sids evolved as members of separate communities. The history of archosaurs seems to have been closely linked with the history of synapsids, not only because they are considered to be derived from pelycosaurs, but also because the fossil record indicates that the first archosaurs were found in the same deposits that yield various synapsids as part of the same general faunal associations.

OSVALDO A. REIG
Instituto de Zoologia Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Caracas

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1 May 1967

Molecular Size of Hagfish Muscle Lactate Dehydrogenase

Abstract. *In contrast to an earlier report, we find that the primitive vertebrate* Eptatretus *possesses a muscle lactate dehydrogenase whose molecular size is like that of lactate dehydrogenases from higher vertebrates. The molecular size of lactate dehydrogenase appears to have remained constant during evolution.*

Hagfishes belong to the most primitive class of vertebrates, the Cyclostomata (*1*). The lactate dehydrogenases (*2*) of the hagfish *Eptatretus* have been studied with respect to their electrophoretic mobility, thermostability, immunological reactivity, and subunit hybridization (*3–5*).

It was recently reported that hagfish lactate dehydrogenases are unusually small molecules, possibly monomers (*6*), and it was suggested (*6*) that the common ancestor of vertebrates may have had a monomeric lactate dehydrogenase that was the evolutionary precursor of the tetrameric form found in all contemporary vertebrates examined, with the exception of the hagfish (*3, 7*). We, however, present evidence that the muscle lactate dehydrogenase of the hagfish is not a small molecule; it appears to have a molecular size like that of lactate dehydrogenase from other species. The molecular weight of lactate dehydrogenase has been previously determined to be approximately 140,000 in several species (*3, 7*).

Hagfishes (*Eptatretus stouti*) were caught off the coast of Southern California in April 1963, by Dr. David Jensen (*8*) and stored at −10°C. Owing to their large size, tongue muscles were used as a major source of lactate dehydrogenase. Portions (1 g) of tissue were ground in 3 ml of 0.25*M* sucrose at 4°C. The homogenates were clarified by centrifugation at 30,000*g* for 15 minutes, and the extracts were stored at −10°C.

Lactate dehydrogenase activity was estimated spectrophotometrically (*9*). The muscle extracts contained 150 enzyme units/ml (*10*), a value which is close to that which we have observed for muscle extracts from other vertebrates. In apparent contrast to certain earlier findings (*6*), hagfish muscle lactate dehydrogenase is rather stable, surviving for 4 years at −10°C with little loss of activity either in tissues or in sucrose extracts. The enzyme is also relatively resistant to denaturation by heat; 50 percent of its activity persists for 20 minutes at 64°C (*5*). No change in heat stability was observed after the enzyme's storage for 4 years at −10°C.

In order to estimate the molecular size of lactate dehydrogenase from hagfish muscle, we carried out gel-filtration studies with a Sephadex G-100 column (53 by 1.5 cm). The degree to which the column separated compounds differing in molecular size was determined by preliminary experiments with proteins of known molecular weight (*11, 12*). Table 1 presents the published molecular weights of the proteins that were used and the elution volumes that were observed in our experiments. Details concerning the experimental conditions are also given in Table 1.

Lactate dehydrogenase from extracts of hagfish muscle was eluted from the Sephadex column as a single symmetrical peak. More than 90 percent of the enzyme activity that was applied to the column was recovered. The elution volume for hagfish lactate dehydrogenase was 38 ml (*13*). The same elution volume was observed in another experiment with crystalline lactate dehydrogenase from chicken H_4; the molecular weight of this enzyme is approximately 140,000 (*7*). No evidence for the existence of a smaller form of lactate dehydrogenase was obtained in either case.

To check whether the elution profiles of these two enzymes were identical, we applied a mixture containing 65 enzyme units of hagfish lactate dehydrogenase from a muscle extract and 4.0 enzyme units of crystalline H_4 lactate dehydrogenase from chicken to the column. Samples of the material eluted from the column were assayed for enzyme activity before and after heating for 20 minutes at 72°C in the presence of 0.05 percent bovine serum albumin. The activity of chicken H_4 lactate dehydrogenase is unaffected

Table 1. Sephadex chromatography with proteins of known molecular weight. The elution volume for each compound was calculated after Sephadex chromatography on a column 53 by 1.5 cm at 4°C. The flow rate in all experiments was maintained at 13 ml/hour. In every case, 0.5 ml of sample was added to the column and 2-ml fractions were collected. The elutant was a buffer containing 0.14 mole of NaCl, 0.01 mole of tris(hydroxymethyl)aminomethane hydrochloride, 5×10^{-4} mole of $MgSO_4$, and 1.5×10^{-4} mole of $CaCl_2$ per liter at a final *p*H of 7.45. Enzymatic assays and absorbance measurements were done at 23°C with a Zeiss spectrophotometer.

Compound	Method of determination	Elution volume from Sephadex G-100 column (ml)	Molecular weight (*11*)
Blue dextran	Absorbance at 650 mμ	27.5	$\sim 2 \times 10^6$
H_4 LDH (crystalline, chicken)	Enzymatic assay (*9*)	38	1.4×10^5
Alkaline phosphatase (crystalline, *E. coli*)	Enzymatic assay (*16*)	46	8×10^4
Malic dehydrogenase (crystalline, pig heart)	Enzymatic assay (*12*)	49	6.2×10^4
Ovalbumin (crystalline, chicken)	Absorbance at 280 mμ	52	4.5×10^4
Cytochrome *c* (crystalline, horse)	Absorbance at 413 mμ	71	1.2×10^4

by this treatment, whereas that of the hagfish enzyme is completely destroyed (*3*, *5*, *14*). Using this property to distinguish between the two enzymes in the mixture, we found nearly identical elution profiles (Fig. 1).

Figure 1 also gives the elution profile for crystalline alkaline phosphatase which had been included in the lactate dehydrogenase mixture. The phosphatase was eluted from the column later than the lactate dehydrogenases, in accordance with the fact that its molecular weight is lower (80,000).

Extracts of hagfish hearts and gills were also subjected to Sephadex chromatography. The elution profiles of lactate dehydrogenase were identical with those observed for extracts of hagfish muscle. Extracts of heart and gill contain two forms of lactate dehydrogenase; one is electrophoretically identical with the enzyme from skeletal muscle, and the other, at *p*H 7, migrates faster toward the anode (*3*, *6*). Owing to the instability of this second form, our experiments with Sephadex probably measured the elution profile of only the muscle-type lactate dehydrogenase.

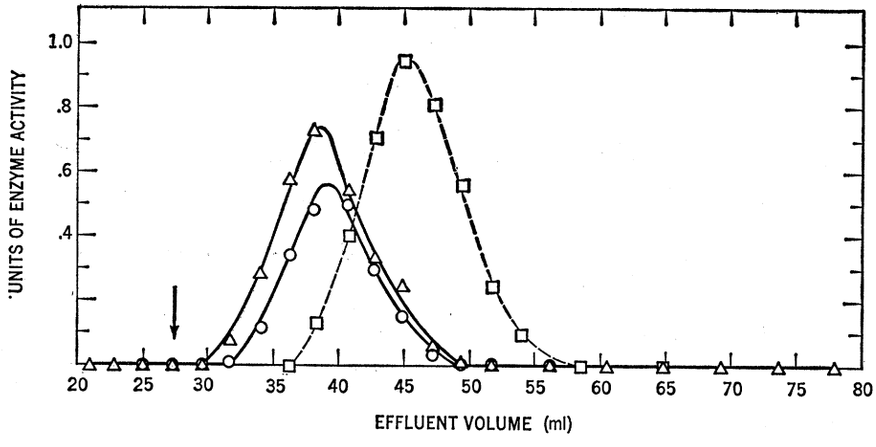

Fig. 1. Chromatography of lactate dehydrogenase from hagfish muscle on the calibrated column of Sephadex G-100 described in Table 1. A mixture (0.5 ml) containing 65 units of hagfish lactate dehydrogenase from a muscle extract, 4 units of crystalline H_4 lactate dehydrogenase from chicken, and 7 units of crystalline alkaline phosphatase from *Escherichia coli* was applied to the column. Elution profiles are given for lactate dehydrogenase before (△————△) and after (○————○) heating at 72°C for 20 minutes in the presence of 0.05 percent bovine serum albumin. The former profile represents predominantly hagfish lactate dehydrogenase, while the latter profile is due entirely to chicken H_4 lactate dehydrogenase. To facilitate comparison of the shapes and positions of the two curves, all assay values for chicken lactate dehydrogenase were multiplied by 10. The alkaline phosphatase elution profile (□————□) is also shown; extracts of hagfish muscle alone do not contain significant levels of alkaline phosphatase activity. The exclusion volume for blue dextran is indicated by the arrow.

In contrast to the report of Ohno *et al.* (*6*), we have shown that lactate dehydrogenase from hagfish muscle is not unusual in molecular size. Crystalline lactate dehydrogenase from muscle of the lamprey *Petromyzon*, another member of the class Cyclostomata, has sedimentation properties like that of all other lactate dehydrogenases examined (*3*, *7*). Also lactate dehydrogenases crystallized from an invertebrate and a bacterium have molecular weights of about 140,000 (*3*). It appears probable that the molecular size of lactate dehydrogenase has remained constant during evolution (*15*).

N. ARNHEIM, JR.
G. T. COCKS
A. C. WILSON
Departments of Biochemistry and Genetics, University of California, Berkeley

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14. Experiments on inhibition by pyruvate (see *9*) confirmed that the lactate dehydrogenase which persisted after the heat treatment was the chicken H_4 enzyme.
15. After completing this work, we learned that contrary to his earlier report, Dr. Ohno now has evidence from hybridization studies that hagfish muscle LDH exists as the tetramer in vivo (S. Ohno, personal communication).
16. Alkaline phosphatase was assayed in a 3-ml volume containing 0.5 mole of tris (hydroxymethyl)aminomethane, *p*H 8.5; 0.001 mole of *p*-nitrophenyl phosphate, and 0.01 mole of $MgCl_2$ per liter at 23°C. One unit of enzyme activity produces a change of absorbance at 410 mμ of 0.1 per minute.
17. Supported by an NIH postdoctoral traineeship to N.A. (GM-367), an NSF predoctoral fellowship to G.T.C., and an NSF grant to A.C.W. (GB-3839). We thank Miss Linda Ferguson for technical assistance.

5 May 1967