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KINETICS AND INHIBITION OF ALKALINE PHOSPHATASES FROM CANINE TISSUES

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SUMMARY

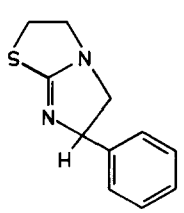
The kinetics and inhibition of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from several tissues of the dog were studied and compared with those of the *Escherichia coli* enzyme. *N*-Ethylaminoethanol proved to be the most appropriate buffer. Only small concentrations of magnesium are needed for optimal activity. There is substrate inhibition for all enzymes at concentrations above 1 mM. In the dog neither substrate specificity, nor K_m, nor the effect of various inhibitors could differentiate the enzymes from kidney, bone, liver, placenta or mammary tumor whereas the intestinal enzyme was clearly different. The bacterial enzyme differs in many ways from the canine enzymes. The anthelmintic levamisole and one of its analogues, R 8231, were found to be very potent inhibitors of alkaline phosphatase from all tissues except intestine.

INTRODUCTION

During an investigation of the biochemical effects of tetramisole*, a potent broad-spectrum anthelmintic[1], preliminary findings indicated that this drug inhibited alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from mammalian tissues. Since no other drug, except aminophylline[2], has been reported to show such inhibitory activity *in vitro* it seemed worthwhile to study this effect in more detail. This paper describes the kinetics and inhibition of alkaline phosphatases isolated from dog tissues**.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

* Tetramisole: (±)-2,3,5,6-tetrahydro-6-phenylimidazo(2,1-b)thiazole hydrochloride.



R 8231: (±)-6(*m*-bromophenyl)-5,6-dihydroimidazo(2,1-b)thiazole oxalate.

** Similar studies are in progress for the enzymes of the rat and man.

MATERIAL AND METHODS

Healthy mongrel dogs were killed by injection of succinylcholine chloride. The tissues were immediately removed, cooled on ice and stored at −21 °C.

Alkaline phosphatase activity was extracted essentially according to the procedure of Morton[3] using *n*-butanol and partially purified by acetone precipitation of the butanol extract.

Intestinal enzyme was isolated from mucosa of the proximal part of the small intestine. Tumor alkaline phosphatase originated from a mammary tumor of the mixed type. Bone alkaline phosphatase was isolated from epiphyseal centers of bones from a 2-day-old pup. Alkaline phosphatase from *Escherichia coli* was kindly supplied by Prof. H. Gutfreund (University of Bristol, U.K.).

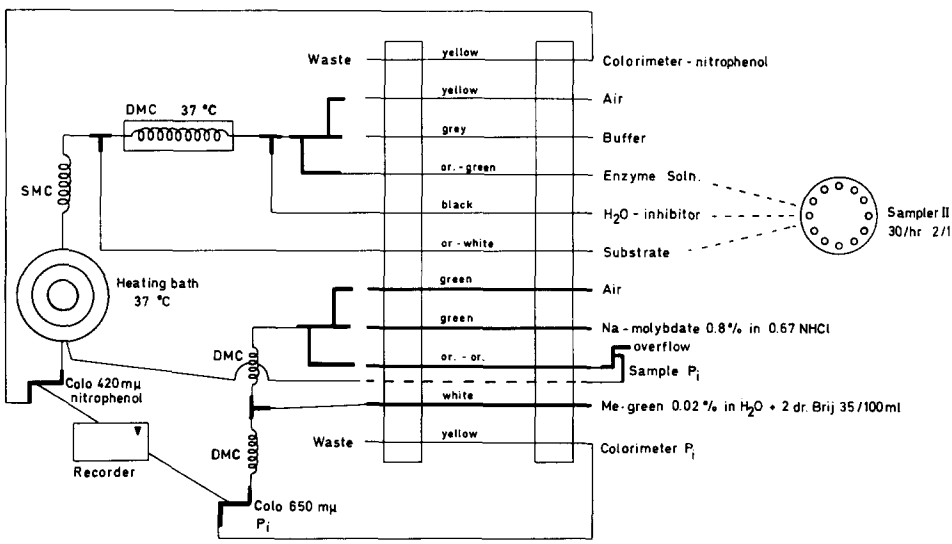


Fig. 1. Manifold for the determination of alkaline phosphatase by nitrophenol release and P_i release (thick lines).

The release of nitrophenol from nitrophenyl phosphate was measured using the Technicon AutoAnalyser and the manifold shown in Fig. 1. The sampler contained either the enzymes or the substrates or the inhibitors, depending on the variables to be measured. Preincubation of the enzymes with inhibitor took 2 min at 37 °C. The reaction time from the addition of the substrate to measurement was approx. 10 min. Using a manual technique it was established that under the conditions of the test the enzymatic reaction is proportional to the amount of enzyme added and proceeds linearly for more than 30 min.

P_i was measured without deproteinisation according to a method previously described[4] (Fig. 1, thick lines).

TABLE I

EFFECT OF BUFFER AND pH ON ALKALINE PHOSPHATASE ACTIVITIES

A, relative activity, nitrophenol release. B, relative activity, P_i release. C, ratio, release nitrophenol/release P_i. Buffers, all 50 mM + 0.5 mM Mg^{2+}; [S] = 0.5 mM.

Buffer	*pH**	*Liver*			*Kidney*			*Bone*			*Placenta*			*Tumor*			*Intestine*		
		A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Tris	9.0	21	31	1.04	24	31	1.18	21	30	1.11	25	33	1.18	22	30	1.21	41	48	1.26
N-Ethylaminoethanol	9.0	25	34	1.11	26	33	1.24	25	33	1.18	27	34	1.26	26	32	1.31	49	57	1.24
	9.3	43	53	1.26	45	52	1.34	43	52	1.32	47	54	1.35	45	52	1.42	70	76	1.33
	9.6	66	77	1.31	68	78	1.37	67	77	1.37	70	78	1.41	68	77	1.44	90	96	1.37
	9.9	94	98	1.46	95	100	1.48	95	99	1.52	97	100	1.53	95	100	1.56	100	100	1.46
	10.2	100	100	1.52	100	100	1.55	100	100	1.59	100	98	1.60	100	100	1.63	80	76	1.51
Bicarbonate	9.0	27	38	1.09	27	34	1.23	27	37	1.16	29	38	1.21	27	34	1.27	57	61	1.35
	9.3	34	47	1.11	34	44	1.22	34	46	1.18	37	49	1.21	34	45	1.26	60	64	1.35
	9.6	39	55	1.09	39	51	1.17	39	52	1.19	42	55	1.21	39	51	1.25	53	58	1.33
	9.9	38	53	1.10	37	49	1.17	38	51	1.18	39	52	1.17	37	49	1.25	39	43	1.31
Glycine	9.9	33	48	1.03	33	45	1.13	33	46	1.15	37	50	1.14	33	46	1.31	46	48	1.40
Diethanolamine	9.9	72	84	1.32	74	86	1.36	72	83	1.39	78	87	1.40	73	83	1.43	85	89	1.40

* pH measured at 22 °C.

RESULTS

Effect of buffer composition and pH

Both release of nitrophenol and release of P_i were studied in several buffering systems.

It is evident from Table I that the *N*-ethylaminoethanol buffer results in higher activities than the $NaHCO_3$–Na_2CO_3 buffer or glycine buffer. Diethanolamine although almost as effective as *N*-ethylaminoethanol is not very suitable for the measurement of nitrophenol because of its yellow color. Tris buffer ($pK_a = 8.08$) cannot be used at pH values above 9.0.

The pH optimum lies around pH 9.9 for all enzymes using *N*-ethylaminoethanol.It tends to be somewhat lower in the carbonate buffer especially for the intestinal enzyme.

There is a considerable phosphorylation (transphosphorylation) of the buffer as may be concluded from the ratio: nitrophenol released/(P_i released). The apparent phosphorylation of the carbonate buffer is due to the presence of Tris in which the enzyme was diluted.

When the enzymes are diluted in Tris buffer without Mg^{2+}, the activity rapidly decreases on storage at room temperature. Thus Mg^{2+} is needed at least for the stability of the enzymes. The concentration of Mg^{2+} for optimal activity is however fairly low especially in *N*-ethylaminoethanol buffer where maximal activity is obtained at 0.25 mM Mg^{2+} while in the bicarbonate buffer 1 mM Mg^{2+} is needed.

Substrate specificity

The release of P_i from 8 substrates at 0.5 mM in *N*-ethylaminoethanol buffer by the various enzymes reveals that phenyl phosphate is most rapidly dephosphorylated under these assay conditions (Table II). With the exception of the intestinal enzyme all other mammalian phosphatases show an almost identical substrate specificity.

TABLE II

SUBSTRATE SPECIFICITY OF ALKALINE PHOSPHATASES

Buffer, 50 mM *N*-ethylaminoethanol (pH 9.9) + 0.5 mM Mg^{2+}.

[Substrate]0.5 mM	*Intestine*	*Liver*	*Kidney*	*Bone*	*Placenta*	*Tumor*
Phenyl phosphate	100	100	100	100	100	100
Nitrophenyl phosphate	45	89	88	85	90	85
β-Glycerophosphate	25	37	33	33	32	31
AMP	37	42	37	37	39	35
ATP	19*	11	6	8	6	5
Thiaminepyrophosphate	28	33	28	30	35	26
P-Threonine	51	38	35	36	35	32
Pyrophosphate	1	10	3	4	4	2

* In the absence of Mg^{2+} the ATPase activity of the intestinal enzyme only is markedly increased and amounts to 61% of the phenylphosphatase activity under identical conditions.

Kinetics

The K_m values for the various enzymes were determined using nitrophenyl phosphate concentrations between 0.05 and 5 mM.

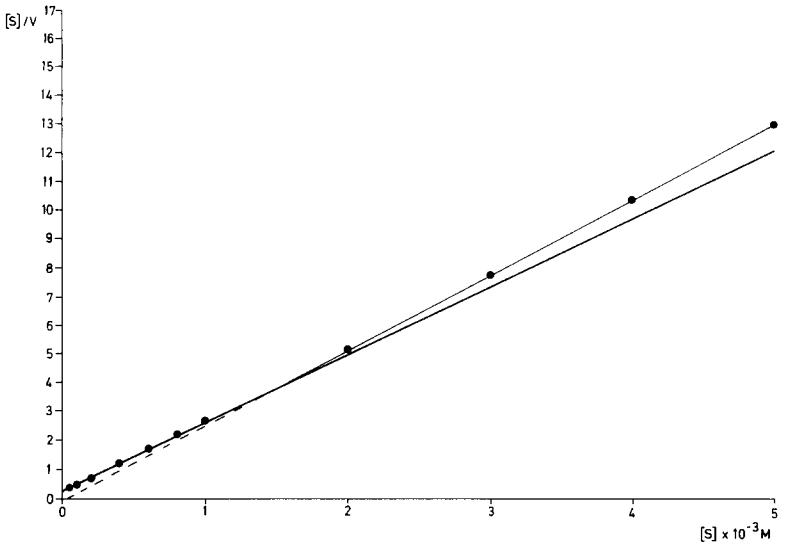


Fig. 2. $[S]/v$ *vs* $[S]$ plot for kidney alkaline phosphatase. Buffer, *N*-ethylaminoethanol (pH 9.9) + 0.5 mM Mg^{2+}. Substrate, nitrophenyl phosphate. ———, straight line through data from 0.05 to 1 mM $[S]$; ———, straight line through data from 2 to 5 mM and — — —, extrapolation to $[S]/v = 0$.

Plotting $[S]/v$ *versus* $[S]$, revealed a slight substrate inhibition at concentrations above 1 mM as is illustrated in Fig. 2 for the kidney enzyme. Consequently the K_m values were calculated from the results obtained at substrate concentrations $\leqslant$ 1 mM using the method of the least squares. The results of a typical experiment are given in Table III.

Here again there is a striking similarity between all enzymes except for the enzymes from *E. coli* and from the small intestine.

TABLE III

DETERMINATION OF K_m VALUES FOR VARIOUS ALKALINE PHOSPHATASES

Buffer, 50 mM *N*-ethylaminoethanol (pH 9.9) + 0.5 mM Mg^{2+}; substrate, nitrophenyl phosphate. ND, not determined.

$[S]$ (10^{-3} M)	$[S]/v$						
	Intestine	*Liver*	*Kidney*	*Bone*	*Placenta*	*Tumor*	*E. coli*
0.05	4.13	3.97	3.38	2.89	3.50	3.97	2.15
0.1	ND	5.15	4.52	3.77	4.85	5.46	ND
0.2	7.09	7.94	6.85	5.85	7.46	8.13	6.49
0.4	10.93	13.25	11.59	9.85	12.78	13.79	12.31
0.6	14.63	18.52	16.44	13.79	18.02	19.42	17.80
0.8	18.39	23.88	21.33	17.90	23.46	25.24	23.74
1	22.22	29.15	26.52	21.83	28.90	30.96	29.24
a	3.2636	2.5908	2.0729	1.8346	2.1343	2.5150	0.7792
b	18.9731	26.5792	24.0792	20.0119	26.6761	28.3698	28.5424
*r**	1.0	1.0	1.0	1.0	1.0	1.0	0.99994
K_m (10^{-3} M)	0.172	0.097	0.086	0.092	0.080	0.089	0.027

* *r*, correlation coefficient (Pearson).

Enzyme inhibition

Effect of common enzyme affectors. The effect of the following substances on the enzymatic activity of alkaline phosphatases from dogs and *E. coli* was studied: the SH-reagents *p*-chloromercuribenzoate (PCMB) and N-ethylmaleimide, the mercapto-compounds glutathione (GSH) and *l*-cysteine, the metal-complexants EDTA and KCN, NaF, beryllium, aminophylline, *l*-phenylalanine and finally the reaction product P_i, all at 1 mM substrate (nitrophenyl phosphate) concentration in *N*-ethylaminoethanol buffer (pH 9.9). The concentration of inhibitor which gave 50% inhibition (I_{50}) was graphically determined. The results are tabulated in Table IV.

All enzymes are rather insensitive towards the SH-reagents except for (l)-cysteine. (l)-Cystine has no effect. EDTA can be regarded as a strong inhibitor of the canine enzymes when keeping in mind that the effective Mg^{2+} concentration in the incubate is 0.540 mM. Since beryllium strongly adsorbs to glass coils, its effect was measured by a manual technique under similar conditions but at 22 °C. In contrast with aminophylline neither caffeine nor papaverine have any effect at 1 mM concentrations.

Effect of tetramisole and its analogue R 8231. The kinetics of the inhibition of canine alkaline phosphatases by levamisole (the levo-isomer of tetramisole) were studied at concentrations of nitrophenyl phosphate ranging from 0.1 to 1 mM and a fixed concentration of the inhibitor ($2 \cdot 10^{-3}$ M for the intestinal enzyme; $1 \cdot 10^{-5}$ M for all others).

Plotting $[S]/v$ *versus* $[S]$ revealed that the inhibition by levamisole is of the uncompetitive type (= coupling inhibition) for all phosphatases. The data for the kidney enzyme are illustrated in Fig. 3A. Fig. 3B illustrates a $[I]\ (1-i)/i$ *versus* $[S]$ plot of the same experimental data. In this type of plotting it is known that only the coupling inhibition results in a hyperbolic-type curve[5].

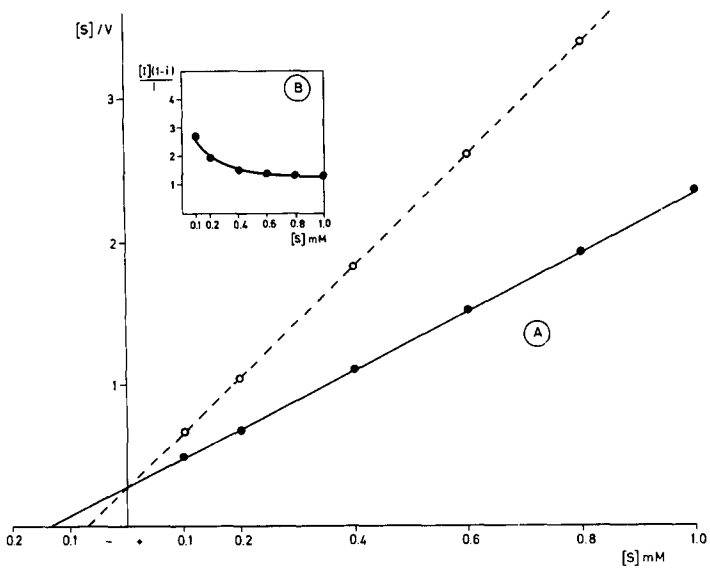


Fig. 3. (A) $[S]/v$ *vs* $[S]$ plot for kidney alkaline phosphatase in the absence (●—●) and in the presence (○---○) of 10 μM levamisole. (B) $[I](1-i)/i$ *vs* $[S]$ plot of the inhibited reaction.

TABLE IV

I_{50} VALUES (mM) OF VARIOUS INHIBITORS OF ALKALINE PHOSPHATASE

Buffer, 50 mM *N*-ethylaminoethanol (pH 9.9) + Mg^{2+} 0.5 mM; substrate: nitrophenyl phosphate, 1 mM. ND, not determined.

Inhibitor	*Intestine*	*Liver*	*Kidney*	*Bone*	*Placenta*	*Tumor*	*E. coli*
N-Ethylmaleimide (2 mM)	No effect	No effect	No effect	No effect	No effect	No effect	No effect
PCMB	1.73	2.21	1.46	1.92	2.52	1.26	> 10 mM
GSH	0.76	2.08	1.94	2.03	2.56	1.72	1.75
l-Cysteine	0.043	0.042	0.043	0.042	0.046	0.043	0.044
EDTA*	0.703	0.596	0.587	0.591	0.593	0.583	15% inhibn at 2 mM
KCN	0.73	0.81	0.64	0.65	0.64	0.62	0.63
NaF (2 mM)	No effect	No effect	No effect	No effect	No effect	No effect	No effect
Beryllium**	$0.28 \cdot 10^{-6}$ M	$0.72 \cdot 10^{-6}$ M	$0.69 \cdot 10^{-6}$ M	$0.66 \cdot 10^{-6}$ M	$0.81 \cdot 10^{-6}$ M	$0.68 \cdot 10^{-6}$ M	ND
Aminophylline	0.201	0.132	0.103	0.123	0.076	0.090	0.550
l-Phenylalanine	2.95	20.9	18.5	19.6	19.1	18.0	No effect at 20 mM
P_i	1.12	6.30	5.40	5.80	6.80	6.00	0.41

* Effective Mg^{2+} concentration in incubate = 0.540 mM.

** Effect measured by manual technique under similar conditions but at 22 °C.

TABLE V

CALCULATED K_i-VALUES FOR LEVAMISOLE AND R 8231 FOR THE VARIOUS ENZYMES

Buffer 50 mM *N*-ethylaminoethanol (pH 9.9) + 0.5 mM Mg^{2+}; [*S*] = 0.1, 0.2, 0.4, 0.6, 0.8 + 1 mM nitrophenyl phosphate; [*I*], levamisole = $1 \cdot 10^{-5}$ M (intestine, $2 \cdot 10^{-3}$ M); R 8231, $1 \cdot 10^{-6}$ M (intestine, $5 \cdot 10^{-5}$ M). In all experiments the correlation coefficient *r* (Pearson) for the calculation of the straight lines was between 0.9995 and 1.0.

	K_i (10^{-3} M)	K_i (10^{-6} M)				
	Intestine	*Liver*	*Kidney*	*Bone*	*Placenta*	*Tumor*
Levamisole	2.94	9.45	10.79	8.90	6.58	7.22
R 8231	0.68	1.12	1.06	0.92	0.57	1.08

K_i values for levamisole and R 8231 for the canine alkaline phosphatases are given in Table V. It is evident that the enzymes studied are very sensitive to levamisole and especially its analogue except for the intestinal enzyme which is inhibited only at very high concentrations. There is no effect of levamisole or its analogue on alkaline phosphatase from *E. coli*.

The dextro-isomer of tetramisole has no effect at all on the enzyme activity in any tissue at concentrations up to $2 \cdot 10^{-3}$ M.

Increasing the Mg^{2+} concentration in the incubate from 0.25 to 2 mM does not affect the inhibition by levamisole.

When using different substrates, the K_i values for levamisole are almost similar. Thus the K_i values for the kidney enzyme were: nitrophenyl phosphate: $10.33 \cdot 10^{-6}$ M ($K_m = 0.1 \cdot 10^{-3}$ M), phenyl phosphate: $8.77 \cdot 10^{-6}$ M ($K_m = 0.092 \cdot 10^{-3}$ M), AMP: $9.86 \cdot 10^{-6}$ M ($K_m = 1.47 \cdot 10^{-3}$ M), β-glycerophosphate: $8.52 \cdot 10^{-6}$ M ($K_m = 2.50 \cdot 10^{-3}$ M) and phosphothreonine: $10.60 \cdot 10^{-6}$ M ($K_m = 1.71 \cdot 10^{-3}$ M).

The inhibition by levamisole of kidney alkaline phosphatase is only slightly affected by decreasing the pH from 9.9 to 8.6 at 1 mM substrate concentration.

Both, levamisole and R 8231 can be used to distinguish intestinal from the other canine alkaline phosphatases studied as is illustrated in Fig. 4. All sensitive enzymes are inhibited to the same extent by levamisole while the placental enzyme is slightly more affected by R 8231 than the enzymes from other sources.

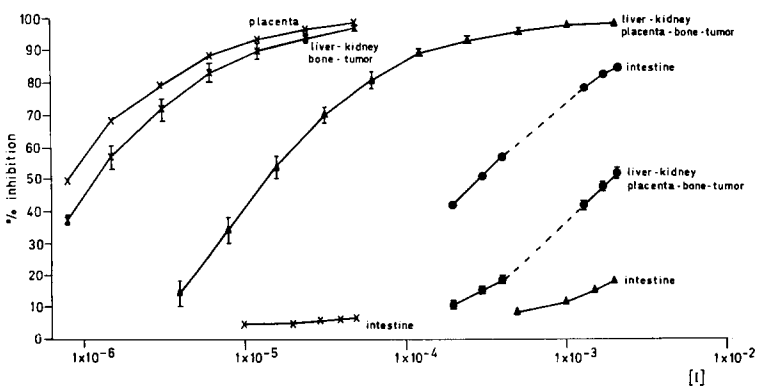


Fig. 4. Organ specificity of inhibition by *l*-phenylalanine (●—●), levamisole (▲—▲) and R 8231 (×—×). Buffer, 50 mM (pH 9.9) + 0.5 mM Mg^{2+}. [*S*] = 1 mM nitrophenyl phosphate.

DISCUSSION

For measuring nitrophenylphosphatase activities, *N*-ethylaminoethanol is the most favourable buffer not only because of its maximal buffering capacity at optimal pH ($pK_a = 9.6$) but also because the enzymatic activity is much higher than in the other buffers and especially in the usual carbonate buffer. This is in agreement with recent findings by McComb and Bowers[6] for human serum. The alkaline phosphatase activation in *N*-ethylaminoethanol and related buffers parallels the phosphorylation of these buffers (transphosphorylation) as shown by Amador[7]. The finding that transphosphorylation of *N*-ethylaminoethanol increases with increasing pH values may explain to some extent why the pH optima are lower for all enzymes in carbonate buffer than in *N*-ethylaminoethanol buffer.

There is no difference as far as substrate specificity is concerned among the canine alkaline phosphatases except the intestinal one which behaves slightly different under the conditions used. The increase in ATPase activity of the intestinal enzyme in the absence of Mg^{2+} (Table IV) confirms the findings by Moss[8] for human intestinal alkaline phosphatase.

Substrate concentrations ranging from 20 to as high as 100 mM have been reported in the literature[9]. Such high concentrations could have a profound effect on the kinetics and properties of the enzymes. A slight but definite substrate inhibition of all alkaline phosphatases was indeed observed in our experiments at rather low substrate concentrations (>1 mM). Thus it was impossible to get K_m values at substrate concentrations from 2 to 5 mM (Fig. 3). K_m values are almost identical for all enzymes ($0.080 \cdot 10^{-3}$–$0.097 \cdot 10^{-3}$ M) except for the intestinal enzyme ($0.172 \cdot 10^{-3}$ M) and the enzyme from *E. coli* ($0.027 \cdot 10^{-3}$ M).

The effect of several inhibitors on alkaline phosphatase activity from different sources is fairly complex. Thus PCMB inhibits enzyme activities by 50% at 1.5–2.5 mM (>10 mM for *E. coli*) whereas *N*-ethylmaleimide, another sulfhydryl reagent, has no effect at 2 mM. The lack of effect of *N*-ethylmaleimide may be due to the short preincubation since *N*-ethylmaleimide has been reported to react fairly slowly with some proteins[10].

l-Cysteine markedly inhibits all enzymes tested without any differentiation ($I_{50} = 4 \cdot 10^{-5}$ M). On the other hand glutathione, although less inhibitory, affects the intestinal enzyme more easily than the others ($I_{50} = 0.7 \cdot 10^{-3}$ and $2 \cdot 10^{-3}$ M, respectively).

The metal chelator EDTA inhibits enzyme activities from all sources except the phosphatase from *E. coli*. Apparently the metal in the mammalian enzymes is much more rapidly chelated than the zinc atoms in *E. coli* which are known to react slowly with EDTA[11]. There is also a pronounced inhibition of all alkaline phosphatases by KCN (I_{50} = 0.6–0.8 mM), presumably by metallo-complexing.

Intestinal alkaline phosphatase is more sensitive to product (P_i) inhibition than the other canine enzymes but less than the enzyme from *E. coli*.

A 77–79% inhibition of both, human intestinal and placental alkaline phosphatases by *l*-phenylalanine at 5 mM concentration has been reported[12]. At the same concentration the inhibition of human bone and liver enzymes is less than 10%. The canine alkaline phosphatases were much less differentiated by *l*-phenylalanine. Indeed, whereas the intestinal enzyme is the most sensitive towards *l*-

phenylalanine (but less than the human enzyme) the other enzymes are also affected to a relatively large extent at similar concentrations of inhibitor. In contrast to the human enzyme, the placental enzyme from the dog does not show the same *l*-phenylalanine sensitivity as the intestinal alkaline phosphatase. It is also remarkable that even at 20 mM *l*-phenylalanine the alkaline phosphatase activity from *E. coli* remains completely unaffected.

More attention should be paid to the inhibition of alkaline phosphatases by aminophylline which is known primarily as an inhibitor of phosphodiesterase (EC 3.1.4.1). However, alkaline phosphatases are inhibited by 50% at $1 \cdot 10^{-4}$ M concentrations whereas the K_i reported for phosphodiesterase is $2.3 \cdot 10^{-4}$ M (ref. 13). Curiously enough, papaverine, another more potent inhibitor of phosphodiesterase, has no effect on alkaline phosphatase.

Levamisole is a potent stereospecific inhibitor of canine alkaline phosphatases. The nature of the inhibition is purely uncompetitive as has been reported for *l*-phenylalanine on human intestinal alkaline phosphatase[14]. However, in contrast to *l*-phenylalanine which acts preferentially on the intestinal enzyme, levamisole has a negligible effect on this enzyme and has a pronounced effect on all other canine phosphatases studied. The way in which this compound inhibits alkaline phosphatase is unknown but it is apparently not due to metal complexing since it is independent of the Mg^{2+} concentration. Changing the pH has only a negligible effect on the inhibition. K_i values are also identical for different substrates. The inhibition by levamisole and especially its analogue R 8231 exceeds by far the inhibition by other known inhibitors except beryllium, a very specific inhibitor of alkaline phosphatase and phosphoglucomutase (EC 2.7.5.1)[15]. Beryllium and levamisole, however, do not act in a similar way since levamisole does not inhibit phosphoglucomutase and has no effect on the bacterial enzyme, whereas beryllium is equally effective on kidney alkaline phosphatase and the enzyme from *E. coli*[16].

From these experiments it can also be suggested that the alkaline phosphatases from canine liver, kidney, bone placenta and mammary mixed cell tumor, are similar enzymes. Indeed, neither kinetic studies nor the various inhibitors tested could reveal any noticeable difference although they showed different mobilities on electrophoresis on cellogel. Thus their physicochemical diversity is apparently not related to their enzymological behaviour. However, there are marked differences between these enzymes and the intestinal enzyme, as illustrated by their inhibition by levamisole.

Levamisole and its analogue R 8231 appear to be the most potent inhibitors of canine alkaline phosphatases now available. If the same applies to other mammalian species, levamisole may become an interesting tool to study the mechanism and the role of alkaline phosphatases. Both compounds may also be used in the diagnosis for intestinal alkaline phosphatase. Indeed, the latter enzyme is only marginally affected at concentrations which completely block all other alkaline phosphatases.

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