The Regulation of Ketogenesis from Octanoic Acid

THE ROLE OF THE TRICARBOXYLIC ACID CYCLE AND FATTY ACID SYNTHESIS*

J. DENIS McGarry and DANIEL W. FOSTER

From the Departments of Internal Medicine and Biochemistry, The University of Texas (Southwestern) Medical School at Dallas, Dallas, Texas 75235

SUMMARY

The regulation of ketogenesis in the rat has been studied both in vivo and in the isolated perfused liver with octanoic acid as substrate. This fatty acid, which is not utilized directly for triglyceride synthesis, was chosen in order to investigate the events that govern the metabolism of fatty acids subsequent to the β-oxidation process.

Experiments in vivo established that for any given load of octanoate, fasted animals produced ketones at a much faster rate than normal animals and that insulin had no inhibitory effect on this response. Qualitatively similar results were obtained with the isolated perfused liver. Livers from alloxan diabetic rats produced ketones at maximal rates independent of the fatty acid concentration in the perfusion fluid. With high concentrations of octanoate, livers from fasted animals, unlike those from normal animals, could be induced to synthesize ketones at a rate approximately equal to that of the diabetic group.

Perfusion studies with octanoate-1-14C revealed that the activity of the tricarboxylic acid cycle, although modestly depressed in the ketotic state, exerted only a minor modulating influence on the rate of ketogenesis. Surprisingly, the depression of lipogenesis accompanying fasting was found to be of sufficient magnitude to account for a major proportion of the increased C3 unit flux into ketone bodies. The block in lipogenesis alone could not account for the markedly elevated rate of ketogenesis seen in the diabetic state in which an accelerated generation of acetyl-CoA appeared to be the dominant feature.

There is little doubt that in the intact animal a sustained high rate of ketone body formation requires an elevated influx of free fatty acids from peripheral fat depots to the liver. On the other hand, a considerable body of evidence has accumulated in recent years indicating that the concentration of nonesterified fatty acids in the blood cannot be the sole determinant of the rate of hepatic ketogenesis (1-4). The studies reported here were designed to evaluate the possible regulatory factors operating in the liver itself and were prompted by the observation that significant differences in ketone body synthesis resulted when livers from normal and fasted rats were perfused with octanoic acid. Since this fatty acid, unlike the physiological long chain fatty acids, does not serve directly as substrate for triglyceride formation (5, 6), it seemed an appropriate tool for the investigation of events occurring in the liver subsequent to the initiation of β-oxidation of fatty acids. In view of the controversy surrounding the possible role of the tricarboxylic acid cycle in the over-all control of ketogenesis (7-12), particular emphasis has been placed on an attempt to assess quantitatively the activity of the cycle in livers from normal and ketotic animals.

The findings outlined below indicate that the observed differences in ketone body synthesis from a given load of octanoic acid cannot be accounted for simply by differences in tricarboxylic acid cycle activity. Surprisingly, a depression of fatty acid synthesis may be of major importance in the ketotic state.

EXPERIMENTAL PROCEDURE

Animals—Male Sprague-Dawley rats weighing 100 to 140 g were used in all experiments. Animals were fed a diet containing 55.5% sucrose, 21% casein, and less than 1% fat (General Biochemicals, Chagrin Falls, Ohio). Fasted rats were deprived of food for 48 hours prior to use. Alloxan diabetes was induced by the intravenous injection of alloxan monohydrate (60 mg per kg of body weight) and animals were used 48 hours later if they proved to be acutely ketotic as determined by an immediate strongly positive urine test for ketones (Keto-stix, Ames Company, Inc., Billerica, Massachusetts).

Experiments in Viva—Rats were anesthetized with pentobarbital, and No. 10 polyethylene catheters were placed in the femoral artery on one side and in the inferior vena cava through the femoral vein on the other. The animals were then placed in individual restraining cages and allowed to awaken from the anesthesia. One to 2 mg of heparin were given to each animal before the experiment. Test materials were infused via the venous catheter and blood samples, generally 0.2 ml, were collected at various time intervals from the arterial catheter into heparinized polyethylene microcentrifuge tubes as previously described (13). The time required to collect a sample in this manner was approximately 1 min. In other experiments only one postinfusion blood sample (2 to 3 ml in volume) was taken.
and this was drawn rapidly from the aorta into a heparinized syringe after first anesthetizing the animal and stopping the infusion immediately before the blood was withdrawn.

Liver Perfusion—The apparatus and technique used (13) have been described in detail by Exton and Park (14). The perfusion medium (initially 80 ml) consisted of washed, aged human erythrocytes suspended to a hematocrit value of 20% in Krebs bicarbonate buffer, pH 7.4, containing bovine serum albumin sufficient to give a final concentration of 4%. The albumin was not treated to remove bound fatty acids. The flow rate of perfusion fluid through the liver was 7 ml per min and the temperature inside the cabinet was maintained at 38°C.

In experiments with unlabeled substrates the gas mixture of 95% O2-5% CO2 flowed into the oxygenator at a rate of 1.5 liters per min and escaped to the atmosphere via an outlet tube. When it was desired to collect metabolically produced 14CO2 the gas flow was increased to 3 liters per min and the effluent stream was passed through two CO2 traps, each containing 200 ml of 2 N KOH. As initially performed it was found that resistance to gas flow caused by the liquid in the traps was so great that the back pressure prevented the flow of perfusion fluid from the liver to the oxygenator. This problem was easily eliminated by placing a manometer (containing dilute H2SO4 plus indicator) between the oxygenator and the first CO2 trap and connecting the outlet from the second trap to a vacuum line. The vacuum was then increased until the back pressure, as indicated by the manometer, was relieved. Best results were obtained with a negative pressure of approximately 12 cm of water. In experiments in which measurement of hepatic components was desired the liver was removed at the end of the perfusion period, frozen in liquid nitrogen, weighed, and stored at -20°C.

Measurement of 14CO2—Each CO2 trap was fitted with a sampling port sealed with a rubber cap through which 0.5 ml of the KOH solution was withdrawn at given intervals with a syringe. This was transferred to a 25-ml center well flask, which contained 1 ml of Hyamine hydroxide (1 N solution in methanol) and a filter paper wick in the center well. The flask was then sealed with a rubber stopper and 0.25 ml of 18 N H2SO4 was injected into the outer compartment. After 45 min of shaking at room temperature the Hyamine and filter paper were removed, the center well was washed with methanol, and an aliquot was assayed for radioactivity in a liquid scintillation counter. The overall efficiency of the CO2 traps and subsequent manipulations were tested as follows. Eighty milliliters of 0.1 N H2SO4 was circulated through the perfusion apparatus with all other conditions exactly as used in experiments. Known quantities of NaH14CO3 were injected at intervals or as a constant infusion and samples were taken from each CO2 trap as described above. The sum of the radioactivity recovered from the two traps was found in every case to be 98 to 100% of that originally injected into the perfusion circuit.

Analyses on Blood, Perfusion Fluid, and Liver—Samples of blood and perfusion fluid were centrifuged and all analyses were carried out on the erythrocyte-free supernatant. Acetoacetate and β-hydroxybutyrate were determined directly on 25 or 50 μl samples as described by McGarry, Guest, and Foster (13). The concentration of octanoate in plasma and cell-free perfusate was determined by gas liquid chromatography after formation of the methyl esters. Because 30 to 40% of the methyl octanoate was invariably lost in the concentration step prior to injection of the sample into the chromatograph, an internal standard of octanoate-1-14C was used. To 1 ml of plasma was added 10 μl of a solution containing 20 μCi per ml of sodium octanoate-1-14C (specific activity, 15 μCi per μ mole). Ten milliliters of a 1:1 acetone-ethanol mixture were then added and, after centrifugation, the precipitated protein was re-extracted with a further 2 ml of acetone-ethanol. The combined extract was evaporated to dryness under a stream of N2 gas and 5 ml of dry methanol and 0.25 ml of concentrated H2SO4 were added to the residue. The mixture was refluxed for 1 hour on a sand bath. Five milliliters of water and 3 ml of chloroform were then added and, after vigorous shaking, the mixture was allowed to stand for 1 hour. The chloroform layer, which now contained 100% of the initial radioactivity, was carefully removed and evaporated to dryness at 0°C under N2 gas. The residue was then dissolved in an appropriate volume of chloroform (generally 50 to 100 μl) and a 5-μl aliquot was assayed for radioactivity. A second 5-μl aliquot was analyzed for its content of methyl octanoate by gas-liquid chromatography. Six-foot glass columns packed with Gas-Chrom P (80 to 100 mesh) coated with 15% diethyleneglycol succinate were used. The column temperature was 50°C and the gas flow was 100 ml per min. The retention time of methyl octanoate was 4 min under these conditions. Quantitative determination was obtained by triangulation of peaks and comparison with a standard curve obtained with authentic samples of the ester. The concentration of octanoate in the plasma sample was calculated after correction for loss of isotope.

In some experiments in which livers had been perfused with octanoic acid-1-14C it was desired to investigate the extent of incorporation of the label into liver lipids. The tissue was frozen in liquid N2 and pulverized in a previously cooled mortar. One gram of the resulting powder was homogenized in a Waring Blender with 20 ml of a 2:1 chloroform-methanol mixture. After centrifugation and removal of the supernatant the residue was re-extracted with a further 20 ml of solvent. Six milliliters of water and 1 ml of 0.2 N KOH were added to the combined extracts and, after shaking, the mixture was allowed to stand for 30 min. After centrifugation the water-methanol phase, which contained any ketone bodies and octanoic acid that were present in the liver sample, was removed. The lower chloroform phase was concentrated to approximately 5 ml and a 1-ml aliquot was streaked along the long axis of a thin layer chromatography plate, 40 × 20 cm, coated with Silica Gel G (0.5 mm in thickness). For identification purposes a standard mixture of known lipid composition was spotted on either side of the plate. The plates were developed in a solvent system of n-hexane-diethyl ether-acetic acid-methanol in a ratio of 90:21:2:3 by volume (15). Lipids in the standard lanes were located by spraying these areas of the dried plates with 2',7'-dichlorofluorescein (0.2% in ethanol). The corresponding areas of the plates containing the sample were scraped and the powder was placed in stoppered tubes. Phospholipids were eluted with 20 ml of a mixture of ethanol-chloroform-water-acetic acid (100:30:29:2 by volume) (16); all other lipids were eluted with 20 ml of a 2:1 mixture of chloroform methanol. Radioactivity of the fractions was determined by gas liquid chromatography after formation of the methyl esters. The over-all efficiency of the CO2 traps and subsequent manipulations were tested as follows. Eighty milliliters of 0.1 N H2SO4 was circulated through the perfusion apparatus with all other conditions exactly as used in experiments. Known quantities of NaH14CO3 were injected at intervals or as a constant infusion and samples were taken from each CO2 trap as described above. The sum of the radioactivity recovered from the two traps was found in every case to be 98 to 100% of that originally injected into the perfusion circuit.
were autoclaved for 30 min at a pressure of 15 p.s.i. When cool, the solutions were acidified with 0.5 ml of 18 N H₂SO₄; the free fatty acids were extracted into 20 ml of hexane and subsequently were converted into their methyl esters in the manner described above for octanoic acid. Aliquots of the final chloroform solutions of the methyl esters were assayed for radioactivity and analyzed by gas-liquid chromatography as described above with the exception that the column temperature was raised to 155°. Radioactivity in the individual fatty acids was determined by passing the effluent gas through glass tubes containing toluene-soaked cotton-wool which was then assayed for radioactivity in a liquid scintillation system.

In some experiments the water-methanol phase obtained by alkaline extraction of the chloroform-methanol solution was further treated to separate ketone bodies from octanoic acid. This was accomplished by acidification and extraction of the latter into hexane.

Determination of Radioactivity in Ketone Bodies—Fifty-microliter aliquots of cell-free perfusate were used for the conversion of 3-hydroxybutyrate to acetoacetate enzymatically as previously described (13). Radioactivity associated with the acetone moiety of the acetoacetate was then measured by preparation and radioassay of the Denigès salt. The isotope content of the carboxyl group was determined by the use of aniline citrate and subsequent assay of the ¹⁴CO₂ liberated. The details of these methods have been given previously (13). Control experiments indicated that the presence of octanoate-¹⁴C did not interfere with these determinations. In all experiments the isotopic content of both the acetone and carboxyl group was added to give total ketone radioactivity.

Materials—Unlabeled octanoic acid was obtained from Sigma and in all experiments was used as a complex of the sodium salt with 4% albumin in Krebs bicarbonate buffer, pH 7.4. Octanoic acid-¹⁴C was purchased from New England Nuclear. Pyridine nucleotides and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were from Boehringer. Glucagon-free insulin was obtained from Lilly. Gas chromatography supplies came from Applied Science Laboratories.

RESULTS

Ketogenesis from Octanoate in Intact Normal and Fasted Rats—Normal and fasted rats were prepared for infusion experiments as described above. After control samples of blood had been obtained sodium octanoate was given intravenously at the rate of 10 μmoles per min and additional samples were taken at the indicated times for acetoacetate and 3-hydroxybutyrate determinations. Other animals were treated identically except that octanoate was omitted from the infusion mixture. While small variations were observed in total ketone response to octanoate the typical pattern shown in Fig. 1 was regularly found. Fasted animals, whose basal levels of plasma ketones were generally lower than those of the normal group, always responded with a dramatic increase in ketone body production in contrast to normal rats in which only a slight increase was noted.

These data suggested that the livers of the fasted animals were better equipped to convert octanoic acid into ketone bodies than those of the normal rats. Before such a conclusion could be drawn, however, it had to be established that under these experimental conditions the livers of both types of animals were exposed to the same concentration of plasma octanoate. For this reason the sodium octanoate solution was infused at varying rates for a period of 20 min, at which time the animals were anesthetized and blood was drawn from the abdominal aorta for analysis of ketone body and octanoate levels. The results are shown in Fig. 2 where the change in plasma ketone concentration has been plotted against the octanoate level. It can be clearly seen that the ketogenic response was more marked in the fasted animals at all concentrations of plasma octanoate.

In view of the known antiketogenic effect of insulin and the fact that the blood level of this hormone is diminished in fasting (17), it was conceivable that the observed differences could have been the result of differences in plasma insulin levels. For this reasons...
reason the experiments with octanoate were repeated after first reversing the starvation ketosis with insulin. As shown in Fig. 3, 0.05 unit of insulin caused a sharp reduction in the level of plasma ketones in the fasted animals. At 30 min a second injection of insulin was given followed immediately by an infusion of octanoate. Despite the presence of insulin sufficient to reverse the original ketosis, the ketogenic response to octanoate was unimpaired.

In order to investigate in more detail the differences in response to octanoate in normal and ketotic animals and the failure of insulin to suppress ketone body synthesis from this fatty acid, a series of experiments in vitro was undertaken.

Ketogenesis from Octanoate in Isolated Perfused Liver—The first experiments were designed to establish whether the observations made in vitro could be reproduced in the isolated perfused liver. To this end livers from fed, fasted, and alloxan diabetic rats were perfused without substrate for 15 min, after which an infusion of sodium octanoate was begun as described in Fig. 4. Samples of the perfusate were removed at intervals and analyzed for acetacetate and β-hydroxybutyrate content. The results indicate striking differences between the three types of liver. In the absence of substrate, livers from diabetic rats synthesized ketones at a remarkably brisk rate compared with the less ketogenic group which generally exhibited 2 to 3 times the endogenous rate found in livers from normal animals. However, the addition of octanoate had little effect on the rate of ketogenesis in livers from diabetic animals whereas it greatly stimulated production in livers from the other two groups. In confirmation of the results obtained in vivo, the response of the livers from fasted rats was considerably greater than that of the normal animals. Between 15 and 30 min of perfusion the rates of ketogenesis in the absence of added substrate were 12, 32, and 260 μmoles/100 g of body weight per hour for livers from normal, fasted, and diabetic animals, respectively. Octanoate infusion caused an increase in these rates to 160, 280, and 320 μmoles/100 g of body weight per hour. It thus appeared that livers from both normal and fasted animals were capable of increasing ketone body output under the influence of an octanoate load while livers from the diabetic rats were operating close to maximal capacity even in the absence of added substrate. In experiments not shown, neither insulin nor glucose, separately or together, had any significant effect on rates of ketogenesis in the presence or absence of octanoate.

In the preceding experiment the volume of perfusion fluid into which the priming dose of octanoate (150 μmoles) was infused was approximately 75 ml. Thus the initial concentration of the fatty acid was approximately 2 mM in the whole perfusion fluid and 2.5 mM in the cell free medium (based on a 20% hematocrit value and the observation that no fatty acid was trapped inside the red blood cells). Inspection of Fig. 2 indicates that in vivo this concentration of octanoate in plasma was sufficient to cause maximum rates of ketogenesis in both normal and fasted animals. In order to compare the ketogenic response of the three experimental groups of liver to a range of concentrations of substrate in vitro, a series of experiments was undertaken in which single doses of 25, 50, 100, 150, or 200 μmoles of octanoate were added to the perfusate in order to bring the initial concentration of the fatty acid in the cell free medium to 0.4, 0.8, 1.6, 2.4, or 3.2 mM. The results are shown in Fig. 5. At all levels of substrate the rate of ketone body production by livers from fasted animals was markedly greater than that in livers from fed rats. Since the activity found in the diabetic group was virtually independent of the amount of fatty acid added, only the results obtained with 0 and 200 μmoles of octanoate are illustrated. When the rates of ketogenesis were calculated for the first 15 min after the addition of octanoate and plotted against the initial concentration of the fatty acid, the curves shown in Fig. 6 were obtained. It will be observed that at high substrate levels the livers from fasted animals were capable of manufacturing ketone bodies at rates comparable with those of the diabetic group (300 to 320 μmoles/100 g of body weight per hour). In contrast, the normal group exhibited maximal rates in the region of 200 μmoles/100 g of body weight per hour, indicating fundamental differences in fatty acid metabolism in the intact livers of ketogenic and nonketotic animals. It is of interest that these data bear a striking qualitative resemblance to the curves obtained with normal and
FIG. 5. The effect of different concentrations of octanoate on the rate of ketogenesis and the \( \beta \)-hydroxybutyrate to acetoacetate ratios in perfused livers from normal, fasted, and alloxan diabetic rats. Livers were perfused without substrate for 15 min, at which time 0 to 200 \( \mu \)moles of sodium octanoate (as indicated by numbers on the curves) was given as a single dose. Each point represents the mean value of two to four perfusions. For reasons of clarity the standard errors, which were very small, have been omitted from the figure.

Included in Fig. 5 are data showing the changes in the \( \beta \)-hydroxybutyrate-acetoacetate ratios during the course of the perfusions. As expected, a close parallel was observed between the rate of ketone body production and the value imposed by the liver on this ratio. The changes in the \( \beta \)-hydroxybutyrate and acetoacetate concentrations reflect changes in the intramitochondrial NADH : NAD ratio which, in turn, is influenced by the rate of fatty acid oxidation (18).

Conversion of Octanoate-\( ^{14} \)C into Ketone Bodies and CO\(_2\) by Perfused Rat Liver—It is a widely held view that one of the central factors controlling the rate of ketogenesis is the intramitochondrial concentration of oxaloacetate, fluctuations of which might be expected to modulate the rate at which acetyl-CoA can enter the tricarboxylic acid cycle (4, 8, 9, 12). If this were the explanation for the differences in behavior between the normal and ketotic rats discussed above, the useful information should be gained by measuring the relative rates of incorporation of isotope into ketone bodies and CO\(_2\) during the metabolism of octanoate-\( ^{14} \)C by the perfused liver. Such an approach would allow an assessment of the total flux of C\(_2\) units into ketone bodies and the tricarboxylic acid cycle. Therefore, experiments were carried out in which livers received a priming dose and then a constant infusion of octanoate-\( ^{14} \)C. The \( ^{14} \)CO\(_2\) evolved during the course of the perfusion was measured, and the amount and specific radioactivity of the ketone bodies formed were determined. The results of these experiments are shown in Table I. Prior to consideration of the data, the method of calculating the carbon flux is discussed.

Calculations in Table I were based on the following considerations. Since the specific activity of the added octanoate-\( ^{14} \)C was known, the amount of this material entering the ketogenic pathway could be calculated from the total radioactivity found in the ketone bodies (acetone plus carboxyl moieties). Multiplication of this figure by 4 represents the micromoles of labeled C\(_2\) units (i.e., C\(_2\) units derived from octanoic acid-\( ^{14} \)C) converted into ketones (Table I, Column A). Similarly, the total number of C\(_2\) units thus converted is given by the total production of ketones multiplied by 2 since 2 molecules of acetyl-CoA are utilized in the synthesis of one molecule of the 4-carbon ketone (Column B). The “specific activity” of the C\(_2\) unit pool from which the ketone bodies arise is defined by dividing Column A by Column B and is listed as Column C. If it is assumed that the acetyl-CoA produced from the metabolism of endogenous substrate and that resulting from the oxidation of octanoate-\( ^{14} \)C enter a common pool prior to the formation of acetoacetate, the value of A/B provides an index of the extent of dilution of labeled C\(_2\) units by unlabeled 2 carbon molecules. The maximum possible value for this ratio would be 1, in which case all of the ketone bodies would have originated from the labeled octanoate.

Because metabolically produced \( ^{14} \)CO\(_2\) was overwhelmingly diluted with nonradioactive CO\(_2\) from the gas phase it was impossible to determine its specific activity under these conditions. However, from the total radioactivity recovered in trapped CO\(_2\) the number of labeled C\(_2\) units from octanoate-\( ^{14} \)C converted into CO\(_2\) could be assessed (Column D). The further assump-

![Diabetic Starved Normal](https://example.com/diabetic_starved_normal.png)

![Initial Ketone Production](https://example.com/initial_ketone_production.png)

![Initial Octanoate Concentration](https://example.com/initial_octanoate_concentration.png)
Livers were perfused in the manner described in the text. Sodium octanoate-1-14C (0.25 μCi per μmole) was given at zero time as a priming dose followed by a constant infusion as indicated. Samples of perfusate and KOH from the CO2 traps were taken for analysis at 15-min intervals. The numbers in parentheses refer to the number of livers. When used, glycerol was added at zero time to give an initial concentration in the cell-free utilizing the group means of Columns A to D.)

The data have been presented in Table I. In the discussion that follows units of C2 flux are frequently omitted but it should be understood that, unless otherwise stated, this refers to micromoles converted into product in 60 min per 100 g of body weight. Consideration was made that a common pool of acetyl-CoA feeds the tricarboxylic acid cycle and the ketogenic pathway, allowing the total number of C2 units oxidized to CO2 to be calculated by dividing the figure in Column D by that in Column C to give Column E.

As shown in Table I, livers were perfused with different quantities of octanoate-1-14C. Samples of perfusate and KOH from the CO2 traps were taken for analysis at 15-min intervals over a period of 1 hour. In all cases the rate of ketone body production were approximately linear throughout the term of the experiment and the specific activity of the ketones was constant. The evolution of radioactive CO2, however, was characterized by an initial lag phase of 0 to 15 min, following which a linear response was observed. The slow initial rate presumably reflects the time taken for isotopic acetyl-CoA to equilibrate with the multiple pools of nonradioactive intermediates of the tricarboxylic acid cycle. These points are illustrated in Fig. 7 which shows data obtained with livers from fasted animals. Qualitatively similar curves were obtained with livers from the normal and diabetic rats and for this reason, in order to simplify presentation of the data, only the values obtained at the 60 min time point have been presented in Table I. In the discussion that follows units of C2 flux are frequently omitted but it should be understood that, unless otherwise stated, this refers to micromoles converted into product in 60 min per 100 g of body weight.

Considering first the response to the highest load of octanoate (Experiment 3) it is seen that livers from normal, fasted, and diabetic rats converted 111, 243, and 273 labeled C2 units into ketones under circumstances in which total C2 flux into this pathway was 144, 341, and 687, respectively. On both counts, then, livers from the fasted animals were more than twice as active as those from normal animals. Total ketone body production in the diabetic group was twice again greater than that in the fasted rats while ketogenesis from octanoate-1-14C appeared to be identical in the two states. It is clear, therefore, that while octanoate had little stimulatory effect on the over-all rate of ketogenesis in livers from diabetic animals (see Figs. 4 and 5), this fatty acid was in fact metabolized at a rate equal to that in livers from fasted rats. From the "specific activity" of the C2 pool (Column C) it is apparent that in livers from the normal and fasted animals the added fatty acid contributed 70 to 77% of the total acetyl-CoA generated but that in the diabetic liver its contribution was only 40%.

The rate of flux of labeled and total C2 units into the tricarboxylic acid cycle and the ketogenic pathway, allowing the total number of C2 units oxidized to CO2 to be calculated by dividing the figure in Column D by that in Column C to give Column E.

Conversion of octanoate-1-14C into ketone bodies and CO2 by perfused livers from normal, fasted, and alloxan diabetic rats

### Table I

<table>
<thead>
<tr>
<th>Experiment and state of animal</th>
<th>Octanoate-1-14C</th>
<th>Glycerol</th>
<th>A. Labeled C2 units ketones</th>
<th>B. Total C2 units ketones</th>
<th>C. &quot;Specific activity&quot; of C2 pool (A/B)</th>
<th>D. Labeled C2 units CO2</th>
<th>E. Total C2 units CO2 (D/C)</th>
<th>F. Labeled C2 units ketones + CO2 (A + D)</th>
<th>G. Total C2 units ketones + CO2 (B + E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal (4)</td>
<td>12.5 0.5</td>
<td>−</td>
<td>23 ± 1.8</td>
<td>61 ± 4.2</td>
<td>0.38</td>
<td>28 ± 2.6</td>
<td>73 ± 4.4</td>
<td>51 ± 3.8</td>
<td>134 ± 7.1</td>
</tr>
<tr>
<td>Fasted (4)</td>
<td>12.5 0.5</td>
<td>−</td>
<td>56 ± 5.6</td>
<td>263 ± 26</td>
<td>0.28</td>
<td>18 ± 0.5</td>
<td>65 ± 2.9</td>
<td>73 ± 5.8</td>
<td>268 ± 29</td>
</tr>
<tr>
<td>2. Normal (4)</td>
<td>25.0 1.0</td>
<td>−</td>
<td>56 ± 1.2</td>
<td>109 ± 3.1</td>
<td>0.81</td>
<td>51 ± 2.8</td>
<td>98 ± 5.5</td>
<td>106 ± 3.7</td>
<td>207 ± 7.9</td>
</tr>
<tr>
<td>Fasted (4)</td>
<td>25.0 1.0</td>
<td>−</td>
<td>138 ± 2.8</td>
<td>273 ± 14</td>
<td>0.50</td>
<td>38 ± 0.6</td>
<td>76 ± 3.8</td>
<td>174 ± 2.7</td>
<td>349 ± 18</td>
</tr>
<tr>
<td>3. Normal (6)</td>
<td>50.0 2.0</td>
<td>−</td>
<td>114 ± 4.1</td>
<td>144 ± 5.9</td>
<td>0.77</td>
<td>87 ± 5.5</td>
<td>113 ± 9.3</td>
<td>195 ± 5.5</td>
<td>257 ± 12</td>
</tr>
<tr>
<td>Fasted (5)</td>
<td>50.0 2.0</td>
<td>−</td>
<td>245 ± 11.4</td>
<td>341 ± 13</td>
<td>0.71</td>
<td>41 ± 3.2</td>
<td>58 ± 4.9</td>
<td>294 ± 10</td>
<td>399 ± 9.8</td>
</tr>
<tr>
<td>Diabetic</td>
<td>50.0 2.0</td>
<td>−</td>
<td>273 ± 18</td>
<td>687 ± 7.4</td>
<td>0.40</td>
<td>18 ± 1.4</td>
<td>46 ± 4.4</td>
<td>291 ± 18</td>
<td>733 ± 7.3</td>
</tr>
<tr>
<td>(4)</td>
<td>50.0 2.0</td>
<td>+</td>
<td>267 ± 5.2</td>
<td>267 ± 5.8</td>
<td>0.92</td>
<td>36 ± 2.3</td>
<td>40 ± 3.9</td>
<td>292 ± 4.0</td>
<td>397 ± 9.2</td>
</tr>
</tbody>
</table>

**Fig. 7.** The conversion of octanoate-1-14C into ketone bodies and CO2 by perfused livers from fasted rats in the absence of glycerol. Livers received a priming dose of 50 μmoles and a constant infusion of 2 μmoles per min of octanoate-1-14C (0.25 μCi per μmole). The data have been taken from Table I, Experiment 3, and represent the means of five perfusions in livers from fasted animals. A, labeled C2 units ketones; B, total C2 units ketones; C, "specific activity" of ketones (A/B); D, labeled C2 units CO2; E, total C2 units CO2. In these abbreviations the arrow means "converted into."
boxylic acid cycle is shown in Columns D and E where it can be seen that in both respects the normal liver was about twice as active as the fasted. Surprisingly, however, the difference in $C_2$ flux into the cycle was numerically insufficient to account for the different flux rates into ketone bodies. This is apparent from the figures in Columns F and G which show the total flux through these two pathways. Had decreased flux into the cycle been primarily responsible for the enhanced ketogenesis in fasting, the sum of the fluxes through both pathways would have been identical in the two conditions and this was not the case. Consistent with this interpretation is the finding that total ketone body production in the diabetic state was double that found in livers from fasted animals without significant further depression of total $C_2$ flux into the tricarboxylic acid cycle.\(^1\)

Studles with lower substrate loads are shown in Experiments 1 and 2 in which only livers from normal and fasted animals were examined. The data show basically the same pattern seen with the higher substrate delivery discussed above. Again ketogenesis in the fasted group was markedly greater than in the normals.

\(^1\) It should be understood that fluxes through the tricarboxylic acid cycle as estimated here represent labeled $C_2$ units from the acetyl-CoA pool recovered as $^{14}CO_2$ and that they may not equal total carbon flow because of dilution by unlabeled intermediates of the cycle. The possibility also existed that the extent of dilution varied in the fed and ketogenic states such that the estimates presented above would be misleading. In an attempt to gain information about these problems, in another set of experiments we determined the specific radioactivity of metabolically produced $CO_2$ by perfusing livers with media freed of carbon dioxide and bicarbonate and with a gas phase of pure oxygen. The effluent gas was passed through freshly prepared $4\% Ba(OH)_2$ which had been filtered immediately before use under a CO$_2$-free atmosphere. Fresh traps were provided at 10-min intervals so that the specific activity of $CO_2$ was determined sequentially and not cumulatively.

Precipitated barium carbonate was immediately collected, washed thoroughly, and dried. The precipitate was transferred to aluminum foil planchettes, 1 cm in diameter, and weighed. The planchet and precipitate were then placed in the outer compartment of a center well flask and acidified, the radioactive $CO_2$ being collected as described earlier. In control experiments, in which the liver was omitted from the system, no barium carbonate was found in the traps.

In all cases the specific activity of the $CO_2$ increased over a 1-hour period without reaching a steady state. The results of two representative experiments follow. In a fed animal the specific activities of the perfusate ketones at the 20-, 40-, and 60-min intervals were 16.3, 16.1, and 16.0 $\times 10^4$ dpm per pmole, respectively. The simultaneously determined specific activities of $CO_2$ were 0.18, 0.92, and 1.30 $\times 10^4$ dpm per pmole. Since the maximal theoretical specific activity of the $CO_2$ would be one-fourth that of the ketones, the specific activity at 60 min was 32$\%$ of maximum (1.3 $\times 4/10$), indicating about a 5-fold dilution of labeled $C_2$ units by unlabeled tricarboxylic acid cycle intermediates at this time point. A similar experiment in an alloxan diabetic animal gave values of 5.02, 6.10, and 7.06 $\times 10^4$ dpm per pmole for the specific activities of ketones at 20, 40, and 60 min while specific activities of $CO_2$ were 0.11, 0.49, and 0.71 $\times 10^4$ dpm per pmole. In this case the observed specific activity at 60 min was 40$\%$ of maximum (0.71 $\times 4/7.06$), suggesting a 2.5-fold dilution by unlabeled intermediates. Two conclusions appear warranted from these results. First, it seems unlikely that major differences exist in the pool sizes of tricarboxylic acid cycle intermediates in ketotic and nonketotic states. Flux rates as calculated in the text can, therefore, be assumed to be reasonable indicators of total cycle activity. Second, since the specific activity of the $CO_2$ was still increasing at the 60-min point (in other experiments an isotopic steady state was not reached at 100 min), the upper limit of total carbon flux through the cycle would be 2 to 3 times that calculated for labeled $C_2$ flux.
of free fatty acids in the blood remained essentially unaltered. In view of the suggestion by these investigators that the mechanism involved in this reversal of starvation ketosis was an elevation of the intramitochondrial level of oxaloacetate which would facilitate oxidation of acetyl-CoA in the tricarboxylic acid cycle, it seemed appropriate to test the effect of glycerol on the metabolism of octanoate-1-14C. According to this hypothesis, the addition of glycerol to the perfused liver metabolizing octanoate-1-14C should have the effect of causing an increase in the rate of 14CO2 evolution and a decrease in labeled ketone formation. As seen in Experiment 3 of Table I, this was found not to be the case. In livers from fasted animals the flux of labeled C3 units into ketones and into CO2 was unaffected by the addition of glycerol. On the other hand, total C3 flux into ketones was decreased from 341 to 267 amoles/100 g of body weight and total C3 flux into CO2 was reduced from 88 to 40. Thus, it can be calculated that the flow of unlabeled C3 units through both pathways was depressed by approximately 80%. These findings indicate that the antiketogenic effect of glycerol resulted from its action on the metabolism of endogenous substrate, presumably long chain fatty acids, while the metabolism of octanoic acid was unaffected. The data, therefore, do not support the concept that glycerol diminishes ketone body synthesis by stimulating tricarboxylic acid cycle activity. They would, however, be consistent with the view that glycerol exerts an inhibitory effect on the generation of acetyl-CoA from long chain fatty acids and that this site of action is not shared by the metabolism of short chain fatty acids. While the precise mechanism by which glycerol exerts its antiketogenic action was not investigated a likely possibility would be that, after its conversion into α-glycerophosphate, it promotes the esterification of long chain fatty acids and thereby diverts them from oxidative metabolism. A similar conclusion was reached by Wioland and Matschinsky (20) who observed that glycerol was strongly antiketogenic toward oleic acid in the perfused rat liver but was without effect when octanoic acid was the substrate. The failure of glycerol to inhibit ketogenesis from octanoate was also reported by Edson (19). It should be noted that glycerol had minimal effects on livers from diabetic animals, for reasons that are not clear.

Uptake of Octanoate by Perfused Liver—As discussed above, differences in the rate of acetyl-CoA oxidation via the tricarboxylic acid cycle in livers from normal and ketotic rats were insufficient to account for the observed differences in ketogenesis from octanoic acid. It was possible that this fatty acid was taken up and oxidized more rapidly in livers from ketotic animals as compared with the normal group. It was necessary therefore to measure the amount of octanoate remaining in the perfusion fluid at the end of these experiments. This was done in two ways. First, in the perfusions referred to in Experiment 3, Table I, aliquots of the 60-min cell-free perfusate were taken through the lipid extraction procedure as described for liver tissue under "Experimental Procedure." It was found that virtually all of the radioactivity present in the initial chloroform-methanol extract was recovered in the alkaline water-methanol phase. After acidification of the latter and extraction with hexane approximately the same amount of radioactivity was removed in all samples. This suggested that all of the perfusion samples contained similar quantities of octanoic acid. In these experiments direct assay of octanoate content was precluded by the fact that octanoate-1-14C had been infused. Additional evidence that the uptake of octanoic acid was equivalent in the livers from normal and ketotic rats was obtained when identical perfusions were carried out with unlabeled octanoate and the 60-min perfusion fluid was analyzed for octanoate content by the method described for plasma. Close agreement was found by the two methods and approximately 70% of the fatty acid was taken up by each group of livers.

Distribution of Isotope from Octanoate-1-14C in Liver Tissue—Since approximately equal amounts of octanoate-1-14C were taken up by the livers from normal, fasted, and diabetic rats while considerably more radioactivity appeared in ketones and CO2 in the ketotic compared to the normal groups, it seemed reasonable to investigate the distribution of isotope from the labeled fatty acid in various liver components. Accordingly, portions of the livers used in Experiment 3, Table I, were extracted as described earlier. The results are presented in Table II and represent a balance sheet showing the percentage distribution of the label from octanoate-1-14C in the livers and perfusion media. Total recovery of the administered isotope ranged from 81 to 91%. The major points of interest are, first, that the incorporation of isotope into phospholipids and triglycerides was markedly diminished in the livers from fasted and diabetic animals compared with the normal group (Column D). Second, when these differences are taken into account it can be seen that the total recovery of radioactivity in ketones, CO2, and lipids was essentially identical in all three groups. Saponification and extraction of the phospholipid and triglyceride fractions established that all of the radioactivity was present as fatty acids. Analyses of the methyl esters of these fatty acids by gas chromatography indicated that virtually all of the radioactivity was present as palmitate and stearate with no isotope detectable in the short chain fractions.

At the present time the precise mechanism involved in the synthesis of long chain fatty acids from octanoate-1-14C is not clear. However, experiments outlined below strongly indicate that the octanoic acid is first metabolized to acetyl-CoA which then participates in the formation of long chain fatty acids partly through a process de novo and partly through a mechanism of chain elongation. Assuming that the acetyl-CoA involved in lipogenesis is in equilibrium with that entering the ketogenic and oxidative pathways it is convenient to translate the data in Table II, Column D, into rates of C3 unit flux into long chain fatty acids as discussed for the data in Table I. This has been done in Table IV in an attempt to gain some insight into the mechanism of long chain fatty acid synthesis from octanoate two livers from normal animals were perfused with octanoate-1-14C as described in Experiment 1, Table I. Two more livers were perfused with acetate-1-14C. The triglyceride fatty acids were isolated and subjected to the Schmidt degradation as described by Goldfine and Bloch (21). The decarboxylation ratios (i.e. the ratio of radioactivity present in the complete fatty acid molecule to that present in the carboxyl group) were found to be 4.4 and 4.6 in the livers perfused with octanoate-1-14C and 3.5 and 4.3 in those perfused with acetate-1-14C. Since the fatty acids synthesized were predominantly C16 and C18 in chain length a decarboxylation ratio in the region of 8 to 9 would have been expected had the fatty acids been synthesized entirely de novo from the acetyl-CoA pool. The lower values obtained suggest that significant quantities of these fatty acids were synthesized by a process involving the elongation of unlabeled precursor free fatty acids or partially completed fatty acid chains with labeled C3 units. In the context of this study the important point is that similar ratios were obtained with octanoate-1-14C and acetate-1-14C as substrates. This provides strong evidence that the biosynthesis of long chain fatty acids from octanoate involves the preliminary oxidation of this fatty acid to acetyl-CoA.
III. When taken together with the data in Columns F and G of Table I, Experiment 3, these figures indicate that the difference in isotopic C₂ flux into ketones plus CO₂ between the normal and ketogenic groups is numerically approximately equal to the differences in labeled C₂ units appearing in long chain fatty acids. Moreover, the increased conversion of total C₂ units into ketones plus CO₂ in the fasted with respect to normal groups is almost fully accounted for by the high rate of fatty acid synthesis in the latter. Although long chain fatty acid synthesis was not measured in livers receiving smaller loads of octanoate (Experiments 1 and 2, Table I), it can be inferred by extrapolation of the data obtained in Experiment 3 that in these cases also the differences between livers from normal and fasted animals in total C₂ flux into ketones plus CO₂ (Column G) reflects primarily the difference in lipogenesis in the two nutritional states. Surprisingly, this metabolic parameter appeared to remain constant (approximately 140 µmoles of C₂ units converted into long chain fatty acid per 100 g of body weight in 60 min), irrespective of the rate of delivery of octanoate.

**DISCUSSION**

The studies of octanoate metabolism reported here allow a number of conclusions to be drawn about the regulation of ketogenesis. The first point to be emphasized is that the relation between the rate of delivery of fatty acids to the liver and the extent of hepatic ketogenesis is not the same in normal and ketogenic animals. This was true both in vivo and in vitro with the intact liver since equivalent concentrations of octanoic acid reaching hepatic tissue resulted in widely disparate rates of ketogenesis in normal and starved rats. This observation was consonant with previous reports that elevation of long chain free fatty acid concentrations in the blood of dogs did not result in ketosis (1, 3) and the fact that fasting ketosis in rats could be reversed under circumstances in which fatty acid levels remained high (4, 22). In the studies cited the possibility existed that the nonparallel relation between free fatty acid levels and ketogenesis was due, at least in part, to variations in rates of esterification of the incoming fatty acids prior to entry into the β-oxidative pathway (10). Since octanoate is known not to be utilized directly for triglyceride synthesis (5, 6), the fact that differences in ketogenesis existed with this fatty acid strongly suggested the operation of regulatory factors beyond the initiation of the β-oxidation sequence.

In an attempt to dissect the mechanisms accounting for these differences, extensive use was made of the isolated perfused liver system which gave responses in ketogenesis to varying loads of octanoate qualitatively similar to the responses found in vivo. Several points of interest emerged from the initial experiments. In contrast to livers from normal and fasted animals, livers from diabetic rats appeared to produce ketone bodies at a maximal rate even in the absence of added fatty acid, confirming the observation of Van Harken, Dixon, and Heimberg (23) who studied ketogenesis in livers perfused with oleic acid. Presumably this lack of dependence of ketogenesis on the influx of fatty acids to the liver reflects the ready availability of fatty acids from the greatly expanded hepatic triglyceride stores (24) which saturate the β-oxidation machinery. It is also noteworthy that livers from fasted animals, unlike those from normal rats, could be induced to produce ketone bodies from octanoate at a rate comparable with that of the diabetic group.

In view of long standing interest in the possibility that diminished Krebs cycle activity is related to the initiation of ketogenesis (4, 8, 9, 12), it seemed warranted to test this concept with the intact liver by assessment of 2-carbon flux through the cycle, with octanoate-1-¹⁴C as the ketogenic substrate. The results indicated modest depression of acetyl-CoA oxidation in the livers from fasted and diabetic animals. Importantly, however, the diminished C₂ flow into the tricarboxylic acid cycle could not in itself account for the elevated flux of C₂ units into ketone bodies. Furthermore, in livers from normal animals, increasing levels of octanoate caused concurrent increases in ketogenesis and cycle activity. Only at high levels of infused fatty acid was accelerated

---

**TABLE II**

Conversion of octanoate-1-¹⁴C into long chain fatty acids by infused livers from normal, fasted, and alloxan diabetic rats

<table>
<thead>
<tr>
<th>State of animal</th>
<th>Conversion into long chain fatty acids</th>
<th>Per cent of infused radioactivity recovered as</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled C₂ units</td>
<td>Total C₂ units</td>
<td>Ketones</td>
</tr>
<tr>
<td>Normal...........</td>
<td>92 ± 13</td>
<td>120 ± 16</td>
<td>20.0 ± 0.7</td>
</tr>
<tr>
<td>Fasted...........</td>
<td>9 ± 1.1</td>
<td>13 ± 1.8</td>
<td>41.8 ± 2.0</td>
</tr>
<tr>
<td>Diabetic........</td>
<td>11 ± 1.1</td>
<td>27 ± 3.8</td>
<td>43.8 ± 3.3</td>
</tr>
</tbody>
</table>

---

**TABLE III**

Distribution of radioactivity after perfusion of livers from normal, fasted, and alloxan diabetic rats with octanoate-1-¹⁴C

Values are expressed as means ± standard error and refer to the percentage of infused radioactivity recovered in the indicated products in Experiment 3 of Table I after livers had been perfused for 60 min with octanoate-1-¹⁴C. Column D represents total radioactivity in the alkali-washed chloroform-methanol extracts of liver. Approximately 90% of this radioactivity was present in phospholipids and triglycerides.
ketogenesis accompanied by diminished C₂ flow into the tricarboxylic acid cycle. Even in livers from fasted animals a similar relationship was observed although with low rates of octanoate infusion cycle activity was lower than in livers from normal rats. On the basis of these results it is quite clear that in the intact liver increased rates of ketone body synthesis do not always demand concomitant depression of tricarboxylic acid cycle activity (11).

The diminished capacity of the liver to synthesize fatty acids in fasting and diabetes is well documented (6) and it has been proposed that this metabolic lesion is a contributory factor in the development of the ketosis characteristic of these conditions (25-27). However, studies of hepatic ketogenesis and fatty acid synthesis in vitro showed that maximal activity of the latter was only a fraction of the rate of ketone formation (23-30). For this reason, at the outset of this investigation, it was felt unlikely that variations in rates of lipogenesis would play an important role in the regulation of ketogenesis from octanoic acid. It was surprising, therefore, that calculation of the rate of conversion of C₈ units into long chain fatty acids in livers from normal rats actually turned out to be equal to or greater than the C₂ flux into ketones or CO₂ over a wide range of rate of octanoate delivery to the liver increased rates of ketone body synthesis do not always demand concomitant depression of tricarboxylic acid cycle activity (11).

In summary, we take the view that the control of ketogenesis is not invested in a single regulatory step (cf. Srere (33)) and favor the interpretation that under given conditions ketone body production may be affected by changes at several reaction sites. In the mild ketosis of fasting underutilization of acetyl-CoA may play the key role whereas severe diabetic ketosis results under circumstances in which the rate of delivery of fatty acid to the liver exceeds its capacity for esterification, resulting in a rate of generation of acetyl-CoA that vastly surpasses the tissue's ability to utilize the latter compound via oxidative and lipogenic pathways.

Acknowledgments—The authors express appreciation for the expert technical assistance of Misses M. Joanne Guest and Petra Contreras.

REFERENCES

The Regulation of Ketogenesis from Octanoic Acid: THE ROLE OF THE
TRICARBOXYLIC ACID CYCLE AND FATTY ACID SYNTHESIS
J. Denis McGarry and Daniel W. Foster


Access the most updated version of this article at http://www.jbc.org/content/246/4/1149

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/4/1149.full.html#ref-list-1