Inherited Disorders of Mitochondrial β-Oxidation

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Inherited abnormalities of mitochondrial oxidation of long chain fatty acids cause a number of clinical syndromes, including sudden infant death, Reye’s syndrome, episodic hypoglycemia, and lipid storage myopathy. Since suberylglycinuria was first described in 1976, there has been a growing recognition that these inherited defects of the mitochondrial oxidation of fatty acids constitute an important group of disorders, some of which are treatable by simple dietary manipulation, such as the avoidance of starvation (1,2). Thus deficiencies of long chain acyl-CoA dehydrogenase (3,4), medium chain acyl-CoA dehydrogenase (5), short chain acyl-CoA dehydrogenase (6,7), ETF or ETF:QO [glutaric aciduria type II, multiple acyl-CoA dehydrogenase deficiency] (8), long chain 3-hydroxyacyl-CoA dehydrogenase (9), and acetoacetyl-CoA thiolase (10) have been described. Also of relevance to the present discussion, disorders of carnitine palmitoyltransferase (11) and of the Lynen cycle of ketogenesis (12,13) have been documented.

Mitochondrial β-oxidation of saturated acyl-CoA esters proceeds by a repeated cycle of four reactions: flavoprotein-linked dehydrogenation, hydration, NAD⁺-linked dehydrogenation, and thiolysis. The three chain-length-specific acyl-CoA dehydrogenases (14) which catalyze the first dehydrogenation step are linked to the respiratory chain by the electron transfer flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO) (Fig. 1). The second dehydrogenation step is catalyzed by two chain-length-specific NAD⁺-dependent 3-hydroxyacyl-CoA dehydrogenases (15). There are two acyl-CoA hydratases (16) and two thiolases (17,18), which vary in their chain length specificity. These nine enzymes are required for the complete oxidation of long chain saturated acyl-CoA's. There are also auxiliary systems for the transport of long chain fatty acids across the inner mitochondrial membrane and for the oxidation of polyunsaturated fatty acids. Carnitine palmitoyltransferase I is located on the inner face of the outer mitochondrial membrane and catalyzes the
formation of long chain acylcarnitine from the corresponding long chain acyl-CoA, which can traverse the inner mitochondrial membrane in exchange for free carnitine via a specific translocase. The matrix acylcarnitine then becomes the substrate for carnitine palmitoyltransferase II to regenerate acyl-CoA. Two enzymes are required for the oxidation of polyunsaturated fatty acids: 2,4-dienoyl-CoA reductase and an isomerase (19,20).

The control of β-oxidation in the mitochondrial matrix occurs at several steps and depends on the redox state and the rate of recycling of CoA (Fig. 1; 21–24). The rate is lowered with reduced states since low NAD+/NADH ratios impair the activity of the hydroxyacyl-CoA dehydrogenase (25,26) and increase the formation of ETFed, which is a potent inhibitor of the acyl-CoA dehydrogenases (27). These changes affect the steady state concentrations of acyl-CoA intermediates, which in turn may change the control strength of other enzymes of the pathway as outlined in Fig. 1.

In liver mitochondria acetyl-CoA produced by each cycle of β-oxidation has four major routes of disposal: ketogenesis, oxidation by the citrate cycle, conversion to acetylcarbonyl, or hydrolysis to acetate, and each of these reactions generates free CoA. During maximum flux through β-oxidation, up to 95% of the mitochondrial CoA pool is acylated (28) and thus the rate of recycling of CoA may partly control β-oxidation (29). Increased steady state concentrations of some acyl-CoA esters may also occur when one or more of the enzymes of β-oxidation is inhibited, as in hypoglycemia, hypoglycemia poisoning (30) or where one or more of the enzymes of the pathway are absent.

In normal subjects under fasting conditions, lipolysis is stimulated and fatty acids are released into the circulation. Following their uptake by tissues, fatty acids are activated to acetyl-CoA esters, which may be utilized for the formation of triacylglycerides or transported into the mitochondrial matrix for oxidation. The acetyl-CoA generated by β-oxidation may then be completely oxidized via the tricarboxylic acid cycle or utilized for ketogenesis. Circulating ketone bodies may then be oxidized by extrahepatic tissues, notably brain (31,32) and muscle (33,34). This system constitutes the Randle cycle and is the mechanism by which glucose utilization is spared.

**FIG. 1.** β-Oxidation of hexadecanoate and its relation to the redox state of the mitochondrion. Each cycle of the mitochondrial β-oxidation of hexadecanoyl-CoA and all 27 intermediates is shown. The flow of electrons between 3-hydroxyacyl-CoA/3-oxoacyl-CoA esters and the NAD+/NADH pool, between the NAD+/NADH pool and the respiratory chain at the level of NADH dehydrogenase, between acyl-CoA/2-enoyl-CoA esters and the ETF/ETFH2, between the ETF/ETFH2 pool and the respiratory chain at the level of ETF-Qo, and electron flow from NADH dehydrogenase and ETF-Qo to O2 along the respiratory chain is indicated by thin lines. Electron flow through NADH dehydrogenase and complexes III and IV of the respiratory chain to O2 is associated with proton ejection from the matrix, and the proton gradient generated is used to drive the synthesis of ATP by complex V. The flux through the respiratory chain is controlled by the ATP/ADP ratio and is maximal in uncoupled mitochondria. Acetyl-CoA is converted to acetoacetate with recycling of CoA.

It can be seen that the flux through β-oxidation depends on the supply of hexadecanoyl-CoA (via CPT I/carnitine-acyl carnitine exchange carrier/CPT II not shown), the [acyl-CoA]/[CoA] ratio, and the redox states of the NAD+/NADH and ETF/ETFH2 pools, which in turn depend on the rate of electron transport through the respiratory chain and are more oxidized when electron transport is fast.
for those tissues, such as brain, which have an obligatory requirement for glucose as a metabolic fuel (35). If fatty acid oxidation is impaired by one of the enzyme defects mentioned above, there are a number of metabolic consequences. The first effect is an impaired ability to withstand fasting, with rapid onset of hypoglycemia as glycogen reserves become depleted. Second, as lipolysis continues and the blood concentration of free fatty acids rises, the fatty acids entering the liver are either partially oxidized to a variety of abnormal metabolites or stored as triglyceride. Third, the impairment of mitochondrial β-oxidation appears to trigger the proliferation of hepatic peroxisomes (36), with resultant stimulation of peroxisomal β-oxidation and further production of chain-shortened intermediates. The abnormal metabolites generated by partial mitochondrial β-oxidation, peroxisomal β-oxidation, and a number of detoxification mechanisms are excreted and can be detected in urine (dicarboxylic acids, hydroxy acids, carnitine esters, and glycine conjugates) (37–40).

Thus β-oxidation defects cause major changes in whole body fuel economy, but changes that may only be apparent in response to metabolic stress such as fasting. We suggest that much of the argument regarding the presence or absence of putative pathognomonic urinary metabolites (see, e.g., ref. 41), the usual mainstay of inborn error diagnosis, arises because the metabolic conditions under which specimens are collected are not defined with sufficient rigor. It is more logical to identify patients with impaired ketogenesis by monitoring the major metabolic fuels during a fast, and only then progressing to secondary investigations such as urine analysis and direct enzyme assay.

In many patients with inherited disorders of β-oxidation, hypoglycemia is the preeminent presenting feature. Hypoglycemia is one of the most common metabolic abnormalities in pediatric medicine. There are numerous causes and several different clinical manifestations, and its occurrence is often intermittent and unpredictable. These aspects, together with the involvement of several biochemical pathways in the maintenance of normoglycemia, has made the condition particularly difficult to investigate and manage. There have been few attempts to define a practical, logical approach to diagnosis. It is further complicated by the continued use of archaic terms such as “idiopathic hypoglycemia,” “asymptomatic hypoglycemia,” and “ketotic hypoglycemia.”

Although the descriptive phrase “ketotic hypoglycemia” cannot be regarded as a final diagnosis, it is important to recognize whether children have hypoglycemia in the presence or absence of ketonemia or ketonuria. Ketotic hypoglycemia occurs when the falling glucose concentration results in mobilization of fatty acids from adipose tissue and generation of ketone bodies by fatty acid oxidation in the liver. Hypoglycemia without ketonuria or ketonemia has been regarded as pathognomonic of hyperinsulinism in view of the anti-lipolytic effect of high circulating insulin concentrations.

It is now recognized that there exist a number of children in whom hypoglycemia is associated with unexpectedly low blood concentrations of ketone bodies due to impaired oxidation of fatty acids in the liver. This may be due to a primary defect
in the enzymes of fatty acid oxidation or a secondary effect on mitochondrial fatty acid oxidation due to a defect elsewhere in intermediary metabolism.

This chapter documents an analysis of metabolic relationships in patients presenting with hypoglycemia associated with varying degrees of ketonemia, and from this we outline a practical, logical, and comprehensive approach to elucidate the underlying biochemical abnormality.

PATIENTS

Twenty-eight patients aged from 1 day to 4 years of age were referred for investigation of symptomatic hypoglycemia. Of the 28 patients, 13 were found on investigation to be suffering from hyperinsulinemic hypoglycemia, one child had fructose-1,6-diphosphatase deficiency, one child had glucose-6-phosphatase deficiency, and 13 had evidence of defects in the β-oxidation of fatty acids. Eight of these 13 patients were subsequently confirmed to have medium chain acyl-CoA dehydrogenase (MCAD) deficiency. In addition, results are presented from patients whose urine specimens and fibroblast cell lines were referred for analysis (cases 29–36).

METHODS

Clinical

In all children referred for investigation of spontaneous hypoglycemia a plastic cannula was inserted into a peripheral vein to allow the withdrawal of free-flowing venous blood samples. A small number of patients were studied by the withdrawal of a single blood sample at the time of an emergency admission to the hospital due to symptoms of spontaneous hypoglycemia. In all other cases, a starvation challenge was performed. The children were starved under careful clinical supervision and with hourly monitoring of blood glucose concentrations. There was an arbitrary limit on the maximum period of starvation allowed for each age group of children. Children during the first month after birth were subjected to a maximum period of starvation of 6 h. Infants between 1 and 3 months of age were starved to a maximum of 8 h; children 3 months to 1 year were starved for a maximum of 12 h. A maximum of 18 h of starvation was allowed for children between ages of 1 and 3 years, with a maximum limit of 24 h for children beyond this age. In all patients hypoglycemia occurred prior to these limits of starvation.

At the onset of symptoms or when the blood glucose concentration fell below 2.5 mmol/liter, a venous blood sample was obtained. An aliquot (0.5 ml) was immediately placed into 5 ml of ice-cold 5% (v/v) perchloric acid, and the remainder was used to prepare a plasma sample. In some of the older children a series of blood samples was drawn at 3-h intervals during the starvation procedure. The urine sample passed immediately after the episode of spontaneous or starvation-induced hypoglycemia was collected and deep frozen immediately.
The results of the metabolic investigations were compared with the profiles of circulating levels of intermediary metabolites in 46 normal children undergoing routine surgical procedures. The samples in these children were obtained immediately prior to the operation and the period of starvation was noted. The investigations performed on the patients and control children were approved by the Newcastle Joint Ethics Committee, and parental consent was obtained.

**Measurement of Blood and Plasma Concentrations of Intermediary Metabolites, Insulin, and Carnitine**

Whole blood concentrations of glucose, lactate, pyruvate, acetoacetate, 3-hydroxybutyrate, glycerol, and alanine were measured fluorometrically (42). Plasma free fatty acid concentrations were also measured by a spectrophotometric enzyme-linked assay using the Cobas Bio Fast Analyzer. Plasma insulin concentrations were measured by a double antibody radioimmunoassay (43). Plasma-free carnitine, short chain acylcarnitine, and long chain acylcarnitine were measured radioenzymatically (44).

**Determination of Urinary Organic Acids and Acylcarnitine Concentrations**

Urinary organic acid analysis was by gas chromatography–mass spectrometry. Acyl carnitines were analyzed by either reversed-phase high-performance thin layer chromatography or high-performance liquid chromatography (46,46).

**Measurement of Fibroblast Acyl-CoA Dehydrogenase Activities**

Acyl-CoA dehydrogenase activities were measured in cultured skin fibroblasts by the ETF-linked fluorometric method (47).

**RESULTS**

**Interrelationships of Metabolic Fuels and Intermediary Metabolites**

The concentrations of blood glucose, lactate, pyruvate, acetoacetate, hydroxybutyrate, glycerol, alanine, and free fatty acids in controls and patients are shown in Table 1. None of the control children were hypoglycemic under the conditions of preoperative starvation.

Thirteen patients could clearly be identified who had hypoglycemia with low concentrations of acetoacetate and hydroxybutyrate together with low concentrations of free fatty acids. All of these children had inappropriate concentrations of plasma insulin for the level of glycemia. One patient (case 14) had hypoglycemia accompanied by hyperlacticacidemia, hyperalaninemia, and hyperglycerolemia. This pa-
### TABLE 1. Blood intermediary metabolites, plasma non-esterified fatty acids and insulin

<table>
<thead>
<tr>
<th>Patient</th>
<th>lact</th>
<th>pyr</th>
<th>ala</th>
<th>β-OHB</th>
<th>AcAc</th>
<th>glu</th>
<th>NEFA</th>
<th>glyc</th>
<th>Insulin</th>
</tr>
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<tbody>
<tr>
<td>Controls (fasted for 24 h; n = 19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>1.26</td>
<td>0.11</td>
<td>0.20</td>
<td>1.98</td>
<td>0.74</td>
<td>3.6</td>
<td>1.58</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.56</td>
<td>0.07</td>
<td>0.06</td>
<td>1.38</td>
<td>0.56</td>
<td>0.60</td>
<td>0.39</td>
<td>0.04</td>
<td>&lt;1.0</td>
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<td>Hyperinsulinemia: patients 1–13</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.07</td>
<td>0.09</td>
<td>0.24</td>
<td>0.29</td>
<td>0.14</td>
<td>2.5</td>
<td>0.58</td>
<td>0.13</td>
<td>9.8</td>
</tr>
<tr>
<td>SD</td>
<td>0.53</td>
<td>0.04</td>
<td>0.08</td>
<td>0.47</td>
<td>0.17</td>
<td>0.9</td>
<td>0.45</td>
<td>0.11</td>
<td>7.6</td>
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<td>Fructose-1,6-diphosphatase deficiency</td>
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<td></td>
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<tr>
<td>14</td>
<td>8.77</td>
<td>0.32</td>
<td>0.60</td>
<td>0.28</td>
<td>0.09</td>
<td>2.6</td>
<td>0.91</td>
<td>0.74</td>
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<td>Glucose-6-phosphatase deficiency</td>
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<td>15</td>
<td>5.61</td>
<td>0.30</td>
<td>0.50</td>
<td>0.04</td>
<td>0.01</td>
<td>2.3</td>
<td>1.30</td>
<td>0.18</td>
<td>1.5</td>
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<td>Medium chain acyl-CoA dehydrogenase deficiency</td>
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<tr>
<td>16</td>
<td>1.45</td>
<td>0.09</td>
<td>0.16</td>
<td>0.67</td>
<td>0.50</td>
<td>2.7</td>
<td>2.58</td>
<td>0.32</td>
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<tr>
<td>17</td>
<td>1.90</td>
<td>0.14</td>
<td>0.16</td>
<td>0.28</td>
<td>0.18</td>
<td>1.9</td>
<td>2.99</td>
<td>0.43</td>
<td>&lt;1.0</td>
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<tr>
<td>18</td>
<td>0.85</td>
<td>0.05</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
<td>2.6</td>
<td>1.93</td>
<td>0.09</td>
<td>&lt;1.0</td>
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<tr>
<td>19</td>
<td>1.25</td>
<td>nd</td>
<td>nd</td>
<td>0.40</td>
<td>nd</td>
<td>2.2</td>
<td>2.21</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>1.00</td>
<td>nd</td>
<td>nd</td>
<td>0.40</td>
<td>nd</td>
<td>3.9</td>
<td>1.86</td>
<td>nd</td>
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<tr>
<td>21</td>
<td>1.66</td>
<td>nd</td>
<td>nd</td>
<td>0.42</td>
<td>nd</td>
<td>1.9</td>
<td>2.46</td>
<td>nd</td>
<td>&lt;1.5</td>
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<tr>
<td>22</td>
<td>0.85</td>
<td>nd</td>
<td>nd</td>
<td>0.39</td>
<td>nd</td>
<td>2.3</td>
<td>1.90</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>23</td>
<td>0.96</td>
<td>nd</td>
<td>nd</td>
<td>0.62</td>
<td>nd</td>
<td>2.8</td>
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<td>Probable β-oxidation defects</td>
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<tr>
<td>24</td>
<td>1.05</td>
<td>0.11</td>
<td>0.10</td>
<td>0.54</td>
<td>0.44</td>
<td>15.0*</td>
<td>1.83</td>
<td>0.08</td>
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<tr>
<td>25</td>
<td>1.26</td>
<td>nd</td>
<td>nd</td>
<td>0.10</td>
<td>nd</td>
<td>1.8</td>
<td>3.09</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>26</td>
<td>1.43</td>
<td>nd</td>
<td>nd</td>
<td>0.10</td>
<td>nd</td>
<td>1.8</td>
<td>2.06</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>27</td>
<td>1.35</td>
<td>0.11</td>
<td>0.17</td>
<td>0.21</td>
<td>0.10</td>
<td>1.8</td>
<td>2.88</td>
<td>0.28</td>
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<tr>
<td>28</td>
<td>6.92</td>
<td>0.32</td>
<td>0.40</td>
<td>0.27</td>
<td>0.09</td>
<td>1.4</td>
<td>2.28</td>
<td>0.30</td>
<td>nd</td>
</tr>
</tbody>
</table>

Patient was shown subsequently to have a deficiency of fructose-1,6-diphosphatase. Case 15 had hypoglycemia with hyperlacticacidemia and low concentrations of ketone bodies. Measurement of glucose-6-phosphatase in her liver biopsy demonstrated that she had glycogen storage disease type 1B. The remaining children had hypoglycemia with normal concentrations of lactate, pyruvate, alanine, and glycerol, and elevated levels of free fatty acids but low concentrations of blood ketone bodies. This finding strongly suggested a defect in the oxidation of fatty acids in the liver.

While these findings provide some clues for further investigation of patients, considerably more information can be obtained by relating the concentration of blood glucose to that of plasma insulin and from the relationship between the concentrations of ketone bodies and fatty acids to blood glucose concentration. Another interrelationship of great clinical value is between plasma free fatty acid concentrations and the concentrations of total ketone bodies (acetooacetate plus 3-hydroxybutyrate). Figure 2 demonstrates a linear relationship with 95% confidence limits between these two variables as derived from the data from normal children. With increasing levels of free fatty acids there is a direct increase in the concentration of ketone bodies.
The relationship of children with hyperinsulinism falls within the 95% confidence limits. Thus, although these children had an inappropriately low concentration of ketone bodies for the level of glycemia, the relationship with free fatty acids was appropriate. The hypoketonemia was therefore due to a failure of free fatty acid release as a result of the antilipolytic effect of insulin on adipose cells.

The child with fructose-1,6-diphosphatase deficiency had an appropriate level of ketone bodies for the degree of free fatty acidemia. However, the child with glycogen storage disease type 1B clearly had an inappropriately low level of ketone bodies for the degree of free fatty acidemia, suggesting a secondary effect on fatty acid oxidation. The children with suspected and proven defects of β-oxidation had high

**FIG. 2.** Relationship of plasma NEFA concentrations to blood ketone body concentrations in controls (the line of best fit and the 95% confidence limits determined by Deming's method are as indicated), patients with medium chain acyl-CoA dehydrogenase deficiency (○), glycogen storage disease type 1B (★), fructose-1,6-diphosphatase deficiency (★), patients with probable β-oxidation disorders (□), and hyperinsulinemic patients (■). The case numbers are as shown in Table 1.
FIG. 3. Relationship of plasma NEFA concentrations to blood ketone body concentrations during the course of fasting in controls (solid lines, no symbols), patients with proven medium chain acyl-CoA dehydrogenase deficiency (●), and a patient with a probable β-oxidation disorder (□).

Concentrations of free fatty acids but inappropriately low concentrations of ketone bodies for that degree of lipolysis.

Figure 3 shows the sequential change in the relationship during the progression of the starvation provocation test between free fatty acids and ketone bodies in five children with medium chain acyl-CoA dehydrogenase deficiency. It is clear that while the relationship is normal at the onset of the starvation, with increasing starvation-induced stress the relationship rapidly becomes abnormal.

Concentrations of Carnitine and Acylcarnitines in Plasma

The results of the assays of plasma carnitine, short chain acylcarnitine, long chain acylcarnitine, and percentage acylated carnitine in patients, adult controls, and nor-
**DISORDERS OF MITOCHONDRIAL β-OXIDATION**

**TABLE 2. Fasting plasma concentrations of free carnitine, short chain acylcarnitine, long chain acylcarnitine, and percentage carnitine acylated**

<table>
<thead>
<tr>
<th></th>
<th>Carnitine</th>
<th>Short chain acylcarnitine</th>
<th>Long chain acylcarnitine</th>
<th>% Acylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (n = 30)</td>
<td>32.8</td>
<td>6.2</td>
<td>3.2</td>
<td>23</td>
</tr>
<tr>
<td>Mean</td>
<td>32.8</td>
<td>6.2</td>
<td>3.2</td>
<td>23</td>
</tr>
<tr>
<td>SD</td>
<td>6.3</td>
<td>2.3</td>
<td>0.7</td>
<td>5</td>
</tr>
<tr>
<td>Normal children aged &lt;5 years (n = 11)</td>
<td>26.2</td>
<td>17.2</td>
<td>3.1</td>
<td>44</td>
</tr>
<tr>
<td>Mean</td>
<td>26.2</td>
<td>17.2</td>
<td>3.1</td>
<td>44</td>
</tr>
<tr>
<td>SD</td>
<td>7.7</td>
<td>5.9</td>
<td>0.8</td>
<td>9</td>
</tr>
<tr>
<td>Hyperinsulinemic patients (n = 8)</td>
<td>24.2</td>
<td>7.5</td>
<td>2.9</td>
<td>32</td>
</tr>
<tr>
<td>Mean</td>
<td>24.2</td>
<td>7.5</td>
<td>2.9</td>
<td>32</td>
</tr>
<tr>
<td>SD</td>
<td>12.4</td>
<td>2.7</td>
<td>1.5</td>
<td>12</td>
</tr>
<tr>
<td>Medium chain acyl-CoA dehydrogenase-deficient patients (n = 7)</td>
<td>11.4</td>
<td>13.4</td>
<td>2.8</td>
<td>58</td>
</tr>
<tr>
<td>Mean</td>
<td>11.4</td>
<td>13.4</td>
<td>2.8</td>
<td>58</td>
</tr>
<tr>
<td>SD</td>
<td>5.9</td>
<td>7.4</td>
<td>1.1</td>
<td>12</td>
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<tr>
<td>Patients with probable β-oxidation disorders</td>
<td></td>
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<tr>
<td>Case</td>
<td>24</td>
<td>7.1</td>
<td>7.2</td>
<td>1.5</td>
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<td></td>
<td>28</td>
<td>34.9</td>
<td>21.7</td>
<td>7.1</td>
</tr>
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</table>

Mal children during ketosis are shown in Table 2. Patients with hyperinsulinism, fructose-1,6-diphosphatase deficiency, and glycogen storage disease type 1B did not demonstrate any abnormality in carnitine metabolism. However, patients with MCAD deficiency had an abnormally high short chain acylcarnitine concentration and a low free carnitine concentration. In two patients with probable defects of β-oxidation there was a total absence of short chain acylcarnitine, which is suggestive of a defect in long chain fatty acid oxidation. The total absence of plasma short chain acylcarnitine is abnormal; taken in conjunction with the hypoketonemic hypoglycemic hyper-fatty-acidemia, it is suggestive of a disorder of long chain acyl-CoA metabolism.

**Urinary Dicarboxylic Acids and Acylcarnitines**

Patients with proven MCAD deficiency excreted dicarboxylic acids, suberylglu-cine, and octanoylcarnitine when hypoglycemic. However, case 17, who presented in the neonatal period, did not excrete suberylglucose at presentation, but did so when fasted at 7 months of age. One patient, case 24, who had normal MCAD activity, also excreted the dicarboxylic acids suberylglucose, and octanoylcarnitine, and as can be seen from the results presented in Table 1, clearly had hypoketotic.
hyper-fatty-acidemic hypoglycemia. The results of urinary acylcarnitine analysis from patients with proven and suspected medium chain acyl-CoA dehydrogenase deficiency are presented in Table 3. Some typical HPLC chromatograms of urinary acylcarnitines are shown in Fig. 4.

**Fibroblast Acyl-CoA Dehydrogenase Activities**

The activities of short, medium, and long chain acyl-CoA dehydrogenases are shown in Table 4.

**DISCUSSION**

The cause of hypoglycemia cannot be determined by the measurement of blood glucose concentration alone. It is essential to examine blood glucose levels in relation to the concentrations of other major metabolic fuels, intermediary metabolites, and metabolic hormones at the time of hypoglycemia. This diagnostic strategy is not always used, and many of these children have experienced recurrent episodes of severe hypoglycemia, with an unacceptable delay in establishing an appropriate diagnosis due to measurement of blood glucose alone. Our data support our contention that it is important to obtain a blood and a urine sample at the time of hypoglycemia, either during a spontaneous attack or during a supervised fast.

We suggest the following protocol for the investigation of children with hypoglycemia:
TABLE 4. Fibroblast acyl-CoA dehydrogenase activitiesa

<table>
<thead>
<tr>
<th>Patient</th>
<th>SCAD</th>
<th>MCAD</th>
<th>LCAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.24</td>
<td>0.10</td>
<td>1.11</td>
</tr>
<tr>
<td>17</td>
<td>0.04</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>19</td>
<td>0.24</td>
<td>0.16</td>
<td>1.34</td>
</tr>
<tr>
<td>22</td>
<td>0.25</td>
<td>0.13</td>
<td>1.37</td>
</tr>
<tr>
<td>29</td>
<td>0.03</td>
<td>0</td>
<td>0.79</td>
</tr>
<tr>
<td>30</td>
<td>0.12</td>
<td>0.05</td>
<td>0.78</td>
</tr>
<tr>
<td>31</td>
<td>0.16</td>
<td>0.19</td>
<td>1.10</td>
</tr>
<tr>
<td>32</td>
<td>0.17</td>
<td>0.08</td>
<td>1.45</td>
</tr>
<tr>
<td>24</td>
<td>0.13</td>
<td>0.63</td>
<td>0.75</td>
</tr>
<tr>
<td>26</td>
<td>0.24</td>
<td>0.94</td>
<td>1.52</td>
</tr>
<tr>
<td>Controls (n = 18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.22</td>
<td>0.95</td>
<td>1.42</td>
</tr>
<tr>
<td>SD</td>
<td>0.10</td>
<td>0.17</td>
<td>0.21</td>
</tr>
</tbody>
</table>

a Units: nmol ETF reduced·min⁻¹·mg protein⁻¹

Stage 1 is the recognition that the child’s symptoms could be explained on the basis of hypoglycemia.

Stage 2 is obtaining a blood and urine sample at the time of spontaneous hypoglycemia to measure the blood concentrations of intermediary metabolites, free fatty acids and hormones, and urinary organic acids and acylcarnitines.

Stage 3 is the determination of all metabolic fuels, metabolites, and hormones during the course of a starvation provocation test.

Since hypoglycemic episodes may be intermittent, we have introduced a starvation provocation test to stress the counterregulatory metabolic and endocrine systems. Over 100 formal starvation tests have now been performed on children of different ages, and within the strict time limits for maximum periods of starvation as outlined above, no adverse sequelae have been documented. However, it is essential to recognize that prolonged starvation could be dangerous for a child with a defect of β-oxidation in view of the elevation of free fatty acid concentrations that can occur under these circumstances. Our approach is to pay meticulous attention to the clinical condition of the child during the starvation, with an indwelling venous canula in situ for the withdrawal of the blood sample and for the immediate correction of hypoglycemia or a deteriorating circumstance by intravenous glucose.

The etiology and management of hyperinsulinemic states due to dysregulation of the islets of Langerhans have been described previously (48,49). It is evident from our series of patients that this cause of hypoketotic hypoglycemia is associated with the lowest concentrations of ketone bodies; in some cases both hydroxybutyrate and acetoacetate are virtually undetectable. This hypoketonemia is associated with low concentrations of free fatty acids, although the relationship between the two substrates is within the normal range. Recognition of this relationship should alert the clinician to the possibility of hyperinsulinism and the patient should then be investigated as outlined previously (49).

The relationship of free fatty acids to total ketone bodies is highly informative. All patients with enzyme-proven medium chain acyl-CoA dehydrogenase deficiency have demonstrated an abnormal relationship as indicated in Fig. 2. Confirmation that this abnormal ratio is due to MCAD deficiency is shown by the measurement of abnormal concentrations of plasma carnitine and its derivatives, and by the demonstration of the excretion of abnormal urine constituents.

However, as is evident from our data, there remains a group of children who demonstrate an abnormally low concentration of ketone bodies for the degree of free fatty acidemia, implying impaired ketogenesis, yet who do not excrete abnormal constituents in their urine. Moreover, some children who do demonstrate abnormal urinary constituents with an abnormal fatty acid/ketone body ratio do not have abnormal activity of MCAD on enzyme analysis of tissue culture samples. These children presumably have defects of the other enzymes of fatty acid oxidation, and further investigations are in progress. Some children with impaired β-oxidation are able to generate ketone body concentrations in the blood but the concentration is inappropriately low for the level of free fatty acids.

We conclude that it is too simplistic to classify children with hypoglycemia into “ketotic” and “hypoketotic” categories. There is a spectrum of relationship which can only be assessed by the direct relationship shown in Figs. 2 and 3. Determination of the concentration of metabolic fuels, intermediates, and hormones during fasting is extremely valuable in the diagnosis of these children.

Finally, it is emphasized that some children may have a secondary impairment of fatty acid oxidation as a result of a primary enzyme defect elsewhere. This is exemplified by the observation that a child with glycogen storage disease type 1B also has an abnormal relationship between free fatty acids and ketone bodies. However, another disorder of gluconeogenesis, fructose-1,6-diphosphatase deficiency, although also characterized by an abnormal elevation of blood lactate levels, was not associated with the secondary disturbance of ketogenesis. Secondary effects on β-oxidation are further illustrated by the data presented in Fig. 5. This shows the acyl CoA intermediates generated by the incubation of [U-14C]palmitate with isolated skeletal muscle mitochondria from a patient with a lipid storage myopathy due to a deficiency of complex I of the respiratory chain. Thus an impaired ability to oxidize NADH results in a slowing of β-oxidation flux (Fig. 6), the accumulation of hexadec-2-enoyl-CoA, tetradec-2-enoyl-CoA, and 3-hydroxyhexadecanoyl-CoA, and lowered production of acetyl-CoA. These observations provide a mechanism for the lipid
FIG. 5. Radiochromatograms of $[^{14}C]$acyl-CoA ester intermediates of the oxidation of $[^{14}C]$-hexadecanoate by skeletal muscle mitochondria from a patient with a deficiency of complex I of the respiratory chain and controls. Analysis was by reversed-phase HPLC with on-line photodiode array and radiochemical detection as described previously (50). The identity of hexadec-2-enoyl-CoA (C16:1) was confirmed by its characteristic UV absorbance spectrum. Tetradec-2-enoyl-CoA (C14:1) and 3-hydroxyhexadecanoyl-CoA (C16-OH) coelute.
FIG. 6. Generation of acid-soluble metabolites from the oxidation of [U-14C]hexadecanoate by skeletal muscle mitochondrial fractions from a patient with deficiency of complex I of the respiratory chain (<2% of controls) and three controls. Mitochondrial fractions (0.55–4.4 mg protein) were incubated with 60 nmol of [U-14C]hexadecanoate and acid-soluble radioactivity determined at the indicated times. The rates of oxidation were: patient (▲) 9.9 nmol-min⁻¹·mg protein⁻¹; controls (●) (mean ± SD) 15.0 ± 1.9 nmol-min⁻¹·mg protein⁻¹.

storage observed in this patient. This clearly illustrates the intimate relationship between β-oxidation flux and mitochondrial redox state depicted in Fig. 1.

REFERENCES

8. Freeman FE, Goodman SI. Deficiency of electron transfer flavoprotein or electron transfer flavoprotein...
DISORDERS OF MITOCHONDRIAL β-OXIDATION

DISORDERS OF MITOCHONDRIAL β-OXIDATION


DISCUSSION

Dr. Wanders: One of the problems, as Professor Van Hoof says, with regard to the mitochondrial β-oxidation defects is that it is difficult to identify them if you look at organic acids in urine. You suggest doing starvation provocation tests, but perhaps there is another possibility and I should like to have your ideas about it. Mitochondrial β-oxidation is expressed in leukocytes and blood platelets. We have been working on this aspect for several months, and what we do now, in our hospital at least, is to take blood for isolation of leukocytes and platelets from any patient with hypoketonotic hypoglycaemia to study octanoate β-oxidation and palmitate β-oxidation, looking at CO₂ production and acid-soluble material. In this group of disorders particularly, in which you should intervene with diet treatment as soon as possible, it is of utmost importance to know straight away whether or not there is a mitochondrial β-oxidation defect. Hence in this case enzyme analysis should not be at the end of the investigations but rather should be one of the first things to do. What is your view on this?

Dr. Bartlett: I agree. I think we have a problem here: If you measure a specific enzyme and you find it is normal, you may then conclude that you are not dealing with a β-oxidation defect.
This is obviously a dangerous assumption. There are two possible solutions. You can either devise a method to measure the flux of the whole process, for example by measurement of total acid soluble radioactivity derived from radiolabeled substrates, or measure all the enzymes of β-oxidation directly. The latter course would be a major undertaking.

Dr. Wanders: But the first is very easy to do. To look at octanoate β-oxidation and palmitate β-oxidation is simple. I would like to make a strong plea for overall β-oxidation testing in leukocytes or platelets.

Dr. Bartlett: We have tried to devise a radiochemical method for the measurement of β-oxidation flux, although we have not yet applied it to routine screening. I should explain that the conventional way to approach this problem is to incubate fibroblasts or leukocytes with [1,\(^{14}\)C]-labeled substrates, for example [1,\(^{14}\)C]octanoate and [1,\(^{13}\)C]palmitate, and to measure \(^{14}\)CO\(_2\). The logic is that if octanoate oxidation is normal and palmitate oxidation is impaired, a long chain disorder is indicated. The problem with this technique is that it is difficult to obtain reliable assays, probably because CO\(_2\) is quantitatively a minor product in these tissues. We would like to have a reliable single method to measure β-oxidation flux. We have tried using universally labeled substrates and measuring CO\(_2\) production and acid-soluble radioactivity. To date we have obtained rather poor discrimination between affected and nonaffected cell lines. Whether or not there is still a place for CO\(_2\) release assays I am not sure.

Dr. Van Hoof: One of the difficulties in the interpretation of results obtained with 1[\(^{14}\)C]-labeled fatty acids is that \(^{14}\)CO\(_2\) production can result both from α- and β-oxidations. I think that Dr. Saudubray has some experience with the use of radiolabeled fatty acids in the diagnosis of mitochondrial β-oxidation defects.

Dr. Saudubray: I have a comment and a question. The comment is about fatty acid oxidation in fresh isolated lymphocytes. To me it is a very convenient method: very rapid and reliable for screening most fatty acid oxidation disorders, but not all. I should like to emphasize the value of performing an in vivo oral loading test with polyunsaturated long chain fatty acids. This procedure is very reliable and very safe; you can perform the test after an overnight fast of 10 to 12 h, with 1.5 g per kilogram of polyunsaturated sunflower oil. Blood ketones are measured at 0, 1.2, and 3 h after the load. We have used this test successfully in every fatty acid oxidation disorder we have investigated, including PCT1, PCTII, long chain, medium chain, and generalized fatty acid oxidation disorders, and also 3-hydroxyacyl-CoA-dehydrogenase deficiency. In addition, we identified four patients with this loading test who had a clinical and metabolic pattern very suggestive of long chain fatty acid disorders (hypoketotic hypoglycemia, Reye-likeencephalopathy), but without any defect identified in vitro in lymphocytes or “film blasts.” These four patients presented with a complete absence of increase of blood ketones after the loading test with the polyunsaturated long chain fatty acids. There was no abnormal plasma carnitine level, no abnormal organic acids in the urine, and fatty acid oxidation was completely normal in fibroblasts and lymphocytes. We are now planning to carry out in vitro fatty acid oxidation experiments in liver biopsy specimens. Because these patients suggest the possibility of fatty acid oxidation defects restricted to the liver, it seems to me at the moment that the best system for screening such patients is the long chain fatty acid loading test.

Dr. Bartlett: We certainly considered that possibility when we started our studies. We came to the conclusion that as a general principle, once one has administered something to a patient it is impossible to remove it if something goes wrong. We concluded that the safest course was a fasting provocation test, because it is straightforward to abort the test by the administration of glucose. So although I take the point that it is possible to stress β-oxidation by the administration of a fat load, we have no direct experience.

Dr. Endres: I think that fasting is a relatively dangerous procedure for young babies or infants.
As a pediatrician I have some difficulty in deciding what is the most suitable screening test for defects in \(\beta\)-oxidation in children with near-miss sudden infant death syndrome. Tanaka proposed that one should just take a random sample of urine and use a stable isotope dilution technique for gas chromatography–mass spectrometry (GC-MS). What is your experience with this method, since the loading with phenylpropionic acid proposed by Seakins and co-workers can be used only for the diagnosis of MCAD deficiency? What do you think about this special technique for GC-MS?

Dr. Bartlett: I believe you are referring to a paper by Rinaldo et al. (1) which describes a stable isotope dilution assay for glycine conjugates in urine. They measured hexanoylglycine, phenylpropionylglycine, and suberylglycine and reported that they could detect abnormal concentrations of some of these during periods of remission; in other words, under normoglycemic conditions. The only problem with this approach is that the technique requires gas chromatography–mass spectrometry, and the stable isotopically labeled internal standards are not commercially available. I cannot say from my own experience whether the technique is reliable, although I am sure Dr. Roe has something to say about the alternatives.

Dr. Roe: That is an interesting point. I think the use of phenylpropionate loading, as Professor Endres indicated, would be relatively restricted to MCAD patients. So it is a rather focused approach, almost like doing an enzyme assay selectively. On the other hand, it should be recognized that phenylpropionyl glycine is not seen in newborns because it is a metabolite produced by established bacterial flora. I would agree with Professor Endres that one should avoid fasting young infants when looking for \(\beta\)-oxidation disorders.

Dr. Bartlett: We have now performed over 200 fasting stress tests, including tests of patients with subsequently proven \(\beta\)-oxidation disorders. In none have we experienced any untoward clinical sequelae. However, it is important to emphasize that these were always carried out under close clinical supervision and that fasts were always terminated within the limits indicated.

Dr. Roe: The Hopkins group lost one child in the course of the fasting, and another child in Denmark was lost.

Dr. Eggermont: In the newborn, ketonuria is very rare. Can you comment on the metabolism of ketone bodies in the newborn and the infant?

Dr. Bartlett: Ketone bodies are a major substrate for brain in the newborn period. There are, of course, some important dietary transitions in early life, from a largely carbohydrate-based economy during fetal life to a high-fat diet in the neonatal period and a further transition to a mixed diet at weaning. Thus in the newborn period it is really not surprising that ketone bodies are extensively utilized, both as metabolic fuels and as lipogenic precursors in the brain.

Dr. Holton: Screening for MCAD has been recommended, particularly in siblings of SIDS patients. It is suggested that the urine metabolites could be picked up during the first couple of days of life, and some groups are doing this in large numbers. Do you think that this is reliable?

Dr. Bartlett: If you assume that about 2–3% of SIDS cases are caused by medium chain acyl-CoA dehydrogenase deficiency, although the precise proportion is still open to debate, and further that it is an autosomal condition, then in order to obtain statistically significant results, large numbers of siblings of SIDS would have to be screened. There is a more serious problem with screening siblings of SIDS. It is accepted that the excretion of informative abnormal metabolites is critically dependent on the metabolic milieu at the time the urine is collected. If conventional gas chromatographic methods are used rather than the stable isotope/GCMS method described by Rinaldo et al. (1), I am not convinced that even if MCAD were present, it would be detected by analysis of random urines, that is, without some form of metabolic stress.
Dr. Duran: I would like to comment on the neonatal urine screening of sibling case of the SIDS. We had the opportunity to study two siblings of proven MCAD-deficient patients in the first week of life and we did not find any abnormal metabolites then. What we then did was a phenylpropionate loading test 2 months later and we found that the result of that loading test was positive. We are content with this loading test because it cannot only pick up the MCAD deficiency but probably also other defects. For example, we showed that we could pick up long chain 3-hydroxyacyl-CoA-dehydrogenase deficiency.

Dr. Hobbs: I realize that your particular assay at the moment requires a large number of cells, but it is possible these days to produce them in culture. There does tend to be a dogma that it is almost impossible to insert extra mitochondrial enzymes into mitochondria, but if you were to grow the fibroblasts of a deficient patient and then add the cells or plasma from a normal subject, the hypothesis could be tested. If, indeed, the normal cells were able to deliver the deficient enzyme into mitochondria of the patient’s cells, you would have a clear indication of future possible treatments.

Dr. Bartlett: Most polypeptide precursors of mitochondrial proteins have a peptide leader sequence that both targets them to the correct intracellular compartment and allows them to traverse the inner mitochondrial membrane. This leader sequence is removed to allow generation of active enzyme. I did not show the data, but we have studied a patient whom we found difficult to explain. She presented with hypoketotic hyper-fatty acidemic hypoglycemia and excreted the metabolites characteristic of medium chain acyl-CoA dehydrogenase deficiency. However, we found normal activity as measured in homogenates of cultured skin fibroblasts. We were puzzled by this until it was pointed out that a mutation in the leader sequence could account for these findings. It is possible that an accumulated precursor peptide could undergo maturation to active enzyme following homogenization. I agree that it may be possible to study enzyme replacement therapy for mitochondrial disorders by such a technique, but I think it would be necessary to use the precursor peptide.

Dr. Krywawych: In those cases where you see dicarboxylic acids, is it only saturated dicarboxylic acids or are unsaturated acids also present? If there are unsaturated dicarboxylic acids, do you think that this is simply a reflection of dietary intake of unsaturated acids, or do you think that this may be used as an indicator of a more specific enzyme lesion in β-oxidation?

Dr. Bartlett: We do see C10 unsaturated dicarboxylic acids. I have always assumed that these are generated from polyunsaturated fatty acids which are dietary in origin.

Dr. Saudabay: I have a question on the 3-hydroxybutyrate-acetoacetate ratio and lactate biorate ratio in the blood. Have you checked these ratios in fatty acid oxidation defects?

Dr. Bartlett: The 3-hydroxybutyrate/acetoacetate and lactate/pyruvate ratios were normal, as were the lactate concentrations in medium chain acyl-CoA dehydrogenase deficiency. However, we have observed raised lactate concentrations in three cases of long chain 3-hydroxyacyl-CoA dehydrogenase deficiency.

Dr. Saudabay: We could expect to find lowered ratios. The fatty acid oxidation defect should lower the production of NADH and FADH in mitochondria, resulting in secondary lowering of lactate.

Dr. Bartlett: I would not anticipate an altered mitochondrial redox state simply because of impaired β-oxidation. There are, after all, plenty of other oxidizable substrates, at least under normoglycemic conditions.

Dr. Krywawych: You have said that control of β-oxidation occurs at the CPT1 stage. Presumably, the carnitine concentration can influence the rate of β-oxidation, as carnitine is a substrate for the CPT1 enzyme. Does the carnitine status, reflected in the plasma carnitine concentrations, alter the plot of plasma concentrations of fatty acids against ketone bodies in
FIG. 1. Triple-quadrupole mass spectrometric detection of palmitoyl-CoA. In the simple FAB-MS spectrum the molecular ion (M-1) is almost undetectable (A). The most intense signal corresponding to a negative ion with a mass of 597 results from the loss of adenosine 3'- and 5'-bisphosphate. Daughter fragments of this 597 ion displayed in (B) are typical. Three among them with masses of 243, 261, and 359 are common to all CoA esters; the other two (499 and 597) vary with the length of the fatty acid, as explained on the formula of a coenzyme A ester, in (C).
normal subjects? Can carnitine deficiency, which may be secondary to some other problem, produce a condition that appears to be a block in \(\beta\)-oxidation?

**Dr. Bartlett:** Early reports of what were thought to be cases of primary systemic carnitine deficiency have been shown to be. I think without exception, secondary to \(\beta\)-oxidation disorders. As far as I am aware, there have been very few substantiated cases of inherited primary carnitine deficiency.

**Dr. Van Hoof:** Important progress in the diagnosis of disorders of mitochondrial \(\beta\)-oxidation has resulted from the development of tandem mass spectrometry. Acylcarnitine in blood and urine reflect the accumulation of acyl-CoA esters in the mitochondrial matrix. I would like to show you that acyl-CoA can be directly analyzed with a tandem mass spectrometer. Figure 1, obtained with the collaboration of S. Ponchaut, as well as E. de Hoffmann from the Department of Mass Spectrometry of the University of Louvain (Louvain-la-Neuve), shows that specific fragments are formed by fast atom bombardment, which allow one to recognize the CoA esters. The upper part of the figure (A) displays a positive ion scan of deionized palmitoyl-CoA dissolved in a mixture of glycerol and 1\% glycolic acid. The molecular ion is barely detectable at a mass of 1004, but there is an intense fragment at mass 597. The daughter scan of this fragment submitted to collision with xenon atoms (B) reveals interesting fragments, some (at mass 499) containing the fatty acid and some (at mass 243, 261, 359) which are common to all CoA derivatives. An explanation of each of these fragments is given in the inferior part of the figure (C).

**Dr. Bartlett:** We have done a similar sort of thing with FAB-MS and intact CoA esters. We have been able to carry out FAB-MS analysis of the complete series of saturated C4–C16 mono-CoA esters of dicarboxylic acids. It also works well for the carnitine esters, of course.

REFERENCE