THE *IN VITRO* EFFECT OF ETHANOL ON RAT LINGUAL EPITHELIUM

BY

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To Stuart and my mother

We dance around in a ring and suppose but the secret sits in the middle and knows

From "The Secret Sits", in *The Poetry of Robert Frost*, edited by Edward Connery Lathem, New York: Holt, Rinehart and Winston, 1971.

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Abbreviations

ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AMPS	ammonium peroxidisulphate
ATPase	adenosine triphosphatase
bis	N′ methylenebisacrylamide
BSA	bovine serum albumin
CBB-G250	coomassie brilliant blue (G250)
CBB-R250	coomassie brilliant blue (R250)
CO_2	carbon dioxide
1-D	one dimension
dH_2O	distilled Water
DMBA	dimethylbenz(a)anthracine
DMEM	Dulbecco′s modification of Eagle′s medium
DMSO	dimethyl sulfoxide
EBSS	Earle′s balanced salt solution
EDTA	ethylenediaminetetraacetic acid
GRP	glucose related protein
HRBP	histadine rich basic protein
HSP	heat shock protein
KD	kilodaltons
MEM	minimum essential medium
MEOS	microsomal ethanol oxidising system
MW	molecular weight
Na/K ATPase	sodium/potassium adenosine triphosphatase
NAD(P)	nicotinamide dinucleotide (phosphate)
O_2	oxygen

PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
S.D.	standard deviation
S.E.	standard error
PAGE	polyacrylamide gel electrophoresis
TEMED	NNN′-tetramethylethylene-diamine

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Preface

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH, CHICAGO, USA, 1993.
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(2) The effect of alcohol and its primary metabolite on oral epithelium *in vitro*. A. Bell, J. S. Rennie and A. S. G. Curtis.
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Declaration

This thesis is the original work of the author

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Summary

Chronic alcohol abuse has been linked to the development of oral cancer, but before studying the role of alcohol in intra-oral carcinogenesis the effect of alcohol alone upon the oral epithelium must be clarified.

Since cells express their individuality through the proteins they produce, alcohol related alterations are likely to be mediated or accompanied by alterations in the epithelial protein pattern. Graham & Rennie (1987) noted alterations in the levels of three proteins from rat lingual epithelium after 102 days of alcohol intake. However, animal models are time consuming and expensive. It was thus the aim of the work reported in this thesis to develop an *in vitro* model to investigate the effects of alcohol on rat lingual epithelium and to compare any changes in epithelial protein profile with those described *in vivo*.

Rat tongue epithelium was initially grown with a 3T3 feeder layer and subsequently passaged onto plastic surfaces (Jepsen, MaCallum & Lillie, 1980). This technique produced cultures which could be given alcohol and any effects examined in the absence of metabolites from other cells.

To confirm that the epithelial cells were metabolising alcohol, cultures were given ethanol and the concentration measured over 48 hours. It was shown that there was a significant difference in ethanol

concentration between flasks with cells and control flasks, indicating that ethanol was utilised by the epithelial cells. Additionally a proportion of the added alcohol was lost by evaporation.

The effect of alcohol on cell viability was investigated using the vital stain fluorescein diacetate (Mishell & Shiigi, 1980). Five percent ethanol was chosen as the upper limit and a range of ethanol concentrations down to 0.25% employed. Concentrations between 5 and 1% killed cells in culture while concentrations of 0.5% and 0.25% ethanol did not significantly reduce cell viability.

Accordingly 0.5% ethanol was chosen as an appropriate concentration and used throughout this thesis. By giving cells 0.5% ethanol every 24 hours the concentration of ethanol was kept between 0.5% and 0.38%. Cells were therefore surrounded by medium with an alcohol concentration similar to that found in the blood of inebriated persons and this system was considered suitable for the study of the effects of alcohol on the oral epithelium *in vitro*.

To investigate the biochemical effects of ethanol the protein profile of cultured rat lingual epithelium was examined using SDS-PAGE. Proteins from cells given 0.5% ethanol were prepared for SDS-PAGE at 1, 3, 5, 7, 14, 21 and 30 days of treatment. Visual and laser densitometer analysis of gels revealed that in cells given alcohol for 7 days or more there was a reduction in the levels of two

high molecular weight (MW) proteins of 230 and 200 kilodaltons (kD) and an increase in the levels of two low MW proteins (30kD and 28kD). There were no significant protein changes noted before 7 days. These protein changes were similar to those observed in the animal model (Graham & Rennie, 1987). The changes were not reversed by removing the alcohol and culturing for a further 7 days.

The protein alterations with ethanol treatment may not occur as a direct result of ethanol damage but may be induced by the primary metabolite, acetaldehyde, as it has been reported to be more toxic to neurones in culture at much lower concentrations than ethanol (Smith *et al.*, 1990). A viability study showed that this was also true for oral epithelium in culture. Cells given 0.5% acetaldehyde died within 24 hours and even at concentrations of 10 and 100 less than ethanol most of the cells were dead by 14 days.

The effects of 0.05% and 0.005% acetaldehyde on cultured epithelial cells were examined using SDS-PAGE at 1 and 7 days. Protein levels altered after 7 days of ethanol treatment were altered after 1 day of treatment with both concentrations of acetaldehyde. This suggested that the changes were acetaldehyde mediated and did not occur as a direct result of ethanol. Increased levels of an additional protein (66-70kD) were observed in acetaldehyde treated cells but not with ethanol treatment.

Graham (1987) suggested that the increased levels of the lower MW proteins could be the result of the synthesis of heat shock proteins (HSP). To investigate the possibility of the protein level increases observed being due to the synthesis of HSPs cells were subjected to a heat treatment of 50°C for five minutes and the protein profile of cells examined immediately, 1 hour, 12 hours and 24 hours after treatment. The results of this study showed that levels of the 66-70kD protein increased at 12 and 24 hours after heat treatment. Heat treatment did not alter the levels of the other four proteins altered by alcohol and acetaldehyde. The increase in levels of the 66-70kD protein appears to be a general stress response while the alterations in levels of the other proteins are specifically related to alcohol/acetaldehyde.

The possibility that the 66-70kD protein was a HSP was further investigated using immunofluorescent staining with an anti-HSP 70kD antibody. Acetaldehyde treated cells and cells at 12 and 24 hours after heat treatment stained positively. A Western blot carried out 24 hours after heat treatment using the anti-HSP 70kD antibody detected a positive band in the 70kD region.

Simple subcellular localisation studies using differential centrifugation and Triton X-100 detergent lysis of cells showed that the three higher MW proteins were present in the insoluble cell fraction while the two lower MW proteins were present in the soluble cell fraction.

The significance of the increased levels of the 30kD and 28kD proteins is not clear, although they do not appear to be HSPs. The 66-70kD protein appears to be a HSP and would therefore assist the epithelial cells resist external stress. The high molecular weights and the presence of the 230kD and 200kD proteins in the insoluble cell fraction suggests that they may be large structural proteins, and as such are probably keratin peptides and polypeptides.

The work presented in this thesis confirms earlier *in vivo* studies (Graham, 1987; Graham & Rennie, 1987) and indicates that the *in vitro* model is a useful system for the future study of the effects of alcohol on oral epithelium. This work has also described the importance of acetaldehyde and emphasises the need for further investigation of the role of acetaldehyde in alcohol related disease processes.

Chapter One

Introduction and Review of Literature

1.1 General Introduction

Alcohol has been strongly implicated as an aetiological factor in the development of aerodigestive squamous cell carcinoma, particularly of the oral cavity. Investigation of the cause of neoplasia is complex as most common cancers have a multifactorial origin. Before any study of the role of alcohol in the aetiology of oral cancer can be carried out it is important to consider what effect alcohol alone has on epithelium. Despite a large amount of literature on the effects of alcohol on hepatocytes and neurones, very few studies of the effects of alcohol on the oral epithelium exist and it was the aim of the work reported in this thesis to examine the effect of alcohol on the oral epithelium.

The current knowledge of the structure of the oral epithelium and of the effects of alcohol abuse is considered as an introduction to the experimental work of this thesis.

1.2 Structure of the Oral Epithelium

1.2.1 Introduction

Oral epithelium is a stratified squamous epithelium which serves many functions acting as a permeability barrier playing a role in eating, drinking, speaking and even breathing. To enable it to perform such diverse functions oral epithelium exhibits a wide range of differentiation patterns.

Human oral epithelium may be classified into three main types (Alvares & Meyer, 1971):

(a) Lining: This epithelium is non-keratinised stratified squamous epithelium and covers the cheeks, lips, floor of mouth, ventral surface of the tongue, soft palate and a portion of the maxillary or mandibular alveolus.

(b) Masticatory: This epithelium is found on the hard palate and is the mucosa which surrounds the teeth and gingiva. This may either be fully keratinised (orthokeratinised) or partially keratinised (parakeratinised) where the surface cells retain pyknotic nuclei.

(c) Specialised: This type comprises the distinctive papillated surface of the dorsum of the tongue. Structurally it is related to the masticatory mucosa but exhibits unique organisation and cell migration (Hume & Potten, 1976, 1980; Dhouailly *et al.*, 1989). Again, this epithelium is ortho or parakeratinised.

In most animals the same basic epithelial structure is exhibited but all of the oral epithelium is keratinised (ortho or para).

1.2.2 Keratinised Epithelium

The keratinised (ortho and para) epithelia are divided histologically into four strata:

1) *Stratum basale* (the basal layer)
2) *Stratum spinosum* (the prickle cell layer or spinous layer)
3) *Stratum granulosum* (the granular layer)
4) *Stratum corneum* (the keratinised layer)

Mitoses occur in the basal cell layer which rests on a basement membrane. Basal cells are cuboidal or columnar in shape. The cells undergo terminal differentiation as they pass from the basal layer to the most superficial layer. Contact with the basement membrane is suggested to suppress signals for keratinocyte differentiation (Lillie, MacCallum & Jepsen, 1988) and when direct contact is broken differentiation begins. The time taken for the passage of cells from the basal to surface layer varies from region to region. Generally, the time taken is shorter in non-keratinising epithelia than in keratinising epithelia, e.g. c.41-57 days for human gingiva (Meyer, Marwah & Weinmann, 1956) and c.25 days for buccal epithelium (Alvares *et al.*, 1972).

In the suprabasal layers cells in the spinous layer are isodiametric in shape and are larger than those of the basal layer. This stratum is so named because of the numerous prominent intercellular bridges which give the cells a spiny appearance in histological preparations. The cells of the *stratum spinosum* differentiate to form the larger but more flattened cells which comprise the granular layer.

Tonofilaments are the most abundant cytoplasmic component and the major structural element of stratified squamous epithelium (Chen & Squier, 1984). Bundles of tonofilaments insert into desmosomes and hemidesmosomes in all oral epithelia. Desmosomes and bundles of tonofilaments begin to increase in the *stratum spinosum*.

This represents the start of an accumulation of fibrous keratin in the cytoplasm.

In the outermost cornified layers the cells are markedly flattened and appear as hexagonal discs or squames, which in orthokeratinised epithelium, lack nuclei, keratohyaline granules and other organelles. They are entirely filled with keratin filaments and also contain an insoluble protein envelope which is closely apposed to the inner surface of the plasma membrane.

In parakeratinised areas pyknotic nuclei, recognisable filaments and numerous lipid droplets are retained in the surface cells (Silverman, 1971).

As a result of the total loss of cellular organelles in the keratinised layer these cells are non-vital.

To summarise, during maturation keratinocytes move to higher layers in the epithelium and tend to increase in size as they go. This is accompanied by a gradual loss of organelles and an increase in the amount of structural protein until the most superficial cells contain no organelles and are densely packed with keratin filaments embedded in a keratohyaline derived matrix (the *stratum corneum*). The end result is a rather inflexible, mechanically tough and chemically resistant surface.

At the end of maturation the most superficial cells are lost by desquamation. Little is known of this process but

hydrolytic enzymes extruded by the membrane coating granules may be involved. Weinstock & Wilgram (1970) suggest that such enzymes break down the extracellular cement holding cells together and with, or without, the assistance of extracellular abrasion, enzymic action leads to the sloughing of the outermost cells.

An alternative explanation suggests that the adhesive between cells has a limited lifespan and its spontaneous deterioration and breakdown, possibly aided by external abrasion, leads to the detachment of cells (Chen & Squier, 1984).

1.2.3 Non-keratinised Epithelium

Non-keratinised epithelium lacks granular and keratin layers and has been divided into basal layer, prickle cell layer, intermediate layer and superficial layer (Squier, Johnson & Hopps, 1976). Another study by Schroeder (1981) proposed a new subdivision for non-keratinised epithelia. The terms *stratum basale* and *suprabasale* have been retained and the terms *stratum filamentosum* and *stratum distendum* have been proposed. *Stratum filamentosum* is the layer of non-keratinised epithelium at the level of the *stratum spinosum* of keratinised epithelium. The term describes the numerous unbundled filaments which begin to appear in this layer. Superficial to this stratum is the *stratum distendum* which represents the most mature cells of a non-keratinised tissue.

Basal cells in non-keratinised epithelium are cuboidal or ovoid. As cells migrate towards the surface they increase in volume. This increase is more marked in non-keratinised epithelium (Meyer & Gerson, 1964). Cells in the suprabasal layer tend to retain many basal cell characteristics.

As cells of non-keratinised epithelium leave the suprabasal layer and enter the *stratum filamentosum* they are characterised by the appearance of an extensive network of enmeshed single filaments which amount to about 35% of the tissue volume of the *stratum filamentosum* (Schroeder, 1981).

In the superficial *stratum filamentosum* membrane coating granules become arranged at the distal aspect of the cells. Most of these granules remain inside these cells as they enter the *stratum distendum*. In this layer they are thought to fuse with the plasma membrane and extrude their contents into the intercellular space (Squier, 1977). This suggested extrusion coincides with the appearance of an intercellular permeability barrier at this level in non-keratinising oral epithelium (Squier, 1973; Squier & Rooney, 1976).

The terminal stage of maturation in non-keratinised epithelium is the *stratum distendum* and consists of cells containing a variety of organelles. Unbundled filaments show a progressive increase through the *stratum distendum* (Schroeder, 1981) and glycogen also shows an increase

within this layer. However, most other organelles show a decrease in relative volume having reached peak values in the *stratum filamentosum*.

The end result of the maturation process in non-keratinised epithelium does not lead to the inflexible, mechanically tough and chemically resistant surface of keratinised epithelia but demonstrates a pattern of differentiation which results in surface cells which are less able to resist damage but remain capable of mechanical distension.

1.2.4 Non-keratinocytes

Within oral epithelium there are cells at various levels which differ quite markedly from the majority of epithelial cells in that they possess a clear halo around the nucleus. These non-keratinocytes can account for up to 10% of the total epithelial cell population and such cells, sometimes referred to as clear cells, include melanocytes (Schroeder, 1969), Langerhans cells (Difranco *et al.*, 1985), Merkel cells (Ness, Morton & Dale, 1987) and lymphocytes. Melanocytes and Merkel cells are basally located while Langerhans cells are predominantly suprabasal and lymphocyte location varies.

None of these cells show any tendency to keratinise and all of them, excepting the Merkel cell, lack tonofilaments and desmosome attachments to adjacent cells. Merkel cells have very small numbers of desmosomes and tonofilaments. It has been suggested that the absence

of desmosomes allows the cytoplasm of the non-keratinocytes to shrink around the nucleus during histological processing and so produce the clear perinuclear halo that gives rise to their name (Breathnach, 1965). A lengthier discussion of the structure and function of the various oral non-keratinocytes can be found in a review by MacKenzie & Binnie (1983).

1.2.5 The Basal Complex

The junction between epithelium and connective tissue is termed the basement membrane. Under the light microscope the basement membrane appears as a continuous but relatively structureless layer some 1-2μm thick (Gersh & Catchpole, 1960). Substances moving from the connective tissue to the epithelium or in the other direction must cross the basement membrane which exerts a filtering effect so that only particles of a certain size can pass through. In this way the basement membrane can supplement the barrier function of the superficial layers of the epithelium.

Electron microscopy has revealed the basement membrane to be essentially similar in ultrastructure in a variety of covering and lining epithelia, including those of skin and the mucous membranes of the mouth, vagina and uterine cervix (Kefalides, Alper & Clark, 1979). Immediately below the basal epithelial cell layer is an electron-lucent zone termed the *lamina lucida* or *lamina rara* and beneath the *lamina lucida* is an electron-dense amorphous

layer called the *lamina densa* or basal lamina. A series of anchoring fibrils form loops, both ends of which insert into the *lamina densa*. These fibrils are narrower than collagen fibrils and cross-band in a regular repeating pattern (Palade & Farquhar, 1965).

The collagen fibrils of the connective tissue form a subjacent layer to the *lamina densa*, and run through the loops of the anchoring fibrils (Chen & Squier, 1984). The network of anchoring fibrils provides a system which interlocks the larger collagen fibres of the connective tissue with the *lamina densa*.

The whole structure constituted by two laminae, the anchoring fibrils and associated collagen is commonly termed the basal complex. Electron microscope studies of oral mucosa stained with the periodic acid-silver methenamine technique (Rambourg & Leblond, 1967) suggest that the basal complex represents the ultrastructural counterpart of the basement membrane seen under the light microscope (Melcher, 1965).

1.3 Biochemistry Of The Oral Epithelium

1.3.1 Introduction

There is very little available data relating specifically to the oral epithelium and much of the information available for stratified epithelium is for epidermis. Therefore, this review on the biochemical features of the oral epithelium is supplemented by data from studies on other stratified squamous epithelia, particularly skin.

1.3.2 Proteins

Keratins

The cells of stratified squamous epithelium synthesize (and retain) a large amount of structural protein. These structural proteins make up the major component of the filaments (tonofilaments), present in all cell layers of both keratinised and non-keratinised epithelium. This heterogeneous group of proteins is termed collectively as keratins.

Tonofilaments vary from 7.5-10.0nm in diameter (Green, 1978). Cross reaction studies (Sun, Shih & Green, 1979; Schlegel, Banks-Schlegel & Pinkus, 1980) and immunofluorescence studies (Saurat *et al.*, 1978; Oberle *et al.*, 1979; Loning *et al.*, 1980) indicate some similarities among filaments in all epithelial cells, especially among the keratins of stratified squamous epithelia. Similarities have also been shown in amino acid and polypeptide composition (Green, 1978).

Tonofilaments can be electrophoretically resolved into at least 7 polypeptides ranging in molecular weight from 40-68 kilodaltons (kD) (Green, 1978). As the epithelial cells migrate toward the surface layers there is a progressive increase in molecular weight of the main components of the filaments. This is the result of the synthesis of new filament proteins (Fuchs & Green, 1979).

Cells of non-keratinised oral epithelium contain only the smaller molecular weight keratins as do cultured

monolayers of oral epithelium (Fuchs & Green, 1980; Tseng *et al.*, 1982; Clausen *et al.*, 1986). Cells from keratinised epithelium contain the higher molecular weight keratins and middle molecular weight keratins are present in both types of epithelium (Tseng *et al.*, 1982).

Tonofibrils are resistant to chemical degradation with those in cells nearer the surface being most resistant (Green, 1978). Cells in the *stratum corneum* are resistant to urea and sodium dodecyl sulphate (SDS), and a reducing agent is required to break the chemical bonds. The chemical resistance is thought to be due to strong attractive forces between, and within polypeptide chains as a result of hydrogen bonding and disulphide bridges. Disulphide bonds increase in number as epidermal cells differentiate and reach the more superficial layers (Susi, 1969).

Other proteins

There is evidence that other fibrous proteins are also present in stratified squamous epithelium. An immunofluorescent study identified actin in human gingival wounds (Goteiner, Krawczyk & Gillon, 1977) and observed staining in basal cells distal from the wound and adjacent to it. Reibel *et al.* (1978) did not identify actin in epithelial cells adjacent to experimental wounds in oral epithelium in primates but did observe it in outgrowths of epithelial cells from rat tongue and human gingiva.

1.3.3 Keratohyaline Granules

Protein and RNA are the major components of epidermal and oral epithelial keratohyaline granules (Ugel, 1971; Singh, McKinney & Kolas, 1975). Biochemical and histochemical studies have also identified carbohydrates, lipids and calcium as components of keratohyaline granules in oral epithelium and epidermis (Cane & Spearman, 1969; Singh *et al.*, 1975).

There are varying reports about the amino acids in epidermal keratohyaline protein. Much of the inconsistency may stem from the different extraction procedures employed which may act on one or other of the two components usually present in the granules (Fukuyama *et al.*, 1976).

The fibrillar component, which is the major component, is extracted by phosphate buffer (Ugel, 1969) mercaptoethanol and deoxycholate (Tezuka & Freedberg, 1972) or urea (Dale & Ling, 1979). The most common amino acids in the fibrillar component are, in decreasing order of abundance serine, glycine, glutamic and aspartic acids, arginine, alanine and histidine (Ugel, 1975; Baden, 1979).

The other component of the granules appears as dense homogeneous deposits. Matoltsy & Matoltsy (1970) extracted a component using detergent in citrate buffer which Fukuyama *et al.* (1976) suggested was the homogeneous deposit. The most abundant amino acids

observed in this component are cysteic acid, proline, glycine and glutamic acid (Matoltsy & Matoltsy, 1970).

It was claimed that the material in keratohyaline granules formed the matrix component of the *stratum corneum* as early as 1959 (Brody). Chen & Squier (1984) supported this by his observation that the orthokeratinised palatal epithelium in rabbits, which has no keratohyaline granules, also has no matrix material in the *stratum corneum*. Many studies on epidermal keratohyaline granules support this view. ^{3}H-labelled histidine is concentrated in granules following injection and is later found in the stratum corneum (Fukuyama *et al.*, 1976; Dale & Ling, 1979).

When keratin filaments and the histidine-rich basic protein (HRBP) of the *stratum corneum* are combined *in vitro* a structure is formed which is similar to the patterns of filaments in the matrix found in the *stratum corneum* (Dale, Holbrook & Steinert, 1978; Dale *et al.*, 1981). This would suggest that the HRBP, now termed filaggrin (Steinert *et al.*, 1981), is the matrix protein of the *stratum corneum*.

There is little available literature to date on the chemical characterisation of the keratohyaline granules that are not associated with tonofilaments (buccal type).

1.3.4 Cell Membranes

As the cells reach the more superficial layers of the epithelium the plasma membrane develops a thick inner

leaflet, the cell envelope, which is the part of the cell that is most resistant to the action of lytic enzymes (Gerson & Harris, 1984). This increased resistance is the result of a number of chemical changes which take place as the membrane thickens.

The cell envelope is composed largely of protein (Sun & Green, 1976) and as it forms, the protein to lipid ratio increases (Gray, 1981). Immunochemical tests have identified a soluble precursor, involucrin (Rice & Green, 1979; Watt & Green, 1981), which first appears in the middle of the spinous layer. Antibodies to involucrin react with the inner leaflet but the amino acid composition of the precursor differ from the product (Rice & Green, 1979). The protein in the envelope is cross-linked by gamma-glutamyl linkages (Rice & Green, 1977). This cross-linkage is extensive and is resistant to proteolytic enzymes (Baden, 1979). Disulphide linkages, also present in the thickened cell membrane may contribute to its chemical resistance (Matoltsy & Matoltsy, 1966; Fukuyama & Epstein, 1969).

As epithelial cells migrate through the different cell layers changes in plasma membrane lipid composition take place. In the deeper cell layers the major membrane lipids are phospholipids and cholesterol while in the *stratum corneum* cholesterol, fatty acids, ceramides and glucosyl-ceramides are the major components of the plasma membrane (Gray, King & Yardley, 1978). Phospholipases

present in the plasma membrane are partly responsible for the changes that take place (Gray *et al.*, 1978).

Some of the glycoproteins of the plasma membrane cover the outer area of the outer leaflet while some are an integral part of the membrane. The latter group are important components of cell membrane receptors (Gray, 1981).

1.3.5 Glycoproteins

Cells of the oral epithelium are surrounded by an intercellular material composed largely of carbohydrate-protein complexes. These increase in amount as the cells migrate towards the surface (Meyer & Gerson, 1964). In all tissues these carbohydrate-protein complexes are synthesised and secreted by the cells which they surround (Bennett, 1963).

Electron microscopic studies using periodic acid-Schiff (PAS) staining show positive material in membrane coating granules of human gingival and buccal epithelium and hard palatal epithelium of rats (Hayward & Hackemann, 1973). This PAS positive material appears on the intercellular aspect of the plasma membrane as a result of the fusion of granules with the plasma membrane and the discharge of their contents into the intercellular space.

The intercellular glycoproteins function as an adhesive and as a lubricant allowing the movement of cells. They

may also play a role as receptor sites and in cell to cell communication.

By virtue of filtration and selective binding they can exert some control over the diffusion of metabolites through the intercellular spaces. In the more superficial cell layers the cells of the epithelium are at some distance from capillaries and the controlling influence of the intercellular material may be greater than in other tissues (Gerson & Harris, 1984).

1.3.6 Lipids

As in all cells, phospholipids and cholesterol are an integral part of the plasma membrane and since cells of the oral epithelium divide and enlarge more rapidly than those in epidermis these membranes must be synthesised at a high rate (Gerson & Harris, 1984).

There are only a limited number of quantitative studies on lipid composition of the oral epithelium. Pig gingival epithelium was investigated by Rabinowitz, Tavares & Marsh (1972) and human hard palatal epithelium was examined by Lekholm and Svennerholm (1977).

In both studies phospholipids are present in higher concentrations than the other lipid components. In pig gingival epithelium cholesterol and cholesterol esters are similar but low in concentration (Rabinowitz *et al.*, 1972). However, in human palatal epithelium cholesterol makes up a larger proportion of total lipid composition

than in pig gingival epithelium and there is very little esterified cholesterol (Lekholm & Svennerholm, 1977).

Hard palate and gingival epithelium are both keratinised and lipid concentration differences may be related to species. High concentrations of glycolipid have been recorded in human palatal epithelium.

No quantitative studies on non-keratinised epithelium are available at present but phospholipids, cholesterol and free fatty acids have been shown to be present in non-keratinised human gingival sulcular epithelium (Anneroth & Ivemark, 1971) and neutral lipids have been identified in all layers of non-keratinised human oesophageal epithelium (Hopwood *et al.*, 1977).

Many changes in lipid composition occur as cells move toward the outer cell layers. As cells mature there is a substantial decrease in all intracellular lipids (Elias, 1981). Accompanying this change there is a substantial increase in sterols and triglycerides (Gray & Yardley, 1975; Elias, 1981).

As the horny cell layer is formed, phospholipids and cholesterol are released because of the breakdown of organelle membranes and changes in the lipids of the plasma membrane. Phospholipases are largely responsible for the breakdown of phospholipids and the release of fatty acids, the result of which is an increase in

esterified cholesterol in the *stratum corneum* (Freinkel & Fiedler-Weiss, 1974).

The role of lipids in energy production is discussed in section 1.4.

1.4 Energy Conversion

1.4.1 Glycolysis and the Krebs Cycle

Unlike most tissues stratified squamous epithelium does not have a direct blood supply. Only cells situated at, or close to, the basement membrane have easy access to the blood supply of the *lamina propria*. The more superficial cells receive nutrients that have passed through or between deeper cells.

The Krebs cycle, which takes place in the mitochondria, is the predominant metabolic pathway in the lower cell layers where the oxygen tension is higher. Mitochondria are more concentrated in basal and suprabasal cell layers, their concentration decreasing as cells move to the more superficial layers. Enzyme studies also support this postulated change in the metabolic cycle as cells progress through the different strata, and although the Krebs cycle does not shut down completely in the more superficial layers, it does decrease. In human and rat oral epithelium levels of activity of enzymes of the Krebs cycle decrease more rapidly towards the surface than those involved in glycolysis (Gerson, Meyer & Mattenheimer, 1966; Gerson & Silverman, 1973).

Freinkel & Traczyk (1976) suggest that less than 2% of glucose consumed by epidermis is oxidised by the Krebs cycle, while 70% is converted to lactate, the end product of anaerobic glycolysis. In human and rabbit oral epithelium, enzymes from the glycolytic pathway are more active than those of the Krebs cycle (Gerson, 1970; Gerson & Silverman, 1973). In keratinised tissues the *stratum corneum* is metabolically inert.

1.4.2 Pentose-Phosphate Pathway

A large part of the glucose taken in by epidermis is metabolised by the pentose-phosphate pathway (hexose-monophosphate shunt) (Freinkel, 1960; Freinkel & Traczyk, 1976). Gerson (1967) and Simpson (1975) reported the presence of this pathway in rat oral epithelium.

The pentose-phosphate pathway increases in importance as cells move more superficially from the basal to the spinous layer and decreases only moderately after this (Gerson, 1967).

The pentose-phosphate pathway occurs to a lesser extent in non-keratinised than in keratinised oral epithelium (Gerson, 1967, 1973) and may play a role in keratinisation.

1.4.3 Other Glucose Utilising Systems

Another pathway for the utilisation of glucose is conversion to glycogen. Deposits of glycogen have been identified in human non-keratinised oral epithelium

(Wislocki, Fawcett & Dempsey, 1951). The significance of glycogen in this tissue is unknown. Wislocki *et al.*(1951) suggest that it may act as a precursor carbohydrate in the synthesis of intercellular ground substance, which is more abundant in non-keratinised than in keratinised epithelium.

1.4.4 Alternative Energy Sources

In stratified squamous epithelium, as in many other tissues, lipids and amino acids can be used as energy sources following their conversion to intermediates of the Krebs cycle.

Cruickshank, Trotter & Cooper (1962) showed, using slices of skin, that lipids are oxidised by stratified squamous epithelium. More oxygen (O_2) was consumed than can be accounted for by the disappearance of carbohydrates or by amino acid metabolism.

In epithelium from the cheek pouch of hamsters the energy produced by fatty acid oxidation is very similar in amount to that produced by glycolysis (Harris & MacKenzie, 1980).

Amino acid oxidation has been shown in epidermis. When whole skin is incubated without glucose more ammonia is produced than when glucose is present, showing that under these conditions endogenous amino acids are utilised as an energy source (Cruickshank *et al.*, 1962).

1.5 Alcohol: Introduction

Ethyl alcohol has been an integral part of most human societies since recorded time. In many cultures the consumption of alcohol is socially acceptable, associated with personal pleasure, social interaction and celebration.

The popularity of alcohol in our society is demonstrated by the figures relating to expenditure on, and consumption of alcohol. In Britain household expenditure on alcohol was estimated at £21,730 million for 1990 and £23,555 million for 1992 (Social Trends, Government Publication, 1992, 1993). In 1983 it was reported that the British adult public consumed the equivalent of 9.91 litres of absolute alcohol per head of the population per year (Annual Abstract of Statistics, 1983). A survey carried out in 1990 showed that this level of alcohol consumption had continued and there was a small but significant increase in the proportion of males drinking more than the recommended safe maximum (21 units per week) (Smyth & Browne, 1990). This survey also reported an increase in the number of younger people (age 18-24 and 25-44 years) exceeding the recommended sensible dose and a 1993 government publication pointed out that the heaviest alcohol drinkers were in the 18-24 year age group (Social Trends, Government Publication, 1993).

Whereas moderate consumption of alcohol is an accepted social custom, excess consumption of alcohol is generally believed to be detrimental to personal and social well-

being, and its addictive potential and drug effects are well recognised.

Heavy alcohol consumption is associated with increased mortality (Marmot, Rose & Shipley, 1981) through cirrhosis, accidents, hypertension, cardiovascular disease and various cancers. Alcohol may be responsible for up to ten thousand premature deaths every year (Taylor, 1981) and it is estimated that alcohol is involved in the deaths of 500 young people each year in England and Wales. This represents about 10% of mortality in persons under 25 (Havard, 1977).

A general household government study for 1990 (Smyth & Browne, 1990) estimated the upper safety limit, before risk of physiological dependence, to be 50 units of alcohol per week (or 25 pints of beer) for men and 35 units (or 17.5 pints of beer) for women. However, these figures were the absolute upper safety limits and recommended sensible maximum amounts were set at 21 units of alcohol per week (or 10.5 pints of beer) for men and 14 units (or 7 pints of beer) for women. Throughout 1990 it was estimated for England and Wales that 27% of males aged sixteen and over exceeded the sensible dose.

Since alcohol consumption is not declining it is unlikely that there will be a decline in the incidence of alcohol related problems. The solution to the problem is far from simple since alcohol has become an intrinsic part of most civilisations and is of considerable economic value. It

therefore must be accepted that alcohol and its associated problems are a part of most societies and these problems must be faced and overcome where possible.

1.6 Alcohol and Disease

1.6.1 Introduction

Alcohol is known to be involved in the development of a number of disease entities. This review will make special reference to the liver, cancer in general and oral cancer. It must be noted that alcohol is involved in the disease processes of other systems such as gastrointestinal, cardiovascular, respiratory and nervous systems but these shall not be specifically dealt with in this thesis.

1.6.2 Alcohol and Liver Disease

The liver is the major site of ethanol metabolism (primarily by alcohol dehydrogenase) and the association of alcohol and liver disease has been recognised for many years. The three most common liver complaints resulting from alcohol abuse are alcoholic fatty liver, alcoholic hepatitis (both of which are potentially reversible) and cirrhosis which is irreversible (Lieber, 1982; O'Brien & Chafetz, 1982).

Despite the low incidence of cirrhosis it is becoming more common. From 1970-1981 the death rate from cirrhosis in Scottish males rose by 104% and by 73% in females (Register General's Report for Scotland : Scottish Health Education Co-ordinating Committee, 1985).

Due to the higher average consumption of alcohol by men cirrhosis is more common in males than females but women appear more susceptible to this disease.

Women have a greater proportion of body fat than men and accordingly less alcohol is absorbed from the circulation leaving higher circulating levels of alcohol. This may explain their higher susceptibility to liver alcohol disease. Sciot & Desmet (1992) describe in detail alcoholic liver disease in their comprehensive review of this problem.

1.7 Alcohol and Cancer

1.7.1 Introduction

The association of alcohol with oral cancer was probably first reported in 1837 by a Boston surgeon who reported a case of lingual carcinoma, attributing it to "the long use of ardent spirits," (Wynder, 1976). Hiatt (1992) noted that the earliest recognition that excess alcohol consumption was common to a series of cases was made in 1910 in France by Lamy, who found 80% of a group of oesophageal cancer cases were men who were heavy alcohol users.

Since these early findings large numbers of epidemiological studies have associated chronic alcohol consumption with an increased risk of cancer of the upper airways and alimentary tract. These associations have been confirmed by studies showing a decreased incidence of these cancers in social groups that abstain from

alcohol, for example, Mormons and Seventh Day Adventists (Tuyns, 1979). Cancer of the liver has also been associated with alcohol consumption, with a 24% risk attributed to heavy drinkers in Japan (Tsukama, Hiyana & Oshima, 1990).

1.7.2 The Oesophagus

Alcohol consumption may be responsible for approximately 75% of all oesophageal cancers in the United States (Rothman, 1980). The risk of developing oesophageal cancer is estimated to be 17 times greater in alcoholics than in the rest of the population.

There are markedly higher rates of oesophageal cancer for some geographical locales. It is particularly high in central Asia and in the West coast provinces of France (Tuyns, 1979). Although there is an increased risk of oesophageal cancer irrespective of the type of beverage consumed, it would appear that these geographical differences may be related to local drinks. The significance of the association with certain types of beverages is that substances in the beverages, besides alcohol, may be partially responsible for the increased risk (Tuyns et al, 1982).

African maize beers and French apple brandies and ciders have been shown to contain high levels of certain carcinogens. However, there are high incidences of oesophageal cancer in geographical locations that cannot be accounted for by alcohol or substances in alcoholic

beverages. For example, Iran, a Muslim state, has a low per capita consumption of alcohol, but has a high oesophageal cancer rate (Gillis & Carter, 1976).

Smoking has also been implicated as a cause of oesophageal cancer and the interaction of both tobacco and alcohol has been shown to be multiplicative. Although both tobacco and alcohol can independently confer increased risk, alcohol appears to be a more important aetiological factor (Wynder & Bross, 1961; La Vecchia & Negri, 1989)

1.7.3 The Pharynx and Larynx

The relative risk for cancer of the pharynx and larynx is increased in the presence of alcohol abuse 12-15 times (Elwood *et al.*, 1984; Tuyns *et al.*, 1988). Laryngeal cancer is common in South West Europe, a region with a very high wine consumption. As with cancer of the oesophagus, carcinogenic contaminants in local beverages may be of importance (Tuyns, 1979). Tobacco has been implicated as a potent aetiological agent in both pharyngeal and laryngeal cancer and a multiplicative interaction between alcohol and tobacco in the aetiology of these cancers has been reported (Tuyns *et al.*, 1988)

1.7.4 The Liver

The liver is another site where cancer has been associated with alcohol consumption, even though undiluted alcohol does not come in direct contact with hepatic tissues. In Western populations primary

hepatocellular cancer is rare. It is more common amongst cirrhotic alcoholics. An estimated 10-30% of this group develop liver cancer (Rothman, 1980). Although it has been suggested that hepatocellular carcinoma is secondary to cirrhosis and is not of a direct consequence of alcohol consumption (Tuyns, 1979), there is evidence that liver cancer occurs in alcoholics even without cirrhosis (Lieber *et al.*, 1979). A study in Japan has calculated an attributable risk of 24% for heavy drinkers (Tsukama, Hiyana & Oshima, 1990) and the estimated attributable risk of liver cancer in heavy drinkers in the United States is similar at 30% (Rothman, 1980)

1.7.5 Other Sites

Alcohol abuse has been associated with the development of cancers of a number of sites other than those already discussed. The stomach is one such site, although alcohol may not be a risk factor for stomach cancer, except perhaps for the region closest to the oesophagus. Most studies that have looked at mortality in alcoholics or heavy alcohol consumers have found no increased risk (Monson & Lyon, 1975; Carstensen, Bygren & Hatschek, 1990).

Results implicating alcohol in the aetiology of colonic and rectal cancer are conflicting (Hinds, *et al.*, 1980; Wu, Paganini-Hill & Ross, 1987). Alcohol does seem to be implicated to some degree in colon and rectal cancer, although the relationship is weak. Evidence for a link between cancer of the pancreas and alcohol consumption is

weak (Robinette, Hrubec & Fraumeni, 1979; Clavel, Benhamou & Anquier, 1989). For alcohol and breast cancer much of the evidence supports a causal relationship, however, the evidence is not entirely consistent (Lowenfels & Zevola, 1989).

Several other cancer sites have been studied with respect to alcohol consumption as a causal factor including lung, bladder, ovary, prostate and cervix. There is no strong support for a causal relationship between alcohol consumption and cancer of any of these sites. A review on this subject has been published by Hiatt (1992).

1.8 Alcohol and Oral Cancer

1.8.1 Oral Cancer: Epidemiology

Oral cancer is largely a disease of the elderly and accounts for some 3-5% of all cancers in most European countries. In 1992 it was estimated to account for 4% of male and 2% of female cancers in Europe (Boring, Squires & Tong, 1992). However, a higher incidence of oral cancer can be found in parts of India and South East Asia, where 40-50% of all cancers are oral (WHO, 1984).

The aetiology of oral cancer is complex and a detailed discussion of the factors involved is outwith the scope of this review, however it does appear that alcohol and tobacco are the most important non-infective aetiological factors. An extensive review on this subject has been published by Binnie, Rankin & MacKenzie (1983).

1.8.2 Association of Alcohol with Oral Cancer

Most studies show that a high proportion of oral cancer patients are heavy alcohol consumers (Monson & Lyon, 1975; Robinette *et al.*, 1979; Prior, 1988) and some authors have concluded that ethanol is the single most important aetiological factor in the development of oral cancer (Vincent & Marchetta, 1963; Elwood *et al.*, 1984)

The incidence of oral cancer among populations who do not normally consume alcohol is extremely low e.g. the Seventh Day Adventists (Lemon, Walden & Woods, 1964; Jensen, 1983) and Mormons (Enstrom, 1980; Lyon, Gardner & West, 1980). This lends support to an important role for alcohol in oral carcinogenesis.

As already mentioned, tobacco is a major aetiological factor for oral cancer and it is difficult in many cases to separate the risk attributable to alcohol and tobacco (Graham *et al.*, 1977; Mashberg, Garfinkel & Harris, 1981; Blot *et al.*, 1988). Where alcohol and tobacco have been examined jointly (Rothman & Keller, 1972; Blot *et al.*, 1988) approximately 75% has been attributed to the effects of alcohol and tobacco together. Rothman (1980) estimated that if each factor is assumed to have independent effects, then perhaps 50% of oral cancer in men and 40% in women could be attributed to alcohol alone.

Most authors show the interaction of alcohol and tobacco to have synergistic effects on cancer risk. Rothman & Keller (1972) reported that the risk for persons who used large amounts of both alcohol and tobacco was several times greater than would be expected on the basis of adding the two factors together. This synergism between alcohol and tobacco has been confirmed by others (Rothman, 1980; Elwood *et al.*, 1984; Blot *et al.*, 1988). It is worth noting that some authors do not agree with a synergistic model and suggest an additive model explains the relationship between alcohol and tobacco in the risk of oral cancer (Graham *et al.*, 1977).

There is a gradient of increasing risk with increasing alcohol consumption although some confusion exists as to whether the type of beverage consumed is important. Mashberg *et al.* (1981) suggests that beer is more carcinogenic than whisky, with beer drinkers having a threefold higher risk of developing oral cancer than whisky drinkers. However, Wynder & Bross (1957) suggest that whisky is more carcinogenic than beer. It is clear, however, that there is an increase in risk regardless of the beverage consumed. All types of alcoholic beverages have been implicated in the aetiology of oral cancer. Blot *et al.*, (1988) reported that consumption of over four drinks per week of either hard liquor, beer or wine increased the risk of oral cancer.

1.9 The Action of Alcohol in Carcinogenesis

1.9.1 Introduction

The way in which alcoholic beverages are carcinogenic is not clearly understood but given the correlation between chronic alcohol abuse and certain cancers it is worth discussing some of the postulated mechanisms for the role of alcohol in carcinogenesis.

1.9.2 Alcohol as a Carcinogen, Cocarcinogen or Promoter

For the purposes of the discussion the following definitions will be used:

Carcinogen: an agent which can cause cancers to develop which would not otherwise have done so.

Promoter: a factor which, when applied repeatedly after a dose of a tumour initiating agent insufficient to produce tumours, then results in tumour formation.

Cocarcinogen: a non carcinogenic agent which augments the action of a carcinogen.

Numerous studies have shown that ethanol is not a carcinogen (Ketcham, Wexler & Mantel, 1963; Garro & Lieber, 1990), although, it can cause chromosomal changes *in vivo* (Obe & Herha, 1975). An early study by Stenback (1969) suggests that ethanol is not a tumour promoter and is most likely acting as a cocarcinogen. Ethanol does not increase the risk of cancer in general, but only at specific sites. Alcohol therefore only affects the sites at which the cancer will occur but not the risk of cancerous growth in general (Schmidt & Popham, 1981).

This role is more typical of cocarcinogen than of a carcinogen or promoter.

There are several postulated mechanisms by which ethanol may act as a cocarcinogen:

Local Action Directly on Tissues Through a Solvent or Cytotoxic Effect

Ethanol may increase the solubility of carcinogens (e.g. tobacco tars) and therefore increase their penetration into susceptible tissues (McCoy & Wynder, 1979). This theory is credible for cancers of the upper digestive tract and respiratory tract. These tissues come into direct contact with undiluted alcohol. In support of a direct effect of ethanol on these tissues is the gradient of decreasing risk, paralleling the successive dilutions of alcohol in the alimentary tract (Lieber, 1982).

Shirazi *et al.*, (1974) and Shirazi & Platz, (1978) demonstrated an increased permeability of canine oesophageal mucosa in the presence of ethanol. However, the association of upper respiratory and digestive cancers with beer, which is a weak alcoholic drink, does not support a direct effect (Garro & Lieber, 1990). This theory cannot be applied to sites with no direct contact with high concentrations of alcohol such as liver, breast, colon and rectum and other explanations must be sought. There is also a suggestion that ethanol increases local conversion of carcinogenic precursors to their carcinogenic form. The nasal mucosa of ethanol fed rats has an increased ability to activate N-

nitrosonornicotine, by alpha carbon hydroxylation (Castonguay *et al.*, 1984).

The Induction of Microsomal Enzymes That Convert Carcinogens Into More Active Forms

Ethanol increases microsomal enzyme activity, which is a systemic effect. This system can convert different chemicals into electrophilic metabolites that are carcinogenic (Garro & Lieber, 1990). Ethanol also causes the liver to release vitamin A either directly or by activating the cytochrome P-450 system to metabolise retinol and retinoic acid (Leo & Lieber, 1985). Vitamin A may have an important anticarcinogenic role (Ziegler, 1986) and its depletion through this mechanism may lead to increased susceptibility to carcinogens.

Murphy & Hecht (1986) demonstrated an increased ability of the microsomal fraction from the cheek pouch epithelium of alcohol fed hamsters to metabolise benzo(a)pyrene to a structure closer to the ultimate carcinogen.

Inhibition of DNA Repair Mechanisms

The DNA repair system contains enzymes that protect cells from chemical carcinogens which affect the integrity of DNA structures and sequences (Craddock, Henderson & Gash, 1982). The enzymes are inhibited by chronic alcohol consumption and the consequent loss of an intact DNA repair system leads to increased risk of somatic mutations and the expression of oncogenes.

The primary metabolite of ethanol, acetaldehyde, affects DNA. It is a potent mutagen and carcinogen (Obe & Ristow, 1977, 1979; Hayes, 1985) and has been shown to cause chromosomal aberrations, sister-chromatid exchanges and cross-linking of DNA.

Immunosuppression

Although most studies involve persons who already have alcoholic liver disease, whose immune system may be affected by associated nutritional deficiencies (Garro & Lieber, 1990), animal studies show that ethanol or its metabolites can impair the immune system in the absence of liver disease (Garro & Lieber, 1990). However, the importance of this system is questionable since the tumours associated with alcohol consumption are not the lymphomas or leukaemias which generally occur in immunosuppressed persons (Garro & Lieber, 1990).

Alteration in Liver Metabolism

A relationship exists between cirrhosis of the liver and head and neck cancers (Keller, 1967). It has been suggested that the diseased liver is unable to metabolise adequately carcinogens leading to raised levels of circulating carcinogens so increasing the risk of cancer. Mascres & Franchebois (1979, 1981) examined histological and histochemical changes in the oral epithelium of cirrhotic humans and found these to be similar to those of the oral epithelium of alcoholic rats. Evidence also suggests that in the presence of ethanol, the liver has an increased ability to activate procarcinogens, again

increasing the circulating levels of carcinogens. Studies with rats show that following ethanol consumption hepatic microsomes have an increased ability to convert the procarcinogen N-nitrosopyrolidine to a mutagen (McCoy & Wynder, 1979). However, it is unlikely that this mechanism can occur in the highly compromised liver of a chronic alcohol abuser. Also, any carcinogens activated by this mechanism would need to be highly site specific, since only some tissues develop tumours.

1.9.3 Malnutrition

A common concomitant of alcoholism is malnutrition. Dietary deficiencies may contribute to increased cancer risk, especially of the oesophagus. Alcoholics, who may consume 30-50% of their calories from alcohol, tend to become malnourished (Rogers & Conner, 1986). Poor diets may lead to dietary deficiencies such as iron, zinc, riboflavin, pyridoxine, vitamin E and lipotropes (Garro & Lieber, 1990). However, this is usually only the case in some alcoholics and the role of dietary deficiencies is not applicable to moderate alcohol consumers who generally maintain a balanced diet (Hiatt, 1992).

1.9.4 Contaminants in Alcoholic Beverages

Although evidence suggests that the risk of developing cancers at specific sites is increased regardless of the beverage consumed (MacSween, 1982), it is clear that certain beverages are potentially more dangerous than others. While the presence of carcinogens in alcoholic beverages is not the whole story, they may play a role in

certain areas of the world (Garro & Lieber, 1990). Carcinogenic contaminants such as fusel oils, polycyclic hydrocarbons, nitrosamines and asbestos fibres have been found in a number of beverages and in French apple brandies and ciders diethylnitrosamine, a potent oesophageal carcinogen has been found (Lieber, 1982). Also, oesophageal cancer among African populations has been ascribed to fermented drink derived from maize (Cook, 1971).

1.10 The Metabolism of Alcohol

1.10.1 Introduction

To understand the action of alcohol it is important to have a working knowledge of the pathways of ethanol metabolism. The three main pathways of ethanol metabolism are the alcohol dehydrogenase (ADH) pathway, the microsomal ethanol oxidising system (MEOS) and a catalase based system.

1.10.2 Alcohol Dehydrogenase

In most circumstances the major enzyme system responsible for the first stage of ethanol metabolism, oxidation to acetaldehyde, is the ADH system (Dawson, 1983). ADH is widely distributed in animals, plants and microorganisms and in mammals it exists as a cytosolic enzyme. The highest concentrations are found in the liver.

There was confusion at first as to why the body should produce an enzyme to metabolise an exogenous compound

consumed as a social drug. However, it is now known that gastric microorganisms produce ethanol as part of their normal metabolism (Krebs & Perkins, 1970) and ethanol has been detected in the blood stream of individuals who had consumed no alcohol. In man 1-10 grammes of ethanol are synthesised daily by intestinal microorganisms and various endogenous pathways (Li, 1977).

Mammalian ADH has a broad substrate specificity and its physiological role appears to be the detoxification and metabolism of various alcohols and aldehydes, of which ethanol is only one. It has the capacity to oxidise primary and secondary aliphatic alcohols, diols, cyclic and aromatic alcohols, W-hydroxylated fatty acids and 2-enoic alcohols and their respective aldehydes. In some cases it can oxidise steroids (Li, 1977). ADH has a pH optimum of 10, although it is sufficiently active at physiological pH to account for observed rates of metabolism. ADH has been shown to be responsible for at least 85% of ethanol metabolism in rats (Goldberg & Rydberg, 1969). Makar & Mannering (1970) observed that the Michaelis constant (Km) for ADH *in vitro* was in agreement with the Km for blood ethanol disappearance *in vivo*.

1.10.3 Microsomal Ethanol Oxidising System

The microsomal ethanol oxidising system (MEOS) is a minor pathway of ethanol metabolism. This auxiliary enzymatic pathway is located in the smooth endoplasmic reticulum. It appears that the contribution of this enzyme system to

ethanol metabolism is at most 10-20%, although the role of MEOS may become more important as the ethanol concentration increases (Mezey, 1985).

The observation that chronic ethanol consumption is associated with proliferation of smooth endoplasmic reticulum membranes (Lane & Lieber, 1966) is evidence in favour of the role of MEOS in ethanol metabolism. This was subsequently demonstrated *in vitro* (Lieber & DeCarli, 1968, 1970). Isolated microsomes have the ability in the presence of NADPH and O_2 to oxidise ethanol.

The MEOS also requires cytochromes to allow activation of the molecular O_2 to free radicals. The enhancement of MEOS activity is primarily due to the induction of a unique microsomal cytochrome P-450 isozyme, which has a high capacity for ethanol oxidation (Lieber *et al.*, 1987). This form of cytochrome P-450 is normally present in microsomes at low levels and increases dramatically after chronic ethanol intake in many species. Induction of microsomal cytochrome P-450 by ethanol is associated with enhanced oxidation of other drugs as well, resulting in metabolic tolerance to these agents. The role of cytochrome P-450 in the conversion of carcinogens to more active forms and oxidation of retinols and depletion of vitamin A from the liver is discussed in the section on the biological mechanisms of the action of alcohol in carcinogenesis (1.9). The interplay between ethanol and liver microsomes has a profound effect on the way heavy

drinkers respond to drugs, solvents, vitamins and carcinogens (Lieber *et al.*, 1987).

1.10.4 Catalase

Catalase is found in the peroxisomes of most cells and in the mitochondria of hepatocytes (Lieber, 1982). Evidence that some ethanol metabolism could not be inhibited by ADH inhibitors led to a search for alternative pathways of ethanol metabolism, one such pathway involved the enzyme catalase.

Catalase metabolises ethanol by the peroxidatic reaction:

$$CH_3CH_2OH + H_2O_2 \longrightarrow CH_3CHO + O_2$$

The best substrates for this reaction appear to be methanol, ethanol and formate with methanol being more important in the rat, at least. It does not appear to be a major contributor to the metabolism of ingested ethanol, accounting for less than 10% of ethanol oxidation (Peters, 1982). It is believed that catalase becomes more important at higher concentrations of alcohol (Li, 1977).

1.11 The Biochemical Effects of Ethanol

1.11.1 Introduction

Following acute or chronic ingestion of ethanol some biochemical changes may result from the direct action of ethanol, although the majority of changes appear to result from the action of various metabolic by-products

of ethanol metabolism e.g. acetaldehyde and the increased levels of reducing equivalents. Little information is available which relates specifically to the oral cavity and information has been drawn from literature on the effects of ethanol at other body sites.

1.11.2 Metabolic Effects

Following alcohol consumption reducing equivalents are produced from the metabolism of ethanol by the following reaction:

$$\text{Ethanol} + NAD^{+} \longrightarrow \text{Acetaldehyde} + NADH$$

The hydrogen produced from this reaction, even at moderate blood alcohol levels, exceeds that which can be utilised and so there is an increase in the NADH/NAD ratio (Lundquist, 1975). This alteration in NADH/NAD ratio leads to an increase in the cellular lactate/pyruvate ratio which can result in hyperlactic acidosis. However, the conversion of pyruvate to lactate is only one of the pathways employed for lowering the NADH/NAD ratio. In normal mitochondria the excess NADH is used for respiratory chain activity in the place of reducing equivalents generated from beta oxidation of fatty acids. This results in a build up of lipids leading to the development of fatty liver and the increased NADH/NAD ratio also leads to an inhibition of adenosine diphosphate (ADP) translocation across the mitochondrial membrane. This accounts for the lowering of respiratory activity and the lack of respiratory control often

observed in the mitochondria of ethanol treated livers (Gordon, 1973). This fat accumulation eventually stabilises around the time the redox state is attenuated (Lieber, 1980).

1.11.3 Carbohydrates and Organic Acids

The altered redox state (increased NADH/NAD ratios) in alcoholics affects carbohydrate metabolism in a number of ways:

(1) Hypoglycaemia

In the presence of excess reducing equivalents a large proportion of pyruvate, the common starting point for gluconeogenesis, is reduced to lactate leading to the inhibition of gluconeogenesis. If the body is in a fasted state when this occurs blood sugar levels will drop resulting in hypoglycaemia. This is a known complication of alcohol abuse (Lieber, 1976).

(2) Hyperglycaemia

In a well fed state alcohol consumption can lead to hyperglycaemia. This results from an increase in glycogenolysis coupled with a reduced peripheral glucose consumption. This effect has also been observed in isolated perfused livers (Lundquist, 1975).

(3) Lactate Metabolism

The increase in lactate/pyruvate ratio, due to the increase in reducing equivalents alongside a reduction in the elimination of lactate in the alcoholic leads to lactic acidosis (Lieber, 1980). This condition results in

an increase in serum uric acid because the ability of the kidneys is impaired. This may be involved in the exacerbation of gout following alcohol consumption (Mezey, 1985).

1.11.4 Proteins

Alcohol is known to affect protein synthesis and/or protein secretion. Under conditions of acute ethanol treatment cellular protein synthesis is decreased both *in vivo* and *in vitro* (Morland & Bessesen, 1977; Lieber, 1980). Lieber (1980) observed a disaggregation of both free and bound polysomes and a detachment of ribosomes from the rough endoplasmic reticulum when perfused rabbit livers were treated with ethanol. It is possible that this may be involved in the decreased protein synthesis.

Ethanol may affect different proteins in different ways. In the liver a stimulus to collagen synthesis may occur simultaneously with a depression of albumin and transferrin synthesis and release. The effects on hepatic protein synthesis may be moderated during chronic exposure (Rothschild, Oratz & Schrieber, 1987).

Cellular transport and secretion may also be altered. Hepatomegaly was previously attributed to the accumulation of lipids but it has now been demonstrated that at least 50% of the increase in liver dry weight is associated with an increase in hepatic protein content (Rothschild *et al.*, 1987). It has been suggested that this increase in protein content results from impaired

secretion of hepatic secretory proteins. There is an inhibition of the secretion of albumin and glycoproteins from the liver following both acute and chronic ethanol administration. The inhibition of secretion is probably mediated at the post-translational level and in the case of glycoproteins it may result from an impaired transport of glycoproteins from the golgi complex to the plasma membrane (Mailliard *et al.*, 1984).

1.11.5 Effects of Ethanol on Biological Membranes

The acute presence of ethanol generally exerts a disordering (fluidising) effect on the lipid bilayer of biological membranes. In contrast, chronic ethanol consumption leads to altered membranes, which are resistant to disordering (membrane tolerance) (Chin & Goldstein, 1977; Waring *et al.*, 1981; Taraschi, Wu & Rubin, 1985). Acute ethanol administration reduces the order parameter (rigidity) of phospholipid model membranes, synaptosomal plasma membranes and erythrocyte membranes (Chin & Goldstein, 1977, 1981).

During chronic exposure of a biological system to ethanol the fluid membrane should become more rigid and adjust its chemical composition to compensate for the continuous presence of the ethanol.

There are many examples of increased membrane order after chronic administration of ethanol. However, the effect is not universal and depends on the tissue, the method of examining the membranes and possibly the conditions of

ethanol administration (Goldstein, 1987). Rottenberg, Waring & Rubin (1981) found that the order of synaptic membranes of ethanol treated mice increased, although Harris *et al.* (1984) found no change in the order of the extracted membrane lipids.

Hepatic microsomal membranes of chronically ethanol treated rats were more ordered than controls (Ponnappa *et al.*, 1982). The increased rigidity which results from chronic ethanol treatment can be accounted for by alterations in the component lipids (La Droitte, Lamboeuf & De Saint-Blanquat, 1984).

Enzymes that reside in membranes are often inhibited by ethanol *in vitro* and become insensitive to it after chronic administration. An increase in their activity may develop after chronic ethanol administration suggesting both tolerance and physical dependence. This is true for the Sodium/Potassium (Na/K) adenosine triphosphatase (ATPase) (Israel *et al.*, 1970). These enzymes have been implicated as the intermediate link between metabolic energy and nerve impulses and so ethanol may exert its primary effect on the brain by altering the action of these synaptosomal enzymes (Sun & Somorajski, 1970). The inhibition of the Na/K ATPase in erythrocyte membranes may also contribute to ethanol brain effects by causing a reduction in oxygen uptake by the brain following alcohol consumption (Lundquist, 1975).

1.11.6 Effects of Ethanol on Enzymes

A number of enzyme systems in the body are affected by ethanol. Most notably affected are those of the muscles and liver.

(1) Muscle Enzymes

Ethanol disrupts the energy balance of skeletal muscle by reducing the activity of a number of enzymes. Amongst the enzymes affected are creatine phosphokinase, lactate dehydrogenase, hexokinase, malate dehydrogenase and succinate dehydrogenase (Suominen *et al.*, 1974), and also triosephosphate dehydrogenase and cytochrome c oxidase (Kiessling *et al.*, 1975).

(2) Liver Enzymes

Ethanol effects on the liver may often lead to enzyme induction and cell lysis, resulting in an increased release of some hepatocellular enzymes into circulation. These enzymes may be used as markers of liver damage and can allow specific diagnosis of the liver disorder. These enzymes include gamma-glutamyl transferase, alkaline phosphatase, aspartate transaminase and alanine transaminase.

1.12 The Effects of Ethanol on the Oral Epithelium

Despite the evidence linking alcohol and oral cancers, experimental studies on the effects of ethanol on the oral epithelium are not common.

Early studies demonstrated that locally applied ethanol reduced the latent period and increased tumour size in hamster cheek pouches painted with dimethylbenz(a)anthracine (DMBA) (Elzay, 1966). It was also demonstrated however, that tobacco was more potent in promoting tumour formation than alcohol and in this experimental system the two factors did not act synergistically (Elzay, 1969). The effect of alcohol on carcinogenesis in hamster buccal pouch was also demonstrated by Freedman & Shklar (1978), who showed that hamsters given alcohol and painted with DMBA, developed leukoplakic lesions and squamous cell carcinoma two weeks before control animals. Similar results were produced using sherry as a promoting agent (Arendt *et al.*, 1981).

Where histological studies have been carried out on the effects of ethanol alone on the oral epithelium results have been conflicting. One study of human alcoholics demonstrated an increased frequency of dysplastic epithelial cells (Anderson, 1972) and a post mortem study of human alcoholics, demonstrated a reduction in epithelial thickness, resulting from reduced cell size in the superficial cell layers (Valentine, *et al.*, 1985). The latter study showed similar histological changes with tobacco usage, although they were more severe with alcohol, and suggested that the changes were non-specific reactions to chronic irritation.

Histological changes have been noted in animal studies. Epithelial dysplasia was observed in rabbit oral mucosa following chronic topical ethanol treatment (Muller *et*

al., 1983). Also, an increase in thickness of the keratin layer of rat lingual epithelium has been demonstrated in alcoholic animals (Mascres &.Joly, 1981).

There are not many biochemical studies in this field, although Mascres & Joly (1981) demonstrated increased levels of acid phosphatase activity and succinate dehydrogenase activity. An increase in SS and SH groups in the keratin layer was also observed. Previous work in the Department of Oral Sciences (Glasgow University), investigating the effects of ethanol on the protein biochemistry of oral epithelium of alcoholic rats showed a reduction in levels of a high molecular weight glycoprotein and an increase in levels two low molecular weight proteins (Graham & Rennie 1987).

1.13 Alcohol and Cell Culture

Cell culture systems should be ideal for the study of the effects of alcohol on epithelium. They are controlled systems in which the effects of alcohol can be examined without interference from external factors.

As with all tissue culture experiments cellular changes induced by ethanol in culture depend largely on the type of cells and the growth conditions. The concentration of ethanol and the duration of exposure are of particular importance (Freund & Forbes, 1976). Normal liver cells in culture metabolise ethanol to acetaldehyde and it has been suggested that the effects may be due to the metabolic products rather than to ethanol (Walker *et al.*,

1974). Acetaldehyde has been shown to be more potent than ethanol when applied to neurones in culture (Smith *et al.*, 1990; Smith & Wubetu, 1991).

The effects of ethanol on growth vary from complete inhibition at high ethanol concentrations to a stimulatory effect at low concentrations (Freund & Forbes, 1976). The toxic effects of low concentrations are apparent only with chronic exposure while at higher concentrations ethanol exerts effects within 24 hours (Siyam, 1987). It has been suggested that ethanol interacts with the plasma membrane and with cellular enzymic systems and these are the main pathways for ethanol induced damage. This has been shown in bacteria (Dombeck & Ingram, 1984). Alcohol has been shown to lead to the synthesis of heat shock proteins in culture. This is a family of proteins which undergo enhanced synthesis in response to environmental stress including heat and ethanol (Burdon, 1986).

1.14 Tissue Culture

1.14.1 Introduction

Animal models have been widely used to evaluate the safety of both medical and commercial compounds in the drug and food industry. The development of tissue culture offered an alternative to animal models in many instances and has become increasingly accepted as a tool for cytotoxicity testing and particularly in the evaluation of cancer chemotherapeutic agents (Wilson, 1986). Cell culture also offers the opportunity for experimental

studies on tissue of human origin which would be impossible *in vivo*.

Tissue culture techniques are particularly attractive in alcohol related research. Animal models are expensive and time consuming as well as the ethical problems faced in using live animals. Tissue culture offers an attractive alternative to animal models and a particular advantage in alcohol related research is that the effects of alcohol on a living tissue/cell can be examined without interference from other compounds such as tobacco and factors such as age, sex and health and from secondary effects produced by the action of alcohol on other tissues.

1.14.2 History and Development of Tissue Culture

One of the earliest recorded attempts at *in vitro* culture of tissue was by Roux (1885) who maintained explanted pieces of chicken embryo in warm saline but Harrison (1907) is widely regarded as the founder of modern tissue culture techniques. He maintained fragments of frog medullary tube in aseptic conditions for up to 4 weeks using a lymph clot hanging drop technique.

Great advances in tissue culture came with the development of more defined growth media. Initially natural media such as plasma clots and extracts were used to supply growth requirements. Early attempts were made to replace this natural media with synthetic media (Lewis & Lewis, 1911; Carrel & Baker, 1926) and it was this work

that led to the development of synthetic media of known composition (Baker, 1936; Fischer *et al.*, 1948; Eagle, 1955; Waymouth, 1956, 1959; Ham, 1963). This development enabled extensive work to be done on specific growth requirements.

Contamination by microorganisms prevented major advances in tissue culture research and it was the discovery and use of antibiotics and antifungal agents which allowed an explosive expansion of tissue culture techniques. Cultures are now routinely maintained in medium containing antibiotics, the most commonly used being penicillin, streptomycin, gentamicin and kanamycin, and antifungal agents such as amphotericin-B and nystatin (Paul, 1970).

There are now a wide range of media available specific for the growth requirements of different cell types *in vitro* and the use of different substrates and non-growing conditional cells such as 3T3 mouse fibroblasts, particularly in the case of epithelial cells (Rheinwald & Green, 1975), have contributed to the success of tissue culture techniques now available for the growth of normal cells.

1.14.3. Tissue Culture General Considerations

Introduction

Many factors must be considered when deciding upon the culture techniques to be employed. After deciding whether to use organ or cell culture, the source of tissue,

medium, gas phase and substrate are but a few of the factors to be considered.

1.Organ Culture

Organ culture has been used in many areas of research and although it has certain advantages in complex structures cell culture is a more flexible tool. This review will concentrate on cell culture which is directly relevant to the work presented in this thesis, although a brief discussion of organ culture and oral epithelium is presented in section 1.15.2. Further details on organ culture are available from Paul (1970) and Freshney (1987).

2. Cell Culture

Introduction

Cell cultures are established from single cell suspensions obtained from embryonic or adult tissue. To disaggregate the tissue the intercellular material is digested or disturbed releasing a single cell suspension. This may be done in several ways:

Mechanical Disaggregation

Some techniques involve the use of filters of different sizes. However, the numbers of cells obtained by straining tissue through these filters tends to be small and the cells released often remain in clusters (Morasca & Erba, 1986).

Mechanical methods such as teasing, slicing and stretching are often used in dermal epidermal separations (Freshney, 1987).

Enzymic Disaggregation

The use of proteolytic enzymes was first reported by Rous & Jones (1916). The enzymes employed in tissue disaggregation generally belong to one of three groups; those used on fibrous tissue e.g. collagenase, those active on mucopolysaccharides e.g. hyaluronidase and non specific proteases e.g. trypsin and pronase (Morasca & Erba, 1986).

Chelating Agents

Chelating agents such as ethylenediaminetetraacetic acid (EDTA) are commonly used. These agents bind to Ca^{2+} and Mg^{2+} which play an important role in maintaining the integrity of cells and extracellular matrix (Paul, 1970; Kuchler, 1977). The enzyme trypsin is commonly used in combination with chelating agents (Kuchler, 1977; Morasca & Erba, 1986) and a concentration of 0.02% EDTA used for 30-60 seconds is effective in removing fibroblasts which contaminate epithelial cell cultures derived from skin and oral mucosa (Rheinwald, 1980; Parkinson & Yeudall, 1992).

Cell Separation

Different cell types may be separated by centrifugation according to their size, density and shape. Ficoll and bovine serum albumin (BSA) are the most commonly used

density gradient media. Ficoll density gradients have been used for separation of cultured human lymphoid cells and human keratinocytes (Everson, Buell & Rogentine, 1973; Sun & Green, 1976). However, the high concentration of most density gradient media may induce osmotic damage, and the high centrifugal force may also damage the cells.

Different Types of Cell Culture

(1) Monolayer Culture Systems

Monolayer culture systems are flexible and are one of the most widely used techniques for most cells. However, there can be problems in controlling homogenicity during the culturing period and it can be a time consuming technique.

There are a variety of systems employed in scaling-up cells in monolayer cultures. The roller bottle system is the most commonly used for this purpose. Cells are attached to most of the inner surface area of the bottle and are bathed and exposed to medium and gas phase alternately by rolling the bottle (House, Shearer & Maroudas, 1972).

(2) Suspension Culture

Many cells are not anchorage dependent and can be grown in suspension and some cells which are anchorage dependent can be adapted into suspension culture (Freshney, 1987). Cells in suspension culture may adhere to the wall of the flask unless it is rotated (Paul, 1970).

(3) Microcarrier Culture

This system brings together the monolayer and suspension systems. The cells are grown on the surface of small solid particles or beads which are suspended in medium. The culture is often agitated to keep the beads in suspension. Modifications such as collagen coated beads and polystyrene (Freshney, 1987) have improved the microcarrier system and often reduce the toxic effect on the bead material.

(4) Multilayered Cultures (Stratified Cultures)

Many modifications of the monolayer culture have allowed the production of multilayered tissue-like structures. Knazek *et al.* (1972) developed a system where the cells grow on artificial capillary propagators and the cells are supported by a vascular network that delivers nutrients and removes waste products.

Epithelial cells grown on collagen as a gel or raft and raised to a gas-liquid interface will stratify and form a differentiated tissue. Subcultivated oral keratinocytes have been grown on collagen matrices and subsequently cultured at a nutrient/medium gas (liquid-air) interface (Lillie, MacCallum & Jepsen, 1980, 1982, 1988). Lillie *et al.* (1982) also formed a basement membrane on the surface of collagen matrices and cultured oral keratinocytes onto the basement membrane. Cultures grown at liquid-air interface resulted in the formation of a stratified squamous epithelium similar to the native epithelium.

Source and Types of Tissue

Tissue is either obtained from embryos, normal adult tissue or malignant tumours. In general, embryonic tissue survives and grows better than adult tissue, but it differs having fewer specialised cells and a higher proportion of stem cells.

Cells derived from malignant tumours are perhaps the easiest cells to grow. They can be readily and quickly grown in simple media and may also be immortal (Paul, 1970).

Cell lines produced from tumours are continuous while those from normal tissue have a finite life span. However, normal cell lines may sometimes transform to become a continuous cell line after several subcultures.

There is some doubt as to how representative continuous cell lines and tumour cells are of normal tissue. It has been suggested that these cell lines retain their abnormal characteristics in culture and are therefore not ideal for study in some areas of cell biology. Bissel (1981) emphasises the need for employing normal cells and tissues in culture.

Selection of Medium

Introduction

The type and composition of medium are essential to the successful culture of cells and tissues (Paul, 1970). The

choice of medium is dependent on the type of cells to be grown and the culture system employed.

The medium must provide cells with an optimum pH, and osmotic pressure (Paul, 1970). The range for most animal cells is pH 6.8-7.8 and most cell lines grow well at pH 7.4 (Freshney, 1987).

Culture media contain a mixture of low-molecular weight nutrients dissolved in a buffered basal salt solution. There are two categories of media, natural and defined media.

(1) Natural Media

Body fluids such as clotted plasma, serum and different types of tissue and embryonic extracts were originally used as culture medium (Harris & Kutsky, 1954; Sanford *et al.*, 1955). The disadvantage in using natural media is the lack of knowledge of its exact composition and so it is impossible to ensure the maintenance of identical conditions from one experiment to another (Paul, 1970).

(2) Defined Media

Defined media vary in complexity from Eagle's minimum essential medium (MEM) (Eagle, 1959), which contains essential amino acids, vitamins and salts, to complex media such as 199 (Morgan, Morton & Parker, 1950), F12 (Ham, 1965) and RMPI 1640 (Moore, Gerner & Franklin, 1967). The complex media contain a large number of different amino acids and vitamins and may often be

supplemented with extra metabolites and minerals. Other commonly used media are Earle's balanced salt solution (EBSS) (Earle *et al.*, 1943), Hank's balanced salt solution (Hanks & Wallace, 1949), Dulbecco's phosphate-buffered salt solution (Dulbecco & Vogt, 1954) and Dulbecco's modification of Eagle's MEM (DMEM) (Dulbecco & Freeman, 1959).

Modern artificial media can maintain tissues for short periods but most would not grow unless serum or components of serum were added. Serum contains most of the low molecular weight nutrients essential for cellular growth and is rich in fibronectin (cold insoluble globulin) which is essential in cell attachment and aids cell spreading across the substrate (Klebe, 1974; Yamada & Olden, 1978). Serum also contains protein carriers for minerals, fatty acids and hormones (Freshney, 1987). Albumin (Iscove & Melchers, 1978; Barnes & Sato, 1980) and globulin (Tozer & Pirt, 1964) are also important. Another protein, transferrin (Guilbert & Iscove, 1976) binds iron making it available to the cell. Although proteins are the major component of serum the function of many of them *in vitro* are as yet unknown.

Serum also contains growth factors and hormones essential for cellular multiplication. It contains insulin, required for the uptake of glucose and amino acids. Hydrocortisone is also present in varying amounts which can promote cell attachment (Fredin, Seiffert &

Gelehrter, 1979) and cell proliferation (McLean *et al.*, 1986)

Serum-Free Media

The addition of serum to media may create problems, as serum components may affect or mimic the action of agents under study. The study of cellular products released into the medium can prove very difficult in the presence of large amounts of serum protein (Barnes & Sato, 1980).

Since the 1950's attempts have been made to culture cells without serum. NCTC 109 (Evans *et al.*, 1956) and Waymouth MB 705/1 (Waymouth, 1959) are examples of media developed to support monolayer growth and clonal growth of permanent cell lines. Hayashi & Sato (1976); Ham, Hammond & Miller, (1977); Barnes & Sato (1980) and Tsao, Walthall & Ham (1982) have all been able to sustain the growth of cells in media in which serum has been replaced with a mixture of hormones, growth factors, enzyme inhibitors, attachment factors and other supplemental nutrients.

The growth of normal diploid cells in serum free medium is difficult, although it is possible to reduce the serum concentration to 2% or less by supplementing it with defined additives (Freshney, 1987).

Although serum free conditions are desirable, the relative simplicity of retaining serum, the lack of reliable sources of most serum-free medium and the time, effort and resources that must go into preparing recipes

for the different cell types under different conditions often act as deterrents to a serum-free medium.

Temperature

Typically cells from warm blooded animals are grown at 37°C although tissue from skin and testes require lower temperatures, between 31°C-33°C (Sarkany, Grice & Caron, 1965; Jensen & Therkelsen, 1981). Tissue such as oral epithelium is often contaminated by fibroblasts. This contamination is greatly reduced by lowering the incubation temperature to 32°C-33°C (Jensen & Therkelsen, 1981; Jepsen, MacCallum & Lillie, 1980).

Gas Phase

The important constituents of the gas phase are oxygen (O_2) and carbon dioxide (CO_2). The O_2 tension varies mainly between organ and cell cultures. O_2 tension is elevated in organ culture. Late stage embryo, newborn, or adult tissues require up to 95% O_2 in the gas phase (Trowell, 1959; Freshney, 1987). Lower O_2 tensions are preferable for most cell cultures (Balin *et al.*, 1976), and particularly for the growth of established cell lines.

Cells at low concentration in an open vessel and cells at very low concentration in sealed flasks require CO_2 in the gas phase. At high cell concentrations the need for CO_2 is not absolute and where there is a high endogenous CO_2 production it is often necessary to slacken flask caps and allow excess CO_2 to escape (Freshney, 1987).

The gas phase therefore, is dependent on the type of medium, whether the culture vessel is open or sealed and the density of cells in the culture (Freshney, 1987).

Substrate

Artificial polystyrene treated plastic is the most commonly used substrate for a wide variety of tissue types. Glass is another commonly used substrate although cells are usually more prolific on plastic than on glass. Lamey, Marshall & Ferguson (1982) found that outgrowth of salivary epithelial sheets was less on glass when compared to plastic.

Feeder layers such as mouse fibroblasts have been used for many years to enhance cell proliferation particularly at low densities (Puck & Marcus, 1955). The cells of the feeder layer may condition the substrate by cell products and may make the surface suitable for attachment by other cells. 3T3 mouse fibroblasts have contributed significantly to the successful growth of normal epithelial cells (Rheinwald & Green, 1975).

Three dimensional matrices have been employed in tissue culture with a resultant improvement in cell adhesion, cell-cell and cell-matrix interaction. Substances such as cellulose sponge (Leighton, 1951; Leighton, Mark & Justh, 1968) and gelatin film (Smulow, Rustigian & Tye, 1961) have been used with cultures of a finite life span.

Collagen has also been used as a film, gel or raft and has been found to improve cellular attachment, inhibit fibroblast overgrowth, promote epithelial adhesion and enhance basal lamina formation (Liu, Eaton & Karasek, 1979; Lillie *et al.*, 1980; Yang *et al.*, 1981).

Growth of oral keratinocytes on a collagen matrix raised to gas-liquid interface resulted in the formation of a stratified squamous epithelium (Lillie *et al.*, 1982).

1.15 Tissue Culture and Oral Epithelium

1.15.1 Introduction

Many of the methods employed in the culture of epidermal keratinocytes have also been employed in the culture of oral epithelium. Epithelial cells obtained from the outgrowths of explanted human skin and buccal mucosa were found to be histologically indistinguishable (Flaxman, Lutzner & Van Scott, 1967).

1.5.2 Organ Culture

Organ culture was the first method used for long term growth of oral mucosa. The period of experimentation spanned the 1960's and is reviewed by Bergenholtz & Thilander (1970) and Hill & Miles (1976). Oral mucosa has been grown in organ culture with the aid of gelatin films (Smulow, Rustigian & Tye, 1961) and using plasma clot and raft techniques (Banoczy & Torok, 1970). Bergenholtz (1969) and Bergenholtz & Thilander (1970) maintained the keratinised palatal mucosa of the cat for up to 6 days using Eagle's minimum essential medium without serum.

Cell division and terminal differentiation was observed prior to epithelial degeneration. The absence of serum combats the problem of epiboly, a significant problem in cultures of both oral mucosa (Powell, 1967) and skin (Chang & Maiback, 1967). Epiboly involves the epithelium at the cut edges of the tissue growing around and encapsulating the explant. Hill & Miles (1976, 1978) and Hill (1978) modified the method of Bergenholtz (1969) by using a different medium (Waymouth's MB 752/1) supplemented with bovine serum albumin and achieved histodifferentiation in neonatal rat palate for up to 28 days with continued cell division.

Hill & Miles (1978) noted the effect of incubation temperature on the survival of explants, with a 25-30% decrease in explant survival when temperature was reduced from 37 to 34 or 30°C.

1.15.3 Cell Culture

Propagation of most epithelial cell cultures, including oral epithelial cells, beyond the initial primary culture proved to be difficult (Prunieras, Delescluse & Regnier, 1976).

Primary keratinocyte cultures from the oral mucosa fall into two categories, namely primary explant cultures and single cell suspensions obtained from the dissociation of tissues prior to culture (MacCallum *et al.*, 1987).

Subsequent culture from both categories involves disaggregating the cells into a single cell suspension prior to subcultivation. It is not easy to subcultivate oral epithelial cells and a major breakthrough in both the successful primary culture and subsequent successful subcultivation of keratinocytes was made by Rheinwald & Green (1975) with the use of mitotically inhibited feeder cells.

The cell line of 3T3 mouse fibroblasts (Todaro & Green, 1963) supports the growth of keratinocytes when added to the culture (Rheinwald & Green, 1975; Rheinwald, 1980). Human oral keratinocytes (Gusterson & Monaghan, 1979; Taichman, Reilly & Grant, 1979) and rat lingual epithelium (Crane *et al.*, 1986) have been successfully grown using this technique. Following one or two passages on 3T3 cells, oral keratinocytes can be successfully subcultivated onto a conventional plastic culture surface or extracellular matrix. The subcultivated cells stratify and exhibit terminal differentiation. Primary cultures and subcultures shed their terminally differentiated cells into the medium.

The growth media used for the culture of oral epithelial cells are often the same as those used to culture epidermal keratinocytes. Dulbecco's modification of Eagle's MEM (DMEM) is widely used and is often used in combination with Ham's F12 medium (MacCallum *et al.*, 1987). 20% Foetal calf serum is often used with rat oral

epithelium while 10% is used for human oral epithelial cell culture (MacCallum *et al.*, 1987).

In addition to growth media, serum, antibiotics and supplements such as hydrocortisone, triiodothyronine, epidermal growth factor, insulin and cholera toxin have been used in small amounts in the successful culture of keratinocytes (MacCallum *et al.*, 1987). These supplements and factors have been found to increase cell proliferation, cloning efficiency and the life span of subcultures. However, the precise mode of action of the supplements on keratinocytes is not completely understood.

Oral mucosa cultured from rodents grows best at 32°C as opposed to 37°C (Jepsen, 1974) while the optimal incubation temperature for human oral keratinocytes is 34°C (Arenholt-Bindslev *et al.*, 1987).

1.15.4 Oral Epithelium Cultured on Defined Matrices

It is believed that differentiation and growth of epithelial cells is strongly influenced by, if not dependent upon, factors derived from connective tissue. Subcultivated oral keratinocytes on matrices composed of various interstitial collagens and subsequently cultured at a nutrient/medium gas (liquid-air) interface results in the formation of a stratified squamous epithelium (Lillie *et al.*, 1980, 1982, 1988). Lillie *et al.* (1982) formed a basement membrane on the surface of the collagen matrices. When keratinocytes are cultured using this

technique the epithelium is similar to that observed *in vivo* and degeneration is rare since viable cells have access to the nutrient medium. Similar histodifferentiation was observed by MacKenzie & Fusenig (1983) using murine oral keratinocytes.

Artificial substrata (nylon membranes) can also be employed to enhance the differentiation of stratified squamous epithelium when the culture is grown at a liquid-air interface (MacCallum *et al.*, 1987).

1.16 Aims and Scope of Study

Despite the strong links between alcohol and oral cancer (section 1.8) there are relatively few studies on the effects of ethanol alone on the oral epithelium. The majority of these studies have demonstrated histological changes as a consequence of chronic alcohol intake.

It is reasonable to assume that alcohol related alterations will be mediated or accompanied by alterations in the epithelial protein pattern since cells express their individuality through the proteins manufactured. Graham & Rennie (1987) carried out a biochemical investigation into the effects of chronic alcohol exposure on the proteins of rat lingual epithelium. A matched-pair feeding technique was used and after 102 days of alcohol intake SDS-PAGE analysis revealed alterations in the levels of three proteins.

Animal models, particularly those involving the use of matched-pair feeding techniques, are time consuming, expensive and involve many practical difficulties. With this in view it was the aim of the work presented in this thesis to set up an *in vitro* model for the study of alcohol effects on the oral epithelium. Rat lingual epithelium was used to provide a fair comparison with the *in vivo* work described above.

The setting up of the *in vitro* alcohol model is described in chapter two. Having achieved this the effect of ethanol on the protein profile of the cells in culture was investigated using electrophoretic techniques. The techniques involved and the results obtained are described in chapter three.

The work presented in chapter three led to the question of whether ethanol or metabolites of ethanol were responsible for the observed changes and a study into the effects of acetaldehyde on the cells grown in culture was carried out. This study is presented in chapter four.

The results of chapter four suggested that a further study involving another stress treatment other than ethanol/acetaldehyde might be worthwhile. This would determine whether any changes observed were specifically related to ethanol/acetaldehyde or simply to a general stress response. The results of this study are presented in chapter five.

In order to gain some knowledge of the cellular location of epithelial proteins, which showed altered levels in the above mentioned treatments simple subcellular localisation studies were carried out and are presented in chapter six. Finally, chapter seven contains a concluding discussion and suggestions for future work.

Chapter 2

Preliminary Studies on Alcohol Utilisation and Tissue Culture Techniques

2.1 Introduction

In vitro methods are now widely used for toxicity testing. One of the major benefits of these techniques is the elimination of the complex systemic interactions which make interpretation of results difficult in animal studies. There are several methods available for the *in vitro* growth of oral epithelium (reviewed by MacCallum *et al.*, 1987). Two of the most widely used systems are the culture of epithelial cells on plastic surfaces and the culture of epithelial cells in the presence of inhibited 3T3 feeder cells.

The aim of the work reported in this chapter was to produce sufficient viable oral epithelial cells for the study of the *in vitro* effects of alcohol on the oral epithelium and to set up a model for the study of alcohol effects on the epithelial cells *in vitro*. The words alcohol and ethanol are synonymous throughout the following chapters.

2.2 Materials and Methods

2.2.1 Choice of Animal and Selection of Tissue

Previous *in vivo* and *in vitro* studies of the effect of alcohol on the oral epithelium in this department used

the lingual epithelium of Sprague-Dawley rats. Graham & Rennie (1987) examined the effect of alcohol on the protein profile of lingual epithelium using the DeCarli & Lieber (1967) matched pair feeding technique to induce alcoholism in Sprague-Dawley rats and Siyam (1987) examined the effect of alcohol on the protein profile of lingual epithelium *in vitro* with epithelial cell cultures derived from Sprague-Dawley rats. The ventral tongue of these animals is a suitable source of epithelium as the rats are readily available, relatively inexpensive and the tongue provides a plentiful supply of oral epithelium. For these reasons and for the purpose of comparing the results of this investigation with those of the previous studies the lingual epithelium of Sprague-Dawley rats was chosen for the work reported in this thesis. The ventral surface of the lingual epithelium was again chosen as it has a relatively simple structure, lacking papillae and having a comparatively uniform anatomical form.

2.2.2 Initial Attempts to Culture Oral Epithelium using a Plastic Substrate

Four young adult male Sprague-Dawley rats were killed by cervical dislocation and the tongues were excised and washed three times by gentle shaking in a universal container containing transport medium (Table 2.1). The wash was repeated three times to remove all hair, debris and blood. The tissue was then transported to the

Table 2.1 Transport medium for rat tongues.

Dulbecco's Modified Eagle Medium (DMEM)$_1$

20% Foetal Calf Serum$_2$

L-Glutamine (200mM)$_1$

Gentamicin (10mg/ml)$_1$

Penicillin/Streptomycin (10000Iu/ml/10000/10000µg/ml)$_1$

Fungizone (Amphotericin B) (250µg/ml)$_1$

1 Gibco
2 Sigma

Table 2.2 Culture medium for epithelial cells.

3 parts Dulbecco's Modified Eagle Medium DMEM$_1$

1 part Ham's F12 (with Glutamax)$_1$

20% Foetal Calf Serum$_2$

1% Gentamicin (10mg/ml)$_1$

1% Penicillin/Streptomycin (10000Iu/ml/10000µg/ml)$_1$

1% Fungizone (Amphotericin B) (250µg/ml)$_1$

Cholera Toxin (8.4ng/ml)$_3$

Epidermal Growth Factor (10.0ng/ml)$_3$

Hydrocortisone (0.4µg/ml)$_3$

Insulin (5.0µg/ml)$_3$

1 Gibco
2 Globepharm
3 Sigma

laboratory in a universal container with chilled fresh transport medium. This part of the procedure was carried out within minutes of killing the animals.

The ventral surface of the tongue was dissected into strips and then into small blocks (1-3mm in diameter). Most of the connective tissue was removed with a sharp scalpel and the remainder of the tongue was discarded.

To completely remove the epithelium from the underlying connective tissue the blocks of tissue were placed in a solution of the enzyme Dispase (Neutral Protease II, Sigma) at a concentration of 0.5mg/ml in phosphate buffered saline (PBS) overnight at 4°C. This enzyme cleaves the basement membrane (Stenn *et al.*, 1989) and allows the epithelium to be separated from the connective tissue with very low fibroblast contamination. The small epithelial sheets were then placed in 0.25% trypsin (porcine pancreas, Sigma) and finely minced with a scalpel. The disaggregated epithelium was left in the trypsin for five minutes and then repeatedly aspirated gently using a pasteur pipette. The cell suspension was centrifuged at 1000rpm for five minutes and resuspended in complete culture medium (Table 2.2). To ensure complete removal of the trypsin this procedure was repeated. The cells were counted using a haemocytometer and seeded at a density of $5X10^5$ cells per $25cm^2$ culture flask, with 10ml of complete growth medium. The flasks

had vented screw cap lids and were incubated at 37°C and 32°C with a gas phase of 5% CO_2 and 95% air in a humidified incubator. The growth medium was replaced three times per week with freshly prepared medium. The epithelial cells reached confluence in 10 days and were then ready for subculture.

Subculture

To remove any contaminating fibroblasts confluent cultures were washed with PBS (Ca^{2+} and Mg^{2+} free) twice and 0.02% ethylenediaminetetraacetic acid (EDTA) pH 8.6 was added and left for 1-3 minutes at 37°C and then vigorously pipetted against the epithelial cells. The intact epithelial sheets were then washed with PBS and 0.25% trypsin/0.02% EDTA (1ml per 25cm^2 flask) added and the flasks incubated at 37°C for 5-10 minutes. Following this incubation any remaining epithelial cells were mechanically removed by pipetting the fluid against the flask surface. The action of trypsin was inhibited with 1ml of foetal calf serum. The cell suspension was then centrifuged for five minutes at 1000rpm and the cells resuspended in growth medium containing serum. This was repeated and the cells suspended in complete growth medium for epithelial cells (Table 2.2). Cells were counted using a haemocytometer and seeded at a density of $5X10^5$ per 25cm^2 culture flask with 10mls of growth medium. The flasks were incubated at 37°C or 32°C with a gas phase

of 5% CO_2 and 95% air, in a humidified incubator. The growth medium was changed three times in a week.

2.2.3 Initial Attempts to Culture Oral Epithelium using a Feeder Layer

Preparation of Feeder Layer

The most commonly used feeder layer is a mouse 3T3 fibroblast feeder layer (Rheinwald & Green, 1975; Rheinwald, 1980). To prevent fibroblast overgrowth these are mitotically inhibited by a sublethal dose of irradiation or by mitomycin-C, an inhibitor of DNA synthesis and nuclear division (Rheinwald & Green, 1975; Taichman et al., 1979).

Mouse fibroblast 3T3 cells were obtained from Professor D. MacCallum, Department of Anatomy and Cell Biology, University of Michigan, USA and grown in 80cm^2 culture flasks with a recommended growth medium (Table 2.3). The medium was changed three times in a week. 3T3 cells are less effective as a feeder layer if allowed to become hyperconfluent before subculture and so they were subcultured when 80-90% confluent. This state of confluence was generally attained in six days.

To subculture the cells, the medium was decanted and the cells washed twice with PBS (Ca^{2+} and Mg^{2+} free). 10ml of trypsin (0.25%) were added and the flasks were incubated at 37°C in a humidified incubator with a gas phase of 5%

Table 2.3 3T3 cell culture medium.

Dulbecco's Modified Eagle Medium[1]

10% Newborn Calf Serum[3]

1% Gentamicin (10mg/ml)[1]

1 Gibco
3 Sigma

CO_2 and 95% air for three minutes. The cells which remained attached to the flask were mechanically removed by pipetting the fluid against the flask. The action of trypsin was inhibited with 1ml of newborn calf serum and the cell suspension centrifuged at 1800 rpm for five minutes. Cells were resuspended in medium containing 10% newborn calf serum (Table 2.3). The cells from one $80cm^2$ flask were reseeded into ten $80cm^2$ flasks and incubated at 37°C in a humidified incubator with a gas phase of 5% CO_2 and 95% air.

Mitotic Inhibition of 3T3 Fibroblasts with Mitomycin-C

3T3 cells were inhibited when 80-90% confluent. The medium was decanted and the cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free). To find a suitable concentration of inhibitor 20mls of 4µg/ml, 8µg/ml and 12µg/ml mitomycin-C (Sigma) in Earle's Balanced Salt Solution (EBSS) were added to the cells (three flasks for each concentration) and incubated in the dark at 37°C for three hours. Flasks were wrapped in foil to keep as much light out as possible since mitomycin-C is very light sensitive.

After three hours incubation some of the cells detached and were washed off using PBS. The remaining cells were detached by incubating them in trypsin (0.25%) for three minutes at 37°C. The cell suspension was centrifuged for five minutes and the cells resuspended in fresh complete

medium for epithelial cells (Table 2.2). This washing was repeated twice to ensure complete removal of the mitomycin-C. The cells were then resuspended in complete medium for epithelial cells and at this stage the cells were ready for seeding with epithelial cells either immediately or after 24 hours incubation.

Storage of 3T3 Fibroblasts

To store 3T3 cells for future use 80-90% confluent cultures were washed twice with PBS (Ca^{2+} and Mg^{2+} free) and the cells detached by trypsinisation as described earlier in this section. The cell suspension was centrifuged at 1800rpm for five minutes, the cells resuspended in 1ml of complete culture medium for fibroblasts (Table 2.3) and a cell count carried out using a haemocytometer.

Following counting the cells were centrifuged for five minutes at 1800rpm and the cells resuspended in 10% dimethyl sulfoxide (DMSO) in culture medium. 1ml of 10% DMSO containing at least $5X10^6$ cells was placed in a cryo-container and frozen at -70°C in a polystyrene box insulated with cotton wool. The frozen cells were stored on a short term basis for up to three months in a -70°C freezer and on a long term basis in liquid nitrogen. To reconstitute the cells the cryo-containers were brought to room temperature and the contents added to an $80cm^2$ culture flask. Complete medium for fibroblasts was added

slowly to the flask and the cells were incubated at 37°C in 5% CO_2 and 95% air until 80-90% confluent. Confluent cultures were then subcultured or inhibited as described above.

Culture of Epithelial Cells using a 3T3 Feeder Layer

Rat tongues were prepared as described in section 2.2.2 and the epithelial cells ($5X10^5$ per $25cm^2$ flask) were added to the flasks containing $2X10^5$ (per $25cm^2$ flask) mitomycin-C treated 3T3 cells in complete growth medium for epithelial cells (Table 2.2). The flasks were incubated at 37°C and 32°C in a gas phase of 5% CO_2 and 95% air in a humidified incubator. This procedure was repeated three times for each concentration of mitomycin-C used.

The medium was changed three times a week and the 3T3 feeder layer was detached and removed once a week and replaced with fresh 3T3 cells. The 3T3 feeder layer was detached by removing the medium, washing the cells twice with PBS (Ca^{2+} and Mg^{2+} free) and adding 0.02% EDTA. The flasks were incubated for three minutes at 37^0C after which time any remaining attached fibroblasts were mechanically dislodged by pipetting the fluid against the flask surface. The epithelial cells were then washed with PBS (Ca^{2+} and Mg^{2+} free) twice and the new medium with freshly prepared 3T3 fibroblasts added. The epithelial

cells reached confluence in 7-10 days and were then ready for subculture.

Subculture of Epithelial Cells

The fibroblast 3T3 cells and any contaminating epithelial derived fibroblasts were removed as described above and the epithelial cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free). 1ml of trypsin (0.25%)/EDTA (0.02%) was then added and the flasks incubated for 5-10 minutes at 37°C until the epithelial cells detached. The action of trypsin was inhibited with 1ml of foetal calf serum and the cell suspension centrifuged for five minutes at 1000rpm. This was repeated and the cells suspended in complete medium for epithelial cells (Table 2.2).

The epithelial cells were counted using a haemocytometer counting chamber and $3\text{-}5X10^5$ epithelial cells were added to $25cm^2$ flasks containing $2X10^5$ mitomycin-C treated 3T3 cells. The cultures were incubated at 37°C or 32°C in 5% CO_2 and 95% air, in a humidified incubator. The medium was changed three times a week. The feeder layer was removed and replaced with fresh 3T3 cells once a week. The epithelial cells reached confluence in 10 days and were then ready for subculture to the second passage.

2.2.4 Culture and Subculture with a Feeder Layer Followed by Subculture on Plastic Alone

Jepsen *et al.* (1980) found that rat oral epithelium could be successfully subcultivated on plastic alone following one or two passages on 3T3 cells.

Cells were cultured with 3T3 cells as previously described in section 2.2.3. They were then subcultured twice onto 3T3 cells (also described). All subsequent passages were onto plastic substrate alone (section 2.2.2). Cells were incubated and the medium changed as described in sections 2.2.2 and 2.2.3.

2.2.5 Technique to Prevent and Control Microbial Contamination

Microbial contamination is a problem in tissue culture and there are a number of ways to reduce and prevent contamination. After excision the tongues were washed in three changes of transport medium containing antibiotics (Table 2.1) to remove the hair, debris and blood and were then kept in fresh transport medium until they were prepared for culture.

The caps of tissue culture flasks are often wet by medium which dries and is a potential area for microbial contamination. To reduce the risk of contamination each cap was cleaned with 70% ethanol, and the alcohol removed with sterile water.

All cultures were fed with growth medium containing 1ml gentamicin (10mg/ml; Gibco), 1ml penicillin/streptomycin (10000Iu/ml and 10000μg/ml respectively; Gibco) and 1ml fungizone (250μg/ml; Gibco) per 100mls of medium. This antimicrobial cocktail largely irradicated microbial contamination.

All incubators were sterilised and all handling of cultures was carried out in clean air cabinets with bunsen flames on. Cabinets were swabbed with 70% alcohol before and after use.

2.2.6 Cell Examination and Identification

Epithelial cells were observed using inverted phase microscopy. Epithelial cells and fibroblasts (3T3 cells and normal rat fibroblasts) were initially identified according to morphological and histological criteria. However, this method of identification is limited, and the epithelial nature of the cells in culture was confirmed using monoclonal antibodies directed against keratin proteins.

Immunofluorescent Staining

Epithelial cells were grown on glass coverslips in petri dishes in the presence of 3T3 feeder cells. The coverslips were removed from the petri dishes between 5-7 days after seeding, placed in a coplin dish, rinsed

three times with PBS and fixed for ten minutes at -20°C in acetone.

The fixed cells were washed three times in PBS (three minutes each wash) and rehydrated in PBS for ten minutes. The coverslips were then drained but not dried, and placed inside a moist chamber. Mouse anti-cytokeratin antibody (Sigma) was placed on the coverslips at a concentration of 1:100. The coverslips were incubated for 60 minutes at 37°C in a humidified chamber and then rinsed thoroughly with PBS (at least 4 washes) before the second antibody was added. Prior to the addition of the second antibody the cells were incubated in rabbit serum for 30 minutes to prevent any cross reaction. Rabbit anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate isomer 1 (Dakopatts) was applied at a dilution of 1:10 for 60 minutes at 37°C. After incubation the coverslips were rinsed four times in PBS and mounted in 10% glycerol buffer. The cells were viewed using a Nikon fluorescence microscope. Fixation alone, non-immune mouse serum applied as the first antibody and second antibody alone were used as controls.

2.2.7 Addition and Measurement of Alcohol in Cultures

Once epithelial cell cultures were established alcohol was given to the cells as part of the culture medium. Before investigating the effect of alcohol on the cells it was necessary to establish whether or not the

epithelial cells utilised the alcohol or whether it was lost by evaporation.

To measure the concentration of alcohol in culture medium an alcohol (ethanol) detection kit (Sigma), which is designed to quantify blood, plasma, serum and urine alcohol levels, was employed. Oral epithelial cells were grown with a 3T3 feeder layer and subcultured onto plastic alone ($25cm^2$ culture flasks). Cells in the third passage on plastic were supplied with full growth medium (Table 2.2) containing 0.5% ethanol. The alcohol in the culture medium was measured before being added to cells and at 24 and 48 hours following addition to cultures. Measurements were also made at the same time periods for control flasks with no cells. There were five cell cultures and five controls for each time point.

At each of the three time points 10µl of the culture fluid were added to a vial containing nicotinamide dinucleotide (NAD), alcohol dehydrogenase (ADH) and buffer salts and incubated for ten minutes. The absorbance was then read on a spectrophotometer at 340nm and the concentration of alcohol either calculated against a standard or read from a standard curve. The principle of the assay is that ADH catalyses the oxidation of alcohol to acetaldehyde with the simultaneous reduction of NAD to NADH (Lundquist, 1957). The consequent increase in absorbance at 340nm is

directly proportional to the alcohol concentration in the sample.

2.2.8 The Effect of a Range of Ethanol Concentrations on Epithelial Cell Viability

As a preliminary study the effect of a range of ethanol concentrations on epithelial cell viability was investigated. Viability was assessed using the fluorescent dye, fluorescein diacetate (Mishell & Shiigi, 1980). Oral epithelial cells were grown with a feeder layer and subcultured onto plastic alone and cells in the third passage on plastic were seeded onto glass coverslips. This was done to facilitate the use of a fluorescent agent as some plastic dishes autofluoresce. After five days in culture ethanol was added to the culture medium to give a range of concentrations and the cells were incubated for varying periods. In a previous *in vitro* study 5% ethanol was added to cultures of rat tongue epithelium for one hour (Siyam, 1987). However, 0.4% (400mg/dL) ethanol is the highest recorded human blood alcohol level and at levels of 0.35% (350mg/dL) and above most individuals are inebriated (American Medical Association, 1968). It would be more realistic to have the medium surrounding the cells at concentrations closer to those found *in vivo*. Initially a range of concentrations were added to cells in culture. 5% ethanol was chosen as the upper limit and concentrations of 4, 3, 2, 1, 0.75, 0.5 and 0.25% ethanol were employed. Control

cultures were given growth medium without alcohol. The time periods selected were 1, 7, 14, 21 and 30 days after the addition of alcohol. Five cultures were set up for each concentration at each time point. The medium was removed from the cultures and coverslips were rinsed three times with PBS. A 5mg/ml solution of fluorescein diacetate in acetone, diluted to 1:500 with PBS, was added to culture dishes completely covering the coverslips. The cultures were incubated at room temperature for 15 minutes and washed at least four times with PBS. Coverslips were mounted in glycerol buffer and examined under a Nikon fluorescence microscope.

Live cells take up the fluorescein diacetate agent and convert it to its fluorescent form. The percentage of viable cells on each coverslip can then be calculated.

To determine if viabilities in ethanol treated cultures were significantly different from those in control cultures a covariance analysis on regression coefficients was carried out (Minitab).

2.3 Results

2.3.1 Growth on a Plastic Surface

Epithelial cells grew well on plastic substrate. The cells were granular, flatly packed squamous like structures with large nuclei containing several nucleoli

(Fig. 2.1.a-c). Multilayers formed in areas as cultures became confluent. Confluence was reached at 10 days.

Subcultured epithelial cells also grew well on plastic, reaching confluence between 12-15 days. Cells grown at 37°C became difficult to maintain in culture after the second or third passage. Reducing the incubation temperature to 32°C increased the life of the culture, allowing cells to be passaged up to six times before cells became senescent and difficult to maintain in culture.

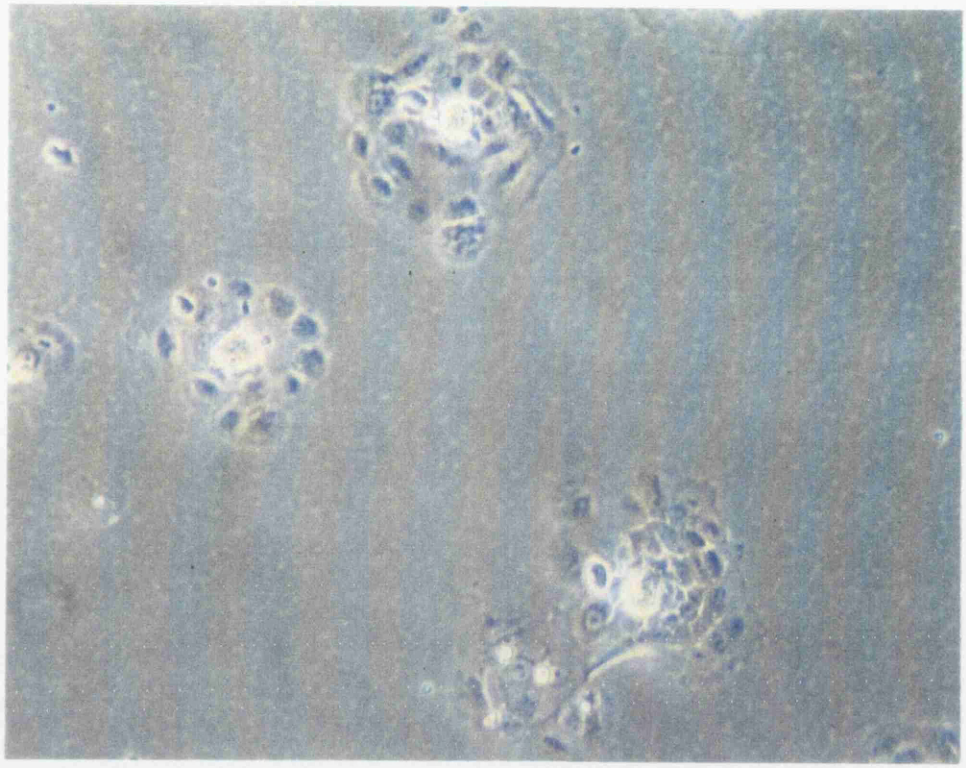
The use of the enzyme Dispase (Sigma) to remove the epithelium from the connective tissue ensured very little fibroblast contamination and by growing the cells at 32°C any rat fibroblast growth was stopped.

2.3.2 Growth with a 3T3 Feeder Layer

Epithelial cells cultured with mitotically inhibited 3T3 fibroblasts grew well. The only difference from plastic substrate alone was that cultures proliferated rapidly coming to confluence in 7-10 days as compared with 10 days on plastic alone.

From the three concentrations of mitomycin-C 8μg/ml applied for three hours was found to be the optimum concentration to inhibit the growth of the 3T3 cells.

(a)



(b)

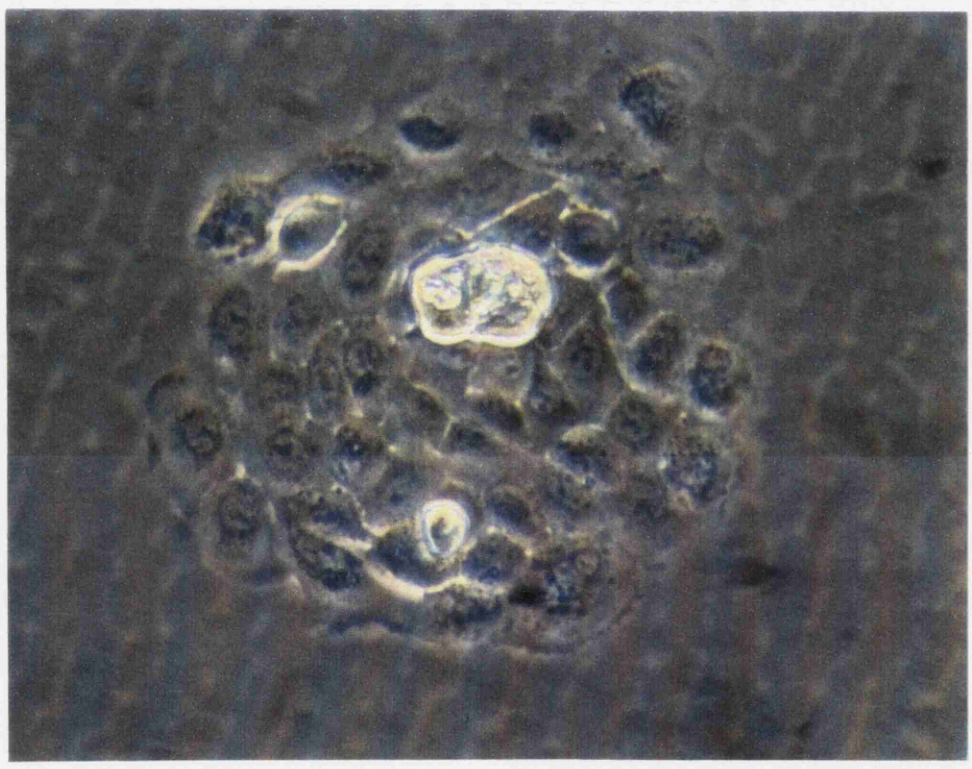


Figure 2.1 (a-c) Phase contrast micrographs of rat lingual epithelial cells grown on a plastic surface. Figure (a) shows epithelial cell colonies forming 3-4 days after cultures were set up (mag. X 100). Figure (b) shows a high power view of a cell colony at 3-4 days (mag. X 400).

(c)

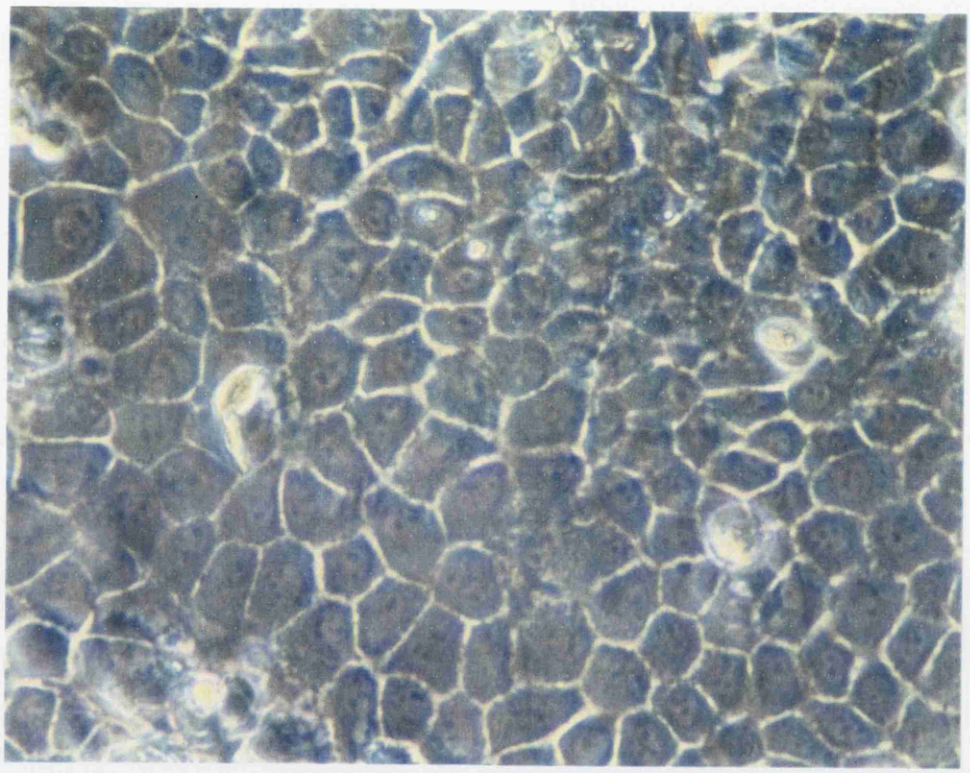


Figure 2.1 (a-c) Phase contrast micrographs of rat lingual epithelial cells grown on a plastic surface. Figure (c) shows a confluent culture after 10 days (mag. X 200).

4μg/ml mitomycin-C applied for three hours did not inhibit 3T3 cells in every case.

Subcultured epithelial cells also grew well supported by a 3T3 feeder layer. Within three or four days following the passage, small colonies of epithelial cells became apparent between the 3T3 cells. As the colonies continued to grow and expand they pushed the 3T3 cells aside (Fig. 2.2). Confluence was reached at 10 days. Cells stratified in areas as cultures reached confluence.

Subculture on 3T3 produced longer lived cells, than on plastic alone, which could be passaged up to 14 times when grown at 32°C. Cells were shorter lived at 37°C and were difficult to maintain after 10 passages.

Rat fibroblast contamination was eliminated when cells were grown with a 3T3 feeder layer at 37 or 32°C.

2.3.3 Culture and Subculture with 3T3 Cells Followed by Subculture on Plastic Alone

As Jepsen *et al.*(1980) found the cells could be grown and successfully subcultured up to 12-14 times on plastic alone following one or two passages with 3T3 cells.

2.3.4 Identification of the Cells

Following immunofluorescent staining the cells stained very positively with the anti-cytokeratin antibody

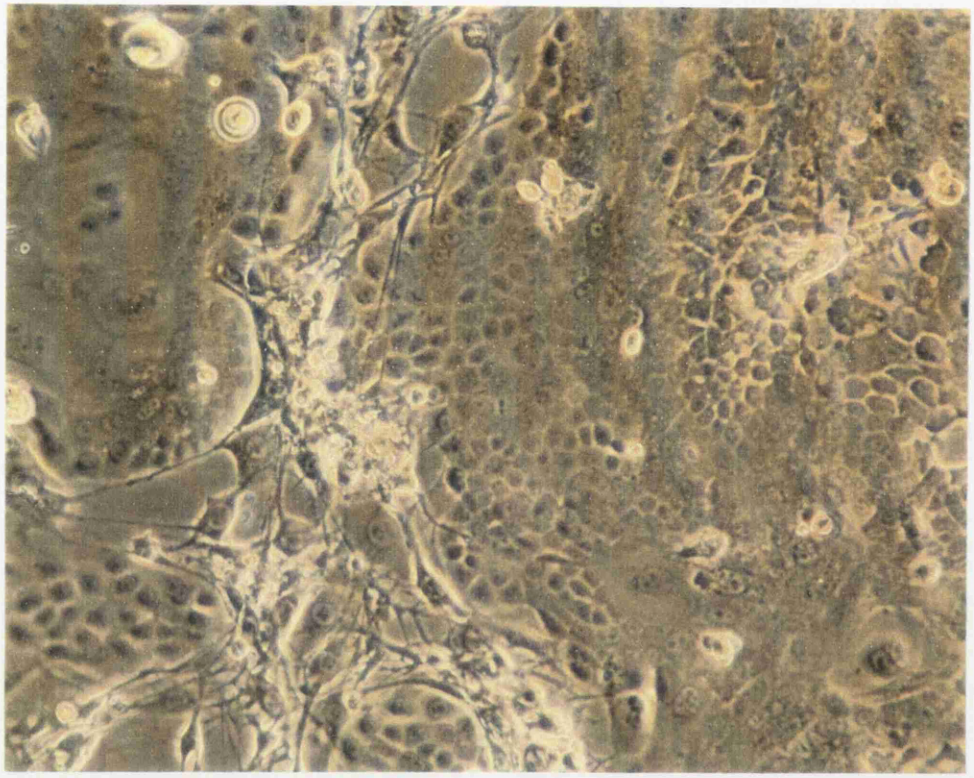


Figure 2.2 Phase contrast micrograph of rat lingual epithelial cells grown for 7 days with a 3T3 feeder layer. The epithelial cells are shown pushing the 3T3 cells aside as the culture approaches confluence (mag. X 100).

confirming their epithelial origin (Fig. 2.3). The entire epithelial colony stained strongly, while the 3T3 feeder layer showed no positive reaction.

2.3.5 Measurement of Alcohol in the Culture Medium

Using the ethanol detection method described in section 2.2.7 it was shown that after 24 hours incubation of cell cultures given 0.5% alcohol there was a 24% decrease in the alcohol concentration, leaving the concentration at 0.38% (n=5, s.e.=0.008). Since five flasks were used at each time point, for the purposes of standard errors n=5. At 48 hours there was a 52% decrease, giving a concentration of 0.24% (s.e.=0.01). In control flasks 14% of the added ethanol was lost after 24 hours, leaving 0.43% (s.e.=0.003) and 36% was lost after 48 hours, resulting in a concentration of 0.32% (s.e.=0.01) (Fig. 2.4).

Using a two-sample t-test (Minitab) the 10% difference at 24 hours and 15% difference at 48 hours between the cells in culture and controls was found to be statistically significant ($p<0.001$).

2.3.6 The Effect Of Varying Ethanol Concentrations On Epithelial Cell Viability

Five cultures were used at each time point so that n=5 for the standard errors. At a concentration of 5% ethanol over 90% of the cells were dead after 24 hours (Fig.

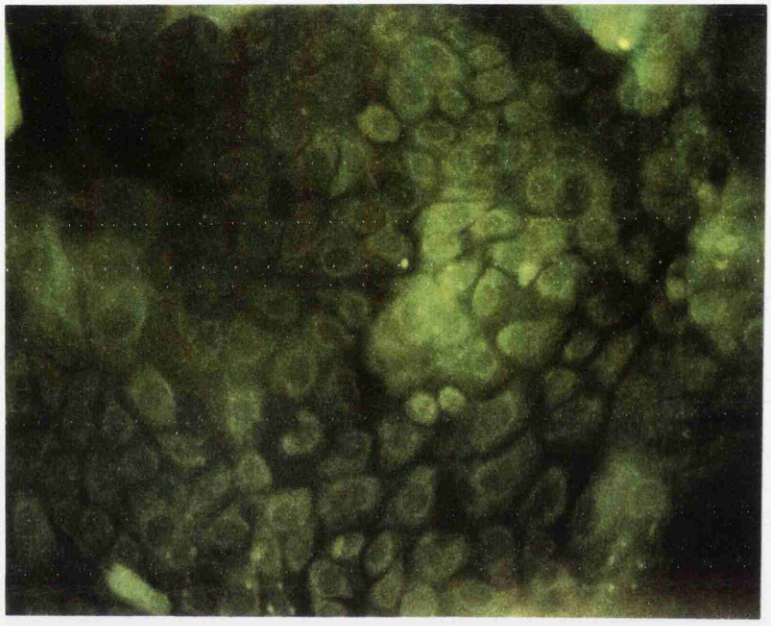


Figure 2.3 Immunofluorescent staining of rat tongue epithelial cells at 7 days with an anti-cytokeratin antibody. The secondary antibody was conjugated to fluorescein. (mag. X 100)

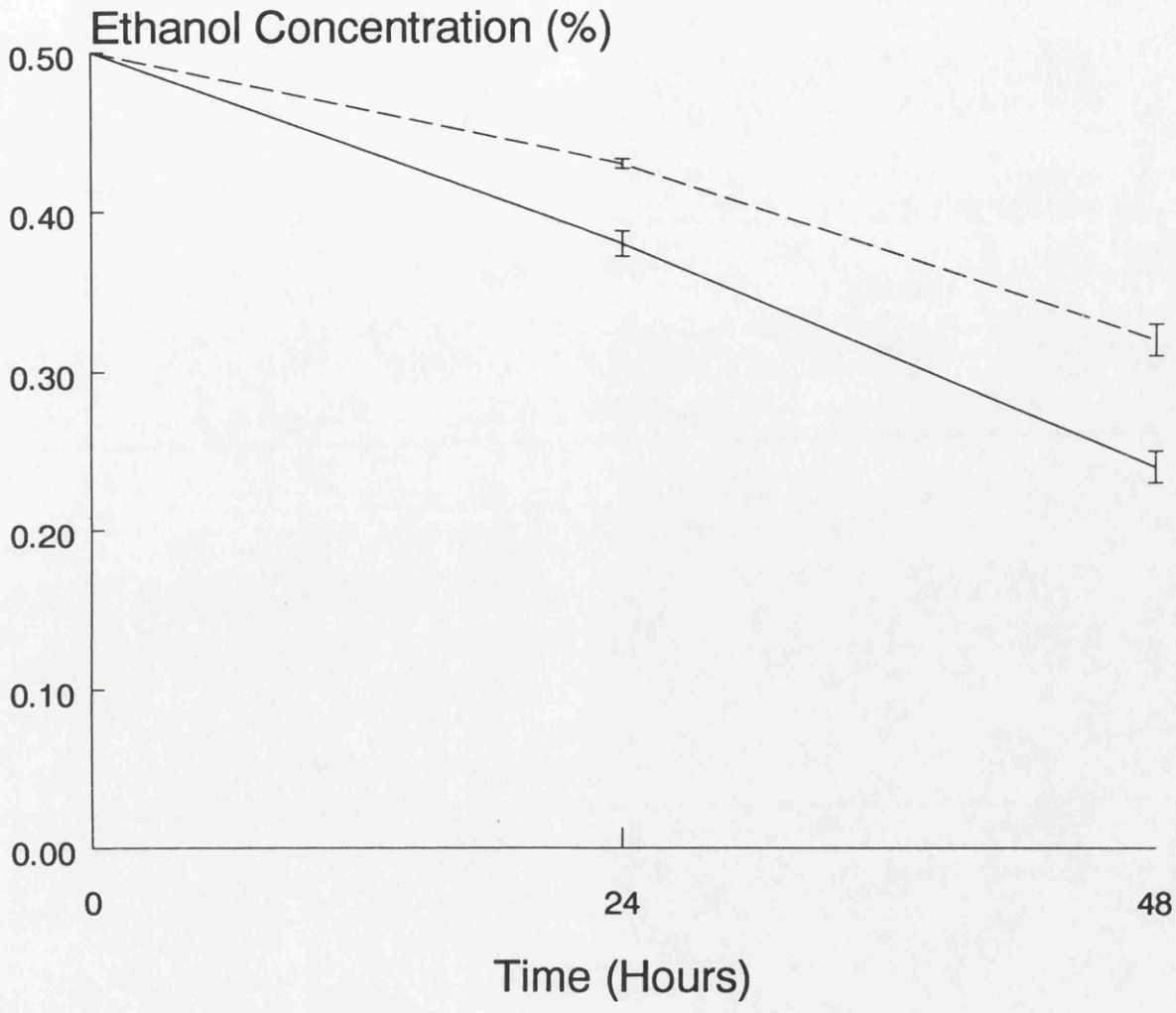


Figure 2.4 Shows the loss of ethanol (%) from the culture medium of cell cultures and control flasks with no cells over a 48 hour period. Each point represents the mean and standard error of five measurements. ---- Control flasks ——— Cells in culture

2.5.a). In 4% ethanol 80% (s.e.=2.50) of the cells were dead by 24 hours. At seven days only 4% (s.e.=0.49) of the cells remained viable and there were no cells surviving by 14 days (Fig. 2.5.b). In 3% ethanol there was a similar pattern of decrease in cell viability, with slightly higher percentages of surviving cells at each time point. After 24 hours 26% (s.e.=4.81) of the cells remained viable, after seven days 5% (s.e.=0.55) were viable and there were none surviving after 14 days (2.5.c). Cells given 2% ethanol showed a different pattern of decrease with 70% (s.e.=2.13) of the cells remaining viable after 24 hours. However, after seven days viability decreased to 4% (s.e.=0.55) and again there were no viable cells after 14 days (Fig. 2.5.d). In 1% ethanol there was a similar pattern of decrease in viability with 73% (s.e.=1.72) of cells viable after 24 hours, 15% (s.e.=3.87) after seven days and 100% cell mortality by 14 days (Fig. 2.5.e).

As alcohol concentrations approached those found *in vivo* cell survival was higher. In 0.75% ethanol 93% (s.e.=1.72) of the cells were viable after 24 hours, and at seven days 90% (s.e.=1.03) were still living. This dropped to 55% (s.e.=1.77) by 14 days, 48% (s.e.=0.86) by 21 days, and by 30 days 40% (s.e.=7.06) of the cells remained viable (Fig. 2.5.f). A covariance analysis on regression coefficients indicated clearly a significant difference in cell viability in ethanol concentrations

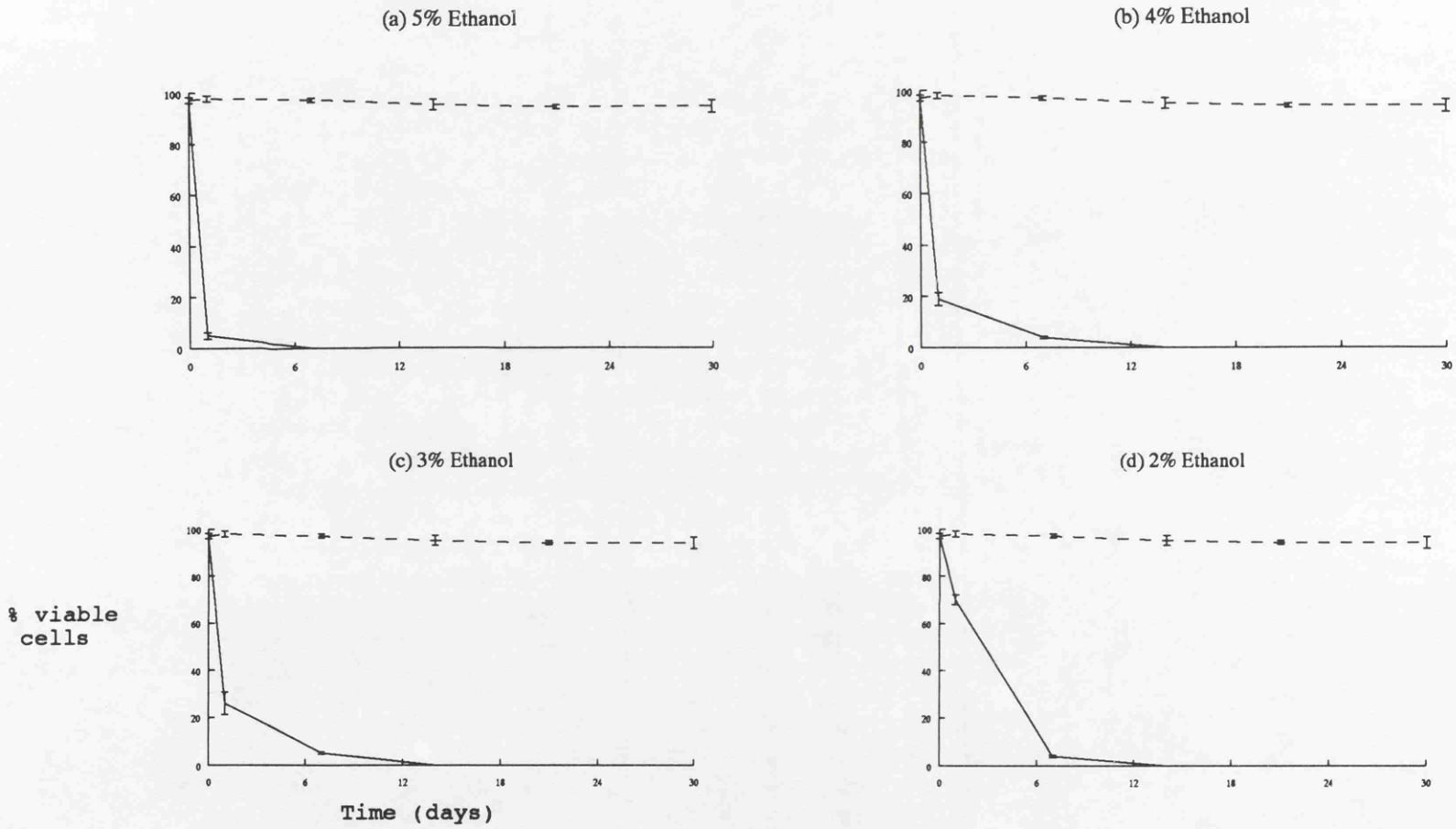


Figure 2.5 (a-d) Shows the effect of a range of ethanol concentrations (%) on epithelial cell viability (%) over a 30 day period. Each point represents the mean and standard error of five measurements. ---- represents 0% ethanol ——— represents ethanol concentration where graph (a) shows 5% ethanol, (b) shows 4% ethanol, (c) shows 3% ethanol and (d) shows 2% ethanol. The x-axis and y-axis legends for all the graphs are identical.

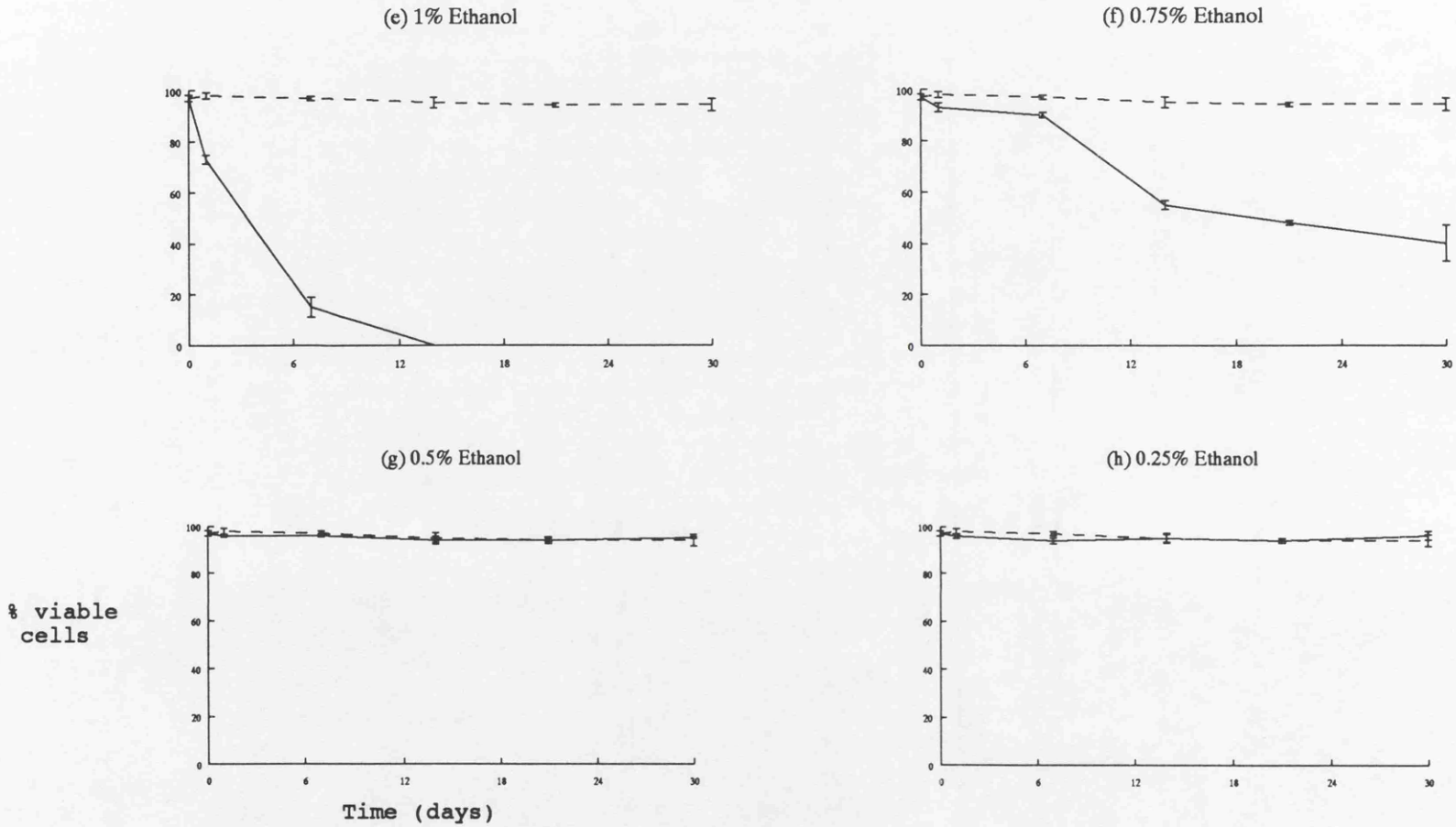


Figure 2.5 (e-h) Shows the effect of a range of ethanol concentrations (%) on epithelial cell viability (%) over a 30 day period. Each point represents the mean and standard error of five measurements. ----represents 0% ethanol ————represents ethanol concentration where graph (e) shows 1% ethanol, (f) shows 0.75% ethanol, (g) shows 0.5% ethanol and (h) shows 0.25% ethanol. The x-axis and y-axis legends for all the graphs are identical.

5 - 0.75% from that of control cultures (F values: 5% F=58.14, 4% F=78.06, 3% F=89.08, 2% F=211.15, 1% F=320.75, 0.75% F=107.72). All were significant at $p<0.001$.

In 0.5% ethanol epithelial cell viability remained just below control values throughout the 30 days (Fig. 2.5.g). After 24 hours in ethanol 96% (s.e.=0.54) of the cells remained viable as compared with 98% (s.e.=1.20) in controls. At 7, 14, 21 and 30 days 96% (s.e.=0.66), 94% (s.e.=1.76) 94% (s.e.=1.56) and 94% (s.e.=0.84), respectively, remained viable, as compared with 97% (s.e.=0.80), 95% (s.e.=2.14), 95% (s.e.=0.37) and 94% (s.e.=2.47) in control cultures. In 0.25% ethanol values were again similar to control values throughout the study and were slightly higher at 21 and 30 days (Fig. 2.5.h). At 1, 7, 14, 21 and 30 days 96% (s.e.=1.03), 94% (s.e.=1.22), 95% (s.e.=1.61), 94% (s.e.=1.02) and 96% (s.e.=1.94), respectively, remained viable, as compared with 98% (s.e.=1.20), 97% (s.e.=0.80), 95% (s.e.=2.14), 94% (s.e.=0.84) and 94% (s.e.=2.47) in control cultures.

A covariance analysis on regression coefficients showed that there was no significant difference in the viability of cells in 0.5 and 0.25% ethanol from that of controls (F values: 0.5% F=1.92, 0.25% F= 1.53).

2.4 Discussion

2.4.1 Epithelial Cell Culture

The use of mitotically inhibited feeder cells (Rheinwald & Green, 1975; Rheinwald, 1980) greatly facilitated subculture of the rat tongue keratinocytes. Cells grown with the 3T3 feeder layer rapidly proliferated coming to confluence in 7-10 days in first culture and 10 days in subcultures as compared with 10 days in first culture and 12-15 days in subcultures for cells cultured on plastic alone. Cultures on plastic substrate alone could only be subcultured a maximum of six times, whereas cells subcultured with 3T3 cells could withstand up to 14 passages. Although the feeder layer system gave longer lasting cultures which proliferated more rapidly than those on plastic alone this system has major disadvantages for the study of the effects of alcohol on epithelial cells. The presence of metabolising, though mitotically inhibited, feeder cells makes it difficult to assess the effects of alcohol on epithelial cells since the 3T3 cells may metabolise some of the alcohol and their metabolic products may influence the epithelial cells. It was therefore decided to adopt a similar technique to that previously used by Jepsen *et al.* (1980). Here the first two passages were with a 3T3 feeder layer and all subsequent subcultures were onto plastic alone. Cells treated in this way withstood 12-14 passages.

All cultures were incubated at 32°C since this temperature increased the lifespan of the cultures and helped eliminate rat fibroblast contamination. This has previously been observed with rat oral epithelium (Jepsen, 1974).

The immunofluorescent staining confirmed that the cultures were pure epithelial cultures (section 2.3.4). It is difficult to produce anything other than epithelial cells in culture as Dispase (Sigma) cleaves the basement membrane allowing the epithelium to be easily separated from the underlying connective tissue with very low fibroblast contamination (Stenn *et al.*, 1989).

These techniques produced a culture system in which the cells could be given alcohol and its effects examined without the presence of metabolites from any other cells.

2.4.2 Measurement of Alcohol in Culture and its Effects on Epithelial Cell Viability

To ensure the cells were metabolising some of the added ethanol the concentration was measured over a 48 hour period in cell cultures and compared with control dishes with no cells. There was a 14% decrease in 24 hours and a 36% decrease in 48 hours in control dishes showing that a proportion of the alcohol was lost from the culture medium by evaporation. However, the significant difference (section 2.3.5) in ethanol loss between cell

cultures and control flasks indicated that at least 10% and 15% of the added ethanol was metabolised by the cells in 24 and 48 hours, respectively.

The large evaporative loss of ethanol from the culture medium was countered by renewing the medium every 24 hours in all studies involving alcohol containing medium.

It has been reported that serum proteins bind alcohol thus reducing its potency (Koerker, Berlin & Schneider, 1976; Smith *et al.*, 1990). To minimise any such effect the medium in control cultures contained serum so that the difference in ethanol loss between cells in culture and controls (that is the ethanol metabolised by the cells) would be over and above any ethanol bound by serum proteins.

Some workers investigating the effects of alcohol on cultured neurones have used serum free medium noting that less alcohol was needed to kill cells than when serum was present (Smith *et al.*, 1990). It took 48 hours to obtain an LD50 with 2% ethanol whereas in earlier studies with serum in the medium it took 24 hours of 9% ethanol treatment to reach an LD50 (Koerker *et al.*, 1976) and after 6 days of 1% ethanol treatment no change in cell viability was observed (Greenberg, Carpenter & Messing, 1987). However, the transformed cell line used by Koerker

et al. (1976) may be less typical of how normal neuronal cells respond.

Serum free media preparation is costly and time consuming and to date it is easier to grow oral epithelium in serum containing medium (section 1.14.3). It was therefore decided to use serum containing medium for the work contained in this thesis.

Since the cells do metabolise some of the alcohol the effect of this alcohol on cell viability was investigated. 5% ethanol was used in an earlier study (Siyam, 1987) and was chosen as the upper limit and varying concentrations down to 0.25% were employed. The alcohol concentrations (5-1%) that killed cells are unrealistic in terms of blood alcohol levels and *in vivo* the oral mucosa would never be subject to such concentrations for a prolonged period of time. 0.5% and 0.25% ethanol are closer to the concentrations of alcohol found *in vivo* after imbibing and do not significantly reduce cell viability of the oral epithelial cells in culture (section 2.3.6).

A concentration in the range of high blood alcohol levels and one in which the cells remained viable was required and since cells in 0.5% remained similar in viability to control cells this ethanol concentration was chosen for future studies.

0.4% ethanol is one of the highest recorded blood alcohol levels and although 0.4% ethanol could be used 0.5% was chosen to combat alcohol evaporation, and by ensuring the cells received 0.5% ethanol every 24 hours they would always be exposed to a range of 0.5 to 0.38% ethanol. This should mimic a state of constant and complete inebriation.

There is very little available work on the effects of alcohol on oral epithelial cells *in vitro*. A large proportion of the *in vitro* alcohol work has been carried out on neurones grown in culture (Koerker *et al.*, 1976; Scott, Petit & Lew, 1986; Greenberg *et al.*, 1987; Smith *et al.*, 1990; Smith & Wubetu, 1991). The results reported in this chapter are not directly comparable with the work on neurones since the cellular effects of ethanol in culture depend on the type of cells and conditions for growth. The concentration of ethanol and duration of exposure are very important. However, as with this study the aim of most of the above mentioned studies was to set up an *in vitro* model to investigate the cellular effects of ethanol. These studies all involved neurotoxicity testing. Since there is a lack of work in the field of oral epithelium it was desirable to set up a basic *in vitro* alcohol model to be employed in the further study of the cellular effects of alcohol on the oral epithelium.

No other study appears to have employed a range of alcohol concentrations in an attempt to set up a model and in cases where alcohol has been given to oral epithelium *in vitro* concentrations have been high and the duration of exposure short (Siyam, 1987). It was the aim of this work to consider human blood alcohol levels found *in vivo*, to mimic concentrations of alcohol close to those found *in vivo* and to maintain the cells in these concentrations so that the effect of ethanol upon the cells could be further examined. These investigations are described in the following chapters.

2.5 Conclusions

The use of a mitotically inhibited feeder layer greatly facilitated subculture of the rat lingual epithelial cells.

The technique of subculturing on plastic alone after two passages with a feeder layer produced a culture system in which the cells could be given alcohol and its effects examined without the presence of metabolites from any other cells.

Rat tongue epithelium *in vitro* utilises alcohol in the culture medium, although there is a high evaporative loss of the added alcohol.

Rat tongue epithelial cells in culture survived in concentrations of alcohol similar to high blood alcohol levels, allowing for further investigation of the *in vitro* effects of alcohol on rat lingual epithelium.

Chapter Three

The Effect of Ethanol on the Protein Profile of Cultured Oral Epithelium

3.1 Introduction

3.1.1 General Introduction

Having reported the initial characterisation of a model for the *in vitro* investigation of the effects of ethanol on oral epithelium, this chapter describes a study of the effects of ethanol on the protein profile of oral epithelium. Although ethanol may evoke a number of biochemical and cellular changes it would be difficult to examine a wide range of effects in the time available. It was therefore decided to be selective and to study the effect of ethanol on one aspect of epithelial biochemistry, namely, the protein profile of the epithelial cells. This had the advantage of complementing the *in vivo* work previously carried out in the Department of Oral Sciences, Glasgow University (Graham & Rennie, 1987).

Cell structure and function is expressed through the proteins produced and any disease related alteration of epithelial morphology or function may have its origin in abnormalities in protein expression. Graham & Rennie (1987) observed protein alterations in the lingual epithelium of alcoholic rats. After 60 days of ethanol intake there was a decrease in levels of an epithelial protein of molecular weight 160-200kD and an increase in levels of a 30kD protein, and after 102 days there was an

increase in levels of a 28kD protein. The model described in chapter 2 was employed to study the protein composition of cultured rat lingual epithelium given ethanol and control cultures given no ethanol. The aim of this study was to detect any protein alterations brought about by ethanol treatment and to compare them to those observed in the *in vivo* study carried out by Graham & Rennie (1987).

3.1.2 Ethanol Dose

Cells remain viable in 0.5% ethanol for periods up to 30 days before showing signs of senescence (section 2.3.6). Graham & Rennie (1987) suggested that some, but not all, of the altered protein profile may be heat shock proteins and it was therefore essential to examine the proteins at short as well as long intervals throughout a 30 day period. Cells were given 0.5% ethanol in the culture medium (and fresh medium added at 24 hour intervals) and control cultures were given medium containing no ethanol. Cells were prepared for analysis at 1, 3, 5, 7, 14, 21 and 30 days of ethanol treatment. Additionally, to investigate the reversibility of any protein alterations, some cultures were given 0.5% ethanol for 7 days followed by ethanol free medium for 7 days.

3.1.3 Method of Epithelial Protein Analysis

Electrophoretic techniques are the most widely used methods for the study of protein populations. Although, there are a number of electrophoretic techniques

available, the one widely regarded as giving the best resolution of proteins in one-dimension (1-D) is discontinuous **sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)** (Laemmli, 1970). Using this method reduced and denatured proteins can be electrophoretically separated from complex mixtures (Hames & Rickwood, 1981).

3.1.4 Preparation of Proteins for Electrophoresis and the Theory of Separation of Proteins by SDS-PAGE

The preparation of proteins for SDS-PAGE involves heating the samples in buffer which includes sodium dodecyl sulphate (SDS) (an anionic detergent) and 2-mercaptoethanol (a reducing agent). The proteins are completely dissociated by heating at 100°C for 2-3 minutes, and allowed to react effectively with the SDS and mercaptoethanol (Deyl, 1979). Mercaptoethanol disrupts any intra- or inter-peptide disulphide linkages, breaking the protein down to individual peptide chains. SDS denatures the protein by disrupting noncovalent bonds and coats the protein in a uniform manner with negative charges. At SDS monomer concentrations higher than $8x10^{-4}$, 1.4g of detergent binds to 1g of protein, resulting in a complex with rod-like conformation.

The length of this rod-like complex varies uniquely with the polypeptide molecular weight. SDS binds to proteins in a constant ratio, producing complexes with constant

charge per unit mass. Glycoproteins are an exception to this general rule. The carbohydrate moieties sterically hinder the binding of SDS leaving the glycoproteins with a reduced SDS:peptide ratio (Weber & Osborn, 1975). The non-specific binding of SDS to a wide variety of proteins produces complexes with the same charge:mass ratio, the complexes would travel through an electric field at similar rates. However, in SDS-PAGE the acrylamide gel acts as a retarding matrix, separating them according to size, with the smallest peptides being retarded least and migrating furthest in the gel (Hames & Rickwood, 1981). The matrix is created by cross-linking acrylamide monomers with a bi-functional crosslinking agent, N-methylenebisacrylamide (bis). The porosity of the matrix can be varied with the concentration of crosslinking agent used and thus the molecular range of peptides resolved by the gel can be varied.

Discontinuous slab gels were used in the present study. These gels consist of two components, the first of which (the upper gel component), has a low porosity and allows peptides to migrate through at an even rate producing a tight protein band in the upper gel. This effect is known as "stacking" and thus the upper gel is called the "stacker gel". When the tight band of proteins reach the lower gel component, the resolving gel, the peptides are retarded and separate out according to their molecular

weight. The resolving gel acts as a molecular sieve and can vary in porosity.

3.1.5 Molecular Weight Analysis on SDS Gels

The molecular weight of a given SDS coated peptide can be determined by comparing its electrophoretic mobility (Rf) with those of known protein markers. A linear relationship is obtained if the logarithms of the molecular weights of standard peptides are plotted against their respective electrophoretic mobilities (Rf). The molecular weight of any other protein for which an Rf value has been obtained can be calculated from this standard curve. An example standard curve for the molecular weight markers used in this study is shown in Figure 3.1. The molecular weight markers are listed in section 3.2.5.

3.2 Materials and Methods

3.2.1 Preparation of Epithelial Cells and Addition of Ethanol

Rat tongue epithelium was cultured onto 3T3 feeder cells and subcultured onto plastic alone as described in section 2.2.4. Cells in the third passage on plastic were allowed to grow for 7 days, until semi-confluent, before ethanol was added.

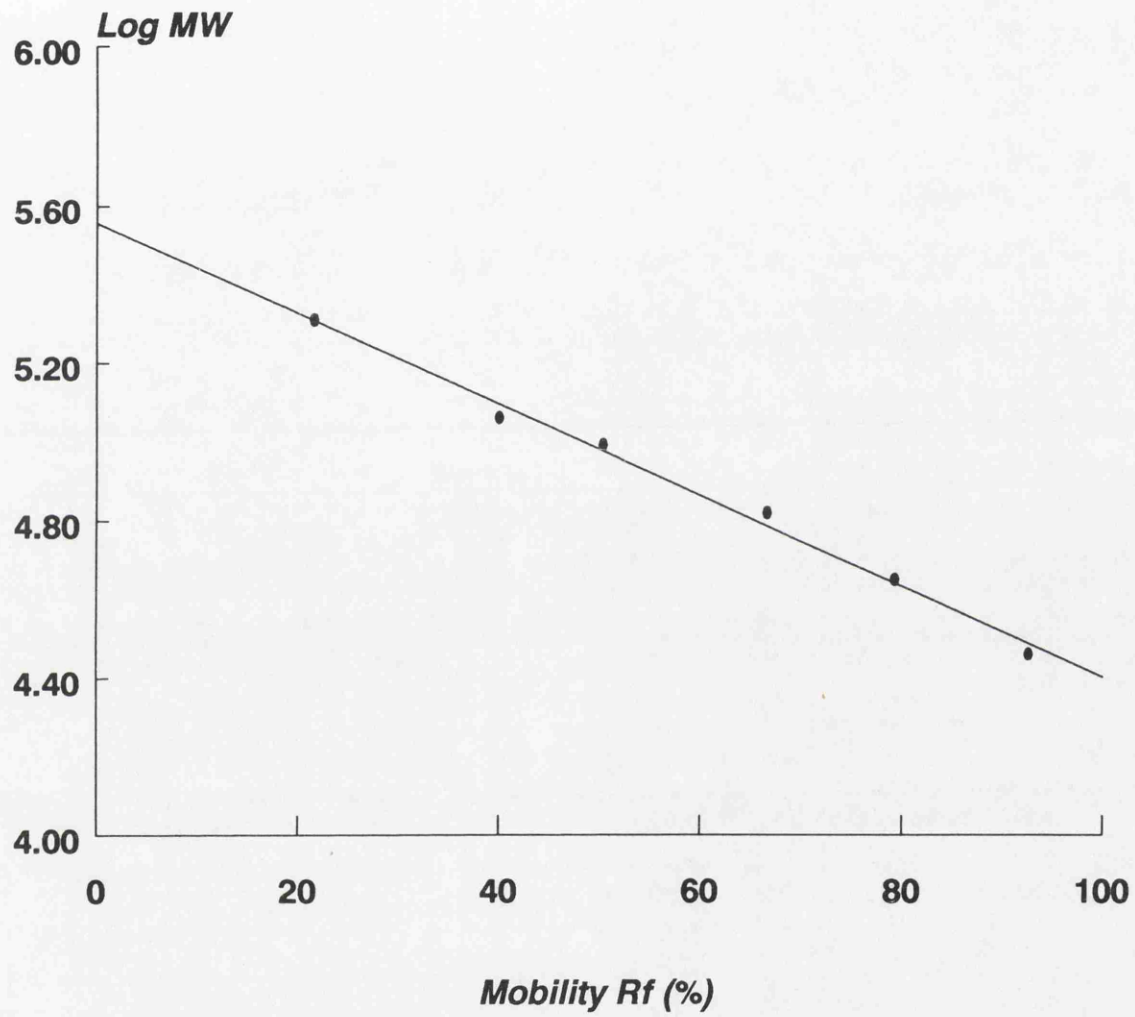


Figure 3.1 A typical calibration line for molecular weight markers in the range of 205 - 29kD on a 10% gel.

Ten 25cm^2 flasks for control and ethanol treatments were used for each time point examined. Epithelial cell culture medium (Table 2.2) without ethanol was given to cells as a control and medium containing 0.5% ethanol was used as the ethanol treatment. Fresh medium was prepared and added to cultures every 24 hours. Both control and ethanol treated cells were prepared for SDS-PAGE at 1, 3, 5, 7, 14, 21 and 30 days. An additional ten flasks were given 0.5% ethanol for 7 days followed by a further seven days of no ethanol treatment and prepared for SDS-PAGE after the 14 days. Control cells were given medium without ethanol for 14 days.

3.2.2 Preparation of Epithelial Cells for SDS-PAGE

At each time point ten "treatment" flasks and ten control flasks were prepared for SDS-PAGE. Following the removal of the culture medium, cells were washed with PBS three times, with two minutes between each wash, to remove all traces of serum. 1ml of distilled H_2O (dH_2O) containing 2mM protease inhibitor (phenylmethylsulfonyl fluoride, PMSF; Sigma) was added to each flask and the cells mechanically removed from the flask surface with the use of a "policeman". A "policeman" is an instrument designed for the mechanical removal of cells from the surface to which they are attached. The cells were scraped from the flask surface into the dH_2O. The cell suspension was then centrifuged at 1000rpm and resuspended in dH_2O with 2mM PMSF. This was repeated and the pellet resuspended in 1ml

dH_2O with 2mM PMSF. This mixture was stored at -20°C prior to SDS-PAGE.

The mixture was removed from -20°C and kept at 0-4°C while the cells were disrupted using an ultrasonic cell disrupter. Cells were disrupted for 15 seconds on 20% power. 40µl of the homogenate were removed and stored for protein concentration analysis.

The homogenate was mixed with a sample buffer containing 2% SDS, 30mM mercaptoethanol, 10% glycerol and 0.1% bromophenol blue in dH_2O. Four parts sample were added to one part sample buffer and the mixture heated to 100°C in a water bath for two minutes. The samples were allowed to cool and then loaded onto the gel for protein analysis.

3.2.3 Protein Concentration Determination

The concentration of protein in each of the epithelial preparations was determined by the method of Bradford (1976). This involves the quantitative binding of coomassie brilliant blue G-250 (CBB-G250) to protein.

Following homogenisation and prior to preparation for SDS-PAGE 40µl of each homogenate were removed for protein concentration determination. The protein concentration analysis was carried out in duplicate. 20µl of the homogenate were placed in a test-tube and 1ml of protein dye reagent added (Bradford, 1976). The contents of the

tube were thoroughly mixed by vortexing. The absorbance of the resulting solution was measured at 595nm in a Pye Unicam SP8-100 spectrophotometer using disposable plastic cuvettes. The protein concentration was read from a standard curve prepared, as above, using 20µl of various concentrations of bovine serum albumin (BSA). An example of a protein standard curve is shown in Figure 3.2.

All measurements were carried out between 2 and 30 minutes after vortexing and the absorbance read against a reagent blank prepared from 20µl of dH_2O with 2mM PMSF and 1ml of protein dye reagent.

3.2.4 One-dimensional Discontinuous SDS Polyacrylamide Gel Electrophoresis

A 10% acrylamide gel solution (Table 3.1) was prepared without SDS, ammonium peroxidisulphate (AMPS) and tetramethylethylene-diamine (TEMED) and degassed under vacuum for 15 minutes in a 100ml capacity side arm flask. This was to remove any O_2 which could have interfered with the polymerisation reaction. The SDS, AMPS and TEMED were then added and the solution mixed by swirling briefly, to avoid the introduction of air bubbles.

The gel was cast between glass plates separated by 1.5mm thick neoprene spacers. The gel solution was poured slowly between the glass plates to within 4cm of the top. The solution was then overlayed with isobutanol (which

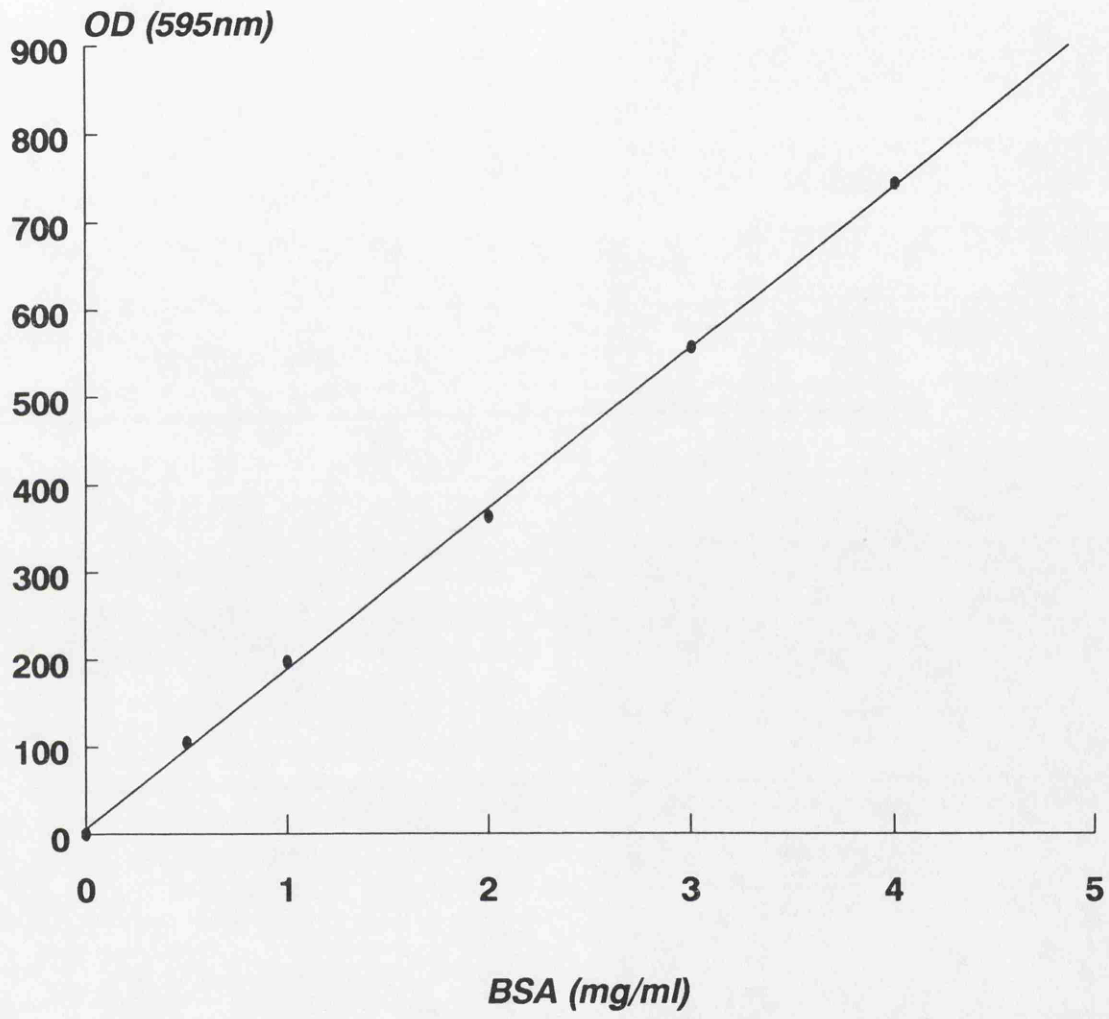


Figure 3.2 An example of a protein (BSA) standard curve. Each point represents the mean of three readings. The standard errors are too small to be detected.

TABLE 3.1 RECIPES FOR POLYACRYLAMIDE GELS.

PERCENTAGE GEL:	5%	7.5%	10%	12.5%	15%
30% ACRYLAMIDE	5.0	7.5	10	12.5	15
1% BISACRYLAMIDE	7.8	5.8	3.9	3.1	2.6
1.5M TRIS pH 8.7	7.5	7.5	7.5	7.5	7.5
20% SDS	0.15	0.15	0.15	0.15	0.15
WATER	9.2	8.7	8.0	6.4	4.4
10% AMPS	0.1	0.1	0.1	0.1	0.1
TEMED	10µl	10µl	10µl	10µl	10µl

STACKING GEL:	5%
30% ACRYLAMIDE	3.3
1% BISACRYLAMIDE	2.7
1M TRIS pH 6.8	2.5
20% SDS	0.1
WATER	8.8
10% AMPS	0.1
TEMED	10µl

N.B. All volumes in millilitres unless otherwise stated.

ensures an even gel surface) and allowed to polymerise at room temperature for 1 hour.

When polymerisation was complete, the stacker gel solution was prepared (Table 3.1), again without the SDS, AMPS and TEMED. The solution was degassed as described above and the SDS, AMPS and TEMED added. Following the removal of the isobutanol the stacker gel solution was applied to the top of the resolving gel. Into this unpolymerised stacker gel was placed a well forming comb (15 wells) around which the gel was allowed to polymerise. Once polymerisation was complete, the comb was removed and the resulting gel was ready for electrophoresis. Gloves were worn at all times during the preparation of the gels as acrylamide, and possibly bis, are potent neurotoxins.

3.2.5 Loading the Gels and Electrophoresis

Electrophoresis buffer (0.025M tris, 0.192M glycine and 0.1% SDS, pH 8.3) was added to the bottom gel tank and to the sample wells of the gels. The epithelial protein preparations were loaded into the sample wells, 50µg in 50µl, and the gels placed in the bottom tank of the electrophoresis apparatus. The top reservoir was then put in place and filled with electrophoresis buffer. Molecular weight markers were included in the run to allow calculation of the molecular weight of any proteins

of interest. The molecular weight marker proteins used in the present studies were:

Carbonic Anhydrase	- 29kD
Albumin (Egg)	- 45kD
Albumin (Bovine)	- 66kD
Phosphorylase 6	- 97kD
B-Galactosidase	- 116kD
Myosin	- 205kD

Sigma Chemicals Ltd., Dorset, England

Electrophoresis was carried out at 30 mAmps per gel until the proteins reached the end of the resolving gel (marked by bromophenol blue dye). Gels were run at 10°C using the cooling system which is part of the electrophoresis apparatus (Hoefer Scientific Instruments).

3.2.6 Staining and Assessment of Protein profile

The coomassie brilliant blue R-250 (CBB-R250) staining method (Hames & Rickwood, 1981) was used to stain the gels.

Following electrophoresis, the gels were carefully removed from between the glass plates and placed overnight in a fixing/staining solution of 0.1% CBB-R250, 25% methanol and 10% acetic acid. To destain, the gels were placed in the same solution without the CBB-R250.

After 1 hour the gels were placed in fresh destain solution and left overnight. A final change of destain solution was given and the gels left until fully destained. The final destained gel was stored in a sealed moist bag.

The CBB-R250 stained gels were analysed on an LKB 2202 Ultroscan laser densitometer (LKB Instruments Ltd, England). The parameter measured was the area percentage which is a measure of the percentage of the total protein represented by a single band (LKB Instruments, 1981). The use of this parameter allowed for quantitative comparisons between levels of individual proteins in different samples. Although protein concentrations were assessed (section 3.2.3) and a concentration of 50µg for each sample loaded, the ethanol treated samples often appeared fainter on the gel and appeared to have lower protein concentrations. However, this variation between samples was counteracted by measuring the percentage of total protein within a sample represented by a band. To assess whether variations in protein concentration between samples might affect the percentage of total protein represented by individual bands within a sample, different total protein concentrations of a single sample were run and analysed on the laser densitometer. Irrespective of the protein concentration the percentage area represented by individual bands remained very similar. Thus any variation in total protein

concentration between samples would not make a difference to the percentage of total protein represented by individual bands.

3.2.7 Statistical Analysis

Differences between the percentage areas from ethanol treated cells and control cells at each day were compared using a Mann-Whitney U test (Minitab). Since four comparisons were made within each day the significance level was set at 0.025 ($0.05/\sqrt{4}=0.025$, where 4= number of comparisons) in order to compensate for multiple comparisons (Brown & Swanson Beck, 1988).

3.3 Results

3.3.1 Comparison of the Protein Profiles of Ethanol Treated and Control Cells

Visual Comparison

Example lanes from the SDS polyacrylamide gels from the lingual epithelial proteins from the ethanol treated and control cells from 1, 3, 5, 7, 14, 21, 30 and 7+7 days are shown in Figures 3.3-3.10. Ten samples were analysed at each time point for both alcohol treated cells and controls. The gel lanes for each sample were very similar and it was decided to show example lanes. Visual comparison between ethanol treated cells and control cells suggested that there were no protein changes at 1,

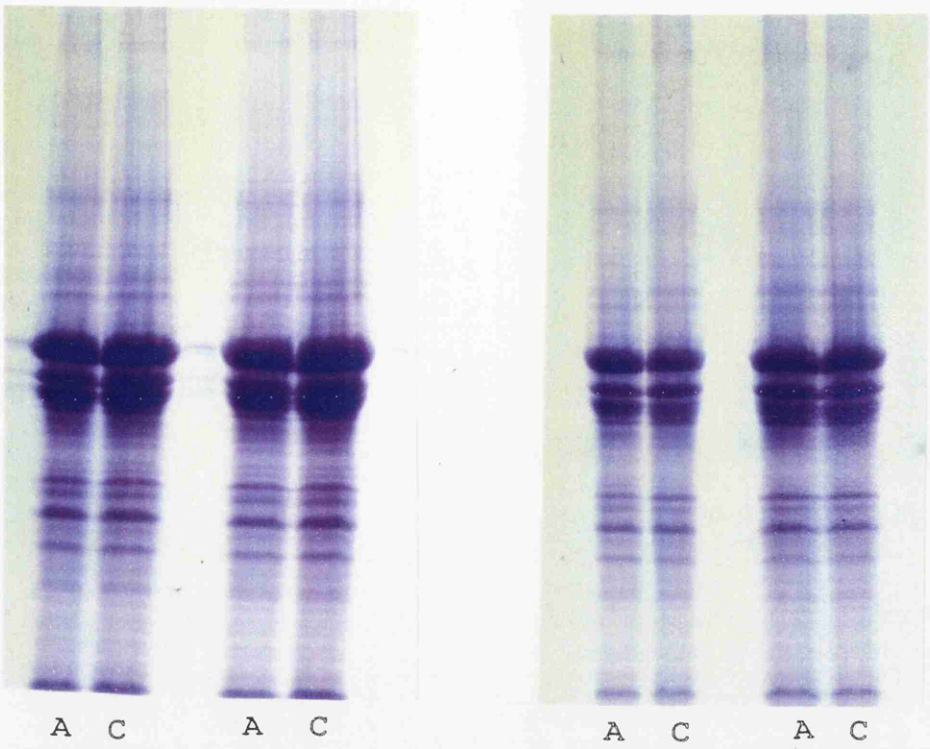


Figure 3.3 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 1 day. A - Alcohol C - Control

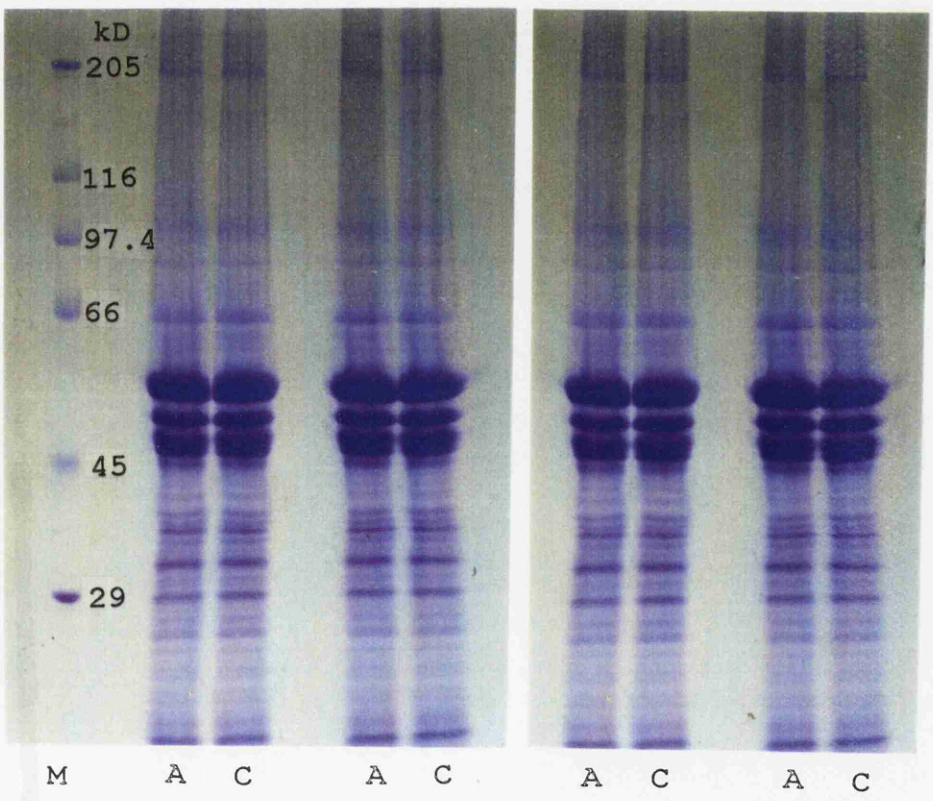


Figure 3.4 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 3 days. A - Alcohol C - Control M - Molecular weight markers.

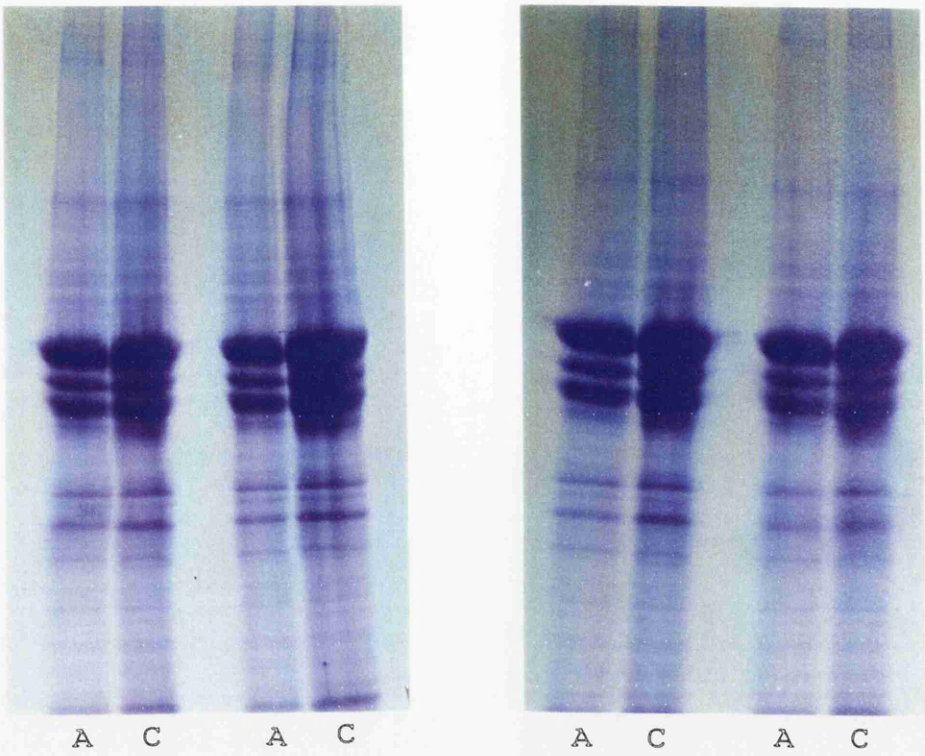


Figure 3.5 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 5 days. A - Alcohol C - Control.

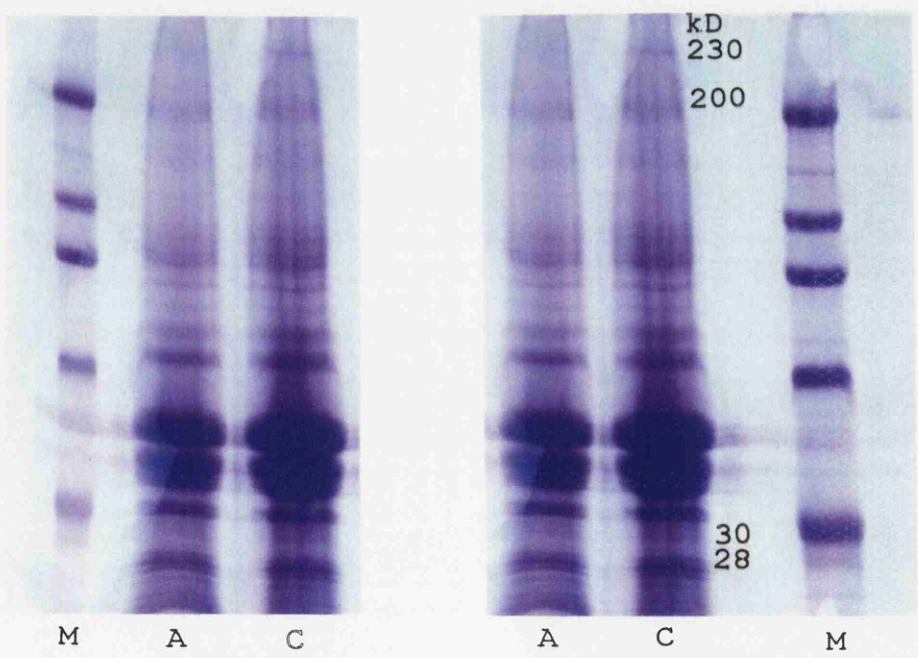


Figure 3.6 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 7 days. A - Alcohol C - Control M - Molecular weight markers.

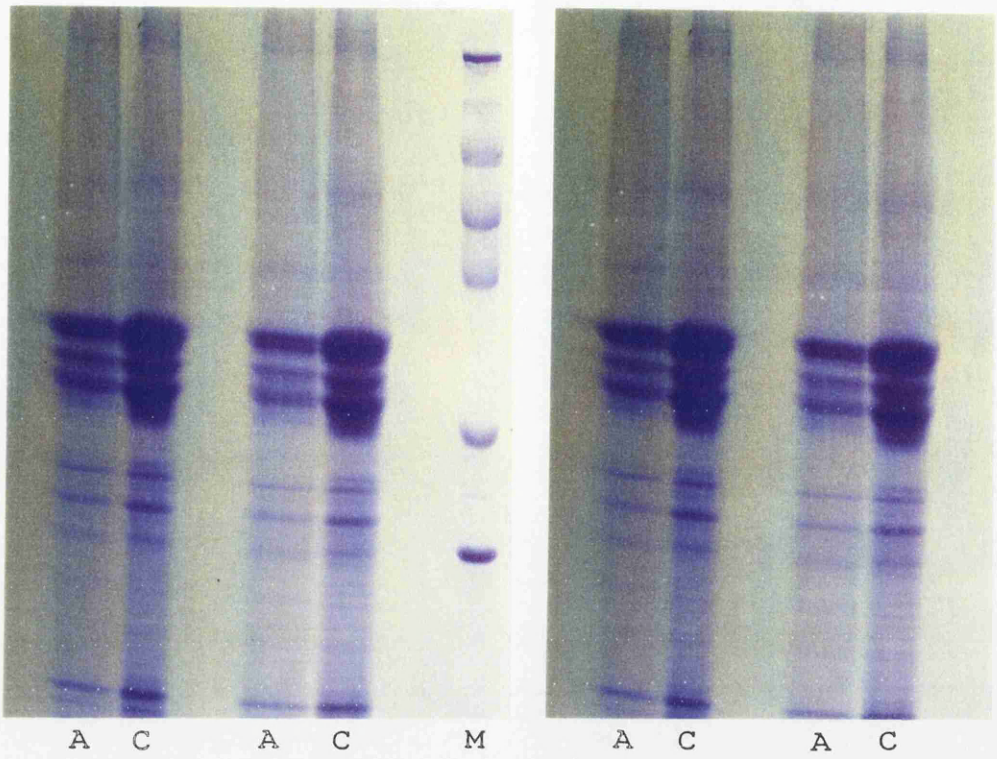


Figure 3.7 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 14 days. A - Alcohol C - Control M - Molecular weight markers.

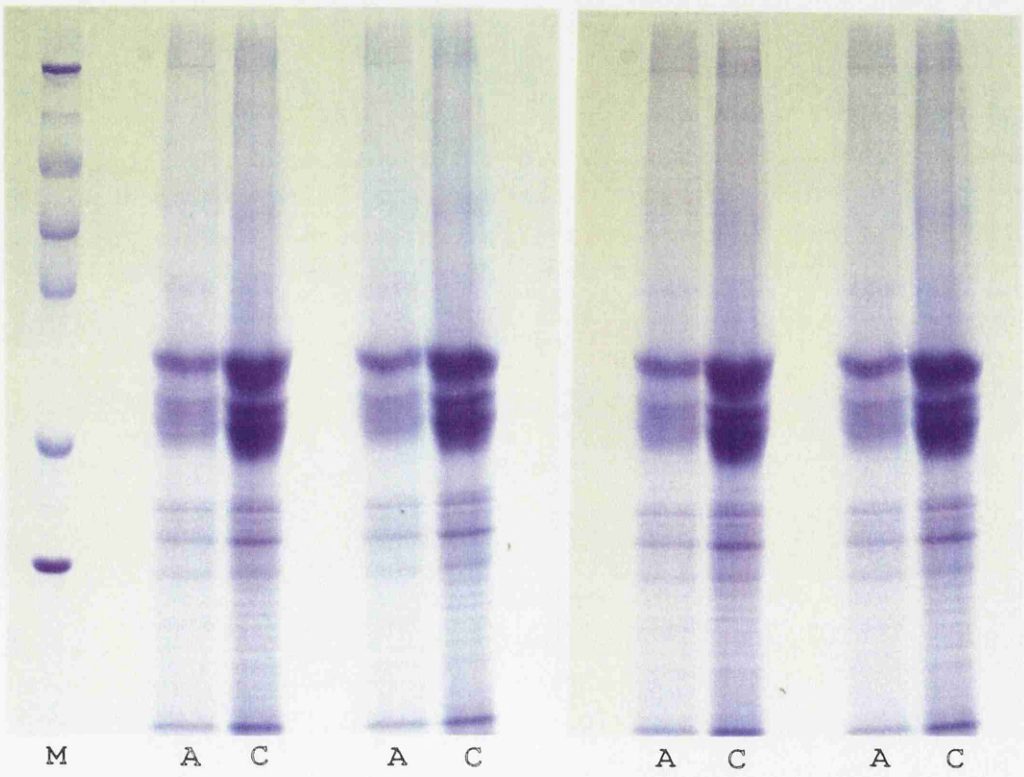


Figure 3.8 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 21 days. A - Alcohol C - Control M - Molecular weight markers.

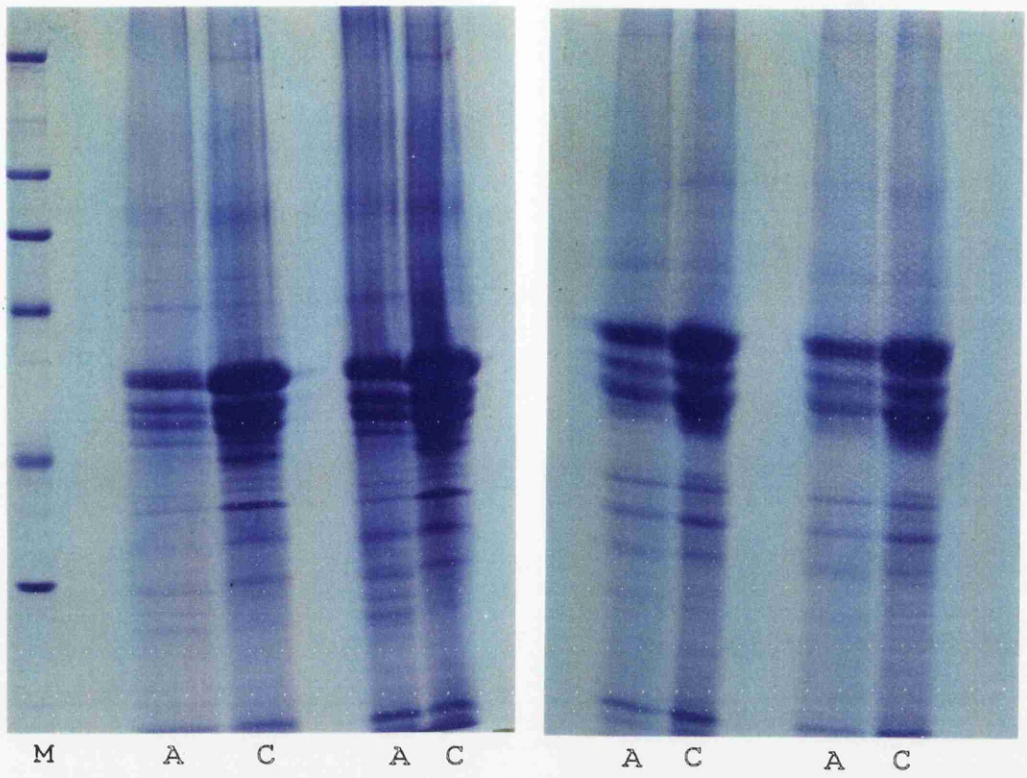


Figure 3.9 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.5% ethanol in the culture medium for 30 days. A - Alcohol C - Control M - Molecular weight markers.

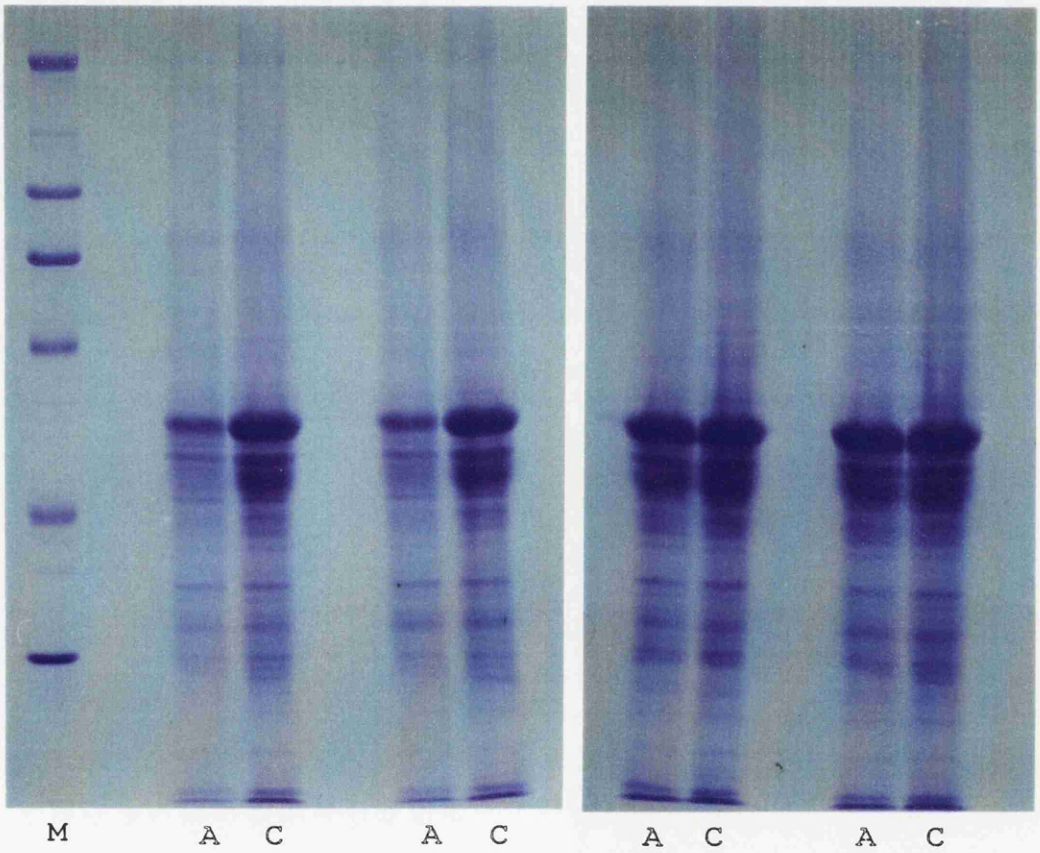


Figure 3.10 Shows example lanes from gels of rat lingual epithelial proteins in cells given 0.5% ethanol for 7 days followed by 0% ethanol and cells given 0% ethanol for 14 days. A - Alcohol/ No Alcohol C - Control M - Molecular weight markers.

3 and 5 days of ethanol treatment in comparison to controls. After 7, 14, 21 and 30 days ethanol treatment and also 7 days ethanol + 7 days no ethanol treatment there appeared to be a reduction in the levels of two high molecular weight (MW) proteins in the ethanol treated cells compared with controls.

No alterations in the levels of low MW proteins were noted by visual analysis.

Densitometric analysis

An example densitometer reading is shown in Figure 3.11(a-c). Densitometric analysis confirmed the visual analysis of reduced levels of 2 high MW proteins at 7, 14, 21 and 30 days of ethanol treatment and also 7 days ethanol + 7 days no ethanol treatment. There was no significant change in protein levels at 1, 3 and 5 days of ethanol treatment.

Despite the lack of obvious visual evidence, densitometric analysis also revealed an increase in the levels of two lower MW proteins in all ethanol treated samples after 7 days. There were no changes noted with shorter exposure to ethanol.

MW analysis of the four proteins revealed that the two lower MW proteins have MWs of 30kD and 28kD. Analysis of

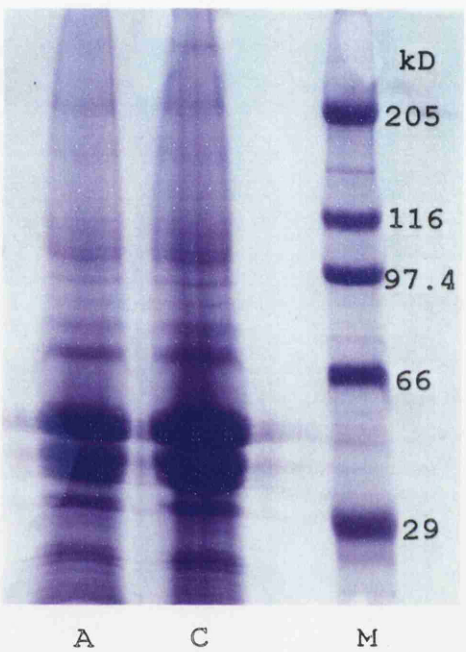
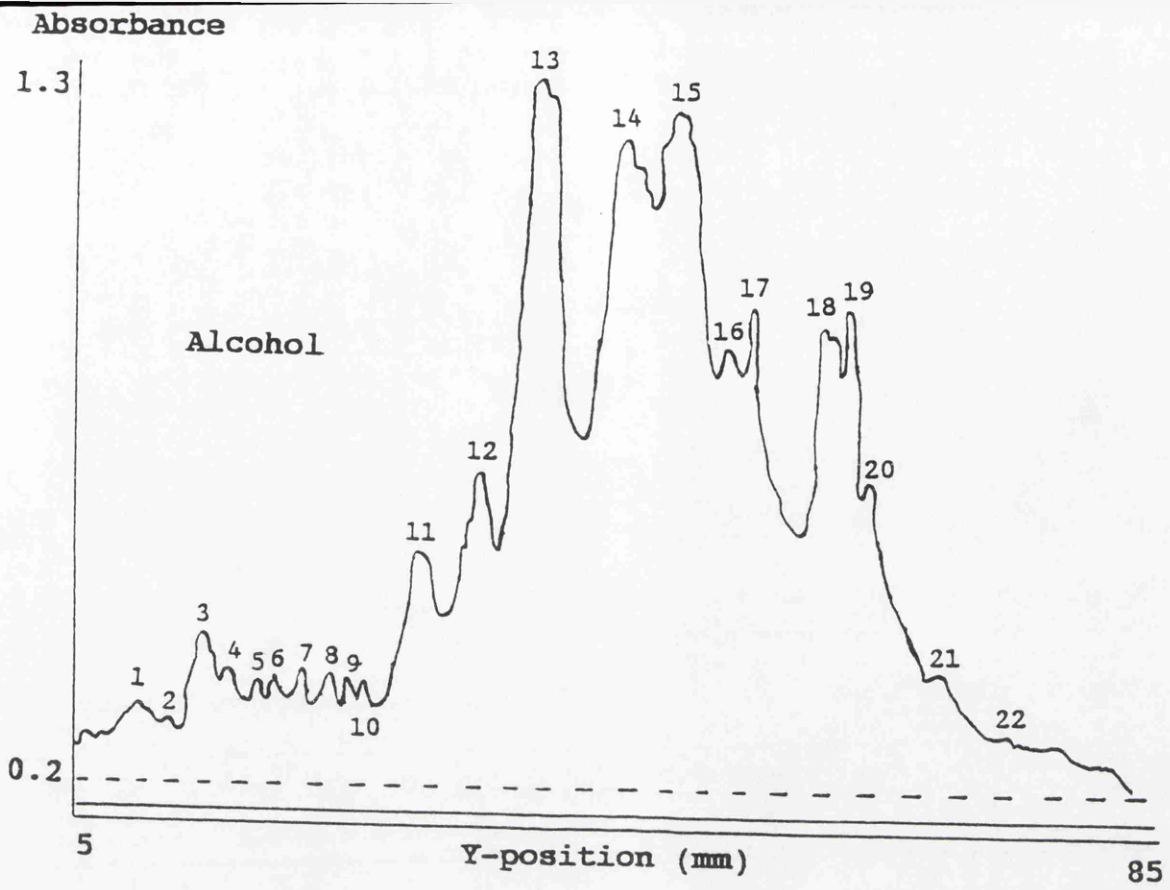


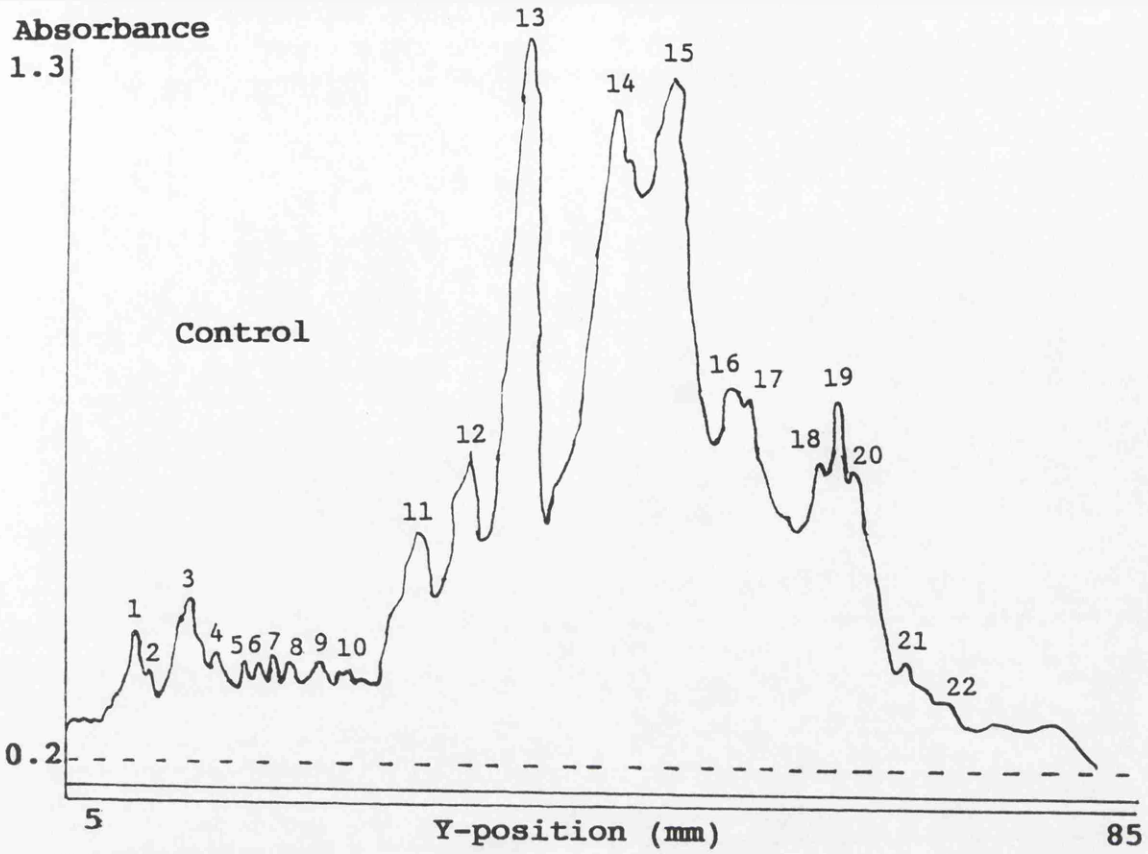
Figure 3.11 (a-c) Example of a laser densitometer reading. Figure (a) shows the gel corresponding to the readings. A - 0.5% Alcohol treated cells (7 days) C - Control cells M - Molecular weight markers.



UltroScan XL

Peak	Position (mm)	Area (AU*mm)	Rel. Area (%)
1	8.00	0.540	0.90
2	12.90	0.312	0.52
3	16.90	1.207	2.01
4	23.00	1.081	1.80
5	28.50	1.051	1.75
6	30.00	1.075	1.79
7	32.10	1.081	1.80
8	33.40	1.069	1.78
9	36.70	1.045	1.74
10	39.40	1.009	1.68
11	42.90	1.940	3.23
12	46.80	2.408	4.01
13	56.00	12.628	21.03
14	61.00	7.806	13.00
15	62.80	9.014	15.01
16	66.00	3.062	5.10
17	67.00	3.700	6.16
18	73.00	3.357	5.59
19	73.90	3.621	6.03
20	75.00	2.408	4.01
21	78.10	0.474	0.79
22	79.00	0.324	0.54

Figure 3.12.b Laser densitometer reading for the alcohol treated cell lane.
Peak 1 - 230kD Protein, 2 - 200kD Protein,
17 - 30kD Protein, 18 - 28kD Protein.



UltroScan XL

Peak	Position (mm)	Area (AU*mm)	Rel. Area (%)
1	8.10	1.315	2.13
2	12.80	0.685	1.11
3	16.40	1.537	2.49
4	22.60	1.167	1.89
5	28.30	1.087	1.76
6	29.40	1.019	1.65
7	32.50	1.105	1.79
8	33.60	1.043	1.69
9	36.80	1.099	1.78
10	39.30	0.975	1.58
11	42.70	1.976	3.29
12	46.40	2.463	3.99
13	55.90	13.293	21.53
14	60.50	8.946	14.49
15	63.00	9.971	16.15
16	66.80	3.081	4.99
17	67.10	2.593	4.20
18	73.00	2.161	3.50
19	74.10	2.840	4.60
20	75.30	2.118	3.43
21	78.20	0.624	1.01
22	80.00	0.586	0.95

Figure 3.12.c Laser densitometer reading for the control cell lane.
Peak 1 - 230kD Protein, 2 - 200kD Protein,
17 - 30kD Protein, 18 - 28kD Protein.

the two high MW proteins revealed them to have MWs of 230kD and 200kD.

1 - 5 Days

Table 3.2 shows the mean values of the area percentages of the 4 proteins after 1 day of 0.5% ethanol treatment. The mean area percentages for each protein were similar to those of the control cells given no ethanol. Statistical analysis (Mann-Whitney U test) revealed no significant differences between the protein levels of ethanol treated and control cells. This was also the case after 3 days of ethanol treatment (Table 3.3) and 5 days of ethanol treatment (Table 3.4).

7 Days

Table 3.5 shows that the mean values of the area percentages for the 230kD and 200kD proteins in the ethanol treated cells were lower than those for the control cells (0.79 and 0.40% in ethanol and 2.68 and 1.90% in controls, respectively). After 7 days this approximate 3 fold decrease in levels of the 230kD protein was statistically significant ($p<0.0002$). The 4 fold decrease in levels of the 200kD protein was also significant at $p<0.0002$.

Table 3.5 also shows that the mean area percentages for the 30kD and 28kD proteins were higher in the ethanol treated cells than controls after 7 days (7.04 and 5.47%

Table 3.2 Relative area percentage of protein bands from 1 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	1.57	1.95	2.16	2.51	5.40	5.59	4.99	4.43
Area	1.79	2.00	2.40	2.68	4.64	4.00	3.98	4.09
	1.65	1.80	2.06	2.24	4.50	4.14	4.78	5.00
	1.83	1.44	1.91	2.03	5.81	5.69	4.00	3.89
	2.00	1.99	2.44	2.51	4.74	4.85	3.99	3.62
	1.80	1.93	2.08	2.31	3.99	4.86	3.55	4.35
	1.78	1.57	1.98	1.70	4.35	5.56	3.65	3.42
	1.53	1.80	1.81	2.30	3.96	3.64	3.46	3.06
	0.98	1.43	1.76	1.82	4.54	4.28	3.09	3.62
	1.70	0.70	2.01	1.60	4.81	3.46	3.70	3.26
X	1.66	1.66	2.06	2.17	4.67	4.61	3.92	3.87
S.D.	0.28	0.40	0.22	0.37	0.58	0.82	0.58	0.60
S.E.	0.09	0.13	0.07	0.12	0.18	0.26	0.18	0.19

n=10

Table 3.3 Relative area percentage of protein bands from 3 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	2.00	2.11	1.97	2.08	4.89	4.82	3.91	4.00
Area	1.99	2.18	2.10	2.27	5.07	4.49	4.30	3.80
	1.87	1.99	2.01	1.98	4.99	5.49	3.98	3.99
	1.91	2.13	2.14	2.29	5.75	4.29	4.21	3.81
	2.48	2.30	2.50	2.55	5.21	4.46	4.22	3.75
	1.96	2.40	2.00	2.49	4.39	4.35	3.65	3.34
	1.89	2.00	2.15	2.41	3.90	3.58	3.00	2.90
	1.55	1.83	2.04	2.36	3.78	3.82	3.09	3.13
	2.01	2.31	2.60	2.75	4.60	5.00	3.87	4.11
	1.53	1.97	2.14	2.25	4.20	4.08	3.95	2.32
X	1.92	2.12	2.17	2.34	4.68	4.44	3.82	3.52
S.D.	0.26	0.18	0.21	0.22	0.62	0.56	0.45	0.58
S.E.	0.08	0.06	0.07	0.07	0.20	0.18	0.14	0.18

n=10

Table 3.4 Relative area percentage of protein bands from 5 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	1.90	2.30	1.67	1.70	4.03	3.83	3.51	3.01
Area	1.97	2.70	2.01	1.99	3.14	3.81	3.00	3.32
	2.00	2.10	1.88	2.03	4.39	4.87	3.95	3.46
	2.30	2.44	2.51	2.67	4.00	4.11	3.32	3.44
	2.13	2.00	1.90	2.10	4.18	4.21	4.00	4.54
	2.40	2.51	2.00	2.41	3.99	4.00	3.18	3.43
	1.70	2.01	1.91	2.03	3.98	4.11	3.56	3.01
	1.91	2.65	2.65	2.70	4.10	4.30	3.96	3.79
	0.85	1.00	1.99	1.87	4.41	3.98	3.41	3.22
	0.90	0.51	1.42	2.00	3.95	3.74	3.48	3.00
X	1.81	2.02	1.99	2.15	4.10	4.20	3.54	3.42
S.D.	0.53	0.72	0.36	0.33	0.35	0.33	0.34	0.47
S.E.	0.17	0.23	0.11	0.11	0.11	0.10	0.11	0.15

n=10

Table 3.5 Relative area percentage of protein bands from 7 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	0.90	2.13	0.52	1.11	6.16	4.20	5.59	3.50
Area	0.90	2.40	0.00	2.00	6.00	3.00	5.00	3.04
	0.91	2.36	0.20	1.57	6.48	3.61	5.53	3.51
	0.96	2.58	0.40	2.40	7.71	3.96	4.96	3.29
	0.60	1.83	0.50	2.09	8.14	4.19	6.90	3.46
	0.97	3.08	0.20	3.22	7.49	4.00	5.14	3.80
	0.25	2.62	0.20	1.23	7.65	3.03	5.96	3.97
	0.31	3.69	0.17	1.98	8.85	4.48	6.72	4.24
	1.02	3.96	0.91	1.65	5.92	4.76	4.03	3.55
	1.12	2.10	0.89	1.75	5.98	3.21	4.85	3.58
X	0.79	2.68	0.40	1.90	7.04	3.84	5.47	3.59
S.D.	0.30	0.70	0.31	0.61	1.06	0.61	0.88	0.34
S.E.	0.10	0.22	0.10	0.19	0.34	0.19	0.28	0.11

n=10

in ethanol compared to 3.84 and 3.59% in controls). The approximate 2 fold increase in levels of the 30kD protein was statistically significant ($p<0.0002$) as was the increase in the 28kD protein ($p<0.0002$).

14 Days

Densitometric analysis revealed a 14 fold decrease in levels of the 230kD protein from 2.30 to 0.16% and almost an 100 fold decrease in levels of the 200kD protein from 1.84 to 0.02% (Table 3.6). These changes were statistically significant ($p<0.0002$).

The mean relative percentage areas for the two lower MW proteins increased in cells given ethanol for 14 days compared with controls (5.66 and 4.62 in ethanol for 30kD and 28kD proteins, respectively and 3.33 and 2.84% for controls). The differences were significant at $p<0.0002$.

21 Days

Table 3.7 shows a decrease in the mean percentage area of the 230kD protein after 21 days of ethanol treatment compared with control cells. This decrease from 2.18 to 0.05% was significant at $p<0.0002$. The 200kD protein showed a marked decrease from 2.18 to 0.04%. This decrease was again significant ($p<0.0002$).

Table 3.7 also shows that levels of the 30kD protein increased significantly ($p<0.0002$) from 3.47 to 6.17% in

Table 3.6 Relative area percentage of protein bands from 14 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	0.00	2.56	0.00	1.20	5.91	3.71	4.36	2.90
Area	0.00	2.40	0.10	1.70	5.66	3.82	4.75	2.98
	0.00	1.89	0.00	1.88	6.56	3.01	4.00	2.30
	0.25	1.70	0.00	2.35	4.98	3.00	4.01	3.11
	0.40	2.58	0.05	1.98	5.45	3.51	5.00	2.99
	0.20	2.96	0.00	2.00	5.29	3.81	5.02	3.41
	0.35	2.00	0.00	1.77	5.70	2.90	4.71	2.33
	0.22	1.83	0.00	0.99	4.50	2.30	3.80	2.15
	0.21	3.00	0.00	2.56	6.00	4.13	5.51	3.35
	0.00	2.10	0.00	2.00	6.50	3.14	5.00	2.91
X	0.16	2.30	0.02	1.84	5.66	3.33	4.62	2.84
S.D.	0.15	0.47	0.03	0.47	0.64	0.56	0.55	0.44
S.E.	0.05	0.15	0.01	0.15	0.20	0.18	0.17	0.14

n=10

Table 3.7 Relative area percentage of protein bands from 21 day alcohol treated cells and control cells.
A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.%	0.00	2.40	0.00	2.00	6.51	3.00	5.50	2.71
Area	0.00	2.51	0.00	1.81	5.98	2.89	4.56	3.71
	0.22	3.11	0.10	3.55	6.00	3.21	5.98	3.92
	0.30	2.85	0.00	3.00	5.95	3.91	4.54	3.59
	0.00	1.11	0.00	1.20	6.15	3.13	5.54	3.51
	0.00	2.00	0.00	2.11	6.44	3.30	4.44	3.31
	0.00	2.11	0.10	1.98	8.78	4.45	3.99	1.99
	0.00	1.95	0.12	2.10	6.85	3.96	3.56	2.50
	0.00	2.03	0.00	2.00	4.99	3.76	3.44	2.70
	0.00	1.71	0.10	2.10	4.00	3.10	2.76	1.55
X	0.05	2.18	0.04	2.18	6.17	3.47	4.43	2.95
S.D.	0.11	0.57	0.05	0.65	1.23	0.52	1.03	0.79
S.E.	0.04	0.18	0.02	0.20	0.39	0.16	0.33	0.25

n=10

the ethanol group after 21 days. Levels of the 28kD protein increased from 2.95 to 4.43%. This increase was statistically significant ($p<0.005$).

30 Days

Following 30 days of ethanol treatment the mean percentage areas for the 230 and 200kD proteins showed a 12 fold and a 50 fold decrease, respectively (Table 3.8). The 230kD protein showed a significant decrease from 2.34 to 0.19% ($p<0.0002$) and the 200kD protein levels decreased significantly from 2.05 to 0.04% ($p<0.0002$).

The mean percentage areas for the 30kD and 28kD proteins increased after 30 days of ethanol treatment (Table 3.8). The increase in levels of the 30kD protein from 3.69 to 6.37% was statistically significant ($p<0.0002$) as was the increase in the levels of the 28kD protein from 2.98 to 4.70% ($p<0.0002$).

7 Days ethanol + 7 days no ethanol

Comparison of the mean area percentages of the proteins in cells given ethanol for 7 days followed by medium containing no ethanol for a further 7 days (Table 3.9) revealed that the levels of the 230kD protein were 3 times lower than those for control cells (0.68 and 2.12%, respectively). This decrease was statistically significant ($p<0.0002$). The mean area percentage for the 200kD protein was almost 6 times lower in the ethanol/no

Table 3.8 Relative area percentage of protein bands from 30 day alcohol treated cells and control cells. A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.% Area	0.00	2.15	0.00	1.89	5.51	3.33	5.00	3.11
	0.40	2.70	0.44	2.00	5.98	3.41	4.00	2.98
	0.39	2.10	0.00	1.99	5.70	3.00	4.11	2.50
	0.10	2.10	0.00	1.95	6.12	3.57	4.01	3.00
	0.20	2.11	0.00	2.00	8.44	4.13	6.99	2.98
	0.11	2.54	0.00	2.11	7.36	4.05	4.76	2.50
	0.10	2.00	0.00	2.04	5.78	3.98	4.00	2.97
	0.09	2.78	0.00	2.44	6.50	3.64	4.98	3.89
	0.30	2.81	0.00	2.30	7.61	4.71	5.14	3.81
	0.18	2.08	0.00	1.78	4.71	3.11	4.00	2.11
X	0.19	2.34	0.04	2.05	6.37	3.69	4.70	2.98
S.D.	0.14	0.33	0.14	0.19	1.12	0.53	0.94	0.55
S.E.	0.04	0.10	0.04	0.06	0.35	0.17	0.30	0.17

n=10

Table 3.9 Relative area percentage of protein bands from 7 day alcohol/7 day no alcohol treated cells and control cells. A - alcohol C - control.

Protein	230kD		200kD		30kD		28kD	
	A	C	A	C	A	C	A	C
Rel.% Area	0.87	2.00	0.40	2.40	6.00	3.13	5.14	2.87
	0.50	2.87	0.31	2.10	6.70	3.56	5.00	2.90
	0.70	2.10	0.25	1.90	5.96	2.70	3.78	2.41
	0.91	2.98	0.40	1.80	5.00	3.19	4.55	3.50
	0.90	2.90	0.10	2.01	5.51	3.50	6.10	3.56
	1.00	2.56	0.21	2.00	7.61	4.00	7.00	3.99
	0.87	2.31	0.40	1.97	8.81	4.90	4.90	2.91
	0.60	2.00	0.50	1.90	5.30	3.00	7.50	3.00
	0.00	0.40	0.00	0.55	7.65	4.41	6.00	3.41
	0.40	1.10	0.51	0.98	6.98	4.50	5.98	3.11
X	0.68	2.12	0.31	1.76	6.55	3.69	5.60	3.17
S.D.	0.31	0.83	0.17	0.56	1.22	0.73	1.14	0.45
S.E.	0.10	0.26	0.05	0.18	0.39	0.23	0.36	0.14

n=10

ethanol treated cells compared with control cells (0.31 and 1.76%, respectively). This decrease was again statistically significant ($p<0.0002$).

Levels of the 30kD and 28kD proteins increased in ethanol/no ethanol treated cells compared with controls (Table 3.9). The increases from 3.69 to 6.55% for the 30kD protein and 3.17 to 5.60% for the 28kD protein were statistically significant ($p<0.0002$).

Graphs summarising the alterations in the levels of the four proteins over the 30 day study are shown in Figure 3.12(a-d).

3.4 Discussion

3.4.1 Ethanol Related Protein Changes

The results demonstrated a significant reduction in the levels of two high molecular weight proteins (230 and 200kD) and an increase in the levels of two lower MW proteins (30 and 28kD) in epithelial cells given 0.5% ethanol for 7 days or more. The proteins in the ethanol treated cells did not differ significantly from those of control cells at the time points examined before 7 days.

The 200, 30 and 28kD proteins are similar in MW to those observed by Graham & Rennie (1987) in an *in vivo* study of the effects of ethanol on rat lingual epithelium. A

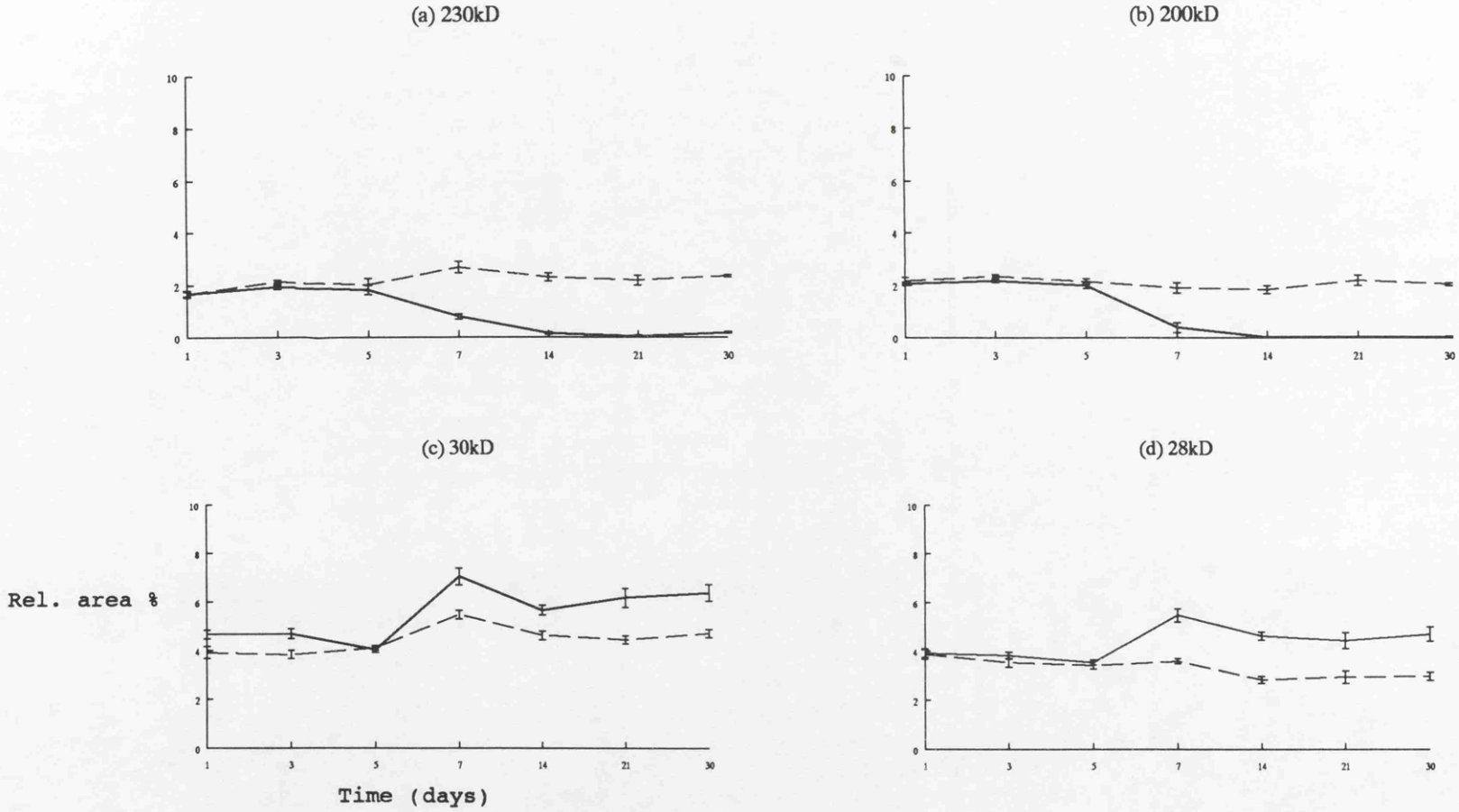


Figure 3.12 (a-d) Graphs (a-d) show the alterations in levels (relative area percentage) of the four proteins over the 30 day period. Each point represents the mean and standard error of ten measurements. – – – – Control cultures ——— Alcohol treated cultures. Graph (a) represents the 230kD protein, (b) represents the 200kD protein, (c) represents the 30kD protein and (d) represents the 28kD protein. The x-axis and y-axis legends for all the graphs are identical.

matched pair feeding technique was employed in which ethanol was part of the experimental animals' diet for periods of 30, 60 and 102 days. Liver enzyme analysis revealed that the procedure was effective in producing a model of tissue damage due to chronic ethanol consumption. After 60 days there was a decrease in levels of a 160-200kD and an increase in levels of a 30kD protein, and after 102 days there was an increase in levels of a 28kD protein. It was concluded that the protein changes were a consequence of chronic ethanol exposure.

The above changes and a decrease in a 230kD protein were observed *in vitro* after only 7 days of ethanol treatment. The cells received ethanol concentrations equivalent to very high blood ethanol levels but it is difficult to extrapolate between an animal model and the situation in culture. Tissue culture techniques have many advantages but they cannot represent the whole animal. Changes may occur in a shorter period of time in culture and the life span of cells in culture is shorter than the life span of the live animal. 60 days is approximately one tenth of the total life span of a laboratory rat whereas the cells in this study began to senesce after 30 days in culture without being passaged. The situation is difficult to interpret and there are no studies relating the life span of oral epithelial cells in culture to the life span of the live animal. Also, there is no liver present to

metabolise ethanol in the culture situation as opposed to the whole animal.

Over the 30 day period levels of the 230kD protein progressively decreased (Fig. 3.12) as did those of the 200kD protein. The 30kD and 28kD proteins showed a more erratic increase over the 30 days. Additionally they did not show a corresponding progressive increase as the larger protein levels decreased. Graham (1987) had originally suggested a link between the breakdown of the high MW protein and the increase in the lower MW proteins, suggesting that they may be related. This was discounted when the two lower MW proteins were shown to arise independently, the 30kD protein at 60 days and the 28kD protein at 102 days. The results of the present study show that the decrease in high MW proteins and the increase in low MW proteins occurs at the same time points. However, since the rise in levels of low MW proteins does not progress as the levels of the high MW proteins decrease but show a more erratic increase it seems likely that there is no direct link between the lowered levels of the high MW proteins and the raised levels of the two lower MW proteins. This is further supported by the findings of Graham (1987) who used peptide mapping to show that the proteins were not related. However, a lot of difficulty was encountered due to the small amount of protein obtained from the rat lingual epithelium. This would be even more of a problem

with cell cultures and it was decided not to pursue this topic further in this thesis.

There was no significant variance in the levels of the four proteins in any of the control cultures. This suggests that the altered levels of protein in the ethanol treated cells can be explained on the basis of ethanol related effects and not on aging of the cultures.

When cells were given ethanol for 7 days followed by medium containing no ethanol for a further 7 days the levels of the two high MW proteins remained significantly lower than controls and levels of the low MW proteins remained significantly higher than controls. There were no significant differences in protein levels after 7 days in 0.5% ethanol compared with values obtained from cells given 0.5% ethanol 7 days/0% ethanol 7 days. This suggests that the protein changes that take place after 7 days ethanol intake are not readily reversible since levels do not return to normal control levels in seven days.

3.4.2 Possible Mechanisms Underlying the Protein Alterations

The first suggestion that the two lower MW proteins were the breakdown products of the high MW proteins seems unlikely but cannot be totally ruled out.

There may not be a direct link between the high and low MW proteins but rather an indirect link. The high MW proteins may normally repress production of the low MW proteins and lower levels of the high MW proteins would result in increased levels of the lower MW proteins. The opposite may also be true, in that lower levels of the low MW proteins control the levels of the high MW proteins and ethanol related raised levels of the lower MW proteins lead to a decrease in the higher MW proteins.

The protein alterations may take place at the level of transcription, translation or post-translational processing.

While it is likely that the effects described in this chapter are due to ethanol it is possible that the protein changes may be due to a metabolite of ethanol rather than ethanol itself and it has been reported (Obe & Ristow, 1979; Hayes, 1985) that acetaldehyde can produce DNA alterations. The next chapter investigates the effects of acetaldehyde on the epithelial cells in culture.

3.5 Conclusions

0.5% ethanol given to rat lingual epithelium in culture for 7 days or more significantly reduces the levels of a 230 and a 200kD protein and increases the levels of a 30

and a 28kD protein. These changes are not reversible after a further 7 days without ethanol.

It is not likely that there is a direct relationship between lowered levels of the high MW proteins and increased levels of the low MW proteins.

The changes observed are similar to those observed using a chronic alcohol animal model (Graham & Rennie, 1987).

Chapter 4

The Effect of Acetaldehyde on Epithelial Cell Viability and Protein Profile

4.1 Introduction

There is no clear evidence from experimental animal studies that ethanol alone can induce neoplastic change (Mufti, 1992), but acetaldehyde, the first product of ethanol produced by oxidative metabolism, is a potent mutagen and carcinogen (Obe & Ristow, 1979; Hayes, 1985) and has been shown to cause chromosomal aberrations, sister-chromatid exchanges and cross-linking of DNA. Additionally, it has been shown that acetaldehyde induces cellular damage and cell death in the liver through the formation of protein adducts. This may result in enzyme inactivation, decreased DNA repair, alterations in microtubules, plasma membranes and mitochondria, glutathione depletion and lipid peroxidation (Sciot & Desmet, 1992). However it has also been suggested that the transient cellular levels of this highly reactive metabolite may not reach a high enough level to pose a carcinogenic threat (Mufti, 1992) but it is recognised that in many alcohol related conditions acetaldehyde plays a major role in cellular changes.

The effects of ethanol on the protein profile of rat lingual epithelium both *in vivo* (Graham & Rennie, 1987) and *in vitro* (chapter 3) may be mediated specifically by ethanol or may be the result of the action of one of the

metabolites of ethanol rather than ethanol itself. The aim of this chapter was thus to examine the effect of the principal metabolite of ethanol on rat lingual epithelium grown in culture. The effect of a range of acetaldehyde concentrations upon epithelial cell viability and upon the protein profile of the epithelial cells in culture were studied.

4.2 Materials and Methods

4.2.1 Concentrations of Acetaldehyde

The volatile nature and transient levels of acetaldehyde in cells makes it difficult to measure cellular levels following ethanol treatment. Most methods of acetaldehyde measurement employ intricate gas chromatography techniques and it was decided that it was beyond the time frame of this thesis to pursue the measurement of acetaldehyde in epithelial cell cultures. Instead it was decided to employ a range of acetaldehyde concentrations and examine their effects upon the viability of the cells in culture. Smith *et al.* (1990) and Smith & Wubetu, (1991), working with nerve cell cultures, suggest that acetaldehyde is more toxic to the cells at 100 times lower concentration than ethanol. Espinet & Argiles (1984) found that the levels of acetaldehyde in the blood of rats, following intravenous ethanol injection was approximately five times less than the amount of ethanol present in the blood. It was therefore decided to use

0.5% acetaldehyde as the upper limit, the same concentration of ethanol employed as chapter 2, and varying concentrations down to 0.005% acetaldehyde were also employed.

4.2.2 The Effect of a Range of Acetaldehyde Concentrations on Epithelial Cell Viability

The effect of a range of acetaldehyde concentrations on epithelial cell viability was assessed using the fluorescent dye, fluorescein diacetate (Mishell & Shiigi, 1980), and as described earlier (section 2.2.4) oral epithelial cells were grown on a 3T3 feeder layer and subcultured onto plastic alone. Cells in the third passage on plastic were subcultivated onto glass coverslips. This was to facilitate the use of the fluorescent agent because some types of plastic autofluoresce.

After five days in culture acetaldehyde was added to the culture medium and the cells were incubated for varying periods. 0.5% acetaldehyde was chosen as the upper limit and concentrations of 0.25%, 0.1%, 0.05%, 0.025%. 0.01% and 0.005% acetaldehyde were also used. Control cultures were given growth medium without acetaldehyde. The time periods selected were 1, 7, 14, 21 and 30 days after the addition of acetaldehyde. Five cultures were set up for each concentration at each time point. The cells were treated with fluorescein diacetate and the percentage of

viable cells at each time point, for each concentration was determined as described in section 2.2.8.

Statistical Analysis

A covariance analysis on regression coefficients was carried out to determine if the viabilities in acetaldehyde treated cultures differed significantly from control values (Minitab).

4.2.3 The Effect of Acetaldehyde on Protein Profile

Preparation of Epithelial Cells and Addition of Acetaldehyde

Rat tongue epithelium was cultured with a 3T3 feeder layer and subcultured onto plastic alone as described in section 2.2.4. Cells in the third passage on plastic were allowed to grow for seven days, until semi-confluent, before adding acetaldehyde. Ten 25cm^2 flasks for controls and acetaldehyde treatments were used for each time point examined. Concentrations of 0.05% and 0.005% acetaldehyde were chosen since these concentrations were 10 times and 100 times lower than the 0.5% ethanol used in chapter 3. Protein analysis was carried out at 1 and 7 days for both concentrations. Epithelial cell culture medium (Table 2.2) without acetaldehyde was given to the cells as a control, and medium containing 0.05% or 0.005% acetaldehyde was given as the acetaldehyde treatment. Fresh control and acetaldehyde medium was prepared and added to cultures every 24 hours.

4.2.4 Preparation of Epithelial Cells for SDS-PAGE

At each time point ten treatment flasks and ten control flasks, for each concentration, were prepared for SDS-PAGE (section 3.2.2) and the concentration of protein in each of the epithelial preparations was determined as described previously (section 3.2.3). Electrophoresis and the staining and assessment of gels were carried out as described in chapter 3 (sections 3.2.4, 3.2.5 and 3.2.6, respectively).

4.2.5 Statistical Analysis

A non-parametric statistical test, the Mann-Whitney U test was used for comparisons of the acetaldehyde and control groups (Minitab). As four proteins were initially compared at each time point the level of significance was set at 0.025 ($0.05/\sqrt{4}=0.025$, where 4=number of comparisons) in order to compensate for multiple comparisons (Brown & Swanson Beck, 1988). A 70kD protein was examined separately and comparisons were made at two time points in two acetaldehyde concentrations. Since the test was carried out four times for this protein the level of significance was again set at 0.025.

4.3 Results

4.3.1 The Effect of Acetaldehyde on Epithelial Cell Viability

Since five cultures were examined at each time point n=5 for the purposes of standard errors. In an acetaldehyde concentration of 0.5% more than 90% (s.e.=1.63) of cells died in 24 hours (Fig. 4.1.a). In 0.25% acetaldehyde 24% (s.e.=3.26) of cells survived for 24 hours but at 7 days only 7% of the cells remained viable (s.e.=2.81) and there were no cells surviving by 14 days (Fig. 4.1.b). In 0.1% acetaldehyde the percentages of surviving cells were slightly higher with 47% of cells viable after 24 hours (s.e.=3.74), 23% viable at 7 days (s.e.=4.82) but none survived after 14 days (Fig. 4.1.c).

In 0.05% acetaldehyde 57% (s.e.=2.50) of cells survived for 24 hours, 40% survived for 7 days (s.e.=3.31) and again there were no viable cells after 14 days (Fig. 4.1.d). Cells given 0.025% acetaldehyde showed a similar pattern of decrease in viability as those of 0.05%, with slightly higher percentages of surviving cells at each time point. After 24 hours 61% (s.e.=2.91) of cells were viable, 44% (s.e.=5.99) were viable after 7 days and 1% (s.e.=0.45) survived for 14 days (Fig. 4.1.e).

In 0.01% acetaldehyde 63% (s.e.=3.81) of the cells survived for 24 hours, 46% (s.e.=5.63) were viable after 7 days and at 14 days 2% (s.e.=1.26) of the cells

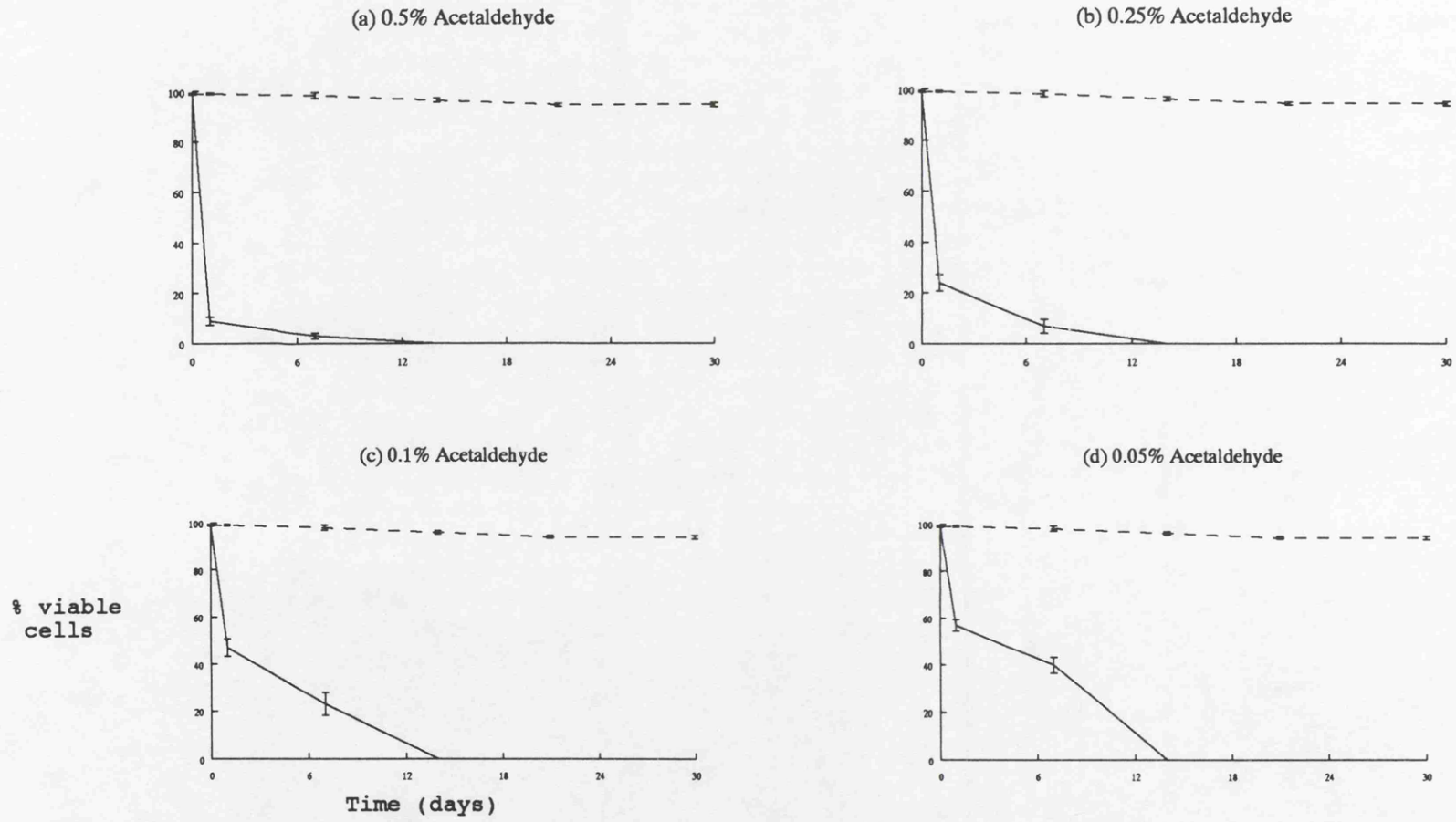


Figure 4.1 (a-d) Shows the effect of a range of acetaldehyde concentrations (%) on epithelial cell viability (%) over a 30 day period. Each point represents the mean and standard error of five measurements. ---- represents 0% acetaldehyde ——— represents acetaldehyde concentration where graph (a) 0.5% acetaldehyde, (b) 0.25% acetaldehyde, (c) 0.1% acetaldehyde and (d) 0.05% acetaldehyde. The x-axis and y-axis legends for all the graphs are identical.

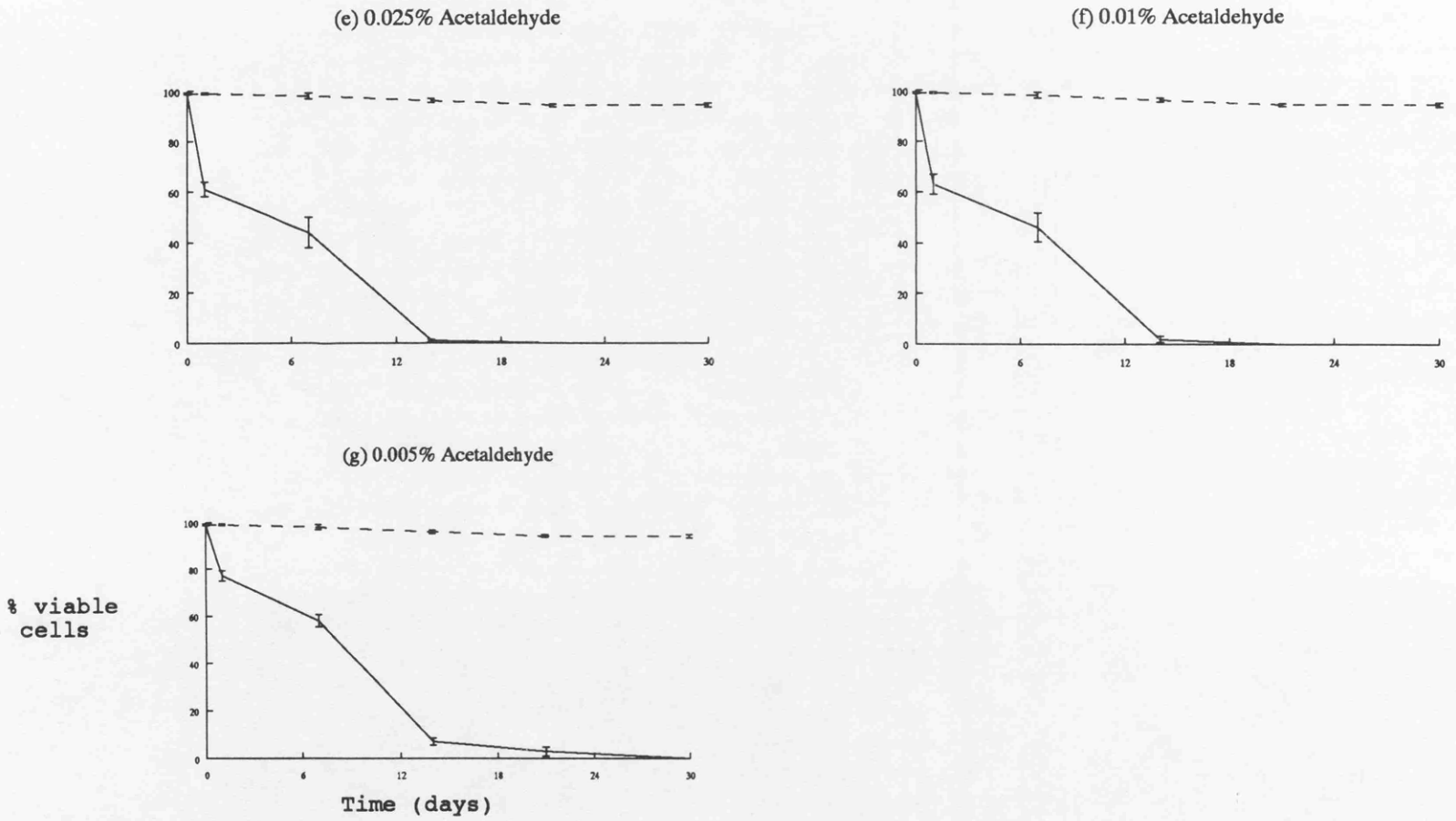


Figure 4.1 (e-g) Shows the effect of a range of acetaldehyde concentrations (%) on epithelial cell viability (%) over a 30 day period. Each point represents the mean and standard error of five measurements. – – – – represents 0% acetaldehyde ——— represents acetaldehyde concentration where graph (e) 0.025% acetaldehyde, (f) 0.01% acetaldehyde and (g) 0.005% acetaldehyde. The x-axis and y-axis legends for all the graphs are identical.

remained viable (Fig. 4.1.f). The pattern of decrease was also similar for cells grown in 0.005% acetaldehyde, but with slightly higher values at each time point. After 24 hours 77% (s.e.=2.18) of cells remained viable, 58% (s.e.=2.60) were viable at 7 days, 7% (s.e.=1.59) survived for 14 days and 3% (s.e.=1.88) of the cells were viable at 21 days (Fig.. 4.1.g). No cells survived for the full 30 day study. A covariance analysis on regression coefficients showed that cell viabilities in acetaldehyde treated cultures were significantly lower than those in control cultures. (F values: 0.5% F=61.65, 0.25% F=85.30, 0.1% F=169.70, 0.05% F=279.17, 0.025% F=269.19, 0.01% F=286.57, 0.005% F=497.30, all were significant at $p<0.001$).

A summary graph showing the time taken for each concentration of acetaldehyde to kill 50% of the cells is shown in Figure 4.2. These values were taken from Figure 4.1(a-g).

4.3.2 The Effect of Acetaldehyde on Protein Profile

The protein profiles of the acetaldehyde treated and control cells were compared by visual and densitometric means.

Visual Comparison

Example lanes from the SDS polyacryamide gels of the lingual epithelial proteins from the 0.05% and 0.005%

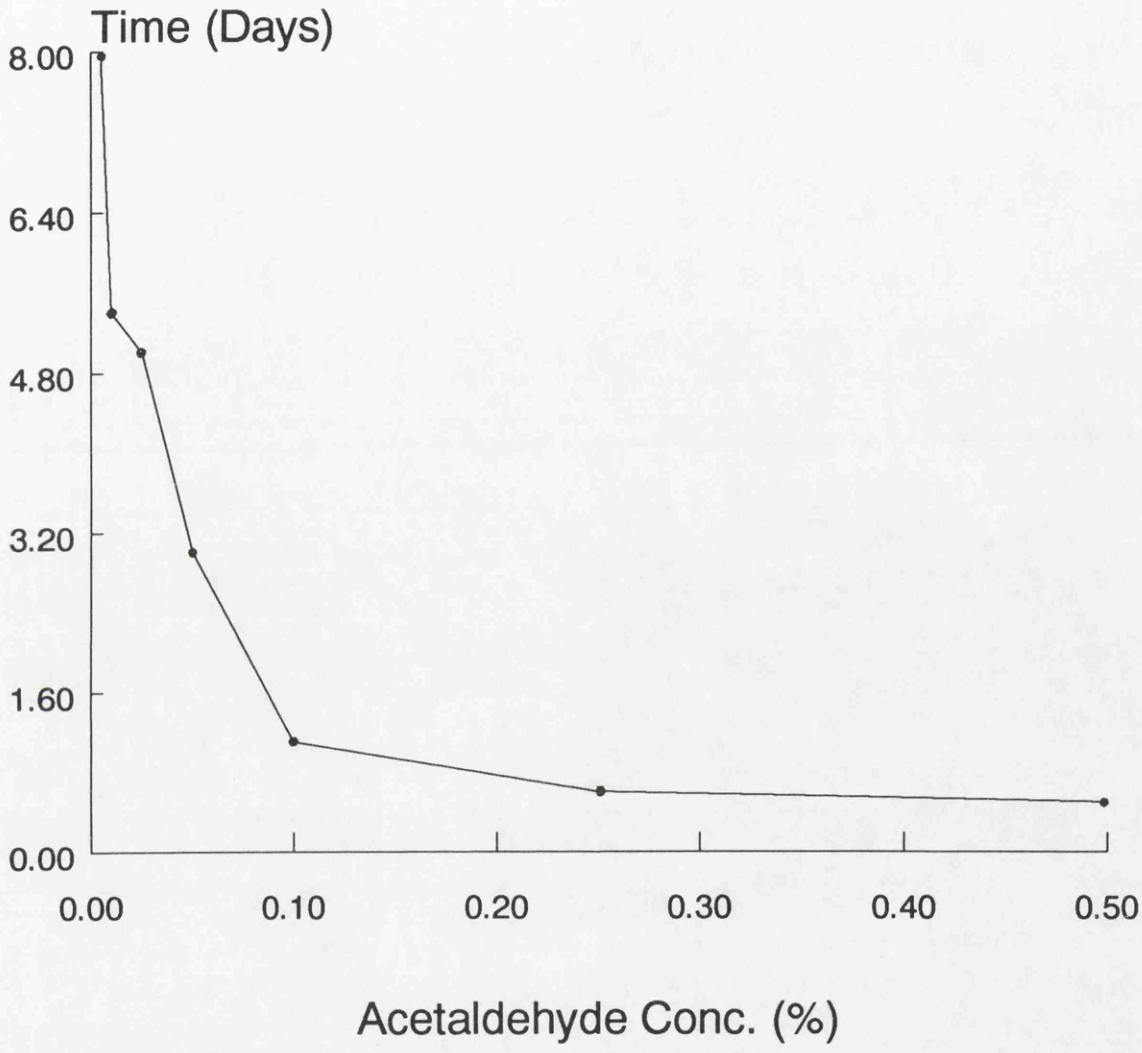


Figure 4.2 This figure presents a summary of the time taken for each concentration of acetaldehyde to kill 50% of the epithelial cells (LT50).

acetaldehyde treated cells and control cells from 1 and 7 days are shown in Figures 4.3-4.6. Visual comparison between acetaldehyde treated cells and control cells suggested a reduction in the levels of a 230kD and a 200kD protein in both acetaldehyde treatments at 1 and 7 days. As with the ethanol protein study no alterations in the levels of low MW proteins were noted by visual analysis.

Visual analysis also revealed an increase in the levels of a 66-70kD protein in the acetaldehyde treated cells when compared with control cells. This protein will be discussed separately from those common to both alcohol and acetaldehyde treatments and will be referred to as the 70kD protein.

Densitometric analysis

Densitometric analysis confirmed the visual analysis concerning the reduction in levels of the 230kD and 200kD proteins in both acetaldehyde concentrations at both time points. It also revealed an increase in the levels of a 30kD and a 28kD protein in both acetaldehyde treatments at 1 and 7 days.

The 70kD protein was also shown to increase in all acetaldehyde treatments at 1 and 7 days when examined densitometrically.

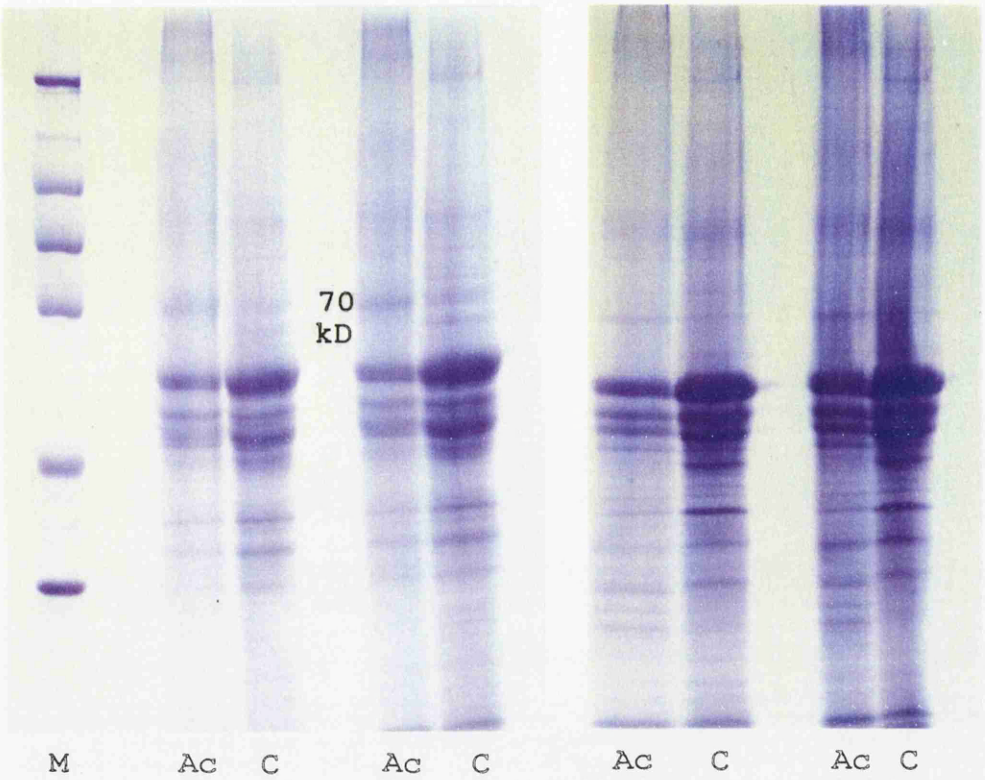


Figure 4.3 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.005% acetaldehyde in the culture medium for 1 day. Ac - Acetaldehyde C - Control M - Molecular weight markers.

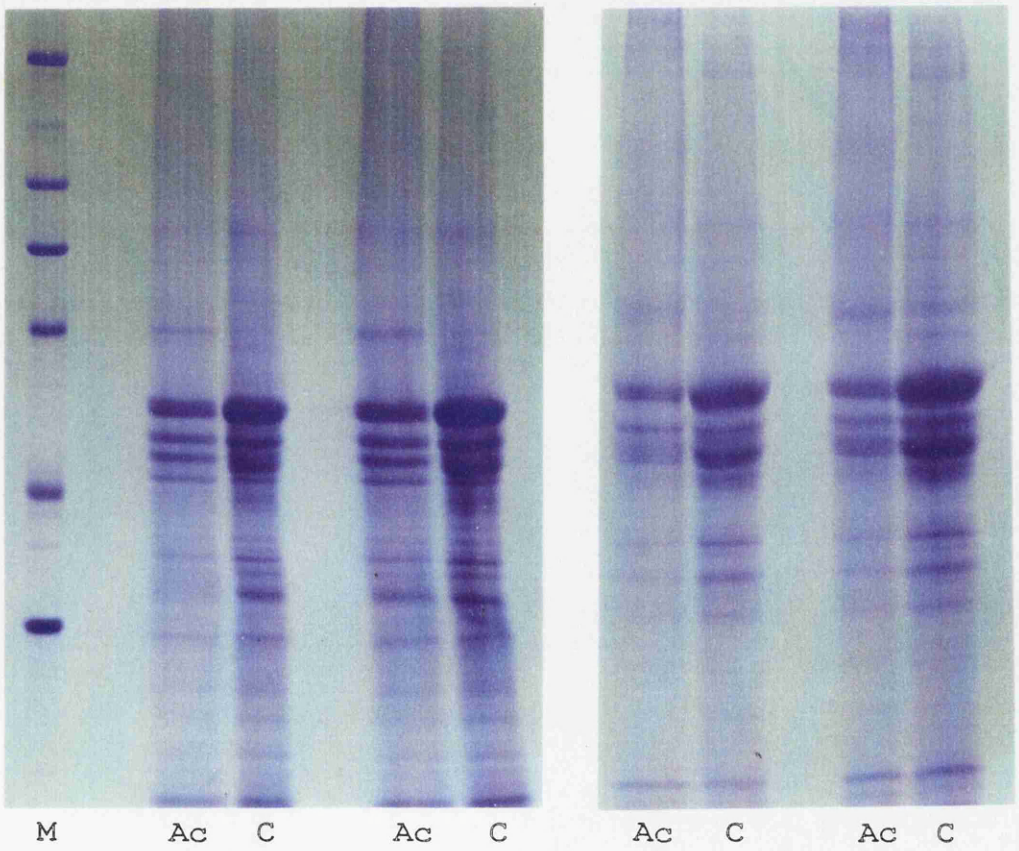


Figure 4.4 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.005% acetaldehyde in the culture medium for 7 days. Ac - Acetaldehyde C - Control M - Molecular weight markers.

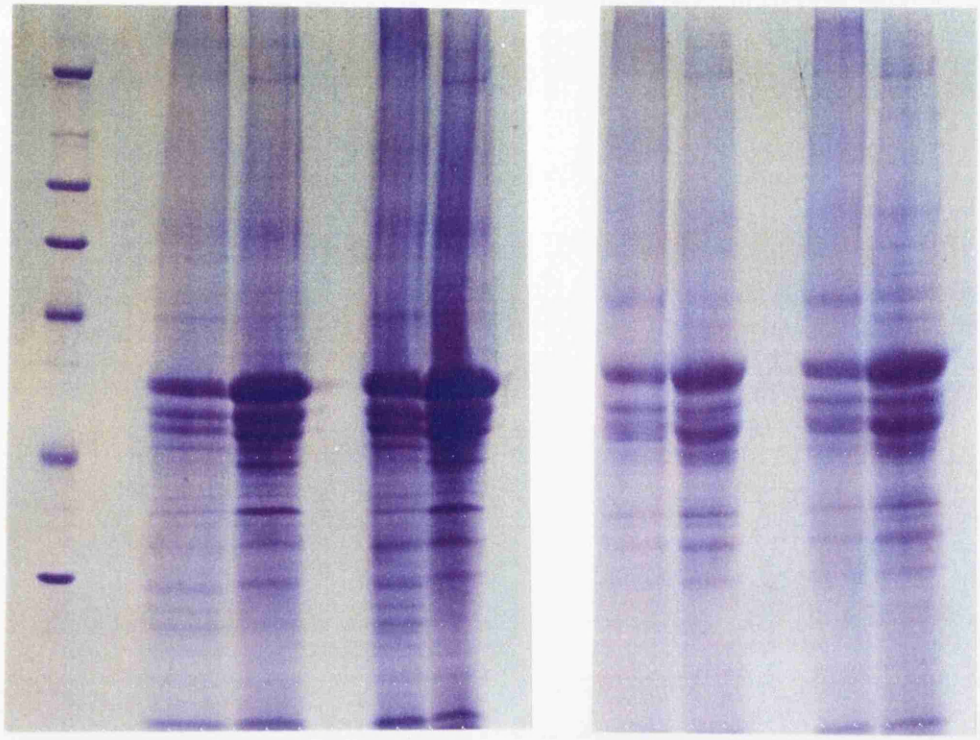


Figure 4.5 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.05% acetaldehyde in the culture medium for 1 day. Ac - Acetaldehyde C - Control M - Molecular weight markers.

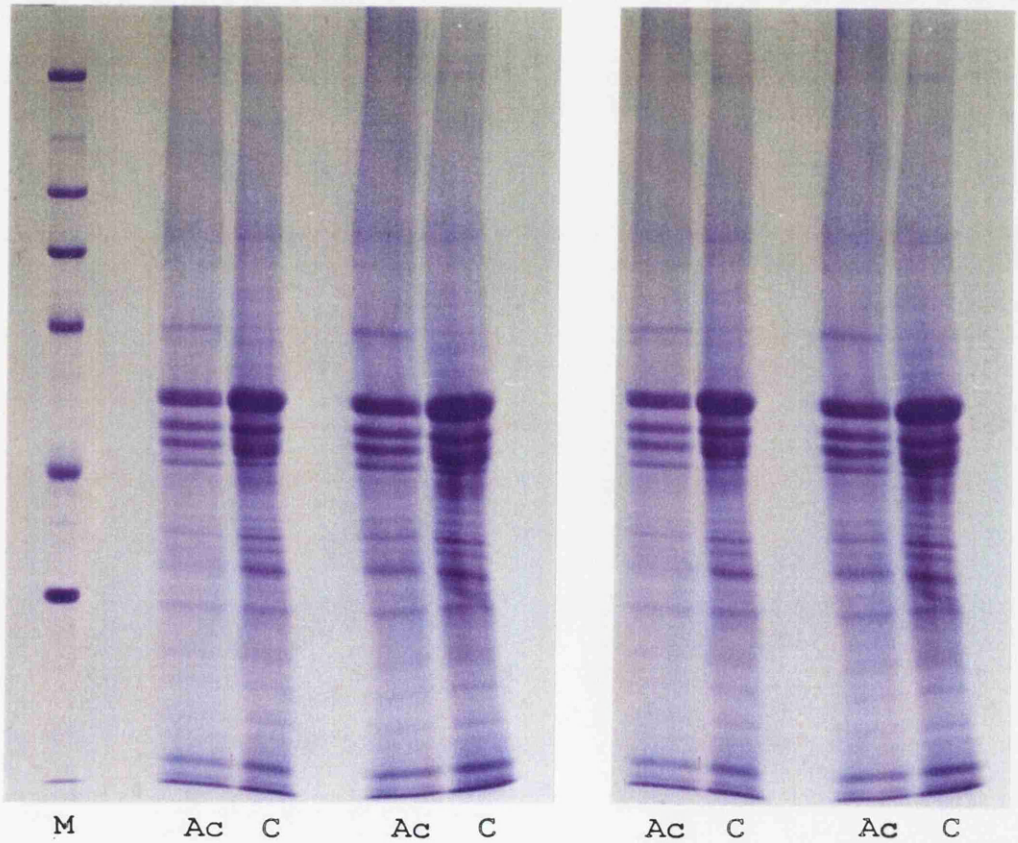


Figure 4.6 Shows example lanes from gels of rat lingual epithelial proteins in the presence and absence of 0.05% acetaldehyde in the culture medium for 7 days. Ac - Acetaldehyde C - Control M - Molecular weight markers.

1 Day 0.005% Acetaldehyde

Table 4.1 shows the mean values of the area percentages of the 230, 200, 30 and 28kD proteins after 1 day of 0.005% acetaldehyde treatment. Levels of the 230kD protein showed a decrease from 1.23 to 0.22% in 0.005% acetaldehyde. Levels of the 200kD protein decreased from 1.68 to 0.13% when cells were given acetaldehyde for 1 day. These differences in levels were statistically significant (230kD, $p<0.005$; 200kD, $p<0.002$).

Table 4.1 also shows that the mean area percentages of the 30kD and 28kD protein were higher in acetaldehyde treated cells than in controls (6.11 and 6.33% in acetaldehyde and 4.43 and 4.00% in controls for 30kD and 28kD proteins, respectively). The increases were statistically significant (30kD, $p<0.006$; 28kD $p<0.0002$).

7 Days 0.005% Acetaldehyde

After 7 days in 0.005% acetaldehyde densitometric analysis revealed a 14 fold decrease in levels of the 230kD protein from 1.50 to 0.11% and a decrease in levels of the 200kD protein from 1.47 to 0.16% (Table 4.2). These changes were statistically significant ($p<0.0002$).

The mean relative percentage areas for the two lower MW proteins increased slightly when cells were given 0.005% acetaldehyde for 7 days (Table 4.2). The 30kD protein increased significantly from 5.12 to 6.98% ($p<0.0006$) and

Table 4.1 Relative area percentage of protein bands from 1 day 0.005% acetaldehyde treated cells and control cells. Ac - acetaldehyde C - control.

Protein	230kD		200kD		30kD		28kD	
	Ac	C	Ac	C	Ac	C	Ac	C
Rel.%	0.01	0.21	0.00	1.00	5.11	4.00	6.01	3.10
Area	0.11	0.41	0.00	0.99	4.10	3.81	5.61	3.00
	0.55	1.51	0.22	2.10	6.17	4.81	6.81	4.79
	0.44	1.70	0.34	2.31	8.10	5.17	7.95	4.98
	0.31	0.30	0.00	0.85	5.48	4.13	6.16	3.91
	0.05	0.33	0.01	0.40	4.44	4.19	5.00	4.00
	0.04	1.91	0.01	2.40	6.16	4.01	6.20	3.99
	0.18	2.00	0.20	2.20	8.22	5.67	6.99	4.10
	0.21	1.87	0.28	2.17	5.91	4.00	6.11	4.15
	0.32	2.09	0.20	2.33	7.36	4.50	6.44	3.98
X	0.22	1.23	0.13	1.68	6.11	4.43	6.33	4.00
S.D.	0.18	0.81	0.13	0.77	1.42	0.61	0.80	0.62
S.E.	0.06	0.26	0.04	0.24	0.45	0.19	0.25	0.20

n=10

Table 4.2 Relative area percentage of protein bands from 7 day 0.005% acetaldehyde treated cells and control cells. Ac - acetaldehyde C - control.

Protein	230kD		200kD		30kD		28kD	
	Ac	C	Ac	C	Ac	C	Ac	C
Rel.%	0.91	1.05	0.99	1.00	7.65	6.99	6.48	5.97
Area	0.09	1.00	0.40	0.98	8.10	7.00	5.40	3.01
	0.06	1.91	0.03	1.97	5.98	5.00	6.14	3.99
	0.00	1.14	0.04	1.10	7.51	4.58	5.94	3.50
	0.04	1.96	0.01	1.30	6.10	3.98	6.20	3.10
	0.00	1.51	0.08	1.42	8.30	5.04	7.14	4.13
	0.01	1.90	0.00	1.70	6.52	5.10	7.00	4.02
	0.03	1.77	0.00	1.71	8.11	4.99	6.96	3.99
	0.00	1.41	0.00	1.90	6.10	4.51	5.78	4.00
	0.00	1.30	0.00	1.61	5.38	3.99	4.50	2.98
X	0.11	1.50	0.16	1.47	6.98	5.12	6.15	3.87
S.D.	0.28	0.37	0.32	0.36	1.07	1.07	0.81	0.87
S.E.	0.09	0.12	0.10	0.12	0.34	0.34	0.26	0.28

n=10

the 28kD protein increased significantly from 3.87 to 6.15% (P<0.0006).

1 day 0.05% Acetaldehyde

Table 4.3 shows that there was a 17 fold decrease in the mean percentage area of the 230kD protein after 1 day in 0.05% acetaldehyde. This decrease from 1.86 to 0.11% was statistically significant (p<0.0002). The 200k protein showed a marked decrease from 2.32 to 0.07%. This decrease was again significant (p<0.0002).

Table 4.3 also shows that levels of the 30kD protein increased significantly from 4.19 to 6.90% in the acetaldehyde group (p<0.0002) and levels of the 28kD protein increased significantly from 3.20 to 5.35% (p<0.0002).

7 days 0.05% Acetaldehyde

After 7 days in 0.05% acetaldehyde the mean percentage areas for the 230kD and 200kD proteins showed a 32 fold and a 22 fold decrease, respectively (Table 4.4). The 230kD protein showed a significant decrease from 0.96 to 0.03% (p<0.002) and the 200kD protein decreased significantly from 1.33 to 0.06% (p<0.002). The mean percentage area for the 30kD protein increased by 1.5 times in 0.05% acetaldehyde (Table 4.4). The increase from 4.53 to 6.83% was statistically significant (p<0.0002). Levels of the 28kD protein increased 3.69 to

Table 4.3 Relative area percentage of protein bands from 1 day 0.05% acetaldehyde treated cells and control cells. Ac - acetaldehyde C - control.

Protein	230kD		200kD		30kD		28kD	
	Ac	C	Ac	C	Ac	C	Ac	C
Rel.% Area	0.10	2.30	0.05	2.90	5.98	4.00	5.11	3.59
	0.21	1.00	0.01	2.00	7.40	4.58	5.00	3.01
	0.00	2.01	0.00	2.44	6.15	4.10	4.99	3.08
	0.05	2.24	0.10	2.56	7.89	3.99	5.21	2.99
	0.20	1.99	0.31	2.65	8.01	5.11	6.15	4.10
	0.19	1.51	0.00	2.01	6.98	4.19	4.03	3.00
	0.09	2.11	0.06	2.41	6.09	4.20	5.58	2.86
	0.11	1.71	0.10	2.00	7.11	3.80	6.01	2.98
	0.05	1.86	0.00	2.08	6.58	3.91	5.90	3.57
	0.10	1.91	0.05	2.10	6.86	4.04	5.54	2.81
X	0.11	1.86	0.07	2.32	6.90	4.19	5.35	3.20
S.D.	0.07	0.38	0.09	0.32	0.72	0.39	0.63	0.42
S.E.	0.02	0.12	0.03	0.10	0.23	0.12	0.20	0.13

n=10

Table 4.4 Relative area percentage of protein bands from 7 day 0.05% acetaldehyde treated cells and control cells. Ac - acetaldehyde C - control.

Protein	230kD		200kD		30kD		28kD	
	Ac	C	Ac	C	Ac	C	Ac	C
Rel.% Area	0.10	1.94	0.04	1.90	5.95	4.31	6.01	3.86
	0.04	1.01	0.08	1.71	8.46	4.59	7.41	3.99
	0.04	1.11	0.01	1.80	6.01	5.00	6.98	3.01
	0.01	1.10	0.00	1.31	7.11	4.48	5.87	3.18
	0.03	0.91	0.00	1.11	6.57	3.98	5.88	3.00
	0.00	0.00	0.00	0.00	7.08	4.50	6.99	4.00
	0.05	0.91	0.08	0.99	8.10	5.00	7.98	4.54
	0.00	0.51	0.00	0.40	7.44	5.54	6.00	4.10
	0.07	1.10	0.00	2.00	5.61	3.99	4.84	3.57
	0.00	1.00	0.41	2.03	6.00	3.89	5.50	3.61
X	0.03	0.96	0.06	1.33	6.83	4.53	6.35	3.69
S.D.	0.03	0.49	0.13	0.70	0.97	0.53	0.96	0.51
S.E.	0.01	0.16	0.04	0.22	0.31	0.17	0.30	0.16

n=10

6.35%. This increase was also statistically significant ($p<0.0002$).

The 70kD Protein

Densitometric analysis confirmed the increase in levels of a 70kD protein in all acetaldehyde treatments compared with controls (Table 4.5). In cells given 0.005% acetaldehyde for 1 day levels of a 70kD protein increased by 2 fold from 2.81 to 6.31%. This increase was statistically significant ($p<0.0002$). Following 7 days 0.005% acetaldehyde treatment the percentage area of the 70kD protein increased from 3.19 to 7.61%. This was statistically significant ($p<0.003$).

Table 4.5 also shows an increase in the levels of this protein after 1 day 0.05% acetaldehyde treatment. The increase from 2.02 to 7.26 was statistically significant ($p<0.0002$). After 7 days 0.05% acetaldehyde treatment levels of the 70kD protein showed a 3 fold increase from 2.48 to 6.85%. This increase was again statistically significant ($p<0.0002$).

4.4 Discussion

4.4.1 The Effect of Acetaldehyde on Cell Viability

There are suggestions that acetaldehyde is more toxic to cells in culture than ethanol, and at lower concentrations (Smith *et al.*, 1990; Smith & Wubetu,

Table 4.5 Relative area percentage of a 70kD protein in 1 and 7 day 0.005% and 0.05% acetaldehyde treated cells and control cells. Ac - acetaldehyde C - control.

	0.005% 1 day		0.005% 7 days		0.05% 1 day		0.05% 7 days	
	Ac	**C**	**Ac**	**C**	**Ac**	**C**	**Ac**	**C**
Rel.%	5.01	3.18	7.71	7.59	5.54	1.98	5.35	2.01
Area	6.11	3.70	8.51	8.33	7.01	0.99	5.21	1.24
	6.90	3.55	7.35	1.99	7.44	1.88	5.91	1.99
	7.45	2.30	7.77	2.03	8.91	2.67	7.00	4.13
	6.08	3.34	8.41	2.19	7.31	1.70	8.55	3.90
	5.90	3.65	8.22	2.04	5.11	0.87	7.98	3.68
	6.44	2.35	8.07	2.30	7.56	2.10	6.64	2.03
	7.15	2.11	8.90	1.99	8.22	3.07	7.04	2.00
	6.10	2.00	5.88	1.87	7.12	1.79	7.11	1.97
	5.98	1.95	5.30	1.59	8.41	3.14	7.68	1.86
X	6.31	2.81	7.61	3.19	7.26	2.02	6.85	2.48
S.D.	0.71	0.73	1.16	2.53	1.19	0.77	1.10	1.01
S.E.	0.22	0.23	0.37	0.80	0.38	0.24	0.35	0.32

n=10

1991). Most studies have been carried out using neurones but the study reported in this chapter shows this is also the case for oral epithelium *in vitro*. Epithelial cells grown in 0.5% ethanol survived for up to 30 days (Fig. 2.5.g) whereas most of the cells given the same concentration of acetaldehyde died within 24 hours (Fig. 4.1.a). This pattern was repeated when cells were given 0.25% acetaldehyde (Fig. 4.1.b), although *in vivo* cells would not be subjected to such high concentrations of acetaldehyde.

Espinet & Argiles (1984) found blood acetaldehyde levels to be five times less than the amount of ethanol injected. In this study this concentration would correspond to 0.1% acetaldehyde, but, at this acetaldehyde concentration 50% of the cells died within 24 hours and there were none surviving by 14 days. If so many cells die in acetaldehyde concentrations recorded in the blood of test animals (Espinet & Argiles, 1984) more damage would be expected after higher alcohol intake than is observed. However it must be noted that levels of acetaldehyde are usually transient and, in the experiment reported by Espinet & Argiles (1984), the ethanol was injected into the rats and not taken orally. Smith *et al.* (1990) and Smith & Wubetu (1991) observed that acetaldehyde was still more toxic to nerve cells at concentrations of 100 times lower than ethanol concentrations and this also seems to be true for oral

epithelium. Although more cells survived in the concentrations 0.05-0.005% (Fig. 4.1.d-g) than in the higher concentrations of acetaldehyde (Fig. 4.1.a-c), the cells were nevertheless dead by 14 days.

From the results reported in this study acetaldehyde appears to be more toxic to oral epithelium grown in culture and at concentrations, 10 and 100 times less than the ethanol concentrations used in the viability and protein studies (section 2.2.8 and 3.1.2).

4.4.2 The Effect of Acetaldehyde on Protein Profile

As reported in chapter 3, it took 7 days of 0.5% alcohol to alter the protein profile of the epithelial cells in culture. The work reported in this chapter shows that concentrations of acetaldehyde 10 and 100 times lower than ethanol concentrations can alter the levels of the same proteins after 1 day.

After 7 days 0.5% alcohol treatment levels of a 230kD and 200kD protein decreased while levels of a 30kD and 28kD protein increased. This was also the case following 1 and 7 days of 0.05% and 0.005% acetaldehyde treatment (Tables 4.1-4.4). The changes in levels of the high MW proteins in acetaldehyde treated cells were of a similar magnitude to those in cells given 0.5% ethanol for 14 days or more, and changes in levels of the lower MW proteins in

acetaldehyde treated cells were of a similar order to those in cells given alcohol for 7 days or more.

It therefore seems likely that the protein changes observed when alcohol is added to cultures do not occur as a direct result of ethanol, but are acetaldehyde mediated. This is in agreement with some *in vivo* studies which have suggested that the primary metabolite of ethanol, namely acetaldehyde, is a potent mutagen and carcinogen (Obe & Ristow, 1979; Hayes, 1985).

There was also an increase in levels of a protein with a MW of approximately 70kD in all of the acetaldehyde treated cultures (Table 4.5). This protein change was not observed in ethanol treatments.

Siyam (1987) treated rat tongue epithelium *in vitro* with 5% alcohol for 1 hour. At 12 and 24 hours following the removal of the alcohol there was an increase in levels of 2 proteins of MW 92.5kD and 71kD. It was suggested that these were heat shock proteins (HSP).

It is possible that the 70kD protein observed in this study could be a HSP. Levels of this protein increased after 1 day in acetaldehyde but did not change with ethanol treatment. The protein changes common to both acetaldehyde and ethanol were also brought about after 1 day acetaldehyde treatment and Graham (1987) had

suggested that the two lower MW proteins which increased in levels in alcoholic animals could be HSPs. This is possible but seems unlikely in this study due to the time scale of the changes in the ethanol treated cells. It took 7 days in ethanol to bring about these protein changes and they were not specific to the more potent acetaldehyde treatment, whereas the 70kD protein changes were. This would suggest that the 30kD and 28kD proteins are not HSPs, whereas the 70kD protein may be a heat shock related protein.

Siyam (1987) used 5% alcohol and as discussed in chapter 2, 0.4% is one of the highest recorded blood alcohol levels. 5% alcohol would therefore have exerted great stress on the cells and could lead to an increase in or appearance of HSPs. The ethanol concentrations used in the present investigations were similar to high blood alcohol levels and would not exert the same stress on the cells as 5% ethanol. Acetaldehyde, which is more potent than the ethanol, would exert more stress and is therefore more likely to induce production of HSPs than the ethanol. The possibility of any of the proteins being HSPs is further explored in chapter 5. In this chapter a study on the effects of heat shock treatment on the protein profile of the cells in culture is presented and any alterations compared with those observed in alcohol and acetaldehyde treatments.

4.5 Conclusions

The primary metabolite of ethanol oxidation, acetaldehyde, is more toxic to oral epithelium grown in culture than ethanol.

Acetaldehyde alters the protein profile of the epithelial cells in culture at lower concentrations and more rapidly than ethanol, but in similar ways.

Acetaldehyde also alters the levels of a 70kD protein that is not altered in ethanol. This protein may be a HSP and this possibility is further investigated in the next chapter.

Although it was not possible to undertake the somewhat difficult methods of acetaldehyde measurement in this thesis it would be worthwhile measuring the levels of acetaldehyde present in the culture medium of oral epithelial cells receiving alcohol.

Chapter 5

The Effect of Heat Shock on Epithelial Cell Viability and Protein Profile

5.1 Introduction

Mammalian cells subjected to elevated temperatures and other stresses often respond by transient production of several families of stress (heat shock) proteins (HSP). HSPs are defined as proteins which increase in levels as a result of an external stress exerted on the cell/organism. Ethanol, heavy metals, amino acid analogues and recovery from anoxia can induce a stress response in cells (Stoklosinski *et al.*, 1992). Li (1983) demonstrated the synthesis of HSPs in hamster fibroblasts in response to *in vitro* ethanol treatment and Rodenhiser, Jung & Atkinson (1985) produced a stress response in mammalian lymphocytes. They reported that heat treatment of over 41°C stimulated the production of several HSPs ranging in MW from 27-110kD. Burdon (1986) has also discussed this stress response to temperature and ethanol, and notes that one of the HSPs expressed in most of the cell types studied has a MW of 70kD. HSPs have also been observed in bacteria and fungi and Zoeger *et al.* (1992) reported that starvation induced the production of several proteins in the fungus *Neurospora crassa*, some of which were HSPs.

These proteins have been reported to be associated with both the cytoplasmic and the membrane/microsomal

fractions of the cell (Pelham, Munro & Lewis, 1986) and are thought to protect cells from the adverse consequences of heat and other stresses (Stoklosinski *et al.*, 1992). They may be important for the development of thermotolerance and/or cell survival under conditions of environmental stress (Rodenhiser *et al.*, 1985). However there is very little information available on how these proteins perform their protective role.

During chronic exposure of a biological system to ethanol the cell membrane may become more rigid (Rottenberg *et al.*, 1981) and may be altered chemically to compensate for the continuous presence of ethanol. This development of tolerance to ethanol may be associated with alterations in the phospholipids and an increase in cholesterol content. However it is not known whether this is solely a lipid response or whether membrane proteins are involved. If they are this may be a heat shock/stress response, although this increase in membrane order has not been consistently observed (Harris *et al.*, 1984).

A common effect of stress conditions within a cell is an alteration in protein folding. A group of stress proteins, known as glucose-related proteins (GRP) are thought to recognise and bind to misfolded proteins in the endoplasmic reticulum to prevent their aggregation (Pelham, 1986) and so serve to protect the cell.

The expression of the genes responsible for the production of HSPs is not limited to cells undergoing some form of stress and some HSPs are already present in the cells, being expressed constitutively (Barnier *et al.*, 1987; Milarski & Morimoto, 1989).

The heat shock response can also include the inhibition of "normal" protein synthesis and can affect cytoskeletal morphology, cell growth and intracellular calcium concentrations (Burdon, 1986; Stoklosinski *et al.*, 1992).

The aim of the work reported in this chapter was to heat shock cells (Stoklosinski *et al.* (1992) to examine the effects on epithelial cell viability and protein profile. The results of protein analysis could then be compared with those from the ethanol and acetaldehyde studies (chapters 3 and 4, respectively) to investigate the possibility that the increased levels of the 30kD, 28kD and 70kD proteins described in these chapters were indeed due to the production of HSPs.

Additionally, a commercially available anti-HSP 70kD antibody (Santa Cruz Biotechnology) was used to study the 70kD protein which increased in levels in acetaldehyde treated cells (chapter 4). This antibody was used with a fluorescein labelled second antibody in order to identify (subjectively), by immunofluorescent staining, increased levels of production of this protein. This would indicate

if the 70kD protein in the cells was the 70kD HSP. Once immunofluorescent staining had been carried out an alternative strategy to further study this problem was to employ Western blotting (Towbin, Staehelin & Gordon, 1979; Burnette, 1981). This is a rapid and sensitive assay for the detection and characterisation of proteins. This technique combines electrophoretic separation of proteins with immunological identification. The technique involves the transfer of the electrophoretically separated proteins to nitrocellulose which can then be used in an enzyme immuno-assay to identify and characterise specific protein bands.

5.2 Materials and Methods

5.2.1 Heat Shock and Determination of Cell Viability

Rat tongue epithelium was cultured with a 3T3 feeder layer and subcultured on a plastic surface (section 2.2.4). Cells in the third passage on plastic were seeded on glass coverslips and allowed to grow for five days, following which time three heat shock treatments were applied to the cells. These treatments were 60°C, 50°C and 45°C. Cultures were placed in incubators at the three temperatures for a five minute period after which time some cultures were examined immediately while the other cultures were allowed to recover for 1 hour, 12 hours and 24 hours at 32°C. Control cultures remained in 32°C throughout the study. Five culture dishes were set up for

each temperature at each time point and cell viability was assessed using the vital stain fluorescein diacetate (Mishell & Shiigi, 1980). The cells were stained with fluorescein diacetate and the percentage of viable cells determined as described in section 2.2.8.

Statistical Analysis

A covariance analysis on regression coefficients was carried out to determine if the percentage of viable cells in heat treated cultures differed significantly from control values (Minitab).

5.2.2 The Effect of Heat Shock on Protein Profile

Preparation of Epithelial Cells and Heat Shock Treatment

Rat tongue epithelial cells were cultured with 3T3 feeder cells and subcultured onto plastic alone as described in section 2.2.4. Cells in the third passage on a plastic substrate were allowed to grow for seven days, until semi-confluent, before heat shock treatment was applied. Ten 25cm^2 culture flasks for both controls and heat shock treatments were used at each time point examined.

The temperature of 50°C was chosen since this was the highest temperature that maintained a reasonably high cell survival (section 5.3.1) and so would give a maximum heat shock effect. Epithelial cells were given a heat shock treatment of 50°C by placing cultures in an incubator at this temperature for five minutes and ten

flasks were prepared for SDS-PAGE analysis immediately after treatment. The other cultures were allowed to recover at 32°C and prepared for SDS-PAGE at 1 hour, 12 hours and 24 hours after the heat shock treatment. The ten control flasks and ten heat shock flasks for each time point were prepared for SDS-PAGE as described in section 3.2.2 and the concentration of protein determined by the method of Bradford (1976).

One-dimensional Discontinuous SDS Polyacrylamide Gel Electrophoresis

Electrophoresis and the staining and assessment of gels were carried out as described in chapter 3 (sections 3.2.4, 3.2.5 and 3.2.6, respectively).

Statistical Analysis

A non-parametric statistical test, the Mann-Whitney U test was used for comparisons of the heat shock and control groups (Minitab). As the test was carried out four times the level of significance was set at 0.025 ($0.05/\sqrt{4}=0.025$, where 4=number of comparisons) in order to compensate for multiple comparisons (Brown & Swanson Beck, 1988).

5.2.3 Investigation of the Acetaldehyde Altered 70kD Protein using Immunofluorescent Staining

To explore further the possibility that the increased levels of the 70kD protein (section 4.3.2) was a HSP,

control cells, heat shock treated cells and acetaldehyde treated cells were incubated with an anti-HSP 70kD antibody raised in mouse (Santa Cruz Biotechnology).

Epithelial cells in the third passage on plastic were cultured on glass coverslips and grown for five days. The cells were then given a 50°C heat shock (section 5.2.1) or acetaldehyde treatment (section 4.2.1). Those given heat shock treatment were fixed and stained immediately, 1, 12 and 24 hours after treatment. Acetaldehyde treated cells, given 0.005% and 0.05% acetaldehyde, were prepared for immunofluorescence at one and seven days. Control cells were fixed and stained at corresponding times for both treatments.

The coverslips were placed in a coplin dish, rinsed three times with PBS and fixed for ten minutes at -20°C in acetone. The fixed cells were washed three times with PBS and left in PBS for ten minutes to rehydrate. The first antibody was placed on the coverslips at a dilution of 1:50 and the coverslips were incubated for 60 minutes at 37°C in a humidified chamber. Following incubation with the anti-HSP 70kD antibody coverslips were rinsed thoroughly with PBS (at least four washes, two minutes per wash). Prior to the addition of the second antibody the cells were incubated in rabbit serum for 30 minutes to block any cross reaction of mouse and rabbit antibodies. After washing in PBS rabbit anti-mouse

immunoglobulin conjugated with fluorescein isothiocyanate isomer 1 (Dakopatts) was applied at a dilution of 1:10 for 60 minutes at 37°C in a humidified chamber. After incubation the coverslips were rinsed four times in PBS (at least two minutes per wash) and mounted on a slide with 10% glycerol buffer. The cells were viewed using a Nikon fluorescence microscope. Fixation alone, non-immune mouse serum applied as the first antibody and the second antibody alone were used as controls.

5.2.4 Investigation of the 70kD Protein using a Western Blot

Due to a shortage of available tissue heat treated cells were used for the Western blot, although it would have been better to examine both heat and acetaldehyde treated cells. Cells were cultured with 3T3 feeder cells and subcultured on plastic surfaces as described in section 2.2.4. Cells in the third passage on a plastic substrate were allowed to grow for seven days before heat shock treatment was applied. The cultures were then given a heat treatment of 50°C as described in section 5.2.2 and the cells allowed to recover at 32°C. Cells were prepared for SDS-PAGE as described in section 3.2.2. Gels which were to be Western blotted were prepared as described in section 3.2.4 but were not stained. Gels containing molecular weight markers were used as controls.

After separation of epithelial proteins by SDS-PAGE, the proteins were electroblotted to nitrocellulose in a Bio-Rad Transfer Blot Cell (Bio-Rad, USA). The gels were transferred to a tray containing blotting buffer (48mM Tris, 39mM Glycine, 0.037% SDS and 20% methanol made up in dH_2O) and allowed to equilibriate. Whatmann 3mm filter paper and nitrocellulose sheets were cut to the same size as the gels and soaked in blotting buffer. They were then transferred to gel holders and a sandwich made in the following order: bottom electrode (the anode), four layers of filter paper, one nitrocellulose membrane, polyacrylamide gel and four layers of filter paper. The sandwich was assembled, taking care not to trap air bubbles between the gels, nitrocellulose membranes and filter paper. The area around the sandwich was dried to remove excess buffer. The upper electrode (the cathode) was placed on top of the stack and the electrodes connected to allow the commencement of transfer. The blot was allowed to run for 1.5 hours at a current of 0.8 mA/cm^2 of gel. After transfer the power was disconnected and the membrane orientation marked. The nitrocellulose membranes were removed and washed three times in blotting buffer, allowing ten minutes for each wash. Gels were stained as described in section 3.2.6 to ensure that proteins had been transferred.

Non-specific binding of the antibody to the membrane was blocked by incubation for 1 hour at room temperature in

blocking buffer (5% fat-free dried skimmed milk (Marvel) with PBS/0.05% Tween-20). The membranes were washed three times (ten minutes per wash) in blotting buffer and incubated for 1 hour in blocking buffer with the anti-HSP 70kD antibody diluted to 1:50.

Excess antibody was removed from the membranes by three washes in blotting buffer (ten minutes per wash). Biotin labelled rabbit anti-mouse antibody (Sigma) was added at a dilution of 1:500 in blocking buffer and incubated at room temperature for 1 hour. The membranes were washed three times in blotting buffer (ten minutes for each wash). They were then incubated for 1 hour at room temperature with extravidin peroxidase (Sigma) at a dilution of 1:200 in blocking buffer. The membranes were washed three times in blotting buffer (ten minutes for each wash) and developed in a substrate solution containing 100mM disodium hydrogen phosphate (Na_2HPO_4), 45mM citric acid (pH 5.0), 0.015% 4-chloro-1-napthol and 0.03% hydrogen peroxide. The results were seen in minutes and the reaction stopped by the immersion of the filters in dH_2O. Membranes were photographed immediately and stored in the dark to prevent the bands from fading.

5.3 Results

5.3.1 The Effect of Heat Shock Treatment on Cell Viability

Five cultures were examined at each time point and so for the purposes of standard errors n=5. In the 45°C heat shock treatment 98% (s.e.=1.16) of the cells remained viable immediately after treatment and after 1 hour 96% (s.e.=1.70) of the cells were viable. This dropped to 72% (s.e.=5.82) after 12 hours and after 24 hours 73% (s.e.=4.35) of the cells were viable.

Immediately after 50°C heat shock treatment 93% (s.e.=3.56) of the cells were still alive and after 1, 12 and 24 hours 69% (s.e.=2.44), 59% (s.e.=3.31) and 54% (s.e.=3.67), respectively, remained viable.

In the 60°C heat shock treatment 91% (s.e.=3.38) of the cells remained viable immediately after treatment. This fell to 54% (s.e.=3.67) after 1 hour and after 12 and 24 hours 43% (s.e.=2.00) and 39% (s.e.=3.31), respectively, of the cells remained alive.

In control cultures given no heat shock treatment 99% (s.e.=0.58), 98% (s.e.=0.66), 98% (s.e.=0.81) and 98% (s.e.=0.84) of the cells were viable at 5 minutes, 1, 12 and 24 hours, respectively (Fig. 5.1). A covariance analysis on regression coefficients showed that cell viabilities in heat shock treated cultures were

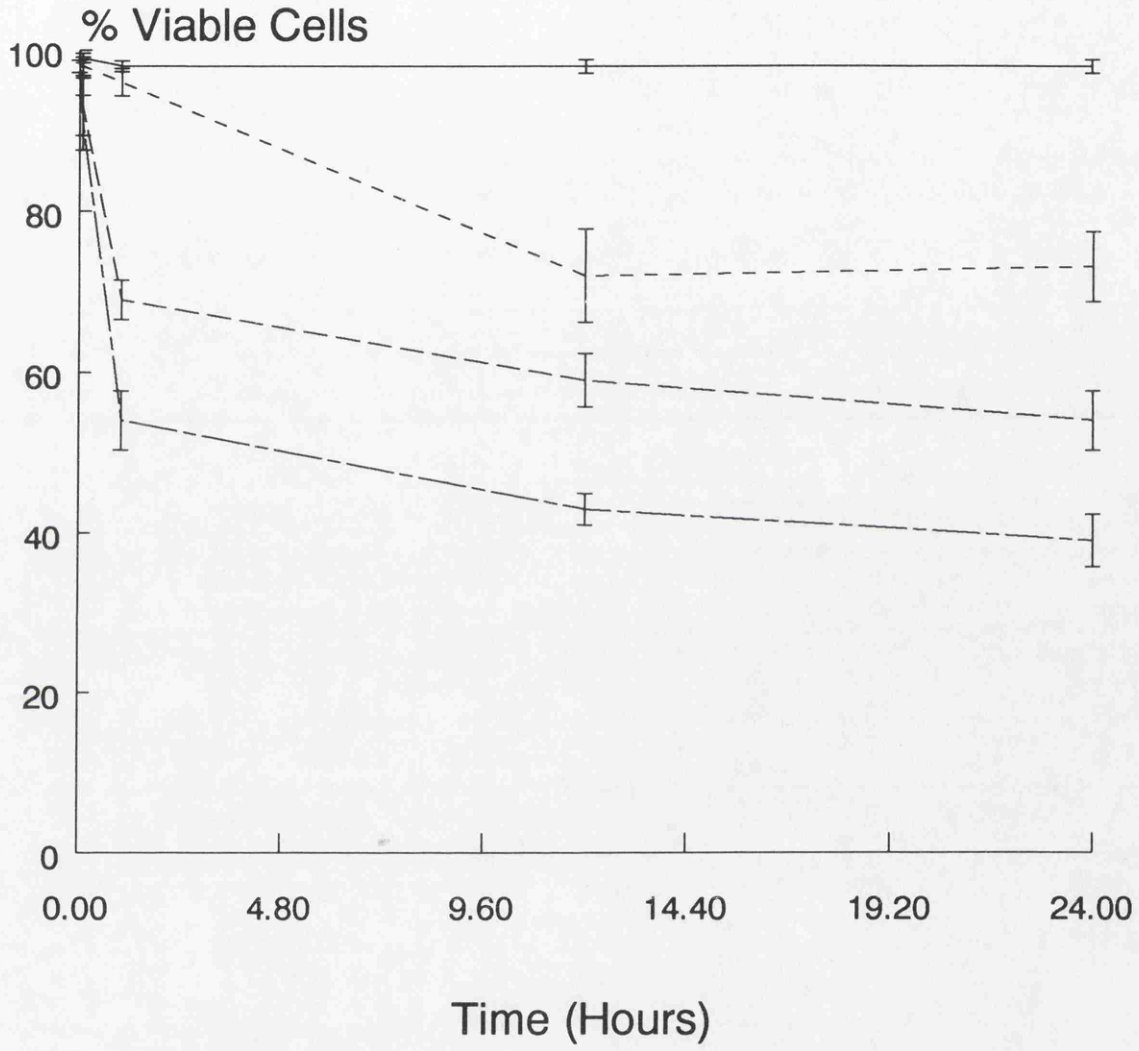


Figure 5.1 Shows the effect of a five minute 45°C, 50°C and 60°C heat treatment on epithelial cell viability (%) over a 24 hour period. Each point represents the mean and standard error of five measurements. ——— Control - - - - - - 45°C— — — 50°C — - — - — 60°C.

significantly lower than those in control cultures. (F values: 45°C F=37.75, 50°C F=41.56 and 60°C F=41.45. All were significant at $p<0.001$).

5.3.2 The Effect of Heat Shock Treatment on Protein Profile

The protein profiles from the heat shock treated and control cells were compared by visual and densitometric analysis.

Visual Comparison

Example lanes from the SDS polyacrylamide gels of the lingual epithelial proteins from heat shock treated and control cells immediately, 1, 12 and 24 hours after treatment are shown in figures 5.2-5.5. Visual comparison between heat shock treated cells and control cells suggested that there were no protein changes immediately and 1 hour after heat shock treatment. After 12 and 24 hours there appeared to be an increase in the levels of a protein with a MW of 66-70kD (this will be referred to as the 70kD protein) in heat shock treated cells compared with controls. This was the only consistent protein change observed.

Densitometric Analysis

Densitometric analysis confirmed the visual analysis of increased levels of a 70kD protein at 12 and 24 hours after heat shock treatment. There were no significant

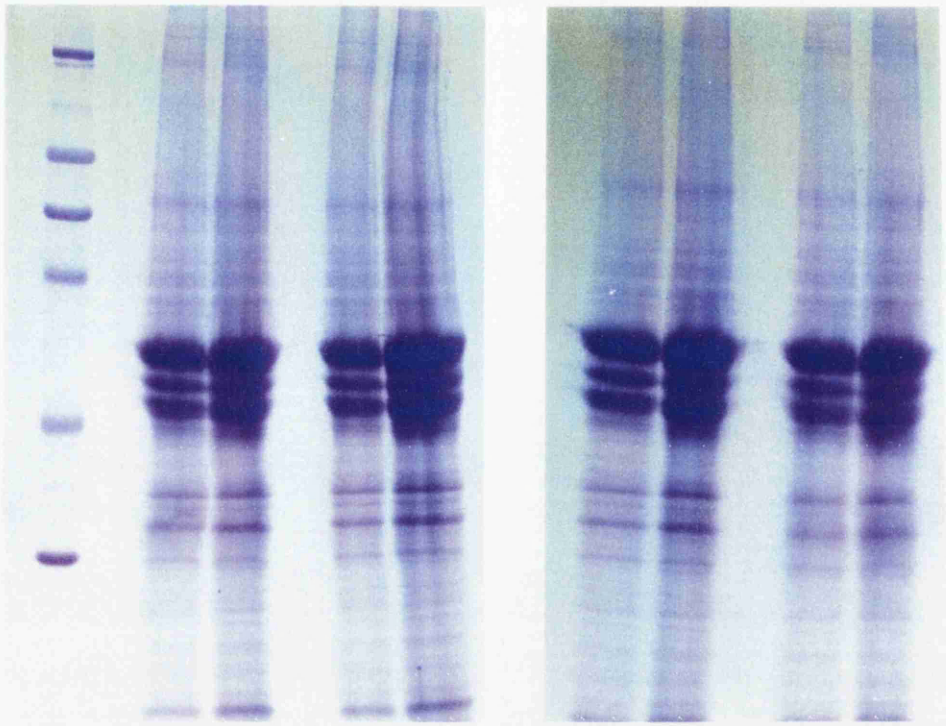


Figure 5.2 Shows example lanes from gels of rat lingual epithelial proteins from cells immediately after a five minute 50°C heat treatment and cells given no heat treatment. H - Heat treatment C - Control M - Molecular weight markers.

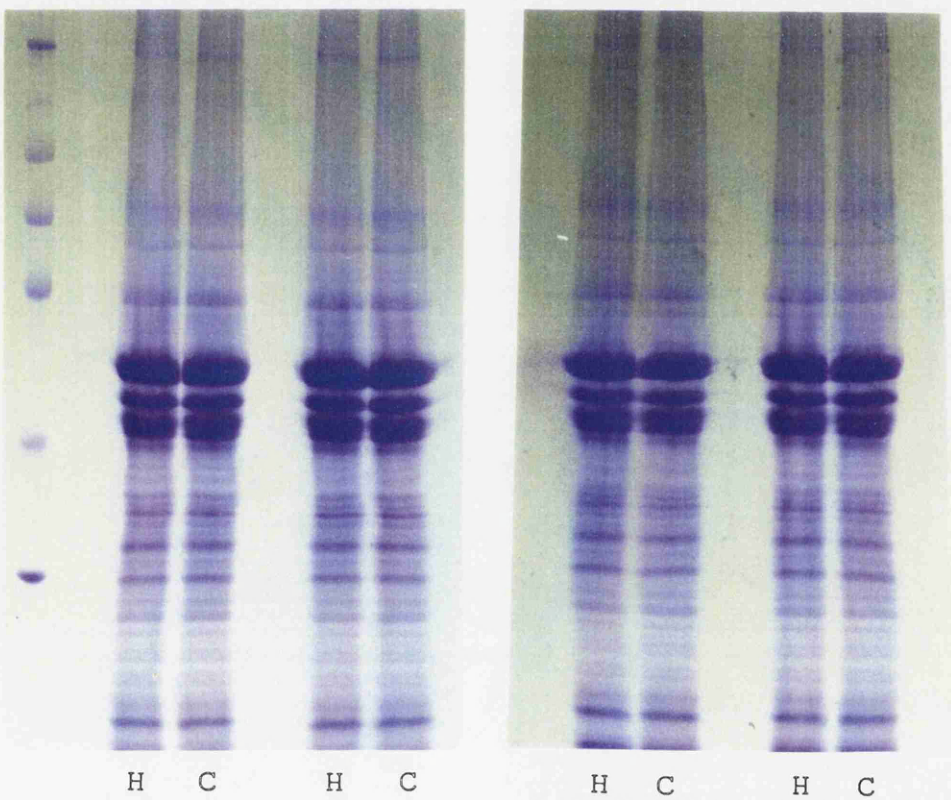


Figure 5.3 Shows example lanes from gels of rat lingual epithelial proteins from cells 1 hour after a five minute 50°C heat treatment and cells given no heat treatment. H - Heat treatment C - Control M - Molecular weight markers.

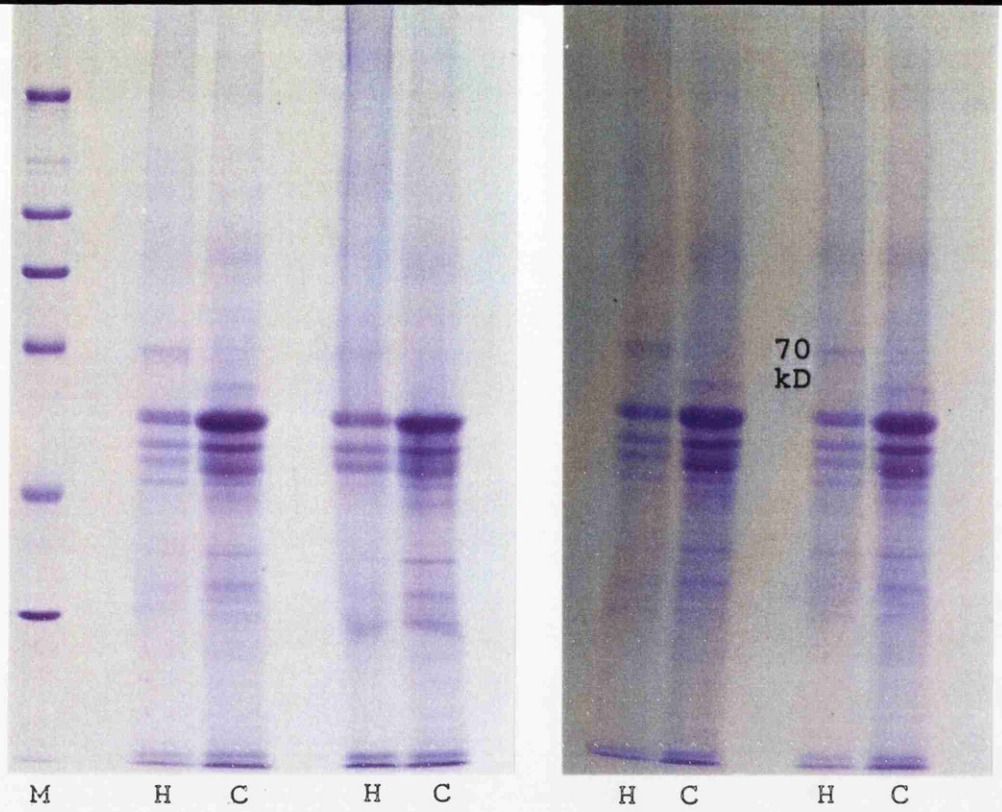


Figure 5.4 Shows example lanes from gels of rat lingual epithelial proteins from cells 12 hours after a five minute 50°C heat treatment and cells given no heat treatment. H - Heat treatment C - Control M - Molecular weight markers.

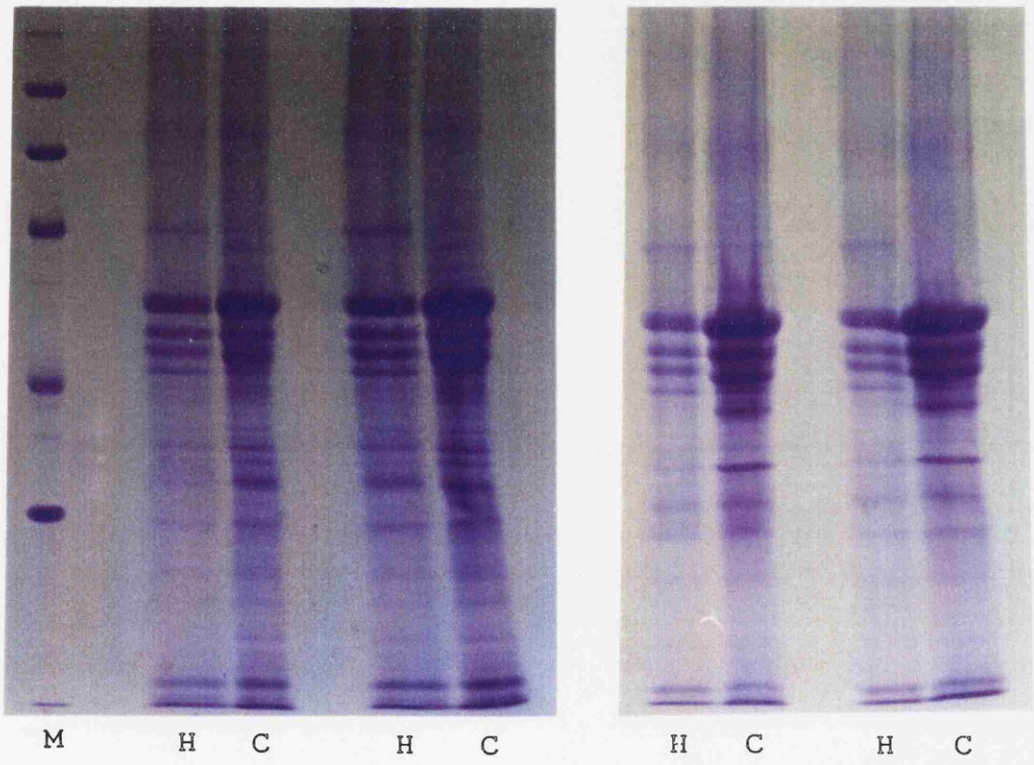


Figure 5.5 Shows example lanes from gels of rat lingual epithelial proteins from cells 24 hours after a five minute 50°C heat treatment and cells given no heat treatment. H - Heat treatment C - Control M - Molecular weight markers.

changes in protein levels immediately and 1 hour after heat shock treatment.

Table 5.1 shows the mean values of the area percentages of the 70kD protein. The mean area percentages immediately and 1 hour after heat shock treatment were similar to those of control cells given no heat shock treatment. Statistical analysis (Mann-Whitney U test) revealed no significant differences between the protein levels of heat shock treated cells compared with controls at this time interval.

Table 5.1 also shows that the mean values of the area percentages for the 70kD protein in heat shock treated cells were higher than those for the control cells at 12 and 24 hours after treatment. The increase from 2.76 to 6.72% after 12 hours was statistically significant ($p<0.0002$) as was the increase from 2.39 to 6.53% after 24 hours ($p<0.0002$).

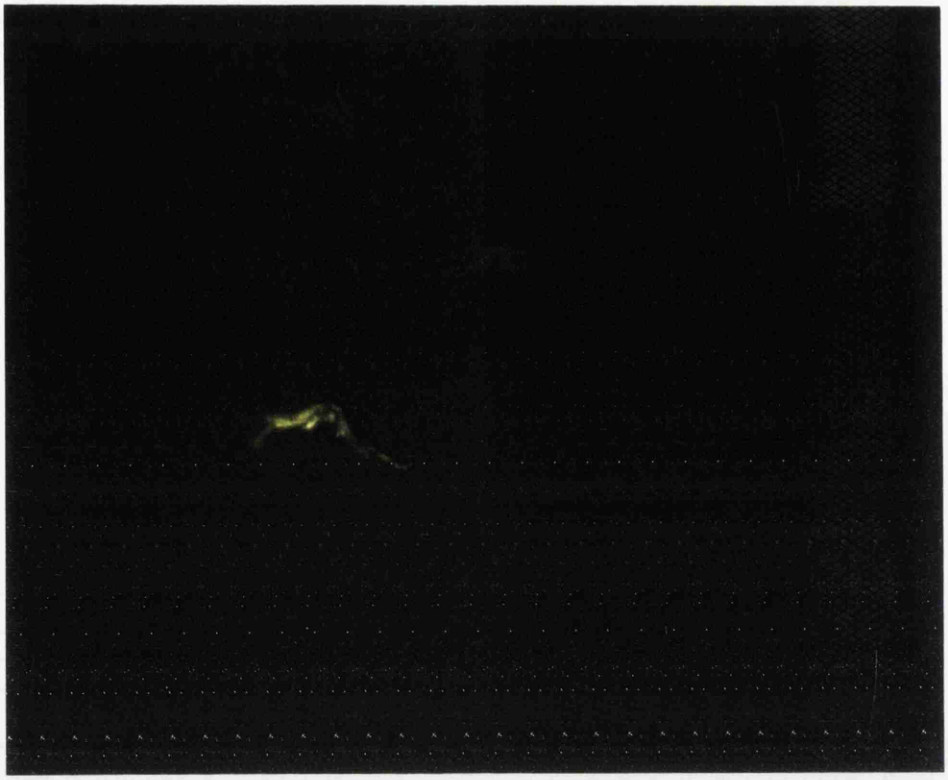
5.3.3 Immunofluorescence

Control cells and heat shock treated cells immediately and 1 hour after treatment stained very faintly (Fig. 5.6.a-b) with the anti-HSP 70kD antibody. Heat shock treated cells at 12 and 24 hours after treatment and all acetaldehyde treated cells stained intensely with the HSP 70kD antibody (Fig. 5.7.a-c). Since all acetaldehyde treated cells stained in a similar manner only 1 day

Table 5.1 Relative area percentage of a 70kD protein immediately, 1 hour, 12 hours and 24 hours after heat treatment. H - heat treatment C - control.

	Immediately		1 hour		12 hours		24 hours	
	H	C	H	C	H	C	H	C
Rel.% Area	2.96	2.51	3.18	2.99	6.58	2.11	6.87	2.43
	3.89	3.11	3.05	2.87	6.70	2.36	6.61	2.50
	2.78	2.44	2.95	2.66	7.11	3.48	6.11	2.18
	3.01	2.85	2.78	2.61	6.21	3.08	5.12	2.08
	2.66	2.31	3.11	3.20	7.13	3.40	7.01	2.37
	2.15	2.97	3.01	3.22	5.89	2.10	6.98	3.00
	2.90	3.10	2.75	3.09	7.09	3.00	6.55	2.22
	2.68	3.04	2.69	2.97	6.95	2.98	5.91	2.08
	2.88	2.97	2.80	3.10	7.00	2.77	7.14	2.44
	2.76	3.14	2.77	2.98	6.50	2.33	6.99	2.58
X	2.87	2.84	2.91	2.97	6.72	2.76	6.53	2.39
S.D.	0.43	0.31	0.17	0.21	0.42	0.51	0.64	0.28
S.E.	0.14	0.10	0.05	0.07	0.13	0.16	0.20	0.09

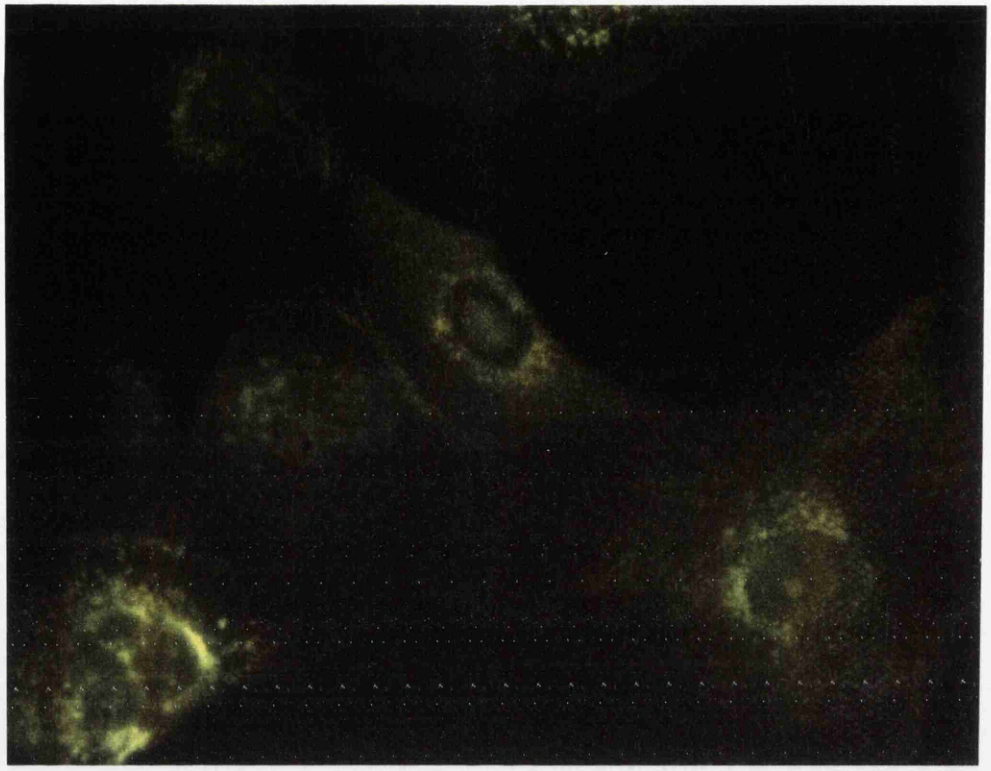
n=10



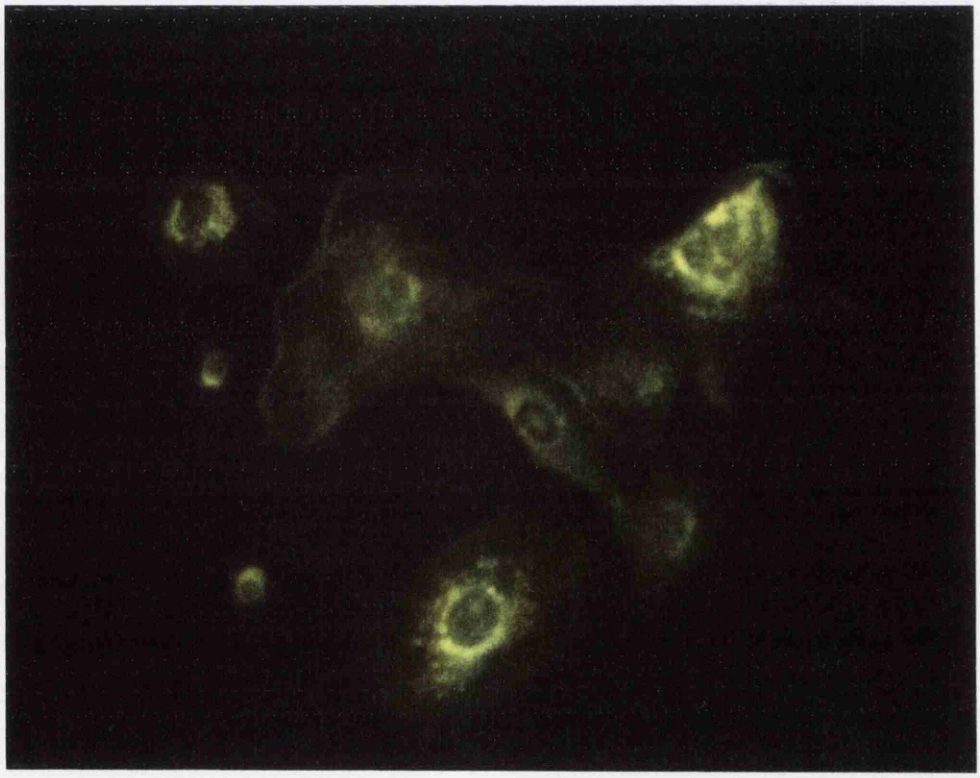
(a)

(b)

Figure 5.6 (a-b) A pictorial representation of the faint fluorescence observed when cells (a) immediately after and (b) 1 hour after heat treatment were stained with an anti-HSP antibody. The secondary antibody was fluorescein labelled. (a) mag. X 200 (b) mag. X 100.

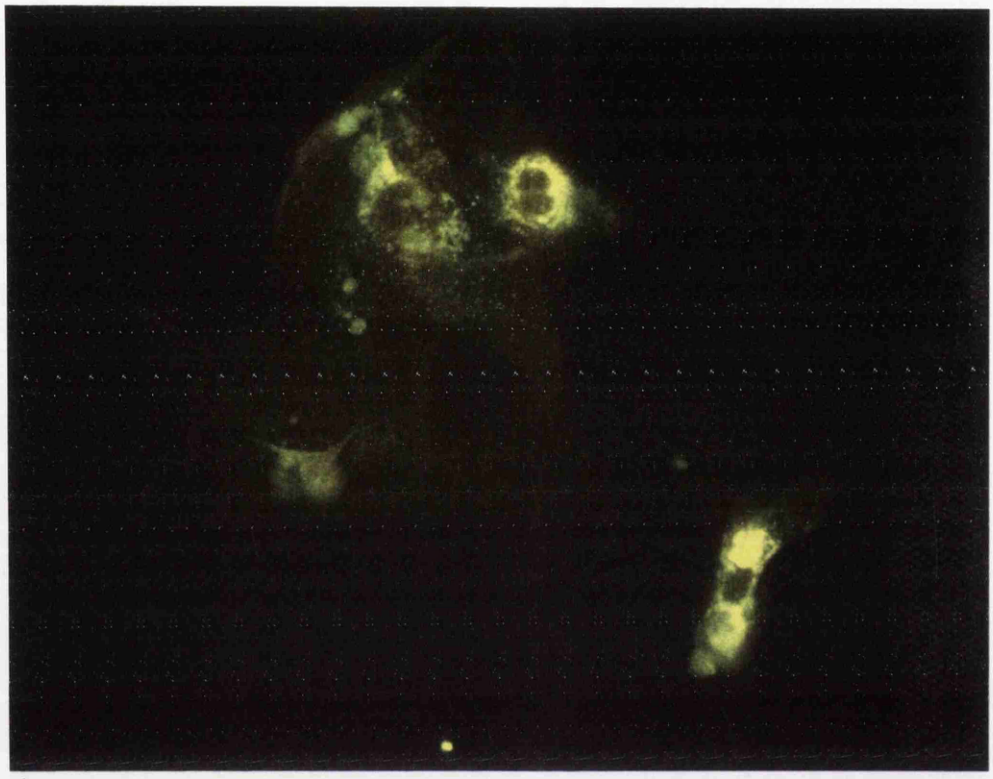


(a)



(b)

Figure 5.7 (a-c) A pictorial representation of the positive fluorescence observed when cells (a) given acetaldehyde for 1 day, (b) 12 hours after and (c) 24 hours after heat treatment were stained with an anti-HSP antibody. The secondary antibody was fluorescein labelled. (a) mag. X 300 (b) mag. X 200 (c) mag. X 200.



(c)

(c) 24 hours after heat treatment (mag. X 200).

0.005% acetaldehyde treated cells are shown as an example.

5.3.4 Western Blot

Figure 5.8 shows an immuno-blot of the proteins from heat treated epithelial cells blotted with an anti-HSP 70kD antibody. Two protein bands in the 70 and 75-80kD region were detected using this technique. Membranes with control molecular weight markers remained blank.

5.4 Discussion

5.4.1 Heat Shock and Cell Viability

Three temperatures, 45°C, 50°C and 60°C, were used to heat shock rat lingual epithelial cells *in vitro*. The aim of the study was to select the highest temperature which would leave sufficient surviving cells to provide enough protein for SDS-PAGE analysis. The highest temperature was selected to give cells the maximum heat shock they could survive as it is likely that this temperature would induce the maximum stress response. With 60°C treatment under 40% of the cells survived 24 hours. This resulted in too few cells to harvest sufficient protein for SDS-PAGE. The cells were better able to cope with 50°C and 45°C treatments as 60% and 70%, respectively, of the cells remained viable after 24 hours. Both treatments yielded enough protein for SDS-PAGE analysis and the higher heat shock treatment was chosen for the protein study.

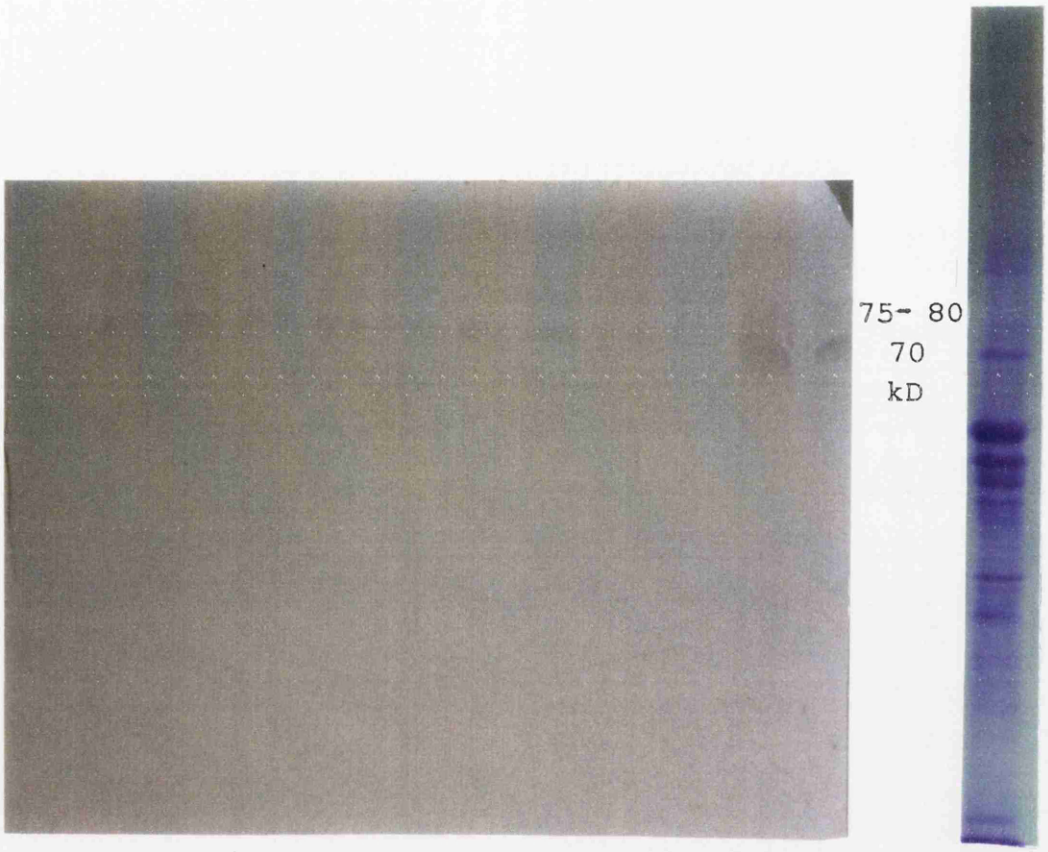


Figure 5.8 This figure represents a Western blot showing the detection of two bands in the 70 and 75-80kD region with an anti-HSP antibody.

5.4.2 Heat Shock and Protein Profile

The results of this study demonstrated an increase in levels of a protein with a MW of approximately 70kD at 12 and 24 hours after heat shock treatment. This was the only consistent change observed. There were no alterations in the levels of this protein before the 12 hour time point.

An increase in the levels of a 70kD protein was also observed after one day in 0.05% and 0.005% acetaldehyde (section 4.3.2). Since heat shock treatment also brings about an increase in the levels this protein the changes observed with acetaldehyde treatment may be a general response to stress rather than a specific acetaldehyde effect.

The 230, 200, 30 and 28kD protein alterations observed after seven days in 0.5% ethanol and after one day in 0.05% and 0.005% acetaldehyde were not brought about by heat shock and so may be more specifically related to ethanol/acetaldehyde treatment than to a general stress mechanism.

Graham (1987) suggested that the 30 and 28kD proteins that increased in levels in alcoholic rats could be HSPs. These protein changes were observed in this study after seven days 0.5% ethanol and after one day 0.05 and 0.005% acetaldehyde treatment, but did not occur after heat

shock treatment. It therefore appears likely that they are not HSPs.

5.4.3 Immunofluorescence

An anti-HSP 70kD antibody was used to investigate further the possibility of the 70kD protein being a HSP. Control cells and heat shock treated cells immediately and 1 hour after treatment stained with the 70kD antibody, however, the staining was very faint (Fig. 5.6.a-b). The cells in all acetaldehyde treatments stained very intensively with the antibody, as did those at 12 and 24 hours after heat shock treatment (Fig. 5.7.a-c).

The results suggest that the 70kD protein is a HSP. The faint fluorescence observed in controls and cells immediately and 1 hour after heat shock suggests that this protein is expressed constitutively in these cells *in vitro* and increases upon heat shock/stress as observed in the increase in intensity of staining in acetaldehyde treated cells and cells at 12 and 24 hours after heat shock.

This is also supported by the presence of the 70kD band on protein gels of control cells and cells immediately and 1 hour after heat shock. The relative percentage area of this band increases after one day in acetaldehyde and at 12 and 24 hours after heat shock treatment.

Some heat shock genes are expressed constitutively and the proteins are present in the cell (Stoklosinski *et al.*, 1992); the levels of these proteins may be increased following stress/heat shock.

5.4.4 Western Blot

The suggestion that the 70kD protein is a heat shock protein is further supported by the results of the Western blot (Fig. 5.8). A protein in the 70kD region was detected in heat treated cells using the anti-HSP 70kD antibody, suggesting that this protein is a HSP.

However another band of approximately 75-80kD was detected in the immuno-blot. This may result from unspecific binding of the antibody or may suggest the existence of two forms of the same protein. The 75-80kD protein could be the glycosylated form of the 70kD protein. It is known that acetaldehyde impairs protein glycosylation (Nalpas & Brechot, 1992) but it is not known if heat shock has the same effect. It may be that raised levels of the 70kD protein are the result of decreased levels of the glycosylated form, although no changes in levels of a 75-80kD protein were detected on gels of heat and acetaldehyde treated cells. Further work is needed to clarify the nature of the 75-80kD protein.

5.5 Conclusions

Heat shock treatment of 50°C for five minutes elicits an increase in the levels of a 70kD protein at 12 and 24 hours after treatment. The levels of this protein are also increased after one day in 0.05% and 0.005% acetaldehyde, although 0.5% ethanol does not alter the levels of this protein. Heat shock treatment does not alter the levels of the 230, 200, 30 and 28kD proteins altered by ethanol and acetaldehyde.

Acetaldehyde treated cells and cells at 12 and 24 hours after heat shock treatment stain positively with an anti-HSP 70kD antibody. Controls stain faintly.

It seems likely that the 70kD protein is a heat shock protein, produced constitutively in the cells, and increased in levels as a result of general stress treatment. This is further supported by a Western blot of proteins from heat treated cells.

The 230, 200, 30 and 28kD protein changes may be more specifically related to ethanol/acetaldehyde treatments and do not occur in response to the heat shock treatment applied in this study.

To investigate the significance of the changes in protein profile reported in chapters 3-5 it is essential to characterise the proteins as far as possible. As an

initial step in the characterisation process attempts were made to assign the five proteins to either the membrane/microsomal or cytoplasmic cell fractions. This study is reported in chapter 6.

Chapter 6

Subcellular Localisation of The 230, 200, 70, 30 and 28kD Proteins

6.1 Introduction

In order to define a role for the observed alterations in protein levels in ethanol (chapter 3), acetaldehyde (chapter 4) and heat shock (chapter 5) treated cells or to suggest mechanisms to explain the alterations in protein levels it is essential to characterise the proteins as far as possible.

There are a variety of biochemical techniques which may be used for protein characterisation such as peptide mapping, chemical solubilisation, purification and amino acid analysis. However such studies are outwith the time constraints of this thesis, but to provide some clues as to the possible functions of the altered proteins simple attempts were made to assign the proteins to specific sites within the cell. As an initial step in the characterisation process the five proteins could be assigned to either the membrane/microsomal or the cytoplasmic fractions of the cell using differential centrifugation and the detergent lysis of cells. If time and resources had been available immuno-electron microscopy techniques would have provided information on the specific cellular locations of the proteins.

The membrane/microsomal fraction of the cell is defined as being the particulate fraction that sediments upon centrifugation at speeds exceeding 10,000g (Tata,1972). Small membranous sheets and membrane vesicles derived from the smooth and rough endoplasmic reticulum, golgi and plasma membranes are found in such fractions (Graham, 1984) and it can be said that this fraction represents the cellular membrane population. Following the removal of the membrane population the supernatant or cytoplasmic fraction can be taken as representative of the soluble cellular components.

Another useful tool for the separation of soluble and insoluble cellular components is detergent lysis of cell membranes. These agents disrupt hydrophobic associations and partially destroy the membrane lipid bilayer. When mixed with membranes the hydrophobic ends of detergents bind to the hydrophobic regions on the exterior of membrane proteins, thereby displacing the lipid molecules and lysing the cell. The soluble cellular components are released into the medium and can be removed leaving the insoluble components. The detergent employed in this study was a nonionic detergent, Triton X-100 (BDH).

6.2 Materials and Methods

6.2.1 Subcellular Localisation by Centrifugation

Cells were grown in culture as described in section 2.2.4. Ten 25cm^2 flasks were used. Cells in the third passage on a plastic substrate were grown for 7-10 days. Cultures were then washed three times with PBS and the cells removed using a "policeman", and homogenised in ice cold water (section 3.2.2). 40µl of each homogenate were stored for protein concentration determination (section 3.2.3) and the remainder centrifuged at 100,000g for 90 minutes in a Beckman L2-65B ultracentrifuge, using a Beckman SW 50L swinging bucket rotor (Beckman Ltd, High Wycombe, England) and polyallomer centrifuge tubes. The supernatant from this spin represented the cytoplasmic fraction of the cells and the pellet represented the membrane/microsomal fraction. The supernatant was carefully removed, the protein concentration determined and the proteins prepared for SDS-PAGE (section 3.2.2).

The pellet was resuspended in ice cold dH_2O and washed by centrifugation at 100,000g for 90 minutes. This wash was repeated and the resulting pellet was prepared for SDS-PAGE (section 3.2.2). All centrifugation was carried out at 0-4°C. Protein concentration was determined as described in section 3.2.3.

Electrophoresis and the staining and assessment of gels were carried out as described in chapter 3 (3.2.4, 3.2.5 and 3.2.6, respectively).

6.2.2 Subcellular Localisation with Triton X-100

Cells were grown with a 3T3 feeder layer and subcultured on plastic alone as described in section 2.2.4. Ten 25cm^2 flasks were used. Cells in the third passage on a plastic substrate were grown for 7-10 days and washed three times with PBS to remove all traces of tissue culture medium. The cells were then bathed in a 0.1% solution of the detergent Triton X-100 for 1-2 minutes during which time the detergent lysed the cells releasing the soluble cellular components into solution. The detergent solution was then decanted, the protein concentration determined (section 3.2.3) and the proteins prepared for SDS-PAGE (section 3.2.2). In these samples not enough protein was present to load 50μg in each well and 25μg were loaded.

The remaining material in the flask represented the membrane/microsomal components of the cell and following three washes with PBS the remaining components of the cells were removed from the flask using a "policeman" (section 3.2.2). This material was then homogenised and the protein concentration of the homogenate determined (section 3.2.3). The proteins were prepared for SDS-PAGE (section 3.2.2) and electrophoresis carried out as described earlier.

6.3 Results

6.3.1 Subcellular Localisation by Centrifugation

Subcellular localisation studies using centrifugation to separate cytoplasmic and membrane/microsomal cell

fractions showed that the high MW proteins were associated with the insoluble fraction of the cell while the lower MW proteins were present in the soluble cytoplasmic cell fraction.

Figure 6.1 shows the protein samples from the supernatant following high speed centrifugation. The 230, 200 and 70kD proteins were absent from these gels, while the lower MW proteins were present. The protein samples from the pellet are shown in Figure 6.2. The 30 and 28kD proteins did not appear on these gels, while the 230 and 200kD proteins were present. It was difficult to assess the 70kD protein because the bands were very faint but there does appear to be a faint banding in the region of the 70kD protein on the insoluble sample gels.

6.3.2 Subcellular Localisation using Triton X-100

Very low protein concentrations were present in the soluble samples, which are shown in Figure 6.3. The 230 and 200kD proteins were absent in these samples but the protein bands were very faint making the gels difficult to assess. The 70kD protein which could not be detected in supernatant extractions in the absence of Triton X-100 (section 6.3.1) was noted on the gels when Triton X-100 was used.

The protein samples from the remaining cell components following treatment with Triton X-100 are shown in Figure

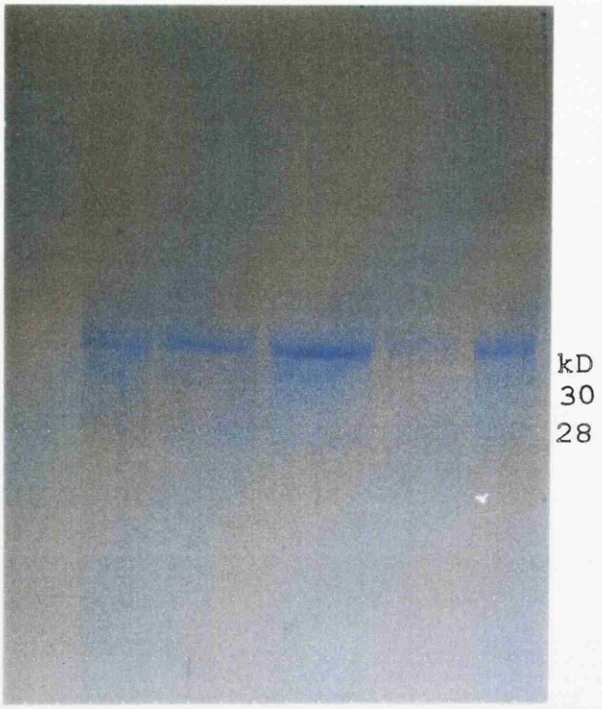


Figure 6.1 Shows gels of the protein samples from the supernatant following high speed centrifugation. The 30kD and 28kD proteins are present in this fraction.

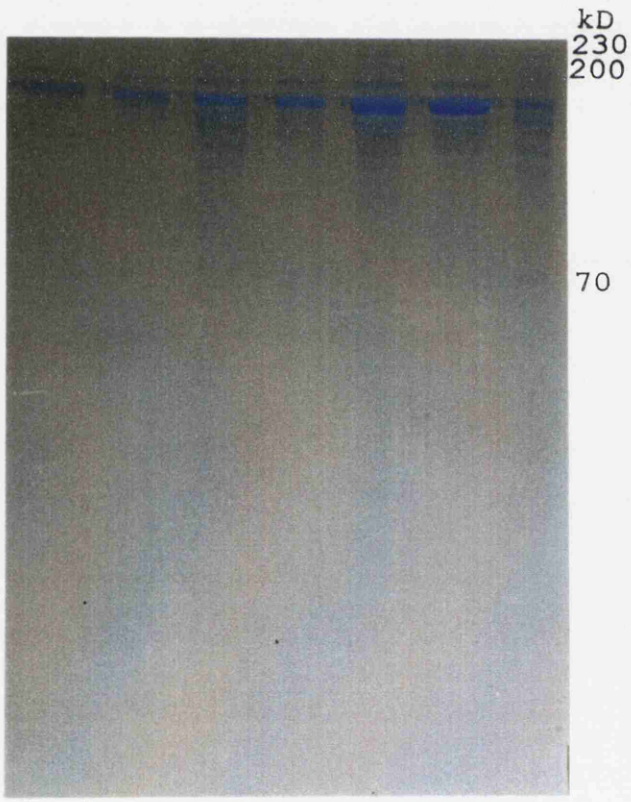


Figure 6.2 Shows gels of the protein samples from the pellet following high speed centrifugation. The 230kD, 200kD and 70kD proteins are present in this fraction.

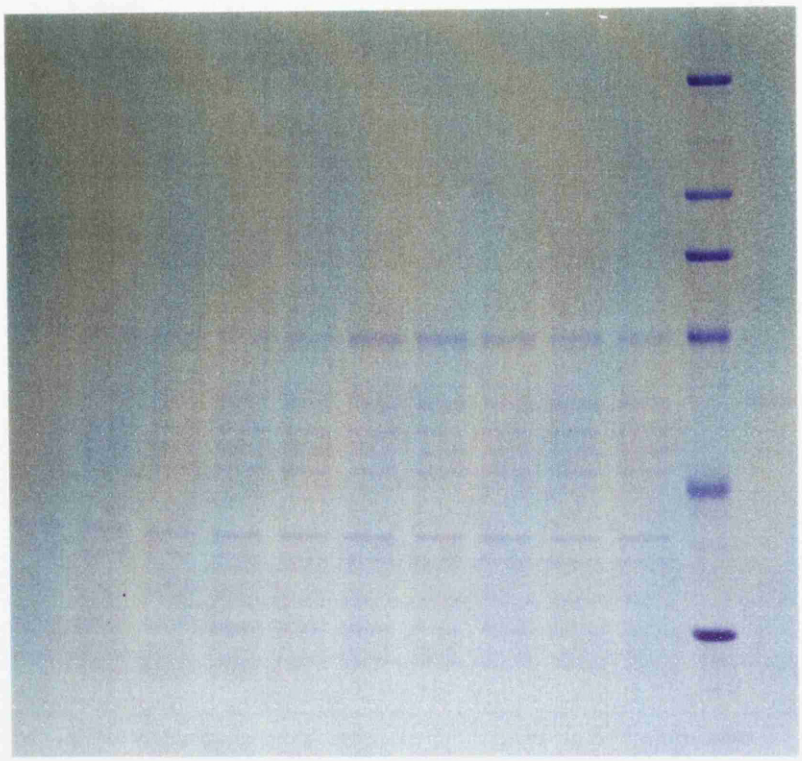


Figure 6.3 Shows gels of the soluble samples from the Triton X-100 lysis study. The 230kD and 200kD proteins were not detected on these gels.

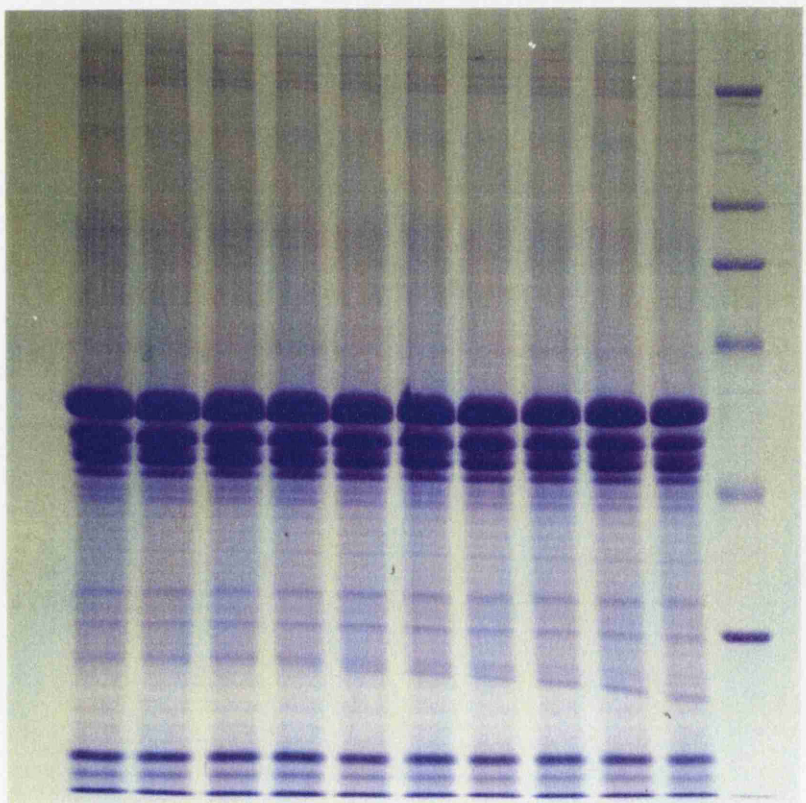


Figure 6.4 Shows gels of the insoluble samples from the Triton X-100 lysis study. All proteins were present in these samples.

6.4. All of the proteins were present in these samples, making it difficult to draw any conclusions concerning the insoluble fraction.

6.4 Discussion

6.4.1 Centrifugation

The insoluble material and membranous/microsomal material of the cell sediments upon centrifugation, at speeds of 10,000g and above (Tata, 1972; Graham, 1984) and analysis of the protein content of this fraction allows for crude assessment of the cellular origin of proteins of interest. The supernatant may then be assessed as the soluble fraction of the cell. This study suggested that the 30 and 28kD proteins were associated with the soluble fraction of the cells while the 230 and 200kD proteins were associated with the insoluble fraction. The 70kD protein was more difficult to assess. It was absent from the soluble fraction and appears to be associated with the insoluble fraction, although it was only faintly visible on gels of insoluble samples.

6.4.2 Triton X-100

The centrifugation method discussed above, although helpful, is a crude method of subcellular localisation and further attempts were made to assign the proteins to specific fractions of the cell using the detergent Triton X-100. However, several attempts at this method gave no

conclusive proof of the location of the proteins within the cells. The 230 and 200kD proteins were absent from the soluble components and present in the insoluble components, although their absence from the soluble samples may be due to the very faint banding observed on these gels. The 70, 30 and 28kD proteins were present in both the soluble and insoluble samples and there appeared to be incomplete lysis and release of soluble components from the cells by the detergent. It is therefore difficult to draw conclusions from the detergent study.

If the 70kD protein is an insoluble component protein as suggested by the centrifuge study its presence in the detergent solution could be explained by the incomplete lysis of the cells and by insoluble parts of cells being decanted alongside soluble components. Incomplete lysis would also explain the presence of the 30 and 28kD proteins in both samples since not all soluble components may have been released. Alternatively, the membrane proteins may have been brought into solution as detergent-protein complexes and removed with detergent instead of being left in the flask. This would explain the presence of the 70kD protein in the soluble fraction but not the presence of the 30 and 28kD proteins in the insoluble fraction. However, Graham (1987) did associate the 30 and possibly the 28kD proteins with the membrane/microsomal cell fraction.

To avoid the possibility of insoluble proteins being removed with soluble proteins in any future work of this nature the decanted detergent solution should be centrifuged to remove any insoluble material which could then be analysed with the insoluble material on the flask surface. The cellular material left in the flask should perhaps also be centrifuged and the supernatant removed to ensure that no soluble cytoplasmic components are left in this sample.

6.4.3 The Possible Nature of the Proteins

The 200kD glycoprotein altered in the *in vivo* alcohol model (Graham & Rennie, 1987) was also found to be in the insoluble fraction of the cells and the 28kD protein appeared to be associated with the soluble fraction, although the results for the 28kD protein were unclear. The main difference was in the localisation of the 30kD protein. Graham (1987) associated this protein with the membrane/microsomal fraction while the results of this study associated the 30kD protein with the soluble fraction. However, as already discussed the results of this study were fairly crude and the study was designed to give some initial clues about the five proteins of interest and further study is needed to clarify this area.

With respect to the cellular location of the 70kD protein the results of this study were inconclusive, although the

centrifuge study suggested an association with the insoluble fraction of the cell.

The exact locations and nature of these proteins cannot be deduced from the work reported in this chapter and as already mentioned techniques such as immuno-electron microscopy might have provided more detailed information. However, Graham (1987) suggested that the 200kD protein may be associated with the cytokeratin fraction of the cells. The high molecular weights and the appearance of the 230 and 200kD proteins in the insoluble fraction is consistent with the proteins being associated with the cytokeratin fraction of the cell and hence playing a structural role.

6.5 Conclusions

This study made an initial attempt to assign the five proteins of interest to the soluble or insoluble components of the cell. The 230 and 200kD proteins were present in the insoluble cell fraction and may have a structural role in the cell. The 30 and 28kD proteins were present in the soluble fraction of the cells. The 70kD protein may be associated with the insoluble fraction but the results concerning this protein were unclear. This protein appears to be a HSP (Chapter 5).

The 200kD protein location was similar to that found *in vivo* by Graham & Rennie (1987). However, there are differences in the 30 and. possibly the 28kD protein results between this study and the *in vivo* study.

The methods employed in this chapter gave only a crude estimation of the protein locations and more accurate characterisation techniques would have to be used to provide conclusive evidence as to the location and nature of these proteins.

Chapter 7

Concluding Discussion

7.1 Introduction

A number of epidemiological (Rothman & Keller, 1972; Tuyns, 1979) and experimental animal (Elzay, 1966, 1969; Freedman & Shklar, 1978) studies have strongly implicated alcohol as an aetiological factor in the development of oral cancer. Before investigating any role that alcohol may have in the development of oral cancer it is essential that its effects on the epithelium should be understood. Relatively few studies on the effects of alcohol on the oral epithelium exist and of those that do the majority report histological changes as a consequence of chronic alcohol intake (Mascres & Joly, 1981; Muller *et al.*, 1983; Valentine *et al.*, 1985). The studies reported in this thesis were carried out with the aim of investigating the effects of alcohol on oral epithelium in an attempt to understand how alcohol may play a role in the pathogenesis of oral malignancy. This chapter summarises the work presented in chapters 2 to 6.

7.2 The *In Vitro* Alcohol Model

Graham & Rennie (1987) carried out a biochemical investigation into the effects of chronic alcohol exposure on the lingual proteins of rats. An isocalorific matched pair feeding technique was used (DeCarli & Lieber, 1967) and while such models are useful they are time consuming, expensive and involve many practical

difficulties. Siyam (1987) made some attempts to set up an *in vitro* alcohol model examining the effects of high concentrations of alcohol over one day. The first objective of this thesis was to develop an *in vitro* model for the study of the effects of alcohol on oral epithelium. The model should be simple to use, produce consistent changes and be analogous to the human situation.

Rat tongue epithelium was grown in culture using the method described by Jepsen *et al.* (1980). In this study, cells were initially grown with a feeder layer and subsequently passaged onto plastic surfaces. Cells cultured in this way can withstand 12-14 passages. All cultures were incubated at 32°C since this temperature increases the life span of cultures and reduces fibroblast contamination (Jepsen, 1974; MacCallum & Lillie, 1987). This method produces a culture system in which oral epithelial cells could be given a range of alcohol concentrations and any effects of alcohol treatment could be assessed without the presence of metabolites from other cells influencing the results.

7.3 Measurement of Ethanol in Culture and its Effects on Epithelial Cell Viability

An alcohol detection kit (Sigma) designed to measure blood, serum, plasma and urine alcohol levels showed that although a large proportion of added alcohol was lost by

evaporation, some of the alcohol was utilised by the cells (section 2.3.5). To counter the large evaporative loss fresh alcohol medium was prepared and added to cultures every 24 hours.

The effect of alcohol on cell viability was investigated using the vital stain fluorescein diacetate (Mishell & Shiigi, 1980). A previous study (Siyam, 1987) used 5% ethanol and this concentration was chosen as the upper limit and varying concentrations down to 0.25% were employed. Concentrations between 5% and 1% killed the cells in culture and are unrealistic in terms of blood alcohol levels. *In vivo* the oral mucosa would not be subjected to such concentrations for a prolonged period of time. Concentrations of 0.5% and 0.25% ethanol are closer to the concentrations of alcohol found *in vivo* after imbibing and do not significantly reduce the viability of oral epithelial cells in culture (section 2.3.6).

One of the highest recorded blood alcohol levels is 0.4% ethanol and although this concentration could have been used 0.5% was chosen to help combat the evaporation. By giving the cells 0.5% ethanol every 24 hours the concentration was always between 0.5% and 0.38% ethanol (section 2.3.5). This would mimic a state of complete inebriation. Since 0.5% approaches high blood alcohol levels and cells remained viable when given this

concentration it was chosen as the concentration most appropriate and used throughout this thesis.

Since the epithelial cells were surrounded by medium with alcohol concentrations similar to those found in the blood of inebriated persons this system was considered a suitable model for the *in vitro* study of the effects of alcohol on the oral epithelium.

7.4 The Effect of Ethanol on Protein Profile

The proteins from cultured rat lingual epithelium were examined using SDS-PAGE. Proteins from cells given 0.5% ethanol and from control cells were compared. Cells were given ethanol over a 30 day period and prepared for SDS-PAGE at 1, 3, 5, 7, 14, 21 and 30 days.

There was a reduction in the levels of two high MW proteins of 230 and 200kD and an increase in the levels of two lower MW proteins of 30 and 28kD in the cells given 0.5% ethanol for 7 days or more. There was a progressive decrease over 30 days in the levels of the high MW proteins in ethanol treated cells, although before 7 days of treatment there were no significant changes in ethanol treated cells compared with control cells at the time points examined (section 3.3.1). The 30kD and 28kD protein levels did not show a corresponding progressive increase as the larger MW protein levels decreased.

Graham (1987) had originally suggested a direct link between the lowered levels of the high MW protein and the increase in levels of the lower MW proteins. However, this was discounted when the levels of the two lower MW proteins were shown to rise independently. It was also shown using peptide mapping that the proteins were not related (Graham, 1987). These findings and the erratic increase in levels of the lower MW proteins, which did not correspond to the decrease in levels of the higher MW proteins, suggest that there is no direct link between the lowered levels of the high MW proteins and the raised levels of the two lower MW proteins.

Since the changes in protein profile were similar to those observed in the animal model (Graham, 1987; Graham & Rennie, 1987) it appears that the model is analogous to the *in vivo* situation.

7.5 The Effect of Acetaldehyde on Cell Viability and Protein Profile

The protein changes observed after ethanol treatment may not occur as a direct result of ethanol damage but may be induced indirectly by a metabolite. Acetaldehyde has been reported to be more toxic to neurones in culture than ethanol (Smith *et al.*, 1990; Smith & Wubetu, 1991). This was also found in the present study as epithelial cells given 0.5% acetaldehyde died within 24 hours. Even at concentrations of 10 and 100 times lower than ethanol

concentrations most of the cells were dead by 14 days (section 4.3.1).

Acetaldehyde also alters the protein profile of the epithelial cells in culture at lower concentrations and more rapidly than ethanol. It took 7 days of 0.5% ethanol to alter the protein profile of the epithelial cells in culture whereas 0.05% and 0.005% acetaldehyde altered the levels of the same proteins after 1 day (section 4.3.2). After 7 days 0.5% ethanol treatment levels of a 230 and 200kD protein decreased while the levels of a 30 and 28kD protein increased. These changes were also observed following 1 and 7 days of 0.05% and 0.005% acetaldehyde treatment. The changes in levels of the high MW proteins were of a similar magnitude to those in cells given 0.5% ethanol for 14 days or more and changes in levels of the low MW proteins were of a similar order to those in cells given ethanol for 7 days or more.

It therefore seems likely that the protein changes observed may be acetaldehyde mediated and do not occur as a direct result of ethanol. This raises the question as to how much "ethanol" related damage is due to the primary metabolite and emphasises the need for further investigation of the role of acetaldehyde in alcohol related disease processes.

Acetaldehyde also alters the levels of a 70kD protein which is not altered in ethanol treated cells. Siyam (1987) observed an increase in a 71kD protein after treating rat lingual epithelial cells in culture with 5% ethanol. It was suggested that this protein was a heat shock protein (HSP). Graham (1987) suggested that the two lower MW proteins of 30 and 28kD, which increased in levels in alcoholic rats, might be HSPs. To investigate the possibility of the presence of HSPs a heat shock study was undertaken (chapter 5).

7.6 The Heat Shock Study

Elevated temperatures and other stresses such as ethanol and heavy metal ions may bring about a stress response in mammalian cells. This response may involve a transient production or an increase in the production of several families of proteins known as HSPs which are involved in the protection of the cell (Li, 1983; Stoklosinski *et al.*, 1992).

Cells were given three heat shock treatments, 60, 50 and 45°C for five minutes, and cell viability assessed using fluorescein diacetate (Mishell & Shiigi, 1980). After 24 hours 73 and 54% of the cells remained viable in the 45 and 50°C treatments, respectively, and 50°C was chosen to give the maximum heat shock treatment which is likely to induce the maximum response (section 5.3.1).

Cells were given a heat shock treatment of 50°C for five minutes and prepared for SDS-PAGE immediately, 1, 12 and 24 hours after treatment. The protein profile was compared with that of cells given no heat shock treatment.

The heat shock treatment brought about an increase in the levels of a 70kD protein after 12 and 24 hours (section 5.3.2). Acetaldehyde treatment increased the levels of this protein but 0.5% ethanol did not. It therefore appears that acetaldehyde exerts enough damage upon the cells to bring about a stress response whereas ethanol concentrations similar to high blood alcohol levels do not exert such stress. The increase in levels of the 70kD protein in acetaldehyde treated cells appears to be a general stress response.

Acetaldehyde treated cells and cells at 12 and 24 hours after heat shock treatment stained positively with a 70kD anti-HSP antibody. Controls stained very faintly (section 5.3.3). This would suggest that the 70kD protein is a HSP and is already present in the cells, increasing in levels after stress treatment.

A Western blot of proteins from heat treated cells also suggested that the 70kD protein was a HSP (section 5.3.4). A protein band in the 70kD region was detected in the immuno-blot using an anti-HSP 70kD antibody.

Heat shock treatment does not affect the levels of the 230, 200, 30 and 28kD proteins which are altered by ethanol and acetaldehyde treatment. These alterations may be more specifically related to ethanol/acetaldehyde and do not occur as a result of general stress treatment. This is contrary to the suggestions of Graham (1987) that the 30 and 28kD proteins were HSPs.

7.7 Subcellular Localisation of Proteins

To define a role for the observed protein alterations it would be essential to characterise the proteins as far as possible. This is a large task in itself and was beyond the scope of this study, but to provide some information simple subcellular localisation studies were carried out.

Cellular homogenate was centrifuged at 100,000g for 90 minutes and the soluble and insoluble fractions investigated with SDS-PAGE. These results suggested that the 230 and 200kD proteins were present in the insoluble cell components. The 70kD protein also appeared to be present in this cell fraction although results concerning this protein were unclear. The 30 and 28kD proteins were present in the soluble material.

The 200kD protein location was similar to that found *in vivo* by Graham (1987). However there were differences in the 30kD and possibly the 28kD protein results. Graham (1987) found the 30kD protein to be associated with the

insoluble fraction and, although it was unclear, suggested that this may also be the case for the 28kD protein.

Another localisation study, using the detergent Triton X-100, gave no conclusive results concerning the location of the proteins.

7.8 Significance of Protein Changes

The results of the studies reported in this thesis indicate changes in the levels of four proteins following alcohol treatment (section 3.3.1) and that these changes may be mediated by acetaldehyde rather than directly by ethanol (section 4.3.2). The protein alterations were not brought about by heat shock treatment (section 5.3.2) and therefore do not appear to be a non-specific stress response. However, a 70kD protein altered by acetaldehyde also shows altered levels in heat treated cells and does appear to be a HSP. Ethanol did not alter the levels of the 70kD protein.

Simple subcellular localisation studies suggested that the 230, 200 and possibly the 70kD protein belong to the insoluble fraction of the cells, whereas the 30 and 28kD proteins are found in the soluble fraction. The studies carried out do not characterise the proteins or shed a great deal of light on the nature and significance of the protein changes. As previously discussed, this would

involve further biochemical investigation which is outwith the time frame of this thesis.

The significance of the 30 and 28kD protein level increase in ethanol and acetaldehyde treatment is not clear. As previously discussed in chapter 5 Graham (1987) suggested that these proteins were associated with the insoluble components of the cell and may be HSPs, playing a protective role. This was not observed in the studies reported in this work (chapter 5 and 6) and further investigation of these proteins will be required before any significance for the increased levels can be suggested.

The 70kD protein appeared to be a HSP (section 5.3.2) and would therefore serve a protective role in the defence of the cell against external stress. There may be two forms of this protein (section 5.4.4) one of which could be glycosylated. Alterations in glycosylation may lead to an increase in the levels of a lower MW form of this protein. However, there is no firm evidence to support this suggestion and further work would need to be undertaken to clarify this area.

Graham (1987) suggested that the 200kD protein was a keratin polypeptide and that decreased levels of this protein may reduce the permeability of the epithelium by reducing the keratin content of the *stratum corneum*, thus

allowing potentially toxic substances to enter the epithelium. Increased permeability is believed to play a role in the development of oral cancer (Squier, 1973) and keratin peptides and the extrusion of keratin in the superficial layers of the epithelium are important in the formation and maintenance of the permeability barrier.

The high MW and the presence of the 230 and 200kD proteins in the insoluble fraction of the cells suggests that they may be large structural proteins within the cell, most of which are keratin peptides and polypeptides. Although confirming the results of *in vivo* work the possibility that these changes lead to reduced permeability cannot be addressed from the results reported in this thesis.

7.9 Future Work

The aims of the work presented in this thesis were to set up an *in vitro* model to study any effects of alcohol on oral epithelium and to compare them with the reports of *in vivo* research (Graham, 1987; Graham & Rennie, 1987). The *in vitro* work undertaken in this thesis has opened up a potentially large area for study concerning the characterisation of the proteins and the further development of the *in vitro* model. Suggestions for future work are described below.

7.9.1 Further Characterisation of the Proteins

Further characterisation of the proteins would be necessary to provide information concerning the function of these proteins and to provide clues as to the mechanisms whereby their levels might have been altered. To do this proteins of interest could be extracted, purified and further examined using amino acid analysis.

It would also be possible to use the purified proteins to produce polyclonal antibodies which could then be used with a second antibody to locate the proteins within the cells. Monoclonal antibody production could be attempted, although this would be a more difficult task.

Studies could also be undertaken to investigate whether the alterations in protein levels take place at a transcriptional, translational or post-translational level. The suggestion that the 70kD protein has two forms, one of which may be glycosylated could also be investigated and the possibility that alterations in glycosylation may lead to an increase in levels of the lower MW form of this protein assessed.

7.9.2 Further *In Vitro* Work

The culture system employed here is not strictly a monolayer system as cells do stratify and show evidence of differentiation. However, they do not terminally differentiate to produce a keratin layer but are sloughed

off into the surrounding medium. Despite the usefulness of this system and the similarities between the results produced here and those observed *in vivo* (Graham, 1987; Graham & Rennie, 1987), it does not fully represent the oral epithelium as a tissue. Cells of the oral epithelium will stratify, differentiate and keratinise when grown at an air-liquid interface on collagen gels. Histologically this tissue is very similar to the *in vivo* situation (Lillie *et al.*, 1980, 1988). This system may help elucidate whether the effect of alcohol on the oral epithelium is systemic or topical, since in this system alcohol can only get to the cells by being taken up from below.

7.9.3 Electron Microscopy

Although not reported in this thesis, time lapse photography was carried out using cultures in alcohol medium and cultures in alcohol free medium. From this work and from general viewing of cultures using phase microscopy it appears that after 3-7 days in alcohol, cells become granular in appearance. This change has also been observed in nerve cells (Smith *et al.*, 1990; Smith & Wubetu, 1991). This change could be further examined using electron microscopy.

The cellular location of the proteins could also be investigated using electron microscopy. Immuno-electron microscopy would provide detailed information on the

specific cellular locations of the proteins. The anti-HSP 70kD antibody could be used to stain cells which could then be examined using electron microscopy. This would allow the specific cellular location of the 70kD protein to be identified.

7.9.4 Studies with Human Tissue

If biopsies of oral mucosa from human alcoholics could be obtained it would be interesting to examine the protein profile of this tissue and compare it with that of epithelium from a control population.

It would also be interesting to grow human oral epithelium in culture to examine the *in vitro* effects of alcohol in a similar fashion to that described in this thesis. Human oral epithelium is more difficult to culture than rat oral epithelium and does not withstand so many passages, although it has been grown very successfully in culture (Arenholt-Bindslev *et al.*, 1987). This area has been reviewed by MacCallum *et al.* (1987).

Such studies would provide important information and lead to a greater understanding of the effects of alcohol on the oral epithelium.

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