[^{13}C]adipic acid as a probe of fatty acid oxidation in human subjects: feasibility study and pilot trial of correction of endemic riboflavin deficiency in The Gambia

Christopher J. Bates, Glynis Edwards,* Robert Downes,† and Andrew Coward

Medical Research Council, Dunn Nutrition Unit, Cambridge, UK, and Keneba, The Gambia, West Africa

Fatty acid catabolism was explored in vivo in human subjects, by a breath [^{13}C]CO_2 test, following a test oral dose of [^{13}C]adipic acid (1,4-butane dicarboxylic acid), 5 mg (34.2 μmol)/kg body weight. Peak enrichment of breath CO_2 occurred around 100 min after the dose, in both adults and schoolchildren. The percentage of the dose which was oxidized within 200 min was greater in the children (mean 11.5%) than in the adults (mean 9.9%). This difference was accounted for by the greater overall CO_2 production rate per unit body weight (or surface area) in the children. The percentage oxidized by lactating women (mean, 10.6%) was greater than that oxidized by pregnant women (mean 8.4%). Correction of endemic riboflavin deficiency, by supplementation with 5 to 10 mg riboflavin per day for 2½ weeks, had no significant overall effect on the rate or amount of [^{13}C]adipic acid oxidized, although a paradoxical result was obtained in one subgroup (schoolgirls). The minimum detectable supplementation effect, averaged over all 89 subjects, would have been ± 14%, for significance at the 5% probability level. Correction of naturally occurring endemic riboflavin deficiency in Gambian subjects thus appears to have no easily detectable effect on their ability to oxidize [^{13}C]adipic acid to [^{13}C]CO_2. Further studies will be needed to seek possible transient supplementation effects, effects in younger subjects, or effects which breath [^{13}C]CO_2 analyses, after oral dosing, may fail to detect. The [^{13}C]fatty acid probe-procedure appears to be acceptably reproducible, and may be applicable to the study of other nutrition-related functional indices.

Keywords: human; riboflavin deficiency; fat oxidation; adipic acid; carbon-13; mass spectrometry

Introduction

The use of [^{13}C]labelled substrates to probe nutritionally sensitive biochemical pathways in human subjects in vivo has major apparent advantages in being non-invasive, safe, and ethically acceptable. Moreover, breath [^{13}C]CO_2 analyses, in particular, are operationally simple, are easily replicated, and can be made very sensitive to small degrees of CO_2 enrichment, even by commercially available isotope-ratio mass spectrometers.[1,2] Important unresolved questions are: Can they reflect the competence of key metabolic pathways at nutrionally sensitive sites?, What are their limitations, in terms of metabolic complexity, biological sensitivity, and reproducibility?, and Can the correction of endemic nutrient deficiencies produce significant changes as proof of in vivo functional benefits?

As part of an ongoing program to address such questions, a study was undertaken of [^{13}C]adipic acid (1,4-butane dicarboxylic acid) catabolism to [^{13}C]CO_2, before and after the correction of endemic riboflavin deficiency, in rural Gambian women and school-

Address reprint requests to Christopher J. Bates, Medical Research Council, Dunn Nutrition Unit, Milton Road, Cambridge, CB4 1XJ, UK.
* Current address: Middlesex Hospital Medical School, Mortimer Street, London, UK.
† Current address: Department of Community Child Health, Newcastle General Hospital, Newcastle-upon-Tyne, UK.
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children. The choice of [^{13}C]adipic acid as a probe of fatty acid catabolism was prompted by recent studies in riboflavin-deficient rats,[3,4] in which it was shown that fatty acid catabolism to CO_2 in vivo was much more responsive if adipic acid (a dicarboxylic acid) was used as the probe, than if a monocarboxylic acid, such as octanoic acid, was substituted.

The choice of Gambian subjects for the human study described here was prompted by our observations of consistent biochemical evidence of riboflavin deficiency, and the evidence of clinical signs of such a deficiency in this community.[5,6,7] The Gambian study was preceded by a feasibility study on a single caucasian subject, in the United Kingdom.

Subjects, materials, and methods

Subjects

Pilot studies were carried out on a well-nourished male subject aged 50 years, in Cambridge, UK. Gambian subjects comprised 48 school children (22 boys; 26 girls) aged 8 to 12 years, and 41 pregnant or lactating women, aged 17 to 40 years, who were living in the rural villages of Keneba and Manduar in the West Kiang region of The Gambia. Of the women, 27 were lactating at the outset of the study, and their infants were aged 0 to 6 months. Fourteen of the women were pregnant (3rd trimester) at the outset, and one of these delivered during the course of the study. Since this subject could not be assigned unequivocally to either the pregnant or the lactating group, her data have been excluded from *Tables 1-4*. The time of year was late November to early December, two months after the end of the rainy season, when previous studies have noted a clear-cut peak in the prevalence of clinical signs of riboflavin deficiency.

Materials

[1,6-$^{13}C_2$]adipic acid, 99% enrichment, was purchased from Isotec Inc, Miamisburg, OH, USA. The manufacturer's claim of > 99% [^{13}C] in carbons 1 and 6 was verified by mass spectrometry of a 1:400 wt/wt dilution of the enriched with unenriched material. This yielded a fractional abundance of 0.010832 [^{13}C] in the unenriched material, and 0.363 [^{13}C] (expected = 0.341) in the enriched material, after correction for the dilution. Tablets containing 5 mg (children) or 10 mg (adults) riboflavin, and matching placebo (lactose) tablets, were donated kindly by Hoffmann-La Roche, Basel, Switzerland.

Methods

Subjects were allocated to the active and placebo tablet groups, so as to achieve a matched distribution based on sex, category (child, pregnant or lactating women), village of domicile, body weight, and age. The pregnant women living in Keneba were receiving a food supplement[8] which contributed ca. 1 mg/d to their riboflavin intake.[9]

Two breath-test sessions, each following an oral dose of [^{13}C]adipic acid, were performed for each subject: one just before the start of the riboflavin supplementation period in late November 1988, and one after 2½ weeks of riboflavin supplementation in mid-December. In addition, four of the subjects attended a breath-test session without the [^{13}C] dose, in order to monitor typical background [^{13}C]enrichment fluctuations, during the 200-min period of testing.

The subjects attended breath-test sessions in the Keneba clinic in small groups during the early morning. No food was permitted during the test, but drinking water was permitted. Physical activity was kept to a minimum, and most of the subjects elected to sit or lie down during the sessions. None of the subjects moved from the room used for the test, except possibly to visit the toilet, which was about 30 yards distant, and the size of the room also ensured that their movement was restricted. It is estimated that none of the subjects deviated more than 15% from the mean in this respect. At the start of a session, each subject received an oral dose of [^{13}C]adipic acid, diluted 1:3 with unenriched adipic acid (Sigma, London, UK), at a total dose of 5 mg (34.2 μmol)/kg body weight. The adipic acid was dissolved in water with simultaneous adjustment to neutral pH by sodium hydroxide, and it was given as a drink (with 50 ml Coca-Cola to improve its palatability). At precisely timed (10 min) intervals, just before, and for 200 min following the dose, 20 ml end-expired breath samples were collected, and were stored in Becton-Dickinson "Vacutainers" for shipping to the United Kingdom. Breath-collection was achieved by asking the subject to blow into a "party blower" device* which enabled the terminal segment of the breath to be retained, and then transferred via a syringe to the Vacutainer. Room temperature and barometric pressure were recorded each day.

In addition to these small-volume breath collections for [^{13}C]enrichment of expired CO_2, timed Douglas bag collections of expired air were made, usually three times at ca 60-min intervals during the session, for the measurement of total CO_2 production rates. The concentration of CO_2 in these samples was measured on-site with an infra-red CO_2 analyzer, and the volume of expired air in the bag was measured by a gas-meter.[10] From these measurements, the molar amount of carbon dioxide at STP in the expired breath, over the 200-min period of testing, was calculated.

During each breath-test session, a heparinized venous or finger-prick blood sample was collected from each subject and was separated into plasma and (saline-washed) red cells. The plasma samples were used subsequently (in Cambridge) for the measurement of free carnitine[11] and the red cells were used for the measurement of riboflavin status, by the activation coefficient of erythrocyte glutathione reductase.[12] Spot urine samples were collected also, at the post-supplement sessions only and were stored frozen, for the measurement of hydroxyproline: creatinine ratios

* (Wiggins HS, personal communication.)

in Cambridge. Creatinine was assayed by the Jaffé procedure, on a Roche Cobas centrifugal analyzer, by a Roche creatinine kit, cat. no. 1421. Hydroxyproline was analyzed on the same centrifugal analyzer, after acid hydrolysis, by a procedure similar to that of Ho and Pang,[13] except that the oxidation step and the chromophore formation were performed by manual addition and heating, before transfer to the analyzer for the final measurement.

The riboflavin (or placebo) tablets were given on a single-blind basis to each subject daily, on six days a week for 2½ weeks, and were seen to be swallowed. ^{13}C-enrichment of the breath samples was measured in Cambridge with a VG SIRA 10 isotope ratio mass spectrometer, related to reference CO_2 gas which had been calibrated against Pee Dee belemnite. This permitted the calculation of μmol $^{13}CO_2$ expired, using each individual's mean CO_2 production rate and the relationship between δ^{13}PDB‰ and percent ^{13}C given by Schoeller et al.[14] The result was then expressed as a percentage of the calculated amount of $^{13}CO_2$ obtainable by complete oxidation of the dose, to give the % of the dose which had been oxidized in 200 min.

Statistical significance of differences between groups was analyzed by one-way ANOVA, followed by Students *t* test, or by paired *t* test, as appropriate.

Ethical permission for the study was granted by the MRC Gambian Ethical Committee, and for the pilot work in Cambridge, by the Dunn Nutrition Unit's Ethical Committee.

Results

Pilot study of [^{13}C]CO_2 production from [^{13}C]adipic acid in Cambridge

The preparations for the Gambian study included a series of trial breath collections after [^{13}C]adipic acid dosing, in a 50-year-old caucasian male subject.

A study of baseline enrichment in the absence of ^{13}C-dose indicated a characteristic diurnal fluctuation of around 3‰, in which the enrichment was highest during the morning and fell progressively throughout the day and night, returning again by early the next morning (*Figure 1*). However, over a 3 to 4 hour period in the morning, the steady drift and fluctuations were within 0.3‰. Following an oral dose containing 1.7 mg (11.6 μmol) [^{13}C]adipic acid (99% enrichment) per kg body-wt, the change in enrichment above the predose value increased progressively to a maximum value of δ^{13}PDB of ca. 9‰ at around 100 min, and then fell to about half this value by 200 min (*Figure 1*). If collections were continued throughout the day, the enrichment continued to decline, but with temporary increases following meals. Periods of physical activity consistently produced a temporary decline in the enrichment, presumably attributable to dilution of the [^{13}C]CO_2 released from the [^{13}C]adipic acid, by unenriched CO_2 arising from oxidation of endogenous substrates in muscular tissues.

When the [^{13}C] adipic acid dose was diluted by increasing amounts of unenriched adipic acid, and the total adipic acid dose thereby was increased by a factor of 6 (i.e., from 103 ± 1.2 SE mg to 604 ± 1.2 SE mg), then the peak became broader, and the % oxidized in 200 min decreased from 14.7 ± 1.05 SE (14 runs) to 6.8 ± 0.58% (7 runs). A small positive enrichment was observed when Coca-Cola alone was substituted for the [^{13}C]adipic acid dose. This contributed ca. 15% of the observed ^{13}C response, and would have been similar for both riboflavin-supplemented and unsupplemented subjects. The results obtained from the Gambian subjects have been corrected for this contribution.

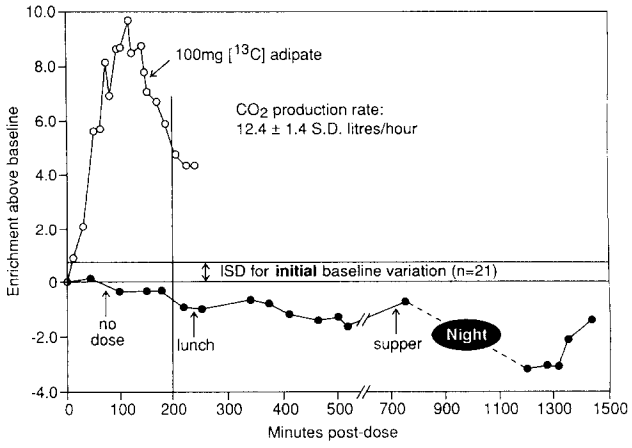

Figure 1 Baseline variation, and response to [^{13}C]adipate, in the single control subject in Cambridge. The units for the values for [^{13}C]enrichment above baseline are δ relative to Pee Dee belemnite (δ^{13}PDB‰). In the absence of any [^{13}C]dose, there was a slow fall in enrichment during daylight hr and most of the night, followed by a rapid increase soon after rising in the morning. The [^{13}C]dose was always given between 8 and 9 A.M. The mean baseline variation (one standard deviation) for 21 experiments is shown, together with a typical enrichment time-curve in one experiment, after dosing with 100 mg (685 μmol) [^{13}C]adipate. The mean CO_2 production rate in this experiment was 12.4 ± 1.4 SD L/hr, and the mean % of dose oxidized during 200 min for 16 experiments on the control subject was 13.19 ± 3.5 SD % of the 100 mg dose.

The main requirements of the Gambian study were perceived to be: a) a period of relative inactivity and fasting following the [^{13}C] dose; b) metabolic stress on the pathways of fatty acid catabolism (which is likely to increase with an increasing weight of adipic acid dose); and c) the minimum [^{13}C] dose for adequate precision of measurement above background fluctuations (the latter being due mainly to natural fluctuations of breath [^{13}C]CO_2 enrichment and very little to measurement error). The conditions chosen to optimize these factors were: a dose of 5 mg adipic acid/kg body weight of which 1.67 mg was the 99% enriched [^{13}C]labelled material, and a collection period of 200 min. Any major effects of the riboflavin supplement on adipic acid oxidation might then be observed, either as a change in elapsed time to peak enrichment, or as a change in the area under the time versus enrichment curve (or % of dose oxidized). The possible disadvantages of a relatively short (200 min) collection period were justified by considerations of subject coopera-

tion, ethical acceptability, and by ensuring a sufficiently large subject sample size (89 subjects). For the caucasian subject, the intra-subject coefficient of variation was 27% (but there was no indication that a smaller CV would have been achieved if the collection period had been doubled or even tripled). [^{13}C]enrichment measurements on urine samples collected after dosing indicated that a significant proportion of the [^{13}C]adipic acid dose which was not oxidized to [^{13}C]CO_2, was excreted rapidly in the urine. This corresponds with previous experience using rats.[3,4]

Gambian study

General characteristics of the Gambian treatment groups

Table 1 shows that the two treatment groups were matched closely with respect to a number of characteristics which might be thought to affect the efficiency of fatty acid oxidation. About three-quarters of the subjects were from Keneba; the ages of the children were between 8 and 12 years; those of the women were between 17 and 40 years. About three-quarters of the women were lactating during the study and were between 0 and 6 months post-partum; the remainder were in the final trimester of pregnancy.

Effect of riboflavin supplementation on riboflavin status

The data in *Table 2* shows that all the sub-groups of subjects who received the active tablets responded by a highly significant improvement (reduction) in their biochemical index of riboflavin status. Indeed, every supplemented subject showed a reduction, whereas none of the placebo groups showed any significant status changes. This confirms that both compliance and status response to the supplementary riboflavin were excellent, (as had been the case in all our previous riboflavin-supplementation studies in this community). Of the women, those with the poorest response to the riboflavin supplement tended to be those who were nearest to parturition (correlation coefficient for quadratic regression of time versus EGRAC = −0.59, N = 7), which also matches the conclusions of our previous studies.

Seven of the children and 13 of the women were found to exhibit oral lesions similar to those associated with clinical riboflavin deficiency. There was no significant difference between these 20 subjects and the rest, with respect to their biochemical indices of riboflavin status, and by chance they were almost equally distributed between the active and placebo supplementation groups (11 to the active and 9 to the placebo group).

Table 1 General characteristics and initial matching of the two treatment groups

Characteristic	Active tablets		Placebo	
	Mean	SE	Mean	SE
Children: *N*	25		23	
Male (%)	48		43	
Age (yr)	10.3	0.2	10.2	0.3
Body weight (kg)	25.0	0.9	24.8	0.8
From Keneba (%)[a]	76		83	
Women: *N*	19		21	
Lactating (%)	68		74	
Months post-partum	2.6	0.5	2.4	0.5
Age (yr)	31.3	1.2	29.0	1.5
Body weight (kg)	52.3	1.8	54.1	1.6
From Keneba (%)[a]	62		74	

[a] The remainder were from the nearby village of Manduar.

Rates of total CO_2 production: comparison between groups, and effects of supplementation

Table 3 compares groups of subjects, before and after supplementation, for total CO_2 production by the Douglas bag analyses. There was no consistent or significant change, attributable to the riboflavin supplement.

Some clear differences in CO_2 production-rate per kg body weight or per m^2 of surface area (calculated by the Dubois equation), existed between the groups of subjects. The highest rates of CO_2 production were seen in the children, although no significant difference was found between the boys and the girls. The overall difference between children and adult women was highly significant (Student's t test: $P < 0.001$) and the clear exponential relationship with body weight is shown in *Figure 2*. CO_2 production rate per kg decreased progressively with increasing body weight or surface area; moreover, the pregnant women had significantly lower production rates than the lactating women ($P < 0.025$).

Conversion of [^{13}C] adipic acid to [^{13}C]CO_2: comparison between groups, and effect of supplementation

The median inter-session differences in pre-dose baseline values of [^{13}C]enrichment (δ^{13}PDB), without respect to sign, within subjects were 0.64‰ (children) and 0.68‰ (women).

Four subjects were followed for the 200-min test period, without being given any [^{13}C] dose. Fluctuations in their CO_2 enrichment around their baseline (predose) levels were small, and in no case exceeded 5% of the mean area under the curves obtained in the dosed subjects.

Table 4 compares groups and sub-groups of subjects before and after supplementation, with respect to recovery of [^{13}C] in [^{13}C]CO_2 in the breath samples (calculated from the area under the [^{13}C] enrichment curves and the mean total CO_2 production rates for each subject at each test session).

Comparisons between subject-groups revealed a highly significant difference (t = 3.30, 86 df, $P <$ 0.005) between the overall mean % oxidation in children (11.54 ± 0.38 SEM) and that of the women (9.88 ± 0.33). This difference is similar to the difference in

Table 2 Riboflavin status by the erythrocyte glutathione reductase test, before and after supplementation with the riboflavin or placebo tablets

Subject group	Activation coefficient of erythrocyte glutathione reductase (ratio)							
	Active tablets				Placebo			
	Before supplement		After supplement		Before supplement		After supplement	
	Mean	SE (*N*)	Mean	SE (*N*)	Mean	SE (*N*)	Mean	SE (*N*)
All children	1.67	0.04 (25)	1.16[a]	0.01 (25)	1.73	0.04 (23)	1.69	0.04 (23)
Keneba children	1.69	0.05 (19)	1.15[a]	0.01 (19)	1.74	0.04 (19)	1.71	0.03 (19)
Manduar children	1.59	0.06 (6)	1.19[a]	0.04 (6)	1.66	0.09 (4)	1.58	0.13 (4)
All lactating women	1.86	0.08 (13)	1.24[a]	0.04 (13)	1.73	0.09 (14)	1.85	0.12 (14)
Keneba lactating	1.85	0.12 (8)	1.22[a]	0.03 (8)	1.75	0.12 (9)	1.87	0.16 (9)
Manduar lactating	1.86	0.04 (5)	1.29[a]	0.14 (5)	1.70	0.13 (5)	1.80	0.16 (5)
All pregnant women	1.81	0.09 (6)	1.27[a]	0.03 (6)	1.69	0.12 (7)	1.83	0.18 (7)
Keneba pregnant	1.86	0.09 (5)	1.28[a]	0.04 (5)	1.75	0.13 (6)	1.87	0.21 (6)
Manduar pregnant	1.55	(1)	1.23	(1)	1.30	(1)	1.58	(1)

[a] Significantly different from the corresponding group before supplementation (paired *t* test) $P < 0.005$.

Table 3 Rates of total CO_2 production

Subject group	CO_2 production rate (μmol per min, per m^2 surface area)							
	Active tablets				Placebo			
	Before supplement		After supplement		Before supplement		After supplement	
	Mean	SE (*N*)	Mean	SE (*N*)	Mean	SE (*N*)	Mean	SE (*N*)
All children	9731	324 (25)	10209	521 (25)	10104	424 (23)	10212	299 (23)
Boys	9909	540 (12)	11225	949 (12)	11106	603 (10)	10207	442 (10)
Girls	9544	390 (13)	9544	390 (13)	9334	510 (13)	10216	420 (13)
All lactating women	7372	472 (13)	7462	514 (13)	7480	507 (13)	7278	433 (13)
Keneba lactating	6803	471 (8)	7534	772 (8)	7213	711 (8)	7086	422 (8)
Manduar lactating	8233	792 (5)	7318	544 (5)	7902	664 (5)	7565	1011 (5)
All pregnant women	6229	229 (6)	6864	451 (6)	6141	595 (7)	6600	387 (7)

Before versus after supplement values by paired *t* test: $P > 0.05$, ns, for all groups and subgroups.
All women versus all children; mean values compared by Student *t* test: t = 7.9, 86 df, $P < 0.001$.
All pregnant versus lactating women, by Student *t* test: t = 2.35, 38 df, $P < 0.025$.

total CO_2 production rates between the women and children. There was no significant difference between the boys and the girls, but the lactating women (10.60 ± 0.50) were significantly different from the pregnant women (8.38 ± 0.60) (t = 2.84, 38 df, $P < 0.01$). This difference was also reflected in differences in the CO_2 production rates.

Comparisons between active and placebo groups revealed no overall difference in the change between pre- and post-supplement tests. If present, such a difference would have indicated an effect of the riboflavin supplement on the oxidation of the labelled adipic acid. The mean change between clinics for all subjects in the active tablet group was −0.54 ± 0.50%, and for all subjects in the placebo group it was −0.41 ± 0.47%. Thus, the mean difference between the intervention groups was only 1.8% of the overall mean % of dose oxidized, whereas a mean difference of 14% would have been required in order to achieve statistical significance at $P = 0.05$, by Student's *t* test.

Surprisingly, one subgroup (the girls) did exhibit a significant active-versus-placebo difference in this analysis (t = 3.2, 24 df, $P < 0.005$). Paradoxically, this difference represented a reduction in the % of the dose oxidized between sessions in the active-tablet group, compared to an increase in the % oxidized in the placebo group (which is the opposite of the predicted result). Offsetting this result, however, was a nearly significant difference in the other direction for the Keneba lactating women (t = 1.95, 16 df, $P < 0.1$).

Analyses based on the time from the dose to the peak $[^{13}C]CO_2$ production rate revealed no significant differences between the active and placebo groups, for any of the group comparisons described in the previous paragraph.

Plasma free carnitine, and urinary hydroxyproline/creatinine ratios

The data in *Table 5* (incomplete data-sets, because only incomplete sample-sets were available for these analyses) revealed no evidence of any significant supplementation effects for either plasma carnitine or uri-

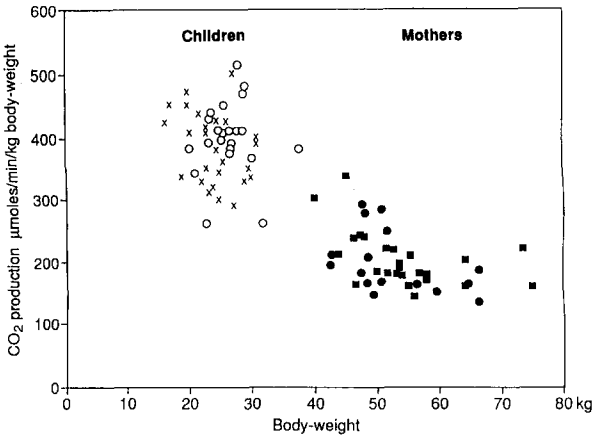

Figure 2 Relationship between total CO_2 production per kg body weight and body weight in Gambian subjects: X = girls; O = boys; ■ = lactating women; ● = pregnant women. See *Table 3* for group values and statistical comparisons, based on surface area calculations.

nary hydroxyproline:creatinine ratios, although the expected age-related differences (mothers versus children) were clearly apparent for both of these indices.

Discussion

Adipic acid is a minor product of the β-oxidation of fatty acids in normal subjects, but can become a major product, appearing in the urine of animals made riboflavin-deficient, and of human subjects with certain defects in the β-oxidation pathway.[4,15] Its formation probably involves both mitochondria and peroxisomes.[15]

The present pilot study has demonstrated that the use of [^{13}C]apidic acid, given orally to a human subject in order to monitor its oxidation to [^{13}C]CO_2 by breath-sampling, is a feasible and reproducible index, and that the amounts of substrate required are acceptable in terms of cost and palatability. These conclusions were confirmed and extended by the Gambian study. (In future studies, the use of an artifically

Table 4 Conversion of [^{13}C]adipic acid to [^{13}C]CO_2 during the 200 min test

	Percentage of [^{13}C]adipic acid dose oxidized and liberated as breath [^{13}C]CO_2 during the 200 min test							
	Active tablets				Placebo			
Subject group	Before supplement Mean	SE (*N*)	After supplement Mean	SE (*N*)	Before supplement Mean	SE (*N*)	After supplement Mean	SE (*N*)
All subjects	10.86	0.48 (44)	10.32	0.44 (44)	10.70	0.49 (44)	10.29	0.49 (44)
All children	11.63	0.66 (25)	10.50	0.68 (25)	11.44	0.64 (23)	11.64	0.64 (23)
Boys	11.45	1.31 (12)	11.72	0.96 (12)	12.21	1.18 (10)	10.72	1.02 (10)
Girls	11.80	0.76 (13)	9.38	0.88 (13)[a]	10.85	0.69 (13)	12.34	0.80 (13)
All lactating women	10.22	0.89 (13)	10.61	0.64 (13)	10.98	0.84 (14)	10.83	1.00 (14)
Keneba lactating	9.08	0.97 (8)	10.47	0.97 (8)	10.31	0.45 (9)	11.40	1.42 (9)
Manduar lactating	12.04	1.49 (5)	10.84	0.74 (5)	10.46	0.76 (5)	9.79	1.16 (5)
All pregnant women	9.07	0.44 (6)	8.95	0.63 (6)	7.68	0.97 (7)	6.44	0.92 (7)
All subjects with mouth signs	12.79	1.03 (11)	10.73	0.70 (11)	9.94	1.14 (9)	8.14	1.20 (9)

Paired *t* test comparison between active and placebo groups, for the change between before- and after-supplement values: [a] $P < 0.005$. One way ANOVA applied to all groups for this change: F = 2.06 (9 + 79 df), $P < 0.05$.

Table 5 Plasma-free carnitine and urinary hydroxyproline/creatinine ratios

	Active tablets				Placebo			
Subject group	Before supplement Mean	SE (*N*)	After supplement Mean	SE (*N*)	Before supplement Mean	SE (*N*)	After supplement Mean	SE (*N*)
Plasma free carnitine (µmol/l)								
Children	33.1	3.2 (12)	28.8	1.9 (14)	26.2	3.7 (11)	24.9	2.2 (13)
Mothers[a]	20.5	1.8 (9)	25.2	2.8 (10)	20.2	2.2 (10)	21.3	2.4 (17)
Urinary hydroxyproline/creatinine (molar ratio)								
Children			0.196	0.016 (24)			0.209	0.017 (22)
Mothers[b]			0.072	0.005 (20)			0.081	0.015 (17)

Significant intergroup differences (children versus mothers) by Student *t* test: [a] t = 3.48, 94 df, $P < 0.001$, [b] t = 3.08, 81 df $P < 0.005$. No significant intergroup differences attributable to the riboflavin supplement (active versus placebo groups) in either index, by paired or unpaired *t* tests.

sweetened soft drink vehicle with low carbonation would be preferable in order to avoid unwanted baseline-disturbances.) The choice of a 200 min sampling period, under fasting conditions, proved to be an appropriate choice, but both meals and exercise clearly affected the enrichment of breath CO_2, after the [^{13}C]substrate dose. The proportion of the dose (mean, ca. 10%) that was oxidized in the 200 min sampling period was large enough for an acceptable accuracy and precision of measurement, but it was also small enough to ensure that the availability of substrate would not be an important limiting factor. Since previous studies on rats[3,4] had indicated clearly that an oral adipic acid dose, coupled with breath testing, was easily able to detect changes in fatty acid turnover that were associated with moderate riboflavin deficiency and repletion, the approach selected for the human study here, appeared promising, in comparison with an animal model, as well as with earlier in vitro studies.[16-18]

The initial riboflavin status (glutathione reductase test) values of the Gambian subjects confirmed that riboflavin deficiency was present and widespread, and the changes in this index after supplementation confirmed the efficacy of the supplementation schedule. The observation that 22% of the subjects showed oral lesions consistent with riboflavin deficiency was further evidence that the endemic riboflavin deficiency state was relatively severe.

The observation that the pregnant women from Keneba had very poor biochemical status, despite their receipt of the antenatal food supplement containing riboflavin, is likely to be due to the fact that they were all approaching parturition, when riboflavin demands are greatest, and that the study was performed at the "worst" time of the year for riboflavin deficiency.[19] The absence of a significant effect on plasma-free carnitine, and on urinary hydroxyproline/creatinine ratios, indicated that in the age groups studied here, these indices are not very riboflavin-sensitive. (Total carnitine might have been a preferable measurement here, but the amounts of plasma available did not permit it.) Previous studies,[20] which had identified hydroxyproline sensitivity to riboflavin status, were performed in much younger children, and in a different country.

The observation that the rate of total CO_2 production per kg body weight was strongly correlated with body weight, is probably attributable to changes, with age and total body weight, in the proportion of lean tissues. In addition, the women were slightly less active, and, therefore, nearer to their resting metabolic rates, than the children.

Overall, there was no indication that riboflavin repletion increased the measured indices of [^{13}C]-adipic acid oxidation: either in terms of % oxidized during the 200 min test period, or in the interval between dose and peak [^{13}C]CO_2-enrichment. The only sub-group which showed a significant supplement-versus-placebo difference was the schoolgirls, where the riboflavin supplement apparently produced a significant reduction in percentage of substrate oxidized compared with the placebo group. Since there was no a priori reason to expect such a result, and since other groups showed a trend in the opposite direction, it seems likely that this single unexpected difference, in a direction opposite from that predicted both by rat studies and by theoretical considerations, was a statistical artifact.

Since the results of the present study have indicated that an increase of 14% in the overall rate of substrate oxidation would have reached just conventional significance, whereas the overall observed difference was less than one-eighth of this amount, it appears that riboflavin-deficient human subjects may not, after all, exhibit the predicted response in their fatty acid oxidation rate after riboflavin-repletion. Some possibilities that need to be addressed in future studies are: a) that functional riboflavin sensitivity may be confined to a different age group (e.g., very young children, or to groups who become deficient as a result of different [e.g., non-dietary] insults); b) that any change in oxidation rate following riboflavin-repletion may be transient, as was apparent from the rat studies[3,4]; or c) that in humans, unlike rats, fatty acyl dehydrogenases may not be rate-limiting for fatty acid oxidation in vivo, so that major changes in dehydrogenation capacity can occur before any decrease in the overall rate of conversion of ingested substrate to breath CO_2 will be observed. Clearly, there are many stages between the two ends of this metabolic chain, and the breath-test procedure is limited in its capacity to probe the intermediate stages. It is also possible that CO_2 that is produced in different compartments in the body may be disposed of in different ways; some may be used more readily for reincorporation into macromolecules. Likewise, some of the adipic acid may be metabolized by pathways which do not result in the liberation of its ^{13}C as $^{13}CO_2$.

The results of the present study do, however, provide important new evidence to suggest that in humans, a riboflavin deficiency does not compromise adipic acid oxidation, at least at low levels of physical activity. This conclusion applies even to "high-risk" groups, such as pregnant and lactating women, and subjects with oral lesions. The use of ^{13}C-labelled fatty acid probes, and particularly the use of ^{13}C-labelled dicarboxylic acids, which can probe segments of the matabolic pathway which are less accessible to monocarboxylic acids,[3,4,5] may, however, prove useful in studying inborn errors of fatty acid metabolism, metabolic effects produced by drugs or training, or exercise-induced changes in fatty acid metabolism.

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