RIBOFLAVIN AND ORAL EPITHELIUM

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THESIS

Submitted for the Degree of Master of Medical Science

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University of Glasgow, 1998

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" this I know, moreover, that to the human body it makes a great difference whether the bread be fine or coarse: with or without the hull, whether mixed with much or little water, strongly wrought or scarcely at all, baked or raw....whoever pays no attention to these things, or paying attention, does not comprehend them, how can he understand the diseases which befall man? For, by every one of these things, man is affected and changed this way, or that, and the whole of his life is subjected to them, whether in health, convalescence, or disease. Nothing else, then, can be more important or more necessary to know than these things."

(Hippocrates II)

SUMMARY

There is conflicting evidence as to the role of riboflavin in cancer. Riboflavin deficiency has been demonstrated to be both a protective factor and potentiating factor in experimental carcinogenesis. In human studies, riboflavin deficiency has been linked to oesophageal cancer, although there is little in the literature regarding the effects of riboflavin deficiency on the oral mucosa. This thesis examines the effects of riboflavin deficiency on the oral epithelium. Chapter 1 is a review of riboflavin, its sources, functions, metabolism and measurement and the effects of riboflavin deficiency, particularly its role in cancer.

It was decided to attempt to induce riboflavin deficiency in the hamster. This animal was chosen with a view to using the frequently used hamster cheek pouch model of experimental oral carcinogenesis in subsequent studies. Chapter 2 describes the method of bleeding of the hamsters and verification that testing of riboflavin levels was possible on the small sample size available. Optimisation of the erythrocyte glutathione reductase test for hamster blood is described. The biochemical criteria for assessing the presence of riboflavin deficiency in the hamster were determined.

Chapter 3 describes the induction of riboflavin deficiency in a group of hamsters together with biochemical monitoring of their riboflavin status. Compartment and cell analysis of the lingual epithelium was undertaken. A number of difficulties were encountered in the experiment. Firstly, both experimental and control hamsters suffered from gastro-enteritis (wet tail) and several subsequently died, reducing the numbers available for analysis. Analysis

of the riboflavin content of the diet indicated that the difference between the experimental and control diets was less than expected.

In Chapter 4 the problems which were met and possible avenues for future research are discussed.

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ACKNOWLEDGEMENTS

I wish to thank numerous people who have given me support throughout the duration of this thesis. I acknowledge the supervisory support of Professor G MacDonald and the moral support of both Professor D Wray and Dr B Doubleday. I also wish to express my gratitude to Joyce Hope whose immaculate preparation of the histological sections was only surpassed by her good humour, to Alan Reid , the biochemist at The Victoria Infirmary who helped on many dark evenings to improve my understanding of things "biochemical" and lastly to my long suffering parents who have probably earned several honorary degrees along the way.

CHAPTER 1

RIBOFLAVIN IN MEDICINE-INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 HISTORICAL INTRODUCTION

The existence of riboflavin (vitamin B_2) was first realised in 1917 when Emmett and McKim showed there was a dietary growth factor for rats in rice polishings. Shortly after thiamine was isolated from yeast concentrates, Emmett and Luros (1920) suggested the presence of a second nutritional factor which unlike thiamine, was a thermostable growth promoting substance. The complexity of this factor was not initially realised, due to the fact that animal assays were used to determine the factors and not all animal species responded similarly. However, once this was realised, the thermostable "factor" was designated "vitamin B_2 complex" and was found to consist of riboflavin (a rat, growth-promoting factor), pyridoxal (a rat, pellagra-preventing factor), pantothenic acid (a chick, antidermatitis factor), and nicotinic acid (a human, pellagra-preventing factor).

Whilst in 1927 the British Medical Research Council proposed the name vitamin B_2 for this heat stable component, in the USA it was known as vitamin G. Attempts to isolate it began in 1932. Within one year, crystals of "oroflavin" were isolated from egg white by T. Wagner-Jauregg at R. Kuhn's Institute. Previous investigators had succeeded in obtaining impure preparations of flavin which were given a variety of names such as lactochrom, cytoflav and ilyochrome, but it was not until 1932 that this substance was recognised as a vitamin.

Warburg and Christian in 1932 isolated a yellow enzyme which contained riboflavin and which helped in the understanding of riboflavin biochemistry. In 1933, György *et al.* isolated pure vitamin B_2 from milk and recognised its growth promoting activity. By 1935 Kuhn *et al.* and Karrer *et al.* had confirmed the structure and achieved the synthesis of riboflavin. By this stage there was some confusion due to the variety of names and reported symptoms arising from deficiency of vitamin B_2 (Guha, 1935). The Council on Pharmacy and Chemistry (1937) agreed on its nomenclature and the name flavin, was given to the water-soluble pigment necessary for the normal nutrition of the rat and for growing chicks. The Conference of the American Chemical Society decided to use the name flavin, not lactoflavin as it was not initially isolated from milk, but from egg white. The name riboflavin was eventually decided upon, as it is a ribose derivative of isoalloxazine. It is also referred to as vitamin B_2 as it was the first member of the "vitamin B_2 complex" to be isolated.

1.2 RIBOFLAVIN IN NATURE

1.2.1 Isolation and Structure

Riboflavin is a fine, orange-yellow crystalline powder which is practically odourless, but which has a bitter taste (Street and Street, 1952). Water solutions give a greenish, yellow fluorescence (Cooperman and Lopez, 1984). Riboflavin is found in nature in three forms; as free riboflavin, riboflavin 5' phosphate (FMN) and flavin adenine dinucleotide (FAD), the latter two of which are protein bound. Most of the riboflavin found in nature is protein bound. Some of the proteins involved have been identified as albumin and immunoglobulins (Merrill *et al.*, 1979 and 1981). One particular binding protein of the immunoglobulin group appears to correlate with pregnancy. Binding to plasma proteins facilitates the delivery of

riboflavin to tissues or sequesters it from glomerular filtration. The different types of flavins belong to the group known as the isoalloxazines, but can also be designated as benzopteridines. Riboflavin itself has the formula 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine (McCormick, 1990). Flavoproteins are ubiquitous, being present in all biological fluids and tissues. FAD has a greater molecular weight than FMN or riboflavin itself. Riboflavin itself has various melting points due to the polymorphism of the crystals.

1.2.2 Functions of Riboflavin

FMN and FAD are found in a large number of systems which function in carbohydrate, fat and lipid metabolism (Cooperman and Lopez, 1984). They serve as agents for the transfer of hydrogen between the nicotinic acid-containing coenzymes I and II and the iron-porphyrin cytochromes. Riboflavin participates in enzymatic reactions through intermediate free radical forms. It is essential for growth and repair in species from protozoa to man. It is important in both the preventive and radical quenching antioxidant defence systems of the tissues and the immune system by being involved in the regeneration of reduced glutathione (Bates and Thurnham, 1991). There appears to be a lower limit of hepatic flavin levels compatible with life (Fass and Rivlin, 1969). Riboflavin is also necessary for the conversion of pyridoxine and folic acid into their coenzyme forms (Cooperman and Lopez, 1984).

1.2.3 Biosynthesis of Riboflavin

Many micro-organisms and plants synthesise riboflavin from simple precursors, such as purine (guanine). This serves as an obligatory precursor with clearance of the imidazole portions of the purine with loss of carbon at position 8, before incorporation of diaminopyrimidine. Unusually large quantities of riboflavin are produced by some species of

micro-organisms such as *Candida species*. Commercially available riboflavin is made by bacterial synthesis.

1.3 RIBOFLAVIN IN THE BODY

1.3.1 Sources

Riboflavin is present to some degree in virtually all naturally occurring foodstuffs. Liver and cooked beef have very high levels and cholorophyll-containing plants such as broccoli, asparagus and other green vegetables also contain a considerable amount. Most riboflavin consumed in Europe is derived from milk, meat, fish and eggs, whereas in developing countries it is principally supplied by cereals or starchy roots, although cereals are a poor source of riboflavin. The riboflavin content of foods can be affected by handling, processing and storing. For example milk loses hardly any of its riboflavin when it is pasteurised but loses 71 percent, after four hours in direct sunlight. This sensitivity to light is important if foods are sun-dried (Hunt, 1975). Faecal bacterial flora are a major vitamin source for coprophagic rodents (Hill, 1997).

1.3.2 Absorption

There appears to be considerable variation in the absorption of riboflavin among different species. In man it has been studied by following the urinary excretion. Riboflavin appears to be rapidly absorbed as shown by the early appearance of peak excretion rates and the parallel decline of the oral and intravenous curves if the vitamin is given orally. It is probable that absorption is limited to the proximal small intestine.

From experimentation, there appears to be a capacity-limited transport process (Jusko and Levy, 1975). FMN is hydrolysed in the upper gastrointestinal tract, giving free riboflavin which enters the mucosal cells of the small intestine. It is then phosphorylated to FMN by flavokinase. FMN enters the portal system bound to albumin and travels to the liver where it is converted to FAD. In experiments where bilateral nephrectomy was carried out and intravenous riboflavin was administered, it was rapidly excreted into the small intestine, especially the duodenum (Selye, 1943).

In animals the absorption of riboflavin appears to be different from man. Early experiments appeared to show that in rats and hamsters, absorption occurs by passive diffusion. This was confirmed by other workers in rats but little information exists on the situation in the hamster (Jusko and Levy, 1975). In the rat, the little absorption that does occur, takes place in the ileum and so this is a poor model for extrapolation to man.

1.3.3 Metabolism in Man

The body tends to facilitate the maintenance of nutritionally adequate levels of vitamin B_2 as well as the rapid elimination of excessive loads. If large doses are given, the easily saturated specialised transport process of the small intestine limits absorption whereas if only small doses are given, absorption is rapid and efficient. If there are high circulating levels there is rapid elimination in urine or bile. Also, if the body levels are high, little is bound to plasma or present as FMN or FAD, whereas if the body levels are low, most riboflavin is protein bound and little is excreted in the urine. An excess of riboflavin leads to only a slight increase in the storage of riboflavin in the liver. Most of the flavin taken in is stored as coenzymes which function catalytically with specific proteins.

1.3.4 Excretion

Riboflavin is phosphorylated to FMN in the intestine and to FAD in the liver. Elimination then occurs primarily by renal excretion, but also by biliary excretion and minor losses with body secretions and metabolic interconversions. Renal excretion in man involves glomerular filtration, tubular secretion and tubular reabsorption. If a large parenteral dose of riboflavin is given, man excretes most of this in the urine (Axelrod *et al.*, 1941). Typical excretion of riboflavin in the urine of an adult is 200μg in a 24 hour period. This decreases to 40-70μg in deficiency states. Dessner (1952) aimed to find a clinically useful method for the standard determination of riboflavin in man. He measured the amount of riboflavin excreted in the urine, using the fluorometric method, following a 5mg dose of riboflavin given intramuscularly. He proposed that if more than 30 percent of the 5mg dose was excreted, there was no deficiency whereas less than 20 percent excretion meant there was a decided deficiency. The Interdepartmental Committee on Nutrition for National Defence (ICNND) lists excretions of less than 27μg riboflavin/gm creatinine as an indicator of deficiency in man (Cooperman and Lopez, 1984).

1.3.5 Requirements

It is difficult to assess what is the correct daily requirement for riboflavin as it is unlikely that clinical signs and symptoms are the most sensitive index of adequate status (Bates and Thurnham, 1991). In humans the daily requirement of riboflavin varies from 0.4mg/day in young children to 1.3-1.7mg/day in adults (Cooperman and Lopez, 1984). The optimum level is obviously that which promotes the highest level of health. However, nutritional requirements may differ according to sex, age, activity or physiological state and can be influenced by drugs, smoking, alcohol and other factors (Sauberlich, 1984). It has been

suggested that the recommended daily allowance in some parts of the world is too high and requires revision (Brun *et al.*, 1990). Antibiotics such as penicillin or tetracycline can reduce the requirements for riboflavin in several species by inhibiting microorganisms in the gut that compete for riboflavin (Cooperman and Lopez, 1984).

1.4 MEASUREMENT OF RIBOFLAVIN IN THE BODY

The status of riboflavin in man has been assessed in a great variety of ways. Microbiological, fluorometric and enzymatic testing have been carried out, the latter now being the most popular although "high performance" liquid chromatography (HPLC) has been used more recently. Assays can be used to measure riboflavin in the urine, plasma, serum, erythrocytes or whole blood. One of the main problems with many of the methods is that the results reflect recent dietary intake rather than body or functional stores.

1.4.1 Microbiological Method

This method can be used to measure riboflavin levels in blood, urine and tissue samples. This method is based on the fact that many lactic acid producing organisms require exogenous riboflavin. *Tetrahymena pyriformis* (a protozoan ciliate), *Lactobacillus casei* and *Leuconostoc mesenteroides* have been used. If blood is to be tested, it is first citrated and frozen. FMN and FAD are deproteinized into their free forms and the supernatant obtained by centrifugation. The supernatant is added to a number of flasks containing basal medium and various quantities of distilled water. Various dilutions of serum are therefore produced. The flasks are inoculated with culture and incubated for three days. Growth is measured in optical density units. The measurements are plotted on a graph and values for riboflavin

calculated from standard curves. The method using *Lactobacillus* is virtually the same as previously described, but as an alternative to measuring the turbidity, the acidity can be measured (Baker and Frank, 1975).

The microbiological method is simple and the required equipment is inexpensive. However, if the subjects have received antimetabolites eg antibiotics, these may interfere with the growth of the micro-organisms. Strong *et al.* (1941) studied the applicability of the bacterial method in assessing the amount of riboflavin in the blood and urine of humans and animals and the effect of varied intake on urinary excretion. They felt that the direct assay of blood furnished a reliable measurement of riboflavin present.

Of the organisms described, *Tetrahymena pyriformis* responds equally well on a molar basis to riboflavin, FMN and FAD, whereas *Lactobacillus casei* uses FMN and FAD, but this is not proportional to the molar concentration of riboflavin in these analogues (Baker and Frank, 1975). Also, *Lactobacillus casei* responds to many other riboflavin analogues that may or may not have biological activity.

Unfortunately, although the microbiological method was thought to be more specific than the fluorometric method (see below), it requires larger quantities of tissues and is cumbersome to carry out (Bamji *et al.*, 1973). It is not the method of choice in most UK hospital laboratories.

1.4.2 Fluorometric Method

With this method the intensity of the natural fluorescence of flavins or the fluorescence of lumiflavins derived from flavins can be measured. Free riboflavin exists in only tiny quantities, therefore, total riboflavin in acid extracts is a measure of that resulting from hydrolysis of FMN and FAD. Riboflavin and FMN have the same fluorescence whereas FAD is only 14 percent as fluorescent. Riboflavin is more readily extracted by benzyl alcohol from aqueous solution than FMN or FAD, whereas FAD is completely hydrolysed to FMN in 5 percent trichloroacetic acid in 20 hours at 37°C. Therefore, by manipulating a sample the fluorescence due to each component can be measured and the total riboflavin calculated (Baker and Frank, 1975).

However, this method is slow and the calculations are laborious. It does have the advantage of delineating the various flavins. The problem with this assay is that there may be interference from other non-specific fluorescent substances. Bamji *et al.* (1973) generally found little difference between the microbiological method and the fluorometric method as regards consistency of results. Guinea pigs did give a slightly higher result than either human or rat samples. They proposed that this was possibly due to the presence of aforementioned non specific fluorescent substances in guinea pig tissues.

1.4.3 Enzymatic Method

This method does not directly measure actual riboflavin levels in the body. The vitamin in its coenzyme form participates in a specific enzymatic reaction. By measuring the activity of an enzyme which requires FAD as a coenzyme, an indirect measurement of riboflavin is obtained. This type of method is used to measure a number of vitamins such as thiamine and

pyridoxine. For riboflavin, erythrocyte glutathione reductase (EGR) activity is examined (Baker and Frank, 1975).

This enzyme catalyses the reduction of oxidised glutathione (GSSG).

$$NADPH + H^{+} + GSSG \xrightarrow{EGR(FAD)} NADP^{+} + 2GSH$$

Erythrocyte glutathione reductase requires as a coenzyme, FAD, a phosphorylated form of riboflavin. EGR activity is altered in vivo by dietary riboflavin, (NADPH-nicotinamide adenine dinucleotide; GSH- reduced glutathione).

When erythrocyte stores of riboflavin are optimal, if additional supplies of FAD are added in vitro, little or no enzyme activity should occur. However, if there is a suboptimal supply with insufficient FAD, addition of FAD to the blood cells could stimulate enzyme activity. Glatzle *et al.* (1970) looked at 190 healthy subjects. They found only five subjects had an activation coefficient >1.27 and 99 percent of the remaining 185 patients were in the range 0.85-1.2. It was therefore decided that activation coefficients of 1.2 or more would be considered as an indication of biochemical vitamin B_2 deficiency. They also advised that the results should be used with care as there may be stimulation of EGR activity when the haemolysate grows old and the presence of impurities may have an inhibiting effect.

Glatzle *et al.* (1973) demonstrated that activation coefficients in blood appeared to reflect quite well the flavin pool in body tissues such as the liver, making this a suitable technique to evaluate riboflavin status. Sharada and Bamji (1972) suggested that the correct interpretation

and practical usefulness of the EGR test depended on exact information regarding the influence of other nutrients. For example, although thiamine, folic acid and vitamin C deficiency had no effect on EGR activity and riboflavin concentration, pyridoxine deficiency in rats caused a decrease in EGR activity while also causing an increase in erythrocyte riboflavin concentration.

An ultramicro enzymatic method has been described and used in field studies in Thailand (Thurnham *et al.*, 1970). It requires only a small volume of blood from a finger prick. Some workers suggest that BGR assay (whole blood glutathione reductase) offers analytical and technical advantages over EGR assays and that it is a reliable and practical method for assessing riboflavin status in man (Garry and Owen, 1976).

1.4.4 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been used more recently, as a method of assessing riboflavin and appears to be reliable, fast and reasonably accurate (Fidanza *et al.*, 1989 & Johansen and Edlund, 1990). There appears to be a good correlation with the EGR method (Fidanza *et al.*, 1989). A wide variety of structural analogues of FMN can be obtained in pure form by HPLC (Nielson *et al.*, 1983). Venous blood samples are taken, centrifuged and following treatment with various reagents, added to a SPE-column (solid phase extraction). There can be much error in the technique due to evaporation of organic solvents. Internal standards need to be used to minimise error and these should be of close structural similarity to riboflavin (Lambert *et al.*, 1985). Botticher and Botticher (1987) described a method of determining vitamins B_1, B_2 and B_6 in serum and whole blood from only one sample preparation.

1.5 RIBOFLAVIN DEFICIENCY

1.5.1 Riboflavin Deficiency in Humans

It was thought at one time that riboflavin deficiency was probably the most prevalent avitaminosis. However, deficiencies of the water soluble vitamins often occur together (Street and Street, 1952). It is unlikely that pure riboflavin deficiency occurs in nature as riboflavin is closely linked with deficiencies of the other B group vitamins. When experimental deficiency is produced in humans, oral abnormalities develop, such as lesions at the angles of the mouth and on the lips. Signs such as dermatitis of the nasolabial folds, vestibule of the nose, ears and eyelids and diminution of the ability to perceive flicker have been reported (Horwitt *et al.*, 1949).

Where riboflavin deficiency is endemic there is soreness and burning of the lips, mouth and tongue. The lips are red, denuded, dry, chapped and fissured. There is angular stomatitis. The tongue is purple and pebbled. Photophobia and burning and itching of the eyes, visual fatigue and dimness of vision are present (Goldsmith, 1975).

Riboflavin deficiency has been reported as providing the host with resistance to malaria, and if riboflavin status is improved the host has increased susceptibility to malarial infection. However, this is more likely to be due to action on the parasite rather than increased defence by the host (Bates *et al.*, 1986). Dutta *et al.* (1988) examined the effects of dietary riboflavin deficiency on the fragility of red blood cells, on the reticulocyte count and on the concentration of ATP and reduced glutathione (GSH) in red blood cells, all of which have been suggested as being involved in the protective mechanism. They demonstrated that the erythrocytes of deficient animals were more resistant to haemolysis by H_2O_2 and

ferriproto-porphyria-IX, that GSH was actually increased and ATP and the reticulocyte count were unaltered. Thus it would appear that the protective mechanism is more likely to be due to direct effects on the parasite rather than the metabolic status of the host.

Biochemical evidence of riboflavin deficiency has been found to be more prevalent in oral contraceptive users (Newman *et al.*, 1978). This does not necessarily mean there are signs and symptoms of deficiency (Sanpitak and Chayutimonkul, 1974). Iyengar (1973) analysed the diets of pregnant women of low income groups in India. Those suffering from angular stomatitis and glossitis were found to have a poor intake of riboflavin. When their diet was supplemented with riboflavin some improved, however there were also some who did not respond until they were also treated with pyridoxine. There have been reports of riboflavin deficiency in chronic diabetics. Reddi *et al.* (1990), in an experiment on streptozotocin diabetic rats, suggested that this may be related to augmented urinary excretion of riboflavin.

Biochemical similarities exist between hypothyroidism and riboflavin deficiency (Rivlin *et al.*, 1968). Two flavoprotein enzymes, in addition to FMN and FAD were depressed in both hypothyroidism and riboflavin deficiency. It was postulated that the reduction in activities of these enzymes was related to the common factor of diminished synthesis of FMN and FAD from riboflavin. Alcoholics are often riboflavin deficient, this probably being due to inadequate intake as they often have multiple vitamin deficiencies (Rosenthal *et al.*, 1973). Copper, zinc, cobalt, manganese, iron and cadmium are thought to chelate with riboflavin and FMN and may induce deficiency (Cooperman and Lopez, 1984).

1.5.2 Experimental Riboflavin Deficiency in Animals

Riboflavin deficiency can be induced experimentally by feeding a riboflavin deficient diet (Kim and Roe, 1985), administering a riboflavin antagonist, such as galactoflavin (Hoppel *et al*, 1979) or by chronic alcohol administration (Kim and Roe, 1985) The effects of riboflavin deficiency in a variety of species of animals have been widely documented. The majority of the work has been carried out in the rat model and this is reflected in the extensive literature available. There is little in the literature specifically describing the effects of riboflavin deficiency in the hamster.

Hamsters appear to require a higher dietary intake of riboflavin than either mice or rats. They need at least 6mg/kg diet as compared with 1-3mg/kg in the mouse and 2.5mg/kg in the rat (Cooperman and Lopez, 1984). Indeed, the recommended levels usually vary between 12 and 20mg/kg of diet (National Academy of Sciences, 1978). Methods of monitoring biochemically the progression of riboflavin deficiency vary, but in an experiment on hamsters reported by Kim and Roe (1985) the erythrocyte glutathione reductase test was utilised. They found that initially there was considerable fluctuation of erythrocyte gluathione reductase activation coefficients. This occurred in both experimental and control animals. They proposed that this was likely to be due to refection, as previous experiments with rats fitted with tail cups had prevented coprophagy and allowed riboflavin deficiency to develop without early recovery. Salley and Weatherred, (1964) carried out studies to obtain basic information on riboflavin deficiency in Syrian hamsters. Forty two animals were divided into three groups; one fed a diet deficient in riboflavin *ad lib*, one pair-fed but supplemented with 1.6mg riboflavin/100g of diet and lastly a group fed a diet with sufficient riboflavin *ad lib*. Two animals from each group were sacrificed at weekly intervals over seven weeks. Using

the microbiological method of analysis they found liver riboflavin levels of near 40μg in the supplemented groups, but these fell to 15μg by the sixth week in the group fed a riboflavin deficient diet. Plasma levels showed no significant variations throughout the experimental period in any of the groups. Clinical signs of deficiency, such as weight loss, lethargy, matted fur and tremors were noted. These effects only became apparent when the liver riboflavin levels fell below 60 percent of the controls. They concluded that riboflavin content of liver provided a quantitative measure of the acute deficiency state before other signs became apparent. This, unfortunately is not a feasible method of monitoring riboflavin levels in experiments which require the animals to survive throughout the experimental period. It must also be noted that the numbers of animals in each group was small. It is difficult to know whether the lack of an effect on the plasma was a reflection of the technique of testing or a true reflection that riboflavin levels are maintained in the plasma despite falling levels in other tissues.

The majority of early experiments which involved riboflavin deficiency in rats or mice, did not monitor riboflavin levels biochemically, throughout the experimental period, often relying on weight loss alone as an indicator of riboflavin status.

Experimental riboflavin deficiency has a number of profound effects on the tissues, especially epithelial. Early in the history of riboflavin studies, Adams (1936) supplied white rats with a riboflavin deficient diet. They developed thinning of the hair and alopecia after 35 days. He concluded that there was a marked reduction in the rate of respiration of the skin cells. No testing of riboflavin levels was carried out. Reported signs in the rat model include, ragged fur, hair of uneven length, scaly skin, areas of alopecia due to separation of the basal portions

of hair shafts from the anchoring cells and conjunctivitis with corneal vascularisation and opacity. Lippincott and Morris (1942) reported that young mice fed on a riboflavin deficient diet, had thin hair about the snout and ears and that they developed cracks and fissures on their snouts and the inner surfaces of their front legs. Histologically there was focal atrophy of the epidermis sometimes with the layer of keratin directly on the collagen fibres of the corium. Myelin was lost in some areas and some animals had cataracts. As these experiments were carried out so soon after the discovery and isolation of riboflavin, doubt must be cast on the validity of the findings. It is likely that the animals were suffering from multiple vitamin deficiencies, rather than pure riboflavin deficiency.

When baboons were fed a riboflavin deficient diet, they developed extensive scaling of the face, arms, hands and legs. Microscopically the cutaneous lesions demonstrated hyperkeratosis, acanthosis, extensive "pseudocarcinomatous" hyperplasia with thickened, branching rete pegs, aberrant individual cell keratinisation and intraepidermal "epithelial pearl" formation. The oesophageal mucosa was thin and pale with penetrating ulcers and similar changes in the oral mucosa (Foy *et al.*, 1972; Foy and Kondi, 1984). Not all the animals were on the riboflavin diet for the same amount of time (8-24 weeks) and in addition one of the animals was fed a riboflavin antagonist, galactoflavin. No subjective differences in histological findings were noted, but no quantitative histology was carried out. What was also of interest was the relative sparing of the ocular and respiratory epithelium, suggesting lower requirements for riboflavin of these tissues. Wynder and Klein (1965) fed mice a riboflavin deficient diet, varying in fat and carbohydrate content for up to nine weeks. Initially, there was atrophy of the squamous epithelium which paralleled the body weight and degree of deficiency, although again, no mention was made of riboflavin monitoring.

Hyperkeratosis also occurred early on in the experimental period. Between the fifth and eighth weeks, a slightly irregular reduction of the epithelium of the tongue occurred, but in the seventh to ninth weeks epithelial hyperplasia occurred. Changes throughout the experimental period occurred more quickly in those animals fed a diet with higher fat content. Shaw and Phillips (1941) had previously found that symptoms of deficiency appeared earlier in rats on a high fat diet. No explanation was proposed for this.

As riboflavin deficiency progresses, the body weight reduces, although deficient rats have been found to consume 15-20 percent more calories to maintain the same weight as controls (Goldsmith, 1975). Young mice fed on a riboflavin deficient diet, failed to grow normally and died in 6-8 weeks while adult mice gradually lost weight and died in 8-10 weeks. The animals moved in a hunched position with a dragging or jerky gait (Lippincott and Morris, 1942). In one of the few experiments involving hamsters and riboflavin deficiency, Kim and Roe (1985) divided 36 hamsters into six groups, varying the riboflavin and alcohol content of liquid diets. They lost six animals during the experimental period of 13 weeks, although there was no explanation why and after the first two weeks of diet, the riboflavin restricted group gained less weight.

Norton *et al.* (1979) induced riboflavin deficiency in rats by administering galactoflavin, an antagonist of riboflavin, together with a riboflavin deficient diet. In the bone marrow, decreasing cellularity was noted as riboflavin deficiency progressed and by the fifth week, proerythroblasts were noted to be absent. There appeared to be asynchrony of rat erythroblast maturation with a decrease in the rate of ribosome turnover. Demyelination of rat sciatic nerve fibres occurred, while nonmyelinated nerve fibres were unaffected. It has

been suggested that the demyelination may be due to altered fatty acid metabolism (Norton *et al.*, 1976).

Riboflavin deficient swine show retarded growth, with dermatitis, inco-ordination, nerve degeneration, corneal opacities and cataracts whereas riboflavin deficient dogs show ataxia, weight loss, weakness and fatty degeneration of the liver (Goldsmith, 1975).

1.5.3 Riboflavin Deficiency and Other Nutritional Deficiencies

It is unlikely that pure riboflavin deficiency is found in the general population. This is partly due to the fact that the foods which contain riboflavin also contain other nutrients and therefore if they were lacking in the diet, there would likely be multiple deficiencies. In addition, riboflavin is involved in the metabolism of other nutrients such as folic acid and pyridoxine. It is common to find deficiencies of several B group vitamins.

Vitamin B_6 (pyridoxine) takes part in the transport of amino acids into and out of cells and red blood cells play an important role in the transport of amino acids (Anderson *et al.*, 1976). Krishnaswamy (1971) treated patients with oral lesions such as angular stomatitis and glossitis with riboflavin and vitamin B_6. Seven out of 13 patients responded to riboflavin, although they required vitamin B_6 for complete resolution of symptoms. Six required vitamin B_6 with only a limited response to riboflavin. In those who had responded mainly to riboflavin, the transaminase activity was higher, with low activity in the group which primarily responded to vitamin B_6. Riboflavin status appeared to modify the transaminase activity considerably. A group of patients with oral lesions and very low EGR activity were administered pyridoxine. There was a subsequent decrease in RBC riboflavin, but an increase

in EGR activity and a good clinical response, suggesting that riboflavin is utilised in pyridoxine metabolism (Nutrition Reviews, 1972). The enzyme concerned with the conversion of pyridoxine to its active form pyridoxal phosphate is pyridoxine phosphate oxidase which is a FMN dependent enzyme (Nutrition Reviews, 1977). The utilisation of vitamin B_6 depends on the conversion of the active compounds, pyridoxine in plants and pyridoxal and pyridoxamine in foods of animal origin, into pyridoxal phosphate (Nutrition Reviews, 1972). If rats are maintained on a vitamin B_6 deficient diet, erythrocyte riboflavin increases. It may be that in the absence of pyridoxine, less riboflavin is utilised. A deficiency of riboflavin may also lead to a deficiency of pyridoxine. Conversion to the active form of vitamin B_6 is increased if red cells are incubated with riboflavin. It may be that a significant proportion of ingested pyridoxine is metabolised by the red blood cells.

The similarities of changes in the neural tissues found between riboflavin deficient animals and humans with vitamin B_{12} deficiency stimulated Frenkel *et al.* (1979) to examine whether the tissue changes were caused by defective vitamin B_{12} function due to the requirement for riboflavin dependent oxidoreductase systems in B_{12} coenzyme synthesis and function. They failed to demonstrate abnormal enzyme reactions or an increase in odd chain fatty acids which is characteristically seen in B_{12} deficient states.

The effect of riboflavin on folic acid metabolism was examined by Dako and Hill (1980). They administered diets with varying amounts of folic acid and riboflavin to groups of rats and then examined various parameters in the blood such as haemoglobin, red blood cells and the haematocrit. The highest values were in those receiving the full dosage of riboflavin and folic acid. Folic acid alone gave lower values. However, if only a fifth of the full dosage of

riboflavin was administered with the folic acid, the haemoglobin values increased significantly. Bovina *et al.*(1969) demonstrated the influence of riboflavin on the utilisation of folic acid for the biosynthesis of coenzyme derivatives, in particular in the reduction steps responsible for the conversion of folate to tetrafolate.

Riboflavin deficiency appears to have some effect on the concentration of iron in the liver due to impaired absorption of dietary iron. Adelekan and Thurnham (1986) demonstrated that there was a lower concentration of liver ferritin-Fe in riboflavin deficient rats with a significant increase in the erythrocyte glutathione reductase activation coefficients. They suggested that the reason iron absorption was impaired was due to the iron being retained more in the tissues, due to a decrease in the activity of the enzyme NADH-FMN oxidoreductase. This enzyme requires FMN which is reduced in riboflavin deficiency. Powers and Bates (1987) tried to determine whether riboflavin was required for iron metabolism. There was a significant increase in packed cell volume when riboflavin and iron were given together to patients suffering from deficiencies. It was suggested that riboflavin may have a role in promoting iron utilisation in the erythroid marrow. Riboflavin deficiency may impair the mobilisation of hepatic iron stores and possibly iron absorption.

A group of 37 patients with iron deficiency anaemia was tested for pyridoxine status and for erythrocyte total riboflavin (Jacobs and Cavill, 1968a). In those patients with oral lesions there was a significant reduction in erythrocyte riboflavin levels, though the authors felt that this was of doubtful pathological significance.

It would appear that there are a number of non specific oral manifestations such as glossitis and angular stomatitis that can be seen in conjunction with a number of nutritional deficiencies such as riboflavin, pyridoxine, folic acid, iron and vitamin B_{12}. This can cause problems therefore in assigning any sign or symptom to a particular deficiency unless all are tested for.

1.5.4 Riboflavin Deficiency and Alcohol

Alcoholics are known to suffer from a variety of vitamin deficiencies. Such deficiencies in alcoholics may be due to low dietary intake, impaired absorption, deficient metabolic transformation due to hepatic hypofunction and impaired tissue storage. Majumdar *et al.* (1981) assessed the vitamin status of 12 chronic alcoholics and found six of the 12 were deficient in riboflavin. Normal levels of riboflavin were reached after treatment with intravenous vitamins. Rosenthal *et al.* (1973) found 11 out of 22 alcoholics admitted to hospital had riboflavin deficiency on testing although they did not exhibit any of the symptoms normally associated with such deficiency. Leevy *et al.* (1965) found significant reductions in the circulating levels of two or more B complex vitamins in more than 40 percent of a group of malnourished alcoholics with liver disease. Although there were no signs of deficiency, electronmicroscopic examination of samples of liver from patients with riboflavin, folic acid, pantothenic acid and pyridoxine deficiency showed reversible enlargement of the mitochondria in the absence of pathological changes at the light microscope level.

Stanko *et al.* (1978) tried to determine whether oral administration of naturally occurring chemical agents known to be hydrogen acceptors and/or necessary for efficient oxidative

metabolism would protect the liver against the fat accumulating effect of ethanol. Riboflavin was found to decrease the concentration of esterified fatty acids and if pyridoxine dihydroxyacetone was used, riboflavin potentiated its effect.

Kim and Roe (1985) studied the sequential development of riboflavin deficiency in hamsters in response to alcohol administration and tried to determine whether it could be prevented by increasing the dietary intake of riboflavin. They found in a group of hamsters, that the most severely affected were the alcohol fed riboflavin depleted group while it appeared that supplementation with riboflavin prevented alcohol-induced riboflavin deficiency. It would appear that alcohol increases the requirement for riboflavin with specific effects on its absorption, metabolism and excretion.

As it is known that acute ethanol consumption and riboflavin deficiency each induces oxidative stress within tissues, Dutta *et al.* (1995) examined whether their combined effects would compromise the major antitoxidative system in the liver, reduced glutathione metabolism. They found that while riboflavin deficiency alone did not compromise hepatic reduced glutathione (GSH) metabolism, ethanol consumption together with riboflavin deficiency depleted hepatic GSH, blunting enzyme activities and may therefore enhance alcohol induced liver injury.

1.6 RIBOFLAVIN AND CANCER

1.6.1 Introduction

It is estimated that greater than 90 percent of cancers are caused by environmental, dietary and lifestyle related factors and as 30 percent are due to tobacco and 40 percent dietary,

about 70 percent could be prevented if people stopped smoking and modified their diets and lifestyles (Prasad, 1990). Exposure to one carcinogenic stimulus does not predestine the individual to develop a neoplasm, because familial, dietary, hormonal and gender related factors modulate the progression (Boyd and Reade, 1988; Cairns, 1981).

Calabrese (1979) challenged the role of the environment in carcinogenesis and felt that the figure of environmental factors initiating or promoting 85-90 percent of all cancers was misleading. He felt the critical role of the individual's health status as a modifying factor was neglected.

Many authors have attempted to correlate differences in food intake with the differences in incidence of various cancers in world populations. It is postulated that dietary factors play a vital role in the genesis of several major neoplasms especially gastric and colonic (Lowenfels and Anderson, 1977). However, it is difficult to assess the contribution of the diet generally to carcinogenesis due to the large number of chemicals with a wide and contradictory range of biological activities. Before recommendations are made to the public, the degree and certainty of risk has to be ascertained (Weinhouse, 1986).

Lyon *et al.* (1983) looked at the problems involved in epidemiological studies of diet and cancer. There appears to be a lack of understanding between nutritionists and epidemiologists, the nutritionists generally being more interested in current intake and nutritional status and epidemiologists more interested in food intake prior to the onset of cancer, which can be over a period of years.

Elements of the diet may be initiators or complete carcinogens or may modulate carcinogenesis. It is difficult to measure the intake of individual food components if they have been consumed years earlier and so, much of the data is from laboratory studies. Unfortunately, due to the varying metabolic pathways in different species it is difficult to extrapolate results. Also, experimental diets usually compare extremes which are not usually applicable to humans, as variables are usually within a more modest range (Higginson and Sheridan, 1991).

Cancer has profound effects on nutritional status (Rivlin *et al.*, 1983). Some tumours are supplied with amino acids at the expense of the host (Henderson and LePage, 1959). The relationship between vitamin metabolism and cancer is very complex, such as the effects of vitamins on the growth of the tumour, the effects of tumours on vitamin metabolism and the effects of vitamins on chemical carcinogens and antitumour chemotherapeutic agents (Nutrition Reviews, 1974). There appears to be a lot of contradictory evidence as to the relationship of riboflavin to cancer. Riboflavin deficiency has been implicated in both protection and causation of cancer. While riboflavin deficiency appears to prolong the survival of tumour-bearing animals it may also accelerate carcinogenesis (Rivlin, 1982).

1.6.2 Riboflavin Deficiency as a Protective Factor in Experimental Carcinogenesis

Several authors have reported that riboflavin deficiency inhibits the number and rate of growth of some spontaneous and transplanted tumours. Riboflavin deficiency has been reported to reduce the size of previously developed lymphosarcoma transplants in mice (Stoerk and Emerson, 1949) and diminish the growth rate of Walker carcinoma in rats (Aposhian and Lambooy, 1951). There is reported to be non-specific inhibition of growth of

Walker rat carcinoma 256 in the presence of diethyl riboflavin, a potent antagonist of riboflavin (Kim *et al.*, 1966). Rivlin *et al.* (1973) tried to determine whether Novikoff hepatoma differs from normal tissue in its adaptation to riboflavin deficiency and whether the presence of the tumour alters the hepatic concentration of riboflavin, FMN and FAD. The survival of the rats in the deficient group was increased by 5.7-10.8 days. The concentration of FAD decreased to one third of its normal in the livers of deficient rats. FAD concentration in the hepatoma was one tenth that of the liver.

Riboflavin deficiency has also been reported to protect mice from the growth and spread of mammary tumours (Morris and Robertson, 1943). They did not monitor riboflavin levels, relying instead on weight loss. It is worth noting that the fat contents of the diets were not the same and as has been previously mentioned, the fat content can affect the rate of attainment of riboflavin deficiency (Shaw and Phillips, 1941). Morris (1947) aimed to determine the influence of vitamin intake on the in vivo growth of tumours already formed and in the genesis of cancer. He looked at the effects of rapid, extreme deficiency and partial depletion on the growth of spontaneous mammary tumours in mice. He found that rapid extreme deficiency of pantothenic acid and riboflavin decreased the rate of growth of spontaneous mammary adenocarcinomas. Deficiency of pyridoxine had no effect and if there was only partial depletion of pyridoxine, thiamine or riboflavin, there was no effect on the growth of the tumours. Riboflavin supplementation actually increased the number of tumours. Unfortunately, a number of the early experiments gave no information on whether the inhibition of growth rates was due to a reduction in number and/ or size of cells.

Baker *et al.* (1981), found that tissue of primary colonic adenocarcinoma had significantly more of the vitamins, riboflavin, B_6, nicotinate, pantothenate, thiamin, biotin and folates, whereas the concentration of B_{12} was almost half that of adjacent normal tissue. They suggested that those tumours with a high vitamin requirement for enhanced growth may be arrested with antivitamins targeted at metabolic sites. Perhaps some of the protective mechanism of vitamin deficiency is due to starvation in the rapidly multiplying tumour of the flavin coenzymes vital for metabolism. These, apparent inhibitory effects of riboflavin deficiency have not been fully elucidated.

It is difficult to identify whether inhibition of growth rate of some tumours may be due to a change in calorie intake and body weight or due to a direct effect of riboflavin deficiency (Rivlin, 1975). In the experiment by Morris (1947) the caloric intake was approximately the same in both groups. Tannenbaum and Silverstone (1953) in an extensive review of the literature, came to the conclusion that varying the level of the B vitamins in the range above minimal needs had little effect on carcinogenesis. Rao *et al.* (1987) found that a 10-20 percent reduction in body weight decreased the incidence of some tumours such as benign mammary tumours in the rat, but did not decrease the incidence of liver tumours.

1.6.3 Riboflavin Deficiency as a Potentiating Factor in Experimental Carcinogenesis

In contrast to spontaneous and transplanted tumours, there have been reports of potentiation of carcinogenesis by riboflavin deficiency, especially those induced by azo dyes.

Kensler *et al.* (1941) reported some protection from liver cirrhosis and cancer by riboflavin in the rat. Miller *et al.* (1941) studied the effects of modifying the diet on the rate of hepatic

tumour formation due to DMBA (7,12-dimethylbenzanthracene). In general, the protective supplements were rich in protein and vitamin B complex especially riboflavin. There has been a suggestion that excess riboflavin protects against hepatomas induced by 4-dimethylaminoazobenzene dye (DAB). It has been proposed that excessive riboflavin enhances the activity of the enzyme, azoreductase which degrades the azo dyes. Lambooy (1976) found if a small amount of riboflavin was added to a diet with a hepatic carcinogen for 18 weeks, carcinogenesis was strongly inhibited and 80 percent of rats did not develop hepatomas.

Schaefer *et al.* (1950) described the influence of variations in dietary levels of riboflavin, pyridoxine, inositol and protein depletion-repletion on the incidence of tumours induced in rats by choline deficiency. Neoplasms were found in all groups apart from a group of eight rats supplemented with 20mg of riboflavin/kg of diet. There also appeared to be less severe cirrhosis induced. Robertson and Kahler (1942) compared the riboflavin content of primary and transplanted hepatomas with rapidly growing non-neoplastic tissue of similar origin and found it to be below that of non-neoplastic liver.

Chan *et al.* (1972) demonstrated that the skin and livers of mice fed a riboflavin deficient diet had a much lower than normal activity of microsomal aryl hydrocarbon hydroxylase. This is part of the microsomal enzyme complex which metabolises a variety of exogenous chemicals including carcinogens, drugs and pesticides. Yang (1974) also demonstrated changes in this enzyme system in riboflavin deficiency. This system has also been considered a detoxification system. Some of the effects may be due to the fact that flavin cofactors are involved in drug and carcinogen metabolism (Rivlin, 1982). Riboflavin is involved in the electron transport

chain and lipid metabolism and also assists in the degradation of drugs and foreign chemicals via microsomal hydroxylation (Rivlin *et al.*, 1983).

Roe (1962) attempted to reduce the incidence of experimental skin carcinomas in mice by administering massive doses of riboflavin and other B group vitamins. Riboflavin had no more than a slight inhibitory effect while other vitamins of the B group had no definite effect. While Wynder and Klein (1965) felt that the early epithelial changes detected in the oesophageal and stomach mucosa of riboflavin deficient mice could not be attributed to riboflavin deficiency alone, but rather to starvation, in later experiments Wynder and Chan (1970) found that pure caloric restriction did not affect tumour induction. They compared the induction of skin tumours due to local application of DMBA and croton oil between mice fed a normal diet and those that had been fed a riboflavin deficient diet for four weeks. Skin tumours were found to develop sooner, and in greater numbers than the controls. Joseph-Bravo *et al.* (1976) carried out an experiment looking at the effects of artificial light and riboflavin on the toxicity of aflatoxin. They demonstrated that light decreased the incidence of aflatoxin induced cancer. Aflatoxin caused blood riboflavin levels to decrease and this was enhanced by irradiation, suggesting that photosensitised riboflavin and aflatoxin form a complex thereby inhibiting its degradation into carcinogenic metabolites.

More recently, it has been suggested that riboflavin has a potential chemopreventive role due to its modulation of carcinogen-induced DNA damage and repair enzyme activity (Webster *et al.*, 1996).

Riboflavin as a "protective factor" would appear to be due the role of flavin dependent enzyme systems which inactivate the azo dyes and may have a role in metabolising and degrading a number of potential carcinogens. It may also be that the aforementioned effects of riboflavin deficiency on the epithelium makes it more prone to carcinogenesis.

1.6.4 Riboflavin and Cancer in Man

The majority of the human studies have centred on the relationship between riboflavin deficiency and oesophageal cancer. This is discussed further in Section 1.7.4. It has been proposed that in man, other neoplasms may be sensitive to riboflavin deficiency. Lane and Smith (1971) induced riboflavin deficiency in a small number of patients with Hodgkins disease, lymphosarcoma and polycythemia vera by giving them a synthetic diet and the riboflavin antagonist, galactoflavin. They managed to bring about partial remission of tumours in one of two patients with Hodgkin's disease and two of four with lymposarcoma. However, treatment times varied between 24 and 152 days and the concentration of galactoflavin also varied. Patient numbers were small and concomitant treatment was not taken into consideration. It may have been expected in the diseases from which these patients were suffering that periods of remission would be expected.

The major carriers of riboflavin have been identified as immunoglobulins. Plasma of 182 patients with different malignant diseases was tested for riboflavin binding. There was a significant increase in riboflavin binding immunoglobulins in those suffering from breast cancer and melanoma compared with controls. This may mean that these immunoglobulins maintain more riboflavin in the circulation which is then available for tumour tissues (Innis *et al.*, 1986). These elevated levels may contribute to the lower urinary levels and clearance of

riboflavin in cancer. This may also imply that if urine has been used as the body fluid to be monitored for riboflavin levels, there may be a false impression of the true body stores.

1.7 RIBOFLAVIN AND ORAL AND OESOPHAGEAL CANCER

1.7.1 Introduction

There have been a number of large epidemiological studies aimed at detecting a link between riboflavin deficiency (amongst other micronutrients) and oesophageal cancer. Oesophageal and oral cancer share some aetiological factors such as smoking and alcohol ingestion. In the study by Wynder and Klein (1965) similar histological changes were noted to occur in oesophageal and oral mucosa. They are likely due to their anatomical juxtaposition to be subjected to the same carcinogens and are both squamous epithelium. As there is little reference in the literature to the role of micronutrients in oral carcinogenesis, it would seem worthwhile to review some aspects of carcinoma of the oesophagus.

1.7.2 Incidence of oesophageal cancer

There is a 500 fold difference in rates of oesophageal cancer in different parts of the world (Ghadirian *et al.*, 1988). This appears to be associated with differences in alcohol intake, tobacco usage, socioeconomic group and poor diet especially of vitamins A, C and B_2. Carcinoma of the oesophagus accounts for approximately 25,000 deaths annually in Europe (approximately 5 percent of all carcinomas), whereas that from oral carcinoma accounts for approximately 2 percent in Western Europe (Soames and Southam, 1993). The 5 year survival rate is reported to be 5 percent (Krevsky, 1995). Men, are in the main, affected more commonly than women except in Scandinavia, UK and India (Mandard, 1989). There

is a high incidence in the Middle East , especially North-East Iran (Warwick and Harington, 1973).

In China, especially in the North, 2000 year old records mention a "dysphagia" syndrome in Henan province and in Linxian *ge shi bing* or "hard of swallowing disease" has long been endemic. There has even existed a "Throat-God Temple" (Yang, 1980). Due to the high incidence of the disease in China, epidemiological studies were initiated in 1959. In the Linxian area 20 percent of the total deaths were due to oesophageal cancer.

1.7.3 Aetiology of Oesophageal Cancer

Many aetiological factors have been implicated in the development of oesophageal cancer, such as alcohol, tobacco, opium, nutritional deficiencies, malabsorption and chronic irritation, to name but a few (Cook-Mozaffari *et al.*, 1979: Ghadirian *et al*, 1985)). Physical damage due to hot liquids and hard foods has also been implicated (Martinez, 1969). Pickled vegetables are often consumed in areas with a high incidence of oesophageal carcinoma and the fact that chickens in these areas also suffer from a high incidence of gullet cancer suggests "common carcinogens" (Ghadirian *et al.*, 1988; Yang, 1980). N-Nitroso compounds including N-nitrosamines have been implicated in oesophageal cancer (Lu and Lin, 1982: Lu *et al.*, 1986).

A low intake of fresh fruit and vegetables has been implicated (Cook-Mozaffari *et al.*, 1979: Guo *et al.*, 1990: Hormozdiari *et al.*, 1975). In areas where sundried food are the norm, riboflavin levels may be severely affected. As previously mentioned, riboflavin levels drop

after exposure to sunlight. In contrast to oral carcinoma, the study by Guo et al (1990) failed to link the incidence of oesophageal cancer to alcohol intake or tobacco usage.

Franceschi *et al.* (1990) examined patients with oral, pharyngeal and oesophageal cancer from Pordenne Province in North East Italy where there is a high incidence of these neoplasms. There appeared to be a significant association between maize consumption and the incidence of neoplasms. These differences could not be explained in terms of education, occupation, tobacco use or consumption of fruit or vegetables. Maize increased the risk if the patients consumed more than 42 drinks per week. It was suggested that the effects were due to maize causing deficiencies of micronutrients such as niacin and riboflavin. Deficiencies of micronutrients have been found in plants in the gardens patients suffering from oesophageal cancer (Burrell *et al.*, 1966). Maize was prone to attack by two fungi toxic to animals, leading to the formation of nitrosamines.

Much of the research into carcinoma of the oesophagus has taken place in Iran due to the high incidence of the disease in that country. Oesophagitis has been implicated as an aetiological factor (Crespi *et al.*,1979). A population survey in a high risk area for oesophageal carcinoma in Russia, showed that 60 percent of those who had oesophagogastroscopy had chronic oesophagitis (Zaridze *et al.*, 1985b).

Just as is the case with oral cancer, it would seem that there is no single factor responsible for oesophageal cancer. It may be, that it is due to deficiencies in the diet making the mucosa more vulnerable to carcinogens.

Paterson-Kelly syndrome (or Plummer-Vinson syndrome) is associated with oesophageal cancer. It appears to occur in females with a history of anaemia and dysphagia (Ahlbom, 1936). Riboflavin has been implicated as a deficiency factor although in one group of 25 patients, no evidence was found that riboflavin deficiency was an important factor in this condition and it did not appear to play a part in causing the associated oral lesions. There was evidence of pyridoxine deficiency (Jacobs and Cavill, 1968b). Wynder *et al.* (1957) carried out a survey to elucidate the causes of cancer of the upper alimentary and respiratory tracts in Sweden. There was a close relationship between Plummer Vinson syndrome and carcinomas of other sites. The high incidence may have been due to the high incidence of Plummer Vinson syndrome in Sweden. Apart from iron deficiency being a primary factor it was thought that other deficiencies were also involved although they were not specified. As riboflavin is involved in the metabolism of iron, a deficiency in riboflavin may indirectly influence the incidence of the syndrome.

1.7.4 Riboflavin Deficiency in Relation to Oral and Oesophageal Cancer

Riboflavin deficiency has been implicated in both oral and oesophageal cancer. It may be, that certain deficiencies are associated with increased susceptibilities to oral and oesophageal cancer. Low levels of riboflavin have been found in several populations with a high incidence of oesophageal cancer (Zaridze *et al.*, 1985a: Thurnham *et al.*, 1985). In Linxian in Northern China, there was a very high mortality rate from oesophageal cancer. Yang *et al.* (1984) analysed the nutritional status of the population of the area and found riboflavin deficiency was present in 90 percent of subjects. Thurnham et al., (1985) in the same population found low levels of riboflavin in those with oral leukoplakia. While low levels of riboflavin have been found, treatment with micronutrients has had mixed results. Munoz *et al.* (1985) treated

subjects with retinol, riboflavin and zinc for one year, but no differences in the incidence of oesophagitis, atrophy or dysplasia were found. It was suggested that the reason for failure to detect changes could have been due to the treatment period being too short or the doses being too small. However, a later examination of a subsample of 200 subjects to evaluate micronuclei formation of cells in the buccal mucosa and oesophagus found a significant reduction in the frequency of micronucleated cells in the oesophageal mucosa, but not the buccal mucosa (Munoz *et al.*, 1987).

Rather than testing populations for deficiency, a number of workers rely on questioning populations as to previous intake of nutrients or extrapolate that recent dietary habits correctly reflect previous intake. This is notoriously unreliable. Ziegler *et al.* (1981) questioned the next of kin of 120 black male residents of Washington who had died of oesophageal cancer and correlated estimates of intake of vitamin A, carotene, vitamin C, thiamin and riboflavin as being inversely associated with relative risk, but as broad food groups rather than as each micronutrient. Analysis of the intake of populations in the Transkei demonstrated less than two thirds of the recommended dietary requirements of nicotinic acid, riboflavin and ascorbic acid were consumed in those with a high frequency of oesophageal cancer (Groenewald *et al.*, 1981).

The effects of oestrogen on riboflavin excretion were examined in a group of patients with oral cancer (Sathe *et al.*, 1958) The excretion levels of thiamine and riboflavin were lower than normal in cancer patients whereas excretion levels of oestrogen were higher. If these vitamins were administered, it led to an increase in the excretion of oestrogens in the cancer patients, but not in the controls. Administration of oestrogen on the other hand decreased

levels of thiamine and riboflavin. It was concluded that oestrogen treatment appeared to be harmful in men with oral cancer as it induced thiamine and riboflavin deficiencies.

Water contaminated with calcium, magnesium, chromium, cadmium, cobalt and traces of petroleum oil has been implicated in oesophageal cancer in a study of the Gassim region of Saudi Arabia (Amer *et al.*, 1990). As mentioned previously some of these elements are thought to chelate with riboflavin and may bring about deficiency (Cooperman and Lopez, 1984).

1.8 AIMS OF THE THESIS

As can be seen, there is conflicting evidence as to the role of riboflavin in cancer. Population studies may give some pointers to possible aetiological factors in cancer of the oesophagus and oral cavity, however, the links are all too often tenuous. It is impossible to separate out the multifactorial nature of carcinognesis in the general population There are a few studies reporting effects of riboflavin deficiency on epithelium, but there is little or no information regarding quantitative histology of the epithelial changes. In addition, most of the early studies fail to adequately monitor riboflavin levels throughout the experimental period.

The aim of the studies reported in this thesis was to investigate the effects of riboflavin deficiency on oral epithelium. Because of the difficulty in studying simple nutrient deficiencies in man, an experimental animal study was undertaken. It was intended that future studies would progress to investigation of experimental carcinogenesis and therefore the hamster, with the well characterised cheek pouch model, was selected as the experimental animal. The

series of experiments included establishment of tests to determine normal riboflavin values for the hamster; administration of an experimental diet deficient in riboflavin with monitoring of riboflavin levels throughout the experiment; and the quantitative evaluation of oral epithelial changes in the experimental riboflavin deficient group compared to a control group.

CHAPTER 2

RIBOFLAVIN TESTING IN HAMSTERS- A PILOT STUDY AND OPTIMISATION OF THE ERYTHROCYTE GLUTATHIONE REDUCTASE TEST FOR HAMSTER BLOOD

2.1 INTRODUCTION

The hamster was the chosen experimental animal. Previous work has been carried out on the manipulation of the hamster diet resulting in various nutritional deficiencies. However, little has been reported in relation to riboflavin deficiency. Riboflavin appears to be a nutritional requirement in the hamster. Riboflavin deficiency can be induced in the hamster by dietary manipulation. Riboflavin can be excluded from the diet or an antagonist such as galactoflavin can be used. Salley and Weatherred (1964) fed hamsters a riboflavin deficient diet and using a microbiological assay, demonstrated significantly lower riboflavin levels in the hamster liver after seven weeks. Kim and Roe (1985) experimented with hamsters fed diets with varying levels of riboflavin and alcohol. They monitored the levels by the erythrocyte glutathione reductase assay, which is the most commonly used method of monitoring riboflavin levels in humans. Kim and Roe (1985) did not report any technical problems using hamster blood, but as only small amounts of blood can be removed regularly from hamsters it was decided to determine whether the laboratory scheduled to carry out erythrocyte glutathione reductase testing was able to obtain reliable data from the small amounts of blood available.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Seven, young adult Syrian hamsters were used in the pilot study. They were fed on standard hamster diet and appeared completely healthy during the experimental period. The animals were bled four times at intervals of four weeks and were sacrificed on the twelfth week.

2.2.2 Collection of Blood Samples

Collecting repeated blood samples from laboratory animals can be problematical. In the rat the tail vein can be used and in the rabbit the ear vein. Unfortunately neither option is suitable in the hamster as the superficial veins are small and the tail is rudimentary. Cardiac puncture can be used, but is unsuitable for repeated sampling in an individual animal because even in the best circumstances there is an associated high mortality. Pansky *et al.* (1961) described a technique for obtaining blood from the retro-orbital sinus in the hamster and this provides the only practicable method for repeated sampling in the individual animal.

Bleeding was undertaken using a 2ml syringe with a 15mm long needle of 0.5mm diameter. Hamster blood clots very readily and to avoid this a small volume of 1:1000 heparin solution was drawn into the syringe and then expelled shortly before bleeding. Using the Pansky *et al* (1961) technique, the aim was to remove 1ml of blood from each of the seven animals on three occasions. At the time of sacrifice a larger volume was collected by cardiac puncture following a lethal overdose of Nembutal. All blood samples were placed in standard lithium heparin containers and were analysed by the Biochemistry Department of Glasgow Royal Infirmary.

2.2.3 Technique of Riboflavin Analysis

The enzymatic method is the most commonly used test for riboflavin levels in hospital laboratories. This test appears to be the most reliable although one result should not be looked at in isolation, but rather as a series of results. There are numerous variations to the technique, using a variety of concentrations of reagents. The technique used at Glasgow Royal Infirmary is the erythrocyte glutathione reductase test as described by Delides *et al.* (1976). The enzyme exists in both inactive and active forms. FAD converts the inactive to the active form and the active form then reduces oxidised glutathione with a corresponding oxidation of NADPH. The amount of NADPH which is oxidised is monitored optically at 340 nm and therefore the degree of activation is calculated which gives an indirect measure of riboflavin status. The percentage activation is calculated from the following equation:-

$$\% \text{ activation} = \frac{(\text{GR activity with FAD} \times 100) - 100}{\text{GR activity without FAD}}$$

In humans a percentage activation of greater than 60 indicates riboflavin deficiency and there are no reports in the literature that suggest a different threshold for hamsters.

2.3 RESULTS

2.3.1 Health of the Animals

Throughout the experimental period, none of the animals showed any sign of distress and there appeared to be no problems with the orbital area following each bleed. There were no features to suggest nutritional deficiency.

2.3.2 Blood Samples

Difficulty was experienced in removing adequate amounts of blood for testing due to the operator's inexperience in the technique. A sufficient volume was not always obtained on each occasion.

2.3.3 Riboflavin Levels

The results of the riboflavin assessment in the pilot study are shown in Table 2.1. Where there is no result listed, there was insufficient sample to carry out the analysis. In the human subject, if the percentage activation is greater than 60 percent there is thought to be deficiency. There appeared to be one result which suggested riboflavin deficiency and that was in hamster seven on the first bleed. Thereafter, however, the results for the animal showed no sign of deficiency. It is advised in humans, to carry out serial testing due to the inconsistency of the results and this appeared to be borne out in this pilot study. Insufficient blood for analysis was collected from animals 2 and 4 on the first bleed and from animal 5 on the second bleed. The samples from the second bleed were analysed twice to check for reproducibility. As can be seen there was some variability. This may have been due to slight differences in dilutions and handling of the samples. As mentioned previously, riboflavin testing, even in man is variable. One of the problems involved in testing hamster blood is that hamster blood clots readily, although this was not a problem in this pilot study. The largest volume that could be supplied to the laboratory was 1ml of sample and usually it was 0.5ml or less, thus making the handling of the sample more difficult. However it was hoped that as experience was gained with the retro-orbital bleeding, more consistent sampling up to 1ml of blood would be achieved.

Alternative methods of riboflavin testing were considered. The use of high performance liquid chromatography was considered. No laboratories approached considered this as appropriate and the technique would normally require 2-3ml of blood.

HAMSTER	WEEK1	WEEK4	WEEK8	WEEK12
1	4	33 16	12	33
2	-	18 20	5	38
3	4	37 21	35	27
4	-	31 19	16	7
5	33	- -	25	31
6	4	54 39	21	6
7	62	7 7	28	25

Table 2.1 Riboflavin results of pilot study. Units are percentage activation

2.4 OPTIMISATION OF THE ERYTHROCYTE GLUTATHIONE REDUCTASE TEST FOR HAMSTER BLOOD

Due to the inconsistent results for riboflavin levels obtained from the laboratory at Glasgow Royal Infirmary in the pilot study and in further experiments (Carmichael and MacDonald, 1992), it was decided to change laboratories and to develop testing of riboflavin using the same basic method of the erythrocyte glutathione reductase (EGR) test, but optimised for hamster blood. This was carried out in the Biochemistry Laboratory at the Victoria Infirmary, Glasgow.

2.4.1 Technique

It was decided to utilise the modifications of the EGR test as described by Thurnham and Rathakette in 1982. This technique was based on the method described by Glatzle *et al.* (1970). It was found when analysing samples of a quality controlled haemolysate at the beginning and end of each batch of samples over a 20 week period that the activation coefficient (AC) was higher at the end of a batch than at the beginning (Thurnham and Rathakette, 1982). It was felt that this was due to suboptimal conditions of the assay. Maximum coupling of FAD to glutathione reductase ($NAD(P)H_2$:glutathione oxidoreductase; *EC*1.6.4.2) occurred with an incubation of 15 minutes at 35° rather than 5-7 minutes as used by most workers. As an alternative, if batches of sample are used, the enzyme and FAD could be preincubated in the reaction mixture for 2 hours at 4° or 1 hour at 25° before incubating for the standard 5 minutes at 35°. There was a slight deterioration of quality control results after 4 weeks, but even over a 20 week period the results remained within 2 standard deviations of initial values.

2.4.2 Optimization for the Hamster Model

Familiarisation with the technique was achieved using blood from human volunteers. Following this, blood samples from normal hamsters were tested. Three normally fed, healthy, Syrian hamsters were sacrificed and bled by cardiac puncture in order to obtain the maximum amount of blood for analysis. As previously described, heparinised needles and syringes were used and the blood was transported in lithium heparin containers. It soon became apparent that hamster blood had a much lower level of activity than human blood and therefore a variety of different concentrations of the reagents were tested to find the optimal conditions. Glutathione reductase catalyzes the reduction of oxidised glutathione (GSSG) with simultaneous oxidation of nicotinamide-adenine dinucleotide phosphate (NADPH). A measure of enzyme activity is provided by the rate of absorbance decrease at 340nm. This activity is then expressed as U/g of haemoglobin in the haemolysate.

Samples from the three animals were tested using combinations of [FAD] of 6, 8 and 10mmol/l;[NADPH] of 40, 80, 100, 120, 160 and 200mmol/l; [Ox.Glutathione] 1 and 2mmol/l. All reagents were supplied by Sigma.

Once the optimum concentrations of all the reagents had been determined, samples from nine hamsters which were being sacrificed for other reasons were also tested and riboflavin levels ascertained. In order to establish a normal range of riboflavin levels, the initial samples from the 25 animals used in the experiment reported in Chapter 3 were also included giving a larger sample size. Quality control samples of hamster blood were included in each batch.

The haemolysate was prepared by centrifuging the venous blood at 3000 rpm for five minutes. The plasma and buffy layer were then pipetted off and discarded and the haemolysate was prepared by mixing equal volumes of red cells and water. These were vortex mixed and then frozen to -20°C. Potassium phosphate buffer was prepared by mixing 18.42g of $K_2HPO_4.3H_20$ and 2.61g KH_2PO_4 made up to 1 litre in distilled water with 1g of K_2EDTA added giving a pH of 7.4. FAD was prepared by mixing 2.488g with 10ml buffer. The optical density of FAD was read at 450nm and the concentration calculated from

$$c = \frac{A}{11300}$$

Oxidised glutathione was prepared by mixing 109mg in 100ml buffer. NADPH was prepared by mixing 20.84mg in 50ml buffer. The frozen haemolysate was then defrosted and 50ml of the haemolysate and 950ml of buffer were vortex mixed. The Cobas-Bio Analyser (Roche Diagnostic Systems) was then programmed to use a total volume of 250 ml made up of 40ml sample, diluent in the form of water of 10ml, GSSG of 150ml and NADPH of 50ml. Cuvettes were set up with 120ml haemolysate with every two cuvettes having 20ml buffer or 20ml FAD. Haemoglobin measurements were carried out on a 2500 Co-oximeter (CIBA-CORNING). The cuvettes were incubated at 37°C for 15 minutes. The activity of glutathione reductase were measured before and after the addition of FAD, respectively, by spectrophotometric determinations of NADPH oxidation. The activation coefficient (AC) was derived by dividing the stimulated EGR activity + FAD by basic EGR activity. Results in humans of less than 1.3 are regarded as normal, 1.3 to 1.5 as marginal deficiency and those above 1.8 as severe deficiency.

2.5 RESULTS

2.5.1 Optimum concentrations of reagents

The optimum concentrations of reagents decided upon were [FAD] 8.0mmol/l, [NADPH] 100mmol/l and [Ox. glutathione] 1.0 mmol/l.

2.5.2 Normal values

34 samples were analysed using the above concentrations. The EGR activity with and without FAD was obtained, EGR activity with and without FAD per g/dl of haemoglobin was calculated and the activation coefficient then obtained. EGR activity was much lower in hamster blood than in human blood, being less than 20 compared with more than 30 in humans. Activation coefficients for the 34 animals are shown in Tables 2.2 and 2.3. The values ranged from 0.92 to 1.23. The mean value for the 34 animals was 1.06 with a standard deviation of 0.07. The values for all animals lay within the range +/- 2.5 standard deviations of the mean. This would suggest an activation coefficient of greater than 1.24 is indicative of riboflavin deficiency in the hamster. The reliability of the test was satisfactory with the quality control for hamster blood over the experimental period having a mean activation coefficient of 1.05 and standard deviation of 0.04. The enzymatic activity of all the quality control samples fell over the experimental period, although it always remained within 2.5 standard deviations of the initial sample. Each quality control sample was tested twice in a session, at the beginning and end of batches. The intra batch variation never exceeded 2 standard deviations.

Only one animal gave a result which approached biochemical deficiency, but as mentioned earlier, results should not be looked at in isolation and repeated sampling is indicated.

2.6 CONCLUSIONS

Using this technique, even small amounts of sample can be tested for riboflavin levels. It was felt that by carrying out the testing ourselves the samples would be handled with more care and perhaps more consistent results would be obtained. It was also felt that the results would be more meaningful as the reagents were now optimised for the testing of hamster blood.

Animal	EGR ACT	EGR+FAD ACT	EGR ACT/Haem	EGR+FAD ACT/Haem	AC
1	14.15	14.89	2.00	2.11	1.05
2	11.57	11.48	1.69	1.67	0.99
3	14.05	14.19	1.92	1.94	1.01
6	8.50	9.99	1.38	1.62	1.18
7	8.33	9.98	1.28	1.53	1.20
8	7.33	8.09	1.17	1.29	1.10
9	7.99	9.79	1.27	1.55	1.23
10	8.75	9.12	1.33	1.39	1.04
11	9.41	10.45	1.35	1.51	1.11
Mean	10.05	10.89	1.49	1.62	1.10
SD	2.7	2.23	0.3	0.26	0.03

Table 2.2 Riboflavin levels of normal hamsters
EGR ACT = erythroctye glutathione activity
EGR+FAD ACT=erythrocyte glutathione + FAD activity
EGR ACT/Haem= erythrocyte glutathione activity per gram of Haemoglobin
EGR+FAD ACT/haem=erythrocyte glutathione + FAD activity per gram of haemoglobin
AC=activation coefficient

Animal	EGR ACT	EGR+FAD ACT	EGR ACT/Haem	EGR+FAD ACT/Haem	AC
1	8.59	9.23	2.82	3.03	1.08
2	9.92	10.04	3.13	3.17	1.01
3	12.04	11.08	2.03	1.87	0.92
4	11.43	11.93	3.00	3.13	1.04
5	8.45	9.95	3.34	3.93	1.18
6	16.83	16.87	2.82	2.83	1.00
7	13.65	13.67	2.51	2.51	1.00
8	6.61	6.85	2.85	2.96	1.04
9	9.64	9.74	2.32	2.34	1.01
10	9.20	9.92	2.21	2.39	1.08
11	17.53	17.10	3.10	3.02	0.98
12	13.97	14.58	2.47	2.58	1.04
13	10.14	11.11	2.39	2.62	1.10
14	8.54	10.11	2.05	2.43	1.18
15	16.85	17.13	2.95	3.01	1.02
16	15.79	15.51	2.67	2.62	0.98
17	12.80	13.23	2.84	2.94	1.03
18	15.88	16.45	2.72	2.82	1.04
19	12.46	12.33	2.57	2.55	0.99
22	5.78	6.11	1.87	1.98	1.06
23	10.10	10.58	2.68	2.81	1.05
24	14.08	14.57	2.71	2.81	1.03
26	15.49	16.05	3.04	3.15	1.04
27	14.79	15.46	3.16	3.31	1.05
28	16.52	16.65	3.11	3.13	1.01
MEAN	12.28	12.65	2.69	2.80	1.04
SD	3.45	3.28	0.39	0.43	0.06

Table 2.3 Initial riboflavin levels of hamsters from Chapter 3. Legend as for table 2.2

CHAPTER 3

RIBOFLAVIN DEFICIENCY IN THE HAMSTER MODEL AND ASSOCIATED ORAL EPITHELIAL CHANGES

3.1 AIMS

Previous studies had led us to believe that the posterolateral surface of the lingual epithelium was significantly reduced in length and in thickness of the cellular compartment in a group of hamsters fed a riboflavin deficient diet. A greater cell density was also found in progenitor areas (Carmichael and MacDonald, 1992). However, problems with the health of the animals meant that it was likely they had been suffering from multiple vitamin deficiencies. In addition, riboflavin results were inconsistent. Now that an optimised technique of riboflavin testing had been developed, the aims of this part of the study were to induce riboflavin deficiency in a group of hamsters; to monitor the riboflavin levels throughout the experimental period using the optimised erythrocyte glutathione reductase test and to determine if any oral epithelial changes were demonstrable in association with the riboflavin deficiency. In particular, to ascertain whether earlier findings were a true reflection of the effect on oral epithelium of riboflavin deficiency.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Twenty five young adult male Syrian hamsters were used in the experiment. These were divided into an experimental group of 15 animals and a control group of 10 animals. Due to the early deaths of some of the animals, a further three animals were recruited into the control group. They were housed in the animal house of the Anatomy Department of

Glasgow University, and were obtained from their inbred colony. There was no evidence of vitamin deficiency prior to commencing the experiment and the colony had no history of large outbreaks of "wet tail". The animals were caged individually in plastic containers. In order to avoid the possibility of riboflavin being obtained from organic bedding material, absorbant non organic bedding material was used to cover the base of each cage. The animals were weighed regularly throughout the experimental period.

3.2.2 Diet

The experimental animals were fed a commercially obtained riboflavin deficient diet (Special Diet Services, Lavender Mill, Manea, Cambridgeshire, PE15 OLU) while the control animals were fed the same diet with a higher riboflavin content. The requested levels of riboflavin were <1mg/kg and >15mg respectively. The recommended riboflavin levels are 12-20 mg/kg of diet. The diet was obtained as a powder which was mixed with a small amount of water to provide a thick paste. Animals were given fresh diet each morning in amounts which exceeded the daily intake and water *ad libitum*.

3.2.3 Bleeding

Up to 1ml of blood was removed at the start of the experiment on week 0 and then on weeks 5 and 11 using the technique described previously in Section 2.2.2. The anaesthetic agent used on these occasions was Halothane. At sacrifice, blood was removed by cardiac puncture in order to obtain the greatest amount of blood possible for analysis.

3.2.4 Technique of Riboflavin Analysis

All the blood samples were taken in lithium heparin containers to the Biochemistry Laboratory at The Victoria Infirmary, Glasgow for analysis as described in Chapter 2.

3.2.5 Killing of Animals and Preparation of Sections for Analysis

The animals were deeply anaesthetised using Halothane and blood was obtained by cardiac puncture. The animals were then killed by cervical fracture. Following sacrifice, the heads were prepared, prior to placement in 10 percent formal saline. The tip of the tongue was removed from each animal in order to allow the remainder to lie undistorted in the oral cavity during fixation. This assisted in the standardisation of the sections taken from tongue. The jaws were propped open by a rubber bung placed between the teeth again to minimise the distortion effects on the tongue.

Following fixation, the tongue was dissected out and blocks were taken from the posterior part where the lateral sides merged with the floor of the mouth and from the body of the tongue which was halved into dorsal and ventral portions. (Fig 3.1) The tissue blocks were paraffin processed. The blocks were cut at 4μm and haematoxylin & eosin stained sections were prepared. In order to obtain consistent sampling the sections of dorsal and ventral tongue epithelia were cut from the keratinised surface down through the epithelium. This standardised the compression effects and minimised splitting of the keratin layer.

3.2.6 Histological Analysis of Sections

Quantitative histological analysis was undertaken using computerised planimetry. This utilised a microscope, computer and bitpad. (Fig 3.2) The part of the microscope section to

be quantified, designated as a field, was delineated with an eyepiece graticule. A light spot on the bitpad mouse was projected into the microscope field using a drawing tube attachment. When this light spot was traced along or around elements of the section, the corresponding movements of the mouse were converted into lengths and areas recorded on the computer.

Three areas of the tongue were chosen for analysis, the posterior lateral surface, the dorsal and the ventral surfaces of the tongue. Large variations in the histology of oral epithelium occur within short distances in hamster tongue, therefore consistent sampling between animals was achieved by the selection of fields for quantification relative to anatomical landmarks.

The area to be measured on the posterior lateral aspect of tongue was that situated three field widths from the reflection of the epithelium of the floor of the mouth. The following three fields were then measured (Fig 3.3). Values from both sides were combined giving values for six fields for each animal. The fields were delineated by the square in an eyepiece graticule and each area of epithelium was orientated such that the surface was parallel to the top of the graticule. The magnification used was x400. Fields were orientated to give columns, running through the epithelium at right angles to the mean epithelial surface. The lengths of the surface and basement membrane and the areas of the cellular and keratinised compartments were measured. The keratinised compartment, which was the stratum corneum, was usually simple to distinguish from the rest of the epithelium. The cellular compartment was that between the basement membrane and the stratum corneum. The width of the sampling square in the eyepiece graticule was 228μm. The compartment area measurements were

divided by the column width of 228μm to provide an estimate of mean compartment thickness, which it was felt was an easier parameter to envisage.

Compartment analysis of the dorsal (Fig 3.4) and ventral (Fig 3.5) surfaces was also carried out, measurements being taken of two adjacent fields commencing two fields widths, one each side of the midline. The field width used for the dorsal surface was 368μm and for the ventral surface 228μm. These differences were due to the different characteristics of the areas. The dorsal surface was thicker and more convoluted due to the papillae and was assessed at a magnification of x250.

3.2.7 Compilation and Analysis

The mean for every parameter of each area in every animal was obtained. Therefore, the values for the parameters of the six fields measured in the posterior lateral surface of tongue were averaged and the mean used for further analysis. Four fields of dorsal and ventral surfaces of tongue were likewise averaged. Statistical analyses using the Mann-Whitney U test were used to compare the values for animals on the control and deficient diets.

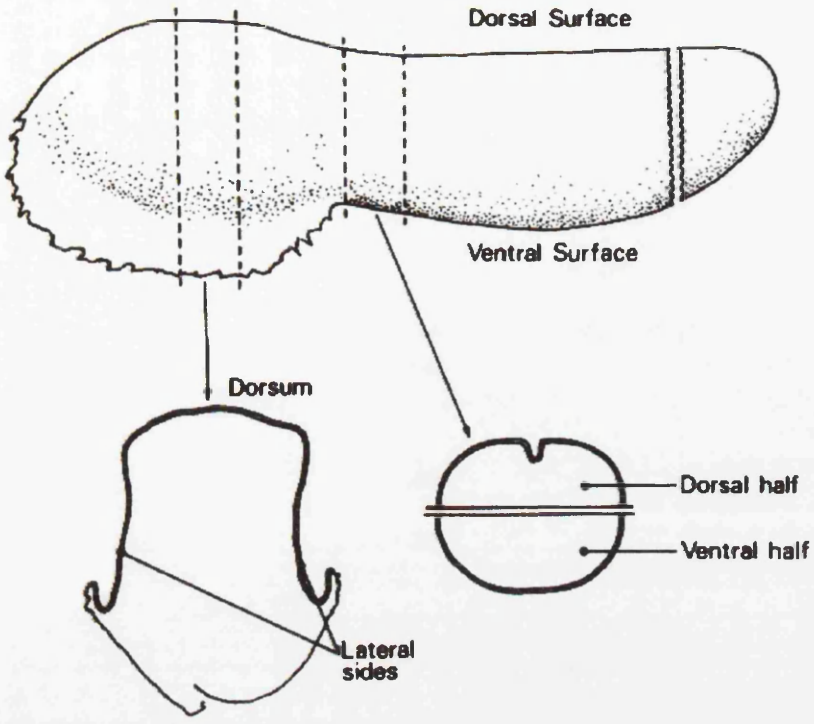

Fig 3.1 Blocks from three areas of the tongue were taken. These were the posterolateral aspect, and the body of the tongue which was divided into dorsal and ventral halves.

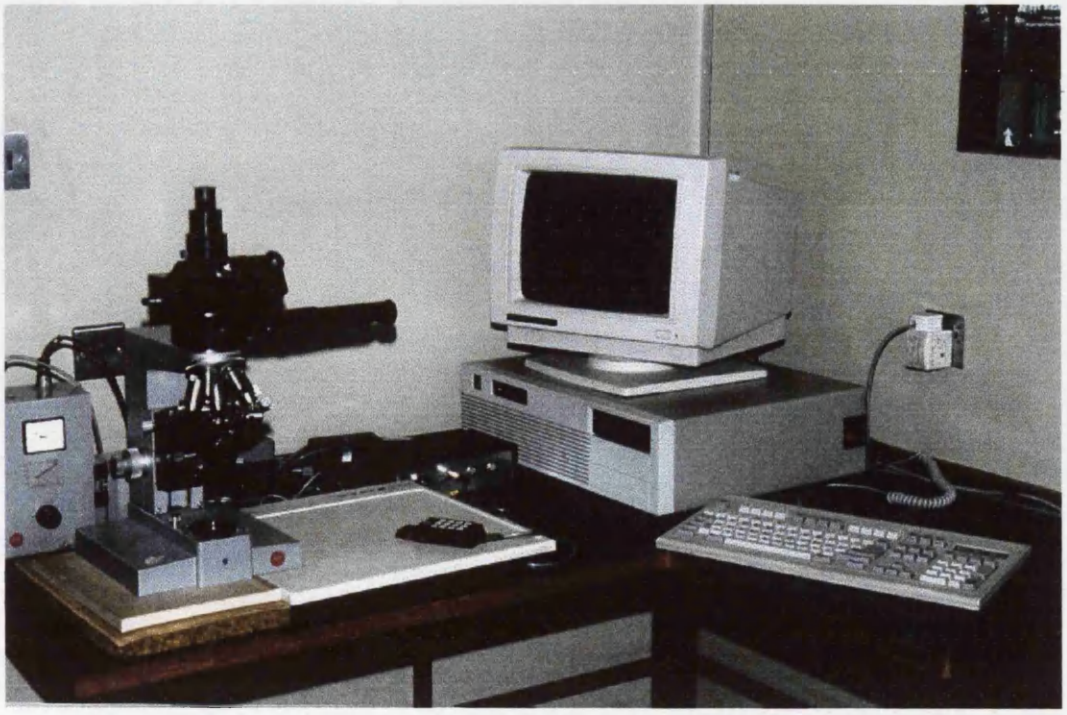

Fig 3.2 Microscope, computer and bitpad used for the quantitative histological analysis.

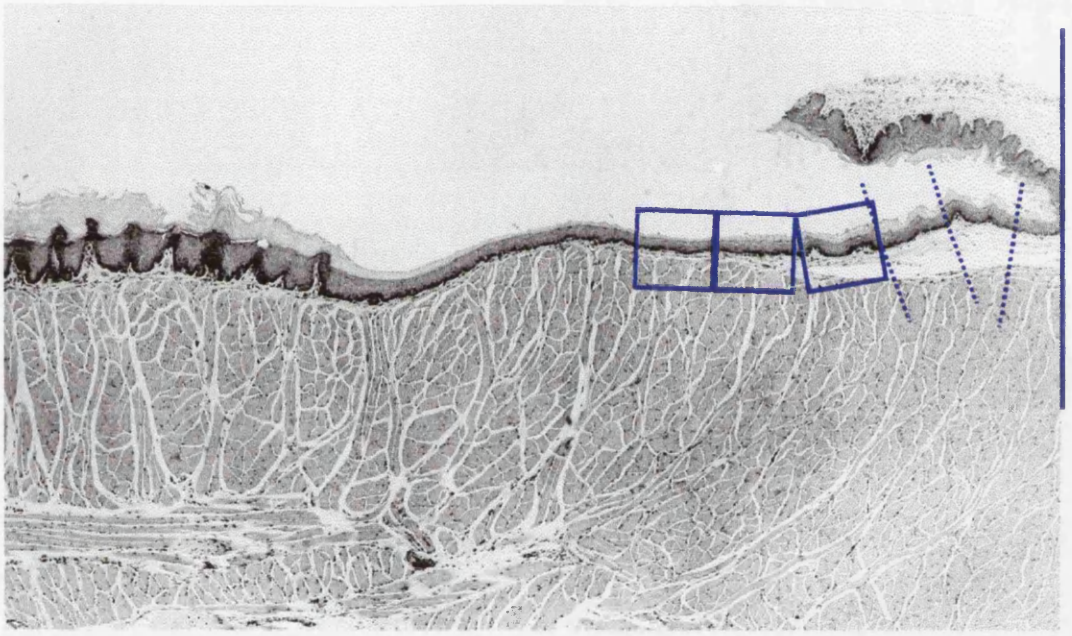

Fig 3.3 Measurements from the posterolateral surface of tongue were made 3 field widths from the reflection of the floor of the mouth. The following 3 fields were measured. The field width was 228 μm.

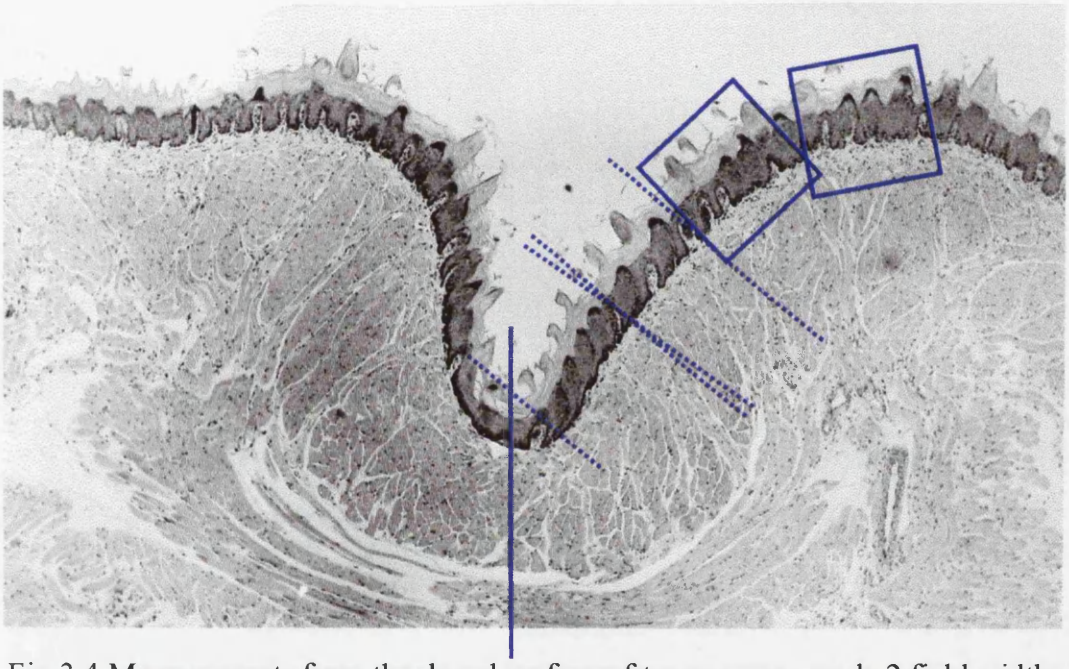

Fig 3.4 Measurements from the dorsal surface of tongue were made 2 field widths from the midline. The following 2 fields were measured. The field width was 368 μm.

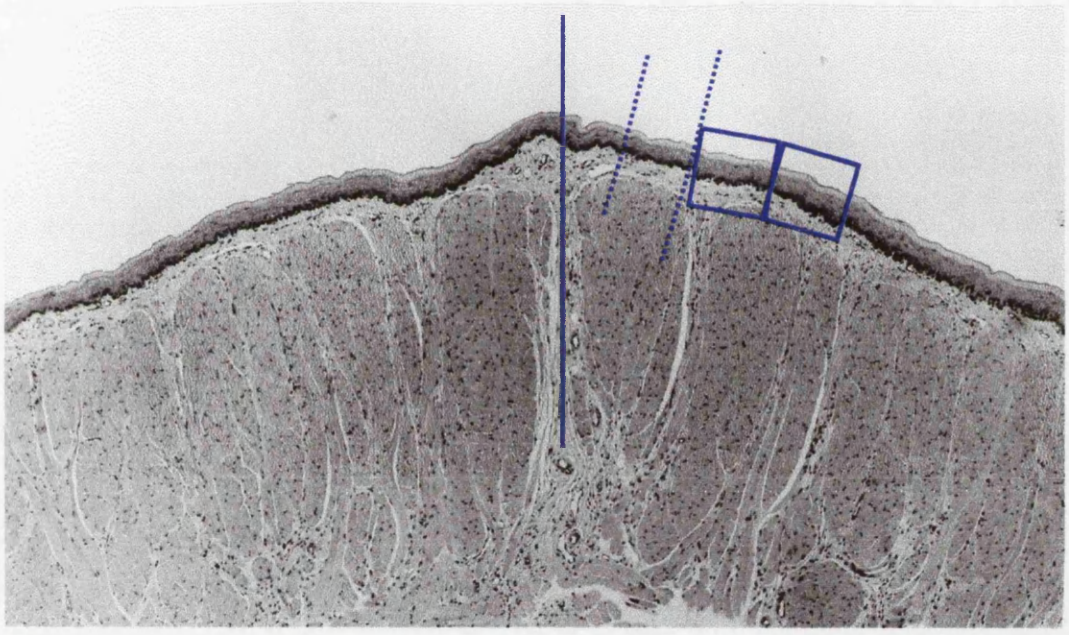

Fig 3.5 Measurements from the ventral surface of tongue were made 2 field widths from the midline. The following 2 fields were measured. The field width was 228 μm.

3.3 RESULTS

3.3.1 Health of the Animals

Unfortunately, several of the animals failed to thrive and due to some early deaths three further animals were recruited into the control group. The animals were diagnosed as suffering from "wet tail". "Wet tail" refers to the appearance that results from diarrhoea and this may be caused by a variety of diseases, but is most often used synonymously with proliferative ileitis. This is a highly contagious disease most likely transmitted by the faecal-oral route and has an associated high mortality. *Campylobacter species* and *Escherichia coli* have been implicated in its aetiology and tetracyclines have been found to be most effective in the treatment of this infection (Frisk, 1987). Antibiotics are also known to affect riboflavin requirements (Cooperman and Lopez, 1984). Three animals in the experimental group and seven in the control group died prior to the end of the experiment. This meant that there were only 12 animals in the experimental group and six in the control group available for analysis. With the deterioration in health of many of the animals including the control group it was decided to terminate the experiment at week 11.

3.3.2 Weights

The weights of the animals are shown in Tables 3.1 and 3.2. There were no significant differences in the weights at the start of the experiment, week 5 or at sacrifice.

3.3.3 Riboflavin Levels

The riboflavin levels are shown in Tables 3.3 and 3.4. Deficiency was deemed to be present if the ratio was greater than 1.24. There was a trend towards deficiency in the experimental group, four of the 12 animals demonstrating biochemical deficiency at sacrifice. However,

one of the six control animals also appeared to be biochemically deficient at sacrifice, although the two previous results for this animal did not suggest a trend towards deficiency. There were no significant differences in activation coefficients between the experimental and control groups at sacrifice although the experimental group had significantly higher activation coefficients at week five ($p=0.01$). The lack of difference at week 11 may have been due to the small number of control group animals. The animals suffering from "wet tail" may have suffered from reduced absorption of nutrients including riboflavin and as there were a greater number of those within the control group this may also have accounted for the lack of a significant difference in activation levels.

3.3.4 Histological Analysis of Posterolateral Surface of Tongue

These sections were analysed as described in Sections 3.2.5 and 3.2.6. The results are shown in Tables 3.5, 3.6, 3.7 and 3.8. There were no significant differences in the length of surface epithelium, length of basement membrane, thickness of the cellular compartment or thickness of the keratinised compartment. The ratios of keratinised compartment:total thickness and cellular compartment:total thickness were calculated and compared in order to ascertain whether the proportions were different. There were no significant differences.

3.3.5 Histological Analysis of Dorsal Surface of Tongue

These sections were analysed as described previously in Sections 3.2.5 and 3.2.6. The results are shown in Tables 3.9, 3.10, 3.11 and 3.12. There were no significant differences in the lengths of the epithelial surface, the lengths of the basement membrane or in the thickness of the cellular compartment. There was a significant difference in the thickness of the keratinised compartment, being significantly greater in the experimental group compared

with the control group. (Mann Whitney U test p=0.04) There were no significant differences in the ratios of keratinised compartment thickness:total thickness or cellular compartment thickness:total thickness.

3.3.6 Histological analysis of Ventral Surface of Tongue

These sections were analysed as described in Sections 3.2.5 and 3.2.6. The results are shown in Tables 3.13, 3.14, 3.15 and 3.16. There were no significant differences in length of surface epithelium, thickness of keratinised compartment or thickness of the cellular compartment. There was a significant difference in the length of the basement membrane, this being greater in the experimental group (Mann Whitney U test p=0.046). There were no differences in the ratios, keratinised compartment:total thickness or cellular compartment:total thickness.

3.4 DISCUSSION

In this experiment, difficulty was experienced in producing biochemically deficient animals. Early on in the experiment there were significant differences in activation coefficients between the experimental group and controls. However, this was not the case at the conclusion of the experiment. Interestingly, the activation coefficients reported in the experiment by Kim and Roe (1985) followed a similar pattern, in that there was an initial increase in all groups over the first two to four weeks followed by a decrease over the next two to four weeks and finally, another increase over the last two weeks of the experiment. A significant difference in the activation coefficients between the groups fed a riboflavin deficient diet and those with riboflavin supplements (no alcohol) only occurred at week two. At the end of the experiment, while there was a trend towards a difference this was not

significant. They considered refection as the most likely cause of the initial variation in values of activation coefficients. This is certainly a possible reason for the variation reported in this experiment as faeces were not collected.

Due to the problems encountered with the health of the animals including the control group, analysis of both diets was carried out to exclude possible bacterial contamination and to identify the nutritional components. The microbiology results indicated that there was no bacterial contamination likely to cause an enteric infection. The riboflavin content of the deficient diet was found to be 2.7mg/kg by Aspland and James Ltd (Independent Analytical Consultancy) and 1.4mg/kg by Special Diet Services. The control diet was found to have 8.4mg/kg riboflavin by Aspland and James Ltd and 5.3mg/kg by Special Diet Services. These levels differed from the levels requested as the recommended dietary level of riboflavin for hamsters is 12-20mg/kg, although 6mg/kg has also been reported as satisfactory (Cooperman and Lopez, 1984) and the suppliers of the diet when contacted, quoted a range of 10-15mg/kg. It was concluded that the levels obtained were often lower than those expected due to the method of testing. A microbiological technique had been utilised. The levels reported were what they would expect following the addition of 10mg/kg of riboflavin to the base diet. This meant that the control animals may have received less than the recommended levels, therefore, there was not such a contrast between the two groups. This may account for the lack of flourishing of the control group, although if this was the only reason, it would have been expected that more of the experimental group would be affected.

The significant differences found in the thickness of the keratinised compartment of the dorsal surface of tongue and length of basement membrane of the ventral surfaces of

tongue were only slight and have to be treated with some sceptism in view of the lack of attainment of convincing riboflavin deficiency in the experimental group. If the differences did truly represent the effect of riboflavin deficiency then it would seem that it causes a thickening of the keratin layer in the dorsal surface of tongue which could offer some protection against topical carcinogens and the increase in the basement membrane length of the ventral surface of tongue would suggest more pronounced rete ridges. This may also afford some protection. Foy *et al.* (1972) noted that baboons fed a riboflavin deficient diet, developed hyperkeratosis and thickened, branching rete pegs of the epidermis. These changes would be consistent with the changes noted in this experiment. In addition, Wynder and Klein (1965) when supplying mice a riboflavin deficient diet noted hyperkeratosis early on in the experiment and epithelial hyperplasia of the tongue in the seventh to ninth weeks. These changes are similar to the findings reported in this study.

Animal	Week 0	Week 5	Final
1	82	75	90
2	82	84	89
3	80	79	87
4	73	78	79
5	50	88	101
6	69	83	87
7	72	83	90
8	68	93	102
9	68	81	95
10	67	86	99
11	77	84	89
12	89	89	88
13	62	67	75
14	64	75	60
15	79	77	74
Mean	72	81	87
SD	9.8	6.6	11.2

Table 3.1 Weights (g) of the animals in the experimental group. All the animals were alive at week 5. Due to the early deaths of animals 13, 14, 15 they were excluded from any further analysis. The remainder of the animals survived to week 11.

Animal	Week 0	Week 5	Final
16	84	93	102
17	81	83	89
18	74	82	95
19	76	76	73
20	57	-	45
21	72	-	55
22	68	75	66
23	67	78	90
24	75	92	105
25	68	-	52
26	-	68	74
27	-	70	68
28	-	72	73
Mean	72	79	76
SD	7.7	8.6	19.2

Table 3.2 Weights (g) of the animals in the control group. Due to the early deaths of animals 19, 20, 21, 22 and 25 which were excluded from further analysis, animals 26, 27 and 28 were added at week 5. Animals 27 and 28 also died early and were also excluded from further analysis.

Animal	Week 0	Week 5	Week 11
1	1.08	1.17	1.25
2	1.01	1.15	1.26
3	0.92	1.15	1.17
4	1.04	1.11	1.31
5	1.18	1.20	1.18
6	1.00	1.11	1.13
7	1.00	1.10	1.18
8	1.04	1.18	1.22
9	1.01	1.08	1.23
10	1.08	1.13	1.14
11	0.98	1.24	1.11
12	1.04	1.05	1.26
13	1.10	1.04	*
14	1.18	1.05	*
15	1.02	1.09	*
Mean	1.05	1.12	1.20
SD	0.07	0.06	0.06

Table 3.3 Riboflavin levels of the experimental group, which are recorded as an activation coefficient. Levels over 1.24 were considered to demonstrate deficiency.

Animal	Week 0	Week 5	Week 11
16	0.98	1.07	1.23
17	1.03	1.03	1.25
18	1.04	1.10	1.02
19	0.99	1.02	*
22	1.06	1.07	*
23	1.00	1.11	1.00
24	1.03	1.14	1.20
26	*	1.04	1.14
27	*	1.05	*
28	*	1.01	*
Mean	1.02	1.06	1.14
SD	0.03	0.04	0.11

Table 3.4 Riboflavin levels in the control group, which are recorded as an activation coefficient. Levels over 1.24 were considered to demonstrate deficiency.

Animal	LSU	LBM	TKC	TCC	TOTAL
1	214.10	236.11	11.48	26.94	38.42
2	274.11	256.78	19.25	40.80	60.05
3	234.96	244.11	14.82	37.84	51.08
4	243.94	238.05	13.72	37.41	51.13
5	263.38	250.27	25.08	44.74	69.82
6	254.02	268.18	17.82	50.62	68.44
7	250.59	283.56	27.18	59.73	86.91
8	258.19	279.82	22.41	53.04	75.45
9	248.32	265.08	29.83	58.30	88.13
10	245.04	262.94	22.97	54.73	77.70
11	245.33	239.19	21.16	43.17	64.33
12	250.29	252.21	14.35	49.81	64.16
Mean	248.52	256.36	20.06	46.30	66.30
SD	14.83	15.94	5.77	9.86	14.83

Table 3.5 Quantitative histological analysis of epithelium from the posterolateral surface of tongue of the experimental group. The values LSU and LBM represent the mean surface length and basement length respectively for the six measured columns of 228 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	LSU	LBM	TKC	TCC	TOTAL
16	248.28	255.18	25.40	53.51	78.91
17	281.94	265.35	21.82	50.03	71.85
18	251.71	255.35	19.45	42.34	61.79
23	246.58	242.28	19.40	48.88	68.28
24	254.62	258.93	25.21	57.60	82.81
26	244.50	252.29	17.42	40.20	57.62
Mean	254.60	254.90	21.28	48.76	70.21
SD	13.87	7.64	3.09	6.59	9.70

Table 3.6 Quantitative histological analysis of epithelium from the posterolateral surface of tongue of the control group. The values LSU and LBM represent the mean surface length and basement length respectively for the six measured columns of 228 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	RATIO KER:TOTAL	RATIO CELL:TOTAL
1	0.30	0.70
2	0.32	0.68
3	0.29	0.71
4	0.27	0.73
5	0.36	0.64
6	0.26	0.74
7	0.31	0.69
8	0.30	0.70
9	0.51	0.49
10	0.30	0.70
11	0.33	0.67
12	0.22	0.78
Mean	0.31	0.69
SD	0.07	0.07

Table 3.7 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the six measured columns of the posterolateral surface of tongue in the experimental group.

Animal	RATIO KER:TOTAL	RATIO CELL:TOTAL
16	0.32	0.68
17	0.30	0.70
18	0.31	0.69
23	0.28	0.72
24	0.30	0.70
26	0.30	0.70
Mean	0.30	0.70
SD	0.01	0.01

Table 3.8 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the six measured columns of the posterolateral surface of tongue in the control group.

Animal	LSU	LBM	TKC	TCC	TOTAL
1	595.47	921.63	54.88	109.53	164.41
2	628.43	725.35	64.03	100.16	164.19
3	523.33	556.53	64.06	110.01	174.07
4	527.99	832.69	59.72	128.19	187.91
5	680.44	707.75	67.84	115.51	183.35
6	607.25	758.97	48.33	99.15	147.48
7	599.77	772.98	51.24	109.27	160.51
8	665.02	743.17	54.49	105.79	160.28
9	500.21	830.06	54.02	119.07	173.09
10	570.62	815.22	56.60	121.37	177.97
11	564.66	698.75	59.36	108.24	167.60
12	638.37	783.01	48.28	101.54	149.82
Mean	591.80	762.18	56.90	110.65	167.56
SD	56.71	90.14	6.78	8.92	12.38

Table 3.9 Quantitative histological analysis of epithelium from the dorsal surface of tongue of the experimental group. The values LSU and LBM represent the mean surface length and basement length respectively for the four measured columns of 368 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	LSU	LBM	TKC	TCC	TOTAL
16	564.72	703.93	53.74	104.71	158.45
17	537.10	796.04	51.13	114.28	165.41
18	614.68	704.88	47.76	106.48	154.24
23	557.46	639.68	57.64	111.93	169.57
24	595.56	716.74	48.49	107.28	155.77
26	605.99	680.98	45.38	114.34	159.72
Mean	579.25	707.04	50.69	109.84	160.53
SD	30.66	51.47	4.46	4.21	5.88

Table 3.10 Quantitative histological analysis of epithelium from the dorsal surface of tongue of the control group. The values LSU and LBM represent the mean surface length and basement length respectively for the four measured columns of 368 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	RATIO KER:TOTAL	RATIO CELL:TOTAL
1	0.34	0.66
2	O.39	0.61
3	0.37	0.63
4	0.32	0.68
5	0.37	0.63
6	0.33	0.67
7	0.32	0.68
8	0.34	0.66
9	0.31	0.69
10	0.32	0.68
11	0.35	0.65
12	0.32	0.68
Mean	0.34	0.66
SD	0.03	0.03

Table 3.11 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the four measured columns of the dorsal surface of tongue in the experimental group.

Animal	RATIO KER:TOTAL	RATIO CELL:TOTAL
16	0.34	0.66
17	0.31	0.69
18	0.31	0.69
23	0.34	0.66
24	0.31	0.69
26	0.28	0.72
Mean	0.32	0.69
SD	0.02	0.02

Table 3.12 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the four measured columns of the dorsal surface of tongue in the control group.

Animal	LSU	LBM	TKC	TCC	TOTAL
1	448.98	490.53	36.66	88.10	124.76
2	449.98	436.48	11.42	60.03	71.45
4	484.01	450.57	26.30	63.57	89.87
6	391.65	460.26	33.21	92.92	126.13
7	390.67	479.29	30.81	92.03	122.84
8	381.50	447.16	23.85	87.38	111.23
9	420.01	493.07	35.67	109.90	145.57
10	530.49	509.72	21.37	83.76	105.13
12	436.72	449.42	22.36	87.07	109.43
Mean	437.11	468.50	26.85	84.97	111.82
SD	48.52	25.34	8.12	15.15	21.78

Table 3.13 Quantitative histological analysis of epithelium from the ventral surface of tongue of the experimental group. The values LSU and LBM represent the mean surface length and basement length respectively for the four measured columns of 228 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	LSU	LBM	TKC	TCC	TOTAL
17	455.38	440.02	23.57	71.78	95.35
18	445.54	419.04	15.58	76.47	92.05
23	478.59	462.02	24.73	101.81	126.54
24	432.19	439.02	25.21	105.07	130.28
26	446.33	407.64	21.04	75.90	96.94
Mean	451.61	433.55	22.03	86.21	108.23
SD	17.21	21.00	3.95	15.88	18.55

Table 3.14 Quantitative histological analysis of epithelium from the ventral surface of tongue of the experimental group. The values LSU and LBM represent the mean surface length and basement length respectively for the four measured columns of 228 μm width. The thickness measurements of the keratinised compartment (TKC), cellular compartment (TCC) and the combination of these (TOTAL) are in μm.

Animal	RATIO OF KER:TOTAL	RATIO CELL:TOTAL
1	0.29	0.71
2	0.19	0.67
4	0.29	0.71
6	0.26	0.75
7	0.25	0.75
8	0.21	0.79
9	0.25	0.75
10	0.20	0.80
12	0.20	0.80
Mean	0.24	0.75
SD	0.04	0.04

Table 3.15 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the four measured columns of the ventral surface of tongue in the experimental group.

Animal	RATIO OF KER:TOTAL	RATIO CELL:TOTAL
17	0.25	0.75
18	0.17	0.83
23	0.20	0.80
24	0.19	0.81
26	0.22	0.78
Mean	0.21	0.79
SD	0.03	0.03

Table 3.16 The mean ratios of the thickness of the keratinised and cellular compartments against the total thickness for the four measured columns of the ventral surface of tongue in the control group.

CHAPTER 4

A DISCUSSION OF THE EXPERIMENTAL FINDINGS

4.1 STATUS OF ORAL EPITHELIUM

The studies reported in this thesis were designed to assess the effect on the oral epithelium in hamsters of a diet deficient in riboflavin. Riboflavin levels were monitored throughout using the erythrocyte glutathione reductase test. Several quantitative histomorphometrical techniques were used to determine whether any changes occurred in the oral epithelium. Several problems were encountered which did not allow final correlation of riboflavin deficiency with oral changes. However, in a pilot study, it was found that the length of the surface epithelium of the posterior lateral surface of tongue was significantly shorter in the experimental group implying that the surface was flatter. The length of the basement membrane in these sections was also significantly shorter implying that the rete ridges were less pronounced, and the cellular compartment was significantly thinner, implying atrophy of the epithelium in the experimental group (Carmichael and MacDonald, 1992). The initial findings seemed to suggest that riboflavin deficiency, like that of some other nutrients, could cause atrophy of the oral epithelium. It is interesting to note that the area affected by atrophy was the posterolateral surface of the tongue. This finding was similar to that for other nutrients (Al-Damouk and MacDonald, 1987). It would appear that the dorsal and ventral surfaces of the tongue are possibly less susceptible to nutritional deficiencies. A number of problems had been encountered in the previous study which caused the validity of these findings to be questioned. These findings were not confirmed in the current study described in Chapter 3.

As was stated in Chapter 1, no quantitative work has been carried out in an animal model on oral epithelial changes and riboflavin deficiency apart from a pilot study (Carmichael and MacDonald, 1972). Epithelial changes have been noted in mice (Wynder and Klein, 1965) and baboons (Foy *et al.*, 1972) but these were purely descriptive. In mice, initially epithelium developed atrophy, followed by hyperkeratosis and then hyperplasia. They proposed that the initial changes seen of atrophy were identical to those seen in starvation, but that later, hypertrophy of the epithelium, which developed during the fifth to eighth weeks could be specifically attributed to riboflavin deficiency. In baboons the oral epithelia showed irregular patchy hyperplasia, thinning and dysplasia. It may have been that the changes seen in the pilot study (Carmichael and MacDonald, 1972) were more consistent with general starvation, rather than due to specific effects of riboflavin deficiency. Certainly the results of the current study showed slightly significant differences ($p<0.05$) in the thickness of keratin of the dorsal surface of tongue, being greater in the experimental group and, in the length of the basement membrane of the ventral surface of tongue, being longer in the experimental group. No significant differences were noted on the posterolateral surface of tongue. These changes were similar to those noted subjectively by Wynder and Klein (1965) and Foy *et al.* (1972). A number of studies have examined the influence of riboflavin deficiency on carcinogenesis and as has been already stated, the apparent effects are contradictory. In the current study, marginal riboflavin deficiency was produced and this had the effect of producing a thicker keratin layer and longer basement membrane. These may be protective features against topical carcinogens and therefore the apparent protective effect of riboflavin deficiency may only occur with chronic, marginal deficiency. In contrast, the apparent potentiating effect of riboflavin deficiency may occur in severe deficiency states, when epithelial atrophy may be produced. If the latter is true, it could be due to the effect of riboflavin deficiency on the

metabolism of other nutrients. This could be an area of future research in the hamster cheek pouch model or in application of carcinogens to the lingual epithelium. In particular, comparing groups with optimum riboflavin levels, marginally and severely deficient. Perhaps utilising a riboflavin antagonist to ensure severe deficiency.

4.2 RIBOFLAVIN TESTING

Chapter 2 described the initial testing of hamster blood for riboflavin levels. A variety of methods have been used in other *in vivo* studies. The microbiological and fluorometric methods have been used in early studies, but usually at the end of the experiment to assess riboflavin levels in liver tissue (Salley and Weatherred, 1964). This would have been unsuitable as a method of monitoring in the current study as it was necessary for all the animals to survive to the end of the experimental period. In previous studies, riboflavin levels were not monitored during the experimental period and weight loss alone was used as a marker of developing deficiency (Wynder and Klein, 1965: Foy *et al*, 1972). In one of the few papers describing riboflavin deficiency in the hamster, the erythrocyte glutathione reductase test was utilised (Kim and Roe, 1985). They did not mention encountering any particular problems with riboflavin testing in the hamster model using this method. The activation coefficients in all their groups after eight weeks on diets containing a varying amount of alcohol and riboflavin were higher than found in all our animals at weeks five and eleven. They did report the activation coefficients as means of groups, rather than as individual animals. It would appear that at least one of their animals had an activation coefficient of over 1.7 which was much higher than any achieved by our animals. This animal was in a group also supplied with alcohol which appeared to induce more severe deficiency.

However, even in their control group, with optimum levels of riboflavin and no alcohol, the mean activation coefficient was higher at 1.32 than the mean for the group fed the deficient diet in our experiment which was 1.2. Kim and Roe (1985) did not mention optimising the technique for hamster blood and as was found in Chapter 2, the activity of FAD in hamsters is much less than in humans which may lead to less precision in calculations. Kim and Roe (1985) also found that there were initial fluctuations in riboflavin levels, with initial increase in AC up to week two followed by a drop in weeks 4-6 and an increase on week 8. They attributed this effect to refection. Coprophagy was not prevented in the current experiment and so this may have had some effect on the results described.

The activation coefficients found in studies on the rat are far higher than those seen in the hamster, or for that matter in humans. Mean values for a group fed a riboflavin deficient diet have been quoted as 2.66 (Adelekan and Thurnham, 1986) and 2.54 (Gomez *et al.*, 1988). It is interesting to note that in these papers, no reference is made to a different threshold for deficiency in the rat, or that the levels were particularly high.

4.3 PROBLEMS WITH DIET

Due to the problems encountered regarding animal health, and the apparent resistance of the animals to becoming biochemically deficient despite considerable time on the diet, the diets supplied were examined for bacterial contamination and at the same time the riboflavin levels were checked. Two separate analyses were carried out, one by the company supplying the diet and by an independent firm. They confirmed that there was no bacterial contamination of the diet. The riboflavin levels measured, differed from those requested. The deficient diet had

higher levels than expected and the levels in the control diet were lower than expected. When the supplier was contacted they stated that these would be the levels expected if 10mg/kg of riboflavin was added to the basal diet. The diets are based on purified raw materials as opposed to natural cereal based ones. They utilise materials such as casein, cellulose powder and cornstarch as these materials have naturally low levels of many components including minerals and vitamins. The use of synthetic diets allows good control over nutritional levels. The supplier felt that any discrepancies were due to the method of analysis. They used a microbiological assay which often gives results lower than expected. The variation is what would be expected between laboratories or even repeated assay by the same laboratory. They also quoted the recommended riboflavin levels for hamsters as being 10-15mg/kg of diet. In view of the smaller difference in dietary riboflavin levels between the two groups, it was not surprising that no significant changes were found in any of the epithelial parameters measured. There were also differences in the measured levels of dietary riboflavin from the two testers. The independent tester measured higher levels than the supplier. This may reflect different methods of testing, or that the diet varied considerably in riboflavin content throughout batches. If the riboflavin content varied to this degree, it may be that other nutritional factors were altered. It may have been that if the diet was old or had been exposed to sunlight this had caused a reduction in riboflavin content. It could be argued that as severe deficiency of a single nutrient is unlikely in the general population, rendering the animals chronically marginally deficient, would be more appropriate to the real life situation. Only one control animal was rendered marginally deficient by week one, whereas four became marginally deficient in the experimental group. It would be interesting to carry out a similar experiment with three different levels of riboflavin in the diet, <1mg/kg, 5mg/kg and

>15mg/kg in order to compare the epithelium in animals severely deficient, marginally deficient and normal.

4.4 HEALTH OF ANIMALS

The health of the animals proved troublesome and the experiment was terminated at 11 weeks. Several animals died and three further animals were recruited into the control group. A number of animals became riboflavin deficient in the experimental group, but so too did three of the control animals.

Hamsters are not resilient laboratory animals. Once an epidemic of "wet tail" starts in a hamster colony, it can be difficult to control. Tetracycline can be used to treat this gastrointestinal infection, but were not in this series as the use of antibiotics can interfere with normal riboflavin levels if bacteria in the gastrointestinal tract compete for riboflavin stores. This can have the effect of raising the animals riboflavin levels if the bacteria are killed or it can have the reverse effect if the antibiotic spares some gut bacteria which then are able to multiply and they have a greater requirement for riboflavin than the normal flora. As the animals were suffering from diarrhoea, transit times would have been altered thus allowing less time for absorption from the gastrointestinal tract. This may also have induced multiple vitamin deficiencies. Kim and Roe (1985) reported in their experiment that six of 36 hamsters were lost. No reason was given for this and we have no way of ascertaining whether these were during bleeding, due to riboflavin deficiency or due to infection.

4.5 CONCLUSION

The studies reported in this thesis confirm that hamsters do have a dietary requirement for riboflavin and that marginal deficiency can be induced by a riboflavin deficient diet. The aim of this group of experiments was to ascertain whether isolated riboflavin deficiency could bring about epithelial changes, particularly atrophy, which could make the oral epithelium susceptible to carcinogens.

Riboflavin deficiency is linked to deficiencies of other nutrients such as folate and it is therefore difficult to identify if any one factor is the cause of atrophy. It is also unusual in nature to have an isolated deficiency of one B group vitamin.

The number of animals dying during the experimental period was abnormally high. No specific bacterial pathogen was ever identified from the dietary source. It may have been that suboptimal nutrition rendered the animals more susceptible to infection.

Another major problem encountered was the unexpected levels of riboflavin found in the diet. The diets were ordered from a reputable supplier and were to have contained specified levels of riboflavin. It was an error not to have confirmed the exact levels by analysis before the experiment. However, the supplier was satisfied that the levels of riboflavin in both the experimental and control diets were as expected.

In view of the findings reported in this thesis, further work is indicated. If it was to be carried out in the hamster model, the diet supplied should be examined carefully prior to use. It may however be possible to carry out a similar set of experiments using an organ culture deprived

of riboflavin, perhaps by using a riboflavin antagonist such as galactoflavin. It would also be of interest to investigate whether riboflavin deficiency alters the rate or number of tumours induced by a chemical carcinogen.

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