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Characterization of Alkaline Phosphatase of the Kidney of Vertebrates. I. Activity of the Enzyme with Steroid-phosphates

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Endocrinologia. — *Characterization of Alkaline Phosphatase of the Kidney of Vertebrates.* I. *Activity of the Enzyme with Steroid-phosphates* (*). Nota di GIOVANNI DELRIO e VIRGILIO BOTTE, presentata (**) dal Socio G. MONTALENTI.

RIASSUNTO. — È stata saggiata l'attività della fosfatasi alcalina, estratta dal rene di diversi Vertebrati, nei confronti di alcuni steroidi fosfati. Sono stati usati reni prelevati da maschi e da femmine di bovino, topo, pollo, lucertola e rana. La fosfatasi renale idrolizza in tutti i casi i substrati coniugati nelle posizioni 21 e 3. Il testosterone 17–fosfato, invece, viene idrolizzato dalla fosfatasi della rana e della lucertola, ma molto meno dall'enzima estratto dagli altri animali. L'estradiolo 17–fosfato viene, invece, scisso solo dalla fosfatasi della lucertola e del topo.

Non-specific phosphatases of animal origin, such as those present in the tissues, in milk and microrganisms, hydrolyze a wide variety of monophosphoric esters. Typical substrates are the phosphate esters of aliphatic alcohols, alcoholic sugars, cyclic alcohols, phenols and, in some cases, phosphate amines. With such substrates, the specificity of this enzyme for the organic portion is very low.

The phosphatases extracted from the tissues of various animals hydrolyze, moreover, some steroid-phosphates (1) (Botte and Koide, 1968; Botte and Delrio, 1968; DiPietro and Zengerle, 1967; DiPietro, 1968). In fact, human placenta phosphatases and those extracted from some tissues of adult female mice hydrolyze hydrocortisone 21–phosphate, dehydroepiandrosterone, androsterone and epiandrosterone 3–phosphates, testosterone and estradiol 17–phosphate. The latter two substrates are mainly hydrolyzed by acid phosphatase.

The alkaline phosphatase of the kidney of the adult female mouse is active with all the steroid phosphates tested; even with estradiol 17–phosphate which, as a rule, is little utilized by alkaline phosphatase of the other tissues (Botte and Delrio, 1968). After such a finding, it seemed interesting to ascertain whether these characters be common to the alkaline phosphatase of

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(**) Nella seduta del 14 dicembre 1968.

(1) Trivial names: Hydrocortisone 21–phosphate: 4–pregnene–11 β, 17 β–diol–3,20–dione 21–phosphate; testosterone 17 β–phosphate: 4–androsten–3–one 17 β–phosphate; dehydroepiandrosterone 3 β–phosphate: 5 α–androsten–17–one 3 α–phosphate; estradiol–17 β–phosphate: 1, 3, 5, (10)–estratrien–3–ol 17 β–phosphate; estradiol 3 β–phosphate: 1, 3, 5, (10)–estratrien–3–ol 3 β–phosphate.

vertebrates' kidney. We have, therefore, tried to determine the activity of the alkaline phosphatase extracted from the kidneys of some vertebrates with various steroids phosphate.

MATERIAL AND METHODS.

The kidneys of adult specimens of both sexes were utilized from the following species of vertebrates: bovine (*Bos taurus*) (1 ♂ and 1 ♀); mouse (Balb/C strain) (21 ♂ and 21 ♀); chicken (local Naples strain) (1 ♂ and 1 ♀); lizard (*Lacerta sicula*) (50 ♂ and 50 ♀) and frog (*Rana esculenta*) (50 ♂ and 50 ♀). The extraction and purification of alkaline phosphatase was performed on homogenates. In the case of the mice, lizard and frogs, it was necessary to collect the kidneys of various specimens to obtain enough enzyme for the successive purifications. The tissues were homogeneized in Tris–HCl 0.05 M buffer, pH 7.4, with a ratio of 2 ml buffer/gram of fresh tissue. The alkaline phosphatase was extracted with *n*–butyl alcohol and partially purified following Morton's method (1954) as modified by Cox and Griffin (1967). The activity of the enzyme was determined with Sigma method (Sigma 104) and expressed in mμmoles of *p*–nitrophenol/mg of proteins of the enzymic solution for 10 minutes incubation at 37° C. The proteins were determined according to the method of Lowry *et al.* (1951).

The activity of the enzyme solutions as regards the steroid phosphates were determined according to the previously mentioned method (Botte and Koide, 1968; Botte and Delrio, 1968). The following modifications were effected in the quantitative determination of some free steroids: testosterone was quantified by gas-cromatography on a Barber Colmann apparatus, Series 5000, column SE 30 1 % on Gaschrom Q, 230° C, argon flow 65 ml/min. (RT, 3,6 min.). The androstenedione derivative was also prepared, and analyzed in turn at the gas-chromatograph (Borgestede *et al.*, 1963). Dehydroepiandrosterone was analyzed by gas-chromatography (same conditions as for testosterone). The identification of this steroid was confirmed by its transformation into its acetylated derivative (Wotiz *et al.*, 1961). Estradiol was quantified by gas-chromatographic analysis of column QF 1,5 %, 230° C flow 66 ml/min., and confirmed by the acetyled derivative (Wotiz *et al.*, 1961).

The enzymic activities were expressed in mμmoles of free steroid/mg of protein of the enzymic solution, for 30 min. incubation at 37° C. Control experiments were made incubating in a medium without substrates. In the various experiments there was a recovery of about 80 %, which was considered in the evaluation of the enzymic activities.

Hydrocortisone 21–phosphate was obtained from Sigma Chemical, St. Louis, USA; the estradiol 17–phosphate was purchased from Mann Research, New York, USA; the testosterone 17–phosphate and dehydroepiandrosterone 3–phosphate were kindly supplied by prof. K. Schubert, Inst. Mikrobiol. exper. Therapie, Jena, Germany; estradiol 3–phosphate was a generous gift of dr. DiPietro, Vanderblit University, Nashville, USA.

Results and Discussion.

Table I shows the activities of alkaline phosphatase of the kidney of various vertebrates, with steroid-phosphates used as substrates. The phosphatases hydrolyze in all cases the substrates phosphates in the positions 21 and 3. Peculiar is the behaviour of the substrates conjugated in the position 17. In fact, testosterone 17–phosphate is hydrolyzed by the alkaline phosphatase of the frog and the lizard in an appreciable rate, but in a much lower degree by the enzyme extracted from the other animals. The estradiol 17–phosphate was hydrolyzed exclusively by lizard and mouse enzyme; in the latter animal such activity is not always detectable.

Table I.

Activity of the alkaline phosphatase of the kidney with steroids phosphate as substrates (*).

Species	Hydrocortisone 21–phosphate	Testosterone 17–phosphate	Estradiol 17–phosphate	Estradiol 3–phosphate	Dehydroepiandrosterone 3–phosphate
Bovine ♂	2.22	traces (**)	0	20.00	5.37
Bovine ♀	2.25	1.06	0	9.25	3.62
Mouse ♂	30.62	traces	0	127.50	39.62
Mouse ♀	22.87	4.37	4.06	51.25	26.25
Chicken ♂	15.75	traces	0	47.75	12.00
Chicken ♀	13.50	traces	0	72.12	36.00
Lizard ♂	20.62	120.00	19.75	125.00	32.62
Lizard ♀	58.75	68.25	36.62	212.50	50.00
Frog ♂	26.00	12.50	0	80.37	28.50
Frog ♀	20.62	15.00	0	87.50	54.00

(*) Enzymic activities are expressed in mμmoles of free steroid/mg of protein for 30 minutes' incubation at 37° C. The assays contained; 2 ml of buffer Tris–HCl 0.05 M, 0.01 $MgCl_2$, pH 9.2; 5 μg of protamine sulfate; 50 μl of the substrate solution (1 mg/ml) and 0.3 ml of the enzyme solutions.

(**) By traces we mean the presence of a hydrolysis lower than 1 mμmole per mg of protein.

In the cases examined, the enzymic activity seems to be different, with the various substrates, in the two sexes and even within a single species. In the lizard, for instance, while testosterone 17–phosphate is better utilized by the phosphatase of the male, the opposite happens with the other substrates. However, the interpretation of such differences appears to be premature.

Peculiar is the behaviour of lizard alkaline phosphatase, since it repeatedly hydrolyzes the estradiol and testosterone 17–phosphates. Such conjugates are, in fact, little utilized by the alkaline phosphatases of the animal tissues studied so far (Botte and Koide, 1968 *a*, *b*; Botte and Delrio, 1968).

TABLE II.

Activity of the kidney alkaline phosphatase with p–nitrophenol phosphate as substrate.

The activity is in μmoles of PNP/mg of protein/10 minutes incubation (SIGMA method)

	SPECIES				
	bovine ♂	bovine ♀	mouse ♂	mouse ♀	chicken ♂
Enzyme activity . . .	5.00	11.48	4.00	3.30	2.05

	SPECIES				
	chicken ♀	lizard ♂	lizard ♀	frog ♂	frog ♀
Enzyme activity . . .	4.33	1.00	1.10	6.00	5.80

Table II shows that the steroid substrates are hydrolyzed at a lesser degree than *p*–nitrophenol phosphate. This finding suggests the presence in the pool of phosphatases of variants specific for steroid phosphates. It is known that the alkaline phosphatase of tissues is in form of different isoenzymes, which often have different affinity for the same substrates (see Wilkinson, 1965). An isoenzyme which is active mainly for estradiol 3–phosphate has been recently purified from the acid phosphatase of human placenta (DiPietro, 1968).

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