CHAPTER-I

GENERAL INTRODUCTION
Nutritional status of the children represents the nutritional level of the community because growing children are the most vulnerable group. Despite several advances in the field of nutrition, malnutrition is widely prevalent in many parts of the world and is the single most important associated factor responsible for the high mortality rate in developing countries. It is one of the greatest international health problems of the day, and is a major public health problem in many of the developing countries of the world. While in technologically developed countries nutritional deficiency diseases have been largely eliminated, in the developing countries like India, nutritional disorders like protein-calorie malnutrition, xerophthalmia due to vitamin A deficiency and mucocutaneous lesions of B-complex deficiency are rampant. Pellagra continues to appear in pockets, and rickets continues to be a clinical if not a public health problem. Happily however, the one-time dreaded diseases like beriberi and scurvy have almost disappeared. Severe forms of malnutrition constitute only a fraction of the whole problem representing the tip of the proverbial "iceberg", larger portion of the population suffering from sub-clinical malnutrition remains unnoticed and is the submerged portion of the "iceberg".

BRIEF HISTORY OF RIBOFLAVIN

Discovery:

Blyth observed a pigment with green fluorescence in milk which he called lactochrome (1). Simultaneously during 1890's, Bijkmann (2) reported that hens fed predominantly on polished rice subsequently
exhibited polyneuritic symptoms that appeared very similar to the neurological symptoms of beriberi in humans. Later studies by some workers revealed that consumption of unmilled rice or milled rice together with its bran, protected the animals against the development of polyneuritis. By 1926, the active portion of the molecule was successfully isolated. Subsequent studies revealed that this anti-beriberi factor (vitamin B) was composed of both a heat labile anti-beriberi factor (vitamin B₁) and a thermostable growth factor (vitamin B₂). Later it was found that vitamin B₂ was also a mixture and in due course its components were isolated (3).

Warburg and Christian isolated a yellow enzyme necessary for cell respiration from yeast in 1932 (4,5), and also discovered that a protein and the pigment were two factors in the enzyme. The next year, Kuhn and co-workers reported the synthesis of riboflavin and also noted the relation of its activity to the green fluorescence, thereby establishing that lactoflavin and the vitamin are identical (6). This was the first example of a vitamin functioning as coenzyme (7).

**Structure and metabolic interconversions:**

Riboflavin is a 7,8 dimethyl-10 (D-ribityl) isoalloxazine ring attached to a ribityl side chain (Fig. 1) (8). The coenzyme derivatives of riboflavin, flavin mononucleotide (FMN) (Fig. 2) and flavin adenine dinucleotide (FAD) (Fig. 3), are synthesized sequentially from riboflavin. In the first step, catalyzed by flavokinase, riboflavin reacts with ATP to form riboflavin-5' phosphate, or FMN (9). In the second biosynthetic step, FMN combines with a
Riboflavin

**FIG 1**

Flavin Mononucleotide

**FIG 2**
Flavin Adenine Dinucleotide

Fig 3
second molecule of ATP to form FAD in a reaction catalyzed by the enzyme FAD pyrophosphorylase (10). The degradation of FAD to FMN is catalyzed by FAD-pyrophosphatase and the further degradation of FMN to riboflavin by FMN phosphatase (11). Thyroxine regulates the conversion of riboflavin to FMN and FAD (12).

Absorption:

Riboflavin and flavin mononucleotide are absorbed in humans by the upper gastrointestinal tract in what is believed to be a specialized transport process involving a phosphorylation - dephosphorylation mechanism, rather than by passive diffusion (13). The flavins in food must be converted to free riboflavin by digestive enzymes before they can be absorbed. Covalently bound flavins appear to be unavailable (14). The main site of absorption is the upper small intestine, and the process is active, saturable, and linear up to about 30 mg riboflavin given with a meal. Bile salts, and anything which increases transit time, increases absorption (15,16). Absorption in the large bowel is much less efficient than that in the ileum (17,18), but the contribution of riboflavin producing microflora may nevertheless be significant. Studies in rabbits evidently showed active transport systems across the blood-brain barrier in the choroid plexus (19,20), and there are specific transport systems also in the liver, kidney and placenta (15,21,22,23). FMN and FAD, the major forms in which flavins occur naturally in the diet, must first be hydrolyzed before absorption. Results from experiments on riboflavin absorption by rat intestine in vitro have been contradictory (24).
Binding and transport by plasma proteins:

The specificity and physiological significance of the binding of riboflavin to plasma proteins have been shown most clearly in the chick. Presence of two specialized riboflavin binding proteins have been observed in laying hens, which have the same polypeptides but differ in carbohydrate and phosphate composition. These riboflavin binding proteins are required for storage of riboflavin in the egg for utilization by the developing embryo (25,26). The riboflavin-binding protein, identified in pregnant rats (25,26,27) and cows (28), has many similarities to the corresponding binding protein of avian eggs. Although the presence of binding protein in human pregnancy has not yet been proved unequivocally, preliminary reports suggest that human cord blood may contain a similar protein (29).

FMN and FAD are rapidly hydrolyzed in the body and presumably it is free riboflavin that traverses the cell membrane. Riboflavin is primarily present in biological fluids and tissues in the coenzymatic forms, so it is presumed that apoflavoenzymes are responsible for the binding and retention of this vitamin in the body and that excess free riboflavin should be excreted (30). In plasma, a considerable fraction of riboflavin is bound to albumin (about 42%), while other plasma proteins, viz. β-globulin, α-globulin, γ-globulin, fibrinogen etc. also bind riboflavin to a significant extent (31). Riboflavin binding to a fraction of immunoglobulin (IgG) in normal human plasma has been demonstrated (32).
Excretion:

In man riboflavin is excreted both in urine and faeces (13). Free riboflavin is excreted in urine. However, when large doses of riboflavin are ingested by humans or animals, certain metabolites of riboflavin are excreted in urine due to bacterial degradation of vitamin by the intestinal flora (33,34).

Occurrence and dietary sources:

Riboflavin is distributed widely in animal and plant kingdom, but the distribution is not even. Riboflavin appears to be formed primarily in the most actively functioning part of the plant, the green leaves and the growing tips (35). Milk from cows receiving an abundance of fresh grass tends to be slightly richer in riboflavin than the milk of cows fed drier and more mature grass (36). Major portion of riboflavin (90%) in milk is in a free dialysable form. In most of the other materials such as liver, yeast and green leaves, it occurs in combination with proteins (37).

Milk, liver, kidney, heart, egg white, and green leafy vegetables are excellent sources of riboflavin. Lean meat, beef, veal, pork, poultry, cheese, apricots and tomatoes furnish valuable amounts. Cereals and legumes are not rich sources of the vitamin, but due to the large quantities consumed, they supply much of the riboflavin in human diets. Milling of grains deprives the flour of much of the vitamin because the major part of vitamin is in the germ and bran. However, germination increases the riboflavin content of cereals and pulses (38).
Effect of cooking on riboflavin content of foods:

The heat stability of riboflavin protects it from destruction with ordinary cooking procedures. Extraction by water and exposure to light during cooking in uncovered dishes leads to a substantial loss. Cooking in an alkaline medium will accelerate the rate of destruction. Processing of foods such as canning, slow freezing, thawing and dehydration also cause loss of riboflavin. The technique of sun-drying as practised in tropical countries for fish, vegetables and other food products leads to considerable destruction of riboflavin (38). Pasteurization of milk does not lower riboflavin content very much but exposure to sunlight destroys large amounts (85%) of the vitamin (39). Pai (40) reported riboflavin losses ranging from 0.15 to 48% in different Indian food preparations. The losses were more by the direct heat method than either by steam cooking or by pressure cooking. Pasricha (41) has reported riboflavin retention in several foods ranging from 23 to 100% during cooking, retention being higher than in the case of thiamin. An average loss of 20-25% can be assumed for riboflavin as a result of cooking.

Clinical signs of riboflavin deficiency:

The 'classical' signs of riboflavin deficiency comprise mainly of lesions of the mucocutaneous surfaces of the mouth (glossitis, angular stomatitis, cheilosis, atrophic lingual papillae, magenta tongue), seborrheic skin lesions, and surface lesions of the genitalia. The occurrence of 'circum-corneal injection' of ocular capillaries (42,43) remains controversial.
The first systematic study of experimental riboflavin deficiency in human subjects was undertaken by Sebrell and Beutler (44) where the dietary ration contained relatively small amount of riboflavin. They observed development of cheilosis in 10 of the 18 women subjects for the period from 94 to 130 days after the initiation of the study. In addition to the lesions on the lips, a fine, scaly, slightly greasy desquamation on a mildly erythematous base appeared in the nasolabial folds, the vestibule of the nose and ears, and on the alae nasi. These lesions disappeared after the administration of riboflavin (0.25 mg/kg body weight). Jelliffe et al (45) observed magenta colour of the tongue (glossitis) and felt, that to be the characteristic clinical sign of riboflavin deficiency. Sydenstricker et al (46) reported on five patients who presented lesions of riboflavin deficiency. They possessed the typical dermatitis and conjunctivitis, which were cured by riboflavin. Occular manifestations of ariboflavinosis were reported by Sydenstricker et al (43). Using a slit lamp, they observed that the earliest lesions were proliferation and engorgement of the limbic plexus, followed by superficial vascularization of the cornea. These ocular symptoms were cured by administration of riboflavin. 

Sebrell (47) described that riboflavin deficiency in man caused reduced visual acuity. Signs of riboflavin deficiency are more common during physiologic and pathologic stress like rapid growth, pregnancy, lactation and trauma including burns and surgical procedures and chronic debilitating diseases like rheumatic fever, tuberculosis, malignancy etc. (48).
The diagnosis of a vitamin deficiency state presents no problem to the clinician when it is present in its classical and overt form. However, attempts have been made to advance the diagnosis of the disease and nutritionists have become interested in the possibility of a sub-clinical deficiency state. This may be defined as a state in which the level of a vitamin in the tissues is low, but in which the classical symptoms and signs of the disorder are not present.

The sequence of events which lead to a classical vitamin deficiency state are: inadequate availability of the vitamin to meet the needs at that particular time. This can result not only from a primary dietary lack, but also from poor absorption, impaired transport or excessive requirements. When the intake is inadequate, then tissue desaturation takes place. Once tissue desaturation has reached a critical level, then the metabolic functions of the vitamin are lost and changes occur in specific enzyme activities. When these in turn reach a critical level, the clinical picture of the vitamin deficiency disorder can be expected to appear.

Requirements of riboflavin:

There are essentially two methods available for estimating human riboflavin requirements: (a) population surveys and (b) controlled depletion-repletion studies.

Minimum requirement is defined as the amount that prevents manifestation of signs of disease and optimum requirement as the amount that provides the individual the best possible health, functional capacity and resistance to disease (49). The requirement for any
nutrient must have a criterion on which it is based. In case of water-soluble vitamins, mostly the tissue saturation will be taken as a criterion. Since B-vitamins are not stored to any appreciable extent by tissues, when the intake is greater than the requirement, the excess is excreted in urine. Urinary excretion of riboflavin and red blood cell levels of riboflavin have, therefore, been used as criteria for the assessment of requirements. The basic work to determine the minimum requirements of the vitamin for healthy young adults was carried out in three major experimental depletion studies soon after the discovery of the vitamin (50,51,52). However, there continues to be some controversy regarding the basis for calculating the requirement. National Research Council (NRC) (53) recommended that the requirement of riboflavin may more correctly be regarded as depending on protein metabolism. Later, FAO/WHO Expert Group (38) suggested that the requirement was best expressed on the basis of calorie intake, since flavin nucleotides take part in energy metabolism also. It has however been shown that the riboflavin requirement calculated on either basis yields similar results. From the available data, FAO/WHO Expert Group (1967) concluded that the minimum requirement of riboflavin is 0.44 mg/1000 Cal. The Group further suggested an intake of 0.55 mg/1000 Cal with an allowance of 20% for individual variation. The NRC of USA (1964) has recommended an allowance of 0.586 mg/1000 Cal. Of factors affecting riboflavin requirements, Roe et al (54) says that active young women need more riboflavin than the current RDA.
Clinical deficiency signs are slow to appear in controlled depletion studies, but a combination of clinical and biochemical evidence suggested a minimum requirement of about 0.5–0.8 mg/day. At intakes between 1.1 and 1.6 mg/day, the renal threshold was reached, and urinary excretion began to rise sharply with increasing intake (55,56).

In terms of total daily intake of riboflavin, clinical evidence of deficiency has developed when the dietary supply of riboflavin was less than 0.56 mg/day (50,57). Lesions attributable to lack of riboflavin have not been observed at levels of intake above 0.75 mg/day (58).

Urinary excretion studies for assessing riboflavin requirements in Indians (59,60,61,62) indicate that an intake of 0.36 mg/1000 Cal is below the requirement level. Intake of 0.50 mg/1000 Cal appeared to be satisfactory. These values are not different from FAO/WHO recommendations. Therefore, riboflavin allowance for Indians have been suggested as 0.55 mg/1000 Cal (63). Recently, Indian Council of Medical Research revised the recommendation to 0.6 mg/1000 Cal (64) for Indians.

Studies on riboflavin requirements in past years based on the near saturation of erythrocyte glutathione reductase with its cofactor FAD, in non-pregnant, non-lactating American women and also depletion repletion studies in Indian population, showed a requirement of 0.5–0.8 mg/1000 Kcal (65,66,67,68).
Prevalence of clinical and biochemical riboflavin deficiency in developing countries:

Riboflavin deficiency is widely prevalent in developing countries, particularly among pregnant women, nursing women, and children, in whom the incidence of clinical signs may be as high as 20 to 25% and biochemical deficiency over 70% (69,70,71). Biochemical deficiency of riboflavin is seen among pregnant women and children even in developed countries (72).

The overall incidence of biochemical riboflavin deficiency among rural children in Southern Italy was only 13 per cent, and the authors pointed out that children who consumed less than 300 ml of milk and diary products per day had biochemical deficiency (73). Forty one percent of the Nigerian school children had biochemical riboflavin deficiency and their diets were grossly inadequate in thiamin and riboflavin (74).

Recent diet surveys conducted by the National Nutrition Monitoring Bureau (NNMB) of the Indian Council of Medical Research in 8 states of India reiterate the point that the diets are very deficient in vitamin A and riboflavin when compared to other vitamins, calories and protein (75) (Table 1). Although average intakes of thiamin, niacin and vitamin c were adequate, diets in some states were deficient in these vitamins too. Surprisingly, a survey from Japan suggests that even in this developed country, the diet is deficient in calories and vitamins particularly riboflavin (76) (Table 1).
Table 1
Food and nutrient intake in rural India and Japan

<table>
<thead>
<tr>
<th></th>
<th>Calories % of RDA</th>
<th>Rice as % of total cereal</th>
<th>Protein</th>
<th>Vitamin A</th>
<th>Thiamin</th>
<th>Riboflavin</th>
<th>Niacin</th>
<th>Vitamin C</th>
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<tr>
<td>Kerala</td>
<td>90</td>
<td>97</td>
<td>100</td>
<td>51</td>
<td>61</td>
<td>64</td>
<td>.83</td>
<td>175</td>
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<tr>
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<td>106</td>
<td>31</td>
<td>82</td>
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<td>86</td>
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<td>61</td>
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<td>103</td>
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<td>42</td>
<td>110</td>
<td>70</td>
<td>96</td>
<td>104</td>
</tr>
</tbody>
</table>

| Japan                      | 91                | --                        | 81      | 81        | 99      | 79         | --     | 263       |

aData from National Nutrition Monitoring Bureau, 1981
bC unit = Consumption unit
cData from Hosoya, 1977 (Ref. 76)
d% RDA, corrected for 2,400 calories.

Source: Ref. 77
The deficiency diseases occur largely among the population groups of low socio-economic status, usually with defective diets and living in poor sanitary environments which increase the hazard of infection. Poor dietary intake, however, appears to be the principal factor. The intake of riboflavin is lowest in Asia and the Far East. The principal sources of this nutrient in the developing regions are either cereals or starchy roots and tubers which contribute about 45 per cent of the total riboflavin in the diet. In Africa, the fruits and vegetables or milk may also contribute to significant amounts. In Europe and North America the principal sources are milk and milk products, while fish, meat and eggs are also important (77).

The typical clinical symptoms of riboflavin deficiency are the triad of orolinguinal lesions - angular stomatitis, glossitis, and cheilosis. The prevalence of angular stomatitis in rural Indian school boys is 41.3% and that of glossitis is 18.2% (69). These authors showed that treatment with B-vitamins daily for one month produced significant reduction in glossitis, but had no effect on angular stomatitis which responded better to topical application of gentian violet. The findings suggested that while glossitis is a manifestation of riboflavin or pyridoxine deficiency or both, angular stomatitis has more complex aetiology perhaps associated with infection. Later studies of Sharma et al (78) also suggest that the seasonal fluctuation in vitamin deficiency signs might be due to other aetiological factors apart from dietary intake of vitamins.
AETIOLOGY OF RIBOFLAVIN DEFICIENCY

The two major factors responsible for the high prevalence of vitamin deficiencies are: (a) inadequate diet due to poverty, ignorance, food taboos, and superstitions; (b) infections - diarrhoea, worm infestation and respiratory infections, due to unhealthy environment, ignorance and unprotected water.

Nutrition surveys carried out by the Indian Council of Medical Research (ICMR) in different parts of India provide information about the nature and magnitude of nutritional disorders and the dietary patterns and nutrient intakes in children belonging to different socio-economic groups (79).

The diet surveys show that while protein requirement can be met in the process of meeting calorie requirement, deficits of vitamins' particularly vitamin A, riboflavin, followed by folic acid and pyridoxine, as well as minerals such as iron and calcium cannot be met through the traditional poor man's diets, unless there is qualitative improvement. Though the primary cause may be inadequate food intake, the root causes are a combination of social, economic and environmental factors typical of under development.

Infectious illness results in anorexia, malabsorption, increased urinary and faecal losses of nutrients and thus a cycle of infection and worsening nutritional status is established (80). The effects of infectious diseases differ according to the nature of the infection, the site affected, the age of the host and the nutritional status. An infection, innocuous for well-nourished children, can precipitate
acute malnutrition and cause death in children of poor communities. Undernutrition decreases immunological resistance and infection reduces intake of food (81).

**Metabolic sequelae of infection:**

It is important to note that both anabolic and catabolic processes occur at the same time, accentuating the magnitude of the metabolic alterations. These processes are generally accompanied by fever, which increases metabolic rate (by about 13% per degree C) (82), and consequently energy requirements at a time when the host is often anorexic. Since carbohydrate stores are inadequate to meet these needs (83) and lipid stores are not effectively used in the infected patient (84), another source of energy is required. In most infections, this turns out to be gluconeogenesis, the production of glucose by the liver from amino acid precursors released from contractile proteins of muscle (85,86,87,88).

There is an apparent coordination of protein and energy metabolism as the branched-chain amino acids released by proteolysis of muscle are oxidized *in situ* for energy and the amino acids reaching the blood stream are taken up by the liver for synthesis of acute-phase protein reactants, other anabolic repairs and stress responses (87). These complex adaptations are aided by increase in circulating insulin, glucagon and growth hormone levels, loss of diurnal variation and elevation in glucocorticoid levels, and functional insulin resistance in muscle (89,90).
Lipid metabolism can be grossly altered as well, depending on the nature of the infectious stress, duration of infection, and its severity (91,92,93). Lipid changes are particularly evident during gram-negative bacillary infections, resulting in hypertriglyceridemia due to defective lipid clearance from serum (94). Tremendous changes also occur in a number of minerals during infection (95).

Far too little is known about the impact of infectious illness on vitamin nutriture of the host. Successive generations of research workers have often postulated that an increased intake of certain vitamins could help to prevent the occurrence of an acute infectious illness (96). Despite several decades of work devoted to this possibility, there is little to suggest that supernormal resistance can be achieved by an excess intake of any vitamin. Rather, host defense mechanisms seem to function optimally when vitamin nutriture is normal. Nevertheless, the popularity of daily mega doses of vitamin C continues to be a phenomenon of our times, and hopes persist that this measure will ward off attacks of the common cold and upper respiratory viral infections.

In studies conducted with healthy volunteers who were receiving recommended daily intakes of vitamins throughout the course of sandfly fever, no detectable changes in the vitamin metabolism was seen with the exception of an increased excretion of riboflavin (97). Heightened metabolic activity due to severe infection and fever undoubtedly accelerated the utilization of various vitamins. However, no changes attributable to the illness were detected in the serum concentrations of vitamins A, E, C or folacin or in the urinary excretion of
vitamin B₁, B₆ or niacin (98). Thus, febrile illness and negative nitrogen balance during sandfly fever appeared to be followed by reduced utilization and retention of riboflavin. Evidence carefully assembled by Scrimshaw and colleagues (96) however shows that overt symptoms of vitamin deficiency can precipitate in some individuals by the occurrence of severe infectious illness.

FUNCTIONAL CONSEQUENCES OF RIBOFLAVIN DEFICIENCY AND MALNUTRITION

Functional consequences of malnutrition are both overt and covert. In addition to some of the frank clinical signs and symptoms, malnutrition is also known to result in subtle functional impairments. In children, growth retardation is one of the most sensitive functional consequences of malnutrition. In animals, deficiency of any single micronutrient results in anorexia and growth retardation. However, in human situations effects of individual micronutrient deficiencies on growth have not been effectively examined, since normally multiple deficiencies exist and it would be unethical to conduct selective deprivation studies in children.

Several workers have examined the consequences of smaller stature due to growth retardation in early childhood on physical performance and work output (99,100). These studies clearly show that small built individuals as well as obese individuals are at a disadvantage. There is an optimal height and weight for an optimal physical performance and work capacity.

Most of these studies deal with calories and protein, there is very little information on deficiency of micronutrients and physical
performance. When diet and fitness are related, the individual aspects of physical fitness must be differentiated. Physical fitness and performance are constituted mostly of morphological prerequisites (body size and composition), energy output (aerobic and anaerobic processes) which depend mostly on the efficiency of cardiorespiratory systems, neuromuscular functions (strength, technique), and finally psychological factors (motivation, tactics). These factors play a more or less dominating role depending on the nature of the physical performance (101,102).

In a study on school aged Columbian boys, marginal malnutrition did not influence the efficacy of treadmill walking (103). Iron deficiency anaemia is known to worsen physical performance (104).

While growth is defined as physical maturation, i.e. increase in body size, development is defined as functional maturation. Psycho-motor performance is an attribute of the latter. Motor development is a continuous process through which a child acquires, basic movement patterns, and skills (105). Reduced motor nerve conduction velocities are commonly observed in severe undernourishment (106,107). The defects in cellular metabolism of the brain due to nutritional deficiencies, lead to electrophysiological changes and finally manifest as behavioural (sensory, motor, intellective and personality), abnormalities. Amongst the B-vitamines, thiamin has received maximum attention. Some of the earlier studies have been reviewed by Brozek (108). In the first Minnesota study conducted by Keys et al (109), restricted intakes of thiamin, riboflavin and niacin did not alter motor performance and any other aspects of fitness.
Short term effects of moderately restricted intakes of nutrients on individual performance have been described by some (110) but denied by others (58,109). Some of the earlier studies reviewed by Brozek and Vaes (108) failed to indicate a deterioration in performance capacity including psychomotor performance. But later studies reveal electrophysiological changes (111), as well as impairment in psychomotor performance in human riboflavin deficiency (112,113).

Amongst the vitamins, the deficiency of thiamin, pyridoxine, folic acid and riboflavin have been shown to affect the psychomotor performance. Deterimental effects of pyridoxine deficiency on central nervous system as reflected in behavioural and electrophysiological changes have been reported in a few animal and human studies (114, 115,116).

Folate deficiency in utero and suckling states impaired maze performance in rats (108) and also brain function in infants fed on milk from mothers with low serum folate levels (111,116). Administration of iron and folate supplements to Indian children of 5-6 years age, produced significant improvement in verbal and performance IQ of WISC (117).

Numerous studies in animals reveal an adverse effect of B-vitamin deficiencies particularly vitamin $B_6$ deficiency on immune mechanisms (118). Elevated morbidity has been reported in deficiencies of vitamins $B_1$, $B_2$, $B_6$ and C in humans (119).

In addition to these functional consequences, malnutrition is also known to have several other functional effects such as wound
healing (120, 121) and cataract formation (122, 123, 124). A study by Bhat (125) has demonstrated lowered riboflavin nutritional status in cataract patients. The physiological responses to inadequate dietary intake of riboflavin are numerous and depend to a certain degree on the extent and duration of the deprivation (30, 126).

ASSESSMENT OF RIBOFLAVIN STATUS

Assessment of riboflavin nutritional status can be done by (a) enquiry into dietary history, (b) clinical examination of subject for the presence or absence of signs of deficiency and (c) laboratory tests on easily available body fluids such as blood and urine.

While the first two methods are quick, they give only a rough idea of the state of nutrition. More precise information on individuals can only be obtained through biochemical tests.

**Dietary intake:**

There is no generally accepted method of measuring the dietary intake of free-living individuals. Yet there is a constant demand by clinicians, epidemiologists, nutritionists, the food industry and others for such measurements to be made. Nutrient intake in a given population group is usually estimated by dietary survey data, which is based on the nutrient composition of raw foods consumed by individuals. Physical and physiological status of the population is generally interpreted in terms of nutrient intake and recommended daily allowances. The validity of dietary surveys largely depends upon the methodology used for collection of information on food intake.
assumptions that (a) processing and cooking losses are insignificant
(b) nutrient contribution of various spices and condiments used in
recipes is nil, and (c) varietal differences especially in the major
foods such as cereals and legumes are not considerable. In recent
years epidemiologists and other medical researchers have become
increasingly interested in the effects that diet may have on health
and disease. The chief impediment to research on nutritional causes
of disease has been uncertainty about the validity of existing dietary
assessment methods and the consequent uncertainty about the results
obtained with them (128). Numerous studies over the past 30 years
have tried to establish the reliability or validity of numerous
methods, but the methods themselves have been variable, and the
criteria against which they were judged were also variable, with the
result that until recently one would have been hard pressed to make
a statement with any confidence about any of them.

Controversies have raged over the 'best' method. With respect
to assessment methods, four general approaches have been used in
dietary studies. Each has its strength and weaknesses. One widely
accepted technique is the dietary history approach developed by Burke
and Stuard (129). It requires an extensive interview by trained
nutritionist. The 24 hr recall method may be administered by persons
with less training, in a shorter time. The subject is asked to recall
his exact intake in the last 24 hrs. In its favour, memory of recent
intake may be more precise and quantities may be estimated with greater
accuracy. On the other hand, individual diets vary greatly from day
to day (130,131,132) so that a single day's intake may not be
representative. The seven-day recall method is an attempt to achieve greater representativeness. However, memories of intake may fade rather quickly beyond the most recent day or two, so that loss in accuracy may exceed gain in representativeness.

A more common approach, and one that appears to rest on firmer ground, is the seven-day record of actual intake, with either weighing, measuring or estimation of portion sizes. Such a method should give a reasonably accurate measurement of actual intake and an average of seven days is more representative of usual intake than a single day. The chief object is that it is impractical for clinical or epidemiological studies since it demands a high degree of cooperation on the part of subjects, and the number who could be induced to participate may be a small and unrepresentative sample.

Histories and seven-day records may be valid, but are time consuming and require much training on the part of the interviewer or cooperation on the part of the subject. 24 hr recalls are valid for group values but are not valid enough for individuals. As a result, a number of investigators have attempted to develop methods which would nevertheless have validity. Other approaches have tended to emphasize more qualitative measures such as frequency of consumption.

Questionnaires which ask only the frequency with which specified foods were eaten in a given interval are capable of being administered quickly in person or by mail to large numbers of people, and so lend themselves to large scale epidemiologic research. Consequently,
number of investigators have attempted to develop such methods (133,134,135,136). Abranson et al (135) compared the results from a 30 minute segment of an interview on frequency and usual intake with those from longer interview. Thus both Abranson et al (135) and Morgan et al (133) found a food frequency interview to provide reliable results. Methods involving frequencies or other types of short questionnaires offer the possibility of great usefulness in epidemiologic studies. Some of these methods do not yield precise milligram of nutrients; nevertheless, for epidemiologic purposes the ability to classify individuals into categories of intake would be of great value.

The oral questionnaire method has the practical advantage of being simple and less time-consuming and can, therefore, be easily applied to individual subjects. However, the reliability of the results obtained by oral questionnaire depends upon the intelligence and co-operation of the subject and the patience and training of the investigator. Despite these limitations, the oral questionnaire method because of its easy practicability has found useful application in clinics and hospitals (137,138).

Thus, the information obtained by surveys is more complex, human, empirical and closer to practical action than information which can be scientifically rationalized. Even though the information provided through dietary surveys does not have the accuracy of the physical and chemical sciences, it can be useful for more careful understanding of scientific facts. Diet surveys are useful particularly at the community level, but often it is difficult to obtain reliable data
and even when available, they provide a rough estimate of the amount consumed, but not the amount absorbed or utilised.

Clinical examination:

Clinical examination has always been, and remains an important practical method for assessing the riboflavin nutritional status of a community. This is categorized as direct nutritional assessment. Essentially, the method is based on examination for changes that can be felt in superficial epithelial tissues. This method of assessment, based on the recognition of certain physical signs, has the advantage of relative inexpensiveness, as neither elaborate field equipment nor a costly laboratory is needed. The cheapness and relatively easy organization of nutritional assessment by means of clinical examination have sometimes led to the assumption that the method is simple, quickly mastered by the beginner, and yields results that are easy to interpret. This is not the case. Like any other form of assessment, the method has its own limitations.

Various non-nutritional environmental influences can sometimes be responsible for identical appearances. For example, the clinical picture of angular stomatitis, often incorrectly considered pathognomonic of riboflavinosis, can result in India from the excessive chewing of betelnut preparations (paan) containing large amounts of irritant lime (69). The association of these signs with biochemical and other tests may help to identify the lacking nutrient or nutrients responsible for a given lesion, but the commonly found
simultaneous deficiency of many nutrients in diets may render the final specific diagnosis difficult.

Laboratory tests for the assessment of riboflavin status:

Riboflavin status can be assessed by measuring urinary excretion of the vitamin in fasting or random or 24 hour specimens, load return tests, measurement of erythrocyte riboflavin concentration and the erythrocyte glutathione reductase test.

a. Urinary riboflavin:

Urinary riboflavin measurements were the most frequently used index of status (139,140,141,142,143,144,145,146,147,148,149). Urinary riboflavin is capable of giving accurate information provided it is recognized that the results could be altered by factors such as physical activity, environmental stress, temperature, nitrogen balance etc. (150,151,152,153,154,155).

Riboflavin is not metabolized extensively by the body and hence metabolites (except those formed by bacteria) are not detected in the urine. Correlations between urinary riboflavin expressed per gram creatinine and dietary intake have been observed in controlled experiments on human volunteers as well as in population surveys (156,157,158,159). This test is particularly useful for comparisons between populations, and for monitoring the course of experimental deficiency, but is less useful for comparisons between individuals.

Ingestion of antibiotics increases urinary riboflavin excretion (160) and use of oral contraceptives lowers urinary excretion of
riboflavin in women (161) and rats (162). This appears to be due to an increased retention of the vitamin by tissues such as liver. Some improvement in the sensitivity of the urinary index can be obtained by using the test dose procedure (proportion of an oral dose which is subsequently excreted), since this is a good measure of body stores, especially for partly depleted subjects. However, it is more difficult to perform in field conditions, since it requires considerable subject cooperation. Recently interest has been renewed in urinary metabolites of riboflavin (163,164), but it is not yet clear whether they are useful for determining the status.

The concentration of riboflavin in urine has been usually estimated by using either fluorimetric (165,166,167,168) or microbiological (169,170,171) assay methods.

b. Blood riboflavin:

Direct measurements of plasma or red cell flavin concentrations have provided another index (149,172,173,174,175,176), but have proved generally less popular than urine assays. Perhaps the spread of values between deficient and saturated subjects is less wide. Plasma and urinary levels as might be expected, tend to reflect recent intake, whereas red cell levels are more closely related to tissue levels, and are mainly in the cofactor forms, riboflavin phosphate or flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Banji (65) found that the red cell flavin levels are insensitive to marginal changes in riboflavin status, but Bates et al (177), Glatzle et al (178), and Floridi et al (175) have found good agreement between RBC riboflavin and the glutathione reductase test.
Measurement techniques for blood riboflavin include: (a) microbiological assay, most commonly using lactobacillus casei (169,179), although the protozoa Tetrahymena pyriformis and Ochromonas danica (180) may have some advantages; (b) fluorescence assay (180,181) with the possibility of a differential analysis of the coenzyme forms, and (c) high pressure liquid chromatography (175,182,183,184) which offers both specificity and sensitivity.

c. Assay of erythrocyte glutathione reductase (EGR) and its in vitro activation (EGR-AC) test:

What is actually needed is knowledge concerning the body reserves and metabolic state of riboflavin in an individual. In recent years, estimations of vitamin nutriture have been based on the participation of a vitamin in its coenzyme form in a specific enzymatic reaction. These approaches not only indicate whether adequate amounts of a vitamin are ingested but also give valuable information concerning the conversion of the vitamin precursor form to its biologically active coenzyme form. The principle of the glutathione reductase status assay, which has now become the most popular tool for the biochemical assessment of riboflavin status, is the in vitro measurement of resaturation of erythrocyte glutathione reductase with its cofactor FAD, after it has become partly depleted in vivo. Recognition that this index might provide an useful source of information about human riboflavin status came initially from the studies of Bamji (65), Beutler (185) and Glatzle et al (186).
Advantages of the test are that it gives an instantaneous measure of tissue saturation, with minimal need for cooperation by the subject; it requires only very small amounts of blood and is therefore applicable, for instance, to small premature infants or small animals. The apoenzyme appears relatively stable for long periods, if stored correctly (179,187). The test is specific for variations in riboflavin status, and animal studies (188) have shown it to be minimally affected by inanition and other deficiencies. Since glutathione reductase is one of the first enzymes to respond, it is highly sensitive to marginal deficiency states (189). It is little affected by short-term unimportant fluctuations in intake and can be performed on non-fasting subjects. It responds rapidly to repletion of body stores during riboflavin supplementation. Infections and Glucose-6-phosphate dehydrogenase deficiency may however increase the saturation of EGR giving lower values for EGR-AC even in riboflavin deficient states (190,191).

Information about riboflavin status can be obtained either from the basal enzyme (unstimulated) activity expressed per unit of haemoglobin, or from the 'activation coefficient' (AC) which is a measure of the ratio of FAD stimulated and unstimulated activity. Most workers currently use the latter alternative, since limits of 'normality' for it have been defined more clearly, and the ratio is potentially less sensitive to confounding factors such as anaemia. Basal activity correlates well with the activation coefficient in riboflavin-deficient populations before and after supplementation (177). Both the criteria for the definition of normal ranges and the values actually quoted have varied somewhat between different studies and
different analytical methodologies. Studies based on Glatzle's
procedure (186,192,193) have generally accepted an upper limit of
1.2 for the normal range of the activation coefficient, but values
below 1.0 seem to occur fairly frequently here. Several other
investigators (177,194,195,196,197) had an upper limit of 1.3 because
most of the values observed in healthy western populations were below
1.3. An upper limit of 1.4 was preferred in the elderly population
of UK (198).

Recent studies in Indian and rural Gambian populations have
yielded mean values for EGR-AC in the range 1.8-2.0 (69,199,200,201,
202) with practically no values below 1.4, unless glucose 6-phosphate
dehydrogenase deficiency is present. This deficiency is fairly common
in some mediterranean, African and Asian regions (191,203,204). In
such subjects, glutathione reductase fails to lose its coenzyme even
in relatively severe riboflavin deficiency, presumably responding
to a compensatory mechanism which protects the overall glutathione
reductase pathway. Correlations between the glutathione reductase
test and clinical deficiency signs between individuals in a homogenous
population have generally been rather weak (65,69,177,187,202,205),
although in a number of studies riboflavin (or multivitamin) supplemen-
tations have produced parallel (though not necessarily simultaneous)
improvements in both the clinical and biochemical indices.

In the development of any disease, biochemical changes precede
clinical changes and hence biochemical tests help to identify the
disease at the subclinical stage. The biochemical machinery of the
body is very complex and the deficiency of more than one nutrient may lead to molecular event(s) which may ultimately be the cause of morphological lesion(s). A mention is essential at this juncture that though more than six decades have passed since the discovery of vitamins, and a great deal is known about the biochemical and clinical lesions of specific deficiencies, for most nutritional deficiency diseases the exact molecular basis is not understood. Nor are the very early symptoms of deficiency recognised.

Glutathione reductase test was used for assessing riboflavin status in five epidemiological studies (159,196,206,207,208). Three of these studies (196,206,208) reported reasonable consistency between the two indices, whereas the other two (159,207) found little correlation.
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SCOPE OF THE WORK

The studies reported in the present thesis were aimed at examining three aspects of the dynamics of riboflavin deficiency.

(a) Verification of the earlier hypothesis that upper respiratory infections mobilise riboflavin from tissue stores and lead to deterioration in riboflavin status, through appropriate studies in children and animals.

(b) To examine the effects of riboflavin supplementation on select parameters of psychomotor and work performance where there was some earlier evidence of correlation with riboflavin status.

(c) To assess the validity of the currently used interpretary guidelines for the glutathione reductase test.

The thesis comprises of six chapters. Chapter I deals with the review of literature. The methodologies used in the investigations are given in Chapter II.

The third Chapter describes the findings of a one year riboflavin supplementation study in a low-income group urban school children.

The fourth Chapter describes a study in mice which was aimed at examining the effects of respiratory infections on riboflavin nutriture.

The fifth Chapter deals with studies carried out to examine the validity of the currently suggested interpretary guidelines for assessing riboflavin status by the EGR-AC test.

The last Chapter summarises the findings and conclusions.