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Characterization of Two Forms of Beef Heart Cyclic Nucleotide Phosphodiesterase†

Robert J. Hrapchak* and Howard Rasmussen

ABSTRACT: A method has been developed to isolate two forms of cyclic nucleotide phosphodiesterase from beef heart. Using a 13,500*g* supernatant fraction as the starting point, the method involves ammonium sulfate fractionation, protamine precipitation, Sephadex G-200 gel filtration, and DEAE-cellulose chromatography. The total enzymatic activity could be accounted for at all stages in the procedure. There were no indications that a cofactor, protein or otherwise, was removed during the isolation. Although the two forms of phosphodiesterase could not be effectively separated by gel filtration, they could be resolved upon DEAE-cellulose into two active species, designated phosphodiesterase I and II. Phosphodiesterase I, representing 70–80% of the the total activity recovered from DEAE-cellulose, has been purified more than 700-fold. This form has a molecular weight of approximately 121,500 g/mol as determined by sedimentation equilibrium measurements. Both enzymes catalyzed the formation of 5′-mononucleotides from the two naturally occurring cyclic nucleotides, cyclic 3′,5′-adenosine monophosphate and cyclic 3′,5′-guanosine monophosphate, although the former was the preferred substrate of both forms. The K_m for cyclic 3′,5′-adenosine monophospate of the major fraction I is 3.6×10^{-5} M; that of the minor fraction II is 6.9×10^{-5} M. Both forms of the enzyme exhibited several active bands on polyacrylamide disc electrophoresis. Although the two forms of the enzyme have been obtained reproducibly upon DEAE-cellulose chromatography by both stepwise and gradient elution techniques, preliminary considerations do not preclude the possibility that the two forms may represent a complex distribution of a single enzymatic species.

Much research concerning the metabolism of cyclic 3′,5′-adenosine monophosphate (cAMP[1]) has centered upon its synthesis from ATP by the enzyme adenylate cyclase (Sutherland *et al.*, 1962; Robison *et al.*, 1970; Levey, 1970; Klainer *et al.*, 1962). However, the extent and duration of a cAMP-induced response also depends upon cyclic nucleotide phosphodiesterase, the enzyme which catalyzes the hydrolysis of cAMP to AMP. Phosphodiesterase has now been studied in heart (Butcher and Sutherland, 1962; Nair, 1966; Beavo *et al.*, 1970; Goren and Rosen, 1971), brain (Cheung, 1970, 1971; Kakiuchi *et al.*, 1971; Thompson and Appleman, 1971a,b); liver (Menahan *et al.* 1969), slime molds (Chang, 1968; Murray *et al.*, 1971), and frog erythrocytes (Rosen, 1970). How-

† From the Department of Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received July 24, 1972.* This work was supported by a grant from the U. S. Public Health Service (AM-09650).

* The data are taken in part from a dissertation submitted to the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy. To whom to address correspondence at the Department of Radiation Biology and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y. 14642.

[1] Abbreviations used are: cAMP, cGMP, cIMP, cUMP, cTMP, and cCMP for cyclic 3′,5′-adenosine, guanosine, inosine, uridine, thymidine, and cytidine monophosphates, respectively.

ever, the function of the enzyme is not clear because of the difficulties in obtaining adequate yields of it and a lack of knowledge about its basic molecular unit of activity.

Most recent research has noted that multiple forms of phosphodiesterase activity are present in various tissues (Thompson and Appleman, 1971a,b; Kakiuchi *et al.*, 1971; Rosen, 1970; Jard and Bernard, 1970), but considerable uncertainty still exists regarding the interrelationships of these various forms. The enzyme exhibits a complex subcellular distribution in several of the systems studied (Butcher and Sutherland, 1962; DeRobertis *et al.*, 1967; Cheung and Salganicoff, 1966, 1967). Furthermore, there are indications that phosphodiesterase may become dissociated from a required protein factor during the course of its isolation (Cheung, 1970, 1971; Kakiuchi *et al.*, 1970; Goren and Rosen, 1971). Finally, the substrate specificities of the various forms of the enzyme in a single source have not yet been completely clarified. Thompson and Appleman (1971a,b) have suggested that one of several forms of the enzyme found in various tissues of the rat may be effectively a cGMP phosphodiesterase under physiological conditions.

This investigation describes the development of an isolation procedure for the soluble (13,500*g* supernatant fraction) phosphodiesterase system of beef heart. A relatively mild isolation scheme is presented in which the total enzymatic activity can be accounted for at all stages of the purification. There were no unexplainable losses of activity as might be due to the dissociation of a required protein factor. The identification of two independently active beef heart phosphodiesterase activities will be discussed. In addition, a comparison of several properties of the two species and their possible interrelationship will be considered. A preliminary report of this work as been presented (Hrapchak, 1971a).

Experimental Section

Materials

Experimental materials were obtained from the following sources: Sephadex G-25 and G-200, Pharmacia; Whatman DE-52 DEAE-cellulose, H. Reeve Angel Inc.; Bio-Rad AG 1-X2 (−400 mesh), Bio-Rad Laboratories; cAMP, cGMP, cIMP, cTMP, AMP, GMP, *Crotalus atrox* venom, adenosine deaminase (intestinal mucosa, type I), protamine (salmon sperm, free base), and bovine serum albumin, Sigma; IMP, [^{3}H]cAMP (16.3 Ci/mmol), and ammonium sulfate (special enzyme grade), Schwarz BioResearch Inc.; cCMP and cUMP, Boehringer Mannheim Corp.; dithiothreitol, Calbiochem. cAMP was obtained as the free acid; other cyclic nucleotides were the sodium salts. All other reagents were of reagent grade quality.

Methods

Enzyme Assays and Kinetic Measurements. Phosphodiesterase activity was measured by three methods.

Assay procedure I was a convenient spectrophotometric method used to determine activity during purification. This assay was a modification of the method of Drummond and Perrott-Yee (1961). In place of the originally described AMP deaminase, adenosine deaminase and *Crotalus atrox* venom were used. The AMP formed from cAMP by phosphodiesterase was converted to adenosine and then to inosine by means of the 5′-nucleotidase of the venom and adenosine deaminase, respectively. The deamination of adenosine to inosine is accompanied by a significant decrease in absorbance at 265 nm. The method was valid since neither the venom nor the deaminase had phosphodiesterase activity. In addition, neither cAMP nor AMP was deaminated by the deaminase. Finally, the absorption spectra of cAMP, AMP, and adenosine are essentially indistinguishable (Kalckar, 1947a,b, and personal observations). Therefore, the reaction reflected the conversion of cAMP, *via* the isolated phosphodiesterase and the coupling enzymes, to inosine. An extinction coefficient of $-8060\ M^{-1}\ cm^{-1}$ was calculated for the complete conversion of cAMP to inosine at 265 nm (Hrapchak, 1971b). The standard assay of 1 ml total volume contained 0.01 M imidazole-HCl (pH 7.5), 1 mM $MgSO_4$, 0.1 mM cAMP, 100 μg of *Crotalus atrox* venom, and 2 μg of adenosine deaminase (approximately 0.4 unit)[2] to which was added an appropriate volume of phosphodiesterase. The reaction was followed on a Gilford recording spectrophotometer using quartz cuvets maintained at 25°. A unit of activity was defined as that amount of phosphodiesterase that produced the hydrolysis of 1 nmol of cAMP/min. Specific activity was expressed as units/milligram of protein. Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. As in many coupled assay systems, the method could not be used for initial rate determinations because an initial lag of 2–4 min occurred before the system reached its steady-state conditions. Nevertheless, when the rates were measured after this lag period, there was a linear relationship between rate and a 16-fold range of phosphodiesterase concentrations. Unless stated otherwise, the evaluation of properties of the two forms of the enzyme was done with phosphodiesterase I and II of specific activities of 2400 and 620 units/mg, respectively, as determined by assay procedure I.

Assay procedure II was used to determine the substrate specificities of the phosphodiesterases. This assay was essentially the method of Butcher and Sutherland (1962) in which the 5′-mononucleotide product of the phosphodiesterase reaction was subsequently hydrolyzed to nucleoside and P_i by means of the 5′-nucleotidase present in *Crotalus atrox* venom. Suitable samples of phosphodiesterase were incubated for 30 min at 30° in 1 ml total volume of medium containing 0.06 M Tris buffer (pH 8.0), 4 mM cyclic nucleotide substrate, and 5 mM $MgSO_4$. The samples were then placed in boiling water for 4 min, cooled, and then incubated for an additional 20 min after the addition of 100 μg of venom. The reaction was terminated by the addition of 0.1 ml of 55% CCl_3COOH, and aliquots of the supernatant were assayed for P_i by the methods of either Fiske and Subbarow (1925) or Baginski and Zak (1960) and Baginski *et al.* (1967). Identical results were obtained when the initial incubation contained snake venom or when the two-step assay was used.

Assay procedure III is essentially the same as the radiochemical method described by Brooker *et al.* (1968). The same procedure as outlined for Assay Procedure II was followed, except that [^{3}H]cAMP was used and [^{3}H]adenosine was the final product assayed. After the incubation with snake venom, 1 ml of a slurry (50% settled volume) of Bio-Rad AG 1-X2 was added. The anion exchanger adsorbed unreacted [^{3}H]-cAMP, while the [^{3}H]adenosine was not bound by the resin. The radioactivity of the supernatant was then determined by liquid scintillation spectrometry.

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed as described by Davis (1964), using 7% acrylamide gels. However, in place

[2] One unit was defined as that amount of enzyme which caused the deamination of 1 μmol of adenosine to inosine per min at pH 7.5 and 25°.

of the spacer gel, 0.25 ml of a suspension of Sephadex G-200 in a 3% solution of sucrose in electrophoresis buffer was used. After completion, the gels were cut in half longitudinally and one half was stained for protein (using Amido Black 10B) and the other half for enzymatic activity. For the determination of enzymatic activity, a modification of a procedure suggested by Dr. Daniel Bikle was used. In this method, the gel section was incubated at room temperature in a solution of 36 mM Tris-HCl (pH 8.0), containing 15 mM $CaCl_2 \cdot 2H_2O$, 1.5 mM cAMP, 1.8 mM $MgSO_4$, and 100 μg of *Crotalus atrox* venom. The AMP formed from cAMP by the action of phosphodiesterase was then hydrolyzed to adenosine and P_i by the 5′-nucleotidase activity in the venom. Areas of phosphodiesterase activity could then be identified by a white precipitate of calcium phosphate.

Sedimentation Equilibrium Ultracentrifugation. In order to obtain an estimate of the molecular weight of the purified enzyme, phosphodiesterase I, a sedimentation equilibrium run was made using a Spinco Model E analytical ultracentrifuge and a Rayleigh interference optical system. A sample of the enzyme (0.85 mg/ml) was run at both 9,000 and 11,000 rpm with the rotor temperature maintained at 20°. Using the meniscus depletion technique of Yphantis (1964), the weight-average molecular weights at the two speeds were calculated from plots of the log of the fringe displacement, ln Y, *vs.* the square of the radial distance, cm^2. In the absence of an amino acid analysis, the value of 0.75 was assumed for the partial specific volume used in approximating the molecular weights. The centrifuge runs were kindly performed by Dr. J. Rosenbloom.

Concentration Procedure. The best method found for concentrating, and thereby stabilizing, the enzymes involved the use of the Schleicher and Schuell collodion bag apparatus. Collodion bags that could retain molecular weight species greater than 25,000 were used. The dilute enzyme samples were adjusted to a final concentration of 1 mM cAMP and added to the bag which was immersed in 1 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$, 1 mM dithiothreitol, and 0.5 mM cAMP. This procedure was necessary since the dilute enzymes were easily denatured at the surface of the bag if not kept in an aqueous environment with their substrate. All procedures were carried out at 4°, and the fractions were concentrated to a volume of approximately 5 ml. Saturated ammonium sulfate (at 4°) was then added to the concentrated samples to a final concentration of 0.6 saturation. After 2 hr, the samples were centrifuged for 1 hr at 100,000g. The precipitates were dispersed in a minimum amount of 10 mM imidazole-HCl (pH 7.5), containing 1 mM $MgSO_4$, 1 mM dithiothreitol, and 0.6 saturated ammonium sulfate and kept as the ammonium sulfate suspensions.

Protein Elution Patterns. The protein elution patterns from the chromatographic columns were determined using the LKB 8300A Uvicord II apparatus.

Results

Purification of Beef Heart Phosphodiesterase

STEP 1. PREPARATION OF SOLUBLE EXTRACT OF BEEF HEART. Fresh beef hearts were obtained from the slaughterhouse and kept on ice for use, usually within 1 hr. All procedures were carried out at 0–4°. After fat and connective tissue were removed, the ventricles were sliced and washed in saline. The slices were ground with a meat grinder and 600–700 g was added to 1 l. of 1 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$. The mixture was blended for 1 min at low speed and 2 min at medium speed on a Waring commercial blendor, and then centrifuged for 60 min at 13,500g. The supernatants were poured through glass wool and pooled, and the pH was adjusted to 7.3 with 1.0 N KOH.

STEP 2. AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate was added to 0.5 saturation, with constant stirring, over 30 min. The pH was maintained at 7.3 by the addition of 1.0 N KOH. After ammonium sulfate addition, the solutions was stirred for 30 min, and then allowed to sit for 45 min prior to centrifugation at 13,500g for 30 min. The supernatant was discarded, and the precipitate was dispersed in a minimum volume of 1 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$ and 1 mM dithiothreitol. If necessary, the redispersed precipitate was adjusted to pH 7.5 with 1.0 N KOH and then centrifuged for 20 min at 27,000g. The supernatant was poured through glass wool and saved. Ammonium sulfate, which interfered with later steps of the isolation, was removed by gel filtration on a column (4 × 45 cm) of Sephadex G-25 equilibrated with 1 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$ and 1 mM dithiothreitol.

STEP 3. PROTAMINE PRECIPITATION. The desalted eluate from the G-25 column was adjusted to 3 mg of protein/ml (*via* the Lowry method) and to pH 7.0 with 1.0 N HCl. Then, with constant stirring, 0.38 ml of a 10 mg/ml of protamine solution in water was added per 100 ml of the enzyme sample. After centrifugation for 30 min at 27,000g, the supernatants were pooled. The protamine step was repeated on the supernatant, and the samples were centrifuged as above. Supernatants were discarded, and the precipitates were dispersed in approximately 10% of the original volume using 0.1 M imidazole-HCl (pH 8.5) containing 1 mM $MgSO_4$, 1 mM dithiothreitol, and 0.1 M KCl. The precipitate was often quite gummy and care had to be taken to disperse the material well. The dispersed precipitates were centrifuged as above and the supernatants were saved. The higher ionic strength provided by the 0.1 M KCl in this buffer was absolutely required to prevent the reprecipitation of the enzyme as a protamine complex.

STEP 4. REMOVAL OF PROTAMINE. The following step was the simplest means of separating the enzymatic activity from the protamine. To the centrifuged supernatant was added, with careful stirring, an equal volume of saturated ammonium sulfate (at 4°) in 1.0 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$ and 1 mM dithiothreitol. The sample was allowed to sit without stirring for 30 min and was then centrifuged at 27,000g for 30 min. The supernatant was discarded, and the precipitate was dispersed in a minimal amount of 0.1 M imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$ and dithiothreitol. In order to remove residual ammonium sulfate, the redispersed precipitates were pooled and dialyzed overnight *vs.* 1 l. of 1 mM imidazole-HCl (pH 7.5) containing 1 mM $MgSO_4$ and 1 mM dithiothreitol.

STEP 5. SEPHADEX G-200 GEL FILTRATION. A column (4 × 60 cm) of Sephadex G-200 was equilibrated with 1 mM imidazole-HCl (pH 7.5) containing 1 mM each of $MgSO_4$ and dithiothreitol. The dialyzed sample from step 4 was then applied to this column. For the best purifications at this step, a sample of 5 ml total volume and approximately 200 mg total protein was maximum. The exclusion volume (V_0) of the column was 240 ml, and the enzymatic activity was eluted from about 320 to 415 ml (V_e/V_0 equal to 1.33–1.73) of eluent. The activity was eluted as a relatively broad, heterogeneous zone, and fractions for further purification were pooled from this zone. The pooled fractions were concentrated by means of the Schleicher and Schuell collodion bag apparatus (see Methods) or by precipitation with an equal volume of sat-

urated ammonium sulfate. Prior to the next step which involved DEAE-cellulose chromatography, the concentrated G-200 sample was dialyzed overnight *vs.* 40 mM potassium phosphate (pH 7.5), containing 1 mM each of imidazole, $MgSO_4$, and dithiothreitol. The activity had to be concentrated prior to DEAE-cellulose because extensive denaturation occurred if a dilute enzyme sample was added directly to the cellulose. In addition, concentration prior to dialysis was necessary since the dilute activity could not be safely dialyzed.

STEP 6. DEAE-CELLULOSE CHROMATOGRAPHY. A column (2.6 × 9.0 cm) of Whatman DE-52 DEAE-cellulose was prepared and equilibrated with 40 mM potassium phosphate (pH 7.5) containing 1 mM each of imidazole, $MgSO_4$, and dithiothreitol. The concentrated and dialyzed sample from step 5 was then added to the column and washed in with 100 ml of the same buffer. Considerable protein, but essentially no enzymatic activity, was eluted at this point. Then the first peak of enzymatic activity, designated phosphodiesterase I, was eluted with 300 ml of 65 mM potassium phosphate (pH 7.5) containing 1 mM each of imidazole, $MgSO_4$, and dithiothreitol. Following this, the second peak of enzymatic activity, designated phosphodiesterase II, was eluted by the application of 100 ml of 300 mM potassium phosphate (pH 7.5), containing 1 mM each of imidazole, $MgSO_4$, and dithiothreitol. Optimum results in purification were obtained when 30–40 total mg of protein was added to the column. Figure 1 shows a typical DEAE-cellulose elution profile of the dialyzed G-200 sample.

ALTERNATE STEP 6. GRADIENT ELUTION FROM DEAE-CELLULOSE. A sample of concentrated and dialyzed activity, purified through step 5, was applied to the DEAE-cellulose column under conditions identical with those described above. The two activities were then eluted with a linear gradient formed with 300 ml each of 40 and 300 mM potassium phosphate buffer (pH 7.5) containing, in addition, 1 mM each of imidazole, $MgSO_4$, and dithiothreitol. The two forms of the enzyme were well separated and showed an identical distribution as when the step-elution technique was used. A typical elution profile is shown in Figure 2.

A summary of the purification procedure, including yields and specific activities of the various fractions, is given in Table I. The soluble form of the activity present in the 13,500*g* supernatant fraction represented only 30–35% of the total homogenate activity. This agrees with the original findings of Butcher and Sutherland (1962) using this source. Considering the original 13,500*g* supernatant fraction as the starting point, the overall purification of the major form of the enzyme, phosphodiesterase I, was about 220-fold; that of the minor form, phosphodiesterase II, was 56-fold. Considering the original whole homogenate, the purification of fraction I was approximately 720-fold, and that of fraction II, 180-fold. In the isolation method described, there were no unexplainable losses of activity; the total enzymatic activity could be accounted for at each step in the procedure. The relatively low total yield was due to the fact that only those fractions exhibiting the best yields and specific activities at a particular step were selected for further purification.

Properties of the Purified Enzymes. In contrast to the observations of Cheung (1971) and Goren and Rosen (1971) studying beef heart extracts, the activities of phosphodiesterase I and II were purely additive when the two forms of the enzyme were assayed together. There was no evidence of a synergistic response as suggested by the previous authors. In addition, snake venom, as used in the assays described in this paper, had no significant effects upon the activity of either form of phosphodiesterase. This is in contrast to the report of Cheung (1969a) describing the activation of bovine brain phosphodiesterase by snake venom.

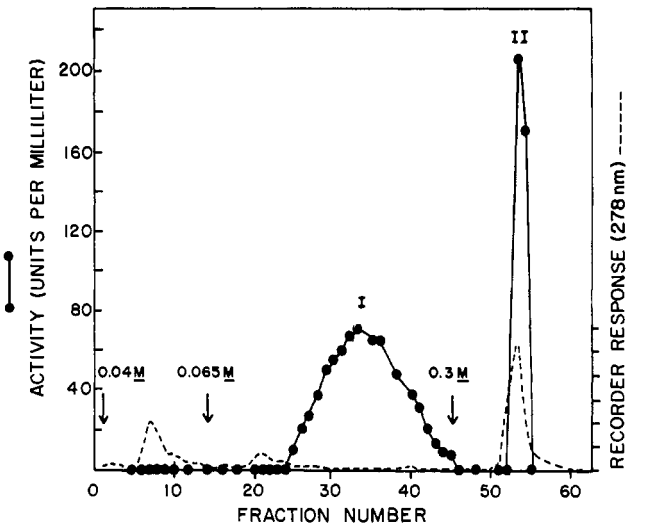

FIGURE 1: DEAE-cellulose elution pattern using a stepwise elution schedule. Conditions: 2.6 × 9.0 cm column; flow rate, 25 ml/hr; 4°; fractions of 6 ml/tube. The arrows indicate the molarities of phosphate buffer, pH 7.5, used. Activity was determined by assay procedure I.

Stability. The phosphodiesterase activity present in the crude (half-saturated) ammonium sulfate fraction (step 2) was stable for at least 1 month at 4°. The activity pooled from the Sephadex G-200 column was stable for at least 1 week at 4° when concentrated to approximately 10 mg/ml or higher by means of the Schleicher and Schuell apparatus. The concentrated G-200 sample could also be frozen at −50° for at least 1 week, with no apparent loss of activity. The concentrated activities of phosphodiesterase I and II, obtained from DEAE-cellulose chromatography (step 6), were stable at 4° for at least 2 weeks when stored as the ammonium sulfate suspension at a concentration of 5–8 mg of protein/ml. In all cases, the

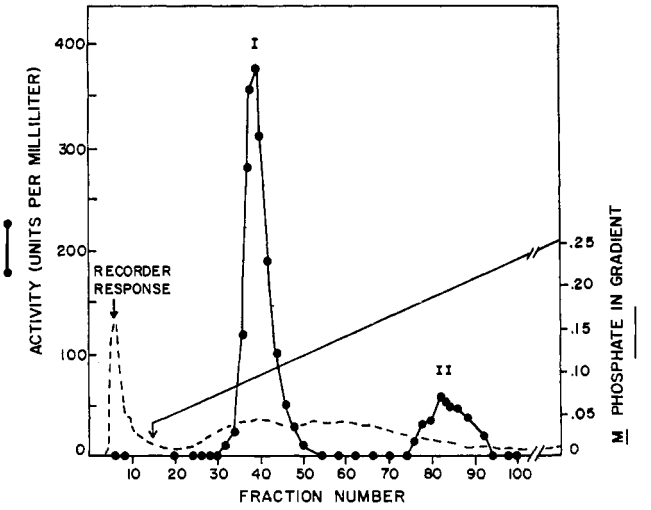

FIGURE 2: DEAE-cellulose elution pattern using a gradient elution procedure. Conditions: 2.6 × 9.0 cm column; flow rate, 30 ml/hr; 4°; fractions of 5 ml/tube. The arrow indicates the point of application of the gradient. Activity was determined by assay procedure I.

TABLE I: Purification of Beef Heart Cyclic Nucleotide Phosphodiesterases.[a]

Step	Fraction	Vol (ml)	Protein (mg)	Enzyme Units	Yield (%)	Sp Act. (Units/mg of Protein)
	Whole homogenate[b]	4470	314,152	1,072,000		3.4
1	Supernatant of homogenate	2630	27,983	308,500	100	11.0
2	0.5 Ammonium sulfate fraction	88	3,608	190,000	61.6	52.7
3	Protamine precipitate	100	265	76,500	24.8	288.7
4	0.5 Ammonium sulfate fraction of protamine precipitate	4.6	176	52,700	17.1	299.4
5	Pooled Sephadex G-200 fractions	48	38.8	24,400	7.9	628.9
6	DEAE-cellulose fractions					
	I	126	2.6	6.355	I + II	2444
	II	12	4.6	2,855	3.0	621

[a] Activities were determined by assay procedure I except for the homogenate and its supernatant post centrifugation. In these cases, assay procedure II was used. The weight of beef hearts extracted was 1.69 kg. [b] Values for the homogenate are given, although the supernatant of the homogenate was considered as the starting point of the fractionation. If the original homogenate is considered as the starting point, the purification factors of phosphodiesterase I and II are, respectively, 720 and 180.

presence of both $MgSO_4$ and dithiothreitol was necessary to maintain enzymatic activity. The enzymes were unstable to repeated freezing and thawing.

Heterogeneity of Phosphodiesterase Activity. GEL FILTRATION CHROMATOGRAPHY. As described in the purification method, the Sephadex G-200 elution pattern showed marked heterogeneity which was especially pronounced in the leading boundary of the activity profile. In some instances, an additional minor fraction of activity was present in the exclusion volume of the gel. However, fractions for further purification were pooled only from the major activity zone. The overall recovery of activity from gel filtration runs was 85–95% of the initial amount applied to the column. Fractions pooled from the columns generally included about 50–60% of the total recovered activity and represented the central portion of the major zone of activity. The amount of phosphodiesterase activity present in the exclusion volume of the G-200 column was variable (approximately 10% or less of the total recovered activity) and a function of whether fresh or frozen beef hearts were used. The amount of activity in this fraction apparently increased when frozen heart was the starting material, but no further evaluation of this effect was attempted.

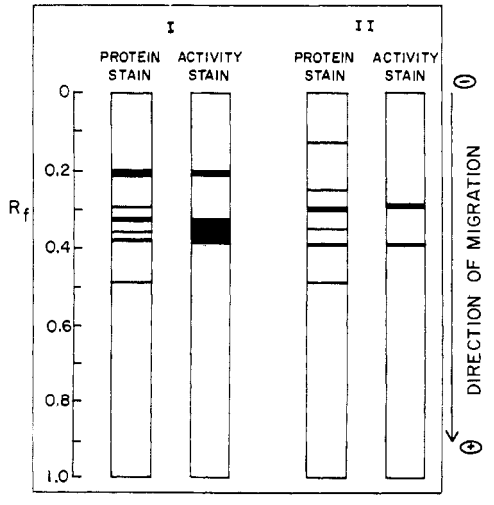

FIGURE 3: Schematic representation of polyacrylamide gel electrophoresis of phosphodiesterase I and II. Conditions of electrophoresis are described in the text. The cathode is the origin and is at the top of the gels.

Heterogeneity of Phosphodiesterase Activity. DEAE-CELLULOSE CHROMATOGRAPHY. When the pooled fractions from Sephadex G-200 were eluted from DEAE-cellulose, using a stepwise elution technique, two active enzymatic species were obtained (Figure 1). To further emphasize the fact that the two forms of the enzyme were separate entities and not artifacts of the stepwise elution method, a similar distribution of the activity was obtained with a gradient elution technique (Figure 2). Again, the two forms of the enzyme were well separated and showed an identical distribution. In both cases, phosphodiesterase I represented 70–80% of the total activity recovered from DEAE-cellulose. Overall recovery of the activity from the ion-exchange columns was always 80–95% of the initial amount applied. The stepwise elution procedure was used routinely because it produced the best purification of the major form of the enzyme, phosphodiesterase I.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed on samples of both phosphodiesterase I and II in order to determine their relative purifications and any structural similarities. A schematic representation of the electrophoresis patterns is shown in Figure 3. Fractions I (145 μg) and II (40 μg) were used of specific activities 1942 and 500 units/mg, respectively, as determined by assay procedure I. The first gel in each pair shows the results of protein localization using Amido Black 10B; the second gel in each pair represents the determination of enzymatic activity. The positions of the various bands are indicated by R_F values which are the ratios of distance of migration of individual bands to the distance of migration of the tracking dye, Bromophenol Blue.

The following points should be noted concerning the electrophoretic results of phosphodiesterase I. On the protein stained gels, bands at R_F positions 0.29, 0.35, and 0.49 were extremely light and hardly discernible. The major protein

TABLE II: Substrate Specificities of Phosphodiesterase I and II.[a]

Compd	Rel Rate of Hydrolysis (%) I	II
cAMP	100.0	100.0
cGMP	30.8	53.0
cIMP	61.5	68.0
cUMP	25.0	26.0
cCMP	<1.0	4.0
cTMP	16.7	14.5

[a] The percentages refer to the rate of hydrolysis of the different compounds relative to the rate of hydrolysis of cAMP under the conditions of the assay. Assay procedure II was used with each cyclic nucleotide present at 4.0 mM.

TABLE III: Effect of Various Compounds on the Activities of Phosphodiesterase I and II.[a]

Compd	Concn (M)	Activity (% Control) I	II
None		100.0	100.0
cGMP	4×10^{-5}	56.7	60.2
cIMP	4×10^{-5}	59.4	79.6
AMP	2×10^{-3}	85.1	82.9
GMP	2×10^{-3}	65.6	78.0
IMP	2×10^{-3}	80.3	91.0
Theophylline	1×10^{-3}	38.1	41.2

[a] Assay procedure III was used; 4×10^{-4} M cAMP was present in each case. The percentages are given for the hydrolysis of cAMP in the presence of the tested compounds relative to the hydrolysis of cAMP in the absence of any additions.

bands at R_F positions 0.21, 0.33, and 0.38 were prominent and always associated with enzymatic activity as determined on the companion gel. In an effort to avoid the difficulties in interpretation caused by diffusion of the calcium phosphate precipitate, a gel of phosphodiesterase I was also segmented and the segments assayed individually for activity. A precipitate was seen in all segments corresponding to regions of protein on a paired gel. Considering the two gels of phosphodiesterase II, the following points were noted: all bands on the protein stained gel were easily detectable; those at R_F positions 0.30 and 0.39 were most prominent. These latter bands corresponded to areas of enzymatic activity on the paired gel. The purification of phosphodiesterase I by this isolation procedure was significant, considering that the fraction I sample contained more than 3.5 times the total protein of fraction II. Most importantly, both species apparently had a similar constituent that migrated to an R_F position approximately 0.38–0.39. This was indicative of a possible close structural relationship between the two forms. However, the additional bands present in I and II did not correspond.

Molecular Weight Determination of Phosphodiesterase I. Since analytical disc electrophoresis showed significant purification of phosphodiesterase I, an estimation of the molecular weight of this enzyme was made using equilibrium ultracentrifugation. From runs at two rotor speeds (see Methods), an average molecular weight of the enzyme was determined to be 121,500 ± 10,500 g/mol. At high values of cm^2, the plots of $\ln Y$ *vs.* cm^2 showed an upward curvature, indicative of a possible aggregation phenomenon. The molecular weight calculated is in the range of several estimates of a basic molecular weight of the enzyme from other sources as determined by gel filtration techniques (Rosen, 1970; Cheung, 1970).

Kinetic Parameters of Phosphodiesterase I and II. The kinetic parameters of the two forms of the enzyme were determined by the method of Lineweaver and Burk (1934) using cAMP as substrate. Assay Procedure III was used. Initial velocities for phosphodiesterase I were determined with 1.6 μg of the enzyme incubated with five concentrations of cAMP ranging from 2.5 to 40 $\times 10^{-5}$ M. The value of K_m was found to be 3.6×10^{-5} M, and the value of V_m was 454.4 nmol of cAMP hydrolyzed/min per mg of protein. Initial velocities for phosphodiesterase II were determined with 3.1 μg of the enzyme incubated with five concentrations of cAMP ranging from 3.2 to 50 $\times 10^{-5}$ M. The value of K_m was found to be 6.9×10^{-5} M, and the value of V_m was 351.8 nmol of cAMP hydrolyzed/min per mg of protein. Hill coefficients were also determined (Atkinson, 1966) for both forms of the enzyme. The values for phosphodiesterase I and II were 1.02 and 0.94, respectively.

Substrate Specificities of Phosphodiesterase I and II. A comparison of the substrate specificities of phosphodiesterase I and II with respect to nucleoside 3′,5′-monophosphates was made. The results of these studies are shown in Table II. Assay procedure II was used with each cyclic nucleotide present at 4.0 mM concentration. Phosphodiesterase I (1.5 μg) and phosphodiesterase II (11.4 μg) were used per assay. The results for the individual nucleotides are expressed as per cents of the rates of hydrolysis of the preferred substrate, cAMP. In agreement with most workers in this area, both forms of the enzyme showed preferences for cyclic nucleotides with purine bases, that is cAMP, cGMP, and cIMP. However, most importantly, the two forms showed variable activity with respect to the hydrolysis of cGMP. In addition, both forms of the enzyme had considerable activity with respect to the pyrimidine cyclic nucleotide, cUMP.

Effect of Various Compounds on the Activity of Phosphodiesterase I and II. Because various compounds have been implicated in the regulation of phosphodiesterase, a study was designed to determine the effects of the major potential inhibitors. The results are shown in Table III. Assay procedure III was used with the substrate, cAMP, present at a concentration of 4×10^{-4} M. Phosphodiesterase I (1.0 μg) and phosphodiesterase II (2.77 μg) were used per assay. The modifiers were present at the concentrations stated. The results for the various compounds are expressed as per cents of the rates of hydrolysis of cAMP in the absence of any modifiers. As indicated in Table III, theophylline, at 1×10^{-3} M, was an inhibitor of both forms of the enzyme. More significant, however, was the fact that cGMP, present at one-tenth the concentration of cAMP, was a potent inhibitor of cAMP hydrolysis by both forms of the enzyme. Unlike the data for beef heart phosphodiesterase described by Goren and Rosen (1971) GMP apparently was an inhibitor of the reaction of both forms of the enzyme. In their work, Goren and Rosen (1971) described GMP as an activator of the enzyme. However, they also reported that activation by GMP was a labile property of the enzyme that was dependent upon both storage conditions and pH.

Discussion

In this isolation method, the total soluble phosphodiesterase activity in the 13,500*g* supernatant fraction of beef heart homogenate can be accounted for at all steps in the procedure. Our data do not preclude, however, the possibility of an additional protein requirement for the activity of either or both forms of the enzyme. It is only stated that, under these conditions, no additional factors became apparent. The data indicate the presence of at least two distinct forms of cyclic nucleotide phosphodiesterase in beef heart. Unfortunately, the relationship between these two forms of the enzyme remains unclear.

Although the phosphodiesterase activity showed distinct heterogeneity upon gel filtration chromatography, it was not possible to obtain better resolution of the main peak of activity unless altered chromatographic procedures were employed. In this regard, preliminary work (Hrapchak, 1971b) indicated that a crude half-saturated ammonium sulfate fraction of the activity could be partially resolved into three peaks of activity upon Sephadex G-200 only in the presence of 0.5 M KCl. A minor peak of activity was present in the void volume of the column as well as at positions approximately equal to molecular weights of 100,000 and 200,000 as determined by the relative elution volume relationship of Andrews (1970). Similar heterogeneity of phosphodiesterase upon gel filtration has been reported by others (Thompson and Appleman, 1971a,b; Cheung, 1969b, 1970; Rosen, 1970; Kakiuchi *et al.*, 1971; Jard and Bernard, 1970). In particular, Thompson and Appleman (1971a) have reported that two of the multiple phosphodiesterase activities present in rat brain have molecular weights of approximately 200,000 and 400,000. Although this integral ratio of molecular weights suggested a possible subunit aggregation relationship, distinctly different substrate specificities indicated the possibility of two distinct enzyme systems.

In the present study, the major zone of activity from Sephadex G-200 could be effectively resolved into two peaks of activity following DEAE-cellulose chromatography under the described conditions. The distribution of the activity into the two forms was reproducibly obtained from DEAE-cellulose using both stepwise and gradient elution techniques, indicating that the presence of two forms was not an artifact of the stepwise elution technique. Furthermore, this distribution was observed with both crude (step 2, purification) as well as more purified preparations, indicating that the presence of the two forms was not an artifact of the isolation method. However, preliminary work does not allow us to preclude the possibility that the two species may represent a complex equilibrium distribution of two forms of a single enzyme. In support of this possibility were the results of rechromatography on DEAE-cellulose of each of the separated enzymes, phosphodiesterase I and II (Hrapchak, 1971b). Although in both cases of rechromatography, the major amount of activity was present in its initial elution volume, minor but significant amounts of the other form were also detected. These data, along with the results of later work with analytical disc electrophoresis and sedimentation equilibrium, suggested the possibility of a complex aggregation phenomenon.

In characterizing the two forms of the enzyme, and in particular the highly purified phosphodiesterase I, several observations were made. On the one hand, possibly significant differences between phosphodiesterase I and II were observed with respect to their values of K_m for cAMP hydrolysis and substrate specificities. On the other hand, the two species exhibited similarities with respect to analytical disc electrophoresis patterns and inhibition characteristics.

Values of K_m for cAMP of phosphodiesterase I and II determined in this study are within the range of published data for the soluble forms of the enzyme from beef heart. Beavo *et al.* (1970) reported a K_m value of $2.5\text{–}4.5 \times 10^{-5}$ M for a partially purified soluble beef heart activity. This figure agrees closely with the K_m value for phosphodiesterase I as determined here, 3.6×10^{-5} M. The enzyme sample used by Beavo *et al.* (1970) was prepared from a 2000*g* soluble extract according to the method of Butcher and Sutherland (1962). In another investigation, Goren and Rosen (1971) have identified a beef heart phosphodiesterase which may require an additional protein factor. The activity was isolated from the 4000*g* supernatant fraction of the homogenate. Whereas the enzyme described by Goren and Rosen (1971) had a K_m for cAMP of 50×10^{-5} M in the absence of the protein factor, the K_m was reduced to approximately 6×10^{-5} M in its presence. This latter value is within the range of the K_m determined for phosphodiesterase II here, *i.e.*, 6.9×10^{-5} M.

It should be noted that other heart phosphodiesterase activities have been reported with values of K_m for cAMP much lower than those described in this report. In one study, Beavo *et al.* (1970) stated that two phosphodiesterase activities were present in a 1000*g* particulate fraction of beef heart which had values of K_m equal to 2.5×10^{-5} M and 0.8×10^{-6} M. However, when a soluble fraction prepared from the 2000*g* supernatant was used, only a single enzyme with a K_m value of $2.5\text{–}4.5 \times 10^{-5}$ M was detected as mentioned above. In another study, Thompson and Appleman (1971b) identified two forms of phosphodiesterase in a sonically disrupted 20,000*g* supernatant fraction of rat heart. These two activities had K_m values of 8.67×10^{-5} M (fraction II) and 3.85×10^{-6} M (fraction III). Furthermore, the fraction III enzyme appeared to exhibit negative cooperativity. These additional data offer intriguing possibilities. First, under the conditions of the kinetic determinations in the present report (cAMP $\geqq 2.5 \times 10^{-5}$ M), lower K_m values for either or both phosphodiesterase I and II may not have been detected. Further studies in this area are desirable. Second, since our isolation uses an initial 13,500*g* supernatant fraction (representing only 30–35% of the total homogenate activity), an enzyme with a lower K_m value may have been lost during centrifugation. It is therefore possible that phosphodiesterase I and II may indeed be different forms of aggregation of a single soluble enzyme. This would agree with the data of Beavo *et al.* (1970) and Butcher and Sutherland (1962), both describing single activities in soluble extracts of beef heart.

In studying the substrate specificities of phosphodiesterase I and II, both forms of the enzyme, and in particular the highly purified fraction I, showed activity with respect to other cyclic nucleotides besides cAMP. The enzymes also had variable activity with respect to cGMP, the only other cyclic nucleotide known to exist physiologically. Phosphodiesterase II was nearly twice as active as phosphodiesterase I with respect to cGMP hydrolysis.

The two forms of the enzyme were quite similar in regard to electrophoretic patterns and inhibition characteristics. Both the highly purified phosphodiesterase I and the cruder phosphodiesterase II showed several bands of activity following electrophoresis, indicating complex structures. In addition, both forms apparently had a similar constituent, indicating possibly a close structural relationship.

Considering the inhibition studies, cGMP inhibited both

phosphodiesterase I and II to the same extent when present at one-tenth the concentration of the substrate, cAMP. Rosen (1970) has suggested that cGMP may function indirectly to alter intracellular cAMP concentrations by inhibiting phosphodiesterase.

In summary, although the two forms of the soluble beef heart phosphodiesterase, I and II, are stable during isolation, evidence indicates that they may be interrelated. Their stability during isolation suggests that a complex equilibrium involving lipids, carbohydrates, or disulfide interchange reactions may be involved in determining their distribution. In this regard, Cheung (1970) has reported that the heterogeneity of beef brain phosphodiesterase may be produced by disulfide interchange reactions. Furthermore, Thompson and Appleman (1971a) reported that one form of the rat brain enzyme showed a tendency to associate with particulate matter in the preparation. They found that sonication disrupted this association. The interrelationships between the several forms of the enzyme found in the two preceding studies have not yet been completely defined.

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