The Oxidation of Fatty Acids Combined with Albumin by Isolated Rat Liver Mitochondria*

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SUMMARY

Long chain fatty acids at concentrations inhibiting mitochondrial respiration were, in the presence of serum albumin, found to produce almost as high a rate of oxygen uptake as α-ketoglutarate, succinate, or acetate. This oxidation was characterized in terms of its coupling to phosphorylation, need for cofactors, and production of different metabolites during the reactions.

Fatty acids were oxidized to carbon dioxide, acetoacetate, β-hydroxybutyrate, and other water-soluble metabolites, tentatively identified as intermediates of the citric acid cycle.

An agent to spark the citric acid cycle and adenosine tri- or monophosphate were necessary for optimal oxidation rate, as described for other fatty acid oxidation systems.

Balance experiments with different amounts of malate were performed with incubations lasting as long as oxygen uptake took place. In the presence of 1 μmole of malate, practically all added palmitic acid was used up and found to be converted primarily to carbon dioxide, acetoacetate, and other water-soluble metabolites of which the major part was tentatively identified as succinate. A significant portion was found in mitochondrial phospholipides. With 10 μmoles of malate some palmitic acid remained in the system, while a comparatively small amount was converted to carbon dioxide, and a major part was found as succinate. Here also incorporation into phospholipides occurred. With no malate added, fatty acid oxidation was much smaller than with malate, although significant conversion to carbon dioxide took place. Only a little succinate and phospholipid were found.

Oxygen uptake was greater than a theoretical value calculated from radioactive balance experiments. It was concluded that albumin contains oxidizable material even after extraction and dialysis. Albumin at high concentrations inhibited both fatty acid and α-ketoglutarate oxidation.

The oxidation of long chain fatty acids in high concentrations in the form of albumin-fatty acid complex was coupled to phosphorylation. Thus P:O ratios above 2 were found as well as evidence for respiratory control.

It was concluded that oxidation of long chain fatty acids by isolated mitochondria occurs from their albumin complex. This process can also be studied at high concentrations of fatty acids, where high rates of oxygen uptake are obtained from oxidation which is coupled to phosphorylation.

The fatty acids of main physiological importance in energy transport are those with a chain length of 16 carbon atoms and more (1). These fatty acids are powerful inhibitors of oxidation and phosphorylation in isolated mitochondria (2–5) and thus are not very suitable for studies of these reactions. They are metabolized differently in several respects as compared with shorter chain fatty acids (6), which so far have generally been used as fatty acid substrates in studies utilizing isolated mitochondria (7).

During studies on the effect of serum albumin as antagonist to the inhibitory effect of fatty acids on respiration and phosphorylation in isolated rat liver mitochondria, it was noted that albumin not only protected the mitochondrial oxidation of α-ketoglutarate or succinate but also, in the presence of fatty acids, caused a marked oxygen consumption, suggestive of fatty acid oxidation (5). This occurred with long chain fatty acids and in concentrations which are within the range of those occurring in blood plasma (8).

Oxidation of fatty acids bound to albumin has earlier been observed in experiments with subcellular fractions (9, 10). This oxidation does not seem to have been studied further with respect to such characteristics as maximal rate, coupling to phosphorylation, production of metabolites, and requirements for cofactors. This is particularly the case when isolated liver mitochondria were used as enzyme source.

Carnitine increases oxidation of long chain fatty acids by subcellular preparations from different tissues (7, 11). This is particularly the case when albumin is present to keep the fatty acids in solution (9). Bode and Klingenberg (12) recently demonstrated that the oxidation of caprinylcarnitine was increased by the addition of adenosine diphosphate to isolated calf diaphragm mitochondria. Coupling of oxidation of fatty acylcarnitine to phosphorylation in vitro has not been reported for longer chain fatty acids; nor have studies been performed in which oxidation of long chain fatty acids by isolated mitochondria has occurred with a demonstrated tight coupling of phosphorylation. This is particularly the case with fatty acids in concentrations of the same magnitude as those occurring in

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blood plasma, from which they rapidly disappear into the cells (13).

The possibilities for studying the mitochondrial oxidation of the physiologically important long chain fatty acids, in physiological concentrations and under conditions in which phosphorylation was coupled to oxidation, seemed to be offered by the fatty acid-albumin complex. This possibility motivated the present investigation, in which this oxidation has been characterized.

MATERIALS AND METHODS

Male rats of a Sprague-Dawley strain (Anticimex) weighing 200 to 330 g were fasted about 18 hours, killed by a blow on the head, and exanguinated by decapitation. The liver was removed and homogenized (1:10) in 0.25 M sucrose + 0.001 M EDTA, pH 7.5, in a test tube with a glass pestle. Mitochondria were then isolated according to the procedure of Schneider (14).

Oxygen consumption was measured by the Warburg technique. Each vessel contained 50 μmoles of P_i, 5 μmoles of ATP, 6 μmoles of MgCl_2, and 260 μmoles of glucose in the main compartment; 0.1 ml of 20% KOH in the center well; and, when acetoacetate was determined colorimetrically, 0.2 ml of 30% tri-chloroacetic acid in the side arm. Other additions are indicated for each experiment. Final volume was 3.0 ml; final pH, 7.5; temperature, 37.4°; and gas phase, air.

When ^14CO_2 was collected, incubations were performed in 50-ml cylindrical flat bottom tubes with a diameter of 25 mm (Hagedorn tubes). These were sealed by a rubber stopper which held 2 glass tubes closed by rubber membranes. After incubation, 0.3 ml of 50% citric acid was injected through one of the membranes of the glass tubes into the incubation mixture and 0.3 ml of Hyamine through the other into a 1-ml beaker which was suspended on a stainless steel wire and contained a folded filter paper. After 90 min of incubation, the beaker and paper were placed in a Packard Tri-Carb counting vial and 0.5 ml of 99.5% ethanol, 10 ml of 0.1% 2,5-diphenyloxazole, and 0.01% p-bis-2-(5' phenyloxazolyl)benzene in toluene were added. Quenching was corrected for by addition of internal standard. This procedure gave a 93% recovery when tested with ^14CO_2 from NaH^14CO_3.

The carbonyl-^14C content of β-keto acids, derived under the present conditions mainly from acetoacetate (9), was estimated by the addition of aniline citrate (15). The ^14C was then collected as described above.

Radioactivity that was not collected as ^14CO_2 or acetoacetate-carbonyl-^14CO_2 was measured by extracting an aliquot of the incubation mixture after the end of ^14C_2 collections in Dole's (8) extraction mixture. In some experiments, aliquots of the heptane or the water-sulfuric acid phase were taken directly from the main vial and added to a scintillation vial. The vial was then adjusted to 7.5 at room temperature by the dropwise addition of excess of potassium hydroxide under slight warming; the pH was then adjusted to 7.5 at room temperature by the dropwise addition, with constant stirring, of hydrochloric acid. The result was a fine, milky, rather stable suspension with oleic acid; the palmitic acid product was flakier, but it was still possible to pipette reproducibly after shaking. Uniformly labeled oleic-^14C and palmitic ^14C acids and sodium acetate 1.2 ^14C were obtained from The Radiochemical Centre, Amersham, England (CFB 39, CFB 37, and CFA 1229). Nucleotides and hexokinase (crystalline) were purchased from Sigma.

RESULTS

Effect of Albumin Addition on Oxidation—The effect of adding increasing amounts of albumin on the respiration of mitochondria in the presence of α-ketoglutarate or oleic acid plus malate, or malate only, is shown in Fig. 1. After 100 mg of albumin, mitochondrial respiration in the presence of α-ketoglutarate was inhibited. As described earlier (5), addition of albumin to oleic acid first increased respiration until a level was reached at a molar ratio of oleic acid to albumin of about 7. Here also an inhibition was noted at higher albumin concentrations. Increasing amounts of albumin in the presence of malate increased respiration.

Products of Fatty Acid Oxidation—Table 1 shows that the oxidation of palmitate, bound to albumin, produced acetoacetate when malate was not present. When the fatty acid-albumin complex was dialyzed after preparation, the oxygen consumption...
decreased somewhat, while acetoacetate production increased. Malate in the same systems caused a higher oxygen uptake and decreased acetoacetate accumulation. Albumin plus malate gave a higher oxygen uptake than malate alone and resulted in formation of acetoacetate. The palmitate-albumin complex gave as high an oxygen consumption as acetate.

Fig. 2 shows the distribution of radioactivity from uniformly labeled palmitate, bound to albumin, in different products after different periods of reaction. Lipid-soluble radioactivity quickly leveled off, while water-soluble radioactivity increased to more than 50% of total after 15 min and then decreased almost linearly. Incorporation of label into carbon dioxide seemed to occur linearly over the period of time during which it was measured. A small part of total radioactivity was found in acetoacetate in this system, which contained 1 pmole of malate. Recovery of label in this experiment was at the lowest 82% (15-min observation).

Table II shows an experiment in which fatty acid with albumin was allowed to be oxidized in the presence of different amounts of malate, or in its absence, as long as measurable oxygen uptake took place in any of the flasks in the experiment. With no malate present, 64.6% of added radioactivity in the form of palmitic acid remained as fatty acid after incubation. The rest of the radioactivity was found primarily as carbon dioxide and acetoacetate, but some of it could also be found on the sites of citric acid cycle metabolites as well as those of β-hydroxybutyrate and phospholipids. Most of the added radioactivity could be accounted for. No oxygen uptake could be measured in this system.

In the presence of 1 µmole of malate, practically all palmitic acid disappeared. More than half of it was found as carbon dioxide and 17.6% as acetoacetate. More than 10% was found...
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Recovery of label from palmitate oxidized by isolated rat liver mitochondria in presence of albumin and different amounts of malate

Mitochondria corresponding to 4.2 mg of protein and, where indicated, 0.25 μ mole of potassium palmitate containing 129,000 cpm of uniformly labeled potassium palmitate-14C; 0.12 μ mole of albumin containing 0.04 μ mole of titratable fatty acids; potassium malate; 6 units of hexokinase with 1 μ mole of malate; and 24 units of hexokinase with 10 μ moles of malate were incubated in Warburg vessels and in Hagedorn tubes for 5 hours. Distribution of radioactivity was then measured in the Hagedorn tubes. Other additions and conditions were as described in "Materials and Methods" except that the Hagedorn tubes contained no KOH. Acetoacetate results are given as 4 times the radioactivity collected as carbon dioxide after addition of aniline citrate.

| Table II
| Recovery of label from palmitate oxidized by isolated rat liver mitochondria in presence of albumin and different amounts of malate

<table>
<thead>
<tr>
<th>Components</th>
<th>Oxygen uptake</th>
<th>Respiratory control</th>
</tr>
</thead>
<tbody>
<tr>
<td>With hexokinase</td>
<td>Without hexokinase</td>
<td>μmoles</td>
</tr>
<tr>
<td>Palmitate + albumin</td>
<td>6.3</td>
<td>9.8</td>
</tr>
<tr>
<td>Palmitate + albumin + malate (1 μmole)</td>
<td>52.0</td>
<td>17.6</td>
</tr>
<tr>
<td>Palmitate + albumin + malate (10 μmoles)</td>
<td>6.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Hexokinase (6 units) + malate (1 μmole)</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td>Hexokinase (24 units) + malate (10 μmoles)</td>
<td>60</td>
<td>33</td>
</tr>
</tbody>
</table>

Influence of albumin on respiratory control of α-ketoglutarate

Each flask contained 25 μ moles of α-ketoglutarate and mitochondria corresponding to 2.5 mg of protein. Albumin and 6 units of hexokinase were added as indicated. Incubation time was 15 min. Other additions and conditions were as described in "Materials and Methods." Respiratory control is expressed as the ratio between oxygen uptake in the presence of hexokinase and in its absence.

| Table III
| Influence of albumin on respiratory control of α-ketoglutarate

<table>
<thead>
<tr>
<th>Components</th>
<th>Oxygen uptake</th>
<th>Respiratory control</th>
</tr>
</thead>
<tbody>
<tr>
<td>With hexokinase</td>
<td>Without hexokinase</td>
<td>μmoles</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>75</td>
<td>36</td>
</tr>
<tr>
<td>α-Ketoglutarate + 70 mg of albumin</td>
<td>130</td>
<td>68</td>
</tr>
<tr>
<td>α-Ketoglutarate + 50 mg of albumin</td>
<td>128</td>
<td>70</td>
</tr>
<tr>
<td>α-Ketoglutarate + 10 mg of albumin</td>
<td>101</td>
<td>37</td>
</tr>
<tr>
<td>α-Ketoglutarate + 5 mg of albumin</td>
<td>100</td>
<td>33</td>
</tr>
</tbody>
</table>

as other water-soluble compounds, primarily in a spot on the chromatoplate corresponding to succinate. A clear incorporation of radioactivity into phospholipids occurred; the traces of radioactivity in the front region of this chromatoplate, however, are of doubtful significance. Practically all radioactivity was accounted for. After respiration had ceased, a total of 6.8 μ moles of oxygen had been taken up.

Addition of 10 μ moles of malate changed the pattern of radioactive products considerably. First, a significant fraction of palmitic acid was left after oxygen uptake in the system had ceased. Half of the radioactivity recovered was found in water-soluble metabolites, primarily in the regions of the chromatoplates corresponding to succinate and malate + citrate. There were also significant amounts of acetoacetate and β-hydroxybutyrate. Carbon dioxide production was much smaller than with 1 μ mole of malate, while incorporation of radioactivity into phospholipids seemed almost as high. A small amount of radioactivity of doubtful significance was found also in the spot containing triglycerides. Total recovery was 80.1%, and total oxygen uptake was 10.9 μ moles.

When the oxygen uptakes for the flasks containing malate only were subtracted from the values for the corresponding flasks with malate + palmitate, 4.8 (1 μ mole of malate) and 2.1 (10 μ moles of malate) μ moles of oxygen taken up remained. These two amounts are higher than the theoretical values of oxygen consumption calculated from the radioactive data.

Phosphorylation Reactions—Lower concentrations of albumin

| Table IV
| Influence of adenine nucleotides on oxidation of α-ketoglutarate or oleate in presence of albumin

<table>
<thead>
<tr>
<th>Components</th>
<th>Oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-Ketoglutarate</td>
<td>106</td>
</tr>
<tr>
<td>Oleate + albumin + malate + ATP (0.5 μ moles)</td>
<td>114</td>
</tr>
<tr>
<td>Oleate + albumin + malate + ATP (5 μ moles)</td>
<td>122</td>
</tr>
<tr>
<td>Oleate + albumin + malate + ATP (25 μ moles)</td>
<td>113</td>
</tr>
<tr>
<td>Oleate + albumin + malate + ATP (50 μ moles)</td>
<td>108</td>
</tr>
<tr>
<td>Oleate + albumin + malate + AMP (5 μ moles)</td>
<td>114</td>
</tr>
<tr>
<td>Oleate + albumin + malate + AMP (25 μ moles)</td>
<td>102</td>
</tr>
<tr>
<td>Oleate + albumin + malate + ATP (5 μ moles) + hexokinase</td>
<td>132</td>
</tr>
<tr>
<td>Oleate + albumin + malate</td>
<td>60</td>
</tr>
<tr>
<td>Oleate + albumin</td>
<td>40</td>
</tr>
<tr>
<td>Malate + ATP (5 μ moles) + hexokinase</td>
<td>12</td>
</tr>
</tbody>
</table>
FIG. 3. Dependence of hexokinase concentration on oxidation of α-ketoglutarate, succinate, and palmitate in the presence of albumin. Each flask in the α-ketoglutarate experiment (●) contained 25 μmoles of α-ketoglutarate, and mitochondria corresponding to 3.3 mg of protein; in the succinate experiment (X) 25 μmoles of potassium succinate, and mitochondria corresponding to 2.9 mg of protein; and in the palmitate experiment (○) 5 μmoles of potassium palmitate, 1.0 μmole of albumin, 1 μmole of potassium malate, and mitochondria corresponding to 3.5 mg of protein. Hexokinase was added where indicated. Incubation time was 20 min. Other conditions and additions were as described in “Materials and Methods.”

are known to increase respiratory control (25); this is confirmed in Table III, which also shows that higher amounts of albumin, similar to the levels used in the present work, do not diminish respiratory control.

In the system used, both α-ketoglutarate and succinate needed the addition of at least 24 units of hexokinase for maximal rate of oxygen uptake (Fig. 3). Palmitate oxidation, however, showed quite different conditions. Oxygen uptake was maximal at about 6 units of hexokinase. At higher amounts, oxygen uptake diminished markedly. The ratio of respiration in the absence of hexokinase to maximal respiration was higher for palmitate than for α-ketoglutarate. In this respect fatty acid oxidation resembled succinate oxidation more than α-ketoglutarate oxidation. The P:O ratio for the oxidation of palmitate with no hexokinase present was 0.2, and with the optimal amount of hexokinase (6 units), 2.1.

As may be seen in Table IV, the addition of adenine nucleotide, whether ATP or AMP, approximately doubled the oxygen uptake of oleate + albumin + malate. Omission of malate from the latter system caused a decrease.

DISCUSSION

It was possible to show that the oxygen uptake rate produced by isolated rat liver mitochondria with substrate fatty acids in the presence of albumin and malate in sparkler concentrations is of the same magnitude as that obtained with α-ketoglutarate, succinate, or acetate (Figs. 1 and 3; Table I). This oxygen consumption parallels oxidation of the albumin-bound fatty acid to carbon dioxide and acetoacetate or other water-soluble intermediates.

There was, however, additional oxygen uptake not accounted for by fatty acid oxidation (Table II). There appear to be several explanations for this. Extracted albumin without added fatty acids produced oxygen consumption. This is in part attributable to an acetoacetate-yielding substrate, possibly the fatty acids which always remain on extracted albumin.

The possibility that this additional oxygen uptake was due to these traces of fatty acid is also supported by the fact that oxygen uptake with extracted albumin shows a dependence on hexokinase similar to that obtained when fatty acids are added. The amount of extracted albumin generally used in the present work was 1 μmole, containing at least 0.1 μmole of long chain fatty acids. This corresponds to an appreciable amount of acetate or oxygen equivalents. This residual fatty acid cannot be removed without denaturation of the protein (23).

Other oxidizable substrates might also be attached to albumin. The finding of a diminished oxygen uptake and increase of acetoacetate production after dialysis of the albumin-fatty acid complex suggests removal of a substance that is able to spark the citric acid cycle. Such a compound could also contribute to the additional oxygen uptake.

It appears unlikely that albumin would be broken down and oxidized by mitochondria because there are no protein-cleaving enzymes present in mitochondria as there are in lysosomes (26). In the mitochondrial fraction used in the present work, there is only a small amount of contaminating lysosomes as judged by analysis of acid phosphatase (26) and by fluorescence microscopy (27).

Goodman (28) found that long chain fatty acids are bound to albumin at different sites with different association constants; these are highest for fatty acids bound in a molar ratio of fatty acids to albumin of less than 2. The fatty acids bound in a
ratio higher than this are readily oxidized by mitochondria, as also described by Fritz and McEwen for subcellular muscle preparations (10) and by Fritz for a liver fraction containing nuclei and mitochondria (9). The fatty acids bound in a lower ratio also seem to be oxidized, as judged from experiments in which complete oxidation was found.

It is not possible to discover from the experiments reported here whether fatty acids are available equally freely from each of these two binding sites, but this question is under study. It might be that the apparent inhibition of oleic acid oxidation at high amounts of albumin, shown in Fig. 1, is caused by an increased binding of fatty acids to a site where they are less available for oxidation. Fritz (9) suggested this mechanism for inhibition of fatty acid oxidation by high concentrations of albumin in tissue preparations which were stimulated by carnitine. The inhibition of α-ketoglutarate oxidation by high amounts of albumin probably has other causes, however. This inhibition was found to vary with different brands of albumin and with different batches of the same brand. Although not systematically tested, bovine serum albumin seems less inhibitory than human. The extraction procedure (23) does not seem to modify the inhibition; this was also observed by Fritz (29).

Water-soluble intermediates accumulated during fatty acid oxidation, particularly in the presence of high amounts of malate, as described earlier (30). In these experiments, radioactivity was found to be situated at the same spot as succinate on chromatoplates. One reason for this might be inhibition of succinic dehydrogenase by oxaloacetate formed from added malate (31).

A short time after the beginning of incubation, the majority of the fatty acid was transferred to a water-soluble metabolite pool, which later decreased and was transformed to carbon dioxide in a linear manner.

In the presence of malate, radioactivity from fatty acids was incorporated into phospholipids. Wojtczak and Lehninger have shown (32) that oleic acid can be incorporated by ATP into mitochondrial membrane phospholipids during the process of contraction. The incorporation occurs not only from free fatty acids but also from fatty acids bound to albumin (33). For optimal conditions α-glycerophosphate has to be added (33). Furthermore, sucrose (32) and albumin (33) inhibit this incorporation. These differences between the conditions used in the present work and those used by Wojtczak (34) probably explain why, in the latter work, more incorporation of fatty acids into mitochondrial phospholipids was found, while in the present work the oxidative pathway for the fatty acids was predominant.

At 24 units of hexokinase, the ADP formed was no longer rate-limiting for α-ketoglutarate or succinate respiration in the coupled system (35), while in the fatty acid system 6 units were optimal, respiration at higher amounts falling rather steeply. The intramitochondrial ATP concentration at amounts of hexokinase higher than 6 units might be too low to allow an efficient activation (36) of the fatty acid, since hexokinase competes for ATP with intramitochondrial reactions (37).

The increase of oxygen uptake by the optimal amount of hexokinase as compared with the increase found in the absence of hexokinase was lower in the fatty acid system than for α-ketoglutarate. It was shown that uncoupling by the comparatively large amounts of albumin did not occur. One possible explanation for the apparent low degree of respiratory control is that the true maximal oxidation rate might never have been reached for the fatty acids because either ATP, for fatty acid activation, or ADP, as phosphate acceptor, or both, was also rate-limiting at the observed optimal hexokinase concentration.

As described earlier by Lehninger and Kennedy (38) for other types of fatty acid oxidation systems by liver particulates, ATP and an agent to spark the citric acid cycle were found necessary for the present system. AM1' could replace ATP (38).

Albumin is probably not an intracellular fatty acid transport vehicle, since its concentration within the cell is very small. It is not possible to discover from the experiments reported here whether fatty acids are available equally freely from each of these two binding sites, but this question is under study. It might be that the apparent inhibition of oleic acid oxidation at high amounts of albumin, shown in Fig. 1, is caused by an increased binding of fatty acids to a site where they are less available for oxidation. Fritz (9) suggested this mechanism for inhibition of fatty acid oxidation by high concentrations of albumin in tissue preparations which were stimulated by carnitine. The inhibition of α-ketoglutarate oxidation by high amounts of albumin probably has other causes, however. This inhibition was found to vary with different brands of albumin and with different batches of the same brand. Although not systematically tested, bovine serum albumin seems less inhibitory than human. The extraction procedure (23) does not seem to modify the inhibition; this was also observed by Fritz (29).

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