***rac*-Glycerol 1:2-Cyclic Phosphate 2-Phosphodiesterase, a New Soluble Phosphodiesterase of Mammalian Tissues**

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1. A soluble phosphodiesterase is present in mammalian tissues which rapidly hydrolyses enantiomorphs of *rac*-glycerol 1:2-cyclic phosphate, producing *rac*-glycerol 1-phosphate.
2. The enzyme has been purified up to 1700-fold by a combination of acetone precipitation and chromatography on DEAE-Sephadex A-50, Sephadex G-150 and hydroxyapatite.
3. The K_m with glycerol cyclic phosphate as substrate is 7.2mM, and the pH optimum broad (6.9–7.5). The molecular weight (by gel filtration) of the enzyme is approx. 35 500.
4. The phosphodiesterase has no requirement for Ca^{2+} or Mg^{2+} , but is stimulated by reducing agents (cysteine, dithiothreitol) and Fe^{2+} .
5. The purified phosphodiesterase preparation also hydrolysed 3':5'-cyclic AMP, producing 5'-AMP exclusively, and 2':3'-cyclic AMP, forming 3'-AMP and 2'-AMP in the ratio 7:3. Bis-(*p*-nitrophenyl) phosphate was slowly hydrolysed, but other phosphodiesterases tested were not attacked.
6. The phosphodiesterase is inhibited by theophylline and *o*-phenanthroline. It is inhibited by P_i and by a variety of phosphomonoesters, of which certain aromatic primary phosphates are particularly effective.

Both glycerol 1:2-cyclic phosphate and glycerol phosphate can be formed through the action of glycerophosphocholine diesterase (EC 3.1.4.2), one of the enzymes involved in the turnover of phospholipids (Clarke & Dawson, 1976). In further experiments (N. Clarke & R. M. C. Dawson, unpublished work), small amounts of glycerol 1:2-cyclic phosphate have been detected in mammalian tissues. We show in the present work that tissues also contain phosphodiesterases that hydrolyse glycerol cyclic phosphate, producing glycerol phosphate. In kidney, one of these phosphodiesterases is associated with the particulate fraction of tissue homogenates. It is strongly inhibited by EDTA and has a pH optimum around 8.9. Another phosphodiesterase is a low-molecular-weight soluble enzyme uninhibited by EDTA and with a pH optimum near neutrality. In the present paper we report the purification of this latter enzyme and an examination of its characteristics. It has not been possible to equate the enzyme with any phosphodiesterase hitherto described. In all tissues, including spleen, the rate at which extracts hydrolyse glycerol cyclic phosphate is appreciably higher than the rate of hydrolysis of the *p*-nitrophenyl ester of thymidine 3'-phosphate, a substrate for soluble phosphodiesterase II (EC 3.1.4.18). In addition, activity towards this latter substrate disappears on purification of the present enzyme.

Experimental

Substrates

sn-Glycerol 2:3-cyclic phosphate. This was prepared by the base-catalysed methanolysis of egg phosphatidylcholine (Maruo & Benson, 1959; Brockerhoff, 1963). Phosphatidylcholine (36mg of P) was dissolved in 10.2ml of chloroform, and 6.8ml of ethanolic 0.3M-KOH was added and the mixture incubated for 50min at 18°C. K^+ cations were removed by adding 10ml of 0.21M- NH_4ClO_4 in methanol/water (43:7, v/v), the insoluble $KClO_4$ being separated by centrifugation at 0°C. The glycerol cyclic phosphate was recovered from the supernatant by preparative paper ionophoresis at pH 3.6 (Dawson & Clarke, 1972). It had a mobility towards the anode slightly faster than P_i (M_{P_i} 1.04), whereas glycerophosphocholine, the other phosphorus-containing product of the methanolysis, was isoelectric.

rac-Glycerol 1:2-cyclic phosphate. This was obtained from butyrylglycerol cyclic phosphate prepared as described by Clarke & Dawson (1976). On paper ionophoresis at pH 3.6 the butyrylglycerol cyclic phosphate gave a single phosphorus-containing spot (M_{P_i} 0.79). On paper chromatography in ethanol/5M- NH_3 (2:1, v/v) the compound had R_F 0.83. It (0.8g of P) was dissolved in 24ml of aq. 18M- NH_3 , and after hydrolysis for 2h at 18°C the reaction mixture was evaporated to dryness *in vacuo*.

at 65°C. The residue was solubilized in 48 ml of dry NH_3 in methanol (5% saturation) and the ammonium salt of glycerol cyclic phosphate precipitated at 18°C with 10 vol. of acetone. This solubilization and reprecipitation were repeated twice. The final product was dissolved in 60 ml of 5%-satd. NH_3 in methanol and treated with an equal volume of diethyl ether. A small precipitate containing glycerol phosphate was removed by centrifugation at 2000g and discarded. The supernatant solution was evaporated to dryness below 50°C and the residue (ammonium glycerol cyclic phosphate) was stored in chloroform/methanol (1:3, v/v) at -28°C. The yield was 60% (based on phosphorus content) and no appreciable decomposition occurred during 8 months. On paper ionophoresis at pH 3.6 the product gave a single phosphorus-containing spot (M_F 1.03), and this homogeneity was also found after paper chromatography in ethanol/5 M- NH_3 (R_F 0.68). Acid hydrolysis (60 min, 0.05 M-HCl, 40°C) completely decomposed the product into glycerol phosphate. This eliminated the possibility of contamination with glycerol 1:3-cyclic phosphate, which is much more stable under acid conditions (Khorana *et al.*, 1957).

Glycerol cyclic [^{32}P]phosphate. The starting point of this synthesis was [^{32}P]phosphatidylcholine or [^{32}P]phosphatidylethanolamine, which were prepared biosynthetically from *Saccharomyces cerevisiae* (Hazlewood & Dawson, 1975). These were deacylated with alkali to give glycerophospho-'base' derivatives (Dawson *et al.*, 1962), which were then hydrolysed in 1 M-HCl (15 min, 100°C) to release glycerophosphoric acid. The glycerol [^{32}P]phosphoric acid was then converted into its cyclic derivative as described above.

Butyrylglycerol phosphate. Butyrylglycerol cyclic phosphate (see above) was dissolved in 0.05 M-HCl at a concentration of 0.5 mg of P/ml and heated at 100°C for 3 min. After neutralization with 0.1 M- NH_3 , the hydrolysate was loaded on a column (10 cm $\times$ 1 cm) of Dowex 1X (OH^- form). Elution of the column with 0.05 M- $(\text{NH}_4)_2\text{SO}_4$ produced an initial phosphorus-containing peak identified as glycerol phosphate. This was followed by the major product, which proved on analysis (Clarke *et al.*, 1976) to contain glycerol, butyric acid and phosphorus in equimolecular ratios. Although this latter product is likely to consist of a mixture of positional isomers of butyrylglycerol phosphate, it nevertheless gave a single spot on paper ionophoresis at pH 3.6 (M_F 0.73) and on paper chromatography in ethanol/5 M- NH_3 (R_F 0.62).

Enzyme assay

Method 1. The usual complete reaction mixture contained, in a total volume of 0.18 ml, a final

concentration of the following reagents: ammonium *rac*-glycerol 1:2-cyclic phosphate (11 mM), glycylglycine/NaOH buffer, pH 7.5 (70 mM), MgCl_2 (10 mM), dithiothreitol (15 mM), ammonium ascorbate (29 mM), FeCl_2 (0.3 mM) and alkaline phosphatase (0.8 unit) (calf intestinal; grade I; Boehringer, Mannheim, Germany). The phosphodiesterase (0.025–50 μg of protein) was added in 50 μl of 0.1 M-glycine/NaOH buffer, pH 8.5, a medium in which the enzyme proved to be relatively stable on storage. With the purified enzyme of high specific activity, consistent results were only obtained if it was supplemented with a solution of bovine serum albumin dialysed against water to act as a protein carrier.

Incubation was for 30 min at 38°C together with independent enzyme and reagent controls. The reaction was stopped by adding 10 ml of a solution containing 10% (w/v) HClO_4 , 0.36% (w/v) ammonium molybdate and 0.0045% (w/v) aminonaphtholsulphonic acid reagent (Bartlett, 1959). After centrifugation at 2000g for 5 min to remove precipitated protein, the P_i released was determined by heating for 10 min at 100°C and measuring the A_{830} of the solution.

When cyclic AMP substrates were used, some decomposition occurred during the colour development. Consequently, with 3':5'-cyclic AMP, heating was shortened to 5 min and the aminonaphtholsulphonic acid reagent was increased to 0.009% (w/v). With 2':3'-cyclic AMP, the HClO_4 concentration was decreased to 7.5% (w/v), the heating time to 2.5 min and the aminonaphtholsulphonic acid reagent increased to 0.0135% (w/v). The hydrolysis of the *p*-nitrophenyl ester of thymidine 3'-phosphate and of bis-(*p*-nitrophenyl) phosphate were measured by determining *p*-nitrophenol liberation (Razzell, 1961).

Method 2. The incubation was as in method 1, with the omission of alkaline phosphatase. At the end of the incubation 0.5 ml of 0.1 M-glycylglycine/NaOH buffer (pH 8.0) containing 15 mM- MgCl_2 was added and the mixture immediately heated at 100°C for 10 min. After cooling to 18°C, 2.0 units of calf intestinal alkaline phosphatase in 0.2 ml of 0.1 M-glycine/NaOH buffer (pH 9.5) were added and the mixture was incubated for a further 20 min at 37°C. Duplicate incubations were carried out for 40 min to ensure that all the phosphomonoesters liberated by the phosphodiesterase had been decomposed. The P_i released was determined as in method 1.

Method 3. The incubation was as in method 1 with omission of alkaline phosphatase. The hydrolysis was stopped by cooling in ice and adding 20 μl of 8 M- NH_3 . A portion was spotted on paper and the reaction products were ionophoresed at pH 3.6 (in pyridine/acetic acid/water, 1:10:89, by vol.) at 48 V/cm. The cyclic phosphate ester substrates were separated from their phosphomonoester products

by ionophoresis for the following times, by using appropriate markers: glycerol cyclic phosphate and its butyryl derivative, 2.5 h; 3':5'-cyclic AMP and 2':3'-cyclic AMP, 6 h. The phosphorus-containing spots were located and measured as previously described (Dawson *et al.*, 1962). This method had to be used with glycerol cyclic [^{32}P]phosphate substrate. The glycerol [^{32}P]phosphate spot separated by ionophoresis was digested and the ^{32}P assayed by Čerenkov counting (Hazlewood & Dawson, 1975).

Enzyme purification

All operations were at 4°C or below. Protein was determined by the method of Lowry *et al.* (1951) with serum albumin as standard. The cortex was taken from the kidneys of two young pigs and homogenized briefly in a Waring blender and then in a homogenizer (Aldridge *et al.*, 1960) in 2.94 vol. of 0.1 M-glycine/NaOH buffer (pH 8.5) containing 20 mM-2-mercaptoethanol. The homogenate was centrifuged for 4 h at 34 000g and the clear supernatant collected.

Acetone precipitation. To 200 ml batches of supernatant was added ammonium ascorbate (0.15 M final concentration) and acetone (38%, v/v). The precipitated protein was centrifuged (34 000g, 3 min) and discarded. Acetone was added to the supernatant to give a final concentration of 50% (v/v) and the precipitated protein collected by centrifuging as before. This 38–50%-acetone fraction was solubilized in 29 ml of 0.1 M-glycine/NaOH buffer (pH 8.5) containing both ammonium ascorbate (0.15 M) and 20 mM-2-mercaptoethanol, and an acetone-precipitated fraction collected between 42 and 50% (v/v). The 42–50%-acetone fraction was resolubilized and dialysed against 50 mM-ethanolamine/HCl buffer (pH 8.5) overnight.

DEAE-Sephadex chromatography. DEAE-Sephadex A-50 (6.7 g) was mixed with 200 ml of 0.5 M-ethanolamine/HCl buffer (pH 8.5) and heated at 100°C for 20 min. After cooling and centrifuging, the gel was washed well with 50 mM-ethanolamine/HCl buffer (pH 8.5). Half the gel was poured to form the base of a column (6.2 cm diam.), and half was mixed with the dialysed 42–50%-acetone fraction containing 0.9 g of protein, which was quickly adsorbed. This gel was poured on the column, which was then eluted with ethanolamine/HCl buffer (pH 8.5) by using a concentration gradient of 50 mM increasing to 160 mM over 2 litres and then remaining constant. Fractions of high specific activity eluted between 150 and 160 mM were pooled and concentrated by adsorbing on a smaller DEAE-Sephadex column (2.5 g) and elution in 12 ml of 3 M-ethanolamine/HCl buffer (pH 8.5).

Gel filtration on Sephadex G-150. The eluate from the DEAE-Sephadex column (see above) (78 mg of

protein in 12 ml) was applied to a Sephadex G-150 column (118 cm $\times$ 2.4 cm) and the proteins were eluted with 0.1 M-glycine/NaOH buffer (pH 8.5). The enzyme emerged as a peak after most of the other proteins had appeared. The active fractions were pooled and dialysed overnight against 20 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.8).

Hydroxyapatite. Bio-Gel HTP (3.3 g) was slurried with 20 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 6.8) and half was poured to form a column (1.1 cm diam.). The enzyme recovered from the Sephadex G-150 column was mixed with the residual half of the Bio-Gel HTP slurry and the remainder of the column poured. Protein was displaced from the column by a stepwise (150 ml) increase in the concentration of the phosphate buffer, 20, 36, 120 and 240 mM. To assay the enzyme it was necessary to remove the phosphate ions from the fractions emerging from the column by dialysing against water. The protein with highest specific activity was eluted at 36 mM-phosphate although some enzyme remained adsorbed up to 240 mM.

Repetition of preceding three steps. By repeating the ion-exchange chromatography, gel-filtration and hydroxyapatite procedures on smaller columns a further purification of up to 3-fold could be achieved, but with poor recovery.

Identification of the products of cyclic AMP hydrolysis

The hydrolysis was allowed to continue almost to completion and a portion of the incubation mixture spotted on paper. With 3':5'-cyclic AMP as substrate the products were separated by anodic paper ionophoresis (pH 3.6, 40 V/cm, 8 h) followed by chromatography in ethanol/1 M-ammonium acetate (7:3, v/v) in the same direction. Under these conditions the migration distances of standards were as follows: 3'-AMP, 32 cm; 5'-AMP, 28.5 cm; 3':5'-cyclic AMP, 37 cm. For 2':3'-cyclic AMP the products were examined by two methods. (1) Paper ionophoresis (pH 3.6, 40 V/cm, 24 h) followed by spraying to detect phosphorus, which was then measured (Bartlett, 1959). In this time 2'-AMP had migrated 86 cm, 3'-AMP 90 cm and 2':3'-cyclic AMP 96 cm. (2) Paper chromatography for 48 h in a two-phase system (Carter, 1950). Spots were located by u.v., cut out, eluted with water and the A_{260} was measured. The R_F values were as follows: 2'-AMP, 0.69; 3'-AMP, 0.62; 2':3'-cyclic AMP, 0.56; adenosine, 0.49.

Reproducibility

Each type of experiment was carried out at least three times with minor variations in the experimental procedure and the results agreed in all essential details.

Results

Glycerol cyclic phosphate phosphodiesterase in various tissues

After the kidneys of a rat were homogenized, both the supernatant and pellet fractions were capable of hydrolysing glycerol cyclic phosphate (Table 1). Although the activity in the supernatant was not inhibited by EDTA, that in the pellet was totally suppressed, suggesting insoluble and soluble phosphodiesterases of different character.

The pH optimum of the insoluble enzyme was alkaline (pH 8.9), whereas it is shown below that the pH optimum of the soluble enzyme is near neutrality. In the tissues examined, the soluble phosphodiesterase was much more active than the insoluble enzyme (Table 1), and in the liver, which showed very active hydrolysis by the soluble enzyme, there was hardly detectable activity in the insoluble fraction. Further studies were therefore concentrated on the soluble phosphodiesterase.

A survey of the soluble phosphodiesterase in various tissues of the rat active against glycerol cyclic phosphate is shown in Table 2. It is clear that the richest sources of the enzyme are the liver,

kidney and intestinal mucosa. All of the other tissues sampled showed some activity, apart from whole blood. The relative distribution of the enzyme appears to be different from that of the phosphodiesterases attacking 2':3'-cyclic AMP (EC 3.1.4.16) and 3':5'-cyclic AMP (EC 3.1.4.17) (Table 2), which as expected were highly enriched in the nervous system (Butcher & Sutherland, 1962; Olafson *et al.*, 1969). Moreover, the highest activity towards the *p*-nitrophenyl ester of thymidine 3'-phosphate, a substrate of soluble phosphodiesterase II (spleen exonuclease, EC 3.1.4.18), was present in the spleen (Table 2). On the other hand, the activity towards bis-(*p*-nitrophenyl) phosphate seemed to bear a fairly constant relationship to the rate of glycerol cyclic phosphate breakdown throughout the various tissues.

To obtain a suitable bulk source of the soluble enzyme for purification and to examine interspecies variability, activities were examined in the livers and kidneys of the mouse, rat, guinea pig, sheep and pig (Table 1). The liver usually had a higher activity per g wet weight than the kidney, apart from in the pig, where activity in the kidney was much higher than that in the liver and approaching that found in rat liver. The pig kidney cortex and medulla showed very similar activity, but, since the medullary material showed more activity towards phosphodiester substrates other than glycerol cyclic phosphate, kidney cortical tissue was chosen as a bulk source of enzyme for purification.

Purification of enzyme

A combination of acetone precipitation and fractionation on successive columns of DEAE-Sephadex anion-exchanger, Sephadex G-150 molecular-filtration gel and hydroxyapatite resulted in a purification that in a number of preparations was 400–500 times the activity in the original supernatant, with a yield of about 5% (Table 3). Repetition of the column-fractionation stages resulted in a maximum purification of 1400–1700 times, in very low yield. Nevertheless sufficient activity was available to investigate those situations, e.g. substrate specificity, that required a high degree of purity.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purest enzyme preparations showed a major band corresponding to a mol.wt. of 36000 and three smaller bands of lower molecular weight. Attempts to elute an active phosphodiesterase from the gel proved unsuccessful, so it was impossible to equate the enzyme with any particular band.

Products of the enzymic reaction

With the purified enzyme and glycerol cyclic phosphate substrate, the sole product detected by

Table 1. *Glycerol cyclic phosphate hydrolysis by soluble and insoluble parts of homogenates of tissues from various species*

Tissues were homogenized in 10 vol. of 0.05 M-glycylglycine buffer, pH 7.5, containing 20 mM-2-mercaptoethanol or in 0.1 M-glycine/NaOH buffer, pH 8.5. The homogenate was centrifuged at 100000g for 90 min, and the precipitate was washed with 10 vol. of water and then recentrifuged at 100000g for 60 min. The soluble portion was dialysed overnight against the buffer, and then both fractions were assayed for glycerol cyclic phosphate hydrolysis (method 1). The soluble part was assayed at pH 7.5 and the insoluble portion at pH 8.9.

Animal	Tissue	Hydrolysis (μ mol/min per g wet wt. of tissue)	
		Soluble	Insoluble
Rat	Kidney (whole)	19.8	1.0
	Liver	27.5	0.05
	Brain (cerebral hemispheres)	2.2	0.11
	Intestinal mucosa	12.9	0.50
Mouse	Kidney*	4.4	—
	Liver*	7.8	—
Guinea pig	Kidney (whole)	13.8	1.2
	Liver	27.1	0.03
	Brain (cerebral hemispheres)	6.8	0.12
Sheep	Brain (cerebral hemispheres)	2.1	0.02
	Kidney (cortex)*	8.3	—
	Liver*	10.4	—
Pig	Kidney (cortex)*	22.8	—
	Liver*	8.3	—

* Glycine/NaOH buffer used.

Table 2. Breakdown of various phosphodiesterases by the soluble phosphodiesterases of rat tissues

The tissues were homogenized in 9 vol. of 0.1 M-glycine/NaOH buffer, pH 8.5, in a Potter-type machine. The homogenates were centrifuged at 100000g for 40 min and the supernatants collected. Incubation conditions were as described in the Experimental section with substrates added at 11 mM concentration (method 1).

Tissue	Substrate ...	Rate of breakdown of glycerol cyclic phosphate ($\mu\text{mol/min per g}$)	Rate of breakdown (% of that for glycerol cyclic phosphate)			
			Bis-(<i>p</i> -nitrophenyl) phosphate	2':3'-Cyclic AMP	3':5'-Cyclic AMP	<i>p</i> -Nitrophenyl ester of thymidine 3'-phosphate
Liver		24.8	2.7	3.9	5.0	0.9
		24.6				
Kidney (adult)		13.0–18.8 (5)	5.1	6.1	8.8	1.4
Kidney (13-day-old rat)		14.7	5.8	8.4	12.8	1.5
Kidney (newborn rat)		3.2	—	—	—	—
		2.3				
Small-intestine mucosa		16.1	—	—	—	—
Colon mucosa		7.4	4.0	7.0	4.5	1.2
Pancreas		11.0	3.4	7.9	6.2	1.0
Heart ventricle		6.5	3.8	4.0	7.6	0.9
Salivary gland		6.4	4.9	9.8	5.7	3.5
Skeletal muscle		4.7	3.3	2.4	4.0	—
Stomach mucosa		3.4	—	—	—	—
Spleen		3.4	4.0	15.0	15.9	47.6
Lung		2.9	4.4	11.5	18.7	—
Brain (cerebrum)		1.5	3.9	128	514	—
Epididymis		3.6	—	—	—	—
Testis		1.9	4.8	9.7	12.5	—
Uterus		2.4	—	—	—	—
Ovary		2.3	—	—	—	—
Blood		0	—	—	—	—

Table 3. Purification of soluble glycerol cyclic phosphate phosphodiesterase from pig kidney cortex

Purification step	Specific activity ($\mu\text{mol/min per mg of protein}$)	Purification (fold)	Yield (%)
(1) Original supernatant	0.188	1	100
(2) 42–50%-acetone precipitation	1.34	7.1	22.7
(3) DEAE-Sephadex A-50 column	3.42	18.1	5.2
(4) Sephadex G-150 column	15.2	80.5	5.0
(5) Hydroxyapatite column	91	485	4.4
(6) Step 3 repeat	90.5	483	2.3
(7) Step 4 repeat	214	1140	1.6
(8) Step 5 repeat	322	1710	0.12

paper ionophoresis was glycerol phosphate. Crude preparations gave some P_i , presumably by secondary phosphomonoesterase activity. A solution of the glycerol phosphate produced by enzymic hydrolysis of *sn*-glycerol 1:2-cyclic phosphate by the phosphodiesterase was examined with glycerol phosphate dehydrogenase (EC 1.1.1.8) (Baldwin & Cornatzer, 1968), and this indicated that 98% was *sn*-glycerol 3-phosphate. Synthetic *rac*-glycerol 1:2-cyclic phosphate gave a glycerol phosphate that assayed as

53% *sn*-glycerol 3-phosphate when subjected to degradation with glycerol phosphate dehydrogenase. This glycerol phosphate reacted with lithium periodate at pH 7.5 producing 1 mol of formaldehyde/mol of glycerol phosphate, whereas authentic glycerol 2-phosphate produced no formaldehyde under the same conditions. These results indicate that no glycerol 2-phosphate had been produced during the enzymic hydrolysis of the glycerol cyclic phosphate and that the phosphodiesterase exclusively acts on the cyclic phosphate ring at its point of attachment to the C-2 position of the glycerol moiety. When *rac*-butyrylglycerol cyclic phosphate was hydrolysed by the enzyme, butyrylglycerol phosphate was the only product identified by paper ionophoresis.

When 3':5'-cyclic AMP was used as substrate, it was shown that 5'-AMP was the only product. With 2':3'-cyclic AMP, both 3'-AMP (69–72%) and 2'-AMP (28–31%) were formed. In additional experiments we found that the same products in a similar ratio were formed by a supernatant of a rat intestinal-mucosa homogenate; Whitfield *et al.* (1955) reported that the main product formed by calf intestinal phosphodiesterase was 3'-AMP. On the other hand we found that rat brain particulate fraction gave 2'-AMP almost exclusively, in agreement with the results of Drummond *et al.* (1962) for bovine brain.

Molecular weight

The purified enzyme was applied with protein standards of known molecular weight (cytochrome *c*, ribonuclease, trypsin inhibitor, whale myoglobin, ovalbumin, bovine serum albumin, calf intestine alkaline phosphatase) to a column of Sephadex G-150 and eluted with 0.02M-NaOH/glycine buffer, pH 8.5. Enzymic activities and A_{280} were measured in collected fractions. Plots of molecular weight against elution volume (Andrews, 1967) gave the phosphodiesterase an approximate mol.wt. of 35 500.

General properties of enzyme

The purified enzyme (1400-fold) had a K_m of 7.2 mM against *rac*-glycerol 1:2-cyclic phosphate when assayed by method 2. A broad pH optimum of 7.4 was recorded in 0.07M-imidazole/HCl buffers (method 1) and 7.5 in 0.1M-glycylglycine buffer (methods 2 and 3). A slightly lower pH optimum (6.9) was observed in a series of 0.1M- $\beta\beta'$ -dimethylglutarate buffers (methods 2 and 3).

Under the conditions of assay, the hydrolysis of substrate was practically linear with time up to 30 min and proportional to protein concentration up to 0.075 μ g of protein (1400-fold-purified enzyme) in the standard assay. Assays carried out by method 1 departed more from the ideal than methods 2 and 3, presumably because the P_i produced during the determination of phosphodiesterase activity with alkaline phosphatase acted as an inhibitor (see below). This inhibition was partially relieved by the Mg^{2+} present in the assay medium.

Stimulatory action of reducing agents and Fe^{2+} ions

The partially purified enzyme was substantially but variably (61–150%) stimulated by the addition of dithiothreitol, the effect being maximal at 10 mM (Fig. 1). The stimulation of the highly purified (1400-fold) enzyme tended to be less, but on aging the stimulation by dithiothreitol increased. Cysteine and 2-mercaptoethanol were almost as effective as dithiothreitol. Ascorbate produced a small stimulation of the reaction (Fig. 1) and when added with dithiothreitol there was an additive effect.

Fe^{2+} cations also produced an additional stimulation of the enzymic activity already stimulated by optimal concentrations of dithiothreitol and ascorbate. An effect was observed at concentrations as low as 5 μ M, but maximal stimulation occurred at 0.3 mM (155% of control); above this concentration precipitation of $Fe(OH)_2$ was observed and the effectiveness declined. In the absence of the other reducing agents, the stimulatory effect of the Fe^{2+} cations was markedly less. Fe^{3+} ions could be substituted for Fe^{2+} without loss of activity; pre-

Fig. 1. Stimulation of glycerol cyclic phosphate hydrolysis by various reducing agents

Incubation conditions were as described in the Experimental section (method 1): 1 μ g of 50-fold-purified enzyme with no reducing agents added hydrolysed 7% of the substrate (11 mM) in 30 min incubation at 38°C. Reducing agents: ●, ascorbate; ○, dithiothreitol; □, ascorbate plus dithiothreitol; △, 2-mercaptoethanol; ▲, cysteine; ■, thioglycollate.

sumably these became reduced to the Fe^{2+} form by the dithiothreitol/ascorbate mixture. Mn^{2+} , Ni^{2+} , Co^{2+} and Cu^{2+} cations failed to produce any stimulation of the reaction.

Substrate specificity

Table 4 shows the specificity of the phosphodiesterase when tested against a variety of phosphodiester substrates. *rac*-Glycerol 1:2-cyclic phosphate and its butyryl ester were attacked most readily. It has been indicated under 'Products of the enzymic reaction' that the phosphodiesterase shows no absolute specificity towards enantiomorphs of glycerol cyclic phosphate; *sn*-glycerol 1:2-cyclic phosphate was readily hydrolysed to completion.

2':3'-Cyclic AMP was hydrolysed at about one-tenth of the rate of glycerol cyclic phosphate, and there was no significant change in the relative rates of attack on these two substrates during a 1700-fold purification. 3':5'-Cyclic AMP was hydrolysed at approximately the same rate as 2':3'-cyclic AMP by the original kidney supernatant, but the activity towards this substrate fell to about 50–70% of the original during purification (Table 4). 3':5'-Cyclic GMP was attacked minimally by the phosphodiesterase, and inositol 1:2-cyclic phosphate not at all. The *p*-nitrophenyl ester of thymidine 3'-phos-

Table 4. *Substrate specificity of glycerol cyclic phosphate phosphodiesterase*

Incubation conditions were as described in the Experimental section (method 1) with ascorbate, dithiothreitol and Fe^{2+} added. Substrates were added at 11 mM concentration: 15–20% of the glycerol cyclic phosphate was broken down in 30 min with the various enzyme preparations used. Results are expressed as percentages of the rate of breakdown of glycerol cyclic phosphate. Degradation of less than 0.5% of the activity towards glycerol cyclic phosphate was shown with *p*-nitrophenyl ester of thymidine 5'-phosphate, and with inositol 1:2-cyclic phosphate, glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoinositol, phosphatidylcholine, phosphatidylinositol, ATP, NADH, PP_i and ADP.

Substrate	Pig kidney enzyme activity (% of rate of breakdown of glycerol cyclic phosphate)		
	Kidney supernatant	Purified 16-fold	Purified 320-fold
Glycerol cyclic phosphate	100	100	100
Butyrylglycerol cyclic phosphate	—	91	58
2':3'-Cyclic AMP	11.6	13	16
3':5'-Cyclic AMP	9.8	6.0	12.5 (purified 1700-fold)
3':5'-Cyclic GMP	2.7	1.4	5.4
<i>p</i> -Nitrophenyl ester of thymidine 3'-phosphate	1.1	0.11	7.1 (purified 1700-fold)
Bis-(<i>p</i> -nitrophenyl) phosphate	4.7	3.6	1.2
			0.1
			4.0

Table 5. *Effect of various substances on the action of purified phosphodiesterase on different phosphodiester substrates*
Substrate concentration was 11 mM unless otherwise stated. Incubation conditions were as described in the Experimental section (method 2, except that method 3 used for inhibition by P_i). The enzyme concentration was adjusted so that 15–20% of substrate was broken down in 30 min.

Phosphodiester substrate	Substance added and concentration (mM)		Purification of enzyme (fold)	Activity (% of original activity)
Glycerol cyclic phosphate	EDTA	0.05	1400	101
		1.0	1400	103
		10.0	20	93,* 95
3':5'-Cyclic AMP		1.0	1400	90
		10.0	20	94
		10.0	20	104
Glycerol cyclic phosphate	MgCl_2	0.25	1400	115
		5	1400	102
		10	20 (enzyme dialysed 11 days)	108
3':5'-Cyclic AMP		12		114
		5	1400	115, 113, 128, 128
		12	20 (enzyme dialysed 11 days)	114
2':3'-Cyclic AMP		12		105
Bis-(<i>p</i> -nitrophenyl) phosphate		12		59
Glycerol cyclic phosphate (7 mM)	P_i	1	20	34
		3	20	17, 12
		10	20	12, 25
3':5'-Cyclic AMP (7 mM)		10	20	14
2':3'-Cyclic AMP (7 mM)		10	20	45
Bis-(<i>p</i> -nitrophenyl) phosphate (7 mM)		10	20	

* $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ present in assay.

phate, a substrate of phosphodiesterase II, was slowly attacked by the original kidney supernatant, but this activity was lost during purification. Bis-(*p*-nitrophenyl) phosphate was hydrolysed by the enzyme at about 4% of the rate of glycerol cyclic phosphate and this activity ratio did not change during purification. All other phosphodiesterases tested were hydrolysed at rates of less than a few per cent of that of glycerol cyclic phosphate.

Effect of bivalent metal ions and EDTA

Apart from Fe^{2+} ions the hydrolysis of glycerol cyclic phosphate was only minimally stimulated by any other metal ions tested. Mg^{2+} stimulated a 20-fold purified enzyme, dialysed for 11 days against 0.1 M-glycine buffer, pH 8.5, by 2% at 10 mM and 8% at 12 mM (assay method 3). With a purer enzyme (1700-fold purified) and in the absence of

added Fe^{2+} ions the stimulation was 4% at 0.25 mM and 15% at 5 mM (assay method 2) (Table 5). With 3':5'-cyclic AMP as substrate the stimulation by Mg^{2+} tended to be somewhat higher (Table 5).

Ca^{2+} (10 mM) did not stimulate glycerol cyclic phosphate hydrolysis, nor did Co^{2+} , Ni^{2+} , Mn^{2+} or Cu^{2+} when tested at 0.3 mM. Zn^{2+} (1 mM) inhibited the hydrolysis by 18%.

EDTA had little effect on the basic phosphodiesterase activity observed in the absence of added Fe^{2+} ions. With a highly purified enzyme preparation (1400-fold), addition of 0.05 mM-EDTA gave an activity that was 101% of control, and 1 mM gave 103%. With a less-pure enzyme preparation (20-fold purified) dialysed for 11 days, 10 mM-EDTA gave activities that were 93–95% of the control (Table 5).

Inhibitors

Theophylline produced some inhibition of the phosphodiesterase acting against both glycerol cyclic phosphate and 3':5'-cyclic AMP substrates, and this was also true for *o*-phenanthroline (Fig. 2). The characteristics of the inhibition produced by theophylline and *o*-phenanthroline were similar for the hydrolysis of both these substrates and quite unlike that of a brain supernatant preparation hydrolysing 3':5'-cyclic AMP (Fig. 2). It can be calculated from the results given in Tables 2 and 4 that the present glycerol cyclic phosphate phosphodiesterase would contribute at most a few per cent of the 3':5'-cyclic AMP breakdown catalysed by the brain supernatant, and therefore it is likely that almost all would occur through the specific cyclic AMP phosphodiesterase (EC 3.1.4.17), which is enriched in brain tissue.

The phosphodiesterase was markedly inhibited by P_i (Table 5); 10 mM lowered the activity against glycerol cyclic phosphate and 2':3'- and 3':5'-cyclic AMP to a low value. In these experiments the enzymic activity had to be assayed by method 3 because of the presence of large amounts of added P_i in the incubation medium. The inhibitory effect of P_i could be partially alleviated by the addition of 10 mM- Mg^{2+} , which presumably decreases the effective concentration of phosphate ions through the formation of magnesium phosphate. NaF (10 mM) and iodoacetate (10 mM) produced a small inhibition of the phosphodiesterase activity against glycerol cyclic phosphate when assayed by method 3 (with dithiothreitol omitted), amounting to 23–28% and 20–21% respectively.

The most potent of the inhibitors of the enzymic hydrolysis of glycerol cyclic phosphate that were examined were certain phosphomonoesters. Although all phosphomonoesters tested produced some inhibition of the reaction, there was wide variation in their effectiveness (Table 6). Thus phenyl phosphate and

Fig. 2. Comparison of the inhibition by theophylline (a) and *o*-phenanthroline (b) of the purified phosphodiesterase hydrolysing glycerol cyclic phosphate and 3':5'-cyclic AMP, and a rat brain supernatant fraction hydrolysing 3':5'-cyclic AMP

The substrates were used at a concentration of 0.4 mM. Phosphodiesterase activity was assayed by method 2 with the incubation medium containing 0.3 mM- Fe^{2+} . Symbols: ○, glycerol cyclic phosphate, kidney phosphodiesterase (1500-fold-purified); ●, 3':5'-cyclic AMP, kidney phosphodiesterase (1500-fold-purified); □, 3':5'-cyclic AMP, rat brain supernatant fraction (Table 2).

α -naphthyl phosphate were highly inhibitory, phosphatidic acid and dibutyl glycerophosphate and AMP were intermediary and phosphocholine and glucose 6-phosphate were much less effective. The inhibition produced by phenyl phosphate was tested for competitiveness. Lineweaver-Burk (1934) plots indicated that phenyl phosphate was competing very effectively with the substrate for the active centre (Fig. 3). Phenyl phosphate itself was not hydrolysed by the enzyme preparation. The phosphodiesterase was inhibited by its hydrolysis product, glycerol 1-phosphate, but the effectiveness of this as an inhibitor was low compared with some of the other phosphomonoesters (Table 6). The hydrolysis of 3':5'-cyclic AMP by the enzyme was also

Table 6. *Inhibition of glycerol cyclic phosphate phosphodiesterase by various phosphomonoesters*

The activity was measured by using method 3 (see the Experimental section) and glycerol cyclic [32 P]phosphate. The phosphodiesterase had been purified 1500-fold. The glycerol 1- 32 P]phosphate produced was separated by ionophoresis and its radioactivity determined.

Inhibitor	Substrate concn. (mM)	Inhibitor concn. (mM)	Inhibition (%)
Phenyl phosphate	11	0.25	58
		0.45	68 (62)*
		1.0	81
α -Naphthyl phosphate	11	0.25	51
		0.45	64
Methyl phosphate	11	0.25	34 (34)*
Ethyl phosphate	11	0.25	34
Phosphatidic acid	11	0.45	43 (49)*
Dibutylglycerol phosphate	11	0.45	40
Pyridoxal phosphate	11	0.25	14
Cetyl phosphate	11	0.45	29
<i>p</i> -Nitrophenyl phosphate	0.4	5.5	95
5'-AMP	11	9.0	88
	11	1.5	48
	11	9.0	76
2'-AMP	0.4	9.0	90
	0.4	9.0	75
	0.4	9.0	68
	11	1.5	19
	11	5.0	16
Glycerol 1-phosphate	11	10.0	25
	11	20.0	34
	0.4	5.0	41
	0.4	10.0	57
	0.4	20.0	66
Glycerol 2-phosphate	0.4	20.0	40
ATP	0.4	9.0	34
Phosphocholine	0.4	9.0	18
Glucose 6-phosphate	0.4	9.0	28

* Inhibition with 3':5'-cyclic AMP (11 mM) as substrate.

Fig. 3. *Lineweaver-Burk (1934) plots for the inhibition of glycerol cyclic phosphate phosphodiesterase by phenyl phosphate*

The phosphodiesterase was determined by using method 3 with glycerol cyclic [32 P]phosphate substrate. Enzyme was 1500-fold purified, assayed in a reaction mixture of 90 μ l total volume. Symbols: Δ , no inhibitor present; $\bullet$, 0.03 mM-phenyl phosphate; $\circ$, 0.2 mM-phenyl phosphate.

properties are more equivalent to those displayed by lysosomal phosphodiesterase II (Flanagan & Zbarsky, 1977), but the latter can readily attack the *p*-nitrophenyl ester of thymidine 3'-phosphate (Razzell, 1961; Erecińska *et al.*, 1969), a substrate not hydrolysed by the present purified enzyme. In conformity with this observation the highest activity displayed towards the *p*-nitrophenyl ester of thymidine 3'-phosphate in simple extracts of various rat tissues was found in the spleen (Table 2), whereas the activity towards glycerol cyclic phosphate was highest in the liver. The ratio between the rates of hydrolysis of the two substrates varied over 50-fold between the two tissues, again indicating that the substrates are broken down by different enzymes.

A soluble phosphodiesterase active at neutral pH in the presence of EDTA has been purified from the tobacco plant (Shinshi *et al.*, 1976). However, unlike the present enzyme this phosphodiesterase readily attacked the *p*-nitrophenyl ester of thymidine 3'-phosphate. Its preferred substrate is bis-(*p*-nitrophenyl) phosphate, a phosphodiester that is attacked by our enzyme at about 4% of the rate of glycerol cyclic phosphate. We have measured the hydrolysis of glycerol cyclic phosphate by extracts of various plants, including tobacco, and found that although this substrate was hydrolysed, bis-(*p*-nitrophenyl) phosphate was broken down at a faster or equal rate.

The ability of the present enzyme preparation to attack enantiomorphs of glycerol cyclic phosphate is in marked contrast to D-*myo*-inositol 1:2-cyclic phosphate 2-phosphodiesterase, which we have

inhibited by phenyl phosphate, phosphatidic acid and methyl phosphate to an extent approximately equivalent to that observed with glycerol cyclic phosphate as substrate (Table 6).

Discussion

The phosphodiesterase studied and substantially purified in the present investigation appears to be different from any in mammalian tissue previously characterized. Many phosphodiesterases in such tissues are membrane-bound, active at an alkaline pH and require added bivalent metal ions, usually Mg^{2+} or Ca^{2+} , for full activity. The present enzyme appears to be substantially soluble, it has a neutral pH optimum and is not inhibited by EDTA. These

shown is specific for the D-enantiomorph of the substrate (Dawson & Clarke, 1972). Although the maximum rate of hydrolysis occurs with glycerol cyclic phosphate and its butyryl ester, the enzyme preparation can also attack 2':3'-cyclic AMP, 3':5'-cyclic AMP and bis-(*p*-nitrophenyl) phosphate at substantial rates. The evidence available suggests that these latter phosphodiesterases are being hydrolysed by the same active centre that is responsible for the activity towards glycerol cyclic phosphate. The ratio between the rates of hydrolysis of glycerol cyclic phosphate and 2':3'-cyclic AMP or bis-(*p*-nitrophenyl) phosphate does not change on purification, whereas the relative activity towards 3':5'-cyclic AMP is decreased slightly after a 1700-fold purification from the supernatant. This does not necessarily imply that the present enzyme when pure cannot hydrolyse 3':5'-AMP; the most likely explanation is that the original supernatant contains cytoplasmic 3':5'-cyclic AMP phosphodiesterase (EC 3.1.4.17) and sufficient Mg^{2+} for this to show some activity towards 3':5'-cyclic AMP under the conditions of the assay. In contrast with the present enzyme, the main mammalian enzyme that hydrolyses 2':3'-cyclic AMP (EC 3.1.4.16) appears to be membrane-bound and of low activity in kidney tissue (Drummond *et al.*, 1962), although soluble phosphodiesterases that hydrolyse this substrate have been reported.

The probability that the glycerol cyclic phosphate phosphodiesterase also attacks 2':3'- and 3':5'-cyclic AMP and bis-(*p*-nitrophenyl) phosphate is also supported by the lack of any requirement for Mg^{2+} or effect of EDTA when these substrates were being attacked. In addition, their hydrolysis, like that of glycerol cyclic phosphate, was markedly inhibited by P_i ions and phosphomonoesters. The hydrolysis of 3':5'-cyclic AMP also showed the same pH optimum (7.3) and was inhibited by theophylline and *o*-phenanthroline in a manner similar to the hydrolysis of glycerol cyclic phosphate and distinct from that of specific 3':5'-cyclic AMP phosphodiesterase. In addition, the ratio between bis-(*p*-nitrophenyl) phosphate and glycerol cyclic phosphate hydrolysis is reasonably constant in the supernatants from various tissues of the rat (Table 2), although it is low in the liver. In additional experiments (N. G. Clarke & R. M. C. Dawson, unpublished work) we have found that in sheep tissues proportionally much more (2.5–3.0 times) of the bis-(*p*-nitrophenyl) phosphate is decomposed by the supernatant fraction compared with the rat tissues, which could indicate that more than one type of phosphodiesterase is involved in the hydrolysis of this substrate.

The complex kinetics of 3':5'-cyclic AMP hydrolysis by many tissue extracts, including kidney (Jard & Bernard, 1970), has been ascribed to multiple forms of the enzyme (e.g. high- and low- K_m forms). However,

unlike the present enzyme, these forms all require bivalent metal ions for activation and are probably multiple molecular aggregates of 3':5'-cyclic nucleotide phosphodiesterase (Pichard & Cheung, 1976).

Buchnea (1973) quoted preliminary experiments that suggested that glycerol 1:3-cyclic phosphate could act as a substrate for purified 3':5'-cyclic nucleotide phosphodiesterase. In additional experiments we have tested the commercially purified enzyme (Boehringer, 0.15 unit/mg) from ox heart and found that *rac*-glycerol 1:2-cyclic phosphate was hydrolysed at about 3% of the rate of 3':5'-cyclic AMP cleavage, quite different from the phosphodiesterase studied in the present investigation. Morishima (1974) has isolated a purified phosphodiesterase from the larvae of the silkworm that can attack both 2':3'- and 3':5'-cyclic AMP in the presence of EDTA. The former substrate was hydrolysed at a rate that was 10 times greater than for the latter, which contrasts markedly with the present enzyme.

The activation of the enzyme by reducing agents such as dithiothreitol, cysteine, ascorbic acid and Fe^{2+} ions could be produced by the reduction of disulphide bonds in the enzyme structure, or alternatively by these changing the polymeric form of the enzyme. Although a number of dehydrogenases contain or have iron intimately associated with them, the stimulation of the phosphodiesterase by Fe^{2+} ions is more likely to be associated with their reducing capacity. Thus compared with such enzymes the phosphodiesterase is insensitive to *o*-phenanthroline inhibition, which would suggest that the phosphodiesterase activity has no absolute requirement for iron. A few reports have occurred describing the activation of EDTA-insensitive 3':5'-cyclic AMP phosphodiesterases by Fe^{2+} or Fe^{3+} (Okabayashi & Ide, 1970; Brewin & Northcote, 1973).

The marked competitive inhibition observed with certain phosphomonoesters is likely to be due to the latter having a higher affinity for the active centre of the enzyme than the substrate itself, although no phosphatase action occurs. That this affinity can exist is perhaps indicated by the product inhibition of the activity by glycerol 1-phosphate, suggesting that the dissociation of the enzyme-product complex may be of significance in determining the K_m value.

It is not known whether glycerol cyclic phosphate is the main physiological substrate of the enzyme. Although this phosphodiester has so far only been shown to be produced from glycerophosphocholine and glycerophosphoethanolamine through the action of glycerophosphocholine diesterase (Clarke & Dawson, 1976), it is conceivable that other enzymes forming glycerol phosphate might also produce small amounts of the cyclic derivative under certain conditions. In preliminary experiments (N. G.

Clarke & R. M. C. Dawson, unpublished work) we have detected low concentrations of glycerol cyclic phosphate in several mammalian tissues. The cyclic derivative can act as a precursor of tissue glycerophospholipids and triacylglycerol (Buchnea, 1973), but presumably only after hydrolysis to glycerol phosphate.

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