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Biochemical Detection, Aetiology and Functional Consequences of Riboflavin Deficiency

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The erythrocyte glutathione reductase test reveals a very high incidence (over 80%) of riboflavin deficiency among low-income group populations in and around Hyderabad. 15–20% show clinical lesions of riboflavin deficiency, such as mucocutaneous lesions of the mouth. Besides dietary deficiency, urinary losses during diseases such as upper respiratory infections and measles may also contribute to the high incidence of riboflavin deficiency.
Skin lesions of riboflavin and pyridoxine deficiency in animals and humans are similar and may have a common molecular bases viz. impaired collagen cross-linking. Riboflavin deficiency impairs pyridoxal phosphate (PLP) synthesis and PLP is essential for collagen cross-linking. Hitherto unrecognized functional consequences of riboflavin deficiency are, delayed wound healing and reduced hand steadiness.

Biochemical Detection of Riboflavin Deficiency

Several years ago we developed a test for the assessment of riboflavin nutrition status, based on the *in vitro* activation of the flavin enzyme, erythrocyte glutathione reductase by its coenzyme FAD (Bamji 1969). The logic in this test is, in riboflavin deficiency, the enzyme activity drops due to reduction in the levels of the coenzyme FAD. However, the enzyme activity can be restored by *in vitro* addition of FAD. *In vitro* activation of glutathione reductase referred to as EGR-AC is inversely related to riboflavin deficiency. Following guidelines for interpretting this test were derived from control depletion-repletion studies, and values seen in subjects suffering from riboflavin deficiency. EGR-AC$<$1.2, normal, 1.2–1.4, marginal deficiency $>$1.4, deficient. Subsequent surveys of population groups, particularly women and school children show a very high incidence of biochemical riboflavin deficiency in and around Hyderabad (Bamji et al. 1979, Bamji et al. 1982, WHO task force on oral contraceptives 1986, Prasad et al. 1987) (table 1).

Aetiology of Riboflavin Deficiency

Diet surveys conducted by the National Nutrition Monitoring Bureau (ICMR) show that vitamin A and riboflavin are among the most limiting nutrients in Indian diets (National Nutrition Monitoring Bureau Report 1981). Dietary riboflavin deficiency is particularly marked in the southern and eastern states of India where rice is the staple diet. Situation improves with a mixed cereal millet diet (table 2). Poverty, ignorance, wrong food preferences and cooking practices all contribute to dietary riboflavin dificiency. We feel that apart from diet, infections also play a significant role in development of riboflavin deficiency. Very often normalization of EGR-AC does not occur despite administration of riboflavin supplements. A study in rural children showed an association between seasonal rise in upper respiratory infections and urinary excretion of riboflavin during winter (Sarma et al. 1981). Subsequent study in urban preschool children showed that during measles and bouts of upper respiratory infections (URI) there is a marked rise in the urinary excretion of riboflavin (Bamji et al. 1987) (table 3). Such metabolic losses may perpetuate riboflavin deficiency and may account for its very high incidence. Similar phenomenon does not seem to operate for other B-complex vitamins. Along with rise in urinary excretion, a transient rise in blood levels also occur suggesting mobilisation of riboflavin from the tissues.

Table 1 *Prevalence of riboflavin deficiency as judged by erythrocyte glutathione reductase activation coefficient (EGR-AC) among low-income group women and children*

		EGR-AC Percentage frequency distribution				
		1.20	1.21–1.40	1.41–1.60	>1.610	Source
	Number	Low risk	Medium risk	High risk		
Rural children 1–5 years	105	21	33	21	25	Unpublished
Rural school boys 5–11 years	114	0	5.2	15.5	79.3	Bamji et al. 1982
Urban school children 7–11 years	103	2	4	9	85	Prasad et al. 1987
Rural women 15–45 years	105	8	21	21	50	Unpublished
Urban women 18–35 years	415	8	10	14	68	Bamji, Prema & Jacob (WHO task force, unpublished)

Table 2 *Riboflavin intake in Rural India* % RDA for 2400 calories/C unit†*

	Riboflavin intake % RDA	Rice as % of total calories
Kerala	64	97
Tamil Nadu	63	98
Orissa	52	97
Andhra Pradesh	59	88
West Bengal	65	86
Karnataka	73	65
Gujarat	92	38
Uttar Pradesh	101	27

*Data from National Nutrition Monitoring Bureau (1981)
†C unit = Consumption unit. For an adult man during sedentary work, C unit is 1. For others a scale of coefficients worked out based on calorie requirements

Table 3 *Effect of infections on urinary riboflavin levels in children*

	Sample size	Riboflavin mg/g creatinine
Control	21	0.39 ± 0.17
Upper respiratory infections	29	1.20 ± 0.73***
Measles	28	1.24 ± 0.15***

***0.001 compared to controls by 't' test

Certain drugs are also known to alter the riboflavin requirement. Our studies show that high dose formulations of oral contraceptives increase the riboflavin requirement due to selective increments in specific flavin enzymes. This leads to an unequal distribution of riboflavin between its enzyme systems creating pockets of deficiency and excess—a state of relative deficiency (Ahmed et al. 1975, Ahmed & Bamji 1976a,b).

Functional Consequences of Riboflavin Deficiency

Figure 1 describes the stages in the development of vitamin deficiency. The molecular basis of most vitamin deficiency diseases is not understood. There are reasons to believe that even at the subclinical stage of malnutrition some subtle functional impairments like changes in psychomotor performance may occur. Even an apparently healthy individual may not be in a state of optimum health.

Subclinical malnutrition can be detected only through appropriate biochemical tests. Our experience shows that while majority of the subjects who show clinical signs and symptoms of vitamin deficiency, show evidence of biochemical deficiency, within the biochemically deficient individuals, there are many who do not show clinical evidence. This is because, disease is a complex phenomenon in which factors other than vitamin deficiency may play a role in tipping an individual from subclinical to the clinical state (figure 1).

Flavin enzymes are essential for biological oxidation reduction and energy transduction reactions. Riboflavin deficiency in animals leads to loss of apetite, skin changes, growth failure and death. However, in human

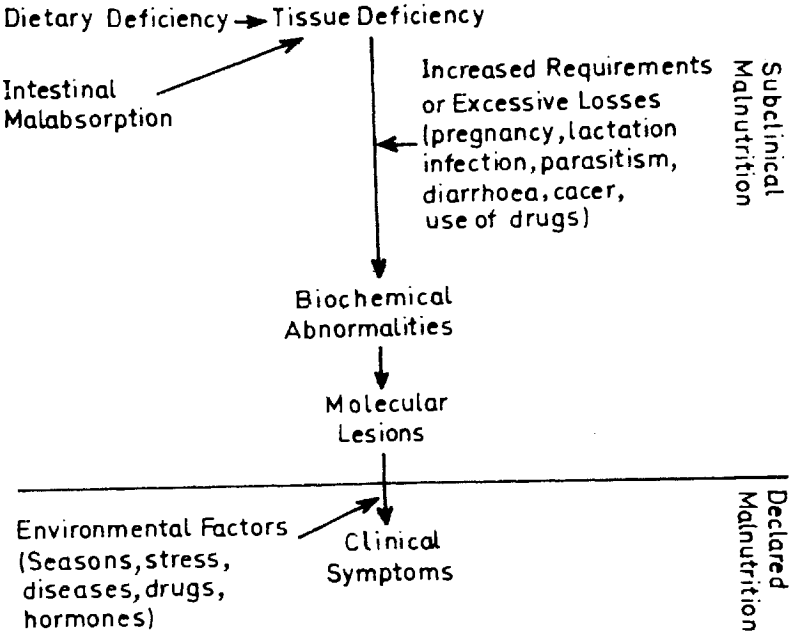

Figure 1 Interrelationship between riboflavin and pyridoxine (vitamins B_2 and B_6). *Source:* M S Bamji 1986

situation, where only sub-total deficiency occurs, the clinical morbidity, of riboflavin deficiency is not as serious as that of some of the other vitamins. The presenting clinical symptoms of riboflavin deficiency in humans are, mucocutaneous lesions of the mouth such as angular stomatitis, glossitis and cheilosis. One possible explanation for the less serious consequences of sub-total riboflavin deficiency as compared to the deficiencies of other B-complex vitamins in humans may be the fact that important mainstream flavoproteins concerned with energy metabolism do not lose their activity till very terminal stage of deficiency because the dissociation constants (*Kd*) for their coenzymes are very low (Burch et al. 1956). It is therefore possible that the pathology of riboflavin deficiency is due to molecular event(s) related to side-stream reactions and hence are of less serious consequences.

Molecular basis of the skin lesions of riboflavin deficiency: Several years ago some of my clinical colleagues observed that the oral lesions regarded to be pathognomic of riboflavin deficiency, sometimes do not respond fully to treatment with riboflavin. However, they respond to treatment with pyridoxine (Krishnaswamy 1971, Iyengar 1973). Experimental deficiencies of vitamins B_2 as well as B_6 have also been reported to produce skin changes in rats and mucocutaneous lesions of the mouth such as angular stomatitis, glossitis, cheilosis and nasolabial dyssebasia in human (Goldsmith 1962, Viter 1962). This raised the possibility of a unified hypothesis explaining the roles of these two vitamins in the development of skin lesions. Wada and Snell (1961) have reported that the conversion of pyridoxine to its coenzyme pyridoxal phosphate depends on the FMN-dependent enzyme, pyridoxaminephosphate oxidase (PPO) in the following reaction sequence:

$$\text{Pyridoxine} \rightarrow \text{pyridoxine phosphate} \xrightarrow[\text{FMN}]{\text{PPO}} \text{pyridoxal phosphate}$$

Based on this, we postulated that the biochemical basis of the skin lesions which respond to riboflavin and/or pyridoxine may be cellular deficiency of pyridoxal phosphate (PLP) caused either by dietary deficiency of pyridoxine *per se* or secondary to impaired synthesis of PLP due to riboflavin deficiency. Since riboflavin is the more limiting vitamin in the diet than pyridoxine, these lesions are normally observed to respond to treatment with riboflavin.

To substantiate this hypothesis we have examined the effects of riboflavin deficiency in rat and humans on the metabolism of pyridoxine to pyridoxal phosphate. These studies showed that in riboflavin deficiency the activity of flavin enzyme pyridoxaminephosphate oxidase is markedly reduced and synthesis of PLP impaired (Lakshmi & Bamji 1974, 1976, 1979) (tables 4,5,6). Additionally it was observed that riboflavin deficiency also raises the cellular requirement of

Table 4 *Effect of riboflavin deficiency on pyridoxal phosphate (PLP) synthesis in rats*

	Control	Deficient
Liver PPO activity*	165 ± 12.1†	27.5 ± 4.3
In vivo incorporation of ^{14}C pyridoxine into liver PLP (% total radioactivity)		
3 min after injection	28.20	6.07
10 min after injection	37.13	1.35

*Pyridoxaminephosphate oxidase activity expressed as PLP formed from pyridoxine phosphate (n mol)/g liver per 30 min
†Mean ± SE

Table 5 *Effect of riboflavin deficiency on* in vivo *PLP synthesis in humans*

No. of subjects	Clinical signs	Biochemical deficiency	PLP ratio
4	absent	absent	8.25 ± 0.72
2	absent	present	3.94 ± 5.03
10 (BT)	present	present	3.61 ± 0.76
7 (AT)	absent	absent	5.60 ± 1.02

PLP ratio = Blood PLP concentration, 15 min after parenateral administration of 5 mg pyridoxine/blood PLP before pyridoxine administration
BT before treatment; *AT*, after treatment with 5 mg riboflavin for 10 days

Table 6 *Effect of riboflavin deficiency on* in vitro *synthesis of PLP in human erythrocytes*

Substrate	Pyridoxine	Pyridoxaminephosphate
	μg PLP formed/ml RBC in 30 min.	
Controls (6)	0.76 ± 0.03	0.69 ± 0.11
Deficient (7)	0.19* ± 0.03	0.20* ± 0.02

*P 0.05 compared to controls
( ) Number of subjects

pyridoxine by increasing the levels of some PLP-dependent enzymes (Lakshmi & Bamji 1975). This leads to a bizarre redistribution of PLP between its enzyme systems creating pockets of deficiency and excess i.e. state of relative deficiency (figure 2).

The next question was, why should absolute or relative PLP deficiency cause skin lesions. PLP was suggested to be a cofactor for the enzyme lysyl oxidase which initiates the process of collagen cross linking (Murray & Levine 1971). Collagen is an important connective tissue protein present in the dermis of the skin. Based on this we hypothesized that the molecular basis of skin lesions in riboflavin deficiency is impaired collagen crosslinking. This would weaken the dermis of the skin, and render the overlying epithelial tissue susceptible to stress and infections. The lesions would be more marked in the areas of constant stress and friction such as the mucocutaneous junctions. Normal collagen is also essential for epidermal growth and differentiation (Bano et al. 1983). Our experiments in rats show that riboflavin as well as pyridoxine deficiency impair cross-linking in skin collagen as judged by several biochemical, biophysical and biomechanical properties of the skin (Prasad et al. 1983, 1986a) (tables 7,8). Thus both vitamins B_2 and B_6 deficiencies reduce skin collagen content and aldehyde levels, increase collagen solubility in non-polar solvents such as NaCl and in denaturing agents, such as urea and potassium thiocyanate. They increase collagen gel reversibility and decrease its tensile strength and shrinkage temperature. All these changes suggest that deficiencies of these vitamins affect collagen cross-linking. Studies with ^3H-proline suggest that collagen synthesis is also affected (Prasad et al. 1986b).

Table 7 *Skin collagen content and properties in vitamins B_2 and B_6 - deficient growing male rats*

	Food restricted (weight matched control)	Vitamin B_2 deficient	Vitamin B_6 deficient
	Percentage of *ad lib*-control rats		
Total collagen	83.1	72.1	78.3
Insoluble collagen	80.7	67.4	70.3
Aldehyde content	94.9	70.8	61.8
α/β subunit ratio	130	142	143
Tensile strength	33.2	22.2	26.5

Table 8 *Effect of riboflavin or pyridoxine deficiency on susceptibility of insoluble collagen to denaturing and proteolytic agents*

Solubility of insoluble collagen by treatment with	Weight control	Riboflavin deficient	Pyridoxine deficient
	(% of total)		
6M urea	44.65	71.32	72.93
2M KCNS	41.83	76.46	74.46
0.0016% pronase	39.48	70.92	66.45

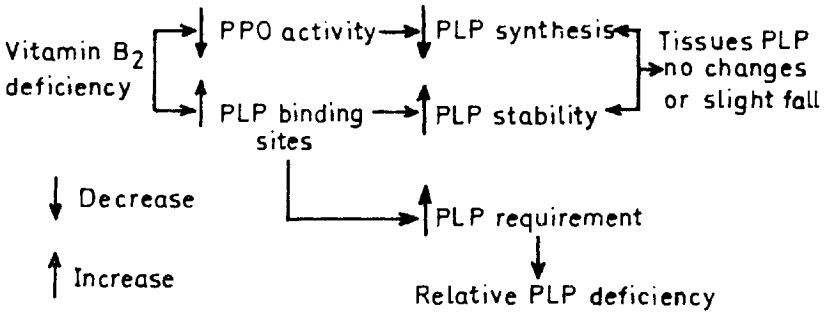

Figure 2 Development of nutritional deficiency disease

The above-mentioned experimental observations support our hypothesis that the molecular basis of skin lesions in riboflavin and pyridoxine deficinecy is impaired dermal collagen structure. Food restriction has similar (though not identical) effects but of lesser magnitude.

Addendum

Recent observations of Williamson and Kagan (1987) question the claim of earlier investigators that PLP is a cofactor for lysyl oxidase. Thus the mechanism of altered collagen properties in the deficiency of vitamins B_2 or B_6 remains to be elucidated. According to one possibility, increased levels of sulfer-containing amino acids like homocysteine in vitamin B_6 deficiency may interfere with collagen cross-link synthesis by binding to the lysine-derived aldehyde groups.

Wound healing: Collagen synthesis is an important event in the process of wound healing. Our recent unpublished observations show that healing of both excised and incised wounds is affected in the deficiency of riboflovin and pyridoxine (Laxmi A V, Laxmi R and Bamji M S, unpublished). Whether impaired collagen synthesis is the only factor responsible or other molecular events are also involved remains to be examined.

Psychomotor function: In two separate studies among rural (Bamji et al. 1982) and urban (Prasad, Lakshmi and Bamji unpublished) children we have observed that riboflavin deficiency impairs hand steadiness. Other psychomotor, performance tests such as cognitive ability, reaction time, finger dexterity (Bamji et al. 1982) and physical work capacity as judged by time taken to run a fixed distance and pulse rate after work, (unpublished observations) are not affected. Hand steadiness is an extra-pyramidal function, dependent on dopamine activity. PLP is an essential cofactor for dopamine synthesis. In view of the earlier mentioned relationship between riboflavin deficiency and PLP synthesis it is possible that dopamine synthesis is impaired in riboflavin deficiency and this leads to subtle extrapyramidal deficits.

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