Synthesis of New Cyclic Nucleotides and Their Differential Stimulatory Effects on Thyroid Function in Mice

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Various 8-substituted cAMP analogues were converted into the corresponding $N^6,O^{2'}$-dibutyryl, N^6-monobutyryl, or $O^{2'}$-monobutyryl derivatives and the parent compounds as well as the new derivatives were tested for TSH-like stimulation of the thyroid function in mice in vivo by means of the McKenzie bioassay. It was found that a pronounced stimulatory effect on the thyroid secretion can be produced by certain cyclic nucleotides. The most potent compounds, $8H_2N$-cAMP, 8MeS-cAMP, and $8N_3$-cAMP at 15–30 mg/kg iv, showed an activity approximately one order of magnitude higher than that of cAMP and comparable to the action of 0.6–1.0 mU of TSH per mouse iv. Radioimmunological determination of thyroxine levels in the serum confirmed these results. Serum T_4 levels in mice injected, for instance, with 8MeS-cAMP (30 mg/kg) rose from 4.85 ± 0.25 to 6.32 ± 0.41 $\mu g/100$ ml in 1 h, the net increase in T_4 being comparable to that produced by TSH (1 mU/mouse) under identical experimental conditions. Introduction of one or two butyryl groups (in the N^6 and/or $O^{2'}$ position) significantly increased or decreased the biological activity, dependent on the parent compound, but no direct correlation between biological activity and degree of butyrylation was apparent. The effect of these compounds on the thyroid seems to be specific for cyclic nucleotides because 8MeS-5′AMP which resembles 8MeS-cAMP but lacks the cyclic phosphate structure is inactive in the McKenzie bioassay and in the T_4 radioimmunoassay.

Considerable evidence[1,2] has been accumulated that adenosine cyclic 3′,5′-phosphate (cAMP) mediates the action of TSH (thyroid stimulating hormone) on the thyroid. It has been found that cAMP and its $N^6,O^{2'}$-dibutyryl derivative (Bt$_2$-cAMP) can reproduce many of the metabolic and morphological responses to TSH in thyroid tissue in vitro.[3]

For example, cAMP and Bt$_2$-cAMP in vitro stimulate the release of radioiodine from mouse[3] or rat[4] thyroid glands, the ratio of the released thyroxine (T_4) and triiodothyronine (T_3) being similar to that produced by TSH, as judged by chromatography. Furthermore, cAMP and Bt$_2$-cAMP stimulate droplet formation in thyroid slices[5] and increase glucose oxidation, phospholipid turnover,[6] RNA, and protein synthesis in thyroid tissue.[7–9]

In contrast to in vitro studies, the results obtained in in vivo systems are conditional and not clearly positive. Bastomski and McKenzie[10] and Ahn et al.[11,12] have reported that in vivo injections of only very high doses of cAMP ($\sim$300 mg/kg) and/or pretreatment of the animals with thyroxine and TSH produced a statistically significant increase in thyroidal radioiodide release or enhanced the synthesis of organically bound iodine in the thyroid.

In the present study we have tested a series of 8-substituted cAMP derivatives, as well as various new butyrylated derivatives thereof, for their in vivo effect on the thyroid, mainly because these 8-substituted analogues of cAMP have been shown, at least in isolated enzyme systems, to be better activators of protein kinases, more resistant to phosphodiesterases, and also to be more active than the parent compound with respect to TSH release in vitro[13] and in other hormone systems.[14]

Experimental Section

Chemistry. Paper chromatography was carried out with (a) 2-propanol–0.5 M ammonium acetate (5:2) or (b) butanol–acetic acid–water (5:2:3). High-voltage electrophoresis was run in 0.05 M triethylammonium bicarbonate buffer, pH 7.5, at 1000 V for 3 h. The electrophoretic mobility is expressed in values relative to cAMP = 1. Uv spectra were recorded in 0.05 M phosphate buffer, pH 7, with a Beckman Acta CIII spectrophotometer.

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Elemental analyses were performed by Galbraith Laboratories.

All 8-substituted cAMP derivatives, i.e., 8-bromoadenosine cyclic 3′,5′-phosphate (8Br-cAMP, 1), 8-thioadenosine cyclic 3′,5′-phosphate (8HS-cAMP, 2), 8-methylthioadenosine cyclic 3′,5′-phosphate (8MeS-cAMP, 3), 8-benzylthioadenosine cyclic 3′,5′-phosphate (8PhCH$_2$S-cAMP, 4), 8-benzyloxyadenosine cyclic 3′,5′-phosphate (8PhCH$_2$O-cAMP, 5), 8-hydroxyadenosine cyclic 3′,5′-phosphate (8HO-cAMP, 6), 8-azidoadenosine cyclic 3′,5′-phosphate (8N$_3$-cAMP, 7), and 8-aminoadenosine cyclic 3′,5′-phosphate (8H$_2$N-cAMP, 8), were prepared according to published procedures[15,16] and obtained in crystalline, analytically pure form after chromatography on DEAE-Sephadex. 8-Methylthioadenosine 5′-phosphate (8MeS-5′AMP, 9), in analogy to 3, was obtained by bromination of adenosine 5′-phosphate, followed by nucleophilic displacement.

The majority of the various butyrylated cAMP analogues were synthesized from the corresponding 8-substituted cAMP derivatives, following essentially the standard methods described by Posternak et al.[17,18] Namely, the $O^{2'}$-monobutyryl derivatives, e.g., $O^{2'}$-butyryl-8-thioadenosine cyclic 3′,5′-phosphate (11) and $O^{2'}$-butyryl-8-methylthioadenosine cyclic 3′,5′-phosphate (14), as well as the $N^6,O^{2'}$-dibutyryl derivatives, e.g., $N^6,O^{2'}$-dibutyryl-8-thioadenosine cyclic 3′,5′-phosphate (10), $N^6,O^{2'}$-dibutyryl-8-methylthioadenosine cyclic 3′,5′-phosphate (13), and 8-benzylthio-$N^6,O^{2'}$-dibutyryladenosine cyclic 3′,5′-phosphate (16), were prepared by treating the corresponding 8-substituted cyclic nucleotides as such or, preferably, in the form of their triethylammonium salts with a large excess of butyric anhydride in pyridine. Good yields of the $O^{2'}$-monobutyryl derivatives were obtained when the reaction was stopped after 2 h at room temperature or 1–2 days at 0–5 °C, whereas exhaustive butyrylation at room temperature for 7–9 days afforded the dibutyryl derivatives as main products, accompanied by small amounts of other unidentified products, presumably the $N^6,N^6,O^{2'}$-tributyryl[19] derivatives. The N^6-monobutyryl-8-substituted analogues, e.g., N^6-butyryl-8-thioadenosine cyclic 3′,5′-phosphate (12), N^6-butyryl-8-methylthioadenosine cyclic 3′,5′-phosphate (15), and 8-benzylthio-N^6-butyryladenosine cyclic 3′,5′-phosphate (17), were produced by partial hydrolysis of the corresponding dibutyryl derivatives in 0.1 N aqueous–alcoholic KOH under carefully controlled conditions (0–5 °C, 1 h). Compounds in which the substituent in position 8 is susceptible to acylation, e.g., 8H$_2$N-cAMP (8) or 8HO-cAMP (6), were butyrylated in the form of a suitable precursor like 8N$_3$-cAMP (7) or a blocked derivative like 8PhCH$_2$O-cAMP (5) and subsequently converted to the desired product, i.e., 8-amino-$N^6,O^{2'}$-dibutyryladenosine cyclic 3′,5′-phosphate (19) or $N^6,O^{2'}$-dibutyryl-8-hydroxyadenosine cyclic 3′,5′-phosphate (18), respectively.

While the synthesis of these butyrylated, 8-substituted cAMP analogues is straightforward, their purification is complicated due to their pronounced instability in aqueous systems; especially the

dibutyryl derivatives tend to hydrolyze when standard workup or isolation procedures are employed. However, analytically pure dibutyryl derivatives, in the free acid form, were obtained by ion-exchange chromatography on DEAE-Sephadex A-25 in the acetate form at pH 4.7 or in the bicarbonate form at pH 7.5, using gradient elution with triethylammonium acetate or triethylammonium bicarbonate, respectively, at 0–5 °C followed by cation exchange over Bio-Rex 70 (H⁺) at 0–5 °C. The same chromatographic procedure, but at room temperature, was also very successfully applied to the N^6-monobutyryl derivatives. The O^2-monobutyryl compounds could be crystallized directly from the reaction mixture.

Method A. N^6,O^2-**Dibutyryl-8-substituted cAMP Derivatives. 8-Benzylthio-N^6,O^2-dibutyryladenosine Cyclic 3',5'-Phosphate (8PhCH₂S-Bt₂-cAMP, 16).** 8-Benzylthioadenosine cyclic 3',5'-phosphate[15] (triethylammonium salt, 1.5 g), suspended in dry pyridine (45 ml), was allowed to react with freshly distilled butyric anhydride (22 ml) at room temperature, protected from light, for 7 days. Excessive anhydride was hydrolyzed by addition of water (20 ml) at 0 °C under stirring for several hours. The mixture was then evaporated (temperature <40 °C) in high vacuum followed by coevaporations with toluene–ethanol to give the crude dibutyryl derivative in the form of a yellow oil. All subsequent operations, unless stated otherwise, were carried out at 0–5 °C. The crude product was dissolved in 0.02 M triethylammonium acetate (pH 4.7), the pH of the solution readjusted to 4.7, and the solution applied to a column (2.5 × 60 cm) of DEAE-Sephadex A-25 (acetate). The column was developed with a linear gradient of 0.02 vs. 0.4 M triethylammonium acetate (3 l.) each. Elution was monitored with a uv monitor. The fractions of the main peak were pooled and lyophilized to furnish the triethylammonium salt of the product. Residual triethylammonium acetate was removed by several evaporations with ethanol (temperature <30 °C). The triethylammonium salt was converted into the free acid by passage through a column of Bio-Rex 70 (H⁺, 100–200 mesh) followed by lyophilization (see also Table I).

N^6,O^2-**Dibutyryl-8-methylthioadenosine Cyclic 3',5'-Phosphate (Bt₂-8MeS-cAMP, 13).** See preparation of 16 and Table I. 13 (Na salt) was precipitated from ethanol–ethyl acetate by addition of ether.

N^6,O^2-**Dibutyryl-8-thioadenosine Cyclic 3',5'-Phosphate (Bt₂-8HS-cAMP, 10).** See preparation of 16 and Table I. 10 (free acid) was crystallized from a large volume of ether by dropwise addition of absolute ethanol.

N^6,O^2-**Dibutyryl-8-hydroxyadenosine Cyclic 3',5'-Phosphate (Bt₂-8HO-cAMP, 18).** 8-Benzyloxyadenosine cyclic 3',5'-phosphate[15] (1 g) was butyrylated as described above and the crude product in 50% ethanol hydrogenated over 10% Pd/C (1 g) at 40 psi and room temperature overnight. The hydrogenation mixture was chromatographed (Table I) and 18 (free acid) obtained in semicrystalline form by trituration with ether.

8-Amino-N^6,O^2-dibutyryladenosine Cyclic 3',5'-Phosphate (8H₂N-Bt₂-cAMP, 19). Crude 8-azido-N^6,O^2-dibutyryladenosine cyclic 3',5'-phosphate obtained by butyrylation of 8N₃-cAMP,[15] uv $\lambda_{max}^{pH\ 7}$ 292 nm, in 0.03 M triethylammonium bicarbonate, pH 7.5, was hydrogenated in the presence of 10% Pd/C at 40 psi for 2 h. Chromatography on DEAE-Sephadex afforded 19 and a major side product, presumably 8-amino-N^6,N^6,O^2-tributyryladenosine cyclic 3',5'-phosphate,[19] uv $\lambda_{max}^{pH\ 7}$ 293 nm (ϵ 12 700) and 255 (7300), alkaline hydrolysis to 8NH₂-cAMP. Anal. (C₂₂H₃₁N₆O₉P·H₂O) C, H, N.

Method B. N^6-**Butyryl-8-substituted cAMP Derivatives. 8-Benzylthio-N^6-butyryladenosine Cyclic 3',5'-Phosphate (8PhCH₂S-N^6Bt-cAMP, 17).** Crude 16 obtained from 4 (1 g) was dissolved in ethanol–water (3:1, 80 ml), cooled in ice, and the solution neutralized to pH ~7 (2 N NaOH). To this solution was added 2 N NaOH (4 ml) to give a final concentration of 0.1 N NaOH. The hydrolysis is allowed to proceed for 2 h at 0–5 °C. Then the pH was adjusted to 7.5 (3 N HCl), the ethanol evaporated (temperature 30 °C), and the solution applied to a column (2.5 × 60 cm) of DEAE-Sephadex A-25 (HCO₃⁻). Elution with a linear gradient of 0.02 vs. 0.2 M triethylammonium bicarbonate, pH 7.5 (3 l. each), afforded the desired product in fractions 115–200 (20 ml each) and a small amount of 4 in fractions 220–250. Fractions 115–200 were pooled and lyophilized and the residue

was evaporated several times with absolute ethanol. The triethylammonium salt of 17 thus obtained was converted into the free acid by passage through a Bio-Rex 70 (H⁺) column at 0–5 °C. The eluate was lyophilized, the residue dissolved in ethanol, and the product precipitated with ether.

N^6-**Butyryl-8-methylthioadenosine Cyclic 3',5'-Phosphate (N^6Bt-8MeS-cAMP, 15).** See synthesis of 17 and Table I. 15 (Na salt) was crystallized from a small volume of ethanol–ethyl acetate (1:1) by addition of ca. 10 vol of ether.

N^6-**Butyryl-8-thioadenosine Cyclic 3',5'-Phosphate (N^6Bt-8HS-cAMP, 12).** See synthesis of 17 and Table I.

Method C. O^2-**Butyryl-8-substituted cAMP Derivatives. O^2-Butyryl-8-methylthioadenosine Cyclic 3',5'-Phosphate (O^2Bt-8MeS-cAMP, 14).** 3 (triethylammonium salt, 0.5 g) in dry pyridine (15 ml) and butyric anhydride (5 ml) was stirred under exclusion of moisture for 2 days at 0–5 °C or 2 h at room temperature. Excess anhydride was destroyed by addition of methanol (10 ml) and stirring for several hours at 0 °C. The mixture was evaporated to dryness (temperature <40 °C), followed by several coevaporations with toluene–ethanol. The residue was triturated with ether to give crystalline 14. The product, after filtration, was resuspended in ca. 20 ml of water, acidified to pH ≈3, filtered, and washed with water, methanol, and ether.

O^2-**Butyryl-8-thioadenosine Cyclic 3',5'-Phosphate (O^2-Bt-8HS-cAMP, 11).** See preparation of 14 and Table I.

McKenzie Bioassay.[10] After weaning, female mice of 14–16 g were put on an iodine-poor diet for 14 days. Then they were injected (sc) with 10 μg of L-thyroxine and 6–8 μCi of ¹²⁵I (ip) and received L-thyroxine (1 mg/l.) in their drinking water for the three following days. On the fourth day, the cyclic nucleotides to be assayed were injected (as sodium salts in aqueous solution, pH 7.4) into the tail vein. The first blood sample (0.1 ml) was taken immediately after injection of the compound (by puncturing the orbital sinus), and a second blood sample was collected in the same way after 2 h. All samples were counted in a γ counter (Beckman) and the increase in blood radioactivity was determined.

Radioimmunoassay of Serum Thyroxin. Mice of 18 g (Swiss male, SPF, EVIC-CEBA) were used. The animals, without any pretreatment, received the cyclic nucleotides injected into the tail vein as described before. Blood samples (50 μl) were collected by orbital puncture at 0 (immediately following the injection), 1, and 2 h, respectively, after the injection. The thyroxine was assayed in 10-μl aliquots of serum by a double antibody radioimmunoassay using [¹²⁵I]thyroxine and an appropriate thyroxine antiserum (Nuclear Medical Systems, Newport Beach, Calif.).

Results and Discussion

The new butyrylated, 8-substituted analogues of cAMP, their method of preparation, and physical properties are summarized in Table I. To correlate structure and TSH-like activity of the various cyclic nucleotides, all new derivatives and the parent compounds were first evaluated in the McKenzie bioassay.

In the McKenzie bioassay,[20] TSH injected into mice pretreated with ¹²⁵I and T₄ produces in 2 h a dose-related increase of blood radioactivity due to an increased secretion of radioiodine from the thyroid. The cyclic nucleotides were injected iv in a series of doses ranging from 6 to 60 mg/kg. The results, expressed as percent increase in cpm with respect to individual blood radioactivity in cpm at zero time, and a rank order of activity are represented in Table II.

As illustrated in Table II, all cAMP derivatives used in this study were found to be more active than cAMP in stimulating the thyroid secretion and most of them were more active than Bt₂-cAMP.

In a limited concentration range, a linear dose–response was demonstrated. The highest stimulatory effects on thyroidal function were produced by 8MeS-cAMP, 8H₂N-cAMP, and 8N₃-cAMP, which exert an effect of approximately one order of magnitude greater than cAMP. In contrast, other 8-substituted cAMP analogues, e.g., 8HS-cAMP and 8HO-cAMP, were toxic at high doses and

Table I. Butyrylated 8-Substituted Derivatives of Adenosine Cyclic 3′,5′-Phosphate

R_1 = SH, SMe, SCH$_2$Ph, OH, NH$_2$
R_2, R_3 = H or Bt

No.	Compd abbrev	Meth-od[a]	Purification[b]	Yield,[c] %	Uv spectra $\lambda_{max}^{pH\,7}$, nm ($\epsilon \times 10^{-3}$)	H.V.E.[d]	Analyses (Formula)
10	Bt$_2$-8HS-cAMP	A	DEAE-Sephadex, 0.05–0.5 M HNEt$_3$OAc	74	322 (29.4), 243 (20.0)	1.8	C, H, N, S (C$_{18}$H$_{24}$N$_5$-O$_8$PS·1H$_2$O)
11	O$^{2'}$Bt-8HS-cAMP	C	Crystd from MeOH	60	297 (23.7), 227 (19.2)	2.0	C, H, N, S (C$_{14}$H$_{18}$N$_5$-O$_7$PS·1H$_2$O)
12	N^{6}Bt-8HS-cAMP	B	DEAE-Sephadex, 0.02–0.25 M HNEt$_3$HCO$_3$	79	320 (22.9), 243 (16.7)	1.8	C, H, S; N^e (C$_{14}$H$_{18}$N$_5$-O$_7$PS·0.5EtOH)
13	Bt$_2$-8MeS-cAMP	A	DEAE-Sephadex, 0.02–0.25 M HNEt$_3$OAc	64	296 (20.6)	1.1	C, H, N, S (C$_{19}$H$_{25}$N$_5$-O$_8$PSNa·0.5EtOH)
14	O$^{2'}$Bt-8MeS-cAMP	C	Crystd from EtOH	43	278 (18.5)	0.9	C, H, N, S (C$_{15}$H$_{20}$N$_5$-O$_7$PS·H$_2$O)
15	N^{6}Bt-8MeS-cAMP	B	DEAE-Sephadex, 0.02–0.15 M HNEt$_3$HCO$_3$	56	295 (19.2)	0.98	C, N, S; H^f (C$_{15}$H$_{19}$-N$_5$O$_7$PSNa)
16	8PhCH$_2$S-Bt$_2$-cAMP	A	DEAE-Sephadex, 0.02–0.4 M HNEt$_3$OAc	58	299 (18.7)	0.8	C, N, S; H^g (C$_{25}$H$_{30}$-N$_5$O$_8$PS)
17	8PhCH$_2$S-N^{6}Bt-cAMP	B	DEAE-Sephadex, 0.02–0.2 M HNEt$_3$HCO$_3$	51	298 (17.5)	0.85	C, H, N, S (C$_{21}$H$_{24}$N$_5$-O$_7$PS)
18	Bt$_2$-8HO-cAMP	A	DEAE-Sephadex, 0.02–0.15 M HNEt$_3$HCO$_3$	44	288 (14.4)	1.2	C, N; H^h (C$_{18}$H$_{24}$N$_5$-O$_9$P)
19	8H$_2$N-Bt$_2$-cAMP	A	DEAE-Sephadex, 0.02–0.1 M HNEt$_3$HCO$_3$	28	295 (16.1), 258 (6.9)	1.1	C, H, N (C$_{18}$H$_{29}$N$_6$-O$_{10}$P·2H$_2$O)

^{a,b} See Experimental Section. ^{c} Overall yield from the corresponding 8-substituted cAMP derivative. ^{d} High-voltage electrophoresis, mobility relative to cAMP = 1. ^{e} N: calcd, 14.67; found, 15.38. ^{f} H: calcd, 4.10; found, 4.65. ^{g} H: calcd, 5.11; found, 6.03. ^{h} H: calcd, 4.98; found, 4.56.

Table II. McKenzie Bioassay^a of Cyclic Nucleotides

Substituents on cAMP^d	Blood ^{125}I levels, cpm (2 h)/cpm (0 h) × 100			
	60 mg/kg	30 mg/kg	15 mg/kg	6 mg/kg
8MeS-		804 ± 52^b	645 ± 55	492 ± 31
8H$_2$N-	818 ± 78^c	607 ± 42	663 ± 76	226 ± 15
8N$_3$-		633 ± 33	594 ± 61	227 ± 26
N^{6}Bt-8HS-	630 ± 36	342 ± 26	139 ± 9	
Bt$_2$-8HO-	582 ± 66	500 ± 57	184 ± 14	
N^{6}Bt-8MeS-	533 ± 74	204 ± 17	121 ± 13	
8Br-	488 ± 68	331 ± 50	284 ± 16	248 ± 30
Bt$_2$-8HS-	238 ± 28	177 ± 14	154 ± 7	
8PhCH$_2$S-N^{6}Bt-	197 ± 3	122 ± 13	146 ± 10	
O$^{2'}$Bt-8HS-	190 ± 0	153 ± 12	155 ± 9	
8PhCH$_2$S-Bt$_2$-	123 ± 7	141 ± 11	170 ± 13	116 ± 7
Bt$_2$-8MeS-	174 ± 23	125 ± 17	150 ± 11	
O$^{2'}$Bt-8MeS-	110 ± 12^c	156 ± 16		
8H$_2$N-Bt$_2$-	164 ± 6	146 ± 7		
8PhCH$_2$S-	Lethal	195 ± 21	183 ± 13	135 ± 14
8HS-	Lethal	Lethal	Lethal	136 ± 26
8HO-		Lethal	114 ± 8	
cAMP	135 ± 10			
Bt$_2$-cAMP	245 ± 22	136 ± 12		
Saline (0.05 ml)	101 ± 1	110 ± 1	107 ± 4	
8MeS-5′AMP		112 ± 2		

^{a} See Experimental Section. ^{b} The data for each dose represent mean values of, on average, six animals. ^{c} 45 mg/kg. ^{d} Listed in order of decreasing effect on thyroid.

almost ineffective at low, nontoxic doses.

Introduction of one or two butyryl groups (in the N^6 and/or O$^{2'}$ position) significantly changed the biological activity but there was no direct correlation between bi-

ological activity and the degree of butyrylation in this test. For instance, if an inactive or moderately active compound like 8HS-cAMP or 8HO-cAMP was converted into its monobutyryl or dibutyryl derivative, then butyrylation resulted in a much more active and less toxic compound. If, however, the very potent derivatives, e.g., 8MeS-cAMP or 8H$_2$N-cAMP, were butyrylated, then the biological activity was diminished.

Generally, we found that the N^6-monobutyryl derivatives were more potent than the corresponding dibutyryl derivatives.

As already mentioned briefly, 8HS-cAMP and 8HO-cAMP were highly toxic (at a dose of 15–30 mg/kg all animals died). Interestingly, the mono- and dibutyryl derivatives were well tolerated (LD$_{50}$ > 200 mg/kg, or in some cases >400 mg/kg).

The effect of these compounds on the thyroid appears to be specific for cyclic nucleotides because 8-methyl-thioadenosine 5′-monophosphate (8MeS-5′AMP) which resembles 8MeS-cAMP, but has an open chain, instead of a cyclic phosphate structure does not show any activity. This observation and our previous findings with 5′-AMP are in contrast to earlier reports[10] where injections of 1 mg of 5′-AMP or ATP gave rise to an increase in blood radioactivity similar to that observed with 5 mg of cAMP.

For better evaluation of the relative potency of the cyclic nucleotides in the McKenzie assay, we determined the log dose–response for TSH (standard NIH-TSH-B6) under exactly the same experimental conditions (Figure 1).

Based on this TSH standard curve, it can be seen that the most potent cyclic nucleotides (8MeS-cAMP, 8H$_2$N-cAMP, and 8N$_3$-cAMP at 15 mg/kg) produced in
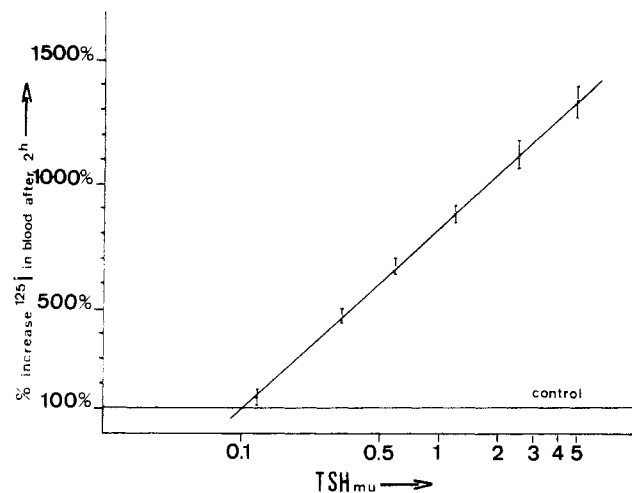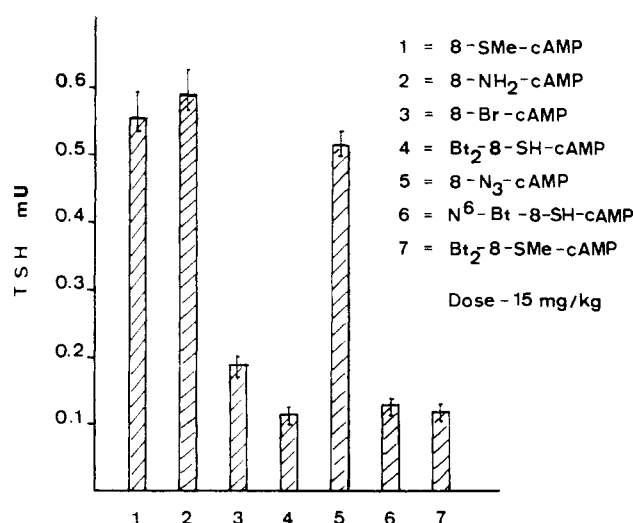
Figure 1. Log dose–response curve for TSH.

Figure 2. Relative potency, expressed in mU of TSH of cAMP derivatives on the thyroidal function.

vivo a stimulatory effect on the thyroid comparable to that of 0.5 mU of TSH (Figure 2).

In another series of experiments we measured serum thyroxine (T_4) by radioimmunoassay[21] to determine if the cAMP analogues were actually causing an increase in thyroxine synthesis and/or thyroxine release.

As shown in Table III, injections of 1 mg of 8MeS-cAMP, $8H_2N$-cAMP, or $8N_3$-cAMP produced in all animals after 1 and 2 h, respectively, a net increase in the T_4 level comparable to that produced by 1 mU of TSH. A significant elevation of serum T_4 was also observed in the case of Bt_2-8HO-cAMP after 1 h, while Bt_2-8HS-cAMP failed to show a marked effect. In the animals receiving NaCl or 8MeS-5'AMP, the T_4 level remained the same for the 2 h. It is worth mentioning that the initial T_4 level varied from animal to animal, but individual T_4 levels remained constant over 2 h in the control groups while they rose sharply in all animals injected with TSH or, e.g., 8MeS-cAMP (Figure 3).

In summary, there is good agreement between the rank order of activity found in the McKenzie bioassay and by radioimmunological thyroxine determination, the RIA data providing direct evidence that certain cyclic nucleotides increase in vivo secretion of thyroxine as opposed to a release of only iodide.

In contrast to the observations made by Ahn et al.,[12] it was not necessary to pretreat the animals with T_4 and TSH
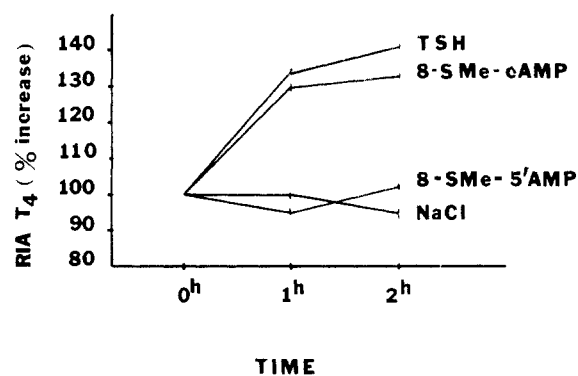

TIME

Figure 3. Change in T_4 levels after administration of 1 mU of TSH, 1 mg of 8SMe-cAMP, 1 mg of 8SMe-5'AMP, or 0.05 ml of NaCl per mouse. Results are expressed as mean values (five animals) of percent increase at 1 and 2 h with respect to the initial, individual T_4 levels at zero time.

Table III. Serum Thyroxine Levels in Mice Measured by RIA[a] before (0 h) and after (1 and 2 h) Iv Injection of (a) TSH Standard (1 mU/Mouse), (b) Various Cyclic Nucleotides (1 mg/Mouse), (c) 8MeS-5'AMP (1 mg/Mouse), and (d) 0.9% Saline Solution (0.05 ml/Mouse)

Compd	T_4, $\mu g/100$ ml		
	0 h	1 h	2 h
TSH	4.57 ± 0.27	$6.15^x \pm 0.47^b$	$6.45^{xx} \pm 0.54$
8MeS-cAMP	4.85 ± 0.25	$6.32^{xx} \pm 0.41$	$6.47^{xx} \pm 0.43$
$8H_2N$-cAMP	5.30 ± 0.31	$6.84^{xx} \pm 0.39$	
$8N_3$-cAMP	4.32 ± 0.30	$6.19^{xx} \pm 0.40$	
Bt_2-8HO-cAMP	4.64 ± 0.29	$5.58^x \pm 0.31$	
Bt_2-8HS-cAMP	4.20 ± 0.25	$4.30^{ns} \pm 0.22$	
Bt_2-cAMP	4.95 ± 0.35	$5.15^{ns} \pm 0.14$	
8MeS-5'AMP	4.64 ± 0.48	$4.44^{ns} \pm 0.41$	$4.78^{ns} \pm 0.46$
NaCl	6.06 ± 0.36	$6.00^{ns} \pm 0.36$	$5.83^{ns} \pm 0.31$

[a] See Experimental Section. [b] Mean ± S.E. Student's t test: ns = not significant, $p > 0.05$; x = significant, $0.05 > p > 0.01$; xx = highly significant, $p < 0.01$.

to demonstrate a marked increase of T_4 secretion in vivo with a cAMP derivative.

It has been shown, mostly by in vitro studies, that cAMP acts as a second messenger or mediator of TRH action on the pituitary[22–24] as well as of the TSH action on the thyroid.[1,2]

At this point, it is not established whether this exceptionally strong stimulation of the thyroidal function in vivo by certain cAMP derivatives is primarily due to their effect on TSH release or on their direct action on the thyroid gland. It is likely that these compounds act simultaneously at more than one level along the hypothalamus–pituitary–thyroid axis and thereby produce their effect in a cascade fashion.

According to Sutherland's second messenger concept,[25] it is generally believed that hormones act as first messengers on target cells by increasing intracellular cAMP levels. The exact mechanism of action of exogenous cAMP on cell functions is not yet established but it has been postulated that exogenous cAMP must penetrate the cell membrane in order to be effective.

The greater biological activity of Bt_2-cAMP has been presumed to result from its more lipophilic character and/or more resistance to hydrolysis by phosphodiesterase (PDE). The idea that Bt_2-cAMP would be transported more readily was first proposed by Posternak et al.[17,18] and is frequently accepted as fact, although there is no experimental evidence to substantiate this suggestion.

We have now synthesized a series of butyrylated derivatives but do not find any correlation between the

degree of butyrylation and biological activity on thyroid function. Evidently other factors besides penetration, e.g., binding requirements of various enzyme systems involved in the mechanism of action of cAMP, will have to be considered to explain our results.

Several groups[26,27] have reported variations in the PDE isoenzyme composition of different tissues and we might speculate that there are also differences in the protein kinase system of various tissues.

Comparing the effect of the various cyclic nucleotides on the thyroidal function with results previously obtained on the release of growth hormone[28,29] or prolactin,[30] we find some degree of tissue specificity. For example, Bt_2-8HS-cAMP, which was one of the most active compounds on growth hormone release, shows only a marginal effect on thyroid function. On the other hand, $8H_2N$-cAMP, which is a very strong stimulator of the thyroid, is one of the least active cAMP analogues with respect to growth hormone release. This observation of relative specificity of certain cAMP derivatives could contribute to a better understanding of the mechanism of action of cAMP and lead to further study of possible therapeutic applications of cyclic nucleotides.

References and Notes

(1) W. Tong, *Handb. Physiol., Sect. 7: Endocrinol.*, **3**, 273 (1974).
(2) W. D. Wicks, *Adv. Cyclic Nucleotide Res.*, **4**, 385 (1974).
(3) J. M. Ensor and D. S. Munro, *J. Endocrinol.*, **43**, 477 (1969).
(4) T. Tonoue, W. Tong, and V. Stolc, *Endocrinology*, **86**, 271 (1970).
(5) F. Rodesch, P. Neve, C. Willems, and J. E. Dumont, *Eur. J. Biochem.*, **8**, 26 (1969).
(6) I. Pastan, *Biochem. Biophys. Res. Commun.*, **25**, 14 (1966).
(7) P. R. Adiga, P. V. N. Murthy, and J. M. McKenzie, *Biochemistry*, **10**, 702 (1971).
(8) B. D. Wilson and R. L. Wright, *Biochem. Biophys. Res. Commun.*, **41**, 217 (1970).
(9) S. Lissitzky, S. Manté, J. C. Attali, and G. Cartouzou, *Biochem. Biophys. Res. Commun.*, **35**, 437 (1969).
(10) C. H. Bastomski and J. M. McKenzie, *Am. J. Physiol.*, **213**, 753 (1967).
(11) C. S. Ahn and I. N. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.*, **60**, 830 (1968).
(12) C. S. Ahn, J. C. Athans, and I. N. Rosenberg, *Endocrinology*, **85**, 224 (1969).
(13) G. Cehovic, *C. R. Hebd. Seances Acad. Sci.*, **268**, 2929 (1969).
(14) G. Cehovic, I. Marcus, H. Gabbai, and T. Posternak, *C. R. Hebd. Seances Acad. Sci.*, **271**, 1399 (1970).
(15) K. Muneyama, R. J. Bauer, D. A. Shuman, R. K. Robins, and L. N. Simon, *Biochemistry*, **10**, 2390 (1971).
(16) G. Weimann, E. Haid, K. Muhlegger, H. U. Bergmeyer, K. Dietmann, G. Michal, and M. Nelboeck-Hochstetter, U.S. Patent 3 712 885 (1973).
(17) T. Posternak, E. W. Sutherland, and W. F. Henion, *Biochim. Biophys. Acta*, **65**, 558 (1962).
(18) J. G. Falbriard, T. Posternak, and E. W. Sutherland, *Biochim. Biophys. Acta*, **148**, 98 (1967).
(19) K. Anzai and M. Matsui, *Bull. Chem. Soc. Jpn.*, **46**, 3228 (1973).
(20) J. M. McKenzie, *Endocrinology*, **63**, 372 (1958).
(21) I. J. Chopra, *J. Clin. Endocrinol. Metab.*, **34**, 938 (1972).
(22) G. Cehovic, I. Marcus, S. Vengadabady, and T. Posternak, *C. R. Seances Soc. Phys. Hist. Nat. Geneve*, **3**, 135 (1968).
(23) J. F. Wilber, G. T. Peake, and R. D. Utinger, *Endocrinology*, **84**, 758 (1969).
(24) T. Posternak and G. Cehovic, *Ann. N.Y. Acad. Sci.*, **185**, 42 (1971).
(25) E. W. Sutherland and T. W. Rall, *J. Am. Chem. Soc.*, **79**, 3608 (1957).
(26) W. J. Cheung, *Adv. Psychopharmacol.*, **3**, 51 (1970).
(27) E. Monn and R. O. Christiansen, *Science*, **173**, 540 (1971).
(28) T. Posternak, I. Marcus, and G. Cehovic, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **271**, 622 (1971).
(29) G. Cehovic, T. Posternak, and E. Charollais, *Adv. Cyclic Nucleotide Res.*, **1**, 521 (1972).
(30) N. B. Giao, G. Cehovic, M. Bayer, H. Gergely, and T. Posternak, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **279**, 1705 (1974).

Synthetic Inhibitors of *Escherichia coli*, Calf Thymus, and Ehrlich Ascites Tumor Thymidylate Synthetase

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In a study of active site binding the inhibition of thymidylate synthetase derived from *Escherichia coli*, calf thymus, and Ehrlich ascites tumor was examined using eight inhibitors. 5-Substituted 2'-deoxyuridine 5'-phosphate analogues used in this study are the hydroxymethyl, methoxymethyl, benzyloxymethyl, formyl, acetyl, allyl, and two potential active site alkylating substituents: 2,3-oxypropyl and the azidomethyl analogues. All compounds were competitive with the substrate, 2'-deoxyuridine 5'-phosphate; the most potent inhibitor was 5-formyl-dUMP (K_i = 0.1, 0.09, and 0.08 μM for the respective enzyme). The 5-hydroxymethyl, 5-benzyloxymethyl, and 5-azidomethyl derivatives of dUMP showed some differential inhibition; these compounds were two to three times more active against the ascites tumor enzyme than against the thymus enzyme.

Thymidylate synthetase catalyzes the conversion of dUMP[1] to dTMP, a reductive methylation reaction utilizing formaldehyde as the carbon source and tetrahydrofolic acid as the reducing agent.[1b] The fluoro analogues of both the substrate (5-fluoro-dUMP) and product (5-trifluoromethyl-dUMP), potent inhibitors of the enzyme, are effective in the treatment of cancer.[2] The primary effect, inhibition of DNA synthesis, is caused by a depletion of dTTP.

An approach to the treatment of cancer that offers greater selectivity is the design of inhibitors that have different affinities for isoenzymes. The key features in