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Received June 5th, 1967

Biochim. Biophys. Acta, 146 (1967) 587–590

BBA 63271

The disappearance of ^{14}C-labelled isoenzyme 5 of L-lactate dehydrogenase from plasma

In a previous investigation it was observed that intravenously injected isoenzyme 5 of lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) disappeared from plasma much more rapidly than isoenzyme 1 (ref. 1). The present experiments were performed using ^{14}C-labelled LDH 5 to find out whether the rapid decline of enzymic activity in plasma following LDH 5 injection represents a genuine disappearance of circulating LDH 5 molecules. Since iodoacetate reacts only with the non-essential thiol groups of lactate dehydrogenase[2], ^{14}C-labelled LDH 5 was prepared by incubating purified sheep muscle LDH 5 with [2-^{14}C]iodoacetate. The radioactive carboxymethylated isoenzyme was injected intravenously into sheep and the rate of disappearance of enzymic activity compared with that for radioactivity.

Preparation of ^{14}C-labelled LDH 5. Total lactate dehydrogenase activity was determined by the method of HENRY *et al.*[3]. The enzyme units are the μmoles of NADH oxidized per min at 25°. LDH 5 was partially purified from minced sheep hind leg muscles as described previously[1]. The remaining impurities were removed by electrophoresis on the vinyl copolymer Pevikon (Shandon Scientific Co. Ltd., London, England) in 0.07 M barbitone buffer, pH 8.6, at 2.5 V/cm for 48 h. The entire purification was performed at 4° in a cold room.

Three batches of purified sheep LDH 5 were incubated at 37° with [2-^{14}C]-iodoacetate, specific activity 7.0 μC/μmole, from The Radiochemical Centre, Amersham, Bucks., England (Table I). The labelled enzyme sedimented in the ultracentrifuge as a single peak ($s_{20,w}$ 7.42) with a trace of a slightly heavier impurity.

Abbreviations: LDH 5, isoenzyme 5 of lactate dehydrogenase; LDH 1, isoenzyme 1 of lactate dehydrogenase.

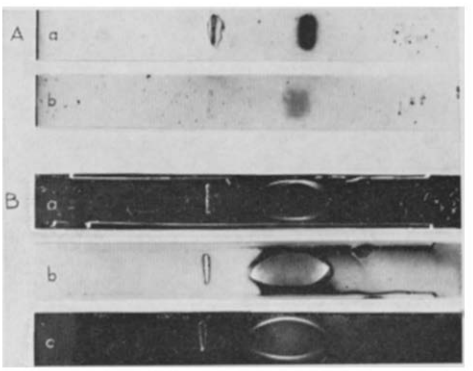
TABLE I

INCUBATION OF PURIFIED LDH 5 WITH [2-^{14}C]IODOACETATE AT 37° IN 0.05 M TRIS BUFFER FOLLOWED BY GEL FILTRATION*

	Before incubation		*After incubation*		
	Activity (U)	*Protein (mg)*	*Activity (U)*	*Protein (mg)*	*Radioactivity (disint./min × 10^{-5})*
A. 18 h at pH 7.6 with 14.3 μM [2-^{14}C]-iodoacetate in 10 ml	26800	76.0	6680	32.0	149
B. 12 h at pH 8.2 with 3.0 μM [2-^{14}C]-iodoacetate in 4 ml	9400	36.5	5510	28.7	65
C. 3 h at pH 8.2 with 3.0 μM [2-^{14}C]-iodoacetate in 5 ml	3630	—	3200	16.0	12

* Using Sephadex G-25 from Pharmacia, Uppsala, Sweden, to remove iodide and unreacted iodoacetate.

After agar-gel electrophoresis[4] of unlabelled LDH 5 a single sharp protein band with lactate dehydrogenase activity was found near the cathode. The labelled LDH 5 was found to be displaced slightly towards the anode, as would be expected after the



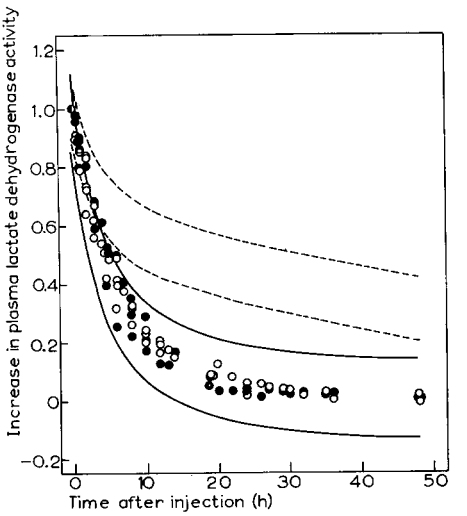


Fig. 1. A. Electrophoresis of (a) unlabelled sheep LDH 5 and (b) more dilute ^{14}C-labelled LDH 5 in agar: stained with amido black. B. Immunoelectrophoresis of ^{14}C-labelled LDH 5 from sheep using rabbit antiserum. (a) Photograph of the precipitin lines. (b) The gel was rinsed for a few hours in a buffer solution and incubated with a substrate–tetrazolium mixture[4] to stain for lactate dehydrogenase activity. (c) A photoautograph was then made from the stained and dried agar gel. The protein nearer the cathode (seen in B(a) is an impurity with no lactate dehydrogenase activity and negligible radioactivity.

Fig. 2. Intravenous injection of purified ^{14}C-labelled LDH 5 into sheep in three experiments. Enzymic activity (●) and radioactivity (○) are plotted as fractions of the increased plasma levels 5 min after injection. The curves are the 95% confidence limits (double exponential curve $\pm 2\times$ S.D.) obtained in previous experiments (see ref. 1) using unlabelled lactate dehydrogenase isoenzymes; continuous lines, LDH 5; broken lines, LDH 1.

carboxymethylation of thiol groups. Both labelled and unlabelled LDH 5 could be detected by agar-gel immunoelectrophoresis using anti-LDH 5 rabbit antiserum. A trace of a slightly more cathodic protein was also detectable, but unlike LDH 5 its precipitin lines showed no lactate dehydrogenase activity when incubated with substrate[4] and only very slight radioactivity after radioautography (Fig. 1).

Antiserum to LDH 5 was obtained from a rabbit injected with partially purified LDH 5 mixed with Freund's adjuvant. After 30-min incubation at room temperature 20 μl of antiserum caused approx. 65% inhibition of 100 mU of either ^{14}C-labelled or unlabelled LDH 5 in a total volume of 0.2 ml. The antiserum did not react with sheep LDH 1 nor with rabbit LDH 5.

Disappearance of ^{14}C-labelled LDH 5 from circulating plasma. Three sheep were injected intravenously with the ^{14}C-labelled LDH 5 (Table II). Heparinised blood samples were collected at intervals before and after injection. The plasma from these samples was assayed for lactate dehydrogenase activity and radioactivity (Fig. 2). To determine radioactivity each 0.2-ml sample of plasma (or urine) was heated with 0.05 ml 50% (w/v) KOH for 10 min at 70°. After cooling, 20 ml of a mixture of 2 parts toluene (containing 4.0 g/l of PPO and 0.05 g/l of POPOP) and 1 part of methanol was added to each phial. ^{14}C counting was then performed using a Tri-Carb

TABLE II

DOSES OF ^{14}C-LABELLED LDH 5 USED IN THE INJECTION EXPERIMENTS
For experimental details see text.

Expt. No.	*Sheep No.*	*Body weight (kg)*	*Batch sample*	*Dose of labelled LDH 5*	
				Activity (U)	*Radioactivity (disint./min × 10^{-5})*
1	43	10.5	A	3430	74.5
2	45	9.7	B	3670	42.2
3	44	12.4	A, C	3900	49.3

liquid scintillation spectrometer (Packard Instrument Company, Inc., Ill., U.S.A.). Using internal standards the counting efficiency was found to be 45% with plasma and 40% with urine.

Following these injections of ^{14}C-labelled LDH 5 both enzymic activity and radioactivity declined in plasma at the same rate as was observed previously[1] with unlabelled LDH 5 (Fig. 2).

In the first injection experiment samples of plasma collected at 2, 10 and 20 h after injection were subjected to electrophoresis on cellulose acetate strips for 2 h at 25 V/cm. The strips were stained for lactate dehydrogenase activity[4] and the ^{14}C distribution determined by liquid scintillation counting of 4.0 cm × 0.5 cm sections. The ^{14}C was entirely confined to the band of LDH 5 activity. Dialysis of plasma samples caused no loss of either radioactivity or lactate dehydrogenase activity.

In the second injection experiment urine was collected and its ^{14}C content was determined. Only about 10% of the injected ^{14}C appeared in urine during the first

24 h after injection, while it can be calculated (from Eqn. 5, ref. 1) that about 90% of the injected ^{14}C-labelled LDH 5 had disappeared from the circulating body fluids during that period. Since dialysing the urine removed all ^{14}C radioactivity, it is apparent that intact ^{14}C-labelled LDH was not excreted in urine.

In all three experiments samples of plasma containing 100 mU of injected LDH 5 were incubated with rabbit antiserum. There was no decrease in the percentage immune inhibition of lactate dehydrogenase activity during the course of an experiment. If present with its antigenic sites intact, inactive circulating LDH 5 would have caused a fall in percentage immune inhibition. A 1:1 ratio of inactive to active LDH 5 would be expected to reduce inhibition from 65 to 55% since that is the effect of decreasing antiserum concentration by half.

It can be concluded that the rapid loss of LDH 5 activity from plasma following its intravenous injection does represent the actual disappearance of the isoenzyme from plasma. If LDH 5 is inactivated in the circulation then the inactive form must be removed very rapidly indeed.

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Received June 6th, 1967

Biochim. Biophys. Acta, 146 (1967) 590–593

BBA 63272

Relative levels of the disulfide-interchange enzyme in the microsomes of the bovine tissues

It has been shown that rat and beef liver microsomes[1,3] and pig, pigeon and chicken pancreas tissue[2] contain an enzyme that catalyzes the reactivation of the inactive fully reduced or of randomly cross-linked forms of bovine pancreatic ribonuclease, lysozyme[3], soy bean trypsin inhibitor[4] and pepsinogen[5]. The enzyme isolated from beef-liver microsomes has been purified and characterized[6,7] and it has been shown to catalyze sulfhydryl–disulfide interchange in proteins[8].

The enzyme may be assayed by measuring the reactivation of fully reduced proteins in the presence of an added oxidizing agent[1,2] or of the inactive forms of ribonuclease and soy bean trypsin inhibitor (in which incorrect disulfide bonds have been introduced) in the presence of low levels of a reducing agent[8].

Since the disulfide interchange enzyme catalyzes the reactivation of several animal and plant proteins and since the enzyme is localized at the site of protein