proteases for protein synthesis. This interpretation emphasizes that it is the joint action of enzyme and substrate that contributes to the specificity of the reaction. This joint action may lead to a self-regulated order of residues. Such regulation, controlled by the simultaneous influence of enzyme and substrate, is here referred to as *zymosequential specificity*.

This concept is related to one proposed by Bergmann to explain a postulated periodicity in protein structure.[13,21] Bergmann stated that the mutual interdependence of enzyme and substrate determined which one of four types of reaction (hydrolysis, synthetic coupling, replacement of two types) occurred. It is now clear that protein structures are not generally periodic[22,23] and it becomes necessary to explain a highly exact ordering of individual residues. The experiments reported here, particularly the pronounced selectivity of coupling of bz-amino acid with glycinanilide, indicate that the particular reactants limit the nature of the possible syntheses. The introduction of a glycine residue into the aminoid component (Tables I and II), furthermore, is seen to alter the synthetic capabilities of the system from one of a broad gradation of preference[7] to a more highly selective situation. Extrapolation to larger molecular intermediates should thus lead to increasingly selective reaction, in the presence of any one protease. The initial reactions in a stepwise synthesis would thus predetermine in the fashion indicated by the results presented here, the identity of the later reactions. This would constitute connotations of zymosequential specificity. In conformity with the observation that different proteases can catalyze different interactions, each protease should selectively interact with its available substrates in its own characteristic way.[4]

(21) M. Bergmann, *J. Mt. Sinai Hosp.*, **6**, 181 (1939).

(22) R. J. Block in C. L. A. Schmidt, "Chemistry of the Amino Acids and Proteins," Charles C Thomas, Springfield, Ill., 1944, p. 1093.

(23) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

The following influences leading to selectivity in reaction can be summarized: a single instance of synthesis from a series of possibilities (Tables I and II), non-reactivity of some components under conditions in which others react and in which they react in some cases when only one amino acid residue is present, selectivity imposed each by the aminoid and carboxoid reactants (Table III and ref. 4) and a replacement of preferential action by specificity in reactions involving two amino acid residues instead of one.

In view of these facts, and inasmuch as proteases have the unique ability among enzymes of functioning also as substrates,[24] the possibility of a converse relationship may be considered. Can substrates function as part of the protease complex? If so, at each step the new protease–substrate complex may become in effect a new enzyme different from that operating at any other step in synthesis. Evidence that may confirm or deny the applicability of such a mechanism to protease–substrate action should clarify concepts related to zymosequential specificity.

Acknowledgments.—The assistance of Jacquetta S. Halverson, Dorothy De Fontaine and Armand J. McMillan is appreciated.

(24) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948.

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[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL, NEW YORK]

Immunochemical Studies on Blood Groups. XIII. The Action of Enzyme from Snail (Busycon) Liver on Blood Group A and O(H) Substances (Hog)[1a]

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RECEIVED JUNE 8, 1953

Enzymes from snail liver were shown to split methylpentose from blood group A and O(H) substances and to increase their cross-reactivity with horse anti-SXIV. Blood group A activity was reduced, but O(H) activity was unaffected. N-Acetylhexosamine was liberated from A substances but not from O(H) substances, suggesting structural differences between these two antigens.

The degradation of chitin by extracts of snail hepatopancreas (*Helix pomatia*, sp.) was first described by Karrer and his associates,[1c] who showed that N-acetylglucosamine was liberated by the hydrolytic action of an enzyme which they named chitinase. Freudenberg and Eichel[2] subsequently showed that similar enzyme preparations reduced the specific blood group activity of polysaccharides obtained from human urine, with the liberation of N-acetylglucosamine and a 25% increase in reducing power. Neuberger and Pitt Rivers[3] and Zechmeister and Tóth[4] have since demonstrated that extracts of whole digestive tract and liver from *Helix pomatia* exhibited β-glucosaminidase activity. More recently, Utusi, *et al.*,[5] and Yosizawa[6] have reported the effects of

(1) (a) This investigation was carried out in part under grants from the National Institutes of Health, Public Health Service (RG34), and the William J. Matheson Commission. (b) Senior Fellow in Virus Diseases of the National Research Council, 1949–1951; Fellow of the National Foundation for Infantile Paralysis, 1951–1952; Markle Scholar in Medical Science, 1952. (c) P. Karrer and A. Hofmann, *Helv. Chim. Acta*, **12**, 616 (1929); P. Karrer and G. von François, *ibid.*, **12**, 986 (1929).

(2) K. Freudenberg and H. Eichel, *Ann. Chem.*, **518**, 97 (1935).

(3) A. Neuberger and R. V. Pitt Rivers, *Biochem. J.*, **33**, 1580 (1939).

(4) L. Zechmeister and G. Tóth, *Naturwissenschaften*, **27**, 367 (1939).

(5) M. Utusi, K. Huzi, S. Matumoto and T. Nagaoka, *Tohoku J. Exp. Med.*, **50**, 175 (1949).

(6) Z. Yosizawa, *ibid.*, **55**, 35 (1951).

enzymes from the species *Helix laeda Gould* on blood group substances obtained from hog gastric mucin. Using a preparation from snail liver fractionated to contain only β- and no α-glucosaminidase, the latter investigator demonstrated maximal hydrolysis of the substrate after three days, at *p*H 4.5 and 37°.

The present report summarizes a series of experiments in which an attempt was made to determine quantitatively the effect of snail enzymes on the immunochemical properties of blood group A and O(H) substances. Evidence will be presented that the effect of these or similar enzymes on the blood group polysaccharides may afford clues to structural differences between the A and the O(H) antigens.

Materials and Methods

Preparation of Enzyme Solutions.—Livers of fresh snails (*Busycon*, sp.) were dissected free of stomach and intestine and were ground with sand in cold acetone. The mixture was filtered and the residue stored in the dry state at 4° for about 3 years. The crude powder was extracted several times at 4° with acetate buffer of *p*H 4.0 and 0.15 ionic strength. The clear yellow extract was then fractionated in the cold with ammonium sulfate. The precipitates obtained at one third saturation (fraction 1) and between one third and one half saturation (fraction 2) were dissolved in acetate buffer of *p*H 4.0 and 0.15 ionic strength. These solutions were dialyzed in the cold against repeated changes of 0.15 *M* sodium chloride, buffered at *p*H 4.0 with acetate, until the last few dialyzates were nitrogen free, and were then stored under toluene at 4° until used.

Three separate preparations of snail liver extract were used in the experiments to be described. Some of the enzymes present are listed in Table I, together with the time elapsing between the preparation of the materials and the performance of assays[7] for α-glucosaminidase,[8] β-glucosaminidase[9] and glucuronidase.[10]

TABLE I

ENZYMES IN FRACTIONS OF SNAIL LIVER EXTRACT

Preparation no.	Fraction	Days stored at 4° under toluene before assay	α-Glucosaminidase, units/mg. N	β-Glucosaminidase, units/mg. N	Glucuronidase, units/mg. N
2	1 (0–1/3 Sat.)	416	5	700	23
3	1 (0–1/3 Sat.)	44	30	1080	100
3	2 (1/3–1/2 Sat.)	44	89	8850	120

Preparation of Blood Group Substances (BGS).—Blood group substances were prepared from commercial hog gastric mucin (Wilson) by phenol extraction and fractional alcohol precipitation.[11] These preparations are described in a separate report.[12] The fraction precipitating from phenol solution between 4 and 8% ethanol was used for experiments 1 and 2 (Hog 6A) and that precipitating between 8 and 10% ethanol for experiment 3 (Hog 7A). These materials contained not more than 50–60% blood group A substance, as determined by quantitative immunochemical methods, the remainder being material of essentially the same chemical composition but showing O(H) activity.[12,13] Blood group A and O(H) substances were also prepared from individual hog stomach linings[13c] by the same alcohol–phenol fractionation procedure.[11,12] In experiments 4 and 5, the fraction precipitated from phenol between 4 and 8% ethanol was used with the exception of hog substance 54, which was the fraction precipitated from phenol between 8 and 10% ethanol. These purified A and O(H) substances were comparable, in their immunochemical properties, with the blood group A and O(H) substances described in earlier reports.[13c,14] The A substances (50 and 52) contained no detectable O(H) activity; and the O(H) substances (38, 42 and 54) contained no detectable A activity.

Immunochemical Methods.—Blood group A antigen was estimated by inhibition of hemagglutination, using group A erythrocytes and human anti-A, and was determined quantitatively by precipitation of anti-A from calibrated human antisera,[15] using the quantitative precipitin method described by Heidelberger and MacPherson.[16] The various fractions were assayed for O(H) activity by measuring their relative capacities to inhibit hemagglutination of group O cells by bovine serum containing anti-O(H), previously absorbed with A_1B erythrocytes.[14] Since no precipitating anti-O(H) serum was available no truly quantitative estimation of O(H) antigen was possible.

The cross reaction of both the A and the O(H) substances with horse antibody to the specific polysaccharide of type XIV pneumococcus was measured by the quantitative microprecipitin technique.[16,17]

Hexosamine was determined by the method of Elson and Morgan,[18] methylpentose by the method of Dische and Shettles,[19] reducing sugar by the method of Hagedorn and Jensen,[20] and nitrogen by the Markham modification of the micro-Kjeldahl procedure.[21] N-Acetylhexosamine was estimated by the Morgan and Elson method as recently modified by Aminoff, Morgan and Watkins.[22]

Experimental

The essential data from five separate experiments are presented in Table II. In experiments 1, 2 and 3, mixtures of A and O(H) substances (6A and 7A), and in experiments 4 and 5, individual A (50 and 52) and O(H) (54, 38, and 42) substances were exposed to active enzyme. Fractions 1 and 2 from the three preparations of enzyme solution were employed in the various experiments. Prior to use in the five experiments, these enzyme solutions had been stored at 4° under toluene for periods of 3, 6, 1 and 10 months, and 3 weeks, respectively.

Sixty to one hundred milligram samples of blood group substance were dissolved in acetate buffer of *p*H 4.0 and 0.15 ionic strength. One of these samples was mixed with active enzyme (Table II, column 3, "BGS + enzyme") and made up to 20 ml. with acetate buffer. Immediately after mixing, 1.0 ml. (5% of the total blood group substance and first aliquot of enzyme) was withdrawn and heated to 100° for 10 min. to inactivate the enzyme (Table II, column 3, "BGS + enzyme, heated"). The other sample of blood group substance, to which no enzyme had been added, was also made up to 20 ml. with acetate buffer (Table II, column 3, "BGS"). Enzyme alone in buffer and, in experiments 4 and 5, buffer alone, were included as additional controls. All solutions were incubated under toluene at 37°. In the first four experiments, the initial periods of incubation were 5, 7, 4 and 7 weeks, respectively. A second aliquot of enzyme solution, equal to the amount initially included in the active enzyme digest, was then introduced into the digest

(7) Enzyme assays summarized in Table I were performed in the laboratory of Dr. Karl Meyer.

(8) S. Roseman and A. Dorfman, *J. Biol. Chem.*, **191**, 607 (1951).

(9) M. E. East, J. Madinaveitia and A. R. Todd, *Biochem. J.*, **35**, 872 (1941).

(10) P. Talalay, W. H. Fishman and C. Huggins, *J. Biol. Chem.*, **166**, 757 (1946).

(11) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Biochem. J.*, **46**, 426 (1950).

(12) C. Howe and E. A. Kabat, in preparation.

(13) (a) W. T. J. Morgan and M. B. R. Waddell, *Brit. J. Exp. Path.*, **26**, 387 (1945); (b) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Nature*, **158**, 879 (1946); (c) A. Bendich, E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **83**, 485 (1946).

(14) A. Bendich, E. A. Kabat and A. E. Bezer, THIS JOURNAL, **69**, 2163 (1947).

(15) E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **82**, 207 (1945); E. A. Kabat, A. Bendich and A. E. Bezer, *ibid.*, **83**, 477 (1946).

(16) M. Heidelberger and C. F. C. MacPherson, *Science*, **97**, 405 (1943); **98**, 63 (1943).

(17) (a) E. A. Kabat, A. Bendich, A. E. Bezer and V. Knaub, *J. Exp. Med.*, **87**, 295 (1948); (b) E. A. Kabat, H. Baer, A. E. Bezer and V. Knaub, *ibid.*, **88**, 43 (1948).

(18) L. A. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).

(19) Z. Dische and L. B. Shettles, *J. Biol. Chem.*, **175**, 595 (1948); H. Baer, Z. Dische and E. A. Kabat, *J. Exp. Med.*, **88**, 59 (1948).

(20) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, **135**, 46 (1923).

(21) R. Markham, *Biochem. J.*, **36**, 790 (1942).

(22) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *ibid.*, **51**, 379 (1952).

TABLE II

IMMUNOCHEMICAL PROPERTIES OF ENZYME-TREATED AND UNTREATED BLOOD GROUP A AND O(H) SUBSTANCES (HOG) AND OF DIALYZABLE SPLIT PRODUCTS

Expt. (1)	Prepn. and blood group act. (2)	Soln. (3)	BGS, mg. (4)	μg. Inhibiting: A anti-A: Soln.[c] (5)	μg. Inhibiting: A anti-A: Reisol. BGS (6)	μg. Inhibiting: O(H) anti O(H): Reisolated BGS (7)	Methylpentose: Orig. BGS, % (8)	Methylpentose: Recovd. in dialyzate: Mg. (9)	Methylpentose: Recovd. in dialyzate: Total, % (10)	Methylpentose: Reisol. BGS,[b] % (11)	Hexosamine: Orig. BGS, % (12)	Hexosamine: Recoved. in dialyzate: Before hydrol., mg. (13)	Hexosamine: Recoved. in dialyzate: After hydrolysis[e]: Mg. (14)	Hexosamine: Recoved. in dialyzate: After hydrolysis[e]: Total, % (15)	Hexosamine: Reisol. BGS,[b] % (16)	N-Acetylhexosamine Recovd. in dialyzate: Mg. (17)	N-Acetylhexosamine Recovd. in dialyzate: Total dialyzable hexosamine,[f] % (18)	Reducing sugar[g]: Orig. BGS, % (19)	Reducing sugar[g]: Recovd. in dialyzate: Before hydrol., Mg. (20)	Reducing sugar[g]: Recovd. in dialyzate: After hydrolysis[e]: Mg. (21)	Reducing sugar[g]: Recovd. in dialyzate: After hydrolysis[e]: Total, % (22)	Reducing sugar[g]: Reisol. BGS,[b] % (23)
1	(6A)	BGS + enzyme	91	12	5	4	9	2.10	26	9	26	0.78	3.47	14	29			59	8.60	7.2	13	57
	A +	BGS + enzyme, heated[a]	5	0.5																		
	O(H)	BGS	97	0.5	0.2	2	9	0		11	26	0	0		33			59	0	0		58
		Enzyme	...					0		0[d]		0	0		0[d]				0.15	0.73		
2	(6A)	BGS + enzyme	94	2.5	5	4	9	0.69	8	12	26	0.41	1.33	6	32	1.3	79	59	3.05	3.78	7	63
	A +	BGS + enzyme, heated[a]	5	0.5																		
	O(H)	BGS	94	0.5	0.2	2	9	0		12	26	0	0		32			59	0	0.96		64
		Enzyme						0		0[d]		0	0		0[d]				0.40	1.89		
3	(7A)	BGS + enzyme	95	2.5	5	14	9	0.94	11	9	31	0.50	2.26	8	33	1.8	64	54	3.15	4.11	8	60
	A +	BGS + enzyme, heated[a]	5	0.3																		
	O(H)	BGS	101	0.1	0.5	14	9	0		10	31	0	0		34			54	0	0.84		63
		Enzyme	...					0		0[d]		0	0		0[d]				0.10	1.08		
4	(50)	BGS + enzyme	96	2.5	5–10		8	0.50	7	9	30	0.37	1.57	5	30	1.0	51	51	3.40	3.93	8	61
	A	BGS + enzyme, heated[a]	5	0.3																		
		BGS	100	0.3	0.5		8	0		9	30	0	0		34			51	0.60	1.59		58
	(54)	BGS + enzyme	88			8	8	0.72	10	9	26	0.10	0.24	1	32	(0.3)		50	2.00	2.58	6	57
	O(H)	BGS + enzyme, heated[a]	5																			
		BGS	83			8	8	0		10	26	0	0		32	0		50	0.60	0.93		55
		Enzyme	...					0				0	0		0[d]	0			0.90	1.14		
		Buffer	...					0				0	0			0			0.43	0.66		
5	(50)	BGS + enzyme	81	11	5–10		8	0.76	12	7	30	0.63	3.12	13	32	1.6	41	51	4.55	5.04	12	58
	A	BGS + enzyme, heated[a]	4	0.4–1																		
		BGS	69	0.7–2	0.5		8	0		8	30	0	0		30	0		51	0.48	0.81		57
	(52)	BGS + enzyme	89	5–14	20		8	0.93	13	8	33	0.69	4.14	14	31	2.0	39	56	5.42	5.64	11	58
	A	BGS + enzyme, heated[a]	5	0.5–1																		
		BGS	91	0.5–1	0.5		8	0		8	33	0	0		34	0		56	0.75	1.05		60
	(38)	BGS + enzyme	87			4–8	7	0.92	15	7	32	0.18	0.50	2	27	(0.6)		53	2.23	2.67	6	62
	O(H)	BGS + enzyme, heated[a]	5																			
		BGS	91			5–9	7	0		7	32	0	0		29	0		53	0.43	0.87		57
	(42)	BGS + enzyme	99			4–8	6	0.79	13	6	27	0.21	0.58	2	31	(0.7)		49	2.63	3.18	7	58
	O(H)	BGS + enzyme, heated[a]	5																			
		BGS	98			2–4	6	0		7	27	0	0		30	0		49	0.58	1.44		59
		Enzyme	...					0				0	0			0			0.48	1.11		
		Buffer	...					0				0	0			0			0.25	0.30		

[a] 5% of initial mixture of BGS + enzyme, heated to 100° for 10 minutes. [b] Precipitate obtained at 16% ethanol from phenol extract of non-dialyzable residue. [c] Figures referable to micrograms BGS originally in solution. [d] Analyses on slight precipitate obtained from non-dialyzable residue by addition of ethanol to 80% by volume. [e] 2 N HCl, 100°, 2 hours. [f] 0.81 of column 21 divided by column 18. [g] Referred to glucose.

mixture, and, except in experiment 1, into the enzyme control. Incubation was continued for another 2–3 weeks, during which no further reduction in blood group A activity was detectable by hemagglutination inhibition. In experiment 5, the total period of incubation was only 30 days, the addition of a second aliquot of enzyme having been omitted. In each of the first 4 experiments, a total of 2.2–3.2 mg. of enzyme nitrogen, and in experiment 5, 0.5 mg. of enzyme nitrogen, had been added to the active enzyme digest and to the enzyme control. In a similar experiment not otherwise detailed herein, blood group A substance was incubated for 30 days with fractions 1 and 2 of enzyme preparation 3. Specific β-glucosaminidase activity in the enzyme control, while readily detectable, was reduced by about 50% at the end of the incubation period.

There was no obvious bacterial contamination in any of the solutions at the end of the total incubation period. Blood agar streak plates made from all solutions in experiments 4 and 5 just prior to dialysis remained sterile.

After incubation, all solutions but the heated enzyme control were dialyzed against 4–6 daily changes of de-ionized water. The dialyzates were concentrated in an atmosphere of nitrogen under reduced pressure at 50–55°. After being made up to 10 ml., they were analyzed for hexosamine and reducing sugar before and after hydrolysis with 2 *N* HCl for 2 hours, and for methylpentose and N-acetylhexosamine.

Ethanol to 80% by volume was added to the non-dialyzable residues. The resulting precipitates obtained from the solutions containing blood group substance were dried and extracted with phenol. Blood group substance was then reisolated from this phenol solution by precipitation with ethanol in a concentration of 16% by volume. This fraction is referred to in Table II as "Reisolated BGS."

Results

The capacity of blood group substance contained in the active enzyme digest, with reference to the concentrations originally introduced into the mixture, to inhibit the agglutination of group A erythrocytes by anti-A was reduced in most instances about 10-fold when compared with the heated enzyme control and the substrate control in the same experiment (Table II, column 5). Likewise the enzyme-treated blood group substances reisolated from the non-dialyzable residues had in all instances become one-tenth to one-twentieth as active as the corresponding untreated controls (Table II, column 6). In contrast, the O(H) antigen appeared to be unaffected by similar exposure to the enzyme (Table II, column 7). The differences in O(H) activity between the reisolated enzyme-treated and untreated BGS in experiments 1 and 2 are not significant, and the O(H) activities of the corresponding fractions containing O(H) substance in experiments 4 and 5 are virtually identical. However, due to the lack of precipitating anti-O(H) serum, no judgment can be made as to whether slight alteration of the blood group O(H) antigen resulted from enzymic treatment.

The reisolated blood group substance from the non-dialyzable residues amounted in most instances to 60% or more of that originally introduced. Recoveries of untreated BGS in experiment 1 and of both enzyme-treated and untreated BGS in experiments 2 and 3 were low because a considerable portion of the non-dialyzable residue in these instances had become insoluble in phenol. However, with the addition of this phenol-insoluble fraction, the recovery of blood group substance in the cases cited amounted to well over 70%.

Twenty-six per cent. of the methylpentose originally present in experiment 1, and up to 15% of that originally present in experiments 2–5, was recovered in dialyzable form from the active enzyme digests. None was recovered from any of the controls (Table II, columns 9–11). Fucose was identified by paper chromatography[17] in the dialyzate of the active enzyme digest in experiment 1. Of the hexosamine calculated to have been present initially, up to 14% was recovered in the dialyzates of digests containing A substance, but no more than 2% in the dialyzates of digests containing O(H) substance. In addition, significant amounts of N-acetylhexosamine[22] were found in the A substance digest dialyzates, and on a molar basis accounted for 39 to 79% of the total hexosamine liberated (Table II, columns 17 and 18). Hydrolysis of these dialyzates with 2 *N* HCl for 2 hours resulted in a 4- to 6-fold increase in the color value for hexosamine. A slight effect was noted with the dialyzates of O(H) substance digests; but the analytical figures were at the lower limit of accuracy of the hexosamine method (Table II, columns 13 and 14).

N-Acetylhexosamine and hexosamine in the dialyzates were differentiated by paper chromatography[23] both on the basis of color reaction and R_f, using butanol–acetic acid–water (4:1:5) as solvent. In experiments 2 and 3, free N-acetylhexosamine was demonstrated in the unhydrolyzed dialyzates of active enzyme digests; after hydrolysis, only hexosamine was detectable. In experiment 5, dialyzates of A substance digests showed N-acetylhexosamine before hydrolysis and hexosamine alone after hydrolysis. No amino sugars were found by paper chromatography in dialyzates of the O(H) substance digests or in dialyzates of any of the controls.

There was no striking alteration in the analytical properties of the various substances as a result of enzyme treatment (in Table II, compare columns 8 and 11; 12 and 16; 19 and 23). Whenever there was a difference between the value for the original substances and those for the reisolated substance (*e.g.*, reducing sugar and nitrogen values for substances 50 and 52 in experiment 5) the change was noted in the untreated as well as in the enzyme-treated BGS, and could be ascribed to small amounts of residual impurities removed by the additional phenol extraction and alcohol precipitation. In experiment 5, samples of reisolated enzyme-treated and untreated hog substance 52 gave acetyl values[24] of 10.31 and 10.62%, respectively.

In the first three experiments, the precipitate obtained at 80% ethanol from the non-dialyzable residues of the enzyme-treated and untreated BGS (80% precipitate) and the phenol-insoluble portion of this precipitate were analyzed for methylpentose, hexosamine, reducing sugar and nitrogen. The analytical figures for these various fractions in each experiment were closely comparable with those for the reisolated BGS obtained as the 16% ethanol precipitate of phenol-soluble material, with the exception of experiment 3, in which

(23) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(24) Determined by Dr. A. Elek, using the method of A. Elek and R. A. Harte, *Ind. Eng. Chem., Anal. Ed.*, **8**, 267 (1936).

the phenol-insoluble fraction from both the enzyme-treated and the untreated BGS were incompletely water soluble.

Quantitative data showing the effect of enzyme treatment on the blood group A antigen are given in Fig. 1. Reisolated enzyme-treated blood group substances with A activity showed a consistently reduced capacity per unit weight to precipitate anti-A when compared with the reisolated untreated substance in the same experiment. The differences, while not great, paralleled the reduction in hemagglutination-inhibiting potency shown in columns 7 and 8 of Table II.

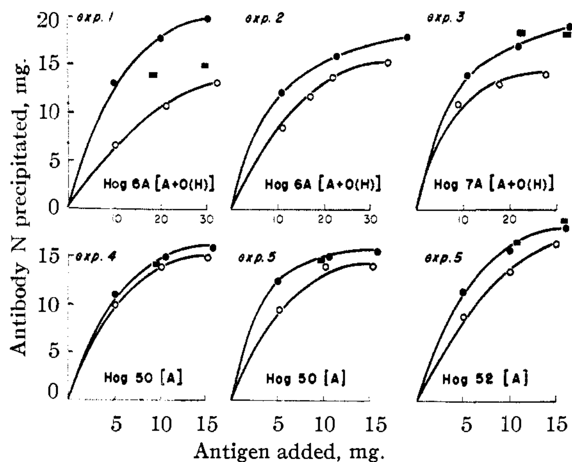

Fig. 1.—Effect of snail enzyme on precipitation of anti-A: O, reisolated enzyme-treated BGS; ●, reisolated untreated BGS; ■, original BGS.

Cross-reactivity with type XIV anti-pneumococcal horse serum was significantly altered by enzymic treatment in all cases. As is shown in Fig. 2, the reisolated enzyme-treated BGS was capable of precipitating significantly greater quantities of anti-SXIV than were similar quantities of either the reisolated untreated BGS or preparations of the same BGS which had been stored in unbuffered aqueous solution or had been freshly dissolved.

Discussion

The values in Table I show that β-glucosaminidase activity was preponderant in all of the enzyme preparations, although slight α-glucosaminidase activity was regularly detectable. There appeared to be more of both enzymes per mg. nitrogen in fraction 2 than in fraction 1. Even after more than one year's storage at ice-box temperatures under toluene there were 700 units per mg. nitrogen of β-glucosaminidase (preparation 2). Other workers have also reported prolonged storage of enzyme solutions without loss of activity, in some cases for periods up to three years.[5] In the present experiments, β-glucosaminidase activity survived 1 month of incubation at 37° in solutions representing tenfold dilutions of the original enzyme preparations.

Preliminary experiments had shown that the enzyme reduced the A activity of hog blood group substance maximally at pH 4.0 and 4.5, in agreement with Freudenberg and Eichel[2] and with

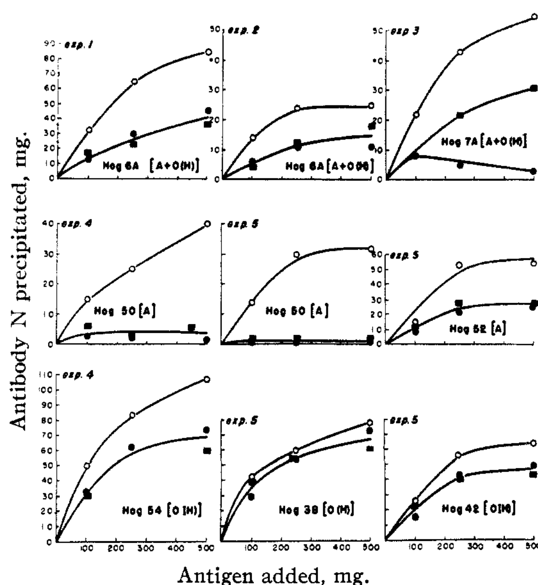

Fig. 2.—Effect of snail enzyme on cross reaction with type XIV anti-pneumococcal horse serum: O, reisolated enzyme-treated BGS; ●, reisolated untreated BGS; ■, original BGS.

Yosizawa,[6] acted very slightly at pH 5.0, and had no detectable effect at pH 6.0 or 7.0. At pH 4.0, the effect obtained after incubation at 37° was equivalent to that obtained at 20°. Using freshly prepared enzyme solutions, reduction in blood group A activity was detectable after only 4 days of incubation; however, with enzyme solutions previously stored for long periods at ice-box temperatures, as in the present experiments, no diminution in A activity could be detected until several weeks had elapsed. In the first four experiments, a second addition of enzyme produced no further reduction. Since the blood group substance in the heated enzyme controls retained full A activity throughout each experiment (Table II, column 5), the immunochemical changes noted in the reisolated enzyme-treated BGS can be ascribed to true carbohydrase activity rather than to any non-enzymic effects of crude snail liver extracts. Results obtained by other workers also suggest that the enzyme or enzymes responsible for altering the blood group A specificity act on the intact substances at a relatively slow rate.[2,6]

It has been shown that blood group substances A and O(H), heated for 2 hours to 100° at pH 1.5 and 1.8, respectively, lost their specific blood group activity and showed increased cross-reactivity with type XIV pneumococcal horse antibody.[17] After dialysis and reprecipitation with ethanol, the non-dialyzable residues, representing about two-thirds of the original blood group substance, showed a greatly reduced methylpentose content, but the concentrations of hexosamine and reducing sugar were approximately the same as in the original substance. The increase in cross-reactivity was ascribed in part to the splitting of methylpentose from the molecule. In the present experiments, reisolated enzyme-treated blood group substance, while exhibiting an equally striking increase in cross-reactivity with anti-SXIV, when compared

with the reisolated untreated substance showed no essential change in methylpentose content, hexosamine, or reducing sugar; the reduction of A activity was slight, and no reduction in O(H) activity was detectable. This apparent difference may be only one of degree, since heating hog A substances to 100° at *p*H 2.90, while still increasing the cross-reactivity with anti-SXIV, diminished the blood group A activity only slightly (*cf.* Table I, ref. 17b). It is not known to what extent methylpentose was split off under these conditions. If increase in cross-reactivity with anti-SXIV be largely related to loss of methylpentose from the molecule, the work reported herein suggests that the amount which must be split off is relatively small. An estimate of this amount should be gained more reliably from determination of methylpentose in concentrated dialyzates than from analysis of the blood group substances themselves before and after enzymic treatment. In these 5 experiments, the methylpentose recovered in the dialyzates varied from 7 to 26% (av. 15%) of the total originally present in the intact blood group A or O(H) substance. Thus a change of this magnitude would not necessarily be apparent on examination of the non-dialyzable residues from which only about 60% of the original weight of material could be reisolated; also, the error in the determined methylpentose value in the latter might be appreciably greater. It is further possible that the splitting of methylpentose from one site on the molecule might have a more pronounced effect on cross-reactivity than its removal from another site. Increase in cross reactivity occurred with both substances, but was somewhat less with the O(H) substances in which the inherent cross-reactivity was appreciable before enzymic treatment. In experiment 3, the significant difference in cross-reactivity (Fig. 2) between the reisolated untreated BGS and the original BGS may have been due to changes following one additional phenol extraction and alcohol precipitation. The cross-reactivity of both controls was still significantly less than that of the reisolated enzyme-treated BGS.

It is also possible that the increased cross-reactivity with anti-SXIV following enzymic treatment or mild acid hydrolysis may have been due in part to the loss or alteration of some constituent other than methylpentose.[17,25] In the present experiments, investigation of the non-hexosamine nitrogen split from the BGS by enzyme was not possible since the enzyme solution was shown by paper chromatography to contain free amino acids after prolonged incubation at 37°. There was no significant difference in acetyl content between reisolated enzyme-treated and untreated blood group substance in one experiment (exp. 5, BGS No. 52); since a snail extract has been reported[6] as lacking in N-deacetylase, this enzyme was probably absent from the present preparations also.

The dialyzable hexosamine recovered from the A substances was 5–7 times that recovered from the O(H) substances in terms of total hexosamine originally present in the BGS–enzyme mixture (Table II, column 15). The reducing sugar recovered in the dialyzate of the enzyme-treated O(H) substances was lower than that recovered from the enzyme-treated A substances (Table II, column 22). From 39–79% of the total hexosamine liberated from the A substances appeared to be in the form of N-acetylhexosamine (Table II, column 18), in accord with the original observations of Freudenberg.[2] Since the method for N-acetylhexosamine[22] tends to give low values, it is possible that more, or even all, of the hexosamine liberated by the enzyme was in the form of free N-acetylhexosamine. These results, together with the finding already noted that enzyme reduced the activity of the A but not of the O(H) antigen, suggest that the linkages in the A substances attacked by enzyme (possibly beta-glucosaminidase[cf. 6]) differ structurally from those in the O(H) substances. It is further possible that the activity of at least two types of enzyme may have to be invoked, one accounting for the fairly uniform loss of methylpentose and increased cross-reactivity in both A and O(H) substances, and the other distinguishing between A and O(H) substances with respect to the form and amount of hexosamine liberated and the effect on blood group specificity.

Finally, Yosizawa[6] noted that, although the release of reducing substance from hog mucin blood group substance by the activity of β-acetylglucosaminidase solution was very slight after 3 days, exposure of the same enzyme to blood group substance, previously heated to 100° for two hours with 0.1 N sulfuric acid, resulted in a fivefold increase in free hexosamine.[6] Since a large proportion of methylpentose and considerable non-hexosamine nitrogen (amino acid nitrogen) has been shown to be liberated by partial acid hydrolysis,[17b] the prior removal of such groups might permit more extensive attack on the remainder of the molecule by the snail enzyme. Further investigation of this point is in progress.

Acknowledgments.—The authors are indebted to Dr. Karl Meyer for carrying out the initial assays, and supplying substrates for additional assays for glucosaminidase and glucuronidase. Miss Louisa Perez performed some of the assays for blood group activity.

NEW YORK 32, N. Y.

(25) S. M. Beiser and E. A. Kabat, *J. Immunol.*, **68**, 19 (1952).