The Oxidation of Protein Thiol Groups by Iodate, Bromate and Persulphate

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The oxidation of some simple thiols by oxidizing agents known to act as improvers in bread manufacture has been investigated (Hird & Yates, 1961). It was shown that when cysteine was oxidized the major product was the disulphide cystine, but that small amounts of cysteinesulphonic acid and cysteinesulphonic acid were minor products (approx. 10%). Cystine, however, was not oxidized by these reagents under the conditions used. It has also been shown that the thiol groups of gluten formed by reduction with sodium borohydride were oxidized (Frater, Hird, Moss & Yates, 1960), but the nature of the oxidation products was not investigated.

The work described in the present paper attempts to define more precisely the action of iodate, bromate and persulphate on the thiol groups of certain protein preparations, particularly those from flour. In the flour being investigated the disulphide content of the total proteins is approximately ten times that of the thiol content when considered on the basis of sulphur atoms (Frater *et al.* 1960). Because of the low thiol content compared with the -S-S- groups already present, accurate estimation of the yield of disulphide when thiol groups are oxidized is difficult. Further, isolation of the proteins is accompanied by atmospheric oxidation of thiol and it has therefore been necessary to reduce the protein preparations with sodium borohydride to obtain preparations with sufficient thiol content for subsequent experiments.

The oxidation of thiolated gelatin by ferricyanide has been studied by Benesch & Benesch (1958) and this substance, because of its high thiol content and suitable solubility properties, has been included in the present investigation.

MATERIALS AND METHODS

The gluten was a commercial sample prepared by acetic acid extraction of flour and was presented by Barretts Ltd., Melbourne. The water-soluble proteins were prepared by extraction of 1 kg. of flour (Victorian FAQ) with 10 l. of

water for 1 hr., followed by freeze-drying of the extract. The thiolated gelatin was a commercial sample obtained from Schwartz Laboratories Inc., Mount Vernon, N.Y. As obtained it contained approx. 16 moles of thiol groups/10⁵ g. of protein.

Sodium borohydride was obtained from Metal Hydrides Inc., Beverley, Mass., U.S.A.

Estimation of thiol and disulphide groups

Thiol and disulphide groups in the proteins were determined amperometrically by estimation of excess of methyl mercuric iodide added to the system at the end of the reaction time. The titration was done at -0.7 v with a dropping mercury electrode with Ag-AgCl reference electrode. A sample of the reaction mixture (10 ml.) was removed at the appropriate time interval, and added to a known excess of methyl mercuric iodide to stop the oxidation reaction. A portion (10 ml.) of aq. 0.25 M-NH₃ soln.-NH₄Cl buffer, 8 M with respect to urea, at pH 9.0, was then added. The temperature of the reaction vessel was kept at 25 ± 0.05°. A separate base line was determined in the absence of mercurial for each sample analysed. The current relative to this at -0.7 v was then measured on the experimental sample, and serial additions of 0.2 ml. of methyl mercuric iodide, 2 mM in 25% dimethylformamide, were added, and the current was determined in each case. The number of thiol groups present was calculated by extrapolation to zero current.

For the estimation of disulphide, the buffer was made 0.2 M with respect to Na₂SO₃, which raised the pH from 9.0 to 9.25 and the extra thiol obtained was taken to be equal to the disulphide in the sample, as in the presence of the mercurial the reaction goes to completion (Leach, 1960). Before estimation of disulphide, the protein was dispersed in a tightly fitting Potter-Elvehjem type homogenizer in the presence of the sulphite and excess of methyl mercuric iodide. These manipulations were done at room temperature (18-20°). The total reaction time with the mercurial was 10-15 min., including the gassing of the reaction vessel. This is in excess of that required for the reaction to go to completion (see also Leach, 1960). Under these conditions cystine disulphoxide is not estimated as disulphide.

The estimation of thiol groups with methyl mercuric iodide involves an assumption of absolute specificity of this reagent. To check this point several samples of gluten were analysed for cysteine+cystine sulphur by the above-described procedure and by the differential-oxidation method of Lugg (1938). For three separate samples the results were 13.0, 12.4 and 13.6 moles of S/10⁵ g. of protein when estimated polarographically and 13.8, 14.1 and

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Table 1. *Formation of disulphide bonds on oxidation of reduced gluten*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μ moles/100 mg. of protein.

Time	Iodate			Bromate			Persulphate		
	-SH	-S-S-	Total S (-SH + -S-S-)	-SH	-S-S-	Total S (-SH + -S-S-)	-SH	-S-S-	Total S (-SH + -S-S-)
0	9.7	3.1	15.9	9.7	3.1	15.9	9.7	3.1	15.9
30 min.	0	6.5	13.0	—	—	—	—	—	—
24 hr.	—	—	—	1.5	5.0	11.5	0	6.8	13.6

15.3 moles of S/10⁵ g. of protein respectively when estimated by Lugg's procedure. There is therefore reasonable agreement between the two methods.

Preparation of reduced proteins

Gluten. Reduced gluten was prepared by dispersing as finely as possible 2 g. in 200 ml. of water in the Potter-Elvehjem homogenizer, the final suspension being made 0.1M with respect to sodium borohydride. Frothing caused by liberation of hydrogen was kept at controllable levels by the use of octanol and reduction was carried out for 30 min. at 50°. Treatment for longer periods was found to result in a decrease in the number of thiol groups. The addition of urea to the system to open up the protein structure was impracticable as it caused difficulties in the isolation of the reduced protein.

After reduction the system was cooled to 0°, brought to pH 6 with HCl and stirred for 30 min. at 0°. This procedure has the dual effect of destroying the excess of borohydride and precipitating the reduced gluten. The protein was isolated by centrifuging, washed twice with 5 mM-EDTA and freeze-dried. Of the original gluten 50–60% was obtained in the reduced state. On reduction the thiol content rose from 1.2 to approx. 11 moles of thiol/10⁵ g. of protein, depending on the preparation.

Water-soluble proteins. With the water-soluble proteins, the reducing system contained 0.2 g. of protein/100 ml. of water. The reduction was carried out at 50° for 30 min. in 0.05M-sodium borohydride. After this time an equal volume of 0.5M-citrate buffer, pH 6, was added and the solution stirred for 30 min. as before. The solution of reduced water-soluble proteins was used immediately after preparation and thus contained metaborate, the decomposition product of the borohydride. It was found to be impossible to reduce completely the water-soluble proteins, a maximum of six thiol groups being obtained out of a possible 12.6.

Oxidation of reduced protein. A suitable quantity of protein (approx. 1 mg./ml.) was dissolved or finely dispersed (for reduced gluten) in 0.5M-citrate buffer, pH 6.0. After addition of oxidizing agent the system was gently shaken in a constant-temperature bath throughout the period of reaction. The reaction was carried out at 28° under oxygen-free nitrogen.

After the appropriate time, 10 ml. of the sample was pipetted into 10 ml. of the appropriate analytical system containing the excess of methyl mercuric iodide and the thiol and disulphide content were estimated.

Oxidation of flour. The oxidation of flour was carried out in an aqueous suspension consisting of 20 ml. of water and 1 g. of flour. The oxidizing agents were added so that the

Table 2. *Molar ratio thiol/oxidizing agent at the equivalence point with reduced gluten*

Concentration of protein: 10 mg./10 ml. Iodate was added to oxidize an increasing percentage of thiol groups. Reaction time: 15 min. Percentage of thiol groups oxidized is given in parentheses. For other conditions see text.

Initial thiol content of system (μ moles)	Iodate added (μ mole)	Thiol oxidized (μ mole)	Thiol groups oxidized/oxidizing agent added
1.18	0.056	0.20 (18%)	3.7
1.18	0.16	0.72 (61%)	4.5
1.18	0.23	0.98 (83%)	4.3

molar ratio of thiol/oxidizing agent was 1/1. After the appropriate reaction time, 1 ml. of aq. NH₃ soln.-NH₄Cl buffer (2M) was added to the system so that the final pH was 9.0, and the thiol content estimated.

RESULTS

Table 1 shows the thiol and disulphide distribution of reduced gluten after oxidation by the reagents used. Oxidation by iodate brings about a definite and an immediate decrease of thiol groups and an increase in the number of disulphide bonds. With bromate and persulphate there is also an increase in the number of disulphide bonds, but the oxidation by these reagents is much slower than with iodate. It can also be seen that there is a decrease in the amount of sulphur recoverable as cysteine plus cystine after oxidation. This is consistent with the formation of higher oxidation products found with cysteine (Hird & Yates, 1961) and also with the experimentally determined molar ratio thiol/iodate of 3.7–4.4 at the equivalence point (Table 2). In these experiments iodate has been used for the convenience of its rapidity of action. It can be seen, too, that the ratio does not change appreciably with the different percentages of thiol groups oxidized (see also Table 6).

Table 3 shows that the thiol groups of the reduced water-soluble proteins are oxidized but less completely than those of reduced gluten (Table 1) and thiolated gelatin (Table 5). This resistance to oxidation parallels the resistance of these prepara-

tions to reduction. The number of thiol groups disappearing is too small for any accurate estimation of the disulphide formed.

As Table 4 shows, 50% or more of the thiol groups of flour suspensions are susceptible to oxidation. It is pointed out, however, that the

number of thiol groups/ 10^5 g. of flour, i.e. 6.0 moles, represents only 40% of the total determined in the presence of 8M-urea (Frater *et al.* 1960).

Table 4 shows the oxidation of the thiol groups of thiolated gelatin by iodate, bromate, persulphate and air. The rates of oxidation found are similar to those obtained with simple thiols (Hird & Yates, 1961; Frater *et al.* 1960), reduced gluten and flour. Oxidation by air is slower than that by the oxidizing agents used. Table 6 shows the appearance of disulphide bonds on oxidation of thiolated gelatin. As with reduced gluten there is formation of disulphide bonds. With increasing concentration of thiol groups there is an increase in the percentage yield of disulphide (Table 7). This is shown not only by the increased recovery of total sulphur as thiol plus disulphide but also by an increase in the moles of thiol oxidized/mole of iodate added. At concentrations of 5 and 10% of thiolated gelatin, gelling occurred.

Table 3. *Oxidation of reduced water-soluble proteins of flour*

Concentration of protein: 1 mg./ml. Oxidizing agents were used in a molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μ moles of thiol/100 mg. of protein.

Time (hr.)	Iodate	Bromate	Persulphate	Air
0	6.0	6.0	6.0	6.0
0.25	3.2	—	—	—
2	—	4.5	4.0	—
4	—	4.3	3.5	—
8	—	4.0	3.4	5.5

Table 4. *Oxidation of thiol groups of flour*

Concentration of flour: 1 g. in 20 ml. of water; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μ moles of thiol/10 g. of flour.

Time (hr.)	Iodate	Bromate	Persulphate
0	6.0	6.0	6.0
1	2.4	5.0	4.8
9	1.3	3.2	3.0

Table 5. *Oxidation of thiolated gelatin*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μ moles of thiol/100 mg. of protein.

Time	Iodate	Bromate	Persulphate	Air
0	15.5	15.0	15.5	16.6
5 min.	0	—	—	—
1 hr.	—	13.4	14.7	—
3 hr.	—	12.0	13.5	—
6 hr.	—	8.0	11.5	13.5

DISCUSSION

The results show that the thiol groups of the reduced proteins of flour and of thiolated gelatin are oxidized by iodate, bromate and persulphate. The rates of oxidation are similar to those obtained with low-molecular-weight thiols (Frater *et al.* 1960; Hird & Yates, 1961), i.e. very rapidly with iodate and slowly with bromate and persulphate.

Taking iodate as the example, the oxidation of a thiol may proceed between the two extremes (Hird & Yates, 1961).

Thus the ratio of thiol oxidized to oxidizing agent at the equivalence point will vary between 6 and 1, and this ratio can therefore be a guide to the reactions occurring. At low concentrations of thiol groups the ratios, obtained with gluten and thiolated gelatin, are between 3.7 and 4.8, values similar to those obtained with cysteine (Hird & Yates, 1961). However, as the concentration of

Table 6. *Formation of disulphide bonds on oxidation of thiolated gelatin*

Concentration of protein: 1 mg./ml.; molar ratio, thiol/oxidizing agent, 1/1. For other conditions see text. Results are expressed as μ moles/100 mg. of protein.

Time	Iodate			Bromate			Persulphate		
	-SH	-S-S-	Total S (-SH + -S-S-)	-SH	-S-S-	Total S (-SH + -S-S-)	-SH	-S-S-	Total S (-SH + -S-S-)
0	16.4	1.6	19.6	16.4	1.6	19.6	16.4	1.6	19.6
5 min.	0	6.5	13.0	—	—	—	—	—	—
24 hr.	—	—	—	6.5	5.5	17.5	0	6.5	13.0

Table 7. *Oxidation of thiolated gelatin*

Concentration of thiolated gelatin was as indicated. Quantities of iodate added were sufficient to oxidize approximately 50 and 75 % of the thiol groups, assuming a molar ratio, thiol/oxidizing agent, 6/1. Ratios were calculated from μ moles of thiol groups oxidized to μ moles of iodate added. Results are expressed as μ moles/100 mg. of protein.

Concn. of thiolated gelatin (%)									
0.1					2.5				
SH oxidized (%)	-SH (μ moles)	-S-S- (μ moles)	Total S (μ moles)	Thiol/oxidizing agent	SH oxidized (%)	-SH (μ moles)	-S-S- (μ moles)	Total S (μ moles)	Thiol/oxidizing agent
Initial	16.6	1.3	19.2	—	Initial	16.3	1.6	19.5	—
45.8	9.0	3.8	16.6	4.5	37.5	10.2	3.4	17.0	5.0
72.4	4.6	6.2	17.0	4.8	54.0	7.5	4.5	16.5	4.9

Concn. of thiolated gelatin (%)									
5.0					10				
SH oxidized (%)	-SH (μ moles)	-S-S- (μ moles)	Total S (μ moles)	Thiol/oxidizing agent	SH oxidized (%)	-SH (μ moles)	-S-S- (μ moles)	Total S (μ moles)	Thiol/oxidizing agent
Initial	16.6	2.4	21.4	—	Initial	16.4	1.9	20.2	—
51.2	8.1	5.7	19.5	6.0	43.3	9.3	5.0	19.3	5.9
74.8	4.2	7.2	18.6	5.9	69.5	5.0	7.5	20.0	6.1

thiol groups is increased, the ratio goes up, corresponding to reaction (1). In the previous work with cysteine and in the present work with proteins the major product is found to be the disulphide but there is the likelihood of the corresponding sulphinic acid and sulphonic acid being formed, as happens with cysteine. The increase in disulphide formation with concentration suggests that the proximity of thiol groups undergoing oxidation will determine the products formed. This further suggests that it is difficult to extrapolate from the present experiments to a complex system such as dough, in which these oxidizing agents are used as improvers.

A further point arises from the type of disulphide formed. It has been shown by Benesch & Benesch (1958) that the oxidation by ferricyanide of thiolated gelatin at a concentration of 0.2 % produces intramolecular disulphide bonds, and at a concentration of 5 % the formation of intermolecular disulphide bonds causes gelling. In the present work iodate has also been shown to bring about gel formation at concentrations of thiolated gelatin of 5 % and 10 %. An increase in the number of intermolecular disulphide bonds resulting from oxidation of thiol groups of dough by improvers would, of course, increase its strength but the mere removal of thiol groups by *N*-ethylmaleimide and other thiol-blocking reagents is also sufficient to cause similar rheological changes in dough (Goldstein, 1957; Bloksma, 1958; Mecham, 1959; Bloksma & Hlynka, 1960; Frater *et al.* 1960; Mecham, Sokol & Pence, 1960; Frater, Hird & Moss, 1961). The

changes in the strength of dough cannot therefore be wholly dependent on the formation of new intermolecular disulphide bonds, although these may make some contribution. It would seem, however, that the major effect of the removal of thiol groups by oxidizing agents is to be sought in the inhibition of exchange reactions involving such groups.

SUMMARY

1. The oxidation of the thiol groups of flour, reduced protein prepared from flour and thiolated gelatin has been studied.

2. Iodate, bromate and persulphate have been shown to oxidize thiol groups in the protein preparations. Iodate has been shown to oxidize at a faster rate than do bromate and persulphate.

3. When reduced gluten and thiolated gelatin were oxidized disulphide has been shown to be a product. When iodate oxidizes thiolated gelatin there is evidence that at lower concentrations of thiol groups oxidation products higher than disulphide are formed. At higher concentrations of thiol groups the product of oxidation is entirely the disulphide. Gelling occurs at these high concentrations.

4. The results are discussed in relation to the action of these oxidizing agents as improvers of the rheological properties of dough.

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A Direct Quantitative Paper Chromatography of Amino Acids and its Application to the Urinary Excretions of some Human Ethnic Groups

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The work of Dent & Harris (1951), Harris (1953), Harris & Warren (1953) and Datta & Harris (1953) has indicated the existence of characteristic patterns of urinary amino acid excretion in human and other mammalian species. As an item in the study of biochemical aspects of human racial differentiation it was planned to examine the excretions among physiologically normal members of ethnologically distinct human groups on Singapore Island and in the Federation of Malaya.

With so potentially extensive a programme it was proposed to employ direct transmission photometry ('minimum transmission' type) of ninhydrin-treated two-dimensional paper partition chromatograms for most of the assays. Established procedures were found to lack delicacy and accuracy, more in respect of colour development and photometry than of chromatography. A brief report of the preliminary work and details of the photometry refinements, which alone render the means of triplicate assays as reliable as those of some twelve replicates otherwise obtained, have been provided elsewhere (Lugg & McEvoy-Bowe, 1957, 1961; McEvoy-Bowe & Lugg, 1958, 1959). This article describes the chromatography, the closely controlled colour-development procedure (which, like the refined photometry, greatly reduces variability of results) and the preliminary treatment of urine samples, and records the results of the exploratory ethnic-group survey. Attention was directed principally to the excretion rates of glycine, taurine, β -aminosobutyric acid, alanine and glutamine, but those of cystine (determined polarographically) have also been included.

EXPERIMENTAL

Chromatography. Square sheets (20 cm. $\times$ 20 cm.) of Whatman no. 1 filter paper were used in two-dimensional ascending-front separations. Perforated at the corners, they were fitted usually 12 at a time with glass spacing sleeves on the rails of a stainless-steel frame resting in a trough of this alloy (cf. Datta, Dent & Harris, 1950), at the bottom of a glass tank (36.5 cm. $\times$ 26.5 cm. base and 24.5 cm. height; as supplied by Shandon Scientific Co. Ltd., London, W. 10) fitted with a glass sealing lid. Aluminium-alloy frames and troughs (as used by Datta *et al.* 1950) were found unsatisfactory. Climatic conditions made it necessary to control, thermostatically, the temperature of the tank enclosure (at $29.5 \pm 0.5^\circ$).

The initial spot was a 5.0 mm. disk with centre 4.5 cm. diagonally inward from a corner, applied as evenly as possible from an Agla (Burroughs Wellcome and Co. Ltd., London, N.W. 1) micrometer syringe, and dried with warm air from a hair-drier. The spot was briefly exposed to ammonia gas and excess blown away.

So-called 'single-phase' solvent systems, claimed in the literature to be advantageous, were used for each 'run'. They appear to function as conventional double-phase systems in association with sheet fibres (Martin, 1949). For the first run, which was stopped with the front approx. 1 cm. from the top, the solvent was prepared from water-saturated phenol by dissolving in it 10% (w/v) phenol, 0.04% (w/v) 8-hydroxyquinoline as heavy-metal chelating agent (Block, 1950), and 0.5% (w/v) 2:6-lutidine. With this solvent the sheets were used as supplied, as prior washing conferred no benefit. For the second run, stopped with the front almost at the top, the solvent (Block, Durrum & Zweig, 1955) was prepared by mixing 55 vol. of redistilled 2:6-lutidine with 25 vol. of 95% ethanol and 20 vol. of water. Diethylamine (1.5 ml.) was placed in the bottom of the tank, as direct addition to the solvent appreciably increased the 'background'.