6. Gros, Audrey T., and Feuge, R. O., J. Am. Oil Chemists' Soc., *31*, 377–383 (1954).
7. Hellman, N. N., Zobel, H. F., and Senti, F. R., J. Am. Oil Chemists' Soc., *32*, 110–114 (1955).
8. Herting, D. C., Ames, S. R., Koukides, M., and Harris, P. L., J. Nutrition, *57*, 369–387 (1955).
9. Jones, E. P., Dutton, H. J., and Cowan, J. C., J. Am. Oil Chemists' Soc., *30*, 609–610 (1953).
10. Lancaster, E. B., Beal, R. E., Jones, E. P., Dutton, H. J., Evans, C. D., and Cowan, J. C., J. Am. Oil Chemists' Soc., *32*, 9–13 (1955).
11. Mattson, F. H., Alexander, J. C., Baur, F. J., and Reller, H. H., J. Nutrition, *59*, 277–285 (1956).

[Received June 11, 1958]

Carboxymethylated Soybean Protein[1]

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TWO MAJOR PROBLEMS in the industrial utilization of vegetable proteins involve their dispersibility at a neutral pH and stability to putrefaction. Soybean protein can be modified during its isolation from hexane-extracted flakes to give a product of improved solubility at a neutral pH by treating the meal extract with alkali at pH 11–12 in the presence of sulfite and lime, heating for 0.5 to 2 hrs. at 50°–70°C., and clarifying the dispersion prior to precipitating with acid at pH 4.5–4.8 (2, 5, 12). Besides bleaching, the sulfite reduces the disulfide bonds, which are then readily attacked by the alkali (7, 9), and eliminates the possibility of decreased solubility because of polymerization through disulfide bond formation. At pH 11–12 phytate is dissociated and insolubilized by the lime, hence it may be removed by clarification (5). Removal of the phytate has been shown to improve solubility of the protein (5, 8). In addition, the mild hydrolytic treatment converts a portion of the nonpeptide amide groups of asparagine and glutamine to carboxyl groups; this allows dispersion of the protein at a lower pH.

Ten per cent of the total nitrogen of isolated soybean protein has been shown to be nonpeptide amide nitrogen (6). Calculations based on nonpeptide amide nitrogen and the reported (11) dicarboxylic acid content of acid-precipitated soybean protein indicate that about 50% exists as asparagine and glutamine residues. That increased solubility of proteins at lower pH values can be achieved by hydrolysis of nonpeptide amide groups to carboxylic acids is shown by experiments on zein (4). Zein, which is insoluble in water below pH 11, was rendered soluble at pH 6–7 when about 40% of the nonpeptide amide groups was hydrolyzed to carboxylic acids.

This paper describes the reaction of soybean protein with sodium chloroacetate to produce a protein derivative containing added carboxyl groups, resulting in increased solubility of pH 6–7 and in resistance to putrefaction; dispersions of the derivative do not gel on adding formaldehyde. The purpose of this study was to determine whether soybean protein would react with sodium chloroacetate in an alkaline medium sufficiently mild not to expect extensive alkali degradation of the protein, to determine the extent of the reaction, if any, and to determine whether addition of carboxymethyl groups to the protein would allow increased solubility at a lower pH than the starting protein does. No attempt was made to carry out an extensive study of the reaction conditions to produce a series of proteins with varying carboxymethyl content for study and evaluation. Such a study should be made on the wet protein curd obtained during its manufacture to avoid drying twice.

[1] Presented at fall meeting, American Oil Chemists' Society, Cincinnati, O., September 30–October 2, 1957.
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Preparation of Carboxymethylated Protein

Materials Used. Protein A was commercial Alpha Protein purchased from the Glidden Company.[3] *Analyses.* P, 0.19%; total N, 15.0; amino nitrogen (Van Slyke), 0.60%; ultracentrifugal analysis: S_{20}, 40% at 1.10, 60% at 3.93.

Protein B was prepared in the pilot plant from undenatured hexane-extracted flakes by extracting with lime at pH 9.6 in the presence of 0.1% sodium sulfite at 50°C. The extract was clarified by centrifugation, and the protein was precipitated from the clarified extract by adjusting the pH to 4.2 with sulfur dioxide. *Analyses.* P, 0.79%; total N, 15.5%; amino nitrogen (Van Slyke), 0.63%; neut. equiv. to pH 9.5, 1430 (dry basis); ultracentrifugal analysis: S_{20}, 2.41.

Reaction with Sodium Chloroacetate. Data for the reaction and isolation of two preparations are shown in Table I. A control for Preparation 1 is included in which the protein was carried through all of the reaction steps without the addition of sodium chloroacetate. No control was carried out for Preparation 2 because this reaction was run to compare the reacted with the unreacted protein.

TABLE I

Preparation and Analyses of Carboxymethylated Soybean Protein

Preparation steps and analyses	Preparation 1	Control for 1	Preparation 2
Preparation, 100 g. protein:			
Source of protein	Protein A	Protein A	Protein B
pH of dispersion	10	10	10
Protein conc., %	14	14	12.5
$ClCH_2COONa$, moles	0.1875	0	0.224
Temperature, °C.	70	70	50
Time, hrs.	5.5	5.5	10
NaOH used, moles[a]	0.139	0	0.150
pH of min. soly.	3.1	4.5	3.1
Pptd. yield, %	86	89	90
Dialysis loss, %	12	12	10
Analyses:[b]			
Total N, %	13.9	15.0	14.1
Amino N, %	0.07	0.60	0.08
$-CH_2COOH$, %[c]	7.3	0	9.0
Neut. equiv. to pH 9.5	590	1250	830
Ultracentrifugal analysis, S_{20}	1.35	....	2.59

[a] Moles of alkali required to maintain pH 10 indicate chloroacetate reacted.
[b] Analyses on dialyzed product, dry basis.
[c] Calcd. from difference in N content.

[3] Mention of this company and its products does not constitute preference by the U. S. Department of Agriculture over similar products manufactured by other companies.

The reaction procedure was carried on thus: 100 g. of soybean protein were slurried in water containing 4.0 g. sodium hydroxide. The dispersion was then adjusted to pH 10 by adding a measured amount of 10% sodium hydroxide. Approximately 2.5 molar solution of sodium chloroacetate was prepared by neutralizing chloroacetic acid with sodium hydroxide. The temperature was not allowed to exceed 20°C. during the neutralization step to avoid hydrolysis of chloroacetate to glycolate. The protein dispersion was contained in an open vessel placed in a water bath for temperature control and was stirred with an electric stirrer. The solution of sodium chloroacetate was added, and the pH of the reaction mixture was maintained at 10–11 by the periodic addition of 3.37 *N* sodium hydroxide. The consumption of alkali afforded a means of following the progress of the reaction (Figure 1).

Carboxymethylated protein (CBM-protein) was recovered from the reaction mixture by adjusting the pH to 3.1 with dilute hydrochloric acid. The pH of minimum solubility of CBM-protein was found by precipitating at different pH values as plotted in Figure 2.

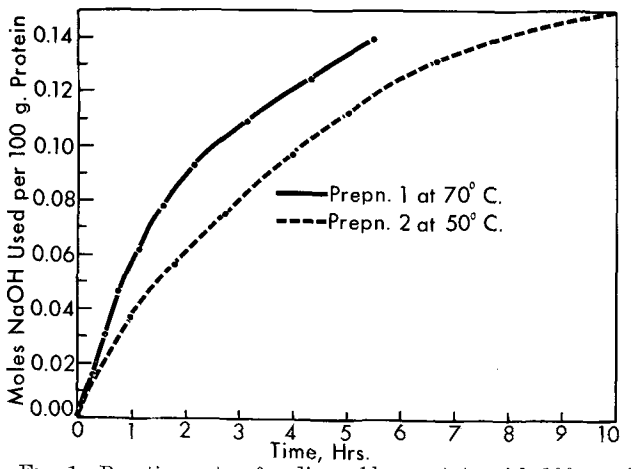

FIG. 1. Reaction rate of sodium chloroacetate with 100 g. of soybean protein dispersed in 700–800 ml. of water at pH 10 as determined by the amount of sodium hydroxide to maintain a constant pH: Preparation 1, 0.187 mole of chloroacetate used; Preparation 2, 0.224 mole of chloroacetate used.

The precipitated curd was removed by centrifugation, twice resuspended in distilled water, and recentrifuged to remove salts. The wet curd, containing 68% moisture, was dried in an air-draft oven at 50°C. to about 10% moisture. During drying the lumps were periodically crumbled by hand to give a pulverized, friable product.

A 20-g. sample of the dried product was dispersed in water at pH 6.6 with sodium hydroxide and dialyzed three days against frequent changes of distilled water. After dialysis the product was precipitated by adjusting the pH to 3.1, and the dialyzed product was used for the analyses reported in Table I. Variations in the two reaction procedures were made in quantities of chloroacetate, temperatures, time, and protein concentrations in the reaction mixtures.

Discussion of the Reaction with Sodium Chloroacetate

Because of the possibility of hydrolyzing chloroacetate to glycolate under the reaction conditions, a control was run for sodium chloroacetate at pH 11 with conditions identical to those of Preparation 1 (Table I) except that protein was not added. The amount of alkali required to maintain a constant pH indicated that only 2% of the reagent was lost through hydrolysis.

Figure 1 indicates that both proteins reacted readily with sodium chloroacetate. Based on calculations from the amount of alkali required to maintain a constant pH, only 74% of the total chloroacetate in the reaction mixture of Preparation 1 and only 67% of that present in Preparation 2 were consumed. Slopes of the two curves, especially Preparation 1 run at the higher temperature, indicate that a greater percentage of the reagent would have been consumed had the reactions been run for a longer time.

Analyses of free-amino groups by the Van Slyke method (Table I) indicated that 88% and 87% of the original free-amino groups reacted with chloroacetate ions in Preparations 1 and 2, respectively. Assuming that one chloroacetate reacted with each free-amino group, only 0.039 mole of chloroacetate per 100 g. of protein is accounted for, whereas calculations based on the difference in nitrogen analyses and the amount of sodium hydroxide used during the reaction indicate that 0.14 and 0.15 moles of chloroacetate reacted with each 100 g. of protein in Preparations 1 and 2, respectively. Biddison (1) has reported that two carboxymethyl groups are added to each free-amino group and guanidino group when sodium chloroacetate is reacted with blood globin. Sulfhydryl groups in the globin molecule also react; however the number of these groups remaining in an alkali-sulfite treated protein is probably insignificant and is not considered here.

Amino nitrogen analyses indicated that proteins A and B contained about 0.045 mole of free-amino groups ($-NH_2$) per 100 g.; reported arginine analysis (11) of isolated soybean protein indicates 0.033 mole of guanidino groups [$-NH-C(=NH)-NH_2$]. If two carboxymethyl groups were added to each of these functional groups, a total of 0.16 mole of chloroacetate would react with 100 g. to give a protein derivative with a carboxymethyl content of about 9.4%. Preparation 1 contained 7.3% carboxymethyl

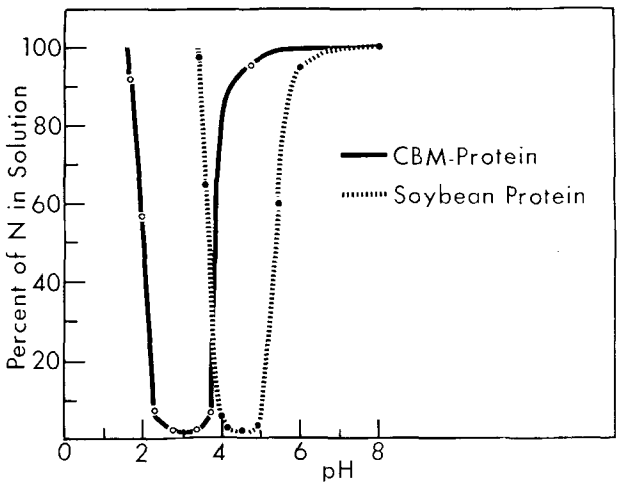

FIG. 2. Shift in pH of minimum solubility on carboxymethylated soybean protein (CBM-protein): solid line is pH solubility curve for Preparation 1 with 7.3% carboxymethyl content; broken line is pH solubility curve for the unreacted soybean protein designated as protein A.

groups, and Preparation 2 contained 9.0%. Because only 87% of the free-amino groups in Preparation 2 reacted and because the reaction had not stopped (Figure 1), it appears that other functional groups may be involved, possibly hydroxyl groups of serine, threonine, and tyrosine. The secondary amino groups of histidine and tryptophan could also be involved. Determination of the functional groups involved in the carboxymethylation of proteins with sodium chloroacetate is beyond the scope of the present investigations. Further study is also required to establish the maximum carboxymethyl content attainable by this reaction.

An attempt was made to react soybean protein dispersed in potassium carbonate solution at pH 8 with sodium chloroacetate. After heating at 50°C. for 8 hrs., there was neither a change in pH nor liberation of carbon dioxide, findings which indicated that no reaction had occurred. Nitrogen analyses of the isolated product also indicated that no reaction had occurred.

Properties of Carboxymethylated Protein

One of the most striking properties of CBM-soybean protein is its resistance to putrefaction. Ammonical dispersions stood in open test tubes in the laboratory for three months, with periodic addition of water to compensate for evaporation, without any sign of putrefaction. Dispersions of unreacted protein under these conditions became putrid within 48 hrs. Mold colonies did appear on the surface of CBM-protein dispersions after one or two months. Inoculation of CBM-protein dispersions with putrefying protein also failed to induce putrefaction. To determine whether the presence of unreacted chloroacetate was acting as a preservative, a dispersion of 50–50 mixture of CBM-protein and unreacted protein was allowed to stand in an open beaker. The mixture was putrid within three days. In another test, sodium chloroacetate was added to a dispersion of untreated protein which putrefied in the same time as the control without added chloroacetate.

Sedimentation constants were taken before and after reacting the proteins with chloroacetate. In Preparation 2, reacted at 50°C., there was substantially no change, indicating no appreciable change in molecular size other than a slight increase of S_{20} from 2.41 to 2.59, which would be expected from adding carboxymethyl groups. Protein A showed two peaks at S_{20} 1.10 and 3.93 before reaction and only one at S_{20} 1.35 afterward. Alkali degradation of the protein during the reaction at 70°C. for 5.5 hrs. at pH 10–11 is the most plausible explanation for the apparent reduction in molecular weight even though amino nitrogen analysis did not indicate hydrolysis.

Figure 2 indicates that the pH of minimum solubility is shifted 1.4 units toward the acid side after carboxymethylation. Titration of Preparation 1 to pH 9.5 required 0.17 mole (neut. equiv., 590) of sodium hydroxide per 100 g. while its control protein required 0.08 mole (neut. equiv., 1250); 0.12 mole (neut. equiv., 830) was required for Preparation 2 while the unreacted protein required 0.07 mole (neut. equiv., 1430). A clear dispersion containing 10% of Preparation 1 was obtained at pH 5.5 with sodium hydroxide while a pH of 7.5 was required for the unreacted protein. A 20% dispersion of Preparation 2 was obtained at pH 6.6 with sodium hydroxide whereas the maximum concentration obtained with the unreacted protein without gelation was 15%, which required a pH of 10. Dispersions of the modified proteins exhibited a slightly lower viscosity than those made from the unmodified proteins.

Alkaline dispersions of CBM-protein did not gel on the addition of formaldehyde as did unmodified protein dispersions. For example, a 15% dispersion of Preparation 1, which contained 0.75% formalin, failed to gel on standing for three months whereas the unreacted protein dispersion gelled within 1 hr.

The CBM-soybean protein was readily hydrolyzed by papain. Approximately one-half of the nitrogen was nonprecipitable after digesting for 3 hrs. at room temperature with this enzyme. The adhesive strength of the CBM-protein was slightly less than that of unreacted proteins when measured by the wax pick test (3). Synthetic latex emulsion paints formulated with CBM-protein had viscosities of 90–100 centipoises compared to 70 centipoises for paints formulated with unreacted protein (10). The addition of formalin to the CBM-protein dispersions used in formulating the paints failed to affect the viscosity and stability of the paints over a three-month observation period. Inoculation of the emulsion paints with a putrefying protein solution also failed to affect the viscosity and stability over a three-month period.

No tests were made on the toxicity of the CBM-soybean protein. However carboxymethylated globin has been repeatedly administered to animals without any evidence of toxicity (1), a finding which indicates that a low order of toxicity might be expected for the soybean protein derivative.

Summary

Soybean protein in aqueous alkaline dispersions at pH 10–11 reacts readily with sodium chloroacetate at temperatures of 50–70°C. to give a protein derivative containing 7–9% carboxymethyl groups. Ultracentrifugal measurements indicate no change in molecular size of the protein when the reaction is carried out at 50°C. but lowered molecular weight at 70°C. The reaction with sodium chloroacetate lowers the pH of minimum solubility from pH 4.5–3.1 and renders the protein more soluble at a neutral pH. Dispersions of carboxymethylated proteins exhibit resistance to putrefaction and do not gel on the addition of formaldehyde.

Acknowledgment

We are indebted to Glenn E. Babcock of this laboratory for the ultracentrifugal analyses.

REFERENCES

1. Biddison, E. A. (Cutter Laboratories), U. S. 2,719,837 (1955).
2. Cone, C. N., and Brown, E. D. (The Glidden Company), U. S. 1,955,375 (1934).
3. Kirkpatrick, W. A., Paper Trade J., *109*, No. 12, 1–4 (1939).
4. McKinney, L. L., and Johnsen, V. L., Trans. Ill. State Acad. Sci., *50*, 90–95 (1957).
5. McKinney, L. L., Sollars, W. F., and Setzkorn, E. A., J. Biol. Chem., *178*, 117–132 (1949).
6. Mecham, D. L., and Olcott, H. S., Ind. Eng. Chem., *39*, 1023–1027 (1947).
7. Nicolet, B. H., J. Am. Chem. Soc., *53*, 3066–3072 (1932).
8. Smith, A. K., and Rackis, J. J., J. Am. Chem. Soc., *79*, 633–637 (1957).
9. Swan, J. M., Nature, *179*, 965 (1957).
10. Uhing, E. H., and McKinney, L. L. (U. S. Dept. of Agr.), U. S. 2,788,336 (1957).
11. VanEtten, C. H., Hubbard, J. E., Mallan, J. M., Smith, A. K., and Blessin, C., Abst. Papers, 134th Meeting, Am. Chem. Soc., Chicago, Ill., Sept. 7–11, 1958.
12. Wahlforss, E., and Satosky, J. A. (The Glidden Company), U. S. 2,233,439 (1941).

[Received October 22, 1957]