THE INFLUENCE OF ACID AND DIRECT AZO DYES AND THEIR INTERMEDIATES ON THE DEGRADATION OF WOOL KERATIN

The characterisation by yarn strength measurements of the degradation of wool under conditions relevant to dyeing and of the keratin degradation products, by fractionation, electrophoresis and amino acid analysis

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SUMMARY

The degradation of wool keratin under conditions relevant to those of wool dyeing was investigated* using the techniques of gel permeation chromatography (GPC), ion exchange gel chromatography, and amino acid analysis.

Physical testing of the treated and untreated wool was also carried out to determine the physical changes occurring, parameters used being percentage elongation at the break, and the breaking strain of the fibre.

Samples of wool keratin were immersed in various aqueous solutions at 100°C for 24 hours and the filtered, aqueous, oxidised extracts were analysed* . The solutions used varied only in the dye, or dye intermediate present in the treatment solution. All treatment baths contained

10% owf 1.02×10^{-2} M Sulphuric VI acid;

10% owf 7.04×10^{-3} M Sodium sulphate VI ;

A 100 : 1 liquor ratio was used in each case.

Some of the dye intermediates showed a marked catalytic effect, particularly in their effect on breaking strain, a decrease of 40% in some cases.

The GPC profiles of the extracted proteins were examined in detail and compared against previous workers' results.

An explanation of the behaviour of the dyes and intermediates was proposed.

The amino acid composition data of the extracted and fractionated proteins were compared against various morphological components extracted by other workers, as was the total gelatin obtained from each treatment.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Classification of Proteins

Proteins are common to all living systems. They are macromolecules, and in common with many naturally occurring macromolecules, they are polymers. They are distinguished from peptides and polypeptides by the arbitrary assignment of a lower limit for their molecular weight which is set at 5000. The term 'polypeptide' is reserved for substances of lower molecular weight, formed from similar units.

Proteins are classified according to their solubility properties. Distinct boundaries, unfortunately, do not exist and, as a consequence, the system is limited (1). Proteins are divided into two general classes:

The simple proteins, defined as those yielding only α-amino acids and their derivatives on hydrolysis, and

the conjugated proteins, defined as those which contain a protein molecule bound to an organic non-protein prosthetic group.

The wool fibre, i.e. wool keratin, is a simple protein and belongs with collagen to a class called the scleroproteins. These are insoluble in water or salt solution but are soluble in aqueous solutions of strong acids or strong alkalis. The keratins which are the major constituents of skin, hair, and other epidermal structures, contain variable amounts of sulphur-containing amino acids and are frequently rich in basic amino acids.

1.2 Occurrence of Keratins

All mammalian hairs belong to the same family of proteins, the keratins, and are closely related in chemical structure to all types of epithelial cells such as horn, skin, and quills of feathers. Some indication of the characteristics of wool are given in table 1.1 which shows the variability in length and diameter which is possible.

TABLE 1.1

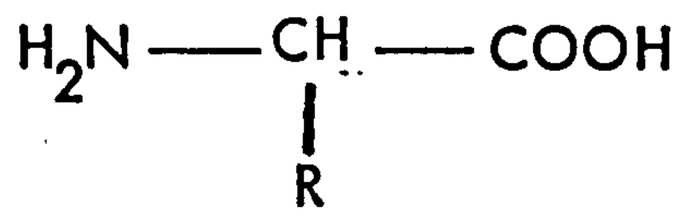
Type	Breed	Average Length (in)	Average Diameter (μ)
Fine	Merino	1.5 - 4	10 - 30
Medium	Cheviot	2 - 4	20 - 40
	Suffolk		
Long	Cotswold	5 - 14	25 - 50
	Leicester		
Crossbred	Corriedale		20 - 40

CHAPTER 2

STRUCTURE OF WOOL

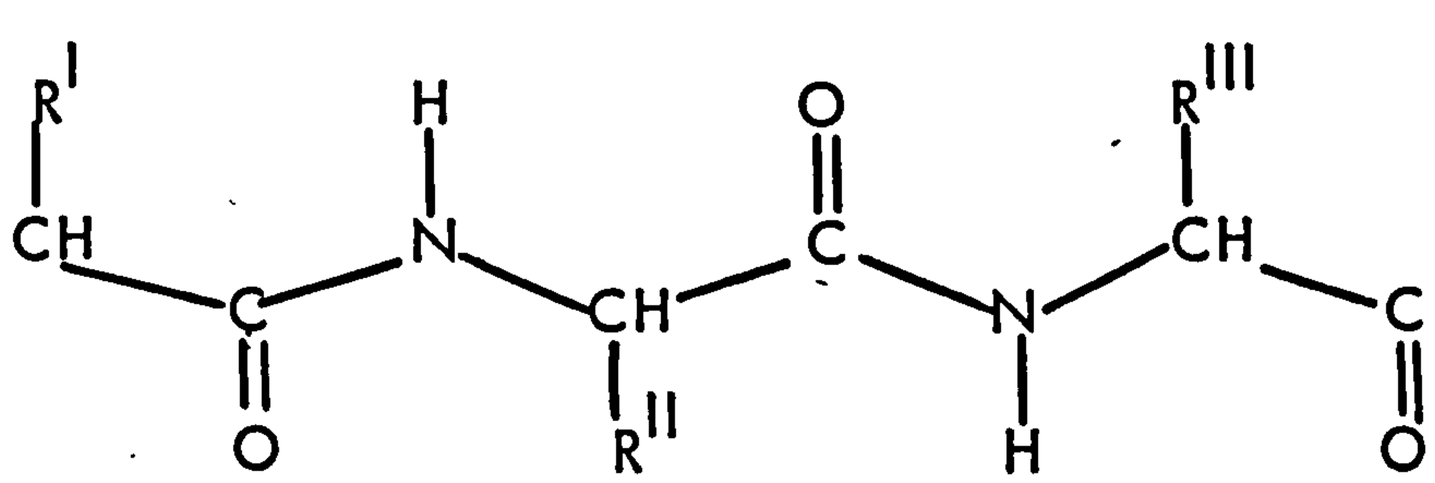
2.1 Chemical Structure

The structure of wool is well documented and many references cover this topic in far more detail than described here (2). Wool keratin is composed of α-amino acids, the general formula of which is



(except for proline and cystine).

The simplest structure of the keratin is that of the polypeptide chain formed by the condensation of α-amino acids, with the residues arranged in the following diagrammatic manner:



etc.

The amino acids which are constituents of wool keratin are listed according to type in table 2.1, which also indicates the nature of the side chain.

When wool is oxidised with peracetic acid and dissolved in dilute ammonia solution, 10% of the keratin remains as an insoluble fraction known as β -keratose. This has a low sulphur content (2.2%) and has been identified with the cortical-cell membranes; 60% of the solution can be precipitated with acid or electrolyte as a fraction with a high molecular weight and a low sulphur content (2.5%) known as α-keratose. The fraction remaining has a low molecular weight and a high sulphur content (6.1%) and is known as γ keratose. The high molecular weight fraction, low in sulphur (α-keratose), has proved to consist of fibrous molecules capable of

regeneration into fibres, whereas the sulphur rich fraction consists of globular molecules (3). Alexander concluded that a keratin fibre was composed of two types of material, one forming the fibrous structure and the other providing the cement for crosslinking in three dimensions. By means of the electron microscope Mercer and Birbeck obtained visual evidence for this theoretical model (4). They found that hair consisted of densely packed microfibrils set in a cross-linked amorphous matrix. Since the pioneering work of Astbury (5) much work has been carried out on the structure of wool keratin using X-rays. Astbury concluded that the long polypeptide chains are thin compared to the length and lie with their axes roughly parallel to the axis of the fibre. He also postulated that these chains were folded in the unstretched state (α-keratin), and that the repeat distance for the folds was 5.14 A. He gained support for this idea by showing that stretched wool has a repeat distance of 3.3 A (very similar to the 3.5 A calculated for the unfolded chains) (5).

As knowledge of the bond distances and interbond angles in polypeptide chains increased, it became clear that the folded chain structure proposed by Astbury was possible. In 1951 Middlebrook (6) suggested a hexagonal closely packed system of chains, and then Corey and Pauling (7) proposed a configuration known as the α helix in which 5 turns occupy 27 A in length and consist of 18 residues. The observation of a 1.5 A spacing, corresponding to the vertical separation of successive amino acid residues gave support for this structure, and this is generally accepted as the basic structure of wool.

This simple helix, however, does not account for the 5.14 A spacing found by Astbury, and in order to overcome this difficulty Corey and Pauling (1956) and Crick (1953) (6) suggested that the structure was in fact a coiled coil, the axis of the helix being itself helical. Corey and Pauling (7) suggested that the coiled

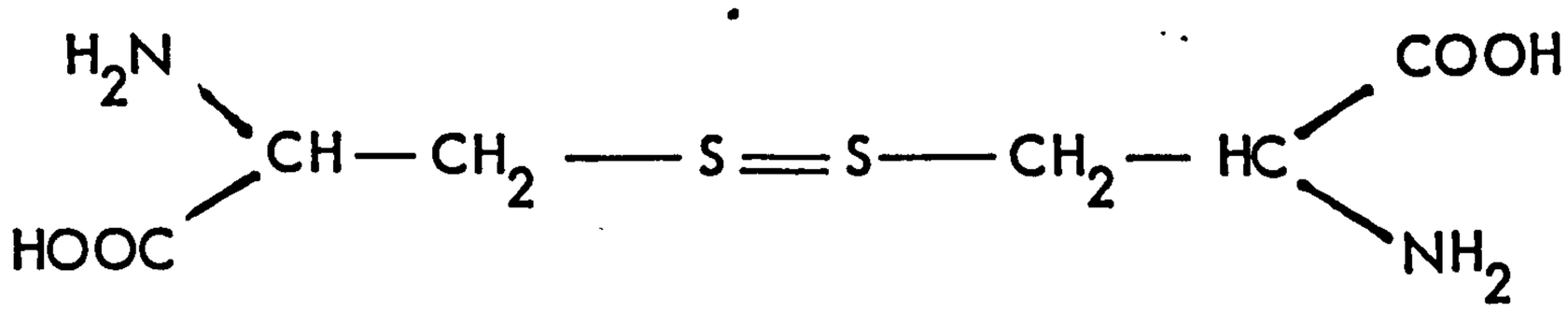
coil was a seven stranded cable, whereas Fraser and McRae (8) have developed Crick's proposal of a three stranded cable wool keratin then, the protein from which the fibre is made, consists of the condensation products of 18 amino acids, in long polypeptide chains. The quantities of the various amino acids change greatly with the type of keratin (37) etc. and Simmonds (10) and Corfield and Robinson (11) have shown that this can also vary with or within a given quantity of wool. There are probably several different types of polypeptide chains in wool keratin having a mean molecular weight of 60,000 (12). Many of the side chains (the amino acid residues) are bulky, and hence close packing of the polypeptide chains as occurs in silk, is prevented to a large extent. This results in a degree of crystallisation of about 30 - 36%.

All polypeptide chains constituting any type of protein are terminated by a free amino group at one end and a free carboxyl group at the other, and are present in roughly equal amounts.

There are three main types of interaction which can take place between polypeptide chains. The acidic and basic side-chains can interact to form the so-called "Salt-links"

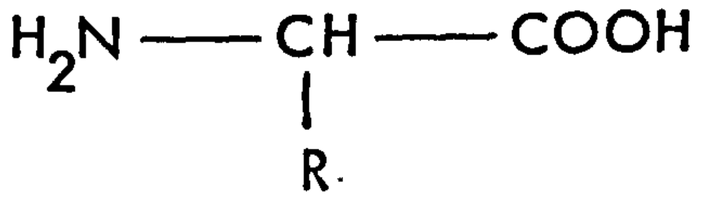
$$\text{—— } RNH_2 \,.\, HOOCR^1 \text{——} \quad \text{——} \quad \text{——} \; RNH_3^{+}\,\overline{O}OC \text{—} R^1 \text{——}$$

In addition, main chains can be bridged by strong covalent crosslinks through the bifunctional amino acid, cystine, which has the formula:



The third type of interaction occurs through hydrogen-bonding. These bonds are weaker than covalent links but stronger than Van der Waals forces between non-bonded atoms. The two previous types of bonding both occur in the same plane,

TABLE 2.1

General formula 

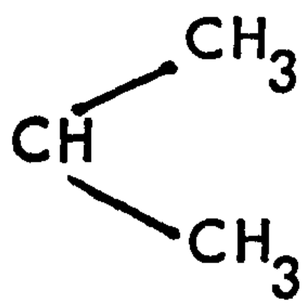
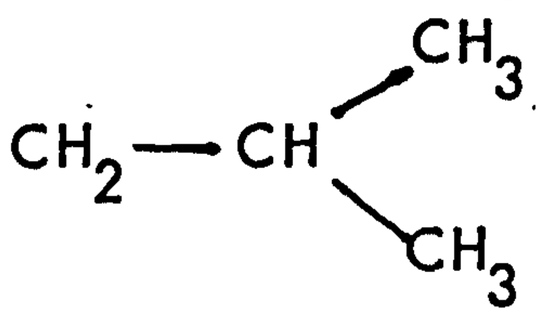
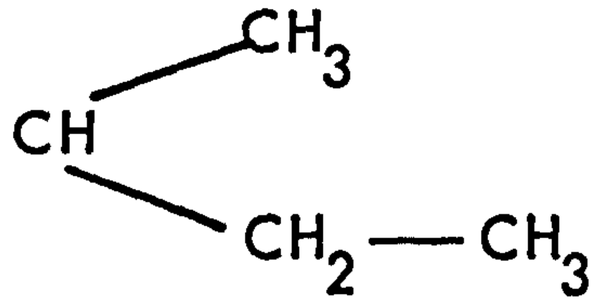
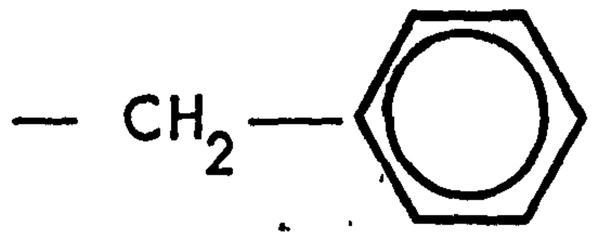
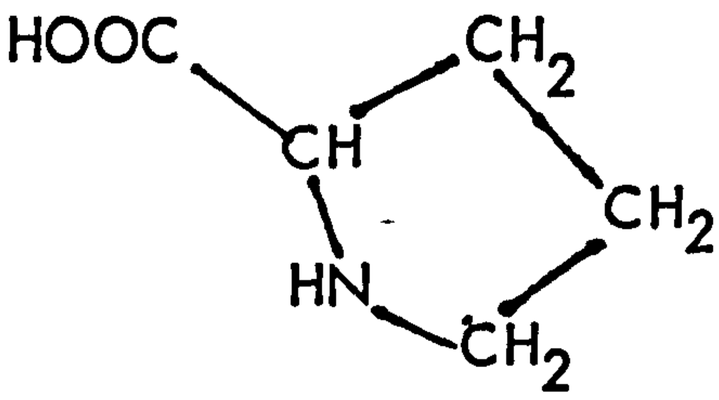
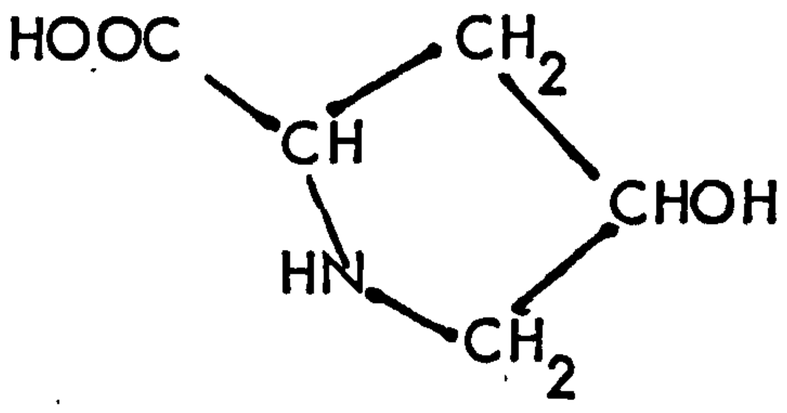
Amino Acid	Formula R =	Molecular Weight
	R = Hydrocarbon	
Glycine	H	75
Alanine	CH_3	89
Valine		117
Leucine		131
Isoleucine		131
Phenylalanine		165
	IMINO ACIDS	
Proline (imino acid)		115
Hydroxy proline (imino acid)		131

Table 2.1 continued

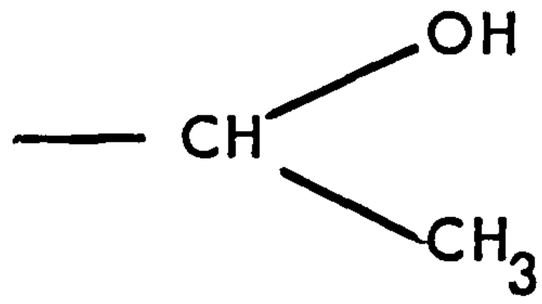
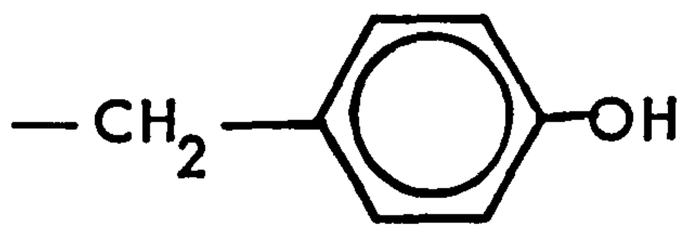
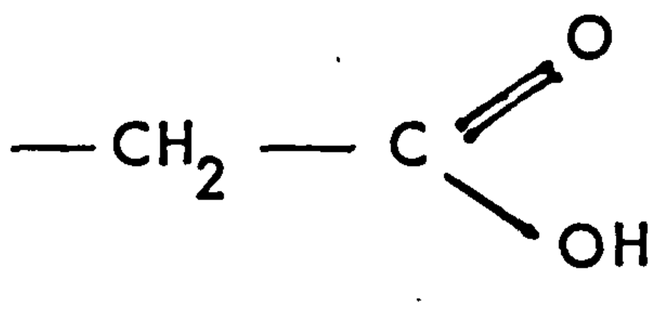
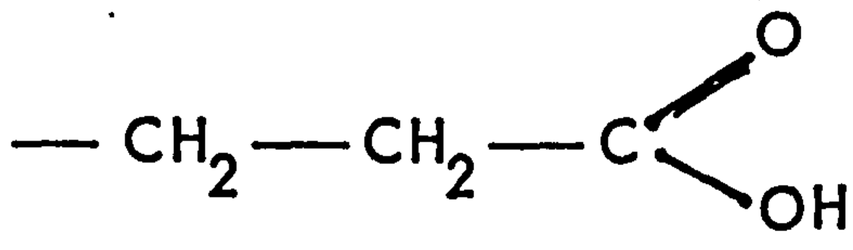
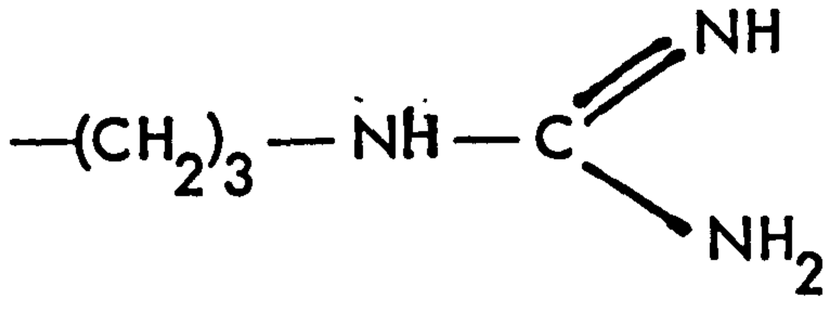
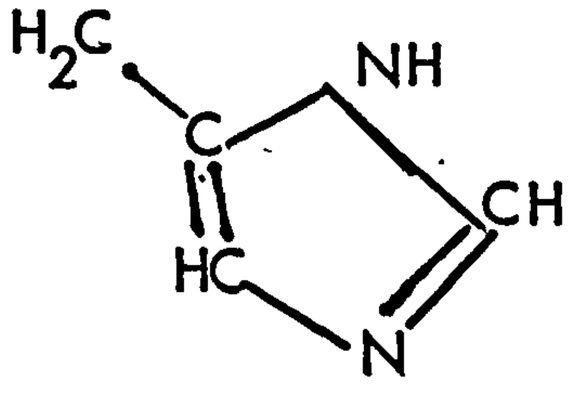
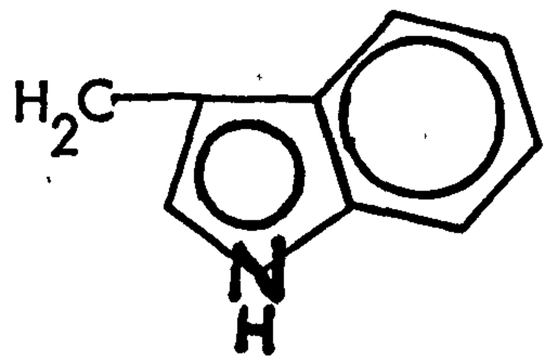
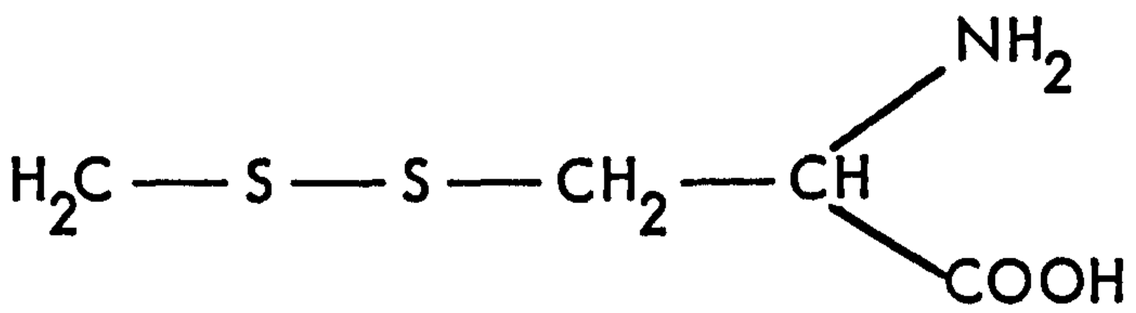
Amino Acid	Formula R =	Molecular Weight
	Hydroxyl containing	
Serine	CH_2OH	105
Threonine		119
Tyrosine		181
	Acidic side groups Free Acid and as the Acid Amide	
Aspartic Acid and Asparagine		133
Glutamic Acid and Glutamine		147
	Basic	
Lysine	$-(CH_2)_4NH_2$	146
Hydroxylysine	$-(CH_2)_2-CHOH.CH_2\ NH_2$	162
Arginine		174
Histidine		154

Table 2.1 continued

Amino Acid	Formula R =	Molecular Weight
	Heterocyclic	
Tryptophan		204
	Sulphur containing	
Cystine		240
Methionine	$-CH_2-CH_2-S-CH_3$	149

with hydrogen bonding however occurring at right angles to this plane (12).

The extreme resistance of keratin fibres to degradation by enzymes or other proteolytic agents is well known and is related to their protective function in nature. This resistance has been attributed firstly to the complex histological structure of fibres, in which the various components tend to be complementary in their inertness toward chemicals. Secondly the disulphide crosslinks produce a compact three dimensional network, the stability of which increases with an increase in the cross-link density from one residue in ten (average for the wool fibre) to one residue in five for the exocuticle from wool.

The discovery of a cross-link between the side chains of lysyl and glutamyl residues in insoluble fibrin (13a, 13b, 13c), has led to its observation in the medulla and in wool fibres (14) . It is clear that the -glutamyl (-lysyl) cross-link is the major source of stabilization of the medulla, which is almost devoid of disulphide bonds. The extent of its effect in components containing disulphide bonds is largely to be determined. It is believed that an important contribution of the disulphide bond is in bridging the polypeptide chains of the microfibrils and the matrix.

Van der Waals forces, a generic term applied to a multitude of interactions, are split into three main types: London, or dispersion forces, Keeson or dipole-dipole interaction, and Debye or induction energy (dipole-induced dipole) (15). The London attraction exists between all atoms as a result of the interaction between instantaneous but non-permanent dipoles. London showed that these dispersion forces (16) tend to bring identical groups into contact. This may be of importance for proteins in connection with the detailed structure and composition of the non-polar micelles which arise from hydrophobic interaction. Thus, one might assume that the aromatic side chains of tryptophan and phenylalanine might interact, and

the aliphatic side chains of leucine, valine and alanine might likewise tend to cluster together. Therefore, those chain configurations which permit segregation of the different types of side chain would be expected to give a more stable or lower energy configuration than those not allowing it. Hydrophobic interactions are those taking place between the non-polar groups in wool on its immersion in an aqueous environment. Approximately 30 - 50% of the residues of most proteins are non-polar (17). Since the non-polar side chains are repelled by water molecules they interact to produce the least possible surface of contact to the water molecules. In this state the free energy change for the system, ΔG, given by the equation

$$\Delta G = \Delta H - T\Delta S ,$$

will be negative. Here the enthalpy change is apt to play a secondary role (18, 19) while the entropy change, $T\Delta S$, acts as the driving force. Approximately 30 - 50% of the side chains in wool are hydrophobic, or behave in a hydrophobic manner (17) i.e. alanine, valine, leucine, isoleucine, phenylalanine, methionine, cystine, cysteine, and tryptophan, even the non-polar portions of those amino acids having polar side chains, glutamic acid and lysine, may take part in hydrophobic interactions. Therefore, it may reasonably be expected that these forces play a large role in both the conformation a protein adopts and the stability it has to a particular environment in which it finds itself (20, 21). Wool, like any protein, takes part in such interactions, but probably on a larger scale. Since it has limited powers of movement, due to its complexity of structure, any changes which take place in its orientation are likely to be small.

Changes which arise producing extra stability are likely to be a rearrangement of the side chains in an effort to attain a low(er)-energy configuration for the environment in which it is to be treated. Since non-polar groups can interact

with each other and with the solvent only by dispersion forces, early discussion of hydrophobic interactions (16) considered it only in terms of these interactions. The interaction of non-polar side chains with water is unfavourable; that is, there is a thermodynamic tendency to contact other non-polar groups, with an accompanying decrease in their interactions with water molecules, rather than remain separated from each other and surrounded by water.

When the hydrophobic interaction occurs the order increases overall, resulting in a favourable entropy change and hence a favourable free energy of formation. All evidence supports the early suggestions of Frank and Evans (17, 18) treated later in more detail by Kauzmann and by Nemettry and Sheraga (19, 20, 21), that structural order increases near non-polar solutes. It has been shown that the presence of highly hydrogen-bonded water near non-polar solutes is favoured because of the chances of increasing the number of attractive (dispersion) forces. This results in a stabilisation of hydrogen-bonded water networks or clusters. Since this effect leads to the immobilisation of more water molecules than in pure water, it results in a negative excess entropy of solution and hence a large positive free energy of solution. Hydrophobic interaction can be considered as the partial or complete reversal of the solution process for a hydrocarbon in water. The stable conformation of a protein in water will be that in which the non-polar groups can come into contact with each other, and are thus partially or completely removed from contact with the water molecules. This process will be accompanied by a large negative free energy change, composed of a large positive entropy change and a zero or negligible positive enthalpy component.

$$\Delta G_{(HI)} = [\Delta H\ (+ve)] - [T\Delta S\ \text{large}\ (+ve)]$$

Hydrophobic Interaction — Large Negative

The free energy describes the tendency of the hydrophobic groups to adhere to each other and thus reflects the strength of the hydrophobic interaction. The character of hydrophobic interactions is evident from their temperature dependence ; at low temperatures, hydrophobic interactions become stronger as the temperature increases. The maximum strength is reached at a certain temperature which is estimated to be about 58°C for aliphatic side chains and 42°C for aromatic side chains (21). A different proposal was put forward by Klotz (22, 23). According to his view the formation of regions of ordered water in ice-like sheaths (hydrofactoids) (22) in the vicinity of non-polar groups is favoured, because such a structure would be stabilised by these non-polar groups in analogy with crystalline gas hydrates. Such ice-like sheaths would lead to the masking of reactive groups. This view experiences difficulties on both thermodynamic and structural grounds. While exposed non-polar groups lead to the ordering of water (as discussed above) the presence of large regions of this kind would lead to the aggregation of the proteins, in analogy with the formation of micelles.

In contrast, the alcoholic side chains of threonine and serine contribute to the hydrophilic nature of the protein. The acidic nature of aspartic and glutamic acids is of considerable importance in determining the chemical and physical properties of the wool, but the corresponding amides take little or no part in interaction except possibly as hydrogen bonding sites. They are, however, very susceptible to hydrolysis. A summary of non-covalent interactions is given in table 2.2 (24).

TABLE 2.2

NON-COVALENT BONDS AND INTERACTIONS IN POLYPEPTIDES

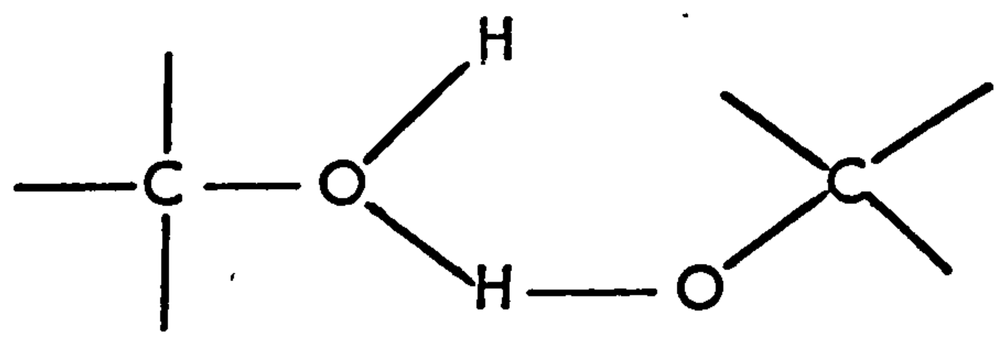
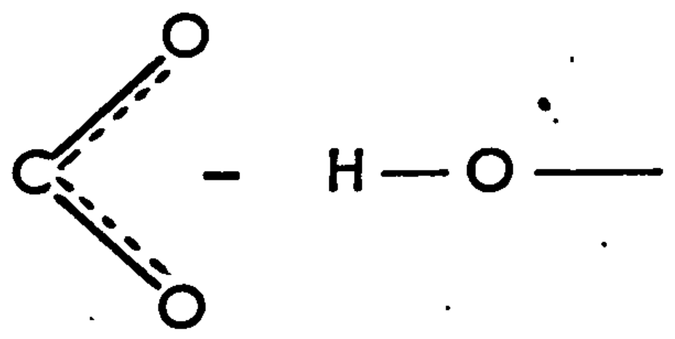
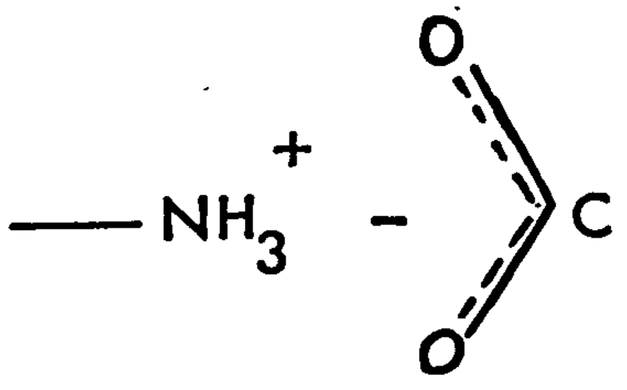
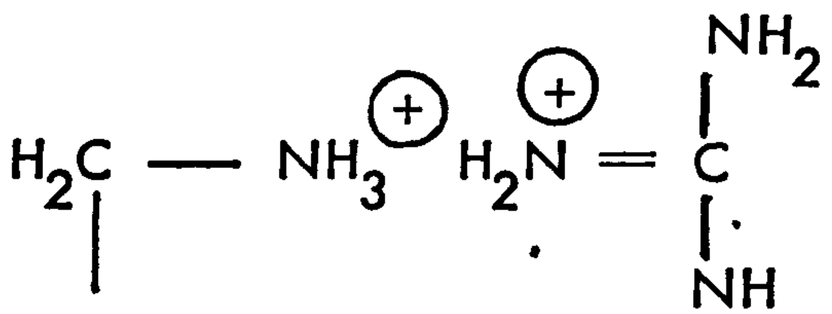
		Approximate stabilisation energy (kJ/mole)
C = O ---- H —— N	hydrogen bonds between peptides	8 - 11
	hydrogen bonds between neutral polar groups	8 - 11
	hydrogen bond between neutral polar and charged groups	8 - 11
C = O ---- HO ——	hydrogen bond between peptide and polar side chain group	8 - 11
	ionic bond between charged groups strongly dependent on distance	< 40

Table 2.2 continued

	repulsive interaction between similarly charged groups; strongly dependent on distance	< - 20

2.2 Physical Structure

2.2.1 Introduction

Morphologically, the wool fibre is complex. Grossly it consists of,

A Cuticle

B Cell membrane complex

C Cortex

D Medulla.

Fine wool fibres contain two types of cells, viz. flattened, external cuticle cells and long, polyhedral cortical cells. In coarse wool fibres and hairs there is a third cellular component, the medulla which forms a central core of interlocking cells. The cuticle cells consist of three layers, epicuticle, exocuticle, and endocuticle, and overlap in the longitudinal direction of the fibre rather like tiles on a roof. They are separated from one another and the underlying cortex by a cell membrane complex similar to that which separates the cortical cells from one another.

In fine wool fibres, the cortex is divided into two sections called the ortho-cortex and paracortex. The structure within each cortical cell is very complex since, apart from the remains of the cellular apparatus of the once living cell, labelled "nuclear remnants" in fig 2.1, there are successively smaller structures, the macro-fibril, the micro-fibril and, the protofibril, the existence of which is still controversial.

2.2.2 The cuticle

The fraction of cuticle present in keratin fibres is likely to vary considerably from one type of fibre to another and estimates have varied from 2% - 10% (25) to 20% (26). However, a more recent estimate for merino 64's fibres, based on the non-uniform distribution of citrulline in the various histological components, in conjunction with citrulline analyses of the whole fibre and the various components,

gives a value of 0.1 $\pm$ 0.03% which agrees with an estimate made from electron microscopy (27).

It has been confirmed by many workers by examination of cross-sections and longitudinal sections that the cuticle of wool is normally only one cell thick (31). The cuticle cells of wool fibres overlap both in a transverse and longitudinal directions; the degree of overlap in the longitudinal direction is about $\frac{1}{6}$ th of the length of the fibre. On the other hand, the degree of overlap with human hair is about $\frac{5}{6}$ ths so the amount of each cuticle cell that is exposed is only $\frac{1}{6}$ th of its surface (31,32). The degree of overlap in the transverse direction has not been studied in detail but can be observed readily in electron micrographs of cross-section of fibres. There is also evidence that cuticle cells can be interlocked to adjacent cuticle cells and to underlying cortex by interdigitating fingerlike projections (28 a,b, 29, 30). The cuticle often remains as an intact continuous sheath when the fibre is subjected to chemical treatment. This fact has supported the conception of a continuous sheath arising from the fusion of the cell membrane of each individual cortical cell. Scale cells are more resistant to chemical attack than are cortical cells and this is comparable with their high sulphur content and the presence of an outer protective membrane.

2.2.2a The Epicuticle

The epicuticle is only 10 A thick and is thought to be the cause of the fibre smoothness and also the basis of much of the protective nature of the cuticle. The existence of a membrane at the surface of the fibre was first shown by von Allwörden (33) who showed that the cuticle formed bubbles on the surface when the fibres were placed in chlorine or bromine water. The formation of bubbles is retarded in damaged fibres, and this has been used as a test for fibre damage. Only after the development of electron microscopy was this membrane recognised as a definite

component. The epicuticle is very resistant to chemical attack but is easily removed or damaged by mechanical handling. It was found to be responsible for reducing the rate of penetration of dyes and Lindberg (34,35) concluded that the extent of damage of the epicuticle is the important factor in determining the rate of diffusion of dyes and acids into the fibre

2.2.2b The Exocuticle

This is the cuticle layer which appears to be on the outside of the fibre since the very thin epicuticle membrane on the surface is not visible. It is considered to represent more than half of the cuticle cell content, and Bradbury and Ley (36) showed it to be about 64%. The outer part of the exocuticle consists of a dense layer, about 0.1 μ thick called the 'a' layer. This is a prominent feature of electron micrographs of stained sections of keratin fibres and has been observed by many workers (37). By using metal-staining techniques, the sulphur content of the exocuticle and endocuticle has been inferred by several workers to be in the order

'a' layer $>$ rest of the exocuticle $>$ endocuticle

This has been confirmed by amino acid analyses of the separated layers.

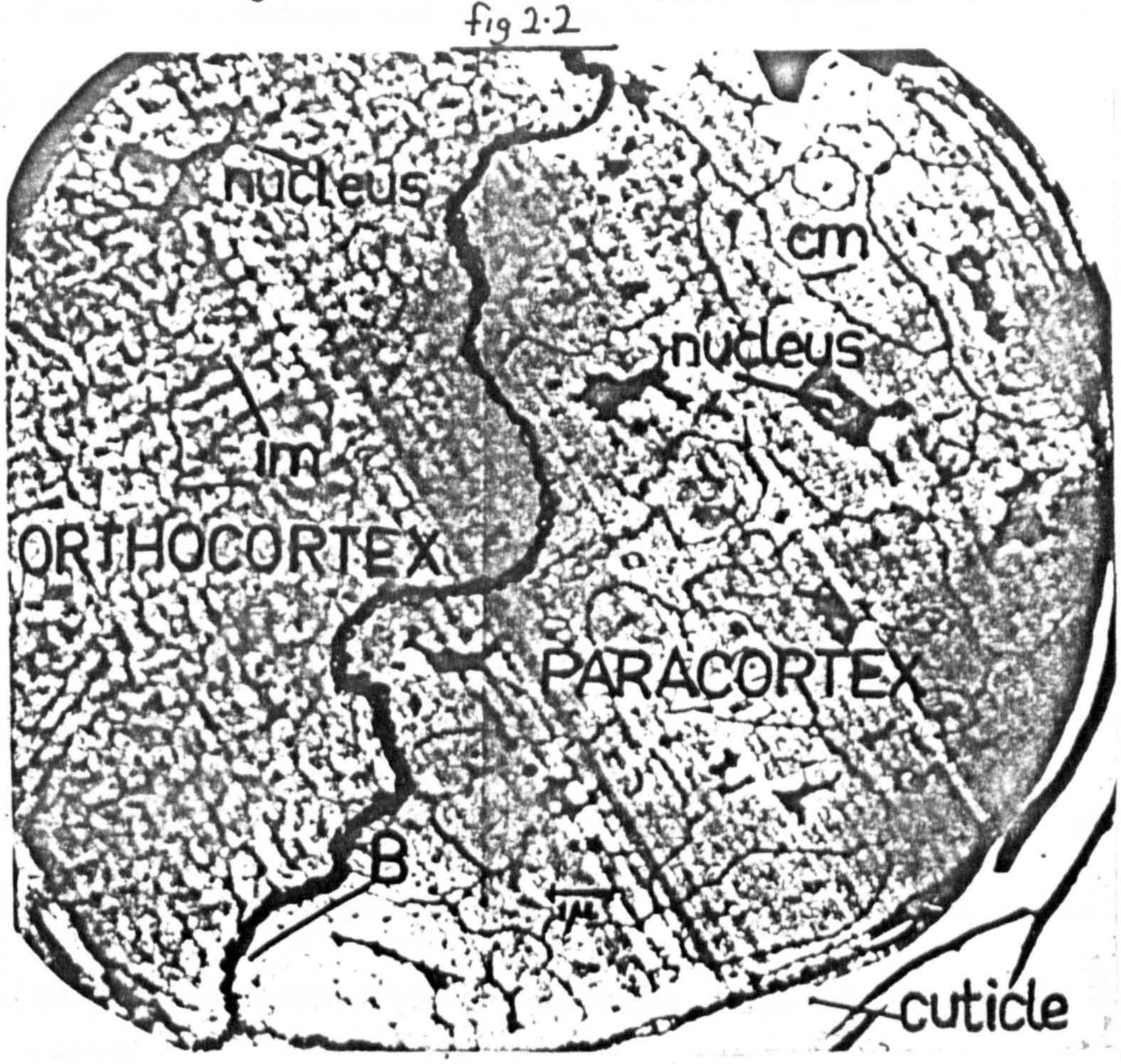
In the early work on the cuticle (38, 39) it was concluded that the exocuticle is dissolved by treatment with enzymes, and this was restated in later literature (35, 26) even after it was shown conclusively by Mercer and Birbeck (40) that this was not the case. They also showed that the exocuticle, apart from the 'a' layer, is dissolved by treatment with peracetic acid and ammonia whereas the endocuticle remains intact.

2.2.2c The Endocuticle

This is a well defined layer below the exocuticle and separated from the next underlying cuticle cell by a cell membrane complex. In merino wool it accounts

for 36% of the cuticle. It has been shown by studies on developing hair fibres that the endocuticle consists of cytoplasmic debris derived from the cytoplasm of the once living cuticle cell and as such is similar to the material labelled cyto-plasmic debris in Fig 2.2 which is intermacrofibrillar material. The latter

fig 2·2



Electron micrograph of a stained cross section of a Merino wool fiber showing the bilateral nature of the cortex. In the paracortex (separated from the orthocortex by the broken line B) the cortical cells are clearly outlined and separated from each other by the cell membrane complex (cm). Many nuclear remnants are observed in the paracortex whereas in the orthocortex the non-keratinous material of the once living cell becomes occluded during keratin synthesis and distributed around the periphery of the macrofibrils, forming inter-macrofibrillar material (*im*), hence making difficult the delineation of cortical cells in the orthocortex. From Rogers (81,82)

represents the remnants of the cytoplasm and nuclei of once living cortical cells, and they both have similar amino-acid compositions.

2.2.3 Cell membrane complex

The cell membrane complex underlies the external cuticle cells and surrounds completely the internally situated cortical cells of the fibres. It forms a network structure the extent of which can be seen using light microscopy (41).

It thus performs the function of "sticking" the cells together. The importance of this is soon realised if the cell membrane complex is partially dissolved using enzymes (42,43,44,45), or by treatment with formic acid (46,47,48), when the individual cells are liberated and the fibres gradually fall apart. The detailed structure of the cell membrane complex is shown in Fig 2.3(a)(b) It is formed in the hair follicle from the two plasma membranes of the living cells which remain separated from one another in the hardened keratin by means of an intercellular cement (see δ on diagram). A less densely stained β region is thought to consist of the original plasma membranes, in a modified state. The membranes are thought to consist of two protein layers interleaved with a lipid bilayer, the presence of which has been proven by X-ray diffraction studies, and also by extraction in formic acid for 24 hours. This treatment rapidly removes lipid from the fibres (46). The X-ray diffraction studies show a sharp 47 A equatorial arc due to the lipid (49) which disappears after the formic acid treatment but is still present after immersion of the wool for 28 days in ethanol at room temperature. The ethanol removes lipid from wool slowly over a long period (50). These experiments tend to confirm the presence of the lipid and the fact that it causes the 47 A reflection, but they do not give information about its site. The identity of the densely stained ρ layer, the intercellular cement, is also a matter of speculation with regard to both its origin as an extracellular material and its chemical composition (51). In fact the various chemical studies, which in some cases have been combined with electron microscopic examination of their effects on the cell membrane complex, have, with one exception, shown simply whether the cell membrane complex as a whole is modified. Thus, direct observations of electron micrographs have shown that the cell membrane complex is disrupted and material is extracted by treatment with,

(1) Boiling aqueous hydrochloric acid at pH2 (52).

(2) Dichloro-acetic acid at room temperature (46).

(3) Formic acid which disrupts the cortical cell membrane but not the cuticle cell membrane (48).

(4) Trypsin (48)

(5) Formamide in the presence of a reducing agent (54).

The rapid attack of formic acid at room temperature on the cortical cell membrane complex whilst leaving the cuticle cell membrane complex unchanged is the first evidence of any difference between them (48). The attack of the cell membrane complex by formic acid and enzymes is confirmed by the release of clean cuticle and cortical cells by such treatments coupled with mechanical agitation.

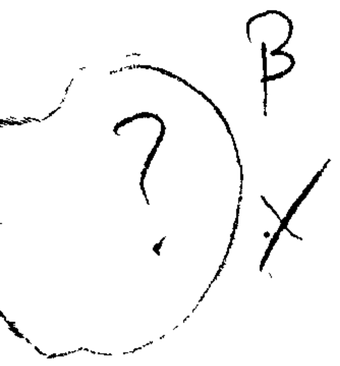
Treatment of wool with formic acid at room temperature modifies the cortical cell membrane complex preferentially as compared with the cuticle membrane complex (48) and removes about 0.8% of lipid (which probably includes some lipid from the nuclear remnants) and 0.7% of a protein of very low cystine content (27,46,56).

A protein of related composition is obtained in 0.4% yield by extraction with 50% formic acid (55) or in 2-3% yield by extraction in formamide in the presence of a reducing agent (54). A residue of about 1.5% of highly resistant membranes is obtained after removal of the rest of the fibre by treatment with performic acid followed by ammonia, and it has been postulated that this material originates from the cell membrane complex (56). Peters (48) has shown that membraneous residues are obtained from both separated cuticle and cortical cells in yields of 2.4% and 1.5% respectively, which confirms that they are distributed throughout the fibre.

It is likely that these three proteins, viz a readily extractable protein (1%), lipid (0.8%), and a highly resistant membrane (1.5%), constitute the cell membrane

complex of wool. Their total amount, about 3.3%, by weight of the fibre, agrees moderately well with a direct estimate 3.7% based on fibre cross sections (56). but is less than earlier estimates of 5 - 7% (57) and 8% (26) of the weight of the fibre. The distribution of these three components within the cell membrane complex is speculative, but one suggestion would be that each β layer contains a resistant membrane located nearest to the cell itself.

This would allow the easily degraded part of the cuticle plasma cell membrane on the exterior surface of the fibre to be lost during growth, thus exposing a resistant cell membrane on the surface. Also in the β layer would be a bilayer of lipid. The intercellular cement would then contain the readily extractable non-keratinous protein.



2.2.4 The Cortex

This is a very complex region of the fibre, far more complex than the region of the fibre discussed above. It constitutes by far the largest amount of the fibre (about 86.5% in fine wool) and is responsible for many of its important physical properties such as elasticity. A cross-section of merino wool fibre as in Fig 2 shows clearly the boundaries of the cortical cells. The paracortex with its boundary, is particularly clear.

It appears that the cortical cells are many-sided polyhedra which pack together in the cortex without leaving any free space. The free cortical cells, which may be liberated chemically, have the genefal shape of a spindle with finger-like processes at their ends, which interdigitate with adjacent cells. Interlocking in the transverse direction also occurs because of the shape of adjacent cortical cells with "horns or arms" as in ___ (58). Cortical cells next to the cuticle appear to be flattened (59) and, in medullated fibres, those adjacent to the medulla have, on the one side of the cortical cell, finger-like trabeculae that separate individual

medullary cells and hold them in place (60). The maximum width and length of cortical cells from various fibres have been examined in detail by Lockart (61) and Chapman and Short (62). There is some variability in length between different breeds and within the one sample but the approximate size for fine wool is length 95 μ and maximum width 5.5 μ.

2.2.4a Segmentation of Cortex

The bilateral segmentation of the cortex of fine wool fibres into two major components, now universally called orthocortex, and paracortex (63,44) is shown clearly in Fig 2. This dichotomy of the fibre was first fully realised by Horio and Kondo (64), who related accessibility to dyes and birefringence of the fibres in sodium hydroxide, with crimping and coiling of wool. Mercer (63) studied the differential digestibility of the fibre by enzymes. The ortho- and para-cortices are approximately hemi-cylinders wound round each other helically in phase with the crimp of the fibre, so that the para-cortex is always placed on the inside, and the ortho-cortex on the outside of the crimp. However, the sense of the helix varies, so there is little net twist (65). The important papers of Horio and Kondo and Mercer (63,64) generated a lot of further work.

The proportion of para-cortex in fine wool fibres is about 30 - 50 % of the total amount of the cortex. It increases with increase in fibre diameter until the bilateral assymetry is replaced by cylindrical assymetry. It is important to note that even in fine wool fibres the assymetry is not always uniformly bilateral.

At the boundary between the ortho-cortex and para-cortex is sometimes found a small percentage (1% - 4% of the cross-sectional area of the cortex) of cells that are intermediate in morphology between ortho-cortical and para-cortical cells. These have been called meso-cortical cells (66, 67, 68).

2.2.4b Differences in fine structure between ortho-cortex and para-cortex

There are two main differences between the ortho-cortex and para-cortex,

(1) The macrofibrils of the ortho-cortex are clearly delineated by the non-keratinous intermacrofibrillar material which surrounds them, whereas the non-keratinous material in the para-cortex is mainly located in a few large areas, the nuclear remnants. Since this non-keratinous material is easily extracted with enzymes and acids, and easily swollen because of its low content of cystine (53), ortho-cortex is much more readily penetrable by liquids than the paracortex.

(2) The microfibril matrix structure is different in the two cortices (see fig 2.4); the arrangement of the microfibril matrix structure is much more regular in the para-cortex than the ortho-cortex, and there is a larger amount of matrix relative to microfibrils in the para-cortex (fig 2.5a,b).

Since the matrix stains more heavily with metals than the microfibrils, it has been argued that the former is more heavily cross-linked with disulphide bonds (69). If this is true one would expect para-cortical cells to have a higher cystine content than ortho-cortical cells. This is, in fact, the case.

(3) A possible difference exists in the cell membrane complex between ortho-cortical and para-cortical cells though the only direct evidence to support this is that ortho-cortical cells are released preferentially by treatment with enzymes.

2.2.4c Differential Dyeing of Cortices

It is widely reported, and accepted, that both acid and basic dyes stain the ortho-cortex more heavily than the para-cortex. This is not a kinetic effect but represents the situation at equilibrium.

Since dyeing with acid dyes and basic dyes is largely a matter of binding to charged sites of opposite sign in the fibre (3), it is clear that additional charged sites in the ortho-cortex would give rise to the observed effect. The results

indicate only a small excess (of about 3%) of charged sites in the ortho-cortex, assuming the content of both asparagine and glutamine to be constant in both cortices. Also it is possible that some of the charged groups in the heavily crosslinked matrix, which predominate in the para-cortex, may be inaccessible to the rather large dye molecules (71).

When fibres are oxidised with peracetic acid, performic acid, or bromine water the para-cortex (owing to its higher cystine content) becomes more heavily charged with $-SO_3^-$ groups than the ortho-cortex, and hence one might expect an increased affinity of the para-cortex to basic dyes but not to acid dyes. This is indeed observed, since with oxidised fibres the para-cortex dyes more heavily than the ortho-cortex with basic dyes, whilst the situation is as normal for the staining with acid dyes (72,73,74). Many acid, basic, and fluorescent and other types of dyes have been used and Chapman has carried out a review of the literature (147). Because of the intrinsic nature of the ortho-cortex (i.e. its more extensive network of intermacrofibrillar material and lower cystine content than the para-cortex), it is more accessible, and more reactive chemically to almost all reagents, than the para-cortex.

This is the case despite the evidence from X-ray diffraction studies that the interchain distance within the microfibrils (which are more abundant in the ortho-cortex) increases much less (5%) in water and methyl alcohol (11%) than does the fibre as a whole (16%) (76). On this basis, the matrix swells more than the ortho-cortex at neutrality. The ortho-cortex, however, probably contains more charged groups than the para-cortex and hence one might expect it to swell more when exposed to conditions of pH well away from the iso-electric point. Furthermore, the rate of chemical reaction is dependent on transport of reactants and products of reaction through the fibre, and this is facilitated in the ortho-cortex

by the intermicrofibrillar network. Ortho-cortex dissolves much faster than the para-cortex on treatment with acids (77, 78, 45), followed by subsequent alkaline extraction using various agents from alkalis in water or ethanol to urea and sodium bisulphite. Many of these treatments, such as that with alkalis cause preferential loss of birefringence of the ortho-cortex, and this has been used very extensively to observe the ortho, and para-cortices. The only treatment that effects the para-cortex more than the ortho-cortex is oxidation with peracetic acid or bromine water, which produces more $-SO_3^-$ groups in the cystine rich para-cortex than the ortho-cortex and causes greater swelling in the para-cortex (74).

The treatment of fine wool fibres with enzymes causes dissolution of part of the cell membrane complex and liberation of ortho-cortical cells in preference to para-cortical cells (63,79). Fibres that have been reduced and ethylated and digested in pepsin, show similar preferential dissociation of the ortho-cortex as do fibre fragments from the gut of insects (80).

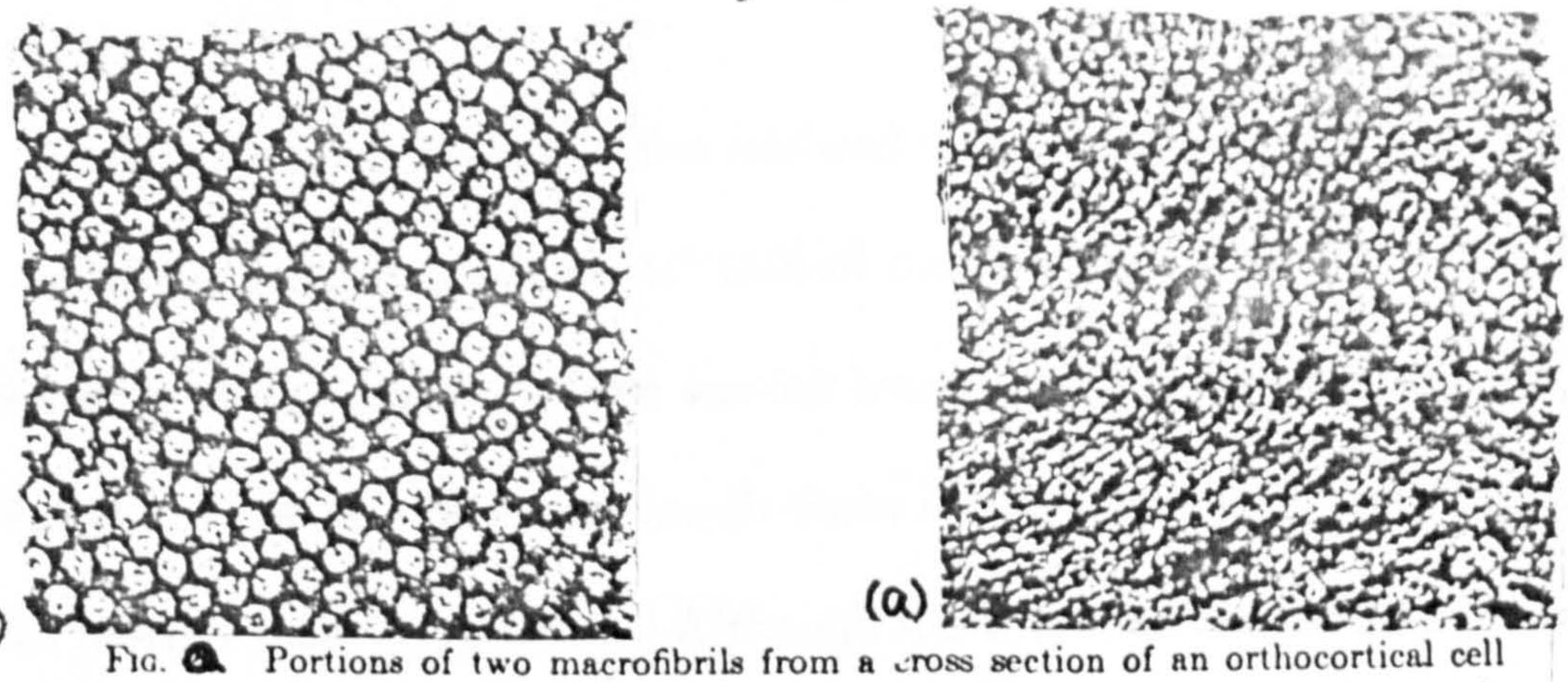
2.2.5 Cortical Components

2.2.5a Macrofibrils, Nuclear Remnants and Intermacro Fibrillar Material

The macrofibrils represent aggregates of microfibrils as observed by electron microscopy of stained cross-sections (see diagrams above). The macrofibrils in the ortho-cortex are well defined because of the abundance of the intermacrofibrillar material that normally separates them from one another. Also the microfibrils are arranged in whorls in the macrofibrils of the ortho-cortex, whereas those of the para-cortex show a common form of close packing(body centred hexagonal close packing - h.c.p.) (see fig 2.4). The macrofibrils of the para-cortex show considerable fusion into larger units (81).

fig 2.4

fig 2.4



(b) (a)

FIG. a. Portions of two macrofibrils from a cross section of an orthocortical cell of wool showing the packing of the microfibrils in cylindrical laminae or whorls with much less matrix evident than in Fig. 19. From Rogers and Filshie

FIG. b. Part of a cross section of a paracortical cell of a wool fiber at high magnification showing the regular arrangement of microfibrils separated by heavily stained matrix protein. There appears to be detail observable (dark spots) within the lightly stained microfibrils. From Rogers and Filshie (70)

The development of macrofibrils in the follicle by lateral aggregation of microfibrils and their fusion by matrix protein causes the trapping of the nuclear remnants and cytoplasmic remnants of the cells in the interstices between the macrofibrils (40,81,82,51). The cytoplasmic debris of the cell thus forms the intermacrofibrillar material, which can be more readily observed in cross-sections after partial extraction of fibres with thioglycollic acid (81,82).

The dendritic structures, which are the nuclear remnants of the cells, are much more evident in the para-cortex than in the ortho-cortex and sometimes extend laterally to the cortical cell boundaries.

2.2.5b Microfibrils and Matrix

The idea of a two-phase structure for keratin fibres, consisting of crystallites which give rise to the specific X-ray diffraction (α pattern), embedded in a matrix of high sulphur content is not new (83).

Early electron microscopy studies led to the identification of the crystallite or microfibril as the primary element of structure. Its size was estimated to be about 100 A in diameter and many times longer (84,85). The diameter is now known to be less than this, but it is important to note that the microfibril is indeed the

primary element of structure.

Fibrous proteins appear in the mid and upper bulb region of the hair follicle as "wispy clumps of filaments" (40) of diameter less than 100 A (86,87,90). The diameter of the microfibrils from various sources appears to be about the same (69,89) i.e. 60 A - 80 A, although there is one report (88) that it is probably smaller in Merino than in Lincoln. Although there is some variability in the degree of resolution and relative intensities, the main features of the X-ray diffraction pattern are common for all α-keratins (92) including all keratin fibres and various quills (26). Finally, the separation of sheets of microfibrils from wool after fission of disulphide bonds (85) and more particularly, the separation of single microfibrils from the follicle (81,9193), confirms that microfibrils possess that integrity of structure which was inferred from the original experiments of Birbeck and Mercer (40). This being the case, the postulation of a two phase structure of microfibrils embedded in a matrix is a logical one. The packing of microfibrils in the paracortex as mentioned above in some areas approximates to body-centred hexagonal close packed structure.

2.2.5c The Protofibril

There has been much argument, and it still continues, about this fundamental unit of the keratin fibres. Evidence from electron microscopy of separated filaments from α-keratin purports to show that the protofibril is a long (1 - 2 μ) structure of diameter 20 A, which consists of two or three polypeptide chains with banded segments due to disordered chains. However, these structures may result from cellulosic contamination and the controversy still rages. The currently favoured model is a two stranded rope with a coherence length of only 50 - 100 A (89).

2.2.5d Arrangement of Protofibrils within the Microfibril

A structure for the microfibril of an outer ring and central core of high electron density with annular ring between them of lower density, the so-called ring-core structure, seems to be generally accepted (94,95,89,86,87). The radius of the ring appears to be about 29 A (95). The arrangement of protofibrils around the ring is still a matter of conjecture; the core presumably consists of one or more protofibrils. The space between the ring and core has an electron density and stain density (in electron microscopy) less than that of ring and core and may contain some of the non-helical material of the low sulphur proteins. A possible structure for the microfibril has been proposed by Fraser and McRae (96).

2.2.6 The Medulla

The medulla does not occur in fine wool fibres but when present forms an axial stream of cells in the centre of the fibre. The central core may range from a small amount of material in Lincoln 36's wool to a large core in other cases, amounting to more than 15% of the weight of the wool fibre in some cases (97).

In contrast to the compact, dense structure of cuticle and cortex, the medulla is of an open texture and contains a large number of vacuoles (60,98). This results from the fact that during growth in the follicle the amount of protein synthesised is inadequate to fill the cell cavities and, during dessication of the cells, intracellular gaps occur, and the final structure becomes open and light but stiff (Mercer, 1961). In turn, this causes the formation of a lighter, bulkier, but stiffer fibre which presumably has advantages for certain animals, such as rodents (51). The medulla appears to be largely amorphous in the electron microscope (99) although there is some evidence of fibrils (98). The protein from the medulla contains a very low content of cystine, and a large content of citrulline (100,60,101).

The medulla is relatively stable toward reagents such as peracetic acid

followed by ammonia and caustic alkali. This is now attributed to the -ε-(γ-glutamyl) lysine crosslinks which have been shown to be present in medulla (37).

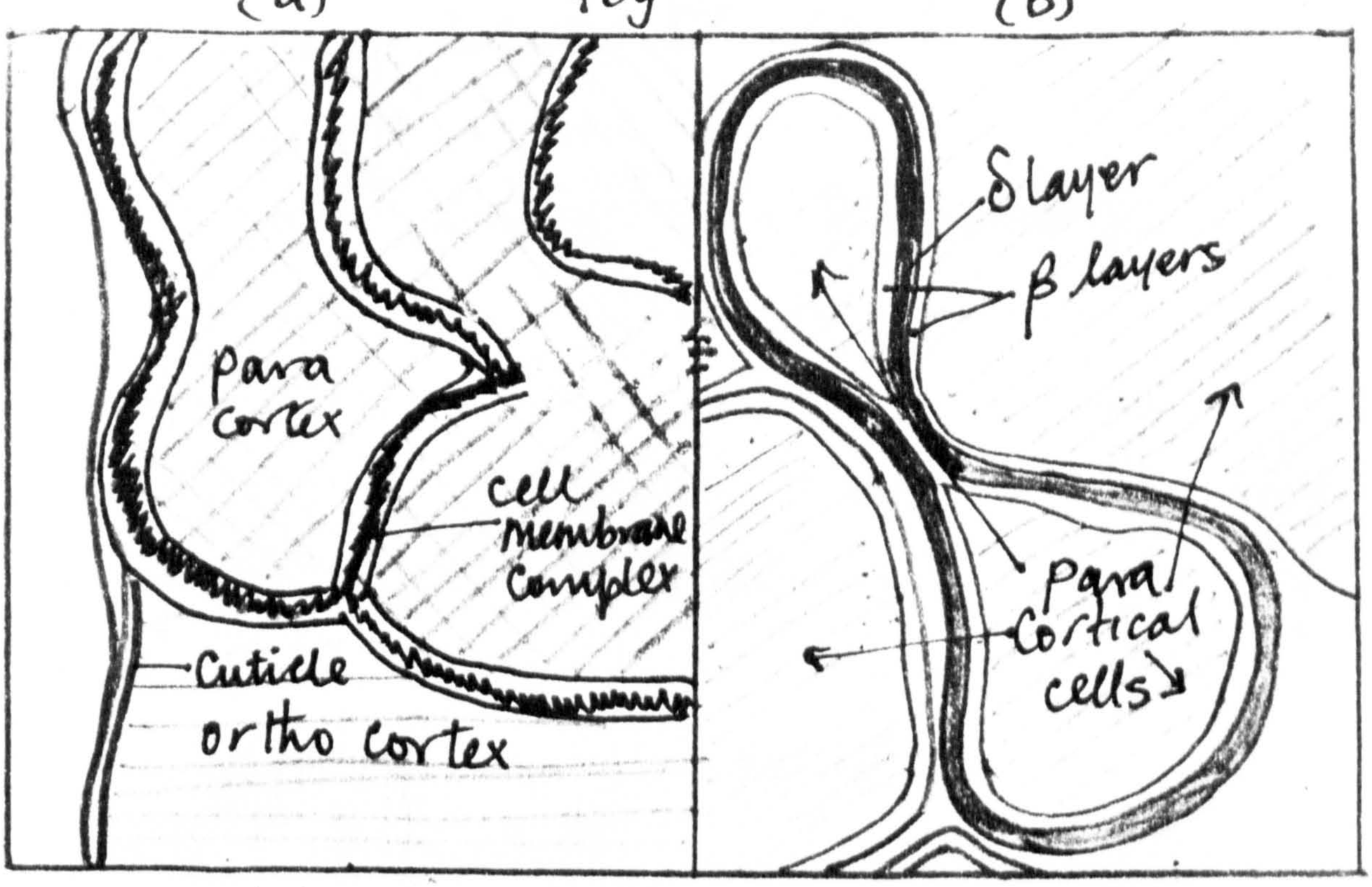


fig 2.3

CHAPTER 3

CHEMICAL PROPERTIES OF THE WOOL FIBRE

3.1 Acid Hydrolysis

The physical properties of wool are changed by treatment with acids and bases. Aspartic acid and glutamic acid, as well as serine, may be split off by partial acid hydrolysis, while most of the wool protein remains unhydrolysed. Tryptophan can be almost completely destroyed and there can be losses in threonine, serine, and cystine. This degradation of wool usually results in a loss of wet strength and, the sensitivity of wool to acid hydrolysis is increased if the cystine is transformed, by oxidation, to cysteic acid, because the peptide bond adjacent to a cysteic acid group is very sensitive to attack (103).

Acid hydrolysis is not a random cleavage of peptide bonds; instead, a degree of specificity is observed (104, 105) with the bonds involving threonine and serine being most labile (106, 107). Bonds formed by the carboxyl groups of valine, leucine and isoleucine are most stable. Synge (108) attributed this to the steric limitations imposed by the iso-propyl and iso-butyl side chains of valine and leucine on the approach of H^+ ions to the peptide bond. Hydrolysis of the peptide linkages produces free carboxyl and amino groups, a fact which is reflected in the increased capacity of the wool to combine with acids. The extent of hydrolysis is increased in the presence of anions which are attracted to the fibre (109) and this effect has been interpreted in terms of the Donnan membrane concept where more acid is present inside the fibre in the presence of neutral salts.

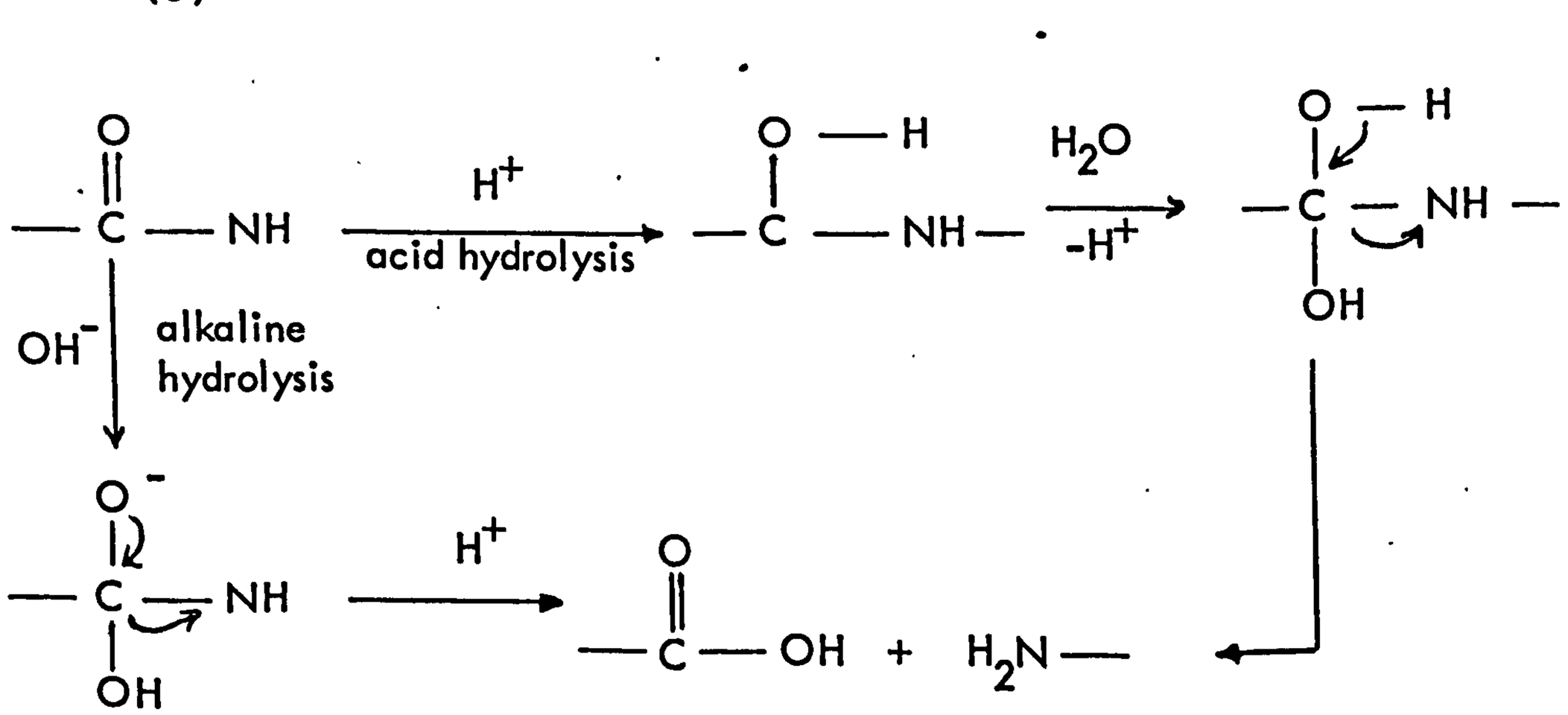
3.2 Alkaline Hydrolysis

Alkaline hydrolysis is less selective than acid hydrolysis and, in fact, 0.1 M sodium hydroxide rapidly dissolves wool at 100°C. The complete destruction of arginine, serine, threonine, cystine, and cysteine preclude the use of this method

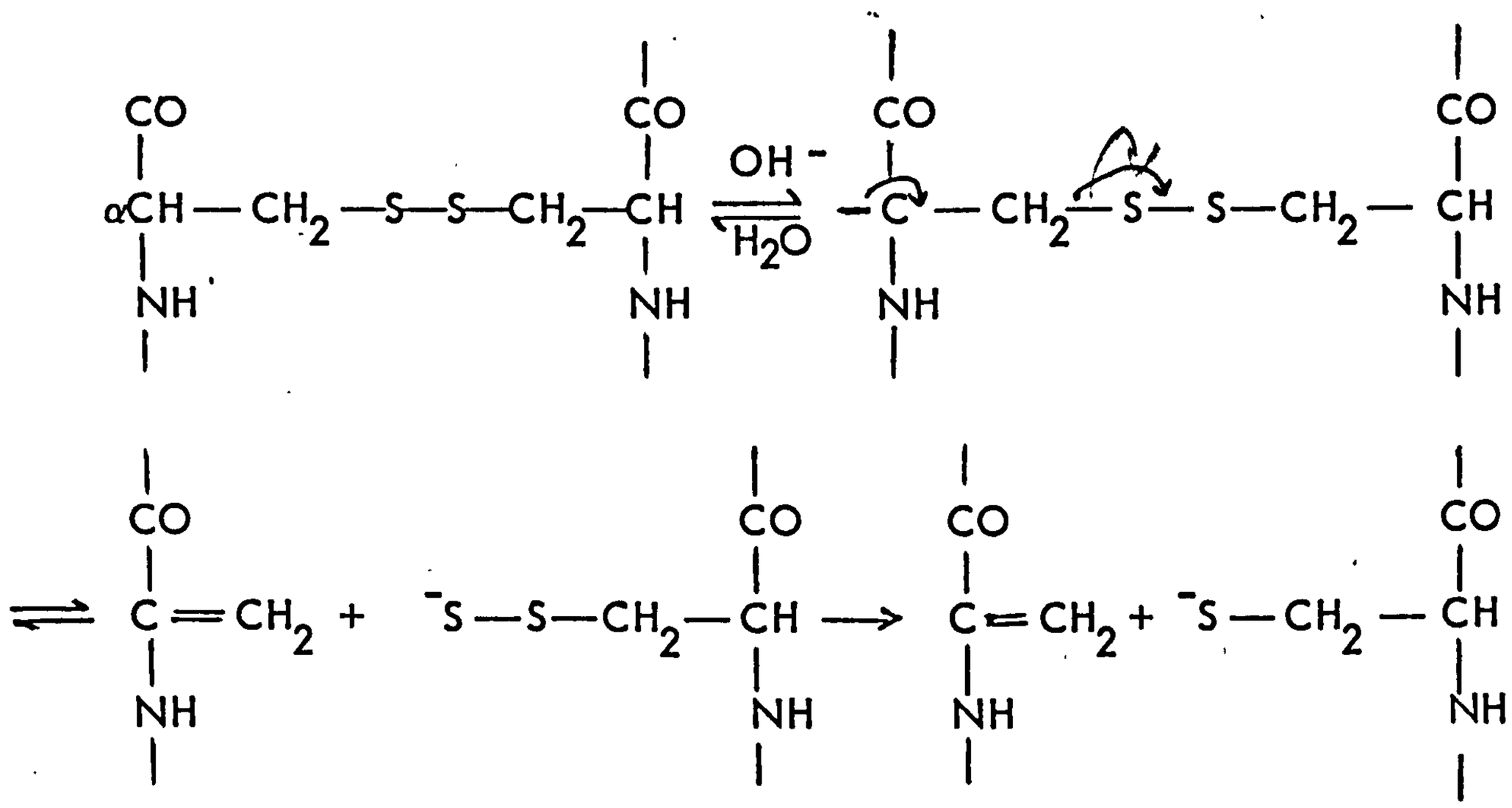
for amino acid analysis. On the other hand, tryptophan is not destroyed in alkali, and analysis of alkaline hydrolysates forms the basis of one method for the quantitative determination of this amino acid.

The extent of the reaction of keratin fibres with alkali depends upon the conditions used, such as, temperature and concentration. From the practical point of view, solubility of the fibre in alkali has been used as a parameter for assessing damage that may have occurred during wet processing. Treatment for one hour in 0.1 M sodium hydroxide at 65°C has been standardised, and the importance of temperature control has been emphasised. Intact keratin fibres exhibit fairly low solubilities. It can also be used to determine, qualitatively, the amount of cross-linking in the fibre. It is important to realise that alkali itself gives rise to new crosslinkages in the fibre so that alkali solubility can not be used to assess damage due to peptide bond hydrolysis occurring in alkaline treatments.

Hydrolysis of the peptide chain involves nucleophilic substitution, in which the —— NH —— group is replaced by —— OH. Under acid conditions hydrolysis involves attack by the water molecule on the protonated amide, whereas under alkaline conditions it involves attack by the strongly nucleophilic hydroxyl ion on the amide itself. It is generally agreed that protonation of the carboxyl oxygen rather than the amide nitrogen is predominant during acid hydrolysis of amides (6).



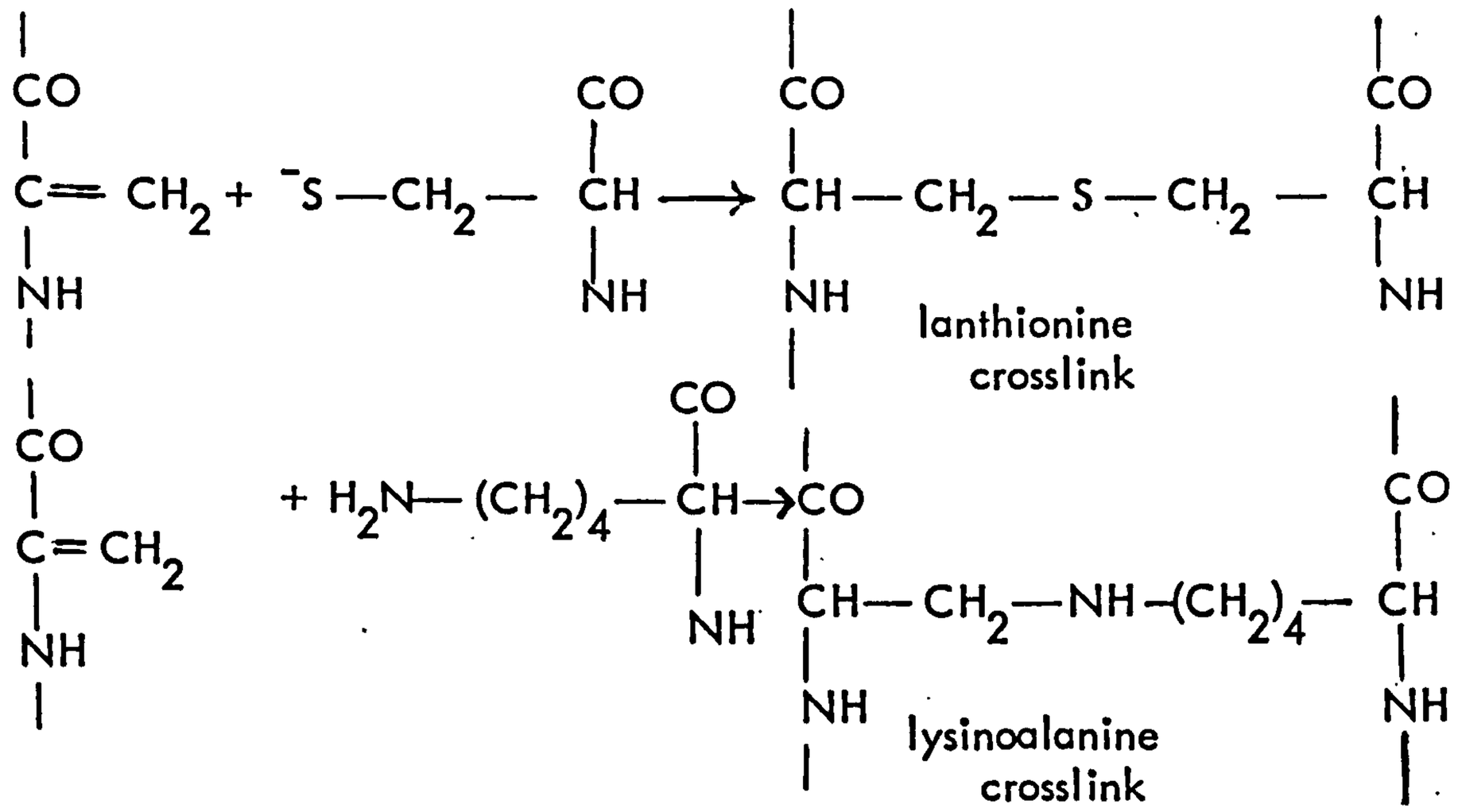
From alkali treated wool, three new amino acids have been isolated, namely, lanthionine, lysinoalanine and β-aminoalanine (111, 112, 113, 114). The most probable mechanism for the formation of these amino acids is by alkali catalysed β-elimination of the disulphide group. This is initiated by a proton abstraction from the α-carbon by the attack of an hydroxyl ion, leading to the formation of a dehydroalanine residue and a S-thio cysteine residue which decomposes to give a bound cysteinate ion and sulphur

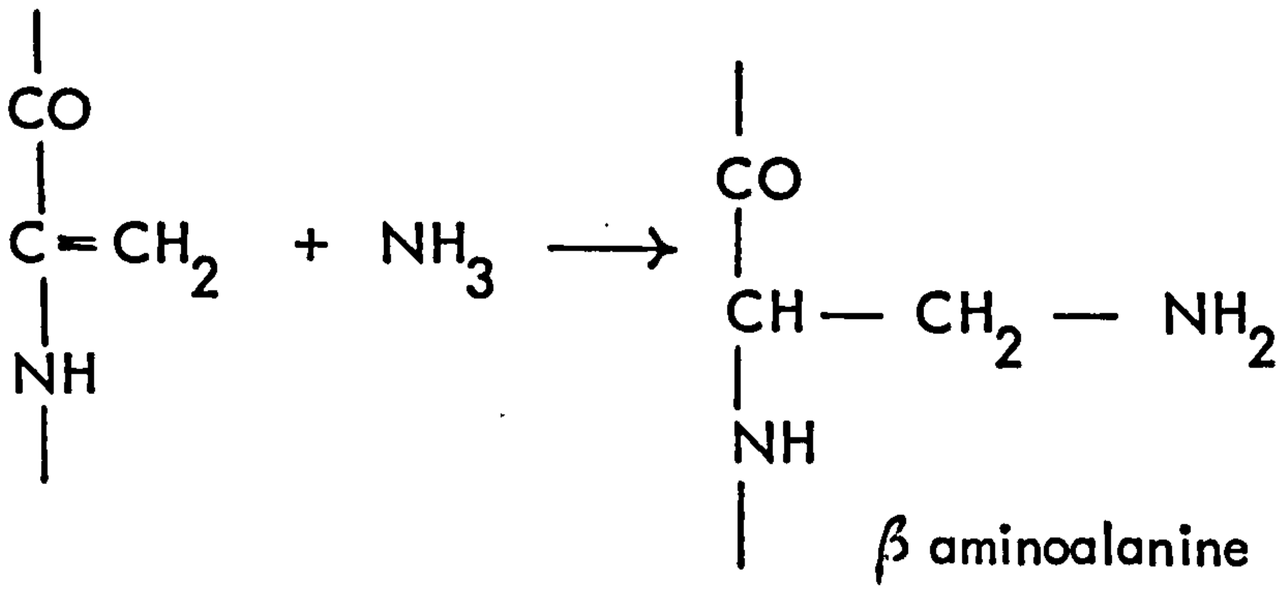


dehydroalanine S-thio cysteine +S

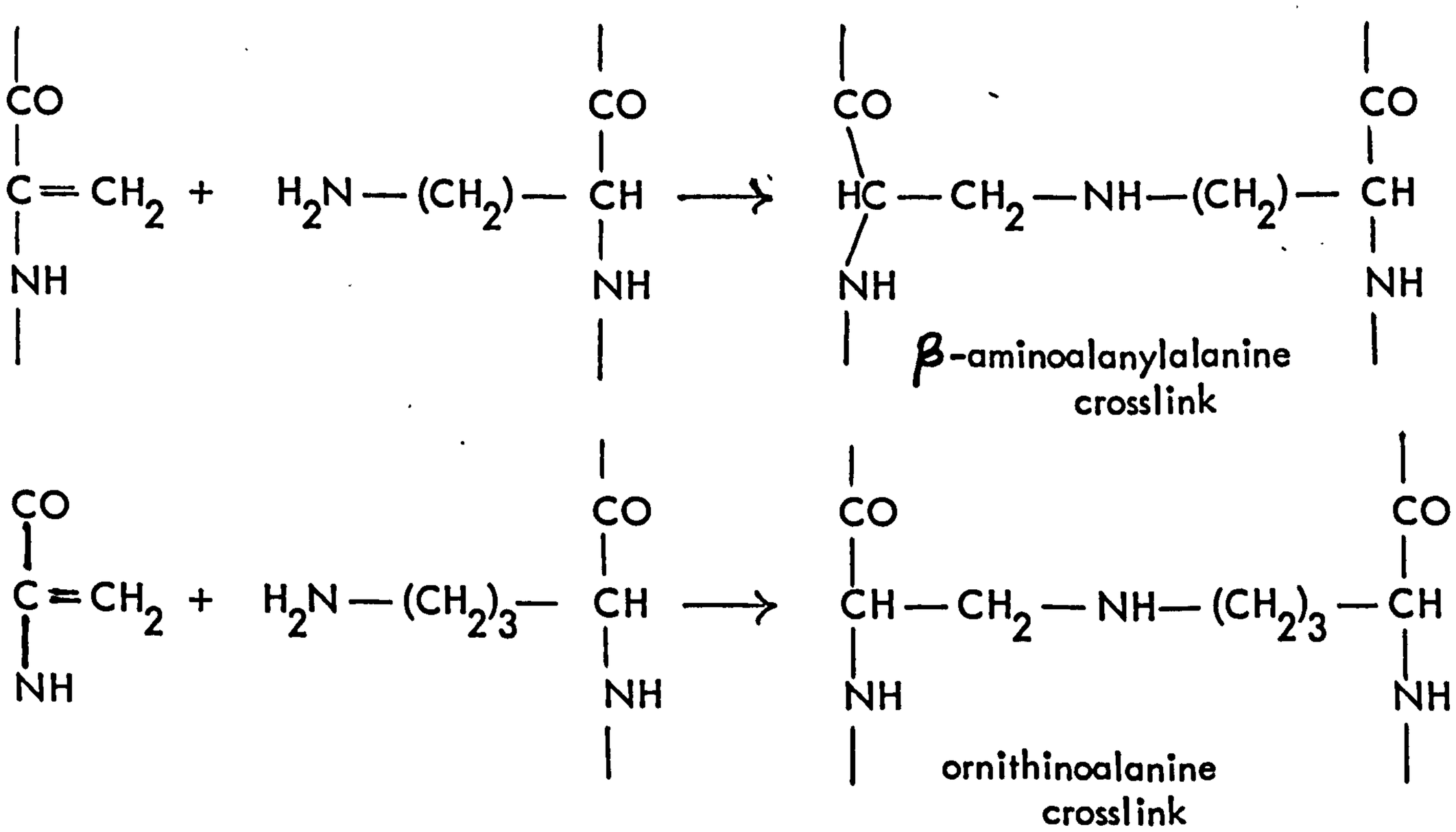
The best evidence supporting this mechanism is the fact that α, α'-dimethyl cystine does not undergo β-elimination since it has no hydrogen on the α-carbon atom, and hence it is not degraded by alkali (115).

The dehydroalanine residue is capable of adding nucleophilic groups across its activated double bond to form new crosslinks.



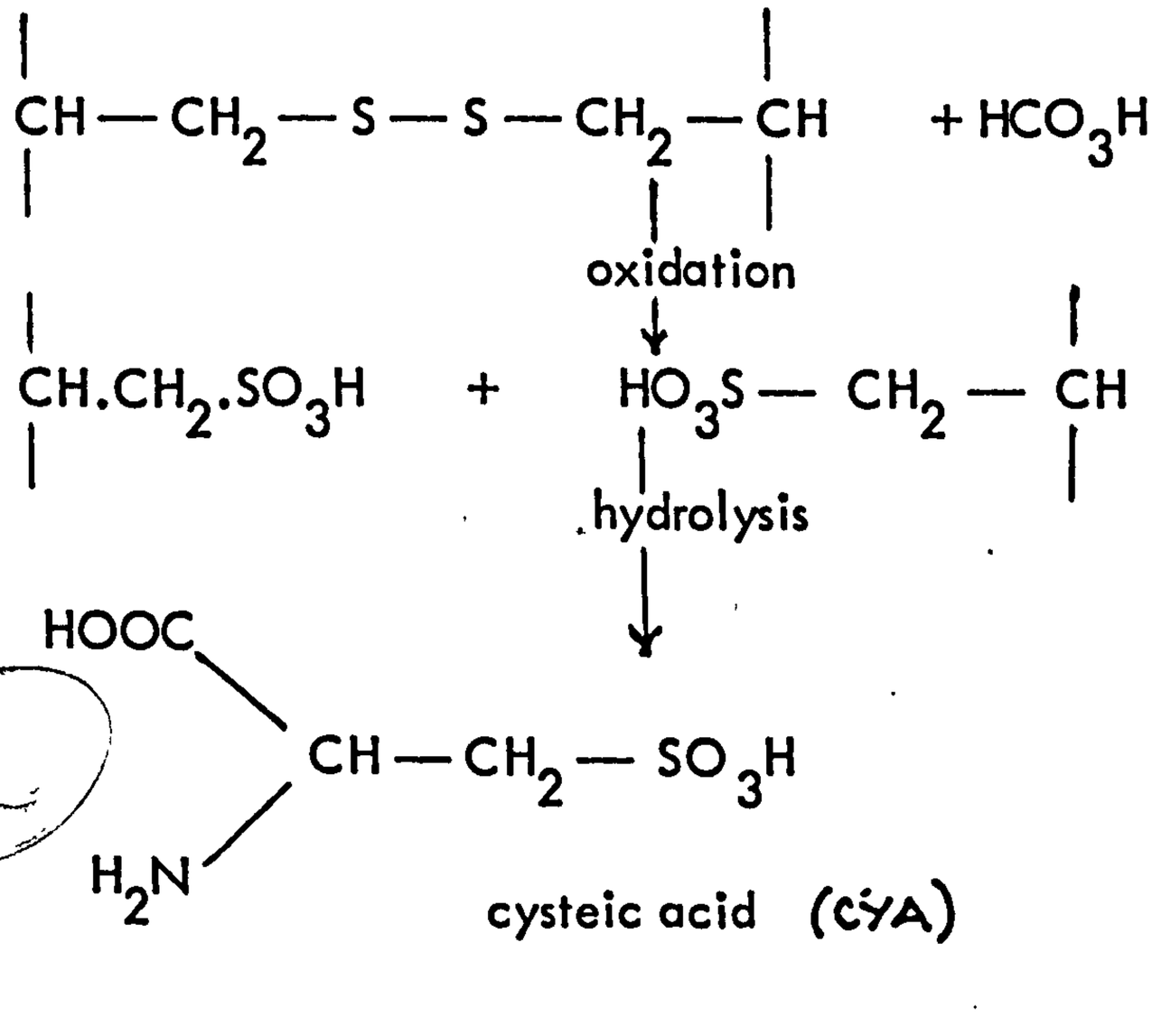


Yet another two new amino acids, β-aminoalanylalanine and ornithinoalanine, may also be formed. Addition to the dehydroalanine residue of the newly formed β-aminoalanine gives β-aminoalanylalanine whilst addition to the dehydroalanine residue of the ornithine residue, resulting from the alkaline degradation of arginine residues, gives ornithinoalanine:



3.3 Oxidation

Oxidation of wool keratin is a well studied reaction. Hydrogen peroxide is used as a bleaching agent and, chlorine, bromine, and potassium permanganate, have all been used to produce 'non-felting' wool. Each of these reagents can convert cystine to cysteic acid but intermediate oxidation products of cystine are formed (116). Organic peracids, although very powerful oxidising agents, do not oxidise amino acids in general, but only react with tryptophan, methionine, and cystine, the last being oxidised quantitatively to cysteic acid. Sanger (117) used performic acid to split the disulphide bond in insulin.without affecting any of the other amino acid residues, and in this way obtained two polypeptide chains. Other organic peracids (e.g. peracetic acid) can be used to oxidise wool without affecting the peptide bonds and hence without main chain degradation. Hydrolysis of such oxidised wool yields cysteic acid.



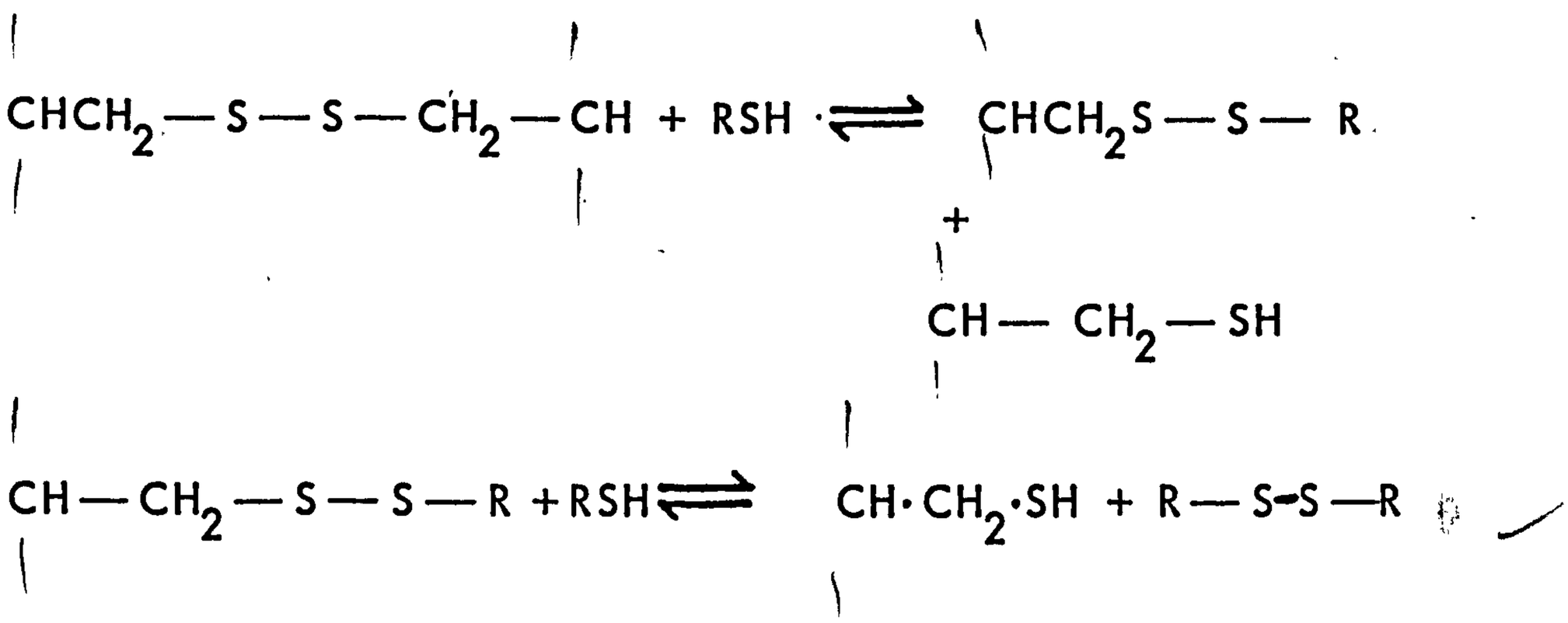
cysteic acid (CYA)

3.4 Reduction

Studies of the effect of reducing agents on wool have been confined almost exclusively to the disulphide bond. The reduction of cystine (Cys) residues to Cysteine (CysH) residues is arguably the most important reaction in wool chemistry. It is a necessary preliminary to the separation and isolation of wool proteins as

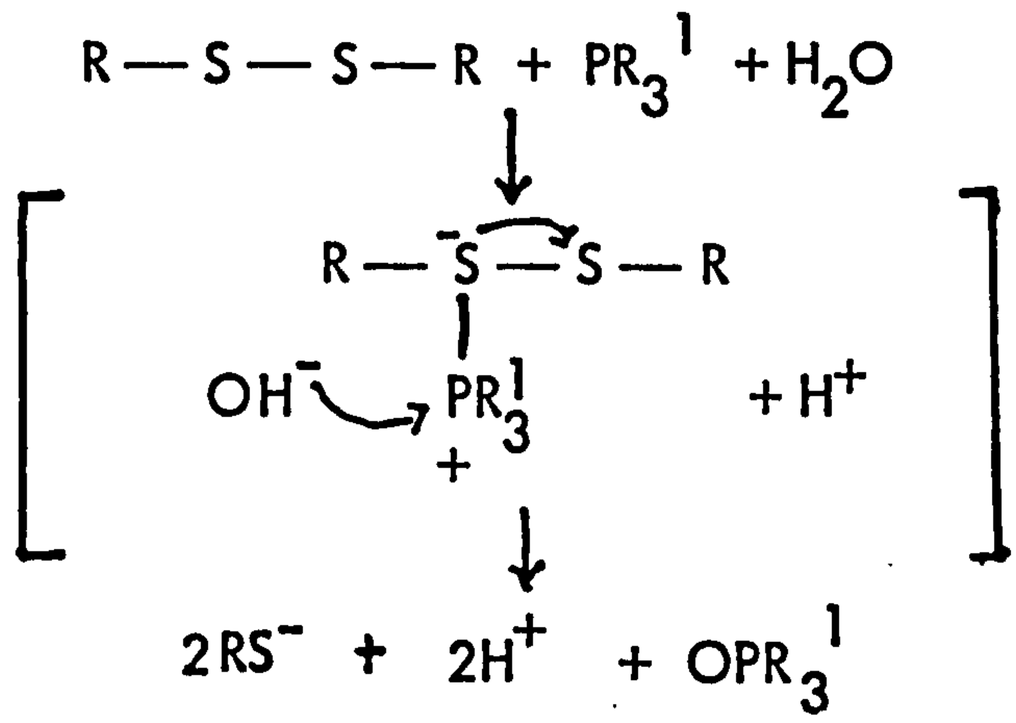
S-carboxymethyl kerateines, to the introduction of many new crosslinks, to the labelling of CysH residues with mercurials for morphological studies, and, most important from the textile point of view, to the promotion of chemical setting and flat pressing.

Most of the work on the reduction of cystine in wool has been carried out using thiols of small molecular weight, where the reaction proceeds by an interchange mechanism involving two sequential nucleophilic attacks by thiol anions:



The equilibrium constants of the reactions are dependent on the electrode potential of the reducing agent and on pH. For most thiols, the equilibrium constants are near unity, and to effect complete reduction a 100 - 400 - fold excess of thiol is required. Because of the reaction mechanism, the reaction should be carried out above the pK value of the thiol being used to ensure it is fully ionised. For this reason, at pH values higher than 7, a rapid increase in disulphide bond cleavage occurs. In practice, it is extremely difficult to effect complete reduction of all the disulphide bonds in wool. Even under conditions of high pH and high concentrations of urea, in which the reduced, denatured wool proteins dissolve, a small amount of unreduced cystine invariably persists. Almost 100% reduction has been claimed by Thomson and O'Donnell (118) using 4 M mercaptoethanol, by MacLaren (119) using 0.1 M benzyl-mercaptan in ethanol/water, and by Leach (120) using electrolyte reduction in the

presence of thiol. Leach (121) also studied the reaction of thiol and disulphide groups with mercuric iodide and methylmercuric iodide. He found that almost 100% reduction could be obtained in a few hours using methylmercuric iodide at pH 9.3. In the presence of 25% dimethylformamide wool samples with zero —S—S— bond content could be obtained. In comparison with the large excesses of thiols required for complete reduction, tributylphosphine has the advantage that it will give the same result with a small excess (122). The possible mechanism involves an initial nucleophilic attack at a sulphur atom by a tertiary phosphine, followed by a nucleophilic displacement:



An alkylating agent must also be present subsequently to block the cystine residues. In the work reported here, since acid hydrolysis, using 6M hydrochloric acid, was used to obtain the free amino acids for subsequent analysis, the advantages of the reduction process, i.e. the non-destruction of methionine and trytophan, were negated. It was therefore decided not to use reduction, but to use performic acid for splitting the disulphide bond.

3.5 Disulphide Exchange

Disulphide exchange takes place when wool is in contact with traces of thiol or some thiol producing agent in an aqueous environment. Thiols catalyse disulphide exchange in neutral or alkaline solutions. In acid solutions the presence of thiols inhibits the reaction.

A mechanism via the sulphenium ion has been suggested:

$$\text{Wool}_1\text{—S—S—Wool}_2 + {+}H^+$$

$$\rightleftharpoons \left[\text{Wool}_1\text{—S—S—Wool}_2\right]^+$$

$$\rightleftharpoons \text{Wool}_1\text{—SH} + \text{Wool}_2\text{—S}^+$$

$$\text{Wool}_2\text{—S}^+ + \text{Wool}_3\text{—S—S—Wool}_4$$

$$\rightleftharpoons \text{Wool}_2\text{—S—S—Wool}_3 + \text{Wool}_4\text{—S}^+$$

$$\text{Wool}_4\text{—S}^+ + \text{Wool}_1\text{—S—S—Wool}_2 \rightleftharpoons \text{Wool}_1\text{—S—S—Wool}_4 + \text{Wool}_2\text{S}^+$$

etc.

Support for this mechanism is available from disulphide protonation in acid solution (123). Spackman, Stein and Moore (124) studied model reactions involving cystine and glutathione and concluded that the disulphide bonds were most stable at pH2. Thus to minimise disulphide exchange in the present work, the filtered aqueous extract from each of the treatments given to the wool keratin was adjusted to pH2 by the addition of 98% formic acid before rotary evaporation.

CHAPTER 4

PREVIOUS STUDIES ON THE PRODUCTS OF WOOL KERATIN DEGRADATION

Steinhardt and Fugitt (125) found that the rates of hydrolysis by dilute acids of both a dissolved protein (egg albumin) and an insoluble protein (wool) depend not only on the temperature and acidity but also on the acid used. When hydrolysed at 65°C by strong monobasic acids of high molecular weight, the amide and the peptide bonds are broken over a hundred times more quickly than when they are hydrolysed with hydrochloric acid. Even among the common mineral acids, large differences appear. These differences in hydrolytic effectiveness parallel differences in the affinities of the acids for protein. They further attributed this effect to the anions, because of the attainment, with anions of high affinity, of a maximum rate of amide hydrolysis at relatively low concentrations, stoichiometrically equivalent to the sum of the amide plus the amino groups. A similar limiting anion concentration on maximum rate of hydrolysis of the much more numerous peptide groups was not observed.

Leach, Rogers and Filshie (52) examined the selective extraction of wool keratin with dilute acid, particularly the chemical and morphological changes which occurred. Wool was extracted with boiling hydrochloric acid at a pH value of 2. These conditions (similar to the treatments used in the work carried out in this project) are highly selective for hydrolysis of peptide bonds adjacent to aspartyl residues and avoid disulphide bond fission. Only the orthocortex passed into solution, the paracortex remained largely unchanged. Most of the material extracted was closely similar in amino acid composition to the low sulphur protein fractions of wool obtained after oxidative disulphide bond fission, and about 50% was non-dialysable or was precipitated at pH 5.5. They found extraction and weight loss ceased when the

orthocortex was completely solubilised. The paracortex appeared to be intact and consisted of the matrix and microfibrils in approximately equal amounts, and although the total sulphur content was high, the residue had an amino acid composition approximately midway between the low and high sulphur protein components of whole wool. To fractionate the paracortical material required oxidative fission of its disulphide bonds. The two fractions had amino acid compositions similar to those from whole wool.

There are advantages in studying the conformation of keratins in the dissolved as well as in the fibrous state. For example, it is possible to measure the sizes of protein molecules, their heterogeneity, content of α-helix, and particularly the changes in each of these properties in response to changes in the solvent environment. Due to the nature of keratins, it is unfortunately not possible to dissolve them without using reagents which cause a great deal of covalent bond breakage.

It has often been the case to extract proteins from wool by oxidative or reductive disulphide bond fission, under conditions chosen to minimise peptide bond fission. Such protein extracts yield valuable analytical information about the size and type of proteins present in the intact fibre. However the information they provide about molecular conformation must be limited since the constraints imposed by disulphide bonds have been removed and the resulting polypeptide chains may be expected to show greater configurational freedom in solution than their parent fibrous proteins.

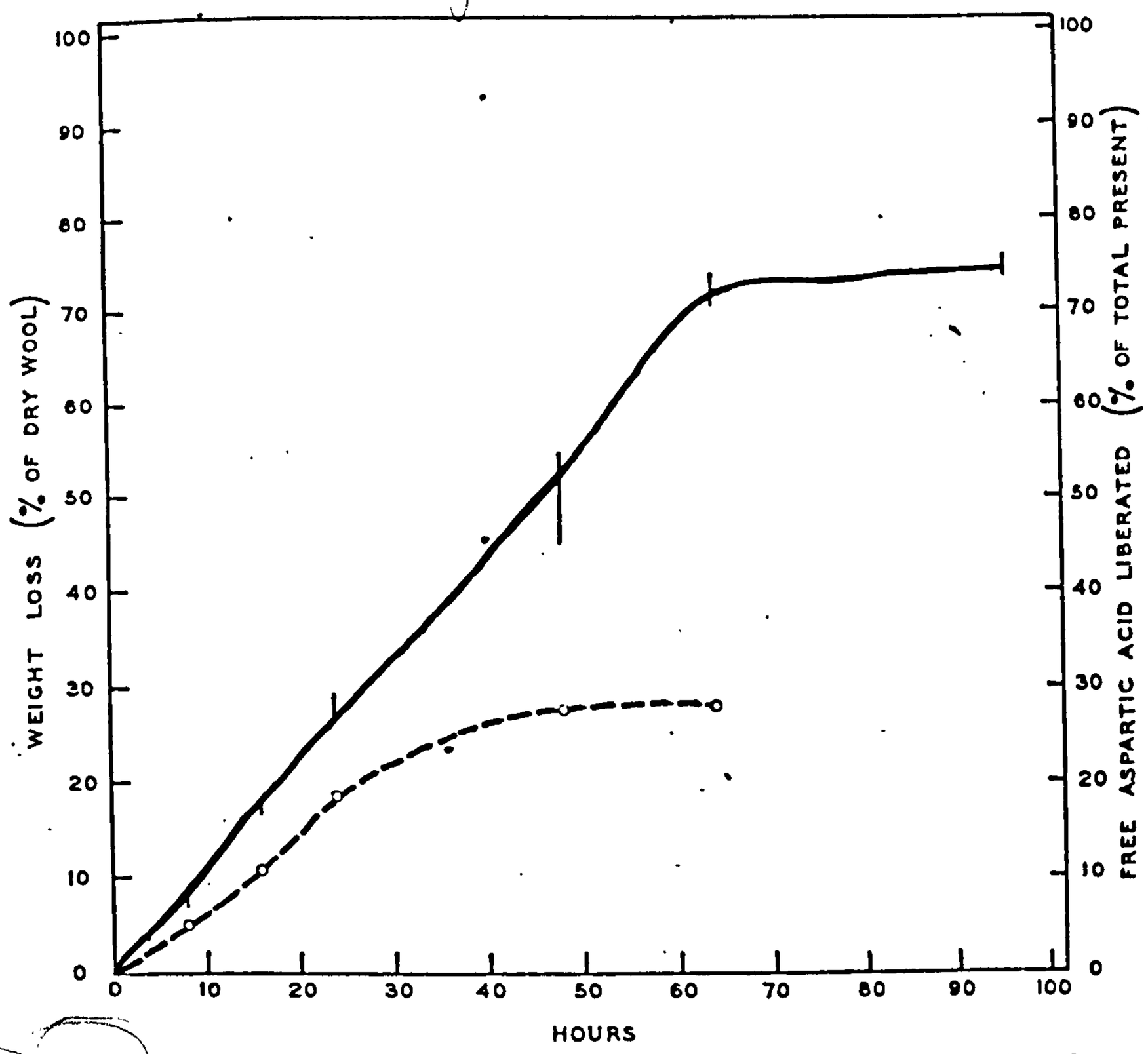
The work in this thesis is concerned with the extraction of such solubilised protein-like fragments, their origin in the wool fibre, molecular size, and the effect of dyes and their intermediates on the cleavage of such proteins from wool. Attempts at hydrolytic extractions have usually utilised strongly acidic solutions. However peptide bond fission under such conditions is not sufficiently selective and the

proteinaceous material obtained by such methods is very heterogeneous. Peptide bond fission in weakly acid solutions (below 0.1 M, or above pH 2 at 100°C) is more selective and it is possible to split out more than 50% of the available aspartic acid before any other free amino acid appears in significant amounts (129). The mechanism of this hydrolysis, a proton transfer from the protonated β·COOH side chain of the aspartyl residue, has been elucidated by kinetic studies on peptides (129, 130, 131, 132). The wool was treated with 0.9 M HCl for 24 hours at 100°C. Initially (after the first 24 hours' extraction)74% of the wool remained intact. The extract (1) (26%) contained equal amounts of dialysable and non-dialysable proteins. Further 24 hour extractions yielded non-dialysable polypeptides and this left the paracortex intact. The latter was subjected to peracid oxidation, and fractionation, giving rise to "α", "β" and "γ" keratoses in the proportion 7%: 2.2%: 10.8%; all percentages refer to the whole wool fibre originally present. Work showed that oxalic acid and dichloroacetic acid (0.1 M) at 100°C, though both having a pH of 1.3, gave different rates of hydrolysis, the former being more effective than the latter. Both acids were less effective than HCl of a similar pH value in solubilising wool protein.

The N-terminal end groups in the residual wool, after extraction, were found to be qualitatively the same as those in an untreated control, with only insignificant traces of new kinds of N-terminal groups. However, the amounts of each end group viz, aspartic and glutamic acids, serine, threonine, glycine, and alanine, increased with time of digestion up to a maximum at 72 hours.

The percentage of wool solubilised showed an almost linear increase with the time of digestion until only about 25% of the wool remained after 64 hours. At this stage digestion virtually ceased. (Fig 4.1)

Fig 4.1



Extraction of wool with HCl (0.01*M*, pH 2) at 100°C. The continuous line shows the weight loss as a percentage of the original wool. The dashed line shows the liberation of free aspartic acid as a percentage of the total acid initially present in the wool. (52)

Leach (52) concluded that there was a similarity in composition between the proteins extracted after 24 hours and the low sulphur component, "α-keratose", extracted from the same sample of wool. Studies on wool keratin indicate a pattern in which microfibrils, containing proteins poor in sulphur, are embedded in a matrix which contains proteins rich in sulphur. The microfibrillar material is considered to be more highly ordered and therefore less penetrable than the surrounding matrix which would appear to be largely amorphous. Microscopic observations showed that the breakdown of the orthocortex first occurred in the macrofibrils. The material which is extracted first appears from its analysis to originate in the microfibrils rather than the matrix. However, the ratio of microfibril to matrix in the orthocortex is thought to be about 4 : 1, so that the analysis of this segment as a whole would not be expected to deviate greatly from that for the low-sulphur proteins. When the extraction nears completion, the composition of the extract changes to that expected for a mixture of both microfibrillar and

matrix components. At this stage, Leach proposed, they could be removing the material which resided at the interface between the matrix and microfibrils, and this interfacial material, of minor amount, was thought to contain possibly disulphide bonds cementing the high and low sulphur regions together. Most of the low sulphur proteins, however, can not be linked to high sulphur proteins by disulphide linkages, since the bulk of the material extracted with an amino acid analysis closely similar to the low sulphur proteins, is removed under conditions designed to leave disulphide linkages intact. Even if only one disulphide link were present per molecule linking the high and low sulphur proteins, it would be sufficient to maintain the linkage between the two kinds of protein and destroy the correspondence in composition between the acid extracted material and the low sulphur "α-keratose".

There are one or two notable deviations in composition between the keratoses of the paracortex and those from the whole wool. For example, both paracortical fractions are richer in sulphur and poorer in glycine than their counterparts from whole wool. It has also been noted that most of the material extracted from the orthocortex by acid, though closely similar in amino acid composition to the low sulphur proteins extracted from whole wool by oxidative and reductive methods, is nevertheless somewhat richer in glycine and tyrosine. These deviations suggest that while the high and low sulphur proteins from the orthocortex are very similar in their overall amino acid composition to the corresponding proteins in the paracortex, they differ with respect to cystine, glycine, and tyrosine. Some of the questions raised by their work were: why is the orthocortex attacked in preference to the para-, and why are the microfibrils therein attacked before the matrix? The answers to these questions were thought to be related. The reasons for the sharp differentiation between the two segments in their behaviour toward acid attack are by no means clear. The greater instability of the orthocortex has been recognised for some years, though the reasons

for the sharp differentiation between the two segments in their behaviour toward acid attack are by no means clear.

Two possible reasons might be suggested; firstly, at all levels of organisation there are well defined differences between the two segments, starting with the macrofibrils and cortical cells down to the microfibrils which differ with respect to size. At the protofibrillar level the characteristic "9 x 2" pattern (now called into question by later workers) (145) (70) is more clearly defined in the para- than in the ortho- segment, and in the latter the one or two central protofibrils frequently do not appear to be present (70). It would therefore not be surprising if there were differences between the two segments even at the molecular level, and the individual polypeptide chains were more closely packed and therefore resistant to chemical attack in the para- than in the ortho-cortex.

The second reason for the preferential attack on the orthocortex may be connected with the fact that it contains a lower proportion of matrix material to microfibrils. Leach suggested that the matrix was more easily dissolved or attacked than the microfibrils. This idea is based upon the fact that the matrix is more heavily stained than the microfibrils after reduction and treatment with heavy metals, and also that the microfibrils containing the "crystalline" protein components, should be more dense and therefore less penetrable than the matrix. Preferential metal staining of the matrix is easily explained in terms of its higher sulphur content. He then cited the evidence of Mason (143) that the matrix was more dense than the microfibrils. For this reason reagents which do not attack the disulphide bonds might reasonably be expected to attack microfibrillar material before the matrix. The paracortex with its higher content of matrix material and its more regular arrangement was thus proposed to be protected by its matrix material against the attack of dilute acid. De Deurwaerder, Dobb, Holt and Leach (134) examined

the properties of the extracted proteins. Fractionation revealed two groups of proteins of similar amino acid composition, one having a continuous distribution of molecular weights of about 5,600 and the other having a large proportion of material with an apparent weight averag molar weight of about 21,000. Sweetman's work (135) also showed the preferential hydrolysis of certain peptide bonds and the formation of lanthionine, particularly above pH 4; the mechanism for this reaction was discussed in the previous chapter. He also studied the amino acid composition of the wool gelatin obtained from the treatment of wool in water at 50 - 100°C. He deduced that peptide bonds were broken during the treatments, and this was made more evident when it was found that the non-dialysable proportion of the wool gelatin decreased with an increase in treatment time. Furthermore, the electrophoretic behaviour of the water soluble proteins on starch gel suggested that peptides were present, which had a low molecular weight (136). The preferential hydrolysis of aspartyl, glycyl and seryl residues occurred. It was noted that arginyl, prolyl, cystyl, lanthionyl, tryptophyl, and to a lesser extent threonyl, lysyl, valyl and isoleucyl contents of the residual wool tended to increase with increasing severity of conditions. These increases were balanced by approximately equivalent decreases in the contents of these amino acids in the water soluble fraction. It also seemed possible that peptide bonds adjacent to the rather bulky arginyl, prolyl and tryptophyl residues might be comparatively resistant to hydrolysis. An alternative explanation for this could be that a high proportion of these residues might be located in particular regions of the wool fibre, like the paracortex, where the extraction of peptides is not as favourable as in, for example, the orthocortex.

Recently (137) at Aachen work has been carried out attempting to clarify the sources of these extracted proteins. Depending on the extraction conditions, (1 hour, pH 2 - 8, 100°C) wool gelatins of differing composition were obtained.

While the low and high molecular weight pH 2 wool gelatins consisted of inter-related proteins, the low and high molecular weight proteins extracted at pH 8 had an entirely different composition as regards amino-acids. Baumann (137) deduced that the pH 2 wool gelatins belonged to the cell membrane-complex proteins, while high molecular weight pH 8 wool gelatins probably originated in the endo-cuticle, and the low-molecular weight material, in the non-keratinous proteins of the fibre stem, which are rich in tyrosine. The determination of the N terminal amino acids in wool showed that at pH 2 selective cleavage of the aspartyl peptide bonds occurred. This degradation took place in a relatively specific way in certain morphological regions of the fibre. The mole percentages of amino acids extracted from wool, were found to vary with both pH and extraction time.

Other work carried out at Aachen involved the study of the degradation of wool under conditions which frequently occur during the wet processing of wool. It was found that two major factors influenced the amount of wool gelatin extracted, namely, pH and electrolyte concentration. Over the pH range 2 - 8 the maximum yield of wool gelatin occurred at pH 2; this decreased to a minimum at pH 4 to 5 and increased again above pH 5. Two buffer systems, hydrochloric acid/caustic soda, and citrate/phosphate, both capable of buffering over the range pH 2 - 8 were used. At pH 2, the amount of wool gelatin obtained was independent of the electrolyte concentration. At pH 4 and pH 5 in citrate buffer, twice the amount of wool gelatin was obtained in comparison with that obtained in the presence of hydrochloric acid/caustic soda. At pH 3 and pH 6 this increased to three times the amount, and at pH 8 to four times the amount. (In terms of ionic strength, the higher the pH value of a phosphate/citrate buffer, the higher the ionic strength in comparison with the hydrochloric acid/caustic soda buffer, whose ionic strength remains constant.) These findings can be classified as follows.

Then please clarify. (1) During treatment in boiling aqueous solutions at various pH values, the molecular structure of wool is partially destroyed and salt bridges, hydrogen bonds, hydrophobic interactions and Van der Waals forces can be broken. Rupture of these bonds leads to unfolding and reorientation of the polypeptide chains in wool, and a number of groups previously inaccessible, become available for chemical interaction. (2) In the acid range wool has a positive net charge which has several consequences: swelling increases and the protein chains repel each other due to their like charges. Also the high hydrogen ion concentration causes a splitting of peptide bonds. All this leads to a high yield of wool gelatin.

(3) With increasing pH the iso-electric point of wool is reached, and if the charges are mutually compensated in the presence of foreign ions, then one speaks of the iso-ionic point. Proteins exhibit a solubility and swelling minimum at this point. The iso-ionic point of wool is 4.9, as determined by Elöd (138), and corresponds to a maximum formation of stabilised salt links and hydrogen bonds, and the maximum possible mechanical resistance of the wool. A minimum yield of soluble gelatin is therefore obtained at the iso-ionic point. Upon further increase in pH values, the wool assumes a net negative charge. Because of the changing conditions, a reorientation of certain protein chains occurs, followed by an increased hydrolytic degradation and a deterioration in mechanical properties. Even in the neutral pH range, hydrolytic degradation of disulphide bonds occurs at the boiling point, and in the weakly alkaline region, this degradation increases to considerable proportions. The natural crosslinks are therefore destroyed but are replaced to a small extent by new crosslinks, such as lysinoalanine and lanthionine, discussed in the previous chapter. Swelling also increases considerably at this pH region. With high electrolyte concentrations, swelling is again increased, causing degradation of both disulphide bonds and peptide links.

Baumann (139) also studied the effect of various acids at pH 2 on the extraction of wool gelatin. Formic, phosphoric, hydrochloric, and citric acid treatments gave similar amounts of wool gelatin but acetic acid gave 50% more while sulphuric acid gave 25% less. Of the six acids used at pH 2, sulphuric acid is likely to have the lowest ionic strength because it is dibasic hence it could be expected to produce the least amount of wool gelatin. With sulphuric acid, the sulphate ion has two effects; it promotes the structure of water and therefore it promotes the helical structure of wool protein. The high yield of wool gelatin from the acetic acid treatment is expected because of the high concentration needed to reach pH 2.

The influence of the sulphate anion on the degradation of wool keratin is complex. Aqueous solutions of sodium sulphate were shown by Botton (140) to inhibit hydrolytic degradation. Yet in the presence of sulphuric acid the electrolyte brings about considerable degradation in comparison to that of the acid alone. This effect could again have been caused by an increase in ionic strength. Alternatively, the known increase in the bisulphate anion concentration, in solutions of sulphuric acid and sodium sulphate, compared to that of sulphuric acid alone (141) could be responsible for this degradation behaviour. Contrary to this, Baumann found that using shorter treatment times, sodium sulphate produced no increase in wool gelatin when present in solutions of sulphuric, hydrochloric, and phosphoric acid at pH 2. Animashaun's work (141) indicated that sulphate ion was not absorbed as such by wool, but that bisulphate ion was. If this is the case one would reasonably expect it to have an affinity for a positively charged wool fibre. Because of its small size and low affinity for wool relative to dyes, it would also take a long time to come to an equilibrium within the whole dyeing system. The above results obtained by Baumann could then conceivably be put down to a kinetic effect.

CHAPTER 5

EXPERIMENTAL PROCEDURES

5.1 Materials

5.1.1 Wool Fibre

Loose Australian merino wool was used for the entire work. Prior to use the wool was degreased by Soxhlet extraction with ether, and then ethanol, each for a period of 24 hours. After air-drying the roots and tips of the wool fibres were clipped off and any vegetable matter removed by hand. (Gloves were worn throughout handling.) It was then repeatedly washed using distilled water, and was then highly squeezed to remove any excess.

After being air-dried, and prior to use in experiments, all wool was stored in vacuo at room temperature over phosphorus pentoxide for at least two weeks.

5.1.2 Reagents

All chemicals used were of Analar grade. The detailed preparation of the reagents is given in the appropriate sections on experimental methods.

5.1.3 Dyes and their Purification

All dyes used were obtained from commercial sources, or were prepared in the laboratory. Prior to use they were purified using a process devised and perfected by the author and colleagues, who tailored the purification process to suit their individual needs. The dyes used are listed in table 5.1.

(i) Method

The dyes to be purified were dissolved in 0.1 M sodium hydroxide and subjected to gel permeation chromatography (G.P.C.) using Sephadex G25 Superfine (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) with 0.1 M sodium hydroxide as the eluant.

Under these conditions the dyes separated to give several bands. The

major component was collected in each case and the isomers or by-products of dye manufacture were removed and discarded. The emergence of the bands was monitored

(a) Visually

(b) Using a spectrophotometer.

The latter was only used initially in order to establish the identity of the dye. After G.P.C. the main band was further purified by neutralising the dye solution in the presence of n-butanol. In most cases this compound was to be a suitable solvent for the dye-acid.

In the presence of n-butanol a two-phase system was established in which the sodium salt of the dye and other electrolytes were soluble in the aqueous phase, while the dye-acid was soluble in the n-butanol layer.

$$RSO_3^-Na + H^+Cl^- \longrightarrow RSO_3H + Na^+Cl^-$$

n-butanol layer — aqueous layer

After separation of the two layers using a separating funnel, the dye-acid was obtained by rotary evaporation under reduced pressure at room temperature and then rinsed repeatedly with distilled water until no n-butanol remained.

Essentially, the first stage of this method removes the organic molecules from the dye sample the second stage removes the ionic molecules present. The dyes were obtained in the sodium form by titrating with sodium hydroxide to pH 7. The dye solution was then rotary evaporated to remove the water and the dye samples were stored under vacuo over phosphorus pentoxide. This method leaves the dyes pure, a fact which was verified for Orange II (C.I. Acid Orange 7) and similar smaller acid dyes by elemental analysis.

For the large dye molecules, the purity could only be established by titration with titanous chloride (titanium III chloride) and by spectrophotometric observation.

(ii) Estimation of dye purity

A quantitative evaluation of the purity of the dyes obtained by the method above was carried out by elemental analysis (Butterworth Microanalytical Consultancy Ltd.), and by titration with titanium III chloride. The latter gave an estimate of the azoic material present, isomeric forms having been removed.

Titanium III chloride procedure

Titanous chloride in the presence of acid behaves as a reducing agent

$$TiCl_3 + HCl \longrightarrow TiCl_4 + H^+$$

$$Ti^{3+} \longrightarrow Ti^{4+} + e^-$$

and providing the conditions are correct, the azo links of a dye can be reduced using an excess of titanous chloride and the unreacted titanous chloride back-titrated. A simple calculation then gives an estimation of the amount of the titanous chloride present in excess. From this the number of azo groups present in a given molecule can be calculated.

Titanous chloride is very susceptible to atmospheric oxidation and must constantly be standardised and care taken to exclude oxygen at all stages of the reaction. Standardisation is carried out using ferric ammonium sulphate.

Compound		Molecular weight	Equivalent weight
Titanous Chloride	$TiCl_3$	154.3	154.3
Ferric Ammonium Sulphate	$Fe_2 \cdot (NH_4) \cdot (SO_4)_4 \cdot 2H_2O$	964.6	$\frac{1}{2} \times 964.6$
Orange II		350.36	$\frac{1}{4} \times 350.36$

Standard titanous chloride solution

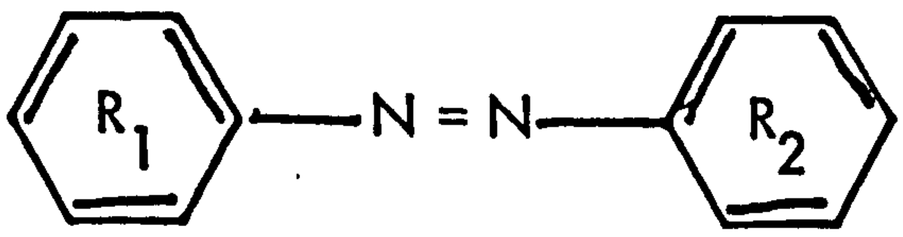
Titanous chloride is available as a 15% solution, and 60 mls of this solution was mixed with 100 mls of concentrated hydrochloric acid and boiled, allowed to cool, and diluted to 2000 mls with distilled deaerated water and stored

in a full bottle. This solution was standardised using ferric alum solution (0.0125 M) made by dissolving 12.0575 g of A·R ferric alum in water, adding to this 600 mls of 2.5 M sulphuric acid and making up to 1000 mls in a graduated flask, 15 ml of 10% potassium thiocyanate added, and titrated with standard titanous chloride until all the red colouration disappeared. Near the end-point of the titration, the last traces of ferric thiocyanate reduce very slowly and consequently, the last drops were added over several minutes to ensure an accurate reading.

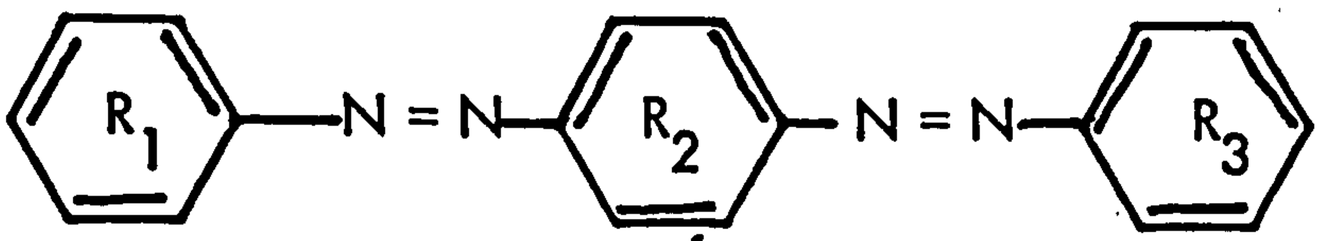
(iii) Estimation of the dyes

Each dye sample (1 gm) was dissolved in boiling water, cooled and made up to 1000 ml with distilled water which had been recently boiled. 100 ml of this was pipetted into a conical flask, and boiled. 10 ml of concentrated hydrochloric acid, and 50 mls of the standardised titanous chloride were added, the boiling being continued until only the faint pink colour of excess titanous chloride remained.

The solution was allowed to cool and was then back-titrated with the ferric alum solution. Assuming the dyes have a structure of type



or



it was possible to establish their molecular weights. By titration of the acid groups of the acid dyes with standard hydrochloric acid, and by combining this with the observed data from the G.P.C. experiments, and the above titanous chloride titration, the identity and purity of the dye samples could be elucidated. This was most important in the case of dyes of high molecular weight such as Evans blue (molecular

weight 980). This dye has four sulphonic acid groups and a high molecular weight, rendering it non-volatile as far as mass spectrometry is concerned, and therefore this method was the only one which was conveniently usable for estimating the purity of Evans blue and other dyes which behaved similarly on mass spectrometry.

5.2 Experimental Methods

5.2.1 Treatment conditions for the wool fibre

10g samples of wool fibre were treated in 1 litre of the following aqueous solutions for 24 hours at the boil in a 2 litre round bottomed flask:

Treatment 1: Distilled water

Treatment 2: 0.00704 M Sodium sulphate (equivalent to 10% o.w.f)

Treatment 3: 0.0102 M Sulphuric acid (equivalent to 10% o.w.f.)

Treatment 4: 0.00704 M Sodium sulphate and 0.0102 M Sulphuric acid

Subsequently treatment 4 was referred to as the blank dyebath. Treatment of the wool was carried out in this blank and solutions of dyes were made up in it.

The flask was stirred at intervals until the fibre was thoroughly immersed and once the treatment was under way the boiling action was vigorous enough to ensure thorough agitation. After each treatment the resultant solution was removed from the water bath and immediately filtered, and a few drops of 98% formic acid were then added to bring the pH to a value of 2 in order to minimise disulphide exchange. The volume was finally reduced by rotary evaporation.

5.2.2 Protein Oxidation

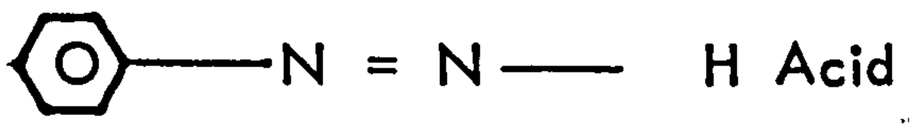
Prior to oxidation the protein degradation products were desalted on a column of Sephadex G25 using 1% w/v aqueous ammonium acetate in order to remove the presence of any chloride ion. This was necessary to prevent the formation of mono-chlorotyrosine. After desalting the proteins were freeze-dried to remove

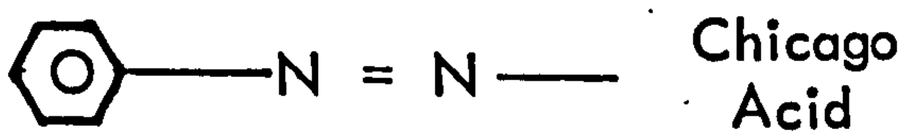
TABLE 5.1

Dyes used

X	Kiton Orange II	CI No 14600
✓	Crocein Scarlet	CI No 27290
	Biebrich Scarlet	CI No 26905
✓	Evans blue	CI No 23860
	Naphthalene Red J	CI No 15620
	Naphthalene Green B	CI No 44090
	Benzyl Red MG	CI No 22890
	Carbolan Yellow 3G	CI No 18961
	Carbolan Blue B	CI No 62075
	Carbolan Red B	CI No 18073

Two other dyes were prepared by the author





Other treatments involved the presence of the following dyestuff intermediates in the dyebath:

✓ H Acid (1,8 amino naphthol -3,6-disulphonic acid)

Chicago Acid (1,8 amino naphthol-5,7-disulphonic acid)

1 Naphthol,-4 sulphonic Acid

Naphthalene-1,5-di-sulphonic acid

Naphthalene-2,7-di-sulphonic acid

Naphthalene-1-sulphonic acid

ammonium acetate, and then dissolved in 98% formic acid (1 : 25 w/v). Performic acid was prepared by reacting 30% hydrogen peroxide and 98% formic acid in the ratio 1 : 9, and the reaction mixture was allowed to stand at ambient temperature prior to use.

Excess performic acid was then allowed to react with the wool gelatin overnight (12 hours) at room temperature. The solution was then diluted with an equal volume of water and rotary evaporated to dryness and washed several times with distilled water. The oxidised wool gelatin was then freeze dried overnight to remove any remaining traces of acid.

5.2.3 Gel permeation Chromatography

(i) Gel Types. For technical data see table 5.2.

Gel permeation chromatography (GPC) was carried out using Sephadex dextran gels which were supplied by Pharmacia Fine Chemicals. Dextran is a polysaccharide built up from glucose residues and is produced by fermentation of sucrose. The micro-organism used for this process is "Leuconostoc Mesenteroides". When the dextran is crosslinked to form a gel, the polysaccharide chains of the gel form a three-dimensional network to produce the commercially available gels.

The crosslinking is carried out using epichlorhydrin and the degree of crosslinking is finely controlled by careful selection of the conditions of the reaction. The gels are of eight different types which differ in their degree of swelling. The various gels are characterised by the letter G followed by a number (Sephadex G10 - G200). The numbers correspond to the moisture regain of the gel multiplied by a factor of 10. Some of the gels are available in more than one particle size. The smaller the particle size the higher the degree of resolution of the mixture applied to the column. For analytical purposes the use of "fine" or "superfine" grade materials is necessary and the flow-rate has an optimum value.

TABLE 5.2

The manufacturer's technical data

Designation		Particle Size μ	Fractionation range mol.wt	Water regain $ml.g^{-1}$ dry gel	Bed volume $ml.g^{-1}$ dry gel
G10		40 - 120	700	1.0 ± 0.1	2 - 3
G15		40 - 120	1,500	1.5 ± 0.2	2.5 - 3.5
G25	coarse	100 - 300	1,000 - 5,000	2.5 ± 0.2	4 - 6
	medium	50 - 150			
	fine	20 - 80			
	superfine	10 - 40			
G50	coarse	100 - 300	1,500 - 30,000	5.0 ± 0.3	9 - 11
	medium	50 - 150			
	fine	20 - 80			
	superfine	10 - 40			
G75	superfine	10 - 40	3,000 - 70,000	7.5 ± 0.5	12 - 15
G100	superfine	10 - 40	4,000 - 150,000	10.0 ± 1.0	15 - 20
G150	superfine	10 - 40	5,000 - 400,000	15.0 ± 1.5	20 - 30
G200	superfine	10 - 40	5,000 - 800,000	20.0 ± 2.0	30 - 40

(ii)a Preparation of the Gel

The gel was allowed to swell for the period of time given in table 5.3. The buffer solution used for this purpose was that for subsequent elution.

TABLE 5.3

Type of Sephadex	Minimum swelling time in boiling eluent (hrs)
G10 - G50	1
G75	3
G100 - G200	5

In order to prevent bacterial and fungal growth in the gels, once swollen, the buffer solution contained 0.02% w/v sodium azide. Except for G10 and G15 gels, superfine grades were used throughout.

(ii)b Eluent buffer

The eluent used was made as follows:

1 g L^{-1} Sodium dodecyl Sulphate/Ammonium Acetate 1% w/v

0.02% (2 g L^{-1}) Sodium azide.

(iii) Column Packing

The columns used for GPC were supplied by Pharmacia Fine Chemicals along with other accessories such as flow adaptors, gel and eluent reservoirs, and valves.

The procedure used was as follows. After the column had been mounted vertically in all planes (using a plumb line) a small amount of eluent was poured into it and all air bubbles were removed by forcing the liquid back and forth through the bottom adaptor with a syringe. A gel reservoir was attached to the top of the column so that all the gel could be added at once and thus minimise any tendency the gel might have to form zones. The slurry of gel and eluent was then prepared using the makers' recommended method and was poured down a glass rod and slowly

stirred to remove air bubbles. The outlet tubing was positioned just below the liquid level in the column and opened to allow the solvent to flow out slowly. The column outlet tubing was gradually lowered until the operating pressure to be used was reached. For G10 - G50 it was not necessary to limit this operating pressure. When the gel had settled the top adaptor was carefully positioned so as not to trap any air bubbles. The homogeneity of the packing was now tested using Blue Dextran 2000, and observing the progress of the coloured zone. A tight zone with parallel edges in a horizontal plane indicated a satisfactory packing. The flow rates were adjusted to give a void volume time of about 4 hours.

(iv) Sample Application

Insert Fig 5.1 ?

Samples were applied using a syringe attached to the valve system as shown in fig 5.1. A maximum of 2 mls was applied at one application. A small air bubble was introduced before and after the sample to prevent diffusion of the sample during application.

The sample was pushed into a sample loop by use of two valves, one before, the other after the loop (see fig 5.1) and then the sample was pumped on to the column as normal. This method assures minimal diffusion and very little pressure change during loading. GPC was carried out using downward flow.

(v) Detection

Proteins and peptides were detected using the LKB 8300A Uvicord II photometer at 280 nm or the LKB 2089 Uvicord III at 206 nm and 278 nm.

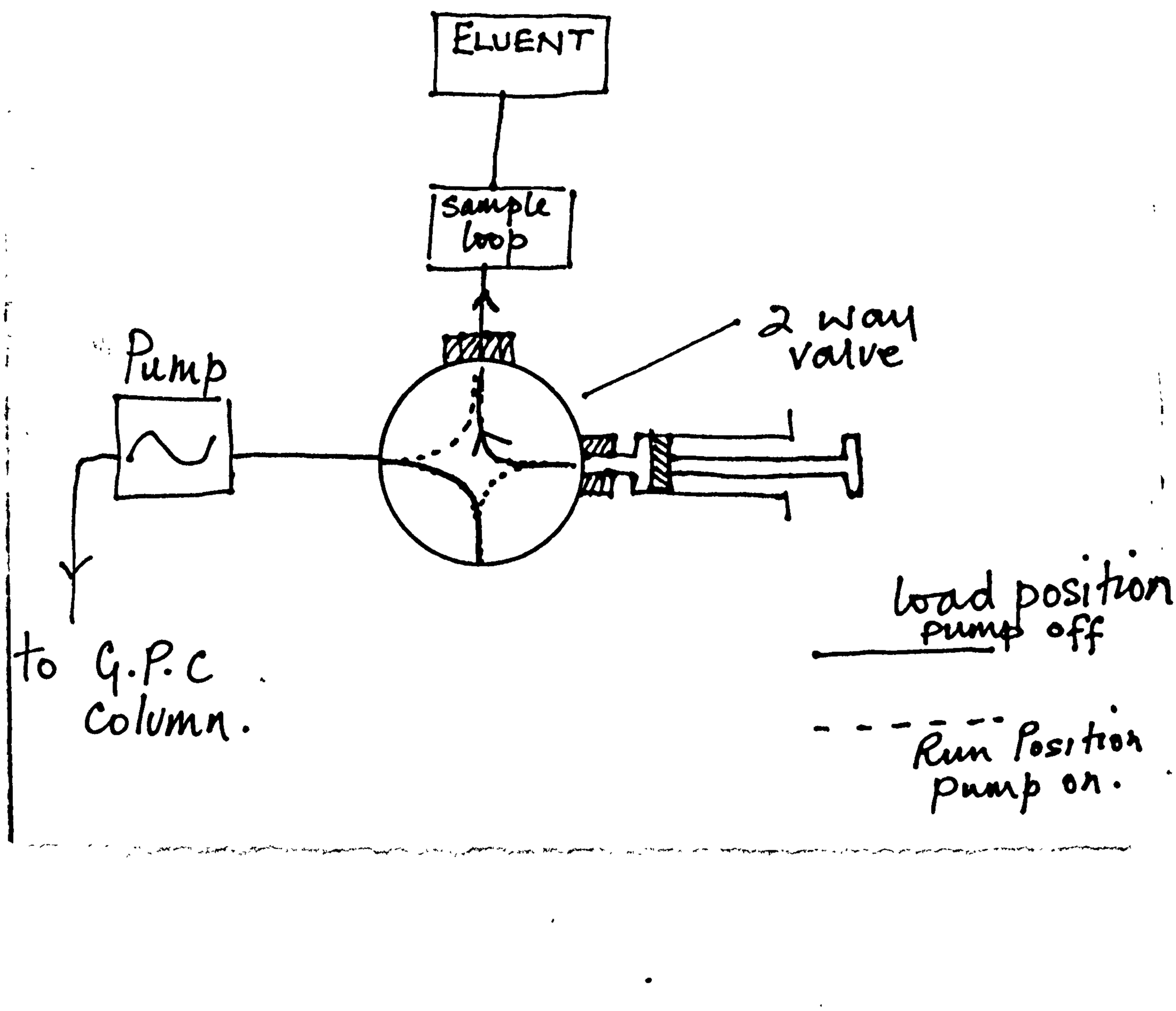
An LKB 6520 chopper-bar recorder was used connected to the photometer and to a fraction collector with event-marker, so that peaks could quickly be localised to specific test-tubes in the fraction collector.

The Uvicord II was mainly used for detection of the fractions from Sephadex G50. The Uvicord III has the advantage over the Uvicord II of using simultaneously two separate channels for detection.

Two narrow band filters select the required wavelength from each beam, namely 206 nm or 278 nm. The use of 206 nm enables the absorption of non-aromatic peptides to be monitored.

Both the Uvicord II and III were used in conjunction with an LKB 7000 Ultravac fraction collector.

fig 5.1



5.2.4 Amino Acid Analysis

(i) Analyser

The analyser used was a Biocal BC100 Auto Analyser, in conjunction with a BC500 auto loader, an Elliot three-point recorder and an amino log.

The sample applied to the top of the column (via the autoloader) was eluted through the cation exchange resin (Biorad Aminex A6) using an aqueous salt buffer with a stepwise pH gradient of 3.25 to 4.25 to 6.45. See table 5.4 for the composition of the solutions. The buffer was pumped at a constant rate (approximately 80 $ml.h^{-1}$) down the column (maintained at a steady 55°C by a thermostatically controlled hot water jacket) by a Milton-Roy micro-pump. This system enables all the amino acids to be separated in about 4 hours.

On leaving the column the eluent passed into a mixing block where it met a stream of aqueous ninhydrin solution. The subsequent mixture was passed through Teflon tubing immersed in a boiling water-bath, and arranged in a coil, where the colour reaction between successive amino acids and ninhydrin took place. The reaction mixture was then passed through flow cells of a colorimeter where the absorbance of the solution was measured continuously, and automatically recorded using photocells and the Elliot recorder. In the colorimeter used there were two flow cells, each having an optical path-length of 3 mm and the absorbence of the solutions was recorded at 570 nm and 440 nm.

(ii) Amino log

To improve on the sensitivity of the instrument an "Aminolog detector amplifier" was incorporated into the system. This had two functions:

The aminolog converted the linear transmission from the photovoltaic cells into a scale expanded linear absorbance.

The aminolog facilitated a range of expansion scales for any given analytical

condition, the recorded signal depended upon the setting of an aminolog scale and the position of the baseline control. The scale settings were:

O position - analyser photometer signals were disconnected from the recorder in order that electrical zeropotentials might be checked.

%T position - performance was similar to that when the aminolog was not used, but the operation was via the aminolog pre-amplifier. The recorder was scaled linearly from 0 - 100 % corresponding to full scale deflection, but the pen deflection was reversed compared to the original.

2A*, 1A*, 0.5A* positions - recorder pen deflection corresponds linearly to 0-2, 0-1, 0-0.5 absorbance units for full scale deflection.

Throughout the range over which Beer-Lambert's Law applied (approximately 0 - 0.8A*) and subject to reaction colour factors, recorder peak areas could be directly related to amino acid concentrations. Peak heights could therefore be measured linearly during manual quantisation. 0.2A* and 0.1A* positions correspond to 0-0.2 and 0-0.1 absorbance units for full scale deflection. The high sensitivity of the system on these settings gave high electrical "noise", so that these settings made peak height estimation extremely difficult.

To calibrate the amino acid analyser with the aminolog, the buffer and reagent solutions were pumped through the system and the aminolog was switched to the scale to be used. The baseline control was then adjusted to bring the recorder pen for both channels near to the zero mark.

(iii) Column-packing, and Regeneration

The resin was suspended in 0.2 M aqueous sodium hydroxide solution in the ratio (1 : 2 w/v) and the column exit closed with a plastic stopper. The resin suspension was poured into the column at the operating temperature of 55°C and was forced to settle by applying pressure from the pump, the plastic stopper having been removed

* A = Absorbance Units

and replaced by the appropriate column end and filter wad. In order to prevent subsequent shrinkage of the column, a flow rate greater than the normal analysis flow rate was used for resin packing. This procedure was stopped when the resin suspension was slightly above the settlement bed; the supernatant liquor was then sucked off and fresh resin suspension poured in. This process was repeated until a suitable column height was obtained. Contamination of the resin can occur and this is obvious by the broadening of some amino acid peaks, the appearance of unknown assymetrical peaks, and the gradual increase in working pressure needed to maintain the same flow rate through the column. If washing of the column in 0.4 M sodium hydroxide gives no improvement, then the column has to be unpacked and the resin washed more thoroughly as follows:

1 M sodium hydroxide at 60°C followed by 1% E.D.T.A. in 1 M hydrochloric acid followed by 1 M sodium hydroxide at 60°C.

(iv) Flow rate

A given pump stroke of the Milton-Roy micropump corresponds to a particular flow rate of the eluent through the entire system including the flow meter. In order to measure the flow rate the tubing was first unscrewed from the inlet of the flow-meter. The discharge of the pump in $ml.hr^{-1}$ could now be ascertained by taking the average discharge per minute and multiplying by 60. The tube was now screwed back on the flow meter and the time taken for a small injected air bubble to travel between two marks was measured several times. The average result was then inserted into the following formula to obtain the flowmeter constant for the equipment.

K = pump discharge in mls/hr x measured time on flowmeter(seconds)

For any other desired flow rate, the time on the flowmeter can now be calculated thus:

$$\frac{K}{\text{desired flow rate}} = \text{seconds on flowmeter}$$

or more usefully,

$$\frac{K}{\text{seconds}} = \text{desired flow rate}$$

In the work carried out the eluent pump was set to deliver 80 ml/hr and the reagent pump half this amount 40 ml/hr.

(v) Sample Preparation and Application

(v)a Sample Preparation

Two types of sample were analysed, the first type were protein fractions from GPC runs, the second type were wool samples that had been dyed. In the first case the fractions from GPC were desalted on G25 using 1% ammonium acetate buffer and were then freeze-dried to remove all traces of ammonium acetate. The samples were then dissolved in 2 mls of 6N hydrochloric acid and were transferred to toughened glass hydrolysis tubes which were then sealed under vacuum by fusing the glass of the neck in an oxy-acetylene flame. The samples were then heated for 18 hrs at 110°C in a thermostatically controlled oven.

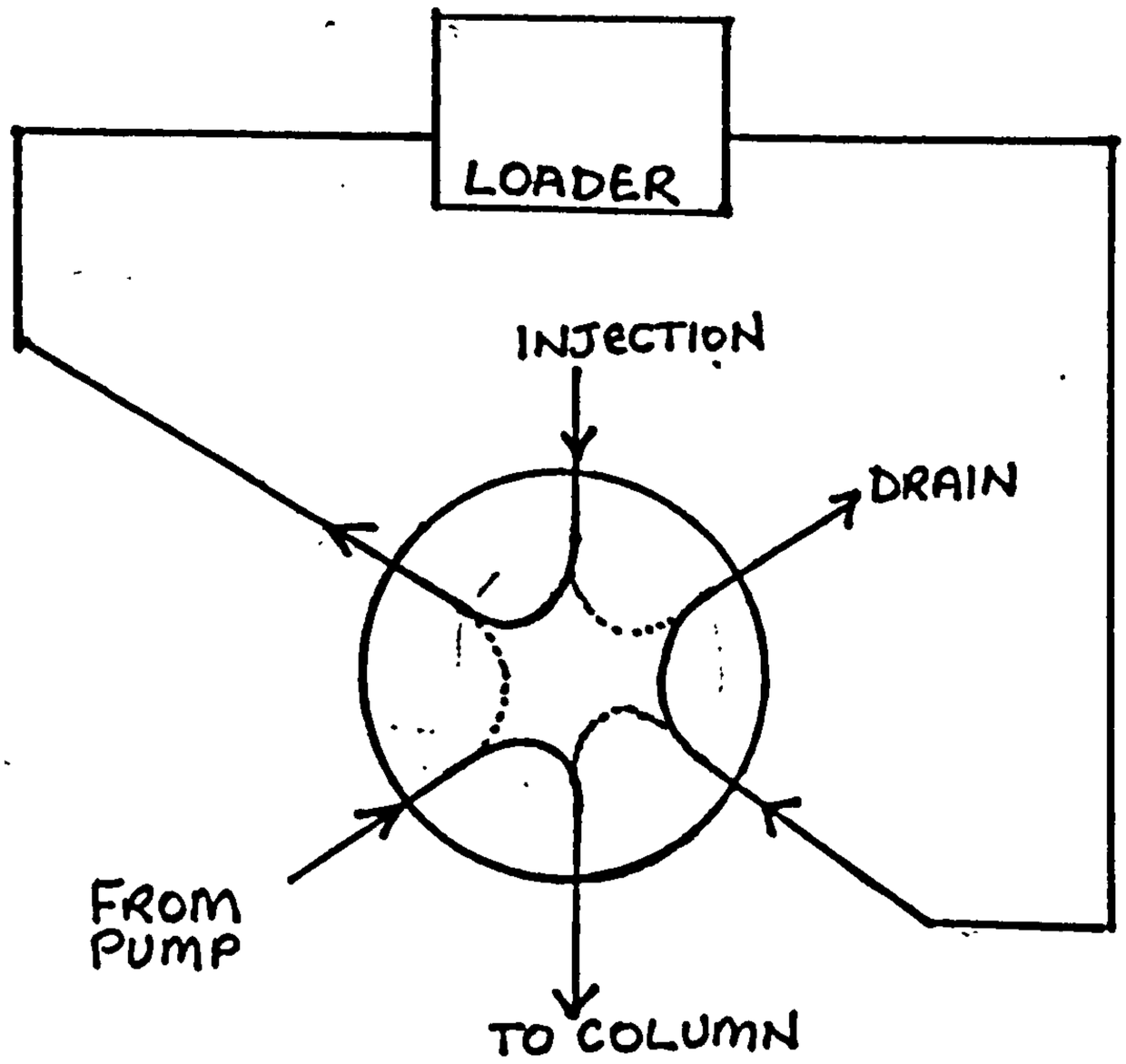
After hydrolysis the samples were freeze-dried and taken up in pH 2.2 citrate buffer to a volume of 10 mls. 1 ml aliquots of these samples were then taken and diluted 1 : 1 with an internal standard solution of 200 nM/ml dl nor-leucine and 20 nM/ml taurine. The mixture was thoroughly shaken and was then applied as below. In the case of wool samples these were hydrolysed in the above way but 2.5 cm (0.002 g) lengths of yarn were measured out and were placed into the hydrolysis tubes directly. The procedure followed after this, was as for the GPC samples.

(v)b Sample Application

All samples were applied to the column through the BC500 automatic loader. This consists of a double, twenty position rotary valve, with a Teflon tubing loop formed between each pair of valve ports. Loops are selected by a rotary switch, the valve rotor being driven through a precision worm drive by a motor. Precise location of the valve is ensured by the gear arrangement, where one complete rotation of the drive system results in exactly 1/20th rotation of the valve.

Samples are first stored in calibrated loops of exactly 1 ml volume and are injected on to the ion exchange resin at the appropriate point in the analytical programme. In this way up to 19 samples may be run consecutively (see fig 5.2)

fig. 5.2



ANALYSIS

—— LOAD POSITION
- - - - ANALYSIS POSITION

(vi) Buffer Solutions

Four aqueous buffer solutions were prepared the compositions of which are given in Table 5.4. A small amount of phenol was dissolved in each buffer solution to prevent bacterial growth. Ethanol may be added to the pH 3.25 buffer in order to increase the separation between threonine and serine, but in the work carried out this was not found to be necessary.

All the buffers were made up to 20 litre stock solutions and were at approximately the correct pH during storage. Prior to use they were adjusted

accurately to within ± 0.005 of a pH unit by the addition of sodium hydroxide pellets or concentrated hydrochloric acid.

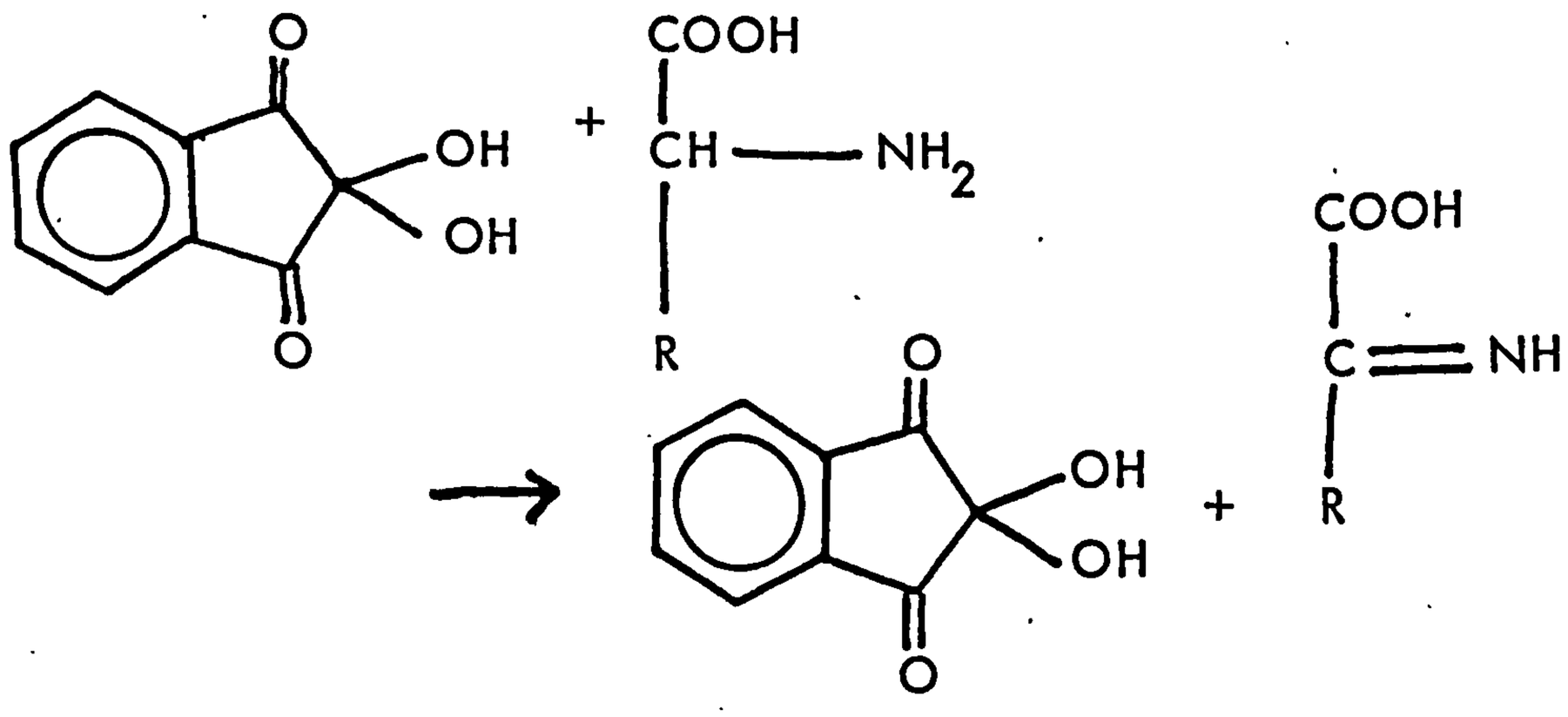
The use of sodium hydroxide, and hydrochloric acid in such concentrated forms kept to an absolute minimum any change in the ionic strength of the solutions.

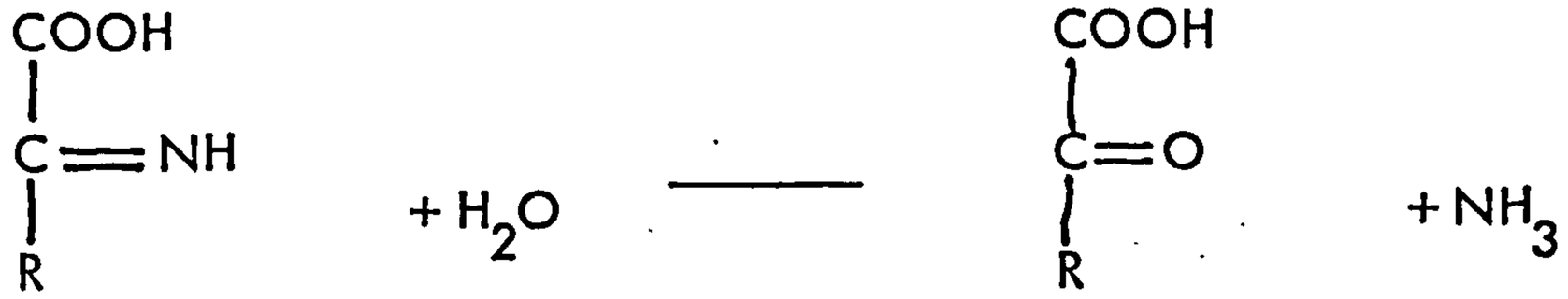
TABLE 5.4

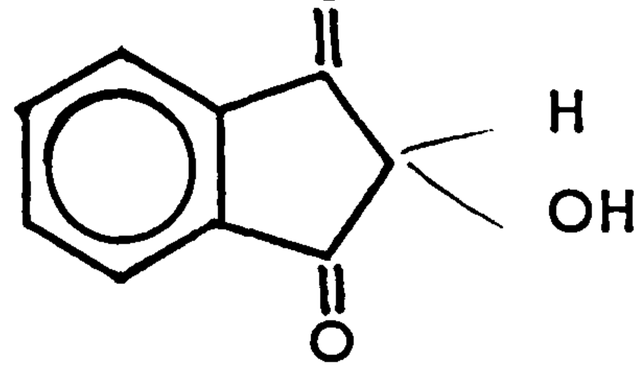
pH + Na(M)	2.2 / 0.2	3.25 / 0.2	4.25 / 0.2	6.45 / 1.2
Citric Acid	192	192	192	192
Sodium hydroxide (g)	20.48	20.48	47.68	207.6
Sodium chloride (g)	207.6	207.6	166	996
Thiodiglycol 25% (mls)	400	400	400	-
Phenol (g)	1	1	1	1
Final Volume (L)	20	20	20	20

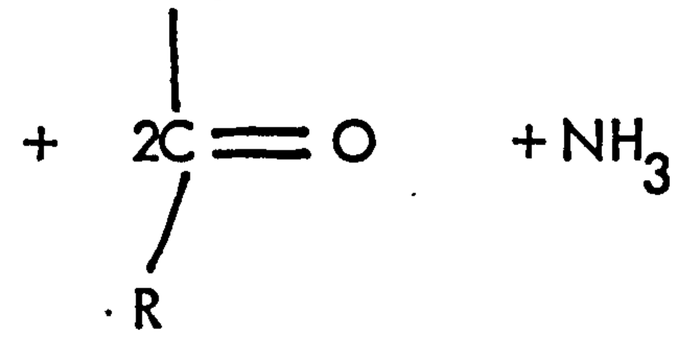
(vii) Detection of Amino Acids

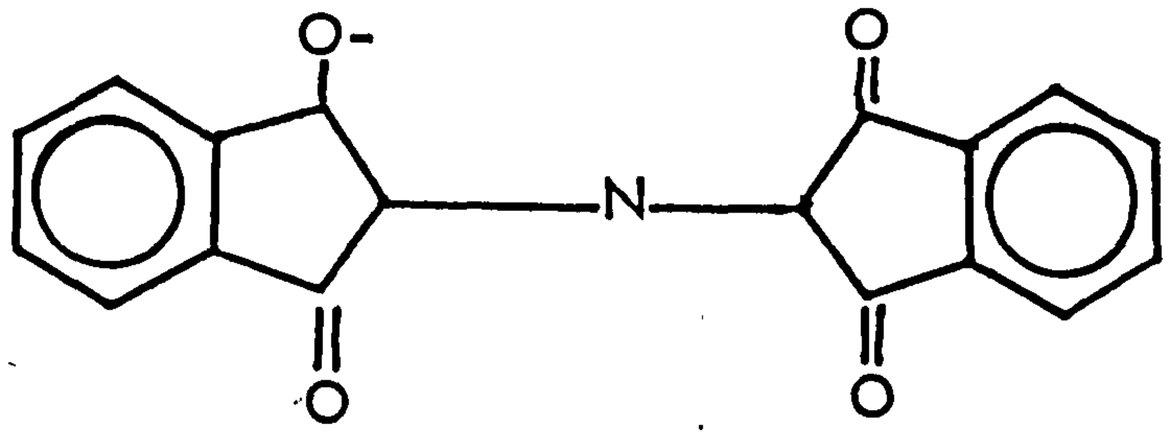
There are several methods available for the detection of amino acids but the one used in the experiments was of the ninhydrin reaction. Ninhydrin (indane 1,2,3-trione-2-hydrate) oxidises the amino acids to ammonia and carbon dioxide and the corresponding aldehydes, giving rise to dihydrides will condense with ammonia, forming a coloured compound.



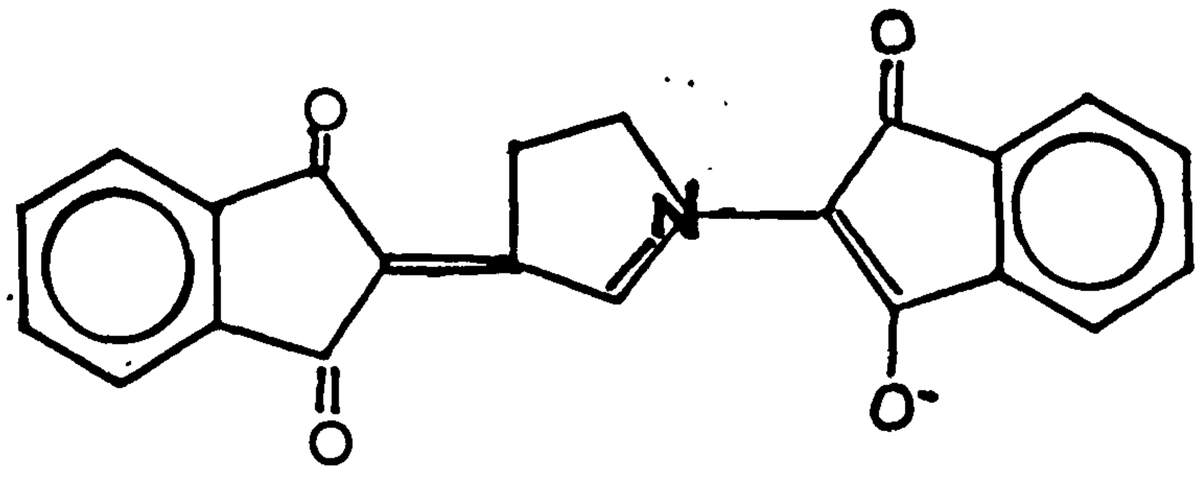








For imino acids the following coloured compound is formed according to Johnson and Macaldin (142) .



(viii) Ninhydrin reagent

This is a solution of ninhydrin dissolved in peroxide-free, 2-methoxy ethanol and sodium/potassium acetate buffer at pH 5.13. The buffer composition was:

5890 g Potassium acetate	80 g Sodium citrate
2720 g Sodium Acetate	2 L Glacial acetic acid

made up to ⟶ 20 L total volume

When required for use the acetate buffer was diluted 1 : 3 with distilled water and the pH was adjusted to 5.13 with either solid sodium hydroxide or concentrated hydrochloric acid. The ninhydrin reagent was prepared in the 5 L storage bottle of the analyser, 2 methoxy ethanol (3.75 L) was poured into the bottle and nitrogen was bubbled through for 15 minutes. 60 g of ninhydrin was then dissolved in this 2 methoxy ethanol and 1.25 L of diluted acetate buffer at pH 5.13 was then added. Finally 12.5 mls of 15% w/v titanium III chloride was added. Prior to use the ninhydrin was left overnight to stabilise. At all stages in preparation, and once prepared, the ninhydrin was continuously purged with nitrogen.

(ix) Evaluation

The amino acids are eluted as symmetrical gaussian peaks unless there is a fault in their separation, or an unknown peak is eluted (as has happened). Quantitative evaluation is based on the curve with the highest absorption values, with most amino acids this is the 570 nm curve; proline and hydroxyproline (imino acids) differ in that they show maximum absorption at 440 nm. Quantitative evaluation was carried out thus:

(a) The exact extinction value of each peak was noted.

(b) The baseline value (H_N) unless zero was subtracted from this value.

(c) The absolute height H was divided by 2 and the value of the baseline was added to this. A thin pencil line was drawn across the amino acid peak at this point.

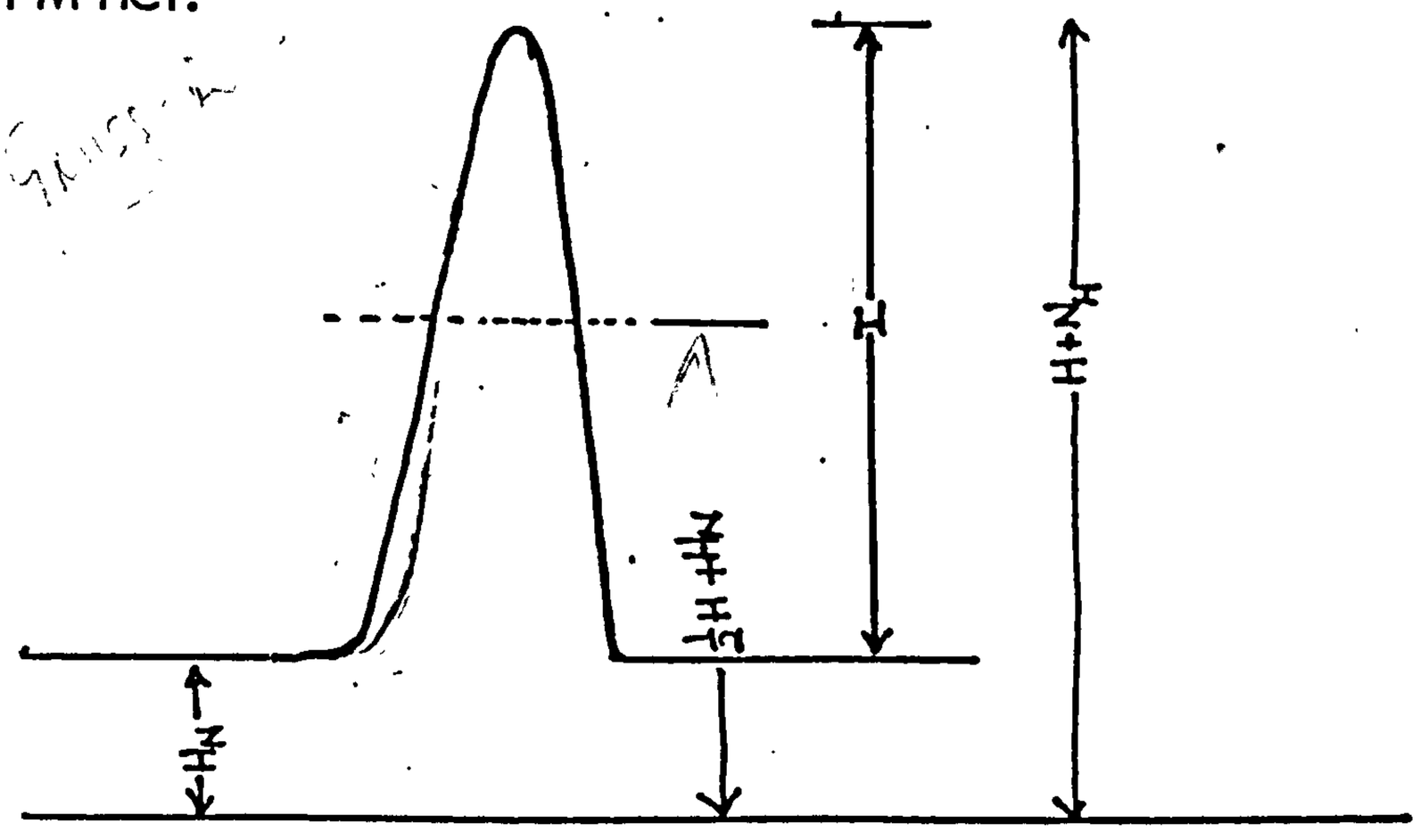
(d) The points above this line were then measured to the nearest 0.1 spacing in order to obtain the width. To simplify this operation the recorder printed every fourth point of the chromatogram in black.

The results were then used to calculate the concentration of the amino acids using the equation

$$\mu \text{ mole} = \frac{H \times W}{C_{HW}}$$

C_{HW} corresponds to the H x W value for 1 μ mole of an amino acid i.e. the molar extinction coefficient.

A large number of samples were run and in order to compare any run with previous, or subsequent runs, on an absolute base, an internal standard was always included. This standard was 200 n mol/ml —— and 20 nm/ml taurine —— in 0.1 M HCl.



Before analysis of samples of unknown composition was carried out, a standard sample of 15 amino acids was run to obtain the relative colour recovery values of each amino acid on a molar basis with nor leucine. In order to speed up the calculation process a computer program was devised to simplify the calculation procedure. Values of peak areas (H x W) were still calculated by hand but the values were then fed into a program. For each analysis the molar concentration of each amino acid was obtained and was then converted to a mol %. Values of mol % were then printed in tabular form together with total concentration of all amino acids present.

The latter was calculated as follows:

$$\text{Total Unit Concentration} = \frac{A_{AA} \times C_{AA}}{A_{NIe} \times C_{NIe}}$$

where A_{AA} is peak area for each amino acid and C_{AA} the colour recovery of each amino acid. A_{NIe} is the peak area for nor leucine and C_{NIe} the colour recovery for nor leucine.

5.2.5 High Voltage Electrophoresis (HVE)

(i) Instrument

HVE was carried out on the degradation products from the treatment of wool using a Locarte instrument. The paper used in the experiments was sandwiched between two metal cooling plates through which passed water from a refrigerating thermostat bath. The plates (105 cm x 15 cm) were insulated from each other, and from the paper by 4 layers of polyethene film 0.05 cm thick. The plates were pressed together by air pressure of 6 p.s.i. applied by a bag underneath the moveable bottom plate. This pressure assured efficient contact, and therefore, cooling.

The electrodes were made of platinum wire and were immersed in a buffer bath (see below for constituents) and brought into contact with the paper by paper wicks.

(ii) Buffer Solution

An aqueous solution of the following composition (pH 1.85) was used at all times.

98% Formic acid	60 ml
Glacial Acetic Acid	140 ml
total volume	2 L

(iii) Sample application

A strip of Whatman 3MM chromatography paper marked with a baseline, (100 cm x 15 cm) was immersed in the above buffer solution and placed on the lower polyethene sheet. A similar sheet of dry paper was laid along its length and superfluous buffer was thus soaked up. This sheet was then discarded. The sample was applied (100 µl) together with an alanine reference standard and the top polyethene sheet was placed in position together with the top cooling plate. Runs were carried out for varying times depending on the samples used. The voltage applied was 6 kV and the current varied from run to run. After each run the paper was dried in an oven at 80°C.

(iv) Development

This was carried out using a cadmium acetate ninhydrin solution of the following composition:

Cadmium acetate	100 mg
Distilled water	10 ml
Glacial acetic acid	2 ml
Acetone	100 ml.
Ninhydrin	1 g

The paper was immersed in this solution, then returned to the oven at 80°C for thirty minutes. $R_{alanine}(R_{ala})$ values were then calculated thus:

$$R_{ala} = \frac{\text{Distance moved by sample}}{\text{Distance moved by alanine}}$$

Most samples appeared as tight bands. However, where the bands were diffuse then the R_{ala} was taken at the front, R_{ala}^{I}, and rear, R_{ala}^{II}, of that band.

5.2.6 Breaking Strength Testing

Yarn strength tests were carried out on a Zellweger Uster A.D. automatic yarn strength machine in a standardised atmosphere maintained at 65% $R_H \pm$ 2% and 18°C ± 1°C.

CHAPTER 6

H ACID IN ACID DYEBATH
EXAMINATION OF RESULTS OBTAINED ON GEL PERMEATION CHROMATOGRAPHY AND SUBSEQUENT AMINO ACID ANALYSIS

A 10 g sample of wool was treated in 1 litre of acid dyebath, the composition of which was,

10% owf (1.02×10^{-2} M) Sulphuric VI acid

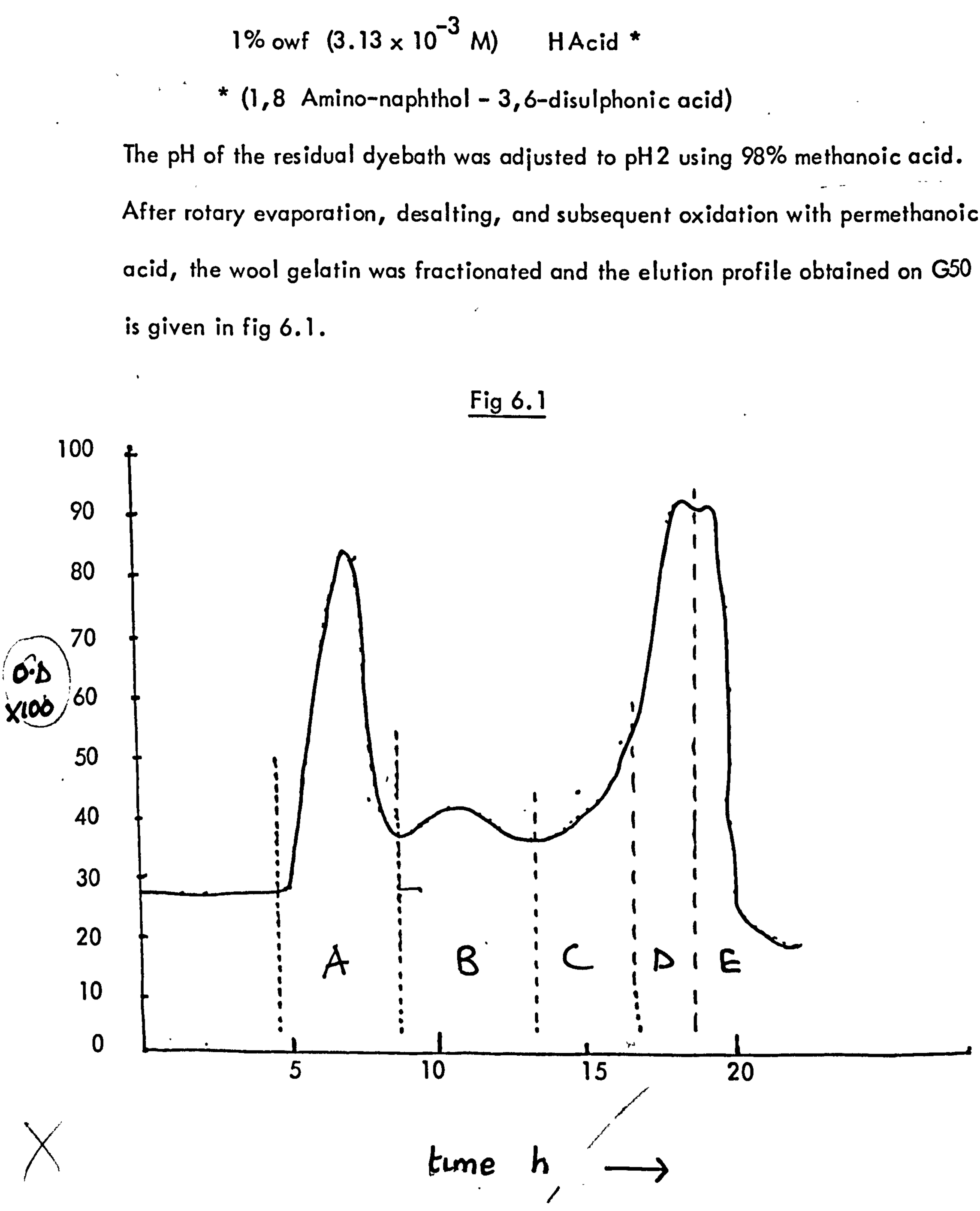
10% owf (7.04×10^{-3} M) Sodium sulphate VI

1% owf (3.13×10^{-3} M) HAcid *

* (1,8 Amino-naphthol - 3,6-disulphonic acid)

The pH of the residual dyebath was adjusted to pH2 using 98% methanoic acid. After rotary evaporation, desalting, and subsequent oxidation with permethanoic acid, the wool gelatin was fractionated and the elution profile obtained on G50 is given in fig 6.1.

Fig 6.1



The fractionated proteinaceous material was divided into 5 separate portions, A - E, which were then subjected to ion-exchange chromatography on A25. The latter used a combined pH and salt gradient which was adjusted by the use of the ultragrad. The pH value was decreased from 9 to 4 and at the same time the concentration of sodium ions was increased from 0.1 M to 1.0 M. The proteins separated by this technique gave the elution profiles illustrated in figs 6.2 to 6.6.

fig 6.2

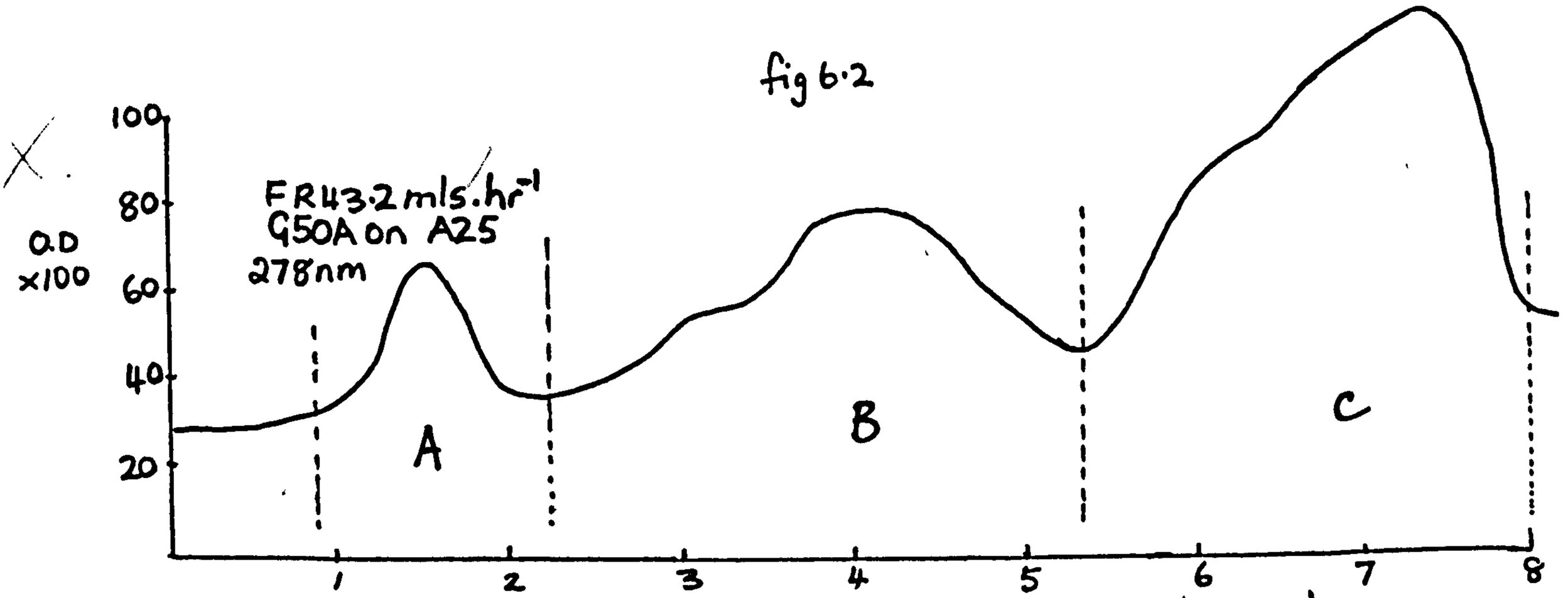


fig 6.3

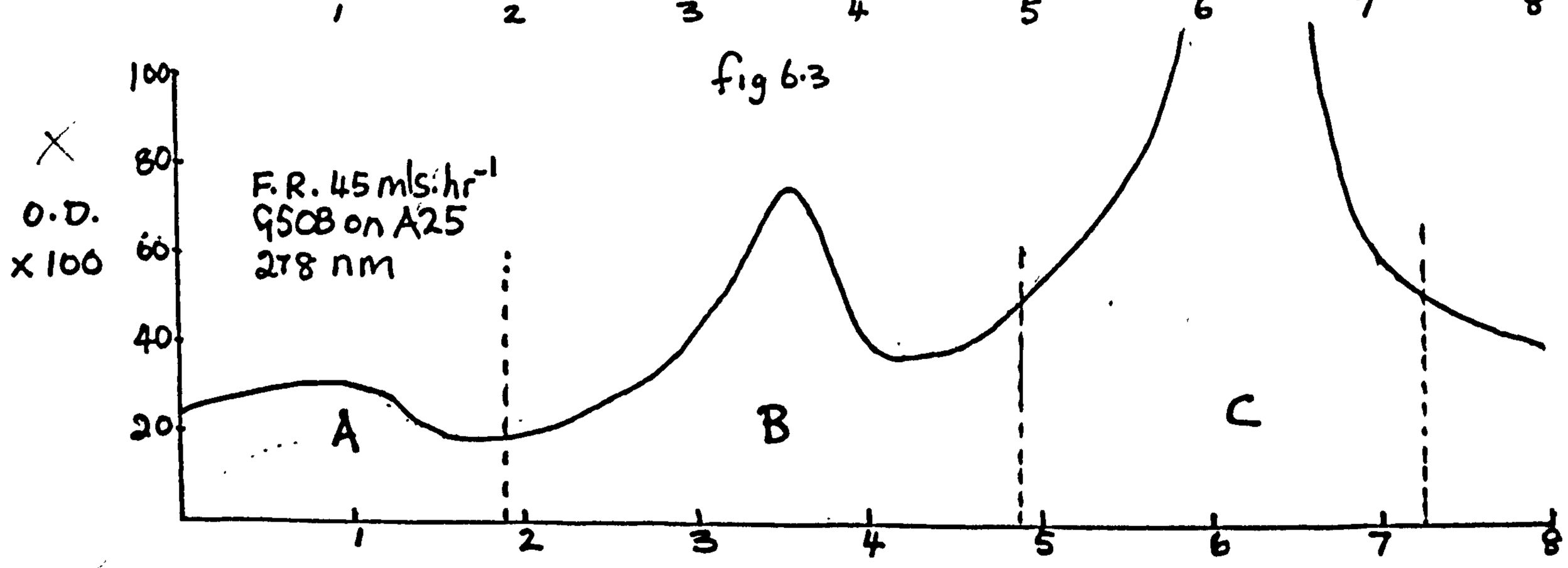


fig 6.4

fig 6·5

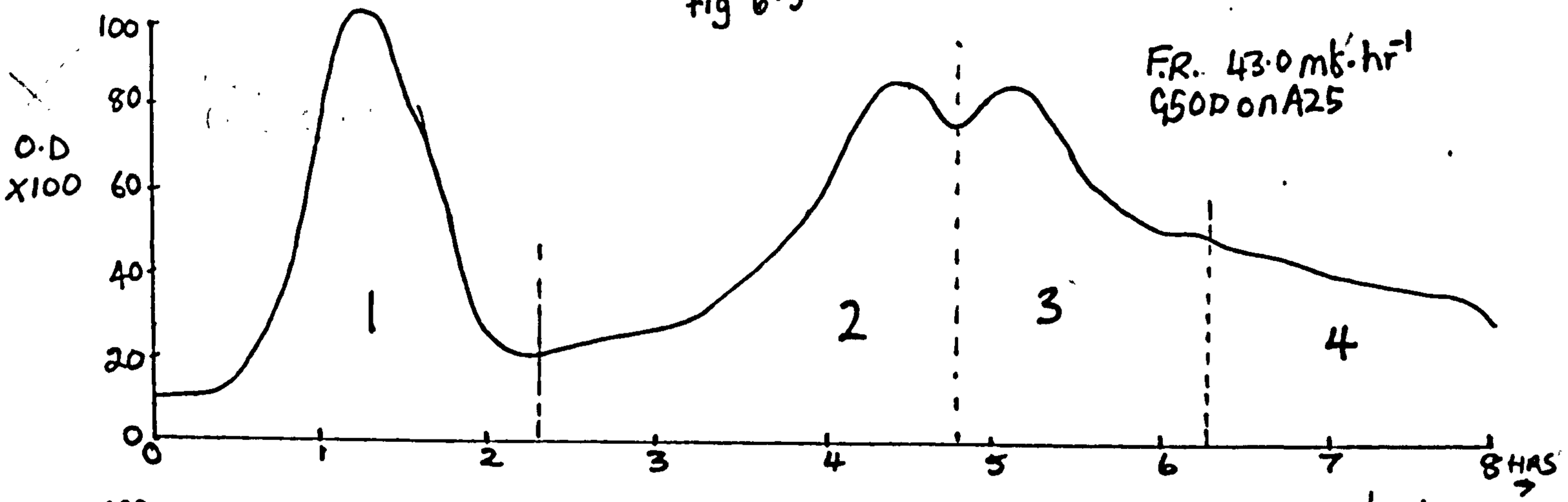
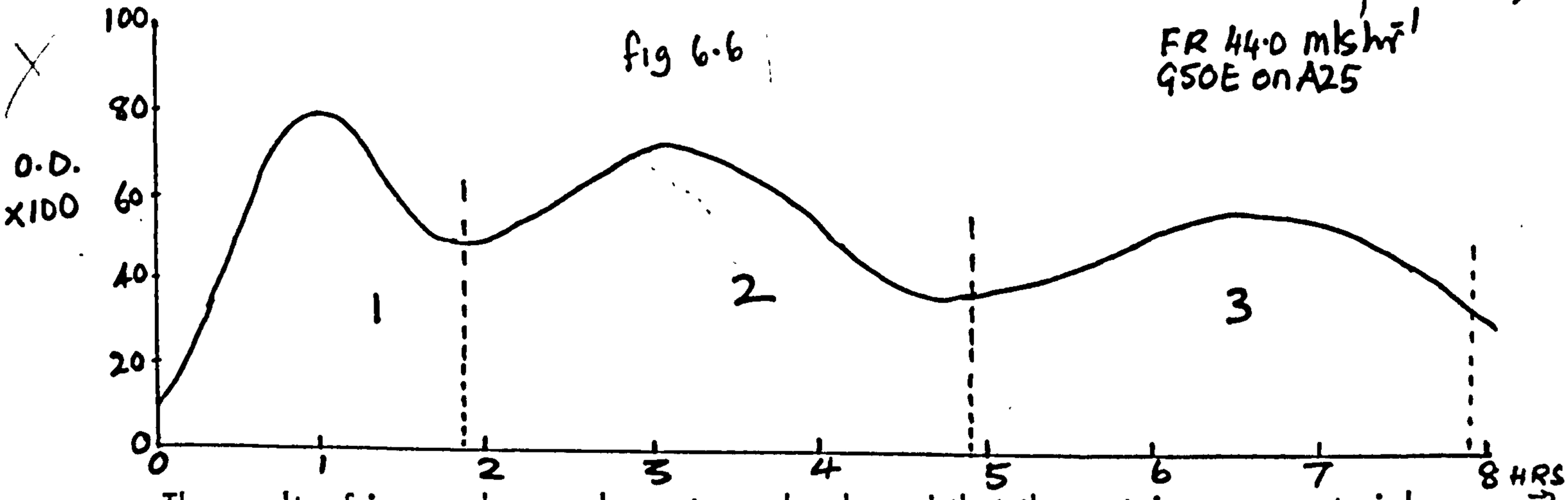


fig 6·6



The results of ion-exchange chromatography showed that the proteinaceous material was of a heterogeneous nature. The lack of many sharp peaks was indicative of this, as a sharp peak would imply a species with a very distinct p I value or a small molecule. Large proteins tend to have a neutral zone much as wool has (pH 4 - 9 approx). The shape of the elution profiles indicates a diverse range of small molecules that have slightly differing p I values, or large molecules with zones such as those in wool and which would, therefore, be eluted over a range of pH values.

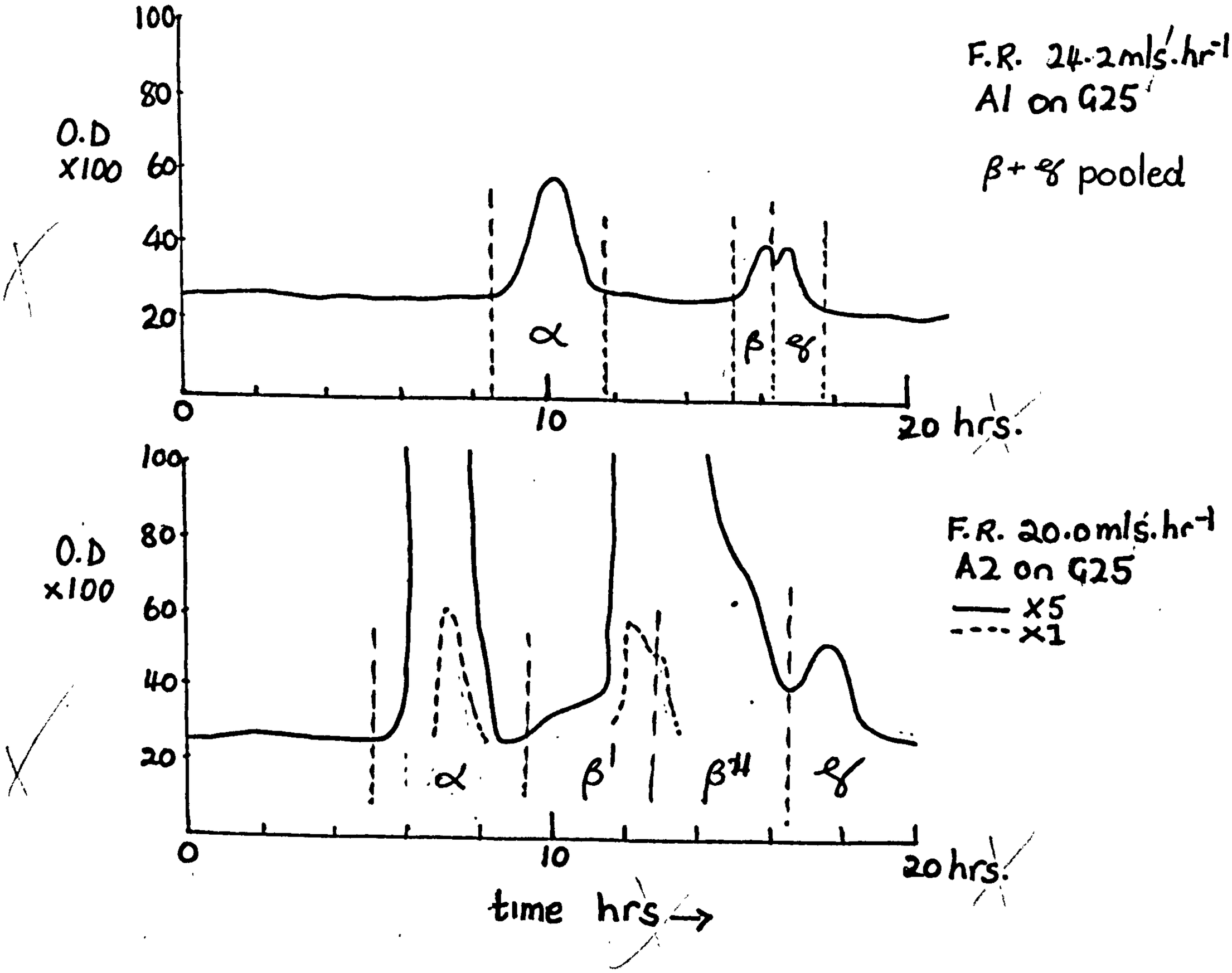
The latter hypothesis would appear to be rejected since the material can be fractionated on G50, and in some cases G25. An upper limit of 30,000 daltons is not a very high molecular weight in terms of proteins or biomolecules.

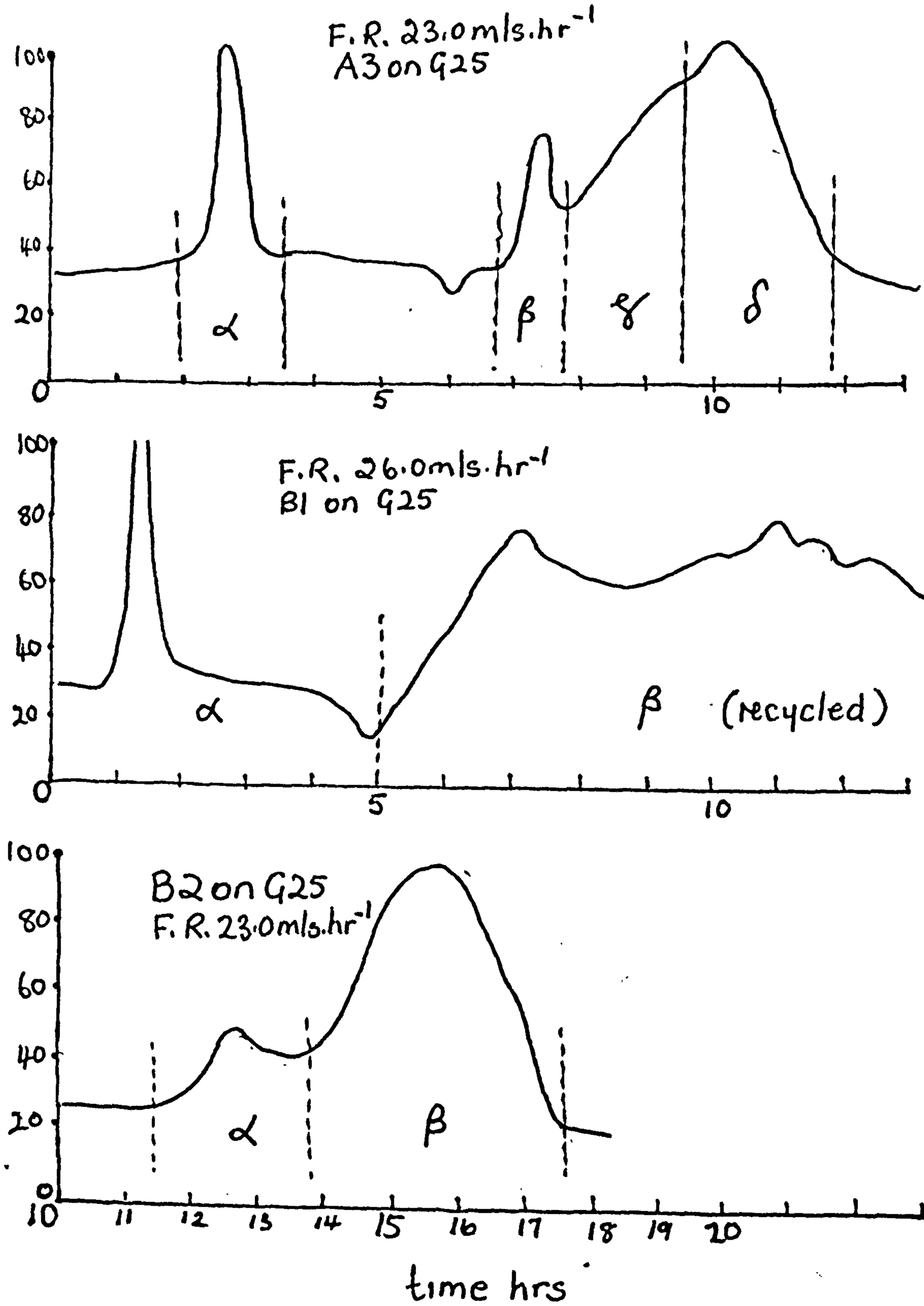
Further to the above, the material appeared to exhibit a spectrum of the entire pH range from pH 9 to pH4. Acidic proteins were always present in large quantities but then so were basic and neutral proteins. After desalting on a 25 cm column of G25 the proteins obtained were then fractionated on G25. Each fraction was then rotary evaporated to dryness and hydrolysed using 6 M hydrochloric acid at 110° C

under reduced pressure.

The profiles from the desalting procedure on G25 revealed the following. The fractions from D and E did not give any trace on G25; even a repeat run was unsuccessful. This desalting and fractionation was monitored by the use of a Uvicord II and the lack of a trace could have been due to the insensitivity of this instrument due to very small molecules being present (perhaps even separate amino acids). Whilst giving a trace at 206 nm, during desalting, fractions D and E did not give any sign of a trace at 280 nm. A, B, C did, however, give elution profiles and it is these results which are recorded, together with the amino acid composition data obtained expressed in mole percent.

Fig 6.7

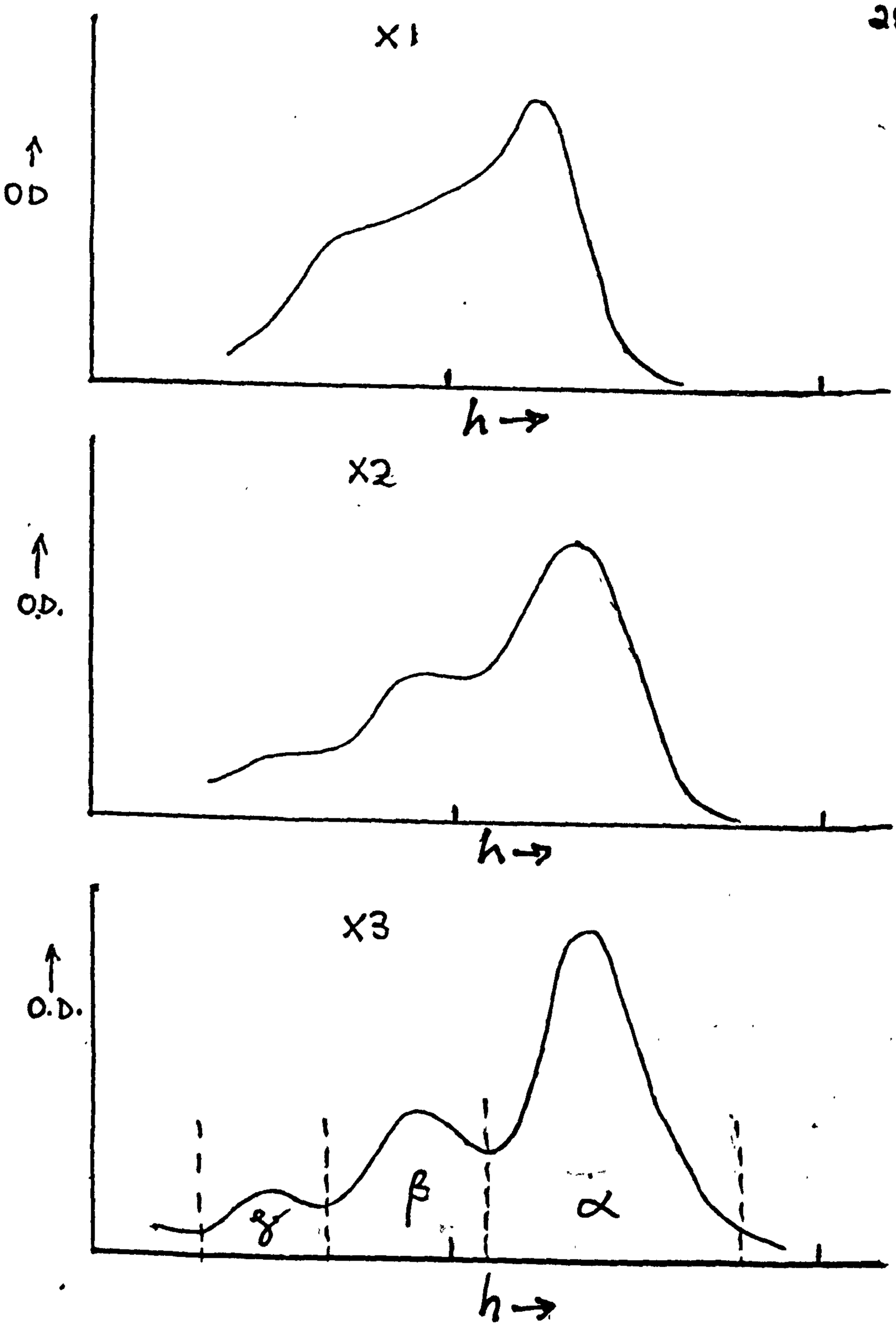


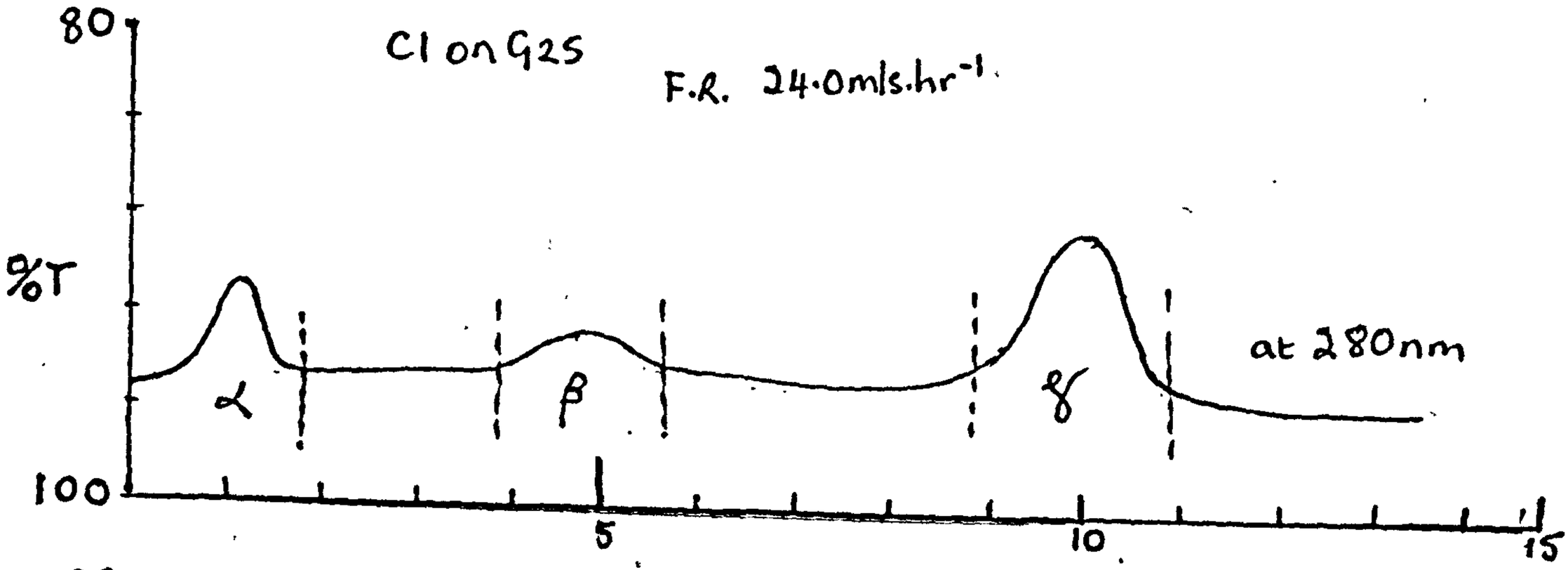

F.R. 23.0 ml/s.hr⁻¹
A3 on G25
100
80
60
40
20
0
5
10
α
β
γ
δ
F.R. 26.0 ml/s·hr⁻¹
B1 on G25
α
β (recycled)
B2 on G25
F.R. 23.0 ml/s.hr⁻¹
α
β
10
11
12
13
14
15
16
17
18
19
20
time hrs

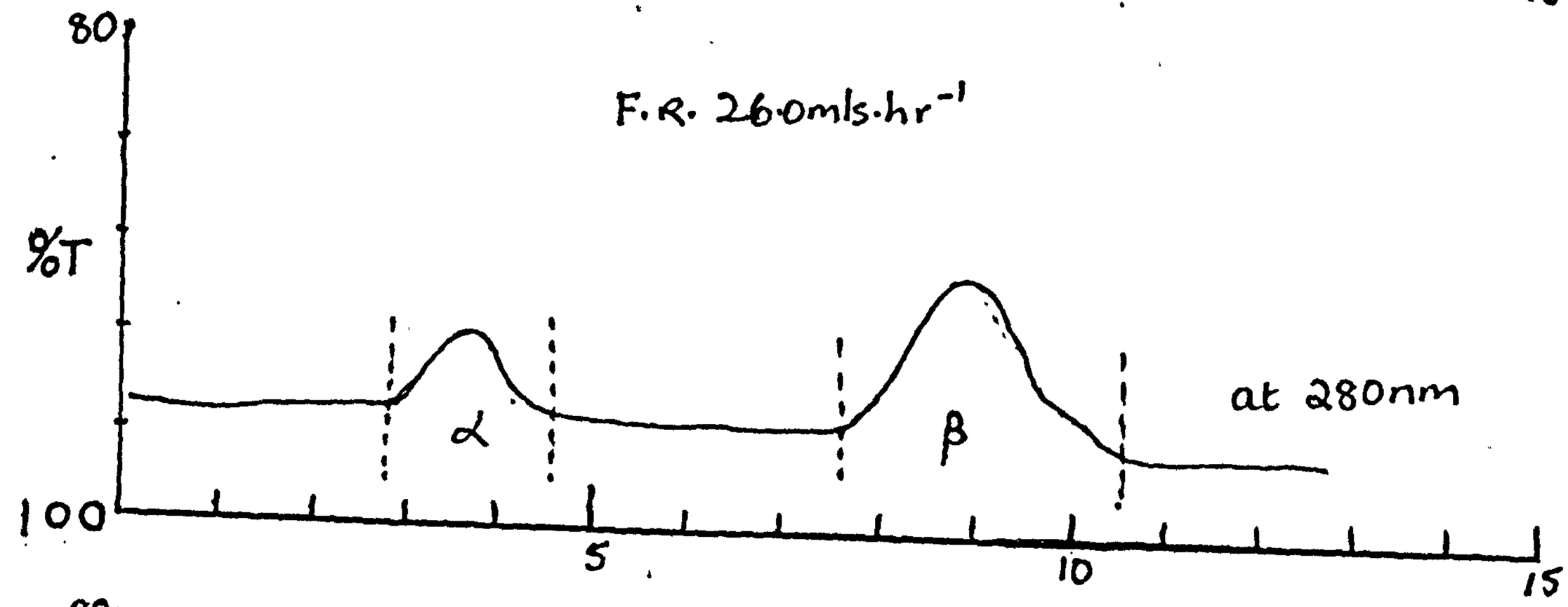
all at 280nm

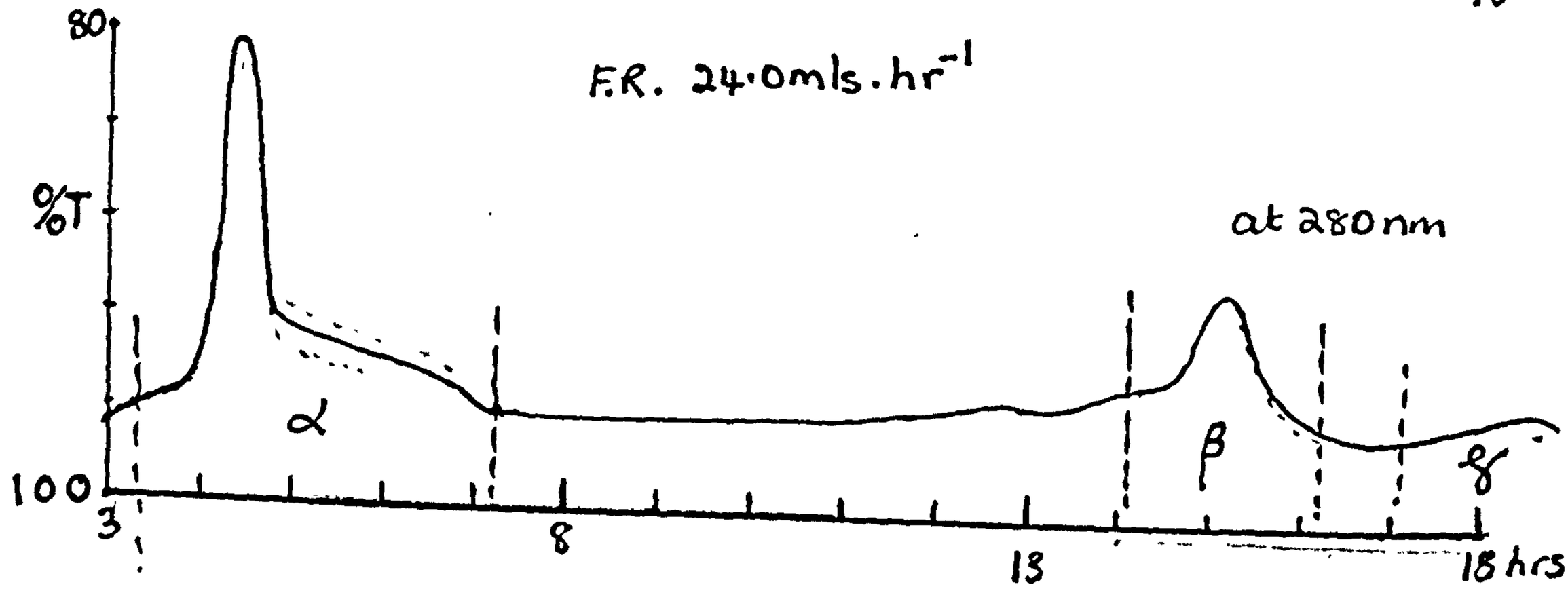
Bl β recycled on G25

F.R. 26·0 ml s.hr⁻¹
280 nm




80
Cl on G25
F.R. 24.0ml/s.hr⁻¹
%T
α
β
γ
at 280nm
100
5
10
15


80
F.R. 26.0ml/s.hr⁻¹
%T
α
β
at 280nm
100
5
10
15


80
F.R. 24.0ml/s.hr⁻¹
%T
α
β
γ
at 280nm
100
3
8
13
18 hrs

time in hrs.

The fractions from the A25 resolution behaved, as can be seen, in a diverse way from each other. Some of the fractions were eluted as fairly sharp peaks indicating the possibility that the material was an individual protein, or a fragment of an individual protein. Other fractions gave less well defined plateaux which would indicate a range of material of similar shape and molecular volume and possibly molecular weight.

This treatment was very time consuming, and the repeated use of rotary evaporation and fractionation, collection, and "pooling" plus the apparent insensitivity of the instrument to some of the smaller fractions, together with the questionable value of examining such, possibly, small fragments, led to the abandonment of ion exchange as the second stage of fractionation. In future all samples were subjected to G50 followed by G25, with subsequent amino acid analysis of the proteinaceous material. The results of the amino acid analysis of the above 24 fractions appear in tables 6.1 to 6.11; all results expressed as mole percent.

From tables 6.9 and 6.10 it can be seen that the character of the proteins differs markedly on each of the parameters tested. Of the 24 fractions 7 had an Acidics/Basics ratio of less than 1, i.e. the basic amino acids predominated; 15 had an Acidics/Basics ratio of greater than one, some markedly so, i.e. A2γ, A3α, B1α (9.1), B1β (7.6), B1δ (5.6), C1α (6.3), C2α (4.9). In A2γ large quantities of aspartic acid and glutamic acid gave rise to the high A/B ratio as in A3α. In B1γ the very high ratio is due to large quantities of cysteic acid, 15.6% and the almost complete absence of basic amino acids. B1β shows a low basics content rather than a high acidics content. C1α had a very high content of aspartic acid (32.6%) as did C1β (16.8%) but in the case of C1β the basic amino acids were present in far greater amounts thus tending to negate the effect on the Acidics/Basics ratio. There was a similar trend with C2β. Most of the protein was fairly low in sulphur with the

TABLE 6.1

H ACID A1

	mole %	α	β	γ
CYA		2.1	2.7	2.8
ASP		9.2	7.9	6.9
THR		6.1	8.8	5.7
SER		9.8	12.3	12.8
GLU		9.6	7.6	10.6
PRO		7.1	4.9	3.2
GLY		12.2	12.5	14.6
ALA		6.1	8.0	6.1
VAL		2.4	7.3	3.1
MET		0	0.7	0.9
ILE		1.3	2.8	2.0
LEU		4.6	6.1	5.3
TYR		1.7	0.2	2.4
PHE		0.9	0.4	0.1
HIS		7.9	2.7	4.6
LYS		8.2	6.3	8.9
ARG		11.9	9.1	10.3
CYS		0	0	0

mole %

TABLE 6.2

H ACID A2

mole %	α	β	γ
CYA	2.3	1.8	3.1
ASP	8.2	9.9	8.7
THR	10.1	5.1	6.1
SER	12.6	14.6	9.6
GLU	9.2	11.2	13.2
PRO	3.5	5.1	6.7
GLY	11.7	15.8	12.6
ALA	9.7	7.2	9.3
VAL	5.4	4.6	5.9
MET	0	0	0
ILE	2.6	1.6	3.3
LEU	7.7	6.2	6.7
TYR	0.8	1.9	2.6
PHE	0.5	0.3	0.9
HIS	4.3	0.9	1.3
LYS	6.1	5.9	6.9
ARG	5.4	7.8	3.4
CYS	0	0	0

TABLE 6.3

H ACID A3

mole %	α	β	γ	δ
CYA	2.4	2.7	5.3	2.9
ASP	10.6	9.2	8.2	8.8
THR	6.3	4.8	4.7	4.2
SER	12.7	10.3	14.2	12.1
GLU	8.1	8.3	6.2	6.7
PRO	3.3	5.7	3.1	4.7
GLY	17.6	12.6	16.1	23.6
ALA	7.9	6.3	5.9	6.1
VAL	6.3	3.6	1.6	3.2
MET	0	0	0	0
ILE	3.1	2.9	2.3	3.9
LEU	8.6	8.6	6.1	7.5
TYR	2.7	3.1	0.9	5.2
PHE	1.6	0.6	0	0
HIS	5.2	1.4	4.4	1.8
LYS	1.3	6.9	6.7	3.9
ARG	2.5	6.5	12.6	5.6
CYS	0	0	0	0
UNK	0	6.9	1.7	0
No. of UNK	0	1	1	0

UNK ≡ Unknown

TABLE 6.4

H ACID B1

mole %	α	β	γ	δ
CYA	15.6	5.1	3.0	5.0
ASP	0	4.3	9.6	30.6
THR	14.1	7.3	5.2	2.1
SER	25.4	15.1	17.6	15.2
GLU	0.1	4.3	7.4	2.6
PRO	4.5	16.6	5.3	6.1
GLY	0.8	20.2	21.6	19.2
ALA	20.2	15.1	6.1	4.3
VAL	7.3	4.5	5.6	3.1
MET	0	0	0	0
ILE	4.7	0	0	0.8
LEU	2.3	0	3.6	3.2
TYR	3.1	1.5	5.3	1.3
PHE	0	4.6	0.8	0
HIS	0	0	0.3	2.1
LYS	1.7	0	1.6	0.6
ARG	0	1.8	6.9	4.1
CYS	0	0	0	0

TABLE 6.5

H ACID B2

	mole % α	mole % β
CYA	3.2	7.6
ASP	10.3	5.7
THR	4.7	4.2
SER	12.6	11.8
GLU	6.1	7.2
PRO	4.8	3.6
GLY	12.1	18.6
ALA	8.8	3.6
VAL	3.6	3.4
MET	0	0
ILE	0.9	3.2
LEU	6.1	8.2
TYR	4.7	6.1
PHE	0.5	0.3
HIS	4.2	3.2
LYS	6.9	7.8
ARG	10.7	5.7
CYS	0	0

TABLE 6.6

H ACID C1

mole %	α	β	γ
CYA	4.2	2.1	2.1
ASP	32.6	16.8	4.6
THR	4.8	5.2	3.2
SER	11.7	11.7	6.1
GLU	3.2	2.5	5.3
PRO	3.4	8.6	4.3
GLY	16.6	14.1	26.4
ALA	4.2	10.6	4.8
VAL	1.6	2.7	5.2
MET	0	0	0
ILE	0	0.6	2.1
LEU	4.4	3.2	8.7
TYR	1.6	0.5	6.6
PHE	2.6	0.8	0
HIS	1.6	4.7	5.2
LYS	2.1	8.2	6.7
ARG	2.6	8.7	8.9
CYS	0	0	0

TABLE 6.7

H ACID C2

	mole %	α	β
CYA		4.2	2.7
ASP		24.1	20.2
THR		2.7	2.6
SER		12.6	7.8
GLU		5.2	2.7
PRO		5.1	4.6
GLY		23.6	23.8
ALA		3.6	2.7
VAL		2.2	1.6
MET		0	0
ILE		1.6	2.7
LEU		3.2	2.8
TYR		5.6	4.7
PHE		0	1.8
HIS		0.8	5.2
LYS		1.3	8.6
ARG		4.6	4.9
CYS		0	0

TABLE 6.8

H ACID C3

	Mole %	α	β	γ
CYA		10.2	2.3	0.9
ASP		6.1	7.6	6.9
THR		3.2	3.1	5.2
SER		1.6	10.2	16.9
GLU		2.8	3.2	6.1
PRO		6.2	6.1	5.4
GLY		24.2	18.6	6.9
ALA		4.7	4.3	8.7
VAL		5.3	7.6	1.4
MET		0	0	0
ILE		6.2	0.9	4.2
LEU		7.3	3.7	8.7
TYR		4.1	5.9	5.2
PHE		0.8	1.1	2.7
HIS		3.6	7.2	1.8
LYS		5.3	12.1	6.2
ARG		8.7	6.1	12.9
CYS		0	0	0

TABLE 6.9

mole %	A1			A2			A3			
	α	β	γ	α	β	γ	α	β	γ	δ
ACIDICS	20.8	18.2	20.3	19.7	23	25	21.2	20.1	19.7	18.31
BASICS	28.0	18.1	23.8	15.8	14.6	11.5	9.0	14.9	23.7	11.3
NEUTRALS	51.2	63.7	55.9	64.5	62.5	63.5	70.0	65	56.6	70.4
CYA/B	0.07	1.5	0.12	0.15	0.13	0.3	0.26	0.2	0.2	0.2
(THR + SER)/ARG	1.75	1.8	2.7	4.2	2.5	4.6	7.7	2.3	1.0	2.9
A/B	0.75	1.0	0.8	1.2	1.6	2.2	2.4	1.3	0.83	1.6

	B1				B2		C1			C2	
	α	β	γ	δ	α	β	α	β	γ	α	β
ACIDICS	15.8	13.7	20	38.2	19.6	20.6	40	21.4	12.0	33.5	25.6
BASICS	1.7	1.8	8.8	6.8	21.8	18.7	6.3	21.7	20.8	6.8	18.8
NEUTRALS	82.5	84.5	71.2	55	58.7	62.8	53.7	56.9	67.2	59.8	55.6
CYA/B	9	2.8	0.34	0.7	0.1	0.5	0.7	0.1	0.1	0.6	0.1
(THR + SER)/ARG		12.4	3.3	4.2	1.6	2.8	21.8	1.9	1.0	3.3	2.1
A/B	9.1	7.6	2.3	5.6	0.9	1.2	6.3	1.0	0.6	4.9	1.4

TABLE 6.10

mole %	C3 α	C3 β	C3 γ
ACIDICS	19.1	23.6	13.8
BASICS	17.6	13.7	21.0
NEUTRALS	63.3	61.6	65.0
CYA/B	0.6	0.1	.04
(THR + SER)/ARG	0.5	2.2	1.7
A/B	1.1	0.5	0.7

TABLE 6.11

mole %	A1			A2			A3			
	α	β	γ	α	β	γ	α	β	γ	δ
GLYCINE	12.2	12.5	14.6	11.7	15.8	12.6	17.6	12.6	16.1	23.6
TYROSINE	1.7	0.2	2.4	0.8	1.9	2.6	2.7	3.1	0.9	5.2
TOTAL	13.9	12.7	17	12.5	17.7	15.2	20.3	15.7	17	28.8

	B1				B2	
	α	β	γ	δ	α	β
GLYCINE	0.8	20.2	21.6	19.2	12.1	18.6
TYROSINE	3.1	1.5	5.3	1.3	4.7	6.1
TOTAL	3.9	21.7	26.9	20.5	16.8	24.7

	C1			C2		C3		
	α	β	γ	α	β	α	β	γ
GLYCINE	16.6	14.1	26.4	23.6	23.8	24.2	18.6	6.9
TYROSINE	1.5	0.6	6.6	5.6	4.7	4.1	5.9	5.2
TOTAL	18.1	14.7	33	29.2	28.5	28.3	24.5	12.1

exception of A3 γ, B1 α , B1 β , B2 β , and C3 α .

The content of glycine and tyrosine is worthy of consideration in determining the origin of the material. Several of the fractions are rich in glycine (A2 β, A3 α, γ, δ ; B1 β, γ , δ ; B2 β ; C1 α , γ; C2 α , β ; C3 α , β ;). Some of these were also rich in tyrosine (A3 δ ; B1 γ; B2 α , β; C1 γ; C2 α , β ; C3 α , β , γ.) There is a marked tendency for those fractions rich in glycine and tyrosine to be of lower molecular weight within a group and indeed the fractions richest in glycine/tyrosine are those which come originally from the lowest molecular weight fractions from G50, C.

Some of the material could come from the cell membrane complex . The intermacrofibrillar material found in wool, a non-keratinous protein material, could also be removed. Analysis of this material by Peters and Bradbury (53) showed it to be rich in aspartic acid, glutamic acid, glycine, serine, leucine and alanine. The protein extracted would appear to come from a variety of sources and one would say that the total protein extracted by the treatment was a composite of all the labile proteins in order of their accessibility. What one is seeing is a prolonged extraction process gradually removing the protein which is accessible and susceptible to extraction. Material rich in glycine and tyrosine from the cell membrane complex and intermacrofibrillar material are extracted after relatively short times, up to 8 hours (Baumann (139)).

It is well documented that, over a period of 24 hours at acid pH values outside the isoionic region, the cortex itself is gradually dissolved, with preferential dissolution of the orthocortex (Elliott and Roberts (78), Elliott et al (77), Leach et al (120), Kulkarni et al (45)). With increasing time the composition of the total protein extracted tends to approach that of the cortex, and on still further increase in time of treatment it approaches that of the whole wool fibre. This is only to be expected

TABLE 6.12

RESIDUAL WOOL 24 HOURS ACID DYEBATH

H ACID

mole %

	0.1%	0.2%	0.5%	1.0%	2.0%	5.0% *
CYA	0.4	0.4	0.3	0.4	0.3	0.6
ASP	6.3	6.2	7.5	7.6	7.6	7.6
THR	5.2	4.8	4.5	4.5	3.9	4.2
SER	7.8	8.1	7.5	7.8	7.3	7.3
GLU	10.0	8.6	8.6	8.4	8.6	8.6
PRO	8.5	8.0	8.2	8.3	9.2	7.8
GLY	6.5	6.1	6.3	6.4	6.0	6.1
ALA	5.1	5.4	5.5	5.6	6.1	5.5
VAL	5.3	4.6	4.6	4.8	4.7	4.6
MET	0	0	0	0	0	0
ILE	2.1	2.8	2.5	2.4	2.6	2.8
LEU	10.4	8.6	8.8	8.3	8.0	8.5
TYR	4.5	4.6	4.2	4.2	4.9	4.7
PHE	3.8	3.9	3.9	4.1	4.1	3.9
HIS	1.8	1.3	1.3	1.4	1.4	1.4
LYS	3.8	4.8	4.2	4.4	4.5	4.4
ARG	12.0	11.6	11.8	11.0	11.2	11.5
CYS	9.6	9.9	10.4	10.4	11.0	10.8

* owf

mol/%

TABLE 6.13

24 HOURS ACID DYEBATH

H ACID

mole %	1%	5%	10% *
CYA	0.4	0.5	0.4
ASP	10.2	10.7	9.8
THR	4.6	4.2	4.8
SER	7.3	6.1	6.5
GLU	8.3	6.1	7.3
PRO	5.6	5.4	5.7
GLY	6.7	9.3	9.6
ALA	5.7	6.3	5.9
VAL	5.9	6.2	6.4
MET	0	0	0
ILE	4.3	4.7	4.5
LEU	8.7	8.9	9.2
TYR	3.8	4.1	3.7
PHE	3.7	3.6	2.8
HIS	1.5	1.8	1.6
LYS	4.9	3.9	4.2
ARG	10.1	9.9	9.6
CYS	8.2	8.3	8.3

* owf

since what is occurring is a gradual hydrolysis of the whole wool.

The effect of altering the concentration of H Acid was not marked. The gelatins were similar although glycine was increased from 1% to 5% to 10%. Aspartic acid showed a slight decrease from 1% to 10% though the 5% treatment showed a slight increase over the 1% and 10% treatments. Leucine showed a slight increase over the range of concentrations. The trends in the data of the residual wool from the treatments with a range of concentrations of H Acid were not marked either. This is more to be expected since the treatment time and pH are probably the major driving force in any changes. The effect of the H Acid would appear merely to modify these major effects.

The total gelatin and residual wool from treatments with H Acid were examined by amino acid analysis. The total gelatin was high in aspartic acid, glutamic acid, leucine, arginine and cystine plus cysteic acid.

The residual wool from the 1% treatment was low in threonine, serine, glutamic acid, glycine, valine, isoleucine. Aspartic acid was slightly increased, while there was also a marked increase in proline above that of untreated wool (5.8 - 8.3% and a slight increase in alanine; leucine was also increased from 6.5 - 8.3% tyrosine and phenylalanine were also increased as were histidine and lysine. Arginine was markedly increased from 8.3% - 11.0 %.

Most of the differences across the range appear to take place from the 0.1% to 0.2% treatment, thereafter changes were not marked. It was as though a threshold value were crossed, after which concentration was not a decisive factor. The handle of the wool was, however, drastically altered, and its breaking strain was noticeably decreased (see chapter 10).

CHAPTER 7

†ORANGE II IN ACID DYEBATH
EXAMINATION OF RESULTS OBTAINED ON GEL PERMEATION CHROMATOGRAPHY AND SUBSEQUENT AMINO ACID ANALYSIS

A 10 g sample of wool was treated in 1 litre of acid dyebath the composition of which was,

10% owf	(1.02×10^{-2} M)	Sulphuric VI acid
10% owf	(7.04×10^{-3} M)	Sodium sulphate VI
1% owf	(3.35×10^{-4} M)	†C.I. Acid Orange 7

The treatment bath was adjusted to pH 2 using concentrated (98%) methanoic acid and was then rotary evaporated to dryness. The material was then desalted on a short G25 column (50 cm of gel) and the proteinaceous material obtained was at this point dissolved in distilled water.

The water was removed by rotary evaporation under reduced pressure at 35°C and the protein was then oxidised using per methanoic acid.

The oxidised proteinaceous material thus obtained was then subjected to gel permeation chromatography using Sephadex G50 followed by G25. The fractions thus obtained were then rotary evaporated and hydrolysed using 6 M hydrochloric acid at 110°C under reduced pressure. Amino acid composition data expressed as moles per 100 moles amino acids was then recorded as shown in tables 7.1 to 7.4. G50 fractionation yielded three fractions (fig 7.1) which were in turn fractionated on G25 after rotary evaporation. The traces then obtained are illustrated in figs 7.2 to 7.4. The G50A fraction yielded 4 peaks (A1 → A4) which commenced elution after 2 hours (fig 7.2). Fraction A1 gave a sharp distinct peak at the void volume time for the column while the other peaks were more diffuse and were therefore probably a large spread of small proteinaceous molecules. The G50B fraction gave a similar peak at the void volume followed by 3 other less well defined peaks (fig 7.3).

fig. 7·1

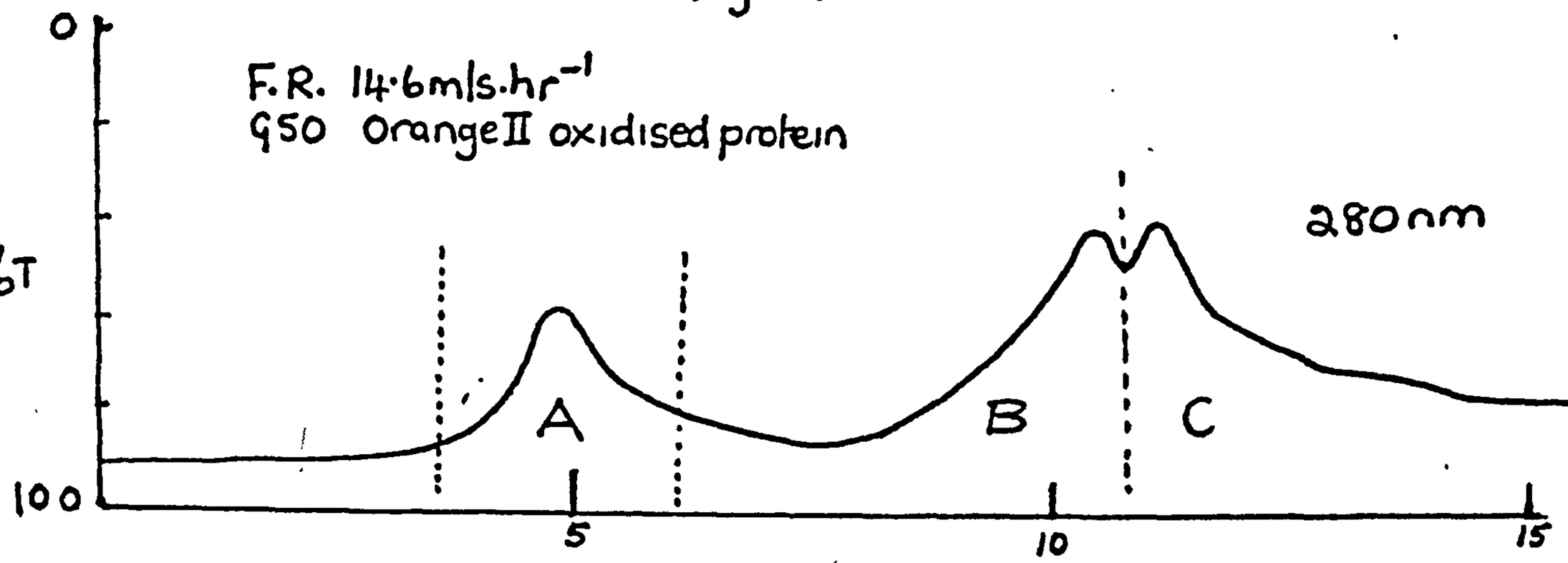
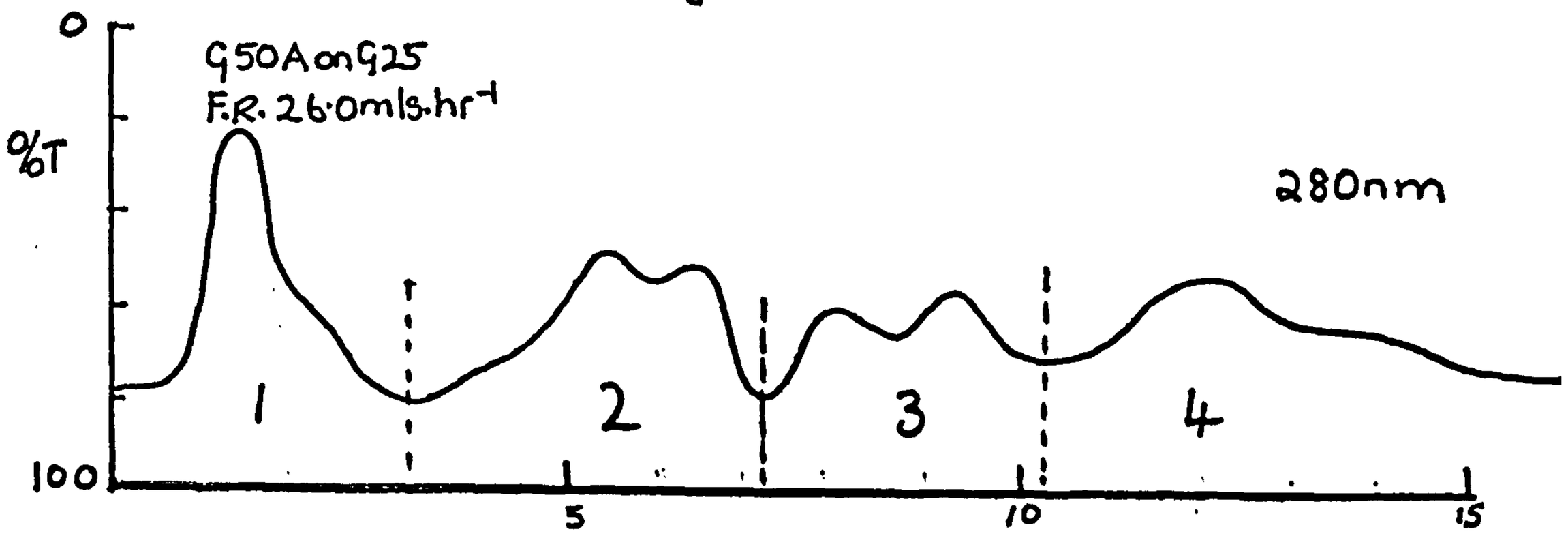


fig. 7·2



V_0 ?

fig 7.3

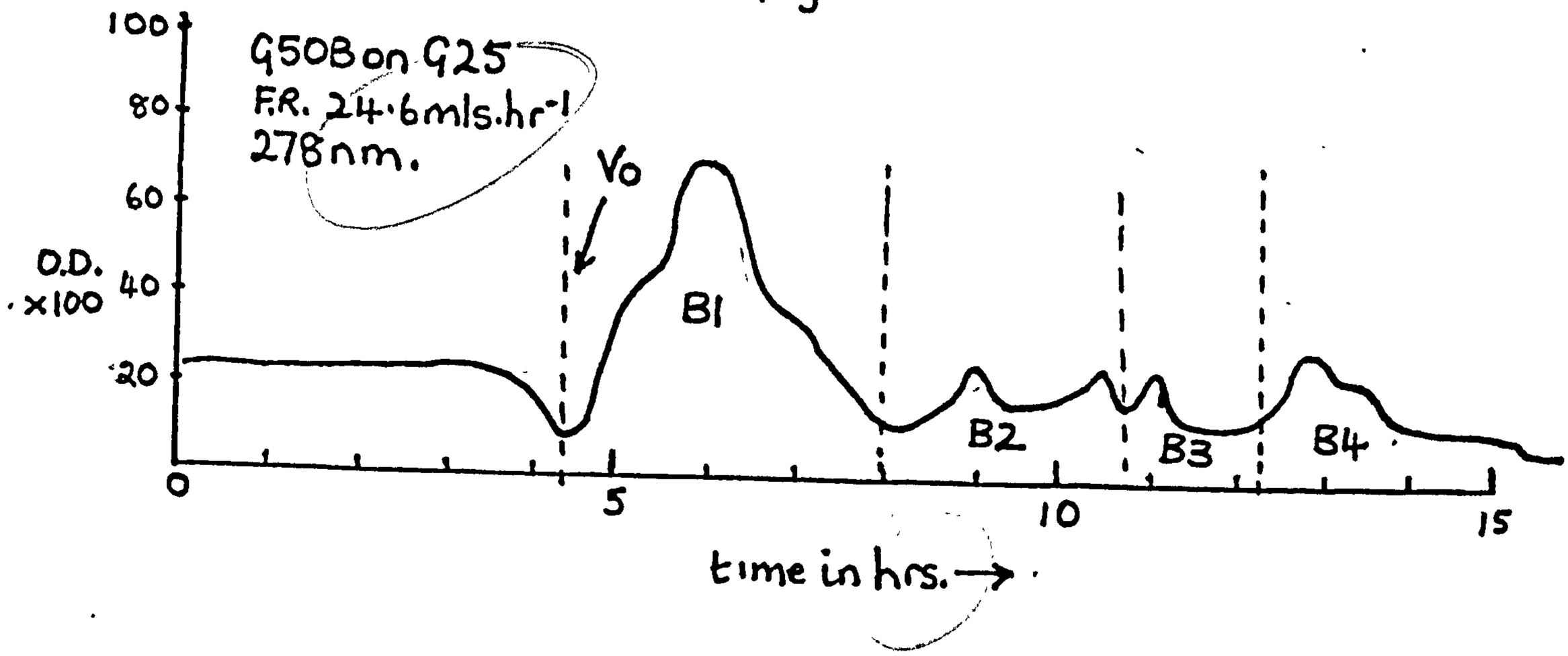
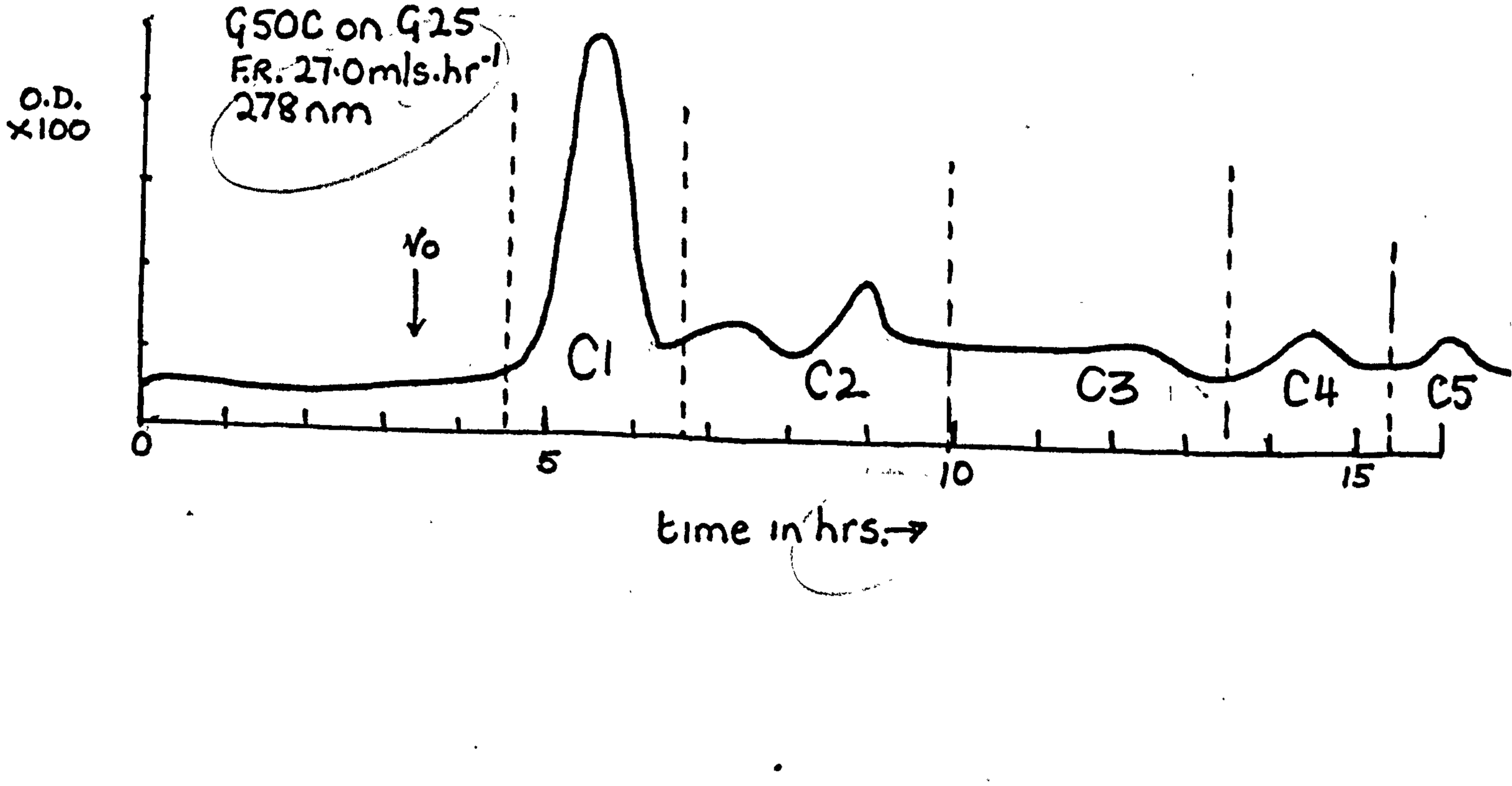

G50B on G25
F.R. 24·6 mls.hr⁻¹
278 nm.
O.D. ×100
100
80
60
40
20
0
5
10
15
Vo
B1
B2
B3
B4
time in hrs.→

fig. 7.4


G50C on G25
F.R. 27·0 ml/s.hr⁻¹
278 nm
O.D. ×100
Vo
0
5
10
15
C1
C2
C3
C4
C5
time in hrs.→

Elution times on G25 of fractions from G50A

peak	time, hrs
A1	2
A2	3-7
A3	7-10
A4	10-15

V_o = 2 hrs

In all a total of 13 fractions was obtained,

A1, A2, A3, A4, B1, B2, B3, B4, C1, C2, C3, C4, C5,

which, on hydrolysis, gave the amino acid data in tables 7.1 to 7.4.

Analysis of the data shows three fractions in which basic amino acids predominated, namely, A1, C2, C(4 + 5). The majority of the protein fractions showed an excess of acidic amino acids, some markedly so, viz.

ACIDIC/BASIC > 1	A2', A2", A3, A4, B1, B2, B4, C1, C3
ACIDIC/BASIC ≥ 2	B3, C1
BASIC/ACIDIC > 1	A1, C2, C(4 + 5).

B3 showed an ACIDIC/BASIC ratio of 3.1 : 1, and C1 a ratio of 2 : 1.

Another parameter worth examining in the context of wool proteins is the % glycine and % tyrosine content. High glycine/tyrosine proteins have been found to exist in the intercellular cement found in the cuticle and in the soluble proteins extracted from cell membranes. Fractions A3 and A4 had high glycine contents while B1 to B4 were very high in glycine and tyrosine, and also high in threonine, serine and aspartic acid.

TABLE 7.1

ORANGE II

۳۹٥٥ %

	A1	A2'	A2''	A3	A4
CYA	3.6	4.7	5.2	3.6	2.9
ASP	9.2	12.6	13.1	10.6	11.7
THR	5.8	5.1	5.3	9.7	14.3
SER	11.3	9.2	7.6	12.2	11.2
GLU	4.7	5.3	6.2	7.3	6.1
PRO	8.2	6.2	7.3	6.6	5.3
GLY	6.7	6.9	9.8	11.3	16.6
ALA	5.3	5.6	3.7	4.1	2.9
VAL	8.2	6.2	4.8	5.2	5.3
MET	0	0	0	0	0
ILE	3.7	4.2	4.6	5.3	3.7
LEU	4.2	5.7	4.6	6.2	2.9
TYR	3.6	4.6	5.2	3.2	4.7
PHE	1.8	3.2	1.9	1.2	0.8
HIS	4.7	5.9	5.3	3.6	0.9
LYS	6.1	7.3	6.8	2.8	2.1
ARG	12.8	7.2	8.7	7.2	8.6
CYS	0	0	0	0	0

TABLE 7.2

ORANGE II

Mole %

	B1	B2	B3	B4
CYA	3.7	3.6	2.7	2.6
ASP	8.6	12.2	11.6	10.7
THR	12.2	14.6	13.3	14.6
SER	11.6	9.7	8.6	9.2
GLU	4.7	5.2	6.3	5.6
PRO	5.2	5.7	7.3	4.3
GLY	17.6	24.7	22.6	19.7
ALA	4.7	3.2	4.7	2.7
VAL	6.2	3.1	3.2	3.2
MET	0	0	0	0
ILE	4.7	4.3	4.2	4.1
LEU	4.3	3.7	2.6	1.9
TYR	5.6	6.2	5.7	4.7
PHE	0.7	0.3	0.7	2.3
HIS	4.7	3.2	2.6	2.7
LYS	2.3	3.7	0.9	2.1
ARG	3.3	5.8	3.2	9.7
CYS	0	0	0	0

TABLE 7.3

ORANGE II

Mole %

	C1	C2	C3	C(4 + 5)
CYA	2.7	2.6	3.7	4.2
ASP	9.6	8.3	12.6	7.8
THR	10.5	6.7	6.8	11.2
SER	9.2	9.6	8.2	6.5
GLU	7.2	4.3	5.8	4.7
PRO	6.4	6.2	4.8	5.8
GLY	17.6	19.8	16.7	19.3
ALA	6.7	4.7	6.2	4.8
VAL	3.2	2.8	0.9	3.7
MET	0	0	0	0
ILE	3.7	3.6	2.9	2.7
LEU	7.2	8.8	6.7	4.7
TYR	4.7	2.7	5.2	3.6
PHE	1.2	3.4	2.8	2.7
HIS	1.6	3.6	2.4	2.8
LYS	4.3	4.7	2.7	5.6
ARG	4.1	8.2	11.6	9.8
CYS	0	0	0	0

TABLE 7.4

ORANGE II

Mole %

	A1	A2'	A2"	A3	A4
ACIDICS	17.5	22.6	24.5	21.6	20.7
BASICS	23.5	20.3	20.9	13.5	11.5
NEUTRALS	59.0	57.0	54.7	64.9	67.8
CYA/B	0.2	0.2	0.2	0.3	0.3
(THR + SER)/ARG	1.3	2.0	1.5	3.1	3.0
A/B	0.7	1.1	1.2	1.6	1.8

	B1	B2	B3	B4
ACIDICS	17.0	21.0	20.6	18.8
BASICS	10.4	12.7	6.7	14.4
NEUTRALS	72.6	66.3	72.8	66.8
CYA/B	0.4	0.3	0.4	0.2
(THR + SER)/ARG	7.2	4.2	6.8	2.5
A/B	1.6	1.7	3.1	1.3

	C1	C2	C3	C4 + 5
ACIDICS	19.5	15.2	22.0	16.7
BASICS	10.0	16.5	16.7	18.3
NEUTRALS	70.5	68.4	61.25	65.0
CYA/B	0.3	0.2	0.2	0.2
(THR + SER)/ARG	4.8	2.0	1.3	1.8
A/B	2.0	0.9	1.3	0.9

Fractions A1 and A2' + A2" showed a high lysine content and fraction A1 a high arginine content as did fractions B4, C2, C3 and C(4 + 5).

Due to the nature of the treatments, involving a temperature of 100°C for 24 hours in aqueous acidic solution, the extraction of proteins, and other materials such as lipids associated with the cell membrane proteins, is a complex affair and probably takes place in a series of steps based on the susceptibility of the proteins or other components to extraction. Once extracted, a protein would then find itself in a hydrolysing medium. This secondary hydrolysis, as opposed to the initial extraction, complicates the interpretation of the data. The overall effect of this persistently hydrolysing and extracting environment is to render the proteins ultimately into a very heterogeneous mixture.

The period of extraction is critical. Prolonged extraction gives heterogeneous proteins that are less and less identifiable with any particular component of the original wool. Ultimately the wool gelatin extracted would be hydrolysed to small peptides and individual amino acids. Even after 24 hours there are some individual amino acids present. One interesting experiment consisted of taking the total gelatin extracted and running it through the amino acid analyser without first hydrolysing in 6 M hydrochloric acid. Substantial quantities of aspartic acid were observed together with glutamic acid and serine. At the dilution used these were the only amino acids present in significant quantifiable concentrations. The concentrated dyebath extract gave a different elution profile. The three amino acids mentioned above were present but other peptides and amino acids were noticeable. Aspartic acid was completely off the scale.

As well as the amino acid analysis of fractions from gel permeation chromatography, amino acid analysis was also carried out on the total gelatin obtained from more extended treatments, and also on the residual wool from these treatments.

The results obtained are given in tables 7.5 and 7.6. The total gelatin was high in glycine, aspartic acid, serine, tyrosine, histidine, lysine and arginine compared to the composition of the wool as a whole. The cystine content was lower than in the original wool as were the contents of threonine, glutamic acid and phenylalanine. As regards the residual wool, the cystine content was reduced, as were the contents of aspartic acid, threonine, serine, glutamic acid (markedly 13.3 to 7.6) glycine, and valine. Proline content of the residual wool was increased as were alanine, isoleucine, leucine, tyrosine, phenylalanine, lysine and arginine.

TABLE 7.5

24 HOURS ACID DYEBATH TOTAL GELATIN

ORANGE II

	1%	5%	10%
CYA	0.3	0.6	0.5
ASP	11.2	7.9	9.3
THR	3.7	4.2	4.3
SER	10.8	9.7	9.2
GLU	6.9	7.1	7.8
PRO	5.3	4.2	5.2
GLY	14.7	17.6	13.7
ALA	4.7	5.9	6.3
VAL	5.2	5.8	6.1
MET	0	0	0
ILE	3.2	3.0	3.7
LEU	6.8	7.2	6.9
TYR	5.3	4.5	5.3
PHE	1.3	0.6	0.9
HIS	2.7	3.8	3.1
LYS	4.2	6.3	5.4
ARG	8.5	5.7	5.2
CYS	6.8	6.2	7.3
CYA) + CYS)	7.1	6.8	7.8

TABLE 7.6

ORANGE II

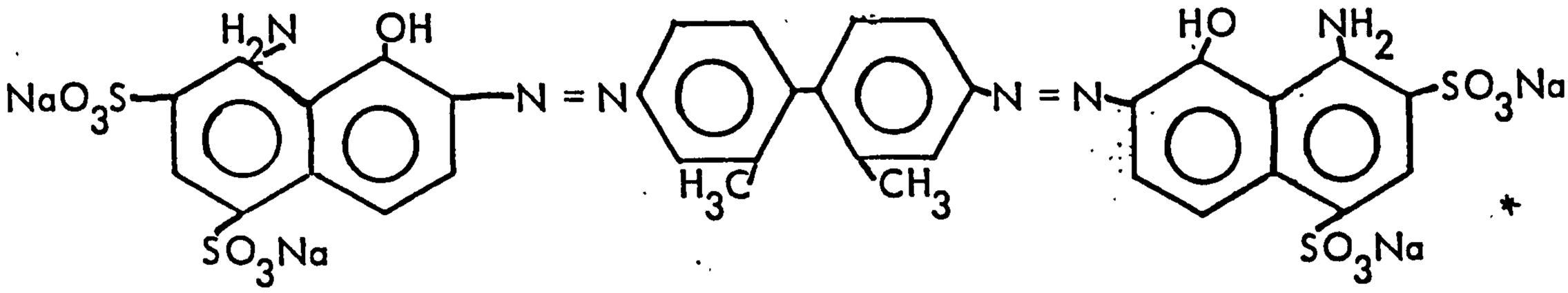
	0.2%	0.5%	1%	2%*
CYA	0.37	0.4	0.4	0.3
ASP	6.1	5.8	5.1	5.5
THR	4.7	4.5	4.6	4.7
SER	6.9	7.4	7.3	7.4
GLU	7.7	7.5	7.6	7.3
PRO	9.2	8.4	8.7	9.4
GLY	4.2	4.5	4.6	4.7
ALA	6.2	6.0	6.0	6.3
VAL	4.5	4.6	4.5	4.5
MET	0	0	0	0
ILE	4.3	4.3	4.2	4.8
LEU	7.0	7.9	7.9	7.5
TYR	5.6	5.6	5.6	5.5
PHE	4.1	4.1	4.0	3.8
HIS	1.1	1.2	1.3	1.1
LYS	4.7	4.6	4.4	4.2
ARG	14.4	14.4	13.0	12.7
CYS	9.2	8.2	9.0	9.0

* % owf

CHAPTER 8

EVANS BLUE IN ACID DYEBATH
EXAMINATION OF RESULTS OBTAINED ON GEL PERMEATION CHROMATOGRAPHY AND SUBSEQUENT AMINO ACID ANALYSIS

A 10 g sample of wool was treated in 1 litre of acid dyebath the composition of which was



10% owf (1.02×10^{-2} M) Sulphuric VI acid

10% owf (7.04×10^{-3} M) Sodium sulphate VI

1% owf (1.00×10^{-4} M) Evans Blue *

* C.I. Direct Blue 53.

The proteinaceous material contained in the above solution after treatment for 24 hours at the boil was isolated by rotary evaporation to dryness, desalted, then finally rotary evaporated to dryness and dissolved in a solution of permethanoic acid. The oxidised protein was then subjected to gel permeation chromatography on G50, and the elution profile illustrated in fig 8.1 was obtained. The fractions A, B and C were further fractionated on G25; the subsequent profiles are illustrated in figs 8.2 to 8.4. Each fraction from G25 was rotary evaporated and subjected to hydrolysis using 6 M hydrochloric acid at 110°C under reduced pressure. The amino acid analyses obtained, together with a summary of the content of each fraction using the parameters as previously, are given in tables 8.1 to 8.4. All the amino acid composition data is in mole percent.

fig 8.2

G25
278 nm
27 ml.h^{-1}

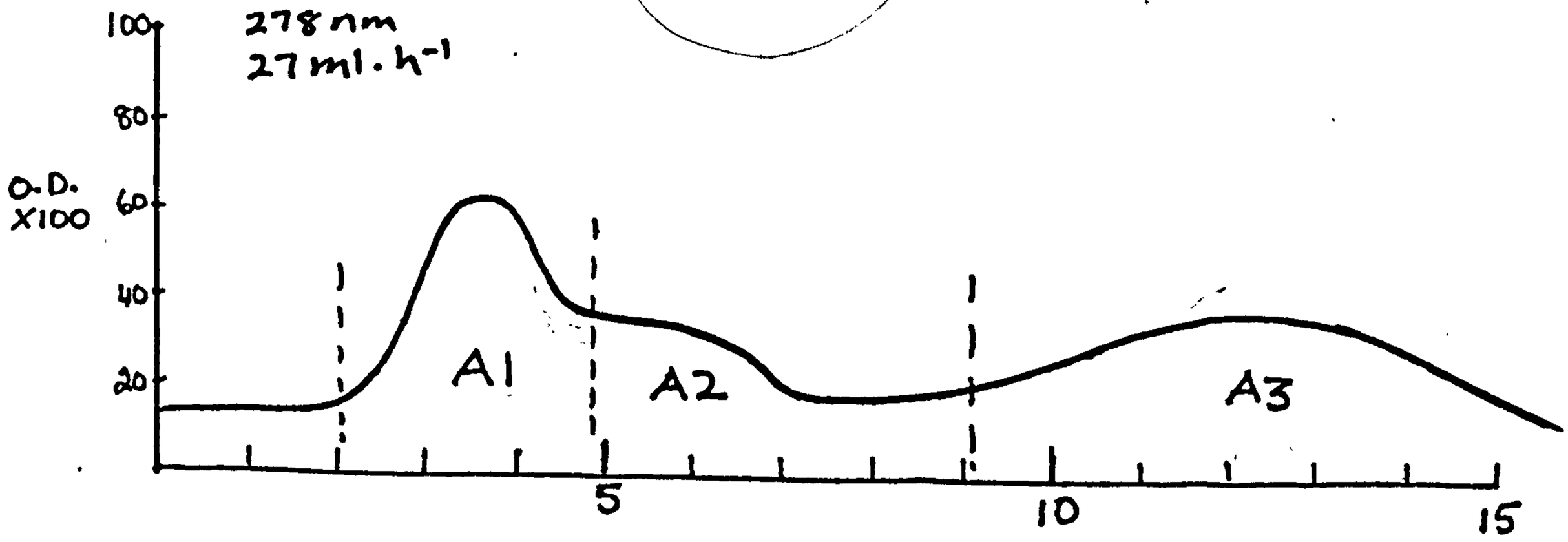
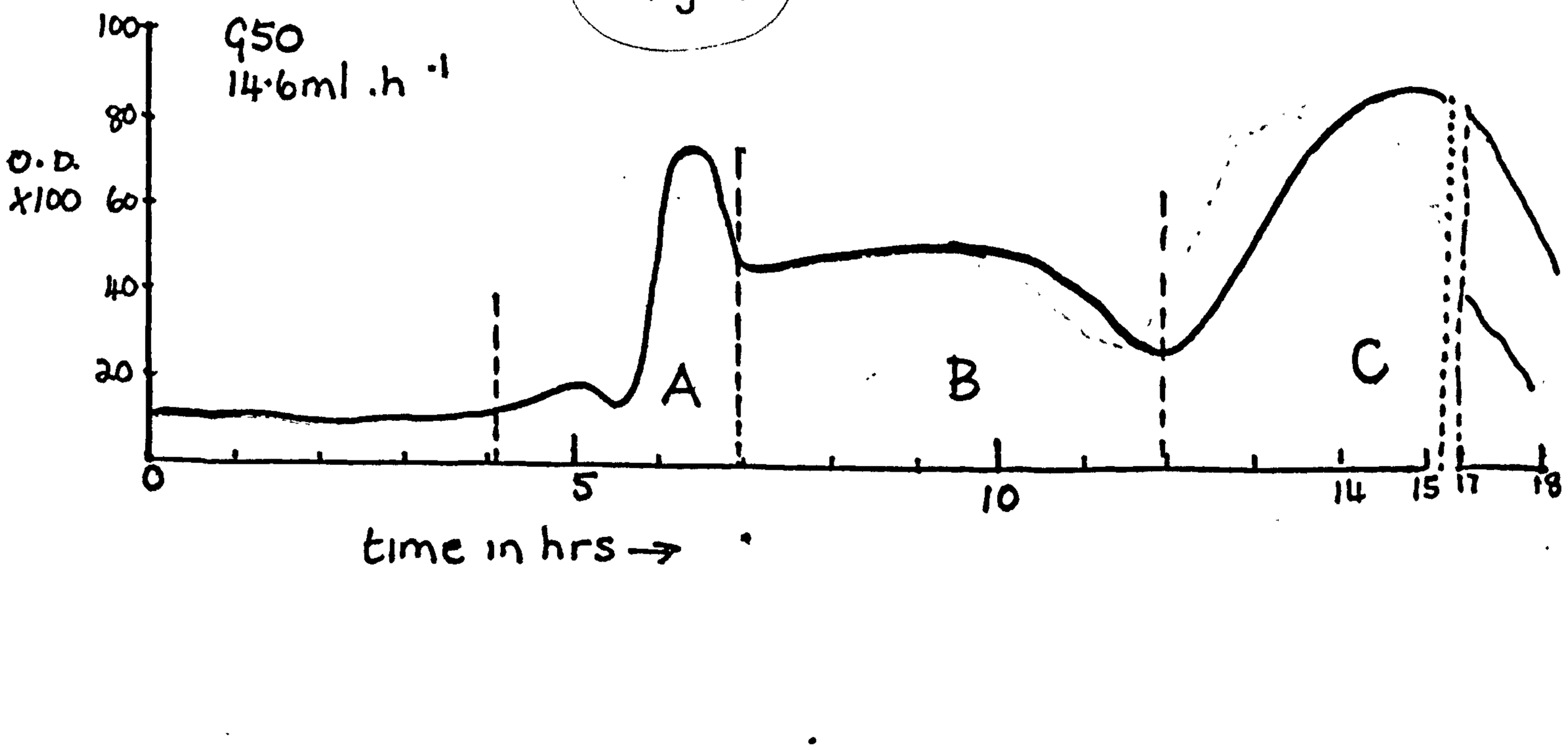


fig 8.1

G50
14.6ml.h^{-1}



Tilbs

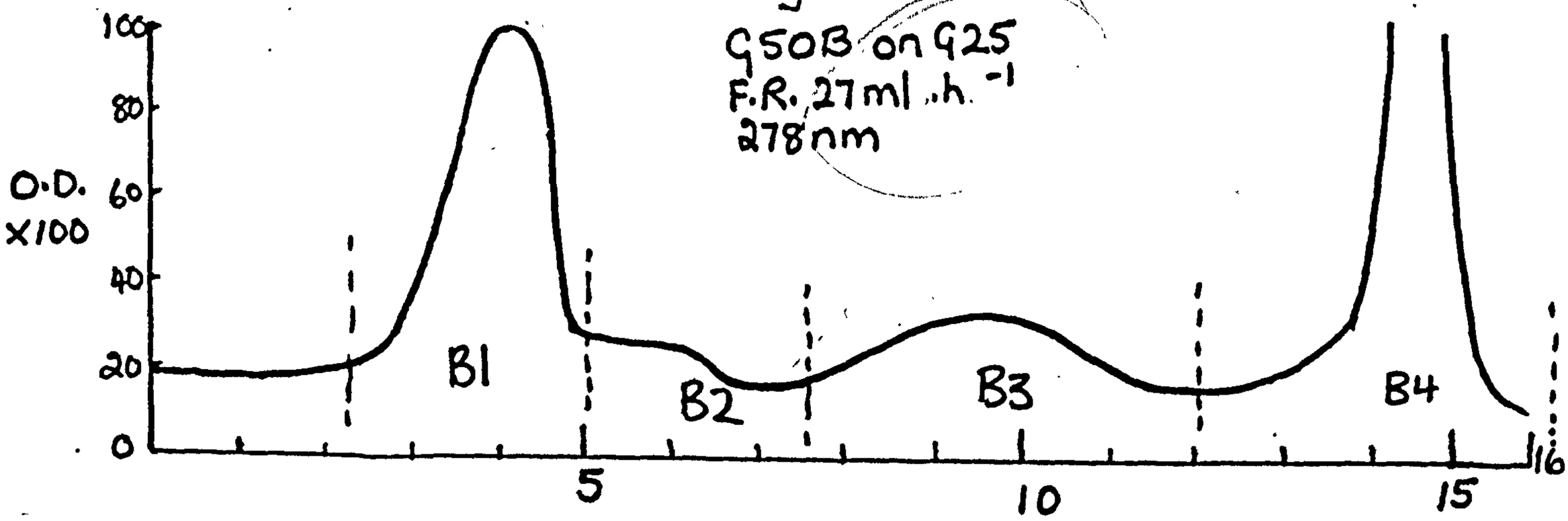
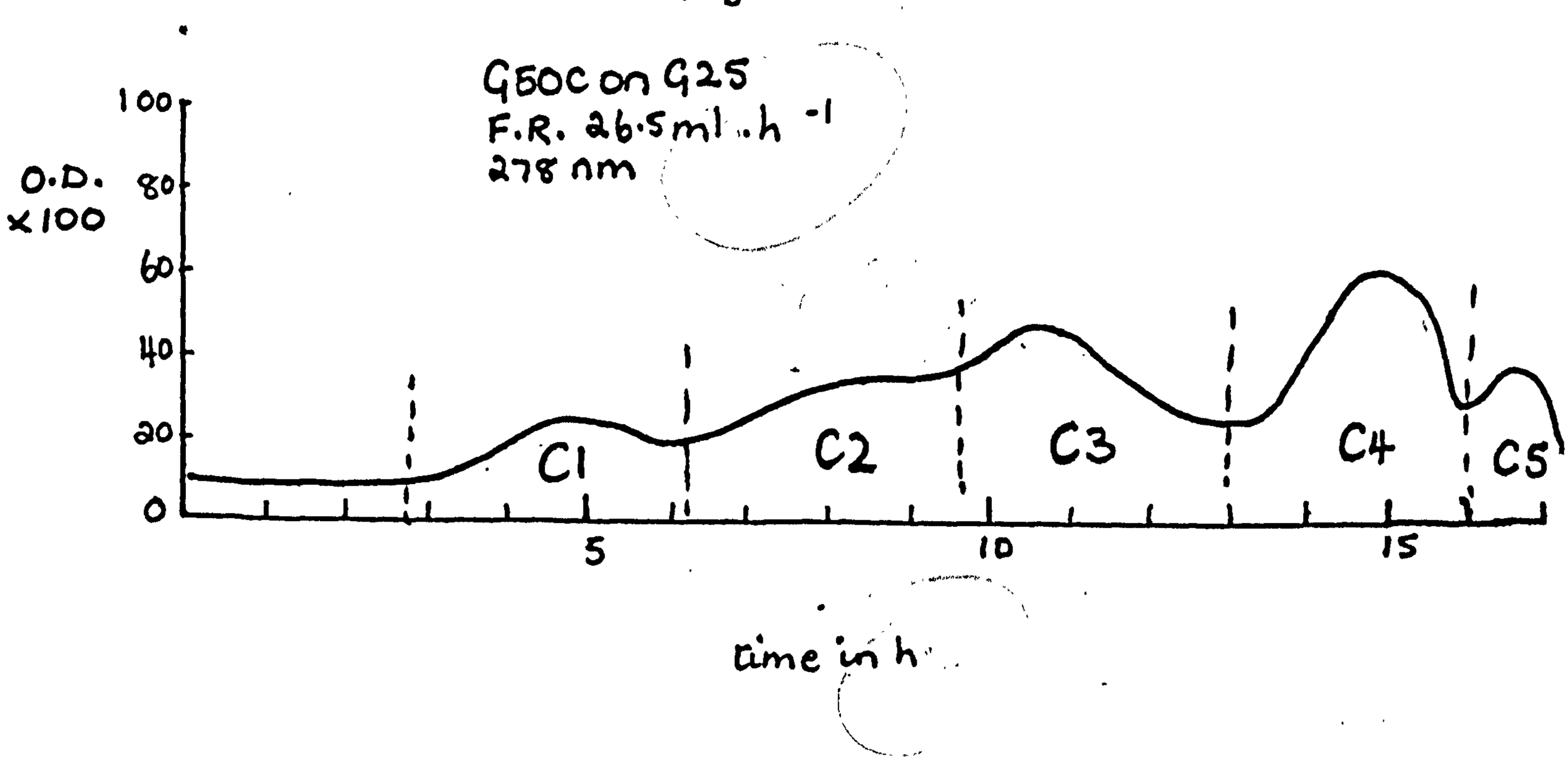


fig 8.3
G50B on G25
F.R. 27 ml .h.$^{-1}$
278 nm

fig 8.4



G50C on G25
F.R. 26.5 ml .h^{-1}
278 nm

time in h

TABLE 8.1

EVANS BLUE

Mole%

	A1	A2	A3
CYA	2.1	1.6	16.2
ASP	12.6	7.2	3.2
THR	8.7	5.1	6.2
SER	8.6	7.8	8.1
GLU	5.2	4.5	2.6
PRO	4.6	5.8	6.2
GLY	8.1	16.9	17.4
ALA	4.2	8.3	7.3
VAL	3.1	6.1	4.2
MET	0	0	0
ILE	3.1	2.8	0
LEU	3.0	4.6	4.2
TYR	8.3	3.8	5.6
PHE	8.2	1.3	4.2
HIS	4.6	4.1	5.3
LYS	5.1	3.6	1.6
ARG	8.7	15.4	7.8
CYS	0	0	0
U	0	5.2	0

TABLE 8.2

EVANS BLUE

Mole%

	B1	B2	B3	B4
CYA	5.3	5.1	11.8	3.8
ASP	2.9	4.7	3.8	15.2
THR	12.6	6.2	3.8	4.1
SER	15.8	15.2	4.2	4.9
GLU	1.3	3.2	5.0	7.1
PRO	6.1	12.3	9.3	6.2
GLY	22.0	26.1	18.6	21.6
ALA	5.3	4.1	7.1	4.7
VAL	4.7	2.3	5.6	5.2
MET	0	0	0	0
ILE	3.2	2.8	1.8	3.1
LEU	4.6	1.7	4.9	2.8
TYR	1.8	4.7	3.4	3.7
PHE	0	0	2.1	2.2
HIS	0	2.1	8.0	5.6
LYS	6.1	3.6	4.4	2,8
ARG	8.3	6.1	2.5	7.2
CYS	0	0	0	0
U	0	0	3.6	0

TABLE 8.3

EVANS BLUE

Mole%

	C1	C2	C3	C4	C5
CYA	3.5	3.7	3.7	4.7	3.6
ASP	9.6	11.4	6.9	8.9	10.2
THR	4.7	5.3	7.6	4.6	5.6
SER	11.8	12.2	8.8	12.2	11.4
GLU	4.7	4.2	4.9	5.8	6.1
PRO	5.2	3.7	3.8	6.1	5.7
GLY	32.1	21.3	19.2	25.2	28.6
ALA	4.7	4.4	4.2	5.8	6.2
VAL	5.3	5.8	6.3	5.2	4.3
MET	0	0	0	0	0
ILE	2.1	3.2	3.7	3.1	4.7
LEU	3.9	3.8	3.2	4.7	3.8
TYR	3.6	5.6	5.6	3.2	5.2
PHE	0	2.1	3.8	1.1	1.9
HIS	0.8	2.7	3.8	1.8	0.3
LYS	2.4	4.7	4.9	1.7	0.5
ARG	5.7	6.2	9.7	6.1	2.2
CYS	0	0	0		

TABLE 8.4

EVANS BLUE

Mdo%

	A1	A2	A3
ACIDICS	19.9	13.3	22
BASICS	18.4	23.1	14.7
NEUTRALS	61.7	63.6	63.3
CYA/B	0.1	0.07	1.1
(THR + SER)/ARG	2.0	0.8	1.8
A/B	1.1	0.6	1.5

	B1	B2	B3	B4
ACIDICS	9.5	13	20.6	26.1
BASICS	14.4	11.8	14.9	15.6
NEUTRALS	76.1	75.2	64.5	58.3
CYA/B	0.4	0.4	0.8	0.2
(THR + SER)/ARG	3.4	3.5	3.2	1.25
A/B	0.7	1.1	1.4	1.7

	C1	C2	C3	C4	C5
ACIDICS	17.8	19.3	15.4	19.4	19.9
BASICS	8.9	13.6	18.4	9.6	3.0
NEUTRALS	73.3	67.1	66.2	71	77.1
CYA/B	0.4	0.3	0.2	0.5	1.2
(THR + SER)/ARG	2.9	2.8	1.7	2.8	7.7
A/B	2.2	1.4	0.8	2.0	6.6

Of the fractions obtained 9 had an Acidics/Basics ratio greater than unity. B4 (1.7), C1 (2.2), C4 (2.0) and C5 (6.6) had by far the highest values for this ratio. The value for C resulted from a very low content of basic amino acids rather than a high acidic content accounting for the high ratio in this case, and also for the high value for another parameter, the (Thr + Ser)/Arg) ratio. A3 and B3 had very high cysteic acid contents of 16.2 and 11.8 respectively. Several of the fractions were rich in aspartic acid particularly, A1, B4, C1, C2 and C5. A1 was also rich in threonine and serine as were A3, B1 and B2. High serine contents were also found in C1, C2, C4 and C5. B4 was rich in glutamic acid, and B2 and B3 were rich in proline. A majority of the proteins were very rich in glycine, some of them very much so, notably B2, C1, C4 and C5, all with values above 25 mole per cent. The amount of alanine in the different samples varied between 4.1 and 7.3 mole per cent and the concentration of valine between 2.3 and 6.3 mole per cent. Tyrosine content varied markedly, between 1.8 in B1 and 8.3 in A1 which also had a low percentage of glycine compared to some of the high glycine fractions. A number of fractions had both high glycine and tyrosine as can be seen in table 8.5. Phenylalanine varied markedly and also apparently quite randomly between 0% and 8.3%. The basic amino acids varied throughout the range of fractions, the major influence on this value being the concentration of arginine present which varied between 2.2 and 15.4 mole per cent.

Two unknowns were found, one in each of A2 and B3; in each case they occurred between cysteic acid and aspartic acid and were therefore acidic in character. They each gave a high reading at both 570 and 440 nm, as though absorbing across a range of wavelengths rather than the usual situation where the product produced from the ninhydrin reaction absorbs specifically at 570 or 440 nm.

TABLE 8.5

	A1	A2	A3
GLYCINE	8.1	16.9	17.4
TYROSINE	8.3	3.8	5.6
TOTAL	16.4	20.7	23.0

	B1	B2	B3	B4
GLYCINE	22	26.1	18.6	21.6
TYROSINE	1.8	4.7	3.4	3.7
TOTAL	23.8	30.7	22.0	25.3

	C1	C2	C3	C4	C5
GLYCINE	32.1	21.3	19.2	25.2	28.6
TYROSINE	3.6	5.6	5.6	3.2	5.2
TOTAL	35.7	26:9	24.8	28.4	33.8

The total gelatin extracted was also examined to determine its amino acid composition. Other treatments were carried out by having variations in dye content and time; the results appear in tables 8.6 - 8.8. The treatments with Evans Blue were in two groups; one group looked at the effect of concentration on the degradation products and the other group looked at the effect of time, which varied from 30' to 24 hours (1440').

The effect of dye concentration did not appear to be marked with regard to the total gelatin obtained. There was an overall reduction in the % aspartic acid and this decreased from the 1% treatment to the 10% treatment. Threonine increased as did serine though the latter showed a 'low' at 5% owf Evans Blue. Glutamic acid decreased from 14.1 to 12.7. Proline, glycine, alanine, and valine all showed an increase from the 1% to the 10% treatment. Isoleucine remained constant at ± 0.2%. Leucine decreased from 9.5 to 8.9% with a 'low' of 8.7% at 5% owf. Tyrosine showed an increase from 3.8 at 1% to 4.2 at 5% owf and 4.1% at 10% owf. Phenylalanine showed a decrease from 4.1 to 3.7%. Histidine and lysine both showed an overall decrease as did cystine. Arginine showed an increase. The effect of time produced noticeably different results that could be split between the short term treatments from 30' - 240' and the long term, 1440', treatment. The author would suggest that different parallel extraction processes were occurring at different rates, and that as extraction proceeded the slower initial processes continued and in the end prevailed as the major process, whilst the more rapid of the initial processes subsided with time. This would suggest different susceptibility to extraction of different proteins or morphological components of the fibre. This is to be expected.

The results of the residual wool (see table 8.8) showed no marked differences with variation in concentration of dye, however, some trends were discernable. This again might be expected, as the results being examined are due to dye concentration,

TABLE 8.6

GELATIN FROM TREATMENT OF WOOL WITH EVANS BLUE 1% owf

mole%	30'	60'	120'	240'	1440'
all treatments in an acid dyebath 10% owf Na_2SO_4 + 10% owf H_2SO_4					
CYA	0	0	0	0.3	0.6
ASP	6.3	7.3	6.7	8.6	9.9
THR	7.6	7.5	6.3	6.7	4.2
SER	6.3	7.2	6.8	6.7	7.1
GLU	11.7	10.7	12.6	12.6	14.1
PRO	4.7	5.2	4.9	4.9	4.1
GLY	12.2	12.9	11.2	10.7	5.1
ALA	6.9	5.3	5.2	4.3	4.2
VAL	5.2	5.1	4.3	4.9	5.1
MET	0	0	0	0	0
ILE	4.3	4.7	5.2	5.1	3.7
LEU	7.3	5.3	5.3	7.6	9.5
TYR	6.7	5.2	5.6	6.2	3.8
PHE	4.3	3.3	4.6	4.7	4.1
HIS	1.9	2.1	2.2	2.1	1.4
LYS	2.5	4.3	4.8	2.1	4.6
ARG	6.1	7.3	7.2	6.8	9.9
CYS	6.2	6.7	7.3	6.2	8.7
ACIDICS	17.9	18.0	13.4	21.0	24.6
BASICS	10.5	13.7	11.5	11.0	15.9
NEUTRALS	71.6	68.3	75	68.0	59.5
THR + SER/ARG	2.3	2.0	2.8	2.0	1.1
CYA/BASICS	0	0	0	0.02	0.04
ACIDICS/BASICS	1.7	1.3	1.2	1.9	1.5

TABLE 8.7

EVANS BLUE

TOTAL GELATIN 24 HOURS ACID DYEBATH

Mole%	1%*	5% *	10% *
CYA	0.6	0.5	0.5
ASP	9.9	9.8	8.7
THR	4.2	4.8	4.6
SER	7.1	6.7	8.1
GLU	14.1	14.3	12.7
PRO	4.1	4.6	4.7
GLY	5.1	6.7	6.2
ALA	4.2	4.7	5.2
VAL	5.1	5.8	5.5
MET	0	0	0
ILE	3.7	3.9	3.8
LEU	9.5	8.7	8.9
TYR	3.8	4.2	4.1
PHE	4.1	3.9	3.7
HIS	1.4	1.3	1.3
LYS	4.6	3.6	3.7
ARG	9.9	9.2	10.3
CYS	8.7	7.6	7.8
ACIDICS	24.6	24.6	21.9
BASICS	15.9	14.1	15.3
NEUTRALS	59.5	61.3	62.8
THR + SER/ARG	1.1	1.25	1.23
CYA/BASICS	0.04	0.03	0.03
ACIDICS/BASICS	1.5	1.7	1.4

* % owf.

TABLE 8.8

RESIDUAL WOOL 24 HOURS ACID DYEBATH

VARYING % (owf)

EVANS BLUE

Mole %

	0.1 *	0.2 *	0.5 *	1 *	2 *	5 *
CYA	Results lost due to	0.1	0.2	0.1	0.0	
ASP	instrument failure	4.4	4.8	4.8	5.2	
THR		4.1	4.3	4.3	4.6	
SER		7.6	8.2	7.8	8.1	
GLU		8.0	7.6	7.5	7.2	
PRO		9.2	9.4	9.7	9.5	
GLY		6.2	6.0	5.7	5.8	
ALA		4.7	4.6	4.3	4.1	
VAL		5.6	5.5	5.2	5.2	
MET		0	0	0	0	
ILE		3.0	2.8	2.7	2.3	
LEU		8.6	8.5	8.8	8.7	
TYR		4.1	4.0	3.7	3.5	
PHE		4.2	4.0	4.1	3.7	
HIS		1.5	1.2	1.5	1.7	
LYS		6.4	6.1	6.3	6.6	
ARG		10.7	11.6	12.3	11.8	
CYS		11.8	11.2	10.8	11.7	

* o.w.f.

which is probably a modifying influence superimposed on the major driving force of the extraction with sulphuric acid and sodium sulphate. Aspartic acid and threonine showed an overall increase as did proline. Most other amino acids showed a decrease on increasing the concentration of Evans Blue.

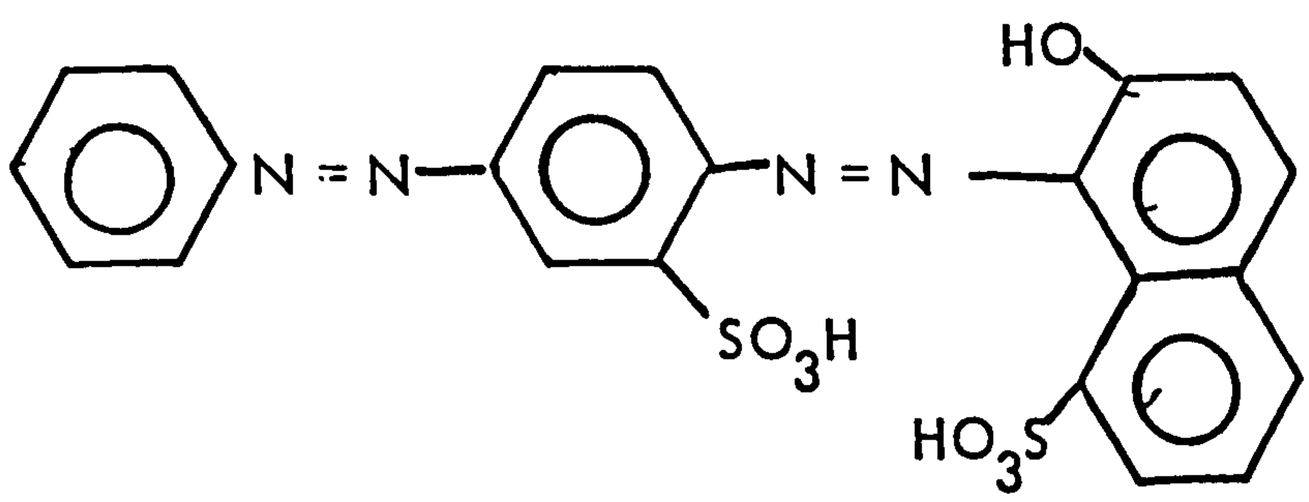
CHAPTER 9

CROCEIN SCARLET IN ACID DYEBATH
EXAMINATION OF RESULTS OBTAINED ON GEL PERMEATION CHROMATOGRAPHY AND AMINO ACID ANALYSIS

A 10 g wool sample was treated for 24 hours at the boil in an aqueous solution containing in 1 litre, the following:

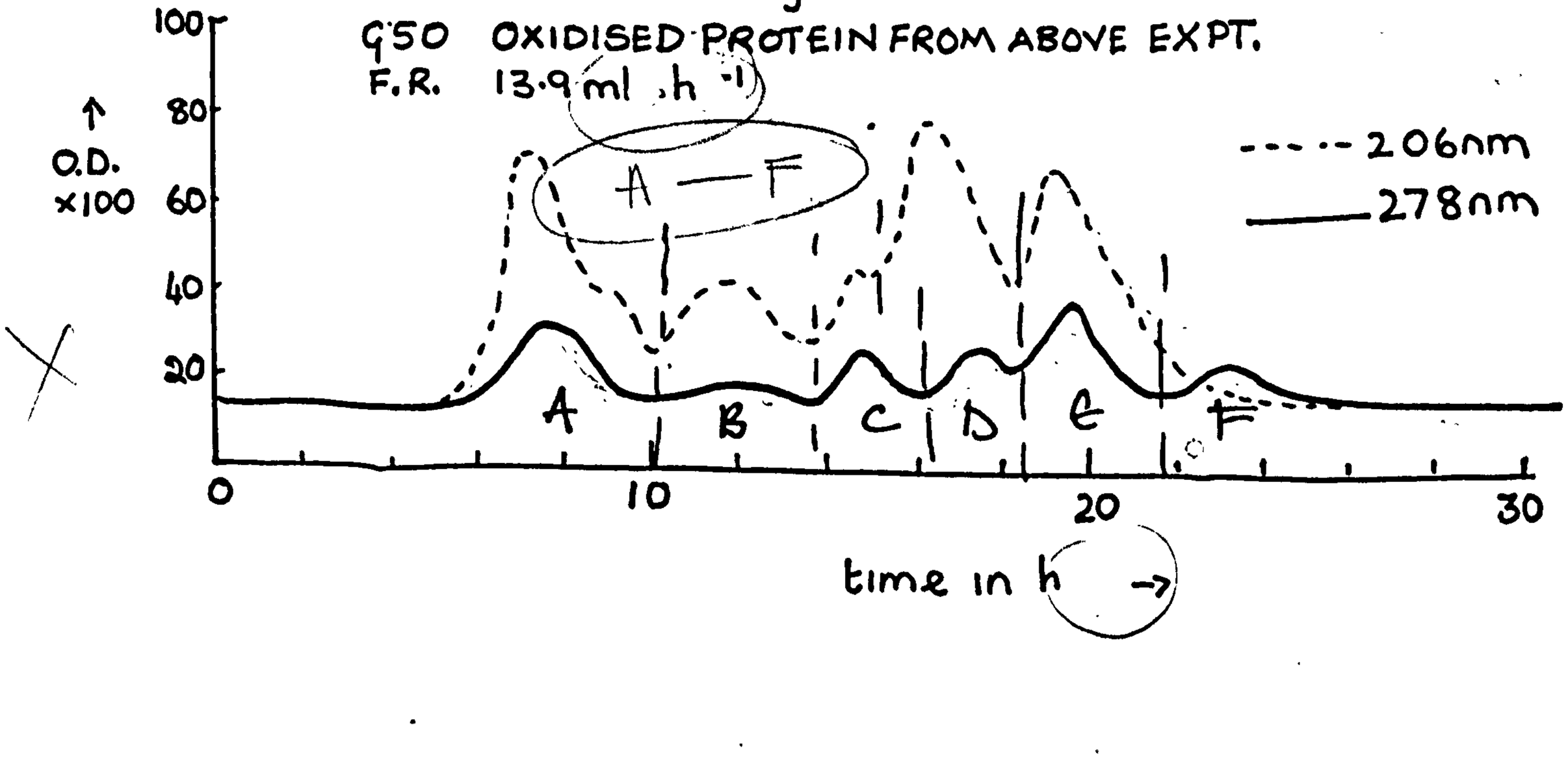
10% owf (1.02×10^{-2} M)	Sulphuric VI acid
10% owf (7.04×10^{-3} M)	Sodium sulphate VI
1% owf (2×10^{-2} M)	Crocein Scarlet

Crocein Scarlet MOO C.I. acid red 73



The elution profiles obtained on gel permeation chromatography on G50 of the proteinaceous material in the dyebath after desalting and subsequent oxidation with permethanoic acid are given in figure 9.1.

fig 9.1



Each of the fractions A – F was rotary evaporated to dryness, dissolved in distilled water and then subjected to further GPC on G25. The profiles of these further fractionations are given in figures 9.2 to 9.6. Fraction G50F gave no results on G25.

Fig 9.2

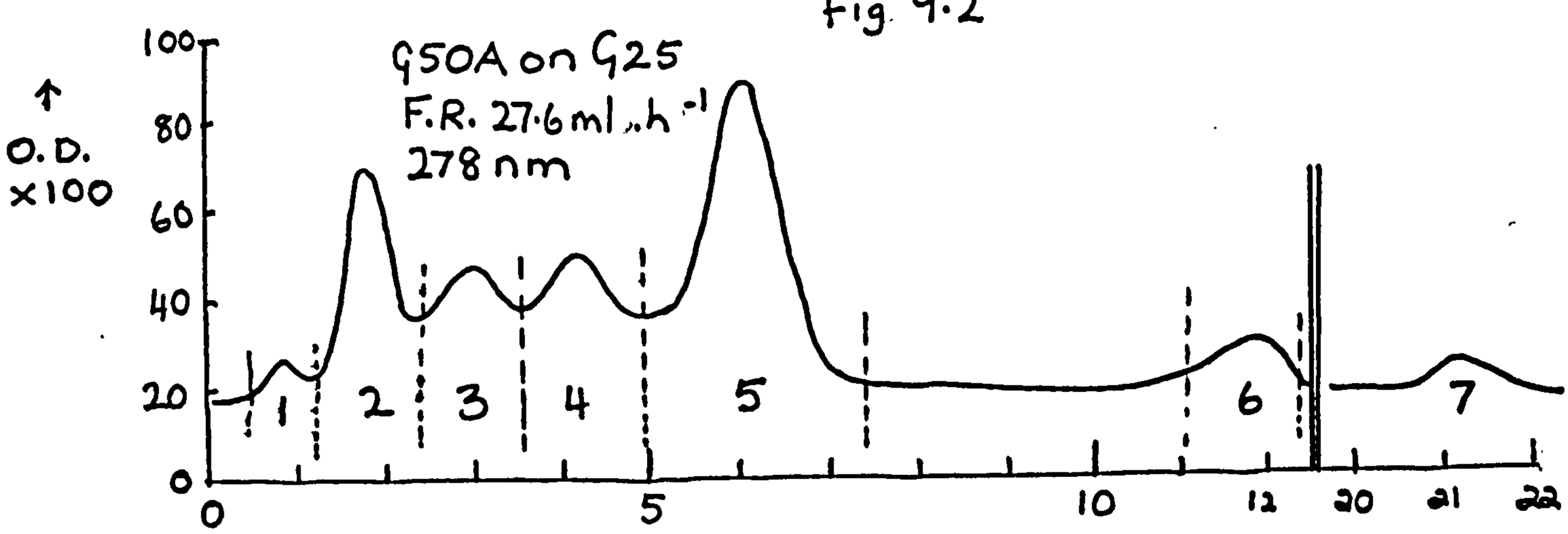


fig 9.3

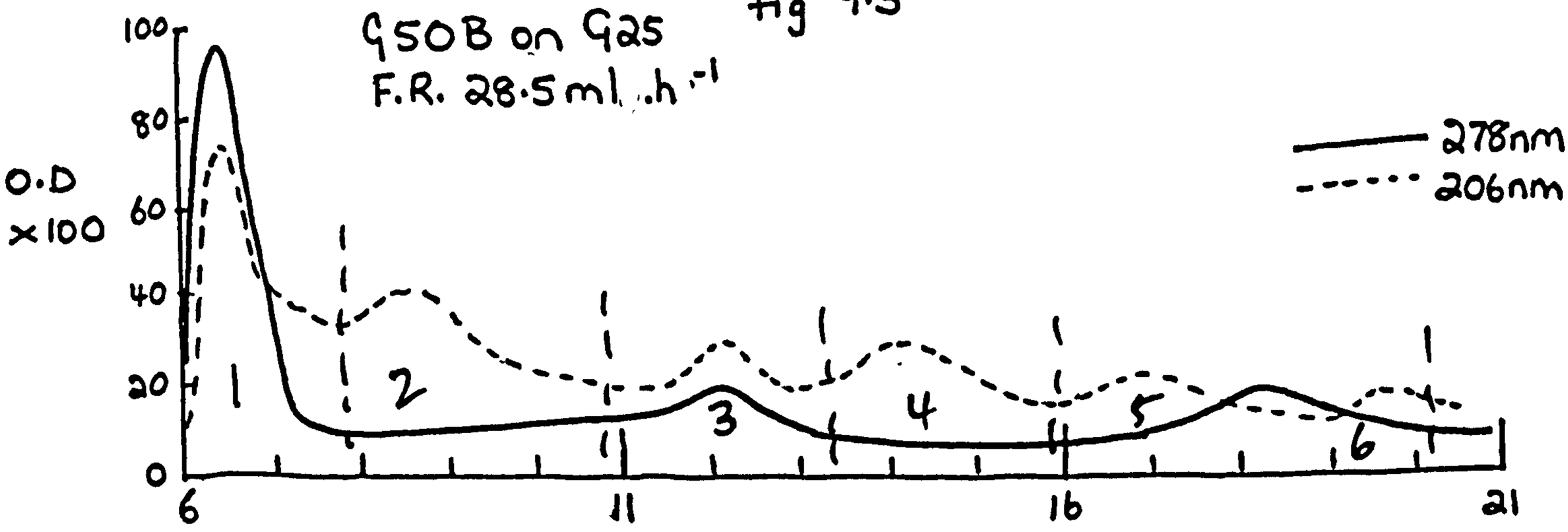


Fig 9.4

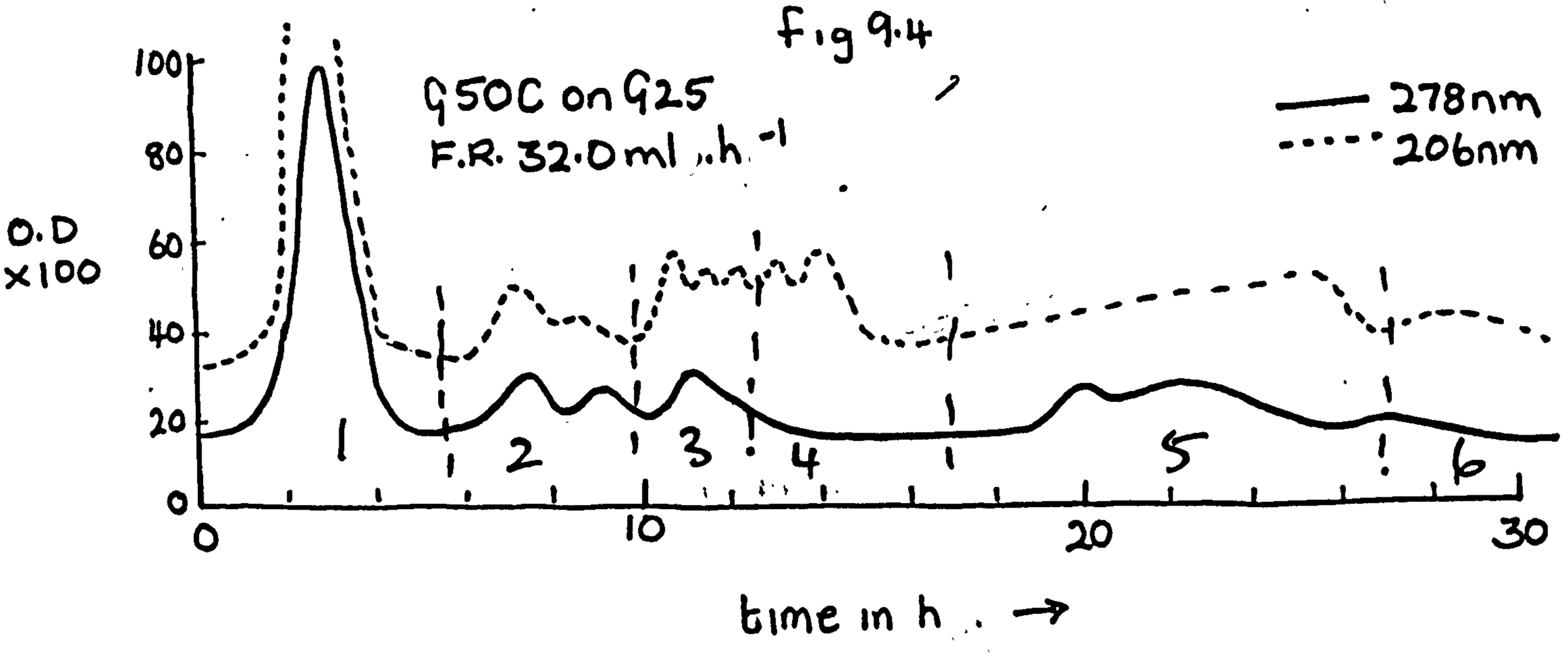


fig 9.5

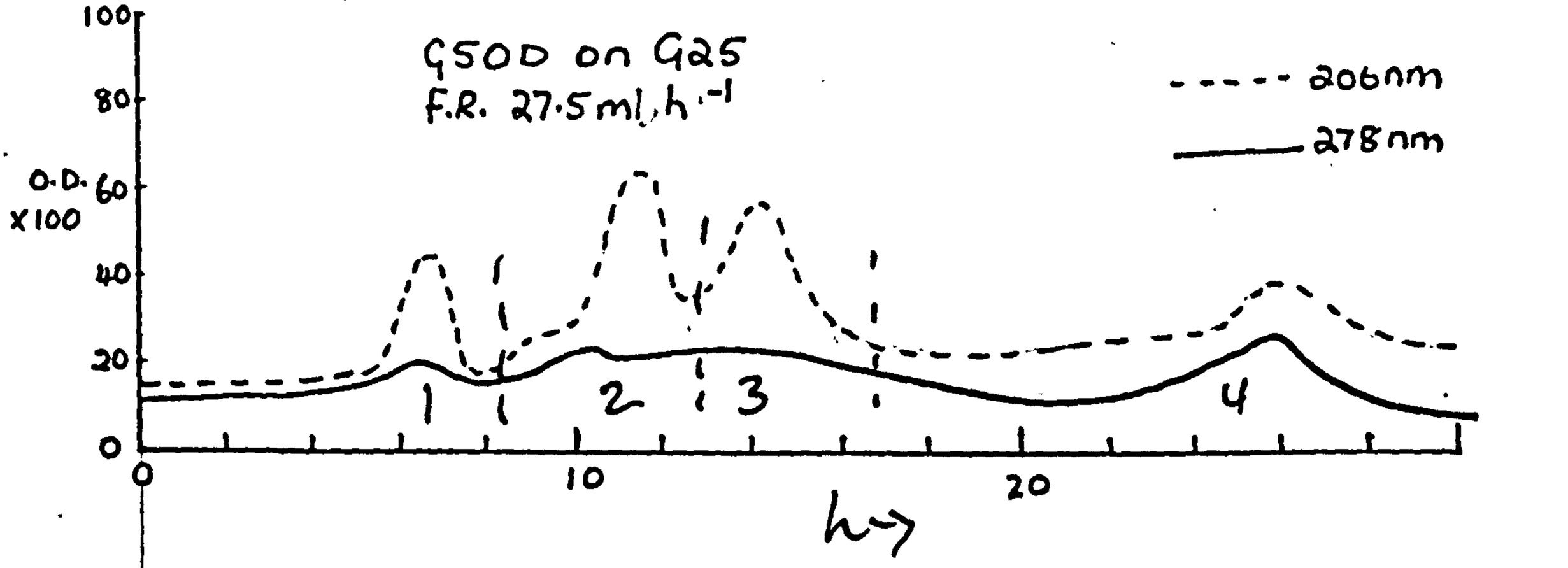
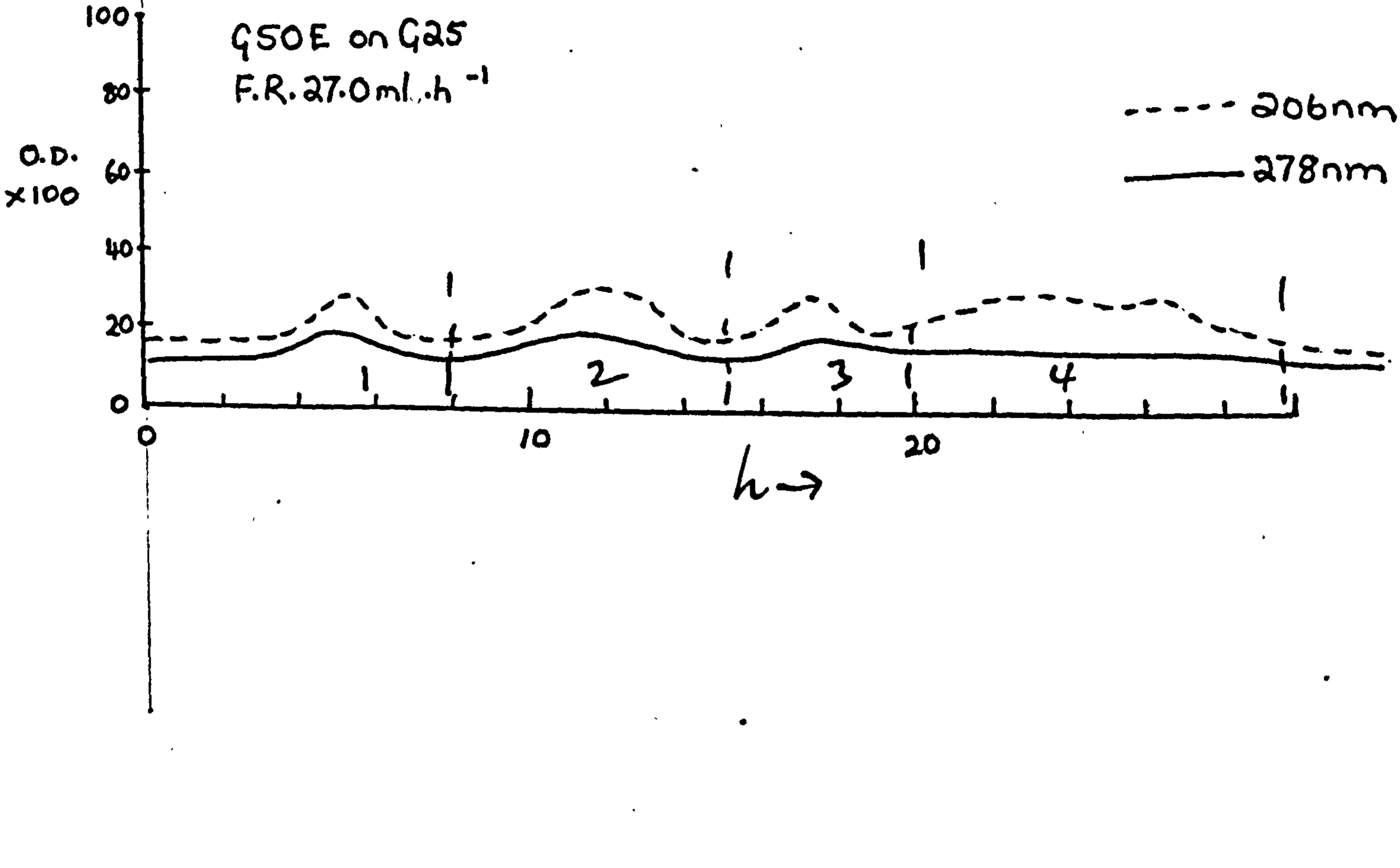

G50D on G25
F.R. 27.5ml.h⁻¹
----- 206nm
——— 278nm
O.D. X100
100
80
60
40
20
0
1
2
3
4
0
10
20
h→

fig 9.6


G50E on G25
F.R. 27.0ml.h⁻¹
----- 206nm
——— 278nm
O.D. X100
100
80
60
40
20
0
1
2
3
4
0
10
20
h→

The fractions obtained from GPC on G25 were rotary evaporated to dryness and were then hydrolysed in 6 M hydrochloric acid under reduced pressure at 110°C. The hydrolysate was in each case freeze dried overnight and subjected to amino acid analysis, the results of which appear in the tables 9.1 to 9.6 expressed in mole percent.

On examination (see table 9.5) the fractions can be seen to be of varying composition. Most of the fractions were acidic in character and had Acidics/Basics ratios varying from 1.03 to 61.3 (this latter has very little meaning because of the very low content of basic amino acids); other high values were 4.9 and 5.2. Three of the fractions, namely A2, C1 and E4 were slightly basic in character. Alanine varied from fraction to fraction with the highest percentage in B4 (15.2%) and the lowest in B1 (0.9%). Valine content varied less markedly, being between 1.5% and 7.0%. Isoleucine varied between 0.4 and 5.9% with the majority of the fractions containing less than 3.0%. Leucine content varied between 0 and 4.6%. Phenylalanine varied between 0.2 and 3.9%. Histidine varied markedly between 0% and 12.6%, and lysine between 0 and 14.9%. Arginine varied between 0 and 12.8%. Overall the content of basic amino acids (see table 9.5) varied very greatly between 0% and 28.1% of the total hydrolysate. The mole % present of all three basic amino acids varied a great deal from fraction to fraction and this variation in all of the basic amino acids, rather than any one anomolous result, caused the total fluctuation. Since the proteins were oxidised with permethanoic acid there was no cystine present in any of the above fractions.

Investigations were carried out on the total gelatin from the dyebath after treatment with Crocein Scarlet under the conditions described at the beginning of this chapter, together with the residual wool from these treatments as given in table 9.7 and 9.8.

TABLE 9.1

CROCEIN SCARLET

Mole %

	A1	A2	A3	A4	A5	A6	A7
CYA		2.1	6.4	4.6	6.2		
ASP		10.8	13.6	12.2	9.7		
THR		12.6	12.7	9.7	4.8		
SER		8.2	14.6	12.6	16.6		
GLU		2.6	3.5	4.3	4.7		
PRO		5.1	4.7	5.2	8.6		
GLY		9.3	12.6	12.7	16.8		
ALA		4.7	3.6	4.1	5.2		
VAL		3.2	4.3	3.6	4.1		
MET	NIL	0	0	0	0	NIL	NIL
ILE		1.7	1.2	3.3	5.7		
LEU		4.6	3.2	3.7	2.6		
TYR		4.1	2.1	4.6	2.8		
PHE		3.2	1.6	2.7	0.2		
HIS		8.6	3.2	2.1	0.3		
LYS		7.3	2.8	3.6	2.2		
ARG		12.2	9.8	11.2	9.6		
CYS		0	0	0	0		

A1, A6, A7 did not give any trace after hydrolysis.

TABLE 9.2

CROCEIN SCARLET

Mole %

	B1	B2	B3	B4	B5
CYA	18.1	8.0	20.2	4.0	6.0
ASP	0.1	7.8	2.7	5.0	5.3
THR	13.7	6.9	5.1	6.1	6.8
SER	26.9	17.2	2.3	14.9	14.6
GLU	0.2	5.7	1.5	3.9	3.6
PRO	3.9	3.8	12.8	17.5	8.0
GLY	26.2	14.7	15.2	17.1	19.8
ALA	0.9	9.3	7.2	15.2	6.7
VAL	3.4	6.2	6.0	4.3	7.0
MET	0	0	0	0	0
ILE	4.2	1.6	0	0	5.9
LEU	0	3.2	0	0	1.5
TYR	1.1	1.2	2.9	1.4	1.3
PHE	1.04	1.6	2.21	3.9	3.0
HIS	0	0	12.6	0	2.2
LYS	0.3	0	5.5	0	2.9
ARG	0	12.8	6.5	6.5	5.3
CYS	0	0	0	0	0
U1	0	0	0.50	0	0
U2	0	0	0.80	0	0
U3	0	0	0.2	0	0
U4	0	0	1.5	0	0

U ≡ unknown

TABLE 9.3

CROCEIN SCARLET

Mole %

	C1	C2	C3	C4	C5
CYA	7.7	2.5	2.3	0.6	5.6
ASP	1.1	14.6	55.0	18.7	11.3
THR	11.5	9.5	2.7	5.3	9.1
SER	22.8	22.8	6.5	10.1	14.1
GLU	0.9	1.7	1.6	2.5	0.7
PRO	5.6	5.6	3.4	8.9	10.0
GLY	20.8	22.8	9.5	13.2	21.8
ALA	4.5	3.9	3.9	10.6	7.9
VAL	4.3	3.8	1.5	3.3	6.0
MET	0	0	0	0	0
ILE	2.2	1.2	0.4	1.0	2.1
LEU	3.6	2.1	1.1	3.0	2.2
TYR	0	2.9	0.3	0.7	1.3
PHE	0	0	0.4	0.6	0.8
HIS	0	1.9	3.8	4.4	2.4
LYS	0	1.1	5.9	14.9	1.1
ARG	15.0	3.4	1.7	1.9	2.93
CYS	0	0	0	0	0

TABLE 9.4

CROCEIN SCARLET

Mole%

	D1	D4	E1	E4
CYA	17.9	0.6	10.4	0
ASP	14.8	34.4	7.6	5.1
THR	14.8	4.8	0.5	4.6
SER	31.2	12.2	1.5	17.2
GLU	0	2.8	3.9	6.0
PRO	0	6.74	12.3	5.4
GLY	15.6	16.2	26.8	6.8
ALA	5.7	4.8	6.0	11.1
VAL	0	0	7.0	1.3
MET	0	1.4	1.3	0
ILE	0	0	1.8	3.8
LEU	0	4.3	2.2	8.3
TYR	0	0.6	0.7	4.7
PHE	0	2.4	1.4	3.8
HIS	0	6.5	0.9	1.9
LYS	0	0.6	2.4	4.1
ARG	0	0.6	12.2	15.8
CYS	0	0	0	0
U	0	0.93	0	0

D2, D3, E2, E3 did not give any trace after hydrolysis.

U ≡ unknown

TABLE 9.5

Mole %

	A2	A3	A4	A5
ACIDICS	15.5	23.5	21.1	20.6
BASICS	28.1	15.8	16.9	12.1
NEUTRALS	56.4	60.7	62.0	67.3
CYA/B	0.07	0.4	0.3	0.5
(THR + SER)/ARG	1.7	2.8	2.0	2.2
A/B	0.55	1.5	1.2	1.7

	B1	B2	B3	B5	B6
ACIDICS	18.4	21.5	24.4	12.9	14.9
BASICS	0.3	12.8	18.6	6.5	10.4
NEUTRALS	81.3	65.7	57.0	80.6	74.7
CYA/B	60.3	0.625	1.1	0.6	0.6
(THR + SER)/ARG		2.1	14.8	42.0	4.0
A/B	61.3	1.7	1.3	2.0	1.4

	C1	C2	C3	C4	C5
ACIDICS	9.7	18.8	58.9	21.8	17.6
BASICS	15.0	6.4	11.4	21.2	7.40
NEUTRALS	75.3	74.8	29.7	57.0	7.5
CYA/B	0.5	0.4	0.2	0.03	0.8
(THR + SER)/ARG	2.3	9.5	5.4	8.1	7.9
A/B	0.6	2.9	5.2	1.03	2.4

	D1	D4	E1	E4
ACIDICS	32.7	37.8	21.9	11.1
BASICS	0	7.7	15.5	21.8
NEUTRALS	67.3	54.5	62.6	67.1
CYA/B	N/A	0.08	0.7	0
(THR + SER)/ARG	N/A	2.2	0.16	1.4
A/B	N/A	4.9	1.4	0.5

TABLE 9.6

GLYCINE/TYROSINE CONTENT OF PROTEIN FRACTION

Mole %.

	A2	A3	A4	A5
GLYCINE	9.3	12.6	12.7	16.8
TYROSINE	4.1	2.1	4.6	2.8
GLY + TYR	13.4	14.7	17.3	19.6

	B1	B2	B3	B4	B5
GLYCINE	0.9	14.7	15.2	17.1	19.8
TYROSINE	1.1	1.2	2.9	1.4	1.3
GLY + TYR	2.0	15.9	18.1	18.5	21.1

	C1	C2	C3	C4	C5
GLYCINE	20.8	22.8	9.5	13.2	21.8
TYROSINE	0	2.9	0.3	0.7	1.3
GLY + TYR	20.8	25.7	9.8	13.9	23.1

	D1	D4	E1	E4
GLYCINE	15.6	16.2	26.8	6.8
TYROSINE	0	4.3	2.2	4.7
GLY + TYR	15.6	20.5	30.0	11.5

Most of the fractions were rich in glycine, with some containing very high contents of this amino acid, e.g. E1 with 26.8%

As in other chapters the effect of dye concentration was not marked. There were differences, but again these were not significant. The effect of the dye would appear to be to modify slightly the overall effect of the extracting media, rather than to be a major force itself. This is indicated by the similar materials which were extracted by the different dyebaths. They would appear to act, possibly, as catalysts for the reactions of extraction, such as hydrolysis. Such a conclusion is not hard to prove when one considers some of the information in chapter 11 which examines, using GPC, the material obtained on extraction using various compounds.

High values for cysteic acid, which would indicate a high sulphur content were given by several fractions, notably, B1 (18.1%), B3 (20.2%) and D1 (17.9%). Several of the fractions showed a high aspartic acid content. These include all of the G50A and G50C subfractions, notably, C2 (14.6%), C3 (55%) and C4 (18.7%) together with D1 (14.8%) and D4 (34.4%). Six of the fractions were rich in threonine, and fourteen were rich in serine. Glutamic acid varied randomly between 0.0% and 6%. Three of the fractions had a very high proline content of greater than 12%; fourteen of the fractions had high glycine contents. Some of the fractions were also high in tyrosine (A2, A4, and E4).

TABLE 9.7

AMINO ACID COMPOSITION DATA FOR THE TOTAL GELATIN FROM THE TREATMENT OF WOOL WITH CROCEIN SCARLET

	mole % amino acid
CYA	0.3
ASP	10.1
THR	4.6
SER	7.3
GLU	13.8
PRO	4.7
GLY	5.7
ALA	4.4
VAL	5.0
MET	0
ILE	3.2
LEU	9.1
TYR	3.3
PHE	4.9
HIS	1.2
LYS	4.2
ARG	10.1
CYS	8.1

TABLE 9.8

AMINO ACID COMPOSITION DATA FOR THE RESIDUAL WOOL FROM THE TREATMENT OF WOOL WITH CROCEIN SCARLET

	mole % amino acid
CYA	0
ASP	4.9
THR	4.3
SER	7.5
GLU	8.2
PRO	9.6
GLY	5.9
ALA	5.1
VAL	5.2
MET	0
ILE	3.3
LEU	7.5
TYR	4.1
PHE	4.2
HIS	1.4
LYS	6.5
ARG	10.9
CYS	11.3

CHAPTER 10

AN EXAMINATION OF THE DATA OBTAINED DURING PHYSICAL TESTING OF WOOL TREATED WITH A VARIETY OF AGENTS

2 g samples of wool yarn were treated for various times in the reagents named and the yarn from these treatments was then tested using the Zellweger Uster A.D. yarn tester. Prior to such testing the wool was rinsed thoroughly in cold deionised water and was then allowed to dry under reduced pressure, over fused calcium chloride in a dessicator. The wool was then allowed to equilibrate under standard conditions (65% RH $\pm$ 2% and 18°C $\pm$ 1°C) for 48 hours; the testing was then carried out. Due to differences in the length of yarn which constituted a 2g hank, the number of tests carried out varied slightly in each case. The results obtained are recorded below together with the results from the testing of untreated wool yarn.

TABLE 10.1

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
10.1a			
Naphthalene 1,5 Disulphonic acid % owf			
1%	7.4	274	25
5%	5.8	294	25
10%	5.4	310	25
10.1b			
Naphthalene 1 Sulphonic Acid % owf			
1%	6.2	330	25
5%	7.8	330	25
10%	5.8	306	25

Treatment	% elongation at the break	Breaking Strain (g)	Number of Tests
10.1c			
Biebrich Scarlet % owf *			
1%	7.7	398.5	20
5%	7.0	326	25
10%	8.48	415.2	25
10.1d			
Naphthol Green B % owf *			
1%	9.2	394	20
5%	7.9	360.5	26
10%	7.5	349	26
10.1e			
Naphthalene Red J* % owf			
1%	7.4	374.6	25
5%	7.8	394	25
10%	8.2	370.	25
10.1f			
Benzyl Red MG* % owf			
1%	Lost	Lost	0
5%	7.12	375.8	26
10%	9.4	438	25

* Dyebath 100 : 1 Liquor ratio 10% owf Sulphuric Acid + 10% owf Sodium sulphate

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
	10.1g		
Carbolan Crimson B * % owf			
0.1%	11.8	438	25
0.2%	11.4	430	25
0.5%	12.6	454	25
1.0%	12.2	446	25
2.0%	12.2	426	25
5.0%	11.4	442	25
	10.1h		
1,8 amino naphthol 2 phenylazo- 3,6 disulphonic acid * % owf			
0.1%	12.6	450	25
0.2%	12.6	458	25
0.5%	13.0	442	25
1 %	12.6	438	25
2 %	13.8	458	25
5 %	15.8	470	25

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
	10.1 i		
1,8 Amino Naphthol 3,6 Disulphonic Acid * % owf			
0.1%	20.4	478	25
0.2%	21.6	466	25
0.5%	22.4	454	25
1 %	22.0	446	25
2 %	21.6	430	25
5 %	19.6	422	25
	10.1 j		
Evans Blue * % owf			
0.1%	10.6	422	25
0.2%	13.0	438	25
0.5%	12.2	406	23
1 %	13.8	458	25
2 %	11.4	434	28
5 %	12.6	458	25
	10.1 k		
Carbolan Yellow G * % owf			
0.1%	18.8	418	25
0.2%	20.4	454	25
0.5%	20.8	458	25
1 %	21.2	458	25
2 %	22.4	450	25

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
	10.1 l		
Carbolan Blue B * % owf			
0.1%	14.2	434	25
0.2%	13.8	442	25
0.5%	13.4	442	25
1%	13.8	470	25
2%	13.0	462	25
5%	13.4	458	25
	10.1m		
Orange II * % owf			
0.1%	12.6	470	25
0.2%	14.6	474	25
0.5%	13.0	474	25
1%	13.8	474	25
2%	-	-	25
5%	13.0	446	25
	10.1n		
Crocein Scarlet * % owf			
0.1%	21.2	494	25
0.2%	21.2	446	25
0.5%	22.0	466	25
1%	20.0	430	25
2%	21.2	466	25
5%	19.6	438	25

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
	10.1o		
1,8 amino naphthol 2 phenylazo * 5,7 disulphonic acid % owf			
0.1%	13.0	450	25
0.2%	12.6	462	25
0.5%	12.2	462	25
2%	21.6	482	25
5%	20.4	487	25
	10.1p		
1 naphthol 4 sulphonic acid * % owf			
1%	7.5	341.6	23
5%	9.8	379	25
10%	10.2	441	22
	10.1q		
naphthalene 2,7 sulphonic acid * % owf			
1%	6.6	350	25
5%	6.2	328	25
10%	6.2	316	25

Treatment	% Elongation at the break	Breaking Strain (g)	Number of Tests
	10.1 r		
Untreated	10.49	506	103
Crocein Scarlet * †	8.03	392	50
Biebrich Scarlet * †	7.2	380	50
H Acid I	9.6	292	50
Evans Blue * I	7.0	368	50
Distilled Water	7.8	332	50

* 100 : 1 LR. Deionised water + 10% owf sulphuric acid + 10% sodium sulphate

† all 1% owf in blank (*) dyebath

The untreated control gave the highest breaking strain of all the samples tested. Most of the dyes tested produce breaking strain results above 350 g. Breaking strain varied widely, as did % elongation at the break, over the whole set of results. The % elongation was in many cases higher than that in the untreated wool, (see tables 10.1 i, k, n, all of which contain % elongations above 20%). Other values were also above 10.49% (untreated wool), though not so drastically. These ranged from 10.6% 10.1 j Evans Blue to 14.6% 10.1 m Orange II and 22.4% H Acid 10.1 i. There was a gradual decrease in breaking strain with increasing concentration of H cid. The % elongation varied randomly between 19.6% and 22.4% with no defineable pattern. The isomeric dyes shown in tables 10.1h and 10.1 o gave very similar results but, though the breaking strains were of the same order, the % elongations did not compare with H Acid (see table 10.1 i).

Interesting results were obtained with some of the simplest reagents tested (see tables 10.1a, b, p, q.). All the breaking strains were markedly lower. The

lowest was 274 g with 10.1a. · Breaking strains were all less than with untreated wool. The compounds in a, b, p, q were all naphthalene sulphonic acids, or naphthol sulphonic acids, relatively small molecules compared to most acid dyes and also fairly strongly acidic. This combination would, therefore, appear to be significant. Several other reagents gave low % elongation and low breaking strains (see tables 10.1c, d, e, f). These molecules are larger but do, however, possess sulphonic acid groups. All of the dyes mentioned in c, d, e, f are poly-sulphonated. This factor could be the major reason behind the similarity of these results.

The significance of these results and any conclusions drawn from them will be discussed later in Chapter 12. —— MS True

It could also be that the results are not significant.

CHAPTER 11

EXAMINATION OF THE G50 PROFILES OBTAINED FROM GEL PERMEATION CHROMATOGRAPHY OF THE TOTAL DYEBATH AFTER VARIOUS TREATMENTS

10 g samples of wool were treated in 1 litre of water at the boil for 24 hours. A variety of treatments were used, the details are recorded below. The soluble extract remaining was adjusted to pH2 using 98% methanoic acid, rotary evaporated to dryness, then treated with permethanoic acid. The products from this reaction were then rotary evaporated to dryness, dissolved in distilled water, and then subjected to GPC on a K100/25 Column containing G50. The profiles obtained are shown in figures 11.1 to 11.7. An examination of the results follows.

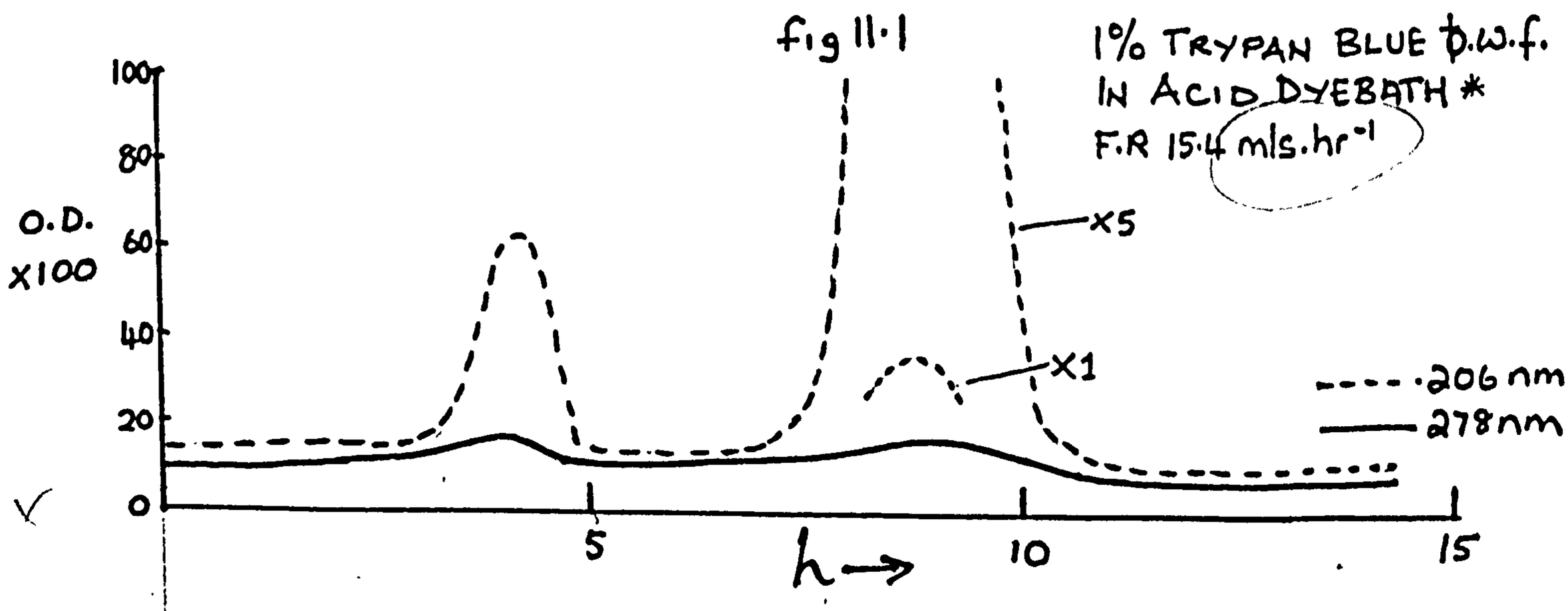


fig 11·2

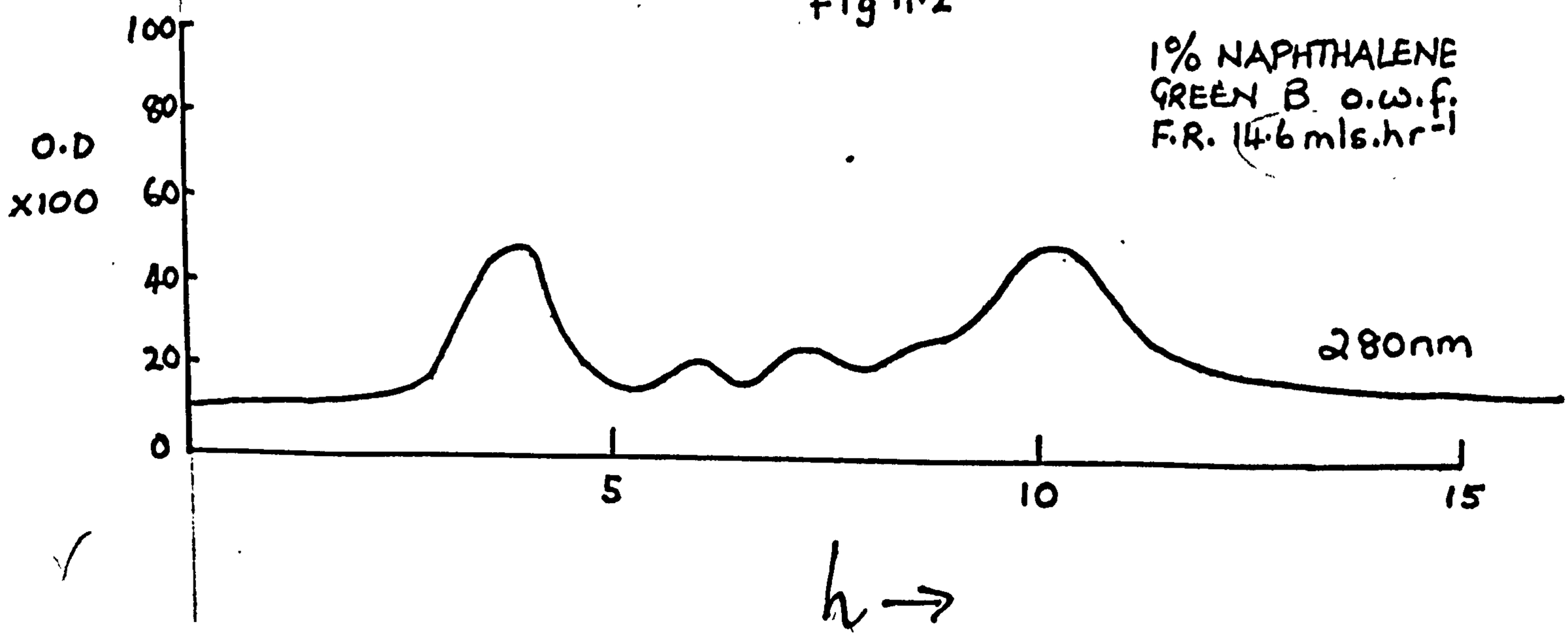


Fig 11.3

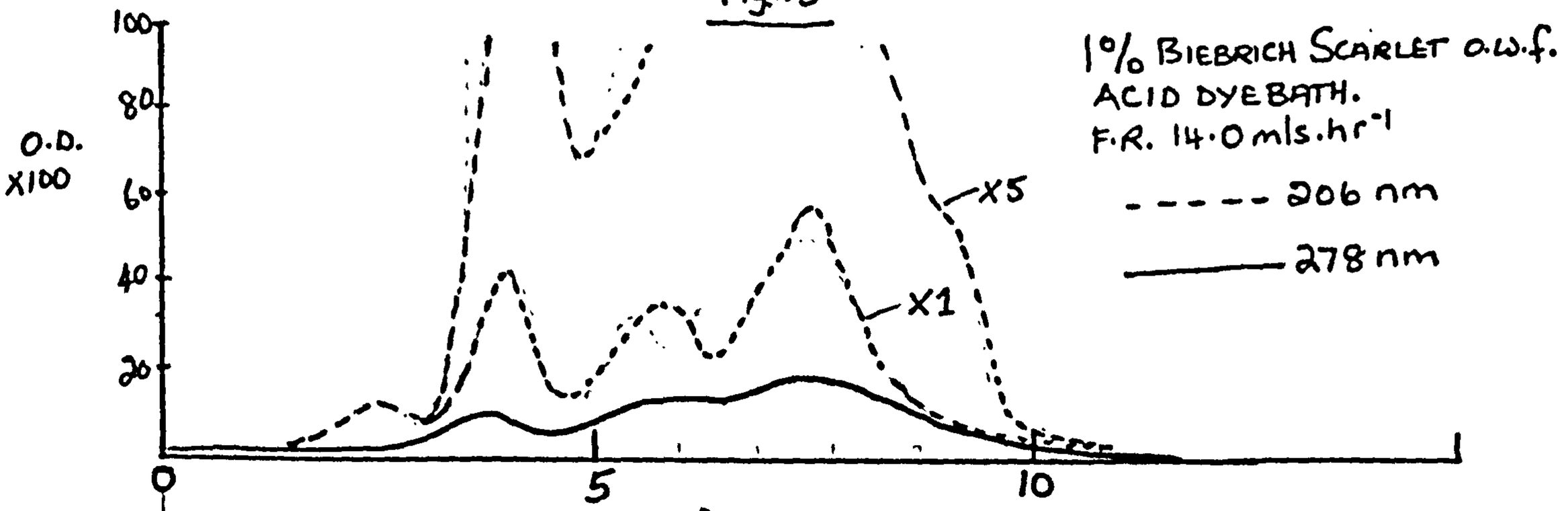


Fig.11.4

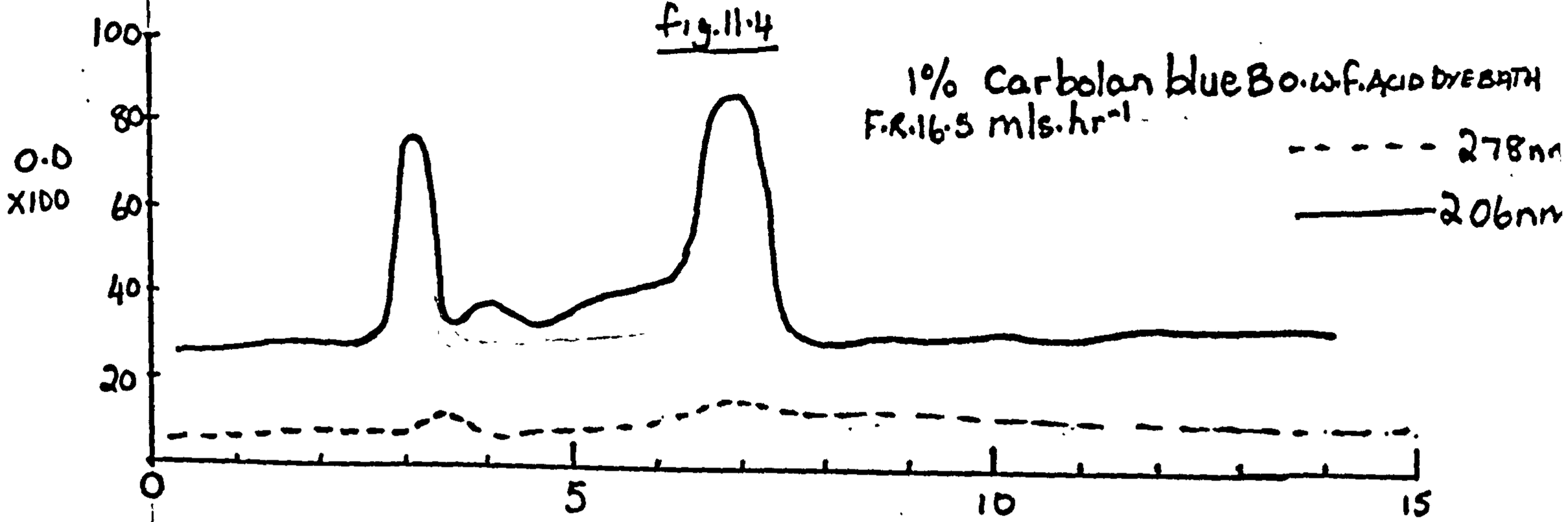


Fig.11.5

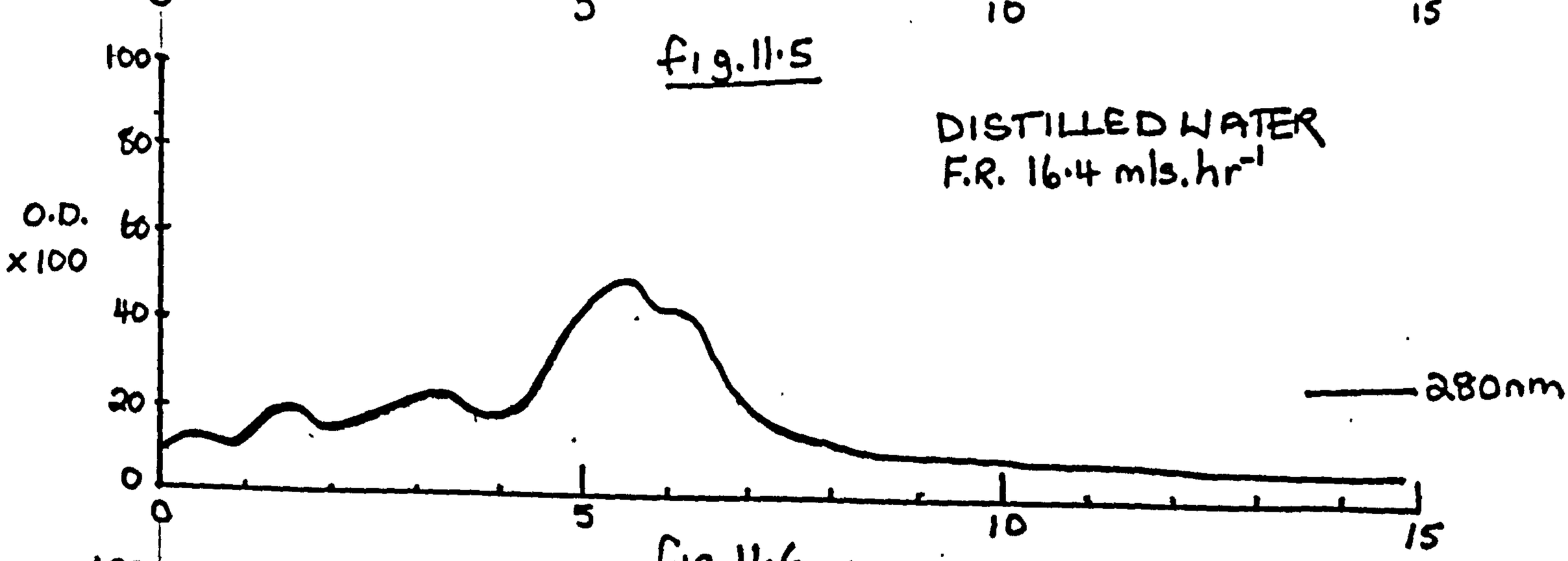


Fig.11.6

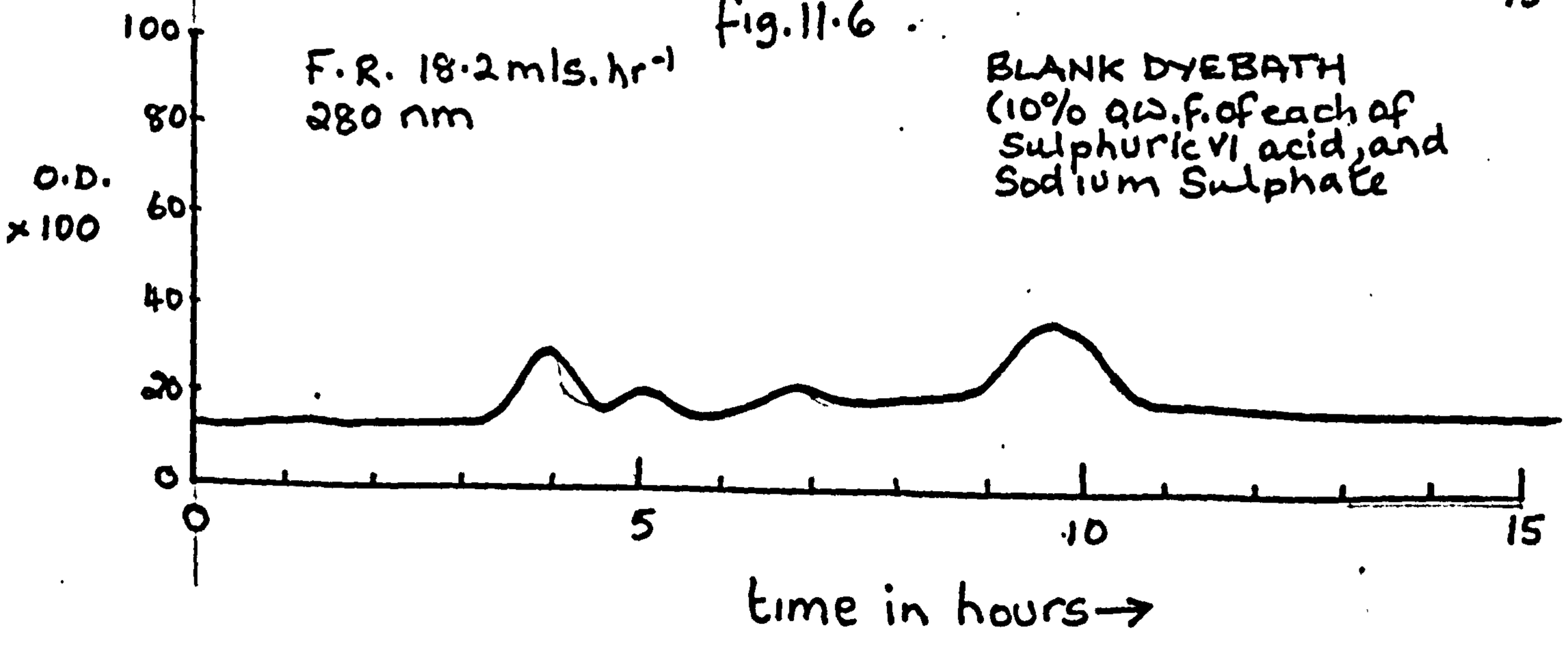
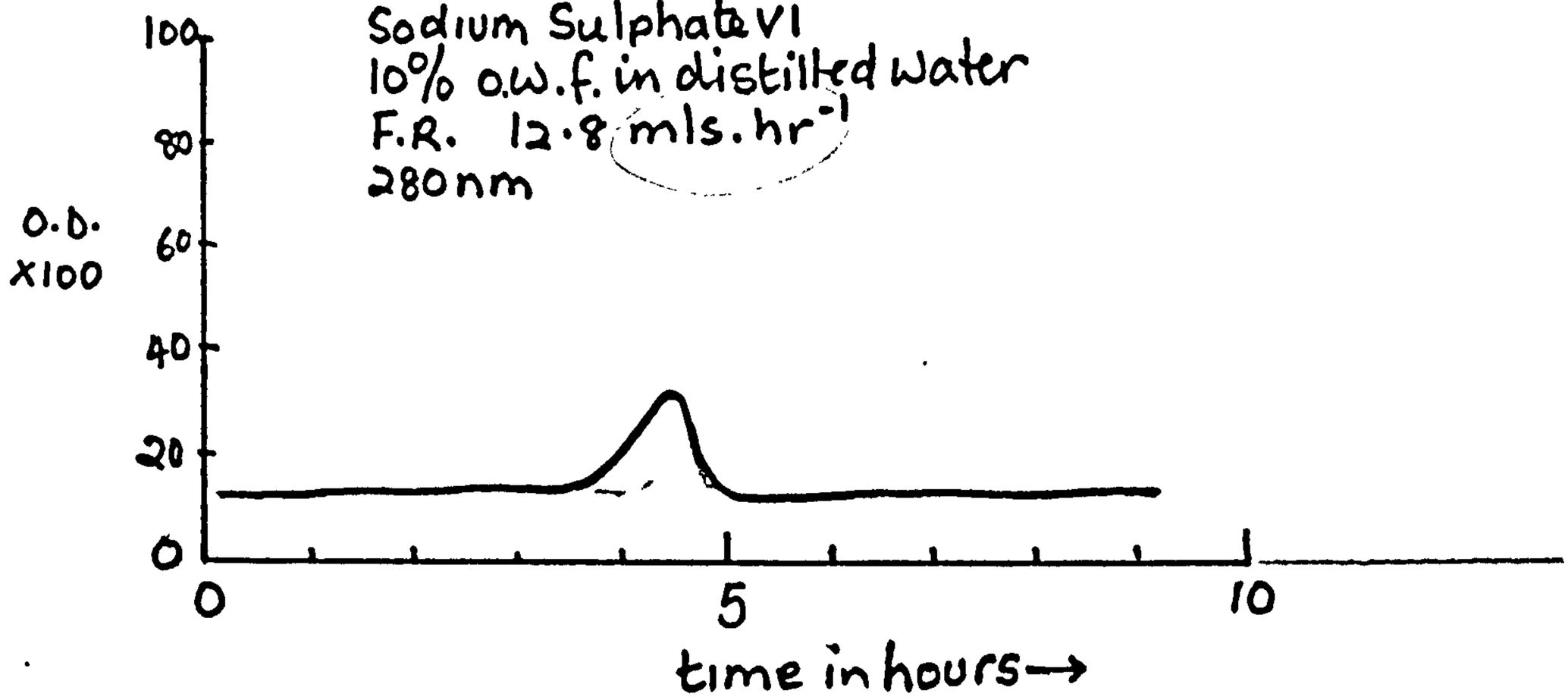


fig 11·7



From the profiles it can be seen that by far the simplest is given in figure 11.7 by treatment of wool with 10% owf sodium sulphate. Figures 11.1, 11.2, 11.3, 11.4 and 11.6 show some broad similarity giving fractionation into two distinct sets of proteins. One, presumably of higher molecular weight, 30,000 daltons approximately, eluted at the void volume time. The other of lower molecular weight eluted at about twice the void volume time. All of the samples in figures 11.1, 11.2, 11.3, 11.4 and 11.6 were treated under acid conditions, the dyebath

containing 10% owf sulphuric acid, rendering the pH $\geq$ 2. The two anomalous results were obtained using conditions which were not acidic (see figs 11:5 and 11.7). It seems reasonable to suggest that pH appears to affect the type of degradation taking place. Baumann (139) and Baumann and Mochel (137) ~~support this~~ had found this

Between the two groups of proteins eluted at Vo and 2Vo there was also in each of the cases 1, 2, 3, 4, and 6, evidence of proteinaceous material of intermediate molecular weight between these two main groups. In each case this took the form of a plateau region with little evidence of discrete peaks, notable exceptions were figs 11.2 and 11.3. The lack of discrete peaks would suggest a spread of molecular weights in the material of these fractions. The longer the treatment time the more this group of proteinaceous material would be expected to increase in concentration, since the author suspects that it is due, at least in part, to degradation of the extracted material once it is in solution. There do, however, appear to be two distinct groups of proteins extracted under acid conditions. These results will be further discussed in Chapter 12.

Your work supports Baumann Work!!

CHAPTER 12

CONCLUDING DISCUSSION

Steinhardt and Fuggit (125) reported in 1942 that the rates of hydrolysis, by dilute acids, of both a dissolved protein (egg albumen) and an insoluble protein (wool) depended not only on the temperature and acidity but also on the acid used. They found that at 65°C certain strong monobasic acids of high molecular weight, e.g. dodecyl sulphuric acid, hydrolysed amide and peptide bonds one hundred times as fast as hydrochloric acid. Even amongst the common mineral acids, there are differences in the rate of hydrolysis of protein.

These differences in hydrolytic effectiveness closely parallel the differences in the affinities of the anions of the acids for the protein undergoing hydrolysis. A further reason for attributing this effect to the anions was the attainment, with anions of high affinity of a maximum rate of terminal amide hydrolysis at relatively low concentrations. A similar limiting anion concentration, or maximum rate of hydrolysis, of the much more numerous peptide groups was not observed.

The compounds used during the work carried out are all acid dyes, direct dyes or intermediates used in their manufacture. The structures of the compounds differ but all have several features in common. They all contain sulphonic acid groups (from one to four). They all contain aromatic rings. Except for the intermediates they all contain diazonium bonds. There are also differences, though these are mainly in the way the above features in common are arranged. The dyes and intermediates are given in Table 5.1.

As the compounds used showed similarities, so too, did the results obtained, particularly when the G50 profiles are examined. These profiles give an indication of the distribution of molecular weights, within the extracted proteins, in the treatment bath, together with the relative proportions of the different proteins obtained.

For those treatments utilising an acid dyebath (10% owf of each of H_2SO_4 and Na_2SO_4) this similarity is quite marked, as indicated by figs 6.1, 7.1, 8.1, 9.1, 11.1, 11.2, 11.3, 11.4, 11.6. Peaks occur at the void volume time and approximately twice the void volume time, and between these two extremes there is a plateau region in which the peaks are less well defined. This would indicate a range of proteins of gradually decreasing molecular weight, although further GPC on G25 sometimes resulted in further resolution. The lack of discrete peaks indicates the heterogeneity of this material as well as the closeness of the molecular weights of the proteins within this portion.

The profiles obtained in figs 11.5 and 11.7, the treatment of wool with distilled water, and 10% (owf) sodium sulphate, respectively, showed different characteristics to those mentioned previously. The author believes that this is due to the fact that these treatments are the only two which were carried out at pH values which were not strongly acidic (i.e. approximately pH2 or below).

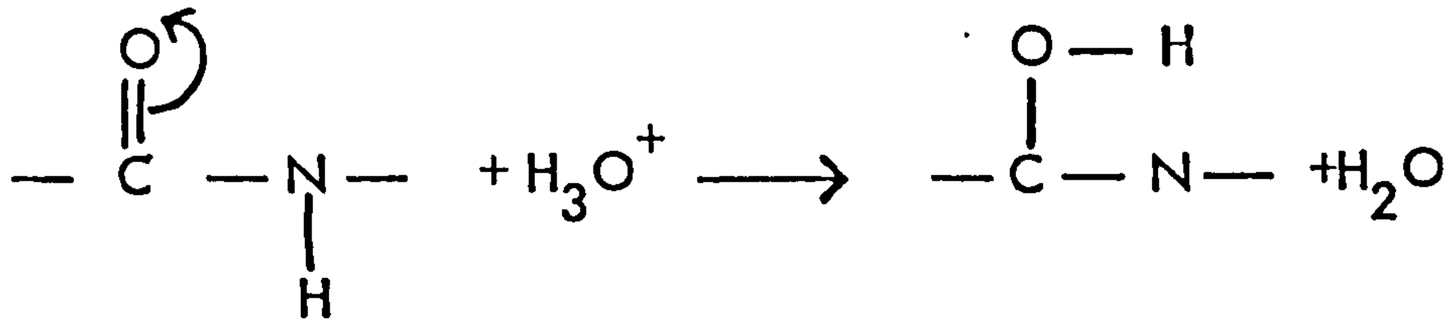
This confirms the work of Baumann (139); the proteinaceous materials extracted during the treatment of wool over a range of pH values were found to differ markedly from each other. The pH values and the effect of electrolytes were studied. In his study two sets of treatments were carried out, one using solutions of low electrolyte concentration, such as dilute hydrochloric acid, and dilute sodium hydroxide and the other utilising citrate-phosphate buffer solutions of high electrolyte concentrations. For both the above treatments, whether with hydrochloric acid, sodium hydroxide, or citrate-phosphate buffer, the yield of wool gelatin that was obtained was dependent on the pH value of the treatment solution.

The maximum yield of wool gelatin was found at pH2, and the yield clearly decreased with increasing pH value, with a minimum between pH4 and pH5,

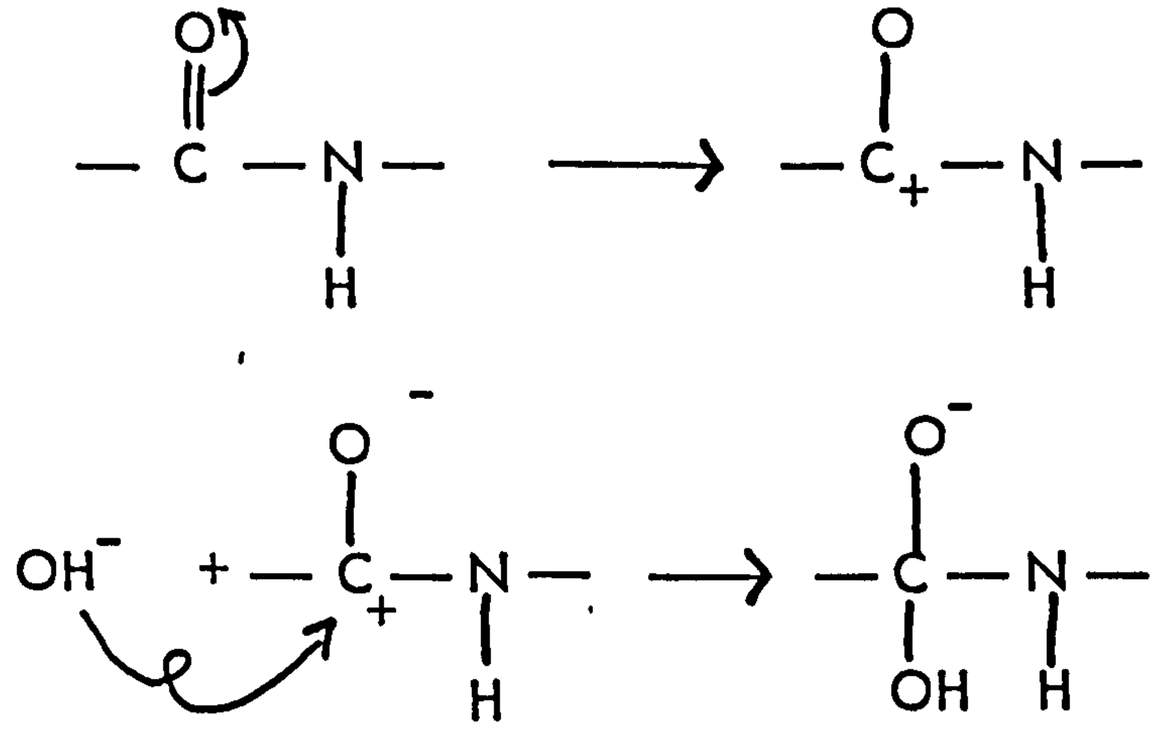
and thereafter with increasing pH value the yield again increased. The yield of wool gelatin obtained at pH2 was the same regardless of the electrolyte concentration. At pH4 - 5 in citrate-phosphate buffer twice the quantity of wool gelatin was obtained in comparison with that obtained in the hydrochloric acid/sodium hydroxide treatment over the same pH range. At higher pH values this difference in yield differed further. At pH 8, four times the quantity of protein was obtained with the citrate-phosphate buffer than with the hydrochloric acid/sodium hydroxide treatment.

In the work reported here, the conditions used were all acidic, with the two exceptions already described above; the concentration of sulphuric acid was 1.02×10^{-2} M and gave a pH value of approximately 2. All dyebaths also contained sodium sulphate (7.04×10^{-3} M) together with a dye or dye intermediate.

Baumann's work showed differences, not only in the quantity of proteins obtained, but also in the amino acid composition of the proteins. This would be expected because of the response of proteins to an alteration in the pH value of their environment. Proteins have the ability to adapt themselves to the prevailing conditions. They can buffer small changes in pH quite easily because of their ability to absorb hydrogen ions, or hydroxyl ions. This is because they possess both acidic and basic side groups. In the presence of small quantities of acid the carboxylic acid groups present can be back titrated. In the presence of small quantities of hydroxyl ions the basic side groups present in the wool can be deprotonated. Under more strongly acidic conditions it is possible to protonate the much more numerous peptide bonds, and this is the first stage in acid hydrolysis of peptides and proteins.



Similarly, under more strongly alkaline conditions the strongly nucleophilic hydroxyl ion attacks the carbonyl carbon atom of the peptide bond.



This is the first stage in the alkaline hydrolysis of proteins.

As well as the chemical attack of the treatment media, other changes take place in the conformation of the wool when it is treated under boiling aqueous conditions. The detailed molecular structure of wool is subjected to a transformation, with the secondary and tertiary structures becoming partially destroyed. Salt bridges, hydrogen bonds, hydrophobic interactions and Van der Waals' forces can all be broken. Rupture of these inter and intrachainic forces of attraction leads to the unfolding and reorientation of the polypeptide chains. Once the conformational constraints of such bonds have been removed the protein would then begin to unfold in such a manner that the groups exposed to the surrounding environment had the greatest degree of compatibility with that environment. Groups previously hidden inside the folded protein would thus become more accessible to chemical agents.

Such structural changes can be detected by using X-ray analysis techniques (143).

At low pH values wool has a net positive charge due to protonation of the ionised carboxyl groups. Adjacent protein chains of like charge repel each other and so the fibre swells. In a typical acid dyebath, wool encounters a medium which contains approximately 10% (owf) Sulphuric acid, 10% (owf) Sodium sulphate, together with varying quantities of acid dyes which are aromatic compounds, and in this work all contain sulphonic acid groups. In industry the dyebath may also contain, levelling agents, impurities from dye manufacture, and additives such as glucose which are introduced for the purpose of standardising the tinctorial strength of the dye. In this work all dyes and intermediates were purified so as to remove all of the above materials.

Previous work in this field by Steinhardt and Fuggit (125), and Botton (140) suggests that the anion has an effect on the rate of hydrolysis. Botton found that when the treatment bath contained a dye, the proteins obtained were both more heterogeneous and obtained in greater quantity, than treatments which did not contain dye. Botton's work differed from Steinhardt's and Fuggit's in that the treatment bath was examined in order to find the detailed protein content. Proteins were subjected to a similar treatment to that carried out in chapter 6 of this work on the proteinaceous material extracted during treatment with H Acid. The proteins were subsequently hydrolysed and the amino acid composition of the proteins studied in detail.

In nearly all previous work the proteins have not been fractionated, but the total protein merely hydrolysed. Some workers such as Sweetman (135), and Leach, Rogers, and Filshie (52), also dialysed the proteins obtained prior to the amino acid analysis procedure. De Deurwaerder et al (134) actually treated wool under similar conditions to those in this work. They used hydrochloric acid at pH2 and treated the wool at 100°C for 24 hours. They also fractionated the protein

on G50, the profiles from which showed a marked similarity to those obtained under similar pH conditions by the author and by Botton (140). Particular reference is drawn to the similarity of the G50 profile in fig 11.1 with those obtained in figs 11.2, 11.3, 11.4, together with those obtained in figs 6.1, 7.1, 8.1, 9.1. In their work De Deurwaerder et al found that sites adjacent to aspartyl residues were preferentially attacked during acid hydrolysis at pH2. They also found that material was removed almost exclusively from the orthocortex. The origin of the proteins extracted was elucidated using electron microscopy.

Their evidence suggested that the extracts originated in the microfibrils of the orthocortex and also contained some matrix material. After 72 hours the paracortex was still virtually undamaged on the evidence of its microscopic appearance.

They found the proteins extracted were heterogeneous. Fractionation of the extracted material produced two main groups of proteins, one of the groups (~~peak 2~~) showed a continuous distribution of molecular weights with a weight-average molecular weight of about 5600, while the other group (~~peak 1~~), though heterogeneous, contained a large proportion of material with an apparent weight-average molecular weight of about 21,000. The amino acid compositions of the two fractions were similar to each other and to the "low-sulphur proteins". The possibility that the smaller fragments were subunits of the larger ones and that both could represent sub-units in the original microfibrils of the keratin was suggested.

On examination of the results obtained in this work, the proteins extracted show broadly similar G50 profiles to those obtained in the work above (134).

There appear to be two groups of proteins one of higher, and the other of lower molecular weight, together with other material of intermediate molecular weight. Subsequent chromatographic separation gave profiles which were less

readily comparable with those in previously published work.

The profiles obtained on G25, and in the case of H Acid (chapter 6), A25, show separation of the extracted proteins into subfractions of relatively low molecular weight, and dissimilarity with other work is, therefore, perhaps not unexpected.

The amino acid data examined was in three parts; the first was on the fractions obtained from GPC, the second on the total gelatin extracted, and the third on the residual fibre. The results from each treatment may be seen in chapters 6 - 9. In chapter 10 the physical testing data is examined. It was found that the most marked effects on breaking strain occurred with the naphthalene sulphonic acids, and the 1-naphthol-4-sulphonic acid. Other reagents such as the polysulphonated Benzyl Red MG and Naphthalene Red J also gave markedly reduced breaking strains.

The breaking strain is a measure of the strength of a fibre. When examined at its most fundamental level it relates directly to the damage, or lack of damage, which is inflicted on the keratin protofibrils by a particular treatment. The percentage elongation gives a measure of the amount of "uncoiling" that takes place in a fibre prior to breaking, and relates to intrachainic and interchainic crosslinking such as disulphide bonds, salt links, polar interactions and hydrophobic interactions, which exist between different coils of the same helix and between adjacent chains.

If these intrachainic bonds are weakened or are broken then more uncoiling will take place before the protofibril peptide chains are put under strain. A low value for both breaking strain and percentage elongation at the break would indicate that the peptide bonds of the protein chains had been hydrolysed. Coupled with this the intra, and interchainic crosslinks would have remained relatively

unaffected.

The lowering of breaking strain and increase in % elongation at the break would tend to indicate a lowering of both intrachainic and interchainic forces within the protofibrils, and, peptide hydrolysis. The reduction in intra, and interchainic forces could conceivably be brought about by conformational changes imposed during aqueous treatments at the boil (142).

The results of chapter 10 show the resilience of wool. Its resistance to the treatments it was subjected to is ~~commendable~~ substantial. Indeed, most dyes gave decreases in breaking strain of less than 20%. The range varied from a 2% decrease (table 10.1n) to a 20% decrease (table 10.1c) though many of the treatments gave breaking strain values which had decreased by much less than this percentage.

The treatments with the dye intermediates used gave much lower absolute breaking strain values. In some cases the decreases in breaking strain values were more than 40% (tables 10.1a and 10.1b). The % elongation at the break increased by a considerable amount, for example from 10.49% for untreated wool to over 23% for Carbolan Yellow G treated wool (table 10.1k).

The significance of the author's GPC and amino acid analysis data, against the background of previous work, will now be discussed.

With Evans' Blue the data obtained on analysis of the total gelatin gave an insight into the effect of time on degradation products. This was the only treatment in which times of treatment were altered. The shorter treatment times, up to 240 minutes, gave similar amino acid composition data (table 8.6). The 1440 minute treatment, using identical treatment bath conditions, gave material which differed in composition when compared to the material from the shorter treatment times. The gelatins extracted in this 24 hour treatment appear to have more in common with other 24 hour treatments with different dyes, than with the

short term treatments with the same dye.

The G50 profiles illustrated in figs 6.1, 7.1, 8.1, 9.1 are not identical; however, they possess broad similarities which would indicate similarities in the extraction mechanism. Since the profiles obtained on G50 differ only in detail and would suggest that the dye is only modifying the overall extraction process.

It must be stated that dye anions are large compared to inorganic mineral acid anions, and they have features of note. They have both aromatic and polar or ionic character and this leads to some interesting properties.

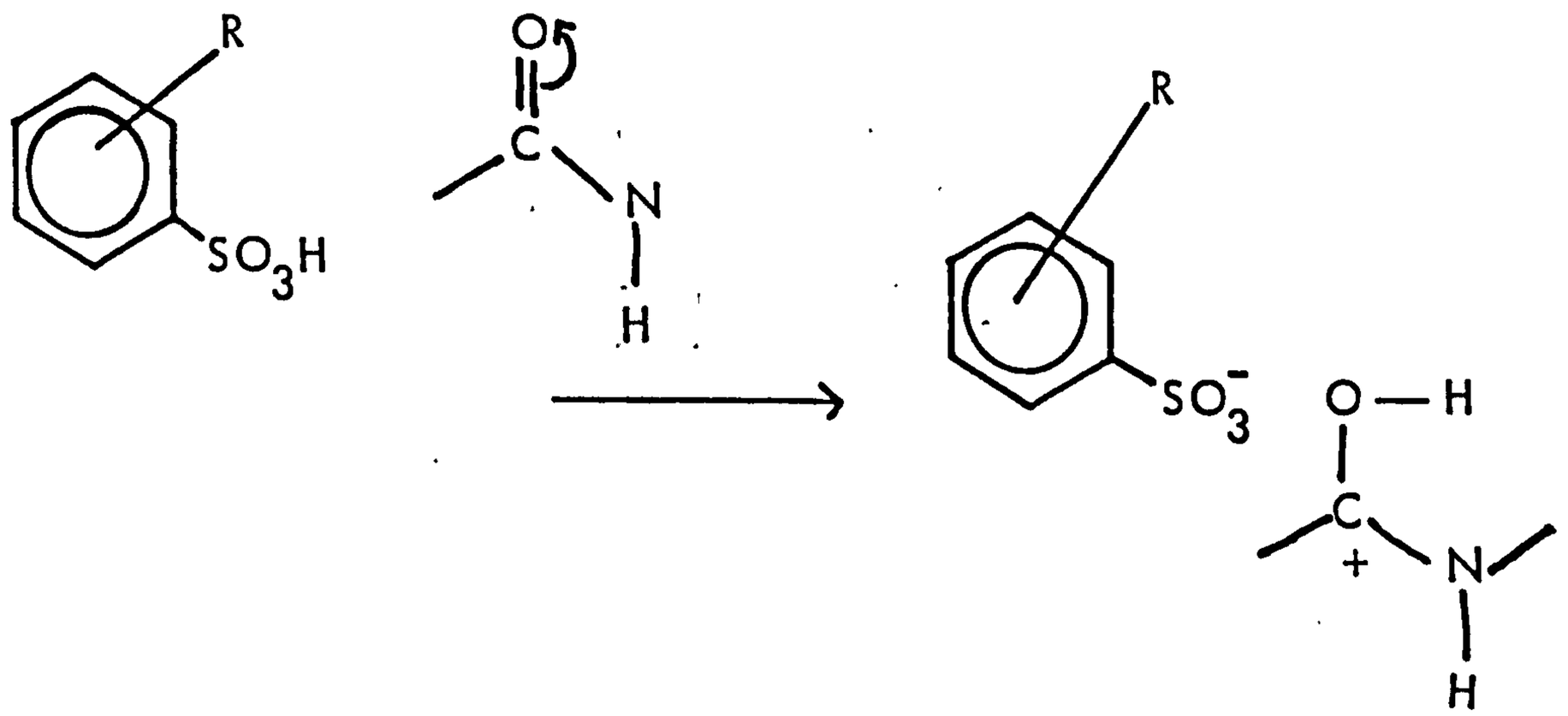
Consider Orange II. It is readily soluble in water as the sodium salt, but in the presence of a strong mineral acid the sulphonic acid group is back titrated and the dye becomes sparingly soluble in water.

The undissociated dye acid is more soluble in n-butanol than water. In an aqueous dyebath the sodium salt of the dye acid will be soluble. In the presence of mineral acids at pH2 or below the dye will form the dye acid. The latter will find itself in an environment for which it has a reduced affinity, and indeed this is one of the driving forces in wool dyeing. The dye acid will have more affinity for wool, with the aromatic portions of the dye having a particular affinity for regions of like character, the non-polar, or hydrophobic regions, of the wool fibre. The net effect is to carry hydrogen ions into the fibre. The dye molecule is therefore a 'carrier' ion. The author has already stated that hydrolysis occurs at pH2 and De Deurwaerder et al showed this hydrolysis occurred at the aspartic acid residues in particular (see also chapter 7). Other sites are also attacked, notably those adjacent to glutamic acid, and the residues of glutamine and asparagine (chapter 7). Whilst the more labile sites are available, little hydrolysis will take place at other sites.

The non-polar regions of wool in particular would under normal circumstances

be little affected by such hydrolytic processes. The dye molecule's ability to penetrate these regions will initiate hydrolytic attack on regions which were previously unaffected.

Further, the dye molecule, in acting as a 'carrier', also slows down the hydrogen ion it carries. In doing this it could conceivably bring about more hydrolysis at sites adjacent to the non-polar regions in wool or at residues which previously had few productive (hydrolytic) collisions with hydrogen ions. One of the roles of a positive catalyst is to increase the number of reactive collisions; some do this by making the reacting species increase their contact time, e.g. vanadium (V) oxide in the contact process. The dye molecule, in binding to protein, has a slower mean relative velocity, and reduced mean free path, and consequently increases the contact time of the attached hydrogen ion with the peptide bonds in the protofibrils. In so doing a carbonium ion is formed.



The ability of dye molecules so to protonate protein chains of wool and thereby to promote hydrolysis is not contentious. Animashaun's work (141) showed that it was another protonated anion which was in fact absorbed by the wool during dyeing in the presence of sulphuric acid; in this case it was the hydrogen sulphate (VI) ion. This ion also acts as a 'carrier' ion for hydrogen ions. This could partly explain the increased degradative effect of sulphuric acid/sodium

sulphate mixtures over the two reagents when they are used separately (140). However, using breaking strain as a parameter, the most effective reagents used in this work were, 1-naphthol-4-sulphonic acid, naphthalene sulphonic acids, and amino naphthol sulphonic acids.

These molecules are small compared to dye molecules but are large when compared to inorganic ions.

Their smaller size compared to dye molecules would mean they had greater mobility or less steric hindrance in the fibre at a given temperature. As smaller molecules they would be more effective in transferring hydrogen ions from the aqueous phase to the solid wool and once within the fibre, in transferring hydrogen ions from site to site.

If the extraction processes involved are similar the total protein extracted in each case will be similar and differences would only arise when the fractions were examined. On examination of tables 6.13, 7.5, 8.6, 8.7 and 9.7 some trends are evident. All the 24 hour treatments gave total gelatin composition data which was not dissimilar. On examination of the above tables there are obvious anomalies but many of the amino acids show similar data throughout all the above tables. Some amino acids are prone to variation, and would, therefore, alter the relative mole percent data for all other amino acids. The amino acids which vary by the greatest amounts are those which are most labile such as aspartic acid, glutamic acid, glycine; though others do vary, their variations are not as marked, and do not, therefore, greatly affect the relative mole percent of other amino acids.

The behaviour of the acid treatment-bath appears to be similar in each case where a 24 hour treatment time is used. With Evans' Blue, however, in table 8.6, the effect of time is shown. The total gelatin extracted after various

shorter times is quite different from the 24 hour treatment. The author has already proposed that this is due to different processes occurring with time, i.e. different material is being extracted from the fibre. The proteins extracted are of two types, namely (i) the intermacrofibrillar cement and cell-membrane complex material, and (ii) the combined degradation products of the cortex and other accessible areas of the wool. The orthocortex was shown to be particularly susceptible to this extraction procedure (37, 134, 135). Based on the evidence of microscopy the paracortex was not affected by anything like the same amount (37). The fractions obtained and their composition data have been examined in chapters 6 - 10 under treatment headings.

The fractionation on G50 gave in all cases a void volume peak which would indicate material with a relative molecular weight of 30,000 units approximately. The other material from fractionation was eluted later and would, therefore, have a lower relative molecular weight. De Deurwaerder found the latter to have a molecular weight in the range 5000 - 6000. In between these two extremes there is a continuous band of proteins of gradually decreasing molecular weight. Whether this diffuse plateau region is due to hydrolysis of extracted proteins in solution was not suggested, though this would be a feasible explanation. The same author suggested that the ~~peak 1 and peak 2~~ material was related and that on standing the low molecular weight material was capable of fibril formation as though it had an inbuilt "structural memory". He further suggested that the protein extracted was similar to the low sulphur proteins.

The total gelatins extracted and the fractions obtained from GPC were compared against low sulphur proteins and also against other groups of proteins obtained from wool by other workers. Some similarities exist when total gelatin is considered from the author's treatments and those of other workers.

No statistical procedure was used in any comparison since the most suitable method, the Y value method (144), could not be rigorously applied, because the relative molar masses of the proteins could not be assessed with sufficient accuracy to determine the number of amino acid residues in each protein. The difference index (145), was not used because of its shortcomings, mainly its lack of sensitivity. Comparison was therefore restricted to examination of composition data on a purely visual basis.

Data on low sulphur proteins, high sulphur proteins, ultra high sulphur proteins and proteins rich in glycine and tyrosine is readily available as indicated by tables 12.1 and 12.2.

From the results obtained in this work it can be seen that the fractionated proteins are very heterogeneous. All the treatments gave some proteins which were high in glycine and tyrosine as indicated in the tables 6.11, 7.1 (A4), 7.2(B1 - B4), 7.3 (C1 - C4/5), 8.5 and 9.6. Some of the proteins (tables 6.13, 7.5, 8.7, 9.7) obtained showed a resemblance to the low sulphur proteins as in table 12.1.

The proteinaceous material extracted showed two distinct groups, those with a resemblance to the low sulphur proteins and those which resembled the high glycine tyrosine fraction which have been associated with the cell-membrane complex proteins. In addition, there were other proteins which were not readily identifiable with any proteins obtained in previous work.

The very nature of the extraction process together with the environment the protein further encounters may lead to heterogeneity because of further hydrolysis and confirmational changes.

The low sulphur proteins obtained in this work showed a resemblance to the whole protein extracted from the orthocortex (37). There is a large amount of evidence to suggest that the orthocortex is susceptible to selective hydrolysis in preference to the para cortex by a solution at approximately pH2 (52).

TABLE 12.1 (after Bradbury, ref.37)

ANALYSES (MOLE PERCENT) OF SOLUBLE PROTEINS FROM WOOL

Amino Acid	Low Sulphur		High Sulphur		Ultra High Sulphur
CYA or SCMC	6.8	5.2	17.9	19.1	29.9
ASP	8.1	8.8	4.1	2.4	0.6
THR	4.4	5.3	10.4	10.7	11.1
SER	7.3	10.4	12.0	14.1	12.7
GLU	14.1	15.4	6.4	7.7	7.9
PRO	4.2	3.8	13.6	13.0	12.8
GLY	8.8	7.3	5.4	6.5	4.1
ALA	6.4	6.8	2.9	3.4	2.0
VAL	5.9	5.6	6.7	5.5	4.3
MET	0.6	NM	0.0	NM	0.0
ILE	3.7	3.5	3.0	2.8	1.7
LEU	10.3	10.3	5.0	3.4	1.4
TYR	4.3	3.4	1.9	1.8	1.9
PHE	3.0	2.7	2.4	1.5	0.5
HIS	0.7	0.6	0.9	1.3	0.1
LYS	4.1	3.2	0.7	0.7	0.9
ARG	7.3	7.3	6.7	6.3	6.9

NM ≡ Not measurable

TABLE 12.2 (after Bradbury, ref.37)

ANALYSES (MOLE PERCENT) OF SOLUBLE PROTEINS FROM WOOL

High-glycine-tyrosine protein

Amino Acid	Zahn and Biela	Gillespie and Darskus	Brunner et al	Parisot and Derminot
ASP	3.4	1.9	4.0	5.1
THR	5.6	2.0	3.2	5.4
SER	15.9	11.3	12.9	16.9
GLU	3.5	1.2	3.4	5.2
PRO	8.4	3.2	4.6	4.6
GLY	19.8	33.1	24.2	22.6
ALA	1.9	0.5	2.1	5.3
VAL	3.7	1.1	2.3	4.1
MET	0.0	0.0	0.0	0.6
ILE	1.4	0.5	0.9	1.9
LEU	5.5	5.7	6.6	5.6
TYR	10.9	19.4	13.1	6.8
PHE	6.4	2.7	7.9	6.2
HIS	0.1	0.1	1.0	1.6
LYS	0.3	0.1	0.7	2.2
ARG	4.8	4.8	5.6	5.9

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