THE EFFECTS OF ALCOHOL ON THE PROTEIN PROFILE OF RAT LINGUAL EPITHELIUM

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DECLARATION

This thesis is the original work of the author.

PREFACE

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ABBREVIATIONS

ADH - Alcohol Dehydrogenase
Alk. Phos.- Alkaline Phosphatase
ALT - Alanine Transaminase
AMPS - Ammonium Persulphate
AST - Aspartate Transaminase

BIS - NN' Methylenebisacrylamide

CBB R-250 - Coomassie Brilliant Blue R-250

DTT - Dithiothreitol

g - Unit of Centrifugal Force
GGT - Gamma Glutamyl Transferase

IU/L - International Units per Litre

K - KiloDalton
kJ - KiloJoule

M - Molar
MEOS - Microsomal Ethanol Oxidising System
MW - Molecular Weight

NAD(P) - Nicotinamide Adenine Dinucleotide (Phosphate)

PAGE - Polyacrylamide Gel Electrophoresis

SDS - Sodium Dodecyl Sulphate

TEMED - NNN'N'-Tetramethylethylenediamine
TRIS/UREA - 0.1 M tris pH 8.0, 4 M urea

SUMMARY

A large volume of epidemiological evidence suggests a strong correlation between chronic alcohol abuse and the development of intra oral squamous cell carcinoma (Rothman & Keller, 1972; Kissin, 1975; Tuyns, 1982).

Despite this evidence linking alcohol abuse and oral cancer, there are few properly controlled studies of the effect of alcohol alone on the oral epithelium. Those that have been properly controlled have demonstrated histological effects of alcohol on the epithelium. Structural or morphological changes in the oral epithelium may have a biochemical basis and thus the aim of this study was to investigate any biochemical abnormalities in the oral epithelium of alcoholic animals. The specific approach to the study involved an assessment of the effects of alcohol on the protein profile of rat lingual epithelium.

A suitable animal model was required for the study and that chosen was the Isocalorific Matched Pair Feeding Technique of Lieber and DeCarli (1973). This involved maintaining one group of experimental animals on a nutritionally adequate liquid diet with ethanol substituted to provide 36 percent of the daily calorific intake. A second group was maintained on the same liquid diet, but with sucrose substituted to provide 36 percent of the animals' calories. By measuring the volume of diet consumed, each pair of animals had their calorific intake closely monitored and matched exactly. A control group maintained on standard laboratory chow was included in both of

the animal studies presented and, in the second study, an additional control group maintained on liquid diet alone was also included.

The usefulness of this animal model as a representation of chronic alcohol consumption was demonstrated by assessing liver damage in the alcoholic animals. Liver damage was demonstrated both enzymically, with Gamma-Glutamyl Transferase being of particular use in this respect, and histologically, with centrilobular fatty infiltration being observed in the livers of the alcoholic animals. Unfortunately, two of the liver enzymes that were measured, Aspartate Transaminase and Alanine Transaminase, appeared to be affected by the liquid diet and as a result were of limited use in assessing liver damage. It was as a means of investigating the effects of the liquid diet on these enzymes that the extra control group was included in the second animal study.

The initial animal study ran for 102 days at the end of which, the animals were sacrificed and the lingual epithelium was removed and prepared for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Results from this protein study revealed, in the alcoholic animals, a reduction in the levels of a high molecular weight (MW) glycoprotein with a molecular weight of c. 160 Kilodaltons (K), and an accompanying increase in the levels of two lower MW proteins (30K and 28K). Visual and densitometric analysis revealed that these protein levels were significantly altered in the alcoholic animals with respect to both the sucrose pair-fed control animals and the animals maintained on standard laboratory chow.

A second animal study was set up to investigate the alterations in the levels of these three proteins, in the alcoholic animals, over fractions of the original 102 day study. The results demonstrated that chronic alcohol consumption was required before alterations could be detected in the levels of these proteins. It was postulated that the two lower MW proteins may be breakdown products of the high MW glycoprotein although there was no evidence of such a relationship in this time-course study, with each of the proteins being capable of expressing themselves at altered levels independently of the other two.

An alternative approach to investigating a possible relationship between the high MW glycoprotein and the two lower MW proteins involved the use of peptide mapping. Results from these studies were inconclusive as the high MW glycoprotein appeared to be resistant to proteolytic digestion.

Characterisation of the two lower MW proteins proved to be difficult as little was known about them, short of their molecular weights. Subcellular localisation studies revealed the 30K protein to be present in the epithelial cells associated with the membrane/microsomal fraction. Unfortunately, the 28K protein was more difficult to study in this respect, as it banded on SDS gels alongside a protein of very similar molecular weight as a closely associated doublet. One member of this doublet was seen to be associated with the membrane/microsomal fraction and the other with the cytoplasmic fraction of the cell. It is not yet clear which member of this doublet is enhanced in the alcoholic animals. Investigation of the

solubility of these two proteins showed them to be maximally soluble in distilled water.

Given the lack of information on these two proteins, it has not been possible to accurately identify them. It is suggested, however, that they may be members of the heat shock family of proteins. This is a family of proteins which are expressed at altered levels following exposure of cells to an environmental stress. Ethanol is known to represent such an environmental stress. Alternatively, since ethanol is an inducer of microsomal proliferation, and at least one of these two proteins is membrane/microsomal associated, the increased levels may simply be the result of an increase in the cellular microsome content.

The high MW glycoprotein has proved easier to study because of a number of unusual properties. For example, it shows anomalous migrational characteristics on SDS gels and appears to be resistant to proteolytic digestion. Subcellular localisation studies have shown that the protein pellets from solution at 3000 g, and solubilisation studies have demonstrated that for effective solubilisation, a denaturing agent, such as urea, is required. On the basis of these two properties, it is suggested that this protein is present in the epithelium associated with a rapidly pelleting structure which pellets from solution at 3000 g and which is held together by hydrophobic forces that can be disrupted by denaturing agents such as urea. Suggested candidates for such a structure include the keratin filaments and possibly the keratohyaline granules of the epithelium.

The high solubility of this protein in solutions of 4 molar urea and its very low solubility in solutions of 1 molar urea, greatly aided its purification. Urea solutions of epithelial proteins (enriched with respect to the high MW glycoprotein) were diluted 1:4 with distilled water and the high MW glycoprotein, along with other proteins not normally soluble in dilute urea solutions, precipitated out and were harvested by centrifugation. This preparation was electrophoresed in a preparative SDS gel and the high MW glycoprotein band was identified using guide strips. This band was cut out and the protein extracted from the gel matrix by re-electrophoresis.

An amino acid analysis was performed on the purified high MW glycoprotein which showed it to be a member of the keratin family. This protein is unusual in this respect in that it has a considerably higher molecular weight than that normally associated with the keratin proteins. It is suggested that this protein represents three keratin peptides crosslinked by an ϵ-(γ-glutamyl) lysine linkage that is not reducible by dithiothreitol, the denaturing agent used in SDS gel electrophoresis.

It can be envisaged that reduced levels of such a high molecular weight keratin protein may compromise the integrity of the epithelial permeability barrier and may thus increase the entry of potentially carcinogenic compounds into the epithelium. In this way, lowered levels of the high MW glycoprotein may account for the observed aetiological relationship between chronic alcohol abuse and the development of intra-oral squamous cell carcinoma.

CHAPTER ONE - INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Ethanol in the form of alcoholic beverages, has played a role in the development of human civilisation for an estimated five to eight thousand years, from the crude fermented meads of stone age cultures, to the ever lucrative alcohol production business's of today.

Ethanol is regarded by most cultures as an acceptable social drug and has thus become entrenched in the social and indeed religious lifes of many diverse societies. The popularity of alcohol in our own society is amply demonstrated by considering the figures relating to expenditure on, and consumption of, alcoholic beverages. It is estimated that in Scotland, up to three and a half million pounds is spent daily on alcohol, accounting for some 7.4 percent of the total consumer expenditure (Annual Abstract of Statistics, 1982). Additionally, according to 1983 statistics, the British adult public consume the equivalent of 9.91 litres of absolute alcohol per head of the population per year. This represents a doubling of the *per capita* consumption of alcohol as compared to the 1950 figures.

The use of any pharmacologically active substance to such a degree, is likely to be associated with some form of harm and the history of alcohol is inseparable from the history of alcohol abuse and alcohol related disease. Although no direct figures exist for Scotland, in England and Wales, it is estimated that alcohol intoxication is involved in the deaths of over 500 young people each year,

representing about 10 percent of mortality in persons under 25 (Havard, 1977) and that it may be responsible for up to ten thousand premature deaths every year (Taylor, 1981).

The many problems associated with alcohol abuse have prompted attempts at defining upper safety limits of consumption above which the consumer is in danger of experiencing biomedical complications. The Royal College of Psychiatrists (1979) have estimated that the absolute upper safety limit is equivalent to eight units (or four pints of beer) for males and six units (or three pints of beer) for females. Despite these apparently liberal safety limits, almost 750,000 people in England and Wales in 1979 were registered alcohol abusers, with up to three million (8 percent of the adult population) having detectable ethanol related biochemical abnormalities.

It is likely, with the recent dramatic increase in the per capita consumption of alcohol, that there will be an accompanying increase in the incidence of alcohol related problems. Indeed, as discussed in Section 1.2.4., a large increase in the incidence of liver cirrhosis has been observed amongst the Scottish population since the 1950's.

Given these statistics, it is surprising that alcohol has largely escaped the wrath of restrictive legal measures and has been allowed to become an intrinsic part of most civilisations, the uisque betha (water of life) and a generally accepted social psychotrope. The answer to this apparent dilemma may lie in the not inconsiderable economic value of alcoholic beverages. In the United Kingdom in 1981 for example, 11.4 billion pounds were spent on alcoholic beverages,

raising some five billion pounds in excise duties and Value Added Tax (V.A.T.). This sum is equivalent to one third of the total cost of running the National Health Service (Scottish Health Education Co-ordinating Committee, 1985).

Thus, for whatever reasons, alcohol is deeply rooted in most societies and we must learn to come to terms with or overcome the many problems associated with it.

1.2 ALCOHOL AND DISEASE - THE LIVER

1.2.1. General Introduction to Alcohol and Disease

Alcohol is known to be involved in the development of a number of disease entities. Sections 1.2-1.5 comprise a review of alcohol and disease with special reference to the liver, the gastrointestinal tract and cancer. Cancer shall be dealt with in two sections, Section 1.4 being concerned with cancer in general and Section 1.5 with oral cancer. Clearly alcohol is involved in the disease processes of other body systems such as the cardiovascular, respiratory and nervous systems, however, these shall not be discussed in this thesis.

The liver is the major site of ethanol metabolism and the relationship between alcohol and liver disease has been recognised for many hundreds of years. There are three main expressions of alcoholic liver disease: fatty liver, alcoholic hepatitis and cirrhosis, each of which will be discussed in turn.

1.2.2. Alcoholic Fatty Liver

Fat accumulation is the earliest and most common liver disorder resulting from alcohol ingestion, usually being evident within 3-7 days of excessive alcohol consumption (Gerber et al., 1973). Fatty liver is generally completely reversible (Lieber & Rubin, 1968).

The minimum alcohol exposure needed to cause fatty liver is not known, although it is clear that the dosage need not be inebriating (Feinman & Lieber, 1974).

Fatty change typically occurs in a centrilobular position but in severe cases it can affect virtually the whole liver lobule. Usually the lipids appear as a single large globule, producing peripheral displacement of the nucleus and other cell organelles and eventually obscuring the normal lobular appearance of the liver. If accumulation is extensive, there may be rupturing of the cell wall between adjacent hepatocytes resulting in the formation of fat cysts (Flemming & McGee, 1984).

Ultrastructural changes include enlarged and distorted mitochondria with shortened cristae which contain crystaline inclusions. Additionally vacuolar dilation and proliferation of the endoplasmic reticulum is noted (Svoboda & Manning 1964).

When sufficient dietary fat is available, the lipids accumulating in the alcoholic fatty liver, closely resemble the dietary fats. However when dietary fat is limited, the fat in hepatocytes tends to be derived largely from endogenously synthesised fatty acids.

A variety of other dietary factors, including total fat intake and protein content, can influence the severity of fatty change. A fuller description of this process is given in a recent review by Lieber et al. (1979).

The biochemical mechanisms underlying the development of fatty liver are described in Section 1.7.2.

1.2.3. Alcoholic Hepatitis

Alcoholic hepatitis is the potentially reversible acute or chronic inflammation of the liver that results from alcohol induced parenchymal damage (MacSween, 1978). Whilst fatty liver is common amongst alcoholics, alcoholic hepatitis develops in only a portion of heavy drinkers, even after decades of abuse (Lieber, 1982). However Lischner, Alexander and Galambos, (1971) suggest that it may arise at any time in the course of a patient's alcoholism, even in the first year. It may also exist quite independently of fatty liver.

Histologically, alcoholic hepatitis is characterised by ballooning degradation of the hepatocytes with parenchymal and portal infiltration by polymorphonuclear leukocytes and varying degrees of steatosis, fibrosis and cholestasis. These changes are seen predominantly in centrilobular areas, becoming more diffuse in more severe cases (Gerber & Popper, 1972).

A further histological feature of alcoholic hepatitis is the presence of alcoholic hyaline (Mallory Bodies) in the hepatocytes. Although strongly suggestive of alcoholic hepatitis, Mallory bodies are seen in a number of other diseases and are not essential for the diagnosis of alcoholic hepatitis if other features of alcoholic liver disease are present (Flemming & McGee, 1984).

In its more extreme forms, alcoholic hepatitis may not be reversible and it has been suggested that cirrhosis may develop from these more extreme forms (Lieber, 1982).

1.2.4. Alcoholic Cirrhosis

Cirrhosis is a condition involving the entire liver in which the parenchyma is changed into a large number of nodules separated from one another by irregular branching and anastomosing sheets of fibrous tissue (MacSween, 1985).

Cirrhosis precipitated by alcoholism takes at least 5-7 years of excessive alcohol abuse to develop. Even then only approximately 10 percent of alcoholics develop cirrhosis, which unlike fatty liver or hepatitis, is completely irreversible (O'Brien & Chafetz, 1982). Death from cirrhosis is believed to result from hepatocellular failure, portal hypertension or a combination of both (MacSween, 1985).

Despite the relatively low occurrence of cirrhosis, it appears from recent data that the disease is becoming more common. The Registrar General's Report for Scotland, shows that from 1970-1981, the cirrhosis death rate in Scottish males, rose by 104 percent and by 73 percent in females (Scottish Health Education Co-ordinating Committee, 1985).

Cirrhosis is more common in males than in females due to the higher average consumption of alcohol by males. Despite this, women appear to be more susceptible to this lesion as well as to alcoholic liver disease in general. One possible reason for this is that women have a greater proportion of body fat than men and thus absorb alcohol less readily from the circulation, leaving higher and potentially more injurious circulating levels of alcohol.

Histologically there is extensive fibrosis, nodule formation with evidence of regeneration and distortion of the lobular and vascular architecture. There are also varying degrees of cholestasis and bile duct proliferation (Flemming & McGee, 1984).

Both alcoholic fatty liver and alcoholic hepatitis may coexist with cirrhosis. However, despite suggestions of a progression from fatty liver to cirrhosis, no definite evidence exists of a precursor relationship between these lesions and cirrhosis. One suggestion is that hepatocellular necrosis may be involved in stimulating fibrinogenesis (Popper & Orr, 1970). The presence of Mallory bodies has also been correlated with the pronounced destruction of the normal liver lobular architecture (Christophersen & Poulsen, 1971).

The fibrous connective tissue involved in cirrhosis has been found to consist of collagen fibrils, which may be stabilised by a mucopolysaccharide-protein complex thus enabling the formation of collagen bundles (Flemming & McGee, 1984). The accumulation of this hepatic collagen and its role in the inflammatory response that frequently accompanies cirrhosis is reviewed in detail by Feinnman and Lieber (1972) and Lieber, (1982).

1.3 ALCOHOL AND DISEASE - THE GASTROINTESTINAL TRACT

1.3.1. The Oesophagus

A number of authors have observed disturbances of the normal oesophageal motility with changes in peristalsis (including the presence of nonperistaltic waves), resulting from alcohol abuse (Winship et al., 1968; Lieber, 1982).

Another alcohol related oesophageal condition is that of reflux oesophagitis which involves the reflux of acid, pepsin and often bile salts from the stomach into the lower oesophagus. The mechanisms underlying reflux oesophagitis are not yet clearly understood. However, delayed gastric emptying and lowered gastro-oesophageal sphincter pressure, both result from alcohol abuse and both may be involved in causing reflux oesophagitis (Lieber, 1982).

The oesophagus may also be damaged in Mallory-Weiss syndrome, (longitudinal tears in the mucosa of the oesophago-gastric junction). This probably results from excessive wretching and vomiting following ingestion of excessive amounts of alcohol, rather than from the direct effects of ethanol (Langman & Bell, 1982).

There is extensive epidemiological evidence implicating chronic alcohol abuse in the development of cancer of the oesophagus. This, however, will be dealt with in more detail later, in the section on alcohol and cancer (Section 1.4.2.).

1.3.2. The Stomach

The first reported studies on the acute effects of ethanol on the gastric mucosa were performed by William Beaumont (Lorber, Dinoso & Chey, 1974) on his now celebrated patient, Alexis St.Martin. It was observed that following acute alcohol ingestion, St.Martin produced classic signs of inflammation of the gastric mucosa, with concomitant erythema and mucopurulent discharge. The mucosa was seen to rapidly return to normal following abstinence. Beaumont's findings, have been confirmed in a more recent study of 34 severely inebriated soldiers, 30 of whom had gastric erosions, petechial haemorrhages and patchy hyperaemia (Palmer, 1954). These changes were completely reversible within 7-20 days.

Animal studies using cats (Grant, 1945) and guinea pigs (Williams, 1956) have confirmed that acute gastritis is associated with acute ethanol ingestion, the extent of the gastritis being dependent on the concentration of the ingested ethanol. The exact aetiology of the damage is unknown, but Glowacka *et al.* (1974), in an *in vitro* study of ethanol treated human gastric mucosa cells, have shown a decrease in galactosamine synthesis (a key component of mucus). This may be the cause of the observed reduction in the mucosubstance of the gastric mucosa following acute ethanol ingestion (Dinoso *et al.*, 1970).

Structural alterations in the gastric mucosa observed by light microscopy following acute ethanol administration, include oedema, increased cell exfoliation and nuclear changes. Electron microscopy revealed clumping of nuclear chromatin, mitochondrial distortion and occasional disruptions of the apical cell membrane (Pitchumoni &

Glass, 1977).

Additionally, it has been repeatedly shown that chronic gastritis is a common finding in patients with a heavy alcohol intake (Teske, Find & Wood ,1955 and Dinoso et al., 1972).

It is widely believed that alcohol is a powerful stimulant of gastric acid secretion, but, whilst alcohol is indeed capable of stimulating gastric acid production, the net physiological effect appears to be one of lowering the gastric acid output (Lieber, 1982). In general, oral administration of alcohol leads to a reduction in gastric acid output, whereas intravenous administration leads to an increase (Langman & Bell, 1982). A discussion of the possible mechanisms behind the increased and decreased acid production is outwith the scope of this Chapter but the topic is widely reviewed by Lieber (1982).

The widespread assumption that ethanol precipitates gastric haemorrhaging, remains unsubstantiated. Indeed, some studies suggest (Goulston & Cooke, 1968; Bouchier & Williams, 1969), that ethanol per se will not cause gastric haemorrhage. Despite the large number of alcoholic patients who have haematemesis and melaena, it is firmly believed that these conditions result from coincidental lesions and are not in fact a direct effect of ethanol (Langman & Bell, 1982). It was, however, noticed that alcoholic patients who also had atrophic gastritis, did show an increased faecal blood loss subsequent to ethanol ingestion (Dinoso, Meshkinpour & Lorber, 1972).

Ethanol displays a concentration dependent effect on gastric emptying, ranging from no effect (or even a slight stimulation) at concentrations of 6 percent or less, to a definite delay at ethanol concentrations of 10 percent or more (Mezey, 1982). The mechanism underlying delayed gastric emptying could simply relate to the hyperosmolarity of the ethanol solution, but is more likely to be mediated by the suppression of gastric motility brought about by chronic alcohol ingestion.

1.3.3. The Small Intestine

A number of reports suggest that there are no microscopic changes in the small intestine as a result of acute alcohol ingestion (Pirola & Davies, 1970), although most authors have noticed haemorrhagic erosion of the villi. These lesions appear to occur rapidly following alcohol ingestion (within ten minutes) and disappear rapidly, following abstinence, to leave intact healthy villi (Baraona, Pirola & Lieber 1974 ; Kerawitt, 1974). The extent of the structural changes are dependent on the ethanol concentration and decrease towards the ileum. Studies using solutions isosmolar to ethanol, have demonstrated that the morphologic effects of ethanol, result at least in part from the hypertonicity of the ethanol solution (Baraona, Pirola & Lieber, 1975).

After chronic ethanol consumption, rat jejunum shows slightly shortened villi, with an accompanying reduction in the number of cells per villus (Baraona et al., 1974). Electron microscope studies have revealed a number of ultrastructural changes including mitochondrial abnormalities, dilation of the endoplasmic reticulum and of the

cisternae of the golgi apparatus and focal cytoplasmic degradation, these latter changes being found in the villus and crypt cells of both the jejunum and the ileum.

Malabsorption of carbohydrates in alcoholics, was first confirmed using the D-Xylose absorption test (Small, Longarini & Zamcheck, 1959) and evidence now suggests that a large number of alcoholics may suffer from carbohydrate malabsorption. Inhibition of D-glucose transport has been demonstrated (Panowicz, 1967) and appears to be a concentration dependent phenomenon, which may be linked to ethanol inhibition of active transport linked sodium pumps.

Ethanol also inhibits the active transport of amino acids in the small intestine (Spencer, Brody & Lutters, 1964; Chang, Lewis & Glazko, 1967 and Israel, Salazar & Rosenmann, 1968), although concentrations of at least 2 percent are required before any inhibitory effects are noticed. The inhibition of active transport of amino acids is immediately reversible upon the removal of the ethanol.

Chronic alcoholism has been associated with a number of vitamin deficiencies (Lieber, 1982), e.g. thiamine, folate, vitamin B12 and vitamin A. Amongst alcoholics, folate is the most common form of hypovitaminosis. Similarly, chronic alcohol consumption is associated with alterations in the uptake of a number of metal ions, notably iron, calcium and magnesium.

Ethanol has been shown to have effects on intestinal motility which result in the accelerated movement of food through the small

intestine. This may help to account for the diarrhoea often associated with chronic ethanol ingestion (Pirola & Davies, 1970; Robles et al., 1974).

1.3.4. The Colon

Haemorrhoids are probably the most common alcohol related lesion of the colon (Williams, 1975) and may result from portal hypertension in cirrhotic patients. Alcohol has also been shown to have direct effects on colonic motility, resulting in a general increase in the propulsive motility (Lieber, 1982). It may also induce alterations in the normal colonic morphology (Brozinski et al., 1978).

The possible association between alcohol and colorectal cancer is discussed elsewhere in this review (Section 1.4.2.).

1.4 ALCOHOL AND CANCER

1.4.1. Introduction and Epidemiology

Perhaps the earliest report of an association between alcohol and cancer was by Warren, a Boston surgeon who reported a case of lingual cancer, commenting that "Predisposition was generated by the long use of ardent spirits." (McCoy & Wynder, 1979). Since this observation, large numbers of epidemiological studies have testified to the strong relationship between chronic alcohol consumption and an increased risk of cancer of the mouth, pharynx, larynx and oesophagus. These relationships have been confirmed by studies showing a decreased incidence of these cancers in social groups that largely abstain from alcohol, for example, Mormons and Seventh Day Adventists (Tuyns, 1979). Alcohol has also been implicated in cancers of a number of other sites (e.g. colon, rectum and liver) although the evidence for such relationships tends to be conflicting. Estimates of the percentage of total cancers attributable to alcohol in the United States vary in general from 3-8 percent (Tuyns, 1979; Rothman, 1980).

1.4.2. The Sites Affected

The effect of alcohol on cancer development in general is difficult to assess, but when cancers are studied with respect to their site of origin, striking positive correlations with alcohol intake do appear (Schmidt & Popham, 1981).

(1) The Oesophagus: It is estimated that the risk of developing oesophageal cancer is up to 17 times greater in alcoholics than in the rest of the population and that almost 75 percent of all oesophageal cancers in the United States are alcohol related (Rothman, 1980).

Of particular interest with regards to oesophageal cancer, is the considerable geographical variation in its occurrence and whilst this cancer is rare in the United States, it is common in central Asia and is surprisingly high in the west coast provinces of France (Tuyns, 1979). These observations suggest that environmental carcinogens may play an important role in the development of oesophageal cancer and whilst the relationship with alcohol has been shown to be independent of the type of beverage used, it is seen to be particularly marked in regions in which local drinks (african maize beers and french apple brandies and ciders) have been shown to contain high levels of certain carcinogens. It is important to note that carcinogens present in alcoholic beverages cannot completely account for the geographical variation in oesophageal cancer incidence. For example Iran, being a Muslim state, has a very low per capita consumption of alcohol, but has a remarkably high oesophageal cancer rate (Gillis & Carter, 1976).

Smoking has also been implicated as an aetiological agent in oesophageal cancer and joint exposure to chronic ethanol and tobacco results in a multiplicative effect.

(2) The Pharynx and Larynx: The relative risk for cancer of the pharynx is increased in the presence of alcohol abuse by factors of up to 12 (Elwood et al., 1984) and for laryngeal cancer by factors of up to 10 (Rothman, 1980). Cancer of the larynx is a frequent disease in South West Europe, a region with a very high wine consumption and as is suggested for cancer of the oesophagus, carcinogenic contaminants in locally brewed beverages may be of importance (Tuyns, 1979).

(3) The Liver: In western populations, primary hepatocellular carcinoma is rare. However, it is considerably more common amongst cirrhotic alcoholics, with an estimated 10-30 percent of this group developing liver cancer (Rothman, 1980). The relationship between alcohol and primary liver cancer is not yet fully understood and it has been suggested that hepatocellular carcinoma is secondary to cirrhosis and not necessarily a consequence of alcohol consumption per se (Tuyns, 1979). Some evidence does exist, however, of a relationship between alcohol and liver cancer even in the absence of cirrhosis (Lieber et al., 1979).

(4) Other Sites: Alcohol abuse has been implicated in the development of cancers of a number of sites other than those mentioned above e.g. stomach, colon, rectum, prostate and pancreas. The evidence substantiating these relationships is sparse and no positive conclusions with regards to these neoplasms can be drawn.

It has also been suggested (MacSween, 1982), that an inverse relationship exists between alcohol and certain cancers, with decreased incidences of lymphomas, soft tissue tumours and female genital tract tumours, being observed in alcohol abusers.

1.4.3. The Mechanisms of Action of Alcohol in Carcinogenesis

Given the positive correlation between chronic ethanol abuse and certain cancers, it is worth discussing the possible role of alcohol in the carcinogenesis. Postulated roles include:

(1) Alcohol as a Carcinogen, Cocarcinogen or Promoter: For the purposes of the following discussion, a carcinogen is defined as being an agent which can cause cancers to develop that would not otherwise

have done. A promoter is defined as being a factor which, when applied repeatedly after a dose of a tumour initiating agent insufficient to produce tumours, then results in tumour formation. A cocarcinogen is a non carcinogenic agent which augments the action of a carcinogen.

The vast majority of studies have failed to show that ethanol alone is a carcinogen (Ketcham, Wexler & Mantel, 1963; Stenback, 1969). Ethanol has, however, been shown to cause chromosomal changes in vivo (Obe & Herha, 1975) and abnormal oral epithelial cells have been reported in human alcoholics (Anderson, 1972). Stenback (1969) has also demonstrated that ethanol is not a tumour promoter.

It has been observed that ethanol does not increase the risk of cancer in general, but only of specific sites. Alcohol thus affects only the site at which the cancer will occur but not the likelihood of cancerous growth in general (Schmidt & Popham, 1981), such a role being more typical of a cocarcinogen than of a carcinogen or promoter.

Ethanol may act by increasing the solubility of carcinogens (e.g. tobacco tars) and thus increasing their entry into susceptible tissue.

The teratogenic potential of ethanol is now well accepted, with excessive alcohol intake during pregnancy being a major cause of mental retardation in infants. One third to one half of babies born to alcoholic mothers have signs of foetal alcohol syndrome (Lieber, 1982).

(2) Contaminants in Alcoholic Beverages: Despite the evidence suggesting that the risk of developing cancers of the upper alimentary and respiratory tracts is present, regardless of the beverage consumed (MacSween, 1982), it is clear that certain beverages are potentially more dangerous than others. Carcinogenic contaminants such as fusel oils, polycyclic hydrocarbons, nitrosamines and asbestos fibres, have been found in a number of beverages and in the apple brandies and ciders implicated in oesophageal cancer, diethylnitrosamine, a potent oesophageal carcinogen has been found (Lieber, 1982).

(3) Ethanol Effects on Epithelia: Ethanol induced local tissue damage may be an important mechanism by which cells are sensitised to chemical carcinogens. In support of a direct effect of ethanol on the tissues is the gradient of decreasing risk, parallelling the successive dilutions of alcohol in the alimentary tract (Lieber, 1982).

The topical effect of ethanol has been demonstrated by Mascres, Ming-Wen and Joly (1984) who reported epithelial atrophy in the oesophageal epithelium of rats fed alcohol for up to 290 days. The authors reported this alteration to be due to a reduction in epithelial cell size.

An increased permeability of canine oesophageal mucosa in the presence of ethanol has been demonstrated (Shirazi et al., 1974; Shirazi & Platz, 1978). Evidence of such alterations in the mucosal permeability lends credence to the suggestion that ethanol aids the process of carcinogenesis by lowering the effectiveness of the epithelial permeability barrier.

There is also evidence to support the claim that ethanol increases the local conversion of procarcinogens to their ultimate carcinogenic form. For example, it has been demonstrated that nasal mucosa from ethanol fed rats has an enhanced ability to activate N-Nitrosonornicotine, by α-carbon hydroxylation (Castonguay et al., 1984).

(4) Alterations in Liver Metabolism: A relationship between cirrhosis and the development of head and neck cancer (Keller, 1967), has been demonstrated, and Protzel, Giardina and Albano (1964), have suggested that the relationship may derive from an inability of the alcohol compromised liver to adequately metabolise carcinogens leading to an increase in circulating carcinogen levels and thus to an increased cancer risk.

Evidence also exists which suggests that in the presence of ethanol, the liver has an increased ability to activate procarcinogens, again leading to an increase in the circulating levels of carcinogens. Studies of ethanol treated rats, support this suggestion demonstrating an increased ability of hepatic microsomes to convert the procarcinogen N-Nitrosopyrolidine to a mutagen (McCoy & Wynder, 1979). This finding is consistent with the observed increase in the microsomal drug metabolising capacity of the alcoholic liver (McCoy & Wynder, 1979) . One objection to this clearly attractive mechanism is that it is highly unlikely to occur in the severely compromised liver of a chronic alcohol abuser. Also any carcinogens that are activated in this way must be highly site specific or else there would be no reason for only some tissues developing tumours.

(5) Malnutrition: Malnutrition, a common concomitant of alcoholism, has been implicated as a promoting agent in the process of carcinogenesis (Lieber et al., 1979). Of particular interest is the observation that chronic alcoholism leads to a decrease in the levels of vitamin A, a compound known to be involved in the process of cellular differentiation. It has been shown that lowered Vitamin A levels can affect the induction of various tumours (Lieber et al., 1979).

1.5 ALCOHOL AND ORAL CANCER

1.5.1 Oral Cancer Epidemiology

Oral cancer is a disease of the elderly and accounts for some 3-5 percent of all cancers in most European countries. The aetiology of oral cancer is complex and an extensive review on this subject has recently been published (Binnie, Rankin & MacKenzie, 1983). A detailed discussion of these factors is outwith the scope of this chapter but it appears that at present, tobacco habits and alcohol are the most important non-infective aetiological factors.

1.5.2. Alcohol and Oral Cancer - Epidemiology

Most studies show that a high proportion of oral cancer patients are heavy alcohol consumers 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 important role of alcohol in oral carcinogenesis is also emphasised by studies on populations not normally consuming large quantities of alcohol e.g. Jews, Mormons and Seventh Day Adventists. Generally, such populations show consistently lower than average oral cancer rates (Binnie et al., 1983).

Another frequently observed factor is the interaction of alcohol and tobacco in oral carcinogenesis. Most authors show (Table 1.1), that the relative risk for oral cancer resulting from exposure to both alcohol and tobacco is greater than the sum of the two independent risk factors and indeed the majority of the figures (Table 1.1) bear out this relationship. It is perhaps worth noting that some authors

do not agree with this synergistic model suggesting that an additive model could also explain the relationship between alcohol, tobacco and oral cancer (Graham et al., 1977).

Alcohol Alone	Tobacco Alone	Alcohol and Tobacco	References
1.23	1.53	5.71	Rothman & Keller, 1972
4.19	2.05	8.58	Elwood et al., 1984
1.70	1.54	2.49	Graham et al., 1977
10.6	5.0	--	Mashberg, Garfinkel & Harris, 1981
2.33	2.43	15.5	Rothman, 1980
5.1	10.0	24.0	McCoy & Wynder, 1979

Table 1.1: ORAL CANCER RELATIVE RISK

There appears to be a gradient of increasing risk with increasing alcohol consumption but confusion exists as to whether the type of beverage consumed is of importance. Mashberg et al., (1981) suggest that some beverages are more carcinogenic than others, with beer drinkers having approximately a three fold higher risk of developing oral cancer than whisky drinkers. Wynder and Bross (1957), on the other hand, demonstrated that whisky rather than beer is associated with the higher oral cancer risk. It is clear, however, that there is a definite increase in risk regardless of the beverage consumed.

1.5.3. Experimental Studies

Despite the convincing evidence linking alcohol and oral cancer, experimental studies on the effects of ethanol on oral carcinogenesis are uncommon. Elzay (1966) demonstrated that locally applied ethanol reduced the latent period and increased tumour size in hamster cheek pouches painted with DMBA. In addition, he demonstrated that tobacco was a more potent promoting agent than alcohol and that in this experimental system alcohol and tobacco did not act synergistically (Elzay, 1969). Further evidence for an effect of alcohol on the process of hamster buccal pouch carcinogenesis came from the study of Freedman and Shklar (1978), who demonstrated that hamsters fed alcohol and painted with DMBA, developed leukoplakic lesions and squamous cell carcinoma two weeks ahead of the control animals. Arendt et al. (1981), using sherry as a promoting agent, produced similar results and showed that tumours developed two weeks earlier in the experimental group than in the controls.

Histological studies of the effects of ethanol alone upon the oral epithelium have produced conflicting results. Anderson (1972) demonstrated an increased frequency of dysplastic epithelial cells in human alcoholics and more recently, in a post mortem study of human alcoholics, Valentine et al. (1985) demonstrated a reduction in epithelial thickness, resulting from reduced cell size in the maturation layer. They also demonstrated similar histological changes resulting from tobacco usage and suggested that the observed alterations may be non-specific reactions to chronic irritation. These authors failed to demonstrate any interaction in the histological effects of alcohol and tobacco.

Histological alterations have been demonstrated in animal systems, with epithelial dysplasia appearing in rabbit oral mucosa, after chronic topical ethanol treatment (Muller et al., 1983). An increased thickness of the keratin layer was demonstrated in the oral epithelium of alcoholic rats (Mascres & Joly, 1981). One study (Margarone et al., 1984), has failed to demonstrate any histological effects of ethanol on the hamster cheek pouch epithelium although the duration of ethanol treatment was very short in this study and may not have allowed time for histological changes to develop.

Biochemical studies are largely lacking in this field although Mascres and Joly (1981) using histochemical techniques, demonstrated increases in acid phosphatase activity and succinate dehydrogenase activity. An increase in SS and SH groups in the keratin layer were also noted.

1.5.4. Possible Mechanisms of Action of Ethanol in Oral Cancer

The mechanisms mentioned previously (Section 1.4.3.) for the role of alcohol in carcinogenesis are also likely to apply in the oral cavity. However there are a number of additional points worthy of note.

It has long been known that a correlation exists between cirrhosis and oral cancer even in the absence of proven alcohol abuse (Keller, 1967). Protzel et al. (1964), demonstrated a markedly enhanced tumour yield and a substantially reduced latent period for benzpyrene related oral tumours in mice with carbon tetrachloride induced cirrhosis. The suggested mechanism is that a cirrhotic liver has a reduced ability to metabolise carcinogens leading to raised circulating levels of benzpyrene and thus enhanced tumour formation. Mascres and

Franchebois (1979 & 1981) studied histological and histochemical changes in the oral epithelium of cirrhotic humans and found these to be remarkably similar to those found in the oral epithelium of the alcoholic rats.

More recently, Murphy and Hecht (1986) have demonstrated an increased ability of the microsomal fraction from the cheek pouch epithelium of alcohol fed hamsters to metabolise benzpyrene to a structure closer to the ultimate carcinogen. It is suggested that in this way alcohol might enhance benzpyrene carcinogenesis in the hamster cheek pouch.

1.6 THE METABOLISM OF ALCOHOL

1.6.1. Introduction

To understand the mechanism of action of alcohol in some of the disease processes in which it is involved, a knowledge of the pathways of ethanol metabolism is important. The following review shall deal with alcohol dehydrogenase, catalase and the microsomal ethanol oxidising system.

1.6.2. Alcohol Dehydrogenase

There is little doubt that under all but the most exceptional circumstances, the major enzyme system responsible for the first stage in ethanol metabolism, oxidation to acetaldehyde, is the alcohol dehydrogenase system (Dawson, 1983). Alcohol dehydrogenase (ADH) is widely distributed amongst animals, plants and microorganisms and in mammals exists as a cytosolic enzyme, the highest concentrations of which are found in the liver.

Initially there was considerable debate over the physiological role of ADH, it being considered unlikely that the body should manufacture an enzyme to metabolise an exogenous compound that is consumed largely as a social drug. More recently, however, it has become apparent that ethanol is not an entirely exogenous compound and that many gastric flora produce ethanol as a result of their normal metabolic function (Krebs & Perkins, 1970). In fact ethanol has been detected in the blood of non drinking individuals and also in the blood of some animals with germ free gastrointestinal tracts (endogenous synthesis). It has been estimated that in man, 1-10 grammes of ethanol are

synthesised daily by intestinal microorganisms and by various endogenous pathways (Li, 1977).

The above evidence coupled with the very broad substrate specificity of all mammalian ADH's has led to the suggestion that ADH probably has a general physiological role in detoxifying or metabolising various alcohols and aldehydes, of which ethanol is only one.

Inhibitor studies using pyrazole and its analogues (competitive inhibitors of ADH) have shown ADH to be responsible for at least 85 percent of ethanol elimination in rats (Goldberg & Rydberg, 1969). Further evidence comes from the observation that the Michaelis constant (Km) for ADH *in vitro* is in agreement with the Km for blood ethanol disappearance *in vivo* (Makar & Mannering, 1970) and that despite having a pH optimum of 10, ADH is sufficiently active at physiological pH to more than account for the observed rates of ethanol metabolism.

The enzyme from humans, horses and rats appears to be a dimer with a molecular weight of 80 Kilodaltons (K) per molecule or 40K per subunit. All enzymes have a broad substrate specificity, having the capacity to oxidise primary and secondary aliphatic alcohols, diols, cyclic and aromatic alcohols, ω-hydroxylated fatty acids and 2-enoic alcohols and their respective aldehydes. In some cases the enzymes can also oxidise steroids (Li, 1977).

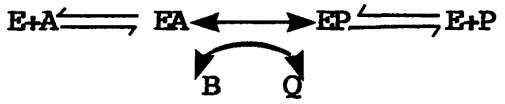
In all cases the enzymes are metalloenzymes containing 4 atoms of zinc per protein molecule or 2 per subunit, and are accordingly inhibited

by chelating agents, e.g. EDTA, 1,10-phenylalanine, indicating that zinc is essential for the activity of the enzyme. Studies on the horse enzyme have shown that only 2 of the 4 zinc atoms are located at the active site of the enzyme, the other two probably being involved in maintaining the 3-Dimensional structure of the enzyme molecule. The enzyme also contains associated nicotinamide adenine dinucleotide (NAD) coenzyme molecules, which are essential for its activity and which are present 2 per enzyme molecule, this being consistent with the presence of 2 active sites.

In the horse and the human, but not in the rat, ADH exists in multiple enzymic forms (isozymes) which differ in a number of respects and which are discussed at length in a recent informative review by Pietruszko (1980).

Also worthy of note is the highly conserved nature of ADH throughout evolution, and in fact approximately 80 percent sequence homology has been found between the human, horse and rat enzymes (Jornvall & Markovic, 1972) and approximately 90 percent between the human and the horse enzymes. Cross reactivity of antibodies raised against ADH from various species also points to the high degree of homology between these enzymes, although a notable exception is the recently discovered pi-ADH (Pietruszko, 1980).

The reaction mechanism by which ADH oxidises ethanol and other substrates, is believed to be of the Theorell-Chance or compulsory order kinetic type, as shown below:



E = enzyme

A = alcohol substrate

P = aldehyde product

B = reduced NAD (NADH)

Q = oxidised NAD

The mechanism involved in the oxidation of more complex alcohols may not be of this type and may in fact be considerably more complex.

1.6.3 Catalase

Catalase is found in most animal tissue, with concentrations being highest in the liver, kidney and erythrocytes. More specifically it is situated in the peroxisomes of most cells (Lieber, 1982).

Early studies, now disproved, indicated that there was insufficient ADH activity in the liver to account for the observed rates of *in vivo* ethanol metabolism (Peters, 1982). This evidence, coupled with the observation that some ethanol metabolising capacity was insensitive to ADH inhibitors, led investigators to search for alternative, minor pathways of ethanol metabolism, one of which involved the enzyme

catalase.

The reaction by which catalase metabolises ethanol (the 'peroxidatic reaction) is as follows:

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

The best substrates for this reaction appear to be methanol, ethanol and formate and it is believed in the rat at least, that catalase may be important mainly for methanol oxidation.

Despite the considerable controversy concerning the role of catalase in ethanol metabolism, it is now agreed that catalase does in fact catalyse ethanol oxidation in vivo. It is apparently not, however, a major contributor to the metabolic flux of ingested ethanol being responsible for less than 10 percent of ethanol oxidation (Peters, 1982). It is generally believed that the contribution of catalase to ethanol oxidation increases progressively as the ethanol concentration increases (Li, 1977).

1.6.4. The Microsomal Ethanol Oxidising System

The search for minor pathways of ethanol metabolism, has also led to the discovery of a microsomal ethanol oxidising system (MEOS), utilising the mixed function oxidases of the smooth endoplasmic reticulum. This system, which is also believed to be responsible for detoxifying many drugs, requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 for its activity and also requires cytochromes to allow activation of the molecular oxygen to

free radicals. The ethanol oxidation reaction is believed to be as follows:

$$CH_3CH_2OH + NADPH + H^+ + O_2 \longrightarrow CH_3CHO + NADP^+ + 2H_2O$$

Evidence in favour of the role of MEOS in ethanol metabolism comes from the observations that ethanol ingestion leads to an increase in the smooth endoplasmic reticulum content of cells and that isolated microsomes have the ability in the presence of NADPH and O_2 to oxidise ethanol.

There is a wealth of convincing evidence, both for and against a major role in ethanol metabolism for the MEOS and it remains an area of some controversy. The available evidence has been reviewed by Li (1977).

It does appear, however, that the contribution of this enzyme system to ethanol metabolism is at most 10-20 percent although, as for catalase, the role of MEOS may become more important as the ethanol concentration increases (Mezey, 1985).

1.7 THE BIOCHEMICAL EFFECTS OF ETHANOL

1.7.1. Introduction

Following acute or chronic ethanol consumption, a number of biochemical changes take place in a variety of cellular systems. Some of these changes result from the action of ethanol per se, but the majority appear to result from the action of the various metabolic by products of ethanol metabolism e.g. acetaldehyde and the increased levels of reducing equivalents.

Unfortunately, little information relating specifically to the oral cavity is available and thus the bulk of this review will concentrate on the effects of ethanol at other body sites.

1.7.2. Metabolic Effects

Ethanol metabolism by alcohol dehydrogenase produces reducing equivalents in the following way:

$$\text{ETHANOL} + \text{NAD}^{+} \longrightarrow \text{ACETALDEHYDE} + \text{NADH}$$

At even moderate blood ethanol levels, the amount of hydrogen generated in this way, exceeds that which can be readily utilised and hence there is an increase in the NADH/NAD ratio in the tissues following alcohol consumption (Lundquist, 1975). This alteration in the NADH/NAD ratio is the mediator for many of the biochemical and metabolic effects of ethanol and leads to an increase in the cellular lactate/pyruvate ratio which can result in hyperlactic acidosis. Lactic acidosis will be discussed in more detail below.

The excess reducing equivalents must be used up and the pyruvate to

lactate conversion is only one of a number of proposed pathways for lowering the NADH/NAD ratio. In normal mitochondria, the reducing equivalents required for respiratory chain activity, arise mainly from β-oxidation of fatty acids. However in the presence of the excess reducing equivalents produced by ethanol metabolism, the β-oxidation of fatty acids is inhibited and the respiratory chain uses the reducing equivalents produced by ethanol metabolism. This results in a build up of lipids (both exogenous and endogenous), leading to the development of a fatty liver and also to an inhibition of adenosine diphosphate (ADP) translocation across the mitochondrial membrane (a process that is inhibited by long chain acyl coA derivatives of fatty acids). This accounts for the lowering of the respiratory activity and the lack of respiratory control commonly observed in mitochondria from ethanol treated livers (Gordon, 1973). This fat accumulation does not continue indefinitely and eventually stabilises around the time the redox state is attenuated (Lieber, 1980).

1.7.3. Carbohydrates and Organic Acids

The increased NADH/NAD ratio in alcoholics also has a number of deleterious effects on carbohydrate metabolism:

(a) Hypoglycemia: Pyruvate is a common starting point for gluconeogenesis and in the presence of excess reducing equivalents, much of the pyruvate is reduced to lactate thus inhibiting gluconeogenesis. If this occurs whilst the body is in a fasted state, then the above effect leads to a lowering of the blood sugar levels and thus to hypoglycemia, a known complication of alcohol abuse (Lieber, 1976).

(b) Hyperglycemia: In the well fed state, alcohol leads to an increase in glycogenolysis and this, coupled with a reduced peripheral glucose consumption, produces hyperglycemia. This effect is also observed in isolated perfused livers and the effect is not wholly dependent on hormones (Lundquist, 1975).

(c) Lactate Effects: As mentioned previously, the excess reducing equivalents produced lead to an increase in the lactate/pyruvate ratio. This increase is enhanced by the reduced elimination of lactate in the alcoholic and often leads to lactic acidosis (Lieber, 1980). Lactic acidosis reduces the ability of the kidneys to excrete uric acid, resulting in an increase in serum uric acid, a factor that is responsible for the observed exacerbation of gout following alcohol consumption (Mezey, 1985).

1.7.4. Protein Effects

Under conditions of acute ethanol administration, both in vivo and in vitro, cellular protein synthesis is decreased (Lieber, 1980). The mechanisms underlying the observed alterations in protein synthesis are as yet obscure, but may relate to the ethanol mediated alteration in the cellular redox potential. It has been noticed that addition of ethanol to perfused rabbit livers produces disaggregation of both free and bound polysomes and detachment of ribosomes from the rough endoplasmic reticulum (Lieber, 1980). It is possible that it is in this way that ethanol, or the resultant rise in reducing equivalents, exerts its effect on protein synthesis

The reaction of cells to chronic ethanol treatment is not clear but there is evidence that the response may be cell specific (Bengstsson,

Smith-Kiellan & Morland, 1984). The results are conflicting, ranging from an observed 30-35 percent inhibition of protein synthesis in rat hepatocytes following chronic ethanol ingestion (Bengtsson et al., 1984), to a virtual doubling of protein synthetic activity in the rat in vivo (Khawaja & Lindholm, 1978).

Despite the belief that hepatomegaly resulted largely from the accumulation of lipids, it has more recently been demonstrated that at least half of the increase in liver dry weight can be accounted for by an increase in hepatic protein content (Baraona, Leo & Borowsky, 1975). The suggested mechanism for this hepatic accumulation of protein, is impaired secretion of hepatic secretory proteins (e.g. albumin) and experimental results have gone some way towards verifying this by demonstrating inhibited secretion of albumin and glycoproteins from the liver following both acute and chronic ethanol administration (Tuma & Sorrell, 1984). The inhibition of secretion is probably mediated at the post translational level and in the specific case of glycoproteins, may result from an impaired transport of glycoproteins from the golgi complex to the plasma membrane (Mailliard et al., 1984).

1.7.5. Vitamins

In alcoholic patients a number of vitamin deficiencies are commonly observed e.g. thiamine, folate, vitamin B6, vitamin A and vitamin D. The mechanism underlying these deficiencies varies and is outwith the scope of this review. The topic is fully discussed by Mezey (1985).

1.7.6. Effects of Ethanol on Biological Membranes

Although a little simplistic, in general, ethanol fluidises membranes and this fluidising property, has been suggested as the mechanism underlying ethanols psychotropic effects (La Droite, Lamboeuf & De Saint Blonquet, 1984). Further evidence substantiating this claim has come from the observation that some membranes isolated from chronically treated animals, which have developed a degree of tolerance to ethanol, are resistant to the fluidising effects of ethanol.

Despite the known fluidising effect of ethanol, it has frequently been observed that ethanol can in fact lead to an increased rigidity of biological membranes. There are a number of mechanisms proposed to explain this phenomenon:

(1) Alterations in the component lipids have been shown to result from ethanol treatment and to lead to an increased rigidity of the membranes (La Droite et al., 1984).

(2) In the specific case of Escherichia coli plasma membrane, it has been demonstrated that the increased rigidity can be accounted for almost entirely by a decrease in the lipid/protein ratio and it is suggested that the regulation of the lipid/protein ratio may be an important adaptive response to ethanol (Dombeck & Ingram, 1984).

Ethanol effects on the membrane sodium/potassium (Na/K) adenosine triphosphatase (ATPase) pumps have also been demonstrated. Despite data showing an increase in the activity of the Na/K ATPases from rat brain following chronic ethanol treatment (Israel et al., 1970), it is generally accepted that ethanol inhibits such enzymes. These enzymes

have been implicated as the intermediate link between metabolic energy and nerve impulses, thus ethanol may exert its primary brain effects by altering the action of these synaptosomal enzymes (Sun & Morajski, 1970). Another possible contributor to ethanol brain effects is the inhibition of the Na/K ATPases in erythrocyte membranes which may account for the decreased oxygen uptake by the brain following ethanol consumption (Lundquist, 1975)

1.7.7. Ethanol and Enzyme Effects

Ethanol affects a number of enzyme systems in the body, most notably, those of the muscles and liver.

(1) Muscle Enzymes: Muscle biopsies from chronic alcoholic patients, show reduced activity of a number of enzymes, resulting in a disturbance of the energy balance of skeletal muscle. Amongst the enzymes affected are creatine phosphokinase, lactate dehydrogenase, hexokinase, malate dehydrogenase and succinate dehydrogenase (Suominen et al., 1974) as well as triosephosphate dehydrogenase and cytochrome c oxidase (Kiessling et al., 1975).

(2) Liver Enzymes: The effects of ethanol on the liver often lead to enzyme induction and cell lysis, resulting in enhanced release of various hepatocellular enzymes into the circulation. These circulating enzymes can be used as diagnostic markers of liver injury and can in fact allow quite specific diagnosis of individual liver disorders. These enzymes include gamma-glutamyl transferase (GGT), alkaline phosphatase (alk. phos.), aspartate transaminase (AST) and alanine transaminase (ALT). This topic shall be dealt with in more detail in Chapter two.

1.8 ORAL EPITHELIUM STRUCTURE AND FUNCTION

1.8.1. Introduction

The oral epithelium is a stratified squamous epithelium and acts as a mechanical barrier which is impermeable to most solutes. Human oral epithelium can be divided on a functional basis into three main types (Squier & Meyer, 1971):

(a) Lining: This epithelium covers the cheeks, lips , floor of the mouth, ventral surface of the tongue and the soft palate, and is largely non-keratinised.

(b) Masticatory: This type is found in the gingivae and hard palate and is largely ortho- or para-keratinised.

(c) Specialised: This type is only found on the dorsal surface of the tongue and is structurally related to the masticatory mucosa but differs in that it contains specialised and extensive papillae, some of which carry taste buds. This epithelium is again ortho- or para-keratinised.

The oral cavity of most animals shows the same basic structure but is nearly always an ortho- or para-keratinised epithelium, of varying thickness.

1.8.2. Structure

The ortho- and para-keratinised epithelia are subdivided into the following strata:

(1) STRATUM BASALE (the basal layer)

(2) STRATUM SPINOSUM (the prickle cell layer)

(3) STRATUM GRANULOSUM (the granular layer)

(4) STRATUM CORNEUM (the keratinised layer)

Mitotic division occurs mainly in the basal layer, with clustering of mitoses at certain points. The time taken for a cell to pass from the basal layer to the most superficial layer varies from region to region, but is generally faster in non-keratinising epithelia than in keratinising epithelia, with values for human gingiva being c.41-57 days (Meyer, Marwah & Weinmann, 1956) and for buccal epithelium being c.25 days (Alvares et al., 1972).

1.8.3. Ultrastructural Features of the Oral Epithelium

Oral epithelial cells have many features in common with epithelial cells at other sites. An extensive review of the ultrastructure of the oral epithelium has been presented by Chen and Squier (1983), and a detailed description is outwith the scope of this review. There are, however, certain features peculiar to keratinising epithelium which are of interest :

(1) The Intercellular Substance: Most cells of the oral epithelium are surrounded by a carbohydrate/protein coat named 'glycocalyx' (Bennet, 1963) which is found to be more abundant between the cells of the buccal than of the palatal epithelium. This intercellular substance may play a role in adhesion but may also act as a lubricant facilitating the sliding of cells past one another. It may also be involved in the control of trans-epithelial permeability.

(2) Tonofilaments: Tonofilaments are the characteristic major structural elements that constitute the most abundant cytoplasmic component of stratified squamous epithelium (Chen & Squier, 1983). Bundles of tonofilaments insert into all desmosome and hemidesmosome

attachment plaques.

(3) Membrane Coating Granules: In the prickle cells, membrane bound electron dense structures begin to appear in the cytoplasm. These are the membrane coating granules, which are present in almost all stratified squamous epithelia. In the upper granular layer, the membrane coating granules extrude their contents into the extracellular space resulting in the formation of a permeability barrier.

There are two major forms of membrane coating granules found in the oral epithelium:

(a) In keratinised epithelium they are ovoid and membrane bound, having a series of parallel internal lamellae (Hayward, 1979).

(b) In non-keratinised tissue, they are typically spherical and membrane enclosed. The core is electron dense and delicate strands can often be seen radiating from it (Squier, 1977).

An intermediate form has been observed at the junction between keratinised and non-keratinised epithelium (Hayward, 1979).

(4) Keratohyaline Granules: There are two types of keratohyaline granules normally found in the oral epithelium (Chen & Squier, 1983):

(a) Irregularly shaped epidermal like keratohyaline granules are found largely in the masticatory mucosa, and have tonofilaments radiating from their surface and occasional ribosomes attached to the surface.

(b) A less common and more regular and spherical form, having numerous attached ribosomes but only few, if any, radiating tonofilaments. Such granules are more common in the para-keratinised

and non-keratinised epithelia.

The association of both types of keratohyaline granules with ribosomes has led to the suggestion that they are synthesised by the ribosomes. The function of keratohyaline granules is to provide an embedding matrix for the tonofilaments during the formation of the keratin layer. It is suggested that the more regular type granules may be involved in membrane thickening, a feature common to the outermost granular layer and the deepest row of squames in keratinising epithelia.

1.8.4. Epithelial Maturation

Although the time sequence and fine details of maturation varies from site to site within the oral cavity, in most oral epithelia, the final product of maturation is a keratinised layer formed by tonofilaments embedded in a matrix of keratin proteins.

An in depth discussion of the various stages of epithelial maturation is beyond the scope of this review although the subject is discussed at length in a recent review by Chen and Squier (1983). Briefly, as the cells move through the epithelium, they tend to increase in size and suffer a gradual and eventually complete (in the Stratum Corneum) loss of organelles. Throughout the maturation process, the amount of structural protein in the cells increases with the most superficial cells containing densely packed filaments embedded in a keratohyaline derived matrix. These cells are dehydrated and flattened (squames) and as a result of the total loss of cellular organelles, are non vital. The final stage of maturation is the loss of the most

superficial cells by desquamation.

Cells in the uppermost layers of all oral epithelia and epidermis are removed by desquamation, a process about which little is known, but which may have links with the hydrolytic enzymes extruded by the membrane coating granules. Such enzymes are suggested to be responsible for the breakdown of the extracellular cement holding the cells together, leading, with the assistance of extracellular abrasion (although this is not essential), to the shedding of the outermost cells (Weinstock & Wilgram, 1970).

Another more simple theory suggests that the intercellular adhesive has a limited life span and that cell detachment results from its spontaneous deterioration and breakdown, a process that is again aided by exposure to and abrasion by the external environment (Chen & Squier, 1983).

1.8.5. The Epithelial Connective Tissue Interface

The interface between the epithelium and the underlying connective tissue, is corrugated, resulting in an increase in the surface area over which the epithelium and connective tissue make contact. This ensures a strong mechanical attachment between the two tissues and also increases the surface area available for the delivery of nutrients to the epithelium, which is dependent on the underlying connective tissue for its blood supply.

Initial light microscopic studies on the interface area, identified a noncellular, periodic acid schiff. staining layer, 1 μm or more in

thickness, which was termed the BASEMENT MEMBRANE. The membrane acted as a barrier to the movement of cells and large particles, and allowed the anchoring of the epithelium to the connective tissue. Subsequent electron microscopic studies have further resolved the basement membrane into two layers (Susi, 1971):

(1) The LAMINA LUCIDA to which the basal cells are attached via hemidesmosomes.

(2) The LAMINA DENSA which binds the epithelium to the underlying connective tissue via anchoring fibrils.

The lamina lucida, lamina densa and the subjacent fibrils together comprise the BASAL COMPLEX which has its origin partly in the epithelium and partly in the connective tissue. It has been suggested that the laminae lucida and densa are epithelial derived and that the subjacent fibrils are connective tissue derived (Chen & Squier, 1983).

1.8.6. Non-keratinocytes

In the oral epithelium, there exists a sub-population of cells, labelled the non-keratinocytes, and characterised by the complete, or almost complete lack of desmosomes and tonofilaments. Such cells can account for up to 10 percent of the total epithelial cell population (Meyer, Alvares & Gerson, 1976), and are represented principally by melanocytes and langerhans cells, which are referred to as clear cells as a result of their tendency to shrink during histological preparation. Merkel cells are also present and are mainly basally situated. The structure and function of the various oral non-keratinocytes are discussed by MacKenzie and Binnie (1983).

1.9 BIOCHEMISTRY OF THE ORAL EPITHELIUM

1.9.1. Introduction

To investigate any biochemical effect of alcohol on the oral epithelium, and to successfully interpret any results obtained, it is essential to have a working knowledge of the biochemistry of the oral epithelium. Unfortunately, data relating specifically to the biochemistry of the oral epithelium, is sparse and to gain an insight into the probable biochemical features of the oral epithelium such data must be supplemented with data from studies on other stratified squamous epithelia, e.g.skin.

1.9.2. The Structural and Biochemical Components

(1) Proteins: An epithelial cell, in passing from the basal layer to the granular layer, increases its dry weight 29 fold (values calculated for the rat) over a period of six to seven days (Meyer,Alvares & Barrington, 1970). It is believed that a significant part of this weight gain is due to the synthesis of the structural proteins.

The structural proteins are the major protein products of stratified squamous epithelia, and their cellular concentrations increase as the cells move more superficially. These structural proteins are present as components of the filaments (tonofilaments), seen in all cell layers, which are the precursors of the fibrous material of the stratum corneum (Rudall, 1952). These tonofilament proteins are an heterogenous group, known collectively as keratins, a family of proteins that are highly insoluble and resistant to protease digestion

(Green, 1979). These properties are believed to be due to the strong attractive forces between and within the peptide chains.

In any stratified squamous epithelium, the keratins are abundant, and it is estimated in the epidermis that 30 percent of the cellular messenger RNA is involved in keratin synthesis (Gibbs & Freedberg, 1980). Keratins show site specificity, which develops postnataly from a uniform neonatal pattern and it is of interest to note that factors affecting the morphology of an epithelium, e.g. hyperplastic agents, will also lead to an alteration of the keratin polypeptide pattern (Odajium, 1984).

In human oral epithelium, the keratin pattern varies according to the degree of keratinisation, with hard palate showing six keratin polypeptides in the molecular weight range 47-67K and the nonkeratinising epithelium generally expressing smaller keratins in the molecular weight range 50-56K (Clausen et al., 1983; Clausen et al., 1986). Despite the oral cavity of rodents being entirely keratinised, the same general trends exist, with the hard palate showing higher molecular weight keratins than the more loosely packed buccal and tongue epithelium, which show a generally lower molecular weight pattern (Dale, Lonsdale-Eccles & Lynley, 1982).

The tonofilaments eventually aggregate to form tonofibrils which form the fibrous material of the stratum corneum. In the stratum corneum the tonofilaments are believed to be held together by a matrix protein that has been labelled Filaggrin (Steinert et al., 1981), or Histidine Rich Basic Protein (HRBP) (Dale et al., 1981). This protein appears

to be derived from a phosphorylated precursor located in the keratohyaline granules.

(2) The Glycoconjugates: In the oral epithelium, carbohydrate-protein complexes are the major macromolecular components of the intercellular material and have been shown to be synthetic products of the cells that they surround (Bennet, 1963). In the human gingival epithelium, proteoglycans are the major extracellular nonfibrous proteins and are able to aggregate into very large macromolecules in contrast with those of the gingival connective tissue which lack aggregating properties and are generally of lower molecular weight (Bartold, Weibkin & Thonard, 1982). In inflamed human gingivae, reduced levels of proteoglycans have been noticed and this is believed to result from catabolism of the peptide, rather than the glycosaminoglycan moiety of the macromolecule (Embery, Oliver & Stanbury, 1979).

Periodic acid schiff sensitive material has also been located in human membrane coating granules in gingival and buccal epithelia and in hard palate epithelium of the rat (Hayward & Hackemann, 1972; Hayward & Hackemann, 1973).

There are a number of postulated roles for the carbohydrate-protein complexes such as adhesives or lubricants facilitating the movement of cells past one and other. Another suggested role for such complexes is in regulating trans-epithelial ion movement. This is accomplished through the numerous hydroxyl groups present on the sugar residues which can bind large numbers of water molecules and can exhibit the

properties of the stationary phase of a chromatography column. In conjunction with this, the charged groups of the acid mucopolysaccharides can exhibit the properties of an ion exchange resin. On a more general level, by virtue of filtration and selective binding, the carbohydrate-protein complexes can regulate the diffusion of many metabolites through the intercellular spaces and can thus control the cellular micro environment (Gerson & Harris, 1983).

As in the cells of most other tissues, it is also assumed that cell surface glycoproteins and glycolipids are instrumental in defining intercellular recognition and antigen specificity.

(3) The Lipids: As is true for proteins, the high turnover rate of the oral epithelium means that lipids must be synthesised at a very high rate and it is believed that most subcellular fractions have the ability to synthesise lipids. It has been calculated that of the total wet weight of pig gingival epithelium, 2-8 percent is due to lipids (Gray & Yardley, 1975). As the cellular layers move more superficially and lose intracellular components, the concentration of cellular lipids decreases.

Apart from being crucial for cell and organelle membrane formation, it is also suggested that lipids may play a role in the process of keratinisation. Evidence includes the observation that the concentration of phospholipids and fatty acids are higher in keratinising than in non-keratinising human gingival epithelium (Anneroth & Ivemark, 1971) and that lipids (e.g. choline) have in fact been found associated with keratin fibrils.

Lipids are also present in the oral epithelium as glycolipids (gangliosides) which have been demonstrated in high concentrations in human palatal epithelium. In view of their ability to inactivate toxins, a protective role is proposed for the glycolipids (Lekholm & Svennerholm, 1979).

1.9.3. The Keratohyaline Granules

Unlike histological studies, biochemical studies fail to distinguish between the morphologic variants of the keratohyaline granules. In general terms, the keratohyaline granules contain large amounts of protein and RNA and are in fact known to be actively synthesising proteins. Carbohydrates, lipids and calcium are also believed to be present although DNA is thought to be totally absent (Gerson & Harris, 1983). Keratohyaline granules and their component structural macromolecules are water insoluble though they will dissolve in concentrated urea solutions or in other chaotropic solvents (Ugel, 1969).

As mentioned previously the keratin matrix protein filaggrin (or HRBP), is located in the keratohyaline granules as a highly phosphorylated precursor. When released from the keratohyaline granules into the stratum corneum, the protein acts as the keratin matrix protein (Dale et al., 1982).

1.9.4. The Cell Membrane

As the cells move more superficially, the membrane is seen to thicken as a result of the formation of an inner leaflet or cell envelope, which confers on the cell, considerable resistance to the action of

lytic agents (Malotsy, 1977). This cell envelope has been shown to be composed largely of protein (mainly INVOLUCRIN) (Watt & Green, 1981), thus accounting for the observed increase in the protein:lipid ratio in the more superficial cellular layers (Gray, 1981).

Plasma membrane lipids change as the epithelial cells mature, with the number of phospholipids decreasing as the cells move into the stratum corneum. This change is largely due to the action of phospholipases located in the plasma membrane (Gray, King & Yardley, 1978). The functions of the plasma membrane glycoproteins have been discussed above (Section 1.9.2.).

1.9.5. Metabolism in the Oral Epithelium

(a) Glycolysis and the Krebbs Cycle: Stratified squamous epithelium, unlike most other tissues, lacks a direct blood supply and the cellular oxygen tension decreases as the cells migrate outwards from the basal layer. One metabolic consequence of this is that glycolysis in the oral epithelium is by and large anaerobic (Langvad & Roed-Petersen, 1969). Aerobic glycolysis, utilising the Krebbs cycle is only found in the lower cell layers where sufficient oxygen is present to maintain this cycle. It is estimated that less than 2 percent of the glucose consumed by the epidermis is oxidised by the Krebbs cycle, whereas as much as 70 percent is metabolised by the anaerobic pathway (Frienkel & Traczyk, 1976). It appears, given the basal and suprabasal location of the Krebbs cycle enzymes, that what energy they produce is probably important largely for cell division and protein synthesis (Jarrett, 1980). The presence of numerous mitochondria in the lower epithelial layers, correlates well with the

above postulated location of the Krebbs cycle which is known to be carried out entirely in the mitochondrial matrix.

(b) The Pentose Phosphate Pathway: Despite being more common in the oral epithelium than in the epidermis, and although there is evidence of a large amount of glucose being metabolised by this pathway, it is not believed to be a major source of energy (Frienkel, 1960). In general terms, the pentose phosphate pathway shows a reciprocal relationship with the Krebbs cycle, the Krebbs cycle decreasing in importance as the cells move more superficially and the pentose phosphate pathway increasing in importance (Gerson, 1967). This inverse relationship is simply a general rule and the precise distribution of the pentose phosphate pathway appears to vary from species to species and even within a species, depending on the degree of keratinisation of the epithelium. This has led to the suggestion (Jarrett, 1980) that the pentose phosphate pathway may play a role in the process of keratinisation.

The major role of the pentose phosphate pathway relates to glycoprotein synthesis (Nicolau, Fova-De-Moraes & Rosa, 1978) and production of reducing equivalents (NADPH) which are important in lipid synthesis.

(c) Other Glucose Utilising Systems: An alternative pathway for glucose utilisation is conversion to glycogen. Glycogen deposits have been identified in human non-keratinising epithelia (Wislocki, Fawcett & Dempsey 1951), but the function of glycogen in the oral epithelium is as yet unknown. It is suggested that it may act as a carbohydrate precursor for the intercellular ground substance which is known to be more abundant in non-keratinised than in keratinised epithelia.

(d) Alternative Energy Sources: Whilst carbohydrates will certainly provide the major part of the energy requirements of a stratified squamous epithelium, work on the epidermis has demonstrated that lipids and amino acids can also be oxidised after conversion to intermediates of the Krebbs cycle. Clearly these sources of energy will be of more importance in the upper strata where the Krebbs cycle is inactive.

1.10 ANIMAL MODELS OF ALCOHOLISM

1.10.1. Introduction

As a result of the many ethical and practical difficulties associated with alcohol research in humans, a suitable animal analogue had to be selected for use in the studies presented in this thesis. In an animal system, it is impossible to reproduce the complexities of human alcoholism and thus there is no completely adequate animal model of human chronic alcohol abuse.

There are, however, a number of animal models available for use in alcohol related studies which, despite being limited representations of the human situation, are adequate for experimental purposes. Animal models of alcoholism can be divided into three basic groups depending on whether the alcohol ingestion is voluntary, semi-voluntary or involuntary (Pohorecky, 1981).

1.10.2. Animal Models

Voluntary Models: Most experimental animals have a marked aversion to ethanol (Freund, 1975) and any animal model of voluntary ethanol ingestion must somehow overcome this aversion. In general this is achieved by designing the model such that the animal does not notice the unpleasant taste and smell of the alcohol. There are two main models of this voluntary type:

(a) Intravenous Self Administration: Animals (especially primates) will self administer alcohol in pharmacologically active amounts by the intravenous route. This leads to extremely high blood alcohol levels and to the development of tolerance and withdrawal

signs. Disadvantages of this model are that the ethanol is not consumed by the oral route and that the animal often becomes so inebriated that it may fall into a comatose state and die (Cicero, 1980).

(b) Intragastric Self Administration: In this case the smell and taste of the ethanol are masked by direct intragastric self administration through a tube inserted into the stomach. Very high blood alcohol levels can be achieved with this model and the animals can also develop signs of tolerance and withdrawal. An advantage of this model is that the ethanol is absorbed through the gastric mucosa, which is the normal route for absorption in humans. Disadvantages include the occurrence of severe gastric irritation produced by the relatively high ethanol concentrations and the formation of gastric ulceration especially in prolonged studies (Cicero, 1980).

Semivoluntary Models: The semivoluntary models involve ingestion of ethanol as part of the daily fluid or food intake of the animal. These models are labelled semivoluntary because the amount of ethanol ingested is not completely controlled by the experimenter. Also, the volume of ethanol consumed by the animal, is not a true measure of the volitional intake of ethanol, since most of the ethanol will be ingested as a means of obtaining either food or water.

A general advantage of these semivoluntary models is that the ethanol is not ingested at prefixed treatment times. In fact, despite some diurnal variation, ingestion is spread evenly over 24 hours and results in high blood levels throughout the day.

The available semivoluntary models are:

(1) Alcohol Administration as Part of the Fluid Intake: This method involves confining the animals fluid intake to aqueous solutions of ethanol of concentrations ranging from 15-30 percent. Even using these high ethanol concentrations, the exposure time required for the development of signs of tolerance and withdrawal has to be relatively long (up to several months). A further disadvantage of this method is the chronic dehydration suffered by the experimental animals (Pohorecky, 1981).

(2) Liquid Diet Techniques: Liquid diet models are perhaps the most frequently used animal models of alcoholism and have the added advantage of allowing for control of nutritional factors (Pohorecky, 1981). Such controls are important in studies of certain alcohol related diseases which are believed to arise from the poor nutritional condition of the alcoholic, rather than from alcohol itself. A liquid diet model was chosen for use in the studies presented in this thesis and this will be discussed in more detail in Section 1.10.3.

Involuntary Models: With the involuntary models, the experimenter is able to completely control the animal's ethanol intake and thus blood ethanol levels can be accurately controlled. Exposure to ethanol according to the involuntary model may be continuous (e.g. by inhalation or by sustained ethanol release tubes) or at regularly spaced intervals (e.g. oral or gastric intubation). These models are reviewed by Pohorecky (1981).

1.10.3. The Model Used in the Present Study

The particular model chosen for this study was the semivoluntary liquid diet pair-feeding model of Lieber and DeCarli (1973), which allows for easy control of nutritional factors. The nutritional control is achieved by pair-feeding.

Pair-feeding was first used with liquid diets incorporating alcohol, by Lieber and colleagues (Lieber et al., 1963), who reported that rats could be maintained on a liquid diet with ethanol comprising 36 percent of the calories.

The pair-fed animal was given the same diet, except that the 36 percent ethanol was substituted with 36 percent sucrose, thus ensuring an identical intake of calories and essential foodstuffs. This ensures that any biomedical alterations are due to the effects of ethanol and not the effects of nutritional irregularities.

Later, Lieber and DeCarli (1973), altered the diet slightly, and demonstrated fatty livers, pharmacologically significant blood ethanol levels and signs of tolerance and withdrawal in the alcoholic rats. None of these signs were noted in the isocalorifically pair-fed control animals.

The choice of 36 percent as the fraction of the total calorific intake to be supplied by ethanol, is based on the assumption that the human alcoholic typically consumes sufficient ethanol to comprise 35-50 percent of his total daily calories. Additionally, rats will not consume significant quantities of a liquid diet in which the ethanol

content amounts to more than 40 percent of the total calories, preferring to starve (Pohorecky, 1981).

This technique has numerous advantages, being ideal for use with rodents, thus allowing the use of large numbers of relatively inexpensive experimental animals which require no special surgical or handling techniques. High blood ethanol levels are achieved, as are high grades of tolerance and withdrawal, all within reasonably short periods of time. Unfortunately as with all other models, this procedure has its attendant disadvantages, not least of which is the high cost of the liquid diets. Another problem is that whilst the animals being maintained on the ethanol diet spread their dietary intake fairly evenly over 24 hours, the pair-fed controls tend to consume their diet in a relatively short period of time and are therefore fasted for a significant part of the day (Cicero, 1980).

The pair-fed animals tend to maintain themselves at approximately 80 percent of their free feeding weight. A common criticism of this model is that it is possible that any effects of alcohol observed under these conditions may be different from those observed under free feeding conditions.

Despite the above disadvantages, this procedure is still the most frequently used model and lends itself well to biomedical studies.

1.10.3. The Animal of Choice

To conduct an alcohol related study one must decide which is the most useful and easy animal to use. A number of considerations are relevant:

(1) Primates and Large Mammals: Large animals such as primates, cats and dogs, tend to be expensive, cumbersome and space consuming, thus automatically reducing the number of animals that any one study can involve. Such animals also require specialised equipment and expertise to allow proper handling and are therefore generally impractical.

(2) Rodents: Clearly rodents are ideal for alcohol related studies, being small, easy to handle and inexpensive. Of the common laboratory rodents, the hamster, mouse and rat can all be used:

(a) The hamster: The hamster is an obvious choice as it has a well characterised oral environment. However, the hamster is unusual in that it has a preference for ethanol solutions (up to 10 percent) over water (Arvola & Forsander, 1961). The hamster also has the ability to consume large amounts of ethanol solutions at concentrations up to 40 percent (w/v) without showing signs of either dependence or withdrawal. The probable reason for this is that the hamster has a very high metabolic capacity for ethanol, thus blood alcohol is not maintained at significantly high levels after ethanol ingestion to produce dependence (McMillan et al., 1977).

The hamster is therefore not ideal, but given the low blood ethanol levels, even after ingestion of highly concentrated solutions, it is possible that the hamster may be useful as a model of purely local oral effects of ethanol.

(b) The mouse: The mouse requires a 30 percent weight reduction before it will ingest significant quantities of an ethanol adulterated liquid diet and as such is not ideal (Pohorecky, 1981).

(c) The rat: The rat adapts well to a liquid diet containing ethanol and is suitable for studies of oral mucosal pathology. It becomes dependent within a reasonably short period of time (a few weeks), demonstrates tolerance and withdrawal symptoms and will consume quantities of ethanol equivalent to those consumed by human alcoholics.

For the studies presented in this thesis, the isocalorific matched pair feeding technique of DeCarli and Lieber was deemed most suitable and, for the reasons described above, the rat was chosen as the most useful experimental animal.

1.11 THE AIMS AND SCOPE OF THE PRESENT STUDY

Given the proven link between alcohol and oral cancer, both in humans and experimental animals (Sections 1.5.2. and 1.5.3.), it is surprising how few studies of the effects of ethanol alone upon the oral epithelium exist. Such studies as have been performed (Section 1.5.3.), have generally been histological in nature and have demonstrated microscopical abnormalities in the oral epithelium.

Since any cell or epithelium expresses its individuality through the proteins that it manufactures, it is reasonable to assume that alcohol related histological alterations (Section 1.5.3.) will be mediated by, or accompanied by, alterations in the epithelial protein pattern. It was thus with a view to investigating the effects of ethanol on the protein biochemistry of the oral epithelium, that the present study was undertaken. It was hoped that the results would provide an insight into the biochemical mechanisms that may underly the observed aetiological relationship between alcohol and oral cancer.

The particular approach adopted, involved the use of electrophoretic techniques in a study of the proteins of the lingual epithelium from alcoholic and control rats. The techniques involved are discussed more fully in Chapter 3.

The results from the first study of the effects of alcohol on lingual epithelial proteins are presented in Chapters two and three. Chapter two relates to the success of the animal model and Chapter three reports the results of the protein studies.

The results from this initial study suggested that a further similar study involving analysis of epithelial protein alterations over a time course, might be worthwhile. The results from such a time course study are presented in Chapters four and five with Chapter four again reporting on the adequacy of the animal model and Chapter five on the results from the protein studies.

Chapters six and seven deal with the biochemical characterisation of epithelial proteins, which show altered levels in the presence of alcohol. Finally, Chapter eight presents a concluding discussion and suggestions for future work.

CHAPTER TWO - THE 102 DAY STUDY

2.1 INTRODUCTION

2.1.1. Introduction to the Study

It is surprising how few studies there are of the effects of alcohol upon the oral epithelium (Section 1.5.3.). Those that have been performed have related almost exclusively to histological and histochemical alterations in the oral epithelium (Mascres & Joly, 1981 and Mascres et al., 1984), and it would appear that studies of ethanol mediated biochemical alterations in the oral epithelium are lacking in the literature. It was with a view to investigating the effects of ethanol on aspects of the biochemistry of the oral epithelium that the present study was undertaken.

An animal model of chronic alcohol abuse was chosen as the best method of controlling alcohol and dietary intake. A small-animal experiment is comparatively inexpensive to run and problems relating to oral habits, smoking and drug ingestion are avoided. Furthermore, such a model avoids the ethical problems that may be associated with the use of human subjects.

It is important to allow enough time for any biochemical changes to occur and thus this initial study was designed to run for approximately 100 days as it was at this time that Mascres and Joly (1981), using a similar animal model, detected the initial histological alterations in the rat oral mucosa. It has been assumed that any biochemical alterations are also likely to be detected at

this time.

The rationale was thus to use a small-animal model of chronic alcohol abuse for approximately 100 days and then to examine the oral epithelium for evidence of biochemical alterations.

This study would be very difficult in human subjects and clearly requires a well controlled animal model to allow for regulation of the alcohol and dietary intake during the study. As discussed in Section 1.10.3., the animal model chosen for use in this study was the isocalorific matched pair feeding method of Lieber and DeCarli (1973). This method allows for close calorific control and has been shown to lead to biomedical complications of the type typically associated with chronic ethanol abuse (Lieber et al., 1963 and Lieber & DeCarli, 1973).

It is important for valid comparisons, to use genetically identical rats and for this reason, an inbred strain, (Sprague-Dawley) was used. Male rats were chosen to ensure that no oestrous cycle alterations in the epithelial structure would complicate the interpretation of the results.

For the isocalorific matched pair feeding model to be an accurate representation of human alcohol abuse, consumption of the ethanol liquid diet must be shown to lead to biomedical complications. Probably the best method of gauging the biological effects of chronic excess alcohol consumption is to assess liver damage by measuring the serum levels of hepatic enzymes known to be raised as a result of alcohol abuse.

2.1.2. Liver Enzyme Analysis-Introduction

The use of serum hepatic enzyme levels as indices of alcohol induced liver damage allows the detection of the very early effects of chronic ethanol consumption. Using Gamma-Glutamyl Transferase, detection of raised levels resulting from enzyme induction, is even possible prior to the development of abnormal liver function (Rosalski, 1984).

The measurement of a spectrum of serum enzymes, including some not normally affected by ethanol ingestion, allows a more specific estimate of alcohol induced liver damage as opposed to other forms of liver damage.

The ease with which blood can be obtained from rats in sufficient quantities to allow serum enzyme analysis, means that the effects of ethanol on liver function can be readily followed at intervals throughout the course of the study without the need for liver biopsy which would cause morbidity and mortality amongst the experimental animals.

The enzymes chosen for measurement in this study were Gamma-Glutamyl Transferase (GGT), Alkaline Phosphatase (alk. phos.), Aspartate Transaminase (AST) and Alanine Transaminase (ALT).

Gamma-Glutamyl Transferase: Although some authors have doubts about the use of GGT as a diagnostic marker of alcohol consumption (Penn & Worthington, 1983), measurement of GGT is generally regarded as the most valuable of all the biochemical procedures for measuring excess alcohol consumption in both animals and man (Teshke et al., 1983).

Increased serum levels of the enzyme have been reported to result from chronic, but generally not from acute (except where existing liver disease is present), ethanol consumption (Rosalski, 1984). Raised values are found in around 75 percent of human alcoholics and appear to correlate well with histological evidence of liver damage. Initially, the raised levels result from an induction of enzyme synthesis, although, with continuing alcohol abuse, hepatocellular damage contributes to the raised serum levels.

Alcoholic rats show raised serum GGT levels even after relatively short periods of alcohol intake (4-6 weeks) (Nishimura & Teshke, 1982; Teshke & Petrides 1982 & Teshke et al., 1983). As in humans, it appears that induction of the enzyme is the major cause of raised serum levels.

Alkaline Phosphatase: Serum levels of this enzyme are elevated in up to 50 percent of human alcohol abusers (Rosalski, 1984) and as for GGT, the mechanism behind the raised levels is one of enzyme induction with hepatocyte damage being a secondary cause (Nishimura & Teshke,

1982). In alcoholic rats, raised levels of alk. phos. are found, but enzyme induction appears to play no part and the major cause of the raised levels is hepatocyte damage (Nishimura & Teshke, 1982; Teshke & Petrides, 1982; Teshke et al., 1983). Alk. phos. is a better index of alcoholic liver damage in rats than in man, although it is not specific and should be used in conjunction with the more specific GGT values.

The Transaminases: Levels of both AST and ALT are elevated in man as a result of alcohol related hepatocellular damage, with AST being a more sensitive marker of alcoholic liver damage than ALT.

In the rat, the situation is different with only ALT showing any increase in either serum or hepatic levels following chronic ethanol consumption. Rat serum AST is raised in some forms of liver damage but shows no increase as a result of alcohol abuse (Teshke et al., 1983). It thus provides a marker for distinguishing alcoholic from non-alcoholic liver damage. The raised hepatic ALT levels (Teshke et al., 1983) imply that the elevated serum ALT levels in rats, following alcohol ingestion, result at least in part from induction of the enzyme.

2.1.3. Liver Histology

Also presented in this Chapter is evidence of the development of fatty liver in the alcoholic animals as shown by histological staining of liver sections obtained after sacrifice. This is perhaps the best method for assessing alcohol induced liver damage although it is of limited value in following the development of such damage throughout the course of a study.

2.2 MATERIALS AND METHODS

2.2.1. Initiating the Study

Forty young male Sprague Dawley rats (Bantin and Kingman, Hull, England) aged 6 - 8 weeks were initially divided into two groups. The first group consisted of ten rats which were maintained on standard laboratory chow (SDS Ltd., Witham, Essex) and water ad libitum for the duration of the study. The second group consisted of thirty rats which were maintained on a nutritionally adequate liquid diet as their sole source of food and water. All the animals were housed individually in wire bottomed cages in order to reduce faecal consumption which might have interfered with the isocalorific pairings. The compositions of the laboratory chow and the liquid diet are shown in Figure 2.1.

CARBOHYDRATE	**CARBOHYDRATE**
58.3 %	**70 %**
FIBRE 22.4 %	**FIBRE 8.9 %**
FAT 2.9 %	**FAT 2.9 %**
PROTEIN 16.4 %	**PROTEIN 18.2 %**
(1) Laboratory Chow	(2) Liquid Diet

FIGURE 2.1: PERCENTAGE COMPOSITION OF THE STANDARD LABORATORY CHOW AND THE LIQUID DIET

The liquid diet was prepared daily at a concentration of 20 g of powdered diet per 100 ml of water and 100 ml of this diet was made

available to each of the thirty rats per day. The diet was prepared in bulk using warm water and a domestic food mixer to thoroughly mix the liquid diet. This mix was then measured out in 60 ml syringes, each animal receiving two 50 ml portions of the diet in its feeding bottle. The high viscosity of the liquid diet made it difficult to clear the full 100 ml of diet from a measuring cylinder or flask and therefore a syringe was used for more accurate dispensing of the diet.

After one week, the thirty rats were further divided into 15 closely weight-matched pairs. One animal in each pair was designated 'alcoholic' and following induction (Section 2.2.2.) was maintained on the liquid diet with ethanol substituted to provide 36 percent of the daily calories. The other member of the pair was designated the 'sucrose control' and was maintained on the liquid diet with sucrose substituted to provide 36 percent of the daily calorific intake. A liquid diet with ethanol comprising 36 percent of the calories is equivalent to a 5 percent v/v solution of ethanol. The reasons for ethanol being supplied as 36 percent of the calories are discussed in Section 1.10.3.

2.2.2. Induction of the Animals onto the Alcoholic Diet

Most animals do not cope well if suddenly presented with a liquid diet containing ethanol as 36 percent of the calories and for this reason, the alcoholic animals were gradually weaned onto the high alcohol diet. They were fed a 10 percent ethanol plus 26 percent sucrose diet for three days, followed by a 20 percent ethanol plus 16 percent sucrose diet for three days and finally a 36 percent ethanol diet for the remainder of the study. The sucrose controls were

maintained on a 36 percent sucrose diet throughout. The recipes for the above diets are shown in Table 2.1.

It will be noticed from this table that the pair-fed diets contained more calories per 100 ml than the liquid diet alone. This was to ensure that even dietary volumes as low as 75 ml would provide 250 KiloJoules (kJ) which is sufficient energy to support growth and maintenance in the rat. This precaution was necessary as although animals will readily consume 100 ml of liquid diet alone, they will not readily consume this volume of the alcoholic diet.

2.2.3. The Pair-Feeding Procedure

Pair-feeding involved giving animals in the alcoholic group 100 ml each of the ethanol-containing liquid diet daily, and 24 hours later, measuring the volume consumed. This was measured by adding 50 ml of water to the feeding bottles, shaking and then measuring the resultant volume. The volume in excess of 50 ml was taken as the volume of diet not consumed by the alcoholic animal and thus the actual volume consumed was calculated. This volume was noted for each animal and the respective pair-fed animal was given the same volume of the isocalorific sucrose-containing diet. This ensured that despite the staggering of the feeding by 24 hours, each member of the pair received the same overall calorific intake. The animals were fed daily between the hours of 9 a.m. and 11 a.m. The pair-feeding commenced from the moment the alcoholic animals were placed on the 10 percent alcohol diet.

All animals were weighed weekly throughout the study.

	ETHANOL (ml)	SUCROSE (g)	DIET (g)	ENERGY (kJ/100ml)
10 % ethanol	24	91	287	332
20 % ethanol	48	56	287	332
36 % ethanol	86	--	287	332
36 % sucrose	--	126	287	332
Liquid Diet alone	--	--	340	252

ALL THE ABOVE DIETS WERE MADE UP TO 1.7 LITRES WITH WARM WATER PROVIDING SUFFICIENT DIET FOR UP TO SIXTEEN RATS.

TABLE 2.1: RECIPES FOR THE EXPERIMENTAL DIETS

ENERGY CONTENT OF THE DIETARY CONSTITUENTS:

Ethanol (Absolute alcohol; A.R. quality, James Burrough Ltd., London, England): 29.8 kJ/g

Sucrose (BDH Ltd., Poole, England): 16.1 kJ/g

Liquid diet (SDS Ltd., Essex, England): 12.6 kJ/g

2.2.4. Collection of Blood

To provide sufficient serum for enzyme analysis, 1 ml of blood was obtained under ether anaesthetic every four weeks to the termination of the study. All the animals were bled at the beginning (at the point of pairing) and at the end of the experiment but at the intermediate points (weeks 4, 8 and 12), only half the pair-fed animals were bled. This still provided enough samples for analysis and had the added advantage of causing minimum distress to the animals, with bleedings being reduced to approximately once every 8 weeks.

Prior to bleeding, the rat tail vessels were dilated by gently warming the animal in a 'light box', and following ether anaesthesia, blood (c. 1 ml) was removed from the animals by amputating the distal 2-3 mm of the tail and collecting the blood in Luckham tubes (Luckam, West Sussex, England). Bleeding was terminated by cauterising the end of the tail. The blood samples were later spun at 3000 rpm for 15 minutes to provide serum for the biochemical analyses.

Considerable care was taken to avoid haemolysis, which might have interfered with the enzyme assays (Czerwek & Bleuel, 1981). The serum was stored frozen at -20° C prior to enzyme analysis.

2.2.5. Sample Collection at Sacrifice

After 102 days, all the animals were sacrificed by an overdose of intraperitoneal barbiturate (Nembutal) and about 10 ml of blood was removed from the aorta to provide serum for liver enzyme analysis. The tongues were dissected free and halved longitudinally, one half

being used for the biochemical studies presented in Chapter 3, and the other half being used for histological analysis. The half for biochemical analysis was placed in Medium 199 (Gibco Europe Ltd., Paisley, Scotland) in a bijou bottle, and placed on ice for transportation from the animal house to the laboratory. The half for histological analysis was placed in 10 percent neutral buffered formalin along with the oesophagus, stomach, liver segments and salivary glands which were also removed from each animal. The palatal mucosa was removed from the rats and stored in liquid nitrogen for future biochemical analysis.

All killings were performed between the hours of 10 a.m. and 12.30 p.m.

2.2.6 Gamma-Glutamyl Transferase Assay

GGT (EC 2.3.2.2.) is found in a number of organs in the body associated with the microsomal/plasma membrane fraction of the cells. The enzyme catalyses the transfer of the gamma-glutamyl (GG) moiety from gamma-glutamyl peptides to amino acids or other peptides ie,

$$\text{L-GG peptide + aa.} \xrightarrow{\text{GGT}} \text{L-GG-aa. + derivative of L-GG peptide}$$

This reaction is believed to be involved in the transfer of amino acids across biological membranes (Orlowski & Meister, 1970).

The assay used was that described by 'The Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology' (1976), which uses gamma glutamyl-4-nitroaniline (glu-4-na) and glycylglycine (glygly) as substrates according to the following equation:

$$\text{glu-4-na + glygly} \xrightleftharpoons{\text{GGT}} \text{glu-glygly + 4-na}$$

The production of 4-nitroaniline is measured at 37° C, by continuously monitoring the absorption of the reaction mixture at 405 nm on an SP8-100 spectrophotometer (Pye Unicam Ltd., Cambridge, England) in paired, glass, semimicro cuvettes, which provide a 1 cm path length for absorption. One hundred microlitres of serum was required per assay.

The spectrophotometer was zeroed on the reaction mixture (without serum), prior to the reaction rate measurement.

The manual assay described above, was used in preference to an automated assay because the GGT levels found in rats were below the

range of the automated assay system.

2.2.7 Alkaline Phosphatase Assay

Alkaline phosphatase (EC 3.1.3.1.) represents a family of enzymes that hydrolyse phosphate esters at alkaline pH according to the following equation:

$$\text{compd-Pi.} \xrightarrow{\text{alk.phos.}} \text{compd.} + \text{Pi.}$$

In man, the enzymes are present in almost all tissues of the body and in the liver are associated with the hepatocyte plasma membrane.

The assay relies on the colorimetric measurement of p-nitrophenol, produced as a result of the following reaction:

$$\text{p-nitrophenolphosphate} + H_2O \longrightarrow \text{phosphate} + \text{p-nitrophenol}$$

The reaction was carried out at 37° C and was started by the addition of the p-nitrophenolphosphate substrate. The rate of the reaction (formation of product) was measured by continuous monitoring at 405 nm against an air blank. Twenty microlitres of serum was required per assay.

The AST, ALT & alk. phos. levels were assayed on an LKB 8600 Rapid Reaction Rate Analyser (LKB Instruments, Ltd., South Croydon, Surrey, England) using automated assay kits supplied by Boehringer Mannheim (BCL Ltd., East Sussex, England)

2.2.8. The Transaminases

In most tissues, the predominant transaminases are Aspartate Transaminase (AST or SGOT: EC 2.6.1.1.) and Alanine Transaminase (ALT or SGPT: EC 2.6.1.2.), both of which catalyse the transfer of an amino group from the respective amino acid to an α-oxy acid.

The assays for these enzymes are as follows;

Aspartate Transaminase Assay

This assay is a linked assay and utilises the production of NAD^+ from the reduction of oxaloacetate, to measure the enzyme activity i.e.:

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + NAD^+$$

(MDH = Malate Dehydrogenase).

The reaction was carried out at 37° C and was started by the addition of the α-oxoglutarate substrate. The course of the reaction was followed at 340 nm against an air blank. One hundred microlitres of serum was required per assay.

The assay kit used was 'CBR GOT opt UV', supplied by Boehringer Mannheim (BCL Ltd.).

Alanine Transaminase Assay

Again, this is a linked assay involving the production of NAD^+ from the reduction of pyruvate, one of the products of the ALT reaction.

$$\alpha\text{-oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^{+}$$

(LDH = Lactate Dehydrogenase).

This reaction was carried out at 37° C and was started by the addition of the α-oxoglutarate substrate. The enzyme activity was again measured at 340 nm against an air blank. One Hundred microlitres of serum was required per assay.

The assay kit was 'CBR GPT opt.' supplied by Boehringer Mannheim (BCL, Ltd.).

Wellcomtrol QC (Wellcome, Dartford, England) quality controls were included with each set of assays to gauge the accuracy of the assay.

2.2.9 Liver Histology

Small pieces of liver were fixed in formalin, trimmed and paraffin processed. Sections were prepared both for routine 'H and E' histology and also, by the frozen section technique, for oil red O staining of fat deposits.

2.2.10 Statistical Tests

Due to the numbers of animals in each group, a non parametric statistical test, the Mann-Whitney U test was used for comparisons between groups. Where appropriate (in comparisons between the matched pairs), the Wilcoxon Matched-Pair Signed Rank Test (Siegel, 1956) was used. The confidence limit was set at 5 percent.

In the presentation of results, when values were shown to be significantly different by both the above tests, neither will be mentioned by name and the upper significance level shall be quoted. When the values were shown to be different by only one of the tests, it shall be named and the appropriate statistical level quoted.

2.3 RESULTS

2.3.1. General Observations

None of the animals died during the course of the study and all appeared to thrive and to gain weight on their given diet (Figure 2.2). The animals maintained on the 36 percent ethanol containing diet (and hence the sucrose controls), initially consumed relatively small amounts of diet, but within 25-30 days were consuming average daily volumes of approximately 90 ml.

2.3.2. Weights of the Animals

At the start of the study, the group to be maintained on the laboratory chow diet showed a mean weight (Table 2.2) of 296.9 g. The alcoholic and sucrose control animals had mean weights of 280.1 g (Table 2.3).

By sacrifice, the mean weights of these groups had risen to 519.4 g, 414.5 g and 435.5 g, respectively. Weight gains throughout the study were thus 222.5 g for the laboratory chow group, 134.4 g for the alcoholic group and 155.4 g for the sucrose control group.

Latterly, the pair-fed animals maintained themselves at approximately 80 percent of the weight of the laboratory chow animals (Free Feeding weight).

2.3.3. Comparison of the Weights

The laboratory chow animals were significantly heavier than either of the two pair-fed groups at all times subsequent to the second week of

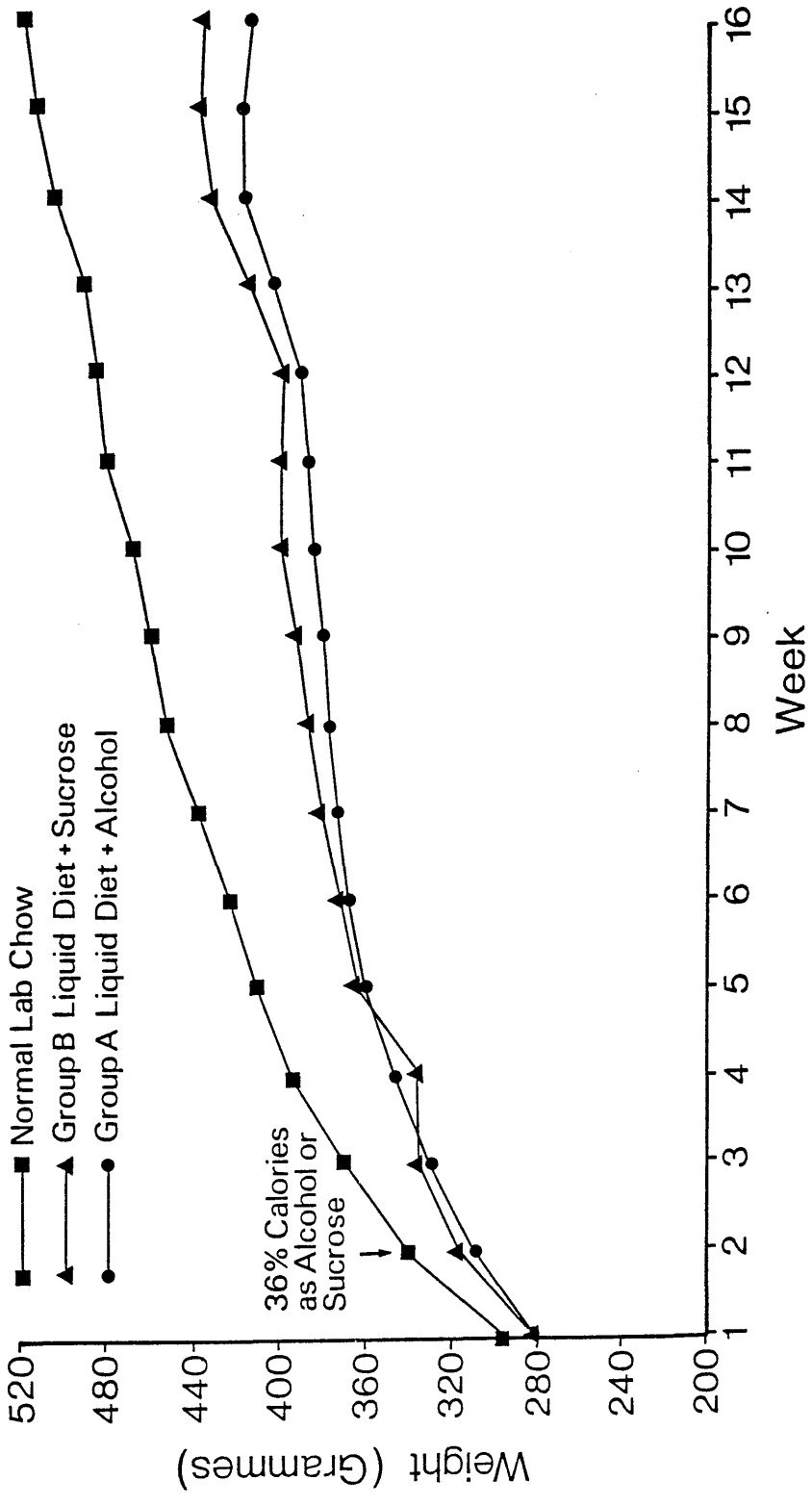


FIGURE 2.2: GRAPH OF MEAN WEIGHTS OF THE EXPERIMENTAL GROUPS OVER THE COURSE OF THE 102 DAY STUDY.

	WEEK															
ANIMAL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	286	315	340	363	372	385	400	411	421	412	431	441	439	451	462	460
2	266	292	324	346	356	368	378	392	396	401	409	424	414	428	431	436
3	309	353	379	408	421	435	445	463	471	487	500	514	512	524	526	537
4	319	355	385	414	451	463	478	500	501	520	536	545	536	550	562	566
5	330	381	415	442	439	454	474	488	504	507	532	556	541	559	569	572
6	320	373	399	423	440	461	477	495	501	513	526	521	552	565	577	581
7	293	341	367	386	408	421	442	453	460	467	484	432	489	496	506	512
8	324	369	393	417	434	452	462	476	475	488	497	484	504	518	526	533
9	290	329	362	383	397	410	424	434	440	443	456	463	468	478	489	493
10	232	309	344	373	394	400	411	427	438	450	453	470	494	500	504	504
Mean	**296.9**	**341.7**	**370.8**	**395.5**	**411.2**	**424.9**	**439.1**	**453.9**	**460.7**	**469.7**	**482.4**	**485.0**	**492.5**	**506.3**	**514.8**	**519.4**
S.D.	**30.5**	**29.8**	**28.8**	**30.1**	**31.4**	**33.5**	**35.0**	**37.0**	**36.7**	**40.3**	**43.9**	**47.1**	**45.3**	**45.7**	**47.2**	**48.0**

TABLE 2.2: WEIGHT IN GRAMMES OF THE LABORATORY CHOW CONTROL ANIMALS FROM THE 102 DAY STUDY.

	WEEK															
ANIMAL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
11A	284	320	336	351	366	375	384	388	385	392	386	404	419	429	422	419
11B	287	323	352	331	376	382	397	394	421	409	410	400	430	437	442	443
12A	249	269	293	308	315	328	332	341	337	358	357	364	373	391	388	379
12B	245	273	296	301	313	319	322	333	336	341	344	343	351	368	369	365
13A	308	338	359	377	395	408	420	427	427	431	440	433	445	473	470	465
13B	308	354	382	375	406	426	439	448	458	463	475	461	489	502	520	512
14A	301	328	356	371	389	399	397	400	400	416	415	431	449	456	454	450
14B	300	332	349	321	365	381	378	388	400	406	396	403	416	433	442	454
15A	246	256	268	281	294	300	309	300	313	321	326	324	340	355	354	347
15B	244	285	291	280	320	342	348	340	342	348	347	340	367	371	370	373
16A	298	329	349	360	373	382	388	390	390	397	403	387	400	430	418	413
16B	298	339	362	366	386	400	418	428	431	438	444	437	453	471	475	478
17A	281	319	341	362	371	375	384	401	397	385	396	397	437	441	441	432
17B	283	325	338	352	371	382	383	394	402	405	411	411	434	439	456	454
18A	252	303	328	346	352	355	358	364	369	373	376	386	396	408	404	410
18B	246	287	303	314	331	335	338	348	350	359	353	368	365	390	395	387
19A	294	318	337	357	363	376	372	380	383	388	379	384	400	412	410	407
19B	294	334	348	348	384	389	402	408	411	416	411	419	436	461	459	459
20A	301	346	370	383	405	411	427	434	437	436	445	442	455	474	472	483
20B	304	356	364	383	400	405	416	424	430	436	443	434	447	471	473	467
21A	274	310	328	342	354	361	370	373	375	383	378	382	392	396	397	395
21B	270	300	315	327	337	345	353	359	365	370	366	369	375	385	397	390
22A	290	325	351	367	381	387	397	394	399	399	399	417	427	439	438	424
22B	287	316	339	345	373	385	401	395	406	414	409	407	440	452	463	462
23A	278	302	321	327	339	349	343	358	351	360	362	360	362	385	387	380
23B	281	322	340	339	369	375	380	393	398	397	396	401	403	424	427	431
24A	283	318	344	366	377	387	393	400	404	410	409	404	424	434	448	438
24B	285	320	347	359	381	395	400	411	411	425	422	432	442	456	459	460
25A	263	295	310	323	338	337	337	344	362	341	349	352	361	368	373	376
25B	270	296	318	326	345	352	361	374	372	379	381	369	386	385	404	398
A MEAN	280.1	311.7	332.7	340.1	360.8	368.7	374.1	379.6	381.9	386.0	388.0	391.1	405.3	419.4	418.4	414.5
S.D.	19.9	24.2	26.6	20.0	29.9	30.8	33.1	34.6	32.5	31.9	32.6	32.6	35.3	35.8	35.3	36.3
B MEAN	280.1	317.5	336.3	337.8	363.8	374.2	382.4	389.1	395.5	400.4	400.5	399.6	415.6	430.3	436.7	435.5
S.D.	21.2	24.8	26.4	27.9	28.4	29.5	32.7	33.3	35.5	35.0	37.8	35.5	39.5	40.6	42.6	43.1

TABLE 2.3: WEIGHT IN GRAMMES OF THE MADELIC (A) AND SUCROSE (B) PAIR-FED ANIMALS FROM THE 102 DAY STUDY

the study (Mann-Whitney, $p<.05$).

Statistical analysis, using the Mann-Whitney test, revealed no significant differences in the weights of the two pair-fed groups at any point in the study although the Wilcoxon test showed the sucrose control animals to be significantly heavier ($p<.05$) than the alcoholic animals at weeks 4, 9, 10, 11, 15 and 16.

2.3.4. Liver Enzyme Analysis

In the subsequent tables of enzyme levels, missing values resulted from insufficient serum being available for the assay. In this study, the small number of laboratory chow values at week 12 was a result of problems in the handling of the animals and thus to minimise distress to the animals, only 5 were bled.

2.3.5. Gamma-Glutamyl Transferase

The results of the GGT assays for all the animals are shown in Table 2.4.

The laboratory chow animals: The initial mean value for the laboratory chow animals was 4.90 IU/L and this level remained constant throughout the study.

The sucrose control animals: The initial mean value for the sucrose control animals was 4.85 IU/L and despite the value for week 12 being lower than expected (4.06 IU/L), the levels remained approximately constant throughout the study.

The alcoholic animals: The initial mean value for the serum GGT from the alcoholic animals was 4.88 IU/L. A significant increase (Mann-Whitney, $p<.02$) in this value was obvious 4 weeks after starting the alcohol diet (7.48 IU/L), and this raised level was maintained throughout the study.

Comparison of the reported values: Statistical analysis of the GGT values revealed that the raised levels in the alcoholic animals were raised, not simply with respect to initial levels, but also with respect to levels in the sucrose control animals ($p<.05$) and the laboratory chow animals (Mann-Whitney, $p<.05$) at all times subsequent to the start of the study.

ANIMAL	SERUM SAMPLE 1(Start)	4	8	12	16(End)
1	4.50	4.88	5.44	3.94	4.12
2	6.00	7.88	7.31	4.12	4.50
3	4.50	5.44	4.69	4.88	6.75
4	4.31	2.62	6.38	4.96	3.00
5	6.56	5.44	6.94	7.12	6.75
6	4.05	5.81	6.75	----	4.72
7	4.12	3.75	3.94	----	5.40
8	6.00	6.56	5.62	----	5.62
9	3.75	3.38	3.75	----	5.92
10	5.25	6.00	4.50	----	4.80
Mean	**4.90**	**5.18**	**5.53**	**5.00**	**5.16**
S.D.	**0.98**	**1.58**	**1.28**	**1.27**	**1.17**
11A	8.44	----	6.38	----	8.62
11B	4.69	----	4.12	----	8.81
12A	3.19	6.00	----	7.12	5.25
12B	10.30	3.79	----	3.56	4.30
13A	3.00	----	7.12	----	5.06
13B	3.00	----	3.19	----	3.75
14A	3.56	----	7.50	----	5.89
14B	5.25	----	4.58	----	8.62
15A	5.25	----	8.62	----	9.30
15B	6.22	----	4.50	----	5.70
16A	3.75	5.25	----	5.62	7.50
16B	4.12	5.25	----	4.20	7.12
17A	3.75	11.62	----	4.88	9.38
17B	5.62	6.75	----	3.75	6.34
18A	3.38	8.25	----	4.88	13.28
18B	4.01	8.25	----	3.94	4.31
19A	3.94	----	11.44	----	8.81
19B	7.12	----	5.62	----	4.80
20A	3.94	9.38	----	7.12	9.07
20B	4.88	6.94	----	4.50	3.94
21A	12.38	7.88	----	7.50	10.30
21B	4.31	5.06	----	4.50	4.69
22A	5.62	----	6.38	----	10.10
22B	3.11	----	7.50	----	6.82
23A	4.12	----	7.12	----	10.50
23B	3.15	----	5.25	----	8.06
24A	5.06	6.19	----	6.08	9.08
24B	3.07	3.19	----	3.68	5.02
25A	3.75	5.25	----	5.06	5.62
25B	3.94	5.25	----	4.31	4.80
A MEAN	**4.88**	**7.48**	**7.80**	**6.03**	**8.52**
S.D.	**2.48**	**2.25**	**1.78**	**1.09**	**2.29**
B MEAN	**4.85**	**5.56**	**4.97**	**4.06**	**5.81**
S.D.	**1.93**	**1.68**	**1.36**	**0.37**	**1.71**

TABLE 2.4: GAMMA-GLUTAMYL TRANSFERASE RESULTS FROM THE 102 DAY STUDY.

There were no significant differences in the enzyme levels of the sucrose control and laboratory chow groups.

2.3.6. Alkaline Phosphatase

The results of the alk. phos. assays for all the animals, are shown in Table 2.5.

The laboratory chow animals: The initial mean value for the laboratory chow animals was 491.8 IU/L and this value decreased significantly over the course of the study to a final value of 196.9 IU/L (Mann-Whitney, $p<.002$).

The sucrose control animals: The initial mean value for the sucrose control animals was 338.2 IU/L and again, this value decreased significantly over the course of the study to a final value of 125.5 IU/L (Mann-Whitney, $p<.002$).

The alcoholic animals: The initial mean alk. phos. value for the alcoholic animals was 320.6 IU/L. This value subsequently decreased significantly (Mann-Whitney, $p<.002$) until, at the termination of the study, it was 161.3 IU/L.

Comparison of the reported values: The alk. phos. values from the laboratory chow group were significantly higher (Mann-Whitney, $p<.02$) than those from the two liquid diet groups at the beginning of the study and also at weeks 8 for the alcoholic group and 8 and 16 for the sucrose control group.

The mean alk. phos. values from the alcoholic animals were higher than those from the sucrose controls at all points subsequent to the start of the study. This difference reached levels of significance at weeks 4 ($p<.019$), 12 (Mann-Whitney, $p<.028$) and 16 ($p<.05$).

	SERUM SAMPLE				
ANIMAL	1(Start)	4	8	12	16(End)
1	485	277	339	---	250
2	498	151	321	238	231
3	465	116	257	153	118
4	421	217	376	---	143
5	498	254	304	244	151
6	521	353	195	---	217
7	590	456	428	---	257
8	376	258	291	---	120
9	508	318	316	---	224
10	556	413	489	---	258
Mean	**491.8**	**281.3**	**331.6**	**211.7**	**196.9**
S.D.	**61.6**	**107.5**	**83.7**	**50.9**	**57.4**
11A	277	---	191	---	204
11B	501	---	185	---	150
12A	409	257	---	214	195
12B	335	217	---	260	159
13A	264	---	155	---	120
13B	323	---	150	---	130
14A	412	---	277	---	---
14B	341	---	153	---	131
15A	330	---	138	---	79
15B	310	---	149	---	130
16A	396	191	---	198	122
16B	349	271	---	118	110
17A	---	323	---	221	113
17B	350	225	---	198	132
18A	---	549	---	286	198
18B	---	238	---	198	118
19A	250	---	143	---	133
19B	272	---	135	---	85
20A	330	297	---	290	219
20B	409	194	---	198	159
21A	---	337	---	271	229
21B	449	201	---	113	98
22A	308	---	250	---	178
22B	264	---	150	---	99
23A	---	---	156	---	125
23B	355	---	152	---	154
24A	230	237	---	134	124
24B	139	237	---	191	111
25A	---	273	---	311	219
25B	---	217	---	141	117
A MEAN	**320.6**	**308.0**	**187.1**	**240.6**	**161.3**
S.D.	**67.1**	**108.1**	**55.4**	**59.3**	**49.4**
B MEAN	**338.2**	**225.0**	**153.4**	**177.1**	**125.5**
S.D.	**88.9**	**24.2**	**15.2**	**49.7**	**23.1**

TABLE 2.5: ALKALINE PHOSPHATASE RESULTS FROM THE 102 DAY STUDY.

2.3.7. Aspartate Transaminase

Results from the AST assays are shown in Table 2.6.

The laboratory chow animals: The initial mean value was 129.5 IU/L and subsequently this value increased significantly (Mann-Whitney, $p<.01$). At week 16, however, the mean value had dropped to 69.3 IU/L, which was significantly lower (Mann-Whitney, $p<.002$) than any of the other values.

The sucrose control animals: The initial value from the sucrose control group was 99.8 IU/L and this value decreased steadily until, at sacrifice, it was 36.5 IU/L (Mann-Whitney, $p<.002$).

The alcoholic animals: The initial AST value from the alcoholic animals was 108.4 IU/L and subsequently, this value decreased significantly with the final mean value being 82.9 IU/L (Mann-Whitney, $p<.002$).

Comparison of the reported values: At week 16 there were no significant differences between the AST values from the alcoholic and laboratory chow animals. However, at all other times in the study, the values from the laboratory chow animals were significantly higher than those from either of the two pair-fed groups (Mann-Whitney, $p<.05$).

Serum AST values from the alcoholic animals were consistently higher than those from the sucrose control animals although this difference only reached the level of significance at week 16 (Mann-Whitney, $p<.002$).

	SERUM SAMPLE				
ANIMAL	1(Start)	4	8	12	16(End)
1	114	166	166	206	66
2	153	337	180	---	70
3	92	221	139	164	70
4	107	162	152	156	68
5	147	92	130	176	53
6	167	147	145	---	73
7	142	249	147	---	63
8	118	204	163	---	65
9	121	169	184	---	81
10	134	166	210	---	84
Mean	129.5	181.0	161.6	175.5	69.3
S.D.	23.1	47.0	24.2	21.9	8.8
11A	73	---	84	---	55
11B	99	---	43	---	32
12A	112	116	---	63	49
12B	94	69	---	61	34
13A	130	---	69	---	68
13B	116	---	66	---	37
14A	114	---	69	---	89
14B	102	---	58	---	31
15A	95	---	61	---	56
15B	105	---	60	---	21
16A	197	124	---	95	113
16B	110	97	---	52	36
17A	112	86	---	53	37
17B	103	139	---	79	59
18A	116	98	---	122	354
18B	115	89	---	103	51
19A	77	---	52	---	49
19B	111	---	85	---	65
20A	92	107	---	62	108
20B	91	75	---	56	36
21A	121	103	---	60	61
21B	122	76	---	61	38
22A	121	---	79	---	54
22B	95	---	63	---	26
23A	109	---	50	---	49
23B	75	---	60	---	27
24A	79	74	---	55	63
24B	73	62	---	64	27
25A	78	67	---	54	39
25B	86	71	---	65	27
A MEAN	108.4	96.9	66.3	70.5	82.9
S.D.	30.7	19.9	12.8	24.8	78.4
B MEAN	99.8	84.8	62.1	67.6	36.5
S.D.	14.4	24.6	12.5	16.3	12.6

TABLE 2.6: ASPARTATE TRANSAMINASE RESULTS FROM THE 102 DAY STUDY.

2.3.8. Alanine Transaminase

The results from the ALT assays are shown in Table 2.7.

The laboratory chow animals: The initial mean value from the laboratory chow group was 29.6 IU/L and this value increased significantly (Mann-Whitney, $p<.02$) to a maximum value of 41.3 IU/L at week 12. The final value represented a return to the initial levels (29.0 IU/L).

The sucrose control animals: The initial mean value from the sucrose control animals was 10.6 IU/L. This value remained approximately constant over the first 12 weeks, although at week 16, it was significantly lower than the initial value (Mann Whitney, $p<.002$).

The alcoholic animals: The initial mean ALT value in the alcoholic group was 10.7 IU/L and this value remained relatively constant throughout the study.

Comparison of the reported values: From the start of, and throughout, the study, the ALT values from the laboratory chow animals were significantly higher than those from the two pair-fed groups (Mann-Whitney, $p<.002$).

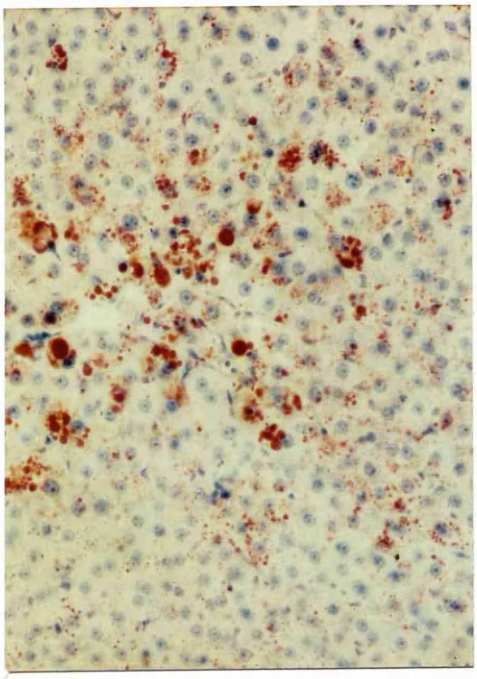
No significant differences were observed in the ALT values from the two pair-fed groups at any point in the study.

2.3.9. Liver Histology

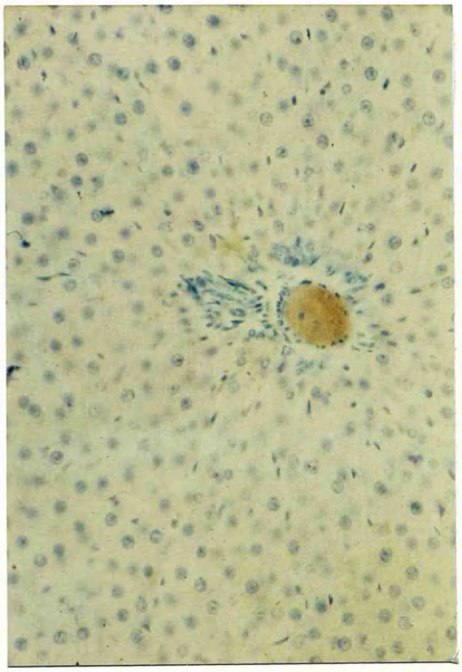
Results from the histological analysis of the liver sections revealed substantially greater centrilobular fatty deposits in the alcoholic animals than in either the sucrose or the laboratory chow controls (Figure 2.3).

	SERUM SAMPLE				
ANIMAL	1(Start)	4	8	12	16(End)
1	34	35	36	---	33
2	35	48	41	---	37
3	30	35	32	38	39
4	31	39	39	44	26
5	29	35	31	42	25
6	26	25	41	---	24
7	37	41	39	---	28
8	30	45	44	---	28
9	30	37	54	---	29
10	14	27	36	---	31
Mean	29.6	36.7	39.3	41.3	29.0
S.D.	6.3	7.1	6.6	3.1	3.9
11A	9	---	10	---	7
11B	9	---	8	---	5
12A	9	12	---	10	9
12B	10	8	---	5	5
13A	12	---	9	---	5
13B	11	---	10	---	6
14A	8	---	8	---	13
14B	11	---	12	---	8
15A	14	---	6	---	7
15B	11	---	13	---	6
16A	---	10	---	12	11
16B	14	8	---	8	8
17A	13	9	---	8	6
17B	13	9	---	10	8
18A	9	12	---	13	38
18B	11	10	---	14	---
19A	9	---	14	---	5
19B	11	---	12	---	8
20A	11	6	---	8	15
20B	12	14	---	8	---
21A	11	12	---	8	7
21B	---	8	---	7	10
22A	13	---	8	---	7
22B	10	---	13	---	8
23A	13	---	8	---	7
23B	8	---	11	---	5
24A	8	9	---	8	7
24B	8	9	---	7	8
25A	11	6	---	6	5
25B	10	9	---	6	7
A MEAN	10.7	9.5	9.0	9.1	9.9
S.D.	1.7	2.0	1.8	2.8	1.6
B MEAN	10.6	9.4	11.3	8.1	7.1
S.D.	2.0	2.5	2.5	2.4	8.3

TABLE 2.7: ALANINE TRANSAMINASE RESULTS FROM THE 102 DAY STUDY.



ALCOHOLIC



CONTROL

FIGURE 2.3: RESULTS FROM THE HISTOLOGICAL ANALYSIS SHOWING SUBSTANTIALLY GREATER FATTY DEPOSITS IN THE ALCOHOLIC LIVER THAN THE CONTROL LIVER

Additionally, a mild, mixed inflammatory infiltrate was noted in the alcoholic animals. No evidence of liver cell necrosis or increased collagen deposition was noted.

2.4 DISCUSSION

2.4.1. Dietary Composition and Consumption

No animals died during this study and the rats gained weight showing no obvious signs of ill health. Comparison of the composition of the laboratory chow and the commercially supplied liquid diet used in this study revealed some major differences, especially in the carbohydrate and fat content. Despite this, the diets used in the present study were nutritionally adequate and allowed the animals to thrive and to gain weight. A further discussion of the dietary composition can be found in Section 4.4.8.

As rats are known to respond poorly to radical alterations in their diet, the alcoholic animals were allowed to adapt gradually to the 36 percent ethanol diet. Even given this weaning period, initial consumption of the 36 percent ethanol diet was low being on average 67 ml per day per animal. It should be noted that volumes of diet as low as 67 ml still contain 222 kJ of metabolisable energy which corresponds to approximately 89 percent of the daily calorific requirements of the rat as recommended by the diet manufacturers (250 kJ).

After approximately 25 days on the 36 percent ethanol diet the average consumption increased and remained above 82 ml (272 kJ) for the remainder of the experiment. This time interval was similar to the length of ethanol exposure required to produce withdrawal symptoms in rats (Freund, 1975) and it may be that the increase in dietary consumption at this time resulted from the development of dependence

or tolerance in the alcoholic animals. This, however, was not measured objectively.

2.4.2. Assessment of the Pair-Feeding Procedure

To ensure a thorough mix and effective suspension of the diet in the water, warm water and a domestic food mixer were required. The diet flowed freely from the feeding bottles and did not show any appreciable settling over 24 hours, thus ensuring that it remained available to the rats for as long as they required.

One drawback of this technique is that it was particularly time consuming and very expensive although the method itself was fairly easy to carry out and allowed for accurate control of nutritional consumption.

Other anticipated problems with the paired feeding procedure i.e. possible evaporation of diet, clogging of the feeding bottles or loss of diet from the bottles, did not arise and, as shown by the similarity of the weights of the matched pairs, there was close calorific control (Figure 2.2.). As reported in Section 2.3.3., the Mann-Whitney U test did not reveal any differences in the weights of the two pair-fed groups although the Wilcoxon Matched Pair Signed Rank Test did reveal some differences in the comparison of the paired animals. These differences are minor compared with the weight differences reported in other pair-feeding studies (Morland et al., 1977 and Gadeholt et al., 1980), and are easily tolerated by this type of model. Such differences between pairs do not detract from the value of the pair-feeding technique.

The higher weights of the laboratory chow group were to be expected, as the liquid based diets were not fed ad libitum and the aversion of the animals to alcohol resulted in a further decrease in the dietary volumes consumed. Throughout the latter stage of the study the pair-fed animals maintained themselves at approximately 80 percent of the weight of the laboratory chow group, this being in general agreement with published results from similar pair-feeding studies (Gadeholt et al., 1980).

The initial weight loss reported in the literature for animals maintained on ethanol adulterated liquid diets, (Banks, Kline & Higgins, 1970; Morland et al., 1977 and Gadeholt et al., 1980) was not observed in the present study. The probable reason for this was that in this study the animals were allowed to adapt gradually to the 36 percent alcohol diet and not placed on it from the start. This further emphasises the importance and value of gradually weaning animals onto radically altered diets.

One drawback that is reported frequently in the literature (Cicero, 1980) and that was observed in this study, is that whilst the alcoholic animals tend to spread their dietary intake over the full 24 hours, the isocalorific pair-fed controls (sucrose control animals) typically consume their full quota of diet in a very short period of time and are thus starved for the major part of the day. This is a common criticism of the pair-feeding method and whilst fractionally dividing the diet over a 24 hour period would go some way to correcting this, it would be too time-consuming to be of any practical value.

The removal of 1 ml of blood, under ether anaesthetic by the tail amputation technique, proved to be a safe procedure and had no associated mortality. During the collection of blood and the weekly weighing of the animals, the alcoholic animals were observed to be more irritable and nervous than the animals in the other groups.

An interesting problem arose after a number of weeks on the liquid diets when fungal and bacterial growths appeared on the walls of the feeding bottles. The growths were considerably heavier in the sucrose control bottles than in the alcoholic bottles probably because of the antimicrobial action of ethanol. As a result the bottles were thoroughly washed daily and changed weekly to discourage the growth of these microorganisms. Analysis of samples of the growths revealed them to be composed largely of Coliforms (Escherichia coli, various Pseudomonas species) and Candidal species (especially Candida krusei).

2.4.3 Gamma-Glutamyl Transferase

The vast body of evidence demonstrates an ethanol-related increase in both hepatic and serum GGT values in rats maintained on ethanol containing liquid diets (Nishimura & Teshke, 1982 and Teshke et al., 1983). This effect is independent of, but may be modified by, dietary effects (Teshke & Petrides 1982).

The enzyme levels reported in Table 2.4 are higher than those previously reported (Nishimura & Teshke, 1982 and Teshke et al., 1983), but this can be accounted for by differences in the experimental method. Teshke et al., (1983) used female rats in their studies and measured GGT activity at 25° C as opposed to 37° C. Both these factors will result in lower GGT values (Boehringer Mannheim, 1982).

The results in Table 2.4 demonstrate a very definite ethanol related increase within 4 weeks of the start of the study, which was maintained throughout. It therefore appears that GGT is a useful and reliable indicator of chronic alcohol consumption in rats maintained on a liquid diet, and one that is not readily distorted by alterations in dietary composition.

The results reported in the present study are in general agreement with the bulk of the literature relating to the effect of ethanol on serum GGT values in rats. They also demonstrate the ability of the ethanol/liquid diet procedure to produce liver damage.

2.4.4 Alkaline Phosphatase

As reported in Section 2.3.6., the alk. phos. values from the alcoholic rats were significantly higher than those of the sucrose control rats at weeks 4, 12 and 16. This suggests that alk. phos. is also a useful marker of chronic alcohol ingestion in rats, although it is perhaps not as sensitive in this respect as GGT. These results are in agreement with those of Nishimura and Teshke (1982) and Teshke et al., (1983), who also demonstrated serum alk. phos. levels to be elevated in alcoholic rats when compared with pair-fed controls.

There was some evidence (Table 2.5) of an effect of the liquid diet on the levels of alk. phos. in the two pair-fed groups. At weeks 1, 8 and 16, the values for the pair-fed groups were markedly lower than for the laboratory chow animals. It is likely that this was an effect of the liquid diet since this was the only nutritional source common to both pair-fed groups. It has been suggested (Teshke & Petrides 1982), that such alterations may stem from carbohydrate repression of alk. phos. synthesis. Comparison of the alk. phos. values from the laboratory chow and pair fed groups at week 12 was difficult due to the small number of values available at this time. There is no clear explanation for the low serum values in the laboratory chow animals at week 4.

A significant temporal reduction in the alk. phos. levels was noticeable in all three experimental groups. Unfortunately, data in the literature (which tends to give a single time-value for activity) is unable to substantiate this, although it appears (Boehringer Mannheim, Technical Dept. Written communication, 1984) that such an

'ageing' effect is normal.

The activity levels reported in Table 2.5 are slightly higher than those reported in the literature (Nishimura & Teshke, 1982; Teshke et al., 1983). However, as previously, the differences in experimental technique would explain these findings.

2.4.5 Aspartate Transaminase

With the exception of week 16 when the alcoholic animals showed significantly higher values than the sucrose controls (Section 2.3.7.), there were no significant differences between the serum AST values from the two pair-fed groups. This single difference was more likely to have been due to the unusually low AST levels recorded in the sucrose controls at that time, than to any direct effect of alcohol. The lack of an effect of alcohol on the rat serum AST values was anticipated on the basis of previous reports (Nishimura & Teshke, 1982; Teshke et al., 1983) and AST values were measured here to ensure that any apparent ethanol effects on other hepatic enzymes could confidently be accredited to ethanol and not to some more general form of liver damage.

There was a very clear liquid diet related effect on the AST values, with the serum levels in the laboratory chow group being significantly higher than those from the pair-fed animals. This effect was very rapid, being noticeable even in the blood samples taken at the time of pairing at which time the animals had only been fed the liquid based diets for one week. Again, this may have been due to components within the liquid diet itself, or to the nutritional restrictions

placed on the pair-fed animals compared to the animals maintained ad libitum on standard laboratory chow. This will be discussed in more detail in Section 4.4.8.

The values found in the serum from the pair-fed animals also appeared to undergo a temporal reduction over the course of the study. Rather than being simply a function of time, this may have been due to a cumulative AST suppression by the liquid diet or by the above mentioned nutritional restrictions. No temporal reduction was noticed in the serum AST levels from the laboratory chow animals.

The serum levels of AST in the laboratory chow animals (Table 2.6), were in general agreement with previously published results (Nishimura & Teshke 1982; Teshke et al., 1983).

2.4.6. Alanine Transaminase

The ALT levels showed a very sharp reduction in the serum of the groups maintained on the liquid based diets, even from the start of the study (Table 2.7). This effect was thought to be due to the intake of liquid diet. As with AST, ALT seemed to be of little value in this study as an index of alcohol-induced liver damage, although it may be of use in demonstrating the absence of non-alcohol related liver disease in the alcoholic animals. The present findings are at odds with those of Nishimura and Teshke (1982) and Teshke et al. (1983), both of whom demonstrated significantly raised ALT values in the serum of alcoholic animals. It is conceivable that the liquid diet used in this study caused a suppression of hepatic ALT to such an extent that any alcohol related effect had only a minimal, and perhaps

immeasurable, effect on the serum enzyme levels. Certainly, whilst the ALT values from the laboratory chow animals were in general agreement with those of the above studies, the liquid diet values were considerably lower.

2.4.7. Liver Histology

The results from the histological analysis of the liver sections clearly demonstrated the presence of a pronounced fat accumulation in the liver of the alcoholic animals. Such fatty deposition was not noticed in the livers of the laboratory chow control animals, but some minor accumulation was seen in the sucrose controls. It can thus be concluded that in this study, ethanol had been consumed by the animals, in quantities sufficient to produce abnormalities of the type commonly observed in human alcoholics.

2.5 CONCLUSIONS

The isocalorific matched pair feeding technique, is an effective method of ensuring alcohol consumption in the rats, and allows for close calorific control between the pair-fed animals. The model worked well throughout the study and would probably have been useful over a considerably longer period.

The present study indicates that both GGT and alk. phos. (in the latter stages), are valuable indices of alcohol related hepatocellular damage in rats maintained on a pair-feeding liquid diet regimen.

Although difficult to interpret accurately, the transaminase values support the view that the increases in GGT and alk. phos. were alcohol related and not due to any other forms of liver damage.

A surprising finding in this study is the low levels of the serum transaminases (and possibly alk. phos.) in the animals maintained on the liquid based diets. These values are not low simply with respect to the laboratory chow animals in the present study, but are also considerably lower than values reported elsewhere in the literature.

Possible explanations of this effect are that the liquid diet was quantitatively or qualitatively different in composition to the laboratory chow and that such differences could have led to enzyme repression, (for example, the carbohydrate effect reported by Teshke and Petrides (1982)), or that simply the ingestion of a liquid diet as opposed to a solid diet may lead to reduced enzyme levels. It is also

conceivable that such a liquid diet related effect arises from the nutritional limitations placed on the animals, since the diet is not made available ad libitum to the experimental rats. The inclusion of a high percentage of the calories (36 percent) as 'empty calories' (i.e. ethanol and sucrose) may again explain the above effect.

This topic is discussed in more detail in Chapter 4, along with an analysis of the effects of liquid diet alone on the serum enzyme levels.

CHAPTER THREE - THE PROTEIN STUDIES: 102 DAYS

3.1 INTRODUCTION

3.1.1. General Introduction

The principal aim of this thesis was to investigate any effect of ethanol alone upon the biochemistry of the oral epithelium. However, ethanol may affect a variety of biochemical systems or cellular components and to attempt a wide biochemical study would be of little value. It was therefore decided to be selective and to study the effect of ethanol on a single aspect of epithelial biochemistry.

Any cell expresses its identity, structure and function through the proteins that it manufactures and any disease related alteration of epithelial differentiation or morphology, may have its basis in alterations in the proteins expressed in the affected cells. Protein abnormalities have been observed in epidermal and epithelial cells following treatment with a variety of chemical agents (e.g. tumour promoters) and thus a study of the protein composition of the oral epithelium from alcoholic and control animals was the considered approach in this study.

The tongue was chosen as the most suitable intra-oral site for study as it provides a large area of epithelium which is reasonably easy to remove and also because the lateral tongue is reported as the major site of occurrence of alcohol related squamous cell carcinoma in man (Wynder, Bross & Feldman, 1957). Additionally, as the ethanol is administered using a drinking bottle, the tongue is in direct contact

with ethanol, more so than any of the other intra-oral sites.

No studies of the effects of alcohol on the protein profile of the oral epithelium have been performed, and indeed, very few studies (Section 1.9.2.) of the protein chemistry of the oral epithelium exist. The aims of this study were firstly, to detect any protein alterations in the oral epithelium brought about by chronic alcohol ingestion and secondly, to adapt and develop biochemical techniques for use in the study of oral epithelial proteins.

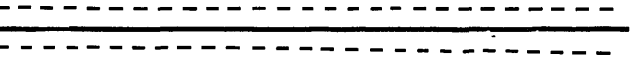
3.1.2 The Method of Choice for Studying Epithelial Proteins

The best methods available for studying protein populations (even total cellular proteins) are electrophoretic techniques. Whilst a number of electrophoretic techniques are available, the one that is widely regarded as giving the best resolution of proteins in one-dimension (1-D) is the discontinuous sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), method of Laemli (Laemli, 1970). This technique allows for the electrophoretic separation of reduced and denatured proteins from complex mixtures (Hames & Rickwood, 1981).

3.1.3. Preparation of the Proteins for Electrophoresis and the Theory of Separation of Proteins by SDS PAGE

The preparation of proteins for electrophoresis involves heating them in a solution of sodium dodecyl sulphate (SDS) (an anionic detergent) and dithiothreitol (DTT), a reducing agent. Heating at 100° C for 2-3 minutes allows the proteins to react effectively with both of these reagents (Deyl, 1979). The DTT breaks the protein down to individual

peptide chains by disrupting any inter- or intra-peptide disulphide bonds. The SDS denatures the protein by disrupting any noncovalent bonds and coats the protein in a uniform manner with negative charges. The resulting protein adopts a rod-like conformation.

i.e. 

The SDS coats peptide chains in the ratio of 1.4 grammes of SDS per gramme of peptide and thus proteins prepared in this manner possess an approximately constant charge:mass ratio. There are some exceptions to this general rule notably glycoproteins, the carbohydrate moieties of which sterically hinder the binding of SDS. These glycoproteins, therefore, have a reduced SDS:peptide ratio (Webber & Osborne, 1975).

The separation of the peptides prepared for SDS PAGE relies on a molecular sieving effect. Given that most SDS coated peptides show the same charge:mass ratio, they will move at similar rates through an electric field. If a retarding matrix, in this case an acrylamide gel matrix, is placed in the path of the peptides, they will separate out according to size, with the smallest peptides being retarded least and therefore migrating furthest in a given time period (Hames & Rickwood, 1981).

The acrylamide matrix is set up by crosslinking acrylamide monomers with a bi-functional cross linking agent, N-methylenebisacrylamide (bis). By varying the concentration of cross linking agent and monomer, gels of differing porosity can be produced and thus the molecular weight ranges of the peptides resolved by the gels can be

varied. The polymerisation reaction is initiated by a free radical generating system involving NNN'N'tetramethylethylenediamine (TEMED) and ammonium persulphate (AMPS).

The gels used in the present studies were slab gels and were of the discontinous type. Discontinuous gels consist of two components, the first (upper) gel component being a very low porosity gel through which peptides will migrate at an even rate regardless of size. This results in the formation of a tight band of protein in the upper gel, and means that high loads of protein may be applied to the gel without encountering any trailing effects. This effect is known as 'stacking' and thus the upper gel is refered to as the 'stacker gel'. The proteins move through the stacker gel as a tight band until they reach the lower gel component, the resolving gel. This is a gel of variable porosity which will retard the movement of protein and will thus result in the separation of the peptides on the basis of molecular size.

3.1.4. Molecular Weight Analysis on SDS Gels

A logarithmic relationship exists between the distance of migration (Rf) of an SDS coated peptide (in comparison with a free moving marker molecule) and its molecular weight. Rf values can be calculated for a number of proteins of known molecular weight, and a calibration curve produced. The molecular weight of any other protein for which Rf has been calculated, can thus be obtained.

3.1.5. Two Dimensional Gel Electrophoresis

More recently, a much higher degree of resolution of protein mixtures has been possible due to the development of a two-dimensional (2-D) electrophoresis system (O'Farrell, 1975). This involves subjecting the proteins to isoelectric focusing (IEF) in one-dimension and then to SDS PAGE in the second dimension. Given a sufficiently sensitive protein detection procedure, this technique can resolve many thousands of proteins (compared with the 50 or 60 resolved by 1-D methods). This 2-D technique was attempted in the present study (Section 3.2.3.) but the evaluation of the gels obtained proved too complex (Figure 3.1) in a study limited by time. For this reason and also because the results from the 1-D gels were of sufficient interest to direct the remainder of the work, the 2-D approach was discontinued and the results are not presented in this thesis.

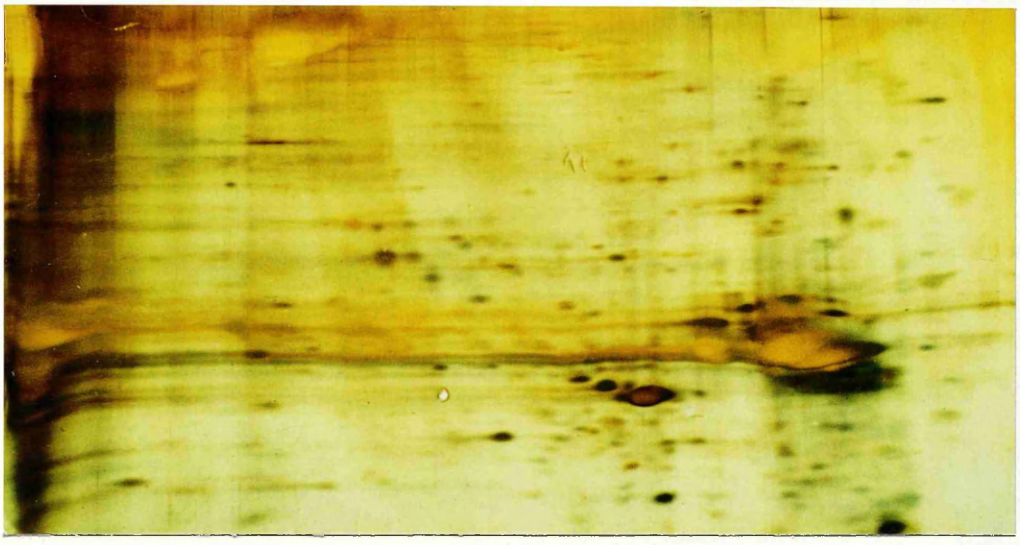


FIGURE 3.1: AN EXAMPLE OF A TWO-DIMENSIONAL GEL OF RAT LINGUAL EPITHELIAL PROTEINS. THIS GEL WAS SILVER STAINED (SECTION 6.2.3.) TO MAXIMISE THE VISUALISATION OF THE PROTEINS.

3.2 MATERIALS AND METHODS

3.2.1. Removal of the Epithelium from the Underlying Connective Tissue and Disruption of Epithelial cells

To study the effects of ethanol on lingual epithelial proteins a method was required to remove the lingual epithelium from the underlying connective tissue. A number of methods were attempted (acetic acid treatment and trypsinisation), but the most effective method tried was that described by Ranieri, Simsiman and Boutwell (1973). This method was originally developed for epidermal studies but proved useful in the present study for removal of lingual epithelium. The technique involved placing rat tongues (halved longitudinally) into ice cold water for 30 seconds and then into water at 55° C for 30 seconds. The tissue was finally placed into ice cold water again for 30 seconds. Following this treatment, the epithelium was removed with ease as an intact sheet (Figure 3.2.).

Epithelial cells have to be disrupted to release the proteins for study and although a number of techniques were tried, only motor assisted homogenisation provided a high degree of cell disruption. Homogenisation of the lingual epithelium was performed as follows.

Epithelial sheets were placed in 1 ml of ice cold distilled water and minced thoroughly with a pair of sharp scissors. This mixture was kept at 0-4° C and subjected to fifteen strokes of a tight fitting glass-teflon homogeniser, driven at full speed (12000 rpm) by a 'Tri-R Stir-R' laboratory motor (Camlab, Cambridge, England). The resulting homogenate was kept on ice prior to preparation for 1-D SDS PAGE

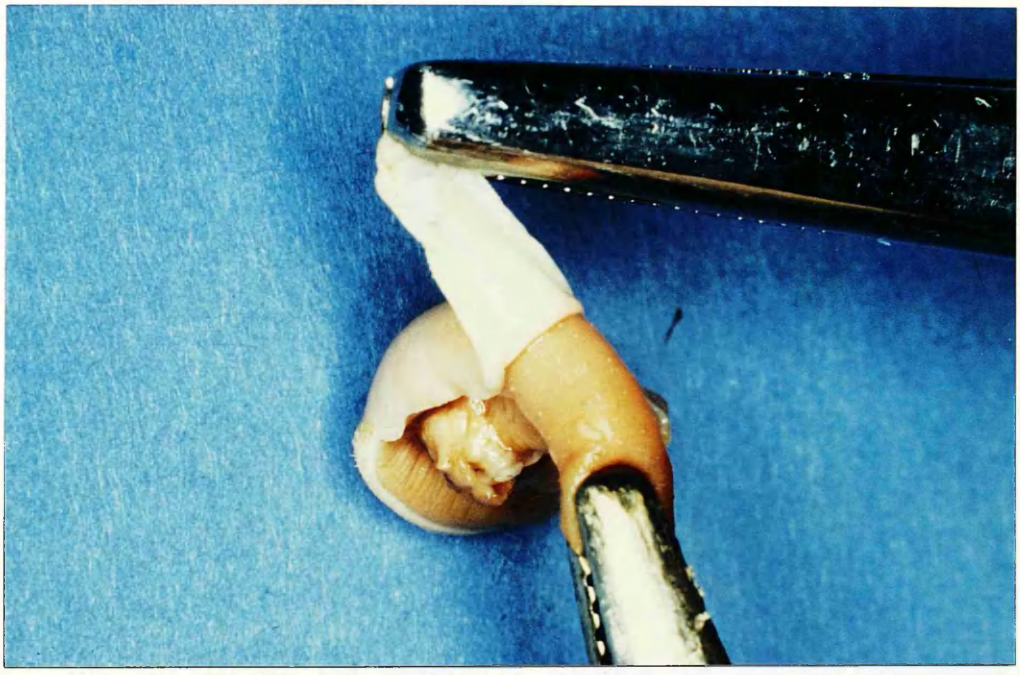


FIGURE 3.2: REMOVAL OF THE LINGUAL EPITHELIUM FROM THE UNDERLYING CONNECTIVE TISSUE BY THE METHOD DESCRIBED IN SECTION 3.2.1.

(Section 3.2.2.).

3.2.2. Preparation of Proteins for One-Dimensional SDS PAGE

The epithelium from tongues of half of the controls (1-5) and half of the pair-fed animals (pairs 11-18) were prepared for 1-D SDS PAGE. The animal numbers and pairing are as in Chapter 2.

The homogenate was brought to 2 percent SDS, 25 millimolar (mM) DTT (by addition of quantities of a 10 x concentrated solution) and heated in a boiling water bath for 2-3 minutes. The resulting preparation was centrifuged at 6000 g for 20 minutes in an MSE High Speed-18 centrifuge (Fisons, Crawley, England) to remove any insoluble material. The supernatant was carefully removed and was set dialysing overnight against 1:4 diluted SDS/glycine electrophoresis buffer (Section 3.2.7.). The following day, the dialysate was brought to 10 percent glycerol (to increase its density) and a small volume of bromophenol blue solution (0.2 percent in ethanol) was added to act as a tracker dye during electrophoresis. All these steps were performed at 0-4° C to minimise proteolysis.

The resulting protein preparation was stored at -20° C prior to electrophoresis.

3.2.3. Preparation for Two-Dimensional Gel Electrophoresis

The remaining animals (6-10 and pairs 19-25) were prepared for 2-D gel electrophoresis essentially as described by O'Farrell (1975). The tissue, prepared as described (Section 3.2.1.) was this time homogenised in O'Farrell lysis buffer (9.5 M urea, 1.6 percent

ampholines pH 5-7, 0.4 percent ampholines pH 3.5-10 and 5 percent β-mercaptoethanol). The homogenate was brought to 2 percent Nonidet P40 following homogenisation.

3.2.4. Preparation of the Two-Dimensional Electrophoresis Samples for One-Dimensional Electrophoresis

As discussed in Section 3.1.5., the 2-D approach was abandoned in this study and to obtain a preparation that might be used for 1-D electrophoresis, the proteins prepared for 2-D electrophoresis were dialysed overnight against two changes of 1:4 diluted SDS/glycine electrophoresis buffer (Section 3.2.7.). The dialysate thus formed was prepared for SDS PAGE exactly as described previously (Section 3.2.2.).

Again, all the procedures were carried out at 0-4° C and all the samples were stored at -20° C prior to electrophoresis.

3.2.5. Protein Concentration Determination

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

Following homogenisation, and prior to preparation for SDS PAGE, 25 μl of each of the homogenates was placed in a test tube and the volume brought to 100 μl by the addition of 75 μl of distilled water. To this tube, 5 ml of the protein dye reagent (Bradford, 1976) was added and the contents of the tube were thoroughly mixed by vortexing. The absorbance of the resulting solution was measured at 595 nm in a Pye

Unicam SP8-100 spectrophotometer (Pye Unicam) using disposable plastic cuvettes. The protein concentration was read from a standard curve prepared, as above, using 100 μl volumes of various concentrations of Bovine Plasma Albumin.

All measurements were performed between 2 and 60 minutes after vortexing and the absorption was read against a reagent blank prepared from 100 μl of the appropriate buffer (O'Farrell lysis buffer or H_2O) and 5 ml of protein dye reagent.

3.2.6. One-Dimensional Discontinuous SDS Polyacrylamide Gel Electrophoresis

The composition of the solutions and the gel 'recipes' are given in Table 3.1.

Following preparation of the chosen gel mixture, (without SDS, AMPS or TEMED), it was degassed under vacuum for 5 minutes in a 100 ml capacity side arm flask. This was to remove any O_2 that may have interfered with the polymerisation reaction. The SDS, AMPS and TEMED were then added and the solution mixed by swirling briefly (this avoids introducing air bubbles).

The gel was cast between glass plates separated by 1.5 mm thick neoprene spacers (Figure 3.3). To ensure no leakage at the bottom corners of the gel, a 'plug' was prepared using a small amount (c. 3 ml) of the above gel solution but with excess AMPS and TEMED. This solution was poured between the glass plates and formed a rapidly polymerising layer which acted as a plug blocking off possible points

GEL RECIPES

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
1.5 M 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	10 μl	10 μl	10 μl	10 μl	10 μl

STACKING GEL: 5 %	
30 % ACRYLAMIDE	3.3
1 % BISACRYLAMIDE	2.7
1 M TRIS pH 6.8	2.5
20 % SDS	100 μl
WATER	8.8
10 % AMPS	100 μl
TEMED	10 μl

TABLE 3.1: RECIPES FOR THE POLYACRYLAMIDE GELS

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

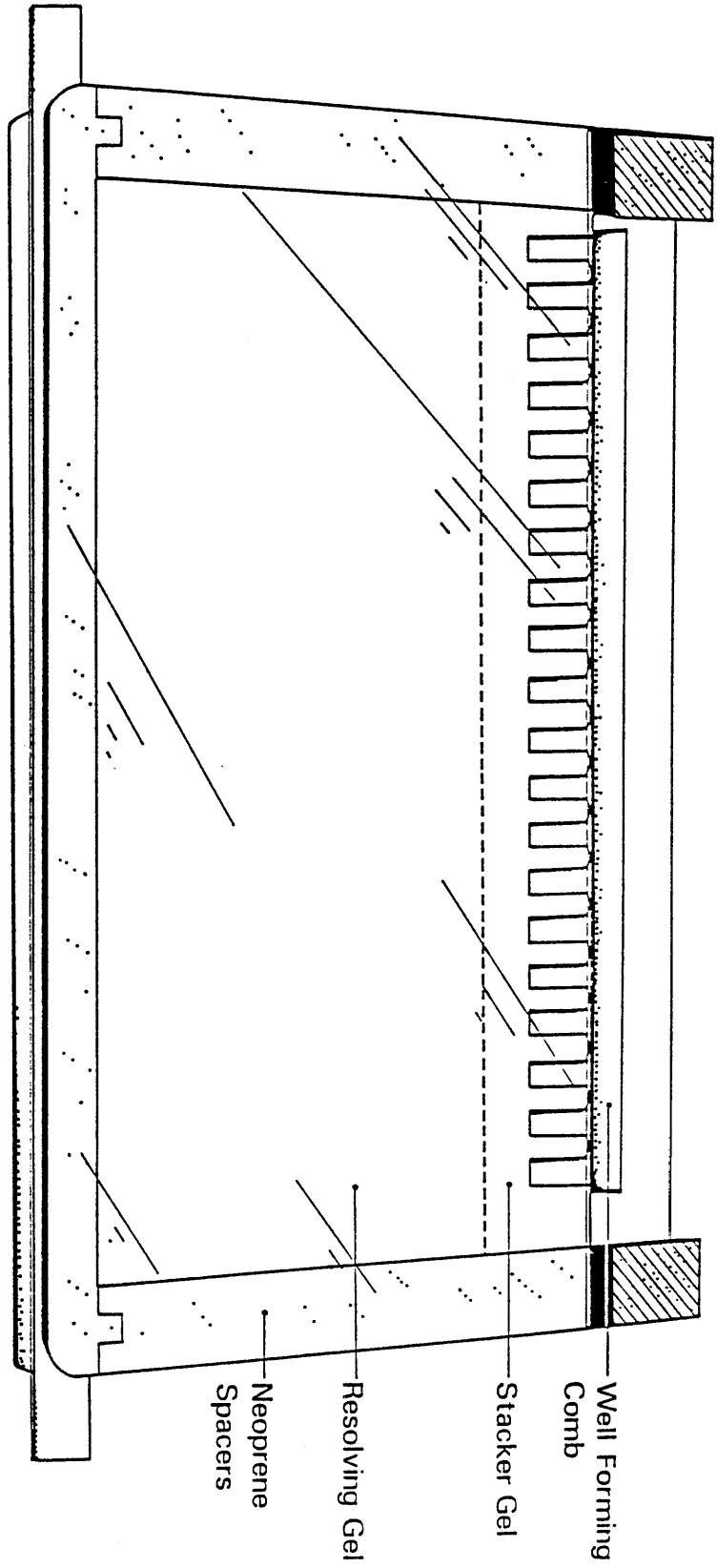


FIGURE 3.3: GEL CAST BETWEEN GLASS PLATES, SHOWING THE NEOPRENE SPACERS AND THE WELL FORMING COMB IN POSITION.

of leakage. Using a Pasteur pipette, the remainder of the gel solution was poured slowly between the glass plates to within 4 cm of the top. The solution was then over-layed with isobutanol (which 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 (see Table 3.1), again omitting the SDS, AMPS & TEMED. The solution was degassed as above, the SDS, AMPS and TEMED added and the mixture applied (after the removal of the isobutanol) to the top of the resolving gel. Into this unpolymerised stacker gel was placed a well forming comb (20 wells) around which the gel was allowed to polymerise. Once polymerisation was complete, the comb and bottom spacer were 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.

3.2.7. Loading the Gels and Electrophoresis

Buffer for the electrophoresis (0.025 M tris, 0.192 M glycine, 0.1 percent SDS, pH 8.3) was added to the bottom reservoir of the electrophoresis apparatus (see Figure 3.4) and the gel was carefully loaded ensuring that no air bubbles formed at the gel/buffer interface. The top reservoir was then filled with electrophoresis buffer and 75 μg of each of the epithelial protein preparations, in a maximum volume of 50 μl were loaded into the sample wells. Molecular weight markers (either high or low) were included with each run to

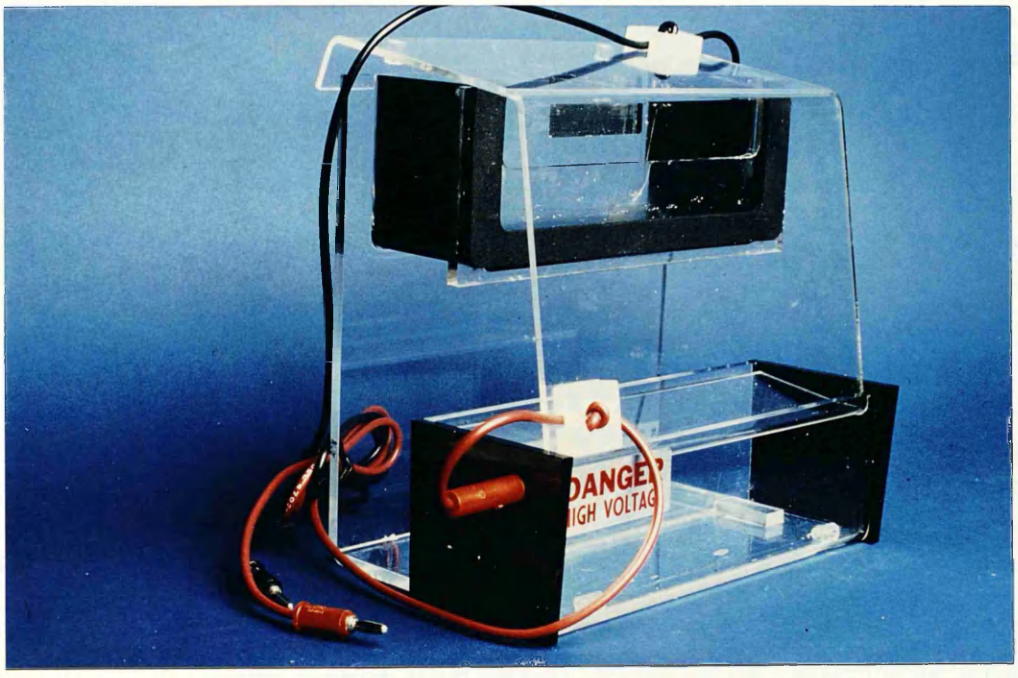


FIGURE 3.4: THE ELECTROPHORESIS APPARATUS USED THROUGHOUT THE STUDY FOR RUNNING SDS SLAB GELS.

allow calculation of the molecular weight of any proteins of interest. The molecular weight marker proteins used are listed in the Appendix.

Electrophoresis was carried out initially at 80 volts until the proteins (marked by the bromophenol blue dye) reached the end of the stacker gel, and subsequently at 160 volts until the bromophenol blue marker dye reached the bottom of the resolving gel. Unless otherwise indicated, gels were run at 0-4° C in a cold room.

3.2.8. Staining of the Proteins

Coomassie brilliant blue R-250 (CBB-R250) staining is the most widely used and most sensitive of the common protein staining methods (Hames & Rickwood, 1981) and was the one used in this thesis.

Following electrophoresis, the gels were carefuly removed from between the glass plates and placed overnight in a fixing/staining solution of 0.1 percent CBB R-250, 50 percent ethanol and 10 percent acetic acid. Destaining was by diffusion against a succession of ethanol/acetic acid solutions (Table 3.2), and the final destained gel was stored in 5 percent acetic acid in which it is stable almost indefinitely with only minimal loss of band intensity (Pharmacia Fine Chemicals, 1983).

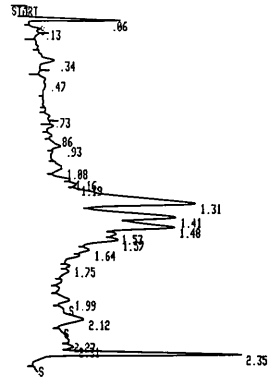
	Ethanol	5 percent Acetic Acid	Water	Time
Stage 1	400	600	---	1-2 hrs.
Stage 2	300	700	---	1-2 hrs.
Stage 3	300	700	---	1-2 hrs.
Stage 4	200	800	---	1-2 hrs.
Stage 5	50	700	250	O/night
Stage 6	--	700	300	Storage

Table 3.2: Destaining Protocol All volumes are in ml.

The CBB R-250 stained gels were, where appropriate, analysed on an 'LKB 2202 Ultroscan Laser Densitometer' attached to an 'LKB 2220 Recording Integrator' (LKB Instruments Ltd., England). The densitometer detects the protein bands using a laser beam of wavelength 632.8 nm and the recording integrator produces a plot and a quantitative analysis of the densitometric readings. A specimen print out is shown in Figure 3.5. The most useful parameter for the purposes of comparison is the area percentage which is a measure of the percentage of total protein represented by a single band (LKB Instruments Ltd., 1981). The use of this parameter allows for direct and quantitative comparisons between levels of individual proteins in different samples.

3.2.9. Statistical Tests

The statistical tests used in the evaluation of the densitometer results were those described in Section 2.2.10.



RUN # 492

AREA%

RT	AREA	TYPE	AR/HT	AREA%
0.06	2807000	DSBB	0.016	2.924
0.13	86587	BB	0.008	0.090
0.34	1251800	VB	0.041	1.304
0.47	842960	PV	0.071	0.878
0.73	123290	PB	0.020	0.129
0.86	240310	PV	0.025	0.250
0.93	1010800	VV	0.041	1.053
1.08	1570200	VV	0.065	1.636
1.16	1155500	VV	0.030	1.204
1.19	1737900	VH	0.034	1.811
1.31	2.0838E+07	SHH	0.074	21.709
1.41	1.4156E+07	SHH	0.059	14.748
1.48	1.3694E+07	SHH	0.057	14.267
1.53	4617400	SHH	0.037	4.811
1.57	6641300	DSHH	0.051	6.919
1.68	7373800	SHH	0.132	7.682
1.91	3511200	SHH	0.225	3.658
1.99	1307629	SHB	0.047	1.362
2.12	3158700	SPB	0.064	3.291
2.27	1400100	SPH	0.066	1.459
2.31	1031800	SHH	0.035	1.075
2.35	7429400	DSHB	0.020	7.740

TOTAL AREA= 9.5986E+07
MUL FACTOR= 1.0000E+00

FIGURE 3.5: A SPECIMEN DENSITOMETRIC 'PRINT OUT' SHOWING THE PROTEIN PROFILE AND THE QUANTITATIVE ANALYSIS PRODUCED BY THE RECORDING INTEGRATOR. NOTE THE AREA PERCENTAGE VALUES IN THE FAR RIGHT HAND COLUMN.

3.3 RESULTS

3.3.1. Separation of the Epithelium from the Connective Tissue and Disruption of the Epithelium

The method of Ranieri et al. (1973) allowed the epithelium to be removed with ease as an intact sheet (Figure 3.2). Very little connective tissue was visible on the separated epithelium and only few basal cells remained on the connective tissue (Figure 3.6).

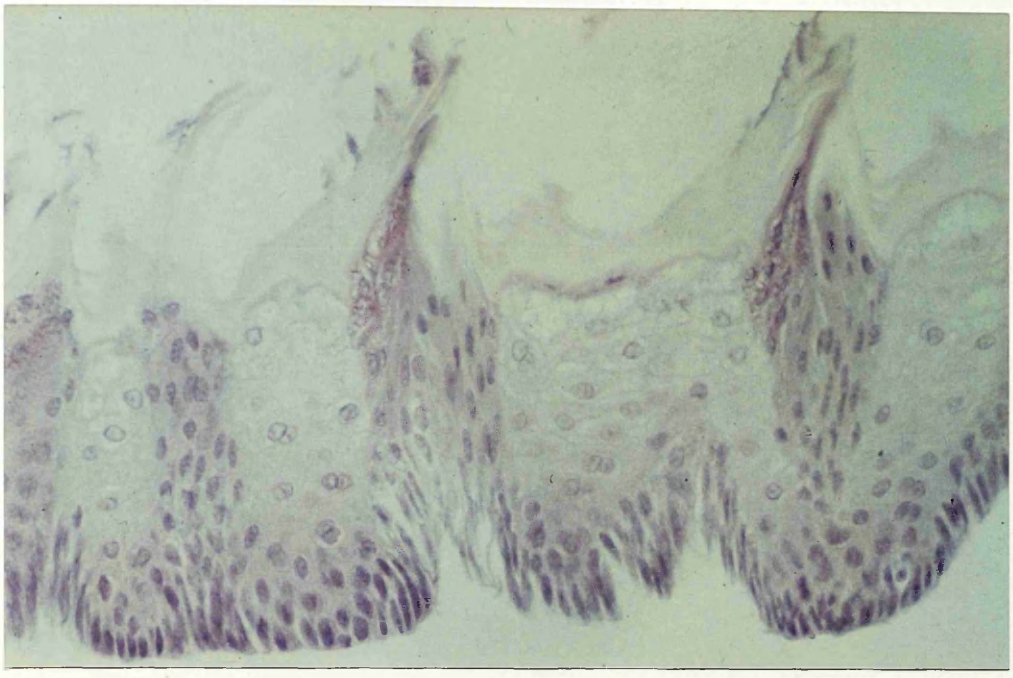
The epithelium is tough and a high shearing force was required to rupture the epithelium and to burst open the individual cells. Neither sonication nor hand-homogenisation could produce such a force and only motor driven homogenisation proved useful and effective in this respect. Following homogenisation, the homogenate contained protein at a concentration that was typically in the range 3-5 mg/ml.

3.3.2. Maximal Protein Loads

Comparison of one-dimensional gels loaded with varying amounts of epithelial proteins, revealed that maximal resolution (without overloading effects) was achieved using c. 75 μg of protein, in a maximum volume of 50 μl (the volume of the sample wells).

3.3.3. Comparison of the Protein Profiles from the Experimental Animals

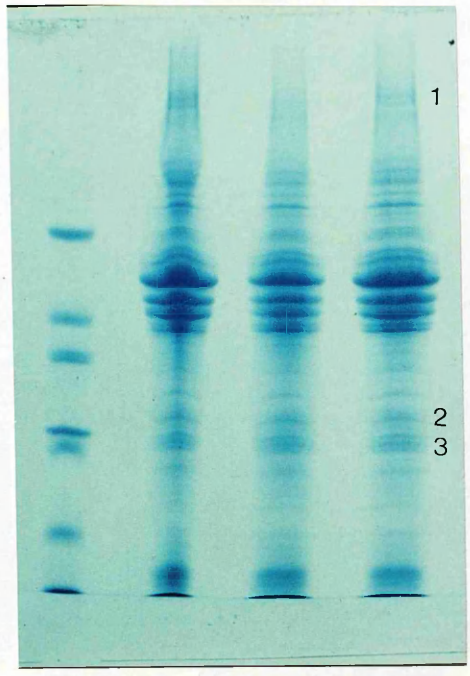
Visual comparison: The 1-D SDS polyacrylamide gels of the lingual epithelial proteins from the experimental animals are shown in Figures 3.7, 3.8, 3.9 and 3.10. Visual comparison between the pair-fed



A

B

FIGURE 3.6: HISTOLOGICAL ANALYSIS OF THE EPITHELIUM (A) AND CONNECTIVE TISSUE (B) SEPARATED BY THE METHOD DESCRIBED IN SECTION 3.2.1.



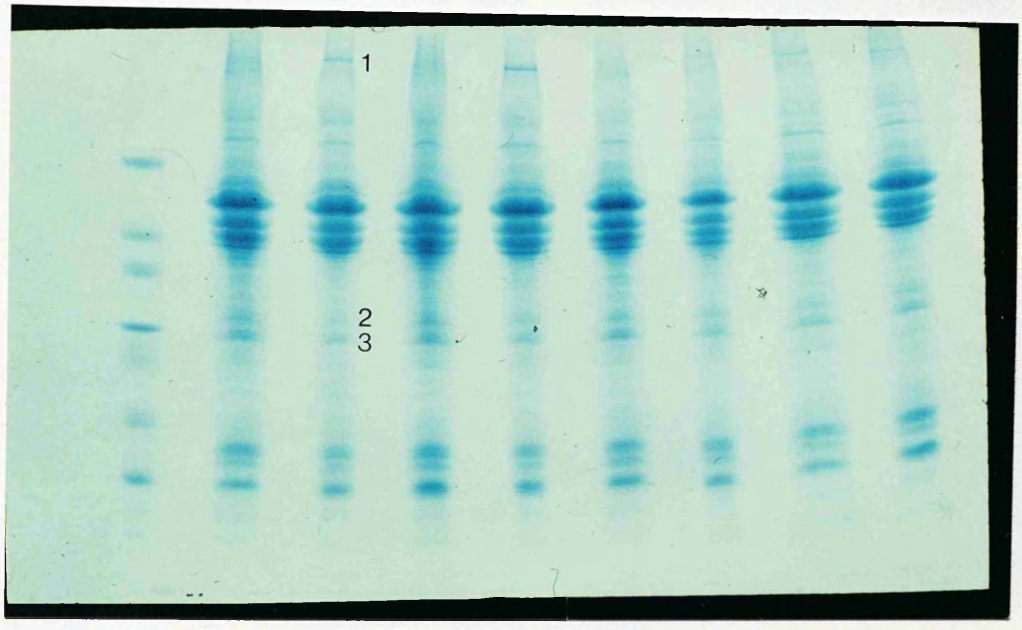
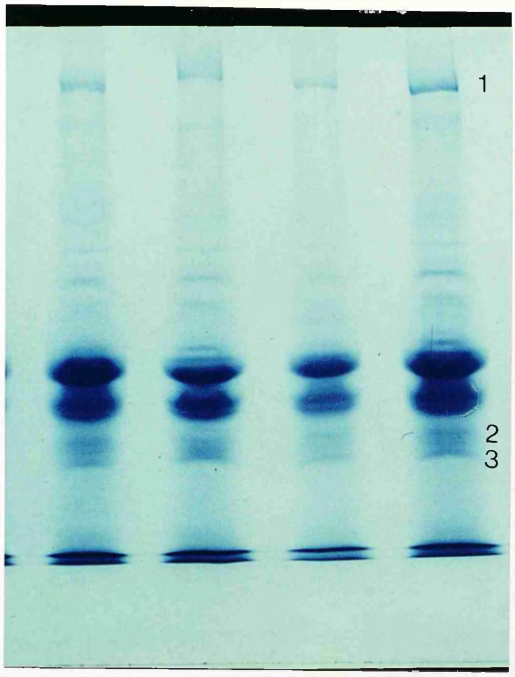
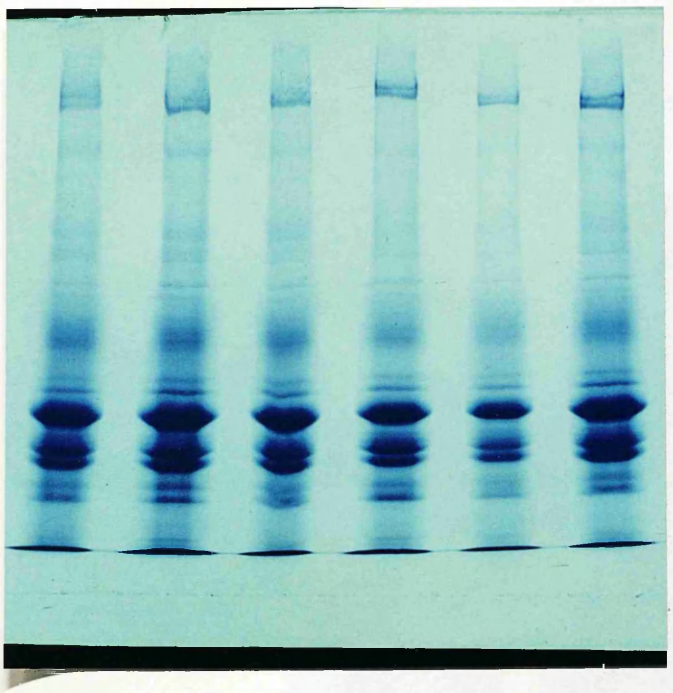


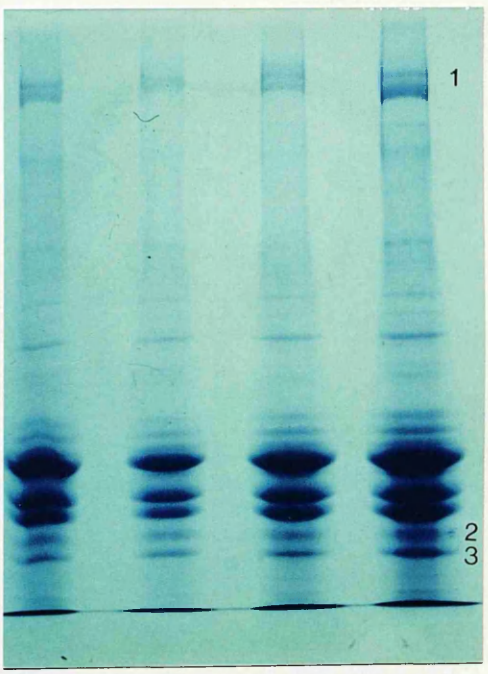
FIGURE 3.7: RESULTS FROM THE PROTEIN GELS OF THE PAIR-FED ANIMALS NUMBERS 11-16.



19*a* 19*b* 20*a* 20*b*



21*a* 21*b* 22*a* 22*b* 23*a* 23*b*



24*a* 24*b* 25*a* 25*b*

FIGURE 3.8: RESULTS FROM THE PROTEIN GELS OF THE PAIR-FED ANIMALS NUMBERS 19-25.

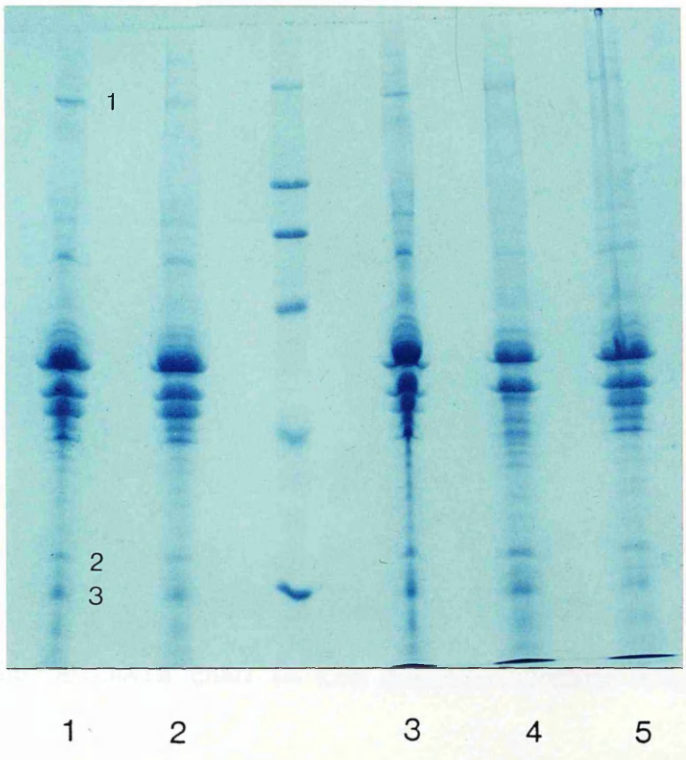


FIGURE 3.9: RESULTS FROM THE PROTEIN GELS OF THE LABORATORY CHOW ANIMALS NUMBERS 1-5.

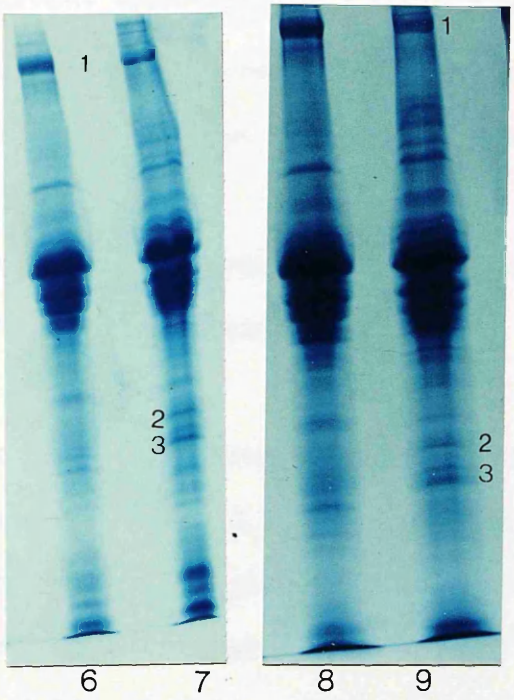


FIGURE 3.10: RESULTS FROM THE PROTEIN GELS OF THE LABORATORY CHOW ANIMALS NUMBERS 6-9.

Sample number 10 was lost during the preparative procedure.

animals (pairs 12-16) revealed (Figure 3.7) a reduction in the levels of a high molecular (MW) protein in the alcoholic animals (protein number 1) compared to the sucrose control animals. This difference was not so apparent in pairs 19-25 (Figure 3.8), which showed higher levels of this high MW protein (these proteins were originally prepared for 2-D electrophoresis). Further visual analysis, best highlighted in Figure 3.7, revealed two lower MW proteins (protein numbers 2 and 3), levels of which appeared to be higher in the alcoholic animals than in the sucrose control animals.

Visual analysis of Figures 3.9 and 3.10, revealed no consistent differences in the lingual epithelial protein profiles of the laboratory chow animals and the sucrose control animals. It was, however, apparent from Figures 3.9 and 3.10, that the differences between the alcoholic and the sucrose control animals also existed between the alcoholic and the laboratory chow groups.

Further visual analysis revealed no additional protein differences between any of the three groups.

Densitometric Analysis: Densitometric analysis (Figure 3.11) confirmed the visual analyses. Table 3.3 shows that the mean value of the area percentage for the high MW protein (protein 1) in the alcoholic group was lower than that for either the sucrose or laboratory chow control groups (0.89, 2.55 and 2.02 respectively). Statistical analysis revealed the levels in the alcoholic group to be significantly lower than those in either the sucrose ($p < .002$) or laboratory chow (Mann-Whitney, $p < .02$) control groups. Thus despite the lack of obvious

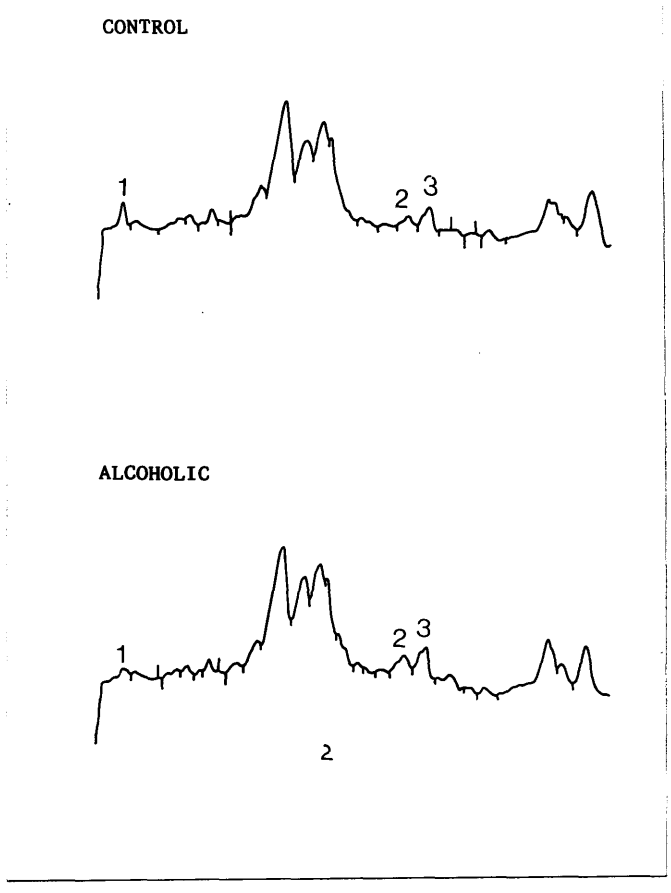


FIGURE 3.11: DENSITOMETRIC COMPARISON OF THE PROTEIN PROFILES OF THE SUCROSE CONTROL AND ALCOHOLIC ANIMALS.

(1) The High MW Protein

(2) The 30K Protein

(3) The 28K Protein

THE LABORATORY CHOW CONTROL ANIMALS

ANIMAL	HIGH MW	30K	28K
1	2.8	2.2	3.4
2	1.6	2.4	3.3
3	1.6	1.5	3.6
4	2.4	3.9	3.6
5	2.1	3.9	4.4
6	2.4	2.5	3.1
7	1.6	3.8	4.3
8	1.8	2.7	2.5
9	1.9	2.3	4.2
10	---	---	---
Mean	**2.02**	**2.80**	**3.60**
S.D	**0.43**	**0.86**	**0.62**

THE ALCOHOLIC (A) AND SUCROSE (B) PAIR-FED ANIMALS

	HIGH MW		30K		28K	
ANIMAL	A	B	A	B	A	B
11	---	2.8	----	4.1	----	1.7
12	0.0	2.2	3.0	2.3	6.7	3.5
13	2.9	4.4	5.9	2.8	6.4	3.0
14	0.0	2.7	2.3	2.1	6.4	2.7
15	0.8	1.9	3.1	2.7	6.4	6.9
16	2.2	2.1	4.2	4.6	4.5	4.4
17	---	---	---	---	---	---
18	---	---	---	---	---	---
19	0.0	1.5	4.0	---	8.5	---
20	0.0	3.2	4.8	3.5	4.2	3.8
21	0.7	1.8	3.5	1.3	7.9	2.2
22	1.1	1.8	2.5	2.6	3.7	5.1
23	1.1	3.2	---	3.5	7.3	3.5
24	0.9	2.0	5.2	2.4	5.2	3.0
25	1.0	3.6	5.1	1.3	5.4	1.5
Mean	**0.89**	**2.55**	**3.96**	**2.77**	**6.05**	**3.44**
S.D.	**0.91**	**0.85**	**1.19**	**1.02**	**1.49**	**1.51**

Table 3.3: AREA PERCENTAGE VALUES FOR THE THREE ABERRANT PROTEINS FROM THE 102 DAY STUDY

Missing values 11A, 17 A & B, 18 A & B and 10 are due to loss of samples during the preparative procedures. Missing values from animals 19 B and 23 A are due to poorly resolved gels.

visual evidence of alterations of this protein in the alcoholic animals of pairs 19-25, densitometric analysis demonstrated that such a reduction had indeed taken place. No significant differences were observed in the levels of this high MW protein in the sucrose and laboratory chow control groups.

Table 3.3 also demonstrates the higher levels of the two lower MW proteins in the alcoholic animals. The mean area percentage values for protein 2 in the alcoholic, sucrose and laboratory chow groups were 3.96, 2.77 and 2.80 respectively. Statistical analysis showed the levels of this protein to be significantly higher in the alcoholic group than in either the sucrose ($p<.05$) or laboratory chow (Mann-Whitney, $p<.05$) control groups. No significant differences were observed in the levels of this protein in the sucrose and laboratory chow control groups.

The mean area percentage values for protein 3 in the alcoholic, sucrose and laboratory chow groups were 6.05, 3.44 and 3.60, respectively. Statistical analysis revealed the levels in the alcoholic animals to be significantly higher than those in either the sucrose ($p<.025$) or laboratory chow (Mann-Whitney, $p<.002$) control groups. No significant differences were observed in the levels of this protein in the sucrose and laboratory chow control groups.

The levels of the above three proteins in the protein preparations initially intended for 2-dimensional gel electrophoresis were not significantly different from those in the one-dimensional protein preparations. This was true for each of the three experimental

groups.

Further densitometric analysis revealed no additional consistent protein differences between any of the experimental groups.

3.3.4. Molecular Weight Analysis of the Aberrant Proteins

Analysis of the molecular weight of these three proteins revealed that the two lower MW proteins (numbers 2 and 3) have apparent molecular weights of 30 Kilodaltons (K) and 28K respectively. Analysis of the molecular weight of the high MW protein (number 1) proved more difficult although initial results revealed it to be between 160 and 200K (this is discussed in more detail in Chapter 6).

3.4 DISCUSSION

3.4.1. Preparation of the Epithelial Proteins for SDS Polyacrylamide Gel Electrophoresis

Removal of the epithelium by the method of Ranieri et al (1973) proved ideal for use in the present study and resulted in a rapid and clean separation of the epithelium from the underlying connective tissue. This method had the added advantage of being effective without prolonged exposure to chemical substances (e.g. acetic acid or trypsin) which may have altered the epithelial protein pattern (Skerrow & Skerrow, 1985) and could, conceivably, have altered any alcohol mediated changes.

Only motor driven homogenisation produced a sufficiently high shearing force to effectively disrupt the epithelium, yielding an homogenate that contained epithelial proteins at a concentration suitable for electrophoresis.

Of the media used for homogenisation, i.e. H_2O and the O'Farrell lysis buffer without Nonidet P40, both yielded high quality protein preparations which differed only in the levels of the high MW protein. The O'Farrell lysis buffer extracted much higher levels of this protein than did simple homogenisation in H_2O. The reason for this (as discussed more fully in Chapter 6) was that the O'Farrell buffer contained 9.5 M urea, a dissociating agent, which markedly enhanced the solubility of this high MW protein.

Interestingly, the area percentage measured for this protein in the

samples prepared initially for 2-D electrophoresis, was not significantly higher than that measured for the same protein in the samples prepared for 1-D electrophoresis despite the higher visible levels of the protein in the 2-D preparations than in the 1-D preparations. This may have been due to the higher background staining that was observed in the protein tracks from the gels of the 2-D preparations.

Studies using various amounts of epithelial proteins revealed that the load of protein which afforded maximal resolution and minimal overloading, was c.75μg of protein. This amount of protein was added in a maximum volume of 50 μl, because this was the limit of the capacity of the gel sample wells. The value of 75μg is in general agreement with the results of Ramsden, Loehren and Balmain, (1983) who suggested an identical load for epidermal proteins.

3.4.2. The Alcohol Related Protein Changes

The results demonstrated a reduction in the levels of a high MW protein (160-200K) and a concomitant increase in the levels of two lower MW proteins (30 & 28K) in the lingual epithelium of the alcoholic animals compared to either of the two control groups. No alterations in the protein profiles were observed in the sucrose control group when compared to the laboratory chow control group and thus the observed effects can confidently be said to be alcohol related.

One complicating factor in the interpretation of the results from the 1-D gels is that the 28K protein appears to band alongside a protein

of very similar molecular weight as a closely associated doublet (Figure 3.7). Densitometer traces have been unable to resolve this doublet and it is not yet clear which member of the doublet is enhanced in the alcoholic animals.

It is conceivable that the two lower MW proteins were breakdown products of the high MW protein, with the alcohol in some way increasing the rate of breakdown of this high MW protein, resulting in enhanced levels of the two lower MW proteins. The techniques used in this Chapter did not allow for assessment of this possibility. Investigations into the relationship between the high MW protein and the two lower MW proteins are presented in Chapters 5 and 6.

Although these changes appeared to be due to alcohol intake, it is not known whether the observed effects were the result of chronic or acute alcohol consumption, or whether this was a systemic or local effect of alcohol. It was not even clear whether the alterations resulted from the effects of ethanol *per se* or from one of the products of its metabolism. The question of whether chronic or acute alcohol exposure is required to cause the observed protein changes is addressed in Chapter 5.

3.5 CONCLUSIONS

Results from this initial study into the effects of alcohol on the protein profiles of rat lingual epithelium revealed a reduction in the levels of a high MW protein (160-200K) and an accompanying increase in the levels of two lower MW proteins (30 & 28K) following chronic alcohol consumption.

It is conceivable that the two lower MW proteins were breakdown products of the high MW protein, increased levels being brought about by alcohol mediated increases in the breakdown of the high MW protein.

CHAPTER FOUR - THE 30 AND 60 DAY STUDY

4.1 INTRODUCTION AND RATIONALE BEHIND THE 30 AND 60 DAY STUDY

The second animal experiment was designed to study the alterations in the lingual epithelial protein profile of alcoholic animals over fractions of the original 102 days. It was hoped, in this way, to discover whether the changes in epithelial proteins, initially observed after 102 days, were the result of chronic or short term ethanol consumption. If the alterations were due to chronic consumption, it was assumed that it would be possible to follow the reduction in the high MW protein and the increase in the two lower MW proteins as a function of time. For this reason, thirty and sixty days were chosen as approximately one- and two-third time points of the original study. This is further discussed in Section 5.1.

In Chapter 2 there were some enzyme results which were difficult to explain and accordingly an additional control group maintained on liquid diet alone, was included in this study.

4.2 MATERIALS AND METHODS

4.2.1. Initiating the Study

Sixty-one young male Sprague Dawley rats (Bantin and Kingman, England) aged 6-8 weeks, were initially divided into two groups. The first group consisted of 14 rats maintained on standard laboratory chow (SDS Ltd., England) and water ad libitum for the duration of the study. The second group, consisting of 47 rats, was maintained on a nutritionally adequate liquid diet (SDS Ltd., England) prepared at a concentration of 20 grammes of diet per 100 ml of water. One hundred millilitres of this diet was made available to each rat per day. All the animals were housed individually in wire bottomed cages.

After one week, the 47 liquid diet animals were divided into a further three groups. Fifteen rats were maintained on the liquid diet alone for the duration of the experiment and the remaining 32 rats were divided into 16 closely weight-matched pairs and fed either liquid diet plus ethanol, or liquid diet plus sucrose, according to the isocalorific matched pair feeding technique described previously (Section 2.2.3.). The alcoholic animals were weaned onto the 36 percent ethanol diet via 10 percent and 20 percent ethanol diets as in the earlier experiment (Section 2.2.2.). The four groups of animals in this study and their respective dietary regimens are summarised in Table 4.1. The dietary compositions are outlined in Table 2.1.

The pair-feeding procedure and the materials used were identical to those described for the 102 day study and all animals were weighed weekly.

GROUP	NUMBER OF ANIMALS	DIET AVAILABLE
Laboratory Chow	14	Ad Libitum
Liquid Diet	15	100 ml
Liquid Diet and Sucrose	16	100 ml
Liquid Diet and Alcohol	16	100 ml

TABLE 4.1: DIETARY REGIMENS OF THE EXPERIMENTAL GROUPS-30 AND 60 DAYS.

Use of unnecessarily large volumes of this expensive diet was not desirable thus the liquid diet was not fed ad libitum to the liquid diet group. One hundred millilitres of this diet, which provided sufficient nutrients and calories to support growth and maintenance, was made available to these animals each day.

4.2.2. Collection of Blood

Blood was removed, as described previously (Section 2.2.4.), at three points in the study. Blood was first removed at the point of pairing, at which time all the animals were bled, and then at the first and second kills. This blood was centrifuged to provide serum for liver enzyme analysis exactly as described in Section 2.2.4.

4.2.3. Sample Collection at Sacrifice

Thirty days after starting the alcoholic animals on the 36 percent ethanol diet, half of the animals from each experimental group were killed by an overdose of intraperitoneal barbiturate (Nembutal) and before death 10 ml of blood was removed from the aorta to provide serum for liver enzyme analysis. The animals were dissected and the tissue samples obtained as described in Section 2.2.5. All samples were taken between 10 a.m. and 12.30 p.m.

Thirty days later the remaining animals were similarly sacrificed and dissected to provide the 60 day samples.

In all groups of animals, samples of liver were obtained and liver histology was carried out exactly as described in Section 2.2.9.

4.2.4. Liver Enzyme Assays

Gamma-Glutamyl Transferase, Alkaline Phosphatase, Aspartate Transaminase and Alanine Transaminase were again chosen as the most suitable enzymes and the assays were performed exactly as described in Sections 2.2.6.-2.2.8.

4.3 RESULTS

4.3.1. Animal Weights

As previously (Section 2.3.1.), none of the animals died during the course of this study and all appeared to thrive and to gain weight on their given diet. A graph of the mean weights of the experimental animals is shown in Figure 4.1.

At the start of the study (i.e at the point of pairing), the laboratory chow group had a mean weight (Table 4.2.) of 305.4 g. The liquid diet group had a mean weight (Table 4.3.) of 284.0 g and the two pair-fed groups had mean weights (Table 4.4.) of 288.5 g.

By 30 days, the mean weight of the liquid diet group had risen to 317.3 g. The sucrose control and alcoholic animals had mean weights of 335.5 g and 332.9 g respectively. Due to destruction of the animal house balance, weights were not available for the laboratory chow group at this time. However, the mean weight for this group the previous week was 355.0 g.

Mean weight gains throughout this period were thus 49.6 g for the laboratory chow group (to week 4), 33.3 g for the liquid diet group, 47.0 g for the sucrose control group and 44.4 g for the alcoholic group.

By 60 days, the mean weight of the laboratory chow animals had risen to 414.3 g and that of the liquid diet group to 381.4 g. The sucrose

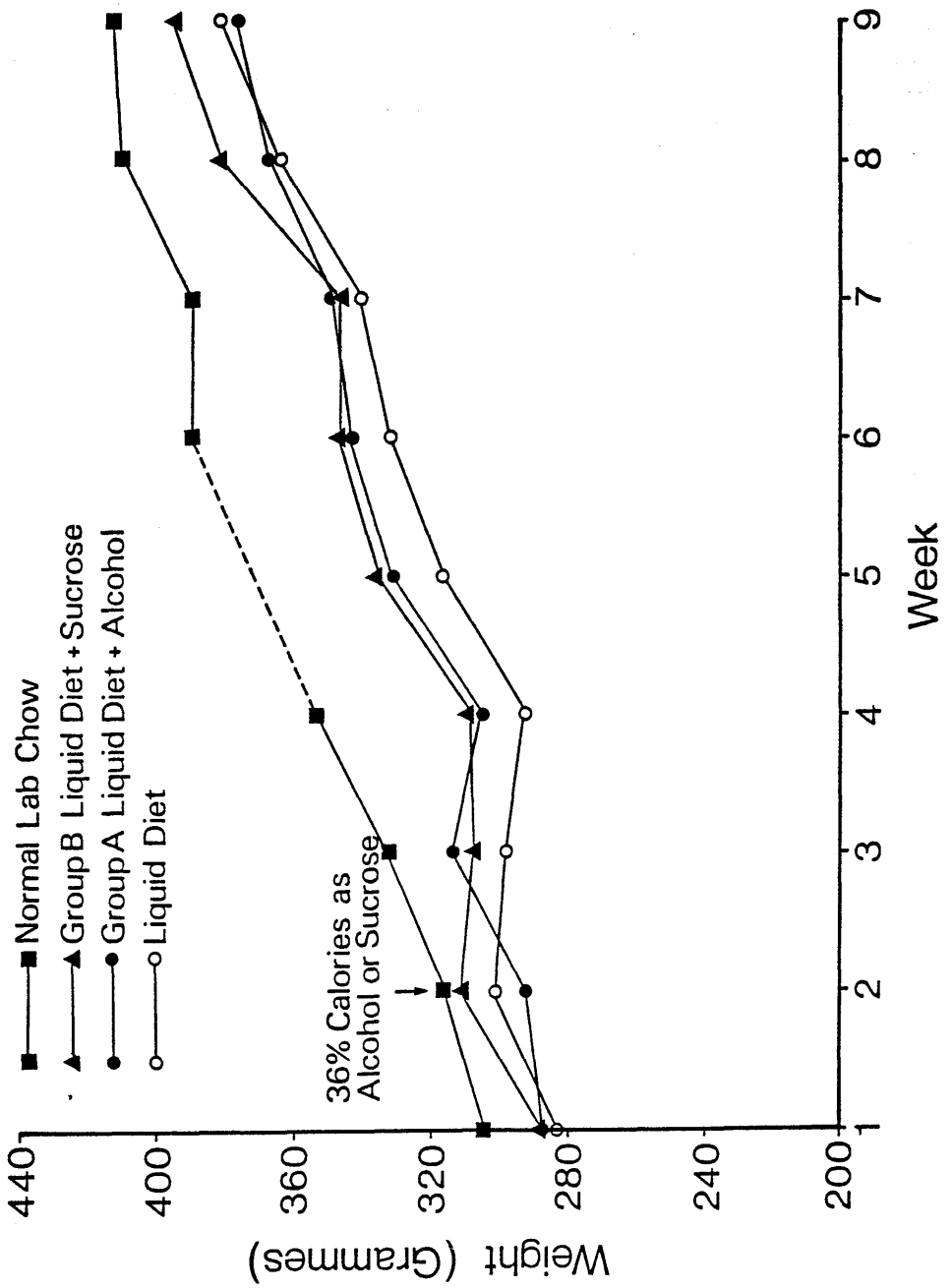


FIGURE 4.1: GRAPH OF MEAN WEIGHTS OF THE EXPERIMENTAL GROUPS OVER THE COURSE OF THE 30 AND 60 DAY STUDY.

	WEEK								
ANIMAL	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
32	330	345	360	380	---	---	---	---	---
33	325	335	350	375	---	---	---	---	---
34	315	320	345	365	---	---	---	---	---
35	310	320	335	360	---	---	---	---	---
36	285	300	315	335	---	---	---	---	---
37	300	310	335	355	---	---	---	---	---
38	290	295	305	320	---	---	---	---	---
39	330	345	350	390	---	438	428	470	472
40	295	305	325	340	---	386	380	400	400
41	290	300	330	350	---	392	388	400	400
42	300	315	340	365	---	386	398	430	418
43	310	320	335	350	---	372	376	400	410
44	290	305	315	325	---	362	362	360	375
45	305	320	340	360	---	404	400	420	425
Mean	**305.4**	**316.8**	**334.3**	**355.0**		**391.4**	**390.3**	**411.4**	**414.3**
S.D.	**15.2**	**16.0**	**15.3**	**20.2**		**24.6**	**21.2**	**33.9**	**30.1**

TABLE 4.2: WEIGHT IN GRAMMES OF THE LABORATORY CHOW CONTROL ANIMALS FROM THE 30 AND 60 DAY STUDY.

Weights for week 5 not available due to extensive damage to the animal house balance.

	WEEK								
ANIMAL	1	2	3	4	5	6	7	8	9
17	260	276	282	272	300	---	---	---	---
18	310	326	320	310	350	---	---	---	---
19	240	260	264	268	284	---	---	---	---
20	246	254	270	258	276	---	---	---	---
21	266	278	276	270	310	---	---	---	---
22	316	334	328	326	350	---	---	---	---
23	272	296	296	292	312	---	---	---	---
24	296	360	298	294	310	---	---	---	---
25	286	278	270	284	300	304	322	330	342
26	284	316	312	308	334	342	354	386	392
27	304	318	324	308	328	342	356	396	410
28	320	332	330	322	348	356	366	390	412
29	300	298	300	288	312	318	328	346	372
30	274	294	292	286	312	330	328	358	368
31	286	320	322	300	334	332	330	356	374
Mean	284.0	302.7	298.9	292.4	317.3	332.0	340.6	366.0	381.4
S.D.	24.4	30.1	22.9	20.2	22.9	17.1	17.5	25.0	25.0

TABLE 4.3: WEIGHT IN GRAMMES OF THE LIQUID DIET CONTROL ANIMALS FROM THE 30 AND 60 DAY STUDY.

	WEEK								
ANIMAL	1	2	3	4	5	6	7	8	9
1A	276	290	318	320	340	---	---	---	---
1B	276	312	302	322	352	---	---	---	---
2A	282	290	310	308	332	---	---	---	---
2B	282	294	294	304	332	---	---	---	---
3A	286	292	296	284	310	---	---	---	---
3B	286	294	298	292	318	---	---	---	---
4A	314	326	340	336	370	---	---	---	---
4B	314	340	350	340	376	---	---	---	---
5A	284	300	334	324	346	---	---	---	---
5B	284	300	302	302	336	---	---	---	---
6A	276	264	---	260	290	---	---	---	---
6B	276	292	280	270	300	---	---	---	---
7A	284	286	306	290	320	---	---	---	---
7B	284	306	300	300	324	---	---	---	---
8A	302	306	320	320	350	---	---	---	---
8B	302	340	326	334	342	---	---	---	---
9A	300	282	296	286	318	314	320	332	334
9B	300	302	296	294	326	320	310	338	350
10A	298	300	312	318	340	348	350	374	368
10B	298	324	300	304	342	344	350	386	398
11A	288	300	310	280	318	338	350	370	384
11B	288	312	324	298	320	328	328	366	380
12A	306	300	354	352	380	394	408	434	442
12B	306	340	340	348	370	392	410	440	456
13A	268	272	300	296	310	324	330	356	356
13B	268	286	288	280	318	324	330	376	388
14A	282	282	302	306	324	332	330	344	366
14B	282	302	320	316	346	352	356	388	406
15A	292	306	320	320	350	354	358	370	382
15B	290	324	330	328	346	364	354	386	400
16A	278	292	306	304	328	342	350	370	382
16B	280	310	300	300	320	330	340	370	384
A:									
Mean	**288.5**	**293.0**	**314.9**	**306.5**	**332.9**	**343.2**	**349.5**	**368.8**	**376.8**
S.D.	**12.5**	**14.6**	**16.7**	**23.2**	**23.2**	**24.2**	**27.1**	**30.3**	**31.2**
B:									
Mean	**288.5**	**311.1**	**309.4**	**308.2**	**335.5**	**344.2**	**347.2**	**381.2**	**395.2**
S.D.	**12.4**	**17.8**	**19.8**	**21.5**	**20.0**	**24.5**	**29.7**	**28.8**	**30.0**

TABLE 4.4: WEIGHT IN GRAMMES OF THE ALCOHOLIC (A) AND SUCROSE (B) PAIR-FED ANIMALS FROM THE 30 AND 60 DAY STUDY.

Animal 6A appeared highly irritable at week 3 and thus was not weighed.

control animals now had a mean weight of 395.2 g and the alcoholic animals had a mean weight of 376.8 g.

Weight gains over the 60 days were 108.9 g for the laboratory chow group, 97.4 g for the liquid diet group, 106.7 g for the sucrose control group and 88.3 g for the alcoholic group.

In general, the pair-fed and liquid diet animals maintained themselves at between 80 and 90 percent of the free feeding (laboratory chow group) weight.

4.3.2. Comparison of the Weights

When comparison was made of the weights of the laboratory chow control animals and the liquid diet and sucrose control animals, in general, the laboratory chow animals showed significantly higher (Mann-Whitney, $p<.05$) weights than did the animals from either of these two liquid diet based control groups. By sacrifice, however, this significant difference was no longer apparent between the laboratory chow and sucrose animals.

At week 5, the liquid diet animals showed significantly lower weights (Mann-Whitney, $p<.05$) than the sucrose control animals. However, at no other point in the study were significant differences observed in the weights of the liquid diet and sucrose control animals.

As expected, throughout the study, the animals in the laboratory chow group showed significantly higher (Mann-Whitney, $p<.05$) weights than did the animals in the alcoholic group. There were no significant

differences noted in the weights of the liquid diet control animals and the alcoholic animals at any point in the study.

With the exception of weeks 2 ($p<.002$) and 9 (Wilcoxon, $p<.03$), there were no significant differences in the weights of the two pair-fed groups.

4.3.3. Gamma-Glutamyl Transferase

The results of the GGT assays are shown in Table 4.5.

The laboratory chow animals: The initial mean value from this group was 4.14 IU/L and this level remained reasonably constant over the course of the study, being 3.65 IU/L at 30 days and 3.94 IU/L at 60 days.

The liquid diet animals: The initial mean value for GGT from this group was 4.44 IU/L. There was no significant difference between this level and the level at 30 days (3.82 IU/L), although by 60 days the level was significantly lower (Mann-Whitney, $p<.05$) than the initial level (3.57 IU/L).

The sucrose control animals: The initial mean value from the sucrose control group was 4.64 IU/L and this level remained approximately constant over the three points of the study, with the value at 30 days being 4.11 IU/L and at 60 days being 4.42 IU/L.

The alcoholic animals: The initial mean serum GGT value from the alcoholic animals was 4.57 IU/L. An increase in this value was evident after 30 days (5.24 IU/L), although it was only after 60 days (5.58 IU/L) that this increase became significant (Mann-Whitney, $p<.02$).

Comparison of the values: No significant differences were observed between the sucrose, the liquid diet or the laboratory chow control groups at any point in the study.

GGT values were significantly raised in the alcoholic group compared with the sucrose control group at both 30 ($p<.05$) and 60 ($p<.03$) days. Similarly, the GGT levels of the alcoholic animals were significantly higher than those of the liquid diet group (Mann-Whitney, $p<.01$ and

SERUM SAMPLE

LABORATORY CHOW ANIMALS				LIQUID DIET ANIMALS			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
32	3.67	3.90	---	17	4.04	3.31	---
33	---	3.31	---	18	3.49	3.12	---
34	4.59	3.12	---	19	2.94	3.67	---
35	4.59	3.31	---	20	5.88	5.33	---
36	3.49	3.67	---	21	3.67	3.31	---
37	3.67	4.59	---	22	3.86	3.86	---
38	3.09	3.67	---	23	6.98	3.90	---
				24	4.19	4.04	---
39	5.14	---	3.49	25	5.51	---	3.67
40	3.31	---	3.67	26	---	---	3.67
41	5.88	---	4.59	27	3.67	---	4.04
43	3.49	---	3.49	29	3.49	---	3.31
44	---	---	3.31	30	5.33	---	3.49
45	3.68	---	5.14	31	5.14	---	3.49
MEAN	**4.14**	**3.65**	**3.94**		**4.44**	**3.82**	**3.57**
S.D.	**0.89**	**0.49**	**0.68**		**1.14**	**0.69**	**0.25**

SUCROSE CONTROLS (B)				ALCOHOLIC ANIMALS (A)			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
1	4.04	3.67	---	1	---	5.14	---
2	2.94	3.86	---	2	4.59	5.86	---
3	3.67	5.51	---	3	4.04	7.35	---
4	---	3.67	---	4	4.96	5.33	---
5	9.92	3.67	---	5	3.86	4.26	---
6	3.49	4.04	---	6	9.55	5.88	---
7	4.04	5.14	---	7	---	3.86	---
8	3.31	3.31	---	8	3.67	4.22	---
9	3.86	---	3.67	9	3.49	---	5.14
10	6.98	---	5.51	10	5.69	---	5.51
11	6.54	---	3.49	11	5.88	---	5.88
12	3.86	---	3.67	12	3.86	---	5.00
13	---	---	5.73	13	4.41	---	6.17
14	3.49	---	4.04	14	3.31	---	7.28
15	4.59	---	5.00	15	2.94	---	5.29
16	4.22	---	3.31	16	3.67	---	4.41
MEAN	**4.64**	**4.11**	**4.42**		**4.57**	**5.24**	**5.58**
S.D.	**1.91**	**0.78**	**1.09**		**1.67**	**1.14**	**0.87**

TABLE 4.5: GAMMA-GLUTAMYL TRANSFERASE RESULTS FROM THE 30 AND 60 DAY STUDY (International Units/Litre).

p<.001) and the laboratory chow animals (Mann-Whitney, p<.005 and p<.004) at 30 and 60 days respectively.

4.3.4. Alkaline Phosphatase

The results from the alk. phos. assays are shown in Table 4.6.

The laboratory chow animals: The initial mean alk. phos. value from this group was 253.3 IU/L and this level remained within a narrow range over the course of the study, being 288.4 IU/L by 30 days and 244.3 IU/L by 60 days.

The liquid diet animals: The initial mean value from this group was 289.0 IU/L and this level was significantly reduced (Mann-Whitney, p<.02) to 195.1 IU/L by 30 days and remained at a significantly lower level (Mann-Whitney, p<.05) for the remainder of the study being 219.1 IU/L at 60 days.

The sucrose control animals: The initial mean value for alk. phos. from the sucrose control animals was 321.1 IU/L. This value subsequently fell to 274.4 IU/L by 30 days, but was not significantly lowered (Mann-Whitney, p<.05) until 60 days when the value was 243.9 IU/L.

The alcoholic animals: The initial mean alk. phos. value for the alcoholic animals was 340.4 IU/L. No statistical difference was evident in this level over the first 30 days (349.8 IU/L), although, after 60 days, this level was significantly lowered (Mann-Whitney, p<.05) to 272.1 IU/L.

Comparison of the values: Comparison of the values from the three control groups showed them to be broadly similar. An exception to this was the value for the liquid diet group at 30 days which was significantly lower than the values for the sucrose control (Mann-

SERUM SAMPLE

LABORATORY CHOW ANIMALS				LIQUID DIET ANIMALS			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
32	---	214	---	17	343	277	---
33	---	254	---	18	353	143	---
34	300	214	---	19	260	221	---
35	297	277	---	20	229	198	---
36	297	391	---	21	240	143	---
37	280	376	---	22	206	128	---
38	248	293	---	23	349	224	---
				24	290	227	---
39	198	---	160	25	191	---	214
40	353	---	343	26	---	---	209
41	244	---	268	27	356	---	235
42	208	---	216	28	369	---	242
43	250	---	249	29	320	---	201
44	138	---	224	30	316	---	283
45	227	---	250	31	224	---	150
Mean	253.3	288.4	244.3		289.0	195.1	219.1
S.D.	57.1	71.4	55.8		62.5	52.3	41.0

SUCROSE CONTROLS (B)				ALCOHOLIC ANIMALS (A)			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
1	356	353	---	1	---	435	---
2	402	152	---	2	415	349	---
3	412	273	---	3	313	389	---
4	214	288	---	4	338	330	---
5	372	260	---	5	270	316	---
6	237	372	---	6	316	254	---
7	250	240	---	7	---	356	---
8	349	257	---	8	270	369	---
9	280	---	250	9	379	---	303
10	336	---	278	10	438	---	273
11	392	---	272	11	231	---	247
12	338	---	186	12	333	---	277
13	227	---	242	13	369	---	244
14	247	---	247	14	478	---	336
15	379	---	254	15	282	---	237
16	346	---	222	16	333	---	260
Mean	321.1	274.4	243.9		340.4	349.8	272.1
S.D.	67.6	68.2	29.1		69.7	53.3	33.5

TABLE 4.6: ALKALINE PHOSPHATASE RESULTS FROM THE 30 AND 60 DAY STUDY (International Units/Litre).

Whitney, p<.02) and the laboratory chow (Mann-Whitney, p<.04) groups at this time. Also, the laboratory chow values at the start of the study were significantly lower than those of the sucrose control group (Mann-Whitney, p<.05). There were no further significant differences in the alk. phos. levels from any of the three control groups.

The alk. phos. values from the alcoholic animals were significantly (Mann-Whitney, p<.02) higher than those from the laboratory chow group at the start of the study, although there were no further significant differences between these two groups. The values for the alcoholic animals were significantly higher than those from the liquid diet animals at both 30 (Mann-Whitney, p<.001) and 60 days (Mann-Whitney, p<.02).

Athough the alk. phos. values were consistently higher in the alcoholic group than in the sucrose control group, this difference did not reach the level of significance at any point in this study.

4.3.5. Aspartate Transaminase

The results from the serum AST assays are shown in Table 4.7.

The laboratory chow animals: The initial mean AST value in this group was 135.6 IU/L. This level was significantly reduced (Mann-Whitney, p<.02) by 30 days (82.0IU/L) and although it increased to 106.7 IU/L by 60 days, this value was still significantly lower (Mann-Whitney, p=.05) than the initial value.

The liquid diet animals: The initial mean value from this group was 154.0 IU/L. By 30 days this value was significantly reduced (Mann-Whitney, p<.002) to 47.8 IU/L and despite a subsequent rise to

SERUM SAMPLE

LABORATORY CHOW ANIMALS				LIQUID DIET ANIMALS			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
32	158	67	---	17	269	63	---
33	86	73	---	18	140	44	---
34	166	151	---	19	169	59	---
35	162	77	---	20	177	46	---
36	123	63	---	21	127	48	---
37	86	76	---	22	197	40	---
38	121	67	---	23	89	34	---
				24	108	48	---
39	137	---	90	25	121	---	105
40	128	---	118	26	175	---	73
41	146	---	122	27	175	---	105
42	122	---	90	28	130	---	65
43	154	---	154	29	83	---	60
44	---	---	89	30	125	---	57
45	174	---	84	31	225	---	89
MEAN	135.6	82.0	106.7		154.0	47.8	79.1
S.D.	28.3	30.9	25.8		51.1	9.5	20.5

SUCROSE CONTROLS (B)				ALCOHOLIC ANIMALS (A)			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
1	138	32	---	1	137	65	---
2	180	55	---	2	178	40	---
3	119	56	---	3	166	48	---
4	118	47	---	4	77	40	---
5	177	52	---	5	159	45	---
6	123	40	---	6	191	31	---
7	169	51	---	7	---	49	---
8	208	40	---	8	164	41	---
9	84	---	46	9	269	---	56
10	142	---	58	10	154	---	61
11	78	---	44	11	187	---	55
12	121	---	49	12	136	---	75
13	---	---	63	13	128	---	36
14	136	---	41	14	115	---	40
15	154	---	35	15	105	---	48
16	186	---	33	16	167	---	36
MEAN	142.2	46.6	46.1		155.5	44.9	50.9
S.D.	37.1	8.5	10.4		44.6	9.9	13.6

TABLE 4.7: ASPARTATE TRANSAMINASE RESULTS FROM THE 30 AND 60 DAY STUDY (International Units/litre).

79.1 IU/L, the level was still significantly reduced at 60 days (Mann-Whitney, $p<.002$) when compared to the initial value.

The sucrose control animals: The initial mean value from the sucrose control group was 142.2 IU/L. Again, this value was significantly reduced (Mann-Whitney, $p<.002$) by 30 days (46.6 IU/L), and remained significantly reduced (Mann-Whitney, $p<.002$) over the next 30 days (46.1 IU/L).

The alcoholic animals: The initial mean AST value for the alcoholic animals was 155.5 IU/L. A significant reduction (Mann-Whitney, $p<.002$) in this value was evident after 30 days (44.9 IU/L) and the value remained significantly reduced (Mann-Whitney, $p<.002$) at 60 days (50.9 IU/L).

Comparison of the values: The enzyme levels for the laboratory chow animals were significantly higher than those from the sucrose control animals at both the 30 (Mann-Whitney, $p<.001$) and 60 day (Mann-Whitney, $p<.001$) measuring times and were higher than those from the liquid diet animals at the 30 day point (Mann-Whitney, $p<.01$) although not at the 60 day point.

At the 60 day point, the liquid diet animals had significantly higher AST values (Mann-Whitney, $p<.003$) than the sucrose control animals although no significant differences were noted between these two groups at any other point in the study.

The AST levels from the laboratory chow group were significantly higher than those from the alcoholic group at both the 30 (Mann-Whitney, $p<.001$) and 60 (Mann-Whitney, $p<.001$) day measuring times. The only significant difference between the alcoholic and the liquid

diet groups was at the 60 day point when the liquid diet animals showed significantly (Mann-Whitney, $p<.02$) higher values.

No significant differences were observed at any of the three measuring times between the enzyme levels in the alcoholic animals and those in the sucrose control animals.

4.3.6. Alanine Transaminase

The results from the ALT assays are shown in Table 4.8.

The laboratory chow animals: The initial mean ALT value from this group was 23.5 IU/L and this value rose significantly (Mann-Whitney, $p<.02$) to 28.0 IU/L by 30 days and to 46.7 IU/L by 60 days (Mann-Whitney, $p<.002$).

The liquid diet animals: The initial mean value from this group was 14.5 IU/L and this value was significantly (Mann-Whitney, $p<.002$) lowered to 7.6 IU/L by 30 days. Over the next 30 days this value rose again to 10.1 IU/L although was still significantly lower than the initial value (Mann-Whitney, $p<.02$).

The sucrose control animals: The initial mean value from this group was 13.9 IU/L and was subsequently lowered to 8.4 IU/L by 30 days and to 6.5 IU/L by 60 days. Both reductions were significant with respect to the initial value (Mann-Whitney, $p<.002$).

The alcoholic animals: The initial mean serum ALT value from the alcoholic animals was 13.8 IU/L. This value was decreased significantly by 30 days (Mann-Whitney, $p<.002$) to 6.4 IU/L although it subsequently rose to 10.9 IU/L, a value that was not significantly lower than the initial value.

Comparison of the values: The enzyme levels in the laboratory

SERUM SAMPLE

LABORATORY CHOW ANIMALS				LIQUID DIET ANIMALS			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
32	23	32	---	17	15	9	---
33	--	34	---	18	20	7	---
34	19	25	---	19	16	9	---
35	23	24	---	20	17	6	---
36	24	28	---	21	15	11	---
37	16	25	---	22	13	6	---
38	22	28	---	23	11	7	---
				24	10	6	---
39	24	---	42	25	4	---	12
40	23	---	52	26	--	---	11
41	29	---	77	27	18	---	10
42	24	---	37	28	18	---	7
43	26	---	43	29	13	---	9
44	25	---	36	30	18	---	11
45	28	---	40	31	15	---	11
MEAN	23.5	28.0	46.7		14.5	7.6	10.1
S.D.	3.4	3.8	14.4		4.2	1.8	1.7

SUCROSE CONTROLS (B)				ALCOHOLIC ANIMALS (A)			
ANIMAL	START	30 DAYS	60 DAYS	ANIMAL	START	30 DAYS	60 DAYS
1	11	7	---	1	16	6	---
2	16	9	---	2	20	8	---
3	18	12	---	3	16	7	---
4	16	6	---	4	12	3	---
5	17	9	---	5	14	6	---
6	11	9	---	6	18	8	---
7	13	5	---	7	7	6	---
8	17	10	---	8	14	7	---
9	13	---	6	9	9	---	6
10	13	---	7	10	12	---	10
11	13	---	5	11	16	---	8
12	16	---	5	12	12	---	12
13	6	---	5	13	11	---	12
14	14	---	7	14	17	---	8
15	17	---	10	15	15	---	13
16	11	---	7	16	12	---	18
MEAN	13.9	8.4	6.5		13.8	6.4	10.9
S.D.	3.2	2.3	1.7		3.4	1.6	3.8

TABLE 4.8: ALANINE TRANSAMINASE RESULTS FROM THE 30 AND 60 DAY STUDY (International Units/Litre).

chow group were significantly higher at all points in the study than the levels in the alcoholic, the sucrose control or the liquid diet groups (Mann-Whitney, $p<.002$).

The only significant difference between the ALT levels in the liquid diet and sucrose control groups was at 60 days, when the liquid diet animals showed significantly higher enzyme levels than the sucrose control animals (Mann-Whitney, $p<.005$).

No significant differences were observed in the enzyme levels from the alcoholic and liquid diet animals at any point in the study.

No consistent pattern emerged in the comparison of the enzyme levels from the alcoholic and the sucrose control animals. At 30 days, the sucrose control animals showed significantly higher enzyme levels than the alcoholic animals (Wilcoxon, $p<.05$) whereas, at 60 days, the reverse was observed ($p<.01$).

4.3.7. Liver Histology

Histological examination of the liver sections of the experimental animals, showed varying degrees of minor liver damage and fat accumulation. These changes were most pronounced in the livers of the alcoholic animals (Figure 4.2), where swollen hepatocytes and some centrilobular fat accumulation was noted. This fat deposition, was confirmed by Oil Red 'O' staining (Figure 4.3), although this was much less than that noted in the 102 day study (Figure 2.3).

Although not present in every animal, some fat deposition was apparent in the livers of occasional alcoholic animals at 30 days (Figure 4.4).

As noted in the 102 day study, a minor increase was found in the quantity of fat present in the liver samples taken from the sucrose control animals. Similarly, the livers of the liquid diet animals also showed a slight increase in fat content. This did not appear to have a particular anatomical distribution in either case.

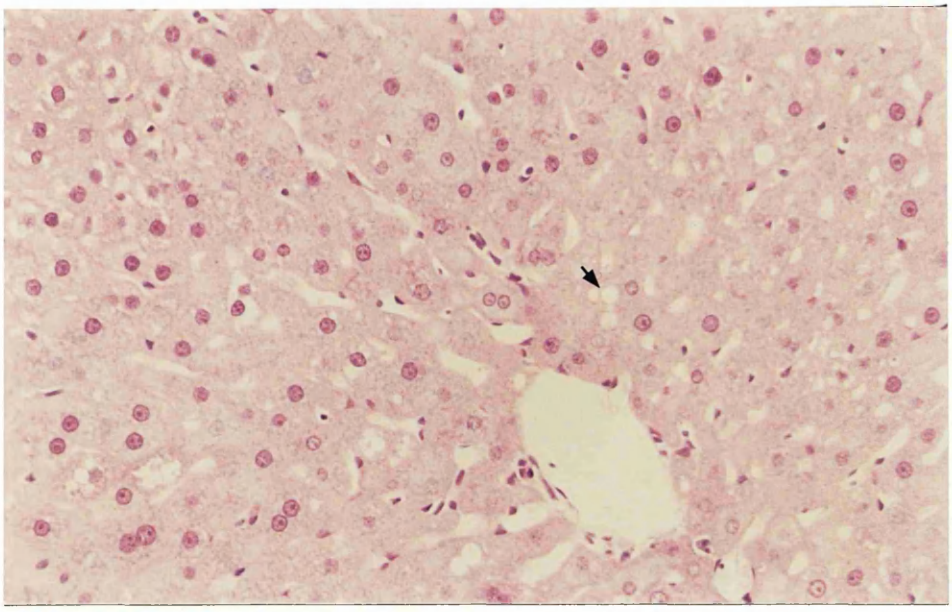


FIGURE 4.2: HEAMATOXYLIN AND EOSIN STAINED LIVER SECTION (x250) OF AN ALCOHOLIC ANIMAL AT 60 DAYS SHOWING SOME DEGREE OF HEPATOCYTE SWELLING AND OCCASSIONAL FAT DROPLETS (↟). INFLAMATORY CELLS WERE RARELY NOTED.

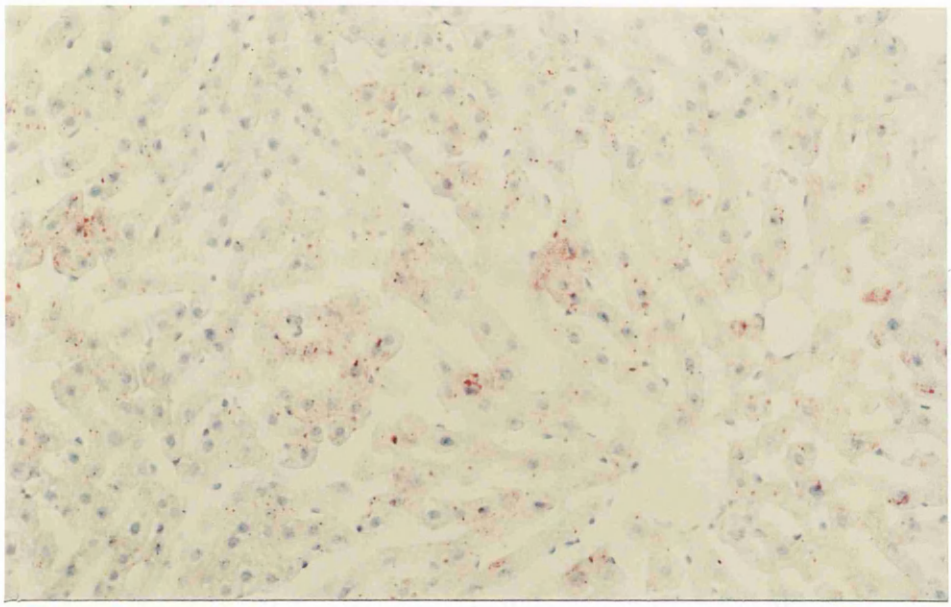


FIGURE 4.3: OIL RED 'O' STAINED LIVER SECTION (x250) FROM AN ALCOHOLIC ANIMAL AT 60 DAYS SHOWING FAT ACCUMULATION.

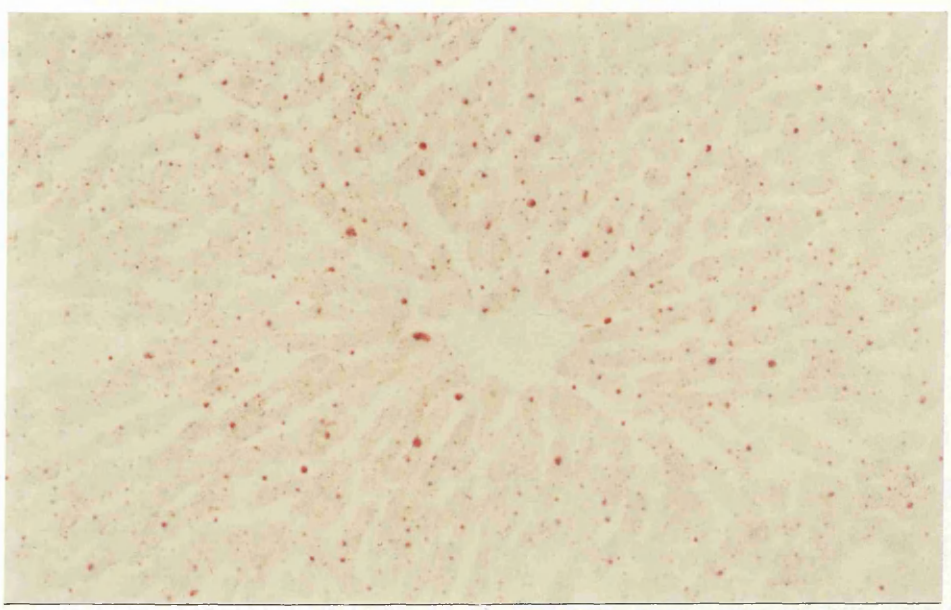


FIGURE 4.4: OIL RED 'O' STAINED LIVER SECTION (x160) FROM AN ALCOHOLIC ANIMAL AT 30 DAYS SHOWING FAT ACCUMULATION WITH A CENTRILOBULAR DISTRIBUTION.

4.4 DISCUSSION

4.4.1. General Observations

As previously, the animals tolerated the experimental procedure well and no mortality was observed during the study. With the exception of some irritability during handling, none of the animals showed external signs of ill health.

4.4.2. Dietary Consumption

The animals were weaned onto the 36 percent ethanol diet as described previously (Section 2.2.2.) although the average initial consumption of this diet was not as low as it was for the 102 day study (82.6 ml as compared to 66.7 ml). There was no obvious explanation for this difference but perhaps minor alterations in dietary composition could have accounted for it.

The mean dietary consumption in this study varied between 80 and 90 ml per day. This volume is similar to that observed for the dietary consumption measured in the 102 day study (Section 2.3.1.).

4.4.3. The Animal Weights

Throughout this second study all the animals gained weight, and the liquid diet and pair-fed animals maintained themselves at c.80-90 percent of the free feeding weight (i.e. that of the laboratory chow group). Again, close calorific control was observed between the matched pairs. At the time of sacrifice, the Wilcoxon test revealed significant differences in the weights of the pair-fed animals although the Mann-Whitney test did not show any significant difference

between the two groups. As mentioned in Chapter 2, the minor weight differences observed between the two pair-fed groups were within the limits of acceptability for a pair-feeding model and did not detract from its value.

The weight gains of each of the experimental groups were significantly (Mann-Whitney, $p<.002$) lower over the first 30 days than weight gains for similar groups over the same period in the previous study. A possible reason for this is that the animals in this second study were housed in a different, slightly colder animal house and the increased metabolism required to maintain body temperature in these animals may have accounted for their slow weight gain. Over 60 days the weight gains for the two pair-fed groups were similar to those of the equivalent groups in the 102 day study but the weight gains of the laboratory chow animals were still significantly less (Mann-Whitney, $p<.002$). The fact that a different laboratory chow was used in this second study may explain this effect.

A number of other factors may also explain the minor weight differences observed between the animals in this study and those in the 102 day study. For example, the animal house used in this second study was being re-decorated during the course of the study. Paint fumes and the continual noise of the workmen may have distressed the animals resulting in a loss of appetite. On top of this, the animal house was considerably busier than that used for the 102 day study and the animals were handled (when required) by a different animal technician.

4.4.4. Gamma-Glutamyl Transferase

As in the 102 day study, GGT appeared to be a good indicator of alcohol related liver damage and showed significantly increased levels in the alcoholic animals compared with any of the three control groups. No obvious effect of the liquid diet was noticed on this enzyme.

The values obtained were lower than those from the 102 day study and the rise in serum GGT as a result of alcohol ingestion was less pronounced. Minor differences in the assay solutions or experimental conditions (which should have been minimal as the QC values did not differ), may have accounted for these differences in the enzyme levels.

Nevertheless, it appears that GGT is a very useful and sensitive indicator of chronic alcohol consumption in the type of animal model reported in this thesis. The results proved that the alcoholic animals consumed alcohol in pharmacologically significant amounts.

4.4.5. Alkaline Phosphatase

Results here were similar to those from the 102 day study with the exception of the initial laboratory chow values which were low. The alcoholic animals again showed consistently higher values than the sucrose control animals although these differences did not reach levels of significance at any point in the study. It is possible therefore, that alcohol had an effect on the serum alk. phos. values, although either the levels or the duration of the ethanol consumption in this study may have been insufficient to significantly raise the

alk. phos. values. It would be of interest to attempt a longer period of alcohol ingestion or to allow the animals access to larger volumes of the alcoholic diet.

It is unfortunate that a number of studies showing raised alk. phos. values in alcoholic rats are reports of single, early measurements and not values taken over a time course (Nishimura & Teshke, 1982; Teshke et al., 1983). It is possible, as in the present study where raised alk. phos. values were demonstrated early in the 102 day and the 30 and 60 day studies, that these early raised values resulted more from an initial liver reaction to ethanol than to any consistent and significant alcohol related increase in the serum alk. phos. values.

In this study, there was no consistent evidence of the liquid diet effect suggested for the 102 day study. On the available evidence, given the lack of consistency in the effect reported for the 102 day study, it seems that the liquid diet had no discernible significant effect on the alk. phos. values.

In each of the groups maintained on the liquid based diets, a time dependent decrease in the alk. phos. levels was noticed. In the laboratory chow group, no such reduction was seen, probably because of the very low initial values from this group rather than because of an actual absence of such an effect. On balance, given the results from this study and the 102 day study, it appears that this temporal reduction is normal for alk. phos. in the rat (Boehringer Mannheim Technical Department, Written Communication, 1984).

In conclusion, it appears that alk. phos. levels were affected to a limited extent by alcohol. The results from the 102 day study suggest that prolonged exposure to ethanol results in more statistically significant increases in the serum alk. phos. values in the alcoholic animals.

This enzyme therefore appears to be of limited use in following chronic ethanol consumption in animal studies such as those presented in this thesis, and was not as sensitive as GGT in this respect.

4.4.6. Aspartate Transaminase

At the 60 day point, the AST levels in the liquid diet group were significantly higher than those in either the alcoholic or sucrose control groups (Mann-Whitney, $p<.003$). No significant differences were observed between the alcoholic and sucrose control groups or between either of these groups and the liquid diet control group at any other point in the study. This was consistent with the literature and confirmed the lack of any non alcohol related liver disease that may have resulted in raised AST values.

As previously, significantly lower enzyme levels were observed in the three groups maintained on liquid based diets, although this time the liquid diet effect was not obvious until the first kill. It is perhaps worth mentioning that whilst in the 102 day study the reduction in the levels in the animals on the liquid based diets was gradual, in the present study the AST values tended initially to fall sharply and subsequently to remain at approximately the same low value.

There was some evidence of a temporal reduction in the laboratory chow animals, with the AST values at 30 and 60 days being significantly lower than the initial values. The results from the 102 day study would, however, argue against such a reduction being a general phenomenon in the laboratory chow animals. The increase noted in the present study between 30 and 60 days would also argue against such an effect.

It is assumed, on the basis of the available evidence, that the temporal reduction is not a normal phenomenon but was in some way related to the consumption of the liquid diet.

The initial ALT levels reported for this 30 and 60 day study were slightly higher and the subsequent values slightly lower than those from the 102 day study. There are no obvious explanations for this finding additional to those postulated for GGT.

4.4.7. Alanine Transaminase

Reported ALT levels for this 30 and 60 day study were similar to those from the 102 day study.

As in the 102 day study, a very rapid reduction in the ALT values from animals maintained on liquid based diets was observed. This decrease resulted from only one week of liquid diet consumption and persisted throughout the study.

Again, this 30 and 60 day study failed to substantiate the reported increase in rat serum ALT levels following chronic alcohol consumption

(Nishimura & Teshke, 1982; Teshke et al, 1983). This was possibly a consequence of the strong effect of the liquid diet masking any alcohol related effect in the present study.

The lack of consistently raised ALT values from any of the liquid diet groups appeared to confirm that no non alcohol related liver disease was present in any of these groups. It should, however, be emphasised that the very low ALT levels in these animals make definite conclusions hard to draw in this respect.

Again, as in the 102 day study (Section 2.3.8.), the ALT values in the laboratory chow group were significantly raised during the course of the study.

4.4.8. Discussion of the Effect of Liquid Diet on the Transaminase Values

There are a number of possible explanations for the observed reduction in the levels of the transaminase enzymes in animals maintained on the liquid based diets.

Comparison of the composition of the laboratory chow and liquid diet (Figure 2.1) revealed the liquid diet to be richer in carbohydrates than the laboratory chow diet. Both Krebbs (1972) and Greengard and Dewey (1973) demonstrated that the feeding of glucose solutions to rats resulted in a very rapid (19 hours) reduction of liver amino acid metabolising enzymes including AST and ALT. Greengard and Dewey (1973) suggested that, with glucose as the major nutrient, the main function of the amino acid catabolising enzymes (i.e. provision of precursors for gluconeogenesis) is redundant and the decreased levels of these enzymes e.g. AST and ALT prevent the wastage of body nitrogen. It is thus conceivable that this glucose effect represents a more general carbohydrate effect in which high carbohydrate diets will lead to a decrease in the levels of the amino acid catabolising enzymes. This could account for the observed rapid reduction in the transaminase values in the present study.

It might be expected that, if this was the case, the high sucrose content of the sucrose control diet would have further reduced the levels of the transaminases. This, however, was not observed, probably because the liquid diet itself was sufficiently high in carbohydrates to suppress the transaminases to a threshold level below which they would not fall.

Interestingly, Greengard and Dewey (1973) pointed out that under conditions of carbohydrate repression, the amino acid catabolising enzymes show diminished responses to various subsequently administered inducers. This may account for the lack of an ethanol effect on the serum ALT levels and may also cast some doubt on the validity of serum AST as a marker of non alcohol related liver damage in the animals maintained on the liquid based control diets.

4.4.9. Liver Histology

As in the previous study, centrilobular fatty deposition was observed in the liver sections from the alcoholic animals although it was not as marked as at 102 days. Again, it can be concluded that the ethanol was being consumed in pharmacologically active amounts, leading to the development of pathological complications of the type commonly observed in human alcoholics.

4.5 CONCLUSIONS FROM THE 30 AND 60 DAY STUDY

In conclusion, the pair-feeding procedure has been successfully carried out and has been associated with a zero mortality rate and close calorific control.

The enzyme assays performed demonstrated the effectiveness of GGT, and possibly alk. phos. as indicators of alcohol related liver damage in rats maintained on the pair feeding model described in this thesis.

The results, along with those of the liver histology, clearly demonstrated (in both the 102 day, and the 30 and 60 day, studies) a degree of liver damage proceeding as far as fatty liver, in the alcoholic animals. Thus the model presented in this thesis was effective as a model of chronic alcohol consumption and the results of the histological and biochemical analysis of liver function revealed that the ethanol was being consumed in pharmacologically active amounts.

It is clear, from the results presented in this thesis, that animal studies of liver function must use diets with carefully controlled composition as diets, high (or low) in carbohydrates, can have a marked effect on hepatic function.

CHAPTER FIVE - THE PROTEIN STUDIES: 30 AND 60 DAYS

5.1 INTRODUCTION

This study was designed to investigate any temporal alterations in the levels of the three aberrant proteins observed in the 102 day study. As discussed in Section 4.1, the animals in this study were killed at two time points representing approximately one- and two-thirds of the original 102 days (i.e. 30 and 60 days).

The rationale behind this approach was to investigate any alteration in the levels of these three proteins over the two time points of the study, and to assess whether or not the alterations were a consequence of chronic alcohol consumption. If the protein alterations, after 30 days, were of similar magnitude to those noted after 102 days, then it could be concluded that the alterations were the result either of short term chronic or even acute consumption and further studies would therefore be required to define the duration of consumption needed to induce the protein changes.

One further advantage of this study was that, if it was indeed possible to follow alterations in the levels of these three proteins over time points of the original 102 days, then it should be possible to study the relative alterations in the high MW protein and the two lower MW proteins with a view to either confirming or refuting a possible precursor/product relationship between them (Section 3.4.3.).

5.2 MATERIALS AND METHODS

Details of the animal housing, feeding and dietary composition are reported in Chapter 4. The only difference between this study and the 102 day study was the inclusion of an additional control group. This group of animals was maintained on liquid diet alone and was included to attempt to elucidate the mechanisms underlying the unusual liver enzyme results discussed in Chapter 2.

As before, the lingual epithelium was prepared for SDS PAGE and the preparative and electrophoretic methods were exactly as described in Section 3.2. Only small pieces of anterior tongue were prepared for histological analysis and thus the larger portion of tongue was available for preparation for electrophoresis.

No attempts were made at 2-dimensional gel electrophoresis in this study and the lingual epithelium of each of the animals was prepared for 1-dimensional SDS PAGE.

5.3 RESULTS

The numerical results quoted refer to the area percentage values measured by the laser densitometer as discussed in Section 3.2.8.

5.3.1. 30 days: High Molecular Weight Protein

Photographs of the gels of the lingual epithelial proteins at 30 days are shown in Figures 5.1, 5.2, 5.3 and 5.4.

Visual analysis of Figures 5.1 and 5.2 revealed apparently normal levels of the high MW protein in each of the laboratory chow and liquid diet animals. Gross visual analysis of Figures 5.3 and 5.4 showed that the previously reported (Section 3.3.3.) reduction in the levels of the high MW protein in the alcoholic animals, relative to those in the sucrose control animals, was only noted in a few of the alcoholic animals (e.g. numbers 1, 2,and 8).

Densitometric analysis (Table 5.1) revealed the mean area percentage levels of the high MW protein to be 1.57 for the laboratory chow animals and 1.66 for the liquid diet animals. The values for the sucrose control and alcoholic animals were 1.90 and 1.30 respectively.

There were no significant differences in the levels of this protein in any of the three control groups (i.e. laboratory chow, liquid diet and sucrose) or between the alcoholic group and any of these control groups. There were no significant differences in the levels of this high MW protein in the laboratory chow, sucrose control or alcoholic animals, at 30 and 102 days.

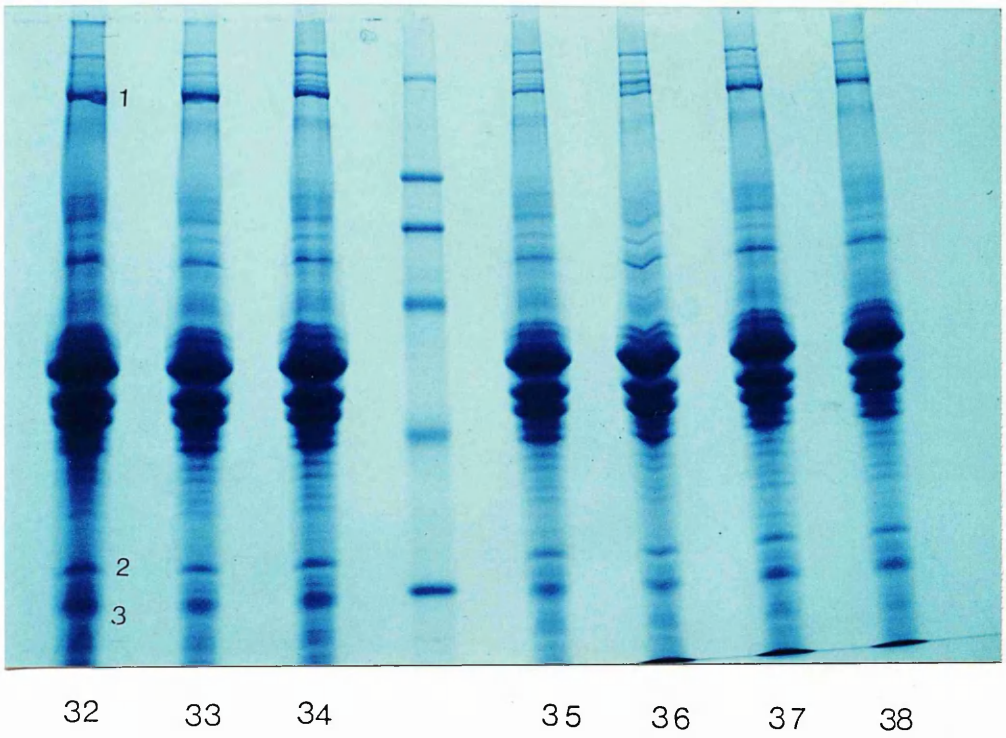


FIGURE 5.1: RESULTS FROM THE PROTEIN GEL OF THE LABORATORY CHOW CONTROL ANIMALS AT 30 DAYS.

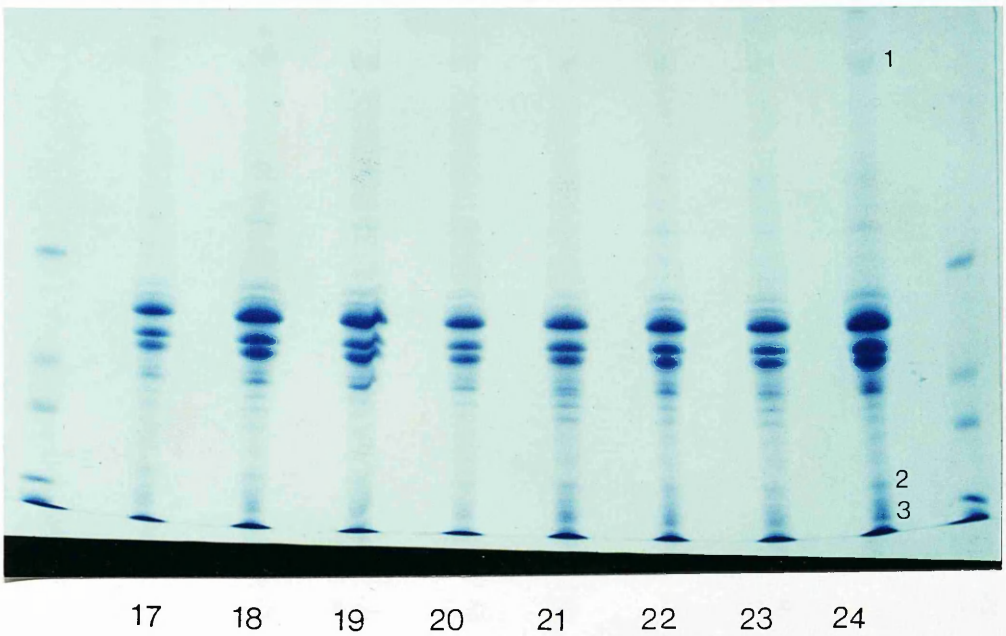


FIGURE 5.2: RESULTS FROM THE PROTEIN GEL OF THE LIQUID DIET CONTROL ANIMALS AT 30 DAYS.

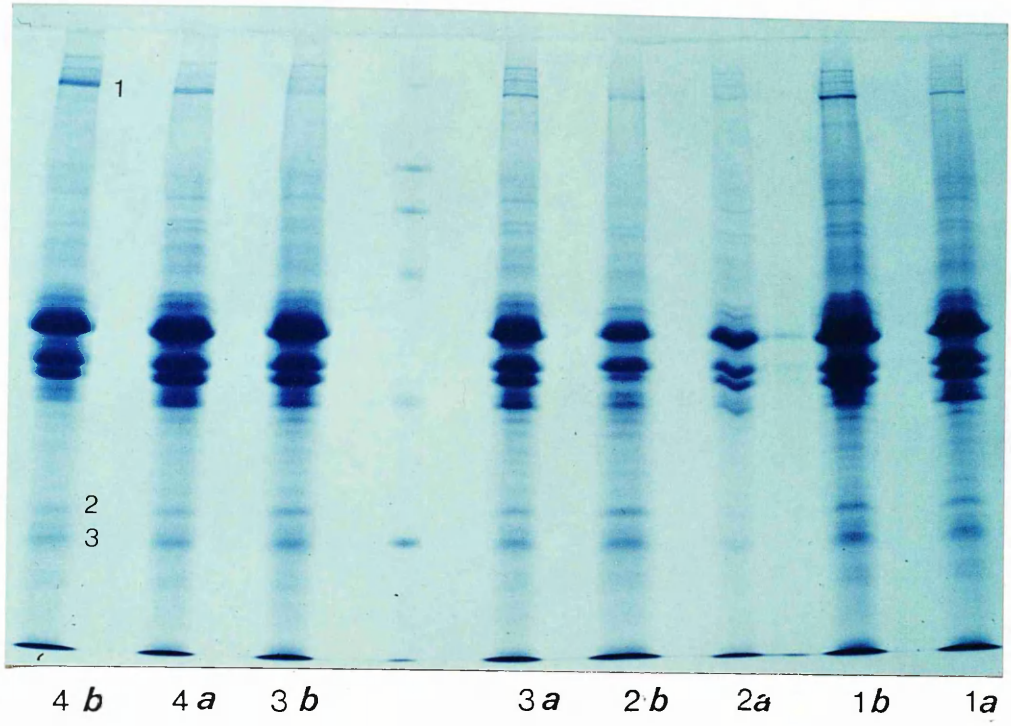


FIGURE 5.3: RESULTS FROM THE PROTEIN GEL OF THE PAIR-FED ANIMALS, NUMBERS 1-4, AT 30 DAYS.

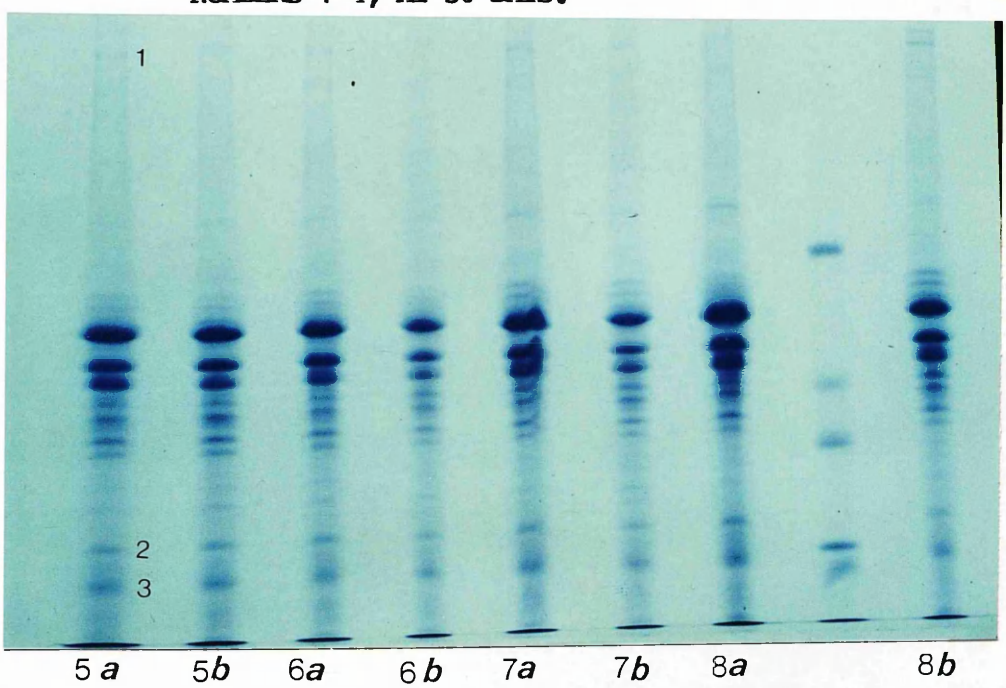


FIGURE 5.4: RESULTS FROM THE PROTEIN GELS OF THE PAIR-FED ANIMALS, NUMBERS 5-8, AT 30 DAYS.

LABORATORY CHOW ANIMALS

ANIMAL	HIGH MW	30K	28K
32	1.7	2.1	5.0
33	0.8	2.5	4.8
34	0.8	2.0	4.3
35	3.0	1.0	2.6
36	2.1	2.1	4.9
37	1.1	1.7	2.3
38	1.5	1.1	3.0
MEAN	1.57	1.78	3.84
S.D.	0.79	0.56	1.17

LIQUID DIET ANIMALS

ANIMAL	HIGH MW	30K	28K
17	2.2	3.4	4.3
18	1.1	1.2	2.2
19	1.3	2.3	3.1
20	1.2	1.9	4.8
21	2.7	4.5	8.1
22	2.5	5.5	8.5
23	0.6	3.8	6.4
24	1.7	3.3	4.0
	1.66	3.24	5.18
	0.74	1.41	2.29

THE ALCOHOLIC (A) AND SUCROSE (B) PAIR-FED ANIMALS

	HIGH MW		30K		28K	
ANIMAL	A	B	A	B	A	B
1	---	1.5	3.0	5.4	5.8	4.7
2	2.6	3.1	8.0	4.1	8.7	3.7
3	1.2	1.8	1.8	1.3	4.2	2.8
4	0.6	2.1	1.8	1.7	4.0	4.0
5	1.6	1.4	1.8	2.1	8.0	4.4
6	0.7	1.0	2.2	1.6	4.9	3.7
7	1.1	1.0	2.4	1.8	3.2	3.6
8	1.3	3.5	8.2	2.1	3.5	5.8
MEAN	1.30	1.90	3.65	2.51	5.29	4.09
S.D.	0.67	0.90	2.78	1.45	2.06	4.09

TABLE 5.1: AREA PERCENTAGE VALUES FOR THE THREE ABERRANT PROTEINS AT 30 DAYS.

Missing Values Are Due To Poorly Resolved Densitometer Scans.

5.3.2. 30 Days:30K Protein

Visual analysis of Figures 5.1-5.4 failed to reveal any obvious differences in the levels of the 30K protein in the animals from any of the four groups.

Densitometric analysis (Table 5.1) revealed the mean area percentage levels of this protein to be 1.78 for the laboratory chow animals and 3.24 for the liquid diet group. The mean values for the sucrose control and alcoholic animals were 2.51 and 3.65 respectively.

Statistical analysis revealed the values from the liquid diet group to be significantly higher (Mann-Whitney, $p<.05$) than those from the laboratory chow group. No further significant differences were noted between the three control groups. There were no significant differences between the alcoholic group and any of the three control groups.

The levels of the 30K protein in the laboratory chow group at 30 days, were significantly lower (Mann-Whitney, $p<.02$) than the levels for the equivalent group in the 102 day study. There were no significant differences in the levels of this protein in the sucrose control or alcoholic animals at 30 and 102 days.

5.3.3. 30 Days: 28K Protein

Visual analysis of Figures 5.1-5.4 failed to reveal any obvious differences in the levels of the 28K protein in the animals from any of the four groups.

Densitometric analysis (Table 5.1) revealed the mean values for the area percentage for the 28K protein to be 3.84 for the laboratory chow group and 5.18 for the liquid diet group. The mean levels for the sucrose control and alcoholic groups were 4.09 and 5.29 respectively.

No significant differences were noted between the levels of this 28K protein in any of the three control groups, nor between the alcoholic group and any of the control groups.

There were no significant differences in the levels of this protein in the laboratory chow, sucrose control or alcoholic groups, at 30 and 102 days.

5.3.4. Other Protein Differences

No consistent differences were observed in the levels of any other proteins in the gels by either visual or densitometric analysis.

5.3.5. 60 days:High Molecular Weight Protein

Photographs of the gels of epithelial proteins from the 60 day kill are shown in Figures 5.5, 5.6, 5.7 and 5.8.

Visual analysis of Figures 5.5 and 5.6 revealed apparently normal levels of the high MW protein in each of the laboratory chow and liquid diet animals. Gross visual analysis of Figures 5.7 and 5.8 again revealed that the reduction in the levels of the high MW protein, observed at 102 days, was only noted in a few of the alcoholic animals when compared with the sucrose controls (e.g. numbers 13 and 15).

By 60 days, densitometric analysis (Table 5.2) revealed the mean area percentage value for the high MW protein from the laboratory chow group to be 1.71 and that from the liquid diet group to be 1.20. The mean values from the sucrose control and alcoholic animals were 1.61 and 0.48 respectively.

No significant differences were found between the levels of this protein in any of the three control groups at this time. The levels in the alcoholic animals, however, were seen to be significantly lower than the laboratory chow group (Mann-Whitney, $p<.002$), the liquid diet group (Mann-Whitney, $p<.004$) and the sucrose control group ($p<.05$).

The values reported for the alcoholic animals were significantly lower than those for the same group at the 30 day kill (Mann-Whitney, $p<.0.03$). There were no significant differences in the levels of this protein in the three control groups at 30 and 60 days, nor between the

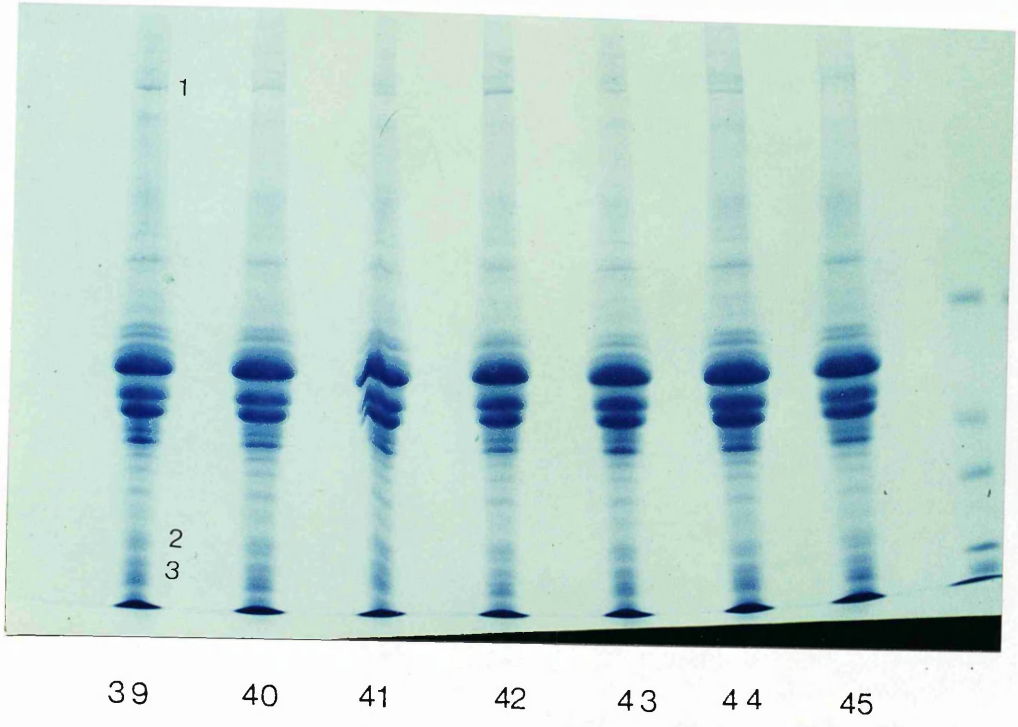


FIGURE 5.5: RESULTS FROM THE PROTEIN GEL OF THE LABORATORY CHOW CONTROL ANIMALS AT 60 DAYS.

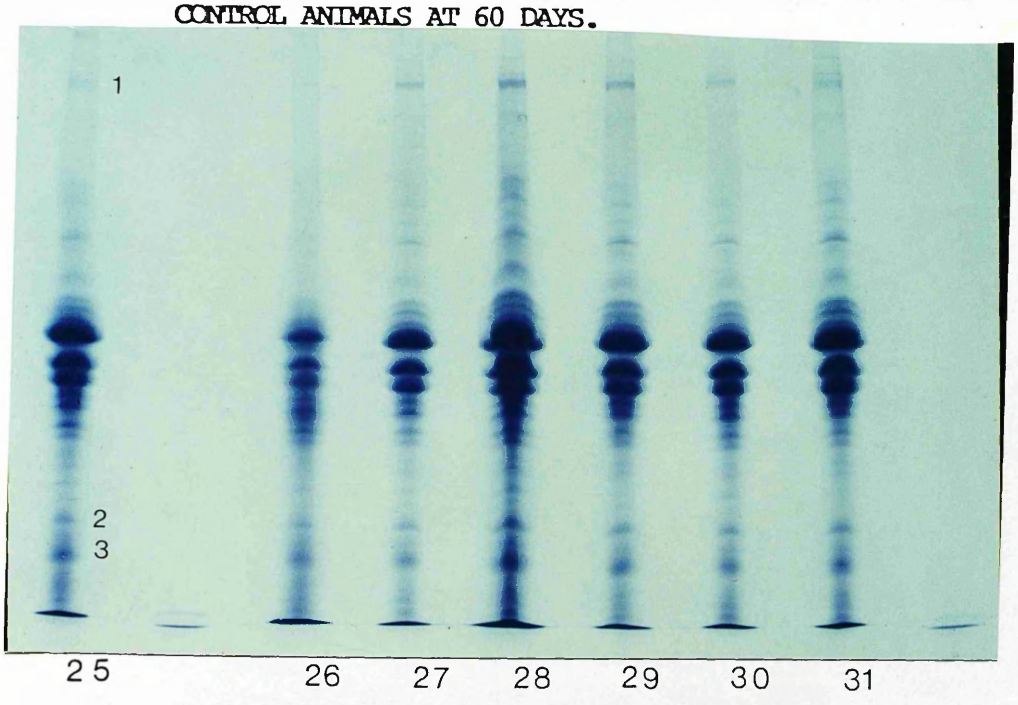


FIGURE 5.6: RESULTS FROM THE PROTEIN GEL OF THE LIQUID DIET CONTROL ANIMALS AT 60 DAYS.

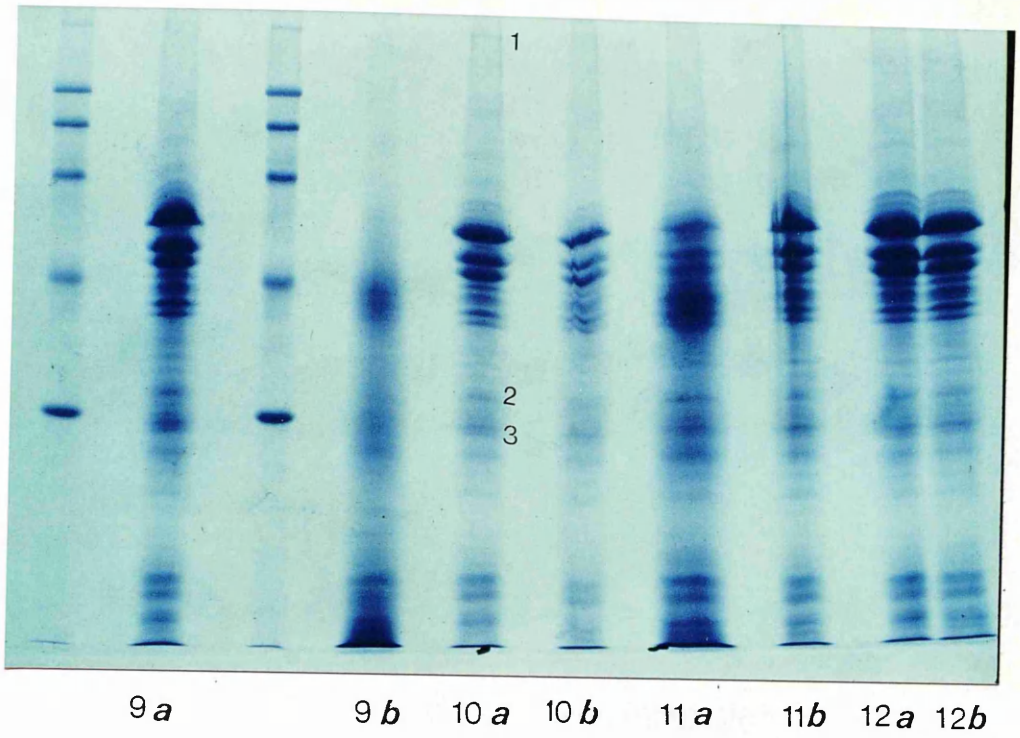


FIGURE 5.7: RESULTS FROM THE PROTEIN GEL OF THE PAIR-FED ANIMALS, NUMBERS 9-12, AT 60 DAYS.

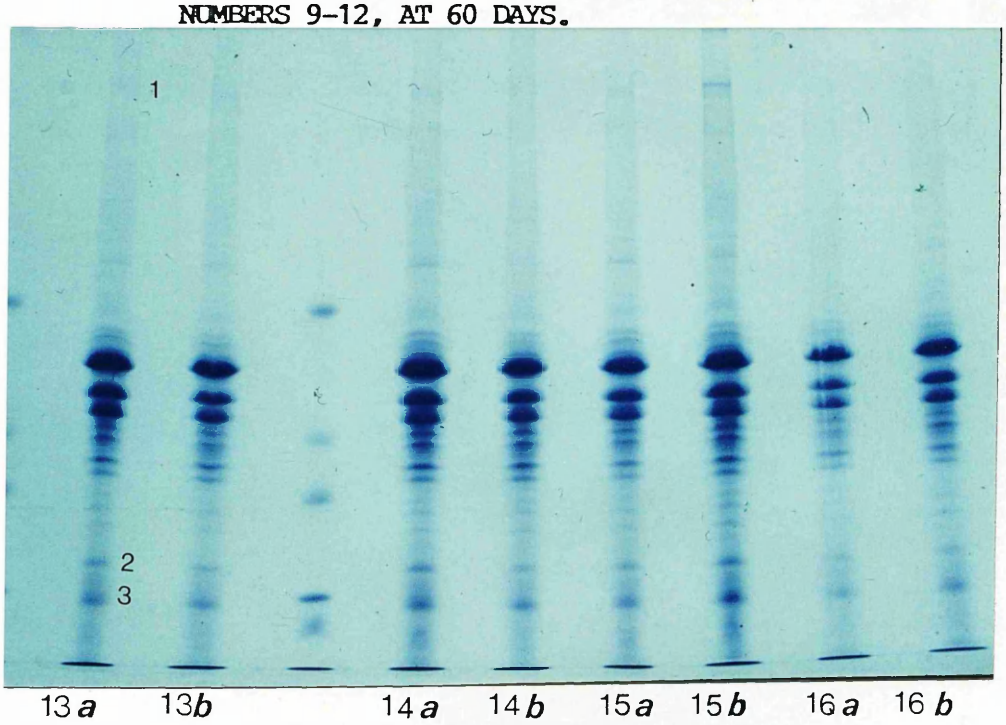


FIGURE 5.8: RESULTS FROM THE PROTEIN GEL OF THE PAIR-FED ANIMALS, NUMBERS 13-16 AT 60 DAYS.

LABORATORY CHOW ANIMALS

ANIMAL	HIGH MW	30K	28K
39	1.4	1.4	4.1
40	1.1	3.8	6.4
41	1.2	1.4	2.5
42	1.3	1.5	3.8
43	1.5	2.2	6.6
44	3.0	3.6	6.9
45	2.5	1.9	3.1
MEAN	1.71	2.26	4.77
S.D.	0.73	1.03	1.82

LIQUID DIET ANIMALS

ANIMAL	HIGH MW	30K	28K
25	1.3	1.3	4.7
26	1.0	5.4	11.2
27	1.3	1.4	4.5
28	0.8	0.9	2.2
29	1.7	1.8	4.5
30	0.9	1.2	4.4
31	1.4	4.7	8.5
	1.20	2.39	5.71
	0.32	1.85	3.05

THE ALCOHOLIC (A) AND SUCROSE (B) PAIR-FED ANIMALS

	HIGH MW		30K		28K	
ANIMAL	A	B	A	B	A	B
9	0.1	---	5.2	---	7.1	---
10	0.3	2.1	3.9	3.8	6.2	6.3
11	0.0	1.0	6.7	1.7	4.8	4.2
12	0.7	2.6	6.2	2.0	6.9	2.7
13	0.3	0.8	4.6	1.9	4.4	3.8
14	0.7	1.7	6.7	2.4	7.0	4.9
15	1.2	2.9	7.1	4.8	7.1	8.7
16	0.6	0.2	2.1	1.5	5.5	3.9
MEAN	0.48	1.61	5.30	2.59	6.12	4.93
S.D.	0.39	0.99	1.72	1.24	1.10	2.00

TABLE 5.2: AREA PERCENTAGE VALUES FOR THE THREE ABERRANT PROTEINS AT 60 DAYS.

Missing Values Are Due To Poorly Resolved Densitometer Scans.

levels in the laboratory chow, sucrose control and alcoholic animals at 60 days and at 102 days (Section 3.3.3.).

5.3.6. 60 Days: 30K Protein

Visual analysis of Figures 5.5-5.8, failed to detect any obvious differences in the levels of the 30K protein in the animals from any of the four groups.

Densitometric analysis (Table 5.2) revealed the mean area percentage levels of this protein to be 2.26 in the laboratory chow group and 2.39 in the liquid diet group. The mean levels in the sucrose control and alcoholic groups were 2.59 and 5.30 respectively.

No significant differences were found in the levels of this protein when the three control groups were compared. The levels of this protein in the alcoholic group of animals were significantly higher than those in the laboratory chow group (Mann-Whitney, $p<.002$), the liquid diet group (Mann-Whitney, $p<.002$) and the sucrose control group ($p<.02$).

Despite the mean levels of this protein in the alcoholic animals being higher at the 60 day point than at the 30 day point, this difference did not reach the levels of significance. No statistical differences were observed between the levels of this protein at 30 and 60 days for any of the three control groups.

No differences were found between the mean values for this protein in the laboratory chow, the sucrose control and the alcoholic groups at

60 and 102 days.

5.3.7. 60 Days:28K Protein

Gross visual analysis of Figures 5.5-5.8 failed to detect any obvious differences in the levels of the 28K protein in the animals from any of the four groups.

Densitometric analysis (Table 5.2) revealed the mean area percentage value for this protein to be 4.77 in the laboratory chow group and 5.71 in the liquid diet group. The mean levels in the sucrose control and alcoholic groups were 4.93 and 6.12 respectively.

Comparison of the values showed that there were no significant differences between the levels of this protein in any of the three control groups. When the alcoholic animals were compared with the liquid diet and sucrose control groups no differences were noted, but the levels in the alcoholic group were significantly (Mann-Whitney, $p<.05$) higher than those in the laboratory chow group.

No significant differences in the 30 and 60 day levels were noticed in any of the four groups. Similarly, no significant differences were observed in the levels of this protein at 60 and 102 days for the laboratory chow, sucrose control or alcoholic groups.

5.3.8. Other Protein Differences

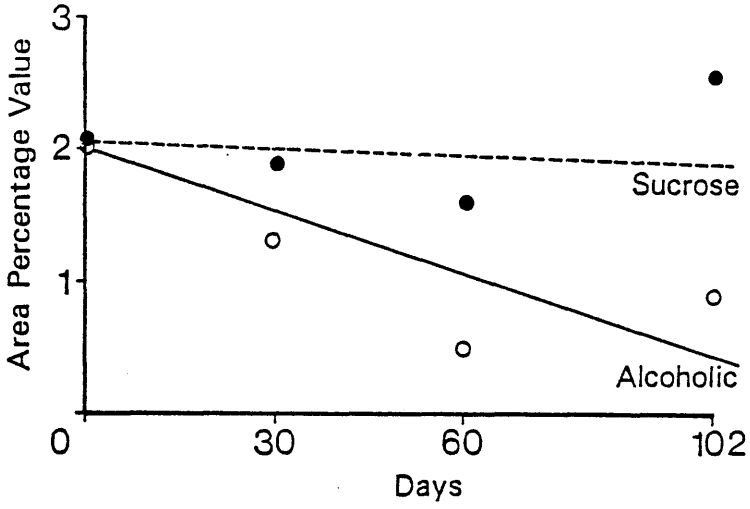
No consistent differences were observed in the levels of any other proteins in the gels by either visual or densitometric analysis.

Graphs summarising the alterations in the levels of the three proteins over 30, 60 and 102 days are shown in Figures 5.9-5.11.

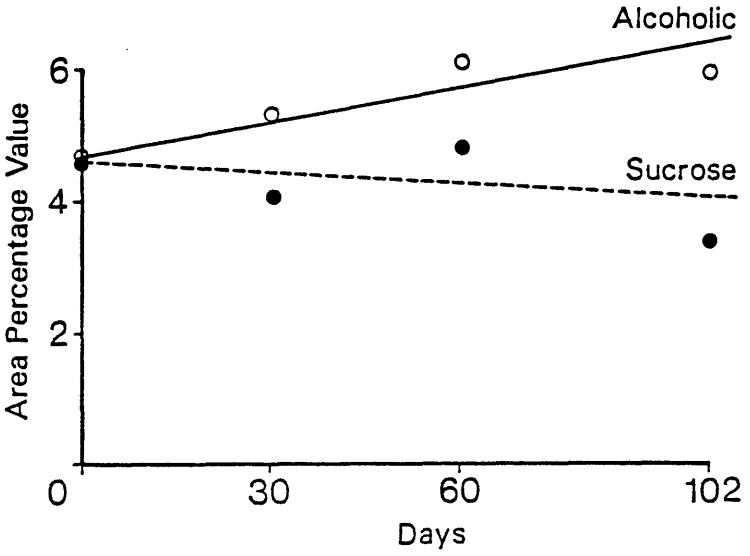
5.3.9. The Relationship Between the High Molecular Weight Protein and the Two Lower Molecular Weight Proteins

Attempts to show a relationship between the reduced levels of the high MW protein and the increased levels of the two lower MW proteins, as measured densitometrically, met with little success. There was no obvious correlation between reduced levels of the high MW protein and enhanced levels of the two lower MW proteins, and it would appear (Tables 5.1 and 5.2) that enhanced levels of the two lower MW proteins can arise independently of lowered levels of the high MW protein. There was also no obvious relationship between the raised levels of the two lower MW proteins both of which could appear either together or independently in raised levels.

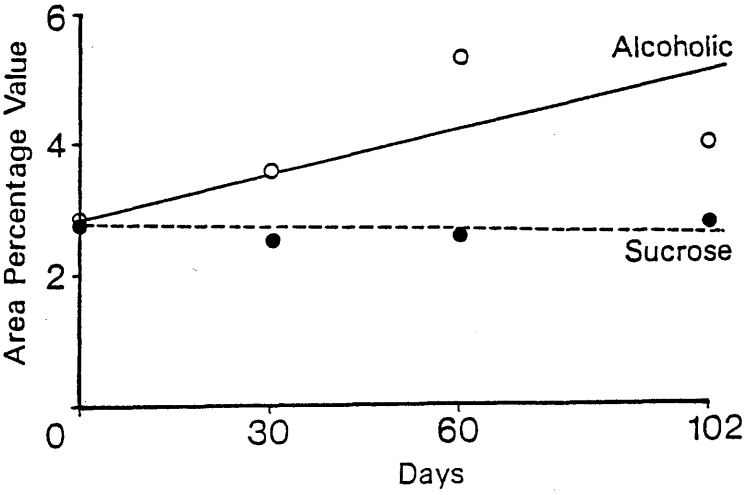
FIGURES 5.9–5.11: THESE THREE FIGURES REPRESENT GRAPHS OF THE ALTERATIONS IN THE LEVELS OF THE HIGH MOLECULAR WEIGHT PROTEIN (FIGURE 5.9), THE 30K PROTEIN (FIGURE 5.10) AND THE 28K PROTEIN (FIGURE 5.11) OVER 30, 60 AND 102 DAYS.


Area Percentage Value
3
2
1
0
30
60
102
Days
Sucrose
Alcoholic

5.9


Area Percentage Value
6
4
2
0
30
60
102
Days
Alcoholic
Sucrose

5.10


Area Percentage Value
6
4
2
0
30
60
102
Days
Alcoholic
Sucrose

5.11

5.4 DISCUSSION

5.4.1. The Case for Chronic Exposure

The results from this 30 and 60 day study clearly demonstrated a time dependent alteration in the levels of the three proteins in the alcoholic animals. It was seen that the alterations in the levels of the high MW protein and the 30K protein became statistically significant by 60 days and the alteration in the levels of the 28K protein became significant by 102 days. Sixty days is approximately one tenth of the total life span of a laboratory rat and consumption of alcohol for 60 days thus represents chronic exposure. It would appear that the alterations in the three proteins can confidently be concluded to be a consequence of chronic, and not of acute, ethanol exposure.

The fact that no significant differences were observed in the levels of these three proteins in any of the three control groups over the time course, indicated that there was no general age, or dietary, related effect on the levels of the proteins. Thus the altered levels in the alcoholic animals could be explained only on the basis of alcohol related effects.

5.4.2. The Relationship between the High Molecular Weight and the Two Lower Molecular Weight Proteins

It would appear from the results that there was no direct link between the lowered levels of the high MW protein and the raised levels of the two lower MW proteins, and that the two lower MW proteins could show raised values either together or independently.

A further test of a possible precursor/product relationship between the high MW protein and the two lower MW proteins, involving peptide mapping by limited proteolysis, is presented in Chapter 6.

5.4.3. Possible Mechanisms Underlying the Observed Protein Alterations

As there seems to be no relationship between the high MW protein and the two lower MW proteins, it is unlikely that the two lower MW proteins result simply from an alcohol mediated increase in the breakdown of the high MW protein (Section 3.4.3.).

An alternative explanation is that alterations at the level of transcription, translation or indeed post-translational processing may be responsible. That ethanol is capable of mediating effects at the nuclear level is demonstrated by its ability to induce chromosomal alterations in vivo (Obe & Herha, 1975) and by the increased numbers of dysplastic cells (with abnormal nuclei) observed by Anderson (1972), in human alcoholics. A teratogenic potential for ethanol is suggested by the observed effects of ethanol on the developing foetus, particularly foetal alcohol syndrome.

Ethanol or one of its metabolites may be capable of exerting effects at the level of transcription or translation and the observed protein alterations could be a consequence of this effect. In this respect, it is perhaps worth noticing that acetate, raised levels of which follow alcohol ingestion, is known to stimulate epidermal RNA, DNA and protein synthesis (Slaga, Bowden & Boutwell, 1975).

One further possible mechanism explaining the altered levels of the proteins, is that there may be repression control of one of these proteins over the others. If, for example, the high MW protein exerts repression control over the expression of one or both of the two lower MW proteins, lower levels of this high MW protein would result in freer transcription of the gene for the repressed proteins. This would lead to higher levels being expressed in the cell. Alternatively, the lower MW proteins may exert a similar control over the levels of the high MW protein. Thus raised levels of the lower MW proteins may lead to lowered levels of the high MW protein. Although this may explain part of the findings, the mechanisms behind the altered levels of the repressor proteins would still have to be defined.

The above discussion is somewhat speculative and definite conclusions cannot be drawn from the present study. It would require considerable additional work to investigate these possibilties. Further work shall be discussed in Chapter 8.

5.5 CONCLUSIONS

Using a similar approach to the 102 day study, temporal alterations in the levels of the three aberrant proteins identified in the 102 day study were investigated. The results demonstrated that levels of the three proteins were only altered after chronic alcohol consumption.

This study also attempted to investigate a possible relationship between the reduced levels of the high MW protein and the increased levels of the two lower MW proteins. The results failed to demonstrate any relationship and it was apparent that altered levels of each of these proteins could arise independently of altered levels of the other proteins.

CHAPTER SIX - INITIAL CHARACTERISATION OF THE THREE ABERRANT PROTEINS

6.1 INTRODUCTION

6.1.1. General Introduction

To define an aetiological role for the observed protein alterations reported in Chapters 3 and 5, or to suggest mechanisms whereby the levels of these proteins might have been altered, it is essential to characterise them as far as possible. To do this, a variety of techniques were used and it is considered of value to discuss the rationale behind some of the experimental methods used.

6.1.2. Peptide Mapping

As discussed earlier (Section 3.4.3.), one possible explanation for the observed increases in the two lower MW proteins is an ethanol related breakdown of the high MW protein. Peptide mapping provides an ideal tool for investigating such a precursor/product relationship.

When a protein undergoes limited proteolysis, and the fragments produced are subjected to electrophoresis, a 'fingerprint' of the peptides is produced. If two proteins are related, their respective peptide 'fingerprints' (or 'maps') will show a degree of similarity. Thus by subjecting the high MW and the two lower MW proteins (cut out as stained bands from a previously run SDS gel), to limited proteolysis, comparison of the peptide maps produced will allow assessment of the degree of similarity between the proteins.

6.1.3. Subcellular Localisation

To assign the three proteins to either the membrane/microsomal or the cytoplasmic fractions of the cell, subcellular localisation studies were performed. By assigning the proteins to a specific site within the cell, clues may be provided to the possible function of these three proteins. Such studies may also prove useful in simplifying subsequent isolation and purification procedures.

6.1.4. Protein Solubilisation Studies

To study proteins, maximisation of their extraction from the source tissue is essential. In addition, it is important to ensure that the extracting medium is one in which the proteins of interest are soluble so that they can be readily separated from cellular debris. For the purposes of the studies presented in this thesis, a protein is defined as being soluble when it is capable of being separated from cellular debris by centrifugation at 10,000 g (kg). It should, however, be emphasised that many of these proteins will not in fact be in true solution.

Solubilisation studies are also useful as knowledge of the chemical properties of the extracting medium will give an indication of the possible chemical properties of the protein and may thus aid characterisation as well as purification and isolation.

6.1.5. Possible Glycoprotein Nature of the High Molecular Weight Protein

The unusual molecular weight results discussed in Section 3.3.4. in relation to the high MW protein, suggested that it bound SDS, or that

it migrated through polyacrylamide gels, in a manner different to that of a typical protein. The apparent molecular weight of this high MW protein, increased with decreasing gel porosity. This is a property that is typical of glycoproteins which, due to the presence of carbohydrate residues attached to the peptide chain, bind less SDS per gramme of peptide than do 'normal proteins' (Weber & Osborne, 1975). The attached carbohydrate residues themselves can alter the electrophoretic mobility either by steric interactions between the carbohydrate groupings and the gel matrix or as a result of the inherent charge of the carbohydrate groupings altering the expected mobility of the peptide in an electrical field (Weber & Osborne, 1975). The methods involved in investigating the possible glycoprotein nature of the high MW protein are outlined in Sections 6.2.6. and 6.2.7.

6.2 MATERIALS AND METHODS

6.2.1. Electrophoretic Methods

Electrophoresis was performed according to the method of Laemli (1970) as described in Sections 3.2.6 and 3.2.7. The percentage acrylamide composition of each gel is mentioned in the appropriate figure legend.

6.2.2. Peptide Mapping Studies

As discussed in Section 6.1.2., peptide mapping studies were undertaken in an attempt to test any relationship between the high MW and the two lower MW proteins. The method employed was that of 'Peptide Mapping by Limited Proteolysis in SDS and Analysis by SDS Gel Electrophoresis' (Cleveland Mapping). The particular method used involved the digestion of proteins in stained gel slices (Cleveland et al., 1977). The proteolytic enzymes used in the present study were Staphylococcus aureus protease V8, papain, chymotrypsin, trypsin and elastase. One exception to the original method was that when using S. aureus protease V8, EDTA was omitted from all buffers as this enzyme requires divalent cations (Mg^{2+} & Mn^{2+}) for activity (Hames & Rickwood, 1981).

Electrophoresis was carried out at room temperature in 15 percent polyacrylamide gels prepared with wider than normal sample wells (0.8 mm). Electrophoresis was interrupted, as described by Cleveland et al. (1977), when the bromophenol blue marker dye reached the end of the stacker gel. Enzymatic digestion was allowed to proceed for 30 minutes regardless of the enzyme used.

6.2.3. Silver Staining

Due to the small amount of protein that was available for proteolysis in the peptide mapping studies, CBB R-250 staining (Section 3.2.8.) was abandoned in favour of silver staining. This stain is regarded as being some 100-fold more sensistive than the CBB R-250 stain.

Silver staining was performed by the method of Morrissey, (1981) in clean plastic sandwich boxes. All water used during the silver staining was double distilled and deionised, with a conductance that was never greater than 1 micromho. The entire procedure was carried out at 37° C, with constant gentle shaking, and disposable plastic gloves were worn at all times.

6.2.4. Subcellular Localisation

The initial approach was designed to show whether the three proteins were associated with either the membrane/microsomal or the cytoplasmic fractions of the cell. These studies involved homogenisation of the epithelial tissue (prepared as described in Section 3.2.1.) in ice-cold distilled water (1 ml per epithelial sheet), followed by centrifugation, at 10 kg for 20 minutes in an MSE High Speed-18 centrifuge (Fisons, England), to remove any insoluble material. Twenty five microlitres of the supernatant produced was removed for protein concentration determination, (Section 3.2.5.) and the remaining volume of supernatant was subjected to centrifugation at 100 kg for 90 minutes in a Beckman L2-65 B ultracentrifuge using a Beckman SW 50L swinging bucket rotor (Beckman Ltd., High Wycombe, England) and polyallomer centrifuge tubes. The supernatant from this 100 kg spin represented the cytoplasmic fraction of the cell and the pellet

represented the membrane/microsomal fraction of the cell. The supernatant was carefully removed, the protein concentration determined (Section 3.2.5.) and the proteins prepared for SDS PAGE (Section 3.2.2.).

The pellet was resuspended in ice cold distilled water and washed by spinning down again at 100 kg for 90 minutes. This wash was repeated and the resulting pellet was prepared for SDS PAGE (Section 3.2.2.).

Subcellular localisation of the high MW protein was performed by differential centrifugation. Samples (500 μl) of epithelial homogenate (in H_2O) were centrifuged, initially at either 1000, 2000 or 3000 g in an MSE High Speed-18 centrifuge (Fisons, England) for 20 minutes. The pellets (consisting largely of insoluble material) were discarded and the supernatants recentrifuged at 10 kg for 20 minutes. The surfaces of the pellets obtained were carefully washed with ice cold distilled H_2O and were then prepared for SDS PAGE (Section 3.2.2.).

Preparation of pellets for SDS PAGE required that they be thoroughly mixed with the SDS/DTT buffer, by homogenisation, prior to heating. Failure to do this tended to result in an incomplete solubilisation of the pellets in this buffer.

All the centrifugations were carried out at 0-4° C.

6.2.5. Solubilisation Studies

In aqueous solution three major factors govern the solubility of

proteins, i.e. temperature, pH and salt concentration (Morton, 1955). Proteins are least soluble at their isoelectric point (pI) and thus solubilisation is best achieved at pH values far removed from the isoelectric point (Palmer, 1985). Accordingly, since the majority of proteins have their pI in the pH range 4-7, they are generally more soluble at alkaline pH values (Pharmacia Fine Chemicals, 1982).

Alterations in the salt concentration (ionic strength) of the medium, can also increase solubilisation of a protein through an effect known as 'salting in'. This involves the addition of small quantities of a neutral salt (typically of the order of 0.15 M), which can cause minor alterations in the charges on the amino acid side chains and can also interfere with the interactions between protein molecules. The overall effect is one of increased interactions between solute and solvent (Palmer, 1985). At higher salt concentrations, the ions compete with the protein for the water of solubilisation and effectively decrease the possibility of protein-water interactions. This effect is known as 'salting out' (Palmer, 1985), and results in precipitation of the protein from solution. This is a technique frequently used in protein purification procedures.

Given the observed increase in solubility of the high MW protein in the O'Farrell lysis buffer (Section 3.4.1.), solubility was also studied in urea solutions of varying concentrations. The urea solutions were buffered in 0.1 M tris pH 8.0 for the reasons outlined in Section 6.4.3.

To study solubilisation, epithelial sheets were homogenised in various buffers (see the legends to Figures 6.4, 6.5 and 6.6). Subsequently the homogenates were centrifuged at 10 kg for 20 minutes in an MSE High Speed-18 centrifuge (Fisons, England) to remove cellular debris. In this way, the solubility of the high MW protein (which usually spins down at 10 kg) and the two lower MW proteins, could be assessed in the relevant buffer. The supernatants thus formed were prepared for SDS PAGE (Section 3.2.2.).

6.2.6. Assessment of the True Molecular Weight of the High Molecular Weight Protein

An estimate of the true molecular weight of glycoproteins can be obtained by the procedure of Segrest and Jackson (1972), in which the apparent molecular weight of the protein of interest, is measured at different gel concentrations. The molecular weight is then plotted out against the acrylamide concentration to obtain curves asymptotically approaching the real value of the molecular weight. The rationale behind this approach, is that at low gel concentrations the reduced binding of SDS by the glycoprotein results in reduced electrophoretic mobility and hence a higher apparent molecular weight for the protein. In more concentrated gels, the molecular sieving by the gel matrix becomes dominant over the protein charge in defining the migration rate and thus a more accurate measure of the molecular weight may be made. Hence, a graph of molecular weight versus gel concentration will asymptotically approach the true molecular weight of the glycoprotein at higher gel concentrations.

Epithelial proteins prepared for SDS PAGE (Section 3.2.2.) were electrophoresed in 5, 7.5, 10, 12.5 and 15 percent polyacrylamide gels. The apparent molecular weight of the high molecular weight protein was assessed at each of these gel concentrations and a graph of molecular weight versus acrylamide concentration plotted.

6.2.7. Glycoprotein Staining

Prior to staining SDS gels of epithelial protein preparations with glycoprotein specific stains, it was necessary to remove all the SDS as this substance reacts with the stains and would have given false positive results. This was achieved by washing the gels in 2.6 litres of 40 percent methanol, 7 percent acetic acid, overnight, and then for a further 8 hours in a fresh volume of this solution (Glossman & Neville, 1971).

Following this treatment, which ensured removal of the SDS from the gels, the gels were stained in one of the following two ways:

Periodic Acid Schiff (PAS) Staining (Glossman & Neville, 1971): The washed gel was oxidised for 1 hour with 1 percent periodic acid in 7 percent acetic acid in the dark. This was followed by a 24 hour wash in several changes of 7 percent acetic acid after which the gel was stained in Schiff's reagent for 1 hour, at 4° C, in the dark.

The gel was then washed three to four times with a 1 percent solution of sodium metabisulphite in 0.1 M hydrochloric acid, and was finally stored in this solution.

Alcian Blue (Wardi & Michos, 1972): Following the initial wash, the gel was placed in 1 percent periodic acid in 3 percent acetic acid, at 4° C, for 50 minutes in the dark. Excess periodate was removed by repeated washing with distilled water after which, the gel was placed in 0.5 percent potassium metabisulphite for 30 minutes. The gel was again washed with distilled water, and then placed for at least 4 hours in 0.5 percent alcian blue in 3 percent acetic acid.

Destaining was by diffusion against 7 percent acetic acid.

Non oxidised controls (i.e. no periodic acid) were included with both the PAS and alcian blue stains.

6.3 RESULTS

6.3.1. Peptide Mapping Studies

Attempts to show a relationship between the high MW protein and the two lower MW proteins, using the peptide mapping procedure discussed above, were unsuccessful (Figure 6.1). This was due to the apparent resistance of the high MW protein to proteolytic digestion by the range of proteolytic enzymes mentioned in Section 6.2.2. Comparison of the peptide maps from the high MW protein and the two lower MW proteins was thus not possible. The peptide maps produced for the two lower MW proteins showed them to be unrelated.

One further complicating factor was that the high MW protein did not stain well with silver stain.

6.3.2. Subcellular Localisation Studies

Subcellular localisation studies (Figure 6.2) revealed that the 30K protein was clearly associated with the membrane/microsomal fraction of the cell.

The 28K protein proved more difficult to study as it bands on SDS gels, alongside a protein of very similar molecular weight, as a closely associated doublet. As reported in Section 3.4.2., densitometer scans failed to sufficiently resolve these two proteins and it was not clear which member of this doublet was being enhanced in the alcoholic animals. To further confuse this issue, subcellular localisation studies have shown one member of this doublet to be associated with the membrane/microsomal fraction, and the other with

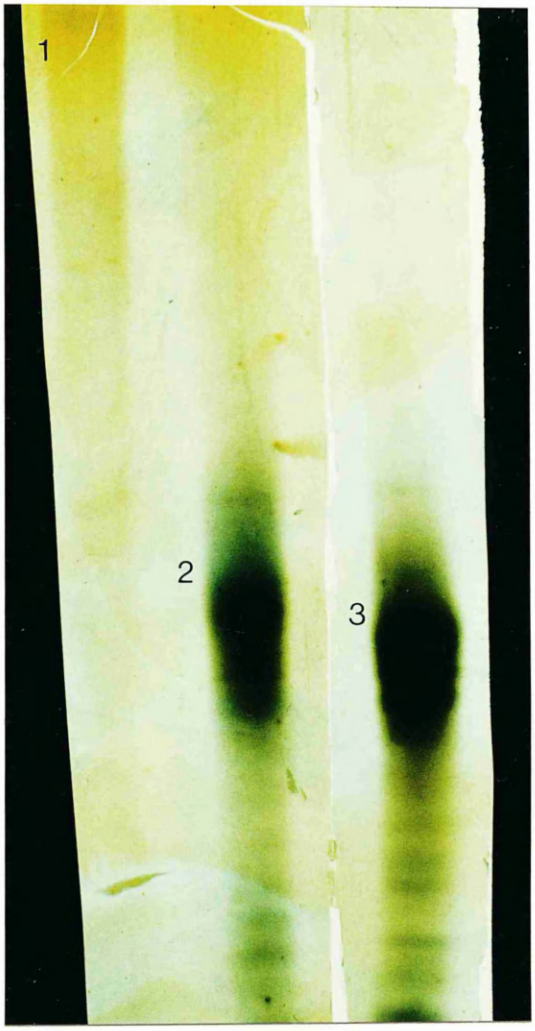


FIGURE 6.1: SPECIMEN RESULTS FROM THE PEPTIDE MAPPING STUDIES.

The gel used was 15 percent acrylamide and the enzyme used was *S. aureus* protease V8 (0.5 μg per protein band).

(1) High molecular weight protein

(2) 30K protein

(3) 28K protein

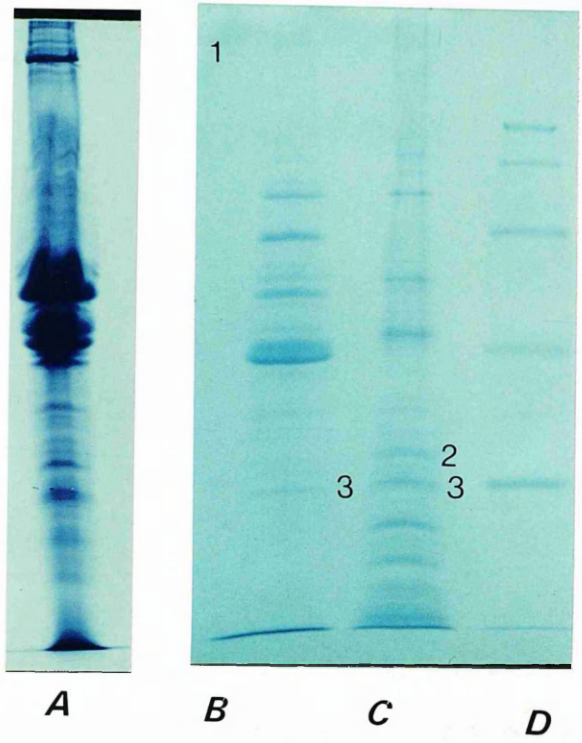


FIGURE 6.2: RESULTS FROM THE SUBCELLULAR LOCALISATION STUDIES.

The gel used was 10 percent acrylamide.

(A) Total cellular proteins

(B) The cytoplasmic fraction

(C) The membrane/microsomal fraction

(D) High molecular weight markers

1. Note the absence of the high molecular weight protein from both the cytoplasmic and the membrane/microsomal fractions.

2. The 30K protein is associated with the membrane/microsomal fraction.

3. The 28K protein is a doublet and one member is associated with the cytoplasmic fraction and the other with the membrane/microsomal fraction.

the cytoplasmic fraction of the cell. It was, thus, not possible to assign the 28K protein specifically to either the cytoplasmic or the membrane/microsomal fractions of the cell.

Also noticeable in Figure 6.2 is the complete absence of the high MW protein in either the cytoplasmic or the membrane/microsomal fractions of the cell. It appeared, therefore, that the high MW protein was pelleting at a centrifugal force of less than 10 kg and thus was removed from suspension by the initial low speed clarifying spin. By subjecting epithelial preparations to centrifugation at various low speeds, as discussed in Section 6.2.4., it was demonstrated that the high MW protein pelleted from solution at a centrifugal force of 3000g (Figure 6.3).

6.3.3. Solubilisation Studies

Initial solubilisation studies demonstrated (Figures 6.4 and 6.5) that buffers of high ionic strength and varying pH had no observable effect on the solubilisation of any of the three proteins.

The higher levels of the high MW protein solubilised by the O'Farrell lysis buffer (Section 3.3.3.), indicated that urea may be an effective solubilising agent for the high MW protein and indeed, homogenisation in various urea solutions (1-8 M in 0.1 M tris buffer, pH 8.0) resulted in markedly higher amounts of this protein being extracted and solubilised from the epithelial tissue (Figure 6.6). The maximal urea concentration for solubilisation appeared to be 4 M with 1 M having little or no effect on the yields of this protein. Urea had no observable effect on the solubilisation of either of the two lower MW

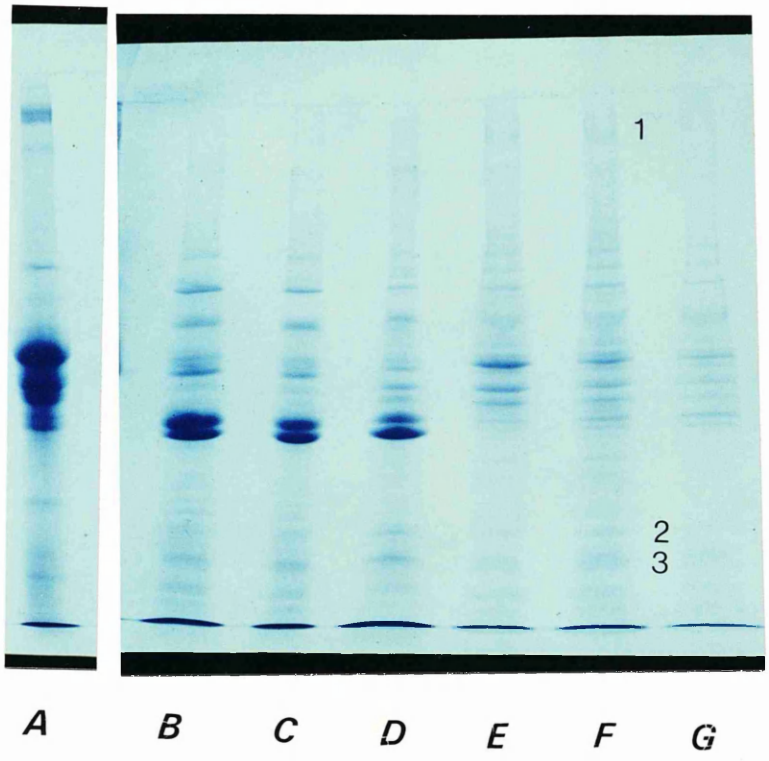


FIGURE 6.3: RESULTS FROM THE DIFFERENTIAL CENTRIFUGATION STUDIES.

(A) Total cellular proteins.

(B) Supernatant from centrifuging the 1000 g supernatant at 10 kg (1 x 10k) - Note the absence of the high molecular weight protein.

(C) Supernatant from centrifuging the 2000 g supernatant at 10 kg (2 x 10k) - Note the absence of the high molecular weight protein.

(D) Supernatant from centrifuging the 3000 g supernatant at 10 kg (3 x 10k) - Note the absence of the high molecular weight protein.

(E) Pellet from the 1 x 10k spin - Note the presence of the high molecular weight protein which, therefore does not spin down at 1000 g.

(F) Pellet from the 2 x 10k spin - Note the presence of the high molecular weight protein which, therefore does not spin down at 2000 g.

(G) Pellet from the 3 x 10k spin - Note the absence of the high molecular weight protein which therefore must spin down at 3000 g.

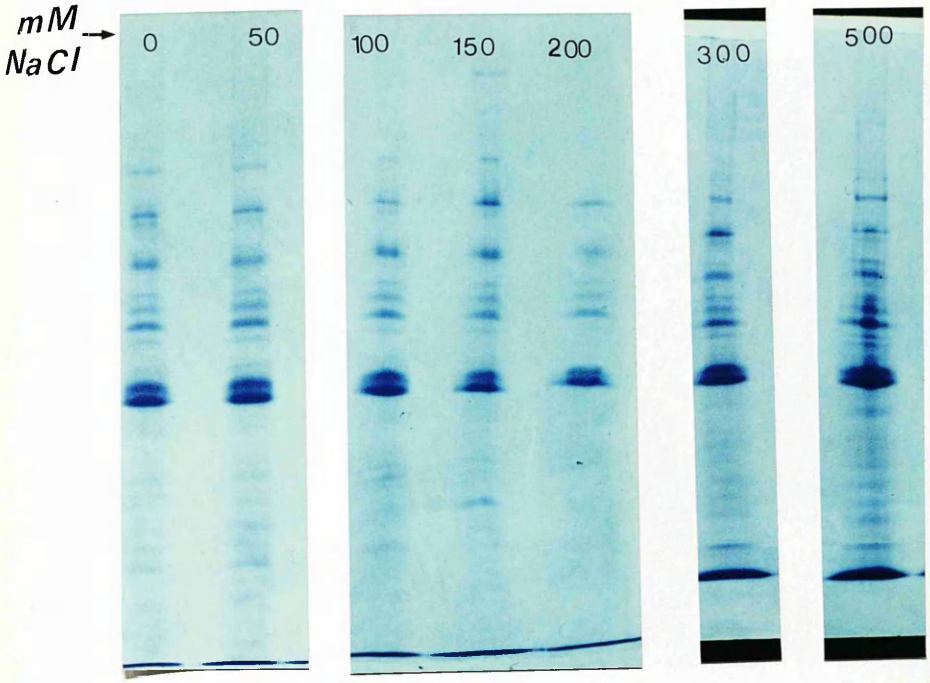


FIGURE 6.4: EFFECTS OF VARYING SALT CONCENTRATIONS ON THE SOLUBILITY OF THE THREE ABERRANT PROTEINS.

The gel used was 10 percent acrylamide.

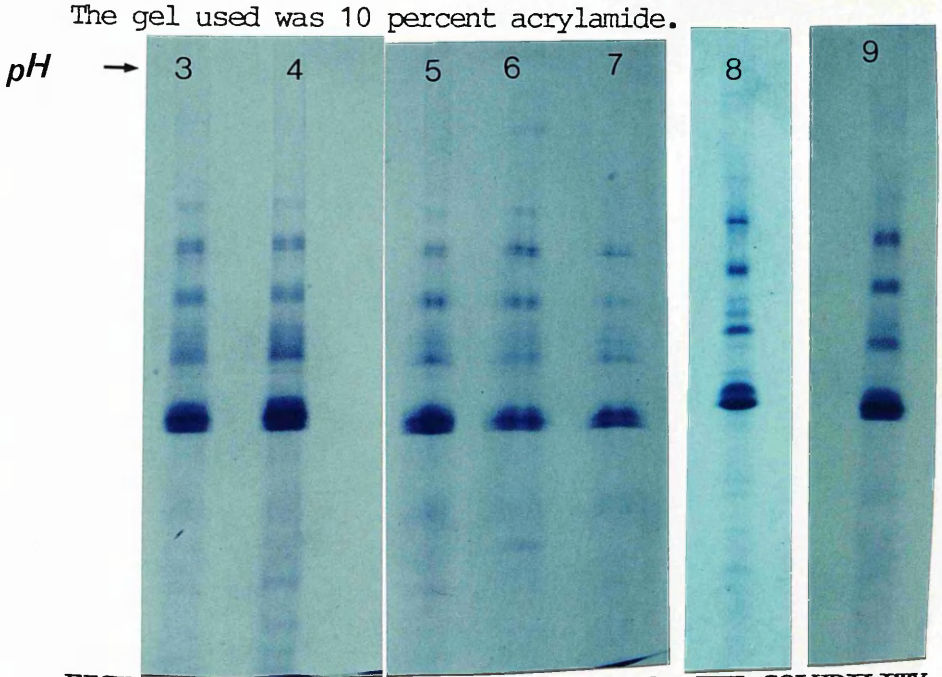


FIGURE 6.5: EFFECTS OF VARYING pH ON THE SOLUBILITY OF THE THREE ABERRANT PROTEINS.

The gel used was 10 percent acrylamide.

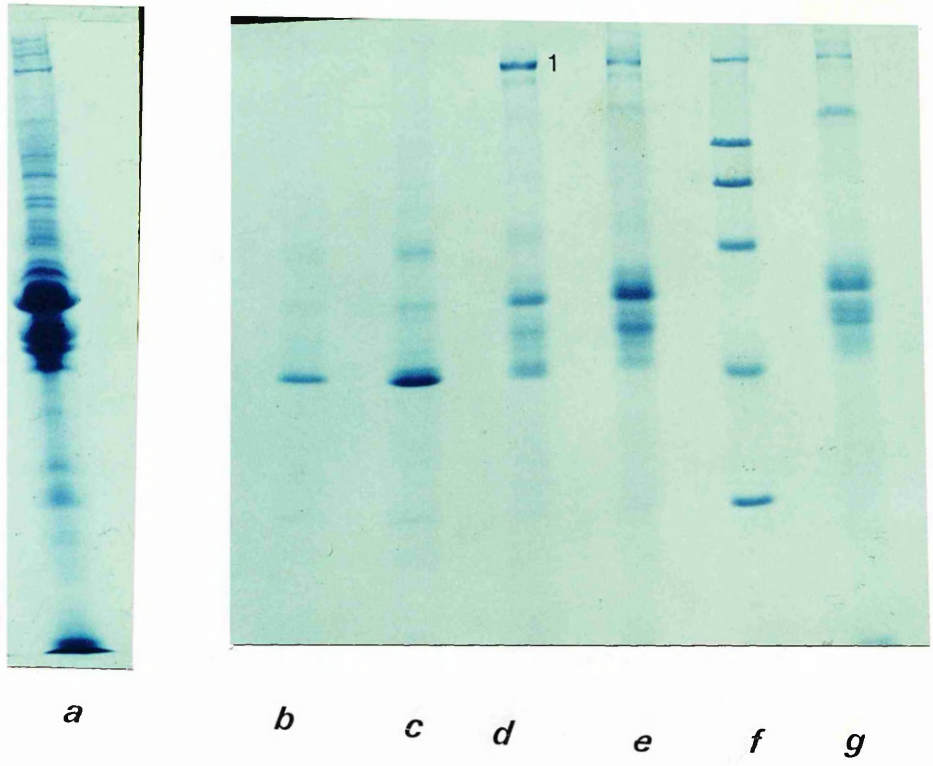


FIGURE 6.6: ANALYSIS OF THE UREA SOLUBLE PROTEINS OF THE LINGUAL EPITHELIUM.

The gel used was 10 percent acrylamide.

1 - The high molecular weight protein.

(A) Total cellular proteins.

(B) Proteins soluble at 10 kg in 1 M urea.

(C) Proteins soluble at 10 kg in 2 M urea.

(D) Proteins soluble at 10 kg in 4 M urea.

(E) Proteins soluble at 10 kg in 6 M urea.

(F) High molecular weight markers.

(G) Proteins soluble at 10 kg in 8 M urea.

proteins.

6.3.4. Results From The Glycoprotein Stains

Figure 6.7 shows the results from the PAS staining procedure, which demonstrated the high MW protein to be a glycoprotein. Non oxidised controls failed to show PAS staining in the region of the high MW protein, confirming that the staining of this band was not artefactual. The identity of this protein as the high MW protein of interest was confirmed by CBB R-250 staining (Section 3.2.5.) which showed it to band at the characteristic position on SDS gels.

The high MW protein shall now be referred to as the high MW glycoprotein.

Alcian blue failed to stain any protein bands in either the oxidised or non-oxidised gels.

6.3.5. Glycoprotein Molecular Weight Estimation

Results from the Segrest and Jackson (1972) analysis of the molecular weight of the high MW glycoprotein are shown in Figure 6.8. The graph of molecular weight versus mobility, asymptotically approached 160K and this was taken as a reasonable estimate of the true molecular weight of the glycoprotein.

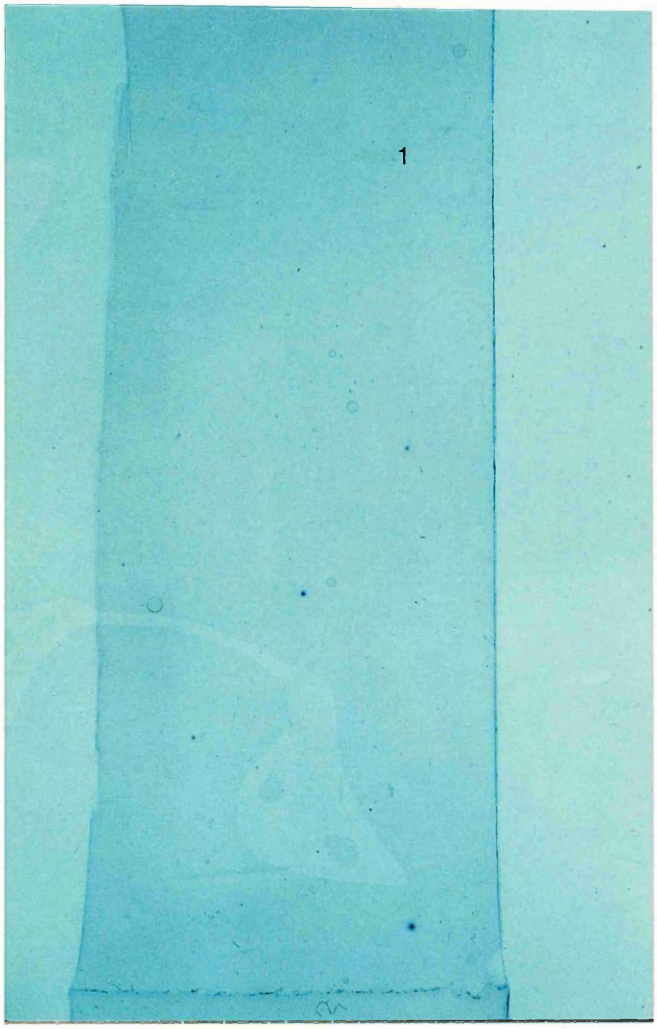


FIGURE 6.7: PERIODIC ACID SCHIFF STAINING OF A LINGUAL EPITHELIAL PROTEIN PREPARATION.

The gel used was 10 percent acrylamide.

1 - The high molecular weight glycoprotein.

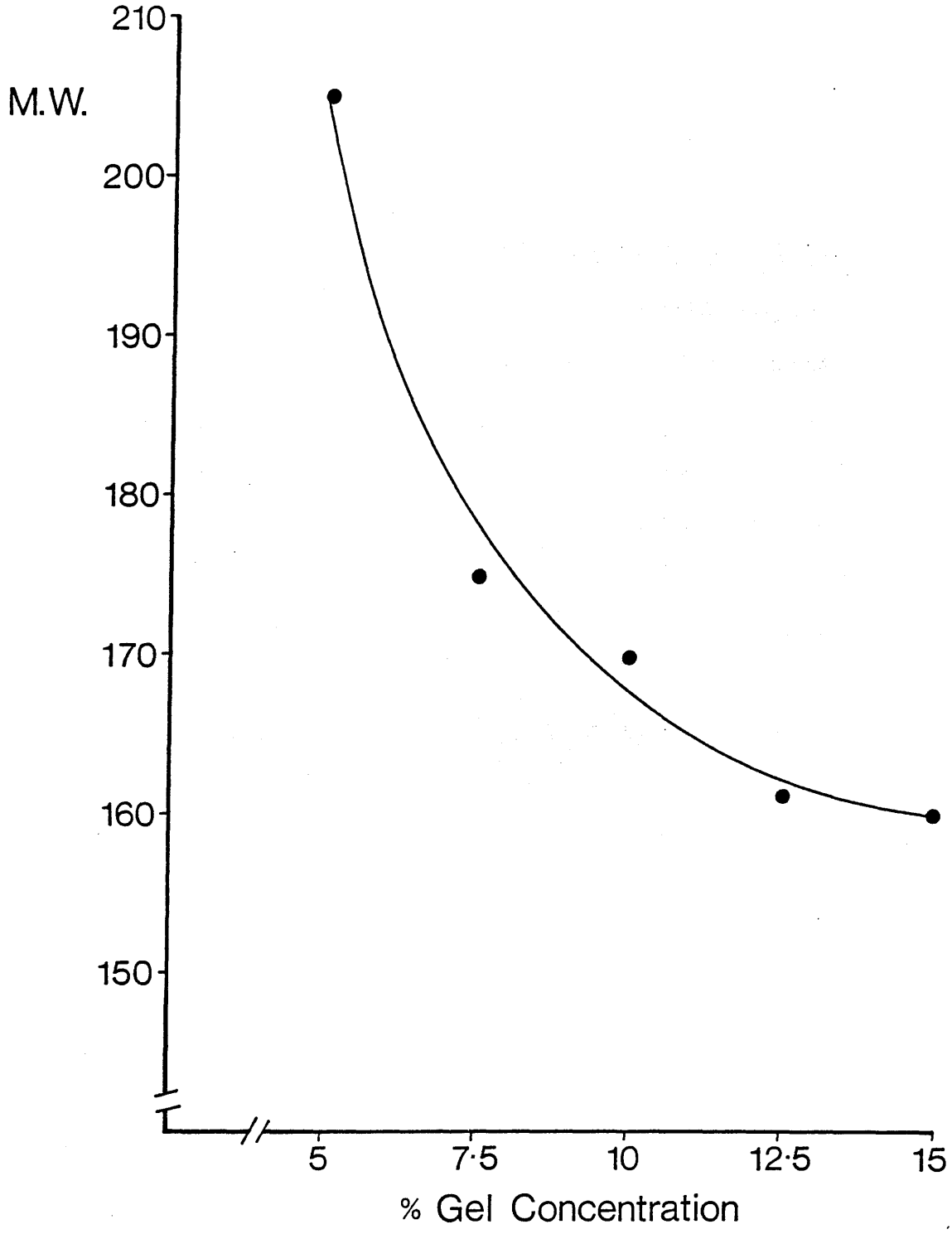


FIGURE 6.8: RESULTS FROM THE ANALYSIS OF THE MOLECULAR WEIGHT OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN.

6.4 DISCUSSION

6.4.1. Peptide Mapping Studies

As discussed in Section 6.1.2., peptide mapping studies were performed in an attempt to relate the high MW glycoprotein to the two lower MW proteins. Unfortunately, comparison of the peptide maps of these three proteins proved impossible due to the apparent resistance of the high MW glycoprotein to digestion by a wide range of proteases. This was despite the concentration of each of these enzymes being sufficiently high to digest the two lower MW proteins. The two lower MW proteins were successfully digested by each of the enzymes, yielding characteristic, but dissimilar, peptide banding patterns.

There are no clear reasons why the high MW glycoprotein has proved so resistant to proteolytic digestion especially over a range of enzymes of differing specificities. Given the glycoprotein nature of this high MW protein, it is possible that the carbohydrate groups sterically block the access of the various proteases to the peptide chain thus inhibiting proteolysis.

One further possibility is that, since the high MW glycoprotein did not stain well with silver stain (Section 6.3.1.), any proteolytic fragments may also have stained poorly and the apparent resistance of this protein to proteolytic digestion could simply be a reflection of the poor silver staining properties of the fragments. Due to the limited quantities of protein available, it was not possible to load sufficient amounts of this protein to allow for CBB R-250 staining (Section 3.2.8.). This limitation is unfortunate as CBB R-250

staining would have allowed visualisation of proteolytic fragments without the need for silver staining.

6.4.2. Subcellular Localisation Studies

The membrane/microsomal fraction of the cell is defined as being the particulate fraction that sediments upon centrifugation at speeds exceeding 10 kg (Tata, 1969). Such fractions contain small membranous sheets and membrane vesicles derived from the smooth and rough endoplasmic reticulum, golgi and plasma membranes (Graham, 1984). Thus, whilst a large amount of plasma membrane material will pellet after the initial 10 kg spin, the membrane/microsomal fraction is still a fair representation of the cellular membrane population. Analysis of the protein content of this membrane/microsomal fraction and of the 100 kg supernatant will allow crude assessment of the cellular origin of the proteins of interest. The cytoplasmic fraction (supernatant after the 100 kg spin) represents the soluble cellular proteins and, given that the clearance of the homogeniser used in this study was of the order of 0.11-0.15 mm, it was assumed that this fraction was largely free of protein derived from nuclei or mitochondria, both of which are too small to be disrupted by the homogeniser. From this study, it became clear that the 30K protein was associated with the membrane/microsomal fraction of the cell although the 28K protein, as mentioned in Section 6.3.2., has proved more difficult to study.

Interestingly, the high MW glycoprotein was absent from either of the two fractions discussed above, and it was concluded that this protein pelleted at centrifugal forces of less than 10 kg. Differential

centrifugation studies revealed that this protein pelleted from solution at a centrifugal force of 3000 g. The high MW glycoprotein must therefore be associated with a rapidly pelleting structure, as such a low centrifugal force would not pellet free, soluble protein molecules. Knowing that the protein did not pellet in large amounts from solution at forces of less than 3000 g, it is assumed that it is not a component of the nucleus. Also, given that none of the protein remained in solution above 3000 g, it can be concluded that it is not associated with mitochondria, which usually require forces of up to 20 kg to pellet completely (Graham, 1984). It is unlikely that this protein is a component of the membrane fraction of the cells as demonstrated by its absence from the membrane/microsomal fraction discussed above.

6.4.3. Solubilisation Studies

In studying the solubility of the three proteins of interest in this study, it became clear that there were no observable 'salting in' effects, and that altering the pH did not noticeably affect the solubilisation of any of the three proteins.

The most striking alteration in the solubility of any of these three proteins, resulted from the use of concentrated urea solutions which markedly enhanced the extraction and solubilisation of the high MW glycoprotein from the epithelial tissue. These urea solutions appeared to have no effect on the yields of the two lower MW proteins. This is surprising given that at least one of these proteins is associated with the membrane/microsomal fraction of the cell, which should have been solubilised to a greater extent by the urea which is

a chaotropic agent.

It is possible that the urea related increased extraction of the high MW glycoprotein in fact represented an increased extraction of a protein of identical molecular weight that comigrated with the high MW glycoprotein. However, the identity of the enhanced protein was confirmed by its resistance to proteolytic digestion, its poor silver staining properties, and by molecular weight analysis, which demonstrated the same asymptotic curve in the graph of molecular weight versus electrophoretic mobility (Section 6.3.5.).

Urea is a chaotropic agent and solubilises proteins by destructuring water and interfering with hydrophilic/hydrophobic bonds and amino acid side chains. Urea also denatures proteins, probably by disrupting the hydrogen bonds that hold the protein structures together. The fact that the high MW glycoprotein is solubilised by urea suggests that in the cell this protein is present, hydrophobically associated with a structure that, according to Section 6.3.2., pelleted from solution at 3000 g. The high MW glycoprotein appears to be released from such a structure by chaotropic agents, and this is confirmed by the ability of SDS (another denaturing agent) to solubilise the protein, although it was not as effective in this respect as urea.

It has been observed by Steinert (1975), that in repeated experiments using unbuffered urea solutions the results obtained were variable with regards to the amounts of epidermal polypeptides solubilised. It was thus decided to buffer the 4 M urea using a 0.1 M tris/HCl buffer,

with a pH of 8.0. As mentioned above, variations in the pH have little effect on the solubilisation of the high MW glycoprotein and the value of 8.0 was chosen as it is near physiological pH, and because it is a pH that has frequently been used in epidermal studies (e.g. Scott & Harding, 1981; Ramsden et al., 1983). Additionally, as the pI of the high MW glycoprotein was not known, and as most cellular proteins have pI values in the range 4-7 (Section 6.2.5.), it seemed reasonable to buffer the urea at a slightly alkaline pH that was not far removed from physiological pH.

The protein preparation produced by homogenisation of the epithelium in the 4 M urea, 0.1 M tris pH 8.0 (tris/urea) buffer, was greatly enriched in the high MW glycoprotein (Figure 6.9), and this initial purification step greatly facilitated the subsequent isolation and purification of this protein. This subject will be discussed in greater detail in Chapter 7.

6.4.4. Possible Subcellular Sites For the High Molecular Weight Glycoprotein

From a knowledge of the ease of pelleting of this protein in aqueous solution, and from an awareness of its solubility in chaotropic/denaturing agents, a number of subcellular localisations may be suggested:

(1) The protein may be present, associated either with itself or with other proteins as a highly insoluble complex which is disrupted by denaturing agents such as urea.

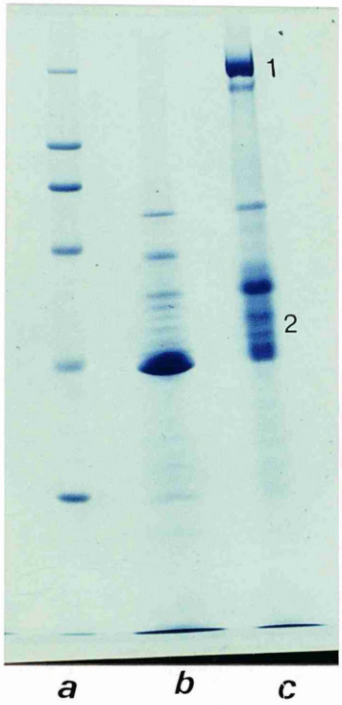


FIGURE 6.9: THE UREA SOLUBLE PROTEINS OF THE EPITHELIUM.

The gel used was 10 percent acrylamide.

(A) High molecular weight markers.

(B) Water soluble epithelial proteins.

(C) Proteins soluble in 4 M urea solutions.

1 The high molecular weight glycoprotein.

2 The keratin peptides.

(2) The protein may also be present hydrophobically associated with a rapidly pelleting subcellular structure. In the oral epithelium, the two most likely candidates for such a structure are the keratohyaline granules and the membrane coating granules (Sections 1.8.3. and 1.9.3.).

(3) It is also possible that the high MW glycoprotein could be associated with the highly insoluble epithelial keratin filaments. That this protein might be derived from the keratin filaments is suggested by the fact that urea not only increased the extraction of this protein, but also of several other proteins in the molecular weight range typical of the keratin proteins i.e. 45-70K (Figure 6.9). In addition, analysis of Figure 6.2 demonstrates that as well as the high MW glycoprotein, the majority of the keratin peptides appear to pellet under low centrifugal forces (e.g. 10 kg). It is of interest to note that Steinert et al. (1981) used a centrifugal force of 2000 g to pellet keratin filaments produced in vitro. This is a similar centrifugal force to that required to pellet the high MW glycoprotein from aqueous solution.

6.4.5. The Glycoprotein Nature of the High Molecular Weight Protein

Initial indications that the high MW protein may be a glycoprotein, came from attempts to analyse the molecular weight, which produced variable results depending on the porosity of the gel used. For this reason, PAS and alcian blue staining were employed. Both are relatively insensitive stains usually requiring more than 2 μg of protein bound carbohydrate in a band to allow detection (Andrews, 1981). The particular PAS staining method used in this thesis has been reported (Glossman & Neville, 1971) to detect as little as 0.5 μg

of bound carbohydrate in a single band, and is thus more sensitive than the alcian blue stain.

The band on the PAS stained gel corresponding to the high MW glycoprotein (Figure 6.7), stained very weakly. This may have been due to the protein having very little bound carbohydrate or may have been because there was insufficient protein in the band to allow for a strong staining reaction. It was not possible to increase the protein load as the load is restricted by the volume of the sample wells (50 μl maximum).

Additionally, higher loads of protein will lead to overloading and distortion of the protein bands, which would interfere with the staining procedure.

The lack of alcian blue staining could be accounted for on the above basis and thus no conclusions can be drawn from the insensitivity of the protein to alcian blue staining.

6.4.6. Possible Identity of the Three Proteins

The high MW glycoprotein is highly soluble in dissociating buffers and pellets from aqueous solution at 3000 g. It also appears to be resistant to proteolytic digestion and has been demonstrated to be a glycoprotein. The identity of this protein will be discussed in more detail in Chapter 7 and the remainder of this discussion will concentrate on the two lower MW proteins.

The 30K protein has been shown to be membrane/microsomal in origin and

it is possible that the 28K protein is also membrane/microsomal in origin. Bearing in mind that ethanol is regarded as an inducer of microsomal proliferation, especially in the liver (Lieber et al., 1979), it is possible that raised levels of these proteins may appear simply as a consequence of the ethanol related increase in the cellular microsome content.

Another, more speculative, suggestion is that one or more of these proteins may belong to the Heat Shock family of proteins. This is a family of proteins that undergo induction, or considerably enhanced synthesis, in response to environmental stress including heat and ethanol (Burdon, 1986). These proteins have been reported to be associated with both the cytoplasmic and the membrane/microsomal fraction of the cell (Pelham, Munro & Lewis, 1986) and it is suggested that they might be important for the development of thermotolerance and/or cell survival under conditions of environmental stress (Rodenhiser, Jung & Atkinson, 1985).

Chronic ethanol exposure of the type described in this thesis clearly represents an environmental stress and thus one or both of the two lower MW proteins may be heat shock proteins. In this way, enhanced levels of these proteins may represent a more general stress response of oral epithelial cells. It would be interesting to investigate whether or not it is possible to induce increases in the levels of these proteins in oral epithelial cells following treatment with alternative forms of stress e.g. heat. This approach would probably be suited more to tissue culture and would be worth investigating should a suitable oral epithelial tissue culture system become

available.

Further circumstantial evidence concerning the possible heat shock nature of one or both of these proteins, comes from studies of hamster fibroblasts, which report heat shock proteins of similar molecular weights (26K and 31K) (Li, 1983) to the two proteins in the present study. A recent report has also shown the existence of a 32K heat shock protein in the rat (Caltabiano et al., 1986).

6.5 CONCLUSIONS

This Chapter has reported on an initial characterisation of the three aberrant proteins involving peptide mapping studies, subcellular localisation studies, solubilisation studies and an assessment of the glycoprotein nature of the high MW protein.

It appears that the 30K protein is present in the cell associated with the membrane/microsomal fraction. The 28K protein may also be associated with this subcellular fraction, although as discussed in Section 6.3.2. definite conclusions cannot be drawn in this respect. Peptide mapping studies have shown these two proteins to be unrelated. Neither of these proteins appeared to show increased solubility in the various solubilising buffers used.

Subcellular localisation studies have shown that the high MW glycoprotein pellets from solution at a centrifugal force of 3000 g. Solubilisation studies showed this protein to be insoluble in water (or simple aqueous buffers) but to be highly soluble in 4 M urea solutions. It is thus suggested that this protein is present in the lingual epithelium associated with a rapidly pelleting structure that is held together by hydrophobic bonds. The fact that a number of proteins in the molecular weight range typical of the keratin peptides, spin down with the high MW glycoprotein, and that such proteins are also solubilised by the 4 M urea solutions, would suggest that the rapidly pelleting structure is most likely to be the epithelial keratin filaments.

CHAPTER SEVEN - CHARACTERISATION OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN

7.1 INTRODUCTION

7.1.1. General Introduction

Of the three aberrant proteins identified in this study, the one that proved most amenable to study was the high MW glycoprotein. It was simpler to obtain information on the characteristics of this protein: its molecular weight, its high solubility in urea solutions, its apparent resistance to proteolytic digestion and its glycoprotein nature. It was thus decided to concentrate on this protein, and to isolate and characterise it as far as possible. Such an approach for the two lower MW proteins would have been impossible in the time available, as the limited amount of information obtained on each of these proteins would make their definitive characterisation, during purification, difficult.

It was the aim of the studies presented in this Chapter, to purify the high MW glycoprotein and to determine its amino acid composition.

7.2 MATERIALS AND METHODS

7.2.1. Electrophoretic Techniques

Throughout this chapter, electrophoresis was performed exactly as described in Section 3.2 and all gels were 10 percent acrylamide.

7.2.2. Preparation of the Lingual Epithelial Extracts for Precipitation of the Urea Soluble Proteins

Given the high solubility of the high MW glycoprotein in urea solutions of 4 Molar (M) and the low solubility of this protein in solutions of 1 M (Section 6.3.3.), it seemed likely that dilution of the 4 M urea solutions of epithelial proteins with distilled water would precipitate this protein, along with other proteins not normally soluble in more dilute urea solutions. These proteins should then be harvestable by centrifugation. This is similar to the method of Ugel (1969) who isolated and characterised the highly insoluble keratohyaline granules on this basis. Two approaches are reported, one involving prolonged dialysis of the urea soluble proteins against distilled water, and the other involving dilution of the urea preparation with distilled water.

In both the methods discussed below (Sections 7.2.3. and 7.2.4.), the lingual epithelium was removed as described in Section 3.2.1. and was homogenised in ice-cold 0.1 M tris/4 M urea pH 8.0 (tris/urea: Section 6.4.3.). This homogenate was centrifuged at 50 kg for 20 minutes at 4° C in a Sorvall RC-5 Superspeed Refrigerated Centrifuge (DuPont, Stevenage, England) to remove insoluble material. The supernatant thus formed (henceforth referred to as the tris/urea preparation) was

ready for precipitation.

7.2.3. Precipitation of the Proteins by Dialysis

The first precipitation method involved extensive dialysis, initially over 24 hours, of the tris/urea preparation against several changes of ice cold distilled water at 4° C. After 24 hours, a white precipitate appeared in the solution and was harvested by centrifugation at 50 kg as described in Section 7.2.2.

In a separate experiment, the above procedure was repeated however, in this instance, phenylmethylsulphonyl flouride (PMSF), a potent inhibitor of serine proteases, was added to the tris/urea preparation and to the water against which this preparation was being dialysed. The final concentration of PMSF in the water was 10 μg/ml. This experiment was included to attempt to gauge the extent of enzymic proteolysis of the precipitated proteins.

The dialysis tubing was prepared by boiling in several changes of distilled, deionised water.

7.2.4. Precipitation of the Proteins by Dilution

The second precipitation method involved taking the tris/urea preparation, and diluting it 1:4 with ice-cold distilled water. The urea concentration in the preparation was now 1 M. This solution was vortexed and left on ice for one hour following which the precipitate (which was not normally visible) was pelleted by centrifugation as described in Section 7.2.2.

The surfaces of the pellets resulting from both of the above procedures were carefully washed with distilled H_2O. The pellets were then gently homogenised in distilled H_2O and 25 μl were removed for the purposes of protein concentration determination (Section 3.2.5.). The resulting volume was prepared for SDS gel electrophoresis (Section 3.2.2.) and the proteins analysed by SDS gel electrophoresis.

7.2.5. Preparative Gel Electrophoresis

To purify the high MW glycoprotein, preparative gel electrophoresis was performed.

Thirty male Sprague-Dawley rats (Bantin and Kingman, England) with a mean weight of 400 g, were sacrificed by cervical dislocation, the lingual epithelium was removed from each rat and the urea soluble proteins were prepared as described in Section 7.2.2. The urea soluble proteins were precipitated by dilution as described in Section 7.2.4. and prepared for SDS gel electrophoresis (section 3.2.2.).

The preparative gels were prepared as described in Section 3.2.6., except this time the well-forming comb was omitted, allowing the formation of a flat stacker gel surface. This allowed direct loading of large volumes (1.5 - 2 ml) of epithelial protein preparations enriched in the high MW glycoprotein (4 - 6 mg total protein loaded per gel). The preparative gel was electrophoresed overnight in a cold room at 75 volts. Such slow electrophoresis tended to result in sharper and straighter protein bands than would have been obtained had the gel been run at a higher voltage as in Section 3.2.6.

The region of the gel containing the high MW glycoprotein was identified by cutting guide strips (Andrews, 1981) from each side of the gel, and staining them briefly in a 0.1 percent solution of CBB R-250 in 50 percent methanol/10 percent acetic acid. The high MW glycoprotein band was usually visible after the staining procedure and destaining was not required. The major part of the gel was covered in 'clingfilm', and stored at 4° C during the staining procedure. The stained guide strips were placed alongside the gel and correctly aligned to show the position of the high MW glycoprotein band in the unstained gel (Figure 7.1). The region of the gel containing this protein was cut out using a fresh razor blade, chopped into small fragments, and transferred into the barrel of a 5 ml syringe. The gel fragments were then minced by passing them through the syringe nozzle. Following this treatment, extraction of the high MW glycoprotein from the minced gel slices was attempted by simple elution (Section 7.2.6.) and by re-electrophoresis (Section 7.2.7.).

7.2.6. Extraction of the High Molecular Weight Glycoprotein from Gel Slices by Simple Elution

It is important, in attempting to elute proteins from preparative gel fragments, to ensure that the buffer into which the protein is being eluted is one in which it is highly soluble. For this reason, the high MW glycoprotein was eluted into tris/urea (Section 6.4.3.).

Gel fragments, prepared as discussed in Section 7.2.5. were placed in a sterile plastic disposable 'universal' and covered with approximately three volumes of tris/urea. This mixture was stirred overnight (12 hours) at 4° C, following which the gel fragments were

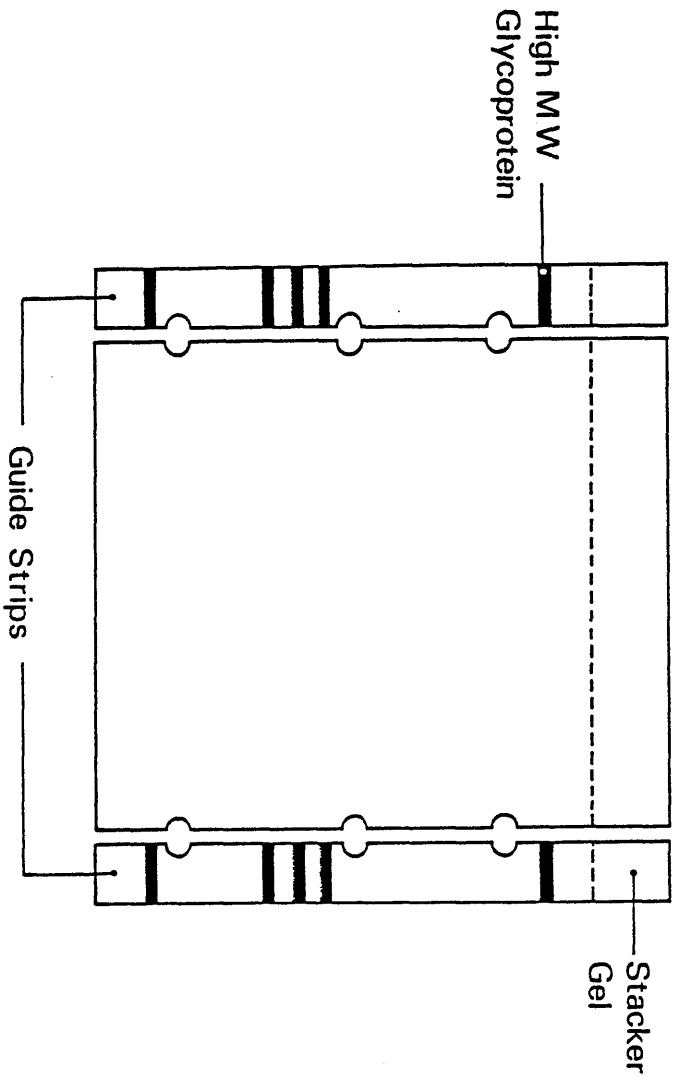


FIGURE 7.1: LOCALISATION OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN IN PREPARATIVE GELS USING GUIDE STRIPS.

pelleted by centrifugation (15 minutes at 1000 g) and the supernatant freeze dried for four hours in a Modulyo freeze dryer (Edwards, Crawley, England).

Elution was also attempted for shorter periods of time, e.g six hours at room temperature, following which the gel fragments were removed by centrifugation and the supernatant freeze dried as described above.

The residue left after freeze drying the supernatants from the above two elution attempts, was prepared for SDS gel electrophoresis (Section 3.2.2.) and analysed on SDS gels.

7.2.7. Extraction of the High Molecular Weight Glycoprotein from Gel Slices by Re-Electrophoresis

Extraction of proteins from preparative gel slices by re-electrophoresis is an extremely elegant and effective method. It involves resuspending fragmented gel slices (containing protein) in a second gel and electrophoresing the protein out of the gel fragments into a dialysis bag. It is a particularly valuable method for extraction from SDS gel fragments as the proteins in these fragments are shrouded in SDS (Section 3.1.3.) and migrate easily into the dialysis bag during re-electrophoresis.

The gel solution used for the re-electrophoresis was a phosphate buffered gel system of the Weber and Osborne (1969) type rather than the Laemmli tris/glycine buffered system (Section 3.2.7.). This change in buffer was required, as both tris and glycine would have interfered with subsequent amino acid analysis of the purified high MW

glycoprotein (Section 7.2.9.).

The Weber and Osborne gel solution was prepared as follows:

Distilled Water	6.44 ml
Phosphate Buffer*	16.5 ml
30% Acrylamide	5.43 ml
1% Bisacrylamide	4.44 ml
10% Ammonium Persulphate	120 μl
TEMED	20 μl

*The phosphate buffer was prepared by dissolving 51.6 g of Na_2HPO_4:12 H_2O in 800 ml of distilled deionised water, and adding NaH_2PO_4:$2H_2O$ to pH 7.1 (c.8.8 g was usually required). The final solution was thus 0.2 M phosphate, pH 7.1 and SDS was added to 0.2 percent. The electrophoresis buffer was a 1:1 dilution of this phosphate buffer with distilled deionised water.

The re-electrophoresis was performed as follows. Gel fragments, prepared as described in Section 7.2.5., were added to a small volume of a polymerising Weber and Osborne gel solution and then rapidly transferred in this solution to glass tubes (6 mm internal diameter). These glass tubes were sealed at one end with dialysis tubing, held in place by a small rubber gasket. This mixture was overlayed with water saturated isobutanol and allowed to polymerise. Polymerisation was normally complete in 20 minutes.

When the gels had polymerised, the dialysis tubing was removed from the end of the gel rods and replaced by small dialysis bags filled with the phosphate electrophoresis buffer (Figure 7.2). The gel rods

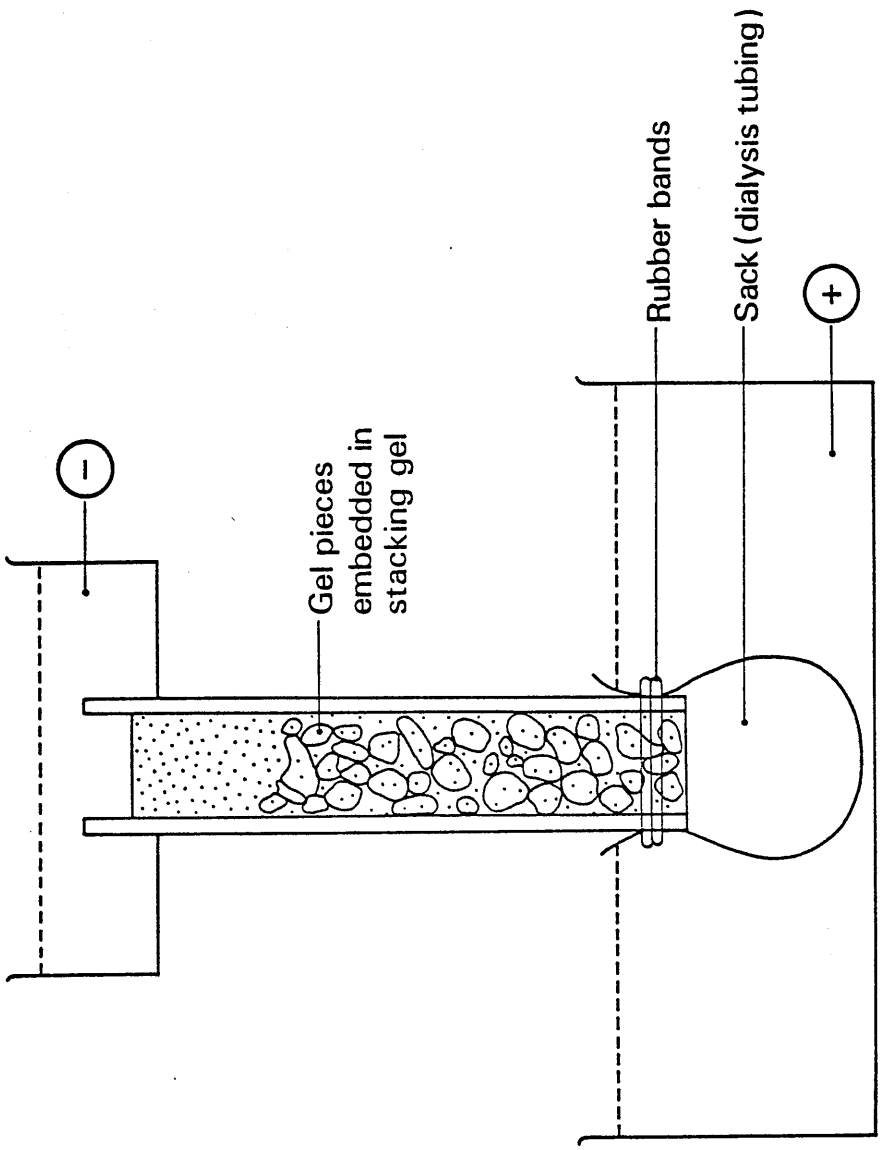


FIGURE 7.2: THE RE-ELECTROPHORESIS APPARATUS.

were then carefully loaded into a Shadon rod gel electrophoresis apparatus (Shandon Ltd., Cheshire, England) which was then filled with electrophoresis buffer. Electrophoresis was carried out at room temperature, at c 80 mA, for four hours. The progress of the electrophoresis was followed by loading a gel rod containing no preparative gel fragments, with a small amount of bromophenol blue marker dye. Electrophoresis was taken to be complete one hour after the bromophenol blue dye had reached the bottom of the gel rod and at this point the current was switched off.

The dialysis bags were carefully removed, the contents were pooled, placed in a second dialysis bag and set dialysing at 4° C for two hours against several changes of 1 percent SDS. This was done in an attempt to reduce the high concentrations of sodium and phosphate ions in the solution.

Following dialysis, the contents of the dialysis bags were freeze dried for approximately four hours. The freeze dried purified glycoprotein was then redissolved in 2 percent SDS and stored frozen at -20° C.

A blank gel rod, i.e. one containing Webber and Osborne gel solution with protein-free preparative gel fragments, was also re-electrophoresed as described above. Again the products of the re-electrophoresis were collected in a dialysis bag, dialysed and freeze dried. This control was included to allow assessment of the amino acid contaminants present in the preparative gel system.

7.2.8. Special Precautions During Re-electrophoresis

Initial results from these purification studies indicated high levels of protein contamination in the purified protein preparations (Section 7.3.4.). These artefacts were eliminated by adopting the following procedures during purification:

-Wearing gloves at all times.

-Preparing all glassware and dialysis tubing by boiling in 5 percent (w/v) sodium bicarbonate followed by five washes in boiling distilled, deionised water (Brown & Howard, 1983).

-All water was double distilled and deionised prior to use.

-The entire procedure was performed in an 'Inter Med microflow pathfinder' clean-air cabinet (Nunc, Stafford, England).

-The top of the re-electrophoresis gels were thoroughly washed with double distilled deionised water prior to re-electrophoresis.

The reasons for this approach shall be discussed in Section 7.4.4.

7.2.9. Amino Acid Analysis

Amino acid analysis was performed on c. 200 μg of the purified high MW glycoprotein in an LKB amino acid analyser (LKB Instruments Ltd., England). Quantitative analysis of the relatedness of the high MW glycoprotein to other epithelial/epidermal proteins of known amino acid analysis, was carried out using the following three equations:

Let A and B be two proteins, with N_A and N_B residues respectively, of which n_{iA} in A and n_{iB} in B are of the i^{th} type of amino acid.

$$(1)\ \mathbf{DI} = 50 \sum \left| \frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right|$$

This equation (Metzger et al, 1968) allows calculation of the difference index (DI), and it is suggested (Cornish-Bowden, 1980), that values < 13, give an indication of relatedness between the two proteins being compared.

$$(2)\ \mathbf{D} = \left[\sum \left(\frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right)^2 \right]^{\frac{1}{2}}$$

This equation (Harris & Teller, 1973) allows calculation of the composition divergence (D), and it is suggested that values < 0.07, give an indication of relatedness between the two proteins being compared.

$$(3)\quad S\Delta Q = 10^4 \sum \left(\frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right)^2$$

This equation allows calculation of the $S\Delta Q$ index of Marchalonis and Weltman, and it is suggested that values < 100, give an indication of relatedness between the two proteins being compared.

7.2.10. Identification of the High Molecular Weight Glycoprotein During Purification

As discussed in Section 6.4.3., it is essential to ensure that it is the protein of interest that is being purified, and not a protein of similar electrophoretic mobility. For this reason, the identity of the purified high MW glycoprotein was checked by confirming its resistance to proteolytic digestion, and its poor silver staining properties. Segrest and Jackson (1972) analysis of the molecular weight of the protein was also undertaken to check for the typical asymptotic curve of molecular weight versus acrylamide concentration (Section 6.2.6.).

7.3 RESULTS

7.3.1. Attempts at Precipitation of the High Molecular Weight Glycoprotein from Urea Solutions by Dialysis

Initial results from attempts at precipitation of the high MW glycoprotein by dialysis against distilled water, proved encouraging (Figure 7.3c). The protein preparation that was obtained from the precipitate was seen to be highly enriched with respect to the high MW glycoprotein and to contain few other proteins. Unfortunately, subsequent attempts at precipitation of the urea soluble proteins by this method proved less encouraging, yielding preparations that showed wide variations in protein content (Figure 7.3d and 7.3e). Also noticed in these subsequent dialysis studies was that very often the high MW glycoprotein was absent from the precipitated proteins (Figure 7.3d). Further analysis also showed it to be absent from the non precipitated proteins remaining in the dialysis bag (Figure 7.3f). It was thus concluded that the high MW glycoprotein was breaking down during the dialysis procedure, even at 4° C. Studies using PMSF in the homogenising solution and the dialysing solutions, showed that this protease inhibitor did not noticeably reduce the observed breakdown of the high MW glycoprotein. The high lability of the high MW glycoprotein, along with the inconsistent nature of the protein preparations, meant that an alternative and more rapid method of protein precipitation was required.

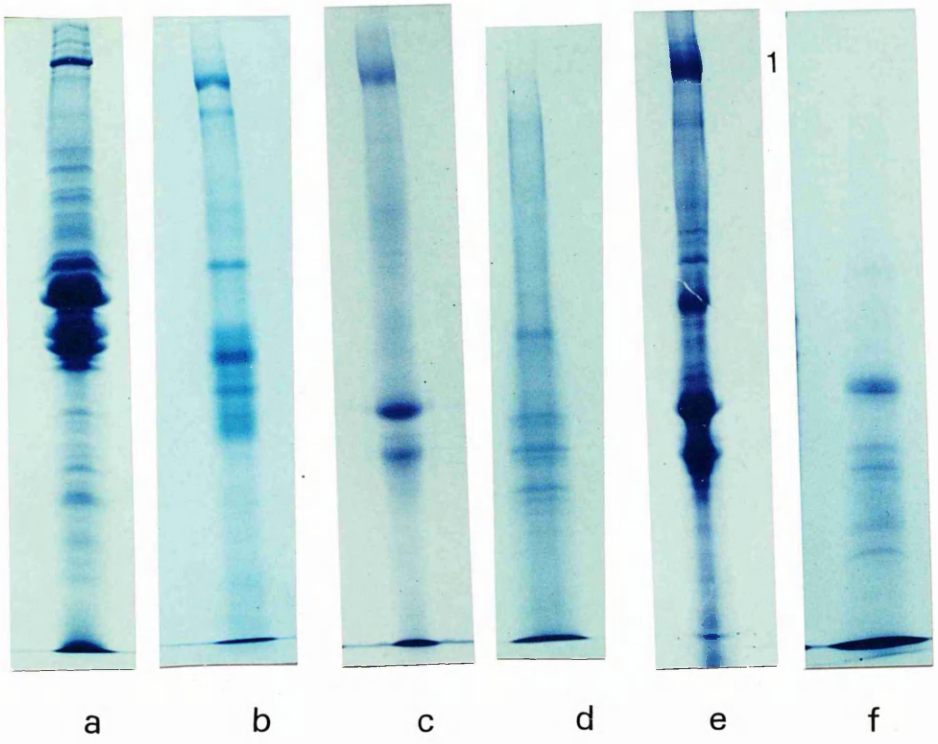


FIGURE 7.3: PROTEIN PREPARATIONS OBTAINED BY PRECIPITATION OF THE UREA SOLUBLE PROTEINS BY DIALYSIS.

(A) Total cellular proteins.

(B) Urea soluble proteins

(C) Urea/H_2O dialysis precipitate.

(D) Urea/H_2O dialysis precipitate.

(E) Urea/H_2O dialysis precipitate.

(F) Non precipitated proteins.

1 - The high molecular weight glycoprotein.

7.3.2. Attempts at Precipitation of the High Molecular Weight Glycoprotein by Dilution

One in four dilution of the tris/urea protein preparation, yielded a fine precipitate (often not visible) which was harvested by centrifugation and studied by SDS gel electrophoresis. The preparation produced was seen to be highly enriched with respect to the high MW glycoprotein (Figure 7.4). This protein preparation was suitable for preparative gel electrophoresis in that, on SDS gels, the high MW glycoprotein was seen to be free from surrounding proteins. This allowed for easy purification of the high MW glycoprotein from gel slices, with no obvious contamination from surrounding proteins.

7.3.3. Preparative Gel Electrophoresis and Extraction of the Glycoprotein from Gel Slices by Simple Elution.

Preparative gels were run as described in Section 7.2.5. and the band containing the high MW glycoprotein was successfully located, and cut out using the guide strip technique.

Attempts at extracting the protein from the minced gel slices (Section 7.2.6.) by simple elution into tris/urea over 12 hours at 4° C, proved unsuccessful. It is possible that the high MW glycoprotein was being eluted from the gel slices but was then breaking down. Shorter extraction times were used in an attempt to minimise the breakdown of this protein, but despite a slight increase in the yields, this method also proved to be of limited use in purifying large quantities of the high MW glycoprotein. This method of purification was therefore abandoned in favour of purification by re-electrophoresis.

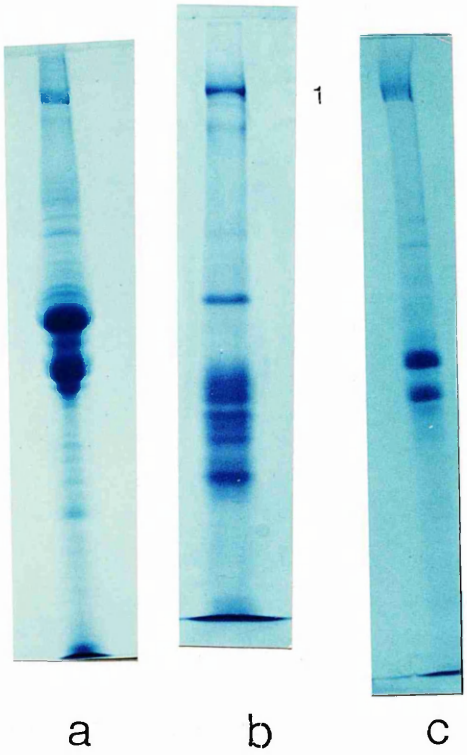


FIGURE 7.4: PROTEIN PREPARATION OBTAINED BY THE PRECIPITATION OF THE UREA SOLUBLE PROTEINS BY DILUTION.

(A) Total cellular proteins.

(B) Urea soluble proteins.

(C) Urea/H_2O dilution precipitate.

1 - The high molecular weight glycoprotein.

7.3.4. Purification by Re-Electrophoresis

Initial attempts at purification by re-electrophoresis demonstrated that it was indeed possible to isolate the high MW glycoprotein from the gel slices in this way. However, analysis of the isolated protein by SDS gel electrophoresis (Figure 7.5), showed two other proteins of molecular weight 67 and 56K in the isolated preparation. These two proteins were also observed to be present in the buffer from re-electrophoresis of the blank gel rod.

Identification of the origin of these proteins was attempted by running gels of concentrated preparations of all of the chemical components used during the purification procedure. These compounds were prepared for SDS gel electrophoresis as described previously (Section 3.2.2.). Silver staining (Section 6.2.3.) revealed the impurities to be present in each of the gel components tested (Figure 7.6). It was noticed, however, that considerably higher levels of these proteins were present in the dithiothreitol (DTT) preparation.

For the reasons discussed in Section 7.4.4., the precautions outlined in Section 7.2.8. were adopted for subsequent purification by re-electrophoresis. As shown in Figure 7.7, this procedure resulted in a highly purified preparation of the high MW glycoprotein, that was free of the above protein artefacts and was therefore suitable for amino acid analysis.

Densitometer traces summarising the various stages of the purification of the high MW glycoprotein are shown in Figure 7.8 a-d.

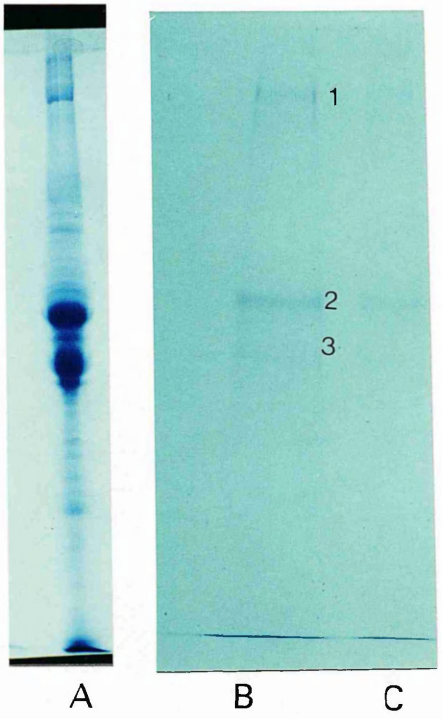


FIGURE 7.5: INITIAL RESULTS FROM ATTEMPTS AT PURIFICATION OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN BY PREPARATIVE GEL ELECTROPHORESIS.

(A) Total cellular proteins.

(B) Purified high molecular weight glycoprotein.

(C) Purified high molecular weight glycoprotein.

NOTE: 1 The purified high molecular weight glycoprotein.

2 The 67K contaminant protein.

3 The 56K contaminant protein.

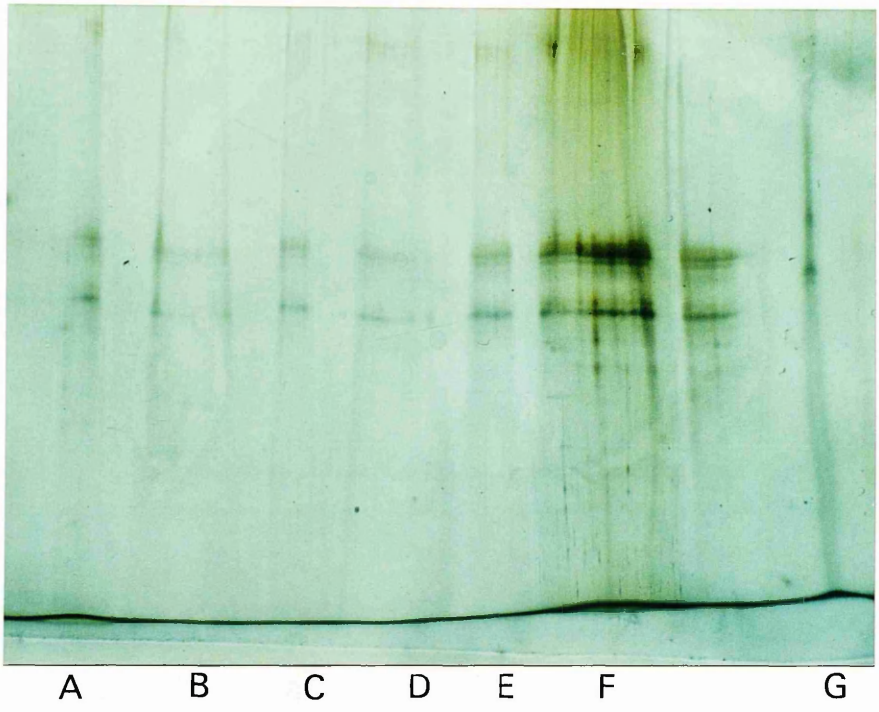


FIGURE 7.6: ANALYSIS OF GEL CONSTITUENTS IN AN ATTEMPT TO LOCATE THE ORIGIN OF THE CONTAMINANT PROTEINS.

(A) Glycerol

(B) Glycine

(C) SDS

(D) Tris

(E) Bromophenol Blue

(F) DTT

(G) Freeze dried water

Note the higher amounts of the contaminant proteins associated with the DTT.

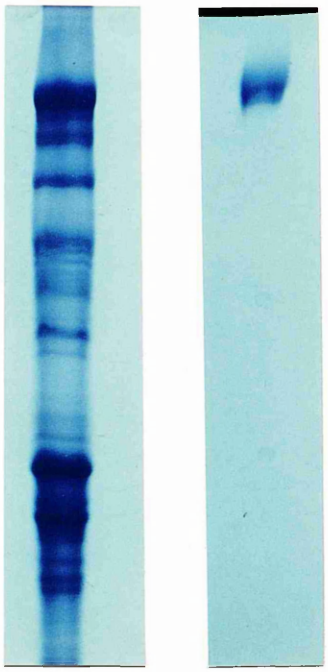
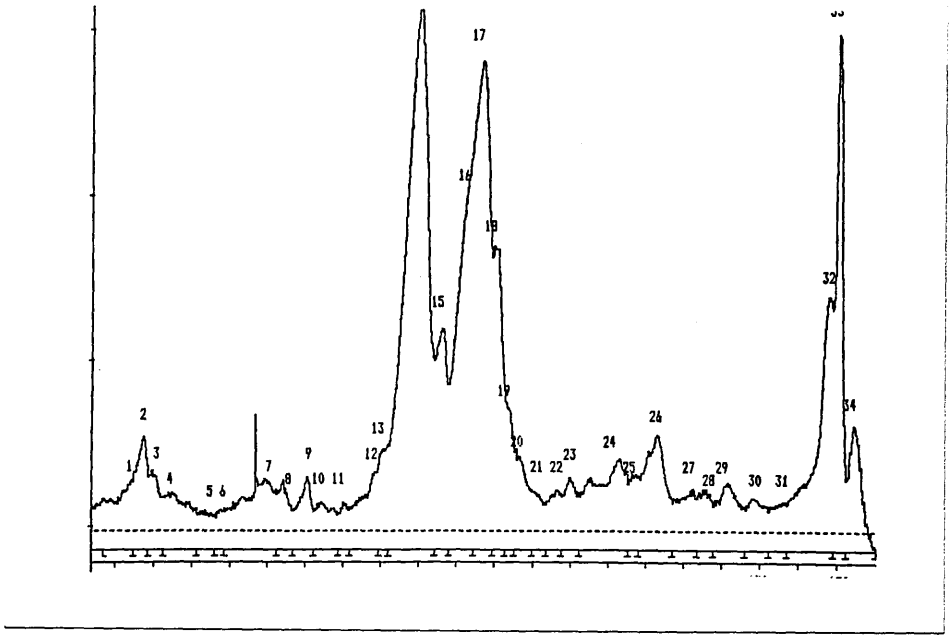


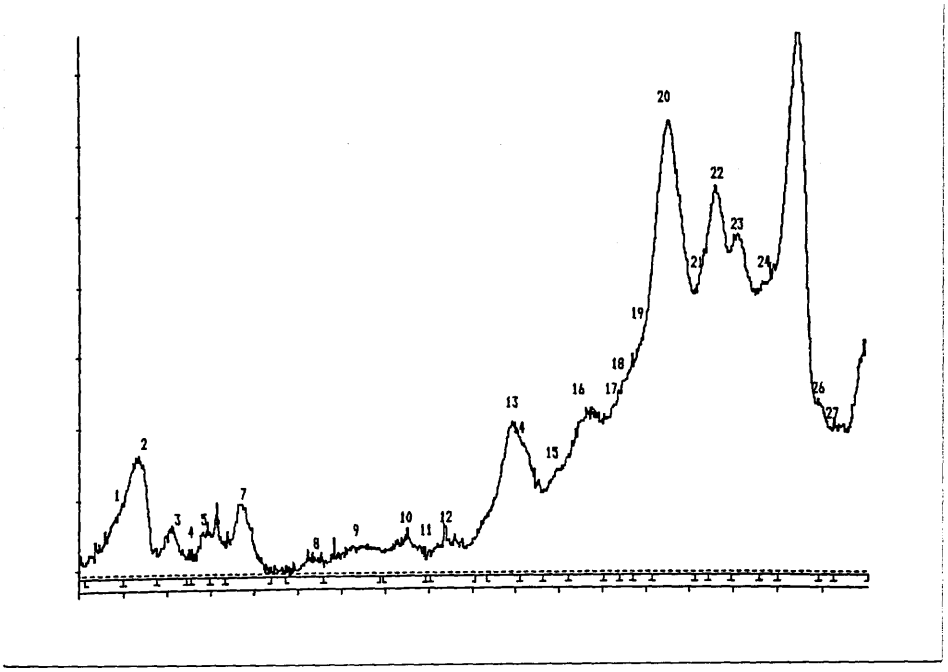
FIGURE 7.7: THE PURIFIED HIGH MOLECULAR WEIGHT GLYCOPROTEIN.

(A) Urea soluble proteins.

(B) The purified high molecular weight glycoprotein.

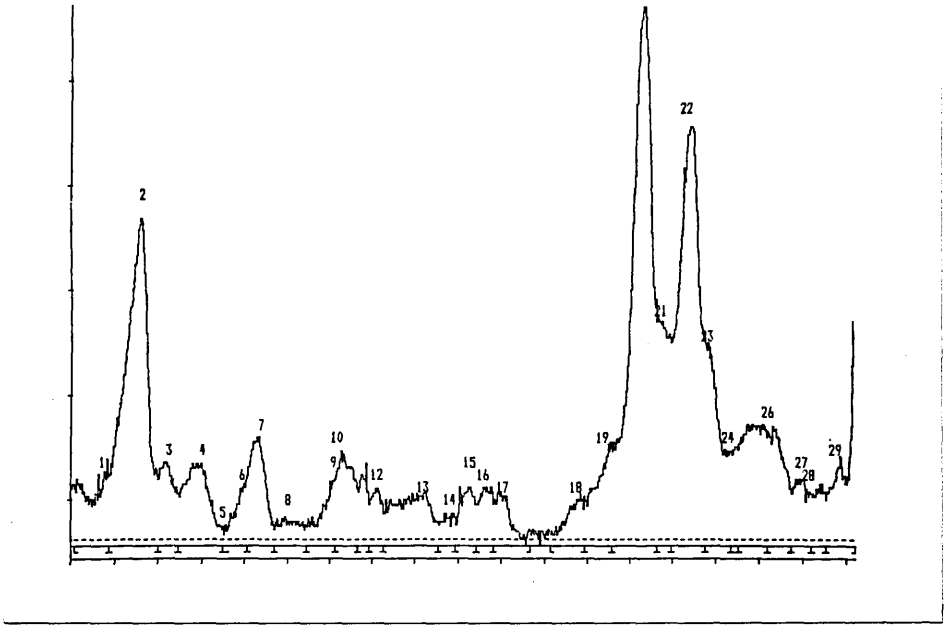


(A) TOTAL CELLULAR PROTEINS.

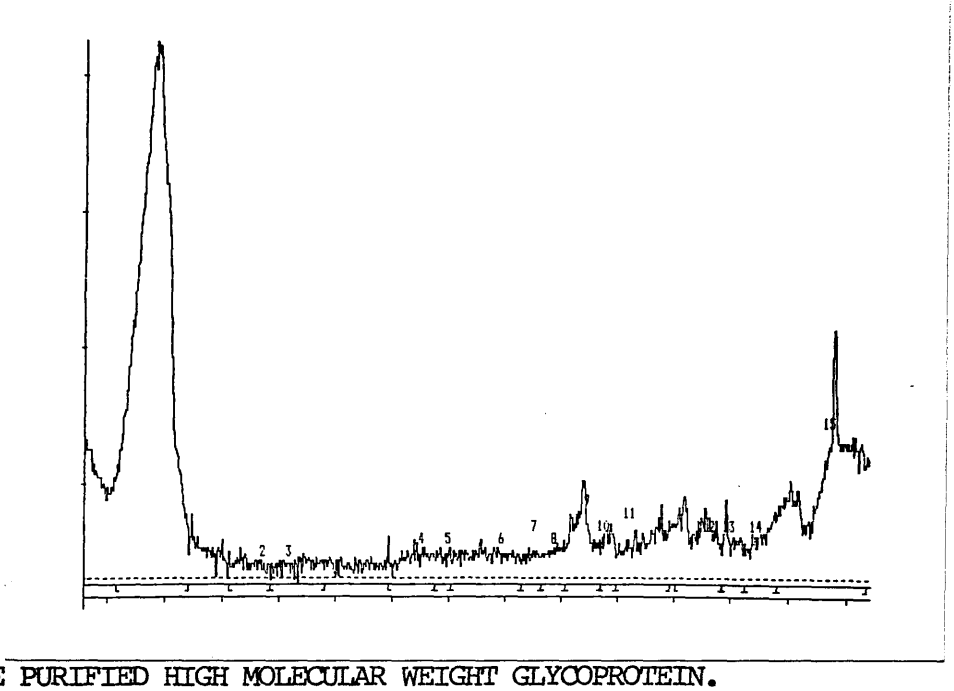


(B) UREA SOLUBLE PROTEINS.

FIGURE 7.8: DENSITOMETER TRACES SUMMARISING THE VARIOUS STAGES IN THE PURIFICATION OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN.



(C) UREA/H_2O DILUTION PRECIPITATED PROTEINS.



(D) THE PURIFIED HIGH MOLECULAR WEIGHT GLYCOPROTEIN.

FIGURE 7.8 (CONTINUED): DENSITOMETER TRACES SUMMARISING THE VARIOUS STAGES IN THE PURIFICATION OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN.

As mentioned in Section 7.2.10., the identity of the high MW glycoprotein was checked and was confirmed at each step of the purification.

7.3.5. Amino Acid Analysis

Analysis of the amino acid composition of the purified high MW glycoprotein is shown in Table 7.1. The results indicated that with the exception of minute amounts of glutamate, glycine and isoleucine, the electrophoresis buffer from the re-electrophoresis of the blank gel rod, was free from protein or amino acid contamination. The high MW glycoprotein appeared to be rich in glycine and the acidic amino acids aspartate and gultamate. Only very low levels of cysteine, histidine and methionine were detected.

The analysis also showed the presence of carbohydrate residues in the protein.

Quantitative analysis (Section 7.2.9.) revealed similarities in the amino acid composition of the high MW glycoprotein, and those of human epidermal fibrous proteins (DI=9.9, D=0.067 and $S\Delta Q$=44.3), bovine epidermal alpha keratin (DI=7.05, $S\Delta Q$=61.8), bovine prekeratin (DI=11.5, $S\Delta Q$=65.7) and rat oral epithelial alpha keratin (DI=12.8, $S\Delta Q$=86.7) (Table 7.2.).

	HIGH MW GLYCOPROTEIN	BUFFER
	(MOLE PERCENT)	(NANOMOLES)
ASPARTATE/ASPARAGINE	10.8	
THREONINE	5.4	
SERINE	6.0	
GLUTAMATE/GLUTAMINE	11.7	0.1
PROLINE	6.9	
GLYCINE	15.7	0.1
ALANINE	4.5	
CYSTEINE	1.3	
VALINE	4.9	
METHIONINE	0.9	
ISOLEUCINE	5.2	0.1
LEUCINE	9.5	
TYROSINE	2.6	
PHENYLALANINE	4.0	
HISTIDINE	1.9	
LYSINE	3.4	
ARGININE	5.4	
CARBOHYDRATE RESIDUES	2.3	

TABLE 7.1: AMINO ACID COMPOSITION OF THE HIGH MW GLYCOPROTEIN AND THE RE-ELECTROPHORESIS BUFFER.

	(1)	(2)	(3)	(4)	(5)
ASPARTATE/ASPARAGINE	10.8	9.2	8.1-9.6	9.2	7.1-7.8
THREONINE	5.4	4.0	3.7-4.6	3.9	3.9-4.7
SERINE	6.0	9.1	9.8-13.9	9.4	13.3-17.4
GLUTAMATE/GLUTAMINE	11.7	13.0	10.6-16.1	14.7	12.7-14.9
PROLINE	6.9	2.7	0.6-1.4	1.6	3.0-3.8
GLYCINE	15.7	18.5	12.4-17.2	15.5	16.5-20.3
ALANINE	4.5	5.1	3.8-6.6	6.4	3.4-4.1
CYSTEINE	1.3	---	-------	1.2	-------
VALINE	4.9	4.9	4.6-6.4	5.2	3.4-4.1
METHIONINE	0.9	1.2	1.1-2.2	1.8	0.7-1.2
ISOLEUCINE	5.2	4.9	3.7-4.4	4.0	2.9-3.2
LEUCINE	9.5	9.8	7.7-10.2	8.8	3.1-7.4
TYROSINE	2.6	2.3	2.1-5.1	3.0	1.9-2.5
PHENYLALANINE	4.0	4.3	2.2-3.7	3.6	2.2-2.8
HISTIDINE	1.9	1.0	1.0-1.4	1.1	2.3-3.0
LYSINE	3.4	5.5	4.4-5.2	5.0	4.0-5.7
ARGININE	5.4	4.5	5.4-7.0	6.1	3.1-4.9

TABLE 7.2: COMPARISON OF THE AMINO ACID COMPOSITION OF THE HIGH MW GLYCOPROTEIN AND THOSE OF OTHER SIMILAR EPIDERMAL/EPITHELIAL PROTEINS.

(1) Amino acid composition of the high molecular weight glycoprotein.

(2) Amino acid composition of the human alpha fibrous proteins (Baden & Goldsmith, 1972)

(3) Amino acid composition of bovine epidermal alpha keratin (Steinert & Idler, 1975)

(4) Amino acid composition of bovine epidermal prekeratin (Steinert, 1975)

(5) Amino acid composition of rat oral alpha keratin (Dale, Stern & Clagett, 1977)

7.4 DISCUSSION

7.4.1. Precipitation of the High Molecular Weight Glycoprotein

One of the major obstacles to the purification of this protein by dialysis, was its extreme lability at 4° C, even in the presence of protease inhibitors. Indeed, the results from the dialysis studies were highly variable, and often the high MW glycoprotein appeared to be totally absent from the precipitated protein population. The possibility that this protein was cold labile and was in fact breaking down at 4° C was tested by carrying out the dialysis procedure at room temperature. The protein appeared more labile at this temperature therefore cold lability could not account for the protein degradation observed at 4° C.

It was thus concluded that the protein was breaking down by spontaneous denaturation which did not require the presence of any proteolytic enzymes.

Periods of dialysis shorter than 24 hours would clearly have been preferable. However, it was only after prolonged dialysis that proteins precipitated from solution in large quantities. Precipitation by dialysis was therefore abandoned in favour of precipitation by dilution.

Precipitation by dilution provided a considerably more rapid alternative (1 hour) and the results did not reveal an obvious breakdown of the high MW glycoprotein. Attempts were made to see if dilution of the urea to concentrations below one molar would increase

the yields of the high MW glycoprotein. Such dilution did not appear to affect the yields of the high MW glycoprotein, but did increase the total number of other proteins precipitated. This clearly represented an unnecessary contamination of the precipitated proteins and thus the 1:4 dilution was adopted.

Studies using various times of precipitation revealed that maximal precipitation of the high MW glycoprotein, from the diluted preparation, was achieved after one hour.

Visual analysis of the proteins precipitated by the above two methods (Figures 7.3 and 7.4), revealed a further advantage of the dilution procedure. Whereas in the dialysis preparation a protein, with a molecular weight just lower than that of the high MW glycoprotein, was seen precipitating from solution, this protein was absent from the dilution preparation. The dilution preparation was therefore ideal for preparative gel electrophoresis.

7.4.2. Preparative Gel Electrophoresis

To allow preparative gel electrophoresis of high loads of the precipitated protein preparation, it proved useful to simply adapt the analytical type gels discussed in Chapter 3. This was achieved by omitting the well forming comb and allowing a flat stacker surface to form.

Various methods were used in attempting to localise the region of the preparative gel containing the high MW glycoprotein. Simple staining with CBB R-250 (Section 3.2.8.) was undesirable as coomassie blue has

been reported to interfere with amino acid analysis (Hames & Rickwood, 1981). Various other methods were therefore attempted to try to localise the high MW glycoprotein in the preparative gel. Little success was achieved with attempts to obtain a fluorescent dansylated derivative of the epithelial proteins (using the method of Talbot and Yphantis, 1971) which would have allowed localisation of the protein in the gel under ultra violet light. Another method attempted, that of visualising the protein bands in the gel by cooling for several hours (Wallace et al, 1974) also proved to be of little use.

The best method for localising the high MW glycoprotein band in the preparative gel appeared to be staining of guide strips. The absence of protein in the neighbouring regions of the gel allowed the high MW glycoprotein band to be cut out from the preparative gel without the problem of contamination by neighbouring proteins.

7.4.3. Extraction of the Glycoprotein from the Gel Slices

Attempts at extracting the high molecular weight glycoprotein from gel slices by simple elution into a buffer in which this protein is known to be soluble, met with little success. Two factors may have accounted for this. Firstly, the protein may have been rapidly broken down during extraction from the gel. Both attempts described (Section 7.2.6.) involved either prolonged elution (12 hours) at 4° C, or shorter periods at room temperature. Both prolonged exposure at 4° C and shorter exposure at room temperature appeared to be harmful to the protein (Section 7.4.1.).

Secondly, this method is at best reported to result in yields of 60 percent and more often in yields as low as 15 - 20 percent (Hames & Rickwood, 1981). Thus, the very low level of protein extracted from the gel fragments by this method, may simply have reflected the inefficiency of the procedure. Extraction was therefore carried out by re-electrophoresis.

After adopting the precautions outlined in Section 7.2.8., re-electrophoresis led to a preparation of the high MW glycoprotein that appeared on SDS gels to be purified to homogeneity. Despite this success, the technique did not result in particularly high yields of protein. This may have been due to the protein breaking down during re-electrophoresis or to the slow migration of the protein from the gel fragments. Longer periods of re-electrophoresis were undesirable because of the inevitable increased breakdown of the extracted protein.

7.4.4. The Protein Artefacts Observed During Re-Electrophoresis

As mentioned in Section 7.3.4., initial attempts at extracting the high MW glycoprotein from gel slices by re-electrophoresis, yielded a preparation that was contaminated by two lower molecular weight proteins (56K and 67K). These proteins were originally thought to be breakdown products of the high MW glycoprotein produced during the re-electrophoresis. However, the presence of these two proteins in the buffer preparation disproved this. Analysis of the possible origin of these proteins indicated that they were in some way related to the presence of DTT. Marshall and Williams (1984) reported similar protein artefacts, and estimated the molecular weights to be 54K and

68K respectively. It appears that these two proteins are common contaminants in protein gels in which a reducing agent, e.g. DTT or β-mercaptoethanol is involved (Ochs, 1983; Marshall & Williams, 1984), although they are generally only observed at the level of silver staining. Their entry into the gel appears to be dependant on the presence of reducing agents and it is suggested that they are in fact keratin peptides (Ochs, 1983) possibly resulting from contamination by skin proteins. Freeze drying of the initial re-electrophoresed protein preparations (Section 7.3.4.) appeared to have concentrated these proteins to such an extent, that when the reconstituted protein preparation was run on an SDS gel, the artefacts were visualised by simple coomassie staining (Figure 7.5). Careful observance of the special precautions outlined in Section 7.2.8. resulted in a protein preparation that was free from these protein artefacts.

7.4.5. The Amino Acid Analysis

Unanticipated problems were encountered during the amino acid analysis because of the presence of large amounts of SDS in the purified high MW glycoprotein preparation. In general, SDS will not interfere with amino acid analysis (Hames & Rickwood, 1981) although in this case it appeared that the levels of SDS were so high that a definite effect, consisting of a shift in the amino acid elution profile, was noticed. This made identification of individual amino acids more dificult than would otherwise have been. Nevertheless, the results presented (Table 7.1) are believed to be correct, and an accurate representation of the amino acid composition of the high MW glycoprotein.

One notable feature of the amino acid composition is the very low levels of cysteine. Chuba and Palchaudhuri (1986) suggested that low levels of cysteine in proteins leads to anomalous silver staining and this may correlate with the poor silver staining properties (Section 6.3.1.) of the high MW glycoprotein.

It appears from the analysis, that although the high MW glycoprotein is indeed glycosylated, it is only lightly so. This may account for the poor PAS staining properties of this glycoprotein (Section 6.4.5.).

7.4.6. The Possible Identity of the High Molecular Weight Glycoprotein

The high MW glycoprotein occasionally bands as a closely associated doublet on SDS gels (usually of low porosity). It also often appears as a very diffuse band and shows anomalous migrational characteristics on SDS gels. The protein is also extremely prone to proteolysis. These properties are typical of Profilaggrin (Lonsdale-Eccles, Haugen & Dale, 1980; Scott & Harding, 1981; Harding & Scott, 1983 and Fleckman, Dale & Holbrook, 1985) a keratin matrix protein found in the epidermis (Scott & Harding, 1981) and epithelium (Smith & Dale, 1986). Profilaggrin is also similar to the high MW glycoprotein in the strategy used for its purification. Despite this considerable circumstantial evidence, the amino acid composition of the high MW glycoprotein showed little similarity to that of rat lingual epithelial profilaggrin.

A review of the amino acid compositions available for other epidermal and epithelial proteins, however, indicated a strong homology (Table

7.2) between the amino acid composition of the high MW glycoprotein and that of the keratin peptides of the epidermis and epithelium. Major differences were only seen in the levels of two amino acids with the levels of serine being lower and the levels of proline being higher than normally reported for the keratin peptides. These similarities were confirmed by the quantitative analyses reported in Section 7.3.5. It has been suggested (Cornish-Bowden, 1980) that such analyses are of limited value in comparison of proteins with markedly different molecular weights e.g. the high MW glycoprotein and the keratin peptides, although they do give an indication of relatedness.

The possibility that the keratinous artefacts were not completely removed during the purification procedure and that the amino acid analysis is affected by these peptides was discounted. As Figure 7.7b shows, these artefacts were not visible in the coomassie stained preparation of the purified high MW glycoprotein. Subsequent silver staining revealed only very small levels of these contaminant proteins which could be accounted for by the presence of dithiothreitol in the buffer used to prepare the purified protein for SDS PAGE (Section 3.2.2.). These proteins were thus, not believed to be present as contaminants in the purified preparation and this was confirmed by the almost total absence of amino acids in the buffer preparation. In addition, it is known (Dayhoff, 1972) that the keratin artefacts have extraordinarily high molar percentages of cysteine (greater than 17 percent), which are clearly not seen in the amino acid analysis of the high MW glycoprotein.

It was therefore concluded that this protein was a member of the keratin family, but was unusual in that it had a much higher molecular weight than that typically associated with the keratin peptides i.e. 45-70K (Steinert & Cantieri, 1983). Keratins are not believed to be synthesised from large precursors (Schweizer & Goerttler, 1980) and any interchain disulphide bonds binding keratin peptides together, would have been disrupted by the DTT used in preparing the proteins for SDS PAGE (Section 3.2.2.). It appeared, therefore, that this protein must represent a number of keratin chains (probably three), covalently bound together by bonds other than the classical disulphide bonds.

Reports have appeared (Steinert & Idler, 1979 and Eichner, Bonitz & Sun, 1984) of an epidermal alpha keratin protein with a molecular weight of 100-200K which is present in the upper layers of the epidermis. Analysis has shown these proteins to be polymers of keratin peptides, covalently bound together by ϵ-(γ- glutamyl) lysine linkages. The existence of this linkage was first suggested by Goldsmith et al (1974) and later confirmed as a covalent binder of epidermal keratins by Abernethy, Hill and Goldsmith (1977). It is suggested (Buxman & Wuepper, 1975), that these linkages are important during the final stages of keratinisation.

The presence of a high MW keratin peptide is apparent in the keratin preparations from human lingual epithelium (Clausen et al, 1986) although this protein is not discussed in the text. Proteins of similar molecular weight are also obvious in the keratin preparations of Fuchs and Green (1980) and Franke et al (1981).

There are no reports of the subcellular localisation of this protein in epidermal or epithelial cells, and thus it is not possible to determine whether or not this protein is a component of the keratohyaline granules or the keratin filaments (Section 6.4.4.).

7.5 CONCLUSIONS

The high MW glycoprotein has been isolated and characterised by preparative gel electrophoresis of a preparation of epithelial protein, highly enriched in this high MW glycoprotein. The glycoprotein has been shown, by amino acid analysis, to be a member of the keratin family of proteins, but to be unusual in this respect in that it is of a considerably higher molecular weight than that typically associated with the keratin peptides. It is believed that this protein is composed of a number (probably three) of normal keratin chains covalently bound to each other via ϵ-(γ-glutamyl) lysine linkages.

CHAPTER EIGHT - CONCLUDING DISCUSSION

8.1 INTRODUCTION

The work presented in this thesis was carried out with a view to investigating the effects of alcohol on the protein biochemistry of rat lingual epithelium. Alcohol is a known aetiological factor in the development of oral cancer and it was hoped that results from these studies would provide clues to the possible role of alcohol in this disease process. This Chapter summarises the work presented in Chapters two to seven.

8.2 THE SUCCESS OF THE ANIMAL MODEL

The animal model described in Chapters two and four, proved to be successful in the two separate studies. No mortality was observed and each of the animal groups appeared to thrive on their given diet. The usefulness of this animal model as a model of chronic alcohol abuse was demonstrated by assessing liver damage in the alcoholic animals. Liver damage was demonstrated both enzymically, with Gamma-Glutamyl Transferase being of particular use in this respect, and histologically, with centrilobular fatty infiltration being observed in the livers of the alcoholic animals.

Thus, the pair-feeding procedure was a valid model of chronic alcohol abuse, ideal for the work presented in this thesis. There is no reason to assume that this technique might not also have been useful over a considerably longer period than that used in the present study.

Indeed, Mascres and Joly (1981) successfully used this same model over 290 days.

There are, however, a number of problems associated with this model. As well as the typical practical difficulties associated with animal experiments, the technique is particularly time consuming and the liquid diet very expensive. In addition, the liquid diet appeared to affect the levels of liver enzymes, especially those of the Transaminase family (AST and ALT), making enzymic assessment of liver damage in the alcoholic animals, more difficult than it might otherwise have been.

8.3 THE PROTEIN STUDIES

8.3.1. Introduction to the Protein Studies

The protein studies represented the major thrust of the work described in this thesis and the particular method employed was that of SDS PAGE in slab gels. Using this technique, the lingual epithelial proteins from alcoholic and control animals were investigated and compared.

8.3.2. Results from the Protein Studies - 102 Days

Initial results from the 102 day study, revealed, in the alcoholic animals, a reduction in the levels of a high MW glycoprotein (160K) and an increase in the levels of two lower MW proteins (30K and 28K). These visible differences were confirmed and quantified by laser densitometry.

8.3.3. Results from the Protein Studies - 30 and 60 Days

The 30 and 60 day study was designed to investigate the alterations in the levels of the three aberrant proteins over one- and two-thirds of the original 102 day study. It proved possible to follow the alterations in the levels of these three proteins, in the alcoholic animals, over the 30 and 60 day time points and it was concluded, therefore, that for the protein alterations to occur, chronic alcohol consumption was required. The first significant protein alterations were noted at 60 days with reduced levels of the high MW glycoprotein and increased levels of the 30K protein being observed in the alcoholic animals.

The levels of the 28K protein were not significantly increased in the alcoholic animals until 102 days.

As discussed earlier (Section 5.4.1.), 60 days is equivalent to approximately one tenth of the total life span of a laboratory rat and alcohol consumption over such a time period clearly represents chronic exposure.

The results indicated that a precursor/product relationship (Section 5.4.2.) between the lowered levels of the high MW glycoprotein and the increased levels of the two lower MW proteins is unlikely. Each of the three proteins were observed to be capable of appearing at altered levels independently of each other.

8.3.4. The Questions Remaining

What these studies did not reveal is whether the protein effects were the result of local or systemic effects of ethanol, or indeed whether they were a direct result of ethanol itself rather than one of its metabolites. The animal model used in this study would be of little use in attempting to answer these questions and an alternative approach would be required.

The use of the hamster as the experimental animal may be of value in assessing whether the effects are local or systemic, as the hamster does not show detectable blood ethanol levels even after consuming ethanol solutions up to 40 percent (McMillan et al, 1977). Intragastric or intravenous administration methods would allow the oral cavity to remain free of the ingested alcohol and would therefore allow assessment of the purely systemic effects of ethanol on the epithelial proteins. Tissue is available from an intragastric intubation ethanol study, carried out at Liverpool Dental Hospital, and shall be analysed at a later date.

8.4 INITIAL CHARACTERISATION OF THE THREE PROTEINS

8.4.1. Introduction

To gain an insight into the possible functions of the three aberrant proteins, and to suggest mechanisms whereby their levels might be altered, it was considered appropriate to characterise them as far as possible. The first characterisation experiment involved attempts to detect a relationship between the high MW glycoprotein and the two lower MW proteins. This involved the use of peptide mapping studies

which were unfortunately not very informative due to the apparent resistance of the high MW glycoprotein to proteolytic digestion. This apparent resistance to digestion may be related to the poor silver staining properties of this protein as silver staining was the method used to detect the proteolytic fragments.

8.4.2. The 30K and 28K Proteins

The 30K protein is a membrane/microsomal associated protein that appears to be maximally soluble in distilled water.

The 28K protein proved more difficult to study as it appeared to band as a closely associated doublet on SDS gels. It is not yet clear which member of this doublet was enhanced in the alcoholic animals; it may even have been both. One member of this doublet is associated with the membrane/microsomal fraction of the cell and the other with the cytoplasmic fraction. Again, as with the 30K protein, this protein appears to be maximally soluble in distilled water.

8.4.3. The Possible Identity of the two Lower Molecular Weight Proteins

As discussed in Section 6.4.6., a number of suggestions can be made regarding the nature of these two proteins. It is conceivable that since at least one of them was associated with the membrane/microsomal fraction of the cell, enhanced levels may have resulted simply as a result of ethanols ability to increase the cellular microsomal content, which presumably would also have increased the levels of associated microsomal proteins.

Another suggestion is that one or both of these proteins are heat shock proteins, whose raised levels may represent a non specific cellular response to an environmental stress, i.e. chronic alcohol consumption. Although heat shock proteins, in tissue culture systems, are manufactured rapidly following exposure to the appropriate stress, in the in vivo situation described in this thesis, it may take considerably longer for an environmental stress to express itself in a highly keratinised tissue such as the rat lingual epithelium. Thus the requirement for chronic alcohol consumption does not necessarily argue against the possibility that one or both of these proteins may be heat shock proteins.

8.4.4. The High Molecular Weight Glycoprotein

The high MW glycoprotein has proved easier to study as a result of a number of unusual properties. In SDS gels, this protein has shown unusual molecular weight characteristics similar to those exhibited by glycoproteins, and staining studies and analysis of the carbohydrate content of the protein revealed that it was in fact lightly glycosylated. This protein appeared to be resistant to proteolytic digestion although, as discussed in Section 8.4.1., this may be a consequence of the poor silver staining properties of the protein.

Subcellular localisation studies indicated that the high MW glycoprotein pellets from solution at 3000 g. Solubilisation studies demonstrated that for effective solubilisation a denaturing agent, such as urea, was required. Upon dialysis, or dilution of the urea soluble protein preparation with water, the high MW glycoprotein (along with other proteins not soluble in water or dilute urea

solutions) precipitated out and was harvestable by centrifugation. It is believed that these results indicate that the protein resides in the epithelium attached to, or part of, a rapidly pelleting structure which pellets from solution at 3000 g and which is held together by hydrophobic forces that can be disrupted by denaturing agents. The most likely candidates for such a structure are the keratin filaments of the epithelium which are known to show similar solubilisation properties to the high MW glycoprotein and which also precipitate from urea solutions following dialysis or dilution (Ugel, 1969). Another possible candidate for this structure is the keratohyaline granules of the epithelium which again show similar solubility properties to the high MW glycoprotein, and which reaggregate and precipitate from urea solution following dialysis or dilution (Ugel, 1969).

The high MW glycoprotein has been purified on the basis of its high solubility in 4 M urea solutions and its very low solubility in more dilute urea solutions. Preparative gel electrophoresis, of protein preparations enriched in this high MW glycoprotein, was carried out and the protein was extracted from the preparative gel slices by re-electrophoresis. An amino acid analysis was carried out on this purified protein which showed it to be a high molecular weight keratin protein which, as mentioned previously, was lightly glycosylated.

Given that keratin peptides are characteristically 45-70K in molecular weight (Steinert & Cantieri, 1983), it appears unlikely that this protein represents a single keratin peptide chain. The inclusion of dithiothreitol in the buffer used to prepare proteins for SDS PAGE

would have ruptured any disulphide bonds which are the common interpeptide chain covalent linkages. It was thus concluded, that some alternative form of covalent bonding was present in this protein. A likely candidate for such a bond, and one that has been described in high molecular weight keratin proteins of a size similar to the protein identified in this study, is the ϵ-(γ-Glutamyl) Lysine linkage. The high MW glycoprotein is therefore seen as being a polymer of keratin peptides, probably three, held together by ϵ-(γ-Glutamyl) Lysine linkages.

8.4.5. The Possible Significance of the Lowered levels of the High Molecular Weight Glycoprotein in the Processes of Oral Carcinogenesis

The permeability of the oral epithelium is governed by a number of interacting factors, including the keratin peptides and the extruded products of the membrane coating granules (Scheuplein & Bronaugh, 1983). Permeability is believed to be an important factor in controlling the development of oral squamous cell carcinoma (Squier, 1980). Lowered levels of the keratin peptides, or alterations in their structural arrangement may compromise the effectiveness of epithelial permeability. In this way, lowered levels of the high MW glycoprotein may lower the integrity of the epithelial permeability barrier and may allow an increased entry of potentially carcinogenic compounds into the epithelium. This may thus explain the observed aetiological relationship between chronic alcohol abuse and oral cancer. It is of interest to note, in this respect, that alterations in mucosal permeability have been suggested by a number of authors as the possible mechanism underlying the relationship between alcohol

abuse and cancer (Squier, Cox & Hall, 1986 and Bjarnason, Ward & Peters, 1984).

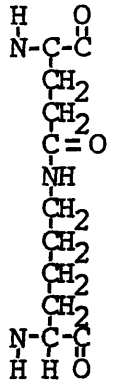
8.5 THE POSSIBLE MECHANISM BEHIND THE LOWERED LEVELS OF THE HIGH MOLECULAR WEIGHT GLYCOPROTEIN

Suggestions as to the possible mechanisms underlying the reduction in the levels of the high MW glycoprotein must necessarily be speculative. There are clearly a number of possible mechanisms that may result in lowered levels of this protein involving, for example, the effects of ethanol, or one of its metabolites, on protein synthesis at the level of transcription or translation.

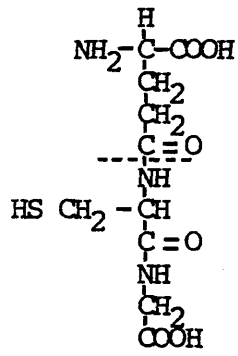
A simpler, although perhaps more realistic, explanation can be suggested without resorting to investigations at the genetic level. As demonstrated in many studies, including those presented in this thesis, raised serum Gamma-Glutamyl Transferase (GGT) levels can be detected in the serum of chronic alcohol abusers as a result of liver damage. GGT in other body sites that are exposed to alcohol may also therefore show increased levels and, given the demonstration of GGT in the epidermis (Chiba & Jimbow, 1986), it is reasonable to assume that GGT will be present in the oral epithelial cells and raised levels may also be found there.

The bond proposed to hold the keratin peptides together in the high MW glycoprotein (i.e. the ϵ-(γ-Glutamyl) Lysine linkage), is structurally very similar to the γ-glutamyl peptide bond that is cleaved by GGT during the amino transferase reaction.

i.e.



(1) ϵ-(γ-Glutamyl) Lysine linkage



(2) Glutathionine, the cellular substrate for gamma glutamyl transferase. NOTE,--- marks the point of cleavage of this peptide

It is possible, therefore, that the ϵ-(γ-glutamyl) lysine bond may be able to act as a substrate for GGT and that increased GGT levels in the oral epithelium may lead to increased cleavage of this bond resulting in lower levels of the high MW glycoprotein being evident in the epithelium. In such a situation, it is probable that a number of keratin peptides of more typical molecular weight, will be produced, although in the total cellular protein preparations investigated in Chapters 3 and 5, such minor increases in the levels of the highly abundant keratin peptides would be very hard to detect.

8.6 FUTURE WORK

8.6.1. Introduction

The work presented in this thesis opens up a potentially large area for study. For example, it would be of interest to attempt to study epithelial proteins in lingual biopsies from human alcoholics. There would clearly be ethical and practical difficulties involved in such a study (Section 1.10.1), although these may not be insurmountable.

Demonstration of protein alterations in the lingual epithelium of alcoholic humans would be of considerable interest, especially if they echoed the findings in the rat model.

8.6.2. Further Animal Studies

Tissue remaining from the animal studies described in this thesis, is available for further work. A histological analysis of the effects of alcohol on the lingual epithelium is currently being carried out in this department using tissue set aside specifically for this purpose from the 102 day study. Similarly, pieces of tissue are available from the 30 and 60 day study, and these shall be analysed at a later date.

Palatal epithelium set aside from the 102 day and the 30 and 60 day studies, and stored in liquid nitrogen, is also available for study. It is intended to isolate and study the keratin peptides from these pieces of tissue with a view to investigating the role of alcohol specifically on the keratin peptides. Such an approach will clearly be of value given the known properties of the high MW glycoprotein.

New animal studies may be of value in attempting to study the local and systemic effects of alcohol. This is discussed in Section 8.3.4.

8.6.3. Tissue Culture Based Studies

The major disadvantages of the animal model are that it is expensive, time consuming and requires a lengthy time period before results can be analysed. For these reasons, it would be of value to develop an *in vitro* model for subsequent alcohol studies.

A simple monolayer tissue culture system would probably be of limited use in such studies as it is unlikely that it would adequately represent the epithelium as an organ. In any case it is probable that all that would be detected using this system would be the heat shock proteins which are known to be synthesised in monolayer cultures following exposure to alcohol (Caltabiano, 1986).

The most valuable *in vitro* model for continuation of the studies in this thesis would be an organ culture system. In such a system, lingual epithelial biopsies are maintained in culture dishes in an adequate medium and can continue to grow for a number of weeks. An organ culture system, to which small amounts of alcohol have been added, would be of considerable use in the further studies of the effects of alcohol on the oral epithelium.

Should such a model prove to be successful and adaptable to use in alcohol studies, it would be invaluable in studies of the effects of alcohol on the molecular biology of the epithelial cells. An attempt could then be made to assess the effects of alcohol at the levels of transcription and translation using a range of molecular biology techniques. Techniques that are envisaged as being of value in such a study are the use of inhibitors of transcription and translation and *in vitro* translation of the messenger RNA populations from alcohol treated and control cells. The production of cDNA libraries from alcohol treated and control cells could also be carried out with a view to investigating any alcohol mediated alterations in gene expression.

Such studies would, if feasible, provide a definitive insight into the effects of alcohol on the oral epithelial cells.

APPENDIX

The Chemicals and Suppliers Used Were as follows:

Bovine Plasma Albumin

Bromophenol Blue

Chymotrypsin

Coomassie Brilliant Blue

Dithiothreitol

Elastase

Molecular Weight Markers (a) High: Carbonic Anhydrase - 29K
Albumin (Egg) - 45K
Albumin (Bovine) - 66K
Phosphorylase b - 97.4K
β-Galactosidase - 116K
Myosin - 205K

(b) Low: α-Lactalbumin - 14.2K
Trypsin Inhibitor - 20.1K
Trypsinogen - 24K
Carbonic Anhydrase - 29K
Glyceraldehyde-3-Phosphate DeH - 36K
Albumin (Egg) - 45K
Albumin (Bovine) - 66K

Nonidet p 40

Periodic Acid

S. Aureus Protease V8

Schiff Stain

TEMED

ALL THE ABOVE WERE PURCHASED FROM SIGMA LTD. POOLE, DORSET.

Acetic Acid

Acrylamide (Electran)

Ammonium Persulphate

Bis (Electran)

Disodium Orthophosphate
Glycerol

Glycine

Hydrochloric Acid

Potassium Metabisulphite

Sodium Bicarbonate

Sodium Chloride

Sodium Dihydrogen Orthophosphate

SDS (Biochemical)

Sodium EDTA

Sodium Metabisulphite

Tris

Urea (Aristar)

ALL THE ABOVE WERE PURCHASE FROM BDH LTD. POOLE DORSET.

Ampholines pH 5-7 and 3.5-10, were purchase from LKB Ltd, England.

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BIOCHEMICAL ABNORMALITIES OF RAT LINGUAL EPITHELIUM FOLLOWING CHRONIC ALCOHOL INTAKE

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Summary—Slab-gel electrophoresis of lingual epithelial protein preparations showed that in alcoholic animals there was a reduction in the presence of a high molecular-weight glycoprotein and a concomitant increase in two low molecular-weight proteins.

INTRODUCTION

Epidemiological evidence suggests a strong correlation between chronic alcohol abuse and the development of intra-oral squamous cell carcinoma (Rothman and Keller, 1972; Tuyns, 1982; Kissin, 1975). Animal studies of the effect of ethanol upon tumour development in the mouth, although largely agreeing with the epidemiological studies, have in general lacked dietary control, alcohol-intake control and convincing evidence of alcoholic liver damage (Elzay, 1966; Freedman and Shklar, 1978; Protzel, Giardina and Albano, 1964). Studies of the effect of alcohol upon oral epithelium are few but Mascrès and Joly (1981) reported epithelial thickening due to an increased thickness of the keratin layer. We have carefully controlled the intake of alcohol by giving animals ethanol as 36 per cent of their calories in an isocalorifically, matched-pair feeding technique (De Carli and Lieber, 1967). Any structural or morphological changes in the oral epithelium are likely to have a biochemical basis and, although occasional reports have noted histochemical changes (Mascrès and Joly, 1981), no true biochemical investigation of oral epithelium following alcohol abuse can be found.

MATERIALS AND METHODS

Forty young adult male (6–8 weeks old) Sprague–Dawley rats were divided into two groups. A group of 10 rats, housed individually in wire-bottom cages, were fed standard laboratory chow and water *ad libitum*. The remaining 30 rats were also individually housed in wire-bottom cages but were fed a commercially supplied, nutritionally adequate, liquid diet (Special Diets Services Ltd, Witham, Essex) as their sole source of food and water. After one week, the 30 experimental animals were divided into 15 closely weight-matched pairs. One of each pair was then fed liquid diet with ethanol substituted to provide 36 per cent of the daily calories. The matched animal is then given liquid diet with sucrose providing the same percentage of calories (De Carli and Leiber, 1967). By measuring the volume of diet consumed, each pair of animals had their calorific intake closely monitored and exactly matched.

All animals were weighed weekly and had 1 ml of blood removed from the tail under ether anaesthesia every 4 weeks. This blood was used for biochemical analysis of serum γ-glutamyl transpeptidase (γ-GT) which was chosen as the best marker of liver damage. After 17 weeks, all animals were killed by an overdose of intraperitoneal barbiturate, and the tongues dissected free and halved longitudinally, one portion being used for biochemical analysis and the other for histological studies.

The tongue epithelium was removed by the method of Raineri, Simsiman and Baitwell (1973) in which the intact tissue is placed in ice-cold water for 30 s, then in water at 55°C for 30 s and finally into ice-cold water again for 30 s. After this treatment, the epithelium can be removed with ease as an intact sheet. Such sheets were immediately placed into ice-cold water (1 ml per epithelial sheet), minced thoroughly with scissors and then disrupted with a power-driven tissue homogenizer at full speed. The resulting suspension was brought to 2 per cent sodium dodecyl sulphate (SDS; BDH Chemicals Ltd, Poole, Dorset), 25 mM dithiothreitol (DTT; Sigma Chemicals Ltd, Fancy Road, Poole, Dorset) and heated at 100°C for 3 min. Any insoluble residue was removed by centrifugation at 6000 *g* for 15 min and the supernatant was dialysed against 1:4 diluted SDS/glycine electrophoresis buffer. The dialysate was brought to 10 per cent glycerol and a small amount of bromophenol blue was added as a tracker dye. Samples were frozen and stored at −20°C prior to electrophoresis.

Determination of the protein concentration of the homogenates was performed by the spectrophotometric method of Bradford (1976) using bovine serum albumin as a standard.

Slab gels of various concentrations were prepared as described by Laemmli (1970) and samples containing approx. 75 μg of protein, in a maximum volume of 50 μl, were loaded and electrophoresed in the SDS/glycine electrophoresis buffer, initially at 80 V until the dye front had reached the resolving gel then at 150 V until the run was complete. Molecular-weight standards were included with every run. Gels were stained using Coomassie blue R/250 in 50 per cent ethanol/10 per cent acetic acid. De-staining was by diffusion against 5 per cent acetic acid.

Glycoproteins were stained selectively using the periodic acid-Schiff stain (Fairbanks, Steck and Wallach, 1971) and all gels were scanned on an LKB 2202

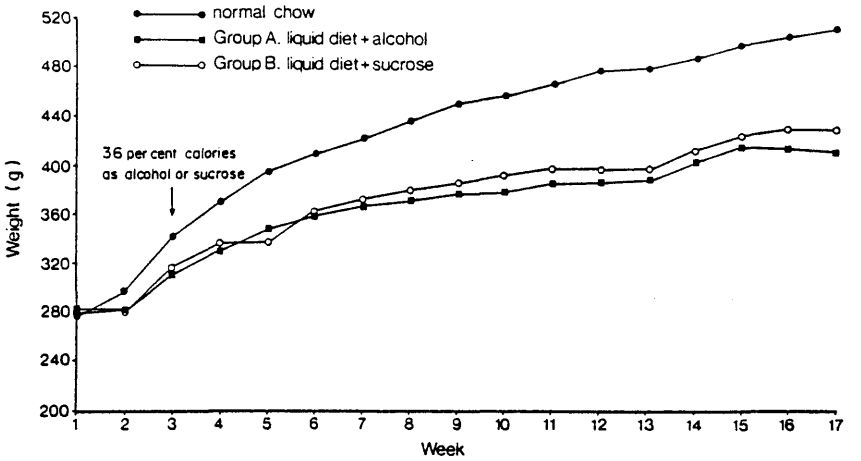

Fig. 1. Graph of mean weights of animal groups throughout the experiment. The standard error of mean for each group was smallest at week one, being 5.00 for normal animals, 4.42 for group A and 4.36 for group B. At week eight, the figures were 11.1 for normal animals, 8.6 for group A and 8.5 for group B. At killing, the values were 15.2, 9.4 and 11.1 respectively.

ultroscan laser densitometer and were then photographed in both black and white and colour.

RESULTS

Throughout, all animals gained weight but the control group gained significantly ($p < 0.001$, Mann–Whitney U-test) more than the experimental animals. There was no statistically significant difference between the weights of the two experimental groups except during week 16 when the animals in the sucrose-fed group gained significantly ($p < 0.05$, Wilcoxon matched pairs sign-ranked test) more weight than the alcoholic group. The reason for this difference is not known; there was no difference between the groups at killing (Fig. 1). Liver damage was demonstrated by enzymic and histological analysis with the alcoholic animals having significantly higher ($p < 0.01$, Mann–Whitney U-test) γ-GT levels than either the normal or the matched-pair group (Table 1). There was no statistical difference in γ-GT levels between the normal animals and the sucrose group (Table 1). Alcoholic animals had extensive centrilobular fatty change substantially greater than either the normal animals or the sucrose-fed animals.

The separation of epithelium from underlying connective tissue was essentially complete as verified by light microscopy although occasional small groups of basal epithelial cells remained on the connective tissue. Of the 40 tissue samples prepared for SDS gel electrophoresis, five were lost during preparation so that protein profiles of only 12 of the 15 pairs of rats could be compared. The SDS gels revealed a reduction in the levels of a high molecular-weight protein in the alcoholic one of each of the 12 experimental pairs. Laser-densitometer scans confirmed this substantial reduction (Fig. 2) and indicated an increase in two smaller protein peaks (Fig. 2). No differences in protein pattern were observed between the animals fed the liquid diet and sucrose and the animals fed normal laboratory chow.

The high molecular-weight protein had an apparent molecular weight of around 185 K and the lower molecular-weight proteins of 30 K and 28 K. The high molecular-weight protein has proved insoluble in non-dissociating buffers but PAS staining showed that it is a glycoprotein.

DISCUSSION

Occasional studies (Mascrès and Franchebois, 1981) have shown minor histochemical changes in buccal epithelium after chronic alcohol consumption and two semi-quantitative studies on buccal and oesophageal epithelium have shown changes in the thickness of the keratin layer (Mascrès and Joly, 1981; Mascrès, Ming-Wen and Joly, 1984).

With the technique used close control of the experimental groups was obtained and there was no mortality.

Our protein gels showed a consistent reduction in the levels of the large glycoprotein and marked

Table 1. Mean γ-GT results for all animals at the start, mid-point and end of the experiment (the range of values is shown in parentheses)

	Mean γ-GT results (IU)		
	Start	Mid point	End
Normal	4.90 (3.75–6.56)	5.53 (3.75–7.31)	5.61 (3.00–6.75)
Alcoholic	4.88 (3.00–12.38)	7.79 (6.38–11.44)	8.52 5.06–13.28)
Sucrose	4.85 (3.00–10.30)	4.97 (3.19–7.50)	5.81 (3.75–8.81)

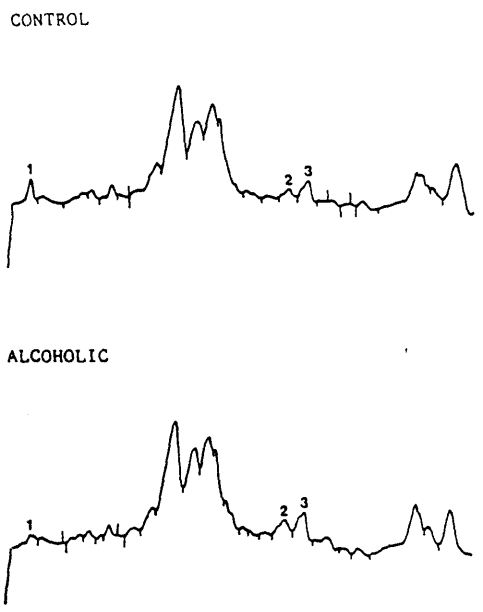

Fig. 2. Laser-densitometer tracings of slab gels from alcoholic and sucrose control animals. The high molecular-weight protein (1) and the two lower molecular-weight proteins (2 and 3) peaks are indicated.

increases in the levels of the two lower molecular-weight proteins in the alcoholic animals. There was no detectable change in the protein profile of the animals fed standard laboratory chow or, more importantly, in the animals fed sucrose up to 36 per cent of their dietary calories.

Further studies are being undertaken to characterize the three proteins which should provide some clues to their possible roles and to the mechanisms behind their altered levels.

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