Enzymatic Deacetylation of Mono- and Dibutyryl Derivatives of Cyclic Adenosine 3',5'-Monophosphate by Extracts of Rat Tissues*

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SUMMARY

Cell-free extracts of several rat tissues contained Nβ-butyryl amidohydrolase and Oα'-butyryl esterase activities for the deacetylation of [3H]butyryl-labeled Nβ, Oα'-dibutyryl cyclic AMP, Nβ-monobutyryl cyclic AMP, and Oα'-monobutyryl cyclic AMP.

The highest rates of deacetylation were in the soluble cytoplasm of all tissues; cyclic AMP phosphodiesterase activities were also highest in the same cell compartment.

Since rates of cyclic AMP phosphodiesterase and 5'-AMP 5'-nucleotidase activities were from one to two orders of magnitude greater than those of the deacylases, deacylation is clearly the rate-limiting process in the catabolism of butyryl derivatives of cyclic AMP to adenosine.

Despite these relatively low rates of deacetylation, they are sufficiently high to produce, even when operating minimally, amounts of cyclic AMP far greater than could be produced endogenously, even under hormonal stimulation.

It is concluded that dibutyryl cyclic AMP could serve as a source of intracellular cyclic AMP and of biologically active monobutyryl cyclic AMP.

The Nβ and Oα'-mono- and dibutyryl derivatives of cyclic adenosine-3',5'-monophosphate (cAMP) have achieved widespread use as substitutes for cAMP in in vitro and in vivo studies with intact animal cell systems (1). They are also being used in treating humans with a variety of endocrinopathies and other disorders suspected of being related to a lack of endogenous cAMP (2-5).

Considerable uncertainty remains as to the basis for the unique effectiveness of these derivatives, and little is known about their catabolism. Henion et al. (6) proposed two explanations for their mechanism of action: (a) the derivatives may penetrate biological membranes more readily than cAMP, and (b) the derivatives may be more refractory to the action of the catabolic enzyme, cAMP phosphodiesterase. In the only direct study of mechanism (a) reported, Kaukel and Hilz (7), in a study published following completion of our work, found that extracellular [3H]cAMP was rapidly and completely degraded by enzymes located on the surface of HeLa cells and in the tissue culture medium to a mixture of H-labeled purine metabolites, these compounds readily entered the cells and, to a small degree, were reconverted to [3H]cAMP. In contrast, ring-labeled Nβ, Oα'-[3H]dibutyryl cAMP resisted extracellular degradation, was rapidly taken up by the cells, and was mostly converted intracellularly to Nβ-[3H]monobutyryl cAMP. With regard to mechanism (b) above, resistance of butyryl derivatives of cAMP to catabolism by cAMP phosphodiesterase has been well documented in several laboratories (6, 8-10).

With regard to the mechanism of action and metabolism of these acyl derivatives of cAMP, many questions remain unanswered, among which are the following. (a) Do these derivatives function biologically as such, or must they first be deacylated? (b) Do deacylases exist in animal cells? (c) If deacetylation is a requisite prelude to function, are rates of deacetylation sufficient to produce sufficient cAMP to account for the observed effects? (d) In the overall metabolism of these derivatives to adenosine, is deacetylation, phosphodiesterase, or 5'-nucleotidase rate-limiting? (e) Are these derivatives merely competitive inhibitors of cAMP phosphodiesterase, and, by this means, do they elevate endogenous levels of cAMP?

Although Posternak et al. (11) inferred from indirect evidence that dog liver extracts contained an esterase capable of converting Bt2-cAMP to Nβ-Bt-cAMP, and although Kaukel and Hilz (7) found Nβ-Bt-cAMP accumulating in HeLa cells following incubation with Bt2-cAMP, no direct experimental evidence has been reported for the existence of Nβ-acyl amidohydrolases and Oα'-acyl esterases catalyzing the conversion of these acyl derivatives to cAMP. Indeed, to the contrary, in our earlier studies with isolated fat cells (12), and in the work of Heerche et al. (13) with fetal rat calvaria, no enzymatic deacetylation of Bt2-cAMP was observed. In this paper, we show directly that cell-free extracts of rat adipose, brain, heart, kidney, and liver con
tain N\textsuperscript{6}-butyryl amidohydrolases and O\textsuperscript{2'}-butyryl esterases which deacylated Bt\textsubscript{2}-cAMP, N\textsuperscript{4}-Bt-cAMP, and O\textsuperscript{2'}-Bt-cAMP to cAMP, but which did so at rates very much lower than those of the cAMP phosphodiesterase in the same tissue compartments. Furthermore, these rates of deacylation were of a magnitude sufficient to produce biologically effective amounts of CAMP. Some of these observations have been reported in preliminary form (5).

**EXPERIMENTAL PROCEDURE**

**Cyclic Nucleotides—N\textsuperscript{6},O\textsuperscript{2'}-[2',3'-3H]Dibutyryl cAMP (Schwarz Lots 6001 and WR-2426) was purified as before (12). N\textsuperscript{4}-[2',3'-3H]Monobutyryl cAMP was produced from [\textsuperscript{3H}]Bt\textsubscript{2}-cAMP by a modification of our original procedure (13) as follows. To [\textsuperscript{3H}]Bt\textsubscript{2}-cAMP (31 nmoles, 5.55 \times 10\textsuperscript{6} dpm) plus unlabeled Bt\textsubscript{2}-cAMP (63 \mu moles) in 1 ml of absolute methanol were added 10 \mu l of concentrated NH\textsubscript{2}OH; the solution was maintained at 25\textdegree C for about 1 hour. The hydrolysis was ended by the addition of 2 ml of water and adjustment to pH 3 with dilute HCl. [\textsuperscript{3H}]Butyric acid was removed from the solution by three extractions with three volumes each of diethyl ether, following which the pH of the aqueous phase was quickly adjusted to 6.5. The hydrolysate was lyophilized, and the nucleotides in the residue were separated and isolated by preparative paper chromatography as we described before (12), modified to maintain the solvent system (ethanol-0.5 \% ammonium acetate, 5:2) at pH 7. Typically, specific radioactivities of product N\textsuperscript{6}-[\textsuperscript{3H}]Bt\textsubscript{2}-cAMP and recovered [\textsuperscript{3H}]Bt\textsubscript{2}-cAMP were 642 and 439 dpm per nmole, respectively.

O\textsuperscript{2'}-[2',3'-3H]Monobutyryl cAMP was synthesized by modifications of the method of Falbriard et al. (14). Cyclic AMP (BMC lot 6499415) and freshly prepared 4-morpholino-N,N' -dicyclohexyl carbamidine (15), each 0.3 \mu mole, were refluxed in 3 ml of dry pyridine until complete dissolution occurred (15 to 30 min); the reaction mixture was then returned to 25\textdegree C. To this solution was added 0.25 \mu mole (12.5 mCi) of [2',3'-\textsuperscript{3H}]butyric anhydride (custom synthesis by Schwarz-Mann, lot XR-2051) and 8.86 \mu moles of unlabeled butyric anhydride, and the mixture was stirred under anhydrous conditions for 17 hours; the reaction was ended by the addition of 1.5 ml of water at ice-bath temperature. The esterification was followed by periodically transferring 1-\mu l aliquots to 4 \mu l of water, and, after 4 hours of hydrolysis, chromatographing this solution on thin layers of cellulose (Machery-Nagel MN 300, A254) in ethanol-1 \% ammonium acetate (7:3). The only radioactive ultraviolet-absorbing spots were those with mobilities (R\textsubscript{f} 0.93 and 0.75, respectively) corresponding to authentic (BMC) Bt\textsubscript{2}-cAMP and Bt-cAMP (N\textsuperscript{4}-Bt-cAMP and O\textsuperscript{2'}-Bt-cAMP have identical mobilities in this system; Fig. 2 and Reference 14). The reaction mixture was dried at 40\textdegree C under reduced pressure (rotary evaporator), and the residue was subjected to preparative paper chromatography (12). O\textsuperscript{2'}-[\textsuperscript{3H}]Bt-cAMP was identified by the following: its mobility (R\textsubscript{f} 0.73) in the thin layer chromatographic system compared to that of authentic N\textsuperscript{4}-Bt-cAMP, its ultraviolet absorption spectrum at pH 7 (Fig. 1, Curve 2) which shows a maximum at 257 nm like that of cAMP but in contrast to the absorption maximum at 274 nm observed with N\textsuperscript{4}-cyclic derivatives (Fig. 1, Curves 3 to 5), its extinction quotients at pH 7 (250/200, 0.55; 280/260, 0.16; 290/260, 0.01 (no literature values available, but compare with the corresponding quotients for N\textsuperscript{4}-Bt-cAMP of 0.73, 1.15, and 0.80, respectively, according to BMC data), and its quantitative conversion to cAMP upon brief exposure to alkali (Fig. 2; cf. Reference 11). The specific radioactivity of a typical preparation was 1570 dpm per nmole.

**Tissue Preparations—**Tissues from male, Sprague-Dawley rats (140 to 200 g, fed ad libitum) were homogenized in 0.25 M sucrose-40 mm Tris-HCl (pH 7.4) in a loose-fitting, all glass, motor-driven (725 rpm) Potter-Elvehjem instrument (Kontes) for 10 to 15 excursions. Homogenates, except adipose tissue, were centrifuged briefly at 100 x g to remove debris, then at 104,500 x g for 30 min (4\textdegree C); particulates were washed three times by homogenization in buffer, and the washings (104,500 x g supernatant fluids) were pooled with the cytosols. In the case of adipose tissue, the homogenate was centrifuged for 10 min at 2000 x g at 4\textdegree C; the infranatant fluid (under the solidified fat cake at the surface) was then subjected to fractionation as for the other tissues.

Protein was determined by a modified biuret procedure (16).

**Enzyme Assays**

**cAMP Phosphodiesterase—**The assay, a modification (17) of that of Butcher and Sutherland (18), measures both high and low Km enzymes. Incubation mixtures contained, in a total volume of 0.5 ml of Tris-HCl buffer (pH 7.5), 0.88 \mu mole of MgSO\textsubscript{4} excess (1 to 2 \mu moles) cAMP, and various amounts of particulate or cytosol protein. Zero order kinetics were obtained with up to 0.15 (adipose), 0.20 (brain), 1.0 (heart), 0.6 (kidney), and 0.3 (liver) mg of protein. After 20 min of incubation at 37\textdegree C, snake venom 5'-nucleotidase (Sigma) was added, and the incubation was continued for another 10 min. The reaction was ended by 50 \mu l of 55% trichloroacetic acid, and aliquots of the deproteinized incubation mixtures were analyzed for Pi as described previously (16).

**Fig. 1.** Ultraviolet absorption spectra of several cyclic nucleotides in water. The spectra were obtained in a Beckman Acta V. Curves 1, 3, and 4 are those of authentic cAMP, Bt\textsubscript{2}-cAMP, and N\textsuperscript{4}-Bt-cAMP, respectively. Curves 2 and 5 are those of synthetic O\textsuperscript{2'}-[\textsuperscript{3H}]Bt-cAMP and synthetic [\textsuperscript{3H}]Bt\textsubscript{2}-cAMP, respectively.
**Butyryl cAMP Deacylation**—The assay of butyryl cAMP deacylation is based upon the quantification of the [3H]butyric acid produced by enzymatic hydrolysis of butyryl-labeled [3H]-

\( \text{Butyryl cAMP} \), \( \text{N}^6\text{-[3H]Bt-cAMP} \), and \( \text{O}^\prime\text{-[3H]Bt-cAMP} \). In a total volume of 0.5 ml of 40 mM Tris-HCl buffer (pH 7.5), incubation mixtures contained 0.88 µmoles of MgSO\(_4\), excess (0.4 to 0.7 µmoles) substrate, and various amounts of particulate or cytosol protein. Generally, zero order kinetics with respect to either substrate were obtained with up to 0.5 (adipose and liver), 0.7 (brain and kidney), and 2.0 (heart) mg of cytosol protein. Incubations were usually for 20 min since linearity of deacylation occurred for at least this period for all preparations. The pH optima for N\(^6\)-butyryl amidohydrolase activity were 7.5 (adipose, heart, and liver) and 8.2 (brain and kidney). Optimal pH values for \( \text{O}^\prime\text{-butyryl esterase} \) activities could not be determined with accuracy because of the lability of this ester bond under alkaline conditions; however, pH 7.5 appeared to be optimal in all cases relative to lower values. The optimum temperature for all deacylase activities was 37°C. Reactions were ended by acidification to pH 2 with 2 N HCl followed by addition of 100 µmoles of carrier butyric acid. Incubation mixtures were rapidly extracted three times with 2 ml portions of diethyl ether. The pooled etheric extracts were treated with 0.1 ml of 1.2 N KOH, and the ether was evaporated by a stream of air or nitrogen. Following acidification of the residue at 0°C with 10 µl of 2 N H\(_2\)SO\(_4\), [3H]butyric acid was extracted into a solution of 1% I-butanol in n-hexane and isolated by chromatography on columns of silicic acid-bromcresol green, as described previously (12). Aliquots (5 ml) of the eluate fractions were counted by liquid scintillation spectrometry in Nuclear-Chicago equipment. Appropriate controls, i.e. incubation mixtures without enzyme carried through the entire procedure, were performed in order to assess the nonenzymatic formation of [3H]butyric acid during the incubation and isolation procedures. This minor correction amounted to, at most (with [3H]But2-cAMP), less than 4% of that produced enzymatically.

**RESULTS**

Although neither intact fat cells (12) nor fetal rat calvaria (13) deacylate But2-cAMP, we found in preliminary experiments (10) that preparations of broken cells from rat liver and epididymal adipose tissues removed butyryl groups from But3-cAMP and N\(^6\)-But3-cAMP. Table I shows typical results with particulate and soluble fractions of five rat tissues for deacylase activities with [3H]But2-cAMP and N\(^6\)-[3H]But3-cAMP as substrates; the tissues studied are targets of hormones whose actions appear to be mediated by cyclic nucleotides. Although \( \text{O}^\prime\text{-[3H]But2-cAMP} \) was not available for these early studies, later work with this compound revealed that the subcellular distribution of \( \text{O}^\prime\text{-butyryl esterase} \) activity resembled that observed with the dibutyryl derivative. In all tissues, it was clear that N\(^6\)-butyryl amidohydrolases were essentially cytosol enzymes. In contrast, although \( \text{O}^\prime\text{-butyryl esterase} \) activities (inferred in Table I by comparing [3H]But2-cAMP with N\(^6\)-[3H]But3-cAMP) were chelically soluble in these tissues, substantial activity also resided in well washed kidney and liver particulate fractions.

The subcellular distribution of cAMP phosphodiesterase was also determined in the same tissues. Cytosol phosphodiesterase activities were substantially greater than particulate values in brain, heart, and kidney, but phosphodiesterase activities were about equal in both subcellular fractions of adipose tissue and liver (Table I).

Since vasopressin-sensitive adenylate cyclase is located mainly in kidney medullary tubules, whereas parathyrogonone-sensitive cAMP synthesis is primarily a cortical phenomenon (19), it was of interest to compare deacylase and phosphodiesterase activities in both anatomic compartments; such an experiment is shown in Table I. As the data reveal, soluble N\(^6\)-butyryl amidohydrolase and \( \text{O}^\prime\text{-butyryl esterase} \) activities appear to be equally distributed between the cortical and medullary regions. In contrast to this symmetric distribution, the specific soluble cAMP phosphodiesterase activity of the medulla was three times that of the cortical enzyme; however, even the cortical enzyme had a specific activity second only to the brain cytosol enzyme. Therefore, intrarenal barriers to the deacylation of butyryl derivatives of cAMP do not appear to exist in the rat.

In order to obtain meaningful rate data for phosphodiesterase and deacylase activities, we performed simultaneous assays of cAMP phosphodiesterase, N\(^6\)-butyryl amidohydrolase and \( \text{O}^\prime\text{-butyryl esterase} \) (using N\(^6\)-[3H]But3-cAMP), \( \text{O}^\prime\text{-butyryl esterase} \) (using \( \text{O}^\prime\text{-[3H]But2-cAMP} \)), and composite deacylation (using [3H]But2-cAMP) in the same...
cysol fraction of each of the five tissues under $V_{\text{max}}$ conditions; the data are given in Table II. It is evident that the activities of both deacylases were remarkably similar among all tissues, ranging from about 10 (adipose, N\textsuperscript{4}-butyryl amidohydrolase) to about 50 (kidney, composite deacylation) nmoles of butyryl groups hydrolyzed per mg of cytosol protein in 30 min. In contrast, phosphodiesterase activities were more variable among tissues, varying over an order of magnitude. The major result to emerge from these assays, however, is the marked contrast between rates of phosphodiesterase activities and those of deacylation. Deacylase activities relative to phosphodiesterase activities ranged from a high of 1:14 (heart, [PH]Bt\textsubscript{2}-cAMP) to a low of 1:185 (brain, N\textsuperscript{6}+[H]Bt\textsuperscript{2}-cAMP).

**DISCUSSION**

Our preliminary (10) and present studies demonstrate directly that cell-free extracts of rat adipose, brain, heart, kidney, and liver contain both O\textsuperscript{6}-butyryl esterases and N\textsuperscript{4}-butyryl amidohydrolases which, when acting sequentially or in concert, can hydrolyze Bt\textsubscript{2}-cAMP to either of its monobutyryl analogues or ultimately to cAMP\textsuperscript{6}. In general, this deacylation was essentially a cytosol phenomenon, although liver and adipose tissue particulate fractions also exhibited substantial O\textsuperscript{6}-butyryl esterase activity. From the point of view of the catabolism of acyl derivatives of cAMP to inactive products, e.g. 5'-AMP, it is significant that cAMP phosphodiesterase activity was also highest in the cytosol fraction of these tissues. Thus, no anatomic barriers appear to be present to the catabolism of these derivatives, i.e. deacylation and opening of the cyclic phosphate ring. Of ancillary interest is our observation that, in adipose tissue and liver, the cytosol and particulate phosphodiesterase activities were similar. Substantial (60\% of the total) particulate phosphodiesterase activity has also been noted in bovine heart homogenates by Sutherland and Rall (20) and in brain by DeRobertis et al. (21); however, in both instances, although the soluble enzyme had the higher specific activity, it did not appear to otherwise differ from the particulate enzyme.

Since butyryl derivatives are ineffective as substrates for cAMP phosphodiesterase, it is clear that deacylation of these derivatives is a prerequisite for further catabolism by phosphodiesterase. Since (Table II) deacylase activities in all tissues examined were lower than phosphodiesterase activities in the same tissue compartments by from one to two orders of magnitude, and since 5'-nucleotidase activities in whole homogenates of at least one of these tissues, namely, liver, averages about 2000 nmoles of 5'-AMP hydrolyzed per mg of protein in 30 min (100-fold greater than deacylase activities), it is also clear that deacylation is the rate-limiting process in the catabolism of di- and monobutyryl derivatives of cAMP to adenosine.

### Table I

**Subcellular distribution of phosphodiesterase and deacylase activities in rat tissues**

| Tissue          | Fraction | Phosphodiesterase | Deacylase |
|-----------------|----------|-------------------|-----------|
|                 |          | PH[Bt\textsubscript{2}-cAMP] | N\textsuperscript{6}+[H]Bt\textsuperscript{2}-cAMP |
|                 |          | nmoles CAMP hydrolyzed/mg protein/30 min | nmoles butyryl groups hydrolyzed/mg protein/30 min |
| Adipose         | Particulates | 340 | 46 | 4 |
|                 | Cytosol   | 110 | 105 | 68 |
| Brain           | Particulates | 1430 | <1 | 5 |
|                 | Cytosol   | 4950 | 34 | 24 |
| Heart           | Particulates | 110 | <1 | <1 |
|                 | Cytosol   | 310 | 29 | 15 |
| Kidney          | Particulates | 110 | 13 | 3 |
|                 | Cytosol   | 1070 | 87 | 10 |
| Kidney cortex   | Cytosol   | 730 | 72 | 26 |
| medulla         | Cytosol   | 2250 | 87 | 32 |
| Liver           | Particulates | 430 | 46 | 11 |
|                 | Cytosol   | 320 | 92 | 56 |

### Table II

**Simultaneous assay of cAMP phosphodiesterase and butyryl cAMP deacylase activities**

The cytosols from five rat tissues (all taken from the same rat or from litter mates) were assayed on the same day for cAMP phosphodiesterase activity and for deacylase activities with all three butyryl derivatives of cAMP; assay conditions and amounts of cytosol protein employed were as shown under “Experimental Procedure” and in Table I. The values shown below are the means and S.E. of from 5 to 10 experiments, each performed in duplicate.

| Tissue          | Phosphodiesterase | Deacylase |
|-----------------|-------------------|-----------|
|                 | PH[Bt\textsubscript{2}-cAMP] | N\textsuperscript{6}+[H]Bt\textsuperscript{2}-cAMP | O\textsuperscript{6}+[H]Bt\textsuperscript{2}-cAMP |
|                 | nmoles CAMP hydrolyzed/mg protein/30 min | nmoles butyryl groups hydrolyzed/mg protein/30 min |
| Adipose         | 395 ± 35 | 21.3 ± 5.4 | 9.4 ± 1.5 | 12.2 ± 5.3 |
| Brain           | 4580 ± 293 | 26.4 ± 5.0 | 24.7 ± 1.5 | 43.7 ± 10.1 |
| Heart           | 314 ± 35 | 22.4 ± 2.6 | 13.6 ± 2.2 | 14.1 ± 1.6 |
| Kidney          | 1146 ± 14.5 | 58.8 ± 14.5 | 24.4 ± 4.3 | 50.9 ± 23.7 |
| Liver           | 437 ± 42 | 20.2 ± 7.0 | 20.7 ± 3.1 | 28.1 ± 13.4 |

\(^{a}\) M. Blecher and C. B. Johnson, unpublished experiments.
In studies with fetal rat calvaria, Heersche et al. (13) observed that \( \text{Bt}_2\text{-cAMP} \) (initial concentration, 0.3 to 0.6 mm; intracellular concentration after 15 min of incubation with intact tissue, 0.2 mm) elevated tissue levels of cAMP 2- to 3-fold during 15 min of incubation from basal levels of about 0.3 nmole per g (wet weight) of tissue. Since deacylation of \( \text{Bt}_2\text{-cAMP} \) under their conditions produced at most 0.2 nmole per g of calvaria (13), and since under certain conditions \( \text{Bt}_2\text{-cAMP} \) (0.1 mm, a 166-fold molar excess over the substrate cAMP) significantly (about 30%) inhibited the cAMP phosphodiesterase activity of calvarian extracts (13), Heersche and co-workers concluded that \( \text{Bt}_2\text{-cAMP} \) acts primarily as an inhibitor (competitive?) of cAMP phosphodiesterase, thus permitting the accumulation of endogenous cAMP. It should be noted that, at high, nonphysiological concentrations of cAMP, others (8) have not seen an inhibition of cAMP phosphodiesterase by equimolar concentrations of \( \text{Bt}_2\text{-cAMP} \).

Although the conclusions expressed by Heersche et al. (13) may be correct, several other possibilities have not yet been eliminated. (a) \( \text{Bt}_2\text{-cAMP} \) may act as an intracellular substitute for cAMP. This possibility is supported by evidence (12, 13) that \( \text{Bt}_2\text{-cAMP} \) functioned in intact cells without detectable enzymatic deacylation, but is refuted by reports (11, 22) that in cell-free, skeletal muscle glycogen phosphorylase and synthetase systems \( \text{Bt}_2\text{-cAMP} \), at low, equimolar concentrations, was only a poor substitute for cAMP. Of course, this latter observation might also be explained by an absence of deacylase activities in the preparations employed (\textit{viva infra}). (b) Di- and monobutyryl derivatives of cAMP might be deacylated to the active species, namely, cAMP itself or intermediate monobutyryl derivatives. Our present experiments make it clear that at least five major organs have the potential of producing from \( \text{di-} \) and \( \text{monoaeryl} \) derivatives of cAMP amounts of cAMP that exceed those produced endogenously following maximal hormonal stimulation of adenylate cyclase. In general, basal concentrations of cAMP in various animal tissues range between 0.1 and 0.5 nmole per g (wet weight) of tissue, or about 0.5 to 2.5 pmole per mg of protein (1); hormones increase these values from 3- to 10-fold, depending upon the tissue and hormone (23), and these concentrations are more than sufficient to produce maximal physiological responses (1). In the present experiments, tissue deacylases were capable of producing at least 10,000 pmole of cAMP per mg of protein in 30 min from butyryl derivatives of cAMP. Clearly, even slight intracellular deacylase activities (perhaps beyond the sensitivity of methods used for detection) would produce amounts of cAMP sufficient to elicit all physiological effects. It would be of interest to determine the factors which elicit the activities of \( \text{N}^4\text{-butyryl amido} \) dehydrolase and \( \text{O}^6\text{-butyryl esterase} \).

Undoubtedly, intracellular cAMP from whatever source, including deacylation of butyryl derivatives of cAMP, is subject either to degradation by cAMP phosphodiesterases or removal from the cell. The extent of degradation is controlled by several factors. For example, it is not yet clear to what extent intracellular cAMP is available to its phosphodiesterases. It has long been recognized (24) that much cAMP is sequestered in intracellular compartments; for example, 60% of rat liver cAMP was found to be associated with particles (25). Furthermore, it has been demonstrated recently that the fraction of skeletal muscle (26) and kidney (27) cAMP bound to a cytoplasmic protein (the regulatory subunit of cAMP-activated protein kinase) was not susceptible to the action of phosphodiesterase. The stability of the cAMP-binding protein complex has been emphasized (26-28), and the rate of dissociation of this complex was shown to be the limiting reaction in the hydrolysis of cAMP by phosphodiesterase. The recent report by Chambart et al. (29) of the presence in rat liver extracts of a cAMP binding protein devoid of kinase activity underscores the possibility that one role of the binding protein is to regulate the availability of free cAMP in the cell. The intracellular concentration of cAMP may also be protected by physiological inhibitors of the cAMP phosphodiesterases. These enzymes are known to be competitively inhibited by the only other known cyclic nucleotide, that is, cyclic GMP (30-33), and there is a recent report of a nondialyzable, heat-stable inhibitor of cAMP phosphodiesterase in \\textit{oxymyctine} cells of frog gastric mucosa (34).

With regard to possibility (a) above, namely, that \( \text{N}^4\text{-Rt-cAMP} \), formed from \( \text{Bt}_2\text{-cAMP} \) by \( \text{N}^6\text{-butyryl amidohydrolase} \), is the true imitator of cAMP, two lines of evidence support this postulate. (a) Posternak et al. (11) found that \( \text{N}^4\text{-Bt-cAMP} \) was second only to cAMP as an activator of glycogen phosphorylase in cell-free extracts of dog liver, whereas \( \text{Bt}_2\text{-cAMP} \) and \( \text{O}^6\text{-Bt-cAMP} \) exhibited only minor amounts of activity; and (b) Kaukel and Hila (7) recently reported that \( \text{N}^4\text{Bt-cAMP} \) had a high affinity for a cAMP binding protein from HeLa cells.

Finally, the identity of the two deacylases should be considered. Since no \( \text{N}^4\text{-acyl} \) derivatives of adenine nucleosides or nucleotides are known in a nature, the \( \text{N}^4\text{-butyryl amido} \) dehydrolase activity observed in the present experiments may be nonspecific. The only \( \text{N}^4\text{-acyl} \) amido\textit{hydrolases} which have been identified in animal tissues are the \( \text{N}^4\text{-acyl} \) amidohydrolase described in kidney by Birnbaum (25), which may be a cytosol enzyme (it appeared in the 4,000 rpm supernatant fluid of homogenates) and which had an optimum pH of about 7. Naturally occurring 2- and 3'-fatty acyl esters of the ribose and deoxyribose moieties of nucleotides have not been found. It is likely, therefore, that in the present experiments deacylation of \( \text{O}^6\text{-Bt-cAMP} \) was carried out by one or more nonspecific esterases. Most animal tissues contain complex mixtures of esterases with overlapping substrate specificities (26), such as the broad specificity carboxylic ester hydrolase (EC 3.1.1.18) or the more specific acetyl ester hydrolase (EC 3.1.1.6); this overlap may be due to an indiscriminate bond-breaking mechanism which will act on any ester that can approach the enzyme active center closely.

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Enzymatic Deacylation of Mono- and Dibutyryl Derivatives of Cyclic Adenosine 3', 5'-Monophosphate by Extracts of Rat Tissues
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