A SPECIFIC PHOSPHODIESTERASE FROM BEEF PANCREAS*

by

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The specificities of phosphodiesterase preparations from a variety of sources have been studied recently by the use of various esters of ribonucleotides. The mechanism of ribonuclease action has been determined largely by the use of simple esters of nucleotides as substrates for the enzyme[1,2]. Enzyme fractions from spleen, intestinal mucosa, potato and rye grass have been shown to hydrolyze the benzyl group from the benzyl esters of 3′ cytidylic and 3′ adenylic acids, but not from the benzyl esters of the corresponding 2′ nucleotides[3]. DEKKER[4] has reported that the venom of *Crotalus adamanteus* hydrolyzes nucleoside-2′,3′ phosphates to the corresponding 3′ nucleotide. WHITFELD, HEPPEL AND MARKHAM[5] have shown recently that phosphodiesterase preparations from calf spleen, calf intestinal mucosa, and rye grass hydrolyze the nucleoside-2′,3′ phosphates. The fractions from spleen and intestinal mucosa produce almost entirely the 2′ nucleotide from the corresponding cyclic diester, whereas the phosphodiesterase in the rye grass preparation apparently hydrolyzes adenosine-2′,3′ phosphate with the formation of approximately equivalent amounts of adenosine-2′ phosphate and adenosine-3′ phosphate.

This communication reports the partial purification from beef pancreas of a phosphodiesterase which is specific for the hydrolysis of purine and pyrimidine nucleoside-2′,3′ phosphates to the corresponding nucleoside-2′ phosphates.

EXPERIMENTAL

Partial purification of phosphodiesterase. Beef pancreas was collected immediately after the death of the animal, trimmed from fat and connective tissue, and dropped into an acetone-dry ice mixture. The remaining steps were carried out at 4°. 500 g of the frozen pancreas were ground into 1 l of cold acetone, stirred for one hour, and centrifuged. The acetone wash and centrifugation were repeated, and the residue was dried overnight *in vacuo*. The residue was then stirred for four hours with 1 l of 0.1 *M* acetate buffer at pH 6.0, and centrifuged. The supernatant solution was fractionated with ammonium sulphate. The material which precipitates at 0.6 to 0.8 saturation contained the major portion of the enzymic activity. This fraction was collected by centrifugation, dissolved in water, and dialyzed for 36 hours against several changes of distilled water. The fraction was then centrifuged, and the supernatant liquid passed through a column of IRC-50 (200–400 mesh; 2.5 × 12 cm) which had been equilibrated with 0.1 *M* acetate buffer at pH 6.0, and washed with water. The enzyme was washed through the column with water, and did not appear to be retained on the column to any marked extent. This step completely removed the ribonuclease which contaminated the fraction. The column eluate was lyophilized. The preparation

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contained a small amount of a monophosphatase which was inhibited by the addition of 0.03 *M* fluoride to the incubation medium. Yield: 300 mg.

Nucleoside-2',3' phosphates. Guanosine-, adenosine-, cytidine-, and uridine-2',3' phosphates were prepared, by the method of BROWN, MAGRATH, AND TODD[6].

Nucleoside-2' and 3' benzyl phosphates. Adenosine-2' benzyl phosphate and adenosine-3'-benzyl phosphate were prepared according to BROWN, HEPPEL AND HILMOE[3]. Cytidine-2' benzyl phosphate and cytidine-3' benzyl phosphate were prepared by the method of BROWN AND TODD[2]. Uridine-2' benzyl phosphate and uridine-3' benzyl phosphate were synthesized by reacting uridine-2' phosphate and uridine-3' phosphate, respectively, with phenyldiazomethane[2]. The benzyl esters were purified by elution from a cellulose column by the use of a solvent mixture of *iso*propanol, ammonia, and water[6]. The uridine benzyl phosphate which was prepared from uridine-3' phosphate contained at least 95% of the 3' isomer, as determined by completeness of ribonuclease action, and the benzyl ester which was prepared from uridine-2' phosphate contained at least 95% of the 2' isomer.

Adenosine-5' benzyl phosphate. 500 mg of adenosine-5' phosphate were suspended in 10 ml of dimethyl formamide and phenyldiazomethane (from 3 g of benzaldehyde hydrazone) was added. The flask was stoppered, and stirred for 18 h at room temperatures. 50 ml of ether were added to the reaction mixture and the precipitate was washed three times with 50 ml portions of ether and finally dried. The dried material was chromatographed on a 5 × 30 cm column of cellulose. The column was developed with the *iso*propanol-ammonia-water solvent of BROWN, MAGRATH AND TODD[6]. The fractions of the major peak were combined, and the solvent removed under reduced pressure. The residue was washed with ether and dried. The material was homogeneous by paper electrophoresis and paper chromatography. In butanol-acetic acid-water solvent[7] the compound exhibited an R_F value of 0.40 which is in agreement with that recorded by BROWN AND TODD[8] for adenosine-5' benzyl phosphate. In phosphate buffer at pH 2 and in sodium bicarbonate at pH 8 the substance exhibited an electrophoretic mobility close to that of adenosine-2' benzyl phosphate. In 0.4 *M* borate buffer of pH 9.2, however, the mobility was 63% greater than that of the 2' ester, owing to the formation of a borate complex with the *cis*-hydroxyl groups of the 5' ester. The substance was stable to 0.5 *M* sodium hydroxide for 24 hours at room temperature, and was degraded readily to adenosine by rattlesnake (*Crotalus adamanteus*) venom.

Ribonucleic acids. Yeast ribonucleic acids were isolated from fresh baker's yeast by the procedure of CRESTFIELD, SMITH AND ALLEN[9].

Deoxyribonucleic acids. Deoxyribonucleic acids were prepared by the method of SIMMONS *et al.*[10] from calf thymus and kindly supplied by Dr. E. L. DUGGAN.

Incubations of the phosphodiesterase preparation with nucleotide esters were carried out at room temperature. Each incubation mixture contained 0.5% of the ester, 0.5% of the enzyme, 0.03 *M* fluoride and 0.1 *M* triethanolamine buffer, at pH 7.4. The total volume of the reaction mixture was 40 μl. Incubations were carried out over periods ranging from 4 to 24 hours.

Enzymic action was determined by subjecting aliquots of the reaction mixtures to paper electrophoresis. The paper electrophoresis apparatus developed by CRESTFIELD AND ALLEN[11] was employed. The conditions for the separation of product from substrate were: 0.2 *M* ammonium bicarbonate, pH 8.4, and a field strength of 25 V/cm for 45 min.

The identification of the isomer of each mononucleotide which was produced by enzymic action was performed by the method of CRESTFIELD AND ALLEN[12].

RESULTS

The phosphodiesterase fraction hydrolyzed guanosine-2',3' phosphate to guanosine-2' phosphate, adenosine-2',3' phosphate to adenosine-2' phosphate, cytidine-2',3'-phosphate to cytidine-2' phosphate, and uridine-2',3' phosphate to uridine-2' phosphate. The percentage of 3' isomer that could have been present and escaped detection would not have exceeded 10% in any of the hydrolysates. The cyclic nucleotides were hydrolyzed completely in 6 hours. The following compounds showed no evidence of hydrolysis under similar conditions after 24 hours: adenosine-2' benzyl phosphate, adenosine-3' benzyl phosphate, adenosine-5' benzyl phosphate, cytidine-2' benzyl phosphate, cytidine-3' benzyl phosphate, uridine-2' benzyl phosphate, uridine-3'-benzyl phosphate, adenosine diphosphate, and adenosine triphosphate. 0.001 *M bis p*-nitrodiphenyl phosphate was not hydrolyzed over a period of 12 h as determined by the spectrophotometric method of SINSHEIMER AND KOERNER[13].

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Action of the enzyme fraction on deoxyribonucleic acids and ribonucleic acids

Deoxyribonucleic acids and ribonucleic acids were incubated with the enzyme fraction for 24 h at 22° in 0.1 *M* phosphate buffer, pH 7.4. The concentrations of substrate and enzyme were each 1%. Thymol was added to inhibit growth of microorganisms. The reaction mixtures were then chromatographed in two dimensions on Whatman #1 paper as described by SMITH AND ALLEN[14]. The deoxyribonucleic acids showed no evidence of hydrolysis by the enzyme. The ribonucleic acid was hydrolyzed slightly. The following percentages of each mononucleotide originally present in the ribonucleic acids were found as free mononucleotides after enzymic action: guanosine-3′ phosphate 3%, adenosine-3′ phosphate 2%, cytosine-3′ phosphate 6%, and uridine-3′ phosphate 5%.

DISCUSSION

Previous phosphodiesterase preparations[1,3,4,5] which hydrolyze nucleoside-2′,3′-phosphates have also exhibited pronounced activity towards other esters of nucleotides or toward nucleic acids or their partial hydrolysates. The inactivity of the present enzyme preparation towards benzyl esters of nucleoside-2′,3′, or 5′ phosphates suggests that previous phosphodiesterase preparations which hydrolyze 3′ or 5′-nucleoside benzyl phosphates in addition to cyclic nucleoside phosphates are mixtures of enzymes of different specificities. This might well be the case with those enzyme preparations which, for example, hydrolyze nucleoside-3′ benzyl phosphates to form the 3′ nucleotide, but split nucleoside-2′,3′ phosphates to produce the 2′ nucleotides[5].

It is considered unlikely from the results reported here that the enzyme which acts upon the nucleoside-2′,3′ phosphates is also responsible for the partial degradation of the ribonucleic acids. WHITFELD *et al.*[5] report that the spleen fraction which hydrolyzes nucleoside-2′,3′ phosphates in two hours degrades the "ribonuclease-resistant core" of ribonucleic acids completely in 7 hours, whereas the pancreas fraction used here splits the nucleoside-2′,3′ phosphates completely in six hours but has no noticeable activity against nucleoside benzyl phosphates and hydrolyzes yeast ribonucleic acids only to the extent of a few per cent in 24 hours. Additional evidence for the existence of enzymes specific for the 2′:3′ nucleoside phosphate diester is that of BROWN, DEKKER AND TODD[1] in which an enzyme preparation from calf intestinal mucosa exhibits strong diesterase activity toward desoxyribonucleic acids but has no effect on cytidine-2′,3′ phosphate.

The slight hydrolysis of yeast ribonucleic acids on prolonged incubation with the pancreas fraction is probably due to the presence of a minute amount of a nuclease. The existence of nucleases which differ in specificity from Kunitz' ribonuclease has been demonstrated many times. JONES[15] reported that all four mononucleotides are present in the hydrolysate when yeast ribonucleic acid is treated with a boiled extract of pig's pancreas. BREDERECK[16] and BOLOMEY AND ALLEN[17] obtained substantial amounts of purine nucleosides from incubation mixtures of yeast nucleic acids and enzyme preparations from sweet almond meal. SCHMIDT *et al.*[18] report that crude spleen and pancreas extracts act on both purine and pyrimidine nucleotide interlinkages. The spleen nuclease preparation of MAVER AND GRECO[19] was shown by VOLKIN AND COHN[20] to degrade ribonucleic acids to the 3′ mononucleotides, and SHUSTER AND KAPLAN[21] found a nuclease to be present in their preparation of *b*

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nucleotidase that hydrolyzes ribonucleic acids completely. HOLDEN AND PIRIE[22] have effected a 230-fold purification of a nuclease preparation from pea leaves that readily degrades the "ribonuclease-resistant core" of ribonucleic acids. Since in the present work both the purine and pyrimidine 3′ nucleotides were liberated from ribonucleic acid, this second enzyme may be similar in its action on ribonucleic acids to the phosphodiesterase from spleen as described by BROWN *et al.*[3]. The separation of the four mononucleotides from the ribonucleic acids reaction mixture calls to mind the hitherto unexplained results of LORING AND CARPENTER[23] who isolated the four mononucleotides from a ribonuclease hydrolysate of yeast ribonucleic acids. A possible explanation is that certain of their ribonuclease preparations were contaminated by traces of a second pancreatic nuclease.

SUMMARY

1. A specific phosphodiesterase fraction from beef pancreas has been obtained which hydrolyzes nucleoside-2′,3′ phosphates to the corresponding 2′ nucleotide.

2. No hydrolysis of nucleoside-2′,3′ or 5′ benzyl phosphates was observed with this fraction.

3. Prolonged incubation of the enzyme preparation with yeast ribonucleic acid yielded traces of the four mononucleotides, which are attributed to the presence of an unknown phosphodiesterase.

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