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-

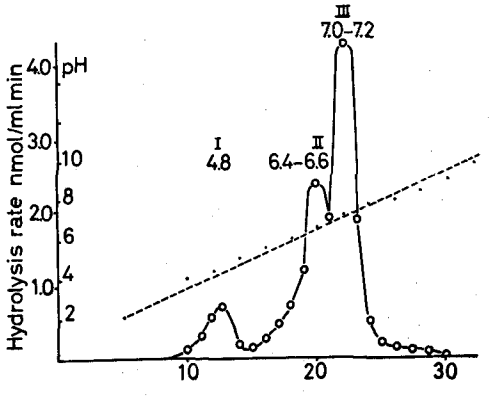

Fig. 1. Isoelectric focusing was made in LKB Ampholine 8100 column using sucrose gradient, pH-range 3.5–10.0 according to the manufacturers instructions. ○—○, Enzyme activity, ---, pH-gradient. Fraction numbers are shown on abscissa and isoelectric points of the fractions given.

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Purification procedure

	Protein (mg/ml)	Specified activity	Purification coefficient	Relative yield
Supernatant of bone homogenate	6.96	1.053	1.00	100
Tissue extract (after treatment at pH 5.6)	2.68	2.670	2.54	80
Isoelectric focusing*				
Peak I	–	–	–	27
Peak II	–	–	–	152
Peak III	–	–	–	118
Sephadex G 50 gel filtration from				
Peak I	0.15	32.260	30.64	29
Peak II	0.12	100.080	95.04	170
Peak III	0.18	86.670	82.31	97

* Because of the presence of Ampholine the protein concentration was not determinated at this stage.

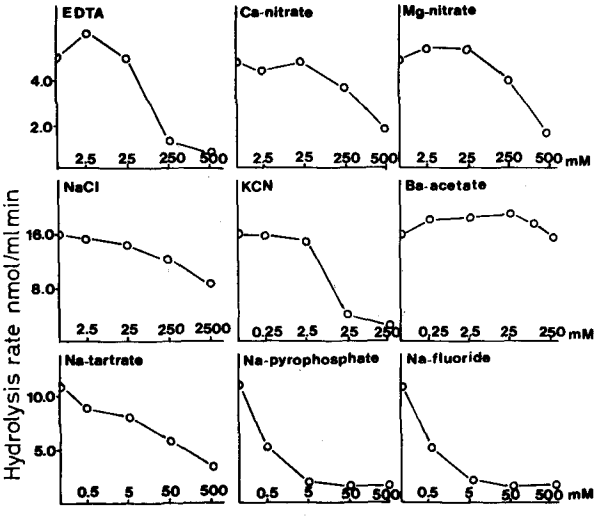

Fig. 2. Effects of selected activators and inhibitors on rat bone arylsulphatase activity on peak II are shown; all components behaved similarly. O—O, Enzyme activity.

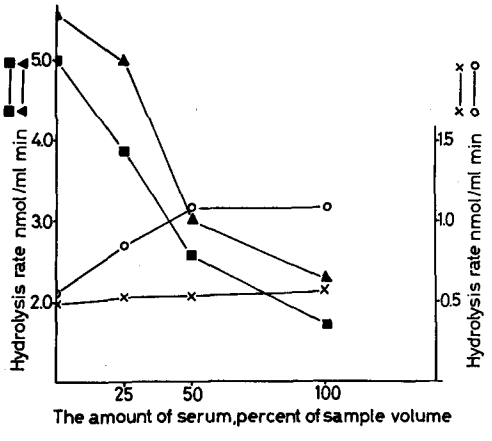

Fig. 3. The effect of serum on the arylsulphatase activity of tissue extract and pools I, II and III. ■—■, Tissue extract; ×—×, pool I; ▲—▲, pool II; O—O, pool III.

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factory results and the simple and constant results producing precipitation at pH 5.6 was chosen as the pretreament.

Supernatant (called as tissue extract) obtained after this precipitation was subjected to preparative electrofocusing. 3 components of enzyme activity were separated (figure 1). Peak I represents 10%, peak II 57% and peak III 33% of the total activity. These peaks were pooled separately and subjected to Sephadex G-50 gel filtration. DEAE chromatography using a semi-open linear NaCl gradient and 0.05 M Tris-acetate buffer (pH 7.7), also fractionated the arylsulphatase into 3 components. Purification coefficients, calculated from the total activity, are given in the table.

The arylsulphatase activity of homogenate or tissue extract was eluted as a single peak corresponding to mol. wt of 67,000–70,000 in Sephadex G-200 gel filtration. After electrofocusing all 3 components both alone and pooled together eluted also as one peak but corresponding to mol. wt of 32,000–36,000. The results were the same in Sephadex G-50 and G-100 gel filtrations. No activity was found corresponding to mol. wt of arylsulphatase A[13] during any steps of purification.

Peaks I and II and the optimum pH at 5.0, peak III at 5.5. Peak I and II had a K_m-value of 3.3 mM, peak III 6.1 mM estimated according to Lineweaver-Burk's method (with 30 min incubation at pH 5.3).

Sodium chloride, a typical inhibitor of arylsulphatase B[14], caused an inhibition on all enzyme components separately. This inhibition was stronger when tested with 3 components pooled together. The effects of selected activators and inhibitors are summarized in figure 2. The problem in studies of simple effectors is that chloride, tartrate, nitrate, and sulphate anions all affect the reaction. 3 components of arylsulphatase have largerly similar pH optima, K_m-values, and mol. wt; also inhibitors and activators have similar effects. This suggests that the 3 forms are closely related, differing mainly in electrical charge. The mol. wt and the effects of Cl^-, $P_2O_7^{=-}$ and F^- ions suggest that the arylsulphatase activity of bone corresponds to sulphatase B[9, 15, 16], although the isoelectric point of peak I is analogous to that of arylsulphatase A in other tissues[17]. Hook et al.[18] and Worwood et al.[19] have also reported the predominance of arylsulphatase B in rat tissues.

When we studied the physiological regulation of arylsulphatase, we discovered a strong inhibition of hydrolysis of p-Nitrocathecol sulphate by serum (figure 3). This effect on pooled enzyme components did not change when diluted serum was warmed at 80 °C for 10 min. The effect on 3 fractions of arylsulphatase was different. Serum strongly inhibited peak II, activated peak III and had no effect on peak I.

During purification, the total activity increased about 3fold when compared to the original homogenate. This suggests a separation of inhibitor(s) or other substrates which could originate from the serum. This type of effect of serum has not been known previously, Okada et al.[9] only briefly mentioned an activatory effect by albumin.

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