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The Thin-Layer Isoelectric Focusing of Lactate Dehydrogenase Isoenzymes in Rabbit Lens Parts and in Intraocular Tissues*

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Summary. Lactate dehydrogenase (LDH) isoenzymes of rabbit lens and other intraocular tissues are separated by thin-layer isoelectric focusing and localized as discrete groups of multiple bands with defined isoelectric points after staining by the tetrazolium method.

In the rabbit lens parts, the predominant isoenzymes are LDH-4 and LDH-5. The bands show microheterogeneity, are composed of 2–4 subcomponents, and the patterns show a distribution of the liver type. The activity of the LDH-4 decreases and that of LDH-5 increases in an order given by the equator, anterior and posterior cortex, and nucleus. LDH-3 remains almost constant in all lens parts. LDH-4 is composed of two subcomponents, one of which, the most cathodic with higher isoelectric point, is almost absent in the lens nucleus.

Of the LDH localized in the rabbit intraocular tissues, only the retina shows a pattern of five isoenzymes also of the liver type. In all intraocular tissues LDH-3, -4, and -5 are very prominent, show also microheterogeneity of their isoenzyme bands, and are each composed of 4–6 subcomponents. LDH-1 and -2 show only one isofocused component.

Species specificity is shown of the LDH isoenzymes in the rabbit, mouse, dog, and calf lens.

Zusammenfassung. Mit dem Verfahren der Isoelektrofokussierung auf Dünnschichtplatten werden die Isoenzyme der Laktatdehydrogenase aus Linse, Hornhaut, Iris, Netzhaut und Aderhaut getrennt. Nach Anfärbung mit der Tetrazoliummethode können verschiedene LDH-Isoenzyme, die aus mehreren Einzelbanden bestehen, aufgrund der definierten isoelektrischen Punkte unterschieden werden.

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In den einzelnen Linsenbereichen sind LDH-4 und LDH-5 die vorherrschenden Isoenzymformen. Die Banden zeigen Mikroheterogenität, bestehen meist aus 2—4 Unterkomponenten und entsprechen im Isoenzymmuster dem Lebertyp. Die Aktivität der LDH-4 vermindert sich in der Reihenfolge: Linsenäquator, vordere und hintere Linsenrinde und Linsenkern; die Aktivität der LDH-5 steigt im Gegensatz dazu an, LDH-3 ist in allen Linsenteilen gleich. Im Linsenkern ist die eine der beiden Unterkomponenten der LDH-4, die am stärksten kathodische Form mit höherem isoelektrischen Punkt, kaum vorhanden. Von den anderen untersuchten Augengeweben zeigt lediglich die Netzhaut ein Isoenzymmuster mit 5 Formen vom Lebertyp. In Hornhaut, Iris und Aderhaut sind die LDH-3, LDH-4 und LDH-5 prominent. Die einzelnen Isoenzymbanden weisen ebenfalls Mikroheterogenität auf, sie bestehen aus 4—6 Unterkomponenten. LDH-1 und LDH-2 treten immer nur als eine einzige Bande auf.

Die Verteilung der LDH Isoenzyme in den Linsen von Kaninchen, Maus, Hund und Kalb zeigt artspezifische Unterschiede.

Introduction

The enzyme lactate dehydrogenase (LDH) (L-lactate: NAD oxidoreductase, EC 1.1.1.27) is generally accepted to exist in multiple forms known as isoenzymes, usually numbered 1 through 5. Several investigators have observed that LDH shows microheterogeneity of the isoenzyme bands after polyacrylamide disc electrophoresis (Dudman and Zerner, 1974), density gradient isoelectric focusing (Susor et al., 1969), disc isoelectric focusing (Chamoles and Karcher, 1970) and thin-layer isoelectric focusing (Thorstenssen et al., 1975; Ross, 1976; Brahma and van der Saag, 1976).

Though much work has been done on the spectrophotometric determination of the LDH isoenzymes in the lens, we refer here mainly to the analysis made of LDH isoenzymes in the rabbit lens by electrophoretic methods. Graymore and McCormick (1966) studied the LDH isoenzymes in rabbit cornea and rat retina by cellogel electrophoresis, and Bernstein et al. (1966) studied those in rabbit lens and cornea by starch gel electrophoresis.

We have made a qualitative and quantitative analysis of the LDH isoenzymes and their subcomponent activities in the whole rabbit lens, in four lens parts, and in the retina, iris, choroid, and cornea. The isoelectric points of the LDH isoenzymes and subcomponents are determined at 4° C, in this way permitting an exact comparison of the samples applied. Preliminary reports have been given (Bours et al., 1975; 1977).

Material and Methods

Tissue Extracts

Rabbit eyes (1 1/2-year-old) were dissected immediately after killing, into cornea, iris, lens, retina, and choroid. The wet weigths were determined and the dry weights after lyophilization (Table 1). The lens was immediately solidly frozen at −20 °C and divided into four parts, i.e. equator, anterior cortex, posterior cortex, and nucleus

Table 1. Isoelectric points and relative activities in per cent of lactate dehydrogenase isoenzymes in whole lens and in various parts of the lens and intraocular tissues of the rabbit eye

LDH isoenzyme No.	Sub-component No.	*Lens*					*Other intraocular tissues*			
		Equator	Anterior cortex	Posterior cortex	Nucleus	Whole lens	Retina	Choroid	Iris	Cornea
		IEP (%)	IEP (%)	IEP (%)	IEP (%)	IEP (%)	IEP (%)	IEP (%)	IEP (%)	IEP (%)
1	1	– – – –	– – – –	– – – –	– – – –	– – – –	4.90 6	4.80 Tr	4.85 Tr	4,85 Tr
2	1	– – – –	– – – –	– – – –	– – – –	– – – –	5.50 7	5.50 Tr	– – – –	5.50 Tr
3	1	– – – –	– – – –	– – – –	– – – –	– – – –	5.80) 19	– – – –	5.80)	– – – –
	2	5.95 8	5.95 5	5.95 7	5.95 5	5.95 9	6.00)	5.95 26	5.90) 40	5.90 31
	3	– – – –	– – – –	– – – –	– – – –	– – – –	6.10) 10	6.15 Tr	– – – –	6.08)
	4	– – – –	– – – –	– – – –	– – – –	– – – –	6.25)	6.25 3	6.25 1	6.35) 27
4	1	6.75 22	6.75 20	6.75 20	6.75 18	6.75 24	6.75 }	– – – –	– – – –	– – – –
	2	6.95 22	6.95 13	6.95 10	– – – –	6.95 14	7.00 } 29	– – – –	– – – –	– – – –
	3	– – – –	– – – –	– – – –	– – – –	– – – –	7.10 }	7.10 24	7.00 20	7.00 22
5	1	– – – –	– – – –	– – – –	– – – –	– – – –	7.60 }	– – – –	– – – –	– – – –
	2	– – – –	– – – –	– – – –	– – – –	– – – –	7,75 }	– – – –	– – – –	– – – –
	3	7.80) 48	7.80) 62	7.80) 63	7.80) 77	7.80) 53	7.85 } 29	7.80) 47	7.85 Tr	– – – –
	4	8.00)	8.00)	8.00)	8.00)	8.00)	8.00 }	8.00)	8.00 39	8.00 20
	5	– – – –	– – – –	– – – –	– – – –	– – – –	8.10 }	8.10 Tr	– – – –	– – – –
	6	– – – –	– – – –	– – – –	– – – –	8.15 Tr	8.20 }	8.20 Tr	– – – –	– – – –
Tissue wet weight (mg)		190	57	62	180	540	57	52	100	85
Tissue dry weight (mg)		–	–	–	–	114	3	3	4	4
Protein content (mg)		56	17	19	69	–	–	–	–	–
H/M ratio		0.18	0.12	0.12	0.08	0.16	0.49	0.26	0.35	0.53
Refer to Figure		1c	1e	1f	1d	1g; (2e)	2a; (1h)	2c	2b	2d

IEP = isoelectric point
(%) = the relative activity of LDH isoenzymes, expressed as a percentage of total activity
– – = absent
– = not carried out
Tr = trace

according to Hockwin and Kleifeld's method (1965). All intraocular tissues and the four lens parts were homogenized in distilled water at 4° C and the homogenates were centrifuged at 38,000 g for 1 h at 4° C, and the supernatants used for further tests. Soluble protein in the lens and lens parts were determined by the method of Waddell and Hill (1956).

Isoelectric Focusing and Determination of the pH Gradient

Thin-layer isoelectric focusing of all tissue extracts was carried out on a Multiphor electrophoresis apparatus (LKB 2117) during 16 h at 100–110 V, 8–0.8 mA and at 4° C according to Bours' method (1975). For determination of the isoelectric points of the single isofocused LDH components, the pH gradient was measured at 4° C (Bours, 1971).

Staining for Lactate Dehydrogenase Activities

After isoelectric focusing, the polyacrylamide gels were incubated for 1.5 h at 37° C in the dark in 111 ml of a medium prepared according to Dietz and Lubrano's method (1967) without any modification. For proper comparison with the LDH patterns obtained from the tissue extracts mentioned above, two references were run simultaneously with the samples: 1. LDH-5, purified from rabbit muscle (Boehringer Nr. 15371); 2. LDH-1, purified from swine heart (Boehringer Nr. 15378). After staining, the gels were washed with 10% acetic acid and preserved by covering them with a solution containing 7% gelatin and 5% glycerol and were dried. The photos were scanned in a Chromoscan densitometer.

Results

LDH isoenzymes are detected by tetrazolium staining after isoelectric focusing. The enzyme activities in the extracts of the whole rabbit lens and of various parts of the lens are shown in Figure 1 and Table 1. In the rabbit lens, and also in the lens parts, the predominant isoenzymes are Nos. 4 and 5, which are each composed of two subcomponents. Compared to the LDH activities shown in the retina which has a distribution of the liver type, those of the lens have a much lower activity of LDH-3 (Fig. 1).

The isofocusing patterns of the activities of the LDH isoenzymes in the other intraocular tissues are shown in Figure 2. In the retina, LDH has five isoenzymes, three of them having a marked microheterogeneity. The isoenzymes Nos. 3, 4, and 5 are very much prominent, and are each composed of four to six subcomponents. The isoenzymes Nos. 1 and 2 show only one component. All other intraocular tissues such as choroid, iris, cornea, and lens contain only LDH isoenzyme activities which have an incomplete number of isofocused components, compared to the retina.

The isoelectric points determined at 4° C and the relative activities, expressed as percentages, of the isoenzymes in the whole lens, in the various lens parts and in the other intraocular tissues of the rabbit eye, are given in Table 1. Going from equator,

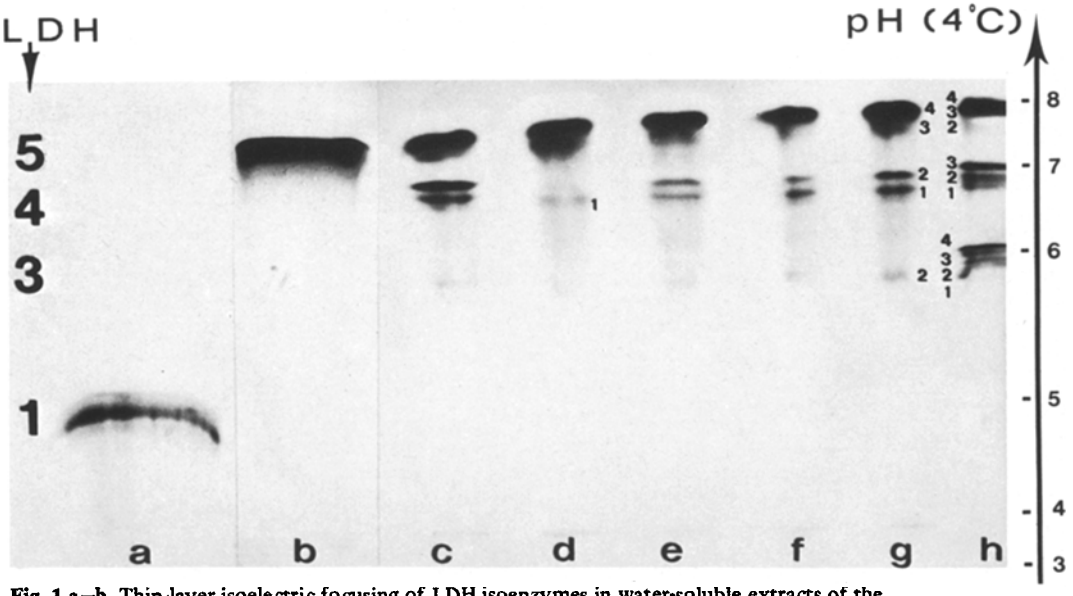

Fig. 1 a–h. Thin-layer isoelectric focusing of LDH isoenzymes in water-soluble extracts of the whole lens and of various parts of the rabbit lens. The amounts of protein determined are given in mg. **c** = equator, 1.0 mg; **d** = nucleus, 1.0 mg; **e** = anterior cortex, 1.0 mg; **f** = posterior cortex, 1.0 mg; **g** = whole lens, 1.0 mg; **h** = rabbit retina, 0.4 mg (reference); **a** = LDH-1 isoenzyme purified from swine heart, 25 μg; **b** = LDH-5 isoenzyme purified from rabbit muscle, 25 μg. The scale on the right shows the pH values along the gel. The isoelectric points are mentioned in the text and in Table 1. The numbers on the left side represent the various LDH isoenzymes

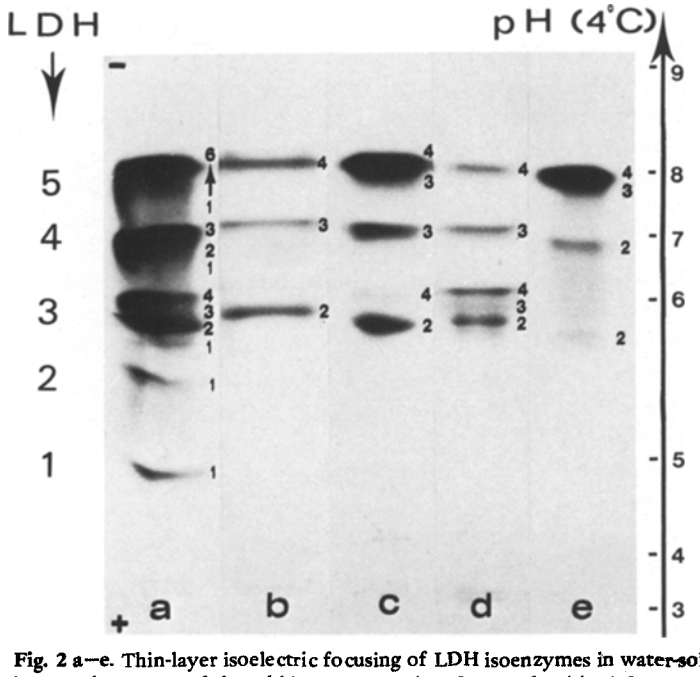

Fig. 2 a–e. Thin-layer isoelectric focusing of LDH isoenzymes in water-soluble extracts of various intraocular tissues of the rabbit eye. **a** = retina, 0.4 mg; **b** = iris, 1.2 mg; **c** = choroid, 1.5 mg; **d** cornea, 2.4 mg; **e** = whole lens, 1.0 mg. The scale on the right side shows the pH values along the gel. Numbers on the left side represent the various LDH isoenzymes

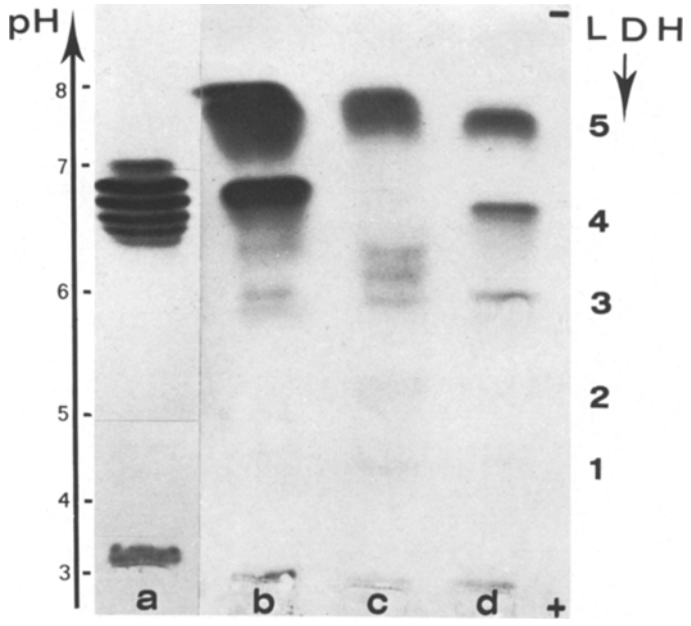

Fig. 3 a–d. Thin-layer isoelectric focusing of LDH isoenzymes of the lens of 4 species. Samples amount to 2 mg of protein. **a** = mouse, 2-month-old; **b** = rabbit, 1-year-old; **c** = dog (beagle), 1-year-old; **d** = calf, 4-months-old. The scale on the left side shows the pH values along the gel, measured at 4° C. Numbers on the right side represent the various LDH isoenzymes

anterior and posterior cortex to the nucleus, the activities of isoenzyme No. 4.2 decrease and those of isoenzyme No. 5 increase, while those of Nos. 3 and 4.1 are fairly constant. Of all intraocular tissues examined, the retina shows the most complete pattern (Figs. 1h and 2a). The pattern of the choroid resembles that of the retina (Fig. 2a and c), while those of the iris and the cornea also show similarities (Fig. 2b and d). Only in the lens is the activity of LDH-3 lower than in any other intraocular tissue (Figs. 1c–g and 2e). The LDH activity in the vitreous body is too low to include (Hoffmann and Wurster, 1974); though the bands were very faint, a pattern similar to that of the iris could be recognized.

It should be stated that for the lens only fresh materials were used, because after freeze-drying the LDH isoenzymes of the lens show a loss of activity of 10–40 %, while the activities of these isoenzymes in the other intraocular tissues seem to remain fairly unaffected by freeze-drying.

The species specificity of lens LDH is illustrated in Figure 3. None of the patterns is alike. The LDH of mouse lens is only composed of one group, while in the dog (beagle) lens the LDH-4 is lacking from the pattern. The LDH activities of rabbit and calf lens to a certain extent show similarities.

Discussion

In the LDH isoenzyme patterns of the 1 1/2-year-old rabbit whole lens, only isoenzymes Nos. 3, 4, and 5 are present (Figs. 1g and 2e). This predominance of activity of LDH 3–5 was also observed in the 1-year-old rabbit lens by Bernstein et al. (1966) with the aid of starch gel electrophoresis. The rabbit lens fiber mass, which is rich in LDH 3–5, belongs to the part of the lens metabolizing under relatively anaerobic conditions, unlike the lens epithelium cells which are slightly more aerobic, due to direct exchange with the aqueous humor. For this reason, the LDH pattern of the rabbit lens epithelial cells shows not only LDH 3–5, but also LDH-1 and -2 (Bernstein et al., 1966). These authors also found much the same lens LDH pattern as we did, going from 2-day-old to 1-year-old rabbit lens, suggesting that the 'aging phenomena' in rabbit lens are not yet strongly expressed after 1 year of age, because the life expectancy of the rabbit is about 8 years. This is in contrast to the LDH activity in the isoenzyme patterns of the aging bovine lens (Bernstein et al., 1966), and to the total LDH activities in the bovine lens, which decrease significantly with age (Hockwin, 1965; Hockwin and Ohrloff, 1973; Hockwin, 1974). This aging phenomenon was also met with in our efforts at isofocusing the LDH isoenzymes of cow lens in which LDH isoenzymes could hardly be visualized or were absent, unlike calf lens LDH (Fig. 3d).

In Figure 3, which has also been published by Bours and Hockwin (1976), the LDH isoenzyme patterns after isoelectric focusing show species specificity for mouse, rabbit, dog, and calf, confirming the species specificity which is also apparent when the LDH isoenzyme patterns of human lens (Yamaguchi and Hamada, 1971) and of chick lens (Maisel et al., 1966) are compared with our results.

The species specificity of the lens LDH isoenzymes is also observed in changes of specific LDH activity with age, which differ from animal to animal. The total LDH activity shows a decrease in the bovine lens (Hockwin, 1965) and in the human lens (Kasavina et al., 1972), and an increase in the chicken and guinea pig lens (Hockwin, 1965). In the rat and mouse lens this activity remains almost constant over a relatively long period of life (Hockwin and Bours, unpublished).

The LDH of mouse lens (Fig. 3a) consists only of isoenzyme No. 4, which is composed of six subcomponents ranging in isoelectric points from 6.12 to 7.01 (4° C) (Bours and Hockwin, 1976). Weller et al. (1973) and Lyra et al. (1976) found that LDH isolated from mouse skeletal muscle consisted of one isoenzyme; this is most probably similar to the one observed in the mouse lens in our experiments, though Weller et al. (1973) measured, at 20° C, a lower isoelectric point of 5.95. Our isoenzyme pattern of the 4-months-old calf lens (Fig. 3d) differs from those of calf lens (age not recorded) as published by Stewart and Papaconstantinou (1966), who found, in addition, the more anodic bands of LDH-2 and -1 by starch gel electrophoresis. The pattern produced from LDH isoenzymes of adult bovine lens cells in tissue culture (Stewart and Papaconstantinou, 1966) was more or less in agreement with our results (Fig. 3d).

In the rabbit lens there is a considerable topographic difference of LDH metabolism in the distribution of the enzyme activities (Table 1), in contrast to more or less comparable data for the calf and the bovine lens, where only small differences in total LDH activity of each age group were found between the equator, anterior and

posterior cortex, and the nucleus (Hockwin et al., 1966). Kasavina et al. (1972) found a substantial topographic difference of LDH activity in the 60-year-old human normal lens, expressed as a four times higher activity in the cortex than in the nucleus.

In the other intraocular tissues of the rabbit, the activity of LDH-3 is more pronounced than in the lens itself (Fig. 2). The rabbit retina also shows activities of LDH-1 and -2, which confirms the results of Quentin and Neuhoff (1972) and Vassileva (1975) for rabbit, and of Futterman and Kinoshita (1959) for bovine retina, of Graymore (1964) and Graymore and McCormick (1966) with the same picture in both papers for rat retina, and of Papadopoulos and Thomas (1969) and Lam et al. (1972) for human retina. Bonavita et al. (1963) showed a different picture for the adult rat retina, with highest activities of LDH-1 and -2.

The rabbit cornea also has a marked activity of LDH-3 (Fig. 2d). This pattern is not in agreement with the results of Moore and Wortman (1959), of Graymore and McCormick (1966), or of Bernstein et al. (1966), who found for the whole rabbit cornea more LDH-1 and -2 activity, which is a pattern similar to that of the bovine corneal epithelium (Futterman and Kinoshita, 1959) – a tissue of relatively more aerobic conditions. Our results for rabbit cornea (Fig. 2d) reflect more those obtained for human cornea by Papadopoulos and Thomas (1969) and for bovine cornea by Bernstein et al. (1966).

However, we have found that each of the intraocular tissues, though comparable to some extent, has a different pattern of LDH isoenzyme components (Fig. 2).

The values of the isoelectric points of the LDH isoenzyme components of the rabbit intraocular tissues (Table 1), are compared with those from two standards: (1) LDH-5 purified from rabbit muscle, pI ranging from 7.6 to 8.2 (4° C); (2) LDH-1, purified from swine heart, pI = 4.8 to 5.2 (4° C). Broadly the same values were measured by Susor et al. (1969) for three LDH-5 components from rabbit muscle: 8.3, 8.4, 8.5 (20° C) and by Weller et al. (1973) for purified rabbit muscle LDH-5: 8.9 (20° C).

Isoelectric focusing always shows a higher resolving power than observed with any other electrophoretic technique, which is shown here in Figures 1, 2, and 3 in the microheterogeneity of the LDH main bands. However, in microisoelectric focusing (Quentin and Neuhoff, 1972) this high resolution is lost, which makes this variation of the valuable isoelectric focusing technique nearly equal to agar electrophoresis which has a low resolving power.

The theory of Markert (1962) and Cahn et al. (1962), whereby any particular LDH pattern made up of the 5 isoenzymes 1 (HHHH), 2 (HHHM), 3 (HHMM), 4 (HMMM), and 5 (MMMM) reflects the ratio H : M monomers present in the tissue of origin, as we calculated in Table 1, is not able to explain the presence of subcomponents in each of the LDH isoenzymes. Kraus and Neely (1964) postulated that tetramers composed of M and M′ subunits are possible. LDH-5 could be composed of 5 tetramers: MMMM, MMMM′, MMM′M′, MM′M′M′, and M′M′M′M′. In a similar arrangement, the LDH-4 through -1 could consist of 4, 3, 2, and 1 isoenzyme band. In this way formation of the multiple bands seen in Figures 1–3 could be better explained. The multiple bands of activity observed in the LDH-5 of rabbit retina (Figs. 1h and 2a) differ by 0.10–0.15 of a pH unit and range in isoelectric points by 0.60 of a pH unit. Therefore, this may well be an example of postsynthetic heteroge-

neity (Bours, 1976), i.e. of postsynthetic charge alteration by deamidation (van Kleef et al., 1976), of a biosynthetically homogeneous protein.

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