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Exonuclease (Phosphodiesterase) from the Testes of Chinook Salmon (*Oncorhynchus tschawytscha*)*

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ABSTRACT: An exonuclease, present in the cytoplasmic fraction of partially mature *Oncorhynchus tschawytscha* testes, has been purified 1000-fold. The enzyme, which has optimal activity at pH 5.5, liberates nucleoside 3′-phosphates from the 5′ termini of oligonucleotides. No other enzymes capable of hydrolyzing phosphate esters were detectable in the purified exonuclease.

The stability of the enzyme and its minimal requirements suggest that it could be a useful tool in nucleic acid biochemistry.

As part of studies on the sexually maturing salmonid (Schmidt *et al.*, 1965; Ingles *et al.*, 1966; Dixon and Smith, 1968; Wilson and Smith, 1968) we are investigating the relationship of the enzymes of nucleic acid metabolism to spermatogenesis. This report is concerned with the purification and catalytic properties of an exonuclease which acts on the 5′-hydroxyl termini of oligonucleotides to release nucleoside 3′-phosphates. The enzyme is therefore analogous in its action to spleen phosphodiesterase and can be classified as a phosphodiesterase of type II (Razzell, 1967). The exonuclease activity is found in association with all of the subcellular fractions obtained by differential centrifugation of disrupted testis cells from *Oncorhynchus tschawytscha*. The enzyme described here was isolated from the soluble fraction. It shows some minor differences from spleen phosphodiesterase in its chromatographic properties and requirements for optimum activity. It is like the spleen enzyme in its ability to synthesize larger oligonucleotides from a dinucleoside phosphate at high substrate concentration.

Materials and Methods

Collection of Testes. Chinook salmon (*Oncorhynchus tschawytscha*) were caught in June and July during their spawning migration along the Fraser River, British Columbia. The fish had an average weight of 6000 g with testes approximately 2% of the body weight. Histological examination (Robertson, 1958) indicated that spermatogonia and spermatocytes were the predominant cell types. The testes were frozen immediately in solid carbon dioxide and were subsequently stored at −80°.

Enzyme Substrates. Thymidine 3′-*p*-nitrophenyl phosphate was synthesized using the procedure of Borden and Smith (1966). Both this substrate and thymidine 5′-*p*-nitrophenyl phosphate are available from Raylo Chemicals Limited, Edmonton, Alberta. Thymidine 3′- and thymidine 5′-2,4-dinitrophenyl phosphates were synthesized by the procedure of von Tigerstrom and Smith (1969). Thymidine 3′-phenyl phosphate was synthesized by reaction of 5′-*O*-monomethoxytritylthymidine with phenyl phosphorodichloridate in pyridine (R. G. von Tigerstrom and M. Smith, unpublished procedure). Thymidine 3′- and thymidine 5′-phosphorofluoridates were synthesized using the procedure of Witmann (1963) except that the nucleotides were purified by chromatography on DEAE-cellulose. The synthesis of dTpdT[1] was by the method of Gilham and Khorana (1958). Adenosine 2′,3′-cyclic phosphate and adenosine 3′,5′-cyclic phosphate were synthesized as described by Smith and Khorana (1963). The dinucleotide pApA was isolated from the embryos of *Euchaeta japonica* (Hepner and Smith, 1967). The oligonucleotides ApA, CpA, UpA, and ApApA were purchased from Miles Laboratories. Other nucleotides and polynucleotides were conventional commercial products.

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[1] Abbreviations used are: ApA, adenylyl-(5′→3′)-adenosine; pApA, adenylyl-(5′→3′)-adenosine 5′-phosphate; CpA, adenylyl-(5′→3′)-cytidine; UpA, adenylyl-(5′→3′)-uridine; ApApA, adenylyl-(5′→3′)-adenylyl-(5′→3′)-adenosine; dTpdT, deoxythymidylyl-(5′→3′)-deoxythymidine.

Paper Chromatography. This was carried out, using the descending technique, on Whatman 40 paper. Authentic standard compounds were chromatographed alongside unknowns. Nucleotides were detected by viewing under ultraviolet light and were eluted for spectrophotometric identification and quantitation using standard procedures (Heppel, 1967). The solvent systems employed were: solvent A, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2); solvent B, 1 M ammonium acetate, pH 6.5–ethyl alcohol (3:7); solvent C, *n*-butyl alcohol–acetic acid–water (5:2:3).

Ion-Exchange Chromatography. Both DEAE-cellulose and CM-cellulose (Whatman CM23) were washed with alkali and acid as described by the manufacturer (Whatman Technical Bulletin, IE 2), and were packed into columns in the presence of a molar concentration of potassium chloride buffered with 0.005 M sodium phosphate of appropriate pH. After the packed column had been washed with this solution, it was washed with water and then with the appropriate starting buffer until the pH and conductivity of the effluent and the buffer entering the column were identical.

Enzyme Assays. Exonuclease activity was measured using procedures which have previously been applied to spleen phosphodiesterase (Razzell, 1967). With thymidine 3′-*p*-nitrophenyl phosphate as substrate, the method described by Razzell (1963) was used. With thymidine 3′,2,4-dinitrophenyl phosphate as substrate, the method used was that of von Tigerstrom and Smith (1969). Unlike *p*-nitrophenol, 2,4-dinitrophenol is ionized at pH 5.5 and can be estimated directly from the absorbance of its anion at 360 nm (von Tigerstrom and Smith, 1969). In order to conserve substrate and enzyme, assays of fractions in column effluents and other multiple assays were carried out in reaction mixtures which contained thymidine 3′,2,4-dinitrophenyl phosphate (0.1 μmole), ammonium acetate (25 μmoles), enzyme solution, and water to a total volume of 100 μl, pH 5.5. After incubation at 37°, usually for 15 min, the mixtures were diluted with water to a volume of 1.0 ml and the absorbance at 360 nm was determined. In assays where the hydrolysis of thymidine 3′,2,4-dinitrophenyl phosphate was followed continuously (*e.g.*, in studies of enzyme inhibitors), the reaction mixture contained substrate (1.0 μmole), ammonium acetate (250 μmoles), enzyme solution, and water to a volume of 1.01 ml, pH 5.5. The release of 2,4-dinitrophenoxide ion at 37° was followed directly at 360 nm.

One unit of exonuclease activity is defined as that amount of enzyme which catalyses the hydrolysis of 1 μmole of substrate in 1 hr at 37°. [For spleen phosphodiesterase, assays of enzyme activity have used thymidine 3′-*p*-nitrophenyl phosphate (Razzell and Khorana, 1961), thymidine 3′,2,4-dinitrophenyl phosphate (von Tigerstrom and Smith, 1969), oligoribonucleotides (Heppel and Hilmoe, 1955), or oligodeoxyribonucleotides (Bernardi and Bernardi, 1968) as substrates. A given amount of spleen phosphodiesterase has a different unit value in each assay. The activity of spleen phosphodiesterase with the substrates oligodeoxyribonucleotides, thymidine 3′-*p*-nitrophenyl phosphate, and thymidine 3′-2,4-dinitrophenyl phosphate is in the ratio 1.0:1.8:2.6, respectively (Bernardi and Bernardi, 1968; von Tigerstrom and Smith, 1969). For the *O. tschawytscha* testis exonuclease with the substrates thymidine 3′-*p*-nitrophenyl phosphate and thymidine 3′-2,4-dinitrophenyl phosphate the ratio is 1.0:1.3.]

The increase in absorbance at 260 nm when the internucleotide bond in ApA is hydrolyzed was also used to measure phosphodiesterase activity.

The exonuclease which hydrolyses thymidine 5′-*p*-nitrophenyl phosphate, *i.e.*, the phosphodiesterase of type I, analogous to snake venom phosphodiesterase (Razzell, 1967) was assayed as described by Razzell (1963).

Ribonuclease activity was measured using a variety of assay conditions selected to detect the types of enzyme which act as endoribonucleases (Razzell, 1967).

The ribonuclease which forms oligonucleotides from polyadenylic acid (Razzell, 1963) was measured using a modification of the published procedure (W. E. Razzell, personal communication, 1960). The incubation mixture contained polyadenylic acid (5 mg), Tris-acetate (25 μmoles), disodium EDTA (1 μmole), potassium phosphate (20 μmoles), β-mercaptoethanol (10 μmoles), magnesium chloride (4 μmoles), and water to a volume of 1.0 ml, pH 7.5. This mixture (80 μl) and the enzyme preparation (20 μl) were incubated at 37° for 1 hr. Then 60% perchloric acid (5 μl) was added and the mixture was cooled at 0° for 10 min. The solution was centrifuged and an aliquot of the supernatant solution (50 μl) was diluted to 1.0 ml with 4% perchloric acid and the absorbance at 260 nm was determined. The unit of activity is expressed as micromoles of adenylic acid residues (ε 14,500 at 260 nm) liberated per hour at 37°.

The ribonuclease activities which degrade RNA to 5′-phosphorylated oligonucleotides at acidic or alkaline pH (Razzell, 1967) were assayed in appropriate buffers (W. E. Razzell, personal communication, 1967). Prior to assay, enzyme preparations were treated with acid to inactivate any endogenous inhibitors (Razzell, 1967). To the enzyme solution (200 μl) was added 1 M sulfuric acid (40 μl) at 0°. After 1 hr, 1 M Tris-acetate, pH 7.5 (10 μl), and 1 M sodium hydroxide (80 μl) were added to neutralize the acid. The stock solution for enzyme assay contained ammonium acetate (80 μmoles), potassium phosphate (80 μmoles), Tris (90 μmoles), high molecular weight RNA (5 mg), and water to a final volume of 5 ml, pH 7.8. The assay at acidic pH used the same mixture adjusted to pH 5.5. The assays were carried out in the same way as the assay of ribonuclease active against polyadenylic acid. The unit of activity is μmoles of nucleotide residue (assuming an average value of ε 10,600 at 260 nm) liberated at 37° in 1 hr.

Alkaline deoxyribonuclease (Laskowski, 1966) was measured using an incubation mixture consisting of Tris-hydrochloride (180 μmoles), magnesium chloride (13 μmoles), calf thymus DNA (0.4 mg), enzyme solution, and water to a volume of 1.25 ml, pH 7.5. One unit of enzyme activity catalyzes, in 1 min at 37°, the release of oligonucleotides having an absorbance of 1.0 at 260 nm.

Acidic deoxyribonuclease (McDonald, 1962) was assayed using the procedure described by Bernardi (1966) for spleen acidic deoxyribonuclease, using a volume of 1.25 ml for the incubation mixture. One unit of enzyme activity, under these conditions of assay, catalyzes, in 1 min at 37°, the release of oligonucleotides having an absorbance of 1.0 at 260 nm.

Acidic phosphatase was assayed using the procedure of Bernardi (1966). Under these conditions, one unit of enzyme activity catalyzes the release of 1 μmole of *p*-nitrophenol in 1 hr at 37°.

Alkaline phosphatase was measured using the procedure

TABLE I: Distribution of Exonuclease Activity in Subcellular Fractions of *O. tschawytscha* Testis Cells.

Fraction[a] (rpm)	Protein (mg/g of Testis)	Units[b] per g of Testis	Sp Act.[c]
2,200 sediment	38.3	79.0	2.0
7,200 sediment	0.5	4.3	9.7
40,000 sediment	7.1	35.2	5.8
40,000 supernatant	17.9	31.7	1.8

[a] Testis tissue (7.6 gm) was disrupted in the medium described in the Experimental Section (32 ml) to yield the whole homogenate (40 ml). This was centrifuged at 2200 rpm for 10 min (SS34 rotor) to sediment the nuclear fraction which was suspended in homogenizing medium (30 ml). The nuclear supernatant was centrifuged at 7200 rpm for 25 min (SS34 rotor). The resulting sediment was suspended in the homogenizing medium (10 ml) and centrifuged at 12,500 rpm for 15 min (SS34 rotor) to yield a mitochondrial pellet which was resuspended in homogenizing medium (17 ml). The supernatant from the 7200-rpm centrifugation was recentrifuged at 40,000 rpm for 90 min (40 rotor). The microsomal pellet was suspended in the homogenizing medium (18 ml). The supernatant solution (22 ml) is the soluble enzyme fraction. [b] The exonuclease activity was assayed using thymidine 3′,2,4-dinitrophenyl phosphate as described in the text. [c] The specific activity is the number of μmoles of substrate hydrolyzed per hr per mg of protein.

TABLE II: Purification of Exonuclease from *O. tschawytscha* Testis.

Step	Procedure[a]	Enzyme Units[b]	Protein (mg)	Sp Act.[c]	Recov (%)
I	Centrifugation	10,600	7,580	1.4	100
II	Streptomycin	10,200	4,970	2.1	97
III	Ammonium sulfate	8,500	3,030	2.8	80
IV	CM-cellulose	3,300	32.5	101	31
V	DEAE-cellulose	2,120	18.2	120	20
VI	Hydroxylapatite	1,060	0.6	1,770	10

[a] Frozen testis (500 g) were treated as indicated in the text. The recovery at each step is calculated on the basis of the amount of enzyme present in the supernatant solution from the 30,000-rpm (30 rotor) centrifugation. [b] The substrate was thymidine 3′,2,4-dinitrophenyl phosphate, the assays being carried out at pH 5.5 as described in the text. [c] The specific activity is the μmoles of substrate hydrolyzed per hr per mg of protein.

described by Nisman (1968). One unit of enzyme activity liberated 1 μmole of *p*-nitrophenol in 1 hr at 37°.

Adenosine deaminase (Winter and Bernheimer, 1964; Ogilvie and Letsinger, 1968) was measured from the decrease in absorbance at 265 nm which occurs when adenosine is converted into inosine (Kaplan, 1955). The incubation mixture contained sodium phosphate (100 μmoles), adenosine (60 μmoles), enzyme solution, and water to a volume of 1.0 ml, pH 6.8. One unit of enzyme activity catalyzed the conversion of 1 μmole of adenosine in 1 hr at 37°.

Spectrophotometric Measurements. All measurements were carried out using a Unicam SP800 recording spectrophotometer equipped with thermostated cell holders and with an absorbance scale-expansion amplifier and recorder. Cells of 1.0-ml capacity with a light path of 10 mm were used.

An absorbance unit is an amount of material which has an absorbance of 1.0 when dissolved in 1.0 ml and measured in a 10-mm cell.

Electrofocusing. The L.K.B. apparatus was used following the manufacturers instructions. The step VI enzyme was applied to a pH gradient of 3–10 at a potential of 500 V, and the experiment run for 3 days. The column was then separated in 2-ml fractions and the pH and enzyme activity of each fraction were determined.

Gel Electrophoresis. Electrophoresis in acrylamide gels and staining was carried as described by Davis (1964) in 0.05 M Tris–glycine buffer, pH 8.5. For assay of enzyme activity in proteins fractionated electrophoretically, the unstained gel was cut into thin slices (2 mm) and each slice eluted with 0.25 M ammonium acetate, pH 5.5 (100 μl), prior to assay of enzyme activity.

Protein Analysis. The method of Lowry *et al.* (1951) was used.

Purification of the Exonuclease. Unless stated otherwise, all operations were carried out at 4°. Centrifugations were carried out in a Serval RC2B refrigerated centrifuge using either a GSA rotor or a SS34 rotor. Ultracentrifugation was carried out in a Beckman-Spinco Model L centrifuge equipped with a 30 roto.

The distribution of the enzyme amongst subcellular fractions is shown in Table I and the procedure for purification from the soluble fraction is summarized in Table II.

Step I. Preparation of Soluble Enzyme. Frozen testis (500 g) were thawed and the major blood vessels were removed. The tissue was then homogenized in three volumes of a medium composed of sucrose (0.25 mole), β-mercaptoethanol (10 mmoles), magnesium chloride (1.5 mmoles), Tris-hydrochloride (2.5 mmoles), and water to a volume of 1 l., pH 7.4. The homogenization, in a Waring blender equipped with an ice–water jacket, was carried out for 1 min at 70 V followed by 5 min at 40 V. The homogenate was centrifuged at 6200 rpm (GSA rotor) for 25 min. The supernatant liquid was decanted and ultracentrifuged at 30,000 rpm (30 rotor) for 2 hr. The supernatant liquid (1 l.) is the step I enzyme fraction.

Step II. Streptomycin Sulfate Precipitation. A solution of streptomycin sulfate (5 g) in the homogenizing medium (100 ml) was added, with stirring, to the step I enzyme (1 l.). The solution was allowed to stand for 10 min and then was centrifuged at 10,000 rpm (GSA rotor) for 10 min. The supernatant liquid is the step II enzyme fraction.

Step III. Ammonium Sulfate Fractionation. Ammonium sulfate (209 g) was slowly added, with stirring, to the step II enzyme. After 1 hr, the mixture was centrifuged at 20,000 rpm (SS34 rotor) for 10 min. The supernatant liquid contained

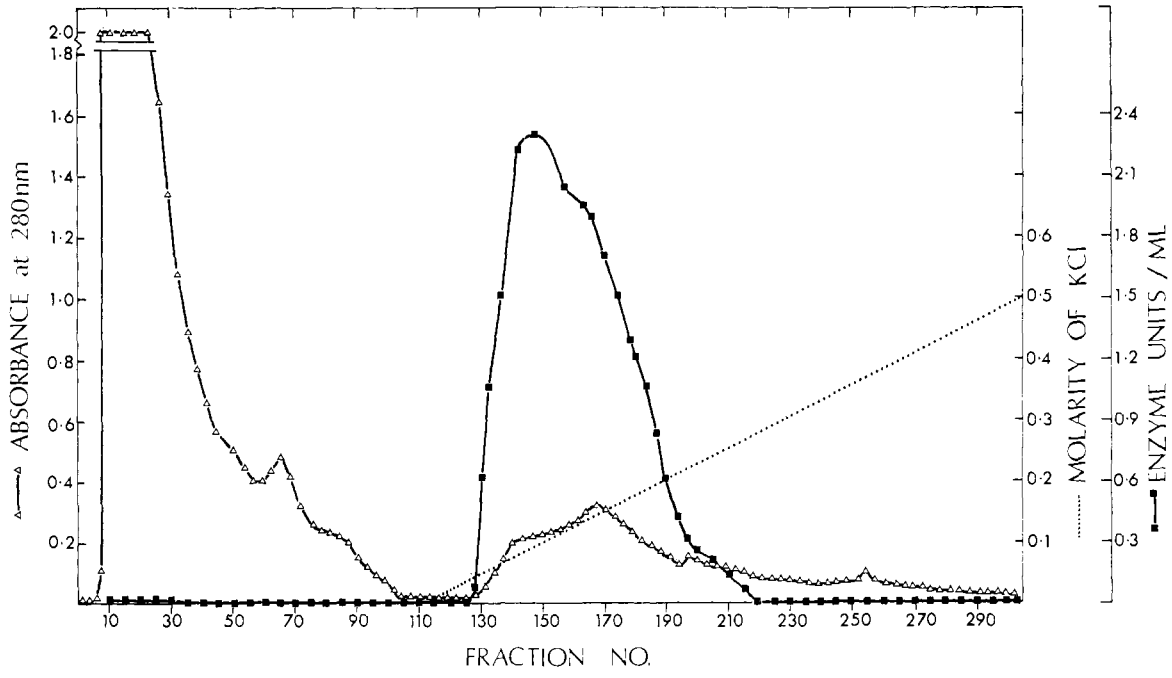

FIGURE 1: Chromatography of *O. tschawytscha* testis exonuclease on CM-cellulose. Step III enzyme (3030 mg of protein) was diluted to a volume of 250 ml with 0.005 M sodium phosphate, pH 6.3, and chromatographed on a column of CM-cellulose (40 × 2.5 cm diameter) as described in the text. The elution of protein was followed at 280 nm. The enzyme activity was assayed with thymidine 3′,2,4-dinitrophenyl phosphate (see text). The estimates of enzyme activity are not quantitative above 1.5 units/ml.

the enzyme. A further amount of ammonium sulfate (319 g) was added slowly with stirring. After 2 hr the mixture was centrifuged at 20,000 rpm (SS34 rotor) for 10 min. The precipitate, containing the enzyme, was dissolved in 0.005 M sodium phosphate, pH 6.3 (20 ml). This solution was dialyzed against two changes of 2 l. of the same buffer. The resulting solution was centrifuged at 20,000 rpm (SS34 rotor) for 10 min to remove a precipitate formed during dialysis. The supernatant liquid is the step III enzyme fraction.

Step IV. Chromatography on CM-Cellulose. The step III enzyme was diluted to a protein concentration of 12 mg/ml with 0.005 M sodium phosphate, pH 6.3 (final volume 250 ml), and applied to a column (40 × 2.5 cm diameter) of CM-cellulose which had been equilibrated with the same buffer immediately prior to loading. Subsequently the column was washed with the buffer until the effluent had an absorbance of 0.02 at 280 nm (approximately 1 l.). The proteins were eluted using a linearly increasing salt gradient with 0.005 M sodium phosphate, pH 6.3 (2 l.), in the mixing chamber and 0.005 M sodium phosphate containing 0.5 M potassium chloride, pH 6.3 (2 l.), in the reservoir. Fractions (20 ml) were collected at 20-min intervals (Figure 1). The enzyme was eluted in a broad peak (fractions 128–200) which also contained material with a pale yellow-pink coloration. These fractions were pooled and the enzyme precipitated with ammonium sulfate (80% saturation). The precipitate was allowed to settle overnight and then was collected by centrifugation at 20,000 rpm (SS34 rotor) for 10 min. It was dissolved in 0.005 M sodium phosphate, pH 7.4 (20 ml), and dialyzed against two changes of the same buffer (2 l.). During dialysis the colored material in the eluate, which was precipitated along with the enzyme by ammonium sulfate, became insoluble and it was removed by centrifugation at 20,000 rpm (SS34 rotor) for 10 min to yield the step IV enzyme fraction (27 ml). The colored material is responsible for the high absorbance of the enzyme fraction relative to the amount of protein in the final fraction.

Step V. Chromatography on DEAE-Cellulose. The step IV enzyme was applied to a DEAE-cellulose column (25 × 1.2 cm diameter) which had previously been equilibrated with 0.005 M sodium phosphate, pH 7.4. The column was washed, after loading, with the same buffer. The enzyme passed directly through the column, fractions (10 ml) being collected at 10-min intervals (Figure 2). Those containing enzyme were pooled and concentrated by ammonium sulfate precipitation (80% saturation). After standing overnight, the precipitate was collected by centrifugation as with the step IV enzyme. It was dissolved in 0.005 M sodium phosphate, pH 6.8 (2.5 ml), and dialyzed against two changes of the same buffer (200 ml) to yield the step V enzyme fraction (3 ml).

Step VI. Chromatography on Hydroxylapatite. Step V enzyme was applied to a column (10 × 1.2 cm diameter) of hydroxylapatite (Bio-Rad) packed in 0.005 M sodium phosphate, pH 6.8. After loading, the column was washed with the same buffer (100 ml), fractions (5 ml) being collected at 5-min intervals. The column was then eluted with a linearly increasing concentration of salt with 0.005 M sodium phosphate (250 ml) in the mixing chamber and 0.4 M sodium phosphate (250 ml) in the reservoir (Figure 3). Fractions containing the enzyme were pooled and the protein precipitated with ammonium sulfate (80% saturation). The precipitate was collected by centrifugation at 20,000 rpm (SS34 rotor) for 10 min and was dissolved in 0.005 M sodium phosphate, pH 6.8 (0.5 ml), to yield the final preparation, the step VI enzyme.

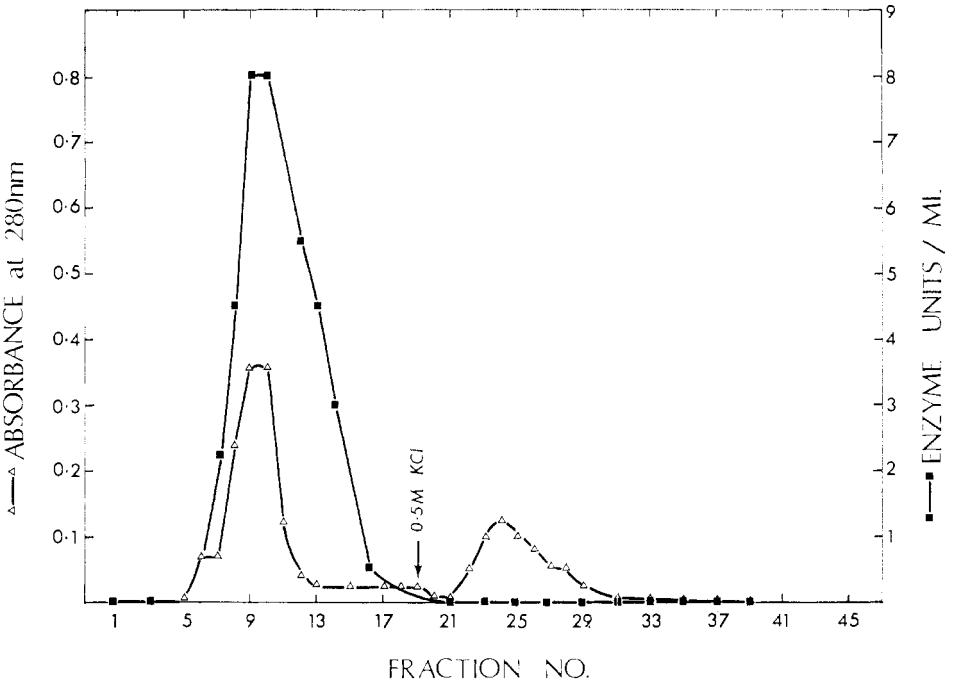

FIGURE 2: Chromatography of *O. tschawytscha* testis exonuclease on DEAE-cellulose. Step IV enzyme (32.5 mg of protein) in 0.005 M sodium phosphate, pH 7.4 (27 ml), was chromatographed on a DEAE-cellulose column (25 × 1.2 cm diameter) as described in the text. Elution of protein was followed at 280 nm. The exonuclease was assayed with thymidine 3′,2,4-dinitrophenyl phosphate (see text). The estimates of enzyme activity are not quantitative above 5 units/ml.

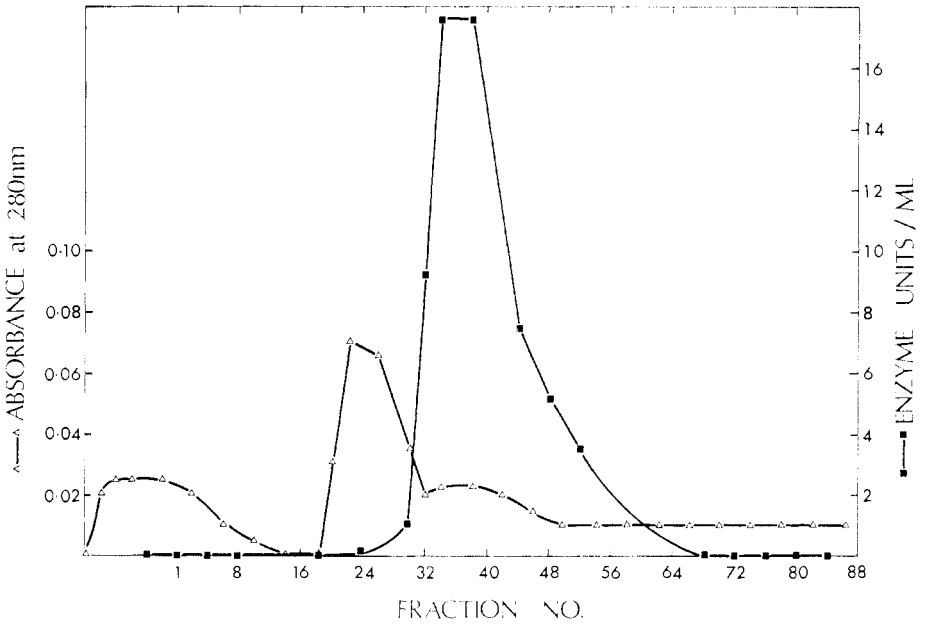

FIGURE 3: Chromatography of *O. tschawytscha* testis exonuclease on hydroxylapatite. Step V enzyme (18.2 mg of protein) in 0.005 M sodium phosphate (3 ml) was chromatographed on a column of hydroxylapatite (10 × 1.2 cm diameter) as described in the text. Elution of protein was followed at 280 nm. Enzyme activity was measured with thymidine 3′,2,4-dinitrophenyl phosphate as substrate (see text).

Chromatography on Sephadex G-200. Step VI enzyme (0.1 ml) was applied to a column (40 × 1.2 cm diameter) of Sephadex G-200, packed in 0.05 M sodium phosphate, pH 7.0. The column was eluted with the same buffer, fractions (1.0 ml) being collected at 3-min intervals.

Results

The step IV preparation of exonuclease was not contaminated with phosphodiesterase, type I, acidic or alkaline ribonuclease, acidic or alkaline deoxyribonuclease, acidic

TABLE III: Comparison of the Amounts of Various Enzyme Activities in the Step I Fraction with Those in the Step VI Fraction.

Enzyme[a] (pH)	Substrate	Specific Activity[b] Step I	Step VI
Exonuclease (9.0)	Thymidine 5′-*p*-nitrophenyl phosphate	0.13	<0.8
Exonuclease (5.5)	Thymidine 3′-2′,4′-dinitrophenyl phosphate	1.77	1770
Phosphatase (5.5)	*p*-Nitrophenyl phosphate	5.8	<1.0
Phosphatase (9.0)	*p*-Nitrophenyl phosphate	0.78	<0.7
Ribonuclease (7.5)	Polyadenylic acid	<0.01	<0.4
Ribonuclease (5.5)	RNA	0.24	<0.5
Ribonuclease (7.8)	RNA	<0.03	<0.5
Deoxyribonuclease (5.5)	DNA	1.8	<0.07
Deoxyribonuclease (7.5)	DNA	0.09	<0.07
Deaminase	Adenosine	0.71	<0.57

[a] The assay conditions are described in the Experimental Section. [b] The units of enzyme activity per mg of protein. The units are defined in the Experimental Section. Where the activity is represented as less than a given value, this indicates the lower level of sensitivity of the assay and no enzyme was detectable.

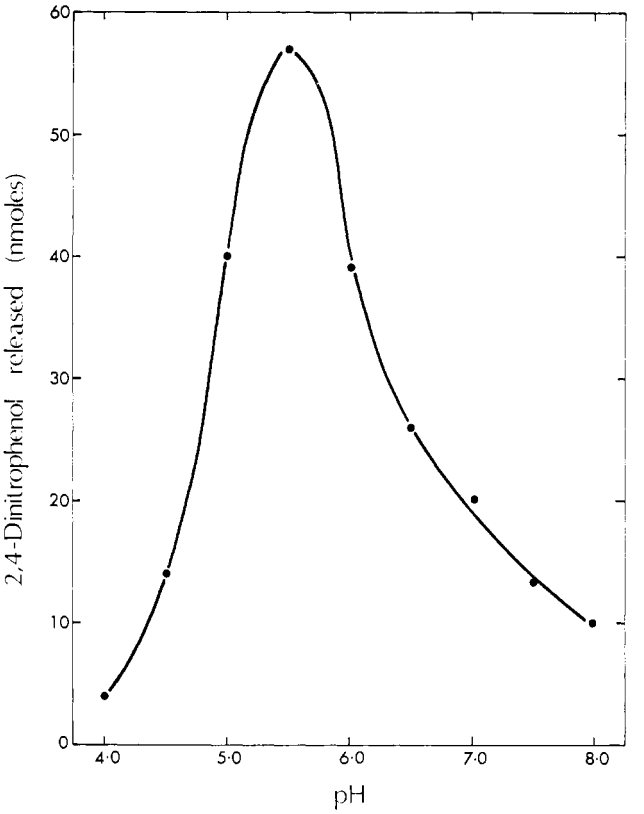

FIGURE 4: The pH optimum of *O. tschawytscha* testis step VI enzyme. Thymidine 3′,2,4-dinitrophenyl phosphate (0.1 μmole), ammonium acetate for pH 4.0 to 5.5, sodium phosphate for pH 6.0 to 7.0, or Tris-hydrochloride for pH 7.5 and 8.0 (25 μmoles), step VI enzyme (0.15 μg), and water to 100 μl were incubated at 37° for 15 min and the released 2,4-dinitrophenol estimated spectrophotometrically (see text).

or alkaline phosphatase, adenosine deaminase, or nucleoside 2,3-cyclic phosphate diesterase (Table III). These enzymes were present in the step I fraction and/or have been found as contaminants of preparations of spleen phosphodiesterase (Razzell, 1963; Winter and Bernheimer, 1964; Ogilvie and Letsinger, 1968).

Properties of Step VI Exonuclease. EFFECT OF pH. With thymidine 3′,2,4-dinitrophenyl phosphate as substrate the pH optimum is 5.5 (Figure 4).

EFFECT OF METAL IONS. The enzyme, assayed in the presence of 0.25 M ammonium acetate, pH 5.5, was neither inhibited nor stimulated by Ca^{2+}, Mg^{2+}, Ba^{2+}, Na^{+}, K^{+}, or Li^{+} at concentrations up to 0.05 M. Over 90% inhibition was produced by 10^{-4} M cupric sulfate. Neither EDTA (up to 10^{-3} M) nor dithiothreitol (up to 10^{-3} M) had any effect on exonuclease activity at pH 5.5.

BUFFER CONCENTRATION. The concentration of ammonium acetate, pH 5.5, had no effect on the enzyme activity over the range 0.1 to 0.5 M. A reduced activity at lower concentrations may have been due to inadequate buffer capacity and a consequent decrease in pH during hydrolysis of substrate.

ISOELECTRIC POINT. The enzyme formed a single zone when electrofocused in a gradient from pH 3 to 10. The fraction containing the greatest activity was at pH 7.0.

SEPHADEX G-200 CHROMATOGRAPHY. The enzyme was eluted as a single symmetrical peak. The position of elution (29.0 ml) from a column of Sephadex G-200 (38 × 1.2 cm diameter) relative to γ-globulin (24.5 ml) and bovine serum albumen (32.0 ml) implies a molecular weight of approximately 100,000.

GEL ELECTROPHORESIS. In acrylamide gels at pH 8.5, the step VI enzyme contained a sharp, slower moving, and a diffuse, faster moving band of protein migrating toward the anode. An unstained acrylamide gel electrophoresis was sliced and the enzyme activity in the slices was assayed. There were two bands of enzyme activity corresponding to the two bands of protein in the stained gel.

STABILITY. The exonuclease was stable for 30-min exposures of temperatures up to 55° (Figure 5). Above that temperature the enzyme was inactivated. In 0.005 M sodium phosphate, pH 6.8, the enzyme (200 μg of protein in 0.5 ml) was stable during 6-months storage at −20° both in the absence of presence of glycerol (10%). The same preparation was also completely stable during storage for 6 months at 0°.

Hydrolytic Properties of the Step VI Enzyme. At pH 5.5, the enzyme catalyzes the hydrolysis of thymidine 3′-phenyl phosphate, thymidine 3′-*p*-nitrophenyl phosphate, and thy-

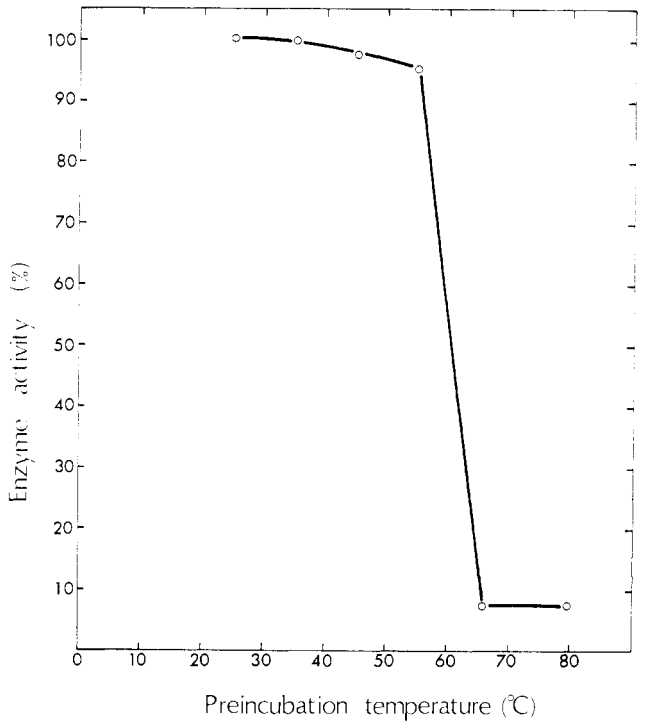

FIGURE 5: Inactivation by heat of step VI enzyme. The enzyme was preincubated in 0.2 M ammonium acetate, pH 5.5, for 30 min at the appropriate temperature prior to assay of enzyme activity in the same buffer at 37° with thymidine 3′,2,4-dinitrophenyl phosphate as substrate.

TABLE IV: Relative Rates of Hydrolysis of Various Substrates by Step VI Exonuclease at pH 5.5 and 37°.

Substrate	Hydrolysis Rate[a] (μmoles/hr per mg of protein)
Thymidine 3′-*p*-nitrophenyl phosphate	1200
Thymidine 3′-2,4-dinitrophenyl phosphate	1700
Thymidine 3′-phenyl phosphate	2970
Thymidine 3′-phosphorofluoridate	1210
Thymidine 5′-*p*-nitrophenyl phosphate	<1
Thymidine 5′-2,5-dinitrophenyl phosphate	26

[a] Enzymic hydrolyses were carried out in 0.25 M ammonium acetate, pH 5.5, with an initial substrate concentration of 10^{-3} M. Hydrolysis of thymidine 3′-phenyl phosphate and thymidine 3′-phosphorofluoridate were followed estimating the released thymidine 3′-phosphate after chromatography in solvent A. The hydrolysis of *p*-nitrophenyl and 2,4-dinitrophenyl esters were followed spectrophotometrically as described in the text.

midine 3′,2,4-dinitrophenyl phosphate but not of thymidine 5′-*p*-nitrophenyl phosphate (Table IV). The hydrolysis of thymidine 5′,2,4-dinitrophenyl phosphate at pH 5.5 is catalyzed at about 1.5% of the rate of thymidine 3′,2,4-dinitrophenyl phosphate. A similar activity is present in spleen phosphodiesterase (von Tigerstrom and Smith, 1969). There is no detectable hydrolysis of any of these compounds at pH 9. Various (3′→5′)-linked dinucleoside phosphates are hydrolyzed to nucleoside 3′-phosphate and nucleoside (Table V). The exonuclease action of the enzyme is demonstrated by the hydrolysis of ApApA which yields only adenosine 3′-phosphate and ApA in the early stages of the reaction (Table VI).

The low molecular weight nucleotides which were not detectably hydrolyzed by the step VI enzyme were, in addition to thymidine 5′-*p*-nitrophenyl phosphate, thymidine 5′-phosphorofluoridate, adenosine 2′,3′-cyclic phosphate, adenosine 3′,5′-cyclic phosphate, and pApA.

Transesterification Catalyzed by Step VI Enzyme. The reaction of the exonuclease with dTpdT, at approximately tenfold substrate concentration relative to conditions used in the study of hydrolytic reactions, is summarized in Table VII. In addition to hydrolysis to deoxythymidine 3′-phosphate and deoxythymidine, there was significant production of longer oligonucleotides.

Inhibitors of the Step VI Enzyme. The effects of various nucleotide derivatives on the rate of hydrolysis of thymidine 3′,2,4-dinitrophenyl phosphate are summarized in Table VIII. The compounds which were inhibitory were adenosine 2′,3′-cyclic phosphate, adenosine 5′-phosphate, pApA, and adenosine 3′-phosphate. The polynucleotides, polyadenylic acid, polyuridylic acid, and yeast transfer RNA had no effect on the enzyme under the conditions of assay.

Discussion

This report describes the isolation of an exonuclease–phosphodiesterase from the soluble subcellular fraction of partially mature testis from a species of Pacific salmon, *O. tschawytscha*. The enzyme is analogous to spleen phosphodiesterase in many of its properties, particularly in its substrate specificity and mode of action (Heppel and Hilmoe, 1955; Khorana, 1961; Razzell, 1963). It releases nucleoside 3′-phosphates from synthetic nucleoside 3′-phosphate esters and from the 5′-hydroxyl termini of oligonucleotides.

Studies on the exonuclease–phosphodiesterases in various vertebrate tissues have generally shown that the amount of phosphodiesterase of type I predominates over type II (Razzell, 1961, 1967). The preponderance of phosphodiesterase of type II in partially developed *O. tschawytscha* testis is striking (Table III). In this context it is of interest that an acid DNase has been isolated in large amounts from salmon testis (McDonald, 1962).

The subcellular distribution of the *O. tschawytscha* testis exonuclease is analogous to that generally found for this type of enzyme (Razzell, 1961), that is, enzyme activity is present in particulate fractions and in the fraction which is not sedimentable (Table I). This latter fraction is the one from which the enzyme was purified. Some preliminary attempts to release the enzyme from particulate fractions did not result in a stable, soluble enzyme preparation.

TABLE V: Hydrolysis of Various Diribonucleoside Phosphates by Step VI Exonuclease.[a]

Substrate	Identity	Product Amount (μmole)	R_F
	A	0.13	0.60
ApA	Ap	0.12	0.20
	ApA	0.21	0.29
	A	0.13	0.59
UpA	Up	0.13	0.28
	UpA	0.28	0.42
	A	0.11	0.61
CpA	Cp	0.10	0.24
	CpA	0.31	0.37

[a] Substrate (approximately 0.5 μmole), ammonium acetate (25 μmoles), step VI enzyme (0.1 μg), and water to a volume of 100 μl, pH 5.5 were incubated for 30 min at 37°. After addition of glacial acetic acid (5 μl) the products were separated by chromatography in solvent B for 20 hr, and then eluted for spectrophotometric identification and estimation. The identity of the adenosine 3′-phosphate released from ApA was confirmed by its resistance to crude snake venom under conditions where adenosine 5′-phosphate was dephosphorylated completely by the venom 5′-nucleotidase.

TABLE VI: Hydrolysis of ApApA by Step VI Exonuclease.[a]

Time (min)	Amounts of Nucleotides (absorbance units at 259 nm)				
	ApApA	ApA	Ap	A	Total
0	5.5	0.2	0.0	0.0	5.7
5	4.3	0.9	0.2	0.0	5.4
10	3.8	1.0	0.9	0.0	5.7
15	3.1	1.0	1.0	0.5	5.6
30	2.5	1.0	1.7	0.7	5.9
60	2.0	0.9	1.8	1.0	5.7
120	1.5	0.8	2.3	1.3	5.9
180	1.0	0.5	2.5	1.9	5.9

[a] The ApApA (58 absorbance units at 259 nm), ammonium acetate (62.5 μmoles), and step VI enzyme (0.5 μg) in water to a volume of 250 μl, pH 5.5, were incubated at 37°. At the intervals indicated above, aliquots (25 μl) were removed, the reaction quenched with glacial acetic acid (5 μl), and then chromatographed in solvent A for 40 hr. The nucleotides were detected under ultraviolet light, eluted, and then estimated at 259 nm.

TABLE VII: Transesterification by Step VI Exonuclease with pTpdT as Substrate.

Product[a]	Relative Molar Amounts
Thymidine	8.5
Tp	4.0
TpT	63.0
TpTpT	7.5
TpTpTpT	1.0

[a] TpT (3.8 μmoles), ammonium acetate (12 μmoles), and step VI exonuclease (0.5 μg) in water (50 μl) at pH 5.5 were incubated at 37° for 30 min and the reaction quenched by addition of glacial acetic acid (10 μl). The products were separated by chromatography in solvent A. The mixture of Tp and TpTpTpT (Razzell, 1961) was resolved by chromatography in solvent C. Nucleotides were estimated at 267 nm after elution with water.

TABLE VIII: Influence of Various Nucleotides on the Rate of Hydrolysis of Thymidine 3′,2,4-Dinitrophenyl Phosphate.[a]

Addition (M)	Hydrolysis Rate
None	100
Thymidine 3′-*p*-nitrophenyl phosphate (10^{-3})	110
Thymidine 5′-*p*-nitrophenyl phosphate (10^{-3})	102
Thymidine 5′-phosphorofluoridate (10^{-3})	100
Adenosine 5′-phosphate (10^{-4})	95
Adenosine 5′-phosphate (10^{-3})	56
Adenosine 3′-phosphate (10^{-4})	95
Adenosine 3′-phosphate (10^{-3})	86
Adenosine 2′,3′-cyclic phosphate (2×10^{-4})	95
Adenosine 2′,3′-cyclic phosphate (2×10^{-3})	49
pApA (10^{-4})	86
pApA (10^{-3})	40
Sodium phosphate (10^{-3})	100
Polyadenylic acid (10^{-3})[b]	102
Polyuridylic acid (10^{-3})[b]	103
Transfer RNA (2×10^{-3})[b]	103

[a] Thymidine 3′,2,4-dinitrophenyl phosphate (0.1 μmole) ammonium acetate (25 μmoles), step VI enzyme (0.15 μg), the appropriate amount of additive and water (100 μl), pH 5.5, were incubated for 15 min at 37°. The released 2,4-dinitrophenol was estimated at 360 nm (see text). [b] The molarity is that of mononucleotide in the polymers.

Purification of the exonuclease followed established procedures (Table II). The most significant chromatographic difference between the *O. tschawytscha* testis enzyme and the analogous enzymes from spleen and *Lactobacillus acidophillus* is that the latter enzymes bind to DEAE-Sephadex A-50 (Bernardi and Bernardi, 1966) or to DEAE-cellulose (Fiers and Khorana, 1963), whereas testis enzyme is not retained by DEAE-cellulose.

The purified step VI enzyme was free of other hydrolytic activities which act on polynucleotides and nucleotides, although many of these activities were present in the crude

extract (Table III). Acrylamide gel electrophoresis revealed that the step VI enzyme contained two protein components, both capable of hydrolyzing thymidine 3′,2,4-dinitrophenyl phosphate. The two components were not resolved on Sephadex G-200 and hence are not likely to be monomer and dimer. The molecular weight of the enzyme, as determined from its position of elution from Sephadex G-200, is approximately 100,000 and this large size is comparable with that of spleen exonuclease which has a sedimentation constant of 4.6 S (Bernardi and Bernardi, 1968). The freedom from other enzymatic activities and the stability of the step VI enzyme suggest that it is a potentially useful tool in nucleic acid biochemistry.

The testis diesterase catalyses the hydrolysis of ApApA, ApA, CpA, and UpA at approximately equivalent rates indicating that there is little, if any, base preference. The hydrolysis of dTpdT and the various synthetic esters of thymidine 3′-phosphate indicate a lack of specificity with regard to pentose. Of interest is the ability of the enzyme to hydrolyze thymidine 3′-phosphorofluoridate to thymidine 3′-phosphate and the lack of interaction with the enzyme of thymidine 5′-phosphorofluoridate. This contrasts with Hela cell nuclei exoribonuclease which is inhibited by thymidine 3′-phosphorofluoridate (Sporn *et al.*, 1969).

The interaction of the enzyme with adenosine 2′,3′-cyclic phosphate and adenosine 3′,5′-cyclic phosphate was investigated since these are nucleoside 3′-phosphate derivatives. Neither was hydrolyzed, however, adenosine 2′,3′-cyclic phosphate inhibited the hydrolysis of thymidine 3′,2,4-dinitrophenyl phosphate.

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