Effects of Guanosine 3',5'-Monophosphate on Glycerol Production and Accumulation of Adenosine 3',5'-Monophosphate by Fat Cells

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SUMMARY

The effects of guanosine 3',5'-monophosphate (cyclic GMP) on glycerol production by isolated fat cells resembled those of adenosine 3',5'-monophosphate (cyclic AMP) but were of lesser magnitude. In Krebs-Ringer phosphate medium, both nucleotides had little or no effect on basal lipolysis. Inhibition of theophylline-stimulated lipolysis by cyclic GMP was less and more variable than that by cyclic AMP. In phosphate 0.8% NaCl medium, with Na⁺ as the only cation, both nucleotides stimulated glycerol production. The increase was directly related to the concentration of each. On a molar basis, cyclic GMP was less effective than cyclic AMP in stimulating lipolysis. Lipolytic effects of cyclic GMP were also observed in the presence of cyclic AMP and theophylline at concentrations that did not maximally stimulate lipolysis.

During incubation of fat cells with cyclic GMP in either medium, accumulation of cyclic AMP was observed. The magnitude of this increase in cyclic AMP was related to the concentration of cyclic GMP over the range of 0.1 to 5 mm. In the presence of 1 mm cyclic GMP, the rate of accumulation of cyclic AMP was essentially constant for 2 hours. After incubation with cyclic GMP, the amount of cyclic AMP in the medium was apparently as high as in cells plus medium. The accumulation of cyclic AMP in the presence of cyclic GMP was not affected by prostaglandin E₁ (5 μg per ml), insulin (12 milliunits per ml) or probenecid (0.1 mm). The latter agent did not alter the distribution of cyclic AMP between cells and medium whether or not cyclic GMP was present. The rate of accumulation of cyclic AMP in the presence of 1 mm cyclic GMP was 10 to 20% of that observed during the first 4 min after epinephrine (1.1 μm) was added to fat cells. During the 4 min after epinephrine was added, the accumulation of cyclic AMP was almost entirely intracellular and was not altered by the presence of cyclic GMP. After incubation for more than 30 min under all conditions, a large fraction of the cyclic AMP in the system was found in the medium.

Cyclic GMP was 30 to 100 times more effective on a molar basis than was theophylline in preventing the degradation of cyclic AMP by extracts from fat cells. Cyclic GMP also inhibited degradation of cyclic AMP by homogenates of rat kidney and liver. This effect may account for the accumulation of cyclic AMP that occurred when kidney cortex, liver, and chicken erythrocytes were incubated with cyclic GMP with or without hormone.

It is generally believed that catecholamines and several polypeptide hormones stimulate lipolysis in adipose tissue by enhancing the activity of adenyl cyclase and thereby increasing the concentration of adenosine 3',5'-monophosphate (1, 2). The mechanism by which cyclic AMP brings about activation of the so-called hormone-sensitive lipase, however, remains to be elucidated. Despite the evidence that cyclic AMP stimulates lipolysis when generated and accumulated within the cell, exogenous cyclic AMP can either stimulate or inhibit lipolysis in fat cells, depending on the cationic composition of the incubation medium. In Krebs-Ringer phosphate medium exogenous cyclic AMP inhibits the lipolytic effects of theophylline or epinephrine, whereas in medium with Na⁺ as the only cation, exogenous cyclic AMP markedly enhances glycerol production by fat cells (3).

Guanosine 3',5'-monophosphate (4, 5) and enzymes for its synthesis (6, 7) and degradation (8) have been found in several tissues. Administration of theophylline to rats caused elevation of cyclic GMP levels in small intestine (9) and in kidney (4) but not in liver (4). Hepatic cyclic AMP content was increased by epinephrine and glucagon with no change in that of cyclic GMP (4). The urinary excretion of cyclic GMP was diminished in hypophysectomized rats, but that of cyclic AMP was unchanged (10). Under other conditions, the excretion of cyclic AMP can be altered without affecting that of cyclic GMP (5).

Effects of cyclic GMP in rat liver (11, 12) and adrenal (11) are quite similar to those produced by cyclic AMP, i.e., both nucleotides stimulated hepatic glucose release, glycogenolysis, gluconeogenesis, and induction of tyrosine aminotransferase and adrenal steroidogenesis. Enhanced glycogenolysis was accompa-

1 The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; cyclic GMP, guanosine 3',5'-monophosphate; cyclic XMP, xanthosine 3',5'-monophosphate; cyclic CMP, cytidine 3',5'-monophosphate; cyclic UMP, uridine 3',5'-monophosphate; cyclic TMP, thymidine 3',5'-monophosphate.
panied by increased phosphorylase activity and decreased glycogen synthetase activity (11, 13). Although the mechanism of these effects remains to be elucidated, it has been reported that cyclic GMP increases the activity of protein kinase (14, 15) and phosphorylase (16) in broken cell preparations.

As reported below, we have found that cyclic GMP, although less effective than cyclic AMP, increased glycerol production in isolated fat cells incubated in phosphate saline medium and had little or no stimulatory effect in Krebs-Ringer phosphate medium. In both media, however, cyclic GMP caused accumulation of cyclic AMP. Several studies undertaken to elucidate the mechanism of this latter effect have led us to conclude that it is secondary to inhibition of cyclic AMP degradation by cyclic GMP. Some of these observations have been reported in abstract form (17).

METHODS

Procedures for the preparation and incubation of rat epididymal fat cells and the determination of glycerol are described in an earlier publication from this laboratory (3). Fat cell weight was estimated from the amount of hexane-extractable, hydroxamate-reactive ester (18) assuming 3 μeq of ester equivalent to 1 mg of cells. Krebs-Ringer phosphate medium was used during the incubation of tissue with collagenase. Cells were washed, suspended, and incubated in this or in medium containing Na+ as the only cation. The latter, referred to as phosphate-0.8% NaCl medium, was prepared by combining 10 ml of 0.1 M sodium phosphate buffer, pH 7.4, and 110 ml of 0.154 M NaCl. Both media contained bovine serum albumin, 30 mg per ml. Cells (25 to 50 mg) were incubated at 37° in polyethylene vials containing vials in a total volume of 2.5 ml of medium. Samples (0.5 ml) of cells in control incubations in both media and in the presence of cyclic plus medium were taken at zero time and at several times thereafter for determination of glycerol. Since glycerol production in supernatant fractions was purified on columns of Dowex 2-Cl, as previously described (19). Eluates of blank portions of papers developed in the solvent systems described above had no effect on the assay system.

In experiments in which cyclic AMP was determined, each incubation vial contained 60 to 100 mg of cells in 2.5 ml of medium. At the end of the incubation period, contents of 2 or 3 vials were pooled for assay of total cyclic AMP (cells plus medium), and treated as described above. The remainder was immediately centrifuged at 1000 x g for 1 min. A sample of medium was then aspirated from beneath the floating fat cells, added to HCl, and heated. Cells were washed twice with fresh medium before addition of HCl. The time between removal of vials from the 37° incubation and addition of acid was about 15 sec for samples of cells plus medium, 2 min for medium, and 5 min for washed cells.

To investigate the degradation of cyclic AMP in fat cell extracts, fat cells were prepared and washed in Krebs-Ringer phosphate medium at 37° as described above. Cells were then washed once with a solution containing 2 mM glycyglycine buffer (pH 7.4), 1 mM MgSO₄, and 0.1 mM dithiothreitol. After centrifugation at 1000 x g for 1 min, the fluid was aspirated from beneath the floating cells. Cells derived from 4 g of adipose tissue were suspended in 4 volumes of the above mixture and frozen, with stirring, in a Dry Ice-ethanol bath. The solidified suspension was then thawed at room temperature and the hard-like fat removed. The remaining infranatant solution was used immediately or stored in small volumes at -80° until use.

Degradation of cyclic AMP was measured using a modification of a previously described method (8). Incubations of [3H]cyclic AMP with fat cell extract were terminated by heating at 100° for 2 min and adding 50 μl of a solution containing 1.5 mM cyclic
AMP and 5 mM 5’-AMP. After cooling, the [3H]5’-AMP formed during the initial incubation was converted to [3H]adenosine by incubating with 50 μl of a solution containing 2 mg per ml of lyophilized Crotalus adamanteus venom in 0.5 mM Tris buffer, pH 8.0, at 37°C for 30 min. At the end of this time, 0.2 ml of water was added and the samples (0.6 ml) were mixed and centrifuged. A sample of the supernatant fluid (0.5 ml) was placed on an anion-exchange column (0.5 x 3.0 cm) of QAE-Sephadex A-25 and followed by addition of 2.0 ml of water. The 2.5 ml of effluent containing [3H]adenosine was collected in a counting vial. Fifteen milliliters of the scintillation mixture described by Bray (20) were added. Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Company. Solutions of L-epinephrine were prepared from the bitartrate. Insulin and glucagon were gifts from Eli Lilly Company, through the courtesy of Dr. O. K. Behrens. Theophylline was purchased from Nutritional Biochemicals. Probenecid was a gift from Dr. C. A. Stone, Merck Institute for Therapeutic Research. Prostaglandin E1 was provided by Dr. J. E. Pike, The Upjohn Company. Lyophilized Crotalus adamanteus venom was purchased from Sigma Chemical Company. Cyclic 3’,5’-nucleotide phosphodiesterase, prepared from bovine heart according to the method of Butcher and Sutherland (22), was kindly donated by Dr. A. G. Gilman.

During the course of this work, several preparations of crude collagenase, purchased from Worthington Biochemical Company and Nutritional Biochemicals, were used. Responsiveness of cells to theophylline or hormones was not related to the source of the collagenase used in their preparation. Cyclic AMP (free acid) was purchased from Calbiochem; ATP and ADP (sodium salts) from Sigma; 2’-GMP and 3’-GMP (sodium salts) from Nutritional Biochemicals; 5’-GMP, GDP, and GTP (sodium salts) from Sigma Chemical Company. Cyclic 3’,5’-nucleotide phosphodiesterase, prepared from bovine heart according to the method of Butcher and Sutherland (22), was kindly donated by Dr. A. G. Gilman.

FIG. 1. Effect of cyclic AMP (cAMP) on glycerol production in Krebs-Ringer phosphate and phosphate saline media. Cells were prepared in Krebs-Ringer phosphate (KRP) medium and divided into two portions. A, cells were washed and incubated in phosphate saline medium with or without cyclic AMP; B, cells were incubated in Krebs-Ringer phosphate medium with or without cyclic AMP for 30 min prior to the addition of theophylline (T). The values for glycerol production are the mean of either two or three incubations. Vertical lines indicate range.

FIG. 2. Effect of cyclic AMP on glycerol production in phosphate saline medium. Cells were incubated with or without cyclic AMP for 60 min. After an initial delay, the rate of glycerol production was essentially constant between 30 and 60 min. The mean values of triplicate incubations for glycerol produced during 15 min of this period are indicated.

RESULTS

Effects of Cyclic AMP and Cyclic GMP on Glycerol Production—When cells were incubated in phosphate saline (all Na+) medium in the presence of 1 mM cyclic AMP, there was usually a lag period of about 15 min before glycerol production reached a maximal rate which was then constant for approximately 60 min (Fig. 1A). This lag period was not observed when cells were incubated in phosphate saline medium for 30 min prior to the addition of cyclic AMP. The maximal rates of glycerol production were similar under both conditions. The relationship between the rate of glycerol production (after it became constant) and the concentration of cyclic AMP in phosphate saline medium is shown in Fig. 2. In this medium apparently...
maximal stimulation of glycerol production was induced by 1 mM cyclic AMP.

In phosphate saline medium, after a lag period of 60 min, cyclic GMP also enhanced the rate of glycerol production (Figs. 3 and 4). As shown in Fig. 3 and Table I, the rate of glycerol production (after it became constant) was directly related to the concentration of cyclic GMP although cyclic GMP was less effective in stimulating lipolysis than was an equimolar concentration of cyclic AMP. The effects of 1 mM cyclic GMP and a concentration of cyclic AMP (0.4 mM) which did not maximally stimulate glycerol production were at least additive, whereas no effect of 1 mM cyclic GMP was observed in the presence of 1 mM cyclic AMP, which itself produced a maximal rate of glycerol production in phosphate saline medium (Figs. 2 and 3). Lipolytic effects of cyclic GMP were also observed in the presence of several concentrations of theophylline (Table I, Fig. 4). At 1 mM, none of the other nucleotides tested (5'-GMP, 2'-GMP, GDP, GTP, 5'-AMP, ADP, ATP) was more than 25% as effective as cyclic GMP in enhancing glycerol production in phosphate saline medium.

In Krebs-Ringer phosphate medium, both 1.0 and 0.1 mM cyclic AMP markedly inhibited glycerol production stimulated by theophylline, and 0.001 mM cyclic AMP decreased the effect of theophylline by about 33% (Fig. 1B). The effects of cyclic GMP in this medium were smaller and more variable than those of cyclic AMP. In the experiment of Fig. 5A, 1 mM cyclic GMP inhibited the initial rate of theophylline-stimulated lipolysis by approximately 24%, 2 mM by approximately 50%. In this experiment 2 mM cyclic GMP also inhibited the stimulatory effect of epinephrine by 40% (data not shown). In the experiments shown in Fig. 5B, inhibition by 1 or 2 mM cyclic GMP was less than 10%, whereas in both experiments, 1 mM cyclic AMP markedly inhibited theophylline-stimulated glycerol production. For these experiments, cyclic AMP (Sigma lot 69B-7420, <0.5% ammonium salt) was dissolved in water and neutralized with NaOH. Similarly variable results were obtained when the free acid was dissolved directly in Krebs-Ringer phosphate medium or when sodium salts from Sigma or Boehringer Mannheim (lot 6508306) were used.

In early experiments, a preparation of cyclic GMP (Sigma lot 77B-7522, supplied as the NH$_4^+$ salt) inhibited the effects of theophylline and several hormones in Krebs-Ringer phosphate medium, stimulated glycerol production in saline phosphate medium, and inhibited the lipolytic effects of cyclic AMP in the latter medium (17). The inhibition of cyclic AMP-stimulated lipolysis in phosphate saline medium has since been shown to be related to the presence of ammonium ion.

![Graph](http://www.jbc.org/)

**FIG. 3.** Effects of cyclic GMP and cyclic AMP on glycerol production in phosphate saline medium. Cells were incubated with or without cyclic GMP for 30 min prior to the addition of cyclic AMP. Glycerol production is presented as the mean of duplicate or triplicate incubations.

**FIG. 4.** Effects of cyclic GMP and theophylline on glycerol production in phosphate saline medium. Cells were incubated with or without cyclic GMP for 30 min prior to the addition of theophylline. Glycerol production is presented as the mean of duplicate or triplicate incubations.

**TABLE I**

| Nucleotide added | Concentration | Effect of theophylline concentration on glycerol production |
|------------------|---------------|----------------------------------------------------------|
|                  | mm            | 0  | 0.05 mm | 0.1 mm | 0.2 mm |
| None             | 0             | 1.5 | 3.2 | 16.3 |
| Cyclic GMP       | 0.4           | 4.2 | 8.8 |
| Cyclic GMP       | 1.0           | 14.2 | 15.8 | 18.5 | 23.9 |
| Cyclic AMP       | 2.0           | 13.3 | 20.8 |
| Cyclic GMP       | 3.0           | 16.4 |
| Cyclic AMP       | 0.4           | 21.0 |

Effects of cyclic GMP, cyclic AMP, and theophylline on glycerol production.

Cells were incubated in phosphate saline medium with or without cyclic GMP for 45 min before theophylline or cyclic AMP was added. Glycerol production was constant for 30 to 60 min after addition of theophylline and cyclic AMP. The values for glycerol production are means of duplicate incubations for 20 min during this period.
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Fig. 5. Effects of cyclic GMP, cyclic AMP, and theophylline on glycerol production in Krebs-Ringer phosphate medium. Cells were incubated with or without cyclic GMP or cyclic AMP for 60 min (A), or 30 or 70 min (B), prior to the addition of theophylline. Glycerol production is presented as the mean of two or three incubations. Since glycerol production in controls or in the presence of cyclic GMP or cyclic AMP alone was less than 1 μmole per g per 60 min, these values are not represented in the figure. O—O, theophylline 0.2 mM; ●—●, theophylline 0.1 mM; ○—○, theophylline 0.1 mM plus 1 mM cyclic GMP; △—△, theophylline 0.1 mM plus 2 mM cyclic GMP; □—□, theophylline 0.1 mM plus 1 mM cyclic AMP.

Fig. 6. Accumulation of cyclic AMP in cells plus medium in the presence of cyclic GMP. The concentration of cyclic GMP was 1 mM. Each point represents the cyclic AMP content of one pool of cells plus medium. Glycerol production in all incubations was less than 3 μmoles per g per 60 min.

Effects of Cyclic GMP on Cyclic AMP Levels—As shown in Fig. 6, when cells were incubated in Krebs-Ringer phosphate medium in the presence of 1 mM cyclic GMP, the amount of cyclic AMP in cells plus medium increased at a nearly constant rate for 60 min, while glycerol production remained unaffected.

Fig. 7. Effect of cyclic GMP concentration on cyclic AMP content of cells plus medium. Cells were incubated for 30 min with or without cyclic GMP as indicated. Data are reported as in Fig. 5.

The amount of cyclic AMP accumulated in 30 min was directly related to the concentration of cyclic GMP (Fig. 7). After incubation with 1 mM cyclic GMP for 1 hour, the concentration (mean ± standard error) of cyclic AMP was 5.9 ± 0.46 μmoles per g (n = 27). In 42 control incubations it was 0.76 ± 0.8. During incubation for up to 2 hours in Krebs-Ringer phosphate medium, there was no significant change in the cyclic AMP content of cells plus medium, whereas in the presence of 1 mM cyclic GMP, the accumulation of cyclic AMP proceeded at a constant rate during this period (e.g. 3.4 μmoles per g in 1 hour, 7.7 in 2 hours). Neither insulin, 12 milliunits per ml, nor prostaglandin E1, 5 μg per ml, concentrations which inhibited the lipolytic effects of epinephrine, altered the effects of cyclic GMP on cyclic AMP accumulation.

Although not a concentration which produces a maximal stimulation of glycerol production in Krebs-Ringer phosphate medium (Fig. 5), 0.1 mM theophylline always enhanced glycerol production, albeit to a variable extent. The effects of 0.1 mM theophylline on cyclic AMP levels were also variable, i.e. 2- to 3-fold increases in cyclic AMP levels were observed in two-thirds of the experiments. On the other hand, although 1 mM cyclic GMP did not significantly stimulate glycerol production in Krebs-Ringer phosphate medium (see legends to Figs. 5 and 6), cyclic AMP levels were always elevated. In the experiments shown in Table II, 0.1 mM theophylline increased cyclic AMP levels approximately 100 to 150% over control values, whereas cyclic GMP increased cyclic AMP levels 8- to 9-fold (Experiments 1 and 2). In the presence of both theophylline and cyclic GMP, cyclic AMP levels were higher than in the presence of theophylline alone and comparable to those in the presence of cyclic GMP alone. Under these conditions, glycerol production was less than that with theophylline alone. In phosphate
saline medium, 0.1 mM theophylline more than tripled the level of cyclic AMP, and the effect of cyclic GMP (or without theophylline) was also greater than that usually observed (Experiment 4, Table II). In another experiment (Experiment 5, Table II) in phosphate saline medium, cyclic AMP levels were increased 8- and 12-fold in the presence of 1 mM cyclic GMP without and with 0.1 mM theophylline, respectively. Thus there was a dissociation between cyclic AMP levels and glycerol production since cyclic AMP levels were elevated whether cyclic GMP stimulated (phosphate saline medium), inhibited, or did not affect glycerol production (Krebs-Ringer phosphate medium).

In Krebs-Ringer phosphate medium (Experiment 3, Table II, and others not shown), 1 mM cyclic TMP, cyclic XMP, cyclic 3'-AMP did not affect glycerol production or cyclic AMP levels. Both cyclic TMP and 5'-AMP inhibited theophylline-stimulated glycerol production but neither nucleotide significantly influenced cyclic AMP levels in the presence of theophylline. 5'-GMP increased glycerol production and cyclic AMP levels to a small and variable extent. Since some of the nucleotides used in these experiments were added as ammonium salts, it should be noted that 1 mM NH₄Cl did not alter cyclic AMP levels in the presence or absence of theophylline. In several experiments both the ammonium and sodium salts of two cyclic GMP preparations were tested. Both salts of both preparations (Sigma lot 77B-7522 and lot 69B-7420) increased cyclic AMP levels in phosphate saline and in Krebs-Ringer phosphate medium, and their effects on cyclic AMP accumulation were destroyed after incubation with a phosphodiesterase preparation from bovine heart.

Distribution of Cyclic AMP Between Cells and Medium—Fig. 8 summarizes data from three experiments in which the cyclic AMP content of medium alone was determined, as well as that of cells plus medium. In control incubations, the percentage of total cyclic AMP in the medium varied widely. The mean (± standard error) percentage in the medium after 1 hour of incubation for 34 min with 1 mM cyclic GMP, 2.9 mmoles of cyclic AMP per g accumulated, essentially all of which appeared in the medium. In the presence of cyclic AMP with or without epinephrine and theophylline, essentially all the cyclic AMP that accumulated during the incubation period was found in the medium. As seen also in Fig. 8, the apparent concentration of cyclic AMP in the medium often exceeded that in cells plus medium after incubation with cyclic GMP. These observations remain unexplained since known quantities of added cyclic AMP were recovered when incubated in Krebs-Ringer phosphate medium (without cells) with or without cyclic GMP or when added just prior to assay to purified fractions prepared from cells plus medium incubated with or without cyclic GMP.

In the experiment summarized in Table III, during incubation for 34 min with 1 mM cyclic GMP, 2.9 mmoles of cyclic AMP per g accumulated, essentially all of which appeared in the medium. When epinephrine, 1.1 μM, was added for the last 4 min of this period, a total of 2.4 μmoles per g was accumulated. The increment of 2.5 μmoles of cyclic AMP per g in the 4 min after epinephrine was added was similar to that (2.2 μmoles per g) produced by the addition of epinephrine to incubations without cyclic GMP. Despite the fact that the total amount of cyclic AMP that accumulated in the system after a 4-min incu-

| Table II |

| Effect of cyclic GMP and other nucleotides on glycerol production and cyclic AMP accumulation in cells plus medium |

Cells were incubated with or without 1 mM nucleotide as indicated, for 30 min. Theophylline, 0.1 mM, was then added where indicated, and the incubation was continued for 30 min more. Glycerol production during the second 30 min (mean of duplicates) is reported. Cyclic AMP levels, cells plus medium, are reported as the mean of duplicate samples which are given in parentheses.

| Additions                       | Glycerol produced | Cyclic AMP content |
|--------------------------------|-------------------|--------------------|
|                                | μmoles/g in 30 min | μmoles/g           |
|                                |                   |                    |
| None                           | 0                 | 1.0 (0.9, 1.1)     |
| Cyclic GMP                     | 0.1               | 9.0 (9.0, 9.0)     |
| Theophylline                   | 32.8              | 2.4 (2.0, 2.9)     |
| Cyclic GMP plus theophylline   | 16.3              | 6.4 (5.4, 7.3)     |
|                                |                   |                    |
| None                           | 0.2               | 0.5 (0.5, 0.5)     |
| Cyclic GMP                     | 0.2               | 4.4 (4.3, 4.4)     |
| Theophylline                   | 15.3              | 1.0 (0.8, 1.2)     |
| Cyclic GMP plus theophylline   | 5.4               | 6.4 (4.8, 8.4)     |
|                                |                   |                    |
| None                           | 1.0               | 0.4 (0.3, 0.5)     |
| Cyclic GMP                     | 0.4               | 10.4 (10.2, 10.6)  |
| Cyclic TMP                     | 0.1               | 0.6 (0.5, 0.7)     |
| Theophylline                   | 21.6              | 0.8 (0.7, 0.8)     |
| Cyclic TMP plus theophylline   | 15.0              | 0.9 (0.8, 1.0)     |
|                                |                   |                    |
| None                           | 0.8               | 1.3 (1.2, 1.4)     |
| Cyclic GMP                     | 1.1               | 23.4 (24.4, 27.3)  |
| Theophylline                   | 8.7               | 4.4 (3.9, 4.9)     |
| Cyclic GMP plus theophylline   | 8.5               | 45.7 (30.1, 61.3)  |
|                                |                   |                    |
| None                           | 0                 | 0.6 (0.7, 0.5)     |
| Cyclic GMP                     | 3.2               | 4.8 (4.6, 5.4)     |
| Theophylline                   | 4.3               | 1.4 (0.9, 1.8)     |
| Cyclic GMP plus theophylline   | 11.0              | 7.2 (6.8, 7.6)     |

* Phosphate saline medium was used in Experiments 4 and 5; Krebs-Ringer phosphate medium in all others.

![Fig. 8. Effects of epinephrine (L-EPI), theophylline (THEO), and cyclic GMP (c-GMP) on cyclic AMP content of cells plus medium and of medium. Cells were incubated in phosphate saline (A) or Krebs-Ringer phosphate medium (B and C) for 30 min with or without 1 mM cyclic GMP. Either theophylline, 0.1 mM, or epinephrine, 1.1 μM (both), was then added where indicated and the incubation continued for 30 min. The hatched bars represent the mean cyclic AMP content of cells plus medium, and the solid bars that of medium alone. Vertical lines indicate the range of duplicates.](http://www.jbc.org/.../by guest on March 24, 2020)
cubulation with epinephrine was similar to that after a 34-min incubation with cyclic GMP, essentially no cyclic AMP accumulated in the medium during 4 min of incubation with epinephrine alone. During this 4-min period, the rate of accumulation of cyclic AMP (cells plus medium) was 550 μmoles per g per min. The rates of cyclic AMP formation were presumably somewhat greater. In incubations with 1 μM cyclic GMP, the rate of accumulation of cyclic AMP (based on Fig. 6 and other experiments) was only 10 to 20% of this figure.

Although the concentration of cyclic AMP in cell water can be calculated by difference when the amount in the medium is not a large fraction of the total, as was the case after brief incubations with epinephrine, the resulting values are undoubtedly not very precise. When most or all of the cyclic AMP appears in the medium, as in incubations with cyclic GMP or epinephrine plus theophylline, such calculations are meaningless if not impossible. We attempted, therefore, to estimate directly the cyclic AMP content of cells incubated with cyclic GMP or cyclic AMP. The observed concentration of cyclic AMP in all samples of washed cells in several experiments was between 4.5 and 10 μM (in cell water) with no significant differences between cells incubated with or without epinephrine or cyclic GMP. We attribute the similarity in measured cell levels after incubation under varying conditions to either degradation or loss (or both) of cyclic AMP during the several minutes required for washing of cells.

Davoren and Sutherland (23) found that probenecid interfered with the movement of cyclic AMP from pigeon erythrocytes into the incubation medium. When fat cells were incubated with or without cyclic GMP, 0.1 mM probenecid did not alter the accumulation or distribution of cyclic AMP. The accumulation and distribution of cyclic AMP under several conditions also was unaltered when cells were prepared with collagenase from two different sources.

Effects of Cyclic GMP on Degradation of Cyclic AMP—Under conditions in which cyclic AMP hydrolysis was proportional to enzyme concentration and time of incubation, crude extracts of fat cells were found to contain cyclic 3′,5′-nucleotide phosphodiesterase activity with at least two apparent Michaelis constants, as shown in Figs. 9 and 10. The higher \( K_m \) varied from 0.02 to 0.12 mM and the lower \( K_m \) was consistently 2 to 5 μM. In experiments in which a single enzyme preparation was tested with a wide range of substrate concentrations, the data yielded double reciprocal plots with an inflection point indicating two apparent \( K_m \) values for cyclic AMP. In these and other experiments, cyclic GMP was 30 to 100 times more effective than theophylline in preventing the degradation of cyclic AMP. Inhibition by cyclic GMP appeared to be competitive or non-

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### Table III

Effects of cyclic GMP and epinephrine on cyclic AMP accumulation and distribution between cells and medium

| L-Epinephrine | Cyclic GMP | Cyclic AMP content | Cells plus medium | Medium |
|---------------|------------|--------------------|------------------|--------|
| 1.1 μM | 1 mM | μmoles/g | 0.7 | |
| 0 | 0 | 1.0 (1.0, 1.1) | 0.7 | |
| 0 | + | 5.8 (3.4, 4.0) | 4.8 | |
| + | 0 | 3.2 (3.2, 3.3) | 0.8 (0.8, 0.8) | |
| + | + | 6.4 (5.5, 7.4) | 5.9 (5.1, 6.7) | |

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3 Assuming that intracellular water equals 50% of cell weight, with 40 μg of cells per ml of medium, there would be only 2 μl of cell water per ml of medium water. Even if the concentration of cyclic AMP in cell water were 100 times that in medium water, 80% of the total cyclic AMP would be in the medium.
TABLE IV

Effect of cyclic GMP on cyclic AMP accumulation in other tissues

| Addition | Cyclic AMP, tissue plus medium |
|----------|-------------------------------|
|          | μmoles/g | μmoles/mg protein |
| Kidney cortex |         |                 |
| None     | 2.8     |                 |
| Cyclic GMP, 1 mM | 4.6     |                 |
| Theophylline, 1 mM  | 2.3   |                 |
| Liver    |         |                 |
| None     | 6.1     |                 |
| Cyclic GMP, 2 mM | 7.2     |                 |
| L-Epinephrine, 11 μM | 9.0     |                 |
| L-Epinephrine + cyclic GMP | 13.0 | |
| Glucagon, 0.5 μg per ml | 3.9     |                 |
| Glucagon + cyclic GMP | 9.5     |                 |
| Erythrocytes |         |                 |
| None     | 0.3     |                 |
| Cyclic GMP, 1 mM | 0.6     |                 |
| L-Epinephrine, 0.1 mM | 1.5   |                 |

Cycloheximide also inhibited breakdown of cyclic AMP by whole homogenates of mouse fibroblasts and rat kidney and liver. Cyclic GMP, 1 mM, increased the accumulation of cyclic AMP in incubations of rat kidney cortex, while 1 mM theophylline was without effect (Table IV). Cyclic GMP (or epinephrine) enhanced accumulation of cyclic AMP in incubations of chicken erythrocytes. While cyclic GMP, 2 mM, had little or no effect on cyclic AMP accumulation in incubations of rabbit liver slices, the effects of either epinephrine or glucagon plus cyclic GMP were greater than those of the hormones alone. Effects of cyclic GMP alone have been observed in preliminary experiments with rat liver slices.

DISCUSSION

Since most tissues contain a variety of types of cells, perhaps all of which contain adenyl cyclase and cyclic AMP, there are obvious pitfalls in attempting to correlate changes in cyclic AMP content of an entire tissue with biochemical events which occur in only a single cell type. For example, prostaglandin E1 inhibits lipolysis and decreases the concentration of cyclic AMP in fat cell incubations, but actually increases the concentration of cyclic AMP in fat pad fragments (2, 25). In addition to the advantages of being an essentially homogenous population, the free fat cells are exposed equally and simultaneously to the medium so that they presumably all participate to the same extent in the uptake of or reaction with its components and likewise can release materials directly into it.

The effects of cyclic GMP on glycerol production by fat cells were qualitatively very similar to those of cyclic AMP albeit of lesser magnitude. Both nucleotides stimulated lipolysis in phosphate saline medium, and these effects were seen and sometimes enhanced in the presence of theophylline. Effects of cyclic GMP were observed in the presence of 0.4 mM cyclic AMP, but not with 1 mM cyclic AMP, a concentration which itself maximally stimulated lipolysis. In Krebs-Ringer phosphate medium, the inhibitory effects of cyclic GMP were smaller and more variable than those of cyclic AMP. The effects of cyclic GMP on lipolysis which, like those of cyclic AMP, depended on the composition of the incubation medium, were clearly not correlated with cyclic AMP levels, which were always elevated when cells were incubated with cyclic GMP.

In considering possible causes of this latter effect of cyclic GMP, it should be noted that conversion of as little as 0.012% of the added cyclic GMP (when present at a concentration of 1 mM) to cyclic AMP would be sufficient to account for the observed increase in cyclic AMP in 1 hour. We therefore incubated fat cells with 1 mM cyclic GMP plus [3H]cyclic GMP (ca. 50 μCi) and found that the apparent conversion of cyclic GMP to cyclic AMP was less than 0.001% per hour, insufficient to account for the observed increase in cyclic AMP in the system. Accumulation of cyclic AMP could result either from increased cyclic AMP formation or decreased degradation. Stimulation of adenyl cyclase by cyclic GMP in intact cells cannot be excluded, but no effect on adenyl cyclase from fat cells has been demonstrated (26). Butcher et al. (1) and Baird (2) found that although prostaglandin E1 and insulin diminished the cyclic AMP content of fat pads or cells exposed to epinephrine (or other lipolytic hormone), they had no effect in the absence of hormonal stimulation. Thus the failure of these compounds to alter cyclic AMP levels elevated by incubation with cyclic GMP would be consistent with the view that cyclic GMP does not stimulate adenyl cyclase activity. In fact, since the rate of accumulation of cyclic AMP in the presence of 1 mM cyclic GMP was only 1.2 to 2.0% of that observed during the first few minutes after cells were exposed to 1.1 mM epinephrine, it could be concluded that any effect of cyclic GMP on adenyl cyclase must have been small compared with that of the hormone, unless cyclic 3',5'-nucleotide phosphodiesterase activity were also enhanced by cyclic GMP. Cyclic GMP, however, inhibited the degradation of cyclic AMP by homogenates of liver and kidney as well as by fat cell extracts. Clearly a similar action of cyclic GMP in the intact cell could cause the accumulation of cyclic AMP which we observed with all of these tissues.

If cyclic GMP facilitated the exit of cyclic AMP from cells or inhibited its re-entry, cyclic AMP might tend to accumulate in the medium (presumably protected from degradation until it re-enters the cell) as we have observed. It is apparent, however, that cyclic AMP also accumulates in the medium in the absence of cyclic GMP. In control incubations or in those with theophylline, 40 to 100% of the total cyclic AMP was found in the medium. When epinephrine and theophylline were present to-
together, essentially all of the cyclic AMP was found in the medium after 30 min. In view of these findings and our failure to obtain evidence for cyclic GMP-induced leakage of other cell components or of degradation products from "Hi-cyclic AMP," we believe that the accumulation of cyclic AMP in the medium in the presence of cyclic GMP is secondary to inhibition of its hydrolysis.

Since a large fraction of the cyclic AMP that accumulated during incubation with cyclic GMP was in the medium, and since some effects of cyclic GMP on lipolysis resemble those of cyclic AMP, could the extracellular accumulation of cyclic AMP explain the effects of cyclic GMP on lipolysis? In control incubations, after 1 hour (with <50 mg of cells per ml) the concentration of cyclic AMP in the medium was 0.01 to 0.02 μM. After the same length of time with cyclic GMP it was 0.1 to 0.4 μM, similar to that observed after 30 min with theophylline plus epinephrine. Such concentrations of cyclic AMP added to the medium would not mimic the effects of cyclic GMP. Since we have been unable to calculate or to measure the intracellular concentration of cyclic AMP after incubation with cyclic GMP, we can draw no conclusions regarding its relationship to the effects of cyclic GMP on lipolysis. Whether cyclic GMP affects lipolysis indirectly by inhibiting degradation of cyclic AMP or acts directly in the hormone-sensitive lipase system is not known. It has been found that cyclic AMP stimulates activation of adipose tissue lipase as does cyclic AMP in a system containing ATP and MgCl₂ and Corbin and Kreb's (14) have reported that a protein kinase preparation from fat cells is activated by cyclic GMP.

It is clear that cyclic AMP does escape or is released from cells in vivo; it is found in urine (5, 22, 28), plasma, and cerebrospinal fluid (16), and its release from avian erythrocytes has been studied (23). Nevertheless, most discussions of the role of cyclic AMP have included, at least implicitly, the assumption that the effects of this nucleotide are confined to the cell in which it is formed. Recent reports suggest that this may not be true in all cell systems and that extracellular cyclic AMP (29) and other cyclic nucleotides (30) may mediate informational exchange in the aggregation and differentiation of slime molds. It remains to be established whether or not this is true in other organisms and in complex tissues. In addition to investigations now in progress concerning the possible role of endogenous cyclic GMP in fat cell metabolism, our attention has been directed toward this problem.

Butcher et al. (1) stated that most of the cyclic AMP that accumulated during incubation of fat cells with epinephrine plus caffeine for 10 min was found in the cells. It has recently been reported, however, that labeled cyclic AMP formed in fat cells from [³²P]adenine was released into the medium in significant quantities (27).

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