CORRELATION OF THE CHEMISTRY
AND FINE STRUCTURE OF WOOL.

A thesis
submitted for the degree of
Doctor of Philosophy
in the
Australian National University

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Department of Chemistry
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I wish to express my sincere appreciation and indebtedness to my supervisor, Dr. J.H. Bradbury, for his guidance, inspiration and kindness towards me.

It is also my pleasure to express thanks to the staff of the Chemistry Department for their unflagging assistance in so many ways.

For permission to use the Electron Microscope facilities of the Zoology Department, I wish to thank Professor J.C. Smyth, Dr. W.L. Nicholas and Mr. J.K. Dodds of the Zoology Department provided valuable instruction and assistance in operation of the microscope, and Mr. I. Fox willingly gave advice on the associated photographic problems.

I certify that this thesis is my own original work, except where reference is made to the work of others.

[Signature]

David E. Peters

The essential financial support was kindly provided by the Australian Wool Board.

Finally I wish to thank my wife Joan, for her constant encouragement, and for typing the manuscript of this thesis.

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Chapter 6 - STUDIES ON GOLD STAINING OF WOOL.

6A Introduction  
6B Materials and Methods  
6C Results and Discussion

Bibliography

Publication
SUMMARY.

A method has been developed for the separation of macrofibrils from cortical cells of wool. Macrofibrils have a similar amino acid analysis to cortical cells. (Bradbury et al., 1965).

Resistant membranes have been isolated from whole wool, cortical and cuticle cells. Their amino acid analysis is not the same as epicuticle (King & Bradbury, 1968) contrary to Bradbury et al. (1971), as they contain less cystine than whole wool. It is suggested that the pronounced insolubility and resistance to enzymic digestion of resistant cell membranes is due to the presence of two types of cross-link (disulphide and ε-(γ-glutamyl)lysine). By comparison of citrulline contents, a considerably higher proportion of ε-(γ-glutamyl)lysine occurs in the resistant cuticle membranes than in the resistant cortical cell membranes. Harding & Rogers (1971a) have found ε-(γ-glutamyl)lysine is closely associated with citrulline containing peptides.

Nuclear remnants from wool have been extracted by an enzymic digestion procedure and the amino acid analysis of their protein component has been determined. It is found to contain a fairly high lysine content consistent with the presence of a basic protein in association with nucleic acids (Howells, 1971).

Soluble proteins from wool have been examined by NMR spectroscopy to observe the effects of various alkylating agents on unfolding of the proteins. This work can be correlated with that of other investigators, and
can be used to predict suitable conditions for reactions which require an unfolded protein, or solvent systems for preferential extraction of wool proteins, or systems for the separation of proteins. Information on the conditions for unfolding proteins is used in gel chromatography to determine molecular weights.

A series of attempts were made to isolate microfibrils from wool in their native form. The empirical approach adopted initially was unsuccessful, but NMR spectroscopy of soluble proteins has indicated conditions which may allow the separation to be achieved.

The separation of gold stained orthocortical and paracortical cells from wool by Chapman & Bradbury (1968) has been confirmed, and model compounds have been employed to establish possible binding sites for chloroauric acid in proteins. The paracortex of wool is shown to be not completely accessible to chloroauric acid at pH 4.5.
ABBREVIATIONS.

DMSO
Dimethyl sulphoxide.

H.S.
High sulphur content.

H.T.
High temperature.

Kerateine
Name given by Goddard & Michaelis (1935) for reduced keratin.

L.S.
Low sulphur content.

NMR
Nuclear Magnetic Resonance.

SCM
S-carboxymethyl.

SCMC
S-carboxymethyl cysteine.

SCMKA
S-carboxymethyl reduced keratin protein - low sulphur content fraction.

SCMKB
S-carboxymethyl reduced keratin protein - high sulphur content fraction.

TBP
Tri-n-butylphosphine.

T.M.S.
Tetramethyl silane.
Figure 0-1 presents a schematic drawing of a wool fibre. The fibre cuticle is divided into a thin, external, sulphur rich layer known as the exocuticle 'a' layer. Beneath this is the keratinous exocuticle 'b' layer, and innermost is the endocuticle which is non-keratinous and enzyme digestible (Ley, 1971). The endocuticle is probably the residual nucleus or nuclear remnant of the cuticular cells (Birbeck & Mercer, 1957).

Surrounding each cell (both cuticle and cortical) is a cell membrane, and where two cells are in contact this forms the cell membrane complex. The membrane complex is composed of two resistant membranes (one from each cell), a quantity of lipid, and an intercellular cement which is rich in aromatic amino acids (Bradbury et al., 1965a, 1971).

Within cortical cells are further components referred to in this thesis as macrofibrils, microfibrils and protofibrils. The microfibrils are embedded in a sulphur rich matrix protein, and Figure 0-1 shows one possible arrangement of microfibril components. Mercer et al., (1963) suggested that macrofibrils, microfibrils and protofibrils should be called fibrils, filaments and protofilaments respectively, but Johnson & Sikorski (1965) regretted the choice of filament as it can be confused with the textile term for a fibre of indefinite length (e.g. nylon filament). This latter term is also in wide and similar use in the polymer industry.

The macrofibril is possibly the smallest component...
of wool which can be separated by mechanical methods. Other chemical treatments, such as those involving alkylation with, e.g., iodoacetic acid, produce proteins called keratoses. Keratins is the name given by Smith and Wood (1935) to reduced keratins which become 5-dioxymethyl keratin (SCMK). The proteins produced by oxidative sulphotreatment of whole wool can be referred to as SCMK. The original separation of the cuticle into cuticular and cortical protein fractions is by no means complete. The proteins which are believed to be the source of the sulphur content are probably the source of the SCMK and of keratoes.
of wool which can be separated by purely mechanical methods. Other smaller components probably require chemical treatment prior to their separation, as they do not possess obvious cleavage planes as do cortical and cuticle cells, and macrofibrils.

Chemical treatments used to prepare soluble proteins from wool often involve reduction followed by alkylation with e.g. iodoacetic acid. This produces proteins called SCM kerateines or SCMK. Kerateine is the name given by Goddard & Michaelis (1935) to reduced keratin which becomes S-carboxymethyl kerateine (SCMK) on alkylation with iodoacetic acid. The proteins produced by oxidative cleavage methods are called keratoses.

SCMK proteins produced from whole wool can be fractionated into proteins of low sulphur content (L.S.) and of high sulphur content (H.S.) which are referred to as SCMKA and SCMKB respectively. The original separation of these two protein types was performed by Goddard & Michaelis (1935). SCMKA is believed to originate in the $\alpha$ helical microfibrillar proteins, and it can be further fractionated into e.g. SCMKAl, SCMKA2. The matrix proteins which are believed to be high in sulphur content are probably the source of the SCMKB group of proteins.
NOTE.

As most photographic copying of originals tends to lose some of the fine detail therein, all electron micrographs in this thesis, except scanning electron micrographs, are printed directly from the original negatives. For this reason, all figure numbers and descriptions of the illustrations appear on the page preceding the electron micrograph.

As an example of the amount of detail sometimes lost on photocopying, compare Chapter 6, Figures 5 and 6 with the illustration in the publication bound into the rear of this thesis.

Dock et al. (1941) showed that the striated appearance of cortical cells under the light microscope is due to the presence of many fine macrofibrils. By use of a micro-dissection technique using glass needles, they were able to separate cortical cells from formic acid treated wool, and also to partially separate these into macrofibrils (Dock et al., 1941). Using the electron microscope, Hook & McCurdie (1943) confirmed the presence of macrofibrils in cortical cells and revealed the presence of SE111 finer components. Similarly, Faworth (1941) demonstrated the presence of fibrillar components by examination of crushed wool fibers, and Zahn (1941) observed that cortical cells from trypsin-digested wool exhibited longitudinal fissures when examined in the electron microscope.

Dock et al. (1943) and Zahn (1943) showed that cortical
CHAPTER 1.

ISOLATION OF MACROFIBRILS FROM CORTICAL CELLS.

1A. INTRODUCTION

In 1886 von Nathusius published a histology of wool which, although it did not cover the fine structure of wool, is still considered to be correct to this day. Some time afterwards, he made the first observation of the fibrillar structure of cortical cells (von Nathusius, 1894).

This early work on keratins was often limited to examination of components produced by mechanical disruption, sometimes following chemical pretreatments, and separation was achieved only by laborious manual techniques.

Hock et al. (1941) showed that the striated appearance of cortical cells under the light microscope is due to the presence of many fine macrofibrils. By use of a microdissection technique using glass needles, they were able to separate cortical cells from formic acid treated wool, and also to partially separate these into macrofibrils (Hock et al., 1943). Using the electron microscope, Hock & McMurdie (1943) confirmed the presence of macrofibrils in cortical cells and revealed the presence of still finer components. Similarly, Reumuth (1942) demonstrated the presence of fibrillar components by examination of crushed wool fibres, and Zahn (1941) observed that cortical cells from trypsin digested wool exhibited longitudinal fissures when examined in the electron microscope.

Elöd & Zahn (1943) and Zahn (1943) showed that cortical
cells can be prepared from wool following pancreatin treatment for 3 days, or pulverization by milling in water, or both. A further 5-8 days digestion of the separated cells with pancreatin followed by pulverization in a mortar produces some macrofibrils (Elöd & Zahn, 1943). An electron micrograph of a single macrofibril was shown; it was about 15 micrometer (µm) long by 0.3 µm diameter. Mercer & Rees (1946), and Auber (1952) both observed that cortical cells consist of closely packed macrofibrils. Mercer & Rees demonstrated the presence of macrofibrils by mechanical grinding of trypsin digested fibres, but did not isolate these macrofibrils. They also observed a less electron dense intermacrofibrillar material which is apparently digestible by trypsin.

Geiger et al. (1941) showed that reduction and ethylation of wool drastically increased its susceptibility to pepsin digestion, but if the digestion is terminated when two thirds of the fibre has been digested and the fibres treated in a Waring Blender, a preparation containing macrofibrils and microfibrils (of 100 to 300 angstroms diameter) is obtained. Hock & McMurdie (1943) (above) and Zahn (1941, 1942) used similar treatments to this in their investigations.

Breakdown of wool into macrofibrils was also noted by Molyneux (1959, 1961) in an investigation of dermoid cysts in sheep. In both in vitro and in vivo cases the decomposition of wool to cortical cells and intracellular macrofibrils was observed.

Burgess (1934) and Mercer & Rees (1946) considered the macrofibrils to be held together by an intermacro-
fibrillar cement which King & Nichols (1933) believed was a non-keratinous protein derived from intercellular fluids trapped during cell growth. Mercer (1953) also supposed the intermacrofibrillar material to be a non-keratinous protein, and that its insolubility was due to the presence of bonds other than disulphide bonds.

Mercury vapour stains the intercellular and intermacrofibrillar cement and this led Rudall (1936) to state that the cements possess a high sulphur content. Analysis of material dissolved by proteolytic digestion (Elöd & Zahn, 1943) and formic acid (Bradbury et al., 1965a) suggests it has a low sulphur content.

Development of macrofibrils in the hair follicle has been observed by Birbeck & Mercer (1957) and by Rogers (1959a). In the upper bulb of the follicle, microfibrils are synthesised in the cytoplasm and these aggregate loosely into bundles. Later, the microfibrils are surrounded by matrix protein and are organised into larger aggregates of macrofibrils. Finally, the macrofibrils enlarge and eventually obliterate the cytoplasm of the cell. The bilateral nature of the fibre is visible in the follicle at this latter stage with merino wool, and there is some evidence of different behaviour in arrangement of microfibrils in the two halves of the cortex prior to this level (Birbeck & Mercer, 1957).

The bilateral nature of the cortex is obvious in electron micrographs of cross-sections, primarily because of the different arrangement of macrofibrils within the orthocortex relative to the paracortex (Rogers, 1959, 1959a; Birbeck & Mercer, 1957; Kassenbeck & Leveau, 1957; Bonès
There is also a different arrangement of microfibrils within the macrofibrils of both orthocortex and paracortex (e.g. Rogers, 1959, 1959a), being arranged in whorls in the orthocortex and in near-hexagonally packed arrays in the paracortex. This different arrangement of microfibrils can be shown by a number of staining methods (e.g. Kassenbeck, 1967). A variety of keratinolytic reagents also show a bilateral attack on the cortex (e.g. Leach et al., 1964; Golden, 1954; Horio & Kondo, 1953). Most of the differences between orthocortex and paracortex have been reviewed by Chapman (1967).

The diameter of macrofibrils appears to be rarely mentioned, although measurements could easily be made from many published electron micrographs (Rogers, 1959, 1959a; Kassenbeck, 1967; Kassenbeck & Leveau, 1957; Bonès & Sikorski, 1967). The more frequently quoted size is 0.1 µm up to 0.4 µm (Lundgren & Ward, 1963; Leach et al., 1964; Elöd & Zahn, 1943). Observation of published electron micrographs indicates that the quoted 0.1 µm diameter probably refers to macrofibrils in the orthocortex and 0.4 to 0.8 µm diameter would be the size of paracortical macrofibrils.

More recently, studies of fibres using the scanning electron microscope have shown macrofibrils. Anderson & Lipson (1970) examined cross-sections of fibres and found that macrofibrils are only obvious after chemical modification e.g. after pepsin digestion, or digestion with papain bisulphite following an aqueous chlorination. Haly et al. (1970) studied 'cut and peeled' fibres and observed assemblies of fibrils of the dimensions of
cortical cells and macrofibrils in the peeled sections. Longitudinal extension of the fibre clearly raised fibrillar material of these dimensions. Kuczera (1969) studied torn, cut and broken hairs and wool, and distinguished a cementing substance joining the various elements. He claimed the presence of five major levels of structural elements and related these to wool fibres. They were (with reference to wool) -

1) The wool fibre itself 15-30 µm diameter.
2) Cortical cells 3-5 µm diameter.
3) Macrofibrils 1-3 µm diameter and a 'subfibril' of 0.08 to 0.3 µm diameter and 30-100 µm long.
4) Microfibrils 75 angstrom diameter.
5) Protofibrils 20 angstrom diameter.

Kuczera divides the latter three structural elements into 2 or 3 subdivisions each, so that the whole system appears rather complex. However it is possible that the level 3) above refers to the two different dimensions of macrofibril observed in cross-sections of orthocortex and paracortex, although the sizes quoted are not in good agreement with figures mentioned previously, the macrofibril being too large.

1B. MATERIALS AND METHODS

(i) Materials.

Formic acid was 98-100% A.R. grade and was redistilled prior to use to ensure absence of residue. All other chemicals were A.R. grade unless otherwise stated. The wool used was virgin Merino 64's from a pen fed
sheep kept at the Division of Animal Physiology, C.S.I.R.O., Prospect, N.S.W. (Fleece No. 3716). This wool was cleaned by first removing the weathered tip of the staple, and then immersing in petroleum spirit (B.Pt. 40-60°C) for 5 min. (This removes the majority of the wool grease.) The fibres were then placed in a cold Soxhlet apparatus and extracted with fresh petroleum spirit for 6 hr.

After air drying, the fibres were washed 5 times in deionised water at 20-30°C. Most of the suint, dirt and skin flakes are removed in the first wash. The fibres were then air dried.

(ii) Preparation of Macrofibrils.

Cortical cells were prepared by Ley's method (Ley, 1971), which is a modification of the process used by Bradbury & Chapman (1964) and produces a greater yield of cortical cells. The method involves ultrasonic disintegration of 1 g. of finely cut wool (approx. 0.5 cm. lengths) in 50 ml. 98-100% formic acid in a Mullard-MSE 500 watt ultrasonic disintegrator for 5 min.. Separation of the formic acid by filtration on a 40 mesh stainless steel sieve removes the small amount of contaminating skin flakes in the filtrate. The wool is then placed in a further 50 ml. of formic acid and finely chopped with a Polytron stirrer operating at its slowest setting (about 10,000 r.p.m.) for 10 min.. The mixture of wool and formic acid is then transferred to the ultrasonic disintegrator and treated for a further 45 min.. Ultrasonic disintegrator settings and the differential screening method used to purify the cortical cells, are described in Bradbury & Chapman (1964).
The crude sample of cortical cells prepared as above was slightly contaminated with smaller particles, cuticle and fragmented cortical cells. As the presence of cortical cell fragments would complicate any attempt to determine the amount of breakdown occurring, it was necessary to remove as many of these contaminants as possible. The method employed was to layer the cortical cells in alcohol on to the top of a mixture of alcohol with 1.6% carbon tetrachloride (v/v) contained in a 2 l. stoppered measuring cylinder (8 cm. x 52 cm.). This blend has the same density as a mixture of alcohol containing 5% (v/v) water, but causes less aggregation of the particles. After allowing the cells to sediment in the cylinder for 6 hr. the supernatant liquid which contains most of the fine material is siphoned off, and the cells which have then sedimented to the bottom of the cylinder are removed and concentrated in a centrifuge. Repetition of this process removes the few remaining contaminants from the cortical cells. This method of separating particles has certain advantages over the sieving technique used by Bradbury & Chapman (1964). When applied to particles of only slightly different size it is easier to remove the fine material from the more coarse particles, and handles much larger quantities (0.5 - 2 g.) in comparison with sieving. The sieving technique in these circumstances, can only handle small quantities (0.050 g.) and even then serious cross-contamination occurs. The disadvantage in this case, is that the sedimentation method can be wasteful of material unless repeated several times.

A sample of purified cortical cells was then placed
in formic acid without drying the cells at any stage. If the cells are dried, they become noticeably more difficult to disperse. These cells were therefore filtered from the alcohol on a No. 3 sintered glass crucible, washing with formic acid and ensuring that at no stage did the liquid meniscus reach the level of the cells. Under these conditions the cells can be immediately redispersed in the liquid without any further agitation. The cells were dispersed in 50 ml. (approx.) of formic acid and treated in the ultrasonic disintegrator for a total of 5 hr..

Separation of the macrofibrils was achieved by firstly centrifuging the formic acid mixture at 1,450 g. for 1 hr. The supernatant after centrifugation was examined in the electron microscope by placing a drop of the mixture directly onto a prepared grid and allowing to dry. This material had the appearance of finely fragmented pieces. There was no regularity of size or shape apparent and no material which could be thought to be microfibrillar or even sheets of microfibrils was obvious. It is therefore doubtful if further ultrasonic disintegration is capable of producing undegraded components of the dimensions of microfibrils or aggregates of microfibrils.

Particles which sedimented under centrifugation at 1,450 g. were washed once with formic acid, once with distilled water and four times with alcohol at the centrifuge.

This material was then layered on to ethanol/1.6% carbon tetrachloride, now contained in a 250 ml. stoppered measuring cylinder (3.5 cm. x 30 cm.) as described
previously. A small amount of fine material was obtained in the supernatant of the first run (overnight). The second run, of 4 hr. duration, gave a very evenly sized grade of particles from the supernatant which were considered to be a pure sample of macrofibrils. The third and fourth runs, each of 1 hr. duration, yielded a pure sample of cortical cells from the material sedimented at the end of the fourth run. A large amount of intermediate sized material was removed at this stage.

(iii) Preparation of Orthocortical and Paracortical Cells.

Gold stained orthocortical and paracortical cells were prepared by the method of Chapman & Bradbury (1968) as modified by Ley (1971). This modification uses a density gradient of 1.310 - 1.356 instead of 1.265 - 1.300.

(iv) Microscopy.

Scanning electron microscopy was performed with the assistance of Dr. B.K. Filshie on a JEOLCO JSM-U3 scanning electron microscope. The electron microscope stub was coated with contact adhesive and macrofibrils placed on it from a drop of alcohol suspension. The samples were then vacuum coated with a thin coating of 70/30 gold/palladium alloy to prevent charging effects.

Light microscopy was routinely performed using phase contrast illumination with a Leitz Dialux microscope. Photomicrographs were made on Ilford Pan F 35mm. film using a Leica M3 camera attached to the microscope. Magnification was determined with a stage micrometer.

(v) Amino Acid Analyses.

Approximately 3 mg. of protein which had been dried under vacuum at 100°C for 1 hr. was weighed accurately
and hydrolysed with 1 ml. of constant boiling hydrochloric acid (about 6 N) for 24 hr. at 110°C. The hydrolysis tube was sealed under vacuum using the method of Crestfield et al. (1963), which is claimed by O'Donnell & Thompson (1964) to result in no degradation of S-carboxymethyl cysteine to cystine.

The hydrolysate was evaporated to dryness and taken up in 2 ml. 0.2 M sodium citrate pH 2.2 containing 12.5% sucrose and taurine, norleucine and α-amino-β-guanidinopropionic acid as internal standards. This solution (0.2 ml.) was loaded on an ion-exchange column of a Technicon amino acid analyser (Piez & Morris, 1960). The analyser has dual columns, and duplicate analyses were obtained and averaged for each determination. Where the ratio of the 440 nm colorimeter trace to the 570 nm trace is less than 6, citrulline is present and can be estimated by the method of Holy (1966), which uses the different ratio of these two colorimeter traces for the pure amino acids. Alternatively, separation of citrulline and proline can be achieved using the method of Bradbury et al. (1966).

1C. RESULTS

(i) Macrofibrils.

The method employed to prepare the pure cortical cells for ultrasonic disintegration means that accurate yields cannot be given. The yields below were obtained by summation of weights of the products as shown.
Recovered weights:

<table>
<thead>
<tr>
<th>Product</th>
<th>Weight (g)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical cells</td>
<td>0.0125</td>
<td>11.5%</td>
</tr>
<tr>
<td>Macrofibrils</td>
<td>0.0012</td>
<td>1.1%</td>
</tr>
<tr>
<td>Products filtered off during various purification stages</td>
<td>0.0823</td>
<td>75.4%</td>
</tr>
<tr>
<td>Soluble material from formic acid</td>
<td>0.0130</td>
<td>12.0%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>0.1090</strong></td>
<td></td>
</tr>
</tbody>
</table>

The large quantity of material removed during the various purification stages is due to variable sizes of much of the product of ultrasonication. There is a considerable quantity of material which is intermediate in size between that of the cortical cells and the macrofibrils, possibly representing cells which are only partly disrupted into macrofibrils. The range of sizes can be seen in Figure 1-3 which was taken after 3 hr. ultrasonic disintegration. Figures 1-1 and 1-2 show the original cortical cell sample used, and that after 15 min. ultrasonic disintegration respectively. A few disrupted cortical cells are visible in Figure 1-1 but these were the only few which could be found in the sample. Figure 1-2 shows a noticeable increase in the number of fragmented cortical cells over those visible in Figure 1-1, and this being after only 15 min. ultrasonic disintegration. Figure 1-3 exhibits as many macrofibrils and disrupted cortical cells as unmodified cortical cells. To stop Brownian movement occurring with many of the smaller particles, the slides were allowed to dry out and then dampened again prior to photographing. The cells and particles are stabilised by this process but a certain amount of aggregation of the
FIGURE 1-1 - UNTREATED CORTICAL CELLS.

FIGURE 1-2 - CORTICAL CELLS AFTER 15 MINUTES ULTRASONIC DISINTEGRATION.

FIGURE 1-3 - CORTICAL CELLS AFTER 3 HOURS ULTRASONIC DISINTEGRATION.
cells occurs.

Figure 1-4 contains two scanning electron micrographs of the same group of macrofibrils. Measurements from these micrographs and the 'stereo-pair' in Figure 1-5 indicate the average length of microfibrils is 10 µm, up to a maximum length of about 20 µm, and the width varies from an average of 0.2 µm up to about 0.3 µm.

(ii) Disruption of Gold Stained Orthocortical and Paracortical Cells.

Ultrasonic disintegration in formic acid of separated, gold stained orthocortical and paracortical cells demonstrates a pronounced difference in the disintegration rate. Amounts of breakdown were roughly estimated from the number of apparently whole cortical cells and the number of fragments at and above macrofibrillar size, visible in any one field of view in the light microscope. This method is biased towards the number of fragments, as one cortical cell is capable of producing many fragments; however it still shows a noticeable difference and should have approximately the same bias for both orthocortical and paracortical cells. The results are shown in Table 1-1.

No whole orthocortical cells are observed after 15 min. ultrasonic treatment, but a few whole paracortical cells are still visible at the end of 3 hr. disintegration. At the end of 1 hr. ultrasonic disintegration in the case of orthocortical cells, and 3 hr. in the case of paracortical cells, the treated cells and fragments were concentrated by centrifugation at 4,600 g. and washed with formic acid. The sedimented material was then photographed.
FIGURE 1-4a - SCANNING ELECTRON MICROGRAPH OF MACROFIBRILS.

FIGURE 1-4b - HIGHER MAGNIFICATION IMAGE OF MACROFIBRILS.
FIGURE 1-5 - STEREO-PAIR OF SCANNING ELECTRON MICROGRAPHS OF MACROFIBRILS.
TABLE 1-1

PERCENTAGE BREAKDOWN OF CORTICAL CELLS ON ULTRASONIC TREATMENT IN FORMIC ACID.

<table>
<thead>
<tr>
<th>Time of Treatment</th>
<th>Orthocortical Cells</th>
<th>Paracortical Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min.</td>
<td>80%</td>
<td>-</td>
</tr>
<tr>
<td>10 min.</td>
<td>90%</td>
<td>-</td>
</tr>
<tr>
<td>15 min.</td>
<td>95%</td>
<td>30%</td>
</tr>
<tr>
<td>30 min.</td>
<td>100%</td>
<td>45%</td>
</tr>
<tr>
<td>1 hr.</td>
<td>100%</td>
<td>50%</td>
</tr>
<tr>
<td>2 hr. 20 min.</td>
<td>-</td>
<td>65%</td>
</tr>
<tr>
<td>3 hr.</td>
<td>-</td>
<td>75%</td>
</tr>
</tbody>
</table>

Formic acid solubles 90% 57%
Residue 10% 43%

Figures 1-6 and 1-7 show paracortical cells before, and after 3 hr. ultrasonic disintegration respectively, and Figures 1-8 and 1-9 show orthocortical cells before, and after 1 hr. ultrasonic disintegration respectively. All four Figures are at the same magnification, but Figures 1-7 and 1-9 are at a much higher concentration of particles following centrifugation.

Owing to the small amount of cells available for this work (10 mg. of orthocortical and 8 mg. of paracortical cells), they could not be purified by the method used on the unstained cortical cells. Figure 1-9 shows that the orthocortical cells were considerably contaminated with cuticle fragments, but there are no whole cortical cells and few cortical cell fragments in comparison with
FIGURE 1-6 - GOLD STAINED PARACORTICAL CELLS.

FIGURE 1-7 - GOLD STAINED PARACORTICAL CELLS AFTER 3 Hr. ULTRASONIC DISINTEGRATION.
FIGURE 1-8 - GOLD STAINED ORTHOCORTICAL CELLS.

FIGURE 1-9 - GOLD STAINED ORTHOCORTICAL CELLS AFTER 1 HR. ULTRASONIC DISINTEGRATION.
the proportion of disrupted paracortical cells visible in Figure 1-7. There is no doubt that orthocortical cells are more fragile than paracortical cells, but it is difficult to obtain a quantitative value of the difference in stability to ultrasonic disintegration of the two cortices.

(iii) Amino Acid Analyses.

Amino acid analyses performed on the macrofibrils and the residual cortical cells left after the ultrasonic treatment are given in Table 1-2 (p. 23). The amino acid analyses are compared with results obtained by previous workers for disrupted cortical cells and orthocortical and paracortical cells.

1D. DISCUSSION

(i) Appearance of the Macrofibrils.

Mercer (1952) observed twisted macrofibrils from shadow-cast specimens in the electron microscope. In 1946, Mercer & Rees looked at cortical cell fragments produced by grinding trypsin digested fibres. Although these were fragments placed directly on the grid, they also appeared to be twisted when viewed in the electron microscope.

This twisting was attributed to supercontraction effects by Rogers (1959), but he also believed the macrofibrils may have originated in the orthocortex. To account for the whorls observed in cross-sections of macrofibrils in the orthocortex, he postulated a helical arrangement of the microfibrils. The paracortex was presumed to possess a parallel arrangement of straight microfibrils.
Macrofibrils having a twisted structure were not observed by Jeffrey et al. (1955) but these workers used Lincoln wool.

Macrofibrils having this characteristic whorl of microfibrils have been observed in cross-sections of hair follicles by Birbeck & Mercer (1957), and this may show up as a twisted macrofibril as suggested by Rogers (1959).

Twisted structures have also been observed by Bradbury & Chapman (1964) in disrupted cortical cells, and by McMurtrie (1886) for intact cortical cells.

Examination of the scanning electron micrographs, Figures 1-4 and 1-5, shows that many of the macrofibrils have a twisted form. (This is particularly noticeable if facilities are available for viewing the 'stereo-pair' in Figure 1-5). Further examination indicates that the length to width ratios of the macrofibrils are of about the same magnitude, (i.e. about 60:1) although there is considerable variation about this figure. The length to width ratio of cortical cells is about 25:1 (see Bradbury & Chapman, 1964). Some of the larger diameter macrofibrils appear to have lower length to width ratios but all of these are of two types. 1. Those with abbreviated ends which may have been cut off during preparation, and 2. those which appear to be incompletely disrupted. A number of the larger macrofibrils in Figures 1-4 and 1-5 show evidence of further splitting. Two or more ends can be seen fraying away in some cases, and hence these macrofibrils are probably not single.

The scanning electron micrographs also show that many of the macrofibrils are not circular in cross-section. This
effect can be clearly seen from cross-sections of whole wool (e.g. Rogers, 1959), but with separated macrofibrils, the asymmetry may also be due to incomplete disruption. Many of the cortical cells remain intact after the ultrasonic treatment and it is also obvious that many are only partly disrupted. This behaviour can reasonably be expected to continue down to macrofibrillar dimensions, therefore the presence of some incompletely disrupted macrofibrils would be anticipated.

The above gives no clear indication of the source of the macrofibrils, although their twisted structure may indicate that they originate from the orthocortex.

(ii) Size of the Macrofibrils.

Few previous workers have quoted sizes for macrofibrils, perhaps due to the incomplete separations achieved. It is not difficult however, to obtain estimates of macrofibril diameters from measurements on published electron micrograph cross-sections of wool fibres.

Mercer & Rees (1946) observed particles of 1,000 - 2,000 angstrom diameter (0.1 to 0.2 µm) in their work. Leach et al. (1964) state a fairly definite and regular size is observed (ca. 0.2 to 0.4 µm diameter), although they also noted that macrofibrils are larger in the paracortex. Előd & Zahn (1943) quoted a size of 0.1 µm diameter but the macrofibril pictured had a diameter closer to 0.3 µm.

Examination of some published cross-sections of wool, e.g. Bonès & Sikorski (1967) for phosphotungstic acid stained wool, Kassenbeck (1967) for silver and uranyl acetate stained fibres, and particularly Rogers (1959)
for osmium stained wool, indicate that values of 0.1 to 0.3 µm would be expected for macrofibrils from the orthocortex. Conversely, the paracortex appears to possess larger macrofibrils with diameters up to 0.8 µm. With wool fibre cross-sections, the maximum observable macrofibrillar diameter is probably the most reliable figure, as smaller diameters could correspond to macrofibrils sectioned at some distance from their largest dimension.

It is important to note that in the case of cross-sections observed in the electron microscope, some swelling is possible, which may increase the sizes obtained. Rogers (1959) comments that in determining the size and spacing of microfibrils, no account was taken of swelling produced by the 35% by weight uptake of osmium by the fibre. He failed to note that the Araldite embedding medium may also cause an unknown amount of swelling. There is no doubt that Araldite penetrates the fibre, as failure to ensure thorough infiltration results in collapse of the fibre sections during sectioning. It is also known that polymers can be deposited in wool fibres, as Alexander et al. (1952) used impregnation with polymers as a method of stabilizing the fibre against supercontraction.

From examination of Figures 1-4 and 1-5, the diameter observed is variable, the maximum diameter is about 0.3 µm, and the average being 0.2 µm. Lengths are still more varied, as noted in Chapter 1D(i), and range from 8 to 20 µm. This variability in size could be due to an actual variation in macrofibrillar size between those from the orthocortex and the paracortex, but it is probably due to...
variation in size of macrofibrils in the orthocortex combined with incomplete disruption of some of these macrofibrils. There may be some macrofibrils which originated from the paracortex, but the range of diameters indicates the orthocortex is a more likely source.

(iii) Cortical Cell Disruption Rates.

Table 1-1, in combination with Figures 1-6 to 1-9 clearly shows that the rate of disruption of gold stained orthocortical cells is much higher than that of gold stained paracortical cells.

From Table 1-1, 1 hr. ultrasonic irradiation is sufficient to cause most of the orthocortical cells to go into solution (90% in 1 hr.), but only just over half this quantity of paracortical cells is dissolved in 3 hr. (57% in 3 hr.). Unstained cells by comparison, only dissolve 12% in 5 hr..

The amount of disruption occurring shows similar behaviour, with the orthocortical cells being almost completely disrupted in 15 min.. Paracortical cells are only 75% disrupted in 3 hr.. For the unstained cells, 11% remain undisrupted after 5 hr., although this latter figure is determined by a different method.

Gold staining is known to cause cleavage of disulphide bonds by their oxidation to cysteic acid (Chapman & Bradbury, 1968). This may weaken the overall adhesion of the macrofibrils to one another, particularly if disulphide bonds are partly responsible for holding them together. The added weight of gold in the cells may change their inertia and affect the rates of disruption. These two effects would be expected to increase the rate of disruption
of gold stained cells over that of unstained cells.

As paracortical cells contain more gold than orthocortical cells, both in the keratin itself and in the nuclear remnant (Chapman & Bradbury, 1968), they would be expected to disrupt at a faster rate than orthocortical cells, all other factors being equal. It is observed that paracortical cells have a lower disruption rate than orthocortical cells, therefore the difference in disruption rates observed for gold stained cells from the two cortices, must be smaller than that which would be observed with unstained orthocortical and paracortical cells. However, Chapman & Bradbury's (1968) results show that there is a higher proportion of cystine remaining in gold stained paracortical cells than in gold stained orthocortical cells (Table 1-2), and this probably contributes slightly to the greater stability of the paracortex.

The light micrographs (Figures 1-2 and 1-3) show that complete disruption is not observed. There is possibly a majority of partially disrupted cells present and this is also indicated by the high degree of wastage observed during purification of the macrofibrils. Increased breakdown could be achieved by longer ultrasonic disintegration times, but this would probably also cause more material to dissolve. Longer ultrasonic disintegration treatments would probably also cause the initially produced macrofibrils to be further disrupted until they finally dissolve. Conversely, shorter ultrasonic disintegration times would be expected to disrupt orthocortical cells only, but in low yield. Therefore, the overall ratio of macrofibrils to cortical cells would probably remain unchanged for longer
treatments.

It is now logical to conclude that the residual cortical cells obtained from ultrasonic disintegration of unstained cells is probably a sample of paracortical cells. The macrofibrils prepared during the same treatment possibly originate from the orthocortex, although there is more chance that these are contaminated with a small quantity of macrofibrils from the paracortex.

Woods (1938) confirmed that cortical cells are the elastic unit of fibres, by measuring length changes of cortical cells isolated from fibres which had been stretched and set. The separated cortical cells also behaved like wool fibres, in supercontraction and the $\alpha-\beta$ transformation as observed by X-ray diffraction. It is likely however, that the basic structural unit of the fibre is not a cortical cell but the macrofibril. The macrofibril being the smallest structural unit in the fibre which possesses most of the properties of the whole fibre. In contrast, the next smaller structural unit, the microfibril, probably does not possess certain whole fibre properties, e.g. swelling behaviour (Gillespie, 1970). Macrofibrils are probably also the smallest structural units which can be separated by purely mechanical means, due to the presence of suitable cleavage planes through the intermacrofibrillar cement. Separation of microfibrils from wool can possibly only be achieved following cleavage of covalent bonds. (See Chapter 5).

(iv) Effect of Formic Acid and Ultrasonic Irradiation.

Table 1-2 shows that the combination of formic acid and ultrasonic disintegration causes modification of some
of the amino acids. Cysteic acid concentration is significantly increased by the treatment and considerably more so in the macrofibrils. Histidine is decreased and methionine shows a decrease in the macrofibrils analysis only.

There is a possibility that the keratin of the macrofibrils has been modified during preparation processes. Bradbury et al. (1965) have examined the effect of dissolution in formic acid of part of the protein from powdered wool on its overall amino acid composition. They concluded the only significant effect of immersion in formic acid was a slight formylation of serine and threonine hydroxyl groups (Kienhuis et al., 1959; Narita, 1959). One third of the formyl groups attached to serine and threonine are lost during 3 days dialysis (Narita, 1959). Solution of a small amount of protein in formic acid, particularly if its amino acid composition was similar to that of whole wool, would have negligible effect on the overall amino acid composition.

Gillespie (1970) was able to demonstrate the inverse relationship between swelling in formic acid and high sulphur protein content of various fibres. He also found the amount of matrix protein was not the only factor controlling swelling. The disulphide content of wool is inversely proportional to diametral swelling in formic acid, and was observed on reduced and alkylated Corriedale wool by Feughelman & Chapman (1966). Immersion of wool in 98% formic acid causes randomisation of the $\alpha$ helical proteins by at least 50% but not the cross-linked protein (Chapman & Feughelman, 1967; Astbury & Haggith, 1953).
This appears to be a reversible effect provided formic acid immersion time is limited (Bendit, 1966; Chapman & Feughelman, 1967).

From the above, the effect of formic acid on the macrofibrils and their amino acid composition is relatively minor and largely reversible. The effect of ultrasonic disintegration on properties of the constituent proteins may not be so mild. Swart & O'Shea demonstrated that ultrasonic irradiation of wool in formic acid prior to extraction of high sulphur and low sulphur proteins results in modification of the gel chromatographic behaviour of the low sulphur protein (O'Shea, 1970). There appears to be more aggregation, or a higher molecular weight low sulphur protein extracted after ultrasonic irradiation. The high sulphur protein is unaffected. O'Shea (1970) has shown degradative effects on ultrasonic irradiation of soluble proteins in formic acid. The severity of degradation increases with increasing molecular weight of the protein and with higher irradiation intensity.

(v) Amino Acid Analyses.

As noted above, the formic acid/ultrasonic disintegration method used to prepare macrofibrils, has resulted in modification of cysteic acid, methionine and histidine in the macrofibrils and residual cortical cells. The results of Bradbury et al. (1965) also show an increase in cysteic acid content and a slight decrease in histidine content on ultrasonic disintegration, but over a considerably shorter treatment time.

The cystine content of the residual cortical cells is marginally higher than that of the macrofibrils. This
### TABLE 1-2

**AMINO ACID ANALYSES OF MACROFIBRILS AND CORTICAL CELLS.**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Macro-fibrils</th>
<th>Residual Cortical Cells</th>
<th>Disrupted Cortical Cells (1)</th>
<th>Cortical Cells (1)</th>
<th>Paracortical Cells (2)</th>
<th>Orthocortical Cells (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{3}HSO\textsubscript{3}</td>
<td>1.69</td>
<td>0.73</td>
<td>0.18</td>
<td>0.19</td>
<td>2.68</td>
<td>2.68</td>
</tr>
<tr>
<td>Asp</td>
<td>7.11</td>
<td>7.39</td>
<td>6.73</td>
<td>6.75</td>
<td>7.34</td>
<td>7.63</td>
</tr>
<tr>
<td>Thr</td>
<td>5.74</td>
<td>5.78</td>
<td>5.57</td>
<td>5.35</td>
<td>6.66</td>
<td>6.21</td>
</tr>
<tr>
<td>Ser</td>
<td>9.33</td>
<td>9.46</td>
<td>10.37</td>
<td>10.64</td>
<td>10.21</td>
<td>10.04</td>
</tr>
<tr>
<td>Glu</td>
<td>12.29</td>
<td>12.37</td>
<td>11.65</td>
<td>11.67</td>
<td>13.06</td>
<td>12.06</td>
</tr>
<tr>
<td>Pro</td>
<td>7.31</td>
<td>5.68</td>
<td>5.94</td>
<td>5.87</td>
<td>6.70</td>
<td>6.13</td>
</tr>
<tr>
<td>Gly</td>
<td>9.05</td>
<td>9.02</td>
<td>9.67</td>
<td>9.62</td>
<td>7.98</td>
<td>9.25</td>
</tr>
<tr>
<td>Ala</td>
<td>6.25</td>
<td>5.67</td>
<td>5.58</td>
<td>5.61</td>
<td>5.73</td>
<td>5.97</td>
</tr>
<tr>
<td>Val</td>
<td>6.68</td>
<td>6.02</td>
<td>5.73</td>
<td>5.85</td>
<td>5.74</td>
<td>5.60</td>
</tr>
<tr>
<td>(\frac{1}{2}) Cys</td>
<td>6.62</td>
<td>6.89</td>
<td>9.72</td>
<td>9.06</td>
<td>6.02</td>
<td>4.85</td>
</tr>
<tr>
<td>Met *</td>
<td>0.24</td>
<td>0.51</td>
<td>0.45</td>
<td>0.47</td>
<td>0.44</td>
<td>0.51</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.56</td>
<td>4.06</td>
<td>3.29</td>
<td>3.33</td>
<td>3.41</td>
<td>3.19</td>
</tr>
<tr>
<td>Leu</td>
<td>8.20</td>
<td>9.49</td>
<td>7.59</td>
<td>7.75</td>
<td>7.72</td>
<td>8.65</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.75</td>
<td>3.29</td>
<td>4.23</td>
<td>4.36</td>
<td>2.87</td>
<td>3.62</td>
</tr>
<tr>
<td>Phe</td>
<td>2.80</td>
<td>3.27</td>
<td>3.04</td>
<td>3.10</td>
<td>2.76</td>
<td>3.09</td>
</tr>
<tr>
<td>Lys</td>
<td>2.85</td>
<td>3.09</td>
<td>2.83</td>
<td>2.82</td>
<td>2.91</td>
<td>2.72</td>
</tr>
<tr>
<td>His</td>
<td>0.16</td>
<td>0.16</td>
<td>0.83</td>
<td>0.82</td>
<td>0.89</td>
<td>0.78</td>
</tr>
<tr>
<td>Arg</td>
<td>6.86</td>
<td>7.19</td>
<td>6.60</td>
<td>6.76</td>
<td>6.85</td>
<td>7.07</td>
</tr>
<tr>
<td>(\frac{1}{2}) Cys +</td>
<td>8.31</td>
<td>7.62</td>
<td>9.90</td>
<td>9.25</td>
<td>8.70</td>
<td>7.53</td>
</tr>
<tr>
<td>C\textsubscript{3}HSO\textsubscript{3}</td>
<td>% #</td>
<td>83.3</td>
<td>84.6</td>
<td>96.2</td>
<td>96.2</td>
<td>99.2</td>
</tr>
</tbody>
</table>

(1) From Bradbury et al. (1965)
(2) From Chapman & Bradbury (1968)
* Includes methionine sulphone
# Recovery of anhydroamino acids
agrees with the proposition that the residual cortical cells originate from the paracortex. Kulkarni et al. (1971), Chapman & Bradbury (1968), Leveau (1959) and Miro & Blade (1965) all quote a higher cystine content of the paracortex relative to the orthocortex. Conversely, the overall cystine plus cysteic acid content shows the reverse of this effect, and cannot be explained on this basis. This difference however may not be significant as it is close to the 10% difference suggested by Corfield et al. (1968) as being the minimum necessary for significance. Chapman & Bradbury's (1968) results show a higher cystine content in the paracortex, and this could well be part of the reason for its higher resistance to disruption. The other cause may be the suggestion by Leveau (1958) of interchain disulphide bonds in the paracortex, and intra­chain disulphide bonds in the orthocortex.

The results in Table 1-2 for amino acid composition of macrofibrils and residual cortical cells indicate no significant differences exist except those mentioned above. Orthocortical and paracortical cells analysed by Chapman & Bradbury (1968) also show little difference, as do the analyses of Kulkarni et al. (1971). The absence of a significant difference in the amino acid composition of macrofibrils and residual cortical cells, probably reflects the lack of difference observed by the other workers mentioned above for orthocortical and paracortical cells. Perhaps most striking is the overall similarity of all amino acid analyses in Table 1-2 including those of Bradbury et al. (1965) and Chapman & Bradbury (1968) who used a different sample of wool to this investigation.
CHAPTER 2.

THE RESISTANT MEMBRANES OF WOOL.

2A. INTRODUCTION

(i) Epicuticle.

The earliest suggestion of a membrane associated with fibres appears to be that of McMurtrie (1886), who believed the scales on the surface of undamaged fibres were held in place by a continuous surface membrane which resists the destructive action of sodium hydroxide. In 1916, Allwörden observed that treatment of fibres with chlorine water raises what he termed 'bubbles' on the fibre surface. Kronacher & Lodeman (1930) observed Brownian movement inside the 'bubbles' after staining with methylene blue, and thus they should be termed 'sacs' (Hock et al., 1941).

Müller (1939) found that Allwörden sacs are formed by a membrane on the surface of the scales, and Lindberg et al. (1948) detached this membrane from the fibre by agitation in chlorine water. After examination in the electron microscope Lindberg et al. (1948) quoted a thickness of 50-100 angstrom for this membrane, which they obtained in only 0.1 - 0.2% yield. One year later, Lindberg et al. (1949) suggested the name 'epicuticular membrane' for the membrane enclosing Allwörden sacs.

According to Müller (1939), chlorine water reacts with protein in the cuticle immediately below the inert semi-permeable epicuticle. The soluble macromolecular products of this reaction then cause swelling of the
membrane due to osmosis. Hock et al. (1941) showed that addition of sufficiently concentrated salt solutions collapses the sacs. Electron microscopic investigations of Ames (1952) indicated that the 'a' layer of the cuticle is the site of attack by chlorine water, and Allwörden sac formation is directly related to the presence of oxidizable sulphur in the underlying protein (Leeder, 1969).

Epicuticle was reported to be composed of lipids (Alexander, 1950; Elliott, 1950), and partly carbohydrate (Lagermalm & Gralen, 1951), but more recently it was found to be predominantly protein (Zahn, 1952; Schuringa et al., 1952; Golden et al., 1955). The first accurate analysis was that of King & Bradbury (1968), and the work of Leeder (Leeder, 1969; Leeder & Bradbury, 1968, 1971a) has shown epicuticle is a cellular membrane and not a continuous sheath over the whole fibre, as was believed by some workers.

The chemical reactivity of epicuticle has been studied frequently, mainly owing to its easy accessibility and separation from the rest of the fibre. Mercer (1953) summarised the resistance of epicuticle by showing that it is the residue remaining after treatments with -

(a) trypsin,
(b) pepsin digestion of reduced fibres,
(c) strong urea solution containing reducing agents,
(d) solution of oxidized fibres in dilute ammonia,
(e) solution of reduced fibres in dilute ammonia,
(f) digestion of reduced fibres by enzymes,
(g) 30% formalin at 130°C,
(h) digestion by papain/bisulphite/urea,
(i) digestion by insects.

Epicuticle is also resistant to some acids e.g. carbon-ization in sulphuric acid has little effect. The membranes raised by chlorine water treatment are altered, as they now become digestible by trypsin (Mercer, 1953). A summary of the chemical resistance of epicuticle is given by Leeder (1969), and Leeder & Bradbury (1971).

Epicuticle comprises only about 0.1% by weight of wool (King & Bradbury, 1968), yet because of its position on the fibre surface it has been implicated in a number of properties of the wool fibre. Most notably it is important in the properties of wool such as frictional behaviour, (i.e. handle) (Gralén, 1950; Leeder & Bradbury, 1971) and wettability (Stewart & Whewell, 1960). It has also been considered to be a barrier to diffusion into the fibre of dyes and other molecules (Millson & Turl, 1950; Lindberg, 1953; Medley & Andrews, 1959).

No direct attempts to examine or separate whole cuticle membranes other than epicuticle appear to have been made, although Leeder's (1969) work indicates epicuticle envelopes the whole cuticle cell.

(ii) Cell Membrane Complex.

In unmodified keratin, the cell membrane complex has a characteristic appearance as described by Rogers (1959a), "The boundaries of cortical cells are separated by a distance of about 250 angstrom. In this gap can be seen a central dense layer (δ) about 150 angstrom wide, with a less dense region (β) on either side of it. Together with the cell membrane boundaries, which are also dense, these structures constitute the cell membrane complex that holds
the cortical cells together". Swift & Holmes (1965) consider the individual cell membrane consists of a bimolecular lipid 'leaflet' sandwiched between two fibrous protein layers. The overall width of this unit being about 75 angstrom; the lipid layer corresponding to the β layer of Rogers (1959a). Two of these unit cell membranes together with a globular protein cement (the δ layer) comprise the cell membrane complex. Apart from in wool, structures similar to that described above have been observed in human hair (Swift & Holmes, 1965), human hair follicle (Birbeck & Mercer, 1957) and feather rachis (Filshie & Rogers, 1962).

The intercellular cement or globular protein cement is easily extractable. Rogers (1964) extracted wool roots with 8 M urea and showed that the intercellular cement had been dissolved leaving the two separated membranes. Filshie & Rogers (1962) extracted feather rachis with thioglycollic acid and also demonstrated the extraction of the intercellular cement. Formic acid was used to extract part of the membrane complex from wool by Bradbury et al. (1965a). Analysis of this material showed it to be low in cystine and hence unlikely to be highly cross-linked. The intercellular cement can also be extracted by enzymes (Burgess, 1934), the extracted material has been analysed (Elöd & Zahn, 1943) and also shows a low sulphur content.

Leeder (1969) and Leeder & Bradbury (1971a) have reviewed and re-examined attempts by previous workers to achieve a resistant membrane residue. Many of these earlier attempts were found to be either stages in the
complete dissolution of keratin and membrane complex, or having residues comprising more components than the resistant membranes alone. They found that a stable resistant membrane residue is achieved using performic acid oxidation at room temperature for 2 weeks followed by 1 M ammonia at room temperature with gentle shaking for 1 week. Treatment with performic acid followed by 8 M urea pH 10 produced a similar percentage residue to the previous treatment, and both were consistent with the calculated membrane content of the fibre (Bradbury et al., 1971).

The cell membrane complex appears to consist of three components, resistant membranes, lipid, and an intercellular cement which has a high content of aromatic amino acids (Bradbury et al., 1971).

(iii) Resistant Cortical Cell Membranes.

Cortical cell membranes have attracted less attention than the epicuticle. In 1937, by observation in the light microscope, Reumuth detected an apparently continuous membrane (the subcuticle) situated between the cuticle and cortical cells. This membrane has been variously termed the 'sub-cutis', 'sub-cuticle membrane', 'epidermis membrane', 'cortex-mantle', and the 'intermediate layer' (Mercer et al., 1949; Mercer, 1953). Lehmann (1941) believed that by treating wool with formaldehyde at 140°C for 2 hr. the cortex and cuticle are dissolved leaving the 'sub-cuticle membrane'. Elöd & Zahn (1944) also prepared the 'sub-cuticle membrane' by supercontracting wool with phenol and then digesting it with trypsin. By careful observation under phase contrast illumination in the light microscope, and examination in the electron
microscope, Mercer et al. (1949) showed that this 'subcuticular membrane' was composed of epicuticle together with adhering cuticle.

Alexander & Earland (1950, 1950a) found that after treatment of peracetic acid oxidized wool with dilute ammonia a thin 'tubular' membrane remained between cuticle and cortex of the wool, (see also Alexander, 1951). Gralen (1950) reported that this membrane was composed of epi-, exo- and endocuticle as well as cortical cell residues, and Manogue & Moss (1953) found it includes boundaries of cortical cells and some macrofibrils. By examination of fibre cross-sections, Mercer (1953) also showed that the residue from peracetic/ammonia treatment of wool consists of cortical and cuticle membranes, nuclear remnants and some macrofibrils. Composed of this number of components, it is not surprising that Alexander & Earland's (1950, 1950a) membranes comprised 10% by weight of the fibre.

Like epicuticle, cortical cell membranes appear to possess considerable chemical resistance. The chemical resistance of the membrane complex of hair has been summarised by Mercer (1965). His table of chemical resistance is reproduced on page 31.

There is doubt concerning the accuracy of the enzymic digestion mentioned by Mercer (1965). Leeder (1969) has shown that when trypsin or pronase solution is applied to membranes isolated after performic acid/ammonia digestion of wool, they dissolve within a few minutes. Conversely, when a 3 week pronase pretreatment is applied to wool (in which 12% by weight is digested), a 'normal' resistant
membrane residue is obtained after performic/ammonia treatment (Leeder, 1969).

There is evidence that resistant cortical cell membranes do exist, as Leeder (1969) obtained a constant resistant membrane yield of 1.5% from whole wool. (Bradbury et al., 1971). It is doubtful that this all originated from the cuticle. Also, resistant cortical cell membranes were isolated by O'Shea (1971) while attempting to raise Allwörden type sacs on separated cortical cells.

During the work described in this Chapter, a method of completely descaling wool was developed. All details of this work are contained in Chapter 2E.
2B. MATERIALS AND METHODS.

Merino 64's wool prepared as described in Chapter 1B was used.

(i) Formic Acid Treated Wool.

To observe the effects of formic acid treatment, samples of wool were placed in 98-100% formic acid (redistilled) at 10:1 formic acid:wool ratio, for various times. The samples were removed and washed in running deionised water until the washings were neutral, and then immersed in deionised water for 15 min. The water remained neutral after this time.

(ii) Preparation of Components and Isolation of Membranes.

Cuticle and cortical cells were prepared by the ultrasonic disintegration of wool followed by differential screening of the products as described in Chapter 1B, and by Bradbury & Chapman (1964).

Initially, cuticle and cortical cells were prepared by a high temperature (H.T.) method as used by Leeder (1969) in his investigation of the properties of epicuticle (Leeder & Bradbury, 1968, 1971a). Ten grams of wool were placed in 500 ml. of redistilled formic acid and boiled for 1 hr. The fibres were screened off on a 40 mesh stainless steel sieve and washed with formic acid. The filtrate contains almost pure cuticle, which can be centrifuged down and washed with water. If the fibres are then placed in formic acid (500 ml.) at ambient temperature and shaken gently overnight (16 hr.), cuticle cells remaining on the fibre are dislodged. Filtration of the fibres and their subsequent washing in deionised water, affects which were used as a source of cortical cells (see Chapter 2B).
water, affords completely descaled fibres which were used as a source of cortical cells (see Chapter 2E).

In all cases, membranes were produced by dissolving the keratin according to a process developed by Leeder (1969) (Bradbury et al., 1971), which leaves membranes as the residue. This method uses performic acid (prepared from 450 ml. formic acid and 50 ml. 30% hydrogen peroxide, mixed 1-2 hr. before use, Thompson & O'Donnell, 1959) at 16-18°C and 100:1 liquor:wool ratio, for 2 weeks, followed by 1 M ammonium hydroxide (100:1 ratio) for 1 week at 16-18°C with gentle shaking (Leeder (1969) used 20°C).

After the performic acid treatment, the residue was centrifuged down and washed once with formic acid, twice with water and once with 1 M ammonium hydroxide before being placed in the ammonium hydroxide for 1 week. Leeder (1969) was able to show that a stable residue weight was reached after this time (Bradbury et al., 1971).

After preparation, the membranes were purified by washing 3 times with formic acid at the centrifuge. They were then layered on to 1,1,1-trichloroethane and centrifuged to remove heavy contaminants, washed again with formic acid and freeze-dried. The membranes were conditioned at laboratory temperature and humidity overnight and dried under vacuum at 100°C for 1 hr. prior to weighing.

(iii) Electron Microscopy.

Preparation of wool fibres for electron microscopy followed the thioglycollic acid/osmium tetroxide method of Rogers (1959). Thioglycollic acid 0.4 M, pH 5.6 was used
to reduce the fibres for 24 hr. at ambient temperature. The fibres were washed in deionised water and stained with 1% osmium tetroxide for 4 to 7 days. Stained wool fibres were again washed, dehydrated with ethanol, then propylene oxide, and embedded in Araldite according to the method of Glauert et al. (1956), using propylene oxide to dilute the initial Araldite mixture for thorough infiltration. Embedded fibres were then sectioned on an L.K.B. Ultrotome I using a diamond knife and collected on carbon stabilised collodion covered grids. Sections were post stained with lead citrate for 20 min. (Reynolds, 1963).

The sections were examined in a Hitachi HU-11C-S electron microscope using the anti-contamination cold trap. Calibration of magnification was achieved using a replica of a 28,800 lines per inch grating. Electron micrographs were taken on Ilford N50 or Agfa SCIENTIA 23D50 plates, and developed in Kodak D19B developer at 68°F.

Reduced fibres alkylated with acrylonitrile were prepared as described in Chapter 5 and stained and embedded as described above.

(iv) Amino Acid Analyses.

Amino acid analyses were performed as described in Chapter 1B.

2C. RESULTS.

(i) Yields of Membranes.

Table 2-1 gives the yields of membrane obtained from the quoted weights of keratin by application of the treat-
ment developed by Leeder (1969). It was found necessary to adhere closely to the conditions used by Leeder, in regard to gentle agitation of the ammonia extraction, to obtain constant residue weights concomitant with minimum treatment time. O'Shea (1971) has observed similar precautions are necessary.

In cases in which the ammonium hydroxide solution was agitated, light microscope examination of the membranes showed no evidence of nuclear remnants, fibrils or endocuticle as observed by Mercer (1953), Golden (1954) or Birbeck & Mercer (1957). This is in agreement with the observations of Leeder (1969). In no case was inorganic or other contamination of the final membranes preparation noted during microscopic examination.

**TABLE 2-1.**

**MEMBRANE YIELDS.**

<table>
<thead>
<tr>
<th>Keratin</th>
<th>Original Weight of Keratin</th>
<th>Yield of Membranes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified wool (agitated) *</td>
<td>2.0</td>
<td>1.60</td>
</tr>
<tr>
<td>Unmodified wool (agitated) (1)</td>
<td>5.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Unmodified wool (agitated) (2)</td>
<td>-</td>
<td>1.50</td>
</tr>
<tr>
<td>Unmodified wool (unagitated)</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Unmodified wool (unagitated) (1)</td>
<td>10.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Cortical cells (agitated)</td>
<td>0.2452</td>
<td>1.47</td>
</tr>
<tr>
<td>Cortical cells (unagitated) (1)</td>
<td>-</td>
<td>2.2</td>
</tr>
<tr>
<td>Cuticle cells (agitated)</td>
<td>0.2990</td>
<td>2.55</td>
</tr>
<tr>
<td>H.T. Cortical cells (agitated)</td>
<td>9.250</td>
<td>0.08</td>
</tr>
<tr>
<td>H.T. Cuticle cells (agitated)</td>
<td>0.6820</td>
<td>0.98</td>
</tr>
</tbody>
</table>

(1) O'Shea (1971)
(2) Leeder (1969), Bradbury et al., (1971)

* Agitated refers to gentle shaking of the NH₃ extraction.
Note that if wool comprises approximately 90% cortex and 10% cuticle (Bradbury & King, 1967) then combination of 90% of the cortical membrane weight percentage with 10% of the cuticle membrane weight percentage should equal the weight percentage of the whole wool resistant membranes. The relevant figures are:

Cortical cell membranes contribution 1.325 %
Cuticle cell membranes contribution 0.255 %

\[ \frac{1.325 \% + 0.255 \%}{1.580 \%} \]

This value of 1.58% compares satisfactorily with the observed value of 1.60%.

(ii) Amino Acid Analyses.

The amino acid analyses of the various membranes together with some pertinent analyses by other workers are given in Tables 2-2 and 2-3. Table 2-2 contains a series of whole wool resistant membrane analyses including the 12% 'membrane' analysis of Corfield et al. (1958). Also included is an average resistant membrane analysis obtained from the results of column 1, Table 2-2 and columns 1 and 3, Table 2-3, as all these results are similar (except in the citrulline/ornithine content), and were obtained from the same sample of wool. The whole wool and cortical cell resistant membranes analyses of O'Shea (1971) also originated from the same wool sample and could have been included in the average although they are different in some respects (below). Table 2-3 contains analyses for resistant membranes prepared from separated cortical and cuticle cells and also, for comparison, the epicuticle analysis of King & Bradbury (1968).

The low recoveries of anhydroamino acids with
resistant membrane preparations have not been accounted for, as the cause of these low recoveries is unknown. Leeder (1969) found 7% ash (maximum) and no carbohydrate in the performic/ammonia whole wool resistant membrane residue, and King & Bradbury (1968) analysed epicuticle and obtained 78% protein, 5% lipid, 4% ash with 13% unaccounted for in the analysis. No tests for lipid or carbohydrate were undertaken on the resistant membrane residues reported in this thesis due to the low weights of membranes recovered. No contamination of any of the residues was visible in the light microscope, and it was considered that the purification procedure employed (Chapter 2B) was sufficient to remove most light or heavy contaminants. Formic acid treatment during preparation should remove most of the lipid, although carbohydrates or lipids only comprise about 5-10% maximum and ash about 10% maximum of the resistant membranes (Leeder, 1969; King & Bradbury, 1968). It is noticeable that recoveries of anhydroamino acids are lower where more manipulation is required to separate components (Table 2-3), indicating that some unknown contamination is introduced during processing.

Further possible causes of low recoveries may be the degradation of certain amino acids by performic acid (Chapter 2D) and losses during hydrolysis. During a 24 hr. hydrolysis with 6N hydrochloric acid at 110°C, King (1967) found losses of 9% of serine, 6% threonine, 6% tyrosine and 3% cystine. In accordance with common practice in this laboratory, these losses were not corrected for.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>HCO$_3$H /NH$_4$OH (2)</th>
<th>HCO$_3$H /NH$_4$OH (1)</th>
<th>HCO$_3$H /8M urea (1)</th>
<th>CH$_3$CO$_3$H /NH$_4$OH (3)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO$_3$H</td>
<td>6.86</td>
<td>5.61</td>
<td>11.60</td>
<td>14.43</td>
<td>6.16</td>
</tr>
<tr>
<td>Asp</td>
<td>7.56</td>
<td>8.82</td>
<td>5.79</td>
<td>5.02</td>
<td>6.66</td>
</tr>
<tr>
<td>Thr</td>
<td>5.54</td>
<td>3.41</td>
<td>5.89</td>
<td>5.57</td>
<td>6.16</td>
</tr>
<tr>
<td>Glu</td>
<td>11.14</td>
<td>12.13</td>
<td>10.43</td>
<td>10.25</td>
<td>10.24</td>
</tr>
<tr>
<td>Pro</td>
<td>6.42</td>
<td>6.83</td>
<td>7.01</td>
<td>7.13</td>
<td>11.20</td>
</tr>
<tr>
<td>Gly</td>
<td>10.22</td>
<td>8.69</td>
<td>13.90</td>
<td>14.43</td>
<td>9.16</td>
</tr>
<tr>
<td>Ala</td>
<td>7.50</td>
<td>8.16</td>
<td>6.90</td>
<td>6.09</td>
<td>6.78</td>
</tr>
<tr>
<td>Val</td>
<td>6.58</td>
<td>7.29</td>
<td>4.66</td>
<td>5.16</td>
<td>6.18</td>
</tr>
<tr>
<td>Met</td>
<td>0.0</td>
<td>0.46</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.70</td>
<td>3.57</td>
<td>2.65</td>
<td>2.46</td>
<td>4.00</td>
</tr>
<tr>
<td>Leu</td>
<td>7.12</td>
<td>8.56</td>
<td>5.08</td>
<td>4.67</td>
<td>8.65</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.0</td>
<td>3.18</td>
<td>0.0</td>
<td>0.0</td>
<td>3.15</td>
</tr>
<tr>
<td>Phe</td>
<td>2.52</td>
<td>3.22</td>
<td>1.53</td>
<td>1.52</td>
<td>3.29</td>
</tr>
<tr>
<td>Lys</td>
<td>8.16</td>
<td>8.39</td>
<td>9.00</td>
<td>7.69</td>
<td>4.52</td>
</tr>
<tr>
<td>His</td>
<td>1.86</td>
<td>1.91</td>
<td>1.29</td>
<td>1.37</td>
<td>1.23</td>
</tr>
<tr>
<td>Arg</td>
<td>5.51</td>
<td>5.69</td>
<td>4.26</td>
<td>4.12</td>
<td>6.22</td>
</tr>
<tr>
<td>Cit</td>
<td>0.37</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orn</td>
<td>0.18</td>
<td>1.29</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cit + Orn</td>
<td>0.55</td>
<td>1.29</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>%</td>
<td>* 71.7</td>
<td>61.1</td>
<td>76.7</td>
<td>86.3</td>
<td>98.2</td>
</tr>
<tr>
<td>Yield †</td>
<td>1.60</td>
<td>1.4</td>
<td>1.50</td>
<td>1.40</td>
<td>12.0</td>
</tr>
</tbody>
</table>

(1) Leeder (1969), Bradbury et al. (1971)
(2) O'Shea (1971)
(3) Corfield et al. (1958)

* Recovery of anhydroamino acids
† % yield of resistant membranes from whole wool.
# Average of column 1, Table 2-2, and columns 1 and 3, Table 2-3, i.e. average resistant membrane analysis.
### TABLE 2-3.

**AMINO ACID ANALYSES OF RESISTANT MEMBRANES FROM HISTOLOGICAL COMPONENTS.**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Cortical Cell</th>
<th>Cortical Cell</th>
<th>Cuticle Cell</th>
<th>Epicuticle Cell</th>
<th>H.T. Cortical Cell</th>
<th>H.T. Cuticle Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CySO₃H</td>
<td>5.79</td>
<td>7.00</td>
<td>7.49</td>
<td>11.58</td>
<td>12.72</td>
<td>10.41</td>
</tr>
<tr>
<td>Asp</td>
<td>7.75</td>
<td>7.50</td>
<td>7.63</td>
<td>5.84</td>
<td>3.60</td>
<td>3.16</td>
</tr>
<tr>
<td>Thr</td>
<td>5.26</td>
<td>4.35</td>
<td>5.23</td>
<td>3.59</td>
<td>3.69</td>
<td>3.69</td>
</tr>
<tr>
<td>Ser</td>
<td>8.62</td>
<td>7.60</td>
<td>9.64</td>
<td>13.65</td>
<td>10.92</td>
<td>10.41</td>
</tr>
<tr>
<td>Glu</td>
<td>11.37</td>
<td>11.38</td>
<td>11.03</td>
<td>10.66</td>
<td>12.01</td>
<td>13.52</td>
</tr>
<tr>
<td>Pro</td>
<td>7.72</td>
<td>5.83</td>
<td>7.43</td>
<td>5.80</td>
<td>6.46</td>
<td>11.43</td>
</tr>
<tr>
<td>Gly</td>
<td>9.01</td>
<td>9.27</td>
<td>9.35</td>
<td>15.35</td>
<td>16.40</td>
<td>21.52</td>
</tr>
<tr>
<td>Ala</td>
<td>7.79</td>
<td>7.47</td>
<td>7.90</td>
<td>4.61</td>
<td>6.09</td>
<td>4.69</td>
</tr>
<tr>
<td>Val</td>
<td>7.18</td>
<td>7.33</td>
<td>7.02</td>
<td>5.72</td>
<td>5.55</td>
<td>5.29</td>
</tr>
<tr>
<td>³Cys</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.33</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Met</td>
<td>0.0</td>
<td>0.30</td>
<td>0.0</td>
<td>0.03</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ileu</td>
<td>4.20</td>
<td>4.49</td>
<td>3.60</td>
<td>2.52</td>
<td>2.22</td>
<td>1.54</td>
</tr>
<tr>
<td>Leu</td>
<td>8.29</td>
<td>8.47</td>
<td>7.94</td>
<td>5.46</td>
<td>4.03</td>
<td>3.68</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.26</td>
<td>0.33</td>
<td>0.36</td>
<td>2.07</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>Phe</td>
<td>3.06</td>
<td>2.85</td>
<td>2.40</td>
<td>1.85</td>
<td>1.28</td>
<td>1.65</td>
</tr>
<tr>
<td>Lys</td>
<td>7.63</td>
<td>7.50</td>
<td>7.19</td>
<td>4.83</td>
<td>10.00</td>
<td>8.20</td>
</tr>
<tr>
<td>His</td>
<td>0.94</td>
<td>1.71</td>
<td>1.12</td>
<td>1.03</td>
<td>1.65</td>
<td>1.99</td>
</tr>
<tr>
<td>Arg</td>
<td>5.27</td>
<td>6.63</td>
<td>4.23</td>
<td>4.27</td>
<td>3.57</td>
<td>2.34</td>
</tr>
<tr>
<td>Cit</td>
<td>0.0</td>
<td>0.0</td>
<td>0.45</td>
<td>0.16</td>
<td>0.08</td>
<td>0.98</td>
</tr>
<tr>
<td>Orn</td>
<td>0.0</td>
<td>0.0</td>
<td>0.99</td>
<td>0.0</td>
<td>0.05</td>
<td>0.83</td>
</tr>
<tr>
<td>Cit + Orn</td>
<td>0.0</td>
<td>0.0</td>
<td>1.44</td>
<td>0.16</td>
<td>0.13</td>
<td>1.81</td>
</tr>
<tr>
<td>%</td>
<td>30.2</td>
<td>33.9</td>
<td>57.9</td>
<td>77.6</td>
<td>61.6</td>
<td>42.5</td>
</tr>
<tr>
<td>Yield †</td>
<td>1.47</td>
<td>2.2</td>
<td>2.55</td>
<td>0.06</td>
<td>0.08</td>
<td>0.98</td>
</tr>
</tbody>
</table>

(1) O'Shea (1971)
(2) King & Bradbury (1968)

* Recovery of anhydroamino acids
† % yield of resistant membranes.
Peracetic acid/ammonia extraction of wool as used by Corfield et al. (1958) leaves 12% resistant membrane residue. Analysis of this residue (Table 2-2) indicates it is probably contaminated with keratin as its amino acid composition approaches that of whole wool and cortical cells (Bradbury et al., 1965).

The whole wool resistant membranes prepared by Leeder (1969) and Bradbury et al. (1971) are higher in cysteic acid and glycine, and lower in phenylalanine than those prepared here (Table 2-2), and correspond more closely to epicuticle (King & Bradbury, 1968) and the H.T. cortical and cuticle cell resistant membranes (Table 2-3). Epicuticle was prepared by chlorine water treatment of wool, and its high cysteic acid content may be due to the presence of some exocuticle 'a' layer which has a high sulphur content (Rogers, 1959a; Ley, 1971). However, the H.T. resistant membrane preparations are probably partly degraded due to their method of isolation and probably represent a very resistant fraction. As Leeder (1969) and Bradbury et al. (1971) used a higher temperature during isolation of resistant membranes, than was used here, it is possible that slight degradation occurring in their case, together with a different wool sample, has resulted in the higher cysteic acid content. Leeder (1969) showed that the temperature of the performic acid extraction step has a considerable effect on the yield of resistant membranes.

The cortical cell resistant membrane analysis agrees quite well with that of O'Shea (1971) (Table 2-3) but there are differences between the whole wool resistant
membrane analysis and O'Shea's analysis (Table 2-2). In both these cases the same wool was used, and better agreement was achieved than with the analyses of Leeder (1969) and Bradbury et al. (1971), particularly in regard to cysteic acid content. O'Shea's (1971) whole wool resistant membrane analysis shows low serine and threonine contents and the presence of methionine and tyrosine. As methionine and tyrosine are almost completely destroyed, and serine and threonine are partly destroyed by performic acid (Chapter 2D(ii)), his results for these particular amino acids should be viewed sceptically.

Tables 2-2 and 2-3 show that the lysine concentration is high for most resistant membrane preparations, and the total cystine content is considerably lower than that of whole wool, cortical or cuticle cells (cystine contents 10.48, 9.06 and 15.31 mole % respectively; Bradbury et al., 1965). These values indicate cystine is not the prime cause of the insolubility of resistant membranes. The glycine content is also high, but the significance of this is not obvious.

Of considerable significance is the presence of citrulline/ornithine (Chapter 2D), although it is easily overlooked on the Technicon amino acid analyser (King, 1967). Citrulline is partially converted to ornithine during hydrolysis (Rogers, 1962), so that the total of citrulline plus ornithine should remain constant for any one material. In both the low temperature and high temperature (H.T.) resistant membrane preparations, citrulline/ornithine occurs in much higher concentration in the cuticle membranes. However, the citrulline/ornithine
content of cuticle resistant membranes only contributes 7% to the whole cuticle citrulline/ornithine content of 0.48 mole % (Bradbury et al., 1966).

(iii) Electron Microscopy.

Figures 2-1 to 2-7 are electron micrographs of wool fibres treated as noted below.

Figure 2-1: Untreated wool fibre at the orthocortex/paracortex boundary. The characteristic arrangements of microfibrils/matrix and macrofibrils are visible in both cortices. Membrane complex structure, distinguishable as the $\beta$ regions (lightly stained) and $\delta$ region (more darkly stained) of Rogers (1959, 1959a) is visible in both cortex and cuticle. It appears that there is a slight difference in the width of the $\beta$ regions at the junction of cortex and cuticle as observed by Rogers (1959). The $\beta$ region seems slightly wider on the cuticle side of the membrane complex. The membrane complex is not always visible along its full length, but the reason for this is not known. Separation of the cuticle cells and between cuticle and cortex is visible, and appears to occur close to the line of the $\beta$ region.

Figure 2-2: Wool fibre after 5 min. immersion in formic acid at room temperature, followed by staining. In this Figure, the most obvious characteristic is the almost complete loss of normal membrane complex appearance in the cortex. Normal membrane complex appearance in the cuticle is retained. This behaviour extends most noticeably to the membrane complex separating cortex from cuticle at 'A'. At this point, the $\beta$ region is almost completely lost on the cortex side, but retained normally on the
FIGURE 2-1

CROSS-SECTION OF AN UNTREATED WOOL FIBRE
(CUTICLE, ORTHOCORTEX AND PARACORTEX).
FIGURE 2-2

CROSS-SECTION OF A WOOL FIBRE AFTER 5 MINUTES IMMERSION IN FORMIC ACID.
(CUTICLE AND PARACORTEX).
cuticle side. Careful examination of the membrane complex at 'B' in the cortex shows some isolated areas where the β region is faintly visible.

Figure 2-3:- Wool fibre after 5 min. immersion in formic acid at room temperature, followed by staining. This clearly demonstrates the 'single line' membrane along the junction of cortex and cuticle, i.e. at 'A'. The microfibril/matrix structure is still visible and although it is not as clear in formic acid treated fibres generally, the overall pattern still appears to be maintained. This possibly reflects the disorientation of microfibrils which is known to occur in formic acid as shown by X-ray diffraction studies (Bendit, 1966; Chapman & Feughelman, 1967). Also in this Figure can be seen the exo-, endo-, and 'a' layers of the cuticle, the separation of cuticle cells near the β layer (at 'B') and the intact appearance of the cuticle cell membrane complex.

Figure 2-4:- Wool fibre after 48 hr. immersion in formic acid at room temperature, followed by staining. In this case there is little difference from that of the 5 min immersion treatment. The only difference appears to be in degree of change, which shows more obvious modification of the membrane complex structure in the cortex. Microfibril/matrix structure is still visible and the 'normal' cuticle membrane complex is still clearly seen.

Figure 2-5:- Wool fibre after 48 hr. immersion in formic acid at room temperature, followed by staining. This Figure shows the single line appearance of the membrane complex along the cortex/cuticle boundary at 'A'.

FIGURE 2-3

CUTICLE REGION OF A WOOL FIBRE AFTER 5 MINUTES IMMERSION IN FORMIC ACID. (ORTHOCORTEX).
FIGURE 2-4

WOOL FIBRE AFTER 48 HOURS IMMERSION IN FORMIC ACID.
(CUTICLE, ORTHOCORTEX AND PARACORTEX).
FIGURE 2-5

WOOL FIBRE AFTER 48 HOURS IMMERSION IN FORMIC ACID.
(CUTICLE AND ORTHOCORTEX).
The orthocortex has not lost microfibrillar structure as is shown here, but unmodified fibres show it more clearly as mentioned above. Exo-, endo-, and 'a' layers of the cuticle are still visible showing little change has occurred there.

Figures 2-6 and 2-7: Wool fibre after 5 min. immersion in formic acid at room temperature followed by a 36 hr. wash in water, then stained.

There is little or no difference between these Figures and Figures 2-2 and 2-3 which did not have the long wash. Splitting between cuticle cells is obvious in Figure 2-6, and note particularly that the δ region has clearly remained in contact with the other layer at 'A'. It is possible that the δ layers of the cortex membrane complex are slightly more visible in isolated positions after the longer washing treatment ('B' in Figure 2-7).

Figure 2-8: Reduced and alkylated fibre. Note that although the internal structure of cortical cells is totally lacking in this example, the δ regions of the membrane complex are still visible at 'A'. Again the membrane complex is more obvious in the cuticle.

Figures 2-11 and 2-12 in Chapter 2E display similar behaviour to the above examples. The fibres in Figures 2-11 and 2-12 have been treated in formic acid at 98°C for 20 min., followed by a 16 hr. immersion in formic acid at room temperature. In Figure 2-11 the normal cuticle membrane complex is visible at 'A'; at 'B' the single line appearance of the membrane complex between cortex and cuticle can be seen, and at 'C' in both Figures 2-11 and 2-12, the δ region of the membrane complex is just visible.
FIGURE 2-6

WOOL FIBRE AFTER 5 MINUTES IMMERSION IN FORMIC ACID, FOLLOWED BY 36 HOURS WASH. (CUTICLE AND PARACORTEX).
FIGURE 2-7

WOOL FIBRE AFTER 5 MINUTES IMMERSION IN FORMIC ACID, FOLLOWED BY 36 HOURS WASH. (CUTICLE, ORTHOCORTEX AND PARACORTEX).
FIGURE 2-8

COMPLETELY REDUCED AND ALKYLATED WOOL FIBRE.
(CUTICLE AND CORTEX).
2D. DISCUSSION.

(i) The Yield of Membranes.

Since 1950, the most commonly employed method of separating membranes has been the peracetic acid oxidation/ammonium hydroxide dissolution procedure developed by Alexander & Earland (1950). Mercer (1953, 1965), Birbeck & Mercer (1957) and Golden (1954) used the peracetic/ammonia method to prepare cell membranes of whole wool, and observed residues composed of nuclear remnants and cell membranes, together with 'fibrils' of unidentified origin. Birbeck & Mercer (1957) made the additional observation that the 'a' layer and endocuticle also remained. Rogers (1959) clearly showed that peracetic acid/ammonia treatment leaves nuclear remnants, cell membranes, endocuticle and epicuticle of the fibres. From Birbeck & Mercer's (1957) study of differentiation of cuticle cells in the hair follicle (also results in Chapter 3 of this thesis, and Ley, 1971), there seems no doubt that endocuticle represents the nuclear remnant of the cuticle, and would also be expected to be insoluble in peracetic/ammonia.

Mercer (1953) describes these nuclear remnants as "somewhat irregular, elongated forms centrally placed within the cell". He also describes the insoluble fibrils as "particulate or short lengths distributed throughout the volume of the cortical cells. In hair these are found uniformly in all cortical cells; in wool fibres, however, their distribution is asymmetrical - they occur in one half of each cross-section". From this, it appears that
Mercer had observed not nuclear remnants and 'fibrils', but two types of nuclear remnants, one from each half of the cortex. Therefore, any treatment which removes nuclear remnants from the membrane preparation should also remove Mercer's 'fibrils' and endocuticle. This has been observed by Golden et al. (1955) who found that if the residue from peracetic acid oxidized wool is agitated in ammonium hydroxide, then the cortical cell nuclei, 'fibrils' and endocuticle are removed, as the residue was composed of cortical cell membranes and epicuticle only.

The only other histological component which cannot be removed by agitation during the peracetic (or performic) acid/ammonia treatment, is medulla, due to its known insolubility in these reagents (Mercer, 1965).

Yields of membranes observed by previous workers are all considerably higher than those obtained here. Leeder (1969) divides resistant membrane preparation methods into two types - specific and non-specific chemical attack. Of the specific methods, the lowest yield is that of the peracetic/ammonia method of Alexander & Earland (1950) at 7 - 10% membranes. Of the non-specific chemical degradation methods, Leeder considered most of these were only a transient stage in the complete dissolution of the fibre. The one exception being the sodium sulphide method of Schuringa et al. (1952, 1952a) which gave a stable residue (0.7%). King & Bradbury (1968) treated separated cuticle with sodium sulphide and obtained a 13% residue (i.e. 1.3% on the weight of wool), indicating the sodium sulphide residue from whole wool originates predominantly from the cuticle.
Yields of epicuticle are more reliable; King & Bradbury (1968) obtained 0.03% epicuticle with a theoretical 0.06 ± 0.02% yield, calculated from an observed 32 angstrom thickness. O'Shea (1971) has obtained yields of 0.115 - 0.177% epicuticle by slightly more vigorous agitation. The true yield of epicuticle is probably in the 0.1 - 0.2% range.

With the performic acid/ammonia treatment used to prepare resistant membranes, it is necessary to agitate the ammonium hydroxide solution gently. Golden et al. (1955) (above) showed this removes the nuclear remnants, and O'Shea (1971) and I obtained comparable and elevated yields when the ammonium hydroxide solution was not agitated (Table 2-1). The reason for the elevated yields is obvious from Leeder's (1969) results which show the yield does not become constant until after 3 days immersion, with agitation, in ammonium hydroxide (Bradbury et al., 1971).

The most gratifying aspect of the resistant membrane yields quoted in Table 2-1 is the close agreement between the contribution of cortical cells and cuticle cells to the whole wool membranes yield. This agreement indicates that a reproducible and stable resistant membrane fraction has been isolated. It is also noticeable that in both the normal and high temperature (H.T.) resistant membrane preparations, the yield of resistant membranes from the cuticle is considerably higher than from the cortical cells. The electron micrographs (Figures 2-2 to 2-7 and 2-11 and 2-12) all indicate that the β region of the cuticle cell membrane complex is almost unaffected by formic acid treatment, whereas the appearance of the cortical cell
membrane complex is considerably modified, although the $\beta$ region can still be seen. For this reason, a higher yield of cuticle cell resistant membranes would be predicted. The H.T. method of preparation however, apparently alters the resistant membranes as shown by the lower yields obtained with both cuticle and cortical cell resistant membranes.

Although the yield of membranes from the H.T. cuticle preparation indicates some modification has occurred, it is probably not excessive, as Leeder (1969) used the same high temperature method to prepare whole cuticle cells. By treatment of these cells with chlorine water, Leeder & Bradbury (1968, 1971a) were able to demonstrate the Allwörden reaction on isolated cuticle cells.

(ii) The Effect of Performic Acid/Ammonium Hydroxide Treatment.

The use of performic acid oxidation stems from the work of Toennies & Homiller (1942) who showed that performic acid at $0^\circ$C reacts specifically with disulphide bonds and is virtually complete within 4 hr. at $0^\circ$C. The long immersion time in performic acid used in this thesis is to ensure maximum solubilisation of keratin proteins from the wool.

Apart from denaturation, there are two possible side effects on proteins from treatment with performic acid - (a) peptide bond splitting, and (b) modification of amino acid residues.

Peptide bond splitting has been investigated both on wool, and various soluble proteins. The initial use appears to have been that of Sanger (1949) using the
method developed by Toennies & Homiller (1942) for separation of the two chains of insulin. No splitting of peptide bonds was observed. Similarly no splitting of peptide bonds was observed with performic acid treatment of lysozyme (Jollès-Thaureaux et al., 1958; Thompson, 1958), ribonuclease (Hirs, 1956), papain (Kimmel et al., 1955) and chymotrypsin (Meadom, 1956).

With wool, Thompson & O'Donnell (1959) have shown that peracetic acid oxidation is incomplete in comparison with performic acid oxidation. They have also shown there is little increase in the dialysable protein content from wool oxidized with performic acid in comparison with peracetic acid oxidation, and treatment with performic acid is not complicated by the presence of free hydrogen peroxide which is known to cleave peptide bonds (O'Donnell & Woods, 1956).

With regard to destruction of amino acid residues, most of the cases mentioned above involved oxidation at 0°C, (except Sanger (1949) who used 15 min. at room temperature) and under these conditions relatively little oxidation of amino acid residues occurs, except for tryptophan, tyrosine and methionine (Toennies & Homiller, 1942; Blackburn & Lowther, 1951). Even with tyrosine and methionine only a small amount of oxidation occurs under these conditions (Thompson & O'Donnell, 1959). At more elevated temperatures, side reactions increase. With peracetic acid (1.6% for 24 hr. at 25°C) Corfield et al. (1958) found losses of tyrosine, phenylalanine and histidine occur. Simmonds (1955) found with performic acid at room temperature, significant losses of serine,
threonine, and histidine occur, with almost total loss of tyrosine and phenylalanine. Cystine is quantitatively converted to cysteic acid in these cases (Toennies & Homiller, 1942; Hirs, 1967). Hirs (1967) claims that oxidation of amino acids with performic acid is most rapid with cystine, then with tyrosine (and presumably methionine) and then with the hydroxy amino acids (serine and threonine).

Apart from dissolving proteins, 1 M ammonium hydroxide probably has little effect on the membrane proteins. It is known however, (Elöd & Zahn, 1943) that concentrated ammonium hydroxide can dissolve wool completely (in 2 years at room temperature).

(iii) Electron Microscopic Observations.

All the electron micrographs of formic acid treated fibres exhibit the same effect - considerable modification of the cortical cell membrane complex with little change of the cuticle cell membrane complex. This effect is particularly noticeable along the boundary between cuticle and cortex, where only half of the β region of the membrane complex is modified. Similar modification of the cortex cell membrane complex is observed on treatment of fibres with dichloroacetic acid (Bradbury et al., 1965a).

It was thought the presence of formic acid in the fibre may affect the staining behaviour of osmium in the region of the cell membrane complex. A long (36 hr.) wash in water was used to remove as much formic acid as possible, but little change in appearance of the cell membrane complex was noted (Figure 2-6 and 2-7 compared with Figures 2-2 and 2-3).

Formic acid treatment of wool fibres does not dissolve
the resistant membranes, as they endure performic acid extraction, also the membrane complex \( \beta \) regions are not removed but are concealed by a change in staining behaviour with osmium. The \( \beta \) regions can still be observed in isolated positions in the cortex, and are quite noticeable in Figures 2-11 and 2-12. This change in staining behaviour is connected with extraction of intercellular cement (Bradbury et al., 1965a; Bradbury & King, 1967), but the precise cause of the change is unknown. This implies that the resistant membranes isolated by performic/ammonia treatment of wool, correspond to the \( \beta \) region of the membrane complex.

Formic acid extracts 1.5% of material from wool, (Bradbury & King, 1967; Bradbury et al., 1971) of which 0.8% is lipid and 0.7% is protein with a high concentration of glycine and aromatic amino acids (Bradbury et al., 1965a). Similar proteins have been extracted with 50% formic acid (Biela, 1967; Zahn & Biela, 1968). The extraction of material from wool by formic acid occurs in three stages; the first very rapid step occurring within 5 min., the material extracted being only 10% protein (Bradbury et al., 1965a; Bradbury & King, 1967). This initial stage obviously corresponds to the modification of the cortex membrane complex as observed with the electron microscope. The origin of the material extracted during the second and third stages is not obvious from electron microscopic examination.

It is therefore most likely that formic acid extracts intercellular cement (i.e. the \( \delta \) region of Rogers, 1959, 1959a) and not the resistant membranes or \( \beta \) regions.
Rogers (1964) dissolved intercellular cement from wool roots with 8 M urea, and De Deurwaerder et al. (1964) extracted an intercellular protein high in glycine, tyrosine and phenylalanine from wool with tris(diethylaminomethyl)phosphine in formamide. In this latter case, although they claim the protein originates from the cuticle membrane complex, a normal membrane complex is visible in the cuticle cells illustrated, and considerable alteration of the cortex membrane complex is clearly visible in their Figure. I suspect the separation of cuticle cells which they observed is largely due to mechanical reasons and not chemical extraction of intercellular cement from the cuticle (e.g. thin sectioning for electron microscopy frequently causes separation of cuticle from the cortex, see untreated fibres in Figures 3 and 4 of Rogers, (1959), and Figure 2-1 in this Chapter).

As noted in Chapter 2C(ii), the resistant membrane preparations contain little cysteic acid (i.e. low in cystine). If, as is generally believed (Rogers, 1959a), osmium tetroxide reacts primarily with thiols, then reduction and alkylation with acrylonitrile (or other coupling agent) should not change the appearance of the \( \beta \) regions of the membrane complex. Figure 2-8 shows the \( \beta \) regions to be still intact and apparently unaltered. These regions are more obvious in the cuticle, again indicating a difference exists between resistant membranes of the cuticle and cortex. That reduction and alkylation affects the staining behaviour of osmium is obvious from the appearance of the cortex in Figure 2-8 in comparison
with any of Figures 2-1 to 2-7.

Rogers (1959a) believes the resistant cell membranes are a darkly stained region inside the β region of the cell membrane complex, i.e. closer to the nucleus of each cell. To be darkly stained with osmium, the membranes would require a high cystine content, but the actual cystine content of membranes is only slightly greater than SCMKA - the low sulphur protein from wool (Crewther et al., 1965). Therefore the resistant cell membrane which is isolated by performic acid/ammonia treatment is probably the β region of Rogers (1959a), and the darkly stained region which he thought represents the membrane, being matrix (high sulphur) protein. Observations that the membrane complex appears to split along this β layer (Rogers, 1959, 1959a), and some of Figures 2-1 to 2-7), led Rogers to suggest it is a mechanically weak layer. However, if the splitting occurs between the β and δ layers, the lightly stained β layer is alongside an edge and becomes very difficult to see in the electron microscope.

The difference in the two halves of the cell membrane complex along the cortex/cuticle boundary after treatment with formic acid is a clear indication that the two β layers are distinct for each type of cell. If the β region of the cell membrane complex were a bimolecular lipid layer as suggested by Swift & Holmes (1965), then it would be necessary to propose two types of lipid layer to account for the behaviour observed at the cortex/cuticle boundary. Lipids are frequently unsaturated compounds (e.g. bacterial cell wall lipids, Fieser &
Fieser, 1961) and as such are well known to react with osmium tetroxide, a process used for preparation of cis diols (Gunstone, 1960). The osmium ester is reasonably stable, and would be expected to show as a stained region in the electron microscope. Therefore the β region is probably not an unsaturated lipid layer.

To summarise, both yields of resistant membranes from cortical and cuticle cells, and electron microscopic observations, indicate that the cuticle and cortical cell resistant membranes are different. Amino acid analyses also indicate these two resistant membranes have a different content of citrulline/ornithine. The electron microscopic observations also indicate there is a change in the staining behaviour of osmium near the cortex cell membrane complex after formic acid treatment.

(iv) The Inertness of Resistant Membranes.

Mercer et al. (1955) claimed that treatment of wool fibres with trypsin produced not cortical and cuticle cells but the keratinised contents of these cells. They believed the membranes, except for the very resistant epicuticle, had been digested. Golden (1954) also makes this claim, but in this case, on membranes which had been oxidized with peracetic acid (see also Golden et al., 1955). Leeder (1969) has shown that these two cases are not identical. Treatment of resistant membranes isolated by performic acid/ammonia, with trypsin, digests the membrane in a few minutes. (Trypsin is a specific enzyme, only cleaving peptide bonds adjacent to lysine and arginine.) A 2 week digestion of wool with the broad specificity enzyme pronase, which digested 12% of the fibre, gave an
apparently normal membrane residue on treatment with performic acid/ammonia (Leeder, 1969).

At least two types of cross-link in the membrane must be proposed to account for the above behaviour. The two most probable cross-links are (1) the disulphide bond of cystine, and (2) ε-(γ-glutamyl)lysine. Evidence for the presence of these two cross-links is presented below.

Although the observed cystine content (as cysteic acid) of membranes is low in comparison with whole wool, they are insoluble to enzymic digestion of unmodified wool (Leeder, 1969, above; see also Chapter 3). On cleavage of the disulphide bond by oxidation to cysteic acid, the membranes are still insoluble in formic acid, which is a good protein solvent (Thompson & O'Donnell, 1959), but have now become easily digestible by trypsin (Leeder, 1969; Golden, 1954). The disulphide bond provides resistance to enzyme digestion, but some other constraint prevents the membrane going into solution in formic acid.

The insolubility of membranes after their disulphide bonds have been cleaved, could also be due to an extremely high molecular weight protein which is not soluble in formic acid. This proposition becomes less likely however, when viewed in the light of recent work by Harding & Rogers (1971, 1971a) (below).

Firstly, it is important to note that medulla has a chemical resistance similar to that of membranes (Mercer, 1965) except that it is readily digestible by enzymes (Stoves, 1945; Rogers, 1964a). Medulla however, has a low content of cystine (average 2.88 mole %) compared with other fibre components, (Bradbury & O'Shea, 1969; Rogers,
1962) and even with resistant membranes (Tables 2-2 and 2-3). Using the digestibility of medullary cells by enzymes to extract medulla protein, Harding & Rogers (1971a) have demonstrated that the insolubility of medulla in other media is probably due to the presence of $\epsilon$-(\(\gamma\)-glutamyl) lysine in the citrulline containing protein fractions of medulla. Harding & Rogers (1971) have shown that the $\epsilon$-(\(\gamma\)-glutamyl)lysine occurs primarily in the citrulline containing peptides isolated from medulla, and have also discovered a transamidase present in hair and wool follicles, which is capable of forming the $\epsilon$-(\(\gamma\)-glutamyl) lysine cross-link.

Presence of a high concentration of lysine together with citrulline/ornithine in the cuticle cell resistant membranes is strong circumstantial evidence for $\epsilon$-(\(\gamma\)-glutamyl)lysine. Cortical cell resistant membranes contain slightly less cystine and little citrulline in comparison with the cuticle membranes, and this may account for their apparently lower chemical resistance. Milligan et al. (1971) have found 2.9 $\mu$M/g $\epsilon$-(\(\gamma\)-glutamyl)lysine in Merino wool and Milligan (1971a) has detected 8 $\mu$M/g in epicuticle prepared by chlorine water treatment of wool. The chlorine water treatment of wool however, may break some of the $\epsilon$-(\(\gamma\)-glutamyl)lysine cross-links as their concentration in a keratose decreased from 38 to 22 $\mu$M/g after treatment with chlorine water (Milligan, 1971a).

Other types of cross-link are possible in resistant membranes, e.g. there are a range of cross-links possible in collagen which are discussed by Harding (1965) and also those involving tyrosine as found in insect cuticle (Prior,
1962). These are all considered less likely than ε-(γ-glutamyl)lysine as they do not yet appear to have been observed in keratin.

As a final point, the report by Swift & Holmes (1965), that epicuticle is soluble in boiling ethanol, does not fit with the established inertness of this membrane as noted previously. Swift & Holmes claimed that ethanol extraction removed epicuticle from human hair, as shown by a 'light' layer observed in the electron microscope, between the cuticle surface and a chromium layer evaporated onto this surface. Examination of the rest of their Figure shows it is clearly underfocused and the 'light' layer also exists on the 'outside' edge of the chromium layer. Swift & Holmes did not show an ethanol extracted fibre for comparison. Further evidence against Swift & Holmes' claim is that Leeder (1969) (Leeder & Bradbury, 1971) was able to raise Allworden sacs on human hair which had been extracted with ethanol, and King (1967) found that epicuticle from wool is insoluble on refluxing for 16 hr. in ethanol.

2E. PREPARATION OF DESCALED FIBRES.

A method of cleanly stripping cuticle from wool was developed while attempting to obtain large yields of cortical and cuticle cells for preparing membranes.

Basically the method involves heating wool under reflux with formic acid, a treatment which removes most of the cuticle as whole cells (Leeder, 1969). The partially descaled fibres are then agitated gently with a laboratory
shaker to remove the last traces of cuticle.

(i) Effect of Variation of the Reflux Time.

Samples of wool (cleaned as in Chapter 1B) were placed in redistilled formic acid (98%) and heated to reflux (formic acid:wool ratio, 250:1). The samples were maintained at reflux for various times, then removed and examined under the light microscope. Observations are listed in Table 2-4. (Percentage removal of cuticle is a visual estimate only).

<table>
<thead>
<tr>
<th>Time of Reflux</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min.</td>
<td>A few scales lifted only.</td>
</tr>
<tr>
<td>5 min.</td>
<td>Most scales lifted - cuticle about 10% removed.</td>
</tr>
<tr>
<td>10 min.</td>
<td>All scales lifted - about 50% of cuticle removed.</td>
</tr>
<tr>
<td>20 min.</td>
<td>About 80% of cuticle removed.</td>
</tr>
<tr>
<td>40 min.</td>
<td>&gt;90% cuticle removed - some cortical cells present.</td>
</tr>
</tbody>
</table>

(ii) Effect of Agitation after Reflux in Formic Acid.

The fibres, after the reflux treatment, were then placed in fresh formic acid and shaken on a laboratory shaker overnight (16 hr.). The results are presented in Table 2-5. Estimates of amount of cuticle removed are again only visual.
**TABLE 2-5.**

**EFFECT OF AGITATION IN FORMIC ACID AFTER FORMIC ACID REFLUX FOR VARIOUS TIMES.**

<table>
<thead>
<tr>
<th>Time of Reflux</th>
<th>Appearance of Fibres after 16 hr. Shaking.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min.</td>
<td>Only 50% of cuticle removed.</td>
</tr>
<tr>
<td>5 min.</td>
<td>About 80% cuticle removed.</td>
</tr>
<tr>
<td>10 min.</td>
<td>About 90% cuticle removed - a few cortical cells present.</td>
</tr>
<tr>
<td>20 min.</td>
<td>About 99% cuticle removed - a few cortical cells.</td>
</tr>
<tr>
<td>40 min.</td>
<td>A large number of cortical cells present.</td>
</tr>
</tbody>
</table>

After this treatment the fibres were washed free of formic acid on a 40 mesh stainless steel sieve with deionised water, and immersed in deionised water for 24 hr. The fibres were then air dried. Leeder (1969) has shown that wool treated in formic acid for 1 hr. at 100\(^\circ\)C loses 11% by weight; this includes cuticle dispersed during the treatment.

Samples of fibres having had 5, 10 and 20 min. reflux followed by 16 hr. shaking in formic acid were supplied to Dr. I.C. Watt (Division of Textile Physics, C.S.I.R.O.), for evaluation. Also supplied to Dr. Watt as a control sample, were some fibres which had been maintained in formic acid at 98\(^\circ\)C (formic acid B.Pt. 100.7\(^\circ\)C) for 20 min. and then stood in formic acid (unagitated) for 16 hr. (Control sample in Table 2-6). These fibres have had effectively the same treatment as the descaled fibres but retain almost all of their cuticle.
(iii) Examination of the Electron Micrographs.

Figures 2-9 and 2-10 are scanning electron micrographs of the 20/16 sample of descaled fibres as supplied to Dr. Watt. Figures 2-11 and 2-12 are transmission electron micrographs of the 'control' sample, to observe effects of the formic acid treatment on the cuticle and cortex.

Figure 2-9a is a low magnification scanning electron micrograph of a descaled wool fibre showing one of the very few traces of cuticle left on the fibre. Note the impressions of scale edges left on the cortex of the fibre (Fraser & Rogers, 1955).

Figure 2-9b is a higher magnification view of the scale patch in Figure 2-9a. In Figure 2-9b a 'flap' of cuticle can be seen lifting. Note particularly the edges of remaining scales; there are fingerlike processes directed towards the fibre tip. Also, one of the scales on the side of the fibre shows longitudinal ridges parallel to the fibre axis.

Figure 2-10a shows a frayed end of a fibre. Note the cortical cells separating and scale edge impressions.

Figure 2-10b is a higher magnification view of a region where cortical cells have been removed. The scale edge impressions are clearly visible.

Figure 2-11 is a transmission electron micrograph of the 'control' sample close to the fibre edge. Different behaviour of the cell membrane complex following formic acid treatment, is visible in cortex and cuticle as observed in Figures 2-2 to 2-7. Note that in this Figure and in Figure 2-12, the B layer of the membrane complex is
FIGURE 2-9a - SCANNING ELECTRON MICROGRAPH OF DESCALED WOOL FIBRE.

FIGURE 2-9b - HIGHER MAGNIFICATION IMAGE OF CUTICLE ON THE FIBRE.
more obvious in the cortex than was observed in Figures 2-2 to 2-7. This is possibly connected with the greater extraction of intercellular cement which probably occurs with the treatment these fibres have received.

Figure 2-12. The orthocortex/paracortex boundary is shown. Microfibril/matrix structure is still visible in the paracortex of both Figures (2-11 and 2-12) but the orthocortex may have lost some fine structure.

'Fibrils' have been observed in cuticle separated after pancreatin treatment by Zahn (1949, 1951) and between the amorphous keratin of exocuticle and endocuticle (Happey & Johnson, 1962, 1965; Lindberg et al., 1949; Lagermalm, 1954; Fraser & Rogers, 1955). The existence of these 'fibrils' was denied by Mercer (1953), and Haly et al. (1970) showed that in scanning electron micrographs of cut and torn fibres, no fibrils are seen and there is no evidence of a fibrous nature of cuticle. A tear in the cuticle travels as readily axially as transversely.

Mercer (1953) did however notice furrows in the exocuticle parallel to the fibre axis which apparently cause ripples in surface replicas of the fibre (Fraser & Rogers, 1955). Birbeck & Mercer (1957) noticed that in hair follicles, keratin in the cuticle forms initially as drops which coalesce into 'bars' and then form a solid mass of exocuticle. Rogers (1959a) observed some aggregation of amorphous protein in the exocuticle which could be the 'bars' of Birbeck & Mercer. It is believed that the longitudinal ridges in the cuticle, parallel to the fibre axis, and observed in Figure 2-9b, correspond with the fibrils, ridges or bars noted by previous workers.
FIGURE 2-11

"DESCALED FIBRE" - CONTROL SAMPLE
SHOWING CUTICLE AND PARACORTEX.
FIGURE 2-12

DESCALED FIBRE SHOWING ORTHOCORTEX-PARACORTEX BOUNDARY.
Rogers (1959, 1959a) observed 'interdigitating finger-like processes' in cross-sections of cuticle which he believed were partly responsible for holding the cuticle cells together. These finger-like processes which are occasionally visible in cross-sections of Merino wool, appear as small circles of material, surrounded by a circular cell membrane complex, and embedded within a cuticle cell (as shown in Figure 2-4). Piper (1966) has observed that cuticle cells of mohair are bound by similar interlocking structures. The finger-like processes noted on scale edges in Figure 2-9b are believed to correspond to the 'interdigitating finger-like processes' of Rogers (1959, 1959a) and Piper (1966).

Apart from changes in the cell membrane complex noted previously (Chapter 2C and 2D) following formic acid treatment, from observation of cross-sections little further damage appears to have occurred to the fibres due to the descaling method used.

The only other attempt to completely descale fibres appears to be that of Blackburn & Lowther (1951) who used a gaseous chlorination treatment followed by a papain digestion and then milling to prepare almost completely descaled fibres. These fibres were then treated with peracetic acid/ammonia to isolate cortical cell membranes. Considerable degradation probably occurred in this case. Other manual methods of descaling fibres have resulted in incomplete removal of the cuticle (e.g. Bradbury, 1959, 1960).


The work described in this sub-section was performed
by Dr. I. C. Watt, who measured the equilibrium water content of some samples of descaled wool. Table 2-6 lists the results obtained by Dr. Watt. The equilibrium water content is expressed as a percentage on the dry weight of wool.

The samples in the Table are arranged such that the samples to the right hand side have less residual cuticle or have had a slightly more severe formic acid treatment. The code 10/16 for instance refers to the formic acid treatment i.e. in this case a 10 min. reflux and a 16 hr. shake in formic acid. The control sample has received the '20/16' treatment but has not been agitated to dislodge the cuticle.

### Table 2-6.

**EQUILIBRIUM WATER CONTENT.**

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Untreated Wool</th>
<th>Control Sample 5/16</th>
<th>Descaled Wool 10/16</th>
<th>Descaled Wool 20/16</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 %</td>
<td>2.6</td>
<td>2.5</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>10 %</td>
<td>3.8</td>
<td>3.1</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>20 %</td>
<td>5.9</td>
<td>5.2</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>35 %</td>
<td>8.5</td>
<td>7.4</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>50 %</td>
<td>11.3</td>
<td>9.5</td>
<td>9.2</td>
<td>9.0</td>
</tr>
<tr>
<td>65 %</td>
<td>14.4</td>
<td>12.1</td>
<td>11.7</td>
<td>11.4</td>
</tr>
<tr>
<td>80 %</td>
<td>18.6</td>
<td>16.6</td>
<td>16.0</td>
<td>15.5</td>
</tr>
<tr>
<td>90 %</td>
<td>23.8</td>
<td>22.7</td>
<td>21.9</td>
<td>21.7</td>
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<tr>
<td>95 %</td>
<td>28.5</td>
<td>29.2</td>
<td>30.1</td>
<td>29.8</td>
</tr>
<tr>
<td>Saturation</td>
<td>34.2</td>
<td>≈40</td>
<td>&gt;40</td>
<td>&gt;&gt;40</td>
</tr>
</tbody>
</table>

Formic acid treatment apparently brings about a reduction in water content in the low and intermediate
regions of the isotherm, and an increased water content at high humidities; the trend is emphasised by the descaling treatment. No differences in kinetics of water sorption were detected. The descaling treatment apparently removes hydrophilic material and also reduces constraints to swelling of the wool (Watt, 1971).

Bradbury et al. (1965, 1966) have shown that Merino wool cuticle is less polar than the cortex and therefore would be expected to absorb a smaller amount (weight for weight) of polar molecules than the cortex. This would have the opposite effect to that observed by Watt, as removal of cuticle without any other modification would increase the equilibrium water content.

Mild peptide bond hydrolysis (produced using 0.01 M HCl) causes increased water uptake above 80% relative humidity (R.H.) but no change below 80% (Watt & Leeder, 1965). In the same paper, Watt & Leeder describe how retention of acid in the fibre (following immersion in 0.1 M HCl at 20°C for 3 days) considerably decreases the equilibrium water content below 80% R.H. and increases it above 80% R.H.. The isotherm produced by this acid treatment is very similar to that obtained with the descaled fibres. On washing the acid treated fibres for 24 hr. Watt & Leeder (1965) found the equilibrium water content isotherm returns to that of the unmodified fibre below 80% R.H., but not above 80% R.H..<br>

One likely explanation for the observed changes in the equilibrium water content isotherm of descaled fibres, is incorporation of formic acid in the wool. Possibly the more severe conditions used to descale wool, in comparison
with those used by Watt & Leeder (1965) in their acid treatment, has resulted in formic acid remaining in the wool after the 24 hr. wash. Behaviour of the descaled fibres at greater than 80% R.H. can possibly be attributed to a slight amount of peptide bond hydrolysis. Another possibility is the increased swelling of wool which is possible on removal of the cuticle. Swelling of the cortex is probably restrained by the cuticle ('straight-jacket'), as immersion in formic acid causes longitudinal cracks in the cuticle (Bradbury & Chapman, 1963). Removal of this constraint possibly allows increased uptake of water.

Fulkarni et al., 1971, attributed the release of cells to an easily digestible intercellular cement which was low in sulphur content. The low sulphur content of this material was shown when Elsd & Bahn (1943) analysed the soluble material produced during separation of cells by pancreatin digestion.

The resistance of native keratins to enzymic digestion is characteristic, yet the simple expedient of cleaving the protein disulphide bonds makes the keratin susceptible to digestion by proteolytic enzymes (Geiger et al., 1941; Middlebrook & Phillips, 1941; Leamon, 1952; Springell, 1964). Similarly the soluble proteins produced from wool following reduction and alkylation of disulphide bonds are also susceptible to enzymic digestion (Springell, 1963; Grether & Barras, 1967). Reduction of wool, followed by oxidation of the fibre proteins to the disulphide form confers resistance to enzymic digestion almost equivalent to that of unreduced fibres (Geiger
CHAPTER 3.

SEPARATION OF NUCLEAR REMNANTS.

3A. INTRODUCTION

Native wool is resistant to attack by proteolytic enzymes (Geiger & Harris, 1942; Elöd & Zahn, 1943; Crewther, 1956), but sustained treatment, particularly with agitation of the fibres, results in separation of cuticle and cortical cells (e.g. Burgess, 1934; Elöd & Zahn, 1943; Mercer & Rees, 1946; Springell, 1963; Kulkarni et al., 1971). Burgess (1934) attributed the release of cells to an easily digestible intercellular cement which was low in sulphur content. The low sulphur content of this material was shown when Elöd & Zahn (1943) analysed the soluble material produced during separation of cells by pancreatin digestion.

The resistance of native keratins to enzymic digestion is characteristic, yet the simple expedient of cleaving the protein disulphide bonds makes the keratin susceptible to digestion by proteolytic enzymes (Geiger et al., 1941; Middlebrook & Phillips, 1941; Lennox, 1952; Springell, 1963). Similarly the soluble proteins produced from wool following reduction and alkylation of disulphide bonds, are also susceptible to enzymic digestion (Springell, 1963; Crewther & Harrap, 1967). Reduction of wool, followed by reoxidation of the fibre proteins to the disulphide form confers resistance to enzymic digestion almost equivalent to that of unreduced fibres (Geiger
et al., 1941; Greenwood & Speakman, 1965).

Mercer et al., (1955) noticed that tryptic digestion of wool also digests nuclear remnants. More recently, Anderson & Lipson (1970) showed scanning electron micrographs of cross-sections of pepsin digested fibres, from which the nuclear remnants had dissolved. Kulkarni et al. (1971) used trypsin digestion followed by ultrasonic disintegration to separate orthocortical and paracortical cells. Electron micrographs of cross-sections of these cells also showed the nuclear remnants and other intracellular debris had been digested.

Fraser & Rogers (1956) claimed that nuclear remnants can be seen in cortical cells prepared by trypsin digestion of wool, but this work was performed by gold shadowing in the light microscope, and the interpretation may be open to some doubt. Peracetic acid oxidation/ammonium hydroxide dissolution does not dissolve nuclear remnants or endocuticle without agitation (Chapter 2), but they are dissolved by trypsin digestion of the residue from this treatment (Mercer, 1953; Golden et al., 1955).

Digestion of endocuticle of hair by enzymes has been observed by Birbeck & Mercer (1957) and Mercer et al. (1955); and Ley (1971) has used enzymic digestion of separated cuticle from Merino wool to determine the amount of endocuticle in cuticle (35%). Both Mercer & Rees (1946) and Ramanathan et al. (1956) wrongly deduced that exocuticle is the enzyme digestible layer.

The susceptibility of medulla to enzymic digestion is well known (Elöd & Zahn, 1944a; Stoves, 1945; Rogers, 1964a). This ready digestibility was used recently by
Harding & Rogers (1971a) to extract medulla for examination for the presence of $\epsilon-(\gamma$-glutamyl)lysine.

Physical modification of the fibre also increases its susceptibility to proteolytic enzymes. Mercer (1953) supercontracted the orthocortex of wool by heating in water at $120^\circ$C. The orthocortex is soluble in trypsin, and the method was used by Golden et al. (1955) to separate the paracortex. Extension of wool by 40\% (i.e. conversion to $\beta$ keratin, Astbury & Woods, 1933) followed by digestion in trypsin for 2 days, only dissolves 4\% by weight, but swelling the fibres in solvents which don't irreversibly affect the $\alpha$ helical portions of the protein appears to make the fibre more susceptible to enzyme attack (Greenwood & Speakman, 1964).

Birbeck & Mercer (1957), during their electron microscopic examination of the growth of hair, concluded that the developing hair follicle cells are rich in ribonucleic acid. This opinion is shared by Hardy (1952) and Montagna (1956). Deoxyribonucleic acid and ribonucleic acid contained in a cell nucleus are generally associated with a basic protein (i.e. rich in lysine and arginine), but the presence of this basic protein may be concealed however, by an acidic protein also present (Howells, 1971).

Leeder (1969) used figures presented by Bradbury & King (1967) and by Lundgren & Ward (1963) to estimate that nuclear remnants comprise 6\% of the wool fibre. While this figure may be correct, enzymic digestion will remove more than this from the whole fibre. Endocuticle is most likely the nuclear remnant of cuticle (Birbeck & Mercer, 1957) and 35\% of cuticle is digestible (Ley, 1971). There
is also digestible material in the intercellular cement (Burgess, 1934; Elöd & Zahn, 1943), and intracellular debris is also digested (Kulkarni et al., 1971). Intercellular cement can be at least partially removed by immersion in formic acid for 5 min. (Chapter 2) or for 2 hr. (Bradbury & King, 1967). The total non-keratinous material in wool is approximately 10% (Mercer, 1961).

Many digestions of untreated wool have resulted in 10 - 20% weight loss of the fibre. Springell (1963) found Merino 64's lose 10 - 20% by weight on digestion with pronase. Leeder (1969) used pronase digestion and showed a maximum of 12% was digested after 6 weeks at 37°C. Only 8% was dissolved by pancreatin in 3 days, but a maximum of 14% was reached at 11 days (Elöd & Zahn, 1946). However, Lennox (1952) found only 2% wool was dissolved at 50°C in 18 hr. with 1% papain.

The amount of digestible material in wool appears to be affected by pretreatment of the fibre. Trypsin dissolves 10% of wool if it is previously swollen in pH 11 buffer for 20 hr. at 20°C, but 17% is dissolved if pH 11 caprate buffer is used (Crewther, 1956).

3B. MATERIALS AND METHODS.

(i) Materials.

Merino 64's wool prepared as described in Chapter 1B was used. To remove intercellular cement from the cell membrane complex, the wool was pretreated for 2 hr. in redistilled formic acid (Bradbury et al., 1965a). Following the formic acid pretreatment, the wool was washed for 24 hr.
in deionised water and then air dried. Cortical cells were prepared by ultrasonic disintegration of wool followed by differential screening of the products as described in Chapter 1B and by Bradbury & Chapman (1964).

Trypsin was 2 x recrystallised, Worthington Biochemical Corp. (Code TRL 71C).

(ii) Enzymic Digestions.

For all enzymic digestions, a pH stat was used to maintain the solution pH to within ±0.1 units of pH 8.5. The pH stat comprised a Pye Autotitrator Controller connected to a Beckman Research pH meter. Only one channel of the Autotitrator Controller was used, as the digestion always becomes acid at this pH, due to ionisation of the carboxyl groups produced by hydrolysis. The unit was charged with 1.0 M ammonium hydroxide to maintain the solution pH. Mechanical stirring of the reaction vessel was necessary to avoid 'overshooting' the pH. All digestions were performed at 37°C.

Optical density measurements were made at 280 nm with a Unicam SP500 UV Spectrometer using 0.5 cm. silica cells. It was found necessary to centrifuge a sample of the solution for 5 min. at 3,000 r.p.m. in a small bench centrifuge, prior to determining the optical density. Optical density at zero time was determined by summing optical densities of the wool suspension prior to trypsin addition, and of a trypsin solution at the same concentration as in the digestion. In all cases, water:wool:trypsin ratio was 10,000:100:1, and normally 1 g. wool was used.

The digestions were stopped by adding hydrochloric
acid to bring the pH to 4; the mixture was then centrifuged in a Beckman L2-50 preparative ultracentrifuge at 45,300 g. The supernatant after centrifugation was concentrated to about 25 ml. on a rotary evaporator, then freeze-dried and weighed.

(iii) Electron Microscopy.

Electron microscopy was performed as described in Chapter 2B(ii).

(iv) Amino Acid Analyses.

These were carried out according to the method given in Chapter 1B(v).

3C. RESULTS.

(i) The Quantity of Nuclear Remnants.

Yields of material extracted by trypsin digestion are given in Table 3-1, and the yields from wool and formic acid pretreated wool are plotted in Figure 3-1. The yields are corrected for the amount of trypsin added.

TABLE 3-1.

YIELD OF TRYPsin DIGESTIBLE MATERIAL.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Digestion Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical cells</td>
<td>36 hr.</td>
<td>15.1%</td>
</tr>
<tr>
<td></td>
<td>84 hr.</td>
<td>14.0%</td>
</tr>
<tr>
<td>Formic acid</td>
<td>100 min.</td>
<td>0.98%</td>
</tr>
<tr>
<td>pretreated wool</td>
<td>30 hr.</td>
<td>4.30%</td>
</tr>
<tr>
<td></td>
<td>84 hr.</td>
<td>6.87%</td>
</tr>
<tr>
<td>Untreated wool</td>
<td>100 min.</td>
<td>1.41%</td>
</tr>
<tr>
<td></td>
<td>21.5 hr.</td>
<td>1.97%</td>
</tr>
<tr>
<td></td>
<td>42.5 hr.</td>
<td>2.93%</td>
</tr>
</tbody>
</table>
FIGURE 3-1

YIELD OF ENZYME DIGESTIBLE MATERIAL FROM UNTREATED AND FORMIC ACID PRETREATED WOOL.

% Dissolved
0 10 20 30 40 50 60 70 80 90

Time of Digestion (hr.)

Formic Acid Treated

Untreated
Yields of extracted material were determined by weighing the total dried extract. This method is considerably more accurate than determining the small weight difference of whole cortical cells (or wool), particularly in view of the difficulty which is experienced in quantitatively collecting digested cortical cells. It also gives a direct correlation with optical density readings, which is necessary to ensure the extracted material obeys Beer's Law, so that the optical density can be used as a measure of extraction for the rate determinations. The only possible contaminant is a small amount of ammonium chloride which is probably removed during the freeze-drying step. As the digest contains proteins (trypsin, at least), polypeptides and probably amino acids, it is important to avoid methods such as chromatography, or dialysis, which may segregate or separate some of these products. For this reason, methods using buffers to maintain pH are unsatisfactory, as the extract is contaminated with buffer salts, and separation of the extract from salts can result in considerable losses of low molecular weight products of the digestion.

The results in Table 3-1 show that 14.5% of cortical cells is digestible by trypsin. This figure is the sum of nuclear remnants plus intracellular cement, and may include some keratin protein. Unfortunately, the total digestible material in wool could not be established by use of the pH stat, as the pH meter became unstable after about 3½ days, and pH could not be controlled beyond this time. It was intended to check the total amount of material extracted from untreated and formic acid pretreated wool.
This would establish if the formic acid treatment necessary to isolate cortical cells, was causing increased digestion of the keratin, possibly by partial denaturation of the proteins. Both Table 3-1 and Figure 3-1 indicate that the rate of digestion of wool is increased by formic acid pretreatment, but give no indication of the final amount extractable.

(ii) Rate of Trypsin Digestion.

Preparation of cortical cells involves a maximum of 2 hr. immersion in formic acid, therefore this is a suitable time to use for treatment of wool prior to enzyme digestion, for comparison with digestion of cortical cells. Bradbury & King (1967) have shown that a rapid removal of intercellular material also occurs within 2 hr. immersion in formic acid.

Using yields of digested material and optical density readings corrected for the trypsin concentration, it was found that the plot of optical density vs. concentration of soluble material was linear. (i.e. the dissolved material follows Beer's Law and its concentration can be determined from the optical density). For extracts from both formic acid pretreated and untreated wool, this relationship was found to be yield (%) = 50(optical density). The optical density readings varied slightly owing to the presence of a 'haze' which did not centrifuge down, thus the relationship is slightly less accurate than could be expected.

For first order kinetics, a plot of $2.303 \log\left(\frac{a}{a-x}\right)$ vs time where $a$=initial concentration and $x$=amount dissolved, is linear. This is inconvenient to use here, but
\[ \log\left(\frac{a}{a-x}\right) = -\log\left(\frac{a-x}{a}\right) = -\log U \] where \( U \) is the percent undissolved.

Figure 3-2 gives the optical density vs. time and \( \log U \) vs. time plots for untreated wool, and Figure 3-3 gives the same plots for formic acid treated wool.

During the first 100 min., with both formic acid treated and untreated wool, there is a rapid dissolution of material from the fibre. Following this initial rapid dissolution, the rate decreases and becomes first order, i.e. the rate depends only upon the concentration of digestible material within the fibre.

The rate plots in Figures 3-2 and 3-3 shows the rate of extraction of trypsin digestible material is increased by a 2 hr. pretreatment in formic acid. The rate constant \( k \) is \( 8.34 \times 10^{-6} \) min.\(^{-1} \) for untreated wool and \( 2.50 \times 10^{-5} \) min.\(^{-1} \) for formic acid treated wool, determined after the digestion rate becomes linear. The initial stage of dissolution is rapid and non-linear in both cases, but the apparently larger amount of material dissolved initially from the formic acid treated wool is not significant as the 'haze' (mentioned above) makes accurate readings difficult during the first 15 min.. Figure 3-1 shows that in fact more material is dissolved from the untreated wool during the first 100 min..

The exact source of material extracted during the first 100 min. is not indicated by electron micrographs of fibres treated for this time, as these show no particular site of attack, but rather a slight overall digestion of nuclear remnants, intercellular cement and endocuticle. Conversely, the material dissolved during
FIGURE 3-3
OPTICAL DENSITY vs. TIME AND RATE PLOTS
FOR TRYPsin DIGESTION OF FORMIC ACID
PRETREATED WOOL.

FIGURE 3-2
OPTICAL DENSITY vs. TIME AND RATE PLOTS
FOR TRYPsin DIGESTION OF UNTREATED WOOL.

The first order kinetic stage, mainly originates from
the nuclear remnants, ecdysonic and intercellular cement.

All expected reactions have been 
shown by Figures 3-6 and 3-7.

Table 3-2 gives analyses of trypsin digests of whole
wool, arranged to show trends in the analysis produced by
increasing digestion time. Apart from, however, these are
a material of substantially constant composition is
extracted, this allows an average analysis to be calculated.

The concentration of hystidine with increases in time, but this may not be
significant as there is a large error inherent in the
determination. Trypsin has a fairly high cystine
content whereas the digestion of hystidine,
therefore small errors in the trypsin-digest reaction,
will result in large errors in the hystidine

Similarly, Table 3-3 gives the analyses of digests
formic acid pretreated with the trypsin reaction,
aim to average the analyses. With both Tables
3-2 and 3-3, hystidine decreases in concentration with
increasing digestion time. This is believed to indicate
the digests are low in cystine content. In Tables 3-2
and 3-3 there is a variation in the arginine content, but
as arginine is the last amino acid eluted on the technique
the first order kinetics stage, mainly originates from nuclear remnants, endocuticle and intercellular cement, as shown by Figures 3-6 and 3-7.

(iii) Amino Acid Analyses.

All amino acid analyses of extracts have been corrected for the presence of trypsin by subtraction of an analysis calculated from the known amino acid composition of trypsin (Dayhoff & Eck, 1968).

Table 3-2 gives analyses of tryptic digests of whole wool arranged to show trends in the analysis produced by increasing digestion time. Apart from Hcystine, there are few trends in amino acid composition with time, indicating that a material of substantially constant composition is extracted; this allows an average analysis to be calculated. There is a trend of decreasing concentration of Hcystine with increased digestion time, but this may not be significant as there is a large error inherent in the Hcystine determination. Trypsin has a fairly high cystine content whereas the digests are fairly low in cystine, therefore small errors in the trypsin:digest ratio used, will result in fairly large errors in the Hcystine content of the digests.

Similarly, Table 3-3 gives the analyses of digests from formic acid pretreated wool, and for the same reason it is possible to average these analyses. With both Tables 3-2 and 3-3, Hcystine decreases in concentration with increasing digestion time and it is believed this indicates the digests are low in cystine content. In Tables 3-2 and 3-3 there is a variation in the arginine content, but as arginine is the last amino acid eluted on the Technicon
TABLE 3-2.

AMINO ACID ANALYSES OF TRYPTIC DIGESTS OF WHOLE WOOL.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>100 min.</th>
<th>21.5 hr.</th>
<th>42.5 hr.</th>
<th>44.5 hr.</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO₃H</td>
<td>0.64</td>
<td>0.23</td>
<td>0.28</td>
<td>0.17</td>
<td>0.33</td>
</tr>
<tr>
<td>Asp</td>
<td>10.89</td>
<td>10.32</td>
<td>11.38</td>
<td>10.79</td>
<td>10.85</td>
</tr>
<tr>
<td>Thr</td>
<td>3.85</td>
<td>4.20</td>
<td>4.36</td>
<td>4.44</td>
<td>4.21</td>
</tr>
<tr>
<td>Ser</td>
<td>9.92</td>
<td>9.03</td>
<td>9.82</td>
<td>6.59</td>
<td>8.84</td>
</tr>
<tr>
<td>Glu</td>
<td>8.85</td>
<td>9.36</td>
<td>9.06</td>
<td>9.92</td>
<td>9.30</td>
</tr>
<tr>
<td>Pro</td>
<td>5.09</td>
<td>5.14</td>
<td>4.86</td>
<td>5.32</td>
<td>5.10</td>
</tr>
<tr>
<td>Gly</td>
<td>13.38</td>
<td>12.58</td>
<td>12.10</td>
<td>12.77</td>
<td>12.71</td>
</tr>
<tr>
<td>Ala</td>
<td>7.17</td>
<td>6.92</td>
<td>7.09</td>
<td>7.44</td>
<td>7.16</td>
</tr>
<tr>
<td>Val</td>
<td>6.68</td>
<td>6.68</td>
<td>6.73</td>
<td>7.15</td>
<td>6.81</td>
</tr>
<tr>
<td>½Cys</td>
<td>4.14</td>
<td>2.88</td>
<td>1.96</td>
<td>2.68</td>
<td>3.92</td>
</tr>
<tr>
<td>Met</td>
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<td>1.23</td>
<td>1.71</td>
<td>1.39</td>
<td>1.35</td>
</tr>
<tr>
<td>Ileu</td>
<td>6.27</td>
<td>5.80</td>
<td>6.45</td>
<td>6.06</td>
<td>6.15</td>
</tr>
<tr>
<td>Leu</td>
<td>6.78</td>
<td>7.50</td>
<td>7.51</td>
<td>7.76</td>
<td>7.39</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.68</td>
<td>3.02</td>
<td>3.62</td>
<td>3.99</td>
<td>3.58</td>
</tr>
<tr>
<td>Phe</td>
<td>1.87</td>
<td>2.33</td>
<td>2.35</td>
<td>1.79</td>
<td>2.09</td>
</tr>
<tr>
<td>Lys</td>
<td>5.87</td>
<td>6.33</td>
<td>6.66</td>
<td>6.84</td>
<td>6.43</td>
</tr>
<tr>
<td>His</td>
<td>1.31</td>
<td>1.41</td>
<td>1.37</td>
<td>1.67</td>
<td>1.44</td>
</tr>
<tr>
<td>Arg</td>
<td>2.59</td>
<td>4.86</td>
<td>3.02</td>
<td>2.86</td>
<td>3.32</td>
</tr>
</tbody>
</table>

% * 59.0 66.0 75.0 75.0 -

* Recovery of anhydroamino acids.

* Recovery of anhydroamino acids.
### TABLE 3-3.

**AMINO ACID ANALYSES OF TRYPIC DIGESTS OF FORMIC ACID TREATED WHOLE WOOL.**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>100 min.</th>
<th>24 hr.</th>
<th>30.3 hr.</th>
<th>84 hr.</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO₃H</td>
<td>0.18</td>
<td>0.20</td>
<td>0.25</td>
<td>0.0</td>
<td>0.16</td>
</tr>
<tr>
<td>Asp</td>
<td>11.01</td>
<td>10.52</td>
<td>10.43</td>
<td>8.02</td>
<td>10.00</td>
</tr>
<tr>
<td>Thr</td>
<td>3.82</td>
<td>5.63</td>
<td>4.73</td>
<td>5.36</td>
<td>4.89</td>
</tr>
<tr>
<td>Ser</td>
<td>7.87</td>
<td>7.14</td>
<td>4.35</td>
<td>8.94</td>
<td>7.08</td>
</tr>
<tr>
<td>Glu</td>
<td>8.76</td>
<td>11.39</td>
<td>11.34</td>
<td>11.99</td>
<td>10.87</td>
</tr>
<tr>
<td>Pro</td>
<td>5.64</td>
<td>4.67</td>
<td>5.31</td>
<td>5.44</td>
<td>5.27</td>
</tr>
<tr>
<td>Ala</td>
<td>7.33</td>
<td>7.57</td>
<td>7.72</td>
<td>7.43</td>
<td>7.51</td>
</tr>
<tr>
<td>Val</td>
<td>6.94</td>
<td>6.13</td>
<td>6.53</td>
<td>6.37</td>
<td>6.49</td>
</tr>
<tr>
<td>½Cys</td>
<td>3.92</td>
<td>1.34</td>
<td>0.35</td>
<td>0.0</td>
<td>1.40</td>
</tr>
<tr>
<td>Met</td>
<td>1.01</td>
<td>1.69</td>
<td>2.01</td>
<td>1.72</td>
<td>1.61</td>
</tr>
<tr>
<td>Ileu</td>
<td>6.16</td>
<td>5.29</td>
<td>5.32</td>
<td>4.51</td>
<td>5.32</td>
</tr>
<tr>
<td>Leu</td>
<td>7.67</td>
<td>8.85</td>
<td>9.71</td>
<td>9.54</td>
<td>8.94</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.99</td>
<td>3.14</td>
<td>3.02</td>
<td>3.38</td>
<td>3.38</td>
</tr>
<tr>
<td>Phe</td>
<td>1.79</td>
<td>3.16</td>
<td>3.33</td>
<td>3.65</td>
<td>2.98</td>
</tr>
<tr>
<td>Lys</td>
<td>6.84</td>
<td>6.62</td>
<td>7.87</td>
<td>6.19</td>
<td>6.88</td>
</tr>
<tr>
<td>His</td>
<td>1.67</td>
<td>1.74</td>
<td>1.89</td>
<td>1.99</td>
<td>1.82</td>
</tr>
<tr>
<td>Arg</td>
<td>2.86</td>
<td>5.18</td>
<td>7.46</td>
<td>6.50</td>
<td>5.50</td>
</tr>
</tbody>
</table>

| % *       | 50.3     | 81.2   | 79.5     | 88.1   |         |

* Recovery of anhydroamino acids.
amino acid analyser it is susceptible to slight variations in processing conditions. Table 3-3 shows decreasing contents of aspartic acid and isoleucine, and increasing content of leucine with time of digestion; no explanation of these trends can be offered.

Table 3-4 compares the average analyses for digests from whole wool, formic acid pretreated wool, cortical cells and cuticle cells (i.e. endocuticle, Ley, 1971). There is excellent agreement between the analyses for digests from cortical cells and formic acid pretreated wool and only slight differences between digests from whole wool and cuticle. Bradbury et al. (1965a) have analysed the material dissolved by a 2 hr. treatment of wool with formic acid, and found it has a high content of glycine (14.5 mole %). In most other respects (apart from tyrosine and phenylalanine) this formic acid soluble material is not very different from the digest analyses in Table 3-4. Extraction from whole wool, of a material with a high glycine content, would account for the lower glycine contents of the extracts from formic acid pretreated wool and cortical cells.

The fairly high lysine plus arginine content of the various digests may be partially attributable to the specificity of trypsin, however Ley (1971) obtained similar high values with endocuticle isolated by pronase digestion and by dissolution of exocuticle with performic acid. The high content of basic amino acids in nuclear remnants can be explained by the suggestion of Howells (1971), who believes a basic protein is associated with nucleic acids in cell nuclei.
TABLE 3-4.

AMINO ACID ANALYSES OF DIGESTS OF WHOLE WOOL, CORTICAL CELLS AND CUTICLE.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Whole Wool Average</th>
<th>Formic Treated Wool Average</th>
<th>Cortical Cells 36 hr.</th>
<th>Cuticle Cells #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CySO₃H</td>
<td>0.33</td>
<td>0.16</td>
<td>0.81</td>
<td>0.95</td>
</tr>
<tr>
<td>Asp</td>
<td>10.85</td>
<td>10.00</td>
<td>9.89</td>
<td>7.43</td>
</tr>
<tr>
<td>Thr</td>
<td>4.21</td>
<td>4.89</td>
<td>4.34</td>
<td>5.61</td>
</tr>
<tr>
<td>Ser</td>
<td>8.84</td>
<td>7.08</td>
<td>7.12</td>
<td>10.70</td>
</tr>
<tr>
<td>Glu</td>
<td>9.30</td>
<td>10.87</td>
<td>11.24</td>
<td>10.26</td>
</tr>
<tr>
<td>Pro</td>
<td>5.10</td>
<td>5.27</td>
<td>4.75</td>
<td>8.82</td>
</tr>
<tr>
<td>Gly</td>
<td>12.71</td>
<td>9.92</td>
<td>9.43</td>
<td>8.18</td>
</tr>
<tr>
<td>Ala</td>
<td>7.16</td>
<td>7.51</td>
<td>7.46</td>
<td>6.64</td>
</tr>
<tr>
<td>Val</td>
<td>6.81</td>
<td>6.49</td>
<td>6.55</td>
<td>7.69</td>
</tr>
<tr>
<td>½Cys</td>
<td>3.92</td>
<td>1.40</td>
<td>2.25</td>
<td>1.96</td>
</tr>
<tr>
<td>Met</td>
<td>1.35</td>
<td>1.61</td>
<td>1.43</td>
<td>0.79</td>
</tr>
<tr>
<td>Ileu</td>
<td>6.15</td>
<td>5.32</td>
<td>5.60</td>
<td>4.01</td>
</tr>
<tr>
<td>Leu</td>
<td>7.39</td>
<td>8.94</td>
<td>8.69</td>
<td>9.50</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.58</td>
<td>3.38</td>
<td>3.11</td>
<td>3.39</td>
</tr>
<tr>
<td>Phe</td>
<td>2.09</td>
<td>2.98</td>
<td>2.98</td>
<td>3.61</td>
</tr>
<tr>
<td>Lys</td>
<td>6.43</td>
<td>6.88</td>
<td>6.51</td>
<td>4.13</td>
</tr>
<tr>
<td>His</td>
<td>1.44</td>
<td>1.82</td>
<td>1.67</td>
<td>1.01</td>
</tr>
<tr>
<td>Arg</td>
<td>3.32</td>
<td>5.50</td>
<td>6.19</td>
<td>5.16</td>
</tr>
</tbody>
</table>

*= Recovery of anhydroamino acids.

# Ley (1971), average of three results.
† From Table 3-2.
‡ From Table 3-3.
The slightly different analysis for endocuticle obtained by Ley (1971) may explain the different staining behaviour of endocuticle with osmium in comparison with nuclear remnants in the cortex. (Rogers, 1959, 1959a, shows it stains lighter than the surrounding keratin while nuclear remnants stain darker). However, the analysis gives no indication of the reason for this difference in staining behaviour with regard to amino acid content of e.g. cystine.

In Tables 3-2 and 3-3 there is a trend of increasing recovery of anhydroamino acids with increased digestion time, and Ley (1971) observed a similar increase with pronase digestion of cuticle. Material extracted during the first 100 min. digestion is more than 50% non-protein as part of the recovery of anhydroamino acids is due to trypsin. Bradbury & King (1967) observed a similar trend of increased recoveries with formic acid extracts of wool and attributed this non-protein material to lipid which is soluble in the formic acid. Tables 3-2 and 3-3 show that formic acid pretreatment does not greatly increase the recovery of anhydroamino acids as would be expected if a large proportion of the lipid is extracted by formic acid. The non-protein material may also consist of nucleic acid fragments, which are soluble in acid and are normally extracted by perchloric acid treatment (Howells, 1971). As both lipids and nucleic acid fragments are acid soluble, it is likely that they are almost completely extracted by formic acid during the preparation of cortical cells, as the cortical cell extract is almost all protein.

(iv) Electron Microscopy. Figures 3-4 and 3-5 are cross-sections of osmium
stained, trypsin digested, paracortical and orthocortical cells respectively, and show that the nuclear remnants are digested. Nuclear remnants are not normally removed during isolation of cortical cells and Figure 6-6 (Chapter 6) shows a gold stained paracortical cell which has retained its nuclear remnant. Figure 6-5 however, shows a gold stained orthocortical cell which has lost the nuclear remnant during separation of the cells (this is unusual).

Figure 3-6 shows the edge, and Figure 3-7 the centre, of a formic acid treated wool fibre which has been trypsin digested for 84 hr. A similar appearance is shown by fibres which have been digested with trypsin, but not formic acid pretreated (Kulkarni et al., 1971). Note that in Figure 3-6, endocuticle, nuclear remnants and intercellular material have been digested. (The black spots are artifacts of the staining process).

Figure 3-7 shows a region of the orthocortex/paracortex boundary (marked on Figure 3-7). The orthocortical cells (at 'O') have almost all separated from one another, the paracortical cells (at 'P') have remained attached, although their nuclear remnants have been digested. This indicates that orthocortical cells are released more rapidly than paracortical cells (Kulkarni et al., 1971).

Figure 3-8 shows a region of a trypsin digested fibre where there is partially digested endocuticle, while the nuclear remnants are more completely digested. This effect indicates that the cuticle is not a barrier to the entry of trypsin molecules into the fibre. The effect is more pronounced on fibres which have been digested for a
FIGURE 3-4

PARACORTICAL CELL AFTER 36 HOURS TRYPsin DIGESTION.
FIGURE 3-5

ORTHOCORTICAL CELL AFTER 36 HOURS TRYPSIN DIGESTION.
FIGURE 3-6

FORMIC ACID PRETREATED WOOL FIBRE
AFTER 84 HOURS TRypSIN DIGESTION.
FIGURE 3-7

FORMIC ACID PRETREATED WOOL FIBRE
AFTER 84 HOURS TRYSIN DIGESTION.
FIGURE 3-8

FORMIC ACID PRETREATED WOOL FIBRE AFTER TRYPsin DIGESTION. CUTICLE SHOWING PARTLY DIGESTED ENDOCUTICLE.
shorter time, as eventually both endocuticle and nuclear remnants are fully digested. Note the cuticle membrane structure is still visible (at 'A') indicating that these membranes are not trypsin digestible, although the intercellular cement may be easily digested, (Chapter 2).

3D. DISCUSSION.

(i) The Quantity of Nuclear Remnants.

The most reliable estimate of the quantity of nuclear remnants in cortical cells obtained in this work is 14.5%, which is the total digestible material in cortical cells and includes intermacrofibrillar cement. This is an upper limit for the correct quantity, as electron micrographs of wool cross-sections clearly show the presence of a non-keratinous intermacrofibrillar material which is also digestible by trypsin (Figure 3-6). As the amount of material extracted from cortical cells is constant after 36 hr. digestion (Table 3-1), it is reasonable to assume that the rate of digestion of nuclear remnants is considerably faster than the rate of digestion of keratin, so that a separation of nuclear remnants has been achieved without much digestion of keratin proteins.

Bradbury & King (1967) have shown that Merino wool comprises 10% cuticle and 88% cortical cells, and Ley (1971) found that cuticle comprises 35% endocuticle and 65% exocuticle. Using the value of 14.5% nuclear remnants, it is possible to calculate a maximum quantity of enzyme digestible material for whole wool i.e. 14.5% x 88% + 2% + 35% x 10% = 18.2%. Leeder (1969) used pronase digestion
of wool and found a maximum of 12% was dissolved in 6 weeks at 37°C; Elöd & Zahn (1946) dissolved 14% in 11 days with pancreatin, and Springell (1963) claims 10-20% is dissolved on digestion with pronase. Apart from Springell's broad range, the figures are lower than that calculated above, indicating that formic acid treatment may increase the amount of keratin digestible by trypsin. The rate determinations (Chapter 3C(ii)) also indicate that formic acid may allow more material to be digested, as the rate is faster after formic acid pretreatment. As these rate determinations could not be continued to completion of the reaction however, it is still possible that only the rate was increased by formic acid treatment, and not the final amount of material digestible.

The rate determinations show that digestion of wool proceeds in two stages. With trypsin digestion, the first is complete within 2 hr. and consists of predominantly non-protein material (Tables 3-2 and 3-3). The second stage extracts a protein fraction which has a high content of lysine plus arginine and may correspond to the basic protein believed to be associated with nucleic acids in cell nuclei (Howells, 1971). The yield of material extracted by trypsin in 100 min. (Table 3-1) decreases after formic acid pretreatment of the fibres indicating that some of the same material is extracted by formic acid and by enzyme digestion. This is probably the non-protein component, as Bradbury et al. (1965a) showed the material extracted by formic acid initially contains a high proportion of non-protein material, and recoveries of anhydroamino acids are higher following formic acid
pretreatment (Tables 3-2 and 3-3). It is difficult to establish how much keratin is dissolved during the enzyme digestion, but the rate of digestion of nuclear remnants is probably considerably faster than the digestion of keratin, as noted previously. It is known that ordered regions of proteins are more resistant to enzyme attack (Harrington et al., 1959; Segal et al., 1967; Lowey, 1968) and denatured proteins are digested considerably faster than native proteins (Line-weaver & Hoover, 1941; Bernheim et al., 1942). Crewther & Harrap (1967) first used this approach to produce a helix rich fraction from the wool low sulphur protein SCMKA, by digestion of the non-helical sections with pronase. Several wool treatments are known to increase disorder in the fibre and thus probably increase the susceptibility of the proteins to enzyme digestion, e.g. dehydration (Fraser, 1961) and formic acid treatment (Chapman & Feughelman, 1967; Feughelman, 1968) and some treatments have been shown to increase the amount digested by enzymes e.g. different pH pretreatments (Meunier et al., 1927; Crewther, 1956) or reversible swelling treatments (Greenwood & Speakman, 1964). In these cases however, it does not seem to have been noted that the apparently greater digestion occurring after treatment may be due to an increased rate of digestion. The apparent difference in susceptibility of bonds to enzyme attack is a rate effect produced by protein secondary structure, and Harrington et al. (1959) have shown that all susceptible bonds are attacked, but at considerably different rates. Gillespie (1970a, 1971) has recently stated that enzymic digestion of wool with
pronase or chymotrypsin results in substantial decreases in molecular weight of the low sulphur proteins, but unfortunately the time of treatment was not specified. Obviously, susceptible bonds in the cross-linked and α-helical keratin proteins have been cleaved, and clearly it is essential to limit enzyme digestions to the minimum time if damage to, and extraction of, keratin proteins is to be avoided.

(ii) Analysis of Nuclear Remnants.

The material extracted from nuclear remnants initially contains a high proportion of a non-protein component, but the protein extracted has a high content of basic amino acids and a low content of cystine. The analyses of extracts from cuticle, cortical cells, formic acid pre-treated and untreated wool are all reasonably similar. This is an indication that the material extracted has a fairly constant composition and is probably not produced by an initial rapid attack on the nuclear remnants followed by a slower attack on the keratin. While there is no doubt that the keratin is slowly digested, and extracted, it probably does not provide a significant proportion of the material analysed from the digestions.
CHAPTER 4.

STUDIES ON SOLUBLE PROTEINS.

4A. INTRODUCTION

The work described in this Chapter was undertaken in order to obtain basic information on the conformation and solubility of soluble proteins from wool. High sulphur content (H.S.) proteins can be extracted preferentially from wool (Harrap & Gillespie, 1963) leaving the low sulphur content proteins in the fibre. These are believed to correspond to the matrix and microfibrils respectively as observed in electron microscopy of cross-sections of wool (Rogers, 1959). Information on properties of these two proteins should be of assistance in developing methods for separating the native microfibrils (Chapter 5).

To examine compounds, including proteins, by high resolution NMR spectroscopy, it is essential that they be soluble. Proteins are extracted from wool by cleavage of the cystine disulphide bond, generally by oxidative or reductive means (Crewther et al., 1965), the latter being used in this case. Although the thiol proteins produced by reductive cleavage have increased solubility over unreduced wool proteins, they are susceptible to autoxidation to form disulphide proteins, particularly under basic conditions (Maclaren et al., 1968). The re-oxidised keratin disulphide proteins are however more soluble than the native keratins (Bhatnagar & Crewther, 1967;
Goddard & Michaelis, 1935). Alkylation of the thiol groups produced by reduction, is necessary to form proteins stable to oxidation, but it also confers increased solubility on the protein if the alkylation reagent is carefully selected. S-carboxymethyl and S-aminoethyl derivatives of wool proteins are more soluble than the thiol protein (Gillespie, 1963). These two alkylation agents sometimes decrease the solubility of proteins which are soluble in their native form, but conversion of positively charged groups to negatively charged groups is one effective means of solubilising proteins in this case (Furka & Sebestyén, 1969).

NMR spectroscopy is basically used for the determination of molecular structure, but this is difficult with proteins because of their large size and correspondingly complex spectra. With proteins, NMR spectroscopy has been used to follow denaturation of e.g. ribonuclease, lysozyme and α-lactalbumin, (Bradbury & King, 1969, 1969a, 1971; Meadows et al., 1967, 1968; McDonald & Phillips, 1969; King, 1970) and Bradbury & King (1969a) have developed a test for complete unfolding of proteins in the NMR.

Possibly the greatest advantages of NMR spectroscopy over other methods of structure determination (e.g. X-ray methods) are that it can follow the behaviour of proteins in solution, and can, under the same conditions, observe the behaviour of different regions of the protein. This facility of NMR spectroscopy permits examination of the four separate histidine residues in ribonuclease to observe their individual pK's (Bradbury & Scheraga, 1966;
Meadows et al., 1967, 1968; King & Bradbury, 1971), or to demonstrate that separate sections of ribonuclease or of lysozyme may denature at different rates (i.e. that denaturation is not necessarily a single step process) (Bradbury & King, 1969). The principle disadvantage of NMR spectroscopy is the high protein concentration employed (10%), which contributes to the possibility of increased aggregation.

In this Chapter, conditions for disaggregation or denaturation of proteins have been studied, as these conditions may be essential before satisfactory separation of some proteins can be achieved (O'Donnell & Thompson, 1961). Interactions between proteins occur even under disaggregating conditions, and are shown in this Chapter to occur with protein mixtures. A corollary of the examination of conditions suitable for denaturation is that they can also be used to define areas of study for renaturation of proteins (Kuehn et al., 1968).

Early work on gel chromatography of soluble proteins in their native form confirmed that elution volume is proportional to molecular weight of the protein (Whitaker, 1963; Andrews, 1964). Thompson & O'Donnell (1965) have observed a similar relationship for reduced and S-carboxymethylated proteins. Andrews (1964) claimed the maximum accuracy obtainable for molecular weight determination of native 'globular' proteins was ±10%, whereas Whitaker (1963) quoted molecular weights of 6,600 and 7,700 for lysozyme in comparison with its correct value of 14,300 (Dayhoff & Eck, 1968). These variations are due to differences in shape and size between the proteins used.
for molecular weight standardisation of the column, and that of the protein under examination (Fish et al., 1969).

Molecular size is related to molecular weight of denatured proteins as they approximate to random coils, and increased accuracy of molecular weight determination has been achieved with denatured proteins (Small et al., 1963; Cebra & Small, 1967).

Davison (1968) and Fish et al. (1969) have both used a 6% agarose gel (Bio-Gel A-5m) with a reducing agent (mercaptoethanol) and 6 M guanidine hydrochloride and shown the log(molecular weight) vs. elution volume plot is linear for fully reduced and denatured proteins. Fish et al. (1969) also showed the same behaviour occurs for reduced and S-carboxymethylated proteins in 6 M guanidine hydrochloride. Gel chromatography on 6% agarose gels in 8 M urea has the advantage over Sephadex G200 of a larger fractionation range in 8 M urea (compare Figure 4-21 with Thompson & O'Donnell, 1965). A recent review of gel chromatography is given by Ackers (1970) and many comprehensive texts are available (e.g. Determann, 1968).

Evidence is presented in this Chapter to show that NMR spectroscopy of proteins can be used to establish conditions for fully unfolding proteins (preparatory to the accurate determination of molecular weight by e.g. gel chromatography), for the prevention of aggregation of proteins, and also to examine the effect on a protein of different alkylating agents.
4B. NMR THEORY.

A number of texts give details of the theory of NMR including its application to organic chemistry (e.g. Bovey, 1969; Emsley et al., 1965; Pople et al., 1959). Abragam (1961) has given a comprehensive account of the theory of NMR. A brief summary of NMR theory will be given in this section.

The nuclei of many elements have an associated magnetic field produced by rotation of the nucleus. Of particular interest with proteins are the common isotopes of carbon, hydrogen, oxygen, nitrogen and sulphur, but of these, only hydrogen and nitrogen have magnetic properties, with hydrogen being far more important than nitrogen.

If a proton is placed in an external magnetic field \( H_0 \) its nuclear field will take up one of two orientations with respect to the applied external field; i.e. aligned with, or opposed to, the external field. The transition between these two orientations or energy states, can be brought about by absorption of a suitable quantum of radiation \( \Delta E = h\nu \) where \( h \) is Planck's constant, and \( \nu \) is the frequency).

The energy difference between the two states is given by \( \Delta E = \frac{\gamma H}{2\pi} \) (where \( H \) is the field at the nucleus and \( \gamma \) is the magnetogyric ratio which is typical of each nucleus), therefore \( \nu = \frac{\gamma H}{2\pi} \). By holding \( H \) constant and varying \( \nu \), energy is absorbed when the resonant condition for the particular nucleus is reached.

In a molecule, the magnetic field at the nucleus \( (H) \) does not correspond to \( H_0 \) as a local opposing field is
generated by rotation of bonding electrons. This shielding effect is represented by a shielding parameter $\sigma$, defined by the equation $H = H_0 (1-\sigma)$, and thus $\nu = \frac{\gamma H_0 (1-\sigma)}{2\pi}$. Protons having different shielding parameters will require different quantities of energy to attain resonance, and will appear at different positions in the absorption spectrum. This is known as chemical shift. If an inductive effect operates, due to the presence of a neighbouring electronegative atom or group, the bonding electrons are partially withdrawn, the shielding magnetic field decreases, and the observed absorption moves down-field (i.e. away from the reference peak, T.M.S.).

Width of the resonance peak is inversely proportional to the average time the nucleus spends in the excited state ($\Delta \nu = \frac{1}{2\pi \Delta t}$ where $\Delta t$ is the lifetime in each state and in the simple case, is assumed to be equal). Loss of energy from the excited state is achieved by spin-lattice and spin-spin relaxation processes, where the energy is transferred to the whole molecule (lattice) or neighbouring nuclei respectively. Spin-lattice relaxation times are affected by the presence of paramagnetic molecules or ions and may cause the peak to become too broad to be seen.

Broadening of the peak can occur via instrumental or natural causes. Magnetic field inhomogeneity is the principal instrumental cause of broadening, as the individual protons sample different field strengths and thus resonate over a small range of frequencies. Natural causes of broadening include the limitation
to the lifetime of a nucleus in a given state imposed by spin-lattice and spin-spin relaxation processes. But with proteins, other factors (below) are of more importance in producing line broadening.

In proteins, several amino acids give rise to characteristic peaks in the NMR spectrum, e.g. the methyl groups of isoleucine, leucine and valine; the aromatic protons of phenylalanine, the S-methyl group of methionine and certain groups in modified amino acids. Alterations to the position or shape of these peaks reflect corresponding chemical or physical changes to that amino acid.

The width of these peaks observed in proteins is the sum of a number of terms (Jardetzky, 1964; Bradbury et al., 1971a) i.e. the peak width in the free amino acid, dipole-dipole interactions, proton exchange, and non-equivalent magnetic environment for otherwise identical protons.

Peak widths in the free amino acid have been covered above. Proton exchange is the same as is observed with small molecules, and is not of importance in this work.

Magnetic dipole broadening occurs due to the imposition of a static local field by an adjacent nucleus or group, e.g. by a paramagnetic nucleus or one with a large quadrupole moment or simply a closely placed proton. Additional magnetic dipole broadening occurs if the nucleus is restrained in one position as e.g. in solids or viscous liquids. Under these conditions, nuclei must be treated as being in a variety of magnetic fields and have a corresponding variation in chemical
shift. Several workers have concluded that the broader peaks of polymers in comparison with monomers, is due to dipole-dipole interactions of this kind (see Markley et al., 1969). Dipole interactions increase peak width in proteins following a decrease in the internuclear distance or an increase in the effective size of the molecule (i.e. by association of rigid molecules, Kubo & Tomita, 1954; Bloembergen et al., 1948).

In a native protein, the sequence of amino acids is such that the same amino acid will experience magnetic environments due to the presence of neighbouring amino acids, which vary with the position of the individual amino acid residue along the protein chain. Each observed peak will therefore consist of a number of separate resonances, each of slightly different chemical shift (McDonald & Phillips, 1969). This non-equivalence of magnetic environments is a major cause of peak broadening in native or folded proteins.

A protein is said to be denatured when the native conformation has undergone a major change without cleavage of a covalent bond (Tanford, 1968). In the cases described in this Chapter, cleavage of disulphide bonds is essential to achieve solubilisation of the proteins. The definition of denaturation must be modified to cover this case by allowing disulphide bonds to be cleaved but no covalent peptide bonds to be broken. This is not restrictive, as the investigation is into properties of the proteins after cleavage of the disulphide bonds.

In their native form, the proteins examined in this thesis are insoluble. Cleavage of disulphide bonds, with
a consequent risk of denaturation of the proteins, is essential to their solubilisation. Additionally, the proteins are probably converted to an unfolded state during extraction prior to examination in the NMR spectrometer. For these reasons it is not possible to state that the proteins regain their native structure during examination. This 'native' state will therefore be termed the folded state, the denatured condition should logically be called the unfolded (or random coil) state.

In a folded protein, many of the shorter amino acid side-chains are held rigidly and rotate with the whole molecule. On unfolding, these side-chains gain considerable freedom of rotation and the observed peak width diminishes. An unfolded protein can be recognised by the relative sharpness of its spectrum, and may be in a random coil state in which rotation about each bond is as free as rotation about the same bond in a compound of low molecular weight (Tanford, 1968). Broadening of peaks has been attributed to folding of the protein (above), but it may also be due to aggregation, which in its most severe form, is observed as a gel.

King (1970) (Bradbury & King, 1969a, 1971), for soluble proteins, defined an 'extent of unfolding' for a single step transition between native and denatured states of the protein, where $h_N$ is the peak height in the native and $h_U$ in the unfolded protein.

At constant peak area, the observed peak height $h$, is inversely related to the peak width, and varies between the different peaks in the spectrum. Using this extent of
unfolding, F, King was able to show that non-equivalence of F for all the peaks observed in a transition region between native and unfolded states, could be interpreted in terms of intermediate stages in the unfolding (King, 1970; Bradbury & King, 1969).

For the proteins examined in this thesis, the native state is not fully characterised as mentioned above, and the fully unfolded state is sometimes not achieved, although a test for the latter can be made (Bradbury & King, 1969a), thus F will not be employed in this thesis as a measure of unfolding.

Proteins vary in molecular weight, but it has been shown that peak width is independent of molecular weight for random coil polypeptides (Bradbury & Stubbs, 1968; Chapman, 1968) and synthetic polymers (Bovey et al., 1959). Peak width is also independent of protein concentration for random coil proteins (Bradbury & King, 1969a).

4C. MATERIALS AND METHODS.

(i) Materials.

Merino 64's wool prepared as described in Chapter 1B(i) was used throughout. Urea was recrystallised from water to remove an impurity giving rise to a peak at 1.05T. Formic acid was redistilled as noted in Chapter 1B(i). For NMR work, approximately 6N hydrochloric acid was prepared by diluting concentrated HCl with an equal volume of D₂O. Approximately 2 M NaOD was prepared by reacting metallic sodium with D₂O. All other
reagents used were A.R. grade.

(ii) Preparation of Proteins.

As wool proteins were to be examined for changes produced by the use of different alkylating agents, it was necessary to use a process which separated the low sulphur (L.S.) and high sulphur (H.S.) proteins prior to alkylation. Methods of separating alkylated H.S. and L.S. proteins are only available for some alkylating agents (e.g. the SCM kerateines and the keratoses, Crewther et al., 1965). Separation of the proteins prior to alkylation obviates criticisms that observed differences are due to the separation method used.

Harrap & Gillespie (1963) reported a method allowing preferential extraction of the L.S. and H.S. proteins, which are only slightly cross-contaminated. In this method, wool (5 g.) is extracted for 18 hr. at 0°C with 150 ml. of 0.8 M potassium thioglycollate containing 0.1 M ammonium hydroxide at pH 10.3. The mixture was filtered (filtrate 1) and the residue treated with a further 150 ml. of the same solution at 0°C for 18 hr.

After filtration (filtrate 2) the residue was placed in 250 ml. of ice-cold water and blended with a Polytron stirrer at its lowest setting (about 10,000 r.p.m.) for 30 sec. A Waring Blender was used in this step by Harrap & Gillespie. Following blending, the mixture was centrifuged on a Beckman L2-50 preparative ultracentrifuge for 15 min. at 45,300 g. The supernatant from this centrifugation contains L.S. protein (contaminated with 8-10% H.S. protein, Harrap & Gillespie, 1963) and the two filtrates contain almost entirely H.S. proteins (Harrap
Alkylation of the L.S. protein was performed at pH 9 by addition of 1.5 times the calculated quantity of alkylating agent, the pH was monitored continuously and maintained at pH 9 as necessary. Stirring and pH maintenance were continued until the nitroprusside test for thiol was negative; if this took longer than 15 min. nitrogen gas was passed over the liquid surface to decrease oxidation. Cysteine was then added (at pH 9) until the nitroprusside test became positive, the mixture was dialysed thoroughly, and then freeze-dried.

Only the protein obtained from filtrate 1 was used in the work described in this thesis, but both filtrates were processed similarly.

The filtrates were dialysed into 2.5 l. of deionised water in a full, sealed measuring cylinder for 24 hr., to decrease the thioglycollate concentration. They were then alkylated as described for the L.S. protein above. In only one case, that of iodoethanol, did the alkylation continue for more than 15 min.. This alkylation was very slow and was therefore continued overnight (about 18 hr.).

The following alkylating agents were used to prepare both L.S. and H.S. proteins:-

<table>
<thead>
<tr>
<th>Alkylating Agent</th>
<th>Kerateine Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetic acid</td>
<td>S-carboxymethyl.</td>
</tr>
<tr>
<td>Acrylic acid</td>
<td>S-carboxyethyl.</td>
</tr>
<tr>
<td>Methyl iodide</td>
<td>S-methyl.</td>
</tr>
<tr>
<td>Acrylonitrile</td>
<td>S-cyanoethyl.</td>
</tr>
<tr>
<td>Iodoethanol</td>
<td>S-hydroxyethyl.</td>
</tr>
</tbody>
</table>

(continued)
In the case of the thiol proteins (kerateines), the H.S. proteins (filtrate 1) were dialysed in a full, sealed vessel, in sufficient water to achieve a final thioglycollate concentration of 0.02 M. Potassium thioglycollate solution was added to the L.S. protein solution after centrifugation until a concentration of 0.02 M thioglycollate was reached. Half of the thiol protein solutions were freeze-dried, and used within 24 hr., being stored at -10°C before use. The other half of each solution was dialysed in tap water for 5 days to produce metakeratin by reoxidation of the thiol groups (Goddard & Michaelis, 1935; Bhatnagar & Crewther, 1969).

With the S-aminoethyl derivative, alkylation according to the method described above produced a H.S. protein which was insoluble in water and also did not redissolve in the 0.8 M potassium thioglycollate reducing solution. This indicates a cross-link may have been formed, other than disulphide. By alkylating at pH 11 instead of pH 9, a protein which is soluble in water is produced. Treatment at higher pH is known to contribute to the formation of lanthionine (Zahn, 1961) and lysinoalanine (Bohak, 1964; Ziegler, 1964) but formation of these would be expected to decrease solubility, not increase it. The L.S. protein alkylated...
at pH 11 remained insoluble.

(iii) NMR Spectroscopy.

Spectra were obtained on a 60MHz Perkin-Elmer R10 spectrometer at 33.4°C with a radio frequency input of 1 mV and at 6.4 Hz per second sweep rate. The signal to noise ratio was improved by use of an on-line Digital Equipment PDP-8/S computer to average the spectra obtained. A spectral-averaging programme was available for this purpose. Other equipment used for spectral-averaging included a real-time clock, an analogue-to-digital converter, an ASR-33 teletype for input and output of commands and some data, and a Tektronix storage oscilloscope to display the current spectrum or averaged spectrum for examination. On completion of accumulation, the averaged spectrum was plotted-out via the R10 spectrometer at 1.6 Hz per second.

Under programme control, correction was made for magnetic field drift by alignment of a reference peak (T.M.S.) in the current spectrum with that of the averaged spectrum prior to further averaging. Sodium 3-trimethylsilyl-1-propanesulphonic acid is normally used as reference for D₂O solutions, but Bradbury et al. (1967) report that this salt sometimes interacts with proteins. An external reference standard was therefore invariably used. This comprised a capillary tube containing a dilute carbon tetrachloride solution of T.M.S..

Also under programme control, corrections were made to the spectrometer controls to eliminate baseline drift. Scans which were unsatisfactory because of severe
baseline drift, magnetic field drift or poor resolution were rejected by the computer. Although the computer programme was capable of calculating peak heights and widths, it was no more accurate than the manual method of Bradbury & Stubbs (1968), which was used routinely.

Solutions were prepared by weighing the solids accurately and then adding a measured volume of solvent to bring the urea concentration to 8 M (if used) and the sample concentration to 10%. Dilution during pH adjustment was checked and found to be less than 3% dilution for adjustment from pH 12 to 3, with a further 2% to reach pH 1. In all cases, the pH reading was adjusted to 11-12 for the first spectrum (sometimes necessary to achieve solution of the protein) and progressively decreased for subsequent spectra. pH's were measured with a Beckman Research pH meter fitted with microelectrodes to allow readings to be made in 0.3 - 0.5 ml. of solution. Values quoted are pH meter readings uncorrected for deuterium isotope effects, as the relationship between pH and pD of protein solutions is not well known (Willumsen, 1968) and there is doubt concerning the significance of pH values of concentrated urea solutions (Simpson & Kauzmann, 1953). The relationship pD = pH meter reading + 0.40 is satisfactory in aqueous solutions of low molecular weight compounds (Glasoe & Long, 1960; Li et al., 1961).

(iv) Gel Chromatography.

Bio-Gel A-5m, a 6% Agarose gel produced by Bio-Rad Laboratories was used for all gel chromatography. It was eluted with a solution containing 8 M urea, 0.1 M tris
(hydroxymethyl)aminomethane (TRIS), 0.01 M ethylenediamine tetra-acetic acid, adjusted to the pH required. No precautions were taken regarding the presence of cyanate in urea as TRIS reacts with cyanate and removes it from urea solutions (Hirs, 1956).

Agarose gels are not cross-linked and precautions must be taken to avoid extremes of pH, the limits suggested are pH 4.5 to 9 (Fischer, 1969). Agents which disrupt hydrogen bonds (e.g. urea) also decrease the mechanical stability of the gel, but this effect can be considerably decreased by use of low temperatures, and after an initial period of time the behaviour of the gel is stable.

The apparatus used consists of a jacketed column 2.54 x 95 cm. maintained at 4°C (lower temperatures crystallise the urea). Upward flow was attempted but severe decreases in the flow rate were observed with time. Downward flow under a head of 20 cm. from a Mariotte flask was very satisfactory, provided the column was upward flowed for a short time after each run, to remove material settled on or clogging the top of the column. This latter precaution was necessary despite the sample being centrifuged at 4,600 g. prior to layering onto the column. The sample was prepared as for NMR spectrometry, but with 100 mg. protein being used in approximately 1 ml. of urea solution containing 10% sucrose and 1-2 mg. Blue Dextran (Pharmacia) as a marker.

Five ml. fractions were collected, commencing immediately the sample was placed on the column. The column effluent was continuously monitored at 280 nm.
with an L.K.B. Uvicord II. Different fractions were indicated by a colour change of the recorder trace.

Blue Dextran eluted after approximately 200 ml. had been passed through the column. Considerably improved reproducability was achieved if all peak maxima were adjusted by the amount necessary to bring the Blue Dextran peak maximum to exactly 200 ml. for each chromatogram.

Calibration of the column was carried out by obtaining elution profiles for SCM insulin, SCM ribonuclease, SCM trypsin, SCM bovine serum albumin and apomyoglobin at pH 8.0. All SCM proteins were prepared by the method of Hirs (1967) and apomyoglobin, prepared by the method of Hanson & Blout (1965), was supplied by Dr.J.M.O'Shea.

Elution volumes for these proteins, with Blue Dextran corrected to 200 ml., were plotted against log(molecular weight) and an almost linear relationship was noted as shown in Figure 4-21. At other pH's only minor variations from the calibration obtained at pH 8.0 were noted, and were all within experimental error (±5 ml.).

(v) Amino Acid Analyses.

Amino acid analyses were performed as described in Chapter 1B. Positions of elution of the various alkylated cysteines in the Technicon system are as follows:-

<table>
<thead>
<tr>
<th>Cysteine</th>
<th>Elution Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCM</td>
<td>Before aspartic acid.</td>
</tr>
<tr>
<td>S-carboxyethyl</td>
<td>Before glutamic acid.</td>
</tr>
<tr>
<td>S-cyanoethyl</td>
<td>Converted to S-carboxyethyl on hydrolysis.</td>
</tr>
<tr>
<td>S-carbamidomethyl</td>
<td>Converted to S-carboxyethyl on hydrolysis.</td>
</tr>
<tr>
<td>S-methyl</td>
<td>Just after proline.</td>
</tr>
<tr>
<td>S-hydroxyethyl</td>
<td>Just before threonine.</td>
</tr>
<tr>
<td>S-aminoethyl</td>
<td>Before lysine.</td>
</tr>
</tbody>
</table>
4D. RESULTS.

(i) Analysis of Proteins Used.

As the proteins used in this investigation were prepared basically by the method of Harrap & Gillespie (1963), it was necessary to compare these proteins with those of Harrap & Gillespie. A different sample of wool was used in this investigation, and Harrap & Gillespie (1963) noted considerable differences in mobility and amino acid analysis for two L.S. proteins prepared from the same wool under apparently similar conditions. The results presented in Table 4-1 are amino acid analyses of the SCMKA and SCMKB prepared as described in this thesis and as prepared by Harrap & Gillespie (1963).

Amino acid analysis of the other proteins prepared, showed that most had similar compositions to those of the L.S. and H.S. proteins given in Table 4-1. The only exceptions were in \( \frac{1}{2} \)-cystine compositions as shown below.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mole % ( \frac{1}{2} )-cystine</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.S., S-hydroxyethyl keratine</td>
<td>5.47</td>
</tr>
<tr>
<td>L.S., S-ethylamino keratine</td>
<td>0.39</td>
</tr>
<tr>
<td>H.S., S-hydroxyethyl keratine</td>
<td>8.80</td>
</tr>
</tbody>
</table>

These values for \( \frac{1}{2} \)-cystine are thought to be due entirely to re-oxidation during the alkylation step. Iodoethanol was very slow to react and ethyleneimine may have a lower reactivity with thiols than iodoacetic acid. The metakeratin and thiol keratine proteins were not analysed.
### TABLE 4-1. AMINO ACID ANALYSES OF SCMKA AND SCMKB.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>This Thesis SCMKB</th>
<th>This Thesis SCMKA</th>
<th>L.S. Protein Prep.A.</th>
<th>L.S. Protein Prep.B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.S. Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CySO$_3$H</td>
<td>0.49</td>
<td>-</td>
<td>0.19</td>
<td>0.74</td>
</tr>
<tr>
<td>Asp</td>
<td>3.40</td>
<td>2.81</td>
<td>7.69</td>
<td>8.64</td>
</tr>
<tr>
<td>Thr</td>
<td>8.94</td>
<td>9.72</td>
<td>5.10</td>
<td>4.59</td>
</tr>
<tr>
<td>Ser</td>
<td>11.33</td>
<td>12.08</td>
<td>8.25</td>
<td>8.29</td>
</tr>
<tr>
<td>Glu</td>
<td>7.48</td>
<td>8.01</td>
<td>13.19</td>
<td>14.52</td>
</tr>
<tr>
<td>Pro</td>
<td>11.94</td>
<td>11.82</td>
<td>5.24</td>
<td>3.87</td>
</tr>
<tr>
<td>Gly</td>
<td>8.59</td>
<td>6.53</td>
<td>8.22</td>
<td>7.54</td>
</tr>
<tr>
<td>Ala</td>
<td>3.26</td>
<td>2.79</td>
<td>5.91</td>
<td>6.62</td>
</tr>
<tr>
<td>Val</td>
<td>6.14</td>
<td>5.36</td>
<td>6.53</td>
<td>6.25</td>
</tr>
<tr>
<td>$\frac{1}{2}$Cys</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>trace</td>
</tr>
<tr>
<td>Met</td>
<td>0.0</td>
<td>0.0</td>
<td>0.48</td>
<td>0.56</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.85</td>
<td>3.39</td>
<td>3.63</td>
<td>3.49</td>
</tr>
<tr>
<td>Leu</td>
<td>4.80</td>
<td>3.63</td>
<td>9.11</td>
<td>9.92</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.84</td>
<td>2.01</td>
<td>4.14</td>
<td>3.67</td>
</tr>
<tr>
<td>Phe</td>
<td>2.29</td>
<td>1.79</td>
<td>3.18</td>
<td>2.95</td>
</tr>
<tr>
<td>Lys</td>
<td>0.87</td>
<td>0.50</td>
<td>3.33</td>
<td>3.86</td>
</tr>
<tr>
<td>His</td>
<td>1.05</td>
<td>0.74</td>
<td>0.87</td>
<td>0.74</td>
</tr>
<tr>
<td>Arg</td>
<td>6.66</td>
<td>5.65</td>
<td>8.06</td>
<td>7.72</td>
</tr>
<tr>
<td>SCMC</td>
<td>18.32</td>
<td>18.02</td>
<td>6.67</td>
<td>6.07</td>
</tr>
</tbody>
</table>

# Harrap & Gillespie (1963)

† Harrap & Gillespie (1963), amino acid analysis from Gillespie & Inglis (1965).
(ii) NMR Spectroscopy.

For each experiment, the solution pH was adjusted to 11-12 and spectra accumulated for 2-3 hr. (60-100 scans). The pH was then re-adjusted in sequence, and further spectra accumulated, until a final spectrum was obtained at approximately pH 2 (if this could be reached). The transition from unfolded to folded states was followed quantitatively by measuring peak heights, and widths at half height. Where a sharp peak is superimposed on a broad region of the spectrum (e.g. the SCM peak), the baseline was taken at the base of the sharp peak. This eliminates problems caused by variations of the underlying resonance produced by conformational changes.

With each protein examined, peak height and peak width have been plotted vs. pH. For any one peak, height and width are inversely related (see Chapter 4B) at constant area, with the height showing progress of the transition, while width indicates proximity to the random coil state (Bradbury & King, 1969a; King, 1970). In the fully unfolded (random coil) state, peak widths approach those of the free amino acids (i.e. for methyl groups, a mixture of isoleucine, leucine and valine has a methyl peak width of $19 \times 10^{-2}$ p.p.m. in 8 M urea, Bradbury & King, 1969a).

Figure 4-1 shows typical spectra obtained in this case with the wool L.S. protein S-methyl kerateine. Assignment of peaks is in accordance with Bradbury & King (1969a), and McDonald & Phillips (1969). In Figure 4-1 the contrast between the unfolded form (pH 11.6) and the folded state (pH 1.9) is very clear. Examination
The protons giving rise to the peaks named in Figure 4-1 are as follows:

- **Phe**: aromatic protons of phenylalanine.
- **Tyr**: aromatic protons of tyrosine.
- **HDO**: solvent protons.
- **Urea**: protons of denaturant.
- **S**: spinning side-bands of HDO.
- **S-Me**: S-methyl protons of substituted cysteine.
- **Ala**: methyl protons of alanine.
- **Thr**: methyl protons of threonine.
- **Methyl**: methyl protons of isoleucine, leucine and valine.
- **TMS**: methyl protons of tetramethylsilane.

The unnamed peaks mostly arise from methylene protons.
FIGURE 4-1
NMR SPECTRA OF S-METHYL L.S. KERATEINE SHOWING DIFFERENCE BETWEEN SPECTRA OF FOLDED AND UNFOLDED PROTEINS.
of the spectrum of the unfolded protein shows no abnormal chemical shifts or excessive peak widths, and the methyl peak width \( (20 \times 10^{-2} \text{ p.p.m.)} \) is close to that of a mixture of the free amino acids. These points indicate that the protein is completely unfolded (i.e. random coil) in 8 M urea at pH 11.6 (Bradbury & King, 1969a). The low pH spectrum shows only a few very broad resonances of small size. This is due to the highly folded nature of the protein (it is a gel), and the considerable decrease in concentration produced by precipitation of the gelled protein.

Figures 4-2, 4-3 and 4-5 are of the S-carboxymethyl proteins SCMKB and SCMKA which have been subjected to considerable examination by other workers (see review by Crewther et al., 1965). In \( D_2O \), (Figure 4-2) the H$_2$ protein SCMKB is unfolded between pH 11.3 and about pH 5. Below pH 5 there is a rapid decrease in all peak heights and a broadening of widths. The decrease of methyl peak height with increasing pH above pH 5.5 may not be significant as there is no corresponding change in peak width. The CH$_2$ of SCMC and the aromatic protons of phenylalanine are the other two resonances plotted. All three peaks change in unison, demonstrating that the regions of the protein represented by these peaks are behaving similarly. In this case, it is obvious that aggregation is occurring as the protein has gelled at pH 4.6.

In the highly aggregated gel, there are differences in the freedom of movement available to certain groups, but in this case the gel is 'solid' or 'hard' as all
FIGURE 4-2

SCMKB IN $D_2O$.

Comparison of Figures 4-2 and 4-3 shows the complex change in behavior brought about by the inclusion of a denaturant (urea). In the presence of urea, no aggregation of the SCMKB leads to gelation occurs, the methyl resonance is almost constant, and the decrease in methyl peak height at low pH is not very significant and it is difficult to assign a cause to these changes of this nature. This shows an example of different effects occurring in separate groups within the protein. Methyl groups such as leucine, isoleucine and valine are affected by pH. CMC are the characteristic responses of phenylalanine. The $C_3$ of SCMC shows a minimum figure at pH 4.9, and some recovery of this mobility occurs below pH 4.0. NMR can also be used to observe pH's of some groups, experimental determined pH's in proteins vary with the state of folding of the protein. With an unfolded protein (as with SCMKB), the pH's for the same group coincide, irrespective of their position along the protein chain. In folded proteins, chemical shifts are altered by charge proximity of other molecules, and thus it is possible to obtain, for example, four pH's for the four histidine residues of ribonuclease by chemical shift of the C-2 and C-4 protons (Bradbury & Schramm, 1969). Figure 4-4 is a plot of pH vs.
peaks decrease to zero. Other examples (e.g. SCMKA - Figure 4-5) show a small amount of mobility is retained by some groups, as peaks are still discernible in the gel state (a 'soft' gel).

Comparison of Figures 4-2 and 4-3 shows the complete change in behaviour brought about by the inclusion of a denaturant (urea). In the presence of urea, no aggregation of the SCMKB leading to gelation occurs, the methyl resonance is almost unaffected by pH. The small decrease of methyl peak height at low pH is not very significant, and it is difficult to assign a cause to small changes of this nature. This case shows an example of different effects occurring in separate positions in the protein. The methyl groups of isoleucine, leucine and valine are unaffected by pH, as are the aromatic protons of phenylalanine. The CH$_2$ of SCMC shows a mobility minimum occurs at pH 4.0 to 4.5 and some recovery of this mobility occurs below pH 4.0.

NMR can also be used to observe pK's of some groups, but experimentally determined pK's in proteins vary with the state of folding of the protein. With an unfolded protein (as with SCMKB), the pK's for the same group coincide, irrespective of their position along the protein chain. In folded proteins, chemical shifts are altered by charge proximity or conformational changes, and thus it is possible to obtain, for example, four separate pK's for the four histidine residues of ribonuclease by observing the chemical shift of the C-2 and C-4 protons (Bradbury & Scheraga, 1966; Meadows et al., 1967, 1968; King & Bradbury, 1971). Figure 4-4 is a plot of pH vs.
The chemical shift for the methyl protons of SCMC in Figure 4-3 indicates a lactam has formed between the distal carbon and the 6 amino group, as protonation of the 6-carboxylic acid of SCMC occurs between pH 4 and pH 2.

Coincidence of the \( pK_a \) of SCMC and the decreasing peak heights in the protein can be explained in terms of protonation of the distal carboxylic acid and protonated there is a consequent decrease in spatially adjacent negative charges which were mutually repulsive.

The protein is able to become more folded in the region of the carboxyls and hence their mobility decreases.

Below pH 4, solubility decreases as was observed for SCMB in various aqueous solutions by Nauspie (1962a, 1963), or to further protonation in development of mutually repulsive positive charges on the protein.

Figure 4-5 shows the behaviour of the L.S. protein in 8 M urea. The gelation point is at pH 6.2 the protein is not completely aggregated to a gel at pH 5.8. Note that in contrast to the solubility of the methyl groups, the solubility of the protein below pH 2.9 may show up here as an increase in aromatic.
chemical shift for the CH$_2$ protons of SCMC in Figure 4-3. The pK$_a$ of the terminal carboxyl is determined from the mid-point of the graph, and is found to be 4.4 ± 0.1. The chemical shift of the distal CH$_2$ of the free amino acid SCMC was also examined vs. pH and no chemical shift of the CH$_2$ is observed except in 2N acid. This probably indicates a lactam has formed between the distal carboxyl and the α amino group, as protonation of the α carboxyl of SCMC occurs between pH 4 and pH 2.

Coincidence of the pK of SCMC and the decreasing peak height in the protein can be explained in terms of protonation of the distal COO$^-$. When these carboxyls are protonated there is a consequent decrease in spatially adjacent negative charges which were mutually repulsive. The protein is able to become more folded in the region of these carboxyls and hence their mobility decreases. Below pH 4, mobility of the CH$_2$ increases, which is not as easily explained. This increase may be connected with an increase in solubility at pH's below 2.9, as was observed for SCMKB in various aqueous solutions by Gillespie (1962a, 1963), or to further protonation causing development of mutually repulsive positive charges sites on the protein.

Figure 4-5 shows the behaviour of the L.S. protein SCMKA in 8 M urea. The gelation point is sharp; at pH 6.2 the protein is in solution, but it is almost completely aggregated to a gel at pH 5.8. Note that in contrast to SCMKB in D$_2$O, this gel allows a small amount of mobility of the methyl groups. Increased solubility of the protein below pH 2.9 may show up here as an increase in aromatic
CHEMICAL SHIFT vs. pH FOR S-CH$_2$-COOH
OF SCMC IN SCMKB (SOLVENT:D$_2$O/8 M UREA).

pK$_a$ 4.4±0.1
and SCMC peak heights observed at pH's above 10, probably due to increased mobility of the side chain of cysteine by one CH₂ group as in 8-carboxymethyl cysteine, slightly decreases the possibility of aggregation at lower pH's in 8 M urea. Figure 4-6 shows the N.M.R. 8-carboxymethyl (8-CH₂) protein and although both the aromatic and methyl protons now show decreased mobility below pH 7 to 8, the 8-carboxymethyl CH₂ protons are not as severely affected as the SCMC CH₂ protons. Preliminary results indicate that protonation of the terminal carboxyl occurs at about pH 4.9 to 5.5. As shown by chemical shifts of the resonances. The chemical shift of the aromatic resonances to overlap and instead of two peaks for the aromatic carboxymethyl resonance only one is observed below pH 5.6, which consequently gives a compressed height. This is indicated by the dotted line in Figure 4-7. The observed resonances do not affect the fact that the minimum mobility is observed at about pH 4.9.

The L.S. 8-CH₂ protein (Figure 4-7) shows similar behaviour to SCMK̂ but again the transition between extended and unfolded states is not as sharp. This abrupt transition is demonstrated by the methyl resonance widths shown in Figures 4-8 and 4-9.

With non-ionic or the bond between a hydrocarbon chain or carboxyl, considerable modification of the protein properties would be expected. This is not clearly observed, however. The aromatic and methyl protein of the carboxyl containing proteins. At high pH, an initial drop
and SCMC peak heights observed at pH 2.2, probably due to increased mobility of the whole molecule.

Extending the side chain of cysteine by one CH₂ group as in S-carboxyethyl cysteine, slightly decreases the possibility of aggregation at lower pH's in 8 M urea. Figure 4-6 shows the H.S. S-carboxyethyl (S-CE) protein, and although both the aromatic and methyl protons now show decreased mobility below pH 7 to 8, the S-carboxyethyl CH₂ protons are not as severely affected as the SCM CH₂ protons at lower pH. With the S-CE resonance, preliminary results indicate protonation of the terminal carboxyl occurs at about pH 4.9 ± 0.5, as shown by chemical shift of the resonance. The chemical shift causes some of the resonances to overlap and instead of two peaks for the S-carboxyethyl resonance only one is observed below pH 4.9, which consequently has an increased height. This is indicated by the dotted line in Figure 4-6. Merging of the observed resonances does not affect the fact that a minimum mobility is observed at about pH 4.9.

The L.S. S-CE protein (Figure 4-7) shows similar behaviour to SCMK4 but again the transition between folded and unfolded states is not as sharp. This slower transition is demonstrated by the methyl resonance widths as shown in Figures 4-7 and 4-5.

With non-ionic groups bonded to the cysteine sulphur instead of carboxyl, considerable modification of the protein properties would be expected. This is not clearly observed, the S-methyl H.S. and L.S. proteins (Figures 4-8 and 4-9) show similar NMR behaviour to that of the carboxyl containing proteins. At high pH, an initial drop
FIGURE 4-6
S-CARBOXYETHYL H.S. PROTEIN
IN 8 M UREA/D$_2$O.

![Graph showing pH meter reading vs. peak height for S-Carboxyethyl, Methyl, and Aromatic groups. The pH meter reading ranges from 2 to 12, with peak height in arbitrary units. The graph indicates a decrease in peak height for S-Carboxyethyl and Methyl groups as the pH meter reading increases, while the Aromatic group remains relatively constant.]
FIGURE 4-7
S-CARBOXYETHYL L.S. PROTEIN
IN 8 M UREA/D$_2$O.
FIGURE 4-8
S-METHYL H.S. PROTEIN
IN 8 M UREA/D_2O.
is noted with both the L.S. and S-proteins for the 7-methyl resonance. A decrease in stability of the unit occurs at higher pH than with the S- and S-CE proteins. Minimum mobility of 7-methyl proteins is at about pH 4, as is shown by the L.S. protein, the S-protein gels at about pH 4. In most cases, a resonance is not sufficiently strong or is submerged in the resonances of other proteins. One example of this is the 7-carnosine methyl resonance (Figures 4-10 and 4-11) in which the carnosine methylene resonance is more prominent in the L.S. protein than in the S-protein. The 7-carbamidomethyl protein (Figure 4-10) show a change from the previous examples. They are more uncharged than the S-protein. At low pH, the 7-carbamidomethyl methylene resonance again shows a more pronounced effect than the 7-methyl aromatic resonances. The L.S. protein (Figure 4-11) shows less effect of the carboximide on the resonances, due to the lower content of this group. The basic behaviour of L.S. protein (i.e., aggregation at about pH 4, and increasing aggregation, or increased solubility, below pH 4) appear to dominate any reverse effect that may be produced by the carboximide group. Glutathione (S-glutathione) is another compound that introduces three disulfide bonds into the protein which gives low solubility and increased solubility in urea solutions, and is more soluble in urea than the S- or S-CE L.S. proteins. A more pronounced effect, in comparison with the
is noted with both the H.S. and L.S. protein for the S-methyl resonance. This probably indicates a decrease in stability of the unfolded form occurs at higher pH than with the SCM and S-CE proteins. Minimum mobility of S-methyl protons is at about pH 4, as is shown by the H.S. protein, the L.S. protein gels at about pH 5.7.

In some cases, a resonance is not sufficiently strong or is submerged in the resonances of other protons. One example of this is the S-carbamidomethyl kerateines (Figures 4-10 and 4-11) in which the carbamidomethyl methylene resonance is observed only in the H.S. protein. This is due to the higher concentration of these groups in the H.S. protein. The S-carbamidomethyl H.S. proteins (Figure 4-10) show a change from the previous examples, as they are more unfolded at low pH, than at high pH. At low pH, the S-carbamidomethyl methylene resonance again shows a more pronounced effect than do the methyl or aromatic resonances. The L.S. protein (Figure 4-11) shows less effect of the carbamidomethyl group, due to the lower content of this group. The basic behaviour of the L.S. protein (i.e. aggregation at about pH 6, and decreasing aggregation, or increased solubility, below pH 4) appears to dominate any reverse effect which may be induced by the carbamidomethyl group. Gillespie (1956) has suggested the S-carbamidomethyl group introduces new hydrogen bonds into the protein which gives low solubility in water but increased solubility in urea solutions, and Figure 4-11 indicates that the L.S. protein is more soluble in urea than the SCM or S-CE L.S. proteins.

A more pronounced effect, in comparison with the
FIGURE 4-10
S-CARBAMIDOMETHYL H.S. PROTEIN IN 8 M UREA/D₂O.

Peak Height (Arbitrary Units)

Peak Width (p.p.m.x10²)

pH Meter Reading
FIGURE 4-11
S-CARBAMIDOMETHYL L.S. PROTEIN
IN 8 M UREA/D₂O.

S-carbamidomethyl protein unfolded with the S-cyanomethyl proteins (Figures 4-12). The folded structure is shown by decreased peak height for all resonances between pH 5.5 and 11. With the S-cyanomethyl protein (Figure 4-13) the effect of the cyanomethyl group is demonstrated by the higher pH at which gelation occurs. This protein also gives viscous solutions at pH 8 which are difficult to handle. In Figure 4-13, the drop in the methyl peak width below pH 8 is due to considerably decreased concentration produced by precipitation of the gel, which makes accurate determination of width difficult. Cyanomethylation of the L.S. protein has increased its aggregation tendency, or decreased its solubility, more than was noticed for the previous substance above.

S-aminoethylation, being a basic substituent, should produce a charged protein which has increased solubility, observed by Gillespie (1963) for the H.S. proteins. Figure 4-14 indicates the methyl and aromatic regions of S-aminoethyl L.S. protein are relatively unaffected, a minimum mobility being noted between pH 4 and 9. Methylene resonances of the S-aminoethyl group indicate that a solubility decrease would be observed below pH 10, as Gillespie (1963) has observed a minimum solubility between pH 5 and 10 in aqueous solution. A shift of the methyl resonances to a position in the spectrum where it is concealed by other methylene resonances. The observed
S-carbamidomethyl proteins, is observed with the S-cyanoethyl proteins (Figures 4-12 and 4-13). Unfolding at low pH (below 5.5) and above pH 11 are apparent with the H.S. protein (Figure 4-12). The folded structure is shown by decreased peak height for all resonances between pH 5.5 and 11. With the L.S. cyanoethyl protein (Figure 4-13) the effect of the cyanoethyl group is demonstrated by the higher pH at which gelation occurs. This protein also gives viscous solutions above pH 8 which are difficult to handle. In Figure 4-13, the dropping-off of methyl peak width below pH 8 is due to considerably decreased concentration produced by precipitation of the gel, which makes accurate determination of width difficult. Cyanoethylation of the L.S. protein has increased its aggregation tendency, or decreased its solubility, more than was noticed for the previous substituents discussed above.

S-aminoethylation, being a basic substituent, should produce a charged protein which has increased solubility, as observed by Gillespie (1963) for the H.S. proteins. Figure 4-14 indicates the methyl and aromatic regions of the S-aminoethylated H.S. protein are relatively unaffected by pH, a minimum mobility being noted between pH 4 and 9. Methylene resonances of the S-aminoethyl group indicate that a solubility decrease would be observed below pH 10, and Gillespie (1963) has observed a minimum solubility between pH 9 and 10 in aqueous solution. A chemical shift of the methylene resonances indicates protonation occurs at about pH 9 to 10, but this same chemical shift moves the resonance to a position in the spectrum where it is concealed by other methylene resonances. The observed
FIGURE 4-12
S-CYANOETHYL H.S. PROTEIN IN 8 M UREA/D₂O.

Methyl

S-Cyanoethyl

S-Cyanoethyl

Methyl

Aromatic

Peak Height (Arbitrary Units)

pH Meter Reading

Peak Width (P.p.m. x 10²)
FIGURE 4-13
S-CYANOETHYL L.S. PROTEIN
IN 8 M UREA/D2O.
As noted in Chapter 4C(ii), the \( S \)-aminoethyl L.S. protein prepared at pH 11 was insoluble in 8 M urea at all pH's between 2 and 12. The reason for this insolubility is not clear, as only a small amount of cysteine was present in the L.S. protein (Chapter 4D(ii)). This insolubility may be produced by amide cross-links between the \( S \)-aminoethyl group and carboxyl groups present in the protein. Like the \( \epsilon \)-[(\( \gamma \)-glutamyl)lysine], these cross-links would not be detected by amide analysis, although participation by the sulphur in the reaction could be possible (Sykes, 1961). Formation of amides is slower, faster in bases than in acids (Hine, 1962; Asquith & Puri (1969, 1970) have suggested homologous forms between \( 3-(\epsilon \)-aminoethyl) disulphhydrolalanine and dehydroalanine (a amino acrylic acid) in analogous solution. There is an equal possibility that the \( S \)-aminoethyl cysteine can form this type of cross-link with any dehydroalanine present.

The NMR spectra of \( \epsilon \)-[(\( \gamma \)-glutamyl)lysine] and \( \epsilon \)-[(\( \gamma \)-glutamyl)lysine] at approximately 6.02 \( \text{ppm} \), cysteine (keratines) was observed in the aliphatic aromatic at about 6 to 6.41, and does not interfere with observation.
change of S-aminoethyl methylene peak height could be due largely to protonation of the amino group, but it does agree with the solubility data of Gillespie (1963), and may well be related to the isoelectric point of this protein.

As noted in Chapter 4C(ii), the S-aminoethyl L.S. protein prepared at pH 11 was insoluble in water and in 8 M urea at all pH's between 2 and 12. The reason for this insolubility is not clear, as only a small amount of cystine was present in the L.S. protein (Chapter 4D(i)). This insolubility may be produced by amide cross-links between the S-aminoethyl group and carboxyl groups present in the protein. Like the ε-(γ-glutamyl)lysine, these cross-links would not be detected by amino acid analysis, but conditions of preparation are not those which would clearly facilitate amide formation or transamidation, although participation by the sulphur in this reaction may be possible (Sykes, 1961). Formation of amides is however, faster in bases than in acids (Hine, 1962; Roberts & Caserio, 1965).

Asquith & Puri (1969, 1970) have suggested a cross-link forms between 3-(2-aminoethyl) disulphenyl alanine and dehydroalanine (α amino acrylic acid) in alkaline solution. There is an equal possibility that S-aminoethyl cysteine can form this type of cross-link with any dehydroalanine present.

The NMR spectra of two thiol proteins (kerateines) were examined in the presence of approximately 0.02 M thioglycollic acid, which shows a strong singlet resonance at about 6 to 6.4t, and does not interfere with observation
of other resonances. Gillespie (1963) demonstrated that the solubility of a H.S. kerateine in 0.1 M KCl, 0.1 M TRIS, 0.05 M thioglycollic acid, decreases rapidly at pH 8.5 and reaches a low value at pH 7. Greater solubility than this would be expected in 8 M urea, but Figure 4-15 shows the methyl resonances are decreasing in height at pH 8.5 and the protein gels at approximately pH 7.3. Harrap & Gillespie (1963) found that a pH greater than 8 is necessary to increase the extraction of thiol L.S. proteins from wool in 8 M urea at 40°C.

Similarity of the behaviour of the L.S. and H.S. kerateines (Figures 4-15 and 4-16) is more pronounced than with any of the substituted proteins discussed previously. The L.S. kerateine produces a 'hard' gel which cannot be examined below pH 6 and is not soluble at acid pH. In contrast, a 'soft' gel, which can be placed into an NMR tube, is produced by the H.S. kerateine. The insolubility of both L.S. and H.S. proteins in the neutral pH region is clear indication of the need for high pH as well as disulphide bond cleavage, to fully solubilise thiol kerateines (Crewther et al., 1965).

Goddard & Michaelis (1935) found that reoxidized (metakeratin) protein which is a mixture of H.S. and L.S. proteins, is relatively soluble in 0.1 M Na₂CO₃ and 0.1 M NH₄OH but insoluble in N/30 HCl. NMR examination of H.S. metakeratin (Figure 4-17) is in agreement with their observations. The H.S. metakeratin is less soluble and aggregates at higher pH than the H.S. kerateine (Figure 4-15), but the L.S. metakeratin is insoluble in 8 M urea over the pH range 1.5 to 12. Bhatnagar & Crewther (1969)
FIGURE 4-16
L.S. (THIOL) KERATEINE IN
8 M UREA/D₂O PLUS 0.02 M TGA.

- Methyl
- Aromatic

Peak Width (p.p.m. x 10²)

Peak Height (Arbitrary Units)

pH Meter Reading
FIGURE 4-17
H.S. METAKERATIN IN 8 M UREA/D_2O.

While the initial increase in aggregation of the proteins in a 50/50 mixture (by weight) of the H.S. and L.S. kerates at pH 10.2, the proteins do not aggregate to form a gel as happened with previous systems and thioglycollic acid. The apparent interaction is due to the formation as the examination was performed in 8 M thioglycollic acid. Specific interactions have also been observed with components of a helix rich fraction prepared by partial proteolysis of BCKA (Creeth, 1971). Due to increasing broadening of the methyl and aromatic resonances together with the relatively lower methyl peak heights, the methyl peak height was obtained below pH 9.5.

Figure 4-18 shows that a 50/50 mixture of BCKA and BCKA in 8 M urea has a spectrum similar to that of the separate proteins in 8 M urea (Figure 4-3 and 4-5). In Figure 4-5, the methyl peak heights are unaffected by pH. With BCKA, (Figure 4-5) all groups are considerably more soluble than the one examined here.

Figure 4-18 shows the NMR behaviour in 8 M urea/0.5 M thioglycollic acid of the M.S. and L.S. kerates in a 50/50 mixture (by weight) of the H.S. and L.S. kerates. The initial increase in aggregation of the proteins in a 50/50 mixture (by weight) of the H.S. and L.S. kerates at pH 10.2, the proteins do not aggregate to form a gel as happened with previous systems and thioglycollic acid. The apparent interaction is due to the formation as the examination was performed in 8 M thioglycollic acid. Specific interactions have also been observed with components of a helix rich fraction prepared by partial proteolysis of BCKA (Creeth, 1971). Due to increasing broadening of the methyl and aromatic resonances together with the relatively lower methyl peak heights, the methyl peak height was obtained below pH 9.5.

Figure 4-18 shows that a 50/50 mixture of BCKA and BCKA in 8 M urea has a spectrum similar to that of the separate proteins in 8 M urea (Figure 4-3 and 4-5). In Figure 4-5, the methyl peak heights are unaffected by pH. With BCKA, (Figure 4-5) all groups are considerably more soluble than the one examined here.
have prepared a metakeratin from low molecular weight components of the H.S. thiol protein from wool which is considerably more soluble than the one examined here.

Figure 4-18 shows the NMR behaviour in 8 M urea of a 50/50 mixture (by weight) of the H.S. and L.S. kerateines. While the initial increase in aggregation of the protein mixture (i.e. decrease in peak height) occurs at higher pH than with the separate proteins (pH 10 compared with pH approximately 8), behaviour of the mixture at lower pH is noticeably different. A faint precipitate appears at pH 10.2 but the proteins do not aggregate fully to form a gel as happened with previous cases when gels occur. This may indicate a specific interaction between the H.S. and L.S. proteins is occurring in 8 M urea. It is doubtful the apparent interaction is due to disulphide formation as the examination was performed in 0.02 M thioglycollic acid. Specific interactions have also been observed with components of a helix rich fraction prepared by partial proteolysis of SCMKA (Crewther & Dowling, 1971). Due to increasing broadening of the methyl resonances together with the relatively low peak height in this case, accurate methyl peak widths were not obtained below pH 9.5.

Figure 4-19 shows that a 50/50 mixture (by weight) of SCMKA and SCMKB in 8 M urea has a different behaviour to that of the separate proteins in 8 M urea (see Figure 4-3 and 4-5). With SCMKB (Figure 4-3) the SCM methylene resonance shows a decrease in mobility occurs for this group at pH 4, while the methyl group is almost unaffected by pH. With SCMKA, (Figure 4-5) all groups are
FIGURE 4-18

50:50 MIXTURE OF H.S. AND L.S. (THIOL) KERATEINES IN 8 M UREA/D₂O PLUS 0.02 M TGA.

[Methyl]

Peak Width (P.P.m.x10²)

Peak Height (Arbitrary Units)

pH Meter Reading
FIGURE 4-19

50:50 MIXTURE OF SCMKA AND SCMKB IN 8 M UREA/D$_2$O.

FIGURE 4-19

50:50 MIXTURE OF SCMKA AND SCMKB IN 8 M UREA/D$_2$O.

Methyl

SCM

Peak Width (P.p.m. x 10$^2$)

20

22

24

26

28

30

6

8

10

12

P.H Meter Reading

Peak Height (Arbitrary Units)

0

2

4

6

8

10

12

2

4

6

8

10

12

Aromatic

SCM

Methyl

It is believed that co-precipitation is occurring in the low ionic strength solution (Gillespie et al., 1962). Although King (1970) has shown that the concentration of a protein acid solution can be determined from the chemical reaction of the aromatic amino acid at pH 3.0 and in NH$_4$Cl we obtained interesting results. However, at pH 7.5 we obtained similar results as the protein acid is increased, peak heights level off as the protein...
affected by pH to approximately the same extent (the protein gels). In the mixture however, it is the methyl group which shows a decrease in mobility at pH 5 while the SCM group is almost unaffected. This greater effect of pH on the methyl resonances of the SCMKA/SCMKB mixture may be evidence that hydrophobic interactions (Némethy, 1967) are important in stabilising these two proteins when in solution. Alternatively, the NMR spectrum may be that of an averaged spectrum of the two separate proteins. If this latter suggestion is correct, the SCM resonance heights should be considerably greater at pH's above 6, as are those of the two separate proteins. S-carboxymethyl kerateine proteins are not fully separated into H.S. and L.S. proteins by precipitation of the L.S. protein at pH 4.1 in 0.1 ionic strength solution (Gillespie, 1962a), but use of pH 4.4, 0.4 ionic strength solution does achieve the separation (Gillespie et al., 1962). It is believed that co-precipitation is occurring in the low ionic strength solution (Gillespie et al., 1962). Although behaviour in 8 M urea is not necessarily comparable with conditions used for the separation of SCMKA and SCMKB, it is possible that a similar interaction between the two proteins is occurring in 8 M urea.

King (1970) has shown that the concentration of a formic acid solution can be determined from the chemical shift of the HDO resonance in the NMR. S-cyanoethylated H.S. protein was suspended in D₂O and an NMR spectrum was obtained following each addition of small amounts of formic acid (Figure 4-20). As the concentration of formic acid is increased, peak heights level off as the protein
Figure 4-20

NMR SPECTRA OF S-CYANOETHYL H.S. KERATEINE. UNFOLDING WITH FORMIC ACID.

When the protein is dissolved in formic acid, the methyl peak width indicates the protein has been fully unfolded (Bradbury & King, 1969). A concentration of 39% formic acid should thus be sufficient to completely dissolve S-cyanoethylated H.S. protein.

The S-cyanoethyl methylene resonances are still present in formic acid and at 17.7% formic acid the aromatic resonance is overlapped by the much stronger resonance of formic acid.

The S-cyanoethylated H.S. protein is insoluble in formic acid or formic acid solutions, although no residual intact protein was found on amino acid analysis of this protein (Chapter 45). Insolubility of proteins has been attributed to conformational changes after freeze-drying (O'Connell & Woods, 1956) and all the proteins studied in this investigation have been subjected to freeze-drying. Conformational changes produced by freeze-drying should still permit valid comparisons to be made.

The growth in formic acid solutions of increasing strength. The solubility of H.S. and insolubility of L.S. S-cyanoethylated keratin can be used as spectroscopic examination of these proteins, indicating that this procedure may be successful in separation of microfibrils and matrix from reduced and cyanoethylated whole wool.
goes into solution (between 2.5 and 7.5\% formic acid).

When the protein is fully dissolved, addition of further formic acid causes unfolding of the protein until at 35\% formic acid, the methyl peak width indicates the protein has become fully unfolded (Bradbury & King, 1969a). A concentration of 35\% formic acid should thus be sufficient to completely dissolve S-cyanoethylated H.S. proteins.

The S-cyanoethyl methylene resonances are offscale at >9\% formic acid, and at 17.7\% formic acid the aromatic proton resonance is overlapped by the much stronger resonance of formic acid.

The S-cyanoethylated L.S. protein is insoluble in formic acid or formic acid solutions, although no residual cystine was observed on amino acid analysis of this protein (Chapter 4D(i)). Insolubility of proteins has been attributed to conformational changes after freeze-drying (O'Donnell & Woods, 1956) and all the proteins used in this investigation have been subjected to freeze-drying. Conformational changes produced by freeze-drying should have affected L.S. and H.S. proteins similarly and should still permit valid comparisons to be made.

One of the methods employed in Chapter 5 to attempt the separation of microfibrils and matrix from whole wool, was to dissolve the reduced and cyanoethylated matrix (H.S.) protein in formic acid solutions of increasing strength. The solubility of H.S. and insolubility of L.S. S-cyanoethylated proteins observed on NMR spectroscopic examination of these proteins, indicates that this procedure may be successful in separation of microfibrils and matrix from reduced and cyanoethylated whole wool.
(iii) Gel Chromatography.

Although examination of NMR spectra of proteins can clearly demonstrate conditions for fully unfolding proteins, it is not as easy to distinguish between a folded or native conformation and an aggregated form. With proteins which are soluble in their native form, the formation of a gel clearly establishes the protein has aggregated. This distinction is not possible with wool proteins which may be insoluble in their 'native' or folded form, although a large proportion of the insolubility of keratins is due to the presence of disulphide bonds in cystine.

There are methods for determination of molecular weight of proteins which require proteins to be extensively unfolded, i.e. in the random coil form (Davison, 1968; Fish et al., 1969; Parish & Marchalonis, 1970). Therefore application of NMR techniques to proteins can establish if they are extensively unfolded under the conditions to be used for molecular weight determination. Results of NMR spectroscopic examination of various wool proteins, obtained in the previous section (Chapter 4D(ii)), can now be applied to the determination by gel chromatography, of the molecular weight of these proteins, in their fully unfolded state.

Alteration of the pH of an Agarose gel column is more difficult to achieve than pH changes in NMR spectroscopy, therefore investigation of the properties of wool proteins was mainly performed at pH 8.0. To observe the effects of pH on gel chromatographic properties, allowing for the limited pH tolerance of Agarose gels (Fischer, 1969), pH's 4.4, 6.0 and 9.5 were also used. Calibration
of molecular weight (M.W.) vs. elution volume was performed at pH 8.0 and the calibration graph is shown in Figure 4-21. At the other pH's used, a brief calibration was carried out which used only 2 or 3 of the proteins employed for calibration at pH 8.0. The slopes and positions of the calibration graphs for these other pH's were within experimental error of that obtained at pH 8.0.

As pH or other variations, produce changes in the size of a protein in solution (i.e. it becomes more or less folded), these will appear as differences in elution volume of the protein. When the conformation of a protein changes from random coil to a partially folded structure, its molecular size decreases (Tanford, 1961), and its elution volume on gel chromatography will increase (Fischer, 1969; Determann, 1968). The increased elution volume will appear as a decreased effective molecular weight (Figure 4-21). Changes in Sephadex G200 gel chromatographic behaviour have been observed on chemical modification of bovine serum albumin, which were attributed to variation of space filling requirements of the protein (Habeeb, 1966).

On gel chromatography at pH 8.0, all of the H.S. proteins showed very similar chromatograms. The chromatograms have three clearly defined peaks and in most cases an inflexion occurs on the leading edge of the first peak (see Figure 4-22) indicating another higher molecular weight component (of M.W. about 23,000) occurs in lower concentration.

Almost identical gel chromatograms were obtained for SCMKB on Sephadex G100 in 0.3 M sodium acetate-acetic acid buffer at pH 4.5 by Joubert & Burns (1967).
FIGURE 4-21

MOLECULAR WEIGHT CALIBRATION GRAPH.

2.54 x 95 cm. AGAROSE A-5m COLUMN
ELUANT 8 M UREA, 0.01 M TRIS,
0.01 M EDTA, pH 8.0.

<table>
<thead>
<tr>
<th>SCM</th>
<th>Molecular Weight (M.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>65,000</td>
</tr>
<tr>
<td>BSA (Dimer)</td>
<td>130,000</td>
</tr>
<tr>
<td>RNase</td>
<td>13,700</td>
</tr>
<tr>
<td>Trypsin</td>
<td>23,300</td>
</tr>
<tr>
<td>Apomyoglobin</td>
<td>16,800</td>
</tr>
<tr>
<td>Insulin (Av. of Chains)</td>
<td>2,750</td>
</tr>
<tr>
<td>SCM</td>
<td>5,700</td>
</tr>
</tbody>
</table>

For the various alkylating agents employed, the alkylation cysteine content of the proteins employed and the molecular weight increase to be expected is only approximately 5.7% (i.e., 23,000 M.W.

Molecular weights of the proteins listed in Table 4-2, only the BSA and 5-carboxymethyl keratins fractions are significantly different, but the others (experimental) peak in the volume determination is ±5 ml. corresponding to 11,000 Dalton at M.W. 65,000. Other molecular weights of low M.W. peaks are also significantly different, but these peaks are mainly non-specific (see later).
For the various H.S. proteins used, the alkylating agents employed vary in molecular weight, but using the cysteine content of the H.S. proteins as 18 mole% (Table 4-1), the maximum molecular weight increase to be expected is only approximately 5.7% (i.e. 23,000 M.W. would increase to 24,300; or 14,000 M.W. to 14,800 M.W.). Values of molecular weight found are given in Table 4-2 together with the molecular weight of the group added by alkylation.

Of the proteins listed in Table 4-2, only the molecular weights of S-methyl kerateine fractions are significantly different from the others (experimental error in the volume determination is ±5 ml. corresponding to ±1,000 Dalton at M.W. 14,000). Molecular weights of the low M.W. peaks are also significantly different, but these peaks are mainly non-protein (see later).

**TABLE 4-2.**

<table>
<thead>
<tr>
<th>Derivative</th>
<th>M.W. of group added</th>
<th>Molecular Weight of Major Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-methyl</td>
<td>15</td>
<td>10,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6,100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,800</td>
</tr>
<tr>
<td>S-aminoethyl</td>
<td>44</td>
<td>14,800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7,200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,700</td>
</tr>
<tr>
<td>S-cyanoethyl</td>
<td>54</td>
<td>14,400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,500</td>
</tr>
<tr>
<td>S-carboxymethyl</td>
<td>59</td>
<td>14,900</td>
</tr>
<tr>
<td>(SCMK)</td>
<td></td>
<td>7,800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,800</td>
</tr>
<tr>
<td>S-carboxyethyl</td>
<td>73</td>
<td>13,300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3,100</td>
</tr>
</tbody>
</table>

The lower molecular weight of S-methyl kerateine can be
explained partly in terms of the low molecular weight of the alkylating agent, and partly in terms of partial folding of the protein in 8 M urea at pH 8. Figure 4-8 shows that H.S. S-methyl kerateine is more folded at pH 8 than at pH 12, but Figures 4-12 and 4-14 indicate similar folding is to be expected with S-cyanoethyl kerateine and S-aminoethyl kerateine respectively, which is not observed on gel chromatography. A major contributing factor to this discrepancy is the increased possibility of protein aggregation occurring at the high concentration (10%) used in NMR spectroscopy. Much lower protein concentrations (approximately 0.1%) are encountered in gel chromatography, and these may be sufficient to allow H.S. S-cyanoethyl kerateine and H.S. S-aminoethyl kerateine to be fully unfolded and unaggregated while H.S. S-methyl kerateine remains partially folded.

Figure 4-22b shows a gel chromatograph trace of SCMKB prepared with a long dialysis prior to alkylation, Figure 4-22a shows SCMKB prepared by the normal method (Chapter 4C(ii)), but with a 1 week dialysis after alkylation. Peak 3 in Figure 4-22b is considerably decreased in comparison with that in Figure 4-22a. Figure 4-22c is a Sephadex G25 gel chromatograph trace of the SCMKB used in Figure 4-22a. Figure 4-22d is a Sephadex G25 gel chromatograph trace of material which passed through the dialysis bag during preparation of the SCMKB in Figure 4-22b. These are all evidence that the low M.W. peak observed in Agarose A-5m gel chromatography of H.S. proteins (peak 3 in Figure 4-22) is retained by the protein, but can be decreased in concentration by dialysis
FIGURE 4-22
GEL CHROMATOGRAPH TRACES OF SCMKB AND A FRACTION FROM SCMKB.

4-22a and b - AGAROSE A-5m/pH 8.0/8 M UREA
4-22c and d - SEPHADEX G25/0.2 M NH₄HCO₃/pH 9.0

SCMKB WITH LONG DIALYSIS AFTER ALKYLATION

Elution Volume (ml.)

- SCMKB WITH LONG DIALYSIS BEFORE ALKYLATION

- SCMKB WITH LONG DIALYSIS AFTER ALKYLATION

MATERIAL CONCENTRATED FROM DIALYSIS OF b.
prior to alkylation.

The two major low M.W. peaks observed in Figure 4-22d have been examined by amino acid analysis (of the SCM derivative). Only 1 to 2% of the material in the low M.W. peaks is protein, and of this protein, approximately 50 mole % is S-carboxymethyl cysteine. S-carboxymethyl cysteine is formed during alkylation of the protein and is probably not significant. If the protein is dialysed repeatedly in distilled water prior to alkylation (method used for preparation of these proteins, see Chapter 4C(ii)) the low M.W. peak (Figure 4-22a & b) can be considerably decreased in size, as demonstrated above. For some reason, possibly because the material is loosely bound to the protein, it does not dialyse out on extensive dialysis after alkylation (1 week at room temperature in running tap water). One possibility is that the low M.W. peak 3 may comprise nucleic acid fragments as Fischer (1969) states that nucleic acids absorb strongly at 260 nm., and they are not observed on amino acid analysis by the Technicon method (Howells, 1971). Gillespie (1971a) has stated the low molecular weight components may be due to the presence of salts or a refractive index change.

The NMR investigations in Chapter 4D(ii) indicate that the four L.S. proteins, SCMKA, S-carboxyethyl keratine, S-methyl keratine and S-carbamidomethyl keratine should all be fully unfolded at pH 8.0. Gel chromatography of these proteins shows they behave similarly and have closely related molecular weight components (Table 4-3).
TABLE 4-3.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Molecular Weight of Major Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-methyl</td>
<td>120,000 50,000 15,000 6,300 3,600 ND#</td>
</tr>
<tr>
<td>S-carbamidomethyl</td>
<td>118,000 53,000 14,000 6,400 3,900 3,100</td>
</tr>
<tr>
<td>S-carboxymethyl (SCMKA)</td>
<td>124,000 47,000 15,200 6,300 4,400 2,600</td>
</tr>
<tr>
<td>S-carboxyethyl</td>
<td>113,000 54,000 14,500 6,900 3,700 2,600</td>
</tr>
<tr>
<td># Not detected.</td>
<td></td>
</tr>
</tbody>
</table>

A typical chromatogram of SCMKA at pH 8 is presented in Figure 4-23a.

The peaks of approximate M.W. 14,500, 6,900 and 3,000 correspond to those observed on chromatography of the H.S. proteins, and Harrap & Gillespie (1963) report that the plasmolysis method of preparing L.S. proteins results in contamination of the protein with about 10% of H.S. protein. As the NMR results presented in Chapter 4D(ii) indicate that both the L.S. and H.S. proteins used, should be almost completely unfolded in 8 M urea at pH 8, at least partial separation of the H.S. proteins from the L.S. proteins is to be expected. Resolution of these peaks is not good, possibly due to the interactions observed between L.S. and H.S. proteins during NMR spectroscopic examination (Chapter 4D(ii)), and no attempt was made to isolate the fractions by repeated chromatography.

Variation in the quantity of material eluted at the excluded volume was observed with the L.S. proteins. This may be due to minor differences in sample preparation.
prior to chromatography, or to the known tendency of these proteins to aggregate (Crewther et al., 1965). Examination by NMR spectroscopy would not detect small quantities of aggregated protein, and therefore this does not disagree with conclusions made from NMR spectroscopy.

The effect of pH on gel chromatographic behaviour was observed on two L.S. and two H.S. proteins. Figure 4-3 indicates SCMKB is unfolded from pH 11.2 to 5.5. Below this point there is evidence that some folding of the protein occurs or that mobility of the SCM methylene protons is restricted. Gel chromatographic results for SCMKB are presented in Table 4-4. Only elution volumes for the major peaks are quoted as the molecular weight of these proteins is not expected to change. Variations in the elution volume in this case are related to molecular size and not molecular weight.

### Table 4-4

<table>
<thead>
<tr>
<th>pH</th>
<th>Elution Volume of Major Peaks (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>409 454 535</td>
</tr>
<tr>
<td>6.0</td>
<td>392 435 525</td>
</tr>
<tr>
<td>8.0</td>
<td>394 438 543</td>
</tr>
<tr>
<td>9.5</td>
<td>384 433 514</td>
</tr>
</tbody>
</table>

Variation in the peak of largest elution volume is probably not significant due to its low protein content, as noted earlier. Table 4-4 shows that elution volume is little changed by pH between 9.5 and 6.0. However, the elution volume increases at pH 4.4, corresponding to a
decrease in effective size of the molecules at this pH. The NMR behaviour of SCMKB (Figure 4-4) only indicated a decrease in mobility for SCM methylene protons, and no change in mobility of methyl or aromatic protons. Gel chromatographic results for SCMKB indicate the molecule has in fact decreased in size, although the only decrease in mobility was associated with the SCM group.

The gel chromatography and NMR spectroscopic behaviour of SCMKB can be summarised as follows:

1. There is no aggregation of SCMKB occurring in 8 M urea between pH 2 and 12.
2. A decrease in size of the molecule occurs, with a minimum size probably at about pH 4 (Figure 4-3).
3. The carboxyl group of S-carboxymethyl cysteine in the protein is involved (possibly primarily involved) in the folding process, but methyl side chains of isoleucine, leucine and valine, and also phenylalanine are not involved in this folding.

The S-carboxyethyl kerateine H.S. protein was only examined at pH 8.0 and 4.4 but the gel chromatographic behaviour indicates that this protein also, is more folded at pH 4.4 (Table 4-5). The difference in elution volumes between pH 8.0 and 4.4 are not as obvious as those of SCMKB, but they have changed in the expected direction.

TABLE 4-5.

<table>
<thead>
<tr>
<th>pH</th>
<th>Elution Volume of Major Peaks (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>418 458 526</td>
</tr>
<tr>
<td>8.0</td>
<td>405 450 535</td>
</tr>
</tbody>
</table>
The L.S. proteins SCMKA and S-carboxyethyl kerateine were also examined, but as these proteins precipitate (or gel) below pH 6.0 they could not be chromatographed at pH 4.4. S-carboxyethyl kerateine commences to gel before pH 6.0 and thus could not be chromatographed at this pH, due to the risk of clogging the gel column. Figures 4-4 and 4-7 indicate that little, if any, difference in elution volumes should be observed between pH 6.0 and 9.5. Only slight differences in elution volumes were observed, although there was considerably more scatter of the results than with the H.S. proteins. Results are given in Table 4-6.

<table>
<thead>
<tr>
<th>pH</th>
<th>Elution Volumes of Major Peaks of SCMKA (ml.)</th>
<th>Elution Volumes of Major Peaks of L.S. S-carboxyethyl Kerateine (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>ND# 287 389 462 ND</td>
<td>215 280 400 467 519 549</td>
</tr>
<tr>
<td>8.0</td>
<td>208 292 390 472 507 550</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>ND 283 380 458 515 553</td>
<td></td>
</tr>
</tbody>
</table>

# Not detected.

Figure 4-23 gives two typical gel chromatograms for SCMKA at pH 6 and 8. Note the variation in the amount of material eluted at the excluded volume, as occurs with most L.S. proteins. These variations do not correlate with pH, i.e. the quantity of material does not
FIGURE 4-23
GEL CHROMATOGRAPH TRACE OF SCMKA AT DIFFERENT pH.

a
pH 8.0

b
pH 6.0
necessarily increase as the pH approaches that at which aggregation occurs, and are probably due to slight variations in sample preparation methods.

NMR spectroscopic behaviour of the mixture of SCMKA and SCMKB (Figure 4-19) indicated that it remains in solution over the full range of pH values examined. Gel chromatography of SCMKA could not be performed below pH 6, as the protein gels, but a 50/50 mixture of SCMKA and SCMKB remains in solution and was chromatographed at pH 6 and pH 4.4. The chromatograms were similar to that of SCMKA (Figure 4-5) but the peaks which correspond to SCMKB at approximately 400, 460 and 530-550 ml. were considerably larger. Interaction between SCMKA and SCMKB must be responsible for maintaining the SCMKA in solution at pH 4.4 in 8 M urea.

4E. DISCUSSION

The method of preparation of proteins examined in this Chapter was selected so that if possible, the variations in properties were due only to the alkylation agent used (i.e. to the alkylated cysteine). One other variable which may cause differences between the proteins is the alkylation step itself. Long alkylation time of reduced whole wool fibres has been observed to decrease the solubility of their component proteins (Maclaren et al., 1968). Conditions used in this step were selected to give rapid reaction with -SH groups and minimum non-specific alkylation of other groups. These conditions were met, except for the slow alkylation observed with iodoethanol,
and possibly with ethyleneimine.

Suitable alkylation times have been published for some of the reagents used. Cecil (1963) found that reaction with iodoacetic acid was rapid and specific for protein thiol groups in solution, and Thompson & O'Donnell (1962) believe it is complete in 30 min. at pH 8.5. Alkylation with ethyleneimine (Raftery & Cole, 1963) is generally complete within 30 min. at pH 8.6 (Cole, 1967).

Acrylonitrile is prone to alkylate ε amino groups of lysine (Weil & Seibles, 1961; Riehm & Scheraga, 1966) but Cavins & Friedman (1968) have shown the reaction of -SH with acrylonitrile is specific and complete in 20 min. Amino acid analysis of the proteins alkylated with acrylonitrile, showed no evidence of non-specific alkylation of lysine. S-cyanoethyl proteins are of interest as the L.S. protein is found to be easier to separate into Components 7 and 8 than is SCMKA (Frater, 1966; O'Donnell et al., 1968). As acrylonitrile introduces no extra charges into the protein, it is believed to cause more efficient fractionation by methods which depend on charge difference (Frater, 1966).

The NMR spectroscopy data for various L.S. and H.S. proteins presented in Figures 4-2 to 4-20 sometimes can be correlated with solubility of these same proteins. Gillespie (1960, 1963) determined the solubility of SCMKB in low ionic strength solutions as a function of pH and showed a solubility minimum occurs at about pH 3. As NMR spectroscopy is undertaken at considerably higher concentrations (10%) than those found at the solubility minimum by Gillespie (approx. 0.1%), then
Figure 4-2 probably demonstrates the equivalent of a decrease in solubility prior to the minimum at pH 3. Figure 4-2 indicates that all sections of the protein are folding or aggregating below pH 5.

However, Gillespie (1963) has shown that the S-carbamidomethyl, S-methyl and S-cyanoethyl H.S. proteins are insoluble over the lower pH range (below pH 9, 11 and 12 respectively) in water. NMR spectroscopy indicates that these proteins are soluble over the whole pH range in 8 M urea with only slight folding at lower pH. Bhatnagar & Crewther (1969) prepared a metakeratin which was considerably more soluble than the one examined in this work (Figure 4-17). Their metakeratin however was prepared from a more soluble protein fraction left after precipitation of part of the reduced H.S. kerateine at pH 8.4.

The latter cases above, indicate NMR spectroscopic behaviour does not always correlate with solubility properties, particularly in different solvents. Random coil proteins are not always more soluble than the folded or native structure, as would generally be indicated from Figures 4-2 to 4-20. Some proteins aggregate or precipitate on unfolding in urea or guanidine hydrochloride or on heating (McDonald & Phillips, 1969). Both serum albumin and ovalbumin are less soluble when heat denatured, than in the native conformation (Tanford, 1961). Denaturation of ovalbumin by urea has been studied (Simpson & Kauzmann, 1953; Frensdorff et al., 1953); the initial unfolding is followed by a slow irreversible aggregation and finally gelation of the protein.

NMR spectroscopy shows where denaturation or unfolding
has occurred (Bradbury & King, 1969a, give the details of an NMR unfolding test) and the unfolding data obtained can be correlated with other work on unfolding of SCMKA and SCMKB. Harrap (1963) determined that SCMKA is 50% α helical in aqueous solution at pH 9.1 (from $b_0$ values) and a reversible transition to the random coil conformation occurs in up to 6 M urea, the $b_0$ values start to decrease when 2 M urea concentration is reached. Figure 4-5 shows that SCMKA should be in the fully unfolded conformation at pH 9.1 in 8 M urea. Bhatnagar & Crewther (1967) examined U.V. absorption spectra and O.R.D. of SCMKA and concluded that 4.3 M urea caused 90% unfolding at pH 8 but only 50% of the α helical content (from $b_0$) is lost under these conditions. They deduced that the chromophoric groups are unfolding first and these are not in the main helical section of the protein. The H.S. protein SCMKB has been shown to be a random coil in aqueous solution (Gillespie, 1962a, 1963), and the NMR results indicate that SCMKB would be expected to remain as the random coil above pH 5 as indicated by Figure 4-2.

The results of NMR spectroscopic examination of the various wool proteins have been summarised in Table 4-7. This table shows the pH ranges over which the particular protein is fully unfolded in 8 M urea. Outside these pH ranges, varying degrees of folding or aggregation are to be expected.

Aggregation occurring at pH 5.5 - 6 for SCMKA and decreased mobility of the SCMC CH$_2$ group in SCMKB at pH 4, may be related to their isoelectric points. Gillespie et al. (1962) quoted pH 4.4 and 2.9 as the
**TABLE 4-7.**

**pH RANGE FOR FULL UNFOLDING**

<table>
<thead>
<tr>
<th>Protein</th>
<th>H.S.</th>
<th>L.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCMKB</td>
<td>5.5 to 12</td>
<td>* 6.2 to 12</td>
</tr>
<tr>
<td>S-carboxyethyl</td>
<td>7 to 12</td>
<td>* 6.5 to 12</td>
</tr>
<tr>
<td>S-methyl</td>
<td>6 to 12</td>
<td>* 7 to 12</td>
</tr>
<tr>
<td>S-carbamidomethyl</td>
<td>2 to 10</td>
<td>* 8 to 12</td>
</tr>
<tr>
<td>S-cyanoethyl</td>
<td>2 to 5.5</td>
<td>* &gt;11</td>
</tr>
<tr>
<td>S-aminoethyl</td>
<td>10 to 12</td>
<td>* insoluble</td>
</tr>
<tr>
<td>Thiol</td>
<td>8.5 to 12</td>
<td>8.5 to 12</td>
</tr>
<tr>
<td>Metakeratin</td>
<td>11 to 12</td>
<td>insoluble</td>
</tr>
</tbody>
</table>

**pH Range in D₂O or Formic Acid Conc.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCMKB</td>
<td>5.5 to 12</td>
</tr>
<tr>
<td>S-cyanoethyl</td>
<td>Fully unfolded in</td>
</tr>
<tr>
<td></td>
<td>&gt;35% formic acid.</td>
</tr>
</tbody>
</table>

* pH 2 to 12 can be used if a small amount of folding can be tolerated.

Isoelectric points of SCMKA and SCMKB respectively, based on the pH of precipitation or minimum solubility. Although minimum solubility of proteins at their isoelectric points is well known (Tanford, 1961), these values may not be a reliable estimate of isoelectric points of proteins from a different sample of wool, and, being a mixture of proteins, prepared by a different method. It is probably significant however, that the difference in isoelectric points between SCMKA and SCMKB (Gillespie et al., 1962) is 1.5 units, and an estimate of isoelectric points from Figures 4-3 and 4-5 gives the same difference (isolectric...
point from the minimum in the SCMC peak heights of SCMKB is pH 4.0 approximately, and that from the various SCMKA resonances is approximately 5.5). While it is appreciated that observations from NMR spectra of proteins are not normally a good indication of their isoelectric points, in SCMKB there is evidence that this procedure may be satisfactory. SCMKB has a large excess of carboxyl groups (mainly of SCMC) over basic groups on the amino acid side chains in the protein (an excess of approximately 1,400 µM/g, calculated from the amino acid compositions quoted in Crewther et al., 1965, p.217, allowing that all the ammonia found comes from side chain amides), and hence the pKₐ of SCMC probably has a controlling influence on the isoelectric point of SCMKB. The excess of acidic over basic side chains, calculated similarly, is only approximately 500 µM/g for SCMKA and a higher isoelectric point would be expected in this case. Problems in determination of pH (or pD) in 8 M urea solutions of proteins (Chapter 4C(iii)), could also contribute to the discrepancy between the isoelectric points suggested by NMR spectroscopy and those of Gillespie et al. (1962).

Gel chromatography of SCMKB in 8 M urea at pH 4.4 (Chapter 4D(iii)), clearly shows that the SCM peak height minimum can be correlated with a decrease in molecular size at this pH. As neither the methyl nor the aromatic resonances are affected by pH in this region, it seems logical to assume the folding is caused by an electrostatic effect, i.e. it is occurring at the isoelectric point. This decrease in molecular size is expected; as the pH drops to that of the isoelectric point, SCM
carboxyl groups are protonated until at the isoelectric point there are an equal number of positive and negative charges on the molecule, and mutual repulsion of like charges will be at a minimum. Below the isoelectric point, further protonation causes an excess of mutually repulsive positive charges and the protein expands again. Similar behaviour is noted with the S-carboxyethyl H.S. protein (Figure 4-6) but observation of pK in this case is complicated by other chemical shifts occurring (Chapter 4D(ii)).

In all of these cases, the observations can also be interpreted as a localised folding occurring in the cysteine regions only, which does not cause folding in the region of the methyl groups. A possible explanation comes from the known fairly high incidence of cysteine-cysteine sequences in H.S. proteins (Blackburn & Lee, 1965; Lindley & Haylett, 1967; Asquith & Shaw, 1968), and mutual interactions between the two cysteines could possibly account for the observations noted.

Neither of these two explanations however, can account for the case of S-methyl H.S. kerateine, which also shows a peak height minimum occurs for the S-methyl group alone. The S-methyl group should behave as a neutral substituent and remain unaffected by pH. No explanation can be offered in this case.

A different explanation is also required for the 50/50 mixture of SCMKA and SCMKB (Figure 4-19), which appears to be folding at lower pH by interactions involving the methyl groups. This could be an example of hydrophobic interactions occurring, as is described by Némethy (1967).
Examples of both electrostatic and hydrophobic interactions occur in insulin dimers and hexamers as determined by X-ray diffraction (Adams et al., 1969). In the dimer, an 'aromatic cage' is formed between the B26 tyrosines and B24 phenylalanines in both molecules, and residues B23 to B28 hydrogen bond in an antiparallel arrangement. In the hexamer (of 3 dimers), the separate dimers are 'hooked' by B1 phenylalanines and close contact (probably H bonded) is observed between all of the B13 glutamic acids.

There are many cases of decreased reactivity of 'buried' amino acid side chains of proteins, where normal reactivity is achieved on denaturation of the protein. Examples include the varying reactivity of cystine in insulin and ribonuclease in the absence of denaturants (Cecil & McPhee, 1959); the variable reactivity of N-bromosuccinimide towards tryptophan has been used as a criterion for 'buried' and 'exposed' tryptophan residues in proteins (Spande & Witkop, 1967); and deuterium exchange is used as a measure of the number of potentially exchangeable protons which are unavailable because of hydrogen bonding in the native structure (Di Sabato & Ottesen, 1967; Tanford, 1961).

Frater (1971) used Hoare & Koshland's (1967) method to estimate the total carboxyl content of reduced and S-cyanoethylated or S-carboxymethylated L.S. proteins from wool. A water soluble carbodiimide (N-(3-dimethylamino propyl) N-ethylcarbodiimide HCl) was used in the presence of a nucleophile (glycine methyl ester HCl, L-argininamide HCl, or L-arginine methyl ester di-HCl)
to react with carboxyl groups in the presence of a
denaturant (6 M guanidine hydrochloride or 8 M urea) at
pH 4.8. Frater found that the reactivity of carboxyl groups
appears to be almost completely lost in the S-cyano-
ethylated protein, but only minor losses in reactivity
were observed for the S-carboxymethylated protein.

It is not possible to draw a direct parallel between
the unfolding observed by NMR spectroscopy for
S-cyanoethyl L.S. proteins in 8 M urea and the work
performed by Frater (1971), as he failed to note the pH
employed, and also used 6 M guanidine hydrochloride. The
original work by Hoare & Koshland (1967) was performed
in 5 M guanidine hydrochloride or 7.5 M urea at pH 4.8,
and it will be assumed that Frater used this pH.

It has been stated that guanidine hydrochloride is
a stronger denaturing agent than urea (White, 1967), but
there are cases where it has been shown to be less
effective than urea (Tanford, 1961; Simpson & Kauzmann,
1953), thus it cannot be assumed per se that guanidine
will be more effective in denaturing proteins than urea.
In fact it is important to realise that generalisations
concerning the complete unfolding of proteins in strong
denaturants are rarely valid. Bradbury & King (1969a)
have shown several cases where proteins retain some
residual folding or other interactions in various strong
denaturants. Insulin, trypsin and myoglobin are
incompletely unfolded in 8 M urea and 6 M guanidine
hydrochloride at pH 4 to 5. Pepsin shows evidence of
interactions in 8 M urea (pH 4 to 5) and myoglobin is
partially folded in both formic acid and deutero-
trifluoroacetic acid but apparently completely unfolded
in dichloroacetic acid (Bradbury & King, 1969a). Hoare
& Koshland (1967) themselves report the low reactivity
of lysozyme with carbodiimides due to incomplete
unfolding in 7.5 M urea, and subsequent reaction of the
carboxyl groups is achieved on complete unfolding of
the lysozyme in 5 M guanidine hydrochloride at pH 4.8.

Examination of Figures 4-4 and 4-13 shows that a
considerable difference exists between the behaviour of
SCMKA and the S-cyanoethyl L.S. proteins under the same
conditions (i.e. 8 M urea, 10% concentration) on NMR
spectroscopic examination. Although the SCMKA is
folded and aggregated at pH 5.8 (Figure 4-5) which is
one pH unit higher than that possibly used by Frater, the
S-cyanoethyl protein has aggregated at pH 8 (Figure
4-13), which is considerably higher than the pH used
by Frater. It is most likely that the combination of
lower concentration and use of 6 M guanidine hydrochloride
permits the SCMKA to remain unfolded at pH 4.8 where the
NMR results indicate it should possibly be folded. How­
ever, it is considered that both 6 M guanidine hydro­
chloride and the lower concentration are not sufficient
to allow the S-cyanoethyl protein to remain unfolded
at pH 4.8, when folding or aggregation occurs at or above
pH 8 in 8 M urea. This folding of the S-cyanoethyl L.S.
protein is probably the most likely cause of the low
reactivity of the carboxyl groups in this protein as
observed by Frater (1971).

Folding of the S-cyanoethyl protein would not prevent
titration of the carboxyl groups as Frater observed,
(Di Sabato & Ottesen, 1967; Tanford, 1961) but would no doubt prevent access of the relatively large carbodiimide molecule. The considerably lower concentration of protein used by Frater (probably 13.3 mg/ml., 1.3%) would decrease the possibility of aggregation of the protein occurring. Aggregation, or folding of proteins does not always cause a visible precipitation and the precipitation may be slow to form (e.g. ovalbumin, Simpson & Kauzmann, 1953; Frensdorff et al., 1953), therefore Frater may have had no indication (by e.g. solution turbidity) that the S-cyanoethyl protein had folded or aggregated at pH 4.8.

This example indicates the advantage of examining a protein by NMR spectroscopy prior to reaction to ensure it is in a suitable conformation for full reactivity of the side chains. It also suggests that had Frater used a slightly lower pH for this reaction, he may have also observed low reactivity of the carboxyl groups in SCMKA.

Gel chromatographic peaks of the proteins examined in this thesis are not fully separated or symmetrical, indicating the estimates of molecular weight obtained may not be completely reliable. However gel chromatographic examination of the SCM proteins has given results in good agreement with those published previously. For SCMKA, Thompson & O'Donnell (1965) obtained 45,000 M.W. for both components 7 and 8 on Sephadex G200 columns in 8 M urea pH 7.4, 5 M guanidine hydrochloride pH 7.0, or 14 M formamide. The values obtained in this work (47,000 and 15,200 for SCMKA with aggregates of about 120,000, Chapter 4D(iii)) are close to this figure. Agreement is more complete when viewed in terms of the proteolysis
experiments of Crewther & Dowling (1971), and Hogg et al. (1971), in which partial digestion of SCMKA gives a helix rich fraction which is thought to comprise 3 sub-units of approximately 13,000 M.W.. Allowing that a certain amount of molecular weight is lost on proteolysis, these values agree satisfactorily with those obtained for SCMKA (Table 4-3).

Molecular weight determinations on SCMKBl and SCMKB2 have given values of 27,000 and 22,000 from sedimentation coefficients (Gillespie, 1963). As noted in Chapter 4D(iii) on gel chromatography of SCMKB in 8 M urea, there is evidence for a protein of M.W. about 23,000, together with others of M.W. 14,000 and 8,000.
CHAPTER 5

APPROACHES TO THE SEPARATION OF MICROFIBRILS FROM WOOL.

5A. INTRODUCTION

High resolution electron microscopic examination of cross-sections of osmium stained wool shows a lightly stained microfibril embedded in a densely stained matrix (e.g. Rogers, 1959, 1959a). This differential staining led Birbeck & Mercer (1957) to suggest these two histological structures are related to the two protein fractions of different sulphur content isolated by Alexander & Earland (1950a), the microfibrils corresponding to the low sulphur protein fraction. Rogers (1959) extracted a high sulphur content protein from oxidized wool with neutral buffer, and found that material was removed from between the microfibrils (i.e. matrix protein). More recently, Gillespie et al. (1964) examined fibres from a sheep which had been fed a sulphur rich diet and noted a clearly observable increase in the amount of matrix, could be seen with the electron microscope.

Filshie & Rogers (1961) examined wool cross-sections at high magnification in the electron microscope and concluded the microfibrils (80 angstrom diameter) are composed of protofibrils (20 angstrom diameter). Nine protofibrils were believed arranged peripherally around a central pair, by analogy with a similar structure which occurs in animal flagella (Fawcett & Porter, 1954; Gibbons & Grimstone, 1960). This arrangement has become known as
Since Filshie & Rogers (1961) suggested the protofibrillar structure of the microfibril, attempts have been made to isolate microfibrils to confirm this suggested structure. Dobb (1964), by ultrasonic disintegration of reduced porcupine quill tips, and Johnson & Speakman (1965) and Dobb et al. (1965) by ultrasonic irradiation of reduced wool, have all isolated material of microfibrillar or protofibrillar dimensions.

Contamination with cellulose has been suggested as a possible source of 'protofibrils' in the investigations mentioned above (Millward, 1969; Fraser et al., 1969). Filshie & Rogers' (1961) 9 + 2 structure has also been criticised as it is only observed in an underfocused condition (Johnson & Sikorski, 1962; Millward, 1970), but a 'ring-core' structure of the microfibril is still observed at true focus (Millward, 1970). Rogers & Clarke (1965) isolated protofibrils of about 20 angstrom diameter from hair follicles of the guinea pig, the amount isolated being greatly increased after a brief ultrasonic treatment in 90% formic acid. Unfortunately this work is still subject to the criticisms of contamination expressed above.

SCMKA from wool has been shown to form aggregates of rods, and filaments (Crewther et al., 1968) and similarly with a 'helix rich' fraction of SCMKA produced by proteolysis of SCMKA with several enzymes (Crewther et al., 1968). This helix rich fraction has a molecular weight of 42,000 and an axial ratio of 8 to 10:1 (Crewther et al., 1968) which corresponds to a triple stranded structure. There is also evidence for chains
containing 2 types of helical unit which interact specifically (Hogg et al., 1971; Crewther & Dowling, 1971). The use of enzymes for isolation of wool protein components requires careful examination however, due to the recent observation that SCMKA isolated from wool after proteolysis of the fibre, shows substantial decreases in molecular weight (Gillespie, 1970a, 1971). Also, Crewther et al. (1968) have suggested the non helical portions of low sulphur proteins are partly responsible for the aggregation to form filaments as observed in the electron microscope.

A clean isolation of microfibrils in their native conformation (preferably in high yield) is one approach to further clarification of the problem of microfibril composition and structure. Another approach has been the recent synthesis of guinea pig hair low sulphur proteins in vitro (Steinert & Rogers, 1971). The synthesised proteins were shown by co-chromatography on Sephadex and co-electrophoresis on acrylamide gel, to be very similar to the native proteins, and even precipitated with anti-sera against the native hair follicle proteins.

Certain of the investigations presented in this Chapter have been of an exploratory nature and are therefore not exhaustive. In the discussion, an attempt has been made to indicate areas which may be fruitful for further examination.
5B. MATERIALS AND METHODS.

(i) Materials.

Wool used was prepared as described in Chapter 1B and was used to prepare cortical cells as also described in Chapter 1B.

Dry dimethyl sulphoxide (DMSO) was prepared by vacuum distillation from over calcium hydride, and was stored in a vacuum desiccator. Aqueous acrylonitrile was prepared by adding water to acrylonitrile until a small amount of water remained in the bottom of the container (about 3.6% water). Formic acid was redistilled A.R. grade as described in Chapter 1B, and all other reagents were A.R. grade and were used without further purification.

(ii) Yields.

Yields were calculated from residues which had been dried by freeze-drying or by evacuation at 0.001 mm. Hg for 1 hr. at 100°C. If the wool fibre or cortical cells had been dried before use these weights were taken, if however the fibres or cells were previously conditioned at room temperature and humidity, the residues were also allowed to equilibrate at room temperature and humidity prior to weighing. With the sodium hydride/DMSO and sodium/liquid ammonia reductions, fibres or cortical cells were in all cases dried at 100°C for 2 hr. under 0.001 mm. Hg.

(iii) Amino Acid Analyses.

Amino acid analyses were performed with the Technicon amino acid analyser as described in Chapter 1B. Estimates of the percentage reduction in disulphide...
content at high levels of reduction, have been made by double loading the amino acid analyser. Having performed one determination under normal conditions it is possible to estimate accurately the residual cystine content by loading double the normal quantity of hydrolysate. Care was taken to exclude oxygen completely during the hydrolysis and it is therefore believed the results represent real quantities of cystine in the protein.

Elution positions of the various substituted cysteines on the Technicon amino acid analyser are given in Chapter 4C(v).

(iv) Microscopy.

Light microscopy was performed as described in Chapter 1B. Electron microscopic techniques used are described in Chapter 2B but negative staining methods were not described.

Dodecatungstophosphoric acid (KPTA) solution (1%) was brought to pH 4.5 by the addition of 10N KOH. A drop of solution to be examined, was placed on a previously prepared carbon coated, collodion covered grid (Chapter 2B) and removed in 1 to 10 seconds by touching with a filter paper. A drop of the 1% KPTA solution was added and allowed to remain on the grid for the same time as the previous solution, and removed similarly. With negatively stained preparations, contamination while in the electron microscope is a problem (Goodchild, 1970) and all possible precautions were taken to avoid contamination (i.e. use of all cold traps).

(v) Reductions.

(a) Sodium hydride/DMSO - The method used was slightly
modified from that of Krull & Friedman (1967). Sodium hydride to cystine ratio used was 5:1 and other reaction conditions are noted in the results section (Chapter 5C). With aqueous acrylonitrile as the alkylating agent, polyacrylonitrile formed and had to be washed from the fibres or cortical cells with fresh DMSO, in which it is soluble. Methyl iodide was used as an alkylating agent in an attempt to overcome this problem, but it reacts with DMSO (Major & Hess, 1958; Smith & Winstein, 1958), and was unsatisfactory.

(b) Tri-n-butylphosphine and Triphenylphosphine - Reductions of wool or cortical cells with tri-n-butylphosphine (TBP) were performed basically in accordance with the sequential one-bath method of Maclaren & Sweetman (1966) (Sweetman & Maclaren, 1966; Maclaren et al., 1968) normally with a TBP to cystine ratio of 2:1, and 10:1 ratio of alkylating agent to potential thiol groups. All reductions were performed under nitrogen, which was used to flush out the flask prior to, and during addition of the TBP. Where modifications to this process occur, they are detailed in the results section (Chapter 5C). Partial reduction of disulphide bonds was achieved by decreasing the TBP to cystine ratio as suggested by Maclaren et al. (1968). After reduction and alkylation the fibres or cells were washed with absolute alcohol and briefly with water (due to the risk of plasmolysis, Harrap & Gillespie, 1963) prior to drying.

Triphenylphosphine ($\Phi_3$P) was used to reduce wool in DMSO instead of in n-propanol/pH 8 buffer, or in formic acid solutions of varying strengths. Humphrey & Hawkins
(1964) claim \( \phi_3 P \) completely reduces disulphide bonds in 0.1N perchloric acid. With the formic acid solutions, the residues were separated from the solution at the centrifuge and washed with formic acid solutions of the same strength. Both residue and supernatant were then dialysed over-night in water, as the formic acid solutions melt during freeze-drying. It was found that \( \phi_3 P \) does not completely dialyse away, but a brief washing with diethyl ether removes the remaining amount of \( \phi_3 P \).

(c) Sodium/Liquid Ammonia - Reductions were carried out according to the method of Benisek & Cole (1965), but at \(-77^\circ C\) instead of \(-33^\circ C\). Other modifications made, were to redistil the liquid ammonia prior to use and to alkylate the reduced disulphide bonds with iodoacetic acid. The liquid ammonia was redistilled by condensing a flow of ammonia gas on a cold finger condenser filled with dry ice/isopropanol. When sufficient liquid ammonia had accumulated (25 ml.) the ammonia supply was stopped and the liquid was then allowed to evaporate and condense on another cold finger condenser over the reaction vessel through which a continuous stream of dry nitrogen was passed. Weighed amounts of metallic sodium were washed with 40°-60° petroleum ether and added to the reaction flask, which was stirred with a glass covered magnetic stirrer bar. After reaction the excess sodium was destroyed by addition of ammonium chloride until the blue colour had been discharged. The liquid ammonia was then allowed to boil off under dry nitrogen, and 25 ml. water (containing a 2:1 mole excess of iodoacetic acid (at pH 8) to potential thiol groups) was added to alkylate the product.
This final mixture was dialysed for 24 hr. and then freeze-dried, or filtered off if fibres were still recognisable in the mixture.

(vi) Solubility Testing.

In all cases, solubility was checked by immersing wool or cortical cells in the particular solvent in the ratio 1:500. Normally 50 ml. of solvent was used with 0.1 g. of fibre or cells and the mixture was ultrasonically disintegrated (Bradbury & Chapman, 1964), vibromixed (Bradbury et al., 1966), gently shaken in a stoppered vessel on a laboratory shaker, or simply allowed to stand for the required time. Times of treatment and solvents are given in the results section (Chapter 5C).

(vii) Extractions and Digestions.

Wool stained with osmium tetroxide was prepared as described in Chapter 2B(ii) for electron microscopic examination.

The high sulphur kerateine was extracted from wool, and wool stained with osmium, by the low temperature preferential method of Harrap & Gillespie (1963). With osmium stained wool, the first extraction was allowed to continue for 21 days at 0-4°C due to the very slow rate of extraction. Protein from the osmium stained wool colours the solution, and extraction was continued until it was estimated no further increase in colour was occurring. Following extraction, the fibres were alkylated with iodoacetic acid at pH 8 in a solution which was 0.5 M in KCl.

Enzymic digestions were performed using a pH stat as described in Chapter 3B. Trypsin digestions were at
pH 8 and pepsin digestions at pH 2, and both were continued for 100 min. To stop the reactions, the trypsin digest was acidified, and the pH of the pepsin digest raised to 4 (Smyth, 1967). The clear supernatant from centrifugation of these digest was negatively stained with KPTA and examined in the electron microscope.

5C. RESULTS.

(i) Reduction of Cystine.

(a) Sodium hydride/DMSO - The results of several reductions with sodium hydride in DMSO are presented in Table 5-1. (Bovine serum albumin = B.S.A.)

TABLE 5-1.

REDUCTION WITH NaH/DMSO

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reduction Time</th>
<th>Alkylating Agent</th>
<th>Alkylation Time</th>
<th>Percentage Reduction #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical</td>
<td>5½ hr.</td>
<td>Acrylonitrile</td>
<td>30 min.</td>
<td>0</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical</td>
<td>15 min.</td>
<td>Acrylonitrile</td>
<td>30 min.</td>
<td>50</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wool</td>
<td>1 hr.</td>
<td>Acrylonitrile</td>
<td>30 min.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wool</td>
<td>2½ hr.</td>
<td>Methyl iodide</td>
<td>30 min.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.S.A.</td>
<td>1 hr.</td>
<td>Acrylonitrile</td>
<td>30 min.</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.S.A.</td>
<td>1 hr.</td>
<td>Methyl iodide</td>
<td>30 min.</td>
<td>44</td>
</tr>
</tbody>
</table>

# Percentage reduction of disulphide bonds determined from amino acid analysis.
<table>
<thead>
<tr>
<th>No.</th>
<th>Protein</th>
<th>Solvent</th>
<th>Mole Ratio</th>
<th>Time</th>
<th>Alkylation</th>
<th>Time</th>
<th>Reduction</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Wool</td>
<td>nPrOH/pH8</td>
<td>5:1</td>
<td>24 hr.</td>
<td>Acrylonitrile</td>
<td>21 hr.</td>
<td>99+</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Cortical cells</td>
<td>nPrOH/pH8</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Acrylonitrile</td>
<td>1 hr.</td>
<td>99.3 D.L.</td>
<td>(7 µM/g)</td>
</tr>
<tr>
<td>3.</td>
<td>Cortical cells</td>
<td>nPrOH/pH8</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Acrylonitrile</td>
<td>1 hr.</td>
<td>98.0 D.L., D.R.</td>
<td>(21 µM/g)</td>
</tr>
<tr>
<td>4.</td>
<td>Wool</td>
<td>nPrOH/pH8</td>
<td>2:1</td>
<td>20 hr.</td>
<td>Acrylonitrile</td>
<td>20 hr.</td>
<td>100 D.L., D.R.</td>
<td>0.7% weight loss.</td>
</tr>
<tr>
<td>5.</td>
<td>Wool</td>
<td>DMSO</td>
<td>5:1</td>
<td>24 hr.</td>
<td>Acrylonitrile</td>
<td>21 hr.</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Wool</td>
<td>DMSO</td>
<td>5:1</td>
<td>88 hr.</td>
<td>Acrylonitrile</td>
<td>21 hr.</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Wool</td>
<td>DMSO</td>
<td>2:1</td>
<td>20 hr.</td>
<td>Acrylonitrile</td>
<td>20 hr.</td>
<td>- D.R., ppt. of polyacrylonitrile.</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Cortical cells</td>
<td>DMSO</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Acrylonitrile</td>
<td>69 hr.</td>
<td>99+</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Wool</td>
<td>DMSO</td>
<td>2:1</td>
<td>48 hr.</td>
<td>Propylene oxide</td>
<td>1 hr.</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Wool</td>
<td>nPrOH</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Methyl iodide</td>
<td>1 hr.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Wool</td>
<td>Ethanol</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Methyl iodide</td>
<td>1 hr.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Wool</td>
<td>90% nPrOH</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Methyl iodide</td>
<td>1 hr.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Wool</td>
<td>Toluene</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Methyl iodide</td>
<td>24 hr.</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Wool</td>
<td>CCl4</td>
<td>2:1</td>
<td>24 hr.</td>
<td>Methyl iodide</td>
<td>24 hr.</td>
<td>- Heavy ppt.</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Wool</td>
<td>H2O</td>
<td>2:1</td>
<td>48 hr.</td>
<td>Iodoacetic acid</td>
<td>1 hr.</td>
<td>95</td>
<td></td>
</tr>
</tbody>
</table>

* Mole ratio of TBP to cystine.

† Reduction calculated from amino acid analysis.

* D.L. = double loading of amino acid analyser.

D.R. = double reduction/alkylation cycle.
As a reducing agent, sodium hydride can exist only in aprotic solvents, and precautions must be taken to avoid including moisture in the reaction mixture. Although the DMSO and proteins were carefully dried prior to use, and dry nitrogen was passed continually through the flask, the results obtained were inconsistent. Reduction of B.S.A. and alkylation with acrylonitrile was an attempt to exactly duplicate the results of Krull & Friedman (1967) to establish if poor penetration of sodium hydride through the fibre was causing the low reduction. It is obvious that the reaction as performed by Krull & Friedman (who found only 4.1% residual cystine under supposedly identical conditions) could not be duplicated. Entry of moisture at some stage, or use of impure reactants may have caused the differences observed. It is also difficult to explain why alkylation with methyl iodide achieved 44% reduction of B.S.A., when it is known that methyl iodide reacts with DMSO (see Chapter 5B(v)). Perhaps this latter reaction is slower than the alkylation reaction.

(b) Tri-n-butylphosphine - Table 5-2 presents conditions used, and percent reduction of disulphide bonds achieved, with a series of TBP reductions of wool and cortical cells. The various solvents used in Table 5-2 are self explanatory, except for nPrOH/pH8 which refers to a 50/50 mixture of n-propanol and pH 8 borate buffer, which was used by MacLaren & Sweetman (1966) in their sequential one-bath process. Different solvents were tried for several reasons; the nPrOH/pH8 system was used as it has been investigated and shown to give high reductions
(Maclaren & Sweetman, 1966). DMSO was used in an attempt to decrease any possibility of peptide bond hydrolysis, although only in one case (Experiment No. 8) was the reduction sufficiently high to be worth further investigation. Reductions in DMSO were also not particularly consistent. Use of a non aqueous solvent also decreases the possibility of weight loss due to solubility of the proteins, although DMSO has been reported to be a protein denaturant (Caldwell et al., 1966), but Tanford (1968, p. 211) has shown it is not a very effective denaturant of proteins.

It has been reported (Feughelman & Snaith, 1964) that alcohols penetrate the microfibrillar structure of keratins better than water. Alcohols were used as solvents to attempt to achieve penetration of the microfibril in case this was restricting reduction, but even in 90% n-propanol, no reduction of disulphide bonds was noted. Conversely, reduction in water should inhibit reaction with disulphide bonds in the microfibrils and thus only reduce these bonds in the matrix. This material (Experiment No. 16, Table 5-2) was however relatively insoluble, dissolving only 45% on vibromixing in formic acid for 1 hr. Reaction in other non aqueous solvents, toluene, n-hexane and carbon tetrachloride, also gave no reduction of disulphide content, the latter two solvents causing precipitation of an unidentified material during the alkylation.

Five to one mole ratio of TBP to cystine was used in an attempt to increase the reduction, but there was no indication that the higher ratio achieved this. Maclaren et al. (1968) state that a 2:1 mole ratio is sufficient
to attain 100% reduction, and there is no evidence from this work that a higher ratio offers increased reduction. Longer reduction times were used to try and increase the reduction attained in cases where it was thought the reaction may be slow. Sweetman & Maclaren (1966) have shown that reduction with TBP is considerably slower in water than in 20% aqueous n-propanol. Increased reduction time appears to be of value with DMSO. (Compare Experiments 5 and 6, Table 5-2).

Acrylonitrile was used as alkylating agent as it does not increase the charge on the proteins, possibly giving lower protein solubility and a better separation of the high and low sulphur proteins, as it does for the L.S. components 7 and 8 (Frater, 1966). Alkylation of lysine by acrylonitrile at high pH has been reported (Weil & Seibles, 1961; Plummer & Hirs, 1964; Riehm & Scheraga, 1966; Cavins & Friedman, 1968), but in most cases little modification of lysine was noted.

Polymerisation of acrylonitrile by TBP sometimes occurs, particularly in DMSO in which the polyacrylonitrile is partially soluble. Propylene oxide was used as it does not react with DMSO (as do alkyl halides) and may not be so prone to polymerisation by TBP. Methyl iodide was employed, as a high percentage reduction was achieved with this alkylating agent in the sequential one-bath process of Maclaren & Sweetman (1966). Maclaren et al. (1968) show that longer alkylation times produce proteins which are less soluble in various solvents, but results presented in this thesis do not further clarify their observations.
Double reduction/alkylation cycles were performed in some cases, as shown in Table 5-2. This procedure was employed to achieve 100% reduction of disulphide bonds, but it is not always a reliable method of obtaining 100% reduction. Experiments 2 and 3 (Table 5-2) were intended to compare the performance of single and double reduction/alkylation cycles. As can be seen in Table 5-2, the double reduction/alkylation cycle produced a 98% decrease in disulphide content, while the single cycle achieved 99.3% reduction. Solubilities of these two keratin samples are in agreement with their disulphide content (Chapter 5C(iii)). In one case (Experiment No.4, Table 5-2) double loading of the amino acid analyser gave no trace of cystine in the hydrolysate. As this particular protein was found to be up to 95% soluble in formic acid (Chapter 5C(iii)), indicating that few disulphide cross-links remain, it is believed that no cystine is produced under the conditions used for hydrolysis (O'Donnell & Thompson, 1964).

This particular sample of wool was stained with ammoniacal silver nitrate and examined in the electron microscope. The microfibril/matrix structure was still visible (Figure 5-4).

(c) Triphenylphosphine (\(\phi_3P\)) - This compound was tried as an alternative reducing agent to TBP, as it is known to react with disulphides (Bartlett & Meguerian, 1956). It was thought it may be superior to TBP in non-aqueous media but this was not found to be the case. Reduction of wool with 2:1 ratio of \(\phi_3P\) to cystine for 96 hr. and coupling with acrylonitrile gave no reduction in
disulphide content of the wool. It has been shown that \( \phi_3P \) reduces disulphides efficiently in acidic media (Humphrey & Hawkins, 1964), and this leads to the possibility of reduction and protein extraction being performed simultaneously (Chapter 5C(ii)).

(d) Sodium/Liquid Ammonia - This procedure, known as the Birch reduction, is capable of cleaving disulphide bonds (Roberts & Caserio, 1965) as well as peptide chains at proline residues (Birch et al., 1955; Benisek & Cole, 1965). As proline is unable to fit sterically into an \( \alpha \) helix (Blout, 1962) and occurs in higher concentration in SCMKB (Crewther et al., 1965) and in the non-helical sections of SCMKA (Crewther et al., 1968), cleavage of disulphides and the peptide chain at proline should result in removal of matrix (non-helical) proteins from wool on mild dissolution.

Cleavage of prolyl peptide bonds has been achieved with lithium aluminium hydride in tetrahydrofuran (Ruttenberg et al., 1964), lithium in methylamine (Patchornik et al., 1964) and sodium hydrazide in hydrazine/ether (Kauffman & Sobel, 1966). A higher degree of cleavage and fewer side reactions (i.e. reduction of phenylalanine, tyrosine, tryptophan and methionine) are observed with sodium in liquid ammonia (Wilchek et al., 1965; Benisek & Cole, 1965). The incidence of side reactions is not altered by the presence of methanol (proton donor) or sodium amide (proton scavenger) during the reduction but is more likely due to excess sodium (Benisek et al., 1967). Atassi & Singhal (1970) claim the side reactions are minimised by careful adjustment of the sodium ratio alone.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Untreated</th>
<th>30 sec.</th>
<th>90 sec.</th>
<th>7 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO₃H</td>
<td>0.26</td>
<td>0.26</td>
<td>0.26</td>
<td>0.20</td>
</tr>
<tr>
<td>Asp</td>
<td>6.96</td>
<td>6.83</td>
<td>7.02</td>
<td>8.02</td>
</tr>
<tr>
<td>Thr</td>
<td>6.20</td>
<td>6.08</td>
<td>6.01</td>
<td>4.31</td>
</tr>
<tr>
<td>Ser</td>
<td>10.81</td>
<td>10.64</td>
<td>10.13</td>
<td>6.13</td>
</tr>
<tr>
<td>Glu</td>
<td>11.99</td>
<td>11.90</td>
<td>12.38</td>
<td>14.50</td>
</tr>
<tr>
<td>Pro</td>
<td>5.84</td>
<td>5.38</td>
<td>5.13</td>
<td>3.36</td>
</tr>
<tr>
<td>Gly</td>
<td>8.10</td>
<td>8.16</td>
<td>7.72</td>
<td>7.52</td>
</tr>
<tr>
<td>Ala</td>
<td>5.52</td>
<td>5.58</td>
<td>5.61</td>
<td>6.23</td>
</tr>
<tr>
<td>Val</td>
<td>5.87</td>
<td>5.74</td>
<td>6.00</td>
<td>6.53</td>
</tr>
<tr>
<td>½Cys</td>
<td>9.34</td>
<td>9.35</td>
<td>7.54</td>
<td>2.96</td>
</tr>
<tr>
<td>Met</td>
<td>0.49</td>
<td>0.52</td>
<td>0.53</td>
<td>0.70</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.33</td>
<td>3.25</td>
<td>3.37</td>
<td>4.25</td>
</tr>
<tr>
<td>Leu</td>
<td>7.77</td>
<td>7.78</td>
<td>7.94</td>
<td>9.44</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.67</td>
<td>3.71</td>
<td>3.53</td>
<td>3.33</td>
</tr>
<tr>
<td>Phe</td>
<td>2.79</td>
<td>2.67</td>
<td>2.36</td>
<td>2.25</td>
</tr>
<tr>
<td>Lys</td>
<td>3.20</td>
<td>3.38</td>
<td>3.36</td>
<td>3.81</td>
</tr>
<tr>
<td>His</td>
<td>0.95</td>
<td>0.92</td>
<td>0.89</td>
<td>1.04</td>
</tr>
<tr>
<td>Arg</td>
<td>7.24</td>
<td>7.41</td>
<td>7.55</td>
<td>7.55</td>
</tr>
<tr>
<td>SCMC</td>
<td>0.0</td>
<td>0.33</td>
<td>2.52</td>
<td>7.83</td>
</tr>
</tbody>
</table>

% * 82.4 80.2 77.6 69.5

* Recovery of anhydroyl amino acids

# Sample was immersed in liquid ammonia for 12 min. at -77°C but without addition of any sodium.
Reaction with SCMC and methionine (Crewther & Nicholls, 1969) is predicted by the work of Birch (Birch, 1950), but is probably minimised by careful control of the sodium concentration.

Reduction for 7 min. resulted in a product lacking any fibrous structure as observed in the light microscope. Physical degradation at the low temperature was not involved, as 12 min. under these conditions in the absence of sodium did not modify the normal appearance of wool. Reduction for 30 sec. or 90 sec. gave fibres with a normal appearance under the light microscope.

Much more developmental work is necessary on this method as it is potentially useful as a reduction procedure. The main disadvantage is degradation of the amino acid adjacent, and on the N-terminal side of proline (Benisek et al., 1967). This is probably demonstrated in Table 5-3 by the decreasing recoveries of anhydroamino acids with increased reduction time. Related to this, some of the amino acids (aspartic acid, glutamic acid and leucine show pronounced effects) increase in apparent concentration with increasing reduction time. Amino acid analyses will not show the reduction of prolyl peptide bonds as proline itself should not be destroyed, but some decrease of proline is noticeable. Cystine concentration also decreases although the total of cystine + SCMC is fairly constant.

Although the analyses in Table 5-3 indicate that reduction of cystine does not occur rapidly (3 mole % is present after 7 min. reduction), it is thought that part of the cystine arises from rapid reoxidation occurring
during the alkylation step. The fibres are almost completely dissolved in liquid ammonia during 7 min. reduction so that the combination of partial disulphide cleavage, and breaking the peptide chain adjacent to proline, is sufficient to allow most of the proteins to go into solution in the liquid ammonia. No estimate of the proportion of cleavage adjacent to proline was made.

(ii) Combined Reduction/Solubilisation Methods.

(a) Partial Reduction - Cortical cells were partially reduced with TBP using a 1.25:1 mole ratio of TBP to cystine, according to the method of Maclaren et al. (1968), and alkylation with iodoacetic acid was carried out by this same method.

The reduced and alkylated cortical cells (containing 0.84 mole percent residual cystine) were then extracted with three separate lots of formic acid at 500:1 solvent: cortical cells ratio for a total of 72 hr. A total of 65% of the cortical cells dissolved in this time. Amino acid analysis of the reduced and alkylated cortical cells, and the soluble material, and insoluble residue, showed no difference in amino acid composition between the three materials. Under these conditions, H.S. and L.S. proteins are being extracted equally, indicating that no selective reduction of disulphides in microfibrillar or matrix regions of the cells has occurred.

(b) Reduction in a Solubilising Medium - Sweetman & Maclaren (1966) found that reduction of wool with TBP (2:1, TBP to cystine ratio) in formamide (100:1, formamide to wool ratio) for 48 hr., resulted in a 14% decrease in weight of the wool. It was thought this extraction could
possibly be increased by use of formic acid as the solvent, but no extraction was observed on reduction for 24 hr.
The results are tabulated in Table 5-4.

**TABLE 5-4.**

**COMBINED REDUCTION WITH TBP AND EXTRACTION.**

<table>
<thead>
<tr>
<th>Solvent to Wool Ratio</th>
<th>TBP to Cystine Ratio</th>
<th>Solvent</th>
<th>Weight Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:1</td>
<td>2:1</td>
<td>Formic acid</td>
<td>0.02%</td>
</tr>
<tr>
<td>500:1</td>
<td>2:1</td>
<td>Formic acid</td>
<td>0.06%</td>
</tr>
<tr>
<td>100:1</td>
<td>2:1</td>
<td>0.1 M Formic acid pH 2.3</td>
<td>0.13%</td>
</tr>
</tbody>
</table>

As noted earlier (Chapter 5C(i)) Humphrey & Hawkins (1964) found that triphenylphosphine ($\phi_3P$) reduces disulphides in acidic media (in their case, in 0.1N perchloric acid), and gave 100% reduction with a 1:1 mole ratio of $\phi_3P$ to disulphide. A series of experiments were performed in which wool was reduced with $\phi_3P$ (2:1 ratio $\phi_3P$ to cystine) in formic acid solutions of various strengths. The results are plotted in Figure 5-1, note that 75% formic acid dissolves only 2-3% of wool in the absence of $\phi_3P$. The lowest concentration formic acid used in Figure 5-1 was 0.1N and 1.4% by weight of wool was dissolved in this medium.

Obviously, covalent bonds, either peptide or disulphide bonds, have been broken to allow solubilisation of almost 80% wool in the 75% formic acid. Amino acid analysis of the material dissolved in 75% formic acid,
FIGURE 5-1

REDUCTION OF WOOL WITH $\phi_3P$ IN FORMIC ACID OF VARYING CONCENTRATION.
indicates it is not significantly different from that of whole wool, although the cystine content was 20% low.

After dialysis, the protein extracted with 75% formic acid was found to be insoluble in 75% and also in 100% formic acid. Had peptide bonds been cleaved, the protein should still be partially soluble in 75% or 100% formic acid. The most likely explanation is that the disulphide bonds were reduced and dialysis was sufficient to allow reoxidation of these bonds, producing a metakeratin (Goddard & Michaelis, 1935) which becomes insoluble in formic acid solutions.

Sweetman & Maclaren (1966) proposed a mechanism for the reduction of disulphide bonds by TBP. There is no reason to believe that \( \phi_3 \)P reduces disulphides via a different mechanism, but the reactivity of TBP and \( \phi_3 \)P varies with the solvent employed. As noted above, TBP reduces disulphides in aqueous solutions down to pH 3 and even slightly in 0.1N HCl (Sweetman & Maclaren, 1966), but not in more acid media. In 0.1N formic acid \( \phi_3 \)P may reduce some disulphide bonds, but not sufficiently to dissolve an appreciable amount of protein, although considerable quantities of protein are dissolved in higher percentage formic acid. These differences can be simply explained in terms of basic strength (or nucleophilicity) of the two phosphines. Henderson & Streuli (1960) gave the pK\(_a\)'s of TBP and \( \phi_3 \)P as 8.43 and 2.73 respectively, and formic acid is therefore probably only acting to increase the apparent basic strength of \( \phi_3 \)P.

(iii) Solubility of Reduced and Alkylated Keratins.

The reduced and alkylated wool from Experiment No.4
(Table 5-2) is thought to have no residual cystine as noted in Chapter 5C(ii). (It will be referred to subsequently as Sample 4.) It is of interest to observe the solubility behaviour of this material and compare it with that of other samples.

On vibromixing Sample 4 in formic acid, there is an initial very rapid dissolution of about 60% of the fibre, followed by a slower stage in which 95% of the fibre dissolves in about 40 hr. These results are plotted in Figure 5-2 and inset is the initial section of the curve plotted on an expanded time scale. Table 5-5 gives amino acid analyses for the soluble and insoluble material obtained after 0.5 and 2 hr. vibromixing in formic acid. It is noticeable that S-carboxyethyl cysteine is concentrated in the insoluble fraction and methionine occurs equally in both fractions. These two points together with the overall similarity in analysis of the separated fractions (with a few exceptions) clearly indicate that preferential extraction of the H.S. protein is not occurring. Amino acid analyses of fractions after 7 hr. and 40 hr. vibromix show no trends in composition and are very similar to the results presented in Table 5-5. The S-carboxyethyl cysteine analyses are high, possibly due to an error in standardisation of this amino acid.

Vibromixing is a reasonably vigorous method of agitation, but a similar rapid dissolution of material from Sample 4 is observed on standing in formic acid or gently shaking in formic acid (Table 5-6).
FIGURE 5-2
PERCENT OF SAMPLE 4 DISSOLVED ON VIBROMIXING IN FORMIC ACID.
TABLE 5-5.

AMINO ACID ANALYSES OF
SOLUBLE AND INSOLUBLE FRACTIONS OF SAMPLE 4.

Mole %

Time of Vibromixing in Formic Acid.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>½ hr. Soluble</th>
<th>½ hr. Insoluble</th>
<th>2 hr. Soluble</th>
<th>2 hr. Insoluble</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO$_3$H</td>
<td>0.13</td>
<td>0.25</td>
<td>0.15</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>Asp</td>
<td>7.34</td>
<td>5.89</td>
<td>7.03</td>
<td>5.46</td>
<td>6.34</td>
</tr>
<tr>
<td>Thr</td>
<td>5.51</td>
<td>5.80</td>
<td>5.84</td>
<td>6.18</td>
<td>5.90</td>
</tr>
<tr>
<td>Ser</td>
<td>8.95</td>
<td>9.71</td>
<td>9.05</td>
<td>10.48</td>
<td>9.58</td>
</tr>
<tr>
<td>Glu</td>
<td>12.92</td>
<td>12.09</td>
<td>13.43</td>
<td>11.27</td>
<td>13.97</td>
</tr>
<tr>
<td>Pro</td>
<td>5.90</td>
<td>8.60</td>
<td>6.86</td>
<td>8.83</td>
<td>5.68</td>
</tr>
<tr>
<td>Gly</td>
<td>8.33</td>
<td>6.87</td>
<td>7.97</td>
<td>6.91</td>
<td>9.66</td>
</tr>
<tr>
<td>Ala</td>
<td>5.32</td>
<td>5.72</td>
<td>5.70</td>
<td>5.83</td>
<td>5.48</td>
</tr>
<tr>
<td>Val</td>
<td>6.02</td>
<td>6.20</td>
<td>5.90</td>
<td>6.37</td>
<td>5.98</td>
</tr>
<tr>
<td>½Cys</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Met</td>
<td>0.48</td>
<td>0.51</td>
<td>0.47</td>
<td>0.54</td>
<td>0.51</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.41</td>
<td>3.41</td>
<td>3.52</td>
<td>3.28</td>
<td>3.17</td>
</tr>
<tr>
<td>Leu</td>
<td>9.63</td>
<td>6.06</td>
<td>6.49</td>
<td>6.34</td>
<td>7.67</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.18</td>
<td>2.88</td>
<td>3.85</td>
<td>2.53</td>
<td>3.36</td>
</tr>
<tr>
<td>Phe</td>
<td>3.01</td>
<td>2.22</td>
<td>3.05</td>
<td>2.01</td>
<td>2.26</td>
</tr>
<tr>
<td>Lys</td>
<td>1.93</td>
<td>2.35</td>
<td>1.94</td>
<td>2.36</td>
<td>1.84</td>
</tr>
<tr>
<td>His</td>
<td>0.83</td>
<td>0.92</td>
<td>0.69</td>
<td>0.98</td>
<td>0.57</td>
</tr>
<tr>
<td>Arg</td>
<td>5.74</td>
<td>4.97</td>
<td>6.43</td>
<td>5.53</td>
<td>7.39</td>
</tr>
<tr>
<td>SCEC #</td>
<td>10.38</td>
<td>15.14</td>
<td>11.52</td>
<td>14.73</td>
<td>10.57</td>
</tr>
</tbody>
</table>

* Recovery of anhydroamino acids.
# S-carboxyethyl cysteine.
Other solvents were used with Sample 4, but all proved to be less effective than formic acid. In this case, ultrasonic disintegration (U.S.) was employed as it is a still more severe method of inducing dissolution of protein. The results are given in Table 5-7. Note that greater than 95% of Sample 4 is dissolved in formic acid under these conditions, but a considerably smaller amount on vibromixing for 4 hr.

### TABLE 5-7. SOLUBILITY OF SAMPLE 4 IN VARIOUS SOLVENTS.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Agitation Method</th>
<th>Treatment Time</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>U.S.</td>
<td>30 min.</td>
<td>Insoluble</td>
</tr>
<tr>
<td>2-chloroethanol</td>
<td>U.S.</td>
<td>45 min.</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>U.S.</td>
<td>1 hr.</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>U.S.</td>
<td>1 hr.</td>
<td>18.5%</td>
</tr>
<tr>
<td>Formic acid</td>
<td>U.S.</td>
<td>1 hr.</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Vibromixing</td>
<td>4 hr.</td>
<td>74.5%</td>
</tr>
</tbody>
</table>

As the various pure solvents failed to dissolve a reasonable proportion of Sample 4 under fairly severe
conditions, it was necessary to attempt to control the effectiveness of formic acid. Gentle agitation or standing Sample 4 in formic acid for 24 hr. dissolves an apparently constant amount of protein (Tables 5-6 and 5-7), therefore various strength solutions of formic acid were used to dissolve Sample 4 on shaking for 24 hr. The results are plotted in Figure 5-3, and indicate the amount of protein extracted depends on the concentration of formic acid used. This in turn may be related to the extraction of separate histological components.

To ensure that the material removed by different concentration formic acid solutions did not originate in separate cellular regions of the fibre (i.e. from cortex or cuticle), two samples of reduced and alkylated cortical cells were prepared (Experiments 2 and 3, Table 5-2). These samples were gently shaken for 24 hr. in formic acid solution in a closed centrifuge tube, the residue 'washed', and then the formic acid concentration increased to the next stage, allowing for the amount of solution remaining from the previous level. As only 40 mg. of cells were used, losses were appreciable, but correspond for the two experiments. Results are given in Table 5-8.

Amino acid analyses of some fractions are given in Table 5-9, but analyses of the material soluble in 25 and 50% formic acid were not performed due to the small quantity recovered. The protein extracted by 75 and 100% formic acid has an analysis approaching that of SCMKA although there are obvious differences. Serine and lysine analyses are considerably lower than for SCMKA, and glutamic acid, total cystine, and valine are higher.
FIGURE 5-3
PERCENT OF SAMPLE 4 DISSOLVED ON SHAKING FOR 24 HR. IN FORMIC ACID SOLUTIONS OF VARIOUS STRENGTHS.
These analyses do not however, resemble that of SCMKB.

**TABLE 5-8.**

SUCCESSIVE EXTRACTIONS WITH FORMIC ACID SOLUTIONS.

<table>
<thead>
<tr>
<th>Formic acid Concentration</th>
<th>Yield</th>
<th>Formic acid Concentration</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>5.3%</td>
<td>25%</td>
<td>1.0%</td>
</tr>
<tr>
<td>50%</td>
<td>8.8%</td>
<td>50%</td>
<td>7.5%</td>
</tr>
<tr>
<td>75%</td>
<td>34.2% #</td>
<td>75%</td>
<td>25.5% #</td>
</tr>
<tr>
<td>100%</td>
<td>8.5%</td>
<td>100%</td>
<td>23.6% #</td>
</tr>
<tr>
<td>Loss</td>
<td>43.2%</td>
<td>Loss</td>
<td>42.4%</td>
</tr>
</tbody>
</table>

# Amino acid analyses of these three components are given in Table 5-9.

The similarity of these analyses with that of SCMKA is sufficient to indicate preferential extraction of the H.S. protein from the L.S. protein could be achieved by refinement of the process, i.e. by selection of other concentrations of formic acid.

The cystine content of these two samples of reduced and alkylated cortical cells were given in Table 5-2. Sample 2 contained only 7 µM/g cystine while Sample 3 contained 21 µM/g (values obtained by double loading the amino acid analyser). During the initial stages of extraction, Sample 2 was considerably more soluble than Sample 3, indicating that even at this low level of
### TABLE 5-9.

**AMINO ACID ANALYSES OF SOLUBLE FRACTIONS FROM TABLE 5-8.**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>SCMKA#</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 3</th>
<th>SCMKB#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75%</td>
<td>75%</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>8.14</td>
<td>8.98</td>
<td>9.48</td>
<td>7.77</td>
<td>0.76</td>
</tr>
<tr>
<td>Thr</td>
<td>4.40</td>
<td>3.88</td>
<td>3.71</td>
<td>3.36</td>
<td>11.26</td>
</tr>
<tr>
<td>Ser</td>
<td>7.31</td>
<td>3.86</td>
<td>3.49</td>
<td>2.04</td>
<td>14.81</td>
</tr>
<tr>
<td>Glu</td>
<td>14.13</td>
<td>17.10</td>
<td>17.40</td>
<td>16.36</td>
<td>9.83</td>
</tr>
<tr>
<td>Pro</td>
<td>4.25</td>
<td>4.65</td>
<td>4.48</td>
<td>7.83</td>
<td>12.33</td>
</tr>
<tr>
<td>Gly</td>
<td>8.82</td>
<td>6.78</td>
<td>7.62</td>
<td>8.14</td>
<td>6.33</td>
</tr>
<tr>
<td>Ala</td>
<td>6.44</td>
<td>7.17</td>
<td>7.18</td>
<td>7.72</td>
<td>3.03</td>
</tr>
<tr>
<td>Val</td>
<td>5.93</td>
<td>7.16</td>
<td>7.35</td>
<td>7.51</td>
<td>4.21</td>
</tr>
<tr>
<td>Met</td>
<td>0.55</td>
<td>0.65</td>
<td>0.54</td>
<td>0.50</td>
<td>0.0</td>
</tr>
<tr>
<td>Ileu</td>
<td>3.66</td>
<td>4.22</td>
<td>4.16</td>
<td>3.87</td>
<td>2.74</td>
</tr>
<tr>
<td>Leu</td>
<td>10.26</td>
<td>8.90</td>
<td>11.28</td>
<td>9.20</td>
<td>1.83</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.28</td>
<td>2.98</td>
<td>3.40</td>
<td>2.25</td>
<td>2.08</td>
</tr>
<tr>
<td>Phe</td>
<td>3.02</td>
<td>2.72</td>
<td>3.00</td>
<td>2.44</td>
<td>0.75</td>
</tr>
<tr>
<td>Lys</td>
<td>4.05</td>
<td>3.14</td>
<td>2.27</td>
<td>1.34</td>
<td>0.48</td>
</tr>
<tr>
<td>His</td>
<td>0.66</td>
<td>0.85</td>
<td>0.66</td>
<td>0.75</td>
<td>0.57</td>
</tr>
<tr>
<td>Arg</td>
<td>7.27</td>
<td>8.36</td>
<td>7.18</td>
<td>6.77</td>
<td>5.06</td>
</tr>
<tr>
<td><strong>Total Cys</strong></td>
<td><strong>6.78</strong></td>
<td><strong>8.52</strong></td>
<td><strong>7.98</strong></td>
<td><strong>13.08</strong></td>
<td><strong>23.65</strong></td>
</tr>
</tbody>
</table>

* Recovery of anhydroamino acids

# From Crewther et al., (1965).
residual cystine, it may be capable of decreasing protein solubility. Sample 3 contains 21 µM/g cystine which is approximately 20 µM/10,000 µM, or one cross-link per 1,000 residues. As a comparison, polystyrene is normally soluble in toluene, but inclusion of 0.02% divinylbenzene (i.e. one cross-link per 5,000 'residues') in the monomer produces a cross-linked polymer which is insoluble in toluene (although considerably swelled by it) (Boyer, 1945).

This small amount of residual cystine probably has a considerable effect on the solubility of keratin proteins and may account for the considerably higher solubilities achieved during this work in comparison with that of Maclaren et al. (1968). As further evidence of the influence of a few residual disulphide cross-links on solubility of keratin proteins, a comparison can be made of the solubilities of Samples 1 and 4 (Table 5-2). Sample 4 had no residual cystine detectable in amino acid analysis, Sample 1 had a maximum of 10 µM/g. During 3 hr. vibromixing of Sample 1 in formic acid, 55.2% dissolves, but 3 hr. vibromixing of Sample 4 dissolves 70 to 75% (see Figure 5-2). Maclaren et al. (1968) dissolved 59% of reduced and S-carboxymethylated wool during 2 days immersion in formic acid, but as shown in Table 5-6, 56% of Sample 4 dissolves during 1 hr. immersion in formic acid and 77% on 24 hr. immersion. Maclaren et al. (1968) plotted percent reduction against percent extraction by formic acid. The percent extraction increases rapidly near 100% reduction, and thus indicates that these last few residual disulphide cross-links may be very important in controlling the degree of extraction of the proteins.
(iv) Other Approaches to the Separation of Microfibrils and Matrix.

The structural entity known as the microfibril is clearly observable by electron microscopy of osmium stained wool cross-sections (e.g. Rogers, 1959, 1959a). Preferential extraction of the matrix protein could leave the microfibril in an unaltered state in the fibre. Observation of microfibrils after extraction of matrix protein in the electron microscope is not simple, as microfibrils are normally observed due to their lower electron density than that of the matrix protein. Preferential extraction of matrix protein from osmium stained wool by the method of Harrap & Gillespie (1963) is slow, as the osmium staining introduces stabilising cross-links (Kay, 1965) and hence slows the extraction of matrix protein.

Examination of cross-sections of the osmium stained and extracted fibres in the electron microscope gave no indication of the existence of microfibrils; no structure was recognisable below the level of cortex and cuticle, the latter being considerably modified by the process.

A reversal of this procedure was also attempted, by preferentially extracting the H.S. protein (Harrap & Gillespie, 1963) and then staining the reduced protein with osmium tetroxide, and examining cross-sections. The fibres did not stain as heavily in this case, and electron microscopic examination again did not show the presence of microfibrils. In these cases, the method of extraction of H.S. proteins may also unfold the \( \alpha \) helical proteins but leave them retained within the fibre cell membranes.
due to their larger size.

Following preferential extraction of the H.S. proteins, some of the fibres were vibromixed in formic acid for 2½ hr., then the mixture was negatively stained with KPTA and examined in the electron microscope. Again, no fibrillar components were obvious.

Harrap & Gillespie (1963) showed that high ionic strength solutions (µ=0.5) completely suppress the liberation of L.S. proteins on plasmolysis of wool from which the H.S. protein has been preferentially extracted. This effect could be produced by osmotic pressure, or the L.S. protein being folded in high ionic strength solutions of salts as occurs with other proteins (von Hippel & Schleich, 1969; von Hippel & Wong, 1964; Berendsen & Migchelsen, 1965). NMR spectroscopy (Chapter 4) indicated that most of the L.S. proteins are folded at low pH, and together with the known resistance of folded proteins to proteolysis (Crewther & Harrap, 1967) leads to a further possible method of separating microfibrils.

By preferential extraction of H.S. protein at 0°C (Harrap & Gillespie, 1963) and subsequent immersion in 0.5 ionic strength solution at low pH, the L.S. proteins may remain in their native folded conformation. (Alkylation with iodoacetic acid was also performed in 0.5 ionic strength solution). Springell (1963) demonstrated that rapid digestion of SCMKB and slow digestion of SCMKA occurs with pepsin at pH 2, digestion of the SCMKB being complete after 100 min.

Extracted and alkylated wool fibres were digested for 100 min. in 0.5 ionic strength solution (KCl) with
pepsin at pH 2. The digestion was halted by raising the pH to 4 (Smyth, 1967) which should not unfold the L.S. proteins (Chapter 4). In this time, the fibres were almost completely digested, and the clear solution after centrifugation was negatively stained with KPTA solution at pH 4.5 and examined in the electron microscope. Again, no fibrillar components nor sheets of microfibrils were visible on examination in the electron microscope.

Jeffrey et al. (1956) obtained material of microfibrillar size by a reversal of this type of approach. They digested Lincoln wool with pepsin, oxidized the liberated cortical cells with peracetic acid and then ultrasonically disintegrated them in lithium bromide solution.

5D. DISCUSSION.

Microfibrils are keratin components which can be observed in most keratins e.g. hair, nails, claws, hoofs and quills, but owing to the natural insolubility of keratin (Crewther et al., 1965) it is essential to break some covalent bonds before any solubilisation or separation of the component proteins can be achieved. Separation of microfibrillar ribbons from keratins by ultrasonic treatment is possible only after progressive breakdown of disulphide cross-links (Jeffrey et al., 1955). Cotton (Zuber & Zahn, 1956) and silk (Rollins et al., 1957) form 'microfibrils' on ultrasonic disintegration, but the same disruption treatment is ineffective after cross-linkage of the fibres.

Keratin proteins can be solubilised by treatment
with strong acids or alkalies (Crewther et al., 1965) but these reagents degrade the proteins by cleaving peptide bonds. A method for specifically cleaving one type of covalent bond is necessary to avoid overall degradation of the proteins.

Where separation of microfibrils and matrix is concerned, the most obvious bond to sever is the cystine disulphide bond. Although cystine is mainly concentrated in the H.S. proteins and non-helical sections of the L.S. proteins (Gillespie, 1967; Crewther et al., 1968), it does occur in the α helix of lysozyme (Dayhoff & Eck, 1968). An even more suitable residue is proline, which due to steric reasons, can only occupy positions at bends in α helical sections or non-helical sections of protein chains (Blout, 1962).

Various reductive and oxidative cleavage procedures for disulphides are well known (Crewther et al., 1965), but the recent development of TBP as a reductant (Maclaren & Sweetman, 1966; Maclaren et al., 1968) offers the advantages of a specific and controllable extent of reduction without the use of high mole ratios of reductant to cystine as is necessary with thiols (Maclaren, 1962).

Recently, Hogg et al. (1971) have shown at least two of the SCMC residues are intimately associated with the α helical portions of SCMKA, thus by selective partial reduction of disulphide bonds of wool, it may be possible to leave these two cystine residues not reduced, which would possibly increase the stability of the α helical sections of protein chain, and allow easier removal of the matrix.
Proline is also concentrated in non-helical regions of the L.S. protein and in H.S. proteins as noted above. Two enzymes which cleave peptide bonds adjacent to proline are known (Grassmann et al., 1929; Davie & Smith, 1957), but both are dipeptidases, not endopeptidases, and will therefore not attack proline in a long protein chain. Sodium in liquid ammonia is a versatile reducing agent known to reduce secondary amides (Birch, 1950; Birch et al., 1955) and consequently it reduces the peptide bond of proline (Benisek & Cole, 1965; Benisek et al., 1967); it has the added advantage of reducing disulphides (Birch, 1950; Roberts & Caserio, 1965). Although the sodium/liquid ammonia reductant causes side reactions (Benisek et al., 1967; Atassi & Singhal, 1970; Wilchek et al., 1965; Crewther & Nichols, 1969), the conditions for reduction have not been thoroughly examined, and there are a number of conflicting reports in the literature (below).

Hofmann & Yajima, (1961) observed cleavage of poly-peptide chains adjacent to proline with sodium/liquid ammonia, but couldn't explain why it occurred. Ramachandran et al. (1965) carefully achieved anhydrous conditions (by redistilling the ammonia from sodium) and found that little cleavage of proline peptide bonds occurs during 30 min. immersion in sodium/liquid ammonia. Evidently in this case, a proton donor was absent, as the reduction normally only requires a few minutes (Benisek & Cole, 1965; Atassi & Singhal, 1970). Together with the increased rate of cleavage, a reduction in the amount of by-products (non-specific cleavage and
reductive modification of aromatic amino acids) may occur in the presence of a proton donor (Wilchek et al., 1965, who used methanol as proton donor, and Guttmann, 1963, who used ammonium chloride as proton donor). Ressler & Ratzkin (1961) suggest reduction is negligible in the absence of proton donors, and Ressler & Kashelikar (1965) achieved specific removal of tosyl blocking groups without cleavage adjacent to proline, by use of anhydrous conditions. (Methanol was necessary to achieve cleavage adjacent to proline). Conversely, Benisek et al., (1967) claimed the presence or absence of methanol (proton donor) or sodium amide (proton scavenger) is not significant and that non-specific reduction of phenylalanine increases in the presence of excess sodium at -78°C. Atassi & Singhal (1970) believe that non-specific cleavage and reduction are due solely to the presence of excess sodium. Evidently, there is room for further investigation of the applicability of this reduction to keratin proteins, and the effect of other metals, anhydrous conditions, excess sodium, and the presence or absence of proton donors, on reduction achieved.

Following specific cleavage of one or more bonds in the keratin, it is necessary to check that microfibrils are still present in the fibre. Sample 4 (Table 5-2) was found difficult to stain for electron microscopic observation, but after placing the fibre in a low pH medium (=pH 1), the nitrile group is hydrolysed and fibres can be stained with ammoniacal silver nitrate (Kassenbeck, 1967) (Figure 5-4). Nitriles are not usually this easily hydrolysed, but it is believed neighbouring group
FIGURE 5-4

FULLY REDUCED AND S-CYANOETHYLATED WOOL FIBRE STAINED WITH SILVER AMMINE (KASSENBECK, 1967).
participation by sulphur is involved (Sykes, 1961).

Having achieved the necessary specific bond cleavage (see comments on completeness of disulphide reduction and solubility, Chapter 5C(iii)), a method of preferentially dissolving the matrix without unfolding microfibrils, is necessary. The empirical approach to this problem employed in part of this chapter has been unrewarding, but observation of protein behaviour in different solvents and pH condition by NMR spectroscopy is capable of solving the difficulty. NMR spectroscopy of keratin proteins (Chapter 4) was not fully examined in this regard, but some areas suitable for further investigation were indicated (e.g. the use of 8 M urea, pH 4 with SCM proteins, and 8 M urea, pH up to 8, for S-cyanoethyl proteins).

Harrap & Gillespie (1963) showed that the amount of L.S. protein released by plasmolysis decreased to zero in 0.5 ionic strength solutions. NMR spectroscopy would show whether this behaviour is due to the L.S. proteins remaining folded in 0.5 ionic strength solutions (von Hippel & Wong, 1964; von Hippel & Schleich, 1969). This possibility has been employed in the work described in Chapter 5C(iv), one advantage being that some enzymes (trypsin, pepsin) are still at least partially active in 0.5 ionic strength solution.

The final stage of this investigation involves examination of the extracted microfibrils in the electron microscope. This is difficult as they are observed in the fibre basically as unstained regions. There is also the possibility of contamination, which has troubled other workers in this field. Possibly the most satisfactory
solution to this problem is to be able to observe microfibrils still within the fibre. A number of stains were tried unsuccessfully in this regard, the best stain being osmium and as noted in Chapter 5C, detail in fibre cross-sections was absent at high magnification. Negative staining of extracts was not thoroughly examined and is peculiar to each system (Kay, 1965), and this, or metal shadowing may solve the problem of observing extracted microfibrils.

In conclusion, to isolate native microfibrils successfully, the NMR spectroscopy of L.S. and H.S. proteins should be examined to determine conditions where the L.S. proteins remain folded and the H.S. proteins are unfolded. Use of this particular solvent system will remove H.S. protein from the microfibrils and allow their separation. Further work is also required to establish suitable conditions for negative staining or metal shadowing of the isolated microfibrils and for staining them within the fibre with stains which will stain the microfibril preferentially.
CHAPTER 6.

STUDIES ON GOLD STAINING OF WOOL.

6A. INTRODUCTION

Two previous advances made in this laboratory have relied upon staining with gold for their success, these were the separation of orthocortical and paracortical cells from wool (Bradbury et al., 1968; Chapman & Bradbury, 1968), and the separation of medulla from keratin fibres (Bradbury & O'Shea, 1969). Both cases used the chloroauric acid staining method of Laxer & Ross (1954).

The separation of orthocortical cells and paracortical cells by Chapman & Bradbury (1968) depends upon the assumption that the heavier, darkly stained cells originate from the darkly stained paracortex of the fibre, and the lighter cells from the orthocortex. Electron micrographs of fibre cross-sections show the gold particles are concentrated in nuclear remnants of the paracortex (Figure 6-1), but some colloidal gold is liberated on ultrasonic disintegration of the fibres in formic acid (Chapman & Bradbury, 1968). It was suggested (Swart, 1969) that the similarity of amino acid analyses obtained by Chapman & Bradbury for orthocortex and paracortex, together with the known resistance of the paracortex to disruption or dissolution (Mercer, 1953; Golden, 1954; Fraser et al., 1954; Leach et al., 1954; Chapman, 1967) indicated an incomplete separation of orthocortical cells and paracortical cells had been
achieved by Chapman & Bradbury. In order to make an unequivocal check that this separation had been attained, the lightly and heavily stained cells were examined by electron microscopy.

Following the initial work by Chapman & Bradbury (1968), several workers in this laboratory have attempted to find some of the reasons for the differential uptake of gold by the various fibre histological components. These attempts were mainly unsuccessful due to the use of absorption studies with fibres (O'Shea, 1970), or amino acids and soluble proteins (Swart, 1969; O'Shea, 1970), which are all fairly complicated systems as regards possible binding sites for gold. Chapman & Bradbury (1968) attribute part of the staining to reduction of gold III (from the 2% HAuCl₄ solution used) to gold by cystine, which is itself oxidized to cysteic acid. The larger particles of gold observed (see Figure 6-1) were considered due to reduction of auric ion by formic acid in the fibre. Bradbury & O'Shea (1969) found that addition of auric ions to solutions of amino acids resulted in the formation of coloured complexes. They concluded that chelation appears to involve primary amino, primary amide or \( +\text{NH}_3-\text{CHR-COO}^- \) groups and not simply carboxyl groups as in poly-L-glutamic acid.

In his thesis however, O'Shea (1970) determined that the binding of gold was due to carboxyl groups present in the fibres. This accounted for the high uptake of gold by medulla, (which contains on average 23 mole % glutamic acid plus glutamine) and that the maximum absorption of Au III from unbuffered solution by kangaroo
fibre occurs between pH 2 and 6, with liberation of protons, but methylation of the fibre did not alter the gold uptake. However, the high uptake of gold by medulla could also be due to reduction of chloroauric acid by formic acid trapped in the medullary cells. More recently, O'Shea (1971) has shown that by lowering the amino content of kangaroo fibres by 67% (by deamination) the uptake of gold from unbuffered solution decreased by 63%.

6B. MATERIALS AND METHODS.

(i) Gold Staining and Electron Microscopy of Cortical Cells.

Preparation of the orthocortical and paracortical cells for microscopy was kindly undertaken by Mr. K. F. Ley (Ley, 1971). This involved preparation by the method described previously (Chapman & Bradbury, 1968) with only minor modification to the density gradients employed (Ley, 1971). The two cell types were then stained with thioglycollate/osmium tetroxide (Chapter 2B) and aligned on a perspex microscope slide by stroking a viscous mixture of the cells in Araldite with a glass rod. The portion of the slide containing aligned cells was embedded in a block and sectioned perpendicularly to the cell axes. Sections were post-stained with lead citrate (Reynolds, 1963).

(ii) NMR Spectroscopy of Model Compounds with Chloroauric Acid.

Cystamine (bis(β-aminoethyl)disulphide) was prepared by the method of Nathan & Bogart (1941) from cysteamine (β-aminoethyl thiol). It was found to be pure by paper
chromatography in n-butanol:acetic acid:water, 60:20:2. All other reagents used were A.R. grade. Molarity of poly-α-amino acids was calculated using the individual residue weights and not the polymer molecular weight.

Except in the two cases where the need to examine the resonance of the α proton required the use of D2O, all NMR spectra were performed in H2O using T.M.S. as an external standard. Values quoted for pH in D2O solution (i.e. for methionine and S-methyl cysteine) are pH meter readings uncorrected for deuterium isotope effects. Other details of NMR spectroscopic techniques are given in Chapter 4C.


Merino 64's wool, cleaned as described previously (Chapter 1B), was immersed in 2% chloroauric acid w/v at pH 4.5 and at ambient temperature for 13 weeks. The 2% chloroauric acid was prepared from B.D.H., L.R. chloroauric acid, using the assay figure to give a solution 2% in Au. The wool was washed briefly with deionised water to remove chloroauric acid solution from the fibre surface, and then dehydrated with alcohol, propylene oxide and embedded in Araldite by the normal procedure (Chapter 2B). Sections were stained with lead citrate (Reynolds, 1963).

6C. RESULTS AND DISCUSSION

(i) Separation of Orthocortical and Paracortical Cells.

The wool fibre cross-section in Figure 6-1 (Chapman & Bradbury, 1968) shows the typical appearance of a fibre
FIGURE 6-1

stained with chloroauric acid by the method of Laxer & Ross (1954). It appears that slightly more gold is deposited in the paracortex itself, but considerably more metallic gold is deposited in nuclear remnants of the paracortex. Partly on the basis of this electron micrograph, Chapman & Bradbury (1968) concluded that the heavier and more darkly stained cells they had isolated, originated from the paracortex, and the lighter and less stained cells, from the orthocortex. The most obvious method of checking the correctness of this assumption is to examine the fine structure of the two types of cells. Orthocortical cells can be identified by the presence of macrofibrils, and the arrangement of whorls of microfibrils within those macrofibrils; and paracortical cells have fewer and larger macrofibrils, and the microfibrils are packed in quasi-hexagonal arrays (Rogers, 1959, 1959a).

A number of sections were cut from the 'light' and 'heavy' cells prepared by Mr. K.F. Ley and examined in the electron microscope. About 400 sections of cells from the light fraction and about 400 sections of cells from the heavy fraction were examined, and any cell section which could not be readily identified in the microscope, was photographed and identification made from the print. Of the total 800 (approximately) sections examined, greater than 99% of the lighter fraction were found to be orthocortical cells, and in the heavier fraction greater than 99% were found to be paracortical cells. If any metacortical cells occur in Merino 64's, then as can be seen from Chapter 6C(iii) these would probably be found in the heavy paracortical cell fraction. It is doubtful if
metacortical cells would be identified as such, due to their probable similarity to paracortical cells.

Typical paracortical and orthocortical cells are shown in Figures 6-2 and 6-3 respectively. The microfibril/matrix structure and large nuclear remnant containing crystals of metallic gold, shown in Figure 6-2 is typical of a gold stained paracortical cell (Rogers, 1959; Chapman & Bradbury, 1968). This picture has been taken at slight underfocus to emphasize the microfibril/matrix structure (Millward, 1970). The orthocortical cell in Figure 6-3 can be identified by the macrofibrils which are observed (Rogers, 1959). In this case, the small nuclear remnant has been partially removed during preparation. The black specks on the perimeter of this cell are thought to be osmium metal produced during the osmium staining procedure. Note that the body of the cell has fewer specks of gold deposited in the keratin itself, in comparison with those visible in the paracortical cell.

As further evidence, it was noted during a study of ultrasonically treated fibres from cortical cell preparations of whole wool, that in no case was a fibre observed in which only part of the cortex was disrupted. Breakdown appears to occur from the fibre ends, and hence orthocortical and paracortical cells should be produced roughly in their correct ratio.

The above results confirm the validity of the separation of orthocortical and paracortical cells which was achieved by Chapman & Bradbury (1968). It also indicates that the amino acid analyses of orthocortex and paracortex are indeed similar, as suggested by Chapman &
FIGURE 6-2

GOLD STAINED PARACORTICAL CELL.
FIGURE 6-3

GOLD STAINED ORTHOCORTICAL CELL.
Bradbury (1968).

(ii) NMR Study of Possible Gold Staining Sites.

A coverage of the theory of NMR spectroscopy was given in Chapter 4B. Only chemical shift and broadening are of particular interest to this investigation, and will be briefly mentioned here.

Causes of broadening which are of interest here, are those due to dipole-dipole interactions produced by the imposition of a static local field. Apart from the presence of an adjacent paramagnetic nuclei, this can also be caused by a nucleus with a large quadrupole moment or even by a closely placed proton, also by restricting rotation of the molecule by e.g. increasing molecular size.

As noted in Chapter 4B, inductive effects acting on a proton cause downfield shifts of the resonance due to withdrawal of the shielding magnetic field produced by rotation of bonding electrons. This occurs when an electronegative atom or group is adjacent to the proton under observation.

The behaviour of a series of model compounds with chloroaauric acid, have been summarised in Table 6-1. These figures do not clearly demonstrate the effects on certain resonances, which occur after the addition of chloroaauric acid to the compound. For this reason, a few NMR spectra have been included to show that these effects can be quite pronounced on some resonances.

In Table 6-1, all chemical shifts of appreciable size have been downfield, indicating that an inductive effect probably operates during interactions with gold. Where broadening of peaks has occurred, this is thought to be
due primarily to interaction with the chloroauric acid. The species produced cannot be determined easily, but broadening will be due to one or a combination of the effects mentioned above. In all cases, broadening and chemical shifts are indicators of an interaction between chloroaurate ion and the molecule under investigation. Generally the group adjacent to the proton(s) under observation, where chemical shift or broadening is most pronounced, is that which interacts with the chloroaurate ion.

**Carbonyl and Carboxyl Groups** - O'Shea (1970) implicated carboxyl groups in aspartic and glutamic acids as the prime site of interaction with chloroauric acid, due partly to the heavy deposition of gold in the medulla of various fibres. Medulla is known to have a high concentration of glutamic acid (Bradbury & O'Shea, 1969). His absorption studies also indicated that the $pK_a$ of the group reacting with chloroauric acid corresponded to that of glutamic or aspartic acids. Table 6-1 shows that none of the carbonyl or carboxyl compounds interact significantly with chloroaauric acid, although the colloidal gold formed with poly-L-aspartic acid cannot be explained, unless a contaminant was present. Poly-L-glutamic acid is insoluble below about pH 6, and was not examined.

**Amide Groups** - These again show no significant reaction with chloroauric acid. No colloidal gold was formed and therefore it is doubtful if the amide group was responsible for the formation of colloidal gold with poly-L-aspartic acid (above).

**Amino Groups** - With both methylamine and poly-L-lysine
## TABLE 6-1

**NMR STUDIES OF GOLD III BINDING SITES OF PROTEINS**

### Chemical Shift and Half-widths for Model Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak Observed</th>
<th>Chemical Shift (±)</th>
<th>Half-width (p.p.m. x 10^3)</th>
<th>pH</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CARBONYL GROUPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone 1.16M</td>
<td>C=O</td>
<td>7.775 (1)*</td>
<td>2.8</td>
<td>4.5</td>
<td>Normal Au colour.</td>
</tr>
<tr>
<td>Acetone 1.16M + Au 0.0662M</td>
<td>C=O</td>
<td>7.78 (1)</td>
<td>2.4</td>
<td>4.5</td>
<td>Normal Au colour.</td>
</tr>
<tr>
<td>Acetic acid 1.16M</td>
<td>C=O</td>
<td>7.95 (1)</td>
<td>1.8</td>
<td>4.6</td>
<td>As Na salt.</td>
</tr>
<tr>
<td>Acetic acid 1.16M + Au 0.0662M</td>
<td>C=O</td>
<td>7.94 (1)</td>
<td>1.8</td>
<td>4.6</td>
<td>Normal Au colour.</td>
</tr>
<tr>
<td>Poly-L-aspartic acid 1.0M</td>
<td>C=O</td>
<td>7.11 (2b)*</td>
<td>20.2</td>
<td>4.75</td>
<td>Insoluble at pH 4.5.</td>
</tr>
<tr>
<td>Poly-L-aspartic acid 1.0M + Au 0.0662M</td>
<td>C=O</td>
<td>7.11 (2b)</td>
<td>20.0</td>
<td>4.75</td>
<td>Red colloidal Au formed.</td>
</tr>
<tr>
<td><strong>AMIDE GROUP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-methyl acetamide 1.3M</td>
<td>CH\textsubscript{3}</td>
<td>8.04 (1)</td>
<td>3.0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>N-methyl acetamide 1.3M + Au 0.0662M</td>
<td>CH\textsubscript{3}</td>
<td>(7.25) (2)</td>
<td>2.7</td>
<td>3.5</td>
<td>Normal Au colour.</td>
</tr>
<tr>
<td><strong>AMINO GROUPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylamine 1.3M</td>
<td>CH\textsubscript{3}</td>
<td>7.31 (1)</td>
<td>2.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Methylamine 1.3M + Au 0.011M</td>
<td>CH\textsubscript{3}</td>
<td>7.31 (1)</td>
<td>5.5</td>
<td>4.5</td>
<td>No Au colour.</td>
</tr>
<tr>
<td>Methylamine 1.3M + Au 0.0662M</td>
<td>CH\textsubscript{3}</td>
<td>7.26 (1)</td>
<td>20.0</td>
<td>4.5</td>
<td>No Au colour.</td>
</tr>
<tr>
<td>Poly-L-lysine HBr 0.78M</td>
<td>CH\textsubscript{3}</td>
<td>8.33 (1b)</td>
<td>37.5</td>
<td>4.3</td>
<td>Immediate bright red precipitate.</td>
</tr>
<tr>
<td>Poly-L-lysine HBr 0.78M + Au 0.006M</td>
<td>CH\textsubscript{3}</td>
<td>(6.90) (2b)</td>
<td>22.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly-L-lysine HBr 0.78M + Au 0.011M</td>
<td>CH\textsubscript{3}</td>
<td>8.34 (1b)</td>
<td>38.5</td>
<td>4.3</td>
<td>Immediate bright red precipitate.</td>
</tr>
<tr>
<td>Poly-L-lysine HBr 0.78M + Au 0.062M</td>
<td>CH\textsubscript{3}</td>
<td>(6.92) (2b)</td>
<td>23.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly-L-lysine HBr 0.78M + Au 0.0662M</td>
<td>CH\textsubscript{3}</td>
<td>8.32 (1b)</td>
<td>37.5</td>
<td>4.3</td>
<td>Deep orange-red precipitate.</td>
</tr>
<tr>
<td><strong>SULPHUR GROUPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiodiglycol 0.57M</td>
<td>CH\textsubscript{3} - S</td>
<td>7.32 (3)</td>
<td>2.0</td>
<td>4.6</td>
<td>No Au colour then red colloidal Au formed.</td>
</tr>
<tr>
<td>Thiodiglycol 0.57M + Au 0.0662M</td>
<td>CH\textsubscript{3} - S</td>
<td>7.04 (3)</td>
<td>3.0</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Methionine 0.47M in D\textsubscript{2}O</td>
<td>CH\textsubscript{3} - S</td>
<td>7.81 (1)</td>
<td>2.4</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Methionine 0.47M in D\textsubscript{2}O + Au 0.0662M</td>
<td>CH\textsubscript{3} - S</td>
<td>7.64 (1)</td>
<td>2.4</td>
<td>4.6</td>
<td>Immediately decomolourised then red colloidal Au formed.</td>
</tr>
</tbody>
</table>

* - Multiplicity of peak: ± b signifies broad peak, m signifies multiplet: ND - Not determined:
### TABLE 6-1 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak Observed</th>
<th>Chemical Shift (τ) (p.p.m.×10^2)</th>
<th>Half-width (p.p.m.)</th>
<th>pH</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SULPHUR GROUPS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-carboxymethyl cysteine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.19M</td>
<td>S-CH(_2)-COOH</td>
<td>6.61 (1)</td>
<td>ND</td>
<td>4.5</td>
<td>Yellow-brown precipitate then colloidal Au formed.</td>
</tr>
<tr>
<td></td>
<td>S(\text{CH}_2)</td>
<td>6.80 (2)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-carboxymethyl cysteine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.19M + Au 0.0662M</td>
<td>S-CH(_2)-COOH</td>
<td>6.56 (1)</td>
<td>ND</td>
<td>4.5</td>
<td>Yellow-brown precipitate then colloidal Au formed.</td>
</tr>
<tr>
<td></td>
<td>S(\text{CH}_2)</td>
<td>6.76 (2)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-methyl cysteine 0.52M</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in D(_2)O</td>
<td>CH(_3)-S</td>
<td>7.80 (1)</td>
<td>2.3</td>
<td>4.3</td>
<td>No Au colour then colloidal Au formed.</td>
</tr>
<tr>
<td></td>
<td>S(\text{CH}_2)</td>
<td>6.83 (2)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aCH</td>
<td>5.96 (3)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-methyl cysteine 0.52M</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in D(_2)O + Au 0.0662M</td>
<td>CH(_3)-S</td>
<td>7.75 (1)</td>
<td>2.3</td>
<td>4.3</td>
<td>No Au colour then colloidal Au formed.</td>
</tr>
<tr>
<td></td>
<td>S(\text{CH}_2)</td>
<td>6.80 (2)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aCH</td>
<td>5.95 (3)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>THIOLS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteamine 0.91M</td>
<td>CH(_2)-NH(_2)</td>
<td>6.67 (5)</td>
<td>ND</td>
<td>4.6</td>
<td>No Au colour.</td>
</tr>
<tr>
<td>NH(_2)(CH(_2))(_2)-SH</td>
<td>CH(_2)-SH</td>
<td>7.04 (3)</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteamine 0.91M + Au 0.0662M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH(_2)-NH(_2)</td>
<td>6.67 (5)</td>
<td>ND</td>
<td>4.6</td>
<td>No Au colour.</td>
</tr>
<tr>
<td></td>
<td>CH(_2)-SH</td>
<td>(6.93 (2b)</td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7.00)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine 0.51M</td>
<td>CH(_2)-SH</td>
<td>(6.80 (2)</td>
<td>4.0</td>
<td>4.3</td>
<td>Immediate pale cream precipitate.</td>
</tr>
<tr>
<td></td>
<td>(6.88)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine 0.51M + Au 0.0662M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH(_2)-SH</td>
<td>(6.80 (2)</td>
<td>4.0</td>
<td>4.3</td>
<td>Immediate pale cream precipitate.</td>
</tr>
<tr>
<td></td>
<td>(6.88)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DISULPHIDE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystamine 0.46M (NH(_2)(CH(_2))(_2)-S(_2))</td>
<td>CH(_2)-S</td>
<td>6.47 (3)</td>
<td>21.0</td>
<td>4.4</td>
<td>Immediate red-brown precipitate.</td>
</tr>
<tr>
<td></td>
<td>CH(_2)-NH(_2)</td>
<td>6.85 (3)</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystamine 0.46M + Au 0.0662M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH(_2)-S</td>
<td>6.48 (3b)</td>
<td>28.0</td>
<td>4.4</td>
<td>Immediate red-brown precipitate.</td>
</tr>
<tr>
<td></td>
<td>CH(_2)-NH(_2)</td>
<td>6.86 (3)</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* - Multiplicity of peak; † - b signifies broad peak, m signifies multiplet; ND - Not determined.
a clear effect is observed on addition of chloroauric acid. Both broadening and a chemical shift are observed with methylamine, and these effects are shown in Figure 6-4.

With poly-L-lysine the effect rapidly decreases in intensity further away from the amino group on the side chain, as would be expected for an inductive effect. Only the ε CH₂ group shows broadening of the peak, following increasing additions of chloroauric acid, and the other CH₂'s in the side chain are unaffected (Figure 6-5).

Numerous cases of interactions of gold with basic groups are to be found in the literature, e.g. complexes with 2,2-bipyridine, 1,10-phenanthroline (Dothie et al., 1939; Harris & Lockyer, 1959), ethylene diamine (Block & Bailar, 1951) and various primary and secondary amines and ammonia (Fowles, 1960; Weitz, 1915) are known.

King (1970) demonstrated that chloroauric acid reacts with the NH groups of lysine and arginine in lysozyme. These NH resonances are visible in the NMR spectrometer, with lysozyme in 8 M urea at pH 2.5 and in H₂O. On addition of chloroauric acid the lysine NH peak at 2.4T progressively diminishes in area as the chloroauric acid concentration is increased, and is replaced by another peak at 2.8T. Similarly the arginine peak is strongly affected by increasing chloroauric acid concentration.

Ion-exchange behaviour of chloroaurate ion in aqueous hydrochloric acid solution has been studied. Korkisch & Klakl (1968) and Butterfield (1971) have both observed that chloroaurate ions are strongly absorbed by
FIGURE 6-4
NMR SPECTRA OF METHYLAMINE WITH VARYING CONCENTRATIONS OF CHLOROAURIC ACID, pH 4.5.

Chemical Shift
FIGURE 6-5

NMR SPECTRA OF POLY-L-LYSINE WITH VARYING CONCENTRATIONS OF CHLOROAURIC ACID AT pH 4.3.

As noted in the introduction, O’Shea (1971) has shown that on decreasing the cation content of resins by demineralization, a corresponding decrease in uptake of chloroauric acid occurs. Thus et al. (1960) have prepared acido-diethylammoniumgold III salts and shown that these compounds behave as if they associated on coordination with less than one equivalent of chloroauric acid per gold, a phenomenon on one of the ethyl groups has a pH of less than one. This salt would similarly be expected to cur on one of the gold atoms. A similar explanation can explain the results of previous investigations by O’Shea (1970), who concluded that the interaction of gold with poly-L-lysine sulfides-—-the result of the reaction of gold with chloroauric acid—-is with the sulfides but not with the thioethers.

The chemical shift of the e-CH₂ group and the β-CH₂ group, adjacent to sulfur, are similar to that of the e-CH₂ group, adjacent to sulfur, in the naturally occurring β-CH₂ group. This indicates that the gold has not reacted with the thiol group, or reacted with the thiol group to any extent, and not due to any reaction with the β-CH₂ group, or any reaction with the ethyl group. The shift observed for the ethyl group is explained in terms of the presence of the gold. The shift observed for the e-CH₂ group is explained in terms of the presence of the gold.
basic ion exchange resins, being bound most strongly by the more basic resins (Butterfield, 1971). Butterfield also found that chloroauric acid is not retained by acidic ion exchange resins (containing carboxyl groups). As noted in the introduction, O'Shea (1971) has shown that on decreasing the amino content of kangaroo fibre by deamination, a corresponding decrease in uptake of chloroauric acid occurs. Baddley et al. (1963) have prepared acido-diethylenetriaminegold III salts, and shown that these compounds behave as if they are acids; on co-ordination with gold, a proton on one of the amino groups has a pK of less than 6. Should similar behaviour occur on binding of gold to amino groups in the fibre, then this would explain the liberation of protons observed by O'Shea (1970), which he concluded was due to interaction of gold with carboxyl groups.

Sulphides - The results in Table 6-1 indicate that chloroauric acid reacts with the sulphide group of thiodiglycol, methionine, S-carboxymethyl cysteine and S-methyl cysteine. With thiodiglycol, the chemical shift of the CH₂ adjacent to the OH is probably due to an inductive effect passing along the carbon chain from the sulphide, and not due to any reaction with the hydroxyl. Similarly with methionine and S-methyl cysteine, the chemical shift is more pronounced at the methylene adjacent to sulphur than at the α CH. This indicates that gold has a greater tendency to complex with, or react with sulphur, than with amino groups.

King (1970) treated ribonuclease in 8 M urea at pH 3 with chloroauric acid, and observed that the NMR resonance for the S-methyl group of methionine disappears, and is
replaced by another peak close to the position of the S-methyl peak of methionine sulfoxide. Reaction of chloroauric acid with sulphides generally proceeds with formation of colloidal gold and conversion of the sulphide to the sulfoxide or sulphone (Teresa, 1943, 1944).

**Thiols** - The results in Table 6-1 indicate that chloroauric acid reacts with cysteamine at the thiol group and probably not at the amino group. However, there is no obvious effect of chloroauric acid on cysteine from the point of chemical shift or broadening of the peaks. In this case it is most likely that all the gold is carried down with the immediate precipitate which is formed. The spectrum is probably that of unmodified cysteine, as its overall intensity decreased to about ¼, following addition of chloroauric acid.

Complexes of gold with thiols are known, the gold being thought to complex preferentially to thiol in the presence of carboxylic acids (Moore & Rapala, 1947; Moore, 1950; Kundu, 1952), and in the presence of various substituted amines and amides (Weiss, 1947) and sulphonamides (Basu & Sikdar, 1947).

**Disulphides** - With cystamine, the results in Table 6-1 show that reaction may also be occurring with the amino group, although it is considerably less pronounced than interaction with the disulphide. This may indicate that sulphur and nitrogen are competing for reaction with chloroaurate ion. However, Sidgwick (1950) states 'the tendency of combined sulphur to act as electron donor increases in the order $\text{SH}_2$, $\text{HSR}$, $\text{SR}_2$, $\text{RSSR}$', indicating even stronger reaction with disulphide than with sulphide.
FIGURE 6-6

NMR SPECTRA OF CYSTAMINE
\((\text{NH}_2-(\text{CH}_2)_2)_2\text{-S}_2\) WITH VARYING CONCENTRATIONS OF CHLOROAURIC ACID AT pH 4.4.

+0.0662 M Au

Chemical Shift \(\tau\)
or thiol. A more probable explanation may be that due to the size of the gold atom, complexing can only occur with one sulphur and one nitrogen on each molecule (i.e. at positions 1 and 5), which then acts as a bidentate ligand to form a 6 membered ring with the gold atom.

In attempting to define metal binding sites on proteins by the use of model compounds, the altered reactivity of amino acid side chains when incorporated into proteins must be appreciated. Protein structure itself can alter reactivity, e.g. although tyrosine residues of ovalbumin do not dissociate protons when titrated with base (Cannan et al., 1941), they can be ionised on protein denaturation (Crammer & Neuberger, 1943). Both 'accessible' and 'buried' tyrosyl residues have been revealed in proteins by spectrophotometric and chemical methods, (Beaven & Holiday, 1952; Wetlaufer, 1962; Simpson et al., 1963; Edsall, 1963; Rioian et al., 1965, 1966, 1967a,b; Sokolovsky et al., 1966). Similar effects have been observed with thiol groups (Mirsky & Pauling, 1936; Neurath et al., 1944) and with lysyl and histidyl residues (Horinishi et al., 1964; Fraenkel-Conrat, 1959). All possible binding sites in proteins need not be either accessible or available for metal binding as would be indicated by studies on model compounds. Such apparent anomalies in the behaviour of amino acid side chains in proteins in comparison with their behaviour in polyamino acids or peptides, probably constitute characteristic features of proteins. Finally, stability constants of protein/metal systems where the metal is merely added to the protein, are much lower than is the case of metallo-
proteins e.g. transferrin, ferredoxin and haemocyanin, where a specific metal binding site exists (Steinhardt & Beychok, 1964). In this latter case the protein generally has no biological activity in the absence of the metal.

The valence state of gold bound to various model compounds remains unknown, and it may exist in the fibre in more than one valence state, or a different one to that in the model compound. There are however, only two stable valence states of gold possible in aqueous systems (I and III), and Au I is considerably less stable in aqueous solution than Au III (or Au) (Moeller, 1952).

This study of gold binding to model compounds does indicate some possible sites in proteins; these are - binding to sulphur in disulphides, sulphides and thiols and to nitrogen in amino groups. There is evidence that gold probably does not bind to nitrogen in peptide bonds (i.e. secondary amides) nor to carbonyl, carboxyl or hydroxyl groups.

(iii) Accessibility of the Fibre to Chloroauric Acid.

As noted in Chapter 6C(i), the gold stained fibre in Figure 6-1 is a typical example of the staining produced by Laxer & Ross' (1954) method. The nuclear remnants are heavily stained with metallic gold, and the paracortex is slightly more heavily stained than the orthocortex. This fibre was prepared by treatment with formic acid prior to immersion in chloroauric acid. Chapman & Bradbury (1968) concluded that the heavy deposit of gold in the nuclear remnant is due to reduction of chloroauric acid by formic acid contained in inter-
stices in the nuclear remnant, which are perhaps produced on dissolution of material by the formic acid.

The origin of the red colour observed in gold stained fibres is probably due to these aggregates of metallic gold particles in the nuclear remnants. Measurements from Figures 6-1 and 6-2 indicate the average size of these particles is about 200 angstrom diameter, although particles of smaller size (20-80 angstrom) are distributed throughout the keratin. Alexander & Johnson (1949) state that the red colour of gold sols is produced by particles of about 400 angstrom diameter. Smaller size particles appear more red, but particles which are not spherical appear more blue (Alexander & Johnson, 1949) so that overall these two effects should compensate, and particles observed in the nuclear remnants are probably the main source of the red colouration.

Figure 6-7 shows a cross-section of fibre which has been immersed in 2% chloroauric acid for 13 weeks, and did not receive any pretreatment or post treatment with formic acid. The orthocortex and paracortex can be easily distinguished, and nuclear remnants in the paracortex are more heavily stained than the surrounding protein. Intermacrofibrillar material can be identified in parts of the orthocortex. Perhaps most noticeable, is the different mode of staining the two types of keratin (i.e. in the orthocortex and paracortex). The dark areas of both orthocortex and paracortex are believed to be regions where chloroauric acid has penetrated into the fibre. The 'white' regions in the paracortex indicate areas where the chloroauric acid has been unable to penetrate. Figures 6-8 and 6-9 are
FIGURE 6-7

WOOL FIBRE STAINED WITH CHLOROAURIC ACID FOR 13 WEEKS.
higher magnification images of the boundary between orthocortex and paracortex. The characteristic appearance of cell membranes can be seen in these cases, the light and dark regions corresponding to the \( \beta \) and \( \delta \) intercellular layers respectively, of Rogers (1959a) (Chapter 2).

The apparent difference in staining behaviour of the orthocortex and paracortex could be due to a number of reasons. These are:-

1. Failure to reach equilibrium in staining i.e. a rate effect.
2. Some difference in the affinity of the proteins in the orthocortex and paracortex for chloroauric acid.
3. Changes in the deposited \( \text{Au} \ III \) produced during electron bombardment.
4. An artifact of the lead citrate section stain used.
5. Dissolution of some protein from the cortex.
6. Different accessibility of regions of the paracortex to chloroauric acid.

Taking these points in order:-

1. Ley (1971) has shown that in 2\% chloroauric acid solution, the fibre increases in weight by 26\%, reaching equilibrium in about 12 hr., demonstrated by a levelling off of the ash content after this time of immersion. If the fibres are washed in water for 3-4 days after initial immersion in chloroauric acid solution, the equilibrium uptake of gold is 14.1\%, and it is reached after an initial immersion in chloroauric acid of only 3 to 6 hr.. Obviously some of the gold is retained in a non-bonded form, and examination of fibres from this treatment in the electron microscope, shows a decrease in the amount of gold
FIGURE 6-8

HIGHER MAGNIFICATION IMAGE OF THE FIBRE IN FIGURE 6-7.
FIGURE 6-9

HIGHER MAGNIFICATION IMAGE OF THE FIBRE IN FIGURE 6-7 SHOWING METACORTICAL CELL.
particles in the nuclear remnants. Additionally, if a rate effect were the cause of differential staining observed in Figures 6-7 to 6-9, a variation in density across each of the two cortices would be expected. Ley's results indicate that 13 weeks immersion should be sufficient to overcome any rate effect on staining.

It is not clear why Ley's (1971) results show staining equilibrium is reached in 12 hr.; but in only 3-6 hr. if the fibre is washed afterwards. Perhaps slow cleavage of disulphides by chloroauric acid (Chapman & Bradbury, 1968) permits a slow entry of more chloroauric acid. Alternatively the gold may bind to co-ordination sites rapidly (Chapter 6C(ii)) but excess gold solution only enters slowly.

2. There are well known differences in the proteins of the orthocortex and paracortex to staining with e.g. phosphotungstic acid (Bonès & Sikorski, 1967), osmium tetroxide (Rogers, 1959, 1959a), potassium permanganate (Rogers & Filshie, 1962) and various dyes, e.g. Horio & Kondo (1953). A review of bilateral stains for wool is given by Chapman (1967). In the case of the metallic stains quoted above however, all show an even distribution of stain within the paracortex totally different to that observed with chloroauric acid. This does not exclude the possibility of a variation in affinity of the two cortices for chloroauric acid, but it does indicate a different mechanism is operating in this case. In Chapter 6C(ii) it was observed that sulphur is a prime binding site for gold. If binding of gold to sulphur were the only cause of the differential staining
observed, then the paracortex should be most heavily
stained as it contains a higher ratio of high sulphur
(matrix) protein than the orthocortex (Rogers, 1959a).
3. Fischer (1954) has shown that only slight morpho-
logical changes occur on bombardment of gold chloride in
the electron microscope. Production of metallic gold is
unlikely as the fibres retain the pale yellow colour of
chloroauric acid indicating the absence of colloidal gold
in the fibre. Also, metallic gold appears as dense spots,
a few of which can be seen outside the cuticle in
Figure 6-8. It is doubtful that the differential staining
is due to changes in the stain produced by the electron
beam, as these should affect both cortices equally.
4. To check the effect of lead citrate section stain
on the observed behaviour, some sections were examined
without post-staining with lead citrate. In this case,
the overall density was extremely low, but the difference
in staining behaviour observed previously was still
evident. Staining with lead does not occur unless the
keratin has been bulk stained previously, and the lead
can be removed with dilute (0.01N) acids or 0.1 M
E.D.T.A. as a chelating agent (Filshie & Rogers, 1961).
5. Solution of sufficient protein from the orthocortex
and paracortex to cause the differential staining is
thought unlikely due to the low solution to wool ratio,
and the neutral pH employed. It is however probable that
some disulphide bonds have been cleaved by the treatment,
due to the known formation of cysteic acid in gold
stained fibres (Chapman & Bradbury, 1968).
6. The most reasonable cause of the differential staining
observed in the two cortices, appears to be a variation in the accessibility of the paracortex to chloroauric acid. Accessibility was suggested to be important for dyes by Rogers (1959). Swift (1965) has shown that silver ammine diffuses evenly into fibres, but the larger molecules of phosphotungstic acid do not diffuse into untreated hair, but enter areas where chemical or physical damage has occurred. Conversely, Bradbury & Leeder (1963) showed this effect may be due to swelling of the fibre as n-pentane, of similar shape but smaller volume than n-hexanol, is absorbed more slowly at 40°C than n-hexanol is at 25°C. However n-hexanol swells wool to a greater extent than n-pentane.

The higher level of staining in the nuclear remnants (Figure 6-7) may be due to the presence of a protein with a high content of lysine plus arginine (Chapter 3) which are a site for binding of gold (Chapter 6C(ii)). It may also be due to the presence of cavities in the nuclear remnants retaining some chloroauric acid solution, as Ley (1971) was able to decrease the gold staining in nuclear remnants by washing fibres after they had been initially stained.

Nucleic acids or their fragments may also exist in the nuclear remnants, and these also have been shown to react with chloroauric acid. Hartman (1967) has shown by infrared spectra that gold chloride at pD 6-8 interacts with various nucleosides and nucleotides. The Soc. Anon. Pour fabricated in ind. Chin. à Bâle (1926) has produced gold compounds of therapeutic value by reaction of gold chloride with nucleic acids. In neutral or alkaline solutions these
are soluble, but become insoluble in acid media.

The lower accessibility of the paracortex to chloroauric acid obviously does not stop penetration of the solution into the nuclear remnants. This infiltration probably occurs either through intermacrofibrillar material, or through the more permeable material of the paracortex which appears darkly stained in Figure 6-7.

Examination of Figure 6-7 shows two or three cells close to the boundary between the orthocortex and paracortex have taken up chloroauric acid slightly more than the rest of the paracortex. One of these cells is shown at higher magnification in Figure 6-9. This could be the metacortex observed by Brown & Onions (1960) who showed anomalous (more basophilic) staining behaviour with dyes in Southdown and Corriedale wools. They noted that the position of their 'metacortex' was close to that of a medulla but it was observed in normally non-medullated fibres, and therefore they considered it part of the cortex. Auber & Ryder (1956) noted that medulla tends to lie within the paracortex, and the metacortex of Brown & Onions occupies this position, as do the cells in Figures 6-7 and 6-9. Brown & Onions found that, like the medulla, the metacortex is deficient in sulphur. Ryder (1966) observed a basophilic strand replacing the discontinued medulla of goat hair, which was about to be shed, and thought it could be the metacortex of Brown & Onions. It is also possible that the 'solid' medulla observed by Nott & Sikorski (1965), which was deficient in sulphur and had intercellular vacuoles, is a meta-
cortex. Again, embedded in the paracortex, Bonès &
Sikorski (1967) observed what they termed a 'mesocortex' in phosphotungstic acid stained wool.

It is concluded that the evidence above indicates that accessibility may be a limiting factor for penetration of some large molecules (particularly chloroauric acid) into the paracortex. The formic acid pretreatment of fibres used by Laxer & Ross (1954), may be required to swell the paracortex sufficiently to allow full penetration of chloroauric acid necessary to achieve the degree of staining desired, or to produce the large gold particles in nuclear remnants, which are probably responsible for producing the colour difference observed between the orthocortex and paracortex.
BIBLIOGRAPHY.

Clarendon, Oxford.


Adams M.J., Blundell T.L., Dodson E.J., Dodson G.G.,
Vijayan M., Baker E.N., Harding M.M., Hodgkin D.C.,


Biochem. J., 52, 177.


86, 449.

Lond., A232, 333.

Acta, 10, 483.


Part C, Polymer Symposia No.20, 187.
Academic, N.Y., 11, 315.
Butterworths, 299.
Crewther W.G., Fraser R.D.B., Lennox F.G. & Lindley H.
Crewther W.G., Gillespie J.M., Harrap B.S., O'Donnell I.J.


Dayhoff M.O. & Eck R.V. (1968) 'Atlas of Protein Sequence and Structure', National Biomedical Research Foundation, Maryland.


Determann H. (1968) 'Gel Chromatography', Springer-Verlag, N.Y.


Gillespie J.M. (1971a) Personal communication.


Milligan B. (1971a) Personal communication to J.H. Bradbury.
Moeller T. (1952) 'Inorganic Chemistry', Wiley, N.Y.
Montagna W. (1956) 'The Structure and Functions of Skin', Academic, N.Y.


Resolution Nuclear Magnetic Resonance', McGraw-Hill,
N.Y.
Prior M.G.M. (1962) 'Sclerotinization' in 'Comparative
Biochemistry' (Ed. H.S.Manson & M.Florkin) Academic,
N.Y., 4, 371.
Comm., 10, 467.
Soc., 87, 2696.
88, 2025.
4, 1758.
Riordan J.F., Sokolovski M. & Vallee B.L. (1967a) Biochem.,
6, 358.
Riordan J.F., Sokolovski M. & Vallee B.L. (1967b) Biochem.,
6, 3609.
Roberts J.D. & Caserio M.C. (1965) 'Basic Principles of
Organic Chemistry', Benjamin, N.Y.
Tanford C. (1961) 'Physical Chemistry of Macromolecules', Wiley, N.Y.


Teresa, Joaquin de Pascual (1944) Anales fíís. y. quím, (Madrid) 40, 222.


Zahn H. (1941) Melland Textilber., 22, 305.
Zahn H. (1952) Text. Rundschau, 7, 305.
A method was developed for the separation of the orthocortex and paracortex of Merino wool [3, 5], which consisted of the preferential staining of the paracortex with gold [6], followed by disruption of the fiber by ultrasonication in formic acid. A clean sample of cortical cells was obtained by a differential screening procedure [2] and the heavily stained, dark red, paracortical cells were separated from the lightly stained, pink, orthocortical cells by centrifugation in a density gradient [5]. The amino acid analysis and content of high-sulfur and low-sulfur proteins from each fraction has been reported earlier [3, 5].

The gold was found by light microscopy to be located preferentially in the paracortex, and electron microscopy of cross sections stained with potassium permanganate showed that the metallic gold particles were concentrated in the nuclear remnants of the paracortex. On disruption of these fibers by ultrasonication in formic acid, a considerable amount of colloidal gold was liberated in the formic acid, but the residual fibers still retained their bilaterally stained appearance. We concluded that the colloidal gold which was liberated came from both orthocortex and paracortex and, hence, the heavily stained cortical cells did, in fact, originate from the paracortex. In order to make an unequivocal check of the correctness of this conclusion we have examined the heavily stained and lightly stained cortical cells by electron microscopy.

Merino 64's fibers were stained with gold, disrupted by ultrasonication, and the lightly stained and heavily stained cortical cells were separated by the method in [5], with only slight modifications (Ley, K. F., unpublished results). The two fractions were stained by the thioglycollate-osmium tetroxide method [8] and aligned by stroking in viscous Araldite on two Perspex microscope slides. The alignment was checked by light microscopy and the Araldite allowed to set hard. That part of each slide which contained the aligned cells was embedded in a block and sectioned perpendicularly to the cells, using an L.K.B. Ullrotome. The sections were post-stained with lead citrate [7] and examined in a Hitachi Model HU-11C-S electron microscope.

Figures 1 and 2 show cross sections of cortical cells from the light and heavy fractions, respectively, which are representative of the cells in these fractions. The cortical cell in Figure 1 originates from the orthocortex as shown by the typical appearance of the small, individual macrofibrils [1, 8]. The cortical cell in Figure 2 is not subdivided into macrofibrils as is an orthocortical cell, the nuclear remnant is large and filled with gold particles [5] and the microfibril-matrix structure is visible to a greater degree than in Figure 1. It is thus readily identified as a paracortical cell [8]. Approximately 800 cell sections from the light and heavy fractions were examined and in greater than 99% of cases the light fraction consisted...
FIG. 2. Cross section of a cortical cell containing gold and originating from the heavy fraction. In confirmation of earlier work [5], this is identified as a paracortical cell on the basis of the absence of small circular macrofibrillar boundaries and the occurrence of large nuclear remnants in which metallic gold is concentrated [5].

of orthocortical cells and the heavy fraction, of paracortical cells. If a small percentage of mesocortical cells [1] occurs in Merino 64's wool, their fate would be unknown, since they may have a different density from either of the two main fractions or may occur in the heavy fraction with the paracortical cells.

These results provide proof of the separation of orthocortical and paracortical cells of fine wool [5]. The way in which the Au(III) is bound to the protein and the mechanism of deposition of gold metal is the subject of a further investigation [4].

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Literature Cited

Manuscript received August 18, 1970.
The following publication was derived from work described in this thesis:


A reprint of this paper is included.