Independence of Cholesterol and Fatty Acid Biosynthesis from Cyclic Adenosine Monophosphate Concentration in the Perfused Rat Liver*

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SUMMARY

Cyclic adenosine 3':5'-monophosphate (cyclic AMP), when added in a concentration of 5 mM to incubations of rat liver slices with [1-14C]acetate or [1-14C]octanoate, was found to depress markedly both cholesterol and fatty acid biosynthesis but not CO2 or ketone body production. The inhibitory effects of the nucleotide were lost when its concentration was reduced to 0.5 mM. These findings, therefore, confirm and extend those made by others. However, it also was observed that perfusion of rat livers with the above-mentioned substrates in the presence of sufficient glucagon to raise the tissue cyclic AMP level by at least 50-fold was totally without effect on rates of cholesterol or fatty acid synthesis. In addition, such treatment of the livers failed to reduce the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in subsequently isolated microsomes. It is concluded that in the intact liver cholesterol and fatty acid synthesis are independent of acutely induced changes in the intracellular cyclic AMP concentration over a very wide physiological range.

The fact that the capacity of animal tissues to synthesize both cholesterol and fatty acids is subject to nutritional and hormonal control has long been recognized (1, 2). In this regard there is good reason to believe that insulin and glucagon, two hormones known to exert directly opposite effects on carbohydrate metabolism, exhibit a similar antagonism in the regulation of these two processes in vivo (3-6). Because of the seemingly ubiquitous role of cyclic adenosine 3':5'-monophosphate as an intermediary in a variety of hormone actions and the well documented ability of insulin and glucagon to influence its concentration in opposite directions in target tissues, the possibility naturally arose that this compound might play a key regulatory role in the synthesis of cholesterol and fatty acids. Indeed, evidence taken to support this concept recently has appeared from a number of laboratories. Thus, it has been shown that cyclic AMP1 or dibutyryl cyclic AMP, when added to rat liver slices incubated with radiolabeled acetate, was capable of greatly diminishing the incorporation of label into both fatty acids and cholesterol (7). Similar observations were made by Allred and Roe (8) who studied the effects of dibutyryl cyclic AMP1 and cyclic GMP on the incorporation of acetate into total lipids in chicken liver slices. In addition, it has been reported by Beg et al. (9) that HMG-CoA reductase activity was diminished in a variety of in vitro liver preparations preincubated in the presence of cyclic AMP. A striking feature of all of these in vitro studies, however, is that the concentrations of nucleotides required to obtain the inhibitory effects mentioned were exceedingly high (generally 10^-4 to 10^-3 M) compared with the known range of cyclic AMP1 concentrations in liver tissue (10^-7 to 10^-3 M) (10). For this reason we felt it worthwhile to re-examine the issue under circumstances where cyclic AMP levels were increased under physiological conditions. The findings outlined below confirm that exposure of rat liver slices to high concentrations of cyclic AMP suppresses both cholesterol and fatty acid biosynthesis. However, they also render questionable the physiological significance of such data since neither lipid synthesis nor the activity of HMG-CoA reductase was affected when cyclic AMP levels in the intact rat liver were raised by some 50-fold with glucagon treatment.

EXPERIMENTAL PROCEDURE

Animals—Male Sprague-Dawley rats weighing 110 to 140 g and fed a standard laboratory chow were used in all experiments. They were housed in a chamber that was lighted from 3:00 p.m. to 3:00 a.m. and were used for experiments at 9:00 a.m.; i.e. at the midpoint of the dark cycle.

Experiments with Liver Slices—These were performed in a manner essentially identical with that described by Dietschy and McGarry (11). Briefly, 300 mg of tissue slices (0.8-mm in thickness) were suspended in 5 ml of Krebs' bicarbonate buffer, pH 7.4, containing 2 μCi of sodium [1-14C]acetate (1.2 mM) or sodium [1-14C]octanoate (1.0 mM). The flasks were gassed with a mixture of 95% O2-5% CO2, stopped, and shaken for 90 min at 37°C at a rate of 150

1 The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; cyclic GMP, cyclic guanosine 3':5'-monophosphate; dibutyryl cyclic AMP, N6,O2-dibutyryl cyclic adenosine 3':5'-monophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
Table I

Effect of cyclic AMP on metabolism of acetate and octanoate in rat liver slices

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Cyclic AMP</th>
<th>CO₂</th>
<th>Cholesterol</th>
<th>Fatty acids</th>
<th>Total ketone production</th>
<th>Relative ketone specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[1-14C]Acetate</td>
<td>5</td>
<td>7800 ± 607</td>
<td>308 ± 19</td>
<td>1417 ± 158</td>
<td>2.48 ± 0.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[1-14C]Acetate</td>
<td>5</td>
<td>8463 ± 325</td>
<td>253 ± 15</td>
<td>249 ± 21</td>
<td>3.01 ± 0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[1-14C]Octanoate</td>
<td>5</td>
<td>9365 ± 701</td>
<td>729 ± 48</td>
<td>249 ± 21</td>
<td>8.99 ± 0.30</td>
<td>76 ± 3</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Octanoate</td>
<td>5</td>
<td>9227 ± 530</td>
<td>354 ± 30</td>
<td>249 ± 21</td>
<td>9.61 ± 0.23</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>[1-14C]Acetate</td>
<td>0.5</td>
<td>7765 ± 712</td>
<td>148 ± 14</td>
<td>1095 ± 108</td>
<td>2.55 ± 0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[1-14C]Acetate</td>
<td>0.5</td>
<td>8146 ± 572</td>
<td>171 ± 33</td>
<td>1111 ± 79</td>
<td>2.44 ± 0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[1-14C]Octanoate</td>
<td>0.5</td>
<td>8155 ± 272</td>
<td>352 ± 27</td>
<td>1111 ± 79</td>
<td>10.75 ± 0.28</td>
<td>86 ± 3</td>
</tr>
<tr>
<td></td>
<td>[1-14C]Octanoate</td>
<td>0.5</td>
<td>8437 ± 540</td>
<td>321 ± 21</td>
<td>1111 ± 79</td>
<td>11.17 ± 0.10</td>
<td>86 ± 4</td>
</tr>
</tbody>
</table>

Common pools of liver slices from animals subjected to light-dark cycling for 1 week and killed in the mid-dark phase were incubated with [1-14C]acetate or [1-14C]octanoate in the presence or absence of cyclic AMP. Details of the incubations, chemical analyses and calculation of the results are given under “Experimental Procedure.” Values represent means ± S.E. for 6 to 12 determinations.

RESULTS AND DISCUSSION

Experiments with Perfused Liver—Livers were perfused with a recirculating medium by the apparatus and techniques described by McGarry and Foster (14). The perfusion medium consisted of washed, aged human erythrocytes suspended to an hematocrit of 20% in 5% bovine albumin (Fraction V, Armour Pharmaceutical Co.) in Krebs' bicarbonate buffer, pH 7.4. The concentrations of substrates and other materials added to the system are given in the text. After 30 min of perfusion livers were quickly removed and analyzed by conventional methods (14) for their content of 14C-labeled lipids, after preliminary extraction with 50 ml of chloroform-methanol (2:1, v/v). In experiments designed to measure the effect of glucagon on hepatic cyclic AMP levels, livers were freeze-clamped in tongs cooled in liquid N₂ and extracted with 3.5 ml of 1N perchloric acid. The concentration of cyclic nucleotide in the neutralized perchloric acid extracts was measured using a competitive protein binding assay with the materials kindly performed by Dr. Michael S. Brown of this department using methods described previously (16).

Because in most previous studies of this kind [14C]acetate was used as the substrate, we, too, have used this material for comparative purposes. However, because of the limitations inherent in the use of [14C]acetate for calculating absolute carbon flow through various metabolic pathways (11), a parallel series of experiments was conducted in which [14C]octanoate served as the substrate. It has been pointed out (11, 14) that because the latter fatty acid is initially metabolized within the mitochondrion, any dilution of the acetyl-CoA pool involved in lipogenesis cannot be made when [14C]acetate is the precursor since under these circumstances the labeled C₂ units formed in the extramitochondrial compartment fail to equilibrate rapidly with the intramitochondrial pool of acetyl-CoA from which the ketone bodies are derived. Thus, in experiments with [1-14C]acetate, rates of C₂ unit flux represent only the rates of conversion of the labeled substrate into products. However, when [1-14C]octanoate served as the labeled precursor, the reported values for C₂ unit flux are derived from the measured rate of incorporation of the fatty acid times 4 times 100 divided by the relative ketone specific activity. In all cases rates of incorporation of C₂ units into cholesterol have been multiplied by a factor of 1.5 in order for loss of 25% of the radioactivity as 14CO₂ during the conversion of [1-14C]acetyl-CoA into sterols.

Materials—All radioactive materials were from New England Nuclear, Boston, Mass. Glucagon, cyclic AMP, and dibutyryl cyclic AMP were obtained from Sigma.

The term “relative ketone specific activity” refers to the absolute ketone specific activity expressed as a percentage of the theoretical specific activity which would be expected if no endogenous dilution of the acetyl-CoA pool occurred.
Livers from animals subjected to light-dark cycling as described under Table I were perfused in the manner outlined under "Experimental Procedure." At zero time either 600 μmoles of sodium [1-14C]acetate (0.05 μCi per μmole) or 150 μmoles of sodium [1-14C]octanoate (0.16 μCi per μmole) were added to the perfusion fluid (80 ml). When used, glucagon was initially present at a concentration of 10^{-6} M and was constantly infused at a rate of 1 nmole per min. Dibutyryl cyclic AMP was added at a concentration of 0.5 mM. After 30 min liver and perfusion medium were taken for analysis as described in the text. The data for biosynthetic parameters were derived from the number of perfusions shown in parentheses. Tissue cyclic AMP levels and HMG-CoA reductase activity were determined in separate experiments in which livers (three in each group) were perfused with nonradioactive octanoate as described above. Values represent means ± S.E.

5 mM cyclic AMP were not simply the result of intracellular dilution of the labeled precursor by endogenously produced acetyl-CoA since a similar effect of the nucleotide at this concentration also was noted on cholesterol synthesis from [1-14C]octanoate, under circumstances where the specific activity of the ketone bodies was unaffected. As was the case with acetate, however, this concentration of cyclic AMP failed to inhibit the oxidative metabolism of octanoate and, when added in a concentration of 0.5 mM, it was again without effect on the rate of cholestereogenesis. Also evident from Table I, and consistent with earlier observations with rat liver slices (11), is the fact that the measured rates of both ketogenesis and cholesterol synthesis were markedly higher with octanoate than with acetate as substrate.

Experiments with Perfused Liver—Because of its failure to alter the activity of the tricarboxylic acid cycle or the enzymes involved in ketone production, it is apparent that the observed inhibitory effects of cyclic AMP on cholesterol and fatty acid synthesis in liver slices cannot be ascribed to a general depression of cellular metabolism. Nevertheless, we were concerned by the fact that the concentration of nucleotide required to exhibit these effects was some 3 to 4 orders of magnitude greater than those present intracellularly in hepatic tissue (10). The question was raised, therefore, as to whether treatment of the intact perfused liver with glucagon, which is known to cause elevations in the cyclic AMP content of this tissue (10), would also result in a diminution of cholesterol and fatty acid synthesis. To this end livers were perfused with [1-14C]acetate or [1-14C]octanoate in the presence or absence of glucagon and the effects of the hormone on a variety of metabolic parameters were determined. The high levels of glucagon employed in these studies were purposely chosen in order to obtain the maximum possible response of hepatic adenyl cyclase to this agent.

A number of interesting points emerged from these experiments. First, it is seen (Table II) that treatment of the livers with glucagon resulted in the expected profound elevation in tissue cyclic AMP levels (0.38 to >16 nmole per g). This increase in cyclic AMP concentration caused a concomitant stimulation of hepatic glycogenolysis which manifested itself in an approximate doubling of the quantity of glucose released into the perfusion medium. As routinely observed in this laboratory, however, the hormone had little, if any, effect on rates of endogenous fatty acid oxidation, as indicated by its failure to alter the relative ketone specific activity or the absolute rate of ketone production.

Although it can be deduced from these data that the much greater ketogenic potential of octanoate compared with acetate, a phenomenon noted above in the slice experiments and discussed in detail previously (11), also held true in the intact liver. A similar situation obtained with regard to the ability of the two precursors to support long-chain fatty acid synthesis. However, interpretation of this finding is hampered by the fact that while fatty acids derived from acetate are presumably synthesized primarily via the de novo pathway, a significant fraction of those formed from octanoate might result from a process of chain elongation (14).

From the standpoint of the initial objectives of the present investigation, the most significant outcome of these studies was the finding that exposure of the perfused rat liver to glucagon in amounts sufficient to raise the intracellular cyclic AMP content at least 50-fold had absolutely no effect on the rates of cholesterol or fatty acid synthesis. This was true whether acetate or octanoate served as the labeled precursor. While the data reported refer only to perfusion studies carried out over a 30-min time period, essentially identical results were obtained in a series of experiments in which livers were exposed to glucagon for 1 hour. Also significant was the finding that when added to the system at a concentration of 0.5 mM, dibutyryl cyclic AMP failed to inhibit either of these processes in livers perfused with radiolabeled octanoate, a result that again contrasts with the reported effects of this compound in rat liver slices (7, 8).

Since we were interested only in showing that a marked rise in tissue cyclic AMP concentration occurred after glucagon treatment, we arbitrarily set an upper limit for the assay at 16 nmol per g of liver. In actuality tissue levels were doubtless much higher than this since Exton et al., whose base-line value of 0.5 nmole per g of liver was similar to that found here, measured tissue concentrations of cyclic AMP as high as 40 nmole per g in livers perfused with 2 × 10^{-8} M glucagon (10).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
<th>C2 units → products</th>
<th>Glucose output</th>
<th>Total ketone production</th>
<th>Relative ketone specific activity</th>
<th>Tissue cyclic AMP</th>
<th>HMG-CoA reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-14C]Acetate</td>
<td>None (6)</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>[1-14C]Octanoate</td>
<td>None (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>Glucagon (12)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Dibutyryl cyclic AMP (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
ment, it was nevertheless desirable to examine the activity of this enzyme directly in microsomes prepared from livers after perfusion in the presence or absence of the hormone. This was felt to be particularly important in view of the recent report by Beg et al. (9) indicating that the activity of the enzyme is diminished in in vitro liver systems preincubated with cyclic AMP. As shown in Table II, the dramatic rise in cyclic AMP concentration seen in the intact liver after glucagon treatment clearly did not result in diminished HMG-CoA reductase activity when this enzyme was subsequently assayed in isolated microsomes.

Finally, certain aspects of these data that relate to the quantitative measurements of cholesterol synthesis in the perfused rat liver deserve some comment. First, it should be emphasized that in all of the experiments depicted in Table II, C₂ unit flux into diglyceride-precipitable sterols (assumed to represent primarily cholesterol) was approximately 60% of that into total nonsaponifiable lipids (data not shown). These findings are reminiscent of those reported by Raskin and Siperstein (17) who showed that when sterol synthesis in rat liver slices was driven at high rates using [4C]mevalonate as substrate, a significant quantity of label was found in precursors of cholesterol that co-chromatographed with squalene and lanosterol. While the significance of these findings remains to be clarified, it would seem reasonable to conclude that the values for C₂ unit flux into cholesterol listed in Table II underestimate the actual flow of carbon through the HMG-CoA reductase step by some 40%. This would indicate, therefore, that in the experiments with [1-¹⁴C]octanoate, the true flow of C₂ units through the pathway was about 6500 nmoles per 100 g body weight per 30 min, or about 2900 nmoles per g of liver per hour, a rate considerably higher than previous estimates of this parameter in the perfused rat liver (18, 19). Further, if a value of 0.4 nmoles per mg of microsomal protein per min is taken for the measured activity of HMG-CoA reductase and it is assumed that microsomal protein constitutes approximately 5% of the wet weight of liver (20), then it may be calculated that the total enzymatic capacity per g wet weight of tissue would be in the region of 1200 nmoles per hour, an activity that could accommodate the flux of 3000 nmoles of C₂ units per g per hour. Thus, under these perfusion conditions it would appear that HMG-CoA reductase was operating at approximately 72% of its maximum capacity. Second, and somewhat unexpected, was the observation that in the intact liver rates of cholesterol synthesis from [1-¹⁴C]acetate were not significantly different from those seen with [1-¹⁴C]octanoate. This contrasts with the data obtained in the present and earlier (11) studies with rat liver slices in which octanoate proved to be a more efficient precursor than acetate for sterol biosynthesis. Although no ready explanation can be offered for this discrepancy between the two systems, the possibility exists, as discussed previously (11), that in liver slices incubated with [1-¹⁴C]acetate there is a partially rate-limiting step in the cholesterol biosynthetic pathway prior to HMG-CoA reductase. Should this be the case it would seem that this limitation on the conversion of acetate into sterols is not operative in the whole organ and would lend further support to the choice of the latter preparation for detailed studies on the quantitative aspects of hepatic cholesterol biosynthesis.

In conclusion, we would like to emphasize that rates of cholesterol and fatty acid biosynthesis in the intact perfused rat liver appear to be totally independent of acutely induced changes in intracellular cyclic AMP concentrations over a very wide physiological range. It remains possible, although we consider it unlikely, that long-term elevations of the nucleotide by hormonal or dietary manipulations might affect these processes in vivo.

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REFERENCES
