PROTEIN VARIATION ASSOCIATED WITH FACIAL ECZEMA RESISTANCE OR SUSCEPTIBILITY IN ROMNEY SHEEP

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TO

MY PARENTS, JOHN, ESTHER AND CYNTHIA

FOR THEIR ENCOURAGEMENT, UNDERSTANDING AND LOVE.

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LIST OF ABBREVIATIONS

Bis-acrylamide	N,N'-methylene-bis-acrylamide
c.p.m.	counts per minute
df	degrees of freedom
DM-POPOP	1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene
d.p.m.	disintegrations per minute
GGT	gamma-glutamyl transferase
GSH	glutathione
g	gravitational field, unit of (in centrifugation)
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IEF	isoelectric focusing
KIU	kallikrein inhibitor units
LD_{50}	median lethal dose
mA	milliamps
μCi	microcuries
mCi	millicuries
mho	reciprocal ohm
M_r	molecular mass
nm	millimicron (10^{-9} m)
1-D IEF	one-dimensional isoelectric focusing
1-D PAGE	one-dimensional polyacrylamide gel electrophoresis
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
pK	negative logarithm of dissociation constant
p.p.m.	parts per million
PPO	2,5-diphenyloxazole

$r_{av.}$	average radius of rotation
SDM	sporidesmin
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylenediamine
TIU	trypsin inhibitor units
Tris	Tris(hydroxymethyl)aminomethane
2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
V·h	volt-hour
v/v	volume per volume
w/v	weight per volume
X^2	chi-square

ABSTRACT

The detection of plasma and liver protein markers for facial eczema resistance or susceptibility in Romney sheep was undertaken. A pooling protocol was used to allow rapid comparison of variation between populations. A 2-D PAGE technique was developed for protein separation. In general, proteins separated by 2-D PAGE were examined on Coomassie blue or silver stained gels. Greater sensitivity was achieved by labelling proteins with radioactive isotopes. Reductive methylation of the free amino groups of proteins with radioactively labelled formaldehyde and sodium cyanoborohydride was used for isotopic labelling of proteins. A double-labelling technique involving ^{14}C and ^{3}H was used to label plasma or liver proteins from facial eczema resistant and susceptible sheep. The labelled proteins were subsequently separated by 2-D PAGE and detected by autoradiography and fluorography. Any detected variation was further analysed for individuals on one-dimensional polyacrylamide gels which allowed more rapid analysis of multiple samples.

No significant difference was detected among the liver proteins of resistant and susceptible sheep. However, among the approximately twenty major plasma protein families visualised on 2-D PAGE gels, significant variation between sheep selected for facial eczema resistance or susceptibility occurred at the transferrin locus. Sheep selected for resistance showed a predominance of acidic transferrins while sheep selected for susceptibility contained a basic transferrin in greater abundance. These results were confirmed and their significance was assessed by transferrin phenotyping on one-dimensional

polyacrylamide gels. The transferrin A allele was more abundant in sheep selected for resistance while the transferrin D allele showed a greater association with facial eczema susceptibility. The A allele frequency was 0.57 in resistants and 0.05 in susceptibles while the D allele frequency was 0.18 in resistants and 0.68 in susceptibles. The results suggest some separation of transferrin A and D alleles between the animals selected for resistance and susceptibility. The basis of this variation is unknown. It may reflect either a physiological association of transferrin alleles with a character of importance in facial eczema resistance, or it may be a phenomenon unrelated to facial eczema resistance produced as a result of the way in which the facial eczema resistant and susceptible flocks were generated. It is expected that subsequent genetic studies will show whether transferrin phenotype can be used as a marker to select for facial eczema resistance as a means of controlling the disease.

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INTRODUCTION

Facial eczema is a mycotoxic disease of livestock in New Zealand. It is due to the ingestion of the mycotoxin, sporidesmin, which is produced by the fungus, Pithomyces chartarum. Sporidesmin is hepatotoxic and causes severe occlusive damage to the bile duct system. The failure of the damaged liver to remove phylloerythrin from the blood of affected animals leads to photosensitisation as an outward symptom of the disease.

Some sheep are resistant to facial eczema, but the biochemical and genetic bases of this phenomenon are not understood. This thesis reports the use of 2-D PAGE in studying the potential association of plasma and liver proteins with facial eczema resistance. Previous work by others in three areas relevant to my investigations, namely, the biology of facial eczema, the potential mechanisms for resistance and the development of electrophoretic systems for the detection of protein variation , is summarised in the following sections.

1-1 Facial Eczema

1-1.1 History

Facial eczema (pithomycotoxicosis) is a mycotoxic disease which occurs in livestock in New Zealand and elsewhere. It is so named because the affected animals develop photosensitivity with serious facial lesions. Facial eczema was first described by Gilruth (1908). The disease was first recognised in lambs which showed exudation and swelling of the skin of the head and ears. Many years later, the facial lesions were recognised as a photosensitisation reaction to sunlight and a symptom of severe liver injury (Hopkirk, 1936).

Sustained research on facial eczema did not begin until 1938 when New Zealand experienced a disastrous outbreak of the disease. The correlation of severely jaundiced livers and conspicuous bile duct lesions with high serum levels of bilirubin and phylloerythrin led Cunningham et al. (1942) to suspect that the photodynamic agent responsible for photosensitisation was phylloerythrin, and this was later confirmed by Clare (1944). Since the disease was found to be non-transmissible and because of its seasonal incidence, it was believed that the cause of the disease was a toxic substance in the pasture. Simpson et al. (1957) showed that toxicity was retained in stored dry pasture. It was further demonstrated that dried toxic pasture produced liver damage in guinea pigs (Evans et al., 1957; Perrin, 1957). White (1958a) using guinea pigs for testing toxicity, was able to prepare a concentrate of the toxic substance from dried toxic pasture by solvent extractions and column chromatography. A substance producing valine and leucine on hydrolysis was found only in toxic pasture samples (White, 1958b). This substance itself was non-toxic but it formed the basis of a chemical test called the 'beaker test' for the toxic pasture (Clare et al.,

1959; Perrin, 1959; Sandos et al., 1959). The chemical properties of the beaker test substance, sporidesmolide (Russell, 1960; Bertaud et al., 1963), resembled products isolated from some fungi and thus indicated the possibility of a link between facial eczema and fungi. Culture of fungal samples from toxic pasture led to the identification of a species of fungus as the cause of facial eczema (Percival and Thornton, 1958). The toxic fungus was originally identified as Sporidesmium bakeri but was later re-classified as Pithomyces chartarum.

Pithomyces chartarum gave a positive beaker test (Percival and Thornton, 1958; Thornton and Ross, 1959), and clinical cases of facial eczema were produced in lambs exposed to Pithomyces chartarum extracts (Percival, 1959). A relationship was demonstrated between the abundance of the spores of Pithomyces chartarum in pasture and outbreaks of facial eczema in sheep in the field (Thornton and Sinclair, 1959, 1960; Crawley et al., 1961; Sinclair, 1961). Thornton and Percival (1959) showed that Pithomyces chartarum contained a toxin capable of producing facial eczema. However, the isolation of the toxin was difficult because of its chemical fragility and erratic occurrence. Finally, success was achieved by a combination of concentration techniques and chromatographic separations which allowed crystals of the toxic material to be isolated from extracts of Pithomyces chartarum cultures (Synge and White, 1959, 1960). The toxin was named 'sporidesmin' from the then current name of the fungus Sporidesmium bakeri, and its structure (Figure 1) and conformation were determined (Fridrichsons and Mathieson, 1962; Ronaldson et al., 1963; Beecham et al., 1966).

1-1.2 Occurrence

Pithomyces chartarum is a saprophytic fungus distributed throughout warm temperate, sub-tropical, and tropical regions of the world (Hughes, 1953; Ellis, 1960; Dingley, 1962). Studies on the morphology of Pithomyces chartarum show the fungus to be an extremely variable species influenced greatly by conditions such as the temperature and the substrate under which it is grown (Di Menna et al., 1977; Sutton and Gibson, 1977). It is found to be a nutritionally undemanding saprophyte with a simple life cycle which colonises among the decaying plant material at the base of pastures (Ross, 1960; Brook, 1963). The decaying plant material provides a warm and humid ground surface mat with a particularly favourable microclimate for growth and sporulation of Pithomyces chartarum. The associations between weather, pasture conditions and facial eczema outbreaks are related to the ecology of the fungus (Mitchell et al., 1959; Mitchell, 1960; Brook, 1964).

Isolated outbreaks of facial eczema have been reported in Australia (Janes, 1959; Hore, 1960; Flynn, 1962; Gardiner and Nairn, 1962; Caple and Van der Graaf, 1976; Edwards et al., 1981), South Africa (Marasas et al., 1972) and America (Parle and Di Menna, 1978). In New Zealand, regions susceptible to facial eczema outbreaks are located in various parts of the North Island. The outbreaks of facial eczema usually occur from January to April, when prolonged hot dry periods with slow pasture growth are terminated by warm rains and high humidity with grass minimum temperatures of 12^{o}C or greater (Filmer, 1958; Mitchell et al., 1959; Smith et al., 1965; Clare, 1969; Di Menna and Baily, 1973; Parle and Di Menna, 1978). Under these conditions, there is a rapid growth and sporulation of Pithomyces chartarum.

There is a close relationship between sporidesmin production and sporulation in different strains of Pithomyces chartarum (Di Menna et al., 1970). The outbreaks of facial eczema are more intense when the pasture is heavily grazed since animals are forced to graze on pasture material which contains Pithomyces chartarum spores. The severity of the disease is related to the number of spores ingested, their sporidesmin content, and the susceptibility of the grazing animal. Sporidesmin levels in spores collected from toxic pastures are on average 0.08 mg of sporidesmin/100 million spores (Clare, 1962). It is estimated that sheep need to ingest several grams of spores to suffer even mild facial eczema symptoms (Brook and White, 1966). Since sporidesmin is readily extracted from the spores of the fungus with water (Clare and Gumbley, 1962; Marbrook and Matthews, 1962), exposure to rain and sunlight may explain the sometimes observed decline in toxicity of pasture despite persistent high spore numbers (Brook, 1964; Smith et al., 1965).

1-1.3 Pathogenesis and Pathology

Facial eczema has been extensively produced and investigated using animals which had been exposed to toxic pasture or orally dosed with sporidesmin (Done et al., 1962; Mortimer, 1962, 1963; Mortimer and Taylor, 1962). It has been shown that ruminants are generally the most susceptible species, and of these fallow deer (Mortimer and Smith, 1981) and sheep (Mortimer and Taylor, 1962) rank highest. All species examined are affected by sporidesmin in a fundamentally similar manner, namely, a severe inflammatory reaction with marked oedema associated with increased capillary permeability. However, different features assume prominance in each species such as the

lungs in the rat and the biliary system in sheep (North and Gwynne, 1960; Mortimer, 1970a).

Sporidesmin is highly active and produces inflammation and destruction of tissues wherever it becomes sufficiently concentrated. After absorption from the intestine, sporidesmin reaches the liver where the highly destructive form of the toxin is excreted into the bile. Over the first 48 h, only minor changes are produced in hepatocytes; mainly vacuolation and minor accumulation of triglycerides (Mortimer et al., 1962). The liver cells are not at this stage seriously injured by the toxin, but the bile ducts which channel the now highly concentrated toxin into the intestine become severely injured with degeneration of the epithelium. (Hopkirk, 1936; Cunningham et al., 1942; Mortimer, 1963). The tissues of the bile ducts react to this severe injury and in the process of repair, connective tissue proliferates around the ducts, eventually causing constriction and occlusion of ducts and preventing bile flow (McFarlane et al., 1959; Done et al., 1960). Biliary obstruction is followed by a second and more severe phase of hepatocyte injury. This is reflected in marked increases in certain serum enzyme activities, and in serum bilirubin, cholesterol, phospholipids, and bile acids (Done et al., 1962; Mortimer, 1962; Ford, 1965, 1974; Leaver, 1968b; Peters and Mortimer, 1970).

Early in the course of the disease, the liver is light coloured and there may be mottled areas. Later, the whole liver becomes mottled and in very bad cases the liver is stained yellow or green with bile pigments (Richard, 1973). When the liver is cut, the bile ducts, not readily apparent in normal tissue, stand out as thickened white areas. The gallbladder may be enlarged and contain thickened bile. Urinary bladder lesions are commonly found and, as a complication, cystitis may occur.

The establishment of duct occlusion from about day 10 onward coincides with the onset of jaundice and photosensitisation. Photosensitisation refers to an injurious skin reaction or photodermatitis. It is produced when a photodynamic agent, aberrantly present in the skin or present in abnormally high quantities, is activated by light energy, causing cellular injury and tissue inflammation (Clare, 1955). In facial eczema, phylloerythrin, a photodynamic porphyrin pigment derived from microbial breakdown of chlorophyll in the digestive tract, accumulates in the peripheral circulation when hepatic excretory function is impaired (Cunningham et al., 1942; Clare, 1944). Phylloerythrin has the peculiar property of photosensitising the skin. Under these circumstances photodermatitis develops rapidly when the skin is exposed to sunlight for periods as short as several minutes (McIlwaine, 1938). The more prolonged the light exposure, the more severe is the skin injury. Phylloerythrin may not itself be involved directly in the reactions that cause the damage to the skin but may transfer energy to some other compound that causes damaging reactions in the skin (Jubb and Kennedy, 1963).

The early sign of photosensitisation is vesicles on the ears due to congestion and oedema underneath the skin. These vesicles soon become broken due to invasion by micro-organisms. The ears, nostrils and eyelids become congested and intensely irritated such that the animal frequently scratches the ears and face on objects such as fences and stumps. This leads to skin abrasions and the whole face may become raw. The affected skin is erythematous, hot and inflammed. Animals seek shade and venture out only in overcast weather or in darkness. The onset of photosensitisation follows the intake of sporidesmin by 10-20 days (Mortimer, 1971). Clinical examination of photosensitised

animals reveals jaundice in the conjunctiva. The urine is deeply coloured with bile pigments. In severely affected flocks, deaths occur from about 10 days after onset of photosensitisation and may continue for several weeks. The severe debilitating stress of photosensitisation and extensive superfacial lesions contributes to the cause of death. In less severe outbreaks, animals can recover but are at greater risk if subsequently challenged.

It is important to emphasise that many affected animals never show photosensitisation. Some suffer liver damage without breakdown of the phylloerythrin excreting mechanism, although other functions of the liver have been badly affected. Such animals represent a subclinical form of facial eczema, and may show no external signs of the disease during an outbreak, yet have liver damage which will affect their subsequent growth and reproduction (Hopkirk, 1936; Leaver and Hore, 1967; Campbell and Wesselink, 1973).

While affected animals can recover from all but severe attacks (Mortimer, 1969), the cost of the disease is high due to loss of condition and increased culling because of liver damage (Smith and O'Hara, 1978). It is difficult to estimate the economic extent of the losses due to facial eczema, but studies have indicated that in a bad year the livestock and production losses on a national scale may be in the region of tens of millions of dollars (Clare, 1969; Bird, 1976).

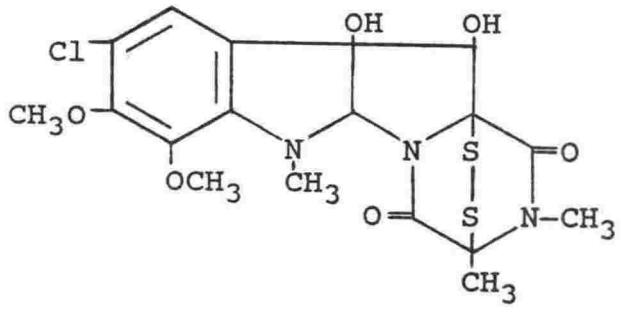
1-2 Sporidesmin

1-2.1 Chemistry and Toxicity

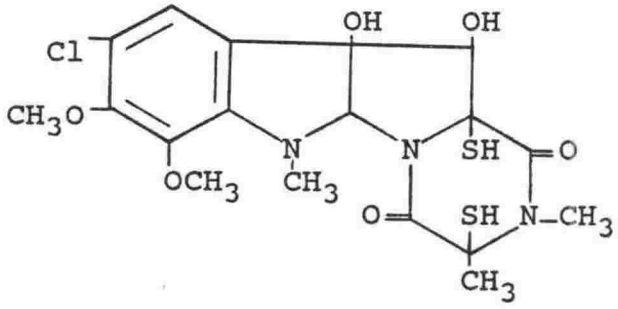
Sporidesmin, sometimes referred to as sporidesmin A (Ronaldson et al., 1963), is one of a closely related group of Pithomyces chartarum metabolites. These compounds are collectively known as the sporidesmins. Sporidesmin represents more than 90% of the total sporidesmins produced by cultures of Pithomyces chartarum (White et al., 1977), the remainder being small amounts of sporidesmin B (Ronaldson et al., 1963; Hodges et al., 1966), sporidesmin C (Hodges and Shannon, 1965), sporidesmins D and F (Jamieson et al., 1969), sporidesmin E (Rahman et al., 1969), sporidesmin G (Francis et al., 1972), and sporidesmins H and J (Rahman et al., 1978). The chemistry of the sporidesmins has been reviewed by White et al.(1977).

The sporidesmins are characterised by a 3,6-epidithia-2,5-dioxopiperazine moiety (Figure 1) with a cage formed by a bridging disulphide group, and an indolopyrrolopiperazine moiety (Figure 1). Toxic activity is associated with the disulphide cage. Sporidesmins D and F, with an opened disulphide bridge, have very low toxicity to cell cultures (Mortimer and Collins, 1968). Unlike sporidesmin, they do not emerge from chromatographic columns where the bulk of the toxic activity is found (White et al., 1977). When the disulphide bridge is chemically eliminated, the resulting degradation products are inactive (Brewer et al., 1966).

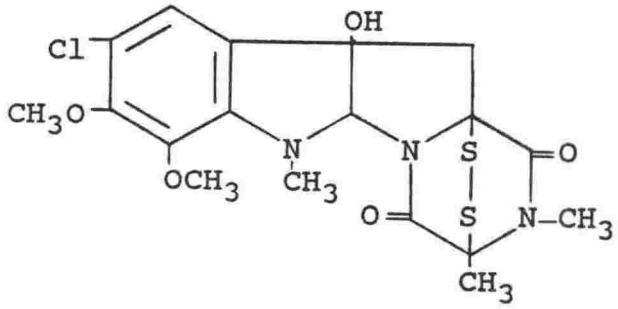
Sporidesmins A and E (Figure 1) are the most toxic sporidesmins. The other sporidesmins B, C, D, F, G, and H have less toxic activity. The toxicity of sporidesmin J is unknown. Sporidesmin is found to be highly toxic to mammalian cells in culture (Murphy and Worker, 1960; Done et al., 1961; Fastier, 1961). The extreme sensitivity of certain cell lines to



A



B



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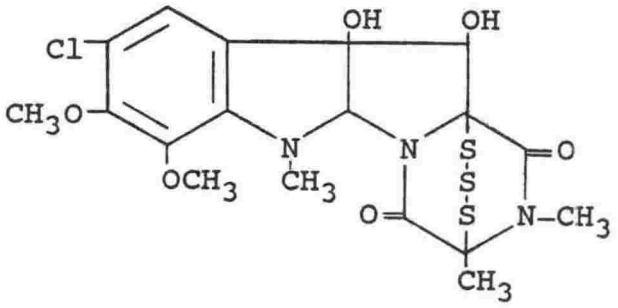

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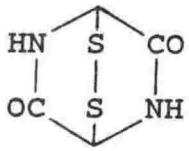


E

FIGURE 1. Chemical Structures of some Epipolythiadioxopiperazines

A) sporidesmin (sporidesmin A);
B) reduced sporidesmin;
C) sporidesmin B;
D) sporidesmin E;
E) 3,6-epidithia-2,5-dioxopiperazine.

Structures from Trown (1968), Taylor (1971), and Cole and Cox (1981).

sporidesmin has found useful application as an assay method (Murphy and Worker, 1960; Done et al., 1961), which has been used to detect sporidesmin in bile, urine, and serum of dosed sheep (Mortimer and Stanbridge, 1968).

Animals have been used to study the pathology of sporidesmin poisoning. Rabbits were found to be susceptible to sporidesmin. An oral dose of 0.5 mg sporidesmin/kg body weight induced icterus, lipaemia and photosensitisation (Mortimer and Taylor, 1962). Guinea pigs were also susceptible to sporidesmin with oral doses of about 2 mg/kg body weight producing severe liver lesions (Synge and White, 1959, 1960). Rats proved more resistant to oral doses of sporidesmin than either the rabbit or guinea pig (Rimington et al., 1962; Slater and Griffiths, 1963; Slater et al., 1964). Slater et al. (1964) produced no liver or bile duct lesions with single oral doses of sporidesmin at 9 mg/kg, but the same dose given by the intraperitoneal route produced ascites, pleural effusions, lung oedema, and death in rats. Mice were found to be considerably more resistant to sporidesmin than rats. Daily oral doses of 0.1 and 0.2 mg sporidesmin per female mouse gave group average survival times of 57 and 35 days, respectively (Mortimer, 1970a). Mortimer (1970a) considered the lethal dose in mice to lie in the range 200-300 mg sporidesmin/kg body weight.

In sheep, sporidesmin at a dose of 0.5-1.0 mg/kg given orally, subcutaneously, or intravenously induced severe facial eczema. At 1 mg/kg given orally to grazing sheep, 80% of the dosed animals died in 24 days, and at 0.5 mg/kg orally, 12% of the animals died in the same period; thus the LD_{50} was between these dose levels (Done et al., 1962; Mortimer, 1962, 1963; Mortimer and Taylor, 1962; Mortimer et al., 1962; Peters, 1963; Peters and Smith, 1964). As for milk-fed calves, 100% of the

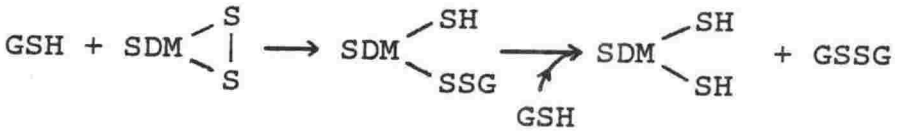
dosed animals died within 3-5 days at a dose of 3 mg/kg. The LD_{50} of sporidesmin in calves was found to be 1.5 mg/kg (Mortimer, 1971). Fallow deer given comparable doses of sporidesmin reacted severely. At a dose of 1.2 mg/kg, all dosed animals died within 7-10 days. At a dose of 0.6 mg/kg, the animals died within 10-14 days, while at a dose of 0.3 mg/kg, the animals died within 14-24 days (Mortimer and Smith, 1981).

1-2.2 Metabolism

Metabolism of sporidesmin was shown to occur in a reaction system containing liver microsomes and cytosol (Aust, 1977; Sissons, 1982). The microsomal metabolism was thought to represent a detoxication step. There has been recent evidence for the formation of a more reactive product from the metabolism of sporidesmin by the cytosol (Sissons, 1982).

Aust (1977) incubated sporidesmin with liver microsomes and various cofactors and found that the metabolism of sporidesmin to more water soluble metabolites did not occur when UDP-glucuronic acid or NADH were added but was substantial in reaction mixtures which had been supplemented with NADPH, thus indicating NADPH-linked mixed function oxidation. Sheep liver microsomes, in which drug metabolising enzymes had been induced by prior exposure of animals to hexachlorobenzene, were incubated with radioactively labelled sporidesmin and an NADPH generating system. At least eight metabolites were isolated and two of these were identified as the monohydroxy and dihydroxy derivatives of sporidesmin (Fairclough et al., 1978; Lauren and Fairclough, 1980). Neither of these metabolites contained sulphur atoms, therefore it was considered that their toxicity would be low and that metabolism which gave rise to such products would represent a detoxication step (Fairclough et al., 1978).

Sissons (1982) showed that incubation of sporidesmin with sheep liver microsomes resulted in an unusual cytochrome P-450 spectrum and suggested an interaction of the sporidesmin sulphur bridge with cytochrome P-450. The reaction of sporidesmin with glutathione was also investigated by Sissons (1982). Sporidesmin disulphide was reduced to sporidesmin dithiol (Figure 1) during incubation with glutathione in a non-enzymic reaction:



Sheep liver cytosol contained a thiol transferase-type enzyme which increased the reaction rate by at least two orders of magnitude. It was estimated that sporidesmin would be rapidly reduced at normal cytosolic levels of glutathione (Sissons, 1982). The sporidesmin dithiol was postulated to generate $O_2^{\cdot-}$ which might initiate the deleterious effects of sporidesmin (Munday, 1982).

1-2.3 Excretion

White et al. (1977) reviewed the information on the absorption and excretion of sporidesmin in animals. The rates of absorption and excretion of sporidesmin in sheep, guinea pigs and rats were found to be rapid. In all species, the bile was the major route of excretion with peak bile concentrations attained within a few hours of dosing and excretion being largely completed in 24 h (Leaver, 1968a; Mortimer and Stanbridge, 1968; Towers, 1970a, b; Towers, 1972). The significance of the circulation of sporidesmin in bile in relation to severity of liver and bile duct lesions was first raised by Worker (1960b) from studies using rabbits. It was demonstrated that bile from

rabbits dosed with crude Pithomyces chartarum extracts was toxic to other rabbits, and facial eczema-type liver lesions were produced when donor bile was added to the duodenum of the recipient animal. Using cannulated sheep and a cytotoxic assay method with cells in culture, Mortimer and Stanbridge (1968) detected sporidesmin activity in bile taken at intervals from 10 min to 24 h after oral administration of sporidesmin (1 mg/kg body weight). Maximum cytotoxic activity, equivalent to 10-20 μg of sporidesmin per ml, was found in bile at 4 h, but after 24 h little sporidesmin activity was detected. The presence of unchanged sporidesmin, which accounted for 10-20% of the administered dose, was confirmed by infra-red spectroscopy after extraction and isolation of the toxin from bile of dosed sheep (Mortimer and Stanbridge, 1968). Results from experiments in which sheep were dosed at a daily rate of 0.08 mg sporidesmin/kg body weight over 3 consecutive days suggested that with an increase in the amount of sporidesmin administered, there was a proportionately greater increase in the concentration of sporidesmin in bile (Fairclough and Smith, 1983).

Cytotoxic activity, equivalent to 0.2-0.4 μg of sporidesmin per ml, was detected in serum up to 12 h after toxin administration. Sporidesmin activity was also detected in the urine of dosed sheep (Mortimer and Stanbridge, 1968). Maximum sporidesmin activity, equivalent to 2-4 μg/ml urine, was detected from 4 to 12 h after oral dosing, but not at 24 h. No sporidesmin activity could be detected in milk from two lactating ewes.

The pattern of excretion of a large single dose of sporidesmin appeared to be similar in other species. Towers (1970a, b) administered ^{35}S-labelled sporidesmin orally to guinea pigs and rats, and found little difference between species in absorption and excretion rates or in the distribution of

radioactivity in tissues including liver, lung and kidney. In both species, 70% of the radioactivity which appeared in the bile did so within 105 min of dosing and the excretion of radioactivity was completed within 7.5 h (Towers, 1972). Two to five times more radioactivity appeared in the bile than in the urine. Highest levels of radioactivity were found in the liver, kidney and spleen. Excreted radioactivity measured in these experiments represented a mixture of sporidesmin and its sulphur-containing metabolites (Towers, 1970b).

The progressive nature of the bile duct lesions leading to facial eczema led Worker (1960a, b) to postulate an enterohepatic biliary circulation of sporidesmin or alternatively retrograde movement of sporidesmin from the gallbladder into the biliary tree. Towers (1972) reported an enterohepatic circulation of sporidesmin or its metabolites in rats and guinea pigs. However, enterohepatic circulation was found to be not necessary for the progression of liver damage in sheep dosed with sporidesmin. It was shown that facial eczema-type liver lesions still developed when all hepatic bile was drained for the first 24 h after dosing (the period of sporidesmin excretion into bile) and the gallbladder functionally isolated (Mortimer and Stanbridge, 1968). Similar results were reported by Leaver (1968a). According to the evidence presented by Mortimer and Stanbridge (1968) and by Leaver (1968a), it was concluded that enterohepatic circulation of sporidesmin would add little to the severity of the hepatobiliary lesions.

1-2.4 Mode of Action

The way in which sporidesmin damages the cells of the liver bile ducts and other tissues to which it is exposed has not been elucidated. It has been suggested that sporidesmin is a membrane

active toxicant which produces general disruption of cell membranes (Slater, 1972; Middleton, 1974a, b). The subcellular distribution of sporidesmin in membrane fractions (Cordiner et al., 1983b) and the plasma membrane damage of hepatocytes as evidenced by loss of microvilli (Bertaud and Mortimer, 1966; Bullock et al., 1974), inhibition of bile acid transport (Cordiner and Jordan, 1983) and changes in hormone sensitivity (Cordiner et al., 1983a), provide support for the hypothesis that sporidesmin damages membranes. Several mechanisms of action have been postulated (Slater, 1972; Middleton, 1974a, b; Sissons, 1982) and these are discussed below.

1-2.4.1 Interactions with Membrane Thiols

Slater (1972) and Middleton (1974b) proposed as the mechanism of action of sporidesmin the interaction of the disulphide bridge with reactive membrane thiol groups which are essential for membrane stability.

Bullock et al. (1974), using isolated perfused rat liver exposed to sporidesmin, reported gross changes in the structural organisation of the biliary canalicular membrane including loss of microvilli and canalicular dilatation. The degeneration of the canalicular membrane was accompanied by an inhibition of bile flow and of bile acid secretion (Bullock et al., 1974). If the disulphide bridge interacts with membrane thiol groups to inhibit membrane transport processes, then thiol compounds which preferentially react with the disulphide of sporidesmin may be useful as protective agents. Cordiner (1984) found no such protection with glutathione, cysteine, methionine, thioglycollic

acid and mercaptoethanol, but partial protection of hepatocytes from sporidesmin inhibition of bile acid uptake was afforded by dithiothreitol and N-ethylmaleimide. These results were similar to those obtained by Middleton (1974b) who found that dithiothreitol protected mitochondria from sporidesmin induced swelling in vitro. Middleton (1974b) proposed that the protection by dithiothreitol and N-ethylmaleimide resulted from chemical interaction with the disulphide bridge of sporidesmin to form non-toxic products. Protection by chemical interactions has also been postulated by Mason and Kidd (1951) who found that inhibition of tumour cell growth by gliotoxin was abolished if gliotoxin was incubated with sulphydryl agents prior to exposure to cells. It was suggested that if disulphide exchange occurred between sporidesmin and membrane thiols, the sporidesmin would be linked to macromolecules through disulphide bridges. Cordiner (1984) investigated the nature of the binding of sporidesmin to the macromolecules of isolated sheep hepatocytes and found that more than 60% of the ^{14}C-labelled sporidesmin which was bound to macromolecules, was released by protease treatment and approximately 50% was released by addition of excess non-radioactive sporidesmin. However, less than 10% of the radioactivity was released following addition of other sulphydryl compounds such as dithiothreitol and mercaptoethanol indicating that sporidesmin might not have been bound through a disulphide linkage.

1-2.4.2 Superoxide Radical Generation

It has been suggested that the generation of superoxide anion radical ($O_2^{\cdot-}$) might initiate the deleterious effects of sporidesmin (Munday, 1982). $O_2^{\cdot-}$ has been shown to inactivate viruses, induce lipid peroxidation, damage membranes, and kill

cells (Fridovich, 1978). The degree of toxicity of $O_2^{\cdot-}$ is uncertain (Fee, 1980; Bannister and Hill, 1982) and there are indications that in many cases $O_2^{\cdot-}$ is not necessarily the species that actually causes the toxic effects, rather it is the precursor of a more potent oxidant, perhaps $OH^{\cdot}$, whose generation depends on the simultaneous presence of H_2O_2 (Fridovich, 1978; Bannister and Hill, 1982; Kakinama et al., 1982).

Munday (1982) postulated the in vitro generation of $O_2^{\cdot-}$ from sporidesmin dithiol. There was no generation of $O_2^{\cdot-}$ from sporidesmin disulphide alone, but when sporidesmin and a thiol (glutathione, cysteine or dithiothreitol) were incubated together, $O_2^{\cdot-}$ was produced.

$O_2^{\cdot-}$ generation from sporidesmin (SDM$\begin{smallmatrix}\diagup\,\mathrm{S}\\ \\ \diagdown\,\mathrm{S}\end{smallmatrix}$) in vitro was postulated to involve autoxidation of the sporidesmin dithiol (SDM$\begin{smallmatrix}\diagup\,\mathrm{SH}\\ \\ \diagdown\,\mathrm{SH}\end{smallmatrix}$) formed from the mycotoxin by thiol-disulphide exchange:

$$\mathrm{SDM}\begin{smallmatrix}\diagup\,\mathrm{S}\\ \\ \diagdown\,\mathrm{S}\end{smallmatrix} + 2\mathrm{RSH} \longrightarrow \mathrm{SDM}\begin{smallmatrix}\diagup\,\mathrm{SH}\\ \\ \diagdown\,\mathrm{SH}\end{smallmatrix} + \mathrm{RSSR}$$

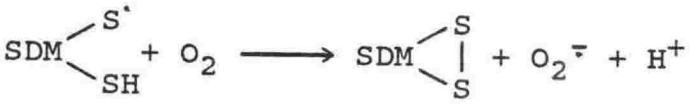
Thiol autoxidation was initiated by electron transfer from the thiolate anion to a transition metal ion in its higher oxidation state:

$$\mathrm{SDM}\begin{smallmatrix}\diagup\,\mathrm{S}^-\\ \\ \diagdown\,\mathrm{SH}\end{smallmatrix} + \mathrm{M}^{n+} \longrightarrow \mathrm{SDM}\begin{smallmatrix}\diagup\,\mathrm{S}^{\cdot}\\ \\ \diagdown\,\mathrm{SH}\end{smallmatrix} + \mathrm{M}^{(n-1)+}$$

$O_2^{\cdot-}$ was produced by re-oxidation of the metal:

$$\mathrm{M}^{(n-1)+} + \mathrm{O_2} \longrightarrow \mathrm{M}^{n+} + \mathrm{O_2^{\cdot-}}$$

and by reaction of the thiyl radical with molecular oxygen:



By dismutation, either spontaneous or enzyme catalysed, $O_2^{\cdot-}$ formed hydrogen peroxide:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Similar reactions have been proposed to occur *in vivo*, producing $O_2^{\cdot-}$ which might account for the cytotoxic action of sporidesmin. Intracellular generation of $O_2^{\cdot-}$ and formation of H_2O_2 have been demonstrated in erythrocytes incubated with sporidesmin (Munday, 1984).

1-2.4.3 Microfilament Disruption

Sporidesmin-induced disruption of cytoskeletal microfilaments has been discussed by Bullock *et al.*(1974), Cordiner and Jordan (1983), Jordan (1986), and Jordan and Pedersen (1986). Actin-containing microfilaments have been found to be closely associated with the plasma membrane of many cells and they have been implicated in the control of the lateral movement of many plasma membrane receptors and in microvillar exocytosis and endocytosis. Such microfilaments appear to be the target of several hepatotoxic agents (Evans, 1980). Phalloidin, a fungal toxin which causes irreversible polymerisation of monomeric actin into microfilaments, produced an increase in the microfilamentous network around bile canaliculi and decreased both basal bile flow and bile flow stimulated by taurocholate infusion (Dubin *et al.*, 1978, 1980). Cytochalasin B, a fungal toxin which disrupts actin microfilaments, competitively

inhibited taurocholate uptake in isolated rat hepatocytes (Reichen et al., 1981).

Sporidesmin induced vacuolation and detachment of cultured adherent cells from tissue culture substrates (Mortimer and Collins, 1968; Mullbacher and Eichner, 1984) and these processes were preceded by disruption of microfilament cables (Jordan and Pedersen, 1986). The cell detachment induced by sporidesmin may thus be the result of microfilament disruption. Such an effect could be due to the direct action of the toxin on microfilament proteins including actin or the actin-binding proteins which regulate microfilament bundling and capping or binding to plasma membranes.

Jordan (1986) used the reaction of an anti-actin antibody with microfilaments to show changes in liver actin microfilament organisation in inbred mice strains exposed to sporidesmin. The loss of actin staining 3 h after sporidesmin exposure was not due to enhanced actin degradation or to depolymerisation of actin filaments. Similarly the enhanced staining at 24 h was not attributable to increased actin synthesis or redistribution of the actin monomer pool into filament actin. Therefore, the changes were suggested to be due either to relocalisation of actin filaments within liver cells or to altered actin filament organisation including filament severing, cross-linking or membrane binding. Microfilament and associated plasma membrane modification may represent a component of the way in which sporidesmin modifies cells leading to the hepatobiliary toxicology seen in sporidesmin poisoning.

1-3 Prevention of Facial Eczema

As there is so far no known cure for facial eczema, prevention of the disease is the only way in which losses can be avoided. There are theoretically several ways in which prevention may be exploited. A combination of measures may be required to ensure absolute protection. However, breeding for resistance may be retarded by using other measures which allow survival and reproduction of susceptible animals.

1-3.1 Recognition of Danger Periods

One of the preventive measures is the recognition of danger periods by on-farm counts of Pithomyces chartarum spores. When weather conditions are conducive to fungal growth, spore numbers are monitored. Regional warnings are broadcasted and farmers are encouraged to count spore numbers on their own individual paddocks and to graze stock in the least affected areas. (Mortimer and Ronaldson, 1983).

1-3.2 Pasture Management and Alternative Crop Use

Restriction of the ingestion of spores by pasture management and alternative crop use are found to be effective in preventing livestock from contracting facial eczema. Frequent rotation of grazing areas reduces the intake of litter and spores, thus decreasing the toxic hazard. The removal of livestock from toxic pasture and the strategic grazing of fodder crops, such as turnips, swedes and kale are found to be effective, but the practice is not widely accepted by grassland farmers (Mortimer and Ronaldson, 1983). Brassica crops and pure white clover swards do not produce facial eczema, so sheep can be grazed safely on them during dangerous periods (Filmer, 1958).

1-3.3 Fungicides

Another preventive measure involves spraying pastures with fungicide to control the growth of Pithomyces chartarum. Early attempts to control the fungus by application of fatty acids (Thornton, 1963; Thornton and Taylor, 1963) and fungicides were unsuccessful (Sinclair, 1967). Often, agents found effective in laboratory were not effective in the field (Janes, 1962; Parle, 1969). Among the fungicides, thiabendazole (Robinson et al., 1964) was reported to prevent seasonal build up of spore numbers for six weeks after application (Sinclair, 1967; Sinclair and Howe, 1968). Thiabendazole was taken up by the pasture within three days and then released again over a much longer period (Parle and Di Menna, 1978). Thiabendazole inhibited Pithomyces chartarum germ tube elongation through blockage of vitamin B_{12} synthesis (Stutzenberger and Parle, 1973). The effectiveness of thiabendazole was related to its high activity in preventing growth of fungus at very low levels (0.2 p.p.m.) and to the fact that prolonged use of sub-lethal doses did not confer resistance (Parle and Di Menna, 1972a). Apart from thiabendazole, other substituted benzimadazoles were also found to reduce spore numbers (Parle and Di Menna, 1972b; Stutzenberger and Parle, 1973). However, no fungicide used has totally eliminated Pithomyces chartarum from pasture. Fungicides only reduced spore numbers therefore it was still possible for sprayed pasture to be toxic (Parle and Di Menna, 1978). There was also difficulty in getting a sufficient amount of the fungicide to the base of the pasture where Pithomyces chartarum was actually growing. Other factors such as its removal by rain or its destruction by light could alter the effectiveness of a fungicide (Bird, 1976). Therefore, even thiabendazole, the most economic of the fungicides, was rather costly to apply over large acreages.

1-3.4 Zinc

It has been shown that the application of trace elements, such as zinc, provided protection from sporidesmin poisoning (Towers et al., 1975). Towers et al. (1975) showed that zinc protected rats from sporidesmin poisoning. The LD_{50} for sporidesmin dosed rats on a zinc-enriched diet was about twice that for rats on a zinc-deficient diet (Towers, 1977). High oral intakes of zinc also gave good protection from sporidesmin poisoning in sheep (Smith et al., 1977) and in dairy cattle (Towers and Smith, 1978). Zinc protection has also been reported in field trials in sheep (Towers et al., 1975) and in a field outbreak in dairy cows (Smith et al., 1978). For effective protection, drenching with zinc oxide emulsion was recommended and had to be given concurrently with sporidesmin intakes and over the period when pastures were toxic. Levels of zinc required were high (calculated as 20-25 mg of elemental zinc per kilogram of live weight per day), 20-30 times greater than nutritional requirements (Towers et al., 1975, 1976; Smith et al., 1977, 1978; Towers and Smith, 1978). While protection was achieved with very high zinc intakes, prolonged dosage at these levels produced toxic effects, mainly expressed as pancreatic necrosis and fibrosis in sheep and cattle (Smith, 1977, 1980; Towers, 1982). High zinc intakes could also adversely affect the metabolism of other minerals (Towers et al., 1981).

The mechanism of zinc protection is not known. Since zinc must be administered prior to, or concurrent with toxin challenge, it seems that zinc exerts its effect at an early stage of sporidesmin poisoning (Towers, 1977). The high zinc doses required for protection may suggest that zinc exerts some effect other than its normal metabolic role (Towers, 1982). The protection from sporidesmin toxic effects was not confined to

hepatobiliary sites, for there was a lower incidence of lesions in the urinary bladder of sheep and cattle and a less marked decrease in milk yield in cows when zinc was given (Smith et al., 1977). The stabilisation of biomembranes by zinc, possibly by interference with lipid peroxidation, has been suggested from studies on zinc protection against carbon tetrachloride-induced hepatic injury in rats (Chvapil et al., 1973). Cagen and Klassen (1980) showed that zinc protected against carbon tetrachloride-induced liver injury in rats through the sequestration of reactive metabolites by metallothionine, a protein in cell cytosol, inducible by high plasma zinc levels. Zinc was reported to stabilise membranes against free radical attack (Chvapil and Zukoski, 1974) and to inhibit $O_2^{\cdot-}$ production from sporidesmin in vitro (Munday, 1982). Munday (1984) has shown that zinc, by virtue of its ability to form a mercaptide with reduced sporidesmin, decreased the rate of $O_2^{\cdot-}$ formation from sporidesmin. It was suggested that this reaction could account for the protective ability of the metal.

1-3.5 Casein

Hove and Wright (1969) found that rats on high protein diets with casein as the protein source were protected from sporidesmin poisoning. Fractionation of the casein indicated that the phosphopeptone moiety was protective and that synthetic D,L-phosphoserine also protected against sporidesmin poisoning. The protective mechanism is not known, but it was suggested that maintenance of cell and membrane integrity by the dietary factor might have counteracted sporidesmin poisoning. However, Mortimer (1970b) found that casein did not protect sheep from sporidesmin poisoning.

1-3.6 Sequestrants in the Intestine

Sheep dosed with sporidesmin developed an accumulation of bile acids in the liver and vascular circulation. In an attempt to establish the significance of the bile acid accumulation in facial eczema, Peters and Mortimer (1966) administered cholestyramine to sheep. Cholestyramine is an anion exchanger that, when orally administered, traps bile acids in the intestine. Cholestyramine modified the course of the sporidesmin-induced disease by prolonging the survival time but the overall mortality was not significantly reduced. Cholestyramine alone had no detrimental effect on normal sheep (Peters and Mortimer, 1966).

Oral administration of activated carbon has been used to prevent absorption of various drugs and toxins from the intestine and to increase their rate of elimination both in man (Neuvonen, 1983) and ruminants (Wilson and Cook, 1970). Wright and Cordes (1972) found that activated carbon protected sheep from sporidesmin poisoning but the protection was only effected with large amounts of carbon.

1-3.7 Immunisation

An early attempt to demonstrate sporidesmin antibodies in the sera of guinea pigs, rabbits and sheep was unsuccessful (Fastier, 1961). Unless sporidesmin could be coupled to a protein, there seemed little likelihood for control by vaccination (Te Punga, 1962). Attempts have been made to couple sporidesmin or its analogues to proteins, bacteria and synthetic polymers or to other high M_r carriers for use as immunogens to stimulate the production of antibodies in animals. Ronaldson (1975) described covalent linking of modified sporidesmin to proteins. Jonas and Ronaldson (1974) reported that a sporidesmin-

poly-L-lysine complex produced sporidesmin-specific low-titre antisera in rabbits. The response was weak, probably due to the low aqueous solubility of sporidesmin which prevented a high percentage substitution of the lysine residues. By using a range of structural analogues of parts of the sporidesmin molecule, the chlorine atom in the sporidesmin molecule was shown to be the important antigenic factor (Jonas and Ronaldson, 1974). A sporidesmin analogue, 2-amino-5-chloro-3,4-dimethoxy benzyl alcohol (ACDMBA), was found to produce a much stronger antibody response in rabbits and guinea pigs when it was coupled to heat killed bacteria than to protein (Jonas and Erasmuson, 1977). ACDMBA is a synthetic molecule similar in structure to the aromatic moiety of sporidesmin. Mice immunised with this ACDMBA-bacteria complex and later challenged with sporidesmin, were apparently more resistant to the bilirubinaemic effects of sporidesmin than were control mice (Jonas and Erasmuson, 1977, 1979). Jonas and Erasmuson (1979) found no relationship between the serum antibody level, determined by complement fixation, and the protection afforded against sporidesmin, thus indicating the involvement of either non-complement fixing serum antibodies or non-serum antibodies in the intestine.

The protective effect was not successfully extended to sheep. On the contrary, immunisation exacerbated sporidesmin toxicity. Sheep immunised with sporidesmin-bovine thyroglobulin complex or ACDMBA-staphylococcus complex were shown to contain antibodies against sporidesmin, but when challenged with sporidesmin, all immunised animals had significantly higher serum cholesterol and bilirubin values and greater liver and urinary bladder damage than the unimmunised controls (Fairclough et al., 1981). Fairclough et al. (1981) concluded that this might have been due to the prolonged half-life of sporidesmin when bound to

antibody, as the extent of liver damage correlated with serum antibody titre. There appears to be little prospect for early field control of the disease by these means although production of sporidesmin-containing immunogens is still an active area of research.

1-3.8 Inducers of Microsomal Drug Metabolism

Hepatic drug-metabolising enzyme activities in sheep are markedly induced by hexachlorobenzene (Turner and Green, 1974). Sheep pretreated with hexachlorobenzene and subsequently challenged, either with doses of sporidesmin or by grazing on toxic pastures, showed significantly less liver injury than the untreated sheep (Mortimer et al., 1978a), and the protection lasted throughout the toxic season. Microsomes from hexachlorobenzene treated sheep gave cytochrome P-450 binding spectra different to microsomes from untreated sheep (Mortimer et al., 1980). These experiments suggested that the hepatic mixed function oxidases might be partly responsible for metabolism and detoxification of sporidesmin. Hexachlorobenzene produced no toxic effects in sheep, but tissue residues in food animals would present an unacceptable hazard to humans (Avrahami and Steele, 1972). Several accepted food additives (antioxidants), animal remedies (antihelminthics) and naturally occurring compounds in possible feed plants were investigated for the ability to induce the mixed function oxidase system in sheep and the most promising inducers were selected for testing against sporidesmin poisoning (Sissons and Mortimer, 1979; Sissons, 1982). Butylated hydroxytoluene showed some degree of protection from liver injury in sheep when pre-injected but not orally pre-dosed (Mortimer et al., 1978b), but both the cytochrome P-450 induction and protection from liver injury varied more among individual animals

than with hexachlorobenzene treatment (Sissons, 1982). Injection of the antioxidant ethoxyquin or the antihelminthic tetramisole afforded no significant protection as compared to controls (Mortimer et al., 1980). Sissons (1982) reported that ethoxyquin decreased liver cytochrome P-450 activities in sheep and increased damage upon exposure to sporidesmin. Brassica crops, known to act as mixed function oxidase inducers, also afforded no significant degree of protection (Mortimer et al., 1980).

Sissons (1982) showed that naphthalene orally administered to sheep shortly before sporidesmin challenge appeared to provide protection from liver damage. It seemed that differences in the rate of metabolism of sporidesmin by hepatic drug-metabolising enzymes might be one of the factors which determined whether sheep were resistant or susceptible to facial eczema. If this was true, then a metabolism assay could be used to select breeding stock for resistance to facial eczema.

1-3.9 Genetic Resistance to Facial Eczema

Campbell et al.(1975) first reported that resistance to sporidesmin liver injury is highly heritable and on the basis of work with 1455 progeny of 160 Romney rams have since estimated the heritability (h^2) to be 0.42 ± 0.09 (Campbell et al., 1981). Such a high heritability offers the possibility of breeding more resistant stock.

The original progeny test for resistance was carried out by dosing the lambs with known amounts of sporidesmin and later slaughtering them to determine the extent of liver damage. A subjective scoring system, based on the extent and degree of damage observed in the excised liver, has been used to monitor the severity of intoxication. The livers have been graded on a

0-5 scale of increasing liver damage (Smith et al., 1977). Although the procedure has been used successfully in trial work for a number of years (Sinclair, 1961; Campbell and Wesselink, 1973; Campbell et al., 1975; Smith et al., 1977), it imposes severe restraints on experimental designs and is subjected to possible variation between trials and between graders. The progeny test, which requires the dosing and slaughter of 10 or so lambs per sire screened, is clearly impractical in the field. Therefore, a more objective measure of liver damage, not requiring liver examination, has been sought. A performance test, based on the elevation of serum GGT following sporidesmin challenge was developed (Ford, 1974; Towers and Stratton, 1978; Campbell and Meyer, 1982). The liver enzyme GGT is released into the blood when the liver is damaged. The increased levels of serum GGT following sporidesmin intoxication are highly correlated with losses in body weight and with the liver-damage scores assigned after slaughter of the animals. The normal GGT levels generally fall within the range 17-50 IU/*l* and values greater than 50 IU/*l* are considered to be abnormal (Towers and Stratton, 1978). As a stable, readily assayed enzyme that remains elevated for several weeks, GGT is well suited to field use. This blood test thus allows assessment of liver damage in live animals and is now widely used. Some commercial stud breeders are already attempting sire selection from resistance shown in spontaneous outbreaks and after sporidesmin administration.

The genetic resistance is highly heritable and is not swamped by a severe toxin challenge. After the challenge, figures for survival and live weight are considerably higher in resistant than in susceptible progeny groups. The biochemical basis for the differing susceptibility to sporidesmin is not clear. There is evidence that sporidesmin resistance in sheep is in part related

to differences in the hepatic microsomal drug-metabolising system (Fairclough et al., 1978). Aust (1977) found a high hepatic microsomal drug-metabolising ability in Merinos, a sporidesmin resistant breed. Sissons and Fairclough (1978) studied the conversion of sporidesmin to polar metabolites in the 10,000 *g* supernatant fraction of liver biopsies taken from rams and discovered that high rates of sporidesmin metabolism were correlated with facial eczema resistance. Sissons (1982) also found that there was an apparent association between inherited susceptibility to sporidesmin and high levels of NADPH cytochrome c reductase in liver. This enzyme, which reduces cytochrome P-450, is low in Merino sheep which have high resistance to facial eczema.

Fairclough and Smith (1983) dosed sheep with sporidesmin at 0.08 mg/kg/day over 3 days and found that susceptible animals showed a marked increase in maximum sporidesmin concentrations in the bile between the first and third dosing, whereas resistant animals showed no marked increase at these times. They suggested that an initial or potentiating dose of sporidesmin might have been required before resistance or susceptibility was expressed.

No practical test has yet been developed to determine the resistance or susceptibility of a particular animal other than exposure to sporidesmin followed by assessment of liver damage. The sporidesmin dosing regime sometimes creates a very severe response if the animals have already been pre-exposed to toxic pastures (Towers and Smith, 1981) and under these circumstances there is uncertainty in the determination of the resistance status. In view of this fact, it is highly desirable to develop a new method by which the facial eczema resistance status can be determined without a sporidesmin challenge. One such method may involve the direct assay of properties which have been shown to

be associated with the resistance mechanism. Another method involves the identification of a trait which is associated with facial eczema resistance. It is this second option that forms the basis for the present study.

1-4 Phenotypic and Genetic Association between Biochemical Polymorphic Characters and Production, Reproductive Traits and Disease Resistance

Polymorphism is defined as the occurrence together of two or more distinct forms of a genetically determined character in the same population at the same time in such proportions that the rarest of them cannot be maintained by mutation alone. Polymorphism is found in many proteins, both enzymes and non-enzymes, from a wide variety of biological fluids and tissues.

It is generally agreed that genetic variability enables a species to evolve in response to changes. It is suggested that variability also allows a population to exploit the environment more efficiently than a single genotype can do.

Since the discovery of inherited biochemical polymorphism in farm animals, interest has centred on the possibility of a relationship between various polymorphic characters and production and reproductive traits, or the resistance of animals to diseases. Such relationships are of great interest in providing biochemical and physiological bases for productive and performance differences and in selecting animals for various desirable traits. Phenotypic associations between a variety of production traits and various blood biochemical polymorphic characters have been reported by Atroshi (1979). Several biochemical polymorphisms have been shown to occur in the red cells and plasma of sheep, and four of these, haemoglobin, glutathione, potassium and transferrin have been the subject of considerable study.

1-4.1 Haemoglobins

There are three different types of haemoglobin normally found in sheep: Hb A, Hb AB and Hb B (Harris and Warren, 1955; Evans et al., 1956). They are distinguished on the basis of their

electrophoretic mobility in starch gels, Hb A having a faster mobility than Hb B (Evans et al., 1956). These haemoglobin types are genetically determined by two co-dominant autosomal alleles which produce three observable phenotypes, namely AA, AB and BB (Evans et al., 1956; Huisman et al., 1958b). In rare cases, additional haemoglobin types, namely Hb C or Hb N and Hb D, are found in sheep. Hb C was reported in animals which were spontaneously anaemic (Blunt and Evans, 1963; Braend et al., 1964; van Vliet and Huisman, 1964; Braend and Efremov, 1965; Arora et al., 1970) or in animals with experimentally induced anaemia (Beale et al., 1966; Moore et al., 1966; Tucker, 1966; Kitchen et al., 1968; Agar and Evans, 1969; Blunt et al., 1969; Neethling et al., 1969; Agar et al., 1970) and also in certain non-anaemic sheep (Wilson et al., 1966; Atroshi et al., 1979). The Hb C was found to have a slower electrophoretic mobility than Hb B (van Vliet and Huisman, 1964). Hb D, with a faster electrophoretic mobility than Hb A, was reported in sheep by Vaskov and Efremov (1967).

Physiological differences in sheep with different haemoglobin types have been reported and the most important difference was with regard to oxygen affinities. Thus, Hb A was shown to have a higher affinity for oxygen than Hb B; Hb AB was intermediate (Huisman et al., 1958a; Meschia et al., 1961a, b; Dawson and Evans, 1962; Naughton et al., 1963; Breathnach, 1964; Sirs, 1966; Horejsi, 1970). The importance of this difference was that Hb B animals were more efficient in transporting oxygen than were Hb A animals because Hb B was able to liberate more oxygen per unit of haemoglobin to the body tissues for a given decrease in oxygen supply (Dawson and Evans, 1965). Dawson and Evans (1962) and Shreffler and Vinograd (1962) have shown that Hb B was less stable than Hb A and was easily denatured and dissociated

into subunits. Manwell and Baker (1970) speculated that sheep with Hb A had an advantage over Hb B sheep because less of their amino acid intake and energy production had to be used for the maintenance of the circulatory haemoglobin levels. This idea was supported by the finding that Hb A sheep had greater fleece weights than Hb B sheep (Watson and Khattab, 1964).

Haemoglobin types have been examined in relation to reproductive performance, weight and lamb mortality, and it was found that Hb A and Hb AB appeared to have an advantage in fertility over Hb B. Ewes of the Hb A type had greater body and wool weights than ewes of Hb AB and Hb B types (Atroshi, 1979). King et al. (1958) have reported that the mortality rate of lambs born to Hb B ewes was lower than that of lambs born to Hb A ewes, while that of lambs born to Hb AB ewes was intermediate.

1-4.2 Glutathione

On the basis of glutathione concentration in the blood, sheep were classified into high (GSH^H) and low (GSH^h) glutathione types (Atroshi, 1979), and it was suggested that the erythrocyte GSH levels were controlled by a single pair of autosomal alleles, the gene for GSH^H being dominant (Tucker and Kilgour, 1970, 1972). It has been shown that ewes with a GSH^H maintained a higher number of lambs than those of GSH^h (Atroshi, 1979). High mortality in lambs was found to be strongly associated with GSH^h. Jaffe (1970) showed that GSH was involved in protecting haemoglobin against irreversible oxidation and denaturation, in guarding membrane lipids against peroxidation and in shielding essential enzymes against inactivation. Therefore, it might be expected that GSH deficient cells would not be able to withstand oxidative stress as well as normal red cells (Tucker, 1977).

Erythrocyte GSH levels have been shown to be associated with milk yield and body size in cattle (Kidwell et al., 1955; Boriskenko, 1961; Slepcov, 1961) and fleece weight in sheep (Saltykov, 1956). Agar et al. (1972) reported a positive correlation between both body and wool weight and GSH^H in Merino sheep. Atroshi (1979) reported that Finnsheep with GSH^H appeared to have greater body weight and more wool yield than sheep with GSH^h. Therefore, it appeared that erythrocyte GSH-types were genetically controlled, and that the GSH concentration within these types was highly heritable (Board et al., 1974).

1-4.3 Potassium

The red cell potassium (K^+) polymorphism found in sheep revealed that some sheep had red cells with high K^+ (HK-type) and some had cells with low K^+ (LK-type). This difference was said to be controlled by an allelic pair of genes, the gene for low K^+ being dominant over that for high K^+ (Evans and King, 1955). There was an indication that the level of potassium might be associated with lamb production (Turner and Koch, 1961). The LK-type sheep appeared to have some superiority over the HK-type sheep in numbers of lambs born. It was also interesting to note that the level of potassium in HK-type sheep was found to be associated with lamb mortality (Atroshi, 1979). In terms of ewe body and wool weights, sheep of LK-type tended to have greater wool and body weights than sheep of HK-type (Watson and Khattab, 1964; Taneja and Ghosh, 1967). Sheep of HK-type had a slightly better birth weight and neonatal growth than LK-type sheep (Atroshi, 1979).

1-4.4 Transferrins

The transferrins comprise a group of iron-binding proteins present in various fluids of vertebrate animals. They have been found in the blood of vertebrates, in the egg white of birds, and in the milk of mammals.

The nomenclature of the transferrins has developed along several different routes. The name ovotransferrin was given to the iron-binding protein in egg white. The milk protein has been called lactotransferrin. The transferrins from blood have been variously called β_1 metal-binding globulin, siderophilin and transferrin. In the present study the term 'transferrin' is used for the iron-binding protein of sheep plasma because this name indicates the major biological function of the protein, and also because it has received wider usage than other names.

The transferrins can be characterised as glycoproteins which contain a single polypeptide chain, having a M_r between 75,000 and 100,000. The heterosaccharide chains may be involved in the binding of transferrins to specific receptors on cells. Transferrin in blood has the vital function of transporting iron to the tissues.

Polymorphism of transferrins has been described in numerous mammalian species, including man (Smithies, 1957; Harris et al., 1958; Roop and Putnam, 1967; Anderson and Anderson, 1979), cattle (Ashton, 1957; Hickman and Smithies, 1957), sheep (Ashton, 1958a; Ashton and McDougall, 1958; Ashton and Ferguson, 1962; Khattab et al., 1964; Cooper et al., 1967; Stormont and Suzuki, 1968; Rasmusen and Tucker, 1973; Chudoba and Jablonska, 1981), goats (Ashton and McDougall, 1958), horses (Ashton, 1958b), pigs (Ashton, 1960; Kristjansson, 1960), mice (Ashton and Braden, 1961; Cohen, 1961), chimpanzees (Boyer and Young, 1960; Buettner-Janusch, 1961), monkeys (Lai and Kirk, 1960; Goodman and Poulik,

1961), reindeer (Gahne and Rendel, 1961), and deer (Lowe and McDougall, 1961). The genetic mechanism, where established, has been shown to be essentially the same in each species. The polymorphism is said to be due to multiple autosomal alleles exhibiting co-dominance (Smithies and Hiller, 1959). Multiple alleles are genes that occupy the identical loci on homologous chromosomes but affect the same trait in a different manner. The number of detectable alleles varies between species and between breeds within the species. Sheep serum transferrin shows the most extreme diversity. Originally, Ashton (1958a, c) identified fourteen distinct transferrin phenotypes in sera from sheep of British breeds. These were regarded as representing all but one of the fifteen possible types from a five allele system (A, B, C, D and E in decreasing order of electrophoretic mobility). A further five alleles (F, G, H, J and K in decreasing order of mobility) were later identified in Merino sheep (Ashton and Ferguson, 1962). An international system for the designation of the transferrin types was established : I, A, G, B, C, M, D, E and P in decreasing order of electrophoretic mobility (Oosterlee and Bouw, 1967). This already complex system was further complicated by the detection of more variants, bringing the total to more than 20 alleles (Guerin et al., 1976). It soon became apparent that even this list of transferrin variants was by no means complete. The assumption of multiple allelism is not proven but it can be supported by several lines of evidence. First of all, no individuals with more than two forms of transferrin have been reported. There appeared to be little, if any, consistent difference in the amount of the two proteins observed in heterozygotes, the total quantity being about the same as that observed in homozygotes. Furthermore, in mixtures of serum obtained from two different homozygotes, the electrophoretic

pattern could not be differentiated from that of a heterozygote. Second, in family studies, the transferrin variants appeared to be inherited as simple Mendelian co-dominant traits. Third, the various transferrins differ from each other in electrical charge, rather than molecular weight. This difference in electrical charge is due to different amino acid compositions of the molecule.

Wang and Sutton (1965) prepared tryptic digests of purified human transferrins C and D_1 and found a difference in the amino composition of one peptide. Amino acid analysis indicated that an aspartic acid residue in transferrin C was replaced by another residue, probably glycine, in transferrin D_1. The authors pointed out that the difference in charge resulting in an aspartic acid to glycine change would be sufficient to account for the electrophoretic behaviour of the two transferrins. Wang et al. (1966) compared the peptide maps of human transferrin B_2 and transferrin C and detected a single peptide difference which was found to be due to the substitution of a glycine residue in transferrin C by a glutamic acid residue in transferrin B_2.

Each transferrin allele causes the production of two or more protein-staining zones on starch or polyacrylamide gels after electrophoresis. The number of electrophoretically distinguishable protein zones produced by an allele is a characteristic of the species. In cattle, each allele produces four zones (Ashton and McDougall, 1958). Three zones are produced by each allele in pigs (Ashton, 1960), mice (Ashton and Braden, 1961), and horses (Ashton, 1958b). Two zones are produced in humans, sheep and goats (Ashton and McDougall, 1958). The reason for this multiplicity of zones from one allele is the different numbers of sialic acid moieties attached to the transferrin molecule (Patras and Stone, 1961).

The widespread gene-controlled polymorphism in serum transferrins has led to consideration of a number of possible selective pressures which might influence type frequencies. Ashton and Fallon (1962) have reported that the transferrin locus in cattle affected fertility. It has been shown that matings between homozygous parents of like genotype had a greater chance of achieving fertilisation than matings between homozygous parents of unlike genotype. Rasmusen and Tucker (1973) have shown that transferrin phenotypes have an effect on reproduction in sheep. In Finnish Landrace, matings of sheep homozygous for transferrin C to those heterozygous for transferrin C gave a significant excess of homozygous male lambs and heterozygous female lambs (Rasmusen and Tucker, 1973). Atroshi (1979) reported that transferrin AD appeared to have a slight advantage over other types in ewe body and wool weight.

1-4.5 Molecular Markers Associated with Disease Resistance in Livestock

Naturally occurring and selectable resistance towards a number of diseases have been found in laboratory animals and in livestock. Since effective measures of disease control are not available for many of these diseases, selection of resistant strains is an attractive control option. In large animals, at least in New Zealand, mannosidosis in Angus cattle has probably been the disease most extensively monitored and selected against (Jolly, 1978). This was possible because of the prior elucidation of the biochemistry of the disease process and the establishment of the blood assays for α-mannosidase which allow detection of heterozygote carriers (Thompson et al., 1976; Jolly et al., 1977; Jolly, 1978).

In New Zealand, heritable resistance to facial eczema in sheep (Campbell et al., 1981) and to bloat in cattle (Cockrem et al., 1983) has similarly been established. There are also marked differences in the susceptibility of cattle breeds to ticks (Wagland, 1975) and sheep have been selected for resistance to particular intestinal parasites. In many cases the biochemistry and physiology of resistance have not been established and an unequivocal molecular marker can not be used to guide selection.

Selection and breeding for resistance to disease is not new especially in the poultry industry where genetic studies have the advantages of prolific progeny and short generation interval. In the case of Marek's disease, chickens with a particular blood group are found to be more resistant to the virus than those without. This blood group, B_{21}, is controlled by a particular gene in the major histocompatibility complex (MHC) of the chicken. Because the MHC is involved in the control of many immune functions, it is likely that the MHC may be involved in disease pathogenesis in larger domestic animals. However, there are other genes not associated with the MHC which also control resistance to some diseases. Resistance to salmonellosis in mice is correlated with antibody levels, thus the ability to produce antibodies may be a potential predictor of susceptibility to some diseases. In other instances, the ability to mount a cellular reponse to infectious organism may provide a clearer marker of disease resistance.

There is an important need to develop simple tests for traits whose molecular basis has not been established. Such tests may merely reflect a genetic or physiological association with the trait. To that extent, the technique of 2-D PAGE which is capable of resolving protein mixtures into several hundred distinct molecular species should be of use in assessing protein

variation linked to disease or productivity characters. Although 2-D PAGE has been used widely to analyse differences in protein composition due to genetic, developmental or environmental variation in gene expression, there appear to be no previous reports of the application of the technique to detection of protein variation linked to disease resistance or productivity in livestock.

1-5 2-D PAGE

Barrett and Gould (1973) and McGillivray and Rickwood (1974) were among the first to introduce the IEF-SDS electrophoresis technique. However, the 2-D PAGE technique did not become popular until 1975 when O'Farrell published the results of two-dimensional fractionation of radioactively labelled proteins from E. coli.

The 2-D PAGE system that combines the techniques of IEF in the presence of urea and a neutral detergent in the first dimension and slab gel electrophoresis under denaturing conditions using SDS in the second dimension, gives the best resolution for separating a complex mixture of proteins (O'Farrell, 1975; Scheele, 1975; Iborra and Buhler, 1976). The separation makes use of two independent protein characteristics: one is the overall charge, which is reflected by the pI, and the other is the M_r, which determines the mobility of the SDS-protein complexes in polyacrylamide gels (Weber and Osborn, 1969). IEF is usually chosen for the first dimension separation because of its ability to concentrate protein species into extremely narrow zones in low concentration polyacrylamide gels. These narrow protein zones serve as ideal sample zones for the second dimension analysis. The most widely used second dimension separation technique is SDS gel electrophoresis (O'Farrell, 1975; Ames and Nikaido, 1976; Fox, 1976; O'Farrell et al., 1977; Piperno et al., 1977; Wilson et al., 1977; Garrels, 1979; Henslee and Srere, 1979; Horst and Roberts, 1979), which in combination with IEF, generates information on both pI values and M_r values. 2-D PAGE is capable of resolving proteins which vary by as little as 0.1 charge unit or by 10 amino acid residues in size (Anderson and Anderson, 1977). The protein map thus produced can be used to distinguish between more than one thousand different proteins on

a single gel and is capable of detecting differences in proteins present in amounts as low as 10^{-5}% of the total protein loaded or a protein containing as little as 1 d.p.m. of either ^{14}C or ^{35}S (O'Farrell, 1975). The reproducibility of the separation is sufficient to allow each spot on different separations to be matched.

O'Farrell (1975) and Ames and Nikaido (1976) have developed high resolution two-dimensional methods for use in analysis of such complex protein mixtures as total E. coli lysates, membrane proteins, and flagellar proteins (Piperno et al., 1977). Anderson and Anderson (1977, 1978 a, b) have further developed the 2-D PAGE for the analysis of human plasma proteins. The 2-D PAGE technique has been used extensively for the analysis of serum and plasma (Latner et al., 1980; Tracy et al., 1982a, b, c; Marshall et al., 1984; Polack et al., 1984; Sprecher et al., 1984; Wheeler et al., 1986). The technique has also been used to demonstrate minor protein variation among cells and cell fractions (Willard et al., 1979), and in body fluids (Burdett et al., 1982), secretory proteins (Scheele, 1975), eukaryotic tissues (Wilson et al., 1977), liver (Henslee and Srere, 1979; Peters and Comings, 1980; Vlasuk and Walz, 1980; Klose and Feller, 1981; Mills and Freedman, 1983; Colbert et al., 1984; Anderson et al., 1985; Neel et al., 1985; Anderson et al., 1986; Rahimi-Pour et al., 1986; Wirth et al., 1986), kidney (Henslee and Srere, 1979), brain (Henslee and Srere, 1979; Klose and Feller, 1981), heart (Henslee and Srere, 1979), skeletal muscle (Henslee and Srere, 1979), and variation due to genetic polymorphism (Walton et al., 1979; McLellan et al., 1983; Neel et al., 1985), and inborn errors of metabolism (Merril and Goldman, 1982). In summary, 2-D PAGE has been used extensively to study protein variation and such differences may reflect either cellular changes, sometimes

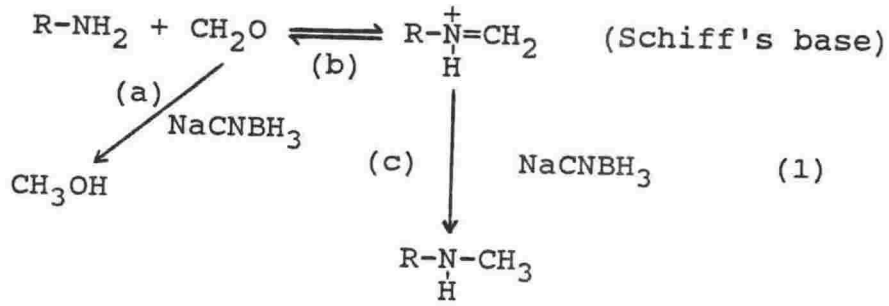
as a result of disease, or the expression of inherited differences.

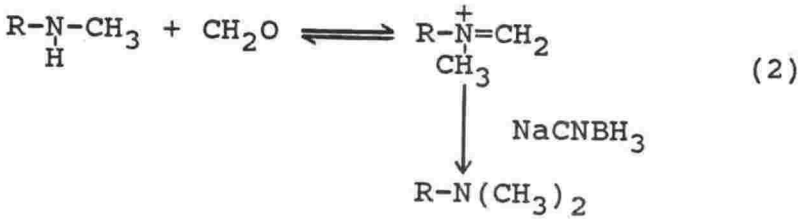
In most of the work with PAGE of proteins, Coomassie blue staining is used as the detection method. Coomassie blue reacts with any protein irrespective of biological activity and the staining procedure is simple. Usually 0.2-0.5 μg of any protein in a sharp band can be detected and staining is quantitative to 15 μg for at least some proteins. Alternatively, silver staining and differential detection of proteins selectively labelled with radioactive isotopes are used to analyse minor variation. The major advantage of radiolabelling is that detection methods for radiolabelled proteins following gel electrophoresis are far more sensitive than staining methods for unlabelled proteins.

1-6 Chemistry of Labelling

There are many ways of modifying proteins to facilitate protein identification. One such way is by attachment of a radioactively labelled group to the side chain residues of the amino acids. Reductive methylation was chosen for the present study because it is rapid, and produces minimal change in the physicochemical properties of most proteins (Means and Feeney, 1968; Geoghegan et al., 1981). Reductive methylation is highly specific. The only groups labelled are the ϵ-amino groups of lysyl residues and the α-NH_2 terminus. Reductive methylation affects amino groups by changing their pK values by about 0.5 pH unit (Means and Feeney, 1968). The mild reaction conditions not only maintain the total charge of the protein, but also the spacial distribution of charges. The dimethyl derivative is still able to be ionised due to the presence of the lone pair of electrons on the nitrogen centre. Hence, it should be possible to use the labelled proteins in place of native ones in many electrophoretic and chromatographic procedures.

The free amino groups of proteins can be converted to the N,N-dimethyl derivatives by reaction with formaldehyde and a reducing agent such as sodium cyanoborohydride. The mechanism of reductive methylation presumably involves the formation of an intermediate Schiff's base, the reduction of which yields the corresponding methyl-derivative as shown in reaction (1).



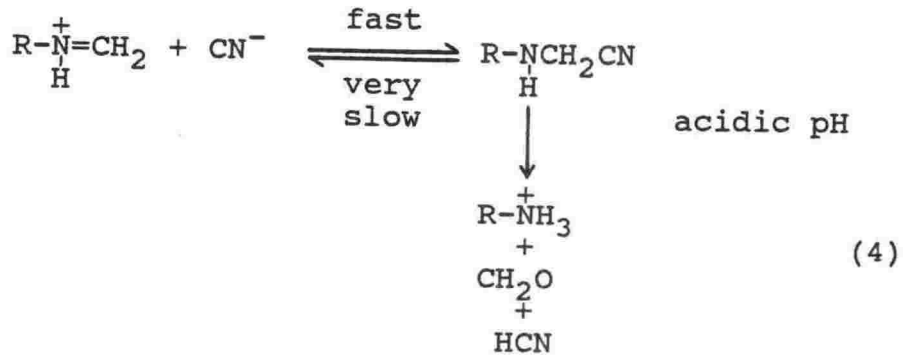


(2)

Since the formation of the Schiff's base is the slow step, an increase in the formaldehyde concentration would be expected to result in greater derivatisation. Reduction, on the other hand, occurs very rapidly and should only require stoichiometric amounts of $NaCNBH_3$. Since both primary and secondary amines can be reductively methylated, the monomethyl amine shown as the product in reaction (1) can undergo a second cycle of reductive methylation to form the N,N-dimethyl derivative as shown in reaction (2).

$NaCNBH_3$, being a good reducing agent for aldehydes as well as for Schiff's bases, reduces formaldehyde directly to methanol (reaction a). Consequently, the efficiency of methylation is lower since it is a function of the relative rates of reaction (b) and (c) compared to reaction (a). Formaldehyde can also participate in undesired side reactions with proteins, the most serious of which is the possible production of intra- or inter-molecular cross-links through formation of methylene bridges. If the formation of a Schiff's base is also a prerequisite for cross-linking reactions, the absence of cross-linking during reductive methylation must be due to the rapid reduction of the Schiff's base by $NaCNBH_3$. Complete derivatisation is never achieved, presumably because of the competitive formation of glyconitrile $HOCH_2CN$ (reaction 3) (Jentoft and Dearborn, 1980).

(3)



(4)

However, this explanation is unconvincing since the final step of reductive methylation (reaction 2) is irreversible while glyconitrile formation is reversible (reaction 3). In fact, low yields are shown to be due to N-cyanomethyl compounds which are formed effectively and irreversibly at neutral pH by the reaction shown in (4) (Gidley and Sanders, 1982). However, exposure to acid or prolonged dialysis at neutral pH, results in hydrolysis of the N-cyanomethyl compounds with regeneration of the starting material. Jentoft and Dearborn (1980) suggested that transition-metal ions, e.g., Ni^{2+}, could be used to increase the yield up to 30% by forming stable coordination complexes with cyanide thus preventing the secondary reaction. The use of Ni^{2+} is effective not because it prevents the formation of the labile glyconitrile, but because it suppresses the more damaging formation of N-cyanomethyl derivatives (Gidley and Sanders, 1982). Recrystallisation of sodium cyanoborohydride to remove cyanide ion is also found to be beneficial.

Substitution of $NaCNBH_3$ for $NaBH_4$ as the reducing agent has a number of advantages: a) the reaction can be performed under mild conditions and at neutral pH, rather than at pH 9 or above. Since formation of Schiff's base is maximal at pH 6 (Borch et al., 1971), a lower pH enhances the extent of methylation, b) the reaction can be carried out at room temperature rather than at $0^{o}C$, c) smaller amounts of formaldehyde are required for

methylation because of the greater stability of formaldehyde at neutral pH (MacKeen et al., 1979), d) much higher levels of labelling can be achieved with $NaCNBH_3$ and a wide variety of proteins can be labelled without any major effect on function.

1-7 Double-labelling

Double-labelling is a technique which can be used to analyse small differences in complex mixture of proteins. With this technique, one protein sample is labelled with one radioactive isotope, e.g., ^{3}H, and the other protein sample with another isotope, e.g., ^{14}C. This can be done by reductive methylation of free amino groups using [^{3}H]formaldehyde and [^{14}C]formaldehyde. After labelling, the two labelled protein samples are mixed together and the individual proteins are subsequently separated by conventional biochemical techniques such as IEF or 2-D PAGE. Once the two protein samples are mixed, the labelled proteins serve as their own internal controls and any condition which affects one type of labelled proteins will affect similarly the other type, thus artificial differences will not be generated. After protein separation, the ^{3}H:^{14}C ratio of each protein can be measured by techniques such as scintillation counting of IEF fractions, or by autoradiography and fluorography of the radioactively labelled proteins separated on polyacrylamide gels. If a protein is present only in one sample and not the other, then this will be manifested as an abnormal ^{3}H:^{14}C ratio.

Both qualitative and quantitative comparisons of ^{3}H-labelled and ^{14}C-labelled protein mixtures are made possible by the high resolution of 2-D PAGE, followed by autoradiography and fluorography. Autoradiographic detection of ^{14}C-labelled proteins can be performed on dried polyacrylamide gel by placing a photographic film against the gel. ^{3}H is not detected by this method since the low energy ß particles of ^{3}H fail to penetrate the gel matrix. The fluorographic method, which relies upon impregnation of the polyacrylamide gel with a scintillant, is used to allow indirect detection of ^{3}H by the light generated

within the gel when the ß particles interact with adjacent fluor molecules (Bonner and Laskey, 1974). Fluorography detects both ^{14}C and ^{3}H while autoradiography detects only ^{14}C, thus a fluorogram contains spots due to both ^{14}C and ^{3}H while an autoradiogram only contains spots due to ^{14}C. This means that those spots due only to ^{3}H can be distinguished by subtracting the spots on an autoradiogram from those on a corresponding fluorogram (McConkey, 1979).

Double-labelled autoradiography and fluorography for comparison of complex protein mixtures after gel electrophoresis has been used in a wide variety of biological investigations including genetics, molecular evolution and metabolism. It has been applied to the comparison of normal and mutant cell proteins (Choo et al., 1980), and for comparison of proteins synthesised by one type of cell in different phases of the cell cycle. Double-labelling has also been used in the comparison of human and mouse liver extracts (Finger and Choo, 1981), and has potential for studies at the molecular level of cell differentiation, tumorigenesis, viral infection and for diagnosis of complex metabolic abnormalities including genetic diseases (McConkey, 1979).

In this thesis, the proteins present in plasma and liver biopsies from facial eczema resistant and susceptible sheep have been compared. The methodology of double-labelling can thus be shown diagrammatically as it applies to the detection of protein markers for facial eczema resistance or susceptibility (Figure 2). In this approach, liver biopsies or plasma samples of resistant sheep are pooled and the proteins are labelled with ^{3}H by reductive methylation of free amino groups. Proteins in pooled liver biopsies or plasma from susceptible sheep are labelled with ^{14}C by the same method. The labelled protein samples are then

mixed and subsequently separated by 2-D PAGE. Pooled protein samples are used to minimise individual animal differences that are not representative of the total population. Analysis of the separated labelled proteins by autoradiography and fluorography will then allow detection of the ^{14}C-labelled proteins from susceptible sheep in both the autoradiogram and fluorogram but detection of the ^{3}H-labelled proteins from resistant sheep only in the fluorogram. Any protein found only in the fluorogram would thus represent a ^{3}H-labelled protein found only in resistant sheep. A reverse-labelling experiment using ^{14}C-labelling of proteins from resistant sheep and ^{3}H-labelling of proteins from susceptible sheep would allow specific detection of proteins found only in susceptible sheep.

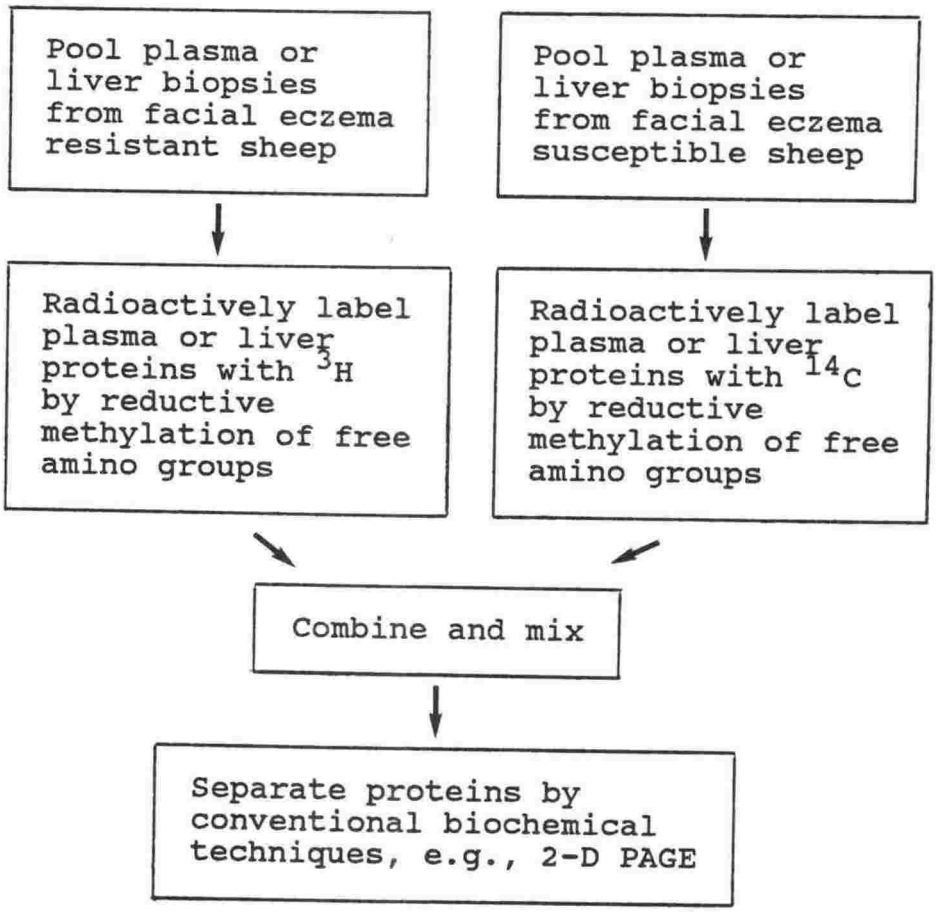


FIGURE 2. Summary of the Double-labelling Technique

Differences in plasma or liver proteins between resistant and susceptible sheep will appear as an abnormal ^{3}H:^{14}C ratio for a particular protein. A protein unique to the resistant sample will have only a ^{3}H-label and a protein unique to the susceptible sample will have only a ^{14}C-label.

1-8 Purpose of this Project

This project involves work on detection of plasma and liver protein markers for facial eczema resistance or susceptibility in Romney sheep. Methodological development was an important component of the project. The techniques developed include IEF and 2-D PAGE for protein separation and analysis. In general, proteins separated by 2-D PAGE were examined on Coomassie blue or silver stained gels for protein variation related to facial eczema resistance or susceptibility. The sensitivity obtained by 2-D PAGE was about 10^{-5}% of the total protein separated. Alternatively, differential detection of proteins selectively labelled with radioactive isotopes was used to analyse minor variation not detectable by the simple staining techniques. To simplify initial analyses, a pooling protocol was used whereby a number of samples from a given population were pooled and examined on a single gel. This technique allowed rapid comparison of variation between populations and any detected variation was further analysed for individuals on one-dimensional polyacrylamide gels which allowed more rapid analysis of multiple samples.

Plasma protein variation between facial eczema resistant and susceptible animals was analysed first because of the convenience of plasma as a source of diagnostic material. However, it was expected that major protein variation related to mechanisms of facial eczema resistance might occur in the liver, either at the level of binding proteins or enzymes affecting the toxicity of sporidesmin, or at the level of liver cell protein targets for sporidesmin action. Therefore, comparison of proteins in liver biopsies from facial eczema resistant and susceptible animals was also undertaken.

MATERIALS AND METHODS

2-1 Materials

Materials	Sources
Acrylamide	(1)
Agarose (type VIII)	(1)
Ammonium persulphate	(2)
Aprotinin (26 TIU/ml, approx. 900 KIU = 1 TIU)	(1)
Bio-Rad silver stain kit	(3)
Bovine pancreatic insulin	(1)
Bovine serum albumin (fraction V)	(1)
Deoxyribonuclease I (bovine pancreas, 76,055 Dornase units/mg)	(4)
Dithiothreitol	(5)
DM-POPOP	(4)
Dowex 1-X8 resin (acetate form)	(2)
$^{59}FeCl_3$ (100 μCi/ml)	(6)
[^{14}C]formaldehyde (53 mCi/mMol)	(7)
[^{3}H]formaldehyde (85 mCi/mMol)	(7)
Fuji RX 100 film	(8)
Hepes	(1)
Horse heart cytochrome c	(1)
Iodoacetamide	(2)
Kodak SB X-ray film	(9)
2-Mercaptoethanol	(1)
N,N'-methylene-bis-acrylamide	(1)
Neuraminidase (from Vibrio cholerae, 1 U/ml)	(10)
N-octyl ß-D-glucopyranoside (Octyl glucoside)	(1)
Ovalbumin	(1)
Paraformaldehyde	(11)
Pharmalytes, 40% (w/v) (pH 3-10, 4-6.5, 5-8)	(1)

Phenylmethylsulphonyl fluoride	(1)
Polyethyleneglycol 6000	(12)
PPO	(2)
Protosol (0.5 M)	(7)
SDS	(2)
Sodium cyanoborohydride	(13)
TEMED	(2)
Toluene	(14)
Tris	(1)
Triton X-100	(1)
Urea	(12)

Sources:

(1) Sigma Chemical Co.; Missouri, U.S.A.

(2) BDH Chemical Ltd.; Poole, England.

(3) Bio-Rad Laboratories; California, U.S.A.

(4) Calbiochem-Behring; California, U.S.A.

(5) Boehringer Mannheim GmBH Biochemica; Mannheim, W. Germany.

(6) Radiochemical Centre Ltd.; Amersham, England.

(7) New England Nuclear Research Products; Massachusetts, U.S.A.

(8) Fuji Photo Film Co. Ltd.; Tokyo, Japan.

(9) Eastman Kodak Co.; New York, U.S.A.

(10) Hoechst-Behring; W. Germany.

(11) May and Baker Ltd.; Dagenham, England.

(12) Merck; Schuchardt, 8011 Hohenbrunn bei Munchen.

(13) Aldrich Chemical Co.; Wisconsin, U.S.A.

(14) J. T. Baker Chemical Co.; New Jersey, U.S.A.

2-2 Animals, Plasma Samples and Liver Biopsy Specimens

The sheep used for initial comparison of protein variation linked to facial eczema were from the facial eczema resistant (R) and susceptible (S) flocks at Ruakura Animal Research Station, Hamilton, New Zealand. The source of these animals has been described by Campbell et al. (1981). The animals had been selected as sires whose offspring were resistant or susceptible to sporidesmin as assessed by criteria including liver damage scores and serum GGT levels following toxin challenge. The most resistant and susceptible rams had been used to generate R and S flocks by mating initially to random ewes and subsequently to F_1 ewe progeny of the resistant and susceptible rams. In each season the selected R and S flock sires had also been mated, along with prospective R and S sires, to a number of random ewes for subsequent progeny challenge so that sire status was continually confirmed. New sires were added to the flocks for six years before the commencement of the present study.

The Romney sheep selected to provide plasma and liver biopsy specimens were ram and ewe hogget offspring of sires previously shown to produce facial eczema resistant or susceptible progeny as assessed by postmortem liver damage score in earlier progeny following challenge with sporidesmin. The sires also had their facial eczema status confirmed in a performance test following sporidesmin challenge. The dams of the selected animals were the progeny of sires selected from progeny-tested sires over several generations. The facial eczema status of the experimental sheep was thus defined by pedigree (Campbell et al., 1981).

The selected animals and their sires are listed in Table 1. The susceptible sheep were the offspring of 5 unrelated sires, but common genes from a resistant progenitor were shared by 2 of

TABLE 1. Romney Ewe and Ram Hoggets from Facial Eczema Resistant and Susceptible Flocks

Resistant		Susceptible	
Animal Number	Sire	Animal Number	Sire
65 (R)		38 (R)	
106 (R)		107 (R)	
207 (E)	FE 85	255 (E)	FE 309
233 (E)		270 (E)	
31 (R)		25 (R)	
34 (R)		68 (R)	
100 (R)	FE 171	117 (R)	ROTO 140
211 (E)		261 (E)	
223 (E)		269 (E)	
126 (R)		253 (E)	
209 (E)	ROTO 123	259 (E)	ROTO 206
213 (E)			
48 (R)		82 (R)	
88 (R)		90 (R)	
125 (R)	ROTO 193	256 (E)	ROTO 601
208 (E)		260 (E)	
224 (E)			
225 (E)			
61 (R)		83 (R)	
105 (R)	ROTO 645	133 (R)	ROTO 658
212 (E)		251 (E)	
218 (E)		254 (E)	

Animals were Romney ewe (E) and ram (R) hoggets from facial eczema resistant and susceptible flocks. Sires prefixed ROTO were from Rotomahana Animal Research Station while those prefixed FE were from the Ruakura Animal Research Station.

the 5 sires of the resistant sheep (resistant animal HF 42 was the sire of FE 85 and the grandsire of FE 171). The ewes were not challenged with sporidesmin because toxin challenge was part of the breeding programme in which only sires were selected. The responses of the rams to sporidesmin challenge are shown in Tables 2-5. The resistant rams were first challenged with a total of 0.25 mg of sporidesmin/kg live-weight, given by stomach tube in equal parts over 3 days (Table 2). Another challenge at 0.33 mg/kg body weight was performed about 6 weeks later. The more resistant animals, which showed only a slight elevation of serum GGT level, were numbers 34, 48, 88, 125, 65, 106 and 61. Animals 48, 88 and 125 were offspring of sire Rotomahana 193 which in earlier trails had consistantly given resistant progeny. The animals from the susceptible flock were challenged once only at 0.066 mg/kg body weight (0.022 mg/day for 3 days) and usually responded with serum GGT levels greater than 300 IU/*l*, except for animals 82 and 133 whose GGT levels were 71 IU/*l* and 162 IU/*l*, respectively (Table 3).

A further group of sheep were used in later studies of the association of transferrin types with facial eczema resistance. For this work, 8 Romney and 3 Coopworth sires previously shown to give rise to resistant offspring were crossed with 223 random Romney ewes to give 159 lambs. The lambs were challenged with sporidesmin, initially at 0.11 mg/kg body weight and later at 0.18 mg/kg body weight, and serum GGT levels were measured to assess the resistance status of the lambs (Table 4).

Plasma samples for electrophoretic studies were prepared from heparinised blood. The samples were stored at -20^{o}C, and were only thawed once before electrophoresis.

The liver biopsy specimens were taken from resistant and susceptible rams which had been performance tested by sporidesmin

TABLE 2. GGT Responses of Resistant Ram Hoggets to Sporidesmin Challenge

Sire	Animal No.	GGT (IU/l) Dose 1 (0.25 mg/kg)			
		Pre-dose	Day 8	Day 14	Day 21
ROTO 123	126	28	49	52	45
FE 171	31	65	78	85	70
	34	42	51	54	51
	100	58	61	64	55
ROTO 193	48	59	68	69	56
	88	53	53	58	54
	125	24	56	66	55
FE 85	65	49	56	60	50
	106		61	76	65
ROTO 645	61	47	57	65	55
	105	52	46	60	48

Sire	Animal No.	GGT (IU/l) Dose 2 (0.33 mg/kg)			
		Pre-dose	Day 8	Day 14	Day 21
ROTO 123	126	42	71	106	109
FE 171	31	64	87	137	126
	34	46	48	62	54
	100	53	73	129	135
ROTO 193	48	51	55	60	59
	88	48	55	49	47
	125	51	54	53	39
FE 85	65	59	54	57	55
	106	51	59	64	60
ROTO 645	61	48	52	55	53
	105	51	91	122	112

The resistant rams were first challenged with a total of 0.25 mg of sporidesmin/kg body weight, given by stomach tube in equal parts over 3 days. Another challenge at 0.33 mg/kg body weight was performed about 6 weeks later. The GGT information was supplied by Ruakura Animal Research Station.

TABLE 3. GGT Responses of Susceptible Ram Hoggets to Sporidesmin Challenge

Sire	Animal No.	GGT (IU/l) Dose (0.066 mg/kg)			
		Pre-dose	Day 8	Day 14	Day 21
ROTO 140	25	62	64	384	
	68	50	112	518	
	117	58	57	349	
FE 309	38	70	76	393	
	107	55	85	653	
ROTO 601	82	61	51	71	80
	90	59	206	626	
ROTO 658	83	41	52	389	
	133	55	65	162	

The susceptible rams were challenged once only at 0.066 mg of sporidesmin/kg body weight, given by stomach tube at 0.022 mg of sporidesmin/day for 3 days. The GGT information was supplied by Ruakura Animal Research Station.

TABLE 4. Resistance of Lambs to Sporidesmin Challenge

Sire		Resistance Grade	Number of Lambs	
			0	1
Coopworth	5		4	11
	7		4	8
	1		10	11
Romney	9		8	8
	2		8	5
Romney	10		14	2
	6		17	0
	8		4	0
Romney	3		18	2
	4		10	1
	11		14	0

The lambs were challenged with sporidesmin, initially at 0.11 mg/kg body weight and later at 0.18 mg/kg body weight, and serum GGT level was measured to assess the resistance status.

Resistance grade 0 - GGT elevated after 0.11 mg/kg challenge.
1 - GGT did not elevate after 0.11 and 0.18 mg/kg challenges.

challenge. The resistant rams were tested by sporidesmin challenge at 0.23 mg/kg body weight and 0.33 mg/kg body weight (Table 5). The susceptible rams were challenged once only at 0.066 mg of sporidesmin/kg body weight (Table 6). The liver biopsies were carried out at Ruakura Animal Research Station and the biopsy tissues were stored at -20^{o}C before analysis.

TABLE 5. GGT Responses of Resistant Rams from which Liver Biopsy Specimens were taken

Sire	Animal No.	GGT (IU/l) Dose 1 (0.23 mg/kg)			GGT (IU/l) Dose 2 (0.33 mg/kg)		
		Day 8	Day 14	Day 21	Day 8	Day 14	Day 21
ROTO 123	126	49	52	45	71	106	109
FE 85	106	61	76	65	59	64	60
ROTO 645	61	57	65	55	52	55	53

The resistant rams were first challenged with 0.23 mg of sporidesmin/kg body weight given by stomach tube and were later challenged with 0.33 mg of sporidesmin/kg body weight. The GGT information was supplied by Ruakura Animal Research Station.

TABLE 6. GGT Responses of Susceptible Rams from which Liver Biopsy Specimens were taken

Sire	Animal No.	GGT (IU/l) Dose (0.066 mg/kg)	
		Day 8	Day 14
ROTO 140	25	64	384
FE 309	107	85	653
ROTO 658	83	52	389

The susceptible rams were challenged once only at 0.066 mg of sporidesmin/kg body weight. The GGT information was supplied by Ruakura Animal Research Station.

2-3 Experimental Methods

2-3.1 Subcellular Fractionation of Sheep Liver

The liver from a 3 month-old Romney lamb was obtained within 5 min of slaughter and was stored on ice for 30 min before homogenisation. The homogenisation and centrifugation medium was 0.25 M sucrose - 0.001 M EDTA, buffered at pH 7.4 with 0.005 M Tris-HCl. Approximately 10 g of liver was minced in 4 volumes of ice-cold homogenisation medium. The medium was decanted and replaced with a further 4 volumes of ice-cold medium. Homogenisation was carried out using a Polytron P20 probe (Kinematica, Switzerland) at speed setting 5 for 30 s. The total volume of the homogenate was measured for the calculation of enzyme activity and a small portion was retained for enzyme assays and 2-D PAGE.

Centrifugation was carried out at 4°C. The homogenate was centrifuged in a Beckman L5-65 centrifuge (Beckman Instruments) with a 60Ti fixed-angle rotor ($r_{av.}$, 6.3 cm) at 750 *g* for 5 min. The sediment was resuspended to the original volume in ice-cold homogenisation medium and centrifuged for 5 min at 750 *g*. The pellet was designated nuclei and cell debris fraction (N). A washed M-L fraction enriched in mitochondria, lysosomes and peroxisomes was obtained by centrifugation of the 750 *g* supernatant at 15,000 *g* for 15 min (Lim and Jordan, 1981). The combined supernatants were centrifuged at 113,000 *g* for 60 min and the resuspended pellet was the microsomal fraction (P). The supernatant was designated the soluble fraction (S). The volume of each fraction was measured in order to calculate the percent of total homogenate activity in each fraction. The activities of subcellular marker enzymes (Table 7) were determined by Dr T. W. Jordan.

TABLE 7. Enzymic Characterisation of Subcellular Fractions obtained from Sheep Liver

Enzyme	Specific Activity (μmol/min/g protein)	Percent Recovery of Activity in Subcellular Fractions			
		N	M-L	P	S
5'-Nucleotidase	24.2	52	8	34	6
Succinate cytochrome c reductase	5.7	38	58	2	2
Glucose 6-phosphatase	2.9	29	10	49	12
Glutathione S-transferase	385.0	3	1	1	95
Protein (mg/g liver)	190.0	44	11	9	36

The enzyme activities were measured as previously described (Lim and Jordan, 1981). The specific activity was that of the homogenate and the percent recoveries of activity in subcellular fractions were calculated with respect to the homogenate. N, M-L, P and S denotes nuclei and cell debris, mitochondrial-lysosomal, microsomal and soluble fractions respectively.

2-3.2 Preparation of Hepatocytes

Hepatocytes were prepared by the collagenase perfusion method of Berry and Friend (1969). The perfusion buffer, which contained 140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM NaH_2PO_4, 25 mM Hepes, 12.5 mM glucose, 3.3 mM sodium pyruvate and 2.3 mM sodium glutamate, was adjusted to pH 7.4 with NaOH. It was maintained at 37°C and continuously gassed with O_2. A 8 mm (external diameter) plastic cannula was inserted into a major blood vessel on the cut surface of a 25 g portion of liver from a 3 month-old Romney lamb. The liver was perfused at 15 ml/min with 500 ml of Ca^{2+}-free perfusion buffer and the perfusate which contained blood cells was discarded. Recycling perfusion was then carried out for 30 min with 100 ml of perfusion buffer containing 50 mg of collagenase and 1.5 mM $CaCl_2$. When perfusate leaked through the surface capsule, the cannula was removed and the liver was transferred to a Petri dish containing fresh perfusion buffer. Mechanical separation of cells was accomplished by combing. The crude cell suspension was passed through 300 μm mesh Nybolt (Swiss silk bolting cloth, Zurich, Switzerland), and the cells were washed twice by centrifugation in perfusion buffer at 50 *g* for 1 min. The washed cell pellet was passed through 60 μm mesh Nybolt and the filtrate, which contained the hepatocytes, was collected. Yields were about 5×10^7 cells/g of liver, with viability greater than 90% as measured by Trypan Blue exclusion. The purified cell suspension contained at least 95% hepatocytes when examined by phase-contrast microscopy. Hepatocytes were stored at -20°C before electrophoresis.

2-3.3 Sample Preparation for Electrophoresis

2-3.3.1 Transferrin Typing

The plasma samples for transferrin typing were mixed with an equal volume of sample buffer containing 0.125 M Tris-HCl buffer, pH 6.8 and 20% (w/v) glycerol. The samples were either used immediately or stored at -20°C before PAGE.

Iron loading of transferrin was effected by adding ferric ammonium sulphate to the plasma in an amount equivalent to about 5 μg of Fe^{3+} per ml. Radioactive iron (^{59}Fe) was bound to transferrin by incubating 100 μl of plasma with 10 μCi of $^{59}FeCl_3$ (100 μCi/ml) and 1 ml of 0.2 M Hepes, pH 7.4, for 1 h at room temperature.

Neuraminidase digestion of transferrin was carried out by incubating 100 μl of plasma at 37°C with 50 μl of 0.2 M sodium acetate-acetic acid buffer, pH 5.5, containing 0.04 M $CaCl_2$, and 40 μl or 80 μl of neuraminidase (1 Unit/ml). Portions were removed at intervals up to 24 h after incubation and were stored at -20°C before PAGE.

The transferrin phenotype standards were received as freeze-dried samples from 1 ml of sheep plasma. They were rehydrated with 1 ml of deionised water and stored at -20°C before PAGE. The transferrin standards were prepared for PAGE in the same manner as described for plasma samples.

2-3.3.2 SDS-PAGE

The liver biopsy specimens were homogenised in 0.25 M sucrose, 0.05 M NaCl, 0.02 M phosphate buffer, pH 7.5, containing 0.001 M phenylmethylsulphonyl fluoride, 0.001 M iodoacetamide and 100 KIU/ml of aprotinin as protease inhibitors, to a protein concentration of 100 mg/ml. The homogenates were subsequently treated with DNase (10^4 unit/ml) at 37°C for 1 h to digest DNA.

An equal volume of 3% (w/v) octyl glucoside was added to solubilise membrane proteins before electrophoresis.

The plasma and liver samples for SDS-PAGE were mixed with an equal volume of SDS sample buffer containing 0.125 M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 0.1 M dithiothreitol and 10% (w/v) SDS, and were heated in a boiling water bath for 3 min. The samples were either used immediately or stored at -20°C before SDS-PAGE.

Protein markers: bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,200), were dissolved in SDS sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 0.05 M dithiothreitol and 5% (w/v) SDS, and were heated in a boiling water bath for 3 min before SDS-PAGE.

2-3.3.3 2-D PAGE

The samples for 2-D PAGE were prepared by mixing 20 μl of plasma or liver homogenate (prepared as described in section 2-3.3.2) with 40 μl of 2-D PAGE sample buffer, followed by heating the solution in a boiling water bath for 3 min. After the solution had cooled to room temperature, 40 μl of IEF sample buffer and 0.057 g of urea were added to give the following final concentrations of reagents in the sample: 2% (w/v) SDS, 16% (w/v) Triton X-100, 9.5 M urea, 0.05 M dithiothreitol, 4% (w/v) Pharmalytes (pH range 3-10, pH range 4-6.5, pH range 5-8; 2:1:1; v/v/v), 0.0625 M Tris-HCl, pH 6.8, and 0.002% (w/v) bromophenol blue. Once the samples had been prepared, they were either used immediately or stored at -20°C for up to 1 month. Frozen samples were only thawed once before electrophoresis.

2-3.4 2-D PAGE for Protein Separation

2-D PAGE was carried out using a modification of O'Farrell's method (1975). The stock reagents were prepared as described in Appendices 6-1 and 6-2.

2-3.4.1 IEF (First-dimension)

Gels were cast in cylindrical glass tubes (130 mm length, 2.5 mm internal diameter) which had been previously soaked in dichromate cleaning solution for 2-24 h, rinsed in deionised water and air dried. The IEF gel system used is given in Table 8. The gel solution was prepared by weighing out 5.62 g of urea in a 250 ml vacuum flask and adding the required amount of 30% (w/v) acrylamide stock, 10% (w/v) Triton X-100 and 0.075 M arginine/lysine/aspartic acid mixture. The urea was dissolved by gentle swirling. A mixture of 4% (w/v) Pharmalytes (pH 3-10, 4-6.5 and 5-8; 2:1:1; v/v/v) and 4.8% (v/v) glycerol was added and mixed thoroughly. The 10% (w/v) ammonium persulphate was added, mixed and the solution was evacuated for 1 min to remove dissolved oxygen. Finally, TEMED was added with mixing and immediately the gel solution was transferred to the gel tubes with a syringe. The tubes were filled to the required gel length. The gel solution was gently overlaid with 10 μl of gel overlay solution and the gels were allowed to polymerise for 2 h. After polymerisation, the overlay solution was aspirated from the top of the gels and was replaced with 20 μl of IEF buffer. The IEF buffer was overlaid with 5 μl of deionised water and the upper surface of the gel was allowed to equilibrate with the IEF buffer for 1 h.

The gels were loaded into the tube gel electrophoresis apparatus which had been modified from the Shandon model. The

TABLE 8. Gel Formulation for IEF

Reagents	Amount
urea	5.62 g
30% (w/v) acrylamide	1.40 ml
10% (w/v) Triton X-100	2.10 ml
arg/lys/asp mixture	1.45 ml
Pharmalytes pH 3-10	0.52 ml
pH 4-6.5	0.26 ml
pH 5-8	0.26 ml
glycerol	0.50 ml
10% (w/v) ammonium persulphate	0.025 ml
TEMED	0.015 ml

The gel solution contained 9 M urea, 4% (w/v) acrylamide/bis-acrylamide (17.52/1, w/w), 2% (w/v) Triton X-100, a mixture of arginine, lysine and aspartic acid (each 0.01 M final concentration), 4% (w/v) Pharmalytes (pH range 3-10, pH range 4-6.5, pH range 5-8; 2:1:1; v/v/v), and 4.8% (v/v) glycerol. Total volume of 10.4 ml were enough to prepare 12 gels.

The stock reagents were prepared as described in Appendix 6-1.

lower reservoir was filled with 0.04 M H_3PO_4 anode electrolyte solution. The gels were overlaid with 20 μl of IEF buffer and then with 0.1 M NaOH cathode electrolyte solution. The upper reservoir was filled with 0.1 M NaOH. The gels were first focused at 300 V (constant voltage) for 1 h to remove excess persulphate ions. The cathode electrolyte solution and overlay were then removed by aspiration, and 200 to 400 μg of protein were loaded onto each gel. The protein samples were overlaid with 10 μl of sample overlay solution. The upper reservoir was refilled with 0.1 M NaOH and IEF was continued for 20 h at 400 V (constant voltage) and then for 1 h at 800 V so that a total of 8,800 V·h was achieved. The final voltage used was 800 V in order to sharpen the protein bands and to reduce streaking.

A syringe with a 23 gauge needle was used to extrude the gels from the glass tubes. The tube gels were marked at the acidic end by injecting a small amount of India ink. The first-dimensional tube gels were either subjected to SDS-PAGE immediately or stored wrapped in aluminium foil at -20°C.

The pH gradient was measured by cutting the gels into 5 mm sections which were placed in individual tubes containing 0.5 ml of boiled, degassed 0.01 M KCl solution. The tubes were capped and shaken occasionally, and the pH of the eluates were measured at 20°C after 2 h using a pH meter with a single electrode.

2-3.4.2 SDS-PAGE (Second-dimension)

SDS-PAGE was carried out using the SDS-discontinuous buffer system of Laemmli (1970). The uniform 12.5% (w/v) acrylamide resolving slab gel system used is given in Table 9. The gel solution was degassed and polymerisation was initiated by the addition of freshly prepared 10% (w/v) ammonium persulphate and TEMED. After polymerisation, the resolving gel was overlaid with

TABLE 9. Gel Formulation for 12.5% SDS-PAGE

Resolving Gel

Reagents	Amount
lower gel buffer	4.00 ml
deionised water	5.22 ml
30% (w/v) acrylamide	6.70 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The resolving gel solution contained a final concentration of 12.5% (w/v) acrylamide, 0.1% (w/v) SDS and 0.375 M Tris-HCl, pH 8.8.

Stacking Gel

Reagents	Amount
upper gel buffer	2.50 ml
deionised water	5.42 ml
30% (w/v) acrylamide	2.00 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The stacking gel solution contained a final concentration of 6% (w/v) acrylamide, 0.1% (w/v) SDS and 0.125 M Tris-HCl, pH 6.8.

The stock reagents were prepared as described in Appendix 6-2.

a four-fold dilution of the lower gel buffer. The gel was allowed to stand overnight at 4°C. A 6% (w/v) acrylamide stacking gel, as described in Table 9, was applied to the top of the resolving gel. The stacking gel was overlaid with a layer of 0.1% (w/v) SDS solution. The slab gel was ready to be used after the stacking gel had polymerised.

The resolving slab gel (0.8 mm thick x 140 mm wide x 110 mm deep) containing a 5-20% (w/v) acrylamide linear gradient (Table 10) or a 10-15% (w/v) acrylamide linear gradient (Table 11) was cast using a gradient maker whose reservoir chamber contained the lower percent acrylamide solution and the mixing chamber contained the higher percent acrylamide solution. The tap between the chambers was opened and the gel solution was pumped into the slab gel sandwich using a Watson-Marlow type MHRE 22 peristaltic pump at a flow rate of 3 ml/min. The gel was overlaid with 0.1% (w/v) SDS solution and was allowed to polymerise until the gel interface was seen as a sharp straight boundary (about 25 min). After 1 h, the overlay and unpolymerised acrylamide were removed by aspiration and replaced with 2 ml of a four-fold dilution of the lower gel buffer. The gel was allowed to stand overnight at 4°C. The stacking gel, as described in Tables 10 and 11, was applied to the top of the resolving gel. The stacking gel was overlaid with a layer of 0.1% (w/v) SDS solution. After the stacking gel had polymerised, the overlay was removed and the top of the gel was washed with deionised water.

The first-dimensional tube gel was equilibrated for 10 min in 5 ml of SDS sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% (w/v) glycerol, 0.05 M dithiothreitol and 5% (w/v) SDS. The tube gel was then attached to the top of the second-dimensional stacking gel with 1% (w/v) agarose. The second-dimensional gel was electrophoresed at a constant current of 20

TABLE 10. Gel Formulation for 5-20% SDS-PAGE

Resolving Gel

Reagents	Amount	
	5% (w/v) acrylamide solution	20% (w/v) acrylamide solution
lower gel buffer	2.000 ml	2.000 ml
deionised water	4.620 ml	
30% (w/v) acrylamide	1.350 ml	5.350 ml
10% (w/v) ammonium persulphate	0.024 ml	0.024 ml
TEMED	0.006 ml	0.006 ml
glycerol		0.620 ml

The resolving gel solutions contained a final concentration of 0.1% (w/v) SDS and 0.375 M Tris-HCl, pH 8.8.

Stacking Gel

Reagents	Amount
upper gel buffer	2.50 ml
deionised water	6.25 ml
30% (w/v) acrylamide	1.17 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The stacking gel solution contained a final concentration of 3.5% (w/v) acrylamide, 0.1% (w/v) SDS and 0.125 M Tris-HCl, pH 6.8.

The stock reagents were prepared as described in Appendix 6-2.

TABLE 11. Gel Formulation for 10-15% SDS-PAGE

Resolving Gel

Reagents	Amount	
	10% (w/v) acrylamide solution	15% (w/v) acrylamide solution
lower gel buffer	2.000 ml	2.000 ml
deionised water	3.300 ml	1.350 ml
30% (w/v) acrylamide	2.670 ml	4.000 ml
10% (w/v) ammonium persulphate	0.024 ml	0.024 ml
TEMED	0.006 ml	0.006 ml
glycerol		0.620 ml

The resolving gel solutions contained a final concentration of 0.1% (w/v) SDS and 0.375 M Tris-HCl, pH 8.8.

Stacking Gel

Reagents	Amount
upper gel buffer	2.50 ml
deionised water	5.72 ml
30% (w/v) acrylamide	1.70 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The stacking gel solution contained a final concentration of 5% (w/v) acrylamide, 0.1% (w/v) SDS and 0.125 M Tris-HCl, pH 6.8.

The stock reagents were prepared as described in Appendix 6-2.

mA for approximately 4 h using 0.192 M glycine, 0.1% (w/v) SDS and 0.025 M Tris, pH 8.3, as the electrode buffer.

2-3.5 PAGE for Transferrin Typing

The Ornstein (1964) and Davis (1964) non-dissociating discontinuous buffer system was used. The preparation of reagents is outlined in Appendix 6-3.

2-3.5.1 Preparation of the Polyacrylamide Gels

The uniform 5% (w/v) acrylamide resolving slab gel system used is given in Table 12. The gel solution was degassed and polymerisation was initiated by the addition of freshly prepared 10% (w/v) ammonium persulphate and TEMED. The gel solution was poured into the slab gel sandwich leaving 3.7 cm at the top for a 2 cm stacking gel and sample wells (1.7 cm deep) to be formed. After polymerisation, the overlay and unpolymerised acrylamide were removed and the resolving gel was overlaid with a four-fold dilution of the lower gel buffer. The gel was allowed to stand overnight at $4^{o}C$.

A 2.5% (w/v) acrylamide stacking gel solution was prepared from the stock solutions described in Table 12. The stacking gel solution was degassed and pipetted onto the resolving gel. A Teflon comb was inserted immediately into the stacking gel solution and the assembly was left undisturbed while the stacking gel polymerised. After polymerisation, the comb was carefully removed to expose the sample wells which were rinsed with reservoir buffer.

2-3.5.2 Sample Loading and Electrophoresis

The samples, prepared as described in section 2-3.3.1, were loaded into each well using a microsyringe. The gel was then

TABLE 12. Gel Formulation for PAGE

Resolving Gel

Reagents	Amount
lower gel buffer	4.00 ml
deionised water	9.22 ml
30% (w/v) acrylamide	2.70 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The resolving gel solution contained a final concentration of 5% (w/v) acrylamide and 0.375 M Tris-HCl, pH 8.8.

Stacking Gel

Reagents	Amount
upper gel buffer	2.50 ml
deionised water	6.59 ml
30% (w/v) acrylamide	0.83 ml
10% (w/v) ammonium persulphate	0.06 ml
TEMED	0.02 ml

The stacking gel solution contained a final concentration of 2.5% (w/v) acrylamide and 0.125 M Tris-HCl, pH 6.8.

The stock reagents were prepared as described in Appendix 6-3.

subjected to a constant current of 20 mA for approximately 3 h using 0.192 M glycine, 0.025 M Tris, pH 8.3, as the electrode buffer.

2-3.6 Staining and Destaining of Gels

2-3.6.1 Coomassie Blue Staining

The slab gels were stained overnight at room temperature in 0.2% (w/v) Coomassie blue R-250 in methanol - acetic acid - water (5:1:4, v/v/v) and were destained in methanol - acetic acid - water (2:1:7, v/v/v). The gels were stored in 7% (v/v) acetic acid before photography.

The IEF tube gels were soaked in 20 ml of 20% (w/v) trichloroacetic acid overnight to precipitate the proteins and to reduce the concentration of Pharmalytes. The tube gels were then washed for 1.5 h with several changes of deionised water before staining in 0.2% (w/v) Coomassie blue G-250 in methanol - acetic acid - water (5:1:4, v/v/v). Destaining was carried out in methanol - acetic acid - water (5:1:4, v/v/v) with several changes for 1 h and finally in methanol - acetic acid - water (2:1:7, v/v/v).

2-3.6.2 Silver Staining

Slab gels were stained using the Bio-Rad Silver Stain which is based on the method of Merril *et al*. (1981). The gels were fixed in methanol - acetic acid - water (4:1:5, v/v/v) for 24 h, followed by washing for 1 h in several volumes of ethanol - acetic acid - water (1:0.5:8.5, v/v/v) to ensure removal of buffer components from the gels before staining. The gels were placed in the oxidising solution, which contained 0.0034 M potassium dichromate and 0.0032 M nitric acid, for 5 min and were washed three times for 15 min each in deionised water before

staining with the silver reagent, which contained 0.012 M silver nitrate, for 20 min. After silver staining, the gels were washed for 2 min with deionised water and then developed to the desired intensity in the developing solution which contained 0.28 M sodium carbonate and 0.05% (v/v) formalin. The developing process was stopped by soaking the gels in 5% (v/v) acetic acid for 5 min. Deionised water, of less than 1 μmho conductivity, was used since contaminants including chloride ions precipitate silver ions thus causing reduced sensitivity and increased background staining.

Gels with high background staining or surface deposits of silver were destained using a photographic reducer such as that described by Switzer et al. (1979). The destain was made up of two solutions: Solution (A) was prepared by dissolving 37 g of sodium chloride and 37 g of cupric sulphate in 850 ml of deionised water. Concentrated ammonium hydroxide was added until the precipitate that formed was completely dissolved to give a deep blue solution, and the volume was adjusted to 1 *l* with deionised water. Solution (B) was prepared by dissolving 436 g of sodium thiosulphate in 1 *l* of deionised water. Equal parts of solutions (A) and (B) were combined and the destain was used immediately. The destaining process was stopped with 10% (v/v) acetic acid.

2-3.7 Development of Protein Labelling Method

2-3.7.1 Purification of Radioactive Formaldehyde

The radioactively labelled formaldehyde (1 mCi [^{3}H]formaldehyde or 0.05 mCi [^{14}C]formaldehyde) was dissolved in 0.2 ml of 0.5 M formaldehyde. This solution was transferred to a small Dowex 1-X8 ion exchange column (acetate form) which removed anions that made up about 30% of the radioactivity. The column

was then washed with 0.2 ml of formaldehyde, followed by 0.2 ml of deionised water. The amount of radioactivity in the combined eluate was measured by liquid scintillation counting of a 20 μl aliquot. A 0.4 ml aliquot of the combined eluate, which contained radioactivity in the order of 10^4 d.p.m., was used for each labelling reaction.

2-3.7.2 Liquid Scintillation Counting

The radioactivity present in radioactively labelled proteins was measured by liquid scintillation counting in a Beckman LSC 80 liquid scintillation counter (Beckman Instruments). The scintillant contained 0.4% (w/v) PPO and 0.01% (w/v) DM-POPOP in toluene-Triton X-100 (2:1, v/v). The counting efficiency for ^3H and ^{14}C was determined by the channels ratio method using a quench curve constructed with nitromethane as the quenching agent. The samples were counted until the 95% confidence interval was less than 1% of the counts.

2-3.7.3 Quantitative Studies of Protein Labelling

Protein was labelled by reductive methylation using $NaCNBH_3$ and radioactive formaldehyde (Jentoft and Dearborn, 1979; Wheeler et al., 1986). The reactants were added in the following order: Hepes, protein, $NiSO_4$ (as required), $NaCNBH_3$ and [^3H]formaldehyde or [^{14}C]formaldehyde. This sequence was used to prevent side reactions occurring between reactants and protein. The reaction was allowed to proceed for 2 h at room temperature and was terminated by addition of 3 ml of 10% (w/v) trichloroacetic acid. Protein precipitation was allowed to proceed for 30 min at room temperature before centrifugation at 1000 *g* for 10 min. The supernatant was discarded and the pellet was washed twice by resuspension in 3 ml of 10% (w/v) trichloroacetic acid and

centrifugation at 1000 *g* for 3 min. The pellet was dissolved in 5 ml of 0.5 M Protosol and the solution was neutralised with 0.1 ml of glacial acetic acid before addition of 10 ml of scintillant prior to liquid scintillation counting. The extent of lysine derivatisation was expressed as a function of the ratio of the number of moles of formaldehyde incorporated per mole of lysyl residue in the protein.

Concentrations of formaldehyde, $NaCNBH_3$, Ni^{2+}, salt and protein were varied to determine the optimal conditions for protein labelling. The effect of formaldehyde concentration on labelling was studied with 50 mg of bovine serum albumin incubated for 2 h at room temperature with a fixed concentration of 0.3 M $NaCNBH_3$, 0.1 M Hepes buffer, pH 7.5 and varying concentrations (0.075 M to 0.3 M) of [^{14}C]formaldehyde. The reactions were terminated by the addition of 10% (w/v) trichloroacetic acid and the precipitated protein was redissolved and the amount of radioactivity was measured.

The effect of $NaCNBH_3$ concentration on protein labelling was studied in reaction mixtures containing 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde, 0.1 M Hepes buffer, pH 7.5, and varying concentrations of $NaCNBH_3$ from 0.01 M to 0.3 M.

The effect of Ni^{2+} concentration was studied with 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$, 0.1 M Hepes buffer, pH 7.5, and Ni^{2+} concentrations ranging from 0.01 M to 0.15 M.

NaCl and $(NH_4)_2SO_4$ were added to study the effect of salt on protein labelling. The labelling reactions contained 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$, 0.1 M Hepes, pH 7.5, and 0.1, 0.5, 1.0 and 2.0 M NaCl or 0.1, 0.5, 1.0 and 2.0 M $(NH_4)_2SO_4$.

The effect of the amount of protein on protein labelling was studied with bovine serum albumin, ovalbumin, cytochrome c and insulin in amounts ranging from 0.1 μmol to 1.0 μmol. The proteins were labelled in reaction mixtures which contained 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$ and 0.1 M Hepes, pH 7.5. Bovine serum albumin, ovalbumin and insulin were prepared for scintillation counting in the normal way while the labelled cytochrome c was decolourised by adding 1 ml of freshly prepared solution of 20% benzoyl peroxide in toluene and incubated at 35^{o}C for 30 min. This oxidised the haeme group and reduced colour quenching in scintillation counting.

2-3.7.4 Quantitative Analyses of the Double-labelling System

Double-labelling was carried out using bovine serum albumin labelled with [^{3}H]formaldehyde and [^{14}C]formaldehyde to determine the reliability and accuracy of the labelling procedure. A 6 ml reaction mixture was used for both ^{3}H- and ^{14}C-bovine serum albumin labelling. After 2 h of reaction, aliquots of the two reaction mixtures were mixed to give ^{3}H:^{14}C ratios of 1:1 (v/v), 1:10 (v/v), 10:1 (v/v), 1:20 (v/v) and 20:1 (v/v). The ^{3}H- and ^{14}C-bovine serum albumin samples were then precipitated, centrifuged and the amounts of radioisotopes in the labelled bovine serum albumin were determined by liquid scintillation counting. The observed ^{3}H:^{14}C ratios were calculated from the measured d.p.m. of the isotopes in the different mixtures. The expected ^{3}H:^{14}C ratios were calculated from the amount of radioactivity incorporated per unit volume of ^{3}H- and ^{14}C-labelled bovine serum albumin. The observed ratios were then compared with the expected ratios to determine the reliability and accuracy of the double-labelling procedure.

2-3.8 Radioactive Labelling of Plasma and Liver Proteins for Electrophoresis

Plasma and liver proteins were labelled by reductive methylation according to the method of Jentoft and Dearborn (1979) as modified by Wheeler et al. (1986). Radioactively labelled formaldehyde (1 mCi [^{3}H]formaldehyde or 0.05 mCi [^{14}C]formaldehyde) was dissolved in deionised water (200 μl for [^{3}H]formaldehyde and 100 μl for [^{14}C]formaldehyde) and acidic contaminants were removed by passage of 100 μl aliquots through a 0.2 ml column of Dowex 1-X8 ion exchange resin (acetate form). The ion exchange resin was washed with 50 μl of deionised water and the purified radioactive formaldehyde was stored at 4^{o}C as 30 μl aliquots (150 μCi ^{3}H or 15 μCi ^{14}C) in sealed glass ampoules.

The extent of incorporation of the radioisotopes into protein was directly proportional to their concentrations in the reaction mixture. Fluorography was found to be about ten times as sensitive for ^{14}C as for ^{3}H (Wheeler et al., 1986); therefore a ten-fold greater concentration of ^{3}H than ^{14}C was used in the reaction mixture to assure the approximately equal contribution of each isotope to fluorography. Under the labelling conditions, the usual level of incorporation of radioisotope into protein was about 40,000 d.p.m./μg for ^{3}H and 4,000 d.p.m./μg for ^{14}C.

The reaction mixture for labelling consisted of 30 μl of radioactive formaldehyde (150 μCi ^{3}H or 15 μCi ^{14}C), 10 μl of 1 M Hepes buffer, pH 7.5, 10 μl of 1 M $NaCNBH_3$ (freshly prepared) and 50 μl of plasma or liver proteins. The ampoule was resealed and the reaction was allowed to proceed for 2 h at room temperature.

The amount of radioactivity covalently bound to the proteins after 2 h was measured by treating an aliquot (4 μl) of the reaction mixture with 3 ml of 10% (w/v) trichloroacetic acid and 0.5 ml of 10 mg/ml bovine serum albumin solution. After

centrifugation, the supernatant was discarded and the precipitate was washed twice by suspension in 3 ml of fresh 10% (w/v) trichloroacetic acid and recentrifuged. The final precipitate was dissolved in 1 M NaOH and was transferred to 5 ml of scintillant. Glacial acetic acid (10 μl) was added to neutralise the pH before the radioactivity was measured by liquid scintillation counting.

The remainder of the reaction mixture was diluted with 2 ml of 0.02 M phosphate buffer, pH 8, and was concentrated to 100 μl by centrifugation in a Centricon-10 centrifugal microconcentrator (Amicon) at 4,000 *g* and 4^{o}C. This procedure was repeated twice and the amount of radioactivity remaining in the microconcentrator was measured by taking a 1 μl aliquot for liquid scintillation counting. A second 1 μl aliquot was used for protein determination by the method of Bradford (1976) (section 2-3.10.2). The concentrated labelled plasma or liver protein samples were stored at -20^{o}C before electrophoresis.

2-3.9 Autoradiography and Fluorography

Slab gels for autoradiography and fluorography were reduced to 12 cm in breadth by gentle agitation at room temperature in 40% (w/v) polyethyleneglycol (Palumbo and Tecce, 1983) and were dried using a gel dryer. The dried gels, with Kodak SB X-ray film applied to one or both sides, were placed in a Kodak X-ray cassette. Autoradiography was carried out for 5 weeks at room temperature. The films were developed for 5 min at room temperature using Kodak liquid X-ray developer Type II. After autoradiography, the gels were rehydrated in water before fluorography

Fluorography was carried out according to the procedure of Skinner and Griswold (1983). The gels were soaked for 15 min in glacial acetic acid to remove water which precipitates PPO. The

gels were then soaked in 20% (w/v) PPO in glacial acetic acid for 45 min and were then exposed to water for 15 min to precipitate the PPO. After this the gels were shrunk in 40% (w/v) polyethyleneglycol and dried. Exposure was carried out at -70°C with Fuji RX 100 film (hypersensitised by flashlight immediately before use) applied to both sides. The films were removed after 1 week and were processed in a Kodak RP automatic processor. The fluorogram was compared with the corresponding autoradiogram of similar sensitivity in the double-labelling analysis.

2-3.10 Protein Determination

Protein concentration was determined by the Biuret method (Mokrasch and McGilvery, 1956) or the Bradford method (1976).

2-3.10.1 Biuret Method

Protein solution diluted in 3 ml of 0.9% (w/v) NaCl was added to 3 ml of biuret reagent and the solution was allowed to stand at room temperature for 30 min before absorbance was measured at 540 nm against a control contained only 0.9% (w/v) NaCl and biuret reagent. Stock biuret reagent which contained 45 g of sodium potassium tartrate, 15 g of $CuSO_4 \cdot 5H_2O$ and 5 g of KI dissolved in 1 *l* of 0.2 M NaOH, was diluted with 4 volumes of diluting solution containing 5 g of KI in 1 *l* of 0.2 M NaOH. Bovine serum albumin standards (1-10 mg of protein) were used for calibration.

2-3.10.2 Bradford Method

1 μl of a solution of protein was added to 99 μl of a solution containing 0.02 M sodium phosphate buffer, pH 8, 1 ml of 0.9% (w/v) NaCl and 1 ml of Coomassie blue reagent which contained 0.01% (w/v) Coomassie blue G-250, 4.7% (w/v) ethanol

and 8.5% (w/v) phosphoric acid. The solution was allowed to stand at room temperature for 2 min before absorbance was measured at 595 nm using a control containing no protein. Bovine serum albumin standards ranging from 1-100 μg were used for calibration.

2-3.11 Statistical Analysis

The Hardy-Weinberg law (Hardy, 1908) was used to test for significant deviation in the distribution of the transferrin phenotypes in the resistant and susceptible flocks. If association between alleles and facial eczema resistance or susceptibility existed, the proportions of different phenotypes observed would be different from those expected from the Hardy-Weinberg formula.

The expected frequency of each allele was calculated by dividing the number of each allele by the total number of alleles in the population. The observed allele frequencies were estimated by the direct counting method from the relationships: $p = (2AA + AB + AC + AD + AG) / 2n$; $q = (2BB + AB + BC + BD + BG) / 2n$; $r = (2CC + AC + BC + CD + CG) / 2n$; $s = (2DD + AD + BD + CD + DG) / 2n$; $t = (2GG + AG + BG + CG + DG) / 2n$, where AA, AB, etc. are the numbers of animals with transferrin phenotypes AA, AB, etc. in the resistant or susceptible flock, n is the total number of animals in the flock, and p, q, r, s and t are the frequencies of the transferrin alleles A, B, C, D and G, respectively. From these frequencies, the expected numbers of the different phenotypes were calculated: $AA = p^2n$, $AB = 2pqn$, $AC = 2prn$, $AD = 2psn$, $AG = 2ptn$, $BB = q^2n$, $BC = 2qrn$, $BD = 2qsn$, $BG = 2qtn$, $CC = r^2n$, $CD = 2rsn$, $CG = 2rtn$, $DD = s^2n$, $DG = 2stn$ and $GG = t^2n$. These expected numbers of the different phenotypes were then compared with the observed numbers by calculating the goodness-

of-fit X^2 ($X^2 = \Sigma [(\text{observed} - \text{expected})^2 / \text{expected}]$). The calculated X^2 value was tested for significant deviation from Hardy-Weinberg equilibrium using the table of X^2 with the appropriate degree of freedom (numbers of different phenotypes - 1).

Publications resulting from this thesis have been LOONG, P. C., JORDAN, T. W., WHEELER, T. T., FORD, H. C., and CAMPBELL, A. G. (1986) Use of Two-dimensional Polyacrylamide Gel Electrophoresis to Detect Plasma Protein Variation between Romney Sheep selected for Facial Eczema Resistance or Susceptibility. N. Z. J. Agr. Res. 29, 449-456 and WHEELER, T. T., LOONG, P. C., JORDAN, T. W., and FORD, H. C. (1986) A Double-label Two-dimensional Gel Electrophoresis Procedure Specifically Designed for Serum or Plasma Analysis. Anal. Biochem. 159, 1-7.
Section 2-3.8 and 2-3.9 of this thesis are based on the methods developed by Mr T. T. Wheeler and myself and are reported in Wheeler et al. (1986).

RESULTS

3-1 Development of Conditions for IEF

The pH gradient in an IEF gel is affected by the anolyte, catholyte and ampholyte composition. In order to achieve reproducible separations at optimal resolution, the most suitable electrofocusing conditions were determined. The following investigations were carried out to establish a suitable IEF system for the study of plasma and liver proteins.

3-1.1 Effect of Electrolytes on the pH Gradient

The effect of electrolyte composition on the pH gradient was investigated in order to establish a suitable pH range for the separation of plasma and liver proteins. In seeking a suitable electrolyte system, only non-volatile electrolytes, with hydronium and hydroxyl ions as migrating species, were tested to avoid possible complications from interactions of proteins and other ions. A number of different combinations of anolyte and catholyte were tested. These were 0.01 M H_3PO_4 and 0.02 M NaOH, 0.02 M H_3PO_4 and 0.05 M NaOH, 0.02 M H_3PO_4 and 0.1 M NaOH and 0.04 M H_3PO_4 and 0.1 M NaOH.

The effect of electrolyte composition on the pH gradient formed during IEF is shown in Figure 3. A non-linear gradient with a narrow pH range, pH 4.5-7, was produced when 0.01 M H_3PO_4 (pH 2.3) and 0.02 M NaOH (pH 12) were used. No improvement in linearity or increase in pH range was obtained when the molarity of the anolyte and catholyte was increased to 0.02 M and 0.05 M respectively. However, when the anolyte was maintained at 0.02 M H_3PO_4 (pH 2.2) but the molarity of the catholyte was raised to 0.1 M NaOH (pH 12.8), the basic range of the pH gradient was extended (Figure 3). Likewise, the acidic range of the pH

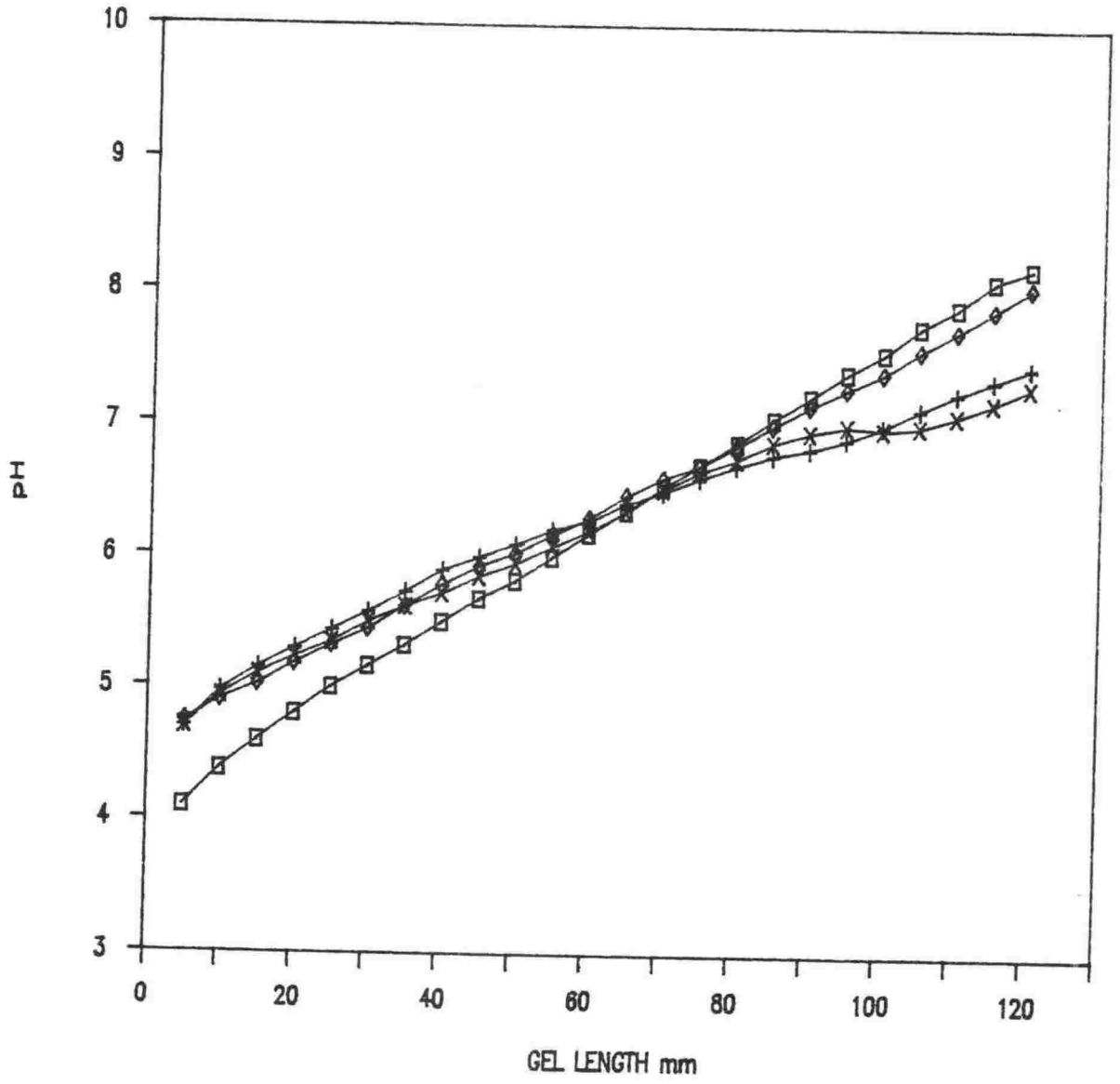


FIGURE 3. Effect of Electrolyte Composition on the Shape of the pH Gradient in IEF Gels

This figure illustrates the pH gradients obtained in IEF gels containing 9 M urea, 2% (w/v) Triton X-100, 0.01 M each of aspartic acid, lysine, arginine and 4% (w/v) Pharmalytes (pH 3-10, pH 4-6.5, pH 5-8; 2:1:1; v/v/v). A number of different combinations of electrolytes were tested: (+) anolyte 0.02 M H_3PO_4 (pH 2.2) and catholyte 0.05 M NaOH (pH 12.5), (◊) anolyte 0.02 M H_3PO_4 and catholyte 0.1 M NaOH (pH 12.8), (□) anolyte 0.04 M H_3PO_4 (pH 1.8) and catholyte 0.1 M NaOH, and (×) anolyte 0.01 M H_3PO_4 (pH 2.3) and catholyte 0.02 M NaOH (pH 12). IEF was carried out for a total of 8,800 V·h at 20°C. The pH gradients were determined by measuring the pH of gel sections equilibrated with 0.01 M KCl solution at 20°C. Results are the means for eight gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

gradient was extended by the increase in the molarity of H_3PO_4 to 0.04 M (pH 1.8). These increases in molarity of anolyte and catholyte provided a stable and linear pH gradient ranging from pH 4 to 8 (Figure 3). Therefore, solutions of 0.04 M H_3PO_4 and 0.1 M NaOH were selected as anolyte and catholyte respectively, and they were used in all subsequent experiments.

3-1.2 Effect of Pharmalyte Concentration on the pH Gradient

Gels containing different Pharmalyte compositions were focused for 22 h and the pH gradients were determined. The concentration of Pharmalytes in the gel affected the shape of the resulting pH gradient (Figure 4). The pH gradient produced in gels containing 4% (w/v) pH 3-10 Pharmalytes was non-linear and shifted towards the cathode (Figure 4). By combining three different pH ranges of Pharmalytes in the following proportions: 0.4% (w/v) pH 3-10, 1.8% (w/v) pH 4-6.5 and 1.8% (w/v) pH 5-8 (total 4% (w/v) Pharmalytes), a more linear pH gradient was established. However, the pH range of the gradient was narrower (pH 4.5-7.5) as the buffering capacity of the Pharmalytes was concentrated between pH 4 and 8. A wider pH gradient was obtained by increasing the pH 3-10 Pharmalyte concentration to 1% (w/v) and decreasing both pH 4-6.5 and pH 5-8 Pharmalyte concentrations to 0.5% (w/v) (Figure 4). Although this composition of Pharmalytes provided a broader pH gradient, the gradient linearity was not achieved. A linear pH gradient from pH 4 to 8 was obtained by increasing the pH 3-10 Pharmalyte concentration to 2% (w/v) and both pH 4-6.5 and pH 5-8 Pharmalyte concentrations to 1% (w/v). Therefore, all subsequent experiments were performed with a Pharmalyte composition of 2% (w/v) pH 3-10, 1% (w/v) pH 4-6.5 and 1% (w/v) pH 5-8 (total 4% (w/v) Pharmalytes).

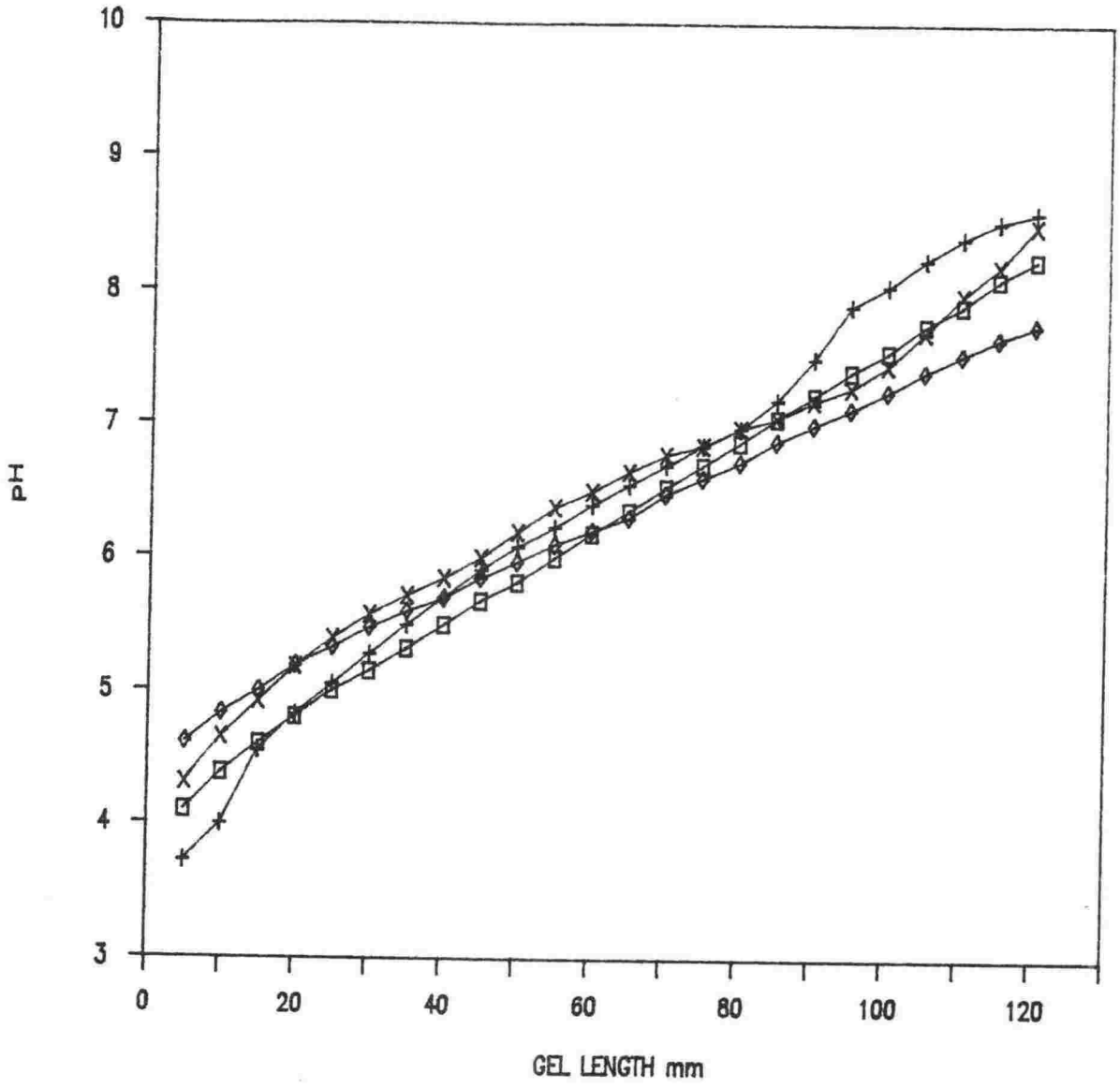


FIGURE 4. Effect of Pharmalyte Concentration on the Shape of the pH Gradient in IEF Gels

Tube gels containing various combinations of Pharmalytes pH 3-10, pH 4-6.5 and pH 5-8 to a total concentration of 2% (w/v) or 4% (w/v), were subjected to IEF for 22 h. (+) 4% (w/v) Pharmalytes (pH 3-10); (◊) 4% (w/v) Pharmalytes (pH 3-10, pH 4-6.5, pH 5-8; 1:4.5:4.5; v/v/v); (X) 2% (w/v) Pharmalytes (pH 3-10, pH 4-6.5, pH 5-8; 2:1:1; v/v/v); (□) 4% (w/v) Pharmalytes (pH 3-10, pH 4-6.5, pH 5-8; 2:1:1; v/v/v). Results are the means of two gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

3-1.3 Effect of Amino Acids on the pH Gradient

The effect of amino acids in broadening the pH gradient formed in IEF gels was investigated. Three amino acids: aspartic acid (pI 2.77), lysine (pI 9.74) and arginine (pI 10.76), were each included in the gel mixture at a concentration of 0.01 M. The pH gradient formed in gels containing amino acids were compared with those formed in gels without amino acids. Figure 5 shows the pH gradients of gels focused with or without amino acids in the gel mixture. In all cases the pH gradients were linear. However, addition of 0.01 M of each of the amino acids: aspartic acid, lysine and arginine, increased the overall range of the pH gradient by nearly one pH unit. Inclusion of lysine and arginine resulted in extending the pH gradient at the basic pH range, while inclusion of aspartic acid extended the pH gradient at the acidic pH end.

3-1.4 Reproducibility of the pH Gradient

Figure 6 illustrates the pH gradient obtained when the standard conditions as described in MATERIALS AND METHODS were used. The pH gradient was linear in the range pH 4-8 and over 90% of the gel length.

Figure 7 and Figure 8 show that a reproducible pH gradient was obtained within each batch and between batches of focused gels. The only noticeable deviation was at the basic pH end where a difference of ± 0.1 pH unit was observed.

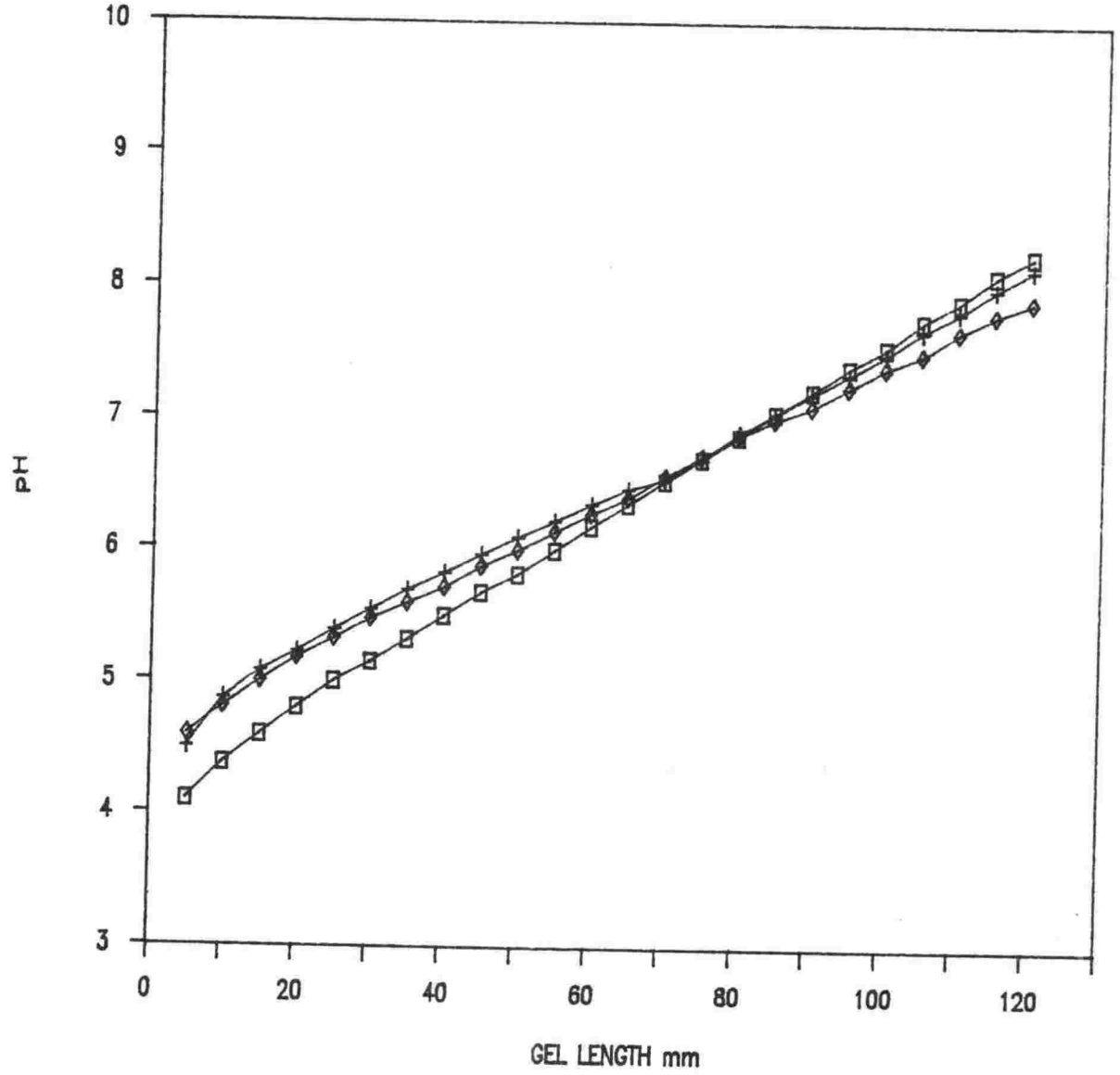


FIGURE 5. pH Gradients of IEF Gels Containing Amino Acids

(◊) pH gradient from a gel prepared without amino acids.
(+) pH gradient from a gel prepared with 0.01 M lysine and 0.01 M arginine.
(□) pH gradient of a gel containing 0.01 M aspartic acid, 0.01 M lysine and 0.01 M arginine.

Results are the means of two gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

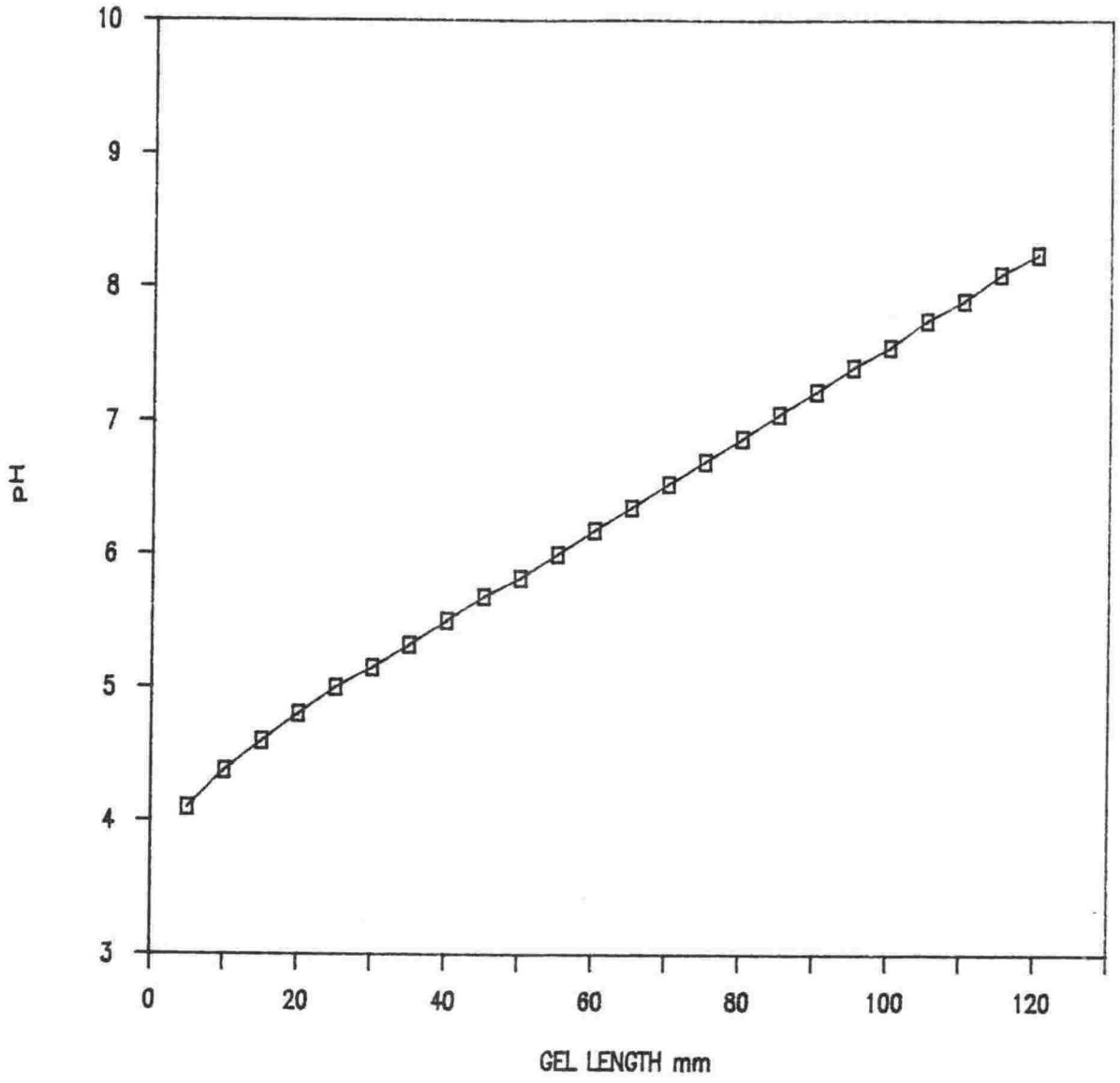


FIGURE 6. pH Gradient of a Typical IEF Gel

This figure illustrates the pH gradient obtained when the recommended conditions were used as described in the text. The IEF gel was cast to contain 9 M urea, 2% (w/v) Triton X-100, 0.01 M each of aspartic acid, lysine, arginine and the following Pharmalytes: 2% (w/v) pH 3-10, 1% (w/v) pH 4-6.5 and 1% (w/v) pH 5-8. IEF was carried out for a total of 8,800 V·h at 20°C. Anolyte was 0.04 M H_3PO_4 and catholyte was 0.1 M NaOH. The pH gradient established was linear over pH 4-8 range. Results are the means of eight gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

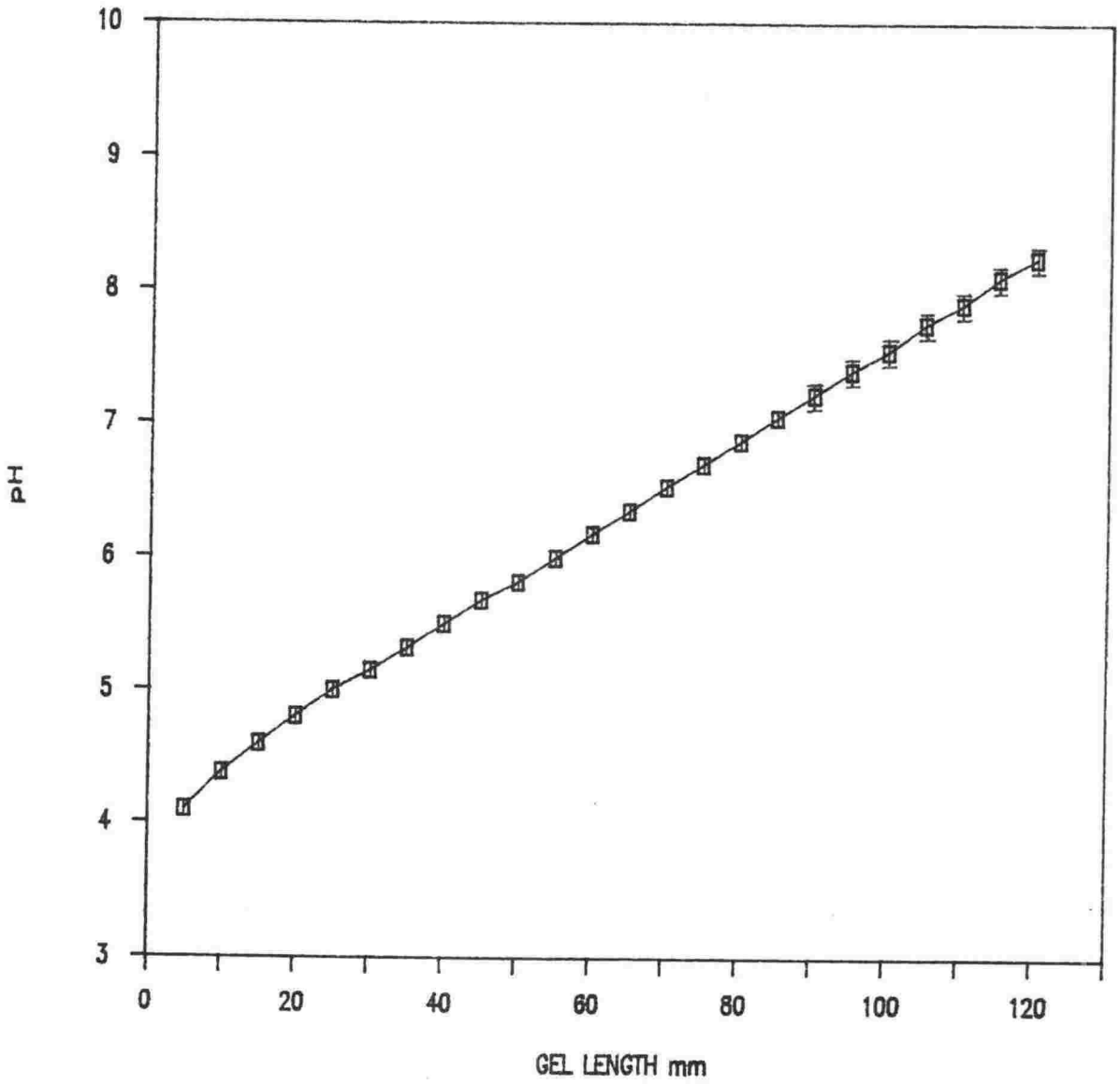


FIGURE 7. Reproducibility of the pH Gradient in Gels Focused at the Same Time

Comparison of the pH gradients of eight gels focused at the same time. Results are means with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

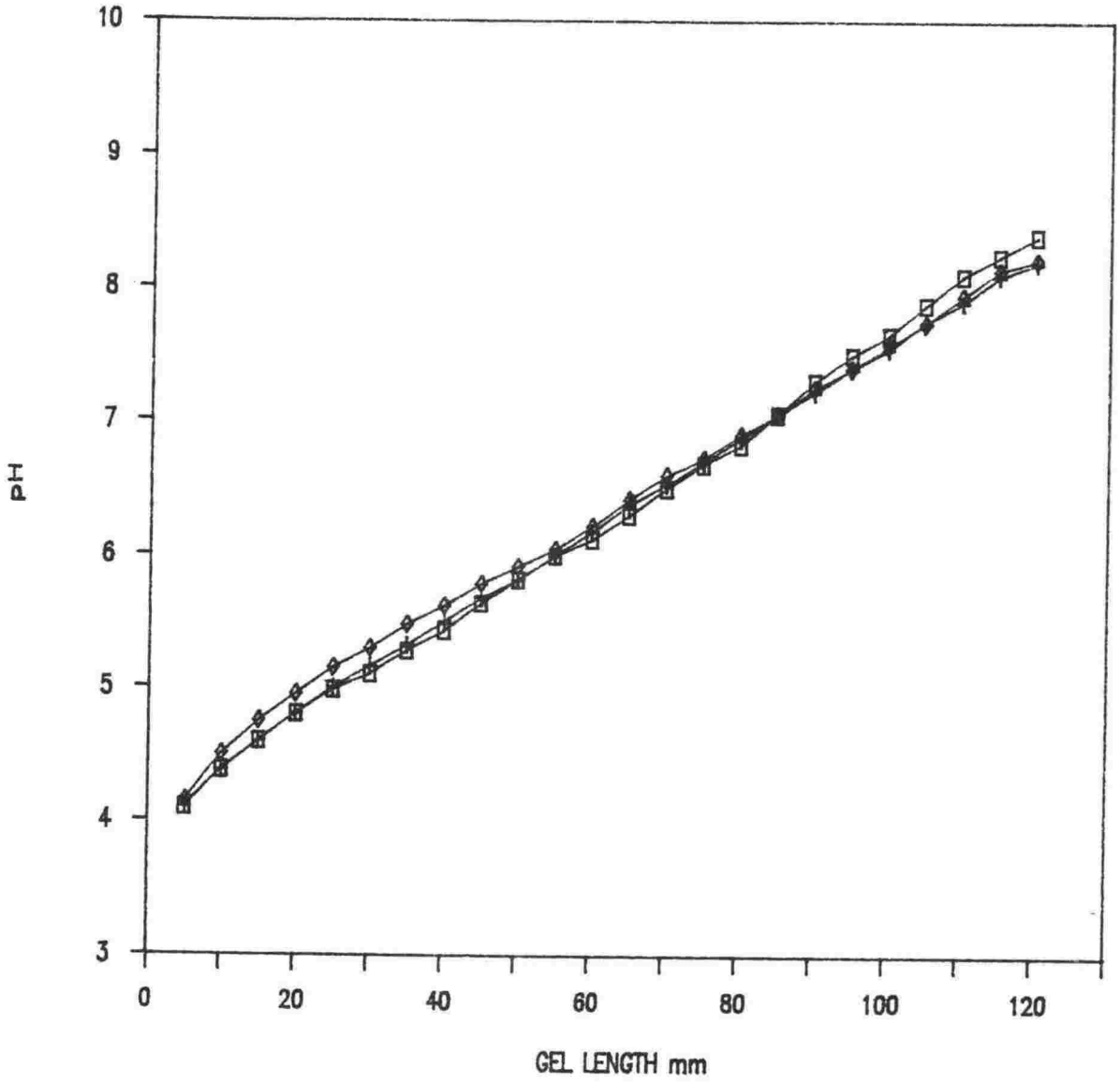


FIGURE 8. Comparison of the pH Gradient Obtained in Gels Focused at Different Times

pH gradients for three gels which had been focused in different experiments using similar experimental conditions.

3-1.5 Effect of Protein Load on the pH Gradient

This experiment was carried out to determine how the presence of relatively large amounts of protein would affect the pH gradient generated during IEF of complex mixtures of protein. Romney sheep plasma was used as the protein source and different amounts of protein were applied to the IEF gels and the resulting pH gradients were compared.

Figure 9 shows that the final pH at the basic end of the gradient was decreased when an increased amount of protein was applied to the gel. The decrease in pH was relatively small (about 0.1 pH unit) when 0.3 mg of protein was focused, but at higher protein load (0.6 mg) the decrease in pH was about 0.5 pH unit.

3-1.6 Effect of 2-Mercaptoethanol and Dithiothreitol on the pH Gradient

2-Mercaptoethanol or dithiothreitol are commonly added to denatured protein samples for the reduction of disulphide bridges. Therefore, the effects of 2-mercaptoethanol and dithiothreitol on IEF pH gradients were investigated.

Figure 10 shows that the presence of 2-mercaptoethanol in the IEF buffer modified the shape and range of the pH gradient. A significant decrease of 0.5 pH unit at the basic end of the pH gradient was observed in gels with IEF buffer containing 1.25 μl of 2-mercaptoethanol. However, when 2-mercaptoethanol was replaced by 0.05 M dithiothreitol, there was little change observed in the pH gradient as compared with the control.

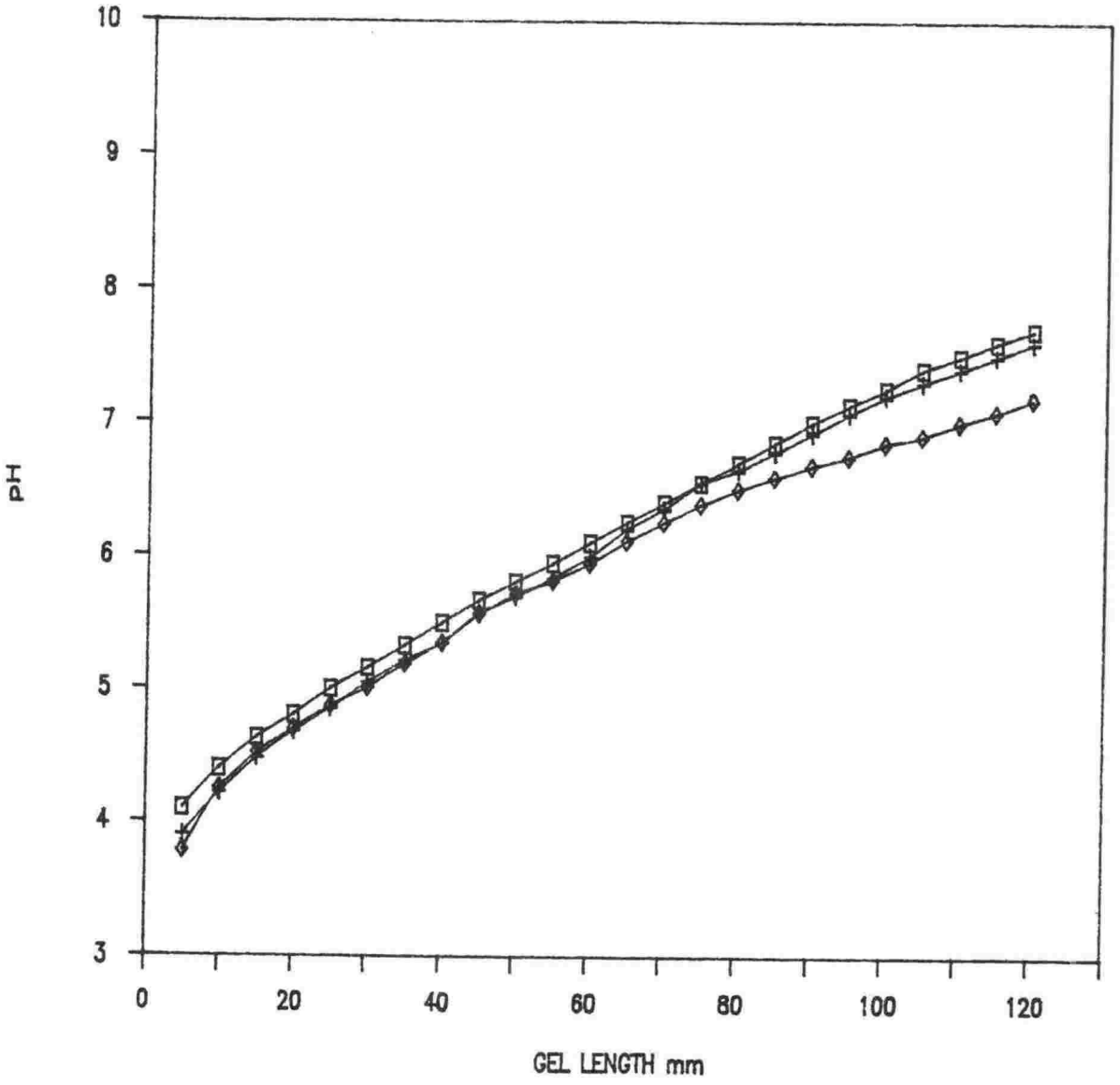


FIGURE 9. Effect of Protein Load on the pH Gradient in IEF Gels

pH gradients in IEF gels with different amounts of protein applied to each gel. The protein sample was sheep plasma. Samples were prepared in 9.5 M urea, 2% (w/v) SDS, 16% (w/v) Triton X-100 and 5% (v/v) 2-mercaptoethanol. (□) no protein; (+) 0.3 mg protein applied; (◇) 0.6 mg protein applied to the IEF gel. The results are the means of two gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

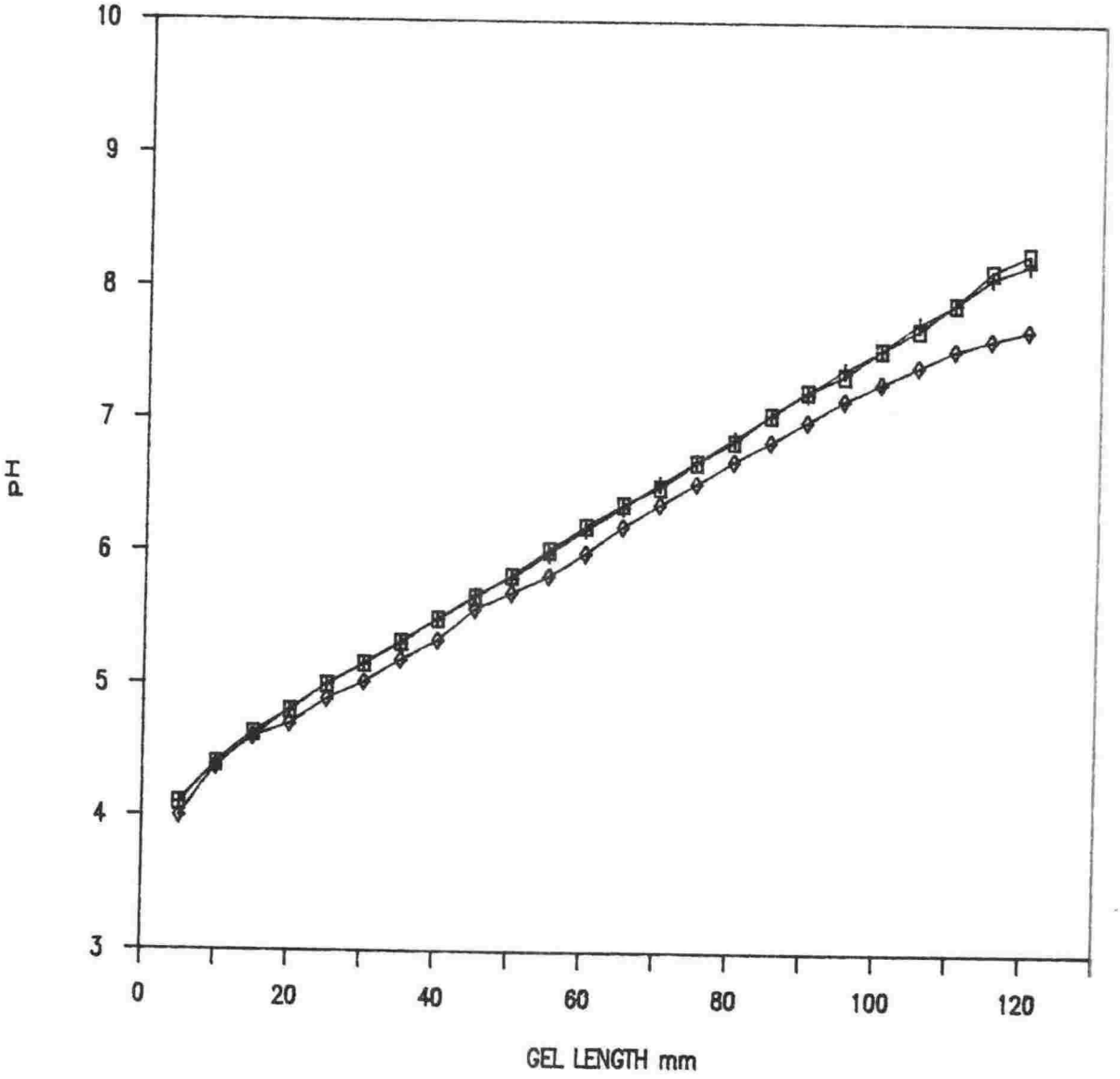


FIGURE 10. Effect of 2-Mercaptoethanol and Dithiothreitol on the pH Gradient in IEF Gels

IEF gels were cast to contain 9 M urea, 2% (w/v) Triton X-100, 0.01 M each of aspartic acid, lysine, arginine and the following Pharmalytes: 2% (w/v) pH 3-10, 1% (w/v) pH 4-6.5 and 1% (w/v) pH 5-8. (□) gel with no IEF buffer as control; (+) gel with IEF buffer containing 9.5 M urea, 2% (w/v) Triton X-100, Pharmalytes: 2% (w/v) pH 3-10, 1% (w/v) pH 4-6.5 and 1% (w/v) pH 5-8, and 0.05 M dithiothreitol, (◊) gel with IEF buffer containing the same components as (+) except dithiothreitol was replaced with 5% (v/v) 2-mercaptoethanol. The results are the means of two gels with standard deviations ± 0.05 pH unit from pH 4 to 7 and ± 0.1 pH unit from pH 7 to 8.

3-2 IEF of Standard Proteins

The resolution of IEF tube gels was examined by analysis of a mixture of bovine serum albumin and human haemoglobin (Plate 1). A number of protein bands, resolved in the pH range 5.5-6.5 and 6.9-7.2, were observed. These proteins were taken to represent multiple forms of bovine serum albumin and human haemoglobin respectively. The resolving power for individual discrete band as estimated from the gel (Plate 1) was in the range of 0.30 ± 0.05 pH unit.

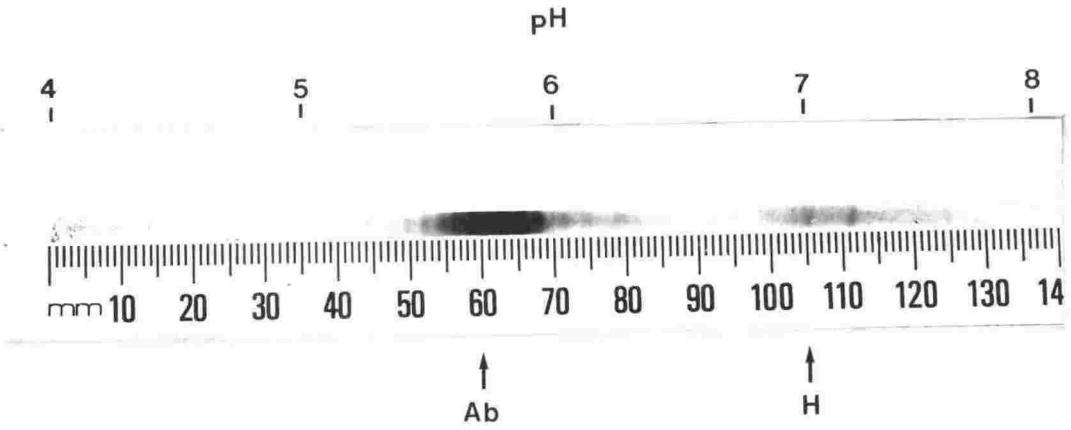


PLATE 1. IEF of a Mixture of Albumin and Haemoglobin

IEF of bovine serum albumin and human haemoglobin in IEF tube gels containing 9 M urea, 2% (w/v) Triton X-100, 4% (w/v) Pharmalytes (pH 3-10, pH 4-6.5, pH 5-8; 2:1:1; v/v/v), 5% (w/v) glycerol, and a mixture of arginine, lysine and aspartic acid (each 0.01 M final concentration) to buffer the pH gradient at each end. IEF was carried out for 20 h at 400 V followed by 1 h at 800 V. The anolyte was 0.04 M H_3PO_4 and the catholyte was 0.1 M NaOH. The amount of protein loaded was 40 μg each of bovine serum albumin (Ab) and human haemoglobin (H). Proteins were detected by staining with Coomassie blue G-250.

3-3 Investigation of the Optimal Conditions for Reductive Methylation of Proteins

Parameters such as the concentrations of formaldehyde, $NaCNBH_3$, Ni^{2+}, salt and amount of protein were investigated to determine the optimal conditions for labelling proteins by reductive methylation. The results were expressed in terms of the total amount of radioactivity incorporated into protein and in terms of the amount of amino groups labelled. In the calculation of the number of amino groups, the M_r of bovine serum albumin was taken as 67,000 and the number of amino groups as 60 (59 lysyl residues and 1 NH_2-terminal aspartic acid residue)(Brown, 1975). Since the majority of the modified amino groups in bovine serum albumin were the ϵ-amino groups of lysyl residues, the results were therefore expressed as a function of the ratio of the number of moles of formaldehyde incorporated per mole of lysyl residue in the protein.

3-3.1 Effect of Formaldehyde Concentration

Figure 11 shows the average number of methyl groups incorporated per lysyl residue in 50 mg of bovine serum albumin. Bovine serum albumin was incubated for 2 h at room temperature with varying amounts of [^{14}C]formaldehyde, 0.3 M $NaCNBH_3$ and 0.1 M Hepes buffer, pH 7.5. An excess of $NaCNBH_3$ was used so that labelling at high concentrations of formaldehyde would not be limited by the amount of reducing agent. The amount of radioactivity in each reaction was about 30,000 d.p.m. and the specific activity of the derivatised protein was about 200 d.p.m./mg protein.

With 50 mg of protein, 75% of the maximum theoretical incorporation of formaldehyde, i.e., 1.5 formaldehyde/lysine, was obtained when formaldehyde concentrations were greater than

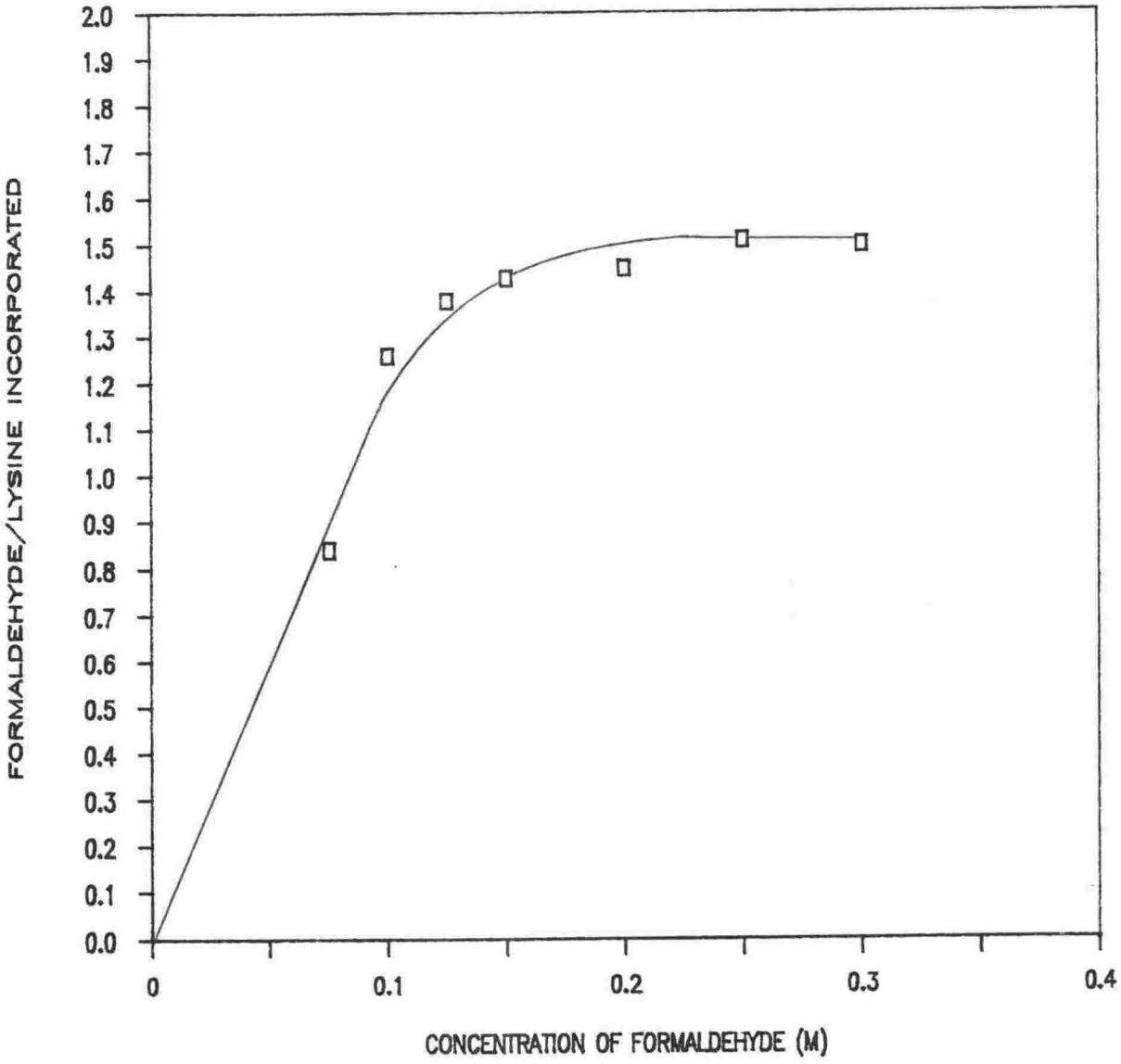


FIGURE 11. Effect of Formaldehyde Concentration on Reductive Methylation

Reaction mixtures containing 50 mg of bovine serum albumin, 0.3 M $NaCNBH_3$, 0.1 M Hepes buffer, pH 7.5, and varying amounts of [^{14}C]formaldehyde were incubated for 2 h at room temperature. The reactions were terminated by the addition of 3 ml of 10% (w/v) trichloroacetic acid and the precipitated protein was collected by centrifugation, redissolved in 5 ml of 0.5 M Protosol, neutralised with 0.1 ml of glacial acetic acid, and the amount of radioactivity was measured by liquid scintillation counting.

0.15 M. Complete derivatisation was not achieved. A concentration of 0.2 M formaldehyde was chosen as optimal for labelling 50 mg of bovine serum albumin.

3-3.2 Effect of $NaCNBH_3$ Concentration

The reaction mixtures containing 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde and 0.1 M Hepes buffer, pH 7.5, were incubated with different concentrations of $NaCNBH_3$ for 2 h at room temperature. Figure 12 shows the maximum observed derivatisation of 1.2 formaldehyde per lysyl residue was obtained when $NaCNBH_3$ concentrations were greater than 0.05 M. A concentration of 0.1 M $NaCNBH_3$ was chosen as optimal for labelling 50 mg of bovine serum albumin.

3-3.3 Effect of Ni^{2+} Concentration

The effect of varying Ni^{2+} concentration on reductive methylation is shown in Figure 13. Increasing the concentration of Ni^{2+} had no substantial effect on the labelling efficiency. There was only a small increase in efficiency at the lowest concentrations of Ni^{2+} (below 0.025 M). This was in contrast with the results obtained using 1 mg of protein (Jentoft and Dearborn, 1979), where Ni^{2+} had a substantial effect on the labelling efficiency. Also the presence of Ni^{2+} at concentrations greater than 0.02 M increased colour quenching during liquid scintillation counting and sometimes resulted in the formation of precipitates which might affect counting efficiency. In consideration of the above factors, Ni^{2+} was not included in subsequent labelling reactions.

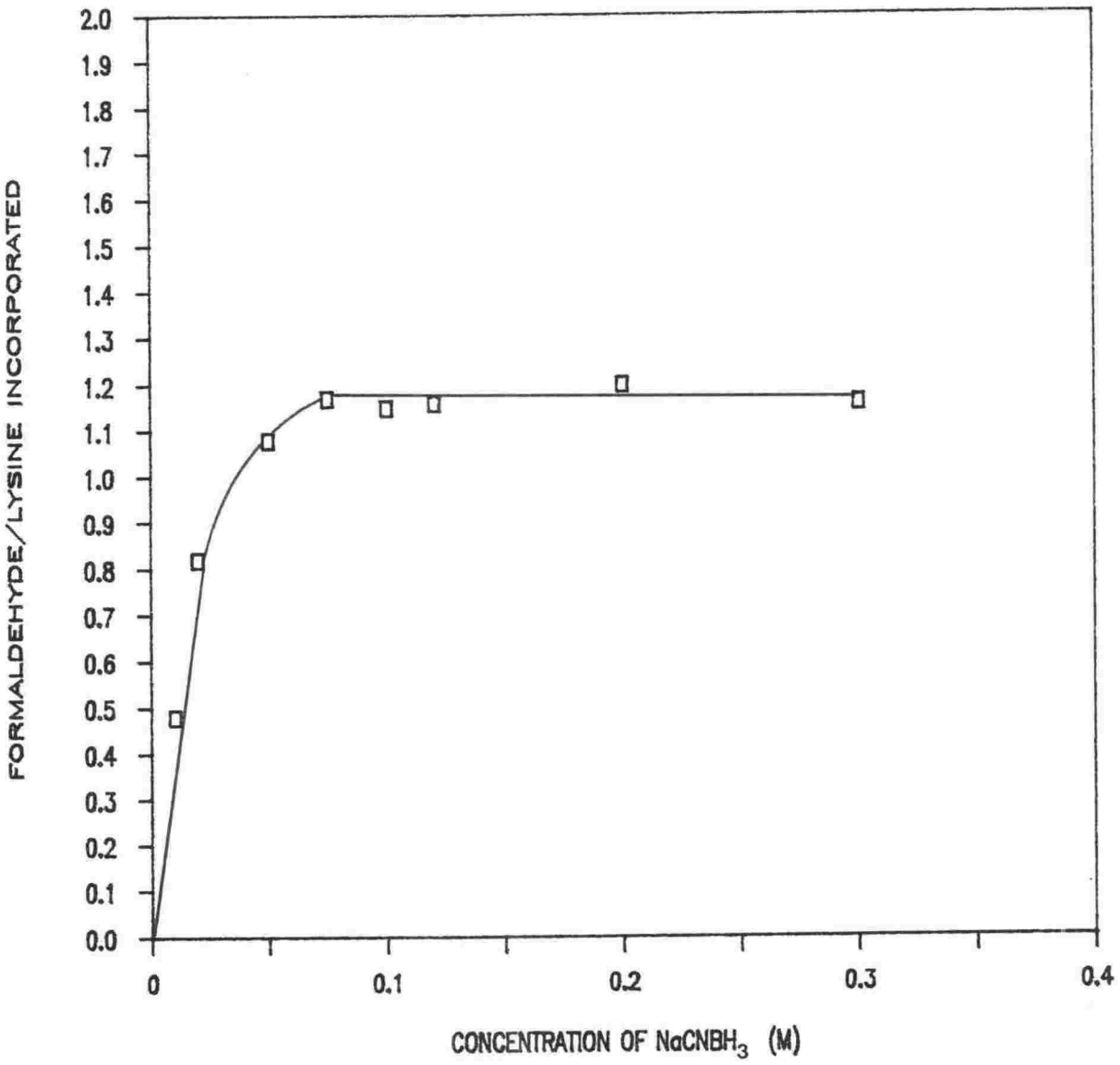


FIGURE 12. Effect of $NaCNBH_3$ Concentration on Reductive Methylation

Reaction mixtures containing 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde and 0.1 M Hepes buffer, pH 7.5, were incubated with different concentrations of $NaCNBH_3$ for 2 h at room temperature. The reactions were terminated by the addition of 10% (w/v) trichloroacetic acid and the precipitated protein was collected by centrifugation, redissolved in 0.5 M Protosol, and the amount of radioactivity was measured by liquid scintillation counting.

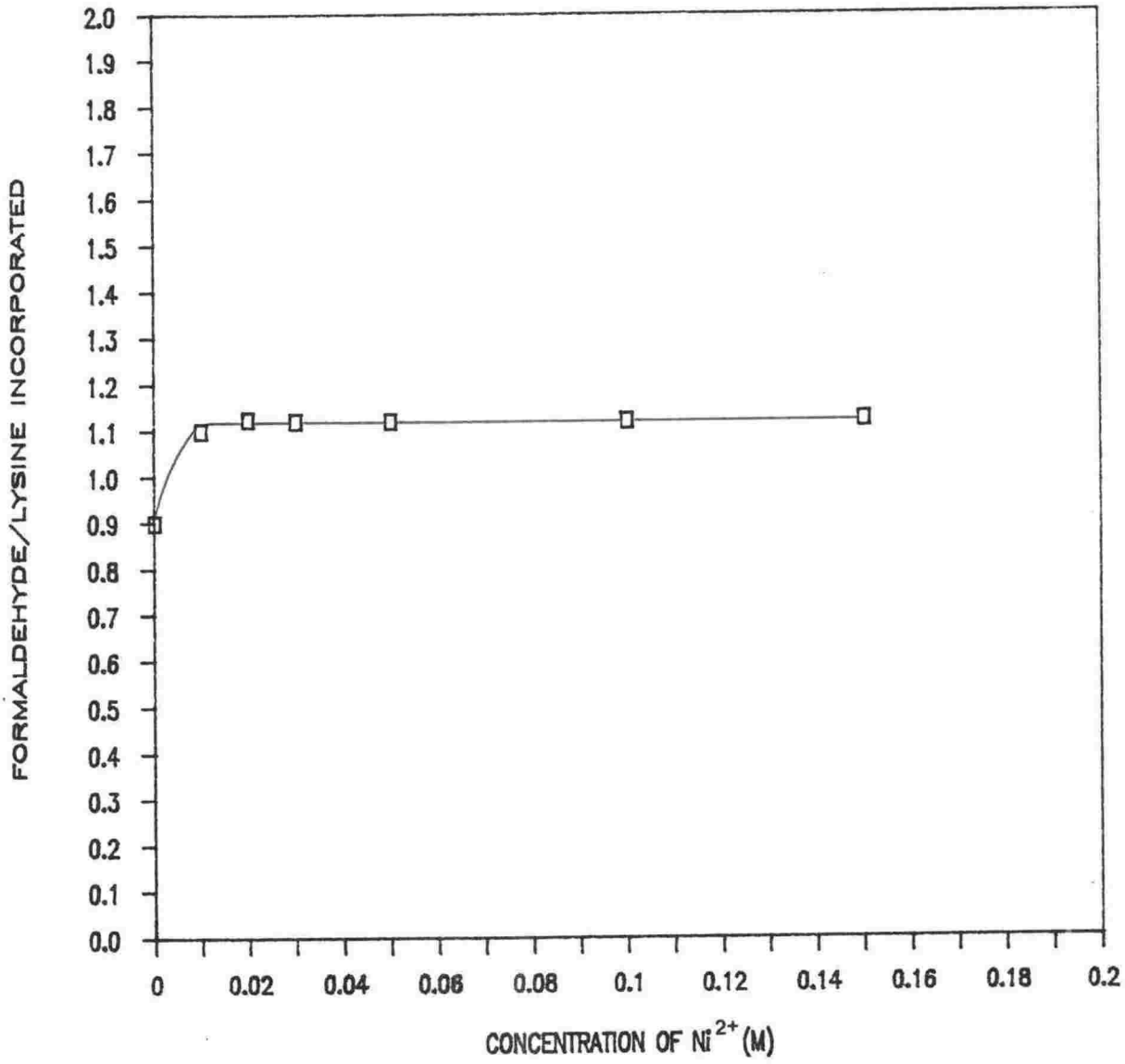


FIGURE 13. Effect of Ni^{2+} Concentration on Reductive Methylation

Reaction mixtures containing 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$, 0.1 M Hepes buffer, pH 7.5, and varying concentrations of Ni^{2+} were incubated for 2 h at room temperature. The reactions were terminated by the addition of 10% (w/v) trichloroacetic acid and the precipitated protein was collected by centrifugation, redissolved in 0.5 M Protosol, and the amount of radioactivity was measured by liquid scintillation counting.

3-3.4 Effect of Salt Concentration

The effects of NaCl and $(NH_4)_2SO_4$ on labelling efficiency were also studied. Figure 14 shows that the labelling efficiency was not affected by NaCl but was inhibited by $(NH_4)_2SO_4$. This indicates that the labelling of plasma proteins would not be affected by the concentration of NaCl present in the plasma but would be affected by $(NH_4)_2SO_4$ which had been used to purify proteins.

3-3.5 Effect of Reaction Time

The time course of reductive methylation conducted at room temperature is shown in Figure 15. At the optimal conditions, the reaction appeared to have reached completion after 1 h. This result was in contrast with that obtained by Jentoft and Dearborn (1979) in which the reaction required at least 2 h for the derivatisation of 1 mg of albumin.

3-3.6 Effect of Amount of Protein

All reactions were carried out at the previously determined optimal conditions which were 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$, 0.1 M Hepes, pH 7.5, and 2 h incubation at room temperature. Each of the four different proteins, bovine serum albumin, cytochrome c, insulin and ovalbumin, was labelled in amounts ranging from 0.1 μmol to 1.0 μmol. The results shown in Figure 16 indicate that apart from some differences seen at low amounts of protein (below 0.3 μmol), all four proteins were labelled to similar extents, i.e., approximately 1.5 formaldehyde/lysine.

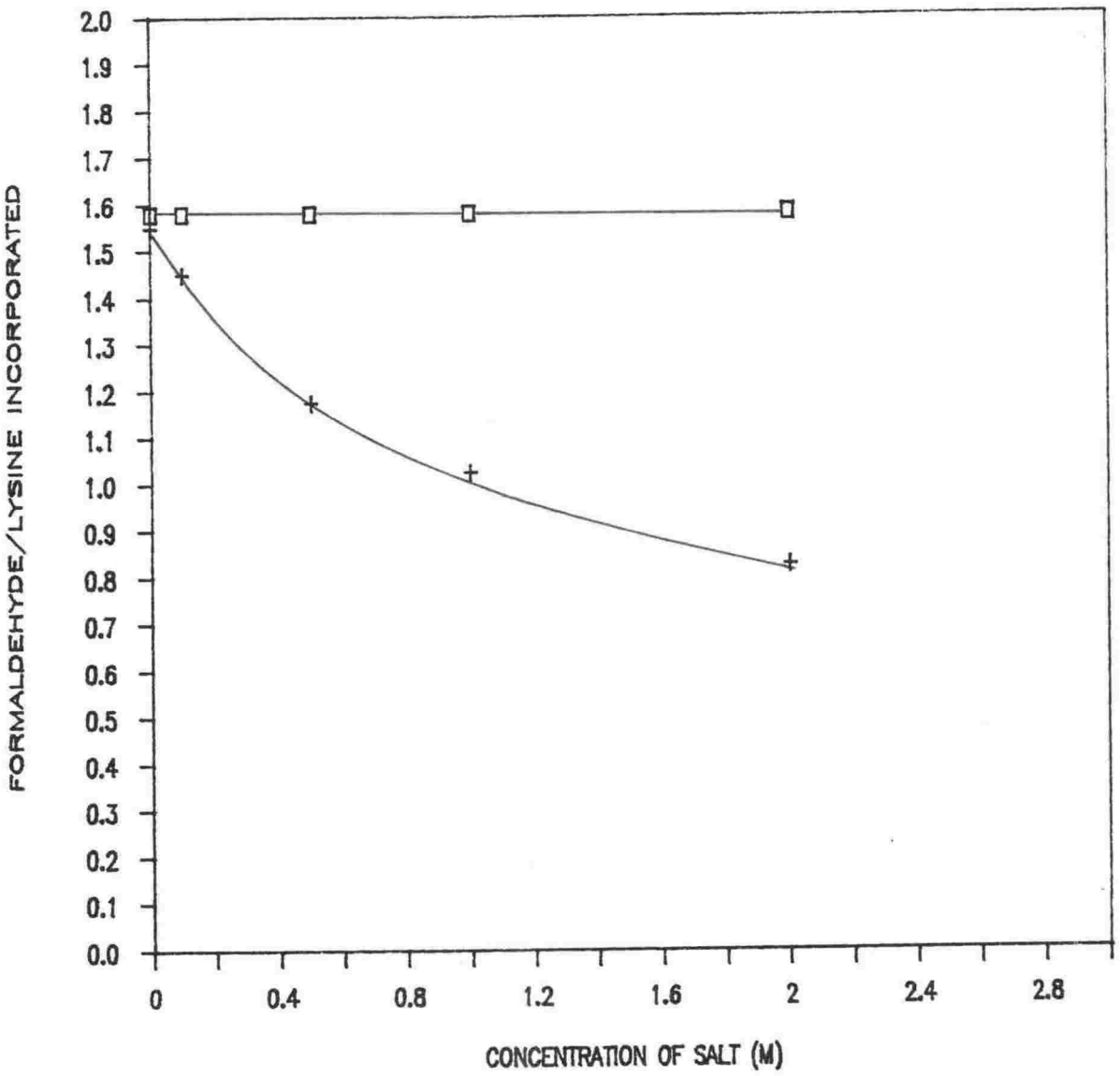


FIGURE 14. Effect of Salt Concentration on Reductive Methylation

Reaction mixtures containing 50 mg of bovine serum albumin, 0.2 M [^{14}C]formaldehyde, 0.1 M $NaCNBH_3$, 0.1 M Hepes buffer, pH 7.5, and varying concentrations of NaCl (□) and $(NH_4)_2SO_4$ (+) were incubated for 2 h at room temperature. The reactions were terminated by the addition of 10% (w/v) trichloroacetic acid and the precipitated protein was collected by centrifugation, redissolved in 0.5 M Protosol, and the amount of radioactivity was measured by liquid scintillation counting.

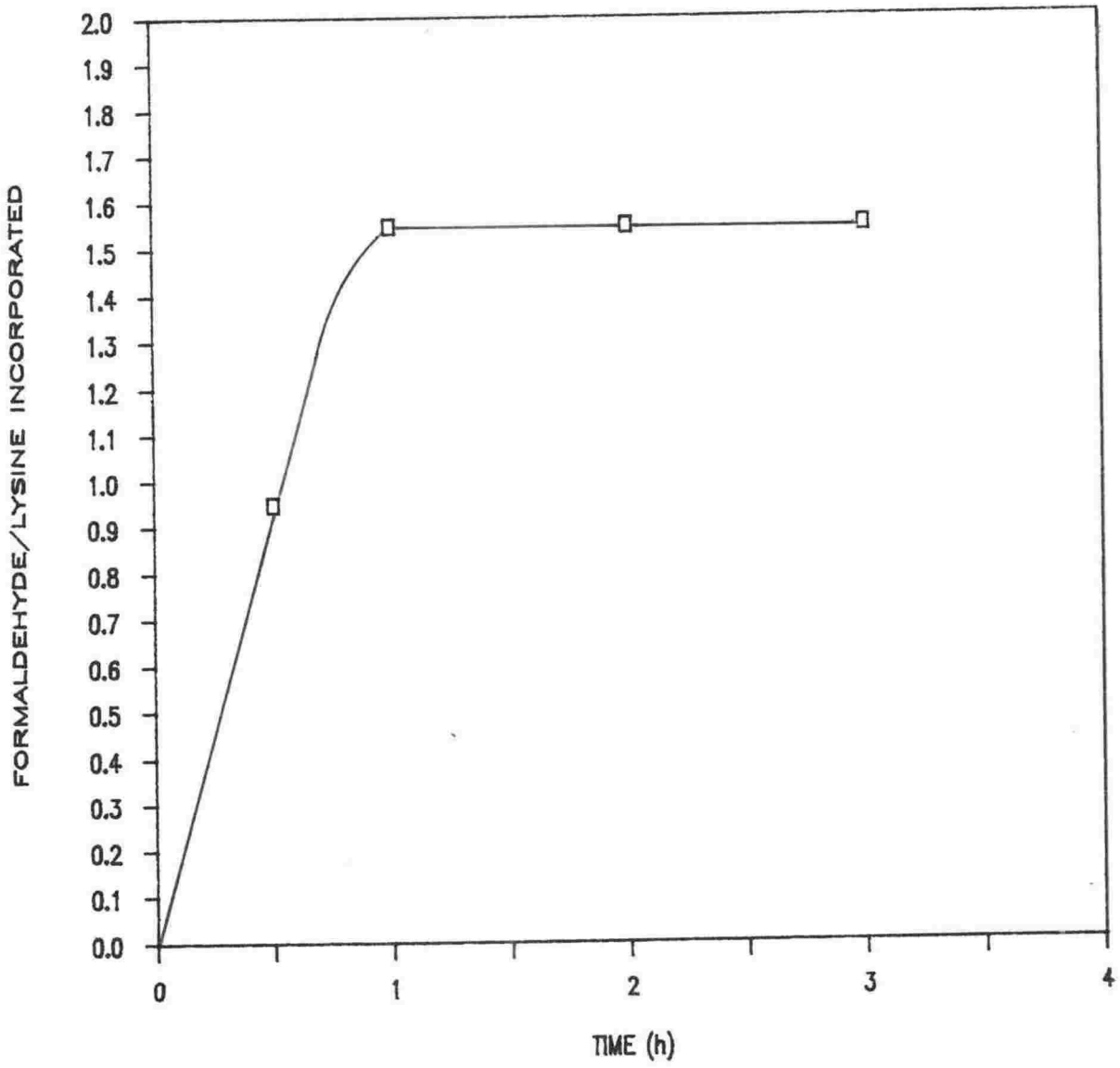


FIGURE 15. Effect of Reaction Time on Reductive Methylation

Reaction conditions were identical to those described in Figure 14 except that assay mixtures contained neither NaCl nor $(NH_4)_2SO_4$.

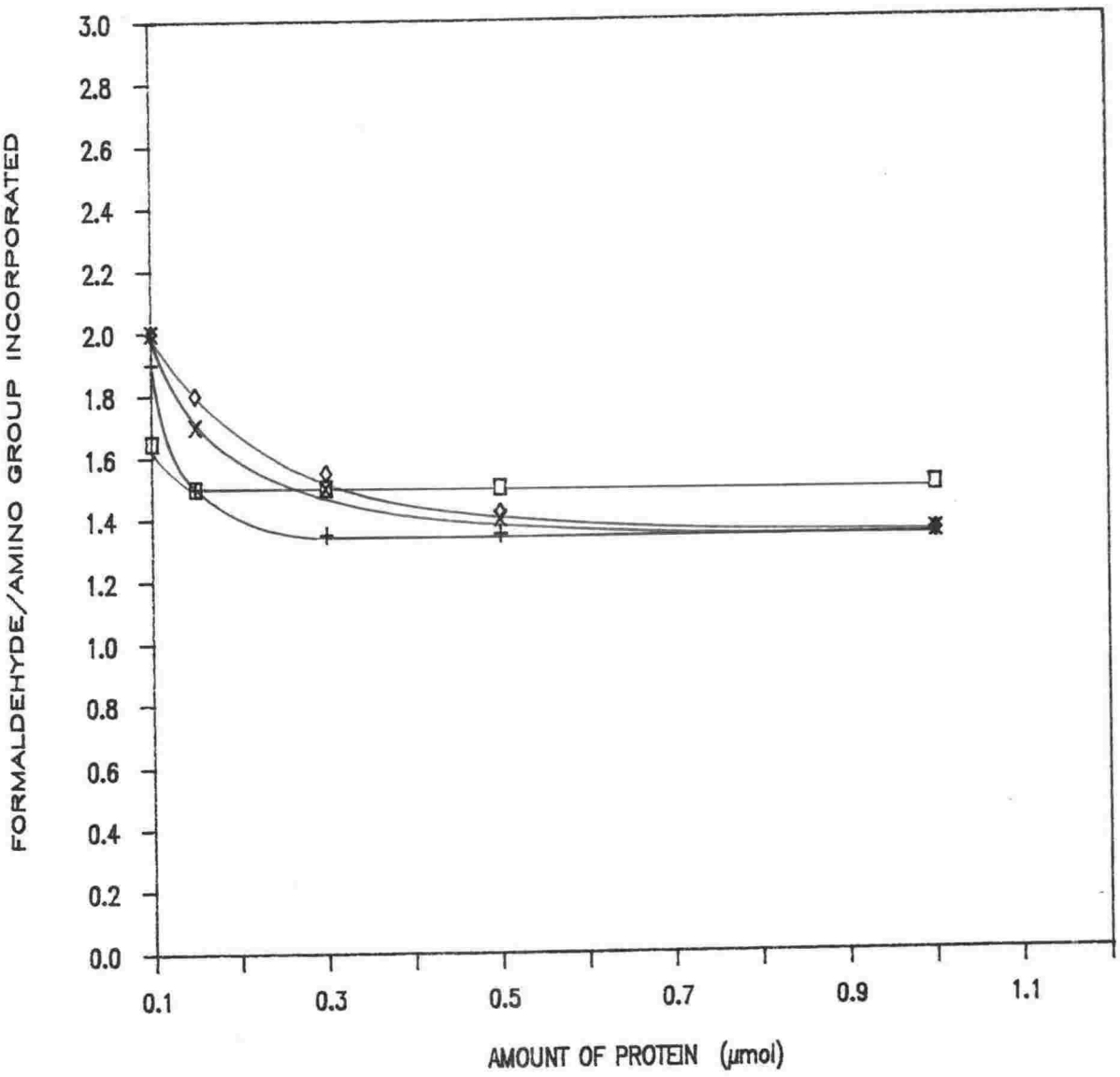


FIGURE 16. Effect of Amount of Protein on Reductive Methylation

Reaction conditions were identical to those described in Figure 14 except that assay mixtures contained neither NaCl nor $(NH_4)_2SO_4$. (◊) bovine serum albumin, (×) ovalbumin, (□) cytochrome c, (+) insulin.

3-4 Quantitative Analyses of the Double-labelling System

Samples of bovine serum albumin were separately labelled with [^{3}H]formaldehyde and [^{14}C]formaldehyde, and aliquots of the completed reaction mixtures were mixed to give the ^{3}H:^{14}C ratios of 1:1 (v/v), 1:10 (v/v), 10:1(v/v), 1:20 (v/v) and 20:1(v/v). The amount of ^{3}H and ^{14}C in each mixture was then measured by liquid scintillation counting. The observed ^{3}H:^{14}C ratios were calculated from the measured d.p.m. values of the isotopes in the different mixtures, e.g., 1156389 d.p.m. of ^{3}H / 5811 d.p.m. of ^{14}C = 199 for ^{3}H:^{14}C ratio of 1:1. The expected ^{3}H:^{14}C ratios were calculated from the amount of radioactivities incorporated per unit volume of ^{3}H- and ^{14}C-labelled bovine serum albumin, e.g., ^{3}H:^{14}C ratio of 1:1 was calculated from the d.p.m. values of 1 ml of ^{3}H-labelled bovine serum albumin and 1 ml of ^{14}C-labelled bovine serum albumin; 1269672 d.p.m. of ^{3}H / 7128 d.p.m. of ^{14}C = 178. The similarity of the expected and the observed ^{3}H:^{14}C ratios (Table 13) demonstrates the reliability and accuracy of the double-labelling and calculation procedures.

TABLE 13. Quantitative Analyses of the Double-labelling System

$^{3}H:^{14}C$ Ratios	Expected Values	Observed Values
1:1	178.0	199.0
1:10	17.8	20.0
10:1	1781.0	1537.0
1:20	9.0	9.9
20:1	3562.0	3075.0

Samples of bovine serum albumin were separately labelled with [^{3}H] and [^{14}C]formaldehyde and aliquots of the completed reaction mixtures were mixed to give the different $^{3}H:^{14}C$ ratios. The observed $^{3}H:^{14}C$ ratios were calculated from the measured d.p.m. values of the isotopes in the mixtures, e.g., 1156389 d.p.m. of ^{3}H / 5811 d.p.m. of ^{14}C gave 199 for $^{3}H:^{14}C$ ratio of 1:1. The expected $^{3}H:^{14}C$ ratios were calculated from the amount of radioactivity incorporated per unit volume of ^{3}H- and ^{14}C-labelled bovine serum albumin, e.g., $^{3}H:^{14}C$ ratio of 1:1 was calculated from the d.p.m. values of 1 ml of ^{3}H-labelled bovine serum albumin and 1 ml of ^{14}C-labelled bovine serum albumin; 1269672 d.p.m. of ^{3}H / 7128 d.p.m. of ^{14}C = 178.

3-5 Analysis of Plasma Protein Variation between Facial Eczema Resistant and Susceptible Sheep

Initial analyses were carried out using a pooling protocol in which the individual samples from the resistant and susceptible populations were pooled and compared in a single experiment. This procedure allowed detection of major variation between populations. Detected variation was then further analysed using individual plasma samples.

3-5.1 IEF of Sheep Plasma Proteins

Pooled plasma from facial eczema resistant and susceptible sheep were compared by one-dimensional IEF. The polypeptides were separated according to their respective pI values along the pH gradient ranging from pH 4 to 8. Over 30 polypeptide zones were detected when the gels were stained with Coomassie blue (Plate 2). The majority of the polypeptides focused between pH 5 and 7. There were no apparent differences when pooled plasma from resistant and susceptible sheep were compared.

3-5.2 SDS-PAGE of Sheep Plasma Proteins

SDS-PAGE of pooled plasma proteins from resistant and susceptible sheep was carried out to search for protein variation between the two populations. Plate 3 shows that about 40 polypeptide zones were detected when the gels were stained with Coomassie blue. The majority of the plasma proteins had an M_r value greater than 20,000. The proteins which were present in large quantities, as indicated by the intensely stained bands, were albumin, IgG heavy chains and IgG light chains with respective M_r values of approximately 67,000, 50,000 and 25,000. There were no apparent differences in protein composition between plasma from resistant and susceptible sheep. The higher

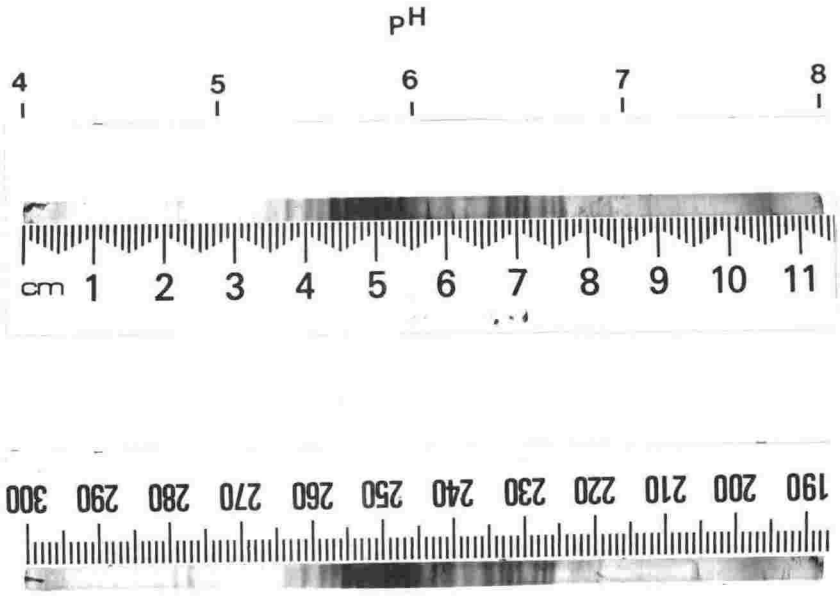


PLATE 2. IEF of Sheep Plasma Proteins

IEF of sheep plasma proteins in gels containing 9 M urea, 2% (w/v) Triton X-100, 0.01 M each of aspartic acid, lysine and arginine, and 4% (w/v) Pharmalytes. IEF was carried out for a total of 8,800 V·h at 20°C with 0.04 M H_3PO_4 as anolyte and 0.1 M NaOH as catholyte. The upper gel contained 250 μg of proteins from pooled plasma of resistant sheep and the lower gel contained 250 μg of proteins from pooled plasma of susceptible sheep. The protein zones were detected when the gels were stained with Coomassie blue G-250.

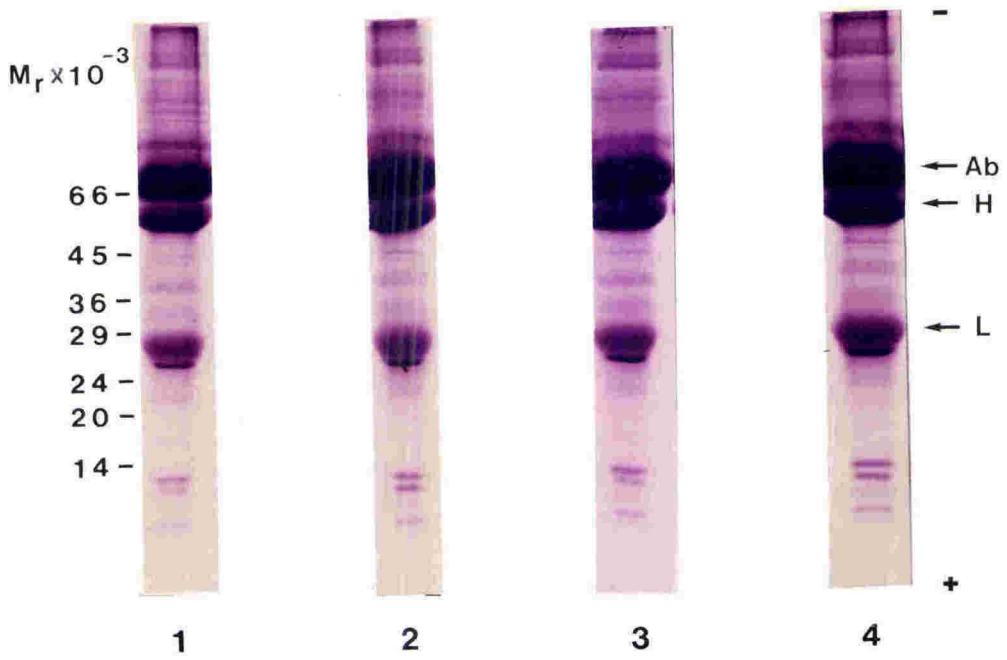


PLATE 3. SDS-PAGE of Sheep Plasma Proteins

Plasma proteins from resistant and susceptible Romney sheep were separated using a 10-15% linear gradient gel electrophoresed at 20 mA for 3 h at room temperature. Polypeptides were detected after electrophoresis by staining with Coomassie blue R-250.
Lane 1, pooled plasma from 11 resistant rams;
Lane 2, pooled plasma from 9 susceptible rams;
Lane 3, pooled plasma from 13 resistant ewes;
Lane 4, pooled plasma from 12 susceptible ewes.
Ab = albumin, H = IgG heavy chains and L = IgG light chains.

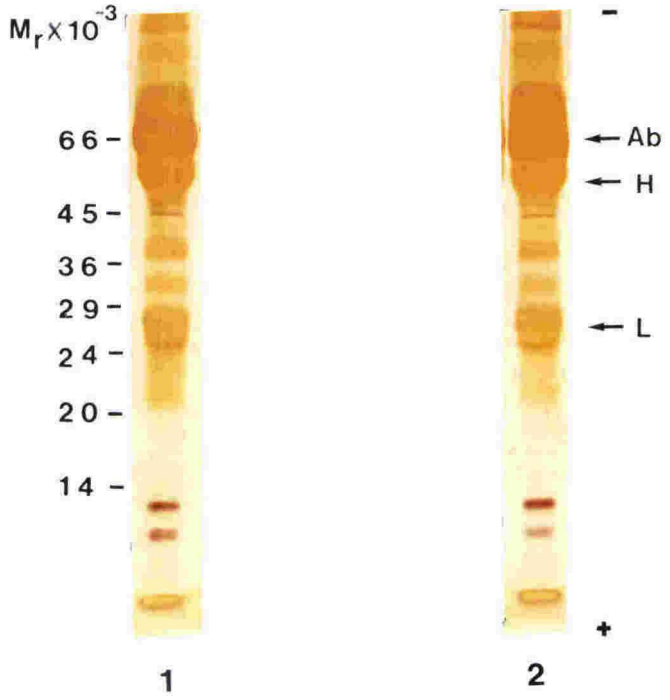


PLATE 4. Silver Stain Detection of Sheep Plasma Proteins Separated by SDS-PAGE

Plasma proteins from 1) pooled resistants, 2) pooled susceptibles, were separated using a 12.5% gel electrophoresed at 20 mA for 3 h at room temperature. Proteins were detected by silver staining.
Ab = albumin, H = IgG heavy chains and L = IgG light chains.

sensitivity detection provided by the silver staining technique also did not reveal any differences in protein composition between the two populations (Plate 4).

3-5.3 2-D PAGE of Sheep Plasma Proteins

Plate 5 illustrates the capability of 2-D PAGE for the separation of sheep plasma proteins. Good resolution of the plasma proteins was obtained by the 2-D PAGE system used in this study. The protein pattern in Plate 5 is a typical representation of 2-D PAGE of sheep plasma proteins. The separations were greater than 95% reproducible when checked by overlaying gels on a light box. However, there was some loss of protein at the acidic end of the gel, most noticeably α_1-acid glycoprotein. It was found that the gradient could easily be manipulated so as to include this part of the pattern, by increasing the concentration of Pharmalytes pH 3-10, the strength of the anolyte, and by decreasing the strength of the catholyte. However, this also resulted in the remainder of the proteins being condensed into a smaller area, thereby decreasing resolution. Furthermore, the proteins at the very acidic end of the gel did not appear to have an association with either facial eczema resistance or susceptibility. Therefore, it was considered acceptable to sacrifice detection of the very acidic proteins for the increased resolution over the rest of the gel.

Over 200 different proteins were detected on Coomassie blue stained 2-D PAGE gels of sheep plasma (Plate 5) and an even greater number of proteins were seen by using autoradiographic and fluorographic detection of radioactively labelled proteins (Plate 6). The proteins are shown separated, left to right as acidic to basic proteins, and from top to bottom as high M_r to low M_r species. Most of the sheep plasma proteins were situated

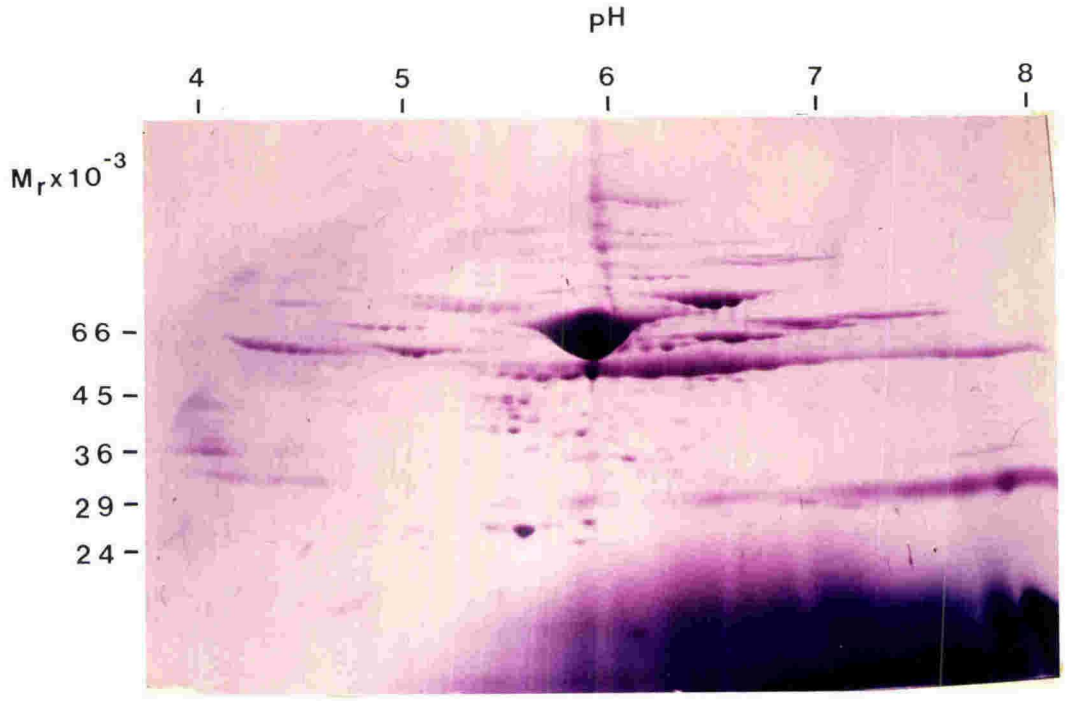


PLATE 5. 2-D PAGE of Sheep Plasma Proteins

2-D PAGE of a gel loaded with 250 μg of plasma proteins. The proteins were focused in an IEF tube gel with a pH gradient ranging from pH 4 to 8 and were then electrophoresed on a 5-20% (w/v) acrylamide linear gradient slab gel. The proteins were detected by staining with Coomassie blue R-250.

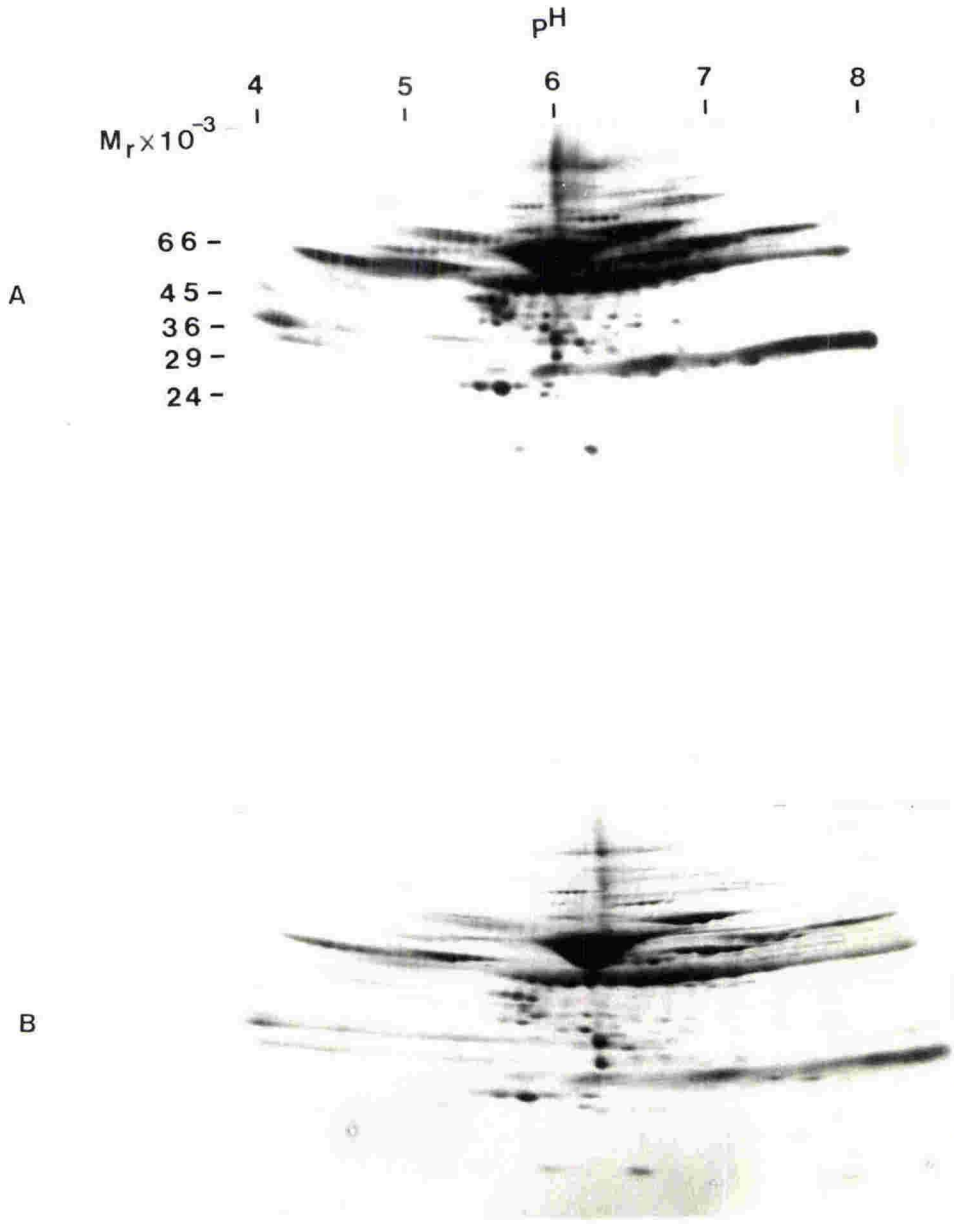


PLATE 6. Autoradiography and Fluorography of Sheep Plasma Proteins

Plasma proteins from facial eczema resistant sheep were ^{3}H-labelled and plasma proteins from susceptible sheep were ^{14}C-labelled. The labelled proteins were pooled and an aliquot containing 1,820,000 d.p.m. of ^{3}H and 255,000 d.p.m. of ^{14}C was electrophoresed. Detection was by A) fluorography (1 week), B) autoradiography (5 weeks).

in the pI range 5-7 and M_r between 24,000 and 70,000 (Plate 5). For most proteins, the pI determined in the IEF-dimension and the M_r determined in the SDS-dimension agreed well with the published values for human plasma proteins (Anderson and Anderson, 1977, 1984). The intensely stained portion at the bottom right of Coomassie blue stained gel was caused by the dye staining Pharmalytes retained in the gel from the first-dimensional separation. These dye staining Pharmalytes thus prohibited the detection of proteins with M_r less than 20,000. Many of the proteins were grouped into families of 4-10 proteins with similar M_r but different pI values. Such families included the IgG heavy and light chains, α_1-antitrypsin and transferrin identified in Figure 17. Although identities can be tentatively assigned to over half of the major protein families by reference to human plasma protein maps (Table 14), unequivocal identification awaits use of either purified marker proteins or antibodies to known proteins. However, despite the lack of formal identification of the protein families, the gels can be used to recognise variation among the plasma proteins of facial eczema resistant and susceptible sheep.

Several polymorphisms are immediately evident. Multiple molecular forms of α_1-antichymotrypsin were detected. The α_1-antichymotrypsin family contained a string of polypeptides comprising at least six spots (Plate 5). Another plasma protein exhibiting genetic polymorphism was α_2HS-glycoprotein. The α_2HS-glycoprotein family contained two parallel strings of polypeptides, each comprising at least four spots (Plates 5 and 6B). These two parallel strings of polypeptides of α_2HS-glycoprotein represented the heterozygous patterns (Anderson and Anderson, 1979). The polymorphism in Gc-globulin was detected as a set of three protein spots which represented the heterozygous

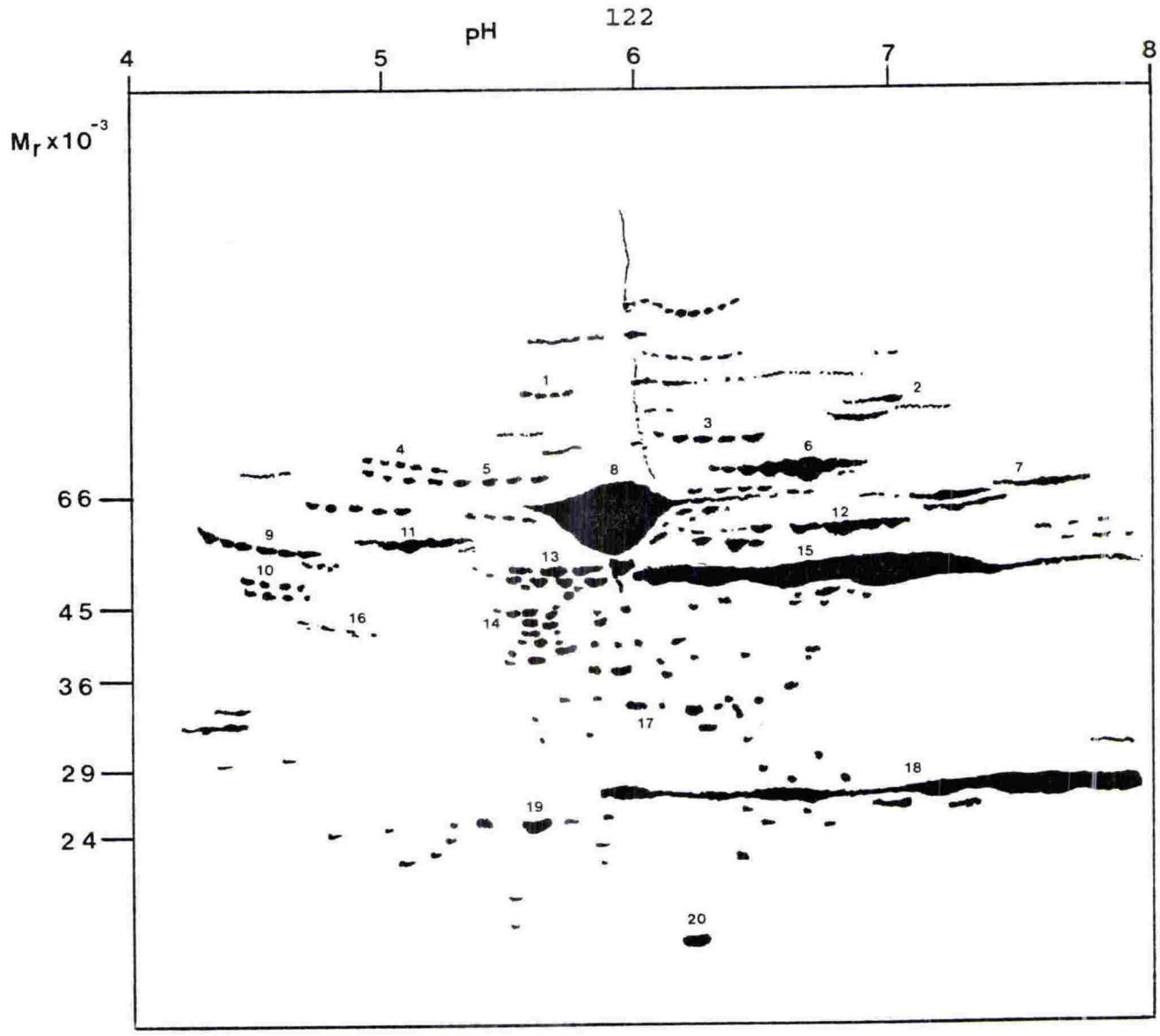


FIGURE 17. 2-D PAGE Map of Sheep Plasma Proteins

This map was drawn from the gel shown in Plate 5, and was labelled to indicate positions of known plasma proteins with reference to Anderson and Anderson (1984). The numbers refer to protein families which are tentatively identified in Table 14.

TABLE 14. Tentative Identification of Sheep Plasma Proteins Separated by 2-D PAGE

		pI
1.	Ceruloplasmin	5.5 - 5.8
2.	Plasminogen	6.4 - 7.0
3.	C_3 activator	6.0 - 6.4
4.	α_1B-glycoprotein	4.9 - 5.2
5.	Hemopexin	5.0 - 5.6
6.	Transferrin	6.1 - 6.7
7.	Fibrinogen α-chain	6.6 - 7.6
8.	Albumin	5.5 - 6.2
9.	α_1-Antichymotrypsin	4.0 - 4.7
10.	α_2HS-glycoprotein	4.3 - 4.7
11.	α_1-Antitrypsin	4.7 - 5.2
12.	Fibrinogen ß-chain	6.2 - 6.7
13.	Gc-globulin	5.4 - 5.7
14.	Fibrinogen γ-chain	5.3 - 5.5
15.	IgG heavy chains	5.9 - 8.0
16.	Haptoglobin ß-chain	4.3 - 4.8
17.	Arginine-rich lipoprotein (LDL)	5.8
18.	IgG light chains	5.7 - 8.0
19.	Apo A-I lipoprotein (HDL)	5.5, 5.3 - 5.8
20.	Haptoglobin α_2-chain	6.2

Identifications were made by comparison with human plasma protein data from Anderson and Anderson (1977, 1984). The pI values were taken directly from Plate 5 and had not been normalised for effects of temperature or other factors that might affect the apparent pI. The pI values were determined under denaturing conditions in the presence of urea. All values were rounded off to one decimal place. When only one major form was present, the value was listed. With multiple forms, the pI range was given with the pI of the predominant component listed first and underlined, e.g., 5.5, 5.3 - 5.8 represents multiple forms with pI values between 5.3 and 5.8, with the major component at pI 5.5.

forms (Tracy et al., 1982a). Polymorphism in other plasma proteins, including α_1-antitrypsin and transferrin, was also detected.

Initial comparison of 250 μg protein loaded 2-D PAGE maps of rams from the facial eczema resistant and susceptible flocks indicated differences among two protein families. A triplet of proteins with a M_r approximately 36,000, was present only in pooled plasma from the resistant rams (Plate 7A) when compared with pooled plasma from the susceptible rams (Plate 7B), but when the ewes were examined, the protein triplet was faintly seen in plasma from both resistant (Plate 7C) and susceptible ewes (Plate 7D). Further analyses of the presence of the protein triplet in individual plasma of ram hoggets from the resistant flock showed that the protein triplet was present in 5 of the animals which were sheep numbers 106 (sire FE 85), 31 (sire FE 171), 88 and 125 (sire ROTO 193) and 61 (sire ROTO 645) (Plate 8). The protein triplet was not detected in plasma from resistant ram numbers 65 (sire FE 85), 34 and 100 (sire FE 171), 126 (sire ROTO 123), 48 (sire ROTO 193) and 105 (sire ROTO 645), even when the protein load was increased from 250 μg to 400 μg. When individual plasma from susceptible rams were analysed, the protein triplet was detected in 1 animal (sheep number 133 from sire ROTO 658) but was not detected in the plasma of the other 8 susceptible rams which were sheep numbers 38 and 107 (sire FE 309), 25, 68 and 117 (sire ROTO 140), 82 and 90 (sire ROTO 601) and 83 (sire ROTO 658) (Plate 8). The identity of the triplet family is unknown and as its presence was not clearly linked to facial eczema resistance, no further studies on its inheritance or identity were carried out.

Variation among a group of proteins, of M_r approximately 80,000 and which were basic with respect to albumin, was more

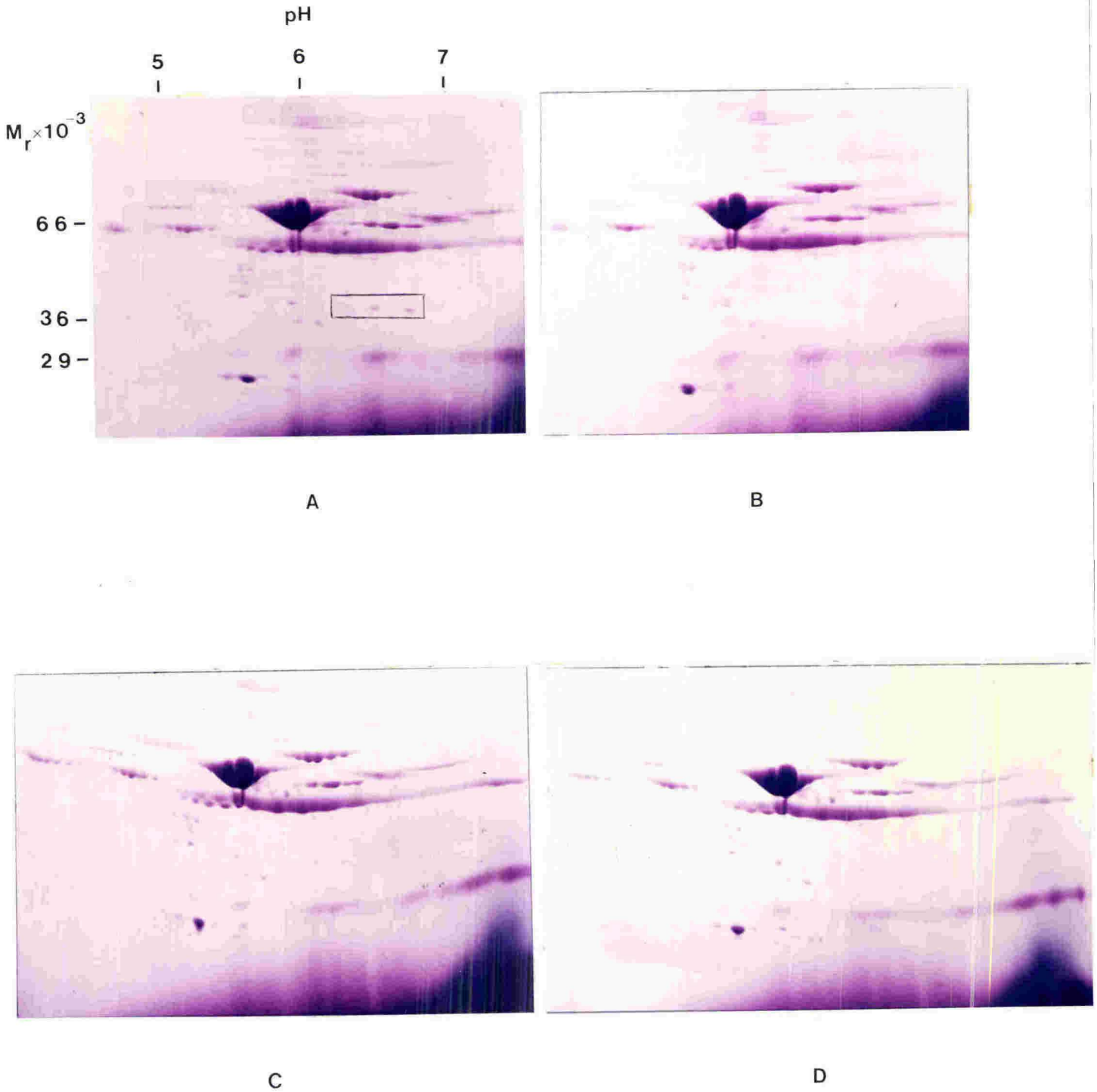


PLATE 7. 2-D PAGE of Plasma Proteins from Resistant and Susceptible Sheep

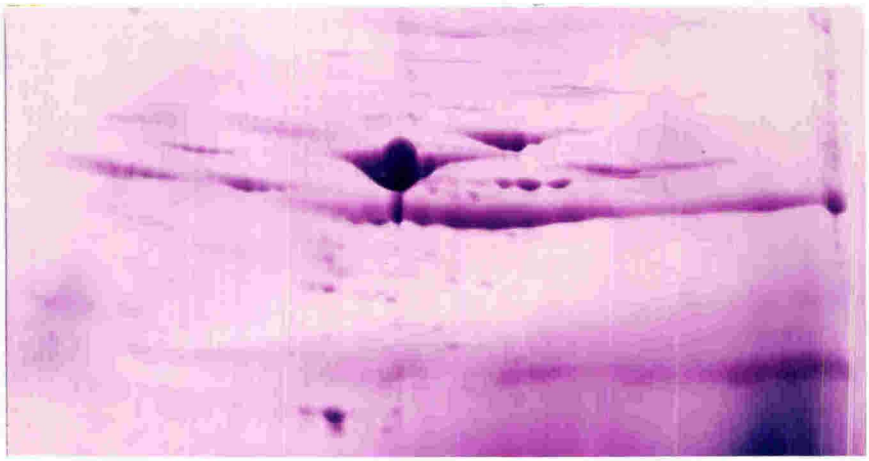
2-D PAGE of plasma proteins (250 μg) using a 5-20% linear gradient gel in the SDS dimension. Boxed spots indicate the protein triplet (M_r - 36,000). A) pooled plasma from resistant rams; B) pooled plasma from susceptible rams; C) pooled plasma from resistant ewes; D) pooled plasma from susceptible ewes.

PLATE 8. 2-D PAGE of Plasma Proteins from Individual Resistant and Susceptible Rams

	Animal Number
Resistant	65
	106
	31
	34
	100
	126
	48
	88
	125
	61
	105
Susceptible	38
	107
	25
	68
	117
	82
	90
	83
	133

The amount of protein electrophoresed was 400 µg except for animal numbers 106, 126 and 82 for which 250 µg was used. Numbers beside gels are the animal numbers previously listed in Table 1.

65



pH

4 6 8

106

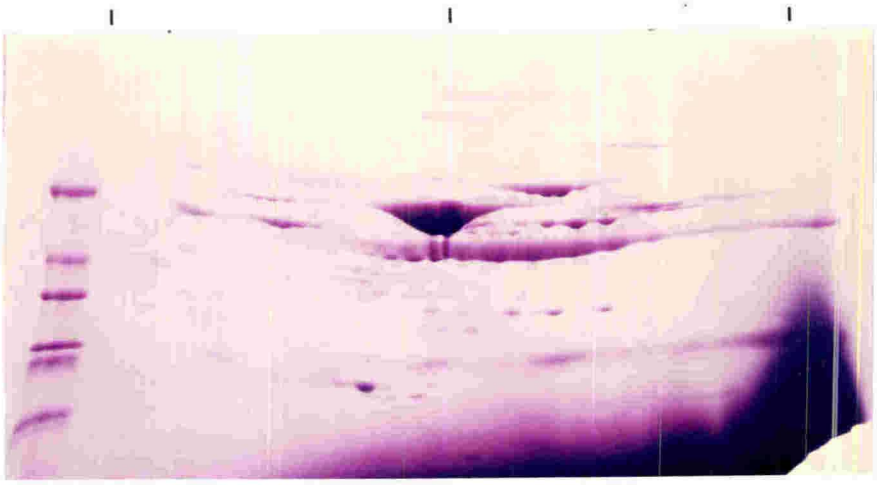
$M_r \times 10^{-3}$

66 -

45 -

36 -

29 -



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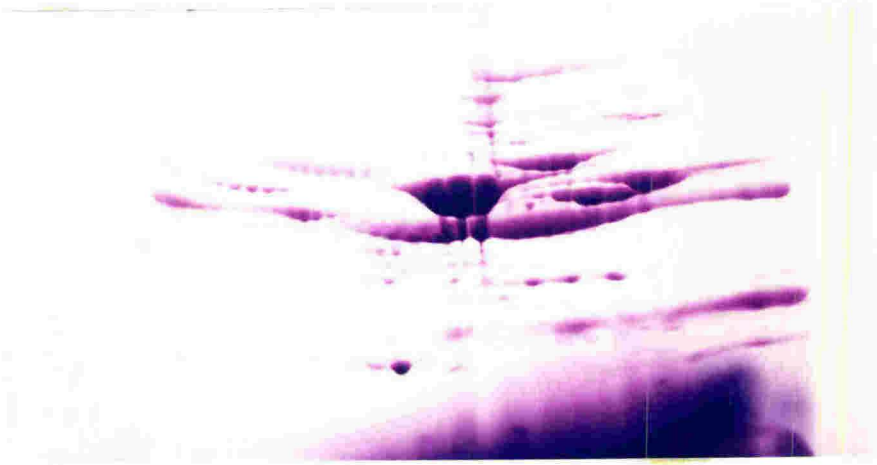

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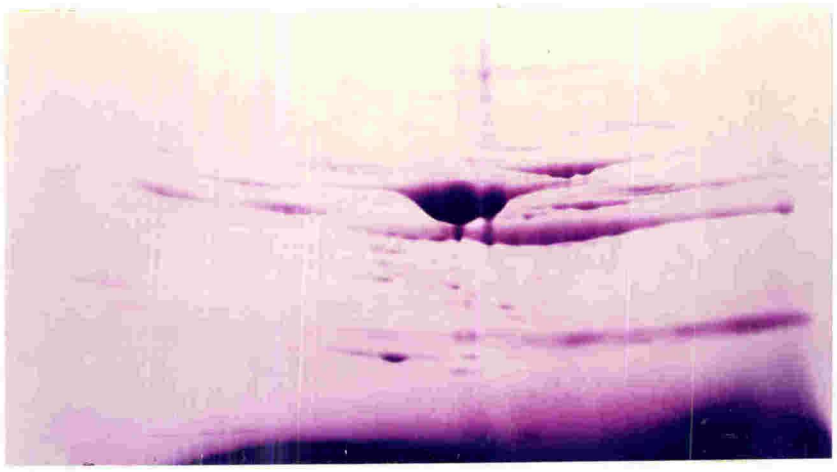

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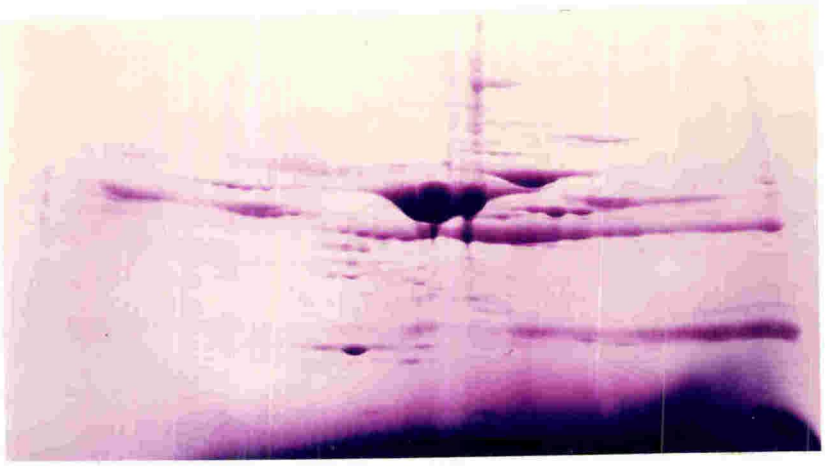

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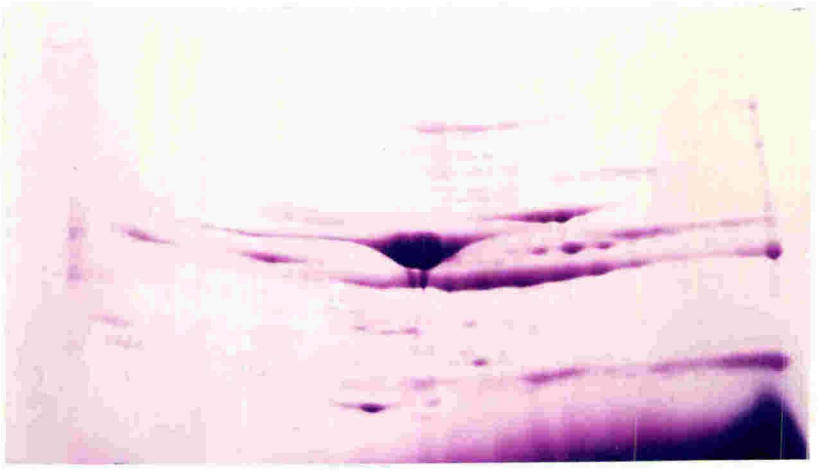

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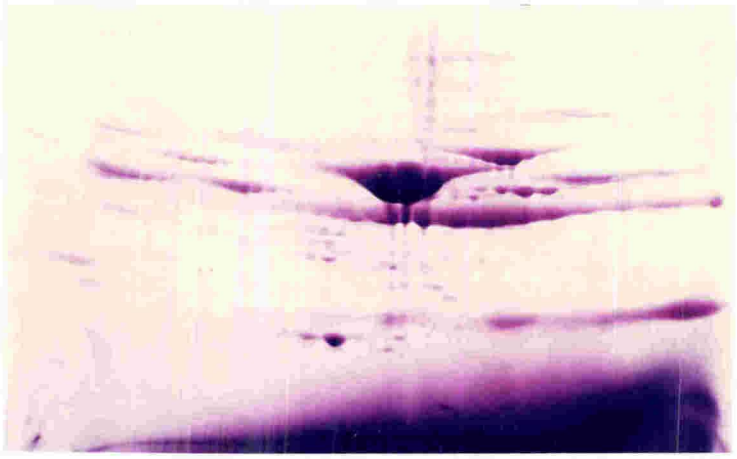

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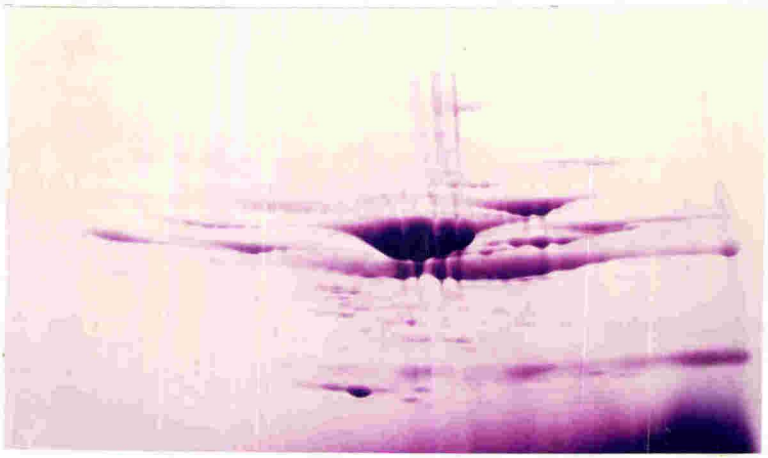

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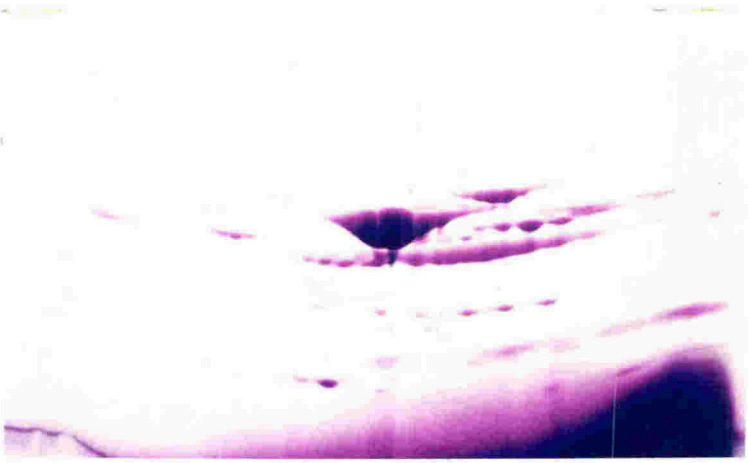

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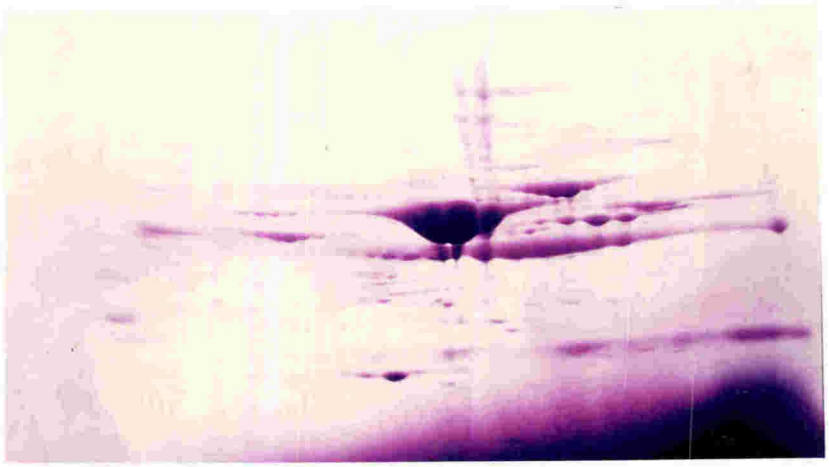

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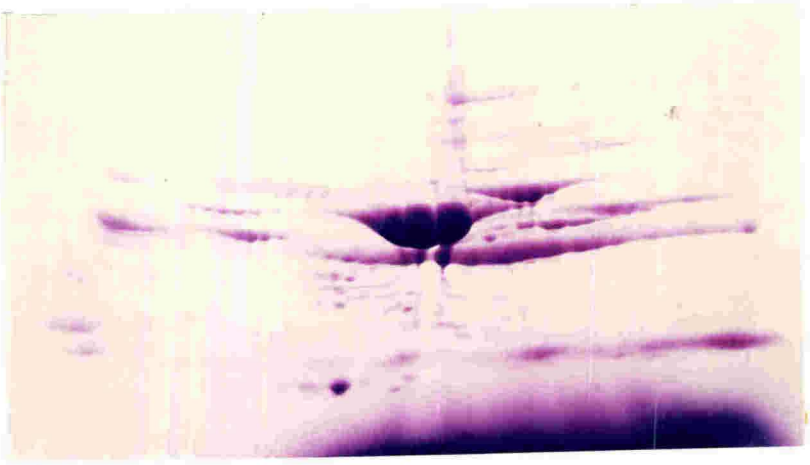

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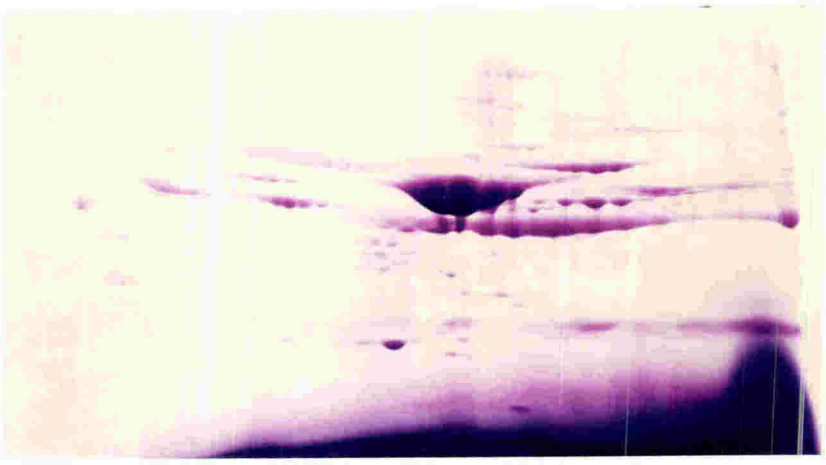

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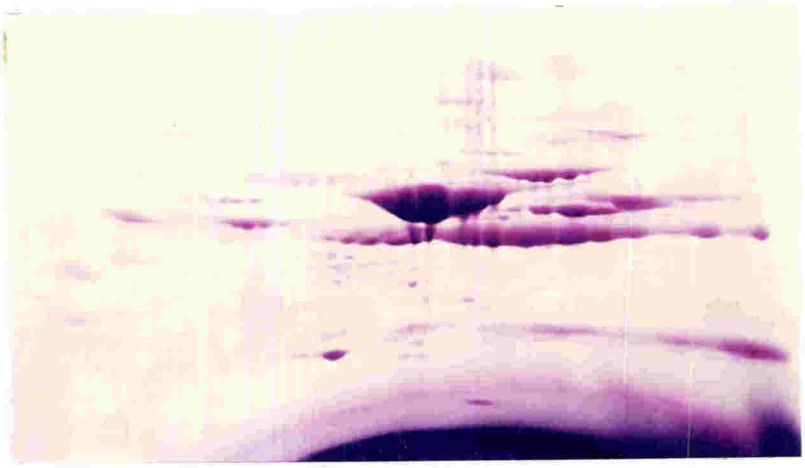

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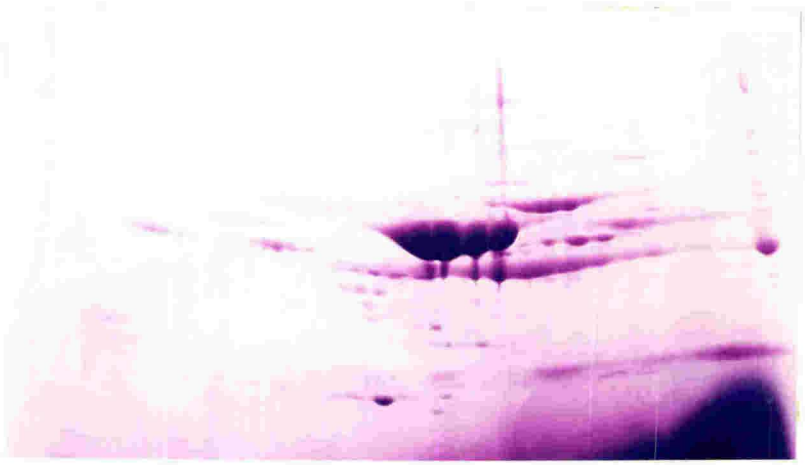

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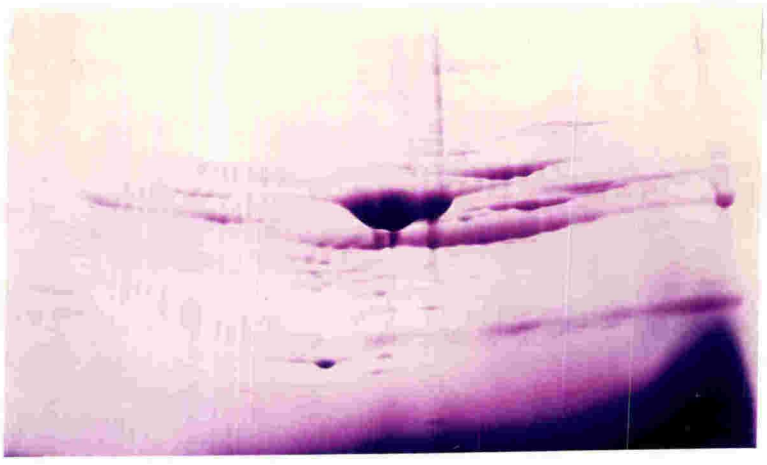

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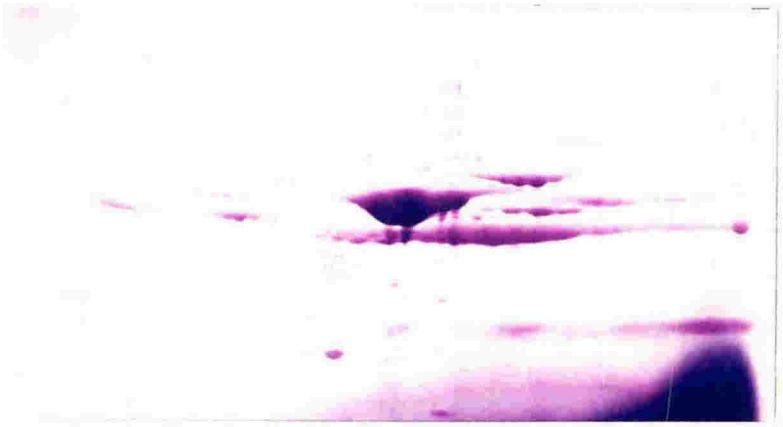

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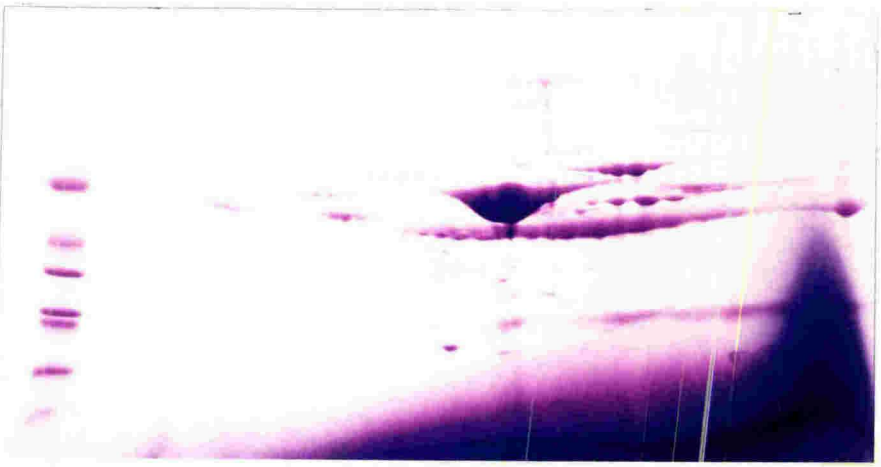

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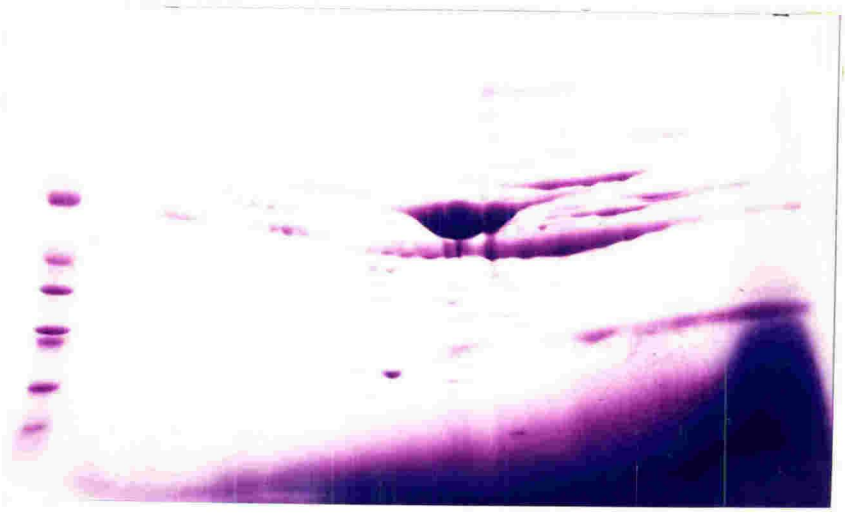

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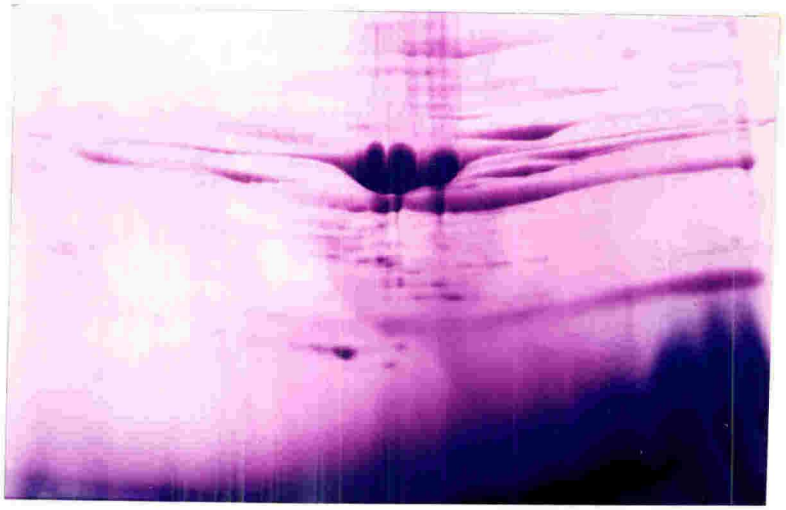


clearly linked to resistance and susceptibility. In this protein family at least five variants, designated I-V from acidic to basic, were observed (Plate 9). For each gel the five variants were positionally characterised with respect to the migration of nearby proteins. Acidic variants (I, II, III) predominated in the pooled plasma of resistant rams (Plate 9A) and resistant ewes (Plate 9C) while a more basic variant (IV) was predominant in susceptible rams (Plate 9B) and susceptible ewes (Plate 9D). When the 2-D PAGE of the individual resistant rams and susceptible rams were characterised for variants I-V, the resistant pattern was present in all but 2 resistant rams, numbers 34 (sire FE 171) and 88 (sire ROTO 193), while the susceptible pattern was present in plasma from each of the 9 susceptible rams (Plate 8). By reference to human plasma protein maps, this protein family was tentatively identified as transferrins.

Differential detection of proteins selectively labelled with radioactive isotopes was used to analyse minor variation not detected by Coomassie blue staining. Plates 10 and 11 show the autoradiograms and fluorograms of a mixture of ^{14}C- and ^{3}H-labelled plasma from resistant and susceptible sheep. In both cases, the resolution of radioactive proteins was less in the fluorograms of 2-D PAGE gels containing double-labelled proteins than in the corresponding autoradiograms. The lack of resolution in the fluorograms was due to over-exposure of the film. In the experiment shown in Plate 10, plasma proteins from resistant sheep were ^{14}C-labelled and the plasma proteins from susceptible sheep were ^{3}H-labelled. The autoradiogram detected only ^{14}C-labelled proteins which were derived from resistant sheep, while the fluorogram detected both ^{14}C-labelled plasma proteins from resistant sheep and ^{3}H-labelled plasma proteins from susceptible sheep. Therefore, any protein detected only on the fluorogram

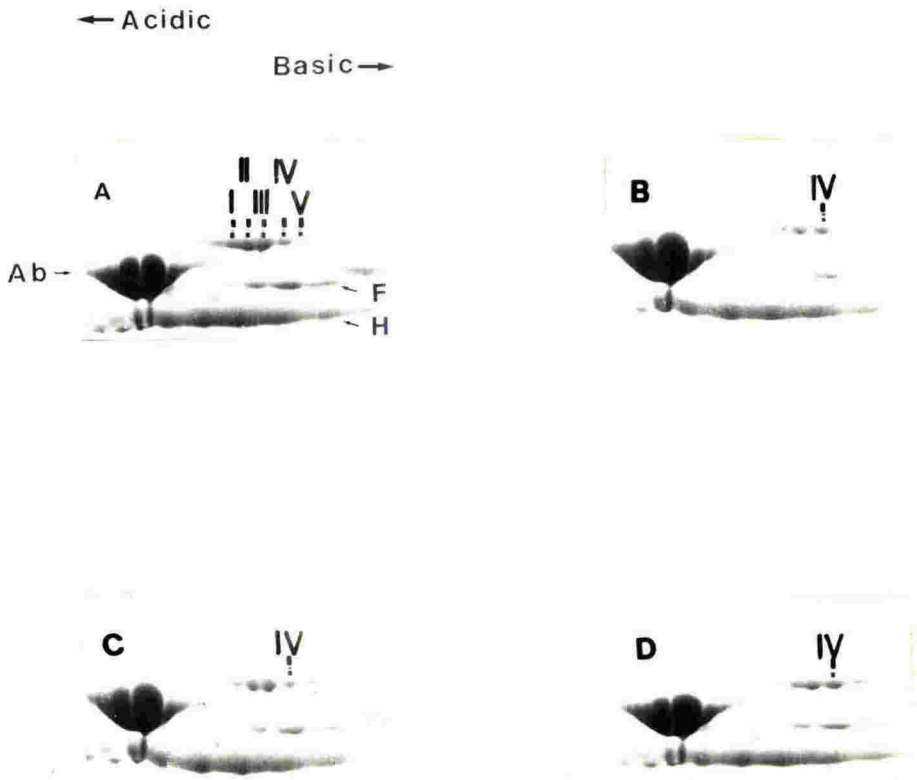


PLATE 9. Sections of 2-D PAGE Gels Showing the Transferrin Variants

The patterns are portions of whole plasma separations: A) pooled plasma from resistant rams; B) pooled plasma from susceptible rams; C) pooled plasma from resistant ewes; D) pooled plasma from susceptible ewes. I-V are transferrin variants. Ab = albumin, H = IgG heavy chains and F = fibrinogen ß-chain.

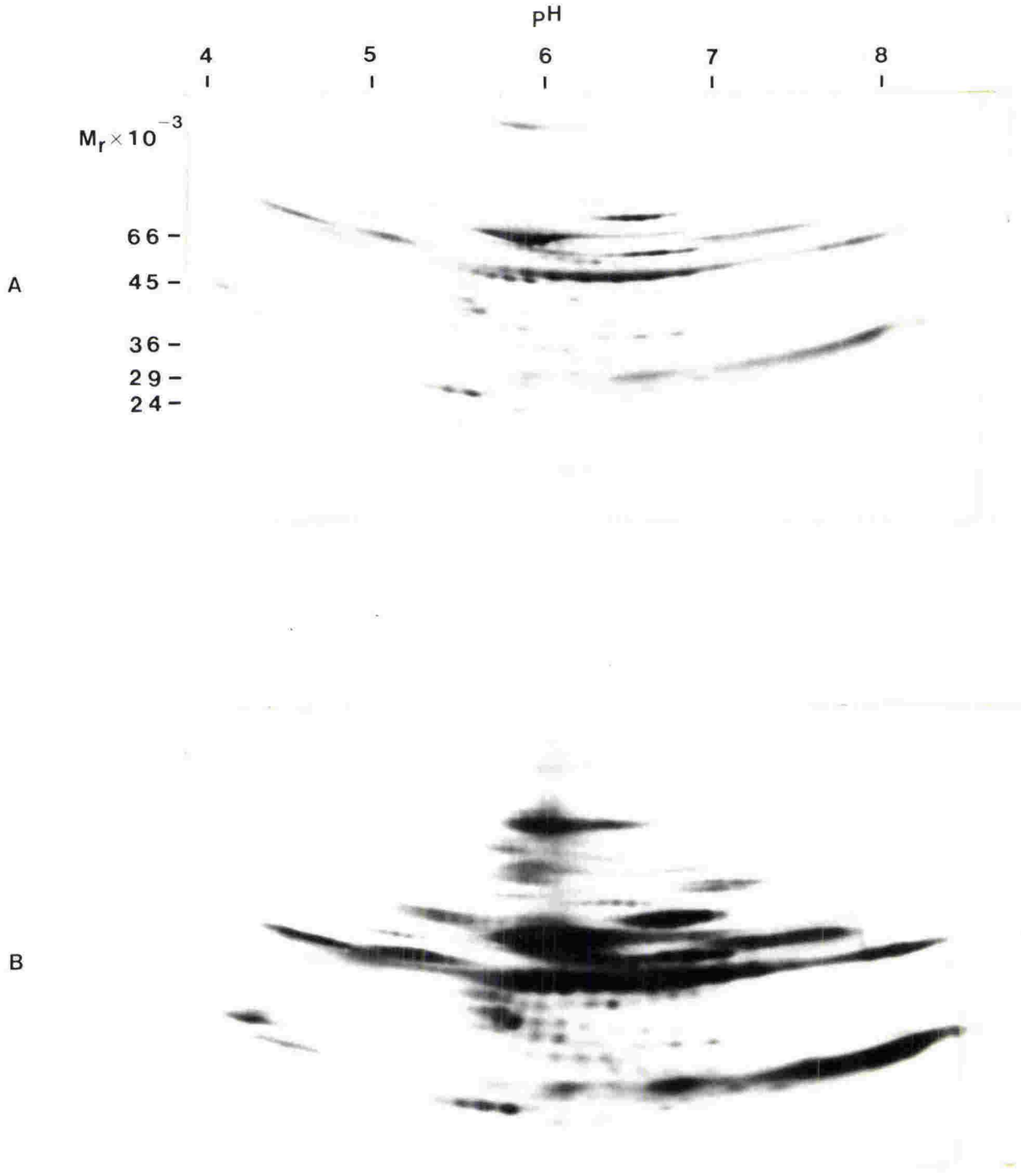


PLATE 10. Autoradiography and Fluorography of Plasma Proteins from Resistant and Susceptible Sheep

The plasma proteins from resistant sheep were ^{14}C-labelled and the plasma proteins from susceptible sheep were ^{3}H-labelled. The labelled plasma were pooled and an aliquot containing 247,210 d.p.m. of ^{14}C and 3,055,440 d.p.m. of ^{3}H was electrophoresed. A) autoradiography (7 weeks), B) fluorography (2 weeks).

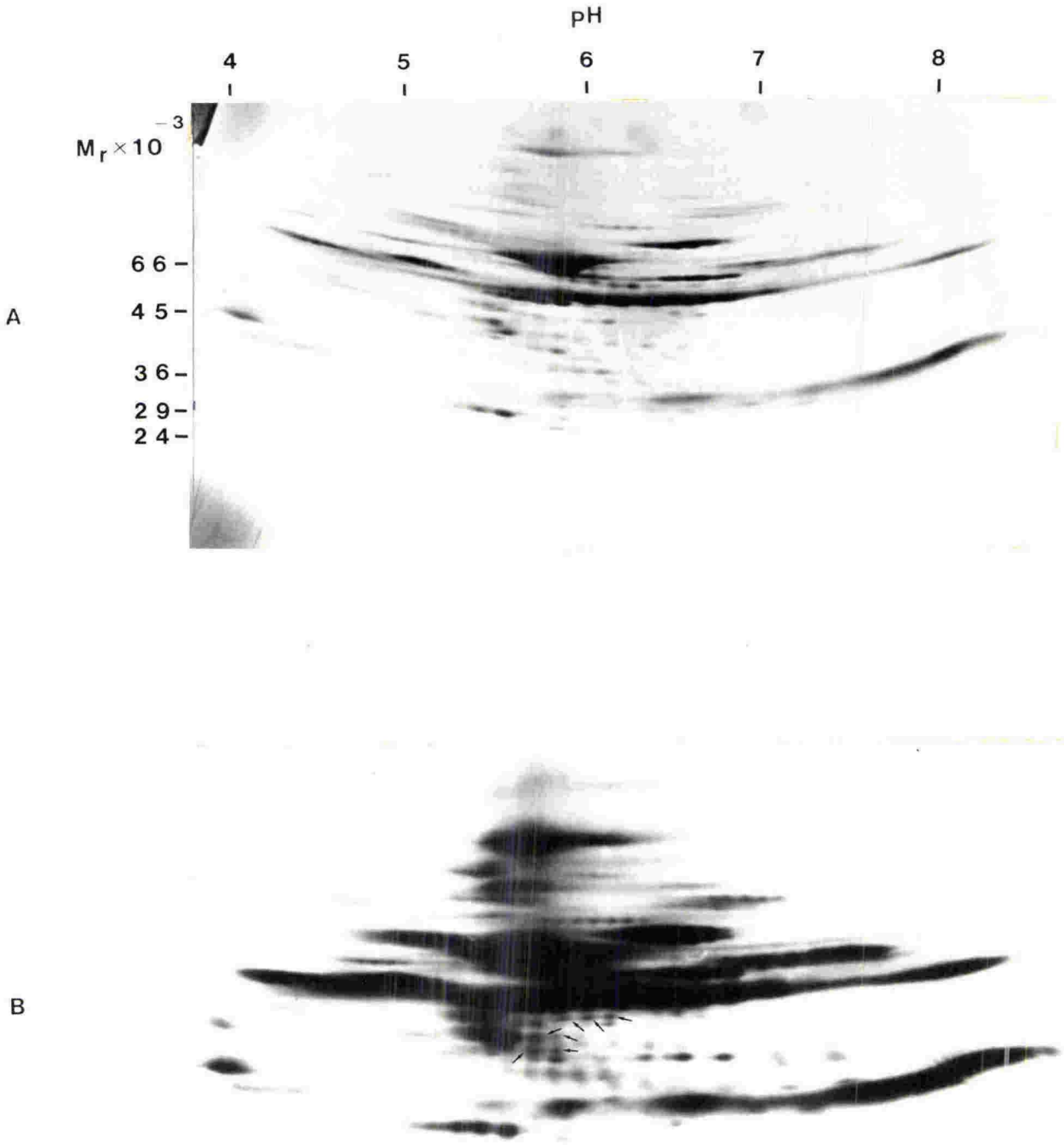


PLATE 11. Autoradiography and Fluorography of Plasma Proteins from Susceptible and Resistant Sheep

The plasma proteins from susceptible sheep were ^{14}C-labelled and the plasma proteins from resistant sheep were ^{3}H-labelled. The labelled plasma were pooled and an aliquot containing 283,237 d.p.m. of ^{14}C and 2,937,607 d.p.m. of ^{3}H was electrophoresed. A) autoradiography (7 weeks), B) fluorography (2 weeks). Additional proteins detected only on the fluorogram are indicated by arrows.

would represent a protein characteristic of susceptible plasma. However, a comparison of the autoradiogram and fluorogram did not reveal any apparent differences between the two plasma populations. The reverse double-labelling, where plasma proteins from resistant sheep were ^{3}H-labelled and the plasma proteins from susceptible sheep were ^{14}C-labelled, was also carried out and the results are shown in Plate 11. Comparison of the autoradiogram and fluorogram revealed a number of extra spots on the fluorogram (Plate 11B). Additional proteins with M_r between 36,000 and 45,000 and pI around 6 (Plate 11B, indicated by arrows), were detected on the fluorogram. These proteins would represent polypeptides specifically associated with resistance. However, such differences were not found for the results presented in Plates 6A, 8 and 10A. Therefore, the protein variations observed in Plate 11B would appear to be artifacts, or perhaps reflecting the greater exposure of the fluorogram.

3-5.4 Identification of Plasma Transferrins on 1-D Polyacrylamide Gels

The identity of the protein variants detected on 2-D PAGE was investigated by 1-D PAGE. Plasma proteins were separated on 1-D polyacrylamide gels and several components were observed in the ß-globulin region (Plate 12). Comparison of pooled plasma proteins from resistant and susceptible sheep again demonstrated that the more acidic proteins, in the family considered to be transferrins, predominated in sheep selected for resistance as compared with those selected for susceptibility to facial eczema.

Evidence of identity as transferrins was sought in experiments in which plasma was incubated with radioactive iron before electrophoresis. In these experiments, 100 µl of sheep plasma, human transferrin (4 µg/µl) or bovine serum albumin (3.2

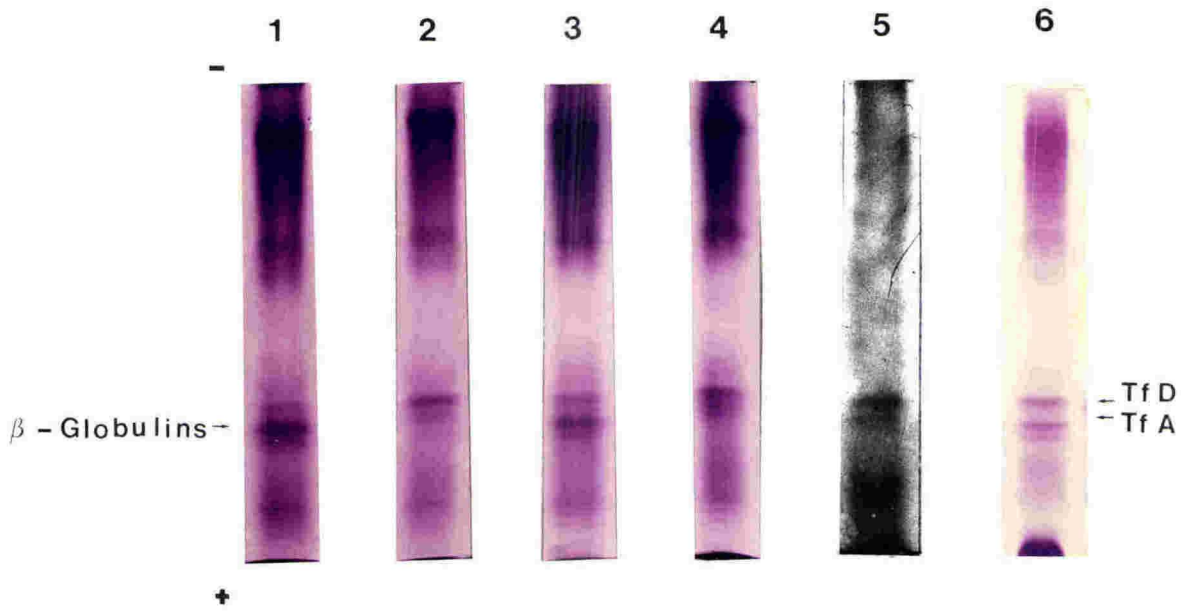


PLATE 12. Plasma Transferrins Separated on 1-D Polyacrylamide Gels

Plasma transferrins from resistant and susceptible Romney sheep were separated on a 5% polyacrylamide gel. Lanes 1-4, Coomassie blue stained gels: 1) pooled plasma from resistant rams; 2) pooled plasma from susceptible rams; 3) pooled plasma from resistant ewes; 4) pooled plasma from susceptible ewes. Lane 5, autoradiogram of ^{59}Fe-loaded pooled susceptible ram plasma. Lane 6, sheep plasma containing transferrin A and D alleles.

$\mu g/\mu l$) were incubated with $^{59}FeCl_3$ before electrophoresis. After incubation, the samples were centrifuged in Centricon-10 centrifugal concentrators (Amicon) to separate protein-bound Fe^{59} from free Fe^{59}. The samples were concentrated to a small volume (approximately 50 μl) and after addition of 2 ml of deionised water, were again concentrated. After centrifugation, more than 90% of the radioactivity was retained in the protein fraction for transferrin and sheep plasma, and less than 5% was associated with albumin. The concentrated protein samples were electrophoresed on a 1-D polyacrylamide gel under non-dissociating conditions and the Coomassie blue-stained gel was subjected to autoradiography to detect the association of ^{59}Fe with individual proteins. Analysis of the autoradiogram showed that radioactivity migrated with human transferrin but not with albumin (data not shown). Radioactivity, which was associated with pooled sheep plasma, migrated with Coomassie blue-stained bands shown on Plate 12 which were thus identified as the iron-binding plasma transferrins. Pooled plasma from susceptible rams contained 2 major zones of ^{59}Fe which migrated with Coomassie blue-stained transferrins (Plate 12, Lane 5). Similarly ^{59}Fe loaded plasma from resistant rams also migrated with the Coomassie blue-stained transferrin zones (data not shown).

Further evidence of identity of the protein variants as transferrins was obtained in an experiment in which plasma from resistant and susceptible sheep were electrophoresed with sheep plasma containing transferrin A and D alleles. The similar electrophoretic mobility between the protein variants and the sheep transferrin A and D alleles provided further evidence for the protein variants as transferrins (Plate 12).

3-5.5 Iron-loading of Transferrins

The effect of iron-loading on the electrophoretic mobility of the transferrin variants was investigated by comparing the electrophoretic mobility of untreated plasma transferrins with that of plasma transferrins which had been treated with ferric ammonium sulphate in an amount equivalent to 5 μg of Fe^{3+} per ml of plasma to effect full saturation of transferrin with Fe^{3+}. The plasma transferrins were separated by PAGE and the iron-saturated transferrins were found to have identical mobility to their untreated counterparts (data not shown).

3-5.6 Neuraminidase Digestion of Transferrins

The effect of neuraminidase digestion on the electrophoretic mobility of transferrins was studied in an experiment in which plasma proteins were incubated at 37^{o}C with 40 μl or 80 μl of neuraminidase before PAGE. Digestion with either 40 μl or 80 μl of neuraminidase (Plate 13) decreased the electrophoretic mobility of the transferrins to a similar extent. In each case, a pattern of three slower moving bands was obtained. All the bands were shifted equally by a similar increment suggesting that the bands represented the removal of a constant amount of sialic acid from the transferrin molecule. The neuraminidase digestion appeared to be complete by 3 h as judged by no further change in electrophoretic mobility after that time (Plate 14).

The decrease in mobility in the horizontal, charge dependent dimension was also observed for other proteins when neuraminidase digested plasma was analysed by 2-D PAGE (Plate 15). The proteins identified as α_1-antichymotrypsin, α_1-antitrypsin, α_1B-glycoprotein, hemopexin, transferrin and fibrinogen ß-chains were all shifted basically after

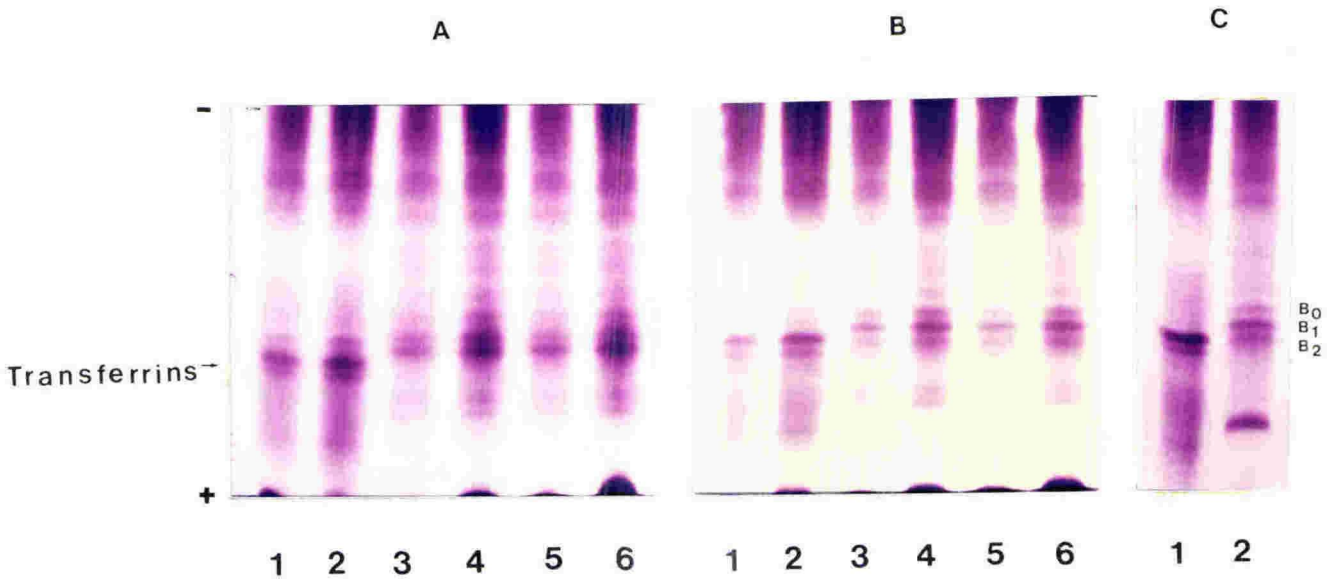


PLATE 13. Effect of Neuraminidase Digestion on the Electrophoretic Mobility of Plasma Transferrins

Plasmas were incubated with neuraminidase at 37°C for 3 h before electrophoresis. A) pooled plasma from resistant rams; B) pooled plasma from susceptible rams. Lane 1, 170 μg of plasma proteins without neuraminidase digestion; Lane 2, 340 μg of plasma proteins without neuraminidase digestion; Lane 3, 170 μg of plasma proteins digested with 40 μl of neuraminidase; Lane 4, 340 μg of plasma proteins digested with 40 μl of neuraminidase; Lane 5, 170 μg of plasma proteins digested with 80 μl of neuraminidase; Lane 6, 340 μg of plasma proteins digested with 80 μl of neuraminidase. C) plasma containing the transferrin D allele; Lane 1, 340 μg plasma protein without neuraminidase digestion; Lane 2, 340 μg plasma protein digested with 40 μl of neuraminidase. Band B_2 represents transferrin with 2 sialic acid residues, band B_1 represents transferrin with 1 sialic acid residue and band B_0 represents the complete removal of sialic acid from the molecule.

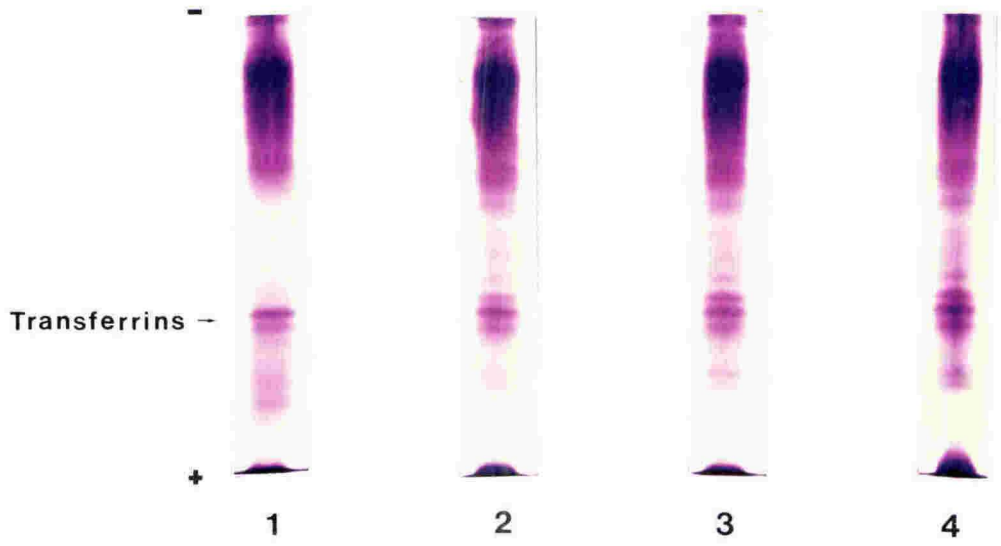
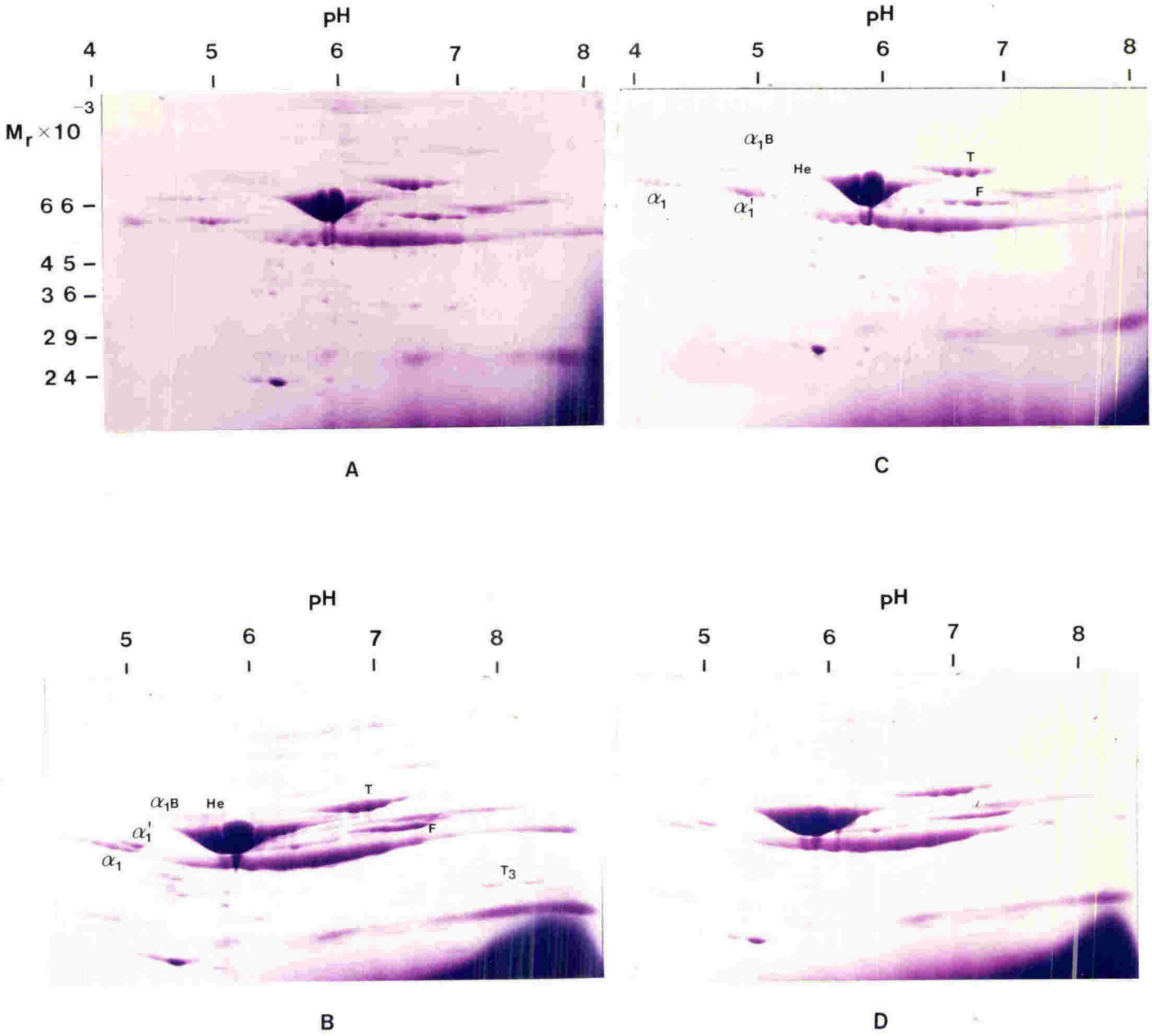


PLATE 14. Effect of the Duration of Neuraminidase Digestion on the Electrophoretic Mobility of Transferrin

Pooled plasma from susceptible rams was digested with 40 μl of neuraminidase for up to 24 h. Lane 1, undigested sample; Lane 2, 3 h digestion; Lane 3, 6 h digestion; Lane 4, 24 h digestion.

PLATE 15. 2-D PAGE of Sheep Plasma Proteins Digested with Neuraminidase

2-D PAGE of plasma proteins digested with 40 μl of neuraminidase for 3 h. A) pooled plasma from resistant rams, B) pooled plasma from resistant rams after neuraminidase digestion, C) pooled plasma from susceptible rams, D) pooled plasma from susceptible rams after neuraminidase digestion. Identifications from standard plasma protein maps are: α_1 = α_1-antichymotrypsin, α_1' = α_1-antitrypsin, α_1B = α_1B-glycoprotein, He = hemopexin, T = transferrin, F = fibrinogen ß-chain, T_3 = triplet of proteins.


pH
4
5
6
7
8
$M_r \times 10^{-3}$
66
45
36
29
24
A
α_1B
He
T
F
α_1
α_1'
C
pH
5
6
7
8
α_1B
He
T
F
α_1'
α_1
T_3
B
D

neuraminidase digestion. α_1-Antichymotrypsin gave a string of at least six protein spots with pI values between 4.0 and 4.7 (Plate 15C). Neuraminidase increased the pI values of α_1-antichymotrypsin to 4.5-5.0 and the spots were displaced towards the position of α_1-antitrypsin (Plate 15D). α_1-Antitrypsin also gave a string of at least six protein spots with pI values between 4.6 and 5.0 (Plate 15A). Following neuraminidase digestion, the string of six protein spots of α_1-antitrypsin were displaced basically to give a single prominent spot with pI about 5 and a less prominent spot with a slightly lower pI (Plate 15B). In the transferrin charge train (pI 6.1-6.7), all subunits shifted equally towards the basic protein side of the gel (pI 6.5-7.1) and the relative proportions of each was retained (Plate 15B and D). The previously identified triplet of proteins with a M_r of about 36,000 and pI values between 6.2 and 6.8 in the resistant plasma was shifted substantially in the direction towards the cathode with resulting pI values between 7.5 and 8.2 after neuraminidase digestion (Plate 15B). This huge reduction in mobility of the triplet of proteins suggested that these proteins contained large numbers of sialic acid residue. Some proteins, including apo A-I lipoprotein, were unaffected by neuraminidase treatment suggesting the absence or inaccessibility to digestion of sialic acid residues.

3-5.7 Transferrin Phenotyping on Polyacrylamide Gels

Transferrin phenotyping was carried out on individual plasma from 22 resistant and 19 susceptible sheep from the facial eczema flocks (Plate 16) so that the distribution of transferrin alleles could be determined. The transferrin phenotypes were assigned by comparison with the electrophoretic migrations of known sheep transferrins which were electrophoresed on each gel.

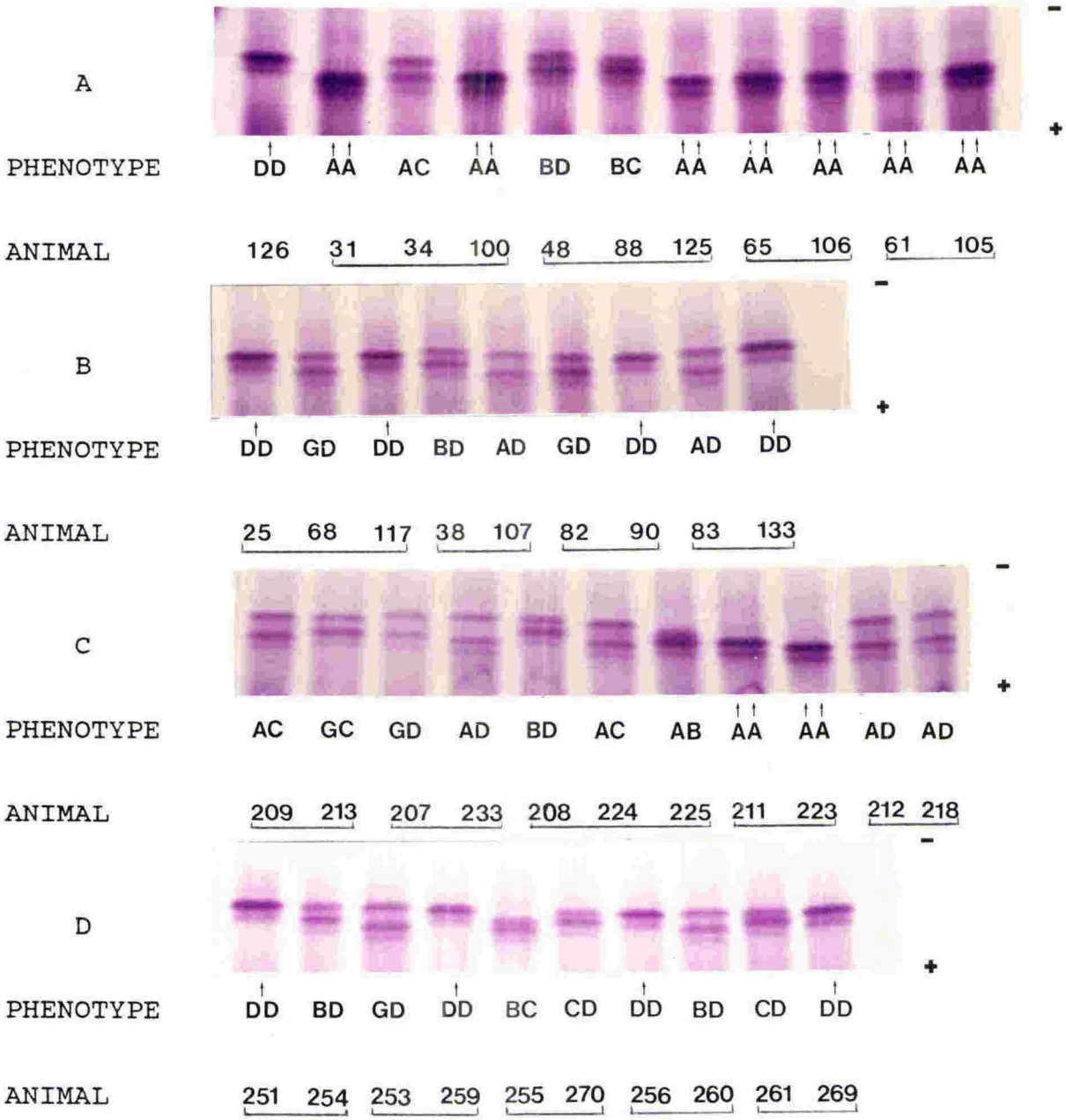


PLATE 16. Polyacrylamide Gels Showing the Transferrin Alleles Detected in the Plasma of Romney Sheep

Transferrin regions from Coomassie blue stained gels illustrating the five transferrin alleles (A, G, B, C and D) detected in individual Romney plasma. Animals grouped in brackets are offspring of a common sire. The detected phenotypes were electrophoresed against known sheep transferrin phenotype standards (A, G, B, C, M and D) for the assignment of transferrin alleles. A) resistant rams, B) susceptible rams, C) resistant ewes, D) susceptible ewes.

Five alleles (A, G, B, C and D in decreasing order of mobility) were detected. Transferrin A was the most rapidly migrating (most acidic) allele and was detected in 16 of the 22 resistant rams and ewes (Plate 16A, C), and was homozygous in 9 of these animals (double arrows, Plate 16A, C). The transferrin A allele was not detected in resistant ewe 207 from sire FE 85, ram 126 and ewe 213 from sire ROTO 123 and rams 48, 88 and ewe 208 from sire ROTO 193. The transferrin AA phenotype was not detected in any of the susceptibles. The most slowly migrating (most basic) allele was transferrin D which was detected in 18 of the 19 susceptible rams and ewes (Plate 16B, D). Ewe 255 from sire FE 309 was the only susceptible animal in which the D allele was not detected. The transferrin D allele was homozygous in 8 of the 19 susceptible animals and in 1 of the resistant animals (single arrow, Plate 16A, B and D).

The frequencies of the transferrin alleles in the resistant and susceptible flocks are shown in Figure 18. In the resistant flock, the frequencies of the 5 alleles were: A 0.57, G 0.05, B 0.09, C 0.11 and D 0.18. The frequencies of the 5 alleles in the susceptible flock were: A 0.05, G 0.08, B 0.10, C 0.08 and D 0.68. From the frequencies of the transferrin alleles, it was apparent that the A allele was most common in the resistant flock while the D allele was most common in the susceptible flock. Therefore, there appeared to be some separation of transferrin alleles between the resistant and susceptible sheep. Assuming random mating and a single population, the expected numbers of the 15 transferrin phenotypes in the resistant flock were calculated and compared with the observed numbers (Figure 19). There were more phenotypes AA, BC, BD, CG and DG observed than were expected, whereas the observed numbers of phenotypes AB, AD, AG and CD were smaller than the expected numbers. However, the

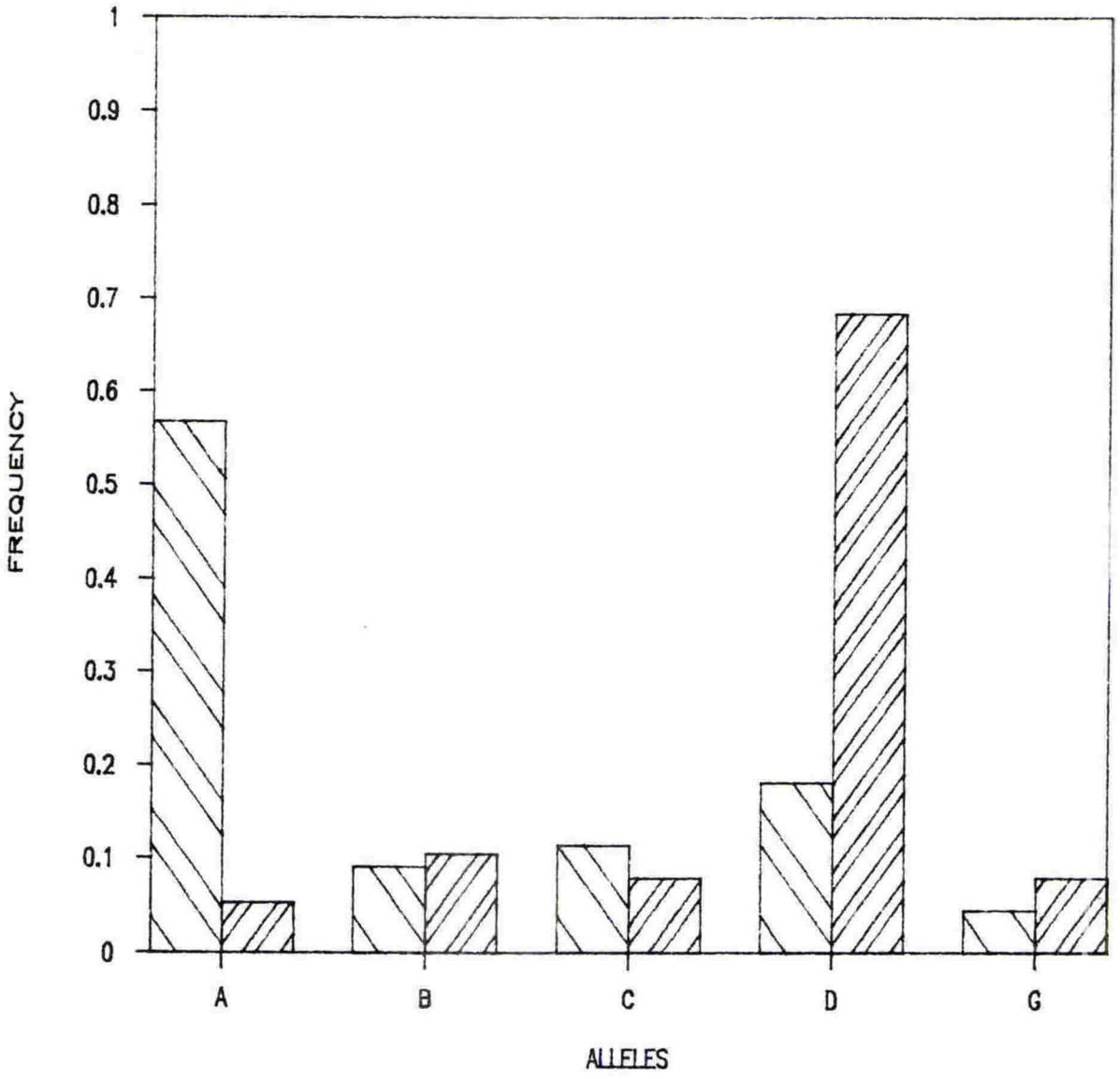


FIGURE 18. Frequency of Alleles in Resistant and Susceptible Flocks

The frequency of the alleles A, B, C, D and G in the resistant and susceptible flocks are shown: ⧅) resistant sheep, ▨) susceptible sheep. The frequency of each allele is given as a decimal fraction of the total alleles in each of the populations sampled. The animals were Romney ewe and ram hoggets from the facial eczema resistant and susceptible flocks as shown in Table 1 and their transferrin alleles are illustrated in Plate 16.

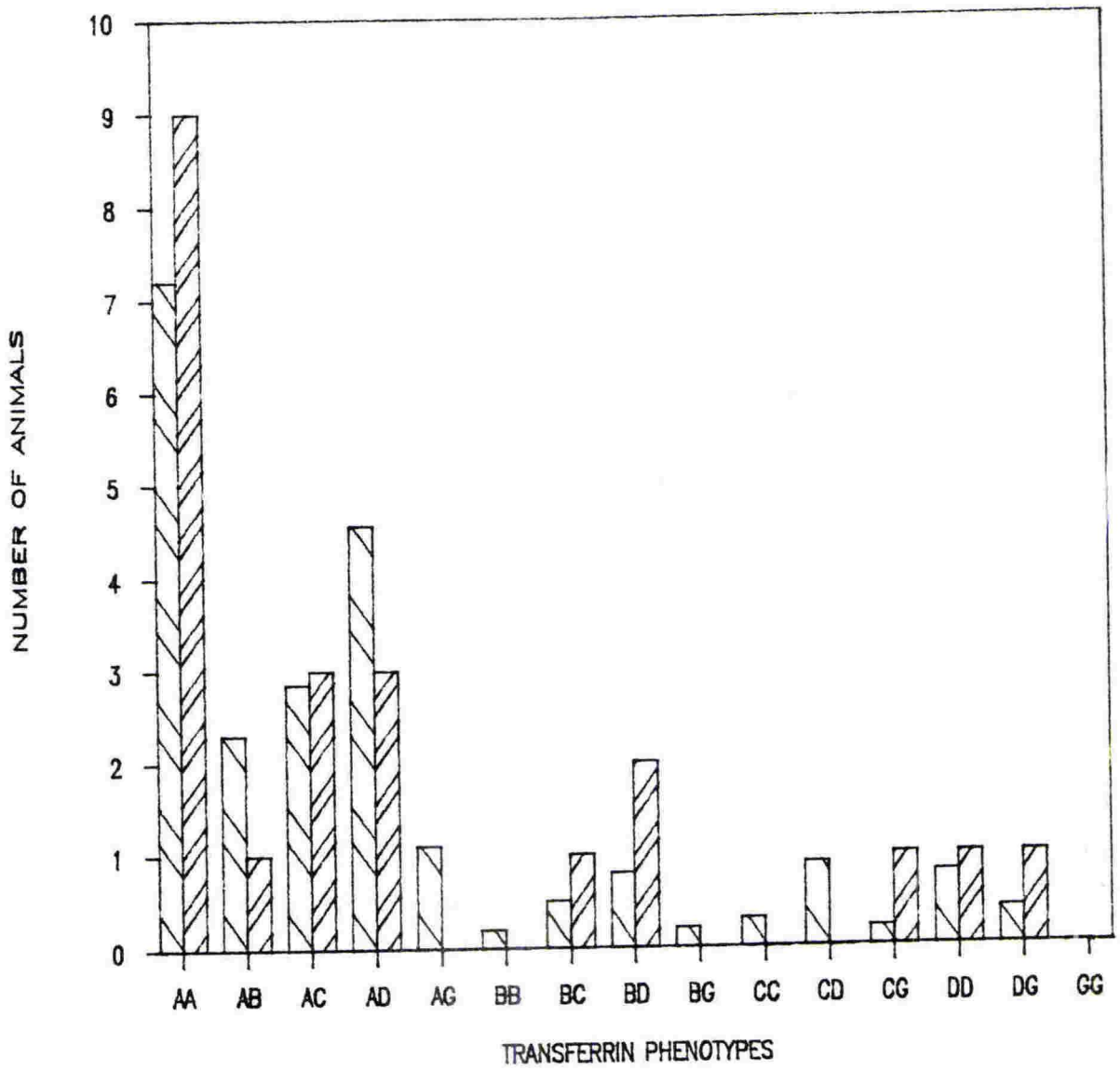


FIGURE 19. Comparison of the Expected Numbers of Transferrin Phenotypes with the Observed Numbers in the Resistant Flock

The expected numbers of the 15 possible transferrin phenotypes from a 5 allele system (A, B, C, D and G) were compared with the observed numbers of the transferrin phenotypes in the resistant flock. ▨) expected numbers in the flock as a single population. ▨) observed numbers in the flock. The deviations between the observed numbers and the expected numbers were small (X^2 = 11.27, 14 df, 0.70 > P > 0.50).

deviations between the observed numbers and the expected numbers were small and were most likely due to chance (X^2 = 11.27, 14 df, 0.70 > P > 0.50). Likewise, the expected numbers of the 15 transferrin phenotypes in the susceptible flock were also calculated and compared with the observed numbers (Figure 20). There were more phenotypes AD, BC and DG but less phenotype DD observed than were expected. The deviations between the observed numbers and the expected numbers were insignificant (X^2 = 3.90, 14 df, P > 0.995). These results indicated that the resistant and susceptible populations had attained Hardy-Weinberg equilibrium.

A further group of sheep, which comprised 159 lambs, were used to study the association of transferrin phenotype with facial eczema resistance. The resistance status of these animals is described in Table 4. A comparison of the distribution of transferrin alleles between the resistant and susceptible lambs is given in Figure 21. In the resistant lambs, the frequency of transferrin A was higher than expected while the frequency of transferrin D was lower than expected. The frequency of all 5 alleles in the susceptible lambs were in good agreement with those expected in the population. Assuming random mating and no differential selection, the expected proportion of the 10 transferrin phenotypes in the resistant and susceptible lambs was calculated from the Hardy-Weinberg formula. Table 15 and Figure 23 indicate that the observed numbers for transferrin phenotypes of the susceptible lambs were not significantly different from the numbers expected on the basis of Hardy-Weinberg equilibrium (X^2 = 6.33, 9 df, 0.80 > P > 0.70). However, the observed numbers for transferrin phenotypes of the resistant lambs showed a departure from the expected (X^2 = 16.56, 9 df, 0.10 > P > 0.05) (Table 15 and Figure 22). This deviation from the expected was significant between the 10% and 5% level of probability. The

departure was due to an excess of phenotypes AA, AD and BB, and a deficiency of phenotype DD. The variation in the distribution of phenotypes again indicated a specific association of transferrin phenotype with facial eczema resistance, but the current sample sizes appeared to be too small to give a more significant result.

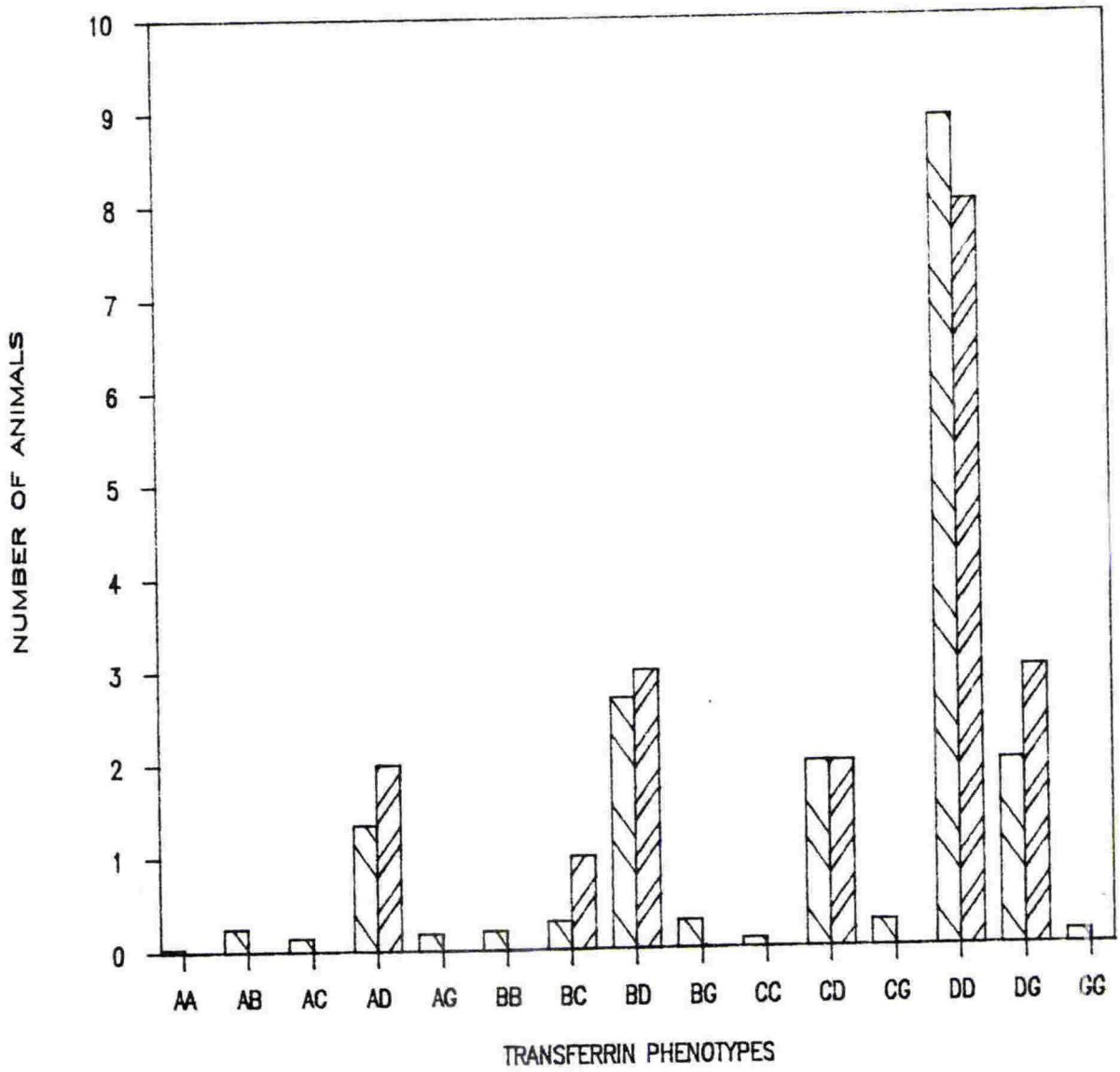


FIGURE 20. Comparison of the Expected Numbers of Transferrin Phenotypes with the Observed Numbers in the Susceptible Flock

The expected numbers of the 15 transferrin phenotypes: AA, AB, AC, AD, AG, BB, BC, BD, BG, CC, CD, CG, DD, DG and GG, were compared with the observed numbers of the transferrin phenotypes in the susceptible flock.

▧) expected numbers in the flock as a single population. ▨) observed numbers in the flock. The deviations between the observed numbers and the expected numbers were insignificant (X^2 = 3.90, 14 df, P > 0.995).

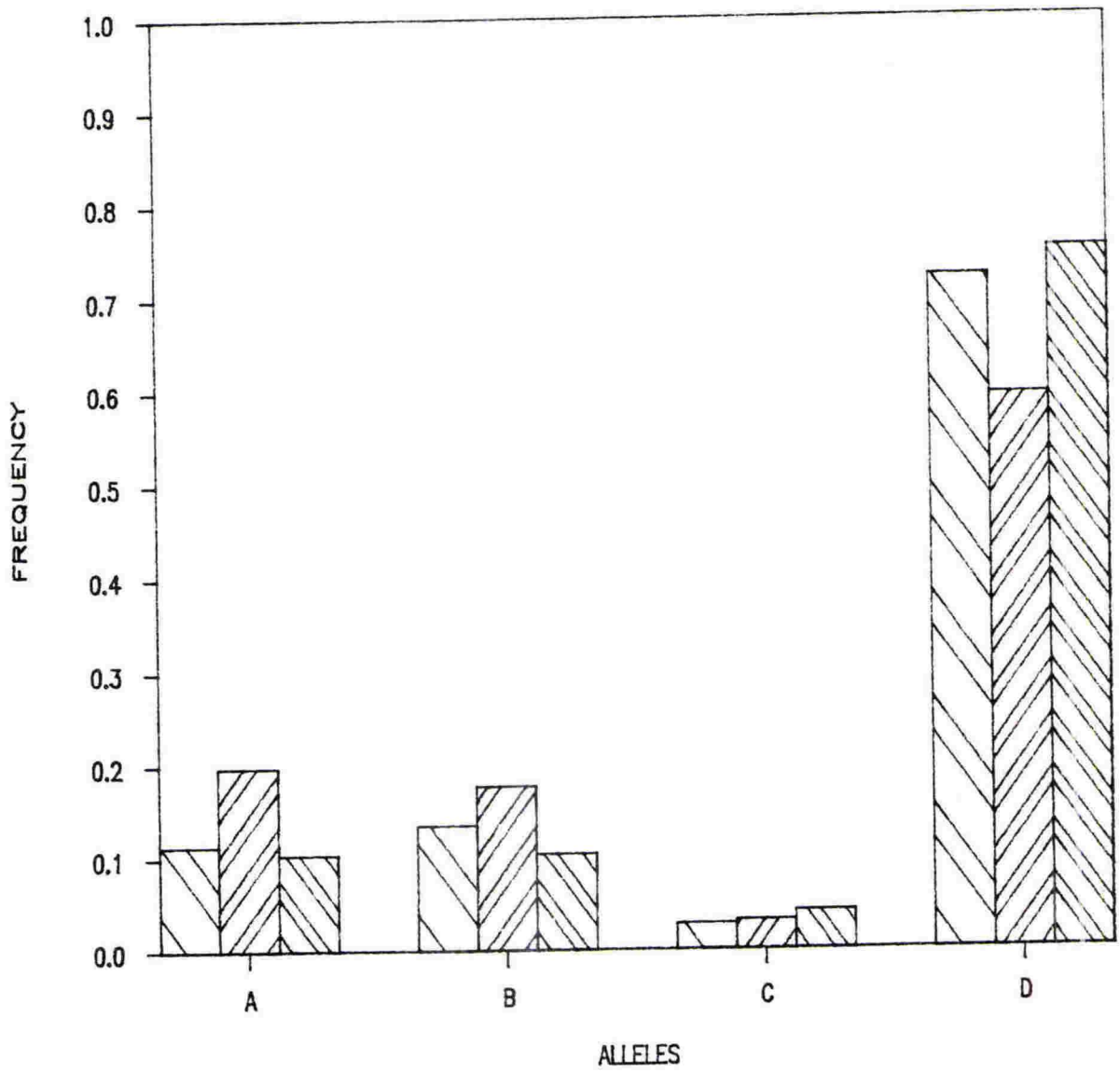


FIGURE 21. Comparison of the Allele Distributions between Resistant and Susceptible Lambs

The frequency of the alleles A, B, C and D in the resistant and susceptible lambs were compared with the expected frequency of each of the alleles in the population.

▨) expected frequency in the population,
▨) resistant lambs,
▨) susceptible lambs.

TABLE 15. Test of Genetic Equilibrium for Transferrin Phenotypes in Resistant and Susceptible Lambs

Transferrin Phenotypes	Resistant		Susceptible	
	Obs.	Exp.	Obs.	Exp.
AA	2	0.61	1	1.42
AB	2	1.46	3	3.39
AC	0	0.30	1	0.70
AD	13	7.84	17	18.14
BB	3	0.87	0	2.02
BC	0	0.36	0	0.84
BD	9	9.37	20	21.67
CC	0	0.04	0	0.09
CD	3	1.94	8	4.49
DD	16	25.09	61	58.02
Total	48	47.88	111	110.78
df	9		9	
X^2	16.56		6.33	

Obs. - observed number of transferrin phenotypes
Exp. - expected number of transferrin phenotypes calculated from the allele frequency and the number of animals in each group.

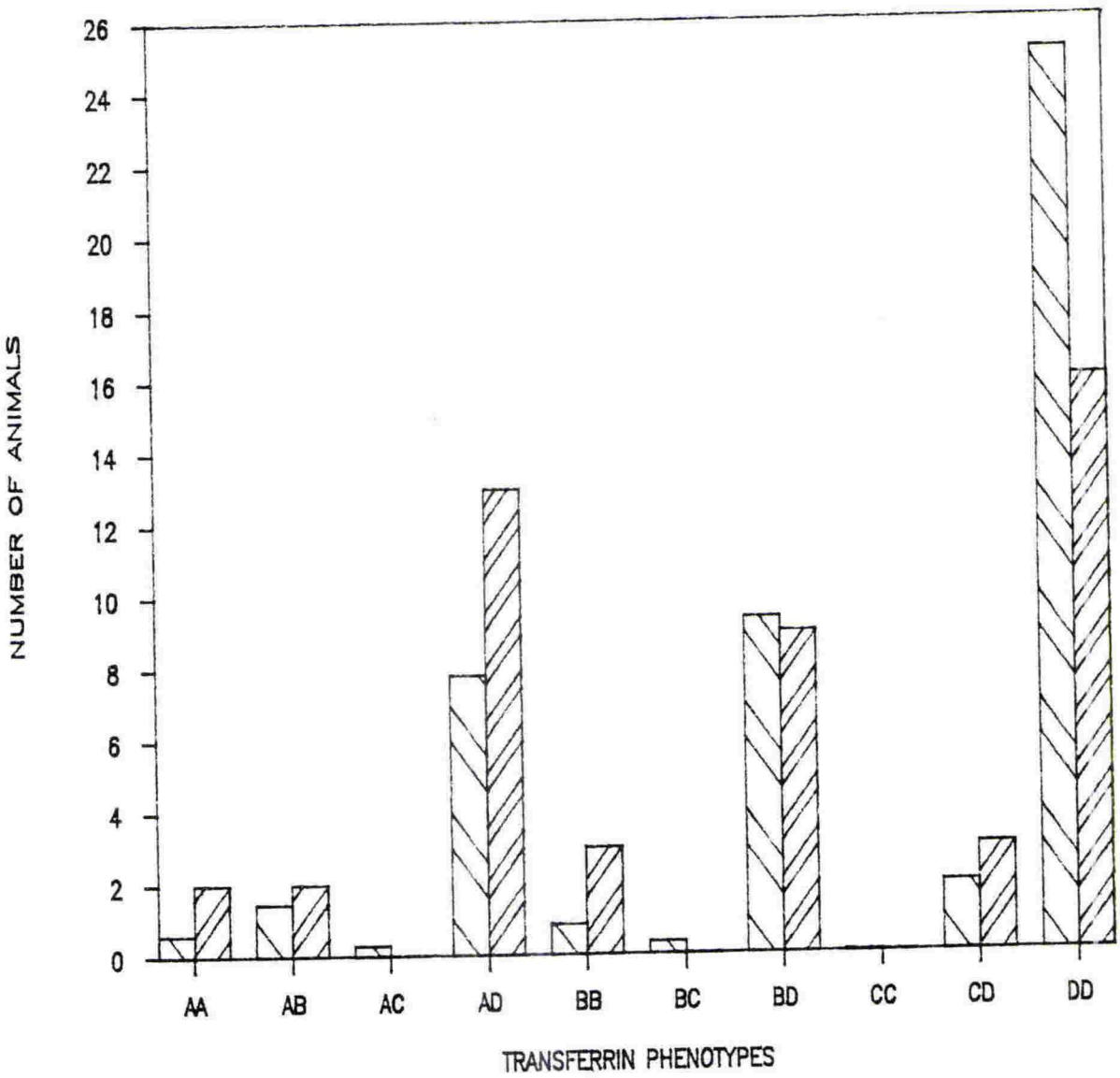


FIGURE 22. Comparison of the Expected Numbers of Transferrin Phenotypes with the Observed Numbers in the Resistant Lambs

The expected numbers of the 10 possible transferrin phenotypes from a 4 allele system (A, B, C and D) were compared with the observed numbers of the transferrin phenotypes in the resistant lambs. ▧) expected numbers, ▨) observed numbers. The deviations between the observed numbers and the expected numbers were significant between the 10% and 5% level of probability (X^2 = 16.56, 9 df, 0.10 > P > 0.05).

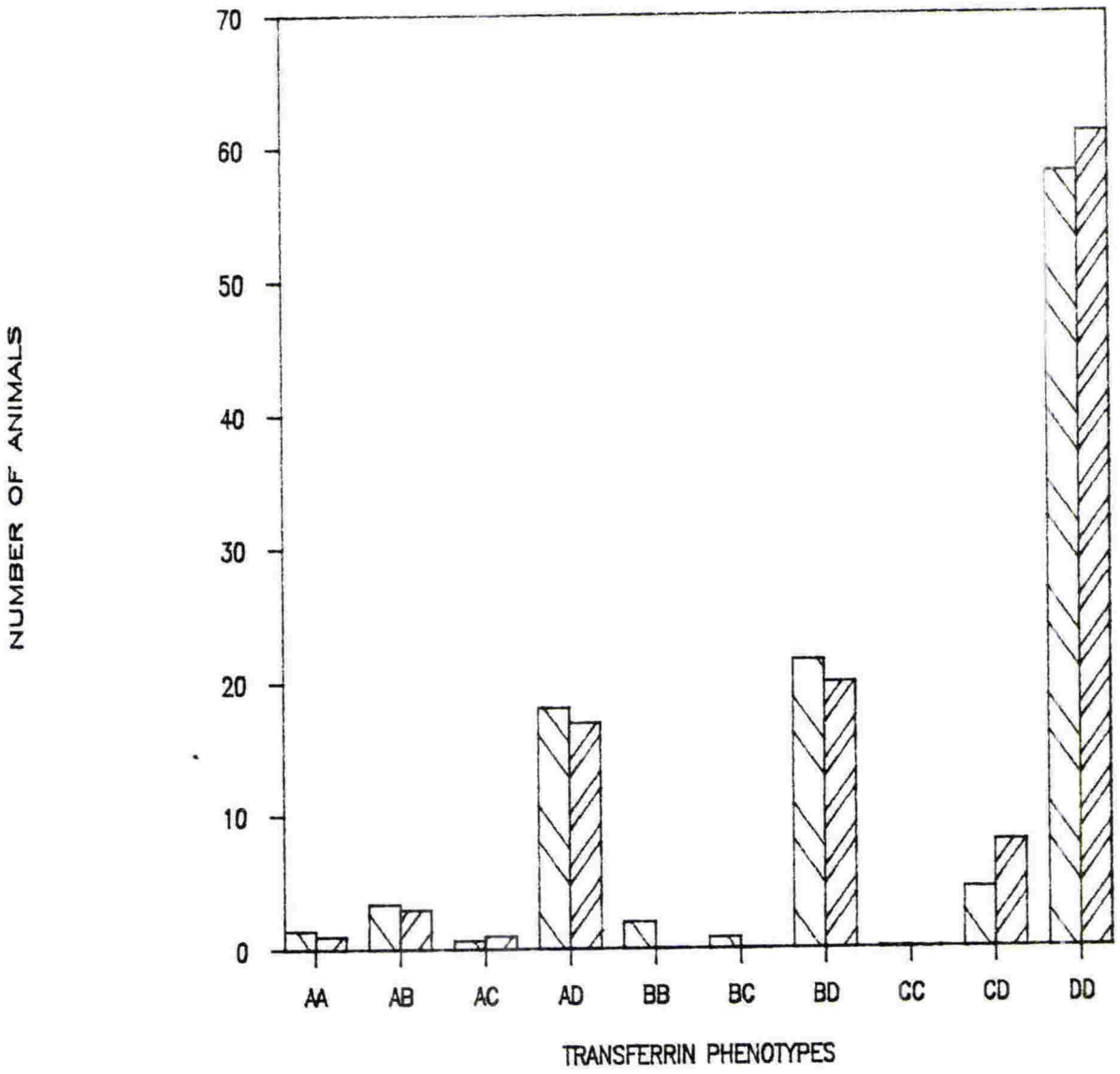


FIGURE 23. Comparison of the Expected Numbers of Transferrin Phenotypes with the Observed Numbers in the Susceptible Lambs

The expected numbers of the 10 transferrin phenotypes: AA, AB, AC, AD, BB, BC, BD, CC, CD and DD, were compared with the observed numbers of the transferrin phenotypes in the susceptible lambs. ▨) expected numbers, ▨) observed numbers. The deviations between the observed numbers and the expected numbers were insignificant (X^2 = 6.33, 9 df, 0.80 > P > 0.70).

3-6 Analysis of Liver Proteins

3-6.1 SDS-PAGE of Sheep Liver Homogenates, Hepatocytes and Subcellular Fractions

SDS-PAGE of sheep liver proteins was carried out to establish conditions for the analysis of liver proteins associated with resistance to facial eczema. The polypeptides of sheep liver homogenates, hepatocytes and subcellular fractions were compared after electrophoresis on a 5-20% gradient gel (Plate 17). The majority of the polypeptides present in liver homogenates had M_r values between 10,000 and 100,000. More than 90% of the hepatocyte polypeptides were of M_r less than 70,000. The polypeptides of the subcellular fractions of sheep liver (nuclei and cell debris, mitochondrial-lysosomal, microsomal and soluble fractions), resolved into 50-60 protein bands with M_r values ranging from 10,000 to 100,000. The resolution was incomplete and many bands, especially microsomal proteins in the M_r range of 45,000 to 60,000, were superimposed. When the subcellular fractions were treated with DNase and octyl glucoside, there was an apparent change in electrophoretic mobility of a number of high M_r proteins (Plate 17, Lanes 7, 9, 11 and 13). This change may reflect the abilities of octyl glucoside to solubilise membrane proteins and of DNase to degrade DNA tightly associated with some proteins.

3-6.2 SDS-PAGE of Liver Homogenate Proteins from Facial Eczema Resistant and Susceptible Sheep

Liver homogenate proteins from individual resistant and susceptible sheep were compared by SDS-PAGE analysis (Plate 18). The liver homogenate proteins, which had been treated with DNase and octyl glucoside to digest DNA and to solubilise membrane proteins, were separated on a 12.5% (w/v) acrylamide gel. The proteins were resolved into 50-60 bands with M_r values ranging

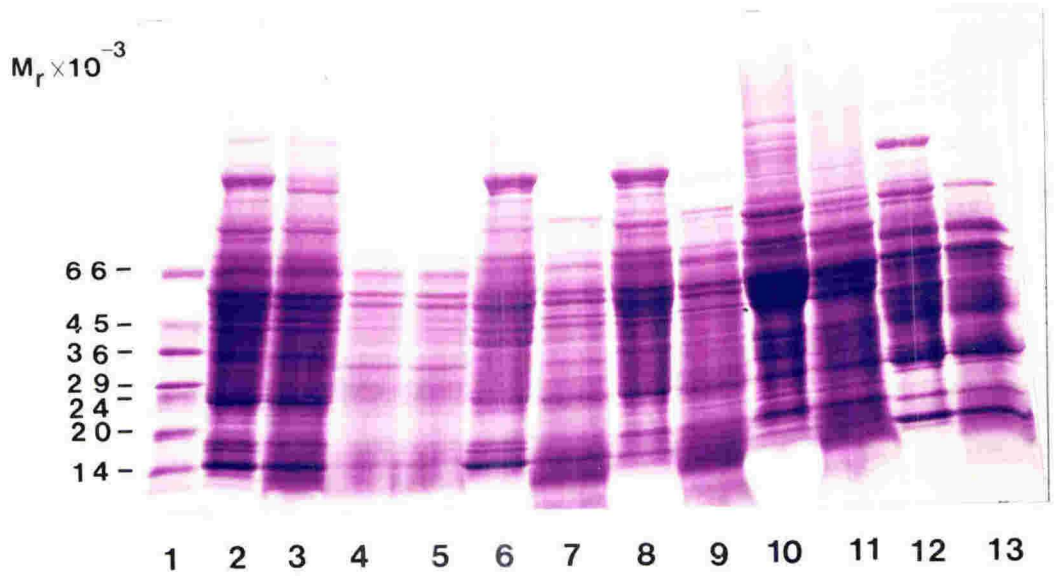


PLATE 17. SDS-PAGE of Liver Homogenates, Hepatocytes and Subcellular Fractions

A protein load of 100 μg from each sample was applied to a 5-20% gradient gel.
Lane 1, M_r standards;
Lane 2, homogenates;
Lane 3, homogenates treated with DNase and octyl glucoside;
Lane 4, hepatocytes;
Lane 5, hepatocytes treated with DNase and octyl glucoside;
Lane 6, nuclei and cell debris fraction;
Lane 7, nuclei and cell debris fraction treated with DNase and octyl glucoside;
Lane 8, mitochondrial-lysosomal fraction;
Lane 9, mitochondrial-lysosomal fraction treated with DNase and octyl glucoside;
Lane 10, microsomal fraction;
Lane 11, microsomal fraction treated with DNase and octyl glucoside;
Lane 12, soluble fraction;
Lane 13, soluble fraction treated with DNase and octyl glucoside.

The polypeptides were detected by Coomassie blue stain.

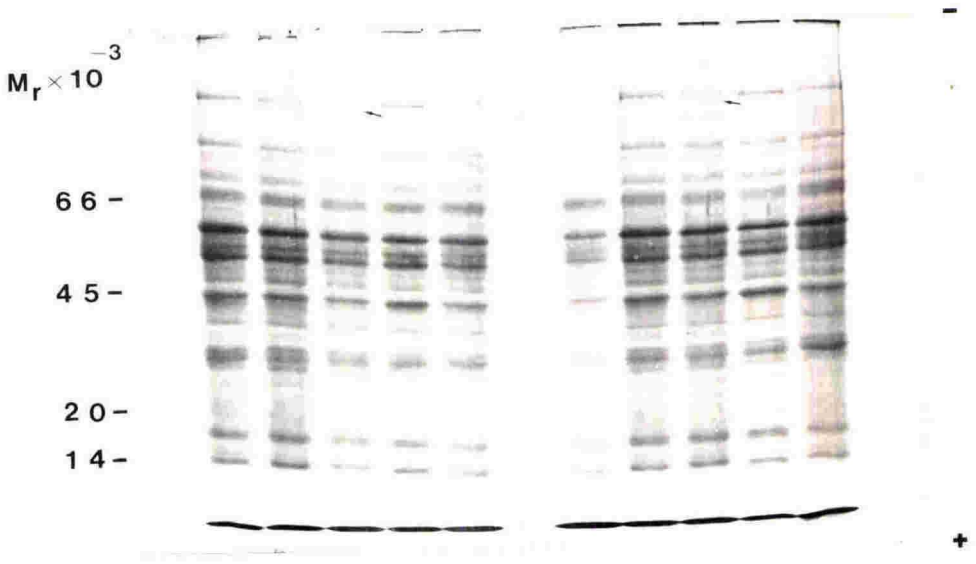


PLATE 18. SDS-PAGE of Liver Homogenate Proteins from Facial Eczema Resistant and Susceptible Sheep

Liver homogenate proteins from 5 individual resistant rams and 5 individual susceptible rams were electrophoresed on a 12.5% (w/v) acrylamide gel. The proteins were detected with Coomassie blue stain. Regions of variation are indicated by arrows.

from 10,000 to 100,000. There were some individual differences in proteins of M_r around 90,000 in both the resistant and susceptible sheep (Plate 18, indicated by arrows). However, these differences did not appear to have an association with either facial eczema resistance or susceptibility.

3-6.3 2-D PAGE of Sheep Liver Homogenates, Hepatocytes and Subcellular Fractions

2-D PAGE of sheep liver proteins has not been reported. Therefore, the polypeptides of sheep liver homogenates, isolated hepatocytes and subcellular fractions were analysed to develop the experimental conditions and to gain some familiarity with the 2-D PAGE map of sheep liver proteins. Plate 19 illustrates the 2-D PAGE separation of Coomassie blue and silver stained polypeptides from sheep liver homogenates. Approximately 240-300 polypeptides, occupying the pH range 4-8 and M_r range 10,000-100,000, were readily visible on the 2-D PAGE gels. This sensitivity is five to six times more than that obtained by one-dimensional SDS-PAGE. Silver staining allowed detection of proteins not visible on Coomassie blue stained gels (Plate 19B).

The 2-D PAGE separation of liver homogenates and hepatocytes are presented on Plate 20. It appeared that all the polypeptides of liver homogenates and hepatocytes resolved as bands in SDS-PAGE were resolved as spots in 2-D PAGE; e.g., comparison of the bands separated by SDS-PAGE (Plate 17) with corresponding apparent M_r positions of 2-D PAGE spots (Plate 20). The electrophoretic patterns of total cellular polypeptides (including both cytosolic and membrane proteins) from liver homogenates and from hepatocytes were similar although numerous qualitative and quantitative differences were noted and higher M_r polypeptides (>66,000) were more apparent in liver homogenates

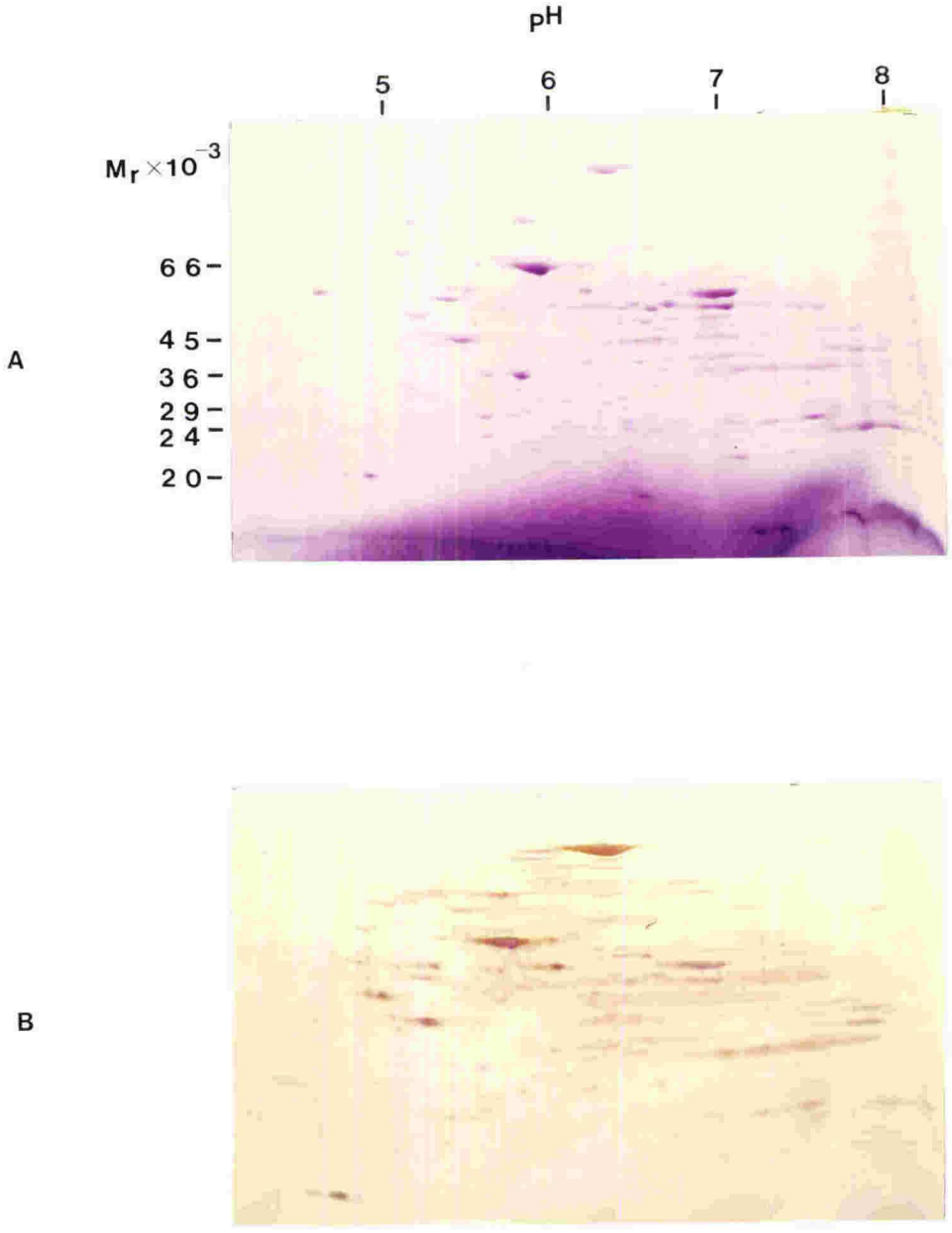


PLATE 19. 2-D PAGE of Sheep Liver Homogenates

2-D PAGE separation of liver homogenate proteins on a 5-20% gradient gel. A) Coomassie blue stained gel (250 μg protein), B) Silver stained gel (150 μg protein).

than in isolated hepatocytes (Plate 20, Figure 24 and Table 16). The qualitative differences are summarised in Figure 24 and Table 16, in which the polypeptides which were not detected on the protein pattern of the homogenates but were detected on those of the hepatocytes and subcellular fractions are labelled by a letter positioned at their expected pI and M_r coordinates. The quantitative differences are indicated with arrows on Plate 20A and B, and they are: polypeptide 52 (5.60 / 58) (designated pI / $M_r x10^{-3}$); polypeptide 68 (6.85 / 52); polypeptide 94 (6.75 / 45); polypeptide 97 (5.25 / 43); polypeptide 138 (6.10 / 32); polypeptide 140 (7.05 / 32); polypeptide 141 (7.30 / 32); polypeptide 142 (7.55 / 32) and polypeptide 143 (7.80 / 32). These polypeptides were detected as more intensely stained spots in the homogenates than in hepatocytes. Polypeptide 53 (6.30 / 58); polypeptide 55 (6.80 / 58) and polypeptide 148 (5.20 / 31) appeared as darker spots in the hepatocyte protein pattern. In addition, there may be other differences which were not detected by this 2-D PAGE system such as polypeptides with pI values greater than 8. A further limitation was imposed by the area of intense staining of Pharmalytes towards the bottom right of the gel which prohibited detection of small polypeptides with M_r less than 20,000.

Protein patterns obtained from the nuclei and cell debris fraction, mitochondrial-lysosomal fraction, microsomal fraction and soluble fraction of sheep liver are shown in Plate 21. The protein patterns of the subcellular fractions each contained about 100 to 150 polypeptides which could be detected by Coomassie blue staining. All polypeptides entered the separating slab gel since no stainable material was found in the original IEF gel and the stacking gel after electrophoresis in the second-dimension. However, not all of the polypeptides were visible as

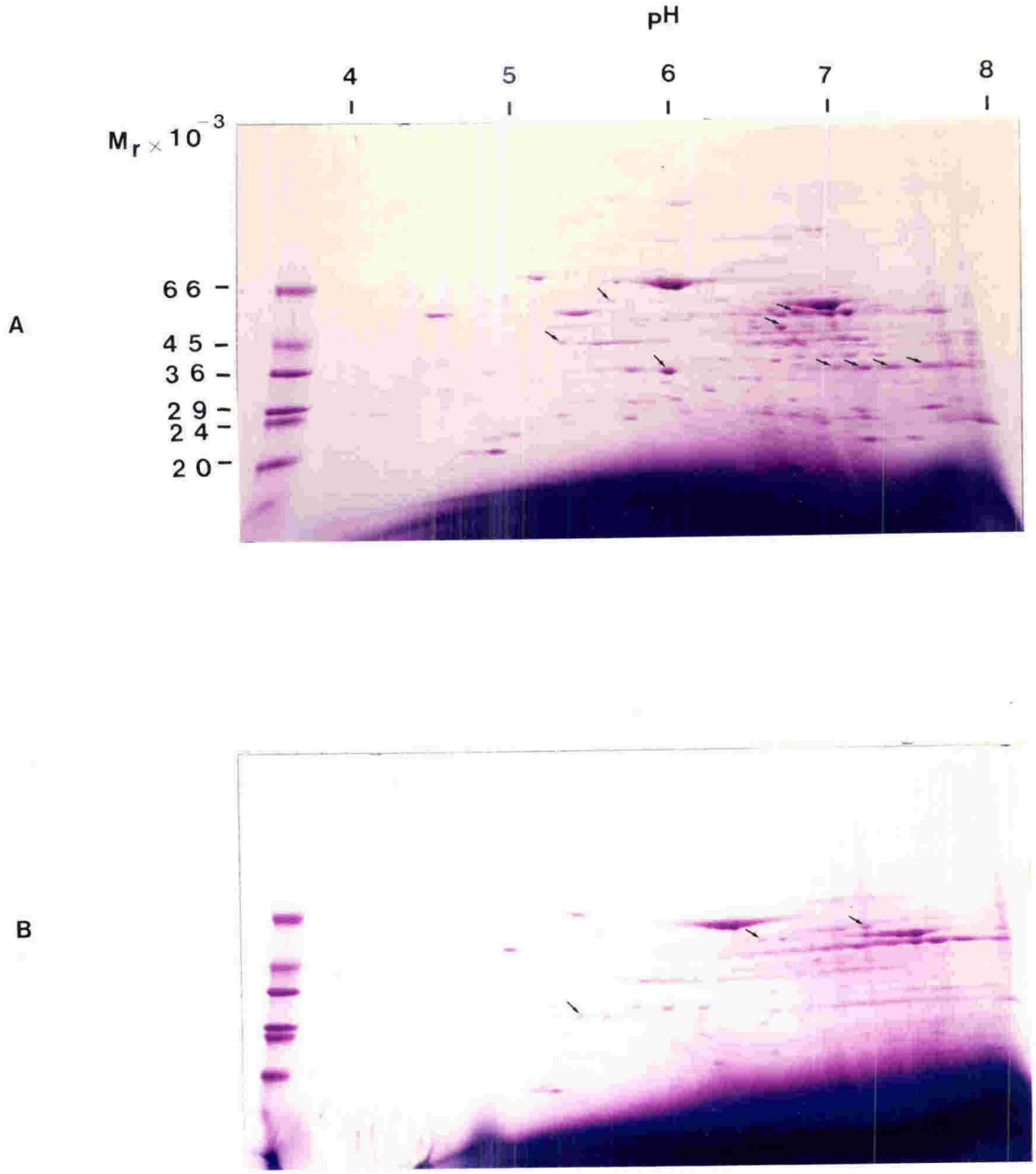


PLATE 20. 2-D Gels of Liver Homogenates and Hepatocytes from Sheep

2-D PAGE separation of liver homogenates and hepatocytes on a 5-20% gradient gel. The protein load was 1200 μg. A) liver homogenates, B) hepatocytes. The positions of quantitative differences are indicated with arrows on each plate (A, B). The qualitative differences are summarised in Table 16.

FIGURE 24. 2-D Gel Map of Sheep Liver Proteins

This map was drawn from the gel shown in Plate 20A The proteins are numbered for reference since form identifications are not available. The proteins which were not detected on the protein pattern of the homogenates but were detected on those of the hepatocytes and subcellular fractions are labelled by a letter at their respective position.

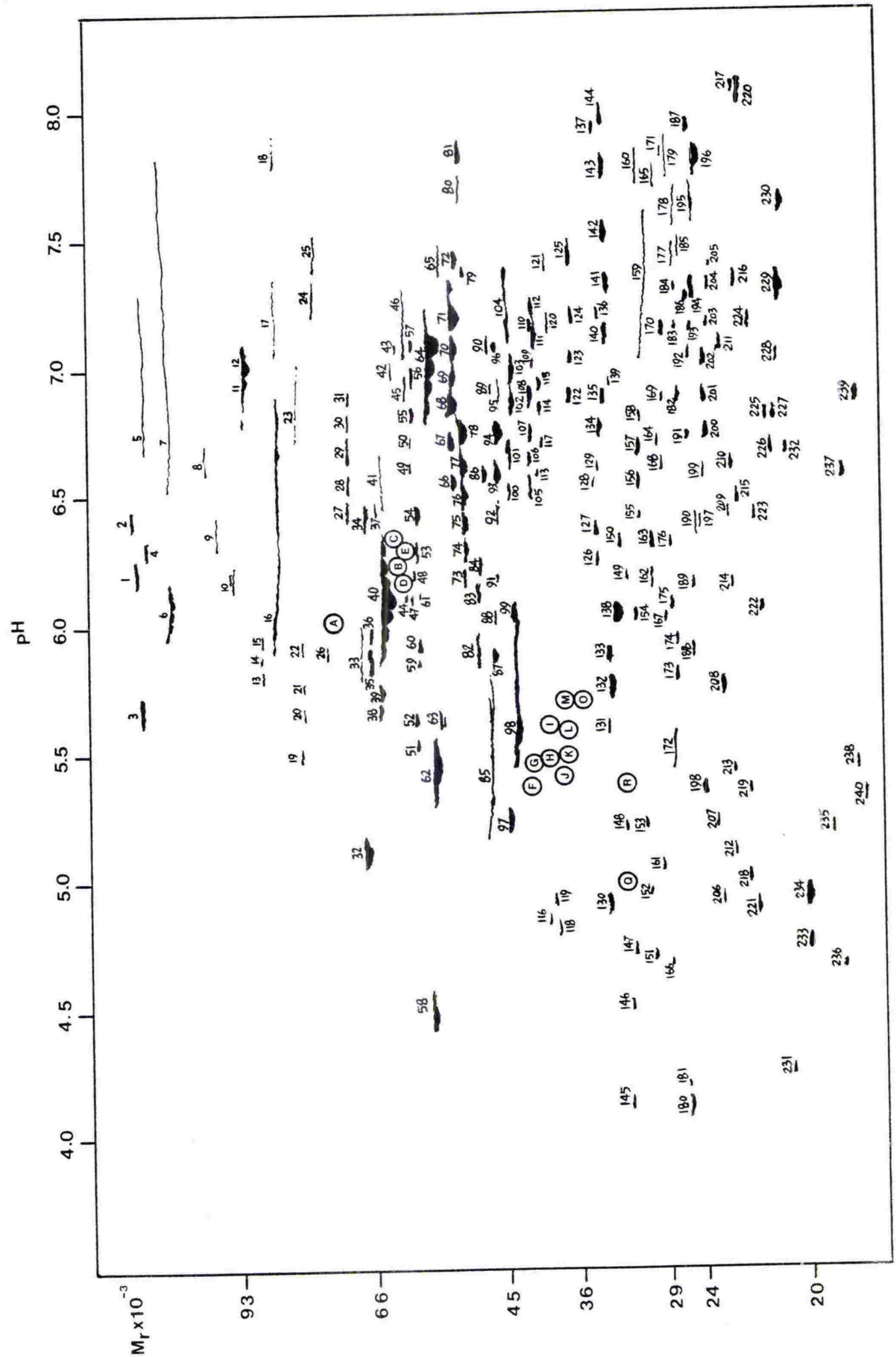

pH
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
$M_r \times 10^{-3}$
93
66
45
36
29
24
20

TABLE 16. Summary of Qualitative Polypeptide Differences between Liver Homogenates, Hepatocytes and Subcellular Fractions

Polypeptide	pI	$M_r x10^{-3}$	Homog.	Hepat.	N	M-L	P	S
1	6.20	112	+					
2	6.45	112	+					
3	5.70	111	+		+	+	+	
4	6.30	110	+					
5	6.70-7.30	110	+	+				+
6	5.90-6.20	106	+	+	+	+	+	+
7	6.50-7.90	106	+	+	+	+		+
8	6.65	100	+					
9	6.45	95	+					
10	6.20	93	+					
11	6.90	90	+				+	+
12	7.00	90	+				+	+
13	5.80	87	+					
14	5.90	87	+					+
15	5.95	87	+					+
16	5.90-6.90	84	+		+	+	+	+
17	7.00-7.40	84	+				+	+
18	7.90	84	+					
19	5.50	80	+					
20	5.65	80	+					+
21	5.80	80	+					+
22	5.95	80	+		+		+	+
23	6.70-7.00	80	+		+	+	+	
24	7.25	76	+					
25	7.45	76	+					
26	5.90	74	+		+	+	+	+
A	6.05	72			+	+		
27	6.45	70	+	+	+	+	+	+
28	6.55	70	+	+	+	+	+	+
29	6.65	70	+	+	+	+	+	+
30	6.80	70	+	+	+	+	+	+
31	6.90	70	+	+	+	+	+	+
32	5.10	68	+		+	+	+	
33	5.90	68	+	+	+	+	+	
34	6.55	68	+	+	+	+		+
35	5.85	66	+		+	+		
36	5.95	66	+		+	+		
37	6.45	66	+		+	+	+	+
38	5.65	65	+			+	+	
39	5.75	65	+			+	+	+
40	5.85-6.30	65	+	+	+		+	+
41	6.45-6.70	65	+	+	+	+	+	+
B	6.25	62			+	+		+
C	6.35	62			+	+		
42	7.00	62	+		+			+
43	7.10	61	+		+			+
44	6.10	60	+			+		+
D	6.20	60			+	+		+
E	6.30	60			+	+		+
45	6.90	60	+	+	+	+		+
46	7.05-7.30	60	+	+	+	+		+
47	6.10	59	+			+	+	
48	6.20	59	+	+	+	+	+	+

49	6.60	59	+	+	+			+
50	6.70	59	+	+	+	+		+
51	5.50	58	+	+	+	+	+	
52	5.60	58	+	+	+	+	+	
53	6.30	58	+	+	+	+	+	
54	6.45	58	+	+	+	+	+	
55	6.80	58	+	+	+	+		+
56	6.95	58	+	+	+	+		+
57	7.10	58	+		+	+		+
58	4.45	57	+	+	+	+	+	
59	5.85	57	+			+		+
60	5.95	57	+		+	+		+
61	6.10	57	+			+		+
62	5.40	56	+	+	+	+	+	+
63	5.65	56	+					+
64	6.80-7.20	56	+	+	+	+	+	+
65	7.40	54	+		+	+		+
66	6.55	52	+	+	+			+
67	6.70	52	+	+	+		+	+
68	6.85	52	+	+	+	+	+	+
69	6.95	52	+	+	+	+		+
70	7.05	52	+	+	+	+	+	+
71	7.20	52	+	+	+	+	+	+
72	7.40	52	+	+	+		+	
73	6.15	51	+	+	+	+	+	+
74	6.25	51	+	+	+	+	+	+
75	6.35	51	+	+	+	+	+	+
76	6.45	51	+	+	+	+	+	+
77	6.60	51	+	+	+	+	+	+
78	6.70	51	+	+	+	+	+	+
79	7.35	51	+					
80	7.70	51	+	+			+	
81	7.85	51	+	+			+	
82	5.90	49	+	+	+	+	+	+
83	6.10	49	+	+	+	+	+	+
84	6.25	48	+	+	+	+	+	+
85	5.10-5.80	47	+			+	+	
86	6.55	47	+					
87	5.90	46	+	+	+	+	+	
88	6.05	46	+	+				+
89	6.90	46	+				+	+
90	7.10	46	+				+	+
91	6.25	45	+			+		+
92	6.40	45	+	+			+	+
93	6.60	45	+	+		+	+	+
94	6.75	45	+	+		+	+	+
95	6.90	45	+				+	+
96	7.10	45	+				+	+
97	5.25	43	+	+		+		+
98	5.45-5.95	43	+	+	+	+		+
99	6.00	43	+	+	+	+		
100	6.50	43	+	+	+	+	+	+
101	6.65	43	+	+	+	+	+	+
102	6.85	43	+	+	+	+	+	+
103	6.95	43	+		+	+	+	+
104	7.10-7.35	43	+		+	+	+	+
F	5.40	41				+	+	
G	5.50	41				+	+	
105	6.55	41	+	+	+	+		+
106	6.65	41	+	+	+	+		

107	6.75	41	+	+	+	+		+
108	6.90	41	+	+	+	+		+
109	7.00	41	+					
110	7.15	41	+	+		+		+
111	7.10	40.5	+	+		+		+
112	7.20	40.5	+	+		+		+
113	6.60	40	+	+	+	+		+
114	6.85	40	+	+	+	+		+
115	6.95	40	+	+	+	+		+
116	4.80	39	+					
117	6.70	39	+	+	+	+		
118	4.80	38	+					
119	4.95	38	+					
H	5.50	38					+	+
I	5.60	38				+	+	+
120	7.15	38	+	+	+			
121	7.40	38	+	+	+			
J	5.45	37					+	
K	5.50	37					+	+
L	5.60	37					+	
M	5.75	37				+	+	
O	5.75	36				+	+	
122	6.90	36	+	+	+	+		+
123	7.05	36	+	+	+	+		+
124	7.15	36	+	+	+	+		+
125	7.45	36	+	+		+		+
126	6.25	34	+	+	+	+	+	+
127	6.40	34	+	+	+	+	+	+
128	6.55	34	+	+		+	+	+
129	6.65	34	+	+		+	+	+
130	4.95	33	+	+		+	+	
131	5.60	33	+	+	+	+		
132	5.75	33	+	+		+		+
133	5.90	33	+	+	+	+	+	+
134	6.75	33	+	+	+	+	+	+
135	6.90	33	+	+	+	+	+	+
136	7.15	33	+	+		+	+	
137	7.95	33	+					
138	6.10	32	+	+	+	+	+	+
139	6.95	32	+	+		+	+	
140	7.05	32	+	+	+	+	+	+
141	7.30	32	+	+		+	+	+
142	7.55	32	+	+		+	+	+
143	7.80	32	+	+				
144	8.00	32	+					
145	4.10	31	+					
146	4.50	31	+	+			+	
147	4.70	31	+					
Q	5.00	31		+				
148	5.20	31	+	+	+	+	+	
R	5.40	31	+	+	+			
149	6.20	31	+	+		+		+
150	6.85	31	+	+				+
151	4.70	30	+					
152	4.95	30	+	+				
153	5.20	30	+	+				
154	6.05	30	+	+		+	+	+
155	6.45	30	+	+		+		
156	6.55	30	+	+		+		+
157	6.65	30	+	+		+		+

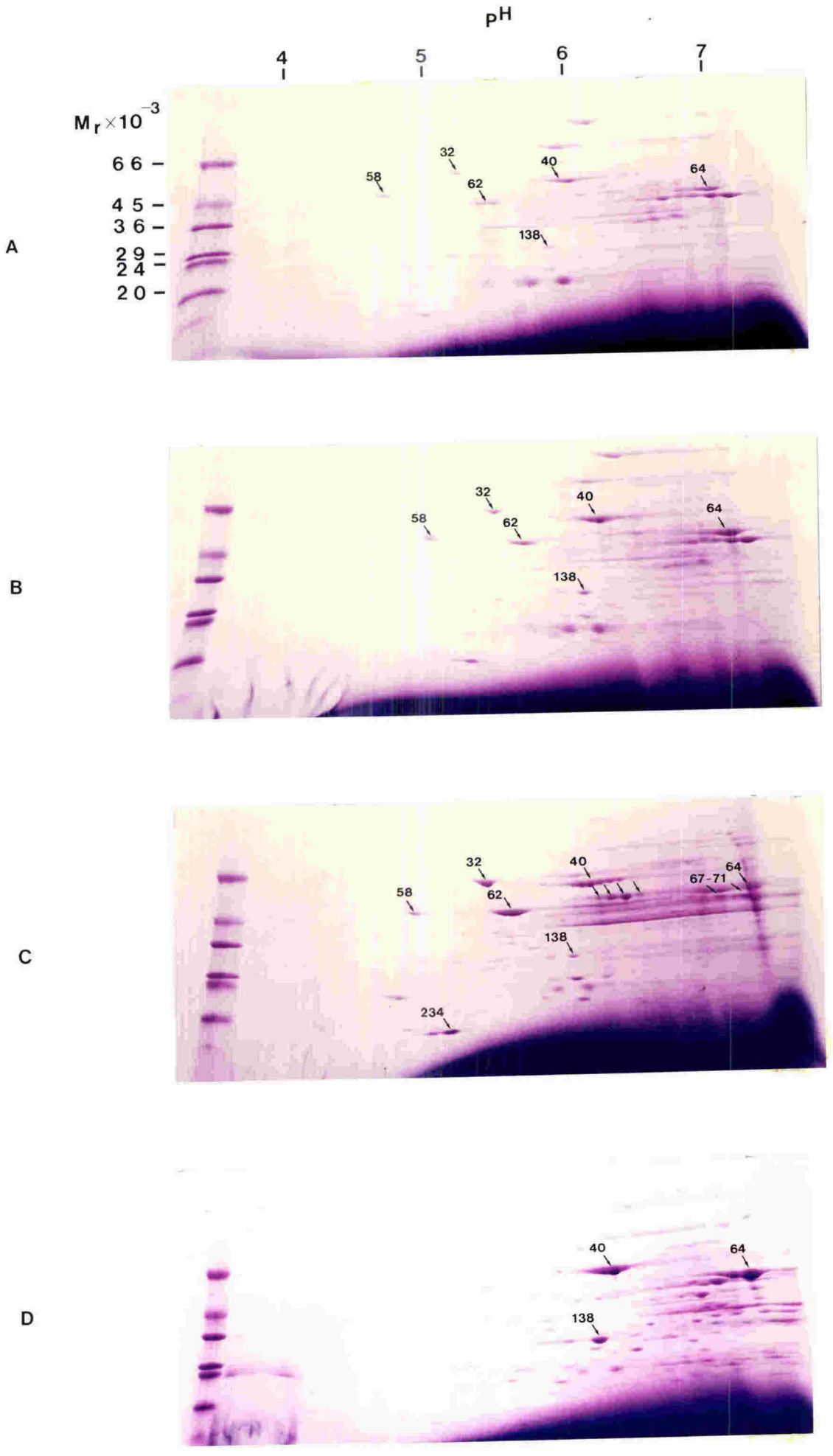
158	6.80	30	+			+		+
159	7.10-7.60	30	+			+		+
160	7.80	30	+					
161	5.10	29	+					
162	6.20	29	+	+				+
163	6.35	29	+	+	+	+		+
164	6.70	29	+			+		+
165	7.75	29	+					
166	4.65	28	+					
167	6.05	28	+	+	+	+	+	
168	6.60	28	+	+	+	+		+
169	6.90	28	+		+			+
170	7.20	28	+			+		+
171	7.85	28	+					
172	5.50	27	+				+	+
173	5.80	27	+	+			+	+
174	5.95	27	+					
175	6.10	27	+	+	+	+	+	
176	6.30	27	+	+		+	+	
177	7.40	27	+					
178	7.60	27	+					
179	7.80	27	+					
180	4.10	26	+					
181	4.20	26	+					
182	6.90	26	+			+	+	+
183	7.15	26	+	+		+		+
184	7.30	26	+	+				+
185	7.40	26	+					
186	7.25	25.5	+	+		+	+	+
187	7.95	25.5	+					
188	5.90	25	+	+		+	+	+
189	6.10	25	+	+	+	+	+	+
190	6.35	25	+					
191	6.75	25	+	+		+	+	+
192	7.05	25	+	+		+	+	+
193	7.20	25	+	+		+	+	
194	7.30	25	+	+		+	+	+
195	7.60	25	+					
196	7.85	25	+					
197	6.35	24	+					
198	5.35	23.5	+		+	+	+	+
199	6.55	23.5	+				+	+
200	6.75	23.5	+	+	+	+	+	+
201	6.90	23.5	+	+	+	+	+	+
202	7.00	23.5	+	+		+	+	+
203	7.20	23.5	+	+		+	+	
204	7.30	23.5	+	+		+	+	+
205	7.40	23.5	+					
206	4.95	23	+					
207	5.20	23	+					
208	5.75	23	+		+	+	+	+
209	6.45	23	+				+	+
210	6.60	23	+				+	+
211	7.10	23	+	+		+		+
212	5.10	22.5	+					
213	5.40	22.5	+			+		+
214	6.15	22.5	+	+	+	+	+	+
215	6.50	22.5	+	+			+	+
216	7.35	22.5	+	+				+
217	8.10	22.5	+					

218	5.00	22	+					
219	5.30	22	+					
220	8.10	22	+					
221	4.90	21.7	+					
222	6.10	21.5	+			+	+	+
223	6.45	21.5	+					
224	7.20	21.5	+			+		+
225	6.85	21.3	+			+		+
226	6.70	21	+			+		+
227	6.85	21	+			+		+
228	7.05	21	+				+	+
229	7.30	21	+	+	+	+		
230	7.65	21	+	+	+	+		
231	4.20	20.7	+					
232	6.65	20.5	+			+		+
233	4.75	20	+			+	+	
234	4.90	20	+		+	+	+	+
235	5.15	19	+					
236	4.60	18.5	+	+		+		
237	6.60	18	+					
238	5.40	17.7	+					+
239	6.90	17.7	+					
240	5.20	17.5	+					

These polypeptides correspond to those shown in Figure 24. The (+) denotes the presence of the polypeptide detected on the protein pattern. Homog., Hepat., N, M-L, P and S denotes homogenates, hepatocytes, nuclei and cell debris, mitochondrial-lysosomal, microsomal and soluble fractions respectively.

PLATE 21. 2-D PAGE of Proteins from Subcellular Fractions of Sheep Liver

2-D PAGE separation of proteins from liver subcellular fractions of sheep on a 5-20% gradient gel. A protein load of 1200 μg was applied. A) nucl and cell debris fraction, B) mitochondrial-lysosoma fraction, C) microsomal fraction, D) soluble fraction. The proteins were detected with Coomassie blue stain. Six major proteins of the liver homogenates (polypeptide numbers 32, 40, 58, 62, 64 and 138) were found in the nuclei and cell debris fraction (A), mitochondrial-lysosomal fraction (B) and microsomal fraction (C). Polypeptide numbers 40 64 and 138 were found in greatest abundance in the soluble fraction (D). In the microsomal fraction (C) polypeptides 32 and 234 were probably NADPH cytochrome P-450 reductase and cytochrome b_5 respectively. Polypeptides 62 and 67-71 could represent the cytochrome P-450s. A series of polypeptides (indicated by arrows in C) appeared to be specific for the microsomal fraction.


pH
4
5
6
7
A
$M_r \times 10^{-3}$
66
45
36
29
24
20
58
32
62
40
64
138
B
58
32
62
40
64
138
C
58
32
62
40
67-71
64
138
234
D
40
64
138

discrete spots. Among the high M_r and more basic polypeptides, horizontal streaking masked nearby spots and thus interfered with the identification of many proteins. Streaking occurred primarily during the IEF-dimension. This problem is generally attributed to either the presence of nucleic acids in the sample, which apparently interfered with proper focusing in that area, or to poor solubility of those proteins in that area. However, the streaking problem was not entirely overcome by the treatment of the liver samples with DNase and octyl glucoside.

When the protein patterns of the liver homogenates and the subcellular fractions were compared, about 44% of the approximately 240 protein spots present in the liver homogenates were found in the protein pattern of nuclei and cell debris fraction, 63% in the mitochondrial-lysosomal fraction, 48% in the microsomal fraction and 62% in the soluble fraction. A large proportion of the homogenate polypeptides were present in all subcellular fractions (54% subcellular fraction - non-specific protein spots) and their common presence can be explained in two ways: (a) the subcellular fractions contained only partially purified organelles so that substantial cross-contamination occurred; (b) certain proteins were present in all subcellular fractions. Table 7 gives estimates of cross-contamination of the subcellular fractions. It can be seen that good cell breakage was obtained since only 3% of the glutathione S-transferase activity was found in the nuclei and cell debris fraction while 95% of the activity of this enzyme was found in the soluble fraction. However, the subcellular fractions appeared to contain only partially purified organelles since only 49% of the microsomal glucose-6-phosphatase and 58% of the mitochondrial succinate cytochrome c reductase were found in the appropriate subcellular fractions. Therefore, cross-contamination of the subcellular

fractions may have accounted for the high percentage of subcellular fraction - non-specific proteins detected in all fractions. Six major proteins of the liver homogenates, namely polypeptide numbers 32, 40, 58, 62, 64 and 138, were found in the nuclei and cell debris fraction (Plate 21A), mitochondrial-lysosomal fraction (Plate 21B) and microsomal fraction (Plate 21C). Of these polypeptides, numbers 40, 64 and 138 were found in greatest abundance in the soluble fraction (Plate 21D). These common polypeptides may represent proteins with a dual soluble-membrane bound organelle distribution in the cell. This is a good illustration of the power of this two-dimensional separation in uncovering differences among proteins which would not be detectable by a one-dimensional separation.

The liver proteins can only be tentatively identified with reference to the known migration of some rat liver proteins on 2-D PAGE. Most information is available for microsomal proteins (Vlasuk and Walz, 1980). The polypeptides which were separated by 2-D PAGE of sheep liver microsomes are shown in Plate 21C. On the basis of their apparent pI, M_r and relative abundance, polypeptide 32 (5.10 / 68) and polypeptide 234 (4.90 / 20) were probably NADPH cytochrome P-450 reductase (Vermillion and Coon, 1978) and cytochrome b_5 (Spatz and Strittmatter, 1971), respectively. In addition, polypeptide 32 focused around pH 5 which is generally consistent with the IEF properties of purified NADPH cytochrome P-450 reductase (Guengerich, 1977). Polypeptide 62 and a series of polypeptides 67-71 could represent the cytochrome P-450s (Vlasuk and Walz, 1980). A series of polypeptides, with pI values between 6.1 and 6.4 and M_r 58,000 (Plate 21C, indicated by arrows), appeared to be specifically located in the microsomal fraction, but their identity is unknown.

3-6.4 2-D PAGE of Liver Homogenate Proteins from Facial Eczema Resistant and Susceptible Sheep

The 2-D PAGE gels of liver homogenate proteins from resistant and susceptible sheep were compared with the goal of detecting proteins specifically associated either with resistance or susceptibility. Plate 22 shows the Coomassie blue stained gels of the electrophoresed liver homogenate proteins. The reproducibility of the separations is very good. The two gels are qualitatively very similar. The horizontal streak at M_r about 30,000 was DNase which was added to digest DNA during the preparation of liver homogenates for electrophoresis (Plate 22A, B, indicated by an arrow). The sensitivity of the method depends on the amount of protein applied. According to O'Farrell (1975), Coomassie blue detects 0.01 μg of protein. However, it is felt that in a 2-D PAGE, the sensitivity is somewhat decreased. Although the interpretation of these gels only allows analysis of abundant proteins, no major differences could be detected when the liver homogenate proteins of resistant and susceptible sheep were compared. Therefore, analysis of liver homogenate proteins labelled with radioactive isotopes was used to further the search for protein differences.

Plate 23 shows the autoradiograms of ^{14}C-labelled liver homogenate proteins from pooled resistant and susceptible liver biopsy specimens (Tables 5 and 6). The labelled proteins, which contained 255,000 d.p.m., were electrophoresed and were then detected on dried gel by autoradiography using Kodak SB X-ray film exposed for 4 weeks. Comparison of the autoradiograms did not reveal any significant difference in the protein pattern between the pooled resistant and susceptible liver biopsy specimens. Further search for protein differences was carried out

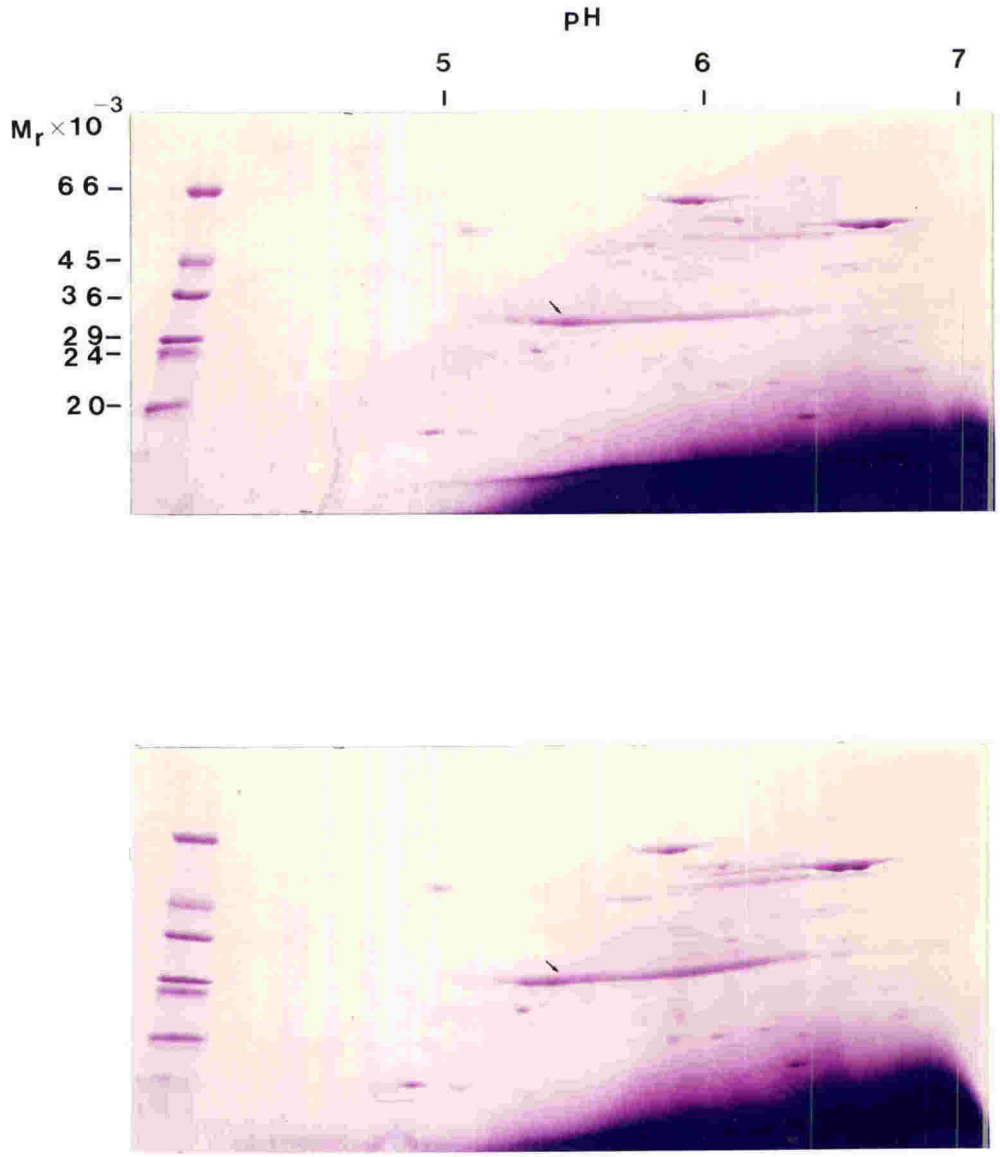


PLATE 22. 2-D PAGE of Liver Homogenate Proteins from Resistant and Susceptible Sheep

2-D PAGE separation of liver homogenate proteins from resistant and susceptible sheep on 5-20% gradient gels. A) 400 μg of liver homogenate proteins from resistant sheep. B) 400 μg of liver homogenate proteins from susceptible sheep. The horizontal streak at M_r about 30,000 was DNase (indicated by an arrow) which was added during preparation of liver homogenates for electrophoresis.

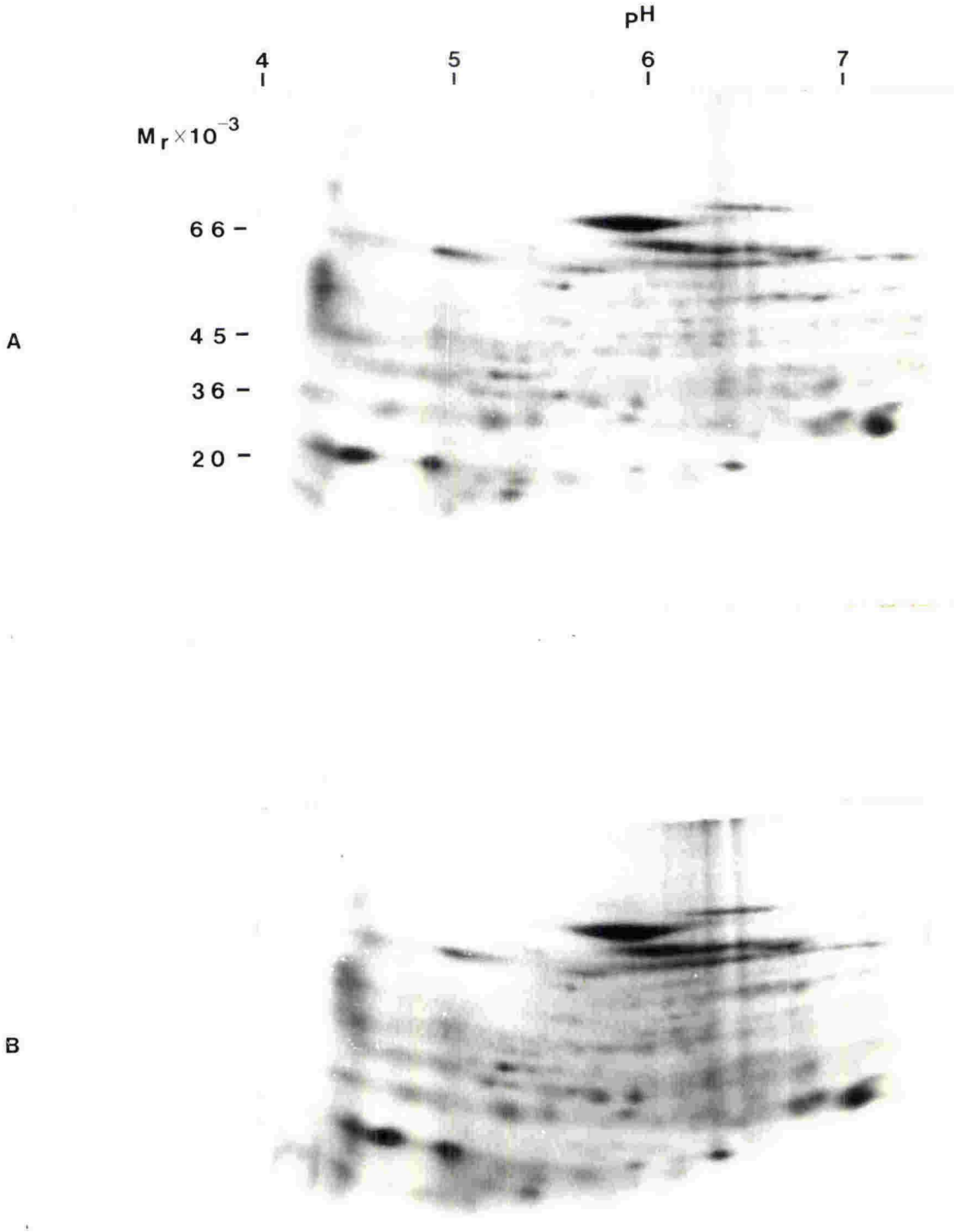


PLATE 23. Autoradiography of Liver Homogenate Proteins from Pooled Resistant and Susceptible Liver Biopsy Specimens

The liver homogenate proteins from pooled resistant and susceptible liver biopsy specimens were ^{14}C-labelled. Radioactively labelled proteins (255,000 d.p.m.) were loaded on the gels and the labelled proteins were detected on dried gels by autoradiography using Kodak SB X-ray film exposed for 4 weeks. A) pooled resistant, B) pooled susceptible.

using double-label 2-D PAGE (Plates 24 and 25). Plate 24 shows the ^{14}C-labelled liver homogenate proteins from resistant sheep and the ^{3}H-labelled liver homogenate proteins from susceptible sheep. The labelled liver samples were pooled and an aliquot, which contained 241,269 d.p.m. of ^{14}C and 2,953,440 d.p.m. of ^{3}H, was electrophoresed. Autoradiography was carried out for 7 weeks at room temperature. After autoradiography, the dried gel was rehydrated and was permeated with PPO. The gel was again dried and was exposed to Fuji RX 100 film at -70^{o}C for 2 weeks fluorography. The developed autoradiogram specifically detected ^{14}C-labelled liver homogenate proteins from resistant sheep while the fluorogram detected both the ^{14}C-labelled liver homogenate proteins from resistant sheep and ^{3}H-labelled liver homogenate proteins from susceptible sheep. Owing to the difference in the duration of exposure of the autoradiogram and fluorogram (Plate 24A, B), comparison of the less abundant proteins was difficult. However, comparison of the more abundant proteins showed a difference among a family of proteins with pI ranging from 5.7 to 6.2 and M_r approximately 66,000 (Plate 24B, indicated by an arrow). This family of proteins were detected only in the fluorogram thus indicating a specific association with the susceptible sheep. However, this difference was not shown in the results of Plate 23 where this particular family of proteins were not detected by autoradiography of liver homogenate proteins from resistant or susceptible sheep.

Further analysis was carried out using reverse double-label 2-D PAGE (Plate 25). In this experiment, the liver homogenate proteins from susceptible sheep were labelled with ^{14}C and the liver homogenate proteins from resistant sheep were labelled with ^{3}H. An aliquot of the pooled labelled liver samples containing 279,240 d.p.m. of ^{14}C and 2,982,017 d.p.m. of ^{3}H was

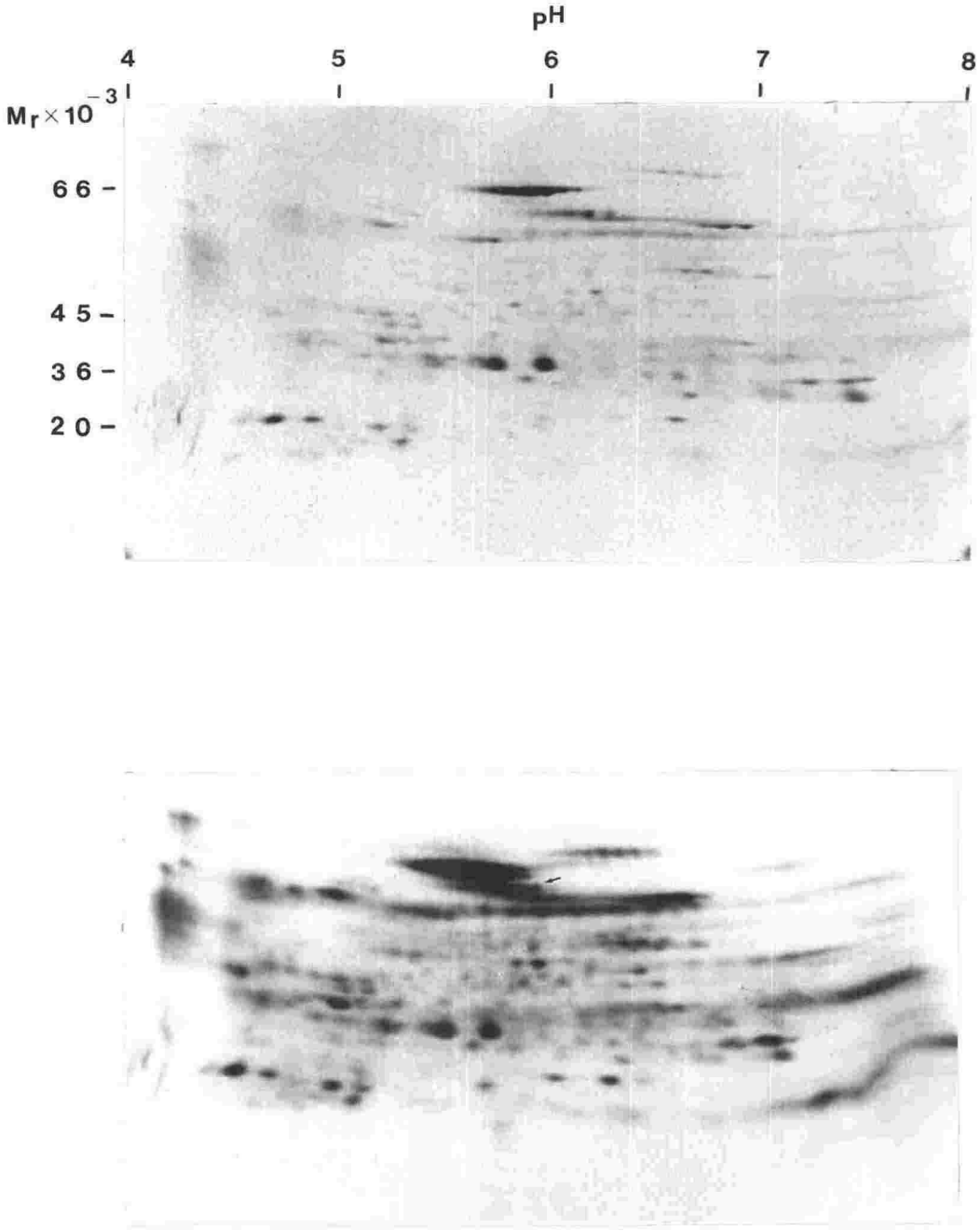


PLATE 24. Autoradiography and Fluorography of Liver Homogenate Proteins from Resistant and Susceptible Sheep

The liver homogenate proteins from resistant sheep were ^{14}C-labelled and the liver homogenate proteins from susceptible sheep were ^{3}H-labelled. The labelled liver samples were pooled and an aliquot containing 241,269 d.p.m. of ^{14}C and 2,953,440 d.p.m. of ^{3}H was electrophoresed. A) autoradiography (7 weeks), B) fluorography (2 weeks). The arrow in (B) indicates protein variation specifically associated with the susceptible sheep.

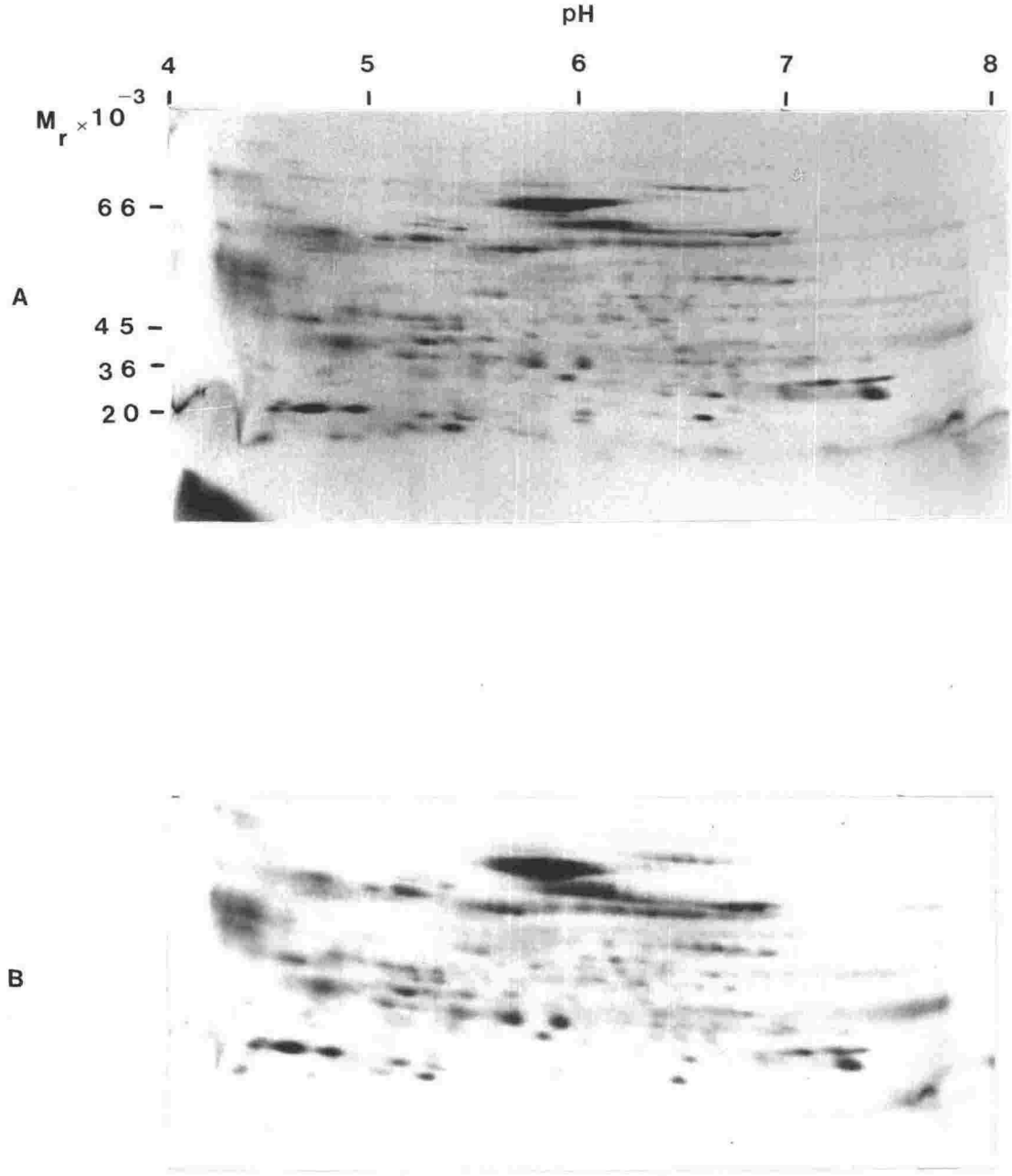


PLATE 25. Autoradiography and Fluorography of Liver Homogenate Proteins from Susceptible and Resistant Sheep

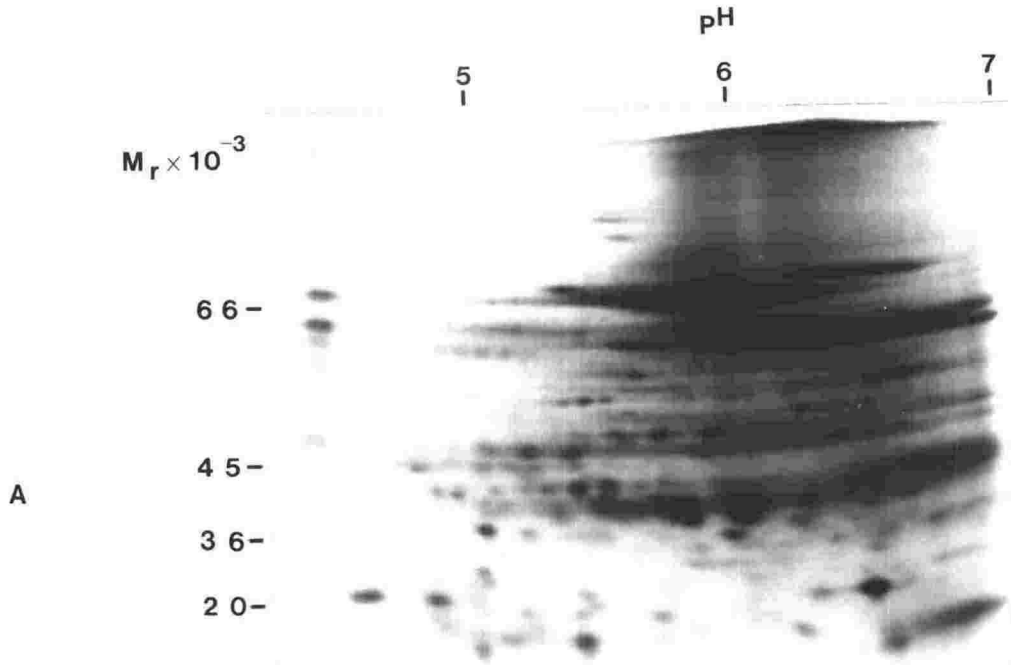
The liver homogenate proteins from susceptible sheep were ^{14}C-labelled and the liver homogenate proteins from resistant sheep were ^{3}H-labelled. The labelled liver samples were pooled and an aliquot containing 279,240 d.p.m. of ^{14}C and 2,982,017 d.p.m. of ^{3}H was electrophoresed. A) autoradiography (7 weeks), B) fluorography (2 weeks).

electrophoresed. Autoradiography was carried out for 7 weeks at room temperature while fluorography was carried out for 2 weeks at -70°C. Comparison of the autoradiogram with the corresponding fluorogram did not reveal any significant difference in the protein map. Also the protein family previously detected in liver homogenates from susceptible sheep (Plate 24B) was not detected on the autoradiogram (Plate 25A).

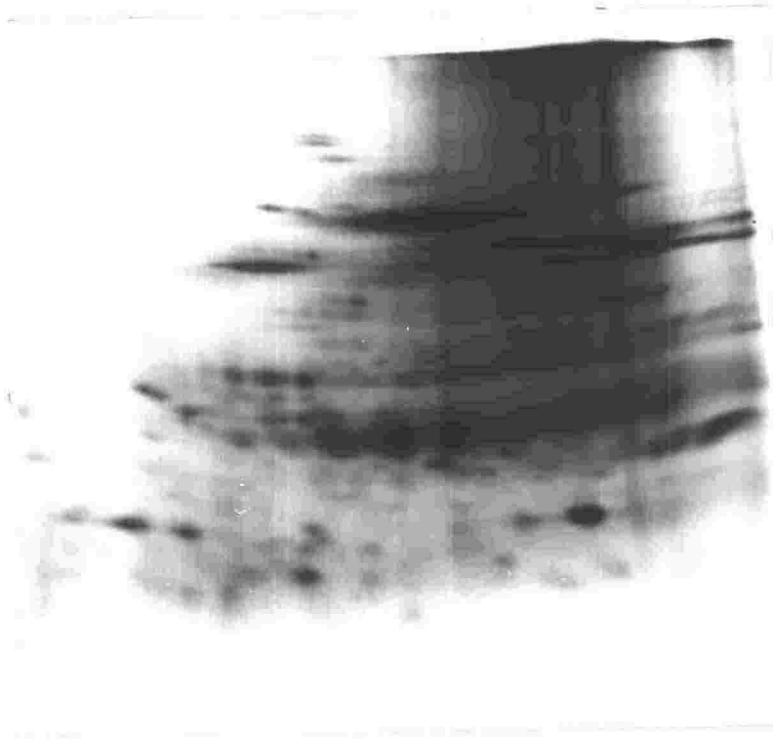
A further examination of liver protein variation was carried out by autoradiography of liver homogenate proteins from individual resistant and susceptible sheep (Plate 26). Liver homogenate proteins from 3 resistant sheep (Table 5) and 3 susceptible sheep (Table 6) were ^{14}C-labelled and liver samples containing 255,000 d.p.m. were electrophoresed. Autoradiography was carried out for 5 weeks at room temperature. Analysis of the autoradiograms revealed only individual differences which could not be viewed as protein variation associated with facial eczema. The protein difference previously detected in the pooled liver homogenate proteins from susceptible sheep (Plate 24B) was not detected among the susceptible individuals. Therefore, the protein difference did not appear to have an association with facial eczema susceptibility.

PLATE 26. Autoradiography of Liver Homogenate Proteins from Individual Resistant and Susceptible Liver Biopsy Specimens

Liver homogenate proteins from individual resistant and susceptible sheep were ^{14}C-labelled. Labelled liver sample containing 255,000 d.p.m. was loaded onto each gel. Autoradiography was carried out using a 5 week exposure period. A) resistant animal number 61, B) resistant animal number 106, C) resistant animal number 126, D) susceptible animal number 25, E) susceptible animal number 83 and F) susceptible animal number 107.



A



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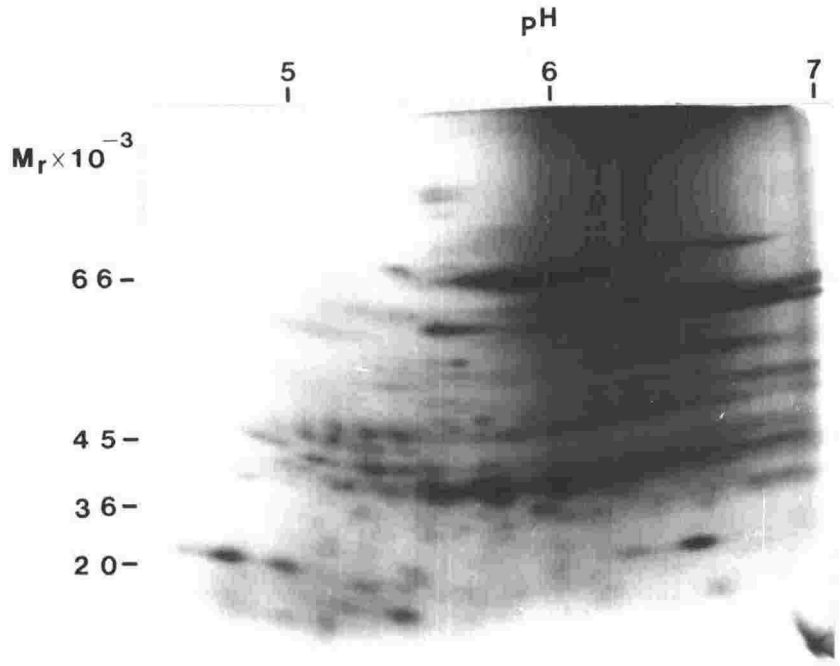
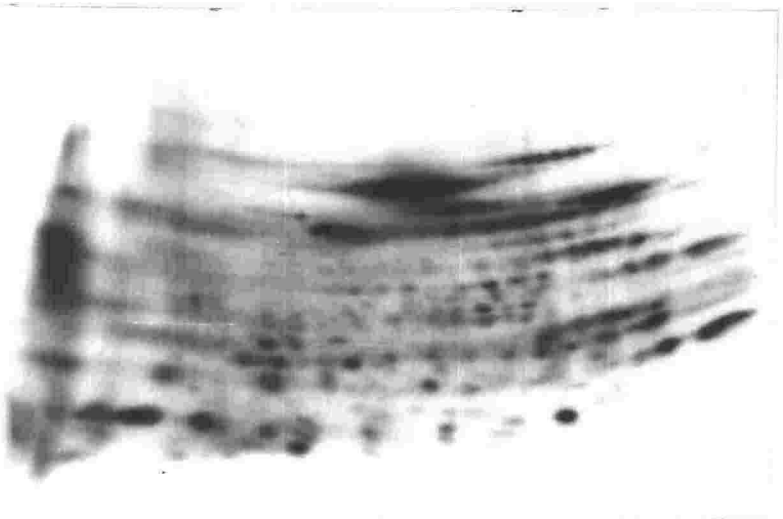
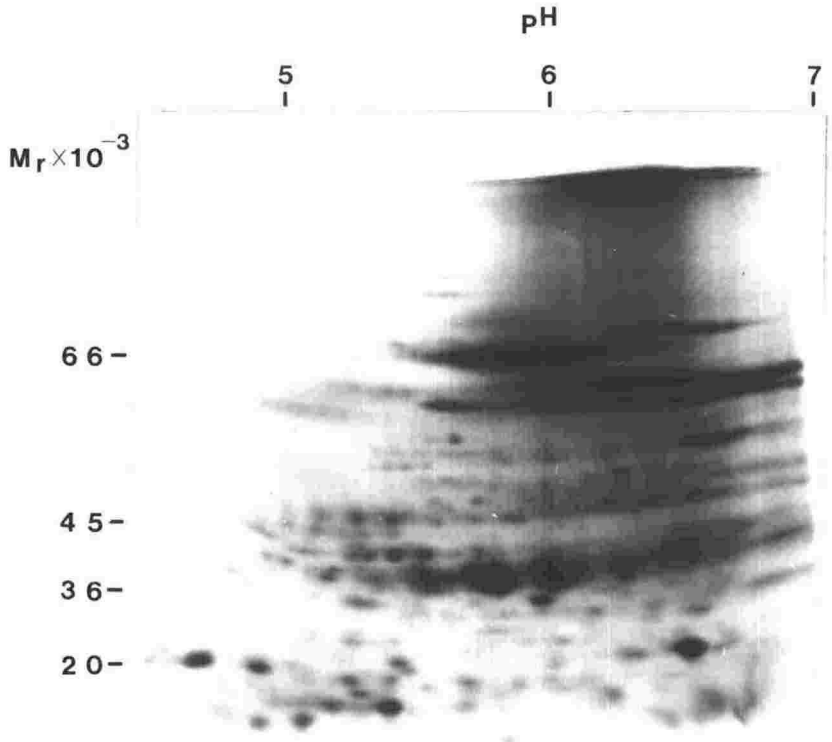


C



D



E

F

DISCUSSION

4-1 Electrophoretic Analysis of Protein Variation

Optimal and reproducible separation systems for the analysis of protein variation were developed so that the plasma and liver proteins of facial eczema resistant and susceptable sheep could be compared.

4-1.1 Formation of pH Gradients in IEF Gels

In IEF, a stable pH gradient increasing progressively from anode to cathode is established by electrophoretic migration of the ampholytes. Under the action of an electric field, charged ampholytes migrate towards the cathode or anode depending on their initial charge. As a result, the ampholytes are aligned along the electric field, from anode to cathode, in order of increasing pI value. In every part of the resulting gradient, the ampholytes cease to migrate at a pH corresponding to their pI where they possess zero net charge and each species maintains a local pH corresponding to its pI by virtue of its strong buffering capacity. Due to overlapping and similarity of the pI of neighbouring ampholytes, the pH interfaces between zones are graduated and an essentially smooth pH gradient is produced.

In this study, the ampholytes used were Pharmalytes and their concentration in the gel was found to affect the shape and linearity of the pH gradient (Figure 4). A linear gradient was obtained by using a total of 4% (w/v) Pharmalytes which contained a high proportion of Pharmalytes in the pH 4-8 region. Higher concentrations of Pharmalytes around the neutral pH region were needed to compensate for the low conductivity in that region because Pharmalytes with pI values close to the neutral pH were less charged as compared to those with pI values in the acidic or

basic pH regions. This may be due to the dilution effect of water molecules concentrated in the pH 7 region of the gradient due to repulsion of H^+ and OH^- ions from the anode and cathode respectively. Water has little buffering capacity and conductivity at its pI would therefore absorb almost all the available voltage, thereby decreasing the focusing effect in other parts of the gradient.

The pH gradient was also affected by the choice of anolyte and catholyte. For example, the use of pH range 3-10 Pharmalytes with 0.04 M H_3PO_4, pH 1.8, as anolyte and 0.1 M NaOH, pH 12.8, as catholyte gave rise to pH gradients with an anodic terminus of pH 4 and cathodic terminus of pH 8 (Figure 3). By comparison, low ionic strength electrolytes provided a narrow pH range (pH 4.5-7) and a non-linear gradient (Figure 3). This may be due to inadequate electrical conductivity supplied by the low ionic strength electrolytes. This is in agreement with the finding reported by Nguyen and Chrambach (1977) that pH gradient stability increases with increasing concentrations of anolyte and catholyte. However, the concentrations of electrolytes were maintained at 0.1 M or less in order to ensure high conductivity in the liquid phase and a minimum of voltage drop between the electrode and the gel extremities.

The addition of aspartic acid (pI 2.77), lysine (pI 9.74) and arginine (pI 10.76) to buffer the pH gradient at each end, increased the range of the pH gradient by about 1 pH unit (Figure 5). This is in agreement with the results reported by Breithaupt et al. (1978). A gel with a broad pH range enables polypeptides at pH extremes to be analysed which otherwise are not detected in a gel prepared with a narrow pH gradient. Subsequently, any specific protein of interest may further be analysed with a higher degree of resolution by decreasing the slope of the pH

gradient. Optimal separation of proteins can thus be obtained by developing the shallowest pH gradient that covers all components of interest.

The pH gradients formed with the conditions specified in this study were highly reproducible and stable (Figures 7 and 8). In addition, the pH gradients were almost linear throughout more than 90% of the length of the gel, so that nearly optimal separations were obtained. The pH gradients were reproducible not just within the same experiment but also in separate experiments operated under similar conditions. The high order of reproducibility allowed direct comparison of proteins separated on different gels to be made without complications due to experimental variation.

4-1.2 Effect of Protein Load, 2-Mercaptoethanol and Dithiothreitol on pH Gradients in IEF

It has been reported that high protein loads produce local changes in the shape of the pH gradients during IEF (Chrambach et al., 1973). Cantrell et al. (1981) have suggested that the flattening of pH gradients at the cathodic end of the gel is directly proportional to the amount of protein applied in the IEF-dimension.

In the present study, there appeared to be a correlation between the amount of protein applied and the decrease in pH at the basic end of the gradient (Figure 9). However, as proteins for IEF were solubilised in buffer containing Triton X-100, SDS, urea and 2-mercaptoethanol, these agents might have affected the stability of the pH gradient.

Righetti et al. (1982) found that cathodic pH gradient flattening was caused by 2-mercaptoethanol in amounts similar to those added to proteins prepared for IEF. The 2-mercaptoethanol

acting as a buffer with pK 9.5 (Sober et al., 1970), ionises at the basic gel end and migrates toward the anode, sweeping away the focused ampholytes, until it loses its charge below pH 8. In the present study, the Pharmalyte concentration was 2% (w/v) in the pH range 8-10. According to Fredriksson (1977a) and Gelsema et al. (1979), this Pharmalyte concentration has a buffering capacity of 3-6 μequiv. pH^{-1} ml^{-1}. In the same pH region, the amount of 2-mercaptoethanol used would have a buffering power of 0.3 μequiv. pH^{-1} ml^{-1}. Fredriksson (1977b) has pointed out that, when a species reaches at least 10% of the buffering power of the surrounding ampholytes, it will alter the pH gradient in IEF. This occurred when 2-mercaptoethanol was applied at the cathodic end of the IEF gel (Figure 10). This may be remedied be applying protein samples containing the sulphydryl compound at the anodic end of the gel where 2-mercaptoethanol has no charge or buffering power. Alternatively, dithiothreitol may be used since it is effective at concentrations much lower than those commonly employed with 2-mercaptoethanol and at such concentrations dithiothreitol does not affect the basic portion of the pH gradient. Dithiothreitol at a concentration of 0.05 M was found to have little effect on the pH gradient (Figure 10).

4-1.3 IEF of Proteins

IEF is essentially an equilibrium electrophoretic method for separating amphoteric macromolecules according to their pI values in stable pH gradients. Due to its very high resolving power and to the sharpening effect of sample zones in their pI position, IEF appears to be particularly useful for the analysis of proteins. Generally, minor components are more readily detected by IEF than by electrophoresis. There are two likely explanations for this. As protein bands separate during

electrophoresis, they become more diffuse so that they may mask minor components. The opposite is true for IEF. If the pH gradient is stable, the separated bands become sharper with time so that their band density is much higher than that usually obtained by electrophoresis. In addition, because the separated proteins form discrete bands, it is possible to overload the gel with major fractions to facilitate the detection of these trace components.

The distribution of proteins depends on the range and shape of the pH gradient in the gel. Optimal separations are obtained with the shallowest pH gradient which covers the extremes of pI values of all components of interest. The pH gradient can be varied by selecting a certain ampholyte pH range. A pH range of about 1 pH unit allows the possibility of separating proteins with a pI of about 0.02 pH unit. This has been confirmed in many applications (Vesterberg and Svensson, 1966; Carlstrom and Vesterberg, 1967; Vesterberg, 1967). Proteins with such a small pI difference are indeed very difficult to separate by other methods.

In this study, the use of different pH ranges of Pharmalytes (Figure 4) were successful in providing a linear pH gradient over the pH range of 4 to 8. With this pH range, it was possible to separate proteins whose pI values differed by 0.30 ± 0.05 pH unit as estimated from the IEF-tube gel (Plate 1).

4-1.3.1 Determination of pI Values of Proteins Separated by IEF

pI values of separated proteins may be determined from the equilibrium position of migration on IEF gels. The pI values of proteins thus determined are temperature dependent and usually decrease with increasing temperature. The same protein measured

at 4°C and 25°C may have a difference in pI as high as 0.5 pH unit, the higher value being obtained at the lower temperature (Vesterberg and Svensson, 1966). This difference is usually more pronounced for basic proteins and for proteins whose pI value is close to the pK of its functional groups. The variation of pK in the range 4-25°C, while being very small for protein carboxyls (dpK = 0.06) is quite large for imidazole (dpK = 0.39) and very large for the ϵ-amino group of lysine (dpK = 0.67) (Tanford, 1962; Bull, 1971). Hence, it is to be expected that neutral and basic proteins may exhibit large dpI/dT increments. It is therefore recommended that the same temperature be used for focusing and pH measurement. The temperature attained during focusing is related to the applied voltage and to the external heating or cooling of the gels. Since the migration rate of the sample and the sharpness of the focused bands at their pI values are proportional to the applied voltage, it is desirable to have the applied field strength as high as possible, while maintaining the heating (which is proportional to the square of the applied voltage) at an acceptable level. The maximum tolerable power, defined as optimum power by Schaffer and Johnson (1973), depends on the conductivity of the system and on its efficiency to dissipate heat. Righetti and Righetti (1975) have shown that a gel which was allowed to dissipate its heat in air had a temperature around 20°C when 0.25 watts/gel was applied. The electrophoretic apparatus used in this study did not have a water cooling system, so narrow gel tubes of 13 cm in length, 0.25 cm in internal diameter and wall thickness of 0.07 cm were used, and the focusing was carried out at room temperature with the electrical load limited to 0.2 watts/gel. Therefore, the temperature used during focusing was taken to be around 20°C and

it was at this temperature that the pI values of the proteins were determined.

The solubility of proteins is often least at pH values close to the pI and precipitation may occur. For this reason, urea at concentrations up to 9 M are sometimes introduced into IEF gels, particularly in the case of membrane and other poorly soluble proteins. Gelsema and De Ligny (1977) and Gelsema et al. (1977, 1978) have stressed that, when using such additives several factors should be introduced to correct the measured pI. Ui (1971) suggested an overall correction factor of 0.42 pH unit to be subtracted from the apparent pI of the protein when using 6 M urea as an additive. A correction factor ranging from 0.7 to 0.9 pH unit has been suggested by Josephson et al. (1971) for 7 M urea solutions. In this study, it was noted that urea caused a shift in the pI of bovine serum albumin. The bovine serum albumin had a pI value between approximately 5.5 and 6.0 (Plate 1), a value of about 1 pH unit higher than the value determined in the absence of urea (Radola, 1973). However, if the measured pI is adjusted for the effect of urea, the difference in pI in the presence and absence of urea becomes insignificant. The elevated pI values of proteins in the presence of urea may reflect conformational transformation of the molecules caused by urea. Such transformations may result in alterations in the degree of shielding and the dissociation of ionisable groups. The pI of a native protein could be different from that of the unfolded molecule so long as the native configuration retains some dissociable groups within the interior of the molecule. However, not all proteins show a shift in pI in the presence of urea. In the presence of 9 M urea, human haemoglobin showed an apparent pI around 6.9-7.2 (Plate 1), a value similar to that determined in the absence of urea (Malamud and Drysdale, 1978). It may either

be that this protein does not contain buried dissociating groups or that the pK values of its ionising groups are generally altered very little by changes in protein conformation. It may also be possible that the participation of two kinds of dissociating groups, which compensate for the variation in charge, results in no detectable shift in pI.

4-1.4 2-D PAGE of Proteins

High resolution 2-D PAGE (O'Farrell, 1975) has proved to be an extremely valuable method for separating proteins present in complex mixtures. The method combines the techniques of IEF in the presence of urea and a non-ionic detergent in the first-dimension and slab gel electrophoresis under denaturing conditions in the second-dimension. The separation relies on two independent properties of proteins. One is charge, which is reflected by the pI, and the other is M_r, which determines the mobility of the SDS-protein complexes in polyacrylamide gels.

IEF separates proteins on the basis of charge and the separation is independent of protein M_r. Therefore, it is possible for two or more proteins to co-migrate in IEF as a single band particularly if the original sample is a complex mixture of proteins. The co-migration of proteins can mask compositional complexity and difference in the amount of the components present in any band. It is for these reasons that 2-D PAGE, which incorporates an IEF separation in one direction followed by a second M_r separation at right angles to the first, has been developed. The superior separation attained using 2-D PAGE is illustrated by comparing Plates 2 and 5 in which plasma proteins were separated by IEF into 30 or more bands (Plate 2), but were separated on the 2-D PAGE gel into more than 200

different proteins whose pI values differed by as little as 0.05 ± 0.02 pH unit (Plate 5).

In general, proteins separated by 2-D PAGE are examined on Coomassie blue stained gels. However, the low sensitivity of the staining technique limits detection of minor proteins on the gel. In addition, there are problems associated with staining gels which contain ampholytes as the ampholytes themselves bind most organic dyes used for detecting proteins. Alternatively, silver staining can be used for protein detection, although care must be taken with each of the individual steps to avoid artifacts and maximise sensitivity. The major loss of sensitivity in silver staining is usually due to inadequate water purity. Deionised water of less than 1 μmho conductivity is required in the preparation of all reagents, including washing and fixing solutions. Contaminants such as chloride ions will precipitate silver ions, thus causing reduced sensitivity and increased background.

The use of radioisotopes provides a much more sensitive method for locating proteins. The sensitivity of autoradiography and fluorography is greater than that of staining methods. A spot on the 2-D PAGE gel that contains approximately 20 ng of labelled protein may be detected (Wheeler et al., 1986). However, the main problem in detecting minor proteins by autoradiography or fluorography is the difference in abundance of the various proteins in the complex mixtures such as plasma. Major proteins may account for much of the radioactivity while less abundant proteins may account for very little. Therefore, when minor proteins are detected using autoradiography or fluorography, the major components will be overexposed, thus producing large spots which result in a considerable loss in the resolving power of the technique.

The comparison of 2-D PAGE protein patterns from different sources usually involves detection of variation in a few proteins present among a large number of proteins. In many cases the differences can be clearly detected by visual inspection when several gel patterns are compared side by side. However, there are cases in which the variations between protein maps are unclear due to small variations in the running conditions that make comparisons very difficult. To circumvent these uncertainties, it is possible to use the double-labelling method in which protein samples to be compared are labelled with different isotopes and co-electrophoresed as described by McConkey (1979), Choo et al.(1980) and Wheeler et al.(1986). The particular value of the double-labelling approach is that complex mixtures from two different sources are resolved together thus eliminating the possibility of differences arising from the resolving procedure itself. The double-labelling procedure used in this study has been specially developed for comparison of serum or plasma proteins in two different samples (Wheeler et al., 1986), but it was also found applicable to the analysis of liver proteins. Therefore, this procedure, with modifications, should be applicable to comparison of proteins from a wide variety of sources.

2-D PAGE holds great promise as a diagnostically useful tool. Its primary advantage lies in the ability simultaneously to resolve and evaluate numerous proteins, many of them clinically significant, in small-volume samples. One genetic application where the use of 2-D PAGE proves advantageous is phenotyping of serum and plasma proteins. Phenotyping of multiple proteins normally requires many individual electrophoretic analyses, but with 2-D PAGE it is possible to determine the phenotypes of many serum or plasma proteins on a single gel. Similarly, 2-D PAGE can

be used to identify 'marker' proteins in diseases where expression of a specific protein can be related to a genetically inherited condition or tissue pathology (Edwards et al., 1982; Willard, 1982). More sophisticated methods of analysing gels by the use of computerised densitometers will hopefully contribute further to the sensitivity and speed of the technique (Garrels, 1979; Lipkin and Lemkin, 1980; Anderson et al., 1981; Vo et al., 1981; Skolnick et al., 1982).

4-2 Radioactive Labelling of Proteins by Reductive Methylation

The reductive methylation of free amino groups with formaldehyde and $NaCNBH_3$ is a simple method for the modification and isotopic labelling of proteins under mild conditions. Reductive methylation not only maintains the total charge of the protein, but also maintains the spacial distribution of charges, thus, proteins can be radioactively labelled with a high degree of efficiency without adversely affecting their biological activities (Means and Feeney, 1968).

It was found that the reductive methylation of bovine serum albumin at pH 7.5 was dependent on the formaldehyde concentration (section 3-3.1). This is to be expected according to the proposed mechanism of the reaction (reaction 1, section 1-6). Since the formation of Schiff's base is the slow step, an increase in the formaldehyde concentration would be expected to result in greater derivatisation. However, complete derivatisation, in which 2 moles of formaldehyde were incorporated per mole of amino group, was never achieved (Figure 11). The derivatisation yield of 1.5 mole of formaldehyde per mole of lysyl residue obtained in this study is in agreement with the reported derivatisation yield obtained by Jentoft and Dearborn (1979). The incomplete derivatisation could have resulted from conversion of formaldehyde to methanol during reductive methylation or from the reductive methylation of contaminating amines in the reaction mixture (Jentoft and Dearborn, 1979). Gidley and Sanders (1982) suggested that the low derivatisation yield was due to N-cyanomethyl compounds formed effectively and irreversibly at neutral pH (reaction 4, section 1-6), and that Ni^{2+} could be used to increase the yield by suppressing the formation of

N-cyanomethyl derivatives. However, Ni^{2+} was found to have no effect on the derivatisation yield in this study (Figure 13).

$NaCNBH_3$, unlike $NaBH_4$, is stable in aqueous solution at pH 7 (Borch et al., 1971). The advantages of using $NaCNBH_3$ for radioactively labelling proteins are that the reaction can be carried out at neutral pH and that greater incorporation of labelled methyl groups into protein can be attained. $NaCNBH_3$ differs from $NaBH_4$ in that it can be used at neutral and mildly acidic pH where the unwanted reactions of formaldehyde with protein side groups have been shown to be much less important than at pH values greater than 8 (Galembeck et al., 1977). However, the hygroscopic nature of the reducing agent may result in appreciable amounts of water being absorbed during weighing which may contribute in part to the observed variation in reducing activity of the weighed sample.

Reductive methylation was found to be suitable for preparing radioactively labelled proteins from plasma and liver since the labelling efficiency was not affected by physiological concentrations of NaCl (Figure 14) and different proteins were labelled to a similar extent (Figure 16). By using either ^{14}C- or ^{3}H-labelled formaldehyde, reductive methylation was used to prepare either ^{14}C- or ^{3}H-labelled proteins and was thus convenient for studies requiring a double label.

4-3 Detection of Protein Polymorphisms and their Association with Facial Eczema Resistance

4-3.1 Search for a Biochemical Marker for Resistance to Facial Eczema

The biochemical basis for the differences in species and strain susceptibility to facial eczema is not clear. However, there is evidence that facial eczema resistance in sheep is in part related to differences in the hepatic microsomal drug-metabolising system (Fairclough et al., 1978). Sissons (1982) also found that there was an apparent association between susceptibility to sporidesmin and high levels of NADPH cytochrome c reductase in liver. Therefore, it is expected that major protein variation between facial eczema resistant and susceptible animals may occur in the liver, either at the level of binding proteins or enzymes affecting the toxicity of sporidesmin, or at the level of liver cell protein targets for sporidesmin action. Since phenotypic and genotypic associations between a variety of production traits and various blood biochemical polymorphisms have been reported (Rasmusen and Tucker, 1973; Atroshi, 1979), it is also possible that plasma protein variation may have an association with facial eczema.

Resistance or susceptibility to facial eczema may involve qualitative and/or quantitative changes in protein gene products. Thus discriminating and sensitive techniques have been developed and applied in order to identify and distinguish minor protein variation linked to facial eczema. 2-D PAGE has good potential for locating protein variation associated with facial eczema. In this thesis, 2-D PAGE analysis of inherited protein variation linked to facial eczema resistance in sheep has been described. In general, proteins separated by 2-D PAGE have been examined on Coomassie blue stained gels. Alternatively, differential detection of proteins selectively labelled with radioactive

isotopes has been used to analyse minor variation not detectable by the simple staining technique. To simplify initial analyses, a pooling protocol was used whereby a number of samples from a given population were pooled and examined on a single gel. This technique allowed rapid comparison of variation between populations. Any detected variation was further analysed for individuals on 1-D PAGE which allowed more rapid analysis of multiple samples.

4-3.2 Analysis of Liver Proteins

The polypeptides present in sheep liver homogenates, isolated hepatocytes and subcellular fractions were analysed by SDS-PAGE and 2-D PAGE to develop experimental conditions and to gain some familiarity with the electrophoretic patterns of sheep liver proteins since this information was not otherwise available. SDS-PAGE resolved the polypeptides of sheep liver homogenates, isolated hepatocytes and subcellular fractions into 50-60 bands but it seemed likely that resolution was incomplete due to superimposition of proteins in several bands (Plate 17). Separation by 2-D PAGE allowed resolution of many of the superimposed groups of proteins (Plate 21). Analysis of liver proteins on 2-D PAGE gels revealed over 200 polypeptides (Plate 20). Many of these polypeptides were present as families of protein spots probably reflecting microheterogeneity. Some basic proteins were not well resolved in the 2-D PAGE system (Plate 21) probably because they entered the IEF gel poorly. Even when more basic ampholytes were included, the extension of the pH gradient was very small because in the presence of urea the basic region of the pH gradient was unstable. Some of the basic proteins that entered the IEF gel under these conditions produced streaks.

Therefore, difficulties existed in the detection of variation among the more basic liver proteins.

The analysis of the sheep liver proteins in relation to their distribution among subcellular fractions showed that some proteins were specifically located in a single subcellular fraction (Plate 21, Figure 24 and Table 16). However, there was a large proportion of polypeptides which were present in all subcellular fractions. This was probably at least in part due to cross-contamination of the subcellular fractions. Since the resistant and susceptible liver biopsy specimens had been stored at -20^{o}C, isolation of subcellular fractions was not possible. In addition, variation among samples introduced by centrifugal fractionation procedures was not desirable. Therefore, liver homogenates rather than subcellular fractions were used for analysis of protein variation associated with facial eczema.

The liver homogenate proteins from resistant and susceptible sheep were initially analysed by SDS-PAGE and 2-D PAGE on Coomassie blue stained gels. Owing to the limited sensitivity of detection, only analysis of the abundant proteins was feasible. No major differences could be detected when the liver homogenate proteins of resistant and susceptible sheep were compared (Plate 22). A further search for protein differences between the resistant and susceptible liver homogenate proteins was carried out with liver homogenate proteins labelled with radioactive isotopes (Plate 23). Double-label 2-D PAGE analysis was used in which the liver homogenate proteins from resistant and susceptible sheep were selectively labelled with ^{14}C and ^{3}H. The labelled liver samples were pooled and an aliquot, which contained ten times as much ^{3}H as ^{14}C, was electrophoresed. The radioactively labelled proteins were detected on dried gels by autoradiography and fluorography. Comparison of the autoradiogram

with the corresponding fluorogram did not reveal any significant difference between the protein maps (Plates 24 and 25). This lack of success might well have been due to the excess of commonly inherited proteins present in whole liver homogenates masking protein differences present in a particular subcellular fraction. For example, microsomal proteins only represented a small fraction (about 9%) of the total protein present in the whole liver homogenates, and did not contribute substantially to the proteins detected after 2-D PAGE of liver homogenates. Therefore, the expected difference between the resistant and susceptible sheep in the hepatic microsomal drug-metabolising enzymes (Fairclough et al., 1978) and in the NADPH cytochrome c reductase (Sissons, 1982) could have been masked by other abundant proteins. Also differences in binding proteins on the cell membrane of the target tissue, e.g., bile duct cells, might well be all hidden by the excess of other proteins. Furthermore, liver protein variation associated with facial eczema may not have been reflected in charge differences but may have been due to amino acid substitutions which did not contribute significantly to the pI or M_r of the protein. However, as such variation may affect enzyme activity, for example in the hepatic microsomal drug-metabolising enzymes, a metabolism assay on the efficiency of hepatic drug-metabolising enzymes may be useful in selection for resistance to facial eczema.

Affinity chromatography of liver proteins using immobolised sporidesmin could be used as another approach to detect protein markers for facial eczema resistance. The availability of radioactively labelled sporidesmin might also allow detection of sporidesmin binding proteins which could be separated by 1-D PAGE or 2-D PAGE and detected by autoradiography.

4-3.3 Analysis of Plasma Proteins

The sheep plasma protein 2-D PAGE maps obtained in this study are similar to those reported for other mammals. Plate 5 shows a typical separation of sheep plasma proteins. Figure 17 is the schematic drawing of this gel and incorporates the numbering system used in Table 14 for the tentative identification of major proteins. Several polymorphisms are immediately evident. These include, but are not limited to, transferrin, fibrinogen chains, α_1-antichymotrypsin, α_2HS-glycoprotein, α_1-antitrypsin, Gc-globulin, haptoglobin and apolipoproteins. Protein families contained variants separating predominantly in the horizontal charge dimension due to amino acid substitutions or glycosylation especially sialylation. The additional sialic acid residues cause the charge train to become progressively more acidic and there is also a slight progressive increase in M_r.

Three types of microheterogeneity have been detected. α_1-Antitrypsin demonstrated charge heterogeneity which was diminished by neuraminidase treatment (Plate 15) and was therefore largely due to charged sialic acid residues at the terminus of the oligosaccharide chains. A second class of charge heterogeneity was exhibited by the heavy and light chains of IgG which showed no response to neuraminidase treatment, indicating genetic polymorphism due to amino acid substitutions. Such genetic polymorphism was also observed with transferrins, as well as with many other plasma proteins, since the charge heterogeneity was maintained after neuraminidase treatment. The third type of microheterogeneity was demonstrated by the fibrinogen α-chains which exhibited heterogeneity with respect to small variations in M_r.

Initial comparison of 2-D PAGE maps of rams from the facial eczema resistant and susceptible flocks indicated differences

among a triplet of proteins of apparent M_r 36,000. However, subsequent analyses of pooled plasma from resistant and susceptible ewes (Plate 7C, D) and individual plasma from resistant and susceptible rams (Plate 8) indicated that inheritance of the triplet was not clearly linked to facial eczema resistance. The major systematic variation between the plasma proteins of Romney sheep selected for facial eczema resistance or susceptibility was among a group of proteins with M_r around 75,000 and pI values between 6.1 and 6.7 (labelled I-V on Plate 9). These proteins were identified as transferrins because of their ability to bind ^{59}Fe. More than 60% of the ^{59}Fe added to sheep plasma migrated in the transferrin zone (Plate 12) and overlaid the protein distribution revealed by Coomassie blue staining. Further evidence of identity of these proteins as transferrins was obtained by their similar electrophoretic mobility to sheep transferrin A and D alleles (Plate 12). It is difficult to relate clearly the transferrin variants I-V seen on 2-D PAGE gels (Plate 9) to the transferrin alleles recognised on 1-D PAGE gels (Plate 12). Transferrin homozygotes which separated by 1-D PAGE as two protein zones (Plate 16) were found to contain several protein spots on the 2-D PAGE gels (Plate 8). In the 2-D PAGE system, the transferrins were separated on the basis of differences in pI values whereas in the 1-D PAGE system, the proteins were separated as a function of charge at pH 8.8 and factors other than pI, including net positive and negative charge distribution, affected the separation. However, the general tendency was the same in both systems in that the more acidic transferrins predominated in sheep selected for facial eczema resistance. The relationship between the transferrin variants separated by 1-D PAGE and 2-D PAGE could be further analysed in an experiment in which transferrin variants separated by 2-D PAGE

were cut out from the gel and separately electrophoresed on a 1-D PAGE gel. The electrophoretic mobility of each of the transferrin variants separated by 2-D PAGE could thus be compared with the electrophoretic mobility of the transferrin zones normally seen on 1-D PAGE gels.

Transferrin is the iron-binding protein of blood plasma. The physiological function of transferrin is to transport iron and to act as a small but easily available iron pool. In many species of mammals, including sheep, transferrin exists as a number of genetically controlled variants and the basis of transferrin polymorphism is well described by Atroshi (1979). In each species so far studied (cattle, horse, pig, sheep and man), the genetic control of transferrins is governed by a number of co-dominant autosomal alleles with full expression of each allele in the heterozygote combinations. Using 1-D PAGE, each sheep transferrin allele gives rise to a pair of electrophoretically distinguishable protein bands, the less abundant and apparently more heavily sialylated form migrating most rapidly towards the positive electrode (Plate 16). Homozygotes are thus represented by one such zone pair while heterozygotes have two equally intensely staining transferrins each with an accompanying more acidic minor form. The transferrins thus recognised by 1-D PAGE behave in family studies as multiple alleles at a single locus.

The results of 1-D PAGE obtained in the present study (Plate 16) can thus be subjected to the following interpretation. Among the 22 ewe and ram hoggets from the resistant flock, 9 (double arrows, Plate 16) were homozygous for transferrin A and a further 7 had heterozygous combinations of the A allele with a more slowly migrating transferrin. Among the 19 ewe and ram hoggets from the susceptible flock, 8 were homozygous for transferrin D (single arrows, Plate 16) and 10 were heterozygotes

containing combinations of the D allele and a more rapidly migrating transferrin. The A allele frequency was 0.57 in resistant sheep and 0.05 in susceptible sheep while the D allele frequency was 0.18 in resistant sheep and 0.68 in susceptible sheep. Thus there appears to be some separation of transferrin alleles between the resistant and susceptible sheep. Analysis of the distribution of transferrin phenotypes in both the resistant and susceptible populations did not show any significant difference between the expected numbers and the observed numbers. This result thus indicates that the resistant and susceptible populations have both attained Hardy-Weinberg equilibrium. The Hardy-Weinberg equilibrium principle states that at equilibrium, both allele and phenotype frequencies remain constant from generation to generation. This occurs in large populations in which mating is random and no selection or other factors affect the allele frequencies. For example, in a large population of unrelated individuals, three phenotypes, G_1, G_2 and G_1G_2 are distinguishable. These phenotypes correspond respectively to three genotypes G_1G_1, G_2G_2 and G_1G_2, where G_1 and G_2 are a pair of alleles. If G_1 and G_2 have respective frequencies p and q in the population, and if the so-called Hardy-Weinberg Law holds, then the proportions of G_1G_1, G_1G_2 and G_2G_2 individuals in the population should be in the ratios $p^2G_1G_1:2pqG_1G_2:q^2G_2G_2$. If a polymorphism is kept in existence by phenotypic advantage, then the proportion of phenotypes observed should theoretically be greater than expected from the Hardy-Weinberg formula. By applying the principles of the Hardy-Weinberg Law, the number and proportion of different phenotypes present in the population can be estimated. The accuracy of this method of analysis depends upon: a) the size of the population, b) the correct determination of the proportions of individuals of different phenotypes and c)

the random mating of individuals in the population for at least one generation. However, this method of comparison could not be used to test whether the A allele was associated with resistant sheep and D allele was associated with susceptible sheep, since the flocks were two separate populations generated by selection of sheep according to their facial eczema resistance status. Therefore, in order to make a valid comparison of the distribution of transferrin phenotypes between the resistant animals and the susceptible animals, it was necessary to obtain a single population in which both resistant and susceptible animals were well represented. A further group of sheep was investigated. These animals were not related to the sheep used in the previous study. The comparison of the expected and the observed distribution of transferrin phenotypes in the resistant animals showed a deviation which was significant below the 10% level of probability but not at the 5% level (X^2 = 16.56, 9 df, 0.10 > P > 0.05). This deviation was due to an excess of phenotypes AA, AD and BB, and a deficiency of phenotype DD (Figure 22). Thus, there appeared to be a relationship between transferrin phenotype and facial eczema resistance, but the sample size was too small to allow for a more statistically significant analysis. A further study using larger numbers of animals is therefore required.

The distribution of phenotypes among the susceptible animals showed a fairly good agreement with the Hardy-Weinberg equilibrium (X^2 = 6.33, 9 df, 0.80 > P > 0.70). There was a high participation of DD homozygotes in the susceptible group (Figure 23). It can be reasoned that high homozygosity resulted merely from a disproportion in the frequency of D alleles in the population. Therefore, any association of the D allele with facial eczema susceptibility would be masked by this disproportion of the frequency of the D allele. The determination

of the association between particular alleles and resistance or susceptibility requires the use of a larger number of animals to increase the population sizes. However, the preliminary evidence obtained in this study suggests that polymorphism at the transferrin locus provides a selective advantage for facial eczema resistance although the nature of this advantage has not yet been demonstrated.

4-3.4 Detection of Transferrin Phenotypes

Some of the difficulties associated with studying transferrin polymorphism arise because electrophoretic analysis of phenotypes is based on small differences in the electrophoretic mobility of the transferrin alleles. In particular, it is difficult to distinguish between the mobility of sheep transferrin C and D alleles. There are other problems in this type of identification. For example, bacterial contamination may bring about a loss of sialic acid residues with a consequent decrease in electrophoretic mobility. Furthermore, since free transferrin and the Fe-transferrin complex have different pI values (Keller and Pennell, 1959), the transferrin zones detected on 1-D PAGE gels may be misinterpreted if the transferrins are not sufficiently saturated with iron.

4-3.4.1 Effect of Neuraminidase on Transferrins and Other Plasma Proteins

Transferrins contain sialic acid residues which behave as relatively strong acids since they possess a carboxyl group with a pK of 2.7 (Blix et al., 1956). The electrophoretic migration of transferrins towards the positive electrode at neutral or basic pH is partly explicable in terms of charge conferred by the ionised sialic acids. Therefore, alteration in the number of

sialic acid residues may affect the electrophoretic mobility of the transferrins. A reduction in electrophoretic mobility of the transferrin bands was observed when sheep transferrin variants were incubated with neuraminidase, an enzyme which cleaves the glycosidic bond joining sialic acid to its adjacent carbohydrate residue. Plate 13 illustrates the result obtained when plasma containing different transferrin phenotypes were incubated with neuraminidase. In each case, a pattern of three slower moving bands was obtained. This reduction in electrophoretic mobility of the transferrin bands reflects variations in the number of sialic acid residues. It appeared that variations in sialic acid content were not involved in the observed genetic variation of sheep transferrins.

The electrophoretic mobility of the major protein band was decreased by two steps for each transferrin allele. These results may be interpreted to indicate that two sialic acid residues were accessible to enzymic hydrolysis in the major transferrin band, and that each sialic acid residue contributed a definite increment to the electrophoretic mobility of the intact protein. As the glycosidic bonds were hydrolysed by the enzyme, an increasing but random removal of sialic acid took place until the two residues had been removed, after which there was no further change in mobility of the transferrin band. The bands represented the stepwise removal of sialic acid from the transferrin molecule. Band B_2 represented transferrin with 2 sialic acid residues and band B_1 represented transferrin with one sialic acid residue, while band B_o appeared to represent the complete removal of sialic acid from the molecule (Plate 13C). This result is in agreement with those reported by Stratil (1973) where the electrophoretic mobility of the major transferrin band was decreased by two steps with a simultaneous slowing of the

migration of the minor band. It was not easy to assess the effect of neuraminidase on the faster and minor band of the untreated phenotype since the identification of its equivalent in the neuraminidase treated transferrin was difficult. In both the present study and the study of Parker and Bearn (1962), neuraminidase treatment did not shift all of the bands to the zero sialic acid form. Possibly more active preparations of neuraminidase would have accomplished this, but bovine and sheep transferrins may be intrinsically more resistant than human transferrin to attack by neuraminidase.

In some plasma, e.g., animal number 133 (Plate 16), normal transferrin types were accompanied by a faint slower-moving component behind the major band. This previously reported 'shadow' band may represent either a naturally occurring heterogeneity in transferrins or the loss of a sialic acid residue from the transferrins as a result of aging or contamination (Boyer and Young, 1960; Harris et al., 1960; Spooner et al., 1975).

It appears that at least two different loci affect the biosynthesis of transferrins. The first is the locus responsible for the polypeptide chain sequence. Variation at this locus determines the transferrin type and is reflected in amino acid substitutions in the polypeptide chain. The second locus affects the proportion of sialic acid in the transferrin molecule either in terms of the number of sialic acid residues contained on each oligosaccharide chain or the number of oligosaccharide chains containing sialic acid. Variation at this locus is responsible for the production of charge variants containing differing amounts of sialic acid which affect the migration of the proteins in the zone pairs of each allele.

After treatment of sheep plasma with neuraminidase, many plasma proteins, including the transferrins, showed charge modification reflected in displacement towards the basic end of the 2-D PAGE gel (Plate 15). These differences probably reflect loss of sialic acid from the oligosaccharide chains of glycoproteins. The subunits of the transferrin charge chain were shifted equally and the relative proportions of each were retained. However, other proteins showed different patterns of charge shift. The triplet of proteins originally observed in pooled plasma from resistant rams had their migration substantially shifted after neuraminidase digestion indicating that the oligosaccharide chains of these molecules were extensively substituted with sialic acid residues. The failure of neuraminidase to yield a single α_1-antitrypsin spot and its total lack of effect upon other proteins, suggests an additional type of charge related heterogeneity to that due to sialic acid. This type of charge heterogeneity is most likely caused by variations in peptide sequences.

4-3.4.2 Effect of Iron on Transferrin Migration

Transferrin has two iron-binding sites which appear to be equal and independent in their iron-binding mode. As iron is bound to these sites, bicarbonate is bound to an anion-binding site in close proximity to each of the iron-binding sites. The result is a red coloured complex with an absorption maximum in the range of 450-465 nm (Feeney and Komatsu, 1964). Three zones, representing human transferrin (or hen egg conalbumin) without iron or with one or two iron atoms per molecule of protein, were demonstrated using free-boundary electrophoresis (Warner and Weber, 1953; Aisen et al., 1966), IEF (Wenn and Williams, 1968; Van Eyk et al., 1969) and starch gel electrophoresis (Stratil,

1967). The separation of these forms correlates directly with the amount of bound-iron. The most slowly migrating zone (nearest to the origin) represents transferrin without iron, the intermediate zone being transferrin with one atom of iron and the fastest zone being transferrin with two atoms of iron per molecule of protein.

Because of these findings, an experiment was carried out to determine whether the different electrophoretic mobilities of the sheep transferrin variants were affected by the amount of iron in the medium. Plasma was incubated with ferric ammonium sulphate in an amount sufficient to effect full saturation of transferrin with iron. The iron-loaded transferrins separated by PAGE had identical mobilities to their untreated counterparts, thus indicating that the mobilities of plasma transferrins were not affected by the availability of Fe^{3+} ions.

One explanation of the apparent association of transferrin phenotypes with facial eczema resistance might concern the iron binding properties of the transferrins, since iron has been shown to provide some protection against facial eczema (Munday and Manns, 1985). However, previous research has indicated little difference among the transferrins in their ability to bind and to deliver iron to tissues. Turnbull and Giblett (1961) reasoned that the transferrin variants might differ in their ability to bind and transport iron, since they are distinguished from each other on the basis of difference in electrical charge. Turnbull and Giblett (1961) therefore added increments of Fe^{59} to human serum containing transferrins C and D_3 and then measured the radioactivity in the two transferrins separated by electrophoresis. They also determined the rate of iron uptake by measuring the radioactivity of the two transferrins sampled at intervals after the addition of a constant amount of Fe^{59}. These <u>in vitro</u> experiments failed to reveal any measurable differences

in the amount and rate of iron-binding by transferrins C and D_3. In vivo tests were also performed by intravenous injection of mixtures of different transferrins selectively labelled with Fe^{55} and Fe^{59}. In all instances, the rates of iron clearance and the percentage of iron delivered by the different transferrins and utilised by the bone marrow for haemoglobin production were essentially the same. It was therefore concluded that any difference among the transferrins in iron-binding and transport must be small.

4-3.5 Association between Transferrin Phenotype and Facial Eczema Resistance or Susceptibility

The association of transferrin alleles with facial eczema resistance or susceptibility may be interpreted in the following ways: a) a physiological role of transferrins in the mechanism of resistance or susceptibility of the animal to facial eczema, or b) linkage of a transferrin gene to a gene conferring facial eczema resistance or susceptibility.

A clear association of transferrin A with a single gene responsible for facial eczema resistance appears unlikely as there were only three A alleles present in the 11 resistant sires used for the random mating experiment. Similarly the existence of resistant animals with a DD phenotype suggests that the mechanism of resistance is not solely determined by the A allele nor that facial eczema susceptibility is determined by the D allele. Therefore, it appears that the mechanism of resistance or susceptibility may be multifactorial and that the A allele or D allele may only be associated with one component of the processes responsible for resistance or susceptibility. The possibility of a linkage between the transferrin genes and a gene which determines facial eczema resistance or susceptibility can not be

overlooked. However, as the transferrins are inherited as multiple alleles at a single locus, it is unlikely that facial eczema resistance is due to a common expression of transferrin A allele and a gene conferring facial eczema resistance.

The physiological role of transferrins in the mechanism of resistance or susceptibility to facial eczema may not be directly related to the iron-binding or transport properties of the molecule but rather to some other unidentified effects, possibly related to interaction with other genes. Such interactions might affect the response of the animal to facial eczema. It is possible for example that transferrin A may have an association with an enzyme which is involved in the detoxication mechanism of sporidesmin, although no other evidence supports this view.

The preliminary results indicate that transferrin phenotypes may provide a means of screening ewe flocks to decrease the proportion of susceptible animals. In selecting animals for facial eczema resistance, it will be important to assess the productivity characteristics of the selected animals. It would be important to assess the effects of transferrin type on lamb number, growth and wool yield. It may be reasonable to sacrifice some production for the sake of increasing resistance to facial eczema; but if facial eczema resistance is genetically linked, for example, to low growth rates or decreased lambing, it would be more sensible to tolerate the disease and try to reduce losses by other preventive measures.

A larger study of the association of transferrin phenotype with facial eczema resistance is in progress. Rams with transferrin phenotypes AA and DD have been crossed with random ewes. The resistance status of the progeny from these crosses will be determined to investigate the association of the A allele with facial eczema resistance and the D allele with facial eczema

susceptibility. If the association can be confirmed, transferrin phenotypes may be of use in breeding programmes for facial eczema resistance.

CONCLUSIONS

Detection and selection of resistant stock would provide a lasting solution to the facial eczema problem. However, the search for the basis of inherited differences between resistant and susceptible animals is probably the most difficult step. If such differences reflect qualitative or quantitative changes in proteins, high resolution 2-D PAGE provides the potential for detection. If it is possible to identify a biochemical marker for resistance, this might provide a means of screening flocks to eliminate susceptible animals, and thus avoid the tedious and time-consuming progeny-testing procedures which are currently used.

Results obtained thus far indicate a possible association of transferrin phenotype with facial eczema resistance. It is not surprising to find such an association as it has been shown that transferrin phenotypes are associated with various production and reproductive traits in sheep (Rasmusen and Tucker, 1973; Atroshi, 1979). If the association between transferrin phenotype and facial eczema resistance can be confirmed, it thus may be of use in breeding programmes for facial eczema resistance. Further studies of the association of liver proteins with facial eczema resistance are also desirable as it may be expected that such change reflects mechanisms responsible for the extent of sporidesmin-induced liver damage.

APPENDIX

6-1 Reagents for IEF

Solutions (recommended volume for each solution is indicated)

1. Dichromate Cleaning Solution

	Amount
Potassium dichromate	15 g
Deionised water	250 ml
Sulphuric acid, concentrated	400 ml

Store solution inside fume cupboard.

2. 30% (w/v) Acrylamide for IEF Gels

	Amount	Final Concentration
Acrylamide	56.76 g	28.38% (w/v)
Bis-acrylamide	3.24 g	1.62% (w/v)

Add deionised water to 200 ml, filter solution through Whatman #1 paper and store solution in opaque bottle at 4°C.

3. 10% (w/v) Triton X-100 Solution

Dissolve 10 g of Triton X-100 in deionised water to a total volume of 100 ml. Triton X-100 is a liquid and must be weighed. Store solution at 4°C.

4. Pharmalytes

40% (w/v) Pharmalyte solutions are used as supplied and are stored at 4°C.

5. 10% (w/v) Ammonium Persulphate

Dissolve 0.1 g of ammonium persulphate in 1 ml of deionised water. Make up fresh solution each week. Store solution at 4°C.

6. Anode Electrolyte Solution (0.04 M H_3PO_4)

Mix 2.25 ml of 85% H_3PO_4 in deionised water to a total volume of 1000 ml. The pH should be around 1.8. The solution can be stored at room temperature.

7. Cathode Electrolyte Solution (0.1 M NaOH)

Boil deionised water for 10 min. Dissolve 2 g of NaOH pellets in 5 ml of deionised water. Add the boiled deionised water to 5 ml of NaOH to make it up to 500 ml. Boil the solution for additional 5 min. Evacuate solution and store under vacuum at room temperature before use on the same day.

8. Arginine / Lysine / Aspartic Acid Mixture

	Amount	Final Concentration
Arginine monohydrochloride	0.158 g	0.075 M
Lysine monohydrochloride	0.137 g	0.075 M
Aspartic acid	0.100 g	0.075 M

Add deionised water to 10 ml and store solution at 4°C.

9. Gel Overlay Solution (8 M urea)

Dissolve 0.48 g of urea in 1 ml of deionised water. Store solution at -20°C.

10. IEF Buffer

	Amount	Final Concentration
Urea	2.850 g	9.50 M
Triton X-100	0.100 g	2.0% (w/v)
Pharmalytes pH 3-10	0.250 ml	2.0% (w/v)
pH 4-6.5	0.125 ml	1.0% (w/v)
pH 5-8	0.125 ml	1.0% (w/v)
Dithiothreitol	0.039 g	0.05 M

Add deionised water to 5 ml and store solution as 0.2 ml aliquots at -20°C.

11. 2-D PAGE Sample Buffer

	Amount	Final Concentration
SDS	0.5000 g	5% (w/v)
Dithiothreitol	0.1950 g	0.125 M
Tris	0.1890 g	0.156 M
Bromophenol blue	0.0005 g	0.005% (w/v)

Add 5 ml of deionised water. Adjust solution to pH 6.8 with concentrated HCl. Add deionised water to 10 ml and store solution at -20°C.

12. IEF Sample Buffer

	Amount	Final Concentration
Triton X-100	0.4000 g	40.0% (w/v)
Pharmalytes pH 3-10	0.1250 ml	5.0% (w/v)
pH 4-6.5	0.0625 ml	2.5% (w/v)
pH 5-8	0.0625 ml	2.5% (w/v)

Add deionised water to 1 ml and store solution at 4°C.

13. Sample Overlay Solution

	Amount	Final Concentration
Urea	0.5400 g	9 M
Pharmalytes pH 3-10	0.0250 ml	1.0% (w/v)
pH 4-6.5	0.0125 ml	0.5% (w/v)
pH 5-8	0.0125 ml	0.5% (w/v)

Add deionised water to 1 ml and store solution at -20^{o}C.

6-2 Reagents for SDS-PAGE

Solutions (recommended volume for each solution is indicated)

1. SDS Sample Buffer

	Amount	Final Concentration
Tris	1.51 g	0.125 M
Glycerol	20.00 g	20% (w/v)
Dithiothreitol	1.56 g	0.100 M
SDS	10.00 g	10% (w/v)

Add to 75 ml of deionised water. Adjust to pH 6.8 with concentrated HCl. Add deionised water to 100 ml and store as 10 ml aliquots at -20°C.

2. Lower Gel Buffer (pH 8.8)

	Amount	Final Concentration
Tris	90.85 g	1.5 M
SDS	2.00 g	0.4% (w/v)

Add to 375 ml of deionised water. Adjust to pH 8.8 with concentrated HCl. Add deionised water to 500 ml. Filter solution through Whatman #1 paper and store solution at 4°C.

3. Upper Gel Buffer (pH 6.8)

	Amount	Final Concentration
Tris	30.3 g	0.5 M
SDS	2.0 g	0.4% (w/v)

Add to 375 ml of deionised water. Adjust to pH 6.8 with concentrated HCl. Add deionised water to 500 ml. Filter solution through Whatman #1 paper and store solution at 4°C.

4. 30% (w/v) Acrylamide for SDS Gels

	Amount	Final Concentration
Acrylamide	116.8 g	29.2% (w/v)
Bis-acrylamide	3.2 g	0.8% (w/v)

Add deionised water to 400 ml. Filter solution through Whatman #1 paper and store solution in opaque bottle at 4°C.

5. 1% (w/v) Agarose in SDS Sample Buffer

Dissolve 0.1 g of agarose in 10 ml of SDS sample buffer (stock SDS sample buffer diluted with equal volume of deionised water) by heating in a boiling water bath. Solution can be stored at 4°C for future use.

6. SDS Electrode Buffer (10 x stock)

	Amount	Final Concentration
Tris	15.15 g	0.25 M
Glycine	72.00 g	1.92 M
SDS	5.00 g	1% (w/v)

Add deionised water to 500 ml. The pH should be 8.3. Do not attempt to readjust pH. If the pH is off by more than 0.2 unit, discard. Buffer is stored at 4°C. Dilute 1:9 with deionised water before use.

6-3 Reagents for PAGE

Solutions (recommended volume for each solution is indicated)

1. Sample Buffer

	Amount	Final Concentration
Tris	1.51 g	0.125 M
Glycerol	20.00 g	20% (w/v)

Add 75 ml of deionised water. Adjust to pH 6.8 with concentrated HCl. Add deionised water to 100 ml and store solution at 4°C.

2. Lower Gel Buffer (pH 8.8)

	Amount	Final Concentration
Tris	90.85 g	1.5 M

Add 375 ml of deionised water. Adjust to pH 8.8 with concentrated HCl. Add deionised water to 500 ml. Filter solution through Whatman # 1 paper. Store solution at 4°C.

3. Upper Gel Buffer (pH 6.8)

	Amount	Final Concentration
Tris	30.3 g	0.5 M

Add 375 ml of deionised water. Adjust to pH 6.8 with concentrated HCl. Add deionised water to 500 ml. Filter solution through Whatman # 1 paper. Store solution at 4°C.

4. 30% (w/v) Acrylamide for Non-dissociating Gels

	Amount	Final Concentration
Acrylamide	116.8 g	29.2% (w/v)
Bis-acrylamide	3.2 g	0.8% (w/v)

Add deionised water to 400 ml. Filter solution through Whatman # 1 paper. Store solution in opaque bottle at 4°C.

5. Electrode Buffer (10 x stock)

	Amount	Final Concentration
Tris	15.15 g	0.25 M
Glycine	72.00 g	1.92 M

Add deionised water to 500 ml. The pH should be 8.3. Do not attempt to readjust pH. If the pH is off by more than 0.2 unit, discard. Buffer is stored at 4°C. Dilute 1:9 with deionised water before use.

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