photomicrograph of a crystalline preparation described as lysozyme but which might be lysozyme acetate, since it was obtained by evaporation of a solution in 0.05 *N* acetic acid. Although crystallographic examination of the material was to have been undertaken, no publication of such results has been found. The shape of the crystals pictured appears to be that of a rhombic dodecahedron and the face angle, measured as carefully as possible on the picture, is 110°, which is correct for that form. The corresponding angle, measured on the crystals of lysozyme chloride, is 144°; thus the two preparations must be different, although they do look somewhat similar. Since the refractive index measurements reported here were made, a preparation of lysozyme chloride containing hydrochloric acid in place of the acetic acid used in the original preparation has been examined. The crystals have the same shape as those grown in the acetate-containing solution. The face angle is the same and the optical properties are the same, so far as has been determined, although the refractive indices were not measured because the preparation was not suitable for that purpose. Attempts to prepare lysozyme acetate have not yielded a crystalline product suitable for crystallographic examination.

Acknowledgment.—Thanks are due Mr. Gordon Alderton for making the lysozyme preparations used in this work, and to Mr. L. M. White for the moisture determinations.

Summary

The optical and crystallographic properties of single crystals of lysozyme chloride have been determined on air-dried and on wet crystals. The refractive indices have been found to vary with moisture content, but the birefringence remains constant.

ALBANY, CALIFORNIA RECEIVED DECEMBER 31, 1945

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Peptic Digestion of Human Gamma Globulin

BY WILBUR B. BRIDGMAN[1]

It has been shown by a number of workers, chiefly Pope,[2] that when horse diphtheria antitoxin is acted upon by pepsin at *p*H 4.0, the product may be separated into a more soluble portion containing the antibody activity and a less soluble portion that is inactive. It was later demonstrated in this Laboratory by sedimentation and diffusion constant measurements[3] that the active portion consists of half the original antibody globulin particle, which has been split in a plane normal to its long axis. Similar results have been obtained with tryptic digestion.[4] In a study of the effect of pepsin on bovine serum globulins[5] it was found that at hydrogen ion concentrations in the neighborhood of *p*H 4.0 the splitting stops with halves of the original particle, while in more acid solutions (*p*H 2) digestion proceeds farther to quarter particles and a much larger proportion of small (dialyzable) fragments.

In the present study human serum globulin has been digested by pepsin. The degree of splitting attained has been followed by ultracentrifugal analysis, and the products have been characterized physico-chemically. The effect of digestion on antibody activity also has been determined.

Methods.—The globulin used was the "Fraction II"[6] obtained by the low temperature alcohol fractionation of human plasma.[7,8,9] Sample II-55 contained 6% albumin on electrophoretic analysis; II-37 contained 10% albumin and 3% beta globulin. Some experiments were also done on electrophoretically pure gamma globulin (99%) obtained from Fraction II by two precipitations of the crude globulin with 40% saturated ammonium sulfate. This has been designated F II-40.

The pepsin used for the digestions was crystallized by the method of Philpot[10] from Parke-Davis or Armour 1:10,000 pepsin, and stored in the cold in acetate buffer at *p*H 5. Its activity was determined by the hemoglobin method of

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(2) C. G. Pope, *Brit. J. Exptl. Path.*, **19**, 245 (1938); **20**, 132, 201 (1939).

(3) M. L. Petermann and A. M. Pappenheimer, Jr., *J. Phys. Chem.*, **45**, 1 (1941).

(4) J. H. Northrop, *J. Gen. Physiol.*, **25**, 465 (1941-1942); A. Rothen, *ibid.*, p. 487.

(5) M. L. Petermann, *J. Phys. Chem.*, **46**, 183 (1942).

(6) Supplied by Dr. E. J. Cohn of the Department of Physical Chemistry, Harvard Medical School. The preparation of this fraction was developed in the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University (*cf.* Ref. 7).

(7) (a) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H., Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944); (b) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, in preparation. The preparations of Fraction II used in the present studies were prepared by the method denoted as Method 2 in the paper of Oncley, Melin, Richert, Cameron and Gross. Some of the later methods described by these authors yielded gamma globulin preparations of 99% purity or better.

(8) J. F. Enders, *J. Clin. Invest.*, **23**, 510 (1944).

(9) J. W. Williams, M. L. Petermann, G. C. Colovos, M. B. Goodloe, J. L. Oncley and S. H. Armstrong, Jr., *ibid.*, **23**, 433 (1944).

(10) J. St. L. Philpot, *Biochem. J.*, **31**, 54 (1937).

Anson.[11] The pepsin used in these particular studies had an activity of 0.29 hemoglobin unit per milligram of nitrogen.

In this study mainly small-scale experiments were conducted. The purpose of the work was to determine the conditions for a maximum yield of half-molecules. The optimum hydrogen ion concentration, pepsin:globulin ratio, and time of digestion were sought.

All digestions were carried out in the cold, at +1 to +4°. The *p*H was fixed by dialysis against citrate buffers or by the addition of solid citric acid. Times of digestion ranged from sixteen hours to eighteen days, most experiments lasting about two days. To stop the process of digestion, samples were dialyzed in Visking tubing against phosphate buffers either of *p*H 8.2 or 7.0. All buffers contained 0.2 *M* sodium chloride.

Total nitrogen was determined by a semi-micro Kjeldahl procedure.

The molecular mass spectra of the non-dialyzable fractions of the digests were obtained by means of sedimentation analysis in the Svedberg oil-turbine ultracentrifuge, according to the usual technique.[12] Scale line displacement–distance diagrams obtained after centrifugation at 60,000 r. p. m. were resolved into their separate components, and the relative amount of each measured (see Fig. 1).

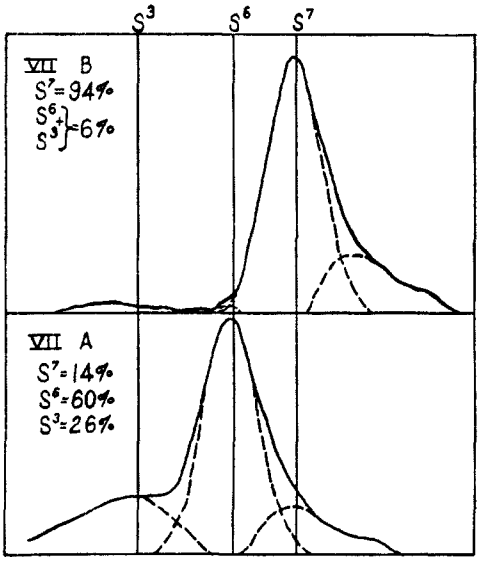


Fig. 1.—Sedimentation diagram showing the action of pepsin on human gamma globulin: VIIB, four days at *p*H 3.5, no pepsin; VIIA, four days at *p*H 3.5, 0.67 unit of pepsin per gram of globulin.

The normal globulins consisted mainly (80%) of material of sedimentation constant, $S_{20} = 7.1$ *S* with some fifteen per cent. of more rapidly sedimenting material and about five per cent. which was lighter in weight (Experiment VII B, Table I and Fig. 1). In the peptic digests various amounts of two new components appeared. These had sedimentation constants of 5.8 and 3.1 *S* (Experiment VII B, Fig. 1). For convenience these various components are referred to in this paper by their approximate sedimentation constants. The normal globulin (including the faster material) is called S^7, and the two components which appear during digestion are called S^6 and S^3. Since the sedimentation rate of denatured pepsin is about 2.7 *S* the small amount of pepsin present is included in the S^3 boundary.[10]

In the analysis of the line displacement–distance diagrams some difficulty was encountered in assigning exact values to the areas under the curves obtained. This was caused by uncertainty in the location of the base lines in the sedimentation diagrams. As a rule the base line was drawn horizontally through the point of minimum scale line displacement. Error from this source is greatest for components present in small amount; for the principal components, S^7 and S^6, it probably does not exceed five per cent. in most cases.

As is often the case with plasma proteins, the area under the sedimentation diagrams accounts for less protein than is indicated by the nitrogen determinations. In the early experiments the value for the refractive index increment for globulin was taken as 0.0022.[13] Our own experiments indicate a low value for this increment, $\alpha = 0.00185$, in agreement with that used by previous workers for horse serum.[14] In all the sedimentation experiments the centrifuge cell was covered with mineral oil. The effect of this oil in lowering the refractive index values obtained from line displacement measurements has been discussed by Pedersen.[15]

All sedimentation analyses were made in a solution which contained 0.20 *M* sodium chloride and phosphate–phosphate buffer of ionic strength 0.02, at *p*H 7.0.

Results

Preliminary experiments were made at acidities of *p*H 4.4, 4.0, 3.5 and 2.9. The largest yield of half-molecules (S^6) was obtained at *p*H 3.5, and this *p*H was used in later experiments. At *p*H 4.4, 70% of the protein was lost through the dialysis bag in eighteen days, but the remainder showed no definite splitting into distinct size classes (see Table I). At *p*H 4.0 it took ten days for 60% of halves to be formed. Digestion for three days at *p*H 3.5 resulted in an apparent state of equilibrium with 60 to 70% of the non-dialyzable protein present as half-molecules. Longer digestion caused a slight increase in the amount of dialyzable protein, but no significant change in the size distribution of the non-dialyzable fraction (see Expts. II, V, VIII and IX). Varying the

(11) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(12) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, 1940.

(13) A. S. Macfarlane, *Biochem. J.*, **29**, 660 (1935).

(14) A. S. Macfarlane, *ibid.*, **29**, 407 (1935).

(15) K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fractions," Uppsala, 1945.

TABLE I

THE PEPTIC DIGESTION OF HUMAN GAMMA GLOBULIN

Expt.	Globulin	Pepsin P. U.Hb per g. globulin	pH	Time, days	% Protein	Sedimentation (non-dialyzable fraction) S^T %	S^6 %	S^3 %	Remarks
I	II—55, 2.2%	?	4.0	0	2	89	7	4	
				2		71	13	16	
				4		41	49	10	
				5.3		33	54	13	
				9.5		26	62	12	
				18	0.7	22	60	18	
II	II—37, 1.8%	1.0	3.5	0	1.4	70	30		
				0.5	1.1	21	47	32	
				1.3	0.9	14	61	25	
				3.0	1.0	10	68	22	
				7		10	68	22	Soluble fraction
				7		no observable peak			Insoluble fraction
III	F I 35,[a] 2.0%	0.8	2.9	0	1.8	100			
				0.3		13	56	31	
				11		about the same as 0.3 days			
IV	F I 35, 2%	.8	4.4	0.3		87	31		
				1.2		93	7		
				18	0.6				
V	F II 40, 6.8%	.68	3.4	3	1.8	16	72	12	
				3	1.6	24	67	9	Soluble in water
VI	F II 40, 3.8%	1.1	3.4	3	1.7	12	70	18	
VII A	F II 40, 6.9%	0.67	3.5	4	1.5	14	60	26	
B	F II 40, 6.9%	0	3.5	4	1.5	94	6		Essentially starting material
VIII	II—48, 10.5%	.67	3.5	3	1.9	15	60	25	pH attained by dialysis
IX	II—48, 6.8%	.67	3.5	3	1.5	13	56	31	pH attained by addition of citric acid
XI	II—72, 19%	1.0	3.5	5	1.6	20	70	10	

									——% distribution total N——			
									S^T	S^6	S^3	Dialyzable
XII	F II 40	0.8	3.5	3								
		.08	3.5	3	1.3	13	58	29	9	41	21	29
		.008	3.5	3	1.3	25	50	15	31	46	13	10
		.0008	3.5	3	1.3	87	10	3	87	10	3	0

[a] Sample F I 35 is globulin II—37 after fractional precipitation with 35% saturated ammonium sulfate.

initial globulin concentrations from 1.5 to 19% did not affect the results.

Digestion proceeded at the same rate when the pH was adjusted to 3.5 rapidly, by the addition of solid citric acid, as it did when the sample was dialyzed against pH 3.5 citrate buffer for twenty-four hours before the addition of pepsin (Experiments VIII and IX). Control experiments in which water was added instead of pepsin showed that the splitting was due to the pepsin and not to acidity alone (Experiment VII). The effect of changing the ratio of pepsin to globulin can be seen in Experiment XII. A reduction from 0.8 to 0.08 unit per gram of globulin did not slow the digestion perceptibly. Even 0.008 unit per gram was quite effective.

Fractionation.—Ultracentrifugal analysis has shown that these globulin systems after digestion contain a number of molecular species. In an attempt to isolate the half-molecules, a number of fractionation procedures have been applied to the digests.

In fractionation with ammonium sulfate at pH 8.0, the bulk of the digest (66%) precipitated between 35 and 45% saturation. The composition of the precipitate, as shown by ultracentrifugal analysis, did not differ significantly from that of the starting material (65% S^6).

Fractionation with ethyl alcohol was next

TABLE II

RESULTS OF FRACTIONATION EXPERIMENTS

Fraction	% C_2H_5OH	% Ppt'd.	Sedimentation S^T	S^6	S^3
Digest V			13–19	72	10–15
Fr. A 1	13	7			
2	20	20	33	49	18
3	24	19	29	58	14
4	29	13	20	75	5
		59			
B 1	22	46	Inconclusive, mostly S^6 or		
2	29	12	heavier		
3	32	13			
4	36	16	18	79	4
		87			

TABLE III

MOLECULAR CHARACTERISTIC CONSTANTS FOR GAMMA GLOBULIN PREPARATIONS

	% Protein	S_{20}	D_{20} a	D_{20} m	D_{20} Av.	Mol. wt.	f/f_0	a/b	Length Å.	Width Å.
Normal globulin (Pedersen)		7.1			4.0	153,000	1.51	9.0	305	33.9
Normal globulin		7.3	3.81	3.57	3.7	170,000	1.58	10.5	350	33.3
Digested globulin	0.8	5.7	4.07	4.67						
Digested globulin	.5	5.9	4.84	4.70						
Digested globulin[a]	.7		4.54	4.67						
Digested globulin[a]	.7		4.50	4.95						
		5.8			4.6	109,000	1.47	8.7	266	30.5
Hypothetical half-molecule		5.7			5.8	85,000	1.26	5.3	175	33.3

[a] Experiments of Dr. Mary L. Petermann of this Laboratory.

tried. Ten cc. of a 5% solution of Digest V (see Table I) was dialyzed against the *p*H 7.0 buffer described above, and ethyl alcohol, 40% by volume, was added slowly with mechanical stirring in an ice-bath. The results are given in Table II, A. The 24 and 29% alcohol fractions were recombined and dialyzed against a *p*H 7.0 buffer containing 0.04 *M* sodium chloride. Again 40% ethanol was added to give the series of new fractions outlined in Table II, B.

Other fractionations were no more successful. Although the undigested globulin is somewhat less soluble than the split products, the difference is not great enough to ensure clear-cut fractionation.

The undigested gamma globulin, F II 40 (99% gamma), could be dialyzed against distilled water without the appearance of any appreciable precipitate. However, dialysis against *p*H 8.0 buffer, or digestion, or both, always altered the protein so that some cloudiness formed on dialysis against water. A sample of Digest V was dialyzed against water and some precipitate separated. The remaining soluble portion had essentially the same size distribution as the original digest (Table I). The precipitate was only partially soluble in *p*H 7.0 buffer of ionic strength 0.22, and may therefore have consisted of denatured material.

Molecular Size Determinations.—Several diffusion experiments were carried out in the Lamm cell.[16] The boundary spreading was measured by the scale-line displacement method. Diffusion constants were calculated by the height and area method (values marked *a* in Table III) and also by the method of moments (values marked *m*). Sedimentation constants obtained for the same preparations at the same protein concentration are also given in Table III. Molecular weights for the undigested and digested globulins were calculated from these sedimentation and diffusion constants and an assumed partial specific volume of 0.718, the value found by Pedersen.[15] Values of molar friction constant, axial ratio and molecular dimensions have been calculated in the usual way by assuming an ellipsoidal particle.[12] Pedersen's data on human gamma globulin are included in the table for comparison.

(16) O. Lamm, *Nova Acta Regiae Soc. Sci. Upsaliensis*, (4) **10**, No. 6 (1937); see also H. Neurath, *Chem. Rev.*, **30**, 357 (1942).

The molecular weight calculated from the experimental values for sedimentation and diffusion constants, 109,000, is larger than would be expected for halves of gamma globulin. The reason for this discrepancy is illustrated in Fig. 1, A. From this it may be seen that the S^6 boundary is well resolved in the ultracentrifuge, so that its sedimentation constant may be determined with considerable accuracy. With the diffusion constant measurement on the other hand the order of accuracy is very much less. The constants obtained represent the average diffusion rate of mixtures which never contained more than 85% of S^6 on ultracentrifugal analysis. The sedimentation and diffusion constants of a hypothetical half molecule have therefore been calculated, and these values are included in Table III. The calculated sedimentation constant agrees very well with the experimental values. The calculated diffusion constant is, as would be expected, considerably higher than any of the measured values obtained.

Immunological Assays.—The globulin preparation II G 72 was tested for antibody activities through the courtesy of Dr. J. F. Enders of the Department of Bacteriology of the Harvard Medical School.[8] The results are given in Table IV. The typhoid "O" agglutinin has been lost while the "H" agglutinin concentration remains unchanged. This agrees with the findings of Rosenheim[17] on horse Typhoid "O" and "H" agglutinins. The concentrations of diphtheria

TABLE IV

IMMUNOLOGICAL ASSAYS ON II G 72 BEFORE AND AFTER PEPSIN TREATMENT. POTENCY IS RELATIVE TO THAT OF A STANDARD GLOBULIN AT THE SAME PROTEIN CONCENTRATION

Immunological tests	May, 1943 Normal	May, 1943 Split	May, 1944 Normal	May, 1944 Split
Typhoid "O" agglutinin	0.7	0	0.7	0
Typhoid "H" agglutinin	0.7	0.7	.5	.5
Diphtheria antitoxin	0.4-0.6	0.4-0.6	.5	.5
Influenza A, Hirst test	0.5	0.5	1.0	.5
Influenza A, mouse protection	1.1	1.0	1.1	.6
Streptococcus antitoxin			1.0	1.0

(17) A. H. Rosenheim, *Biochem. J.*, **31**, 54 (1937).

and streptococcus antitoxins and of anti-influenza A likewise are substantially unchanged.

It will be noted as well that the immunological assay values have not fallen off during a one year period.

Discussion and Conclusions

The best conditions for the production of half molecules of human gamma globulin appear to be digestion at *p*H 3.5 for three days with at least 0.05 P. U. Hb per gram of globulin. About 70% of the protein remains in nondialyzable form; of this material 60 to 70% is present as halves of the normal globulin.

If the pepsin ratio is reduced to 0.01 unit per gram of globulin, an equivalent amount of half molecules is formed, but about 20% of unchanged globulin remains. Also, with reduced relative amounts of pepsin, periods of more than three days are necessary for "equilibrium" to be attained at temperatures near 0°.

The advantages of digested gamma globulin antibodies lie in their smaller particle size. The viscosity of concentrated (16%) solutions is much less than that of the unsplit globulin in solution of like concentration. This makes the final product much easier to handle. The smaller molecules may also be presumed to have an increased rate of diffusion, not only through body fluids, but also across membranes.

Acknowledgment.—This work was made possible by a grant from the Rockefeller Foundation. It is also a pleasure to acknowledge the kindness of Drs. Mary L. Petermann and J. W. Williams whose helpful discussions have been of great aid in its performance.

Summary

1. Human gamma globulin is split by pepsin into molecules of half size. Further digestion leads to the formation of smaller particles and ultimately to dialyzable fragments.

2. The yield of half-molecules is greatest when digestion is carried out at *p*H 3.5 for three days, in the cold, with at least 0.05 hemoglobin unit of pepsin per gram of globulin.

3. Attempts to separate the half molecules from both unsplit globulin and smaller fragments by fractionation with ammonium sulfate or ethanol were partially successful.

WORCESTER, MASS. RECEIVED DECEMBER 1, 1945

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

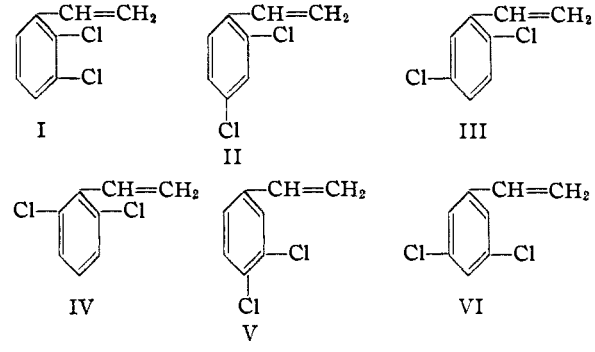
The Preparation and Polymerization of the Six Nuclear Isomeric Dichlorostyrenes[1,2]

BY C. S. MARVEL, C. G. OVERBERGER, R. E. ALLEN, H. W. JOHNSTON, J. H. SAUNDERS AND J. D. YOUNG

Introduction

The recent article of Michalek and Clark[3] reporting the boiling points, refractive indices and densities of the six-nuclear substituted dichlorostyrenes and the properties of one unidentified polydichlorostyrene has prompted us to present here some experiments which have been carried out on these products in our laboratory. Except for the above report, nothing has been found in the literature concerning the 2,3- (I), 2,4- (II), 2,6- (IV) and 3,5- (VI) dichlorostyrenes.

Brooks[4] has reported the synthesis of the 2,5 isomer (III) from 2,5-dichlorobenzaldehyde and the 3,4 isomer (V) from 3,4-dichloroacetophenone. Michalek and Clark[3] state that the 2,5 and 3,4 isomers have been made from the corresponding dichlorobenzaldehyde without giving experimental details. The 2,5 isomer (III) used in our experiments was obtained as a gift from the Monsanto Chemical Company. We have made all



of the other isomeric dichlorostyrenes from the corresponding aldehydes by the Grignard reaction followed by dehydration of the methyl carbinol. These processes are described below. In addition other methods of synthesis are described for certain of the monomers and a brief description and characterization of the polymers of these isomeric dichlorostyrenes is included in this paper.

Experimental

2,3-Dichlorotoluene

Our synthesis of this monomer is outlined below.

We used the method of Hadfield and Kenner[5] to convert *o*-toluidine to 3-nitro-2-aminotoluene. Cohen and

(1) Most of the work described in this manuscript was done under the sponsorship of the Office of Rubber Reserve, Reconstruction Finance Corporation, in connection with the Government Synthetic Rubber Program.

(2) This is the twenty-first communication on vinyl polymers. For the twentieth see THIS JOURNAL, **68**, 736 (1946).

(3) Michalek and Clark, *Ind. Eng. Chem., News Ed.*, **22**, 1559 (1944).

(4) Brooks, THIS JOURNAL, **66**, 1295 (1944).

(5) Hadfield and Kenner, *Proc. Chem. Soc.*, **30**, 253 (1914).