AN INVESTIGATION OF PHOSPHODIESTERASE ACTIVITIES IN PLANT SOURCES

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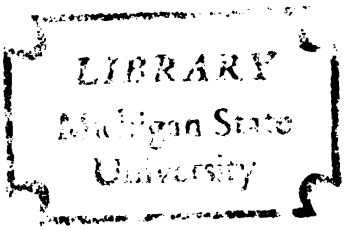
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ABSTRACT

AN INVESTIGATION OF PHOSPHODIESTERASE ACTIVITIES IN PLANT SOURCES

by Richard E. Jagger Jr.

An investigation of the phosphodiesterase activity in plant sources has been described. p-Nitrophenylnucleoside-5'-phosphates and p-nitrophenylthymidine-3'-phosphate used as substrates for the phosphodiesterase were synthesized chemically. Purine and pyrimidine oligonucleotide substrates for phosphodiesterase assays were prepared from DNA by chemical methods. Assay systems for measuring the hydrolysis of the above substrates by crude tissue extracts were developed. An investigation of the distribution of the phosphodiesterases in extracts from a variety of plant seedlings was conducted, and the possible specificity of the phosphodiesterase I activity of the extracts from plant seedlings was examined.

Two phosphodiesterase activities were found in all the plant sources tested. A nonspecific phosphodiesterase activity was found with a pH optimum of 5.0, and a phosphodiesterase I activity was found with a pH optimum of 8.9, the activity of the latter being variable among differing sources but having an average specific activity of 4.3 units per milligram protein. A preference in the rate of hydrolysis of the pyrimidine nucleotide derivatives over

the purine nucleotide derivatives was shown by all tissue extracts. A high rate of hydrolysis of p-nitrophenylthymidine-5'-phosphate over the other nitrophenyl compounds was exhibited by most plant and animal tissue extracts. Two members of the family Cucurbitaceae, muskmelon and cucumber, showed an unusual phosphodiesterase I activity. The pyrimidine/purine ratios of 2.70 and 3.29 determined by the ratio of the rates of hydrolysis of the p-nitrophenylnucleoside-5'-phosphates were two fold higher than that of the plant average, 1.37.

No correlation was found between the phylogenetic position in the plant kingdom and the specificity or distribution of phosphodiesterase activity from plant seedling extracts. For crude extracts of phosphodiesterase I from rabbit kidney and muskmelon, there was no correlation between the K_m values, and thus the affinity of the enzyme for a substrate, and the relative rates of hydrolysis of the nitrophenyl substrates.

AN INVESTIGATION OF PHOSPHODIESTERASE ACTIVITIES IN PLANT SOURCES

By

Richard E. Jagger Jr.

A Thesis

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To my parents and to Carole

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INTRODUCTION

Phosphodiesterases are a group of enzymes which hydrolyze the bond between a phosphoryl function and one of two ligands linked to it, the latter possessing an alcoholic function through which the phosphate ester bond is formed. Although there is an abundance of phosphodiesterase activities in nature, the investigation of plant phosphodiesterases has been minimal. In the metabolism of nucleic acids, there are enzymes which hydrolyze inter-deoxyribonucleotide bonds, those which hydrolyze inter-ribonucleotide bonds, and those which hydrolyze both of these. Hydrolysis of both inter-ribo and inter-deoxyribonucleotide with the stepwise formation of 3' or 5' nucleotides is characteristic of a class of phosphodiesterases or exonucleases, the latter term used somewhat interchangeably with the former, of which phosphodiesterase I and phosphodiesterase II are primary examples.

Phosphodiesterase I, using the nomenclature of W. E. Razzell (1), exhibits an absolute specificity for a nucleoside with a 5' phosphoryl residue and a 3' hydroxyl function. This enzyme liberates 5' mononucleotides from a number of substrates which include DNA, RNA, coenzymes and a number of synthetic substrates including nucleotide polymers and p-nitrophenylnucleoside 5' phosphates. The hydrolysis of

the nitrophenyl-pdX* demonstrate unequivocally the presence of phosphodiesterase I activity in mixtures containing other polynucleotidases (1).

The first report of phosphodiesterase I activity was in 1932 by Uzawa (2) who found this activity as a component of snake venom. An enzyme which liberated 5' nucleotides from DNA and RNA was prepared by W. Klein in 1933 from intestinal mucosa of calves (3). Until the late 1950's the primary work on phosphodiesterase I was concerned with the purification and properties of the enzymes from these two sources, snake venom (4-12) and intestinal mucosa (13, 14). Recently, the distribution of phosphodiesterase I was examined in several other sources; hog kidney (15, 16), animal tissue (17), hog liver (16), rat liver (18), peas, corn and potato (19, 16), malt (20) and carrot (21). The activities from malt and carrot are the only recent examples of phosphodiesterase I purified from plant sources. Phosphodiesterase I from snake venom has still been the principal source of the enzyme for investigation. Numerous approaches to the purification to homogeneity and examination of the properties of this enzyme have been reported (22-27).

Although the preparations of phosphodiesterase I from all of the various sources are still far from homogeneous, numerous properties of these enzymes have been described. The reaction catalyzed by phosphodiesterase proceeds with

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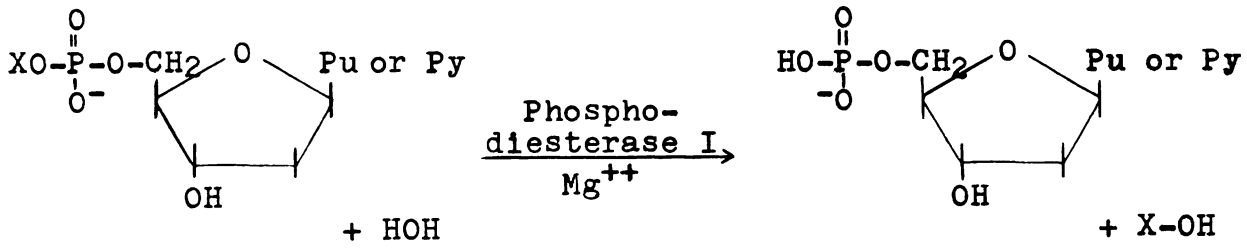
*The abbreviations used in this manuscript are those adopted by the Journal of Biological Chemistry 241, 527 (1960).

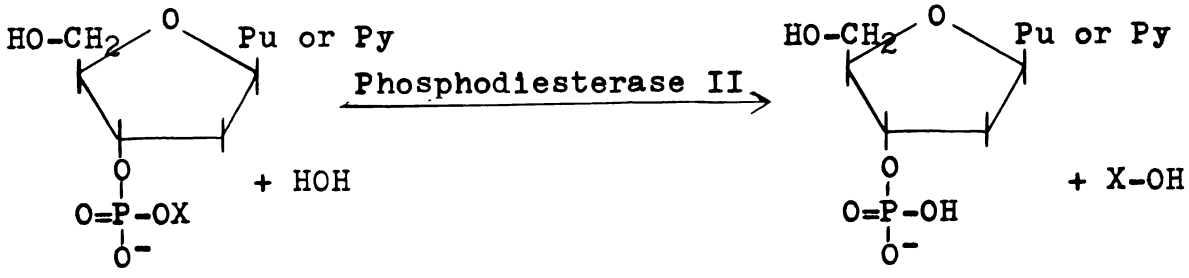
the liberation of 5' mononucleotides from substrates of the form Xp-nucleoside according to the equation:



Using synthetic nucleotides, Razzell and Khorana (28) have shown the mononucleotides to release sequentially from the 3' termini. In the same work, the enzyme was found to be little affected by the substituent attached to the 5' nucleotide. The reaction has a high pH optimum for activity between pH 8.9 and 9.5 for the activity from most sources. Phosphodiesterase I activities have been shown to be enhanced by the presence of Mg^{++} and inhibited by EDTA. Other properties of these enzymes are considered in reviews of phosphodiesterases (1, 29-32).

Phosphodiesterase II is the complementary exonuclease to phosphodiesterase I. The substrates must have a free 5' hydroxyl function and the products liberated are 3' nucleotides. This type of enzyme was originally found in extracts from calf spleen (33). Since 1953 the enzyme has been partially purified and its properties have been reported (1, 34-39). Phosphodiesterase II has also been found localized in several tissues of animals (17) as well as in extracts

from Lactobacillus acidophilus (40). The general properties of the bacterial enzyme have been studied (41). The exonuclease activity can be measured by the release of acid soluble products from DNA or RNA oligonucleotides (36, 37, 39) or by release of nitrophenol from Tp-nitrophenyl. The latter assay is used to demonstrate the unequivocal presence of this activity (38). The pH optimum of the enzyme activity in different sources is rather broad, from pH 5.8-7.8 and the activity is not inhibited by the presence of EDTA. The general equation for the reaction is:



The enzyme activity is not independent of the group (X) attached to the 3' phosphoryl moiety. The rate of hydrolysis is considerably slower when a nucleotide or oligomer is attached to the 3' phosphoryl group in place of the nitrophenyl group. The spleen enzyme is much less sensitive to the ligand attached to the nucleotide. The rate of hydrolysis of p-nitrophenyl nucleotides and of oligonucleotides are within a factor of two (38), with the natural substrate being hydrolyzed most rapidly. Phosphodiesterase II hydrolyzes oligonucleotides stepwise from the 5' termini

liberating 3' mononucleotides (38). There are no marked differences in the rates of hydrolysis with either enzyme, due to the presence of the ribose in place of the deoxyribose moiety or the nature of the base present. Again the homogeneity of these enzymes has not been established and although there are no reliable values for many of the properties of phosphodiesterase II, several reviews include some general properties (1, 27-29).

Because of the absence of a general survey of the phosphodiesterase activities in the plant kingdom, it was considered desirable, as well as interesting, to conduct an investigation of the various phosphodiesterase activities present in plants, in hope of discovering an enzyme of unusual specificity which could be used in nucleotide sequence studies. In the broad sense, the aim of this study was to gain further insight into the nature of phosphodiesterases found in the plant kingdom.

METHODS AND MATERIALS

Substrates

The nucleotides and nucleosides as well as the deoxyribonucleic acid used in these syntheses were commercial products of Nutritional Biochemicals Corp. or Sigma Chemical Company. The diethylaminoethyl cellulose (DEAE-cellulose) was purchased from Bio Rad Laboratories. Organic reagents were obtained from Distillation Products Industries or Mallinckrodt Chemical Works. The hydrazine was purchased from Matheson Coleman and Bell. Other chemicals were reagent grade obtained from commercial sources. All reaction solvents were dried over calcium hydride or Linde 4-A Molecular Sieves. Di-p-nitrophenyl phosphate and nitrophenyl-pT, sodium salt, were purchased from Calbiochem.

Synthesis of p-Nitrophenylthymidine-3'-phosphate

The synthesis of Tp-nitrophenyl was carried out by the reaction of p-nitrophenyl phosphorodichloridate with 5'-O-tritylthymidine. The phosphorylating agent was prepared as described by G. M. Tener (42). Twenty grams of powdered sodium-p-nitrophenoxide was added slowly to 125 ml of ice cold phosphorus oxychloride in a round bottom flask equipped with a stirring apparatus and reflux condenser. After addition of the sodium salt the reaction mixture was kept in ice for one hour after which the sodium chloride was

removed by suction filtering. Phosphorus oxychloride was removed under reduced pressure and the product was maintained at 0.01 mm for 90 minutes. The p-nitrophenyl phosphorodichloridate was distilled using a shortpath apparatus (bp = 130 at 0.02 mm). 5'-O-Tritylthymidine was prepared by the method of P. T. Gilham and Khorona (42); 456 mg of thymidine was dissolved in 13 ml dry pyridine and treated subsequently with 536 mg of triphenylmethyl chloride. After standing 7 days in the dark the solution was poured into 100 ml cold water. The amorphous white solid formed was recrystallized from benzene. The yield was 68%, based on original thymidine.

Five hundred forty mg of 5'-O-tritylthymidine was dissolved in 2.5 ml of anhydrous dioxane and added dropwise to a solution of 500 mg of p-nitrophenyl phosphorochloridate in 2.5 ml dioxane and .35 ml dry pyridine (43). The addition was completed and the reaction solution was stirred for an additional hour in the exclusion of moisture. The reaction was quenched by adding .3 ml pyridine and 1.5 ml of water. The crude product was extracted with chloroform, the latter being washed twice with 1 M pyridine-HCl pH 5.5. The organic solvent was evaporated to dryness. The gum was taken up in 10 ml of 80% acetic acid and hydrolyzed 20 minutes at 100°C. The solution was taken to dryness and redissolved in 10 ml of water. After standing 18 hours at 4° the triphenyl carbinol was filtered off and the solution was lyophilized. An overall yield of 10% was obtained, based on 5'-O-tritylthymidine.

Alternate Synthesis of p-Nitrophenylthymidine-3'-phosphate

An alternate method used for the preparation of Tp-nitrophenyl in later work was that described by Borden and Smith (44). 5'-O-Mono-p-methoxytritylthymidine was prepared by the following procedure. Three grams of thymidine was taken up in 20 ml of dry pyridine and 3.85 grams of monomethoxytrityl chloride was added. This mixture was shaken in the dark for 7 days with the exclusion of moisture. The reaction was stopped by the addition of 50 ml of ethanol and the solution evaporated to a small volume in a rotary evaporator. A suspension of 100 ml water and 100 ml chloroform was added to the gum. The organic layer was washed twice with 100 ml of water and dried over Na_2SO_4 for 24 hours. The dry chloroform solution was evaporated to remove the residual pyridine, and the gummy residue was taken up in chloroform. This procedure was repeated twice. The final concentrate was applied to an alumina column (100 g, 10% deactivated), and the 5'-O-monomethoxytritylthymidine was eluted with 100 ml of chloroform and then with 5% methanol in chloroform. Fifteen ml fractions were collected. The product was found in tubes 20-50. These were pooled and the solution was lyophilized. The yield was 80% based on the starting material, thymidine.

Sodium p-nitrophenyl phosphate (1.3 g) was passed through a Dowex 50-8X column (pyridinium form, 1.5 x 25 cm). The pyridinium salt of the compound present in the effluent was taken to dryness. Dry pyridine was added and the compound subsequently dried two additional times. Then 1.35 g

of 5'-O-monomethoxytritylthymidine was added. Addition of dry pyridine and subsequent flask evaporation was carried out twice. Ten ml of dimethylformamide (DMF) and 5 ml of dry pyridine were added to the 5'-O-monomethoxytritylthymidine and pyridinium p-nitrophenylphosphate, as well as 250 mg of Dowex 50-80X (H^+ form), dried at 130° for 24 hours.

Condensation of p-nitrophenylphosphate and 5'-O-monomethoxytritylthymidine commenced upon addition of 2.06 g of dicyclohexylcarbodiimide (DCC). The reaction mixture was shaken 3 days in the dark, after which a fresh quantity (0.50 g) of DCC was added. After additional 2 days of shaking in the dark, 10 ml of water was added to stop the reaction. The mixture was evaporated to a gummy residue and then dissolved in ethanol. The solution was filtered to remove the insoluble dicyclohexylurea and the resin. The supernatant solution containing p-nitrophenylphosphate, 5'-O-methoxytritylthymidine and p-nitrophenyl-5'-O-monomethoxytritylthymidine-3'-phosphate was applied to the top of a 3 x 40 cm column of DEAE cellulose (carbonate form) previously equilibrated with 50% ethanol. The column was washed with 1 liter of 50% ethanol, 500 ml water, and 1 liter of 0.1 M NH_4HCO_3. A gradient of 50% ethanol to 0.1 M NH_4HCO_3 in 50% ethanol (3600 ml total volume) was applied to the column. Twenty ml fractions were collected. Fractions 39-145 containing the product were pooled and lyophilized. The yield was 50% based on the 5'-O-monomethoxytritylthymidine. Treatment with 80% acetic acid for 6 hours at 37° liberated

the precipitate of mono-p-methoxycarbinol. The final yield of product based on 5'-O-monomethoxytritylthymidine was 35%.

Synthesis of p-Nitrophenylnucleoside-5'-phosphates

The nucleoside-pdX compounds were prepared originally by first acetylating the 3' hydroxyl group and the amino group of the nucleotide with acetic anhydride in an analogous procedure to that described by Ralph and Khorana (44). Ten ml of water and 1.0 ml of pyridine were used to dissolve 165 mg of dCMP and the solution was then lyophilized to a powder. The powder was suspended in 5 ml of dry pyridine and 1.5 ml of acetic anhydride. This mixture was shaken in the dark for 18 hours. To the acetylated dCMP solution, 20 ml of water was added and the solution was kept 1.5 hours at 35°. The solvents were removed in a rotary evaporator and the gummy residue was dissolved in water. The solution of the acylated dCMP was evaporated to dryness and the solids redissolved in water. These steps were repeated twice more. The aqueous N-3'-O-diacetyl dCMP was lyophilized to a powder.

The condensation reaction was improvised as a modification of the procedure of Moffatt (4, 46) and Khorana and Smith (47). The lyophilized N-3'-O-diacetyl dCMP powder was added to 19 ml of dry pyridine. To this solution, 700 mg of p-nitrophenol and 1.03 g of DCC were added. The entire mixture was placed in the dark for 7 days. The pyridine and DCC were removed by evaporation in vacuo. The p-nitrophenyldiacetyl dCMP was dissolved in 20 ml of concentrated NH_4OH and

was kept at room temperature 48 hours. The NH_4OH was removed by evaporation. The nitrophenyl-pdC was dissolved in 20 ml of water and the pH was adjusted to 3.5 with HCl. The excess p-nitrophenol was removed by several extractions with ether. The aqueous solution was then lyophilized to a powder. The impure powder containing nitrophenyl-pdC weighed 670 mg. A column technique for purification of this compound was developed following a private communication from M. Smith. The nitrophenyl-pdC powder was dissolved in 30 ml water and the sample was applied to a DEAE cellulose column (1.5 x 30 cm, carbonate form). A gradient of water (1.5 l) to 0.15 M NH_4HCO_3 (1.5 l) was applied to the column and 10 ml fractions were collected. Fractions 41-80, which contained the desired product were pooled and the salt was removed by repeated evaporations. The yield was 13% of the theoretical value based on the starting material, dCMP. Successful results were also obtained from this synthesis using dAMP and dGMP as the starting nucleotides. Yields averaged 10-14%. The low yield was attributed to the concominant hydrolysis of the p-nitrophenyl ester during the hydrolysis of the acetyl group from the p-nitrophenyl-diacetyl dCMP.

Alternate Synthesis of p-Nitrophenylnucleoside-5'-phosphates

A much more convenient synthesis of the nitrophenyl-pdX compounds was found to be a modification of the procedure of Borden and Smith (44). This procedure makes use of the coupling of the nucleotide and p-nitrophenol without

laborious and time consuming removal of protecting groups. In a method similar to the previous procedure, 218 mg of dGMP was passed through a Dowex 50-8X column (1.5 x 25 cm, pyridinium form). The pyridinium dGMP was evaporated to dryness. The dry powder was dissolved in 10 ml of dry pyridine and evaporated to dryness. This procedure was repeated three times. To the dry pyridinium dGMP, 0.7 g of p-nitrophenol was added. These two compounds were dissolved in 10 ml of dry pyridine and evaporated to a gummy residue. To the residue, 3 ml dry pyridine, 3 ml dry DMF, 0.5 ml tributylamine, and 1.03 g of DCC were added. The entire mixture was shaken 24 hours in the dark at room temperature. The solvents were removed by rotary evaporation in vacuo. A mixture of 50 ml water and 25 ml ether was added to the dry residue. The suspension was shaken several minutes and the aqueous phase was washed several times with ether to remove excess p-nitrophenol. The insoluble dicyclohexylurea, one of the reaction products, was filtered from the solution. The filtrate was then washed onto a DEAE-cellulose column (3 x 35 cm, carbonate form) and a gradient of water (1.5 l) to 0.15 M NH_4HCO_3 (1.5 l) was applied. Twelve ml fractions were collected. Fractions 50-71, which contained the nitrophenyl-pdG were pooled and evaporated several times to remove the NH_4HCO_3.

Nitrophenyl-pdG was dissolved in water and lyophilized to a powder. The yield based on dGMP was 28% of the theoretical value. Using this technique, the following deoxynucleo-

tide and ribonucleotide derivatives were prepared: nitrophenyl-pdG, nitrophenyl-pdC, nitrophenyl-pdA, nitrophenyl-pA and nitrophenyl-pU. The yields averaged between 25 and 40 percent.

Each of the nitrophenyl-pdX compounds prepared according to these procedures, as well as Tp-nitrophenyl, migrated as a single spot in two solvent systems; isopropanol:water: ammonia (7:2:1) and n-butanol:acetic acid:water (5:2:3). The ultraviolet spectra of these compounds were in agreement with the spectra reported for each of the nitrophenyl derivatives by Borden and Smith (44). The synthesized Tp-nitrophenyl chromatographed identically to a sample of the same compound graciously provided by Dr. Michael Smith.

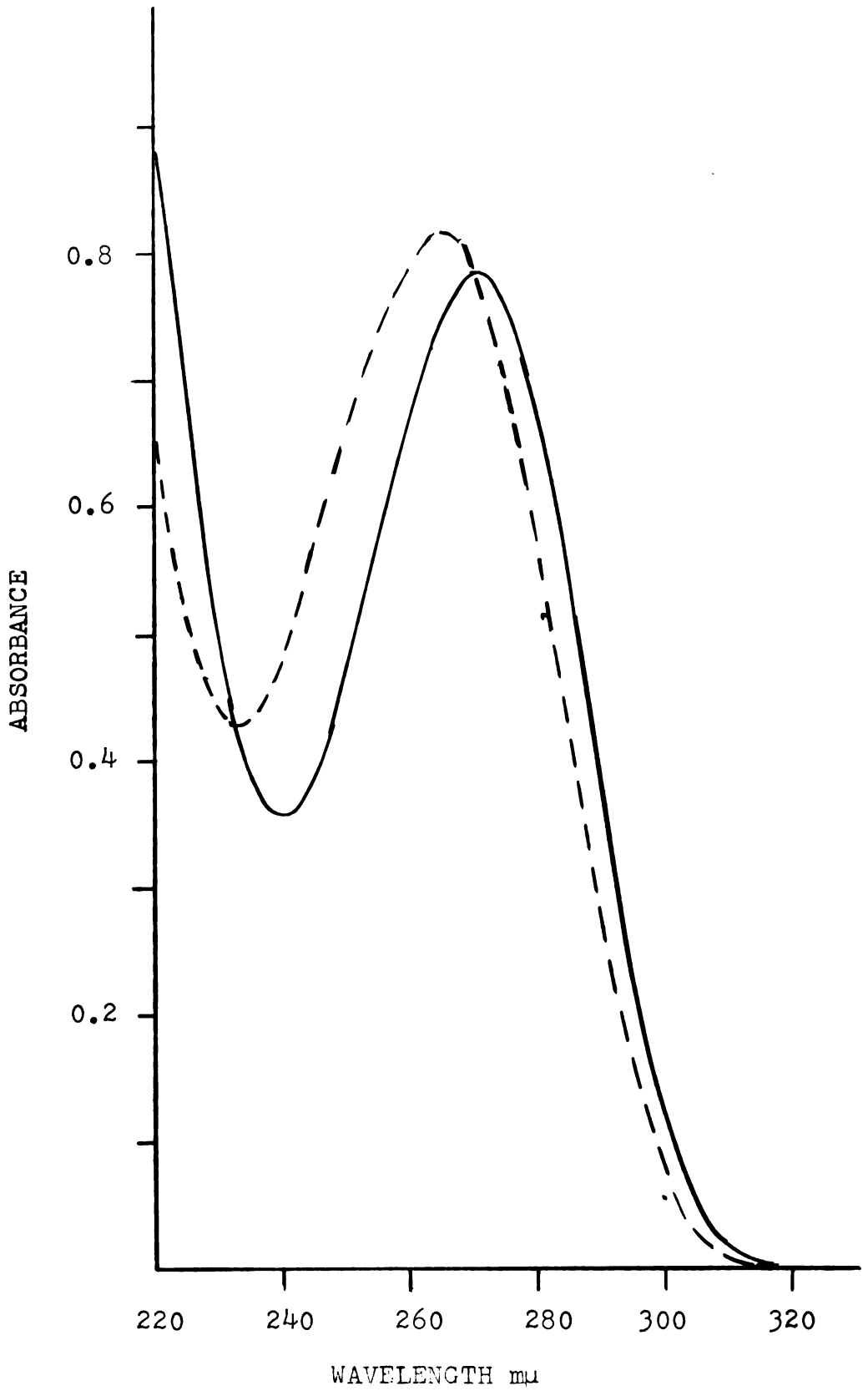
Owing to the tedious procedure of introducing and removing protecting groups from deoxynucleosides, the syntheses of dXp-nitrophenyl compounds have posed somewhat greater problems. Benzoyl chloride, used for protection of amino functions of the heterocyclic bases, is a partially nonspecific reagent. Mono-p-methoxytritylchloride and di-p-methoxytritylchloride, used for protection of the 5'-hydroxyl function of the deoxyribose moieties, form trityl derivatives which are very labile. The di-p-methoxytritylchloride is not commercially available and must be synthesized via a Grignard type reaction with p-anisylmagnesiumbromide and methylbenzoate (48). After initial attempts to synthesize these 3' p-nitrophenyl derivatives provided poor yields of the intermediate protected nucleosides, the further synthesis was suspended.

A much easier synthesis could be accomplished by the coupling of 3' deoxynucleotides and p-nitrophenol by use of DCC. The 3' deoxynucleotides could be formed by exhaustive hydrolysis of DNA by micrococcal nuclease and spleen phosphodiesterase and separation of the products on a Dowex-1-Cl^- column (49).

Preparation of Pyrimidine Oligonucleotides

The preparation of pyrimidine oligonucleotides was carried out by modifications of the procedures of Spencer and Chargaff (50), H. Shapiro (51), Hall and Sinsheimer (52) and Burton and Petersen (53). Two grams of salmon sperm DNA were dissolved in 80 ml of 0.1 M H_2SO_4. The flask was sealed and the mixture was hydrolyzed in a 100° water bath for 35 minutes. After cooling, the suspension was passed through a 0.45 μ membrane filter to remove the acid-insoluble material. The hydrolysate was then either passed through a Dowex 50-8X column (100-200 mesh, H^+ form, 4 x 5 cm) or treated with 12.5 g of Dowex 50-8X batchwise to remove the purine bases from the solution. This normally results in the recovery 45-48% of the total A_{260} from the hydrolysate (20,000 A_{260}). The spectra of the solution after treatment with Dowex-50 shows a shift to higher wavelength indicative of removal of purine bases (Figure 1). The hydrolysate (150 ml) was neutralized with NH_4OH and diluted to 1 liter with 0.01 M lithium acetate pH 5.0. The entire sample was then applied to a DEAE-cellulose column (4.0 x 60 cm) previously equilibrated with 0.01 M LiAc pH 5.0, the starting buffer. The purine bases not removed by Dowex-50 treatment were eluted in the wash through peak.

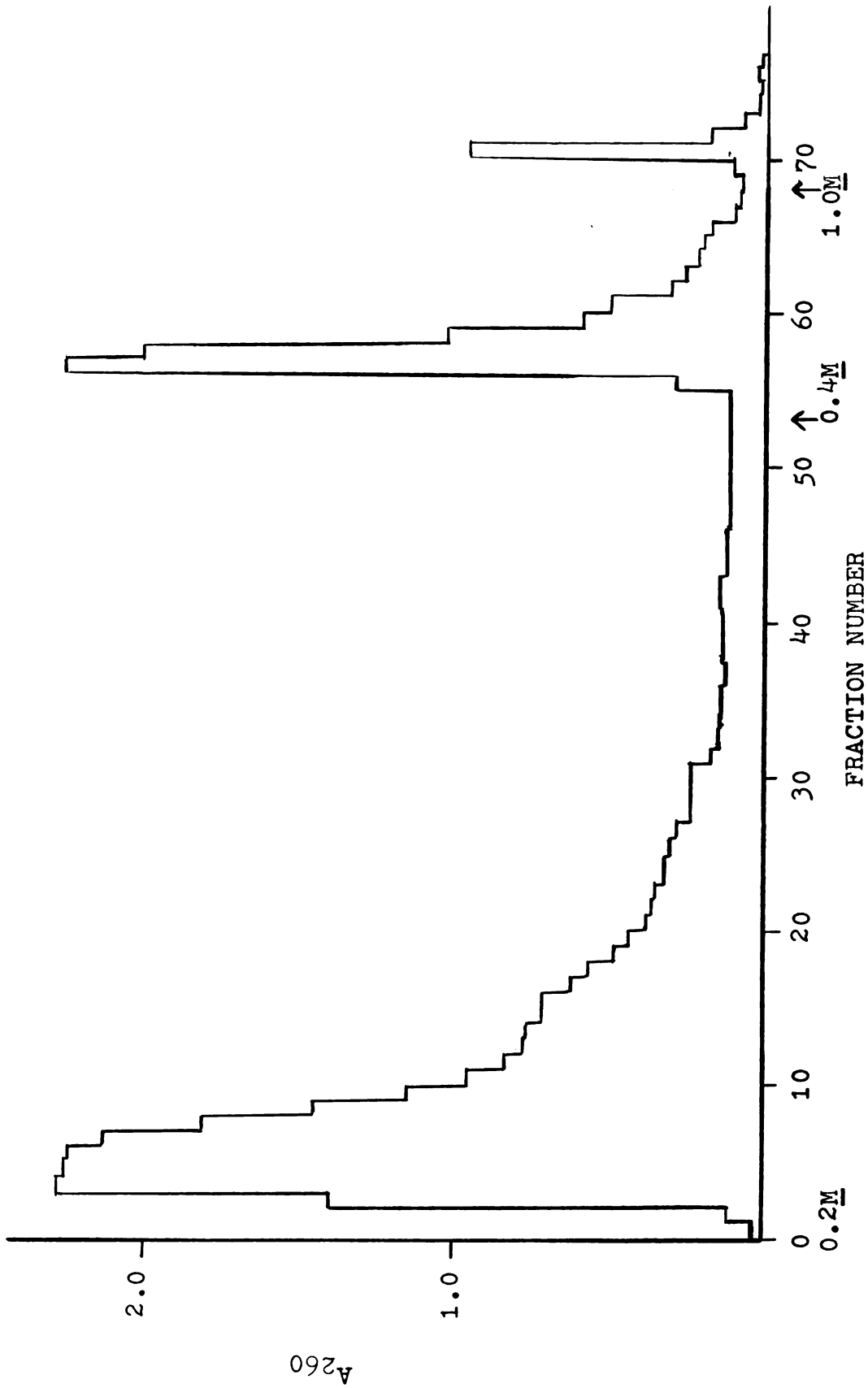
Figure 1: Spectral shift in DNA acid hydrolysates following Dowex 50-8X treatment. The figure is a tracing of spectra run on a Beckman DB spectrophotometer. The pH of the solution is 1.0. (——) Spectrum of DNA hydrolysate is a 1:500 dilution of an aliquot of the solution following the removal of the acid insoluble material. (----) Spectrum of a 1:100 dilution of an aliquot of the solution after treatment with Dowex 50-8X. A shift toward longer wavelength is indicative of removal of the free purine bases from solution by the cation resin.


0.8
0.6
0.4
0.2
ABSORBANCE
220
240
260
280
300
320
WAVELENGTH mμ

A step gradient of lithium chloride was used. The column was washed with starting buffer until the A_{260} was below 0.15. The buffer was changed to 0.2 M LiCl in 0.01 M LiAc pH 5.0 (8 l) and peak I was eluted. When the A_{260} dropped below 0.12, 0.4 M LiCl in the starting buffer (3 l) was applied to the column thereby eluting peak II. The concentration of LiCl in the final step was 1.0 M. This eluted peak III from the column, (Figure 2).

The recoveries of A_{260} based on the original acid hydrolysates were: peak I 31%, peak II 5.8%, peak III 0.8%. The fractions composing peak II were combined and evaporated to a 200 ml volume. This solution was dialyzed against 3 five-liter changes of distilled water. The volume was again reduced (10 ml) and the solution was made 0.5 M in Tris Cl pH 8.5. The oligonucleotides were then incubated at 37° with five units of alkaline phosphatase which had been taken through a 90° heat step. After 24 hours, an additional 2 units of alkaline phosphatase were added. No further release of inorganic phosphate from the oligonucleotides could be detected after 48 hours using the isobutanol-xylene extraction method of Dreisbach for phosphate determination (54). The salt was removed by dialysis (3 five-liter changes of distilled water). The final yield of the desired pyrimidine oligonucleotide was 1,250 A_{260} units. This represents a 3% yield based on the A_{260} of the original acid hydrolysate.

Figure 2: Elution pattern of pyrimidine oligonucleotides from a DEAE-cellulose column (3 x 60 cm). A solution containing 6,500 A_{260} units in 0.01 M lithium acetate pH 5.0 was washed onto the column. This represented 15% of an acid hydrolysate from 2.0 g of salmon sperm DNA. The nucleosides were eluted in the wash-through effluent. A step gradient was applied to the column in the following concentrations of LiCl in starting buffer: 0.2 M, 0.4 M and 1.0 M as indicated. Fractions of 110 ml were collected every 60 minutes.


A_{260}
2.0
1.0
0
0.2M
10
20
30
40
50
0.4M
60
70
1.0M
FRACTION NUMBER

Preparation of Purine Oligonucleotides

The purine oligonucleotides were prepared in a manner similar to that of Sedat and Sinsheimer (55), Chargaff et al. (56), Shapiro (57) and Habermann (58). To 200 ml of dry salmon sperm DNA, 3.0 ml of absolute hydrazine was added and the flask was sealed. The absolute hydrazine was prepared by refluxing 97% hydrazine with KOH and subsequent distillation (60). Hydrazinolysis was carried out for twelve hours at 60°. The mixture was poured into 15 ml ice-cold benzaldehyde very slowly with stirring. The resulting suspension was extracted four times with 15 ml portions of water. The extract was washed several times with ether to remove the residual benzaldehyde. The apyrimidinic acid was made 0.3 N in KOH and heated to 100° for one hour. The hydrolysate was dialyzed 3 times against 5 l of water, neutralized with HCl and diluted 5 fold with 0.01 M lithium acetate pH 5.0. The solution was then applied to the top of a DEAE-cellulose column. Subsequent steps were identical with the isolation of the pyrimidine oligonucleotides. The yield of the desired purine oligonucleotides was 540 A_{260} units. Assuming equal molar concentrations of guanine and adenine nucleotides, a purine molar extinction coefficient of 13,500 at 260 mμ, and an average molecular weight of these residues of 340, this represents 13.6 mg. This gives a yield of 6.8% based on the dry weight of the DNA.

The concentration of the pyrimidine oligonucleotides prepared as described earlier was 0.018 μmoles of nucleotide/

µl determined spectrophotometrically at 260 mµ, assuming equal molar amounts of cytidine and thymidine residues were contained therein. Assuming the average chainlength to be 5 mononucleotide units in length because of the position of elution in ion exchange chromotography, this would result in a substrate concentration of 0.0036 µmoles/µl. A 100 µl aliquot used in assay III would then contain 0.36 µmoles. The concentration of the prepared purine oligonucleotides was 0.0038 µmoles of nucleotide per µl which was also determined at 260 using similar assumptions. The purine oligonucleotide substrate concentration used in assay III was 0.38 µmoles per 100 µl aliquot.

Preparation of the Enzymes

Enzyme solutions to be examined for phosphodiesterase activity against the various substrates described in the previous section were prepared from a variety of plant and animal sources. The enzyme extracts from plants were prepared by the following procedure, a modification of the procedure of Razzell (19). A variety of seeds, purchased from Michigan Seed Foundation or Ferry Morse Seed Co., were germinated on moist newsprint in the dark at 37°C until the shoots and roots had reached a length of 1-2 cm. The radicle and plumule were removed, as well as the cotyledons if present, and placed in ten ml of Tris Cl pH 7.5 per g of wet tissue. This mixture was homogenized in an Omnimixer (16,000 rpm) for 2 minutes followed by centrifugation at 27,000 x g for 20 minutes at 4°. The enzyme was kept at 4° for subsequent

experiments. Protein concentrations of the enzyme extracts were usually 3.0-4.0 mg/ml as determined by the method of Lowry (61).

In some experiments, sodium acetate buffer 0.1 M, pH 5.5, replaced the Tris Cl buffer as the extracting medium to determine if a higher specific activity could be obtained and if any activity was present which was not solubilized at pH 7.5. The specific activities of Triticum vulgaris extracts at pH 5.5 and 7.5 were nearly equal, 1.83 and 1.86 respectively, and the pH optima of the activities were the same. The general use of Tris Cl, pH 7.5 was consequently retained.

Enzyme solutions were prepared from certain animal sources in a similar manner. Kidneys and livers were removed from male New Zealand white rabbits. Sections of these tissues were homogenized as above but in ten ml of 0.25 M sucrose per gram of wet tissue. Subsequent steps were identical with those of plant tissue. Protein concentrations averaged 20 mg/ml.

Venom phosphodiesterase from Crotalus adamanteus (VPH) obtained from Worthington Biochemical Corp. was diluted to five mg/ml with water and passed through a column of Dowex 50-8X, H^+ form, previously equilibrated with 0.005 M NaAc buffer pH 5.8, to remove the 5' nucleotidase activity (62). The pH of the solution was brought to 7.5. The enzyme was kept frozen. Protein concentration of the solution was 320 µg/ml.

A purified preparation of phosphodiesterase I from

Daucus carota sativa (48.8 U/mg of protein), 300 fold purified, was kindly furnished by Dr. C. L. Harvey. This enzyme was used for comparison of rates of hydrolysis upon the nitrophenyl compounds.

Alkaline phosphatase from E. coli (BAP-C) was obtained from Worthington Biochemical Corp. This chromatographically purified enzyme was taken up in a 10 fold dilution of 1.0 M Tris Cl pH 8.0 and 10^{-2} M $MgCl_2$. The enzyme solution was then heated to 90° for twenty minutes to inactivate the contaminating diesterases in accordance with the procedure of Garen and Levinthal (62). The enzyme displayed no diesterase activity over blank value when assayed overnight at 37° with bis-p-nitrophenylphosphate.

Assays for Phosphodiesterase Activity

Two different spectrophotometric assays for phosphodiesterase activity were utilized. Assay I, used primarily for determining plant phosphodiesterases was carried out by a procedure similar to that of Razzell and Khorana (38). The incubation mixture contained in a final volume of 0.3 ml, 0.25 μmoles of nitrophenyl-pT or Tp-nitrophenyl substrate, 100 μmoles of appropriate buffer, 12 μmoles of EDTA or $MgCl_2$ and 100 μl of the enzyme solution previously diluted with water to an appropriate activity. The mixture, minus enzyme, was preincubated for 5 minutes at 37°. The enzyme was then added. Subsequently 0.05 ml aliquots were removed at timed intervals and placed in 1.0 ml of 1.0 N NaOH. The absorbancy was determined at 400 mμ in a Beckman DB spectro-

photometer. Assuming the molar extinction of p-nitrophenol to be 12,000 under these conditions (38), an increase in the absorbancy of 0.2 represents the release of 0.1 μmole of nitrophenol in the original reaction mixture.*

Assay II, primarily used for comparing the rates of hydrolysis of the various nitrophenyl-pdX substrates, was based on the procedure of Razzell and Khorana (4). The reaction was carried out in a Gilford Recording Spectrophotometer equipped with a 37° constant-temperature cell compartment. The substrate solution, consisting of 0.25 μmoles of substrate, 50 μmoles of Tris pH 8.9, and 6 μmoles of $MgCl_2$ in a 0.45 ml volume, was preincubated 10 minutes at 37°. The reaction was commenced by the addition of 0.05 ml of enzyme solution and the increase in the absorbancy was followed with time at 400 mμ. Here an absorbancy increase of 1.2 indicates the hydrolysis of 0.1 μmole of nitrophenyl-pdX. Cuvettes with the entire reaction mixture but without enzyme were used as blanks and showed no change in OD after 20 minutes with each of the nitrophenyl-pdX substrates.

Assays used for the examination of the specificity of venom phosphodiesterase were carried out with two procedures. The assay III substrate solution contained 0.37 μmoles of purine or pyrimidine oligonucleotide substrate, 3 μmoles of $MgCl_2$ and 0.05 units of alkaline phosphatase in a total volume of 210 μl. These were preincubated for 15 minutes to remove any terminal phosphate which may not have been removed in prior dephosphorylation steps in the isolation of the

*A unit is defined as the hydrolysis of one μmole of substrate in the original reaction mixture in one hour. Specific activity is defined as units per mg of protein. Mea

substrates. Ten µl of the enzyme was added to the reaction and the reaction was allowed to proceed for 0, 15 and 30 minute periods. Termination of the reaction was insured by the addition of 10 µl of concentrated HCl. Inorganic phosphate released by the alkaline phosphatase was determined by a modification of the method of Dreisbach (54). Zero time assays were used as a blank in these experiments.

A titrimetric assay, assay IV, was performed as a check on assay III and was a modification of the assay used by Razzell and Khorana (4). A 1.0 ml unbuffered solution containing 1 µmole of oligonucleotide substrate and 3 µmole $MgCl_2$ was adjusted to pH 8.5 and preincubated at 37° for 5 minutes. Fifty µl of the venom enzyme was added to initiate the reaction. The mixture was kept at pH 8.5 by the addition of 0.02 M NaOH delivered from a 0.5 ml barrel syringe on a Radiometer titrimeter equipped with microelectrodes. The temperature was maintained by the use of jacketed reaction vessels and Haake constant temperature bath.

Paper Chromatography of Reaction Products

To determine whether the reaction products were in fact the expected mononucleotides and p-nitrophenol, paper chromatography was carried out with the descending technique using the isopropanol:ammonia:water (7:1:2) solvent system. The filter paper used was either Whatman 3 MM paper which had previously been washed with 0.1 M citrate buffer followed by a water wash, or Whatman No. 40 acid-washed paper depending

upon the nature of the sample. Assay mixtures, each consisting of 10 μmoles of Tris pH 8.9, 0.50 μmoles of nitrophenyl-pdX substrate, and enzyme in a total volume of 0.05 ml, were incubated for 0, 30 and 60 minutes with each substrate. Ten μl of each was placed in 3 μl of glacial acetic acid to stop any reaction. The solutions were then spotted and developed in the system described above.

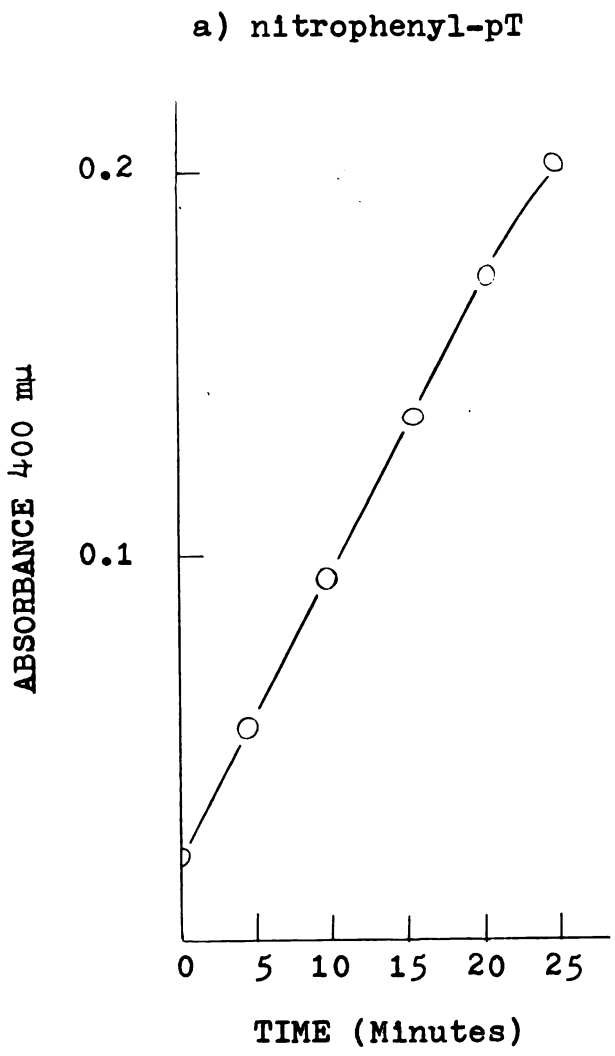
RESULTS

Validity of the Assays

The release of p-nitrophenol from nitrophenyl-pT and Tp-nitrophenyl (Figure 3) in assay I was found to be linear with respect to time and to an OD of 0.12 and linear over a wide range of enzyme concentrations (Figure 4). The spectrophotometric assay II, used for comparing the rates of hydrolysis of several substrates by phosphodiesterase type I enzymes at pH 8.9, was similarly linear with time to an OD of 1.0 and linear with enzyme concentration with both animal and plant extracts on nitrophenyl-pT (Figures 5 and 6). Assay III was linear with time for both purine and pyrimidine oligonucleotides as determined by the inorganic phosphate released.

Evidence that the reaction being examined was in fact the desired reaction was derived from several types of experiments. Assays were carried out to ensure that the reaction was liberating p-nitrophenol and nucleotide products. Paper chromatography of the products of a 30 and 60 minute hydrolysis demonstrated that the products of the reaction have identical R_f values with standard nucleotides and p-nitrophenol. The chromatographs showed no detectable modification of the p-nitrophenyladenosine-5'-phosphate to a deaminated p-nitrophenylinosine-5'-phosphate. The possible presence of a

Figure 3: Linearity of assay I with time for nitrophenyl-pT and Tp-nitrophenyl. The assay conditions are described in the Methods and Materials Section. Aliquots of 0.05 ml were removed at designated intervals and placed in 1.0 ml of 0.1 N NaOH. The solutions were read at 400 mμ. Owing to the slow hydrolysis of the Tp-nitrophenyl, longer intervals between removal of aliquots were used. Crude extracts (50 μl) from T. vulgaris was used in each assay.


a) nitrophenyl-pT
ABSORBANCE 400 mμ
0.2
0.1
0
5
10
15
20
25
TIME (Minutes)

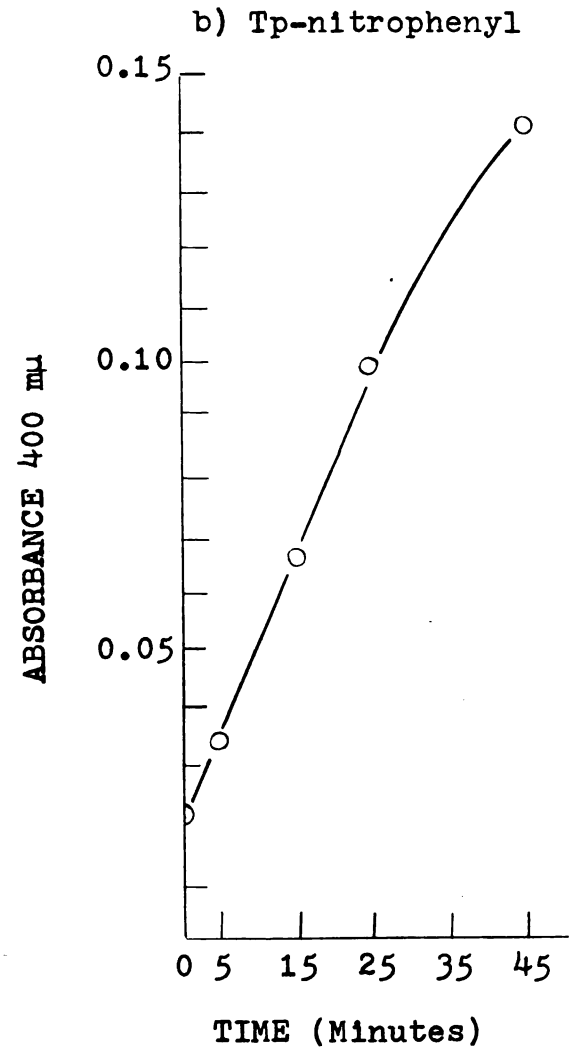
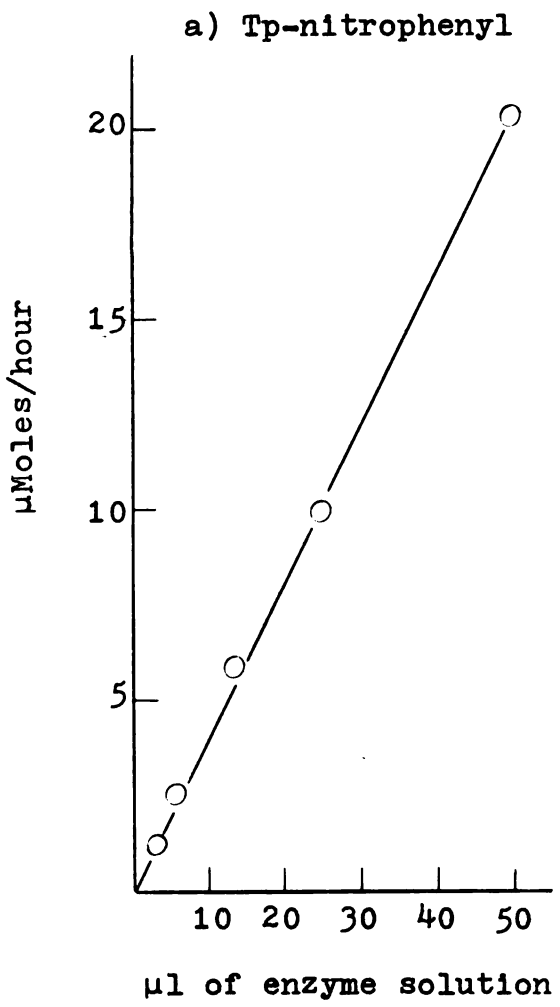

b) Tp-nitrophenyl
ABSORBANCE 400 mμ
0.15
0.10
0.05
0
5
15
25
35
45
TIME (Minutes)

Figure 4: Linearity of the pH optimum assay (assay I) as a function of enzyme concentration. The assay conditions are described in assay I in the Methods and Materials Section. The values for the initial velocity (μmoles of p-nitrophenol liberated) were obtained from plots similar to those found in Figure 3. Extracts from T. vulgaris were used in both determinations.


a) Tp-nitrophenyl
µMoles/hour
20
15
10
5
10 20 30 40 50
µl of enzyme solution

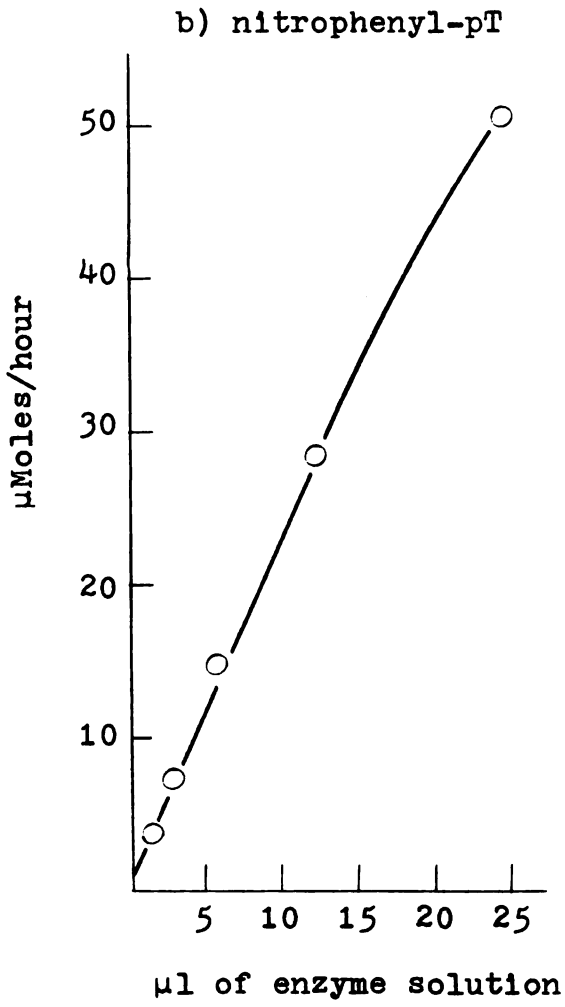

b) nitrophenyl-pT
µMoles/hour
50
40
30
20
10
5 10 15 20 25
µl of enzyme solution

Figure 5: Linearity of assay II with time for three different substrates. Assay conditions are described in assay II in the Methods and Materials Section. Extracts (25 µl) from T. vulgaris was used in all cuvettes except the blank. The cuvettes contained as substrate, 0-0-0 nitrophenyl-pT, □-□-□ nitrophenyl-pdG, X-X-X nitrophenyl-pdC and △-△-△ nitrophenyl-pT. The latter minus enzyme was used as a blank. This diagram is a reproduction of a tracing obtained from a Gilford Spectrophotometer described in assay II.

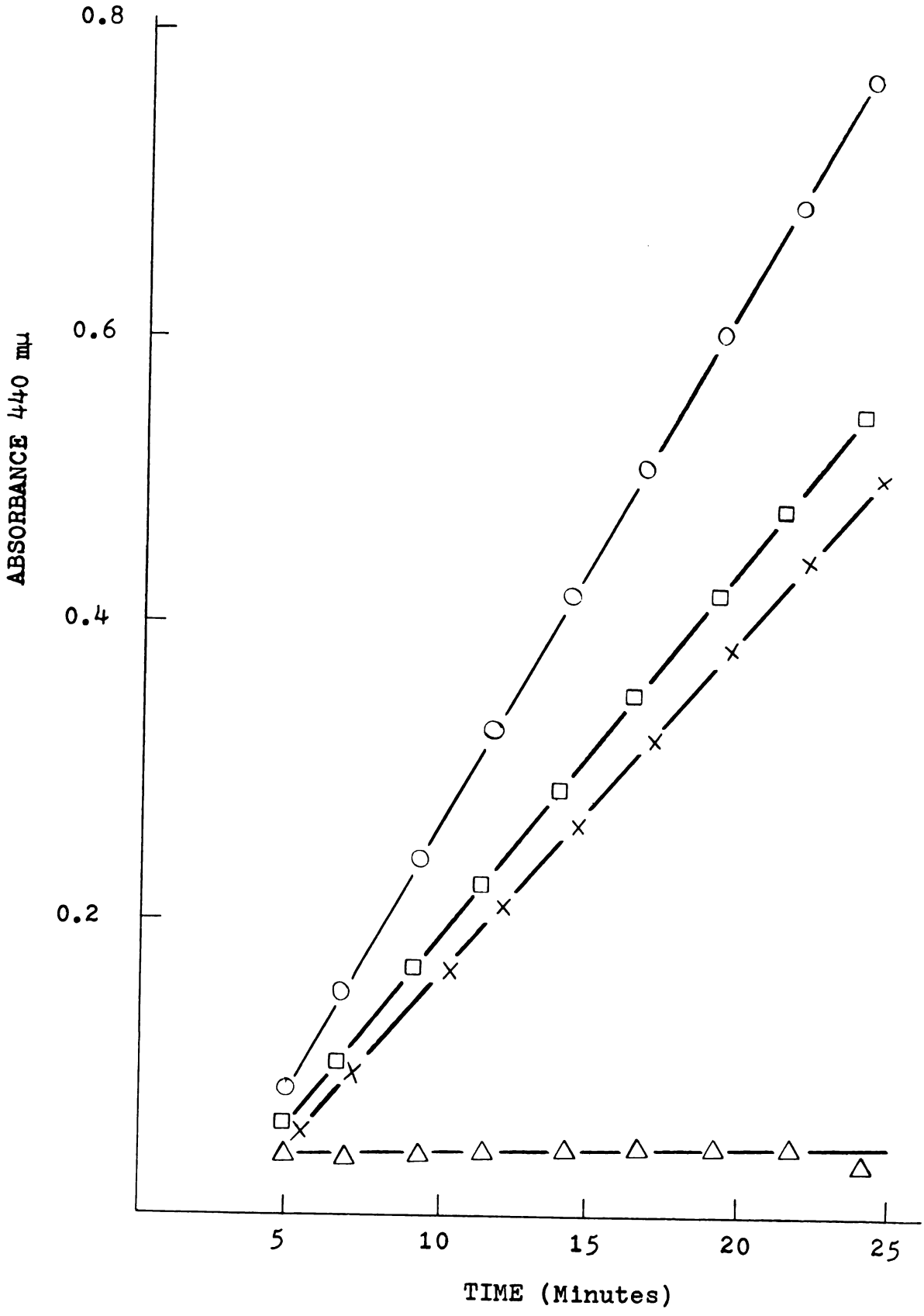
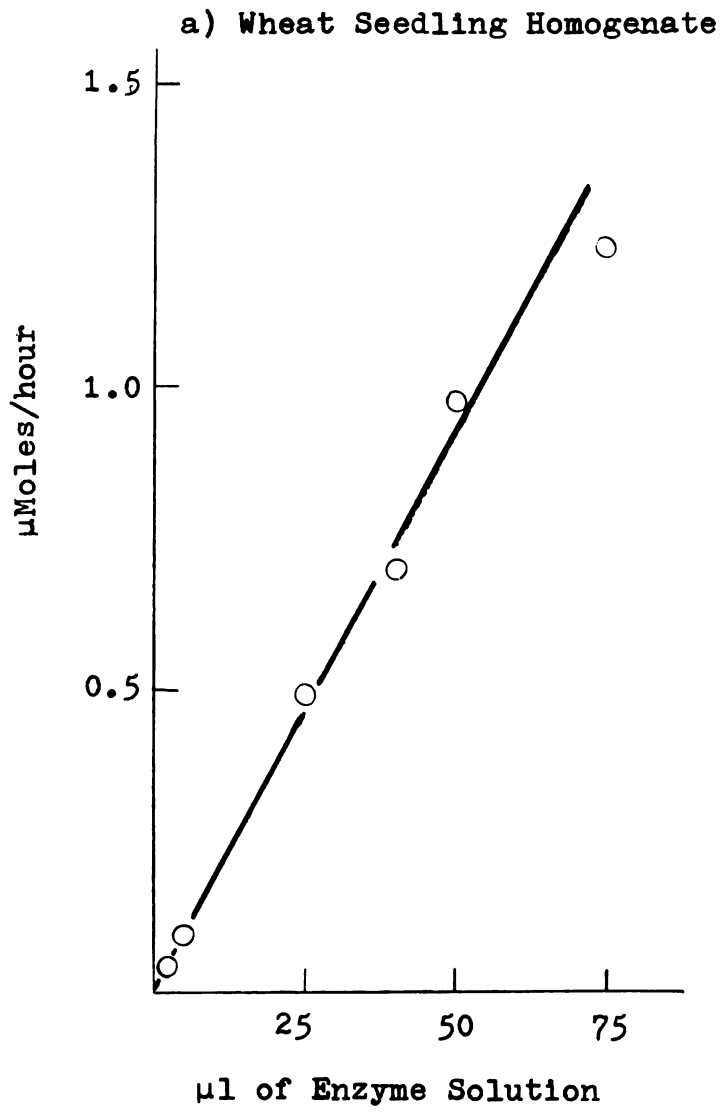
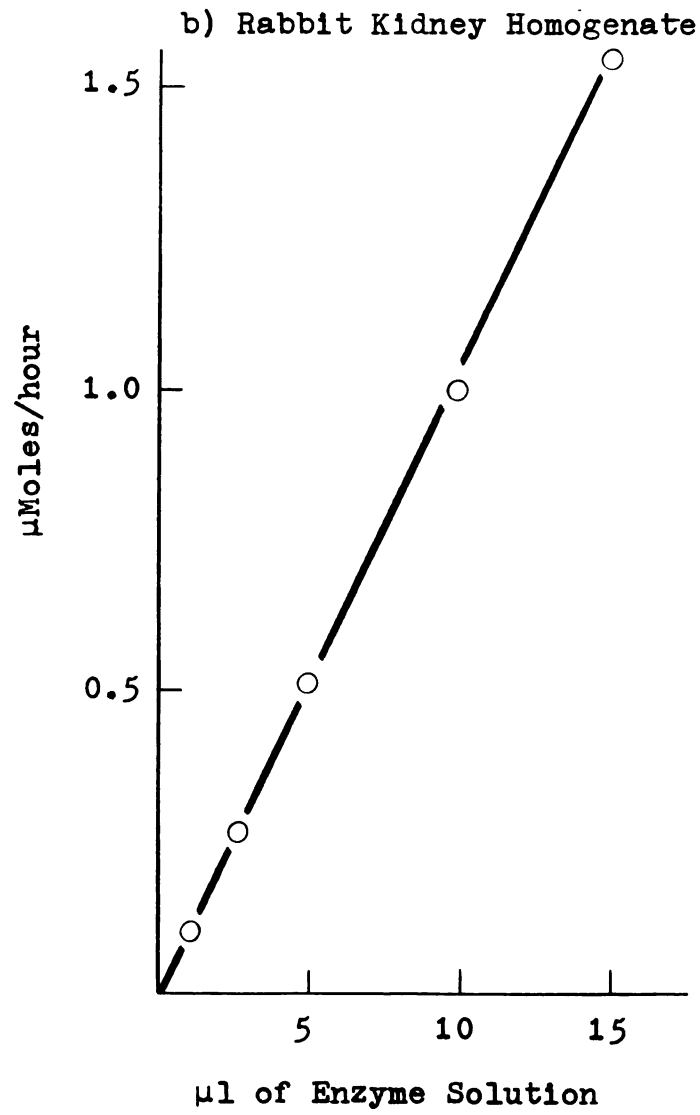

0.8
0.6
0.4
0.2
ABSORBANCE 440 mμ
5
10
15
20
25
TIME (Minutes)

Figure 6: Linearity of assay II as a function of enzyme concentration. The assay conditions are described in assay II in the Methods and Materials Section. Values for initial velocities, μmoles of p-nitrophenol liberated per hour, were calculated from plots similar to those found in Figure 5.


a) Wheat Seedling Homogenate
1.5
1.0
0.5
µMoles/hour
25
50
75
µl of Enzyme Solution


b) Rabbit Kidney Homogenate
1.5
1.0
0.5
µMoles/hour
5
10
15
µl of Enzyme Solution

contaminating adenylate deaminase which might cause the deamination of nitrophenyl-pdA and thus an altered rate of hydrolysis was also examined by the method of Smiley and Seulter (63). No detectable decrease in the absorbance of a 10^{-5} M solution of dAMP at 265 mμ after a 30 minute incubation with enzyme extract suggests no appreciable deamination of nitrophenyl-pdA.

It was considered that the presence of a specific phosphomonoesterase for one nucleotide might cause a distortion in the rates of hydrolysis of the nitrophenyl-pdX substrates by removing the products from the reaction. To examine this possibility, 0.05 units of alkaline phosphatase was added to the assay prior to adding the enzyme extract. The presence of a specific phosphatase would have been diluted, but the ratios of the rates of hydrolysis toward the differing bases did not change more than 6%.

The possibility of the presence of an inhibitor in the crude extracts which would distort the ratio of the rates of hydrolysis was examined in a final validity experiment. The rates of hydrolysis upon the four nitrophenyl-pdX compounds were established for C. adamanteus venom phosphodiesterase I and wheat seedling phosphodiesterase I, the enzyme activities of these two sources being adjusted such that they were similar. Fifty percent mixtures of the two enzymes were added to the assay cuvettes and the rates of hydrolysis against the four substrates were determined. The ratio of the rates of hydrolysis obtained experimentally agreed within

10% of the ratio calculated from the average of the values independently determined.

pH Optima

The pH profiles of the activities of several plant phosphodiesterases (Table I) were similar to previously obtained by several investigators in the examination of animal phosphodiesterases. The activity towards Tp-nitrophenyl was found to occur primarily in the wide range between pH 4 and 8, with an indication of separate peaks at 5 and 7 in several instances. The activities at these two pH's are listed for a variety of plant extracts in Table I. This table also gives the results for nitrophenyl-pT. In all cases, activity was maximal at pH 8.9, the highest pH tested. The addition of EDTA to a concentration of 0.02 M did not appreciably affect the activity against Tp-nitrophenyl; however, activity against nitrophenyl-pT in the alkaline range was essentially abolished by this treatment. Figure 7 is provided to illustrate in more detail the results obtained in these experiments with one of the plants, in this case wheat seedlings.

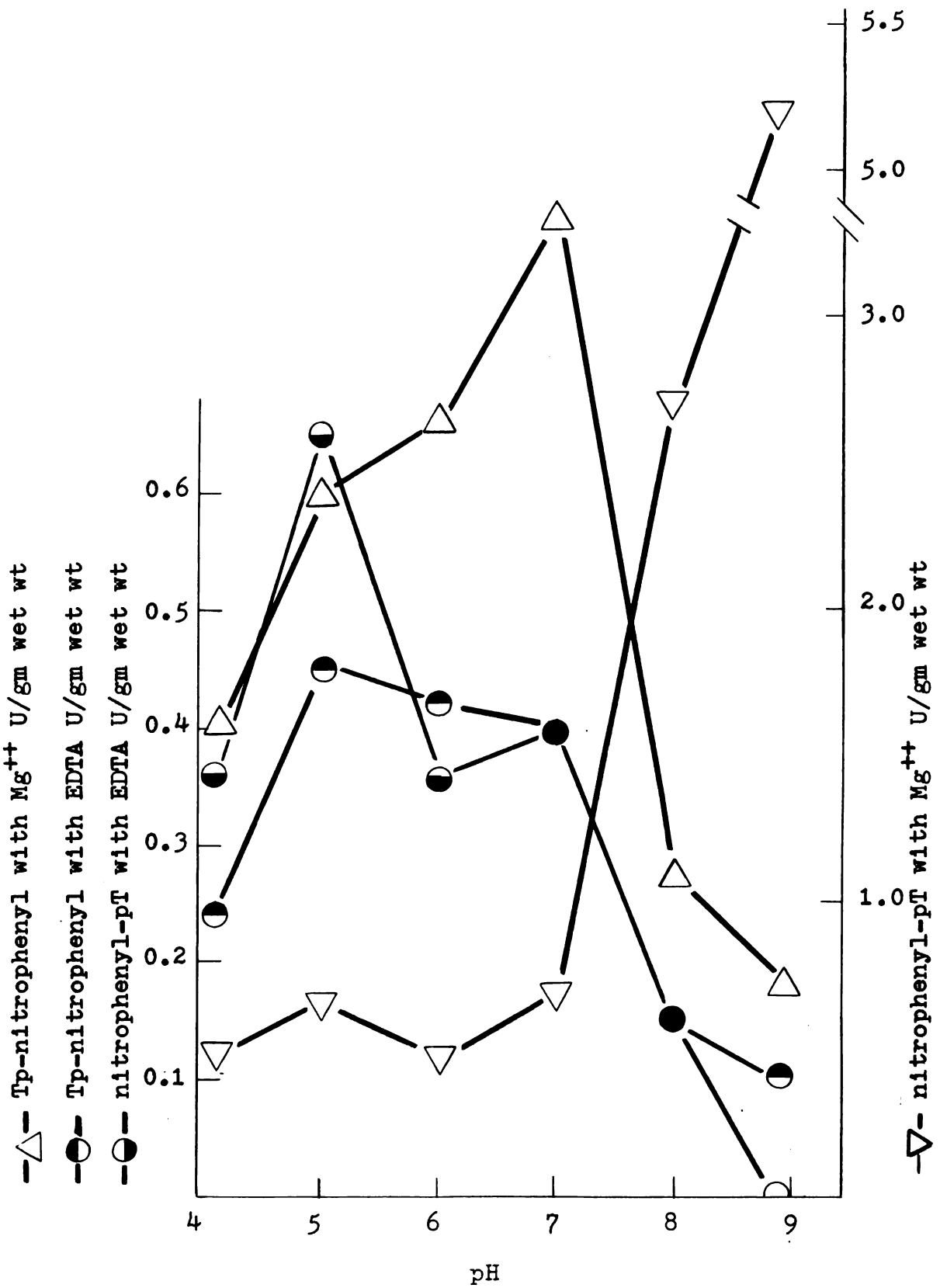
It should be noted that the activities of plant extracts were generally similar in terms of mass specific activity, with corn seedling extract having an unusually high activity against nitrophenyl-pT. Muskmelon and kidney bean extracts had the highest ratios of activity against nitrophenyl-pT at pH 8.9 compared with activity at pH 5.0.

TABLE I

Plant phosphodiesterase activity upon synthetic substrates. The activity is expressed in U/g wet tissue. Specific activity at pH 8.9 is in U/mg protein in presence of 10^{-2} M Mg^{++}. Figures in parenthesis denote the activity in the presence of 10^{-2} M EDTA while others denote the activity in the presence of 10^{-2} M $MgCl_2$. All assays were carried out using assay I described in the Methods and Materials Section.

Source	pH	Tp-nitrophenyl		nitrophenyl-pT			
		5.0	7.0	5.0	7.0	8.9	SA 8.9
Triticum vulgaris (wheat)		.48 (.45)	.85 (.40)	.65 (.65)	.69 (.39)	5.20 (00)	1.86
Zea mays (corn)		2.28 (2.07)	2.01 (1.56)	2.07 (2.16)	4.00 (1.25)	25.00 (00)	10.05
Cucumis melo (muskmelon)		.32 (.26)	.24 (.04)	.46 (.42)	.81 (.18)	11.60 (00)	4.75
Cucumis sativa (cucumber)		.48 (.44)	.30 (.16)	.33 (.25)	.24 (.12)	2.80 (.04)	4.38
Cucurbita maxima (squash)		.56 (.52)	.42 (.14)	.45 (.54)	.37 (.24)	4.44 (.18)	2.41
Raphanus sativus (radish)		.28 (.19)	.22 (.04)	.30 (.46)	.48 (.36)	1.90 (.06)	0.33
Beta vulgaris (beet)		2.60 (1.80)	2.10 (1.52)	6.30 (2.90)	3.72 (.36)	9.60 (00)	3.52
Phaseolus vulgaris (kidney bean)		.42 (.28)	.48 (.24)	.40 (.28)	.64 (00)	11.04 (00)	6.90

Figure 7: The effect of pH on Triticum vulgaris phosphodiesterase activity using two different substrates. Assay conditions are described in assay I using a wide range of pH buffers. The activities are plotted as mass specific activity, μmoles of p-nitrophenol released in the original assay mixture per hour per gram of wet tissue. The nitrophenyl-pT and Tp-nitrophenyl were assayed in the presence either of 10^{-2}M Mg^{++} or 10^{-2}M EDTA.


—△— Tp-nitrophenyl with Mg++ U/gm wet wt
—◐— Tp-nitrophenyl with EDTA U/gm wet wt
—◑— nitrophenyl-pT with EDTA U/gm wet wt
—▽— nitrophenyl-pT with Mg++ U/gm wet wt
0.1
0.2
0.3
0.4
0.5
0.6
1.0
2.0
3.0
5.0
5.5
4
5
6
7
8
9
pH

Base Specificity

The utilization in this work of p-nitrophenyl derivatives of the four nucleotides found in DNA proved an easy method of determination of the base specificities of phosphodiesterase I activities in plant sources. Phosphodiesterase I activities were examined primarily because of their presence in all plant sources and secondly because of the relatively large amounts of activity present. This investigation was carried out using assay system II at pH 8.9. A summary of the results of these rate studies are found in Table II.

These values, which are ratios of the rates of hydrolysis based on nitrophenyl-pT as 1.0, show a pronounced preference of the phosphodiesterase I of several sources for nitrophenyl-pT. It may be noted that the nitrophenyl-pdA has the lowest hydrolysis rate of any of the nitrophenyl derivatives tested. The nitrophenyl-pT + nitrophenyl-pdC/ nitrophenyl-pdG + nitrophenyl-pdA ratios (Py/Pu) are generally similar with the exception of Cucumis melo and Cucumis sativus.

Michaelis Constants

The Michaelis constants found for the four nitrophenyl-deoxyribonucleoside 5' phosphates, nitrophenyl-pU and dinitrophenylphosphate with the enzyme preparations from C. melo and rabbit kidney are presented in Table III. The K_m values of Tp-nitrophenyl for phosphodiesterase I from rabbit kidney and muskmelon, 6.5×10^{-5} M and 9.7×10^{-5} M respectively, can be

TABLE II

Rates of hydrolysis of four substrates by plant phosphodiesterases. The reactions were performed as in assay II in the Materials and Methods Section. Twenty-five µl of crude extract was used for all plant sources. Five µl of extract from animal sources was used. All values are an average of 3 or more determinations. The values are based on the rate of hydrolysis of nitrophenyl-pT at pH 8.9 as 1.00.

Source	Nitrophenyl-pdA	Nitrophenyl-pdC	Nitrophenyl-pdG	Py/Pu
Zea mays (corn)	.51	.81	.91	1.28
Triticum vulgaris (wheat)	.68	.65	.70	1.22
Helianthus annus (sunflower)	.51	.94	.83	1.45
Cucumis melo (muskmelon)	.26	1.05	.50	2.70
Cucumis sativus (cucumber)	.18	1.14	.47	3.29
Raphanus sativus (radish)	.66	.88	.60	1.45
Beta vulgaris (beet)	.56	.58	.73	1.23
Daucus carota (carrot)	.67	.84	.39	1.62
Peptostreptococcus elsdenii	.92	1.94	.72	1.80
Crotalus adamanteus venom	1.04	1.11	.64	1.31
Rabbit kidney	.80	.87	.69	1.21
Rabbit liver	.75	.84	.68	1.21

TABLE III

K_m values for different substrates. The assays were performed as described in assay II in the Materials and Methods Section. Modifications are noted in Figure 8. All values were obtained from plots similar to those shown in Figure 8.

Source	Substrate	K_m	Activity (Relative)
Cucumis melo	nitrophenyl-pT	9.7×10^{-5} M	1.00
	nitrophenyl-pdA	6.7×10^{-5} M	0.26
	nitrophenyl-pdC	8.0×10^{-5} M	1.05
	nitrophenyl-pdG	6.3×10^{-4} M	0.50
	nitrophenyl-pU	3.1×10^{-4} M	0.84
	di-p-nitrophenylphosphate	2.4×10^{-4} M	0.09
Rabbit kidney	nitrophenyl-pT	6.5×10^{-5} M	1.00
	nitrophenyl-pdA	8.3×10^{-5} M	0.80
	nitrophenyl-pdC	3.5×10^{-5} M	0.87
	nitrophenyl-pdG	9.1×10^{-5} M	0.69
	nitrophenyl-pU	5.4×10^{-5} M	0.60
	di-p-nitrophenylphosphate	7.4×10^{-4} M	0.32

compared with that of Crotalus adamanteus venom 5×10^{-4} M (4), Hemachatus haemachates 2×10^{-5} M (27), Daucus carota 2.0×10^{-5} M (21), and hog kidney 4.6×10^{-5} M (15). With the exception of dinitrophenylphosphate, there was little variation in the values from compound to compound and between the two enzyme sources. No correlations between K_m values and relative rates of hydrolysis of substrates were found.

Experiments with Phosphodiesterase from C. Adamanteus Venom

Early experiments with purified venom phosphodiesterase I from C. adamanteus using assay system III exhibited a preference for pyrimidine oligonucleotides prepared from salmon sperm DNA over its counterpart, the purine oligonucleotides. The hydrolysis rate measured by release of phosphomonesterase sensitive phosphate from pyrimidine oligonucleotides was 81.5 μmoles/hr/mg protein, while the rate on the purine oligonucleotides was 63.1 μmoles/hr/mg protein, thus a Py/Pu ratio of 1.29. Similar hydrolysis experiments measured by titrimetric assay IV yielded a Py/Pu ratio, calculated from initial rate of hydrolysis slopes of 1.34.

DISCUSSION

This concise investigation into the activity and specificity of phosphodiesterases from plant sources yielded several interesting and significant properties of these enzymes. The assays for the pH optima of plant extracts are quite similar to the results obtained by W. E. Razzell in his survey of plant phosphodiesterases (19). Corn phosphodiesterase I shows comparable activity (25 U/g wet wt. vs 36 μmoles/hour/g wet weight obtained by Razzell) upon nitrophenyl-pT in the presence of 10^{-2} M Mg^{++}. The average value for the mass activity from the eight sources tested was 8.9 U/g wet weight. This value appears low but this may be misleading in view of the presence of cellular wall material and starch which makes up 10% of the bulk weight. The average specific activity of the sources tested is 4.3 U/mg protein, with the corn extracts displaying the highest specific activity of 10.0. This is much higher than the phosphodiesterase I specific activities from rat and human tissue homogenates which average 2.7 and 1.2 U/mg protein respectively (17).

A rather broad pH optimum of phosphodiesterase I activity from pH 4 to pH 9 deserves some discussion. This activity is apparently nonspecific in nature. The relatively equal hydrolysis rates on Tp-nitrophenyl and nitrophenyl-pT

in the presence of Mg^{++} or EDTA suggests that the phosphodiesterase activity may be due to the same enzyme(s). An extremely high rate of hydrolysis of di-p-nitrophenylphosphate with a broad activity maximum centered at pH 5.0 (5.5 Units/g wet weight) tend to reinforce the nonspecific character of this activity. The activity at pH 7.0 seems to be a reflection of the decrease in the activities at pH 8.9 and 5.0 in most cases rather than a separate activity. To label the activity present at pH 7.0 as nucleotide pyrophosphatase without further purification would probably be erroneous. It is well to note that no activities were found at pH 7.0 which had equal rates of hydrolysis on nitrophenyl-pT with and without $MgCl_2$ or EDTA in the sources tested as Razzell has shown earlier for peas and corn (19).

There is generally little difference to be found in the base specificity of phosphodiesterase I in the various plant and animal sources tested (Table II). Plant phosphodiesterase I activities obtained from several sources show an average Py/Pu ratio of 1.37. This average, however, does not include the unusually high Py/Pu ratios of C. melo and C. sativus which distort this average considerably. This average reflects the preference for a pyrimidine base attached to a deoxyribose moiety over that of its purine counterpart. This preference can also be seen in the animal tissue and bacterial extracts, so it is common not only to plants. Purified carrot phosphodiesterase I displays a much higher Py/Pu ratio, 1.62 which may be indicative of either

removal of contaminating activities from the crude extract or a difference in the specificity or nature of the enzyme from carrot itself. The latter may in fact be more probable since the Py/Pu ratios of C. sativus and C. melo are also quite high, 3.29 and 2.70 respectively.

The nitrophenyl-pdA/nitrophenyl-pT (dA/T) ratios are somewhat varied among the sources tested. The average for the plant extracts is 0.50 while the average of all sources is 0.62. The average value shows the rather high dA/T ratios of animal and bacteral extracts and the much lower dA/T ratios obtained from plant extracts. The extremes, C. adamanteus venom with an dA/T ratio of 1.04 and the two plant sources C. melo and C. sativus with dA/T ratios of 0.26 and 0.18 respectively represent a four fold difference in the specificity of phosphodiesterase activities. An interesting note is the low dA/T values of 0.75 and 0.80 for rabbit liver and kidney which contrast with the dA/T values of 1.13 and 1.06 obtained from purified hog liver and kidney by Razzell (16). The dA/T ratio of 0.67 for purified carrot phosphodiesterase I is also larger than that of the plant average. In light of the dA/T values obtained from purified animal enzymes and the purified carrot enzyme, a high dA/T ratio may be indicative of greater purity of the enzyme.

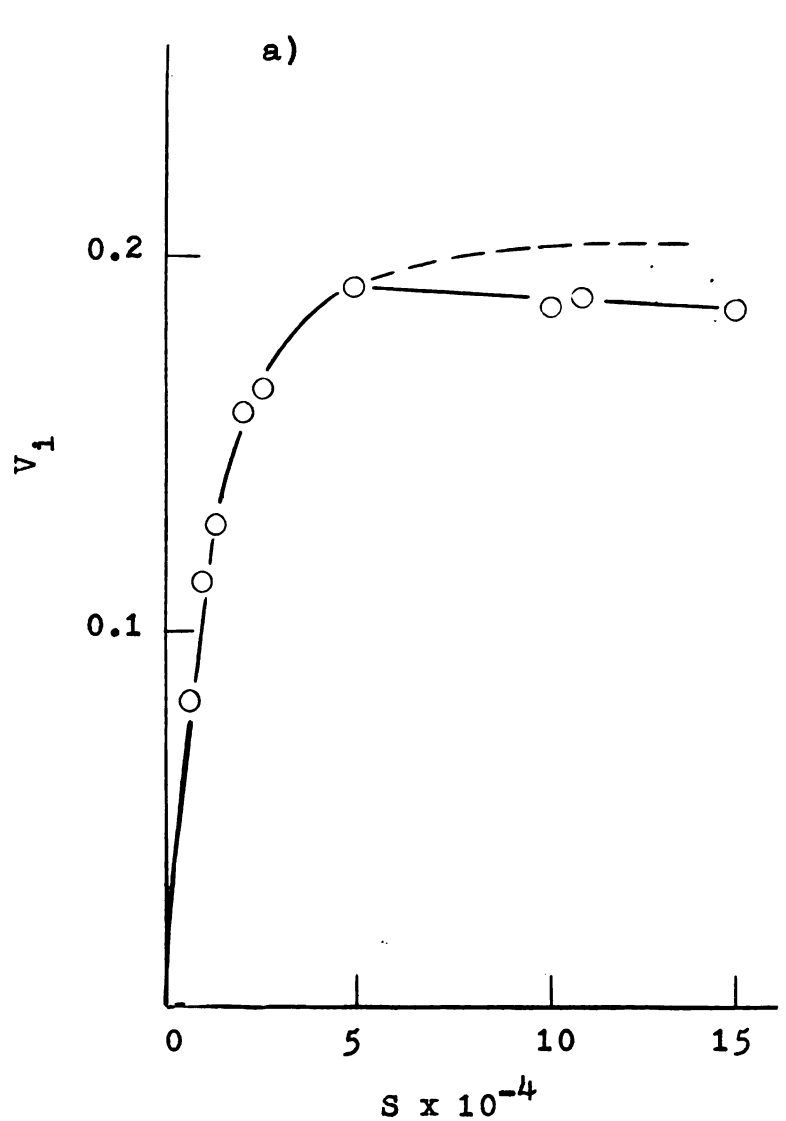
With the arrangement of plants in Table I and Table II according to their phylogenetic position in the plant kingdom (64, 65) it can be seen that no real correlations

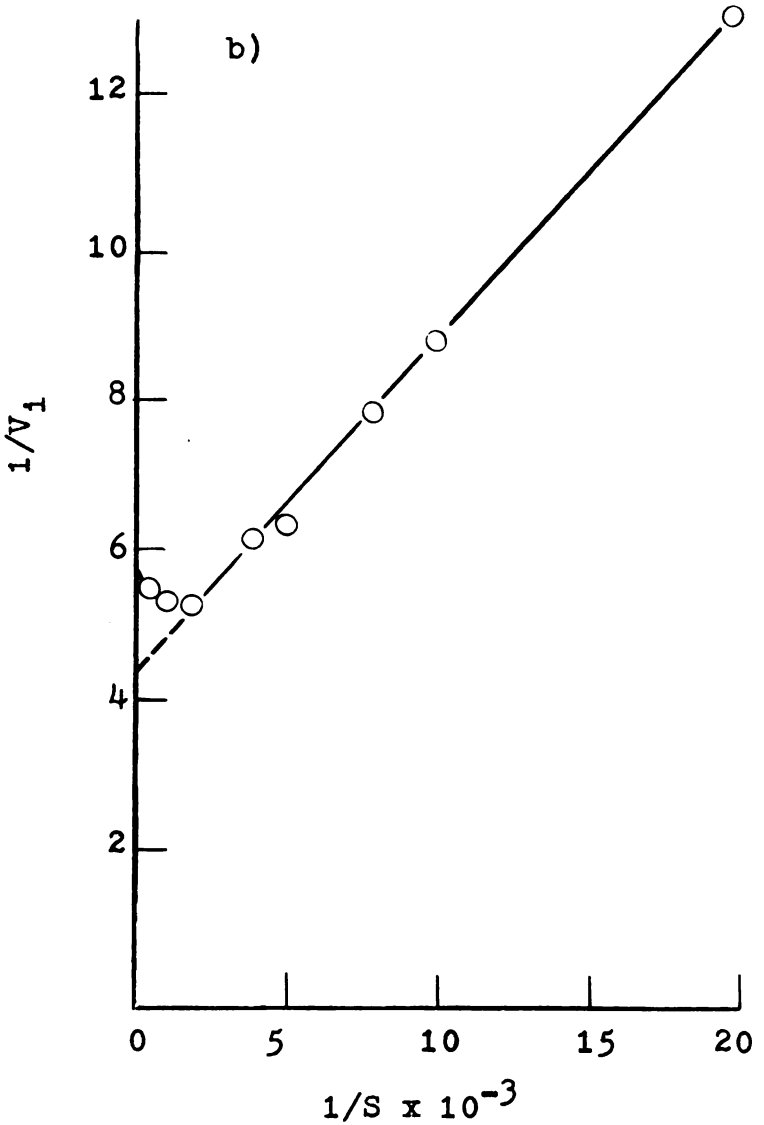
can be drawn as to the advancement of certain plants in the plant kingdom and patterns of phosphodiesterase activity or specificity of phosphodiesterase I of these plants. The division into monocots and dicots of class Angiospermae can be seen to have no real correlation with any change in the activities found in these two groupings. In retrospect, however, the unusually large Py/Pu ratios and extremely low dA/T ratios of C. melo and C. sativus present a possibility that the phosphodiesterase I properties of these extracts may be representative of a peculiar activity common to the family Cucurbitaceae of which they are members.

It would be worthwhile to mention here the possible effect of a contaminant present in one of the substrate preparations. This possibility cannot be overlooked since Khorana and Razzell have been troubled for several years by the presence of an inhibitor in their preparations of nitrophenyl-pU. The presence of an inhibitor in one of the substrates might modify the ratios considerably. No evidence has been found, however, for the actual presence of an inhibitor in the substrates used here.

As clearly shown by Fiers and Khorana (40) in their work with Lactobacillus acidophilus exonuclease, substrate inhibition occurs at high concentrations of the p-nitrophenyl substrates as well as TpT dinucleotides. Phosphodiesterase I from C. melo displayed this property with all six synthetic substrates used in the K_m determinations (Figure 8). The maximum substrate concentration which could be attained with-

Figure 8: Rates of hydrolysis by Cucumis melo as a function of nitrophenyl-pT concentration. The assay was essentially identical with assay II in the Methods and Materials Section. Nitrophenyl-pT concentration was varied over the range $K_m/2$ to greater than 10 K_m. The data is presented a) as a relationship of substrate concentration and reaction velocity, and b) as the Lineweaver-Burk plot. The velocity is in μmoles of p-nitrophenol released per hour and the substrate concentration is in moles per liter.


a)
0.2
0.1
V_1
0
5
10
15
S x 10^{-4}


b)
12
10
8
6
4
2
$1/V_1$
0
5
10
15
20
1/S x 10^{-3}

out deviation from linearity in most cases was 5×10^{-4} M. Very similar results were obtained with extracts from rabbit kidney, Table III. There is little correlation between the K_m values, and thus the affinity of the enzyme for the substrates, of the different nitrophenyl-pdX compounds and the relative rates of hydrolysis. It can be noted, however, that both a heterocyclic base as well as a ribose or deoxyribose moiety is needed for efficient binding and hydrolysis of the phosphate diester linkage. This is clearly shown by the high K_m values and low rates of hydrolysis of di-p-nitrophenylphosphate by C. melo and rabbit kidney extracts.

The use of pyrimidine and purine oligonucleotides as substrates for phosphodiesterase assays require a purified preparation of the enzyme free of the numerous polynucleotidases found in crude extracts. The Py/Pu ratio of 1.29 obtained from hydrolysis of these oligonucleotides by snake venom phosphodiesterase, determined in assay III, is in close agreement with the Py/Pu ratio of 1.31 obtained by the rates of hydrolysis of p-nitrophenyl compounds in assay II. The titrimetric assay IV gave a Py/Pu ratio of 1.34 and was in agreement with the other assays within 5%. The implications of these results are two-fold. The relative rates of hydrolysis of the nitrophenyl-pdX compounds are consistant with the values obtained from other methods and thus provide evidence that assay II is a valid method of determination of the specificity of the enzyme. Secondly, the Py/Pu ratios obtained from the synthetic substrates in fact reflect the

ratios of the rates of hydrolysis of higher polymers. This suggests that the nature of the nucleotide being hydrolyzed is the primary controlling factor of the ratio of the rates of hydrolysis and that large runs of purine or pyrimidine oligonucleotides play little if any control over this ratio. Thus cooperative effects in this instance do not play a major role.

In summary, the examination of the distribution and specificity of phosphodiesterases has led to several interesting observations. In all the plant sources tested, the presence of two phosphodiesterase activities was noted, a phosphodiesterase I activity at pH 8.9 and a nonspecific phosphodiesterase activity at low pH. There was no correlation between the distribution or specificity of plant phosphodiesterases and the phylogenetic position of the plant in the kingdom. The most significant finding was the preference of the phosphodiesterase I activity of the plant and animal sources tested for pyrimidine over purine bases attached to the deoxyribotide moiety as well as a marked preference for the hydrolysis of nitrophenyl-pT as compared with the other nucleotide derivatives. No correlation between K_m values and the relative rates of hydrolysis could be established for phosphodiesterase I from rabbit kidney and muskmelon. On the basis of Py/Pu and dA/T activity ratios, an unusual phosphodiesterase I activity was discovered in the two members of the family Cucurbitaceae. This observation warrants further investigation of the phosphodiesterases of this family by enzyme purification and additional specificity studies.

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