THE ORAL MUCOSA IN IRON DEFICIENCY

Volume 1 of Two Volumes

JAMES STARK RENNIE

B.D.S., F.D.S.R.C.P.S. (GLASG)

THESIS

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Department of Oral Medicine and Pathology

Glasgow Dental Hospital and School

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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PREFACE

The work described in this thesis was undertaken in the Department of Oral Medicine and Pathology, University of Glasgow, during the period October 1973 to September 1976. This work was supported by a Clinical Research Fellowship from the Medical Research Council.

Some of the techniques used in this thesis are modifications of previously published work and some are original techniques developed by the author in conjunction with the supervisors. 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 technical staff under the direct supervision of the author.

Parts of this study have been presented at scientific meetings:

1. "Experimental Iron Depletion in the Hamster"
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   International Association of Dental Research,
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2. "Changes in Human Oral Mucosa in Iron Deficiency"
   British Society for Oral Pathology, Glasgow,
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3. "Experimental Iron Deficiency with Anaemia in the Hamster" with D. G. MacDonald.
International Association for Dental Research, Manchester, April 1976.

4. "Oral Epithelial Cell Kinetics in Iron Deficiency" with D. G. MacDonald.
International Association for Dental Research, Sheffield, April 1979.

SUMMARY

In many parts of the world iron deficiency is the most common nutritional deficiency. The oral manifestations of this disease are well known but objective quantitative studies of the oral mucosa in iron deficiency are lacking. The purpose of this study was to investigate and quantify any changes occurring in the oral mucosa in iron deficiency.

The initial work in this study was an investigation of buccal mucosa from patients with established iron deficiency anaemia. Using stereological techniques a significant reduction in the thickness of buccal epithelium from anaemic patients was found and marked alterations in the composition of the epithelium were demonstrated. Some difficulty was experienced in obtaining uniform control and experimental groups placing limitations on the conclusions which could be drawn from this study.

To overcome these problems it was desirable to use an experimental animal model. An experimental animal model of iron deficiency using the hamster was developed and an analysis of the ventral tongue epithelium from animals with different stages of iron deficiency was carried out. Significant alterations in the composition of the epithelium were demonstrated and these changes were similar to those seen in man.

In order to investigate the nature of the structural changes demonstrated in iron deficiency an in vitro cell kinetic study was undertaken. An increased cell production rate was found in the epithelium of animals with iron deficiency anaemia.

The correlation between the cell kinetic and stereological findings was discussed and the application of the quantitative methods used in this study to other related aspects of iron deficiency and the oral mucosa were described.

GENERAL INTRODUCTION

Iron deficiency affects a large proportion of the world's population and the oral manifestations of this disease are well known. However, subjective and semi-quantitative histological investigations have revealed differing results and have failed to explain the nature of the oral manifestations of iron deficiency.

The aim of the present study was to investigate and quantify any changes occurring in the oral mucosa in iron deficiency. The study is reported in five chapters (chapters 2-6) which describe a progression of the research questions being asked.

In Chapter 1 a general review of the literature about iron, iron metabolism and iron deficiency is presented.

Chapter 2 contains a brief discussion of stereological methods and their application to the quantification of oral mucosa. The results of a study of the buccal mucosa in iron deficiency are reported and the problems associated with human studies highlighted.

To minimise the difficulties associated with human investigations an animal model of iron deficiency was developed. Chapters 3 and 4 describe the development of this model using the hamster and the results of a quantitative

analysis of the epithelium of the ventral surface of tongue during the different stages of iron deficiency are reported.

Chapter 5 contains a short discussion of cell kinetics, the methods of investigation of cell production and their application to studies of the oral mucosa. The results of a preliminary in vitro cell kinetic investigation in the hamster are presented.

Chapter 6 records a more thorough investigation of the cell kinetics of the epithelium of the ventral surface of tongue. The data obtained allows an assessment of the experimental techniques to be made and provides information on the cell renewal in normal and iron deficient hamsters.

In Chapter 7 a general discussion of the experimental findings is presented.

CHAPTER 1

IRON IN MEDICINE

INTRODUCTION AND REVIEW OF LITERATURE

1.1 HISTORICAL INTRODUCTION

Iron is one of the most commonly occurring elements in the universe. It is believed that iron is present in significant amounts in interstellar dust and may make up 40 per cent of the weight of certain meteorites (Neilands, 1974). Within the earth's crust aluminium is the only metal to occur in greater quantity than iron.

Iron exists, as do the other transition metals, in more than one valency state. The commonly occurring forms are ferrous which is divalent (Fe^{++}) and ferric (Fe^{+++}) which is trivalent. It is the stability and morphology of these forms, together with their ability to donate and to accept electrons, that allows iron to serve in electron transport and electron coupling systems (Spiro and Saltman, 1974). So great is the diversity and frequency of iron in biological systems that it is unlikely that life as we know it could exist in the absence of iron (Fairbanks et al, 1971).

Iron has been used in medicine for at least 3000

years. The Ebers Papyrus, an early Egyptian manuscript dating from around 1500 B.C. consists largely of a collection of prescriptions, and records several remedies containing iron. These are the oldest written prescriptions for iron (Bryan, 1931).

The early Greek physicians who used iron for a variety of ailments, believed it to be of heavenly origin and attributed its properties to the war god Mars.

The association between iron and blood appears to have been drawn first from the colour similarities of blood stains and rust or iron ores. Exactly when this analogy arose is uncertain, although Arabic physicians described "blood iron stone" in the 10th-century A.D. (Cumston, 1921).

According to Christian (1903) definite evidence linking iron and blood did not appear until the 17th-century when two English physicians Sydenham and Willis in 1681 found iron salts to be of value in the treatment of anaemia. This observation was substantiated in the early part of the 18th-century by Lemery and Geoffrey, who demonstrated that iron was present in ashed blood. Mengheni, in the middle of the 18th-century showed that the iron content of blood was increased in animals fed an iron rich diet. Further confirmatory evidence was provided in 1812 by Foedisch who demonstrated

that the iron content of blood from patients with iron deficiency anaemia was lower than that of non-anaemic individuals.

By the early 19th-century iron was recognised as an aetiological factor and as a therapeutic agent in many diverse disorders. At the same time, a clearer concept of the role of iron in biological systems was developing. Funke in 1851 identified haemoglobin, and Hoppe-Seyler in 1862 obtained haemoglobin in crystalline form. Following the observations by MacMunn (1886, 1887) on iron and cell respiration, Keilin (1925) demonstrated that iron, through its presence in the cytochrome system, was concerned with cellular respiration in all living cells. The description of the flavoprotein system (Richert and Westerfield, 1954) revealed another intracellular electron transport system having iron as an essential and active component.

Around the same time several groups of workers, (Fontes and Thivolle, 1925; Warburg and Krebs, 1927) demonstrated the presence of 'easily split-off' iron in plasma. Barkan (1927) indicated that this plasma iron was bound to a protein. However, identification and characterisation of the structure of this globulin had to wait until the mid-1940's when, Holmberg and Laurell (1945), Schade and Caroline (1944, 1946), and Surgenor et al (1949) all contributed greatly to the knowledge of the iron-binding protein transferrin.

The presence of iron within ribonucleic acid (RNA) was described by Wacker and Vallee (1959) and a role was suggested for the metal in the transfer of genetic information. Robbins and Pederson (1970) showed that the nucleolus was a repository for protein bound iron and an essential role for iron in cell division was suggested. The concept of the role of iron has thus changed from "a singular preservative against all poysons, sorcerers or enchantments" (Plinius, 1601) to that of a fundamental constituent of many different biological systems.

Before discussing the effects of iron deficiency upon the oral epithelium, it is thought necessary to describe some of the general principles of iron metabolism.

1.2 IRON - SOME PHYSIOLOGICAL CONCEPTS

1.2.1 Distribution of Body Iron

Normal adult males have around 50 mg of iron per kilogram of body weight (Cecil and Loeb, 1974; Harrison, 1974). Women, with a smaller red cell mass and lower iron stores have an iron concentration of about 35 mg per kilogram and consequently a smaller total body iron. In general total body iron varies from 2 g in small women to 6 g in large men (Cecil and Loeb, 1974). Similar tissue concentrations of iron have been described in the rat and differences between species do not appear to be great (Moore and Dubach, 1962), (Table 1.1).

Approximately 70 per cent of body iron is found in functional forms, particularly haemoglobin, while about 30 per cent exists in the storage forms haemosiderin and ferritin. Most of the functional iron is present within the red blood cells as haemoglobin. Myoglobin, a molecule about a quarter the size of haemoglobin, contains much of the remaining functional iron. The smallest functional iron compartment consists of the intracellular iron containing enzyme systems and the iron transport system (Cecil and Loeb, 1974), (Table 1.2). There are some species differences in the distribution of body iron. For example myoglobin rich species such as horse and dog have a smaller proportion of body iron as haemoglobin and more in the form of myoglobin (Hahn, 1937).

1.2.2 Functional Iron Compartments

Haemoglobin

Haemoglobin is a complex of a basic protein, globin, surrounding four ferro-protoporphyrin or haem groups. Each of these haem groups is a polypeptide of about 150 amino acids. Normal adult haemoglobin has a molecular weight of around 68,000 daltons and contains 3.4 mg of iron per gram of haemoglobin (Underwood, 1956; Fairbanks et al, 1971). The exact method by which iron becomes incorporated into protoporphyrin is not known. However, reticulocytes take up iron from transferrin by an active process, and following uptake iron is reduced and incorporated into haemoglobin (Morgan, 1974). Within

haemoglobin, iron is stabilised and the capacity of iron to undergo reversible bonding to oxygen, allows haemoglobin to function as an oxygen carrier (Underwood, 1956).

Myoglobin

Myoglobin is the red pigment of muscle. It has a molecular weight of about 17,000 daltons. Myoglobin is related in structure to the monomeric units of haemoglobin. It consists of a single globin chain attached to a haem group and carries a single iron atom (Dallman, 1974). Myoglobin acts as an oxygen reservoir for use during muscle contraction.

Enzyme Iron

Although enzyme iron is the smallest iron compartment it is of fundamental importance in cell respiration. An idea of the importance of the iron enzymes can be gained from the fact that 11 of the enzymes or co-factors involved in aerobic glycolysis contain or require iron for normal function (Fairbanks et al, 1971).

Iron enzymes are generally found in the inner mitochondrial membrane (Nicholls and Elliott, 1974). Within these enzyme systems the ability of iron to undergo reversible valence changes allows it to play an important role in electron transport.

The iron enzymes may be divided into three groups (Fairbanks et al, 1971).

1. Haem Proteins

The most important of the haem proteins are the cytochromes which are electron carrying and transferring proteins containing iron-porphyrin groups. They act principally in the transfer of electrons originating from various dehydrogenase systems, and undergo reversible Fe^{++} - Fe^{+++} valence changes during their catalytic cycles. The cytochromes are found only in aerobic cells and although other haem enzymes such as peroxidase and catalase are present in mammalian cells the cytochromes are the most important.

2. Flavoproteins

These enzymes function in the oxidative degradation of pyruvate, fatty acids and amino acids as well as in electron transport. The principal metalloflavoproteins are succinic dehydrogenase and NADH.

3. Enzymes Requiring Iron as a Co-factor

There are several enzymes in this group with a variety of functions but aconitase is perhaps the best known. The role of aconitase in the conversion of citrate within the Krebs cycle requires iron, Fe^{++}, which forms a stable complex with citric acid.

Iron and Nucleic Acids

Most intracellular iron is present within mitochrondria (Alfaro and Heaton, 1974) but significant amounts are

present in the nucleus. Robbins and Pederson (1970) indicated that the nucleolus stored iron within the interphase nucleus. Further, it was suggested that a transfer of iron to the chromosomes occurred during mitosis and that iron was crucial in the initiation and maintenance of deoxyribonucleic acid (DNA) synthesis (Robbins and Pederson, 1970). Iron has also been shown to have an effect upon the stability of DNA structure (Eichhorn, 1962).

Wacker and Vallee (1959) demonstrated that iron was tightly bound to RNA. Its function appears to be in stabilising the secondary and tertiary structure of the RNA molecule and in such a capacity it plays a part in the expression of genetic information.

Iron Transport

Iron within the blood stream is completely bound to the β globulin transferrin. Transferrin is not a single molecule but a species of proteins with variable, genetically controlled, amino acid sequences. Each transferrin molecule can carry two atoms of iron (Fe^{+++}), but normally only about one third of plasma transferrin binds iron.

The iron bound to transferrin is not static, but is turning over continuously (Bothwell and Finch, 1962), however, at any time only 3-4 mg of iron are present in serum. Although this is a small amount relative to total body iron

this fraction occupies a central role in iron metabolism since all iron being transported within the body must pass through this compartment.

The prime function of transferrin is to carry ionic iron to sites where it will be utilised, particularly to the reticulocytes. However, transferrin and related proteins may also play a part in host defences as they have been reported to be bacteriostatic and fungistatic agents (Schade and Caroline, 1946).

1.2.3 Iron Stores

Normally iron is stored in the liver, spleen and bone marrow. Adult males have about 1 g of iron stored as ferritin or haemosiderin.

Ferritin is a water-soluble complex and consists of a core of ferric ions surrounded by a shell of the protein apoferritin (Harrison et al, 1974). Ferritin may contain up to 20 per cent by weight of iron (Underwood, 1956). By contrast, haemosiderin is an insoluble, amorphous compound the exact nature of which is not known. It may represent iron-laden organelles or an aggregation of breakdown products of ferritin (Harrison et al, 1974). Haemosiderin contains slightly more iron than ferritin (up to 35 per cent by weight) (Underwood, 1956).

There appears to be a close functional relationship

between ferritin and haemosiderin. Ferritin normally accounts for slightly more than half of the stored iron. However, if iron stores increase, the balance alters and haemosiderin increases relative to ferritin (Bothwell and Finch, 1962).

Iron can be mobilised from both storage sources when required. Ferritin stores iron in a soluble form and acts as a short and medium term reserve. Haemosiderin is a less metabolically active compound, and stores iron as a long term reserve (Harrison et al, 1974).

1.2.4 Iron Absorption

Absorption of iron is a complex phenomenon influenced by a considerable number of factors. Excellent reviews of this subject have been presented by Bothwell and Finch, (1962) Fairbanks et al (1971) and Turnbull, (1974).

Iron is absorbed mainly from the duodenum and jejunum although small amounts may be absorbed from the stomach (Dagg et al, 1967). The ileum has a limited capacity for iron absorption and in laboratory animals some absorption may occur in the colon (Wheby et al, 1964).

Iron absorption takes place in three stages. Iron uptake by the cells of the mucosa is followed by transfer through the cell and then release into plasma. Both iron uptake by the cell and transfer of iron across

the cell are active processes (Jacobs et al, 1966).

The mechanisms regulating and effecting iron absorption still remain a mystery although the bulk of the evidence suggests that the concentration of iron within the mucosal cells is important. Recent work has identified iron-binding proteins in the cell wall of the intestinal epithelium but the method by which they work is not yet clear (Worwood and Jacobs, 1971). Most of the iron absorbed remains within the epithelium of the gut and is lost when these cells are shed (Underwood, 1956). A small part of the iron taken up by the cells of the small intestine is transferred to the plasma, but how this transfer is achieved is not known.

Only about 5-15 per cent of dietary iron is absorbed in man (Moore and Dubach, 1956). The proportion absorbed is affected by age, iron status, general health and by pathological conditions within the gastro-intestinal tract. The amount of dietary iron available and its ionic form are important. The ferrous form is more readily absorbed, probably because of its high solubility at the pH of the small intestine. The quantities and composition of other inorganic and organic components of the diet are critical for iron absorption. For example, phytates bind iron and decrease absorption while ascorbic acid increases iron uptake (Bothwell and Finch, 1962).

1.2.5 Iron Excretion

The body has only a limited capacity to excrete iron (McCance and Widdowson, 1937, 1938), due mainly to the intracellular location of iron and the tight binding of iron to transferrin. Small but significant amounts of iron are lost daily, for example, normal adult males lose between 0.6 and 1.0 mg of iron per day (WHO, 1970).

The main sources of iron loss are desquamated epithelial cells, hair and perspiration accounting in total for about 0.5 mg (Bothwell and Finch, 1962). From cells of the gastro-intestinal tract, bile and blood, around 0.5 mg is lost and less than 0.1 mg is lost in urine (Dagg et al, 1966a). Thus, although iron is bound avidly within the host unavoidable losses do occur, and if neglected these losses can lead to deficiency of iron in situations of marginal iron balance.

1.2.6 Iron Requirements

Iron requirements are determined by the amount needed for haemoglobin formation, for tissue growth and to compensate for essential losses. The average daily British diet contains 12 - 15 mg of iron (Moore and Dubach, 1956) and between 5 - 10 per cent of this dietary iron is absorbed. With normal losses it is thought that such a diet should supply an adequate quantity of iron. However, because of variations in absorptive capacity, dietary make up, and individual losses, it is difficult to estimate precise

physiological requirements. WHO have recommended an optimal dietary iron level and these figures are in general agreement with other expert groups (WHO, 1970; Committee on Iron Deficiency, Council on Foods and Nutrition, 1968). (Table 1.3)

There are three periods of life when iron requirements are increased, early childhood, at adolescence and during the child-bearing period in women. During these periods iron balance is particularly delicate but normal individuals with an adequate diet will increase their absorption of dietary iron and maintain balance. However, when intake is inadequate or losses occur outwith physiological ranges then negative balance ensues. If negative balance continues till iron stores are exhausted then iron deficiency develops.

1.3 IRON DEFICIENCY

1.3.1 The Development of the Concept of Iron Deficiency as a Primary Deficiency Disease

Iron deficiency in common with iron therapy was described first in the Ebers Papyrus (Bryan, 1931). Reference is made in this manuscript to the AAA disease which is thought to be iron deficiency anaemia (Fowler, 1936). Hippocrates described a condition associated with the onset of puberty and with menstrual irregularities which might now be attributed to iron deficiency anaemia (Stengel, 1896).

Credit for the first clinical description of anaemia is given to Johannes Lange (1520) who accurately described the signs and symptoms of severe anaemia as follows:-

> "Is somehow as if exsanguinated sadly paled; the heart trembles with every movement of her body, and the arteries of her temple pulsate, and she is seized with dyspnoea in dancing or climbing the stairs, her stomach loathes food particularly meat, and the legs, especially at the ankles, become edematous at night" (Major, 1945).

Chlorosis was the name given to the disease by Jean Vavandal in 1620 and chlorosis was clearly defined by Hoffman in 1731 as a separate clinical entity. Chlorosis derives from the Greek word meaning green and patients were described as having a greenish hue although not all workers believed this description (Cabot, 1915). Ashwell in 1838 classified chlorosis as a disease of the blood, although Willis in 1681 had previously remarked upon the watery consistency of chlorotic blood. Foedisch in 1832 showed the blood of chlorotics to have reduced iron content, and Popp (1845) noted the pale hue of individual red cells. Johann Duncan in 1867 credited his superior with having observed microscopically a decrease in the pigmentation of red cells in chlorosis, but added that the number of red blood cells was essentially the same as in

normal blood. Duncan (1867) also noted a slow erythrocyte sedimentation rate concluding that this was due to a reduction in the heaviest constituent of the corpuscles, their "Ferrunginous colouring matter".

Hayem, in the early part of this century, confirmed hypochromia as a constant characteristic of chlorosis and demonstrated that the average size of the erythrocytes in chlorosis was reduced (Fowler, 1936). Around the turn of the century the diagnosis of chlorosis gradually gave way to that of hypochromic anaemia. Bloomfield (1932) recognised the similarities between chlorosis and idiopathic hypochromic anaemia and suggested they were parts of a spectrum of the same disease. Although the relationship between iron, haemoglobin and the red cell was becoming clearer, the exact nature of hypochromic anaemia was not recognised. Wintrobe and Beebe (1933) listed fifteen synonyms for idiopathic hypochromic anaemia in their comprehensive review of 498 cases. However, Heath and Patek (1937) in a review of anaemias of different aetiology related most of the idiopathic hypochromic anaemias to deficient dietary intake and, or, excessive loss of iron.

With advances in biochemistry more specific tests became available, in particular the measurement of serum iron (Heilmeyer and Plotner, 1936). The discovery of the correlation between low serum iron (Moore et al, 1937), the absence of marrow iron and the existence of anaemic

states (Rath and Finch, 1948; Davidson and Jennison, 1952) supported and advanced deficiency of iron as the primary nutritional defect.

The concept has thus emerged that iron deficiency is the primary disorder and anaemia is a late manifestation of this deficiency (WHO, 1968). The term iron deficiency is used in preference to iron deficiency anaemia as even mild anaemia is not the earliest sign of deficiency of iron and the purpose of therapy is to correct the underlying deficiency rather than merely its manifestation (WHO, 1968).

1.3.2 Natural History of Iron Deficiency

The development of iron deficiency is the result of disturbed iron balance. When iron absorption and iron stores are unable to meet iron requirements or to compensate for iron losses then negative iron balance results. Although the haematological changes induced by negative iron balance may vary according to the rate of development of negative balance it is simplest to consider the changes occurring by gradual depletion of iron stores.

When iron absorption is insufficient to meet an individual's requirements, iron stores in the form of ferritin and of haemosiderin become progressively reduced. This earliest stage of iron deficiency is referred to as iron depletion or latent iron deficiency and as long as stores remain adequate for marrow requirements no change

in serum iron, transferrin or haemoglobin levels is noted. Diagnosis of this stage relies upon the demonstration of a marked reduction or the absence of marrow iron stores (Beutler et al, 1958; Bothwell and Finch, 1962).

When iron stores are exhausted or so reduced that the requirements of marrow for haemopoiesis are not satisfied, plasma iron levels fall and the level of transferrin in serum rises. The proportion of transferrin being used to carry iron consequently falls and haemoglobin synthesis may be impaired (Bainton and Finch, 1964). If deficiency of iron persists anaemia results. Initially the anaemia may be normochromic and normocytic but eventually the classical blood picture of microcytic hypochromic anaemia is seen (Table 1.4

When iron is lost more rapidly, the sequence of events differs. Conrad and Crosby (1962) withdrew 500 mlof blood weekly from human volunteers and observed a fall in the haemoglobin concentration followed by a decrease in plasma iron level. The transferrin saturation fell and finally gross red cell morphological changes appeared.

1.3.3 Investigation of Iron Status and Iron Metabolism

There is a considerable number of tests available for the assessment of iron status. The importance and efficiency of individual tests varies but most may be described as tests which measure iron stores, evaluate transport iron systems or assess erythropoiesis. There is

also a group of miscellaneous tests which, although not directly useful for diagnostic purposes have contributed greatly to the knowledge of the physiology of iron metabolism. The bulk of these tests involve the use of radioisotopes or specific markers of iron. The iron tolerance test (Moore et al, 1937), radioactive isotopes of Cobalt (Valberg et al, 1972) and chelating agents, can and have been used to investigate iron deficiency. However, they are principally research tools and do not now have a practical diagnostic role. The following discussion will be limited to the investigation of iron deficiency, but many of the tests discussed are also used in the assessment of iron overload states and of other haematological disorders.

1.3.4 Measurement of Iron Stores

Marrow and Tissue Iron

The reticuloendothelial cells of the marrow contain the majority of storage iron. Examination of marrow aspirates as smears or as sections has consistently proved a reliable and accurate method for assessing iron stores (Rath and Finch, 1948; Davidson and Jennison, 1952; Beutler et al, 1958). Semiquantitative methods of estimating the haemosiderin present in marrow are available and results correlate with other parameters in the measurement of iron deficiency (Beutler et al, 1958). In addition marrow sections have the advantage of allowing assessment of the morphology of red cell precursors.

Examination of bone marrow aspirates is the best available method for assessing iron stores and is the only way of confidently diagnosing latent iron deficiency states. It has the disadvantages of being expensive and from the patient's standpoint is uncomfortable and carries a very slight but definite risk of mortality.

Ferritin

Small amounts of ferritin are normally present in serum. There is a marked sex difference in levels of circulating ferritin; males having considerably higher levels than females. This probably reflects differences in iron storage levels. The development of an accurate immunoradio-assay for the measurement of serum ferritin (Addison et al, 1972) and the close correlation between serum ferritin levels and iron stores, (Walters et al, 1973) has resulted in an accurate test for direct assessment of iron stores. As yet this test is not widely used.

1.3.5 Assessment of Iron Transport Systems

Serum Iron, Transferrin Level and Percentage Saturation of Transferrin

Iron is present in serum bound to transferrin and the level of circulating transferrin represents the potential maximum amount of iron that can be carried in serum. Consequently the concentration of transferrin in serum is termed the total iron binding capacity and the concentration of iron bound to transferrin the serum iron.

Measurement of these parameters provides sensitive and specific information about iron status. The normal serum transferrin level is between 45 and 75 μmol/l and the normal serum iron is between 10 and 35 μmol/l. There is a sex difference in serum iron level, women usually being 3 μmol/l lower than men. This difference first appears at puberty and is not altered by iron therapy (Verloop et al, 1959).

The plasma iron concentration is markedly decreased in established iron deficiency (Heilmeyer and Plotner, 1937; Beutler et al, 1958). The level may also be low in mild anaemia and early iron deficiency. There are several dangers in using the serum iron alone as a measurement of iron deficiency. There is a marked diurnal variation (of around 30 per cent) in thephysiological normal levels, which are higher in the morning. This variation is constant and reversed in night workers (Hamilton et al, 1950). Women taking oral contraceptives have low values (Mardel and Zilva, 1967) and variations in serum iron levels have been reported during the menstrual cycle (Zilva and Patston, 1966). Values in the lower part of the normal range have been reported in cases of mild iron deficiency and low values have been recorded in other forms of anaemia (Beutler et al, 1958). As an isolated measurement the serum iron is not of great value but it can help to rule out iron deficiency if high levels are found, as these are very unlikely to be associated with iron deficiency.

Serum iron measurements are frequently used in conjunction with assessments of the level of transferrin. Serum transferrin levels (total iron binding capacity, T.I.B.C.) are increased in iron deficiency (Laurell, 1947; Rath and Finch, 1948) but may be within the normal range in early deficiency (Beutler et al, 1958).

When the serum iron is divided by the transferrin level and multiplied by one hundred the percentage saturation of transferrin is calculated.

$$\frac{\text{Serum iron}}{\text{T.I.B.C.}} \times 100 = \% \text{ saturation}$$

Normally this value lies between 20 and 40 per cent. Values of 16 per cent or less have been shown to be indicative of low iron stores and associated with ineffective erythropoiesis (Bainton and Finch, 1964). This estimation is probably the most commonly employed test of iron status. The measurement of serum iron, transferrin and percentage saturation provides useful easily obtainable information and will detect 75 per cent of cases of iron deficiency. Yet these parameters lack complete specificity and normal results should not exclude a diagnosis of iron deficiency (Garby et al, 1969).

1.3.6 Assessment of Erythropoiesis

Blood Film and Haemoglobin Estimation

In severe iron deficiency interpretation of a

classical blood film showing hypochromic microcytic erythrocytes presents little problem to the trained observer. However, in mild iron deficiency anaemia the blood film may show few morphological abnormalities, (Wintrobe et al, 1974) and diagnosis of iron deficiency from the film alone may be impossible. When mild morphological changes are present there appears to be a considerable variation in diagnostic accuracy between observers and even inconsistencies by the same observer in repeated examinations. This is especially true in the absence of a clinical history (Beutler et al, 1958; Fairbanks et al, 1971). Similarly, estimation of the haemoglobin concentration will show very low values in cases of severe iron deficiency. However, as already noted there may be no alteration in haemoglobin level until late in the deficiency disease. There is also a considerable variation in the physiological normal. WHO (1972) defined the normal haemoglobin concentration and laid down values below which anaemia is said to exist (Table 1.5).

As diagnostic tests the strength of the haemoglobin estimation and of blood film examination lies in the diagnosis of severe iron deficiency but not in the diagnosis of the early stages of the deficiency.

Red Cell Indices

These indices are representative of the size (mean corpuscular volume) haemoglobin content (mean corpuscular

haemoglobin) and haemoglobin concentration (mean corpuscular haemoglobin concentration) of the "average" erythrocyte. They are calculated from the haemoglobin concentration, the red cell count and the volume of packed red cells.

The value of red cell indices has been doubted by several authors (Beutler, 1959; Fairbanks, 1970; Robertson and McLean, 1970; Rose, 1971). They have however, some advantages. They are easy to obtain and are objective observations. In severe iron deficiency they are usually altered but like the haemoglobin concentration and blood film may be entirely normal in mild deficiency.

Erythrocyte Protoporphyrin Estimation

The final step in the biosynthesis of haem involves the incorporation of iron into protoporphyrin. Protoporphyrin is present in small amounts within erythrocytes and greatly increased levels are noted in iron deficiency anaemia (Prato, 1968), but high levels may occur in other conditions (chronic infection; sickle cell anaemia). Measurement of erythrocyte protoporphyrin in iron deficiency without anaemia has revealed significantly increased levels and when taken with the transferrin saturation allows accurate diagnosis of iron deficiency (Dagg et al, 1966). Until recently measurement of erythrocyte protoporphyrin required a large venous blood sample and techniques were time consuming. However, a micro-method has been described (Piomelli et al, 1976) and used as a screening test for iron

deficiency in populations at risk.

From consideration of the tests available diagnosis of iron deficiency should be possible from estimation of bone marrow iron content, of haemoglobin level, of serum iron and of the percentage saturation of transferrin. These tests are accurate, simple to carry out and will result in correct assessment of iron status in the vast majority of cases.

1.3.7 Aetiology of Iron Deficiency

As previously noted iron deficiency usually results from prolonged negative iron balance. There are basically four factors leading to the development of negative balance and subsequent iron deficiency. They are, inadequate dietary intake of iron, impaired absorption of iron, increased blood loss and increased requirements of iron. In many cases a combination of two or more of these factors may contribute to the development of iron deficiency.

Dietary Deficiency

Ingestion of an adequate diet leads to sufficient iron intake (1.2.6) although the form of the diet plays an important part in the availability of iron for absorption (Moore and Dubach, 1956). In advanced countries iron deficiency is rarely caused by uncomplicated dietary deficiency but in underdeveloped regions of the world inadequate diet and religious or social customs may lead to an insufficient

intake of iron. Pica, the habitual ingestion of unusual material such as earth or clay (geophagia) or even ice (pagophagia) may be a symptom of severe iron deficiency. More often, pica, a not uncommon practice in some areas of Egypt, Turkey, Iran or Negro areas of the U.S.A., results in a diet deficient in iron or prevents the absorption of ingested iron (Wintrobe et al, 1974).

Dietary insufficiency as a sole cause of iron deficiency is now rare. However, in cases of marginal balance, poor, or unusual diets, deficiency may result when requirements for iron are increased due to physiological or pathological causes (Fairbanks et al, 1971).

Impaired Absorption

The most important condition leading to impaired absorption of iron is gastric surgery. The decreased iron absorption is due to reduced gastric acidity impairing the release of iron from food and the formation of the more readily absorbed ferrous state. Transit time of food through the duodenum is also decreased in these patients (Bothwell and Finch, 1962; Wintrobe et al, 1974). Chronic gastritis often develops in partial gastrectomy patients and may further impede the absorption of iron. The relationship between gastritis and iron deficiency is discussed further in section 1.4.3.

Coeliac disease has a high incidence of iron

deficiency, indeed deficiency of iron may be the presenting symptom of the disease. Deficiency is due to impaired iron absorption although increased desquamation of mucosal cells and blood loss may play a part (Croft, 1970).

Blood Loss

Bleeding from the gastro-intestinal tract is the most common cause of iron deficiency in men. Any lesion causing haemorrhage within the gastro-intestinal tract is likely to cause iron deficiency but loss of small amounts of blood over a long period are likely to remain undetected and can result in severe iron deficiency. In Great Britain and U.S.A., haemorrhoids, peptic and duodenal ulceration are by far the commonest cause of gastro-intestinal blood loss (Beveridge et al, 1965). However, ulcerative conditions of the colon and malignancy are not uncommon causes of intestinal bleeding (Wintrobe et al, 1974). Therapeutic doses of salicylates may cause erosive gastritis and give rise to daily blood losses of 2 - 3 ml although larger doses may result in greater losses.

On a global scale the major cause of iron deficiency is hookworm infestation. Hookworm is endemic in many areas and epidemic in some (Beaton, 1974), indeed, twenty per cent of the world population are said to be infected by the parasites Ancyclostoma duodenali or Necator americanus. These parasites attach to the intestinal mucosa and remove blood from submucosal vessels. The amount of blood lost

is proportional to the worm load but deficiency may be aggravated by an inadequate diet (Fairbanks et al, 1971; Wintrobe et al, 1974).

In women excessive menstrual loss is the most important single cause of iron deficiency. It appears that 80 ml is the critical level of blood loss and above this, deficiency is likely to develop. In a Swedish study (Hallberg et al, 1966) 67 per cent of women with blood loss of 80 ml or more had iron deficiency anaemia.

Donation of blood imposes an added burden upon existing iron stores and a high incidence of iron deficiency has been reported in regular donors (Bateman, 1951). However, with precautions taken at centres iron deficiency as a result of blood donation is becoming less common but donations could precipitate deficiency in someone with a delicate iron balance or with marginal stores (Wintrobe et al, 1974).

Increased Requirements

Iron deficiency may develop during infancy, childhood and adolescence because of the increased requirements of iron for growth. In children the frequency of iron deficiency has been reported as being as high as 50 per cent (Fairbanks et al, 1971) but the exact incidence is difficult to ascertain. Iron deficiency appears to be temporary in these groups and the significance

of this deficiency in terms of overall health and development is not known (WHO, 1972).

Pregnancy and the inevitable transfer of maternal iron to the foetus often results in low stores of iron being converted into frank deficiency states. Many studies of the incidence of iron deficiency in pregnancy have been carried out and the reported incidence varies between 15 and 80 per cent (Fairbanks et al, 1971; Wintrobe et al, 1974). In certain regions of India 10 - 40 per cent of maternal deaths have been reported as due to anaemia (Bothwell and Finch, 1962). The World Health Organization considers iron deficiency in pregnancy a major public health problem and has embarked upon trials of iron supplementation in pregnancy (WHO, 1972).

1.3.8 Epidemiology of Iron Deficiency

Although deficiency of iron is not associated with as high a mortality as many other deficiency diseases it is probably the most common nutritional deficiency found in both developing and advanced countries. The concepts and meanings of the terms used to describe iron deficiency are widely understood, but the setting of numerical values for the definition of categories in epidemiological studies has been somewhat arbitrary and variable. In addition laboratory techniques and standards differ and consequently estimations of the prevalence of iron deficiency vary and it is difficult to give exact data on the incidence of iron

deficiency. Despite these uncertainties the WHO (1968; 1972) has attempted to define criteria for the standardisation of diagnosis (Table 1.5). On a world wide basis, haemoglobin and packed cell volume estimations are the usual diagnostic tests employed, resulting in severe iron deficiency being detected but milder degrees of deficiency being missed. This fact is recognized by WHO and the use of more specific tests when facilities and expertise allow is recommended (WHO, 1972).

In underdeveloped regions severe iron deficiency is very prevalent. Studies of populations in India and certain regions of Africa reveal that fifty per cent of the adult population suffer from anaemia (Gosden and Reid, 1948; Ramalingaswami and Patwardham, 1949; Venkatachalam, 1968). Early studies failed to indicate the exact aetiology of the anaemia, but it is likely that the majority were iron deficiency in origin. Around fifty per cent of an adult population group in Mauritius were anaemic (WHO, 1959) and ninety five per cent of these cases were due to iron deficiency. No evidence is available relating to less severe degrees of iron deficiency in such underdeveloped areas, but it is apparent that a substantial proportion of the adult population has iron deficiency.

In more affluent societies the incidence of severe iron deficiency is considerably less and more work has been undertaken on the occurrence of the earlier stages of iron

deficiency. A study of various ethnic groups in the U.S.A. revealed a surprisingly high incidence of anaemia (Beaton, 1974). A recent review of the literature (Garby, 1973) indicates that 18 - 25 per cent of women in Sweden are deficient in iron. Similarly the results of a British survey (Kilpatrick and Hardisty, 1961) showed that 14 per cent of women and 3 per cent of men had low haemoglobin levels. In Glasgow the incidence of iron deficiency anaemia in women attending general practitioners was found to be 9.2 per cent. In the same survey iron deficiency without anaemia was present in 22.2 per cent of all patients and 16.3 per cent of the general population (MacFarlane et al, 1967).

The problems of standardisation of normal haematological parameters and of survey methods make accurate assessment of the prevalence of iron deficiency difficult but there can be no doubt that deficiency of iron constitutes a major public health problem in many regions. The severity of this disease has prompted the WHO to recommend controlled trials with the aim of eradicating anaemia, and assessing the effects of eradication upon morbidity, growth, and mortality in mothers and children (WHO, 1972).

1.4 EFFECTS OF IRON DEFICIENCY

1.4.1 Introduction

In common with other nutritional deficiencies iron deficiency causes widespread and diverse tissue changes but

although it causes great morbidity iron deficiency is essentially a benign disorder rarely terminating fatally. Detailed post-mortem reports of deaths due to severe iron deficiency are few (Suzman, 1933) and this in part accounts for the lack of specific histopathological information. However, the morphological changes occurring in the peripheral blood stream and in bone marrow are well documented and in addition to the description of these changes mention will be made of the abnormalities occurring in other specific organ systems.

1.4.2 Effects of Iron Deficiency upon Non-epithelial Tissues

Severe iron deficiency is characterised haematologically by a reduction in the circulating haemoglobin concentration. The number of circulating red cells is usually reduced although it is possible for the red cell count to remain within normal limits (Hardisty, 1974). The red cells are small (microcytic) and poorly haemoglobinised (hypochromic). Bizarre shapes (poikilocytosis) and target cells may be found. Except in the presence of active bleeding or iron therapy, reticulocytes are absent from the peripheral blood (Wintrobe et al, 1974; Hardisty, 1974). There is increased breakdown of the abnormal red cells and the mean survival time of the red cell is reduced (MacDougall, 1968). Further, there appears to be an increased destruction of newly formed red cells and red cell precursors within the marrow and this is

termed ineffective erythropoiesis (Robinson and Kveppel, 1971). Disturbances in glycolysis and decreased enzyme activity within the erythrocyte have been suggested as causes of this ineffective erythropoiesis (Dallman, 1974).

Depressed collagen synthesis has been reported in iron deficiency (Chvapil et al, 1968; Whitson and Peacock, 1969; Prockop, 1971). Absence of iron prevents the hydroxylation of proline and lysine in protocollagen and collagen synthesis is impaired.

Studies of muscle in iron deficiency have revealed reduced levels of enzymes of the cytochrome system aconitase and succinic dehydrogenase (Beutler, 1957, 1959a). However, the effect of these low enzyme levels upon function is not clear. Reduced work capacity or impaired muscular activity have not been clearly attributed to decreased enzyme levels. In fact no change was noted in the amplitude or duration of skeletal muscle contraction when measured in isolated preparations (Dallman, 1974).

Neurological disturbances can occur in severe iron deficiency but little is known about the biochemical response of the brain to iron deficiency. Total brain iron does not parallel the decrease of other body iron stores and the concentration of cytochrome C in rat brain remains normal unless measured under extreme unphysiological conditions (Dallman, 1974). Reduced body monoamine oxidase

activity in iron deficient rats is reported and it has been postulated that this may provide a basis for the neurological manifestations of iron deficiency (Dallman, 1974).

Iron deficiency results in a decreased or defective synthesis or haem enzymes and impairment of other iron dependent systems in many tissues (Beutler, 1960), but there does not appear to be a consistent pattern of depletion for individual enzymes in iron deficiency. Similarly different tissues show a variety of patterns of enzyme depletion which are unrelated to the severity of iron deficiency and result in different functional and structural abnormalities.

1.4.3 Epithelial Changes in Iron Deficiency

Historical description of the changes occurring in finger nails are particularly well documented. The nails become brittle, thinned and flattened. Finally spoon-shaped forms appear, this being known as koilonychia. The reported incidence of koilonychia in iron deficiency varies from being rarely seen in the United States to a not uncommon complaint in Great Britain and Scandinavia (Fairbanks et al, 1971).

Considerable attention has also been paid to the reported occurrence of gastritis and achlorhydria in iron deficiency (Witts, 1930; Wintrobe and Beebe, 1933). In studies by Davidson and Markson (1955), Joske et al (1955), Lees and Rosenthal (1958), Cheli et al (1959), and Coghill

et al (1965) more than 60 per cent of cases of patients with iron deficiency anaemia had gastritis. Badenoch et al (1957) found abnormalities in 43 of 50 cases but noted no correlation between the severity of anaemia and the incidence of achlorhydria, although koilonychia, glossitis and dysphagia were more common in those cases with severe gastric changes. Shearman et al (1966) suggested that perhaps two categories of patients exist. Firstly, a group of patients who develop gastric atrophy due to longstanding iron deficiency and a second group in whom iron deficiency results from the presence of abnormal gastric mucosa preventing or hindering iron absorption.

Approximately one third of subjects with iron deficiency anaemia and histamine fast achlorhydria have serum antibodies to gastric parietal cells (Dagg et al, 1965) and it has been suggested that in a proportion of cases the auto-antibody results in the production of chronic atrophic gastritis (Dagg, 1972). Until the role of gastric auto-antibodies is elucidated and more carefully controlled studies of the histological changes in gastric epithelium are carried out it is impossible to state whether iron deficiency is caused by atrophic gastritis or whether the deficiency leads to the development of gastric atrophy. It seems likely that in a considerable number of patients gastric epithelial atrophy and achlorhydria are sequelae of iron deficiency.

Less information is available on changes occurring elsewhere within the gastro-intestinal tract but in the small intestine Naiman et al (1964) showed that severe iron deficiency caused stunting of the villi which reverted to normal after iron therapy. However, Rawson and Rosenthal (1960) noted no abnormalities of the small intestine in severely deficient patients despite the presence of gastric abnormalities. Valberg et al (1961) commented that the epithelial changes in the small intestine might only develop after longer periods of deficiency and that the severity of the deficiency was likely to be important. As with gastric changes no certain conclusions can be drawn from the available data on the effects of iron deficiency upon the small intestine and further work is required to answer this problem.

1.4.4 Paterson-Kelly Syndrome (Sideropaenic Dysphagia)

In 1919 Brown-Kelly and Paterson speaking at the same meeting, independently described the syndrome which now bears their names. The syndrome, occurring mainly in women, consisted of dysphagia and post-cricoid oesophageal stricture. Associated features were longstanding anaemia koilonychia, glossitis and fissures at the corners of the mouth. Brown-Kelly noted the occasional occurrence of carcinoma of the oesophagus and the syndrome now includes these additional findings. Paterson examined the buccal and tongue mucosa histologically and noted thinning of the epithelium and infiltration of the tunica propria with

chronic inflammatory cells. Vinson (1922) whose name is associated with the same syndrome quoted Plummer (1914) when describing a hysterical dysphagia, but noted absence of oesophageal abnormalities and did not mention the presence of oral lesions. Confirmation of the sex incidence and of the glossitis, cheilitis and anaemia was made by Moersch (1926) who also noted the occurrence of achlorhydria. Cameron (1929) described oral and oesophageal changes. He concluded that the glossitis and oral mucosal changes were due to anaemia and deprivation of essential foods, especially iron. He also warned that "malignant proliferation" might supervene.

Suzman (1933) and Savilahti (1946) reported the histological findings of both tongue and oesophagus in Paterson-Kelly syndrome. Autopsy examinations of tongue revealed atrophic changes, absence of papillae and a plentiful lymphocytic infiltrate. The most distinctive changes were in the oesophagus where the epithelial cells were vacuolated with pyknotic nuclei. The changes were thought to represent marked destruction of superficial and middle layers of the epithelium while the "cells of the basal layers were in a state of rapid division". These changes were thought to be "pre-cancerous".

The possibility of malignant change occurring in cases of Paterson-Kelly syndrome has been discussed since the syndrome was first described. Ahlbom (1936) produced

evidence of a 70 per cent incidence of oral, pharyngeal and oesophageal carcinomas in Paterson-Kelly syndrome. Simpson (1939), Videbaek (1944) and Jones (1961), all confirmed Ahlbom findings and added that the whole of the upper alimentary tract may become "pre-cancerous".

The incidence of malignant change in Paterson-Kelly syndrome seems to have a large regional variation and figures stating an incidence of 10 - 90 per cent of selected populations have been quoted (Ahlbom, 1936; Wynder et al, 1957). Some of these differences may be attributed to selection of patients and lack of consistent haematological data. Further in a general population study in Wales dysphagia was present in only 5 per cent of women and no evidence of anaemia or iron deficiency was found in these cases (Elwood et al, 1964). Wright et al (1968) contradicted these findings when all but 6 of 82 patients with oesophageal webs had evidence of current or past iron deficiency. Chisholm and Wright (1967) emphasised that unless great care was taken iron deficiency might be missed and that Elwood had not accounted for the possibility of iron deficiency having been present at an earlier date. Whether or not iron deficiency leads to dysphagia and premalignant changes in the upper alimentary tract cannot be resolved on the basis of available data although the weight of evidence would suggest a predominant role for iron deficiency in the aetiology of these lesions.

1.4.5 The Oral Epithelium in Iron Deficiency

The oral manifestations of iron deficiency anaemia have been known for many years. Stengel (1896) described angular cheilitis and glossitis in chlorosis. Kelly (1919) attributed pale oral mucous membranes and an atrophic tongue to anaemia. Paterson (1919) was perhaps the first to describe the microscopical appearances of the oral mucosa in anaemia. He noted atrophy of the epithelium and an apparent thinning of the underlying "tunica propria".

Suzman (1933) and Savilahti (1946) reported the histological findings from a number of anaemic patients with Paterson-Kelly syndrome. In addition to the oesophageal and laryngeal changes they described areas of atrophy and of hyperkeratinisation and a pronounced lymphocytic infiltrate. Lewis (1930) reviewed the available literature and described the lingual changes in anaemia of varying aetiologies. He was unable to ascertain the cause of these changes but stressed that clinically the oral changes were a valuable index of health.

Waldenstrom (1938) realised the importance of deficiency of iron in the production of the epithelial lesions of hypochromic anaemia. He noted that koilonychia and glossitis could occur in cases of iron deficiency without anaemia. Darby (1946) indicated that there was still a lack of understanding of the role of iron deficiency in the production of the epithelial lesions of anaemia, and

proceeded to demonstrate by clinical methods that lack of iron alone could be responsible for lingual atrophy, glossitis and angular cheilitis.

A more detailed description of the microscopic features of the oral epithelium was provided by Boddington (1959) who described the cytology of buccal cells in iron deficiency anaemia. Although Graham and Rheault (1954) and Boen (1957) had described the cytological changes in buccal cells in pernicious anaemia Boddington (1959) was the first to describe these changes in iron deficiency anaemia. Smears of exfoliated cells taken from the saliva of iron deficient subjects were stained by the Papanicolaou method. A significant reduction in cytoplasmic diameter of cells from deficient subjects was reported. In cases with a smooth tongue occasional abnormally large nuclei were seen. Cheli et al (1959) noted no clinical or histological correlation between lingual, oesophageal and gastric changes in iron deficiency anaemia although lingual changes were found to be more common when severe gastric changes were present.

An approach to the quantitation of cytological changes in iron deficiency was described by Jacobs (1959). Smears from cheek were stained by the Papanicolaou technique and the degree of cytoplasmic "acidophilia" shown by the cells was termed the "cornification index". A highly significant increase in cornification index was noted in cases of idiopathic hypochromic anaemia. No change of

cornification index was noted in post-haemorrhagic iron deficiency anaemia, in cases of the anaemia of leukaemia, or in pernicious anaemia. Jacobs (1959) assigned this difference to the chronicity of idiopathic hypochromic anaemia and to low tissue iron stores, commenting that the anaemia itself had little to do with the changes. The cornification index returned to normal in some of the patients when they were treated with iron.

Monto et al (1961) examined smears from the dorsal surface of tongue and from the buccal mucosa, before, during and after iron therapy. Smears from untreated iron deficient patients confirmed Boddington's (1959) finding of a reduced cytoplasmic diameter, an increased nuclear size and consequently an altered nucleo-cytoplasmic ratio. Monto et al (1961) also noted an increased number of nucleoli, the presence of double nuclei and of bizarre nuclear patterns in untreated iron deficient patients. These changes were reversed by iron therapy. Histological examination of lingual biopsies revealed absent papillae, thinning of the epithelium accompanied by an increased nuclear diameter and decreased cell size. The changes all returned to normal after iron therapy. The author suggested that the changes might be due to a defect in cell maturation. Walker (1962) reviewed the uses of oral cytology in the anaemias and concluded as did Osborn (1953) that operative, technical and staining procedures were so variable as to invalidate the use of oral cytology in the assessment of

anaemia.

Jacobs (1960) examined biopsies of cheek obtained by a Wood's suction tube. There was no significant decrease in epithelial thickness but the absence of melanin and the presence of a chronic inflammatory infiltrate were described. In the majority of cases differentiation between normal and iron deficient biopsies was impossible on histological grounds alone but this method of biopsy would obscure any structural or morphological abnormality.

Beveridge et al (1965) noted clinically the epithelial changes of glossitis and angular cheilitis in a substantial proportion of patients with severe iron deficiency anaemia. Jacobs (1961a, 1961b) in an attempt to explain the epithelial changes in iron deficiency noted decreased cytochrome C levels in buccal mucosa from anaemic patients. Dagg et al (1966b) confirmed this finding in patients with iron deficiency anaemia and in patients with iron deficiency without anaemia but no correlation between the epithelial atrophy, the symptoms and the degree of enzyme depletion was found by either author. Kalinin (1970) reported low enzyme levels in buccal epithelium of patients with iron deficiency and stated that tissue deficiency of iron plays a prominent role in the development of the oral epithelial changes.

The vast majority of available evidence supports

deficiency of iron as the prime aetiological factor in the development of the epithelial lesions of iron deficiency. It is recognised, however, that in certain cases co-deficiencies of essential nutrients and vitamins may arise and complicate the situation. Jacobs (1963) in a mainly retrospective study of severely anaemic Africans found a very low incidence of epithelial lesions. Consequently it was suggested that iron deficiency plays a secondary or even incidental role in the aetiology of epithelial lesions and a role for pyridoxine deficiency in the aetiology of the epithelial lesions in iron-deficiency anaemia was proposed (Jacobs, 1968). Drinnan (1969) supported this view. Using anaemic rats and sex linked anaemic mice he found no change in the oral mucosa when comparing severely deficient and normal animals. The influence of multiple secondary factors was thought to be the cause of the epithelial changes.

In view of the voluminous literature reporting the epithelial manifestations of severe iron deficiency the lack of accurate reliable quantitative data is surprising and has undoubtedly contributed to the conflicting views of the effects of iron deficiency upon the oral mucosa. Carefully controlled human and animal studies providing objective data are lacking and until these are available it will not be possible to accurately define the effects of iron deficiency upon the oral mucosa.

1.4.6 Related Deficiencies

Several other deficiencies have been reported as occurring with iron deficiency. In particular folic acid, pyridoxine, vitamin B_{12} and protein deficiency have been related to iron deficiency and to the lesions found in iron deficiency.

Folic acid deficiency is a common finding in pregnant women who are also iron deficient. There is considerable debate as to whether or not the deficiency of folic acid is a manifestation of the iron deficiency. Vitale et al (1966) reported the effects of an iron deficient diet on rats given folic acid supplements and concluded that folate deficiency could be induced by dietary iron deficiency. Similarly Toskes et al (1974) and Saraya et al (1971) reported reduced serum folate levels in iron deficiency. Chanarin et al (1967) reported that pregnant patients who received iron supplements had a reduced frequency of megaloblastic erythropoiesis but increased red cell folate levels have been reported in iron deficiency (Omer et al, 1970). In a comprehensive review of this problem Hershko et al (1975) attributed the variations in findings to poor controls and to the use of serum folate estimations instead of red cell folate levels. Further, in reporting results of his own work, he stated that erythrocyte folate measurements were a better indicator of folate status than serum folate and that the folate level of erythrocytes was unaffected by a co-existing iron deficiency.

The assessment of these conflicting results is difficult but certainly indicates a possible relationship between low folate levels and iron deficiency. The significance of these findings is not clear and the effects upon oral epithelium are not known.

It has been suggested that iron is required for optimal incorporation of vitamin B_{12} into red cells (Doscherholmen et al, 1974). Harrison (1971) described decreased erythrocyte vitamin B_{12} associated with iron deficiency. Iron therapy returned vitamin B_{12} levels to normal. As is the case with folate levels the significance of low vitamin B_{12} levels is not known in the aetiology of the tissue manifestations of iron deficiency.

Iron deficiency is part of the protein malnutrition syndrome and is the most common factor in the pathogenesis of the anaemia of this syndrome. However, iron deficiency is only one aspect of this syndrome of nearly total malnutrition (Fairbanks et al, 1971) and thus the effects of iron deficiency are impossible to assess.

1.4.7 Iron Deficiency, Immunity and Infection

Iron is essential for the in vivo and in vitro growth of many species of micro-organism. The importance of iron is illustrated by the enhancement of bacterial growth following administration of iron (Jackson and Burrows, 1956; Martin et al, 1963; Caroline et al, 1964). This may

be more than simply the requirement for an essential growth factor, as the protective effect of specific antisera can be completely abolished by simultaneous injection of iron (Bullen, 1974; Elin and Wolff, 1974). Clinical evidence also suggests that the availability of iron is important in the establishment of infections. Patients with acute leukaemia who develop systemic candidiasis have high levels of serum iron (Caroline et al, 1969). A high incidence of infections has been reported in patients with haemolytic anaemia and iron therapy may aggravate malaria in severely iron deficient patients (Masawe et al, 1973, 1974).

Similarly it is thought that iron lack may contribute to resistance to infection. Infection often causes an immediate fall in serum iron and a consequent increase in unbound transferrin levels (Elin and Wolf , 1974). This increased resistance to infection is said to result from high serum levels of transferrin and low levels of free iron. In support of this concept, Masawe et al (1974) reported a decreased susceptibility to infection in iron deficient patients; in addition transferrin has been shown to be bacteriocidal and fungistatic (Schade and Caroline, 1944, 1946).

Iron deficiency has been reported as depressing the ability of neutrophil polymorphonuclear leukocytes to kill bacteria (Chandra, 1973) and as reducing lymphocyte response to antigenic stimulation (Joynson et al, 1972;

Fletcher et al, 1975). Higgs and Wells (1972) reported that patients with chronic mucocutaneous candidiasis and iron deficiency had skin lesions that responded to iron therapy and postulated that epithelial and lymphocyte abnormalities caused by iron deficiency played a predominant role in the aetiology of certain types of chronic mucocutaneous candidiasis (Higgs and Wells, 1972).

In attempting to summarise the role of iron in infection it can be said that iron is essential for bacterial growth and when iron is not available due to deficiency or to the presence of iron binding agents such as transferrin, bacterial growth is inhibited. However, iron deficiency appears to depress the immune response, particularly neutrophil and lymphocyte function. The presence of high serum iron levels as in iron therapy, acute leukaemia and haemolytic anaemias may predispose to infection but haemochromatosis is not particularly noted for troublesome infections. It is certain that the iron status of an individual influences the host response to infection but the mechanisms by which the host response is influenced are not yet clear.

CHAPTER 2

PRELIMINARY INVESTIGATION OF HUMAN BUCCAL EPITHELIUM IN SEVERE IRON DEFICIENCY

2.1 INTRODUCTION

The oral manifestations of iron deficiency have been reported frequently, but histological assessments of the epithelial changes have been subjective and have provided conflicting results (1.4.3). In some cases semi-quantitative investigation of the oral epithelial changes in iron deficiency has demonstrated a reduction in the thickness of the buccal epithelium (Jacobs, 1960), but on the whole meaningful quantitative data is not available. In addition it is not known whether the reported reduction in thickness of oral epithelium is due to a uniform reduction in each epithelial compartment or to a reduction in the thickness of a specific functional compartment.

One method of obtaining such quantitative data is by employing stereological techniques and before reporting this study a brief description of the principles and techniques of stereology is included.

2.2 PRINCIPLES OF STEREOLOGY

2.2.1 Principles of Analysis of Histological Sections Using Stereological Techniques

Morphometry can be defined as the use of quantitative

data to describe structural features. Stereology is a type of morphometry involving a geometric and statistical analysis of structures. The principles of stereology were originally described in 1847 by Delesse, a French geologist. Delesse (1847) showed that the volume of one constituent within a larger volume is on average equal to the area of that component as seen on sections of the material. The relationship A = V holds and can be proven mathematically, provided a representative sample of the material is examined. Although Delesse (1847) applied his findings to the investigation of minerals, the geometric reasoning is equally applicable to the study of organs, and for estimation of the volumes of various structural components of a tissue. Using the principles of stereology it is thus possible to derive three dimensional information of structures from two dimensional tissue sections (Fig. 2.1).

2.2.2 Methods of Stereological Analysis

There are basically two methods used to obtain area measurements in stereology. Rosiwal (1898) demonstrated that if a test line, or a series of lines, was superimposed upon a cross section of the sample the area of components within the sample could be estimated by measuring the length of the test line overlying each component. Glagoleff (1933) showed that by using a lattice marked with points, and counting the points overlying the component to be quantified an area estimation could be obtained (Fig. 2.1). One of the first to use these techniques in biological

systems was Chalkley (1943) who used a point counting method on histological sections to calculate the nucleo-cytoplasmic ratio of cells.

In addition to the above methods for area measurements, stereology can also be used for the estimation of profile lengths. The length of a curved line or boundary can be obtained using a grid of parallel lines. The number of intersect points between the grid lines and the curved line gives an estimation of the length of the line. The absolute length can be calculated using the formula.

$$L = \frac{\Pi}{2} \times I \times d$$

where L = length

I = number of intersect points

d = distance between the grid lines

(Weibel et al, 1966, Weibel, 1969).

2.2.3 Principles of Sampling

As stereology is based upon geometrical and statistical reasoning it is essential to the theory that the areas to be sampled and quantified are representative of the tissue. The validity of the technique depends upon random sampling of the areas to be measured, and care must be taken at all stages in the collection and preparation of material to ensure bias free random sampling (Weibel, 1969).

When the components of a tissue are randomly

distributed, a random procedure is carried out at all stages from the selection of tissue blocks through the selection of sections to the selection of the area to be measured. To ensure adequate dispersion of the sample when non-randomly distributed structures in tissues are to be quantified, stratified sampling by examining several sections at selected levels has been advocated. This is termed systematic or stratified random sampling (Weibel, 1969).

In many tissues the structural components are orientated in a preferential direction and this feature is known as anisotropy. Within lining epithelia the axis of anisotropy is at right angles to the surface. It is recommended that sections of tissues exhibiting anisotropy should be orientated along the axis of anisotropy (Weibel, 1969) which in oral epithelium is perpendicular to the surface. This orientation also allows recognition of histological strata and will contain the component parts of the epithelium in proportion to their frequency.

The size of the sample to be examined is important and it is obvious that the larger the sample size examined, the more reliable the data collected is likely to be. However, the size of the sample is limited by the time available for sampling, the nature of the tissue and perhaps most importantly by the limits of accuracy required (Weibel, 1969).

There are several methods for calculating the smallest reliable sample. These methods depend on the fact that as the size of the sample increases so the sampling error decreases (Chalkley, 1943; Weibel, 1969). The method used in this study is that of the accumulative means test (Chalkley, 1943). An initial number of point counts is performed and the progressive mean for increasing numbers of counts is calculated (Fig. 2.2). The data is then plotted to show the accumulated mean values for the parameter against the number of points counted (Fig. 2.2). As a range of less than 10 per cent of the final accumulative mean is usually acceptable for biological purposes the sample size required to achieve and remain within 10 per cent of the final accumulative mean can be taken as the minimal sample size for that parameter (Weibel, 1969).

2.2.4 Application of Stereology to Oral Epithelium

Schroeder and Munzel-Pedrazzoli (1970) were the first to use stereological techniques to examine oral epithelium. MacDonald (1971) discussed the application of stereological methods to the oral epithelium and described test systems for the intercept and point counting techniques. Warnakulasuriya (1976) applied such stereological methods as part of his study of the kinetic and morphometric analysis of human oral epithelium. Franklin (1974, 1977) using a variety of related techniques applied stereology to the investigation of the changes occurring in neoplasia and hyperplasia in the hamster cheek pouch.

2.2.5 Aims of this Study

The aims of the study reported in this chapter were to examine the buccal epithelium from normal subjects and from patients with severe iron deficiency, and by the use of stereological techniques quantify any changes arising in iron deficient buccal epithelium.

2.3 MATERIAL AND METHODS

2.3.1 Normal Subjects

Five healthy adult male volunteers were used as controls for this study. To assess the haematological status of the control patients, a blood film was examined and estimations of the haemoglobin, serum iron, total iron binding capacity, serum folate and vitamin B_{12} were carried out. All investigations were performed by the Departments of Biochemistry and of Haematology at Glasgow Western Infirmary as part of their routine diagnostic services. The five volunteers had a thorough oral examination and attention was paid to the oral hygiene and periodontal status of the subjects.

2.3.2 Selection of Patients

Material for this study was obtained from inpatients at the Department of Medicine, Western Infirmary, Glasgow. The patients had been admitted to hospital for investigation of iron deficiency anaemia. Thirteen patients were included in this study of whom four were male and nine female. As with the control subjects a thorough oral

examination was carried out and the patients oral hygiene and periodontal status were assessed. If a prosthesis was worn the age, fit, and condition of the appliance were described. Patients were asked about past or recent episodes of dysphagia, and habits likely to effect the oral mucosa such as smoking or excessive alcohol intake were noted.

2.3.3 Biopsy Procedure

The site chosen for biopsy was the buccal mucosa opposite the lower first molar or second premolar tooth. Care was taken to site the biopsy below the occlusal line and to avoid stretching the buccal mucosa unnecessarily. Anaesthesia was obtained by infiltration of the long buccal nerve in the lower second molar region and by infiltrating around the mental nerve in the lower first premolar region anterior to the biopsy site. A 2 per cent lignocaine hydrochloride solution without vasoconstrictor provided suitable anaesthesia.

A 3 mm trephine type punch was used to make a circular incision (Fig. 2.3) and to prevent loss of surface cells great care was taken not to rub or damage the surface of the mucous membrane. The base of the wound was carefully cut free using a pair of fine scissors. Only minimal bleeding was encountered and suturing was not required (Fig. 2.4). Healing was rapid and uneventful with minimal discomfort.

2.3.4 Section Preparation

The biopsy was immediately transferred to a fixative solution of 2.5 per cent glutaraldehyde in cacodylate hydrochloride buffer at pH 7.2 for 2 hours. Glutaraldehyde was used as a fixative to enable part of the biopsy to be prepared for future electron microscopic investigations which will not be reported in this thesis.

To aid cutting the biopsy at right angles to the epithelial surface the specimen was trimmed and the block orientated using a Vickers stereoscopic microscope. Processing was carried out in a Histokine* and the tissues were then paraffin embedded. After processing 5 μm thick sections were cut on a rotary microtome. From each block an early section was examined to check that the epithelium was orientated at right angles to the surface. Thereafter two ribbons of five sections each were prepared and stained with haematoxylin and eosin (H & E).

2.3.5 Identification of the Compartments of Buccal Epithelium

Human buccal epithelium may be divided histologically into two compartments. These compartments are characterised by the morphology, function and location of the constituent cells. Thus the cells engaged in new cell production are termed progenitor cells and form the progenitor cell compartment. A proportion of these cells undergo maturation and form a compartment of functional cells, the maturation compartment.

*Appendix 1

In human epithelium it is not possible to differentiate precisely between progenitor and mature cell compartments and distinction had unfortunately to be made subjectively. The features used to differentiate between compartments were the orientation, location and staining characteristics of the epithelial cells on haematoxylin and eosin sections. Progenitor cells are smaller than mature cells, appear to have a larger nucleo-cytoplasmic ratio, stain darker than mature cells and are limited to the deepest cell layers (Fig. 2.5). The morphology and orientation of individual cells are also valuable features for differentiating the two compartments. Progenitor cells are elongated at approximately right angles to the basement membrane while maturing cells appear flattened, and are in the main orientated parallel to the surface and at right angles to the progenitor cells.

Having identified the compartments it is essential that consistent separation is achieved. To ensure this, a number of sections was examined on several occasions at varying intervals of time until a consistently high degree of reliable interpretation was achieved.

2.3.6 Stereological Analysis of Compartment Sizes

A Leitz Ortholux microscope fitted with a projection head (Fig. 2.6) was used and a column of epithelium projected between two vertical lines drawn on the projection screen was examined (Fig. 2.7). As oral

epithelium is an ordered structure the sections were orientated along the axis of anisotropy, (2.2.3) that is with the surface of the epithelium at right angles to the lines delineating the edges of the column. The analysis was carried out using a x 25 objective which gave a magnification of x 312.5 on the projection screen.

With a stage micrometer the distance between the edge of the screen and the vertical column on the screen was measured. This distance was 0.17 μm. To avoid damaged areas at the edge of sections and to satisfy the criteria for stratified random sampling (2.2.3) the area analysed was in each case positioned 0.17 μm from the edge of the section. To aid subsequent counting of points falling on each compartment, the line of demarcation between compartments was drawn on the projected image using the criteria described in section 2.3.5. A transparent perspex grid with 1.0 cm squares was placed over the projection screen, and the intersection of the lines used as points for the analysis (Fig. 2.7). The grid was superimposed upon the projected image and the points falling on each cell compartment counted.

From point counts obtained during the analysis the accumulative means test was performed to establish the minimum number of counts required per field (Fig. 2.8) and for the mean number of fields required per biopsy (Fig. 2.9). These tests were performed for each compartment but only

the tests performed for the total epithelial thickness are illustrated. It can be seen that six counts per field and four fields per biopsy gave a sufficient number of point counts.

Two histological sections from each biopsy were used for the analysis. The middle section from each ribbon of five was examined, unless this section was damaged in which case an adjacent section was utilised. Thus sections at least 25 μm apart were employed. To minimise the effects of anisotropy and periodicity inherent in the system and to provide the required number of point counts, on each section the grid was rotated into six positions where the angle between the grid positions was 30^{o} except in one instance where a 20^{o} angle was used to avoid overlapping of grid positions. A simple laboratory counter was used to record the points counted for each cell compartment.

2.3.7 Conversion of Point Counts to Absolute Measurements

The number of points falling on each compartment is proportional to the area of the compartment, thus the areas made up by the progenitor and maturation compartments can be calculated (Fig. 2.10). As measurements were taken from sections orientated at right angles to the surface and a constant width of epithelial column measured the area measurements can be converted to mean thickness estimations by dividing the area by the column width (Fig. 2.10).

2.4 RESULTS

2.4.1 Normal Subjects. Clinical and Haematological Examination

The volunteers were between the ages of 31 and 40 years, had excellent oral hygiene and periodontal status, and were non-smokers. The results of the haematological and biochemical investigations are shown in Tables 2.1 and 2.2 and all values were within the normal ranges.

2.4.2 Anaemic Patients. Clinical and Haematological Examination

The patients ages ranged from 17 - 74 years (Table 2.3) and all had severe iron deficiency anaemia with a haemoglobin of less than 11 g/dl and transferrin saturation of less than 16 per cent (Table 2.4). Although the aetiology of this deficiency varied, in most cases chronic blood loss was the principal causative factor. The aetiology of the iron deficiency and clinical diagnoses are shown in Table 2.3. Peripheral blood film examination confirmed the diagnosis in every case. In most patients bone marrow examination was performed as part of the investigative procedure and when done, the marrow findings confirmed severe iron deficiency. As part of routine investigations, vitamin B_{12} and folic acid levels were obtained and these values are also shown in Table 2.4. When the actual values of folic acid or vitamin B_{12} were reported then these are shown in Table 2.4 but commonly the values were recorded only as within normal limits and the levels not reported.

2.4.3 Compartment Analysis of Buccal Epithelium from Normal Subjects

The average number of points counted per grid position in each case is shown in Table 2.5 and the absolute measurements of buccal epithelial thickness and of compartment thickness are shown in Table 2.6. It is evident from Table 2.6 that normal human buccal epithelium varies greatly in thickness (range 341.0 - 496.5 μm) and it may be that the absolute thickness of an epithelium is not a meaningful estimation. As an additional parameter the proportion of the total epithelial thickness formed by each compartment was calculated. Since the epithelial thickness is proportional to the area and as the stereological point counts are also proportional to the area the ratios of the progenitor and maturation compartment to total epithelial thickness may be estimated directly from point counts. Table 2.7 records the proportion of the total epithelial thickness formed by the progenitor and maturation compartments and the progenitor/maturation compartment ratio. Using the information supplied in Table 2.7 it is apparent that the progenitor cell compartment makes up on average 6.4 per cent of the total epithelial thickness and that the maturation compartment comprises the remaining 93.6 per cent of normal human buccal epithelium (Fig. 2.11).

2.4.4 Compartment Analysis of Buccal Epithelium from Anaemic Patients

The mean number of points counted per field for the iron deficient patients is shown in Table 2.8. Using the

same methods as described earlier (Fig. 2.10), the areas and subsequently the thicknesses of the epithelium and of each epithelial compartment were calculated (Table 2.9). The proportions of the epithelium formed by each compartment are shown in Table 2.10. The progenitor cell compartment in buccal epithelium from anaemic patients makes up about 14.1 per cent of the total epithelial thickness and the remaining 85.9 per cent is formed by the maturation compartment (Fig. 2.12).

2.4.5 Comparison of Buccal Epithelium from Normal Subjects and Anaemic Patients

It was not possible to obtain closely age and sex matched controls but for the purposes of this preliminary study the results of the stereological analysis of the epithelium from control subjects were initially compared with the results from the whole group of anaemic patients. Due to the small number of subjects in the control group the Mann-Whitney U Test, a powerful nonparametric statistical test, was used (Siegel, 1956) (Fig. 2.13).

Comparison of the two groups revealed a highly significant ($P = .001$) reduction in the total epithelial thickness of the anaemic group of patients. This reduction in thickness was due to a highly significant reduction in the thickness of the maturation compartment ($P = .001$). There was however, no significant change in the thickness of the progenitor cell compartment ($P = .26$). The ratio of progenitor cell compartment to total epithelial

thickness and the ratio of progenitor cell compartment to the maturation compartment were significantly greater (P = .003) in the anaemic group indicating that the progenitor cell compartment was making up a larger proportion of the total epithelial thickness. The ratio of maturation compartment to total epithelial thickness was significantly reduced (P = .003) in anaemic patients.

2.4.6 Comparison of Buccal Epithelium from Normal Subjects and Anaemic Male Patients

Comparison of the normal subjects and anaemic male patients revealed that there was a significant (P = .02) reduction in the total epithelial thickness of anaemic patients due to a significant (P = .02) reduction in the maturation compartment size. There was no difference in the thickness of the progenitor cell compartment. As a result of the decrease in epithelial thickness of anaemic male patients the proportion of the epithelium made up by the progenitor cells was significantly increased (P = .03) and the maturation compartment formed a significantly (P = .03) smaller proportion of the epithelium in anaemic patients. The ratio of progenitor compartment to maturation compartment was significantly (P = .03) increased.

2.4.7 Comparison of Buccal Epithelium from Normal Subjects and Anaemic Female Patients

The results of stereological analysis of buccal epithelium from normal subjects were compared against those from the anaemic female patients. The results showed a

highly significant ($P < .001$) reduction in the total epithelial thickness of anaemic female patients and that this was due to a highly significant reduction in the maturation compartment size ($P < .001$). There was no significant difference in the progenitor cell compartment thickness but the ratios of progenitor compartment to total epithelial thickness and progenitor compartment to maturation compartment were significantly increased ($P < .01$) and the ratio of maturation compartment to total epithelial thickness was significantly decreased ($P < .01$) in the anaemic female patients.

2.4.8 Comparison of Buccal Epithelium from Anaemic Male and Female Patients

Although there were significant differences between the epithelial parameters of normal subjects and anaemic females it may be unreasonable to assign these changes to iron deficiency alone and it was felt that comparison of the epithelium from iron deficient males and females might reveal whether or not the differences were indeed due to iron deficiency. No significant difference was noted in the thickness of the progenitor cell compartment, in the proportion of the epithelium formed by the progenitor cell compartment or in the progenitor cell compartment to maturation compartment ratio. The epithelium of the iron deficient males was significantly thicker ($P < .03$) than that of the iron deficient females and this was due to a significantly thinner ($P < .03$) female maturation compartment. The proportion of the epithelium made up by

the maturation compartment was also significantly less ($P = .05$) in female patients. The epithelium of the female patients appears to be thinner than that of the male patients and this is due to a decrease in the thickness of the maturation compartment.

2.5 DISCUSSION

The results of this study although limited by the nature of the control group have suggested certain interesting features of iron deficiency. It appears that iron deficiency causes a reduction in the total epithelial thickness principally as a result of a reduction in the maturation compartment size. The patterns of epithelial change are the same in both iron deficient male and female patients. Although the epithelium of female patients is thinner than that of the iron deficient male patients there is no difference in the proportion of the epithelium formed by the progenitor cell compartment and the ratio of progenitor cell compartment to maturation compartment is unaltered.

This suggests that the compartment changes found in the female patients with iron deficiency anaemia when compared with normal subjects are due to the iron deficiency and not to the sex difference.

2.6 CONCLUSIONS

In a human study the effect of environmental factors and other variables are very difficult to assess and place limitations upon the interpretation of data. However, the results of this study suggest that iron deficiency has a profound effect upon the structure of the oral mucous membrane. Further investigation under the strictly controlled conditions of an animal experiment are required to minimise the effects of variables and to determine whether or not the epithelial changes reported in this study are primarily a result of iron deficiency.

CHAPTER 3

DEVELOPMENT OF AN ANIMAL MODEL OF IRON DEFICIENCY. STEREOLOGICAL ANALYSIS OF VENTRAL TONGUE EPITHELIUM FROM NORMAL AND EXPERIMENTAL ANIMALS

3.1 INTRODUCTION

In Chapter 2 the difficulties experienced in controlling the variables inherent in the investigation of iron deficiency in man were highlighted and the need for a more carefully controlled study was stressed. The effects of variables may be minimised by increasing the number of patients included in the study, or by laying down more rigorous criteria for the inclusion of patients. An alternative method of overcoming these difficulties would be to use an experimental animal model. A suitable animal model would allow the development of iron deficiency independent of other diseases such as neoplasia and would enable such local factors as smoking, poor oral hygiene and alcohol ingestion, which could have an effect upon the oral epithelium, to be eliminated. It would also be possible to control the severity and duration of the deficiency and the exact aetiology of the deficiency would be known. Chronic blood loss, often complicated by a low dietary iron content, is the most common cause of iron deficiency in man. Thus the development of an animal model of iron deficiency using a combination of repeated blood loss and low dietary iron content would be analogous to the human situation.

The aims of the study reported in this chapter were to develop an animal model of iron deficiency using a combination of low dietary iron and chronic blood loss and, by the use of stereological techniques, to quantify any changes occurring in the oral epithelium of experimental animals.

3.2 MATERIALS AND METHODS

3.2.1 Selection of Laboratory Animal

Animal models of iron deficiency commonly involve rearing animals on a diet with a low iron content. Usually young or weanling animals are raised on an iron free synthetic diet and after a period of some months iron deficiency develops. This method has certain disadvantages. Schmidt (1926) showed that severe iron deficiency in young animals results in aplasia of the thymus and other severe systemic abnormalities. It would perhaps be unreasonable to compare the epithelium of such animals with normal animals and assign any epithelial abnormalities to iron deficiency alone. In addition, most laboratory animals require adequate amounts of dietary Vitamin C, Vitamin B_{12} and folic acid and although supplements can be added to synthetic diets, deficiency of these vitamins cannot be easily excluded (1.4.6).

The hamster was chosen as a suitable experimental animal. It does not appear to require dietary Vitamin C, Vitamin B_{12} or folic acid and deficiency of these vitamins

should not arise and complicate the study (Granados, 1951). The hamster has proved a hardy, reliable experimental animal and suitable for the study of diseases of the oral cavity (Shklar, 1972).

3.2.2 Experimental Design

Twenty black eared lemon yellow hamsters from a line bred strain were obtained *. Male hamsters were chosen to avoid any possible epithelial changes due to the cyclical effects of circulating oestrogens (Trott, 1957). Young adult males approximately 8-10 weeks of age were caged individually in plastic cages to avoid the animals obtaining iron from extra-dietary sources. Although trace amounts of iron may have been obtained from water or bedding material, the small amounts received in this manner were considered insignificant.

The animals were divided into three groups. A control group (Group 1) of five animals was fed a normal diet, an experimental group also of five animals (Group 2) were maintained on an iron reduced diet +, and a second experimental group (Group 3) of ten animals were maintained on an iron reduced diet and in addition had 1 ml of venous blood removed weekly. All animals had water and food ad libitum. The animals were weighed at the start of the

* Coombenhurst Breeding Station, Basingstoke, Kent.

\+ Diet supplied by Cooper Nutrition Products Ltd. Stepfield, Witham, Essex.

experiment and weekly thereafter to help assess whether or not the diet or the experimental procedure had a measureable systemic effect.

The iron reduced diet had less than fifty per cent of the total iron content of a normal commercial laboratory chow. The iron content of the diet was checked by flame spectrophotometry. Powdered diet was hydrolysed in hydrochloric acid in iron free glassware. The acid was gradually evaporated, and the residue dry ashed overnight in an oven at 800°C. The ash was then dissolved in a neutral buffered saline solution and the iron content measured. The results agreed closely with the specification of the manufacturer; the diet having between 19-25 mg of iron per kilogram.

3.2.3 Method of Obtaining Blood Samples

Blood samples may be obtained from small animals by several methods. Cardiac puncture may be undertaken under general anaesthesia. A needle is introduced directly into one of the chambers of the heart usually without opening the thorax. This method carries a high mortality and is obviously unsuitable for repeated removal of blood samples. The tail or ear veins, particularly in rats and rabbits respectively, are easily accessible and are suitable sites for repeated venesection, but the hamster has only a rudimentary tail and the veins of the ear are small and thin walled, making venesection difficult.

Pansky et al (1961) described a method of obtaining blood from the retro-orbital venous sinus of the hamster and this technique was used in the present study. If care is taken repeated samples can be conveniently withdrawn. The animals may be venesected either with or without general anaesthesia and it is possible to obtain blood samples without the help of an assistant. In practice, it is easier if the animal is lightly anaesthetised with ether and held by an assistant who retracts the lower eyelid. The operator retracts the upper eyelid and inserts a heparinised needle (gauge 25) about half-way along the supra-orbital ridge between the eyelid and the eye (Fig. 3.1). The needle is passed downwards at an angle of 45^{o} keeping close to the medial wall of the orbit (Fig. 3.2). Gentle aspiration confirms entry to the venous sinus and up to 4 ml of blood can be obtained. As hamsters' blood coagulates very quickly, heparinised needles and syringes must be used or blockage of the needle by clot occurs.

Using the above method venous samples were taken from the normal group, and from the group of animals maintained on the iron reduced diet at the beginning of the experiment and after eight weeks, to provide control values for the haematological parameters being measured. Experimental animals had 1 ml of blood removed weekly for 14 weeks. Estimations of haemoglobin and of serum iron were made weekly from the 1 ml of blood removed.

3.2.4 Sample Collection at Sacrifice

The results of serum iron estimation at 14 weeks suggested that the animals were developing iron deficiency (Fig. 3.3). Due to laboratory difficulties it was not possible to sacrifice the animals at 15 weeks but the animals were sacrificed at 16 weeks by overdose of intra-peritoneal barbiturate (Nembutal). At sacrifice in order to obtain the maximum amount of blood for biochemical analysis the abdomen of the hamsters was opened and blood samples taken from the inferior vena cava (Fig. 3.4). Using this method it proved possible to obtain up to 5 ml of venous blood.

In addition to the blood withdrawn at sacrifice a sample of femoral marrow was aspirated. A femur from each animal was dissected free, fractured, and the marrow removed using a wide bore needle. Smears of femoral marrow were stained by the Prussian blue reaction for iron.* A semi-quantitative system (Beutler et al, 1958) was used to estimate the amount of iron present in the marrow of the animals in the three groups. If on examination of fifty oil immersion fields no iron was seen as blue granules or blue staining reticulum cells, then the iron content of the marrow was scored as absent. If less than three fields contained iron the content was marked as trace. More than three fields, but less than ten containing iron was scored as moderate and more than ten normal.

* Appendix 2

3.2.5 Analysis of Blood Samples

The haemoglobin concentration was measured by a microcyanomethhaemoglobin method.* All measurements were made on fresh venous blood samples and care was taken to avoid haemolysis of samples at all stages in the procedure. After the haemoglobin concentration had been measured the remaining blood was centrifuged, the serum collected, and if not used immediately stored at - 20°C until required. The serum iron estimations were conducted using a modification of the technique described by Giovanniello, (1966) +. The samples were measured as part of the routine diagnostic biochemistry, Department of Veterinary Biochemistry, University of Glasgow.

3.2.6 Selection of Biopsy Site and Biopsy Procedure

The ventral surface of tongue was chosen as a suitable biopsy site. This area is readily accessible and has anatomical features which allow consistent selection of comparable sites in different animals. In addition the ventral surface of tongue has a relatively simple histological composition aiding the subjective differentiation of epithelial compartments.

At sacrifice the tongue was carefully removed from each animal. Resection at the posterior reflection of the

* S.P. 60 Spectrophotometer, Ortho Diagnostics

\+ Technicon Autoanalyser

floor of the mouth allowed most of the tongue to be removed intact. The tongue was placed on a fresh piece of thin dental wax and by use of a razor blade a strip of tongue to one side of the midline was delineated. Using fine forceps and scissors this strip of ventral mucosa was removed. The portion of tissue held in the forceps was discarded and the remaining tissue trimmed into 1 mm cubes. The 1 mm cubes were placed in tissue culture medium and then cultured in vitro in the presence of a radioactive isotope. After in vitro labelling the tissue was fixed in Bouins' solution. The rationale and details of the culture technique are reported in Chapter 5.

3.2.7 Section Preparation

After fixation in Bouins' solution for approximately two hours the material was processed in a Histokine and paraffin embedded. To check the orientation of each block, an early section was taken and stained with haematoxylin and eosin. The subsequent sections were cut on a rotary microtome at 3 μm and mounted on slides treated with a thin layer of gelatin (Rogers, 1973). Four ribbons of five sections from each block were mounted across the slides. These ribbons were the serial sections at levels 1-5, 8-13, 17-21 and 25-29. Sections at intervening levels were mounted separately and used as spares in the event of the first sections being unsatisfactory.

3.2.8 Identification of Compartments Making up Hamster Ventral Tongue Epithelium

The ventral surface of hamster tongue consists of three cell compartments, the progenitor, the maturation and the keratinised cell compartment (Fig. 3.5). Separation of progenitor and maturation compartments has to be made subjectively and the criteria used for identification of these compartments are as reported in section 2.3.5. It is somewhat simpler to distinguish the maturation compartment from the keratinised compartment. Within the keratinised compartment there is loss of stainable nuclear chromatin, loss of cell outlines and it is not possible to distinguish keratohyaline granules (Fig. 3.6).

3.2.9 Stereological Analysis of Compartment Size

The method used was similar to that reported in section 2.3.6. Four sections from each biopsy were used; the middle section from each ribbon of five being examined. A 0.5 cm grid was used and the grid positions were as reported in section 2.3.6. Hamster epithelium is thinner than human buccal epithelium and in order to increase the number of points counted a second field was examined in each section. To ensure that sufficient points were being counted the accumulative means test, as in section 2.3.6 was applied. The lines of demarcation between compartments were again drawn on the projection screen and a x40 objective gave a projected magnification of x500.

3.3 RESULTS

3.3.1 Animal Weights

During the course of the experiment two animals in Group 1 and two animals in Group 3 died. Post mortem examination of the two normal animals failed to reveal the cause of death. The animals dying in Group 3 failed to recover from a general anaesthetic and overdose of ether was considered the cause of death. The weekly weights of the individual animals in the three groups are shown in Table 3.1. Due to the small numbers of animals in each group, non-parametric statistical tests were used for comparison of the groups. Using the Mann-Whitney U Test no significant differences were found in the starting weights of the three groups. The weights of the groups at sacrifice are also shown in Table 3.1 and there was no significant difference between the weights of Groups 1, 2 and 3 at sacrifice.

As minor differences in starting weight may disguise any effect of the experimental procedure the weight gain of Group 1 over the course of the experiment was compared to the weight gains of Group 2 and 3. There was no significant difference in weight gain between Group 1 and Groups 2 and 3, and it appears that neither the diet nor the repeated venesection produced a measurable adverse systemic effect.

3.3.2 Normal Animals Haemoglobin and Serum Iron Values

The haemoglobin value for each animal at the three sample times is shown in Table 3.2. At the start of the experiment, the range of haemoglobin concentration for Group 1 animals was 13.5 - 15.5 g/dl. Although within the normal range these are rather low haemoglobin values, but the values of haemoglobin concentration at week 8 and at sacrifice accord well with the figures reported by other authors (Friedell and Bannon, 1972).

The serum iron values for the animals in Group 1 are shown in Table 3.3 and vary little throughout the course of the experiment. The results of serum iron estimations are within the limits quoted as normal by other authors (Bannon and Friedell, 1972).

3.3.3 Experimental Animals Haemoglobin and Serum Iron Values

The haemoglobin concentrations for Group 2 are shown in Table 3.4. The range of these values is within normal limits and the slight increase in the haemoglobin concentration noticed over the course of the experiment appears to be a normal finding in adult hamsters (Friedell and Bannon, 1972). The serum iron values for animals in Group 2 are within normal limits at all sample times (Bannon and Friedell, 1972) Table 3.5.

The individual haemoglobin values of the animals in Group 3 are shown in Table 3.6. At the start of the

experiment the haemoglobin concentration of the animals of Group 3 ranged from 14.5 - 17.5 g/dl. Around week 9 haemoglobin values of individual animals had risen outwith the normal range for male hamsters of this age (Friedell and Bannon, 1972) and a peak haemoglobin concentration was reached at weeks 10 and 11. This rise in haemoglobin concentration probably reflects a maximal haemopoietic activity in response to repeated venesection. At sacrifice individual values had again risen outwith the normal range as a result of marrow recovery during the interval of fourteen days between the last venesection and sacrifice.

The weekly serum iron levels of animals in Group 3 are shown in Table 3.7. The values of individual animals fell during the course of the experiment to a low of 10.7 μmol/l at week 14. This value is below the normal range for hamsters of this age but by sacrifice the serum iron values had returned to normal (Fig. 3.3).

3.3.4 Total Iron Binding Capacity

It was intended to include an estimation of the total iron binding capacity of Groups 1, 2 and 3 but due to unforeseen technical difficulty it proved impossible to measure the iron binding capacity of the animals in this study.

3.3.5 Comparison of the Haemoglobin and Serum Iron of Normal and Experimental Animals

Haemoglobin: Using the Mann-Whitney U test the three groups were compared at the start, mid point and end of the experiment.

Comparison of Groups 1 and 2 showed that animals in Group 1 had a significantly lower ($P = 0.01$) initial haemoglobin concentration but at week 8 and at sacrifice there was no significant difference in the haemoglobin concentrations. The relevance of the difference in starting haemoglobin is not known but as the week 8 and sacrifice values did not differ the initial difference is probably of little importance and may be the result of experimental error. The similarity of the midway and sacrifice values suggests that the iron deficient diet produced no measurable effect upon haemoglobin concentration.

Groups 1 and 3 did not differ in the initial haemoglobin estimation but by week 8 the animals in Group 3 had a significantly lower ($P = 0.01$) haemoglobin concentration. At sacrifice this difference had altered and there was a slightly higher haemoglobin value in Group 3. It is likely that the low dietary iron content and repeated venesection produced the reduction in haemoglobin concentration at week 8, but the fourteen day period before sacrifice was sufficient for recovery of the haemoglobin of animals in Group 3.

Comparison of Groups 2 and 3 showed no significant difference in the initial, mid point or sacrifice haemoglobin concentrations.

Serum Iron: Comparison of Groups 1 and 2 revealed no significant difference in the levels of serum iron during the course of the experiment. There was no significant difference in serum iron concentration between Groups 1 and 3 and between Groups 2 and 3 at the start of the experiment, at week 8 or at sacrifice.

The results of the serum iron measurements are illustrated in Figure 3.3. It appears that the animals of Group 3 were able to cope with the repeated blood loss and low dietary iron for about 12 weeks. After this period the serum iron fell but the two week period before sacrifice allowed the serum iron to return to normal levels.

3.3.6 Marrow Examination

Using the method described in section 3.2.4 the marrow of each animal was examined for iron without prior knowledge of the animals' number or group. Animals in Group 3 had absent or trace amounts of marrow (Fig. 3.7). Normal amounts of iron were noted on all smears from the animals of Groups 1 and 2. This absence or reduction of body iron stores in the presence of serum iron and haemoglobin values within the normal range is by definition termed iron depletion.

3.3.7 Compartment Analysis of Ventral Epithelium from Normal Animals

The total number of points counted per cell compartment for animals in Group 1 is shown in Table 3.8 and the absolute measurements of epithelial thickness and of compartment thickness are shown in Table 3.9. The proportions of the epithelium made up by each compartment are shown in Table 3.10. Ventral tongue epithelium has a mean thickness of 63.4 μm (range 52.3 - 75.6) and the progenitor, maturation, and keratinised compartments make up 18.8, 55.4 and 25.8 per cent respectively of the total epithelial thickness.

3.3.8 Compartment Analysis of Ventral Epithelium from Animals in the Iron Deficient Diet Group

The number of points counted in each compartment is shown in Table 3.11. The compartment thicknesses and total epithelial thickness are shown in Table 3.12 while the proportions of the epithelium made up by each compartment are tabulated in Table 3.13. The mean epithelial thickness for this group is 69.3 μm (range 58.8 - 89.7). The progenitor cell compartment comprises 18.3 per cent, the maturation compartment 55.0 per cent and the keratinised compartment 26.7 per cent of the epithelium.

3.3.9 Compartment Analysis of Ventral Epithelium from Animals in the Iron Deficient Diet and Bleeding Group

The total number of points counted for each animal is shown in Table 3.14 and the compartment and epithelial

thickness in Table 3.15. The proportions of the epithelium made up by each compartment are shown in Table 3.16. The mean epithelial thickness is 58.9 μm (range 47.2 - 76.7) and the progenitor, maturation and keratinised compartment make up 21.6, 52.0, and 26.4 per cent of the total epithelial thickness.

3.3.10 Comparison of the Compartment Analysis of Normal and Experimental Animals

Groups 1 and 2: Comparison of the results of stereological analysis of the ventral epithelium of animals in Groups 1 and 2 failed to reveal any significant difference in the total epithelial thickness, in the thickness of the individual compartments, or in the proportion of the epithelium formed by each compartment.

Groups 1 and 3: There was no significant difference in the thickness of the epithelium or in the size of individual compartments. The progenitor cell compartment made up a significantly greater proportion ($P = 0.01$) of the epithelial thickness in Group 3 animals. There were no significant differences in the proportions of the epithelium formed by the maturation and keratinised compartments.

Groups 2 and 3: There was no significant difference in the total epithelial thickness or in the thickness of individual compartments. The progenitor cell compartment again made up a significantly greater proportion of the

epithelium in Group 3 animals (P = 0.002). There were no changes in the proportions of the epithelium formed by the maturation and keratinised compartments. As Groups 1 and 2 showed no difference in the epithelial parameters measured and in order to increase the numbers of animals in the control group it was thought reasonable to combine Groups 1 and 2 and compare them with Group 3.

Groups 1 + 2 and 3: There was no significant change in the size of the progenitor and keratin compartments and the total epithelial thickness did not alter significantly. The maturation compartment was significantly thinner (P = 0.05) in animals in Group 3. The progenitor compartment again formed a significantly higher proportion of the total epithelial thickness (P = 0.001). There were no changes in the proportions of the epithelium formed by the maturation and keratinised compartments.

3.4 DISCUSSION

The initial attempt to produce iron deficiency in hamsters has revealed certain interesting findings. The ability of the experimental animals to withstand repeated venesection, and to remain well with a relatively low mortality (2 out of 10) over a sixteen week period has proved the value of the hamster as an experimental animal in this study.

The iron stores of the young adult males appear to be readily mobilised and to be greater than expected. Further, to withstand the loss of about twice their blood volume in a relatively short period of time these animals must be able to absorb virtually all the available dietary iron, and alone, a diet low in iron would be unlikely to produce iron deficiency.

A blood film with a reticulocyte count might have been useful in confirming a maximal haemopoietic response. This was attempted using no anticoagulant, citrate, and sequestrene as anticoagulant. However, all proved unsatisfactory in the operator's hands and the samples clotted immediately on contact with air. In addition it was thought reasonable to base the diagnosis of iron deficiency upon the results obtained from marrow examination, haemoglobin value and serum iron estimation.

The results of stereological analysis of the ventral epithelium showed no overall change in the epithelial thickness. However, even at this early stage in the development of iron deficiency changes were apparent in the make up of the epithelium. The maturation compartment was significantly thinner in iron depleted animals and although there was no significant change in progenitor cell compartment thickness there was a significant increase in the proportion of the epithelium formed by the progenitor cell compartment.

3.5 CONCLUSIONS

The hamster appears to be a suitable animal for the study of iron deficiency. The animal is hardy enough to withstand repeated venesection of about one tenth of its blood volume every week, and by a combination of low dietary iron and repeated venesection it is possible to induce the earliest measurable stage of iron deficiency, that is iron depletion. At week 13 and 14 the low serum iron values obtained from individual animals leads to the conclusion that a longer experimental period and/or more frequent venesection would lead to the development of the further stages or iron deficiency.

Changes are present in the ventral epithelium of iron depleted animals and quantitative examination of the epithelium of animals with the further stages of iron deficiency is thought likely to reveal more marked changes in the structure of the epithelium.

CHAPTER 4

DEVELOPMENT OF IRON DEFICIENCY IN THE HAMSTER. STEREOLOGICAL ANALYSIS OF VENTRAL TONGUE EPITHELIUM IN NORMAL AND EXPERIMENTAL ANIMALS

4.1 INTRODUCTION

In Chapter 3 the results of the initial attempt to produce iron deficiency in hamsters were discussed and the results of the quantitative analysis of ventral tongue epithelium from normal and iron depleted animals were reported. It was apparent that despite a weekly loss of 1 ml of blood hamsters were able to maintain their haemoglobin and serum iron levels for about 12 weeks. By increasing the amount of blood withdrawn and extending this repeated blood loss over a longer experimental period it should be possible to further stress the iron balance of the experimental animals and to produce the later stages of iron deficiency.

Epithelial abnormalities were noted in the lingual epithelium of iron depleted animals and it is likely that quantitative analysis of epithelium from animals with iron deficiency without anaemia and from animals with iron deficiency anaemia will reveal further epithelial abnormalities.

The aims of the study reported in this chapter

were to develop the further stages of iron deficiency using a method similar to that reported in Chapter 3 and to examine and quantify the changes occurring in the ventral tongue epithelium of experimental animals.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design

The hamster has proved to be a suitable animal (3.5) for this type of experimental procedure and was used in this study. Thirty eight adult male Syrian Golden hamsters * were divided into a control group of twelve, and an experimental group of twenty six animals. The experimental animals were fed an iron reduced diet + and the control animals were given standard laboratory chow. Both groups had food and water ad libitum and were housed in standard plastic hamster cages.

All animals were weighed weekly to assess whether or not the extended experimental procedures were having a marked systemic effect. Experimental animals had 1 ml of blood withdrawn, under ether anaesthesia, from the retro-orbital venous sinus (3.2.3) three times every two weeks for a total of twenty six weeks. Normal animals had blood withdrawn at the start, mid point and end of the experiment to provide control values. After twenty seven weeks the

* Coombenhurst Breeding Station, Basingstoke, Kent.

\+ Cooper Nutrition Products Ltd., Stepfield, Witham, Essex.

animals were sacrificed by an overdose of intra-peritoneal barbiturate and blood was removed from the inferior vena cava as described in section 3.2.4. Marrow smears and samples of ventral tongue were also obtained as described in section 3.2.4. During the course of the experiment two control and four experimental animals died. These animals have been excluded from the analysis.

4.2.2 Laboratory Methods

The tissue removed from the ventral surface of tongue was cultured, fixed, processed and slides prepared as in sections 3.2.6 and 3.2.7. The estimations of serum iron, total iron binding capacity and haemoglobin were performed by the Department of Veterinary Biochemistry, University of Glasgow. The method of stereological analysis was described in section 3.2.9 and a x 40 objective was employed giving a total magnification of x 500.

4.3 RESULTS

4.3.1 Animal Weights

Control animals were weighed weekly during the experiment and the weekly weights are shown in Table 4.1. Control animals had a mean starting weight of 99.9 g (S.D. 7.3) and by sacrifice this value had risen to 109.3 g (S.D. 9.4). There was a mean weight gain of 9.4 g during the experimental period.

Experimental animals were also weighed weekly and

the weights are shown in Table 4.2. Experimental animals had a mean starting weight of 101.4 g (S.D. 10.6) but by sacrifice this had fallen to a mean of 95.6 g (S.D. 16.5). There was a mean weight loss of 5.8 g during the experimental period.

The weights of control and experimental animals were compared at the start, midpoint and end of the experiment. The weights of the animals used for comparison are actual weights and are measured on a ratio scale. More than thirty animals were used in this experiment and the weights were normally distributed about the mean. The data was therefore amenable to analysis by parametric methods (Siegel, 1956) and the Students' "t" test was used for the analysis.

There was no difference between the animal weights at the start and at the mid point of the experiment but at sacrifice the experimental animals were significantly lighter than control animals ($P = .006$). It appears that the experimental procedure extended over 26 weeks produced a marked systemic effect with experimental animals being significantly lighter than control animals. The fall in weight for experimental animals only becomes apparent after 24 weeks and this weight loss is accompanied by a marked drop in the haemoglobin concentration (Table 4.6). There is a significant correlation between the weight loss of experimental animals and the falling haemoglobin over

the last four weeks of the experiment. It is therefore thought reasonable to compare epithelium from normal and experimental animals assigning any changes found in the epithelium to the low haemoglobin and not simply to a change in animal size.

4.3.2 Haemoglobin and Serum Iron Values of Control Animals

The results of the haemoglobin estimations for normal animals are shown in Table 4.3. The mean haemoglobin of control animals was 15.6 g/dl (S.D. 1.4) at the start of the experiment with a mean value of 15.0 g/dl (S.D. 1.1) at sacrifice. These results are within the limits of normal. The serum iron values are shown in Table 4.4. The initial mean serum iron was 57.8 μmol/l (S.D. 8.1) and although there was a fall in the value at sacrifice to 46.1 μmol/l (S.D. 11.2) the results are within normal limits.

4.3.3 Total Iron Binding Capacity Values and the Transferrin Saturation of Control Animals

After estimation of the serum iron and the haemoglobin at weeks 1 and 13 there was insufficient remaining serum to allow measurement of the T.I.B.C. It was only possible to measure the T.I.B.C. at sacrifice when a larger volume of blood was available.

Some difficulty was encountered in the measurement of the T.I.B.C. of hamster serum. The nature of the

protein and the high protein content of hamster serum made the separation of bound transferrin difficult. The relatively small amounts of serum left after haemoglobin and serum iron estimation added further to the technical problems and because of these difficulties the T.I.B.C. was not completed in every case. The results of the T.I.B.C. measurement for control animals are shown in Table 4.5.

The ratio of serum iron to T.I.B.C. expressed as a percentage (transferrin saturation) is used as a reliable estimation of iron stores (1.3.5) and the transferrin saturation results for control animals are shown in Table 4.5.

4.3.4 Haemoglobin and Serum Iron Values of Experimental Animals

The haemoglobin values for experimental animals are shown in Table 4.6. At the start of the experiment the mean haemoglobin was 16.3 g/dl (S.D. 1.0) and over the experimental period this gradually fell to 13.7 g/dl (S.D. 1.5) at sacrifice. The mean serum iron for experimental animals was 57.5 µmols/l (S.D. 6.0) at the start of the experiment and this fell to a mean of 23.6 µmols/l (S.D. 10.1) at week 27. The weekly serum iron values are shown in Table 4.7.

4.3.5 Total Iron Binding Capacity Values and the Transferrin Saturation of Experimental Animals

The results of the T.I.B.C. measurement for

experimental animals are shown in Table 4.8. The mean T.I.B.C. for experimental animals was 88.1 μmols/l (range 60.1 - 113.1). The transferrin saturation for experimental animals is also shown in Table 4.8. The mean transferrin saturation for experimental animals was 25.5 per cent (range 6.4 - 49.1).

4.3.6 Marrow Examination of Control and Experimental Animals

Using the techniques described in section 3.3.6 the marrow smears from each animal were prepared and examined. On examination of fifty oil immersion fields all control animals had normal amounts of marrow iron. Quantitation of the marrow smears from experimental animals revealed absent or only trace amounts of marrow iron.

4.3.7 Comparison of the Haematological and Biochemical Results of Experimental and Control Animals

Using the Students' 't' test the haemoglobin and serum iron of control and experimental animals were compared at the beginning, midpoint and end of the experiment. There was no significant difference between the initial and midpoint haemoglobin values of experimental and control animals. At sacrifice, the haemoglobin of experimental animals was significantly less than the haemoglobin of control animals ($P = .01$).

Comparison of the serum iron values revealed no initial difference between the groups but the serum iron

of experimental animals at week 13 and at sacrifice was significantly lower than that of control animals (P = .003 and P = .00001). In addition the total iron binding capacity of experimental animals was significantly lower than that of normal animals (P = .037). The transferrin saturation was also significantly lower in experimental animals (P = .0001).

4.3.8 Interpretation of the Haematological and Biochemical Results

From the data it is apparent that the experimental procedure resulted in a significant drop in the haemoglobin, serum iron and transferrin saturation of experimental animals. It is also clear that individual animals vary greatly in their response to the repeated venesection (Tables 4.6 and 4.7). By using the criteria described in section 1.3.2 and employed in section 3.3.6 it is possible to divide the experimental animals into three groups representing the different stages of iron deficiency.

Animals with absent or trace amounts of marrow iron, and a haemoglobin less than 12 g/dl were described as anaemic. Experimental animals having a haemoglobin within the normal range, absent or trace amounts of marrow iron stores, and a transferrin saturation less than 20 per cent were defined as iron deficient without anaemia. Animals with a normal haemoglobin, normal transferrin saturation but absent or trace amounts of marrow iron were

described as iron depleted. The results of this classification are shown in Tables 4.9, 4.10, 4,11, and this method of grouping the animals was used for the quantitative analysis of the ventral epithelium.

4.3.9 Compartment Analysis of Ventral Tongue Epithelium from Normal Animals

Over 2000 point counts were made per animal and an example of the method used to calculate the absolute thickness from the point count is shown in Figure 4.1. The total thickness of the epithelial compartments for normal animals are shown in Table 4.12. It can be seen that the mean thickness of the ventral epithelium was 53.7 μm (range 44.3 - 70.1 μm) and that these figures compare well with the values recorded in Table 3.9. The proportion of the epithelium made up by each compartment is also shown in Table 4.12. The progenitor compartment makes up 28 per cent (range 24.4 - 30.2) the maturation compartment 44.2 per cent (range 40.6 - 48.7) and the keratin 27.8 per cent (range 24.4 - 31.3) of the epithelium of control animals.

4.3.10 Compartment Analysis of Ventral Tongue Epithelium from Anaemic Animals. Comparison with Normal Animals

The values for the compartment analysis of anaemic animals are shown in Table 4.13. The progenitor compartment had a mean thickness of 14.6 μm (range 13.0 - 16.1) and the maturation compartment measured 18.0 μm (range 16.5 - 19.8).

The mean keratin thickness was 18.2 μm (range 14.3 - 20.9) and the mean total epithelial thickness 50.8 μm (range 44.1 - 56.8). The proportions of the epithelium made up by each compartment are also shown in Table 4.13.

Due to the smaller number of animals in each category it was not possible to use the Students' "t" test and non parametric statistical analysis had to be employed. The Mann-Whitney U test was used.

There was no significant difference in the total epithelial thickness of normal and anaemic animals or in the thickness of the progenitor compartments. The maturation compartment of anaemic animals was significantly thinner than that of the normal animals ($P < .002$). Comparison of the keratinised compartments showed that the anaemic animals had a thicker keratinised layer ($P = .054$). Although this value is not significant at conventional limits of significance used in biological studies, the size of the P value for the statistical test suggests this difference is a real observation and it may be that the small numbers of animals in the anaemic group are influencing the level of P. Accordingly for this parameter the anaemic group and the iron deficiency without anaemia group of animals were combined and the keratinised compartment values compared with normal animals. The keratinised compartment of the experimental group was significantly thicker than that of the controls ($P < .05$).

There was no change in the ratio of progenitor cell compartment to total epithelial thickness but the maturation compartment formed significantly less ($P < .002$) and the keratinised compartment significantly more of the epithelium in anaemic animals ($P < .002$).

4.3.11 Compartment Analysis of Ventral Tongue Epithelium from Animals with Iron Deficiency without Anaemia. Comparisons with Normal and Anaemic Animals

The absolute values of compartment and epithelial thickness for iron deficient but not anaemic animals are shown in Table 4.14. The mean total epithelial thickness of this group of animals was 54.5 μm (range 45.2 - 66.1). The progenitor compartment made up 28.9 per cent of this total and the maturation and keratinised compartments formed 39.3 per cent and 31.8 per cent respectively, (Table 4.14).

Comparison of control animals with animals in the iron deficiency without anaemia group showed no difference in the total epithelial thickness or in any of the individual compartment sizes. There was no difference in the proportion of the epithelium made up by the progenitor cell compartment but the maturation compartment formed a significantly smaller part of the epithelium ($P < .002$) and the keratinised compartment a significantly larger part of the epithelium ($P < .02$) in iron deficient animals.

Comparison of iron deficient but not anaemic animals

and anaemic animals revealed no difference in total thickness nor in the thicknesses of the progenitor and keratinised compartments. The maturation compartment of anaemic animals was significantly thinner than that of the iron deficient group ($P = .037$). The ratio of progenitor to total epithelial thickness did not differ but the maturation compartment formed significantly less of the epithelium ($P = .009$) and the keratin significantly more of the epithelium ($P = .005$) in the anaemic group.

4.3.12 Compartment Analysis of Ventral Tongue Epithelium from Iron Depleted Animals. Comparisons with Normal, Anaemic and Iron Deficient but not Anaemic Animals

The values for the total epithelial thickness and for compartment thicknesses are shown in Table 4.15. The mean epithelial thickness of iron depleted animals was 54.6 μm (range 41.4 - 71.2). The progenitor compartment made up 32.1 per cent, the maturation compartment 38.9 per cent, and the keratin formed 29 per cent of the epithelium, (Table 4.15).

Comparison of iron depleted and normal animals revealed no significant difference in total epithelial thickness or in the thickness of the individual compartments. The progenitor compartment formed a significantly larger part of the epithelium ($P < .002$), and the maturation compartment made up a significantly smaller part of the epithelium ($P < .02$) in iron depleted animals.

When iron depleted animals were compared with anaemic animals there was no difference in total epithelial thickness nor was there any change in the maturation or keratinised compartment thicknesses. The progenitor cell compartment was significantly larger in iron depleted animals ($P < .05$) and the progenitor compartment formed a significantly greater part of the epithelium in iron depleted animals ($P < .02$). There was no difference in the proportion of the epithelium formed by the maturation compartment. The keratin layer formed significantly less of the epithelium ($P < .002$) in iron depleted animals.

Comparison of iron depleted and iron deficient but not anaemic animals showed no significant change in the total epithelial thickness or in the compartment sizes. The progenitor cell compartment formed a significantly larger proportion ($P < .02$) and the keratinised compartment a significantly smaller proportion of the epithelium ($P < .05$) in iron depleted animals.

4.4 DISCUSSION

The results reported in this study fail to show any change in the total thickness of the epithelium of the ventral surface of tongue from animals with varying degrees of iron deficiency. There are however, significant differences in the composition of the epithelium and these differences become more pronounced as the severity of iron

deficiency increases.

Anaemic animals have a significantly smaller maturation compartment than normal animals but the reduction in this compartment does not lead to a reduced total epithelial thickness as the keratinised compartment is significantly increased in size (Fig. 4.2).

Animals with iron deficiency without anaemia have no change in compartment size but the maturation compartment forms a significantly smaller proportion of the epithelium, and the keratinised compartment a significantly larger proportion of the epithelium than in normal animals.

Iron depleted animals show a significant reduction in the proportion of the epithelium made up by the maturation compartment and a significant increase in the proportion made up by the progenitor cell compartment.

Thus as iron deficiency develops there is an initial increase in the proportion of the epithelium made up by progenitor cells and a decrease in the proportion formed by maturing cells. With increasing degrees of deficiency the maturation compartment forms less, and the keratinised compartment more of the epithelium until at the stage of anaemia there is a significant reduction in the maturation compartment and a significant increase in keratin thickness (Table 4.16).

Although the differences between the experimental groups highlight these changes, this type of quantitative analysis provides no information about the pathogenesis of these structural changes. Differences in the compartment sizes could be due to a decrease or an increase in the size of the constituent cells and/or to a decrease or an increase in cell numbers. Further, any alteration in cell numbers will be the result of alterations in the cell kinetics and it is likely that an investigation of the cell kinetic parameters of iron deficient epithelium would reveal valuable information on the development of the structural abnormalities.

4.5 CONCLUSIONS

The hamster has proved a suitable animal for studies of iron deficiency and an animal model of iron deficiency disease has been developed. Quantitative analysis of ventral tongue epithelium revealed no difference in the total epithelial thickness in experimental animals but showed marked differences in the structure of iron deficient epithelium. The magnitude of these differences increased with increasing degrees of iron deficiency.

CHAPTER 5

PRELIMINARY INVESTIGATION OF THE CELL KINETICS OF EPITHELIUM OF THE VENTRAL SURFACE OF TONGUE FROM NORMAL AND IRON DEPLETED HAMSTERS

5.1 INTRODUCTION

In Chapters 2, 3 and 4 the results of quantitative morphological studies of oral epithelium in iron deficiency were reported. The stereological analysis provided an insight into the structural changes occurring in iron deficiency, but information pertaining to the pathogenesis of these structural changes is difficult to obtain by stereological methods alone.

Iron has been reported as being essential for the synthesis of DNA and for spindle formation prior to cell division (1.2.2). It is likely that deficiency of iron results in abnormalities of cell metabolism and cell renewal and that these abnormalities may be manifest as the structural changes reported in earlier chapters. It was felt that a study of cell kinetic parameters would provide an insight into the development of the oral epithelial abnormalities occurring in iron deficiency.

Before reporting the methodology and results of this study a brief review and discussion of the general concepts of cell renewal as applied to oral epithelium and

of the methods of studying cell kinetics will be presented.

5.2 BASIC PRINCIPLES AND TECHNIQUES FOR THE STUDY OF CELL KINETICS IN ORAL EPITHELIUM

5.2.1 Characteristics of the Cell Kinetics of Oral Epithelium

The stratified squamous epithelium of the oral cavity falls into a group of epithelia known as renewing cell populations (Leblond and Walker, 1956; Leblond, 1964). Epithelia in this group synthesise DNA and cells undergo division in numbers exceeding that required for growth. Normally a steady state prevails and cell production is balanced by an equivalent cell loss. Cells arise from stem cells, undergo a period of maturation and are then shed. Cameron (1970) reviewed the characteristics of renewing populations and divided them into two main groups. These were classified as slow or rapid systems depending upon whether renewal occurred in more or less than 30 days. As oral epithelium renews in less than 30 days it is classified as a rapid renewal cell population.

The cell cycle of progenitor cells has been divided into four phases and these are illustrated in Figure 5.1. Mitosis or cell division represents the most readily recognisable stage of the cell cycle. Before the normal somatic cell can enter mitosis duplication of the nuclear material must occur and the period taken for this synthesis of DNA is designated the synthesis or S phase. Incorporation of specific radioactive DNA precursors during

DNA synthesis allows, by the use of autoradiography, identification of the nuclei of cells in this phase. The gap after synthesis of the duplicate DNA and before mitosis is designated G_2 and the gap between mitosis and synthesis, G_1. The presence of a fifth stage of the cell cycle G_0 has been proposed as a resting or non cycling phase before G_1, but evidence for the existence of G_0 is not yet conclusive.

As discussed in section 2.3.5 human buccal epithelium consists basically of two compartments; a progenitor cell compartment and a maturation compartment. By definition only the progenitor cell compartment produces new cells. Once cells have passed to the maturation compartment they do not return to the progenitor compartment and cell loss occurs from the oral aspect of the maturation compartment. In certain areas of the human oral cavity a third compartment consisting entirely of dead cells, the keratinised compartment, is present and this type of epithelium consisting of three recognisable compartments makes up the majority of the oral epithelium of laboratory animals. Within the oral epithelium there are small numbers of other specialised cells for example, melanocytes and Langerhan s cells but because of the relatively small numbers of these specialised cells for the purposes of this study their presence will be ignored.

At present there are several methods available for the study of cell proliferation. These have been

comprehensively reviewed by Leblond (1959), and by Baserga and Wiebel (1969). MacDonald (1971a) and Skougaard (1970) have reviewed the methods of study of cell renewal with particular reference to oral epithelium. However, a brief discussion of available methods will be undertaken to help explain the choice of methods employed in this study.

5.2.2 Methods for Studying Cell Renewal

There are basically three methods of estimating cell kinetic parameters; mitotic indices, mitotic arrest techniques and radioactive labelling techniques.

Estimations of mitotic index involve counting the number of cells undergoing mitosis and relating the figure obtained to a reference unit. The most commonly employed reference unit is the number of cells in a particular area. Hence the mitotic index is often expressed as the number of mitosis per 100 or 1000 viable cells. Mitotic counts have also been expressed in terms of progenitor cell numbers, unit epithelial surface length, and unit basement membrane length.

There are disadvantages and inaccuracies in mitotic counts irrespective of the reference system used. The principal disadvantages are the time and tedium involved in counting sufficient mitoses to give reliable results and the difficulty in detecting the early stages of mitosis. Furthermore the number of mitoses present in sections is a

function not only of the rate of entry of cells into mitosis but also of the duration of mitoses, and unless this is known in all tissues under investigation meaningful comparisons cannot be made.

Mitotic arrest techniques employ a variety of agents to prevent completion of mitosis. Drugs such as colchicine and vinblastine prevent formation of the spindle and stop the cell proceeding through metaphase. High doses of irradiation have been employed for the same purpose and using such techniques it is possible to estimate mitotic duration, mitotic rate, and mitotic index.

Radioactive labelling techniques involve the incorporation of specific DNA precursors, labelled with radioactive isotope, into nuclei undergoing replication of DNA. Cells in the S phase take up the radioactive precursor and by autoradiographic techniques identification of labelled cells is possible. Radioactive labelling techniques have certain advantages over mitotic index and mitotic arresting techniques. Identification of labelled cells is simpler and more accurate than mitotic recognition and as the S phase is longer than mitosis, labelled cells are more frequent than mitoses. Labelling techniques provide an accurate and versatile method of investigating cell kinetics and consequently DNA labelling methods will be used in this study to investigate the nature of any changes occurring in iron deficiency.

5.2.3 DNA Labelling for Cell Kinetic Studies

Labelling techniques can be discussed under three main headings; pulse labelling, double labelling and continuous labelling. Pulse labelling involves presenting a labelled precursor, for a short period of time, to the tissue under investigation with the aim of labelling all cells in S phase at that particular time. The information obtained from a pulse label depends upon the time between administration of the precursor and sampling of the tissue. When sampling is carried out shortly after administration of the radioactive isotope counting of labelled cells will provide a labelling index. By sampling at longer intervals after administration of the isotope, information may be obtained on cell migration and estimations may be made of the duration of the S phase.

Double labelling techniques make use of either two different isotopes, for example carbon 14 and tritium, or alternatively two different doses of the same isotope may be used. The methods are basically similar and involve an initial pulse with a weak dose of isotope (or the first isotope) followed after an interval by a second pulse of a heavier dose of isotope (or the second isotope). The double labelling technique using a weak and heavy dose of tritium was described by Baserga and Lisco (1963) and used by Galand et al (1968) who distinguished lightly and heavily labelled cells by the grain density over nuclei in autoradiographs. The number of grains seen over nuclei is related to the

strength of the radioactive source and thus nuclei containing substantially different amounts of isotope can be distinguished (Rogers, 1973). The advantage of double labelling techniques is that counts of weakly and heavily labelled cells (W.L.C. and H.L.C.) may be used in the formula given below to estimate the duration of the S phase, T_s, where t is the time between pulses (Wimber and Quastler, 1963).

$$\frac{T_s}{t} = \frac{H.L.C.}{W.L.C.}$$

Continuous labelling by the administration of repeated doses of labelled precursor and then repeated sampling at intervals, allows the increase in the proportion of labelled cells to be calculated. By plotting the percentage increase in labelled cells against time, this graph or continuous labelling curve allows an estimation of the rate of flow of cells into S phase and provides a means of determining the proportion of cells engaged in cell production.

Radioactive isotopes can be incorporated either in vivo or in vitro. The in vivo route is suitable for experimental animal work and although in vivo techniques have been used for the investigation of cell kinetics in man the in vitro method is generally preferred for labelling human tissues.

With the exception of methods employing a scintillation counter DNA labelling techniques depend upon autoradiography for visualisation and localisation of the radioactive label. Consequently a brief discussion of the principles of autoradiography will be undertaken.

5.2.4 Autoradiography

The principles behind autoradiography are similar to those of photography and radiography. A source of energy, in this case ionising radiation, is directed onto a sensitised film closely apposed to the tissue containing the source of radiation. The film in autoradiography is a layer of emulsion consisting of silver bromide crystals embedded in gelatin. The emissions from the disintegration of the radioactive isotope result in negative charges collecting at structural faults in the silver bromide crystals. This accumulation of negative charges causes positively charged silver ions to migrate to these sites. The aggregation of silver ions is termed the latent image, the intensity of which is dependent upon the radioactivity of the source, the length of exposure and the emulsion used. After a period of exposure in darkness the latent image is developed by reducing the silver bromide to silver. This reaction is catalysed by silver ions and where collections of silver ions exist metallic silver is deposited. The sites of silver deposition can thus be related to sites where radioactivity exists in the tissues. Unreduced emulsion is removed during fixation and the collections of

metallic silver are visible by microscopy. An autoradiograph is therefore an image produced in a nuclear emulsion by emissions from a radioactive substance which is kept in close contact with the emulsion.

5.2.5 Rationale of Present Study

DNA labelling techniques have been used for experimental purposes in human subjects. However, the dangers inherent in the use of radioactive isotopes have restricted the use of labelled precursors very largely to experimental animals and to in vitro techniques. As a continuation of the work presented in this thesis will involve a study of the kinetics of human oral mucosa an in vitro method was preferred. To eliminate differences of method in any comparison of human and animals results, the in vitro technique was also used for the animal experiments. This had the added advantage of involving less expense as much smaller quantities of isotope are required for in vitro work.

In vitro methods appear to give results similar to in vivo techniques (LaChapelle and Gillman, 1969) and allow single pulse and double labelling techniques to be performed on tissue from the same animal reducing the numbers of animals required. In addition in vitro techniques allow accurate control of the time of exposure to the isotope and the concentration of isotope is more readily maintained.

For studies on oral epithelium tritiated thymidine (^{3}H thymidine) is probably the most useful labelled DNA precursor. Tritium, a β particle emitter has a suitable half life ($12\frac{1}{2}$ years), energy range (0.018 MeV) and path length (1-2 μm) for use in oral epithelium. ^{14}C has also been used, but its larger path length, about 10-60 μm, makes it difficult to obtain accurate autoradiographic resolution (Rogers, 1973).

For a study of the cell kinetics of oral epithelium an in vitro labelling technique was considered to be most suitable. Tritiated thymidine was selected as the most useful radioactive isotope and a double labelling technique employing two different doses of tritiated thymidine was chosen as likely to provide the most useful information.

5.2.6 Aims of the Experiment

The aims of the study reported in this chapter were to apply an in vitro double labelling technique to the investigation of any cell kinetic changes occurring in the epithelium of the ventral surface of hamster tongue in iron depletion. In addition it was hoped to correlate any cell kinetic abnormality present with the structural abnormalities revealed by the concomitant stereological analysis.

5.3 MATERIALS AND METHODS

5.3.1 Experimental Animals

The animals used are described in Chapter 3 and the methods of housing, feeding, experimental procedures and sacrifice are all reported in section 3.2.2. The methodology and the results of cell kinetic studies on the animals used in Chapter 4 will be reported in Chapter 6.

5.3.2 Tissue Sampling

The method of biopsy and tissue trimming was described in section 3.2.6. The 1 mm blocks for in vitro labelling were placed in tissue culture medium 199 in sterile Bijou bottles and transferred to the laboratory in a vacuum flask containing ice. In every case incubation with tri^{ti}ated thymidine was begun within one hour of sacrifice.

5.3.3 Incubation and Labelling Procedures

The method used in this study was essentially that described by Warnakulasuriya (1976). The specimens of tongue were removed from the transport medium and placed in sterile universal containers holding 5 ml of medium 199 of pH 7.0-7.2 at 37^{o}C. A sterile glass tube was passed into each universal container and a continuous gaseous mixture of 95% oxygen 5% Co_2 was supplied. The gas was passed through the culture medium at a rate of about one bubble per second which was sufficient for adequate oxygenation and also provided gentle agitation of the specimens.

A second hole in the top of the container allowed escape of the gas. The containers were incubated in a water bath maintained at 37-37.5°C (Fig. 5.2). 5 μCi of ^{3}H thymidine was added to the containers with the specimens of lingual mucosa giving a concentration of 1 μCi/ ml in the medium. After 15 minutes with this first pulse of label the blocks were randomly divided into two batches. The first group was transferred to label free medium rinsed for 10 minutes then fixed. This group represented the single pulse labelled material.

The rest of the tissue was placed in 5 ml of label free medium in a fresh sterile container and incubated as before. After one hour the tissue was transferred to a further 5 ml of medium and 50 μCi of ^{3}H thymidine added. This gave a concentration of 10 μCi/ml in the medium. After 15 minutes incubation with the heavy dose of isotope the tissue was transferred to label free medium and rinsed for 10 minutes. The tissue was finally fixed in Bouin's solution, processed and paraffin embedded. This will be referred to as the double labelled material.

5.3.4 Processing, Embedding and Sectioning

After fixation in Bouin's solution for approximately two hours the material was processed in a Histokine * and paraffin embedded. Sections were cut on a rotary microtome

* Appendix 1

at 3 μm to allow satisfactory resolution of nuclei and of silver grains in autoradiographs with an oil immersion lens. To check the orientation of each block, an early section was taken and stained with haematoxylin and eosin. As in section 3.2.7 the later sections were mounted on slides treated with a thin layer of gelatin and four ribbons of five sections from each block were mounted across the slides. These ribbons were the serial sections at levels 1-5, 8-13, 17-21 and 25-29. Sections at intervening levels were mounted separately and kept in case the first sections proved unsatisfactory.

5.3.5 Slide Preparation

Autoradiographic techniques require scrupulously clean glass slides and to achieve this, slides were immersed for 24 hours in a 5 per cent solution of a non-foaming biological detergent (Alconox) *. After rinsing overnight in running water the slides were rinsed in two changes of distilled water for a further 6-8 hours. The slides were dried with a clean cloth and finally polished with a clean duster.

To aid the adherence of the autoradiographic emulsion and of the section to the slide, a coating of subbing solution consisting of gelatin, chrome alum and distilled water (Rogers, 1973) was prepared fresh and

* Alconox Inc., New York, NY 10003, U.S.A.

filtered before coating each batch of slides. The clean slides were dipped once in the solution, one side wiped clear and then the slides were allowed to dry in a microflow laminar flow cabinet to ensure a dust free environment. The slides were then stored in plastic or wooden boxes till required.

5.3.6 Autoradiographic Techniques

The technique used to coat the slides with emulsion was essentially that described by Rogers (1973). Sections mounted on the subbed slides were dewaxed in xylene and brought to water. To help reduce negative chemography caused by excess fixative the slides were immersed in lithium carbonate for 15 minutes then washed thoroughly for another 15 minutes. All subsequent procedures were carried out under minimal safelight conditions in the darkroom. Dipping the slides in emulsion was effected using the equipment shown in Figure 5.3.

Shreds of Ilford K5 * emulsion gel were placed in a measuring cylinder in the water bath at 43-47^{o}C. When the emulsion had melted, 15 ml were transferred to a cut down measuring cylinder containing 15 ml of distilled water and 0.6 ml of glycerol. The 1:1 mixture of emulsion and distilled water was stirred and allowed to stand for five minutes in the water bath. A check was made for the

* Ilford Ltd., Essex, England.

presence of air bubbles and the uniformity of the emulsion layer by dipping a clean slide and examining the result under the safe light. When a bubble free uniform layer of emulsion was obtained, the emulsion was ready for use.

Within autoradiographs, certain factors other than ionising radiation may cause false positive silver grain deposition (positive chemography) and may also cause loss of silver grains (negative chemography). Rogers (1973) describes the control of factors likely to lead to chemography and the methods of assessing what positive and negative chemographic effect is present.

To assess positive chemography a non-radioactive specimen was coated with emulsion, then exposed and developed with the experimental material. After staining, any silver grain deposition over nuclei can be visualised and this is referred to as the cold control. Negative chemography is revealed by coating with emulsion a slide containing radioactive tissue and exposing this slide to white light. This hot control is then exposed and developed with the experimental material and any loss of silver grains can be assessed (Fig. 5.4). Both hot and cold controls were prepared and exposed with the autoradiographs from the experimental animals.

5.3.7 Procedure for Quantifying Autoradiographs

The number of labelled nuclei counted in an

autoradiograph is expressed in terms of a reference unit. The most commonly employed reference units are the total viable nuclear count and unit surface or basement membrane length. In this study the labelling index was expressed as the number of labelled nuclei per 100 viable nuclei and as the number of labelled nuclei per unit surface length (field).

In hamster ventral tongue epithelium the surface of the epithelium can be considered as a straight line (Fig. 5.5) and an eyepiece graticule square was used to obtain measurements of the surface length. All autoradiographs were orientated with the surface of the epithelium parallel to the edge of the graticule and labelled cells within this square were recorded. The number of graticule squares (fields) examined was noted and for simplicity the labelling index is expressed in terms of fields examined. At the same time the total viable nuclear count was recorded by counting all viable nuclei and nuclear fragments within the graticule square. To avoid damaged areas at the edges of the specimen the graticule was moved a fixed distance from the edge of the section (one field) in every case before counting was begun.

Autoradiographs were examined using the oil immersion lens (x100). All nuclei showing four or more silver grains were considered as labelled (Fig. 5.6). This criterion was established by examining the cold controls

which showed that the maximal background labelling over nuclei was not more than two silver grains. The total viable nuclear count, the number of labelled cells and the number of fields counted were recorded on a simple laboratory counter.

In the autoradiographs of double labelled tissue two labelled cell populations corresponding to the 1 μCi/ ml and 10 μCi/ ml doses of ^{3}H thymidine have to be identified. On the basis of the work of Warnakulasuriya (1976) and the examination of lightly labelled material a cell having between 4 and 25 silver grains over its nucleus was considered to be lightly labelled and all nuclei with more than 25 silver grains were considered as heavily labelled cells (Fig. 5.7).

5.4 RESULTS

5.4.1 Labelling Index and T_s Values for Control Animals

Pulse labelled tissue for animal 1 and animal 8 was crushed and tissue from these animals had to be omitted from the analysis leaving only two animals in the normal group. In Chapter 3 no differences were found in the total epithelial, the compartment thickness, or in the compartment ratios of normal animals and animals in the iron deficient diet group. For statistical reasons these groups were combined to give a larger control group of six animals.

The results of examination of the single pulse

labelled (1 μCi) material are shown in Table 5.1. The mean labelling index for control animals was 7.39 labelled cells per one hundred nucleated cells, (range 4.82 - 9.29). When the labelling index was alternatively expressed as labelled cells per unit surface length the mean labelling index was 3.78 (range 2.55 - 5.45). The results of the double labelled material are shown in Table 5.2. The mean T_s for control animals was 3.22 hours, (range 2.03 - 6.94).

5.4.2 Labelling Index and T_s Values for Experimental Animals

The results of the single pulse label are shown in Table 5.3. The mean labelling index was 5.95 cells per one hundred viable cells (range 4.19 - 7.66) and 2.89 cells per unit surface length (range 1.94 - 3.82). The mean T_s for experimental animals was 3.82 hours, Table 5.4.

5.4.3 Comparison of Control and Experimental Animals

The labelling index per 100 viable nuclei of experimental animals was significantly less ($P = .04$) than the labelling index of control animals. The labelling index per unit surface length was also significantly less ($P = .05$) in experimental animals. There was no significant difference in T_s ($P = .221$) between the groups.

5.5 DISCUSSION

The number of animals in the control group was

reduced by the deaths of two normal animals. As tissue from a further control animal was unsuitable for autoradiographic assessment there remained for analysis, material from only two untreated control animals. For statistical purposes this number was considered inadequate and to increase the number of control animals the untreated control group was combined with the animals in the iron deficient diet group. From the results reported in Chapter 3 it is apparent that the iron deficient diet produced no measureable systemic effect. In addition stereological analysis failed to reveal any significant differences in the epithelium of control animals and animals fed on the iron deficient diet. It was therefore thought unlikely that there would be cell kinetic abnormalities between these groups and that it was valid to combine them into a single control group.

The mean labelling index of 7.39 labelled nuclei per 100 viable nuclei for animals in the combined control group compares well with the results reported in previous in vivo and in vitro work (Warnakulasuriya, 1976). The labelling index for iron depleted animals was significantly reduced and as this reduction was present with both reference systems used it suggests the change was a real difference.

The T_S estimation resulted in a mean value of 3.8 hours which is lower than the range 5-9 hours quoted by Warnakulasuriya (1976). It is likely that minor variations

in autoradiographic technique could contribute to this difference. In addition the assumed cut off point of 25 silver grains for heavily labelled material might be too high for the material in this study. An accurate estimation of the total number of silver grains occurring in pulse labelled 1 μCi/ml and 10 μCl/ml material before examination of the double labelled material would establish the range of silver grain counts for lightly and heavily labelled tissue. However, for the purpose of comparisons the T_s estimations are valid as all material was identically treated.

A reduced labelling index without an alteration in the T_s indicates a reduction in the cell production rate. This reduced cell production rate could explain the significantly thinner maturation compartment found in iron depleted animals. The proportional increase in size of the progenitor compartment may be due to an increase in the number of cells in the presynthetic phase (G_1) of the cell cycle or to an increase in the size of individual progenitor cells.

Although it was not considered necessary for this preliminary cell kinetic investigation all data derived from counts of nuclei should be corrected for nuclear fragments (Abercrombie, 1946). The correction factor takes into account possible variations in nuclear size and section thickness as alterations in either of these

parameters will affect the number of nuclei or fragments of nuclei seen on histological sections.

5.6 CONCLUSIONS

The conclusions that can be drawn from this preliminary study are limited by the size of the control groups and by the nature of the cell kinetic data. Nevertheless the results suggest that the in vitro method of investigating cell kinetics is a useful technique giving results similar to those reported by other workers. A more accurate assessment of the cut off point for heavily labelled nuclei would be an advantage and measurement of nuclear diameter will allow correction of nuclear counts and an assessment of cell size to be made. However, the results of this preliminary study indicate that cell kinetic abnormalities are present in iron depletion and suggest that further investigation would be worthwhile.

CHAPTER 6

INVESTIGATION OF THE CELL KINETIC CHANGES IN THE VENTRAL TONGUE EPITHELIUM DURING THE DIFFERENT STAGES OF IRON DEFICIENCY IN HAMSTERS

6.1 INTRODUCTION

The data presented in Chapter 5 were limited by the small number of animals in the control groups but indicate that there are cell kinetic changes present in hamster ventral tongue epithelium in iron depletion. Examination of epithelium from animals with the further stages of iron deficiency is therefore likely to reveal more marked cell kinetic abnormalities.

Certain difficulties were encountered in the autoradiographic techniques used in Chapter 5. The division between heavily and lightly labelled material was based on the work of Warnakulasuriya (1976) and it is possible that an alteration in the number of grains required for a cell to be classed as heavily or lightly labelled could markedly affect the value of the T_s. Thymidine is known to stimulate mitotic activity (Blenkinsopp, 1967) and it is possible that the concentration of isotope used for the heavy pulse might influence the cell kinetics of the experimental material. By modifying the method and including tissue cultured in the presence of a single pulse of a heavy dose of isotope any effect of the isotope and

the range of grain counts for heavily labelled material could be more accurately assessed. In addition comparison of the labelling index obtained from a single pulse of isotope and the labelling index obtained from the heavy pulse of the double labelled material would allow the effect of the 1 hour culture period to be assessed.

The structural abnormalities reported in Chapter 4 may not be explained by the cell kinetic data alone and measurement of the size of the epithelial cells would help to clarify the cell kinetic and structural changes reported.

The aims of the study reported in this chapter were:

1. To assess any effect of long culture time and heavy dose of radioisotope upon the cell kinetics of ventral tongue epithelium.
2. To investigate any cell kinetic abnormalities occurring in the ventral tongue epithelium in iron deficiency anaemia, iron deficiency without anaemia, and iron depletion.
3. To investigate any changes occurring in epithelial cell size in the three stages of iron deficiency disease.

6.2 MATERIALS AND METHODS

6.2.1 Laboratory Procedures and Autoradiographic Technique

The animals used in this study, and the experimental procedures are described in Chapter 4. The haematological,

biochemical and stereological results are also reported in Chapter 4.

The 1 mm cubes of tissue removed at sacrifice (4.2) from experimental and control animals were incubated in 5 ml of tissue culture medium 199. Six pieces of tissue per animal were used; four 1 mm cubes were cultured in the presence of an initial isotope concentration of 1 μCi/ml and two incubated with a concentration of 10 μCi/ml in the culture medium. After 15 minutes incubation in the presence of isotope two lightly labelled and the two heavily labelled cubes were removed, washed in label free medium for 10 minutes and fixed in Bouin's solution. The remaining 1 μCi/ml labelled material was incubated for 1 hour in 5 ml of label free medium and then 50 μCi ^{3}H thymidine added, giving a concentration of 10 μCi/ml of radioactive label in the culture medium. This tissue was incubated for 15 minutes in the presence of the heavy dose of label and then washed for 10 minutes in label free medium and fixed in Bouin's solution.

The techniques of slide and section preparation were as reported in Chapter 5, and sections were cut for the 1 μCi/ml, 10 μCi/ml, and the 1/10 μCi/ml labelled tissue. The sections were coated in Ilford K5[+] emulsion, controls prepared, and all autoradiographs exposed in a refrigerator at 4°C.

[+] Ilford Ltd., Essex, England.

At daily intervals after 4 days, test sections of lightly and heavily labelled material were removed, autoradiographs developed and stained with haematoxylin and eosin. The grain density of autoradiographs was assessed and it was found that 6 days was a suitable exposure time giving satisfactory numbers of silver grains. At 6 days all autoradiographs were developed, fixed and stained as described in Chapter 5.

6.2.2 Procedure for Counting Labelled Cells

On examination of the autoradiographs, cells which had four or more silver grains over the nucleus were considered to be labelled (Fig. 6.1.). As in section 5.3.7 this was established by examining the autoradiographs from cold controls to determine the maximal background labelling occurring over cell nuclei.

When normal tissue and pathological lesions are being compared the effects of alteration of compartment thickness, cell size and cell density upon labelling index values should be considered. This subject has been discussed by Loe and Karring (1969), MacDonald (1971a) and Karring and Loe (1972). To minimise these problems it is recommended that the labelling index is expressed in terms of different reference units and for the purposes of this study the labelling index will be expressed as labelled cells per 100 progenitor cells, per 100 total viable cells and per unit surface length.

Using an oil immersion objective (x100) autoradiographs of the light (1 μCi/ml), heavy (10 μCi/ml), and double labelled material were examined. All labelled nuclei were counted and the numbers of nuclei in the progenitor and maturation compartments were recorded. An eyepiece graticule was again used to obtain measurements of surface length and it was therefore possible to express the labelling index as labelled cells per unit surface length, per 100 total viable cells and per 100 progenitor cells.

Wherever possible the middle section from the ribbon of five was examined and if this section was damaged an adjacent section was used. Four sections per animal were counted. The first and last fields in each section were omitted to avoid damaged areas at the edges of the section and when possible every field in a section was counted. Any damaged or folded areas were omitted and when these omissions were combined with minor differences in the size of the tissue blocks the result was a variation in the number of nuclei counted per animal. However, every attempt was made to count over one thousand nuclei per animal for the lightly, heavily and double labelled material.

A simple laboratory counter was used to record the total number of cells, and the numbers of progenitor and mature nuclei were separately noted. After establishing grain counts for the single labelled material the double labelled material was examined and counted.

One of the difficulties with autoradiographic experiments of this nature is standardisation of exposure, development, and fixation conditions, as all of these factors and others, may alter the numbers of silver grains appearing on autoradiographs. To avoid any possible variations in autoradiographic technique or conditions only data derived from autoradiographs produced under identical conditions at the same time will be presented. Accordingly small variations in the number of animals in each group occur. This is the result of omitting crushed tissue, damaged sections or poor autoradiographs from the analysis.

6.2.3 Measurement of Nuclear Diameter and Correction to Cell Counts

One of the aims of this study was to determine whether or not there are changes in the sizes of epithelial cells in iron deficiency. The determination of cell size from histological sections is complex (Weibel, 1969) and for the purpose of this study an alternative technique was employed. This method involves estimating nuclear size and then determining the number of nuclei in unit area. Although not providing an exact measurement of cell size this method allows the estimation of nuclear size, of the number of nuclei per unit area (cell density) and of the nucleo-cytoplasmic ratio of cells in histological sections.

With an oil immersion objective (x100) the nuclear diameters of all nuclei in two randomly selected cell columns, were measured. A calibrated eyepiece graticule,

which also delineated the width of the cell columns, was used to measure the diameter of nuclei within the cell columns. The graticule was orientated at right angles to the surface of the epithelium and all nuclei in the epithelial columns were examined. Two cell columns provided sufficient nuclei for consistent estimation of nuclear diameter and this was checked using the accumulative means test. The nuclear diameter was obtained by recording the distance between two vertical imaginary lines touching the widest points of the nucleus and the measurements of nuclei of progenitor cells and of mature cells were recorded separately.

This nuclear measurement was used to apply a cell count correction factor. Cell counts made by counting nuclei in histological section are biased in favour of large nuclei and may include nuclear fragments which could result in an incorrect estimation of cell numbers. The method used for correcting cell counts was that described by Abercrombie (1946) (Fig. 6.2).

Abercrombie (1946) suggested that for nuclear measurements the block should be recut at right angles to the plane of section used for cell counting, and the nuclear diameter remeasured. However, because of the small block size this was not possible and for the purposes of this study it was assumed that the nuclear diameter would be the same in both planes of section.

All sections used in this study were cut on the same microtome at the 3 μm setting. This microtome was used exclusively for research purposes and had previously been checked for accuracy of section thickness. At this setting the mean section thickness was found to be 3.5 μm (Warnakulasuriya, 1976).

The corrected numbers of progenitor and mature cells were obtained for 1 μCi/ml, 10 μCi/ml and the 1/10 μCi/ml material. As measurements of the surface length had already been recorded (6.2.2) for this material the corrected number of cells per unit surface length could be estimated.

The eyepiece used for estimation of surface length delineated a 90 μm wide column of epithelium under the oil immersion lens. As the thickness of the progenitor cell and mature cell compartments are already known (4.3.9) the number of cells per unit area of section for each compartment can be calculated using the formula:

$$N = \frac{\text{Number of cells/column}}{\text{Area of the column}}$$

$$= \frac{P}{90 \times t} \times 100$$

N = No. of cells per 100 sq. μm.

P = No. of cells per epithelial column.

t = Compartment thickness in μm.

6.3 RESULTS

A considerable volume of data was obtained from this study and the results are presented in the same sequence as the objectives for this experiment. Firstly the values obtained for nuclear diameters are presented as these measurements allow corrections of nuclear counts to be made (6.3.1 and 6.3.2). Secondly the data which allows the validity of the technique to be assessed is presented (6.3.3 and 6.3.4) and the results of the investigation of cell production parameters are subsequently detailed (6.3.5 to 6.3.10). Finally the estimations of cell size are reported (6.3.11 and 6.3.12).

6.3.1 Nuclear Diameters of Epithelial Cells from the Ventral Surface of Tongue of Control Animals

The mean nuclear diameters for the progenitor and mature cells of control animals are shown in Table 6.1. The mean progenitor nuclear diameter was 5.31 μm ranging from 4.69 μm to 5.99 μm. The nuclear diameter of mature cells was greater and ranged from 7.44 μm to 8.79 μm with a mean of 8.13 μm. Table 6.1 also shows the values of the standard deviations for progenitor and mature cell nuclei. From the uniformity of the means and the small standard deviation in each group it is apparent that there is minimal or no overlap of the two cell compartments and that they form two distinct cell populations.

6.3.2 Nuclear Diameters of Epithelial Cells from the Ventral Surface of Tongue of Experimental Animals. Comparison with Control Animals

The mean nuclear diameters of progenitor cells and mature cells for each of the experimental groups are shown in Table 6.2. The mean diameter of progenitor cells for the entire experimental group was 5.13 μm with a range of 4.31 μm to 6.03 μm. The mean nuclear diameter for mature cells was 8.44 μm and ranged from 7.50 to 9.63 μm. The small standard deviation again suggests that the progenitor cell and mature cell compartments formed two distinguishable cell populations.

Using the Mann-Whitney U Test the mean nuclear diameters for progenitor and mature cells of experimental animals were compared with those of control animals. There was no significant difference between the nuclear diameters of control animals when compared with anaemic, iron deficient but not anaemic, and iron depleted animals. In addition there were no significant differences found when the nuclear diameters of three experimental groups were compared.

6.3.3 Labelling Indices of Control Animals. Comparison of Lightly and Heavily Labelled Material

Labelling indices were calculated for the lightly and heavily single pulse labelled tissue. These labelling indices are derived from examination of different tissue blocks from the same animal and it was therefore unnecessary to apply Abercrombie's correction and the comparison was

made using uncorrected nuclear counts.

The labelling indices for control animals expressed in terms of 100 progenitor cells, 100 total viable cells and per unit surface length were obtained using the methods described in section 6.2.2. The mean labelling indices for lightly labelled material were 5.36 labelled cells per 100 progenitor cells, 4.03 labelled cells per 100 viable cells and 1.34 labelled cells per unit surface length, (Table 6.3). For heavily labelled material these indices were 5.37, 4.14 and 2.64 labelled cells respectively, (Table 6.4).

There were no significant differences between the labelling indices obtained from the lightly labelled and the heavily labelled material. The heavy dose of isotope would thus appear to produce no significant alteration in the in vitro labelling of hamster ventral tongue epithelium.

To assess whether or not the one hour culture period produced an effect upon the labelling of cells, the labelling indices derived from the heavy dose of isotope given as a second pulse in the double labelled tissue, will be presented, and compared with the labelling indices obtained from the single lightly labelled material. Due to the small number of animals in each group the Mann-Whitney U Test was again used for the statistical analysis.

6.3.4 Labelling Indices of Control Animals. Comparison of Lightly Labelled Material and Heavily Labelled Material from Double Labelled Tissue

From this point Abercrombie's correction factor has been applied to all labelling index data and for simplicity only the data obtained from corrected cell counts will be presented. An example of the method used to correct the cell counts is shown in Fig. 6.2. Abercrombie's correction factor was calculated for each animal and as no apparent difference was observed in the nuclear diameters of labelled cells the correction factor for each animal was also applied to the counts of labelled nuclei. For estimation of the T_s no correction factor is necessary as lightly and heavily labelled cells are obtained from the same animal and would be multiplied by the same factor. From the lightly labelled material the mean corrected labelling index per 100 progenitor cells for normal animals was found to be 5.34 labelled cells; per 100 viable cells the mean value was 4.29 labelled cells and per unit surface length the value was 0.54 labelled cells. The results are detailed in Table 6.5.

The corrected cell counts and the labelling index expressed as heavily labelled cells, per 100 progenitor cells, per 100 total viable cells and per unit surface length are shown in Table 6.6. The mean values for the labelling indices were 6.49, 5.26 and 0.99 labelled cells respectively. There were no significant differences between the labelling indices derived from the double labelled material when

compared with the labelling indices from the lightly labelled material.

It was thought unnecessary to tabulate the results of the labelling indices for the different isotope doses for experimental animals but no significant differences were found when the labelling indices obtained from the 1 μCi/ml, the 10 μCi/ml and the heavy dose of the double labelled material from each experimental group were compared.

These results indicate that neither the one hour culture period nor the heavy dose of ^{3}H thymidine produced a significant effect upon the in vitro labelling of ventral tongue epithelium.

The labelling indices obtained from the 1 μCi material will be presented for normal and experimental animals and comparisons of normal and experimental animals will be made using the values obtained from this material.

6.3.5 Labelling Indices of Control Animals

The labelling indices for control animals are detailed in Table 6.5. The mean labelling index per 100 progenitor cells was 5.34 labelled cells. The values expressed as per 100 total viable cells and per unit surface length were 4.29 and 0.54 labelled cells respectively.

6.3.6 Labelling Indices of Anaemic Animals Comparison with Control Animals

The values for the labelling indices of anaemic animals are recorded in Table 6.7. The mean labelling indices for anaemic animals were 3.54 labelled cells per 100 progenitor cells, 2.96 labelled cells per 100 total viable cells and 0.44 labelled cells per unit surface length.

When the three labelling indices of the anaemic animals were compared with the labelling indices of the control animals no significant differences were found.

6.3.7 Labelling Indices of Animals with Iron Deficiency without Anaemia. Comparison with Control and Anaemic Animals

Table 6.8 details the results of the labelling index estimations for animals with iron deficiency without anaemia. The mean labelling indices expressed as per 100 progenitor cells, per 100 total viable cells and per unit surface length were 5.48, 4.23 and 0.47 labelled cells respectively.

No significant differences were noted when each of the labelling indices for animals with iron deficiency without anaemia were compared with the labelling indices of control and anaemic animals.

6.3.8 Labelling Indices of Iron Depleted Animals. Comparison with Control, Anaemic and Iron Deficiency without Anaemia Animals

The mean labelling indices of iron depleted animals were 6.64 labelled cells per 100 progenitor cells, 5.20

labelled cells per 100 total viable cells and 0.76 labelled cells per unit surface length, (Table 6.9).

When compared with control animals no significant differences in the labelling index values of iron depleted animals were found.

When compared with anaemic animals iron depleted animals had a significantly higher labelling index ($P < .02$). This observation was true for the three reference systems used.

There was no difference between animals with iron deficiency without anaemia and iron depleted animals when the labelling index per 100 progenitor cells and per 100 total viable cells were compared. The labelling index per unit surface length was significantly greater ($P < .02$) in iron depleted animals when compared with iron deficiency without anaemia animals.

6.3.9 Grain Counts of Lightly and Heavily Labelled Material

To establish the range of grain densities and then the cut off point between lightly and heavily labelled nuclei, approximately 800 labelled nuclei from autoradiographs of lightly and heavily pulse labelled tissue were examined and the number of grains per nucleus recorded. The distributions of the grain counts for lightly (1 μCi) and heavily (10 μCi) labelled nuclei are shown in Fig. 6.3.

It can be seen that 88.3 per cent of "lightly" labelled cells have less than 30 grains per nucleus and 89.4 per cent of "heavily" labelled nuclei have more than 30 silver grains. On this evidence 30 was considered to be a suitable division between lightly and heavily labelled nuclei. It is also evident that the majority of heavily labelled nuclei (79.5 per cent) have greater than 50 silver grains and that the distribution of the grain count is uneven in this group. This distribution is explained by the difficulty experienced in counting more than 40 grains over one nucleus and by the apparent coalescing of individual grains into dense masses at grain numbers of this magnitude. When such coalescence occurred the grain count was scored as greater than 50 (Fig. 6.1).

6.3.10 T_s Estimation for Normal and Experimental Animals

Using the equation illustrated in section 5.2.3 the T_s values for normal animals and for each experimental group were calculated. The results of the T_s estimations are illustrated in Tables 6.10, 6.11, 6.12, 6.13.

The mean T_s for control animals was 4.93 hours and for anaemic animals the T_s value was 3.20 hours. When the T_s values of control and anaemic animals were compared the T_s of anaemic animals was found to be significantly shorter than that of control animals ($P < .05$).

The T_s estimation of animals with iron deficiency without anaemia revealed a mean T_s of 3.25 hours (Table 6.12).

Comparison of the T_s with control animals revealed that animals with iron deficiency without anaemia had a significantly shorter T_s ($P < .02$). There was no difference in the T_s between animals with iron deficiency without anaemia and anaemic animals.

The mean T_s of iron depleted animals was 5.32 hours (Table 6.13) and comparison with normal animals revealed no significant difference in the T_s estimation. The T_s values of anaemic animals and animals with iron deficiency without anaemia were significantly shorter ($P < .02$ and $P < .001$ respectively) than the T_s of iron depleted animals.

6.3.11 Cell Density of Normal Animals

Using the method described in section 6.2.3 the cell densities for normal animals were calculated. Table 6.14 shows the number of corrected progenitor cells per 100 sq μm in normal animals. The mean number of progenitor cells in 100 sq μm was 0.814 (range .662 - .960) Table 6.15 illustrates the number of mature cells per unit area and in normal animals the mean was 0.121 cells (range .086 - .139).

6.3.12 Cell Density of Experimental Animals. Comparison with Control Animals

The cell densities for each of the experimental groups was also calculated using the method described in section 6.2.3 and the values for the numbers of progenitor and mature cells per unit area are listed in Tables 6.16 and 6.17 respectively.

Comparison of the progenitor cell density of normal and anaemic animals revealed an increased cell density in anaemic animals. The value for P of 0.06 was just outside the conventional levels of significance but the size of P values suggests that this is a true observation. There was no difference between the progenitor cell density of normal animals and the progenitor cell density of animals with iron deficiency without anaemia and animals with iron depletion.

Anaemic animals had a significantly greater progenitor cell density than iron deficient without anaemia and iron depleted animals ($P = .008$; $P = .024$). There was no difference between the progenitor cell densities of iron deficiency without anaemia animals and iron depleted animals.

Comparison of the mature cell density of normal animals with the mature cell density of anaemic animals revealed a significantly greater cell density in anaemic animals ($P < .05$). There was no difference between the mature cell densities of normal and iron deficient animals but iron depleted animals had a significantly greater mature cell density than normal ($P < .05$).

No significant differences were found between the mature cell densities of the experimental groups.

It appears that there is an increase in the number of progenitor and mature cells per unit area in anaemic animals. Although the exact cell sizes were not obtained it is highly probable that there is a reduction in the epithelial cell size in iron deficiency anaemia and as there is no change in the nuclear size of these cells there must be an increased nucleo-cytoplasmic ratio of the epithelial cells in anaemia.

6.4 DISCUSSION

The close similarity between labelling indices derived from lightly and heavily pulse labelled material indicates that over the short incubation period used in this study the heavy dose of radioactive thymidine produced no significant alteration of the in vitro labelling of ventral tongue epithelium. In addition the close correlation between the labelling indices obtained from the lightly labelled material and the heavily labelled material of the double pulse indicates that the culture period between the two pulses had no apparent deleterious effect.

When the number of grains overlying the nuclei of heavily and lightly labelled material is counted the distinction between the two doses of isotope can be assessed with confidence. By establishing the cut off point at 30 grains the mean T_s for normal animals in this experiment came close to the levels recorded by other authors

(Warnakulasuryia, 1976). It would seem advisable that in experiments of this nature grain counts of lightly and heavily labelled material should be undertaken to assess accurately the differentiation between lightly and heavily labelled nuclei.

The duration of the S phase in anaemic animals and in animals with iron deficiency without anaemia was significantly shorter than normal. As there was no change in the number of cells in or entering the S phase (labelling index) there must be a net increase in cell production rate in these animals.

There was a significant increase in the number of progenitor and mature cells per unit area and as these cells showed no alteration in nuclear size the nucleo-cytoplasmic ratio of progenitor and mature cells must be increased in iron deficiency anaemia. Although not an exact measurement of cell size this strongly suggests a reduction in the size of progenitor and mature cells in iron deficiency anaemia.

6.5 CONCLUSIONS

The in vitro double labelling technique is a satisfactory method of investigating the cell kinetics of oral epithelium giving consistent reproduceable results.

Cell kinetic abnormalities are present in the ventral

tongue epithelium of animals with iron deficiency anaemia, and iron deficiency without anaemia. It appears that the principal effect of marked deficiency of iron is to cause a decrease in the time taken for DNA synthesis but the number of cells in or entering the S phase is unaltered. There is therefore a net increased rate of cell production in iron deficiency anaemia.

The number of cells per unit area is increased in iron deficiency anaemia indicating a net decrease in the size of individual epithelial cells. As the nuclear diameter is unaltered, the nucleo-cytoplasmic ratio of epithelial cells in iron deficiency anaemia is increased.

CHAPTER 7

A DISCUSSION OF THE EXPERIMENTAL FINDINGS

7.1 INTRODUCTION

The studies reported in this thesis examine the changes occurring in human and animal oral epithelium in iron deficiency. Several quantitative techniques were used to measure the changes occurring in iron deficiency and the purpose of this chapter is to compare, contrast and correlate the results from human and animal experiments.

7.2 STEREOLOGICAL ANALYSIS OF HUMAN AND HAMSTER ORAL EPITHELIUM IN IRON DEFICIENCY

7.2.1 Stereological Analysis of Human Buccal Epithelium in Iron Deficiency

Investigation of the changes occurring in human buccal epithelium highlighted the difficulties of obtaining closely comparable control and experimental material. The stereological investigation revealed that buccal mucosa from normal adult males has a mean thickness of 437.5 μm with a range of 341 to 496.5 μm. Buccal epithelium from patients with iron deficiency anaemia has a mean thickness of 235.3 μm with a range of 110.8 to 383.1 μm.

Comparison of the normal and anaemic subjects revealed that the epithelium from anaemic patients was significantly thinner than normal and that this reduction

was due to a reduction in the thickness of the maturation compartment (Fig. 7.1). These observations have to be interpreted with caution as there are differences in the ages and sex of subjects in this study, but comparison of a sex matched group (2.4.6) revealed the same pattern of epithelial changes. It is therefore likely that the reduction in total epithelial and maturation compartment thickness are related to iron deficiency anaemia.

It is not possible from a study of this nature to be entirely sure whether the changes demonstrated in the epithelium are a result of anaemia (hypoxia) or deficiency of iron. However, as changes are present in the epithelium before anaemia develops it is likely that deficiency of iron and not anaemia is the causative factor.

The presence of a reduced epithelial thickness has been noted subjectively by other workers (Paterson, 1919; Monto et al, 1961) and supports the generally believed clinical impression that epithelium from anaemic patients is thinner than normal.

7.2.2 The Animal Model

A useful small animal model of iron deficiency has been developed and after some initial problems with animal handling and anaesthesia it was possible to remove 1 ml of blood on 36 occasions over a 27 week period. Few complications arose as a result of the repeated venesection

and the animals remained well with a relatively low mortality (4 out of 26). The sequence of haematological changes seen as iron deficiency anaemia developed is similar to that seen in man.

There are certain features which make the hamster a difficult animal to use for studies of iron deprivation. The normal young adult male hamster appears to have large iron stores and is probably able to absorb all available dietary iron. Even by removing around 25 per cent of the animals blood volume every two weeks it still takes about six months to render the animals anaemic. This time could perhaps be reduced by the use of metabolic cages and a diet totally free of iron. Hamster blood coagulates quickly and despite repeated attempts by the author using a variety of anticoagulants it was not possible to produce blood films suitable for microscopical evaluation.

7.2.3 Stereological Analysis of Hamster Ventral Tongue Epithelium in Iron Deficiency

One of the problems associated with comparative quantitative analysis of epithelia is ensuring that sections are cut at right angles to the surface of the epithelium when preparing a histological section. To help overcome this problem great care was taken to trim blocks at right angles to the epithelial surface and to embed the tissue specimens carefully. All embedding of blocks was done with the aid of a dissection microscope and an early section from each block was examined to check the orientation.

At this point it is convenient to consider what degree of tilting of the tissue block would result in a significant change in epithelial thickness. The effect of changing the angulation of the tissue is that the ratio of the thickness of the epithelium at the two different angles of orientation expressed as a percentage is the degree of lengthening of the epithelium on a histological section. This ratio is the cosine of the angle between the plane of section obtained, and the ideal plane of section at right angles to the epithelial surface. The values of the cosine of various angles from the vertical are shown in Table 7.1. It can be seen that the orientation of the tissue specimen has to be approximately 30^{o} to the vertical before there is greater than a 10 per cent alteration in epithelial thickness. It is considered extremely unlikely with the precautions taken that such a degree of tilting would occur or go unnoticed on histological sections.

Stereological analysis of the epithelium from the ventral surface of hamster tongue failed to reveal any significant change in the total epithelial thickness in iron deficiency. There were however, significant alterations in compartment thickness and in the proportion of the epithelium formed by each compartment.

Iron depletion is the first measureable stage in the development of iron deficiency. The first change noted in the epithelium was a relative increase in the progenitor

cell compartment and a relative decrease in the maturation compartment size (Fig. 7.2). When normal animals were compared with iron depleted animals these changes were consistent for both sets of experimental animals examined.

Quantitative analysis of epithelium from animals with iron deficiency without anaemia failed to reveal any change in compartment or total epithelial thickness. The maturation compartment again formed a significantly smaller proportion of the epithelium. There was no alteration in the proportion formed by the progenitor compartment but the keratinised compartment formed a significantly greater part of the epithelium (Fig. 7.3) in iron deficiency without anaemia animals.

Analysis of epithelium from animals with iron deficiency anaemia revealed no change in the total epithelial thickness. There were however, changes in the thickness of epithelial compartments. The maturation compartment was significantly thinner and the keratinised compartment significantly thicker in anaemic animals. The lack of an alteration in the total epithelial thickness of anaemic animals was unexpected in view of the atrophy found in human iron deficiency anaemia. This event may be a late epithelial change. The experimental animals were sacrificed as soon as anaemia was seen to be developing and a longer period of iron deficiency anaemia may be required before an overall decrease in epithelial thickness develops. The

increase in thickness of the keratin layer demonstrated in anaemic animals may have disguised atrophic changes in the cellular layers. When the values for the progenitor cell and maturation compartment thickness were combined and comparisons of anaemic and control animals made anaemic animals were found to have a thinner cellular layer than control animals ($P < .07$). Although this value is not significant it lends support to the idea that atrophic changes are developing and that a longer period of iron deficiency anaemia would result in significant epithelial atrophy (Fig. 7.4).

Epithelial changes are present in iron deficiency and these changes appear progressive, parallelling the development of increasing degrees of iron deficiency. The only significant change in the first measureable stage of iron deficiency is an increase in the thickness of the progenitor cell compartment relative to the rest of the epithelium. As the deficiency develops the maturation compartment forms proportionately less of the epithelium and the keratin proportionately more of the epithelium until at the stage of iron deficiency anaemia there is a reduction in the maturation compartment thickness and an increase in the keratin layer.

7.2.4 Comparison of the Haematological and the Structural Changes found in Human and Hamster Oral Epithelium in Iron Deficiency

Iron deficiency develops in the same sequential

manner in humans and hamsters. With increasing negative iron balance there is a loss of storage iron from marrow accompanied by a fall in the serum iron and a reduction in the transferrin saturation. Iron deficiency anaemia develops after prolonged negative iron balance and is the last measureable manifestation of the deficiency.

The epithelial changes in iron deficiency anaemia are similar in man and hamsters. The most striking feature is the consistent reduction in the thickness of the maturation compartment. This was accompanied in man by a reduced total epithelial thickness but it is likely that a longer period of iron deficiency anaemia would result in a reduced epithelial thickness in anaemic animals. There was an increased thickness of the keratin layer in anaemic animals but buccal epithelium lacks a keratin layer and comparison of this parameter is not possible. However, it is of interest to note that several authors (Suzman, 1933; Savilahti, 1946) have noted hyperkeratinisation in iron deficiency anaemia in man and recent clinical observations have shown a relationship between iron deficiency and some hyperkeratotic lesions in the floor of the mouth. These lesions regressed with iron therapy (Fig. 7.5) (MacDonald, 1979).

7.3 THE CELL KINETICS OF THE VENTRAL SURFACE OF TONGUE EPITHELIUM FROM HAMSTERS WITH IRON DEFICIENCY

7.3.1 The In Vitro Labelling Technique

The absence of significant differences for the

labelling indices obtained for experimental animals using different isotope concentrations indicates that the heavy dose of ^{3}H thymidine used did not significantly alter the uptake of radioactive precursor from the culture medium. In addition the similarity between the labelling indices derived from the lightly labelled tissue and the heavy pulse of the double labelled tissue indicates that neither the extended culture period nor the heavy dose of ^{3}H thymidine given after the one hour culture period produced any significant measureable effect.

It therefore appears that this method of in vitro labelling is a valid one giving reproduceable results and allowing objective data to be obtained.

7.3.2 The Cell Kinetics of Ventral Tongue Epithelium in Iron Depletion

The labelling index estimations from the preliminary and second experiment provided differing results. The results of the preliminary study suggested there was a reduction in the number of epithelial cells synthesising DNA. This observation was not confirmed by the second experiment in which the labelling index obtained from three separate pulses showed no differences from normal. In view of the consistent observations of the second experiment the results of the preliminary investigation must be viewed with some doubt. In addition the nature of the control groups and the interval between the last venesection and sacrifice may have affected the results of the preliminary

study.

Cell kinetic investigations of the ventral tongue epithelium showed no alteration in the duration of the S phase in iron depleted animals and this observation was confirmed in both studies performed.

No change was noted in the nuclear diameter of progenitor and mature cells of iron depleted animals. The progenitor cell density did not change and nucleo-cytoplasmic ratio of progenitor cells is therefore unchanged. There was a significantly increased cell density of mature cells suggesting an increased nucleo-cytoplasmic ratio of mature cells in iron depleted animals.

7.3.3 The Cell Kinetics of Ventral Tongue Epithelium in Iron Deficiency without Anaemia

No significant alteration was noted in the labelling index of the epithelium from the ventral surface of tongue in hamsters with iron deficiency without anaemia.

The duration of the S phase was significantly shorter than normal for animals in this group. The reduced T_s in the presence of a normal labelling index indicates an increased cell production rate in the epithelium of animals with iron deficiency without anaemia.

No alteration in the nuclear diameter or in the cell density of progenitor or mature cells was noted in iron

deficiency without anaemia.

7.3.4 The Cell Kinetics of Ventral Tongue Epithelium in Iron Deficiency Anaemia

As with the other experimental groups no significant differences were found in the comparisons of the labelling indices of epithelium from normal and anaemic hamsters.

The S phase was significantly shorter than normal in anaemic animals again indicating an increased cell production rate in the epithelium of anaemic animals.

There were no alterations in the nuclear diameters of progenitor or mature cells but the cell density of both progenitor and mature cells was greater in anaemic animals. There was thus a reduced progenitor and mature cell size in anaemic animals and since the nuclear diameters were unchanged the results suggest a increased nucleo-cytoplasmic ratio in anaemic animals.

The findings of the cell kinetic and cell size investigations indicate that as iron deficiency develops there is an accompanying increase in the rate of new cell production and a decrease in the size of the epithelial cells.

7.4 CORRELATION OF THE STEREOLOGICAL AND CELL KINETIC DATA

7.4.1 Iron Depletion

The change in the proportion of the epithelium

formed by the progenitor cell compartment may be the result of alterations in cell production parameters. There were no statistically significant differences when the labelling indices of iron depleted animals and control animals were compared but in each case the labelling indices of iron depleted animals were greater than normal. In addition when the labelling index per unit surface length for iron depleted animals was compared with normal the P value of 0.06 approached significance. Together with the observation that iron depleted animals had significantly greater labelling indices than anaemic animals the results indicate an increased labelling in iron depletion.

The T_S for iron depleted animals was unchanged. The increase in the labelling index therefore suggested a net increase in cell production. As there was no change in progenitor cell size, the proportional increase in the progenitor cell compartment could have been the result of an increased production of progenitor cells. However, the same results could have arisen from an imbalance between the production of new progenitor cells and their subsequent migration. A delay in passing to the maturation compartment followed by a rapid transit through the maturation compartment could have produced the same stereological and cell production results.

It is not possible to know which of these mechanisms is operative in iron depletion but the cell kinetic results

substantiate the stereological finding that there is an increased progenitor cell compartment in iron depletion.

7.4.2 Iron Deficiency without Anaemia

Stereological analysis has revealed an increase in the proportion of the epithelium formed by the keratinised compartment and a decrease in the proportion formed by the maturation compartment. Investigation of cell production parameters has shown a net increased cell production rate in iron deficiency without anaemia.

There are several factors which could have been involved in the increase in the relative thickness of the keratin layer. If cells had been lost from the surface at the normal rate in iron deficiency without anaemia and passed rapidly through the maturation compartment then the increase in cell production would account for the thicker keratin layer. The time taken for cells to pass through the maturation compartment is a function of a spatial movement through the compartment and a sequence of biochemical steps leading to the formation of keratin. It can be seen that an imbalance in either of these parameters could lead to proportional variations in epithelial architecture.

7.4.3 Iron Deficiency Anaemia

The epithelium of animals with iron deficiency anaemia had a significantly thinner maturation compartment

and a significantly thicker keratin layer. As in iron deficiency without anaemia there was a net increased cell production rate but there was also a decrease in the size of progenitor and mature cells.

The alterations in compartment size could have been the result of disturbances of cell transit or biochemical abnormalities although neither of these parameters would result in an increased cell production rate. It is possible that a combination of decreased maturation cell size and reduced maturation compartment thickness result in decreased amounts of cell cycle regulatory chalones being formed or released by maturing epithelial cells. Such a decrease in G_1 inhibitor would result in an increased cell production rate.

It is therefore possible to explain in part the structural abnormalities demonstrated in ventral tongue epithelium in iron deficiency in terms of cell production abnormalities. The cause of the cell production disturbances is not clear but it is considered highly probable that dysfunction of one or more of the many iron dependent enzyme systems present in epithelia is responsible for these abnormalities.

7.5 FURTHER WORK

It is intended to continue the work presented in

this thesis to involve a wide variety of related clinically orientated research involving both human and animal studies. An in vitro investigation of the cell kinetics of the buccal epithelium in human iron deficiency anaemia is in progress and by careful selection of patients and controls a uniform group of experimental material is being accumulated.

A third animal experiment is proposed to investigate the effects of extended periods of iron deficiency anaemia upon the oral epithelium and to determine the effects of iron therapy upon the epithelial abnormalities.

In the long term it is hoped to investigate the reported relationship between iron deficiency and the development of oro-pharyngeal malignancy. The effects of carcinogen application upon the oral epithelium of animals with established iron deficiency will be studied. Using a combination of quantitative techniques developing lesions will be investigated and particular attention will be paid to the sequence of changes occurring in early lesions.

Other interesting related avenues of research are the associations between Candida albicans infection, iron deficiency and the development of oral neoplasia and preneoplastic lesions. A preliminary study has been completed and it has proved possible to induce Candida albicans infection in anaemic animals.

It is likely that alterations in the permeability of the epithelium are important in the development and persistence of fungal infection and perhaps in the development of oral cancer. Using recently described in vitro techniques the relationship between mucosal permeability and iron deficiency will be investigated.

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