Maurer, W. (1972) *Biochemistry 11,* 3507–3512.
Jencks, W. P. (1975) *Adv. Enzymol. 43,* 219–410.
Knowles, J. R. (1976) *CRC Crit. Rev. Biochem. 4,* 165–173.
Nicely, V. A., & Dye, J. L. (1971) *J. Chem. Educ. 48,* 443–448.
Ogasawara, N., & Inoue, Y. (1976) *J. Am. Chem. Soc. 98,* 7048–7053.
Osterman, H. L., & Walz, F. G. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 906.
Peller, L., & Alberty, R. A. (1959) *J. Am. Chem. Soc. 81,* 5907–5914.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4,* 647–806.
Rüterjans, H., & Pongs, O. (1971) *Eur. J. Biochem. 18,* 313–318.
Rüterjans, H., Witzel, H., & Pongs, O. (1969) *Biochem. Biophys. Res. Commun. 37,* 247–253.
Takahashi, K. (1970) *J. Biochem. (Tokyo) 63,* 833–839.
Takahashi, K. (1976a) *J. Biochem. (Tokyo) 80,* 1173–1176.
Takahashi, K. (1976b) *J. Biochem. (Tokyo) 80,* 1267–1275.
Takahashi, K., Stein, W. H., & Moore, S. (1967) *J. Biol. Chem. 242,* 4682–4690.
Van Batenburg, O. D., Voskuyl-Holtkampt, T., Schattenkerk, C., Hoes, K., Kerling, K. E. T., & Havinga, E. (1977) *Biochem. J. 163,* 384–386.
Walz, F. G. (1976) *Biochemistry 15,* 4446–4450.
Walz, F. G. (1977a) *Biochemistry 16,* 4568–4571.
Walz, F. G. (1977b) *Biochemistry 16,* 5509–5515.
Walz, F. G., & Hooverman, L. (1973) *Biochemistry 12,* 4846–4851.
Walz, F. G., & Terenna, B. (1976) *Biochemistry 15,* 2837–2842.
Wilkinson, G. N. (1961) *Biochem. J. 80,* 324–332.
Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175,* 558–564.

5′-Nucleotide Phosphodiesterase: Isolation of Covalently Bound 5′-Adenosine Monophosphate, an Intermediate in the Catalytic Mechanism†

Michael Landt and Larry G. Butler*

ABSTRACT: 5′-Nucleotide phosphodiesterase was purified from bovine intestine, employing L-histidyldiazobenzylphosphonic acid–agarose (an affinity resin) to remove copurifying alkaline phosphatase. Stoichiometric amounts of enzyme were incubated with the substrate 3′,5′-[^{3}H]cAMP, quenched and analyzed for bound [^{3}H]AMP. Incubations were quenched with liquified phenol, which quantitatively extracted the protein into the phenol phase as shown by experiments with ^{125}I-labeled enzyme, while unbound substrate and product were removed by multiple aqueous washes. Active phosphodiesterase incorporated [^{3}H]AMP in a form that could not be removed by extensive washing, and migrated with the protein on gel filtration under unfolding conditions. The amount of radioactivity incorporated per milligram of protein was proportional to the specific activity of the enzyme and increased as the pH was lowered or the incubation period was shortened. Labeling was drastically diminished when the usual incubation mixture contained a competitive inhibitor. One-hour incubation time, which permitted complete hydrolysis of substrate, abolished the incorporation of label. These observations suggest the formation of a covalent enzyme–substrate intermediate in the hydrolysis of substrate. The bond between enzyme and the [^{3}H]AMP intermediate was acid stable but labile at pH 13. Addition of active phosphodisterase to labeled (denatured) enzyme also released label from the protein. The pH stability data and susceptibility of the label to phosphodiesterase cleavage suggest that the [^{3}H]AMP is bound to phosphodiesterase through a phosphoester bond to either a serine or threonine residue.

The enzyme 5′-nucleotide phosphodiesterase (EC 3.1.4.1) from bovine intestine catalyzes the hydrolysis of a wide range of phosphoesters. In addition to the hydrolysis of nucleic acids at the 3′ end to liberate 5′-nucleotides, the enzyme cleaves NAD,[1] ADP, ATP, and cyclic 3′,5′-AMP (cAMP) to 5′-AMP (Kelly et al., 1975). Also substrates are phosphonate esters such as 4-nitrophenyl phenylphosphonate, which provides a convenient and specific assay for this enzymatic activity (Kelly and Butler, 1975).

Bovine intestine is a rich source of this enzyme (Dardinger, 1974). It has been estimated that the small intestine of one yearling contains more than 1 g of 5′-nucleotide phosphodiesterase. Previous reports from this laboratory described purification and characterization of this enzyme (Kelly et al., 1975). 5′-Nucleotide phosphodiesterase was reported to have a molecular weight of 108 000, to contain 21% carbohydrate, and to consist of identical subunits with a dimeric structure likely (Kelly et al., 1975).

Kelly and Butler (1977) have investigated the mechanism of 5′-nucleotide phosphodiesterase by determining kinetic constants of systematically varied substrates and by pre-

† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907. *Received February 21, 1978.* This is journal paper no. 7062 from the Purdue University Agriculture Experiment Station. This work was supported by Grant No. GM 23028 from the National Institutes of Health.

[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; cAMP, cyclic 3′,5′-adenosine monophosphate; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/78/0417-4130$01.00/0

steady-state kinetics. They proposed a ping-pong type of mechanism with participation of a covalent enzyme intermediate.

Demonstration of the proposed covalent intermediate required larger quantities of enzyme than was obtainable with the original method of purification. The main difficulty in purification was due to the close resemblance of 5′-nucleotide phosphodiesterase to alkaline phosphatase, which caused them to copurify (Dardinger, 1974). Development of an affinity chromatographic method for alkaline phosphatase (Landt et al., 1978) combined with improvements in the existing protocol allowed purification of substantial amounts of enzyme. These preparations were successfully employed to isolate a covalent intermediate of 5′-nucleotide phosphodiesterase.

Experimental Procedures

Materials. Triton X-100, cAMP, 4-nitrophenyl phosphate, and DEAE-cellulose were purchased from Sigma Chemical Co. 4-Nitrophenyl phenylphosphonate, NH_4^+ salt, was obtained from Regis Chemical Co. Sephacryl S-200 was a product of Pharmacia Co. [2,3-^{3}H]cAMP with a specific activity of 46.5 Ci/mmol was purchased from ICN Pharmaceuticals. Carrier-free [^{125}I]NaI and [U-^{14}C]NAD were purchased from Amersham Corp. Ammonium sulfate, ultrapure grade, was a product of Schwarz/Mann Co. Proteinase K was a product of E. Merck Co.

Assay of Enzyme Activities and Protein. 5′-Nucleotide phosphodiesterase and contaminating alkaline phosphatase were determined spectrophotometrically in discontinuous assays at 30 °C by following release of nitrophenol at 400 nm using 18 320 M^{-1} cm^{-1} as the extinction coefficient (Kelly and Butler, 1975). The phosphodiesterase substrate was 1.00 mM 4-nitrophenyl phenylphosphonate in 100 mM Tris-HCl (pH 8.0). Alkaline phosphatase activity was measured in the same buffer with 1.30 mM 4-nitrophenyl phosphate. One unit of enzyme activity is the amount of enzyme catalyzing the production of 1 μmol of product per minute at 30 °C under standard conditions. Protein concentration was determined from optical density at 280 nm and the mass extinction coefficient of 0.98 OD mg^{-1} mL^{-1} previously reported (Kelly et al., 1975).

Purification of 5′-Nucleotide Phosphodiesterase. The enzyme was purified from fresh calf intestine by a modification of the method of Kelly et al. (1975). The contents and mucosa of 7–12 m of small intestine were expelled and stirred at room temperature for 4 h with an equal volume of 0.1% Triton X-100 in 100 mM Tris-HCl (pH 8.0). Centrifugation for 30 min at 9000*g* produced approximately 4 L of supernatant containing the enzyme. All succeeding steps were conducted at 0–6 °C.

The extract was shaken with a 1/3 volume of water-saturated 1-butanol and centrifuged to separate the layers. The aqueous layer was reextracted with the same volume of 1-butanol and again centrifuged, and the aqueous layer was retained. Solid ammonium sulfate was added until 35% of saturation was attained, and the precipitate was discarded. Ammonium sulfate was added to 80% saturation, and the precipitate was collected by centrifugation. The pellets were dissolved in 500 mL of 100 mM Tris-HCl (pH 8.0) and exhaustively dialyzed against 30 mM Tris-HCl (pH 8.0).

The dialyzed enzyme was chromatographed on a 4.0 × 40 cm column of DEAE-cellulose equilibrated with 30 mM Tris-HCl (pH 8.0). The column was washed with equilibrating buffer until protein which was not bound had been removed, and then the enzyme was eluted in 300 mL with 100 mM Tris-HCl (pH 8.0). If the specific activity of 5′-nucleotide phosphodiesterase was less than 12 units mg^{-1}, the enzyme was precipitated with ammonium sulfate and the DEAE-cellulose step was repeated.

Enzyme from DEAE-cellulose fractionation was made 1.5 M in ammonium sulfate and chromatographed on a 1.5 × 30 cm column of L-phenylalanylagarose (Doellgast and Fishman, 1974) equilibrated with 1.5 M ammonium sulfate in 100 mM Tris-HCl (pH 8.0). Enzyme, bound completely at this high solute concentration, was eluted with a linear decreasing gradient (200 mL total volume) of ammonium sulfate from 1.5 to 0.65 M in 100 mM Tris-HCl (pH 8.0). Fractions containing activity were combined, dialyzed against 100 mM Tris-HCl buffer, and concentrated by ultrafiltration in an Amicon 8 MC device. Concentrated enzyme (2–6 mL) was further fractionated on a 2.5 × 33 cm column of Sephacryl S-200 in 100 mM buffer, eluting just behind the void volume.

The final step utilized L-histidyldiazobenzylphosphonic acid–agarose which specifically bound contaminating alkaline phosphatase (Landt et al., 1978) but did not retard phosphodiesterase. Fractions from the Sephacryl column containing activity were combined, dialyzed against 10 mM Tris-HCl (pH 8.0), and passed through a 1.0 × 17 cm column of the affinity resin equilibrated with 10 mM Tris-HCl (pH 8.0); bound alkaline phosphatase activity was eluted after each passage by including 10 mM Na_2HPO_4 in the wash buffer. Purified enzyme was stored at 4–6 °C in 10 mM Tris-HCl (pH 8.0).

Electrophoresis. The procedure of Davis (1964) was employed, omitting the use of a stacking gel, to prepare 7% polyacrylamide gels and examine phosphodiesterase preparations for homogeneity at pH 8.3. Gels were stained for protein with Coomassie blue G-250 in 12.5% trichloroacetic acid.

Labeling of 5′-Nucleotide Phosphodiesterase with ^{125}I. The method of Greenwood et al. (1963) was used to iodinate 50 μg of enzyme with incorporation of ca. 450 μCi of ^{125}I. The labeled protein was diluted twofold with unlabeled protein.

The Phenol-Extraction Technique for the Isolation of Covalently Labeled Enzyme. The method presented below is based on the procedure of Butler (1964), modified to accommodate the appreciable solubility of nucleotide substrates in phenol. In a typical experiment, a 15-mL conical centrifuge tube containing 450 μL of enzyme solution consisting of 0.9–1.0 mg of protein in 10 mM Tris-HCl (pH 8.0) was incubated for 30 min to allow the protein solution to reach room temperature (23–25 °C), and then 50 μL of 2.0 M imidazole–acetate buffer (pH 4.5–7.0) was added with rapid mixing on a vortex mixer. With continued rapid mixing, a 20-s period was allowed for the pH adjustment, and then 500 μL of substrate solution in 0.2 M imidazole–acetate buffer of the appropriate pH was added; the solution contained 100 nmol of cAMP and 1.0 μCi of [2,3-^{3}H]cAMP. Final concentrations were 8.3 μM enzyme, 100 μM cAMP, and 1.0 μCi mL^{-1} [^{3}H]cAMP in 0.20 M buffer. The labeling reaction was stopped at times of 1–20 s by the addition of 500 μL of phenol solution saturated with wash solution.

Wash solution contained 5 mM tetrasodium pyrophosphate and 5 mM Na_2EDTA. Crystalline phenol was added to this solution in excess of saturation, and the phases were allowed to separate in the refrigerator overnight. The phenol layer was removed and employed to stop covalent-labeling incubations. The upper aqueous layer (wash solution) was added in 5.0 mL volumes to stopped labeling experiments, mixed on a vortex mixer twice for 15-s periods, centrifuged to promote separation of the phases, and the top aqueous layer was removed by pipet and discarded. Addition of wash solution with mixing and centrifugation was repeated a total of eight times to remove

excess radioactive substrate, while the phenol layer containing the protein decreased in volume to 100–200 μL.

Aliquots of the wash (upper) layers were counted along with the entire phenol layer in labeling experiments, as well as controls containing inactivated enzyme (i.e., phenol was added to the enzyme solution before the addition of substrates, in reverse of the normal order of addition of reactants). Samples of up to 1.0 mL were counted in 15.0 mL of Bray's solution. The presence of phenol produced considerable quenching; data were routinely converted to dpm units with the aid of the external standard reference index provided by the Beckman LS 100C spectrometer.

Gel Filtration of Labeled Enzyme. A 1.5 × 30 cm column of Sephacryl S-200 was equilibrated with 100 mM Tris-HCl (pH 8.0) containing 0.10% $NaDodSO_4$ and 0.10% 2-mercaptoethanol. Phenol layers from labeling experiments at pH 4.5 (1-s incubation) were dissolved in 0.80 mL of 100 mM Tris-HCl (pH 8.0) containing 2.0% $NaDodSO_4$ and 2.0% 2-mercaptoethanol. After 30 min at room temperature, the sample was applied to the column and eluted with the equilibrating buffer. One-milliliter fractions were collected, checked for optical density at 280 nm, and counted.

Measurement of the Stability of the Covalent Intermediate. Phenol layers from pH 4.5 1-s reactions, each containing about 1.0 mg of 5′-nucleotide phosphodiesterase labeled with [^{3}H]cAMP, were diluted by the addition of 0.10 M imidazole–acetate (pH 7.0) to form a phenol–H_2O emulsion on mixing. Aliquots of 200 μL containing 200–300 μg of labeled protein were transferred to conical 15-mL centrifuge tubes. One milliliter of acetone–methanol (33:67, v/v) was added to each tube with mixing. The protein precipitated overnight and was pelleted by centrifugation in a bench-top centrifuge. The pellets were dissolved in 500 μL of various solutions and incubated for up to 10.5 h at 55 °C in an oven or at 30 °C in a water bath. At predetermined times, tubes containing labeled protein were neutralized with 200 μL of 2.0 M imidazole–acetate (pH 7.0) followed by the addition of 200 μL of phenol (saturated with wash buffer) and 2.30 mL of wash solution (see labeling methods) with repeated mixing after each addition. Centrifugation produced an aqueous layer containing [^{3}H]-AMP hydrolyzed from the covalently labeled protein and a phenol layer containing the protein plus remaining label. Aliquots of both layers were counted.

Inhibition by Imidazole. Assays of enzymatic activity in the presence of 50–200 mM imidazole–acetate (pH 6.0) were performed at 30 °C with 4-nitrophenyl phenylphosphonate as substrate and 15 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH (pH 6.0) as buffer. Substrate concentration ranged from 2.5 to 20 mM. Discontinuous assays were performed with an incubation time of 60 s; the reaction was shown to be linear for several minutes under these conditions.

Results

Purification of 5′-Nucleotide Phosphodiesterase. The protocol described under Experimental Procedures is a substantial modification of the method of Kelly et al. (1975). Previously, a large fraction of the phosphodiesterase activity eluted from columns of DEAE-cellulose was sacrificed due to overlapping elution with alkaline phosphatase. Development of an affinity resin for alkaline phosphatase allowed complete resolution of these enzymes at the last step. Extraction of the solubilized extract with 1-butanol reduced variability in the behavior of the enzyme in subsequent steps, as well as resulting in considerable purification.

Results of a typical purification of 5′-nucleotide phosphodiesterase from 10 m of small intestine are presented in Table I. Estimations of activity are unreliable for fractions preceding DEAE-cellulose chromatography due to the presence of a low-molecular-weight inhibitor (Ackerman and Butler, unpublished results). Experience with several preparations (data not shown) indicated the described procedure will typically result in 30% recovery of starting activity in the homogeneous product. The best specific activity obtained with this procedure, 71 units mg^{-1}, was a 40% increase above previously reported preparations (Kelly et al., 1975).

TABLE I: Purification of Bovine Intestinal 5′-Nucleotide Phosphodiesterase.

fraction[a]	total act. (μmol min^{-1})[b]	total protein (mg)[c]	sp act. (μmol min^{-1} mg^{-1})	yield (%)
centrif extr	4650			100
butanol extr	3600			77
$(NH_4)_2SO_4$ ppt	3400	10 500	0.3	75
DEAE-cellulose	6000	225	27	129
phenylalanylagarose	4720	94	46	102
Sephacryl S-200	4650	71	65	100
L-histidyldiazobenzyl-phosphonic acid–agarose	3970	56	71	85

[a] The data presented were measured subsequent to the indicated step in purification. [b] Activity was determined at 30 °C in 100 mM Tris-HCl (pH 8.0) by following the liberation of 4-nitrophenol from 4-nitrophenyl phenylphosphonate. [c] Protein was determined from the absorbance at 280 nm. The presence of Triton X-100 in solutions before precipitation with $(NH_4)_2SO_4$ precluded protein determination.

Homogeneity of the purified enzyme was indicated by a high specific activity and the results on a polyacrylamide gels following electrophoresis. A single, sharp band was evident following staining with Coomassie blue G-250.

Solubilities of 5′-Nucleotide Phosphodiesterase and Nucleotide Substrates in Phenol–H_2O Two-Phase Systems. In order to test the applicability of the phenol-extraction technique for the isolation of a phosphodiesterase covalent intermediate, the differential solubilities of enzyme and nucleotide substrates (NAD or cAMP) in phenol–H_2O bilayers were determined. These experiments were performed with the same extraction methods as in later labeling experiments. Aliquots of the aqueous layers from seven washes were counted to determine the counts removed with each wash; finally, the phenol layer was counted.

Despite its bound carbohydrate (Kelly et al., 1975), which might increase its solubility in the aqueous phase, 5′-nucleotide phosphodiesterase exhibited a marked preferential solubility in phenol; 95% of the ^{125}I counts remained in the phenol layer following seven washes with buffer. Disregarding the initial wash, the apparent partition coefficient of the enzyme in these conditions was 2800 (phenol–H_2O). Conversely, nucleotide substrates were almost completely removed by repetitive washes of the phenol layer with buffer; less than 0.6% of NAD and 0.25% of cAMP radioactivity remained. Approximately two-thirds of the remaining nucleotide was removed in each of the early extractions; less efficient extraction in the last washes suggests the presence of phenol-soluble impurities. Zero time control samples (phenol added to enzyme before addition of substrate) were run in each labeling experiment to establish this background level of nonextractable radioactivity, which was subtracted out in calculating levels of isotope incorporation into enzyme. Calculation of the expected counts resulting from labeling experiments with 1.0 mg of enzyme showed that la-

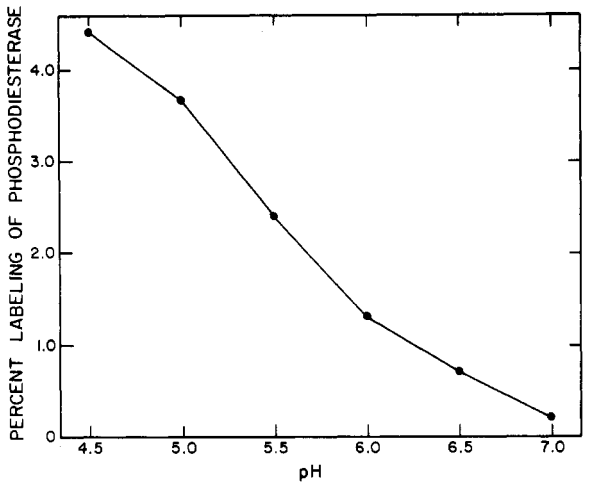


FIGURE 1: Percent labeling of phosphodiesterase as a function of pH. Conditions were 200 mM imidazole-acetate as buffer and 100 μM cAMP as substrate. The points were graphically determined from time courses at the indicated pH (see Figure 2). The percentages were calculated using a molecular weight of 107 000 for phosphodiesterase.

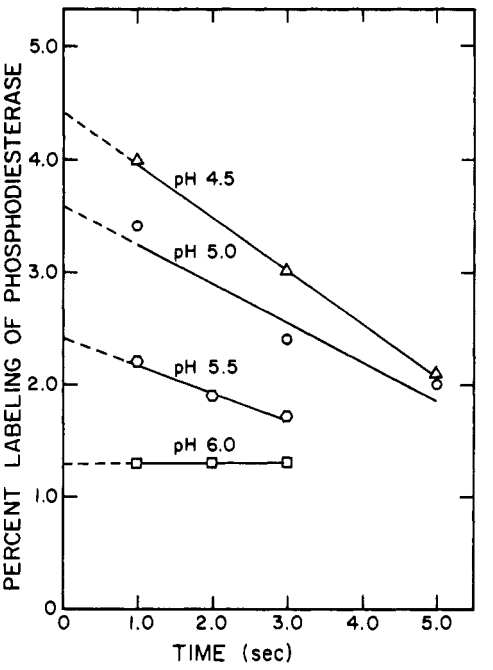


FIGURE 2: Percent of phosphodiesterase labeled vs. incubation time. Labeling experiments were performed with incubations of 1-5 s followed by inactivation and extraction of the enzyme with phenol. These time curves for each pH were extrapolated to zero time in order to obtain comparable values plotted in Figure 1; it should be noted that in reality there is zero labeling at zero time.

beling of 1% of the phosphodiesterase multimers could be readily detected in this system.

Labeling of 5′-Nucleotide Phosphodiesterase. Initially, the search for incorporation of nucleotide substrates into phosphodiesterase was without success despite varying pH, buffer, and incubation time. However, when the labeling experiment was conducted in imidazole hydrochloride buffer at pH 7.0 [the same conditions which had been shown by Kelly and Butler (1977) in stopped-flow experiments to produce a pre-steady-state "burst" of product], a barely detectable but reproducible 0.2% of the phosphodiesterase was labeled. Subsequent experiments in 0.20 M imidazole-acetate showed that incorporation of substrate increased with increasing acidity. The effect of pH on the degree of labeling is shown in Figure 1. A maximal labeling of 4.0% (100 nmol of [^{3}H]cAMP/nmol of phosphodiesterase) was obtained at pH 4.5, the most acidic condition tested, with this enzyme preparation.

Incubation time was also varied from 1 to 5 s in experiments at several pHs. The incorporation of label was very rapid, reaching a maximum within 1 s and then decreasing almost linearly (Figure 2). The rapidity of denaturation by mixing with phenol in the above short incubation experiments was tested by comparing the control radioactivity (phenol injected several seconds before substrate) to an experiment at pH 4.5 where phenol and substrate were injected simultaneously. The values agreed within experimental variation, indicating that denaturation by phenol was very rapid.

As shown in Table II, a competitive inhibitor, 5′-AMP [K_i = 1.2 μM; K_m of cAMP = 100 μM, Kelly et al. (1975)], at a concentration of 100 μM decreased the amount of label incorporated to only 10% of the level observed in the absence of inhibitor. Also, incubation of the enzyme with substrate for 1 h abolished labeling, presumably because the cAMP substrate had been completely hydrolyzed to 5′-AMP. The enzyme retained catalytic activity throughout this period.

The preceding experiments were performed with enzyme having a specific activity of 38 units mg^{-1} (some inactivation had occurred on storage). When experiments at pH 4.5 with 1-s incubation times were performed with enzyme with a specific activity of 63 units mg^{-1}, the percent labeling increased from 4.0 to 7.3%. The improvement in percent labeling very closely paralleled the increase in specific activity.

Inhibition Kinetics of Imidazole. The presence of the inhibitor imidazole (or imidazolium ion) appeared to be necessary in order to obtain measurable labeling near neutral pH. A Lineweaver-Burk plot of phosphodiesterase inhibition by imidazole-acetate at pH 6.0 produced a pattern of lines indicative of noncompetitive inhibition with K_i = 65 mM. K_m for the substrate 4-nitrophenyl phenylphosphonate was 1.6 mM at pH 6.0, eightfold lower than at pH 8.0 (Kelly and Butler, 1977).

TABLE II: Labeling Experiments.

conditions[a]	total dpm	labeling (%)
control (0 s)	3700	0
1 s + AMP[b]	4000	0.2
1 s	6400	1.8
2 s	6100	1.7
3 s	6000	1.6
3600 s	3500	0

[a] Labeling experiments were conducted at pH 6.5 in 200 mM imidazole-acetate at room temperature with 100 μM cAMP as substrate, with the indicated incubation times. [b] The AMP concentration was 100 μM, equal to the substrate concentration. AMP is the product of cAMP hydrolysis.

Gel Filtration of Labeled Phosphodiesterase. The radioactive materials which extracted into phenol in labeling experiments and controls were characterized by fractionation on a Sephacryl S-200 column equilibrated with 0.1% NaDodSO$_4$ in 100 mM Tris-HCl (pH 8.0). Fractions from the column were analyzed for optical density at 280 nm and radioactivity, producing the elution profiles presented in Figure 3. In the labeling experiment, 85% of the eluted radioactivity was associated with the protein peak (eluting at fractions 18-22), whereas no radioactivity was associated with the protein peak from the control experiment. Moreover, the 13% of the eluted radioactivity in the labeling experiment associated

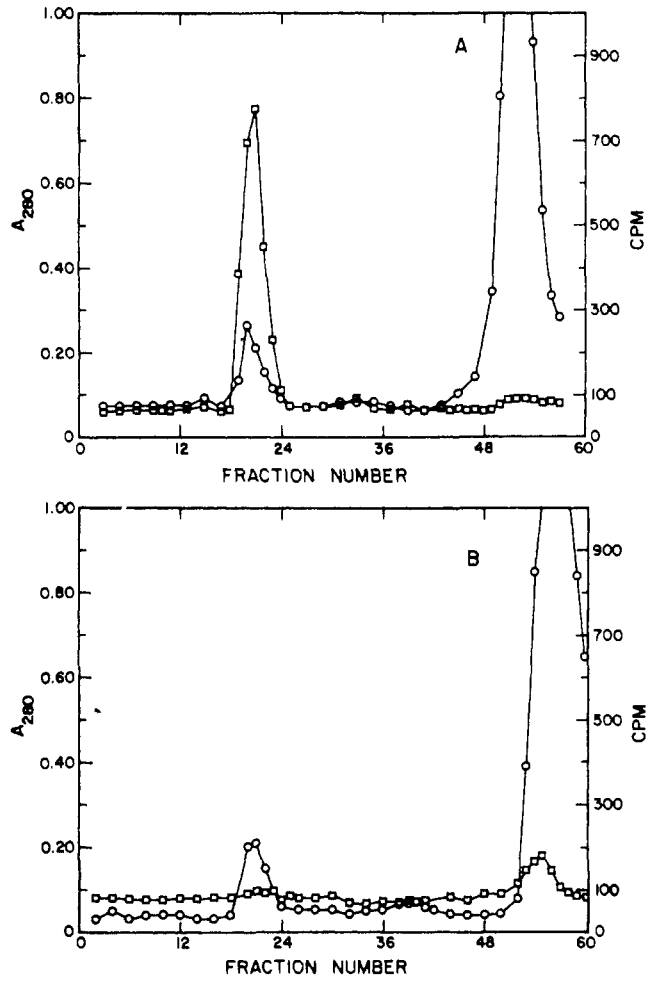


FIGURE 3: Gel-filtration elution profiles of 1-s incubation and control phenol layers from a labeling experiment. Phosphodiesterase, 1.0 mg, was incubated in 100 μM cAMP + 1.0 μCi of [^{3}H]cAMP in 200 mM imidazole-acetate (pH 4.5) at room temperature. After 1 s, phenol was added and the phenol layer washed to remove unbound substrate. A control was treated in precisely the same manner, except that phenol was added *before* substrate. The phenol layers were dissolved in 2% $NaDodSO_4$ and fractionated on a 1.5 × 30 cm column of Sephacryl S-200 equilibrated with 0.1% $NaDodSO_4$ + 0.1% 2-mercaptoethanol in 100 mM Tris-HCl (pH 8.0). Figure 3A is the 1-s incubation profile; Figure 3B is the control profile: radioactivity (□); absorbance (○).

with low-molecular-weight material (eluting at fractions 53–60; largely phenol) was expected because the zero-time control counts were 14% of the labeling experiment counts. Eighty-two percent of the total applied counts were recovered.

The Stability of the Enzyme–Intermediate Bond. To test the stability of the enzyme–intermediate bond, labeled phosphodiesterase, after precipitation from phenol, was dissolved in 100 mM solutions of HCl, NaOH, or imidazole-acetate (pH 7.0) and incubated for periods of up to 10.5 h at 55 °C. After reextraction of the protein with phenol and addition of wash buffer to remove unbound label, the counts present in the phenol layer were due to the remaining label bound to the protein. The results indicate that the bond is stable at acid and neutral pH with over 95% of the radioactivity retained by the protein but labile at pH 13 with over 50% loss of label in 4.9 h at this pH.

If, as anticipated, the isolated intermediate is attached to labeled enzyme by a phosphodiester linkage, it should be a substrate for phosphodiesterase. Labeled phosphodiesterase, digested with proteinase K to make the label more accessible, was exposed to active enzyme at 30 °C in 100 mM Tris (pH 8). Nearly total loss of label was observed at 1 h, whereas the bond was completely stable under these conditions in the control (proteinase K, 100 μg, added but phosphodiesterase omitted). Native phosphodiesterase appeared completely resistant to proteinase K attack based on separate activity measurements at these conditions.

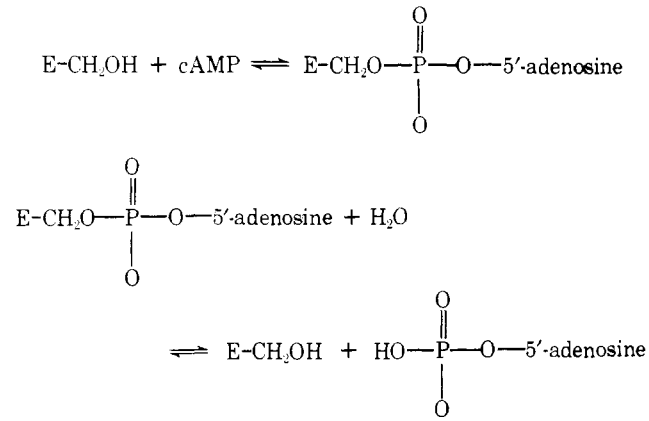
Discussion

A mechanism involving a covalent intermediate was recently proposed for 5′-nucleotide phosphodiesterase by Kelly and Butler (1977) on the basis of the kinetics of systematically varied substrates and pre-steady-state kinetics using bis(4-nitrophenyl) phosphate as substrate. Analogous mechanisms have been proposed or established for several phosphohydrolases (Razzell and Khorana, 1961; Reid and Wilson, 1971; Hickey and Van Etten, 1972; Fernley and Walker, 1970).

Phenol and phenol-H_2O systems have been successfully used to isolate covalent intermediates of other enzymes (Butler, 1964; Boyer and Bieber, 1967; Feldman and Butler, 1972; Lai et al., 1974) by exploiting the powerful denaturing properties and solubility of protein in phenol. However, not all proteins are preferentially soluble in phenol (LeStourgeon and Rusch, 1973). Experiments with [^{125}I]phosphodiesterase showed that phenol nearly quantitatively extracted and retained the enzyme through extensive washing which removed unbound substrate. The efficient separation of enzyme and substrate with this system was crucial to demonstration of a covalent intermediate.

Scheme I represents a simple reaction sequence for the hydrolysis of cAMP by bovine intestinal 5′-nucleotide phosphodiesterase which is in accord with the present observations.

SCHEME I



Incubation of phosphodiesterase with cAMP in imidazole-acetate buffer resulted in incorporation into the enzyme of radioactivity which could not be removed by repeated washing and migrated with the enzyme under unfolding conditions on gel-filtration columns. Control samples of identical composition but opposite order of addition, so that the enzyme was inactivated before exposure to labeled substrate, contained no bound radioactivity.

These experiments suggest that the radioactive species bound to the enzyme is not merely mechanically trapped or tightly associated with the enzyme but instead is covalently bonded to the protein.

Acid pH and the presence of imidazole increased the extent of labeling measured after inactivation of the enzyme with phenol. Shorter incubation times also produced greater incorporation of substrate. Imidazole (or imidazolium ion) was shown to be a noncompetitive inhibitor of phosphodiesterase

with a K_i = 65 mM at pH 6.0. Increased labeling with these conditions might be due to a higher prevailing substrate concentration at the point of addition of phenol, since (1) the initial amount of substrate would last for only a few seconds at the maximal catalytic rate [the labeling mixture contains a 12-fold excess of cAMP over phosphodiesterase; turnover number for cAMP is 13 s^{-1} at pH 8.0, Kelly et al. (1975)] but (2) acid pH, imidazole, and short incubation inhibit the degradation of substrate to product. However, comparison of time courses of reactions at several pH values indicates that the steepest decreases in labeling with time occurred at the most acidic pH values where the overall reaction is slowest. At pH 6.0 where the overall reaction is comparatively rapid (Kelly et al., 1975; Kelly, 1974) and substrate would be exhausted rapidly, the extent of labeling decreased relatively little. The observed decrease in labeling with time at acid pH is more consistent with decay of a transiently elevated level of intermediate as the steady state is approached; the increase in labeling on lowering the pH would thus be due to a slower approach to the steady state at lower pH. This explanation is analogous to a kinetic scheme developed by Mardh and Lindfahl (1977) for a system in which a step subsequent to hydrolysis of a covalent intermediate in a multistep catalytic scheme is rate limiting. Kelly and Butler (1977) have previously proposed dissociation of nucleotide as the rate-limiting step for this enzyme using nucleotide substrates.

The extent of labeling of phosphodiesterase was quite low in comparison to the labeling achieved with alkaline phosphatase, a similar phosphohydrolase (Engstrom 1961). However, high labeling of alkaline phosphatase occurred under conditions when the rate-limiting step of catalysis was discharge of the intermediate, which previous experiments have indicated is not the case for phosphodiesterase with nucleotide substrates (see above).

The parallel increase in labeling of phosphodiesterase with specific activity is consistent with the assertion that the observed covalent label is a catalytically competent intermediate. This claim is strengthened by the observed repression of labeling due to (1) inclusion of a competitive inhibitor in the labeling mixture or (2) use of a long incubation time to allow the enzyme to exhaust the substrate.

When gel-filtration experiments were performed to identify the radioactive species present in phenol layers from labeling experiments, all of the radioactivity in excess of the control radioactivity emerged with the phosphodiesterase peak. These experiments demonstrated that the apparent labeling was not due to reaction products or artifacts. Since the protein was dissolved and chromatographed in strongly unfolding conditions, coelution of the protein and radioactive label indicated a strong, probably covalent enzyme–intermediate bond.

Exposure of labeled enzyme to a range of pH conditions at 55 °C showed the covalent intermediate–enzyme bond to be stable at acid or neutral pH but to decompose in alkaline solution. Phosphoramidates (e.g., phosphohistidine) and *O*-alkyl phosphoesters (e.g., phosphoserine) have opposite stabilities with varying pH; phosphoramidates are unstable in acid, whereas phospho-*O*-alkyl esters are unstable at basic pH (Wong and Rose, 1976). An acyl phosphate, formed by (Na^+, K^+)ATPase, is unstable in acid conditions (Bader et al., 1966). The stability of the phosphodiesterase covalent label at acid pH rules out a phosphoramidate or acyl phosphate linkage and suggests serine or threonine as the residue bearing the phosphoryl intermediate.

In experiments where active enzyme was added to denatured labeled enzyme, the ability of phosphodiesterase to hydrolyze the intermediate provides evidence for the expected phosphodiester linkage to the enzyme through the hydroxyl of a serine or threonine.

The evidence presented here continues a considerable catalog of similarities between bovine intestinal 5′-nucleotide phosphodiesterase and alkaline phosphatase. The formation of a covalent intermediate and the action of acid pH to promote higher levels of intermediate are important characteristics of both enzymes. Investigations now under way are aimed at identification of the residue bonded to the intermediate and the sequence of the active-site peptide.

References

Bader, H., Sen, A. K., and Post, R. L. (1966), *Biochim. Biophys. Acta 118,* 106.

Boyer, P. D., and Bieber, L. L. (1967), *Methods Enzymol. 10,* 768.

Butler, L. G. (1964), Ph.D. Dissertation, University of California, Los Angeles, Calif.

Dardinger, D. E. (1974), M.S. Thesis, Purdue University, West Lafayette, Ind.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Doellgast, G. J., and Fishman, W. H. (1974), *Biochem. J. 141,* 103.

Engstrom, L. (1961), *Biochim. Biophys. Acta 54,* 179.

Feldman, F., and Butler, L. G. (1972), *Biochim. Biophys. Acta 268,* 698.

Fernley, H. N., and Walker, P. G. (1970) *Biochem. J. 116,* 543.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963), *Biochem. J. 89,* 114.

Hickey, M. E., and VanEtten, R. L. (1972), *Arch. Biochem. Biophys. 152,* 423.

Kelly, S. J. (1974), Ph.D. Thesis, Purdue University.

Kelly, S. J., and Butler, L. G. (1975), *Biochem. Biophys. Res. Commun. 66,* 316.

Kelly, S. J., and Butler, L. G. (1977), *Biochemistry 16,* 1102.

Kelly, S. J., Dardinger, D. E., and Butler, L. G. (1975), *Biochemistry 14,* 4983.

Lai, H.-Y. L., Butler, L. G., and Axelrod, B. (1974), *Biochem. Biophys. Res. Commun. 60,* 635.

Landt, M., Boltz, S. C., and Butler, L. G. (1978), *Biochemistry 17,* 915.

LeStourgeon, W. M., and Rusch, H. P. (1973), *Arch. Biochem. Biophys. 155,* 144.

Mardh, S., and Lindahl, S. (1977), *J. Biol. Chem. 252,* 8058.

Razzell, W. E., and Khorana, H. G. (1961), *J. Biol. Chem. 236,* 1144.

Reid, T. W., and Wilson, I. B. (1971), *Enzymes, 3rd Ed. 4,* 373.

Wong, L.-J., and Rose, I. A. (1976), *J. Biol Chem. 251,* 5431.