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Purification and radioimmunoassay of rat lactate dehydrogenase A and B subunits

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We have developed procedures for purifying lactate dehydrogenase isoenzymes from rat tissues that involve two affinity-chromatography steps and that facilitate the isolation of milligram quantities of highly purified proteins within 2–3 days. Antibodies raised against pure A and B subunits in rabbits and hens were used in radioimmunoassays and showed negligible cross-reactivity with heterologous subunits. The radioimmunoassays provide a sensitive method for measuring nanogram amounts of A-subunit and B-subunit polypeptides in tissue homogenates and were employed to characterize the enzyme purification procedures.

The lactate dehydrogenase enzymes of mammalian tissues have been well studied for many years, and much is known about them. Somatic cells contain up to five isoenzymes, each functional molecule being a tetramer of two polypeptide subunits arranged in the five possible combinations A_4, A_3B, A_2B_2, AB_3 and B_4. Different tissues exhibit different isoenzyme patterns as a result of variable expression of the *A* and *B* genes, extreme examples being liver (almost all A_4 isoenzyme in the rat) and kidney cortex (predominantly B-rich isoenzymes) (see, e.g., Fine *et al.*, 1963; Nadal-Ginard, 1978). A-subunit and B-subunit polypeptides are each 331 amino acids long, with extensive sequence homology and a high degree of evolutionary conservation (Eventoff *et al.*, 1977). We became interested in lactate dehydrogenase because it is a constitutive enzyme in all tissues of higher organisms and nevertheless clearly demonstrates a pattern of control in relation to development and tissue differentiation. In the present paper we describe the development of a purification procedure that permits the isolation of large quantities of isoenzymes in a short time and, for the first time, radioimmunoassays for both A and B subunits that can be used to measure nanogram amounts of these polypeptides in crude tissue homogenates.

Experimental

Materials

Tissues were obtained from adult Wistar rats bred in this Department. Antisera were raised in New Zealand White rabbits or Light Sussex hens. 5'-AMP–Sepharose 4B, Blue Sepharose CL-6B, Sephadex G-10 and G-100 and DEAE-Sephadex A-25 were from Pharmacia, Uppsala, Sweden. Phenylmethanesulphonyl fluoride, Nitro Blue Tetrazolium, phenazine methosulphate, bovine serum albumin (radioimmunoassay grade), ω-aminohexyl-Sepharose 4B, potassium oxalate, 1-(3-dimethylaminopropyl)-3-ethyl carbodi-imide, NAD^+ and NADH were from Sigma Chemical Co., Poole, Dorset, U.K. [^{125}I]Iodide (carrier-free) was from Amersham International, Amersham, Bucks., U.K. Donkey anti-rabbit precipitating serum was from Wellcome Research Laboratories, Beckenham, Kent, U.K.

Oxamate–Sepharose 4B was prepared as described by O'Carra & Barry (1972), without adjustment of the pH to 4.5 (Spielmann *et al.*, 1973).

All materials for gel chromatography columns were washed after use with 3 M-NaCl and stored at 4°C in appropriate buffers containing 0.1% NaN_3, under which conditions they were stable for at least 12 months and re-usable at least 50 times.

Purification of lactate dehydrogenase A_4

Liver tissue was washed to remove blood and blended at 4°C in 10 vol. of 20 mM-Tris/HCl buffer, pH 8.6, containing 1 mM-phenylmethanesulphonyl fluoride and 1 mM-2-mercaptoethanol (buffer A). The homogenate was centrifuged at 10000 ***g*** for 15 min, and the supernatant was retained and re-centrifuged at 27000 ***g*** for 15 min. The final supernatant was filtered through four layers of muslin and then applied directly to a 3 cm × 10 cm column of Blue Sepharose pre-equilibrated in buffer A, all at a flow rate of 100–150 ml/h. The column was washed with buffer A until all unbound proteins

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were removed, and then with 100ml of 20mM-sodium phosphate buffer, pH 6.8, containing 0.15M-NaCl (buffer B). Bound proteins were then eluted with buffer B containing 1mM-NADH, and fractions containing lactate dehydrogenase activity were pooled for storage overnight at 0°C.

The Blue Sepharose eluate was then applied to a 2.5cm × 6.0cm column of oxamate–Sepharose pre-equilibrated in buffer B containing 0.2mM-NADH, and allowed to run through at 30–50ml/h. The column was then washed with buffer B containing 0.2mM-NADH until absorbance measurements at 260nm showed a steady trace, at which point the column was eluted with buffer B alone. Fractions containing enzyme activity were pooled, and $(NH_4)_2SO_4$ was added to 0.5g/ml. After the mixture had been kept at 0°C for 15min, precipitated protein was obtained by centrifugation at 30000 *g* for 20min.

Pellets were redissolved in 0.5–1.0ml of 10mM-sodium phosphate buffer, pH 7.3 (buffer C), and desalted by passage through a 2.5cm × 14cm column of Sephadex G-10 equilibrated in the same buffer. Finally, the eluate protein was passed through a 2cm × 10cm column of DEAE-Sephadex A-25 in buffer C, and unbound material was concentrated again by precipitation with $(NH_4)_2SO_4$. The purified enzyme was resuspended in and dialysed extensively against 10mM-sodium phosphate buffer, pH 6.8, containing 50mM-NaCl and 50% (v/v) glycerol before storage at −20°C.

Purification of lactate dehydrogenase B_4

Kidney, heart, testes and brain tissues were carefully cleaned of extraneous fat and blended in 10vol. of 20mM-Tris/HCl buffer, pH 7.4, containing 50mM-NaCl and 1mM-2-mercapthoethanol (buffer D). The homogenate was subjected to centrifugation and filtration as described for isoenzyme A_4, and applied to a 2.5cm × 50cm column of AMP–Sepharose 4B pre-equilibrated in buffer D. After the column had been washed through at 50–100ml/h with excess of buffer D until absorbance of the eluate was down to background values, proteins were eluted with buffer D containing 0.2mM-NADH. Fractions containing lactate dehydrogenase activity were stored overnight at 0°C.

The AMP–Sepharose 4B eluate was adjusted to 0.45M-NaCl, and fresh NADH was added to 0.2mM. This was then run through an oxamate–Sepharose column as described for isoenzyme A_4 except that NaCl was maintained at 0.45M throughout. Enzymes were similarly concentrated by precipitation with $(NH_4)_2SO_4$, desalted on Sephadex G-10 and run on to a column of DEAE-Sephadex A-25. For these latter steps, buffer C was adjusted to 25mM-NaCl, and enzymes bound to the DEAE-Sephadex A-25 column were eluted with a linear 100ml gradient of 0.025–0.25M-NaCl in buffer C. Absorbance at 260nm was monitored and fractions corresponding to the B_4 peak were pooled, precipitated with $(NH_4)_2SO_4$, dialysed and stored as described for isoenzyme A_4.

Operations during the purification of isoenzymes A_4 and B_4 were conducted at 0–4°C, except for the affinity-column steps, which were usually performed at room temperature.

Enzyme and protein assays

Lactate dehydrogenase activity was measured by mixing samples of enzyme with 1ml of 30mM-Tris/HCl buffer, pH 8.6, containing 110mM-lithium lactate, 8mM-NaCl, 3mM-$NaHCO_3$ and 0.2mM-NAD^+ and recording change of absorbance at 340nm (Carlotti *et al.*, 1974). One unit of activity corresponds to the production of 1μmol of NADH/min (Fritz *et al.*, 1970). Protein was determined by the method of Lowry *et al.* (1951), with γ-globulin as standard.

Electrophoresis of lactate dehydrogenase

Samples were made up to 20% (v/v) glycerol or 20% (w/v) sucrose in total volumes of 25–30μl and layered on to 0.6cm × 6.0cm 5.5% polyacrylamide gels set in glass tubes. Electrophoresis (under non-denaturing conditions) was performed at 4°C for 2h and at 2.5mA/tube (Dietz & Lubrano, 1967). Gels were then removed and stained either for protein with 0.1% Coomassie Blue or for lactate dehydrogenase activity with a buffered mixture of sodium lactate, NAD^+, phenazine methosulphate and Nitro Blue Tetrazolium (Dietz & Lubrano, 1967). In some experiments preparative gels (1cm × 5cm) were loaded with 200μg of lactate dehydrogenase, electrophoresed for 2h at 4mA/gel, isoenzymes were identified by brief staining and the bands containing activity were cut out. These were then homogenized for antibody production (see below), or were re-fixed in gel tubes and the enzyme was electrophoresed out into dialysis bags tied around the tube ends.

Preparation of antisera

Antibodies to A subunits were raised by subcutaneous injection of rabbits with 3mg of isoenzyme A_4 in Freund's Complete Adjuvant followed by a booster of 1mg in Freund's Incomplete Adjuvant after 5 weeks and bleeding from an ear vein 5 days thereafter (Carlotti *et al.*, 1974). Regimes for raising antibodies to subunit B included the above method, serial injection of 0.5mg samples at 14-day intervals and serial injection of isoenzyme B_4 with polyacrylamide as adjuvant (Spielman *et al.*, 1974). Alternatively, a chicken was injected with isoenzyme B_4 with use of the protocol employed in raising anti-(subunit A) antibodies in rabbits. Anti-hen serum was prepared by injecting rabbits with

1ml samples of hen serum in adjuvant, also by following the procedure used in production of anti-(subunit A) antibodies.

Blood was allowed to clot for 2h at room temperature, centrifuged and kept at 4°C overnight before the serum was decanted. γ-Globulins were precipitated with the addition of $(NH_4)_2SO_4$ to 33% saturation, resuspended in volumes equivalent to that of the original serum of 20mM-sodium phosphate buffer, pH6.8, containing 50mM-NaCl and dialysed extensively against the same buffer before storage in 0.5ml portions at −70°C.

Alternatively, antiserum was obtained from the yolks of chicken eggs. Four yolks from eggs laid 4–7 days after injection were cut, and their contents pooled and made up to 200ml with 40mM-Tris/HCl buffer, pH7.5, containing 0.15M-NaCl (buffer E). After thorough mixing and centrifugation at 2000*g* for 20min, 22ml of 10% (w/v) dextran sulphate and 50ml of 1M-$CaCl_2$ (both in buffer E) were added to the supernatant, left for 30min at room temperature and centrifuged as above. The final supernatant was made up to 400ml with buffer E, 80g of anhydrous Na_2SO_4 was added and the mixture was stirred for 1h at room temperature. Precipitated protein was collected by centrifugation, and pellet volumes were measured and made up in buffer E to a final volume of 50ml. Na_2SO_4 was added from a 36% (w/v) stock solution to a final concentration of 9% (w/v) (allowing for salt in the pellets) and kept a further 1h at room temperature before centrifugation. Finally, the Na_2SO_4 concentration in the supernatant was raised to 14% (w/v), the precipitation and centrifugation were repeated and the protein pellets were redissolved in 10ml of 10mM-sodium phosphate buffer, pH7.3, containing 50mM-NaCl for storage at −70°C.

Iodination of lactate dehydrogenase

Iodination was performed by using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril as iodogen under relatively mild conditions (Fraker & Speck, 1978). Samples containing 15–30μg of protein were made up to final volumes of 65μl in 0.5M-Tris/HCl buffer, pH7.6, and added to tubes containing iodogen. Carrier-free [^{125}I]iodide (0.25mCi) was added immediately and the reaction allowed to proceed for 10min at room temperature (with shaking) followed by termination with 0.5ml of 50mM-sodium phosphate buffer, pH7.6. The material was then fractionated on a 1.5cm × 15cm column of Sephadex G-100 equilibrated in 50mM-sodium phosphate buffer, pH7.6, containing 0.1% NaN_3, 0.1M-$(NH_4)_2SO_4$, 0.05% bovine serum albumin and 25% (v/v) glycerol, and iodinated protein was pooled, made 50% (v/v) with respect to glycerol and stored at −20°C.

Radioimmunoassay procedures

Assays were set up in duplicate or triplicate in final volumes of 0.5ml. Each contained [^{125}I]-iodinated lactate dehydrogenase A_4 or B_4 (10000–20000c.p.m.), a suitable dilution of anti-(subunit A) or anti-(subunit B) serum [usually 0.005% for anti-(subunit A) antibodies raised in rabbits and 0.02% for anti-(subunit B) antibodies raised in hens] to give 25–40% precipitation of iodinated protein, various amounts of unlabelled isoenzyme A_4, isoenzyme B_4 or tissue homogenate sample and either anti-rabbit precipitating serum (Wellcome) at 1:250 dilution or anti-hen precipitating serum at 1:7 dilution, all in 40mM-sodium phosphate buffer, pH7.4, containing 0.15M-NaCl, 10mM-EDTA, 0.1% NaN_3 and 0.5% bovine serum albumin. Tubes were set up without iodinated protein and precipitating serum and were kept at room temperature for 30min before these two components were added, mixed thoroughly and left for 65h at 4°C. Assay mixtures were then centrifuged at 200*g* for 30min and the supernatants were discarded. Radioactivity in the complete assays and in residual pellets was determined with a Wallac γ-radiation counter.

Results

Purification of lactate dehydrogenase isoenzymes

As an initial step before attempting bulk isolation of lactate dehydrogenases we re-investigated the relative amounts of the various forms in a number of the more abundant rat tissues. The results of such an analysis, with tube gels and the specific stain for lactate dehydrogenase activity, are shown in Fig. 1. As expected, liver and skeletal muscle were rich in A subunits, and heart muscle and kidney were rich in B subunits (Fine *et al.*, 1963). In addition, however, we noted that brain and testes also contained large amounts of B subunits. On the basis of these studies we decided to use liver as the sole source of A-subunit polypeptide and a mixture of kidney, heart, brain and testes for B-subunit polypeptide. Testes have in addition an extra form of lactate dehydrogenase subunit (C), but this has charge properties similar to those of subunit A and the testicular isoenzyme can be readily resolved from isoenzyme B_4 by gel electrophoresis (Fig. 2). Heavily loaded gels stained for protein revealed material only in the isoenzyme-A_4 and isoenzyme-A_3B regions; after a final step (in this case with a preparative gel) isoenzyme A_3B was eliminated and only isoenzyme A_4 was seen. Rat isoenzyme A_4 exhibited a lower electrophoretic mobility than the analogous bovine enzyme (results not shown) and tended to aggregate and form a broad band on gels, as seen in Fig. 2. This was especially true after extensive purification. A-subunit-rich isoenzymes had a high affinity for Blue Sepharose but a relatively low affinity for

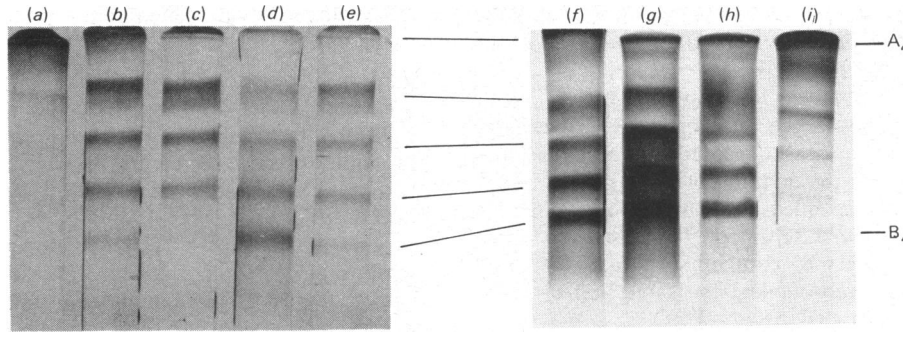

Fig. 1. *Lactate dehydrogenase isoenzymes in rat tissues*

Tissues were homogenized in 2–5 vol. of 20 mM-Tris/HCl buffer, pH 8.6, containing 1 mM-phenylmethanesulphonyl fluoride and, 1 mM-2-mercaptoethanol, filtered through four layers of muslin and centrifuged at 2000 *g* for 20 min. Then 20 μl samples of the supernatants were each mixed with 20 μl of 40% (w/v) sucrose, electrophoresed and stained for enzyme activity as described in the Experimental section. Tissue: (*a*) liver; (*b*) skeletal muscle; (*c*) thyroid; (*d*) brain; (*e*) lung; (*f*) kidney; (*g*) heart; (*h*) testes; (*i*) blood.

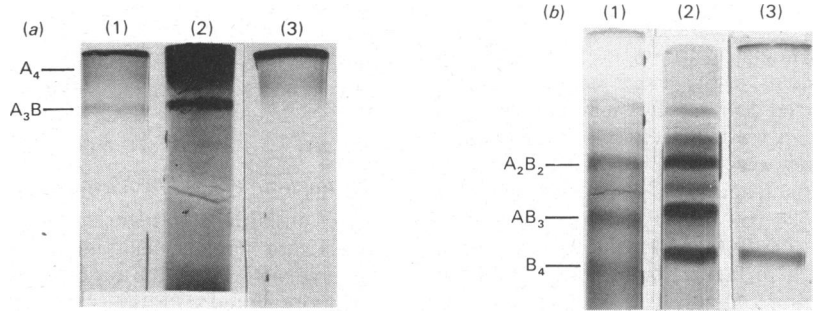

Fig. 2. *Electrophoresis of purified lactate dehydrogenases*

Enzymes were purified either from liver or a mixture of kidney, heart, testes and brain as far as the oxamate–Sepharose step (see the Experimental section) and samples were electrophoresed under standard conditions. (*a*) Liver enzyme stained for lactate dehydrogenase activity (1), protein (2) and enzyme activity after extraction of the A_4 band from a preparative gel followed by re-running under analytical conditions (3). (*b*) Kidney/heart/testes/brain enzymes stained for activity (1), protein (2) and activity after extraction and re-running of the B_4 bands. Loads were 50 μg [(*a*)(1) and (*a*)(2)], 36 μg [(*b*)(1) and (*b*)(2)] and 5 and 15 μg [(*a*)(3) and (*b*)(3)].

oxamate–Sepharose, and to obtain consistently good recoveries we found it necessary to use salt concentrations no higher than 0.15 M in the running buffers. Isoenzyme A_4 does not bind to DEAE-Sephadex at near-neutral pH even in the complete absence of salt, and passage through this resin was the most convenient method for removing traces of the other isoenzymes (all of which do bind).

Fig. 2 also shows an electrophoretic analysis of enzymes from B-subunit-rich tissues after purification to the oxamate–Sepharose step. Isoenzymes A_2B_2, AB_3 and B_4 showed up prominently by staining for either protein or enzyme activity. Isoenzyme A_3B is also detectable, as well as one or two other bands of uncertain nature. These may include traces of testicular isoenzymes C_4 and AC_3 (Ansari, 1981) that have persisted through the oxamate–Sepharose step. When isoenzyme B_4 was removed from a preparative gel and re-electrophoresed, no contaminating activity from other isoenzyme forms was detectable (Fig. 2). Similar results were obtained for both isoenzyme A_4 and isoenzyme B_4 when DEAE–Sephadex chromatography was used as the final step and when gels were stained for protein rather than for enzyme activity. Isoenzyme B_4 is the last isoenzyme to be eluted from DEAE–Sephadex when a salt gradient is applied, being displaced by about 0.15 M-NaCl at pH 7.3. We could not find conditions in which rat isoenzyme B_4 would bind to Blue Sepharose, and hence AMP–Sepharose was substituted as the initial affinity step. On the other hand, isoenzyme B_4 bound

very tightly to oxamate–Sepharose, and higher (more discriminating) salt concentrations could be used in the running buffer. Isoenzymes A_4 and B_4 ran as single bands in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown).

Characterization of antisera

Production of antisera to A subunits was straightforward, as has been found by other workers (e.g. Carlotti *et al.*, 1974). B-subunit polypeptides are, however, well known to be poor antigens (Spielman *et al.*, 1974), and we experienced difficulty in raising antibodies of reasonable titre. Only one out of three rabbits eventually produced an active preparation that inhibited isoenzyme B_4 and not isoenzyme A_4, but large amounts were needed to cause more than a 50% decrease in enzyme activity (Table 1). Injection of isoenzyme B_4 into a hen produced antisera of somewhat better quality rather more readily, and this, together with the ease of immunoglobulin isolation from egg yolk, rendered hens the animals of choice for the production of anti-(subunit B) serum.

The abilities of the various antisera to precipitate iodinated lactate dehydrogenases are shown in Fig. 3. Anti-(subunit A) serum from rabbits was extremely effective, a final dilution of 1:15000 causing 50% of the labelled isoenzyme A_4 to sediment during low-speed centrifugation. On the other hand rabbit anti-(subunit B) serum was very poor, dilution of 1:2500 causing no more than 19% precipitation. Antisera to subunit B raised in hens was significantly better, 30–40% precipitation usually being achieved by about 1:3000 dilutions.

Radioimmunoassay of A and B subunits

Both isoenzyme A_4 and isoenzyme B_4 were iodinated with low efficiency in our hands, and the products were enzymically inactive. Under conditions in which bovine serum albumin incorporated more than 80% of the carrier-free ^{125}I added, 6 times as much lactate dehydrogenase incorporated only up to 40% with the use of a relatively gentle iodination procedure (Fraker & Speck, 1978), in both instances. Also, the alternative techniques that we have tried gave even lower incorporation. This

Table 1. *Effects of antisera on lactate dehydrogenase activities*

(*a*) Duplicate assay mixtures containing 0.1 μg of pure lactate dehydrogenase, alone or with 1 μl of antiserum, were incubated at 37°C for 30min in 80 μl of 20mM-sodium phosphate buffer, pH 7.3, containing 0.15M-NaCl. Then 75 μl samples were tested for enzyme activity (see the Experimental section). Variation between duplicates was no more than 5%. (*b*) Duplicate assay mixtures containing 0.1 μg of pure lactate dehydrogenase were incubated as described above for (*a*) with various amounts of anti-(subunit B) serum raised in rabbit C or in a hen. Samples were then tested for enzyme activity. N.D., Not determined.

(*a*) Effects of antisera to lactate dehydrogenase subunits A and B raised in rabbits

Source of serum	Antibodies raised against subunit	Average change in A_{340}/min: Isoenzyme A_4 in assay mixture	Average change in A_{340}/min: Isoenzyme B_4 in assay mixture
None (control)	–	0.060	0.103
Rabbit 1	A	0.012	0.113
Rabbit 2	A	0.008	0.102
Rabbit 3	A	0.016	0.102
Rabbit A	B	N.D.	0.098
Rabbit B	B	N.D.	0.131
Rabbit C	B	N.D.	0.091

(*b*) Effects of antisera to lactate dehydrogenase subunit B raised in rabbits and hens

Source of serum	Average change in A_{340}/min: Isoenzyme A_4 in assay mixture	Average change in A_{340}/min: Isoenzyme B_4 in assay mixture
None (control)	0.077	0.134
+ 1 μl, rabbit C	N.D.	0.114
+ 10 μl, rabbit C	N.D.	0.057
+ 50 μl, rabbit C	0.092	0.044
+ 1 μl, hen	N.D.	0.082
+ 10 μl, hen	N.D.	0.036
+ 30 μl, hen	0.074	0.018

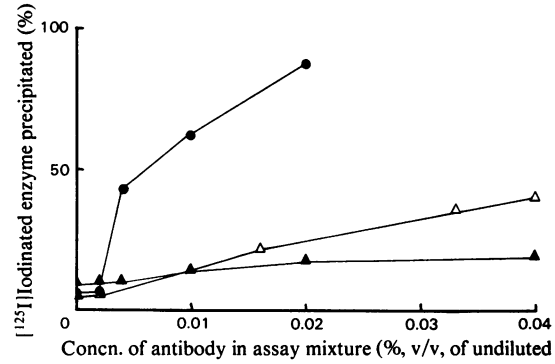

Fig. 3. *Antibody precipitation of iodinated lactate dehydrogenases*

Radioassays were performed as described in the Experimental section, with fixed amounts of [^{125}I]-iodinated isoenzyme A_4 or [^{125}I]iodinated isoenzyme B_4 and various dilutions of antisera. ●, [^{125}I]-Iodinated isoenzyme A_4 with rabbit anti-(subunit A) serum; ▲, [^{125}I]iodinated isoenzyme B_4 with rabbit anti-(subunit B) serum; △, [^{125}I]iodinated isoenzyme B_4 with hen anti-(subunit B) serum.

degree of iodination was, however, quite sufficient for radioimmunoassay work. Storage of the iodinated enzymes in 50% glycerol at −20°C was important in preventing aggregation and precipitation, and in this condition the proteins could be used in assays for 3–4 weeks after preparation.

A characterization of the radioimmunoassay procedure is shown in Fig. 4. We only obtained good results in the absence of carrier serum, and background precipitation in the absence of antibody was in the range 1–5%. Precipitation of [^{125}I]iodinated isoenzyme A_4 was competed by unlabelled isoenzyme A_4 mainly over the range 10–100 ng and not at all by isoenzyme B_4. A 50% competition was caused by about 20 ng of isoenzyme A_4 or 100 ng of isoenzyme AB_3; A subunits complexed with B subunits were therefore somewhat less effective than when complexed together in the competition assay, in this extreme case ($1 \times A + 3 \times B$) needing 5 times as much protein rather than the 4 times predicted if the subunits were acting completely autonomously. The radioassay for B subunits was equally specific, no competition being caused by isoenzyme A_4. A 50% competition was evident with about 22 ng of isoenzyme B_4 or 28 ng of isoenzyme AB_3, close to the predicted relative values from the different contents of B subunits. Fig. 4 also shows the results obtained with different amounts of liver and heart muscle homogenates, known to be rich in A and B subunits respectively. Competition slopes were

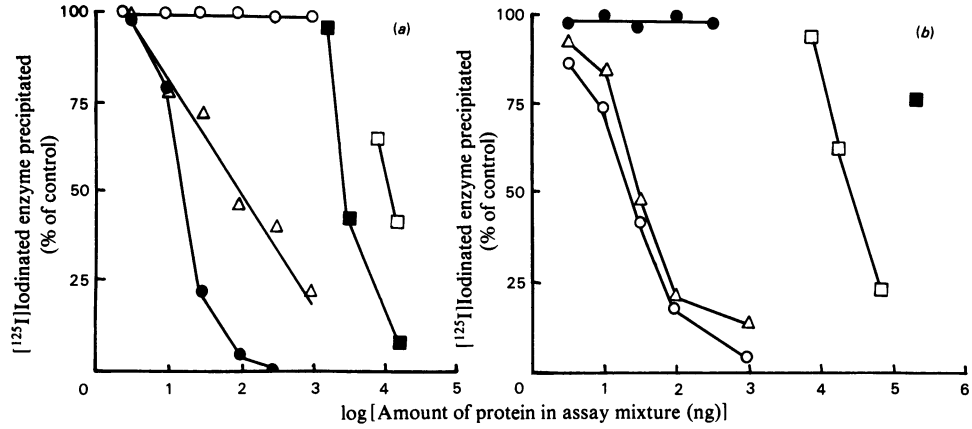

Fig. 4. *Competition specificity of radioimmunoassay*

Radioimmunoassay mixtures contained standard amounts of [^{125}I]iodinated isoenzyme A_4 (*a*) or [^{125}I]iodinated isoenzyme B_4 (*b*), together with various amounts of unlabelled isoenzyme B_4 (○), isoenzyme A_4 (●), or isoenzyme AB_3 (△). In addition, samples of liver (■) and heart (□) homogenates were tested for A-subunit and B-subunit polypeptide with the use of different dilutions in assays.

Table 2. *Characterization of lactate dehydrogenase purification*

Lactate dehydrogenases were isolated from 11.5 g of liver and from 6.5 g of mixed kidneys, heart, testes and brain as described in the Experimental section. Samples of the primary homogenates (H1), homogenates after centrifugation and filtration (H2), eluates from Blue Sepharose (B) or AMP–Sepharose (A) chromatography and eluates from oxamate–Sepharose chromatography (O) were retained for determination of total protein, lactate dehydrogenase activity and lactate dehydrogenase protein (by radioimmunoassay). In the last case A and B subunits were determined independently in assays with a range of pure standards (see Fig. 4). All values given are the averages of duplicates, and variations were always not more than 8%.

Tissue source	Stage of purification	Total protein (mg)	Enzyme activity (units)	Enzyme protein (mg)		Specific activity	
				Subunit A	Subunit B	Apparent (units/total protein)	True (units/enzyme protein)
Liver	H1	1896	492	5.8	0.04	0.26	84.25
	H2	1235	666	4.6	0.03	0.54	143.84
	B	28	476	4.6	—	17.00	103.48
	O	<3	337	2.4	—	122.33	140.42
Kidney/heart/testes/brain	H1	592	73	0.23	2.5	0.12	26.74
	H2	404	77	0.21	2.5	0.19	28.41
	A	15	91	0.18	2.6	6.07	32.73
	O	2	77	0.11	1.8	38.50	40.31

similar to those for the controls and in the expected relative positions. Liver had so little B subunit that only one point (undiluted homogenate) was on scale.

Purification characterization

The purification procedures for isolating lactate dehydrogenases were checked by monitoring enzyme activity, total protein and enzyme protein at four stages, in the latter type of measurement making use of the radioassays. Results are shown in Table 2. Liver apparently contained about 0.5 mg of lactate dehydrogenase/g wet wt., 99% of which was constituted by A subunits. The largest losses occurred at the oxamate–Sepharose step, but overall yield was nevertheless over 40%. True specific activity apparently increased after the initial centrifugation, but did not show any consistent subsequent change. Specific-activity measurements were critically dependent on temperature, and only relative values during purification were of concern in the present study. Overall amount of protein after oxamate–Sepharose chromatography, as measured by the Lowry procedure, was within 20% of that indicated as pure lactate dehydrogenase by the radioimmunoassay. Bearing in mind the variable reaction of individual pure proteins to the Lowry test (Peterson, 1979), this result is compatible with an essentially complete purification.

On average, kidney, heart, testes and brain contained about 0.45 mg of lactate dehydrogenase (excluding form C) per g of tissue. Recovery was better than 70% and in the crude homogenate there was about 8.5% A subunits; this decreased to less than 6% after oxamate–Sepharose chromatography. True specific activity apparently increased steadily through the purification, but remained much lower than for the A-subunit-rich isoenzymes as measured in the NAD^+ + lactate assay. The data were again compatible with complete purification after the oxamate–Sepharose step.

Discussion

Many procedures for the purification of lactate dehydrogenases have been published, and all the individual steps described in the present paper have been used before (e.g. O'Carra & Barry, 1972; Brodelius & Mosbach, 1973; Ryan & Vestling, 1974; Chang *et al.*, 1979). However, we found that single rounds of affinity chromatography were insufficient to yield pure enzymes, and the combinations of methods used in the present study are thought to be novel. For example, the radioimmunoassay confirmed our initial observations with gels (results not shown) that Blue Sepharose could not by itself yield pure lactate dehydrogenase from liver homogenates, and this was true even if an NAD^+/lactate wash was performed before final elution (Ryan & Vestling, 1974). In our hands, only 15–20% of proteins binding to Blue Sepharose were lactate dehydrogenases. A comparable result was obtained with AMP–Sepharose with tissue extracts containing B-subunit-rich isoenzymes. Gradient elution (with increasing NADH concentration) of these columns improved the situation, but not anywhere near to the point of producing homogeneous proteins. Use of oxamate–Sepharose is a fundamentally more powerful technique, relying as it does on the ordered binding of first substrate (NADH) and secondly substrate analogue (oxamate). It has been used to achieve high degrees of purity at a single step in analytical studies (e.g. by Nadal-Ginard, 1978), but in our experience this resin was not stable if used on a preparative scale at early stages with large volumes of crude homogenates. The ion-exchange properties of the oxamate–Sepharose tended to result in a large amount of non-specific protein binding even at 0.45 M-NaCl, and this resin was more satisfactory for use after an initial step with one of the other affinity resins.

Resolution of the individual isoenzymes after the oxamate–Sepharose step was best accomplished by DEAE-Sephadex chromatography. By using the combinations of methods described it was readily possible to purify milligram amounts of isoenzymes A_4, B_4 and AB_3 within 2–3 days. It remained difficult to obtain large amounts of isoenzymes A_2B_2 and A_3B owing to the relative scarcity of these isoenzymes in the tissues used.

The problems involved in raising antisera to B subunits are well documented (e.g. Spielman *et al.*, 1974). Protein modification (acetylation) yields a better antigen, but antibodies precipitate only the modified protein (Rajewsky, 1966). We have, however, been able to develop radioimmunoassays of sufficient specificity and sensitivity to measure nanogram amounts of A-subunit and B-subunit polypeptides in crude tissue extracts without resort to monoclonal antibodies. Other workers have used antibodies to A and B subunits (see, e.g., Kumar *et al.*, 1980), but the present paper is the first report that we know of concerning the development of a radioimmunoassay method. Other methods for quantifying relative amounts of the two subunits in various tissues (gel electrophoresis, DEAE-cellulose chromatography and activity effects of different pyruvate concentrations) can lead to very variable and inconsistent results (Fritz *et al.*, 1970), and it is hoped that the radioassays will provide a more precise probe in the study of lactate dehydrogenase gene expression. Although the radioimmunoassay may slightly underestimate subunits complexed with a majority of the heterologous type, the discrepancy is small and does not lead to serious miscalculation in comparison with the alternative methods available.

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