MALNUIRITION

AND

EXPERIMENTAL ORAL CARCINOGENESIS

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THESIS

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To

My Father

C O N T E N T S

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DECLARATION

This thesis is the original work of the author

J.D. Al-Damouk

PREFACE

The experimental work reported in this thesis was undertaken while the author was completing postgraduate studies in Glasgow supported by a grant from the Ministry of Higher Education and Scientific Research of the Republic of Iraq.

The research was undertaken in the Department of Oral Medicine and Pathology, Glasgow Dental Hospital and School, Glasgow from January, 1985 to January 1988. Animals for the first part of the work were caged in the Animal House of Gartnavel General Hospital, Glasgow. Animals for the three following parts of the work were caged in the Animal House of Glasgow Royal Infirmary, Glasgow.

Diet preparation, experimental work and histological analysis of tissues from all experiments were undertaken solely by the author. Blood analysis was partly done by the author and partly by the Departments of Haematology in Gartnavel General Hospital and Glasgow Royal Infirmary, Glasgow.

Some of the techniques used in this thesis are modifications of previously published work and some are techniques developed by the Oral Pathology Unit of Glasgow Dental Hospital and School. The application of the techniques described in the present study was undertaken by the author personally. The preparation of the

histological sections was carried out by the technical staff of Glasgow Dental Hospital and School under the direct supervision of the author and his supervisor.

Data reported in Chapter 4 of this thesis has been presented at the annual meeting of the British Society for Oral Pathology, 1987 and at a Scientific Forum in Glasgow Dental Hospital and School, 1987.

SUMMARY

This work was undertaken to examine the effects of nutritional deficiencies on cancer induction. Two of the most common and widely distributed nutrients, iron and folic acid, were examined to evaluate the effects of their deficiency on animals. The Syrian golden hamster was the animal model for all experimental work.

In the first part of the study an attempt was made to induce iron deficiency in young adult male hamsters by feeding iron deficient diet coupled with repeated venesection of 1.5ml every two weeks. Following twelve weeks on this regime a superficial biopsy was taken, on week 13, from the medial wall of one pouch in each hamster in order to evaluate the effect of iron depletion on the epithelial compartment thicknesses. After allowing the biopsy sites to heal for two weeks, a solution of 0.25% DMBA in acetone was painted, three times per week, for eight weeks, on a defined one square centimetre area in each pouch of each hamster of the experimental and control groups. The hamsters were then maintained on the same dietary regimes for twelve weeks before being killed at the beginning of week 37 for analysis.

Iron deficiency anaemia could not be induced in the experimental animals of this study. The effect of the iron deficient diet on epithelial compartment thicknesses at the stage of the biopsy was not clear. However, restriction of iron intake did cause animals

to develop significantly fewer grossly seen tumours and histologically identified carcinomas than control animals.

In the second part of this thesis an attempt was made to investigate alternative hamster dietary components that have less iron contamination than the diet given in the first part of this thesis. Casein and calcium lactate were the main contributers to iron in the hamster diet. Casein could not be substituted by another source of protein for hamsters. However, other sources for calcium with less iron contamination were available and therefore investigated in this part of the study.

Three groups of young adult male and female hamsters were given the fully nourishing powdered diet used in previous studies. However, calcium lactate was substitute for by either calcium acetate, calcium chloride or calcium sulphate in each group. None of the three diets was accepted by the animals and many of them died of starvation. When calcium salts were replaced by calcium lactate the surviving animals accepted the diet and recovered quickly afterwards. This study proved that calcium lactate could not be substituted by any other calcium salt with less iron content and therefore iron contamination in the hamster diet could not be further reduced by this method.

In the third part of this thesis the effect of nutritional folate deficiency on cancer induction was studied. A group of young adult female hamsters was given folate deficient diet for four weeks. On week 5, DMBA in acetone at a concentration of 0.25% was painted on

a defined one square centimetre area of the medial wall of each pouch in each hamster in folate deficient and control groups. The carcinogen was applied three times per week for eight weeks following which animals were maintained on the same dietary regimes for a further 13 weeks before being killed at the beginning of week 27 for the final analysis of the study.

It was found that nutritional folate deficiency had significantly reduced the number of animals developing grossly counted tumours and histologically identified carcinomas. The folate deficient animals also developed significantly less tumours and carcinomas compared to control groups.

In the last part of this thesis, the effect of combined iron and folate deficiency was examined for its role in carcinogenesis of the hamster cheek pouch. Two groups of young adult male hamsters were fed powdered diet lacking iron and folic acid and a third group was fed diet lacking iron only. One of the combined deficiency groups and the iron deficiency group were bled 1.0-1.3ml every week. On week 6 of the study DMBA in acetone at a concentration of 0.25% was painted three times per week for eight weeks on the same area of the pouch used in the previous studies. The animals were then maintained on the same experimental regimes for a further eleven weeks before being sacrificed, on week 25, for the final analysis of the study.

In this study, iron deficiency anaemia was induced in animals of the bleeding groups. Animals in the group with combined

iron and folate deficiency without bleeding showed low normal folate levels and normal haemoglobin levels. The two groups that were bled repeatedly showed iron deficiency anaemia. In all groups, the numbers of tumours counted grossly and the numbers of carcinomas identified histologically were significantly reduced compared to control animals in the previous studies. The folate deficient diet did not appear to influence the induction of iron deficiency.

The studies reported in this thesis proved that nutritional folate deficiency not only reduces the incidence, but it also reduces the numbers of tumours and carcinomas in the hamster cheek pouch. Iron deficiency anaemia was also found to significantly reduce the numbers of tumours and carcinomas of the hamster cheek pouch. It was not possible to produce combined iron and folate deficiency under the conditions of these studies. However, animals fed on a diet lacking iron and folic acid had significantly reduced numbers of grossly seen tumours and histologically identified carcinomas in the cheek pouch in response to DMBA applications.

In each of the reported studies, the nutritional deficiency of iron and folic acid, whether individually or combined was found to significantly reduce the growth rate of affected animals.

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 GENERAL INTRODUCTION

It is often said that cancer is a multifactorial disease. However, it is necessary to distinguish between cancer caused by several factors, changes in any one of which may modify its incidence or geographical distribution and those cancers where a single major aetiological factor predominates and in its absence cancer would not develop. The latter situation appears to be true for several cancers identified in man while in others the role of secondary exogenous factors is considered important but the extent to which such factors can cause significant variations in incidence and geographical variations of cancer patterns has not yet been determined. Heavy cigarette smoking can increase the risk of developing lung cancer probably by as much as tenfold. However, not all heavy cigarette smokers develop lung cancer. Furthermore, the latent period in lung cancer victims varies widely. Even in bladder cancer caused by 2-naphthylamine, where almost 100 per cent of exposed individuals develop neoplasia (Higginson, 1973) the latent period is variable. Animals exposed to strong carcinogens have been found to develop tumours at varying rates. Therefore, even with obvious strong carcinogens, secondary factors either external or internal may have modifying effects.

The variations in the latent period and the fact that not all individuals exposed to carcinogenic stimuli develop cancer can be interpreted as follows. Firstly, although only a small number of uncommon cancers such as retinoblastoma are known to be genetically determined, there could probably be a significant genetic element in individual susceptibility to cancer. Secondly, there might be some differences in carcinogen exposure which is a possibility that could not be excluded in prolonged exposure to low doses of a carcinogen. Thirdly, unidentified environmental factors whether carcinogenic or non-carcinogenic may play a significant modifying role in cancer development. These factors might be responsible for the individual susceptibility and geographic variations of cancer incidence. The increasing evidence that many major diseases are related to environmental factors, particularly those involving lifestyle variables is based largely on the marked differences in incidence rates between populations for major diseases such as cancer and cardiovascular disease.

Many of the molecules that come into contact with cells are derived from food. The average human being eats 1-3 kg of food per day. Food is an inseparable part of man's culture and constitutes an important part of his social customs just as do dancing, drinking and the use of herbs and spices. Such integration of food with other aspects of living makes it difficult to isolate single factors in food that could be related to cancer aetiology.

As far as research on food and cancer is concerned, the components of diet which have attracted most study are general nutritional inadequacy, contamination with carcinogenic substances and factors in the food that alter metabolic pathways and so alter response to carcinogenesis. Other factors such as the interaction between different dietary components and between the dietary components and microorganisms in the host are also found to have some significance.

1.2 AETIOLOGICAL FACTORS IN CANCER

1.2.1 Major Aetiological Factors in Cancer

Identification of mechanisms involved in the conversion of a normal cell into a cancer cell has always been a major problem in medical research. A major advance was made in 1775 when Percival Pott observed a high incidence of cancer of the scrotal skin among chimney sweeps in Britain (Pott, 1775). This was the first indication that the development of cancer might not be a spontaneous process, but rather the result of contact with **chemical carcinogens.** The carcinogenic action of coal tar was demonstrated on laboratory animals and proved to be effective in producing cancer (Selkirk, 1980). Subsequently, a large number of chemicals, for example, in drugs and foodstuffs were shown to have carcinogenic potential and were routinely screened for this purpose.

Exposure to **ionising radiation** is another factor in cancer production. It became apparent when a high incidence of cancer of the skin was noticed in early radiologists who used their hands to calibrate their X-ray machine performance (Anderson, 1985). The effects of ionising radiation were emphasized when a high incidence of leukaemia was also observed in these radiologists. Cancer of the skin in fair-skinned people exposed to sunlight for a long time has also proved the association of ultraviolet light with cancer.

Genetic factors have for a long time been suspected as having an influence on the risk of developing cancer. This theory was studied in experimental animals and subsequently applied to man as for example in retinoblastoma (Anderson, 1985). However, the mode of inheritance was not clear and susceptibility to exogenous factors was suspected as having an influence on the outcome of cancer development in people with an appropriate genetic background (Reif, 1981). Epidemiological studies suggested that approximately 2 per cent of cancers were directly related to hereditary factors alone (Higginson and Muir, 1979).

Recent advances in cancer research have involved the discovery of cellular oncogenes. It provided an acceptable explanation for the role of the environmental factors in cancer induction. Cellular oncogenes can be activated by a variety of mechanisms including environmental factors (Paul, 1984).

1.2.2 Minor Environmental Factors and Cancer

The correlation of incidence and mortality rates with the prevalence of environmental agents in various areas has provided useful information on the role of such environmental factors in the aetiology of cancer. However, there is uncertainty regarding the exact meaning of the word "environment" which has led to misinterpretation of the proportion of cancers that might be environmentally determined (Editorial, 1977; Higginson, 1979). MacLure and MacMahon (1980) thought that the word environment generally referred to any factor other than that of genetic background inherited from an individual's parents. Accordingly, when the geographic distribution of cancer was used as an index of the prevalence of environmental factors, it was found that almost all cancers are caused by exogenous factors.

Comparison of cancer rates between regions indicated that as much as 70-90 per cent of cancers were environmentally induced (Wynder and Gori, 1977). However, no matter what percentage environmental factors were given in cancer aetiology, the consistency of the findings suggested that environmental rather than genetic factors were playing a predominant role in cancer aetiology in man.

The mechanisms by which environmental carcinogens could cause 70-90 per cent of cancers have been explained by Weisburger and Horn (1982). They stated that a change in genetic material could happen through several mechanisms such as by radiation, chemicals or viruses. The production of abnormal DNA by any of these factors is

the first step on the way that could terminate in a malignant neoplasm. The duplication process of altered cells, which is the next step towards an obvious tumour formation, will depend on certain endogenous and exogenous controlling elements which would either promote the growth of cancer or retard or even inhibit its growth. Therefore, in any specific human cancer consideration needs to be given both to agents leading to an abnormal genome and those that are involved in the growth and development of the resulting neoplastic cells and their further progression to clinical malignancy (Weisburger and Horn, 1982).

The two basic types of environmental agents distinguished were the consumable agents and contaminant agents. Among consumable agents there were tobacco, alcohol beverages, food and food additives and drugs(Wynder et al., 1956 and 1957a,b; Wynder and Bross, 1961; MacSween, 1982; Weisburger and Williams, 1982). Among contaminants there were atmospheric pollutants, biologic contaminants and radiation. Other aspects of life such as diet and dietary inadequacy were investigated later and also found to be important in the aetiology of many cancers (Reddy et al.,1980).

A widespread interest in the role of human diet as a probable aetiological factor in cancer has developed only recently. A striking correlation was found between death rates from certain cancers and the total consumption of particular dietary items in different countries (Graham et al., 1977; Rose and Boyar, 1986). The study of migrant groups such as the Japanese in the United States

showed changes in cancer risks following their adoption of a more Western diet (Wynder and Hirayama, 1977). Estimations of the extent to which cancer in a number of sites may be attributed to nutritional factors have suggested values as high as 50 per cent in cancers of breast, corpus uteri and colon (Wynder and Gori, 1977; Miller, 1980).

1.2.3 Diet, Nutrition and Cancer

Research on the role of diet in the aetiology of cancer in man has been influenced by the great variety of mechanisms by which diet may exert its carcinogenic effect (Vitale, 1975; Miller, 1980; Shamberger, 1984). The simplest and most obvious mode of dietary involvement in cancer aetiology would be the ingestion of small amounts of carcinogenic substances for example asbestos which is found in many water supplies (Shamberger, 1984). Substances such as cycasin in the cycad nut, safrole in sassafras and extracts of coltsfoot and bracken fern were found to be carcinogenic in experimental animals (Doll and Peto, 1981). However, only bracken fern has been related to cancer in humans. Japanese who ingested bracken fern daily were found to have a three times higher risk of developing cancer of the oesophagus compared to Japanese who avoided it (Hirayama, 1979).

Another possible source of carcinogens is their production during cooking (Shamberger, 1984). Humans are the only animals that cook their food and it has been known for many years that benzo-α-pyrene and other polycyclic hydrocarbons can be produced by pyrolysis when meat or fish is broiled or smoked or when any food is

fried in fat that has been used repeatedly (Hunt, 1980; Miller, 1980). Sugimura et al. (1977) have demonstrated that pyrolysis of food as for example in charcoal-broiled steaks produces powerful mutagens in addition to the production of benzo-α-pyrene. However, no substantial epidemiological evidence has been found to confirm that food cooked in this way causes, for example, gastric cancer which was suggested in the United States as being so related (Dungal and Sigurjonsson, 1967).

Another recognised mechanism is the production of carcinogens in stored food by the action of microorganisms (Doll and Peto, 1981). Aflatoxin, a product of the fungus Aspergillus flavus that commonly contaminates peanuts, beans, corn and other staple carbohydrate food stored in hot and humid climates was found to be a major factor in the production of liver cancer in certain tropical countries (Hunt, 1980; Armstrong, 1977). Experimentally, aflatoxin was found to be among the most powerful liver carcinogens for some animal species and possibly for humans (Armstrong, 1977). The incidence of primary liver cancer which is the commonest type of cancer in inhabitants of some parts of Africa was found to be significantly higher in those parts where the amount of dietary aflatoxin was high (Linsell and Peers, 1977). However, infection with hepatitis B virus was found to be an important predisposing factor and it is possible that both aflatoxin and hepatitis B virus contribute to the risk of liver cancer by acting synergistically (Doll and Peto, 1981).

The possible formation in the body of carcinogenic compounds from precarcinogens has also been studied (Doll and Peto, 1981). N.nitroso compounds are powerful chemical carcinogens. They were found to be present in small amounts in the resting gastric juice and could be formed in the digestive tract and possibly in the infected bladder by the reaction between nitrites and various nitrosable compounds such as amines or N.substituted amides (Fine et al., 1977).

Nitrosable compounds were found to be present naturally in many foods particularly in fish and meat. They could also be ingested as pesticide residues or drugs and they could be formed in the colon from amino acids (Fraser et al., 1980). Nitrite could be derived directly from food to which it has been added either as colour preservative or flavour enhancer, or more probably as a result of its production from nitrate ingested in vegetables and to lesser extent in drinking water (Fine et al., 1977). However, there is no sound epidemiological evidence to support this theory. Cancer of the oesophagus and stomach which were suggested as being relevant to nitrosamine carcinogenesis (Fraser et al., 1980) were not affected and could perhaps be prevented by the high vegetable-containing diet which was a major source for the suggested carcinogen (MacLennen et al., 1977; Lu and Lin, 1982).

1.2.4 Macronutrients and Carcinogenesis - Dietary Fat

Many researchers believe that the main influence of ingested food upon carcinogenesis might not be a direct one, but instead it

could be a modifying effect by providing a more favourable environment for cancer development. Experimental and epidemiological studies showed that excessive fat intake was correlated with higher incidences of colon, endometrial and prostate cancer (Hunt, 1980; Kerr, 1983; Doll and Peto, 1981), cancer of the corpus uteri and ovary (Armstrong and Doll, 1975) and also cancer of the breast (Hirayama, 1979; Rose and Boyar, 1986). Miller (1980) found that high total fat intake in Canada increased the risk for breast cancer by at least 27 per cent.

Dietary intake of fat typical of the Western diet, accounting for about 40 per cent of calories, together with a relatively low fibre consumption was found to increase the amounts of bile acids and cholesterol metabolites in the gut. Animal experiments showed that rats given several types of colon carcinogens had a higher incidence of large bowel cancer when fed fat at 20 per cent in the diet (equal to 40 per cent of calories) than animals receiving 5 per cent fat (Weisburger et al., 1982).

It has been suggested that certain fats might contribute to the production of carcinogens in the body by increasing the amounts of bile acids and cholesterol metabolites that come into contact with the gastrointestinal tract mucosa (Shamberger, 1984). Reddy et al. (1980) reviewed the literature and found that these substances especially deoxycholic and lithocholic acids, were present in higher amounts in the faeces of people on Western type diets and in whom colorectal cancer is more common than in people on Asian or African types of diet and in whom colorectal cancer is uncommon. Deoxycholic and

lithocholic acids were also found at higher levels in the stools of patients with adenomatous polyps of the colon than in patients with other diseases or healthy controls. The amounts of these acids could be increased experimentally in humans by a high-fat, high-meat diet (Reddy, et al., 1980).

Wynder and Hirayama (1977) found that the faecal content of cholesterol metabolites, bile acids and bacterial beta-glucuronides in Americans eating high-fat diet was significantly higher than in Japanese who are a low risk group. Experimental work on animals showed that high-fat diets increase the faecal excretion of the same group of bile acids and increase the incidence of colon cancer induced by a variety of colon carcinogens (Shamberger, 1984). It was felt that colesterol and bile acids were likely to be acted upon by bacteria in the colon to produce carcinogens that either acted locally on the colon or elsewhere in the body (Kerr, 1983; Armstrong, 1977).

Other experimental studies on animals suggested that dietary fat influences the promotional stage of carcinogenesis rather than the initiation stage (Carroll, 1975). It was observed that the yield of mammary gland tumours induced in rats by 7,12-dimethylbenz-α-anthracene (DMBA) could be enhanced by feeding a high-fat diet after, but not before, the carcinogen application. The mechanism by which dietary fat could stimulate mammary carcinogenesis is not clear but it could be due to impared DNA repair systems, immunocompetence or membrane structure and function (Carroll, 1981).

1.2.5 Macronutrients and Carcinogenesis - Dietary Fibre

Dietary fibre is a non-starch polysaccharide substance. It is the most recent of a number of dietary components to be investigated in relation to cancer of the large bowel (Armstrong, 1977; Weisburger et al.,1977). Dietary fibre is seen as a protective factor against bowel cancer and thought to act by neutralising any promotional effects of fat and protein (Reddy, 1986).

It is possible that dietary fibre reduces cancer incidence of the colon by reducing the duration or concentration of carcinogens present in contact with the gut epithelium by increasing the bowel motion and the bulk of stool. Dietary fibre could also alter the total numbers of different bacterial species in the bowel which might be responsible for the production of carcinogenic metabolites (Shamberger, 1984; Graham, 1980). However, investigations regarding the possible role of various types of dietary fibre in animal models have not produced consistent results (Cummings, 1981; Reddy, 1986).

1.2.6 Micronutrients and Carcinogenesis

Research on nutrition and cancer has long regarded malnutrition as the most important way diet could influence carcinogenesis. Research has dealt with this relationship in two distinct ways. The overall nutritional status of the individual and specific nutriet deficiencies. Malnutrition exerts its effect by

suppressing the host's cell mediated immune response to tumour cells (Shamberger, 1984). It was observed that malnourished children were more susceptible to infection than normal children, due to impaired immunocompetence (Chandra, 1974) and that cell mediated immunity in particular was consistently depressed. When T lymphocytes were tested for their frequency of rosette-formation, delayed hypersensitivity responses and for the rate of DNA synthesis under certain conditions (Chandra, 1974) it was found that these functions had been significantly depressed in malnourished children compared to healthy children. These functions reverted to normal following nutritional correction.

The second type of research has dealt with the problem of the relationship between specific nutrient deficiencies and cancer by investigating separately the common nutrient deficiencies in certain populations and their significance to carcinogenesis. Vitamins and some minerals have always been regarded as good indicators of the nutritional status of individual. Vitamins, in particular folic acid and B12 and minerals such as iron have been routinely checked in patients attending clinics for a variety of medical problems. This is done mainly due to their frequent depletion in the body and the serious clinical implications of their deficiencies. In cancer research folic acid and iron were amongst the first nutrients tested for their role in carcinogenesis. These are discussed separately in sections 1.3 and 1.4. However, other vitamins have also been found to be important in cancer research.

Vitamin A and its retinoid derivatives were amongst the widely investigated vitamins in terms of their possible role in the process of carcinogenesis. Vitamin A was found to play an important role in the control of growth, differentiation, maturation and function of epithelial tissues (McLaren, 1978). While excess of vitamin A causes epithelial thinning (Chisholm et al., 1978), deficiency was observed to result in hyperkeratosis of the skin and squamous metaplastic changes in the epithelia of the gastrointestinal, respiratory and urogenital tracts (Clayson, 1975).

Epidemiological studies on nutrition and cancer in man suggested that vitamin A plays a protective role against cancer in general. Vitamin A deficiency was associated with higher risk of cancer especially that of the stomach, nasopharynx and respiratory tract (Shamberger and Willis 1977; Bjelke, 1975). Animal studies on mice showed that animals inoculated with breast carcinoma cells had a 10-50 per cent reduction in incidence of developing tumours when supplemented with vitamin A compared to deficient animals. Those vitamin A supplemented mice that developed tumours showed a considerably prolonged latent period, slower tumour growth rates and infrequent metastases (Seifter et al., 1984). Vitamin A was also found to inhibit the induction of cervical cancer and tumours of the respiratory tract in rats and hamsters induced by DMBA carcinogenesis (Shamberger and Willis, 1977; Seifter et al., 1984). Squamous metaplasia and bronchogenic carcinoma caused by benz pyrene were also inhibited by oral supplementation of vitamin A (Shklar, 1984; Bjelke, 1975).

Several mechanisms are thought to be involved in the anticarcinogenic effect of vitamin A (Genta et al., 1974; Shamberger, 1986). Mechanisms such as the inhibition of microsomal mixed-function oxidases that metabolise polycyclic aromatic hydrocarbons were found to be important. Vitamin A could decrease binding of benz pyrene to DNA of hamster cells of the trachea and thus prevent its potential damage to DNA. It has also been suggested that vitamin A could enhance growth and maturation of cells that regulate various immune functions such as that of T lymphocytes and so decrease tumour growth.

Group B vitamins, in spite of the little available data, were found to influence carcinogenesis in animals and probably man (Boutwell et al., 1949; Tannenbaum and Silverstone, 1952). Riboflavin, and to a lesser extent thiamine and pyridoxine, were the best investigated examples of this group. The role of **riboflavin** deficiency in the aetiology of cancer has been noted since the early observation that Paterson-Brown Kelly syndrome, which is frequently associated with oesophageal cancer, was often accompanied by riboflavin deficiency (Wynder et al., 1957a). Riboflavin deficiency was later found to cause atrophy of epithelial tissues of the oesophagus and forestomach in mice (Wynder, 1971). Epidemiological studies in high and low risk areas for oesophageal cancer in China showed a positive correlation between riboflavin deficiency and increased cancer incidence (Yang et al., 1982). Ninety-seven per cent of the high risk population were found to have biochemical evidence of riboflavin deficiency although only one per cent of them showed clinical signs such as chronic oesophagitis evident by endoscopic

examination (Thurnhan et al., 1982). Zaridze et al. (1985) in a similar epidemiological study on high and low risk populations for cancer in the Central Asian Republic of the Soviet Union found a similar correlation between riboflavin deficiency and cancer of the oesophagus. They suggested that such deficiency may increase the susceptibility of the oral and oesophageal epithelium to the action of N-nitroso compounds derived from nass* smoking which is common among the high risk populations studied.

The effect of riboflavin deficiency on oesophageal and liver carcinogenesis has been studied in rats. Riboflavin deficiency induced by dietary means, in addition to retarding growth, facilitated oesophageal carcinogenesis induced by nitrosamines and liver carcinogenesis induced by azo dyes (Peizhong et al., 1985).

Vitamin C, which is important for the normal synthesis of collagen is also able to inhibit hyaluronidase and strengthen the ground substance of connective tissues (Vilter, 1978). Ascorbic acid is required in significantly high levels in cancer patients (Cameron et al., 1979). Experimental work on animals showed high ascorbic acid content of diet reduced mouse skin papillomas induced by DMBA and bladder carcinomas induced by 3 hydroxyanthranilic acid (Shamberger and Willis, 1977). Dietary sodium ascorbate was also tested in rats by Reddy et al. (1980) who found that higher levels of the vitamin were associated with lower incidences of colon and kidney tumours.

* nass is a mixture of tobacco, ash, cotton oil and lime.

Vitamin C deficiency could influence carcinogenesis through interuption of the mechanical barrier of the connective tissue stroma of tumours causing cancer cells to grow faster and invade the surrounding tissues more easily (Cameron et al., 1979). Ascorbic acid could also inhibit the promotion of certain precarcinogens to active carcinogens (Clayson, 1975; Shamberger, 1986).

Vitamin D, which is an essential substance for the absorption of calcium and phosphate from the intestine and their renal tubular reabsorption (Keele et al., 1982), was noted to inhibit chemical carcinogenesis of the hamster cheek pouch when applied simultaneously with the carcinogen (Rubin and Levij, 1973). The suggested mechanism was the induction of alkaline phosphatase synthesis by vitamin D and facilitation of the flow of different metabolites through the cytoplasmic membrane.

Vitamin E deficiency in man was observed to be associated with few clinical signs such as reduced red cell total lifespan (Underwood, 1978). Vitamin E significantly delayed the development of experimental hamster pouch carcinomas (Shklar, 1984) and reduced the number of DMBA induced skin papillomas in mice (Shamberger and Willis, 1977). Shamberger (1986) suggested that vitamin E could prevent tumour formation through its antioxidant action and that it is more effective than vitamin C in this respect.

1.3 FOLIC ACID AND FOLATE DEFICIENCY

1.3.1 General Considerations

Folic acid has also been called vitamin Bc, vitamin M and pteroylglutamic acid. The term folic acid was first used when the active material was extracted from leafy vegetable "foliage" (Mitchell et al., 1941). Subsequently the terms folic acid or folate were used as general terms for any member of the folic acid family.

Folic acid was first discovered in 1930 as a factor present in an autolysed yeast preparation able to cure nutritional megaloblastic anaemia occurring among pregnant women in India (Davis, 1986). The active agent in that preparation was subsequently identified as folic acid which is chemically known as pteroylglutamic acid.

Folic acid is essential for normal growth and proliferation of all animal cells. Deficiency of this vitamin produces megaloblastic anaemia which is characterised by increased size and slow DNA synthesis in proliferating cells (Herbert and Das, 1976) and macrocytosis of red blood cells.

1.3.2 Chemical Composition and Properties of Folates

Pteroylglutamic acid, the parent compound of all folates, has a molecular weight of 441.4 Daltons and consists of the pteridine or pterin portion, para-aminobenzoic acid and L-glutamic acid. When crystalised from an aqueous solution it appears as yellow spear-shaped crystals. These crystals are poorly soluble in water (500mg/l at 100°C) and organic solvents but as the disodium salt they are freely soluble. Solutions of pteroylglutamic acid are stable in the dark while exposure to ultraviolet light or sunlight results in cleavage of the molecule at certain positions to give pteridine and a free aromatic amine (Chanarin, 1979).

Pteroylglutamic acid as such has not been found in food or in the human body in significant concentrations. It is not biochemically or metabolically active although it becomes so after reduction (Herbert, 1968a; Herbert and Das, 1976). In contrast, other compounds of pteroylglutamic acid are widely distributed in nature and are in the active reduced form. These natural folates are readily oxidised and become inactive in rates varying directly with oxygen concentration, temperature, exposure to light and concentrations of cupric and ferric ions (Chanarin, 1979).

Folate in the diet, in contrast to pteroylglutamic acid, is composed mainly (80-90 per cent) of multiple glutamate residues or polyglutamate called 5-methylpteroylpolyglutamate while the rest was identified as monoglutamate analogues mainly in the form of

5-methyltetrahydrofolate and pteroylglutamic acid (Weir, 1974; Herbert and Das, 1976; Davis, 1986)

The transport form of folate which is present in serum and tissue fluids is mainly the monoglutamate 5-methyltetrahydrofolate. Intracellular folates occur as conjugates with 2-7 glutamic acid residues and thus they are pteroylpolyglutamates. It has not been confirmed whether these intracellular pteroylpolyglutamates are the storage form of folic acid or in fact the active coenzymes of the biochemical reactions required for cell metabolism (Herbert and Das, 1976).

1.3.3 Availability and Human Requirements of Folate

Folates are widely available in nature (Herbert, 1968b). They are found in large quantities in plants such as spinach, peas, lettuce, orange juice and most fresh green vegetables. They are present in animal tissues such as liver and meat and also in egg white and milk. Large quantities are also present in yeasts (Chanarin, 1979). Although folate synthesizing microorganisms were confirmed to be present in the human gut (Davis, 1986), the amount of folate they produce was found to be substantially too low for human needs. As a result humans are dependent on food as the major source of the vitamin.

The suggested minimal daily adult requirement of folate is 50μg (Herbert, 1968c; Davis, 1986). This amount should be increased

with any rise in the daily metabolic rate or cell turnover rate as in hyperthyroidism, pregnancy and haemolytic anaemia (Herbert, 1968a). More recently the World Health Organisation expert group (WHO, 1972) has recommended that the daily dietary intake of folate should be 40-50μg in infants up to six months of age, 120μg for children between seven and twelve months, 200μg for children of one to twelve years and 400μg for over thirteen years. For pregnant women, the daily intake should at least be 800μg which can be reduced to 600μg during lactation. The British Department of Health and Social Security recommended in 1979 a daily folic acid intake of 200μg for adults, 500μg for pregnant women and 400μg during lactation. However, these recommended amounts for British people were withdrawn on the basis that the requirements for folic acid in healthy adults were not firmly established (Davis, 1986).

1.3.4 Absorption and Transport of Folate

Both active and passive mechanisms have been suggested to be operating in the absorption of folate from the alimentary tract (Herbert, 1968a). The active mechanism takes place primarily in the upper third of the small intestine in the presence of small amounts of folic acid in food. Passive absorption was found to operate, probably by diffusion, along the entire length of the small intestine when large amounts of the vitamin were present in the diet.

Before absorption, folates in the diet mainly in the form of polyglutamates have to be degraded to the folate monoglutamate form through the removal of the extra glutamic acid residues by an intestinal conjugase (Weir, 1974). The conjugase is thought to be present in the intestinal juice and mucosal cells (Herbert, 1968b; Davis, 1986). During absorption all available folate undergoes reduction and methylation before entering the portal circulation. This alteration in the folate molecule was thought by some investigators (Perry and Chanarin, 1970 and 1973) to be necessary for absorption. However, other workers found that these alterations were not necessary or rate limiting steps for folate absorption (Weir, 1974) and that reduction of folate may occur entirely in the enterocytes, in a process catalysed by di- and tetrahydrofolate reductase, before being transported to the portal circulation (Herbert, 1973a).

1.3.5 Utilisation of Folate

Folate in the serum, mainly in the form of 5-methyltetrahydrofolate, is transported to sites of utilisation by the help of certain folate-binding proteins (Chanarin, 1979). Part of this circulating folate is delivered across the cell membrane in a process thought to be vitamin B12 dependant (Herbert 1973a; Herbert and Das, 1976) while the other part of the serum folate remains in the circulation and is eventually excreted unchanged in the urine.

Intracellularly, folate is a coenzyme concerned in the transport of one carbon units in different metabolic reactions. These reactions are mainly purine, pyrimidine and methionine synthesis, serine-glycine conversion, histidine degradation and initiation of protein synthesis (Herbert and Das, 1976). The carbon unit may be required in a fully reduced state as methyltetrahydrofolate in methionine synthesis. It may also be required at the oxidation level of formaldehyde in thymidylate synthesis or at the oxidation level of formate in purine synthesis (Chanarin, 1979).

1.3.6 Storage and Excretion of Folate

The exact amount of folate in the human body has not yet been determined, but it was thought that it could be 5-10mg (Herbert, 1968b). A man whose body stores are normally saturated but whose diet is deficient in folate could last 3-6 months before megaloblastic changes would occur in the bone marrow (Herbert, 1962; Herbert, 1973b). On the other hand if the storage was reduced or the liver, which is the main site of storage folates, was metabolically inhibited from creating the storage form of folate then a folate deficient diet would induce the onset of megaloblastic changes much sooner (Weir, 1974).

As mentioned before (1.3.4), dietary folate is present in the form of folate polyglutamate. However, before absorption the glutamic acid residues are removed and folate monoglutamate is absorbed and transported in the blood, probably after methylation and

reduction, to the liver. In the liver folate is resynthesised into the polyglutamate form by the action of a ligase enzyme and stored in the liver cells. When the body stores are saturated excess dietary folate is excreted largely unchanged. Thus the storage capacity will determine the amount of folate retained in the body (Weir, 1974).

It has been suggested that in a normal subject the amount of folate excreted unchanged from the body following ingestion of dietary folate is 50 per cent (WHO, 1972). Estimations of the excretion pathways showed that the major part of excreted folate was through urine (30-50%). Biliary excretion was a very minor pathway. The folate retained in the body is partly degraded in tissues and partly exchanged with the body stores (Chanarin, 1979).

1.3.7 Folate Binding Proteins

Studies on folate metabolism suggested that folate-binding proteins are important mediators for absorption and transport of folate to sites of utilisation. One type of such proteins is thought to be present in the brush border of rat enterocytes and could be present in the same site in humans (Leslie and Rowe, 1972). This folate binder has more affinity for monoglutamates than longer chain folate analogues. The purpose of such protein is not certain but it could be important in the transport of folate from the intestine to the enterocyte (Davis, 1986).

The second group of folate-binding proteins is present in the blood and tissue fluids and it is concerned in the transport of folate to sites of utilisation. This group consists of two types of binders. The first is a nonspecific binder with low affinity for folate binding while the second is specific with high affinity but a low capacity for folate binding due to the presence of few binding sites (Chanarin, 1979).

The nonspecific folate binding protein is mainly albumin (Elsborg, 1972; Hall and Rappazzo, 1974; Soliman and Olesen, 1976) although transferrin and an α 2-macroglobulin may also bind folate under certain conditions (Markkanen and Peltola, 1971; Markkanen et al., 1972a,b; and 1973). Folate molecules are negatively charged at physiological pH. They can attach themselves to these positively charged nonspecific binders. This binding does not follow saturation kinetics and approximately two-thirds of added folate is bound to these nonspecific folate binders. Decrease in the level of these binders has been demonstrated in folate deficiency, pregnancy, in women taking contraceptive pills and in epileptics receiving diphenylhydantoin (Dilantin).

The specific folate binding protein is a glycoprotein with a molecular weight of 40 kilodaltons (Fernandes-Costa and Metz, 1979; Colman and Herbert, 1976). It has more affinity for oxidised folate than for the normally reduced physiological form of folate. High levels of this high affinity binder were detected in folate deficient sera, in cirrhotic patients, in uraemic sera and in pregnancy (Davis,

1986) and in patients with metastatic carcinomas (Eichner et al., 1978).

Other binding proteins have been described in different tissues under different conditions. They were found in leukaemic leucocytes, hepatic cells, hog kidney and other tissues such as lymph nodes, lung, thymus, breast, ovary, pancreas and thyroid (Chanarin, 1979). Folate binding proteins have also been detected in milk where their function was thought to enhance absorption of folate in breast-fed infants and probably to protect folate against utilization by intestinal bacteria (Colman et al., 1981).

1.3.8 Folate Deficiency in Man

Folic acid deficiency is one of the most common and widely spread vitamin deficiencies in the world particularly in underdeveloped countries (WHO, 1972). Southern India is thought to be the most severely affected area in the world with up to 40 per cent of its population being folate deficient (Herbert, 1968b; Weir, 1974). Folate deficiency is a common finding in Africa and Asia especially in pregnant women and people suffering from tropical sprue which is endemic in these countries.

In the developed countries, the incidence of dietary folate deficiency has decreased greatly during recent years. There are still certain groups of people such as the elderly (WHO, 1972; Grinblat, 1985) and heavy alcohol drinkers (Wu et al., 1975) who suffer from

folate deficiency, although only a small percentage of these individuals have clinical signs and symptoms.

Human beings usually have stores of folate that can be mobilised when dietary intake is insufficient to meet tissue requirements. However, under such circumstances storage folate will provide less folate to the serum than under normal conditions. Continued negative balance between availibility and demand for folate may gradually result in clinical and/or biochemical signs and symptoms which terminate in megaloblastic anaemia (WHO, 1972).

Folate deficiency can be the result of inadequate intake, malabsorption, defective metabolism and utilization, or increased demand. A combination of two or more of these factors is a common feature in folate deficiency.

1.3.9 Aetiology: Inadequate Ingestion of Dietary Folate

Although folates are widely distributed in nature, dietary regimen and socioeconomic factors may reduce availability of useful analogues of folate as well as other nutrients. It was estimated that up to 40 per cent of the population of Southern India are folate deficient mainly due to consumption of a diet containing little folate where rice is the staple food (Weir, 1974). Although this type of food has adequate amounts of folate when in a raw state, folate is rapidly destroyed by cooking (Herbert, 1973b). When food is finely

cut and cooked, especially by boiling in large quantities of water, it may lose as much as 95 per cent or more of its folate content. Diet lacking fresh uncooked fruits or vegetables has little folate content (Herbert, 1968b and 1973b).

In Western countries, as in the underdeveloped countries, disability, chronic disease and poverty are the main reasons for folate deficiency in elderly people. These people either lack the basic dietary knowledge or are unable to obtain and prepare a well-balanced diet. They often tend to overcook their food because of dental problems thus destroying much of the folate in their food. It has been estimated that as much as 10-15 per cent of the healthy elderly persons in Britain suffer from some degree of folate deficiency (WHO, 1972). However, clear evidence of megaloblastic anaemia was only evident in one per cent of these people (Mollin, 1960). Dietary folate deficiency per se can also produce malabsorption syndrome by causing morphologic and enzyme changes in intestinal cells and thus reduce the ability of these cells to absorb folic acid. A vicious circle is then set up.

Research as well as epidemiological studies have shown that chronic excessive alcohol consumption is often accompanied by folate deficiency. 23-44 per cent of chronic alcoholics have low serum, red cell and liver folate levels (Herbert et al., 1963; Eichner and Hillman, 1971; Lundy et al., 1975; Wu et al., 1975). Many factors are implicated in this deficiency state, but of these socioeconomic factors are the most important causes of deficiency (Eichner and Hillman, 1971). The factors implicated in folate deficiency in

alcoholics are nutritional deficiency of food folate (Wu et al., 1975; Eichner et al., 1972); malabsorption secondary to the effect of nutritional deficiency (Grinblat, 1985); the direct effect of ethanol on the gastrointestinal mucosa (Herbert and Tisman, 1975); reduced folate stores secondary to liver damage (Weir, 1974); direct interferance of ethanol with folate metabolism (Davis, 1986); and defects in the storage mechanism and release of storage folate from the liver (Cherrick et al., 1965; Eichner and Hillman, 1973; Lane et al., 1976). Although these factors may cause folate deficiency, the resulting megaloblastic anaemia is thought to be largely due to a direct inhibitory effect of alcohol on the bone marrow haemopoetic cells and reduced folate utilisation (Wu et al., 1975).

Other nutritional deficiencies such as thiamine, magnesium and iron deficiency as well as pyridoxine, pantothenic acid, riboflavin and zinc deficiency have also been encountered in chronic alcoholics due to the effect of ethanol on their absorption, metabolism, storage and excretion (Lieber et al., 1979; Morgan, 1982). Deficiency of some of these nutrients may have direct or indirect effects on the levels and availibility of folate (El Banna et al., 1983).

1.3.10 Aetiology: Inadequate Absorption of Folate

Tropical sprue which is endemic in the West Indies, India and Southeast Asia produces variable and often severe atrophy of the

jejunum, the primary site for folate absorption. This tropical sprue together with inadequate dietary intake and increased demand for folate as in pregnancy may be the primary cause of folate deficiency in these countries (Herbert, 1968b; Herbert, 1973b).

Tropical sprue is almost unknown in the developed countries and instead coeliac disease, jejunal resection, Crohn's disease and the extremely rare cases of specific congenital folate malabsorption syndromes are the usual causes of folate malabsorption (Chanarin, 1979; Davis, 1986). In Ireland coeliac disease, which occurs in one per 300-400 live births, is the commonest cause of folate malabsorption (Mylotte et al., 1973). Coeliac disease may reduce absorption of folate due to disruption of the jejunal mucosa, destruction of the carrier system of folate derivatives, increased catabolism of folate due to increased jejunal mucosal cell turnover and a defect in the intestinal conjugase enzyme system (Weir, 1974).

Many drugs have been suggested as interfering with folate absorption (Herbert, 1973b). Diphenylhydantoin (Dilantin), barbiturates, contraceptive agents and ethanol are among the common drugs that may cause such malabsorption.

1.3.11 Aetiology: Inadequate Utilisation of Folic Acid

Among the many different factors that could affect utilisation of folic acid by cells and tissues of the body, Herbert (1973b) suggested that folic acid antagonists such as methotrexate and

trimethoprin are important. The so called folic acid antagonists are in fact substances acting not on folic acid itself but on the enzymes which play a vital role in folate metabolism (Chanarin, 1979). Tetrahydrofolic acid is the active enzyme form of folic acid in humans, animals, protozoa, and some bacteria. The reduction of dihydrofolate to tetrahydrofolate is catalised by the enzyme dihydrofolate reductase which acts by binding to the pyrimidine protein of the pteridine ring. The basic action of folic acid antagonists is to tightly bind the enzyme dihydrofolate reductase and prevent it from participating in folate metabolism for DNA synthesis. Methotrexate was one of the first researched folic acid antagonists to be used clinically (Trier, 1962) and in cancer treatment (Hooton and Hoffbrand, 1977; Allegra et al., 1986).

Vitamin B12 has for a long time been regarded as an important factor for adequate folic acid utilisation (Herbert, 1971). Folic acid in the form of tetrahydrofolate, which is the principle compound concerned in the transfer of one carbon-unit reactions, is generated from 5-methyltetrahydrofolate. This conversion occurs by the vitamin B12-dependent homocysteine-5-methyltetrahydrofolate transferase reaction (Herbert and Das, 1976). Therefore, in vitamin B12 deficiency, cells cannot utilise available folate for their metabolism. Tisman and Herbert (1973) demonstrated that bone marrow cells from patients with vitamin B12 deficiency had defective 5-methyltetrahydrofolate uptake compared to cells from normal subjects. This process was corrected by adding vitamin B12 to the incubation medium of deficient cells in culture.

A direct correlation was also thought to exist between iron and folate levels (Toskes et al., 1974; Hershko et al., 1975). In a study on patients with multiple nutrient deficiencies, Saraya et al. (1971) found that low serum folic acid levels were associated with severe iron deficiency. They concluded that severe iron deficiency (0-10 per cent saturated transferrin) may lead to depletion of circulatory folate which is most likely to be due to poor release of hepatic folate into the circulation.

Experimental studies on rats Vitale et al., 1965; Vitale et al., 1966) suggested that a secondary folate deficiency, as estimated by urinary folate, urinary formiminoglutamic acid and serum folate levels, can be induced by dietary deficiency of iron. The defect in folate metabolism was related to the decreased activity of the enzyme formimino-transferase the optimal activity of which is dependent on iron.

Other suggested factors that could affect folate at the utilization stage are enzyme deficiencies whether congenital or aquired, anticonvulsant drugs such as Dilantin, diuretics such as Triamterene, certain antibacterial agents such as Trimethoprim, and probably ascorbic acid deficiency (Herbert, 1973b).

1.3.12 Aetiology: Increased Requirement for Folate

Needs for folic acid fluctuate according to age, sex, medical condition and other factors. Pregnancy and lactation are the most important conditions where folate is needed in large quantities (WHO, 1972). Pregnancy is associated with a negative folate balance and folate deficiency including low serum and red cell folate levels, rapid clearance of intravenous folate from plasma, and evidence of megaloblastic marrow changes (Chanarin, 1979). These changes are reversible with regular folate supplementation. The deficiency is thought to arise when the increased folate requirement during pregnancy exceeds the amount available from the body stores and from dietary sources. Folate deficiency is more pronounced in pregnant women from lower socioeconomic groups. The increased requirement during pregnancy is due to many factors such as increased blood volume, growth of uterus and placenta and growth of the foetus and subsequently for maintaining lactation (Davis, 1986).

In a study on the nutrient levels in pregnant women in Sweden, Qvist et al. (1986) found that during gestation almost half the women had subnormal plasma folate levels which persisted during the post-partum follow-up. The value of red cell folate was 10 per cent below normal during pregnancy and 30 per cent below normal two months after delivary.

Patients with malignant diseases have been found to have abnormal folate levels. This may be due to increased demand by a

rapidly growing tumour (Saleh et al., 1981) or interference with the production of enzymes required in the processing of folate (Ratanasthien et al., 1977; Pheasant et al., 1983). The requirement for folate is correlated with the approximate mass of the tumour (Saleh et al., 1982). However, most folate abnormalities reported have been found to be associated with the active stage of the disease. Patients in remission usually have normal serum folate levels provided that their dietary intake of the vitamin is adequate. A combination of increased requirement and low intake is the usual cause of folate depletion in this group of people (Davis, 1986). Magnus (1967) on the other hand reported that while up to 85 per cent of patients with malignant disease have low serum folate, most of them had normal erythrocyte concentrations of the vitamin.

Other situations that require more dietary folate intake are infancy, blood diseases such as haemolytic anaemia, leukaemia and multiple myeloma, inflammatory diseases such as rheumatoid arthritis, tuberculosis and psoriasis and also metabolic disorders such as thyrotoxicosis (Herbert, 1973b; Weir, 1974).

1.3.13 Clinical Manifestations of Folate Deficiency

The sequence of progressive, experimentally induced dietary folate deprivation in a healthy young adult male was described by Herbert (1962, 1967 and 1968b) and Jeejeebhoy et al. (1968). It was shown that following three weeks of dietary folate deprivation, serum folate levels fell below normal. This was followed two weeks later by

hypersegmentation of the nuclei of polymorphonuclear leukocytes in the bone marrow and then in the peripheral blood after another two weeks (7th week). On week 14 of the experiment, the urinary output of folate analogues had increased above normal limits. However, red cell folate fell below normal levels only on week 17 of the experiment. This was followed by macrocytosis (week 18), megaloblastic marrow (week 19) and frank anaemia (week 20). The biochemical abnormality underlying megaloblastic anaemia due to folate deficiency and vitamin B12 deficiency was thought due to interference with precursors needed to form new DNA during the S phase of the cell cycle (Hoffbrand, 1983).

The features of folic acid deficiency anaemia seen in the peripheral blood are pancytop enia, macrocytosis of red blood cells and hypersegmentation of polymorphonuclear leukocyte nuclei (Herbert, 1968b; Doscherholmen et al., 1974). This picture is identical to that of vitamin B12 deficiency in man (Chanarin, 1979). However, when folate deficiency is accompanied by iron deficiency, the mean corpuscular volume (MCV) of red cells may not be elevated. Hypersegmentation of nuclei of polymorphonucliar leukocytes is recognised as a good indicator on the presence of folate deficiency because it cannot be masked by the iron deficiency (Herbert, 1968b).

Megaloblastic anaemia is the main marker for folate or vitamin B12 deficiency, but it is not unique to these conditions. Failure of pyrimidine synthesis may produce a potentially fatal megaloblastic anaemia (Huguley et al., 1959). Failure to reutilise

purine bases which cannot be converted into the corresponding nucleotides produces a moderately severe megaloblastic anaemia (Van Der Zee et al., 1970). There are a few other conditions such as erythroleukaemia which can cause megaloblastic anaemia, but these are rare in comparison to folate and vitamin B12 deficiencies. The common feature to most of these conditions is that there is interference with deoxyribonucleic acid (DNA) synthesis.

Mental retardation has been described in inborn errors of folate absorption or metabolism (Arakawa et al., 1963, 1966 and 1967). However, less severe symptomes such as sleeplessness, forgetfulness and irritability have been described in adult-onset folate deficiency (Hoogstraten et al., 1964; Herbert, 1968b).

1.3.14 Oral Manifestations of Folate Deficiency

The major oral changes of the nutritional deficiency anaemias are essentially the same in all kinds of anaemia. However, individual variations exist even amongst patients with identical deficiencies. Clinically, generalised pallor of the oral mucosa is regarded as the most common sign of anaemia (Dreizen, 1962). In folic acid deficiency, stomatitis, glossitis, ulceration and angular cheilitis are the common findings (Chisholm et al., 1978). Stomatitis is characterised by painful hyperaemic and occasionally ulcerated areas throughout the oral mucosa in addition to hypersensitive gingivae and glossitis. Glossitis is manifested by an early disappearance of the filiform papillae and prominent, red fungiform

papillae giving the tongue a strawberry-like appearance. In more advanced conditions the fungiform papillae are lost to leave a smooth, red and burning dorsum of tongue. Nonspecific ulceration is an early sign of folate deficiency and probably the earliest indication of the toxic effect of folic acid antagonists (Dreizen 1962). Recurrent oral ulceration is also a common finding in patients suffering from folate deficiency (Wray et al.,1975; Challacombe et al., 1977; Tyldesley, 1983). Angular cheilitis which is a frequant finding in human folate deficiency was also induced experimentally in 53 per cent of the white cotton marmosets fed a folic acid free diet (Dreizen and Levy 1969; Dreizen et al., 1970).

Morphological changes in the epithelial cells of the oral mucosa in folate deficiency have been studied in human and animal buccal smears. These studies showed large variations in the response of tissues to the deficiency which varied between a slight increase in nuclear sizes to increased nuclear and cytoplasmic components of cells and the presence of multinucleate cells (Staats et al., 1969).

Quantitative histological compartment studies in hamster tongue epithelium (MacDonald et al., 1982) showed that in the dorsum of tongue, the cellular compartment was slightly thinner and the keratinized compartment was significantly thicker than in normal controls. Similar changes in the hamster dorsal tongue epithelium in folate deficiency were seen by Al-Damouk (1984). Furthermore, in the lateral margin of the tongue posteriorly, progressive atrophy corresponding with the degree of folate deficiecy was noticed. The

cellular compartment was affected first and then followed by the keratinized compartment. The progenitor cells showed significantly increased nucleocytoplasmic ratio in folate deficiency (MacDonald et al., 1982).

1.3.15 Folic Acid Deficiency and Cancer

Folic acid plays an important role in cell development through its action on DNA synthesis. An increased requirement for this vitamin and a reduction of its level in the serum of cancer patients were observed by many investigators (Shamberger and Willis, 1977; Saleh et al., 1982; Allegra et al., 1986). This change in folate levels could be due to increased demand by a rapidly growing tumour (Allegra et al., 1986) or to interference with the production of enzymes required in the processing of folate (Davis, 1986). Urinary excretion of folate following an oral dose of radioactive folate was reduced in patients with malignant disease (Saleh et al., 1982). The remaining radioactive folate was thought to be taken up by the tumour tissue. This reduction in excreted folate was more pronounced in patients with more advanced malignant disease (Saleh et al., 1981).

Folic acid antagonists have been successfully employed in the treatment of many cancers especially the rapidly growing types such as leukaemia and squamous cell carcinomas that have been disseminated throughout the body (Shklar et al., 1966).

Although these factors have been known for a long time, the role of folic acid in carcinogenesis has not yet been determined. Experimental work on hamsters treated with folic acid antagonists (Shklar et al., 1966) showed that in those animals given injections of methotrexate, dimethylbenzanthracene (DMBA) induced carcinomas of the buccal pouch appeared more rapidly than in controls. The tumours were also more anaplastic and of greater size in methotrexate treated animals.

The suggested role of folic acid in cancer development was that it could aid in the expression of steroid hormone effects on target tissues (Butterworth, 1981). This hypothesis was found possible in some types of cervical cancers. Dai et al. (1986) have also suggested that many fragile sites on human chromosomes could be demonstrated by growing in tissue culture medium that is relatively deficient in folic acid. Although there is no clear evidence that fragile sites are involved in cancer formation, their existance offers a mechanism whereby specific mutations could occur in target tissues (Butterworth, 1981).

1.4 IRON

1.4.1 General Introduction

The nutritional need for iron in man and all living organisms is derived from the central role this metal performs in the energy metabolism of every living cell. The immediate energy requirements in most living cells are met by the oxidation of organic substances with molecular oxygen. Highly developed organisms such as man rely in addition to diffusion on a specific carrying protein in the blood and cells for oxygen distribution which itself requires iron for its biological activity (Eichhorn, 1964).

Iron is element number 26 in the periodic table and has an atomic weight of 55.85 Daltons. Iron is the fourth most abundant element and the second most abundant metal in the earth's crust (Bothwell et al., 1979). In nature, iron exists in two oxidation states, ferrous (Fe++) and ferric (Fe+++) and rarely if ever, under physiological conditions, exists as a free ion. Ferrous ion is readily oxidised to ferric ion which is the most common state of iron under all but acidic and anaerobic conditions and in some haem complexes (Christopher et al., 1974). Ferric iron at neutral pH is much less soluble than ferrous iron and is almost completely useless for biological consumption (Bothwell et al., 1979).

Many biological systems evolved mechanisms for secreting chelates to solubilise this metal from insoluble minerals and mobilise iron to their cell surfaces. Microorganisms such as Escherichia coli are capable of secreting low molecular weight organic chelating agents into the environment to bind ferric iron and move it into their cells. Higher plants that grow in alkaline soils secrete, from their roots, organic acids such as malonic and citric acids into the soil to depolymerise and solubilise ferric hydroxide so that iron can be utilised by the plant (Christopher et al., 1974).

A large proportion of iron in the food that man consumes is in the insoluble ferric state. In addition adverse interaction between the dietary components further reduces its availability for human utilization (Skikne et al., 1983; Stekel et al., 1983). However, these factors have been compensated for by man's unique ability to conserve body iron. In addition, the amount of iron absorbed each day in order to meet physiological requirements is usually far less than that present in the diet.

Iron also exists in the organic form which is largely incorporated in haem molecules of haemoglobin, myoglobin and in the haem enzymes. This form of iron although rapidly and almost completely absorbable when ingested, is not as widely and easily available as the non-haem iron.

The fact that a high percentage of iron salt present in vegetable staple foods such as rice and beans is a non-haem iron which is not readily absorbed while the well absorbed haem iron present in

meat is not available to many people particularly in the developing countries, is the principal factor behind the high prevalance of iron deficiency in the world.

1.4.2 Biochemical Considerations of Iron

Iron is one of the most versatile metals in that its atomic structure enables it to perform a wide range of biochemical activities (Eichhorn, 1964). It has two oxidation states, the more stable ferric form and the soluble ferrous form. Such structure enables iron to catalise the oxidation-reduction reactions involved in electron transport. Both haem and non-haem iron are involved in these processes which are fundamental to the utilization of oxygen by living cells (Green and MacLennan, 1967; Giorgio, 1970). Additionally, iron can form six covalent bonds four of which serve to bind the metal to the porphyrin ring to form haem. One of the two remaining coordinating positions of iron helps bind the haem to a histidine residue of globin in haemoglobin and the other serves, in the ferrous state, to reversibly bind molecular oxygen. Non-haem iron is required in certain enzymes such as aconitase and succinic dehydrogenase of the Krebs tricarboxylic acid cycle as well as a number of flavin and pyridine-linked oxido-reductases (Green and MacLennan, 1967).

1.4.3 Availability of Iron in the Diet

It is generally believed that iron is so well distributed among foods that there is little need to increase the iron intake by food selection (Wretlind, 1970). Hunt et al. (1976), reported that nutritional education had no effect on iron intake of low-income pregnant women. The Interdepartmental Committee on Nutritional Defense (1963) in their surveys of over 20 countries reported that the actual iron content of diets might be as much as double those expected on the basis of calculations from the constituent foods. The extra iron came from food containers or from dirt contaminating the diet. These reports indicate that iron is present and ingested in good quantities in the daily diet but it was almost completely in the non-haem form which is biologically of little use (Hofvander, 1968; Layrisse et al., 1969).

Studies on the bioavailability of dietary iron showed that the haem iron is readily and almost totally absorbed regardless of the composition of diet (Stekel et al., 1983). The non-haem iron is of low bioavailability. Only 1-7 per cent of the iron in vegetables and staples such as rice, maize, black beans, soya beans and wheat was absorbed if they were consumed as single items (Martinez-Torres and Layrisse, 1973). The poor bioavailability of non-haem iron can further be complicated by other ingredients of the meal such as carbonate, oxalate, phytate, bran, tea and egg yolk (Layrisse et al., 1968; Waddell, 1974; Stekel et al., 1983).

1.4.4 Intestinal Iron Absorbtion in Man

The amount of iron absorbed from food depends on many factors. The most important of these are the saturation of body iron stores, availability of iron in the ingested diet and the type of iron avialable. A male with iron stores saturated at about 1000mg would absorb about 6 per cent of the iron present in the average United States diet while a female with her body iron stores of 300mg would absorb about 15 per cent (Finch et al., 1950 and 1977; Roche and Layrisse, 1966). It was estimated that only 0.5mg/day is needed to replace obligatory iron losses and 3.0mg/day would be used for red cell production.

Iron exists in foods mainly as ferric hydroxide complexes in which the trivalent iron is directly bound to proteins, amino acids and organic acids. It is also present as haem-iron complexes bound to protein (globin). Although the mechanism whereby haem iron is taken into the intestinal enterocytes differs from that of inorganic and non-haem iron, iron from both sources is thought to be handled in the same way once it is in the enterocyte (Bothwell et al., 1979).

Part of the ingested non-haem iron is solubilised and ionised largely by acid gastric juice, reduced to the ferrous state and chelated before it is taken up by the intestinal enterocytes. Previous studies with iron salts showed that ionic iron is absorbed entirely in the ferrous form. Therefore, for iron to be absorbed it must be split from its complex compounds and chemically reduced.

Additionally, normal gastric secretion contain a chemically unidentified stabilizing substance, probably an endogenous chelate, which together with gastric acid could solubilise and chelate some ferric iron and make it absorbable (Beutler, 1980).

Iron in haem-protein complexes such as haemoglobin and myoglobin is thought by some to be absorbed largely intact into the intestinal epithelial cells and only then the haem portion is released from globin. Alternatively, haem is taken up by the enterocytes only after it has been released from its globin combination under the effect of the duodenal proteolytic enzymes (Bothwell et al., 1979).

Absorption of both forms of iron occurs at any level of the small intestine but it is more efficient in the upper part of the small intestine and decreases progressively in the more distal segments of the ileum (Beutler, 1980).

Normal iron balance is largely maintained by the regulation of its absorption rather than by its availability (McCance and Widdowsen, 1938). The concepts of enterocyte regulation of iron absorption and balance have changed over the last few years. It was thought that the concept of a "mucosal block" of iron absorption was the main regulating mechanism. In that concept intracellular ferritin was regarded as the mediator of absorption and that uptake continued until the intracellular concentration of ferritin blocked further absorption. However, that concept was replaced by more recent work (Moore, 1960) which suggested that the columnar mucosal cells in the

crypts of the intestinal villi contain variable amounts of transferrin-derived iron. The size of this intracellular deposit could regulate the quantity of iron absorbed. The cellular iron may enter the body according to need or remain within the cells and be lost when the cells are sloughed from the tips of villi at the end of their 2-3 day lifespan (Beutler, 1980). According to this concept, if little iron is incorporated from transferrin into mucosal cells as in iron deficient people, absorption is enhanced while larger amounts of that iron would reduce absorption from the intestinal lumen.

1.4.5 Factors Affecting Intestinal Iron Absorption

Factors that possibly affect iron absorption in humans were found to mainly affect the non-haem iron (Stekel et al., 1983). There are three major groups of factors; endogenous, intestinal and food factors. Endogenous factors are the status of iron nutrition of the body and its iron stores, erythropoietic activity and haemoglobin concentration (Bothwell et al., 1979). The intestinal or intraluminal factors are influenced by gastric secretory factors and hydrochloric acid (Esko, 1985) and to a lesser extent by pancreatic secretions (Giorgio, 1970). They also include gut transit time, mobility and digestive capacity (Skikne et al., 1983). The third major group of factors that affect iron absorption are the food factors of the iron content of food, the different molecular forms of iron found and the chemical properties of the different components of the daily meal (Stekel et al., 1983).

Some substances are more frequently discussed in relation to iron absorption. Gastric acid has an important role in reducing iron to the divalent state and maintaining optimal pepsin digestion (Heath and Patek, 1937). Therefore, patients with achlorhydria absorb iron less efficiently than those with normal gastric secretion even under the demand of iron deficiency.

Ascorbic acid when given orally in large amounts enhances absorption (Cook et al., 1984; Hallberg et al., 1986). Ascorbic acid was found to help in the reduction of trivalent iron to the divalent state. It also combines with the trivalent iron in low pH media, such as that of the stomach, to form a soluble ascorbate chelate. This chelate will then remain soluble even in the high pH media such as that of the duodenum (Schade, 1974). Ascorbic acid is present in some foods and in gastric and duodenal secretions in amounts sufficient to chelate several milligrams of ferric food iron per day (Malone et al., 1986).

Alcohol has been shown to increase iron absorption from trivalent salts probably through increasing gastric acid flow (Charlton and Bothwell, 1970). Alcohol has no effect on divalent salts or in subjects with achlorhydria. Pancreatic exocrine secretions have been suggested as playing an important role in reducing iron absorption due to their alkaline nature and high phosphate content. However, the effects of alcohol and pancreatic exocrine secretions were later found to be insignificant (Beutler, 1980).

1.4.6 Transport of Iron in the Body

The principal substance involved in iron transport is transferrin. Transferrin is a beta globulin carrier protein with a molecular weight of 80 kilodaltons and a biologic halflife of 8-10.5 days (Harris and Aisen, 1975). There are at least 19 genetic variants of transferrin in the human blood which are all thought to function in the same way. Transferrin has two binding sites each of which accept one atom of ferric iron. At normal physiological pH, iron is tightly bound to transferrin and exchange with body cells occurs at specific receptor sites such as those of developing red blood cells (Bothwell et al., 1979).

Transferrin is present in the intravascular and extravascular spaces and serves a dual function in the transport of iron. It accepts iron from the intestinal cells or from sites of storage or haemoglobin destruction and delivers it mainly (70-90 per cent) to the bone marrow for haemoglobin synthesis, and also to reticuloendothelial cells for storage and to all cells for iron-containing enzyme synthesis (Beutler, 1980). Transferrin under normal physiological conditions is only 30 per cent saturated with iron. Although only 3-4mg of iron is present in the plasma at any particular time, some 30-40 mg of iron is transported in the plasma every day (Fletcher, 1970).

A trace amount of iron (2-20 μg/100ml) in the form of ferritin is present in the plasma. It was suggested that it does not represent a form of transport iron but it is rather the result of leakage from various body cells (Jacobs et al., 1972). However, the measurement of serum ferritin levels serves as a useful means for the estimation of total body iron stores (Walters et al., 1973; Siimes and Dallman, 1974; Jacobs and Worwood, 1975; Krause and Stolc, 1979).

1.4.7 Distribution of Body Iron

The total quantity of iron present in the human body varies with body weight, haemoglobin concentration, sex and size of storage compartments (Beutler, 1980). A wide range of individual values exists with an average of 50mg/kg of body weight in adult men (total of 4-5g) and 35mg/kg in adult females (total of 2-5g) (Moore, 1960).

Two functional compartments of body iron are recognised. An active compartment constituting about 70 per cent of the total body iron. This amount is distributed as haemoglobin (85 per cent), myoglobin (5 per cent) and haem enzymes such as cytochromes, cytochrome oxidase, peroxidase and catalase enzymes and in cofactors which together constitute about 10 per cent of this compartment. A small amount (about 4mg) also exists as transport iron bound to transferrin in the plasma.

The non-functional or storage compartment of iron, predominantly found in the liver, spleen and bone marrow, constitute about 30 per cent of total body iron and exist in almost equal amounts as ferritin and haemosiderin. The absolute value of this compartment under normal physiological conditions ranges between 300-1000mg in the adult female to about 500-1500mg in the adult male. It has been established that both forms of storage iron are capable of being mobilised for haemoglobin synthesis when iron is needed (Beutler, 1980).

1.4.8 Physiological Iron Losses and Requirements

Iron obtained from the diet serves under normal circumstances to replace obligatory losses from the body, to provide for body growth and to establish reserve stores. The obligatory losses take place from the skin, gastrointestinal and genito-urinary tracts. They have been estimated in the adult male to amount to 12-14 μg/kg/day in an individual with normal iron balance (Bothwell and Finch, 1968). The amount of iron normally lost through these routes is largely determined by the amount of iron lost in urine which is dependent on plasma iron concentration. The other major part of iron loss was found to be due to exfoliation of epithelial cells from the skin and gastrointestinal tract and to a lesser extent in the bile (Moore, 1955; Roche et al., 1957). The minor part of iron loss is due to a normal extravasation of red cells into the intestinal lumen (Bothwell et al., 1979). Iron losses could fluctuate between

8μg/kg/day in iron deficiency and 20μg/kg/day in iron overload (Dubach et al., 1955; Green et al., 1968).

The second factor that determines the amount of iron needed by the human body is the iron stores. There is effectively no reserve store of iron between the ages of six months and two years (Rios et al., 1975; Smith et al., 1955). The stores are gradually built up to about 5mg/kg during childhood and maintained at this level in females until menarche while in males the stores are further developed between the age of 15 and 30 years to about 12-15mg/kg.

The third factor that determines the amount of iron needed is the amount required to support growth. This amount was estimated in the infant to be about 80mg/kg of body weight per day (Josephs, 1956; Widdowson and Spray, 1951; Giorgio, 1970) and later in life such as in the child, adult female and adult male is approximately 30, 33 and 37mg/kg/day respectively.

In adult life, physiological losses in the male are about 1mg/day with an additional 0.2mg/day needed between the ages of 18 and 30 years to allow for the accumulation of the normal iron stores. In adult females with an average menstrual blood loss of about 30ml, the daily requirement will increase to 1.6mg/day. However, in women with more blood loss during menstruation daily iron requirement could reach 2.0mg.(Beaton, 1974; Rybo, 1973)

During pregnancy, the requirement for iron usually increases especially during the second half of pregnancy. This is because iron

is needed for the growing foetus and placenta in addition to the expansion of the maternal red cell mass (Delbarre, 1960). Studies have shown a 45 per cent increase of total blood volume (Lowenstein et al., 1962; Lund and Donovan, 1967) which is probably related to the increased oxygen consumption during pregnancy (Flanagan et al., 1966). The total amount of iron required to maintain needs during pregnancy without affecting iron stores was found to be 1190mg which is equivelant to 4mg/day. However, this extra 4 mg/day needed is not evenly distributed between the three trimesters. The pregnant woman initially require's 0.8mg/day less iron than before conception because menstruation has ceased. Her requirements increase to 4mg/day during the second trimester and 6mg/day during the third trimester (Pritchard, 1965; Rovinsky and Jaffin, 1965).

1.4.9 Iron Deficiency

Iron deficiency is the state of diminished total body iron content. It may range in severety from a mild reduction in iron stores due to a temporary inbalance between intake and demand for iron to severe iron deficiency manifested by anaemia or even by deficiency in tissue iron enzymes as happens in cases of severe and persistant bleeding.

Iron deficiency is probably the most common nutritional deficiency both in developing and developed countries (Charlton and Bothwell, 1970). Its exact incidence is difficult to define since

different criteria have been used for diagnosis. Traditionally, haemoglobin values have been applied but these only indicate the severe stages of deficiency (Charlton and Bothwell, 1970). When haemoglobin concentration is taken as the dividing line in studies of the prevelence of iron deficiency, the wide range of normal haemoglobin values even between normal subjects causes difficulty of interpretation. Levels which might be taken as deficient in one person would be normal for another (WHO, 1968; WHO, 1972).

There are three biochemically recognizable stages of iron deficiency that can reflect certain clinical as well as haematological signs (Bothwell et al., 1979). These stages are iron depletion, iron deficient erythropoiesis and iron deficiency anaemia.

Iron depletion refers to a simple decrease in iron stores without any effect on essential body iron. The most practical laboratory method of identifying such depletion is by measuring the plasma ferritin concentration which closely parallels body iron reserves. This linear relationship suggested that each μg/l plasma ferritin corresponds to about 8 mg storage iron. Normal men with apparently normal marrow haemosiderin stores have a mean ferritin concentration of 159μg/l (100$\pm$60μg/l). Men with reduced stores have 51μg/l (20-100μg/l), those with no visible marrow haemosiderin have 6μg/l (10-20μg/l). Values lower than 10-12μg/l indicate complete exhaustion of body iron stores (Bothwell et al., 1979). Other methods of assessing body iron stores such as the estimation of the haemosiderin content of aspirated marrow are thought to be unsuitable for the detection of mild iron deficiency.

Iron deficient erythropoiesis occurs when the iron supply to the bone marrow is inadequate to meet normal haemopoiesis. It represents anaemia which is too mild to be detected by some arbitrary value for haemoglobin which is used to separate normal from anaemic states. It can be identified by detection of a plasma ferritin concentration, as described above, which is less than 12μg/l. The reduced concentration of plasma ferritin will be reflected in the amount of iron bound to transferrin and the percentage saturation of transferrin will be reduced. In normal subjects the percentage saturation of transferrin is 35$\pm$5 which is not affected in iron depletion, but is reduced to less than 15 per cent in iron deficient erythropoiesis and less than 10 per cent in iron deficiency anaemia. Although the measurement of transferrin saturation is a sensitive indicator for diagnosis, it is thought that it could be too sensitive to be taken as a routine method of diagnosis as it could be decreased by acute inflammatory states such as upper respiratory infection and repeated measurement is recommended. Other methods of recognising iron deficient erythropoiesis are available but are less commonly used. The measurement of the rate of disappearance of plasma radio-iron can be valuable. The number of marrow sideroblasts also decrease from a normal value of about 35 per cent to below 10 per cent when transferrin saturation decreases to a level below 16 per cent. Increased red cell protoporphyrin concentration from a normal of 30μg/dl RBC to 100μg/dl RBC was also found to be of diagnostic value in estimating iron uptake by red blood cell precursors.

Iron deficiency anaemia is the stage of severe reduction in iron supply to the bone marrow resulting in a fall of haemoglobin concentration below some arbitrarily selected value. It is recognised on a blood film as microcytic hypochromic anaemia and identified by a rise in the percentage of microcytes, decreased mean cell volume (MCV) from normal 90±5fl to below 85fl, reduced mean cell haemoglobin (MCH) below 27pg and reduced mean cell haemoglobin concentration (MCHC) below 32g/dl. Plasma ferritin is less than 10μg/l, transferrin saturation less than 10 per cent and total iron binding capacity increased from normal 330±30μg/dl to over 410μg/dl.

Studies on the prevalance of iron deficiency (Garby, 1973) have shown that 30 to 50 per cent of the population in certain developing countries suffer from some degree of iron deficiency. In developed countries 40 per cent of women in the fertile age period in Sweden were iron deficient and 25 per cent had iron deficiency anaemia (Hallberg, 1970). In Canada, the analysis of ferritin values showed that iron deficiency was present in 25 per cent of children, 30 per cent of menstruating women, 60 per cent of pregnant women and only 3 per cent in men. However, iron deficiency anaemia was noted only in 2 per cent of the studied population. In Australia, 4.5 per cent of women between the ages of 16 and 61 years were anaemic, 98 per cent of these anaemias were due to iron deficiency (McEwin et al., 1974). In the United Kingdom, 14 per cent of women and 3 per cent of men were found to be anaemic, almost entirely of the iron deficiency type (Kilpatrick and Hardisty, 1961; Kilpatrick, 1970). Similar results were obtained from other developed countries such as Norway (Dresch, 1970; France (Seibold, 1970) and Germany (Vellar, 1970).

1.4.10 Aetiology of Iron Deficiency

Prolonged negative iron balance can terminate in iron deficiency. This negative balance can be the result of either decreased iron intake or increased body demands. Decreased intake of iron could be further divided into inadequate iron nutrition in the diet or due to inadequate absorption from the intestine. Increased demand for iron can also be subdivided into increased utilization of iron, as in certain physiological or pathological conditions such as pregnancy and rapidly growing cancer or could be due to increased losses of body iron as a result of bleeding. Combinations of two or more of these factors are a common feature of iron deficiency and iron deficiency anaemia.

1.4.11 Clinical Manifestations of Iron Deficiency

Manifestations of iron deficiency are generally mild. Iron deficiency may be discovered as an incidental finding where the presenting signs and symptoms are related to the disease which led to the deficiency (for example, peptic ulcer). In other cases, manifestations of both the underlying disease and of iron deficiency itself are found. However, some patients seek medical attention for symptoms related to iron deficiency alone. It was found that 63 per cent of people seeking medical help were complaining from the symptoms

of anaemia alone. Such symptoms could take up to eight years before patients would seek medical help. In addition, 16 per cent were complaining from the disease causing the anaemia while in the remaining 21 per cent the anaemia was discovered incidentally (Wintrobe et al., 1974).

The most important manifestation of iron deficiency is the depressed haemoglobin synthesis which eventually results in anaemia. Anaemia is not a disease but it is a sign of disease and manifests itself by the reduced oxygen carrying capacity of the blood to tissues and organs of the body. Such reduced oxygen carrying capacity is partially compensated for by increased cardiac output and can result in cardiac symptoms. More commonly, patients complain of fatigue, irritability, palpitations, dizziness, breathlessness and headache. However, these symptomes are thought to appear only when haemoglobin reaches significantly low levels (7-8g/dl). Other manifestations of iron deficiency are defects in cell mediated immunity (Joynson et al., 1972), defective polymorph function (Ward et al., 1986) and other haemopoietic tissue cell defects (Hershko et al., 1970).

Wintrobe et al. (1974) reported the incidence of changes in other tissues in iron deficiency. These were changes in the nails such as flattening and koilonychia in 28 per cent of cases, soreness and papillary atrophy of tongue in 39 per cent, other oral epithelial changes such as angular stomatitis in 14 per cent. Achlorhydria and gastritis were present in 74 per cent of cases and dysphagia in seven per cent.

1.4.12 Oral Manifestations of Iron Deficiency

Structural and functional abnormalities of oral epithelial tissues in iron deficiency are well documented (Jacobs, 1982; Rennie and MacDonald, 1984). These abnormalities are thought to be the result of basic metabolic changes in the epithelial cells which are particularly susceptible to minor variations in blood concentrations of nutrients and minerals (Tyldesley, 1975).

Clinically, atrophy of oral epithelium is the underlying abnormality (Boddington and Spriggs, 1959). The lingual epithelium is mainly affected and may show atrophy or loss of the filiform papillae (Lewis, 1930). Generalised soreness and ulceration of the oral mucosa which may sometimes be in the form of recurrent oral aphthae have also been described (Wray et al., 1975; Challacombe et al., 1977). Patients with iron deficiency anaemia may also be more susceptible to fungal and bacterial infections. Angular cheilitis and candidal infections are more frequent among iron deficient patients even before the appearance of anaemia (Jacobs and Cavill, 1968; Higgs and Wells, 1972; Fletcher et al., 1975).

Histologically, Jacobs (1959 and 1960) has examined the buccal mucosa in iron deficient patients and found significantly increased epithelial keratinisation, higher levels of mitotic activity in the basal layer, decreased melanin production and increased subepithelial inflammatory cell infiltration. No significant

epithelial atrophy was recorded in the subjective observations by Jacobs (1960). However, reliable quantitative data using stereological techniques have provided more accurate information on the epithelial changes in iron deficiency (Rennie et al., 1984). Rennie et al. (1982a) showed a highly significant reduction in the total epithelial thickness of human buccal epithelium from patients with iron deficiency anaemia. The decrease in thickness was due to reduction in the thickness of the maturation compartment of the epithelium (essentially the prickle cell layer). Ranasinghe et al. (1985) found in addition to significantly reduced mean epithelial thickness of hamster cheek pouch, the respiration of epithelial cells cultured from the cheek pouch was significantly reduced compared to control animals.

Cytological studies of exfoliated buccal cells obtained from the saliva (Boddington, 1959; Boddington and Spriggs, 1959) showed that the diameters of cells obtained from iron deficient subjects were significantly smaller than in normal controls. The nuclear diameter was within normal limits except for a few cases where the nuclei were significantly enlarged.

Changes in epithelial cell sizes have also been studied by Rennie and MacDonald (1985) who reported a reduction in the size of the maturation compartment in subjects with iron deficiency anaemia due to a reduction in the sizes of individual cells. As in the Boddington (1959) study, no changes were found in nuclear sizes which resulted in increased nucleocytoplasmic ratio of cells in iron deficiency anaemia.

Experimental studies on animals have shown similar result to human studies. Work on sex-linked anaemic (SLA) mice using quantitative techniques, (Steele et al., 1981) noted thinning of the epithelium in the anterior dorsum of the tongue. Stereological studies of hamster ventral tongue epithelium (Rennie and MacDonald, 1982) showed decrease in the thickness of the maturation compartment and increase in the thickness of the keratinized layers in iron deficiency anaemia. These changes in the ventral tongue epithelium were also present in hamsters having iron deficiency without anaemia. However, in hamsters with less pronounced iron deficiency (iron depletion), the maturation compartment was significantly thinner while the progenitor cell compartment was significantly thicker.

Cell kinetic studies in iron deficiency in hamsters (Rennie and MacDonald, 1984) reported a reduction in the time of the DNA synthesis phase of the cell cycle. This reduction was present in both iron deficiency with and without anaemia which resulted in increased cell production rates. The authors concluded that as the iron deficiency develops, there is an accompanying increase in the rate of new cell production and decrease in size of the epithelial cells.

1.4.13 Iron Deficiency and Cancer

Paterson (1919) and Brown Kelly (1919) at a meeting of the Royal Society of Medicine (1919) gave the first comprehensive

description of a syndrome consisting of postcricoid web, dysphagia, anaemia and other occasional conditions such as glossitis, dyspepsia, diarrhoea, cheilosis and koilonychia. Their names have been given to the syndrome in Britain but in the United States of America it is usually referred to as the Plummer-Vinson syndrome while in Scandinavia the term siderop enic dysphagia is used (McNab, 1961). Three causative factores have received most attention from writers on this syndrome, namely, primary dysphagia, riboflavin deficiency and iron deficiency (McNab, 1961; Chisholm and Wright, 1967). However, the majority of authors agree that iron deficiency is the basic cause and that iron deficiency may not be accompanied by frank anaemia (Witts, 1931; Watts, 1961; Jacobs and Kilpatrick, 1964).

Ahlbom (1936 and 1937) was the first to publish an investigation of 250 cases of carcinoma of the upper alimentary tract in women which showed that 70 per cent had preceding Paterson-Brown Kelly syndrome or achlorhydric anaemia. Among women with cancer of the mouth, Ahlbom (1936) reported that signs of this syndrome were present in about 50 per cent. The incidence of malignant changes in the oral, pharyngeal and oesophageal mucosae in patients with Paterson-Kelly syndrome has varied in different studies between 10 and 90 per cent (Ahlbom, 1936; Wynder et al., 1957a).

Experimental studies on anaemic rats (Prime et al., 1983) showed that the incidence of squamous cell carcinomas induced by painting 4-nitroquinoline-oxide was similar to that induced in normal rats but tumour development occured significantly earlier in the iron deficient animals (mean 183 days) compared with controls (mean 229

days). These results are compatible with previous experimental work on rats (Vitale et al., 1978) where liver tumours induced by dimethylhydrazine (DMH) developed much earlier in the iron deficient rats than in controls. The early development of these cancers was thought due to the effect of iron deficiency on liver enzymes involved in the inactivation of dimethylhydrazine (DMH) within the hepatocytes.

At the present time, there seems to be no obvious cause-and-effect relationship between chronic iron deficiency and malignant transformation in epithelium. However, there appears to be little doubt that there is a relationship but the exact nature of this is not yet known.

The suggested modes of action of iron deficiency in the induction of cancer in the upper gastrointestinal tract have been divided into direct and indirect effects. The direct role is where iron deficiency affects target cells or tissues to make them susceptible to carcinogenesis. For example, iron deficiency could affect the iron-containing enzyme system of epithelial cells and increase their susceptibility to local application of chemical carcinogens. It could structurally change the epithelium by reducing its thickness and thus make it more permeable to chemical carcinogens (Steele et al., 1981; Rennie and MacDonald, 1982). The increased mitotic activity demonstrated by kinetic studies (Rennie and MacDonald, 1984) may also be a reasonably important local factor.

The indirect effect of iron deficiency on the other hand is also important. Impaired cell mediated immunity in iron deficient patients (Joynson et al., 1972; Binnie et al., 1983) has been suggested as a possible important factor that could predispose to the development of overt tumours. Rennie et al. (1983) reported increased susceptibility of iron deficient patients to persistant oral candidal infection. While Prime et al. (1983) found that the distribution of rat palatal tumours was more in areas of increased dental plaque accumulation and concomitant bacterial colonisation. This led to speculation that altered oral flora in iron deficiency may have an influence on carcinogenesis.

1.4.14 Iron Overload and Cancer

Iron overload has also been suspected as a possible factor in human and animal carcinogenesis (Weinberg, 1981). It has been reported that the 3.5 fold increase of primary liver cancer in Swedish women was related to iron fortification of flour introduced some 15 years earlier Weinberg (1986). Mice injected with L1210 leukaemia cells and stressed with iron dextran developed a 4-6 fold greater tumour load and died significantly faster than controls who received tumour cells but no iron. It was suggested that in addition to serving as an essential nutrient for the growth of the host immune system, iron also supported the growth of invading cancer cells.

1.5 CHEMICAL CARCINOGENESIS IN LABORATORY ANIMALS

1.5.1 Introduction

Yamagiwa and Ichikawa (1918) discovered that skin cancer can be induced by tar application to the rabbit ear. This was later attributed to the presence of the carcinogenic hydrocarbon, benzpyrene. Hundreds of chemical compounds have subsequently been studied for their carcinogenic potential. Most research on chemical carcinogenesis has been directed to cancers other than that of the mouth despite the fact that cancer of the oral cavity contributes to eight to ten per cent of all human cancers and four per cent of cancer deaths in the United States of America (Salley, 1954).

Early attempts to produce experimental malignant oral tumours were largely unsuccessful until 1950 when Levy and Ring succeeded in producing sarcomas of the oral connective tissue by implanting crystalline 9,10-dimethyl 1,2-benzanthracene in subgingival tissue in hamsters. However, since 90 to 95 per cent of oral malignancy is epithelial in origin attempts were directed towards producing this type of cancer. In 1954, Salley succeeded in producing malignant epithelial tumours (squamous cell carcinoma) of the cheek pouch in most of his hamsters using different kinds of carcinogenic polycyclic hydrocarbons dissolved in acetone or benzene.

1.5.2 Chemical Compounds with Carcinogenic Potential

In an extensive review, Coombs (1980) reported that over 3000 pure chemical compounds are known to be carcinogenic in experimental animals and that this number will increase as there are about 200,000 new organic compounds reported every year. Chemical carcinogens are known to belong to widely diverse structural types which include a number of natural products. The most common and widely used carcinogenic compounds are described below.

Polycyclic hydrocarbons: Polycyclic hydrocarbons are the products of incomplete combustion of coal, oil, gas, wood and other organic materials. They are widely distributed environmental pollutants. A number of these polycyclic hydrocarbons are highly carcinogenic or promoters of other carcinogenic agents (Weibel and Gelboin, 1980). Examples of this family of compounds are benzo(α) pyrine (3:4BP) and 9,10-dimethyl-1,2-benzanthracene (DMBA) which are very strong carcinogenic substances. Topical application of micrograms of DMBA is sufficient to induce skin tumours in the mouse.

Aromatic amines and azo compounds: The highly elevated incidence of bladder cancer formerly found among workers in dyestuff factories was found to be related to their daily contact with aromatic amines such as benzidine and 2-naphthylamine produced during the dyestuff manufacture. Bladder cancer was also a health problem in sections of the rubber industry where aromatic amines were used as anti-oxidants. Another member of this family of compounds is 2-acetamidofluorene (AAF) which was introduced as an insecticidal

agent but was found to be a powerful carcinogen that can produce a variety of tumours in rodents such as those of the liver, external acoustic meatus and mammary glands (Coombs, 1980).

N-Nitroso compounds: This is a relatively recently discovered family of potent carcinogenic compounds which includes the nitrosamines and nitrosamides. They were formerly used in industry as solvents but caused liver cirrhosis in the workers. They were later found to be highly carcinogenic causing 100 per cent cancer of the liver in rats (Coombs, 1980). Of the 100 nitrosamines tested, 90 per cent of them were found to be carcinogenic and could cause cancers of the liver, lung and kidney (Coombs, 1980). The nitrosamides are direct acting carcinogens that do not require biological activation before they can exert their biological potential.

Biological alkylating agents: These are direct acting carcinogens including 2,2-dichlorodiethyl sulphide, the active agent of mustard gas which causes cancer of the bronchus, larynx and nasal sinuses. Replacement of the sulphur atom by nitrogen gives a nitrogen mustard which is used in many anti-cancer drugs such as cyclophosphamide and chlornaphazin. Many of these drugs have been found to be weak or moderately strong carcinogens in animal tests and may be involved in the induction of human tumours (Coombs, 1980).

Carcinogenic natural products: The aflatoxins such as aflatoxin B1 are a family of complex lactones produced by the common mould Aspergillus flavus which contaminates peanuts and certain

grains. This compound is thought to be the most potent carcinogen known, producing liver tumours in rats fed with 1 μg per day and local sarcomas in mice after subcutaneous injection of 10 μg (Coombs, 1980). Bracken fern is another natural carcinogen that causes cancer of the stomach in cattle.

Chemical carcinogens are not confined to organic compounds. A number of metallic elements are known to be active in producing various types of tumours in man and animals, either as pure metals or in the forms of their salts or oxides. Workers in the chromate and nickle industry suffer from high incidences of lung cancer (Coombs, 1980). Asbestos is by far the most important inorganic carcinogen which causes the rare tumour mesothelioma, but also appears to act synergistically with cigarrette smoking in causing lung cancer.

1.5.3 Carcinogenesis

Testing for carcinogenic activity in chemical substances requires extensive and expensive research which can sometimes be dangerous as well. Boyland (1958) has suggested the use of groups of 20 animals each for such purposes. Currently, the scientists of the National Cancer Institute in the United States of America administer chemical compounds under investigation at various dose levels to groups of 50 male and 50 female animals of two species usually mice and rats for the greater part of their natural lifetimes usually 24 months (Boyland, 1980). The number of chemicals that were tested for

their carcinogenicity by 98 institutes in 20 countries during the years between 1958 and 1978 was only 990 (Boyland, 1980).

The potency or activity of a carcinogen, which is defined as the dose that induces cancer in half the treated animals and is referred to as the median effective dose (ED), varies form one chemical to another. Boyland (1980) reported that the difference in potency between dibenz(α, λ)anthracene and 4-dimethylaminoazobenzene was 25,000 fold. As more chemical carcinogens became known the scale of potency for these chemicals has increased resulting in a more accurate estimation of the carcinogenic activity in these chemicals. For example, the difference in potency between aflatoxin B and saccharin which is a weak carcinogen is more than a million-fold. It was also found that this activity can vary with age, sex, species, quality and quantity of diet, dosage schedule, route of administration and vehicle or solvent used (Boyland, 1958 and 1978). The carcinogenic activity can also vary in relation to the induction period, maximum tumour incidence and the degree of malignancy.

Animals, including man, deal with toxic hydrocarbons in either of two ways. The compound might either be converted into more active pathogenic substances or be detoxicated by conversion into some harmless end product (Boyland and Levi, 1935). Generally this metabolic conversion reduces toxicity and protects the organisim from the toxic effects of chemicals in the environment. However, some carcinogens are metabolised to chemically reactive products by enzymes normally involved in detoxication (Boyland, 1980).

Studies of cancer incidence in relation to age, dose and time of carcinogenic onset in animals and humans have shown that the process of carcinogenesis involves several stages, two of which are particularly distinct, initiation and promotion. The early stage is the modification of the cellular DNA by intercalation or chemical reaction with a carcinogen resulting in mutation in cellular DNA. This can result in base mispairing during subsequent cell divisions. Such change has been termed initiation (Rous and Kidd, 1941).

Initiated cells could either be repaired or die and the process of carcinogenesis would be aborted. However, if this does not happen, the initiated cells under some circumstances can continue to behave normally but if a promoting agent either chemical or physical is applied some initiated cells can be transformed into tumours. This later stage is called promotion (Rous and Kidd, 1941; Scribner and Suss, 1978). Coal tar, in low doses, applied to the ears of rabbits or to the skin of mice was the first substance to be considered as an initiating agent and subsequantly pure polycyclic hydrocarbons such as 7,12-dimethylbenz(α)anthracene have been used in later experiments. Croton oil and croton resin were the first chemical promoting agents investigated by Berenblum (1947). Physical irritation and wounding of the skin were investigated by Rous and Beard (1935) as possible tumour promoting influences.

1.5.4 The Metabolic Activity of Chemical Carcinogens

It is now widely accepted that potentially carcinogenic chemicals usually undergo some form of metabolic activation before they induce cancer. Chemically, it is thought that many carcinogens are unlikely to react with macromolecules such as nucleic acids and proteins of the cells of animals without the catalising effect of certain enzyme systems (Sims, 1980). Experimental work on mutagenicity in bacterial systems (Ames et al., 1973) showed that carcinogens were only active if the tests were carried out in the presence of metabolising systems. The administration of specific inhibitors of such enzyme systems either before or at the same time as the carcinogens were introduced often significantly altered the pathways by which the carcinogens were metabolised and activated (Wattenberg, 1978). Although it is widely accepted now that such reactions do occur, little is known of their relationship to the subsequent development of malignancy.

A number of chemical carcinogens are direct-acting alkylating agents and are thus in reactive, electrophilic forms when administered to animals (Lawly, 1976). These compounds are often weak carcinogens, possibly because they react with small nucleophiles such as water or glutathione rather than with critical target sites within cells. These weak activities are in contrast to those of many indirect-acting carcinogens that require metabolic activation. Many of these indirect-acting carcinogens such as aflatoxin B1 and some polycyclic hydrocarbons are potent carcinogens probably because the

reactive species are generated within cells in close proximity to target sites.

The processes by which these and some other chemical carcinogens are activated are often complex and may involve two or more metabolic steps. The products formed in the early steps were called the proximate carcinogens whereas the final products that are directly concerned in reactions with cellular macromolecules are called ultimate carcinogens (Miller and Miller, 1976). The electrophilic compounds generated within cells usually arise through reactions catalysed by enzymes that are part of the detoxification mechanisms of the cells. However, most of these compounds are converted into inactive products by secondary metabolic processes (Sims, 1980). Only those products that escape the secondary processes are likely to react with cellular macromolecules to produce malignant changes perhaps because they are produced faster than they can be further metabolised to harmless products (Sims, 1980). The first stage in the metabolism of many foreign compounds whether carcinogens or non-carcinogens involves mono-oxygenases that are present in the cells of most tissues in man and animals especially in liver cells. Cytochromes P-448 and P-450 are examples of this type of enzyme. The enzyme concerned in the second stage of carcinogen activation is the enzyme epoxide hydratase (Sims, 1980).

Of the many chemical carcinogens that have been regularly used in experimental work on cancer induction in animals, the polycyclic aromatic hydrocarbons such as naphthalene and anthracene and more recently benzo(α)pyrene are the most widely used. The

aromatic amines for example 2-naphthylamine and 2-acetylaminofluorene and the aromatic nitro compounds for example 2-nitronaphthaline, 4-nitrobiphonyl and 4-nitroquinoline N-oxide have also been used in experimental carcinogenesis in animals (Sims, 1980).

1.5.5 Animal Models of Intraoral Chemical Carcinogenesis

Animal models that have been used in experimental carcinogenesis are many. The choice of one animal model for a particular type of research depends on several factors amongst which are the type of cancer to be produced (whether epithelial or mesenchymal) the carcinogen to be tested or used and the target tissue or organ under examination (whether a lining or glandular epithelium). Other factors such as the personal preference and housing facilities available are also important.

Animal research on intraoral chemical carcinogenesis has been restricted to a small number of animal species. The most popular of these species have been rats, mice and golden hamsters although rabbits and monkeys have also been used to a lesser extent. Chemical carcinogens that have been regularly used in animal experiments of intraoral carcinogenesis are mostly polycyclic hydrocarbons. The most common carcinogens used have been 9,10 dimethyl-1,2-benzanthracene (DMBA), 4-nitroquinoline N-oxide (4NQO), 3,4 benzpyrene (3,4 BP) and to a lesser extent 20 methylcholanthrene (20 MC). Experimental changes have usually been measured in terms of criteria such as the

time for induction of tumours, the number of animals developing tumours and the number of animals lost in the process of carcinogenesis.

Rats: Initial attempts to induce oral tumours in the rat by DMBA painting were not satisfactory because of a long induction time and low tumour yield. This situation was improved both in the time of induction and tumour incidence when animals were desalivated before carcinogen applications (Wallenius and Lekholm, 1973). However, experimental induction of carcinoma by DMBA was only possible in the rat palatal mucosa. In 1973, Wallenius and Lekholm introduced an alternative chemical carcinogen that was able to produce cancer in the palate as well as in other sites of the oral cavity such as the tongue and gingiva and in a reasonably short time. The new carcinogen was the water soluble 4 NQO applied in propylene glycol. Since then, 4 NQO has been the carcinogen of choice for this model. Successful results were obtained when 4NQO at 0.5 per cent concentration was painted three times per week for 16-24 weeks (Wong and Wilson, 1983; Steidler et al., 1985). A recent study by Ohne et al. (1985) reported 100 per cent incidence of squamous cell carcinoma in the oral mucosa (tongue, palate and gingiva) when 4NQO was given in the drinking water of rats with only a rare tumour incidence in other organs.

Mice: Although cancer induction in mice is thought to be difficult (Eveson, 1981), squamous cell carcinoma has been induced by a variety of chemical carcinogens. Cancer of the dorsal tongue mucosa in mice was induced by 20 MC injections into the submucosal tissue (Levy, 1958). Tumour yield was improved when the salivary glands were

removed prior to carcinogen administration (Goldhaber, 1957). Oral carcinogenesis in mice was also satisfactory with 3,4 BP painting but was significantly improved when combined with experimental induction of severe liver damage (Protzel et al., 1964). Similarly, 4 NQO when painted three times per week for 16 weeks produced carcinoma in 100 per cent of mice after 50 weeks (Steidler and Reade, 1984 and 1986). However, this carcinogen was more successful when continuous physical irritation of the painted mucosa was used during carcinogen paintings (Fujino et al., 1965).

Hamsters: Adler in 1931 was the first to introduce the golden hamster as an animal model in biologic laboratories (Adler, 1948). Since then hamsters have been used in increasing numbers in a variety of research work. The hamster is unique among the common laboratory animals for certain characteristics such as its cheek pouch and skin (Homburger, 1969) and has proved to be especially valuable in cancer research particularly in chemical carcinogenesis (Emminger and Mohr, 1982). In 1954, Salley succeeded for the first time in producing squamous cell carcinoma of the cheek pouch in this model. Repeated application of one of several carcinogenic polycyclic hydrocarbons, DMBA, 20 MC and 3,4 BP dissolved in either acetone or benzene. Each of these carcinogens was painted three times per week for 16 weeks and the animals were observed for an additional 9 weeks (Salley, 1954).

The distinct phases observed during the course of Salley's (1954) experiment were inflammation, degeneration and necrosis followed by regeneration and healing. The mucosa subsequently passed

through four histologically recognisable stages namely hyperplasia, papilloma formation, carcinoma in situ and invasive squamous cell carcinoma. Salley concluded that DMBA in acetone was the most effective carcinogen for use in the hamster cheek pouch.

Following extensive research on intraoral chemical carcinogenesis in the hamster model, Morris (1961) concluded that young hamsters, up to nine weeks of age, were more prone to develop cancer than older animals and that five weeks of age was the optimum age for such experiments to be started. A 0.5% solution of DMBA in acetone when painted three times per week for twelve weeks would produce the maximum tumour yield (100 per cent) with a relatively shorter latency period and maximum survival of the animals. Higher concentrations of the carcinogen, such as 1.5 per cent, were toxic and associated with high morbidity and mortality while low concentrations such as 0.1 per cent solution were ineffective.

Morris and Reiskin (1965) used varying durations of carcinogen painting (up to 21 weeks) in 13 groups of young adult male and female hamsters each group consisting of 13 animals. They used similar concentrations of DMBA in acetone to that used by Salley (1954) and Morris (1961). Each group was painted for a defined period of time and then left to live until the twenty first week of the experiment. Animals which had been painted with the carcinogen for one week (three applications) developed tumours in 31 per cent of cases while carcinogen application for two weeks produced tumours in 54 per cent of the animals. All animals painted with the carcinogen for three weeks or more developed tumours (100 per cent). Morris and

Reiskin (1965) also showed that the latent period was the same in animals painted with the carcinogen for five to twelve weeks and the maximum tumour number was the same for animals painted for eight weeks or more.

In 1978, MacDonald showed that by using a 0.25 per cent solution of DMBA in acetone instead of the usual 0.5 per cent concentration, he could improve localisation of tumours in the painted areas of the pouches without significantly reducing the proportion of pouches developing tumours in his animals. However, a longer latent period was required for the tumours to develop.

1.5.6 Promotors and Cocarcinogens of Chemical Carcinogenesis in the Hamster

By no means all irritants have a cocarcinogenic or promoting effect on carcinogenesis. Some of them even have a distinctly anticarcinogenic effect while others fail to modify the carcinogenic response (Berenblum, 1947). Many examples on the enhancing effect of irritants are documented. Renstrup et al. (1961 and 1962) showed that chronic irritation by wires attached to the teeth could reduce the latent period of tumours developing in the cheek pouches of hamsters. Although tumour incidence was the same in the irritated and nonirritated pouches, tumours appeared much earlier in the animals with irritated pouches (four weeks after the beginning of the treatment with the carcinogen) than in the nonirritated pouches (ten

weeks after beginning the treatment with the carcinogen). They suggested that chronic irritation was acting as a cocarcinogen for the development of tumours.

Maeda and Kameyama (1986) also reported that excisional wounding of DMBA pretreated mucosa of the tongue enhanced cancer induction when further DMBA application was made. These investigators concluded that excisional wounding acts as a promoting agent. Similar conclusions were reached by Tsiklakis et al. (1986) who found that injury by several incisions of the tumours after an incisional biopsy would enhance tumour growth and invasiveness.

MacDonald and Pospisil (1981) found that prior treatment with cryosurgery did not alter the susceptibility of cheek pouch mucosa to chemical carcinogenesis. However, when premalignant lesions of hamster cheek pouch induced by DMBA were partially treated by cryosurgery, a potentiating effect on subsequent tumours was noticed (Pospisil and MacDonald, 1981).

Perkins and Shklar (1982) showed that aspirin and indomethacin given systemically by the oral route delayed the development of carcinoma of the cheek pouch induced by DMBA. Tsiklakis et al (1986) and Shklar (1966) also found that cortisone when given simultaneously with the carcinogen DMBA decreasesd the growth rate of exophytic lesions but that tumours became more invasive into the deeper tissues of the pouch.

1.5.7 Influence of Dietary Composition on Experimental Chemical Carcinogenesis in the Hamster

Andreou and Morgan (1981) studied the effect of dietary restriction on DMBA-induced carcinogenesis in the hamster cheek pouch. They suggested that dietary restriction of 25% increased the latent period for carcinogenesis and retarded tumour growth. However, no statistically significant differences were present between experimental and control animals and the influence of a specific nutritional deficiency resulting from such dietary restriction on carcinogenesis was not investigated and was dismissed as being unlikely.

Salley et al. (1962) showed that the latent period for tumour development in chronically thiamine-deficient hamsters treated with DMBA was significantly shorter than that for the control group. Zinc deficiency has also been investigated in relation to hamster pouch carcinogenesis but results have not been consistent (Eveson, 1981).

Vitamin E was found to be capable of delaying tumour formation and reducing chemical carcinogenesis in the hamster cheek pouch. Animals given vitamin E produced tumours that were less invasive into the deeper tissues of the pouch and had less surface necrosis compared to tumours of control animals (Weerapradist and Shklar, 1982).

Goodwin et al. (1986) demonstrated a significant delay in the induction time of lingual carcinoma in hamsters by selenium and retinoic acid. Lingual carcinomas were delayed for three weeks in animals given selenium, six weeks in animals given retinoic acid and five and a half in animals given selenium plus retinoic acid compared to animals deficient in these nutrients.

1.5.8 Metastasis of Chemically Induced Oral Carcinoma in the Hamster

Metastasis of oral squamous cell carcinoma in the hamster is uncommon. When metastasis occurs it is through lymphatic spread. No haematogenous spread has been reported in the literature. Salley (1954) reported metastasis of squamous cell carcinoma, produced by 0.5 per cent DMBA in acetone, in all the surviving animals of two separate experiments. However, since then only very few such metastasis has been reported by investigators in spite of the large number of experiments dealing with oral carcinogenesis in the hamster model. Craig (1980) showed that metastasis to cervical lymph nodes was achieved in 48 per cent of his experimental animals by extending the duration of the tumour bearing period following removal of exophytic tumours while they were small and allowing the development of more invasive tumours. Tsiklakis et al. (1986) showed that metastasis to cervical lymph nodes was present in 15 to 38 per cent of their animals and there were no significant differences in the percentage of metastases between animals that only had the DMBA paintings and those

treated with either cortisone or multiple incisions. However, they have stated that such metastasis was only achieved after extending the tumour-bearing period to 20 weeks or more.

More recently, metastasis to cervical lymph nodes from carcinoma of the tongue in the hamster has been studied by Shingaki et al. (1987) who stressed the facts that cervical lymph node metastases can be enhanced by either extending the tumour bearing period, the addition of mechanical trauma to the primary carcinoma or the use of systemic cortisone.

Metastases of carcinoma of the cheek pouch to regional lymph nodes reported in the literature has varied between 95 per cent in Salley's study (1954) which lasted 25 weeks to 48 per cent in Craig's study (1980) that lasted 65 weeks. Metastasis of carcinoma of the tongue has also varied in the literature between 14 and 22 per cent in the Japanese literature where experiments lasted between 18 and 53 weeks. Safour et al. (1984) reported metastasis to the cervical lymph nodes in 53 per cent of his animals during the 21 weeks duration of his experiment. However, such high incidences of metastasis in the hamster were probably due to the nature of the experiment which was designed to implant malignant cells into incisional wounds made deep into the underlying tissues in different sites of the hamster's body.

1.6 AIMS AND DESIGN OF THE PRESENT STUDY

The aims of the present research were to investigate the effects of deficiencies of iron and folate both individually and combined, on chemically induced oral carcinogenesis. The Syrian golden hamster which has been used in previous studies and has been found to be a suitable animal model for dietary induced folate deficiency and for cheek pouch chemical carcinogenesis, was chosen as the animal model for the present research. The hamster model of folate deficiency has been well characterised but the effects of iron deficiency are less well standardised.

This research was designed to induce folate deficiency by feeding folic acid deficient diet or iron deficiency by feeding a diet, lacking iron, coupled with repeated venesection. The carcinogen DMBA in acetone, at a concentration of 0.25 per cent, was chosen for inducing carcinoma of the cheek pouches. Experiments were designed to induce the nutritional deficiency first, followed by application of the carcinogen for eight weeks and maintenance of the animals on the specific nutrient deficient diets for several weeks in order to give time for tumours to develop before sacrifice. After sacrifice tumours were to be counted and measured grossly. The carcinogen treated tissues were then to be examined histologically to identify individual foci of squamous cell carcinoma.

CHAPTER 11

HAMSTER CHEEK POUCH CARCINOGENESIS IN IRON DEFICIENCY

2.1 INTRODUCTION

Chronic blood loss, often complicated by a low dietary intake of iron-containing food, is the most common cause of iron deficiency in man. The development of an animal model of iron deficiency using a combination of repeated blood loss and low or no dietary iron content would be analogous to the human situation. The hamster cheek pouch and tongue models using DMBA (Salley, 1954) and the rat palate model using 4-nitroquinoline-N-oxide (4NQO) appear to be the most suitable models of oral carcinogenesis (Eveson, 1981). However, each of the two animal models has certain advantages and disadvantages depending on the information which the investigator is seeking. Such variables as accessibility of the intraoral site, the histology of that site and its similarity to the equivalent human site and the time required for cancer induction are important.

The rat palate was found to fully satisfy the histological requirements of a model of oral chemical carcinogenesis (Eveson, 1981). Iron deficiency is readily induced in rats (Prime et al.,

1983). However, the time required to induce cancer in the rat was found to be significantly longer, and the percentage of rats developing tumours was low compared to the hamster model (MacDonald, 1973). In addition, Rennie et al. (1982b) found that iron deficiency could be induced in the hamster although this is more difficult than in the rat. Ranasinghe et al. (1983) reported the induction of severe iron deficiency anaemia in the golden hamster in a relatively short period of time. However, the diet these authors used contains less than the recommended level of folic acid.

The experiment reported in this chapter was designed to further develop the hamster as an animal model of iron deficiency and to examine the effects of such deficiency on chemical carcinogenesis. The protocol of the experiment was firstly, to induce iron deficiency in young adult hamsters by a combination of repeated venesection and restricted dietary intake of iron; secondly, to induce cancer in the cheek pouches of these animals and two diet control groups by repeated DMBA painting (0.25% in acetone). The third aim was to compare the yield of tumours in the iron deficient animals to that of the control groups.

2.2 MATERIALS AND METHODS

Animals of this study were started on the experimental regime when they were 6-7 weeks old. During the first twelve weeks of the study one experimental group was maintained on an iron deficient powdered diet and another on an iron supplemented powdered diet. Both experimental groups were bled weekly in order to induce iron deficiency. During weeks 12 and 13, a 5mm square superficial biopsy was taken from the anterior medial wall of one cheek pouch of each animal in the experimental groups and the two control groups to evaluate epithelial compartment changes at an early stage of iron deficiency. Biopsy wounds were allowed to heal for two weeks before the carcinogen was applied, on week 16. A 0.25 per cent solution of DMBA in acetone was painted three times per week for eight weeks on a defined area of the medial wall of each pouch of each animal. The animals were then maintained on the same dietary regimes for another twelve weeks before being sacrificed at the beginning of week 37 for the final analysis of the study.

2.2.1 Animals

The animals used in this experiment were 50 male Syrian golden hamsters (Mesocricetus auratus) which were 6-7 weeks old at the start of the experiment. In order to standardise the animals and reduce possible variation in response to the carcinogen, a line bred strain of hamsters was used and all animals were males. Animals were weighed and divided into four weight-matched groups. Groups 1 and 2

were the control groups each consisting of 12 hamsters. Group 1 received standard laboratory chow ad libitum while Group 2 had locally prepared powdered diet supplemented with all required nutrients, minerals and vitamins. The other two groups (Groups 3 and 4) were the experimental groups. Group 3 consisted of 14 hamsters which received powdered diet similar to that given to Group 2 but "free" of iron while Group 4 consisting of 12 hamsters received powdered diet similar to that of the control group (Group 2). In addition, both experimental groups had 1.5 ml. of blood removed every two weeks to assist induction of iron deficiency.

All groups had distilled water to drink and they were kept on Sorboil (the name has changed later to Klensorb) bedding material(supplied by D.F.Wishart and Co., Edinburgh) which is an absorbant non-organic material (Appendix 1). Animals were caged individually in plastic cages that had aluminium tops. They were arranged in a cage-rack in such a way that Group 3 hamsters which received the iron-free diet was in the top of the rack in order to prevent their access to any iron containing diet from cages of other groups.

2.2.2 Diet

A powdered diet was prepared to provide the nutrient requirements of the golden hamster recommended by the National Academy of Sciences (1978). This diet consisted of the bulk nutrients which

are protein, carbohydrate, dietary fibre and fat as shown in Table 2.1. Corn oil (Mazola cooking oil) was the dietary source of fat. The diet also included the minerals shown in Table 2.2. The quantities of individual minerals were assessed according to their atomic weights in the chemical compounds used and were added to and mixed with the bulk components of the diet. The third component was vitamins, both oil soluble and water soluble vitamins, shown in Table 2.3. The oil-soluble vitamins (A, D, E, and K) were dissolved in the corn oil before it was mixed with the rest of the bulk diet powder. The water-soluble vitamins were dissolved in the water that was added to the powder dispensed daily for each animal. The water soluble vitamins were prepared and mixed with water every week in order that they should remain fresh throughout the experiment. Group 1 animals received the standard laboratory chow, shown in Table 2.4, which contained all the nutrients, minerals and vitamins required by the golden hamster.

The daily ration for each hamster was prepared by mixing approximately 10g of the diet (this amount was increased to 13g later in the course of the experiment) with 8ml of distilled water containing the water-soluble vitamins. This mixture formed a rather dry paste which was found to be more acceptable to the animals than the dry powder diet which was also very wasteful due to spillage. A scoop was used to measure the amount of powdered diet required and a calibrated fluid dispenser was used to measure the mixing water.

Four samples of the iron deficient diet were analysed for iron content in the Department of Biochemistry, Glasgow Royal

Infirmary. The mean values of the duplicate determinations were 8.5, 9.0, 9.1 and 11.9 (±0.4mg/kg) of iron (average 9.6mg/kg diet). This amount of iron was present as a contaminant of some of the dietary components such as casein (3.3-3.7 mg/kg iron) and minerals, particularly calcium lactate (4.28mg/kg iron), which was used as the dietary source of calcium. Calcium carbonate was later substituted for calcium lactate in the diet of Group 3 animals only for two weeks during the course of the experiment but this was unsatisfactory, as explained in Section 2.3.2. Two samples of the calcium carbonate-containing diet were analysed in the same Department of Biochemistry, for their iron content and found to have 6.8 and 7.5 mg/kg of iron.

2.2.3 Weighing and Bleeding

Hamsters were weighed at the start of the experiment, weekly thereafter and at sacrifice.

Blood samples (1.0ml) for corrected whole blood folate and haemoglobin estimations were taken at the start of the experiment, after 4 weeks, 10 weeks and at sacrifice. These samples were sent to the Department of Haematology, Gartnavel General Hospital, Glasgow for analysis. These four main blood samples were taken from all hamsters in all groups.

Additional 1.5ml samples of blood were taken every two weeks from hamsters in Groups 3 and 4 (0.5ml was taken in one week and

1.0ml in the next week and so on) in order to facilitate the induction of iron deficiency in these groups. Blood withdrawal continued for the first 12 weeks of the experiment after which 1.0ml of blood was taken every four weeks until the end of the experiment. This was done in order to try and maintain low blood levels of haemoglobin. Haemoglobin estimation in samples other than those made for folate estimations was carried out by the author using an American Optical portable haemoglobinometer.

The method described by Pansky et al. (1961) was used to obtain the blood samples from the retro-orbital sinus. The animals were lightly anaesthetised with ether and held by the operator who could also retract the upper and lower eyelids with the fingers of the same hand. With the other hand, a heparinised needle was inserted about halfway along the supra-orbital ridge between the eyelid and the eyeball. The needle was passed downwards at an angle of 45° keeping close to the medial wall of the orbit (Fig 2.1). Gentle aspiration confirmed entry to the venous sinus and blood was then withdrawn. A heparanised needle and syringe were used to obtain the blood samples because otherwise hamster blood coagulates very readily.

2.2.4 Biopsy

An epithelial biopsy, measuring approximately 5mm square, was taken from the anterior-medial wall of one pouch of each hamster. Due to the large number of animals, the biopsy was taken from one group of animals each day starting at the last two days of week 12 and

finishing after the first two days of week 13 of the experiment. The biopsy was taken in order to quantify epithelial changes in the different groups. Alternating right and left pouches were chosen in consecutive hamsters in the different groups. All biopsies were taken after the hamsters were lightly anaesthetised with ether first and then by an intraperitoneal injection of Sagatal. A superficial incision (5mm long) was made on one side of the square using a scalpel while the other 3 sides of the square were cut with a pair of fine scissors. No suture was required after the biopsy and healing was achieved fairly quickly (within one week) without serious complications such as infection and haemorrhage. The sequence of the healing process is shown in Figs. 2.2, 2.3 and 2.4.

The biopsy, after removal from the pouch, was laid down gently on a piece of filter paper with the deep surface down in order to avoid distortion during fixation. The biopsy was fixed in formal acetic methanol for 24 hours and transferred to formalin thereafter. This method of fixation was found to cause less tissue shrinkage and blocks from tissue fixed in this way were readily cut (MacDonald, 1973).

2.2.5 Sampling of the Biopsy

Following fixation the biopsy was cut horizontally into upper and lower halves and subsequently paraffin processed using a 24 hour cycle on a Histokinette 2000 processer. The blocks were then

embedded on their upper edges and orientated such that the knife met the keratinised surface first. This procedure was found to minimise splitting of keratin layers thus making quantitation easier (Al-Damouk, 1984). Sections were cut at 5μm thickness and stained with haematoxylin and eosin.

2.2.6 The Computerised Planimetry Technique

Tissue sections obtained from the biopsies at 13 weeks were analysed for their epithelial compartment thicknesses. Two compartments, the keratinised compartment and the cellular compartment were identified and measured and the total epithelial thickness was calculated. The aims of this quantitative analysis were to identify the effects of low levels of iron on pouch epithelium in the experimental group and compare any changes in the epithelium with those of the control groups. A computerised planimetry technique was used for this purpose.

The features of the computerised planimetry system are shown in Fig. 2.5. This consisted of a microscope with a drawing tube attachment which allowed a light spot on the probe to be projected into the microscope field while the probe was held in contact with a bit-pad. When lengths or areas of the section were traced with the light spot of the probe, a corresponding length or area was traced on the bit-pad. The bit-pad was connected to a North Star Advantage

graphics computer which provided a visual representation of the tracing and also allowed the lengths and areas to be expressed directly in units related to the original section.

Certain conditions need to be satisfied in order to have a valid quantitative study. Conditions such as representative areas of the structure of the tissue under examination are required as are randomly distributed samples of the tissue to be quantified (Weibel 1969). However, tissues such as oral epithelium show anisotropy in that there is a non-random orientation of their constituent elements. Such anisotropy requires selective techniques of sampling which take account of the axis of anisotropy. These selective techniques include consistency of the sampling method in different hamsters, consistency in cutting and staining the histological sections to avoid differences due to the microtome pressure during cutting and differences in the intensity of staining which may affect the identification of the tissue compartments. The selective techniques also include accuracy in selecting the same microscopic fields to be analysed in different sections through identification of fixed anatomical structures in the section such as a major blood vessel, nerve or a duct. This last condition was overlooked in the present study due to the small size of the biopsy.

The 5 μm thick, paraffin processed sections were mounted on the circular revolving stage of a Leitz Dialux microscope and the section was aligned so that it was oriented with the mean epithelial surface running horizontally. The width of the epithelial column to be analysed was identified by the vertical lines of a square eyepiece

graticule which divided the graticule into five columns of equal widths.

The computer was calibrated for the microscope magnification used and the areas of the keratinised compartment and of the cellular compartment were measured by tracing the light spot around them. Spaces in these compartments due to splitting of the sections were measured in the same way and subtracted immediately by the computer from the areas of the compartments.

2.2.7 Quantitative Histological Analysis of the Biopsy

The cheek pouch biopsy was halved horizontally and each half was blocked saparately (Section 2.2.5). One 5μm section was taken from each block, stained with haematoxylin and eosin and analysed for the thickness of its keratinised compartment, cellular compartment and total epithelial thickness. Three microscope fields were analysed in every section and the mean values were calculated. The width of the epithelial column measured varied between one-fifth and a whole width of the eyepiece graticule. This depended on the amount of epithelium available in the section that could satisfy the criteria for selecting areas for compartment analysis such as the uniformity of epithelium, absence of epithelial foldings, minimal keratin splitting and good quality staining (Fig. 2.6).

2.2.8 Carcinogenesis

Dimethylbenzanthracene (DMBA) has been shown to be the most effective chemical carcinogen for use in hamster cheek pouch (Sally, 1954). In this experiment 9,10-dimethyl-1,2-benzanthracene was used at a concentration of 0.25 per cent. Acetone was chosen as the solvent in preference to benzene or mineral oil because it causes less tissue necrosis, less mortality of animals and it is easier to control the spread within the pouch (MacDonald, 1973).

A fresh solution of the DMBA in acetone was prepared every two weeks. Any residue was discarded at the conclusion of carcinogen applications for those 2 weeks. The freshly prepared carcinogen solution was stored in small Bijoux bottles (approximately 5ml). Each bottle was enough for one carcinogen application to all animals and any residue was discarded after each session. The sequence of painting of the animals was changed for every session (i.e. if Group 1 was painted first in one session followed by Group 2, 3 and 4, in the following session Group 2 was painted first followed by Group 3, 4 and 1 and so on). Using small bottles, preparing fresh carcinogen every two weeks and changing the sequence of animals during successive painting sessions were done in order to avoid any variation due to changes in the concentration of the carcinogen due to evaporation of acetone during the course of painting.

2.2.9 Immobilisation and Carcinogen Application

The device illustrated in Fig. 2.7 was based on that described by Moss et al. (1965) and modified by MacDonald (1973). The animal was anaesthetised with ether and placed quickly in the detached chamber of the restrainer with its head through the hole in the top of the chamber. This chamber was mounted on the base and the hamster's mouth was opened and held by rubber bands over the incisor teeth. The tension of the rubber bands was checked carefully to avoid damage due to excessive opening of the jaws. Retractors were inserted into the cheek pouches and these provided good exposure of the medial walls of the pouches as shown in Fig. 2.8. When the retractors were placed in the pouches they were held by an assistant while carcinogen applications were made to each cheek pouch.

The anterior part of the medial wall of each pouch was cleaned and dried by a piece of cotton wool before carcinogen application. The carcinogen was applied, in all hamsters, with a specially modified camel-hair brush to an area in the anterior medial wall of the cheek pouch bounded anteriorly by a prominant blood vessel. The brush was 1cm in width and the bristles were trimmed to 1cm in length. The brush was dipped in the carcinogen solution and wiped against the side of the bottle to remove excess solution before application to the pouch. A gentle air jet from a hand air syringe was used to evaporate the acetone on the cheek pouch before releasing the animal from the restrainer. This reduced spread of carcinogen to

adjacent areas in the cheek pouch. The carcinogen was applied to both right and left pouches three times every week (Monday, Wednesday and Friday) in the morning for eight weeks (weeks 16-24).

2.2.10 Safety Precautions

Precautions were taken during carcinogen preparation and application to prevent exposure of the operator and the assisting staff to the carcinogen. DMBA was dissolved and diluted to the required concentration and dispensed into 5ml bottles in a fume cupboard. Surgical gloves, face mask and laboratory coat were used during carcinogen preparation and application. Care was taken to avoid spillage of the DMBA solution and dripping from the loaded brush. Empty bottles and containers were disposed of according to the current safety regulations for laboratory carcinogens.

2.2.11 Techniques of Preparing Tissue for Tumour Counting

The animals were left to live for 12 weeks after the last carcinogen application, after which time they were sacrificed by cervical dislocation following ether anaesthesia. During the course of the experiment, 13 animals died (8 before and 5 during carcinogen painting) and were excluded from the final analysis. After sacrifice, the medial wall of the check pouch was exposed by cutting the outer wall of the pouch with a pair of scissors. The mouth was kept open by inserting a rubber stopper between the upper and lower incisor teeth.

One coloured and one black and white photograph were taken for each pouch from which a coloured transparency and a black and white print were made. The hamster's head was then removed with scissors and fixed in formal acetic methanol for 24 hours following which it was transferred to buffered formalin prior to detailed sampling. The body was dissected and examined grossly for any pathological abnormalities. The viscera were taken out and fixed in formalin for further use in the future if needed.

Following a few days of fixation in formalin a block of epithelium measuring one square centimetre was dissected out from the area of cheek pouch originally painted with the carcinogen (Section 2.2.9). The depth of the block was made to include part of the Masseter muscle. All blocks were paraffin processed using the 24 hour cycle on the Histokinette 2000 processer. The tissue was then trimmed into four smaller blocks in a horizontal direction. Each block was embedded in paraffin on its inferior surface and orientated in such a way that the keratin met the knife first during section cutting. One 5um thick section was obtained from each block and stained with haematoxylin and eosin.

2.2.12 Naked Eye Counting of Tumours

Unlike human oral carcinomas, many malignant epithelial tumours in the hamster cheek pouch arise in squamous cell papillomas of a reasonably large size and then invade the underlying connective

tissue. Accordingly all exophytic tumours can be considered potentially malignant although histological proof of invasion is required to confirm a diagnosis of carcinoma.

Each cheek pouch was photographed twice (Section 2.2.11). A large (15 x 20 cm) black and white print and a 35mm coloured transparency was available for each pouch. A ruler was included in each photograph of the pouch which aided in recording the magnification of the prints. The prints were random numbered. The square centimetre painted with DMBA was identified and tumours within it were counted. Only lesions that measured 1mm or more in the original size and were discretely elevated above the surface were counted as tumours (Fig 2.9). After counting the tumours on the black and white prints the transparencies were projected on a screen and tumours in the painted area of the pouch were counted again to confirm that these had been correctly identified. The tumours were classified into three size categories; small tumours (1-2mm diameter), medium size tumours (2-5mm diameter) and large size tumours (5mm or more in diameter) as described by Pospisil and MacDonald (1981). After counting had finished the random numbers were revealed.

2.2.13 Histological Counting of Carcinomas

Squamous cell carcinoma of the lining epithelium of the hamster cheek pouch usually occurs in a pre-existing squamous cell papilloma. The papilloma often reaches a reasonably large size before invasion from the surface epithelium starts. At the beginning,

invasion is confined to the stroma of the papillomatous growth in which case the carcinoma can be described as exophytic (Fig 2.10). Subsequently, invasion may extend from the exophytic tumour to involve adjacent connective tissue and probably muscle of the cheek pouch. At this stage the carcinoma is called endophytic (Fig 2.11). However, malignant transformation and invasion, occasionally, occurs directly from apparently normal pouch epithelium without the formation of a papillomatous growth. This is also called endophytic carcinoma (Fig 2.12).

Therefore, squamous cell papillomas are regarded as potentially malignant tumours and accordingly were counted grossly. The diagnosis of squamous cell carcinoma was made exclusively on the histological evidence of the invasion. A carcinoma that was present in one block and extending into adjacent blocks was counted as one regardless of the number of blocks involved. Analysis of tumour yield in this study was done on the basis of grossly seen tumours and histologically identified carcinomas.

2.3 RESULTS

During the course of the study 13 hamsters died prematurely. Eight of these died before commencement of carcinogen applications and five died during carcinogen painting. Most deaths were due to severe diarrhoea (wet tail). Postmortem examination did not reveal any gross pathology. These animals were excluded from the final analysis of the

study. Another eleven hamsters were sacrificed early due to the presence of large haemorrhagic tumours in their cheek pouches which interfered with their feeding. However, these hamsters were included in the final analysis of the study. All other animals were sacrificed at the beginning of week 37 of the experiment. The number of animals examined for the purpose of this experiment was 37. Most of the animals that died prematurely and were not included in the final analysis were in the iron supplemented diet group (experimental Group 4) in which 8 of 12 hamsters were lost. Two hamsters were in the iron deficient diet experimental Group 3 and another three were in the powdered diet control Group 2. None of the Group 1 hamsters which were maintained on laboratory chow died prematurely.

2.3.1 Weight Results

The weekly weights of individual animals are shown in Table 2.5 for Groups 1 and 2 hamsters and Table 2.6 for Groups 3 and 4 hamsters. The statistical comparisons between the different groups when a Mann-Whitney U test was used are shown in Table 2.7.

Both experimental and control animals gained weight steadily from the start of the experiment until the stage when tumours started to develop. Group 1 controls maintained on laboratory chow gained less weight, and were significantly smaller ($P<.002$) than the powdered diet controls (Group 2). Group 1 animals were also significantly smaller than and either of the experimental groups ($P<.05$ and $P<.02$ for Groups 3 and 4 respectively) during week 3 of the study. Group 1

animals remained significantly smaller than the powdered diet controls thoughout most of the experimental time and until tumours started to enlarge.

The iron deficiency groups (Groups 3 and 4) maintained on powdered diet had similar weight gain to the powdered diet control animals in Group 2 during the early weeks of the study. No statistically significant differences were present at this stage, either between the experimental groups themselves or between either one of them and the control animals in Group 2. However, both experimental groups were significantly larger than the pellet control animals in Group 1. When calcium carbonate was exchanged for calcium lactate in the iron deficient diet of Group 3 animals on week 9 in an attempt to reduce the iron content of their diet, these animals lost weight so rapidly (average loss was 18g per week) that in week 10 they became significantly lighter than all other groups. Weight loss continued during the two weeks calcium carbonate was given as hamsters reduced their dietary intake significantly (Table 2.8). It was thought that keeping the hamsters on the calcium carbonate diet longer would only lead to animal deaths and accordingly calcium lactate was reintroduced in the diet of Group 3 hamsters. When calcium lactate was given, hamsters started to feed normally and gained weight so rapidly that on week 12 they were of a similar mean weight to the pellet diet controls (Group 1). Two weeks later they were of a similar mean weight to the powdered diet groups (Group 2 and 4). When the carcinogen application was started on week 16 of the study, no

significant differences were present beween the weights of animals in the powdered diet groups (Groups 2, 3 and 4).

Weight gain in the bleeding groups (Groups 3 and 4) was slower than that of the powdered diet animals in Group 2 during the second half of the study. Group 4 animals receiving the iron sufficient diet were significantly smaller ($P<.05$) than Group 2 animals during week 21 while Group 3 animals receiving iron deficient diet were significantly smaller ($P<.05$) than Group 2 controls on week 24. The two experimental groups remained smaller than Group 2 controls until week 30 of the study after which time tumour development affected the weights of all animals.

2.3.2 Haemoglobin Estimations and Corrected Whole Blood Folate Assays

Normal haemoglobin values in the hamster are not established with certainty. Most investigators refer to the human haemoglobin range as a guide for hamster haemoglobin values. Some investigators draw an arbitrary line for the normal range depending on their experimental findings (Ranasinghe et al., 1983) while others take the haemoglobin range in the control groups of their experiments as the normal range for that particular experiment (Rennie et al., 1982b). Moreover, there are large veriations in normal haemoglobin values even in human blood as discussed in Section 1.4.9. Taking these factors into consideration, McPherson (1987) and Tomson and Wardrop (1987) suggested a normal hamster haemoglobin range of 16.8g/dl for males and

16.0g/dl for females ± a standard deviation of 1.2 in males and 1.5 in females. These values are closely similar to the values 13.3-17.5 g/dl reported by Rennie et al. (1981) and 14.3-18.8 g/dl reported by MacDonald (1988). In this study and all other studies reported in this thesis, the range of haemoglobin values in the control hamsters was taken as the normal range for the corresponding study. In the study reported in this chapter, Groups 1 and 2 animals were the control groups. The value of the mean at any week minus two times the standard deviation was calculated. This gave the least value of 12.8g/dl in Group 1 on week 4 of the study (week 37 was disregarded due to the effects of tumour presence in the cheek pouches). This value of 12.8g/dl was taken as the lower limit of the normal haemoglobin range for hamsters in this study.

Haemoglobin levels of control Groups 1 and 2 were normal and ranged between 14.1 and 18.4 g/dl except for three hamsters in the pellet diet controls (Group 1) during weeks 1 and 4 of the study. On sacrifice, haemoglobin levels were considerably lowered probably due to the presence of tumours (Table 2.9). The experimental groups which were bled regularly every week during the first 12 weeks and every 4 weeks afterwards (as discussed in Section 2.2.3) started within the normal range and maintained similar or even higher levels than normal throughout most of the experimental time. Haemoglobin levels only went below normal values when tumours appeared in the cheek pouches after week 21, as shown in Table 2.10. Although statistically significant differences were present between haemoglobin levels of the

different groups (Table 2.11), they were all within the limits of the normal range of the control groups.

The lower limits of the normal range of folic acid estimations are not yet known with certainty. Data on hamster serum folate level published by Cohen et al. (1971) and obtained by microbiological assay gave a value of 2.0 μg/100ml (20ng/ml). Al-Damouk (1984) reported that compartment changes of the hamster tongue and cheek pouch epithelium were encountered when corrected whole blood folate levels reached levels below 90ng/ml as measured with Becton and Dickinson radioassay kits. Using the same folate estimation kit, the human corrected whole blood folate range is 75-400ng/ml. In this and the following experiments reported in this thesis the lower limit of corrected whole blood folate was taken as 100ng/ml provided that the human range was 75-400ng/ml.

Estimations of corrected whole blood folates made in blood samples take on weeks 1, 4, 10 and at sacrifice were all within the limits of normal as shown in Table 2.12.

2.3.3 Quantitative Histological Studies of Hamsters Cheek Pouch Epithelium

The quantitative histological data from the compartment analysis of the biopsy during weeks 12 and 13 of the experiment are summarised in Tables 2.13 and 2.14. The comparisons between corresponding parameters of epithelium are shown in Table 2.15.

The compartment analysis studies showed that the iron deficiency group (Group 3) had the thinnest epithelium while the iron supplemented experimental group (Group 4) had the thickest epithelium amongst all groups. In spite of the significant difference ($P<.02$) between animal weights and the difference in the physical properties of diet between the two control groups (Groups 1 and 2), no statistically significant differences in either total epithelial thickness or in the thicknesses of the constituent compartments were present.

Animals in Group 4 which received an iron supplemented diet and were bled repeatedly had significantly thicker epithelium ($P<.02$) than the pellet diet control animals (Group 1). The difference was due to significantly thicker cellular and keratinised compartments ($P<.05$ and $P<.02$ respectively). The weight difference between the two groups was not statistically significant.

Experimental Group 3 animals maintained on an iron deficient diet and bled repeatedly had significantly thinner epithelium than either of the powdered diet animals in experimental Group 4 and control Group 2 animals ($P<.02$ and $P<.002$) respectively. This thinner epithelium was due to significantly thinner cellular and keratinising compartments. However, animals in Group 3 were significantly smaller than Groups 2 and 4 ($P<.002$ and $P<.02$ respectively).

Correlations were made between the total epithelial thickness or the thicknesses of individual compartments and animal weights at week 12 of the study in all groups, individually and combined, and the results are shown in Table 2.16. The mean epithelial thickness was correlated significantly with the animal body-weight when all animals were considered together using the Pearson correlation coefficient test, ($P<.01$). When individual groups were tested separately, using Spearman rank correlation tests, only epithelium in the pellet diet controls in Group 1 was found to correlate significantly with the body weight ($P<.02$). This was thought to be due to the type of diet these animals were receiving (pellet diet). However, when individual compartments were examined, only the cellular compartment was correlated with weight in Groups 1. No significant correlation between body-weight and compartment thicknesses was found either in animals of the iron deficiency group (Group 3) which were the smallest of all animals or in the iron supplemented animals in Group 4 which had the thickest epithelial compartments.

On the basis of these results it was considered that the differences in epithelial compartment thicknesses between animals in the iron deficiency group and each of the iron supplemented animals (Group 4) and the powdered diet controls (Group 2) were the result of nutritional differences between the groups.

2.3.4 Gross Counting of Tumours of the Hamster Cheek Pouch

The results of the gross counting of tumours and their size distribution in the cheek pouches of experimental and control animals are shown in Table 2.17.

In the control groups, eleven of twelve hamsters in the pellet diet control group (Group 1) developed a total of 52 tumours and eight of nine hamsters in the powdered diet control group (Group 2) developed a total of 36 tumours. In the iron-deficient animals of Group 3, ten of twelve hamsters developed a total of 24 tumours while the four surviving animals of the iron supplemented diet in Group 4 developed 14 tumours.

Analysis of the gross counting of tumours showed no statistically significant differences either in the number of animals developing tumours or in the actual number of tumours present either between the two control groups or between the two experimental groups. Also no significant differences were found between the number of animals developing tumours in the experimental groups compared individually to controls. However, when the numbers of tumours were compared between the experimental and control animals it was found that the iron deficient animals (Group 3) developed very significantly fewer tumours than each of the control groups ($P<.01$ in each case) while the iron supplemented group (Group 4) animals did not differ from either of the control groups. The size observation of tumours in each group showed no obvious differences between the groups.

* Comparisons between groups were made using the X^2 test. The data are summarised in Table 2.19.

2.3.5 Histological Counting of Squamous Cell Carcinomas of the Hamster Cheek Pouch

The histological identification of individual foci of squamous cell carcinoma in the control and experimental groups with their types of invasion are shown in Table 2.18.

In the control groups, eleven of twelve animals in the pellet diet group (Group 1) developed 35 carcinomas while eight of nine animals in the powdered diet control group (Group 2) developed 20 carcinomas. In the experimental groups eight of twelve animals in the iron deficiency group (Group 3) and all four surviving animals in the iron supplemented diet (Group 4) developed 15 and 11 squamous cell carcinomas respectively.

Comparisons between the groups* showed no statistically significant differences in the number of animals developing carcinomas either between the two control groups or between the two experimental groups. Also no statistically significant differences were present between either of the experimental groups compared to each of the controls. When the numbers of carcinoma counted in each group were compared to other groups no significant differences were present between the control groups. However, the iron deficient animals developed significantly fewer carcinomas than the pellet diet controls ($P<.01$) but not statistically less than the powdered diet controls. The iron deficient animals also differed significantly from the iron

supplemented diet animals in experimental Group 4 (P<.05). The iron supplemented diet animals did not differ significantly from either of the control groups.

The pattern of invasion was also studied in all groups as shown in Table 2.18. No obvious differences were seen between the groups.

2.4 DISCUSSION AND CONCLUSIONS

Despite a very low iron-containing diet (9.6 ±0.4mg/kg) and repeated blood withdrawal (1.5ml every two weeks) over a period of 12 weeks, iron deficiency anaemia, as estimated by haemoglobin levels, could not be achieved in the experimental animals. However, despite a normal haemoglobin range in all groups, significant statistical differences were seen between experimental and control groups. Such differences would suggest that if experimental animals were maintained on the same experimental regime for a longer time they would eventually develop haematological evidence of iron deficiency. Such extention of time was not possible if cancer was to be induced in the cheek pouches in sufficiently high incidence and in a reasonably short time.

Animals fed the powdered diet in the experimental and control groups gained more weight than the pellet diet controls in Group 1 and were significantly larger on week 3 of the study.

However, weight gain in experimental Group 3, which was maintained on iron deficient diet and bled repeatedly, was significantly reduced during the second half of the study and rendered animals in this group significantly smaller than each of the control groups. Animals in the iron supplemented diet (Group 4) continued to gain weight despite repeated blood withdrawal and did not differ from either of the control groups. Therefore, the reduced body weight in Group 3 animals could have been due to the effect of iron deficiency without anaemia.

The corrected whole blood folate results were within the limits of the normal range. However, on week 10, folate levels were significantly higher in Group 4 animals, which had been maintained on an iron sufficient diet with repeated venesection, compared to other groups. This increase was interpreted as being reactive and probably due to an increase in the amount of transferrin in the blood.

Compartment analysis of pouch epithelium in the biopsies taken during weeks 12 and 13 of the study showed that the weight difference between the two control groups did not infuence the epithelial parameters. The significant differences between the epithelial compartments of experimental Group 4 and control Group 1 were present in the absence of significant differences between animal weights of the two groups. Therefore, the differences in epithelial thicknesses between animals in the iron deficiency group (Group 3) and either that of the controls in Group 2 or the experimental animals in Group 4 animals was thought unlikely to be simply due to weight differences.

The correlation between the epithelial thickness and animal weight in the pellet diet controls (Group 1) was thought to be due firstly to the physical properties of the diet received by these animals. However, this might be expected to influence the keratinised rather than the cellular compartment. Therefore the significant reduction in epithelial compartment thicknesses was thought more likely to be due to animal weight differences.

No significant correlation was found between the animal weight and epithelial compartment thicknesses either in the iron deficiency group (Group 3) which had the smallest animals, the iron supplemented group (Group 4) which had the thickest epithelium or in the powdered diet controls (Group 2) which had the largest animals. These results suggested that the significantly reduced weight and epithelial compartment thicknesses of animals in the iron deficiency group were the result of iron depletion. However, the effect of the rapid and severe loss of weight experienced by animals in this group when the diet was changed during the preceding two weeks before the biopsy can not be competely excluded.

Analysis of tumour yield in the different groups showed that 91.7 and 88.8 per cent of animals in control Groups 1 and 2 respectively developed tumours. This was despite the fact that these animals were 20-21 weeks old at the start of carcinogen application and only 0.25 per cent DMBA in acetone was used. This finding is not in agreement with what is currently known about the optimum hamster age for cancer induction discussed in 1.5.5.

Gross analysis of tumours showed no significant differences in the number of animals developing tumours between experimental and control animals. However, the iron deficient animals in Group 3 developed significantly fewer tumours than either the control groups or the iron supplemented experimental animals. The iron sufficient animals in Group 4 did not differ from control animals despite repeated bleeding.

Histologically, no differences in the numbers of animals developing squamous cell carcinoma were present between experimental and control animals. When iron was supplemented in the diet of experimental Group 4 animals, the number of carcinomas seen did not differ from that in either of the control groups. However, animals in the iron deficiency group (Group 3) developed significantly fewer carcinomas than either group of control animals (Groups 1 and 2) or the iron sufficient animals in Group 4.

These findings would suggest that the iron deficient diet and probably iron deficiency have a retarding effect on chemical carcinogenesis in the hamster cheek pouch. The results of this study have supported the findings in the rat that iron deficiency does not affect the number of animals developing carcinoma (Prime et al., 1983) although an earlier development of tumours was not obvious in the iron deficient diet animals.

This experiment failed to induce iron deficiency anaemia in hamsters. This was thought mainly due to insufficient blood withdrawal from the animals even when iron contaminating the diet was present in a low concentration. Another possibility is that concurrent use of a low folate diet may be required to produce iron deficiency.

RECOMMENDED DIET OF THE GOLDEN HAMSTER			DIET GIVEN IN THIS EXPERIMENT		
Nutrient	**Amount**	**Unit**	**Nutrient**	**Amount**	**Unit**
Protein	15.0	%	Casein	200	g/kg
Carbohydrates and Digestible Energy	4.2	kcal/g	Starch Sucrose	400 200	g/kg g/kg
Fat	5.0	%	Corn oil	54	ml/kg
Fibre	-	-	Cellulose	50	g/kg

TABLE 2.1 The bulk nutrients in one kilogram of hamster diet.

RECOMMENDED MINERALS			DIET GIVEN IN THIS EXPERIMENT		
Mineral	**Amount**	**Unit**	**Chemical Compound**	**Amount**	**Unit**
Calcium	0.59	%	Calcium lactate	42.80	g/kg
Magnesium	0.06	%	Magnesium sulphate	3.42	g/kg
Phosphorus	0.30	%	Monobasic sodium phosphate	2.33	g/kg
Sodium	0.15	%	Sodium chloride	2.94	g/kg
Potassium	0.61	%	Potassium orthophosphate	10.57	g/kg
			Potassium citrate	8.60	g/kg
Cobalt	1.10	mg/kg	Cobaltous carbonate	2.22	mg/kg
Copper	1.60	mg/kg	Cupric chloride	3.38	mg/kg
Fluoride	0.02	mg/kg	Sodium fluoride	0.06	mg/kg
Iodine	1.60	mg/kg	Sodium iodate	2.72	mg/kg
Manganese	3.65	mg/kg	Manganese carbonate	7.64	mg/kg
Selenium	0.10	mg/kg	Selenium sulphide	0.18	mg/kg
Zinc	9.20	mg/kg	Zinc acetate	68.50	mg/kg
Iron*	140.00	mg/kg	Ferrous sulphate*	380.81	mg/kg

TABLE 2.2 Mineral content of the hamster diet.
* Iron was given to control animals only.

Fat-soluble vitamins	Amount	Unit
A (retinoic acid)	2.0	mg/kg
D3 (cholecalciferol)	2484.0	IU/kg
E (dl-α-tocopherol acetate)	3.0	mg/kg
K1	4.0	mg/kg

Water-soluble vitamins	Amount	Unit
Biotin	0.6	mg/kg
Choline	2000.0	mg/kg
Inositol	100.0	mg/kg
Niacin	90.0	mg/kg
Pantothenate	40.0	mg/kg
Riboflavin	15.0	mg/kg
Thiamin	20.0	mg/kg
Pyridoxine	6.0	mg/kg
Cyanocobalamin	10.0	μg/kg
Ascorbic acid	7.0	mg/kg
Folic acid	2.0	mg/kg

TABLE 2.3 Vitamin requirements of the golden hamster as recommended by the National Academy of Sciences (1978).

Proximate Analysis	Amount	Unit
Crude Oil	2.4	%
Crude Protein	18.1	%
Crude Fibre	3.6	%
Calcium	0.8	%
Phosphorus	0.7	%
Salt	0.7	%
Carbohydrate	57.0	%
Metabolisable Energy	2855.0	kcal

Amino Acids	Amount	Unit
Threonine	0.6	%
Glycine	0.9	%
Valine	0.8	%
Cystine	0.2	%
Methionine	0.3	%
Isoleucine	0.7	%
Leucine	1.4	%
Tyrosine	0.6	%
Phenylalanine	0.8	%
Lysine	1.0	%
Histidine	0.4	%
Arginine	1.2	%
Tryptophan	0.2	%

Trace Elements	Amount	Unit
Manganese	25.0	ppm
Copper	7.0	ppm
Cobalt	0.4	ppm
Iron	30.0	ppm
Iodine	1.3	ppm
Magnesium	102.0	ppm

Fat-soluble Vitamins

Vitamin	Amount	Unit
A	8000	iu
D3	1000	iu
E	60	iu
K	10	mg

Water-soluble Vitamins

B1	4	mg
B2	8	mg
B6	6	mg
B12	12	µg
Nicotinic Acid	50	mg
Pantothenic Acid	12	mg
Choline Chloride	200	mg
Folic acid	10	mg

TABLE 2.4 Diet CRM manufactured in 3/8" (9.6mm) pellet size. Amounts are measured as per kilogram of diet. Data supplied by Labsure, Lavender Mill, Manea Cambredgeshire.

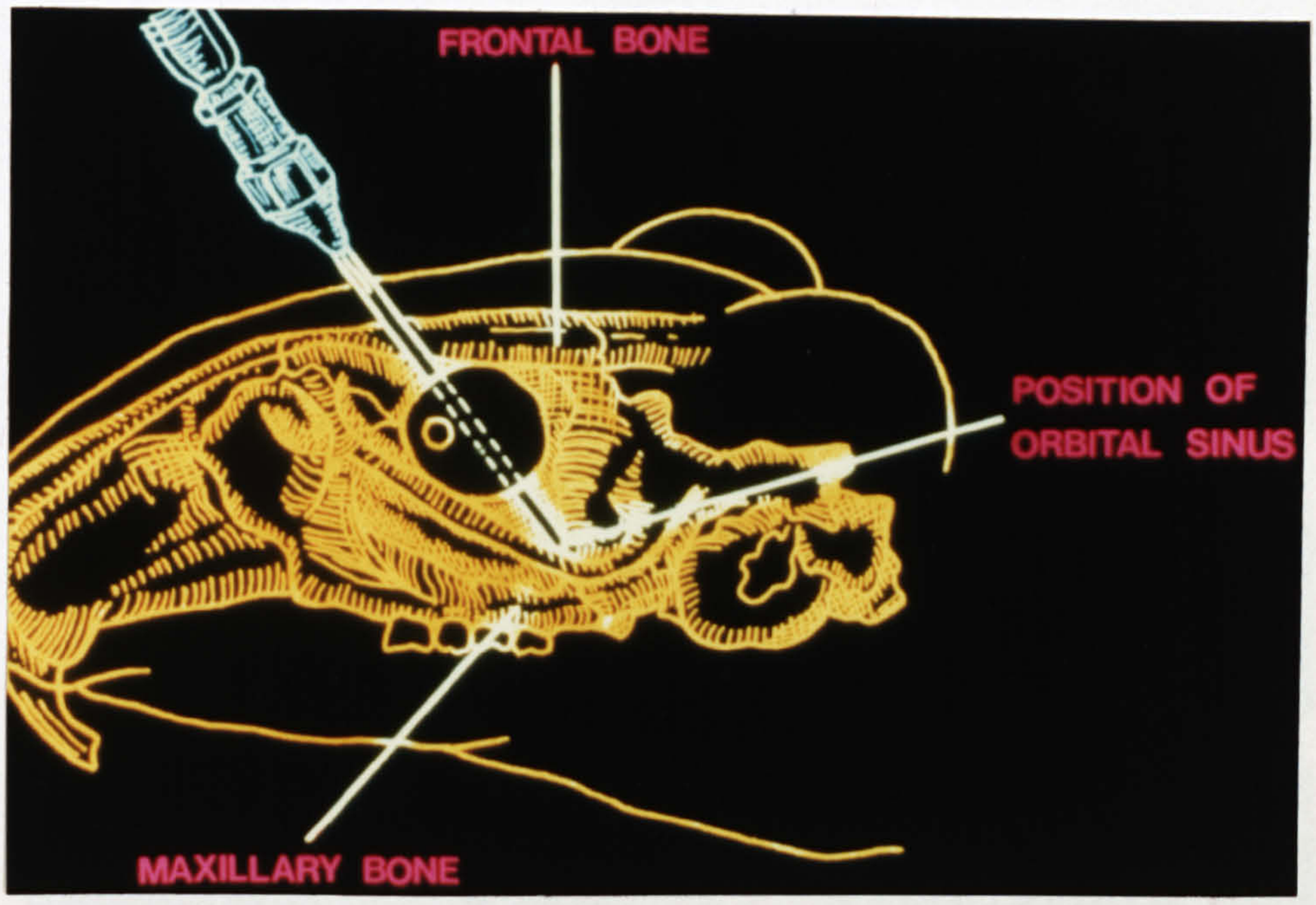

FIGURE 2.1 A diagram showing the method of obtaining blood samples from the retro-orbital venous sinous in the hamster (Pansky et al., 1961).

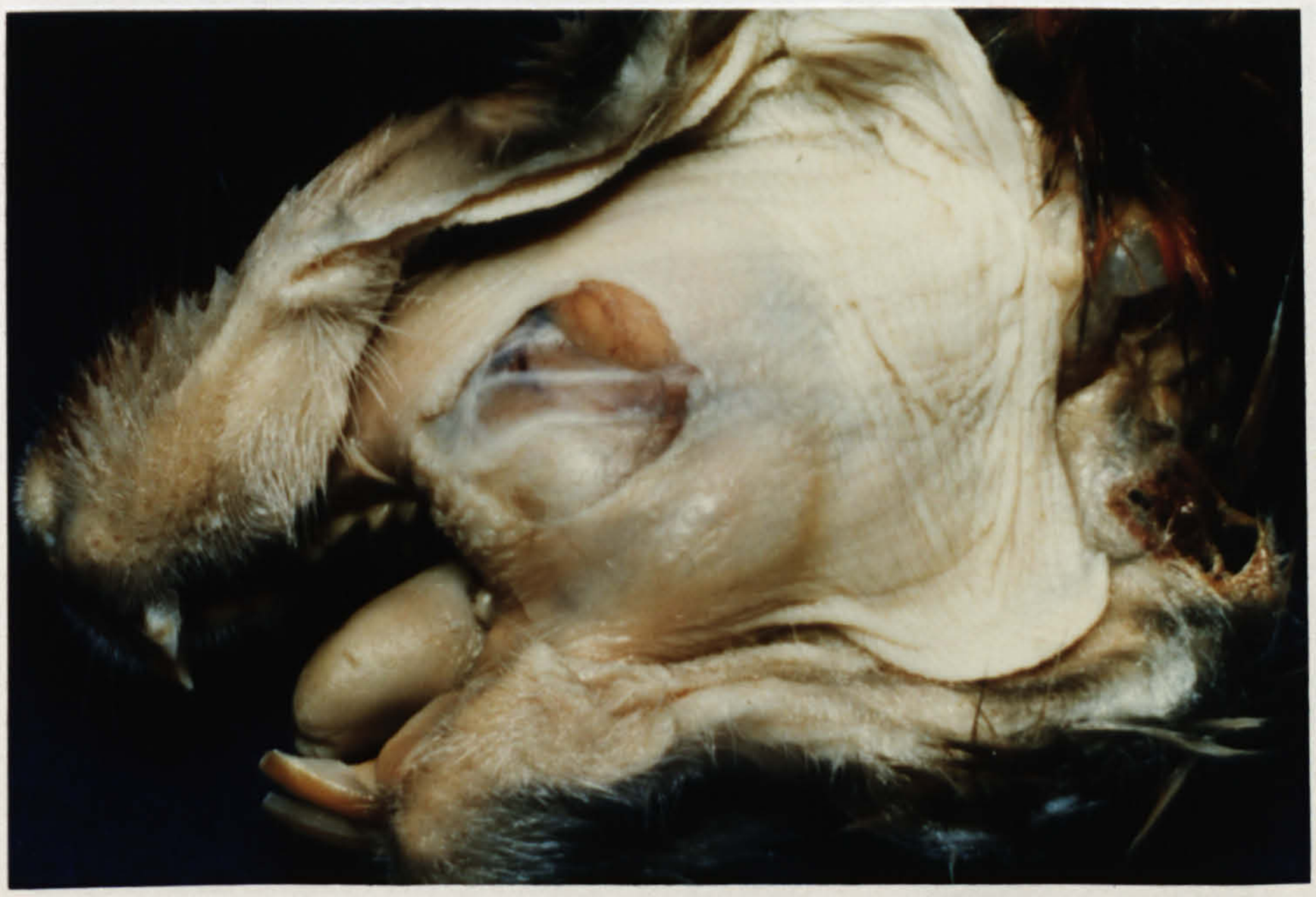

FIGURE 2.2 The medial wall of the hamster cheek pouch showing the surgical wound of the biopsy in a hamster which died later on the same day.

FIGURE 2.3 The biopsy site two days following surgery in a hamster died from wet tail.

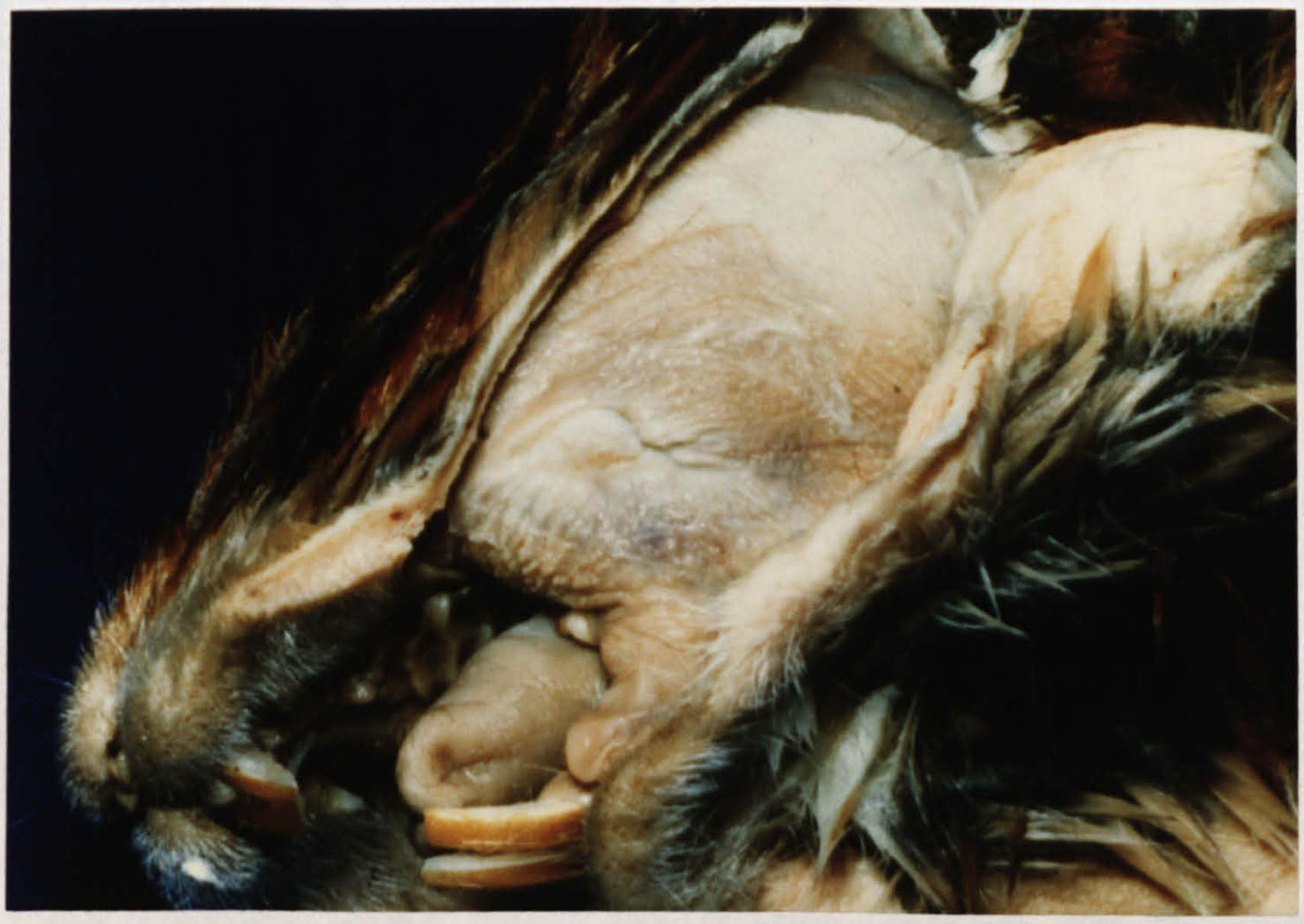

FIGURE 2.4 The biopsy site has almost completely healed four days after surgery in a hamster which failed to recover from the anaesthetic following examination of the pouches.

FIGURE 2.5 The features of the computerised planimetry system used for the compartment analysis of the hamster cheek pouch epithelium.

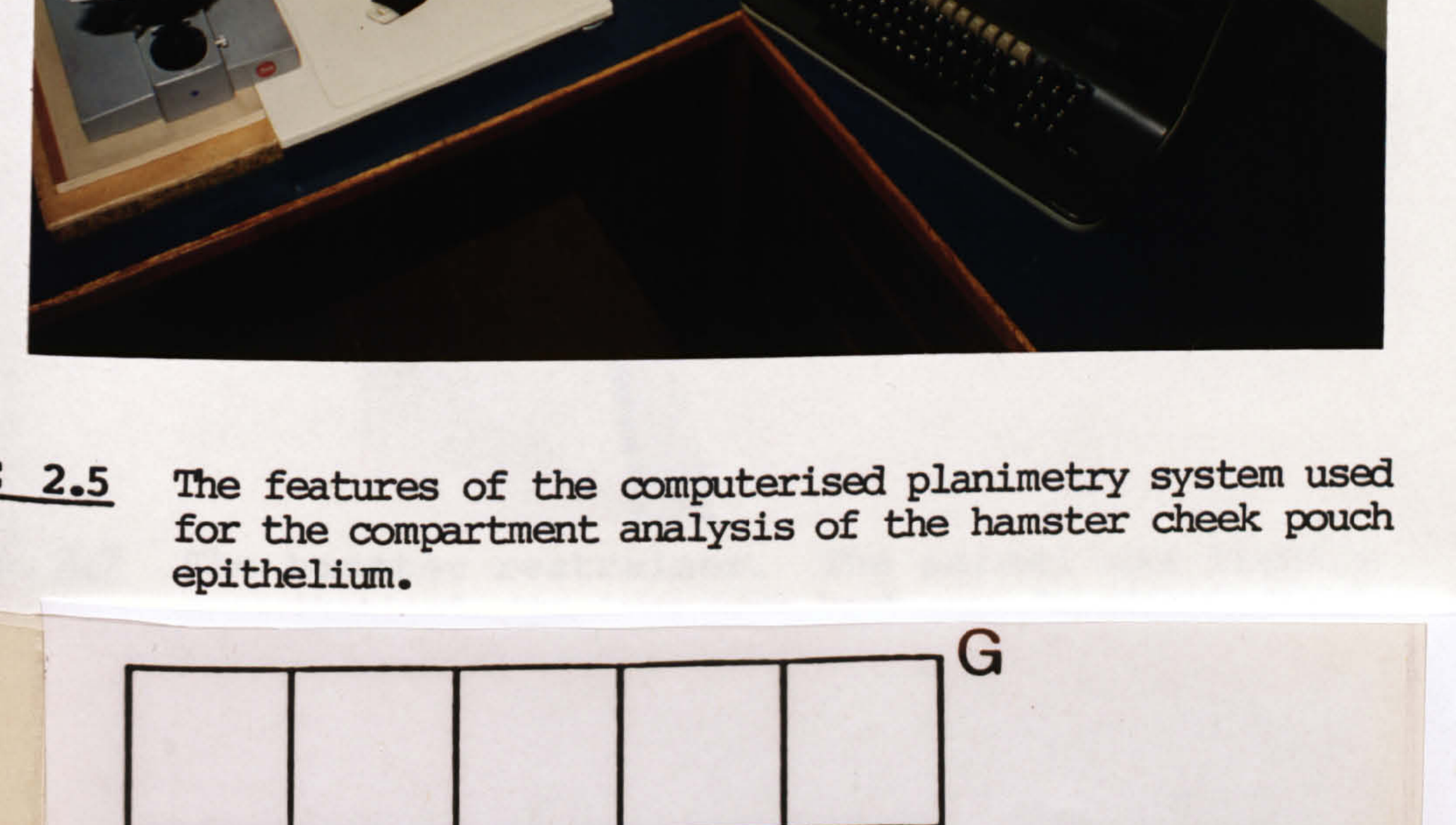

FIGURE 2.6 The hamster cheek pouch epithelium showing how an area of epithelium was limited by the parallel lines of the eye-piece graticule in the microscope. The epithelium was divided into the keratinizing compartment and cellular compartment. H&E x486.

FIGURE 2.5 The features of the computerised planimetry system used for the compartment analysis of the hamster cheek pouch epithelium.

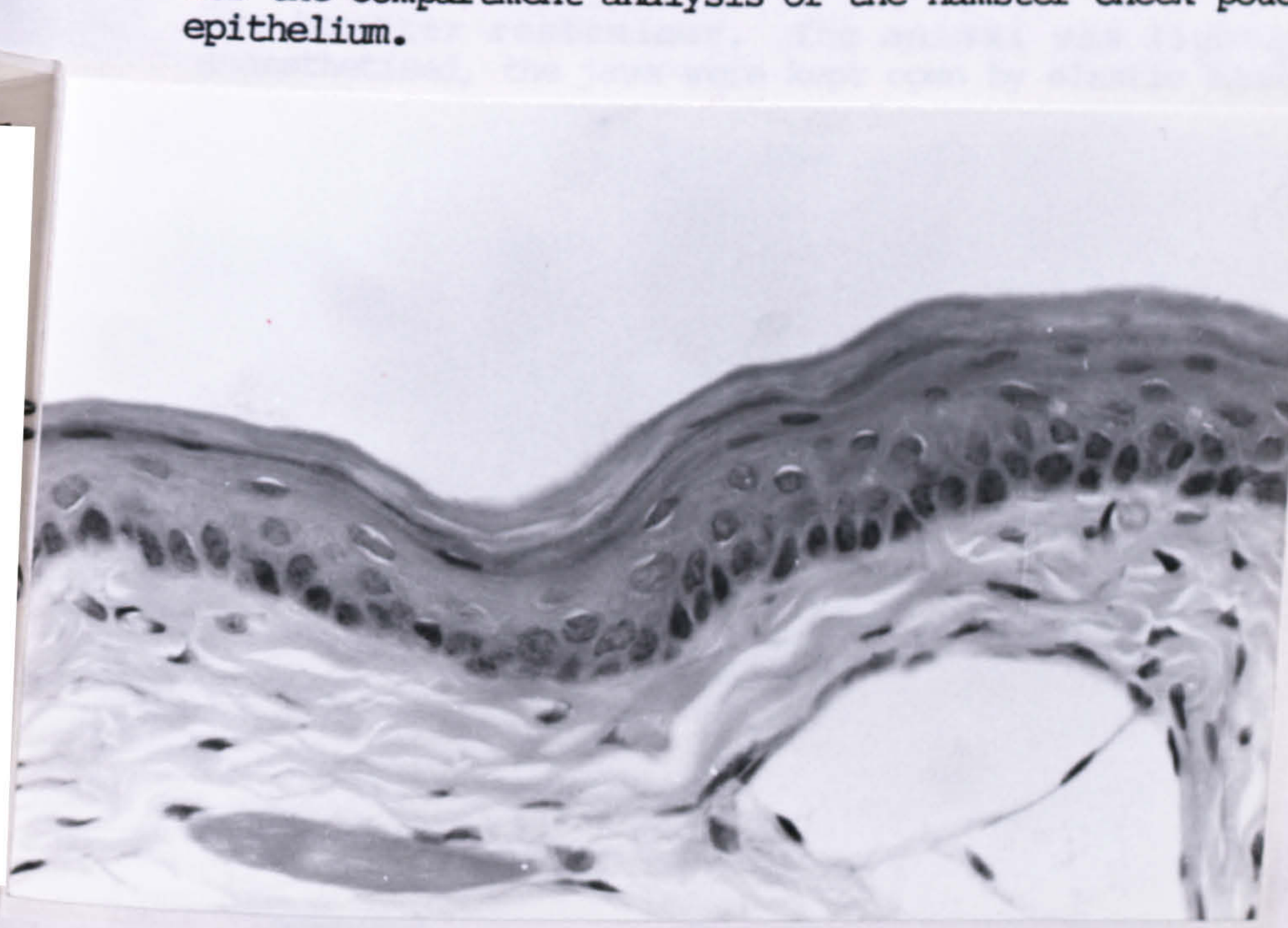

FIGURE 2.6 The hamster cheek pouch epithelium showing how an area of epithelium was limited by the parallel lines of the eye-piece graticule in the microscope. The epithelium was divided into the keratinizing compartment and cellular compartment. H&E x486.

FIGURE 2.5 The features of the computerised planimetry system used for the compartment analysis of the hamster cheek pouch epithelium.

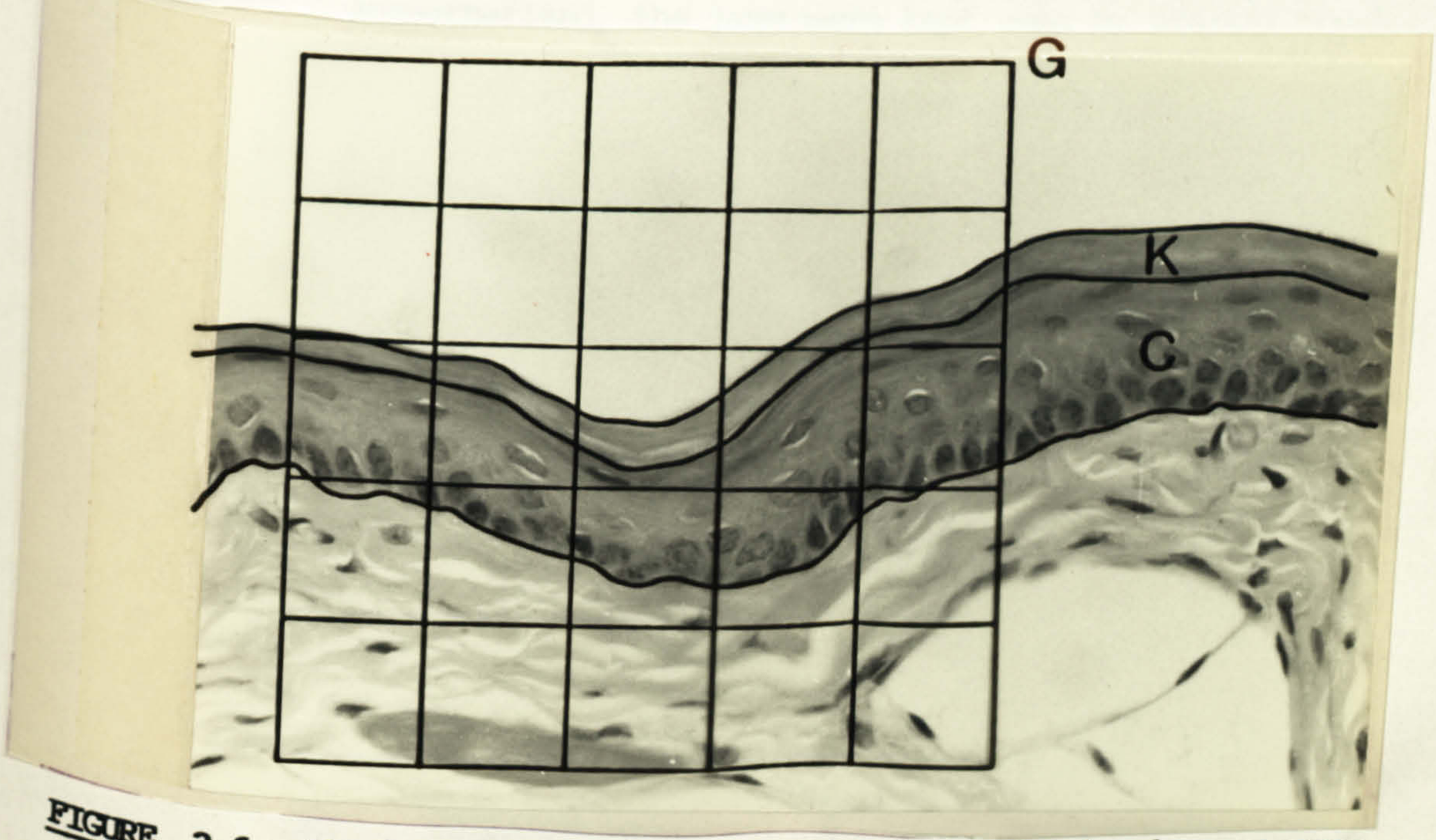

FIGURE 2.6 The hamster cheek pouch epithelium showing how an area of epithelium was limited by the parallel lines of the eye-piece graticule in the microscope. The epithelium was divided into the keratinizing compartment and cellular compartment. H&E x486.

FIGURE 2.7 The hamster restrainer. The animal was lightly anaesthetised, the jaws were kept open by elastic bands hooked over the upper and lower incisor teeth and the medial walls of the pouch were exposed by retracting the lateral walls.

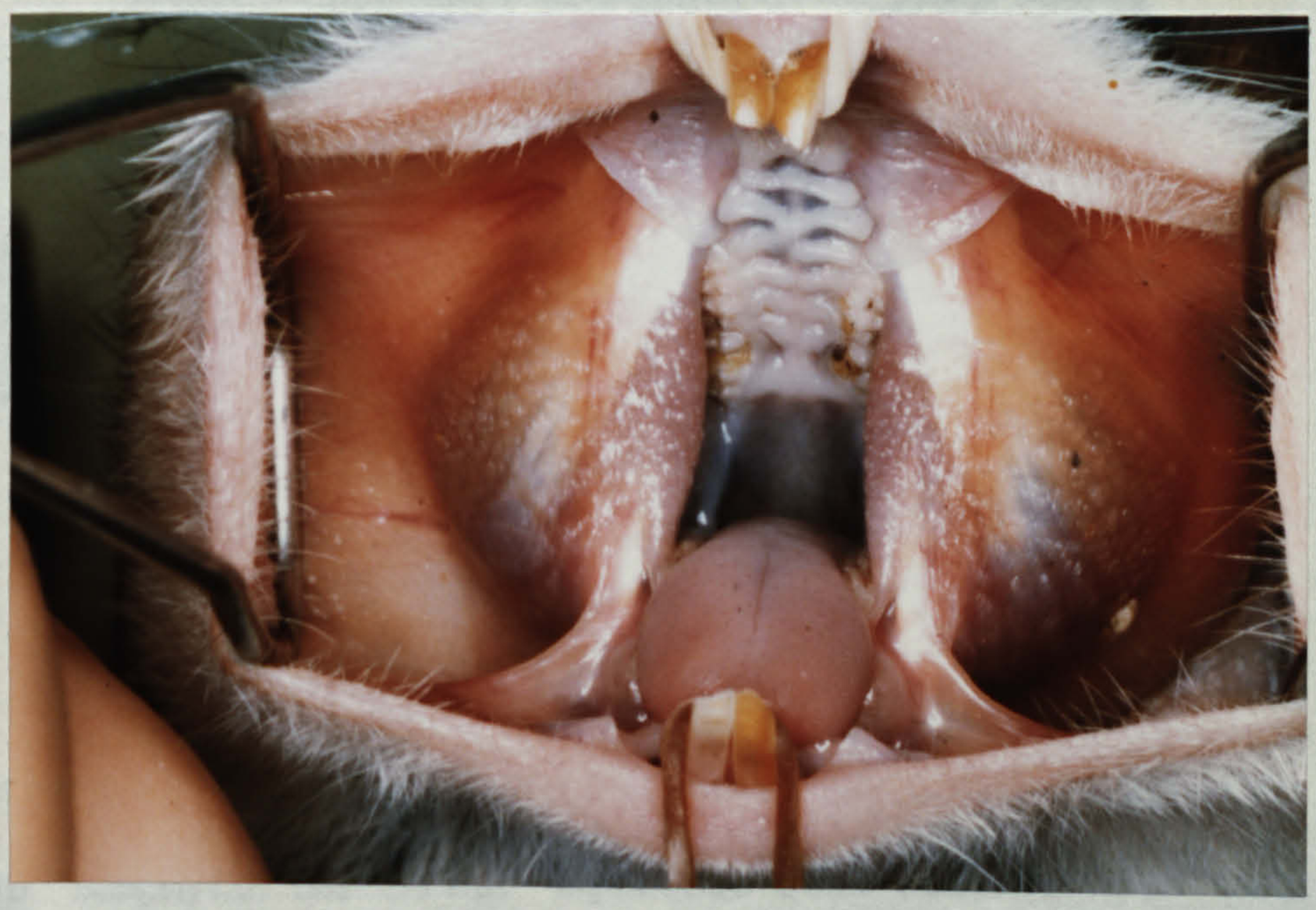

FIGURE 2.8 Well exposed medial walls of hamster cheek pouches after retracting the lateral walls.

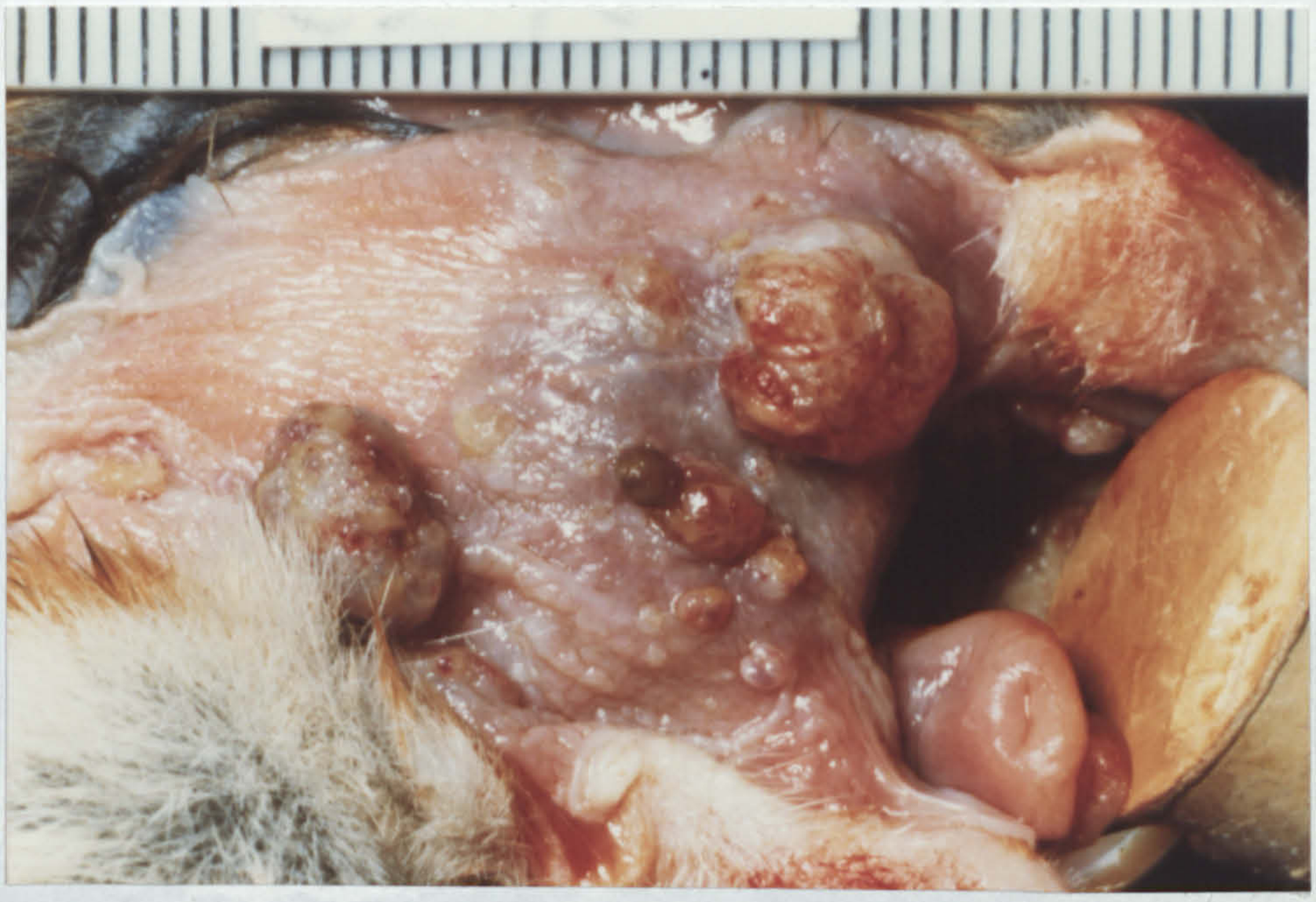

FIGURE 2.9 Tumours in the medial wall of the hamster cheek pouch. The tumours were counted and measured with reference to the scale included in the photograph.

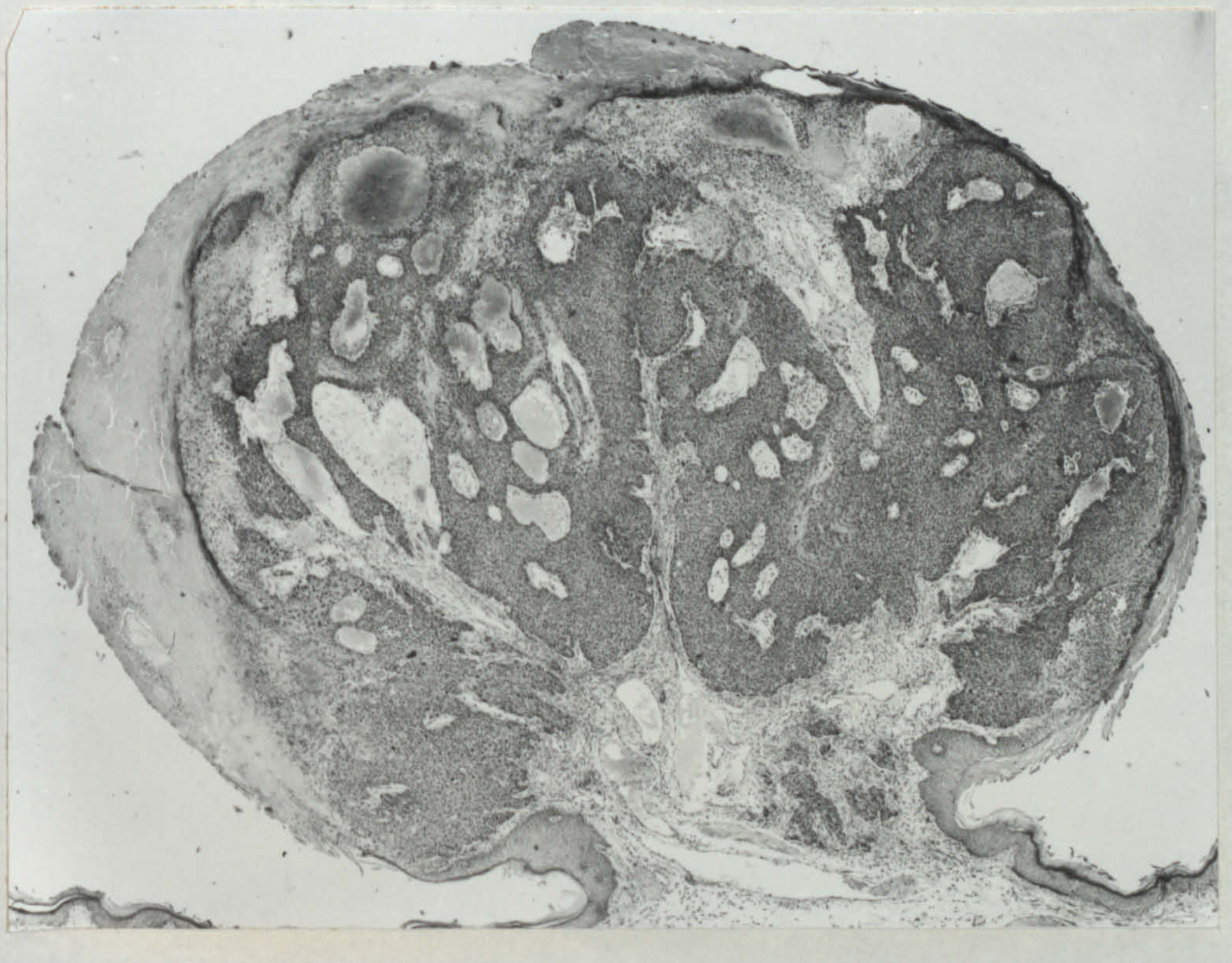

FIGURE 2.10 Exophytic carcinoma: Squamous cell carcinoma arising in, and confined to, a pre-existing squamous cell papilloma. H&E x25.

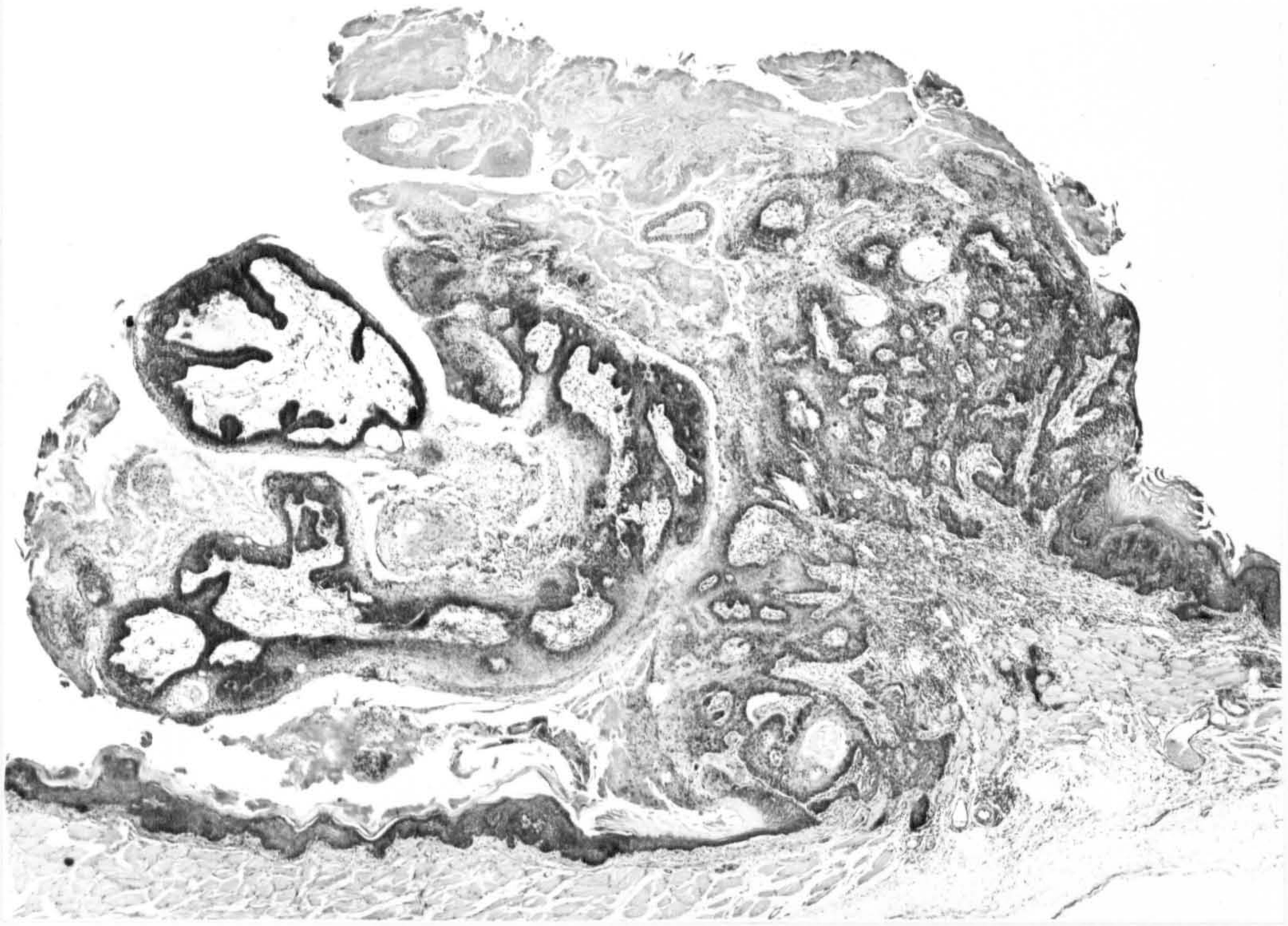

FIGURE 2.11 Endophytic carcinoma: Squamous cell carcinoma arising in a pre-existing papilloma and extending into the deeper tissue of the pouch. H&E x25.

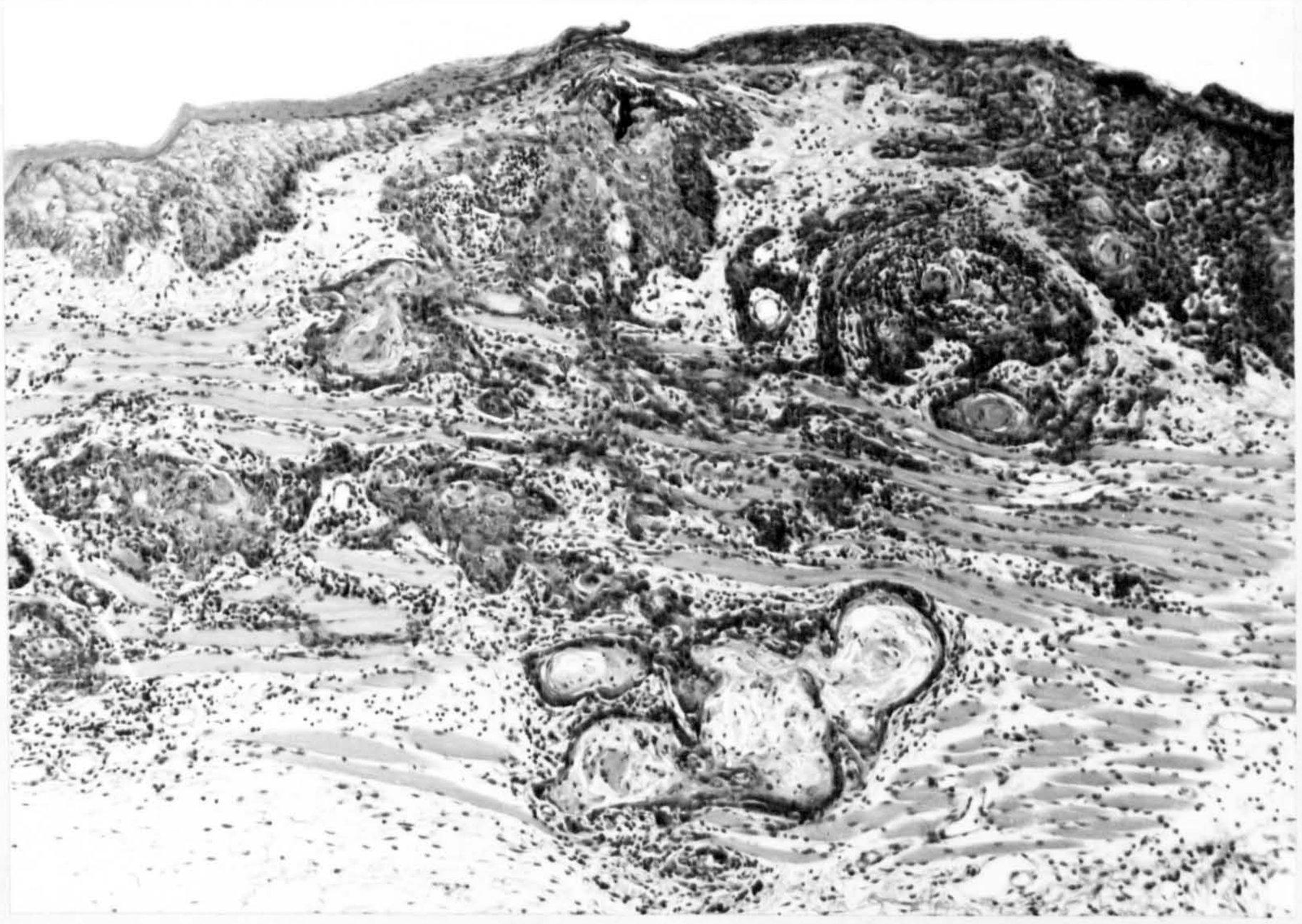

FIGURE 2.12 Endophytic carcinoma: Squamous cell carcinoma arising from the surface epithelium of the pouch without forming a papillomatous growth. H&E x76.

	Week 1	4	7	10	13	16	19	22	25	28	31	34	37
Group 1													
1	74	90	97	108	112	114	110	110	116	104	94	84	78
2	79	102	115	127	125	137	124	127	124	135	132	139	143
3	75	94	111	125	134	132	124	131	135	147	140	131	122
4	73	84	94	104	102	108	97	101	97	103	92	91	92
5	74	96	109	123	129	134	117	130	140	141	133	127	121
6	68	84	92	108	113	113	107	107	116	120	126	116	101
7	82	95	109	120	123	129	110	129	137	135	126	120	77
8	60	100	122	132	130	134	122	129	134	130	120	–	–
9	70	97	117	131	134	134	137	145	146	150	147	135	132
10	69	94	116	131	126	125	134	139	145	157	142	128	–
11	83	109	127	135	139	141	121	117	126	133	125	–	–
12	87	109	121	137	143	141	140	145	146	150	120	–	–
mean	74.5	96.2	110.8	123.4	125.8	128.5	120.2	127.4	130.2	133.7	124.7	117.9	108.2
S.D.	7.4	8.1	11.3	11.2	11.9	11.2	12.9	14.7	14.9	17.4	17.1	20.3	24.9
Group 2													
13	74	113	141	149	156	145	148	151	158	163	151	136	127
14	80	107	130	141	150	151	154	150	156	150	136	–	–
15	68	117	144	153	156	124	139	149	148	155	150	140	123
16	61	102	124	135	139	137	130	136	137	141	135	123	109
17	67	106	134	145	150	148	143	148	150	161	143	125	111
18	73	104	126	133	136	–	–	–	–	–	–	–	–
19	71	102	134	144	145	151	156	159	158	162	157	146	131
20	79	113	132	140	154	150	138	138	140	141	133	128	113
21	82	120	139	143	–	–	–	–	–	–	–	–	–
22	74	99	122	132	131	129	112	113	117	120	102	104	101
23	76	104	96	119	94	–	–	–	–	–	–	–	–
24	88	115	144	147	159	159	151	145	140	142	130	–	–
mean	74.4	108.5	130.5	140.1	142.7	143.8	141.2	143.2	144.9	148.3	137.4	128.8	116.4
S.D.	7.3	6.8	13.1	9.2	18.5	11.4	13.8	13.3	13.2	14.0	16.2	13.8	10.8

TABLE 2.5 Weights (g) of pellet diet (Group 1) and powdered diet (Group 2) control animals.

	Week 1	4	7	10	13	16	19	22	25	28	31	34	37
Group 3													
25	66	105	139	120	142	153	152	151	141	140	128	120	120
26	81	113	140	118	131	138	135	141	134	132	132	135	134
27	74	108	133	114	127	136	134	132	121	121	128	132	134
28	61	98	128	118	140	146	147	157	155	150	146	142	139
29	77	99	116	102	112	–	–	–	–	–	–	–	–
30	80	112	135	118	131	127	111	114	114	119	106	–	–
31	73	100	125	114	138	143	144	147	140	131	130	109	102
32	68	108	130	110	126	132	129	136	134	131	128	114	126
33	83	116	125	122	137	135	131	126	117	123	117	–	–
34	77	95	117	108	134	142	139	140	140	143	144	–	–
35	76	102	134	118	108	119	110	100	84	87	–	–	–
36	73	103	123	105	108	119	113	118	115	123	115	122	125
37	71	96	117	107	120	121	–	–	–	–	–	–	–
38	77	106	127	115	131	134	144	142	140	145	132	–	–
mean	**74.1**	**104.3**	**127.8**	**113.5**	**127.5**	**134.2**	**132.4**	**133.7**	**127.9**	**128.7**	**127.8**	**124.8**	**125.7**
S.D.	**6.1**	**6.5**	**7.9**	**6.1**	**11.4**	**10.6**	**14.4**	**16.6**	**18.8**	**16.6**	**11.8**	**11.9**	**12.3**
Group 4													
39	81	117	131	135	142	152	151	142	132	128	111	106	106
40	64	108	146	140	138	123	124	113	–	–	–	–	–
41	86	121	137	138	–	–	–	–	–	–	–	–	–
42	80	97	121	100	–	–	–	–	–	–	–	–	–
43	70	107	120	138	133	121	104	120	117	–	–	–	–
44	73	114	133	140	145	–	–	–	–	–	–	–	–
45	64	90	99	98	108	104	101	98	–	–	–	–	–
46	72	96	118	130	135	134	116	133	134	132	132	117	104
47	76	117	131	140	140	119	–	–	–	–	–	–	–
48	75	117	133	143	162	160	142	141	138	136	129	120	122
49	78	112	140	143	129	–	–	–	–	–	–	–	–
50	72	104	124	122	120	113	104	116	120	126	114	101	99
mean	**74.2**	**108.3**	**127.7**	**130.6**	**135.2**	**128.2**	**120.3**	**123.3**	**128.2**	**130.5**	**121.5**	**111.0**	**107.7**
S.D.	**6.6**	**9.9**	**12.4**	**15.9**	**14.5**	**19.2**	**19.8**	**16.2**	**9.2**	**4.4**	**10.5**	**9.0**	**9.9**

TABLE 2.6 Weights (g) of experimental animals maintained on iron deficient (Group 3) and iron sufficient (Group 4) powdered diet with repeated venesection.

	Week 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Group																			
1	74.5	79.7	88.1	96.2	100.5	105.7	110.8	115.3	118.8	123.4	122.6	124.4	125.8	125.8	128.2	128.5	130.8	123.0	120.2
2	74.4	84.6	98.0	108.5	115.7	122.9	130.5	134.5	135.1	140.1	139.7	137.2	142.7	145.3	143.1	143.8	147.4	143.4	141.2
3	74.1	82.6	93.6	104.3	111.3	119.6	127.8	135.7	133.1	113.5	104.2	118.1	127.5	129.8	132.8	134.2	139.1	137.0	132.4
4	74.2	83.2	97.0	108.3	116.0	122.3	127.7	131.2	128.2	130.6	134.5	132.6	135.2	127.7	128.0	128.2	126.7	117.6	120.3
1 v 2	NS	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.02	<.02	<.02	<.02	<.02
1 v 3	NS	NS	<.05	<.02	<.02	<.002	<.002	<.002	<.002	<.02	<.002	NS	NS	NS	NS	NS	NS	NS	NS
1 v 4	NS	NS	<.02	<.02	<.002	<.002	<.002	<.02	<.02	<.05	<.02	<.05	<.05	NS	NS	NS	NS	NS	NS
2 v 3	NS	NS	NS	NS	NS	NS	NS	NS	NS	<.002	<.002	<.002	<.002	<.02	NS	NS	NS	NS	NS
2 v 4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	<.002	NS
3 v 4	NS	NS	NS	NS	NS	NS	NS	NS	NS	<.002	<.002	<.002	NS	NS	NS	NS	NS	NS	NS

	Week 20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
Group																		
1	123.5	127.4	127.4	125.8	128.8	130.2	132.1	132.3	133.7	127.3	125.0	124.7	125.6	122.0	117.9	116.0	111.9	108.2
2	142.2	145.0	143.2	144.9	146.7	144.9	145.5	146.0	148.3	143.1	141.2	137.4	134.0	129.4	128.8	123.8	120.8	116.4
3	135.7	138.0	133.7	131.7	129.2	127.9	126.2	127.3	128.7	124.0	125.6	127.8	126.5	122.0	124.8	122.3	122.6	125.7
4	124.0	127.0	123.3	132.6	130.6	128.2	127.7	125.0	130.5	124.2	122.7	121.5	119.0	118.0	111.0	113.0	112.7	107.7
1 v 2	<.02	<.02	<.02	<.002	<.002	<.002	<.02	<.02	<.02	<.02	<.002	<.02	NS	NS	NS	NS	NS	NS
1 v 3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1 v 4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2 v 3	NS	NS	NS	NS	<.05	<.05	<.02	<.02	<.05	<.05	NS	NS	NS	NS	NS	NS	NS	NS
2 v 4	NS	<.05	<.002	<.009	<.02	<.05	<.05	<.05	<.05	<.05	NS	NS	<.036	NS	<.021	NS	NS	NS
3 v 4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

TABLE 2.7 Mean weekly weights (g) of control and experimental animals with the statistical relationships when Mann-Whitney U tests were applied to individual values.

Group 3	Week 7	8	9	10	11	12	13	14	15	16
25	139	147	143	120	114	125	142	146	148	153
26	140	143	137	118	112	121	131	135	137	138
27	133	140	132	114	110	121	127	126	131	136
28	128	139	148	118	115	128	140	142	142	146
29	116	126	121	102	94	111	112	-	-	-
30	135	140	133	118	109	125	131	134	134	127
31	125	137	133	114	109	125	138	139	141	143
32	130	135	128	110	108	116	126	129	132	132
33	125	131	139	122	115	128	137	137	137	135
34	117	129	127	108	94	122	134	136	140	142
35	134	144	143	118	94	96	108	108	116	119
36	123	126	122	105	85	100	108	101	113	119
37	117	125	124	107	98	115	120	123	125	121
38	127	138	133	115	102	120	131	131	131	134
mean	**127.8**	**135.7**	**133.1**	**113.5**	**104.2**	**118.1**	**127.5**	**129.8**	**132.8**	**134.2**
S.D.	**7.9**	**7.2**	**8.2**	**6.1**	**9.6**	**9.8**	**11.4**	**12.9**	**10.1**	**10.6**

TABLE 2.8 Weights (g) of animals in Group 3. Calcium carbonate was given instead of calcium lactate during weeks 9 and 10; the biopsy was taken during weeks 12 and 13 and carcinogen painting was started on week 16 of the study.

	Week 1	4	10	37
Group 1				
1	14.5	15.3	16.4	9.8
2	15.1	15.9	16.6	13.8
3	15.0	16.8	17.4	10.5
4	14.1	15.1	17.2	10.3
5	14.4	13.0	17.7	15.0
6	15.1	14.3	16.7	10.8
7	15.1	15.2	16.9	6.2
8	15.0	15.5	16.5	8.0
9	13.2	14.5	16.3	10.2
10	15.7	12.9	18.4	9.5
11	15.5	16.7	16.9	8.8
12	15.3	17.3	17.2	-
mean	14.8	15.2	17.0	10.3
S.D.	0.7	1.4	0.6	2.4
Group 2				
13	15.3	16.2	17.0	14.8
14	14.5	15.9	18.4	8.2
15	14.7	15.0	17.0	11.2
16	16.0	17.0	16.7	10.6
17	16.0	17.1	16.8	12.4
18	14.8	16.9	16.5	-
19	14.8	15.3	18.5	11.6
20	15.6	14.9	17.9	9.3
21	14.1	17.4	17.1	-
22	15.2	16.2	16.4	15.7
23	15.7	16.8	17.8	-
24	15.7	16.0	18.0	10.4
mean	15.2	16.2	17.4	11.6
S.D.	0.6	0.8	0.7	2.4

TABLE 2.9 Haemoglobin values (g/dl) of control Groups 1 and 2 hamsters with their means and standard deviations (S.D.).

	Week 1	2	3	4	5	6	7	8	9	10	13	17	21	25	29	37
Group 3																
25	15.0	15.8	15.5	14.5	15.6	16.5	15.7	15.1	17.1	15.5	14.3	17.0	15.6	14.6	12.3	12.0
26	13.9	15.0	15.0	15.9	15.5	15.1	14.8	15.1	13.1	16.0	15.2	15.5	16.1	14.0	12.5	11.4
27	13.8	14.3	15.4	14.5	14.7	16.3	15.2	15.2	15.1	14.8	14.6	17.0	13.5	14.7	14.0	14.4
28	13.2	14.0	14.2	15.1	15.0	16.8	14.6	15.9	15.8	15.6	15.5	16.5	15.1	14.2	13.8	14.4
29	14.7	14.1	13.3	14.0	13.5	15.5	12.6	14.8	17.0	15.9	-	-	-	-	-	-
30	14.6	14.6	14.6	14.1	14.6	16.5	14.5	14.5	15.9	15.4	15.3	16.5	16.5	13.8	10.1	7.2
31	14.0	14.8	13.3	15.0	15.5	19.5	16.0	16.4	17.1	15.7	15.1	16.0	16.3	10.5	10.9	14.5
32	13.6	14.1	13.1	15.3	14.6	16.0	13.8	15.1	16.9	15.8	14.5	16.0	15.5	14.5	14.2	15.7
33	15.0	13.8	13.3	14.3	14.1	15.8	13.8	16.1	16.4	16.1	17.0	16.0	16.2	14.8	9.2	6.3
34	15.3	16.3	14.3	15.5	15.9	15.6	14.3	15.4	17.5	16.8	15.6	17.5	17.1	16.2	10.6	12.4
35	15.0	17.0	13.1	14.5	15.3	15.9	15.0	15.3	16.4	19.1	15.5	17.0	14.2	13.8	8.8	-
36	15.0	15.4	15.2	15.8	15.5	14.5	14.4	16.9	17.1	16.4	15.3	16.5	15.7	13.1	12.2	17.0
37	13.8	13.6	13.4	13.5	14.2	14.5	14.6	16.7	17.3	15.7	15.2	16.5	-	-	-	-
38	13.2	14.0	13.5	14.3	14.1	14.5	15.1	15.5	16.9	16.8	14.0	16.5	14.8	13.1	11.3	10.8
mean	14.3	14.8	14.1	14.7	14.9	15.9	14.6	15.6	16.4	16.1	15.2	16.5	15.5	13.9	11.7	12.4
S.D.	0.7	1.0	0.9	0.7	0.7	1.3	0.8	0.7	1.2	1.0	0.7	0.5	1.0	1.4	1.8	3.3
Group 4																
39	15.2	14.8	13.8	15.9	15.5	17.0	16.0	15.7	16.5	16.9	15.7	14.0	14.6	13.5	12.8	10.4
40	13.7	15.6	14.0	14.5	15.7	16.9	15.2	16.3	15.4	16.6	14.1	13.5	14.1	-	-	-
41	15.4	14.8	13.5	15.2	14.4	16.0	14.6	15.0	14.9	15.7	-	-	-	-	-	-
42	14.1	13.6	14.0	14.6	14.9	14.8	14.0	15.3	15.8	13.8	-	-	-	-	-	-
43	14.7	13.9	13.6	14.2	14.4	15.0	14.5	16.2	18.0	16.9	17.1	14.5	12.0	-	-	-
44	15.0	15.3	15.0	16.1	15.6	16.2	14.4	15.7	17.5	16.1	-	-	-	-	-	-
45	13.3	14.5	14.5	15.9	16.0	15.1	14.2	16.0	16.6	15.5	14.9	15.0	14.5	-	-	-
46	13.3	13.8	14.4	16.3	15.4	16.2	15.0	15.2	16.1	16.6	15.0	15.5	14.6	12.1	12.0	12.3
47	14.9	16.2	15.5	15.8	16.1	17.2	16.2	16.7	16.3	18.6	15.7	13.0	-	-	-	-
48	14.6	14.9	13.9	16.0	15.2	17.0	14.1	17.7	15.5	15.7	16.5	16.0	15.1	14.1	10.3	7.4
49	14.0	14.7	14.1	15.4	14.5	16.0	14.9	16.8	16.0	16.5	-	-	-	-	-	-
50	15.1	14.0	14.7	16.2	15.5	16.1	13.3	16.2	16.3	16.5	15.7	15.0	13.9	13.8	13.4	14.1
mean	14.4	14.7	14.2	15.5	15.3	16.1	14.7	16.1	16.2	16.3	15.6	14.6	14.1	13.4	12.1	11.0
S.D.	0.7	0.8	0.6	0.7	0.6	0.8	0.8	0.8	0.9	1.1	0.9	1.0	1.0	0.9	1.3	2.9

TABLE 2.10 Haemoglobin values (g/dl) of experimental Groups 3 and 4 animals.

Group	Week 1	4	10	37
1	14.8(13.2-15.7)	15.2(12.9-17.3)	17.0(16.3-18.4)	10.3(6.2-13.8)
2	15.2(14.1-16.0)	16.2(14.9-17.4)	17.4(16.4-18.5)	11.6(8.2-15.7)
3	14.3(13.2-15.3)	14.7(13.5-15.9)	16.1(14.8-19.1)	12.4(6.3-17.0)
4	14.4(13.3-15.4)	15.5(14.2-16.3)	16.3(13.8-18.6)	11.0(7.4-14.1)

Mann-Whitney U Test

Group	Week 1	2	3	4
1 v 2	NS	NS	NS	NS
1 v 3	<.05	NS	<.02	NS
1 v 4	NS	NS	<.05	NS
2 v 3	<.02	<.002	<.002	NS
2 v 4	<.05	<.05	<.02	NS
3 v 4	NS	<.02	NS	NS
1 v 3+4	<.04	NS	<.002	NS
2 v 3+4	<.004	<.001	<.001	NS
1+2 v 3+4	<.002	<.02	<.001	NS

TABLE 2.11 Means and ranges of haemoglobin values of control (Groups 1 and 2) and experimental (Groups 3 and 4) animals with the statistical relationships when Mann-Whitney U tests were applied to individual values.

CORRECTED WHOLE BLOOD FOLATE

Group 1	Week 1	Week 10	Week 37	Group 2	Week 1	Week 10	Week 37
1	501	285	600	13	371	231	167
2	546	264	318	14	320	174	600
3	595	383	600	15	402	206	600
4	590	222	393	16	349	266	600
5	383	276	181	17	361	326	420
6	350	236	600	18	297	420	-
7	501	244	600	19	406	202	600
8	654	324	600	20	349	287	600
9	379	219	600	21	359	214	-
10	680	305	600	22	375	265	317
11	390	258	600	23	304	210	-
12	467	222	-	24	433	224	600
mean	**503.0**	**269.8**	**517.5**	**mean**	**360.5**	**252.1**	**500.4**
S.D.	**112.5**	**49.0**	**149.3**	**S.D.**	**41.0**	**67.7**	**162.3**

Group 3				Group 4			
25	682	223	402	39	290	289	470
26	368	178	351	40	683	510	-
27	682	223	402	41	333	579	-
28	368	178	351	42	339	267	-
29	481	263	439	43	491	420	-
30	481	182	220	44	425	555	-
31	476	319	-	45	425	760	-
32	387	227	600	46	405	894	542
33	520	366	275	47	407	783	-
34	545	183	120	48	365	891	600
35	317	160	600	49	485	813	-
36	498	238	600	50	346	766	154
37	484	180	-				
38	372	189	171	**mean**	**416.2**	**627.2**	**441.5**
				S.D.	**103.7**	**222.5**	**198.9**
mean	**475.8**	**222.1**	**377.6**				
S.D.	**110.7**	**59.4**	**164.9**				

TABLE 2.12 Corrected whole blood folate results (ng/ml) with their means and standard deviations (S.D.) in control (Groups 1 and 2) and experimental (Groups 3 and 4) animals.

Group 1	Weight (g)	THICKNESS (μm) Keratin	Cells	Total	THICKNESS (%) Keratin	Cells
1	110	5.80	21.93	27.73	20.92	79.08
2	123	5.66	22.03	27.69	20.44	79.56
3	132	7.51	25.51	33.02	22.74	77.26
4	102	4.93	15.36	20.29	24.30	75.70
5	127	5.94	24.30	30.24	19.64	80.36
6	110	5.78	21.28	27.06	21.36	78.64
7	120	5.94	19.54	25.48	23.31	76.69
8	132	5.67	18.73	24.40	23.24	76.76
9	131	5.33	23.55	28.88	18.46	81.54
10	128	5.39	29.00	34.39	15.67	84.33
11	138	4.37	26.79	31.16	14.02	85.98
12	140	7.67	32.01	39.68	19.33	80.67
mean	124.40	5.83	23.33	29.17	20.29	79.71
S.D.	11.85	0.94	4.59	5.06	3.12	3.12
Group 2						
13	151	7.27	24.74	32.01	22.71	77.29
14	144	6.52	25.39	31.91	20.43	79.57
15	151	5.65	22.22	27.87	20.27	79.73
16	132	6.27	28.73	35.00	17.91	82.09
17	147	6.02	32.33	38.35	15.70	84.30
18	134	8.41	24.60	33.01	25.48	74.52
19	147	6.24	23.13	29.37	21.25	78.75
20	149	8.26	32.80	41.06	20.12	79.88
22	133	5.03	24.46	29.49	17.06	82.94
23	95	7.99	21.49	29.48	27.10	72.90
24	150	5.56	24.55	30.11	18.47	81.53
mean	139.40	6.66	25.86	32.51	20.59	79.41
S.D.	16.45	1.16	3.80	4.12	3.46	3.46

TABLE 2.13 Compartment analysis of cheek pouch epithelium in Groups 1 and 2 control hamsters during weeks 12 and 13 of the experiment.

		THICKNESS (μm)			THICKNESS (%)	
	Weight (g)	Keratin	Cells	Total	Keratin	Cells
Group 3						
25	125	5.16	25.45	30.61	16.86	83.14
26	121	6.35	23.78	30.13	21.07	78.93
27	121	4.67	19.68	24.35	19.18	80.82
28	128	6.13	24.72	30.85	19.87	80.13
29	111	5.35	20.65	26.00	20.77	79.23
30	125	6.09	23.00	29.09	20.93	79.07
31	125	5.47	25.58	31.05	17.62	82.38
32	116	4.88	19.73	24.61	19.83	80.17
33	128	6.52	22.98	29.50	22.10	77.90
34	122	6.87	21.96	28.83	23.83	76.17
35	96	6.07	18.92	24.99	24.29	75.71
36	100	6.54	23.82	30.36	21.54	78.46
37	115	5.21	23.96	29.17	17.86	82.14
38	120	5.12	22.84	27.96	18.31	81.69
mean	**118.10**	**5.74**	**22.65**	**28.39**	**20.29**	**79.71**
S.D.	**9.83**	**0.70**	**2.17**	**2.41**	**2.24**	**2.23**
Group 4						
39	138	7.30	26.39	33.69	21.67	78.33
40	133	6.90	20.74	27.64	24.96	75.04
43	130	8.27	28.70	36.97	22.37	77.63
44	139	10.26	34.11	44.37	23.12	76.88
45	104	7.89	27.02	34.91	22.60	77.40
46	130	6.82	26.66	33.48	20.37	79.63
47	143	9.29	30.69	39.98	23.24	76.76
48	153	6.65	24.85	31.50	21.11	78.89
49	142	4.43	28.33	32.76	13.52	86.48
50	114	8.61	30.96	39.57	21.76	78.24
mean	**132.60**	**7.64**	**27.84**	**35.49**	**21.47**	**78.53**
S.D.	**14.40**	**1.62**	**3.68**	**4.84**	**3.07**	**3.07**

TABLE 2.14 Compartment analysis of cheek pouch epithelium of experimental Groups 3 and 4 animals during weeks 12 and 13 of the study.

Group	Weight	THICKNESS (μm) Keratin	Cells	Total	THICKNESS (%) Keratin	Cells
1	124.40	5.83	23.33	29.17	20.29	79.71
2	139.40	6.66	25.86	32.51	20.59	79.41
3	118.10	5.74	22.65	28.39	20.29	79.71
4	132.50	7.64	27.84	35.49	21.47	78.53
Group						
1 v 2	<.02	NS	NS	NS	NS	NS
1 V 3	NS	NS	NS	NS	NS	NS
1 V 4	NS	<.02	<.05	<.02	NS	NS
2 V 3	<.002	<.05	<.05	<.02	NS	NS
2 V 4	NS	NS	NS	NS	NS	NS
3 V 4	<.02	<.002	<.002	<.002	NS	NS

TABLE 2.15 Mean values of compartment analysis of the cheek pouch epithelium with the statistical relationships when Mann-Whitney U tests were applied to individual values.

Group	Weight	Total	Correlation Coefficient	Significance
1	124.4	29.17	0.660	<.02
2	139.4	32.51	-0.055	NS
3	118.1	28.39	0.522	NS
4	132.5	35.49	-0.176	NS
All Groups	127.7	31.06	0.442	<.01

Group	Weight	Keratin	Correlation Coefficient	Significance
1	124.4	5.83	0.148	NS
2	139.4	6.66	-0.192	NS
3	118.1	5.74	0.201	NS
4	132.5	7.64	-0.201	NS
All Groups	127.7	6.38	0.138	NS

Group	Weight	Cells	Correlation Coefficient	Significance
1	124.4	23.34	0.688	<.02
2	139.4	25.86	0.100	NS
3	118.1	22.65	0.485	NS
4	132.5	27.84	-0.097	NS
All Groups	127.7	24.68	0.489	<.001

TABLE 2.16 The correlation of mean epithelial thickness and compartment thickness values with animal weights. Pearson correlation coefficient tests were used when groups were considered together while Spearman rank correlation coefficient tests were used when individual groups were considered separately.

		NUMBER OF TUMOURS GROSSLY			TUMOUR SIZE (mm)		
	Animal No.	L Pouch	R Pouch	Total	1-2	2-5	5 or more
GROUP 1	1	3	3	6	2	2	2
	2	2	3	5	3	2	
	3	2	3	5	1	1	3
	4	2	-	2		1	1
	5	1	1	2	1		1
	6	1	2	3	1	1	1
	7	4	-	4		2	2
	8	4	1	5	3	1	1
	9	3	5	8	3	2	3
	10	2	1	3	1	1	1
	11	5	4	9	3	4	2
	12	-	-	-			
Total	12	29	23	52	18	17	17
GROUP 2	13	2	-	2	1		1
	14	3	1	4		1	3
	15	1	3	4		1	3
	16	4	3	7	3	2	2
	17	4	2	6	2	1	3
	19	2	2	4		3	1
	20	2	2	4	2		2
	22	-	-	-			
	24	2	3	5	1	3	1
Total	9	20	16	36	9	11	16
GROUP 3	25	1	-	1			1
	26	1	1	2		1	1
	27	-	-	-			
	28	-	1	1	1		
	30	1	1	2		2	
	31	1	2	3	1	1	1
	32	-	1	1	1		
	33	2	3	5	1	2	2
	34	4	-	4		1	3
	35	-	-	-			
	36	1	-	1	1		
	38	1	3	4	3	1	
Total	12	12	12	24	8	8	8
GROUP 4	39	1	4	5	2	2	1
	46	1	1	2		2	
	48	3	2	5		3	2
	50	1	1	2		2	
Total	4	6	8	14	2	9	3

TABLE 2.17 Grossly counted tumours and their size categories in Groups 1, 2, 3 and 4 animals.

	Animal No.	NUMBER OF CARCINOMAS					
		L Pouch		R Pouch		Total	
		Exo.	Endo.	Exo.	Endo.	Exo.	Endo.
GROUP 1	1	-	-	-	2	-	2
	2	-	2	-	-	-	2
	3	1	1	2	-	3	1
	4	1	1	-	-	1	1
	5	1	-	-	-	1	-
	6	-	1	-	-	-	1
	7	1	2	-	-	1	2
	8	2	1	-	1	2	2
	9	3	2	1	2	4	4
	10	-	-	1	1	1	1
	11	2	2	1	1	3	3
	12	-	-	-	-	-	-
Total	12	11	12	5	7	16	19
GROUP 2	13	1	-	-	-	1	-
	14	-	2	1	-	1	2
	15	1	-	2	1	3	1
	16	1	1	2	-	3	1
	17	2	1	-	-	2	1
	19	-	-	1	1	1	1
	20	-	-	1	-	1	-
	22	-	-	-	-	-	-
	24	1	-	1	-	2	-
Total	9	6	4	8	2	14	6
GROUP 3	25	1	1	-	1	1	2
	26	-	-	-	2	-	2
	27	-	-	-	-	-	-
	28	-	-	-	1	-	1
	30	1	-	-	-	1	-
	31	1	1	-	1	1	2
	32	-	-	-	-	-	-
	33	1	-	-	-	1	-
	34	2	-	-	-	2	-
	35	-	-	-	-	-	-
	36	-	-	-	-	-	-
	38	1	-	-	1	1	1
Total	12	7	2	0	6	7	8
GROUP 4	39	-	2	1	-	1	2
	46	1	-	-	1	1	1
	48	1	1	-	2	1	3
	50	-	-	1	1	1	1
Total	4	2	3	2	4	4	7

TABLE 2.18 Histologically identified squamous cell carcinomas and types of invasion in Groups 1, 2, 3 and 4 animals.

Chi-Square 2x2 Tests applied for :

Comparison of the number of animals with grossly seen tumours.

Group	1 v 2	NS
Group	1 v 3	NS
Group	1 v 4	NS
Group	2 v 3	NS
Group	2 v 4	NS
Group	3 v 4	NS

Comparison of the number of pouches with grossly seen tumours.

Group	1 v 2	NS
Group	1 v 3	NS
Group	1 v 4	NS
Group	2 v 3	NS
Group	2 v 4	NS
Group	3 v 4	NS

Comparison of the number of grossly counted tumours.

Group	1 v 2	NS
Group	1 v 3	P<.01
Group	1 v 4	NS
Group	2 v 3	P<.01
Group	2 v 4	NS
Group	3 v 4	NS

Comparison of the number of animals with carcinomas.

Group	1 v 2	NS
Group	1 v 3	NS
Group	1 v 4	NS
Group	2 v 3	NS
Group	2 v 4	NS
Group	3 v 4	NS

Comparison of the number of pouches with carcinomas.

Group	1 v 2	NS
Group	1 v 3	NS
Group	1 v 4	NS
Group	2 v 3	NS
Group	2 v 4	NS
Group	3 v 4	NS

Comparison of the number of carcinoma identified histologically.

Group	1 v 2	NS
Group	1 v 3	P<.05
Group	1 v 4	NS
Group	2 v 3	NS
Group	2 v 4	NS
Group	3 v 4	P<.05

TABLE 2.19 Comparisons of the number of animals and pouches with tumours or carcinomas and also of the numbers of tumours and carcinomas in Groups 1, 2, 3 and 4.

CHAPTER III

IRON CONTAMINATION OF THE HAMSTER DIET

3.1 INTRODUCTION

Failure to induce iron deficiency in the hamster in the previous experiment (discussed in Chapter 2) led to consideration of possible alternative ways to reduce haemoglobin levels in the hamster blood. Achieving this reduction was thought to be possible either by increasing the amount of blood removed on repeated venesection or by further reduction in the iron content present as impurities of the different components of diet.

Rennie et al. (1982b) had found that by bleeding the hamster 1ml three times every two weeks it was possible to induce iron deficiency anaemia in spite of a high level of iron contamination in the diet. On the other hand, Ranasinghe et al. (1983) were also successful in inducing severe iron deficiency in the hamster, in a relatively short time, by feeding hamsters a diet containing a very low level of iron and bleeding them 0.5ml of blood every week.

It was thought more practical to induce iron deficiency in the hamster by further reduction in the amount of iron impurities of diet rather than by increasing blood withdrawal. This study was

designed to investigate alternative sources of dietary components that contained less iron impurities than the diet used in previous studies but, at the same time, were acceptable to the hamster.

3.2 DIETARY SOURCES AND IRON IMPURITIES IN HAMSTER DIET

The powder diet described in Section 2.2.2 and shown in Tables 2.1, 2.2 and 2.3 of Chapter 2 was found to have 9.6mg iron per kilogram of diet (this figure was derived from analysis of the diet used in the study reported in Section 2.2.2). This amount of iron was found to come from two major sources, the minerals and bulk nutrients. The iron content of minerals in the diet, calculated from the manufacturers assessments of contaminents were found to be 4.53mg/kg of diet (Table 3.1). This iron was mainly (94.4 per cent) present in calcium lactate which was used as the dietary source of calcium for the hamster. The rest of the iron impurities (3.97-7.37mg/kg of diet) were present in the bulk nutrients which are the casein, starch, sucrose, corn oil and cellulose. It was not possible to find other suitable sources for the bulk nutrients that contained less iron impurities than those used before. Accordingly, it was decided to find a substitute for calcium lactate to fulfil the hamster requirement for calcium in the diet.

Among the list of calcium compounds available for laboratory use (Windholz, 1986), only four were thought suitable for animal

consumption and had low iron impurities. These compounds were calcium acetate, calcium carbonate, calcium chloride and calcium sulphate. Calcium carbonate, however, had been unsuccessfully tried in the previous experiment (2.2.2 and 2.3.2) and was therefore excluded from this study.

3.2.1 Calcium Lactate

The calcium lactate used in hamster diet of this study was Ca(CH3.CHOH.COO)2.4H2O, produced by BDH Chemicals Ltd., Poole, England. This had a molecular weight of 218.22 and contained 0.01 per cent iron impurities. In order to fulfil the hamster requirement for calcium recommended by the National Academy of Sciences (1978) which is 0.59 per cent of the total hamster diet, calcium lactate should be used in an amount that it would contribute 4.28 mg of iron per kilogram of diet (Table 3.2).

Calcium lactate is prepared commercially by neutralization of lactic acid, from fermentation of dextrose, molasses, starch, sugar or whey with calcium carbonate. The pentahydrate form of calcium lactate is almost odourless, slightly efflorescent granules or powder. It is slowly soluble in cold water, quickly soluble in hot water and almost insoluble in alcohol. Calcium lactate is used in the food and beverage industries and dentrifrices. Therapeutically, calcium lactate is used for calcium replenishment and may be used for hypocalcaemic states in animals (Windholz, 1986).

3.2.2 Calcium Acetate

The compound used in this study was "dried calcium acetate" Ca(CH3COO)2, produced by BDH Chemicals Ltd., Poole, England. It had a molecular weight of 158.17 and contained 0.002 per cent iron which would contribute 0.47mg of iron per kilogram of diet when calcium was required at 0.59 per cent as shown in Table 3.2. Calcium acetate is supplied as very hygroscopic rod shaped crystals.

Calcium acetate is soluble in water, slightly soluble in methanol and practically insoluble in ethanol. It is used in the manufacturing of acetic acid and acetone, in dyeing, tanning and curing skins and as a food stabilizer (Windholz, 1986).

3.2.3 Calcium Chloride

The compound used in this experiment was "calcium chloride fused coarse powder" CaCl2, produced by BDH Chemicals Ltd., Poole, England. It had a molecular weight of 110.99 and contained 0.002 per cent iron impurities that would contribute to 0.33mg of iron per kilogram diet (Table 3.2).

Calcium chloride is obtained as a byproduct of the ammonia-soda process and as a joint product from natural salt brines. It is freely soluble in water and alcohol and is mainly used as a drying and

dehydrating agent for organic liquids and gases. Therapeutically, calcium chloride is used for electrolyte replacement and has also been used as a diuretic, urinary acidifier and antiallergic compound. It could also be used intravenously, in animals, in hypocalcaemic states such as milk fever (Windholz, 1986).

3.2.4 Calcium Sulphate

The compound used in this work was "calcium sulphate anhydrous" CaSO4, produced by BDH Chemicals Ltd., Poole, England. It had a molecular weight of 136.14 and contained 0.005 per cent iron which amounted to 1.00mg of iron per kilogram diet when the compound was used to supply the 0.59 per cent calcium required by the hamster (Table 3.2).

The natural form of anhydrous calcium sulphate is an orthorhombic crystal with variable colours such as white with blue, gray or reddish tinge and it is soluble in water. Chemically calcium sulphate is used in the manufacturing of Portland cement, in soil treatment to neutralize alkali carbonate and to prevent loss of volatile and dissolved nitrogenous compounds. Therapeutically, it is used as pharmaceutic aid and plaster casts (Windholz, 1986).

3.3 MATERIALS AND METHODS

This experiment involved 18 young adult male and female golden Syrian hamsters caged separately in standard plastic hamster cages. They were divided into three weight matched groups of six hamsters each with similar numbers of males and females. All groups were fed the standard powdered diet used in previous studies and described in Section 2.2.2. However, calcium lactate was substituted by calcium acetate for Group 1 hamsters, by calcium chloride for Group 2 and by calcium sulphate for Group 3 hamsters. All groups had food ad libitum and distilled water to drink. Four weeks after commencement of the experiment, Several animals died as they refused to eat any of their diet. Therefore on week 5 all surviving animals in all groups were given the calcium lactate containing diet instead of their original experimental diet and they started to feed normally. All animals were weighed weekly throughout the study.

3.4 RESULTS

The average amount of powdered diet a hamster would eat, as established from previous experiments with hamsters, was 10-14g per hamster per day (Al-Damouk, 1984). However, in this experiment hamsters did not eat more than 3g/hamster/day and they were losing weight rapidly. When the old calcium lactate diet was introduced after the forth week of the study, all surviving hamsters gained weight steadily.

In Group 1, hamsters maintained on the calcium acetate containing diet there were two deaths in the first week and two more in the second week of the study. The average weight loss during the first week was 16.1g. The two surviving hamsters when transferred to the calcium lactate diet gained weight rapidly (Table 3.3).

Group 2 hamsters maintained on the calcium chloride diet had two deaths during the first week of the study while the average weight loss was 26.9g. During the second week, three more hamsters died. The only hamster which survived in this group after the fourth week of the study, gained weight rapidly when put on the calcium lactate diet (Table 3.4)

Group 3 hamsters which were on the calcium sulphate diet tolerated their diet better than the other two groups and only one hamster died in the first week and a second one died in the second week. The average weight loss during the first week was 20.8g. The four hamsters surviving after the fourth week of the study were transferred to calcium lactate diet and gained weight rapidly (Table 3.5).

Postmortem examination of animals which died during the course of this study showed no gross pathology.

3.5 DISCUSSION AND CONCLUSIONS

This study failed to demonstrate a suitable substitute for calcium lactate in the hamster diet. It was concluded that unless a new source of diet with less iron impurities was used, the option of achieving low haemoglobin levels remained to be through an increased amount of blood withdrawal. The only other alternative that has not been tested in the hamster before, is to suppress iron metabolism through other nutrient or vitamin deficiencies that are known to influence iron metabolism, such as folic acid. Ranasinghe et al (1983) suspected a low folic acid-containing diet as being a contributery factor when they succeeded in inducing severe iron deficiency anaemia in the hamster. Therefore, it was thought that this hypotheses would be the reasonable step in further developing the hamster as an animal model for iron deficiency.

RECOMMENDED MINERALS			DIET GIVEN IN THIS EXPERIMENT			IRON IMPURITIES	
Minerale	**Amount**	**Unit**	**Chemical Compound**	**Amount**	**Unit**	**Per cent**	**mg/kg**
Calcium	0.59	%	Calcium lactate	42.80	g/kg	0.0100	4.2800
Magnesium	0.06	%	Magnesium sulphate	3.42	g/kg	0.0001	0.0034
Phosphorus	0.30	%	Monobasic sodium phosphate	2.33	g/kg	0.0005	0.0116
Potassium	0.61	%	Potassium orthophosphate	10.57	g/kg	0.0020	0.2114
			Potassium citrate	8.60	g/kg	0.0005	0.0113
Sodium	0.15	%	Sodium chloride	2.94	g/kg	0.0003	0.0088
Cobalt	1.10	mg/kg	Cobaltous carbonate	2.22	mg/kg	0.1000	0.0022
Copper	1.60	mg/kg	Cupric chloride	3.38	mg/kg	0.0500	0.0017
Fluoride	0.02	mg/kg	Sodium fluoride	0.06	mg/kg	0.5ppm	--
Iodine	1.60	mg/kg	Sodium iodate	2.72	mg/kg	--	--
Manganese	3.65	mg/kg	Manganese carbonate	7.64	mg/kg	0.0100	0.0008
Selenium	0.10	mg/kg	Selenium sulphide	0.18	mg/kg	0.0060	0.0001
Zinc	9.20	mg/kg	Zinc acetate	68.50	mg/kg	0.0020	0.0014
Total				**70744.70**	**mg/kg**		**4.5327**

TABLE 3.1 Iron impurities in the mineral component of hamster diet.

Source of Calcium	Amount per kg of diet	Iron content	Total Iron per kg diet
Calcium Lactate	42.80g	0.010%	4.28mg
Calcium Acetate	23.28g	0.002%	0.47mg
Calcium Chloride	16.34g	0.002%	0.33mg
Calcium Sulphate	20.04g	0.005%	1.00mg

TABLE 3.2 Calcium compounds and their iron contents per kilogram diet which would fulfil the hamster requirement for 0.59 per cent calcium.

		Calcium Acetate				Calcium Lactate			
		Week 1	2	3	4	5	6	7	8
Animal	Sex								
1	M	116.6	105.9	113.3	105.0	95.6	91.0	96.0	109.5
2	M	97.0	79.0	-	-	-	-	-	-
3	M	103.4	82.1	-	-	-	-	-	-
4	M	125.0	118.0	124.7	123.0	108.4	108.0	111.0	121.1
5	F	121.2	-	-	-	-	-	-	-
6	F	111.8	-	-	-	-	-	-	-
Mean		112.5	96.4	119.0	114.0	102.0	99.5	103.5	115.3

TABLE 3.3 Weights (g) of animals in Group 1 maintained on diet containing calcium acetate as the source of calcium.

		Calcium Chloride				Calcium Lactate			
		Week 1	2	3	4	5	6	7	8
Animal	Sex								
1	M	114.7	-	-	-	-	-	-	-
2	M	96.0	81.0	-	-	-	-	-	-
3	M	107.3	83.6	-	-	-	-	-	-
4	M	114.2	87.0	-	-	-	-	-	-
5	F	110.8	86.4	96.0	92.0	84.2	81.5	86.5	102.5
6	F	128.0	-	-	-	-	-	-	-
Mean		111.8	84.9	96.0	92.0	84.2	81.5	86.5	102.5

TABLE 3.4 Weights (g) of animals in group 2 maintained on diet containing calcium chloride as the source of calcium.

		Calcium Sulphate				Calcium Lactate			
		Week 1	2	3	4	5	6	7	8
Animal	Sex								
1	M	115.8	93.4	-	-	-	-	-	-
2	M	95.0	72.0	84.0	88.0	74.0	70.0	73.0	98.1
3	M	108.0	78.6	90.3	92.0	88.1	87.7	91.4	105.5
4	M	108.2	96.0	105.7	100.0	91.9	90.3	94.6	109.5
5	M	97.0	-	-	-	-	-	-	-
6	F	121.0	92.0	107.0	107.0	102.0	98.0	104.0	112.9
Mean		107.5	86.7	96.7	96.7	89.0	86.5	90.7	106.5

TABLE 3.5 Weights (g) of animals in Group 3 maintained on diet containing calcium sulphate as the source of calcium.

CHAPTER IV

HAMSTER CHEEK POUCH CARCINOGENESIS IN FOLATE DEFICIENCY

4.1 INTRODUCTION

The histological changes in human and animal oral epithelium in folate deficiency are discussed in Section 1.3.14. Quantitative analysis of such epithelia in hamsters, also discussed in Section 1.3.14, showed atrophy of the cheek pouch epithelium and more severe and progressive atrophy of epithelium in the lateral margins of the tongue corresponding to the degree of folate deficiency. Epithelial atrophy has been suggested as a predisposing factor for oral cancer (MacDonald, 1975; Pindborg, 1972). On the other hand, cancer patients in general have an increased requirement for folic acid; and a reduction in the level of serum folate has been observed in cancer patients by many investigators. Methotrexate, which is a folic acid antagonist has been successfully employed in the treatment of many cancers. The role of folic acid in the aetiology of tumours has not yet been determined.

Although the hamster pouch is a good experimental model for chemically induced oral carcinomas, until relatively recently the hamster was regarded, like the rat and mouse, as a self sufficient animal for folic acid which does not need a dietary supply of this nutrient because sufficient is produced by its gut bacteria. Therefore, methotrexate administration or antibiotic administration were regarded as the only ways to induce folate deficiency in order to test the effect of folate deprivation on cancer induction.

However, more recently the hamster has been investigated for its dietary requirements for folic acid. It was found that folate deficiency can be induced by dietary means alone and can result in quantifiable epithelial changes (Al-Damouk, 1984). These findings about the hamster model have suggested that the use of methotrexate to induce folate deficiency may not simulate the effect of naturally occuring folate deficiency. Furthermore the administration of methotrexate could influence carcinogenesis in this model.

The experimental work reported in this chapter has attempted to investigate the role of folic acid deficiency on chemical carcinogenesis of hamsters oral epithelium. Folate deficiency was induced by a dietary control of intake of this nutrient.

4.2 MATERIALS AND METHODS

The study reported in this chapter consisted of three groups of young adult female hamsters. One group was maintained on a folate deficient diet and there were two control groups. Animals in all groups were maintained on their dietary regimes for four weeks. On week 5, a solution of 0.25 per cent DMBA in acetone was painted, three times per week for eight weeks, in each pouch of each animal of the three groups. The animals were then maintained on the same diets for 13 weeks before being sacrificed, at the beginning of week 27, for the final analysis of the study.

4.2.1 Animals

The animals used in this experiment were 31 female Syrian golden hamsters (Mesocricetus auratus) which were 9-10 weeks old at the start of the study. As in the previous study (Chapter 2) where there was a requirement to standardise animals a line bred strain of hamsters was used. Female hamsters were selected due to their greater susceptibility to folate deficiency (Cohen et al., 1971). Animals were weighed initially and divided into three weight-matched groups. The experimental group (Group 1) consisted of ten hamsters which received a specially prepared powdered diet containing all the required nutrients and vitamins with the exception of folic acid. These animals were given distilled water to drink. Group 2 was a

powdered diet control group and consisted of ten hamsters which received the same powdered diet given to Group 1 animals but had folic acid supplementation in their drinking water. A second control group (Group 3) consisted of eleven hamsters receiving standard laboratory chow which contained all the necessary nutrients, minerals and vitamins and had distilled water to drink. Group 2 under these conditions served as a control group for folate deficiency while Group 3 served as a control group for the powdered diet.

Animals were caged individually in standard hamster plastic cages and were kept on "Klensorb"(previously called Sorboil) bedding material (supplied by D.F. Wishart and Co., Edinburgh). This is an inorganic absorbent material (Appendix 1) which was chosen in preference to organic bedding materials as these might have been a source of folates. The cages were arranged on a cage rack in such a way that Group 1 hamsters were in the top in order to prevent their access to folate supplied to other groups.

4.2.2 Diet

A similar powdered diet to that used in previous experiments (Chapters 2 and 3) was given in this experiment to Groups 1 and 2 hamsters. The diet was prepared without folic acid. Folic acid was given to Group 2 animals in their drinking water in a concentration of 10mg/100ml of water which is much more than the amount normally required by the hamster. This high amount of folate was given in

order to ensure sufficient intake of folate by control animals. Group 3 hamsters received standard laboratory chow the constituents of which are shown in Table 2.4.

4.2.3 Weights and Blood Samples

Hamsters were weighed at the start of the experiment, weekly afterwards and at sacrifice. Blood (0.5ml) was taken from each hamster in all groups at the start of the study, every three weeks afterwards and also at sacrifice. All samples were sent in sterilised heparinised containers to the Department of Haematology, Glasgow Royal Infirmary, Glasgow for corrected whole blood folate estimations. Serum folate levels were also estimated in samples taken at sacrifice. An extra 0.5ml of blood was taken from each hamster in Group 1 on the last two occassions of bleeding. These samples were sent in heparinised containers to the Department of Haematology, Gartnavel General Hospital, Glasgow, for folate estimations. All blood samples were obtained by the method used by Pansky et al. (1961) which was discussed in Section 2.2.3 and all samples were taken under ether anaesthesia. Blood samples were sent to two different hospitals using similar folate estimation kits in order to ensure consistent interpretation of folate levels.

Haemoglobin estimations were done, by the author, at the time blood was withdrawn using an American Optical portable haemoglobinometer. Folic acid estimations were done on all samples of blood using Becton and Dickinson radioassay kits.

4.2.4 Carcinogenesis

The carcinogen used in this study was 0.25 per cent 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone which was prepared in the same as described in Section 2.2.8 and applied to animal pouches as described in Section 2.2.9. One square centimetre in each pouch of each animal of the different groups was painted three times every week. Carcinogen painting was done at the same time of the day and on the same days of the week (Monday, Wednesday and Friday) for eight weeks. Painting was started on week 5 of the experiment and completed on week 13. Light ether anaesthesia was used every time DMBA was painted and safety precautions were observed throughout the time carcinogen was handled (see Section 2.2.10).

Following carcinogen application, all animals were examined weekly for 13 weeks, under light ether anaesthesia, for tumour development. During the observation time, three tumours were removed from three different animals at different days of the experiment in order to study the effects of their removal on folate levels in the corresponding hamsters. The tumours removed from the three hamsters were taken from areas outside the painted square centimetre They were all in control animals (hamster number 16 in the powdered diet control Group 2 and hamsters number 21 and 27 of the pellet diet control Group 3). Folate levels in these animals were very high compared to other animals in the same groups. It was not clear whether such high folate

levels were related to the growing tumours present in the pouches or to other unknown reasons.

4.2.5 Techniques of Preparing Tissue for Tumour Counting

Thirteen weeks after carcinogen applications had finished, all surviving animals were sacrified by intraperitoneal injection of a high dose of barbiturate (Sagatal). The pouches were exposed, cleaned and photographed. The head was separated from the rest of the body and fixed in formal acetic methanol for 18 to 24 hours before being stored in 10 per cent buffered formaldehyde solution. A few days later, the area painted with the carcinogen in each hamster pouch was desected out as one block. Each block was paraffin processed and divided horizontally into four equal blocks which were embedded separately in wax. One section was taken from each of the four blocks of each pouch and stained with haematoxylin and eosin for histological examination. The details of all technical procedures were discussed in Section 2.2.11.

4.2.6 The Gross Counting of Tumours and the Microscopic Identification of Carcinomas

The same criteria previously described in Sections 2.2.12 and 2.2.13 were followed in this study to determine the number of tumours and carcinomas in the $1cm^2$ experimental area of each cheek pouch. Histological sections that did not show a complete layer of

epithelium on the surface were recut and the first section with a complete epithelial surface was selected for detailed examination.

4.2.7 Statistical Methods

The Mann-Whitney U test was used for all comparisons done between weight results, haemoglobin estimations and folate assays reported in this chapter. The Chi-Square 2x2 test was applied for comparisons between animals developing tumours and carcinomas in different groups and also for comparing the numbers of tumours and carcinomas counted in the different groups. The Fisher exact probability test was also applied for the same purpose where the numbers in individual cells were low. The Wilcoxon matched-pair signed-ranks test was used in the study reported in this chapter to compare whole blood folate estimations obtained from two haematology departments. The least squares fit test was used for the derivation of the equation used for the correction of whole blood folate values obtained for the study reported in this chapter in order to compare them with values used in other studies (Siegel, 1956).

4.3 RESULTS

During the course of the study, only one hamster in Group 1 died prematurely, at week 8 (i.e. four weeks after commencing carcinogen painting). This hamster was excluded from the final

analysis of the study. Seven more hamsters were sacrificed 4-5 weeks before the end of the experiment due to the presence of large haemorrhagic tumours in their pouches. These hamsters were counted for the purposes of this work.

4.3.1 Weights

Animals in the different groups gained weight steadily during the course of the experiment as shown in Tables 4.1 and 4.2. Groups 1 and 2 animals, maintained on powdered diet, gained more weight and were significantly larger than the pellet diet control animals in Group 3 during the second week of the study (Table 4.2). The powdered diet control animals in Group 2 gained weight faster than animals in Group 1 which had also received powdered diet although this lacked folic acid. The weight differences between Groups 1 and 2, which started during the second week, reached a statistically significant level on week 10 of the study ($P<.05$). However, this difference in weight gradually decreased with maturity of the animals and was not significant by week 17. Group 3 hamsters maintained on the pellet diet remained smaller than the powdered diet hamsters and did not reach the weight achieved by the powdered diet animals even after maturity. Weight results with their means and standard deviations and also the statistical relationships between the different groups using the Mann-Whitney U test are shown in Table 4.2.

4.3.2 Haemoglobin Estimations

The haemoglobin estimations of all animals with their means and standard deviations are shown in Table 4.3.

Normal haemoglobin values in the hamster are discussed in Section 2.3.2. The lower limit of normal haemoglobin for the present study as obtained from week 8 in Group 3 animals was 12.1g/dl (13.9 - 2S.D.). Blood analyses showed that the mean haemoglobin values of all groups were within normal limits throughout the study. Haemoglobin values of individual animals were also within the normal limits except for animals developing rapidly growing tumours such as animal number 16 in the powdered diet controls (Group 2) and numbers 21 and 27 in the pellet diet control animals (Group 3). No statistically significant differences were present between experimental and control groups.

4.3.3 Comparisons Between Folate Assays Undertaken in Two Different Haematology Departments

The lower limit of normal corrected whole blood folate for hamsters is not yet known as discussed in Section 2.3.2. However, in a previous study (Al-Damouk, 1984) it was found that tissue changes became manifest at folate levels below 90ng/ml. Six to nine weeks on folate free powdered diet were required to induce that level of folate deficiency in the hamster. The haematological investigations for

previous experiments were carried out in the Department of Haematology, Gartnavel General Hospital, Glasgow (G.G.H.). In the present study haematological investigations were carried out in the Department of Haematology, Glasgow Royal Infirmary (G.R.I.). Although both laboratories used similar techniques for analysis they produced different absolute values. The normal range for human corrected whole blood folate in G.G.H. was 75-400ng/ml whereas that for G.R.I. was 106-614ng/ml.

In order to establish a correlation between results obtained in this investigation with those from the previous study, two blood samples from each animal in Group 1 were taken at the same time during week 20 of the study. One sample was sent to Gartnavel General Hospital and the other was sent to Glasgow Royal Infirmary for corrected whole blood folate estimates. When the results were compared using the Wilcoxon matched-pair signed-ranks test, it was found that results obtained from GGH were significantly lower ($P<.001$). The differences were approximately proportional to the difference between the lower level of normal ranges for human values used by the two laboratories. A similar statistical difference was obtained when the same procedure of double blood-sample estimation was repeated for the same animals in week 27.

The comparisons of the values from G.R.I and G.G.H. are shown in Table 4.4. In order to obtain the G.R.I. value equivalent to the lower limit of normal as assessed by G.G.H. a least squares fit test was applied. The equation obtained was **GRI value = 1.31 x GGH value + 2.56.** For a G.G.H. value of 90ng/ml this value gave a G.R.I.

value of 120ng/ml, and this was therefore taken as the lower limit of normal corrected whole blood folate for this study.

4.3.4 Corrected Whole Blood Folate of Hamsters in the Experimental Group

The results of corrected whole blood folate estimations with their means and standard deviations and those of serum folates are shown in Table 4.5.

The corrected whole blood folate as estimated by radioassay technique in blood samples taken from Group 1 animals at the start of the study showed normal levels with no statistically significant differences present between them and those of control groups. During the next three weeks folate levels decreased rapidly in Group 1 animals and became significantly lower ($P<.002$) than those of control groups. By week 8 folate levels were even lower and two hamsters were folate deficient. However, a slight and temporary rise of folate level was recorded in week 11 of the experiment which was overcome during the following three weeks. Folate deficiency was achieved in most animals during week 14 and remained so until the end of the study. Serum folate estimation at sacrifice showed normal or slightly lower than normal levels as discussed in Section 2.3.2.

The temporary rise in folate of the experimental group during week 11 of the experiment was investigated during the time when

it had happened. Samples of water in the drinking bottles of hamsters numbers 5 and 9 and water in the main container together with samples from the diet were taken for analysis to determine their folate contents. The laboratory in charge of folate assays in the Department of Haematology, GRI, investigated possible laboratory sources of folate that might have influenced folate results. Their samples included the distilled water, tap water, ascorbic acid and sterile water for ascorbic acid. Ascorbic acid solution is used to release folate polyglutamate from red cells in order to be split by an endogenous conjugase.

Folate assay of the diet gave unsatisfactory results as the laboratory was not equipped to deal with solid or not completely soluble specimens such as the powdered diet in use. Water from hamsters 5 and 9 drinking bottles showed low folate contamination (0.5ng/ml and 0.9ng/ml respectively). Water from the main container had 0.9ng/ml folate. Samples taken from the laboratory in charge of the folate assays showed folate contents of 0.4, 0.35, 0.27 and 0.5 ng/ml for the distilled water, tap water, ascorbic acid and sterile water for ascorbic acid respectively (folate sensitivity of the laboratory was 0.25ng/ml).

From these results it was concluded that the drinking water was the main exogenous source of folate for the experimetal hamsters in Group 1. This could either be due to folate contamination of water given to the experimental animals from bottles previously used for control animals or could have been derived from metabolites of bacteria colonising water containers. Accordingly it was decided to

mark water bottles used for the folate deficient animals in Group 1 and only use them for this group throughout the experiment. In addition, water bottles of both experimental and control animals were sterilised weekly by boiling. Water stored in the main container for more than a week was also avoided. Such measures were successful in bringing down folate levels in the experimental hamsters and induced folate deficiency until the time of sacrifice.

4.3.5 Corrected Whole Blood Folate of Hamsters in the Control Groups

Folic acid estimates in blood samples taken from control animals (Groups 2 and 3) were within normal limits throughout the experiment as shown in Table 4.5. An unusually high whole blood folate was encountered in three hamsters (hamster 16 in Group 2 and hamsters 21 and 27 in Group 3). These were the same animals that showed very low haemoglobin levels during the same weeks of the study. These high whole blood folate and very low haemoglobin levels had coincided with the presence of large and rapidly growing tumours in the cheek pouches of these hamsters.

The tumours in hamster 16 of Group 2 and in hamsters 21 and 27 of Group 3 were outside the area that was painted with the carcinogen. The opportunity was taken to examine the effect of these tumours on blood folate reported in the literature (1.3.15) and tumours were removed under ether anaesthesia. The tumour in hamster

16 was removed on week 16 while tumours in the two hamsters of Group 3 (numbers 21 and 27) were removed on week 19 of the study. Neomycine ointment was painted on excision areas to prevent infection. The involved hamsters were prevented access to food for the rest of that day to prevent contamination of the wound site. When folate assays were done a week later in each case, folate levels had gone down dramatically. However, when the tumour in hamster number 16 recurred during the following weeks, a very high corrected whole blood folate level of 2626ng/ml was recorded (normal human range 106-614ng/ml) and was accompanied by a high serum folate level of 66.7 ng/ml (normal human range 2.2-11.4ng/ml).

4.3.6 Clinical Observations on Tumour Development in the Cheek Pouch

Tumours started to appear in the cheek pouches of a few hamsters as early as weeks 11 and 12 of the experiment (i.e. 6-7 weeks after commencing carcinogen painting). These animals were numbers 14 and 20 of the powdered diet controls in Group 2 and numbers 21, 24 and 27 of the pellet diet controls in Group 3. Tumours in hamsters of the folate deficiency group (Group 1) were noticed during week 16. However, during the last five weeks of the study small tumours in the pouches of the folate deficient animals in Group 1 were noticed to decrease in size gradually until some of them had disappeared completely.

4.3.7 Gross Counting of Tumours in the Cheek Pouch

The results of the gross counting and size distribution of tumours are shown in Table 4.6.

The gross count of tumours showed that four of the nine hamsters in the folate deficiency group (44.4%) had a total of eight tumours of different sizes in their pouches. Most of these tumours were in the medium size category. In Group 2 controls eight out of the 10 hamsters (80%) developed 36 different sized tumours. In Group 3 all eleven hamsters developed a total of 49 tumours.

4.3.8 Comparison of Grossly Counted Tumours Between Experimental and Control Animals

Analyses of data obtained from the gross counting of tumours, using Chi-Square 2x2 tests, showed that the number of animals developing tumours in the folate deficient animals in Group 1 was significantly less ($P<.02$) than that in the pellet diet controls in Group 3. No statistically significant differences in the number of animals developing tumours were present either between the folate deficient animals in Group 1 compared to the powdered diet controls in Group 2 or between the two control groups (Groups 2 and 3).

When each of the pouches was assessed separately, it was found that six out of 18 pouches in Group 1 hamsters, 15 out of 20

pouches in Group 2 and 19 out of 22 pouches in Group 3 had manifested tumours. Under such considerations the Chi-Squared 2x2 test showed statistically significant differences between Groups 1 and 2, and Groups 1 and 3 ($P<.05$ and $P<.01$ respectively). No significant difference was found between pouches of control Groups 2 and 3 hamsters (Table 4.8).

Comparisons were also made between the number of tumours present in each group. The folate deficient animals (Group 1) developed very significantly less tumours than either of the control groups ($P<.001$ in each case) as shown in Table 4.8.

4.3.9 Histological Counting of Squamous Cell Carcinoma in the Cheek Pouch

Data from the histological counting of carcinomas and their types of invasion are shown in Table 4.7.

Histological counting involved identification of squamous cell carcinomas and classifying them into exophytic and endophytic carcinomas (2.2.13). Two animals in Group 1 (22.2%) developed a total number of six squamous cell carcinomas all of which were endophytic. In Group 2 nine out of ten hamsters (90%) developed 23 squamous cell carcinomas, ten of which (43.5%) were exophytic and 13 were endophytic carcinomas. In Group 3 ten out of the eleven hamsters (90.9%) developed a total of 39 squamous cell carcinomas 19 of which (48.7%) were exophytic while 20 were endophytic carcinomas.

4.3.10 Comparison of Histologically Counted Carcinomas Between Experimental and Control Animals

Comparison between the groups, using Chi-square 2x2 tests, showed that the number of animals developing carcinomas in the folate deficient animals in Group 1 was significantly less than in either of the control groups ($P<.02$ and $P<.01$ for Groups 2 and 3 respectively). No statistically significant differences in the numbers of animals developing tumours were present between the control groups.

When individual pouches were counted separately it was found that three pouches out of 18 in Group 1 (16.7%), twelve out of 20 pouches in Group 2 (60%) and 17 out of 22 pouches in Group 3 (77.3%) developed squamous cell carcinomas. When Chi-Square 2x2 tests were applied, it was found that the number of pouches manifesting carcinomas in the folate deficient animals in Group 1 was significantly less than in either of the control groups ($P<.02$ and $P<.001$ for Groups 2 and 3 respectively).

Comparisons were also made between the numbers of carcinomas seen in the different groups. The folate deficient hamsters in Group 1 developed very significantly fewer carcinomas than either the powdered diet controls in Group 2 ($P<.01$) or the pellet diet controls in Group 3 ($P<.001$) as shown in Table 4.8.

4.4 DISCUSSION AND CONCLUSIONS

Hamsters in all three groups gained weight steadily during the course of the experiment. Hamsters fed the powdered diet in Groups 1 and 2 gained more weight and were significantly larger than Group 3 hamsters maintained on pellet diet and they remained heavier until the end of the study. The folate supplemented powder diet hamsters in Group 2 were significantly larger than Group 1 animals as from week 11 and remained so until week 17 of the experiment. Although the difference between these two groups was not significant after week 17, the folate deficient animals in Group 1 remained smaller than those of Group 2 until the end of the study. These results suggested that folate deficiency has a suppressive effect on growth of animals.

The haematology results showed that folate levels were significantly reduced in a short time (4 weeks) and were at the deficiency level during week 14 of this study. Haemoglobin estimations were normal in most animals of the three groups throughout the course of the experiment. However, few animals reacted severely to the developing tumours in their pouches. These animals showed low haemoglobin levels and died earlier than other animals. No statistically significant differences were found in the mean haemoglobin estimations of the groups. Such results suggest that haemoglobin levels are not affected by folate depletion when an adequate supply of iron in the diet was provided.

The gross analysis of tumours showed that all eleven of the pellet diet control hamsters developed tumours whereas only four of the folate deficient animals developed tumours. This difference was significant. However, the difference in the number of animals developing tumours in the folate supplemented powder diet Group 2 as opposed to the folate deficient group was not statistically significant. Comparisons of the number of tumours observed grossly in the different groups showed that only 8 tumours were present in the folate deficiency group and this was very significantly less than in either of the other two groups.

The data from the histological analysis identifying individual carcinomas showed that only two of the folate deficient animals had malignant tumours and in these animals six carcinomas were seen. By contrast many more animals in the other groups had carcinomas. The folate deficient animals differred significantly from either of the other two groups but the difference between the control groups was not significant.

This study has shown that nutritional folate deficiency influences experimental carcinogenesis in the hamster cheek pouch. Folate deficient animals developed fewer tumours than control animals but there was a difference in the pattern of invasion in that the tumours in the folate deficient animals appeared to be more invasive into the deeper tissues of the pouch.

	Week								
	1	5	8	11	14	17	20	24	27
Group 1									
1	90	116	124	137	147	146	-	-	-
2	80	112	128	139	150	165	177	132	-
3	80	115	115	125	137	141	144	132	126
4	80	129	137	143	156	162	162	156	141
5	80	115	102	110	121	132	142	126	-
6	90	131	132	-	-	-	-	-	-
7	85	105	105	114	117	120	125	118	116
8	85	112	115	120	128	139	139	129	124
9	85	85	111	119	137	147	150	143	130
10	75	93	97	100	103	110	113	108	101
Mean	**83.0**	**111.3**	**116.6**	**123.0**	**132.9**	**140.2**	**144.0**	**130.5**	**123.0**
S.D.	**4.8**	**14.2**	**13.4**	**14.4**	**17.2**	**17.9**	**20.0**	**14.6**	**13.5**
Group 2									
11	90	129	137	145	155	158	162	158	148
12	85	118	136	155	162	159	149	145	138
13	85	123	124	141	150	153	149	147	137
14	85	117	124	134	151	147	144	133	131
15	75	104	103	116	126	129	126	114	110
16	90	124	124	138	143	142	-	-	-
17	80	110	125	138	154	159	154	141	126
18	90	130	120	133	153	154	153	141	132
19	80	117	121	138	157	164	163	141	134
20	80	115	134	149	164	178	184	172	161
Mean	**84.0**	**118.7**	**124.8**	**138.7**	**151.5**	**154.3**	**153.8**	**143.6**	**135.2**
S.D.	**5.2**	**8.1**	**9.8**	**10.5**	**10.8**	**13.2**	**15.7**	**15.9**	**14.1**
Group 3									
21	75	80	86	86	90	91	88	-	-
22	80	102	108	112	116	124	118	112	89
23	80	95	104	112	112	115	113	108	105
24	80	99	96	107	111	115	116	112	111
25	80	85	97	109	113	108	97	97	94
26	70	101	117	131	147	140	133	128	127
27	85	96	106	117	119	120	111	-	-
28	75	90	101	120	124	125	128	119	118
29	90	99	106	116	117	114	109	111	113
30	80	83	88	100	108	101	115	103	-
31	85	85	91	100	102	106	107	102	105
Mean	**80.0**	**92.3**	**100.0**	**110.0**	**114.4**	**114.5**	**112.3**	**110.2**	**107.7**
S.D.	**5.5**	**7.9**	**9.4**	**12.0**	**14.1**	**13.2**	**12.6**	**9.3**	**12.4**

TABLE 4.1 Weekly weights (g) of animals in experimental Group 1 and control Groups 2 and 3 with their means and standard deviations.

Group	Week 1	2	3	4	5	6	7	8	9	10	11	12	13
1	83.0	90.6	103.3	105.8	111.3	119.2	120.4	116.6	115.2	120.3	123.0	127.1	131.1
2	84.0	94.6	108.4	113.6	118.7	125.3	127.9	124.8	128.5	134.2	138.7	144.7	148.1
3	80.0	82.4	85.4	89.6	92.3	98.1	99.7	100.0	103.7	107.4	110.0	111.0	113.8
1 v 2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	<.05	<.05	<.05
1 v 3	NS	<.02	<.002	<.002	<.02	<.002	<.002	<.02	NS	<.05	NS	<.05	<.05
2 v 3	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002

Group	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	132.9	136.4	137.6	140.2	138.4	142.3	144.0	143.0	143.2	138.1	130.5	129.4	125.4	123.0
2	151.5	153.7	155.3	154.3	152.0	149.9	153.8	150.9	149.6	146.6	143.6	144.5	143.1	135.2
3	114.4	117.3	117.1	114.5	115.5	113.7	112.3	110.7	113.0	110.6	110.2	111.7	110.9	107.7
1 v 2	<.02	<.05	<.05	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1 v 3	<.02	<.05	<.02	<.02	<.02	<.002	<.002	<.002	<.02	<.02	<.02	<.003	NS	NS
2 v 3	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002.	<.002	<.002	<.002	<.002

TABLE 4.2 Mean weight (g) of the folate deficient animals (Group 1) and controls (Groups 2 and 3). The table also shows the statistical relationships between individual values when the Mann-Whitney U test was applied.

	HAEMOGLOBIN (g/dl)						
	Week						
	1	8	11	14	17	20	27
Group 1							
1	16.1	15.5	15.1	16.0	14.5	-	-
2	15.5	15.7	13.9	14.9	14.6	15.5	-
3	16.3	14.4	14.6	15.0	15.3	15.2	15.0
4	15.2	14.5	14.4	14.7	15.1	14.1	14.6
5	16.7	14.7	14.9	15.6	15.6	15.3	-
6	14.5	14.2	-	-	-	-	-
7	14.7	14.1	13.5	13.5	14.7	14.5	14.0
8	15.5	15.5	13.9	13.4	15.0	15.0	14.7
9	14.2	14.2	14.1	15.1	14.6	15.9	14.8
10	14.1	14.6	14.2	15.3	15.5	15.5	13.8
Mean	**15.3**	**14.7**	**14.3**	**14.8**	**15.0**	**15.1**	**14.5**
S.D.	**0.9**	**0.6**	**0.5**	**0.9**	**0.4**	**0.6**	**0.5**
Group 2							
11	16.6	14.7	14.9	16.4	15.6	14.6	15.4
12	14.7	14.4	15.5	15.9	14.0	13.6	13.7
13	15.6	12.1	14.4	15.1	15.3	15.5	16.2
14	15.9	15.0	13.9	14.8	14.7	15.9	15.7
15	14.6	13.6	14.2	14.5	14.9	16.0	16.4
16	15.1	15.4	14.5	12.4	12.0	6.0	-
17	15.6	13.6	14.3	15.4	15.6	16.2	15.9
18	16.0	14.0	15.1	14.9	15.8	17.0	16.3
19	15.9	14.1	14.8	15.1	15.6	15.8	15.0
20	15.5	15.1	13.0	13.5	14.1	14.7	12.6
Mean	**15.5**	**14.2**	**14.5**	**14.8**	**14.8**	**15.5**	**15.2**
S.D.	**0.6**	**1.0**	**0.7**	**1.1**	**1.2**	**1.0**	**1.3**
Group 3							
21	17.9	14.3	13.2	14.4	9.5	11.4	-
22	15.5	13.6	15.2	14.6	15.5	15.6	16.8
23	16.1	13.1	15.7	15.7	15.0	16.0	15.2
24	15.0	15.0	15.0	15.4	15.7	15.6	13.0
25	14.2	12.9	14.5	14.9	14.4	15.5	12.8
26	13.6	14.6	14.8	15.6	14.7	15.6	10.9
27	15.9	12.9	14.8	13.3	7.8	10.6	-
28	15.4	15.4	15.2	14.8	15.0	16.5	15.9
29	16.1	14.6	15.0	15.5	15.8	15.9	14.8
30	16.0	12.8	14.1	15.0	15.5	14.9	-
31	15.7	14.2	13.1	15.1	15.8	15.8	13.9
Mean	**15.6**	**13.9**	**14.6**	**14.9**	**14.1**	**14.9**	**14.2**
S.D.	**1.1**	**0.9**	**0.8**	**0.7**	**2.7**	**2.0**	**1.9**

TABLE 4.3 Haemoglobin values with their means and standard deviations in experimental Group 1 and control Groups 2 and 3.

Week	GLASGOW ROYAL INFIRMARY		GARTNAVEL GENERAL HOSPITAL	
	20	27	20	27
Group 1				
1	-	-	-	-
2	139	-	109	-
3	174	94	120	72
4	142	83	116	140
5	102	-	66	-
6	-	-	-	-
7	76	62	56	39
8	102	56	81	35
9	125	91	103	91
10	80	62	63	42
mean	**117.5**	**74.7**	**94.1**	**79.6**
S.D.	**33.6**	**16.6**	**53.7**	**45.3**

TABLE 4.4 Comparison between corrected whole blood folate assays of the folate deficiency animals (Group 1) in Glasgow Royal Infirmary (GRI) and Gartnavel General Hospital (GGH).

	CORRECTED WHOLE BLOOD FOLATE (ng/ml)								SERUM FOLATE (ng/ml)
	Week								
	1	5	8	11	14	17	20	27	27
Group 1									
1	578	282	200	171	118	126	-	-	-
2	416	162	142	167	118	127	139	-	-
3	536	309	150	210	148	144	174	94	3.1
4	639	370	236	226	168	204	142	83	1.7
5	458	267	99	226	159	114	102	-	-
6	514	366	261	-	-	-	-	-	-
7	481	179	111	175	94	104	76	62	2.8
8	534	214	178	184	116	101	102	56	1.3
9	458	343	216	256	162	179	125	91	4.7
10	523	200	188	149	94	122	80	62	1.5
Mean	**513.7**	**269.2**	**178.1**	**196.0**	**130.8**	**140.7**	**117.5**	**74.7**	
S.D.	**64.7**	**77.6**	**52.8**	**35.1**	**28.9**	**36.5**	**33.6**	**16.6**	
Group 2									
11	550	583	589	598	468	497	618	451	>30.0
12	806	709	580	523	417	564	912	702	>30.0
13	415	525	636	635	372	529	415	347	>30.0
14	582	558	637	699	472	688	513	371	>30.0
15	410	313	363	505	370	375	334	298	>30.0
16	607	557	432	526	1052	780	2626	-	-
17	547	702	699	564	430	639	588	564	>30.0
18	581	471	403	555	394	434	356	422	>30.0
19	570	510	289	697	442	429	318	232	26.7
20	473	392	565	479	398	556	591	984	>30.0
Mean	**554.1**	**532.0**	**519.3**	**578.1**	**481.5**	**549.1**	**727.1**	**423.4**	
S.D.	**112.9**	**122.9**	**137.0**	**77.5**	**203.6**	**125.6**	**690.6**	**150.7**	
Group 3									
21	576	651	590	656	534	1356	934	-	-
22	562	592	593	578	473	408	392	227	26.0
23	437	579	526	466	396	346	332	224	>30.0
24	694	616	493	526	413	423	474	749	>30.0
25	571	347	459	417	426	332	355	270	>30.0
26	500	778	574	528	452	388	301	447	24.9
27	514	609	614	565	423	1246	870	-	-
28	328	581	501	537	463	364	384	228	>30.0
29	507	454	431	418	353	287	352	222	27.1
30	455	583	574	457	367	340	571	-	-
31	393	391	429	482	384	437	366	466	29.8
Mean	**499.3**	**561.9**	**525.8**	**511.8**	**425.8**	**538.8**	**484.6**	**354.1**	
S.D.	**91.7**	**121.9**	**67.5**	**72.7**	**52.5**	**380.1**	**219.6**	**189.2**	

TABLE 4.5 The corrected whole blood folate and serum folate levels of experimental Group 1 and control Groups 2 and 3 animals.

		NUMBER OF TUMOURS GROSSLY			TUMOUR SIZE (mm)		
		L Pouch	R Pouch	Total	1-2	2-5	5 or more
Group 1							
	1	1	-	1		1	
	2	2	1	3		1	2
	3	1	1	2	1	1	
	4	-	-	-			
	5	2	-	2		2	
	7	-	-	-			
	8	-	-	-			
	9	-	-	-			
	10	-	-	-			
Total	9	6	2	8	1	5	2
Group 2							
	11	6	2	8	5	2	1
	12	1	4	5	1	2	2
	13	-	-	-			
	14	-	3	3	2	1	
	15	1	1	2	2		
	16	1	2	3	1	2	
	17	2	2	4	1	1	2
	18	-	-	-			
	19	1	1	2		2	
	20	5	4	9	3	3	3
Total	10	17	19	36	15	13	8
Group 3							
	21	3	2	5		1	4
	22	3	-	3	2	1	
	23	1	1	2	1	1	
	24	4	3	7	1	4	2
	25	4	1	5	3	1	1
	26	3	6	9	3	4	2
	27	4	5	9	4	3	2
	28	1	1	2	1	1	
	29	1	-	1		1	
	30	-	3	3		2	1
	31	1	2	3	2	1	
Total	11	25	24	49	17	20	12

TABLE 4.6 Grossly counted tumours with their sizes in the folate deficient animals (Group 1) and controls (Groups 2 and 3).

		NUMBER OF CARCINOMAS					
		L pouch		R pouch		Total	
		Exo.	Endo.	Exo.	Endo.	Exo.	Endo.
Group 1							
	1	-	-	-	1	-	1
	2	-	2	-	3	-	5
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
	5	-	-	-	-	-	-
	7	-	-	-	-	-	-
	8	-	-	-	-	-	-
	9	-	-	-	-	-	-
	10	-	-	-	-	-	-
Total	9	0	2	0	4	0	6
Group 2							
	11	-	-	-	2	-	2
	12	-	1	3	-	3	1
	13	-	1	-	-	-	1
	14	-	-	1	1	1	1
	15	-	-	1	-	1	-
	16	-	2	-	-	-	2
	17	1	1	-	1	1	2
	18	-	-	-	-	-	-
	19	-	-	-	1	-	1
	20	2	3	2	-	4	3
Total	10	3	8	7	5	10	13
Group 3							
	21	1	3	1	3	2	6
	22	2	-	1	-	3	-
	23	-	1	1	1	1	2
	24	2	-	2	3	4	3
	25	2	-	-	-	2	-
	26	-	-	3	3	3	3
	27	1	1	1	2	2	3
	28	-	-	-	-	-	-
	29	1	-	-	1	1	1
	30	-	-	-	1	-	1
	31	-	1	1	-	1	1
Total	11	9	6	10	14	19	20

TABLE 4.7 Results of the histological count of squamous cell carcinomas and their types of invasion in the folate deficient animals (Group 1) and controls (Groups 2 and 3).

Chi-Square 2x2 Tests applied for :

Comparison of the number of animals with grossly seen tumours.

Group	1 v 2	NS
Group	1 v 3	P<.02
Group	2 v 3	NS

Comparison of the number of pouches with grossly seen tumours.

Group	1 v 2	P<.05
Group	1 v 3	P<.01
Group	2 v 3	NS

Comparison of the number of grossly counted tumours.

Group	1 v 2	P<.001
Group	1 v 3	P<.001
Group	2 v 3	NS

Comparison of the number of animals with carcinomas.

Group	1 v 2	P<.02
Group	1 v 3	P<.01
Group	2 v 3	NS

Comparison of the number of pouches with carcinomas.

Group	1 v 2	P<.02
Group	1 v 3	P<.001
Group	2 v 3	NS

Comparison of the number of carcinoma identified histologically.

Group	1 v 2	P<.01
Group	1 v 3	P<.001
Group	2 v 3	NS

TABLE 4.8 Comparisons of the number of animals and pouches with tumours or carcinomas and also of the numbers of tumours and carcinomas in Groups 1, 2 and 3.

CHAPTER V

HAMSTER CHEEK POUCH CARCINOGENESIS IN COMBINED IRON AND FOLATE DEFICIENCY

5.1 INTRODUCTION

In Chapter 2, animals maintained on diet containing an average of 9.6mg/kg of iron for 37 weeks and bled 1.5ml every two weeks for ten weeks during the peak growth period of their lives failed to develop iron deficiency anaemia (as measured by haemoglobin estimations). Ranasinghe et al. (1983) reported success in inducing severe iron deficiency anaemia in the Syrian golden hamster in a relatively short period of time. However, the diet they utilised contained less than the recommended level of folic acid. It is known that folate deficiency, by its action on the small intestine, can cause defective iron absorption. It was therefore felt that a combination of iron deficiency and folate deficiency might be required to cause iron deficiency anaemia.

In the light of the experience gained from the previous study reported in Chapter 2 another attempt was made to induce severe

iron deficiency in the golden hamster and to examine the effect of such deficiency on chemical carcinogenesis. The present study also attempted to examine the effect of a folic acid deficient diet on the induction of iron deficiency in the hamster and to record the combined effects of such deficiencies on chemical oral carcinogenesis in this animal model.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Experimental Protocol

The animals used in this study were 40 male Syrian golden hamsters which were 8-10 weeks old at the start of the experiment. The animals were weighed and divided into three weight matched groups. Group 1 consisted of 14 hamsters. Because of constraints of space in the Animal House, the first six animals were caged individually and the rest were caged in pairs. This group received a specially prepared powdered diet containing all necessary nutrients, minerals and vitamins but lacking added iron and folic acid. Group 2 also consisted of 14 hamsters, the first six of which were caged individually and the rest in pairs. This group received the same diet given to Group 1 but in addition had 1-1.3 ml of blood removed every week to deplete iron stores. Group 3 consisted of 12 animals the first four caged individually and the rest in pairs. This group received the same diet as the other two groups but had folic acid

supplementation in the drinking water and had 1-1.3 ml of blood removed every week during the time of the experiment.

The present study was planned to induce low haemoglobin levels without risking the animal's lives. The level of haemoglobin selected was in the region of 10g/dl. When such levels were reached blood withdrawal would only be required to maintain such a low haemoglobin level and prevent it from rising up again.

The animals were caged in standard hamster cages made of plastic with aluminium tops. Klensorb (Appendix 1) non organic absorbent chips were used on the cage base for all hamsters. During the last three weeks of the experiment Klensorb could not be obtained from suppliers. Another type of absorbent chip material called Roebuck Absorbent Granules (Appendix 2) had to be substituted. This is closely similar to Klensorb, but has a higher iron content.

The cages were put on a cage rack with Group 1 on the top shelves and Group 3 on the bottom shelves. This was in order to prevent access of animals in Groups 1 and 2 to folic acid which might have resulted from spillage of drinking water from Group 3 animals.

5.2.2. Diet

The diet given in this experiment was a powdered diet made according to the nutrient requirements of the golden hamster recomended by the National Academy of Sciences (1978). It was similar

to the diet used in the previous experiments, and described in Section 2.2.2. It consisted of the bulk nutrients shown in Table 2.1.

The mineral mix of the diet was given without iron (ferrous sulphate). Calcium lactate was replaced by tri-calcium phosphate with the intention of reducing iron contamination in the diet (see Chapter 3). This necessitated other changes in the minerals. Tri-calcium phosphate substituted for the phosphate present in monobasic sodium phosphate and potassium dihydrogen orthophosphate which were used in previous mineral mixtures. Therefore these latter minerals were not used. The amounts of sodium and potassium present in other compounds of the mineral mix were adjusted accordingly, as shown in Table 5.1. However, three weeks after the start of the experiment it was apparent that the animals were not thriving and calcium lactate was used in the diet instead of tri-calcium phosphate. The mineral constituents of the diet were made similar to those shown in Table 2.2.

The diet also contained the fat-soluble and water-soluble vitamins shown in Table 2.3. However, folic acid was given in the drinking water of Group 3 animals only and at a concentration of 100mg/l water. Assuming a daily fluid intake of 10ml this is equivalent of a folic acid content of 10mg/kg diet and greatly exceeds the daily requirement.

Animals in all groups were given distilled water to drink ad libitum from standard glass bottles. Folic acid was added to the water of Group 3 hamsters only. All water bottles were autoclaved

weekly and those for Group 3 hamsters were marked and kept for this group only throughout the time of the experiment. The powder diet required by all hamsters of all groups was mixed with distilled water in a clean container to make a thick paste. It was distributed to individual animals in glass dishes. These glass dishes were washed and autoclaved daily.

5.2.3 Weights and Blood Withdrawal

Hamsters were weighed at the start of the experiment and weekly afterwards. Additional weighing of individual animals that looked unwell or lost excessive weight suddenly was also done in order to monitor their health.

A blood sample of 1.0-1.3ml was taken from Groups 2 and 3 animals every week in order to induce iron deficiency anaemia in these animals. Blood was not taken from Group 2 animals on weeks 20 and 21 of the study due to severe deterioration of their health.

Blood samples of 1ml were taken from Group 1 animals every three weeks. Together with the corresponding samples for Groups 2 and 3 animals, these were sent to the Department of Haematology, Gartnavel General Hospital, Glasgow for the estimations of corrected whole blood folate and haemoglobin values.

All blood samples were taken under short ether anaesthesia. Heparinised syringes and needles were used for collection of the blood

samples and the method of Pansky et al. (1961) was adopted for that purpose as described in Section 2.2.3. Myciguent ophthalmic ointment (neomycin) was used for all hamsters after each blood withdrawal as a prophylaxis against eye infections.

The other blood samples obtained weekly from animals in Groups 2 and 3 were taken to Glasgow Dental Hospital. Haemoglobin estimation was made by the author on each sample by using the American Optical portable haemoglobinometer used in the previous experiments. These samples were then centrifuged at 1800 r/min. to separate plasma from the red blood cells. The plasma from each sample was then taken out and put in 1ml polypropylene microtubes (by Sarstedt-Leicester, England). Each container was marked with the animal number and date of bleeding and frozen at -20°C for further use. Plasma samples were kept with the intention that they might be needed for the study of carrier proteins in iron and folate deficient hamsters.

5.2.4 Carcinogenesis

The carcinogen used in this experiment was 0.25 per cent 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone. DMBA was prepared and handled as described in Sections 2.2.8 and 2.2.10 and applied, under ether anaesthesia, to a defined one square centimeter area in the anterior medial wall of both pouches in each animal of the three groups.

The carcinogen painting was started at week 6 of the experiment. DMBA was painted three times per week for eight weeks and the method described in Section 2.2.9 was used for the applications. After paintings had finished, the animals were maintained on the same experimental regimes for a further eleven weeks before being killed, on week 25, for the final analysis of the study.

5.2.5 Tissue Preparation for Tumour Counting

At the begining of week 25 of the experiment all surviving animals were killed by an overdose of intraperitoneal barbiturate (Sagatal). The medial wall of each pouch was exposed by cutting the lateral wall of the pouch with scissors. The pouch was rinsed under running water then dried with cotton wool. The jaws were propped open by inserting a rubber bung between the upper and lower incisor teeth and the pouch was then photographed. Colour transparencies were taken for each pouch of each animal. Each hamster's head was then removed and fixed in formal acetic methanol. The carcass was dissected and examined for gross abnormalities and tissue samples from different organs and bone marrow were kept for future study. Particular attention was made to identify any cervical lymph node involvement by tumour metastasis.

Following 18-24 hours fixation in formal acetic methanol, the heads were transferred to 10 per cent buffered formalin before dissection. A few days later the one square centimetre area of the pouch painted by DMBA was removed in a block, processed to paraffin

and divided horizontally into four blocks of equal thickness. These were then blocked out on their inferior surfaces as described in Section 2.2.11.

5.2.6 Naked Eye Counting of Tumours and Histological Identification of Carcinomas

Large, 15x20cm, black and white prints were prepared from the coloured transparencies of each pouch. The black and white prints were random numbered and tumours in the painted area of each pouch were identified and counted. After counting, they were classified into three size categories of 1-2, 2-5 and over 5mm in diameter in the same way described in Section 2.2.12. In cases of doubt tumours were measured on the projected transparencies when codes of random numbers had been revealed.

The histological counting of carcinomas was undertaken in the way described in Section 2.2.13 after slides had been random numbered. The tumours were classified as exophytic or endophytic according to the type of invasion.

After counting and classifying all tumours seen grossly and histologically codes for the random numbers were broken and groups were identified and compared to each other.

5.3 RESULTS

During the course of this study six animals died prematurely, one from Group 1 died on the second week of the experiment, four from Group 2 died on weeks 14, 17, 18 and 20 respectively. One hamster from Group 3 died on week 17 of the experiment. All six animals were excluded from the study because their deaths were not due to the presence of tumours and occurred too early to be included in the final analysis of the study. Although five more animals in the different groups died before the end of the study but after week 22, they were included in the final analysis.

During the time that tri-calcium phosphate had been used animals did not like the diet and ate much less than expected. The biochemical analysis of tri-calcium phosphate showed that the iron impurity level was 0.0129 per cent which was more than that of the calcium lactate used in previous experiments. Therefore calcium lactate was added to the diet instead of tri-calcium phosphate and given to the animals as from week 4 of the experiment.

5.3.1 Weight Results

Animals in the three groups gained weight steadily throughout most of their lives (Table 5.2). However, animals in the bleeding groups (Groups 2 and 3) gained less weight than animals in Group 1 (non bleeding group). Table 5.3 shows the comparisons between the groups when the Mann-Whitney U test was applied. Group 3 hamsters

were significantly smaller than Group 1 animals on week 4 (P<.02) while on week 6 of the study animals of both bleeding groups (Groups 2 and 3) were very significantly smaller than Group 1 animals (P<.002 in each case) and remained so until the end of the study. No significant differences in weight were seen between Group 2 and 3 animals throughout the experimental time.

5.3.2 Blood Results

Haemoglobin values with their means and standard deviations are shown in Tables 5.4, 5.5 and 5.6 for Groups 1, 2 and 3 respectively. The results of the Mann-Whitney U tests applied for comparisons between the three groups are shown in Table 5.7.

The lower limit of the normal range of haemoglogin in the hamster is not yet known with certainty. Haemoglobin values vary with age and sex (see Section 2.3.2). In the present experiment Group 1 animals formed the main control group. The values of the mean on any week minus two times the standard deviation were calculated. This gave the least value of 13g/dl on week 10. This value was therefore taken as the lower limit of normal and animals with haemoglobin values below 13g/dl were assessed as having iron deficiency anaemia. There is further support for this value in the data of McPherson (1987) who gave the haemoglobin value of male hamsters as 16.8g/dl with a standard deviation of 1.2g/dl. A value of 13g/dl would thus be definately below the normal range.

The results showed that the non-bleeding group (Group 1) animals, with the exception of one suspiciously low value in week 10, maintained normal haemoglobin levels throughout the time of the experiment despite their intake of the diet deficient in iron and folate. Animals in the bleeding groups (Groups 2 and 3) had significantly lower haemoglobin levels than those of Group 1 animals during the fourth week of the study ($P<.002$ in each case). They were anaemic at week 7 and remained so until the last few weeks of the study.

The haemoglobin level increased in Group 2 animals when bleeding was stopped during weeks 20 and 21 and became less significantly different from Group 1 animals ($P<.02$). When Klensorb was substituted by the other absorbent material in the cage base which had more iron contamination, haemoglobin levels of Groups 2 animals further increased and became not significantly different compared to those of Group 1 animals. Haemoglobin levels in Group 3 animals were also improved by the new bedding material but remained significantly lower than those of Group 1 animals. No significant differences were present between the two bleeding groups (Groups 2 and 3) thoughout the experimental time except when bleeding was stopped for Group 2 animals.

The corrected whole blood folate values with their means and standard deviations are shown in Tables 5.8, 5.9 and 5.10 for Groups 1, 2 and 3 respectively. The results of comparisons between the three groups when Mann-Whitney U tests were applied are shown in Table 5.11.

On the basis of previous experiments (Al-Damouk, 1984) the lower limit of normal for corrected whole blood folate as assessed by the haematology Department in Gartnavel General Hospital was 100ng/ml (Section 2.3.2).

Group 3 animals, which had folate supplementation in their drinking water, showed high normal folate levels throughout the experiment. The folate values for Group 2 (folate and iron deficient diet plus bleeding) were lower than Group 3 but never dropped below the normal range although there was a sharp drop between weeks 19 and 22 when bleeding was discontinued. The folate values in Group 1 (folate and iron deficient diet but no bleeding) were lower than in the other two groups but were very variable. On only four occassions were values below 100ng/ml obtained.

5.3.3 Gross Analysis of Tumours of the Cheek Pouch

The results of the gross analysis of tumours of the cheek pouches are shown in Table 5.12. Seven of the 13 surviving animals in Group 1 developed tumours. Two of these animals had tumours in both pouches. Nine animals of the surviving 10 in Group 2 had tumours and of these three had tumours in both pouches. In Group 3, seven hamsters of the remaining 11 had tumours; five of which had tumours in both pouches. There was a total of ten tumours in Group 1 animals, 19 in Group 2 and 22 in the iron deficiency group (Group 3) animals.

5.3.4 Comparison of Grossly Counted Tumours Between the Different Groups

Comparisons between the three groups using Fisher exact probability tests showed that the number of animals developing tumours was significantly less in Group 1 than in Group 2 (P<.001). No statistically significant differences were noted in the numbers of animals developing tumours as counted grossly between Groups 1 and 3 or between Groups 2 and 3.

Chi-Square tests showed that the total number of grossly counted tumours was significantly less in Group 1 animals (folate and iron deficient diet but no bleeding) compared to animals in Groups 2 (P<.02) and Group 3 (P<.01). No statistically significant differences in the number of grossly counted tumours were present between Groups 2 and 3 animals. No significant differences were found between the three groups when tumour sizes were compared.

5.3.5 Results of the Histological Analysis of Cheek Pouch Carcinomas

The results of the histological analysis of tumours are shown in Table 5.13. Five animals in Group 1 developed a total of 8 carcinomas. In Group 2, five hamsters developed a total of 7 carcinomas. In Group 3, which consisted of eleven hamsters, seven of these had a total of 13 carcinomas.

5.3.6 Comparison of Histologically Identified Carcinomas Between the Different Groups

Despite the differences between the groups when tumours were counted grossly, histological analysis of carcinomas showed no statistically significant differences either in the number of animals with and without carcinomas, in the number of carcinomas in each group or in the type of invasion of individual carcinomas.

5.4 DISCUSSION AND CONCLUSIONS

Despite the fact that animals in all groups gained weight steadily during the course of the experiment there were significant differences between the groups. The non-bleeding animals in Group 1 gained more weight and were significantly larger than Groups 2 or 3 by weeks 6 and 4 of the study respectively. The statistical level of difference between Group 1 and each of Groups 2 and 3 remained almost the same throughout the study. No statistically significant weight differences were present between Groups 2 and 3.

Groups 2 and 3 animals which were bled weekly showed significantly reduced haemoglobin levels during week 4 of the study compared to Group 1 animals. By week 7 animals of these two groups

were clearly showing iron deficiency anaemia with haemoglobin values below 13g/dl in all but three animals. When blood withdrawal was stopped for Group 2 animals during weeks 20 and 21 of the study due to deterioration in their general health and when the new bedding material which contain more iron contamination than the Klensorb was used for all groups on week 21, increased haemoglobin levels were recorded in Groups 2 and to a lesser extent in Group 3 animals. This emphasises the difficulty of producing and maintaining iron deficiency anaemia in hamsters.

There was an apparent relationship between animal weights of Groups 2 and 3 animals and their haemoglobin values. The increase in haemoglobin levels of Groups 2 and 3 animals during the last few weeks of the study corresponded to the slight increase of the animals weights during the same period of time.

The corrected whole blood folate results showed a very interesting pattern of fluctuation during this study. Folate estimations of the individual groups differed significantly from each other. Group 1 animals maintained on an iron and folate deficient diet with no blood withdrawal had the lowest values of folate. Animals in Group 2 which had the same diet but in addition had 1-1.3ml of blood withdrawn every week showed significantly higher folate levels than Group 1 animals. When blood withdrawal was stopped for Group 2 animals during weeks 20 and 21 of the study resulting in increase in haemoglobin levels of this group, folate values dropped significantly as shown in blood samples taken on week 22. When blood withdrawal was resumed on week 22, folate levels increased and

returned to their previous levels despite the fact that haemoglobin remained higher than the previous levels for this group. Group 3 animals maintained on an iron deficient diet and bled every week showed very high folate levels (up to 4438ng/ml). Such high folic acid levels have not been noted in the literature before.

The gross analysis of tumours showed significantly less animals developed tumours in Group 1 than in Group 2. No significant differences in the number of animals developing tumours were present between Groups 1 and 3 or between Groups 2 and 3. The number of tumours observed grossly in Group 1 animals was significantly less than in either Group 2 or Group 3 animals. No statistically significant differences in the number of grossly counted tumours were seen between Groups 2 and 3. Also no statistically significant differences either in the number of animals developing carcinomas, the number of carcinomas or in the patterns of invasion were seen between the different groups when histologically examined.

Comparisons of the results of this experiment with those of earlier experiments are discussed in Chapter 6.

RECOMMENDED MINERALS			DIET GIVEN IN THIS EXPERIMENT		
Mineral	**Amount**	**Unit**	**Chemical Compound**	**Amount**	**Unit**
Calcium	0.59	%	Tri-calcium phosphate	15.22	g/kg
Phosphorus	0.30	%			
Potassium	0.61	%	Potassium citrate	16.91	g/kg
Magnesium	0.06	%	Magnesium sulphate	3.42	g/kg
Sodium	0.15	%	Sodium chloride	3.81	g/kg
Cobalt	1.10	mg/kg	Cobaltous carbonate	2.22	mg/kg
Copper	1.60	mg/kg	Cupric chloride	3.38	mg/kg
Fluoride	0.02	mg/kg	Sodium fluoride	0.06	mg/kg
Iodine	1.60	mg/kg	Sodium iodate	2.72	mg/kg
Manganese	3.65	mg/kg	Manganese carbonate	7.64	mg/kg
Selenium	0.10	mg/kg	Selenium sulphide	0.18	mg/kg
Zinc	9.20	mg/kg	Zinc acetate	68.50	mg/kg

TABLE 5.1 Mineral content per kilogram of the hamster diet.

	Week								
	1	4	7	10	13	16	19	22	25
Group 1									
1	91	98	106	113	123	132	145	149	157
2	79	99	118	128	130	135	143	154	163
3	60	87	112	120	126	135	142	138	-
4	74	-	-	-	-	-	-	-	-
5	75	98	112	118	124	131	139	139	141
6	92	108	124	130	149	144	134	130	129
7	87	103	117	110	119	128	125	134	133
8	74	100	115	123	128	132	143	146	140
9	89	106	119	128	138	144	153	163	154
10	90	115	136	145	156	159	163	171	169
11	86	105	115	120	122	127	126	137	134
12	90	112	127	138	143	154	170	179	179
13	83	107	121	127	131	138	143	143	143
14	79	101	113	123	132	140	152	158	150
Mean	**82.1**	**103.0**	**118.1**	**124.8**	**132.4**	**138.4**	**144.5**	**149.3**	**149.3**
S.D.	**9.1**	**7.2**	**7.7**	**9.5**	**11.1**	**9.7**	**12.9**	**14.9**	**15.5**
Group 2									
15	84	91	92	94	99	122	136	142	136
16	81	91	102	103	105	-	-	-	-
17	74	75	79	67	75	77	80	89	-
18	77	94	101	108	116	123	123	136	133
19	80	88	90	95	84	105	93	107	115
20	83	102	112	111	121	126	120	120	115
21	82	100	106	113	115	124	125	133	128
22	86	95	101	103	98	103	101	-	-
23	92	116	122	130	140	144	134	140	142
24	100	108	108	112	107	91	79	74	-
25	86	104	98	101	104	109	101	119	-
26	85	113	112	114	105	70	-	-	-
27	90	102	108	115	116	119	121	140	138
28	95	99	104	103	97	69	-	-	-
Mean	**85.4**	**98.4**	**102.5**	**104.9**	**105.9**	**106.3**	**110.3**	**120.0**	**129.6**
S.D.	**7.0**	**10.6**	**10.7**	**14.3**	**15.9**	**23.5**	**20.4**	**23.5**	**10.8**
Group 3									
29	101	115	122	125	137	140	138	139	133
30	76	89	96	95	96	108	116	131	142
31	79	96	102	105	107	118	125	139	141
32	71	89	99	97	97	107	-	-	-
33	77	88	105	108	114	127	137	142	137
34	82	101	95	105	110	120	110	116	123
35	88	97	106	114	116	137	139	147	142
36	94	99	102	110	109	113	119	119	119
37	84	100	100	105	115	126	133	131	124
38	83	92	97	94	96	92	78	65	-
39	76	95	102	119	124	119	100	89	78
40	72	79	85	86	88	101	105	108	95
Mean	**81.9**	**95.0**	**100.9**	**105.2**	**109.1**	**117.3**	**118.2**	**120.5**	**123.4**
S.D.	**8.9**	**8.9**	**8.6**	**11.1**	**13.6**	**14.1**	**19.1**	**25.1**	**21.5**

TABLE 5.2 The weekly weights (g) of animals in Groups 1, 2 and 3 with their means and standard deviations (S.D.).

Group	Week 1	2	3	4	5	6	7	8	9	10	11	12	13
1	84.1	89.8	97.7	103.2	104.7	114.4	118.3	119.8	121.4	125.2	128.0	129.8	132.6
2	85.6	93.7	98.3	98.6	98.0	101.4	102.8	104.4	104.9	105.0	106.5	105.7	106.6
3	82.1	90.2	95.7	95.1	94.5	99.7	101.1	103.7	104.7	105.4	106.5	106.8	109.4
1 v 2	NS	NS	NS	NS	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002
1 v 3	NS	NS	NS	<.02	<.02	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002
2 v 3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Group	Week 14	15	16	17	18	19	20	21	22	23	24	25
1	130.6	133.9	138.3	141.5	144.2	144.7	147.0	148.0	149.5	149.2	149.5	149.5
2	103.5	105.6	106.5	110.5	112.6	110.5	113.1	118.6	120.2	130.6	127.5	129.6
3	109.6	112.5	117.5	121.1	122.1	118.3	120.3	120.2	120.9	125.3	124.0	123.6
1 v 2	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.02	<.05	<.02	<.02
1 v 3	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.02	<.02	<.02	<.02	<.02
2 v 3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

TABLE 5.3 The mean weekly weight (g) of Groups 1, 2 and 3 animals with the statistical relationships between the groups when Mann-Whitney U tests were applied to individual values.

	Week								
	1	4	7	10	13	16	19	22	25
Group 1									
1	16.6	16.7	13.5	14.1	16.3	15.0	17.4	15.9	17.2
2	15.8	16.5	14.4	14.5	16.9	16.1	16.6	16.5	18.2
3	14.4	17.1	15.1	15.3	16.7	16.4	17.2	15.4	-
4	14.2	-	-	-	-	-	-	-	-
5	15.5	16.9	14.9	15.1	17.2	16.4	15.5	16.5	16.6
6	16.2	19.1	16.0	14.3	17.9	18.3	19.0	18.1	17.1
7	15.1	16.4	14.7	12.8	15.8	16.1	15.3	16.3	16.3
8	15.2	20.3	16.7	16.9	16.1	17.1	16.3	13.9	11.4
9	16.2	15.3	14.5	14.9	15.3	16.8	15.6	17.8	17.3
10	15.3	15.5	15.4	16.2	16.7	17.4	16.7	18.6	16.9
11	16.4	17.1	15.7	15.5	14.1	14.3	13.2	15.7	15.6
12	16.1	17.1	14.2	14.3	16.5	15.3	16.4	16.9	16.6
13	16.9	18.5	16.6	15.7	16.8	16.4	17.2	19.4	20.1
14	15.3	17.2	15.5	14.9	15.9	16.6	16.3	17.4	16.5
Mean	15.7	17.2	15.2	15.0	16.3	16.3	16.4	16.8	16.6
S.D.	0.8	1.4	0.9	1.0	0.9	1.0	1.4	1.5	2.0

TABLE 5.4 Haemoglobin values (g/dl) of Group 1 hamsters maintained on an iron and folic acid deficient diet.

	Week								
	1	4	7	10	13	16	19	22	25
Group 2									
15	16.4	15.2	10.5	10.5	12.4	12.5	12.6	16.6	17.4
16	16.8	13.5	11.8	10.9	10.8	-	-	-	-
17	16.0	12.3	10.4	9.1	9.4	11.3	10.2	12.4	-
18	15.9	14.5	10.9	10.8	13.4	11.4	11.4	16.1	16.0
19	16.0	13.6	10.5	10.1	8.7	11.8	9.6	13.5	14.4
20	16.8	14.6	13.0	12.7	14.8	15.4	14.8	17.4	17.8
21	16.8	14.0	12.2	11.8	15.0	11.7	10.9	14.9	15.3
22	16.1	14.7	11.4	11.1	11.5	9.8	9.6	-	-
23	15.7	14.6	12.6	12.8	14.1	12.8	11.0	13.8	14.4
24	15.2	13.5	12.1	11.1	10.6	8.7	7.9	10.7	-
25	16.9	14.4	12.2	12.0	11.3	11.4	9.5	12.8	-
26	15.7	14.8	12.2	11.0	11.3	8.3	-	-	-
27	16.3	16.2	12.5	11.6	13.5	11.1	12.0	12.3	13.6
28	15.5	13.5	11.1	10.0	10.2	7.8	-	-	-
Mean	16.1	14.2	11.7	11.1	11.9	11.1	10.9	14.0	15.6
S.D.	0.5	0.9	0.9	1.0	2.0	2.1	1.9	2.1	1.6

TABLE 5.5 Haemoglobin values (g/dl) of Group 2 hamsters maintained on an iron and folic acid deficient diet combined with repeated venesection.

Group 3	Week 1	4	7	10	13	16	19	22	25
29	15.8	15.7	13.2	13.1	14.1	14.8	10.4	16.8	16.5
30	15.7	14.0	11.0	9.8	12.7	10.6	11.1	11.7	13.3
31	17.1	13.3	11.6	10.5	12.7	12.1	11.4	11.7	16.1
32	16.1	14.1	12.6	10.9	12.4	10.7	-	-	-
33	16.6	13.7	11.5	10.7	11.9	11.7	12.4	12.6	13.2
34	16.1	14.7	10.8	9.3	9.3	13.1	12.7	12.1	15.4
35	17.0	14.5	12.3	10.7	13.4	12.4	13.0	12.1	14.1
36	16.4	14.4	11.2	9.9	10.7	9.9	10.2	12.5	14.2
37	15.4	14.6	11.4	10.9	13.2	12.0	11.7	12.3	-
38	16.1	13.2	10.8	8.2	8.4	10.5	10.5	11.6	14.9
39	15.7	14.6	13.0	11.7	11.9	12.1	10.4	10.1	10.1
40	15.0	14.2	12.8	10.7	14.3	12.6	11.9	11.8	14.4
Mean	16.1	14.2	11.8	10.5	12.1	11.9	11.4	12.3	14.2
S.D.	0.6	0.7	0.9	1.2	1.8	1.3	1.0	1.6	1.8

TABLE 5.6 Haemoglobin values (g/dl) of Group 3 animals maintained on iron deficient diet and repeated venesection.

Group	Week 1	4	7	10	13	16	19	22	25
1	15.7	17.2	15.2	15.0	16.3	16.3	16.4	16.8	16.6
2	16.1	14.2	11.7	11.1	11.9	11.1	10.9	14.0	15.6
3	16.1	14.2	11.8	10.5	12.1	11.9	11.4	12.3	14.2
1 v 2	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.02	NS
1 v 3	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002
2 v 3	NS	NS	NS	NS	NS	NS	NS	<.05	NS

TABLE 5.7 The mean heamoglobin values (g/dl) of Groups 1, 2 and 3 animals with the statistical relationships when Mann-Whitney U tests were applied to individual values.

	Week 1	4	7	10	13	16	19	22	25
Group 1									
1	549	340	239	264	65	121	155	141	125
2	756	381	308	442	233	119	249	269	272
3	425	361	219	281	178	146	208	219	-
4	682	-	-	-	-	-	-	-	-
5	742	320	251	276	126	123	152	171	159
6	579	406	232	143	137	117	132	75	70
7	490	296	262	169	160	135	236	173	134
8	583	291	248	147	104	89	161	238	312
9	541	294	254	214	213	147	243	153	111
10	675	286	193	143	176	119	167	165	206
11	467	345	239	214	163	167	220	250	239
12	509	327	229	178	166	130	188	224	252
13	459	311	311	161	220	133	237	206	179
14	713	355	282	240	183	167	230	206	205
Mean	583.6	331.8	251.3	219.5	163.4	131.8	198.3	191.5	188.7
S.D.	111.4	37.2	33.5	82.8	47.1	21.4	40.8	52.4	72.2

TABLE 5.8 The corrected whole blood folates (ng/ml) of Group 1 animals (iron and folate deficient diet).

	Week 1	4	7	10	13	16	19	22	25
Group 2									
15	476	357	354	210	205	176	268	146	240
16	590	530	367	514	328	-	-	-	-
17	691	479	247	216	106	189	338	182	-
18	635	544	487	641	572	622	887	346	365
19	579	520	299	229	293	367	569	161	518
20	484	503	350	382	478	233	394	107	189
21	616	485	361	492	273	250	474	210	555
22	611	478	280	437	276	345	1103	-	-
23	458	411	360	502	500	322	276	261	340
24	545	394	318	233	288	355	352	146	-
25	534	523	297	292	170	143	206	225	-
26	635	595	331	488	164	223	-	-	-
27	658	486	374	457	326	329	309	276	829
28	590	367	426	438	277	252	-	-	-
Mean	578.7	476.6	346.5	395.1	304.0	292.8	470.5	206.0	433.7
S.D.	70.8	70.0	60.6	136.6	133.3	123.0	282.1	72.7	219.5

TABLE 5.9 The corrected whole blood folates (ng/ml) of Group 2 animals (iron and folate deficient diet and repeated venesection).

Group 3	Week 1	4	7	10	13	16	19	22	25
29	566	1223	851	715	852	858	1129	487	580
30	693	964	914	1146	977	966	1078	958	825
31	505	824	959	1166	1031	1097	1033	973	741
32	749	752	825	873	842	753	-	-	-
33	561	781	867	983	1016	1079	1477	835	929
34	558	825	1045	1037	1412	1684	1545	988	708
35	612	709	867	897	745	925	1481	948	883
36	583	722	890	1096	1075	1019	1461	856	780
37	640	735	1150	1157	815	939	1454	939	1762
38	577	830	980	1390	1100	1149	2220	1336	-
39	796	883	1096	1249	1226	2383	4438	2345	3157
40	577	1032	937	1214	1032	1295	1530	966	899
Mean	**618.1**	**856.7**	**948.4**	**1076.9**	**1010.3**	**1178.8**	**1713.3**	**1057.4**	**1126.4**
S.D.	**86.3**	**151.2**	**102.5**	**186.2**	**186.9**	**448.7**	**958.7**	**469.8**	**782.5**

TABLE 5.10 The corrected whole blood folates (ng/ml) of Group 3 animals (iron deficient diet and repeated venesection).

Group	Week 1	4	7	10	13	16	19	22	25
1	583.6	331.8	251.3	219.5	163.4	131.8	198.3	191.5	188.7
2	578.7	476.6	346.5	395.1	304.0	292.8	470.5	206.0	433.7
3	618.1	856.7	948.4	1076.9	1010.3	1178.8	1713.3	1057.4	1126.4
1 v 2	NS	<.002	<.002	<.002	<.002	<.002	<.002	NS	<.002
1 v 3	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002
2 v 3	NS	<.002	<.002	<.002	<.002	<.002	<.002	<.002	<.002

TABLE 5.11 The mean values of corrected whole blood folates (ng/ml) of Groups 1, 2 and 3 animals and comparisons between individual values using Mann-Whitney U tests.

	GROSSLY COUNTED TUMOURS			TUMOUR SIZE (mm)		
	L Pouch	R Pouch	Total	1-2	2-5	5 or more
Group 1						
1	-	-	-			
2	-	-	-			
3	-	2	2	1		1
5	-	1	1			1
6	-	-	-			
7	1	-	1		1	
8	1	1	2		1	1
9	-	-	-			
10	1	1	2		2	
11	1	-	1		1	
12	-	-	-			
13	-	1	1	1		
14	-	-	-			
Total	**13**	**4**	**6**	**10**	**2**	**5**	**3**
Group 2						
15	1	2	3	1	2	
17	-	3	3		2	1
18	2	1	3	2	1	
19	1	-	1	1		
20	1	2	3	1	1	1
21	-	-	-			
23	-	2	2			2
24	-	2	2	1	1	
25	-	1	1			1
27	-	1	1			1
Total	**10**	**5**	**14**	**19**	**6**	**7**	**6**
Group 3						
29	-	-	-			
30	-	-	-			
31	-	-	-			
33	2	1	3	1	1	1
34	2	1	3	2	1	
35	3	2	5	2	3	
36	-	1	1	1		
37	2	2	4	1	2	1
38	-	-	-			
39	-	2	2		2	
40	2	2	4	1	1	2
Total	**11**	**11**	**11**	**22**	**8**	**10**	**4**

TABLE 5.12 The results of the gross counting of tumours and their size categories in Groups 1, 2 and 3 animals.

		NUMBER OF CARCINOMAS					
		L Pouch		R Pouch		Total	
		Exo.	Endo.	Exo.	Endo.	Exo.	Endo
Group 1							
	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	1	-	1
	5	-	-	-	1	-	1
	6	-	-	-	-	-	-
	7	-	-	-	-	-	-
	8	1	-	-	1	1	1
	9	-	-	-	-	-	-
	10	-	2	-	1	-	3
	11	1	-	-	-	1	-
	12	-	-	-	-	-	-
	13	-	-	-	-	-	-
	14	-	-	-	-	-	-
Total	13	2	2	-	4	2	6
Group 2							
	15	-	-	-	2	-	2
	17	-	-	-	1	-	1
	18	-	-	-	-	-	-
	19	-	-	-	-	-	-
	20	-	-	1	-	1	-
	21	-	-	-	-	-	-
	23	-	-	2	-	2	-
	24	-	-	-	-	-	-
	25	-	-	-	1	-	1
	27	-	-	-	-	-	-
Total	10	0	0	3	4	3	4
Group 3							
	29	-	-	-	-	-	-
	30	-	-	-	-	-	-
	31	-	-	-	-	-	-
	33	-	1	2	-	2	1
	34	-	1	-	-	-	1
	35	1	-	-	1	1	1
	36	-	-	-	-	-	-
	37	-	1	-	-	-	1
	38	-	-	-	-	-	-
	39	-	-	-	1	-	1
	40	-	1	1	2	1	3
Total	11	1	4	3	5	4	9

TABLE 5.13 Histologically identified squamous cell carcinomas and types of invasion in Groups 1, 2 and 3 animals.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

6.1 ANALYSIS OF WEIGHT RESULTS

Comparison of animal weights in different experiments is restricted by many factors. Animals age or weight at the start of the experiment, their sex, living conditions in the animal house, the physical and nutritional properties of diet, and even the season during which the experiments start are importent. These conditions, with the exception of sex matching, have been fulfilled when animals in the folate deficiency study reported in Chapter 4 and the combined iron and folate deficiency study reported in Chapter 5 were compared to each other. Although it has been suggested that female hamsters have a slightly higher weight range than male hamsters (McPherson, 1987), there is a big overlap between them. In addition, comparisons between the pellet diet control hamsters in Group 1, Chapter 4 (males) and Group 3, Chapter 4 (females) showed similar weight ranges and means with no statistically significant differences between them. This observation was further confirmed when the powder diet control hamsters in Group 2, Chapter 2 and Group 2, Chapter 4 were compared to each other using the Mann-Whitney U tests.

Comparison of animal weights in the powdered diet groups of Chapters 4 and 5 at the start of each study showed that animals in both experiments started with similar weights with no statistically significant differences present between the groups. On week 10 the mean weight of the powdered diet control animals in Group 1, Chapter 4 was 134.2g. This is compared to 120.3g in the folate deficient animals (Group 1, Chapter 4), 125.2g in the combined iron and folate deficiency (Group 1, Chapter 5), 105.0g in the bleeding group of the combined iron and folate deficiency (Group 2, Chapter 5) and 105.4g in the iron deficient animals (Group 3, Chapter 5).

Comparisons between these groups are shown in Table 6.1. Animals expressed iron deficiency anaemia in each of Groups 2 and 3 of Chapter 5 were very significantly smaller than any of the other groups ($P<.002$ in each case). Although animals in the combined iron and folate deficiency without bleeding in Group 1, Chapter 5 which showed low folate level and normal haemoglobin levels had a slightly larger mean weight value on week 10 than the folate deficient animals in Group 1, Chapter 4, they were significantly smaller than the powdered diet controls. The folate deficient animals of Group 1, Chapter 4 were significantly smaller than the powdered diet control animals on week 11. This is due to a more uniform distribution of weight values in the combined deficiency group than the folate deficiency group.

It is concluded that iron deficiency has more influence on animal weights than folate deficiency. However, both iron and folate deficiencies individually influence growth rate of animals

significantly. The combined iron and folate deficiency groups (Groups 1 and 2, Chapter 5) influenced animal growth rates according to the single deficiency developed in each one of them. None of the groups of this study developed a true combined iron and folate deficiency and therefore the effect of such combined deficiency on the animal weight was not determined in this study.

6.2 ANALYSIS OF FOLATE RESULTS

Comparison between folate estimations in the folate deficient animals of Group 1, Chapter 4 and animals in the combined iron and folate deficiency without bleeding in Group 1, Chapter 5 are shown in Table 6.2. Diet lacking folic acid alone resulted in a faster development of more severe folate deficiency than diet lacking both iron and folic acid which only caused low but not deficient folate levels. Animals maintained on combined iron and folic acid deficient diet coupled with repeated venesection (Group 2, Chapter 5) showed significantly higher folate levels ($P<.002$) than animals receiving combined iron and folic acid deficient diet without bleeding (Group 1, Chapter 5).

Interpretation of such opposite trends between folic acid and haemoglobin levels is difficult. However, it is reasonable to suggest that folic acid deficiency as assessed by corrected whole blood folate can be masked by an accompanied iron deficiency or chronic blood loss.

6.3 ANALYSIS OF HAEMOGLOBIN RESULTS

Diet deficient in folic acid alone (Group 1, Chapter 4) or combined iron and folic acid without bleeding (Group 1, Chapter 5) did not result in iron deficiency. Iron deficient (Group 3, Chapter 2) or iron sufficient (Group 4, Chapter 2) diet each coupled with repeated blood withdrawal of 1.5ml every two weeks also failed to induce iron deficiency anaemia in the hamster. Only when blood withdrawal was increased to 2.3ml every two weeks was there resultant iron deficiency anaemia in animals receiving an iron deficient diet. This iron deficiency occured regardless of the presence or absence of folic acid in the diet. However, haemoglobin levels did not go below 10.5g/dl at any week of the study. Haemoglobin levels were also found to be very sensitive to trace amount of iron in the diet or to a temporary reduction of blood loss as discussed in Section 5.3.2.

It was concluded that the presence or absence of folic acid in the diet did not affect haemoglobin levels of hamsters developing iron deficiency. Results obtained from the studies reported in Chapters 2, 4 and 5 suggest that the weekly amount of blood withdrawn from the hamster has the major influence on haemoglobin levels when iron is deficient in the diet. These results are in agreement with the results obtained by Rennie et al. (1982b). The severe iron deficiency anaemia in the hamster reported by Ranasinghe et al. (1983) could not be achieved in this study even when folic acid was completely removed from the diet and blood withdrawal increased to

2.3ml every two weeks which is more than double the amount removed in Ranasinghe's study. It is possible that this difference is a result of differet strains of hamsters being used.

6.4 TUMOUR YIELD IN THE DIFFERENT NUTRITIONAL DEFICIENCIES

Analysis of the results of the gross counting of tumours in the pellet diet control groups of Chapters 2 and 4 shows that 11 of 12 (91.7%) hamsters of Group 1, Chapter 2 developed a total of 52 tumours whereas all eleven hamsters (100%) of Group 3, Chapter 4 develpoed a total of 49 tumours. In the powdered diet control groups 8 of 9 hamsters (88.8%) in Group 2, Chapter 2 and 8 of 10 hamsters (80%) in Group 2, Chapter 4 developed a total of 36 tumours each.

The result of the histological identification of carcinomas in the pellet diet control groups showed that 11 of 12 (91.7%) of hamsters in Group 1, Chapter 2 developed a total of 35 carcinomas and 10 of 11 animals (90.9%) in Group 3, Chapter 4 also had 35 carcinomas. In the powdered diet control groups 8 of 9 (88.8%) of hamsters in Group 2, Chapter 2 developed 20 carcinomas while 9 of 10 (90%) of animals in Group 2, Chapter 4 had a total of 23 carcinomas.

No statistically significant differences in the numbers of animals developing tumours were found between control groups receiving pellet diet or between control groups receiving the powdered diet in Chapters 2 compared to Chapter 4. Also no statistically significant differences in the number of animals developing tumours were present

between control animals fed the powdered diet compared to controls who received pellet diet either in the same experiment or between the two experiments reported in Chapters 2 and 4. The were no statistically significant differences in the numbers of tumours counted grossly or the numbers of carcinomas identified histologically between these control groups when compared to each others. However, in both studies of Chapters 2 and 4 the powdered diet controls developed fewer tumours and carcinomas than the pellet diet controls.

A possible explanation for the higher incidence of tumours in animals on pellet diet could be due to greater physical irritation from the diet. The experimental evidence recording the effect of physical irritation was discussed in Section 1.5.6.

The results of the gross counting of tumours were compared between the folate deficient animals (Group 1, Chapter 4) and the pellet diet control groups of Chapters 2 and 4. It was found that only four (44.4%) of the folate deficient animals developed tumours whereas 91.6 per cent and all of the pellet diet controls in Chapters 2 and 4 respectively developed tumours. The folate deficient animals differed significantly from the controls of Chapter 4 ($P<.02$) and significantly from controls of both studies in Chapters 2 and 4 when individual pouches were considered separately ($P<.01$ in each case). The folate deficient animals developing tumours were also significantly fewer than those in the powdered diet controls of Chapters 2 and 4 ($P<.01$ and $P<.05$ respectively) when the numbers of pouches were considered separately. The folate deficient animals also

developed only eight tumours which is very significantly less than in any of the four control groups of Chapters 2 and 4 ($P<.001$ in each case).

Histologically, only two animals in the folate deficiency group had malignant tumours and in these animals six carcinomas were seen. By contrast many more carcinomas were seen in the control animals. When folate deficient animals were compared to controls it was found that the folate deficient animals developing carcinoma were significantly fewer than in either the pellet diet controls ($P<.01$ in each case) or the powdered diet controls ($P<.02$ in each case) of Chapters 2 and 4 respectively. The numbers of carcinomas seen in the folate deficient group were also significantly less than in either of the pellet diet controls ($P<.001$ in each case) or the powdered diet controls ($P<.01$ in each case) of Chapters 2 and 4 respectively.

The iron deficient animals in Group 3, Chapter 5 were compared to animals in the control groups of Chapters 2 and 4. There were no statistically significant differences either in the number of animals developing tumours or in the number of animals developing carcinoma between the iron deficient animals and any of the control groups. However, the number of tumours seen grossly in the iron deficient animals (22 tumours) was significantly less than in either of the pellet diet controls ($P<.01$ in each case) or in the powdered diet controls ($P<.01$ and $P<.05$) of Chapters 2 and 4 respectively. Histologically, the number of carcinomas recorded in the iron deficient animals (13) was significantly less than the number carcinomas present in either of the pellet diet controls ($P<.01$ and

P<.001) of Chapters 2 and 4 respectively. However, no statistically significant differences in the number of carcinomas were present between the iron deficient group and powdered diet controls of Chapters 2 and 4.

Comparison was also made between the folate deficient and iron deficient animals (Group 1, Chapter 4 and Group 3, Chapter 5 respectively). No statistically significant differences either in the number of animals with grossly seen tumours or in the number of animals with histologically confirmed carcinomas were present between the two groups. Despite the number of grossly counted tumours being significantly more in the iron deficient than in the folate deficient animals (P<.05), the number of carcinomas was not significantly different.

Animals in the combined iron and folate deficiency without bleeding (Group 1, Chapter 5) which showed low folate and normal haemoglobin levels showed a similar but less prominent trend to that of the folate deficient animals of Group 1, Chapter 4 when compared to control groups in Chapters 2 and 4. Grossly, significantly less animals in this group (7 in 13) developed tumours than the pellet diet controls of Chapter 4 (P<.05). When pouches were considered separately, there were significantly fewer pouches with tumours (9 in 26) in the combined iron and folate deficiency group than in either pellet diet (P<.01 and P<.001) or powderd diet (P<.01 and P<.02) control groups of Chapters 2 and 4 respectively. There were also very significantly less tumours in the combined deficiency group (10

tumours) than in any of the four pellet or powdered diet control groups ($P<.001$ in each case).

Histologically, there were significantly fewer animals with carcinomas in the combined deficiency group without bleeding (5 in 13 animals) than in either of the pellet diet controls of Chapters 2 and 4 ($P<.02$ and $P<.05$ respectively) or the powder diet control group of Chapter 4 ($P<.05$). The number of carcinomas seen in this group (8 carcinomas) was also very significantly less than in either of the pellet diet ($P<.001$ in each case) or the powdered diet ($P<.01$ and $P<.001$) control groups of Chapters 2 and 4 respectively.

The combined iron and folate deficiency group without bleeding (Group 1, Chapter 5), which only showed reduced folate levels, did not differ from the folate deficiency group (Group 1, Chapter 4) either in the numbers of animals with grossly identified tumours or in the number of animals with carcinoma. There were also no statistically significant differences between these two groups when the numbers of tumours counted grossly or the number of carcinomas identified histologically were considered.

Animals which received diet lacking iron and folic acid and were bled repeatedly (Group 2, Chapter 5) developed iron deficiency anaemia but had normal folate levels. When these animals were compared with the normal control groups of Chapters 2 and 4 it was found that there were no statistically significant differences in the numbers of animals developing grossly counted tumours or histologically identified carcinomas between these groups. However,

the number of tumours counted grossly was significantly less in the combined deficiency group than in either of the pellet diet ($P<.01$ in each case) or the powdered diet ($P<.01$ and $P<.05$) control groups of Chapters 2 and 4 respectively. Histologically, there were significantly less carcinomas in the combined deficiency group than in either of the pellet diet ($P<.001$ in each case) or powdered diet ($P<.01$ in each case) control groups of Chapters 2 and 4 respectively.

Comparisons between the bleeding animals in the combined iron and folate deficiency group (Group 2, Chapter 5) and animals in the iron deficiency group (Groups 3, Chapter 5) which have both shown iron deficiency anaemia and normal folate levels revealed no statistically significant differences between them either in the numbers of animals developing tumours or carcinomas or in the actual numbers of tumours or carcinomas. However, each of these two groups had significantly more grossly-counted tumours ($P<.02$ and $P<.01$ respectively) than in the non-bleeding group of the combined iron and folate deficiency (Group 1, Chapter 5) which showed normal iron and reduced folic acid levels.

6.6 FINAL CONCLUSIONS AND FUTURE STUDIES

It has been confirmed that folic acid deficiency is readily inducible in the golden hamster. Iron deficiency is difficult to induce in this animal model. Chronic blood loss is the single most important factor that determines the time required for such induction provided that the iron content of the diet is kept to a minimal level. Deficiency of folic acid in the diet has no real effect on the severity of iron deficiency anaemia, while an iron deficient diet prevents folate levels decreasing to clinically detectable deficiency levels. The mechanism of such action is not known.

Both iron and folic acid deficiencies whether individually or combined significantly suppress animal growth. Iron deficiency affects weight more severely than folic acid deficiency while the combined dietary deficiency of iron and folic acid influences growth to a degree depending on the single deficiency that predominates.

An iron deficient diet resulted in thinner oral epithelium as estimated by quantitative compartment analysis. However, it is not known whether such thin epithelium was the result of a direct action of iron deficiency on the metabolism of the epithelium itself or the result of generalised suppresion of tissue growth as indicated by the reduced animal weight. Whether the effect is direct or indirect, iron deficiency reduces the epithelial compartment thicknesses.

As iron and folic acid support tissue growth in general, therefore it is expected that deficiency in either one of them or both of them combined would suppress tumours growth as well. However, in this study it was found that each of iron and folic acid deficiency individually or combined suppressed tumour development and reduced cancer incidence in the hamster cheek pouch. Carcinoma induced under such deficiencies developed earlier and invaded the deeper tissues of the pouch faster than under normal circumstances. Moreover, in iron deficient animals carcinoma in frequent cases was noticed to invade the deeper tissues without passing through the stages of papilloma and exophytic growth usually seen in the hamster cheek pouch carcinogenesis.

It was noticed that the physical properties of the diet influence tumour and cancer incidence in the hamster cheek pouch epithelium. The use of a powder diet resulted in significantly fewer tumours than in the pellet diet control animals.

Further work is required to develop the golden hamster as an animal model of iron deficiency. Also more work is required to duplicate the findings reported in this research on the effect of iron deficiency and combined iron and folic acid deficiencies on carcinogenesis. There is also a requirement for study of the effects of different stages of iron deficiency on carcinogenesis.

		MEAN WEEKLY WEIGHT (g)											
		Week 1	2	3	4	5	6	7	8	9	10	11	12
NUMBER	ANIMALS												
1	G1 CH4	83.0	90.6	103.3	105.8	111.3	119.2	120.4	116.6	115.2	120.3	123.0	127.1
2	G2 CH4	84.0	94.6	108.4	113.6	118.7	125.3	127.9	124.8	128.5	134.2	138.7	144.7
3	G1 CH5	84.1	89.8	97.7	103.2	104.7	114.4	118.3	119.8	121.4	125.2	128.0	129.8
4	G3 CH5	82.1	90.2	95.7	95.1	94.5	99.7	101.1	103.7	104.7	105.4	106.5	106.8
		MANN-WHITNEY U TEST											
1 v 2		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	<.05	<.05
1 v 3		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1 v 4		NS	NS	<.05	<.05	<.02	<.02	<.02	<.02	<.05	<.02	<.02	<.02
2 v 3		NS	NS	<.02	<.02	<.002	<.02	<.02	NS	NS	<.05	<.05	<.02
2 v 4		NS	NS	<.02	<.002	<.002	<.022	<.002	<.002	<.002	<.002	<.002	<.002
3 v 4		NS	NS	NS	<.02	<.02	<.002	<.002	<.002	<.002	<.002	<.002	<.002

TABLE 6.1 The mean weight values (g) of animals in Groups 1 and 2 of Chapter 4 and Groups 1 and 3 of Chapter 5 with their statistical relationships when Mann-Whitney U tests were applied to individual values.
G = Group CH = Chapter

	MEAN WEEKLY FOLATE (ng/ml)							
Week	1	4-5	7-8	10-11	13-14	16-17	19-20	25-27
ANIMALS								
G1 CH4	513.7	269.2	178.1	196.0	130.8	140.7	117.5	74.7
G1 CH5	583.6	331.8	251.3	219.5	163.4	131.8	198.3	188.7
	MANN-WHITNEY U TEST							
	NS	NS	<.002	NS	<.05	NS	<.002	<.002

TABLE 6.2 Comparisons between corrected whole blood folate levels in animals maintained on folic acid deficient diet (Group 1 Chapter 4) and animals receiving diet lacking both iron and folic acid (Group 1 Chapter 5).
G = Group CH = Chapter

Group & Chapter	Animals with Tumours Grossly	Number of Tumours Grossly			Number of Animals with Carcinoma	Number of Carcinomas		
		L Pouch	R Pouch	Total		Exophytic	Endophytic	Total
CONTROLS								
G1 CH2 pellet	11/12	29	23	52	11/12	16	19	35
G3 CH4 pellet	11/11	25	24	49	10/11	19	20	39
G2 CH2 powder	8/9	20	16	36	8/9	14	6	20
G2 CH4 powder	8/10	17	19	36	9/10	10	13	23
FOLATE DEFICIENT DIET-NO BLEEDING (FRANK FOLATE DEFICIENCY)								
G1 CH4	4/9	6	2	8	2/9	0	6	6
COMBINED IRON AND FOLATE DEFICIENT DIET-NO BLEEDING (SIGNIFICANTLY LOW FOLATE BUT NORMAL HAEMOGLOBIN LEVELS)								
G1 CH5	7/13	4	6	10	5/13	2	6	8
COMBINED IRON AND FOLATE DEFICENT DIET-WITH BLEEDING (IRON DEFICIENCY ANAEMIA AND NORMAL FOLATE LEVELS)								
G2 CH5	9/10	5	14	19	5/10	3	4	7
IRON DEFICIENT DIET-WITH BLEEDING (IRON DEFICIENCY ANAEMIA)								
G3 CH5	7/11	11	11	22	6/11	4	9	13

TABLE 6.3 Tumours and carcinomas seen in animals from different groups of the studies reported in Chapters 2, 4 and 5.

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APPENDIX 1

Klensorb (Sorboil): B & D Clays and Chemicals Ltd.

Klensorb is composed of Illite and montmorillonite with other trace minerals. The combination of the two clays results in a material with an extraordinarily porous structure, thereby producing large absorption and adsorption capacity. The chemical composition of Klensorb is shown below;

Typical ChemicalAnalysis

Silicon	Silicon dioxide	60.8
Magnesium	Magnesium oxide	20.4
Aluminium	Aluminium oxide	4.6
Potassium	Potassium oxide	1.1
Calcium	Calcium oxide	1.2
Sodium	Sodium oxide	0.4
Iron	Ferric oxide	1.2
Ignition loss	Largely water	10.3

General Characteristics:

Colour	light cream
Average dimensions of particles	800μm x 25μm x 4μm
Porosity vol/vol	17%
Specific weight	2-2.1 g/cc
Melting point	1550°C
Specific surface	330m^2/g
ph in aqueous suspension (10%)	-8/9

Health and safety Information

No special precautions are necessary when this product became in contact with eyes, skin or was ingested. Inhalation of its dust should be kept as low as is reasonably practicle.

Handling, Storage and Disposal

Storage	Indefinite shelf life but store in dry conditiona.
Handling	Avoid creating dust in working atmosphere.
Spillage	Vacuum or wash away with water.
Disposal	No special precautions.

APPENDIX 2

Roebuck Absorbent Granules

Roebuck Absorbent Granules consists of attapulgite/montmorillonite aggregate and some other minerals as listed below;

Typical Chemical Analysis (dry basis)

Silicon	Silicon dioxide	63.4%
Magnesium	Magnesium oxide	10.8%
Aluminium	Aluminium oxide	9.3%
Potassium	Potassium oxide	0.2%
Calcium	Calcium oxide	8.4%
Carbon	Carbon dioxide	5.0%
Iron	Ferric oxide	2.9%

General Characteristics

Colour	White
Granulometry	0.5-4mm (5/35 mesh)
Density	Approx. 520kg/cubic meter.
Absorption	water 105% oil 70% (using Westinghouse method)

Health and Safety Data

Inflammability	None
Skin irritation	None irritant
Eye irritation	Extremely low order
Acute oral toxicity	Minimal
Inhalation	Very low. Dust levels should be kept as low as possible.