Flavin metabolism during respiratory infection in mice

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(*Received 3 July 1995 – Revised 6 November 1995 – Accepted 21 December 1995*)

Previous control studies carried out in children showed that respiratory infection alters riboflavin metabolism and leads to excessive urinary losses of the vitamin. In order to understand the nature of biochemical changes in riboflavin metabolism during respiratory infection, a study was carried out using the mouse as the experimental model, and *Klebsiella pneumoniae* as the infective organism. Mice were fed on either a low(0·5 mg/kg)- or high(13·3 mg/kg)-riboflavin semi-synthetic diet. Infection resulted in a 5–6-fold higher excretion of riboflavin in the urine of mice fed on the low-riboflavin diet. Higher erythrocyte FAD levels and lower liver FAD levels were also observed during infection. Of the four enzymes involved in the synthesis and breakdown of the flavin coenzymes studied, the activity of hepatic flavokinase (ATP: riboflavin 5′-phosphotransferase; *EC* 2.7.1.26) was significantly lower, and that of FAD synthetase (ATP: FMN adenylyltransferase; *EC* 2.7.7.2) was higher during riboflavin restriction and infection. The activity of FMN (acid) phosphatase (*EC* 3.1.3.2) was unchanged, whereas FAD (nucleotide) pyrophosphatase (*EC* 3.6.1.9) activity was significantly higher both with the low-riboflavin diet and during infection. Thyroid hormone is known to modulate flavokinase activity and, hence, thyroid status was assessed. Plasma triiodothyronine (T3) levels were not affected, but thyroxine levels were lower in the mice fed on the low-riboflavin diet. However, plasma T3 was significantly lower during infection, suggesting a mechanistic role for the hormone in the reduction of flavokinase activity.

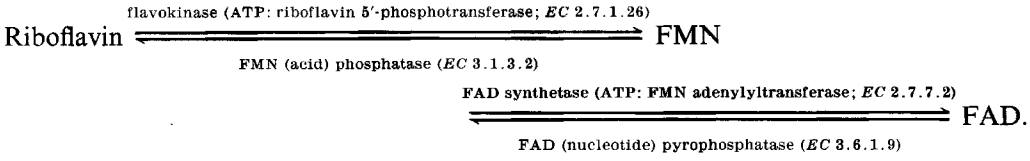
Riboflavin: Respiratory infection

Studies in developing countries, for example India, have revealed a very high incidence of biochemical riboflavin deficiency, particularly in women and children, as judged by the activation of erythrocyte glutathione reductase (*EC* 1.6.4.2; EGR) with its coenzyme, FAD (Thurnham *et al.* 1971; Bamji *et al.* 1979, 1981, 1982; Bates *et al.* 1981; World Health Organization Task Force and Oral Contraceptives, 1986). Investigations carried out with children suggest that the aetiology of riboflavin deficiency in this population may be more complex, and apart from low dietary intake of riboflavin, repeated respiratory infections may also play a role (Bamji *et al.* 1982).

A control study carried out using children with respiratory infection showed an increase in erythrocyte and urinary levels of riboflavin and a decrease in values for EGR activation coefficient (stimulated : basal activity; AC). However, after recovery from infection there was a deterioration in riboflavin status, as judged by the previously mentioned variables (Bamji *et al.* 1987). Similar changes in erythrocyte riboflavin and EGR-AC values were observed in a study carried out using mice infected with *Klebsiella pneumoniae* (Padmaja *et al.* 1991). In this study also there were changes in the activities of the riboflavin-dependent hepatic enzymes, acyl-CoA dehydrogenase (*EC* 1.3.99.3) and pyridoxamine-phosphate oxidase (*EC* 1.4.3.5).

* For reprints.

The objective of the present study was to determine whether the increase in blood and urinary levels of riboflavin observed during infection is due to changes in the synthesis and/or breakdown of the riboflavin coenzymes, FMN and FAD. The investigation was carried out in mice using *Klebsiella pneumoniae* as the infective organism. Activities of the enzymes involved in the synthesis and breakdown of riboflavin coenzymes (FMN and FAD) were measured in the liver during infection and after recovery.



Total flavins, free riboflavin, FMN and FAD levels in the erythrocytes and liver, and urinary excretion of riboflavin were also measured.

Since thyroid hormone is known to influence riboflavin metabolism by modulating flavokinase activity (Rivlin & Langdon, 1966; Lee & McCormick, 1985), thyroid-hormone status of the animals was assessed during infection and after recovery.

MATERIALS AND METHODS

Male weanling mice (128) of the Swiss/NIN strain (mean body weight 12 g) were divided equally into low- and high-riboflavin groups. The animals were housed individually in screen-bottomed cages. Care was taken to maintain the animals at optimal temperature (22–25°) and humidity ($55 \pm 10\%$), and a 12 h light–dark cycle. They were fed *ad libitum* on a purified diet containing (g/kg): sucrose 700, vitamin-free casein (Sigma Chemical Co., St Louis, MO, USA) 200, peanut oil 50, salt mixture 40 and vitamin mixture 10. The composition of the salt and vitamin mixtures was that described previously by Lakshmi *et al.* (1994). The riboflavin content of the low-riboflavin diet was 0·5 mg/kg. This level of riboflavin would be expected to simulate the human situation in developing countries where riboflavin is one of the limiting nutrients. The high-riboflavin diet contained 13·3 mg riboflavin/kg.

After 18 d of feeding the mice with the experimental diets, thirty-two animals in each group were infected with a sub-lethal dose ($6{\cdot}15 \times 10^6$ cells) of *Klebsiella pneumoniae* in 0·5 ml distilled water administered intraperitoneally. At this dose no deaths were observed in preliminary experiments carried out to determine the 50% lethal dose. The organism was isolated from the lung of the rat (NIN/WISTAR strain) and purified. The remaining mice in both groups received the same amount of distilled water intraperitoneally and they served as uninfected controls. Mice were kept in metabolism cages and urine was collected over toluene for 72 h.

At 72 h after injection the infected animals showed signs of acute infection, such as loss of appetite and reddening of the snout. At this stage, half the number of animals in the low- and high-riboflavin, uninfected and infected groups were killed by decapitation after withdrawing a sample of blood from the ocular plexus. The remaining animals were killed 15 d later when the infected animals had recovered completely. The liver was perfused with cold saline (9 g NaCl/l) to remove blood completely and then excised. Due to the paucity of material from a single mouse, blood, urine and tissues respectively of two animals from each group were pooled for a single sample. An homogenate (200 mg/ml) in potassium phosphate buffer (50 mM), pH 7·4, (prepared from 50 mM-K_2HPO_4 and 50 mM-KH_2PO_4) was prepared using a Potter-Elvehjem homogenizer.

Samples (erythrocytes and liver) for HPLC analysis were prepared according to the

method of Batey & Eckhert (1990) and the analysis for free riboflavin, FMN and FAD by the method of Ohkawa *et al.* (1983).

Erythrocyte and liver free riboflavin, FMN and FAD levels were measured by HPLC using fluorescence detection and a Shimadzu (Kyoto, Japan) LC-6A system with two pumps. Separation was carried out on a reversed-phase column (ODS; Showa, Denko K. K., Tokyo, Japan) of size 4·6 mm × 250 mm; 5 μm particle size. A guard column was used to protect the column. The solvents used were: A methanol, B methanol in 10 mM-NaH_2PO_4, pH 5·5, (300 ml/l); the buffer was filtered, mixed with methanol and degassed just before use. Elution was carried out using a linear gradient rising from 300 ml solvent B/litre solvent A to 860 ml solvent B/litre solvent A within 8 min, and this condition was maintained for 5 min. The flow-rate was 0·8 ml/min. The fluorescence intensities of the eluate were measured as excitation at 440 nm and emission at 530 nm using a Shimadzu RF-535 HPLC fluorescence spectrophotometer. The detector signal was recorded and the peak area quantified using a Shimadzu CR3A integrator. The retention times for free riboflavin, FMN and FAD were 8·0, 5·7 and 3·9 min respectively. The CV for this HPLC method for free riboflavin, FMN and FAD was 4·2, 3·14 and 4·6% respectively. The recoveries of the added free riboflavin, FMN and FAD ranged between 94 and 96%.

The EGR-AC in erythrocytes was measured by the method of Bayoumi & Rosalki (1976). Urinary riboflavin was measured fluorimetrically (Morell & Slater, 1946).

Enzyme assays

Flavokinase. Flavokinase activity was determined in the 18500 ***g*** supernatant fraction from liver homogenate as described by Merrill & McCormick (1980) with the following modifications: radioactive riboflavin was not used and the reaction time was 30 min. FMN formed was quantified by the differential-extraction method of Kearney & Englard (1951). Enzyme activity was expressed as nmol FMN formed/mg protein per 30 min at 37°. The CV for the flavokinase assay was 5·02%.

FAD synthetase. FAD synthetase activity was determined in the 100000 ***g*** supernatant fraction from liver homogenate according to the method of McCormick (1964). Quantification of FAD formed was done by the method of Ahmed *et al.* (1981) using apo D-amino acid oxidase (*EC* 1.4.3.3). Enzyme activity was expressed as nmol FAD formed/mg protein per 60 min at 37°. The precision of enzyme assay was 4·21%.

FMN (acid) phosphatase. Preparation of the enzyme was similar to that for flavokinase assay. Incubation of the enzyme with FMN was carried out according to the method of McCormick (1961). Riboflavin formed was quantified by the differential-extraction method of Kearney & Englard (1951). Enzyme activity was expressed as nmol riboflavin formed/mg protein per 30 min at 37°. The CV for this method was 3·2%.

FAD (nucleotide) pyrophosphatase. For reproducible quantification it was found necessary to purify the enzyme partially. A liver homogenate (200 mg/ml) was centrifuged at 800 ***g*** for 7 min to remove cell debris, and the supernatant fraction was centrifuged at 18000 ***g*** for 1 h. The enzyme was partially purified from the pellet and assayed using FAD as substrate (Krishnan & Appaji Rao, 1972). Enzyme activity was expressed as pmol FMN formed/mg protein per min at 37°. The precision of this enzyme assay was 3·9%.

Protein was estimated by the Folin-Lowry method (Lowry *et al.* 1951), urinary creatinine by the alkaline picrate method (Oser, 1965) and plasma triiodothyronine (T3) and thyroxine (T4) were measured using commercially-available kits from the Board of Radiation and Isotope Technology, Bombay, India.

Statistics

Values are presented as means with their standard errors. Homogeneity of variances was tested using the Bartlett test. If this test was found to be significant ($P < 0.05$), then the actual values were transformed into logarithmic values and tested by means of ANOVA with the least significant difference (LSD) multiple-range test. One-way ANOVA was carried out for urinary riboflavin and erythrocyte free riboflavin, FMN and FAD. Specific differences were identified by the LSD multiple-range test at a significance level of $P < 0.05$. The remaining results were compared by two-way ANOVA ($P < 0.05$) with the LSD multiple-range test (Snedecor & Cochran, 1967).

RESULTS

There was a non-significant reduction in feed intake on days 1 and 2 after infection. Infection did not significantly affect body weights of the low- and high-riboflavin-fed groups. However, riboflavin restriction *per se* reduced weight gain (Table 1).

Riboflavin, FMN and FAD

Urinary excretion of riboflavin was significantly higher in the high-riboflavin-fed group when compared with that of the low-riboflavin-fed group. Infection was associated with a 5–6-fold increase in urinary excretion of riboflavin in the low-riboflavin group (Table 1). Although there was a trend towards higher urinary excretion of riboflavin in the high-riboflavin infected group, the difference was not statistically significant, probably because of smaller sample size (due to physical constraints, only three pooled urine samples (six animals) could be collected from this group).

Erythrocyte FMN and FAD levels were significantly lower in the low-riboflavin-fed group when compared with those of the high-riboflavin-fed group. During infection, FAD and total flavin (free riboflavin, FMN and FAD) levels were significantly higher in erythrocytes from both low- and high-riboflavin-fed groups (Table 2).

After recovery from infection, only erythrocyte total flavins were measured and the values were similar to those of the corresponding uninfected groups (Table 2).

EGR-AC values reflected the changes observed in the erythrocyte FAD levels. In the lcow-riboflavin-fed group, EGR-AC values were lower during infection but after recovery from infection they were similar to those for the corresponding uninfected group. Infection had no effect on the EGR-AC values in the high-riboflavin-fed group (Table 2).

Hepatic free riboflavin, FMN and FAD levels at 3 weeks (period 1) were 58, 44 and 24% lower respectively in the mice fed on the low-riboflavin diet when compared with those for mice fed on the high-riboflavin diet (Table 3). Total flavin and FAD levels were significantly lower during infection in the low- and high-riboflavin-fed animals when compared with values for the corresponding controls (Table 3). Infection did not affect free riboflavin and FMN levels.

Liver FAD levels after recovery from infection were similar to those of the corresponding controls (Table 3), whereas total flavin levels continued to be lower in the low-riboflavin infected group when compared with those for the corresponding uninfected control group (Table 3).

Flavokinase

Flavokinase activity was 50% lower in the mice fed on the low-riboflavin diet compared with the high-riboflavin diet at 3 weeks (period 1). No further changes were observed at

Table 1. *Effect of infection with* Klebsiella pneumoniae *on body weight and urinary riboflavin of mice fed on high- and low-riboflavin diets**

(Mean values with their standard errors)

	Low riboflavin						High riboflavin					
	Uninfected			Infected			Uninfected			Infected		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Body wt (g)												
Period 1	16	$18{\cdot}67^{ax}$	0·48	16	$17{\cdot}78^{ax}$	0·48	16	$24{\cdot}03^{bx}$	0·52	16	$22{\cdot}86^{bx}$	0·32
Period 2	14	$21{\cdot}41^{ay}$	0·79	16	$20{\cdot}77^{ay}$	0·49	14	$28{\cdot}08^{by}$	0·75	14	$29{\cdot}28^{by}$	0·52
Urinary riboflavin (nmol/g creatinine)												
Period 1	6†	$4{\cdot}17^{a}$	0·40	6†	$24{\cdot}91^{b}$	5·28	3†	$51{\cdot}97^{c}$	2·82	3†	$62{\cdot}80^{c}$	3·80

[a, b, c] Mean values with unlike superscript letters were significantly different between groups ($P < 0{\cdot}05$).
[x, y] Mean values with unlike superscript letters were significantly different between periods ($P < 0{\cdot}05$).
Period 1, during peak period of infection; period 2, after recovery.
* For details of experimental procedures, see pp. 454–455.
† No. of pooled samples where each sample represents two animals.

Table 2. *Effect of infection with* Klebsiella pneumoniae *on erythrocyte flavin and the activation coefficient (stimulated: basal activity AC) of erythrocyte glutathione reductase (EC 1.6.4.2; EGR) in mice fed on high- and low-riboflavin diets**

(Mean values with their standard errors; *n* represents pooled samples where each sample represents two animals)

	Low riboflavin						High riboflavin					
	Uninfected			Infected			Uninfected			Infected		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Erythrocyte flavin												
Free riboflavin (nmol/l)												
Period 1	8	59·51	10·09	7	60·92	10·27	7	58·75	4·58	7	77·21	5·24
FMN (nmol/l)												
Period 1	8	$228{\cdot}03^{a}$	21·97	7	$247{\cdot}67^{a}$	23·66	7	$462{\cdot}96^{b}$	39·96	7	$486{\cdot}92^{b}$	12·71
FAD (nmol/l)												
Period 1	8	$344{\cdot}75^{a}$	9·18	7	$447{\cdot}15^{b}$	17·05	7	$504{\cdot}85^{c}$	21·10	7	$613{\cdot}05^{d}$	31·65
Total flavins (nmol/l)												
Period 1	8	$636{\cdot}41^{ax}$	34·27	7	$755{\cdot}73^{bx}$	32·13	7	$1023{\cdot}52^{c}$	46·49	7	$1192{\cdot}17^{d}$	52·86
Period 2	6	$923{\cdot}98^{ay}$	29·22	6	$864{\cdot}77^{ay}$	37·67	7	$1212{\cdot}42^{b}$	56·05	7	$1239{\cdot}90^{b}$	24·65
EGR-AC												
Period 1	5	$1{\cdot}25^{a}$	0·03	5	$1{\cdot}07^{b}$	0·02	5	$1{\cdot}08^{b}$	0·02	5	$1{\cdot}04^{b}$	0·02
Period 2	4	$1{\cdot}30^{a}$	0·04	5	$1{\cdot}21^{a}$	0·06	4	$1{\cdot}05^{b}$	0·04	4	$1{\cdot}06^{b}$	0·02

[a, b, c, d] Mean values with unlike superscript letters were significantly different between groups ($P < 0{\cdot}05$).
[x, y] Mean values with unlike superscript letters were significantly different between periods ($P < 0{\cdot}05$).
Period 1, during peak period of infection; period 2, after recovery.
* For details of experimental procedures, see pp. 454–455.

Table 3. *Effect of infection with* Klebsiella pneumoniae *on liver flavins of mice fed on high- and low-riboflavin diets**

(Mean values with their standard errors; *n* represents pooled samples where each sample represents two animals)

	Low riboflavin						High riboflavin					
	Uninfected			Infected			Uninfected			Infected		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Free riboflavin (nmol/g tissue)												
Period 1	7	1·43[a]	0·23	8	1·23[a]	0·09	8	3·45[b]	0·15	8	4·02[b]	0·24
Period 2	7	2·24[a]	0·25	7	2·12[a]	0·22	8	3·80[b]	0·57	8	3·38[b]	0·37
FMN (nmol/g tissue)												
Period 1	7	15·73[ax]	0·96	8	17·05[a]	1·90	8	28·09[b]	1·71	8	28·14[b]	2·25
Period 2	7	25·98[ay]	1·33	7	21·63[a]	0·75	8	34·20[b]	1·73	8	29·84[b]	0·91
FAD (nmol/g tissue)												
Period 1	7	34·10[a]	1·59	8	26·14[b]	1·11	8	44·78[c]	2·25	8	35·42[a]	1·93
Period 2	7	33·57[a]	1·06	7	31·29[a]	1·42	8	43·31[b]	2·31	8	40·95[b]	1·24
Total flavins (nmol/g tissue)												
Period 1	7	50·89[ax]	2·26	8	44·41[bx]	2·42	8	76·36[c]	3·30	8	67·60[d]	2·33
Period 2	7	62·01[ay]	3·40	7	55·05[by]	1·78	8	80·48[c]	1·89	8	74·18[c]	1·95

[a, b, c, d] Mean values with unlike superscript letters were significantly different between groups ($P < 0·05$).
[x, y] Mean values with unlike superscript letters were significantly different between periods ($P < 0·05$).
Period 1, during peak period of infection; period 2, after recovery.
* For details of experimental procedures, see pp. 454–455.

5 weeks (period 2). During infection, flavokinase activity of the low-riboflavin-fed mice was 42% lower than that of the corresponding uninfected mice. There was no comparable change in activity in the high-riboflavin-fed animals. After recovery from infection the flavokinase activity of the low-riboflavin-fed group was significantly higher than that of the corresponding uninfected group (Table 4).

FAD synthetase

Riboflavin restriction *per se* resulted in higher enzyme activity at 3 weeks as well as at 5 weeks (periods 1 and 2 respectively). During infection the FAD synthetase activity of the low-riboflavin-fed group was 70% higher and that of the high-riboflavin-fed group 90% higher than that of their respective uninfected control group. After recovery the enzyme activities were similar to those of the corresponding uninfected control groups (Table 4).

FMN (acid) phosphatase

The FMN (acid) phosphatase activity was not affected by either the riboflavin content of the diet or infection (Table 4).

FAD (nucleotide) pyrophosphatase

The enzyme activity was 30% higher in the low-riboflavin-fed group when compared with high-riboflavin-fed group at 3 weeks (period 1). There was an age-related rise in the activity

Table 4. *Effect of infection with* Klebsiella pneumoniae *on flavin-metabolizing enzymes in the liver of mice fed on high- and low-riboflavin diets**

(Mean values with their standard errors; *n* represents pooled samples where each sample represents two animals)

	Low riboflavin						High riboflavin					
	Uninfected			Infected			Uninfected			Infected		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Flavokinase† (units‡/mg protein)												
Period 1	8	5·14^{a}	0·43	8	3·00^{bx}	0·32	5	9·08^{c}	0·47	4	8·44^{c}	0·96
Period 2	6	5·05^{a}	0·63	5	6·77^{by}	0·29	4	10·10^{c}	0·52	3	10·58^{c}	0·38
FAD synthetase§ (units‖/mg protein)												
Period 1	8	1·35^{a}	0·13	7	2·31^{bx}	0·11	4	1·02^{c}	0·09	4	1·94^{d}	0·17
Period 2	5	1·37^{a}	0·08	5	1·20^{ay}	0·10	3	0·82^{b}	0·08	3	0·98^{ab}	0·07
FMN (acid) phosphatase¶ (units**/mg protein)												
Period 1	8	978·80	38·41	8	894·94	43·18	5	993·62	44·31	4	1043·60	78·41
Period 2	5	875·37	94·34	5	949·10	73·32	3	947·64	131·27	3	998·94	83·17
FAD (nucleotide) pyrophosphatase†† (units‡‡/mg protein)												
Period 1	6	7·35^{a}	0·41	7	11·11^{b}	0·81	7	5·51^{cx}	0·19	6	10·83^{b}	0·58
Period 2	7	9·79^{a}	0·56	7	11·17^{a}	0·53	6	11·49^{ay}	0·71	6	10·54^{a}	0·79

$^{a, b, c, d}$ Mean values with unlike superscript letters were significantly different between groups ($P < 0·05$).
$^{x, y}$ Mean values with unlike superscript letters were significantly different between periods ($P < 0·05$).
Period 1, during peak period of infection; period 2, after recovery.
* For details of experimental procedures, see pp. 454–455.
† ATP: riboflavin 5′-phosphotransferase (*EC* 2.7.1.26).
‡ One unit represents the formation of 1 nmol FMN/30 min at 37°.
§ ATP: FMN adenylyltransferase (*EC* 2.7.7.2).
‖ One unit represents the formation of 1 nmol FAD/60 min at 37°.
¶ *EC* 3.1.3.2.
** One unit represents the formation of 1 nmol riboflavin/30 min at 37°.
†† *EC* 3.6.1.9.
‡‡ One unit represents the formation of 1 pmol FMN/min at 37°.

of this enzyme. During infection the enzyme activities of the low- and high-riboflavin-fed groups were respectively 50 and 96% higher than those of their respective controls (period 1). At 5 weeks (period 2), FAD (nucleotide) pyrophosphatase activities were similar for all four groups (Table 4).

Plasma triiodothyronine and thyroxine

The low-riboflavin diet did not alter plasma T3 and T4 levels at 3 weeks (period 1), but at 5 weeks (period 2), plasma T4 levels of the low-riboflavin-fed group were 45% lower than those of the high-riboflavin-fed group. During infection, plasma T3 levels of the low-riboflavin-fed group were 25% lower and those of the high-riboflavin-fed group were 23% lower than those of their respective controls. Infection had no effect on plasma T4 concentration. After recovery from infection, T3 levels were similar to those of the corresponding uninfected controls (Table 5).

Table 5. *Effect of infection with* Klebsiella pneumoniae *on thyroid hormone status of mice fed on high- and low-riboflavin diets**

(Mean values with their standard errors; *n* represents pooled samples where each sample represents two animals)

	Low riboflavin						High riboflavin					
	Uninfected			Infected			Uninfected			Infected		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Plasma triiodothyronine (nmol/l)												
Period 1	7	2·88^{a}	0·26	8	1·97^{b}	0·09	7	2·54^{a}	0·13	7	1·95^{b}	0·08
Period 2	7	2·67	0·16	7	2·55	0·12	8	2·43	0·16	7	2·47	0·15
Plasma thyroxine (nmol/l)												
Period 1	8	27·19	3·75	8	27·99	4·25	8	33·85^{x}	4·25	8	26·66^{x}	2·73
Period 2	8	28·68^{a}	3·61	7	34·02^{a}	5·74	7	50·75^{by}	3·35	6	38·37^{ay}	4·23

^{a,b} Mean values with unlike superscript letters were significantly different between groups ($P < 0·05$).
^{x,y} Mean values with unlike superscript letters were significantly different between periods ($P < 0·05$).
Period 1, during peak period of infection; period 2, after recovery.
* For details of experimental procedures, see pp. 453–455.

DISCUSSION

The results of the present study for the low-riboflavin infected group confirm the earlier observations made in children that respiratory infection raises the level of riboflavin in blood and urine (Bamji *et al.* 1987).

The increase in erythrocyte flavin was associated with the major metabolite, FAD. This would explain the reduction in EGR-AC reported previously (Bamji *et al.* 1987; Padmaja *et al.* 1991), as well as that found in the present study.

Reduction in total flavin and FAD levels during infection suggests a shift of riboflavin from tissues into the circulation, facilitating its elimination through urine during infection. The data on enzyme activities provide some insight into the mechanism(s) involved.

The low-riboflavin diet *per se* was found to lower the activity of flavokinase, the first enzyme of the pathway towards FAD synthesis. A similar finding has been reported by other workers (Fass & Rivlin, 1969; Prentice & Bates, 1981; Lee & McCormick, 1983) and may be due to instability of the enzyme in the absence of the substrate (Rivlin & Langdon, 1966; Fass & Rivlin, 1969).

Klebsiella pneumoniae infection affected flavokinase but only in the low-riboflavin-fed group. This effect may be due to lower plasma T3 levels. This hormone is known to convert the less-active form of flavokinase to a more-active form (Lee & McCormick, 1985). Reductions in plasma T3 levels were also reported during respiratory infection in children (Hashimoto *et al.* 1994) and bacterial infection in mice (Burgi *et al.* 1986) and this was attributed to increases in cytokines such as tumour necrosis factor (TNF)-α, interleukins (IL)-1 and -6 (Hashimoto *et al.* 1994). Injection of TNF-α (Pang *et al.* 1989) or IL-1 (Fujii *et al.* 1989) resulted in a reduction in plasma thyroid hormone levels in animals.

In contrast to flavokinase, FAD synthetase levels tended to be higher in riboflavin-restricted mice as well as in infected mice. This could have been a compensatory effect to maintain liver FAD levels, but the increase in the enzyme activity was probably not sufficient to compensate, since FAD levels were lowered. The reported effects of riboflavin deficiency on FAD synthetase are variable (Fass & Rivlin, 1969; Prentice & Bates, 1981;

Lee & McCormick, 1983). Fass & Rivlin (1969) observed a peak increase in the FAD synthetase activity after 50 d of riboflavin deficiency. A similar increase in the activity of this enzyme was also observed by Prentice & Bates (1981) after 3 weeks of riboflavin deficiency. However, Lee & McCormick (1983) observed a moderate decrease in the activity of this enzyme after 5 weeks of riboflavin deficiency in rats.

While neither riboflavin restriction nor infection affected FMN (acid) phosphatase activity, FAD (nucleotide) pyrophosphatase levels were significantly increased by infection in the riboflavin-restricted group as well as in the high-riboflavin-fed groups compared with values for the uninfected controls.

An earlier study using a similar experimental design showed that infection lowered the activity of the hepatic FAD-dependent mitochondrial enzyme acyl-CoA dehydrogenase, but not that of D-amino acid oxidase (*EC* 1.4.3.3), whereas the activity of FMN-dependent pyridoxamine-phosphate oxidase increased (Padmaja *et al.* 1991). These observations can be explained on the basis of the changes in FAD and FMN levels reported in the present study. The fall in liver FAD levels during infection observed in the present study may explain the reduction in acyl-CoA dehydrogenase which has been shown to be one of the most sensitive flavin enzymes to riboflavin deficiency (Hoppel *et al.* 1979). A more-severe deficiency may be required to bring about a change in D-amino acid oxidase which is relatively less-sensitive to riboflavin deficiency. Although FMN-dependent pyridoxamine-phosphate oxidase showed an increase in activity, FMN levels were unchanged during infection. The increase in pyridoxamine-phosphate oxidase could be a response to the greater demand for pyridoxal phosphate to metabolize the amino acids liberated as a result of the catabolic effect of infection.

It is possible that respiratory infections inhibit the synthesis of some FAD-dependent flavoproteins, making free FAD available for enzymic degradation. The higher FAD (nucleotide) pyrophosphatase activity observed during infection could be a consequence of higher substrate availability or may be attributed to an independent factor. Reduction in flavokinase may further restrict the availability of riboflavin for coenzyme synthesis. The net effect would be a shift of riboflavin from the liver into the circulation. During infections the demand for riboflavin can be expected to go up in blood cells, particularly in the leukocytes, to sustain some of the oxidation–reduction reactions mediated by FAD-dependent enzymes, such as glutathione reductase and NADPH oxidase. The observed shift of riboflavin from the liver into the circulation may be in response to this demand. In severe riboflavin deficiency this process may be impaired and the defence system compromised. Indeed, in one of our earlier studies phagocytic activity by peritoneal leukocytes was reduced in riboflavin-deficient rats (Lakshmi *et al.* 1994).

Thus, it may be concluded that respiratory infections would exacerbate riboflavin deficiency in a population already suffering from dietary deficiency of this vitamin.

The authors are grateful to Dr G. Radhaiah for his valuable guidance in the statistical analysis of the data.

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Printed in Great Britain