THE CHEMICAL COMPOSITION OF THE EPICUTICLE OF WOOL

I. THE PROTEIN CHARACTER OF THE EPICUTICLE

by

G. J. SCHURINGA, J. ISINGS, AND A. J. ULTÉE JR

Vezelinstituut T.N.O. (Fibre Research Institute), Delft (Netherlands)

INTRODUCTION

In 1948 Swedish workers[14] discovered that wool fibres are covered with a very thin and very resistant membrane, which they called the epicuticle. This layer may have an important bearing on surface phenomena such as the frictional properties of the fibres and the diffusion of dyes into the fibres. Although objections[20] can be raised against the name epicuticle, in this series of articles we shall make use of it to indicate the thin surface layer just mentioned and also the membranes obtained from it by the methods of the Swedish workers, *viz.* (1) by dissolving the wool keratin in a solution of sodium sulphide, the membranes remaining undissolved[11], and (2) by treating the wool with chlorine or bromine water, followed by shaking, a suspension of membranes being formed[12]. It is hoped that in the near future, by agreement among workers in this field, a nomenclature of the different layers of the wool fibre may be accepted that will eliminate the confusion existing today.

The chemical nature of the epicuticle is as yet unknown. Some authors have put forward suggestions based on the few chemical properties published, but have been unable to prove these speculations for lack of experimental evidence. There are several reasons why the chemical investigations have advanced so little up to now. (1) The epicuticle forms only about 0.1% by weight of the wool fibre[14], so that much work on a micro-scale is necessary. (2) Insoluble impurities which cannot be removed from the wool by previous washing cannot be removed by the methods used for isolating the epicuticle either. This accounts for the high ash-content which is encountered sometimes. Finally, (3) on account of the great resistance of the membranes, it is possible to decompose them into soluble products by drastic means only, the decomposition then often proceeding much too far.

It should be mentioned here that the chemical composition of the membranes is affected by the method of isolation. For example, we have found that bromine is chemically bound by the membranes prepared with the aid of this agent, and that sodium sulphide may lower the sulphur content without dissolving the epicuticle. Therefore, in discussing the chemical composition of the membranes, it has to be borne in mind that at the moment the epicuticle can only be isolated by chemical means and that these are by no means harmless. The chemical compositions of both kinds of prepa-

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rations are not the same, and therefore their properties may differ, *e.g.*, their resistances to chemical agents and enzymes. An example of this will be given below.

A second point to be considered is that the epicuticle is a biological material which may be a very complex mixture with a varying composition. A similar problem is met with in the investigation of wool itself, where, for example, cystine and tyrosine contents show considerable variations according to the origin and treatment of the wool used.

For these reasons the aim of the experiments to be described was to obtain only a general insight into the question of what chemical component or structure is responsible for the curious properties of the epicuticle reported in the literature.

We shall now mention briefly what is known about the chemical properties and composition of the epicuticle.

The discoverers (LINDBERG, GRALÉN and co-workers) report that it is resistant to the action of chlorine, bromine, strong acids and enzymes[9,15]. The resistance to sodium sulphide and alkali seems to be considerable, but prolonged action causes perforation of the membranes; it is stated that alcoholic potassium hydroxide strongly damages the epicuticle[11,12,13], or even removes it[2,7]. ALEXANDER[2] and co-workers state that it can also be removed by prolonged extraction of the wool by ether. The epicuticle does not give any reaction with PAULY's reagent[9,15], which led to the conclusion that it does not contain tyrosine or tryptophan residues.

GRALÉN[9] has published an X-ray diagram of epicuticle obtained by bromination of wool. He stated that no sign of an α-diagram was found, but indications of a β-diagram of keratin are superimposed in the diagram. According to the legend of one of his photographs, other reflections do not belong to the ordinary keratin diagram; the sample contained about 75% "foreign matter", *viz.* keratin.

Most authors agree that the epicuticle cannot consist of ordinary keratin, or even of an ordinary protein. However, MERCER AND ROADKNIGHT[18] assume that it is a hardened protein containing the same amino acid residues as wool but in different ratios.

ZAHN[21] states that a considerable part of the epicuticle consists of protein, because, by hydrolysis and paper chromatography, he obtained most of the amino acids present in wool, with the exception of cystine, tyrosine and histidine.

Contrary to these theories, it has been suggested by other workers that the epicuticle may be "waxy"[1] or at least may contain a waxy component[6,7]. ELLIOTT[7], especially, develops a theory of the origin of the epicuticle: "Steroid materials produced by the sebaceous glands may play an important part. The cholesteryl and other steroid esters found in wool grease support this theory, that the extrusion of such steroid materials on the thiol containing fibre shaft just prior to its exit from the hair-sheath, may have the following results: (a) oxidation of the thiol type sulphur to disulphide sulphur, (b) binding of a sterol or sterol ester complex to the fibre by weak chemical bonds, (c) this frail but oxidation resistant and non-proteinaceous epicuticle protects the fibre against action of air oxidation and water-hydrolysis."

Furthermore, it is supposed[6] that the protein part should contain less cystine and less tyrosine and tryptophan than the ordinary wool keratin.

Suggestions have also been made about the presence of a carbohydrate, and a chitin-like structure has been mentioned too (see GRALÉN[9]).

We may say, however, that most of these theories are lacking in experimental evidence, which will be seen from the following to contradict several of the speculations put forward.

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THE ORIGIN OF THE EPICUTICLE

As stated above, some authors assume that the composition of the epicuticle is "waxy"[1]; ELLIOTT[7] has developed a theory that the origin of this layer is the sebaceous gland.

In connection with these hypotheses and with our own experiments on the chemical nature of the membrane, we thought it worth while to make an investigation into the origin of the epicuticle.

In the literature there is one report, based on experiments by HOCK, RAMSAY AND HARRIS[10], mentioning that the ALLWÖRDEN reaction takes place only on the "shaft" of the hair which does not give a positive thiol reaction; there is no membrane on the "root", the reaction beginning at the scales. They carried out their experiments on complete hair roots with sodium nitroprusside as an agent for the thiol reaction.

This reaction, however, does not give a true picture of the site of the SH-groups, because it is not sufficiently specific. For several reasons, in our experiments we have used a somewhat modified method. We have abandoned the sodium nitroprusside reaction[8] on thiol groups, and determined the presence of cysteine by the method of CHÈVREMONT AND FRÉDÉRIC[4], using $Fe[Fe(CN)_6]$ as a reagent. In this way the exact position of the cysteine-cystine transition can be determined histochemically. These experiments were carried out independently of the ALLWÖRDEN reaction. The nuclei were demonstrated with haemalum (MAYER), which stain is not affected by bromine water.

Our experiments have been divided into two groups: one with hair roots of bats, and one with hair roots of foetal pigs.

For the first series we used the species *Myotis mystacinus (Kühl)* — the Whiskered Bat; *Pipistrellus pipistrellus pipistrellus (Sch.)* — the Common Bat; and *Rhinolophus hipposideros hipposideros (Bechst)* — the Lesser Horseshoe Bat. Bats' hair was chosen because of the extraordinarily prominent scales.

For these experiments the hair roots were plucked off the skin of the bats and immersed in diluted bromine water; the nuclei were not stained. The ALLWÖRDEN reaction appeared very clearly after about 10 seconds, but after 30–40 seconds all the sacs burst and no membrane remained visible.

In the case of the species *Myotis mystacinus (Kühl)* the reaction was somewhat slower than in the other species and therefore it was possible to take photographs. It was evident from these experiments that the ALLWÖRDEN reaction extends below the place where the scales become visible (Figs. 1–4). The orifice of the sebaceous gland, however, lies much higher, and these experiments seem to be in contradiction with the theory of HOCK *et al.*[10] and with that of ELLIOTT[7]. We therefore thought it necessary to obtain more definite evidence on this point.

A second series of experiments was carried out, this time with plucked hair roots of foetal pigs, used in our laboratory for histochemical examinations. Before the experiment the hair roots were stained with acid haemalum (MAYER[19]). By this method it was possible to determine definitely the place from where the ALLWÖRDEN reaction takes place.

Figs. 5 and 6 show that the ALLWÖRDEN sacs are visible on the upper part of the hair root, which is stained dark blue in consequence of the presence of many nuclei.

The place from where the reaction becomes visible could be determined inde-

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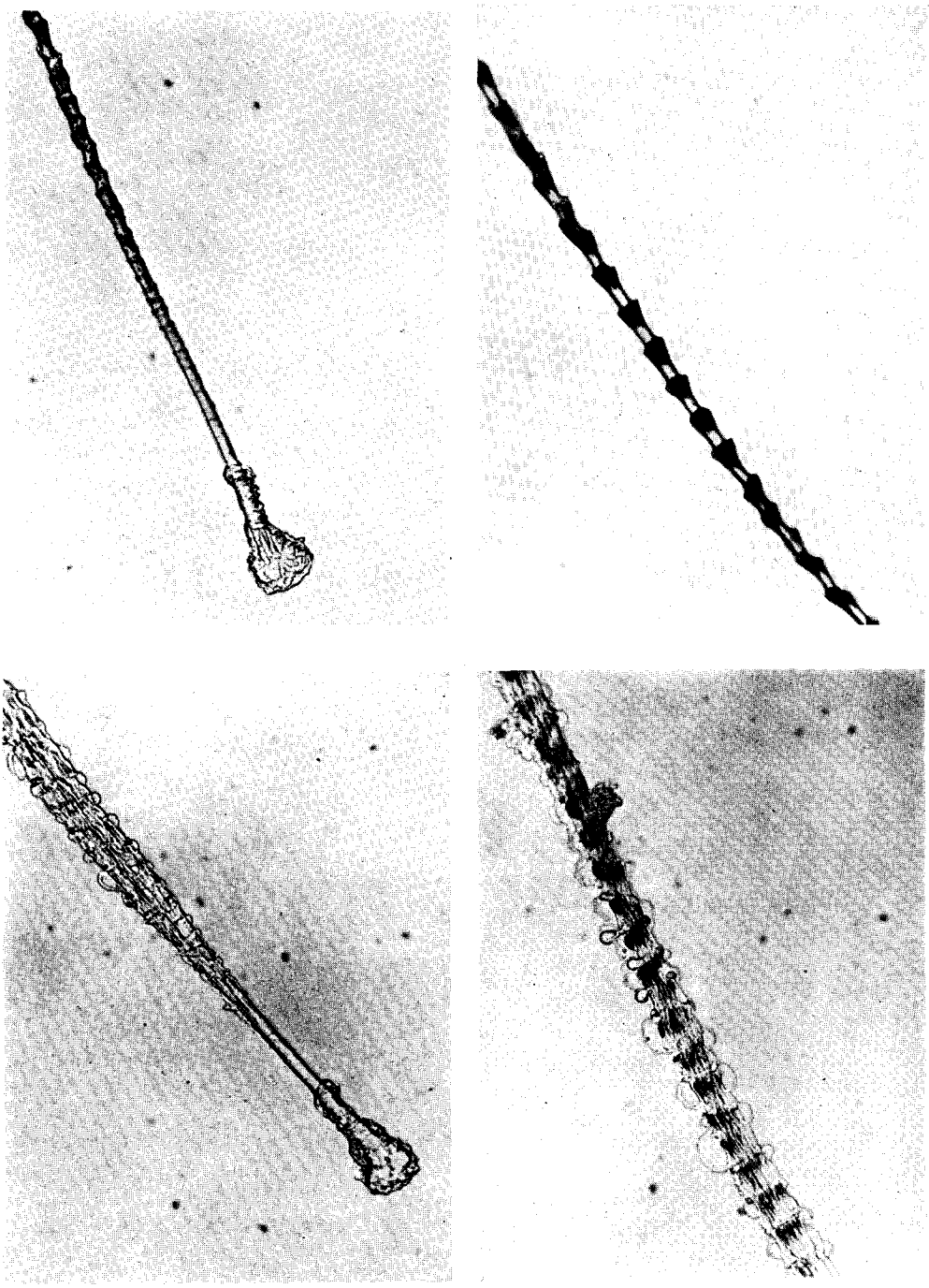
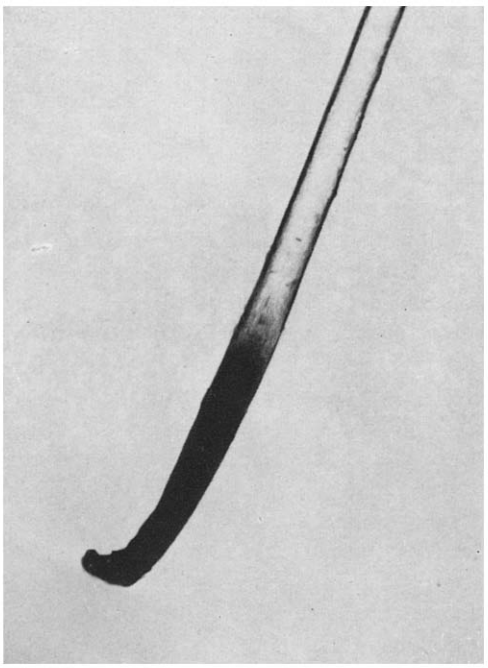
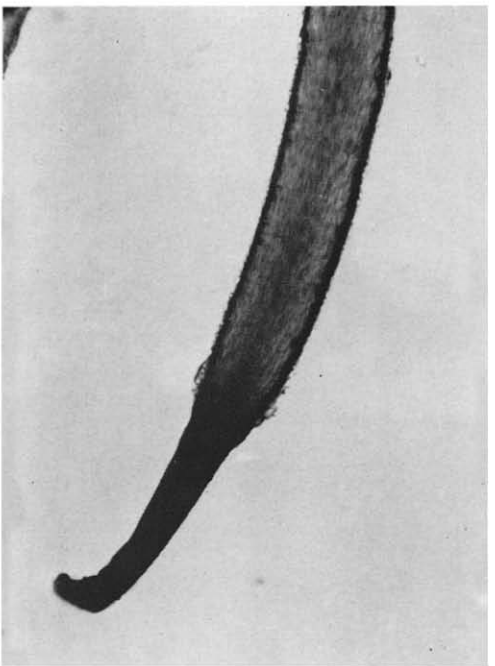


Figs. 1–4. Hair root and hair shaft of Whiskered Bat (*Myotis mystacinus* (*Kühl*)):
1 and 2: before treatment with bromine water,
3 and 4: after treatment with bromine water.





Figs. 5–6. Hair root of a foetal pig, before and after treatment with bromine water.

pendently by means of histochemical methods. The skin of the pig was used as soon as possible after the death of the animal, pieces of the skin being fixed in BOUIN fluid for 8–12 hours. The fixed tissue was enclosed in paraffin wax after dehydration via the alcohol-xylene series in less than 4 hours, and sections were cut at 6 microns. These sections were stained according to the method of CHÈVREMONT AND FRÉDÉRIC[4] with potassium ferricyanide and ferrisulphate (ratio 1:3) in three consecutive baths for 10, 10, and 5 minutes, respectively. The zone of positive thiol reaction was clearly visible by the blue colour (Fig. 7 between arrows). The photograph shows that this zone is sharply defined and much smaller than the zones indicated by the sodium nitroprusside reaction[8, 16, 17].

After staining the sections, we observed the nuclei with the phase contrast

Fig. 7. Histochemical demonstration of cysteine in the hair root of a foetal pig. Positive reaction between the arrows.

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microscope. A comparison between the nuclear forms which change in the various zones of the hair root and those of the plucked hair roots used for the ALLWÖRDEN reaction enabled us to prove that the place where the ALLWÖRDEN reaction becomes visible is the same as that where the cysteine zone begins. Histologically, at this place the hair becomes detached from the HUXLEY layer.

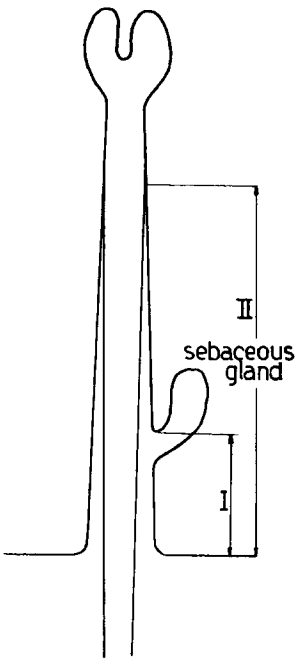

Fig. 8. Distance between epidermis and sebaceous gland orifice (I) and distance between epidermis and place where the ALLWÖRDEN reaction becomes visible (II).

The distance between the outside of the epidermis and the lowest place of the sebaceous gland orifice (I) and the distance between the former and the place where the thiol reaction becomes visible (II) were measured on 125 hair roots of 40 different foetal pigs (see Fig. 8). The mean value for I was 229 μ and for II 639 μ. The ratio II: I was determined in each case, giving a mean of 2.85.

The distances I and II as well as the ratio II: I are shown in the diagrams (Figs. 9 and 10). In the most extreme case the distance between the sebaceous gland and the place where the ALLWÖRDEN reaction (II–I), and also the thiol reaction, becomes visible, was still 212 μ while the mean distance is about 400 μ. The 68% range in these diagrams is 193–265 μ for I, 575–703 μ for II, and 2.43–3.27 for II:I. The standard deviations for I, II and II:I were 36 μ, 64 μ, and 0.42, respectively.

THE PREPARATION OF THE EPICUTICLE

(1) 40 grams of washed and degreased wool was treated with 1.25 litre sodium sulphide solution (0.15 *M*) for 7 days at 40° C. The suspension obtained was separated by centrifugation, and the precipitate was washed several times with distilled water until no more sulphide ions were present. After decanting the supernatant liquid the precipitate was suspended in *N* hydrochloric acid and treated

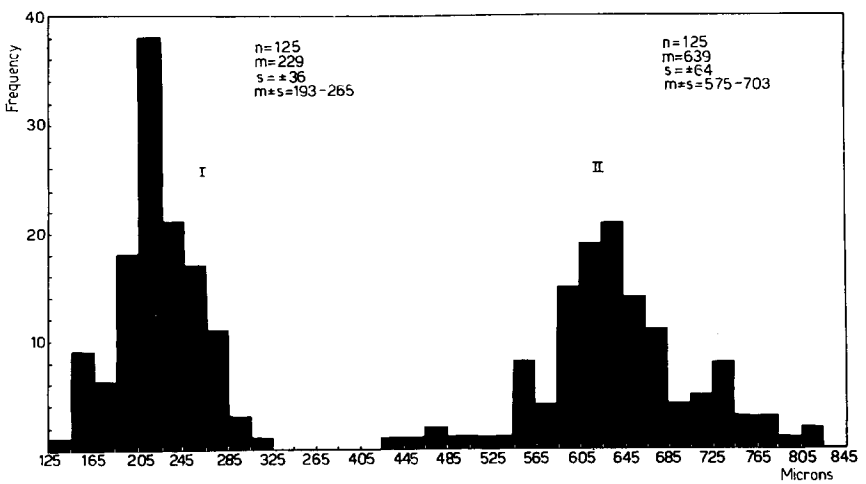

Fig. 9. Frequency distributions of the distances I and II.

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for 15 minutes at 50° C, no sugars being split off. After the acid treatment the residue was washed with distilled water several times until chloride ions could no longer be detected in the washing liquid. Then the digestion with sodium sulphide was repeated for 24 hours in order to remove any free sulphur. The treatment with hydrochloric acid was also repeated. Finally, the membranes were washed carefully and dried. In this way about 300 mg of slightly hygroscopic membranes was obtained.

(2) 40 grams of washed and degreased wool was immersed in water and shaken in order to remove any loose particles. The wool was subsequently treated with 2.5 litres saturated bromine water, and this was carefully replaced by 5 litres of a solution containing 2.5 litres saturated bromine water, the whole bromine treatment taking about 75 minutes. The wool was carefully washed until the washings contained no more bromine; then it was shaken with water again. The suspension formed was filtered through a coarse sinter-glass filter. The membranes contained in the filtrate were precipitated and washed by centrifugation. After drying the yield was about 90 mg.

The purity of the preparations was checked by examination under the electron microscope.

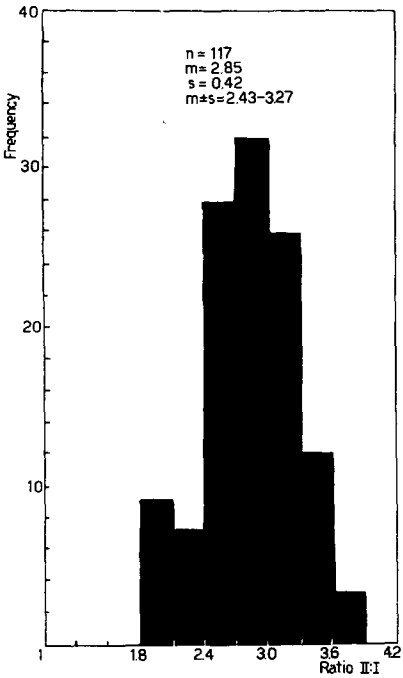

Fig. 10. Frequency distribution of the ratio II:I.

PROPERTIES OF THE EPICUTICLE

Resistance to acids and bases

Contrary to data mentioned in the literature[9,15], the epicuticle can be dissolved by the action of strong acids, only some inorganic material remaining undissolved.

We were able to confirm the slow attack of alkali which is also mentioned[11,12,13]. However, it is improbable that real holes are formed. It was found that the ALLWÖRDEN reaction of wools treated according to LINDBERG[13] is not negative, but only slow. The formation of sacs would not be possible if the membranes were perforated. The rate of the ALLWÖRDEN reaction is shown in the diagram (Fig. 11).

Resistance to enzymes

The epicuticle has a great resistance to enzymic decomposition. In our laboratory, however, ALGERA[3] found that the membranes prepared with bromine could be dissolved completely by trypsin. On the other hand, the membranes obtained with sodium sulphide are not digested by this enzyme.

Resistance to prolonged ether extraction

Wool was extracted for 96 hours with ether. The ALLWÖRDEN reaction was not diminished by this treatment.

Ash content

The preparations showed a great variation in ash content. As a rule the membranes obtained by the use of bromine contain more ash (7–10%) than those obtained with sodium sulphide (2–5%). The amount of membranes yielded by the latter method is, however, several times as high as that obtained by the former. It seems that most of the ash components are particles of foreign matter sticking to the outside of the wool

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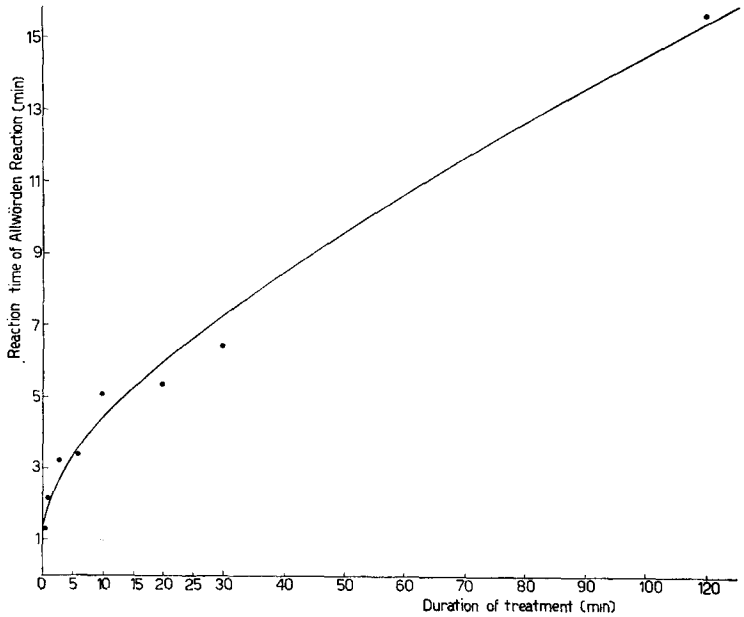

Fig. 11. Relation between the reaction time of the ALLWÖRDEN reaction and the duration of treatment of wool with alcoholic potassium hydroxide.

fibres, these particles occurring in the preparations made by either method. X-ray examination proved that the ash consists for the greater part of quartz. It contains also some alkali salts, as shown from the slightly higher carbon content of the membranes when the sample is burned in the presence of lead chromate, especially in the case of the preparations with high ash content.

Elementary composition

We do not consider the insoluble inorganic substances to be an essential part of the epicuticle. For this reason the percentages found for the elements C, H, N, S and Br were calculated with respect to the ash-free organic residue. For the different preparations of both kinds the values obtained in this way are in striking agreement as to the elementary composition. The mean values are recorded in Table I.

TABLE I

ELEMENTARY COMPOSITION* OF THE EPICUTICLE MEMBRANES AND OF WOOL KERATIN

	C	*H*	*N*	*S*	*Br*	*O (by difference)*
1. Prepared with sodium sulphide	48.9	7.2	13.4	2.4	—	28.1
2. Prepared with bromine	44.0	6.1	13.0	2.8	8.1	26.0
3. Wool keratin**	50	7	16	3.5	—	23.5

* The microanalyses were carried out partly by Miss N. FRANSEN (Org. Chem. Inst. T.N.O., Utrecht) and partly by Mr P. J. HUBERS (Org. Chem. Lab. Gem. Univ. Amsterdam).

** American Wool Handbook.

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X-ray analysis

Several X-ray diagrams were made for us by Dr P. M. DE WOLFF of the Technical Physics Department T.N.O., Delft, using copper K α-rays and a fourfold focussing camera, which suited our purpose because all samples investigated were oriented randomly. The samples were mounted between cellophane. Photometer curves, made with a direct intensity recording photometer, are reproduced in Fig. 12, for the region of 3.5 to 20 A; at shorter spacings the only maxima present were those of quartz (see below). The scattering of the cellophane and of the air is shown by curve (e). The background in the other curves is in any case smaller than this scattering (on account of absorption by the specimen).

All epicuticle diagrams show the presence of quartz roughly proportional to the ash content. The diagram of the membranes obtained by bromination (a) shows broad maxima corresponding to atomic spacings at 4.0–4.6 A and 9.8 A. This confirms roughly the values given by GRALÉN[9]. The diagram of the membranes obtained by the action of sodium sulphide (b) shows a pronounced maximum at 10.9 A apart from a broad maximum at 3.8–4.5 A. For comparison, photometer curves from X-ray diagrams of disoriented keratin are shown. These samples consisted of wool (c) and goose feather barbs (d), respectively, disintegrated by being so finely cut that they become a powdered consistency. These curves resemble each other very much and it may be concluded that the crystalline structure of these materials has been destroyed by the disrupting procedure.

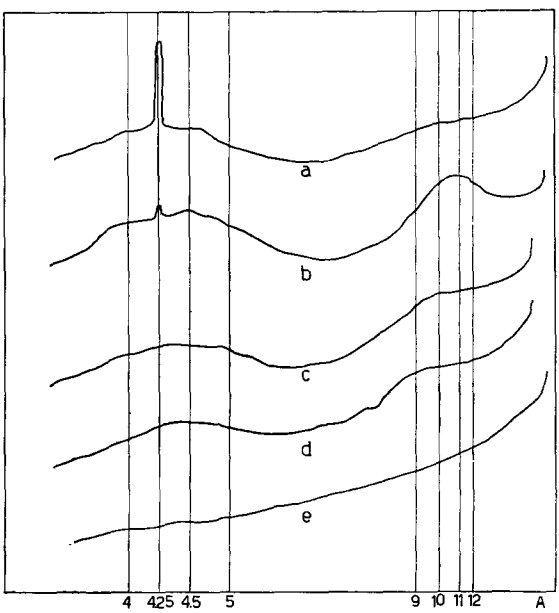

Fig. 12. Photometer curves of X-ray diagrams of epicuticle (a, b) and of disorientated keratin (c, d), compared with a control experiment (e). Maxima at 4.25 A are caused by quartz.

Hydrolysis

The protein character of the epicuticle was confirmed by hydrolysis and paper chromatography. We used concentrated hydrochloric acid according to the standard procedure of CHIBNALL: The membranes were treated with 5 volumes of concentrated hydrochloric acid at 37° C until dissolved. The mixture was diluted with an equal volume of water and heated for 24 hours at 115° C. The hydrochloric acid was removed in vacuo. The residue was dissolved in water and examined by means of paper chromatography; in agreement with ZAHN, we found that it contained the same amino acids as wool hydrolysates.

The hydrolysis can also be effected by boiling with alkali, *e.g.* barium hydroxide; and, in the case of membranes prepared with bromine, also by the action of trypsin as mentioned above.

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DISCUSSION

The histochemical evidence put forward in this paper indicates that the ALLWÖRDEN reaction starts at a much lower level in the hair root than where the orifice of the sebaceous gland is situated. Furthermore, it seems improbable that the steroid material of this gland moves downward against the direction of hair growth; the scales are all turned upward and match closely a set of scales of the HUXLEY layer[5].

The evidence also points to the conclusion that the place of initiation of a positive thiol reaction coincides with that of a positive ALLWÖRDEN reaction. Objection could be made to our histochemical demonstration of thiol groups, because fixatives were used. In our opinion, however, thin tissue sections are better for histochemical demonstrations than complete hair roots, the picture of the latter being confused by absorption.

In view of the amount of nitrogen present it seems probable that the main constituent of the epicuticle is a protein. However, a nitrogen content of 13% is low for a pure protein; such low values can only be found if the protein contains many amino acids with bulky side chains. We therefore think it better to infer the presence of other constituents besides protein. From the values for carbon and hydrogen it appears that these other constituents are not "fatty" or "waxy", but must contain much oxygen. For this reason, the presence of a carbohydrate or glucoprotein corresponds better with the experimental data.

The sulphur values are low in comparison with those of wool keratin. It must be taken into account, however, that by the action of sodium sulphide cystine may be converted into lanthionine with the removal of half its sulphur.

In comparing the sulphur contents of the membranes prepared with sodium sulphide and those prepared with bromine it was evident that in the first case sulphur had been removed. Moreover, during the bromination bromine is introduced into the membranes, resulting in a higher total weight of the preparation; therefore, the sulphur content calculated with respect to the original weight of the preparations would be still higher than indicated in Table I.

In our opinion the experiments discussed above prove that the theory of ELLIOTT is not correct.

HOCK *et al.* consider the presence of –S–S– bonds to be essential for the ALLWÖRDEN reaction, because of its beginning on the "shaft" where the thiol reaction is negative. We found, however, that this reaction is also positive where thiol groups are present.

ZAHN reports that no cystine, tyrosine and histidine are present in epicuticle hydrolysates. This statement applies only to epicuticle prepared with chlorine. It is known, however, that these amino acids are oxidized by halogens.

The oxidation of the disulphide sulphur to sulphonic acid groups is probable also from the behaviour of the bromine-treated membranes towards trypsin which has an optimum in alkaline solutions.

The X-ray evidence seems to confirm the protein character of the epicuticle. Most amorphous proteins show diffuse rings corresponding to spacings of about 4.5 and 10 A, the latter distance being susceptible of considerable variation. It seems that the action of bromine has destroyed the regularity at 10 A for the greater part, possibly by breaking disulphide linkages. Sodium sulphide, however, may convert these linkages partly to thioether linkages. In this case, a pronounced maximum was obtained at 10.9 A.

A subsequent publication of this series will deal with the amino acids, and especially with the sulphur-containing amino acids present in hydrolysates of epicuticle.

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CONCLUSIONS

1. The epicuticle is already present in the zone of the positive thiol reaction.

2. The biochemical evidence indicates that the origin of the epicuticle is not a secretion of the sebaceous gland.

3. The chemical evidence and X-ray analysis suggest that the epicuticle for the greater part consists of protein.

ACKNOWLEDGEMENT

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SUMMARY

The origin of the epicuticle has been investigated with the aid of the ALLWÖRDEN reaction on plucked hair roots. The extension of the ALLWÖRDEN reaction in the direction of the follicle has been studied histochemically. The zone of positive thiol reaction has been determined by the method of CHÈVREMONT AND FRÉDÉRIC; the nuclei were stained with haemalum.

Furthermore, the chemical composition of the epicuticle has been investigated by means of elementary analysis, paper chromatography and X-ray analysis.

RÉSUMÉ

L'origine de l'épicuticule a été étudiée à l'aide de la réaction d'ALLWÖRDEN appliquée aux racines du poil arrachées. L'extension de la réaction dans la direction de la follicule a été recherchée d'un point de vue histochimique. La zône de la réaction thiolique positive a été déterminée d'après la méthode de CHÈVREMONT ET FRÉDÉRIC; les nucléoles ont été teints à l'aide d'haemalun.

En outre, la composition chimique de l'épicuticule a été étudiée à l'aide de l'analyse chimique élémentaire, de la chromatographie sur papier et de l'analyse aux rayons X.

ZUSAMMENFASSUNG

Eine Untersuchung nach dem Ursprung der Epicuticula wurde mittels der ALLWÖRDEN'schen Reaktion an ausgezogenen Haarwurzeln ausgeführt. Die Ausdehnung dieser Reaktion in der Richtung des Follikels wurde histochemisch studiert. Die Zone einer positiven Thiolreaktion wurde nach der Methode von CHÈVREMONT UND FRÉDÉRIC bestimmt; die Kernen wurden mit Haemalaun gefärbt.

Ferner wurde die chemische Zusammensetzung der Epicuticula mittels Elementaranalyse, Papierchromatographie und Röntgenanalyse untersucht.

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