POLAROGRAPHIC BEHAVIOR OF GLUTEN AND THIOSULFOGLUTEN[1]

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ABSTRACT

The protein wave of gluten and thiosulfogluten — gluten in which a part of the disulfide bonds was cleaved to thiosulfo groups (SSO_3^-) by sulfite — was estimated polarographically. The behavior of those protein waves was similar to those of other proteins in the experiment with various concentrations of proteins, ammonia, cobaltic ion, and cobaltous ion. The protein waves shown by thiosulfogluten were presumed to originate from the thiosulfo group, as the behavior in alkali and during sulfitolysis differed from that of gluten. The polarographic method was also found effective for the study of the thiosulfo group in gluten as well as for the study of disulfide bonds.

Since Brdicka found a polarographic catalytic reduction wave of a protein solution in 1933, many papers have been published on so-called

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"protein waves" (1). Some dealt with proteins in food materials such as egg (2), milk (3), and beef (4); others with the nature and origin of the waves (5,6).

As yet, the relation between the disulfide-sulfhydryl system of proteins and the protein wave (PW) has not been clarified satisfactorily. However, when the results of some workers (7,8,9) are considered, a very intimate relationship seems to exist.

If polarographic experiments are carried out under carefully controlled conditions, the PW's of gluten can be a sensitive indicator, at least comparatively, of a sulfhydryl-disulfide system of a flour, which has been regarded as playing an important role in the physical properties of dough. Because it is a simple and rapid procedure requiring only a small amount of sample, the polarographic method has an advantage over other analytical procedures.

Sullivan (10) introduced polarography in 1941, having detected a substance which showed an anodic wave from gluten treated with sodium hydroxide. Hintzer and DeLange (11) estimated sulfhydryl groups with polarographic protein waves (PPW's) in an electrolyte of ammoniacal buffer containing cobaltous chloride. However, application of this wave to such a quantitative determination is debatable, because the wave was also related to disulfide linkages and various conditions.

Recently Dōguchi (12) studied the effect of irradiation on wheat flour with the polarographic procedure. In this paper, the PPW's are related to the disulfide groups and thiosulfo groups of gluten.

Materials and Methods

Gluten Dispersion. The flour used was unbleached, improver-free, straight grade, commercially milled from a blend of Canadian hard red spring wheat. It had 12.5% protein and 0.52% ash on 13.5% moisture basis.

The gluten was washed out from the dough with distilled water, and dispersed in 0.01*N* acetic acid with a Waring Blendor at final concentration about 1.5%. The dispersion was centrifuged for 5 min. at 3,000 r.p.m. before and after heat-treatment for 3 min. at 95°C., and used as a sample.

Preparation of Thiosulfogluten. Thiosulfogluten was prepared with the following reaction mixture, with air bubbling into the medium at 30°C. for 3 hr. at pH 8.7: Gluten dispersion in 0.01*N* acetic acid, 50 ml. (containing 1.5% of protein); urea, 30 g.; sodium sulfite, 1 g.

In this thiosulfogluten, 51% of the original disulfide bond re-

mained intact. The rest of the bonds presumably were cleaved to thiosulfo groups.

Alkali Treatment. Sodium hydroxide, 1*N*, was added to the reaction medium up to the pH's indicated in Table IV (fifth experiment). The medium was kept at 50°C. for 1 hr. It was analyzed polarographically after being neutralized with *N*-hydrochloric acid.

Polarography. An electrolyte was prepared by mixing 1 ml. of protein dispersion in 0.4*M* urea with 1 ml. of buffer containing 2.4×10^{-6} mol of cobaltous chloride (Co++), or cobaltamine (hexamine cobaltic chloride) (Co+++), 0.4×10^{-3} mol of ammonium chloride, and 0.1×10^{-3} mol of ammonium hydroxide.

The protein dispersion was prepared by dilution of the original gluten dispersion or dialyzed thiosulfogluten from 3 mg.% to 62 mg.% with urea solution; final concentration, 0.4*M*.

Samples treated with sulfite were not dialyzed in later experiments (fourth and fifth; Tables III and IV). Sulfite and urea which came from the reaction mixture had a minute effect on the polarogram at diluted concentrations, but experiments were comparative in the same electrolyte.

A Yanagimoto (Japan) pen-recording polarograph (model PA-102) was used under the conditions shown in each experiment. The experimental temperature was 15°–20°C. and fixed during the same kind of experiment: t = 4.7~5.0 sec. per drop of mercury (mercury weight per drop was 2.7 mg.). Potentials are expressed as voltage against a mercury pool electrode. It was +0.033 volt against a saturated calomel electrode.

Experimental conditions were the same in all trials. Oxygen was eliminated from the electrolyte by bubbling oxygen-free hydrogen through the medium for 3 min.

Determination of Disulfide and Sulfhydryl Groups. The procedure outlined by Carter (13) was used with some modifications. Gluten dispersions, from 0.5 to 1.5%, which were $2 \times 10^{-3}M$ with respect to EDTA and 6*M* with respect to urea, were treated with 1.6 g. sodium sulfite per 100 ml. The sulfhydryl group released by this sulfitolysis was titrated amperometrically by Matsumoto and Hlynka (14) with 0.002*M* silver nitrate in an electrolyte containing 0.13*M* tris (tris hydroxymethyl aminomethane), 0.01*M* potassium chloride, 0.11*M* nitric acid, and 4*M* urea (final concentrations).

Determination of Reducing Substance Released by Alkali Treatment. Amperometric titration with 0.002*N* potassium iodate was used as described by Cunningham and Anderson (15), in an electrolyte of 1*N* acetic acid, with the aid of 2×10^{-3} mol of potassium iodide.

Results

PPW's of Original Gluten and Thiosulfogluten at Various Concentrations. Results (Fig. 1) are typical PW's, the first wave at −1.4 volt and the second at −1.65 volt against the mercury pool electrode. The waves increase in height with increasing protein concentrations.

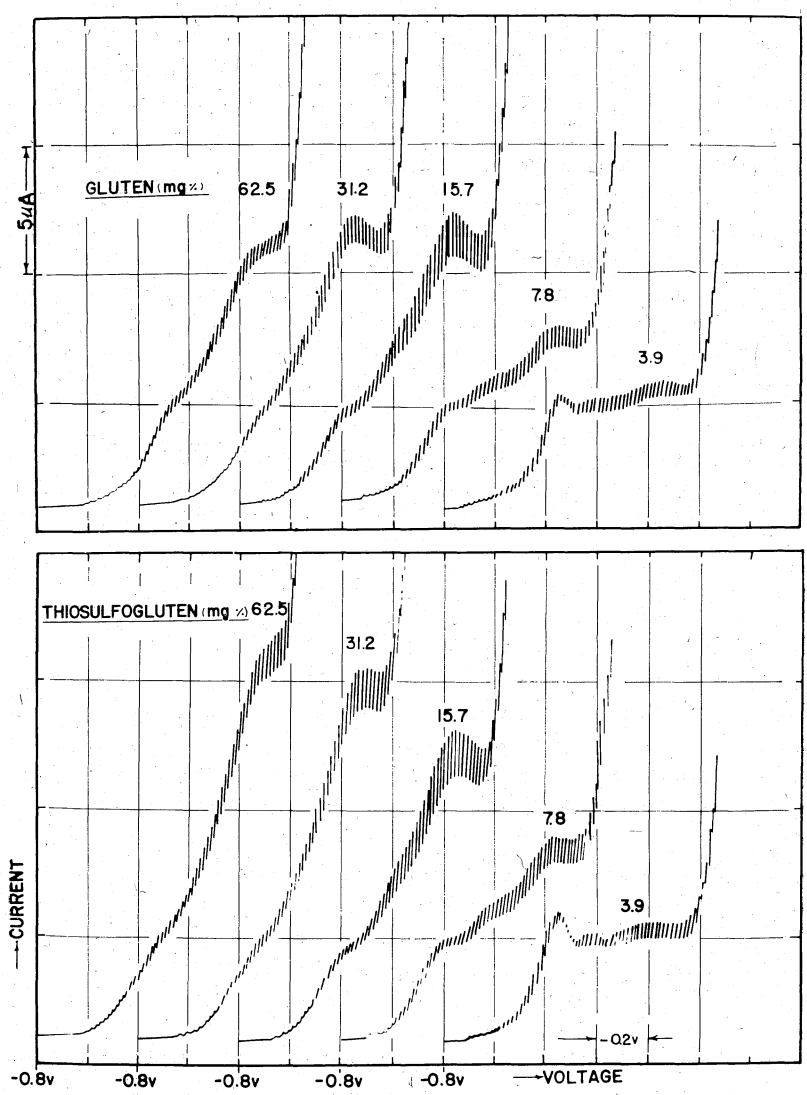

Fig. 1. Polarographic protein waves of various concentrations of gluten and thiosulfogluten. Electrolyte; $1.2 \times 10^{-3}M$ $CoCl_2$, $0.2N$ NH_4Cl, and $0.05N$ NH_4OH in $0.2M$ urea at pH 9.4. Protein concentration indicated in the figure. Potential was expressed against mercury pool electrode.

Results with cobaltamine were quite similar to those with cobaltous chloride and are not presented.

Stability of PPW's of Gluten and Thiosulfogluten in the Electrolyte.

TABLE I

EFFECT OF REST PERIOD IN ELECTROLYTE ON POLAROGRAPHIC WAVES OF GLUTEN AND THIOSULFOGLUTEN

TIME	HEIGHT OF PROTEIN WAVES			
	Original Gluten		Thiosulfogluten	
	I[a]	II[a]	I	II
min.	*μamps.*	*μamps.*	*μamps.*	*μamps.*
0	1.7	4.4	1.4	4.0
10	1.4	4.0	1.2	3.6
20	1.4	3.8	1.2	3.6
30	1.4	3.7	1.2	3.4
40	1.3	3.6	1.2	3.6
50	1.2	3.2	1.2	3.4
60	1.2	3.2	1.2	3.4

[a] I, the first protein wave at —1.40 volt. II, the second maximum protein wave at —1.65 volt. Electrolyte $1.2 \times 10^{-3}M$ $CoCl_2$, $0.2N$ NH_4Cl, and $0.05N$ NH_4OH in $0.2M$ urea. Protein concentration: original gluten, 15.4 mg.%; thiosulfogluten, 14.0 mg.%.

TABLE II

EFFECT OF EACH ELECTROLYTE CONCENTRATION ON THE POLAROGRAPHIC PROTEIN WAVES OF THESE TWO GLUTENS WITH COBALTOUS CHLORIDE OR COBALTAMINE[a]

	HEIGHT OF PROTEIN WAVES							
	Original Gluten				Thiosulfogluten			
	Co^{++}		Co^{+++}		Co^{++}		Co^{+++}	
	I	II	I	II	I	II	I	II
mol.	*μamps.*	*μamps.*	*μamps.*	*μamps.*	*μamps.*	*μamps.*	*μamps.*	*μamps.*
	Cobaltous chloride or cobaltamine							
0.5×10^{-3}	1.8	3.2	2.8	5.0	2.8	4.2	3.8	6.0
1.5×10^{-3}	3.2	7.6	5.2	14.4	4.4	10.2	6.2	16.2
2.0×10^{-3}	5.0	11.8	7.6	15.2	4.8	12.2	9.4	20.4
3.0×10^{-3}	4.2	12.6	10.6	17.0	5.0	14.2	11.2	28.0
	Ammonium hydroxide							
0.02	3.1	5.8	3.7	7.7	4.2	6.5	3.3	7.9
0.05	3.1	6.7	3.6	7.6	3.3	6.9	3.7	8.4
0.10	3.4	7.2	4.2	8.5	3.9	7.4	4.0	9.4
0.20	5.3	8.6	5.1	9.6	5.6	7.9	5.0	10.3
0.40	7.9	10.9	8.1	12.0	7.9	10.7	8.2	15.1
0.60	9.9	14.8	9.2	15.7	9.8	13.6	9.7	15.3
	Urea							
0.05	3.6	7.8			3.4	7.8		
0.10	3.7	7.8			3.4	7.9		
0.20	3.6	7.8			3.4	7.9		
0.30	3.6	7.9			3.4	7.9		
0.50	3.4	7.9			3.7	8.6		
1.00	3.9	7.7			3.8	8.9		

[a] Standard electrolyte: Same as Table I except for the component indicated in the table. Protein concentration: 15.4 mg.%. I, first wave at —1.40 volt; II, second wave at —1.65 volt.

Seven polarograms of these solutions were recorded at various times after the protein was added to the electrolyte in the cell (Table I). A definite decrease of the PW in 10 min. was followed by a gradual decrease. The PW of thiosulfogluten was more stable than that of the original gluten. Experiments were carried out, without a rest period,

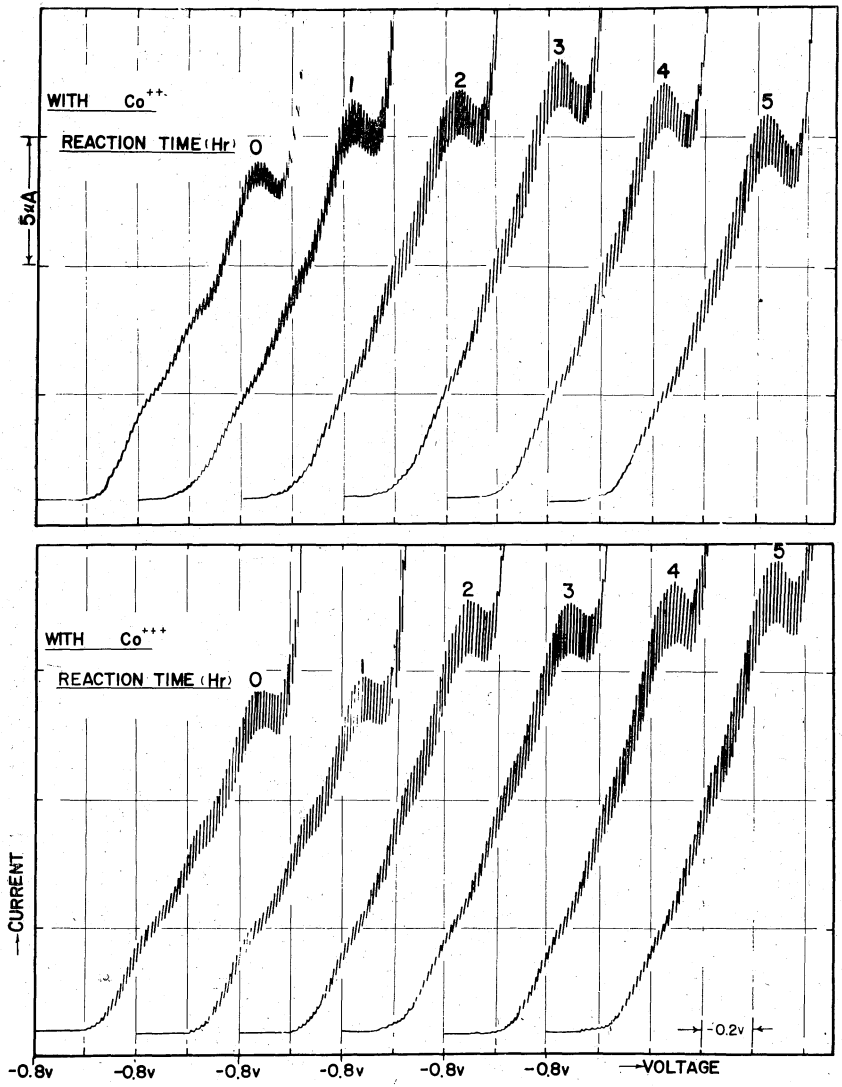

Fig. 2. Variation of polarographic protein waves during sulfide treatment of gluten. Conditions the same as shown in Fig. 1. Co^{++}: $CoCl_2$, Co^{+++}: $Co(NH_3)_6Cl_3$. Protein concentration: 15.6 mg.%.

as soon as the hydrogen gas stopped bubbling. Data were reproducible.

Effects of Cobaltous Chloride, Cobaltamine, Ammonium Hydroxide, and Urea on PPW's of Gluten and Thiosulfogluten. PW's increased in height with increasing amount of cobalt ion, ammonium hydroxide, and urea, whereas only a small change was observed in original gluten with urea (Table II).

Variation of PPW's during Sulfite Treatment of Gluten. The reaction of sulfite on gluten in the preparation of thiosulfogluten was followed at hourly intervals for 5 hr. with the polarograph (Fig. 2).

With hexamine cobaltic chloride (Co^{+++}), the second wave at −1.65 volt increases with time, whereas with cobaltous chloride (Co^{++}) wave height reaches a maximum at 3 hr. Equivalents of disulfide remaining and sulfhydryl groups during sulfite treatment are presented in Table III.

Effect of Alkali Treatment on PPW's of Thiosulfogluten and Original Gluten. Thiosulfogluten and original gluten were incubated, at various pH's, for 1 hr. at 50°C. They were analyzed polarographically after being neutralized with 1*N* hydrochloric acid. Results are in Fig. 3 and Table IV.

TABLE III

REMAINING DISULFIDE LINKAGE AND SULFHYDRYL GROUPS IN GLUTEN DURING SULFITE TREATMENT WITH AERATION [a]

REACTION TIME	REMAINING -SS- [b]	-SH CONTENT [c]
hr.	$\times 10^{-5}$ *eq./g. N*	$\times 10^{-5}$ *eq./g. N*
Original	94	2.0
0.5	78	3.1
1	78	3.1
2	56	4.8
3	48	3.8
4	34	6.2
5	6	2.5

[a] Composition of reaction mixture: Gluten dispersion in 0.01*N* acetic acid (1.5% protein), 50 ml.; urea, 30 g.; sodium sulfite, 1 g.; under bubbling air at 30°C.
[b, c] See "Materials and Methods" section.

TABLE IV

REMAINING DISULFIDE BOND AND REDUCING SUBSTANCE RELEASED BY ALKALI TREATMENT OF GLUTEN AND THIOSULFOGLUTEN

pH	ORIGINAL GLUTEN		THIOSULFOGLUTEN	
	Remaining -SS- [a]	Reducing Substance [b]	Remaining -SS- [a]	Reducing Substance [b]
	$\times 10^{-5}$ *eq./g. N*		$\times 10^{-5}$ *eq./g. N*	
9.0	75.4	6.8	32.8	17.1
10.3	71.0	7.5	30.2	20.6
11.3	70.0	7.5	27.6	23.0
11.8	23.8	18.0	22.0	29.1
13.5	8.8	20.0	21.4	39.5

[a, b] See "Materials and Methods" section.

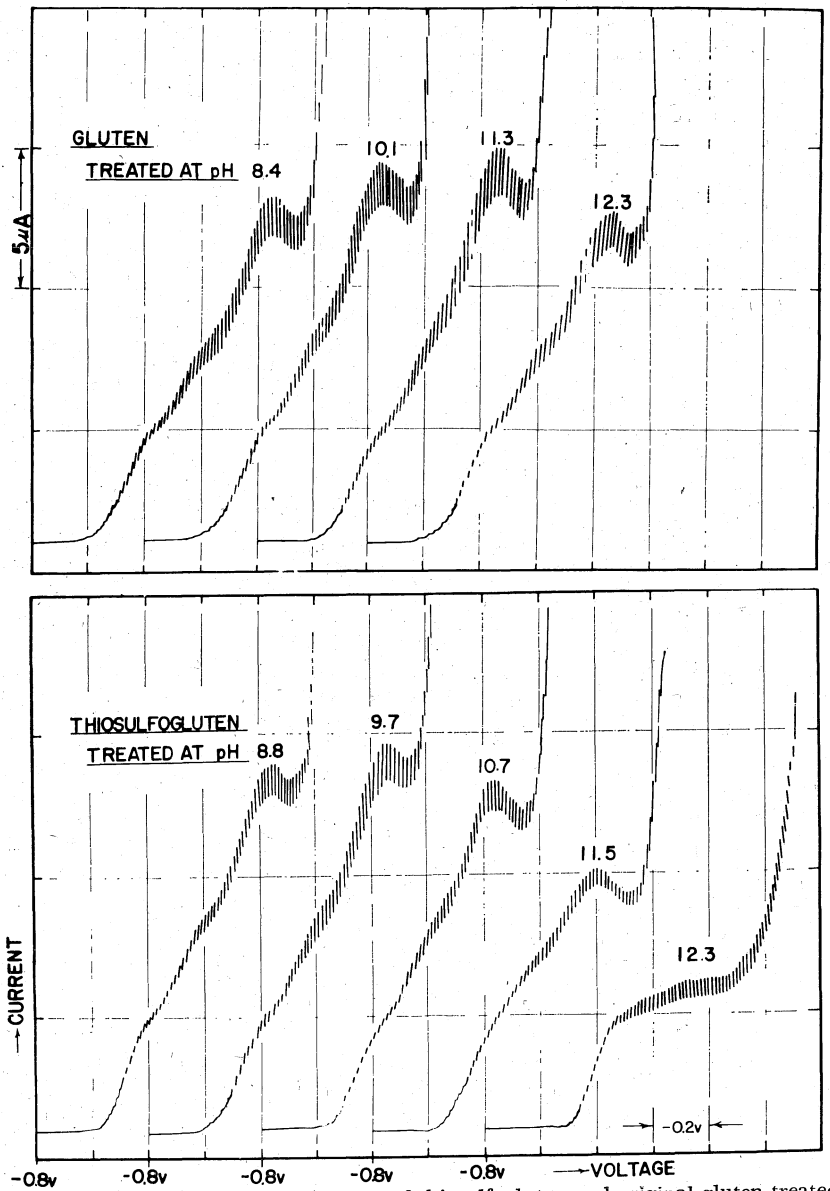

Fig. 3. Polarographic protein waves of thiosulfogluten and original gluten treated at various pH's. Conditions the same as shown in Fig. 1. Protein concentration: gluten, 15.3 mg.%; thiosulfogluten, 13.0 mg.%.

Protein waves of the original gluten were enhanced from pH 8.4 to pH 11.3 and then decreased, whereas waves of thiosulfogluten were gradually suppressed from pH 9.7 to 12.3.

Discussion

That the PPW's require the presence of a disulfide bond in the molecule for generation, at least indirectly, is supported by the following:

1. Gelatin and beta-casein, lacking cystine, exhibit no PW (15), but thiolated gelatin, in which thiol groups are introduced, showed the wave as shown by Hata (7).

2. The height of PW's, especially the second, of various proteins had a parallel relation with cystine content, as shown by Ito (8,16) and Maeno *et al.* (9).

In other reports, the wave was influenced by various factors such as free amino groups (6), molecular size and configuration (2), free amino acids (17), and the condition of electrolysis.

Thus, an explanation of the catalytic PW's requires careful consideration of factors other than the sulfhydryl-disulfide system. In this experiment, the protein sample was free from small molecular compounds in wheat flour.

The first experiment indicated that these samples gave catalytic waves similar to those of various proteins previously reported; wave height was related to protein concentration in a manner analogous to a Langmuir absorption isotherm (Table I).

The effect of other electrolyte components (Table II) indicated that these findings were in good agreement with those published by Millar (18), Maeno *et al.* (3), Hata (7), Obara and Ogasawara (4), and Dōguchi and Okada (12).

Urea was a specific component in this study. Table II indicates no marked effect on the PW at the concentration used. Thus it can be disregarded.

The characteristic change of the second wave during sulfitolysis of gluten was possibly brought about by one or more of the three following factors.

1. Negatively charged thiosulfite groups introduced in the molecule of gluten, as indicated in equation 1, may change the catalytic reduction properties over the surface of the electrode.

2. The effect of sulfhydryl groups, also indicated in equation 1, cannot be disregarded, though they were oxidized to a minute quantity during aeration (Table III).

$$\begin{array}{c} \overset{\downarrow}{\text{PSSP}} + \text{SO}_3^{=} \xrightarrow{\quad\text{O}\quad} \overset{\downarrow}{\text{PS}^-} + \text{PSSO}_3^- \end{array} \qquad (1)$$

3. There is a decrease of molecular size through cleavage of disulfide linkages, as reported by Nielsen, Babcock, and Senti (19) by the sedi-

mentation procedure. Millar (18) observed that the large aggregate size depressed the protein current by interfering with free diffusion of cobalt-ammonia complexes. A similar effect of molecular size was observed by Hata (2) and by Obara and Ogasawara (20) with ovalbumin and beef protein digested by trypsin and pepsin, respectively, at least for the first 0.5 hr.

All of these three factors should be considered, because the samples contained disulfide bonds and thiosulfo-sulfhydryl groups even at the end of the reaction time (Table III).

An increase of the PW during reaction time was observed with Co+++; with Co++ a maximum of the wave was observed at 3 hr. The former phenomenon, which was different from that observed in digestion with enzyme by Hata (2) and Obara and Ogasawara (20), indicated that the origin of the PW in thiosulfogluten may be specific.

The PW of thiosulfogluten presumably originated from additional thiosulfo groups over the remaining disulfide linkages, as shown by the following results.

1. The height of the PW of thiosulfogluten with Co+++ increased even though disulfide bonds and sulfhydryl groups decreased during sulfite treatment (Table III).

2. The catalytic reduction waves with cobaltic and cobaltous ions were also observed by the authors at −1.65 volt with thiosulfocysteine and thiosulfopolyvinyl acetate which had the thiosulfo group in the molecule (unpublished data).

3. The PW of thiosulfogluten also can be distinguished from the normal PW by its behavior in alkali. Alkali treatment increased the height of the second wave of original gluten from pH 8.4 to 11.3, whereas it decreased the height of the second wave of thiosulfogluten from pH 9.7 to 11.3 (Fig. 3). The former increase may be brought about by alkali denaturation, as Hata and Matsushita (21) observed with ovalbumin, and the latter decrease seemed to come from the release of reducing substances such as thiosulfite (unpublished data) from the molecule (Table IV).

A peculiar suppression of the PW was observed at pH 12.3 even for the original gluten, and at pH 10.7 for the thiosulfogluten. The former phenomenon may be due to the disappearance of disulfide linkages (Table IV).

The occurrence of PW's caused by thiosulfo groups may be explained by the following equations:

$$RSSO_3^- + 2e \longrightarrow RS^- + SO_3^= \quad (2)$$

$$RS^- + H^+ \longrightarrow RSH \quad (3)$$

$$RSH + e \longrightarrow RS^- + H \quad (4)$$

After the reduction of thiosulfo groups to ionized thiol on the electrode, according to equation 2, the protein wave may be produced by the same mechanism as that of sulfhydryl and disulfide (equations 3 and 4). The authors expected to obtain different behavior of thiosulfogluten from that of the original gluten in the second and third experiments. Attempts were also made in these experiments with Co^{++} and Co^{+++}, but no specific qualitative difference was detected. Further studies are required to clarify the properties and origin of PW's of thiosulfogluten.

In conclusion, these results indicated that the polarographic behavior of gluten and thiosulfogluten can be as sensitive and effective an indicator in the study of thiosulfo groups as it had been in the study of disulfide groups of gluten and various proteins.

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