THE CHEMICAL COMPOSITION OF THE EPICUTICLE OF WOOL

II. CROSS-LINKING IN EPICUTICLE PROTEIN

by

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INTRODUCTION

In the first publication of this series[27] it was shown that the organic matter of epicuticle preparations consists for the greater part of protein, although the presence of carbohydrates was also suspected. In fact LAGERMALM AND GRALÉN[13] showed a sugar to be present in epicuticle hydrolysates.

If the average diameter of the wool fibres is assumed to be 0.02 mm, it can be calculated that an epicuticle layer of a thickness of about 100 A would represent 0.2% of the wool. As indicated in our first publication, about 0.7% of the wool is insoluble in sodium sulphide and therefore the epicuticle preparations obtained in this way contain other material apart from the thin membranes visible under the electron microscope. Although the epicuticle preparations obtained by treating wool with bromine and shaking off the epicuticle account for only about 0.2% of the wool weight, we do not consider these preparations to be purer, for a considerable part of the remnants of the ALLWÖRDEN sac membranes remains on the fibre during the shaking-off operation. Moreover it is very difficult to obtain these preparations completely free from scales, the latter being somewhat loosened by the action of the bromine. (Tippy wool is also known[7] to lose scales during shaking.)

The protein constituents of the epicuticle possess the same chemical resistance as the epicuticle itself. The weight of the preparations obtained by the action of sodium sulphide is not reduced by renewed treatment and scarcely so by trypsin digestion. As more than 99% of the wool keratin dissolves in sodium sulphide solution it seems probable that the protein of the epicuticle preparations has a different chemical structure. A more complete knowledge of this structure would be very helpful in elucidating the chemical composition of the epicuticle as a whole.

Insolubility of proteins can be attributed to extensive cross-linking. Commonly recognized cross-linkages between protein chains are hydrogen bonds, VAN DER WAALS' bonds, salt linkages and covalent disulphide bonds. The latter are chiefly responsible for the insolubility of the keratins. The action of sodium sulphide, which breaks the disulphide bonds by reduction and at the same time acts as a dispersing medium by cleaving secondary linkages[12], leaves only very few keratins undissolved. Apart from the epicuticle protein, the substance of the egg-shell membranes ("ovokeratin") belongs to this class[12] and has therefore been included in the present investigation.

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Other possible interchain bonds are peptide, thio-ether, ether and ester linkages[15]. We were especially interested in the presence of thio-ether linkages, because the epicuticle preparations obtained with sodium sulphide contained lanthionine residues. It was of interest to investigate whether these had been formed from cystine residues by the alkaline treatment, or that at least some thio-ether linkages had been present in the original epicuticle protein. Hydrolysates of tippy wool also contain lanthionine[7], probably formed from cystine residues during the "weathering" of the tippy parts of the fibres. As was shown in our first publication the epicuticle is also present on those parts of the hair that are still inside the skin and have not yet been exposed to the air and therefore thio-ether linkages formed during "weathering" cannot be held responsible for the curious properties of the epicuticle layer.

We prepared wool samples with known lanthionine contents by treating wool with potassium cyanide so as to compare these with our epicuticle preparations. In this connection the resistance of the lanthionine-containing products to sodium sulphide and to papain was investigated.

In bromine-treated epicuticle preparations bromine would have oxidized any lanthionine residues present in the original epicuticle. To know more about possible oxidation products we investigated hydrolysates of bromine-oxidized lanthionine-containing wool. A further object of the present investigation was to study the amino acid composition of the epicuticle protein itself and to search for any ninhydrin-colouring substances other than those normally occurring in wool hydrolysates. It was found by ZAHN[29] that hydrolysates of chlorine-treated epicuticle membranes contain all amino acids usually encountered except cystine, tyrosine and histidine, which are known to be easily oxidized; they were found by us in the hydrolysates of sodium sulphide-treated epicuticle.

CHEMICAL ANALYSIS OF EPICUTICLE PROTEIN

Hydrolysis of epicuticle preparations

We took special precautions to avoid humin formation during hydrolysis[19]. 10 mg samples of the membranes were suspended in 5 ml five times distilled, constant-boiling hydrochloric acid and shaken in sealed pyrex tubes at 120° C for 24 hours. The resulting solutions were evaporated to dryness below 40° C and the excess hydrochloric acid removed by repeated distillations with water at reduced pressure.

Paper chromatography of epicuticle hydrolysates

Descending chromatography on Whatman No. 1 filter paper was used throughout this investigation. Phenol-ammonia and butanol-acetic acid-water (4:1:5) proved satisfactory solvent systems to separate the amino acids present in the hydrolysates. For one-dimensional chromatograms 5 μl of a 2% solution of hydrolysate was applied and for two-dimensional work this was repeated three times. The spots were revealed by the usual ninhydrin spraying technique. The obtained chromatograms were compared with chromatograms of hydrolysates of untreated wool, potassium cyanide-treated wool before and after bromination, and of ovokeratin.

Amino acids present in epicuticle hydrolysates

Hydrolysates of epicuticle preparations contained most of the amino acids usually encountered in wool hydrolysates, some of them in different proportions. In certain cases modifications could be explained as a result of the chemical treatment used for the separation of the membranes.

A. *Bromine-treated epicuticle preparations* furnished cysteic acid as an oxidation product of cystine. If the epicuticle had contained lanthionine, its oxidation products

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might have shown up in two-dimensional chromatograms, but no such spots could be detected. We expected them to be present in chromatograms from brominated lanthionine-containing wool, but they were not found in this case either. Probably the oxidation products of lanthionine are destroyed during subsequent hydrolysis and chromatography.

Tyrosine and histidine were absent, but a bromination product could be found, which moved somewhat faster than glutamic acid in both solvent systems used. The same spot was detected in chromatograms from brominated wool and brominated silk. The main bromination product of tyrosine was shown to move much faster, as could be concluded from experiments with brominated tyrosine itself and brominated silk.

B. *The hydrolysates of sodium sulphide-treated epicuticle preparations* contained 3.5% cystine (determined by the method of FOLIN[10] and MARENZI). (In the chromatograms cystine was always completely destroyed during the phenol-ammonia run.) A very strong lanthionine spot was observed in the chromatograms. We were able to separate this spot from the others successfully by one-dimensional chromatography using the phenol-ammonia system. The lanthionine content was roughly estimated by running alongside each other chromatograms of epicuticle hydrolysates and of hydrolysates of potassium cyanide-treated wool samples with known lanthionine contents. The former gave a stronger lanthionine spot than the reference sample with highest content, *viz.*, 4.4% lanthionine (see below).

Serine gave a weaker spot than glycine, contrary to the picture of wool hydrolysates. It is known, however, that the hydroxy amino acids are not stable to alkali[5] and they might have given rise to increased amounts of glycine and alanine. (The chromatograms of bromine-treated epicuticle hydrolysates showed glycine and serine spots of about equal intensity.)

A tyrosine spot was detected in the chromatograms. The presence of aromatic amino acid residues in sodium sulphide-treated epicuticle preparations was confirmed by an examination of the ultraviolet absorption spectra, which showed absorption bands in the 290 mμ region in contrast to the flat curve of the bromine-treated membranes.

Histidine, usually not seen in ninhydrin-developed chromatograms of wool hydrolysates, showed up as a fairly strong spot. The presence of rather large amounts of this amino acid was confirmed by spraying the chromatograms with PAULY's reagent. The lysine spot was also stronger than in chromatograms of wool; this applies to both kinds of epicuticle preparations. Thus it can be concluded that the epicuticle protein contains different relative amounts of the basic amino acids as compared to those of ordinary keratin, which are reported to occur in the proportions arginine : lysine : histidine 12:4:1.

Resistance to sodium sulphide solutions

In the preparation of epicuticle obtained by dissolving wool in sodium sulphide about 0.7% of the weight of the wool remained undissolved. It seemed interesting to investigate whether thio-ether linkages can be responsible for the insolubility of proteins on sodium sulphide attack. To this purpose potassium cyanide-treated wool samples containing 5 to 8% lanthionine were incubated with 0.15 *M* sodium sulphide solution in the same way as the wool used for epicuticle preparations. Only about 2% of the samples remained undissolved under these conditions. Moreover, it appeared that the proteins dissolved by the action of sodium sulphide on untreated wool and reprecipitated

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by addition of acetic acid to the dialyzed solution furnished considerable amounts of lanthionine on hydrolysis.

Ovokeratin, which is known to withstand the dispersing action of several reducing denaturants[12], was also subjected to the action of sodium sulphide under the conditions used to prepare epicuticle from wool. In this case we found that 13% remained undissolved. The residue contained 13% nitrogen.

Resistance to papain attack

As it has been reported that epicuticle preparations are extremely resistant to the action of enzymes, it seemed interesting to investigate whether thio-ether linkages are responsible for this behaviour.

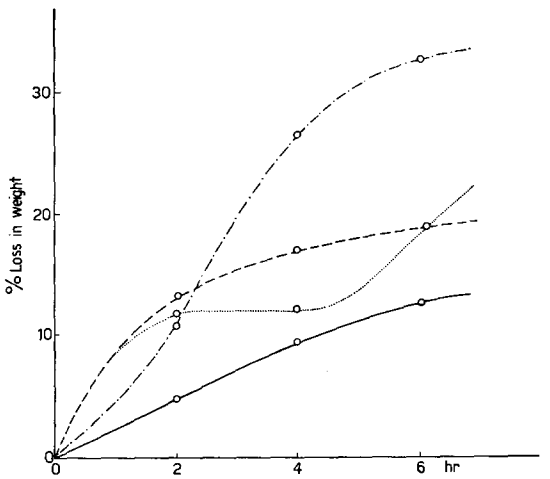

Fig. 1. Influence of Time of Treatment with Papain on the Loss in Weight of the Wool
–·–·– untreated wool
– – – – – wool boiled at pH 8 for 4 hours
····· wool boiled at pH 10 for 1 hour
——— wool boiled with 0.1 *M* potassium cyanide for 30 minutes

GEIGER *et al.*[11] introduced thio-ether linkages in wool by treating reduced wool with an aliphatic dihalide and showed that the stability of these products toward enzymes is greatly enhanced.

The reaction of wool with papain was studied by MIDDLEBROOK AND PHILLIPS[18], SCHÖBERL AND HAMM[25], GEIGER *et al.*[11] and BLACKBURN[3]. MIDDLEBROOK AND PHILLIPS[18] used sodium bisulphite to activate the papain and determined the optimum conditions for digestion by crude papain preparations. SCHÖBERL AND HAMM[25] studied the action of cyanide-activated papain on wool. According to GEIGER *et al.*[11] neither untreated wool nor wool alkylated after reduction is attacked by solutions of crystalline papain. They used cyanide as an activator, though not at optimum conditions of pH and temperature. The temperature especially seems low, *viz.* 20° to 25° C. These authors stress the importance of preventing bacterial growth. BLACKBURN[3] made an examination of the products formed when wool is hydrolysed by papain and sodium bisulphite, following the procedure of MIDDLEBROOK AND PHILLIPS. He states that wool which has

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TABLE I

	Total S before papain treatment	*Cystine-S before papain treatment*	*Time of papain treatment*	*Loss in weight of wool (as % of original)*	*Total N in filtrate (mg)*	*Amino N in filtrate (mg)*
Normal wool	3.38	2.98	—	—	—	—
			2	11	20	4.7
			4	27	41	8.3
			6	33	49	9.6
Wool boiled at pH 8 for 4 hours	2.79	1.60	—	—	—	—
			2	13	22	4.4
			4	17	27	5.3
			6	19	31	7.1
Wool boiled at pH 10 for 1 hour	2.24	0.96	—	—	—	—
			2	12	20	3.2
			4	12	21	3.4
			6	19	31	4.6
Wool boiled with 0.1 *M* KCN for 30 min	1.92	0.75	—	—	—	—
			2	5	9	2.0
			4	10	16	3.3
			6	13	21	3.7
Papain solution	—	—	—	—	3	1.5

been treated with alkali to convert some of the disulphide linkages into lanthionine linkages is attacked by papain and bisulphite at an increased rate. It seemed of interest to us to repeat the experiments of BLACKBURN and include an examination of the behaviour of potassium cyanide-treated wool toward papain and bisulphite. Potassium cyanide specifically converts the disulphide bonds into thio-ether linkages in contradistinction to the more complicated action of alkali[8].

1 g samples of wool tops (untreated, boiled at pH 8 for 4 hours or at pH 10 for 1 hour and boiled with 0.1 *M* potassium cyanide for 30 min) were treated with 30 ml of a 0.067% papain solution, containing 1% sodium bisulphite, at a pH of 6.8 and a temperature of 65° C for 2, 4 and 6 hours. The papain used had been purified by the salting-out procedure of BALLS AND LINEWEAVER[1]. To prevent bacterial growth a crystal of thymol was added to the papain solution. At the end of the treatment the wool samples were filtered off, washed in water and dried to determine the loss in weight. Aliquot samples of the filtrate were used to determine the nitrogen and amino nitrogen in solution by standard Kjeldahl and Van Slyke procedures.

The results obtained are summarized in Table I and Fig. 1. It can be seen that the increased rate of papain attack on alkali-treated wool as observed by BLACKBURN[3] is restricted to the first two hours. Afterwards the rate slows down considerably, especially in wool pretreated at pH 10, and remains far behind the rate of attack of untreated wool. Potassium cyanide-treated wool dissolves much slower still. It can be seen further, that about 18% of the dissolved nitrogen is in the form of amino nitrogen; BLACKBURN[3] found 15% for the products soluble at pH 4. The nitrogen content of the dissolved products remains fairly constant and equals the nitrogen content of the wool. This has also been found by SCHÖBERL AND HAMM[25] who used cyanide-activated papain.

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PREPARATION OF WOOL SAMPLES WITH KNOWN LANTHIONINE CONTENT

In order to obtain wool samples with known lanthionine content we treated wool with potassium cyanide. According to the literature[8,9,24] this reaction proceeds smoothly as follows:

$$R\text{–}CH_2\text{–}S\text{–}S\text{–}CH_2\text{–}R + KCN \rightarrow R\text{–}CH_2\text{–}S\text{–}CH_2\text{–}R + KCNS \qquad (1)$$

For each reacted cystine bond equivalent quantities of lanthionine residues and thiocyanate are formed.

As determination of lanthionine itself is very difficult, we determined the decrease of total sulphur and of cystine sulphur of the wool and the amount of thiocyanate formed in the solution.

The wool was treated as described by FARNWORTH, NEISH AND SPEAKMAN[9,26]. 5 g samples of degreased wool yarn were treated with 150 ml 0.1 molar potassium cyanide (pH 10.6) at 66° C for 30, 60 and 120 minutes. In another experiment 5 g tops were boiled with 0.1 molar potassium cyanide during 30 minutes. The wool was filtered off and its sulphur[2] and cystine[10] contents were determined. The filtrates contained thiocyanate which was determined by a modified ferric thiocyanate procedure. The excess cyanide did not influence colour formation. This is in agreement with a statement of WOODS AND MELLON[28]. We used an excess of iron (III) nitrate in diluted nitric acid to obtain the best results[16]. The colour was measured at 450 mμ because the absorption maximum is shifted to this shorter wave length by using an excess of iron. The nature of the coloured complexes of ferric iron and thiocyanate has formed the subject of much controversy in the past, but has been cleared up by numerous publications of the last decade[17].

CUTHBERTSON AND PHILLIPS[8] showed already that the thiocyanate formed during treatment of wool with potassium cyanide is roughly equivalent in amount to the sulphur lost by the wool.

The results of our determinations are summarized in Table II. As can be seen from this table there exists a remarkable agreement with the amounts expected according to equation (1). It has to be taken into account that part of the wool dissolves during the treatment (about 3% of the weight after two hours at 66° C) and also that the values of the decrease of disulphide sulphur may be low because of loss of cystine during hydrolysis[4]. Therefore, the value of the thiocyanate sulphur may be the most reliable for the estimation of the lanthionine content. The nitrogen content of the wool was found to remain constant during the reaction. It seems that no side reactions take place next to the reaction of equation (1).

TABLE II

Duration of treatment in min	*Temperature ° C*	*Decrease of total S*	*Decrease of disulphide-S*	*Thiocyanate-S in solution*	*Lanthionine %*
30	66	0.35	0.62	0.33	2.1
60	66	0.50	0.88	0.49	3.1
120	66	0.78	1.20	0.65	4.4
30	100	1.31	2.28	1.20	7.9

SOME PROPERTIES OF OVOKERATIN

Egg-shell membranes consist for the greater part of a very resistant type of protein. They do not readily dissolve in sodium sulphide solutions[12] and resemble the epicuticle

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preparations in this respect. Most of the information on the properties of the egg-shell membranes can be found in the book by ROMANOFF AND ROMANOFF[23].

The chemical composition of the membranes has been studied extensively. According to CALVERY[6] they consist of pure keratin, because arginine, lysine and histidine occur in the proportions 12:4:1. However, different figures for these amino acids are given by PLIMMER AND ROSEDALE[21]. MORAN AND HALE[20] state that the egg-shell membranes contain mucin apart from keratin, on account of histochemical evidence. According to ROMANKEWITSCH[22] several authors believe that the membranes contain chitin. Probably the controversies arise from different methods of purification of the membranes: some investigators wash them only in water or salt solution, whilst others clean them thoroughly, *e.g.* by enzyme treatment.

No special precautions were taken to purify our preparations. Under the polarizing microscope some patches of birefringent protein could be observed adhering the non-birefringent sheets of ovokeratin fibres in these samples. Preliminary histochemical experiments carried out in our laboratory by J. ISINGS with cross-sections of the inner and outer membrane seemed to confirm the observations of MORAN AND HALE.

The nitrogen content was found to be 15.9% (the values reported in the literature vary from 13.2 to 16.6%).

The amino-acid composition was studied by paper chromatography of the hydrolysates. The results agreed with the older amino-acid analyses mentioned in the literature[23], but the following additional information was obtained:

In our paper chromatograms a weak tyrosine spot was detected. It has been assumed by several authors that tyrosine is absent, because the membranes do not react with Millon's reagent. Others state that 2.5–3% tyrosine is present.

Serine and threonine were observed by us in fair amounts and the valine spot was very strong. We found a rather high histidine content, visible after ninhydrin spraying and appearing very strongly with Pauly's reagent. We believe that the ovokeratin contains several per cents. of this amino acid, contrary to the value of 0.9% given by CALVERY[6].

No lanthionine was found in the hydrolysates. Indications of the presence of sugars were found, but none of glucosamine.

DISCUSSION

The chemical evidence obtained by paper chromatography of hydrolysates of epicuticle protein shows that this protein resembles wool keratin in many respects. The preparations obtained by dissolving the non-epicuticle parts of the wool in sodium sulphide solution contain tyrosine. It has been suggested by others that this amino acid does not occur in the epicuticle itself, because the epicuticle is not stained by PAULY's reagent. However, we do not think a staining of the extraordinary thin membranes to be visible. From the well-known fact that wool damage can be ascertained with PAULY's reagent it can merely be concluded that the undamaged epicuticle is impermeable to diazotized sulphanilic acid. On the other hand, we found evidence of a rather high histidine content of our epicuticle preparations and also of ovokeratin, which has also been reported to give a negative reaction with PAULY's reagent. The high histidine content of our epicuticle preparations might be explained by assuming that it originates from the least soluble part of the wool keratin, because the basic properties of histidine

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residues might hamper its dissolving in the (alkaline) sodium sulphide solution. However, the much more basic arginine residues apparently are not enriched in our epicuticle preparations. The rather high histidine content of the—also very resistant—ovokeratin seems interesting in connection with the composition and properties of the epicuticle protein.

We attempted to demonstrate the presence of lanthionine residues. So far this has not been successful, because the sodium sulphide-treated epicuticle preparations contain thio-ether linkages formed by the action of the sodium sulphide, whilst in the bromine-treated preparations any lanthionine residues present would have been oxidized and converted into unstable oxidation products.

It does not seem very likely that lanthionine residues are responsible for the resistance of epicuticle preparations to sodium sulphide, as can be concluded from our experiments with potassium cyanide-treated wool. On the other hand, the rate of papain digestion of such wools remains far behind that of untreated wool. The increased rate of decomposition of alkali-treated wool by papain during the first two hours could be explained by the increased accessibility resulting from swelling during the pretreatment. The observed resistance of sodium sulphide-treated epicuticle toward enzymes might, at least partly, be due to the presence of thio-ether linkages.

It has been found in our laboratory that resistance toward trypsine is quite different in the two types of epicuticle preparations studied[27].

For the estimation of the lanthionine content of potassium cyanide-treated wool, the estimation of thiocyanate liberated during preparation has proved very useful. It seems as if the reaction of wool with potassium cyanide proceeds unambiguously according to equation (1), at least during the first stages.

A subsequent publication of this series will deal with the carbohydrate part of the epicuticle preparations, and an overall discussion will be postponed until then.

SUMMARY

An investigation was made of the amino-acid composition of epicuticle protein and of ovokeratin. Paper chromatograms of hydrolysates of epicuticle preparations resemble those from wool keratin. One of the differences was a higher histidine content.

As a result of modifications during the chemical separation of the epicuticle membranes, the presence of lanthionine residues in the original epicuticle protein could not be ascertained.

Wool samples with known lanthionine content were prepared by treatment of wool with potassium cyanide. The behaviour of these samples toward sodium sulphide and toward papain was studied.

RÉSUMÉ

On a étudié les acides aminés dont se compose la protéine de l'épicuticule et de l'ovokératine. A l'aide de la chromatographie sur papier on a pu constater que les hydrolysats de l'épicuticule et de la laine se ressemblent fortement; cependant ceux de l'épicuticule contiennent plus d'histidine.

A cause des modifications que la substance protéinique subit pendant la séparation chimique de l'épicuticule, il a été impossible de constater la présence de résidues lanthionine dans l'épicuticule originale.

A l'aide du cyanure de potassium des échantillons de laine contenant un pourcentage connu de lanthionine ont été préparés. La résistance de ces échantillons contre le sulfure de sodium et la papaine a été étudiée.

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ZUSAMMENFASSUNG

Das Eiweiss der Epikutikula der Wolle gleicht in seiner Aminosäurenzusammensetzung dem Wollkeratin, wie durch Papierchromatographie der Hydrolysaten nachgewiesen wurde. Eine der Differenzen war ein höheres Histidingehalt. Zum Vergleich wurde auch Ovokeratin untersucht.

Infolge chemischer Umwandlungen während der Isolierung der Epikutikulamembranen lässt sich nicht aussagen ob das ursprüngliche Epikutikulaprotein Lanthioninbrücken enthält.

Durch Behandlung mit Kaliumcyanid wurden Wollproben mit bekanntem Lanthioningehalt dargestellt. Das Verhalten derartiger Wollproben gegen Natriumsulfid und gegen Papain wurde untersucht.

REFERENCES

1 A. K. BALLS AND H. LINEWEAVER, *J. Biol. Chem.*, 130 (1939) 669.
2 S. BLACKBURN, *Int. Wool Text. Org., Tech. Comm. Proc.*, 2 (1948) 72.
3 S. BLACKBURN, *Biochem. J.*, 47 (1950) 443.
4 S. BLACKBURN, *Chemistry and Industry*, (1950) 718.
5 T. WIELAND AND L. WIRTH, *Ber.*, 82 (1949) 468;
J. M. BREMMER, *Nature*, 168 (1951) 518.
6 H. O. CALVERY, *J. Biol. Chem.*, 100 (1933) 183.
7 R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN, *Biochem. J.*, 40 (1946) 580.
8 W. R. CUTHBERTSON AND H. PHILLIPS, *Biochem. J.*, 39 (1945) 7.
9 A. J. FARNWORTH, W. J. P. NEISH, AND J. B. SPEAKMAN, *J. Soc. Dyers and Col.*, 65 (1949) 447.
10 O. FOLIN AND A. D. MARENZI, *J. Biol. Chem.*, 83 (1929) 103;
F. K. SHINOHARA, *J. Biol. Chem.*, 112 (1936) 683.
11 W. B. GEIGER, W. I. PATTERSON, L. R. MIZELL, AND M. HARRIS, *J. Res. Nat. Bur. Standards*, 27 (1941) 459.
12 C. B. JONES AND D. K. MECHAM, *Arch. Biochem.*, 3 (1943) 193.
13 G. LAGERMALM AND N. GRALÉN, *Acta Chem. Scand.*, 5 (1951) 1209.
14 H. LINDLEY AND H. PHILLIPS, *Biochem. J.*, 39 (1945) 17.
15 H. P. LUNDGREN, *Advances in Protein Chem.*, V (1949) 316.
16 P. KARSTEN, Thesis Groningen 1934.
17 J. Y. MACDONALD, K. M. MITCHELL, AND A. T. S. MITCHELL, *J. Chem. Soc.*, (1951) 1310, 1574.
18 W. R. MIDDLEBROOK AND H. PHILLIPS, *J. Soc. Dyers and Col.*, 57 (1948) 137.
19 R. MONIER AND M. JUSTISZ, *Bull. soc. chim. biol.*, 32 (1950) 228.
20 T. MORAN AND H. P. HALE, *J. Exptl. Biol.*, 13 (1936) 35.
21 R. H. A. PLIMMER AND J. L. ROSEDALE, *Biochem. J.*, 19 (1925) 1004.
22 N. A. ROMANKEWITSCH, *Z. Zellforsch. u. Mikrosk. Anat.*, 16 (1932) 208.
23 A. L. ROMANOFF AND A. J. ROMANOFF, *The Avian Egg*, John Wiley & Sons Inc., New York 1949.
24 A. SCHÖBERL AND E. LUDWIG, *Ber.*, 70B (1937) 1422.
25 A. SCHÖBERL AND R. HAMM, *Biochem. Z.*, 318 (1948) 331, 355.
26 G. J. SCHURINGA, C. J. SCHOONEVELDT, AND T. KONINGS, *Text. Res. J.*, 21 (1951) 281.
27 G. J. SCHURINGA, J. ISINGS, AND A. J. ULTÉE Jr, *Biochim. Biophys. Acta*, 9 (1952) 457.
28 J. T. WOODS AND M. G. MELLON, *Ind. Eng. Chem., Anal. Ed.*, 13 (1941) 551.
29 H. ZAHN, *Melliand Textilber.*, 32 (1951) 419.

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