The Regulation of Lipogenesis by Cyclic Nucleotides in Intact Hepatocytes Prepared by a Simplified Technique*

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DAVID M. CAPUZZI,† VICKI ROTHMAN, AND SYMION MARGOLIS

From the Clayton Laboratories, Department of Medicine, and Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

An improved, simplified procedure is described for the isolation of liver cells from rat or chicken. Livers were perfused in situ and then incubated in a solution of collagenase and hyaluronidase. Greater than 50% of the liver mass was converted into a suspension of intact parenchymal cells which excluded a vital stain and were undamaged when viewed with the electron microscope. The cells actively incorporated labeled precursors into lipids and proteins without specific cofactor requirements in both simple, ionic, and complex culture media. The chicken hepatocytes synthesized lipids and proteins for several days in suspension culture, but suspended rat hepatocytes had minimal biosynthetic activity after 24 hours.

Both dibutyryl cyclic AMP (0.1 mM) and adenosine 3':5'-monophosphate (cyclic AMP) (0.1 mM) produced a similar striking inhibition of [1-14C]acetate incorporation into lipids in chicken hepatocytes. Dibutyryl cyclic AMP was less inhibitory to lipid synthesis by rat liver cells and cyclic AMP had no effect on this process. The inhibitory actions of dibutyryl cyclic AMP were probably not due to isotopic dilution of the label within the cells. Neither cyclic nucleotide had a lipolytic effect in vitro on hepatocyte lipids prelabeled in vivo. The inhibitory action of dibutyryl cyclic AMP on lipogenesis was significantly greater than the effect of twice the concentration of sodium butyrate, and 5'-AMP did not diminish hepatic lipid synthesis. It is postulated that hepatic lipid synthesis is inhibited specifically by cyclic nucleotides, and that hormonal stimulation of adenylate cyclase regulates hepatic lipid synthesis.

Many reports describe techniques for isolation of rat liver cells and the metabolic characteristics of the resultant cells. These techniques have varied in their complexity and in the yield and metabolic characteristics of the cells. Until recently it has not been sufficiently appreciated that the conditions of cell preparation have a profound effect on the structure and biochemical activity of the cells (1, 2). An ideal method for isolation of hepatocytes should be technically uncomplicated and should furnish a high yield of metabolically active cells that display only those alterations inherent in tissue disorganization. The present report describes a simple method which provides a high recovery of cells that rapidly incorporate precursors into lipids and proteins, respond to hormones, and retain ultrastructural integrity (3).

This technique was also extended to isolation of cells from chicken liver in order to allow a direct comparison of biochemical processes in different species under similar conditions. Such comparisons are particularly crucial in studies of the control of lipid metabolism which varies with species (4, 5). Thus in both primate and avian species, the liver is by far the predominant site of lipogenesis whereas the adipose tissue is the major site of lipid synthesis in the rat (5–7). Metabolic studies with avian liver cells may provide information on the regulation of lipogenesis that may be similar to mechanisms operative in primates. Striking dissimilarities were found in the physical and metabolic characteristics of chicken and rat hepatocytes, and in their response to cyclic nucleotides. In addition, the chicken liver cells could be maintained in suspension culture for several days while the rat liver cells were not viable after 24 hours of culture under the same conditions. A preliminary report of this work has been published (3). The cell preparation described was used to explore the effects of cyclic nucleotides on hepatic lipogenesis. Evidence is presented that the antilipogenic effect of adenine nucleotides is specific for the cyclic compounds; is at least in part independent of the butyryl groups and pool dilution effects; and is likely to occur prior to acetyl-CoA formation.

EXPERIMENTAL PROCEDURE

Materials

White Rock cockerels (3 to 6 weeks old) were obtained from Truslow Farms, Chestertown, Md., and were fed Purina chicken mash. Male Sprague-Dawley rats (200 to 250 g) were obtained from Moore Research Suppliers, Baltimore, Md., and were fed Purina laboratory chow. All feeding was ad libitum. Collagenase (type 1), bovine testicular hyaluronidase (type 1),

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† Present address, Department of Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pa. 19104.
and dibutyryl cyclic 3':5'-AMP (Bt$_2$-cAMP)$^1$ were purchased from Sigma Chemical Co.; nigrosin from Fisher Scientific Co., and 5'-AMP, cyclic 3':5'-AMP (cAMP), and theophylline from Calbiochem. The glucose oxidase kit (Glucostat) was bought from Worthington Biochemical Corp.; agar culture plates, bovine, fetal calf, and chicken serum were supplied by Microbiological Associates. L-[U-14C]Serine (125 mCi per mm) and sodium (14C)acetate (2.0 mCi per mm) were purchased from New England Nuclear Corp. Sterile saline (0.9% NaCl solution) minibottles (150 ml) and Volutrole intravenous infusion sets were from Cutter Laboratories; butterfly infusion sets were from Abbott Laboratories; and spinner flasks (250 ml) were from the Kontes Glass Co. Bell-stir magnetic stirrer for suspension culture was a product of the Belco Glass Co. The sources of reagents and other products have been detailed previously (8, 8, 0).

**Methods**

**Preventive Measures against Contamination**—In experiments that involved cell culture, all instruments, glass, and plastic ware were autoclaved before use; enzyme solutions and incubation media were sterilized by passage through a Millipore filter (0.45 μ). The preparation of cells was carried out under sterile conditions. Sterility of the cultures was tested periodically by streaking samples of the cell suspension on sheep blood agar and Sabouraud's agar plates. Procedures were performed at room temperature except where otherwise indicated.

**Preparation and Culture of Chicken Liver Cells**—A sterile saline minibottle (150 ml) was inverted on a support 80 cm above the operating surface and attached to an intravenous infusion set that consisted of a flexible, Volutrole bag (100-ml capacity), plastic tubing with an adjustable clamp, and a butterfly infusion set. Air bubbles were removed by passage of saline through the tubing.

After chickens were anesthetized by a slow injection of sodium pentobarbital (60 mg per kg) into a wing vein, a transverse abdominal incision was made just below the carina. The incision was extended on both sides of the midline to reach the caudal angles of the sternum. The distal tip of the sternum was severed. Then 50 ml of collagenase (120 units per ml) and hyaluronidase (0.10%) in Ca$^{++}$- and HCO$_3$-free Hanks' medium, pH 7.4 (10), were added to the Volutrole bag through a side arm. The liver was covered with warm, saline-soaked gauze pads while the enzyme solution was infused at a rate of about 4 ml per min. The temperature of the infusion was maintained at 37° by submerging the tubing in a water bath. After 10 to 15 min of infusion the liver was carefully excised, trimmed, and rinsed with serum-free Eagle's medium (11). The liver, which was very soft at this point, was gently minced in a plastic beaker that contained 25 ml of the collagenase-hyaluronidase solution. This mixture was transferred to a plastic Erlenmeyer flask (125 ml), an additional 20 ml of enzyme solution was added, and the mixture was stirred magnetically at 30 to 40 rpm for 1 hour at 37°. The resultant incubation mixture was filtered through 100-mesh silk cloth and centrifuged in plastic conical tubes at 100 x g for 5 min. The supernatant contained only cellular debris and was discarded. The cells were resuspended in 3 volumes of serum-free Eagle's medium or HCO$_3$-free Hanks' solution, and were centrifuged as described above. The resultant packed cells were resuspended in 40 volumes of either Eagle's medium containing 10% chicken serum (complex medium) for spinner culture or HCO$_3$-free Hanks' solution, pH 7.4 (simple medium) for immediate metabolic studies. Cells cultured in stoppered spinner flasks were maintained in suspension by magnetic stirring at 60 to 90 rpm under 5% CO$_2$-95% air. Cells were routinely counted with a hemocytometer, and their viability was assessed with nigrosin dye (0.05%). The medium was not renewed during the culture period.

**Preparation of Rat Liver Cells**—Insertion of the butterfly needle (No. 21 gauge) was much simpler in rats because of their wider and more accessible portal vein. In the rat liver perfusions, the infusion of enzyme solution (25 ml) was preceded by an initial passage of 50 ml of Ca$^{++}$-free Locke's solution (12) containing 0.027 m sodium citrate (37°) at 4 ml per min. The citrate infusion was added for rat liver since Berry and Friend reported that a chelation step augmented cell yield with the recirculating perfusion method (13). The minced rat liver which was about half the weight of the bird liver, was then stirred magnetically in 25 ml of the enzyme solution for 1 hour. Subsequent steps were the same as those described for the chicken liver cells except that the complex medium contained 5% bovine serum and 5% fetal calf serum instead of chicken serum.

Although the open perfusion method as employed subjects the liver to hypoxic conditions for the 10- to 15-min period of perfusion, this feature of the technique did not appear to impair the quality of the resultant cells. Preliminary oxygenation of the perfusion medium and doubling of the flow rate did not provide any additional improvement in the percentage of intact cells obtained or in their biosynthetic activity. However, it was essential to initiate the perfusion while the hepatic circulation was intact.

**Incubation Conditions**—Aliquots of suspended cells (4 ml) were incubated under air at various intervals in complex medium or were gently centrifuged and resuspended for incubation in a similar volume of simple medium. Sodium [1-14C]acetate or [U-14C]serine were used for determination of rates of incorporation into cellular lipids or proteins, respectively. Incubations were carried out at 37° in 25-ml plastic Erlenmeyer flasks in a rotary water bath shaker at 130 rpm. At the end of the incubation period, the flasks were placed on ice, and 0.1 ml of unlabeled 10% sodium acetate or 0.1 ml of unlabeled serine solution (0.3 M) was added to each flask. Cells were separated from medium by centrifugation at 2000 x g for 10 min. Labeled cellular lipids were extracted with chloroform-methanol (2:1) by the method of Folch et al. (14) and counted in 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis[2-(5-phenyloxazoyl)]benzene in toluene) as previously described (8). To isolate labeled protein, the packed cells

$^1$ The abbreviations used are: Bt$_2$-cAMP, dibutyryl cyclic 3':5'-AMP; cAMP, cyclic 3':5'-AMP.
were lysed in 2% Triton; cell lysates were applied to filter paper discs, washed, and counted (9).

RESULTS

Cell Yield and Morphological Characteristics—The recovery of hepatocytes (milliliters of packed cells per g of liver) from both rat and chicken livers was 40 to 70%. The chicken livers were consistently very friable after enzyme perfusion, and in some experiments a cell yield of about 90% was obtained. When the packed cells were suspended in 40 volumes of incubation medium, the density of chicken hepatocytes ranged from 2.4 to 4.1 \times 10^6 cells per ml and the range for rat hepatocytes was 0.6 to 1.2 \times 10^6 cells per ml. Nearly all the cells isolated were hepatic parenchymal cells. The proportion of intact cells isolated varied with the quality of various collagenase batches and with the proficiency with which the cells were prepared. Batches with high collagenase and low proteinase activity yielded greater percentages of active, intact liver cells. The two most important technical requirements for optimal recovery of undamaged cells were adequate perfusion of the liver and gentleness in mechanical manipulation of the liver. More than 90% of the cell preparation obtained from either species consisted of single cells which were granular in appearance. Cell debris was minimal. Nigrosin stain was excluded by at least 80% of fresh rat hepatocytes and by nearly all of the chicken hepatocytes. Of those cells that took up nigrosin, the majority stained only in the nucleus.

At least 50% of the cells from either species were undamaged and under optimal conditions, greater than 90% of the cells had intact cellular structure as viewed under the electron microscope (Figs. 1 and 2). Subcellular organelles in the isolated cells closely resembled in their appearance those found in organized hepatic tissue. The plasma and nuclear membranes and the rough endoplasmic reticulum were intact and well delineated. Ribosomes were studied along the rough endomembrane which formed a parallel array, and mitochondrial morphology was normal. Aggregated glycogen granules, occasional lipid inclusions, and distinct nucleoli were visible. The average diameter of the rat hepatocyte was about twice that of the chicken hepatocyte (Table I). The larger average size of rat hepatocytes was evident by light and electron microscopy and by comparison of cell counts from similar volumes of packed cells from the two species. Cell volumes and dry weights of the rat hepatocytes were about 10-fold greater than the same parameters of the chicken hepatocytes, while the protein content of the rat hepatocytes was 15-fold greater.

Characteristics of Suspension Cultures—The results in Table II depict typical fluctuations in concentration of potential substrates in the medium and the proportion of viable cells during culture of chicken hepatocytes. Levels of free fatty acid declined rapidly and were undetectable from 19 to 32 hours after initiation of cell culture. Thereafter, free fatty acid reappeared in the medium and reached preincubation levels by 72 to 96 hours. The reappearance of free fatty acid in the medium may reflect the lipolytic action of an hepatic lipase (16) that acts either within the cells or extracellularly. Similarly, glucose levels in the medium diminished rapidly; after 24 to 32 hours the glucose concentration fell to about 145 mg/100 ml and then gradually rose over the next 64 hours. This decline and subsequent rise in glucose level may define a critical concentration at which gluconeogenesis is triggered in the chicken hepatocyte. There were no significant changes in the cholesterol concentrations in the medium. As further shown, greater than 80% of the cells excluded nigrosin dye for the first 3 days of culture. After this period, however, increased numbers of cells stained with dye, so that by the end of 1 week only 30% of the cells completely excluded the vital stain.

![Fig. 1 (top). Isolated chicken hepatocyte (× 10,000), prepared for electron microscopic examination as previously described (1).](image1)

![Fig. 2 (bottom). Isolated rat hepatocyte (× 5,000).](image2)

**Table I**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Rat</th>
<th>Chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter/cell (μ)</td>
<td>21.0–25.0</td>
<td>11.0–12.5</td>
</tr>
<tr>
<td>Protein (mg/10^6 cells)</td>
<td>5.6–5.9</td>
<td>0.38–0.40</td>
</tr>
<tr>
<td>Dry weight (mg/10^6 cells)</td>
<td>18.0–20.3</td>
<td>1.8–1.9</td>
</tr>
<tr>
<td>Yield (cells \times 10^6/g liver)</td>
<td>8–36°</td>
<td>51–159°</td>
</tr>
</tbody>
</table>

* Determined by visual measurement of cells fixed for electron microscopy.

* Determined by the method of Lowry et al. (15).

* Ranges based on cell counts from 10 rats and 10 chickens.
Cells were maintained in suspension culture in complex medium (Eagle's medium containing 10% chicken serum) as described under "Methods." At various intervals aliquots of suspended cells were removed for assessment of viability with 0.05% nigrosin stain. Medium was separated from other cell aliquots by centrifugation, and the concentrations of various metabolites in the medium were determined.

<table>
<thead>
<tr>
<th>Time after preparation of cells</th>
<th>Glucose</th>
<th>FFA*</th>
<th>Cholesterol</th>
<th>Visible cells</th>
<th>Cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>mg/100 ml</td>
<td>mg/l</td>
<td>mg/100 ml</td>
<td>%</td>
<td>cells/ml X 10^-5</td>
</tr>
<tr>
<td>0</td>
<td>240</td>
<td>175</td>
<td>68</td>
<td>100</td>
<td>2.4</td>
</tr>
<tr>
<td>3/2</td>
<td>234</td>
<td>75</td>
<td>64</td>
<td>100</td>
<td>2.2</td>
</tr>
<tr>
<td>5/2</td>
<td>200</td>
<td>20</td>
<td>60</td>
<td>90</td>
<td>2.9</td>
</tr>
<tr>
<td>10</td>
<td>170</td>
<td>0</td>
<td>66</td>
<td>90</td>
<td>4.3</td>
</tr>
<tr>
<td>24</td>
<td>150</td>
<td>0</td>
<td>60</td>
<td>90</td>
<td>5.7</td>
</tr>
<tr>
<td>32</td>
<td>145</td>
<td>0</td>
<td>60</td>
<td>90</td>
<td>6.1</td>
</tr>
<tr>
<td>43</td>
<td>222</td>
<td>50</td>
<td>88</td>
<td>90</td>
<td>6.5</td>
</tr>
<tr>
<td>72</td>
<td>213</td>
<td>125</td>
<td>88</td>
<td>90</td>
<td>6.8</td>
</tr>
<tr>
<td>96</td>
<td>244</td>
<td>200</td>
<td>68</td>
<td>70</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Free fatty acids.

Distribution of [1-14C]acetate incorporation into lipid classes in rat and chicken hepatocytes

Freshly prepared liver cells were incubated in simple medium. After 60 min of incubation, cells were separated from medium and extracted with chloroform-methanol (2:1). Lipid classes were separated by thin layer chromatography on Silica Gel G using hexane-diethyl ether-glacial acetic acid (70:20:1; v/v) and counted in a liquid scintillation spectrometer. The data are derived from three rat and three chicken livers.

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Distribution of label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>13.1 ± 2.2*</td>
</tr>
<tr>
<td>1,2 Diglycerides</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>52.2 ± 5.4</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>9.1 ± 4.0</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>10.6 ± 3.0</td>
</tr>
<tr>
<td>Recovery</td>
<td>72 ± 4</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of three experiments.

of chicken liver cells consistently increased during the initial period of culture so that by the end of the first 24 hours they had approximately doubled in number. Changes in cell number over the subsequent 7 days were minor.

By contrast, rat liver cells did not multiply at any time, and after 24 hours of culture, virtually all the rat hepatocytes stained with nigrosin. Omission of serum or substitution of rat or chicken serum for the bovine and fetal calf sera in the complex medium failed to prolong the longevity of the rat hepatocytes. Thus, it is unlikely that the decline in viability and metabolic activity resulted from cell damage produced by heterologous serum constituents (17).

Distribution of Label among Lipid Classes—When the labeled lipids of liver cells from each species were separated by thin layer chromatography, there was a striking difference in the distribution of labeled acetate among the major lipid classes (Table III). The percentage of label in phospholipids was similar in both types of hepatocyte. In the rat hepatocytes about half of the incorporated [1-14C]acetate was found in cholesterol whereas two-thirds of the incorporated [1-14C]acetate was found in triglycerides in chicken cells.

Species Differences in Pattern of Hepatic Lipogenesis—Labeled acetate was incorporated into cellular lipids by both chicken and rat hepatocytes at a linear rate for 60 min, but approached a plateau by about 60 min (Figs. 3 and 4). The pattern of incorporation was similar when label was added at any time during the first 24 hours of cell culture. The plateau was not due to limiting amounts of substrate since a 10-fold increase in acetate concentration did not prolong the linear period of incorporation. The initial rates of [1-14C]acetate incorporation per cell did not differ significantly between the two species when incubations were carried out in simple medium (Fig. 3). However, when suspended in complex medium (Fig. 4), chicken hepatocytes incorporated labeled acetate 20- to 30-fold more rapidly than rat hepatocytes.

Species Differences in Pattern of Hepatic Protein Synthesis—Rat liver cells incorporated [U-14C]serine into cellular protein 10- to 15-fold more rapidly than chicken hepatocytes in either medium (Figs. 5 and 6). In simple medium the rate of incorporation was constant for 3 hours in both species (Fig. 5), in complex medium the incorporation of [U-14C]serine by rat liver cells decreased after 20 min while incorporation by chicken hepatocytes remained constant for 4 hours (Fig. 6). Labeled serine incorporation was greater when fresh cells from either species were incubated in simple rather than complex medium.

Species Differences in Duration of Metabolic Activity—Lipid synthesis from [1-14C]acetate by chicken hepatocytes in suspension culture continued for about 96 hours although the rate of incorporation diminished after the first 24 hour period (Fig. 7). The degree and duration of active lipogenesis in chicken
hepatocytes differed with two concentrations of cells in the initial suspension. When the cell concentration was between 2 and 4 million per ml the rate of labeled acetate incorporation was greatest immediately after cell preparation, and then declined over the next 72 hours. However, when the initial cell concentration was between 1 and 1.5 million per ml, [l-14C]acetate incorporation rose to a peak over the first 20 hours of culture and then fell over the next 76 hours. At each cell concentration, the rate of decline in lipogenesis was greatest during the second 24-hour period.

In contrast, the rate of [U-14C]serine incorporation by chicken hepatocytes in complex medium improved steadily during culture, so that by 72 hours the rate of cellular incorporation was similar in simple and in complex medium. Chicken hepatocytes incorporated [U-14C]serine into protein for at least 7 days (Fig. 8). At each interval tested, the rate of amino acid incorporation per cell was 2- to 3-fold greater in the more dilute cell suspension. Bhargava and Bhargava (18) have previously shown that the incorporation of amino acids by liver cells is inversely proportional to cell density.

Species Differences in Response to Adenine Nucleotides—The effects of Bt2-cAMP and cAMP on cellular lipogenesis were
Fig. 8. Changes in rates of protein synthesis during culture of chicken liver cells. The cells used were from one of the two batches from which the data in Fig. 7 were derived. Cells were maintained in optimum flasks at two concentrations as described under Fig. 7. Similar aliquots of cells were taken for 30 min incubations with [U-14C]serine (1.6 μCi) in complex medium at various times after initiation of culture. Protein radioactivity was determined as described under “Methods.” Each point is the mean of duplicate values expressed in protein counts per min per 10^7 cells.

**Table IV**

Comparative effects of adenine nucleotides and theophylline on lipogenesis from [1-14C]acetate by rat and chicken hepatocytes

Duplicate aliquots of liver cells were incubated with nucleotide or theophylline for 30 min in simple medium. [1-14C]acetate was then added and incubation continued for an additional 30 min. Cellular lipids were extracted and counted as described previously. Data for controls, Bt2-CAMP, and cAMP are mean values from separate experiments with cells derived from four rats and five chickens. The 5'-AMP and theophylline data were obtained in hepatocyte incubations from two rats and three chickens. Relative incorporation values are means ± S E.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (mM)</th>
<th>Relative [1-14C]acetate incorporation (cpm/10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rat %</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Bt2-CAMP</td>
<td>0.1</td>
<td>68.5 ± 5.5a</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.1</td>
<td>98.3 ± 1.7</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>0.1</td>
<td>96.0 ± 6.5</td>
</tr>
<tr>
<td>Theophylline</td>
<td>1.0</td>
<td>114.3 ± 9.9</td>
</tr>
</tbody>
</table>

* p < 0.01.

**Table V**

Effect of Bt2-CAMP on distribution of radioactivity among lipid classes of rat and chicken hepatocytes

Freshly prepared liver cells from one rat and one chicken liver were incubated for 30 min in simple medium with either no additions or with 0.1 mM Bt2-CAMP. Then [1-14C]acetate (4 μCi) was added to each flask, and the incubation was continued for an additional 30 min. Labeled lipids were then extracted, separated by thin layer chromatography, and counted as described in Table III. In the experiments below, the degree of inhibition of lipogenesis by Bt2-CAMP were 89% (rat hepatocytes) and 86% (chicken hepatocytes).

**Table VI**

Effect of unlabeled sodium acetate on inhibition of lipid synthesis from [1-14C]acetate by Bt2-CAMP

Liver cells from one rat were incubated with Bt2-CAMP for 30 min where indicated. Then [1-14C]acetate was added to each flask and unlabeled sodium acetate was added where indicated to give final concentrations of 0.3 and 3 mM respectively. The incubation was continued for an additional 45 min. Cellular lipid radioactivity was determined as previously described.

<table>
<thead>
<tr>
<th>Addition</th>
<th>14C-lipids inhibition by Bt2-CAMP %</th>
<th>cpm/10^7 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>18,560 ± 2,375a</td>
</tr>
<tr>
<td>Bt2-CAMP</td>
<td>(0.1 mM)</td>
<td>8,878 ± 344</td>
</tr>
<tr>
<td>Sodium acetate (3 mM)</td>
<td></td>
<td>7,096 ± 195</td>
</tr>
<tr>
<td>Bt2-CAMP (0.1 mM) + sodium acetate (3 mM)</td>
<td></td>
<td>3,105 ± 573</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of duplicate samples.

**Table VII**

Examination of Mechanism of Inhibition of Lipogenesis by Bt2-CAMP

The magnitude of inhibition of [1-14C]acetate incorporation by Bt2-CAMP was not diminished when the concentration of acetate in the medium was increased 10-fold by the addition of unlabeled acetate (Table VI). An inhibition of slightly greater than 50% was observed after 45 min of incubation. Thus, diminished incorporation of labeled acetate into lipids was probably not due to isotopic dilution of label within the cells. This conclusion is corroborated by the finding of Bloxham and Akhtar that CAMP produced a similar inhibition of lipogenesis from 3H2O and from [2-14C]acetate in rat liver slices (20).

To determine whether the apparent antilipogenic effect of Bt2-CAMP might actually reflect a lipolytic effect of the nucleotide, lipids of rat liver were labeled in vivo as described in Table VII. Prolabeled liver cells were isolated and then incubated...
TABLE VII
Effects of adenine nucleotides on loss of radioactivity from prelabeled lipids of rat hepatocytes

A rat was given an intraperitoneal injection of 20 μCi of [1-14C]-acetate 60 min before killing. Aliquots of labeled liver cells were then incubated for 60 min with the above nucleotides at a concentration of 0.1 mM. Cellular lipids were extracted and counted as described under "Methods."

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cellular 14C-lipids</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4,672 ± 330*</td>
<td>100</td>
</tr>
<tr>
<td>Bt-CAMP</td>
<td>4,566 ± 35</td>
<td>98</td>
</tr>
<tr>
<td>cAMP</td>
<td>4,495 ± 195</td>
<td>96</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>4,454 ± 344</td>
<td>96</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of duplicate samples.

DISCUSSION

The utility of dispersed, intact liver cells as an experimental tool for the study of metabolic regulatory mechanisms is quite evident. Such a preparation preserves a high degree of subcellular organization that is lacking in liver homogenates, and avoids to a large extent the surface cell damage, cellular heterogeneity, and permeability barriers found in liver slices. However, until recently, liver cells were obtainable in large yield only by mechanical or chemical methods that produced severe cell alterations (1, 8). Cells that were structurally intact and active in lipogenesis (1) could be prepared by enzymatic digestion of liver by the method of Howard et al. (23, 24), but the small yield of cells obtained by this method limits its usefulness for metabolic studies. Berry and Friend (13) provided an important methodological advance when they showed that a prolonged perfusion of the liver at 37° with collagenase and hyaluronidase prior to tissue disaggregation improved cell recovery considerably, but did not increase cell damage. Cells prepared by this method have been used successfully to study the processes of protein synthesis (25), gluconeogenesis (26), and fatty acid metabolism (27, 28).

The principle advantage of the presently described technique is a marked simplification of the preparative procedure without sacrifice in cell yield or integrity. The procedure of Berry and Friend, which entails a closed circuit perfusion of the liver, is technically difficult and requires the use of highly specialized and costly equipment. The nonrecirculating perfusion of the liver by gravity as described in the present report is far simpler to carry out, requires no special mechanical equipment, and produces similar results. In previous studies in this laboratory, cells prepared by a mechanical method were obtained in large yield, but showed ultrastructural evidence of damage to plasma membranes and organelles (1, 8, 29). Cofactor supplementation was essential for lipid synthesis by such cells (8, 29), but is unnecessary with cells prepared by the enzymatic technique described. In our experience, lipogenesis from labeled acetate has been a very sensitive indicator of the biochemical integrity of isolated liver cells. A further indication of their integrity is the observation that their protein content and dry weight were greater than that reported for cells prepared by other methods (23, 30). Cells dispensed by the present procedure respond to hormones in vitro in a fashion similar to that of perfused liver (3), and metabolic studies can be conducted for several hours with rat hepatocytes and for days with chicken hepatocytes. The chicken liver cells appeared to synthesize lipids more rapidly than did rat hepatocytes, although differences in incorporation of label might also be explained by variation in intracellular dilution of label in hepatocytes from each species. The greater capacity of the rat hepatocyte for labeled serine incorporation into proteins is in keeping with its larger size and may reflect a greater requirement for synthesis of structural protein.

Successful culture of hepatic parenchymal cells from nonembryonic livers has been an elusive experimental aim. Our results indicate that liver cells prepared from 3- to 6-week-old chickens can be maintained in suspension culture for at least 7 days while those prepared from mature rats were metabolically active for only about 24 hours. This difference may be due in part to relative anoxia since the larger rat hepatocyte presents a more limited surface area per unit mass than the chicken...
hepatocyte. McElmam et al. (31), reported that the viability of cultured liver cells was critically dependent upon the rate of cellular oxygen diffusion. However, other conditions of suspension culture may limit the longevity of rat liver cells in suspension culture. Type (32) described a monolayer culture of enzyme-dispersed rat hepatocytes which appeared to be parenchymal cells; however, the cells changed in morphology after a few days. Although previous reports indicate maintenance of rat liver cells after isolation with tetraphenylboron (33) or with citrate chelation and mechanical disruption (34), we have found that cells prepared by either of these methods showed severe structural damage. Moreover, rates of cellular lipid and protein synthesis which were very low initially (1), failed to improve during subsequent suspension culture.

Although inhibition of fatty acid and cholesterol synthesis by Bt$_2$-cAMP and cAMP has been observed in liver slices (20, 35) there is little information on the mechanism of this inhibition. Using a particle-free soluble fraction from rat liver, Bloxham and Akhtar suggested that cAMP inhibited cholesterol synthesis just prior to cholesterol-7-en-3-$\beta$-ol formation (36). Bricker and Levey (35) observed inhibition of lipogenesis in hepatic slices by Bt$_2$-cAMP or cAMP but found no effect on lipogenesis in rat hepatoma slices (37). Allred and Roehrig (38) also observed an inhibition of lipogenesis by cyclic nucleotides in chicken and rat liver slices. More recently, they demonstrated an inhibition of acetyl-CoA carboxylase by cAMP or Bt$_2$-cAMP in rat liver homogenates, and suggested that a major part of the antilipogenic effect of cyclic nucleotides occurs at this step (39). However, this mechanism would not account for the similar reduction of [1-14C]acetate incorporation into both fatty acids and cholesterol that we (19) and others (20, 35) have observed. There is evidence against the existence of a "malonyl-CoA pathway" for cholesterol synthesis in rat liver (40). Moreover, activation of this enzyme in particle-free extracts of rat liver homogenates (46) is not a cyclic nucleotide.

Previous reports of Bt$_2$-cAMP and cAMP effects on lipid synthesis have not characterized the specificity of the nucleotide inhibition. The present studies show that 5'-AMP does not inhibit hepatic lipogenesis in either rat or chicken hepatocytes, and indicate a requirement for an intact diester ring. In addition, since one or both butyryl groups can be cleaved from Bt$_2$-cAMP in aqueous media (43), in Hela cells (22), and in liver homogenates (44, 49), comparison of the effects of Bt$_2$-cAMP with sodium butyrate was essential. The significantly greater inhibition of [1-14C]acetate incorporation by Bt$_2$-cAMP than by twice the concentration of sodium butyrate indicates that butyryl cleavage alone cannot entirely account for action of Bt$_2$-cAMP, although differing cellular uptake rates of the two compounds could also affect the relative inhibition obtained. Moreover, the effectiveness of cAMP itself in inhibiting lipogenesis in liver slices (20, 35, 38) and in chicken liver cells suggests that these effects of Bt$_2$-cAMP are not due solely to its butyryl groups.

Since the pathways for fatty acid and cholesterol biosynthesis differ after the formation of acetyl-CoA (40), the similar inhibition of synthesis of both lipid classes by Bt$_2$-cAMP suggests that cyclic nucleotides may inhibit lipogenesis at a prior step. Acetyl-CoA synthetase, which has a contrasting subcellular distribution in rat (40) and chicken (47) liver, is a plausible site of inhibition. Murthy and Steiner have reported evidence for its role in the regulation of hepatic lipid synthesis (48). More recently, they demonstrated an increase in free acetate concentration in livers of fasted rats, and suggested that impaired conversion of acetate to acetyl-CoA may occur under conditions of inhibited lipogenesis (49). On the other hand, the antilipogenic action of cyclic nucleotides may represent the cumulative inhibition of several cellular enzymes as suggested by Tepperman and Tepperman (50). In either case, the in vitro studies described in this report add further weight to the growing evidence that cyclic nucleotides may play a significant role as physiological mediators of the hormonal and dietary effects on hepatic lipogenesis that occur in vivo.

REFERENCES

49. Murthy, V. K., and Steiner, G. (1973) Metabolism 22, 81–84