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RAT INTESTINAL PHOSPHODIESTERASE II

PROPERTIES OF THE HIGHLY PURIFIED ENZYME AND ITS INACTIVATION BY IODOACETIC ACID

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Summary

A highly purified preparation of rat intestinal phosphodiesterase II (oligonucleate 3′-nucleotidohydrolase, EC 3.1.4.18) has been studied using a synthetic substrate, thymidine 3′-(2,4-dinitrophenyl) phosphate. The enzyme was most active between pH 6.1 and pH 6.7 and was inhibited by Cu^{2+} and Zn^{2+} but unaffected by EDTA, Mg^{2+}, Ca^{2+}, Co^{2+}, and Ni^{2+}. The reaction rate decreased at high levels of enzyme because of competitive inhibition by deoxythymidine 3′-phosphate, a reaction product, which showed a K_i of $2 \cdot 10^{-5}$ M. The molecular weight of the enzyme by gel-filtration was 150 000—170 000. In electrofocusing experiments multiple peaks of activity were found at pH 3.4, 4.2—4.5 and 7.2. Polyacrylamide gel electrophoresis of freshly purified phosphodiesterase II showed up to 10 protein bands in the gels. If the preparations were stored at 4° C for some time only one or two bands appeared. Investigation of the reaction of rat intestinal phosphodiesterase II with a number of possible phosphodiesterase substrates indicated that the enzyme required a nucleoside 3′-phosphoryl residue for the initiation of hydrolysis. Thus compounds such as NAD, ATP, bis-(*p*-nitrophenyl)phosphate, thymidine 5′-(*p*-nitrophenyl)phosphate, glycerylphosphorylcholine, guanylyl-(2′ → 5′)-adenosine and 3′,5′-cyclic AMP which contain phosphodiester bonds, nevertheless were not substrates for the enzyme. The enzyme was inhibited reversibly by *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate and inactivated irreversibly by iodoacetic acid. Activity of the phospho-

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Abbreviations: dTN_2PhP, thymidine 3′-(2,4-dinitrophenyl)phosphate; A-G, adenylyl-(3′ → 5′)-guanosine; G-A, guanylyl-(3′ → 5′)-adenosine; G2′-5′A, guanylyl-(2′ → 5′)-adenosine; dTP, deoxythymidine 3′-phosphate.

diesterase II was reduced to 50% by incubation with $2.0 \cdot 10^{-3}$—$5.0 \cdot 10^{-3}$ M iodoacetate for 20—30 min at 24°C at pH 5.0—6.1. Iodoacetamide had no effect. The degree of inactivation by iodoacetate was reduced by the presence of a substrate for the enzyme or, more effectively by deoxythymidine 3′-phosphate, a competitive inhibitor. It is concluded that iodoacetic acid alkylates an essential residue at the active centre of the enzyme.

Introduction

Phosphodiesterase II (oligonucleate 3′-nucleotidohydrolase, EC 3.1.4.18) is the enzyme which catalyses the exolytic hydrolysis of 5′-hydroxyl-terminated oligonucleotides to 3′-mononucleotides [1]. The enzyme, which has been extensively purified from calf spleen [2,3], hog spleen [4] and salmon testis [5], has proved to be particularly useful in studies of oligonucleotide sequence (cf. ref. 6). Less well known, however, are details of the structure of the enzyme, its mechanism of action and its function in vivo.

We have studied previously the subcellular location of intestinal phosphodiesterase II in rat and guinea pig and compared the enzymes from the two sources [7,8]. We found the rat enzyme was much more easily obtained in soluble form [7] and could be purified to a much greater degree than that from guinea pig [8]. Rat intestinal phosphodiesterase II has been purified over 500-fold by a novel chromatographic procedure involving its adsorption on agarose and the present paper describes in some detail the properties of this highly purified preparation. Iodoacetic acid inactivated the enzyme irreversibly. The inhibition was reduced in the presence of the competitive inhibitor of the enzyme, deoxythymidine 3′-phosphate indicating that iodoacetate alkylates an essential residue at the active centre of the enzyme molecule.

Materials and Methods

Materials. The synthesis of the substrate, dTN_2PhP, which conveniently enables the activity of phosphodiesterase II to be measured directly and continuously in a spectrophotometer cuvette, has been previously described [7,9]. Bis-(*p*-nitrophenyl)phosphate, cytochrome *c* and calf thymus DNA were purchased from the Sigma Chemical Co. and yeast RNA and RNA "core" were from Calbiochem. Adenylyl-(3′ → 5′)-guanosine, guanylyl-(3′ → 5′)-adenosine, guanylyl-2′ → 5′)-adenosine and deoxythymidine 3′-phosphate (bis-triethylammonium salt) were obtained from Raylo Chemicals Ltd. Polyadenylic acid (poly (A)) was from Miles Laboratories Inc., and any other nucleotides were purchased from P-L Biochemicals Inc. Sephadex G-150 (superfine) was obtained from Pharmacia and Bio-Gel A-0.5m from Bio-Rad Laboratories. *Escherichia coli* alkaline phosphatase, catalase, ribonuclease A, chymotrypsinogen A, ovalbumin and aldolase were all Worthington products and Coomassie Brilliant Blue, myoglobin and apoferritin were products of Schwarz/Mann. Acrylamide, methylene bis-(acrylamide) and *N,N,N′,N′*-tetramethyl ethylenediamine were obtained from Eastman Kodak. *Crotalus adamanteus* venom was purchased from Ross Allen's Reptile Institute, Silver Springs,

Fla., U.S.A. and bovine serum albumin (fraction V) from the Armour Pharmaceutical Co. *N*-Ethylmaleimide, *p*-chloromercuriphenylsulfonic acid and theophylline were purchased from Sigma Chemical Co. and *p*-chloromercuribenzoate and dithiothreitol from Calbiochem. Iodoacetamide and iodoacetic acid were products of Eastman Kodak.

Enzyme assays. The assays of phosphodiesterase II, acidic and alkaline phosphatases have been given previously [7]. Acidic ribonuclease II and adenosine deaminase were assayed as described by Menon and Smith [5] and phosphodiesterase I by the method of Hynie and Zbarsky [10]. The incubation mixture for 5′-nucleotidase contained in final concentration 0.1 M glycine, 10 mM $MgCl_2$, 3 mM adenosine 5′-phosphate and enzyme in a final volume of 1 ml at pH 9.0. After a reaction period of 15 min, 0.5 ml of 10% trichloroacetic acid was added and the suspension allowed to stand for 5 min at 0° C. The liquid was clarified by centrifugation and the resulting supernatant assayed for P_i by the method of Ames [12]. Deoxyribonuclease I was assayed in a volume of 1.25 ml containing 0.1 M morpholinoethanesulphonate buffer, pH 6.8, 0.1 mg DNA, 0.1 M $MnCl_2$ and enzyme solution. After a 15 min incubation period, the reaction was stopped by adding 0.25 ml of 12% trichloroacetic acid. The cloudy solution was allowed to stand at 0° for 10 min, clarified by centrifugation and the absorbance of the resulting supernatant measured at 260 nm. Deoxyribonuclease II was determined in a similar fashion except that the assay mixture contained 0.1 M sodium acetate buffer, pH 5, 0.2 mg DNA, 0.01 M EDTA and enzyme.

Protein was determined by the method of Lowry et al. [11] using bovine serum albumin as the standard.

Except for deoxyribonuclease one unit of enzyme is the amount producing 1 μmol of product per h. One unit of deoxyribonuclease, on the other hand, causes the hydrolysis in 1 min of acid-soluble oligonucleotides having an $A_{260\ nm}$ of 1.0.

Enzyme preparation. The purification of rat intestinal phosphodiesterase II has been described elsewhere [8]. The specific activities of the preparations used in these experiments varied between 200 and 450 units per mg/protein.

Nucleotide hydrolysis. The hydrolysis of G-A, G2′-5′A, A-G and poly (A) was monitored by descending paper chromatography using Whatman No. 31 paper and ethanol/1 M ammonium acetate, pH 7 (7 : 3, v/v) as solvent. Nucleotide and nucleoside spots were visualized on the dried chromatograms under ultraviolet light. The ultraviolet-absorbing areas were cut out and the absorbing material eluted from the paper by the centrifugation method of Markham (described by Heppel [13]). The identity of nucleoside 3′-phosphates was confirmed by their resistance to the powerful 5′-nucleotidase activity present in crude *C. adamanteus* venom [5].

Gel filtration. This was performed using Bio-Gel A-0.5m or Sephadex G-150. In each case the column size was 2.5 × 35 cm; the eluting buffers were 50 mM sodium acetate and 0.5 M NaCl (pH 5) and 20 mM sodium phosphate and 0.2 M NaCl (pH 7.2), respectively. Non-enzymic protein molecular weight markers were dissolved in the appropriate eluting buffer at a concentration of 2—5 mg/ml and the elution of these was estimated by their absorbances at 280 nm. Enzymic marker proteins were used in much smaller amounts since their

elution was monitored by their enzymic activities.

Electrofocusing. An LKB 110-ml electrofocusing apparatus was used but the manufacturer's procedure was modified slightly since the recommended volumes did not completely fill the column. The anodic solution (bottom) contained 1% sulphuric acid, the cathodic solution 2% ethanolamine and the protein sample was applied in the middle of the sucrose gradient. The whole apparatus was kept at 5—7°C by passing cold water through the outer jacket of the column. Experiments were run at a constant voltage of 300 V for periods of time between 24 and 90 h; the initial current was about 6 mA which decreased over the course of the experiment to 0.8—0.4 mA.

Polyacrylamide gel electrophoresis. The system used was that of Davis [14] except that no sample or spacer gel was used and the separating gel consisted of 5.25% total acrylamide; the methylene bis(acrylamide) concentration was 0.25%. Fixation and staining of the separated proteins was accomplished by a procedure adapted from an ORTEC manual [15]. The gels were incubated at 65°C for 30 min in 0.25% Coomassie Blue dissolved in methanol/acetic acid/water (45 : 10 : 45, v/v). Destaining, also at 65°C, was carried out by placing the gels first in ethanol/acetic acid/water (25 : 10 : 65, v/v) for 10 min, then for three additional periods of 20 min each in fresh portions of the same solution and finally for 20 min in 10% acetic acid. The remaining unbound stain was removed by placing the gels overnight in 10% acetic acid at 24°C and the destained gels were stored in this solution.

For detection of phosphodiesterase II activity after electrophoresis the gel was cut into 2-mm slices using a device similar to that described by Chrambach [16]. Each piece was frozen (—20°C) and then extracted in 0.5 ml of buffer containing 5 mM Tris and 5 mM NaH_2PO_4 (pH 7.6) for 24 h at 4°C. Portions of these extracts were then assayed for phosphodiesterase II.

Results

The hydrolysis of dTN_2PhP. When relatively large amounts of purified phosphodiesterase II were assayed the production of 2,4-dinitrophenol was proportional to time for only an initial period. The fall-off was not corrected by increasing the dTN_2PhP concentration so it was not the result of substrate depletion but probably was due to either enzyme inactivation or end-product inhibition of the reaction. Experiments which provide evidence that the latter possibility was more likely will be discussed later. In any event the overall non-linearity of the hydrolysis reaction with time did not preclude measurement of the rate since it could be shown that the initial velocity of the reaction was proportional to the amount of enzyme present.

Effect of pH and divalent cations. As shown in Table I, rat intestinal phosphodiesterase II exhibited maximal activity at a pH around 6.5 with dTN_2PhP as substrate, although the optimum pH varied in different buffer systems. Bernardı and Bernardi [4] found spleen phosphodiesterase had optimal activity at pH 5.5 in 0.1 M succinate or phosphate buffer with acid deoxyribonuclease hydrolysate as substrate. It was also noted in the present work that the intestinal enzyme had 27% less activity in phosphate than in succinate buffer and 20% greater activity in imidazole · HCl.

TABLE I

PHOSPHODIESTERASE II ACTIVITY IN A VARIETY OF BUFFERS

Phosphodiesterase II (0.07 unit) was assayed as described in the legend to Fig. 1 except that the buffer (at a concentration of 0.1 M) was varied as indicated.

Buffer	Optimum pH	Activity (%)
Sodium succinate	6.1—6.6	100
Sodium morpholinoethanesulphonate	6.6	103
Imidazole · HCl	6.6	120
Sodium phosphate	6.7	73

Divalent cations, which were found to be generally inhibitory for spleen phosphodiesterase [2], had no such effect on the intestinal enzyme, with the exception of Cu^{2+} and Zn^{2+}, as shown in Table II. This is in accord with the report of Bernardi and Bernardi [4] that 0.02 M Mg^{2+} did not inhibit the spleen enzyme although it may cause a shift to a lower pH optimum. EDTA (0.1 M) had no effect on the activity of the intestinal enzyme.

Molecular weight. This was determined by gel filtration [17] using two gel systems, Bio-Gel A-0.5m and Sephadex G-150 and values of 150 000 and 170 000, respectively, were obtained. These experiments are illustrated in Fig. 1. A molecular weight of 100 000 has been reported by Menon and Smith [5] for salmon testis phosphodiesterase II and the spleen enzyme has been shown to elute from Sephadex G-100 after acid phosphomonoesterase [4] which has a molecular weight of 100 000 [18]. Thus it appears that rat intestinal phosphodiesterase II is considerably larger than the enzymes from other sources.

Isoelectric point. The results of two electrofocusing experiments are shown in Fig. 2. In the first (Fig. 2a) the peak of phosphodiesterase II activity was found at pH 4.2. However, when the electrofocusing was carried out using a narrower pH gradient for an extended period of 90 h only 11% of the enzymic activity was recovered and the bulk of this was found at the extremes of the pH gradient, at pH 3.4 and pH 7.2 (Fig. 2b). Salmon testis phosphodiesterase II has been reported to have an isoelectric point of pH 7.0 [5].

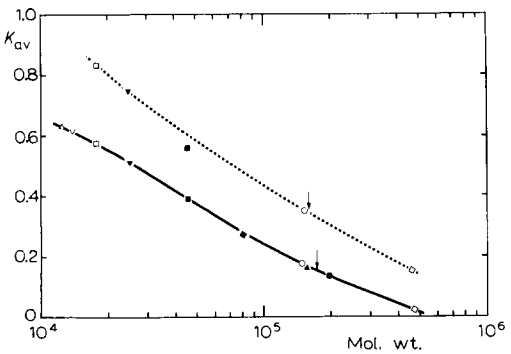
Polyacrylamide gel electrophoresis. Gel electrophoresis of fresh preparations

TABLE II

EFFECT OF DIVALENT CATIONS AND EDTA ON PHOSPHODIESTERASE II ACTIVITY

Phosphodiesterase II (0.11 unit) was assayed using method 2 [7] in the presence of the salts shown.

Addition	Concentration (mM)	Activity (%)
None	—	100
$MgCl_2$	10	96
$CaCl_2$	10	101
$MnCl_2$	10	95
$CoCl_2$	10	95
$NiCl_2$	10	99
$ZnCl_2$	10	76
$CuSo_4$	1	74
$CuSo_4$	10	34
EDTA	100	97



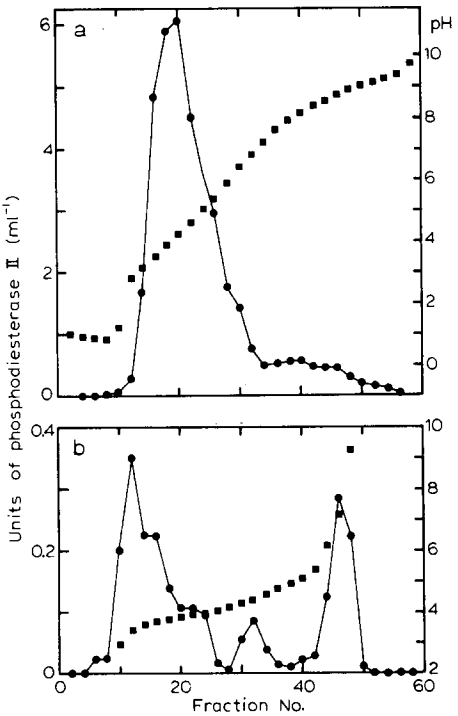


Fig. 1. Estimation of molecular weight of phosphodiesterase II by gel filtration chromatography. The experiments were carried out as described in Materials and Methods, using Bio-Gel A-0.5m (hatched line) and Sephadex G-150 superfine (solid line). The protein markers and their molecular weights were: △, cytochrome *c* (12 400); ▽, ribonuclease A (13 700); ○, myoglobin (17 800); ▼, chymotrypsinogen A (25 000); ■, ovalbumin (45 000); ◆, *E. coli* alkaline phosphatase (80 000); ○, yeast alcohol dehydrogenase (151 000); ▲, aldolase (158 000); ●, catalase (195 000 (although the true molecular weight of catalase is about 240 000, Andrews [38] has shown that the enzyme behaves as a protein with a molecular weight of 195 000 in gel filtration experiments)); ◇, apoferritin (480 000). The K_{av} [27] values of phosphodiesterase II are indicated by the arrows.

Fig. 2. Electrofocusing of phosphodiesterase II. In a, pH 3—10 carrier ampholytes were used and the experiment was carried on for 31 h, whereas in b pH 3—5 carrier ampholytes were empolyed and the electrofocusing period was 90 h. The recovery of phosphodiesterase II activity in a and b was 75 and 11%, respectively. ■, pH; ●, phosphodiesterase II.

of purified phosphodiesterase II yielded 7—10 bands of material stainable with Coomassie Blue as detected by scanning the gels at 500 nm in a Gilford spectrophotometer. A typical scan is illustrated in Fig. 3. The phosphodiesterase II was associated largely with the fastest migrating protein bands. A slower moving protein with much lower enzyme activity was also observed. A number of experiments was also carried out with an enzyme preparation which had been stored at 4°C for a month during which the specific activity of the enzyme decreased from 450 to 200 units/mg protein. Electrophoresis of this fraction gave only two protein bands, with R_F values of 0.37 and 1.00, respectively. The enzyme activity was associated only with the slower moving band.

One explanation for the above observations is that fresh preparations of purified phosphodiesterase II may contain several active or inactive aggregates

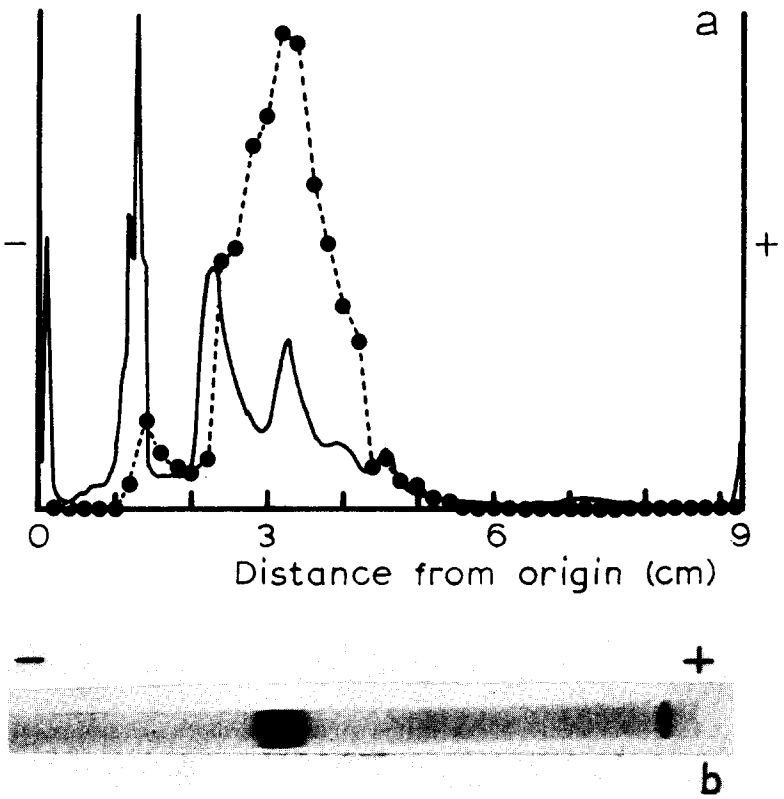


Fig. 3. Polyacrylamide gel electrophoresis of phosphodiesterase II. (a) Samples (50 μg of protein) of a freshly purified preparation were each applied to two identical gels. After electrophoresis one gel was stained with Coomassie Blue while the other was sliced and analysed for phosphodiesterase II activity (●- - - - -●), arbitrary units). The stained gel was scanned at 500 nm in a Gilford spectrophotometer equipped with a linear transport device (solid curve). (b) Photograph of stained gel after electrophoresis of phosphodiesterase II stored for 1 month at 4° C.

of the enzyme which tend to dissociate on storage to give a predominant single activity. The results could also be explained by the possible presence of a weak protease in the purified preparation. A small amount of protein was observed to co-electrophorese with the bromphenol blue which marked the front and this might have arisen from proteolysis of the other proteins. Further, preparations of the intestinal phosphodiesterase II stored at 4° C for 1 month lost up to 40% of their specific activity, an effect which is not inconsistent with proteolytic degradation.

Contaminating enzyme activities. Table III lists the specific activities of a number of polynucleotidases and phosphatases present in homogenates of rat intestinal mucosal scrapings and in the purified phosphodiesterase preparation. Only deoxyribonuclease II and ribonuclease II activities were detectable in the purified fraction in addition to phosphodiesterase activity, but the enrichment of their specific activities was much lower than that observed for phosphodiesterase II.

Hydrolysis of phosphodiesters. The rate of hydrolysis by phosphodiesterase II of a number of compounds which contained phosphodiester bonds is shown

TABLE III

CONTAMINATION ENZYME ACTIVITIES IN PURIFIED PREPARATIONS OF PHOSPHODIESTERASE II

The enzyme purification, from an homogenate of rat intestinal mucosal scrapings, was carried out as previously described [8]. The specific activities (units/mg protein) of the enzymes were determined in the homogenate (A) and in the purified preparation (B).

Enzyme activity	A	B	Enrichment (B/A)
Phosphodiesterase II	0.77	420	550
Phosphodiesterase I	0.61	0	0
Deoxyribonuclease I	0.10	0	0
Deoxyribonuclease II	0.08	0.6	7.8
Acidic ribonuclease II	4.0	110	27.5
Acidic phosphatase	0.97	0	0
Alkaline phosphatase	0.89	0	0
5′-Nucleotidase	1.4	0	0
Adenosine deaminase	4.8	0	0

in Table IV. RNA 'core' which is largely a mixture of oligoribonucleotides and both the *p*-nitrophenyl and the 2,4-dinitrophenyl esters of thymidine 3′-phosphate were readily hydrolyzed by the enzyme though at slightly different rates. Poly (A) and two natural dinucleoside monophosphates, A-G and G-A were hydrolyzed more slowly. It was of interest that a nucleotide with a 2′ → 5′ linkage (instead of the normal 3′ → 5′) was not hydrolyzed by the enzyme and that a "non-specific" phosphodiesterase [19] substrate, bis(*p*-nitrophenyl)phos-

TABLE IV

THE RATE OF HYDROLYSIS BY INTESTINAL PHOSPHODIESTERASE II OF SOME COMPOUNDS CONTAINING PHOSPHODIESTER BONDS

Phosphodiesterase II (0.9 unit in Expt. 1 and 0.8 unit in Expt. 2) was incubated with each compound in 0.1 M sodium succinate buffer, pH 6.1, or, in the case of G-A, A-G and poly(A) in 0.1 M sodium acetate buffer, pH 6.0. The final concentration of each compound was 1 mM except for RNA 'core' (0.5 mg/ml) and poly(A) (1 mg/ml). The rate of hydrolysis of the 2,4-dinitrophenyl and *p*-nitrophenyl esters and RNA 'core' was determined spectrophotometrically; that of G-A, G2′-5′A and A-G and poly(A) by paper chromatography as described in Materials and Methods and that of ATP by measuring the P_i formed.

Compound	Rate of hydrolysis (μmol/h per mg protein)	
	Expt. 1	Expt. 2
dTN_2PhP	311	270
Thymidine 3′-(*p*-nitrophenyl)phosphate	213	—
RNA 'core'	490 *	—
G-A	130	66
G2′-5′A	<10	—
A-G	—	39
Bis(*p*-nitrophenyl)phosphate	1.1	—
Thymidine 5′-(*p*-nitrophenyl)phosphate	<0.1	—
ATP	<0.5	—
Poly(A)	—	15

* Assuming an average ϵ_M value of 10 600 at 260 nm.

phate, was hydrolyzed at less than 0.5% the rate of dTN_2PhP. No hydrolysis of thymidine 5′-(*p*-nitrophenyl)phosphate or of ATP was detectable. The former compound is a substrate for phosphodiesterase I [20] and the latter nucleotide is a substrate for nucleoside polyphosphatase [21].

No evidence was found that rat phosphodiesterase II was able to carry out the "transfer" reactions which Razzell and Khorana [3] and Menon and Smith [5] have described for the spleen and testis enzymes, respectively. Even when concentrations of G-A as high as 8 mM were incubated with the rat enzyme, the only detectable products were guanosine 3′-phosphate and adenosine.

Effect of phosphomonoesters and phosphodiesters on dTN_2PhP hydrolysis. These experiments were carried out to determine the effectiveness of a variety of compounds as likely competitive inhibitors of phosphodiesterase II. Such information might be useful to indicate the nature of the structure most effectively bound by the enzyme at its active center. The results are shown in Table V.

Among the phosphodiesters tested only A-G and G-A inhibited dTN_2PhP hydrolysis to any degree. Interestingly, A-G was a less potent inhibitor of dTN_2PhP than was G-A, a finding which is in accord with the observation that A-G is hydrolysed more slowly than G-A by phosphodiesterase II (Table IV).

Similar experiments with phosphomonoesters are summarized in Table VI. Of the mononucleotides tested, deoxythymidine 3′-phosphate inhibited the intestinal phosphodiesterase II most markedly, 62% at a concentration of 1 mM. dTMP had almost no effect. Adenosine monophosphates inhibited the enzyme only at much higher concentrations. Adenosine 3′-phosphate at a concentration of 10 mM inhibited phosphodiesterase to the same degree as 1 mM deoxythymidine 3′-phosphate. These results suggest that adenosine 3′-phosphate is not bound very tightly to the enzyme which would explain why A-G is even less effective than G-A as a substrate for the enzyme and as an inhibitor in the hydrolysis of dTN_2PhP. The very slow hydrolysis of poly (A) might also be a consequence of the lower degree of binding of adenine 3′-phosphate.

As expected, the inhibition by deoxythymidine 3′-phosphate and by G-A

TABLE V

EFFECT OF PHOSPHODIESTERS ON THE RATE OF dTN_2PhP HYDROLYSIS BY PHOSPHODIESTERASE II

Phosphodiesterase II (0.14 unit) was assayed in the presence of the compounds shown.

Addition	Concentration (mM)	Activity (%)
None	—	100
A-G	1	63
G-A	1	37
Poly(A)	1 (mg/ml)	111
NAD	1	100
ATP	1	100
Glycerylphosphorylcholine	1	95
3′,5′-Cyclic AMP	1	99
3′,5′-Cyclic AMP	10	93

TABLE VI

EFFECT OF PHOSPHOMONOESTERS ON THE RATE OF dTN_2PhP HYDROLYSIS BY PHOSPHODIESTERASE II

Phosphodiesterase II (0.10 unit) was assayed in the presence of the mononucleotides shown.

Addition	Concentration (mM)	Activity (%)
None	—	100
AMP	1	99
AMP	10	63
Adenosine 3'-phosphate	1	92
Adenosine 3'-phosphate	10	28
Adenosine 2'-phosphate	1	98
Adenosine 2'-phosphate	10	47
dTP	1	38
dTMP	1	96

was found to be competitive and an inhibition constant (K_i) for the former compound was calculated from a Dixon plot to be $2 \cdot 10^{-5}$ M. This value, which was of the same order of magnitude as the K_m value for dTN_2PhP [8] indicates a strong degree of interaction with the enzyme and explains the fall-off in 2,4-dinitrophenol production noted earlier in enzyme assays.

Effect of inhibitors and alkylating reagents. The results in Table VII show

TABLE VII

EFFECT OF A NUMBER OF POSSIBLE ENZYME INHIBITORS ON PHOSPHODIESTERASE II ACTIVITY

Phosphodiesterase II activity (0.14 unit) was determined spectrophotometrically [9,10] in the presence of the compounds shown. Except where noted the enzyme was preincubated with the added compound for 5 min after which the substrate, thymidine 3'-(2,4-dinitrophenyl)phosphate, was added to start the reaction.

Addition	Concentration (mM)	Activity (%)
None	—	100
KF	5	103
Theophylline	1	101
Theophylline	5	93
Mercaptoethanol	1	104
Mercaptoethanol	5	106
Dithiothreitol	5	103
Dithiothreitol	10	93
N-Ethylmaleimide	5	84
p-Chloromercuribenzoate	1	30
p-Chloromercuriphenylsulfonate	1	62
p-Chloromercuriphenylsulfonate	5	37
p-Chloromercuriphenylsulfonate	5	37 *
p-Chloromercuriphenylsulfonate	5	31 *
Iodoacetate	1	86
Iodoacetate	5	23
Iodoacetate	5	0 **
Iodoacetamide	1	99
Iodoacetamide	5	97
Iodoacetamide	5	98 **

* Preincubated for 15 s instead of 5 min.

** Preincubated for 30 min instead of 5 min.

the effects of a number of possible enzyme inhibitors on phosphodiesterase II activity. The enzyme remained completely active in the presence of reducing agents and was unaffected by fluoride or theophylline, which are potent inhibitors for acid phosphodiesterase [22] and cyclic nucleotide phosphodiesterase [23].

Thiol reagents such as *N*-ethylmaleimide and the organic mercurials were quite inhibitory as was iodoacetate but iodoacetamide showed no effect. The inhibition by the mercaptide-forming reagents, *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate, was reversible, the enzyme activity being restored when the reagent was removed by dialysis. Binding of *p*-chloromercuriphenylsulfonate was apparently very rapid since preincubation of the compound with the enzyme for only 15 s was sufficient for its inhibitory action. The degree of inhibition by iodoacetate was clearly dependent on the period of contact with the enzyme and was irreversible. This finding is consistent with the well-known alkylating effects of this reagent [24]. The lack of inhibition by iodoacetamide agreed with results obtained with other nucleases [25–28]. The difference in the inhibitory effects of the two reagents is illustrated in Fig. 4 which shows the results of an experiment where phosphodiesterase II was incubated with either iodoacetate or iodoacetamide and aliquots of the incubation mixture were removed at various times and assayed for remaining enzyme activity. Clearly, iodoacetamide had no effect whereas

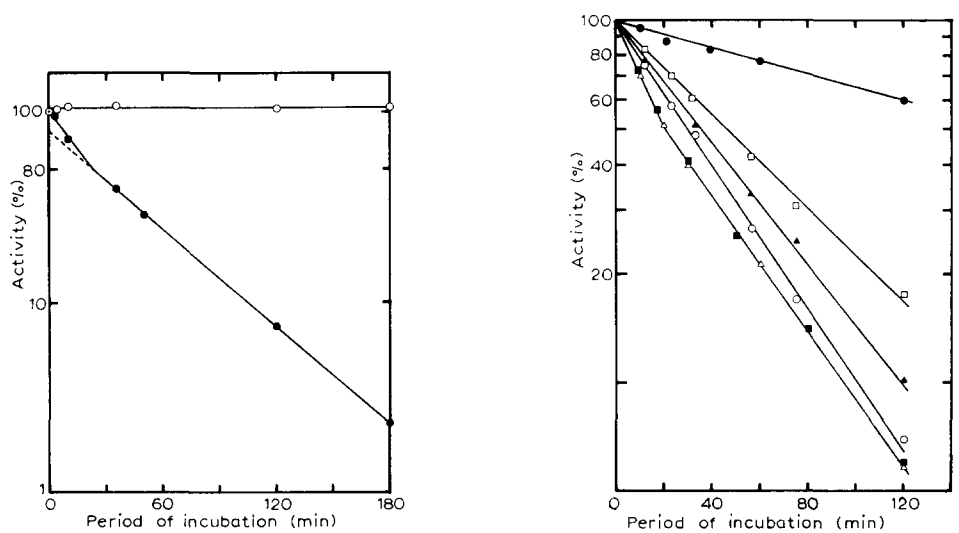


Fig. 4. Inactivation of rat intestinal phosphodiesterase II by iodoacetate. Phosphodiesterase II (8.4 units) was incubated at 24°C in 0.1 M sodium succinate pH 6.1 (1.0 ml) in the presence of either 2 mM iodoacetamide (○) or 2 mM iodoacetic acid (●). Aliquots (0.1 ml) were removed from the incubation mixture at intervals and assayed for remaining phosphodiesterase II activity. Extrapolation (hatched portion) of the slower inactivation rate intersected the ordinate at a point equal to 79% activity.

Fig. 5. The pH dependence of inactivation of rat intestinal phosphodiesterase II by iodoacetate. Phosphodiesterase II (4 units) was incubated with 5 mM iodoacetic acid at 24°C in buffer mixtures containing 25 mM sodium acetate/25 mM sodium phosphate/25 mM sodium succinate/25 mM sodium borate adjusted to different pH values. Aliquots (0.1 ml) of the mixtures were removed at the indicated times and assayed for residual phosphodiesterase II activity. ●, pH 9; □, pH 8; ▲, pH 7; ○, pH 6.6; ■, pH 6.1; △, pH 5.

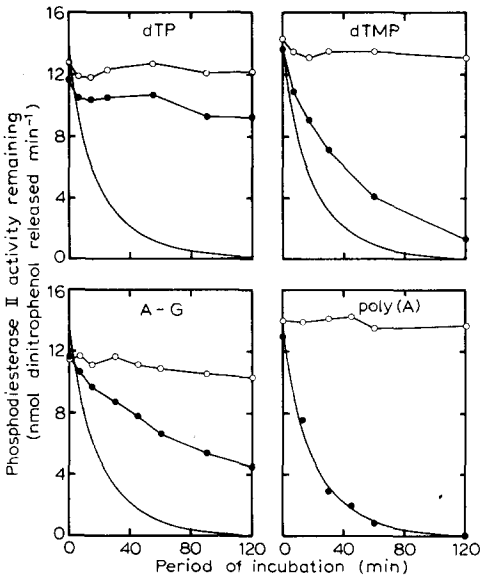


Fig. 6. Protection of phosphodiesterase II against inactivation by iodoacetate. Phosphodiesterase II (8.4 units) was incubated at 24°C in 0.09 M sodium succinate buffer, pH 6.1 (1.0 ml). The effects of dTP, dTMP, A-G (all 1 mM) and poly(A) (1 mg/ml) were investigated by adding each compound to reaction mixtures in the absence (○) or presence (●) of 5 mM iodoacetate. Samples of these mixtures were then removed at the indicated times and assayed for remaining phosphodiesterase II. The smooth, exponentially decreasing curve in each diagram represents the decay of phosphodiesterase II activity in the presence of 5 mM iodoacetic acid alone.

in the presence of iodoacetate phosphodiesterase II was inactivated with approximately first-order kinetics. Evidence that more than one reacting species may have been present can be inferred from the non-linear nature of the curve; approx. 21% of the phosphodiesterase II activity was destroyed at a slightly faster rate than the remaining 79%.

Fig. 5 shows that the inactivation of phosphodiesterase II by iodoacetate was also pH dependent. In the presence of 5 mM iodoacetate the enzyme had a half-life of about 160 min at pH 9 but only a little more than 20 min at pH 6.1 or lower.

Since it was possible that iodoacetate was acting by alkylating an essential active site group, experiments were carried out to test the effectiveness of some nucleotide compounds in protecting the enzyme against the reagent. The results in Fig. 6 show that dTP, A-G and dTMP all afforded some degree of protection whereas poly (A) was ineffective. It is interesting that, with the exception of dTMP, the effectiveness of these compounds was proportional to the affinity with which they bound to the enzyme (Tables V and VI). The best protection was given by dTP, a competitive inhibitor with a high degree of interaction with the enzyme.

Discussion

Generally, the rat intestinal phosphodiesterase II has been shown to be similar to enzymes of this type from other sources [2–5,29,30]. Oligo-

nucleotides (RNA core), dinucleotides and synthetic chromogenic phosphodiesters were hydrolysed rapidly by the enzyme at pH values between 6 and 7. It was shown previously by Razzell and Khorana [3], Fiers and Khorana [29] and Menon and Smith [5] that phosphodiesterase II hydrolyses oligonucleotide chains exolytically in a 5′ → 3′ direction to yield 3′-mononucleotides. Since the intestinal phosphodiesterase II was quite active in attacking oligonucleotides but was inactive toward or did not interact with such compounds as glycerophosphorylcholine, NAD, bis-(*p*-nitrophenyl) phosphate, thymidine 5′-(*p*-nitrophenyl)phosphate, ATP, guanylyl-(2′ → 5′)-adenosine and 3′,5′-cyclic AMP, it is likely that a terminal nucleoside 3′-phosphoryl group is required for enzyme activity. Therefore it was not surprising that the products of enzyme action, nucleoside 3′-phosphates, were found to inhibit the reaction competitively. The K_i value for deoxythymidine 3′-phosphate indicates a high degree of affinity for the enzyme and implies that care should be taken to ensure that initial rates are measured in the routine determination of the activity with substrates that liberate deoxythymidine 3′-phosphate.

The results of experiments designed to determine the degree of homogeneity of the enzyme preparation were somewhat equivocal. Electrofocusing of the enzyme for 31 h with a pH gradient of 3—10 gave one peak of activity at pH 4.2, but when electrofocusing was carried out for 90 h with a pH gradient of 3—5 two peaks of activity were found, at pH 3.4 and 7.2, but only 11% of the enzyme activity would be recovered. It was noticed that the enzyme may precipitate and aggregate during purification, the preparations sometimes becoming cloudy at acid pH. Haglund [31] has indicated that proteins which precipitate during electrofocusing can move from the region in which they are focused to other parts of the column, thus giving rise to artifactual results. The results of the electrophoresis experiments have been discussed earlier. It was also found that the preparations of phosphodiesterase II used in these experiments contained activities which hydrolyzed RNA and DNA. The deoxyribonuclease activity could be reduced by passing the preparation through a column of DNA-cellulose (Flanagan, P.R. and Zbarsky, S.H., unpublished). It would be interesting to know whether the residual activity toward RNA was due to a persistent ribonuclease or to an intrinsic polynucleotidase activity of the phosphodiesterase, since purified phosphodiesterases from spleen [4] and testis [5] did not contain any deoxyribonuclease or ribonuclease. Razzell [32] has commented on the difficulty in distinguishing ribonuclease II activity from that of phosphodiesterase II. An answer in the present case must await a detailed examination of the products released by rat intestinal phosphodiesterase II from RNA and DNA.

Since *N*-ethylmaleimide, *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate all inhibited phosphodiesterase II activity to some extent it might be concluded that sulfhydryl groups are involved somehow in the catalytic behaviour of the enzyme. This is supported by the strong inhibition observed wit Cu^{2+} since copper is a common poisoner of sulfhydryl enzymes [33].

A more potent and irreversible inhibition was observed with the alkylating reagent, iodoacetic acid. At reagent concentrations of $2 \cdot 10^{-3}$—$5 \cdot 10^{-3}$ M, 50% of the phosphodiesterase II activity was destroyed in 20—30 min at 24° C

and at pH values between 5 and 6.1, conditions under which the enzyme is normally completely stable [34]. It is unlikely that essential thiol groups were being alkylated. If thiol groups were involved the alkylation should proceed more rapidly at alkaline pH. In fact, the opposite was found (Fig. 5). Further, iodoacetamide, a more reactive thiol alkylating reagent [24], was without effect. In addition to cysteine, iodoacetate can alkylate the ϵ-amino groups of lysine residues, the imidazole groups of histidine residues, the phenolic hydroxyls of tyrosine residues, the thioether sulfur atoms of methionine residues and the β and γ carboxyl groups of aspartic and glutamic acids, respectively. However, alkylation of lysine residues is most rapid at higher pH values [25] and the inactivation of phosphodiesterase II at neutral or slightly acidic pH values was probably too rapid to support the possibility that a methionine was being alkylated [35].

Of the remaining possibilities the most likely is reaction at a histidine by analogy with observations that alkylation of an essential histidine inactivates ribonuclease A [25,36,37], deoxyribonuclease I [26] and deoxyribonuclease II [27]. In each of these cases iodoacetamide, a neutral alkylating agent, was ineffective as it was in the present case against phosphodiesterase II. The present observation that iodoacetate inactivated phosphodiesterase II whereas iodoacetamide did not suggests that the inactivating alkylation process is similar to that for ribonuclease A as reported by Stark et al. [36]. These workers proposed that there is a positively charged group in or close to the active site of the enzymes which attracts the iodoacetate anion toward the active site where it carboxymethylates the appropriate residue. The pH dependence of the inactivation of phosphodiesterase II by iodoacetate supports the idea that such a positively charged group could be the imidazolium portion of a histidine residue since increasing the pH above 7 lowered the alkylation rate markedly. Such a mechanism has already been indicated for ribonuclease A [36] where one imidazole attracts the negatively charged alkylating agent while the other provides the electrons to displace iodine from it and therefore becomes carboxymethylated.

It is interesting that inhibitory reactions with iodoacetate have now been observed for nucleases such as ribonuclease A [25], deoxyribonuclease I [26], deoxyribonuclease II [27], ribonuclease T_1 [28], and rat intestinal phosphodiesterase II. It is likely that despite the widely different specificities of these enzymes they display similarities at their active site regions.

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