VIII. THE ACTION OF ENZYMES ON ANTIBODIES

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Experiments on the action of pepsin, trypsin and papain on antibodies form the main subject of the present paper. The results are dealt with in considerable detail since they seem to have some bearing on the nature of the chemical changes in globulin induced by immunization.

The antibodies chosen for these experiments were the flagellar and somatic typhoid agglutinins, known as the H and O agglutinins respectively. They are precipitated with the globulin fraction of antityphoid serum.

Sera from three horses, M, C and K, were available in quantities sufficient for the present investigation. The horses were immunized at the Serum Department of the Lister Institute by Dr G. F. Petrie and his colleagues on behalf of Dr A. Felix. The immunizing courses consisted of series of intravenous doses of suspensions of certain strains of *B. typhosus*, living, or killed by various methods. Horses M and C were also immunized for a short period with formalized extracts of these strains. Sample I from each horse was normal serum drawn prior to the first course of immunization. Samples of immune serum obtained subsequently at successive bleedings were numbered from II upwards. These samples were stored at 0° without addition of antiseptic. At the time of testing some of these samples had been stored for a few weeks, others for various periods up to or exceeding a year.

Globulin fractions were precipitated from a number of serum samples with 19 volumes of 22·6 % Na_2SO_4 at 37°. The precipitates were washed with saturated Na_2SO_4 and taken up in normal saline (0·85 % NaCl) to give solutions of titre corresponding to a 1 : 5 or 1 : 10 dilution of the original serum. Euglobulin fractions were precipitated from serum by addition of 19 volumes of 14·2 % Na_2SO_4 or by dilution with distilled water and acidification. The antibodies were found to be distributed between the euglobulin and pseudoglobulin fractions.

In each experiment on enzyme action unfractionated serum, or the globulin fraction, was incubated in a water-bath at 37° together with enzyme solution, buffer of suitable *p*H and saline to give a final concentration equivalent to 1 : 10 or 1 : 20 serum. Chloroform or toluene was usually added. Samples were withdrawn initially and at suitable intervals for estimation of the titres of H and O agglutinins and the degrees of protein hydrolysis. The effects of buffer, *p*H, enzyme activators and inhibitors on agglutinin titre were carefully controlled.

H and O agglutinins were tested for separately with the technique described in a previous paper [Rosenheim, 1935]. Formalized suspensions of *B. typhosus* of the motile strains "Mrs S." and "Watson" were used for the detection of H agglutinins and the non-motile strain "O 901" for O agglutinins. Most of the sera used contained the so-called Vi antibody [Felix & Pitt, 1934] in addition to the H and O agglutinins. As the titre of Vi agglutination is very much lower

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than that of H or O agglutination, the presence of Vi antibody in varying amounts did not interfere with the quantitative estimation of H and O antibodies.

The percentage of H agglutinin destroyed was calculated from a comparison of the end-titres of control and experimental solutions. Quantitatively comparable titres were taken as a basis for comparison rather than end-titres in any experiment in which the degree of agglutination was not the same at the end-titre of every series.

An indication of the extent of protein hydrolysis was usually obtained by estimating the increase in free amino-groups by the Van Slyke method using a micro-Van Slyke apparatus. In a few experiments the increase in free COOH groups was estimated by titration with alcoholic KOH [Willstätter & Waldschmidt-Leitz, 1921]. The total N in each serum sample or globulin fraction was estimated by the micro-Kjeldahl technique and the increase in free amino-N was expressed as percentage of the total N.

Experimental

I. *The action of enzymes on the antibodies in serum obtained from three horses after the first immunizing course with H and O antigens*

Pepsin. If the agglutinins are exposed to a *p*H lower than 4·6 they are gradually inactivated. Experiments on peptic action were therefore carried out at *p*H 4·6–4·8. Although this *p*H range is far removed from the optimum for pepsin the enzyme was sufficiently active to destroy both the H and O agglutinins at a remarkably rapid rate. Its action on the immune globulin was, however, slow, particularly at the higher *p*H. At *p*H 5·6 the agglutinins and the globulin were not attacked. The experiment described below and summarized in Table I is typical of many and will serve to illustrate the technique adopted and the method of estimating the results.

The globulin precipitated from serum C II was taken up in saline to give a solution which corresponded in titre to a 1 : 5 dilution of the original serum and contained 7·6 mg. of protein per ml. A 6·1 % solution of B.D.H. granular pepsin was used in concentration calculated to give 4 mg. pepsin per mg. of globulin.

The following mixtures were prepared:

	Globulin control ml.	Globulin + pepsin ml.	Pepsin control ml.
Globulin	3	3	0
N/10 Na citrate, *p*H 4·7	1·5	1·5	1·5
Pepsin solution, *p*H 4·7	0	1·5	1·5
Water	1·5	0	3
Chloroform	1 drop	1 drop	1 drop

1·5 ml. of each solution were withdrawn immediately and peptic action was inhibited by neutralization with *N*/40 NaOH from a micro-burette. The volume was made up to 3 ml. with saline, solutions of globulin equivalent to a 1 : 20 dilution of serum being thus obtained.

The three mixtures were placed in a water-bath at 37°. Further samples of each were withdrawn, neutralized and diluted as above after 2, 5 and 22 hours and were stored at 0°. Duplicate Van Slyke amino-N estimations were carried out on 1 ml. samples of the neutralized solutions. The values obtained for the pepsin control series showed that there was no increase of amino-N due to pepsin autolysis during the period of enzyme action.

Table I. *Action of pepsin on the globulin fraction of antityphoid serum C II**

	H titre								O titre							
	Globulin control				Globulin + pepsin				Globulin control				Globulin + pepsin			
Duration of hydrolysis hours ...	0	2	5	22	0	2	5	22	0	2	5	22	0	2	5	22
Titre:																
1: 800	+++	+++	+++	+++	+++	++±	++	+	+++	+++	+++	+++	+++	++±	++	(±)
1: 1,600	++±	++±	++±	++±	++±	++	+±	(±)	++±	++±	++±	++±	++±	++	+±	.
1: 3,200	++	++	++	++	++	+±	+	.	++	++	++	++	++	+±	+	.
1: 6,400	+±	+±	+±	+±	+±	+	(±)	.	++	++	++	++	++	+±	+	.
1: 12,800	+±	+±	+±	+±	+±	±	.	.	+±	+±	+±	+±	+±	+±	±	.
1: 17,066	+	+	+	+	+	±	.	.	+±	+±	+±	+±	+±	+	.	.
1: 20,480	+	+	+	+	+	(±)	.	.	+±	+±	+±	+±	+±	+	.	.
1: 25,600	±	±	±	±	±	.	.	.	+	+	+	+	+	±	.	.
1: 34,133	(±)	(±)	(±)	(±)	(±)	.	.	.	+	+	+	+	+	(±)	.	.
1: 51,200	.	.	.	.	.	.	.	.	±	±	±	±	±	.	.	.
1: 68,266	.	.	.	.	.	.	.	.	(±)	(±)	(±)	(±)	(±)	.	.	.
1: 102,400	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Destruction of agglutinin (%)	.	0	0	0	.	40	81	95	.	0	0	0	.	50	75	99
Increase in amino-N as % of total N					.	1·3	2·6	5·0					.	1·3	2·6	5·0

* For details see text.

Agglutination tests on the globulin control tubes served to control the effect of incubation at *p*H 4·7 on the agglutinin titres. The control and experimental solutions were tested simultaneously for agglutinin content with the usual technique using formalized suspensions of the strains "Watson" and "O 901". Besides the usual dilutions in geometric series, 1 : 400, 1 : 800, 1 : 1,600 etc., additional intermediate dilutions were made between 1 : 12,800 and 1 : 102,400 to make possible a closer estimate of the degree of agglutinin destruction.

The customary symbols $+++$, $++\pm$, $++$, $+\pm$, $+$, $\pm$, $(\pm)$ are used to cover the range of floccular (H) and granular (O) agglutination from complete agglutination to a trace not visible without magnification.

The experiment summarized in Table I illustrates (1) the progressive destruction of the agglutinins with time of hydrolysis, (2) the approximately parallel rates of breakdown of the H and O agglutinins and (3) the relatively slight degree of protein breakdown which accompanied an almost complete loss of agglutinating power.

In another experiment in which a lower concentration of pepsin relative to globulin was allowed to act on an immune globulin solution for 19 hours at *p*H 4·8 there was destruction of 97 % of the H agglutinin and 100 % of the O agglutinin with hydrolysis of protein giving an amino-N increase of only 1·74 % of the total globulin-N.

Samples of pepsin from three different firms have been used in this work with essentially similar results. A sample of May and Baker's "Powdered Pepsine B.P." was further purified along the lines used by Northrop [1930] for the crystallization of pepsin and was then active in proportionately lower concentration. The possibility that agglutinin destruction was due to combination with, or the action of, some impurity in the commercial pepsin may therefore be excluded.

Trypsin. Whereas peptic action can readily be arrested at any stage of a hydrolysis by neutralization, no satisfactory method has been found of inhibiting tryptic action without simultaneously destroying the H and O antibodies. It was not possible to obtain more than an approximate comparison of protein and antibody contents in successive samples of tryptic hydrolysis mixtures, since the conditions essential for the agglutination test favour further enzyme action. It has, however, been established that the destruction of the agglutinins by trypsin is very rapid.

In the few experiments in which a comparison of the degree of protein hydrolysis and the degree of antibody destruction was attempted, the technique was complicated and less satisfactory than that used in experiments with pepsin. All the agglutination tests in any one experiment were performed under strictly comparable conditions but not all at one time. Each sample withdrawn from the hydrolysis mixture was immediately diluted in series for agglutination and incubated at 50° together with a control series containing no trypsin. Van Slyke estimations were performed on samples withdrawn at the same time. In this way values were obtained for the increase in amino-N in x hours at 37° and the percentage destruction of the agglutinins by trypsin in x hours at 37° plus the incubation period at 50°. A comparison of 0 hour control samples with and without trypsin showed that approximately 50 % of the H agglutinin was destroyed by trypsin during the 2-hour incubation period at 50°.

In a sample of globulin from C II serum the H and O agglutinins were completely destroyed in 4 hours at 37° plus the incubation period at 50°. The increase in amino-N in 4 hours at 37°, *p*H 8·7, was 11·6 % of the total N.

Papain. At *p*H 7·0 the H and O typhoid agglutinins were destroyed by activated, but not by non-activated, papain. From a typical experiment summarized in Table II it will be seen that KCN, cysteine and H_2S were equally

Table II. *Action of activated and non-activated papain on the H and O agglutinins in the globulin fraction of serum sample II from horse M*

6 hours. 37°. *p*H 7·0

	A	B	C	D	E	F
	ml.	ml.	ml.	ml.	ml.	ml.
Globulin*	0·5	0·5	0·5	0·5	0·5	0·5
M/3 phosphate buffer, *p*H 7·0	0·17	0·17	0·17	0·17	0·17	0·17
Saline	0·33	—	—	—	—	—
2% non-activated papain	—	0·33	—	—	—	—
2% KCN	—	—	0·33	—	—	—
2% KCN-activated papain	—	—	—	0·33	—	—
2% cysteine-activated papain	—	—	—	—	0·33	—
2% H_2S-activated papain	—	—	—	—	—	0·33
Initial H titre	25,600	25,600	25,600	25,600	25,600	25,600
Final H titre	25,600	25,600	25,600	800	400	<400
% destruction of H agglutinin	0	0	0	97	98·5	> 98·5
Initial O titre	12,800	12,800	12,800	12,800	12,800	12,800
Final O titre	12,800	12,800	12,800	3,200	<400	400
% destruction of O agglutinin	0	0	0	75	>97	97

* In a solution of concentration equivalent to a 1 : 5 dilution of the original serum.

effective as activators. For this experiment 4 g. of B.D.H. papain were extracted with 100 ml. of water for 1½ hours on a mechanical shaker. Part of the clear yellow filtrate was brought to *p*H 7·0 and diluted to give a 2% extract. Aliquot parts of this extract were then activated either by bubbling washed H_2S through for 1 hour or by incubation at 37° with 0·1 ml. of a 16% cysteine hydrochloride solution for 30 min. A cyanide-activated solution of comparable strength was prepared by incubating at 37° equal volumes of a neutralized 4% KCN solution and the 4% papain extract.

The composition of the experimental solutions is given in Table II. Papain extract equivalent to 2 mg. of solid enzyme preparation was added for each mg. of serum protein. After 6 hours at 37° enzyme action was arrested by addition of an equal volume of *M*/250 sodium iodoacetate to solutions B–F. Mixture A was equally diluted with saline to serve as a standard of comparison. It will be seen from B that non-activated papain was without action on the agglutinins and that iodoacetate exerted no destructive action, from C that KCN alone did not affect the titre and from D, E and F that papain activated with KCN, cysteine and H_2S respectively almost completely destroyed the agglutinating properties of the immune globulin in 6 hours at *p*H 7·0.

The experiments quoted in Table III show that the destruction of the agglutinins by cysteine-activated papain was progressive and rapid and give an indication of the degree of globulin hydrolysis which accompanied this destruction. For these experiments 20 ml. of a mixture of cysteine-activated papain, globulin and phosphate buffer at *p*H 7·0 were incubated at 37°. Samples were withdrawn initially and at intervals, diluted with an equal volume of *M*/250 iodoacetate and used for agglutination tests and duplicate amino-N determinations.

Under the conditions chosen activated papain was found to be more active proteolytically than pepsin and less active than trypsin for comparable degrees of agglutinin destruction.

Since the work of Willstätter & Grassmann [1924] on the activation of papain by cyanide, the view has become general that the range of specificity of natural papain is extended by activation, and that whereas before activation

Table III. *Action of cysteine-activated papain on the H and O agglutinins in the globulin fraction of serum sample II from horse C*

Extract of 2 mg. papain per mg. protein. *p*H 7·0

Exp.		Control globulin	Duration of hydrolysis: 0 hour	½ hour	1 hour	2 hours
a	H titre	19,200	19,200	12,800	9,600	6,400
	% destruction of H agglutinin	—	0	33	50	66
	O titre	102,400	102,400	76,800	51,200	12,800
	% destruction of O agglutinin	—	0	25	50	87·5
	Increase in amino-N as % of total N	—	—	—	1·7	5·1
b	H titre	3,200	3,200	—	800	150
	% destruction of H agglutinin	—	0	—	75	>95
	O titre	12,800	12,800	—	3,200	1,600
	% destruction of O agglutinin	—	0	—	75	87·5
	Increase in amino-N as % of total N	—	—	—	1·75	3·5

protein molecules only are attacked, after activation protein degradation products such as polypeptides are also split. The recent work of Bergmann and his co-workers [1935; 1936], which promises to be of great significance in elucidating the mechanism of papain action, has necessitated a revision of this view. It is premature as yet to present Bergmann's hypothesis in detail, but a point with direct bearing on the interpretation of the present results must be referred to.

Bergmann & Zervas [1936] found that comparatively simple substrates such as benzoyl*iso*glutamine and hippuramide, as well as gelatin, were attacked by natural non-activated papain. The action of HCN on papain produced no change in specificity but accelerated the splitting. According to the generally accepted theory of the action of activators on papain the logical interpretation of the finding that the H and O agglutinins are attacked by activated and not by non-activated papain under the given experimental conditions would be that antibodies are of a polypeptide and not of a globulin nature. In view of Bergmann's findings, which are based on a considerable amount of evidence, the suggestion that the agglutinins are of a non-globulin nature need not be entertained as a result of the experiments here described.[1]

The results obtained in the experiments with yeast dipeptidase and aminopolypeptidase summarized below provide additional evidence for the generally accepted view that antibodies are not peptides intimately associated with the globulin fraction of immune serum, but an integral part of that fraction.

Dipeptidase. This enzyme was prepared from brewer's top yeast by the method of Macrae [1933]. The preparation was free from proteinase and contained only a trace of polypeptidase. The activity was such that 30 mg. acting on 56 mg. of *dl*-leucylglycine at *p*H 7·8 produced an increase of COOH equivalent to 0·54 ml. of *N*/20 KOH in 1 hour at 40°. 30 mg. acting on 21 mg. of immune globulin of initial H titre 12,800 and O titre 10,240 produced no change in titre and no increase in titratable COOH in 24 hours at *p*H 7·8 and 40°.

Aminopolypeptidase. The activity of a solution of yeast aminopolypeptidase, prepared from brewer's top yeast by the method of Macrae [1933], was such that

[1] Bergmann's recent work was published after completion of these experiments. As his estimations of papain activity were carried out at *p*H 5·0 a few additional experiments were undertaken in which papain was allowed to act on the agglutinins at this *p*H instead of at *p*H 7·0 as before. It was found that under these conditions the agglutinins were destroyed to a slight extent by non-activated papain, and that destruction was accelerated by activation with KCN.

4 ml. acting on 61 mg. of *dl*-leucylglycylglycine at *p*H 7·0 and 37° produced in 1 hour hydrolysis equivalent to an increase of 0·52 ml. of *N*/20 KOH. 4 ml. acting on 10% gelatin at *p*H 7·0 produced no liquefaction in 40 hours at 37°. The solution, although not purified, was therefore free from proteinase. 4 ml. of this solution incubated at *p*H 7·0 with 21 mg. of immune globulin had no effect on the titres of H and O antibody in 24 hours at 37°.

It should be emphasized that the results of all the experiments described in this section relate only to the action of enzymes on those samples of serum (or the globulin fractions thereof) obtained from each of three horses after a first immunizing course with H and O antigens. It is of interest to compare them with those of Macrae [1935] who studied the action of enzymes on antibodies having a specific affinity for malignant cells. After complete destruction of these antibodies by pepsin, trypsin and papain more than 80% of the euglobulin, with which they were associated, could be recovered apparently unchanged. Yeast proteinase caused destruction of the antibodies. Yeast dipeptidase and amino-polypeptidase were without effect.

II. *The action of enzymes on the antibodies in serum obtained from three horses after each of a succession of immunizing courses*

The results of the experiments described in Part I of this paper point to the conclusion that the H and O agglutinins in antityphoid serum are rapidly destroyed by proteolytic enzymes. Further work, however, necessitated a modification of this view. In an experiment with sample V of serum from horse C it was noticed that the H antibody was not destroyed by pepsin as was that in sample II under identical conditions. Any facts indicative of changes in the immune globulin during the course of immunization, and particularly such as are susceptible of a chemical interpretation, are of great interest. This point was therefore investigated in numerous experiments by allowing pepsin, trypsin or activated papain to act under strictly comparable conditions on samples of serum obtained at different times from the same horse. These samples were drawn after each of a succession of immunizing courses spread over several months.

The antigens used for immunization, the dates of bleeding and the variation in titre from sample to sample may be seen by reference to Table IV, in which the results of three such experiments with pepsin are summarized.

Essentially similar results were obtained with serum from each of the three horses.

The outstanding points in the table are:

(1) The marked difference in the percentage reduction by pepsin action of the H agglutinin titre in early and later serum samples.

(2) The uniformity in the percentage reduction of the O agglutinin titre in all samples.

The results suggest that the H agglutinin becomes increasingly resistant to peptic hydrolysis with each successive immunizing course. This interpretation cannot, however, be accepted without certain reservations.

It must be borne in mind that the nature of the agglutination test is such that the backward displacement of the end-titre by one or, at the most, two tubes in a long series of dilutions of a high-titre serum (expressed as a 50% reduction in titre) represents a far greater reduction in agglutinin units than a backward displacement by several tubes in a low-titre serum. On this account the reduction in titre through peptic action is expressed in Table IV in arbitrary units—the difference between initial and final titres—as well as in percentages.

Table IV. *Action of pepsin on the H and O agglutinins in serum samples obtained from horses M, C and K at successive bleedings*

20 hours. *p*H 4·7. Approx. 4 mg. B.D.H. granular pepsin per mg. protein

	Horse M					Horse C								Horse K						
Serum sample ...	II	III	IV	V	VI	II	III	IV	V	VI	VII	VIII	IX	II	III	IV	V	VI	VII	VIII
Suspension of *B. typhosus* used for immunization	Heated 58° 1½ hours	Living smooth virulent	Formalized extract smooth virulent	No injections	Living smooth virulent	Heated 58° 1½ hours	Living smooth virulent	Formalized extract smooth virulent	No injections	Living smooth virulent	No injections	O from smooth "O 901" heated 58° for 1½ hours H from living rough "Ty 441"		Smooth "O 901" heated 58° 1½ hours*	As C VIII and C IX				Alcohol-treated smooth virulent "Ty 2"	
Date of bleeding	2. vii. 34	23. vii. 34	13. ix. 34	22. xi. 34	15. xii. 34	2. vii. 34	23. vii. 34	13. ix. 34	22. xi. 34	15. xii. 34	16. i. 35	4. ii. 35	4. iv. 35	13. iii. 35	20. v. 35	25. vi. 35	9. x. 35	30. xi. 35	5. i. 36	6. iii. 36
H titre before peptic action	51,200	102,400	102,400	102,400	204,800	51,200	51,200	102,400	25,600	102,400	102,400	102,400	102,400	—	6,400	12,800	25,600	51,200	51,200	25,600
H titre after peptic action	3,200	25,600	Not tested	102,400	204,800	1,600	6,400	102,400	25,600	102,400	102,400	102,400	102,400	—	800	3,200	20,000	51,200	51,200	25,600
Reduction in H agglutinin units	48,000	76,800	—	—	—	49,600	44,800	—	—	—	—	—	—	—	5,600	9,600	5,600	—	—	—
% reduction in H titre	**94**	**75**	—	**0**	**0**	**97**	**87·5**	**0**	**0**	**0**	**0**	**0**	**0**	—	**87·5**	**75**	**20**	**0**	**0**	**0**
O titre before peptic action	25,600	12,800	3,200	1,600	25,600	51,200	25,600	12,800	3,200	51,200	51,200	25,600	25,600	25,600	—	12,800	—	12,800	—	12,800
O titre after peptic action	200	200	Not tested	200	800	1,600	1,600	800	400	3,200	3,200	3,200	1,600	800	—	400	—	400	—	3,200
Reduction in O agglutinin units	25,400	12,600	—	1,400	24,800	49,600	24,000	12,000	2,800	48,000	48,000	22,400	24,000	24,800	—	12,400	—	12,400	—	9,600
% reduction in O titre	**99**	**98**	—	**87·5**	**97**	**97**	**94**	**94**	**87·5**	**94**	**94**	**87·5**	**94**	**97**	—	**97**	—	**97**	—	**75**

* Suspension devoid of flagellar antigen.

A closer examination of Table IV will show that in the case of horse K the initial H titre increased progressively in samples III–VI in which the percentage of H agglutinin destruction by pepsin decreased, and that in the case of horse M the initial H titre was lower in sample II than in samples III, IV, V and VI. It will also show that the O titre of any given sample was usually lower than the H titre.

On the other hand:

(1) A reduction of H titre in M V by the same amount as in M II (48,000 units), in M VI by the same amount as in M III (76,800 units) and in C IV–IX by approximately 50,000 units as in C II and C III could not fail to be reflected in the results of the agglutination test. Reductions of this order in the H titre of the later samples would have appeared as a 50 % decrease in titre. There was no evidence of such a decrease.

(2) The initial H titre of C V, apparently unaffected by peptic action, was actually lower than the initial H titre of C II which was reduced by 97 % under comparable conditions.

(3) An H titre of 102,400 in sample M V was unaffected, whereas the same titre in sample M III was 75 % reduced.

(4) In no sample of horse K serum did the initial H titre exceed that of samples M II, C II and C III, and yet in the later samples there was apparently marked resistance to peptic action.

Where the H and O titres in any one sample of serum were the same the difference in the effect of pepsin on the two agglutinins was most clearly revealed. In samples C XIV, C XV and C XVI (not shown in the table) the O titre rose to 102,400. This high titre was considerably reduced by peptic action. The H titre, also 102,400, was unaffected.

From a consideration of these points and the evidence to be presented it would appear that increase in titre is not alone responsible for the apparent increase in the resistance of the H agglutinin to peptic action. There was no evidence to indicate that the degree of resistance of the H agglutinin was influenced by the period of storage.

Results essentially similar to those obtained with whole serum were obtained with the globulin fractions precipitated by saturation with Na_2SO_4, the H agglutinin in the globulin fractions from the later serum samples being completely resistant to peptic action under the conditions studied.

Four experiments in which comparison was made of the action of pepsin on various preparations of globulin from serum C II and from the low-titre serum C V are summarized in Table V. The concentration of enzyme relative to globulin and the period of hydrolysis varied considerably in these experiments but in each case the H agglutinin in C II globulin was attacked and that in C V globulin was not attacked.

In one experiment, summarized in Table VI, evidence on the relation of titre to resistance was sought as follows. The H titres of samples II, III, V, VIII and X of horse C serum were estimated and the samples were diluted from 1·5 to 24 times to give solutions of approximately the same H titre. The same amounts of pepsin and citrate buffer at *p*H 4·7 were added to each. Samples for the agglutination tests were withdrawn and neutralized immediately, and again after incubation for 19 hours at 37°. It will be seen that while the H agglutinin in serum C II was almost completely destroyed there was a marked increase in resistance through the series C II–C X. The results gain in significance through the relatively far higher proportion of pepsin to protein in samples from the later bleedings.

Table V. *Action of pepsin on the H agglutinin in globulin from serum C II and C V*

			Serum ...	C II				C V				
			Globulin preparation	G_7	G_9	G_{11}	G_2	G_8	G_6	G_6	G_5	G_6
			Exp. ...	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*d*
			Initial H titre	51,200	9,600	6,400	51,200	25,600	12,800	6,400	12,800	34,133
Exp.	mg. enzyme per mg. globulin	Duration of hydrolysis hours										
a	4	5	H titre after	25,600	—	—	—	25,600	—	—	—	—
b	6	5	peptic	—	1,600	—	—	—	12,800	—	—	—
c	2	18	action	—	—	200	—	—	—	6,400	—	—
d	4	23		—	—	—	12,800	—	—	—	12,800	34,133
			% reduction in H titre	50	87	97	75	0	0	0	0	0

Table VI. *Action of pepsin on the H agglutinin in a "non-resistant" and in diluted "resistant" serum samples from horse C*

19 hours. 37°. *p*H 4·7

Serum sample ...	C II	C III	C V	C VIII	C X
Dilution factor ...	0	1·5	1·5	6	24
H titre before peptic action	6,400	6,400	6,400	6,400	4,266
H titre after peptic action	266	1,066	4,266	3,200	3,200
% destruction of agglutinin	96	83	33	50	25

The experiments summarized in Tables V and VI serve to emphasize the existence of some essential difference in the H agglutinin obtained from horses after one and after several immunizing courses, a difference evidently not attributable to differences in titre alone.

One of several experiments on the tryptic hydrolysis of serum samples obtained from the same horse at successive bleedings is set out in Table VII. It will be seen that the apparently increased resistance of the H agglutinin to tryptic digestion at *p*H 8·6 is nearly as marked as that to peptic digestion at an acid reaction. The resistance of the O agglutinin to tryptic digestion did not increase.

The significance of these results is emphasized by the finding that the resistance of the H agglutinin to destruction by activated papain did not increase in the same way as did its resistance to pepsin and trypsin. The mode of attack of activated papain on the globulin molecule, which is known to differ markedly from those of pepsin and trypsin, is presumably such as will inactivate the H agglutinin even in the "resistant" state. The four experiments summarized in Table VIII illustrate this point. The corresponding O titres, which were almost completely reduced in every case, are not included. It will be noted that the times of hydrolysis varied from 2 to 20 hours and that various activators were employed.

In Exps. *a* and *b* the hydrolysis mixtures and controls without papain were exposed to the same conditions of *p*H and temperature before comparison of the H titres. In Exps. *c* and *d* samples withdrawn from the hydrolysis mixtures at the start of the hydrolysis period and immediately treated with iodoacetate served as controls for samples withdrawn at the end of the period.

Table VII. *Action of trypsin on the H and O agglutinins in serum samples obtained from horse C at successive bleedings*

6 hours. 37°. *p*H 8·6. Approx. 2 mg. enzyme per mg. serum protein

Serum sample ...	II	III	IV	V	VI	VIII	XI
H titre of control without trypsin	51,200	51,200	102,400	25,600	102,400	102,400	204,800
H titre after tryptic action	800	4,800	51,200	12,800	102,400	102,400	204,800
Reduction in H agglutinin units	50,400	46,400	51,200	12,800	0	0	0
% reduction in H titre	98	90	50	50	0	0	0
O titre of control without trypsin	51,200	25,600	12,800	3,200	102,400	51,200	51,200
O titre after tryptic action	<400	400	400	<400	800	3,200	800
Reduction in O agglutinin units	>50,800	25,200	12,400	>2,800	101,600	48,000	50,400
% reduction in O titre	100	98	97	94	99	94	98

Table VIII. *Action of activated papain on the H agglutinin in serum samples obtained from horses M and C at successive bleedings*

*p*H 6·8–7·0. 37°

Exp.	Activator	Duration of hydrolysis hours	Source of agglutinin		Serial number of bleeding II	III	IV	V	VI	VII	VIII	XI
a	KCN	2	Serum from horse M	H titre of control	6,400	25,600	—	51,200	—	409,600	409,600	—
				H titre after papain action	600	6,400	—	25,600	—	204,800	204,800	—
				% reduction	90	75	—	50	—	50	50	—
b	H_2S	5½	Serum from horse C	H titre of control	25,600	25,600	102,400	25,600	102,400	—	—	204,800
				H titre after papain action	—	800	25,600	6,400	25,600	—	—	25,600
				% reduction	100	97	75	75	75	—	—	87·5
c	H_2S	6	Globulin fraction of serum from horse M	H titre 0 hours	12,800	12,800	102,400	—	51,200	—	204,800	—
				H titre after papain action	400	1,600	25,600	—	3,200	—	12,800	—
				% reduction	97	87·5	75	—	94	—	94	—
d	Cysteine	20	Serum from horse C	H titre 0 hours	51,200	51,200	102,400	34,000	—	—	—	—
				H titre after papain action	100	400	25,600	6,400	—	—	—	—
				% reduction	100	99	75	80	—	—	—	—

Although the percentage reduction in H titre was highest in serum drawn after the first immunizing course (II) it was considerable in all samples. Even with high-titre serum such as M VIII, Exp. *a*, there was 50 % reduction in titre in as short a period as 2 hours. Results obtained with globulin fractions prepared from horse M serum (Exp. *c*) were similar in every way to those obtained with whole serum.[1]

Experiments were undertaken to determine whether the globulin fractions of serum containing the so-called "resistant" H agglutinin were themselves more resistant to peptic and tryptic hydrolysis than the globulin fractions in which the agglutinin was rapidly destroyed. The results of these experiments, summarized in Table IX, show conclusively that this is not the case. They do not, however, prove that the globulin molecules carrying resistant H agglutinin groups are not

[1] A precipitate tended to form in activated papain solutions during the period of incubation at 37°. Tests were made to determine whether the observed reduction in titre were due to adsorption of antibody on this precipitate. After the hydrolysis period the titre of samples containing the precipitate in suspension, the filtered samples and the supernatants after centrifuging were compared. The H titre at *p*H 7·0 was not affected by the presence of the precipitate. The O agglutinin titre was slightly lower in the supernatant than in the suspension, but the loss of agglutinin by adsorption on the precipitated papain was not sufficient materially to influence the results. At *p*H 5·0 adsorption was more marked.

themselves resistant to enzymic attack, since the number of immunologically active globulin molecules may constitute such a small proportion of the total serum globulin that chemical changes in this fraction of the whole would be undetectable by the methods used, being masked by hydrolysis of the normal globulin molecules.

Table IX. *Action of pepsin and trypsin on the globulin fraction of various serum samples obtained from horse C*

	Pepsin						Trypsin					
Exp. ...	*a*						*b*			*c*		
Conditions of hydrolysis ...	*p*H 4·6, 1·2 mg. purified pepsin per mg. protein						*p*H 8·6 (*p*H 7·4 after 3 days) 2 mg. trypsin per mg. protein			*p*H 8·4 (*p*H 7·4 after 3 days) 2 mg. trypsin per mg. protein		
Serum ...	C II			C XII			C II			C XV		
Globulin preparation	G_{11}			G_{13}			G_{11}			G_{12}		
mg. N per ml. of globulin solution	6·7			5·4			6·7			6·3		
Initial H titre	12,800			51,200			12,800			102,400		
Initial O titre	51,200			6,400			51,200			25,600		
Duration of hydrolysis hours	% decrease in titre H	% decrease in titre O	Amino-N increase as % total N	% decrease in titre H	% decrease in titre O	Amino-N increase as % total N	% decrease in titre H	% decrease in titre O	Amino-N increase as % total N	% decrease in titre H	% decrease in titre O	Amino-N increase as % total N
2	—	—	—	—	—	—	87·5	87·5	7	—	—	—
4	—	—	—	—	—	—	94	96	11·6	0	75	11·1
24	97	97	14	0	>94	15	100	99	26	Trace	87·5	24·5
48	—	—	15·5	0	—	16·7	—	—	33	<50	94	36
72	—	—	—	—	—	—	—	—	36	—	—	—

The figures in Table IX, which show amino-N increase as percentage of the total N, give an indication of the extent of protein hydrolysis. It will be seen that in Exp. *a* this was approximately the same for samples of globulin from C II and C XII serum acted on by pepsin, and in Exps. *b* and *c* for samples of globulin from C II and C XV serum acted on by trypsin. The globulin was more completely broken down by trypsin than by pepsin under the conditions investigated. It appears that an increase of amino-N equivalent to 36% of the total globulin nitrogen represents the full extent of trypsin action under these conditions. This increase was attained in approximately 48 hours and was accompanied by a slight destruction of the H agglutinin in the resistant globulin. In no case has a comparable degree of protein breakdown been achieved with pepsin. In two experiments with pepsin, lasting 4 days, the H titre of a resistant globulin sample remained unchanged; in a third, lasting 5 days, there was 25% destruction of the H agglutinin accompanied by an increase in amino-N equivalent to 12% of the total N. Addition of extra pepsin at intervals did not lead to increased proteolysis or destruction of agglutinin, a result to be expected in view of the high proportion of pepsin to globulin originally present. In one of these experiments the H agglutinin in the control non-resistant globulin from C II serum was 97% destroyed in 19 hours, with an amino-N increase of only 1·7% of the total N.

It was thought that the altered resistance of the H agglutinin to peptic and tryptic digestion might be accounted for by a shift of agglutinin from the euglobulin to the pseudoglobulin fraction, or vice versa. No definite evidence of such a shift was obtained by estimating the percentage of the total H agglutinin present in the euglobulin fractions of serum from early and later bleedings. Moreover the H agglutinin in both euglobulin and pseudoglobulin fractions of C II serum was rapidly destroyed by pepsin, that in both fractions of C VIII, C IX, M V and M VII serum being completely resistant under the same conditions.

III. *Possible effect of the source and method of preparation of the antigen on the resistance of the typhoid H agglutinin to proteolytic enzymes*

In this paper emphasis has been laid on the difference in the resistance to proteolytic enzymes of the H agglutinin produced by the first of a series of immunizations and that produced in response to later courses. This difference in resistance was not observed in the case of the O agglutinin formed simultaneously. Reference to Table IV will show that stages of intermediate resistance appear to exist between the so-called "non-resistant" and the fully "resistant" H agglutinin.

The question then arises whether the resistance of the antibody to proteolysis is related to the strain and method of preparation of the immunizing suspension of *B. typhosus*, or to the length of the immunization period.

A consideration of the following points (see Table IV) suggests that the latter explanation is the more plausible:

(1) The least resistant H antibody in horse K serum (sample III) was produced in response to immunization with a living, rough strain of *B. typhosus*, which is devoid of O antigen.[1] The same strain was used for the three subsequent courses of immunization and the resistance of the H antibody increased progressively over a period of 3 months.

(2) The same strain was used in horse C for the first time 7 months after the first immunizing course, and immediately prior to bleeding VIII, and all the H antibody then present was resistant.

(3) A living, smooth, virulent strain was used for the immunization of horses M and C prior to bleedings III and VI, at intervals of 5 months, and the H agglutinin in samples III and VI of the serum responded very differently to peptic and tryptic actions, the former being comparatively non-resistant and the latter resistant.

It is, of course, not possible to decide from the available data what percentage of the total antibody in serum obtained at any given bleeding is formed in direct response to the antigen used immediately prior to that bleeding. Absorption tests with the appropriate antigens might have provided information on this point, but interruption of the present work has unfortunately precluded their performance.

The results of the experiments with pepsin and trypsin here described suggest a more rapid and direct means of determining whether the strain and method of preparation of the antigen can affect the chemical properties of the antibody, namely the immunization of rabbits with antigens prepared in various ways and an investigation of the action of proteolytic enzymes on the H antibody formed in response to each. To obtain reliable data it would be necessary to use

[1] The suspension used for the first course of immunization in this horse (sample II) was devoid of H antigen.

Table X. *Preliminary experiments on the action of pepsin and trypsin on the H agglutinin in the serum of rabbits immunized with* B. typhosus

Rabbit			27			75	84			85			100			
Suspension of *B. typhosus* used for immunization			Heated 60°, 1 hour			Living smooth virulent	Formalized extract smooth virulent			Formalized extract smooth virulent			Rough living			
Cf. samples of horse serum obtained after immunization with a similar suspension			C II M II			C III, VI M III, VI	C IV M IV			C IV M IV			C VIII–XIII K III–VI			
Rabbit serum sample			II	III	IV	II	II	III	V	II	III	V	II	III	IV	V
Date of bleeding			18. xii. 33	20. xii. 33	2. i. 33	—	19. vi. 34	22. vi. 34	3. vii. 34	19. vi. 34	22. vi. 34	3. vii. 34	17. i. 35	21. i. 35	26. i. 35	28. i. 35
Exp.	Details of experiment	H titre														
a	Pepsin	Before peptic action	25,600	—	—	51,200	—	—	51,200	—	—	102,400	—	—	—	—
	10 mg. per mg. protein	After peptic action	6,400	—	—	12,800	—	—	51,200	—	—	102,400	—	—	—	—
	23 hours	% reduction	**75**	—	—	**75**	—	—	**0**	—	—	**0**	—	—	—	—
b	Pepsin	Before peptic action	12,800	—	—	51,200	—	—	51,200	—	—	51,200	6,400	—	—	—
	9 mg. per mg. protein	After peptic action	6,400	—	—	12,800	—	—	51,200	—	—	51,200	800	—	—	—
	23 hours	% reduction	**50**	—	—	**75**	—	—	**0**	—	—	**0**	**0**	—	—	—
c	Pepsin	Before peptic action	25,600	25,600	12,800	—	100	1,600	51,200	400	1,600	51,200	—	—	—	—
	9 mg. per mg. protein	After peptic action	6,400	6,400	6,400	—	50	200	51,200	50	400	51,200	—	—	—	—
	20 hours	% reduction	**75**	**75**	**50**	—	**50**	**87·5**	**0**	**87·5**	**75**	**0**	—	—	—	—
d	Pepsin	Before peptic action	—	—	—	—	—	—	—	—	—	—	9,600	4,266	25,600	34,128
	14 mg. per mg. protein	After peptic action	—	—	—	—	—	—	—	—	—	—	800	>3,200	25,600	34,128
	22 hours	% reduction	—	—	—	—	—	—	—	—	—	—	**92**	**<25**	**0**	**0**
e	Trypsin	Before tryptic action	—	—	—	—	200	1,600	25,600	—	—	—	—	—	—	—
	2 mg. per mg. protein	After tryptic action	—	—	—	—	25	400	25,600	—	—	—	—	—	—	—
	4 hours	% reduction	—	—	—	—	**87·5**	**75**	**0**	—	—	—	—	—	—	—

several rabbits to test each preparation of antigen, to use one antigen only for each rabbit and to standardize the size and spacing of the immunizing doses and the intervals between successive bleedings.

The results of a few preliminary experiments with samples of rabbit serum, prepared at various times for other purposes and made available through the kindness of Dr Felix, indicate that this investigation would provide information of value. They bear out the impression received from experiments with horse serum that the nature of the H antibody apparently changes progressively in the direction of increased resistance to proteolytic enzymes during prolonged immunization, and that time, rather than the source and method of preparation of the H antigen, is the operative factor. It would seem from these results, which are summarized in Table X, that the only completely resistant H agglutinin was that formed in serum obtained at the IVth and Vth bleedings of rabbit 100 and the Vth bleeding of rabbits 84 and 85.[1] The results are, however, in no way conclusive and must be accepted with reservation, particularly in view of the low initial H titre of certain of the samples. They are reproduced here only because interruption of the present work has prevented the performance of further and more comparable tests.

Discussion

The conflicting reports in the literature on the effect of proteolytic enzymes on antibodies [Marrack, 1934] may be reconciled to some extent by the results of the experiments here described, since it has been shown that in the globulin from certain samples of antityphoid serum slight proteolysis is accompanied by complete destruction of the typhoid H and O agglutinins, while in that from others considerable proteolysis may occur without appreciable change in H titre. The antisera used in earlier investigations on this subject may well have contained H agglutinins of varying resistance.

Among the authors who found that the typhoid H agglutinin was not destroyed by trypsin was Frankel [1932]. He claimed to have obtained this agglutinin free from protein by adsorption on kaolin followed by specific elution, but in experiments with various samples of rabbit serum his claim could not be confirmed [Rosenheim, 1935]. The H agglutinin in these serum samples was rapidly destroyed by trypsin. In order to determine whether Frankel's work could be repeated with H agglutinin resistant to tryptic digestion, an experiment was undertaken with serum M V and one with the globulin fraction of serum C V, using the technique of adsorption on $Al(OH)_3$ and elution with alkaline glycine buffer previously employed. No concentration of resistant H agglutinin relative to protein was obtained, and there is, therefore, no indication that the resistant, any more than the non-resistant, H agglutinin can be separated from serum globulin by this method.

Although the exact interpretation of the results presented in this paper must await extension of the work and a fuller knowledge of enzyme and protein chemistry, various possible explanations suggest themselves. These are discussed below.

It is clear that if an increase in the number of immune globulin molecules containing an H antibody group similar to that in samples C II, M II and K III were the only effect of prolonged or repeated immunization with H antigen, samples from later bleedings would have a higher H titre, but, with proteolytic

[1] Each rabbit received one course of intravenous injections about a week before bleeding II; a further injection of the same antigen was given on the day following bleeding III.

enzymes present in excess, as in these experiments, the same percentage reduction in titre would be obtained in the first and later samples under the same conditions. This was not obtained. Moreover increase in resistance was not invariably accompanied by increase in titre.

After the action of pepsin and trypsin on serum from later bleedings each molecule of immune H globulin was still able to exert its agglutinating effect. It would therefore seem that there is a progressive modification in the structure of these immune globulin molecules during prolonged immunization. This is the main conclusion to be drawn from the experiments described.

In the course of this modification the whole globulin molecule may be rendered resistant to attack by certain enzymes, or the antibody groups may themselves become more resistant through an alteration in chemical structure or spatial arrangement, or the number of antibody groups in each molecule may be so increased that after enzymic destruction of one or more there would still be a receptor group present in each molecule to unite with antigen and produce the same amount of agglutination as before.

In accordance with the last suggestion the formation of antibody groups might be conceived as occurring in the terminal or exposed amino-acids in the normal molecule during the early stages of immunization, and in the more central parts during the later stages. As pepsin and trypsin are known to attack long-chain protein molecules at the terminal amino-acids of the chain, the surface antibody groups would then be among the first attacked in all serum samples, and in those samples obtained after a first immunizing course complete destruction of antibody activity would result. In samples obtained after subsequent courses the initial stages of globulin hydrolysis and H agglutinin destruction would expose other and more deep-seated antibody groups, so that extensive proteolysis might be required to reduce the H titre significantly.[1]

The observation that even after prolonged immunization the resistance of the H antibody to papain did not increase as did its resistance to pepsin and trypsin provides some indirect evidence in support of this conception, since papain attacks the globulin molecule not at the terminal amino-acids only, but at any peptide linkage.

As increased resistance of the O agglutinin to proteolytic enzymes was never encountered, a corollary would be that all the O antibody groups are accessible to these enzymes both in the early and later stages of immunization.

In this connexion reference may be made to the work of Joffe [1935] who found variations in the physico-chemical properties of the typhoid O agglutinin which were not correlated with changes in titre. Bacteria were maximally sensitized with homologous immune serum from rabbits immunized with the rough and smooth variants of *B. typhosus* "O 901". Not only did the sensitizing antibodies to the two variants differ in isoelectric point and electrokinetic potential difference, but with increasing time of immunization the isoelectric point of the antibody shifted progressively to the alkaline side. Although the same properties of the H agglutinin were not investigated a possible analogy suggests itself between changes in the immune globulin molecule leading to increased resistance of the antibody to enzyme action, and changes in the electrical properties of the antibody groups on the surface. A further analogy may exist between the type of variation here discussed and the few cases of variation in antiserum at different stages of immunization for which there is immunological evidence in the literature. Browning & Wilson [1911] noted that

[1] If this conception is correct the application of the terms "non-resistant" and "resistant" to the antibody groups themselves might not be entirely justifiable.

the immune body present in a haemolytic antiserum at different stages of immunization varied in its capacity for leading to complement fixation when red cells were sensitized with multiple doses. Thiele & Embleton [1913–14] describe various qualitative changes in the development of immune body, and Fildes [1920] found differences in antimeningococcal serum in rabbits at different stages of immunization. It would be of considerable interest to determine if the resistance to proteolytic enzymes of other antibodies, particularly antitoxins and antibacterial substances in therapeutic sera, varied in the course of immunization as did that of the typhoid H agglutinin.

Summary

1. The O agglutinins in all serum samples from three horses immunized with *B. typhosus* were rapidly destroyed by pepsin, trypsin and activated papain.

2. The H agglutinins in serum samples obtained from each of three horses after a first immunizing course were rapidly destroyed by proteolytic enzymes. Those in samples obtained after several immunizing courses were not appreciably destroyed under identical conditions.

3. The H agglutinins which were apparently resistant to pepsin and trypsin were not resistant to activated papain.

4. The globulin fractions of serum obtained after the first and subsequent immunizing courses were hydrolysed to approximately the same extent by proteolytic enzymes.

5. Peptic digestion giving an increase in amino-N of less than 5% of the total N was accompanied by complete destruction of the H and O agglutinins in the globulin fraction of serum obtained after a first immunizing course.

6. Peptic digestion giving an increase in amino-N of 17% of the total N was not accompanied by an appreciable reduction of H agglutinin titre in the globulin fraction of serum obtained after a later immunizing course. Tryptic digestion giving an increase in amino-N of 36% of the total N was accompanied by less than 50% reduction of H agglutinin titre.

7. The H and O agglutinins in the globulin fraction of serum obtained after a first immunizing course were not destroyed by yeast dipeptidase at *p*H 7·8 or by yeast aminopolypeptidase at *p*H 7·0.

8. The action of proteolytic enzymes on the H agglutinin in serum from rabbits immunized with suspensions of different strains of *B. typhosus* prepared in various ways, was investigated in a few preliminary experiments.

9. The significance of the results obtained and their bearing on the chemical nature of antibodies is discussed.

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