rats or on glyconeogenesis by liver slices from fasted rats.

4. Of the substances mentioned, only choline chloride was oxidized by rat-liver slices. $2 \times 10^{-2} \text{M}$.

Choline chloride also inhibited endogenous respiration by about 30 %.

It is desired to thank the Medical Research Council for an apparatus grant, and Mr W. R. Ferrier for technical assistance.

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The Reaction of Oxidizing Agents with Wool

1. THE DIVISION OF CYSTINE INTO TWO FRACTIONS OF WIDELY DIFFERING REACTIVITIES

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(Received 4 July 1949)

Most processes for preventing the felting of wool make use of strong oxidizing agents (Speakman, Nilssen & Elliott, 1938; Freney, 1946), and the reaction of wool with these reagents is thus of technical as well as of scientific interest. The disulphide bond of cystine is one of the most reactive centres in the wool structure and it is therefore not surprising that oxidizing agents act preferentially upon it. Smith & Harris (1936) showed that hydrogen peroxide oxidizes the disulphide bond in wool slowly, whereas fluorine (Hudson & Alexander, 1946) reacts rapidly and in the initial stages specifically. Consden, Gordon & Martin (1946) identified cysteic acid by paper chromatography in the products of reaction between chlorine or bromine and wool. Speakman (1947) deduced from changes in the mechanical properties

of individual fibres, assuming that cystine cross-links the polypeptide chains, that the disulphide bond is attacked by many oxidizing agents.

Several workers have observed that the disulphide bond of cystine in wool exhibits varying reactivities. Phillips and his co-workers showed in a series of detailed papers (summarized by Phillips, 1946) that the cystine in wool can be divided into four fractions, which differ from one another both in the reaction products formed and in the rate of reaction on treatment with alkali, formaldehyde, bisulphite and thiolacetic acid. Elöd & Zahn (1947) found that even after a substantial proportion of the disulphide bonds had been broken the mechanical properties remained almost unaltered. On the basis of this evidence he proposed that the cystine is incorporated

in the wool molecule in three distinct molecular arrangements and that at least one of the fractions does not play a part in holding the molecules together by cross-links.

To investigate differences in reactivity on oxidation of the disulphide bond, a quantitative study has been made of the cystine content of wool treated with different oxidizing agents, all of which react rapidly with free cystine dissolved in water.

METHODS

The wool used was thoroughly extracted with ether and ethanol. It was a knitted fabric made from an all botany wool yarn which had received no prior treatment other than scouring to remove wool grease and dirt. The sample of horn keratin was taken from the tip of a cow's horn and thoroughly extracted with distilled water; it was cut into small pieces and ground to a fine powder.

Chlorination. The wool sample of known weight was attached to a glass frame and rotated at 200 rev./min. in a closed vessel with a mercury seal. The solution/wool ratio was 150 : 1 and the reaction was carried out in an ice bath at 0°, a thermostat at 25° or a water bath at 80 ± 2°. The wool was wetted out in a buffer solution of the same pH as the hypochlorite subsequently used. A stock hypochlorite solution was prepared by the method of Cattania & Ranucci (1915), and samples of this were diluted and brought to the appropriate pH with 0.1M-phosphate buffer or, for experiments at pH 2, with H₂SO₄. Samples of the solution were periodically withdrawn by means of an automatic pipette and were analysed for Cl with KI and Na₂S₂O₃. The reaction was considered complete when 95% of the available Cl had reacted. The wool was withdrawn, rinsed with distilled water for approx. 30 min. until free from electrolyte and dried to constant weight at 105°. In the experiments with horn the powder was kept well stirred and at the end of the reaction was filtered off in a glass crucible, rinsed until free from electrolyte, dried and weighed. Not all the horn was recovered and the cystine loss was calculated on the weight of horn removed after treatment.

Treatment with permanganate. The calculated amount of KMnO₄ (A.R.) was weighed out and dissolved in either 0.5N-H₂SO₄ or 0.1N-Na₂B₄O₇, and the reaction was carried out as for the chlorinations. At the end of the reaction the sample was withdrawn and the deposited MnO₂ removed by immersion in 0.1N-sodium metabisulphite at room temperature for 1 min. It was shown (Alexander, Carter & Hudson, 1949) that such a brief treatment with bisulphite did not reduce any detectable amount of cystine. The wool was then rinsed and dried.

Treatment with peracetic acid. A solution consisting of 45% (w/v) peracetic acid was prepared from 90% (w/v) H₂O₂ by the method of Greenspan (1947). This solution was diluted 25 times with water and the wool sample, fixed on a rotating frame, was treated with the solution at room temperature for varying lengths of time, removed, rinsed and dried. The total quantity of peracetic acid which reacted was small compared with the amount in solution and hence the solution remained sensibly constant in strength even in experiments of long duration.

Esterification. In an unpublished investigation it was found that epoxy compounds, and notably epichlorhydrin,

esterify the carboxyl groups in wool. The reaction consists in treating the agitated wool sample in a solution of 5% (w/v) epichlorhydrin in water at 60° for 2 hr. The extent of the esterification was assessed by determining the acid combination with 0.02N-HCl. The ordinary value of 0.82 equiv./kg. of wool was reduced to 0.32 equiv. and this corresponds to 60% esterification of the carboxyl groups. After treatment the wool was rinsed, dried and subsequently used in the same way as untreated wool.

Acetylation. The free amino groups were acetylated by the I. G. Farben process (Alexander & Whewell, 1947). From the decreased acid-binding figures it was calculated that 70% of the basic groups were blocked. The wool was not degraded so long as the conditions of treatment described, in particular the temperature, were strictly followed.

Supercontraction. The sample of wool was kept in a 100% (w/v) LiBr solution at 100° for 3 hr., then placed in dilute phosphate buffer (pH 8) for 5 min. at room temperature, and finally rinsed in distilled water until free from halide. After treatment the fabric was reduced in area by 30%. Alexander (1949) has shown that wool fibres treated in this manner contracted approx. 20% in length but underwent no appreciable disulphide bond hydrolysis.

Determination of cystine. The wool sample was hydrolysed in 5N-HCl at 120° for 5 hr. in a sealed tube. With horn the reaction was not quite complete in 4 hr. and had to be continued for 8 hr. The cystine content of the hydrolysate was determined colorimetrically with phosphotungstic acid (Shinohara, 1935). Untreated wool samples were periodically analysed and their cystine content found to be in every case 10.0 ± 0.2%. This figure is low compared with that for root wool, but compares well with figures for whole wool fibres (i.e. including tip wool exposed to moisture and light), the cystine content of which varies from batch to batch. In some of the oxidations a considerable weight loss occurs and the loss of cystine is in each case expressed as percentage of the cystine content of the wool sample before oxidation. This was done as none of the degradation products left in the solution contained a disulphide bond, since all the reagents used react almost instantly with cystine in solution.

RESULTS

Reaction with chlorine solution at different hydrogen-ion concentrations. Wool was treated with different quantities of chlorine and hypochlorite at pH 2 and 10 respectively and the cystine content of the wool determined (see Fig. 1). The quantity of oxidizing agent which reacted in each case is expressed as a percentage (g. chlorine/100 g. of wool; i.e. NaOCl is expressed as Cl). The rate of reaction between chlorine solutions and wool at different pH values has been recorded elsewhere (Alexander, Gough & Hudson, 1949). With high concentrations of available chlorine the reaction was usually complete at 0° in about 20 min. at pH 2, and at pH 10 in 2–3 hr. at 0° and about 30 min. at 80°. It is seen clearly that chlorine at pH 2 can, in sufficient concentration, oxidize all the cystine in wool. Hypochlorite solutions, however, can only oxidize about 25% of the available cystine, and reagent in excess of that required to oxidize this fraction must react with other

groups in the molecule. It is interesting to note that the amount of wool degraded and going into solution is quite small until quantities of chlorine are used in excess of those necessary for oxidizing most of the cystine. With acid chlorination, where all the cystine is oxidized, this point is reached when more than

Fig. 1. Removal of cystine and loss of weight of wool oxidized at pH 2 and 10 with different quantities of chlorine. ○, chlorination at pH 2 and 25°; ●, chlorination at pH 10 and 25°; ◐, chlorination at pH 10 and 80°; —, loss of cystine; ---, loss of weight.

20 % of chlorine is used, but with hypochlorite, where only 25 % of the cystine is capable of oxidation, the weight loss becomes appreciable after more than 5 % of chlorine is used. It would appear from this observation that chlorine solutions begin to break down the main chains probably by stepwise removal of the terminal amino groups.

Wool was treated with different quantities of hypochlorite at 80° in order to see if the fraction of oxidizable cystine was increased. From Fig. 1 it is seen that the amount of cystine removed rose from 23 to 30 %, but it is doubtful whether any significance can be attached to this small increase, which may be due to an additional small amount of purely alkaline degradation.

Table 1. Percentage of cystine in wool oxidized by chlorine solutions buffered at different pH values at 0°

Chlorine reduced (g./100 g. of wool)	Cystine lost (% of that originally present)				
	pH 2	pH 4	pH 6	pH 8	pH 10
5	23.1	16.7	—	13.7	22.2
10	40.8	37.9	—	21.3	22.4
20	73.0	58.2	—	25.6	23.4
50	90.0	96.0	96.3	47.5	24.5

It was found (see Table 1) that hypochlorite solutions at pH 2, 4 and 6 behaved identically and were

all capable of oxidizing substantially all the cystine in wool. Chlorine at pH 8 was still capable of removing all the cystine, but more chlorine was required to do this than with solutions at a lower pH. In Fig. 2 the change in constitution of a chlorine solution with varying hydrogen-ion concentrations is shown. It is seen that the bath consists at pH 2 largely of chlorine, but changes almost entirely to hypochlorous acid between pH 4 and 6. At the higher pH values the ionization of hypochlorous acid

Fig. 2. Variation in constitution of a solution of 0.05 mol. of chlorine dissolved in water at different hydrogen-ion concentrations. x---x---, Cl₂; x—x—, HOCl; —x—, OCl⁻.

to OCl⁻ ions increases, until at pH 10 virtually only OCl⁻ ions are present. It appears that both chlorine and hypochlorous acid can oxidize all the cystine in wool, and OCl⁻ ions only 25 % of the cystine. At pH 8 obviously some of the oxidizing reagent is used in the non-cystine reaction of the OCl⁻ ion, and hence greater quantities of available chlorine than at pH 2 are required to remove the same proportion of cystine.

Table 2. Oxidation of cystine in wool by chlorine at pH 10

(The wool having previously been treated with chlorine at pH 2 at 25°.)

Cystine content after different chlorinations at pH 2 (%)	Cystine content after reaction with 20 g. Cl/100 g. wool at pH 10 (%)
8.66	7.49
5.44	5.40
3.40	3.20

It is seen from Table 2 that the cystine fraction capable of being oxidized by hypochlorite is the first to be attacked by acid chlorination. Thus, when 4 % of chlorine was used at pH 2, 18 % of the cystine was oxidized and in a subsequent treatment with 20 % of chlorine at pH 10 only a further 6 % was removed, bringing the total cystine oxidized to 24 % of that

originally present. Alkaline chlorination removed no further cystine when it succeeded an acid chlorination which had oxidized more than one-quarter of the cystine. Similarly, wool which had received a 20% chlorination at pH 10 lost no more cystine in a further similar treatment.

Oxidation with permanganate. Acid permanganate treatment is similar to alkaline chlorination in that it oxidizes only a fraction of the available cystine (see Table 3). In this case the fraction removed is somewhat greater, 29%, but it seems unlikely that the

Table 3. *Cystine in wool oxidized by potassium permanganate at 25°*

pH	KMnO ₄ reduced (g./100 g. of wool)	Cystine oxidized (% of that originally present)
2	6.25	18.6
2	12.50	21.8
2	16.70	30.7
2	25.00	29.6
9.2	6.25	<3.0
9.2	25.00	17.7

difference is significant. This fraction is identical with that oxidized by alkaline hypochlorite, as this reagent did not remove any appreciable quantity of cystine from a wool sample which had already reacted with 16% of potassium permanganate (i.e. 16 g. KMnO₄/100 g. of wool). The reaction at pH 2 proceeds quite rapidly and is complete in about 30 min. Even when very large quantities of permanganate were used the wool only lost approximately 3% in weight during the reaction. At pH 9.2 permanganate reacted very slowly with wool and the reaction with 25% of permanganate took about 12 hr. to reach completion.

The damage to the wool in the alkaline permanganate solutions is severe and after the 25% treatment the wool was so fragile that it fell to pieces. It is surprising that none of the cystine (see Table 3) should be oxidized by reaction with 6% of permanganate in alkali. Experiments with a solution of the free amino-acid showed that the disulphide bond was rapidly oxidized by alkaline permanganate, almost as rapidly as in acid solution.

Oxidation with peracetic acid. Toennies & Hommiller (1942) showed that performic acid reacts only with the amino-acids cystine, methionine and tryptophan. We found that aqueous solutions of performic acid were unstable, although peracetic could be kept for days. Preliminary experiments indicated that peracetic acid exhibits the same specificity as performic acid so that in the reaction with wool only the oxidation of cystine is significant. It is possible (see Table 4) to oxidize all the cystine in wool with this reagent, but the first 75% is removed much more rapidly than the remaining 25%. From this it should not be concluded that the 25% is more re-

sistant to oxidation by peracetic acid. It seems more probable that the lack of reaction is due to slow diffusion of the reagent into the interior of the fibre. Rate of diffusion does not affect any of the experiments with other oxidizing agents as the reaction was continued in every case until the solution was exhausted.

Table 4. *Cystine in wool oxidized by 1.6% (w/v) peracetic acid for different times at room temperature*

Time of reaction	Cystine oxidized (% of that originally present)
5 min.	19.8
30 min.	46.3
1 hr.	71.1
2 hr.	75.0
4 hr.	77.7
25 hr.	91.7

Oxidation of modified wool samples. The proportion of cystine accessible to alkaline hypochlorite is not affected either by esterifying the carboxyl groups in wool or by acetylating the amino groups, and the maximum amount of cystine which can be oxidized

Table 5. *Cystine oxidized by 20 g. chlorine/100 g. wool at pH 10 and 25° in wool samples which had received different pretreatments*

Pretreatment of wool	Cystine content of wool		Cystine oxidized by chlorine solution (% of that originally present)
	After pre- treatment (%)	After alkaline chlorination (%)	
None	10.17	7.80	23.4
Esterification	9.84	7.23	26.5
Acetylation	9.92	7.81	21.3
Supercontraction in lithium bromide	10.10	7.35	27.0
Boiling in pH 10.2 buffer for 30 min.	4.45	2.86	33.0

Table 6. *Cystine oxidized by chlorine at pH 10 at 25° in wool which had previously been boiled in distilled water for 3 hr.**

Chlorine used (g./100 g. of wool)	Cystine oxidized (% of that originally present)
5.0	21.4
10.0	30.2
20.0	32.1

* The 3 hr. boiling reduced the cystine content of the wool to 9.1%.

remains at approximately 25%. Similarly, removing the cystine sub-fractions (A + B) of Phillips (1946) by boiling wool in pH 10 buffer does not alter appreciably the proportion of cystine which can be oxidized by hypochlorite (see Table 5). To investigate the possibility that the fraction of the cystine which does not react (75%) is inaccessible for morphological reasons, wool was boiled for 3 hr. in distilled

water to disrupt the exterior membrane, which was first recognized by Lehmann (1943) and shown by him to be broken by water at a temperature greater than 80°. Again the percentage of the cystine oxidized remained the same (see Table 6). Similarly, wool when supercontracted by 20% in a lithium bromide solution still retained 75% of its cystine in an unreactive state (see Table 5).

Horn. Results of the chlorination of horn with acid and alkaline hypochlorite are recorded in Table 7. Again, part only of the cystine is accessible to the latter reagent, whereas the whole is oxidized by chlorine in acid solution.

Table 7. *Cystine in powdered horn keratin oxidized by 50 g. chlorine/100 g. keratin at pH 2 and pH 10 at 25°*

Treatment	Cystine content (%)	Cystine oxidized (% of that originally present)
None	13.92	—
Chlorine at pH 10	11.01	21.0
Chlorine at pH 2	2.66	80.9

Products of oxidation. The oxidation product of the cystine was shown by partition chromatography to consist in every case at least partly of cysteic acid. A quantitative analysis of the oxidized wools is now being made in an attempt to isolate other oxidation products and to determine which other amino-acids these oxidizing agents attack. Only in the case of peracetic acid has it been established that cysteic acid is the sole product and that, except perhaps after very prolonged treatments, no other amino-acid is attacked. These investigations will form the subject of another communication.

DISCUSSION

Morphological considerations appear at first to offer the most probable explanation for the division of the cystine in wool into two fractions of widely differing reactivity; a portion of the fibre might be physically inaccessible to hypochlorite and permanganate. The membrane which lies between the scales and the cortical cells (Lehmann, 1943) has been considered to act as a barrier to penetration. It seemed attractive to assume that the reagents capable of oxidizing all the cystine could penetrate the membrane and that those capable of oxidizing only 25% of the cystine could not, being thus able to react only with the outside layer of the wool fibre. However, boiling in water, which was clearly shown to break the membrane, did not increase the fraction attacked by hypochlorite. Further, it has been shown that in powdered horn and supercontracted wool a fraction of the cystine, of the same size as that found in wool, is not oxidized by alkaline hypochlorite. Horn is similar in molecular arrangement to wool, although morphologically the two proteins differ considerably.

Supercontracted wool has undergone chain rearrangement without chemical change.

The difference between the two fractions must thus be found in the molecular environment of the cystine and its position in the polypeptide chain. Lindley & Phillips (1947) suggested that their two major cystine fractions (*A + B*) and (*C + D*) differ from one another in being surrounded by polar and non-polar side chains respectively. Cockburn, Drucker & Lindley (1948) showed that the reaction between cystine and alkali differed in deaminated wool from that in untreated wool. However, both esterified and acetylated wool show the same proportion for the cystine fractions considered in this paper and the differences cannot be ascribed to the proximity or otherwise of charged groups.

The Phillips (1946) sub-fractions, although differing in their rates of reaction, are identified by their giving different reaction products with alkalis, bisulphite and formaldehyde. The two fractions discussed in this paper are identified solely by reactivities. They are entirely unrelated to the Phillips sub-fractions, since, after fractions (*A + B*) have been removed by boiling in pH 10 buffer, hypochlorite still attacks, as in untreated wool, only about a quarter of the total cystine present. It thus appears as if alkali reacts with the two fractions to approximately the same extent, leaving their ratio unaltered.

Smith & Harris (1936) and Rutherford & Harris (1939) showed that alkaline hydrogen peroxide and ultraviolet light, respectively, will in time oxidize all the cystine in wool; these reagents thus fall into the same class as chlorine, hypochlorous acid and peracetic acid. Acid permanganate and alkaline hypochlorite, which can oxidize only 25% of the cystine in wool, form a second group; there is no information in the literature about other oxidizing reagents which might fall into this group. In the first group the oxidizing agent is an uncharged molecule, whereas in the second it is a negatively charged ion and it appears attractive to ascribe their differing behaviour to this difference. However, the failure of the negatively charged ions to react with 75% of the cystine cannot be due to repulsion by negatively charged carboxyl groups, since these groups would be uncharged in acid permanganate solution, and also because 60% esterification has not changed the ratio of the two fractions. The effect also cannot be attributed to steric hindrance since the OCl^- ion and the HOCl and Cl_2 molecules are all of similar sizes.

A possible explanation is that the reactivity of the disulphide group in cystine is highly dependent on induced electrical effects from neighbouring groups. That inductive effects of this kind are capable of producing great changes in reactivity is well illustrated by the fact that alkaline permanganate, which readily oxidizes cystine in solution, does not oxidize

the disulphide bond in the wool at all until considerable degradation has occurred.

Simple cystine polypeptides of varying compositions are now being synthesized to determine in which stereochemical arrangement if any the cystine disulphide bond cannot be oxidized by hypochlorite and permanganate.

SUMMARY

1. A study has been made of the reaction of the disulphide bond in wool with different oxidizing agents.

2. Chlorine, hypochlorous acid and peracetic acid are capable of oxidizing all the cystine in wool, whereas acid permanganate and alkaline hypo-

chlorite are only capable of oxidizing 25% of the cystine present. The cystine in wool may therefore be divided into two fractions, one of which is oxidized by the latter two reagents, and the other not.

3. The difference in reactivity of the two cystine fractions cannot be ascribed to morphological causes since the cystine of physically modified wools and powdered horn shows the same division.

4. The two fractions are not related to the Phillips (1946) cystine fractions. Possible explanations for the differences in reactivity are discussed.

The authors wish to thank Messrs Wolsey Ltd., Leicester, for financial support which made this investigation possible, Prof. H. V. A. Briscoe for his help and interest, and Mr D. Gough for carrying out some of the preliminary experiments.

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The Utilization of Non-protein Nitrogen in the Bovine Rumen*

5. THE ISOLATION AND NUTRITIVE VALUE OF A PREPARATION OF DRIED RUMEN BACTERIA

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Pearson & Smith (1943) showed that when the liquid contents of the bovine rumen were incubated *in vitro* at 39° in the presence of a soluble carbohydrate such as starch or maltose, the non-protein nitrogen (N.P.N.) content of the liquid decreased markedly

* Previous papers in this series were published under the general title 'The Utilization of Urea in the Bovine Rumen'; see Smith & Baker (1944).

within 4 hr., suggesting that significant amounts of protein were formed. Subsequent work by Smith & Baker (1944) confirmed this suggestion and showed that the formation of protein could be attributed to the growth and multiplication of the bacteria which abound in the rumen liquid. In growing and multiplying, many of these bacteria not only synthesized protein, but were found also to build up and store a