A further study of LDH isozymes in the *Rana esculenta* complex

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Summary. Electrophoretic analyses of LDH isozymes in the 3 types of European green frogs by using the buffer system of Williams and Reisfeld demonstrated the occurrence of 4 allelic genes coding for the B subunits. Based on the distribution of these alleles and results from dissociation and recombination of the subunits, the hybrid nature of Rana esculenta is confirmed.

In a previous report on the isozymes of lactate dehydrogenase (LDH) in the 3 types of European green frogs, we described the presence of 3 electrophoretic patterns in Rana lessonae, 3 in Rana ridibunda and 4 in Rana esculenta[2]. Judging from the equal mobility of the enzyme bands, pattern III has been considered to be identical in R. lessonae and R. ridibunda, though band 2 was consistently missing in the latter. Recently, by employing a different buffer system of low pH, distinct differences in anodal migration of the isozymes could be observed, suggesting that this pattern is not the same in the 2 frog types. In addition, by experiments of in vitro hybridization, we were able to confirm our earlier conclusion that the 5 most anodal isozymes in patterns II and IV are tetramers formed by random assembly of 2 different B subunits. These results will be dealt with in the present report.

Materials and methods. For separation of the LDH isozymes in polyacrylamide gel, we have previously used a modified system of Davis[3] as given in Maurer[4] (2.5% sample gel, pH 6.7; 5.5% separation gel, pH 8.9; running buffer, pH 8.3). The new buffer system was that of Williams and Reisfeld[5] (sample gel, pH 5.5; separation gel, pH 7.5; running buffer, pH 7.0). Enzyme samples were prepared from hearts of adult frogs. Since no adult R. esculenta with pattern III was available, tail pieces from metamorphosing larvae were used.

For in vitro hybridization, dissociation of the LDH subunits were effected by freezing and thawing[6]. Tissues (kidney, heart or larval tail piece) from animals of the desired genotype were individually homogenized in a phosphate buffer (0.25 M, pH 7.0) containing 1 M NaCl. Following centrifugation, equal volumes of the supernatant were mixed. The mixture was frozen overnight in a deep freezer (−22 °C) and then warmed gradually to room temperature prior to electrophoresis.

Results and discussion. The zymograms in figure 1 illustrate the major differences between LDH patterns separated by the 2 buffer systems. As can be seen, subsequent to separation at pH 8.3 the isozymes of pattern DIII showed the same anodal mobility in the 3 types of green frogs, but could clearly be distinguished at pH 7.0 (figure 1, a). This is especially true for the most anodal bands which moved distinctly faster in ridibunda than in lessonae and at an intermediate rate in esculenta. Apparently the B subunits involved in the production of the tetramers are different. The subunit in lessonae is now designated B^c and that in ridibunda B^d. The corresponding allelic genes are termed LDB^c and LDB^d. The intermediate mobility in esculenta is no doubt due to the hybrid combination LDB^c/LDB^d.

A similar situation has been found for pattern DII in lessonae and esculenta (figure 1, b) and pattern DIV in ridibunda and esculenta (figure 1, c). In both cases, clear-cut differences in the migration of the 5 most anodal bands could be observed only by the system of Williams and Reisfeld[5] at pH 7.0.

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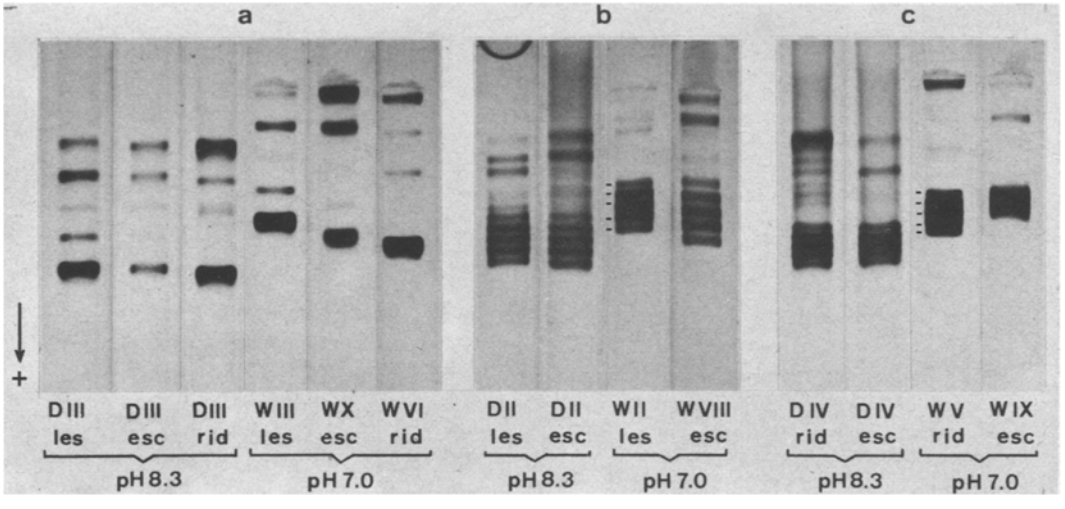

Fig. 1. Zymograms showing differences in the anodal mobility of LDH isozymes in R. lessonae (les), R. ridibunda (rid) and R. esculenta (esc) separated by the system of Davis[3] at pH 8.3 (patterns DI to DVI, see Vogel and Chen[2]) and by the system of Williams and Reisfeld[5] at pH 7.0 (patterns WI to WX, see figure 2 in this paper).

Our overall results are summarized in figure 2. The following conclusions can be drawn: a) A total of 10 LDH patterns are distinguishable, each of which has its origin in a different genotype with regard to the B subunits. b) There are altogether 4 alleles at the genetic locus coding for the subunits B^a, B^b, B^c and B^d. The 2 alleles LDB^a and LDB^c occur only in lessonae, the 2 alleles LDB^b and LDB^d only in ridibunda, whereas all 4 alleles are present in esculenta. c) The homozygous genotypes LDB^a/LDB^a and LDB^c/LDB^c as well as LDB^b/LDB^b and LDB^d/LDB^d, which are characteristic for lessonae and ridibunda respectively, have never been found in esculenta. The same is true for the heterozygous combinations LDB^a/LDB^c and LDB^b/LDB^d. d) In all 4 genotypes found in esculenta, one allele has its origin from lessonae and the other one from ridibunda. The last 2

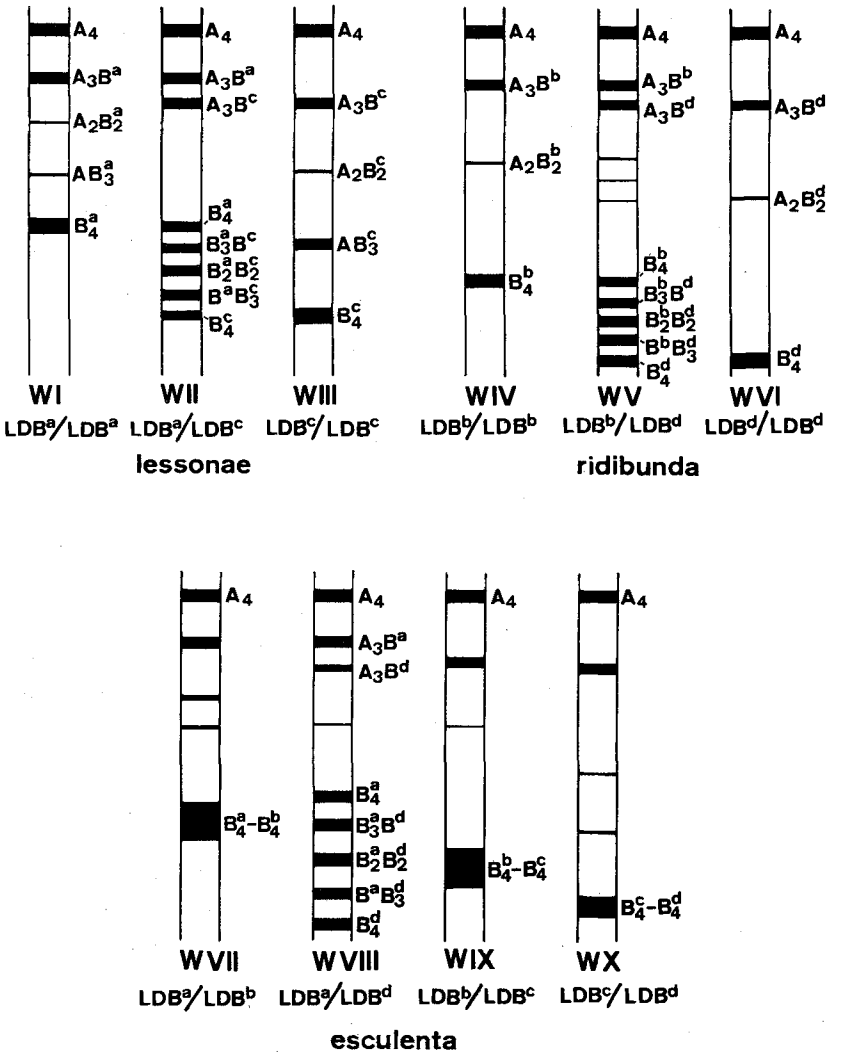

Fig. 2. Diagrams illustrating subunits involved in the formation of the 10 LDH patterns in the 3 types of green frogs identified by the system of Williams and Reisfeld[5]. Genotypes of the B alleles (LDB^a to LDB^d) are given for each pattern. Because of the low resolving power of this system, the 5 most anodal tetramers in patterns WVII, WIX and WX of R. esculenta form each a single broad band.

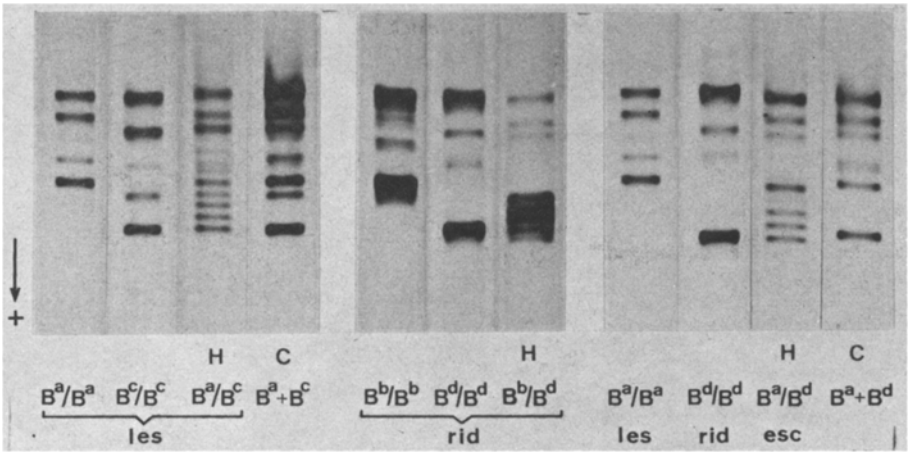

Fig. 3. Formation of heterozygous LDH isozyme patterns (H) by in vitro hybridization of samples prepared from homozygous genotypes. C indicates control zymograms of mixed enzyme samples, the subunits of which were not dissociated by freezing and thawing prior to electrophoresis.

points provide unequivocal evidence for the hybrid nature of R. esculenta, as already postulated from morphological and cytological studies and analyses of the serum proteins (for specific references see Vogel and Chen[2]). Because of its low pH, the system of Williams and Reisfeld[5] has a rather limited resolving power. Thus, the 5 anodal isozymes in patterns WVII, WIX and WX each form a single broad band (figure 2). This is obviously also the reason why pattern WX exhibits only 5 enzyme bands, despite the presence of 3 different subunits (A, B^c and B^d). As shown in figure 3, the heterozygous patterns in lessonae, ridibunda and esculenta could be produced by in vitro hybridization of LDH samples prepared from the proper homozygous genotypes. Although only one hybrid zymogram for esculenta (esc) is included in figure 3, we were able to produce the other 3 patterns by mixing the corresponding enzyme extracts from lessonae and ridibunda. Furthermore, from the control zymogram in figure 3, it is clear that mixed LDH samples without being subjected to freezing and thawing prior to electrophoresis yielded only additive patterns. In conclusion, the results of in vitro hybridization are in excellent agreement with the molecular-genetic implications revealed by our analysis of the LDH patterns.

Uzzell and Berger[7] reported the occurrence of 5 B subunits from their investigation of the LDH patterns in the R. esculenta complex. One of these was found only in 1 lessonae and 4 esculenta from the vicinity of Vienna, although they examined also animals collected from other parts of Europe. Apparently this allele is of only limited geographical location. They performed no extensive cross experiments to clarify the inheritance of the LDH phenotypes identified by them.

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Arylsulphatase in growing bones of rat[1]

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Summary. Soluble arylsulphatase purified from growing bones of rats was fractionated into 3 components with mol. wt of 32,000–36,000, and characteristics similar to arylsulphatase B of other tissues. Serum strongly inhibited the 2nd component, slightly activated the 3rd and had no effect on the 1st.

'Soluble' arylsulphatases A and B (EC 3.1.6.1.) are located in lysosomes[3–5]. Sulphatase A possibly acts as cerebroside sulphatase, sulphatase B is thought to function as a chondroitin sulphatase[6,7], keratan sulphatase[8], or dermatan sulphatase[9].

Arylsulphatase activities has been estimated from bone and articular cartilage[10,11], but characteristics of the enzymes in bone have not been reported previously. In this study we characterized 'soluble' arylsulphatases from growing rat bones. Only B-type of activity was found. 3 components of the enzyme were purified and an inhibition of hydrolysis of p-Nitrocathecol sulphate by serum was discovered.

Material and methods. The femurs of rats aged 22–28 days were cleaned from marrow tissue and homogenized in deionized water using 'Ultra-Turrax' (Janke & Kunkel KG) and centrifuged at 20,000 × g for 30 min. Part of the nonspecific protein of the supernatant was precipitated at pH 5.6 and removed by centrifugation as above. The arylsulphatase activity was assayed using a modified method of Roy[12]. The reaction mixture contained: 0.2 ml of 10 mM p-Nitrocathecol sulphate in 0.1 M Na-acetate buffer, pH 5.3, and 0.2 ml of sample. After a 30 min incubation at +37 °C, the reaction was stopped in an ice-bath adding 0.6 ml of 1 M NaOH. The liberated p-Nitrocathecol was measured at 515 nm. The rate of hydrolysis was linear with time and enzyme concentrations up to 60 min of incubation.

Results and discussion. Since arylsulphatase activity may be bound to lysosomes or microsomal membranes[4], sonication, autolysis overnight at 37 °C or treatment with Triton X-100 were tested. Neither constant increase in yields, nor new components of the enzyme were obtained. Precipitation with ammonium sulphate gave unsatis-

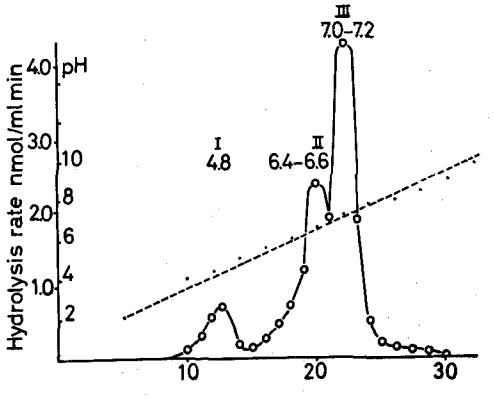

Fig. 1. Isoelectric focusing was made in LKB Ampholine 8100 column using sucrose gradient, pH-range 3.5–10.0 according to the manufacturers instructions. ○—○, Enzyme activity, ---, pH-gradient. Fraction numbers are shown on abscissa and isoelectric points of the fractions given.

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