Weanling rats were fed a simple riboflavin-deficient diet or the same diet supplemented with galactoflavin. Oxidative phosphorylation was studied in isolated hepatic mitochondria. Throughout the course of the experimental diets, a total of 8 weeks, there were no alterations in the respiratory control ratios or in the ADP/O ratios of the isolated mitochondria. Succinate dehydrogenase activity decreased in the first 3 weeks of deficiency, but oxidation of succinate remained at a constant level. After 3 weeks, the State 3 oxidation rate for succinate and $\beta$-hydroxybutyrate declined, while oxidation of pyruvate, glutamate, and $\alpha$-ketoglutarate was unaffected.

The most dramatic mitochondrial effect of riboflavin deficiency was on fatty acid oxidation. As early as 1 day after the deficient diet was begun, palmitoyl-$L$-carnitine and hexanoate oxidation was decreased by 35%; by Day 5, this reduction was 55%, and by Day 28 it was 75%. These effects could not be reproduced by total starvation of the rats. To determine the biochemical basis of the decrease in the ability of mitochondria to oxidize fatty acids, the specific activity of the enzymes involved in fatty acid oxidation was measured. The mitochondrial defect depended in part on a reduction in acyl-CoA dehydrogenase activities. Of these dehydrogenases, butyryl-CoA dehydrogenase activity was depressed to the greatest extent. Alterations in electron transport flavoprotein may also be involved in decreased capacity for fatty acid oxidation. Unlike the acyl-CoA dehydrogenases, the other enzymes of fatty acid oxidation were variably affected by riboflavin deficiency. Of these, carnitine palmitoyltransferase, $\beta$-hydroxyacyl-CoA dehydrogenase, and $\beta$-ketothiolase showed as much as a 2-fold increase in specific activity.

Flavin coenzymes mediate the oxidation of a variety of substrates by hepatic mitochondria. Such flavoproteins utilize either FMN or FAD as their prosthetic groups. Under normal conditions of alimentation, the ultimate source of the latter two compounds is dietary riboflavin (vitamin B2). When rats are deprived of this vitamin, hepatic levels of the flavin coenzymes are markedly diminished (1). Although several groups of investigators have examined this phenomenon, no consensus has been arrived at concerning the effect of lowered flavin content on the oxidative capacity of rat liver mitochondria. With respect to both maximal rates of oxygen consumption and integrity of respiratory control, mitochondria from riboflavin-deficient rats have been reported as either showing noteworthy defects (2) or as being entirely unaffected (3).

The basis for these disparate findings may lie in the fact that animals of different ages were used in the various experiments. We have found that weanling animals show the maximal response to arboflavinosis. In the present study, weanling rats were fed a riboflavin-deficient diet and the progressive alterations in hepatic mitochondrial oxidative metabolism followed for 8 weeks. The mitochondria showed a dichotomous response to riboflavin deficiency. Oxidation of fatty acids was severely impaired within 1 day after the diet was initiated. In contrast, oxidation of non-lipid substrates varied with the particular substrate but usually required a minimum of 3 weeks of deficiency to become evident.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male Wistar strain weanling rats (21 days old) weighing 35 to 50 g were obtained from Carworth Farms (New York, N. Y.) or Charles River (Wilmington, Mass.) and kept in large, wire-bottomed cages. The animals were randomly divided into several groups. In each of five series of experiments there was a control group which was fed Purina Rat Chow ad libitum, and a group that was fed a similar diet which was deficient only in riboflavin (Nutritional Biochemical Corp., Cleveland, Ohio). One series included a third group fed the riboflavin-deficient diet supplemented with the riboflavin antagonist, galactoflavin, to a level of 60 mg/kg. In another series, a group of animals was starved for 2 days and then killed. In a final series, a group of rats was fed a riboflavin-deficient diet supplemented with 22 mg of riboflavin/kg of diet; the amount of diet made available to this group was just sufficient to match weight changes in rats on the simple riboflavin-deficient diet. In sum, controls for the first 3 days of riboflavin deficiency experiments included ad libitum feeding of normal diet, total starvation, and a restricted diet to limit weight gain. All animals had free access to water.

**Mitochondrial Preparation**—At the time of killing, the animals were stunned by a blow to the head and decapitated. The liver was extirpated, rinsed, blotted, and weighed. All subsequent procedures were carried out on ice. The livers from several animals in each group were pooled, minced, and homogenized in 4 volumes of 220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4 (MSM buffer), in a Potter Elvehjem homogenizer with a loose fitting pestle. After four rapid passes, the crude homogenate was diluted with MSM buffer to a concentration of 2 mM. Nuclei, unbroken cells, and cell debris were removed by centrifugation at 400 x g for 10 min. Mitochondria were
then isolated from the supernatant by centrifugation at 7000 \( \times \) g for 10 min and washed twice with MSM buffer. The final mitochondrial pellets were resuspended in MSM buffer and adjusted to a protein concentration of 50 mg/ml.

**Oxidation Experiments**—The oxidation experiments were carried out in a Clark-type oxygen electrode in a 1-ml chamber, as previously described (4). The incubation medium was 80 mM KCl, 50 mM Mops, 5 mM Ph, 1 mM EGTA, and 1 mg of defatted, dialyzed bovine serum albumin/ml. The final pH was 7.0 and the temperature was 30°C. Albumin was omitted from the medium in dinotrophin-uncoupled incubations. Substrate concentrations are given in the figures and tables. Respiration was initiated by addition of substrate to the incubation medium after depletion of endogenous mitochondrial substrates either by dinotrophin or small amounts of ADP. The designations “State 3” for ADP-stimulated respiration and “State 4” for ADP-limited respiration are those used by Chance and Williams (5). Respiratory control ratios RCR and ADP/O ratios were determined by the method of Klotz (6). The ratio of consumed oxygen to palmitoyl groups oxidized (\( \Delta O/\Delta P \)) was determined by dividing the amount of oxygen consumed (in nanogram atoms) in the complete oxidation of a small amount of palmitoyl-l-carnitine by the number of nanomoles of palmitoyl-l-carnitine added (7).

**Electron Microscopic Observations**—Mitochondrial pellets derived from pooled livers were fixed in cold 2% osmium tetroxide buffered with phosphate (8). The pellets were rinsed in saline and were soaked overnight in 0.5% uranyl acetate. After a second rinse in saline, the pellets were dehydrated in ethanol and embedded in Maraglas-D.E.R. 732 (9). Thin sections were sequentially stained with uranyl acetate (10) and lead tartrate (11) and examined in a Siemens Elmiskop I electron microscope.

**RESULTS**

**Gross Changes**

After an initial increase during the first 3 or 4 days, the average weight of the animals on the riboflavin-deficient diet did not change appreciably during the remainder of the 57-day study period. These riboflavin-deficient animals supplemented with galactoflavin showed no initial weight gain, but maintained a constant weight for 21 days. During the 1st week, the livers of riboflavin-deficient animals were infiltrated with fat, resulting in a decreased mitochondrial yield per g of tissue. In preparations made after 7 days of riboflavin deprivation, the yield of mitochondrial protein per g of liver was higher from experimental animals than from controls. After approximately 6 weeks on the experimental diet, the riboflavin-deficient animals showed the epidermal changes and hindquarter spasticity characteristic of severe ariboflavinosis (26). These changes were not accelerated in the galactoflavin group. The livers of the rats on the special diets were heavier per 100 g of body weight than were those of controls. Similar changes have been reported previously (2, 26-28).

**Electron Microscopic Observations**

Mitochondrial pellets obtained at Day 22 were typical in appearance and showed little evidence of contamination by other cellular constituents (Fig. 1). From the 4th week, a few mitochondria in the isolated preparations appeared to possess clongated cristae sometimces arranged in stacks that are similar to those in vivo organelles (Fig. 2).

**Mitochondria Integrity**

In all experiments, mitochondria from the experimental animals exhibited initial ADP/O ratios and respiratory control. Uncoupling of respiration with dinophrophenol neither relieved the depression of palmitoyl-carnitine oxidation nor affected the normal handling of glutamate. Table I, which presents data obtained using animals after 43 days on the experimental diet, shows that even at an advanced stage of ariboflavinosis, when the clinical syndrome was already apparent and there were marked reductions in some respiratory capacities, the mitochondrial respiratory control and ADP/O ratios were unchanged.

**Substrate Oxidation**

**Substrates Undergoing \( \beta \) Oxidation**—A sharp decrease in oxidation rates of long chain and intermediate chain fatty acid substrates was noted in all experiments using livers from riboflavin-deficient animals (Fig. 3). The rate of decline was most rapid during the 1st week of the experiment and continued at a slower pace throughout the balance of the experiment. After 8 weeks, more than 60% of the ability to oxidize fatty acids was lost. This defect was consistently seen in all three series of experiments. The depression was independent of the transport mechanism for the fatty acid substrate since similar results were obtained using enzymatically transported substrates, palmitoyl-CoA and palmitoylcarnitine, and a freely permeable, intermediate chain fatty acid, hexanoate (Fig. 3).

The changes were not due to an alteration in the final product of incubation. When ratios of consumed oxygen to added palmitoyl groups were determined in incubations containing malate, both control and deficient mitochondria showed ratios of 20 to 24, indicating that citrate was the chief product in both instances (Table II). Direct measurement of citrate formation after 21 days of riboflavin deprivation confirmed this finding by showing that 96 and 89% of the two carbon units added as palmitate could be accounted for by increased citrate concentration during incubations with control and experimental mitochondria, respectively.

We further investigated these phenomena by partially isolating the acyl-CoA dehydrogenase step of \( \beta \) oxidation, which...
is flavin-dependent and does not use NAD\(^+\) as an electron acceptor. Using the method proposed by Bremer and Davis (29), mitochondria were incubated with palmitoylcarnitine in the presence of rotenone and oxalacetate. This rate was greatly depressed in mitochondria from riboflavin-deficient animals. Results from test animals after 21 days of riboflavin deprivation and from controls are shown in Table II and are typical of the findings on all days. Incubations with each preparation of mitochondria exhibited the same rate of palmitoyl group utilization under both conditions described in the table.

**Substrates That Do Not Undergo \(\beta\) Oxidation**—The ox-

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Animals</th>
<th>Oxygen consumption State 3</th>
<th>State 4</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 (\mu)M palmitoyl-l-carnitine + 2.5 mm 1-malate</td>
<td>Control</td>
<td>71.4</td>
<td>15.6</td>
<td>4.6</td>
<td>2.20</td>
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<td></td>
<td>B(_2)-deficient</td>
<td>27.2</td>
<td>5.4</td>
<td>5.0</td>
<td>2.21</td>
</tr>
<tr>
<td>10 mm glutamate</td>
<td>Control</td>
<td>57.5</td>
<td>10.9</td>
<td>5.3</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>B(_2)-deficient</td>
<td>42.3</td>
<td>1.2</td>
<td>20</td>
<td>2.71</td>
</tr>
<tr>
<td>10 mm pyruvate</td>
<td>Control</td>
<td>34.9</td>
<td>1.2</td>
<td>20</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>B(_2)-deficient</td>
<td>31.9</td>
<td>5.7</td>
<td>5.6</td>
<td>3.12</td>
</tr>
<tr>
<td>20 mm (\beta)-hydroxybutyrate</td>
<td>Control</td>
<td>43.9</td>
<td>6.6</td>
<td>6.8</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>B(_2)-deficient</td>
<td>26.7</td>
<td>1.9</td>
<td>13.8</td>
<td>2.96</td>
</tr>
<tr>
<td>10 mm succinate + 3.75 (\mu)M rotenone</td>
<td>Control</td>
<td>103.4</td>
<td>16.0</td>
<td>6.5</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>B(_2)-deficient</td>
<td>77.8</td>
<td>27.7</td>
<td>2.8</td>
<td>1.49</td>
</tr>
</tbody>
</table>
Oxidative Metabolism in Riboflavin Deficiency

**Fig. 3.** Fatty acid oxidation by rat liver mitochondria during riboflavin deficiency. Experimental details are described in the text. The rates during riboflavin deficiency (O) are compared to those obtained from chow-fed littermate controls (C). The substrate concentrations are: (a) 40 μM palmitoyl-l-carnitine, 2.5 mM L-malate, and 2.5 mM ADP; (b) 0.4 mM hexanoate, 2.5 mM L-malate, 1 mM ATP, 2.5 μg of oligomycin/ml, and 100 μM dinitrophenol; (c) 8.6 μM palmitoyl-l-carnitine, 2.5 mM L-malate, and 100 μM dinitrophenol; and (d) 8.6 μM palmitoyl-l-carnitine, 10 mM oxalacetate, 3.75 μM rotenone, and 2.5 mM ADP.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Animals</th>
<th>Oxygen consumption</th>
<th>ΔO/ΔP</th>
<th>Rate of palmitoyl utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6 μM palmitoyl-l-carnitine + 2.5 mM L-malate + 100 μM dinitrophenol</td>
<td>Control</td>
<td>120.2</td>
<td>20.7</td>
<td>6.04</td>
</tr>
<tr>
<td>8.6 μM palmitoyl-l-carnitine + 10 mM oxalacetate + 3.75 μM rotenone + 2.5 mM ADP</td>
<td>B6-deficient</td>
<td>40.2</td>
<td>6.95</td>
<td>2.66</td>
</tr>
</tbody>
</table>

**Fig. 4.** Oxidation of non-fatty acid substrates by rat liver mitochondria during riboflavin deficiency. Experimental details are in the text. The rates during riboflavin deficiency (O) are compared to those obtained from chow-fed littermate controls (C). The substrate concentrations are: (a) 10 mM L-glutamate and 100 μM dinitrophenol; (b) 10 mM L-glutamate and 100 μM ADP; (c) 20 mM D,L-β-hydroxybutyrate and 103 μM ADP; (d) 10 mM succinate, 3.75 μM rotenone, and 103 μM ADP; (e) 10 mM pyruvate, 2.5 mM L-malate, and 103 μM ADP; and (f) 10 mM α-ketoglutarate, 10 mM malonate, and 100 μM dinitrophenol.

Riboflavin Deficiency Compared to Starvation—The early changes produced by riboflavin deficiency were not seen in animals starved for a similar time period. Table III compares data obtained during the first 2 days of both starvation and vitamin deprivation. It is apparent that only riboflavin deprivation in the presence of food intake produced the alterations seen.

Mitochondria Enzymatic Activities

The oxidation of fatty acids involves the series of reactions diagrammed in Scheme 1, which uses palmitoyl carnitine as an example. The activity of carnitine palmitoyltransferase, which is involved in a transfer of long chain acyl groups from the

Riboflavin deficiency compared to starvation—The early changes produced by riboflavin deficiency were not seen in animals starved for a similar time period. Table III compares data obtained during the first 2 days of both starvation and vitamin deprivation. It is apparent that only riboflavin deprivation in the presence of food intake produced the alterations seen.

Mitochondria Enzymatic Activities

The oxidation of fatty acids involves the series of reactions diagrammed in Scheme 1, which uses palmitoyl carnitine as an example. The activity of carnitine palmitoyltransferase, which is involved in a transfer of long chain acyl groups from the

Controls for long term deficiency studies were ad libitum fed rats. Burch et al. (2) have previously demonstrated that in hepatic mitochondrial oxidation experiments related to riboflavin deficiency there are no significant differences between controls fed ad libitum diet and those that were pair-fed.
Oxidative Metabolism in Riboflavin Deficiency

Extramitochondrial space to the mitochondrial matrix, was unaltered in the early stages of deficiency but underwent a 2-fold increase in specific activity by the 2nd week of deficiency (Fig. 5). The dehydrogenation of acyl-CoA involves three enzymes; to determine the specific activities of these enzymes, we employed four different substrates (Fig. 6). In the case of the controls fed ad libitum, there was a marked alteration in activity with time for each substrate; other types of controls were not monitored after 3 days. The dehydrogenation of palmitoyl-CoA was relatively stable for the first 2 weeks postweaning; between the 2nd and 3rd week, the specific activity decreased about 40%, remaining at this level for the rest of the experiment (Fig. 6a). The specific activity of lauroyl-CoA and octanoyl-CoA dehydrogenase showed a transient increase at the end of the 1st week, returned to its original level by the 3rd week, and then rapidly increased to a level substantially above its previous high (Fig. 6, b and c). The specific activity of butyryl-CoA dehydrogenase practically tripled in the 1st week after weaning and then exhibited minor fluctuations during the next 5 weeks (Fig. 6d).

After 1 day of riboflavin deficiency, the specific activity of palmitoyl-CoA dehydrogenation was slightly lower than that in controls. There was a precipitous decline during the next day and then no further significant changes during the next 40 days although the means tended to slowly decrease. Lauroyl-CoA and octanoyl-CoA dehydrogenase activity was significantly depressed on the 2nd day of deficiency, was unchanged over the next 4 weeks, and then increased. Butyryl-CoA dehydrogenase activity was depressed at the earliest time point and showed a transient rise that did not keep pace with that of controls; then it fell to extremely low levels at 3 and 4 weeks of deficiency and finally returned to initial levels at 6 weeks. To ensure that the early changes in the acyl-CoA

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**Fig. 5.** Carnitine palmitoyltransferase activity in rat liver mitochondria during riboflavin deficiency. Experimental details are described in the text. The rates during riboflavin deficiency (●) are compared to those obtained from chow-fed littermate controls (○). Data are expressed as mean ± S.D. (n = 3).

**Fig. 6.** Acyl-CoA dehydrogenase activity by rat liver mitochondria during riboflavin deficiency. Experimental details are given in the text. The rates during riboflavin deficiency (●) are compared to those obtained from chow-fed littermate controls (○). Data are expressed as mean ± S.D. (n = 3). a, palmitoyl-CoA; b, 200 μM dodecanoyl-CoA; c, 200 μM octanoyl-CoA; and d, 200 μM butyryl-CoA.

**Fig. 7.** Mitochondrial enzymatic activities of β-oxidation. Experimental details are given in the text. The rates during riboflavin deficiency (●) are compared to those obtained from chow-fed littermate controls (○). Data are expressed as mean ± S.D. (n = 3). a, enoyl-CoA hydratase; b, β-hydroxyacyl-CoA dehydrogenase; and c, β-ketothiolase activities.
TABLE III
Comparison of the effects of 48 h of either starvation or riboflavin deprivation on uncoupled respiration in rat liver mitochondria

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Control</th>
<th>Starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 mm hexanoate + 1 mm ATP + 2.5 μg of oligomycin/ml + 2.5 mm L-malate</td>
<td>64.3</td>
<td>56.1</td>
</tr>
<tr>
<td>10 mm α-ketoglutarate + 10 mm malonate</td>
<td>23.2</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Citrate synthase in control rat liver mitochondria in these experiments did not fluctuate greatly during the observation period. Initially the activity was slightly depressed in the deficient rats and returned to normal by 2 weeks (Fig. 8a).

In control rats, succinate dehydrogenase activity was doubled by the 5th day after weaning, then progressively declined to initial rates by the 28th day, and then rose once again (Fig. 8b). In contrast to the controls, the increase in succinate dehydrogenase-specific activity during the 1st week of deficiency was severely limited. From this point, the activity fell and then began to rise (Fig. 8b).

DISCUSSION

The data in this paper, in association with previously reported experiments on the riboflavin-deficient mouse (30, 31), point out the importance of assaying for disturbances in biochemical function throughout the period required to produce a severe deficiency state. While Beyer et al. (3) demonstrated that the flavin content of hepatic mitochondria decreased 50% during the 1st week of riboflavin deprivation and changed only slowly thereafter, virtually all other biochemical studies have dealt chiefly with animals deficient more than 8 weeks.

The earliest and most significant biochemical change produced by riboflavin deprivation was a severe lessening of the ability to oxidize fatty acids. Both long chain and intermediate chain fatty acids, which enter the mitochondria by different mechanisms and which are converted to intramitochondrial acyl-CoA's by different enzymes, yielded the same pattern of depressed respiration when used as substrates by the riboflavin-deficient rat hepatic mitochondria. Experiments we will report in a separate communication show that the levels of free CoA within these mitochondria were not decreased. These findings suggest that the defect in fatty acid oxidation lies either in faulty β oxidation or in citrate formation. The normal ΔO/ΔP ratios and measured total citrate formation, the normal citrate synthase activity, and the near normal oxidation of pyruvate, which also proceeds via acetyl-CoA to citrate, indicate that β oxidation is the principal process altered in the early stages of riboflavin deficiency. When mitochondria are incubated with rotenone, which inhibits NADH oxidation, the rate of palmitoyl group utilization is depressed to the same extent as is overall β oxidation, indicating that dehydrogenation of acyl-CoA is the most vulnerable step to riboflavinosis.

The dehydrogenation of acyl-CoA is catalyzed by three enzymes with different chain length specificity, all containing the flavin, FAD, as the prosthetic group (32). The electron acceptor for the dehydrogenase is another flavoenzyme, electron transfer flavoprotein, which also requires FAD (33). Fig. 9 summarizes the relationship between oxidation of palmitoylcarnitine and acyl-CoA dehydrogenase activity. The activities of palmitoyl-, dodecanoyl-, and octanoyl-CoA dehydrogenase fall initially and appear to reach a plateau at the same time that the rate of palmitoylcarnitine oxidation is falling. In contrast, the decline in the rate of butyryl-CoA dehydrogenase is more rapid than the decline in the rate of β oxidation of palmitoylcarnitine. Based on these findings, it seems reasonable to conclude that the step involving butyryl-CoA dehydrogenase is the most susceptible to riboflavin deficiency. This effect is quite pronounced at 3 weeks. During the 1st week, however, there is no differential response among the three acyl-CoA dehydrogenases. The decrease in oxidation of palmitoylcarnitine cannot be totally explained by a corresponding decrease in a single acyl-CoA dehydrogenase activity, imply-
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ketoglutarate oxidation was unchanged. Beyer et al. (2) reported that a-ketoglutarate oxidation was decreased 50% during riboflavin deficiency. In our present study, no changes were noted in respiratory control ratios or in oxidative phosphorylation as measured by ADP/O ratios. In contrast, using these same techniques, marked disturbances in both parameters have been observed in mice that were severely riboflavin-deficient (31). It is possible that this species difference in mitochondrial behavior is the basis for the longer survival of the rat after riboflavin deprivation.

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REFERENCES

Riboflavin and rat hepatic cell structure and function. Mitochondrial oxidative metabolism in deficiency states.
C Hoppel, J P DiMarco and B Tandler


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