Comparison of the inhibition of avian and mammalian bone alkaline phosphatases by levamisole and compound R8231[1]

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Summary. Alkaline phosphatases from mammalian bone are inhibited much more than chick bone alkaline phosphatase by levamisole and compound R8231. Doses of R8231 (10^{-4} to 10^{-5} M) that almost completely inhibit mammalian alkaline phosphatases do not inhibit the growth of embryonic rat femurs in vitro. R8231 should be an excellent biological probe for the function of alkaline phosphatase in bone metabolism.

In most mineralizing tissues the first detectable deposition of mineral occurs in extracellular membrane-bound matrix vesicles derived from the cell membrane[2]. In an in vitro study of the forming bone collar in embryonic chick femurs[3] pyrophosphate was found to be a powerful stimulator of calcium uptake. It was proposed[3] that initial crystal formation depends upon alkaline phosphatase acting as a pyrophosphatase[4] to boost the phosphate concentration within matrix vesicles.

Levamisole (L-tetramisole[5]) is a broad spectrum anthelmintic[6] and this compound and analogues of it have been described as potent specific inhibitors of alkaline phosphatase[7]; there are important cytochemical uses for these inhibitors[7–10]. In this report levamisole and an analogue, R8231, have been tested for their effectiveness as inhibitors of avian and mammalian bone alkaline phosphatases and for any possible growth inhibitory effects on chick and rat embryonic femurs in vitro.

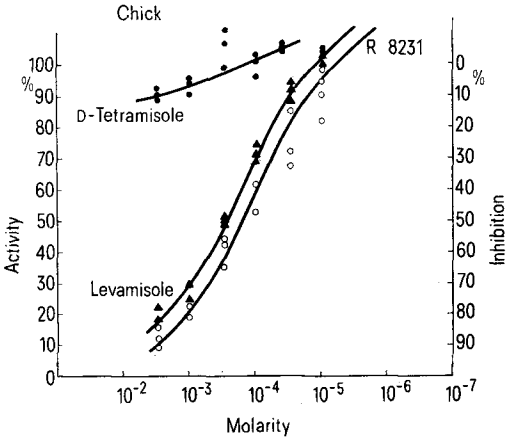

Fig. 1. Inhibition of chick bone alkaline phosphatase by levamisole and R8231.

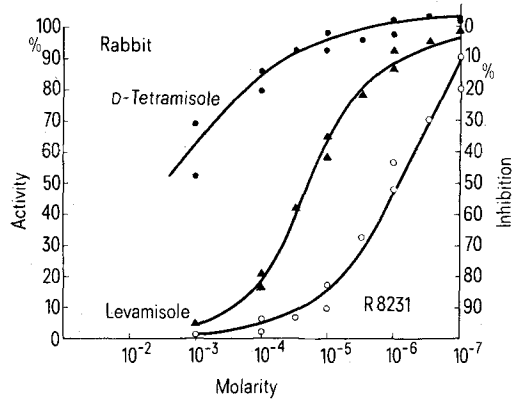

Fig. 2. Inhibition of rabbit bone alkaline phosphatase by levamisole and R8231.

Materials and methods. Preparation of alkaline phosphatase extracts from bone. 1. Chick. Femurs were dissected from either day-old chicks or from those just about to hatch. The femurs were freed of soft tissue and chopped in saline; the extract was left for 2 h at 4 °C to remove blood and marrow. 1 g of washed bone was added to 5 ml of physiological saline, buffered to pH 7.0 with 0.01 M Tris-HCl, and the whole homogenized in a hand glass-homogenizer. The homogenate was spun at 20,000 × g for 10 min to remove cellular and other debris; about 50% of the activity remained in the pellet. The supernatant was stored at 4 °C with 0.02% NaN_3 to prevent bacterial growth. 2. Mouse. This preparation was made as for chick except that adult mice were the source of femurs. 3. Rabbit. An extract from embryonic bone (20 days gestation) was prepared as for chick. Adult rabbit femurs were obtained from the slaughter house. About 20 g of shaft was throroughly washed in 250 ml saline to remove blood. Clean bone was then ground in a mortar and pestle at 4 °C, and the resulting fragments were added to 100 ml buffered saline (see above). This suspension was kept at 4 °C for 1 h with occasional stirring and then centrifuged as for chick. 4. Rat. An extract from embryonic femurs was prepared as for chick.

Assay of alkaline phosphatase. The procedure is based on that of van Belle[11]. The buffer was 50 mM n-ethylamino ethanol and the substrate was 4-nitrophenol phosphate (sodium salt; 10 mg/ml). For assays, 20 µl of bone extract was added to 1.48 ml buffer and 0.5 ml of substrate solution; inhibitors were added as described. The mixtures were incubated for 10 min at 40 °C and the optical density measured at 410 µm. D- and L-tetramisole and R8231

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5 Abbreviations. Tetramisole: (±)-2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b) thiazole hydrochloride. R8231: (±)-6(m-bromophenyl)-5,6-dihydroimidazo (2,1-b) thiazole oxalate.
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were the generous gift of Dr Borgers, Janssen Pharmaceutica, Beerse, Belgium. Inhibitors were dissolved in the assay buffer and kept as 10^{-2} M stock solutions. For assay 0.2 ml of inhibitor solution replaced the equivalent volume of buffer.

Culture of bone rudiments. Chick embryonic femurs were cultured as described previously[3]. For cultures of rat bones, femurs from embryos of 17-day pregnant rats were used in a similar manner to chick explants.

Results. Chick bone alkaline phosphatase can be progressively inhibited with increasing concentrations of either levamisole or R8231 (figure 1). These 2 compounds are equally inhibitory for chick bone alkaline phosphatase and the 50% inhibition level is about 10^{-4} M. The other isomer of tetramisole (D-tetramisole, dexamisole) is without significant effect at concentrations up to 10^{-3} M.

Levamisole and R8231 are much more potent in inhibiting the alkaline phosphatase of adult rabbit bone, as compared with chick (figure 2). Additionally figure 2 demonstrates that R8231 is more than 10 times as potent as levamisole[11]; 50% inhibition by R8231 is achieved at 10^{-6} M. R8231 is a racemic mixture therefore it is likely that the active isomer is even more potent than data suggest. Doses of dexamisole above 10^{-4} M cause considerable inhibition.

Inhibition of avian and mammalian bone alkaline phosphatases by tetramisole, an analogue (R8231) and beryllium sulphate

Source of bone alkaline phosphatase	Percentage inhibition of bone alkaline phosphatase by dexamisole (D-tetramisole)	levamisole (L-tetramisole)	R8231	$BeSO_4$
Chick	5	10	11	79
Rabbit (adult)	5	36	84	72
Rabbit (embryonic)	0	33	75	74
Mouse	6	24	81	57

The values are averages of duplicate or triplicate estimations. All test substances were used at a concentration of 10^{-5} M.

The dose-response curves for levamisole and R8231 together with either embryonic rabbit, or mouse or embryonic rat bone alkaline phosphatase were essentially the same as for adult rabbit (figure 2). Beryllium sulphate[11] is more effective than either levamisole or R8231 as an inhibitor of chick bone alkaline phosphatase (table) and comparable when tested on mammalian enzymes. Because of the toxicity of beryllium salts we did not consider them further as a biological probe. When added to the culture media both isomers of tetramisole and R8231 caused more inhibition of growth of chick explants than rat, in terms of length and weight during 6 days in vitro. Above 10^{-4} M all these compounds were growth inhibitory for chick explants; since there is only 50% inhibition of alkaline phosphatase by either levamisole or R8231 at 10^{-4} M these inhibitors have limited usefulness for studies with chick explants. Much more importantly, with rat bone explants a dose of 10^{-4} M, which for R8231 blocks alkaline phosphatase activity almost completely, was without any growth inhibitory effects.

Discussion. These data fit well other results[7-11] that levamisole and R8231 are potent inhibitors of mammalian alkaline phosphatases. However, chick bone alkaline phosphatase is much less susceptible to inhibition by these compounds; doses needed to almost completely inhibit chick bone alkaline phosphatase were growth inhibitory when tested on bone explants in vitro. The difference between the inhibitory effects of levamisole and analogues on avian and mammalian alkaline phosphatase has also been noted for chick and pigeon liver and serum (M. Borgers and H. van Belle, personal communication), as compared with serum alkaline phosphatase from man, dog and rat. Majeska and Wuthier[12] also suggest that levamisole is not a potent inhibitor of chick alkaline phosphatase but showed that both the phosphatase and pyrophosphatase activities of the enzyme were equally blocked by levamisole.

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Effect of germination on the glycoprotein of mash (Phaseolus mungo) seeds

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Summary. The soluble carbohydrates and insoluble proteins of *Phaseolus mungo* seeds decreased considerably up to 96 h of germination, whereas the soluble proteins remained nearly constant. The carbohydrates content of glycoprotein also remained constant. This suggests that a negligible change took place in the glycoprotein during the initial period of mash seed germination.

Glycoproteins are ubiquitous in the plant kingdom. They are a rich source of many important substances such as hemagglutinins[2], toxins[3] and some enzymes[4,5]. Glycoproteins are also known to play an important physiological role in the transportation and storage[6] of carbohydrates. During germination the metabolic processes become active and the role played by glycoproteins in germinating seeds is still not fully known. Thus, in the present investigation, the changes in glycoprotein fraction in the germinating mash seeds have been reported.

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