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# ADENOSINE 3',5'-CYCLIC PHOSPHATE PHOSPHODIESTERASE FROM BOVINE THYROID: ISOLATION AND PROPERTIES OF A PARTIALLY PURIFIED, SOLUBLE FRACTION

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SUMMARY

1. Adenosine 3',5'-cyclic phosphate (cyclic AMP) phosphodiesterase was partially purified from a 10 000 × *g* supernate of bovine thyroid; the enzyme preparation so obtained was free of 5'-nucleotidase and deaminase activity and yielded [$^3$H]-adenosine 5'-phosphate (AMP) as the end product of enzymatic hydrolysis of cyclic [$^3$H]AMP.

2. Using an assay procedure that relied on physiologic substrate concentrations (*i.e.* conforming to known intracellular levels of cyclic AMP in thyroid), the partially purified thyroid phosphodiesterase was kinetically characterized and compared with a highly purified beef heart phosphodiesterase preparation. Double reciprocal plots of cyclic AMP hydrolysis yielded two apparent Michaelis constants: the lower in the $10^{-6}$ M and the higher in the $10^{-5}$ M range. Two apparent $K_m$ values were also obtained for $Mg^{2+}$ which was required for expression of maximal enzymatic activity. Other bivalent cations ($Mn^{2+}$, $Co^{2+}$, $Ca^{2+}$) activated the enzyme to a significantly lesser extent; $Zn^{2+}$ was stimulatory at $10^{-4}$ M and inhibitory at $\geq 10^{-3}$ M.

3. A number of cyclic nucleotides and acylated derivatives thereof interfered with the enzymatic hydrolysis of cyclic [$^3$H]AMP to varying degrees; of these the mono- and dibutyryl derivatives of cyclic AMP were the most potent inhibitors. The inhibition was competitive in nature with $K_i$ values for dibutyryl cyclic AMP, $N^6$-monobutyryl cyclic AMP, and $O^{2'}$-monobutyryl cyclic AMP of $1.6 \cdot 10^{-4}$ M, $1.1 \cdot 10^{-4}$ M and $2.6 \cdot 10^{-5}$ M, respectively.

4. Methylxanthines and quazodine strongly inhibited thyroid phosphodiesterase whereas sulfonylureas were weakly inhibitory.

5. High concentrations of pituitary thyrotropin *in vitro* enhanced thyroid phosphodiesterase activity slightly while heat-inactivated thyrotropin, long-acting thyroid stimulator immunoglobulin G, and the prostaglandins $E_2$ and $F_{1\alpha}$ were ineffective; prostaglandin $E_1$ was weakly inhibitory. The prostaglandin antagonists 7-oxa-13-prostynoic acid and polyphloretin phosphate effected significant inhibition of thyroid phosphodiesterase, possibly accounting for the agonist properties of these agents occasionally observed *in vitro*.

6. Upon complexing with a cyclic AMP-binding protein prepared from beef kidney, cyclic [$^3$H]AMP became completely resistant to enzymatic breakdown by thyroidal phosphodiesterase.

## INTRODUCTION

Adenosine 3′,5′-cyclic phosphate (cyclic AMP) has been identified as an important intracellular mediator of the action of thyroid stimulators[1]. Activation of thyroidal adenylate cyclase by pituitary thyroid stimulating hormone[2,3], the long-acting thyroid stimulator of Graves' disease[4,5] and the prostaglandins[6,7] has been conclusively demonstrated. The regulation of intracellular levels of cyclic AMP in the thyroid may, however, also be achieved by changes in the activity of 3′,5′-cyclic nucleotide phosphodiesterase[8]. Previous studies of thyroidal phosphodiesterase activity have failed to demonstrate a direct *in vitro* effect of thyrotropin[2,3,9], prostaglandins[6] or long-acting thyroid stimulator[9], whereas prolonged thyroid stimulation by thyrotropin *in vivo* appeared to enhance the enzymatic hydrolysis of cyclic AMP[10]. However, each of these studies relied on the use of crude homogenates or extracts of thyroid which, in addition to phosphodiesterase, contain other hydrolytic enzymes of nucleotide metabolism (*e.g.* 5′-nucleotidase, deaminase) as well as cyclic AMP-binding protein(s). Since these enzymes and proteins may have materially influenced the results obtained, one must be cautious in assuming that such studies yielded unequivocal assessment of direct effects on thyroid phosphodiesterase activity. It seemed desirable, therefore, to purify thyroidal phosphodiesterase to a point where it was free of interfering enzyme activity and to utilize such a preparation for the reevaluation of direct effects of agents known to influence thyroid function and thyroidal cyclic AMP levels *in vitro*. Further, we undertook the kinetic characterization of the partially purified enzyme and compared some of its properties with those of a commercial beef heart PDE preparation.

## MATERIALS AND METHODS

[8-$^3$H]Adenosine 3′,5′-monophosphate (lot No. WR-2111, spec. act. 14.2 Ci/mmole) and unlabeled cyclic AMP (free acid) were obtained from Schwarz/Mann. The labeled nucleotide was 97% pure (as determined by thin-layer chromatography[11]) and was used as supplied by the manufacturer without further purification. Other cyclic nucleotides were purchased from Boehringer Mannheim, Sigma and P–L Biochemicals. Beef heart phosphodiesterase, prepared according to the procedure of Butcher and Sutherland[12], was a product of Boehringer Mannheim (No. 6219208). Tubercidin 3′,5′-cyclic phosphate, tolbutamide (Orinase), chlorpropamide and quazodine (6,7-dimethyoxy-4-ethylquinazoline) were generous gifts of Upjohn, Pfizer, and Mead Johnson, respectively. The prostaglandins $E_1$, $E_2$ and $F_{1\alpha}$ used in this study were kindly provided by Dr John Pike of Upjohn, 7-oxa-13-prostynoic acid by Dr Josef Fried of The University of Chicago, and polyphloretin phosphate and related compounds by Dr B. Högberg, Aktiebolaget Leo Research Laboratories, Hälsingborg, Sweden.

*Determination of phosphodiesterase activity*

The assay procedure was based on the $BaSO_4$–$Zn(OH)_2$ coprecipitation of [$^3$H]-AMP, the end product of enzymatic breakdown, and subsequent liquid scintillation counting of unhydrolyzed cyclic [$^3$H]AMP substrate remaining in the supernate[13]. Into each assay tube was added 50 $\mu$l of substrate Solution A containing 40 mM Tris–HCl, pH 8.0, 1 mM sodium 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 0.12 mM AMP, $3 \cdot 10^{-7}$ M cyclic [$^3$H]AMP and, unless otherwise indicated, 6 mM $MgCl_2$ followed by 50 $\mu$l of Solution B containing, in 40 mM Tris–HCl, pH 8.0, three times the desired final concentration of unlabeled substrate, inhibitor or other test material. After mixing on a Vortex the tubes were placed in a water bath at 30 °C. The reaction was initiated by the addition of 50 $\mu$l of Solution C containing enzyme diluted in 40 mM Tris–HCl, pH 8.0, to a concentration sufficient to hydrolyze 40–50% of the cyclic [$^3$H]AMP present initially (*i.e.* 6.0–7.5 pmoles) during a 10-min incubation at 30 °C. The reaction was terminated by the addition of 0.2 ml of 5% $ZnSO_4$ followed by 0.2 ml of 0.15 M $Ba(OH)_2$ and 50 $\mu$l of water*. After thorough mixing on a Vortex the tubes were allowed to stand at room temperature for 10 min then centrifuged at 1200 $\times$ *g* for 20 min. Precipitation with 0.2 ml each of the $ZnSO_4$ and $Ba(OH)_2$ solutions was repeated, care being taken not to disturb the original precipitate. 0.5 ml of the final supernate was counted in 10 ml of Bray's dioxane-based counting solution[14] in a Packard Tri-Carb liquid scintillation counter. The rate of hydrolysis was estimated by subtraction of the residual cyclic [$^3$H]AMP radioactivity from the total, *i.e.* initial, cyclic [$^3$H]AMP radioactivity. In the early phase of this work the rate of hydrolysis so obtained was corroborated and the distribution of end products determined by thin layer chromatography of acid extracts of incubation mixtures on PEI cellulose plates in 0.3 M LiCl[11].

*Preparation of the enzyme*

Fresh bovine thyroid glands were obtained from a local abattoir and kept at 4–5 °C through all subsequent steps. They were freed of connective tissue and fat, minced, then extracted and processed as described for skeletal muscle phosphodiesterase by Huang and Kemp[15], up to the first DEAE-Sephadex A 50 chromatography step. Three successive, pooled fractions eluted from the column were dialyzed and contained 31.2, 64.3 and 37.9 mg/ml protein, respectively; the specific activity (pmoles cyclic [$^3$H]AMP hydrolyzed/10 min per mg protein) of each fraction was essentially the same. The first of these fractions was used throughout this work; at a dilution of 1:10 it hydrolyzed approximately 40–50% of the cyclic [$^3$H]AMP substrate under the conditions of the assay described above. While the crude $(NH_4)_2SO_4$ fraction used as the starting material for DEAE-Sephadex chromatography yielded predominantly inosine and hypoxanthine (80.0% and 13.6%, respectively) as hydrolytic end products, only negligible amounts ($< 5\%$) of end products other than AMP accumulated during incubation with the column-treated fraction, indicating removal of 5'-nucleotidase and deaminase activity. Aliquots of the dialyzed enzyme preparation were stored at −80 °C; they retained full activity for several months.

Immunoglobulin G from normal and long-acting thyroid stimulator-positive

* It was demonstrated in the initial phase of this work that the amount of $ZnSO_4$ used for coprecipitation of AMP completely arrested the phosphodiesterase reaction.

sera was prepared by the method of Baumstark *et al.*[16]. Biological activity was determined by the mouse bioassay of McKenzie[17]. A crude cyclic AMP-binding protein from beef kidney was isolated as described by Cheung[18]. Protein determinations were carried out by the method of Lowry *et al.*[19].

Kinetic plots of the data were obtained and $K_i$ values for competitive inhibition were calculated according to Dixon and Webb[20].

*t*-tests for statistical significance of differences between experimental observations were calculated according to Dixon and Massey[21].

## RESULTS

Under the conditions of the assay procedure used and up to 40–50% cyclic [$^3$H]-AMP hydrolysis, the rate of the reaction was proportional to time and enzyme concentration. The pH optimum of the enzyme was between 8.0 and 8.4 (Fig. 1).

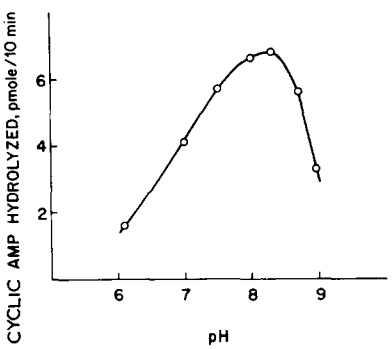


Fig. 1. Effect of pH on cyclic [$^3$H]AMP hydrolysis by partially purified bovine thyroid phosphodiesterase. Assay carried out as described under Materials and Methods except that 50 mM Tris–maleate buffer was substituted for 40 mM Tris–HCl. Initial cyclic [$^3$H]AMP concentration was $10^{-7}$ M.

When the rate of hydrolysis was determined at substrate concentrations ranging from $10^{-7}$ to $6.7 \cdot 10^{-5}$ M, kinetic plots of the data yielded two distinct slopes and $1/v$ intercepts, hence two $K_m$ and $V$ values (Fig. 2). The apparent $K_m$ value obtained at $[S] = 2.3 \cdot 10^{-7}$ M to $4.4 \cdot 10^{-6}$ M was $4 \cdot 10^{-6}$ M; at $[S]$ ranging from $6.8 \cdot 10^{-6}$ to $6.7 \cdot 10^{-5}$ M the apparent $K_m$ was one order of magnitude greater, *i.e.* $4 \cdot 10^{-5}$ M. The two $K_m$ values obtained for the heart enzyme under identical experimental conditions were in the order of $10^{-6}$ M and $10^{-4}$ M (plots not shown).

$Mg^{2+}$ or other bivalent cation was required for activation of the enzyme. Only a minute quantity of cyclic [$^3$H]AMP was hydrolyzed in a $Mg^{2+}$-free medium; hydrolysis increased sharply with increasing concentrations of the cation; a maximal rate was achieved at approximately 4 mM $Mg^{2+}$ (Fig. 3). Double reciprocal plots of $1/v$ *vs* $1/[Mg^{2+}]$, again, yielded two straight lines with two distinct $K_m$ and $V$ values. Other bivalent cations affected enzymatic activity in a more complex manner; $Mn^{2+}$, $Ca^{2+}$ and $Co^{2+}$ were ineffective or inhibitory at 0.1 mM and stimulatory, although to a lesser extent than $Mg^{2+}$, at 1 mM. $Zn^{2+}$, on the other hand, activated the enzyme at the

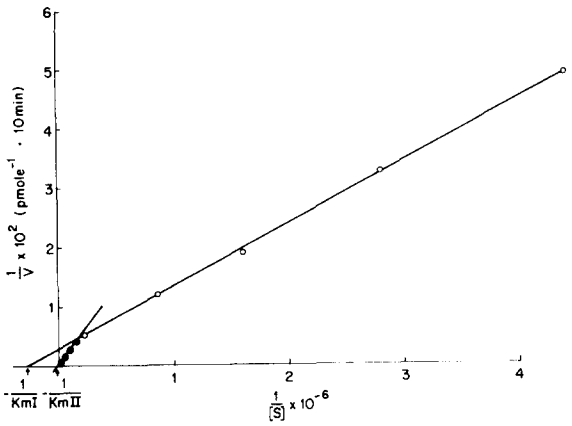


Fig. 2. Double-reciprocal plot of cyclic [$^3$H]AMP hydrolysis by bovine thyroid phosphodiesterase. Assay carried out as under Materials and Methods. Cyclic [$^3$H]AMP concentration was $10^{-7}$ M throughout; [$S$] was varied by the addition of progressively increasing quantities of unlabeled cyclic AMP. Values for $K_m$ I at [$S$] = $2.3 \cdot 10^{-7}$ to $4.4 \cdot 10^{-6}$ M and $K_m$ II at [$S$] = $6.8 \cdot 10^{-6}$ to $6.7 \cdot 10^{-5}$ M were calculated from the $-1/K_m$ intercepts on the abscissa.

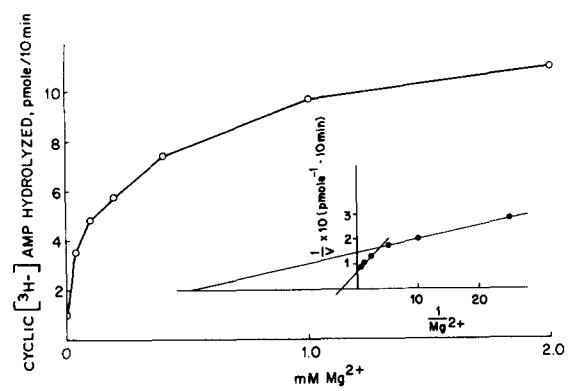


Fig. 3. Kinetic plots of $Mg^{2+}$ activation of cyclic [$^3$H]AMP hydrolysis by bovine thyroid phosphodiesterase. Assay was carried out as described under Materials and Methods except that Solution A contained no $Mg^{2+}$; instead, $MgCl_2$ was added to Solution B at three times the desired final concentration. Initial cyclic [$^3$H]AMP concentration was $10^{-7}$ M.

lower and inhibited it at the higher concentration tested (Table I). The monovalent cations $Na^+$, $K^+$, $Li^+$ and $NH_4^+$ were completely ineffective.

Acylated derivatives of cyclic AMP as well as several other cyclic nucleotides brought about varying degrees of inhibition of cyclic [$^3$H]AMP hydrolysis, although none of the nucleotides tested was as effective in this regard as unlabeled cyclic AMP itself (Table II). Only the pyrimidine-based uridine 3′,5′-monophosphate (cyclic UMP) was completely ineffective.

The kinetics of the inhibition displayed by the butyryl derivatives of cyclic AMP were further explored. Kinetic plots in the presence of $10^{-4}$ M monobutyryl or dibutyryl cyclic AMP established that all three derivatives gave a competitive-type inhibition in both substrate concentration ranges studied (Fig. 4). $K_i$ values for di-

TABLE I

EFFECT OF BIVALENT CATIONS ON CYCLIC [$^3$H]AMP HYDROLYSIS BY PARTIALLY PURIFIED BOVINE THYROID PHOSPHODIESTERASE

Assay conditions were as described under Materials and Methods, except that $MgCl_2$ and EGTA were omitted from Solution A and three times the desired final concentration of bivalent cation was added to Solution B. Initial substrate concentration was $10^{-7}$ M.

| *Addition* | *pmoles cyclic [$^3$H]AMP hydrolyzed/10 min** | | |
|---|---|---|---|
| | *0* | *0.1 mM* | *1.0 mM* |
| None | 1.17 ± 0.16 | — | — |
| $MgCl_2$ | — | 2.92 ± 0.42 | 4.59 ± 0.13 |
| $MnCl_2$ | — | 1.02 ± 0.16 | 3.67 ± 0.15 |
| $CaCl_2$ | — | 0.72 ± 0.24 | 1.78 ± 0.23 |
| $CoCl_2$ | — | 0.72 ± 0.04 | 3.27 ± 0.24 |
| $ZnSO_4$ | — | 1.88 ± 0.24 | 0.45 ± 0.26 |

* Results are the mean ±S.D. of triplicate determinations.

TABLE II

EFFECT OF BUTYRYL DERIVATIVES OF CYCLIC AMP AND OF OTHER CYCLIC NUCLEOTIDES ON CYCLIC [$^3$H]AMP HYDROLYSIS BY BOVINE THYROID PHOSPHODIESTERASE

| *Additions* | *Concn. (M)* | *Phosphodiesterase activity (% cyclic [$^3$H]AMP hydrolyzed/10 min*)* | |
|---|---|---|---|
| | | *Thyroid* | *Heart* |
| Cyclic AMP | $10^{-6}$ | 70 | — |
| | $10^{-5}$ | 23 | — |
| | $10^{-4}$ | 13 | — |
| 2′,3′-Cyclic AMP | $10^{-4}$ | 88 | — |
| | $10^{-3}$ | 54 | — |
| $O^{2'}$-Monobutyryl cyclic AMP | $10^{-4}$ | 27 | 42 |
| | $10^{-3}$ | 16 | 8 |
| $N^6$-Monobutyryl cyclic AMP | $10^{-4}$ | 57 | 68 |
| | $10^{-3}$ | 20 | 43 |
| $N^6$,$O^{2'}$-Dibutyryl cyclic AMP | $10^{-4}$ | 75 | 72 |
| | $10^{-3}$ | 33 | 45 |
| Cyclic GMP | $10^{-4}$ | 89 | — |
| | $10^{-3}$ | 21 | — |
| $N^2$,$O^{2'}$-Dibutyryl cyclic GMP | $10^{-4}$ | 80 | — |
| | $10^{-3}$ | 48 | — |
| Cyclic IMP | $10^{-4}$ | 86 | — |
| | $10^{-3}$ | 79 | — |
| $O^{2'}$-Monobutyryl cyclic IMP | $10^{-4}$ | 85 | — |
| | $10^{-3}$ | 72 | — |
| Cyclic UMP | $10^{-4}$ | 100 | — |
| | $10^{-3}$ | 88 | — |
| 7-Deaza-3′,5′-AMP** | $10^{-4}$ | 71 | — |
| | $10^{-3}$ | 31 | — |

* Initial cyclic [$^3$H]AMP concentration = $10^{-7}$ M; 6.7 pmoles of cyclic [$^3$H]AMP hydrolyzed/10 min in the absence of unlabeled nucleotide taken as 100. Results are the mean of triplicate determinations.

** Tubercidin 3′,5′-cyclic phosphate.

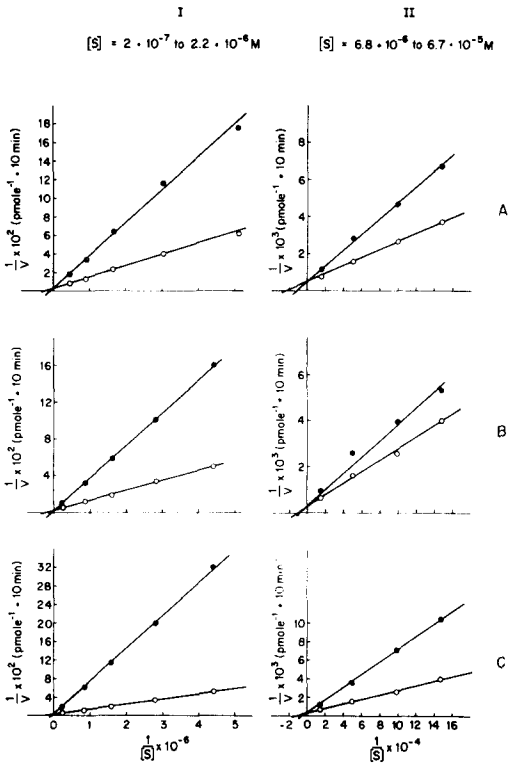


Fig. 4. Kinetic representation of the inhibition of bovine thyroid phosphodiesterase by the butyryl derivatives of cyclic AMP. Assay carried out as for Fig. 2. Inhibitors: (A) $N^6$,$O^{2'}$-dibutyryl cyclic AMP; (B) $N^6$-monobutyryl cyclic AMP; (C) $O^{2'}$-monobutyryl cyclic AMP. ○—○, no inhibitor; ●—●, inhibitor added to a final concentration of $10^{-4}$ M.

butyryl cyclic AMP, $N^6$-monobutyryl cyclic AMP and $O^{2'}$-monobutyryl cyclic AMP were calculated as $1.6 \cdot 10^{-4}$ M, $1.1 \cdot 10^{-4}$ M and $2.6 \cdot 10^{-5}$ M, respectively.

Thyroid phosphodiesterase was strongly inhibited by the methylxanthines, theophylline and caffeine (Table III). Aminophylline, a more soluble complex of 2 moles of theophylline and 1 mole of EDTA, was more inhibitory than theophylline proper; at a concentration of $10^{-2}$ M, that commonly used in *in vitro* studies of intracellular cyclic AMP levels[22], it completely inhibited the enzymatic hydrolysis of cyclic AMP by phosphodiesterase. Quazodine, a compound structurally similar to theophylline, and recently reported to be a more potent inhibitor of phosphodiesterase from a variety of tissues than the latter[23], elicited a greater degree of inhibition in our system as well. Only a modest degree of inhibition was observed upon addition of the sulfonylureas, chlorpropamide and tolbutamide; these agents were, however, equally ineffective in inhibiting bovine heart phosphodiesterase.

Addition of high concentrations of pituitary thyrotropin brought about a slight but significant increase of thyroidal phosphodiesterase activity; biologically inactive thyrotropin, on the other hand, was ineffective (Table IV). The heart enzyme was similarly affected by thyrotropin. Immunoglobulin G from either normal or long-acting thyroid stimulator-positive serum was without effect as were the prostaglandins

TABLE III

EFFECT OF PHOSPHODIESTERASE INHIBITORS ON THYROID AND HEART PHOSPHODIESTERASE ACTIVITY

Assay of enzyme activity as described under Materials and Methods.

| *Additions* | *Concn (M)* | *Enzyme activity (% cyclic [$^3$H]AMP hydrolyzed/10 min*)* | |
|---|---|---|---|
| | | *Thyroid* | *Heart* |
| Caffeine | $10^{-4}$ | 81 | 74 |
| | $10^{-3}$ | 50 | 29 |
| Theophylline | $10^{-4}$ | 73 | 51 |
| | $10^{-3}$ | 32 | 26 |
| Aminophylline | $10^{-5}$ | 90 | — |
| | $10^{-4}$ | 60 | — |
| | $10^{-3}$ | 12 | — |
| | $10^{-2}$ | 0 | — |
| Quazodine | $10^{-4}$ | 41 | 5 |
| | $10^{-3}$ | 11 | 0 |
| Chlorpropamide | $10^{-4}$ | 91 | — |
| | $10^{-3}$ | 87 | — |
| | $5 \cdot 10^{-3}$** | 73 | — |
| Tolbutamide | $10^{-4}$ | 92 | — |
| | $10^{-3}$ | 85 | — |
| | $5 \cdot 10^{-3}$** | 72 | — |

* Initial cyclic [$^3$H]AMP concentration = $10^{-7}$ M; 7.2 and 6.0 pmoles of cyclic [$^3$H]AMP hydrolyzed/10 min in the absence of inhibitor taken as 100. Results are mean of triplicate determinations.

** $5 \cdot 10^{-3}$ M solution was saturated under these assay conditions.

$E_2$ and $F_{1\alpha}$. A slight but significant inhibition was obtained in the presence of prostaglandin $E_1$ and the structurally related prostaglandin antagonist, 7-oxa-13-prostynoic acid[24]. Polyphloretin phosphate, another antagonist of prostaglandins in some systems[25], was also inhibitory; the inhibition appeared to be especially pronounced when a low molecular weight fraction[25] of this compound was employed.

Preliminary experiments with a cyclic AMP-binding protein from beef kidney[18] showed that following preincubation of cyclic [$^3$H]AMP with binding protein at 0 °C for 60 min the remaining and, presumably, protein-bound substrate was completely resistant to hydrolysis by subsequently added thyroidal phosphodiesterase (Fig. 5). One unexpected result of this experiment was the demonstration that the rate of the phosphodiesterase reaction at 0 °C was equal to that measured at 30 °C. The recent report of Cheung[18] suggests a similar lack of temperature dependence for a bacterial phosphodiesterase preparation.

## DISCUSSION

Thyroidal phosphodiesterase has heretofore not been characterized in detail. As was indicated earlier, previous reports dealing with the measurement of thyroid phosphodiesterase activity must be considered somewhat inconclusive because of the relatively crude thyroid fractions employed.

In contrast, the present study has relied on the use of a purified thyroid enzyme

TABLE IV

EFFECTS OF THYROID STIMULATORS AND ANTAGONISTS THEREOF ON CYCLIC [$^3$H]AMP HYDROLYSIS

| *Additions* | *Concn* | *Enzyme activity (% cyclic[$^3$H]AMP hydrolyzed/10 min*)* | |
|---|---|---|---|
| | | *Thyroid* | *Heart* |
| Thyrotropin** | 500 munits/ml | 110.1 ± 0.7*** | 110.0 ± 4.9† |
| | 100 munits/ml | 106.4 ± 0.6† | 109.7 ± 4.2† |
| | 50 munits/ml | 105.7 ± 2.0 | 104.8 ± 2.5 |
| | 10 munits/ml | 105.3 ± 2.6 | |
| | 500 munits/ml (heat inactivated††) | 97.8 ± 0.65 | 108.1 ± 6.7 |
| Normal immunoglobulin G | 5 mg/ml | 97.8 ± 5.2 | — |
| | 1 mg/ml | 101.2 ± 3.6 | — |
| | 0.2 mg/ml | 100.1 ± 2.2 | — |
| Long-acting thyroid stimulator immunoglobulin G††† | 5 mg/ml | 102.6 ± 0.8 | — |
| | 1 mg/ml | 101.7 ± 3.7 | — |
| | 0.2 mg/ml | 100.6 ± 4.6 | — |
| Prostaglandin $E_1$ | $10^{-4}$ M | 85.3 ± 4.2† | 83.6 ± 4.0† |
| Prostaglandin $E_2$ | $10^{-4}$ M | 91.0 ± 4.8 | 87.8 ± 3.9† |
| Prostaglandin $F_{1\alpha}$ | $10^{-4}$ M | 93.3 ± 4.4 | 75.1 ± 2.9*** |
| 7-Oxa-13-prostynoic acid | $10^{-4}$ M | 78.8 ± 4.4*** | — |
| Polyphloretin phosphate | 100 μg/ml | 72.4 ± 1.0*** | — |
| | 25 μg/ml | 83.9 ± 0.7*** | — |
| Leo 1281 (low mol. wt fraction of polyphloretin phosphate) | 100 μg/ml | 17.7 ± 8.7*** | — |
| | 25 μg/ml | 34.8 ± 2.6*** | — |
| | 10 μg/ml | 65.1 ± 5.6*** | — |

* Initial cyclic [$^3$H]AMP concentration = $10^{-7}$ M; 7.4 and 6.1 pmoles of cyclic [$^3$H]AMP hydrolyzed/10 min in the absence of test agent taken as 100. Results are the mean ± S.D. of triplicate determinations.

** Thytropar (Armour Pharmaceutical Co.).

*** Denotes $P < 0.01$ (*cf.* no additions).

† Denotes $P < 0.05$ (*cf.* no additions).

†† Incubated at 70 °C for 1 h.

††† Bioassay response = 1173%/2.5 mg per mouse.

preparation, free of contamination by other hydrolytic enzymes of nucleotide metabolism; hence, one that lends itself to detailed kinetic characterization. Further, the present communication is based on an assay procedure which measures cyclic AMP degradation at substrate concentrations ($10^{-7}$ M) comparable to those found in the isolated thyroid cell under basal conditions[11,22]. Thus, this assay procedure has enabled us to observe a second Michaelis constant in the $10^{-6}$ M range, one that would not have been apparent at the higher substrate concentrations employed in some previous studies[3,10]. Moreover, the higher $K_m$ value obtained in the present study was one order of magnitude lower than that obtained in a crude fraction of rat thyroid by Bastomsky *et al.*[10]. These considerations emphasize the need for studies using a purified enzyme.

With respect to its kinetic properties, the purified enzyme from thyroid closely resembled phosphodiesterase from other tissues. These similarities included two $K_m$ values, a finding previously reported for the brain, muscle, kidney and adipose tissue enzymes[26]; a bivalent cation requirement, including the biphasic action of $Zn^{2+}$ (ref.

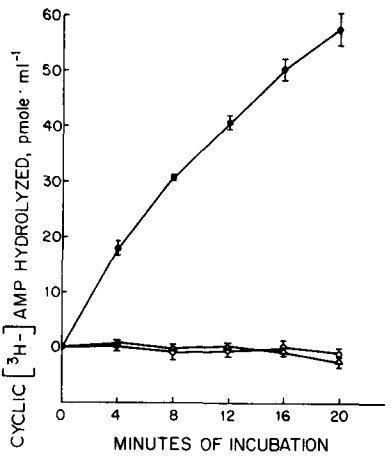


Fig. 5. Effect of a cyclic AMP-binding protein from bovine kidney[18] on bovine thyroid phosphodiesterase activity. 1.5 ml incubation mixture contained, in addition to phosphodiesterase and binding protein as indicated, cyclic [$^3$H]AMP, $MgCl_2$, EGTA and AMP at the same concentrations as in the phosphodiesterase assay and Tris–HCl, pH 7.6 at 25 mM. Incubation at 0 °C was carried out in two stages. During the first 60-min stage cyclic [$^3$H]AMP substrate was preincubated with binding protein; thyroid phosphodiesterase was then added and 100-$\mu$l aliquots were removed at 4-min intervals and added to an equal volume of ice-chilled 12% trichloroacetic acid. The distribution of end products in the acid extract was determined by thin layer chromatography on PEI cellulose. (Chromatographic analysis of samples taken at 0 min of the second stage indicated that approx. 35% of the cyclic [$^3$H]AMP present had been hydrolyzed to inosine and hypoxanthine during the first 60-min stage. No further hydrolysis occurred during the second 20-min stage of incubation). ●—●, phosphodiesterase, 4 mg/ml; ○—○, binding protein, 11 mg/ml; △—△, binding protein *plus* phosphodiesterase. Values represent mean and range of duplicate determinations.

27); and marked inhibition by the methylxanthines[8] and quazodine[23]. In addition, the "specific activity" of our enzyme preparation did not increase during the course of partial purification, a situation perhaps analogous to that described by Cheung[27] for brain phosphodiesterase which progressively loses activity during purification. In contrast to other recent studies[28], only slight inhibition of phosphodiesterase activity was obtained upon addition of the sulfonylureas, chlorpropamide and tolbutamide. It should be noted, however, that the sulfonylureas were similarly ineffective in parallel measurements of heart phosphodiesterase activity, owing, possibly, to our inability to dissolve these compounds under our assay conditions at concentrations exceeding 5 mM. Although the sulfonylureas have been implicated as pharmacological agents affecting thyroid function[28], this action does not appear to be related to an inhibition of thyroidal phosphodiesterase.

Our failure to obtain an activation of the enzyme by $NH_4^+$ and other monovalent cations at concentrations ranging from 10 to 100 mM is in contrast to the finding of Nair[29], who reported a marked activation of dog heart phosphodiesterase by $NH_4^+$.

The degree of inhibition of cyclic [$^3$H]AMP hydrolysis by other cyclic nucleotides is presumably related to the ability of these compounds to interact with the active site of the enzyme; the failure of cyclic UMP to affect enzymatic activity suggests a requirement for the purine structure. The strong competitive inhibition of thyroid phosphodiesterase by the mono- and dibutyryl derivatives of cyclic AMP is of con-

siderable interest since these compounds are capable of mimicking hormone action in the thyroid[30] and numerous other tissues[31]. (Solomon[32] recently reported inhibition of adipose tissue phosphodiesterase by dibutyryl cyclic AMP; the results of his study are entirely analogous to those reported herein.) It is possible that an inhibition of the hydrolysis of endogenous cyclic AMP accounts, at least in part, for this action of the butyryl derivatives, as suggested by Klein and Raisz[33]. Further, the competitive interaction effected by these derivatives suggests that the free $-NH_2$ group in the 6-position and the free –OH group in the 2′-position are not required for binding to the active site of the enzyme.

Chronic *in vivo* stimulation of rat thyroid by 6-*n*-propylthiouracil or low-iodine diet leads to an increase in phosphodiesterase activity of thyroid homogenates[10], while acute *in vivo* administration fails to do so. Direct *in vitro* addition of thyrotropin also fails to affect phosphodiesterase activity in bovine[2], canine[3] and human[9] thyroid homogenates. In the present study with the purified thyroid enzyme, we demonstrated a small but significant increase in enzymatic activity upon *in vitro* addition of biologically active thyrotropin; the heat-inactivated hormone was without effect. This finding would tend to support the hypothesis of Bastomsky *et al.*[10] of an increased turnover of cyclic AMP in the stimulated thyroid gland. However, the small magnitude of the thyrotropin effect leaves its physiological significance open to question. Immunoglobulin G with long-acting thyroid stimulator activity was ineffective, in accord with the findings in human thyroid homogenates by Miyai *et al.*[9], as were the prostaglandins $E_2$ and $F_{1\alpha}$. A slight inhibition of phosphodiesterase activity was obtained with prostaglandin $E_1$, an effect which would tend to potentiate the elevation of intracellular cyclic AMP levels brought about by this agent through the activation of adenylate cyclase[6,7].

The prostaglandin antagonists 7-oxa-13-prostynoic acid and polyphloretin phosphate, inhibitors of thyrotropin- and prostaglandin-induced cyclic AMP accumulation in thyroid gland[34,35], were, paradoxically, also found to be inhibitors of the phosphodiesterase reaction. This inhibition may be relevant in certain *in vitro* experiments with thyroid cells where prostaglandin antagonists occasionally exhibit agonist properties[34,35]. It must, however, be remembered that the correlation between phosphodiesterase inhibition in cell-free systems and pharmacological action in intact cells is not necessarily a direct one[36]; factors such as protein binding, lipid solubility and intracellular distribution of the inhibitor may determine the ultimate pharmacological response. Indeed, our previous studies with intact thyroid cells suggest that aminophylline and dibutyryl cyclic AMP inhibited the degradation of labeled cyclic AMP therein to a much lesser extent than when the purified enzyme was employed[11].

As was reported for phosphodiesterase from other sources[18,37], the purified enzyme from thyroid did not attack the protein bound form of cyclic AMP. The interpretation of these results is somewhat complicated by the endogenous hydrolytic activity (*i.e.* phosphodiesterase, 5′-nucleotidase and deaminase) of the crude binding protein preparation (Szabo and Burke, unpublished observations). Further work with a more highly purified binding protein is thus needed to establish this point conclusively.

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