useful for distinguishing α- from ω-exonuclease are 2,4-dinitrophenyl esters of 3′- and 5′-phosphates of thymidine (*80*). A comprehensive review (*81*) on histochemical aspects has been published.

V. Other Venom Enzymes That Hydrolyze Phosphate Esters

Two monophosphatases of venom are known (*31*). Both have pH optima of about 9 and thus represent dangerous contaminants when exonuclease is used to identify a nucleoside among a large number of mononucleotides.

One of the monophosphatases, 5′-nucleotidase, is quite specific and attacks only 5′-*mono*nucleotides. This enzyme has been used to determine the amount of mononucleotides in a mixture of 5′-monophosphates of various chain length. It was also used to distinguish between 3′- and 5′-mononucleotides in a mixture of both.

Venom endonuclease is 3′-monoester former with a preference for the Gp–G bond and an optimal pH of 5 (*12*). It represents the most dangerous contaminant of exonuclease in experiments designed for the identification of terminals in long chains bearing 3′-monophosphates because it leads to false terminals.

More details concerning the properties of these contaminating enzymes may be found in reviews (*17–21*) and books (*22–24*) devoted to nucleases.

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Spleen Acid Exonuclease

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I. Introduction

Spleen acid exonuclease is an enzyme particularly useful in sequence studies of oligonucleotides, derived from both ribonucleic acid and deoxyribonucleic acid, since it splits off, in a sequential way, nucleoside-3′-phosphates starting from the 5′OH end.

The enzyme has also been called spleen phosphodiesterase (*1*, *2*) and phosphodiesterase II (*3*). We prefer to use the term *phosphodiesterase* as a general name for the broad group of enzymes hydrolyzing phosphodiester bonds whether between nucleosides or not (*4*). Table I gives a few examples of such enzymes. The term *phosphodiesterase II* (*3*), intended to mean an enzyme releasing nucleoside-3′-phosphates, seems to be an unhappy one, like that of deoxyribonuclease II (*5*) from

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5. G. Bernardi, "The Enzymes," 3rd ed., Vol. IV, p. 271, 1971.

which it is derived. In fact, to mention just one of several criticisms which can be raised, roman numerals are more and more used to indicate new enzymes both of bacterial (*6*) and animal (*7*) origin and have little bearing on the position of phosphate in the products of digestion. An alternative, shorter, nomenclature for the enzyme is *spleen exonuclease*.

After the initial and fundamental work of Heppel and Hilmoe, and Razzell and Khorana, already reviewed in the previous edition of "The Enzymes" (*8*) and in two other articles (*3, 9*), the major advances have been the preparation of spleen exonuclease in a very highly purified form (*10, 11*), and the recognition that the enzyme has no endonucleolytic activity and that it can attack oligonucleotides carrying a terminal phosphate in the 5′ position (*12*); this represents, however, a strong rate-limiting step.

II. Isolation, Purity, and Physical Properties

A method for the partial purification of spleen exonuclease was described by Heppel and Hilmoe in 1955 (*13*) and by Hilmoe in 1960 (*14*); this was later improved by Razzell and Khorana (*15*) and Richardson and Kornberg (*16*). In 1966, we described a novel purification procedure (*10*) leading to an enzyme preparation with a specific activity comparable to that of the best preparation of Razzell and Khorana (*15*). Enzyme yields were, however, low; the method was therefore modified and satisfactory results were obtained (*11*). The new method involves the preparation of a crude enzyme obtained essentially as in the case of acid deoxyribonuclease (*5, 17*). The main differences are that acidification to pH 2.5 is avoided and $(NH_4)_2SO_4$ fractionation is done between 35 and 60% saturation. The crude enzyme is then purified by chromatography on CM-Sephadex, hydroxyapatite, Sephadex G-75, and rechromatography on hydroxyapatite. The final product may be freeze-dried with only a small loss in activity. The enzyme is usually stored as a frozen solution or in 50% glycerol at −15°.

The enzyme obtained by this purification procedure (*11*), when tested for contaminants under very stringent conditions, was found to be completely free from phosphatase, DNase, ribonuclease, and adenosine deaminase activities.

The sedimentation coefficient of spleen exonuclease, measured by centrifugation in a sucrose density gradient, using cytochrome c as the reference protein, was 4.6 S (*11*). The enzyme is eluted from Sephadex G-100 between acid phosphomonoesterase ($s = 5.6$ S) and acid DNase ($s = 3.4$ S).

The thermal inactivation curve of the enzyme in 0.15 *M* acetate buffer–0.01 *M* ethylenediaminetetraacetate (EDTA), pH 5.0, showed that a 50% inactivation was obtained by heating for 20 min at 56° (*11*).

III. Catalytic Properties

A. Activity on Natural Substrates

1. *Mechanism of Action*

Spleen exonuclease is active on the 5′-OH oligonucleotides of both the ribo and the deoxyribo series. These are sequentially split from the 5′-OH end with formation of 3′-mononucleotides. It has been suggested that an enzyme–product intermediate may exist in the form of nucleoside-3′-phosphoryl–enzyme complex (*3*) since transfer of nucleoside-3′-phosphate to available 5′-hydroxyl functions (or other alcoholic functions) occurs at high substrate (or acceptor) concentrations (*15, 18*).

Bernardi and Cantoni (*12*) have investigated in detail the mechanism of action of spleen exonuclease on tRNA. They showed that at pH 4.8 the enzyme is practically unable to digest phosphorylated tRNA, whereas it can digest dephosphorylated tRNA perfectly well. In contrast, at pH 6.2 the enzyme attacks phosphorylated tRNA though at a slower rate than dephosphorylated tRNA. Since no inorganic phosphate is liberated in the degradation of phosphorylated tRNA, it is likely that in this case the enzyme begins its attack by releasing the terminal nucleotide as a diphosphate. Following the release of labeled serine at the opposite end of

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17. G. Bernardi, A. Bernardi, and A. Chersi, *BBA* **129,** 1 (1966).
18. L. A. Heppel and P. R. Whitfield, *BJ* **60,** 1, (1955).

the initial enzyme attack permitted to see that exonuclease degrades tRNA molecules "jumping" from one substrate molecule to another; in fact practically no serine is liberated up to 50% digestion, indicating that the enzyme digestion is progressing at the same rate in all molecules. In the case of phosphorylated tRNA, clearly the splitting of the terminal nucleotide represents a strong rate-limiting step since, once this obstacle is overcome, the resulting dephosphorylated substrate molecules are rapidly digested to the end and therefore the liberation of labeled serine takes place early in the digestion course.

The mechanism of action of spleen exonuclease is similar to that seen for venom exonuclease (*19–21*) but different from the processive type of attack exhibited by *E. coli* RNase II, sheep kidney exonuclease, and polynucleotide phosphorylase (*22, 23*), in which cases each polynucleotide molecule is completely degraded before the enzymes attack a new molecule. The results of Bernardi and Cantoni (*12*) contradict the previous beliefs that the enzyme has an intrinsic, though weak, endonucleolytic activity (*8*) and that a phosphate group in a terminal 5′ position makes a polynucleotide chain completely resistant to the enzyme (*15, 24, 25*).

2. *Effect of Secondary Structure*

The enzyme is very sensitive to the secondary structure of the substrate. Native calf thymus DNA is quite resistant to enzymic attack by spleen exonuclease, being split at less than 4% the rate at which alkali-denatured DNA is split (*11*). Long deoxyribonucleotides (average chain length 20–50), which still have complementary double-stranded structure, are rather resistant to the enzyme (*26*). Some limited results obtained with synthetic polyribonucleotides (*11*) are rather puzzling since poly C was found to be completely resistant, whereas poly A, poly I, and poly U were degraded at comparable rates. In the solvent used (0.15 *M* acetate buffer–0.01 *M* EDTA, pH 5.0), poly A and poly C are believed to have

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a similar double-helical structure with co-parallel strands, whereas poly U is in a disordered configuration (*27*).

3. *Effect of Glucosylation and Other Chemical Modifications*

Glucosylated oligonucleotides obtained from T4 phage DNA by acid DNase digestion are resistant to spleen exonuclease (*28*). It has been reported that acetylation of the 2′-OH groups of tRNA completely inhibits the action of the enzyme, whereas venom exonuclease is not affected (*29*). The naturally occurring methylation of sugars and bases in tRNA does not seem to hinder the action of spleen exonuclease.

B. Activity on Artificial Substrates

Other substrates for spleen exonuclease are the *p*-nitrophenyl esters of nucleoside-3′-phosphates and bis(*p*-nitrophenyl) phosphate, which is split only very slowly. These substrates are also split by enzymes having quite different natural substrates (Table I) (*30–37*). In fact, not only phosphodiesterases, in a broad sense, such as acid DNase, micrococcal nuclease, spleen and venom exonucleases, and cyclic phosphodiesterase but also enzymes such as nucleoside phosphoacyl hydrolase and nucleoside polyphosphatase split these substrates. As pointed out by Spahr and Gesteland (*36*), this may be explained by the fact that these substrates are not true diesters but rather mixed phosphoanhydrides because of the acidic character of the phenolic OH. It is evident that the use of the synthetic substrates, advocated by Razzell (*3*) as specific substrates for exonucleases, may be very misleading. Table II shows the distinctive characters of three spleen enzymes active on bis(*p*-nitrophenyl) phosphate which are present in the crude extracts from which acid exonuclease is prepared.

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TABLE I
SOME ENZYMES ACTIVE ON *p*-NITROPHENYL PHOSPHODIESTERS

Enzyme	Ref.	Natural substrate	Bis(*p*-nitrophenyl) phosphate	*p*-Nitrophenyl derivatives of 3′-Nucleotides	*p*-Nitrophenyl derivatives of 5′-Nucleotides
Acid DNase	(*30*)	DNA	+	+	−
Micrococcal nuclease	(*31*)	DNA, RNA		+	+[a]
Spleen exonuclease	(*15*)	5′-OH oligonucleotides	+	+	−
Snake venom exonuclease	(*32*)	3′-OH oligonucleotides	+	−	+
Cyclic phosphodiesterase	(*33*)	Nucleoside-2′, 3′-cyclic phosphates	+		
Nucleoside polyphosphatase	(*34*, *35*)	ATP, ADP	+	+	−
Nucleoside phosphoacyl hydrolase	(*36*)	Aminoacyl adenylate	Competitive inhibition		+
Pancreatic phosphodiesterase	(*37*)	?[b]	+		+

[a] Split with liberation of *p*-nitrophenyl phosphate.
[b] Nucleic acids and oligonucleotides are resistant.

It should be pointed out that the successful purification of spleen exonuclease (*11*) was greatly helped by use of a DNA hydrolyzate produced by spleen acid DNase as the substrate, since the synthetic substrates are nonspecific, and RNA "core" (the water-undialyzable ribooligonucleotides obtained by exhaustive digestion of RNA with pancreatic RNase) is also hydrolyzed by both acid and basic spleen ribonucleases (*38*, *39*). Spleen exonuclease is unable to hydrolyze cyclic phosphates (*14*).

C. pH–Activity Curves—Activators and Inhibitors

Using an acid DNase hydrolyzate as the substrate and 0.1 *M* succinate and phosphate buffers as the solvents, the pH optimum was found to be close to 5.5; a higher value, between pH 6 and 7, was found in 0.1 *M* acetate. The addition of 0.02 *M* Mg^{2+} did not affect very sensibly the pH–activity curves, although a shift to lower values could be detected. These

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TABLE II
PROPERTIES OF SPLENIC ENZYMES ACTIVE ON BIS(*p*-NITROPHENYL) PHOSPHATE[a]

Properties	Acid DNase	Exonuclease	Nucleoside polyphosphatase
1. Sedimentation coefficient[b]	3.4	4.6	3.2
2. pH optimum[c]	5.6–5.9	5.8	6.8
3. Substrates			
Bis(*p*-nitrophenyl) phosphate	+	+	+
p-Nitrophenyl esters of			
thymidine-5′-phosphate	−	−	−
thymidine-3′-phosphate	+	+	+
Native DNA	+	−	−
ATP, ADP, etc.	−	−	+
3′-Phosphate oligonucleotides	−	+	−
4. Inhibitors[c]			
HPO_4^{2-}	+	−	+
SO_4^{2-}	+	−	−
Polyribonucleotides	+	+[d]	−
5. Thermal inactivation (50%)	60°	57°	
6. Chromatographic properties			
DEAE-Sephadex (pH 6.8)	0.05 *M* KP[e]	0.05–0.1 *M*	0.05 *M* KP
Hydroxyapatite (pH 6.8)	0.3 *M* KP	0.12 *M* KP	0.12 *M* KP
CM-Sephadex	pH 6.8; 0.2 *M* KP	pH 6.3; 0.2 *M* KCl	pH 5.7; 0.11 *M* KP
7. Kinetics (V vs. S)	Sigmoid	Hyperbolic	Hyperbolic

[a] From Bernardi and Bernardi (*35*).
[b] As determined by sedimentation in sucrose gradient, using cytochrome c as a reference protein; enzymic assays were done on both bis(*p*-nitrophenyl) phosphate and the natural substrates; the results were the same.
[c] Using bis(*p*-nitrophenyl) phosphate in 0.25 *M* succinate buffer as the substrate.
[d] The enzyme degrades polyribonucleotides to 3′P mononucleosides; these are inhibitory.
[e] Eluting molarity; KP is potassium phosphate buffer.

results (*11*) are in general agreement with those of Hilmoe (*14*), which were obtained on RNA core using lower salt concentrations.

The pH-activity curve using bis(*p*-nitrophenyl) phosphate as a substrate showed an optimum at pH 5.8, but considerable activity could be detected between pH 6 and 7 (*11*).

Using RNA "core" as the substrate, EDTA and sulfhydryl reagents are activators; Mg^{2+}, Mn^{2+}, and, more effectively, Cu^{2+}, Hg^{2+}, and Zn^{2+} are inhibitors; arsenite and fluoride are weak inhibitors (*14*). Deoxyribonucleoside-3′-phosphates are competitive inhibitors of the activity on acid DNase digests.

IV. Distribution and Intracellular Localization

It has been claimed that spleen acid exonuclease has its counterpart in other tissues (*3*, *40*). It is very likely that this claim is correct, in spite of the fact that it was based on the wrong assumption that a hydrolytic activity at pH 6.0 on *p*-nitrophenyl thymidine-3′-phosphate could be equated with acid exonuclease activity (see Tables I and II). In fact, an acid exonuclease activity has been shown in fish muscle (*41*, *42*) and, using as a substrate an acid DNase digest, in rat liver (*43*). In this latter case, it was checked that the activity on *p*-nitrophenyl thymidine-3′-phosphate was not inhibited by SO_4^{2-} and that it was lower than on thymidylyl (3′ → 5′) thymidine (*44*).

As far as the intracellular localization of the acid exonuclease activity is concerned, Razzell (*40*), using the synthetic substrate, found it in both the mitochondrial-lysosomal fraction and in the supernatant. Van Dyck and Bernardi (*45*) found that an enzymic activity on acid DNase hydrolyzates could be extracted from rat liver lysosomes and that the sedimentation coefficient of this activity, as determined by sucrose density gradient centrifugation, was the same as for spleen acid exonuclease. Subsequent work (*43*, *44*) confirmed the idea that acid exonuclease is a lysosomal enzyme like other acid hydrolases of nucleic acids [see Table IV and related discussion in Bernardi (*5*)]. For a recent review on the problem of intracellular localization of exonucleases, the reader is referred to Shugar and Sierakowska (*46*).

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