Alkaline Phosphatase and 5′-Nucleotide Phosphodiesterase from Bovine Intestine Are Cross-Reactive†

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ABSTRACT: Polyclonal antibodies to native alkaline phosphatase and to native 5′-nucleotide phosphodiesterase were found to strongly cross-react with both enzymes. The antibodies also cross-react with both denatured enzymes, with glycopeptides from 5′-nucleotide phosphodiesterase, and with the oligosaccharides remaining after Pronase E digestion of the phosphodiesterase. They do not cross-react with either enzyme after their oligosaccharides have been modified or removed by periodate or trifluoromethanesulfonic acid treatment. Antibodies to denatured 5′-nucleotide phosphodiesterase do not bind to the native phosphodiesterase or alkaline phosphatase but do cross-react with denatured alkaline phosphatase even after removal or modification of the carbohydrate moieties. These results suggest that antibodies to denatured 5′-nucleotide phosphodiesterase may recognize amino acid sequence homology between alkaline phosphatase and 5′-nucleotide phosphodiesterase. However, antibodies to native enzymes apparently recognize cross-reactive determinants of the native enzymes which are carbohydrate in nature. This is the first report of antimammalian alkaline phosphatase antibodies which recognize the carbohydrate moieties of the enzyme.

Nonspecific phosphomonoesterases and phosphodiesterases are abundant in nature although their functions are unknown (McComb et al., 1979; Kelly & Butler, 1975). These enzymes may be functionally related; the products of phosphodiesterases are generally substrates for phosphomonoesterases. The enzymes may also be structurally similar. The product binding site of a phosphodiesterase must be similar to the substrate binding site of a phosphomonoesterase. Indeed, there are several reports of both phosphomonoesterase and phosphodiesterase activities present in the same protein (Yamane & Maruo, 1978; Matsuzaki & Hashimoto, 1982; Yoshida et al., 1981).

The catalytic properties of a pair of phosphohydrolases from bovine intestinal mucosa, alkaline phosphatase (APase)[1] (EC 3.1.3.1) (a phosphomonoesterase) and 5′-nucleotide phosphodiesterase (PDase) (EC 3.1.4.1), are strikingly similar (Table I). Both enzymes are membrane-bound, glycoprotein Zn^{2+} metalloenzymes with alkaline pH optima (Culp, 1983). The catalytic mechanism of both enzymes involves a covalent phosphoryl enzyme intermediate (Engstrom, 1961; Landt & Butler, 1978). Our results demonstrate that bovine intestinal APase and PDase are structurally related in both their protein and their carbohydrate components. The occurrence of antibodies to carbohydrate moieties of these enzymes suggests that conclusions about relationships of the protein moieties of APase isozymes on the basis of antigenic cross-reactivity (McKenna et al., 1979; Firestone & Health, 1981; Badger & Sussman, 1976; Ghosh & Ustegui-Gomez, 1969; Hiwada & Wachsmuth, 1974; Gogolin et al., 1981) must be interpreted cautiously.

Experimental Procedures

Enzyme Purification, Characterization, and Modification. APase and PDase were purified from fresh bovine intestines by the procedure of Landt & Butler (1978). The enzyme activities and protein concentrations were measured as described previously (Culp et al., 1985c).

Table I: Comparison of Properties[a]

characteristic	5′-nucleotide phosphodiesterase	alkaline phosphatase
phosphohydrolase	diesterase	monoesterase
pH optimum	>9.0	>9.0
covalent intermediates	+	+
ferrate inactivation	+	+
theophylline inhibition	+	+
imidazole inhibition	+	+
inactivation by EDTA	+	+
activity-restoring metals	Zn^{2+}, Co^{2+}	Zn^{2+}, Co^{2+}
location	plasma membrane	plasma membrane
glycoprotein	+	+
sialic acid	–	–
isoelectric point	4.5	4.4
resistant to proteases	+	+
resistant of glycosidases	+	+
molecular weight	107 000	140 000
subunit structure	dimer	dimer

[a] From Culp (1983).

SDS–polyacrylamide gel electrophoresis (12% acrylamide gel) was performed according to the procedure of Laemmli (1970). Protein was detected with a silver stain (Bio-Rad). Carbohydrate was detected by using the periodate–fuchsin stain (Fairbanks et al., 1971). The concentration of protein in antibody preparations was determined by the method of Lowry et al. (1951).

PDase was reduced, alkylated, and cleaved with cyanogen bromide as described previously (Culp et al., 1985b). Peptides were chromatographed on Sephadex G-75, and peak fractions were pooled (Culp, 1983).

PDase was labeled with [^{14}C]formaldehyde by using a modification of the procedure of Jentoft & Dearborn (1979). Trichloroacetic acid precipitated 97% of the total radioactivity in the isolated [^{14}C]PDase, indicating that essentially all the

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[1] Abbreviations: TFMS, trifluoromethanesulfonic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; APase, alkaline phosphatase; PDase, 5′-nucleotide phosphodiesterase; anti-PDase (native), antibodies to native PDase; anti-APase (native), antibodies to native APase; anti-PD (denatured), antibodies to denatured PDase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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label was covalently bound to the protein. All the detectable radioactivity on SDS–polyacrylamide gel electrophoresis of the labeled enzyme was located in the protein-staining band migrating identically with PDase (Culp, 1983). Antibodies to PDase did not distinguish between labeled and unlabeled enzyme (Culp, 1983).

PDase (4 mg) in 0.4 mL of 0.1 M Tris-HCl, pH 8.0, was digested with Pronase E (*Streptomyces griseus*, Sigma, 4 units/mg) added to a final ratio of 1% (w/w PDase). Toluene [1% (v/w)] was added as a preservative, and the solution was stirred at 37 °C. At 24-h intervals, additional Pronase [0.5% (w/w) PDase] was added, and the pH was readjusted to 8. After 96 h, the mixtures were stored at −20 °C. Pronase-treated PDase had no activity remaining and was used without further treatment in competition studies with a second antibody. Peptides were not detected on SDS–polyacrylamide gel electrophoresis, indicating complete digestion of PDase had occurred.

PDase and APase (5 mg of each) were reacted with 0.05 M sodium periodate in 0.5 mL of 0.1 M sodium acetate, pH 5.5. Sodium borohydride in a 0.1 N NaOH solution was added to reaction mixtures in a 10-fold molar excess over periodate. The reaction was stopped by the addition of 1 N acetic acid until all the hydrogen gas had been evolved. The mixtures, including some precipitated material, were dialyzed against water and lyophilized.

APase and PDase were treated with TFMS to chemically remove their carbohydrate moieties according to the procedure of Edge et al. (1981).

Immunodiffusion. Ouchterlony double-diffusion analysis (Ouchterlony, 1949) was performed in a 0.6% agarose matrix buffered with 11 mM sodium phosphate, pH 7.4. Pictures were taken against a black background with a Polaroid Land camera and Kodak type 55 film using illumination from underneath to make white precipitin bands visible.

Amino Acid and Carbohydrate Analysis. Proteins were hydrolyzed as described previously (Culp et al., 1985b). All analyses were performed on a Durrum D-500 amino acid analyzer in the laboratory of Dr. Michael Laskowski, Jr., Chemistry Department, Purdue University.

Neutral and amino sugars were analyzed by gas chromatography of the alditol acetates (Mehansho & Carlson, 1983) in the lab of Dr. Don Carlson, Biochemistry Department, Purdue University. Amino sugars were also detected by amino acid analysis as described above.

Production of Antibodies. Homogeneous APase and PDase (each migrated as a single band on SDS–polyacrylamide gel electrophoresis stained with Coomassie blue and contained no grater than 0.1% contaminating activity) were used to inject New Zealand White rabbits (Culp, 1983). To generate antibodies to denatured enzyme, 0.25 mg of the same PDase preparation used to make antibodies to native PDase was diluted to 1 mL with 10 mM sodium phosphate, pH 7.4, and 0.9% NaCl and denatured by heating in a boiling water bath for 10 min. No enzyme activity was detected after this treatment although the denatured enzyme remained soluble. The denatured enzyme was injected as described elsewhere (Culp, 1983).

Nonimmune control serum was obtained from rabbits bled before injection began. Antibodies were partially purified from serum by precipitation with 40% ammonium sulfate (Andrews et al., 1982).

Antibody Assays. In competition assays, the amounts of immunogen and antibody were adjusted to give approximately 70% precipitation in the absence of competitor. Competing substances were added to the antibodies (7 μg of anti-APase, 20 μg of anti-PDase) to a final volume of 250 μL in 100 mM Tris-HCl, pH 8.0, and 10 μM $ZnCl_2$ containing 4% bovine serum albumin. Samples were kept at 4 °C. Immunogen (1 μg) was added the next day, and a second antibody was added the third day. On the fourth day, samples were centrifuged, and supernatant aliquots were assayed for enzyme activity or radioactivity by using liquid scintillation counting.

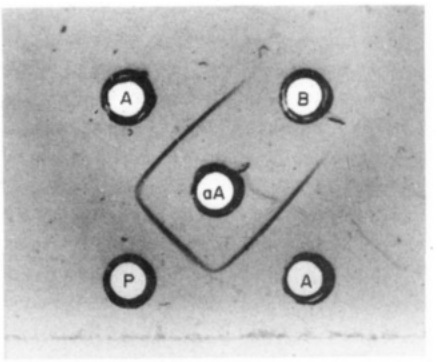

FIGURE 1: Immunodiffusion of anti-APase (native) antibodies with PDase and APase. Immunodiffusion was carried out for 24 h at room temperature as described under Experimental Procedures. (P) 20 μg of PDase; (A) 5 μg of APase; (aA) 125 μg of anti-APase (native); (B) 100 mM Tris-HCl and 10 μM $ZnCl_2$, pH 8.0.

The percent enzyme activity precipitated was calculated as the difference in supernatant activities between nonimmune serum and anti-enzyme antibodies divided by the difference in supernatant activities, in the absence of competition, between nonimmune serum and anti-enzyme antibodies. In this way, sample without competitor, in which enzyme activity was approximately 70% precipitated, were normalized to 100%. Values for control samples containing nonimmune serum were constant.

Antibody Reaction with Enzymes after Carbohydrate Modification. Approximately 0.1 mg of lyophilized sample was dissolved in 10 μL of SDS–polyacrylamide gel sample buffer (Laemmli, 1970), denatured by boiling for 1 min, and spotted onto nitrocellulose (0.45-μM, Schleicher & Schuell) strips (1 × 10 cm). The strips were air-dried and washed with distilled water. One strip was stained for protein with amido black (Schaffner & Weissmann, 1973). The remaining strips were placed in 3 mL of Tris–saline buffer (10 mM Tris-HCl and 0.15 M NaCl, pH 7.4) containing 4% bovine serum albumin, and the solution was rotated on a Lab Quake for 1 h at room temperature. Antibody (30 μL, undiluted) was added, and the solutions were rotated as before for 3 h. Strips were washed with 40 mL each of distilled water, 2% Triton X-100 in Tris–saline buffer, and Tris–saline buffer alone. Goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (Sigma) were diluted 1:200 into Tris–saline buffer containing 4% bovine serum albumin, added to each strip, and rotated in the dark for 4 h. After being washed as before but in the dark, strips were placed on a transilluminator, and pictures were taken with a Polaroid Land camera and Kodak type 55 film.

RESULTS

Immunodiffusion. After 24 h in Ouchterlony double-diffusion experiments (Ouchterlony, 1949), the reaction of anti-APase (native) with APase and with PDase showed an immunological line of identity (Figure 1). The same held true for the reaction of anti-PDase (native) with PDase and with APase (Figure 2). Tests were run by using many different dilutions of antibody and antigen with the same result: only one precipitin line was formed. A criss-cross spur pattern was

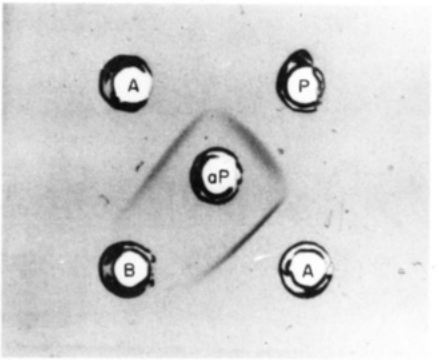

FIGURE 2: Immunodiffusion of anti-PDase (native) antibodies with PDase and APase. Immunodiffusion was carried out for 24 h at room temperature as described under Experimental Procedures. (P) 5 μg of PDase; (A) 20 μg of APase; (aP) 100 μg of anti-PDase (native); (B) Tris buffer.

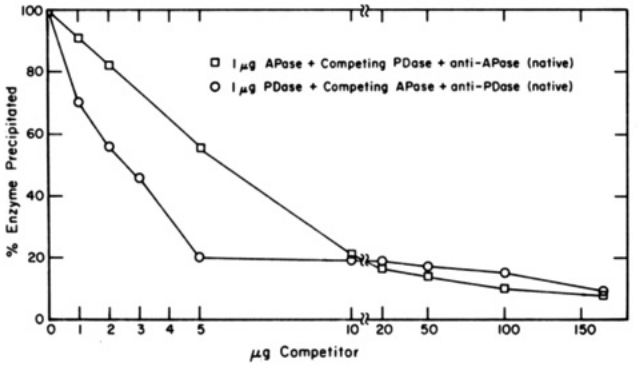

FIGURE 3: Competition between APase and PDase for anti-PDase (native) and anti-APase (native) antibodies. Competition experiments were conducted as described under Experimental Procedures except all incubations were for 1 h at 37 °C. (□) APase activity precipitated with DPase competing; (○) PDase activity precipitated with APase competing.

not detected, consistent with the homogeneity of the immunogens (Crowle, 1973).

Competition Experiments. Cross-reacting enzyme was preincubated with antibody before immunogen was added in order to optimize the interaction between competitor and antibody resulting in precipitation. APase strongly competed with PDase for anti-PDase (native), and PDase strongly competed with APase for anti-APase (native) (Figure 3).

Titration of Denatured [^{14}C]PDase with Antibodies. Denatured [^{14}C]PDase, which remained soluble, was titrated as described under Experimental Procedures except the competitor was omitted and the amounts of antibody and second antibody were varied while maintaining a constant ratio (Figure 4). Antibodies to native enzymes and antibodies to denatured PDase precipitated PDase after its conformational determinants were destroyed by denaturation. However, anti-PDase (denatured) did not precipitate native PDase or native APase, even with a second antibody present, suggesting that determinants in denatured PDase are not accessible to antibody when the enzyme is in the native conformation (Culp, 1983).

Competition Experiments with PDase Cyanogen Bromide Peptides. PDase CNBr peptides, resolved by chromatography on Sephadex G-75 (Culp, 1983), were reacted with anti-PDase (native) (Table II) in the presence of APase or PDase. The larger carbohydrate-containing peptides and the fraction containing the active-site peptide competed with native enzymes for anti-PDase (native) The smallest peptides, which did not contain carbohydrate, did not compete at detectable levels. These results indicated that cross-reacting antigenic

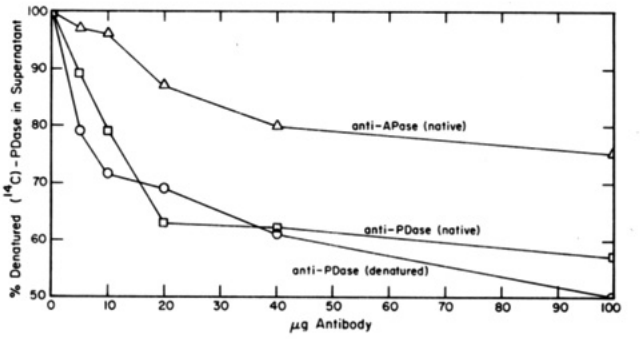

FIGURE 4: Precipitation of denatured [^{14}C]PDase with anti-PDase (native), anti-APase (native), and anti-PDase (denatured) antibodies. Precipitation was conducted without competitor, supernatant was mixed with scintillation fluid, and the radioactivity was quantitated as described under Experimental Procedures. (△) Anti-APase (native); (□) anti-PDase (native); (○) anti-PDase (denatured).

Table II: Antibody Reactivity and Carbohydrate Analysis of Pooled Fractions from Gel Filtration of PDase CNBr Peptides[a]

peak	% APase pptd	% PDase pptd	mol of GlcNAc/mol of Asx	antibody binding
I	41	68	0.94	+
II	19	70	0.79	+
III	40	60	0.71	+
IV	58	79	0.26	+
V	92	97	0.18	–
VI	100	100	0.00	–
VII	100	100	0.00	–

[a] PDase CNBr peptides, eluting from Sephadex G-75 in several peaks (Culp, 1983), were lyophilized. Competition was conducted as described under Experimental Procedures using 100 μg of competing peptide dissolved in 0.1% TFA and 40 μL of anti-PDase (native) per sample. Control samples contained nonimmune serum and TFA without peptide. *N*-Acetylglucosamine and Asx were determined by amino acid analysis. Antibody binding to immobilized antigen was detected as described under Experimental Procedures using anti-PDase (native) or anti-APase (native) (same results for both).

determinants were preserved in large carbohydrate-containing peptides of PDase. PDase CNBr peptides competed with APase more efficiently than with PDase (Table II).

Pronase Digest. To destroy all possible antigenic determinants except for carbohydrate determinants (Momoi et al., 1980), PDase was treated exhaustively with Pronase E (*Streptomyces griseus*). Pronase-treated PDase (100 μg) inhibited precipitation of 0.5 μg of native PDase by anti-PDase (native) by 41%. The same amount of Pronase-treated PDase inhibited by 55% the precipitation of native PDase by anti-APase (native). These results indicate that carbohydrate determinants on PDase are recognized by anti-PDase (native) and anti-APase (native).

Antibody Reaction with Enzymes after Modification of Carbohydrate. The terminal residues of typical complex carbohydrate chains, *N*-acetylglucosamine and galactose, are likely to be part of any carbohydrate antigenic determinant. In an attempt to remove carbohydrate residues, both enzymes were treated with a variety of glycosidases, including endo-β-*N*-acetylglycosaminidases D and H, β-glycosidases from almond emulsin, peptide:*N*-glycosidase, and mixed glycosidases from *T. cornatus*. The glycosidases were used in various combinations under conditions recommended by the manufacturer and the literature. None of these treatments had any effect on the mobility of either enzyme in polyacrylamide gel electrophoresis or in the carbohydrate staining of the resulting bands.

Because some of the carbohydrates contain vicinal hydroxyl groups, periodate treatment may destroy any carbohydrate

Table III: Carbohydrate Analysis of Native and Periodate-Treated 5′-Nucleotide PDase and APase

	native APase[a]	periodate-treated APase[a]	native PDase[b]	periodate-treated PDase[b]
fucose	4.8	1.2	26	3.2
mannose	4.0	1.8	26	15
galactose	7.9	1.3	41	16
N-acetylglucosamine	12	2.8	58	42

[a] Results are expressed as moles of carbohydrate residue per mole of APase monomer [63 000 g/mol (Fosset et al., 1974)]. [b] Results are expressed as moles of carbohydrate residue per mole of PDase protein [85 320 g/mol (Kelly et al., 1975)].

Table IV: Antibody Reaction with Periodate- and TFMS-Treated Enzymes[a]

	denatured APase	periodate-treated APase	TFMS-treated APase	denatured PDase	periodate-treated PDase	TFMS-treated PDase
anti-APase (native)	+	+	+	+	–	–
anti-PDase (native)	+	–	–	+	–	–
anti-PDase (denatured)	+	+	+	+	+	+

[a] Negative reaction was obtained with nonimmune serum.

antigenic determinants. Carbohydrate analysis indicated that only 25% of the carbohydrate residues of APase and 50% of the PDase residues survived the periodate treatment (Table III). On SDS–polyacrylamide gel electrophoresis, both periodate-treated samples had the same average mobility as the native enzymes, and there was no evidence of polypeptide bond cleavage. The levels of all amino acids analyzed (cysteine and tryptophan were not analyzed) remained constant except that the levels of tyrosine and lysine showed small decreases in periodate-treated samples.

Periodate treatment abolished all detectable reaction of PDase with antiserum to native PDase and APase (Table IV). Antibodies to denatured PDase still reacted with determinants in both enzymes modified by periodate treatment.

Trifluoromethanesulfonic acid (TFMS) was used to remove all the sugar residues of complex carbohydrate chains under conditions successfully employed for other glycoproteins (Edge et al., 1981). Amino acid analysis demonstrated that all the *N*-acetylglucosamine was removed from TFMS–PDase and Sephadex-purified TFMS–APase. Significant amino acid destruction for either enzyme was not detected, but significant polypeptide cleavage on TFMS treatment of APase was detected by using silver-stained SDS-polyacrylamide gel electrophoresis (Culp, 1983). The major APase band was the least mobile and had an apparent molecular weight 12% lower than the native enzyme. This difference was similar to the weight of the total carbohydrate of APase (9%), suggesting that the major and possibly only effect of TFMS on this fraction of APase was to remove the carbohydrate. This highest molecular weight form was further purified on a Sephadex G-75 column (2.5 × 100 cm) equilibrated with 9% formic acid and was used for subsequent analysis. The major PDase band, which had the lowest mobility, had an apparent molecular weight 15% lower than that of the native enzyme. Carbohydrate accounts for approximately 20% of the weight of PDase, however, comparison of these values is difficult due to the anomalous behavior of glycoproteins on SDS–polyacrylamide gel electrophoresis (Leach et al., 1980).

APase and PDase depleted of carbohydrate reacted with antibodies exactly as the periodate-treated enzymes (Table IV). Some of the antibodies to these native enzymes apparently are directed against cross-reactive carbohydrate determinants whereas antibodies against denatured PDase appear to recognize similar amino acid sequence determinant(s) present in both enzymes.

Discussion

Antibodies to denatured enzymes are particularly useful because they may detect amino acid sequence buried in the highly conserved protein interior (Gabay et al., 1983) that antibodies to native enzymes cannot detect (Zakin et al., 1978; Schlesinger, 1965, 1967). Antibodies to denatured PDase have been used to detect possible internal amino acid sequence similarities between PDase and APase. Anti-PDase (denatured) is directed against internal amino acid sequence determinants exposed upon denaturation. This is demonstrated by the binding of anti-PDase (denatured) to denatured PDase and denatured APase (Table IV) and the absence of interaction between anti-PDase (denatured) and the native enzymes (Culp, 1983). The cross-reaction of anti-PDase (denatured) with denatured APase is strong evidence for amino acid sequence similarities between PDase and APase.

The active sites of PDase and APase may be similar in amino acid sequence because of the catalytic similarities of these enzymes; the product binding site of PDase must be similar to the substrate binding site of APase. However, no significant amino acid sequence similarities between the active-site peptides of PDase (Culp et al., 1985b) and APase (Culp et al., 1985c) were detected by the computer method of Argos et al. (1981). Therefore, the site(s) of amino acid sequence similarity between PDase and APase detected by cross-reactive anti-PDase (denatured) must be in a region other than that of the active-site residue.

Elucidation of the immunological determinant(s) of a native glycoenzyme is complicated by the presence of the potentially antigenic carbohydrate moiety. Any cross-reactivity between APase and PDase could be due to carbohydrate. In fact, antibodies to APase and PDase appear to cross-react with carbohydrate structures on these enzymes because (a) a portion of antibodies to native enzymes cross-reacts with the denatured enzymes (Table IV) (conformational determinants are destroyed), (b) periodate and TFMS treatment destroyed cross-reactive determinants on denatured enzymes (Table IV), (c) anti-APase recognizes Pronase E digested PDase in which only oligosaccharides remain intact, (d) anti-APase cross-reacts with only those CNBr peptide fractions which contain glycopeptides, and (e) although antibodies to native enzymes precipitated their respective in vitro translated products, cross-reactivity was not detected with translated products in the absence of posttranslational modification (Culp et al., 1985a).

Previous reports in which the carbohydrate determinants of APases were investigated suggest that at least some mammalian APase antibodies were directed against protein determinants (McKenna et al., 1979; Firestone & Heath, 1981; Badger & Sussman, 1976; Ghosh & Ustegui-Gomez, 1969;

Hiwada & Wachsmuth, 1974; Gogolin et al., 1981). Gogolin et al. (1982) suggest, however, that monoclonal antibodies to human placental APase may react with both carbohydrate and protein determinants although direct evidence was not presented. Here, we report anti-mammalian APase antibodies which recognize the carbohydrate moiety of the enzyme. Because of this evidence, previous evidence purporting to demonstrate differences among the protein moieties of APase isoenzymes may have to be reexamined.

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Registry No. APase, 9001-78-9; PDase, 9025-82-5.

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