Effects of 6- and 8-substituted Analogs of Adenosine 3':5'-Monophosphate on Phosphoenolpyruvate Carboxykinase and Tyrosine Aminotransferase in Hepatoma Cell Cultures*

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SUMMARY

A variety of 6- and 8-substituted analogs of cAMP (cyclic adenosine 3':5'-monophosphate) have been tested for their ability to increase the activity of tyrosine aminotransferase (EC 2.6.1.5) in cultured Reuber H35 hepatoma cells. Some analogs, particularly the 8-thio-substituted ones, produced effects approximately equivalent to those generated by cAMP, whereas three other analogs actually depressed the activity of the aminotransferase. Changes in enzyme activity generated by the various analogs were paralleled closely by changes in the relative rate of aminotransferase synthesis.

An excellent correlation was found to exist between the ability of any given analog to influence the activity of tyrosine aminotransferase and that of phosphoenolpyruvate carboxykinase (EC 4.1.1.32). A similar correlation was found to exist between the ability of various analogs to elevate the activity of these enzymes and to inhibit reversibly the growth of H35 cells.

Only one of five inhibitors of cAMP phosphodiesterase activity tested produced any increase in aminotransferase activity when added alone. All of the 6- and 8-substituted analogs tested, including noninducers, stimulated f1 histone phosphorylation in crude rat liver extracts with approximately equal potencies. On the other hand, dibutyryl cAMP was only a weak activator of protein kinase in vitro, even though it is a potent enzyme inducer. A possible resolution of this apparent discrepancy has been provided by preliminary analyses of site-specific f1 histone phosphorylation in whole cells. Only compounds active as aminotransferase inducers are capable of stimulating phosphorylation of the serine-37 residue of endogenous f1 histone (3- to 10-fold).

Previous reports from this laboratory have described the stimulatory effect of N6,02'-dibutyryl cyclic adenosine 3':5'-monophosphate (BtzcAMP) on the synthesis of tyrosine aminotransferase (EC 2.6.1.5) and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) in cultured rat hepatoma cells (1-4). The evidence to date in this system suggests a post-transcriptional site of action of the cyclic nucleotide, perhaps at the polysomal level (2-4). One of the questions we would like to address is whether a cAMP-dependent protein kinase plays a central role in this process. As one means of exploring this possibility, we have made use of a variety of new analogs of cAMP for studies with intact cells. If a protein kinase does mediate the effects of cAMP analogs on enzyme induction, then one would expect, for example, to find a good correlation between the effects of any given analog on the activity of the aminotransferase and that of the carboxykinase.

In searching for nonbutyrylated analogs of cAMP that might be active as enzyme inducers, we turned to the 6- and 8-substituted derivatives prepared recently by several groups (7-10). A number of these compounds have been found to be at least equipotent to BtzcAMP in several biological systems (7-14). Most significant is the finding that many of these analogs are capable of activating protein kinase without apparent structural modification (8, 9, 13, 14), in contrast to the dibutyryl derivative (15-18). In addition, many of the compounds are resistant to hydrolysis by cAMP phosphodiesterase and several are potent inhibitors of this enzyme (8, 9, 13, 19).

It has proven difficult to establish the involvement of protein kinase in more than a small number of cAMP mediated regulatory processes (20-22). Evidence for or against the participation of this enzyme in enzyme induction under clearly physiological conditions, therefore, would be highly desirable. As will be seen, the use of a variety of cAMP analogs with diverse abilities to...
minimize the effects of Bt2cAMP has provided a means of addressing the question of protein kinase involvement in enzyme induction. Preliminary studies of the ability of these analogs to stimulate f1 histone phosphorylation in whole cells have yielded results consistent with the participation of protein kinase as a mediator of cAMP action in these cells. The present results suggest that a number of these analogs deserve consideration as possible alternatives to Bt2cAMP in other systems.

EXPERIMENTAL PROCEDURES

Methods

Cell Culture

Reuber H35 leukaemia cells (H4-11-E-3) were maintained in monolayer culture in 25-cm² Falcon plastic flasks as previously described (1-4). The growth medium consisted of Eagle's basal medium enriched 4-fold with vitamins and amino acids and contained 10% calf serum and 5% fetal calf serum but lacked penicillin and streptomycin. Under these conditions the generation time of H35 cells was 20 to 24 hours. HTC cells were grown under identical conditions and exhibited a generation time slightly shorter than H35 cells. For all but the growth experiments, cells near confluency (usually 4 to 6 days after subculture) were placed in serum-free growth medium containing penicillin G (100 units per ml) and streptomycin (100 µg per ml) for 10 to 18 hours prior to addition of cAMP analogs. Tests for Mycoplasma involving either selective culture techniques or autoradiography with [3H]-thyminidine have been negative to date.

Enzyme Assays

Tyrosine Aminotransferase and P-enolpyruvate Carboxykinase—After overnight incubation of cells in serum-free medium, various additions were made, and at the times indicated the cells were harvested. Harvesting was accomplished by removing the medium, washing the cells once with phosphate-buffered saline, and scraping the cells off the plastic surface with a rubber policeman. After brief centrifugation, the cell pellet was suspended in 0.15 m KCl-1 mM EDTA and the cells were lysed by three cycles of freezing and thawing. The lysate was then centrifuged at 20,000 × g for 30 min, and the resulting supernatant fraction was assayed for aminotransferase or carboxykinase activity, or both, as described previously (5). In experiments in which only the aminotransferase was assayed, the cells were lysed by the addition of 0.1% Non-ident 40. Soluble protein was determined by the procedure of Lowry et al. (22). Basal enzyme activities for the two enzymes during the course of these experiments ranged from 50 to 100 µg of p-hydroxyphenylpyruvate formed per 10 min per mg of soluble protein for the aminotransferase and 100 to 200 mmoI of NaH14CO3 fixed per min per mg of soluble protein for the carboxykinase.

Protein Kinase—The incorporation of [γ-32P]ATP into purified f1 histone was used as the assay for cAMP-dependent protein kinase activity. Incubation of cell or tissue extracts was continued for 20 min at 37° in the presence of 50 µM Tris (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, [γ-32P]ATP, and f1 histone in the amounts indicated. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 5%. After centrifugation (10,000 × g for 10 min), trichloroacetic acid was added to the supernatant fraction to a final concentration of 25% to precipitate f1 histone. The suspension was poured onto a Millipore filter and washed several times with 25% trichloroacetic acid. The filter was then dried and counted in a gas flow counter. Appropriate blanks were run without added histone and with trichloroacetic acid added before the enzyme preparations. Under the incubation conditions employed, the incorporation of radioactive into f1 histone was linear with respect to both time and amount of enzyme in the presence or absence of cyclic nucleotides.

The primary source of protein kinase was the supernatant fraction of rat liver resulting from centrifugation (at 20,000 × g for 30 min) of a 25% homogenate prepared in 0.15 m KCl-1 mM EDTA. For studies of protein kinase activity in H35 cells, confluent cells were placed in serum-free medium overnight. After washing once with phosphate-buffered saline, the cells were removed by scraping and collected by centrifugation. The cells were then lysed in 0.15 m KCl-1 mM EDTA by brief exposure to a Polytron homogenizer. The supernatant fraction after centrifugation of lysates at 20,000 × g for 5 min was used as the source of enzyme.

Cell thymus f1 histone was purified by Method 1 of Johns (24). [γ-32P]ATP was prepared by minor modifications of the procedure of Glynn and Chapell (25). The procedure for the preparation and isolation of the tryptic peptide containing the serine-37 residue of f1 histone has been described by Langan (20).

Rate of Aminotransferase Synthesis Measurements

Confluent cells were placed in serum-free medium overnight. Two hours before the addition of analogs, the medium was removed and fresh serum-free medium containing one-fourth the usual leucine concentration was added. Three and one-half hours after the addition of the analogs, 20 µ Ci of [4,5-3H]leucine were added. Thirty minutes later the cells were washed and harvested by scraping as above.

The pelleted cells were suspended in 0.15 m KCl-1 mM EDTA and lysed by addition of 0.1% Non-ident 40. Cell debris was removed by centrifugation (20,000 × g for 30 min). The aminotransferase was partially purified and then precipitated with specific antibodies. Details of these procedures have been extensively described previously (6, 7).

Radioactivity in total soluble protein was determined in 25-µl samples of the supernatant fraction by the filter paper disc procedure of Maas and Novelli (27), which includes incubation of the discs with 5% trichloroacetic acid at 85°.

Growth Studies

Plastic flasks (25 cm²) were inoculated at a density of 0.3 to 0.5 × 10⁴ cells. The analogs were first added 24 hours after subculture and they were added again 48 hours later with a change of medium. The next day (4 days after subculture, just past the mid-log phase), the cells were harvested as described above and lysed with 0.15 m KCl-1 mM EDTA containing 0.1% Non-ident P40. After centrifugation at 3000 × g for 10 min, the pellets were suspended in 0.1 m NaHCO₃ and assayed for their DNA content by the modified diphosphoramine procedure (28). From hemocytometer analyses coupled with DNA assays, H35 and HTC cells have been found to contain, on the average, 14 pg of DNA per cell (29).

This value was used in calculations of cell number.

Materials

All tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, N. Y. Plastic flasks used for cell growth were from Falcon Plastic Co. The various cyclic nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. (8Br-CAMP, N₈ and O₂'-monobutyryl CAMP, adenosine 2',3'-monophosphate, cAMP, cGMP, and cCMP); Boehringer-Mannheim Co., New York, N. Y. (Bt2cAMP) or were generously provided by Dr. M. Stout of the Squibb Institute. Chromatographic analysis (thin layer chromatography in two solvent systems) revealed that the 6- and 8-substituted analogs were at least 90 to 95% pure. The various phosphodiesterase inhibitors were obtained from Nutritional Biochemical Co., Cleveland, O. (theophylline, theophylline N-oxide, 8-methyltheophylline); G. D. Searle Co., Chicago, Ill. (1-methyl-3-isobutylxanthine); or were generously provided by Dr. F. F. Giarusso, of the Squibb Institute. Various cAMP analogs were obtained from Shell International Co., Ltd., London, United Kingdom. Carrier-free [3H]orthophosphoric acid and [4,5-3H]leucine were obtained from New England Nuclear Corp., Boston, Mass. Na14CO₃ was purchased from Amersham-Searle Corp., Chicago, Ill. Glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase were obtained from Boehringer-Mannheim Co., New York, N. Y. Diphenylamine was purchased from J. T. Baker Co. and was used without recrystallization.
trichloroacetic acid was used in the f1 histone experiments. All other biochemicals used were obtained from Sigma Chemical Co.

RESULTS

Time Course of Effects of cAMP Analogs on Tyrosine Aminotransferase Activity—We have previously reported that Bz2cAMP causes a rapid, nearly linear increase in tyrosine aminotransferase activity in cultured H35 cells (1-4). As shown in Fig. 1A, careful analysis has revealed that a slight lag period actually appears to precede full development of the response to the dibutyryl analog, which is eliminated when Na-monobutyryl cAMP is employed. In contrast to these active butyrylated analogs, O2'-monobutyryl cAMP is totally ineffective at 0.5 mM, although significant activity can be detected at 2 mM (Table I). Similarly, cAMP itself is only marginally effective and only at very high concentrations. Theophylline, a phosphodiesterase inhibitor (8), does potentiate this effect to a modest extent, suggesting that it may be truly ascribed to a CAMP-mediated phenomenon. A reduced response is actually obtained with the other cyclic nucleotides, including cGMP and its 8Br derivative. All analogs were added at 0.5 mM and cells were harvested for assay at the times indicated, as described under "Experimental Procedures." Each value represents the average of four to six observations, with standard errors of 5 to 10%.

The intracellular concentration of CAMP tested (20 mM). Among other inhibitors (8), does potentiate this effect to a modest extent, suggesting that it may be truly ascribed to a CAMP-mediated phenomenon. A reduced response is actually obtained with the other cyclic nucleotides, including cGMP and its 8Br derivative. All analogs were added at 0.5 mM and cells were harvested for assay at the times indicated, as described under "Experimental Procedures." Each value represents the average of four to six observations, with standard errors of 5 to 10%.

The maximal-fold increase (i.e. 100% of the maximal response) in aminotransferase activity observed with each analog is given in brackets below. A: O-...O, Na-monobutyryl cAMP (2.8); A, O2'-monobutyryl cAMP (2.7); •, O' monobutyryl cAMP (2); , Bz2cAMP (2.8); O-...O, 8MeS-cAMP (8-SC6H5) (2.6); O-...O, 8EtS-cAMP (8-SC6H5) (2.5); •...•, 8PhCH&cAMP (8-SC6H5) (2.6) [8.5].

Cyclic nucleotide added

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Tyrosine aminotransferase activity % increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>O' monobutyryl cAMP</td>
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<tr>
<td>O' monobutyryl cAMP</td>
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<tr>
<td>cAMP</td>
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<tr>
<td>cAMP</td>
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<tr>
<td>cAMP</td>
<td>10</td>
</tr>
<tr>
<td>+ theophylline</td>
<td>1</td>
</tr>
<tr>
<td>cAMP</td>
<td>20</td>
</tr>
<tr>
<td>+ theophylline</td>
<td>1</td>
</tr>
<tr>
<td>+ theophylline</td>
<td>1</td>
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<tr>
<td>Adenosine 2':3' monophosphate</td>
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</tr>
<tr>
<td>cIMP</td>
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</tr>
<tr>
<td>ethMP</td>
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</tr>
<tr>
<td>8Br-cAMP</td>
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</tr>
<tr>
<td>cMP</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*The values shown are the average percentage increase in aminotransferase activity observed in one or two experiments, with the number of separate observations in parentheses.

In this study was suboptimal (see Fig. 2), and with higher concentrations the effect persists for a longer period of time. From previous studies, it is clear that H35 cells are capable of metabolizing both Bz2cAMP and Na-monobutyryl cAMP (30), which presumably accounts for the eventual decline in the response.

The maximal elevation of enzyme activity with all of the analogs was found to occur at 3 to 4 hours. This interval is somewhat less than that expected for achievement of the maximal increase in aminotransferase activity if its rate of synthesis had been increased at time zero or shortly thereafter (31), since this enzyme has a half-life of about 17 hours (12). A true steady state condition was not achieved with most of the analogs employed. A decline in elevated enzyme activity occurred after 3 to 4 hours, for reasons as yet unknown. Increasing the concentration of analog has not been found to alter the time of peak enzyme response or its duration when this has been tested.

Dose-Response Relationships—Fig. 2 illustrates the dose-response relationships among the effective analogs for elevating tyrosine aminotransferase activity (measured at 4 hours). The three 8-thio-substituted analogs exhibited identical dose-response curves, which have been combined for simplicity. Bz2cAMP is comparably potent (1), but the Na-monobutyryl and 8Br analogs proved to be considerably less so. On the other hand, 8MeS-cAMP possesses a potency only slightly lower than the S-thio analogs. It should be noted that minimal effects can be observed with the most potent analogs, including Bz2cAMP (1), at concentrations of 5 to 10 μM. The intracellular concentration range expected to result is thus well within that of cAMP generated in rat liver by concentrations of glucagon that produce significant metabolic responses (33). In preliminary experiments, a newer analog, N4-benylamino-cAMP, has been found

A reduced response is actually obtained with the other cyclic nucleotides, including cGMP and its 8Br derivative. All analogs were added at 0.5 mM and cells were harvested for assay at the times indicated, as described under "Experimental Procedures." Each value represents the average of four to six observations, with standard errors of 5 to 10%.
FIG. 2. Dose-response relationships among CAMP analogs for effects on tyrosine aminotransferase activity in H35 cells. Analogs were added at the final concentrations indicated, and cells were harvested 4 hours later for assay, as described under "Experimental Procedures." Each value represents the average of six separate observations, with average standard errors of 5 to 10%. The maximal-fold increase (i.e. 100% of the maximal response) in aminotransferase activity is given in brackets below. The curves for the 8MeS-, 8EtS-, and 8PhCH₃-CAMP analogs (8-SCH₃, 6-SC₂H₅, and 8SC₆CH₃, respectively) were indistinguishable and have been combined [8MeS-CAMP, 2.6; 8EtS-CAMP, 3.4; 8PhCH₃-CAMP, 2.5] (O——O); ■ --- ■, N⁺-monobutyryl CAMP [4.3]; ● — ●, 8-Br-CAMP [5.5].

TABLE II

Effect of analogs of cAMP on relative rate of synthesis of tyrosine aminotransferase in cultured H35 cells

Confuent H35 cells were placed in serum-free medium for 14 hours and then into serum-free medium containing one-fourth the usual leucine concentration. Two hours later the various analogs listed were added at 0.5 mM. Three and one-half hours later 25 pCi of [3H]leucine were added and 30 min later the cells were harvested. After lysis, tyrosine aminotransferase were partially purified and incubated with antiaminotransferase antibodies. The resulting immunoprecipitate was washed three times with 0.15 M NaCl and then counted.

<table>
<thead>
<tr>
<th>Analog added</th>
<th>Tyrosine aminotransferase activity</th>
<th>Radioactivity in Tyrosine aminotransferase</th>
<th>Total soluble protein</th>
<th>Relative synthetic rate</th>
</tr>
</thead>
</table>
|                      | % of control | cpm | A/B X 10⁴ | cpm X 10⁴ | (
| None                 | 100 ± 9 (5) | 1009 ± 112 (5) | 532 ± 22 (5) | 3.0 ± 0.3 (5) |
| BtzcAMP              | 358 ± 69 (2) | 6081 ± 370 (2) | 732 ± 11 (2) | 9.1 ± 0.7 (2) |
| 8MeS-cAMP            | 288 ± 40 (2) | 4503 ± 68 (2) | 712 ± 14 (2) | 6.3 ± 0.2 (2) |
| N⁺-Monobutyryl-cAMP  | 219 ± 35 (2) | 2888 ± 453 (2) | 547 ± 47 (2) | 5.3 ± 0.3 (2) |
| 8PhCH₃S-cAMP         | 190 ± 7 (2) | 396 ± 174 (2) | 699 ± 28 (2) | 4.6 ± 0.1 (2) |
| 8EtN-cAMP            | 97 ± 11 (2) | 878 ± 115 (2) | 470 ± 6 (2) | 1.8 ± 0.2 (2) |
| 8HSEHN-cAMP          | 63 ± 2 (2) | 730 ± 6 (2) | 436 ± 4 (2) | 1.6 ± 0.0 (2) |

* The values shown are the means ± deviation, with the number of separate observations in parentheses. Each value was obtained with four pooled flasks.

to be 5 times more potent than BtzcAMP and 8-thio-substituted analogs.

Rate of Synthesis Measurements—BtzcAMP has been shown to produce its effect on tyrosine aminotransferase activity by increasing the relative rate of synthesis of this enzyme in organ culture (5), adult rat liver (6), and H35 cells (1). It was of interest, therefore, to determine whether the other active analogs of cAMP produced their effects by the same mechanism. (We have not tested all of the analogs in this regard, but the results to date leave little doubt that different mechanisms are operative.)

An excellent correlation was found to exist between the degree of effect of each analog tested on enzymatic activity and on the relative rate of enzyme synthesis (Table II). Two of the analogs tested actually depressed the activity of the enzyme and also depressed incorporation of [3H]leucine into both the aminotransferase and total soluble protein. These results suggest that the active analogs owe their ability to regulate the activity of this enzyme to the same mechanism as that influenced by BtzcAMP. Possible changes in the rate of enzyme degradation produced by by these analogs have not been tested, but BtzcAMP has been shown to be without detectable effect on this process (1).

Correlation of Analog Effects on Tyrosine Aminotransferase and P-Enolpyruvate Carboxykinase—BtzcAMP has been shown to induce both tyrosine aminotransferase and P-enolpyruvate carboxykinase in adult rat liver (6, 34) and in H35 cells (1, 2). In addition, hormones which act by way of promoting cAMP synthesis produce similar increases in the activity of both enzymes in fetal and adult rat liver (5, 6). In an effort to determine whether induction of these two enzymes is mediated by a common intracellular factor, we were interested in determining whether analogs active in elevating aminotransferase activity are also capable of influencing carboxykinase activity to a comparable extent.

With the use of a constant concentration and a fixed time interval, it was found that an excellent correlation does exist be-

* A time interval of 5 hours was employed in these studies because the activity of the carboxykinase does not reach a new
of the analogs begins to drop off by 5 hours (see Fig. 1B), this suggests that they act directly by activating protein kinase in these cells. An increase in intracellular CAMP which, in turn, could bring about the dissociation of the holoenzyme upon lysis, thereby increasing the activity of the enzyme induce by AMP in vivo. Although this relationship has not been extensively studied under a wide variety of conditions, no deviation from the observed correlation has ever been observed. The correlation coefficient for the relationship in Fig. 3 is 0.98, with a p value of <0.001. As are seen below, the dose-response relationships for increases in the activity of both enzymes generated by AMP were similar and 2-fold for the carboxykinase. The values for Analogs 1, 2, and 3 are given as percentage of decrease in control activity. 1, 6HETH-cAMP; 2, 5, 10; HET-cAMP; 3, 6HE-cAMP; 4, 6Me-cAMP; 5, 8HOcAMP; 6, 6Br-cAMP; 7, 8PhCH3S-cAMP; 8, 6EtS-cAMP; 9, 8MeS-cAMP.

TABLE III

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>Concentration</th>
<th>Tyrosine aminotransferase % increase in activity</th>
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<tr>
<td>Theophylline</td>
<td>0.1</td>
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<tr>
<td>Papaverine</td>
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</tr>
<tr>
<td>Methylisobutylxanthine</td>
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<tr>
<td>RO-20-1724</td>
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<td>32%</td>
</tr>
<tr>
<td>SQ-20006</td>
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</table>

* Actually caused a decrease in aminotransferase activity.

Effects of CAMP Analogs on Protein Kinase Activity—The other likely possible mechanism of action of CAMP analogs, namely the direct activation of protein kinase, has been tested initially with rat liver extracts for the most part. It has been reported that all of the analogs used in these studies do activate protein kinases from various tissues in vitro (8, 9, 13, 14). The activity of protein kinase (with calf thymus f1 histone as a substrate) in H35 cells extracts is stimulated by CAMP, but the degree of effect is quite variable for reasons that have not been accounted for as yet. From studies by Hohmann and Langan, and our own preliminary results, it is known that phosphorylation of the serine-37 residue of f1 histone in intact H35 cells is stimulated 3- to 10-fold by Bt2cAMP. These results demonstrate the presence of functional CAMP-dependent protein kinase activity in whole cells. The inconsistent stimulation of H35 protein kinase activity by CAMP in vitro appears to result from variable dissociation of the holoenzyme upon lysis, thereby increasing basal enzymatic activity and proportionately reducing the CAMP dependence of f1 histone phosphorylation. This explanation is supported by the fact that the ability of crude extracts from H35 cells to phosphorylate f1 histone is essentially identical with that observed in H35 cells extracts.

not the mediator of CAMP action on enzyme induction. As an indirect test of the first possibility, a number of known inhibitors of phosphodiesterase activity have been tested for their ability to elevate tyrosine aminotransferase activity.

Theophylline is capable of modestly elevating the activity of the aminotransferase in organ culture (35) and adult liver (5) and it potentiates the effects of hormones and CAMP in these systems (5, 35), but it has little or no effect in H35 cells when added alone (Table III), as reported previously (1). It is able, however, to potentiate weakly the effects of exogenous CAMP, as seen in Table I. Among other inhibitors tested, most were totally inactive, but RO-20-1724 (11, 13, 36) had a consistently modest effect on aminotransferase activity. None of these compounds, however, was capable of generating the degree of increase in enzymatic activity observed with any of the active CAMP analogs, even when concentrations greater than those indicated were employed.
Activation of rat liver protein kinase by analogs of cAMP

Fifty microliters of a 1:50 dilution of a rat liver postmitochondrial supernatant preparation were used as the enzyme source (25 mg of protein per ml, undiluted). Purified calf thymus f1 histone (0.25 mg per tube) was used as the protein substrate. All of the nucleotides were added at 1 pg. Incubation was continued for 20 min with [γ-32P]ATP (10,520 cpm per nmol; 1.29 × 10^8 cpm total), 50 mM Tris (pH 7.5), 5 mM MgCl₂, and I mM dithiothreitol at 37°C.

### TABLE IV

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Incorporation of labeled ATP into f1 histone</th>
<th>Increase in kinase activity</th>
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<tr>
<td>None</td>
<td>428 ± 24 (7)</td>
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<tr>
<td>cAMP</td>
<td>1630 ± 47 (7)</td>
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<tr>
<td>N6-Monobutyryl cAMP</td>
<td>1666 ± 22 (4)</td>
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<td>Bt6cAMP</td>
<td>1928 ± 26 (2)</td>
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<td>8MeS-cAMP</td>
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<td>8H₂N-cAMP</td>
<td>1662 ± 26 (2)</td>
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* Each value is the mean ± standard error, with the number of observations in parentheses.

### DISCUSSION

The results of these studies strongly suggest that the structural specificities for cyclic nucleotides of the intracellular components involved in the regulation of tyrosine aminotransferase and phosphocreatine carboxyl kinase synthesis by cAMP are

hours later with a change of medium. The cells were harvested 24 hours later and assayed for DNA content, as described under "Experimental Procedures." Each value is the mean of four observations in a deprived confluent cells were exposed to the analogs for 5 hours of less than 10%. For studies of carboxykinase activity, serum-deprived confluent cells were exposed to the analogs for 3 hours. Each value was obtained with four to eight separate flasks in at least two experiments.

Figure 4. Dose response relationships for the effects of Bt2cAMP and 8MeS-cAMP on growth rate and P-enolpyruvate carboxykinase activity in H35 cells. In the growth experiments the analogs were first added 24 hours after subculture and again 48 hours later with a change of medium. The cells were harvested 24 hours later and assayed for DNA content, as described under "Experimental Procedures." Each value is the mean of three observations in two separate experiments, with standard errors of less than 10%. For studies of carboxykinase activity, serum-deprived confluent cells were exposed to the analogs for 5 hours prior to harvest and assayed as described under "Experimental Procedures." Each value is the mean of four observations in a single experiment, with average standard errors of 10 to 15%.

The maximal-fold increase (i.e. 100%) of the maximal response in carboxykinase activity is given in brackets below. A, Bt2cAMP [3.3]; B, 8MeS-cAMP (8-SCH3-CAMP) [2.4].

Table VI

<table>
<thead>
<tr>
<th>Analog added</th>
<th>Effect on</th>
<th>Concentration</th>
<th>Cell density</th>
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<tr>
<td></td>
<td>Growth rate</td>
<td>Tyrosine aminotransferase</td>
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<td>Bt2cAMP</td>
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</tr>
<tr>
<td>8MeS-cAMP</td>
<td>Lethal</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

TABLE VII

Effects of analogs of cAMP on growth of cultured HTC cells

<table>
<thead>
<tr>
<th>Analog added</th>
<th>Concentration</th>
<th>Cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>7.1 ± 0.1 (3)</td>
</tr>
<tr>
<td>Bt2cAMP</td>
<td>0.5</td>
<td>8.3 ± 0.1 (4)</td>
</tr>
<tr>
<td>8MeS-cAMP</td>
<td>0.5</td>
<td>7.5 ± 0.4 (3)</td>
</tr>
<tr>
<td>8PhCH2S-cAMP</td>
<td>0.5</td>
<td>7.4 ± 0.3 (4)</td>
</tr>
</tbody>
</table>

1. All of the analogs act by inhibiting cAMP phosphodiesterase activity to various degrees, and the subsequent rise in intracellular cAMP brings about the observed response.

Although this possibility is difficult to eliminate totally, a number of facts argue against it. First of all, no rise in intracellular cAMP has been observed in preliminary experiments with H35 cells exposed to an analog active as an inducer. Second, known inhibitors of cAMP phosphodiesterase have little effect and only weakly potentiate the effects of exogenous cAMP. As in most other systems (8, 12, 13), the active analogs produce much greater responses than phosphodiesterase inhibitors in the absence of exogenous cAMP. Although permeability problems may account in part for the poor effectiveness of these inhibitors (42), it is difficult to imagine that they are all subject to the problem at the same time that all of the active analogs are not. Third, an analog that has been found to be totally inactive as an inhibitor of the partially purified phosphodiesterase from two tissues (12, 13), 8HO-cAMP, is as effective as Bt2cAMP in provoking a rise in aminotransferase activity in H35 cells and markedly enhances glucose production in liver slices (14). Finally, O2'-monobutyryl cAMP has been shown to be a better inhibitor of phosphodiesterase from four tissues than either N6-monobutyryl cAMP or Bt2cAMP (17), and yet it is much less effective as an enzyme inducer.

2. The analogs act by stimulating protein kinase directly, and (a) a number of protein kinases exist that exhibit different substrate specificities but possess similar structural specificities for cyclic nucleotide activation; or (b) a single protein kinase (or two or three closely related isozymes) is activated by the various analogs to differing degrees, and the resulting catalytic subunit phosphorylates different protein substrates specific for the particular process being regulated (i.e. induction of the aminotransferase, induction of the carboxykinase, inhibition of growth, etc.), which ultimately produces the observed differing degrees of response.

These possibilities are also difficult to evaluate with currently available information. The first seems unlikely, since more metabolic processes are influenced by cAMP in liver than exist identified multiple forms of hepatic protein kinase (1, 22, 33, 43-50). In addition, no markedly distinguishable differences in substrate or nucleotide affinity or specificity exist among the two or three reported hepatic forms (20-22, 46-50). The second possibility is most consistent with the known facts but is supported by circumstantial evidence at best. The preliminary observation that analogs active as enzyme inducers (a total of nine have been tested to date) produce marked stimulation of specific I histone phosphorylation in whole cells, whereas the three noninducing analogs tested are completely without effect.

Highly similar, if not identical. A similar, although less well documented, correlation appears to hold for cAMP analog effects on growth regulation and enzyme induction. At least two possible explanations can be envisioned to account for the observed correlations:

* Based on cell densities at 4-day cultures grown in the presence of 0.5 mM of each analog. Analogs were first added 24 hours after subculture and re-added on Day 3 with fresh medium.

* Based on the increase in tyrosine aminotransferase activity after exposure of confluent stationary phase cells to 0.5 mM of each analog for 3 hours. Each value was obtained with four to eight separate flasks in at least two experiments.

* Defined as irreversible cytotoxicity as judged by a continual decline in cell number even after withdrawal of the analog. Cells released into the medium were no longer viable, as judged by their inability to re-attach and grow after transfer to medium devoid of analogs.
on this process, provides the strongest and most direct evidence in support of protein kinase involvement. (It should be pointed out that this analysis is viewed as providing an index of the degree of activation of protein kinase in vivo rather than as implying that the process of histone phosphorylation per se is a prerequisite for induction of these two enzymes.)

The fact that cAMP-dependent phosphorylation of histone exhibits the same site specificity in H35 cells in vivo and in vitro as it does in rat liver suggests that the cAMP-dependent protein kinase present in these cells is very similar to, if not identical with, that in normal liver (20-22, 26, 39, 40, 46-50). The variability reduced dependence of this enzyme on cAMP in cell-free extracts may well be a technical problem. This suggestion is supported by the fact that the degree of protein kinase activation by cAMP analogs in intact H35 cells (3- to 10-fold) is similar to that produced by Bt2cAMP in rat liver (29, 96, 39, 40). It is possible, however, that these cells do possess a variably reduced quantity of the regulatory subunit of protein kinase, as suggested for HTC cells (51).

This model predicts that variants may be found which have lost or reduced cAMP-dependent protein kinase activity, in which case all of the responses of cAMP analogs should be altered correspondingly. Attempts to select for such variants by continuous growth of H35 cells in Bt2cAMP or other analogs have been unsuccessful to date. However, Daniel et al. (52) have isolated a variant lymphoma line, resistant to the cytolytic effects of Bt2cAMP, in which cAMP-dependent protein kinase activity is markedly reduced. In the resistant cells, the activity of cAMP phosphodiesterase is not elevated by exposure to Bt2cAMP, an event which is readily demonstrable in the sensitive cells (53).

The concept of a single protein kinase (or two or three closely related forms) and multiple protein substrates also predicts that cellular variants should be found in which one or more responses to cAMP analogs are altered or missing. The reduced response of the aminotransferase in MHC cells can be explained on this basis (30). In addition, the facts that the aminotransferase can be partially induced in HTC cells by Bt2cAMP and SMcS-cAMP (26, 34) but that the rates of growth or DNA synthesis are not inhibited by these analogs are also explicable on this basis (26, 30).

The phosphorylation of rat liver ribosomal-associated proteins has been shown to be stimulable by glucagon and Bt2cAMP in vivo (55) and by cAMP in vitro (50, 56, 57), but there is no evidence that such an event triggers alterations in ribosomal function (58). Since cAMP analogs do not seem to have any consistent major effect on overall hepatic protein synthesis (5, 6), it is possible that phosphorylation-induced changes in ribosomal function will only be demonstrable when the synthesis of a specific protein is examined. It remains to be seen whether this represents the mechanism by which cAMP analogs regulate specific protein synthesis at the translational level.

The fact that the three analogs that are lethal to H35 cells also kill HTC cells suggests that this effect can be ascribed to events that are apparently unrelated to cAMP-mediated phenomena, since the growth of HTC cells is not inhibited by cAMP analogs active as enzyme inducers. 8-Amino analogs of cAMP are also toxic to nongrowing cells, since they inhibit protein synthesis in H35 cells deprived of serum for ~20 hours (see Table III). The nature of the mechanism by which 8-amino derivatives exert their toxic effects is not clear. In the case of 6H8-cAMP, this derivative is not resistant to phosphodiesterase attack (9) and in the process is converted to 6-thioinosinic acid, a cytotoxic agent (59). It is of interest that this analog is more effective as an activator of the cGMP-dependent protein kinase from lobster tail muscle than it is as an activator of the cAMP-dependent protein kinase (9).

GMeS-cAMP is the only exception to the observed correlations in that it is a moderate enzyme inducer and yet is irreversibly toxic to H35 cells. Since the experimental conditions are different, it is conceivable that this analog could produce cAMP-like effects in nongrowing cells, leading to increases in aminotransferase and carboxykinase activities, whereas, in growing cells over an extended period of time, additional non-cAMP-mediated effects could occur. This suggestion is supported by the fact that 6MeS-cAMP does markedly inhibit the rate of growth of HTC cells and produces severe cytotoxic effects at 0.5 mM similar to those in H35 cells. These non-cAMP effects would result from formation of the corresponding 6MeS-adenylate, which is structurally related to the cytotoxic 6-thioinosinic acid and also inhibits tumor growth (59). The toxic effects of 6MeS-cAMP would be expected to predominate even though protein kinase might be activated, since the latter event apparently leads only to a reversible slowing of the growth rate (3, 29, 41).

It should be pointed out that, had we relied exclusively upon in vivo assay of protein kinase activation by the various analogs, it would have been concluded long ago that this enzyme was not likely to participate in the response of the cells to cAMP analogs. This is illustrated by the fact that 8HN-cAMP does not induce the aminotransferase but is a good activator of protein kinase in vivo (see Table II and Refs. 8 and 13 to 14). Because of the problems of possible differences in uptake or metabolism (or both) of various analogs to compounds which have different abilities to activate protein kinase or to inhibit phosphodiesterase (as appears to be the case with the 8HN-cAMP and Bt2cAMP derivatives), the effects of the analogs on protein kinase and phosphodiesterase activities have been assessed primarily in whole cells, thereby circumventing these problems. Studies of the uptake and metabolism of various analogs are planned as soon as the appropriate radioactive derivatives are synthesized.

3'5'-MonobutylcAMP and Bt2cAMP appear to derive their enhanced efficacy in whole cells, relative to cAMP itself, from their resistance to phosphodiesterase attack and possible inhibition of this enzyme in addition (8, 9, 13, 19). The activity of 3'5'-monobutylcAMP both in vivo and in vitro suggests that intracellular production of this analog may well account, in most cases, for the metabolic activity of Bt2cAMP in whole cells, since the latter is much less effective in vivo (15-17, 19). Addition of tritiated Bt2cAMP to H35 cells leads to the intracellular accumulation of both labeled N5- and O5'-monobutylcAMP and free cAMP (30). Similar results have been reported for other cultured cells (19, 60), although at least one exception

* A rough correlation has been found to exist between the relative potencies of a series of cAMP analogs as activators of the partially purified bovine brain kinase and their ability to increase tyrosine aminotransferase activity in H35 cells (4, 17). Although such a correlation is certainly compatible with the concept of protein kinase involvement in enzyme induction, unfortunately it is difficult to see how potency studies with a partially purified brain kinase preparation can adequately assess possible differences in the uptake, metabolism, and inherent ability of the various analogs to activate protein kinase in intact cultured hepatoma cells. As indicated, the best correlation analyses would seem to be that using histone phosphorylation in whole cells as an index of protein kinase activation.
Cyclic nucleotides have been noted (61). The weak effects of O'-monobutryryl cAMP in H35 cells may be the result of poor cellular penetration or the fact that it is apparently not active until converted to cAMP (which is not resistant to attack by phosphodiesterase), or both (15-17, 19). Preliminary experiments have revealed that cAMP penetrates H35 very slowly, if at all. These results presumably account for the very weak ability of cAMP as an enzyme inducer. Others have also reported that cAMP is poorly taken up and rapidly degraded in perfused liver (62) and in other tissues (18,10,60,63).

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