

Liberation of $^{14}\text{CO}_2$ from [^{14}C]adipic acid and [^{14}C]octanoic acid by adult rats during riboflavin deficiency and its reversal

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The purpose of the present study was to test the hypothesis that the already well-established mitochondrial lesion in fatty acid oxidation in riboflavin-deficient experimental animals, might be accompanied by an alteration *in vivo* in the kinetics of oxidation of labelled adipic acid. This dicarboxylic acid was chosen for testing as a metabolic probe because a block in its oxidation was already apparent from urine analysis of riboflavin-deficient animals, whereas the oxidation of medium- or long-chain monocarboxylic acids seemed to be little affected by deficiency *in vivo*. Female adult Norwegian hooded rats fed on purified diets containing either 15 mg riboflavin/kg diet (controls) or about 0.4 mg/kg (riboflavin-deficient) received an intragastric dose of either [1,6- ^{14}C]adipic acid or [1- ^{14}C]octanoic acid. Expired carbon dioxide was then collected in an alkaline trap over 3 h, for determination of radioactivity. This test was repeated at intervals for up to 2 weeks following riboflavin repletion of the deficient animals, and in riboflavin-dosed controls. Whereas the rate and extent of [^{14}C]octanoic acid oxidation was not significantly affected by the deficiency or repletion, the extent of [^{14}C]adipic acid oxidation was markedly and significantly increased during repletion of the deficient animals. The time-course indicated a temporary overshoot, followed by a slow return to the control values over 1–2 weeks. Adipate oxidation was also much less affected by a preceding period of overnight starvation, than was octanoate oxidation. Thus, adipic acid (or a related metabolic probe) may have appropriate properties for the design of a functional test of fatty acid oxidation efficiency, during riboflavin deficiency or allied metabolic conditions in human subjects.

Fatty acid oxidation: Riboflavin deficiency: Rat

Observations with isolated mitochondria from riboflavin-deficient rats have shown clearly that a deficiency of this vitamin can produce a rapid and far-reaching decrease in fatty acid oxidation (Hoppel *et al.* 1979; Olpin & Bates, 1982; Sakurai *et al.* 1982; Duerden & Bates, 1985*b*; Veitch *et al.* 1985, 1988; Brady *et al.* 1986, 1988; Ross & Hoppel, 1987). Likewise, there is evidence from studies of urinary fatty acid metabolites in riboflavin-deficient rats, that the metabolic disturbance of fatty acid oxidation pathways has important implications for the living animal (Goodman, 1981; Gregersen & Kolvraa, 1982; Draye *et al.* 1988). Paradoxically, however, the production of labelled carbon dioxide from medium- and long-chain monocarboxylic fatty acids in riboflavin-deficient rat pups was not found to be significantly affected by riboflavin deficiency *in vivo* (Patterson, 1987; Patterson & Bates, 1989).

During the search for a more responsive probe, *in vivo*, evidence from a variety of sources suggested that the oxidation of dicarboxylic acids might possess characteristics which could enable them to fulfil this role (Mortensen & Gregersen, 1982; Vamecq *et al.* 1985*b*; Draye *et al.* 1988).

One clue was that dicarboxylic acids, or their closely related metabolites, appear consistently in the urine of riboflavin-deficient animals (Goodman, 1981; Gregersen & Kolvraa, 1982; Draye *et al.* 1988), suggesting that an important metabolic block exists in their catabolism, during riboflavin deficiency. Another was that dicarboxylic acids are

inherently less readily metabolized than monocarboxylic acids (Preiss & Block, 1964). The competition between their oxidation and urinary excretion routes should, therefore, reflect quite moderate changes in the efficiency of the oxidative pathway.

The purpose of the present study was to compare the responses to riboflavin repletion between the oxidation of adipic acid (a typical medium-chain dicarboxylic acid) and of octanoic acid (a typical medium-chain monocarboxylic acid).

MATERIALS AND METHODS

Animals and diets

Female DNL (Norwegian) hooded rats, mean starting body-weight 157 g, were housed individually in suspended wire cages at 22°, with a 12 h light–12 h dark cycle. Coprophagy was minimized by wide-meshed wire cage floors. The composition of the basal diet was (g/kg): sucrose 706, casein 210, arachis oil 30, mineral mixture 50, choline chloride 2, cystine 1.5, thiamin 0.004, pyridoxine hydrochloride 0.009, niacin 0.025, pteroylglutamic acid 0.001, cyanocobalamin 5×10^{-5} , biotin 0.001, calcium pantothenate 0.02, retinyl acetate 2.1×10^{-3} retinol equivalents, α -tocopherol 0.25, cholecalciferol 7.5×10^{-6} , menadione 0.009. The mineral mixture (Greenfield *et al.* 1969) comprised (g/kg): CaHPO_4 325, CaCO_3 205, KCl 205, Na_2HPO_4 185, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 70, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 4.5, $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$ 4.125, ZnCO_3 0.75, CuSO_4 0.375, KIO_3 0.25. The riboflavin content of this 'deficient' diet was about 0.4 mg/kg, from the casein component (Duerden & Bates, 1985*a*). This proved to be just sufficient for maintenance (without growth) of adult female rats. The control, riboflavin-supplemented diet contained additional riboflavin: 15 mg/kg diet which, in the author's previous experience, was sufficient to support maximum growth rates and saturation of riboflavin-requiring enzymes. Eight animals as controls and six as 'deficients' were maintained on these diets for 13 months, all fed *ad lib*.

Measurement of $^{14}\text{CO}_2$ liberation from ^{14}C -labelled fatty acids, and repletion with riboflavin

The labelled fatty acids were: (a) [1,6- ^{14}C]adipic acid (obtained from Amersham International plc, Amersham, Bucks) which was diluted with unlabelled adipic acid and then neutralized with sodium hydroxide (to pH 7.0) to give 0.5 μCi and 87 μmol (12.7 mg) adipic acid/ml aqueous solution; and (b) [1- ^{14}C]octanoic acid, sodium salt (obtained from New England Nuclear Research Products, Stevenage, Herts) which was diluted with unlabelled octanoic acid and then neutralized with sodium hydroxide, to give 0.2 μCi and 868 μmol (125 mg) octanoic acid/ml aqueous solution.

Except where otherwise indicated, each animal was fasted overnight before the breath test. It was then lightly anaesthetized with diethyl ether, and was given an intragastric dose of labelled fatty acid: 2 ml/kg body-weight CO_2 in expired air was collected quantitatively during the subsequent 3 h, by passing air (flow-rate 1.5 litres/min) through a sealed Perspex chamber containing the animal and passing the outflow through 100 ml of a solution containing (ml/l): ethanolamine 40, methanol 250, water 710. The CO_2 trap solution was changed every 20 min, and 5-ml portions were then assayed for radioactivity in a scintillation counter, with automatic external quench correction. The total ^{14}C in each 100 ml was converted to μmol fatty acid oxidized. Except where otherwise stated the quantitative comparisons between groups were based on the cumulative production of $^{14}\text{CO}_2$ between 0 and 80 min after dosing, and were by Student's *t* test, since normal distributions were observed. To obtain an approximate estimate of relative urinary excretion of labelled material, the total ^{14}C content of urine voided during the 3 h period was also measured.

Following a pre-repletion series of fatty acid oxidation measurements, both groups of animals received an intragastric dose of flavin mononucleotide, 10 mg/kg body-weight, in water. This was estimated as being sufficient to achieve saturation of the tissues within a short period after dosing, and the riboflavin-deficient group thereafter received the control diet. Breath tests were repeated at intervals following repletion. One deficient and two control animals were killed before repletion; the remaining five in each group were killed at 30 d after repletion, to measure their liver:body-weight values.

Estimation of riboflavin status by erythrocyte NAD(P):glutathione oxidoreductase (EC 1.6.4.2)

Blood was collected from the tail vein under diethyl ether anaesthesia, shortly before repletion. Another sample was collected by cardiac puncture when the animals were killed. Erythrocyte NAD(P):glutathione oxidoreductase activation coefficients were measured on a Cobas Bio centrifugal analyser, as previously described (Powers *et al.* 1983).

RESULTS

Body-weight changes, and liver:body-weight ratios

During development of riboflavin deficiency the mean body-weight of the animals receiving the deficient diet remained nearly constant (162 (SE 3.4) g, *n* 6) at the start and 177 (SE 8.2) g after 13 months) whereas the controls increased from 153 (SE 3.1) g (*n* 7) at the start to 323 (SE 6.5) g after 13 months. The deficient group remained apparently healthy, however, apart from some deterioration in the condition of the fur.

During the 4 weeks following repletion the control animals gained about 1 g/week, whereas the previously deficient animals gained about 18 g/week, and their fur condition improved rapidly, with the exception of one animal which responded atypically in certain other ways (see pp. 557–558).

Shortly before repletion, one deficient animal was found to have a liver:body-weight ratio of 0.056, whereas two control animals had a value of 0.028. At 4 weeks after repletion five previously-deficient animals had a mean liver:body-weight ratio of 0.045 (SE 0.0027) compared with 0.033 (SE 0.0017) for five controls; this difference was significant (*t* test) at $P < 0.01$.

Riboflavin status

Just before repletion, six deficient animals had a mean NAD(P):glutathione oxidoreductase activation coefficient of 1.86 (SE 0.14) while the seven control animals had a mean value of 1.17 (SE 0.03), this difference being significant (*t* test) at $P < 0.001$. The five animals in each group which were killed 30 d after repletion had mean activation coefficients of 1.16 (SE 0.14) (previously deficient) and 1.24 (SE 0.18) (controls) respectively.

Oxidation of adipic acid

Fig. 1(a) shows that an overnight fast had very little influence on the extent and pattern of adipic acid oxidation, in contrast to the corresponding patterns with octanoic acid (Fig. 1(b)) which were profoundly affected by an overnight fast (control animals).

Before repletion, the cumulative recovery of the adipic acid dose between 0 and 80 min after dosing was 9.0 (SE 1.6)% of that administered to five deficient animals, and 9.4 (SE 0.4)% of that administered to five control animals. These values did not differ significantly (*t* test, $P > 0.05$). Fig. 2(a) illustrates the effect of riboflavin repletion of the deficient group on the pattern of oxidation of adipic acid, compared with a parallel time-course for the control group (Fig. 2(b)). Whereas the control values varied over only a small

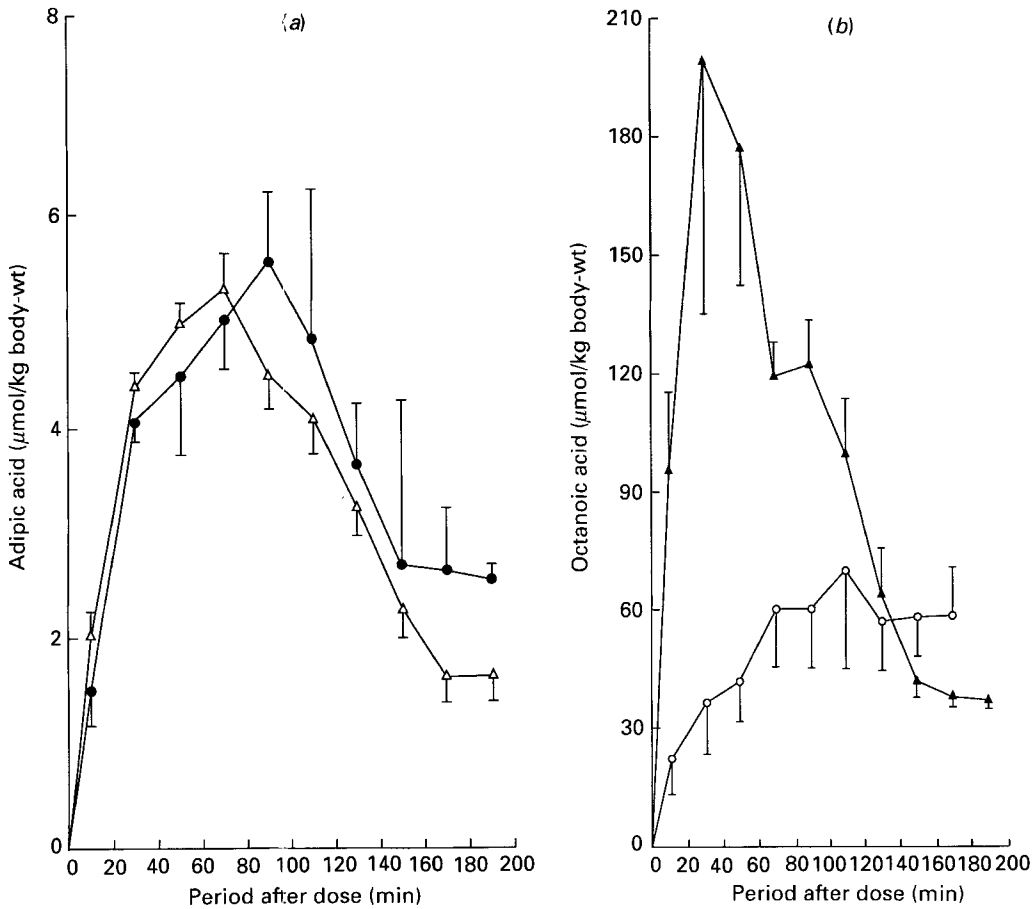


Fig. 1. Effect of a 16 h (overnight) fast on the patterns of oxidation of [^{14}C]adipic acid and [^{14}C]octanoic acid in control rats. Fatty acid oxidized ($\mu\text{mol}/\text{kg}$ body-weight) during each 20 min period was calculated from the appearance of ^{14}C in expired air and is plotted against the midpoint times (min) of $^{14}\text{CO}_2$ samples. Each measurement represents a 20 min collection period. Values are means with their standard errors represented by vertical bars.

(a) Oxidation of [^{14}C]adipic acid to $^{14}\text{CO}_2$: (\triangle — \triangle), after an overnight fast (n 3); (\bullet — \bullet), without an overnight fast (n 6). Mean values for fasted and non-fasted animals (cumulative $^{14}\text{CO}_2$ liberated, 0–80 min) were not significantly different: t 1.54, $P > 0.05$.

(b) Oxidation of [^{14}C]octanoic acid to $^{14}\text{CO}_2$: (\blacktriangle — \blacktriangle), after an overnight fast (n 8); (\circ — \circ), without an overnight fast (n 3). Mean values for fasted and non-fasted animals (cumulative $^{14}\text{CO}_2$ liberated, 0–80 min) were significantly different: t 3.49, $P < 0.01$.

range, the deficient group showed a massive increase in rate of oxidation within 1 d after repletion, followed by a gradual decline during the following 2 weeks. Little change took place thereafter (not shown). The differences between pre- and post-repletion results were significant (t test, $P < 0.001$) at 1 d post-repletion, and they remained significant ($P < 0.05$) until 3 d post-repletion. The increased rate was not a reflection simply of increased growth rate, since the animals continued to grow rapidly even after the initially increased oxidation rate had subsided.

The mean percentage of dose excreted in the urine during the 3 h $^{14}\text{CO}_2$ collection period (excluding the small proportion of animals which did not urinate) was 39.8 (SE 3.3) % for

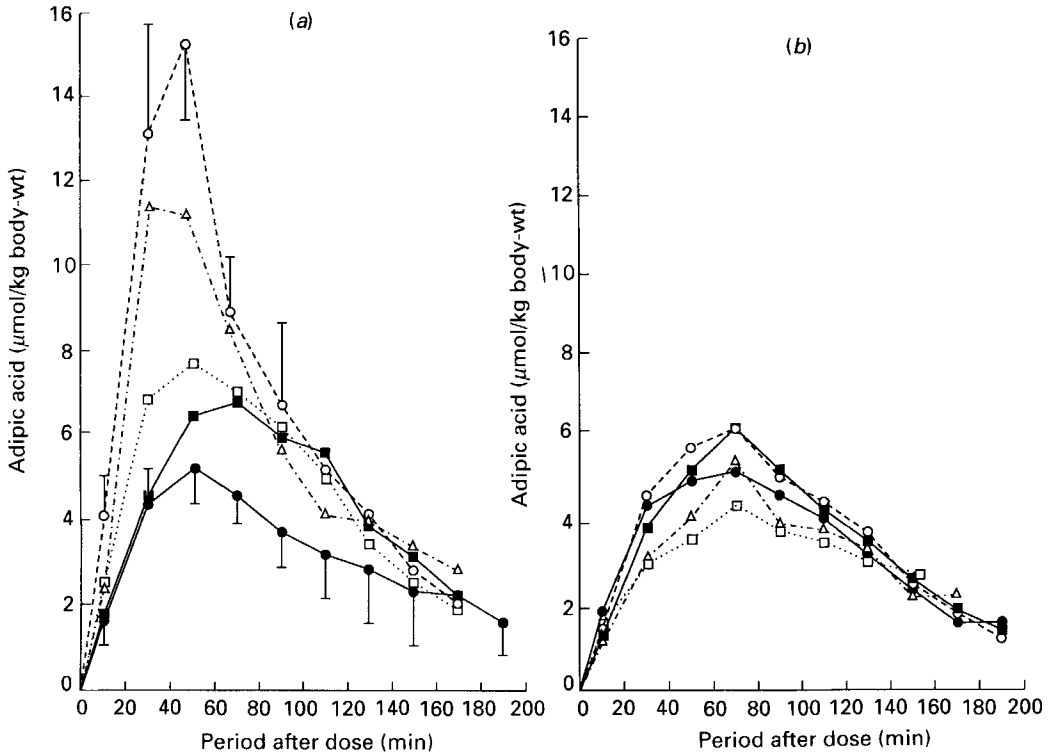


Fig. 2. Changes in $^{14}\text{CO}_2$ production from [^{14}C]adipic acid during repletion of riboflavin-deficient rats. Adipic acid oxidized ($\mu\text{mol/kg body-weight}$) during each 20 min period is plotted against the midpoint times of $^{14}\text{CO}_2$ samples. Oxidation values are means, with individual standard errors represented by vertical bars where space permits. The pooled SE for all time points before repletion for the deficient animals was 0.49 (n 5); for the control animals before repletion it was 0.16 (n 8).

(a) Deficient animals during repletion: before repletion (n 5) (●—●), and 24 h (n 3) (○---○), 3 d (n 2) (Δ ···· Δ), 8 d (n 4) (\square ···· \square) and 14 d (n 5) (■—■) after riboflavin dosing. Mean values for cumulative $^{14}\text{CO}_2$ were significantly different between pre-repletion and post-repletion values at the following time-points only: at 24 h, t 3.74, P < 0.025; at 3 d, t 2.71, P < 0.05.

(b) Control animals treated similarly: before repletion (n 8) (●—●), and 1 d (n 3) (○---○), 3 d (n 4) (Δ ···· Δ), 8 d (n 5) (\square ···· \square) and 14 d (n 5) (■—■) after riboflavin dosing.

twenty-seven measurements on control animals; 39.3 (SE 9.0) % for seven measurements on deficient animals and 32.3 (SE 6.5) % for ten measurements on repleted deficient animals. Clearly, as expected, urinary excretion was a major pathway of disposition of adipic acid, but in the present study, no significant differences emerged between control and deficient animals, nor was the excretion pattern changed markedly during the early stages of repletion of the deficient animals.

Two riboflavin-deficient animals exhibited an anomalous metabolic response towards the [^{14}C]adipic acid probe, at different points during the present study. In both cases, the extent of oxidation suddenly fell to a very low value (less than 3% of the dose being oxidized during the 3 h collection period). One of these two animals (non-repleted) died soon after the measurement, and this was the only animal which succumbed to the riboflavin deficiency. A second animal (after repletion), whose body-weight had responded only slowly to the repletion schedule, also showed a temporary decline in its adipic acid oxidation capacity, to about 1% of the dose in two adjacent measurements. However, it

then recovered rapidly and completely in growth and its adipic acid metabolism. Thus, a sudden fall in the utilization of adipic acid may herald a serious failure in metabolic stability, from which the animal may or may not recover. These anomalously low values were excluded from the analyses.

Oxidation of octanoic acid

Cumulative recovery of the octanoic acid dose between 0 and 80 min after dosing was 29.4 (SE 6.3) % of the dose for six control animals and 32.6 (SE 4.3) % of the dose for three non-repleted deficient animals; these values did not differ significantly (*t* test, $P > 0.05$).

Fig. 3 illustrates the effect of riboflavin repletion on the pattern of oxidation of octanoic acid. No significant change in extent of oxidation was observed following repletion. The areas under the curves for the deficient group were generally greater per unit body-weight than for those of the control group (not shown), which is consistent with their larger liver:body-weight ratio. This difference did not diminish during the 4 week observation period following repletion.

The mean percentage of the [^{14}C]octanoic acid dose which was excreted in the urine during the 3 h $^{14}\text{CO}_2$ collection period was 0.79 (SE 0.15) % for eight measurements on control animals, and 1.06 (SE 0.38) % for seven measurements on deficient animals. Clearly the urinary disposition route is a very minor one for octanoic acid, unlike adipic acid. There was no significant difference between control and deficient animals (*t* test, $P > 0.05$).

DISCUSSION

Choice of the amount of fatty acid given in each dose was dictated partly by the need to achieve an easily measurable, but less than complete, oxidation of the dose during a convenient (3 h) measurement period. The greater inherent rate of oxidation of octanoic acid than of adipic acid dictated the larger molar dose of the former (Bates, 1989).

The results of the present study are fully consistent with previously reported observations (Patterson, 1987; Bates, 1989; Patterson & Bates, 1989), which have suggested that the oxidation rate of adipic acid can reflect the course of recovery from riboflavin deficiency, whereas medium-chain monocarboxylic acids such as octanoic acid and long-chain monocarboxylic acids such as palmitic acid cannot. In the present study, the oxidation of octanoic acid was compared directly in parallel with that of adipic acid, in a group of adult rats during recovery from a long-term, moderately severe period of riboflavin deficiency.

When the acyl-CoA dehydrogenases of β -oxidation which had been depleted of their essential flavin cofactors during riboflavin deficiency, were restored by riboflavin repletion, there was a massive increase in extent of oxidation of adipic acid, followed by a gradual return towards the lower rate seen in already riboflavin-replete (control) animals. In a related study (Bates, 1989) the repletion of riboflavin-deficient animals consistently resulted in an increase in adipic acid oxidation rate, whereas their oxidation rate before repletion exhibited a less clearcut difference from the control group. One possible explanation for this less-consistent reduction before repletion could be the stimulation of adaptive mechanisms to offset the deleterious effects of riboflavin deficiency. One such adaptation is the increase in relative liver weight which invariably occurs in riboflavin-deficient animals.

The observed time-course of the changes in adipic acid oxidation suggested a rapid restoration of riboflavin-sensitive oxidation mechanisms, followed by a slower adjustment in which some spare capacity for oxidation was eliminated. The mechanism of this re-adjustment is not known.

Although the repletion changes with the octanoic acid probe showed an apparent trend in the same direction as those with adipic acid, they failed to reach statistical significance.

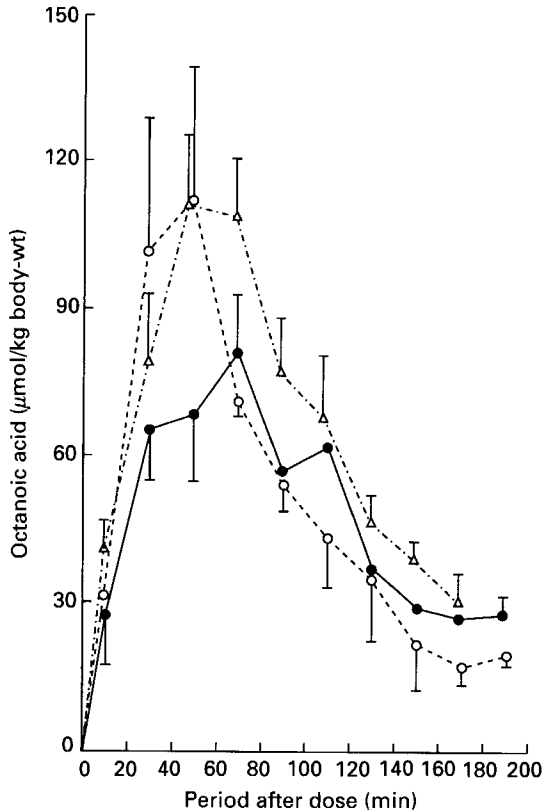


Fig. 3. Changes in $^{14}\text{CO}_2$ production from [^{14}C]octanoic acid during repletion of riboflavin-deficient rats. Octanoic acid oxidized ($\mu\text{mol}/\text{kg}$ body-weight) during each 20 min period is plotted against the midpoint times (min) of $^{14}\text{CO}_2$ samples. Oxidation values are means, with individual standard errors represented by vertical bars where space permits. The pooled SE for all time points before repletion was 1.01.

Mean values for cumulative $^{14}\text{CO}_2$ were not significantly different between pre-repletion and post-repletion values at the following time-points: before repletion (n 3) (●—●), and 1-3 d (n 3) (○---○) and 28 d (n 5) (△- - -△) after riboflavin dosing.

One practical problem encountered with octanoic acid was its much greater sensitivity to pre-dose fasting than was seen with adipic acid (Fig. 1). Mortensen & Gregersen (1982) likewise found only a small effect of fasting on the whole-body oxidation rate for adipic acid; however, the reason for this contrast is not entirely clear. A much greater coefficient of variation of oxidation rates was observed between matched (control) animals when using the octanoic probe, than with adipic acid. Others (Brady *et al.* 1986, 1988; Ross & Hoppel, 1987) have reported a partial reversal of the lowering of acyl-CoA dehydrogenase activity in riboflavin-deficient rat mitochondria following 24-48 h of starvation. For this and other reasons, the fact that adipic acid oxidation in control animals is comparatively insensitive to fasting could prove important for the design of a fatty acid oxidation probe for animals, and human subjects.

In contrast to its clearcut effect on mitochondrial fatty acid oxidation, the effect of riboflavin deficiency on peroxisomal fatty acid oxidation is less certain. Sakurai *et al.* (1982) observed no effect of riboflavin deficiency on peroxisomal oxidation; Veitch & van Hoof (1985) reported that riboflavin-deficient animals possess 'very few' hepatic peroxisomes,

but Draye *et al.* (1988) recorded an increase in hepatic peroxisomal dicarboxyl-CoA oxidase activity in riboflavin-deficient animals. From the observations and suggestions of Vamecq *et al.* (1985a), Kolvraa & Gregersen (1986) and Draye *et al.* (1988), it appears that a possible route for the oxidation of medium-chain dicarboxylic acids, such as glutaric acid, adipic acid and suberic acid, could be (1) peroxisomal conversion to CoA esters and thence carnitine esters, (2) transfer of the carnitine esters to the mitochondria, followed by (3) mitochondrial β -oxidation to CO₂. This complex pathway is necessary, apparently, because the peroxisomes are unable to oxidize dicarboxylates beyond the chain-length of adipic acid (Kolvraa & Gregersen, 1986). Stimulation of the mitochondrial segment by restoration of riboflavin might thus have a major influence on adipic acid, whereas for octanoic acid, other factors may be rate-limiting.

It is difficult to predict precisely how different dicarboxylic acids will respond to riboflavin deficiency *in vivo*, and this clearly needs further study. Increased urinary excretion of dicarboxylic acids, including that of adipic acid, has been reported during riboflavin deficiency, and during several other types of metabolic stress, in rats (Goodman, 1981; Mortensen, 1981; Gregersen & Kolvraa, 1982; Mortensen & Gregersen, 1982; Bergseth *et al.* 1988; Draye *et al.* 1988). Draye *et al.* (1988) found that the excretion of short- and medium-chain dicarboxylic acids in urine was increased during riboflavin deficiency, when dodecane-dioic acid or hexadecane-dioic acid were used as metabolic probes. No studies of riboflavin repletion were reported by them, however. From their studies, adipic and suberic acids differ from longer-chain dicarboxylic acids in the efficiency with which their CoA and carnitine esters are formed in the peroxisomes, and this may also help to determine their suitability as metabolic probes.

The development of probes for fatty acid oxidation defects in riboflavin-deficiency, or in other nutritional insults which affect fatty acid oxidation, has considerable potential for human investigations. The use of non-radioactive, ¹³C-labelled fatty acid probes and simple breath tests with mass spectrometric analysis of ¹³C:¹²C ratios, permits the measurement of these pathways in a non-invasive and ethically acceptable manner. Recent developments in isotope-ratio mass-spectrometry instrumentation for biological studies promise to expedite this approach. Riboflavin-deficient populations (Bates, 1987), riboflavin-dependent subjects with inborn errors of fatty acid metabolism (Gregersen *et al.* 1982, 1986; Harpey *et al.* 1983; Mooy *et al.* 1984; Green *et al.* 1985; Gregersen, 1985; de Visser *et al.* 1986), and infants at increased risk of sudden infant death syndrome due to an abnormality of hepatic acyl CoA dehydrogenases (Howat *et al.* 1985; Allison, 1987), could benefit from such investigations.

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