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PURIFICATION OF RAT HEART CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ON COLUMN OF IMMOBILIZED PHENYLBUTENOLIDE INHIBITOR

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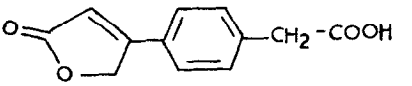
SUMMARY : Cyclic nucleotide phosphodiesterase was purified over 200-fold in a single step from the rat heart cytosolic fraction, using affinity chromatography on phenylbutenolide inhibitor immobilized to AH Sepharose. After elimination of the contaminating proteins by washing with the loading buffer and then with 0.4 M KCl buffer, without any loss in enzymatic activity, the cyclic nucleotide phosphodiesterase was eluted in good yields with a linear KCl gradient from 0.4 M to 1.8 M. Enzymatic activity determination performed with both cyclic AMP and cyclic GMP as substrate, either at low (0.25 µM) or at high (25 µM) concentration, pointed out the presence of several phosphodiesterase forms with different substrate specificities, in the elution profiles.

INTRODUCTION : Since the early work of Cuatrecasas et al (1), the affinity chromatography has been extensively used for the purification of various macromolecules and specially enzymes. This affinity technique has been already employed in the purification procedure of cyclic nucleotide phosphodiesterases from several tissular and species origins. Three types of ligands, linked to a solid matrix, have been reported for phosphodiesterases purification : dyes, such as Cibacron blue F_3GA (2) and related dyes (3), calcium binding proteins as troponin c-like protein (4) or calmodulin (CDR) (5,6,7), cyclic nucleotide phosphodiesterases inhibitors related to papaverine and methylisobutyl xanthine (8) or doxantrazole (9). Blue dextran-Sepharose gel not only retains enzymes which bind cyclic nucleotides, as phosphodiesterases do, but also various other proteins which bind linear nucleotides

Abbreviations : phenylbutenolide acetic acid : (2-oxo,2,5-dihydro,4-furyl)4-phenylacetic acid



A-H-Sepharose 4B : aminohexyl Sepharose 4B ; EDC, HCl : 1-ethyl-3-(-3-dimethylamino-propyl)carbodiimide, HCl ; DMF : N-N dimethylformamide ; Tris : Tris (hydroxymethyl)-aminomethan ; DTE : (erythro-2,3-dihydroxy)-1,4-butan dithiol ; EGTA : ethylene glycol 2 (2-aminoethyl) tetracetic acid ; cAMP : adenosine 3'5' cyclic monophosphate ; cGMP : guanosine 3'5'cyclic monophosphate ; CDR : calcium dependent regulator (calmodulin)

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such as ATP, NAD or coenzyme A, as substrates or effectors. Similarly, CDR-Sepharose not only retains cyclic nucleotide phosphodiesterases but also several other CDR-dependent proteins. Klee et al (10) showed that phosphodiesterases are only a minor component of the proteins specifically retained on a column of CDR-Sepharose in the presence of Ca^{++} and released by EGTA. The CDR-Sepharose affinity step usually gives a 20-40-fold phosphodiesterase purification with a 40% recovery (4,10). It is noteworthy that this affinity technique allows only the purification of the CDR-dependent forms of the phosphodiesterases but does not purify the independent forms. Only few attempts of phosphodiesterases purification by affinity chromatography on columns of immobilized inhibitors were reported. Mohindru et al (8) using STMP-agarose and MIX-agarose isolated two forms of cyclic nucleotide phosphodiesterases(one Ca^{++} and CDR dependent, the other independent) from bovine heart. However this technique only yielded low purification (3 to 4-fold) with a 40% recovery. With a doxantrazole analog as ligand, Wrigglesworth (9) obtained an excellent purification of the total guinea pig lung phosphodiesterase activity but did not follow the eventual various forms in presence. As it was pointed out by several authors (11-12), heart cyclic nucleotide phosphodiesterase is a possible target for several cardioactive drugs. One of our aims is to further investigate the eventual contribution of phosphodiesterase inhibition to the inotropic effect of various synthetic compounds related to cardenolides, with a phenylbutenolide backbone (13-14), and their selective effects on cardiac enzyme. Among the moderate competitive phenylbutenolide inhibitors previously described (15), carboxylic derivatives proved convenient potential ligands since they could be easly linked to ω-aminoalkyl Sepharose via amide linkage. This report describes the preparation and use of a phenylbutenolide acetic acid - AH Sepharose 4B conjugate to highly purify, in a single step, all the multiple forms present in the cyclic nucleotide phosphodiesterases from rat heart cytosolic fraction.

MATERIALS AND METHODS : A-H Sepharose 4B was obtained from Pharmacia Fine Chemicals. EDC-HCl, snake venom (Ophiophagus hannah), unlabelled cyclic nucleotides were purchased from Sigma Chemical Co. AG 1 x 2 resin (200-400 mesh) was from Bio-Rad Laboratories. [^{3}H] cAMP (20-30 Ci/mmol), [^{3}H] cGMP (10-30 Ci/mmol), [^{3}H] adenosine (20-25 Ci/mmol), [^{3}H] guanosine (5-15 Ci/mmol) were supplied by the Radiochemical Centre Amersham. N-N dimethylformamide acetic acid, EGTA were obtained from Carlo Erba-Milano. All other chemicals were reagent grade.

Preparation of cytosolic rat heart phosphodiesterases prior to chromatography. Hearts from Sprague Dawley rats (250 g) were rapidly removed and perfused with 0.32 M saccharose in Tris-HCl buffer 1 mM (pH 7.4) to remove blood. The fresh muscle was homogenized with a glass-glass potter in 3 v/w of the

above-mentioned buffer. The homogenate was centrifuged at 20,000 g for 15 min.. The supernatant was then centrifuged at 105,000 g for 1 h. The 105,000g supernatant fraction was stored at -20°C and used as a source of phosphodiesterases.

Preparation of a phenylbutenolide acetic acid substituted gel. A-H Sepharose 4B (5 g) washed following the manufacturer's instructions, was suspended in 20 ml of deionized water ; 500 mg (2 mM) of phenylbutenolide acetic acid (2-oxo, 2,5-dihydro,4-furyl)-4 phenyl acetic acid) were dissolved in 100 ml N.N-dimethylformamide and added to the A-H Sepharose 4B suspension. EDC-HCl (3 g) in 30 ml of deionized water was added dropwise. The pH was brought to 4.5 with dilute hydrochlorid acid and the reaction allowed to proceed for 48h at room temperature with continuous gentle mecanical stirring. pH was frequently readjusted to 4.5 during the first 24 h. A further overnight incubation in presence of 2 mM acetic acid was performed in order to block any remaining activated groups. The resulting substituted gel was washed with a N.N-dimethylformamide-water (2:1) mixture (1.5 l) to remove unreacted ligand, (the disappearance of unbound phenylbutenolide acetic acid in the wash was monitored by determination of absorbance at 280 nm.), with 500 ml 1 M K_2HPO_4, 500 ml of deionized water and finally with 500 ml of storage buffer (50 mM Tris, 5 mM $MgCl_2$, 0,1 mM DTE pH 7.4). It was suspended in Tris buffer as a 2 : 1 slurry and stored at 4° untill use. In order to perform several control experiments, acetic acid - substituted gel was alternatively prepared following an identical procedure. In this case the coupling reaction was allowed to proceed overnight.
The concentration of phenylbutenolide acetic acid attached to the gel was estimated to be 19 µmoles/g dried resin when determined from the ultraviolet spectrum of the conjugate measured as a suspension in polyethylenglycol and 21 µmoles/g/dried resin when determined from the ultraviolet spectrum of the hydrolysate (6N HCl hydrolysis at 120° for 2 h.).

Chromatography procedure. Chromatography was performed as indicated in legends of figures 1 and 2. Gradient elution was followed by extensive washes of the column with 2 M KCl in Tris buffer. This procedure allows a good regeneration of the affinity column which can be reused at least three times with reproducible cyclic nucleotide phosphodiesterase elution patterns and constant recovery.

3'5'-cyclic nucleotide phosphodiesterase assay. cAMP and cGMP phosphodiesterase activities were assayed according to the method of Thompson and Appleman (16) as modified by Boudreau and Drummond (17). Percentages of adenosine and guanosine fixed on the resin were evaluated with tritiated nucleosides, and the phosphodiesterase activities corrected for these yields. No more than 15% of substrate was hydrolyzed in an assay. Assays were performed on an aliquot of each eluted fraction. On account of sample dilution, KCl concentration did not exceed 0.45 M in the assay mixture. KCl, up to 0.5 M was shown to not significatively affect cAMP phosphodiesterase activity of the original cytosolic preparation when measured with both 0.25 µM and 25 µM substrate concentration ; cGMP phosphodiesterase sensitivity to KCl dependedon assay conditions : whereas it was only slightly inhibited (20% with 0.5 M KCl) when assayed at 25 µM cGMP concentration or at 0.25 µM cGMP in presence of calcium + calmodulin, basal activity measured at 0.25 µM cGMP in presence of EGTA was decreased about 20% with 0.25 M KCl and 50% with 0.5 M KCl. Percent inhibition were determined up to 0.5 M KCl and basal cGMP phosphodiesterase recovery was corrected for these values. A boiled 105,000 g supernatant of rat cerebral cortex was used as calmodulin source (18).

RESULTS : Cyclic nucleotide phosphodiesterase of the rat heart cytosolic fraction was entirely bound both to the acetic acid substituted gel and to the phenylbutenolide acetic acid substituted gel. The bulk of contaminating proteins (50-60% of the applied material) without detectable phosphodiesterase activity was recovered in the first wash with the loading buffer (Fig.1 and 2) Cyclic nucleotide phosphodiesterase bound to the acetic acid-Sepharose column was eluted by a linear KCl gradient as a single peak of activity at 0.45 M KCl (Fig.1). Pooling the active fractions of this peak resulted in a 75% yield with a 10-15 fold purification. The majority (about 85%) of the recovered activity was eluted in a narrow range of KCl concentration from 0.3 to 0.6 M. Cyclic nucleotide phosphodiesterase was more tightly bound to the inhibitor coupled Sepharose since no enzyme activity was detected in the eluate before 0.45 M KCl and 10% only of the recovered activity were eluted before 0.6 M KCl in the same assay conditions (data not shown). The whole

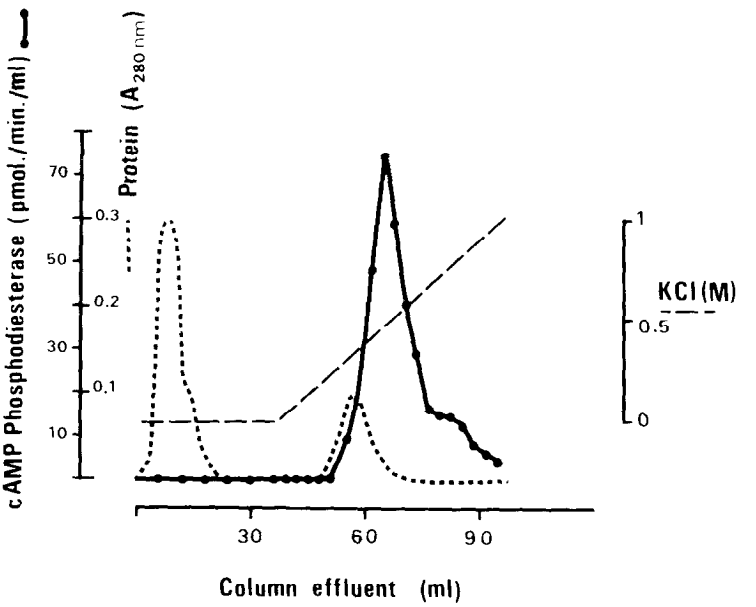
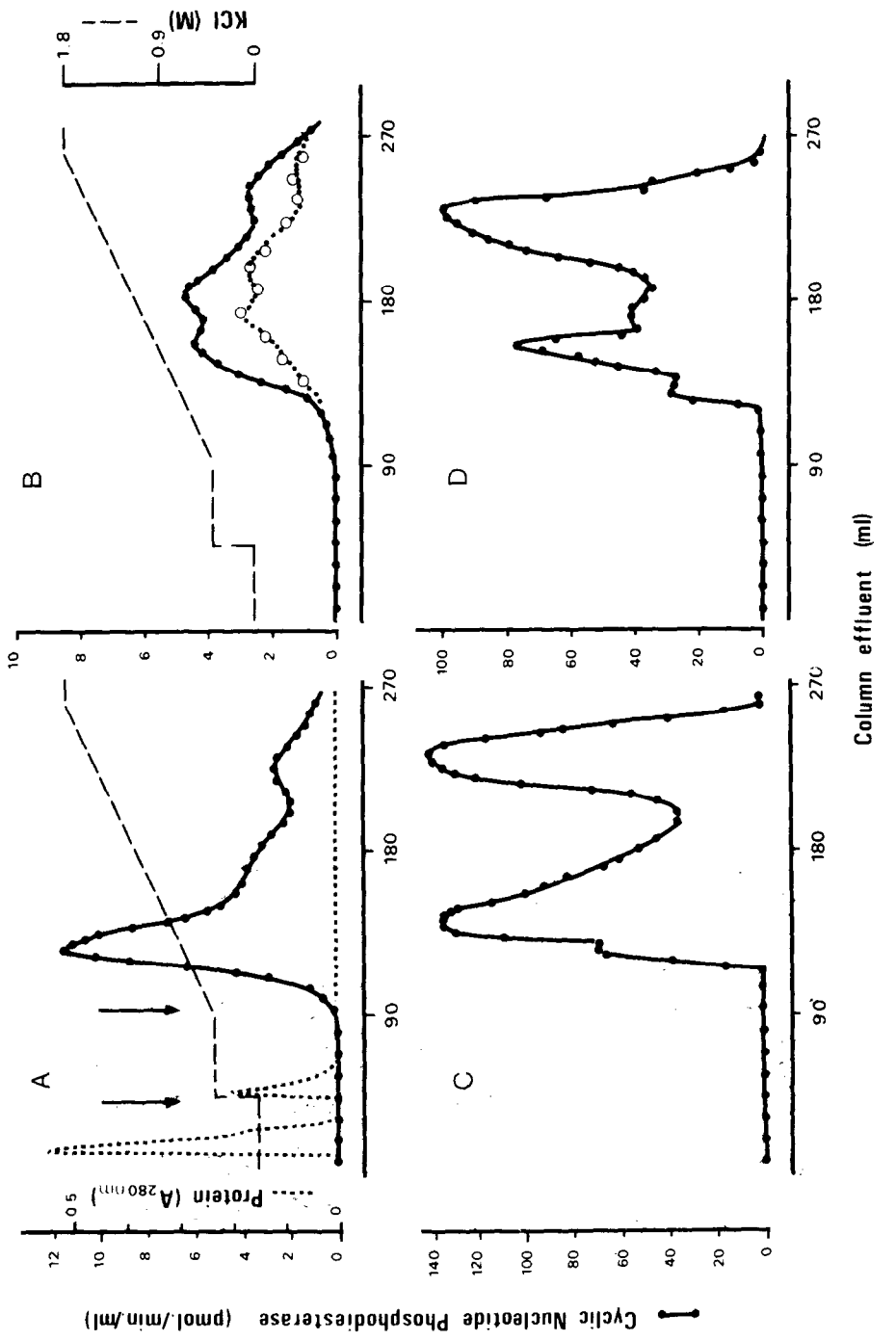


FIGURE 1 : Chromatography using the acetic acid-AH Sepharose 4B conjugate. The acetic acid-substituted gel was packed into a 1.0 x 2.0 cm column, washed and equilibrated with 50 mM Tris buffer containing 5 mM $MgCl_2$, 0.1 mM DTE, 0.1 mM $CaCl_2$,pH 7.5.Enzyme samples (0.5 ml) containing 5.7 mg proteins were applied to the column. After sample application, the column was washed with 36 ml of Tris buffer. 6 ml fractions were collected. The column was then eluted with a linear 0-1 M KCl gradient in Tris buffer (30-30 ml) at a flow rate of 25-30 ml/hour. 3 ml fractions were collected. cAMP phosphodiesterase activity (•—•) was determined in each eluted fraction on 100 µl aliquot using 25 µM cAMP as substrate. Assays were performed in triplicate as described in MATERIALS AND METHODS. Absorbance (------) of effluent fractions was monitored at 280 nm. Proteins were also determined according to Lowry et al. (25) using bovine serum albumin as standard.


A
B
C
D
Protein (A_{280nm})
KCl (M)
Cyclic Nucleotide Phosphodiesterase (pmol./min./ml)
Column effluent (ml)

cyclic nucleotide phosphodiesterase activity was eluted from the inhibitor coupled Sepharose column between 0.5-1.8 M KCl (Fig.2). Washing the affinity column with 0.4 M KCl, prior to the linear KCl gradient elution, still removed a substantial amount of contaminating proteins (10-20% of the applied material) devoid of phosphodiesterase activity. Then, no detectable 280 nm absorbing material was found in the elution profiles from 0.4 to 1.8 M KCl.

As it was generally observed with other phosphodiesterases separation techniques (for review see 19), enzyme elution profiles were strikingly dependent upon assay substrate conditions. When measured at low cAMP concentration (0.25 μM) rat heart phosphodiesterase was resolved into two well separated peaks of activity : a major peak (65% of the recovered cyclic AMP phosphodiesterase activity) at 0.7 M and a minor peak at about 1.5 M (Fig. 2A). In these substrate conditions, the bulk of cAMP phosphodiesterase activity was recovered in 67% yield with more than 200-fold purification (table 1). The most part of cGMP phosphodiesterase activity assayed at low cGMP concentration (0.25 μM) was eluted from 0.8 M KCl (Fig.2B). Two broad overlapping peaks were eluted at 0.925 and 1.15 M KCl and a minor peak appeared at about 1.5 M. The total recovery in cGMP phosphodiesterase activity was about 45% with a 150-fold purification (Table 1). At high substrate level (25 μM) cAMP and cGMP phosphodiesterase showed practically the same elution pattern which proved very different from the 0.25 μM profiles (Fig. 2C and 2D). Cyclic nucleotide hydrolyzing activity emerged as two large peaks with the same relative importance at about 0.9 and 1.5 M KCl. Pooled fractions from these two peaks gave a 68% recovery in cAMP phosphodiesterase activity and a 44% recovery in cGMP phosphodiesterase activity with more than 200-fold and 150-fold purification respectively (Table 1).

FIGURE 2 : Chromatography using the phenylbutenolide acetic acid - AH-Sepharose 4B conjugate. The phenylbutenolide acetic acid-substituted gel was packed into a 1.0 x 3.0 cm column, washed and equilibrated with the same Tris buffer as described in the legend to Figure 1. The 105,000 g supernatant of the rat heart phosphodiesterase preparation (1.5 ml , 7 - 16 mg proteins) was applied to the column. The column was washed with 42 ml of Tris buffer followed by 48 ml of 0.4 M KCl in Tris buffer (as indicated by arrows). 6 ml fractions were collected. After these two washes, the column was eluted with a linear KCl gradient from 0.4 to 1.8 M (84-84 ml) at a flow rate of 25-30 ml/hour. 3 ml fractions were collected. Cyclic nucleotide phosphodiesterase activity (•—•) was measured at the following substrate concentrations : panel A, 0.25 μM cAMP ; panel B, 0.25 μM cGMP ; panel C, 25 μM cAMP ; panel D, 25 μM cGMP. In panel B, (•—•) activity was determined in presence of 0.1 mM $CaCl_2$ plus calmodulin in a saturating amount (40 μg protein/assay) ; (o·····o) activity was determined in presence of 1 mM EGTA. All assays were performed in triplicate on 100 μl of each eluted fraction. Absorbance (------) was measured at 280 nm. In order to avoid large losses in enzyme activity which occurred upon storage at -20°C, the purified fractions were stored at +4°C in presence of bovine serum albumin (1 mg/ml). Before BSA addition, aliquots of each fraction were taken up for protein determination.

TABLE 1 : Purification of rat heart cyclic nucleotide phosphodiesterase.

Assay conditions	Enzyme activity units (pmoles/min)		Total (c) proteins mg		Specific activity (pmoles/min./mg)		Yields %	Purification -fold
	S (a)	E (b)	S	E	S	E		
cAMP 25 µM	7970	5425			506	120 556	68	238
cAMP 0.25 µM	980	656			62	14 578	67	235
cGMP 25 µM	7500	3275	15.75	0.045	476	72 778	44	153
cGMP 0.25 µM + calmodulin + 0,1mM $CaCl_2$	1240(d)	560(e)			78.7	12 445	45	158
cGMP 0.25 µM + 1mM EGTA	680	294			43.2	6 534	43	151

(a) S : 105,000 g supernatant fraction of rat heart phosphodiesterase preparation
(b) E : 0.4-1.8 M KCl gradient eluate
(c) total protein content of the whole 0.4-1.8 M KCl gradient eluate was determined according to Lowry et al (25) with standard curves of bovine serum albumin in the appropriate buffer, after concentration of pooled aliquots from each eluted fraction, using a minicon B 15 concentrator.
(d) inhibition of the original phosphodiesterase preparation by 1 mM EGTA : 45%
(e) inhibition of the elu ate phosphodiesterase activity by 1 mM EGTA : 47,5%

The original phosphodiesterase preparation was found to be nearly insensible to exogenous calmodulin + Ca^{2+} adjunction in every substrate condition employed. In contrast, its sensitivity to EGTA was dependent on assay conditions. At 25 μM substrate concentration both cAMP and cGMP phosphodiesterase activities were slightly inhibited (about 20%) by 1mM EGTA ; at 0.25 μM, cAMP hydrolysis was unmodified (data not shown) while cGMP hydrolysis was inhibited by 45% (Table 1). On account of the above results, the sensitivity of the eluted phosphodiesterase to EGTA was therefore only investigated with 0.25 μM cGMP as substrate, and was shown to be in the same range of importance as that observed prior to chromatography (Fig.1B, Table 1).

DISCUSSION : The affinity chromatography on phenylbutenolide acetic acid-Sepharose reported here is a convenient procedure for a rapid one step purification of whole cyclic nucleotide phosphodiesterase, with good yields in enzyme recovery (68% and 45% for cAMP and cGMP phosphodiesterases, respectively). The purification obtained (more than 200-fold and 150-fold for cAMP and cGMP phosphodiesterases respectively) is far better than that given by classical procedures : 10-40-fold with DEAE cellulose (20-21), 2-5-fold with gel filtration (20). If one considers yields in cyclic nucleotide phosphodiesterase activity obtained in each substrate condition , it can be presumed that no major enzymatic form was lost during the chromatographic procedure. Especially, EGTA inhibition of the eluted cGMP phosphodiesterase attests that calcium-dependent forms are well recovered and that no substantial proteolysis (which decreases calcium-dependence (22-23)) occurred. Cyclic AMP phosphodiesterase was eluted from the affinity column before cyclic GMP phosphodiesterase activity. The first eluted peak (0.7 M KCl), more cyclic AMP-specific (A/G ratio about 4), and of major importance at low substrate level (0.25 μM), only appears as a shoulder of both cAMP and cGMP phosphodiesterase profiles determined at 25 μM substrate concentration. This form presents some similitude with the D III form described by Appleman and Terasaki (24) from rat heart and with the F III form obtained by Hidaka and Asano (20) from human platelet, using DEAE cellulose chromatography as separation procedure. More recently, a high affinity cyclic AMP phosphodiesterase was purified to apparent homogeneity by Thompson et al (21). Further separation and characterization studies will be needed to specify if our 0.7 M KCl fraction is a parent form of the high affinity cyclic AMP phosphodiesterase previously described by others (20,21,24). The active fractions eluted at KCl concentration higher than 0.8 M hydrolyzed both cyclic nucleotides at low substrate concentration. The first part eluted from 0.8 to 1.05 M hydro-

lyzed as much cAMP as cGMP (A/G ratio about 1) while the 1.05-1.4 M zone preferentially hydrolyzed cGMP (A/G ratio = 0.5). This bulk of phosphodiesterase activity might represent either a cAMP-cGMP form of enzyme or a cGMP specific form contaminated by the previously eluted cAMP specific peak, or both. The last eluted phosphodiesterase peak particularly important at high substrate level (50 and 60% of the cAMP and cGMP recovered phosphodiesterase activity) hydrolyzed both cyclic nucleotides. This fraction may be compared to the cAMP-cGMP D II form described by Appleman and Terasaki (24) and to the P II form reported by Hidaka and Asano (20).

Since the elution of cyclic nucleotide phosphodiesterase activity from the phenylbutenolide acetic-acid-Sepharose gel was achieved by use of a "deforming buffer", we can't rule out the possibility that phosphodiesterase adsorption to the gel may have been nonbiospecific. However, the chromatographic behavior of phosphodiesterase on acetic acid-Sepharose,"a mock affinity gel" carrying no recognizable ligand at all, proved very different from its behaviour on inhibitor-coupled gel (phosphodiesterase being less strongly bound to AcOH-Sepharose gel, as related in part results). So, we could assume that non biospecific adsorption caused by hydrophobic spacer arms of aminohexyl Sepharose only accounted for a small part in our chromatographic procedure. Attemps of bioelution, either with cyclic nucleotides or with phenylbutenolide inhibitors stronger than phenylbutenolide acetic acid will bring further informations on the nature of the enzyme-support interactions and might allow a better separation of the multiple forms with higher purification.

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