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The Effect of Digestion with Papain and Pepsin upon the Antitoxic Activity of Rabbit Antibody

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Summary. Rabbit antibodies to the α and ε toxins of *Cl. welchii* have been hydrolysed with papain. It has been found that in some sera there is a loss of molar antitoxic activity which may be as high as 50 per cent. Partial digestion with pepsin does not affect the molar antitoxic activity. Reaction with anti-globulin sera increases the antitoxic activity of the digested globulins. It is suggested that the changes in activity are due to changes in the effective size of the antitoxic molecules and a consequent variation in the extent of steric inhibition of the toxins.

INTRODUCTION

Porter (1959) showed that rabbit γ-globulin could be digested with papain to give three fractions with very little loss of protein nitrogen. Fractions I and II were shown to carry the original antibody activity while the dominant antigenic groups of the original globulin were located on fraction III.* The capacity of fractions I and II to combine with the hapten from the homologous antigen is the same as that of the intact globulin (Karush, 1959; Nisonoff, Wissler and Woernley, 1960c; Velick, Parker and Eisen, 1960). Rabbit γ-globulin may also be split by digestion with pepsin to give a divalent antibody fraction which, on reduction with cysteine, yields two monovalent fractions with properties very similar to those obtained by papain digestion (Nisonoff, Wissler, Lipman and Woernley, 1960b). As far as we know, however, no study has been made of the effect of papain or pepsin digestion upon the reaction of antibody with a naturally occurring toxin. It was therefore of interest to repeat Porter's work using antibody against such a biologically active protein whose activity could be accurately determined. It was found that up to 50 per cent of the antitoxic activity† of the original globulin may be destroyed by splitting the molecule into univalent fragments. The origin of this loss has been investigated and it has been attributed to the loss of a steric component from the activity of the intact globulin.

* While the work described in this paper was in progress, it was reported that fractions I and II originated in different molecules of globulin. The structure of rabbit antibody is therefore I–I–III or II–II–III depending on its mobility (Palmer, Mandy and Nisonoff, 1962).

† Throughout this paper the activity of the rabbit antibody and the fractions I and II as measured in neutralization tests will be referred to as 'antitoxic activity'. This is not necessarily the same as, or even proportional to, the total capacity to react with the toxin molecule which will be referred to as 'combining power'. Antitoxic activity will be expressed as specific antitoxic activity, i.e. units per mg. protein.

MATERIALS AND METHODS

Preparation of γ-Globulin

Rabbits were immunized with partially purified preparations of either the α toxin (lecithinase) from *Clostridium welchii* type A or the ε toxin from *Cl. welchii* type D. The first two injections were given intramuscularly in Freund's complete adjuvant (Difco) a month apart and the rabbits were bled 10 days after the second injection. Further bleedings were taken at intervals of 6–8 weeks, each being preceded by a short course of intramuscular injections of alum-precipitated toxin.

The γ-globulins were isolated by chromatography on DEAE cellulose (Sober, Gutter, Wyckoff and Peterson, 1956), by the rivanol method (Horejsi and Smetana, 1956) or by fractional precipitation with alcohol (Deutsch, 1952). The purity of the isolated globulins was checked electrophoretically on cellulose acetate membranes (Kohn, 1958). The yield of antitoxic activity varied between 40 and 60 per cent in the chromatographic and rivanol methods and between 60 and 85 per cent in the alcohol method.

Determination of Antitoxic Activity

α Antitoxic activity was determined *in vitro* against a routine preparation of *Cl. welchii* toxin in blocking tests in which either sheep erythrocytes (Glenny, Barr, Llewellyn-Jones, Dalling and Ross, 1933) or egg lecithin (Macfarlane, Oakley and Anderson, 1941) was used to detect excess toxin. The test was carried out at the 0·1 unit level and had a coefficient of variation of ±6 per cent. ε Antitoxic activity was determined by the intravenous test in mice (Batty and Glenny, 1947) at the one unit level. The coefficient of variation of this assay was ±5 per cent.

Determination of Protein

Concentrations of intact globulin and the digest fractions were determined from the optical absorption at 280 mμ using a Unicam S.P. 500 spectrophotometer. The $E^{1\%}_{1cm}$ employed was 13·0 for intact globulin and 14·0 for fractions I and II or the divalent fraction produced by peptic digestion (Porter, 1959). As a check on contamination by non-proteinaceous material the absorption at 260 mμ was compared with that at 280 mμ. If the ratio OD_{260} : OD_{280} was greater than 0·6 : 1·0 the assay was not made.

Digestion of Antitoxin

(a) *Papain.* The papain used routinely was prepared from Zimmerman papain (Hopkins and Williams) by the method of Kimmel and Smith (1957) and recrystallized as the mercuri-derivative. The final product had an activity of 0·0375 Anson (haemoglobin) units/mg. Mercuri-papain was also obtained from Mr. C. Gardner of the Wellcome Research Laboratories Biochemical Department, and a third sample, which was purchased from the Worthington Biochemical Corporation, was crystallized as the mercuri-derivative before use. These preparations were compared with a sample of crystalline mercuri-papain given to us by Professor R. R. Porter.

(b) *Pepsin.* Pepsin was purchased from the Worthington Biochemical Corporation and used without further purification.

(c) *Conditions of digestion.* Porter's conditions (Porter, 1959) for digestion with papain were used routinely, that is to say the ratio of the weights of globulin to papain was 100 : 1 and the digestion mixture contained 0·1 M phosphate buffer, pH 7, 0·01 M cysteine and 0·002 M EDTA (diaminoethane tetra-acetic acid). The digestion was at first carried out

for 16 hours at room temperature but for most of the work 2 hours at 37° was found to be adequate. Digestion was stopped by dialysis at 2° against a large volume of 0·01 M sodium acetate/hydrochloric acid buffer, pH 5·6.

Pepsin digestion was carried out as described by Nisonoff *et al.* (1960b). The ratio of the weights of globulin to pepsin was 100 : 3, the mixture was buffered at pH 4·5 with 0·01 M phosphate and the reaction allowed to proceed for 6 hours at 37°. The digestion was stopped by raising the pH to 8. A portion of the mixture was then reduced by adding solid cysteine to a concentration of 0·01 M and incubating for a further 16 hours at 37°. The divalent antibody fraction was isolated by differential precipitation with sodium sulphate (Nisonoff, Wissler and Lipman, 1960a), the material soluble in 12·5 per cent sodium sulphate and precipitated when the concentration was raised to 19 per cent being taken. All samples were dialysed exhaustively against 0·1 M phosphate buffer, pH 7, before determination of specific activity.

Chromatography

The digestion mixture was dialysed against sodium acetate/hydrochloric acid buffer, 0·1 M, pH 5·6, for 24–48 hours at 2° and chromatographed as described by Porter (1959). Fraction I was eluted by running a volume of 0·01 M buffer through the column equal to about twenty times the volume of the fractions collected from the column. Fractions II and III were eluted by a gradient from 0·01 M sodium acetate, pH 5·6, to 0·9 M sodium acetate, pH 5·6. The gradient was produced by running the stronger buffer from a conical flask into a spherical mixing chamber containing the weaker buffer, both vessels emptying simultaneously. The ratio of the volumes of cone to sphere was 1 : 2. The appropriate tubes were pooled, dialysed against 0·01 M phosphate buffer, pH 7 (fractions I and II), or 0·04 M sodium acetate/hydrochloric acid buffer, pH 5 (fraction III), and freeze dried. The losses during dialysis, freeze drying and reconstitution were up to 5 per cent for fraction I and up to 10 per cent for fraction II.

In many experiments a fourth fraction was obtained from the chromatogram emerging from the column between fractions II and III. This only reached significant proportions when the globulin to papain ratio was increased to 1000 : 1 and it was presumed to be undigested globulin.

Anti-Rabbit Sera

(a) *Sheep anti-rabbit.* The serum (Wellcome Brand) was obtained from a sheep which had received five injections of alum precipitated rabbit serum evenly spaced throughout the previous 18 months.

(b) *Guinea-pig anti-rabbit.* Guinea-pigs were injected intramuscularly with 0·2 ml. of a 1 per cent solution of rabbit γ-globulin or of purified digest fraction in Freund's complete adjuvant; a month later they received a second injection and after 10 days were bled from the heart. A further three bleedings were taken at monthly intervals and the animals were then bled out.

RESULTS

LOSS OF ANTITOXIC ACTIVITY DURING PAPAIN DIGESTION

When antitoxic γ-globulin was digested with papain 20–60 per cent of the activity was lost. There was also a loss of non-dialysable protein which in most experiments was about

20 per cent. There was very little correlation between the two and with the exception of experiment 6 the loss of activity was two to eight times that of the loss of protein. This discrepancy is reflected in a reduction of the specific activity* of the dialysed material (Table 1).

TABLE 1

THE EFFECT OF PAPAIN DIGESTION ON ANTITOXIC ACTIVITY

Expt.	*Antitoxin*	*Conditions of digestion*				*Before digestion*	*After digestion and dialysis*	*% loss*
		Ratio globulin:papain	*Time (hr.)*	*Temp.*				
1	ε	100 : 1	16	Room	Weight (mg.) Sp. activity†	123 1·95	95 1·0	23 49
2	α	100 : 1	16	Room	Weight (mg.) Sp. activity	122 1·1	104 0·39	15 64
3	α	100 : 1	16	Room	Weight (mg.) Sp. activity	238 1·58	193 0·95	19 41
4	α	1000 : 1	2	37°	Weight (mg.) Sp. activity	256 1·65	240 0·85	6·3 48
5	α	100 : 1	2	37°	Weight (mg.) Sp. activity	406 1·68	344 1·27	15 24
6	ε	100 : 1	2	37°	Weight (mg.) Sp. activity	350 5·7	292 5·2	17 9

† Specific antitoxic activity in units/mg.

Since the loss of antitoxic activity was higher than had been expected (Karush, 1959; Nisonoff *et al.*, 1960b; Velick *et al.*, 1960) the conditions of digestion were re-examined. It was thought that the active fragments were being further digested by other proteolytic enzymes present as contaminants in the papain (Jansen and Balls, 1941). Samples of papain from four sources were therefore compared. All four samples gave similar results and so the mercuri-papain prepared in this laboratory was used for all the subsequent experiments.

TABLE 2

THE EFFECT OF REDUCING THE RELATIVE CONCENTRATION OF PAPAIN ON THE LOSS OF ANTITOXIC ACTIVITY DURING DIGESTION

Ratio of globulin to papain	*% loss of activity*
100 : 1	46
1000 : 1	40
10,000 : 1	33

As a further check on the possibility of over-digestion the ratio of globulin to papain was increased to 10,000 : 1 using a 2 hour digestion at 37°. There was a progressive reduction in the loss of antitoxic activity as the relative concentration of the enzyme was reduced but the reduction was small (Table 2). It was later found that the specific activity

* The term 'specific activity' refers to units of antitoxic activity per mg. protein, no change in immunological specificity is implied. 'Molar activity' means the activity per gram equivalent taking the valency of the intact globulin and the pepsin digest as 2 and the papain digest fragments as univalent.

of the antibody fractions produced with a globulin : papain ratio of 1000 : 1 was no higher than that of those produced by digestion under the usual conditions; as judged from the chromatogram moreover, the digestion was incomplete so that the yields of fractions I and II were reduced (expt. 3, Table 3). It was therefore concluded that the conditions of digestion were not responsible for the excessive loss of the antitoxic activity.

AVIDITY

The belief that papain digestion does not reduce the combining power of rabbit antibody is based upon determination of the dissociation of complexes of antibody or digest fractions with hapten or with substituted protein (Nisonoff *et al.*, 1960c; Karush, 1959; Velick *et al.*, 1960). The techniques used could not be applied to the toxin–antitoxin system but an approach could be made by determining the specific activity of the antitoxin before and after digestion at widely different concentrations (Glenny and Barr, 1932). Any dissociation of the toxin–antitoxin complex would reduce the activity of the antitoxin at high dilutions.

Antitoxins were selected which had lost a large proportion of their activity on digestion. The ε antitoxin (expt. 1, Table 1) was determined at 50 unit and 0·5 unit levels and the α antitoxin (expt. 3, Table 1) at 1 unit and 0·01 unit levels. In neither case was there any apparent loss of activity at the lower concentration.

ISOLATION OF ANTIBODY FRACTIONS

Since the loss of antitoxic activity from the digest could not be accounted for by the loss of non-dialysable protein, the fractions of digested globulin were isolated by chromatography on CM cellulose (Porter, 1959) and their antitoxic activity was estimated. Two experiments were carried out with ε antitoxin and two with α antitoxin, in one of which the globulin : papain ratio was increased to 1000 : 1. The results are summarized in Table 3.

The manipulative loss of the combined antibody fractions in experiments 1, 2 and 4 was 5–20 per cent which, considering the number of operations involved, was regarded as satisfactory. The elution diagram obtained in experiment 3 showed a considerable residue of undigested globulin, the low yields of fractions I and II in this experiment were therefore due to the low concentration of papain used and the consequentially incomplete digestion. If the antitoxic activity of the undigested globulin is added to the activities of fractions I and II and if, after making the appropriate correction for fraction III, the weight is also added, then the loss of activity on chromatography is 13 per cent and of weight 10 per cent.

It will be seen that in addition to the loss of material during chromatography and concentration there was also a further loss of activity presumably due to disruption of secondary structure which was not reflected in the physicochemical parameters which govern chromatographic properties. This loss was variable and was superimposed upon the loss of activity during digestion.

The theoretical specific antitoxic activity of fractions I and II may be calculated from that of the original globulin by multiplying it by the ratio of the molecular weights divided by the valency of the globulin. Taking the molecular weight of the digest fractions I and II as 52,000, that of γ-globulin as 187,000 (Charlwood, 1959) and the valencies of the fractions and the original globulin as 1 and 2 respectively, we have a factor of 3·6/2.

TABLE 3

RECOVERY OF ANTITOXIC PROTEIN AND OF ANTITOXIC ACTIVITY AFTER CHROMATOGRAPHY OF PAPAIN DIGESTS

Expt.	*Toxin*	*Digestion and analysis*			*Chromatography*					*Losses due to digestion and dialysis* (%)
			Before	*After*	*Applied to column*		*Recovered*		*Calculated values*	
1	ε	Wt. (mg.)	123	95	Wt. (mg.)	83·5	Fr. I Wt. (mg.)	17·6		
		Sp. activity	1·95	1	Sp. activity	1	Sp. activity	1·3		
							Fr. II Wt. (mg.)	22		
							Sp. activity	1·6		
							Fr. I and II Wt. (mg.)	39·6	46·5*	20
							Av. sp. activity	1·45	3·5†	39
2	α	Wt. (mg.)	406	344	Wt. (mg.)	328	Fr. I Wt. (mg.)	86		
		Sp. activity	1·68	1·27	Sp. activity	1·27	Sp. activity	2·1		
							Fr. II Wt. (mg.)	59		
							Sp. activity	1·6		
							Fr. I and II Wt. (mg.)	145	182	13
							Av. sp. activity	1·85	3·0	26
3	α	Wt. (mg.)	256	240	Wt. (mg.)	221	Fr. I Wt. (mg.)	29·4		
		Sp. activity	1·65	0·85	Sp. activity	0·85	Sp. activity	1·2		
							Fr. II Wt. (mg.)	48		
							Sp. activity	1·45		
							Fr. I and II Wt. (mg.)	77·4		
							Sp. activity	1·33	3	39
4	ε	Wt. (mg.)	350	292	Wt. (mg.)	272	Fr. I Wt. (mg.)	77		
		Sp. activity	5·7	5·2	Sp. activity	5·2	Sp. activity	10·5		
							Fr. II Wt. (mg.)	63·4		
							Sp. activity	9·4		
							Fr. I and II Wt. (mg.)	140·4	150	10
							Sp. activity	10	10·3	0

* Weight calculated from intact globulin, corrected for the weight of material applied to column.
† Specific activity calculated by multiplying the value for intact globulin by 1·8 (see text).

If this calculation is applied to the fractions isolated in experiments 1, 2 and 3 (Table 3) it will be seen that the activities observed were roughly half the theoretical value. While it is possible that denaturation also occurred during the digestion which was not reflected in the chromatographic properties of the molecule the results of experiments on digestion conditions make this appear unlikely. It was thought that the loss of specific activity during digestion which occurred in three experiments out of four was due to the reduction in size and valency, both of which might reduce the effectiveness of the antibody.

THE EFFECT OF ANTI-RABBIT SERUM ON THE ANTITOXIC ACTIVTIY OF FRACTIONS I AND II

In order to increase the effective size of fractions I and II and so obtain an indication of the influence of molecular size on antitoxic activity, the isolated fractions were allowed to react with a sheep anti-rabbit serum or a guinea-pig serum against purified rabbit γ-globulin. The activity of the product was then estimated in the usual way. The experiments were carried out with α antitoxin since it was thought that an *in vitro* test was less likely to be affected by dissociation of the antibody than one carried out *in vivo*. The quantity of antitoxin in each tube varied inversely with its activity between 50 and 150 μg. The amount of anti-rabbit serum added was calculated from preliminary experiments to produce the maximum change in activity. The two were allowed to react for 30 minutes at 37° before the toxin was added; there was no visible precipitate under these conditions. The determination of the α antitoxin was then carried out as usual using sheep erythrocytes as an indicator. The results (Table 4) have been expressed as an apparent change in the specific activity of the rabbit antitoxin.

TABLE 4

THE EFFECT OF ANTI-RABBIT SERA ON THE ANTITOXIC ACTIVITY OF INTACT γ-GLOBULIN AND PAPAIN DIGEST FRAGMENTS

Antitoxin	*Percentage change in specific antitoxic activity*			
	Anti-rabbit serum		*Normal sera*	
	Sheep	*Guinea-pig*	*Sheep*	*Guinea-pig*
γ	−38 ± 4*	−38 ± 6	0	0
Fr. I	0	+12 ± 4	0	0
Fr. II	+28 ± 3	+30 ± 4	0	0

* Coefficient of variation.

It will be seen that the changes in activity are well outside the experimental error. Both anti-rabbit sera depress the activity of the whole globulin by about 40 per cent and both increase the activity of fraction II by about 30 per cent. The sheep anti-rabbit serum appeared not to react with fraction I while the guinea-pig anti-rabbit serum, which was produced with the aid of Freund's adjuvant, reacted to produce a 12 per cent increase in activity. Normal sheep or guinea-pig sera were without effect and neither the normal nor the anti-rabbit sera had any α antitoxic activity. Similar results were obtained when lecithin was used as an indicator. The reduction and the enhancement of antitoxic activity were observed with four samples of γ-globulin and three pairs of digest fractions respectively, although the magnitude of the change varied somewhat with different samples of anti-globulin serum.

Since anti-globulin sera were found to precipitate with all three fractions from the papain digest, though the reactions with fractions I and II were weak, the inhibition of γ-globulin activity could have been due either to the strong reaction with the inactive part of the molecule or to the weak reaction directly with the antibody sections. Guinea-pig antisera were therefore prepared against fractions I, II and III and their effects on the activity of intact globulin were determined and compared with their effects on fractions I and II. The results are shown in Table 5.

TABLE 5

THE EFFECT OF GUINEA-PIG ANTI-GLOBULIN AND ANTI-FRACTION SERA ON THE PERCENTAGE CHANGE IN SPECIFIC ANTITOXIC ACTIVITY OF INTACT ANTITOXIN AND UNIVALENT FRACTIONS

Antitoxin	*Antiserum against*			
	γ-Globulin	*Fraction I*	*Fraction II*	*Fraction III*
γ-Globulin	−63	−58	−70	−30
Fraction I	+15	−25	−25	+15
Fraction II	+30	−15	−30	+10

It will be seen that, in its effect on the activity of intact globulin, anti-globulin is quantitatively more like anti-I and anti-II than anti-III but while the activities of the antitoxic fractions are depressed by anti-I and anti-II they are enhanced by anti-globulin and anti-III. There does not seem to be a complete explanation for this, but from the reactions of I and II with the four antisera, it appears that the homologous or closely associated antisera inhibit, while the more weakly cross-reacting ones enhance activity.

THE EFFECT OF PEPTIC DIGESTION ON ANTITOXIC ACTIVITY

It had been established that reduction of the size and the valency of rabbit antitoxin resulted in a considerable reduction in molar antitoxic activity and that the activity could be partially restored by an increase in the effective size of the antitoxic molecule. It was interesting therefore to determine the effect of a reduction in molecular size without a reduction of valency. Nisonoff *et al.* (1960a) have reported that the product of peptic digestion of rabbit γ-globulin is a divalent fraction with a molecular weight of about 106,000, the capacity to combine with homologous antigen is retained and, since the fraction is divalent, precipitates are still formed.

Peptic digests were made of two samples of rabbit α antitoxin and one sample of ε antitoxin. The digests were either fractionated with sodium sulphate and then reduced, the fraction precipitating between 12·5 and 19 per cent (w/v) sodium sulphate being taken as the divalent material, or were run on a CM cellulose column under the same conditions as the papain digests. Nisonoff *et al.* (1960b) have reported that chromatography on CM cellulose reduces the divalent molecule and two univalent fractions are obtained. Our results are in general agreement with this but the chromatographic analysis of the pepsin digest suggests that the products are more complex than has been reported. The results of these experiments are summarized in Table 6. In the first experiment the peptic digest was fractionated once and the specific activity was found to be 30 per cent below the theoretical level; in the second experiment after a repeated fractionation, the discrepancy had been reduced to 15 per cent. It seems likely that the divalent fraction retains the molar

activity of the intact globulin and that its specific activity is close to the theoretical. In both experiments it was found that when the purified material was reduced by treatment overnight with 0·01 M cysteine the specific activity fell significantly, to 40 per cent and to 37 per cent below the theoretical level. These values are what would be expected from papain digest fractions of a γ-globulin with a specific activity of 1·4. The fact that material with this specific activity was obtained by simple reductive splitting of the divalent fraction in the absence of proteolytic enzymes substantiates the evidence already given that the reduced activity of the papain digest fractions is not due to denaturation.

TABLE 6

THE EFFECT OF PEPTIC DIGESTION AND OF SUBSEQUENT REDUCTION ON ANTITOXIC ACTIVITY

Expt.	*Antitoxin*	*Digestion*			*Reduction and dialysis*		*Chromatography*	
		Before	*After Na_2SO_4 fractionation*				*Specific activity*	
		Specific activity	*Specific activity*	*% loss**	*Specific activity*	*% loss*	Fr. I	Fr. II
1	α	1·4	1·77 (×1)	30	1·5	40		
2	α	1·4	1·8 (×1)	30				
			2·1 (×2)	15	1·57	37		
3	α	1·33			1·35	42	1·4	IIa 1·45
								IIb 1·55
4	α	1·57					1·3	1·4
5	ε	17					15	10

* Based on the theoretical value = specific activity of the intact globulin ×1·8.

In experiment 3 the peptic digest was reduced, exhaustively dialysed and then chromatographed on CM cellulose without preliminary purification of the divalent fraction. The original globulin was a sample from the same batch as had been used for experiments 1 and 2, the difference in specific activity (5 per cent) is within the limits of experimental error. The lower specific activity of the reduced digest (1·35 u./mg.) as compared with the isolated fractions indicates the presence of inactive non-dialysable protein which was removed by chromatography.

Chromatography of the reduced digest showed a second smaller peak emerging close behind fraction II. Since this material was non-precipitating antitoxin with a specific activity roughly equal to that of the two main fractions it was assumed to be a monovalent unit and was not investigated further. A similar subdivision of fraction II was also observed in experiment 5. Since the globulin used in experiment 4 had previously been digested with papain and fractionated on CM cellulose (expt. 3, Table 3), it was gratifying to find that the specific activity of the univalent fractions obtained by the two methods was the same. The specific activity of the two univalent fractions of ε antitoxin obtained in experiment 5 were 50 and 66 per cent below the theoretical level confirming again that the phenomenon was not confined to the α antitoxin.

It was concluded that, notwithstanding the effect of anti-globulin serum on the antitoxic activity of the univalent fractions, the activity of the intact globulin was more closely dependent upon divalency than upon the size of the molecule. In order to complete the analogy however the effect of anti-globulin upon the activity of the divalent fractions was determined.

THE EFFECT OF SHEEP ANTI-RABBIT SERA ON THE ANTITOXIC ACTIVITY OF THE DIVALENT FRAGMENT FROM PEPTIC DIGESTION

The antitoxic activities were determined as before using the haemolytic test with and without the addition of sheep anti-rabbit serum. The results are shown in Table 7. The

TABLE 7

THE EFFECT OF SHEEP ANTI-RABBIT SERA ON THE ANTITOXIC ACTIVITY OF THE DIVALENT FRAGMENT FROM PEPTIC DIGESTION

Expt.	*Antibody*	*Sheep anti-rabbit serum*	*Percentage change in specific activity (u./mg.)*
1	Intact globulin 1	A	−52
	Divalent fraction		+30
2	Intact globulin 2	A	−52
	Divalent fraction		+30
3	Intact globulin 1	B	−27
	Divalent fraction		0
	Fr. II		+25

antitoxic activity of the intact globulin was reduced in each experiment but the activity of the divalent fraction was increased by only one of the two anti-rabbit sera used (serum A). Serum B which was without effect on the divalent fraction in experiment 3 still enhanced the activity of monovalent fraction II prepared from it. Anti-rabbit serum does therefore enhance the activity of the divalent fraction, the molar activity of the complex being greater than that of the intact globulin. Not all anti-rabbit sera react however; it is perhaps significant that the anti-rabbit serum B also had the lesser effect on the intact globulin.

DISCUSSION

Previous studies of the products of papain digestion of rabbit γ-globulin have been concerned with antibodies raised against synthetic haptens (Nisonoff *et al.*, 1960c; Velick *et al.*, 1960). These studies showed that the power to combine with free hapten is unchanged by papain digestion. Apart however from the observations that the univalent fractions prevent precipitation of macromolecular antigens by the intact globulin, no studies have been reported of the effect of papain digestion upon the reaction with native protein antigens.

The experiments described here have shown that up to 50 per cent of the antitoxic activity of rabbit γ-globulin may be lost after digestion with papain. Since the losses were unaffected by variation of the digestion conditions or by the use of papain from different sources it was thought that they were unlikely to be artifacts. When the digests were chromatographed on carboxymethyl–cellulose (Porter, 1959) the recoveries of the digest fractions I and II were good but the specific antitoxic activity was reduced in proportion to that of the unfractionated digest. In only one experiment, in which the loss of activity upon digestion was small (9 per cent), did the specific activity of the isolated fractions agree with that calculated from the original globulin. Since it is difficult to conceive of an increase in

activity under these conditions, this agreement has been taken as verification of the assumption that the activity of the fractions could be calculated by multiplying the activity of the globulin by the ratio of the change in molecular weight to the change in valency (3·6/2).

The reports of previous workers that the combining power of rabbit antibody was unchanged by papain digestion were based on estimates of combination with free hapten and of the degree of dissociation before and after digestion. Determination of the 'avidity' of an antitoxin serves the same purpose, though it may not be so accurate, and the finding that though the activity was reduced the avidity was unchanged is therefore in agreement with these results. It is also further evidence against the possibility of extensive denaturation.

Peptic digestion under controlled conditions has been shown not to affect the molar activity of rabbit antitoxin (Table 6). Reduction of the digested antibody however, either with cysteine or by chromatography on CM cellulose, yields two monovalent fractions very similar to those produced by papain digestion (Nisonoff *et al.*, 1960b) and having similar antitoxic activities. Thus the loss of antitoxic activity observed during digestion with papain can be reproduced by non-proteolytic cleavage of the molecule (Table 6). Moreover when two samples of the same antitoxic globulin were digested, one with papain and the other with pepsin, the specific antitoxic activity of the fractions isolated by chromatography was the same (expt. 3, Table 3; expt. 4, Table 6). It seems therefore very unlikely that the reduced activity of the monovalent fractions is due to denaturation or to excessive proteolysis. It must be concluded that it is a consequence of the reduced size and valency of the molecule and that the divergence from the results obtained with free hapten reflects the influence of these parameters on the inactivation of lecithinase and of ε toxin.

The experiments with anti-rabbit serum were intended to determine the effects of changes in size without a simultaneous change in valency but the results are conflicting. On the one hand any effect on either monovalent or divalent fractions is to increase the antitoxic activity, and on the other hand under the same conditions, the activity of the intact globulin is always reduced. The reduction in size from a molecular weight of 180,000 to 106,000 during peptic digestion did not affect antitoxic activity, but if the size of the digest fragment was increased by reaction with anti-rabbit serum its activity was increased by about 30 per cent. Combination with anti-globulin antibodies would of course result in a very much larger change in size than occurred during digestion and evidently the difference is significant.

Since it was found that the sheep anti-rabbit did not react with fraction I, an attempt was made to couple this to fraction III and to determine the effect of anti-rabbit serum on the activity of the coupled material. The results of the coupling were equivocal but in two out of five experiments antitoxic material was obtained, whose activity was increased in the presence of sheep anti-rabbit serum. The increases were 200 and 2000 per cent respectively. It seems that the phenomenon of enhanced activity in the presence of anti-globulin requires that the anti-globulin should react at some distance from the active centre of the anti-toxin. The size of the enhancement indicates that the results of direct reaction with the antitoxic fragments may be the net products of simultaneous inhibition and enhancement.

From a consideration of the results three facts emerge:

(1) Reduction of the molecular weight of antitoxin from 180,000 to 106,000 makes little difference to its molar activity, provided that the molecule is still divalent.

(2) Reduction of the molecular weight to 50,000 with a concomitant reduction of the valency from 2 to 1 may reduce the molar antitoxic activity by as much as 40 per cent, but there is considerable variation from one anti-serum to another.

(3) Increase in the effective size of either mono- or divalent fractions of antitoxin increases the activity by about 30 per cent although under the same conditions the activity of the intact globulin is reduced.

A mechanism may be suggested for both the changes in activity on digestion and for the effect of anti-globulin serum. Antibodies formed in response to toxin may be broadly classified into two types (Fig. 1), those which react with the active centre of the toxin

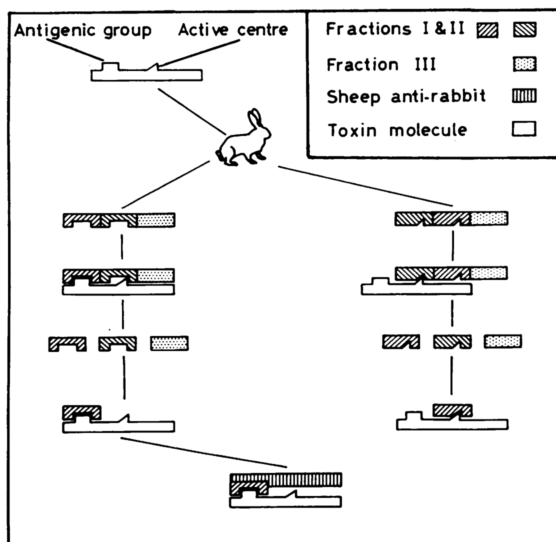

Fig. 1. Schematic representation of the effect of antibody size on antitoxic activity.

or perhaps at a point very close to it and those which react with other antigenic groups on the toxin molecule at some distance from the active centre. The former would inhibit by direct combination with the active centre, the latter inhibit, if they do so, by virtue of the steric effects of the large antibody molecule. No doubt the steric effects would be partly dependent on valency also. Digestion to univalent fractions would leave activity of the first type unchanged but the second type would be largely inactivated. The net result would depend upon the composition of the original antitoxic globulin, that is it would depend upon the animal from which it was obtained. The variation in the results obtained with different batches of globulin which appears in Tables 1 and 3 may be attributed to this. Reaction with anti-globulin by increasing the size of the antitoxic fraction would tend to restore the activity of the second type of globulin.

The same argument can be applied to the divalent fractions produced by peptic digestion except that one would have to suppose that in this case the indirect antibodies were unaffected by digestion and that the addition of anti-globulin either made them more effective, or possibly enabled antibodies on more remote sites of the toxin molecule, which had been ineffective, to exert a steric effect.

Branston and Cinader (1961) have shown that the inhibitory effect of anti-ribonuclease

on the reaction of ribonuclease with poly-ribonucleotides is due to a steric effect, the degree of inhibition varying inversely with the size of the substrate molecule. The hypothesis proposed here to explain the effects of antibody size may be regarded as a natural corollary of this where the size of the substrate is kept constant and the size of the antibody is varied.

Other factors may well be involved such as the degree of aggregation of the toxin–antitoxin complex and the effect of anti-globulin on it. Normally, visible precipitates are not formed when anti-globulin is added to the digest fractions in the assay systems that were used but of course it is quite possible that soluble aggregates are formed. It seems more likely however that the inhibiting effect of anti-globulin on intact anti-toxin is due to the formation of aggregates, indeed the reaction of anti-globulin with the larger and less soluble intact globulin would lead more readily to the formation of complexes whose bulk would interfere with the subsequent reaction with the toxin.

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