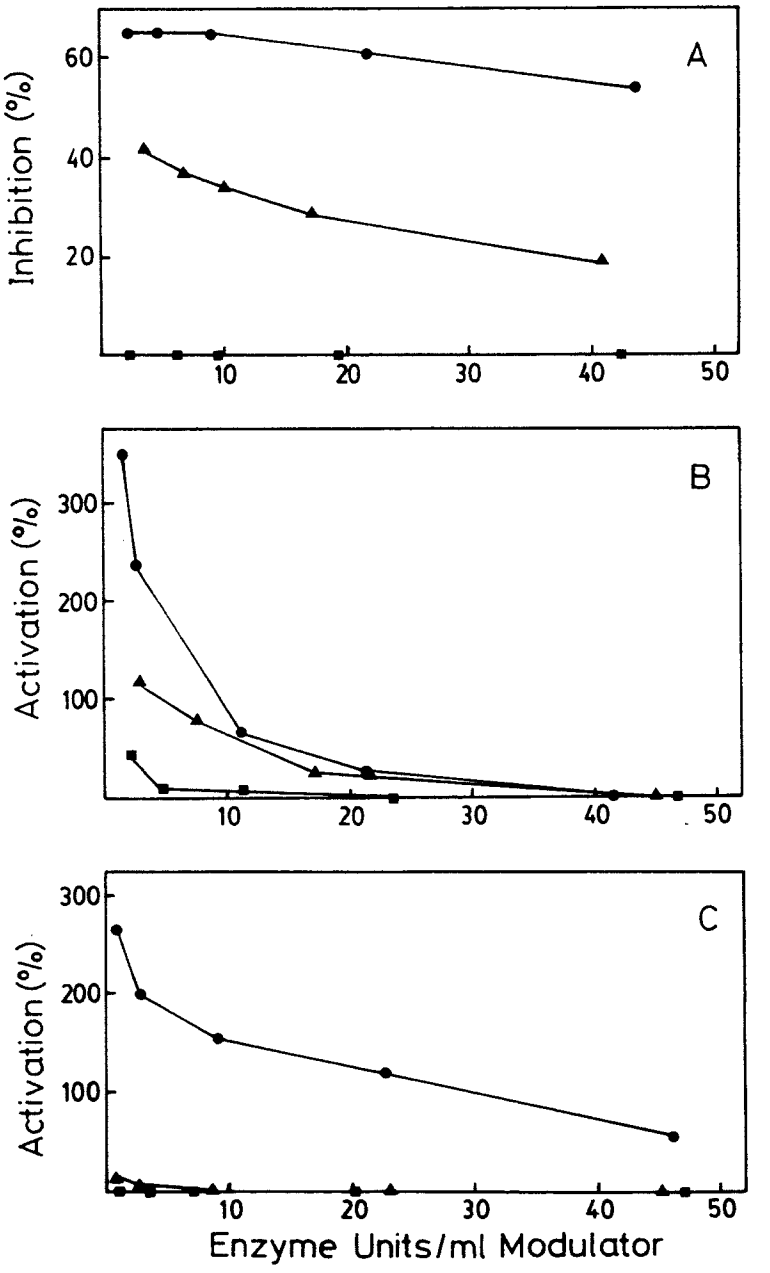

FIGURE 2 Isoenzyme specificity of lactate dehydrogenase modulators. Cytosoluble lactate dehydrogenase from: (●) rabbit muscle, (▲) chicken liver and (■) beef liver. Purified lactate dehydrogenase modulators from: (A) rabbit muscle, (B) beef liver and (C) chicken liver.

extracts. All the lactate dehydrogenase modulator preparations showed one predominant band of high anodic electrophoretic mobility to which was associated the presence of the rabbit muscle inhibitor.[20,21] In all cases, this band together with the modulatory capacity-disappeared when under the same conditions described in Table I mitochondrial extracts were heated for 4 h at pH 4.3. No modulatory capacity was observed in supernatants of purified preparations raised to 80% saturation with $(NH_4)_2SO_4$ and centrifuged. It was shown (Fig. 1) that the electrophoretic runs of the extremely heat-stable rabbit muscle inhibitor and chicken liver activator decreased with an increase in the heat-treatment time. This would account for structural modifications in the rabbit muscle inhibitor and chicken liver activator proteins related to their net charges during the heat-treatment. On the contrary, the electrophoretic run of beef liver activator is not modified with an increase in the heat-treatment time although it is the least heat-stable of the three modulators. This suggest that the heat-treatment of beef liver activator does not modify the protein net charge. These findings indicate that the inhibitor and the activators could be analogous but not identical low molecular weight proteins which have an acidic pI.

The isoenzymatic specificity of mitochondrial extracts modulatory action is shown in Figure 2. Cytosoluble lactate dehydrogenase from rabbit muscle, beef liver and chicken liver (obtained as indicated in Materials and Methods) were selected for their different isoenzymatic composition and electrophoretic mobility as indicated in Figure 3 (Rabbit muscle lactate dehydrogenase: M-type predominance with pI > 7. Beef liver lactate dehydrogenase: H-type predominance with pI < 6. Chicken liver

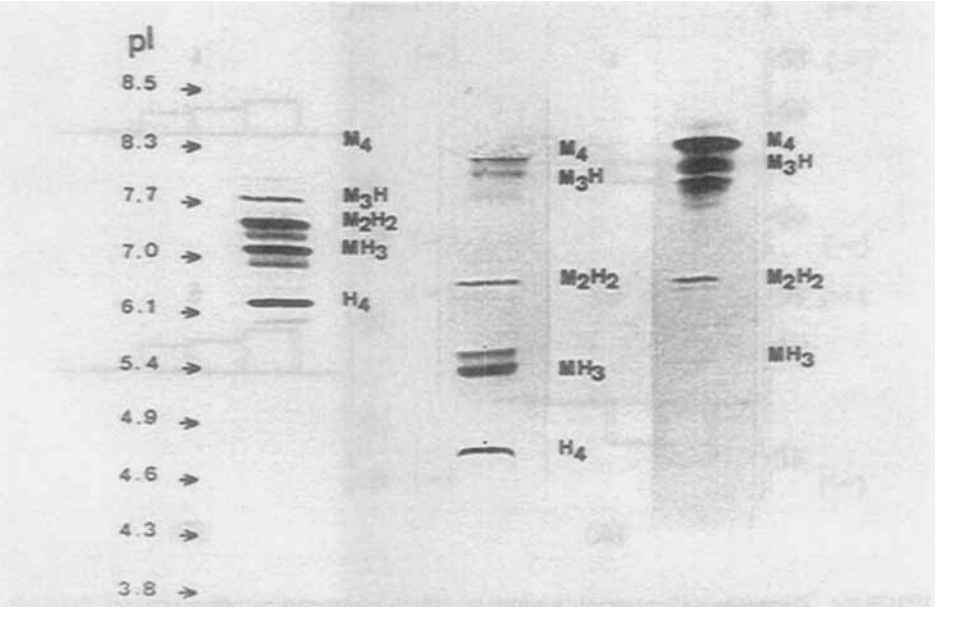

FIGURE 3 Separation of several sources of lactate dehydrogenase isoenzymes by agarose gel IEF. IEF was carried out as described in Materials and Methods. pH range bottom to top: 3–10. Cytosoluble fraction of (left to right): chicken liver, beef liver and rabbit skeletal muscle.

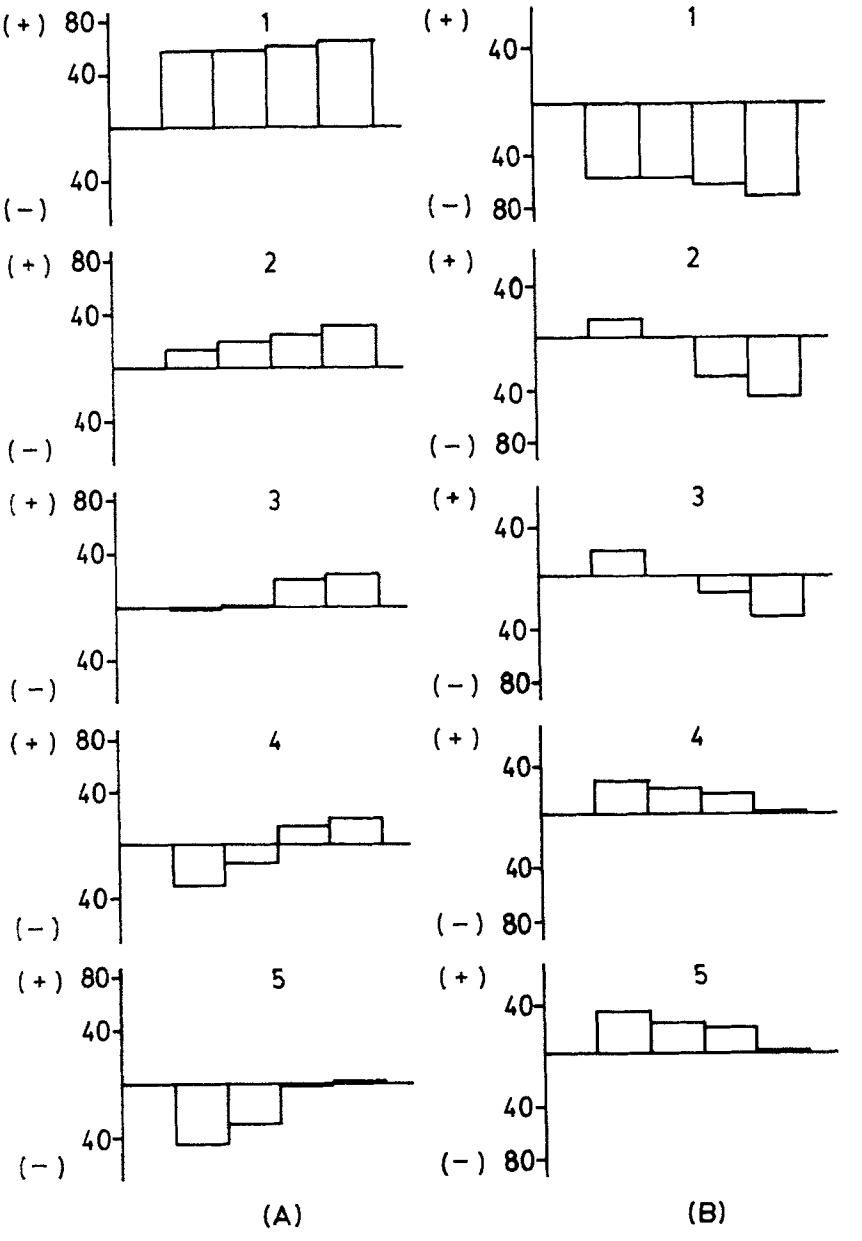

FIGURE 4 Competence of antagonist modulators. Spectrophotometrical cells contained: (A) (Left to right): 0.4, 0.6, 0.8 and 1.0 ml of beef liver activator extract and (1 to 5): 0, 0.2, 0.5, 0.7 and 1.0 ml of rabbit muscle inhibitor extract. (B) (left to right): 0.2, 0.5, 0.7 and 1.0 ml of rabbit muscle inhibitor extract and (1 to 5): 0, 0.4, 0.6, 0.8 and 1.0 ml of beef liver activator extract. Rabbit muscle lactate dehydrogenase in each cell 1.55×10^{-2} U. (+) = activation %; (−) = inhibition %.

lactate dehydrogenase pI $\approx$ 7). When rabbit muscle purified mitochondrial modulator was used, inhibition was observed with rabbit muscle or chicken liver cytosoluble fractions but, under the same conditions, no inhibition was detected with beef liver cytosoluble fraction. On the contrary, when beef and chicken liver purified mitochondrial modulators were used, activation of rabbit muscle cytosoluble enzyme was observed and little or nil effects were detected using cytosoluble livers enzyme. The modulatory action always increases when the units of activity of cytosoluble lactate dehydrogenase decrease for a fixed amount of mitochondrial extract. Activation and inhibition are elevated when the pI of the lactate dehydrogenase isoenzymes increases, independent of whether the M and H subunits are predominant. Nil effect is always observed when the lactate dehydrogenase pI is lower than 6. This shows the higher affinity of mitochondrial modulators for alkaline pI lactate dehydrogenase isoenzymes indicating that activity modulation depends on isoenzymes aminoacid composition. These results are in good agreement with earlier results obtained with the lactate dehydrogenase inhibitor[19]

Competence of Antagonist Modulators for Lactate Dehydrogenase

So far we have shown that the effect of rabbit muscle modulator is antagonist to that exhibited by chicken and beef liver modulators. Since all modulators show greater

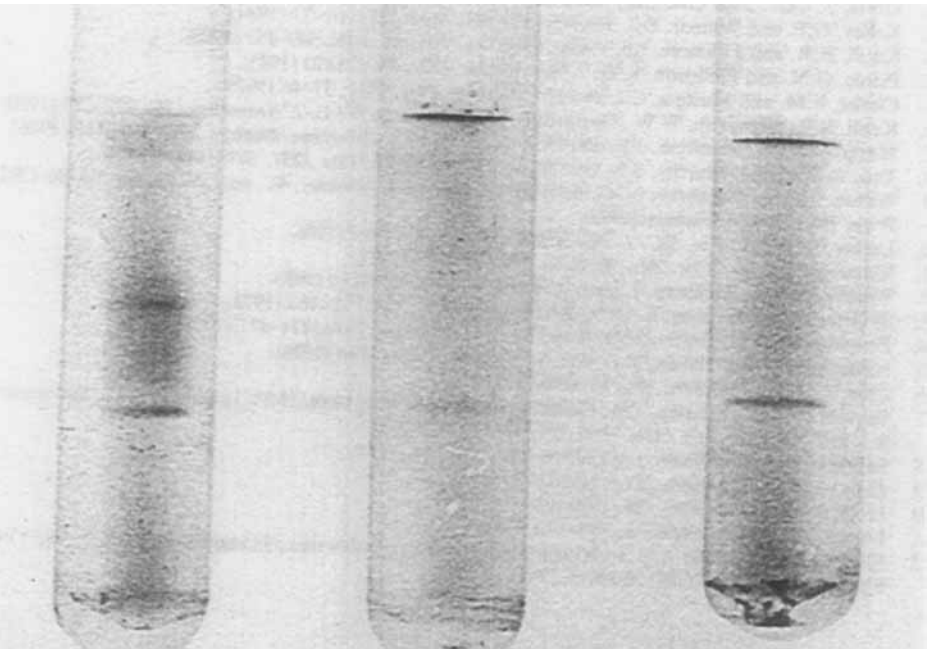

FIGURE 5 Electrophoretic behaviour of a mixed rabbit muscle inhibitor and beef liver activator. Discontinuous polyacrylamide disc gel electrophoresis was carried out as described (see Materials and Methods). Top to bottom direction means anodic mobility. Applied samples were: (A) 400 μl of beef liver activator (A_{280} = 0.520). (B) 400 μl of rabbit muscle inhibitor (A_{280} = 0.325) and (C) 200 μl of beef liver activator and 200 μl of rabbit muscle inhibitor preincubated 10 min.

affinity for basic pI lactate dehydorgenase isoenzymes all of them should compete for the enzyme molecule. To verify this competence we have studied the modulator effect produced by rabbit muscle inhibitor and beef liver activator together on rabbit muscle cytosoluble lactate dehydrogenase. The results obtained are shown in Figure 4. Obviously the rabbit muscle inhibitor and the beef liver activator compete for lactate dehydrogenase, since for a fixed amount of the activator a decrease in its activator effect is observed when the amount of the inhibitor increases (Figure 4A, 1 to 5), until the opposite effect is attained (Figure 4A, 4 and 5). Moreover when the relative amounts of the inhibitor and the activator were similar the modulatory capacity was mullified (Figure 4A, 3 and 5). Analogous results can be observed in Figure 4B.

Figure 5 shows a comparison of the pherograms of rabbit muscle inhibitor and beef liver activator alone and together, when mixed in the same proportion. Since, all samples showed only one band of high anodic electrophoretic mobility and the modulatory capacity for sample C was nil our previous suggestion regarding the analogy between but not the identity of both modulators is reinforced and it may be concluded that there probably exists analogous proteins in the different mitochondrial fractions which modify lactate dehydrogenase activity.

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