14. THE ACTION OF SULPHITES ON THE CYSTINE DISULPHIDE LINKAGES IN WOOL

2. THE INFLUENCE OF TEMPERATURE, TIME AND CONCENTRATION ON THE REACTION

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A STUDY of the interaction of wool at room temperature with buffered sulphite solutions of different pH [Elsworth & Phillips, 1938, 1] showed that only a fraction of the total disulphide-S in the wool reacted at pH 5 according to equation (1) and much smaller amounts in solutions of lower or higher pH.

(R_1 and R_2 , adjacent polypeptide chains).

The present investigation is concerned with the influence of other factors on this reaction, with the explanation of why it is apparently restricted to only a portion of the total disulphide-S of the wool, and also with the determination of the maximum proportion of the disulphide-S that will react in this manner.

EXPERIMENTAL

The influence of time on the reaction. Pieces of loosely woven, thin woollen fabric, prepared from 64–70's wool, were immersed for the times given in Table 1 in thirty times their weight of buffered NaHSO_3 solutions at room temperature containing respectively 1.0 and 2.5 % SO_2 ; the pH was 4.5. The wool fabric was similar to that used in the previous investigation and the buffered NaHSO_3 solutions were also prepared in a similar manner. To avoid oxidation, the reactions were carried out in closed flasks. The bisulphited wools were rinsed in water to remove buffer salts and the bulk of the free bisulphite, dried at room temperature and then allowed to reach equilibrium with a controlled atmosphere of 21° and 70 % R.H. Reaction (1) is reversible and bisulphited wool loses NaHSO_3 slowly when rinsed in water. For this reason, no attempt was made to wash bisulphited wools until they were free from NaHSO_3 . The presence of free NaHSO_3 does not interfere with the methods of analysis employed [Elsworth & Phillips, 1938, 1, 2]. The conditioned wools were cut up into small pieces, their moisture contents determined by Barritt & King's method [1926] and their thiol-S contents, after hydrolysis for 4 hr. in 5*N* HCl (20 ml. per g.), by Shinohara's [1935, 1] modification of Folin & Marenzi's method [1929]. Two thiol groups arise in the hydrolysate of the wool for each disulphide group which reacts with the bisulphite, since the *S*-cysteinesulphonate group (equation (1)) is converted into a thiol group when the bisulphited wool is hydrolysed [Elsworth & Phillips, 1938, 1].

Table 1. *The influence of time of treatment on the reaction between wool and solutions of NaHSO₃ at pH 4.5 containing respectively 1.0 and 2.5 % SO₂*

Time of treatment hr.	Thiol-S in hydrolysate, % of dry wool	
	(a) From solution containing 1 % SO ₂	(b) From solution containing 2.5 % SO ₂
0.25	0.40	—
0.50	0.44	0.52
1	0.50	0.58
2	0.53	0.64
4	0.55	0.66
7	0.61	0.68
24	0.72	0.73
48	0.69	0.85
96	—	0.83
120	0.64	—
168	—	0.82
288	0.84	—
336	—	0.94

The results (Table 1) show that the rate of reaction is high in the early stages, but then gradually decreases. In both solutions, the reaction proceeds very slowly after 0.7 % of disulphide-S (calculated on the dry wool) has reacted.

The influence of temperature on the reaction. Pieces of the woollen fabric, after previous wetting-out, were heated for 30 min. with forty times their weight of buffered NaHSO₃ solutions of pH 4.9 containing 1 % of SO₂. They were then rinsed and dried, as described above, before analysis. Besides the thiol-S, the total SO₂ contents of the wools were determined by distillation with dilute H₃PO₄ (1 ml. H₃PO₄, sp. gr. 1.75 in 500 ml.; initial pH 2.1) [Elsworth & Phillips, 1938, 2]. The rinsed wools could not be considered to be free from NaHSO₃, so that a small amount of the total SO₂ returned by this method was derived from this source. The bulk of the SO₂ arose from the decomposition of *S*-cysteine-sulphonate groups according to equation (3).

Table 2. *The influence of temperature on the reaction between wool and a solution of NaHSO₃ at pH 4.9 containing 1 % SO₂*

Temp. ° C.	Total SO ₂ , % of dry wool	Thiol-S in hydrolysate, as % of dry wool
20	0.72	0.45
30	0.83	0.62
40	0.75	0.65
50	0.83	0.69
60	0.97	0.72
70	0.98	0.74
80	1.01	0.76
90	0.91	0.76
100	0.75	0.66

From Table 2 it will be seen that increase in temperature up to 60° assists the reaction. At higher temperatures the wool was weakened, and at 100° it is probable that other chemical changes occurred in the wool besides the bisulphite reaction and caused the observed decrease in the thiol-S in the hydrolysate. Theoretically, since the weight of 2S atoms is the same as that of the SO₂ molecule, the weights of SO₂ obtained from these bisulphited wools should have

equalled the weights of thiol-S found in the hydrolysates. In every wool, the total SO_2 is greater than the thiol-S because of the incomplete removal of the uncombined NaHSO_3 before analysis.

The influence of the concentration of NaHSO_3 . Pieces of the woollen fabric (10 g.) were immersed for 24 hr. at room temperature in 300 ml. of buffered NaHSO_3 solutions (pH 5.3–5.7) containing 0.1–5.0 % SO_2 . The subsequent treatment of the wools before the determination of the thiol-S contents of their hydrolysates was as previously described. In Fig. 1 the thiol-S contents of the

Fig. 1. SO_2 , % in solution, pH 5.3–5.7. $\times$ — $\times$ Commercial fabric. $\square$ — $\square$ Australian 64's.

hydrolysates of the bisulphited wools are plotted against the concentrations of the solutions in which the wools were treated. It will be seen that the extent of the reaction increases at first, but reaches a maximum when the solution contains 8 % SO_2 (13 % NaHSO_3).

These observations were extended using a virgin wool which contained very nearly the same percentage of disulphide-S as the wool of the commercial fabric. This virgin wool was prepared from a 64's Australian merino fleece by extracting a picked portion three times with benzene at 40° and then removing dirt, vegetable matter and discoloured fibres by hand. The remaining wool, after it had been extracted three times with benzene and twelve times with water, was finally washed in an 0.05 % solution of saponin followed by twelve rinses in water. After drying at room temperature and allowing to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H., the clean wool contained 3.42 % total S and 3.09 % disulphide-S (calculated on its anhydrous weight). Samples of this wool were immersed for 24 hr. in fifty times their weight of solutions of NaHSO_3 of pH 5.7 containing respectively 8 and 16 % SO_2 . The thiol-S values found in the hydrolysates of these bisulphited wools are plotted in Fig. 1, and it will be seen that they fall on the curve drawn through the points given by the wool of the commercial fabric. This wool contained 3.60 % S and 2.99 % disulphide-S. The curve in Fig. 1 possibly gives an exaggerated idea of the regularity of the reaction. A wider variety of different wools needs to be examined before the precise fraction of the disulphide-S that reacts with NaHSO_3 can be stated definitely.

After both these wools had been bisulphited, their original disulphide-S could be almost entirely accounted for either as thiol-S or as disulphide-S: hence during bisulphiting at room temperatures no conversion of disulphide-S into non-disulphide-S¹ took place. For example, after exposure for 24 hr. to the

¹ The term non-disulphide-S is used throughout this paper to describe S not returned either as thiol-S or as disulphide-S by Shinohara's method or as methionine-S by Baernstein's method.

NaHSO_3 solution containing 8% SO_2 , the virgin wool when hydrolysed gave 1.01% thiol-S and 1.93% disulphide-S, thus accounting for 2.94% S or nearly all the total of 3.09% S originally present as disulphide. This conclusion is of interest in the light of analytical results obtained when more drastic conditions for bisulphiting wool were used. Samples of the virgin wool were immersed in NaHSO_3 solutions at pH 5.7 containing 6.2% SO_2 which were maintained at 40°. After 5½ and also after 19 days' exposure to this solution, the wools gave 1.18 and 1.12% thiol-S respectively. These percentages are somewhat higher than those produced by the treatment of wool with NaHSO_3 at room temperature. It is doubtful, however, whether this increase in thiol-S is due to an increase in the reaction between the wool and NaHSO_3 according to equation (1). At the higher temperature, other reactions occur which may increase the amount of thiol-S produced.

The occurrence of such reactions is shown by the conversion of disulphide-S into non-disulphide-S which took place. Thus the original wool contained 3.09% disulphide-S, but after 5½ days' treatment in NaHSO_3 at 40° only 2.76%, and after 19 days only 2.49% of S in the wool could be accounted for either as disulphide- or thiol-S after hydrolysis.

The treatment of wool with concentrated solutions of NaHSO_3 (containing 8% or more of SO_2) at room temperatures thus appears to lead to a straightforward reaction in which about one-third of the disulphide-S is converted into thiol and *S*-cysteinesulphonate groups. If the temperature of the NaHSO_3 solution is raised, even to 40°, other changes take place slowly and disulphide-S is converted into S which is neither thiol-S nor S present in *S*-cysteinesulphonate groups.

The reaction between NaHSO_3 and the cortical cells of wool. Although wool is attacked very slowly by a solution of papain, it has been found [Phillips, Middlebrook & W.I.R.A., 1939] that when NaHSO_3 is added to the papain solution, the wool is rapidly reduced to a mass of cortical cells, the intercellular protein and scales being digested. Cortical cells produced by this method were used to obtain evidence that they react with NaHSO_3 . A 64's merino wool, which had been scoured commercially, was extracted with alcohol and then rinsed repeatedly in water. A sample of the wool was immersed for 30 min. in forty times its weight of solution of NaHSO_3 at pH 5.7 containing 2.0% SO_2 . The wool and solution were maintained at 65°. The bisulphited wool was rinsed with water and then analysed for thiol-S, free and combined SO_2 and for sulphate-S. A further sample of the wool (40 g.) was digested at 65° in 1 l. of 0.05% papain solution containing 1% NaHSO_3 . After 17 hr., the digest was centrifuged and the insoluble material digested for a further 17 hr. with a fresh enzyme solution. The cortical cells that remained were centrifuged repeatedly with water until the supernatant liquor no longer gave a turbidity on the addition of H_2O_2 and BaCl_2 . After drying *in vacuo* over P_2O_5 to constant weight, they were analysed for total S and thiol-S. They did not contain either free or combined SO_2 , indicating that the bisulphite which they acquired in the enzyme solution had been removed completely. A sample of the cortical cells was then bisulphited under exactly the same conditions as the wool. The bisulphited cells were removed by centrifuging the solution and were washed with water. After drying *in vacuo* over P_2O_5 , they were analysed again, the results of this analysis and those of the previous analyses being given in Table 3 as a percentage of the dry wool and cells. The total and 'free' SO_2 were determined by Elsworth & Phillips' methods [1938, 2].

Table 3. *Analysis of wool and wool cortical cells before and after treatment with NaHSO₃*

	Total S %	Thiol-S in hydrolysate %	Free SO ₂ %	Combined SO ₂ %	Sulphate-S %
Wool	3.64	0.10	—	—	0.08
Wool after bisulphiting	4.21	0.76	0.0	0.74	0.28
Cortical cells	4.12	0.17	0.0	0.0	—
Cortical cells after bisulphiting	5.09	0.75	0.23	0.76	0.66

The analyses of thiol-S, after hydrolysis, and the combined SO₂, show that very nearly the same percentages of the disulphide-S of the wool fibre and of the cortical cells react with NaHSO₃. This agreement may be accidental, since changes in the disulphide-S of the wool and cells, leading to its conversion into unreactive non-disulphide-S, may have occurred at unequal rates when the wool and cells were warmed with NaHSO₃ at 65° for 30 min. In addition, during their isolation the cortical cells were exposed for two periods of 17 hr. to 1 % NaHSO₃ at 65°.

The data of Table 3 provide some evidence as to the S content of the cortical cells and how this compares with that of the whole wool fibre. The S content of the cells can be calculated from the analyses after sulphiting, since these include a determination of sulphate-S by Mease's [1934] method. Allowing for sulphate-S, and S present as free and combined SO₂, the S content of the cells is 3.94 % calculated on their anhydrous weight. This exceeds the organic S, namely 3.56 %, of the wool from which they were isolated. Other evidence is available showing that the S content of the cellular components of the wool fibre exceeds that of the intercellular material. Stakheyewa-Kaverznewa & Cavrilov [1937] obtained an extract by the pancreatin-enterokinase digestion of wool which contained less S than the residual cells. They concluded that wool contained some 10 % of an intercellular phase of low S content. Speakman & McMahon [1938] digested the root portions of non-medullated Australian wool fibres with trypsin (thus avoiding the presence of light-damaged fibres with might be preferentially attacked by the enzyme) and found that the residual wool had a higher S content than the original wool. Their figures agreed with the presence of some 9 % of S-free protein in wool.

The reaction between alkali-degraded wool and NaHSO₃. On the hypothesis that NaHSO₃ reacts with a labile fraction of the disulphide-S of wool, it was thought possible that such S could be removed preferentially by alkali and the wool would not then react to the same degree, if at all, with NaHSO₃. The wool for these experiments was prepared by immersing pieces of the wool fabric for 30 min. in boiling buffer solutions of pH 8–10. The buffer solutions were borax-boric acid buffers and were 0.2 M in borate ion, except those of pH 9.6 and 10.0, which were 0.15 and 0.12 M respectively. The total S contents of the untreated and treated wools, after these had been rinsed thoroughly with water, were determined by the Denis-Benedict method as applied to wool by Barritt [1934]. The disulphide-S remaining intact in the wools was determined by Shinohara's method [1935, 2]. The pieces of fabric (about 10 g.) were then immersed for 20 hr. in 2800 ml. of a solution of NaHSO₃ at pH 5.0 containing 1.5 % SO₂. After passing through a wringer, the wools were immersed in water for 1 hr., allowed to dry in air and then to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H. They were then analysed for thiol-S, and free and total SO₂, from which the combined SO₂ content was calculated.

Table 4. *Analyses of alkali-treated wools before and after reaction with NaHSO₃*

(i) pH of buffer with which wool was boiled	(ii) Total-S, % after treatment	After alkaline treatment followed by reaction with NaHSO ₃			(vi) Thiol-S in hydrolysate %
		(iii) Total SO ₂ %	(iv) Free SO ₂ %	(v) Combined SO ₂ %	
(Untreated)	3.60	0.79	0.23	0.56	0.61
10.0	2.52	0.45	0.21	0.24	0.22
9.6	2.90	0.47	0.21	0.26	0.28
9.2	3.05	0.53	0.21	0.32	0.37
8.9	3.29	0.55	0.09	0.46	0.45
8.5	3.56	0.64	0.17	0.46	0.47
8.1	3.51	0.69	0.16	0.53	0.53

An important deduction from these results (Table 4) is that the amount of reaction that occurs is proportional to the disulphide-S contents of the wools as recorded in Table 5. Thus the thiol-S values in the hydrolysates of the wools (Table 4, col. (vi)) are approximately one-fifth of the disulphide-S contents of the wools (Table 5, col. (ii)), suggesting that no matter how small the disulphide-S becomes, one-fifth will react with NaHSO₃ under the conditions of these experiments. The residual disulphide-S of alkali-treated wools thus appears to react as freely with NaHSO₃ as the disulphide-S of untreated wool.

Another significant feature of these results is that for each thiol group in the alkali-treated and bisulphited wools there is one *S*-cysteinesulphonate group. This is indicated by the very good agreement between the figures for the combined SO₂ and the thiol-S in the hydrolysates (Table 4, cols. (v) and (vi)). When the extent and possible nature of the chemical alteration produced in the wool by alkalis is taken into consideration, this agreement is remarkable. For example, most of the chemical changes which it has been suggested that wool undergoes when treated with alkalis are assumed to give rise to free thiol groups. Speakman [1933] has suggested that mild treatment with alkali may cause hydrolysis of the disulphide linkage, followed, under certain conditions, by condensation between the sulphenic acid groups and adjacent amino-groups.

These changes ((4) and (5)) occur without loss of S. When S is lost (as in (6)) Schöberl [1933] has suggested that the sulphenic acid groups decompose giving aldehyde groups and H₂S. Race *et al.* [1938] have obtained evidence that a decomposition similar to that postulated by Schöberl occurs in the tips of wool fibres when they are exposed to light and moisture during growth. They found that such light-damaged wool contained aldehyde groups, but less S than the corresponding root wool. Speakman & McMahon [1938] titrated wool from the tips and from the root ends of a fleece and from the differences between the titration curves concluded that the tips of the fibres contained appreciable quantities of thiol groups, whereas the root ends contained only traces. The evidence for the presence of thiol groups in wool which has undergone chemical change in aqueous solutions is not so strong. Crowder & Harris [1936] caused wool to lose half its S, in accordance with the Schöberl reaction, by exposing it to a current of aqueous NaOH. They were unable to find any cysteine in the hydrolysate of the wool and assumed that this was due to the formation of thioacetals during the hydrolysis.

Assuming that disulphide-S is destroyed by the reactions suggested by Speakman and Schöberl, an estimate of the amounts of thiol-S which were formed in the alkali-treated wools can be made from the analyses recorded in Tables 4 and 5. In these analyses, the difference between the values of the disulphide-S and total S minus the value of the methionine-S (found to be 0.10 % using Baernstein's method [1932] for the determination of methionine) gives the amount of S present in the wools as non-disulphide-S. In the untreated wool, the non-disulphide-S amounts to 0.51 % on the weight of the anhydrous wool, and is considered to have arisen without loss of S. This may not be true, since the wool may have lost S by exposure to air, moisture and light during growth, but the amount is unlikely to be large, since the total S of the wool is 3.60 %, a reasonably high value. The total non-disulphide-S in the alkali-treated wools is subdivided by assuming that for each atom of S lost by the wool, one atom of non-disulphide-S is produced. The non-disulphide-S produced with loss of S is then subtracted and figures for the non-disulphide-S produced without loss of S are obtained.

Table 5. *The modification of the disulphide-S of wool when it is treated with boiling buffer solutions*

pH of buffer with which wool was boiled	Disulphide-S, % after treatment	Non-disulphide-S, % produced without loss of S	Non-disulphide-S % produced with loss of S
—	2.99	0.51	—
8.1	2.59	0.73	0.09
8.5	2.36	1.06	0.04
8.9	1.87	1.01	0.31
9.2	1.57	0.83	0.55
9.6	1.31	0.79	0.70
10.0	1.04	0.30	1.08

It will be seen (Table 5) that when wool is treated with boiling mildly alkaline solutions relatively large amounts of non-disulphide-S are produced without loss of S: in the more strongly alkaline solutions, non-disulphide-S is produced with loss of S. The significance of these changes will be more fully discussed in a subsequent paper. Here we need only point out that if the non-disulphide-S was produced according to the Speakman and Schöberl reactions, part of it should have been found as thiol-S. For example, the hydrolysate of the wool boiled with the buffer of pH 9.2 should have contained 0.42 % thiol-S arising by the Speakman reaction and 0.55 % thiol-S by the Schöberl reaction, a total of 0.97 %, calculated on the weight of wool hydrolysed. Experimentally none was found. On the other hand, when this alkali-treated wool was bisulphited, and determinations of the combined SO_2 indicated that theoretically the hydrolysed wool should contain 0.32 % thiol-S, 0.37 % was actually found.

In some instances, it is not possible to attribute the failure to find the thiol-S to the presence of aldehydes in the hydrolysate of the wool. For example, the non-disulphide-S in the wool increased from 0.51 to 1.06 % when it was boiled in buffer of pH 8.5, but the wool lost only 0.04 % S. If the additional non-disulphide-S arose mainly by the Speakman reaction, 0.25 % thiol-S was produced without the simultaneous production of aldehyde groups. In spite of this, no increase in the thiol-S of the hydrolysed wool was observed. It appears probable therefore that if disulphide cross-linkages are destroyed according to the Speakman and Schöberl reactions, the thiol-S produced must become linked to other atoms in the keratin molecule.

DISCUSSION

The present investigation has shown that whilst the reaction between wool and NaHSO_3 proceeds quickly at first, there is a point at which it is difficult to cause further reaction to take place. This is brought out most clearly in Fig. 1, which shows the effect of increasing the concentration of the NaHSO_3 . In solutions containing up to 8% SO_2 at room temperatures about one-third of the disulphide-S of the wool reacts and a twofold increase in concentration produces no further reaction. At higher temperatures, although increased amounts of disulphide-S disappear from the wool, there is evidence that the wool has not combined with equivalent amounts of NaHSO_3 .

It is, however, important to emphasize that all the bisulphited wools mentioned in this paper were analysed after they had been rinsed with water. It is known [Elsworth & Phillips, 1938, 1] that NaHSO_3 combined with wool is not readily removed by water, but further work is necessary to prove whether all the NaHSO_3 removed when bisulphited wool is rinsed with water is free NaHSO_3 and not NaHSO_3 less firmly combined with some additional fraction of the disulphide-S.

Nevertheless sufficient evidence has been obtained to suggest that the reactivity of one-third of the disulphide-S of wool towards NaHSO_3 differs from that of the remaining two-thirds. This does not necessarily mean that one-third of the disulphide-S of wool is more reactive than the remaining two-thirds. If this were so, we should expect to be able to remove the more reactive fraction by means of alkali, and obtain a wool which would not react with NaHSO_3 . Experimentally it is found, however, that the residual disulphide-S in alkali-treated wools is as reactive towards NaHSO_3 as the disulphide-S of untreated wool.

A more likely hypothesis appears to be that whilst all the disulphide linkages of wool are equally reactive towards NaHSO_3 , after this reagent has reacted with and broken some of these linkages, stresses on the remaining linkages are dissipated and their reactivity is altered. In agreement with this suggestion, it is known that the physical properties of hair are profoundly changed by boiling it for 30 min. with a 5% solution of NaHSO_3 . Speakman [1936] found that such treatment enabled human hair to supercontract 30% in length when it dried. Assuming that human hair reacts with NaHSO_3 in the same manner as wool, it would appear that this supercontraction becomes possible because about one-third of the disulphide linkages in the hair have been broken. These linkages may therefore be responsible for maintaining the keratin in the α -configuration: when they are broken by NaHSO_3 , the keratin molecule can assume a more folded and shorter configuration [Astbury & Woods, 1933].

Unstretched human hair that has been boiled with a 2% solution of borax (pH 9.2) does not contract in length when boiled with 5% NaHSO_3 . If it is first boiled with dilute HCl and then with 5% NaHSO_3 , it contracts 15% in length [Speakman & Stoves, 1937]. These authors assume that for each disulphide linkage destroyed by the borax another linkage is produced. This may either be a $-\text{S}-\text{NH}-$ linkage arising without loss of S, or, when S is lost, a $-\text{CH}=\text{NH}-$ linkage arising, as suggested by Phillips [1936], by condensation between the aldehyde group and an adjacent amino-group. Boiling the hair in dilute HCl may break the $-\text{CH}=\text{N}-$ cross-linkages, so that after additional linkages have been broken by NaHSO_3 , the total number of linkages broken is then sufficient to enable some supercontraction to take place.

SUMMARY

1. The disulphide-S of wool reacts with NaHSO_3 and thiol and *S*-cysteine-sulphonate groups are formed which are not readily removed by rinsing the bisulphited wool with water. By increasing the temperature, time of reaction and concentration of NaHSO_3 not more than one-third of the total disulphide-S of the wool can be caused to react in this manner.

2. The cortical cells, isolated by digestion of wool with papain in the presence of NaHSO_3 , contain more organic S than the fibre as a whole. The keratin of the cells reacts with NaHSO_3 to give thiol and *S*-cysteinesulphonate groups.

3. The residual disulphide-S in wool that has been boiled with alkaline solutions of pH 8 and higher reacts with NaHSO_3 in the same proportion as the disulphide-S of untreated wool.

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REFERENCES

- Astbury & Woods (1933). *Philos. Trans. A*, **232**, 333.
Baernstein (1932). *J. biol. Chem.* **97**, 663.
Barritt (1934). *J. Soc. chem. Ind., Lond.*, **53**, 291 T.
— & King (1926). *J. Text. Inst., Manchr.*, **17**, 386.
Crowder & Harris (1936). *J. Res. Nat. Bur. Stand.* **16**, 475.
Elsworth & Phillips (1938, 1). *Biochem. J.* **32**, 837.
— — (1938, 2). *J. Text. Inst., Manchr.*, **29**, 219 T.
Folin & Marenzi (1929). *J. biol. Chem.* **83**, 103.
Mease (1934). *J. Res. Nat. Bur. Stand.* **13**, 617.
Phillips (1936). *Nature, Lond.*, **138**, 121.
— Middlebrook & W. I. R. A. (1939). B.P. 513,919.
Race, Rowe, Speakman & Vickerstaff (1938). *J. Soc. Dy. Col., Bradford*, **54**, 141.
Schöberl (1933). *Liebigs Ann.* **507**, 111.
Shinohara (1935, 1). *J. biol. Chem.* **109**, 665.
— (1935, 2). *J. biol. Chem.* **112**, 671.
Speakman (1933). *Nature, Lond.*, **132**, 930.
— (1936). *J. Soc. Dy. Col., Bradford*, **52**, 335.
— & McMahon (1938). *Nature, Lond.*, **141**, 118.
— & Stoves (1937). *J. Soc. Dy. Col., Bradford*, **53**, 236.
Stakheyewa-Kaverznewa & Cavrilov (1937). *Bull. Soc. Chem. (Mémoires)*, **4**, 647.