molysis, the greater the number of such complexes/cell the greater the opportunity will be for C′ to combine with such an antibody complex. Consequently, if many complexes are present/cell, less C′ will be required to produce hemolysis. Therefore, it follows that concentration of C′ required for lysis in a particular hemolytic system is a measure of efficiency of sensitization of the cell.

Each of the hemolytic systems in which we have compared C′ activity to amounts of serum required for optimal and maximal sensitization were subject to accumulative effect of various factors which influence sensitization of the cell. The fact that a correlation exists between amount of C′ required for lysis and relative amounts of antibody required for both optimal and maximal sensitization, when expressed in hemolytic activity, indicates that certain general basic factors are involved in sensitization of the cell.

In considering possible factors which influence sensitization of the cell, those which determine the number of antigen-antibody reactions/cell would be of basic consideration. The number of antigenic sites on the cell would be a fundamental limiting factor, which determines the opportunity of antibody to combine with the cell. Specificity of the antibody would effect the opportunity for antigen-antibody reactions to occur, by involving antigenic sites of different specificities. In addition, antibodies which differ in their hemolytic efficiency, but with the same specificity(7), would effect sensitization due to their competition for the same antigenic sites.

Summary. Methods are given for determination of amount of serum required for optimal and maximal sensitization of a hemolytic system. These values were determined for each of 4 hemolytic systems which differ in amounts of C′ required for lysis. When amounts of serum required for optimal and maximal sensitization in each system were expressed in hemolytic units, a correlation was found between these values and C′ activity. This correlation indicated that certain general basic factors are involved in sensitization of the cell by antibody. Possible factors and their role in cell sensitization are discussed.

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Enzymatic Digestion of Rabbit Globulin.* (24780)

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Antibodies can be enzymatically digested under favorable conditions so that antibody activity is maintained even though molecular weight is greatly reduced. Much work has been done with digestion of horse globulins but relatively little with rabbit globulins. An abbreviated summary of enzymatic digestion of rabbit antibodies is as follows. Pepsin or papain destroyed antiurease activity while trypsin did not(1). All typhoid "O" and most "H" agglutinins were destroyed by trypsin(2), although it was later found that "H" agglutinins became increasingly resistant to digestion as immunization progressed(3). Trypsin digestion of agglutinins, heated either alone or in presence of albumin, resulted in release of agglutinating antibodies postulated to be reduced in size(4). Following digestion

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with various enzymes and 12 N HCl, antibody against ovalbumin was still capable of flocculation; following digestion with papain, antibody no longer flocculated with antigen but was capable of blocking its combination with undigested antibody and was estimated to be about one-quarter original size(5). Three fractions of another papain digest contained antibodies of about one-third original molecular size, based on ultracentrifugal data. Two of these fractions showed blocking activity but would not combine with goat antibodies to rabbit gamma globulin. The third fraction showed no blocking activity but could be crystallized and would combine with goat antibody(6). Preliminary results of digestion experiments with anti-tumor globulin fractions have been presented(7). Rabbit antibody globulin is commonly used experimentally, and occasionally therapeutically. Therefore many properties of digested rabbit antibodies would be of interest including *in vivo* distribution and ability to cross capillary, blood-brain, or placental barriers in normal, pregnant, diseased or tumor-bearing animals. Smaller digested antibody fragments might cross such barriers more readily than larger undigested antibodies. (However, digested horse diphtheria antitoxin apparently did not cross the human placental barrier as readily as the undigested(8)). Rabbit typhoid "H" agglutinins were used in the initial experimental digestion system. The following experiments suggest that it was possible to digest partially rabbit antibody globulin and to recover digest fractions with no loss of agglutinating activity.

Materials and methods. Antisera were obtained from New Zealand albino rabbits immunized with *Salmonella typhosa* (Rawlings). Antigen was prepared by adding formalin saline to a 24 hour growth on agar, scraping off organisms, washing with saline in the centrifuge, and adjusting concentration with saline to give 70% transmittance at 560 mμ on Klett colorimeter. This suspension was also used for agglutinin titer determinations. Animals were injected on day 1 and 7 with 1 ml antigen and .5 ml Freund's adjuvant subcutaneously in the back; on day 4 and 11 with .25 ml antigen I.V.; and on day 14 with 1 ml antigen only subcutaneously in the back. Seven days after last injection, all rabbits were bled by cardiac puncture several times in 4 days, and sera obtained were pooled. After 7 more days rabbits were injected I.V. and beginning 7 days later were again bled. This schedule was followed for 5 bleedings; a sixth injection and bleeding were carried out after delay of 2 months. All pooled sera had "H" agglutinin titers of 25,600. Each pooled sera was fractionated separately by adding saturated ammonium sulfate solution. Successive fractions were precipitated and removed at 30, 33, 50, and 75% saturation. The first 3 fractions were reprecipitated at their respective saturations 5 times. Fractions were dialyzed and lyophilized. The 33% fractions were used for digestion experiments and were largely gamma globulin (Fig. 1). The digestion method was somewhat modified from that used for digestion of human gamma globulin(9). Fifty mg of the 33% fractions were dissolved in 10 ml of saline and pH adjusted to 3.6 with 0.0005 N HCl. Twice crystallized pepsin (Nutritional Biochemicals Corp.), in solution in con-

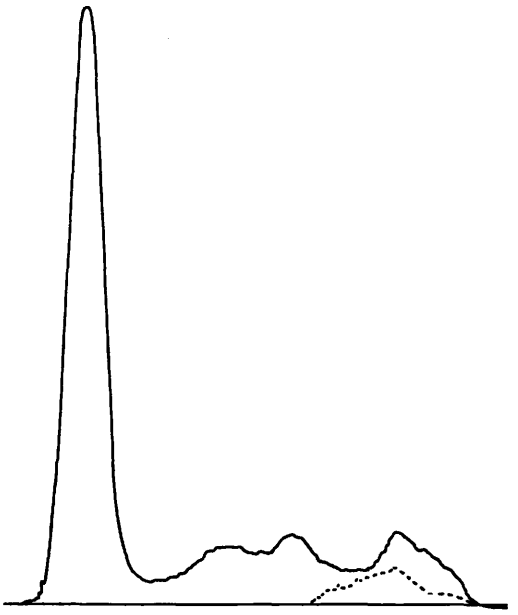


FIG. 1. Electrophoretogram—paper electrophoresis: pooled sera from bleeding 4 and the 33% fraction (dotted line) prepared from it. 0.075 Γ/2, pH 8.6 veronal buffer, room temperature, 16 hr, 6 ma. Staining with bromphenol blue.

centration of 1 mg/ml, was added to give final pepsin:protein ratios of 1:20, 1:100, or 1:200; no pepsin was used in controls. Several digests with different pepsin:protein ratios were usually processed in one experiment. pH was then adjusted to 3.4 by adding more acid, and volume was brought up to total of 30 ml with saline. Digestion was carried out at 0°-5°C for 72 hours. 0.0005 N NaOH was added to bring pH to 7.0. Super-Cel was added to the digest, final concentration 0.5%, to remove pepsin. Following settling and centrifugation the precipitate was discarded. The digest was then fractionated by adding saturated ammonium sulfate solution and removing precipitates at 40% and 60% saturation; digest in the 60% supernate was the third fraction. The 3 fractions were dialyzed and lyophilized. To obtain some estimate of extent of digestion, 1 ml aliquots of the digest were removed at 0, 4, 24, 48, and 72 hours. Each aliquot was added to trichloracetic acid, final concentration 10%, the mixture centrifuged, and the supernate examined for tyrosine-containing digest fragments by use of Folin-Ciocalteau Phenol Reagent. (Molecules soluble in 10% trichloracetic acid are dialyzable (10)). Optical density was compared with that of standard curve prepared previously from known amounts of tyrosine. Results were expressed as 10^{-5} milliequivalents of tyrosine liberated from each mg of protein present at beginning of digestion. "H" agglutinin titers were determined by dissolving a lyophilized globulin and its digest fractions and diluting to equal concentration based on Kjeldahl values. Protein concentration was obtained by multiplying by 6.25. Following doubling dilution in veronal buffer pH 7.3 a constant amount of antigen was added and the mixture incubated 18 hours at 48-55°C. Duplicate samples were run. Titer was determined by last dilution tube in which agglutination occurred. Several of the 33% globulin and digest fractions were analyzed in the ultracentrifuge.†

Results. Table I shows results of representative digestion experiments. Primary interest centered upon digest fractions precipitated at 60% saturation. It was hoped that precipitation of digest at 40% saturation would remove large molecules, and that smaller molecules with antibody activity would then be precipitated at 60%. The titer of the 60% digest fraction was equal to, or perhaps in some cases greater than, the titer of the undigested 33% globulin. This might be expected if antibody activity of the globulin were maintained while part was removed by digestion. Similar results were obtained with all 3 pepsin:protein ratios used. Double-dilution agglutinin titers have a possible inherent error of 100% which may partially explain the 4 fold titer increase of the 60% digest fraction in bleeding 2. This should also be considered in comparing change of titer in different bleedings. Also, as sera from each bleeding were pooled, 33% fractions might vary in composition and ease of digestion.

In Table I only bleeding 2 and bleeding 6 digests were from one experiment. In any one experiment, in which several pepsin:protein ratios were used, digestion was roughly proportional to amount of pepsin. There was markedly less correlation between the same pepsin:protein ratios and amount of digestion in different experiments. In all digests the relatively rapid initial rate of digestion tapered off around 48 hours. No tyrosine was liberated in control digests where pepsin was absent. The minimum amount of globulin digestion can be roughly estimated. Assuming that rabbit globulin contains about 6.8% tyrosine somewhat evenly distributed throughout the molecule; then for every 10^{-5} meq of tyrosine in dialyzable digest products/mg protein produced during digestion (Table I), about 2.7% of the globulin was digested. Thus in bleeding 3, with 7.3×10^{-5} meq tyrosine at 72 hours, there was at least 20% globulin digestion. (One milliequivalent of tyrosine is 181.2 mg, therefore 1×10^{-5} meq is 0.001812 mg. If 1 mg globulin contains 6.8% tyrosine, or 0.068 mg, then 1 meq of tyrosine is $0.001812 \div 0.068 = 0.027$, or 2.7% of tyrosine present. $2.7 \times 7.3 = 20\%$ globulin digestion). The true amount of digestion

† Ultracentrifugation was carried out under supervision of Walter D. Block, Dept. of Biol. Chem. and Dermatol., Univ. of Michigan Med. School.

TABLE I. Typhoid "H" Agglutinin Titers of Rabbit Serum Globulins and Three Fractions of Pepsin Digests Prepared from Them.

Bleeding	Pepsin:Protein ratio	Hr digestion	Extent of digestion (a)	Fraction (b)	Titer (c)
2				33% serum glob.	320
2	1:20	0	6.9		
		4	8.4	Digest 40%	1280
		24	11.7	60	1280
		48	11.7	" sup.	40
		72			
3				33% serum glob.	1280
3	"	0	2.9		
		4	5.5	Digest 40%	2560
		24	5.9	60	2560
		48	7.0	" sup.	
		72	7.3		
4				33% serum glob.	2560
4	"	0	9.6		
		4	14.8	Digest 40%	2560
		24	16.6	60	2560
		48	18.1	" sup.	20
		72	19.5		
6				33% serum glob.	640
6	1:100	0	2.0		
		4	4.6	Digest 40%	640
		24	7.9	60	640
		48	9.4	" sup.	80
		72	9.6		
4				33% serum glob.	2560
4	0 pepsin (control)	0	.0		
		4	.0	Digest 40%	2560
		24	.0	60	
		48	.0	" sup.	
		72	.0		

(a) Digestion products soluble in 10% trichloracetic acid—meq tyrosine $\times 10^{-5}$/mg protein originally in digest. (b) Precipitated at % ammonium sulfate saturation shown. (c) Initial antibody protein concentration in first tube: Bleeding—2, .550 mg/ml; 3, .825 mg/ml; 4, .750 mg/ml; 6, .350 mg/ml.

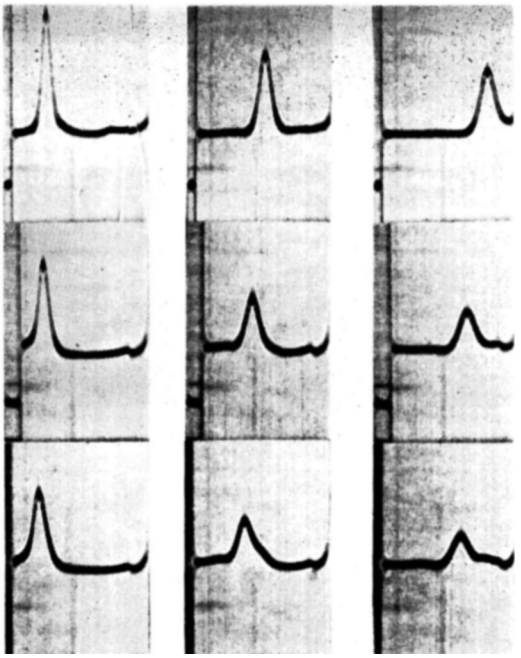
* Trace only recovered after lyophilization.

was probably greater and not uniform for all molecules.

In the ultracentrifuge a 33% globulin fraction of bleeding 2 migrated essentially under a single peak. Following digestion with 1:100 or 1:200 pepsin:protein ratios (minimal digestion 15% and 11% respectively), the major component of the 60% digest fractions moved more slowly (Fig. 2). This is suggestive evidence that these digest fractions were made up largely of smaller sized molecules, although in absence of diffusion data molecular weights cannot be determined. The 60% digest fractions were not entirely homogeneous, similar difficulties being encountered by previous investigators in attempting to obtain homogeneous digest fractions. It is very unlikely that undigested antibody in digest fractions was responsible for agglutinin activity. The electrophoretogram obtained with the 60% digest fraction was similar to that of the 33% globulin fraction, but the pattern was somewhat broader and extended more into the slow gamma globulin area.

During immunization, typhoid "O" agglutinins were also produced. "O" agglutinin activity could be recovered in some of the 60% digest fractions, however this was often less than the activity of the 33% serum globulin. Further characterization and investigation of digested antibodies is continuing in our laboratory.

Summary. Rabbit gamma globulin antibody fractions, prepared by ammonium sul-



30 min. 60 min. 90 min.

FIG. 2. Ultracentrifugation patterns of 33% globulin fraction, bleeding 2 (top), and digests prepared from it: 60% fraction of 1:100 pepsin:protein (middle), and 60% fraction of 1:200 pepsin:protein (bottom). 59,780 rpm, 26°C, 1% protein in 0.9% NaCl.

fate fractionation of pooled sera of rabbits immunized with formalin-killed *Salmonella typhosa*, were digested with pepsin. Extent of digestion was estimated by tyrosine present in dialyzable digest fragments. Digest fractions, which precipitated between 40%-60% ammonium sulfate saturation, had agglutinin titers equal to, or perhaps greater than, titers of undigested globulin. The major component of the 40%-60% fraction also migrated more slowly in the ultracentrifuge. This suggests that it has been possible to digest rabbit antibody globulin so that smaller molecules are obtained in a digest fraction which still maintains its agglutinin antibody activity.

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Effect of Zymosan on Endotoxin Toxicity in Mice.* (24781)

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Animals made tolerant to the lethal effects of endotoxin by pretreatment with these bacterial extracts were found to show an increased phagocytic capacity of the reticulo endothelial system (R.E.S.)(1,2). These findings, together with reports that endotoxin tolerance to fever(3) and lethal effects(4) were abolished in rabbits by reticulo endothelial blockade, suggested(1,2) that the reticulo endothelial system, which phagocytizes these macromolecular toxins(5), plays an important role in protecting against their toxicity. However, Suter *et al.*(6) and Halpern *et al.*(7) reported recently that mice infected with B.C.G. become 50 to 100 times more susceptible to the lethal effects of endotoxin, despite the fact that R.E.S. responds to the infection with increased phagocytic activity(8). These observations emphasize that factors other than phagocytic activity of the R.E.S. are concerned with the resistance to endotoxin. The increase in susceptibility to toxic effects of endotoxin in tuberculous animals, when first observed, was believed by Bordet(9) to be related to presence of inflam-

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