CHAPTER--IV

EFFECT OF RESPIRATORY INFECTIONS ON RIBOFLAVIN ECONOMY OF MICE
The interaction between nutrition and infection has been established for decades both in animal and human studies. Infections contribute to undernutrition through several factors: increased tissue catabolism, nutrient losses in urine, reduced appetite, vomiting, decreased absorption, protein loss in gastrointestinal tract, rapid sequestration of nutrients and their utilization in the production of acute phase reactant proteins, antibodies, complements and other host-protective factors (1).

Credit for the first observations in the field of infection and nutrition goes to O'Shanghnessy, who in 1831 measured the electrolyte content of stool in an effort to decipher the pathogenic mechanisms leading to death in patients with acute virulent Asiatic Cholera (2).

Infectious diseases have adverse effects on practically every aspect of human life, from food production and economic development to nutritional status and host metabolism (3,4,5,6,7,8). Rosenberg et al (9) reported nutrient wastage and weight loss during infection. During infection, in addition to reduced food intake, an increase in the protein metabolism is reported to result in negative nitrogen balance (5,10,11).

There are few reports on the interaction between infection, nitrogen balance, and urinary excretion of riboflavin. Pollack and Bookman (12) observed that individuals in nitrogen equilibrium retained more than half of the ingested riboflavin, whereas those in negative nitrogen balance retained less than that amount. Conditions which
lead to negative nitrogen balance such as starvation, infection, surgery, raise in body temperature increase urinary excretion of riboflavin. Oldham et al (13) observed an increase in urinary excretion of riboflavin in two children suffering from upper respiratory infections during a study to determine the vitamin requirements for children.

Experimental induction of sandfly fever in humans resulted in increased urinary excretion of riboflavin, whereas excretion of other water soluble vitamins like thiamin, pyridoxine or niacin were not affected (14). A study in preschool children suffering from upper respiratory infections and measles showed increased urinary and blood levels of riboflavin during acute phase of infection (15). In the same study, the EGR-AC values decreased during infection suggesting greater saturation of erythrocyte glutathione reductase with FAD.

The data reported in Chapter III confirm the earlier findings regarding change in glutathione reductase activation during infections in school children. As mentioned earlier, one of the non-dietary factors which may be responsible for the high incidence of riboflavin deficiency in children could be repeated infections. It was postulated that repeated bouts of respiratory infections may cause mobilisation of tissue riboflavin and thereby aggravate the overall riboflavin nutriture (15,16).

The present study was designed to test the above hypothesis under controlled condition in an animal model, using mice as the experimental animal and klebsiella pneumoniae as the infectious organism, riboflavin
economy of the body was examined before, during and after infection by measuring liver and erythrocyte riboflavin levels and select flavin enzymes.

Before initiating the study, sub-lethal conditions for infecting the mice with the organism klebsiella pneumoniae were established. This organism was isolated from the rat colony in our animal facility. Since rats had become resistant to acute infection, mouse was tested and found to be suitable as an experimental model.

EXPERIMENTAL DESIGN

Sixty two male weanling mice, of Swiss/NIN strain, having mean body weight of 12.02 ± 0.18 g were divided equally into two groups — infected and uninfected. The animals were kept individually in screen-bottomed cages at 22-25°C room temperature under 12 hour light, 12 hour darkness cycles. They were fed a semipurified diet containing 70% sucrose, 20% vitamin free casein, 5% groundnut oil, vitamins and minerals (17). Vitamin and salt mixture composition is given in Appendix III. Riboflavin content of the diet was 0.5 mg/kg diet. This level is just sufficient to provide minimum requirement of riboflavin for mice but lower than the Recommended Dietary Allowance (RDA) for mice (18). The lower level of dietary riboflavin would be expected to simulate human situation in developing countries where riboflavin is one of the most limiting nutrients in the diet. To ascertain the extent of deficiency induced by the low-riboflavin diet, 12 mice (control group) were fed a diet containing RDA level of riboflavin. This group did not include infected animals.
After two weeks of feeding the experimental diet, all the animals in the "infected group" were injected with a sub-lethal dose (7.25 x 10^6 cells) of klebsiella pneumoniae in 0.5 ml of distilled water, intraperitoneally. The number of cells to be injected was decided on the basis of a preliminary study to determine the LD 50 (19). The uninfected mice (control and low riboflavin) received the same amount of distilled water intraperitoneally.

Within 24 hours of infection, the infected animals showed signs of acute infection such as loss of appetite and reddening of the snout. After 72 hours of infection, half the number of mice in each group (control, low-riboflavin uninfected and low-riboflavin infected) were sacrificed by cervical dislocation after drawing a sample of blood from the ocular plexus. The remaining animals were sacrificed 15 days later when the infected animals had recovered completely. There were no mortalities during that period.

Liver was removed and analysed for riboflavin and the following enzymes as per the methods described in Chapter II. Mitochondrial Acyl-CoA dehydrogenase with palmitoyl CoA as the substrate; D-amino acid oxidase in liver homogenate with DL-alanine as the substrate. Assays of Acyl-CoA dehydrogenase and D-amino acid oxidase were done immediately after killing. Due to paucity of tissue, livers from two animals were pooled for the assay of enzymes and riboflavin. Similar pooling was done for the measurement of RBC riboflavin.
Blood samples were examined for basal EGR activity and its stimulation with FAD (EGR-AC). Erythrocyte and liver riboflavin content were measured fluorometrically.

Details of the methods are given in Chapter II.

Statistical analysis of the data was done by analysis of variance and Duncan's Multiple Range Test (20).

RESULTS

The presence of klebsiella pneumoniae in the lungs of infected mice was confirmed (courtesy, Dr. Suresh) by culturing the lung smears (21). This organism was absent in the lung smears obtained from control and deficient uninfected animals. Infection resulted in a transient loss of appetite for one or two days but did not affect body weight (Table 23).

Effect of feeding low riboflavin diet on body weight and riboflavin status:

Body weight was significantly lower in the low riboflavin-fed animals compared to the control group at the younger age of 5 weeks (Phase I). However this difference was not significant at the older age of 7 weeks (Phase II).

Feeding low-riboflavin diet resulted in a small reduction in erythrocyte glutathione reductase basal activity and a significant rise in the EGR-AC values of the uninfected mice. Red blood cell and liver riboflavin levels were also lowered in mice fed low riboflavin diet compared to the control group.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th></th>
<th>Low-riboflavin uninfected</th>
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<th>Low-riboflavin infected</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
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<td>II</td>
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<tr>
<td>Weight (g)</td>
<td>20.2±1.87a</td>
<td>23.9±3.35a</td>
<td>16.4±1.31b</td>
<td>21.94±3.53a</td>
<td>15.5±1.80b</td>
<td>21.23±2.65a</td>
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<td>(14)</td>
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<td>(15)</td>
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<tr>
<td>BGR - Basal activity*</td>
<td>492.05±55.84a</td>
<td>495.20±31.04ab</td>
<td>458.96±44.89b</td>
<td>425.12±47.60b</td>
<td>526.59±139.10a</td>
<td>434.10±82.10b</td>
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<td>(13)</td>
<td>(11)</td>
<td>(14)</td>
<td>(14)</td>
</tr>
<tr>
<td>BGR - total activity</td>
<td>611.58±66.50</td>
<td>615.50±60.13</td>
<td>615.21±60.53</td>
<td>568.63±30.79</td>
<td>636.54±133.52</td>
<td>617.44±75.82</td>
</tr>
<tr>
<td>BGR - AC</td>
<td>1.25±0.158a</td>
<td>1.25±0.544a</td>
<td>1.33±0.158b</td>
<td>1.35±0.108b</td>
<td>1.24±0.108a</td>
<td>1.44±0.178c</td>
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<tr>
<td>RBC riboflavin (μg/100 ml)</td>
<td>69.30±13.31a</td>
<td>65.60±7.50ab</td>
<td>57.40±6.40b</td>
<td>56.21±7.97b</td>
<td>59.75±3.20b</td>
<td>42.78±6.60c</td>
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<tr>
<td>Liver riboflavin (μg/g)</td>
<td>25.0±3.12a</td>
<td>24.37±4.30a</td>
<td>21.83±5.39ab</td>
<td>20.31±2.06b</td>
<td>19.86±1.38b</td>
<td>21.12±1.30ab</td>
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<tr>
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</tbody>
</table>

Values are Mean ± SD; Values not sharing a common superscript are significantly different by analysis of variance and Duncan's Multiple range test; No. of measurements in parentheses denote number of samples.

* umoles of NADPH oxidized/hr/g Hb

I - During acute phase of infection in the infected mice

II - After recovery
Acyl-CoA dehydrogenase levels increased with age as seen by the difference in values between Phase I and Phase II (Table 24). Dietary riboflavin did not influence the activity of this enzyme.

Effect of age was not seen on either pyridoxaminephosphate oxidase or D-amino acid oxidase activity. Both these enzymes however were influenced by the low-riboflavin diet as seen by the comparison of the control group with the low-riboflavin uninfected group.

Effects of infection:

Though infection reduced food intake for a couple of days, the body weights were not affected, suggesting that the degree of infection was indeed mild.

Infection tended to increase the EGR basal activity and reduced EGR-AC values significantly. EGR total activity was however not affected (Phase I). Though RBC riboflavin registered a small increase, and liver riboflavin a small decrease in the infected compared to the uninfected mice, these differences were statistically not significant. On recovery (Phase II), the EGR-AC values tended to increase beyond the levels seen in uninfected mice suggesting exacerbation of riboflavin deficiency. This was also reflected in the insignificantly lower values for RBC riboflavin. EGR basal activity was also significantly lowered in the infected recovered compared to infected sick animals, but EGR basal activity did not fall below the levels seen in the uninfected mice. Liver riboflavin levels did not show a significant change though lowest values were seen in the infected sick mice (Phase I).
## Table 24
Effect of infection on the activities of some flavin enzymes in the liver

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Low-riboflavin uninfected</th>
<th>Low-riboflavin infected</th>
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<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
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<tr>
<td>Acyl-CoA dehydrogenase</td>
<td>18.01±8.30&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.57±4.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.86±4.69&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(7)</td>
</tr>
<tr>
<td>Pyridoxamine-phosphate oxidase</td>
<td>95.83±33.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>99.64±4.78&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>76.55±26.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
<td>(7)</td>
</tr>
<tr>
<td>D-amino acid oxidase</td>
<td>2.34±0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.59±0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.52±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
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</tbody>
</table>

Values are Mean ± SD. Values not sharing a common superscript are significantly different. Number in parentheses denotes number of samples analysed. Liver from 2 mice were pooled to make one sample.

1. nmole of Cyt.C reduced/minute/mg mitochondrial protein
2. μg/g liver /30 min
3. nmole/g liver /5 hrs

I - During acute phase of infection in the infected mice
II - After recovery
Infection produced a marked and significant reduction in Acyl-CoA dehydrogenase activity. The enzyme activity was restored on recovery. On the contrary, infection increased the pyridoxamine-phosphate oxidase activity and its recovery was not as clear cut as in the case of Acyl-CoA dehydrogenase activity. D-amino acid oxidase activity was not altered by infection.

DISCUSSION

The reduction in erythrocyte glutathione reductase activation coefficient (EGR-AC) seen during acute klebsiella pneumoniae infection in mice is in harmony with observation reported earlier in preschool children suffering from upper respiratory infections and measles (15) and in school children suffering from respiratory infections (Chapter III).

One of the possible mechanisms for the artefactual improvement in EGR-AC values could be a rise in blood riboflavin due to mobilization of riboflavin from tissues during infection due to tissue breakdown or selective turnover of flavoproteins. Of the several B-complex vitamins studied during sandfly fever, the only significant illness-related derangement in vitamin metabolism detected was a progressive increase in daily losses of riboflavin (14,5). In the present study, urinary excretion of riboflavin in mice could not be measured due to the difficulties in urine collection.

An increase in leukocyte glutathione reductase activity has also been reported to occur during phagocytosis and bactericidal activity
(22). There may be an increased demand for reduced glutathione (GSH) during infection to protect the cell from hydrogen peroxide and free radicals formed during the bactericidal activity.

Thus, during infection the demand for riboflavin in the blood cells may increase and this may result in some mobilization of riboflavin from tissues such as liver to the blood cells. The significant rise in EGR-AC and reduction in RBC riboflavin after recovery in the infected mice seen in the present study is in line with the hypothesis that respiratory infections aggravate riboflavin deficiency.

The reduction in Acyl-CoA dehydrogenase activity during the klebsiella pneumoniae infection suggests an adverse impact of infection on this flavin enzyme involved in fatty acid oxidation. It is surprising that D-amino acid oxidase, which is sensitive to changes in dietary riboflavin is not affected by infection. Rather than producing a generalized lowering of riboflavin status, infection may selectively influence flavin enzymes due to altered metabolism. The overall effect may be a greater requirement of riboflavin.

Though the effect of infection on liver riboflavin level was equivocal, the lowest values were observed in the infected mice during infection. Liver riboflavin concentration and enzyme activities after recovery are not suggestive of overall tissue depletion though red cells show such a trend. It may, however, be pointed out that these animals were exposed to only one episode of infection and their diet was not grossly deficient in riboflavin as is often the case in some human situations.
Infection is known to result in muscle breakdown (5). This may increase the demand for pyridoxal phosphate for metabolizing the amino acids released (23). The rise in pyridoxaminephosphate oxidase activity in the liver of infected mice during acute infection may be in response to this demand, since pyridoxaminephosphate oxidase is a key enzyme for pyridoxal phosphate synthesis.

In conclusion, the data reported here and the earlier findings in humans, suggest that riboflavin requirement may be enhanced during infection due to increased demand of some flavin enzymes for coenzymes and this may result in alterations in the profile of flavin enzymes. Due to reduced availability of coenzyme in riboflavin deficiency, there may be an altered distribution of coenzyme between tissues and amongst its enzyme systems within a tissue and consequently alterations in enzyme activities.

From these studies it appears that ECR-AC test would be an unreliable method to assess riboflavin status during certain ailments like upper respiratory infections.
REFERENCES


