Relationship of Adenosine 3',5'-Monophosphate to Growth and Metabolism of *Tetrahymena pyriformis*

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The concentration of adenosine 3',5'-monophosphate (cyclic AMP) and the activity of adenylyl cyclase were determined for the first time in conjunction with cyclic 3',5'-nucleotide phosphodiesterase (phosphodiesterase) during the growth cycle of *Tetrahymena pyriformis*. High levels of cyclic AMP observed during early exponential and late stationary phases were associated with elevated adenylyl cyclase and decreased phosphodiesterase activities. Adenylyl cyclase and cyclic AMP were decreased and phosphodiesterase was increased in cells grown in glucose-supplemented medium. In contrast to findings in mammalian liver, cyclic AMP was decreased during active gluconeogenesis in *Tetrahymena*. This suggests a different modulation of carbohydrate metabolism in the two species. The results illustrate that both the content of cyclic AMP and its action as a regulatory agent in *Tetrahymena* are uniquely suited to the metabolism of this organism.

The ciliated protozoan, *Tetrahymena pyriformis*, offers a model system for the study of the evolutionary development of adenosine 3',5'-cyclic monophosphate (cyclic AMP) as a regulatory agent in intermediary metabolism. This unicellular organism synthesizes and stores large glycogen reserves through gluconeogenesis or glycogenesis if the growth medium is supplemented with glucose. In addition, the presence of an intracellular metabolic control system, perhaps similar to that in mammals, is suggested by the finding of both catecholamines (10) and serotonin (9) in *Tetrahymena*, and by the fact that glycogen content and phosphorylase activity are affected by several adrenergic reactive drugs (2). In this communication, cyclic AMP and adenylyl cyclase are identified for the first time in *Tetrahymena*, and the presence of a cyclic 3',5'-nucleotide phosphodiesterase (phosphodiesterase) is confirmed (3). Changes in the level of cyclic AMP in *Tetrahymena* during growth indicate the similarities to, as well as important differences from, higher animals as to the role this nucleotide plays in the regulation of cell metabolism.

(A portion of this work is from a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the Univ. of Wisconsin, Madison, 1971, and was presented at the 1972 Federation of American Societies for Experimental Biology meetings, Atlantic City, N.J.)

**MATERIALS AND METHODS**

**Growth of cells.** *T. pyriformis* strain E was grown axenically in the dark at 26 C under standing conditions in 2-liter flasks filled to 20% capacity with a proteose peptone base medium. In some experiments, the medium contained 1.0% glucose. Growth of the cultures was followed by determination of dry weight, optically at 600 nm or by counting cells with a Coulter counter. Cells were collected by low-speed centrifugation at 4 C at intervals corresponding to various phases of the growth curve.

**Cyclic AMP assay.** Cyclic AMP was identified in neutralized, desalted 0.6 N perchloric acid extracts of washed *T. pyriformis* by co-chromatography with 3H-cyclic AMP and other nucleotides on diethylaminoethyl-Sephadex A-25 in the chloride form (17). The product eluted with the 3H-cyclic AMP standard from the column and also from Bio-Gel P-2 (50–100 mesh) was hydrolyzed by rat brain and beef heart phosphodiesterases. The concentration of cyclic AMP in the peak eluted with the 3H-cyclic AMP was determined by the method of Brooker et al. (4), with the following modifications. The Mg concentration was 12 mM, and the AG I-X (200–400 mesh) resin was prepared in the chloride form as suggested by Jost (12).

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Cyclic 3',5'-nucleotide phosphodiesterase assay. Packed cells suspended in three volumes of 40 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.85) were treated with ultrasound for 15 s at 100 W (Sonifer Cell Disrupter, model W185D). The phosphodiesterase activity was measured by the method of Butcher and Sutherland (5) in a 22,500 × g supernatant fraction.

Adenylate cyclase activity. Adenylate cyclase activity was determined by measuring the conversion of α-32P-adenosine triphosphate (ATP) to cyclic 3',5'-AMP by a 20,000 × g precipitate fraction of a broken cell preparation homogenized and suspended in 50 mM Tris-hydrochloride (pH 7.4) and 5 mM MgCl2. The composition of the assay mixture was as follows: 1.2 mM ATP containing α-32P-ATP (sp act 20–100 Ci/mmol), 25,000 to 30,000 counts/min, 5 mM MgCl2, 60 mM theophylline, 0.04% serum albumin, 50 mM Tris-hydrochloride (pH 7.4), 50 mM phosphoenolpyruvate 0.05 mg of pyruvate kinase, and 0.4 to 0.7 mg of extract in a final volume of 0.065 ml NaF, when added, was at a 10 mM concentration. The incubation was performed for 3 min at 37 C and was terminated by rapid addition of 0.1 ml of a diluting solution which contained 100 mM ATP, 50 mM cyclic AMP, and 30,000 counts/min of 3H-cyclic AMP (sp act 10 to 20 Ci/mmol) followed by heating at 100 C for 3 min. Cyclic 3',5'-AMP was isolated from other 32P-labeled compounds by chromatography on Dowex 50W-X2 (200–400 mesh), and two BaSO4-supernatant fluids were counted in a Nuclear Chicago Mark II liquid scintillation spectrometer. Total cyclic AMP was corrected on the basis of 3H-cyclic AMP recovery, which ordinarily was between 35 to 45%. A blank for each experiment containing boiled extract and α-32P-ATP was subtracted from each experimental value. Results are expressed as the average of the triplicate assays.

Phosphopyruvate carboxylase assay. Phosphopyruvate carboxylase was assayed, by a previously described method (18), on the cytosol fraction prepared by centrifuging 0.25 M sucrose homogenates of cells at 100,000 × g for 60 min. Glycogen, which was precipitated by adding 95% ethanol to a boiled alkaline hydrolysate of washed cells, was determined by the anthrone procedure (14). Protein was measured by the biuret method (8).

Materials. 3H-cyclic AMP was prepared from Schwarz BioResearch, Inc., α-32P-ATP was obtained from New England Nuclear Corp., cyclic AMP was obtained from CalBiochem, and rat brain and beef heart cyclic nucleotide phosphodiesterase and snake venom phosphatase were obtained from Sigma Chemical Co. Resins for chromatography were obtained from BioRad Laboratories and proteose peptone was obtained from Difco. All other reagents were of the highest grade commercially available and all were prepared in sterile, deionized water.

RESULTS

Cyclic AMP was measured at intervals during the growth curve of Tetrahymena cultures (Fig. 1). The concentration of the nucleotide which was high during linear growth (doubling time 10.5 h) declined sharply and then gradually increased again during the late stationary phase of the growth curve. Cyclic AMP was 14.7 ± 1.79 pmol/10⁶ cells in young dilute cultures, decreased to 3.4 ± 0.54 pmol/10⁶ cells in early stationary culture (2.2 × 10⁸ cells/ml), and rose in late stationary cultures to 8.4 ± 1.90 pmol/10⁸ cells. Experiments were started by inoculating media with sufficient 36-h-grown cells to give an initial concentration of 3 × 10⁸ cells/ml. This would infer a low level of cyclic AMP at zero time.

Additional experiments were done to more precisely determine the relationship of adenylate cyclase and phosphodiesterase with cyclic AMP in these cultures (Fig. 2). Adenylate cyclase declines during early linear growth (as do intracellular cyclic AMP levels) and reaches its nadir at about 72 h when the culture is at the early stationary phase on the growth curve (Fig. 1). A subsequent sharp increase in activity then
occurs, and the high levels are reached again late in the stationary phase of the growth curve. Conversely, phosphodiesterase activity increases early in growth and subsequently declines, reaching a low point at the time adenylate cyclase activity is again elevated. This inverse relationship between the two enzymes is consistent with levels of intracellular cyclic AMP measured at the same time intervals. Variable amounts of cyclic AMP were consistently detected in the medium after the removal of cells by centrifugation. Further studies are necessary, however, to determine whether this represents leakage due to the isolation procedure or is an integral part of the metabolism of the organism.

Accelerated glycogenogenesis is a metabolic characteristic of Tetrahymena grown in proteose peptone under standing conditions. Glycogen stores reach a high level as the cells enter the stationary phase of the growth curve (Fig. 1). Cyclic AMP, by contrast, is highest during early and late stages on the growth curve when glycogen is not being synthesized. The subsequent rise in cyclic AMP during the late stage of culture growth (Fig. 1) may be reflected in the activation of the phosphorylase enzyme.

Data shown in Table 1, in which the level of cyclic AMP is compared with the activity of a key gluconeogenic enzyme, phosphopyruvate carboxylase, provide additional evidence demonstrating the inverse relationship between the nucleotide and active gluconeogenesis in Tetrahymena. As shown here, as well as in previous studies with Tetrahymena (18) and rat liver (19), the cytosol enzyme increases concomitantly with overall gluconeogenesis. Administration of cyclic AMP or the dibutyl derivative to rats causes a marked increase in the liver enzyme (12, 21). Consistent with the present studies, addition of this nucleotide to Tetrahymena cultures at various stages of growth did not stimulate phosphopyruvate carboxylase activity (results not shown).

To gain insight into factors which might regulate levels of cyclic AMP in Tetrahymena, the nucleotide was measured in conjunction with adenylate cyclase and phosphodiesterase in cultures grown on glucose-supplemented medium (Table 2). These cultures have elevated glycogen levels, and cyclic AMP is decreased at least fivefold compared to that of control cultures. Both decreased formation as well as increased destruction of cyclic AMP in glucose-grown cultures are indicated by the lower activity of adenylate cyclase and higher activity of phosphodiesterase. Although the adenylate cyclase of animal tissues can be activated directly by several hormones (20), there is little evidence other than the present experiments to suggest that the enzyme is responsive to catabolite repression by glucose. In fact, studies in Escherichia coli indicate that adenylate cyclase and phosphodiesterase activities do not vary in response to glucose concentration (16).

### DISCUSSION

The importance of cyclic AMP as an effector of cell metabolism in most organisms, including bacteria, is well established (20). Cyclic AMP in mammals is postulated to act through activation of a protein kinase which, in turn, phosphorylates and thereby activates or inactivates a critical enzyme. In the extensively studied lac operon system of E. coli, however, cyclic AMP does not appear to prevent catabolite repression of β-galactosidase by such a mechanism (15). In bacteria, cyclic AMP combines with a specific receptor protein similar to the regulatory subunit of protein kinase, and this complex apparently affects protein synthesis at the transcription level.

Except for the effects on catabolite repression in bacteria (15) and the aggregation phenomena in the slime mold (1), the overall physiological function of cyclic AMP and control of its metabolism in unicellular organisms have not been extensively studied. For the most part, such studies have been carried out in multicellular animals, and, within the concept of the second messenger hypothesis, one of the chief functions of cyclic AMP seems to be to promote mobilization of energy-yielding substrates from their sites of storage (20). The most striking effects of its actions are activation of glycogenolysis, lipolysis, and gluconeogenesis. The close functional similarity of cyclic AMP in lower and higher animals is implied, however,

### Table 1. Comparison of cyclic AMP levels with phosphopyruvate carboxylase activity during glycogenogenesis in Tetrahymena

<table>
<thead>
<tr>
<th>Time of growth (h)</th>
<th>Cyclic AMP</th>
<th>Phosphopyruvate carboxylase</th>
<th>Glycogen (mg/100 mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/10⁶ cells</td>
<td>pmol/g wet wt</td>
<td>(nmol per min per mg of protein)</td>
</tr>
<tr>
<td>12</td>
<td>14.7 ± 1.79</td>
<td>3612 ± 438</td>
<td>91 ± 3.8</td>
</tr>
<tr>
<td>48</td>
<td>6.8 ± 1.01</td>
<td>645 ± 52</td>
<td>385 ± 10.3</td>
</tr>
</tbody>
</table>

* Determinations were carried out on cells grown for 12 and 48 h in 2% protease peptone medium.

*Standard error. Average of at least five values.
by evidence showing lower levels of this nucleotide in organisms grown in glucose-supplemented media (13) as well as in rat liver perfused with glucose and insulin (11).

In *Tetrahymena* the temporal relationship of elevated adenylate cyclase and cyclic AMP level in early exponential growth imply an important effect of this nucleotide on some metabolic parameter associated with protein synthesis and cell division. In recent studies (unpublished data), the activity of a cyclic AMP-dependent protein kinase has been found to follow the same pattern as cyclic AMP during the growth curve shown here.

In the context of the proposed physiological function of cyclic AMP in mammals, the present results depicting its reciprocal relationship to carbohydrate synthesis may initially appear anomalous, but, on further examination, are actually consistent with the metabolism of *Tetrahymena*. In mammals, high levels of cyclic AMP are necessary to maintain blood glucose by gluconeogenesis and glycolysis. The in vitro addition of this nucleotide to perfused liver stimulates the synthesis of glucose (7), whereas injection into the intact animal induces formation of certain glucoenzcogenic enzymes (12). However, in *Tetrahymena*, glycogen, not glucose, is the final product of gluconeogenesis. High levels of cyclic AMP during gluconeogenesis would inevitably inhibit the glycogen synthetase enzyme, thereby acting as an inhibitor of net glycogen formation. Thus, cyclic AMP remains low until late stationary growth when glycogen is utilized for energy and the nucleotide is necessary to stimulate the phosphorylase enzyme system. It thus appears appropriate for *Tetrahymena* that regulation of carbohydrate metabolism by cyclic AMP occurs at the level of the glycogen synthetase and phosphorylase enzymes. The positive effect of cyclic AMP on liver gluconeogenesis somewhere between pyruvate and phosphopyruvate (7) with its concomitant induction of the key glucoenzcogenic enzyme phosphopyruvate carboxylase (12) has been demonstrated in mammals. This control system appears to have occurred later in evolution with the development of discrete organs to carry out various metabolic sequences. Unless there was adequate compartmentation, it would be inappropriate for a unicellular organism, such as *Tetrahymena*, to have cyclic AMP act as a positive effector on gluconeogenesis at the level of phosphopyruvate formation while its negative effect occurs simultaneously at the glycogen synthetase step.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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**Table 2. Effect of glucose on parameters of cyclic AMP metabolism in Tetrahymena**

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Glycogen (mg/100 mg of protein)</th>
<th>Cyclic AMP (pmol/g wet wt)</th>
<th>Adenylate cyclase</th>
<th>Cyclic nucleotide phosphodiesterase (nmol per min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose absent</td>
<td>26 ± 8.5*</td>
<td>3500 ± 325</td>
<td>Basal 11.6 ± 1.2</td>
<td>21.1 ± 2.1</td>
</tr>
<tr>
<td>Glucose present</td>
<td>86 ± 4.5</td>
<td>668 ± 45</td>
<td>NaF 2.6 ± 0.3</td>
<td>30.0 ± 3.8</td>
</tr>
</tbody>
</table>

* Cells were grown either in 2% proteose peptone alone or supplemented with 1% glucose. Determinations are representative of cells from cultures in early exponential growth.

* Standard error. Average of at least five values.