EFFECT OF AEROSOLIZATION UPON BOVINE SERUM ALBUMIN

by

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ABSTRACT

The effects of aerosolization upon bovine serum albumin (BSA) at relative humidities of 43%, 62% and 85% were studied by exclusion chromatography, absorption spectrography and electrophoresis. Seven sources of chicken antisera (prepared against reference BSA and BSA from Andersen samplers at 43%, 62%, and 85% R.H. and the atomizer contents at the same three humidities) were used to study their reactions with the seven sources of BSA antigens. Immunoelectrophoresis, Ouchterlony gel diffusion, and Preer precipitin tests were employed to test for differences. The atomizer and the dehydration and rehydration resulting from aerosolization altered the properties of BSA with respect to apparent molecular weight, isoelectric point, and absorption spectra in the visible light region. The precipitation of the antigen-antibody complexes appeared homogeneous. Limited evidence indicated quantitative differences in the antigen-antibody precipitation which are related to interactions exerted upon the protein by relative humidity, treatment of antigen (aerosol or atomizer) and the various antisera.

INTRODUCTION

There have been numerous research efforts in the application of aerosols to immunize man and animals (9, 19, 22, 27). Many of these efforts have been promising, particularly in immunization against infectious diseases acquired by the respiratory route. Administration of a small amount of antigen has been found to elicit a significant increase in protective antibodies. Direct application to the upper respiratory tract by aerosol route of inactivated, live influenza virus vaccines composed of type A2 strains has been shown to result in the formation of antibody of 11S γA immunoglobulin (IgA) class in the respiratory tract (10, 23). The titers and duration of such antibody were greater than those achieved following parenteral vaccination.

Sawyer and associates (20) have noted that the aerosol microenvironment is unfavorable for most microorganisms, as evidenced by a "biological decay." Webb (24) suggested that the death of the airborne microbe results from the movement of water molecules in and out of the cell, in an equilibrium system, resulting in a collapse of the natural structure of cellular protein.

Ion control and protein synthesis studies summarized by Andersen and Cox (2) suggest that viable bacteria recovered from aerosols are not unchanged, rehydrated forms of the original bacterium. No evidence is presented, however, that would permit differentiation between cellular alterations occurring as a result of aerosolization (dehydration), duration of the airborne state, and the rehydration which occurs during sampling and recovery of the airborne microbes.

The extent of cellular damage that occurs during aerosol generation in the natural environment is unknown and can only be inferred from experimental production of microbial aerosols. Indeed, any theoretical treatment based only on behavior of ideal solutions or perfect gases, fails to consider such additional variables as:

1. Adsorption of vapor on surfaces
2. Denaturation of proteins
3. Crystallization of dissolved salts and the resulting competition for water molecules with protein and nucleic acids

Lapresle, Kaninski and Tanner (12) have shown that antibodies are produced against digested proteins which are not present in antiserums against native protein. Bartel and Campbell (3), using a dissociable hemocyanin as an antigen, demonstrated that antiserum from rabbits immunized

with hemocyanin contain antibodies which reacted with the dissociated molecular units, but not with the intact associated hemocyanin molecule.

If dehydration and subsequent rehydration of aerosolized microbes alters the integrity of proteins and other complex substances in or on the surface of the microbes, such changes might also result in alteration of the antigenic properties of the protein. Since no description of such studies has been found in the literature, this investigation was undertaken to determine if the antigenic properties of a protein model bovine serum albumin would undergo change as a result of aerosolization.

MATERIALS AND METHODS

Experimental Animals

Male, 28-day-old Hubbard White Mountain chickens were used. These animals were obtained from the colonies of The University of Arizona Poultry Science Research Center. Their weights ranged from 615-900 grams and the average weight was 752 grams. The animals were provided standard growth ration, and water ad libitum.

Antigen Preparation

Crystallized Bovine Serum Albumin (BSA) was obtained from Pentex Corporation, Kankakee, Illinois. A 2% solution of this protein was made in sterile 0.15 M NaCl, pH 7. Small aliquots of this solution were stored in tubes at -60 C until used. This stock solution of BSA is hereafter referred to as "native" antigen (Nat).

Aerosol Apparatus

The aerosols were generated with a 3-jet Collison atomizer (5, 8) at an air pressure of 15 p.s.i. housed in an environmental safety cabinet (14). The aerosols were directed through a 78 liter chamber to an exit port to which the aerosol sampling device was attached. Adjusting the relative humidity of the air inside the environmental

enclosure which contained the atomizer permitted aerosols to be studied at three levels of relative humidity (R.H.). These were 43%, 62%, and 85% R.H. as determined by standard wet and dry bulb thermometers. Dry bulb temperatures were maintained between 23-25 C.

Aerosol Sampling

The aerosols were collected with a model 0203 Andersen sampler (1) modified to an 8-stage device with addition of stages 0 and 7, which were obtained from Medi-Comp Research and Development, Salt Lake City, Utah. To assure collection of particles of at least 0.1 micron in size, the sampler was operated at 2 ft^3/min, or twice its normal flow rate. Before sampling, all eight plates were coated with a thin film of glycerol to aid adherence of the aerosolized particles. Thirty to fifty ml of 2% BSA were aerosolized at each different R.H. condition (43%, 62%, and 85%). After each aerosolization of the BSA for the alotted time, the atomizer was turned off and the aerosol apparatus was air-washed by continued sampler operation. Periodically, small samples of the atomizer contents, including foam, were pooled during each aerosol trial and preserved as described below. These samples are referred to later as atomizer (At) for 43%, 62%, and 85% R.H. The aerosol samples deposited on the Andersen plates were removed by a single-edged razor blade. Pooled collections of BSA were

dissolved with 4 ml of sterile 0.15 M NaCl. The solution was centrifuged at 1700 x G for 30 minutes. Samples of these supernatant fluids were adjusted to predetermined concentrations and preserved as described below, and represented Andersen sampler collections (As) for 43%, 62%, and 85% R.H.

Five-hundredths ml volumes from the Andersen sampler solutions and from the solutions obtained from the atomizer were assayed for total protein. Solutions of 20 mg/ml and 10 mg/ml, in sterile 0.15 M NaCl pH 7, were made of these solutions and preserved in sterile tubes at -60 C until needed.

Total Protein Assay

The samples removed from the collection plates and atomizer were tested for total protein with Folin Ciocolteau procedure (15). The colorimetric reaction was read at 660 mμ with either a Spectronic 600 or Spectronic 20.

Preparation of Anti-Bovine Serum Albumin Chicken Serum

Anti-BSA chicken sera were prepared by a single intravenous injection of 20 mg BSA/kg of body weight. BSA from each of the six treatments (As 43%, As 62%, As 85%, At 43%, At 62%, At 85%) were inoculated into groups consisting of three birds. Nat BSA was inoculated into nine birds. All animals were bled by cardiac puncture one week after

sensitization. After removal of lipids by centrifugation at approximately 1030 x G for 30 min at 0 C the serum samples were stored in sterile containers at -20 C until needed.

Precipitating Antibody Technique

Precipitating antibodies were determined with a modified Preer technique (18). In all tests 50 µl of an antigen dilution was separated from 25 µl of the appropriate antiserum by a 5 mm agar gel layer in 45 x 4 mm internal diameter tubes. The composition of the agar gel consisted of 1% Noble agar, 8% NaCl, 0.01% Merthiolate and buffered to pH 7.0 with 0.02 M sodium phosphate (26). The antigen solutions were made by diluting the 10 mg/ml of BSA stock solution to obtain concentrations of 0.05 µg, 0.025 µg, and 0.0125 µg protein/ml. To obtain a sharp interface between antigen, antibody and agar solutions, the antigen was placed in the bottom of the tube, frozen at -20 C, and the melted agar layered on top to a depth of 5 mm. Antiserum was placed on the top of the agar as soon as it had solidified. After incubation for 3 days at room temperature the position of the precipitin line was recorded and indicated as a P value (distance of precipitin line from antisera-agar interface divided by the length of the agar column).

Exclusion Chromatography

One-ml volumes of 20 mg/ml of each of the seven treated BSA samples (i.e., native and Andersen sampler, and atomizer at 43%, 62%, and 85% R.H.) were fractionated by gel filtration on a column of Sephadex G-200 in duplicate. A column with the dimension of 50 x 2 cm was prepared according to the method of Flodin and Killander (6). Glass wool was placed in the bottom and covered with 1 g of glass beads. Eighty-six ml of G-200 were added and equilibrated for 24 hours with a solution of 0.1 M tris pH 8.0 in 0.1 M NaCl. A 1 ml volume of dextran blue was passed through the column to determine the packing of the column and the void volume (V_O). After the column had stabilized, 1 ml of the solution of 20 mg/ml of BSA diluted 1:3 was applied. The tris-NaCl buffer described above was allowed to flow through the column and elution was carried out at room temperature with a flow rate of 15 ml/hour. One hundred twenty 1 ml fractions were collected. The protein was determined by measuring the optical density at 280 mμ on the Spectronic 600. Individual peak effluent fractions were characterized by gel diffusion.

Ouchterlony Gel Diffusion

All antisera were tested for possible differences in antibodies against BSA (identity, partial identity or nonidentity). Ouchterlony plates (17) were prepared by

pouring into petri dishes 10 ml of 1% Noble agar (Difco Laboratories) of pH 7.4, containing 0.01% Merthiolate, and modified to contain 8% NaCl (4). Antiserum was placed in the central well and the antigens in the surrounding wells. Precipitin lines were allowed to develop at room temperature for 1 to 3 days in a moist atmosphere. The preparations were washed for 3 days with 1% NaCl and distilled water. They were then dried and stained with amido black 10B.

Electrophoresis

Gel electrophoresis (7) of the antigens was performed using phosphate buffers of pH 6, 7 and 8 and ionic strength of 0.06 for a duration of 0 to 3 hours. A 1% Noble agar, containing phosphate buffers pH 6, 7 and 8, was used as a medium for electrophoretic separation under a potential gradient of 50 v. BSA from the seven treatments (i.e., native and Andersen sampler, and atomizer at 43%, 62%, and 85% R.H.) in amounts of 1 to 2 microliters were placed in wells at the middle of the slide. After electrophoretic separation was accomplished, they were dried, and stained with amido black 10B.

Absorption Spectra

Absorption spectra of the atomizer contents, Andersen samples, and reference solutions in 1 cm

absorption cells were obtained using a Spectronic 600 spectrophotometer and a 10-inch laboratory potentiometric recorder in the ultraviolet and visible light ranges.

EXPERIMENTAL RESULTS

Exclusion Chromatography

Samples of the seven different sources of BSA were passed through Sephadex G-200 columns in duplicate runs. The 1 ml fractions collected during the runs were analyzed in the Spectronic 600 for protein concentration. The parameters inherent in each column run are summarized in Table 1. The K_d values were transformed to arcsin $\sqrt{K_d}$ and analyzed by analysis of variance using a completely randomized design. The means of the transformed K_d values for the seven treatments accorded the BSA are presented in Table 2. The results of the analysis are presented in Table 3. The treatment mean square was significant at the 0.025 level. Plotting the mean of the transformed K_d values for each of the seven experimental conditions, as shown in Fig. 1, indicate that the values for As 85% and At 85% were significantly lower than the transformed values for Nat and At 62%. Omitting the values for Nat BSA found in Table 2, the values of the remaining six treatments were analyzed using a completely randomized 3 x 2 factorial arrangement of the experimental conditions (source and R.H.) The results of the factorial analysis are presented in Table 4. The single effect of relative humidity was the

Table 1. Parameters of the Sephadex G-200 Column Chromatography of Bovine Serum Albumin Samples*

Sample trials	V_o (ml)[c]	V_e (ml)[d]	K_d[e]
Nat-1	58	97	0.453
Nat-2	55	96	0.476
As43-1	62[a]	65	0.035
	62[b]	89	0.329
As43-2	63[a]	65	0.023
	63[b]	91	0.323
As62-1	62	91	0.336
As62-2	60	96	0.418
As85-1	72	101	0.338
As85-2	76	96	0.232
At43-1	68	101	0.384
At43-2	74	101	0.314
At62-1	60	98	0.442
At62-2	59	97	0.442
At85-1	76	96	0.232
At85-2	71	95	0.278

*For all samples V_i (inner volume) = 86 ml

[a]First peak
[b]Second peak
[c]V_o = void volume
[d]V_e = elution volume
[e]$K_d = \frac{V_e - V_o}{V_i}$

Table 2. Mean Transformed K_d Values of Duplicated Chromatographic Separations of Bovine Serum Albumin Samples with Sephadex G-200*

Nat	As43%	As62%	As85%	At43%	At62%	At85%
42.30	35.00	35.43	35.55	38.29	41.67	28.76
43.05	34.63	40.28	28.78	34.08	41.03	31.82

*Arcsin $\sqrt{K_d}$ transformation

Table 3. Summary of Analysis of Variance of Transformed K_d Values in Table 2 Using Randomized Block Design

	Sums of Squares	Degree of Freedom	Mean Squares	F Ratio
Total	295.720092900	13		
Treat.	247.10304290	6	41.18384	5.93*
Within.	48.61705	7	6.94529	

*$P \leq 0.025$

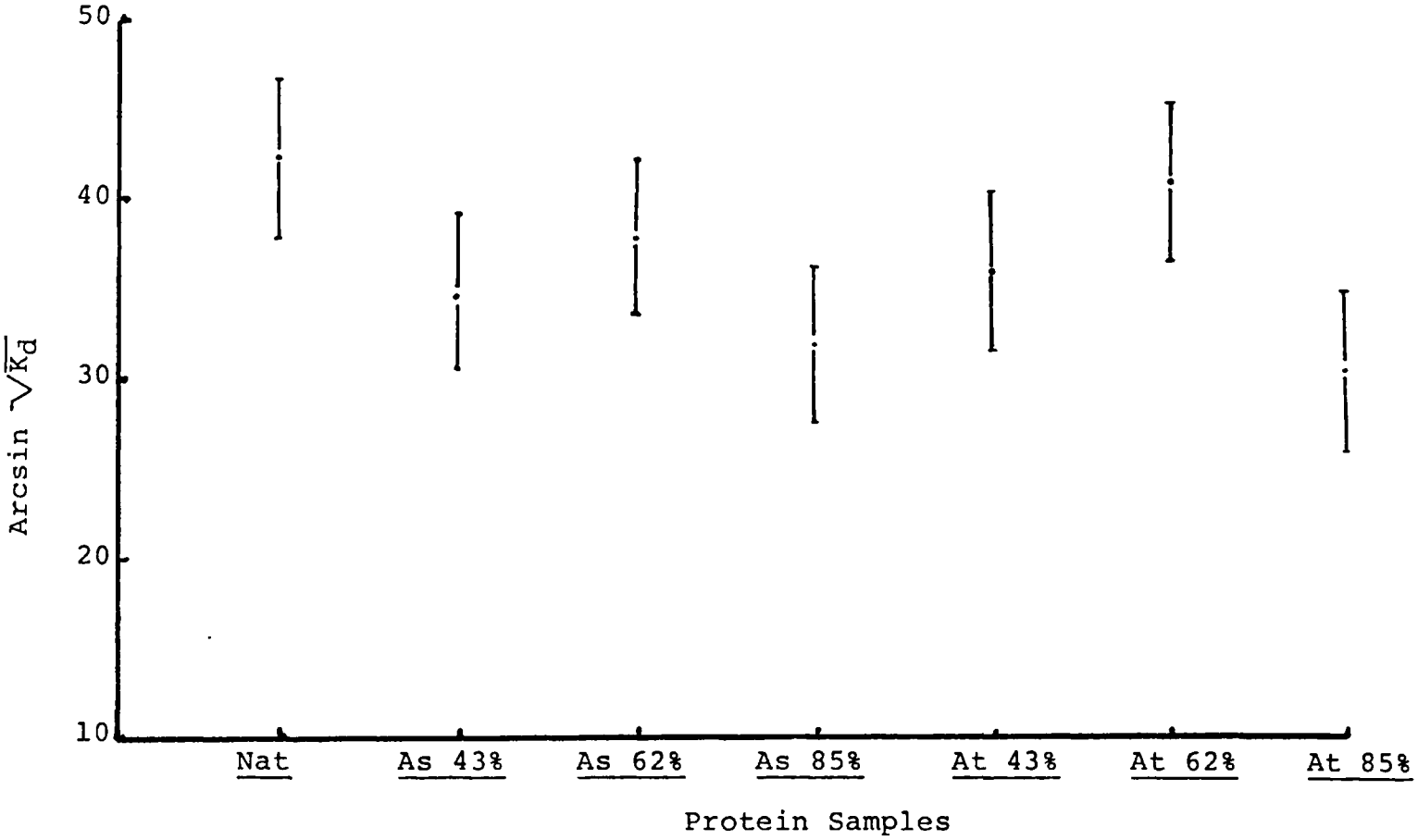

Fig. 1. Mean Transformed K_d Values of Chromatographic Separation with Sephadex G-200 of BSA Samples: Averaged by Source and Relative Humidity

Vertical lines indicate 95% confidence limits.

Table 4. Summary of Analysis of Variance of Transformed K_d Values in Table 2 Using Factorial Arrangement of Variables**

	Sums of Squares	Degree of Freedom	Mean Squares	F Ratio
Total	205.8694667	11		
All treats.	157.5336667	5		
Source	3.00000	1	3.00000	
R.H.[a]	139.96351670	2	69.98176	8.687*
Source x R.H.	14.57015	2	7.285075	ns
Error	48.3358	6	8.0560	

*$P \leq 0.025$

**Native BSA sample data omitted to provide balanced factorial arrangement

[a]R.H. = Relative Humidity

only experimental condition which showed a significant mean square. Plotting the mean transformed K_d values for the six experimental conditions included in the factorial analysis, as shown in Fig. 2, showed that the mean value at R.H. 85% was significantly lower than the mean value at R.H. 42%. The mean transformed K_d value for native BSA has been added to Fig. 2 for comparative purposes. The mean transformed K_d value for R.H. 85% is significantly lower than the mean transformed K_d for native BSA.

The protein concentration was normalized for each fraction by converting the raw protein concentration to per cent of total protein recovered from the column. These relative concentrations are presented in Appendix A. The arcsin $\sqrt{\text{per cent}}$ transforms of the data were analyzed by a 3-way factorial analysis of variance, omitting the data on native protein runs to maintain a completely balanced design which was required by the computer program (21). The three factors represented R.H. (at 3 levels), source of antigen (atomizer or Andersen sampler), and fraction number (59 levels). The 59 fraction levels included the 62nd to the 120th volume fractions in each case. Replication of content for each volume fraction (duplication of column run) for each treatment condition was processed by the computer program as a fourth (dummy) factor having two levels. The results of this computer analysis are summarized in

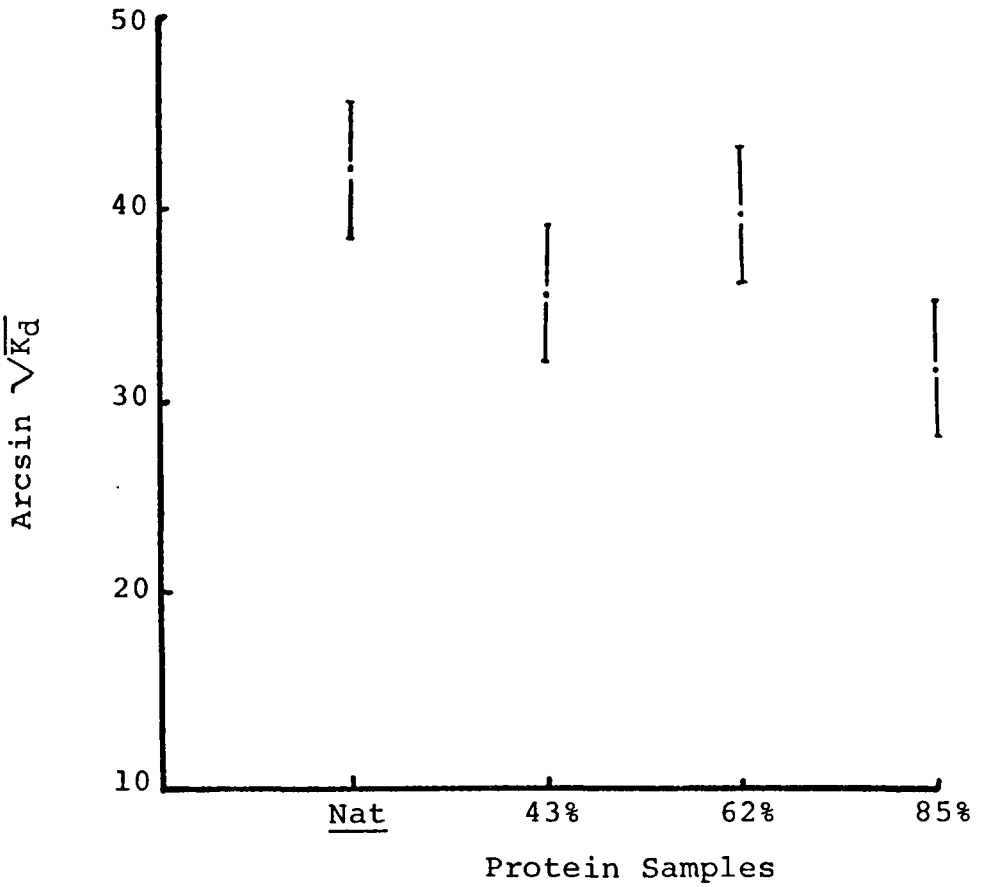

Fig. 2. Mean Transformed K_d Values of Chromatographic Separation with Sephadex G-200 of BSA Samples: Averaged by Source for Each Level of Relative Humidity

Table 5. The fragmented interactions of the dummy factor (replication) have been recombined to provide the error term shown in the table.

As a main effect, source of antigen was insignificant in its contribution to variation over all levels of fraction volumes. Fraction volumes, as expected, was significantly different over all levels of R.H. and source of antigen. Relative humidity was also significant over all levels of source and fraction volumes. Of greatest interest is the significant mean square of the three-way interaction of fraction number, R.H., and source of antigen. The normalized means of fraction protein concentration of duplicate column runs are illustrated in Figs. 3 and 4. The samples from the atomizer (Fig. 3) deviate significantly from each other at the three relative humidities and from the native BSA as well. The samples derived from Andersen sampler collections deviated significantly from each other at the three humidities as well as from the native BSA.

Since the data on native BSA could not, for reasons explained later, be included in the factorial design, the data were analyzed again with the inclusion of the native BSA using a completely randomized design with seven experimental treatment conditions arranged in a factorial manner with fraction numbers. The same multipurpose computer

Table 5. Summary of Analysis of Variance of Sephadex G-200 Chromatography of Bovine Serum Albumin Samples: Factorial Design with Native BSA Omitted

Source of Variation	Degree of Freedom	Sums of Squares	Mean Squares	F Ratio
R.H.[a]	2	21.82217	10.9109	6.52
Source of Ag.[b]	1	2.1424	16.3538	1.28
Fraction No.[c]	58	16068.0385	277.0351	135.67
R.H. x Source of Ag.	2	32.7075	16.3538	9.77
R.H. x Fraction No.	116	1848.5117	15.9354	9.52
Source of Ag. x Fraction No.	58	1311.7883	22.6170	13.51
Source of Ag. x Fraction No.	116	2533.9441	21.8443	13.05
Error	354	592.3853	1.67	

[a]R.H. = Relative Humidity

[b]Ag. = Antigen

[c]No. = Number

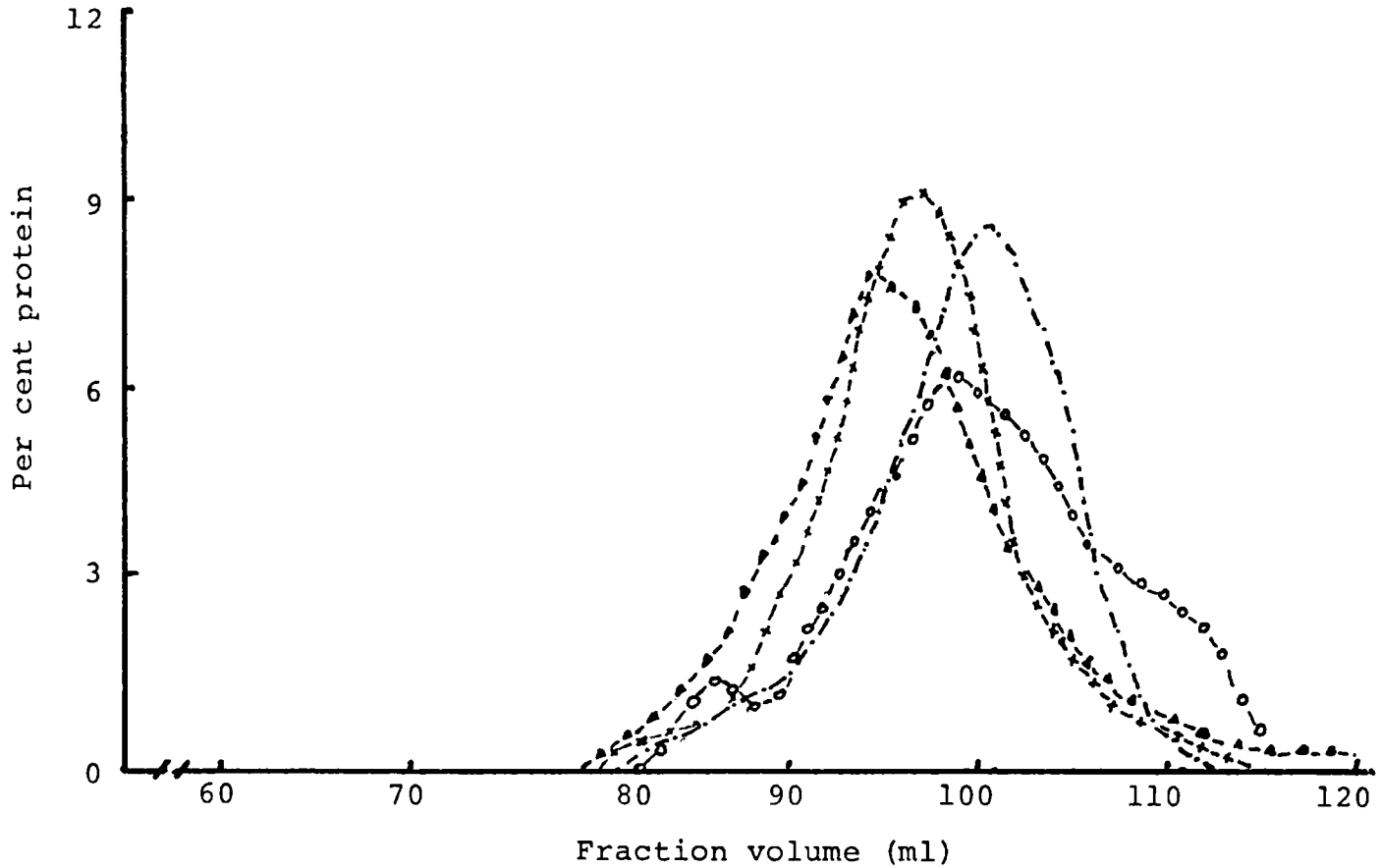

Fig. 3. Chromatography of Native and Atomizer Samples of BSA with Sephadex G-200*
▲–▲= Native, •–•= R.H. 43%, ×–×= R.H. 62%. and
o–o= R.H. 85%

*Curves represent means of duplicate column runs

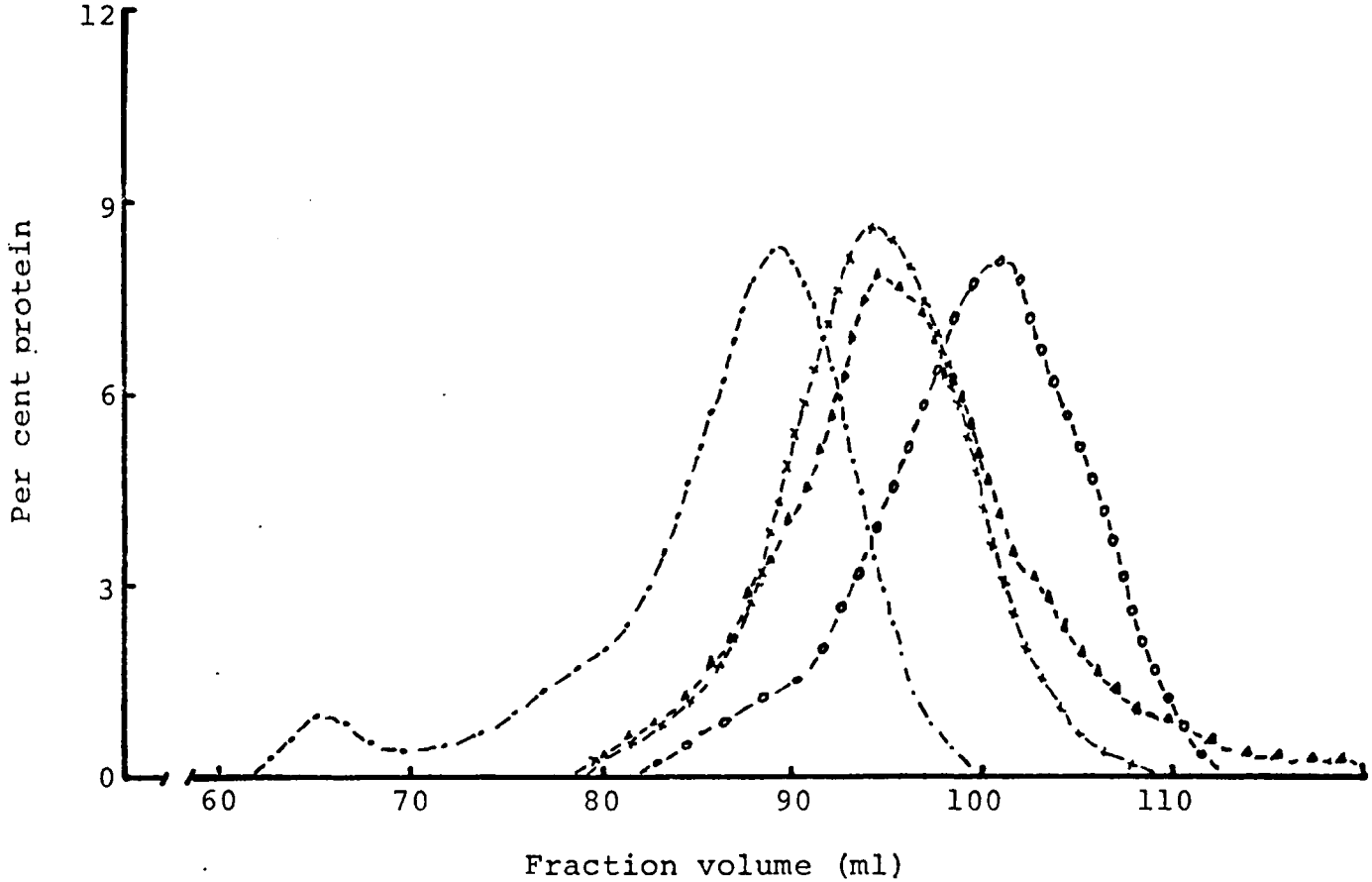

Fig. 4. Chromatography of Native and Aerosol Samples of BSA with Sephadex G-200*
▲—▲= Native, ·–·= R.H. 43%, ×–×= R.H. 62%, and ●—●= R.H. 85%

*Curves represent means of duplicate column runs

program was used in a manner described by Sokal and Rohlf (21). The results of this second analysis are summarized in Table 6. They confirm that significant interaction occurs between the experimental conditions applied to the BSA, and the fraction number. In the first factorial analysis, wherein data for native was necessarily omitted, the standard error was found to be approximately 0.88. In the second analysis, wherein data for native BSA was included, but omitting interactions between source and R.H., the standard error was approximately 1.03.

Table 6. Summary of Analysis of Variance of Sephadex G-200 Chromatography of Bovine Serum Albumin Samples: Factorial Design with Native BSA Included

Source of Variation	Degree of Freedom	Sums of Squares	Mean Squares	F Ratio
Experiment treatment	6	57.1515	9.5252	4.49
Fraction No.	58	19272.9019	332.2914	156.49
Fraction No. x Expt. Treatment	348	5853.7175	16.8210	7.92
Error	413	876.9532	2.12	

Absorption Spectra

The visible differential spectra of samples of native BSA and atomizer contents at the three different R.H. conditions are presented in Fig. 5. It is apparent that solutions in the atomizer at 62% and 85% R.H. are

Fig. 5. Visible Absorption Spectra of Atomizer BSA Samples

△—△ spectrum of the Nat, ○—○ spectrum of At 43%
◎—◎ spectrum of At 62%, ✳—✳ spectrum of At 85%

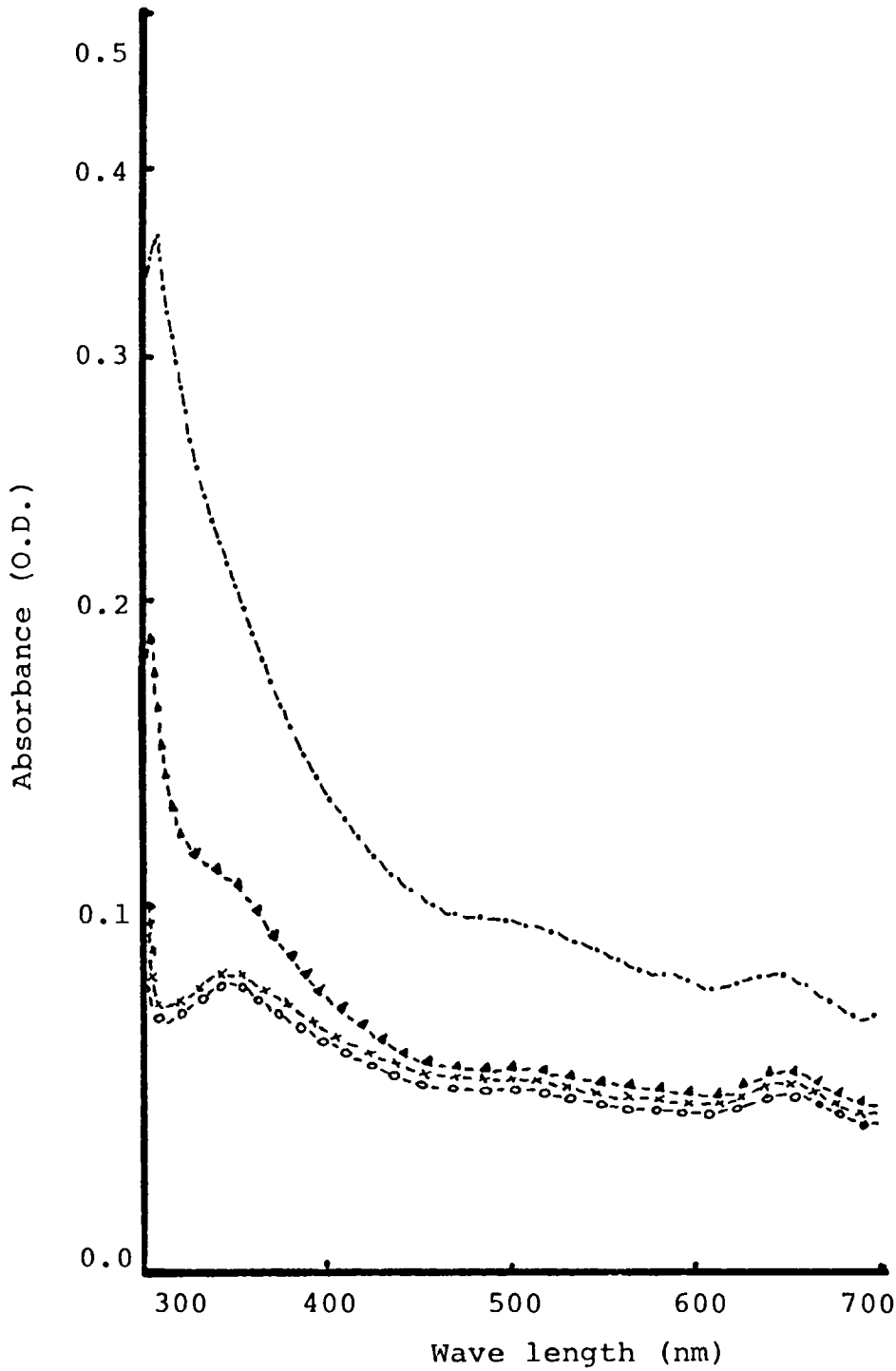

Fig. 5. Visible Absorption Spectra of Atomizer BSA Samples

virtually identical and differ from native BSA. However, the spectra for the atomizer solution at R.H. 43% differs greatly from native BSA and from the solutions at 62% and 85% R.H.

Shown in Fig. 6 are visible absorption spectra from the solutions comprised of Andersen sampler collections at 43%, 62%, and 85% R.H. They show absorption maxima at 310 nm and 355 nm respectively. The solution of BSA representing As 43% R.H. appeared opalescent. The native BSA and As 62% had a light buff or cream color. The solution representing As 85% appeared yellow. These results suggest that all three curves are significantly different from each other and significantly different from native. No differences were observed in the ultraviolet absorption spectra among the native and treated BSA samples.

Electrophoresis

A pattern obtained by electrophoresis at pH 6 is shown in Fig. 7. It is apparent that Nat BSA sample is different from the treated BSA samples because it did not migrate as far as the others. These were characterized by narrow discrete bands with some additional small strong bands. At pH 7 and 8, no difference between native and treated BSA samples was observed.

Fig. 6. Visible Absorption Spectra of Andersen BSA Samples

∘—∘ spectrum of As 43%, ∘-∘ spectrum of As 62%,
*-*spectrum of As 85%. △-△ Nat

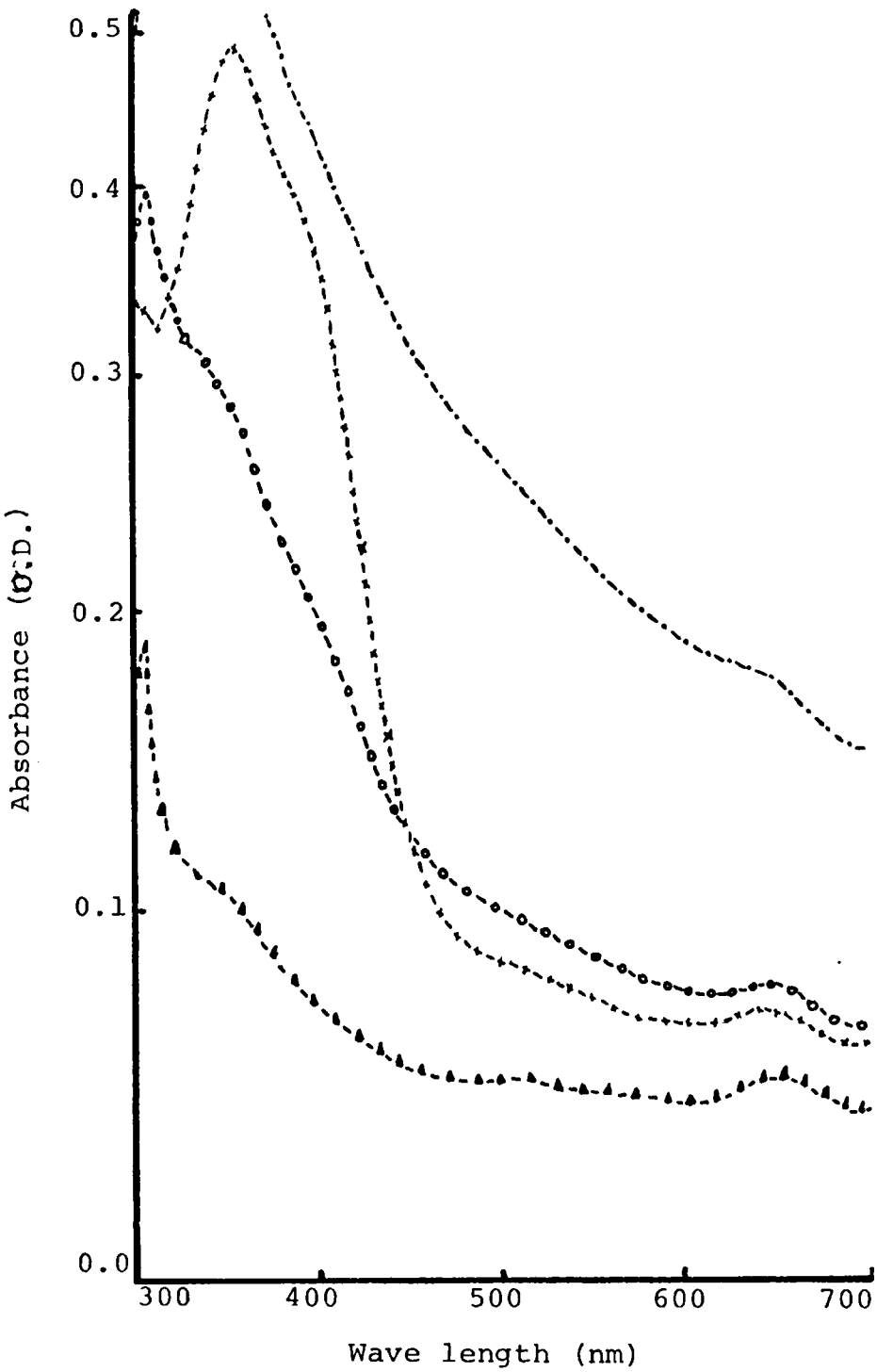

Fig. 6. Visible Absorption Spectra of Andersen BSA Samples

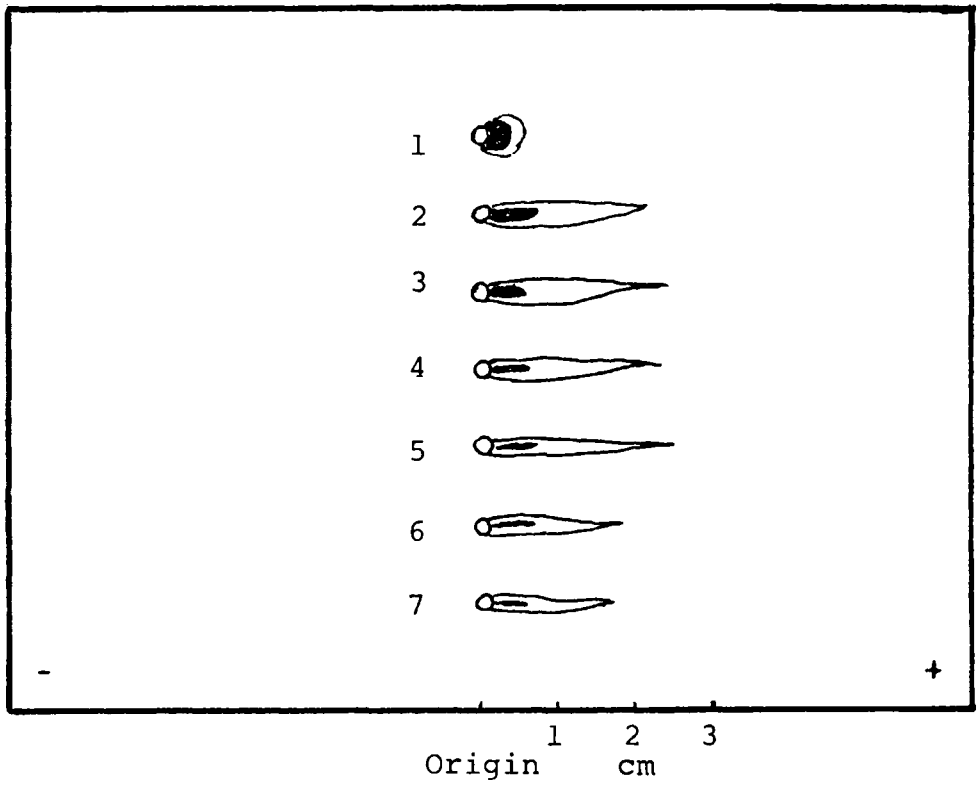

Fig. 7. Electrophoretic Patterns of Chromatographic Separation of BSA in Agar Gel (pH 6). --1- Nat, 2- As 43%, 3- As 62%, 4- As 85%, 5- At 43%, 6- At 62%, 7 At 85%

Ouchterlony

The seven kinds of anti-BSA sera were compared by Ouchterlony immunodiffusion analyses. Native and treated BSA antisera showed formation of precipitin lines against the varied BSA preparations. A single band of precipitate, without spur formation, was observed between each antigen and antiserum. This demonstrated the cross-reactive properties of those antibodies with regard to BSA of varied sources. Also, the single band of precipitate between the different kinds of BSA suggest that all are directed to identical antigenic determinants.

Preer Test

The results of the Preer test were more consistent using a concentration of 0.025 µg protein/ml as the test antigens than when the test antigens contained 0.05 and 0.0125 µg protein/ml. Birds inoculated with At 85% antigen reacted with low P values over all test antigen concentrations. In reviewing the protein assays of antigens administered to the birds, it was discovered that those birds were inadvertently inoculated with far lower amounts of BSA than was intended. The data for antisera from these birds (At 85%) were therefore not included in the statistical analysis.

The P values obtained from the Preer test using test antigens at 0.025 µg/ml are listed in Table 7 for each

Table 7. P Values Resulting from Preer Test of Seven Sources of BSA with Chicken Antisera Prepared Against Those Antigens

Antisera	Bird No.	Test Antigen*					
		Nat	Nat	Nat	As43%	As62%	As85%
Anti-Nat	2	.600	.500	.422	.600	.330	.090
	9	.000	.000	.000	.000	.000	.000
	11	.000	.295	.200	.166	.018	.142
	12	.330	.300	.130	.630	.170	.020
	21	.660	.340	.380	.140	.071	.000
	23	.400	.330	.330	.290	.100	.010
	24	.500	.330	.360	.063	.083	.020
	27	.000	.220	.300	.330	.125	.020
	28	.500	.130	.300	.260	.166	.170
Anti-As43%	20	.660	.500	.385	.000	.100	.170
	22	.660	.440	.200	.029	.290	.036
	29	.600	.330	.300	.140	.100	.290
Anti-At43%	15	.500	.330	.330	.170	.100	.071
	18	.330	.500	.110	.200	.100	.220
	26	.600	.400	.380	.330	.100	.076
Anti-As62%	17	.400	.250	.300	.285	.025	.020
	19	.000	.220	.130	.000	.020	.000
	25	.500	.100	.250	.000	.020	.000
Anti-At62%	7	.400	.290	.380	.062	.140	.025
	14	.500	.330	.380	.200	.100	.010
	15	.500	.300	.330	.000	.018	.100
Anti-As85%	5	.500	.380	.380	.285	.170	.180
	8	.000	.290	.380	.000	.140	.025
	10	.000	.250	.130	.330	.017	.000
Anti-At85%	1	.000	.000	.000	.000	.000	.000
	4	.040	.000	.250	.000	.000	.020
	13	.000	.000	.000	.000	.000	.000

Table 7, (Continued)

Antisera	Bird No.	Test Antigen*		
		At43%	At62%	As85%
Anti-Nat	2	.500	.400	.200
	9	.000	.000	.000
	11	.000	.240	.083
	12	.200	.440	.270
	21	.500	.330	.250
	23	.000	.420	.270
	24	.330	.300	.250
	27	.400	.380	.022
	28	.200	.300	.330
Anti-As43%	20	.400	.420	.300
	22	.200	.330	.300
	29	.330	.330	.420
Anti-At43%	15	.000	.200	.100
	18	.400	.500	.300
	26	.020	.380	.250
Anti-As62%	17	.250	.300	.300
	19	.017	.300	.025
	25	.000	.200	.200
Anti-At62%	7	.000	.220	.130
	14	.200	.500	.270
	15	.013	.380	.200
Anti-As85%	5	.400	.500	.300
	8	.000	.400	.170
	10	.200	.170	.083
Anti-At85%	1	.000	.000	.000
	4	.000	.176	.083
	13	.000	.000	.000

*Test antigens contain 0.025 μm protein/ml

bird's serum. With the exception of those birds receiving the At 85% antigenic stimulation, only one subject (bird no. 9) failed to respond with demonstrable precipitating antibody after being stimulated with native antigen. In the statistical analysis of these data, the response of serum from bird no. 9 was deleted.

The P values were transformed to arcsin $\sqrt{P}$ before they were analyzed by analysis of variance. The data were analyzed with a randomized block design to account for differences between animals and to test for significant differences between the nine column treatments as listed in Table 7. The results of this analysis are summarized in Table 8. The mean transformed P values for each of the seven experimental conditions, as shown in Figs. 8 and 9, indicate the differences in the way antisera prepared against the seven sources of antigen reacted with these antigens in the test. With reference to Fig. 8, antiserum prepared against native antigen showed a significantly lower P value against As 85% than it did against native antigen. Antiserum prepared against As 43% antigen reacted with a significantly higher P value against native BSA than against all other Andersen sampler test antigens. Antiserum prepared against antigen As 62% showed a significantly lower P value against As 62% than against native. With reference to Fig. 9, antiserum prepared against As 85%

Table 8. Summary of Analysis of Variance of Data in Table 7*

Source of Variation	Degree of Freedom	Sums of Squares	Mean Squares	F Ratio
Animals	22	9306.5173	423.0253	3.97**
Treatment	8	13097.4562	1637.1820	15.38**
Error	176	18747.2463	106.5184	

*Data of Birds No. 9, 1, 4, and 13 omitted. Data in Table 7 transformed to arcsin $\sqrt{P}$ value

**$P \leq 0.001$

Fig. 8. Mean Transformed P Values Obtained from Reactions of Seven Sources of Antigen with Antiserum Prepared Against: Nat BSA, As 43% and As 62%

Test Antigens: A = Nat C = As 62% E = At 43% G = At 85%
B = As 43% D = As 85% F = At 62%

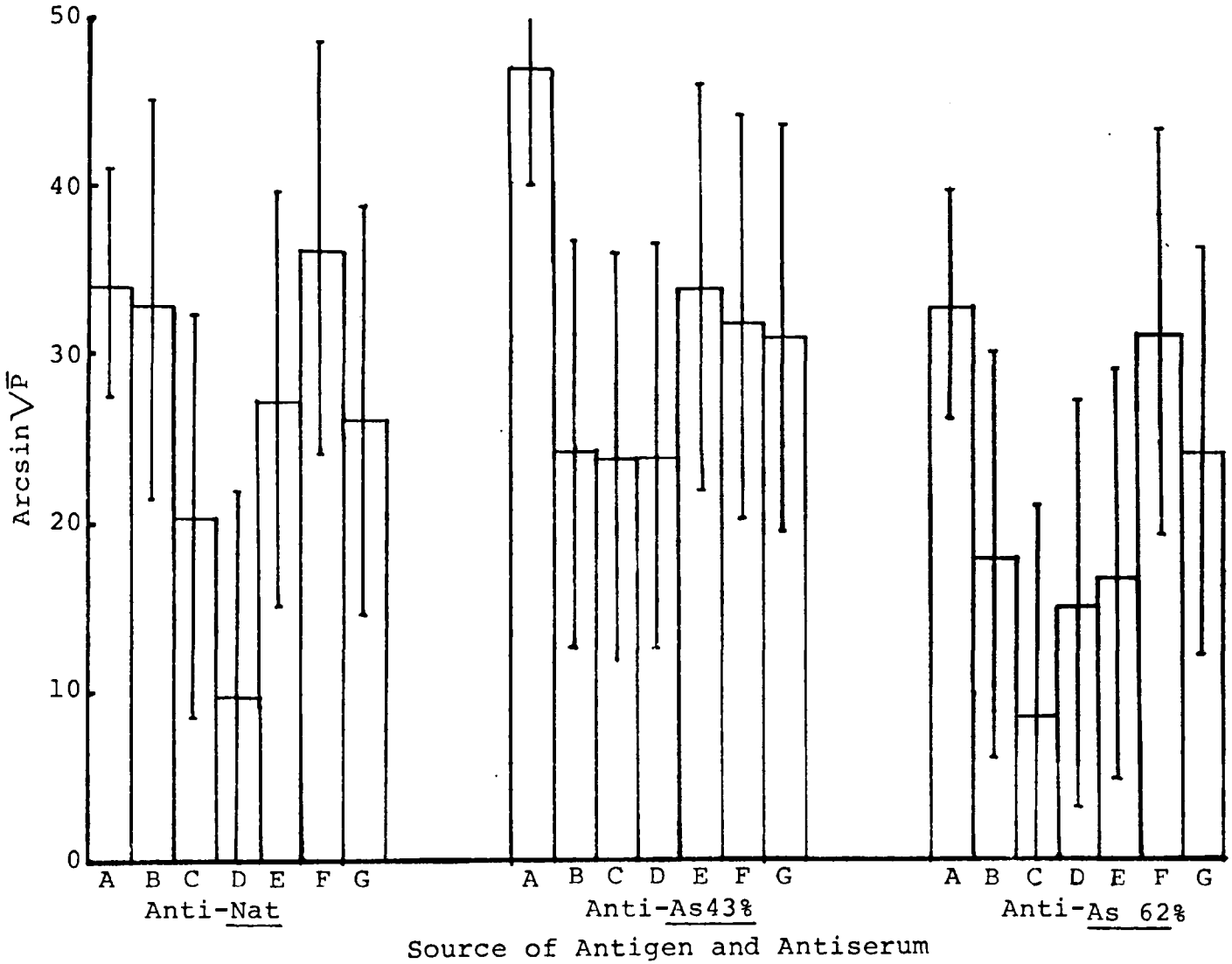

Fig. 8. Mean Transformed P Values for Nat BSA, As 43% and As 62%

Fig. 9. Mean Transformed P Values Obtained from Reactions of Seven Sources of Antigen with Antiserum Prepared Against: BSA As 85%, At 43%, and At 62%

Test Antigens: A = Nat C = As 62% E = At 43% G = At 85%
B = As 43% D = As 85% F = At 62%

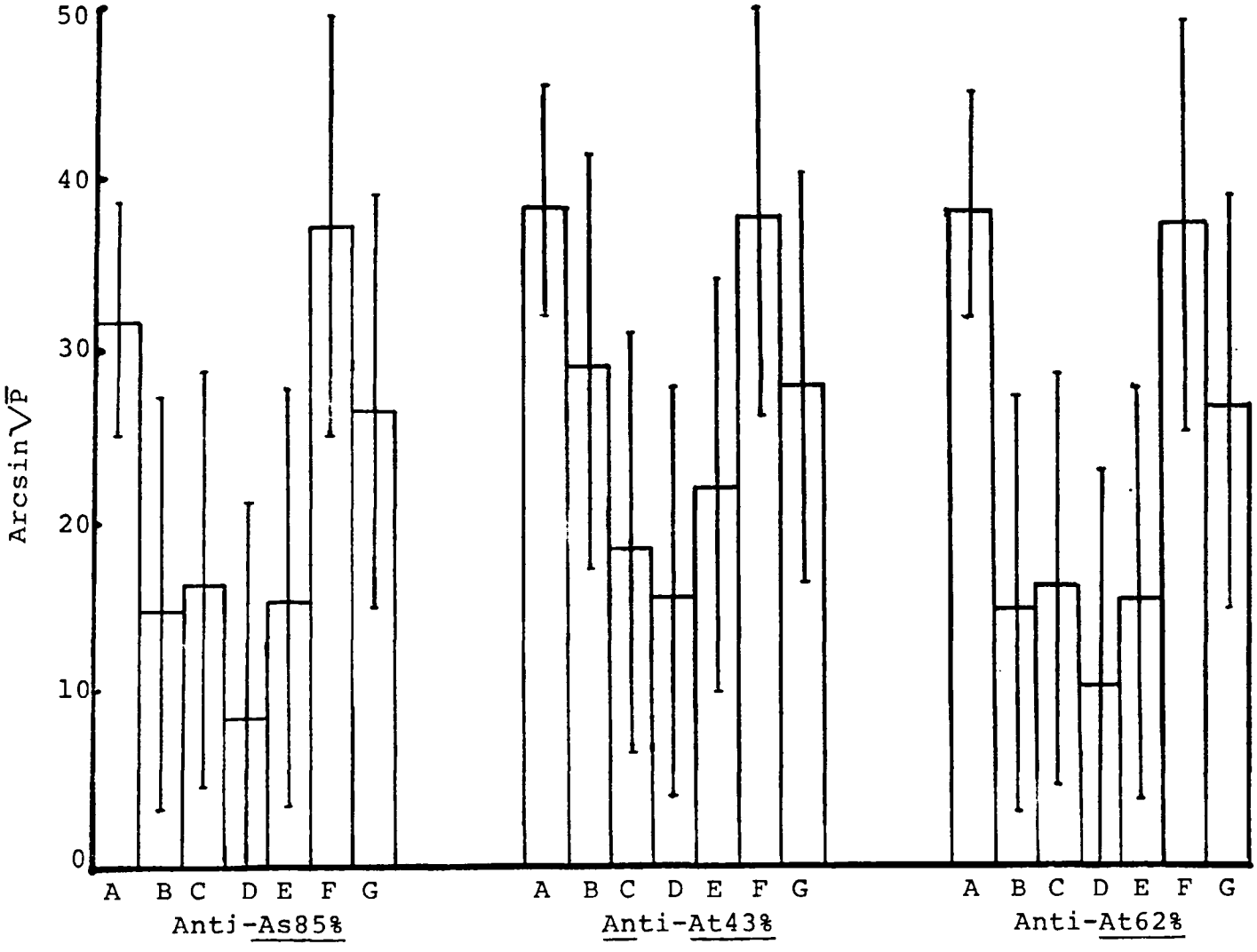

Fig. 9. Mean Transformed P Values for BSA As 85%, At 43% and At 62%

reacted with a significantly lower P value against As 85% than against native test antigen. Antiserum prepared against At 43% reacted with significantly lower P values against As 62% and As 85% test antigens than against Nat test antigen. Antiserum prepared against At 62% reacted with significantly lower P values against As 43%, As 62%, As 85%, and At 43% test antigens than it did against native, and reacted with significantly lower P values against As 85% than against At 62% test antigen.

DISCUSSION

Bovine serum albumin is a blood protein with a molecular weight of about 69,000. It has a prolate shape (16) with an isoelectric point of pH 4.64, and in normal physiological pH ranges, migrates toward the anode. It has three distinct antigenic sites (11, 13).

The experimental approach taken in this study assumed that possible changes in the properties of BSA might be attributed to (a) the violent refluxing and foaming that occurred in the Collison atomizer; (b) the dehydration and subsequent rehydration of finely dispersed aerosol particles, and (c) the duration of the airborne state and its concomitant relative humidity.

Physical limitations of the aerosol techniques and existing equipment necessitated maintaining a constant average cloud age of approximately 1.37 min. It was possible to vary the humidity over three levels, 43%, 62%, and 85% R.H. The particles collected in the Andersen sampler at 62% and 85% R.H. adhered tenaciously to the stainless steel collecting plates, thereby permitting aerosol generation times of from 5 to 7 min without overloading the sampler. In this fashion, it was possible to accumulate sufficient protein at each of these relative humidities with the recovery of from 25 to 35 samples.

In collecting the BSA from aerosols with 43% R.H., the aerosol particles deposited on the steel plates as a finely-divided white powder, and samples could not exceed 4 min generation time without suffering severe wall loss and loss to the vacuum source of collected deposits which were dislodged by the sampling airstream. For this reason, more than 60 separate samples were required to accumulate sufficient protein at this low R.H. This necessitated operating the atomizer for approximately twice the duration of the trials at other humidities.

Some change in the protein can be attributed to the atomizer itself, namely, a change in the isoelectric point. There was a change in the absorption spectrum, but this change was not apparent to the eye. The results of Sephadex chromatography showed that At 43% was different from native and the other atomizer conditions. Relative humidity should play no role upon the environment within the atomizer. The primary compressed air to the device was the same in all trials and the relative humidity is achieved by the moisture content of the air which mixes with the aerosol as it exits the atomizer. Therefore, the likely cause for At 43% being different from native and the other atomizer samples must be attributed to the longer operation interval of the atomizer at 43% R.H. This apparent reduction of molecular weight must be caused by the

shearing stresses and foaming in the atomizer operation. There were no differences in the Preer test with respect to native and atomizer samples except in one case: At 43% test antigen reacted with a significantly lower P value with antiserum against At 62% test antigen than did native test antigen. With all antisera prepared against BSA, with exception of anti-Nat and anti-As 43%, At 43% test antigen reacted with consistently lower P values than At 62% and At 85% test antigens. Severe agitation and the foaming effect of the Collison atomizer are inherent in this device; others, such as the vibrating reed, do not exert this effect (25).

Lower P values suggest that the treated antigens employed in the test are reacting more weakly, but are still capable of stimulating animals to produce antibody. The antisera were generally comparable in their reaction pattern with the various test antigens and the antisera all reacted with moderately high P values against native BSA. These results are interpreted as evidence that all the treated BSA samples were comparable in their immunogenesity, that is, their ability to induce the production of antibody when administered to the chickens.

A slight reduction of the isoelectric point of BSA was observed after the protein was agitated in the atomizer. The airborne state at each of the three conditions

of humidity did not cause any further apparent change in the isoelectric point.

Changes were distinct in absorption spectra as well as visible color of both the aerosol deposits and their reconstituted solutions. None of these changes in color were evident in the foam or fluids contained in the atomizer. Furthermore, the particular color or absorption spectra were related to the relative humidity of the aerosol.

Sephadex G-200 exclusion chromatography of As collections over all humidities differed significantly from each other as well as from native (Fig. 4). As 43% eluted more rapidly than As 62%; As 85% eluted slightly more slowly than native. This was interpreted as indicating that the average molecular weight for BSA aerosolized at 43% R.H. was increased, with an early peak at fraction nos. 65 and 66, suggesting some polymerization may be occurring. The BSA collected from the aerosol at 85% R.H. eluted more slowly, which would imply that it had a slightly lower molecular weight than the Nat BSA. This might occur if strong physical shearing forces in the atomizer were effective against the protein. Why a similar change was not observed in the elution curve for At 62% BSA cannot be explained. The analysis of K_d values, as illustrated in Fig. 1, agrees with the general analysis of fraction

volumes as illustrated in Figs. 3 and 4 with the exception of the average transformed K_d value for As 85%, which would imply a larger molecular weight than for the Nat BSA, whereas the curve in Fig. 4 suggests otherwise.

Results of the Preer test, which was an attempt to obtain quantitative precipitation information on the antigen-antibody reactions, were so variable that considerable reservation is required in interpreting the data. A corresponding increase in P values was not observed as the concentration of antigen used in the test was decreased. Also, as can be seen in Table 7, the P values for 24 tests representing three replicate P values estimated for precipitation occurring between anti-Nat sera (birds nos. 2 through 28, omitting no. 9) and Nat test antigen varied from 0.000 to 0.660.

However, since the accumulated tests represented considerable inherent replication, an attempt was made to analyze the data in Table 7 with omission of the anti-At 85% sera. As 85% reacted similarly to all antisera; so also did As 62% and As 43%. But As 85% reacted with significantly lower P values against anti-Nat, anti-As 43%, anti-As 85%, anti-At 43% and anti-At 62% than did native test antigen. As 62% reacted with significantly lower P values versus anti-As 43%, anti-As 62%, anti-At 43% and anti-At 62% than did native antigen.

As 43% reacted with significantly lower P values against anti-As 43% and anti-At 62% than did native. Fractions from the early peak obtained in the Sephadex chromatography of As 43% was tested by the Preer test, Ouchterlony plates, and immunoelectrophoresis against anti-Nat and anti-As 43% sera; no precipitation reaction was observed.

Changes in BSA which were observed as the result of aerosolization at different humidities were manifested as differences in absorption spectra, isoelectric point, apparent molecular weight and perhaps solubility of antigen-antibody complexes. From an immunological viewpoint, these alterations in the antigen must be rather subtle, since the observed reactions in the Ouchterlony plates were homogeneous. These results using a relatively simple and purified protein as a model suggest that similar and perhaps more profound immunological alterations would be expected to occur if more complex antigens became airborne under certain conditions. The antigenic surface components of viruses and bacteria, and perhaps common allergens, would undoubtedly serve as examples of such complex antigens.

It is not known how severe the changes in surface proteins must be in microbial agents before viability is adversely affected. Numerous studies have shown that for many airborne agents, viability is strongly influenced by relative humidity and the composition of the suspending

media. Phenotypic variations, which are separable from death of the microbes recovered from the airborne state, have been observed. One such variation, diminished infectivity, is of considerable epidemiological importance (20). It would seem plausible that for some agents, aerosolization could alter the immunological properties, albeit only temporarily, without necessitating the death of the organism. In the antigenic surface structure of a microbe, which can be imagined as a mosaic complex, a very subtle alteration of one or more components could conceivably lead to transient but significant changes in the overall immunological properties.

SUMMARY

The effects of the airborne state upon bovine serum albumin, aerosolized from a Collison atomizer at relative humidities of 43%, 62%, and 85% were studied by exclusion chromatography, electrophoresis, immunoelectrophoresis, Ouchterlony and Preer precipitin tests, and absorption spectrography. Analysis of the data acquired by these techniques indicated that:

1. The agitation and foaming of the protein by the atomizer resulted in a slight reduction of its isoelectric point. Upon prolonged atomizer operation, the protein remaining in the device had (a) a slightly lower molecular weight as judged by chromatography with Sephadex G-200; (b) an altered absorption spectrum in the visible light region, and possibly a decreased (c) efficiency in inducing immunological response of sensitized birds as judged by the results of the Preer test.

2. Finely divided aerosol particles of bovine serum albumin, before and after rehydration, acquired color differences and alterations of the absorption spectrum in the visible light region. These changes were related to the relative humidity of the aerosols.

3. Aerosolized bovine serum albumin, when reconstituted into solution, possessed differences in apparent

molecular weight as judged by chromatography with Sephadex G-200. If aerosolized at 43% R.H., the reconstituted protein exhibited a larger apparent molecular weight than the original protein and possessed a distinct fraction which was excluded earlier from the chromatographic column. If aerosolized at 85% R.H., the reconstituted protein exhibited a slightly smaller than normal apparent molecular weight. If aerosolized at 62% R.H., the protein exhibited an average molecular weight slightly larger than normal apparent molecular weight.

4. As judged by the results of the precipitin tests, significant interactions were observed between source of antigen (aerosol or atomizer), relative humidity of the aerosols, and the antisera which were prepared against the atomizer, aerosol, and normal (native) BSA samples.

5. Antisera prepared against the untreated and treated BSA produce an homogeneous precipitate reaction against all the BSA antigens.

APPENDIX A

TABLES SHOWING RELATIVE PROTEIN CONCENTRATIONS IN 1-ML FRACTION VOLUMES FROM CHROMATOGRAMS OF BOVINE SERUM ALBUMIN SAMPLES WITH SEPHADEX G-200

Table A-1. Protein Content of Native BSA Fractions*

Native No. 1		Native No. 2	
Volume (ml)	Protein Concentrations	Volume (ml)	Protein Concentrations
80	.1	82	.4
81	.3	83	.7
82	.5	84	.7
83	.5	85	1.4
84	.8	86	1.4
85	.8	87	2.0
86	1.0	88	2.5
87	1.3	89	2.5
88	1.5	90	5.0
89	1.7	91	5.7
90	2.0	92	6.4
91	2.4	93	8.7
92	3.2	94	10.1
93	4.0	95	8.0
94	4.7	96	8.0
95	5.0	97	7.2
96	6.2	98	5.7
97	7.5	99	4.7
98	6.7	100	2.9
99	6.7	101	2.2
100	6.2	102	1.5
101	5.0	103	.7
102	5.1	104	.7
103	4.2	105	.1
104	4.0	106	.1
105	3.2		
106	2.7		
107	2.2		
108	2.0		
109	1.5		
110	1.6		
111	1.3		
112	1.0		
113	.5		
114	.5		
115	.5		
116	.5		
117	.5		
118	.2		
119	.2		
120	.1		

*Data rounded to nearest 1/10th decimal point.

Table A-2. Protein Content of BSA Fractions at 43% R.H.*

As 43%			At 43%		
Volume (ml)	Protein Concentrations		Volume (ml)	Protein Concentrations	
	No. 1	No. 2		No. 1	No. 2
62	.3	.1	81	.1	.1
63	.6	.3	82	.1	.2
64	.9	.7	83	.3	.4
65	1.3	.7	84	.3	.6
66	1.3	.5	85	.3	.8
67	.9	.2	86	.6	.9
68	.7	.2	87	.9	1.0
69	.5	.2	88	1.1	1.4
70	.5	.4	89	1.3	1.6
71	.5	.5	90	1.7	1.8
72	.6	.5	91	1.9	1.9
73	.7	.5	92	2.2	2.1
74	.9	.7	93	2.6	2.8
75	1.0	.8	94	2.9	3.2
76	1.1	1.2	95	3.9	4.1
77	1.5	1.3	96	4.4	5.3
78	1.6	1.5	97	5.1	6.3
79	1.9	1.7	98	5.9	7.4
80	2.1	1.9	99	7.3	8.6
81	2.5	2.3	100	7.8	8.6
82	2.7	2.7	101	8.2	8.9
83	3.5	3.3	102	7.9	7.8
84	4.3	3.9	103	7.5	7.2
85	5.4	5.0	104	7.1	6.2
86	6.4	5.7	105	5.6	4.4
87	7.5	6.8	106	4.1	3.0
88	7.9	7.2	107	2.9	1.9
89	8.8	7.5	108	2.0	1.3
90	7.7	7.7	109	1.3	.5
91	6.4	7.0	110	.8	.2
92	3.9	5.7	111	.5	
93	2.9	4.8	112	.1	
94	1.8	3.7			
95	1.1	2.8			
96	.5	1.9			
97	.3	1.3			
98	.2				
99	.2				

*Data rounded to nearest 1/10th decimal point.

Table A-3. Protein Content of BSA Fractions at 62% R.H.*

As 62%			At 62%		
Volume (ml)	Protein Concentrations		Volume (ml)	Protein Concentrations	
	No. 1	No. 2		No. 1	No. 2
79	.2	.1	80	.1	.1
80	.4	.4	81	.3	.4
81	.4	.5	82	.4	.4
82	.7	.5	83	.4	.4
83	.8	.9	84	.5	.7
84	1.2	.9	85	.5	.7
85	1.5	.9	86	.6	.8
86	2.6	1.1	87	1.0	1.3
87	3.7	1.2	88	1.2	1.6
88	3.5	1.2	89	1.8	2.4
89	7.5	1.5	90	2.4	3.2
90	7.6	2.5	91	3.1	3.6
91	9.8	3.3	92	4.2	4.4
92	9.7	5.3	93	4.3	6.4
93	9.7	7.5	94	6.2	7.4
94	9.0	7.7	95	7.0	8.3
95	7.5	8.8	96	7.8	9.8
96	7.0	9.9	97	8.3	10.7
97	5.1	9.1	98	8.4	9.1
98	4.4	8.4	99	8.0	7.6
99	3.0	7.1	100	7.4	6.4
100	1.9	5.6	101	6.2	4.4
101	1.4	5.0	102	5.0	2.4
102	.5	3.5	103	4.0	1.6
103	.4	2.5	104	3.1	1.3
104	.4	1.7	105	2.4	1.0
105	.1	1.2	106	1.7	.8
106		.9	107	1.1	.7
107		.5	108	1.0	.6
108		.3	109	.6	.8
109		.1	110	.3	.4
			111	.3	.2
			112	.1	.2
			113	.1	.1
			114	.1	

*Data rounded to nearest 1/10th decimal point.

Table A-4. Protein Content of BSA Fractions at 85% R.H.*

As 85%			At 85%		
Volume (ml)	Protein Concentrations		Volume (ml)	Protein Concentrations	
	No. 1	No. 2		No. 1	No. 2
82	.2	.2	80	.3	.3
83	.4	.2	84	.4	.7
84	.4	.5	85	1.0	1.2
85	.4	1.0	86	1.4	1.4
86	.6	1.1	87	.8	1.7
87	.7	1.3	88	.6	1.3
88	1.3	1.3	89	.8	1.3
89	.9	1.7	90	.8	2.3
90	1.3	1.7	91	1.9	2.5
91	1.4	2.0	92	2.5	3.1
92	1.7	3.3	93	3.2	3.3
93	2.0	3.3	94	4.9	3.3
94	3.1	4.0	95	5.2	1.6
95	4.4	5.1	96	5.8	4.5
96	3.9	6.0	97	5.7	4.5
97	3.9	7.7	98	6.1	5.4
98	6.6	7.8	99	6.3	6.1
99	7.1	7.9	100	6.1	6.1
100	7.9	7.7	101	5.7	5.0
101	8.8	7.5	102	5.2	5.4
102	7.1	7.2	103	4.9	5.4
103	7.0	5.5	104	4.5	4.6
104	6.7	4.6	105	3.8	4.1
105	6.3	3.3	106	3.4	3.3
106	5.5	3.0	107	3.2	3.0
107	4.4	2.1	108	3.1	3.0
108	2.6	1.3	109	2.8	2.8
109	1.7	1.0	110	2.6	2.8
110	1.1	.5	111	2.4	2.3
111	.4	.1	112	2.1	2.1
112	.1		113	1.4	2.0
			114	.7	1.4
			115	.3	.7
			116	.1	.3
			117		.1

*Data rounded to nearest 1/10th decimal point.

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