Action of the Oral Hypoglycemic Agent 2-Tetradecylglycidic Acid on Hepatic Fatty Acid Oxidation and Gluconeogenesis

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Gene F. Tutwiler† and Paul Dellevigne

From the Department of Biochemistry, McNeil Laboratories, Fort Washington, Pennsylvania 19034

Using isolated hepatocytes from fasted rats, the oral hypoglycemic agents, 2-tetradecylglycidic acid (McN-3802) and its methyl ester (McN-3716) inhibited (concentrations down to $5 \times 10^{-7}$ M) the oxidation of palmitate to CO$_2$ and ketones but not the oxidation of octanoate or palmitylcarnitine. The antiketogenic effect which occurs at concentrations as low as $10^{-7}$ M was accompanied by a lowered $\beta$-hydroxybutyrate/acetoacetate ratio and a significant drop in long chain acylcarnitine levels with unchanged levels of total or free acid-soluble carnitine. The fall of long chain acylcarnitines taken together with an observed increase of long chain fatty acyl coenzyme A derivatives suggests that the carnitine acyltransferase I is the site of action of McN-3802 (or its metabolites). Furthermore, it is unlikely that long chain fatty acyl-CoA synthetase is the site of action since McN-3802 inhibited the microsomal synthetase only at high concentrations and since at these concentrations nonhydropglicemic analogs of McN-3802 were also inhibitory. McN-3802 and McN-3716 were found to be converted to acyl CoA thioesters by this enzyme.

McN-3802 produced in hepatocytes from normal and diabetic rats a concentration-dependent inhibition of gluconeogenesis from pyruvate and a lowered lactate/pyruvate ratio which paralleled its inhibitory effect on ketogenesis. Closely related nonhypoglycemic analogs failed to produce these effects even though these analogs were converted to CoA esters by acyl-CoA synthetase. The inhibition of gluconeogenesis when pyruvate was added to liver cells could not be reversed by addition of CoA + carnitine, or by elevating the lactate/pyruvate and $\beta$-hydroxybutyrate/acetoacetate oxidation-reduction couples by addition, respectively, of ethanol and $\beta$-hydroxybutyrate. That the inhibition of gluconeogenesis was secondary to inhibition of long chain fatty acid oxidation seems certain since this effect was almost completely reversed upon addition of 1 to 2 nM octanoate. The potential sites where McN-3802 may be inhibiting gluconeogenesis are discussed. McN-3802 produced much less of an effect on total production of glucose using hepatocytes from fed rats.

Although the discovery of coenzyme A almost 30 years ago (1) and the isolation of the $\beta$ oxidation enzymes shortly thereafter (2), understanding of the organization and regulation of free fatty acid oxidation is still deficient. This is not surprising when one considers the difficulties. The fatty acid derivatives in oxidation (CoA and carnitine esters) occur only in small amounts, are in more than one cellular compartment, and bind tightly to proteins. Inhibitors of fatty acid oxidation such as 4-pentenoic acid, hypoglycin, a-bromopalmitate and (+)-decanoylcarnitine (3-6) have improved our understanding of the regulation of fatty acid oxidation; however, these agents inhibit enzymes unrelated to fatty acid oxidation (3, 4, 7, 8) or are toxic, or both (3, 4, 6). Therefore, a specific and effective inhibitor is still needed for studies of the regulation of fatty acid oxidation. The discovery of such an agent becomes even more important since considerable evidence suggests that depression of muscle glucose utilization and the stimulation of gluconeogenesis by the liver observed in conditions of high lipid mobilization such as diabetes are caused by the products of fatty acid oxidation (3, 9, 10). Therefore, the use of specific inhibitors of fatty acid oxidation may help to define the role of excessive fatty acid oxidation in diabetes and other diseases and might be used therapeutically.

Therefore, 2-tetradecylglycidic acid (McN-3802) and its methyl ester (McN-3716) were synthesized and tested as part of a program directed at generating analogs of fatty acids which would be orally effective, specific inhibitors of fatty acid oxidation. In this report, these compounds will be shown to be potent specific inhibitors of the carnitine-dependent oxidation of long chain free fatty acids by isolated hepatocytes and evidence as to the site of inhibition will be presented. Also, the effects of McN-3802 and McN-3716 on gluconeogenesis were studied since it seems well established that fatty acid oxidation is essential for maximal rates of gluconeogenesis. Such an effect might, therefore, be implicated as a major mechanism responsible for the oral hypoglycemic effect of these compounds (11).

**EXPERIMENTAL PROCEDURES**

**Materials**

Biochemicals and fat-free albumin were purchased from Sigma Chemical Co. Collagenase (150 to 200 units/mg) was obtained from Worthington Biochemical Corp. and all other enzymes and acylcarnitine from Boehringer Mannheim Biochemicals. All radioactive compounds and the scintillation mixture Aquasol were purchased from New England Nuclear. The Aldrich Chemical Co. supplied 4,4'-di-thiodipyrindine; Rohm and Haas Co., Triton X-100, and the Chemical Procurement Co., a-bromomalonic acid.

**The abbreviations used are:**

- McN-3802, 2-tetradecylglycidic acid
- McN-3716, methyl ester of McN-3802

†To whom reprint requests should be addressed.

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Methods

Hepatocyte Isolation and Incubation—Unless otherwise indicated, rats received only water for 48 h prior to the day of experiment. Preparation of hepatocytes was based on the method of Berry and Friend (12). Livers were perfused with 150 to 200 ml of oxygenated (13), calcium-free, Krebs-Ringer bicarbonate buffer at 37°C at a flow rate of 30 ml/min. Cells were harvested following 45 min of recirculating perfusion with Krebs-Ringer bicarbonate buffer containing 3% dialyzed fatty acid-free bovine serum albumin and collagenase (50 units/ml). Viability was determined by trypan blue exclusion which routinely exceeded 95%. Dry weight was measured after precipitation with trichloroacetic acid (14). For comparative purposes, dry weights were converted to fresh wet weights using a conversion factor of 3.77 (14).

Incubations of 2 to 3 mg wet weight/ml of all cell suspensions were carried out in Beckman Poly Q vials in Krebs-Ringer bicarbonate buffer containing 1% dialyzed fatty acid-free bovine serum albumin. Vials were gassed with 95% O2, 5% CO2, sealed, and incubated with shaking at 37°C for 60 min, unless indicated otherwise. Using these conditions, 1 to 2 mM substrate was sufficient to maintain constant rate of 32 ml/min. Cells were harvested following 45 min of recirculation with Krebs-Ringer bicarbonate buffer containing 1% dialyzed fatty acid-free bovine serum albumin and 0.6 N HClO4, and assays of metabolites were performed following neutralization of the supernatant with KOH. Unless otherwise indicated, the sodium salt of McN-3716 was added to a stirring solution of 18% dialyzed fatty acid-poor bovine serum albumin at 55°C and diluted further to 1% bovine serum albumin prior to addition to incubation flasks. Since, in preliminary studies, higher fatty acid/bovine serum albumin ratios were needed for the greatest inhibitory effect of McN-3716, 0.7 mM oleate was used in most hepatocyte experiments.

Preparation of Long Chain Fatty Acid:CoA Ligase (Trivial Name Acyl-CoA Synthetase; EC 6.2.2.3)—Rat liver microsomes were prepared and lyophilized by the method of Bar-Tana (15). Enzyme activity was studied immediately after dissolution of the crude enzyme to 0.5 mg dry weight/ml in 0.2 ml Tris/HCl buffer, pH 7.2, containing 4 mM EDTA, 50 mM MgCl2, and 0.5 mg/ml of Triton X-100. The specific activity at this stage of purity was 16 to 20 nmol of palmitoyl-CoA formed/min/mg. Activity was determined by the disappearance of a CoA sulfhydryl group using 4,4'-dithiobispyridine as described by Grasseti and Murray (16), or by the formation of [14C]palmitoyl-CoA (15).

Substrate Oxidation Studies—The production of 14CO2 from radioactive substrates incubated with rat hepatocytes me measured as described previously (17).

Analytical Methods—Protein (18), glucose (19), acetoacetate (20), ß-hydroxybutyrate (21), lactate (22), and pyruvate (23) were determined by the methods described previously (17). P-hydroxybutyrate (21), lactate (22), and pyruvate (23) were determined by the methods described previously (17).

RESULTS

Effect of McN-3802 on Oxidation of Palmitic Acid, Octanoic Acid, and Palmitoyl-L-Carnitine to CO2 by Isolated Hepatocytes—In preliminary studies, rat hepatocytes oxidized 14C-fatty acids to 14CO2 at a constant rate through 2 h. As presented in Table I, McN-3802 was found to be an extremely potent inhibitor of the oxidation of palmitic acid to CO2 but not the oxidation of palmitoyl-l-carnitine or octanoic acid. The inhibition was concentration-dependent. At 10−4 to 10−2 M, palmitate oxidation was not inhibited by the known fatty acid oxidization inhibitors, a-bromomalonic acid and 4-pentenoic acid (not shown). At 10−5 M, the methyl ester (McN-3716) of McN-3802 inhibited palmitate oxidation to the same extent as McN-3802. However, only McN-3802 was used for most of the following in vitro studies since it is probably the circulating species in vivo following administration of McN-3716.

Effect of McN-3802 on Ketogenesis using Rat Hepatocytes—As expected, the inhibitor of fatty acid oxidation results in a decrease of acetocetate and ß-hydroxybutyrate production. Fig. 1, shows that the production of ketones by hepatocytes incubated with exogenously added oleate was completely inhibited by McN-3802. Significant inhibition by McN-3802 was recorded as early as 4 min after the addition of oleate and McN-3802. This inhibitory effect of McN-3802 on ketogenesis could not be duplicated by the nonhypoglycemic analogs, 1-tetradecenylcyclopropane carboxylic acid and methyl-trans-3-tridecylxirane carboxylate.

As shown in Fig. 2, McN-3802 inhibited ketone body formation significantly from added oleate but not octanoate. Along with the inhibition of ketone output, McN-3802 prevented the increase of the ß-hydroxybutyrate/acetocetate ratio which occurred upon addition of oleate. As this oxidation-reduction couple is considered to reflect the mitochondrial oxidation-reduction equilibrium, this represents an evident shift towards a more oxidized state of the mitochondrial compartment (28, 29) as a result of the inhibitory effect of McN-3802 upon fatty acid oxidation. These effects produced by McN-3802 on ketogenesis and the oxidation-reduction state were not reversed (not shown) by the addition of carnitine (1 to 8 mM) or carnitine (1 mM) + CoA (2 mM). With octanoate as substrate (Fig. 2), the ß-hydroxybutyrate/acetocetate ratio and total ketone body production did fall slightly upon addition of McN-3802. These changes, however, reflect the inhibition by McN-3802 of ketone body output from endogenous hepatocyte lipids. It should also be noted (Fig. 2) that

<table>
<thead>
<tr>
<th>Substrate</th>
<th>McN-3802 added</th>
<th>14CO2 formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-14C]Palmitate</td>
<td>0</td>
<td>105 ± 4 (8)</td>
</tr>
<tr>
<td>1 × 10−6</td>
<td>22 ± 2* (8)</td>
<td></td>
</tr>
<tr>
<td>1 × 10−6</td>
<td>56 ± 2* (8)</td>
<td></td>
</tr>
<tr>
<td>5 × 10−7</td>
<td>78 ± 4* (8)</td>
<td></td>
</tr>
<tr>
<td>1 × 10−7</td>
<td>102 ± 2 (8)</td>
<td></td>
</tr>
<tr>
<td>[1-14C]Octanoate</td>
<td>0</td>
<td>383 ± 20 (4)</td>
</tr>
<tr>
<td>1 × 10−5</td>
<td>443 ± 28 (4)</td>
<td></td>
</tr>
<tr>
<td>1 × 10−4</td>
<td>361 ± 40 (4)</td>
<td></td>
</tr>
<tr>
<td>5 × 10−1</td>
<td>359 ± 22 (4)</td>
<td></td>
</tr>
<tr>
<td>[1-14C]Palmitoyl carnitine</td>
<td>0</td>
<td>101 ± 6 (4)</td>
</tr>
<tr>
<td>1 × 10−5</td>
<td>127 ± 5 (4)</td>
<td></td>
</tr>
<tr>
<td>1 × 10−6</td>
<td>109 ± 10 (4)</td>
<td></td>
</tr>
</tbody>
</table>

*Significance of McN-3802 effects compared to control cells to which 10 μl of Me2SO was added was determined by Student's t test (p < 0.001; all other values were p > 0.05)
glucose formation from endogenous precursors was not inhibited by McN-3802.

Oxidation suggests that McN-3802 could be inhibiting the carnitine-dependent oxidation of long chain fatty acids. There-

fore, in an attempt to elucidate the site of action of McN-3802, the levels of fatty acylcarnitine, carnitine, CoA, and acetyl-CoA in hepatocytes were measured.

Carnitine was extracted by the method of Pearson and Tubbs (30) yielding perchlorate-soluble and -insoluble fractions of carnitine and acylcarnitines. The total acid-soluble fraction contains free carnitine and short chain acylcarnitine, whereas the acid-insoluble fraction contains long chain acylcarnitines. As shown in Table II, McN-3802 produced a highly significant drop in long chain acylcarnitines levels of hepatocytes which could not be explained by depletion of free carnitine.

In other experiments (not shown), the effects of McN-3802 (0.01 mM) on the contents of CoA and acetyl-CoA in liver cells were measured following incubation for 15 min. In a typical experiment, McN-3802 increased free CoA from 111 ± 16 nmol/g to 242 ± 20 nmol/g and caused a marked depletion of acetyl-CoA from 108 ± 6 nmol/g to 41 ± 5 nmol/g.

Activation of McN-3802 and McN-3716 to CoA Esters—As shown in Fig. 3, both McN-3802 and McN-3716 were converted to acyl-CoA thioesters by microsomal acyl-CoA synthetase. The enzyme, however, reached saturation at much lower concentrations of these compounds than was the case when palmitic acid or methyl palmitic acid were used as substrates. The nonhypoglycemic analogs, 1-tetradecylcyclopropane carboxylic acid and methyl-trans-3-tridecyloxirane carboxylate were also found (not shown) to be used, as well as McN-3716 and McN-3802, as substrates for this enzyme. Therefore, differences in CoA ester formation probably cannot explain why only McN-3716 and McN-3802, but not other closely related analogs, produce hypoglycemia (11). An analog (2-tetradecyloxiranemethanol) with an epoxide function identical with McN-3716 but containing a terminal alcohol rather than a carboxyl group did not increase the disappearance of a CoA sulfhydryl group (Fig. 3). This suggests that the reaction of CoA with McN-3716 and McN-3802 results in the formation of acyl-CoA derivatives rather than an alkylolation

In collaboration with Dr. John Williamson, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia. More extensive studies on the effects of McN-3802 on the contents of CoASH and CoA derivatives in liver cells from fasted rats have been completed. McN-3802 caused a marked depletion of acetyl-CoA and acid-soluble acyl-CoA derivatives and an accumulation of long chain fatty acyl-CoA compounds from 345 nmol/g dry weight to 651 nmol/g dry weight. These results will be presented later in a paper dealing with the metabolism of branched chain α-keto acids.
product from interaction of a CoA sulfhydryl group with the epoxide ring. The formation of the thioester was confirmed using the hydroxamate assay.

Effect of McN-3716 and McN-3802 on Microsomal Long Chain Acyl-CoA Synthetase—Using palmitic acid (200 μmol) as substrate, the addition of either McN-3802 or McN-3716 inhibited (not shown) microsomal acyl-CoA synthetase activity measured either by following the disappearance of a CoA sulfhydryl group or by the formation of [%]palmitoyl-CoA measured either by following the disappearance of a CoA sulfhydryl group (see "Methods") after 20-min incubation at 37°C. Substrates (C—C, palmitic acid; Δ—Δ, methylpalmitate; •—•, 2-tetradecyloxiridic acid; O—O, methyl 2-tetradecyloxiranemethanol; and •—•, 2-tetradecyloxiranemethanol) were added in 6 μl of absolute ethanol. Rates for all substrates were not significantly inhibited by increasing substrate concentration to 5 × 10⁻⁴ M. 

**TABLE III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration McN-3802 (mM)</th>
<th>Glucose[n]</th>
<th>Total ketones</th>
<th>β-OHB/A (nmol/g/min)</th>
<th>Lactate (nmol/g/min)</th>
<th>Pyruvate (nmol/g/min)</th>
<th>Lactate/pyruvate</th>
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<tr>
<td>Pyruvate</td>
<td>0</td>
<td>665 ± 37</td>
<td>65</td>
<td>1.39</td>
<td>3665 ± 228</td>
<td>99 ± 6</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>236 ± 93</td>
<td>65</td>
<td>0.85</td>
<td>953 ± 30</td>
<td>63 ± 4</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>366 ± 67</td>
<td>45</td>
<td>0.49</td>
<td>1240 ± 130</td>
<td>484 ± 4**</td>
<td>2.6</td>
</tr>
<tr>
<td>Lactate</td>
<td>0</td>
<td>675 ± 65</td>
<td>70</td>
<td>1.39</td>
<td>3665 ± 228</td>
<td>99 ± 6</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>207 ± 22</td>
<td>70</td>
<td>0.57</td>
<td>7346 ± 90</td>
<td>236 ± 34**</td>
<td>16</td>
</tr>
<tr>
<td>Proline</td>
<td>0</td>
<td>154 ± 8</td>
<td>7</td>
<td>1.85</td>
<td>84 ± 5</td>
<td>6 ± 1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>146 ± 2</td>
<td>7</td>
<td>0.44</td>
<td>31 ± 9**</td>
<td>20 ± 3**</td>
<td>1.6</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>0</td>
<td>318 ± 4**</td>
<td>53</td>
<td>0.41</td>
<td>1002 ± 7**</td>
<td>194 ± 1**</td>
<td>5.2</td>
</tr>
<tr>
<td>Fructose</td>
<td>0</td>
<td>1530 ± 40</td>
<td>47</td>
<td>1.3</td>
<td>1311 ± 52</td>
<td>150 ± 4</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>810 ± 30</td>
<td>47</td>
<td>0.3</td>
<td>2244 ± 35**</td>
<td>538 ± 14**</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Glucose formation in absence of substrates in all experiments was 30 to 100 nmol/g/min in the presence or absence of McN-3802.

** % I = per cent inhibition.

* Significance of McN-3802 effects determined by Student's t test: **p < 0.01; ***p < 0.001; all other values, p > 0.05.

**TABLE IV**

Effect of octanoate addition on the inhibitory effect of McN-3802 on glucose formation from pyruvate using isolated hepatocytes from 48-h fasted rats

All results are the mean ± S.E. of quadruplicate flasks. Conditions were the same as described in Table III. Initial concentrations of pyruvate, octanoate, and McN-3802 were 2.0, 2.0, and 0.01 mM, respectively.
Mechanism of Hypoglycemic Action: 2-Tetradecylglycidic Acid

Discussion

The oral hypoglycemic agent 2-tetradecylglycidic acid (McN-3802) was demonstrated to be an exceedingly potent and specific inhibitor of long chain fatty acid oxidation and gluconeogenesis. For these in vitro effects to be invoked as the cause of the hypoglycemia, they must be specific only for hypoglycemic compounds or their unique metabolites and they must be obtained at possible in vivo concentrations. This was found to be the case for the above in vitro effects of McN-3802 and its methyl ester, McN-3716. The closely related nonhypoglycemic analogs, 1-tetradecylcyclopropane carboxylic acid and methyl-trans-3-tridecyloxirane carboxylate failed to inhibit ketogenesis or gluconeogenesis at concentrations where these effects were seen with McN-3802. Furthermore, the concentrations of McN-3802 used for these in vitro studies (Tables I and III) could well be reached after the oral administration of hypoglycemic doses (11) of these compounds (minimum effective dose, 4 mg/kg, p.o.). Furthermore, the

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>McN-3802</th>
<th>Glucose found</th>
<th>Lactate/pyruvate</th>
<th>β-Hydroxybutyrate found</th>
<th>Acetoacetate found</th>
<th>β-OHB/AcA</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>862 ± 9</td>
<td>5.6</td>
<td>78 ± 3</td>
<td>149 ± 18</td>
<td>0.52</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.0</td>
<td>803 ± 30</td>
<td>6.3</td>
<td>55 ± 5**</td>
<td>88 ± 31</td>
<td>0.63</td>
</tr>
<tr>
<td>Pyruvate + oleate</td>
<td>0.0</td>
<td>1320 ± 90**</td>
<td>3.3</td>
<td>79 ± 4</td>
<td>172 ± 12</td>
<td>0.44</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.0</td>
<td>976 ± 5*</td>
<td>3.2</td>
<td>78 ± 2**</td>
<td>180 ± 10**</td>
<td>0.43</td>
</tr>
<tr>
<td>Alanine + oleate</td>
<td>0.0</td>
<td>1030 ± 30</td>
<td>4.0</td>
<td>85 ± 3</td>
<td>217 ± 8**</td>
<td>0.39</td>
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<tr>
<td>Fructose</td>
<td>0.0</td>
<td>843 ± 9**</td>
<td>2.6</td>
<td>76 ± 5**</td>
<td>174 ± 22**</td>
<td>0.44</td>
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<tr>
<td>Fructose + oleate</td>
<td>0.0</td>
<td>1400 ± 20**</td>
<td>6.5</td>
<td>86 ± 4</td>
<td>186 ± 2</td>
<td>0.46</td>
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<tr>
<td>Fructose + oleate</td>
<td>0.0</td>
<td>1600 ± 30**</td>
<td>12.6</td>
<td>64 ± 4**</td>
<td>104 ± 5**</td>
<td>0.62</td>
</tr>
<tr>
<td>Fructose + oleate</td>
<td>0.0</td>
<td>1100 ± 30**</td>
<td>0.1</td>
<td>77 ± 3**</td>
<td>153 ± 20**</td>
<td>0.50</td>
</tr>
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</table>

* Significant compared to appropriate controls determined by Student’s t test: * p < 0.05; ** p < 0.001; all other values were p > 0.05.

β-OHB/AcA was corrected (not shown) by addition of the extramitochondrial electron donor, ethanol (2 mM) and glyceraldehyde (2 mM) was reversed, but not the inhibition from pyruvate, lactate, or alanine. This effect of ethanol could not be duplicated by the mitochondrial electron donor, β-hydroxybutyrate (2.0 mM). Ethanol addition did not reverse, however, the inhibited production of ketones and the lowered β-hydroxybutyrate/acetoacetate ratio produced by McN-3802.

Balance studies from four experiments using lactate or pyruvate as substrate revealed that greater than 90% of the substrate used by the hepatocytes could be accounted for as glucose and lactate- or pyruvate-formed. The higher the concentration of McN-3802 (greater inhibition of ketogenesis), the greater was the amount of substrate that was unaccounted for as metabolites. This greater proportion of substrate unaccounted for presumptively results from enhanced oxidation.

Also shown at the top of Table III, McN-3802 inhibited glucose formation from pyruvate in a concentration-dependent manner at concentrations ranging from 10^-5 to 10^-7 M. This effect paralleled the concentration-dependent inhibition of ketogenesis which can be taken as an index of the rate of fatty acid oxidation. Thus, the inhibition of gluconeogenesis may be a secondary result of the inhibition of fatty acid oxidation. This conclusion was supported by the results shown in Table IV where the inhibition of glucose formation from pyruvate was almost completely reversed upon the addition of oleate whose oxidation is not inhibited by McN-3802. Furthermore, it was found (not shown) that the inhibitory effect of McN-3802 on gluconeogenesis could not be duplicated by the nonhypoglycemic analogs, 1-tetradecylcyclopropane carboxylic acid and methyl-trans-3-tridecyloxirane carboxylate which fail to inhibit fatty acid oxidation. The inhibition of gluconeogenesis was probably not the result of depletion of cofactors (CoA and carnitine) which are necessary for fatty acid oxidation, since the inhibition produced by McN-3802 of glucose formation from pyruvate was not reversed by the addition of carnitine (1 to 8 mM) and CoA (2 mM).

Effect of McN-3802 on Formation of Glucose and Ketones by Isolated Hepatocytes from Fed Rats—Previously, we have reported (11) that McN-3802 lowers the blood glucose of rats and dogs in the fasted but not the fed state. Presumably, this lack of effect in the fed state reflects the fact that energy needs in this nutritional state are being derived mainly from carbohydrate rather than fat. For this reason, it was of interest to determine whether McN-3802 would have less of an effect on gluconeogenesis using hepatocytes from fed rats. An example of our findings is shown in Table V. In the absence of added oleate, McN-3802 produced little effect on the concentrations of glucose and lactate/pyruvate (not shown) in the cell suspensions when these metabolites were generated endogenously from glycogen or from added fructose, alanine, or pyruvate. Also, the lactate/pyruvate and β-hydroxybutyrate/acetoacetate oxidation-reduction couples were not lowered by McN-3802 addition. However, when oleate was added and ketogenesis increased, a greater inhibitory effect on glucose and ketone body production and lowered lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios were seen upon addition of McN-3802.

**TABLE V**

Effect of McN-3802 on formation of glucose, β-hydroxybutyrate (β-OHB) and acetoacetate (AcA) in isolated rat hepatocytes from fed rats incubated with various substrates

Results are expressed as nanomoles/g wet weight/min, mean ± S.E. of at least three experiments. The concentrations used for pyruvate, alanine, and fructose were 2 mM and of oleate, 0.7 mM.
assumption that hypoglycemia might be the result of the inhibition of gluconeogenesis was supported by the finding (not shown) that the administration of the methyl ester of McN-3802, McN-3716 (10 to 20 mg/kg, p.o.), to fasted rats in vivo inhibited (approximately 50%) conversion of [3-14C]pyruvate to blood [14C]glucose. McN-3802 and McN-3716 inhibited the oxidation to CO₂ and ketones of the long chain fatty acids, palmitate and oleate but not the short chain fatty acids, octanoate and butyrate (not shown). The same findings have also been found using rat hemidiaphragm (32) and perfused heart (33). Both short and long chain fatty acids are oxidized intramitochondrially by enzymes of the β oxidation sequence, but their transport into the mitochondria occurs by different mechanisms. The above findings, as well as the inability to inhibit the oxidation of palmitoylcarnitine and the lowered production (Table II) of hepatocyte long chain acylcarnitines, suggest that McN-3802 is inhibiting at an enzyme in the fatty acid pathway prior to β oxidation which shows a specificity with respect to the fatty substrate. However, it seems unlikely that long chain acyl-CoA synthetase is the site since microsomal acyl-CoA synthetase activity was inhibited at concentrations of McN-3716 and McN-3802 which were nearly 100 times higher than those required to inhibit hepatocyte palmitate oxidation, ketogenesis, or gluconeogenesis. Furthermore, that long chain acyl-CoA derivatives accumulate following addition of McN-3802 to hepatocytes⁶ and that increased quantities of triglyceride are found in the livers of McN-3716-treated fast-fed rats⁶ attests to the integrity of this enzyme in vivo and in vitro.

Thus, by circumstantial evidence, the carnitine acyltransferase I is implicated as the site of inhibition. We have found, using isolated mitochondria, that this enzyme can be inhibited with McN-3802 in the presence of Mg²⁺ and ATP.⁷ However, studies with this enzyme are not complete since there exists a possibility that the actual inhibitor is a metabolite of 2-tetradecylglycidic acid, i.e. carnitine or CoA ester. As shown in Fig. 3, both McN-3802 and McN-3716 can indeed be converted to acyl-CoA thioesters by microsomal acyl-CoA synthetase. The metabolism of these compounds is under current study in our laboratory and will be reported later.

It seems reasonable that the inhibitory effect of McN-3802 on fatty acid oxidation is the sole result of inhibition of the formation of long chain acylcarnitines, since this step has been suggested to be rate-limiting in the overall process (34, 35). The intramitochondrial carnitine acyltransferase II which converts fatty acylcarnitine into CoA esters does not appear to be inhibited since McN-3802 failed to inhibit palmitoylcarnitine oxidation. Hypoglycin and 4-pentenoic acid have also been reported to inhibit long chain fatty acid oxidation at a site of the carnitine-dependent transport of long chain fatty acid into mitochondria (36, 37). This effect has been attributed to their ability to cause depletion of tissue levels of coenzyme A and carnitine in vivo and in vitro (36–40) and their in vivo effects have been reported to be reversed by carnitine administration (36, 37). The present results do not support that McN-3802 inhibits fatty acid oxidation through a similar depletion of CoA and carnitine. The inhibition of ketogenesis and gluconeogenesis produced by McN-3802 could not be reversed by the addition of CoA or carnitine at concentrations 100-fold higher than the concentration of McN-3802. Furthermore, the results in Table II show that McN-3802 produced a highly significant drop in long chain acylcarnitine levels and ketogenesis which could not have resulted from depletion of carnitine. Free coenzyme A levels of cells treated with McN-3802 also were found not to be lowered.⁸

In recent years, the intimate relationship between fatty acid oxidation and gluconeogenesis has been well established. This relationship has been adequately reviewed by others (10, 41, 42) and will not be discussed in detail here. In rat liver, fatty acid oxidation appears to be essential for maximal rates of gluconeogenesis. In fact, the importance of fatty acid oxidation has been underlined by the reports that α-bromopalmitate, 4-pentenoic acid, and (+)-acylcarnitine derivatives, inhibitors of fatty acid oxidation, decrease gluconeogenesis (3, 6, 10). Therefore, as expected, McN-3802 was found to inhibit glucose formation from lactate, pyruvate, etc. in isolated hepatocytes from fasted rats. Glucose formation from endogenous substrates using hepatocytes from fed or fasted rats was not inhibited. That this inhibitory effect of McN-3802 on gluconeogenesis was reversible and secondary to its inhibition of long chain fatty acid oxidation was supported by the finding (Table IV) that the addition of medium chain fatty acid, octanoic acid (1 mm), almost completely reversed this effect. Furthermore, under conditions where McN-3802 inhibited ketogenesis only slightly (Table V) using hepatocytes isolated from livers of fed rats, it also exhibited little effect on glucose production from added fructose, alanine, or pyruvate. But, when oleate was added to hepatocytes from fed rats and rates of ketogenesis increased, McN-3802 produced a greater inhibitory effect on both ketogenesis and glucose formation. Presumably, some of the energy or cofactors used to support gluconeogenesis in these experiments was being generated from the oxidation of the added oleic acid and McN-3802 reversed this effect.

The mechanism(s) responsible for the inhibition of gluconeogenesis produced by McN-3802 requires much additional study; however, there exist several obvious possibilities that should be commented upon. First, McN-3802 could be inhibiting gluconeogenesis solely by limiting ATP production via fatty acid oxidation. However, even though McN-3802 lowered¹ the ATP content of hepatocytes in the absence of gluconeogenic substrates, no decrease was observed in the presence of lactate or pyruvate. Obviously, these data do not preclude that McN-3802 may be limiting a small pool of ATP which is used specifically to support gluconeogenesis.

Pyruvate carboxylase also seems like an obvious site for inhibition, since the activity of this enzyme is enhanced under conditions of high fatty acid oxidation presumably as a result of increased intramitochondrial levels of acetyl-CoA. Thus, the observed lowering of acetyl-CoA when McN-3802 was added to hepatocytes from fasted rats¹ might indirectly inhibit gluconeogenesis by lowering pyruvate carboxylase activity. Also, it does not appear that McN-3802 is directly inhibiting other enzymatic steps in the pathway above pyruvate carboxylase since McN-3802 failed to inhibit glucose production from proline (Table III) which enters the gluconeogenesis pathway at oxalacetate via the Krebs cycle.

The finding mentioned earlier that McN-3802 produced a decrease in the quantity of pyruvate remaining following incubation with hepatocytes that could not be accounted for as glucose or lactate production is consistent with an increase in pyruvate utilization via pyruvate dehydrogenase. Pyruvate dehydrogenase is known to be inhibited under conditions of high fatty acid oxidation (43, 44) and therefore, it seemed likely that the activity of this enzyme would be enhanced when fatty acid oxidation was inhibited with McN-3802. In preliminary experiments⁶ this indeed was found to be the

³ G. F. Tutwiler and P. Dellevigne, unpublished results.
⁴ G. F. Tutwiler, unpublished data.
⁵ Lowered acetyl-CoA levels have also been measured under conditions where gluconeogenesis from lactate was inhibited with McN-3802 (See Footnote 2).
⁶ Dr. John Williamson, personal communication.
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10. The presence of 2 μM 2-ketoisocaproate decreased the percentage of pyruvate dehydrogenase in the active (nonphosphorylated) form from 21.7 to 9.6%. This decrease was not observed when 6 × 10^{-6} M McN-3802 was also added to the incubation medium.

Finally, McN-3802 inhibited glucose formation and increased lactate and pyruvate formation from fructose, dihydroxyacetone, and glyceraldehyde-3-phosphate dehydrogenase. McN-3802, by inhibiting fatty acid oxidation, limits NADH generation and, as a consequence, gluconeogenesis would be expected to be limited only insofar as triose-phosphate is diverted from glucose formation to lactate and pyruvate formation. The fact that ethanol reversed McN-3802 inhibition with McN-3802 and the postulated restraint at triose-phosphate is expected to be limited only insofar as triose-phosphate is diverted from glucose formation to lactate and pyruvate formation.

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Action of the oral hypoglycemic agent 2-tetradecylglycidic acid on hepatic fatty acid oxidation and gluconeogenesis.

G F Tutwiler and P Dellevigne


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