Paukert, J. L., Straus, L. D., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 5104–5111.
Paukert, J. L., Williams, G. R., & Rabinowitz, J. C. (1977) *Biochem. Biophys. Res. Commun. 77*, 147–154.
Priest, D. G., Happel, K. K., Mangum, M., Bednarek, J. M., Doig, M. T., & Baugh, C. M. (1981) *Anal. Biochem. 115*, 163–169.
Rabinowitz, J. C. (1981) in *Folyl and Antifolyl Polyglutamates* (Goldman, I. D., Chabner, B. A., & Bertino, J. R., Eds.) pp 75–83, Plenum Press, New York.
Schirch, L. (1978) *Arch. Biochem. Biophys. 189*, 283–290.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Tan, L. U. L., & MacKenzie, R. E. (1977) *Biochim. Biophys. Acta 485*, 52–59.
Tan, L. U. L., Drury, E. J., & MacKenzie, R. E. (1977) *J. Biol. Chem. 252*, 1117–1122.
Wasserman, G. F., Benkovic, P. A., Young, M., & Benkovic, S. J. (1983) *Biochemistry 22*, 1005–1013.

Purification and Characterization of a Human Platelet Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: A cyclic nucleotide phosphodiesterase was extensively purified from the 100000*g* supernatant fraction of human platelets. The purification was 2500–3000-fold with 30% recovery of activity. The enzyme was isolated by DEAE-cellulose chromatography followed by adsorption to blue dextran–Sepharose and elution with cAMP. The protein has a molecular weight of 140 000 as determined by gel filtration. On $NaDodSO_4$-containing polyacrylamide gels the major band is at 61 000 daltons, suggesting that the enzyme may exist as a dimer in solution under nondenaturing conditions. The enzyme requires Mg^{2+} or Mn^{2+} for activity. The calcium binding protein calmodulin does not stimulate hydrolysis of cAMP by this enzyme. The purified enzyme hydrolyzes both cAMP and cGMP with normal Michaelis–Menten kinetics with K_m values of 0.18 μM and 0.02 μM, respectively. The hydrolysis of cGMP, however, is only one-tenth as rapid as the hydrolysis of cAMP. Cyclic GMP does not stimulate cAMP hydrolysis but instead is a potent competitive inhibitor of cAMP hydrolysis. The enzyme is also competitively inhibited by the phosphodiesterase inhibitors papaverine, 3-isobutyl-1-methylxanthine, and dipyridamole. The enzyme did not cross-react with an antibody raised to a cAMP phosphodiesterase isolated from dog kidney, indicating that the enzymes are not immunologically related. The inhibition of cAMP hydrolysis by cGMP suggests a possible regulatory link between these two cyclic nucleotides. One of the roles of cGMP in platelets may be to potentiate increases in intracellular cAMP by inhibiting the hydrolysis of cAMP by this enzyme.

Cyclic nucleotide phosphodiesterase (EC 3.1.4.17), the catabolic enzyme for the important regulatory nucleotides cAMP and cGMP, has been reported to exist in multiple forms in a wide variety of tissues and cell types [for recent reviews, see Wells & Hardman (1977), Strada & Thompson (1978), Vaughn et al. (1981), Appleman et al. (1982), and Beavo et al. (1982)]. These varieties of the enzyme differ in their substrate specificities, kinetic characteristics, and physical properties and in their response to natural and pharmacologic regulators. The physiological significance and function of these various enzymatic species are not well understood. Whether this diversity is due to the existence of unique enzymes, interconvertible molecules, or common subunits is still unclear. Several cyclic nucleotide phosphodiesterases from different species and sources have been purified and characterized (Miki et al., 1975; Ho et al., 1977; Morrill et al., 1979; Thompson et al., 1979a; Martins et al., 1982). In addition, antibodies have been raised against some of these purified phosphodiesterases (Tucker et al., 1981; Hansen & Beavo, 1982; Mumby et al., 1982; Sarada et al., 1982). Biochemical and immunological characterization of purified cyclic nucleotide phosphodiesterases should facilitate our understanding of the specific functions, interrelationships, and regulation of the multiple forms of cyclic nucleotide phosphodiesterase within cells.

In human platelets an increase in intracellular levels of cAMP is associated with the inhibition of platelet responses such as shape change, aggregation, adhesion, and release of granule contents [for reviews, see Salzman & Weisenberger (1972), Haslam (1973), and Mills (1982)] and thus may regulate platelet participation in physiological hemostasis and pathological thrombosis. Phosphodiesterase inhibitors such as the methylxanthines, papaverine, and dipyridamole have been shown to inhibit platelet activation (Ardlie et al., 1967; Markwardt et al., 1967; Vigdahl et al., 1971). Furthermore, such inhibitors potentiate the inhibition of platelets by such adenylate cyclase agonists as PGE_1, PGI_2, and adenosine (Markwardt et al., 1967; Mills et al., 1970; Mills & Smith, 1971; Jorgensen et al., 1979).

Hidaka and co-workers (Hidaka & Asano, 1976; Asano et al., 1977) reported the separation of three forms of cyclic nucleotide phosphodiesterase from human platelets by DEAE-cellulose chromatography. One enzyme appeared to be specific for cGMP, the second relatively nonspecific, and the third relatively specific for cAMP. In this study we report

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the extensive purification of the latter cyclic nucleotide phosphodiesterase from human platelets and characterization of the purified enyzme.

Materials and Methods

Materials. Florisil and bovine heart phosphodiesterase activator were purchased from Sigma Chemical Co. Human erythrocyte calmodulin was purchased from Calbiochem-Behring. [^{3}H]cAMP (38.1 Ci/mmol) and [^{3}H]cGMP (10.4 Ci/mmol) were purchased from New England Nuclear. DEAE-cellulose (DE-52) was purchased from Whatman, Ltd.

Preparation of Blue Dextran–Sepharose. Blue dextran–Sepharose was prepared by using a procedure modified from that of Ryan & Vestling (1974). Five grams of cyanogen bromide activated Sepharose 4B was rehydrated in 25 mL of 1 mM HCl for 30 min at room temperature. The gel was then filtered, washed repeatedly with 1 mM HCl (1 L total), and then washed repeatedly with 0.1 M $NaHCO_3$, pH 8.5 (250 mL total). The gel was then resuspended in 37.5 mL of 0.1 M $NaHCO_3$ containing 0.35 g of blue dextran 2000 (Pharmacia). The suspension was mixed on a rotating table for 5 h at room temperature. The blue dextran solution was removed by filtration, the gel was resuspended in 50 mL of 0.1 M $NaHCO_3$ containing 1 M ethanolamine, and the suspension was stirred for 1.5 h at room temperature. The gel was filtered, washed repeatedly with water, and stored at 0–4 °C in 50 mM Tris-HCl (pH 7.5)–20 mM $MgCl_2$–0.02% sodium azide.

Protein Determination. Protein determinations were done using the method of Schaffner & Weissmann (1973). Standard curves were prepared by using BSA as the standard protein.

Subcellular Fractionation of Platelets. Subcellular fractionation of platelets was performed as described by Fukami et al. (1978) with minor modifications. Platelet-rich plasma was prepared from freshly drawn blood collected in 0.1 volume of 3.8% sodium citrate containing 100 mM benzamidine, 50 mM EACA,[1] 20 mM EDTA, 0.2% soybean trypsin inhibitor, and 500 kallikrein inhibitory units/mL aprotinin. The citrated blood was centrifuged at 200*g* for 15 min at 20 °C in a Sorvall HS-4 rotor. The platelet-rich plasma was carefully removed and centrifuged at 3000*g* for 10 min at 4 °C. The platelet pellet was resuspended in Tyrode's buffer, pH 6.5, without Ca^{2+} and containing 1 mM EDTA, 0.1% bovine serum albumin, and 2 mM $MgCl_2$. Rotenone (8 μg/mL) and 2-deoxyglucose (0.8 mg/mL) were added and the platelet suspension was incubated at 37 °C for 20 min. After incubation the platelet suspension was centrifuged at 3000*g* for 10 min at 4 °C. The platelet pellet was resuspended in 10 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM $MgCl_2$, 10 mM benzamidine, 5 mM EACA, 2 mM EGTA, 0.2 mg/mL soybean trypsin inhibitor, 50 kallikrein inhibitory units/mL aprotinin, 5 mM 2-mercaptoethanol, and 2 mM phenylmethanesulfonyl fluoride. The platelets were disrupted by using nitrogen bomb decompression (Paar Instrument Co., Moline, IL) after pressurization to 1200 psi for 15 min. The disrupted platelet suspension was centrifuged at 1000*g* (3000 rpm) in a Sorvall SS34 rotor for 20 min. The supernatant was centrifuged at 12000*g* (10000 rpm) for 20 min. The supernatant from this centrifugation was centrifuged at 80000*g* (35000 rpm) for 70 min in a Beckman type 40 rotor. The pellet from the first centrifugation contained large cellular debris and unbroken cells. The second pellet contained granules and mitochondria. The third pellet contained membranous material (Fukami et al., 1978). Each pellet was resuspended in the disruption buffer and made 0.1% in Triton X-100. Each fraction was assayed for cAMP phosphodiesterase activity at 1 μM cAMP.

Purification of Cyclic Nucleotide Phosphodiesterase. (*1*) *Preparation of Platelets.* Outdated platelet concentrates (72–120 h old) were obtained from the local American Red Cross blood bank. Each unit of platelet concentrate was derived from 1 unit (450 mL) of blood and contained approximately 10^{11} platelets. The suspensions were made 1 mM in EDTA and centrifuged for 2.5 min at 3000 rpm (1500*g*) at 5–10 °C in a Sorvall GS3 rotor to remove most of the contaminating erythrocytes. The platelets were then pelleted from the supernatant liquid by centrifugation for 20 min at 8000 rpm (10000*g*) in the GS3 rotor. Platelets were removed from any remaining red cells by carefully removing the upper part of the pellet with a rubber policeman, leaving the lower portion of the pellet containing red cells undisturbed. The platelets were resuspended in 100 mM Tris–acetate (pH 6.0)–1 mM EDTA–0.15 M sodium chloride and recentrifuged, and the procedure was repeated. After a third centrifugation the platelet pellets were frozen in dry ice–methanol and stored at −80 °C.

(*2*) *Disruption of Platelets.* The frozen platelets from 150 to 250 units of blood were resuspended in 200 mL of 100 mM Tris–acetate, pH 6.0, containing 20 mM $MgCl_2$, 20 mM benzamidine, 10 mM EACA, 4 mM EGTA, 0.4 mg/mL soybean trypsin inhibitor, 0.4 unit/mL hirudin, 50 kallikrein inhibitory units/mL aprotinin, 10 mM diisopropyl fluorophosphate, 4 mM phenylmethanesulfonyl fluoride, 40 μM TLCK, 20 μM leupeptin, and 10 μM pepstatin A. All succeeding steps were conducted at 0–4 °C unless indicated.

The platelet suspension was placed in a nitrogen bomb, pressurized to 1300 psi, and held at pressure for 45 min with stirring. The sample was then released from the bomb and centrifuged at 15000 rpm (27000*g*) for 10 min in a Sorvall SS34 rotor. The supernatant was removed, and the pellet was washed with approximately 50 mL of buffer and recentrifuged. The supernatants were combined and centrifuged for 1 h at 50000 rpm in a Beckman Ti60 rotor. The supernatant (100000*g* supernatant) was removed, taking care to avoid the cloudy region just above the pellet.

(*3*) *DEAE-cellulose Chromatography.* The 100000*g* supernatant was applied to a 150–200-mL DEAE-cellulose column equilibrated in 50 mM Tris–acetate, pH 6.0, containing 20 mM $MgCl_2$, 10 mM benzamidine, 5 mM EACA, 2 mM EGTA, 0.02% soybean trypsin inhibitor, 20 μM TLCK, 10 μM leupeptin, and 5 μM pepstatin A. After addition of the 100000*g* supernatant, the column was washed with 2–3 bed volumes of the above buffer at a flow rate of about 1 mL/min. A 1-L linear sodium acetate gradient with a limiting concentration of 1 M was applied, and 4-mL fractions were collected at a flow rate of 1 mL/min. The column fractions were assayed at 1 μM and 100 μM cAMP and the fractions containing the low K_m activity (peak II) pooled.

(*4*) *Blue Dextran–Sepharose Cromatography.* The pooled fractions from the DEAE-cellulose column were applied directly to a 10-mL column of blue dextran–Sepharose equilibrated with 50 mM Tris-HCl, pH 7.5, containing 20 mM $MgCl_2$, 10 mM benzamidine, 5 mM EACA, 2 mM EGTA,

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PIPES, 1,4-piperazinediethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EACA, ϵ-amino-*n*-caproic acid.

20 μM TLCK, 10 μM leupeptin, and 5 μM pepstatin A at a flow rate of 0.5 mL/min. The column was washed with 50–100 mL of the equilibrating buffer. A 100-mL linear gradient of cAMP from 0 to 1 mM in the same buffer was then applied, and 2 mL fractions were collected. The fractions were then assayed for activity, and the active fractions were pooled, placed in dialysis tubing, and concentrated approximately 10-fold in dry sucrose. The concentrated enzyme was then stored at 0–4 °C.

For certain experiments platelets were isolated from freshly drawn blood. The blood (900 mL) was collected into 0.1 volume of 3.8% sodium citrate, platelet-rich plasma was prepared, and platelets were collected and washed as described above. Disruption of the platelets and purification of the cyclic nucleotide phosphodiesterase were done according to the scheme outlined above. The washed platelets were resuspended in 5 mL of buffer and disrupted by nitrogen decompression. The 100000*g* supernatant was applied to a 10-mL DEAE-cellulose column and eluted with a sodium acetate gradient of 140 mL at a flow rate of 0.1 mL/min. Peak II was pooled and applied to a 2-mL blue dextran–Sepharose column and eluted with a 50-mL cAMP gradient at a flow rate of 0.1 mL/min. The peak of activity was pooled, concentrated against dry sucrose, and stored at 4 °C.

Gel Filtration of Purified Cyclic Nucleotide Phosphodiesterase. A sample of the purified cyclic nucleotide phosphodiesterase in 0.5 mL was applied to a column (0.9 × 60 cm) of Sephadex G-200 equilibrated in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5 M NaCl, and 0.2 mg/mL BSA and eluted with the same buffer at about 4 mL/h. Fractions of 0.25 mL were collected and assayed for activity.

Polyacrylamide Gel Electrophoresis. Samples were run on $NaDodSO_4$-containing polyacrylamide gels according to the method of Laemmli (1970). Samples were incubated for 2 min at 100 °C with or without 2-mercaptoethanol and then cooled before application to the gel.

Samples of the purified cyclic nucleotide phosphodiesterase were analyzed under nondenaturing conditions on 7.5% polyacrylamide gels by using buffers identical with those in the Laemmli system (1970) except $NaDodSO_4$ was eliminated from all solutions and the gel and sample solutions were made 30% in ethylene glycol. Disc gels were run at 8 mA/gel for 4.5 h with a 50 mM Tris–3% glycine running buffer at pH 8.3. One gel was stained with Coomassie Blue. A parallel gel was analyzed for phosphodiesterase activity by slicing the gel into 1-mm slices, mincing the slices, and incubating each slice overnight at room temperature in 0.2 mL of a reaction mixture containing 125 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM dithiothreitol, 1 mg/mL BSA, and 250000 cpm of [^{3}H]cAMP (27 Ci/mmol) per slice. After incubation 1 mL of 0.5 M phosphate buffer, pH 7.0, was added to each incubation. The whole mixture was then placed on a Florisil column, eluted, and analyzed for phosphodiesterase activity as described below.

Cyclic Nucleotide Phosphodiesterase Assays. Phosphodiesterase activities were assayed at 30 °C in a 0.1-mL reaction volume containing 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mg/mL BSA, and appropriate levels of tritiated cAMP or cGMP (usually about 50000 cpm/assay). Column fractions were assayed for cAMP phosphodiesterase activity by using a $BaSO_4$ precipitation as described by Sinha et al. (1977). The assay was stopped by the addition of 0.2 mL of 0.2 M $ZnSO_4$, 0.2 mL of 0.2 M $Ba(OH)_2$ was then added, and the $BaSO_4$ precipitate was collected by centrifugation for 3 min at 12000*g* in an Eppendorf centrifuge. The AMP produced by hydrolysis binds to the $BaSO_4$ and is removed from solution. An aliquot of the supernatant was removed for counting in a liquid scintillation counter. The degree of hydrolysis was determined by difference from a control assay that contained no enzyme. Determinations of kinetic constants in reactions utilizing cAMP as the substrate were performed using the Florisil column assay described by Sinha & Colman (1981) except the Mg^{2+} concentration was 20 mM and the reaction volume was 0.1 mL. All reactions were terminated before hydrolysis exceeded 10% of the cAMP in the reaction by the addition of 10 μL of 50% trichloroacetic acid. The assay mixture was diluted with 1.0 mL of 0.5 M phosphate buffer, pH 7.0, and the entire sample was applied to a 1-mL column of Florisil equilibrated in the same phosphate buffer. The column was eluted with additional aliquots of buffer. The AMP eluted first while cAMP remained bound to the column. The fractions containing AMP were counted in a liquid scintillation counter to determine the degree of cAMP hydrolysis.

Assays involving cGMP hydrolysis were performed using the anion-exchange resin column separation procedure described by Thompson et al. (1979b) except that the reaction volume was 0.1 mL with reaction conditions as described above.

Immunologic Analysis of Purified Cyclic Nucleotide Phosphodiesterase. Samples of the purified cyclic nucleotide phosphodiesterase from human platelets and a crude sample of cAMP phosphodiesterase from dog kidney were subjected to electrophoresis on a 10% $NaDodSO_4$ containing polyacrylamide gel according to the method of Laemmli (1970). The gel was blotted by using the western blot technique of Towbin et al. (1979). Proteins were transferred electrophoretically (2 A, 20 V, 15 h) to nitrocellulose paper in a Tris–glycine–methanol buffer (pH 8.3). The bands of cAMP phosphodiesterase were detected by autoradiography after equilibration with ^{125}I-labeled antibody (0.5 mol of ^{125}I/mol of antibody) raised against purified cAMP phosphodiesterase from dog kidney (Sarada et al., 1982). ^{125}I-Labeled human transferrin (M_r 81000), BSA (68000), ovalbumin (44500), chymotrypsinogen A (24100), and lysozyme (15200) were used as standard marker proteins in the gel.

Results

Subcellular Fractionation of Platelets. In studies of the subcellular localization of the cyclic nucleotide phosphodiesterase activity, platelets from freshly drawn blood were incubated with the metabolic inhibitors 2-deoxy-D-glucose and rotenone to prevent secretion of granule contents before homogenization and fractionation of the platelets (Fukami et al., 1978). In addition, differential centrifugation was performed in the presence of a mixture of proteolytic inhibitors to prevent changes due to proteolysis during lysis and centrifugation. About 20% of the cAMP phosphodiesterase activity measured at 1 μM cAMP was in the low-speed pellet (1000*g*) containing large debris and unbroken cells. This fraction has not been further characterized. Virtually no activity was found in either the granular fraction (12000*g* pellet) or the membrane-containing fraction (100000*g* pellet). The soluble fraction (100000*g* supernatant) contained 75–80% of the phosphodiesterase activity measured at 1 μM cAMP. This activity was examined further.

Purification of cAMP Phosphodiesterase. The soluble fraction from platelets was applied without further manipulation directly to a DEAE-cellulose column equilibrated with buffer containing proteolytic inhibitors. Elution of the column with a sodium acetate gradient resulted in the resolution of two peaks of cAMP phosphodiesterase activity (Figure 1).

Table I: Purification of cAMP Phosphodiesterase

	total act. (nmol min^{-1})	sp act.[a] (nmol min^{-1} mg^{-1})	recovery (%)	purification (x-fold)
100000g supernatant	2780	0.98	100	1
DEAE-cellulose	1560	5.8	56	5.9
blue dextran–Sepharose	873	2530	31	2580

[a] Specific activity was determined at 1 μM cAMP.

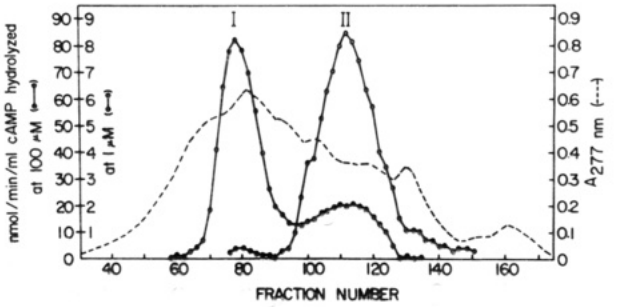

FIGURE 1: DEAE-cellulose chromatography of the platelet 100000g supernatant. The 100000g supernatant from 250 units of outdated platelet concentrate was analyzed as described under Materials and Methods. Fractions (4 mL) were collected and assayed at 100 (●) and 1 μM (○) cAMP. Protein was monitored at 277 nm (---) with an LKB Uvicord S.

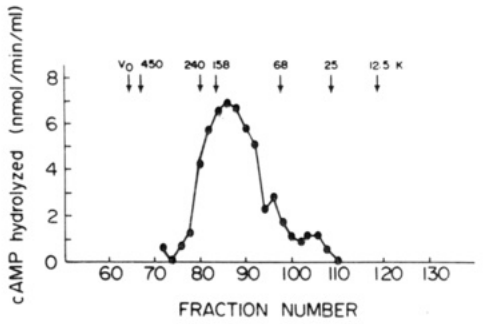

FIGURE 2: Sephadex G-200 gel filtration of purified cyclic nucleotide phosphodiesterase. The purified enzyme was applied to a Sephadex G-200 column as described under Materials and Methods. Fractions (0.25 mL) were collected and assayed at 1 μM cAMP. The markers used to calibrate the column were blue dextran 2000 (V_0), ferritin (450 000), catalase (240 000), aldolase (158 000), BSA (68 000), chymotrypsinogen (25 000), and cytochrome c (12 500).

Peak I was eluted at about 0.3 M sodium acetate and peak II at about 0.45 M sodium acetate. Peak I had a higher K_m for cAMP hydrolysis than peak II as can be seen by comparing the relative ratios of the peaks when activity was measured at 100 and 1 μM cAMP. Peak II was chosen for further purification and study.

The column fractions containing peak II were pooled and applied directly to a blue dextran–Sepharose column. After being washed, the column was eluted with a cAMP gradient. A single peak of activity was eluted, pooled, and concentrated in a dialysis bag against dry sucrose and stored at 0–4 °C.

A typical purification resulting from these procedures is shown in Table I. The overall purification ranged from 2500- to 3000-fold with a specific activity of about 2500 nmol of cAMP hydrolyzed min^{-1} (mg of protein)$^{-1}$ when the activity was measured at 1 μM cAMP. The overall recovery was about 30% of the cAMP phosphodiesterase activity measured in the 100000g supernatant at 1 μM cAMP. This value underestimates the actual recovery of this enzyme since part of the activity measured in the 100000g supernatant is due to the high K_m enzyme in peak I on the DEAE-cellulose column. The enzyme prepared in this manner is quite stable with activity

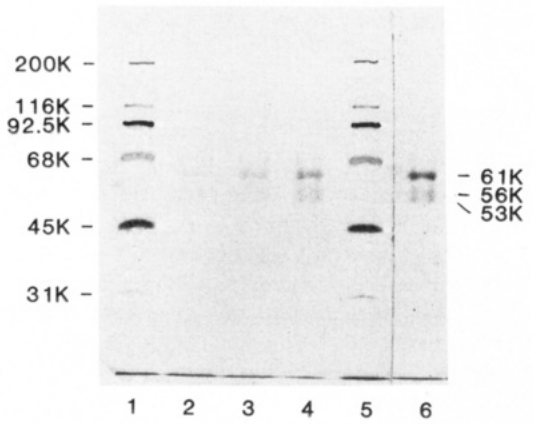

FIGURE 3: NaDodSO$_4$–polyacrylamide gel electrophoresis of purified cyclic nucleotide phosphodiesterase. Electrophoresis was performed in 7.5% gels according to Laemmli (1970) and stained with silver according to Wray et al. (1981). Lanes 1 and 5 contained standard proteins (Bio-Rad Laboratories). Lanes 2, 3, and 4 contained 0.24, 0.6, and 1.2 μg of purified enzyme (reduced), respectively. Lane 6 contained 1.2 μg of purified enzyme, and the staining was allowed to develop longer than that for lanes 1–5 to accentuate the minor bands.

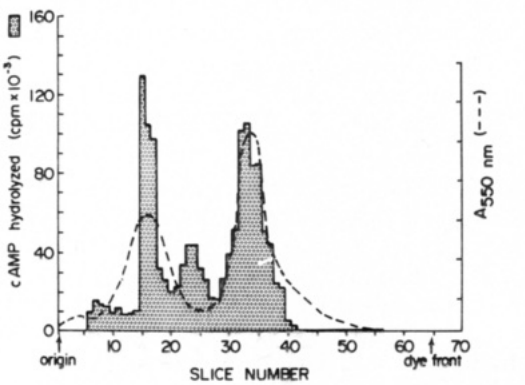

FIGURE 4: Acrylamide gel electrophoresis of purified cyclic nucleotide phosphodiesterase on nondenaturing gels. The purified enzyme was electrophoresed on 7.5% acrylamide gels containing 30% ethylene glycol and analyzed for activity as described under Materials and Methods. The absorbance tracing is of a gel stained with Coomassie Brilliant Blue R 250.

remaining essentially constant for periods as long as 6 months when stored at 0–4 °C.

Gel Filtration and NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Gel filtration of the purified enzyme on a calibrated Sephadex G-200 column gave a single peak of activity with an apparent molecular weight of 140 000 (Figure 2). However, when the same preparation was subjected to electrophoresis on NaDodSO$_4$-containing polyacrylamide gels (Laemmli, 1970), no bands approaching this molecular weight were observed (Figure 3). The major band with both reduced and nonreduced samples is at M_r 61 000, and minor bands are seen at M_r 56 000 and 53 000. Nonreduced samples consistently stained less intensely than reduced samples, and the bands were also more diffuse. These results suggest (but do not prove) that the enzyme may exist as a dimer in solution with a monomer molecular weight of 61 000.

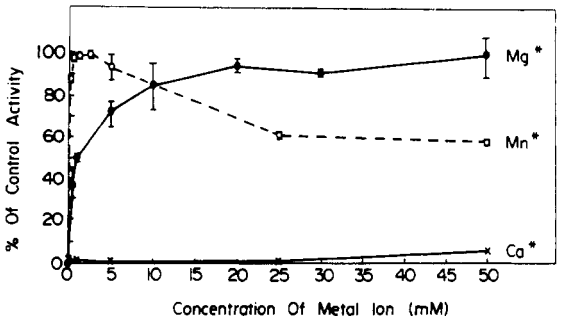

FIGURE 5: Effect of metal ions on the activity of purified cyclic nucleotide phosphodisterase. Aliquots of $MgCl_2$, $MnCl_2$, or $CaCl_2$ were added to a dialyzed sample of the purified phosphodiesterase, and the activity was measured at 1 μM cAMP. The activity was compared to that of an undialyzed sample. The error bars represent the range of activities seen at each point in three experiments.

Examination of the purified enzyme preparation on nondenaturing polyacrylamide gels yielded two bands on staining with Coomassie Blue (Figure 4). The faster migrating band stained more intensely than the slower migrating band. Slicing of a parallel gel for the analysis of phosphodiesterase activity yielded two peaks of activity that were coincident with the stained bands (Figure 4). The presence of two bands both with enzymatic activity suggests that the enzyme constitutes most of the protein present.

To determine if Ca^{2+} might be involved in the formation of a dimer to produce the M_r 140 000 species seen on gel filtration, the enzyme was incubated with 2 mM EGTA at 30 °C for 10 min and applied to a gel filtration column equilibrated in buffer containing 2 mM EGTA. No change in the mobility of the peak of activity was observed, indicating that Ca^{2+} is apparently not responsible for the apparent dimer formation.

Metal Ion Requirements. The enzyme required Mg^{2+} ion for activity with maximum activation between 15 and 20 mM (Figure 5). Manganous ion substituted for Mg^{2+} with activation to similar levels of activity at 0.5 mM (Figure 5). Calcium ion did not stimulate the enzyme in the concentration range of 0.1–100 mM (Figure 5) and had no effect on the activity of the enzyme in the presence of Mg^{2+}. The enzyme was inhibited by the presence of Zn^{2+}, being inhibited at least 80% by 5 mM.

Calmodulin Sensitivity. The sensitivity of the purified cyclic nucleotide phosphodiesterase to stimulation by the calcium binding protein calmodulin was tested with calmodulin from bovine heart and human erythrocytes. The response of the purified phosphodiesterase to calmodulin was examined by measuring cAMP hydrolysis under a range of reaction conditions. Assays were performed at 5 or 20 mM Mg^{2+} with 1–5 ng of purified phosphodiesterase. Calmodulin levels ranged from 0.1 to 10 μg/assay in the presence of 10 μM–10 mM Ca^{2+}. None of the combinations that were tested stimulated cAMP hydrolysis. Furthermore, no inhibition of activity was observed when the enzyme was incubated with EGTA, indicating that the calcium–calmodulin complex had not copurified with the cyclic nucleotide phosphodiesterase.

pH Maximum. The activity of the purified enzyme was measured over the pH range of 6.0–9.0 by using the buffers PIPES (6.0–7.0), HEPES (7.0–8.0), and Bicine (8.0–9.0). The pH maximum determined at 30 °C was about pH 8.0.

Kinetics of Cyclic Nucleotide Hydrolysis. The purified enzyme hydrolyzed cAMP with normal Michaelis–Menten kinetics between 0.025 and 100 μM cAMP with a K_m of 0.18 μM (Table II). The V_{max} was about 3000 nmol of cAMP hydrolyzed min^{-1} (mg of protein)$^{-1}$. The purified enzyme also hydrolyzes cGMP with normal Michaelis–Menten kinetics and a K_m of 0.02 μM. The K_m for cGMP indicates a higher affinity for cGMP than for cAMP; however, the V_{max} for cGMP hydrolysis is 1 order of magnitude slower than that for cAMP hydrolysis (Table II).

Table II: Kinetic Properties of Cyclic Nucleotide Phosphodiesterase

K_m for cAMP (μM)	0.18 ± 0.055
V_{max} for cAMP (pmol min^{-1} μg^{-1})	3000
K_m for cGMP (μM)	0.020 ± 0.007
V_{max} for cGMP (pmol min^{-1} μg^{-1})	275–345
K_i for cGMP (with cAMP as substrate) (μM)	0.035
K_i for cAMP (with cGMP as substrate) (μM)	0.052[a]

[a] Approximate value only.

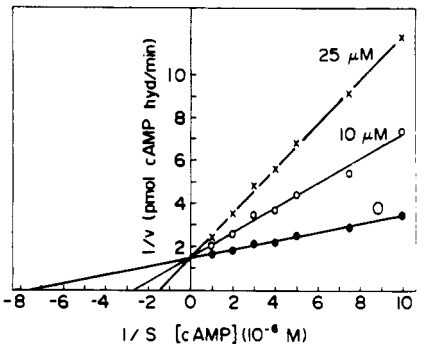

FIGURE 6: Inhibition of purified cyclic nucleotide phosphodiesterase by dipyridamole. Double-reciprocal plot of hydrolysis rate vs. concentration of cAMP in the presence of 0 (●), 10 (○), and 25 (×) μM dipyridamole.

Some cAMP phosphodiesterases have been reported to be stimulated by low concentrations of cGMP. However, cGMP was a potent competitive inhibitor of cAMP hydrolysis by this enzyme with a K_i of the same order of magnitude as its K_m (Table II). Cyclic AMP also inhibits cGMP hydrolysis in a competitive manner although the K_i value reported here has to be considered only an approximation due to the rapid hydrolysis of cAMP by the enzyme during the assay.

Phosphodiesterase Inhibitors. The purified enzyme is inhibited by the phosphodiesterase inhibitors papaverine and 3-isobutylmethylxanthine in a competitive manner with inhibitory constants of 0.26 μM and 0.70 μM, respectively. Dipyridamole has been reported to be a noncompetitive inhibitor of this enzyme in a partially purified preparation (Asano et al., 1977). However, the results from a double-reciprocal analysis of the inhibition by dipyridamole of cAMP hydrolysis by the purified enzyme (Figure 6) indicate that dipyridamole is a competitive inhibitor of the purified enzyme with a K_i of 5.7 μM.

The enzyme is not inhibited by the product of hydrolysis, AMP, at concentrations as high as 1 mM nor by ADP at concentrations up to 0.5 mM.

Immunologic Analysis. In a preliminary study the purified cyclic nucleotide phosphodiesterase was tested for immunologic cross-reactivity to an antibody raised to a purified, high-affinity cAMP phosphodiesterase from dog kidney (Sarada et al., 1982). The dog kidney enzyme has a M_r of 60 000, has a low K_m for cAMP, and shows two peaks of activity on nondenaturing gels (Thompson et al., 1979a). A number of tissues have been tested with this antibody for the presence of immunologically cross-reactive species. Many of the tissues that have been tested have contained a cross-reactive protein with M_r 60 000 (Pratt et al., 1982; Sarada et al., 1982). However, when this antibody was used in a western blot analysis of the purified cyclic nucleotide phosphodiesterase from human

platelets, no cross-reactivity was observed, suggesting that these enzymes are immunologically unrelated.

Comparison with Enzyme from Fresh Platelets. Since our preparation of cyclic nucleotide phosphodiesterase began with outdated platelets, the enzyme could have been altered by proteolysis or some other process during storage of the platelet concentrates. To address this possibility the enzyme was prepared from platelets isolated from freshly drawn blood. The chromatographic and kinetic properties of the enzyme from fresh platelets were the same as those of the enzyme purified from outdated platelets (e.g., K_m for cAMP = 0.14 μM and for cGMP = 0.024 μM compared to 0.18 μM and 0.020 μM, respectively, from outdated platelets). The enzyme from fresh platelets was also insensitive to calmodulin and EGTA. $NaDodSO_4$–polyacrylamide gels showed the same bands seen with the enzyme preparation from outdated platelets. The kinetic and physical properties of the enzyme do not appear to have been altered by the storage of the platelet concentrates.

Discussion

In these studies we have extensively purified a cyclic nucleotide phosphodiesterase from the cytosolic fraction of human platelets that hydrolyzes both cAMP and cGMP. This enzyme is responsible for a major portion of the hydrolytic activity for cAMP in platelets. In the cytosol this phosphodiesterase provides 90% or more of the hydrolytic activity at 1 μM cAMP and about 25% of the activity at 100 μM cAMP (Figure 1).

The cyclic nucleotide phosphodiesterase we have purified appears to be the form called FIII by Hidaka & Asano (1976). They purified the enzyme about 55-fold from a 105000*g* supernatant of sonicated platelets by a combination of gel filtration, hydroxylapatite, and DEAE-cellulose chromatography. In comparison, we have purified the enzyme 2500-fold from a 100000*g* supernatant of platelets. The enzyme we have purified appears to behave the same as FIII on DEAE-cellulose chromatography eluting at approximately the same salt concentration. The K_m and K_i values for the purified enzyme are somewhat lower than those reported by Hidaka & Asano (1976) for FIII, especially for cGMP. The molecular weight determined by gel filtration of the purified enzyme is somewhat smaller than Hidaka & Asano (1976) reported for FIII (140000 vs. 180000). Whether this discrepancy is due to differences in the gel filtration columns used or to removal of some associated protein has not been determined. The data from the $NaDodSO_4$ gels and the gel filtration suggest that the enzyme may exist as a dimer in solution with a M_r 61000 subunit. However, the apparent dimer formation is not influenced by the presence of Ca^{2+} since EGTA did not affect the mobility of the enzyme on gel filtration.

The purified enzyme requires the presence of Mg^{2+} or Mn^{2+} for activity. However, the high level of Mg^{2+} required for maximum activity (Figure 5) raises questions as to which metal ion is the cofactor for the enzyme in the platelet.

The purified enzyme appears to be more strongly inhibited by dipyridamole than the crude enzyme (K_i = 0.57 μM vs. 19.3 μM) (Asano et al., 1977). In addition, the inhibition of the purified enzyme by dipyridamole is competitive (Figure 6) rather than noncompetitive as reported for the less purified preparation (Asano et al., 1977).

Since our preparations of the cyclic nucleotide phosphodiesterase usually employed platelets collected from outdated platelet concentrates, it was important to determine whether the properties of the enzyme were those of the native enzyme or whether the enzyme had been altered during storage of the platelet concentrates. The cyclic nucleotide phosphodiesterase prepared from platelets collected from freshly drawn blood appeared to be identical with that from outdated platelets kinetically and chromatographically. $NaDodSO_4$ gels also contained the same bands, indicating that the properties of the purified enzyme are not due to alterations in a native enzyme during storage of the platelet concentrates.

This enzyme appears to be similar in some respects to other high-affinity cAMP phosphodiesterases such as that isolated from dog kidney (Thompson et al., 1979a). The enzyme from platelets is similar to the dog kidney enzyme in molecular weight, low K_m for cAMP, and calmodulin insensitivity. However, it differs markedly in its high affinity for cGMP (as shown by the very low K_m and K_i for cGMP) and its greater V_{max} for cAMP. The failure of the platelet enzyme to react with antibodies raised against the dog kidney enzyme also indicates that this enzyme belongs to a different class of cyclic nucleotide phosphodiesterases.

Cyclic nucleotide phosphodiesterase activities similar to that of this enzyme from platelets have recently been reported in two other cell types. In a mutant strain of S49 murine lymphoma, Brothers et al. (1982) reported the appearance of a cyclic nucleotide phosphodiesterase activity that hydrolyzed cAMP and cGMP with K_m and K_i values nearly identical with those determined for the purified enzyme from platelets. In addition, the enzyme from the mutant lymphoma cells hydrolyzed cAMP about 10-fold more rapidly than cGMP as does the purified enzyme from platelets (Table II). Brothers et al. (1982) suggest that this new activity in the mutant lymphoma cells is probably due to the increased expression of a cyclic nucleotide phosphodiesterase different from the predominant cAMP hydrolyzing activity in wild-type S49 cells rather than a mutation within the predominant cAMP phosphodiesterase in wild-type cells. Martins et al. (1983) reported (in abstract form) a cGMP-inhibited cAMP phosphodiesterase in cardiac tissue after the cGMP-stimulated and calmodulin-stimulated phosphodiesterases had been removed by immunoadsorption. The K_m for cAMP hydrolysis for this enzyme appears to be nearly identical with that of the platelet enzyme.

The purification scheme outlined above is rapid (2 days), yielding extensive purification with good recovery of activity (Table I). The enzyme purified by this procedure has remained stable on storage at 0–4 °C for periods of at least 6 months. The preparation is not completely homogeneous when examined on $NaDodSO_4$-containing polyacrylamide gels. The major band is at M_r 61000, but minor bands are also seen at M_r 56000 and 53000 (Figure 3). The identity of these bands is not known; however, the association of enzyme activity with both stained bands seen on nondenaturing gels (Figure 4) indicates that the enzyme constitutes the majority of the protein present. The existence of two bands on nondenaturing gels and a single major band on $NaDodSO_4$-containing gels suggests that the enzyme preparation contains charge isomers, similar to the findings of Thompson et al. (1979a) for the cAMP phosphodiesterase from dog kidney. However, the distribution of activity in each form of the enzyme is not clear due to the fact the procedure used to determine activity in the gel slices measured the extent rather than the rate of cAMP hydrolysis. In addition, the relative recovery of activity from the gel and the stability of the different forms of the enzyme are unknown.

The data from both types of gel electrophoresis suggest that the M_r 61000 band is probably the basic subunit of the enzyme. The bands at 56000 and 53000 daltons may be proteolytic products of the M_r 61000 band. During an attempt to further purify the enzyme, the lability of the enzyme activity was markedly increased. When this more labile preparation

was reexamined on $NaDodSO_4$-containing gels, the M_r 56 000 band appeared to be enhanced at the expense of the M_r 61 000 band, suggesting that the M_r 56 000 band is a derivative of the M_r 61 000 band. However, we have not eliminated the possibility that these minor bands may be other cAMP binding proteins that have been eluted from the blue dextran–Sepharose.

The presence of a cAMP phosphodiesterase activity in platelets that is strongly inhibited by cGMP suggests a possible regulatory link between these nucleotides. In platelets, an increase in the intracellular level of cAMP leads to the inhibition of platelet responses such as shape change, aggregation, adhesion, and secretion of granule contents (Salzman & Weisenberger, 1972; Haslam, 1973; Mills, 1982). Compounds such as sodium nitroprusside, the S-nitrosylated derivatives of *N*-acetylpenicillamine, cysteine, and β-D-thioglucose (guanylate cyclase agonists), and 8-bromo-cGMP (a cell penetrating form of the cyclic nucleotide) act synergistically with adenylate cyclase agonists to inhibit platelet responses (Mellion et al., 1982; Davidson & Haslam, 1982; Haslam & Davidson, 1982). These results are very similar to reports of phosphodiesterase inhibitors acting synergistically with adenylate cyclase agonists to inhibit platelet responses (Markwardt et al., 1967; Mills et al., 1970; Mills & Smith, 1971; Jorgensen et al., 1979). These results together with the isolation of this cGMP-inhibited phosphodiesterase suggest that one of the roles of cGMP in platelets may be to potentiate increases in the intracellular level of cAMP by inhibiting the hydrolysis of cAMP by this phosphodiesterase, thus modulating platelet responsiveness.

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Registry No. cAMP, 60-92-4; cGMP, 7665-99-8; cyclic nucleotide phosphodiesterase, 9040-59-9; papaverine, 58-74-2; 3-isobutylmethylxanthine, 28822-58-4; dipyridamole, 58-32-2.

References

Appleman, M. M., Ariano, M., Takemoto, D. J., & Whitson, R. H. (1982) *Handb. Exp. Pharmacol. 58*, 261.

Ardlie, N. G., Glew, G., Schultz, B. G., & Schwartz, C. J. (1967) *Thromb. Diath. Haemorrh. 18*, 670.

Asano, T., Ochai, Y., & Hidaka, H. (1977) *Mol. Pharmacol. 13*, 400.

Beavo, J. A., Hansen, R. S., Harrison, S. A., Hurwitz, R. L., Martins, T. J., & Mumby, M. C. (1982) *Mol. Cell. Endocrinol. 28*, 387.

Brothers, V. M., Walker, N., & Bourne, H. R. (1982) *J. Biol. Chem. 257*, 9349.

Davidson, M. M. L., & Haslam, R. J. (1981) *Thromb. Haemostasis 46*, 149.

Fukami, M., Bauer, J. S., Stewart, G. J., & Salganicoff, L. (1978) *J. Cell Biol. 77*, 389.

Hansen, R. S., & Beavo, J. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2788.

Haslam, R. J. (1973) *Ser. Haematol. 6*, 333.

Haslam, R. J., & Davidson, M. M. L. (1982) *Circulation 66* (*2*), 291.

Hidaka, H., & Asano, T. (1976) *Biochim. Biophys. Acta 429*, 485.

Ho, H. C., Wirch, E., Stevens, F. C., & Wang, J. H. (1977) *J. Biol. Chem. 252*, 43.

Jorgensen, K. A., Dyerberg, J., & Stofferson, E. (1979) *Pharmacol. Res. Commun. 11*, 605.

Laemmli, U. (1970) *Nature (London) 227*, 680.

Markwardt, F., Barthel, W., Glusa, E., & Hoffman, A. (1967) *Naunyn-Schmiedeberg's Arch. Pharmacol. 257*, 420.

Martins, T. J., Mumby, M., & Beavo, J. A. (1982) *J. Biol. Chem. 257*, 1973.

Martins, T. J., Harrison, S. A., & Beavo, J. A. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1846.

Mellion, B. T., Horowitz, P. M., Hyman, A. L., Ignarro, L. J., & Kadowitz, P. J. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1304.

Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320.

Mills, D. C. B. (1982) *Handb. Exp. Pharmacol. 58* (*2*), 725.

Mills, D. C. B., & Smith, J. B. (1971) *Biochem. J. 121*, 15.

Mills, D. C. B., Smith, J. B., & Born, G. V. R. (1970) in *Platelet Adhesion and Aggregation in Thrombosis: Countermeasures* (Mammen, E. F., Anderson, G. F., & Barnhart, M. I., Eds.) p 165, Schattauer, Stuttgart.

Morrill, M. E., Thompson, S. T., & Stellwagen, E. (1979) *J. Biol. Chem. 254*, 4371.

Mumby, M. C., Martins, T. J., Chang, M. L., & Beavo, J. A. (1982) *J. Biol. Chem. 257*, 13283.

Pratt, M. L., Strada, S. J., & Thompson, W. J. (1982) Abstracts of the XII International Congress of Biochemistry, Perth, Australia, p 410.

Ryan, L. D., & Vestling, C. S. (1974) *Arch. Biochem. Biophys. 160*, 279.

Salzman, E. W., & Weisinberger, H. (1972) *Adv. Cyclic Nucleotide Res. 1*, 231.

Sarada, K., Epstein, P. M., Strada, S. J., & Thompson, W. J. (1982) *Arch. Biochem. Biophys. 215*, 183.

Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502.

Sinha, A. K., & Colman, R. W. (1981) *Anal. Biochem. 113*, 239.

Sinha, A. K., Shattil, S. J., & Colman, R. W. (1977) *J. Biol. Chem. 252*, 3360.

Strada, S. J., & Thompson, W. J. (1978) *Adv. Cyclic Nucleotide Res. 9*, 265.

Thompson, W. J., Epstein, P. M., & Strada, S. J. (1979a) *Biochemistry 18*, 5228.

Thompson, W. J., Terasaki, W. L., Epstein, P. M., & Strada, S. J. (1979b) *Adv. Cyclic Nucleotide Res. 10*, 69.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350.

Tucker, M. M., Robinson, J. B., & Stellwagen, E. (1981) *J. Biol. Chem. 256*, 9051.

Vaughn, M., Danello, M. A., Manganiello, V. C., & Strewler, G. J. (1981) *Adv. Cyclic Nucleotide Res. 14*, 263.

Vigdahl, R. M., Mongin, J., Jr., & Marquis, N. R. (1971) *Biochem. Biophys. Res. Commun. 42*, 1088.

Wells, J. N., & Hardman, J. G. (1977) *Adv. Cyclic Nucleotide Res. 8*, 119.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197.