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Attempts to Find Creatine Phosphokinase and 5′-Nucleotidase Activity In Canine Prostatic Fluid.* (31056)

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A partially purified acid phosphatase from human prostate has been found to transfer phosphate from β-glycerylphosphate and phosphocreatine to carbon 6 of glucose as well as to hydrolyze organic phosphates(1). Newton and Rothschild(2) identified phosphocreatine in bull spermatozoa and Yanagisawa(3) demonstrated this phosphamide and its related transferase, creatine phosphokinase, in sea urchin sperm. No report has appeared on the examination of prostatic fluid for these substances although the absence of creatine and creatinine in canine prostatic secretion has been reported(4).

Of relative pertinence is the 5′-nucleotidase activity demonstrated in human semen(5) and purified from the seminal vesicle secretion of the bull(6). The optimal pH of this enzyme was 8.5, similar to that of alkaline phosphatase. A confusing factor is that purified acid phosphomonoesterase has some hydrolytic activity against 5′-nucleotides(7).

The overlap in phosphatase activities and lack of knowledge on creatine phosphokinase suggested an exploration of canine prostatic fluid for these enzymes. The dog with a cystopreputiostomy fistula provides a particularly suitable opportunity for such an investigation since it yields an ample supply of prostatic fluid without vesicular or testicular contamination(8). Furthermore, purified fractions of canine prostatic enzymes are available for comparison with whole prostatic fluid (9); and canine prostatic fluid is suited for studies of phosphate release through any mechanism since at the most only a trace of endogenous orthophosphate has been detected in this fluid(4).

Materials and methods. Creatine phosphokinase catalyzes the transfer of a phosphate group from phosphocreatine to adenosine diphosphate (ADP). The reaction may be followed in either direction by determining the appearance or disappearance of creatine. Neither the internal anhydride, creatinine, nor phosphocreatine give a colored product with the diacetyl reagent. However, it is possible that under acid conditions a small amount of creatine could be released from phosphocreatine by hydrolysis(10). Since the enzyme reaction was to be terminated with 10% trichloroacetic acid (TCA), an estimation of non-enzymic hydrolysis of phosphocreatine was obtained in the presence of TCA. The satisfactory application of the diacetyl procedure for determination of creatine in the presence of prostatic fluid and its components was evaluated by addition of varying amounts of exogenous creatine to prostatic fluid.

The details of the incubation system are as follows: 5 ml of a mixture containing 6 ml 0.4 M glycine-NaOH buffer with 0.1 M magnesium sulfate at pH 9.0 were mixed with 2 ml 0.005 M ATP and 100 μg creatine in 0.1 ml water(11). The enzyme source was 0-3.0 ml canine prostatic fluid or 10-60 mg of a purified canine prostatic fluid fraction. The purified fractions were the insoluble, non-dialyzable acid phosphatase (C-material); the insoluble, non-dialyzable acid phosphatase produced through a preliminary gel filtration

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(GFEP); and the proteolytic proteins not trapped by the gel beads (GFS)(9). When the enzyme reaction was run in the reverse direction, ADP was substituted for ATP and phosphocreatine for creatine.

The incubation was carried out for 1 hour at 37°C under air in 12 ml centrifuge tubes. The reaction was stopped by addition of 3 ml of 30% TCA, the samples centrifuged for 7 minutes at 3000 rpm and 3 ml aliquots of the supernatant taken for creatine analysis. The colored product was developed with the addition of 2 ml alkaline α-naphthol and 1 ml of diacetyl; and 20 minutes at room temperature was allowed for full color development (10). During the addition of alkaline α-naphthol, occasionally a precipitate formed due to the presence of magnesium in the buffer. Centrifugation successfully removed the turbidity without loss of color so that the product could be measured accurately in a spectrophotometer.

The determination of 5′-nucleotidase activity was performed in a glycine buffer of pH 8.5 fortified with magnesium(12). The substrate was 3 μmoles of AMP, or ATP and, for control purposes, β-glycerylphosphate. In an attempt to differentiate acid, alkaline and 5′-nucleotide phosphatase activity, the hydrolysis of AMP and β-glycerylphosphate was compared in barbital, glycine and tris-acetate buffer systems between pH 4.5-9.0.

The incubation system contained 0.2 ml buffer, 0.2 ml of 0.1 M magnesium chloride, 3 μmoles substrate and unfractionated prostatic fluid or 10 mg of a partially purified prostatic fluid protein fraction in a final volume of 2.4 ml. After a 60-minute incubation period at 37°C, 2.4 ml 10% TCA was added to stop the reaction. The liberation of orthophosphate was followed by the usual Fiske-SubbaRow test.

Three separate experiments in triplicate were carried out for the measurements of creatine phosphokinase and 5′-nucleotidase.

Results and discussion. The creatine-diacetyl color reaction was found to be linear and recovery of exogenous creatine was complete. Additions of phosphocreatine revealed that 10% non-enzymic hydrolysis occurred in 10% TCA. Incubation of 1-4 ml canine prostatic fluid with 100 μg creatine resulted in less than 8% disappearance of the substrate. A similar finding was obtained with 10-60 mg of dialyzed, lyophilized protein fractions from the prostatic fluid. When phosphocreatine was employed as the substrate, less than 10% dephosphorylation occurred.

Complete recovery was obtained of exogenous inorganic phosphate added to canine prostatic fluid. The results outlined in Table I are representative of 3 separate experiments and show values for the preferred barbital buffer at pH 5.0 for acid phosphatase and the glycine buffer pH 8.5 for the 5′-nucleotidase system. Results obtained with tris-acetate buffer were essentially the same. For all buffer systems there was a similar progressive decline in phosphate liberated as the pH was increased from 4.5 to 9.0.

From the data in Table I it may be observed that the barbital buffer was more efficient than glycine buffer for estimating acid phosphatase activity. Under optimal conditions at pH 5.0 AMP hydrolysis was approximately 40% that of β-glcerylphosphate. With β-glycerylphosphate as a substrate, no alkaline phosphatase (pH 8.5) activity could be demonstrated in canine prostatic fluid within the limits of error of the method. A small but reproducible increment in phosphate liberation from AMP appeared at pH 8.5 and was more noticeable in the glycine buffer. This possible 5′-nucleotidase activity fractionated with purified preparations of prostatic acid phosphatase. No activity was apparent in the proteolytic fractions.

In one experiment comparing AMP hydrolysis with that of cyclic 2′,3′-AMP, ADP and ATP it was found that no detectable phosphate was liberated from cyclic AMP and that ADP and ATP were less suitable as substrates than AMP. The hydrolysis of β-glycerylphosphate and AMP was inhibited 98% by tartrate and 95% by sodium fluoride but these inhibitors provided no differentiation between the acid phosphatase and a 5′-nucleotidase.

By and large 5′-nucleotidase activity has been associated with the secretion of the seminal vesicle(3).

Summary. An attempt was made to estab-

TABLE I. Attempts to Demonstrate 5′-Nucleotidase Activity in Canine Prostatic Fluid.

Enzyme source	Substrate	pH	PO_4 released, mg/ml Barbital buffer	Glycine buffer
Whole prostatic fluid pool (specific activity based on protein nitrogen was 2.3)	β-glycerylphosphate	5.0	1.61	1.24
	Adenosine-5′-phosphate	5.0	.65	.48
	β-glycerylphosphate	6.5	1.22	.80
	Adenosine-5′-phosphate	6.5	.36	.23
	β-glycerylphosphate	8.5	.08	.09
	Adenosine-5′-phosphate	8.5	.22	.19
Precipitate upon dialysis of whole fluid (C-material with a specific activity of 10.6)	β-glycerylphosphate	5.0	3.88	2.92
	Adenosine-5′-phosphate	5.0	1.51	1.13
	β-glycerylphosphate	8.5	.08	.10
	Adenosine-5′-phosphate	8.5	.25	.40
Gel filtration extract, precipitate upon dialysis (specific activity was 85)	β-glycerylphosphate	5.0	32.98	25.10
	Adenosine-5′-phosphate	5.0	12.75	9.67
	β-glycerylphosphate	8.5	.11	.10
	Adenosine-5′-phosphate	8.5	.22	.41
Gel filtration supernatant (proteolytic enzyme fraction)	β-glycerylphosphate	5.0	.42	.32
	Adenosine-5′-phosphate	5.0	.04	
	β-glycerylphosphate	8.5	.04	.05
	Adenosine-5′-phosphate	8.5	.05	.07

lish the presence of creatine phosphokinase and 5′-nucleotidase activities in canine prostatic secretion. Pooled prostatic fluid and purified enzyme fractions were utilized as sources of enzyme. Transferase activity was followed by phosphorylation of creatine and dephosphorylation of phosphocreatine, and 5′-nucleotidase activity by hydrolysis of adenosine-5′-phosphate. No creatine phosphokinase activity could be detected in the sources of enzyme but a small amount of 5′-nucleotidase activity may have been observed. The latter could not be distinguished from prostatic acid phosphatase by purification procedures or inhibition with tartrate and sodium fluoride.

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