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

The Splitting of Human Gamma Globulin Antibodies by Papain and Bromelin

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Introduction

The early effect of papain on horse diphtheria antitoxin and beef serum globulin[1a] is quite similar to that of pepsin[2,3] and trypsin.[4] The molecules are split into halves, in a plane normal to the long axis. One of the halves of diphtheria antitoxin flocculates with toxin and is soluble at 58° at *p*H 4.2 in the presence of 5% sodium chloride. On prolonged digestion these halves are further split into quarters. None of the quarters is precipitable by toxin, but some of them, presumably from the antitoxin halves, are soluble at 58°. Since the action of pepsin on human gamma globulin antibodies also results in halves,[5] a similar effect might be predicted with papain. The results obtained are quite different from what was expected. Even in the early stages of digestion the chief products are not half molecules, but quarters. The same is true of bromelin digests.

Methods

The gamma globulin antibodies used were the Fraction II prepared by alcohol fractionation of human plasma.[6,7,8] They consisted of from 80 to 97% gamma globulin. The preparation on which papain digest CY-9 was done contained 14% albumin and 3% beta globulin. The globulin used for digest CY-10 contained 3% albumin and 3% beta globulin. Bromelin digest Br 6 was done on a globulin containing 3% beta. According to Pedersen[16] human gamma globulin has a molecular weight of 153,000. Its sedimentation constant is $s_{20} = 7.1\ S$; its diffusion constant, 4.0×10^{-7} sq. cm./sec.; and its partial specific volume is 0.718.

The papain source material was the dry powder, Papain Merck. Its activity was measured by the hydrolysis of hippuryl amide, as described by Balls and Lineweaver.[9] In the first two experiments the powder was ground with buffer at the desired *p*H, kept at 37° in a stoppered flask for two hours after addition of the activator, cooled, and added to the globulin. The large amount of insoluble material present was removed after the digestion. In later work the activation was carried out in a stoppered centrifuge tube. After the suspension had been cooled, the heavier insoluble material was removed by centrifugation and an aliquot of the still cloudy supernatant added to the globulin. Cyanide, hydrogen sulfide or cysteine was used as activator. Details of the digestion are given in Table I.

Bromelin was prepared from the juice of fresh pineapples by the method of Greenberg and Winnick.[10] No unit of bromelin activity has appeared in the literature. The activity was therefore compared with that of papain by the hemoglobin method of Anson.[11] The blue color developed was measured in an Evelyn photoelectric colorimeter with filter no. 720. The amount of digestion obtained in ten minutes with a given amount of enzyme varied from one hemoglobin preparation to another. Good agreement, however, was obtained on any one hemoglobin solution on different days; the same preparation was therefore used for comparing the papain and bromelin. An aqueous extract of papain containing 2.4×10^{-3} [Pa.U.]^{HA} per gram contained one hemoglobin unit per gram. The whole bromelin contained only 0.2 hemoglobin unit per gram, which would be equivalent to 0.5×10^{-3} [Pa.U.]^{HA} per gram. When the papain powder was extracted with salt solutions activity about three times that of the aqueous extract was obtained—7.5×10^{-3}[Pa.U.]^{HA} per gram. Since crude papain is known to be a mixture of enzymes these comparisons indicate only a general order of magnitude.

The dry bromelin was mixed with an equal weight of cysteine hydrochloride and ground in a mortar with phosphate buffer, *p*H 7, ionic strength 0.2. The suspension was activated at 37° for two hours in a stoppered flask. It was then cooled to the digestion temperature and mixed with approximately 20% globulin. Enough phosphate was added to give a final globulin concentration of 5%. The digests stood for periods up to twenty-two days. Further details of these experiments are shown in Table II.

The progress of each digestion was followed by measuring the increase in nitrogen soluble in 10% trichloroacetic acid. One-cc. samples of digest were added to 9 cc. of 11% trichloroacetic acid and the solution filtered after standing for fifteen minutes.

Total and non-protein nitrogen were determined by the micro-colorimetric method of Johnson.[12]

Samples were also withdrawn for ultracentrifugal analysis. These were dialyzed in very slack bags against cold 0.15 *M* sodium chloride containing 0.3% hydrogen peroxide to inactivate the enzyme. The bags were opened occasionally to release the oxygen formed from the peroxide by the small amount of catalase present in these globulins. After dialysis the samples were cleared in an angle-head centrifuge. The digests were studied in the Svedberg oil-turbine ultracentrifuge at 60,000 r. p. m. and their molecular mass spectra determined by analysis of line displacement-distance diagrams. Some experiments were also made at 50,400 r. p. m. These boundaries were photographed by the diagonal knife edge method. Diagrams typical of various stages of digestion are shown in Figs. 1 and 2. Sedimentation constants were calculated in the usual way.[13] Normal human gamma globulin has a sedimentation constant, s_{20} of about 7*S*. The halves have s_{20} of about 6*S*, and the quarters $s_{20} = 4S$. For convenience the components of a digest are therefore referred to as S[7] (normal globulin), S[6] (halves), and S[4] (quarters). The small amount of material smaller than S[4] which has not been removed by dialysis is referred to as S[2].

Diffusion constant measurements were made in a Lamm cell[14] on solutions containing 0.6–0.7% protein in 0.15 *M* sodium chloride. Line displacement-distance diagrams obtained after 48,000 to 94,000 seconds of diffusion were analyzed by the height and area method and the method of moments.

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TABLE I

THE DIGESTION OF HUMAN GAMMA GLOBULIN BY PAPAIN

Expt.	Globulin, g.	Papain U/g.[a] × 10⁵	Activator	pH	Temp., °C.	Time, days	ΔNPN, mg./cc.[b]	Sedimentation analyses, % Protein S⁷	S⁶	S⁴	Total nitrogen S³	S⁷	S⁶	S⁴	S³	NPN
CN-1	0.6	4[c]	0.1 *M* CN	5	2	3		64	14	22						
						5		35	19	46						
CN-2	0.5	10[c]	0.1 *M* CN	5	2	6		12	7	81						
CN-3	0.5	10[c]	0.1 *M* CN	7	2	6		8	5	87						
H_2S-1	1.0	13	H_2S	5	2	1	0.33									
						2	.50									
						4	.66									
						6	.75	9	5	82	4	8	6	73	4	9
H_2S-2	1.0	13	H_2S	5	2	6	.63	3	4	89	4	3	4	79	4	10
			Cysteine·HCl													
CY-1	0.4	13	HCl-8 mg.	5	2	3	.58									
						7	.78	8	3	80	9	7	3	72	8	10
CY-2	1.0	13	HCl-19 mg.	5	2	1	.29									
						2	.45									
						5	.74									
						16	1.45									18
CY-3	10.0	17	HCl-238 mg.	5	2	3	0.70									
						10	1.29									
						13	1.61	2	3	86	9	2	2	69	7	20
CY-4	2.0[d]	13	HCl-47 mg.	7	25	1	3.17									
						2	4.20	1	1	92	6	1	1	68	4	26
						5	7.31	1	4	81	14	1	2	43	8	46
CY-5	6.0	13	HCl-114 mg.	7	2	6	0.80									
						8	.90	2	5	89	4	2	4	79	4	11
CY-6	2.0	8	HCl-24 mg.	7	25	2	.84	7	6	80	7	6	5	71	6	12
						3	.92	6	9	79	6	5	8	69	5	13
						4	.96	4	4	86	6	3	3	75	5	14
CY-7	2.0	8	HCl-24 mg.	7	25	3	1.15									18
						4	1.19	3	4	87	6	2	3	71	5	19
CY-8	2.6	8	HCl-32 mg.	7	25	4	1.19	1	1	90	8	1	1	70	6	22
CY-9	10	8	HCl-120 mg.	7	25	3	1.46									
						4	1.67	8	6	77	9	5	4	52	6	33
CY-10	20	8	HCl-230 mg.	7	25	2	0.74									
						4	1.07	6	5	85	3	5	4	67	2	21

[a] [Pa.U.]^{HA} [b] Increase over initial value. [c] Whole papain suspension. [d] Digested in ten per cent. solution.

Electrophoretic analyses were made in a buffer which was 0.05 molar in diethylbarbituric acid and 0.00765 molar in sodium citrate, and was adjusted to *p*H 8.6 with sodium hydroxide. Some analyses were also made at *p*H 5.0 in sodium acetate–acetic acid buffer, ionic strength 0.10.

From Figs. 1 and 2 it is apparent that the whole digests were not sufficiently homogeneous for accurate diffusion constant measurements. Some fractionations were therefore undertaken.

Three ammonium sulfate fractionations were carried out in the cold room at about +2°. Ammonium sulfate saturated at this temperature was added to the cold protein solution, and the precipitates obtained at 40, 50, 70 and 100% saturation separated in a Sorvall centrifuge.

A second series of fractionation experiments was done by an adaptation of the low temperature ethanol procedures recently developed.[6] Here very sharp differences in solubility were found. When a solution of the digest proteins containing 1 to 3% protein and 0.05 *M* sodium chloride, at *p*H 6, was taken to 25% alcohol by the addition of 80% ethanol, over half the protein precipitated. After the removal of this precipitate at −5°, the supernatant was taken to 45% alcohol, by the addition of precooled 95% ethanol at −5°. Surprisingly, no further precipitate appeared. As the ethanol addition was continued the solution became turbid at about 50% alcohol. The precipitate was removed at a final alcohol concentration of 65–70% at −5 to −8°. Both precipitates were suspended in ice water, shell-frozen and dried *in vacuo*. Further details of these fractionations are given in Table III.

Results

The results of the papain digestions are shown in Table I and Fig. 1. A typical sedimentation diagram for undigested human gamma globulin is given in Fig. 1, A. In the first experiment only 4×10^{-5} unit of papain per gram of globulin was used. The digestion went very slowly, and after three and five days gave the interesting results shown in Fig. 1, B and C. The half molecules, S^6, are quickly split into quarters, S^4, so that at any stage of the digestion only a small proportion of halves is present. When the non-protein nitrogen has reached 10% (Table I) almost 90% of the remaining protein is already in quarters. As digestion proceeds farther (Experiment CY-4) the amount of non-protein material increases but the size distribution of the protein left remains about the same. A typical sedimentation diagram is shown in Fig. 1, D.

TABLE II

THE DIGESTION OF HUMAN GAMMA GLOBULIN BY BROMELIN

Expt.	Globulin, g.	Bromelin U/g.[a] × 10⁵	Activator	pH	Temp., °C.	Time, days	ΔNPN, mg./cc.[b]	Sedimentation analyses, % Protein S⁷	S⁶	S⁴	S³	Total nitrogen S⁷	S⁶	S⁴	S²	NPN
1	1.0	[c]	0.07 *M* CN	7.5	2	6		51	13	36						
			Cysteine as, mg.													
2	0.4	5	HCl-47	7	2	2	0.09									
						6	.16	19	9	72		18	8	68		6
						12	.18	25	9	66		24	8	62		6
3	1.0	5	HCl-100	7	25	1	.22									
						2	.23									
						3	.42	8	11	81		7	10	72		11
						6	.57	10	8	82		8	7	70		15
4	1.0	10	HCl-200	7	2	1	.12									
						3	.16									
						6	.19	18	10	72		17	10	69		4
						16	.39	9	9	82		8	8	75		9
5	1.0	5	HCl-100	7	25	2	.24									5
						6	.59									13
					2	18[d]	.91	2	4	88	5	2	3	70	4	21
						32[d]	.99	3	10	84	3	2	8	66	2	22
6	12.2	5	HCl-1220	7	25	6	.26									3
						13	.35	12	4	79	5	12	4	76	5	4
						22	.51	11	9	78	2	10	8	74	2	6

[a] One unit has same hemoglobin activity as one [Pa.U.]^{HA}. [b] Increase over initial value. [c] First preparation, activity unknown. [d] Days after removal to cold room.

Relatively large amounts of the crude powder are needed—400 mg. per gram of globulin. Of this about one-third, or 130 mg., was soluble protein. The sedimentation constant of papain is 2.7*S*,[15] so the papain boundary is probably hidden by the quarter molecule boundary. Much smaller

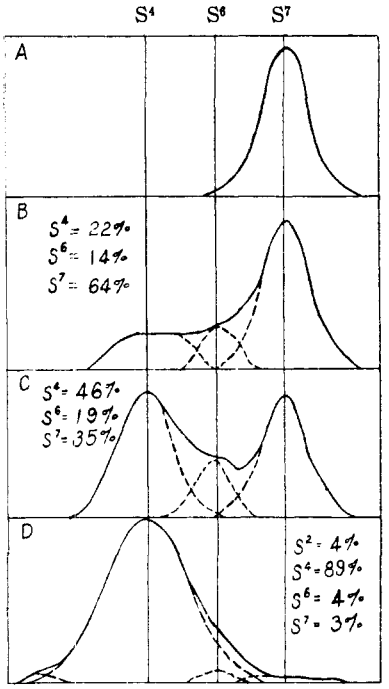

Fig. 1.—Sedimentation diagram showing the digestion of human gamma globulins by papain-CN at *p*H 5: A, undigested; B, digested 3 days at 2° (Expt. CN 1); C, digested 5 days at 2° (Expt. CN 1); D, digested 6 days at 2° (Expt. H_2S 2).

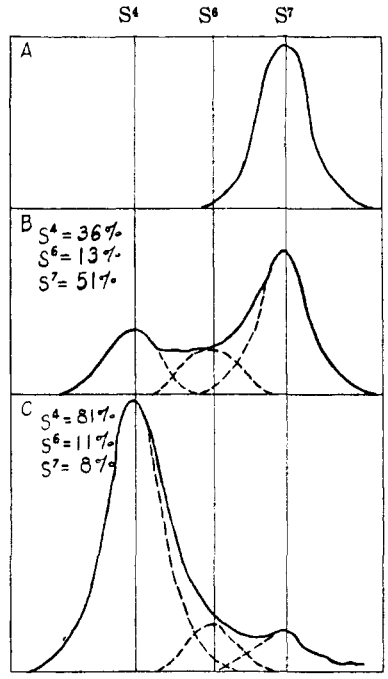

Fig. 2.—Sedimentation diagram showing the digestion of human gamma globulins by bromelin at *p*H 7: A, undigested; B, digested 6 days at 2° (Expt. Br 1); C, digested 3 days at 25° (Expt. Br 3).

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amounts of crystalline papain or chymopapain would suffice.

The results of the bromelin digestions are shown in Table II and Fig. 2. Before 10% of the globulin has been reduced to non-protein nitrogen, 80% has been split into quarters. An equilibrium is then reached, and further digestion results in a continuous increase in non-protein nitrogen but little change in the relative amounts of quarters, halves and unsplit globulin.

From the data in Tables I and II several conclusions may be drawn. For both enzymes the maximum yield of the S^4 component is obtained when the digestion is stopped at about a 10% increase in non-protein nitrogen. An increase in temperature, from 2 to 25°, is more effective in hastening the splitting processes than an increased amount of enzyme. There appears to be some correlation between the rate of formation of non-protein nitrogen and the amount of albumin in the globulin. Thus the highest amount of non-protein nitrogen, 33%, was obtained with papain digest CY 9, which contained 16% albumin; the lowest amount, 4% with bromelin digest 6, which contained no albumin.

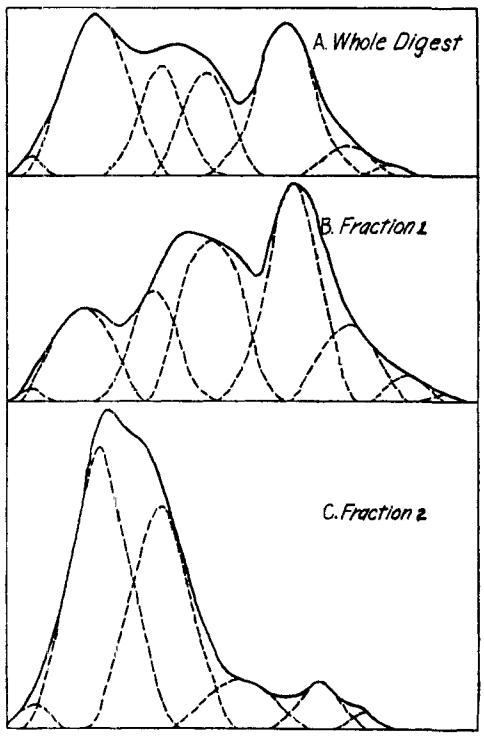

Fig. 3.—Electrophoretic patterns of bromelin digest 6 fractionated by alcohol: descending boundaries; *p*H 8.5.

The electrophoretic analyses of papain and bromelin digests gave unexpected results. It has been observed in this Laboratory that the halves formed from human gamma globulin by pepsin have the same mobility at *p*H 8.6 as the original globulin. On the other hand, the quarters formed by papain and bromelin have quite different mobilities. An electrophoretic diagram of a bromelin digest is shown in Fig. 3A. The papain digests were quite similar, giving the same five principal boundaries and in addition a small boundary of positive mobility which may be ascribed to the papain. Because of the high diffusion rate of these small particles the boundaries could not be separated, even by prolonged electrophoresis. The per cent. of each component could not, therefore, be determined very precisely. Fairly good agreement was obtained between the analyses of the ascending and descending boundaries, however, and the two sets of values have been averaged. At *p*H 5.0 80% of the protein moved in one boundary, and no detailed analysis could be made.

The details of the fractionation procedure and the results obtained are given in Table III. The components found have been lettered in order of mobility at *p*H 8.6. A is papain, J is albumin and the intermediate components are derived from gamma globulin. Since the mobility of gamma globulin at *p*H 8.6 is similar to that of component B, the amount of unsplit globulin (obtained from ultracentrifugal analysis, column 8) has been subtracted from the amount of B, to give the figures

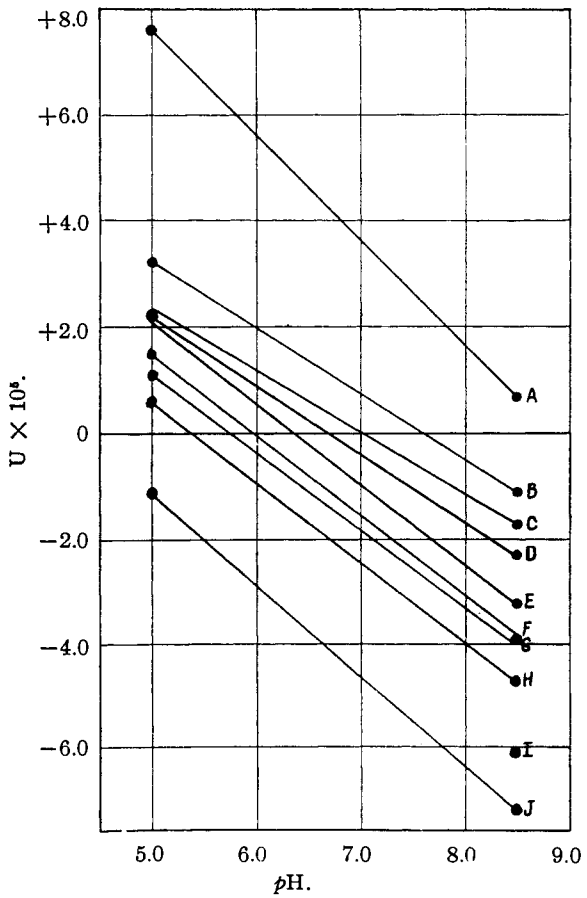

Fig. 4.—Electrophoretic mobilities of components of papain and bromelin digests.

TABLE III

THE FRACTIONATION OF GLOBULIN DIGESTS

Expt.	Fract. no.	% Sat. am. sulf.	% Protein pptd.	Sedimentation analysis S[7]	S[6]	S[4]	S[3]	Electrophoretic analysis A	B	C	D	E	F-G	H	I	J
				A. Ammonium Sulfate												
PaCY 6	Whole															
	1	40	15	6	8	80	6									
	2	50	36	3	4	90	3									
	3	69	27	3	3	90	4									
	4	82	4													
	5	100														
PaCY 9	Whole			8	6	77	9	2	38(30)	14	11	21	8	1	1	5
	1	40														
	2	50[a]		8	3	86	3	0	31(23)	12	14	27	13	2	1	1
	3	70[a]		0	1	94	5	0	60(60)	12	8	4	3	1	3	9
Br 5	Whole			3	7	86	4									
	1	43	10													
	2	50	37													
	3	70	32													
	4	100	3													
				B. Alcohol												
		Alcohol, %														
PaCY 6	Whole			4	4	86	6									
	1	30	47	15	5	77	3									
7	Whole			3	4	87	6									
	1	25	*ca.* 50													
	2	60	*ca.* 10	Trace	4	93	3									
8	Whole			3	3	90	4									
	1	25	55	10	4	84	2									
	2	60	17	1	1	95	3									
10	Whole			6	5	85	3	1	35(29)	15	15	21	9	1	1	3
	1	25	60	15	8	70	7	2	14(0)	9	26	32	14	2		
	2	64	28	3[b]	3	80	5	1	37(34)	42	8	8	3	1		
Br 6	Whole			11	9	78	2	..	27(16)	20	20	22	8	3		
	1	25	67	16	3	77	4	..	13(0)	16	25	31	11	3	1	
	2	70	25	3	4	90	3	..	47(44)	38	9	5	1	0		

[a] Precipitated twice. [b] Nine per cent. inhomogeneous heavy material also present.

in parentheses. Since the mobility of the halves is not known, no correction can be made for them.

The fractionation obtained with ammonium sulfate was never very clear cut. A preliminary experiment on digest PaCY 6 indicated that most of the whole and half molecules had been precipitated at 40% (see Table III). The precipitate obtained between 40 and 50% saturation contained considerably less unsplit globulin than the whole digest. Even the 50–70% precipitate was, however, still not homogeneous in the ultracentrifuge. The experiment was therefore repeated on a larger digest, PaCY 9. Both the 40–50 and the 50–70% fractions were dissolved in water and reprecipitated. The 40–50% fraction still contained a significant amount of unsplit globulin, but the 50–70% fraction was 94% quarters.

Electrophoretic analysis of these fractions showed that no very sharp separations had been made. Most of components E and F-G were found in the 40–50% fraction. Components C and D were fairly evenly distributed, and B was mainly in the 50–70% fraction, with J, the albumin.

A small bromelin digest (Br 5) was also fractionated with ammonium sulfate. Nitrogen analyses indicated a distribution of the digest among the various fractions obtained similar to that found for the papain digests.

The alcohol fractions gave much more clear cut results. Fraction 1 (25% alcohol) contained almost all the undigested globulin and about 50% of the halves (see Table III). There was also a sharper separation of the electrophoretic components. The total yield in milligrams of each fraction has been plotted in Fig. 5. All the B component in Fraction 1 can be accounted for as unsplit globulin; this has been marked γ in the diagrams. Most of components D, E, F-G and H are found in Fraction 1. Fraction 2 contains a small amount of γ, and is otherwise mainly B and C. The supernatants were not analyzed, but must have contained large amounts of B. The losses in other components probably represent experimental errors, since in some cases (areas below the base line) over 100% yields were obtained. The distribution of component C is hard to explain. In the PaCY 10 fractionation the

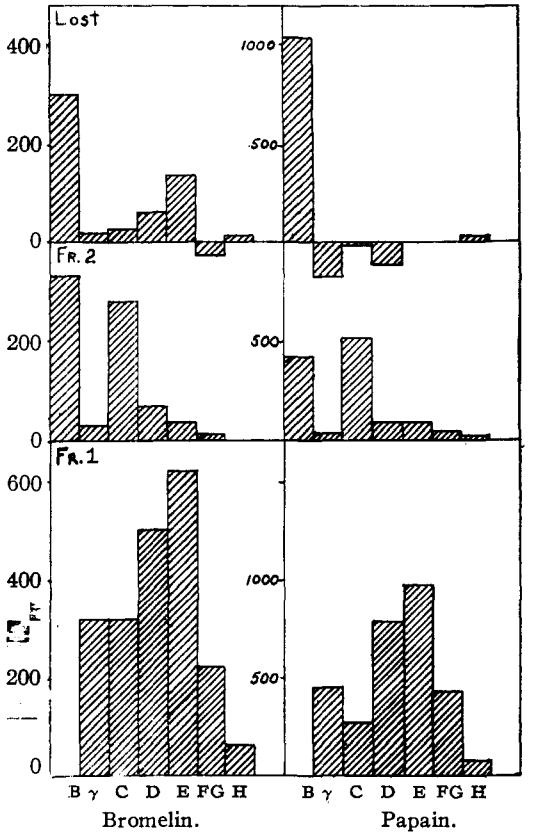

Fig. 5.—The alcohol fractionation of gamma globulin digests.

amount of C in fraction 1 corresponds with the amount of halves found on sedimentation; this correspondence is not so clear with the bromelin digest fractionation. In any case component C must consist of two separate entities of very different solubilities. Electrophoretic patterns of the two fractions are shown in Fig. 3, B and C. In the fractionation of the papain digest the supernatant from Fraction 1 was acidified to *p*H 4.7 before the alcohol concentration was raised. Even at 65% alcohol the supernatant from Fraction 2 was cloudy and, as shown in Fig. 5, much of component B was left in solution. When the electrophoretic analyses were made the reason for this became apparent. As shown in Fig. 4, component B has an isoelectric point near *p*H 7.5, and is therefore much more soluble in acid than near neutrality. In the fractionation of the bromelin digest no acid was added, and more of B was precipitated. If the supernatant from Fraction 1 were adjusted to a *p*H of 7.5 the yield of B might have been even further improved. In the ultracentrifuge Fraction 2 of the papain digest showed 9% of heavy, inhomogeneous material. The combination of low *p*H and 65% alcohol had evidently caused some denaturation.

Sedimentation Constant.—Sedimentation constants were calculated in the usual way.[13] From Fig. 6 it may be seen that the change in sedimentation rate with protein concentration is slight. The value 4.1*S* was therefore used in calculating the size of the quarters for both papain and bromelin digests.

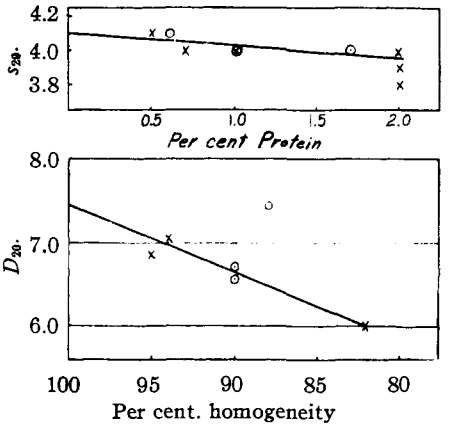

Fig. 6.—Molecular kinetic constants of papain and bromelin digests: **X**, papain digests and fractions; ⊙, bromelin digests and fractions.

Diffusion Constant.—Diffusion constant measurements were made on two whole digests, Pa-H_2S 1 and Br 5. Since these both contained over 10% of material not S^4, the results must be considered as only approximate. The other four sets of measurements were made on the most soluble fractions of digests. PaCY 9-3, prepared by ammonium sulfate fractionation, contained mainly component B. The other samples, prepared by alcohol fractionation, contained component C as well as B. The results of the diffusion constant determinations are shown in Fig. 6B. The value 7.5×10^{-7} sq. cm./sec. was taken as most nearly representative of the S^4 component alone.

Molecular Weight.—From a sedimentation constant $s_{20} = 4.1S$, diffusion constant $D_{20} = 7.5 \times 10^{-7}$ sq. cm./sec., and a partial specific volume of 0.718[16] the molecular weight of the S^4 component was calculated by the formula

$$M = RTs/D(1 - v\rho) \qquad (13)$$

to be 47,000.

Immunological Assays.—Immunological assays were made on some of these globulins, before and after digestion, through the courtesy of Dr. J. F. Enders of the Department of Bacteriology, Harvard Medical School. The methods used have recently been described.[7] The results are given in Table IV. Although 90% of the globulin has been split into quarters, much antibody activity remains. On the whole the action of bromelin appears to be milder than that of papain. For the same degree of splitting the bromelin has caused somewhat less loss of antibody activity. The retention of diphtheria antitoxin activity is particularly interesting. The assays of fractions were done on PaCY 10 and Br 6. Only very small amounts of these fractions were available for testing, and the results are

TABLE IV

IMMUNOLOGICAL ASSAYS. ACTIVITY IS EXPRESSED RELATIVE TO THAT OF A STANDARD GLOBULIN AT THE SAME PROTEIN CONCENTRATION

	Papain				Bromelin			
	Undigested	Whole digest	Fr. 1	Fr. 2	Undigested	Whole digest	Fr. 1	Fr. 2
Diphtheria antitoxin	0.7	0.5			0.35	0.35		
		0.63	0.14	0.49		0.27–0.46	0.17	....
Influenza A mouse protection	1.0	<0.2			0.8	0.3		
		0.75	<0.37	<0.75		0.75	0.75	<0.75
Influenza A Hirst test	1.0	0.4			0.5	0.36		
		0.25	0.06	0.13		0.25	0.25	0.25
Typhoid "H" agglutinin	1.0	<0.3			0.18	0.06		
		0.19	0.10	<0.06		<0.06	<0.06	<0.06
Typhoid "O" agglutinin	3.0	2.0						
		clouds	<2.0	clouds		<2.0	<2.0	<2.0

therefore only suggestive. There appears to be more activity in Fraction 2 (at least for the papain digests) than in Fraction 1. Since Fraction 2 contains only about 5% of unsplit globulin, the activity must be contained in the quarter molecules.

Discussion

The chief product of papain or bromelin action on human gamma globulin, the S^4 component, has been assumed to be one-fourth the size of the original globulin particle. If this is the case its molecular weight should be only 38,000 instead of the value 47,000 obtained from the sedimentation and diffusion constants. A critical examination of the accuracy of these constants is therefore necessary. It can be calculated[13] that a hypothetical particle the same width as the gamma globulin molecule but only one-fourth as long should have a sedimentation constant, $s_{20} = 4.0$-S. This is close to the experimental value of 4.1*S*. Since the S^4 boundary is well resolved in the ultracentrifuge, its sedimentation velocity can be determined with considerable accuracy.

The diffusion constant of this supposed quarter would be 9.0×10^{-7} sq. cm./sec. This is considerably higher than the value 7.5×10^{-7} sq. cm./sec. obtained by extrapolation of the experimental values to 100% homogeneity. This discrepancy may represent experimental error, perhaps caused by the presence of high molecular weight protein not detected in the ultracentrifuge. Pedersen[16] has found this to be the case with other serum proteins. On the other hand the concept of the human gamma globulin molecule as split by these enzymes in planes perpendicular to its long axis may be a gross oversimplification of the true circumstances. Which alternative is correct cannot be decided without much further experimental work.

The possibility that the different electrophoretic mobilities of the various digest components are due to combination of the globulin fragments with enzyme must be considered. It seems unlikely for several reasons. First, the same electrophoretic components were obtained with papain and bromelin. Second, combination of any gamma globulin fragment with the highly basic papain should give a complex of very low mobility at *p*H 8.5 (see Fig. 4). This might be true of component B but all the others have higher mobilities than gamma globulin. That component B is not a complex is indicated by its homogeneity in the ultracentrifuge at *p*H 7. In the ammonium sulfate fractionation of digest PaCY 9 (see Table III) Fraction 3, which is 60% component B, is 94% S^4 component.

Another possibility to be considered is that of changes in the globulin or its split products due to the reagents used: the activators (cyanide, hydrogen sulfide or cysteine) and the hydrogen peroxide. That these cause no change in the unsplit globulin perceptible in the ultracentrifuge is shown by the results of experiments in which insufficient enzyme was used. Thus in experiment PaCN 1, 64% of the globulin remained unsplit after three days of digestion. As shown in Fig. 1B, the sedimentation characteristics of this globulin differed in no way from those of the untreated protein. The same is true in experiment Br 1, where 51% of unsplit globulin remained after six days. In the hydrogen sulfide and cysteine experiments digestion was more complete, but the small amount of unsplit globulin left retained its normal sedimentation characteristics. In the ammonium sulfate and alcohol fractionations, the unsplit globulin showed the same solubility as has been found for untreated human gamma globulin. The split products have the same sedimentation characteristics no matter which activator is used. It is of course realized that only major changes in size or shape are reflected in the sedimentation behavior.

No electrophoretic analyses were performed on other than cysteine digests, nor were any done on globulin treated only with activators and peroxide. Whether the increase in the acidic nature of the split products is in part due to chemical changes cannot be said. It is a distinct possibility that in the presence of hydrogen peroxide

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

disulfide bonds are formed between the protein sulfhydryl groups and the cysteine present in the digestion mixture.

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Summary

1. Human gamma globulin is split by papain or bromelin into particles of one-quarter size.
2. The splitting is accompanied by only a small increase in non-protein nitrogen.
3. In the ultracentrifuge the split products give rise to a single boundary of sedimentation rate $s_{20} = 4.1S$ in dilute solution.
4. Electrophoretic analysis of the digests reveals the presence of a number of separate components derived from the globulin.
5. These components differ in their solubility in ammonium sulfate and in ethyl alcohol.
6. The diffusion constant of the split products is 7.5×10^{-7} sq. cm./sec. The molecular weight is about 47,000.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF WINTHROP CHEMICAL CO., INC.]

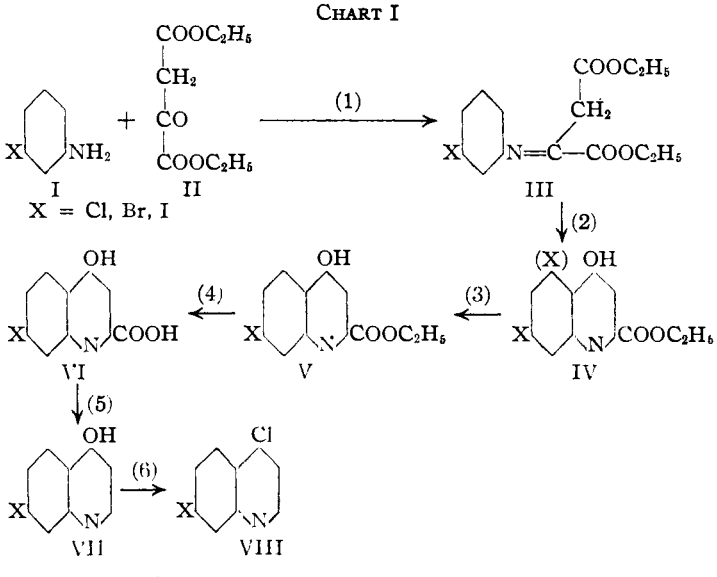
Some 7-Substituted 4-Aminoquinoline Derivatives

BY ALEXANDER R. SURREY AND HENRY F. HAMMER

In continuation of the search in these laboratories for effective antiparasitic agents, several 4-dialkylaminoalkylamino-7-halogen quinoline derivatives have been prepared. Although there have been several papers published recently[1,2,3] on 4-dialkylaminoalkylamino-6-methoxyquinolines, only the patent literature[4] on the synthesis of the corresponding 7-substituted compounds is available. In view of the high therapeutic activity reported for the 6-substituted quinolines on the asexual form of various protozoal organisms, it seemed desirable to study further the preparation of the 7-derivatives for a comparison of activity. Although some of these compounds have been reported in the patent, the preparation of the necessary intermediates has not been described in detail.

The 4-chloro-7-halogen quinolines have been prepared by a series of reactions (Chart I) starting with that of substituted anilines and ethyl ethoxalylacetate in glacial acetic acid.[5,6,7] In some experiments it was found to be advantageous to use an excess of the aniline (I) in order to obtain better yields (50–80%). Practically all of unreacted I can and should be removed in the isolation of the anils (III) inasmuch as small amounts of I interfere with the expected reaction in step 2. The formation of the crystalline quinoline derivative (IV) in yields of 50–80% resulted from the ring closure

CHART I



of III in medicinal mineral oil at 250°. The ethyl alcohol which formed during this reaction was collected by condensation. The quantity obtained served as an indication of the completeness of the reaction. This procedure eliminated any prolonged heating which would cause considerable decomposition. Practically no ethyl alcohol was collected until the reaction mixture reached 235°.

Where ring closure can give a mixture of isomers (as with the *m*-substituted anilines) the

(1) Magidson and Rubtsov, *J. Gen. Chem.* (*U. S. S. R.*), **7**, 1896 (1937).
(2) VanArendonk and Shonle, THIS JOURNAL, **66**, 1284 (1944).
(3) Bachman and Cooper, *J. Org. Chem.*, **9**, 302 (1944).
(4) U. S. Patent 2,233,970; March 4, 1941.
(5) Conrad and Limpach, *Ber.*, **20**, 944 (1887).
(6) Limpach, *ibid.*, **64**, 969 (1931).
(7) Rubtsov and Lizgunova, *J. Gen. Chem.* (*U. S. S. R.*), **13**, 697 (1943).