Chemical and Physical Properties of a 7S Protein in Soybean Globulins[1]

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ABSTRACT

A 7S protein of soybean globulins, pure ultracentrifugally, electrophoretically, and chromatographically on hydroxylapatite and Sephadex G-200, was found to have molecular weight of 180,000 to 210,000 by four separate methods based on different principles. Molecular weights were 193,000 from sedimentation ($S^0_{20,w} = 7.92 \times 10^{-13}$ sec.) and viscosity ($[\eta] = 0.0638$ dl/g.) according to Scheraga-Mandelkern's formula; 186,000 from the Archibald method; 180,000 from the Yphantis procedure; and 210,000 from gel filtration with Sephadex G-200 by the method of Siegel and Monty. The integral numbers of amino acid residues per molecule calculated on the basis of 180,000 were as follows; Try_3, Lys_{86}, His_{19}, Arg_{91}, Asp_{191}, Thr_{43}, Ser_{116}, Glu_{251}, Pro_{71}, Gly_{68}, Ala_{75}, Val_{78}, Met_3, $Ileu_{88}$, Leu_{141}, Tyr_{36}, Phe_{81}, CyS_4, amide-$ammonia_{181}$, $mannose_{38}$, and $glucosamine_{12}$. The numbers of N-terminal residues of this 7S protein to the nearest integer were found to be Asp_1, Ala_1, Gly_1, Val_1, Ser_2, Tyr_1, Glu_1, and Leu (Ileu)$_1$.

In previous papers (1,2) it was reported that a 7S globulin had been prepared which was pure ultracentrifugally, electrophoretically, and chromatographically on hydroxylapatite and Sephadex columns.

This paper deals with some chemical and physical properties, molecular weight estimations by various methods, N-terminal amino acid compositions, and amino acid compositions, in the purified 7S protein.

MATERIALS AND METHODS

Materials

The sample of purified 7S protein was prepared from soybean globulins by the methods described in previous papers (1,3). Proteins for the determination of molecular weight (MW) by gel filtration were obtained as follows: gamma-globulin (bovine, fraction II) from Tokyo Kasei Kogyo Co., Tokyo, Japan; catalase (bovine liver) from Sigma Chemical Co., St. Louis, Mo.; fibrinogen (bovine) from Armour Pharmaceutical Co., Kankakee, Ill.; albumin (bovine, fraction V) from Nutritional Biochemicals Co., Cleveland, Ohio; and hemoglobin (bovine) from Koso Chemical Co., Tokyo.

Potassium phosphate-sodium chloride buffer (0.035*M* potassium phosphate buffer, 0.4*M* sodium chloride containing 0.01*M* 2-mercaptoethanol, pH 7.60, 0.5 ionic strength (4)) was used as the standard.

Physical Analyses

Sedimentation analysis was performed with a Hitachi UCA-1 centrifuge at 55,430 r.p.m. Routine assay runs were made in standard buffer at 20°C. Viscosity measurements were made with an Ostwald viscometer at 20°C. and standard buffer.

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[1]Contribution from Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan; Part V of Studies on Soybean Proteins. This paper is part of a presentation at the 52nd Annual Meeting, Los Angeles, Calif., April 1967.

Chemical Analyses

Nitrogen determinations were carried out by the micro-Kjeldahl method. Protein concentration was routinely determined by light absorption at 280 mμ with a Hitachi P-1 spectrophotometer, and turbidity at 420 mμ with a Klett-Summerson photoelectric colorimeter (5). Phosphorus content was determined by the methods of Allen (6) and Nakamura (7). Ash content was measured by the modified method of the Japan Pharmacopeia.

The phenol-sulfuric acid method according to Dubois *et al.* (8) and the Elson-Morgan method (9) were used for determining total carbohydrate and hexosamine content, respectively. Uronic acid was estimated by the reactions of naphthoresorcinol (10) and carbazol sulfuric acid (11).

Molecular Weight

MW of the 7S protein was determined by the following four methods:

Gel Filtration. Recently, Siegel and Monty (12) indicated that the elution position of a protein upon chromatography with Sephadex G-200 was not correlated with MW but was a function of the Stokes radius (molecular radius). Applying a Stokes radius measured by the chromatographic method and a sedimentation coefficient determined by analytical ultracentrifugation to equation 1, they estimated the MW of a protein.

$$M = 6 \cdot \pi \cdot \eta \cdot N \cdot a \cdot s/(1 - \bar{V} \cdot \rho) \quad (1)$$

where M = molecular weight; a = Stokes radius; s = sedimentation coefficient; $\bar{V}$ = partial specific volume; η = viscosity of medium; ρ = density of medium; and N = Avogadro's number (6.023×10^{23}).

Albumin, gamma-globulin, fibrinogen, and catalase were assayed by light absorption at 280 mμ, and hemoglobin at 415 mμ. Twenty milligrams of each protein dissolved in 2 ml. of the standard buffer was chromatographed on a Sephadex G-200 column (2.5 × 55 cm.) with standard buffer. Stokes radii of these standard proteins were calculated from their MW's in the literature (12).

The gel filtration data of Stokes radius were estimated by the method of Laurent and Killander (13).

Intrinsic Viscosity and Sedimentation Coefficient. The second method used to determine the MW of the 7S protein was that of Scheraga and Mandelkern (14). The MW was calculated from their formula (15),

$$M = 4{,}690 \cdot [S_{20,w}]^{3/2} \cdot [\eta]^{1/2} / (1 - \bar{V}\rho)^{3/2} \quad (2)$$

In equation 2, a value of β, 2.16×10^6, was used and $[\eta]$ is intrinsic viscosity.

Sedimentation Equilibrium. MW was determined by sedimentation equilibrium by two methods:

1. The Archibald method (16) is the approach to the sedimentation equilibrium method; it was carried out in a 13-mm., 4° sector cell at 12,290 r.p.m. and 16.8°C. with 0.20% 7S protein dissolved in standard buffer. The apparent MW at the meniscus was calculated according to the method described by Schachman (17, p. 70).

2. The Yphantis method (18) is the sedimentation equilibrium method; it uses a multichannel cell. The MW was calculated from

$$M = 1/\bar{r} \cdot 1/C_0 \cdot (dC/dr)_{r=\bar{r}} \cdot RT/\omega^2 \cdot 1/(1 - \bar{V}\rho) \quad (3)$$

where r = distance from center of rotation; C_0 = original concentration of protein which is determined with a synthetic boundary cell; ω = angular velocity; T = absolute temperature, and R = gas constant (8.314×10^7 ergs/mole/degree). The Yphantis procedure was conducted with a 12-mm. eight-channel cell at 10,490 r.p.m. and 11.6°C. in standard buffer.

Hydrolysis and Amino Acid Analysis

The protein samples were hydrolyzed *in vacuo* for 22, 44, and 66 hr. at 110°C. with 6*N* hydrochloric acid. Amino acid analysis was performed with a Hitachi KLA-2 automatic amino acid analyzer. For determination of tryptophan and methionine, hydrolysis was carried out separately for 8 hr. at 120°C. with 5*N* baryta and for 6 hr. at 110°C. with 3.5*N* hydrochloric acid, respectively.

For cystine analysis, the protein oxidized with a mixture of 88% formic acid and 30% hydrogen peroxide (9:1 by volume) by the method of Thompson (19) was hydrolyzed for 24 hr. at 110°C. with 6*N* hydrochloric acid. The decomposition for cystine by this procedure was about 30%.

Independent determination of the amide-ammonia in this protein was made by the modified procedure of Bailey (20).

N-Terminal Analysis

For detection of N-terminal amino acid residues in 7S protein, dinitrophenylation was carried out as described by Sanger (21). The dinitrophenyl (DNP) protein was hydrolyzed *in vacuo* for 13.5 hr. at 110°C. with 6*N* hydrochloric acid. The content of protein in DNP-protein was determined by amide nitrogen content. The ether-soluble DNP-amino acids were identified by two-dimensional thin-layer chromatography (TLC) on Silica Gel G film (22); the solvents used were toluene-pyridine-ethylene chlorohydrin-0.8*N* ammonium hydroxide (100:30:60:60 by vol.) and chloroform-benzyl alcohol-acetic acid (70:30:3 by volume) (23,24). The water-soluble DNP-amino acids were identified by one-dimensional TLC with the upper layer of the solvent, butanol-acetic acid-water (4:1:5 by volume) (25). Quantitative analyses of DNP-amino acids were done by colorimetric determination (24, 25).

RESULTS

Chemical Composition

Chemical composition of the 7S protein is shown in the table below.

Analytical Results of 7S Protein of Soybean Globulins

	g./100 g. protein
Nitrogen	15.91
Phosphorus	.....
Ash	.....
Hexose (measured as glucose)	
Measured by phenol-H_2SO_4 method at 490 mμ	4.67
Measured by orcinol-H_2SO_4 method at 420 mμ	4.87
Hexosamine (measured as glucosamine)	1.19
Sialic acid	.....
Uronic acid	.....

The nitrogen content was similar to that of the protein reported by Roberts

and Briggs (26), which contained about 10% 11S protein. It seems unlikely that the 7S protein consists of lipid-protein complex or lipoprotein, because no appreciable change of sedimentation pattern for this protein was observed in a medium of high density (16% sodium chloride solution, d. 1.12) (Fig. 1). It is quite obvious from the absence of organic phosphorus that no phospholipids exist in this 7S protein.

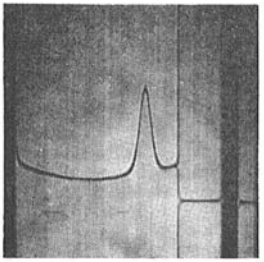

Fig. 1 (left). Ultracentrifugal pattern of the 7S protein in 0.01*M* potassium phosphate buffer, 16% sodium chloride containing 0.01*M* 2-mercaptoethanol, pH 7.80. Photograph was taken after 50 min. of centrifugation at 55,430 r.p.m. at 70° bar angle with 0.90% protein concentration.

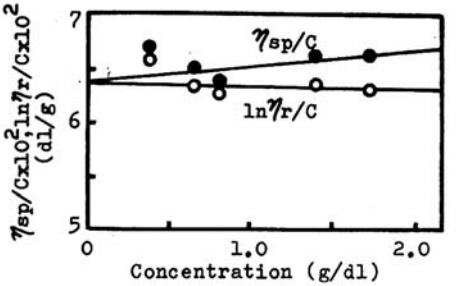

Fig. 2 (right). Reduced viscosity (η_{sp}/C) and inherent viscosity ($\ln\eta_r/C$) of 7S protein in standard buffer.

Some Physical Characteristics of the 7S Protein

Viscosity. Viscosity measurements were made at five different concentrations of the protein at 20°C. (Fig. 2). The value of intrinsic viscosity, $[\eta]$, was 0.0638 dl/g. Both the reduced viscosity (η_{sp}/C) and inherent viscosity ($\ln\eta_r/C$) are used in this case (Fig. 2).

Partial Specific Volume. It has been found for many proteins (17, p. 70) that the partial specific volumes obtained by density measurements are in good agreement with those calculated from the amino acid composition by the following equation:

$$\bar{V} = \Sigma V_i \cdot W_i/\Sigma W_i \qquad (4)$$

where W_i = % by weight of residue and V_i = specific volume of residue. The partial specific volume of this protein calculated from the sum of individual amino acid and carbohydrate residues was 0.725 ml./g. Literature values (17, p. 70; 27) were used for specific volumes of amino acid and carbohydrate residues.

Sedimentation Coefficient. The concentration dependence of the sedimentation coefficient extrapolated to zero concentration was 7.92S (Fig. 3). This value closely corresponded to that given by Roberts and Briggs.

Molecular Weight Determination. 1. By gel filtration. The correlation of K_{av} with Stokes radius is shown in Fig. 4. The value of K_{av} is very close to that of K_d, which is the parameter defined in gel filtration data by Gelotte (28). The relation between these parameters is as follows:

$$K_{av} = (V_t - V_g - V_o)/(V_t - V_o) \cdot K_d = (V_e - V_o)/(V_t - V_o) \qquad (5)$$

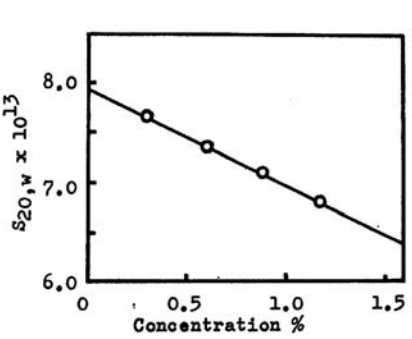

Fig. 3 (left). Sedimentation coefficients of 7S protein in standard buffer. Protein concentrations were 0.30, 0.59, 0.89, and 1.18%.

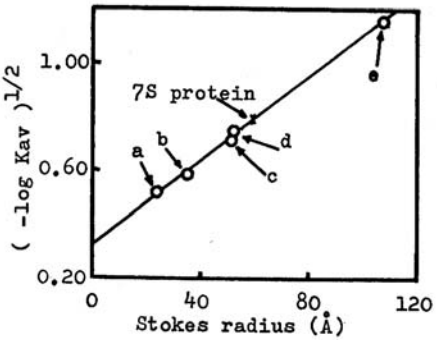

Fig. 4 (right). Correlation of K_{av} with Stokes radius by the method of Laurent and Killander: a, hemoglobin; b, serum albumin; c, gamma-globulin; d, catalase; e, fibrinogen.

where V_e = elution volume corresponding to the peak concentration of a solute; V_o = void volume of the column; V_g = volume not accessible to solvent; and V_t = total volume of the gel bed. The Stokes radius of this protein was estimated to be 59 A. The molecular weight of the 7S protein computed from equation 1 was 210,000 with the values $S_{20,w} = 7.92 \times 10^{-13}$, $\bar{V} = 0.725$, $\eta = 1.0406 \times 10^{-2}$, and $\rho = 1.0159$ (described previously in this paper).

2. By viscosity and sedimentation. The MW from sedimentation coefficient and intrinsic viscosity was obtained according to equation 2. With $S_{20,w} = 7.92 \times 10^{-13}$, $[\eta] = 0.0638$ and $\bar{V} = 0.725$, the MW of the protein is calculated to be 193,000.

3. By sedimentation equilibrium. Since a very long time was required to attain sedimentation equilibrium for the macromolecule protein solution, the following two methods of sedimentation equilibrium were applied.

First, the Archibald method was used; this is one of the most common

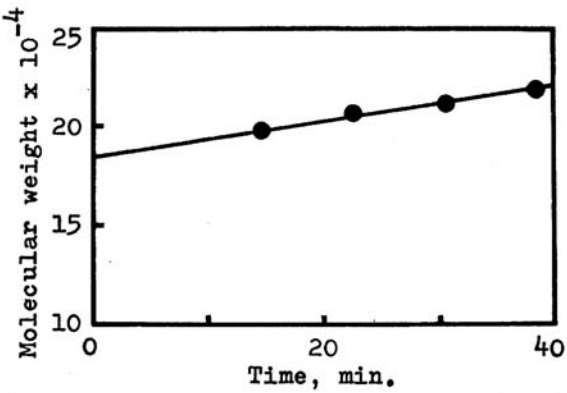

Fig. 5. Variation of the apparent molecular weight with time by the Archibald method. The apparent molecular weights were calculated at the meniscus.

methods of approach to sedimentation equilibrium for measuring MW with an analytical centrifuge. As shown in Fig. 5, the curve of time dependence on the MW showed a positive slope, and the MW of 186,000 was obtained by extrapolating the apparent MW to zero time.

Secondly, the Yphantis procedure of sedimentation in the ultracentrifuge was used. Approximately 64 min. was required to attain equilibrium. The MW was calculated from equation 3. Extrapolation to zero concentration of the protein gave MW value of 180,000 (Fig. 6).

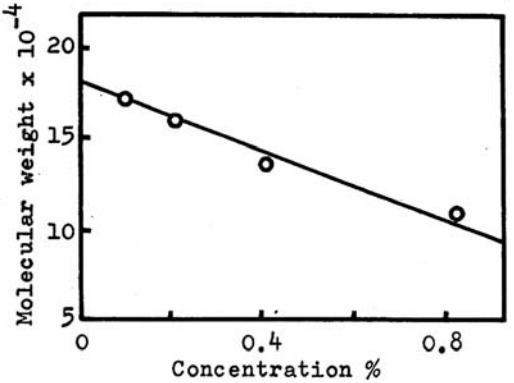

Fig. 6. Dependence of molecular weight on protein concentration for the 7S protein by the Yphantis method. Protein concentrations were 0.104, 0.207, 0.414, and 0.829%.

TABLE I

SUMMARIZED PHYSICAL PROPERTIES OF THE 7S PROTEIN

PROPERTY	METHOD AND SYMBOL	VALUE
Isoelectric point	Electrophoresis	pH 4.90
Sedimentation coefficient	$S^0_{20,w}$	7.92 S
Intrinsic viscosity	$[\eta]$	0.0638 dl/g.
Partial specific volume	$\bar{\nu}$	0.725 ml./g.
Diffusion coefficient[a]	$D_{20,w}$	3.85×10^{-7}
Frictional ratio[b]	f/f_o	1.37
Absorption maximum		278–279 mμ
Absorption minimum		251–252 mμ
$E^{1\%}_{1\text{ cm. at }280\ m\mu}$		5.47
Molecular weight:	Yphantis	180,000
	Archibald	186,000
	$S^0_{20,w}$ $[\eta]$	193,000
	Siegel	210,000

[a]Calculated from viscosity and molecular weight by the method of Yphantis.
[b]Calculated from sedimentation coefficient and molecular weight by the method of Yphantis.

The physical properties of the purified 7S protein are summarized in Table I. The values of diffusion coefficient and frictional ratio were calculated from the next equations (17, p. 103; 29) with MW of 180,000.

$$M = 6.58 \times 10^{-16} / (D_{20,w})^3 \cdot [\eta] \quad (6)$$

$$f/f_o = 1.19 \times 10^{-15} \cdot M^{2/3} \cdot (1 - \bar{\nu}\rho) / S_{20,w} \cdot \bar{\nu}^{1/3} \quad (7)$$

Equation 6 is that calculated by using the value of 2.16×10^6 as beta.

N-Terminal Amino Acids of the 7S Protein

The ether-soluble N-terminal amino acids in the 7S protein, which were found on the thin-layer chromatogram, were serine, glutamic acid, valine, alanine, glycine, aspartic acid, leucine (isoleucine), and tyrosine (Fig. 7). On the chromatogram, one unknown spot was found but was not

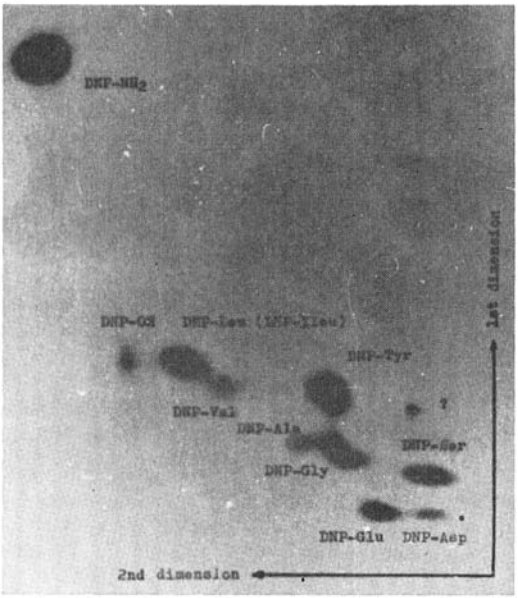

Fig. 7. Two-dimensional thin-layer chromatogram of DNP-amino acids of N-terminal in 7S protein, photographed under irradiation of ultraviolet light.

considered to be that from DNP-amino acid because its position did not correspond to that of any DNP-amino acid. No water-soluble N-terminal amino acids were found. The results of N-terminal analyses in the 7S protein are given in the table below. Recovery of DNP-amino acids during hydrolysis

N-Terminal Amino Acid Composition of 7S Protein

	moles/mole 7S protein	
Aspartic acid	0.63	1
Alanine	0.82	1
Glycine	1.15	1
Valine	0.62	1
Serine	1.55	2
Tyrosine	0.85	1
Glutamic acid	0.79	1
Leucine (isoleucine)	0.73	1

was determined by hydrolysis of the mixture of authentic DNP-amino acids and DNP-7S protein in equal amounts. The numbers of N-terminal residues to the nearest integer are shown in the last column of the table, calculated on the basis of 180,000 as MW.

Amino Acid Composition

Results of amino acid analyses are shown in Table II. Serine, threonine,

TABLE II

AMINO ACID COMPOSITION OF THE 7S PROTEIN

AMINO ACID		INTEGRAL NUMBER[a]	AMINO ACID		INTEGRAL NUMBER[a]
	g./100 g. protein			*g./100 g. protein*	
Tryptophan	0.32	3	Valine	5.08	78
Lysine	7.01	86	Methionine	0.25	3
Histidine	1.67	19	Isoleucine	6.40	88
Arginine	8.82	91	Leucine	10.25	141
Aspartic acid	14.13	191	Tyrosine	3.61	36
Threonine	2.81	43	Phenylalanine	7.39	81
Serine	6.77	116	½-Cystine	0.26	4
Glutamic acid	20.50	251	Amide ammonia	1.71	181
Proline	4.33	71	Mannose	3.75[b]	38
Glycine	2.85	68	Glucosamine	1.19[c]	12
Alanine	3.70	75		113.00	1,676
			M calculated		176,766[d]

[a]Nearest integral number of residues per molecule; M = 180,000.
[b]See ref. 30.
[c]See ref. 31.
[d]Nine moles of water was added from the results of N-terminal amino acid analysis, in which the 7S protein was considered to be composed of nine peptide chains.

and tyrosine were decomposed with increased time of hydrolysis; therefore the values for those amino acids were obtained by extrapolation to zero time. Moisture content of the lyophilized protein was 13.69% and calculated nitrogen content was 15.85%. Nitrogen recovery was 99.62% and total weight of amino acid residues was 99.36 g. per 100 g. protein. Integral numbers of amino acid residues per molecule were calculated on the basis of MW of 180,000. From the nearest integral numbers of residues, MW was calculated as 176,766.

DISCUSSION

Roberts and Briggs determined the MW of the 7S protein as 330,000, by application of the Trautman plots (32), an approach to the equilibrium method which uses the Archibald principle (16), for a 7S preparation which contained approximately 10% of 11S protein. In the present experiments, however, MW of 180,000–210,000 was obtained by four separate methods based on different principles.

The following three possibilities should be considered for the very marked difference between the MW's in this paper and those of Roberts and Briggs (26). The first is that the measurements were made with different 7S preparations; the second is that the differences stem from dissimilar methods of measurement used; and third, the differences may be attributed to contamination by 11S in the preparation of Roberts and Briggs. The first possibility, however, can be excluded, because two lines of evidence indicate that the 7S protein isolated by Roberts and Briggs may be the same protein as this purified 7S protein; one is on the purified 7S⇄9S isomerization behavior and the other is the N-terminal analysis. Both preparations underwent the 7S⇄9S isomerization with change in ionic strength; and further, the results of N-terminal analysis are almost identical.

The Yphantis procedure for MW determination has been used very often

recently because of its rapidity and high reliability. It should be noted that the value for the MW of 7S protein in this paper might be more reliable than that obtained by Roberts and Briggs, owing to the substantially complete agreement of data among the four different methods employing the Yphantis method.

The data of N-terminal amino acid analysis agreed well with those obtained by Roberts and Briggs as described above. There exists, however, the slight difference with regard to the number of the N-terminal glutamic acid residues, that is, 1 mole in the present experiment as compared to 2 moles in that of Roberts and Briggs. This disagreement may be due to the difference in estimation for MW of the protein. Fractional moles of alanine, tyrosine, and aspartic acid found in the 7S protein of Roberts and Briggs were not observed in the present experiments. Decomposition of DNP-aspartic acid, DNP-alanine, DNP-glycine, DNP-tyrosine, and DNP-serine during hydrolysis was considerably greater than that of the other DNP-amino acids found in this 7S protein. The occurrence of the fractional moles of these DNP-amino acids in the data of Roberts and Briggs might be ascribed to error arising from decomposition of these DNP-amino acids during hydrolysis.

It can be mentioned from the present data and data obtained by other investigators (26,33) that the 7S protein consists of more kinds of subunits than the 11S protein.

Although tryptophan and cystine were not reported by Roberts and Briggs for their 7S preparation, these amino acids were present in my preparation. Their existence was also supported by microbioassay with *Lactobacillus arabinosus* ATCC 8014 and *Leuconostoc citrovorum* ATCC 8081, respectively.

All the SH groups in this 7S protein will exist in the form of the S–S bond, since no free SH groups were found in the sample by Boyer's spectrophotometric procedure for determination of SH groups with *p*-chloromercuribenzoic acid (34,35) (Fig. 8).

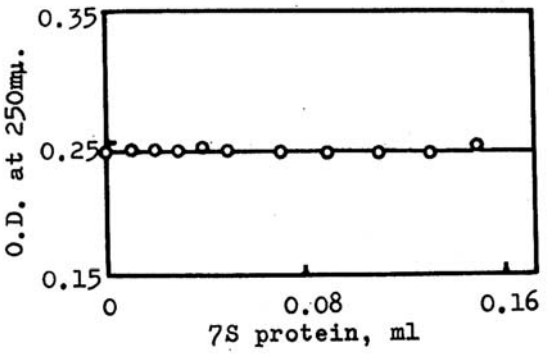

Fig. 8. Determination of SH group in 7S protein. The protein concentration, 17.93 $\times$ $10^{-4}M$. PCMB concentration, 5.96 $\times$ $10^{-6}M$. The protein was treated for 1 hr. with 8.9*M* urea.

Acknowledgments

The author wishes to express his sincerest thanks to Prof. Y. Sakurai of the Japan Women's University and Prof. M. Fujimaki of the University of Tokyo for their kind guidance and encouragement throughout this work. Great indebtedness is acknowledged to Prof. K. Arima and Prof. Y. Ikeda of the University of Tokyo, and Dr. W. J. Wolf of the Northern Regional Research Laboratory, Peoria, Illinois, for their valuable advice and suggestions. Thanks are due to Mr. K. Nomura for operation of the amino acid analyzer; to Dr. M. Mogi, Dr. D. Fukushima, and Dr. N. Iguchi for their helpful discussions and encouragement; and to Miss T. Ichikawa for her kind assistance.

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[Received May 10, 1967. Accepted January 16, 1968]