increased three- to fourfold between pH 7.0 and 8.4–9.2. This discrepancy suggests that the assumption of a simple reversible equilibrium between ferrous HRP and CO may be over simplified.

The dissociation of carbon monoxide from hemoglobins and myoglobins is relatively rapid (half-time less than 1 min) (Gibson, 1959; Antonini, 1964, 1965; Wittenberg *et al.*, 1965) and in fact is rapid even in the case of the *Ascaris* hemoglobins which dissociate oxygen very slowly (Gibson and Smith, 1965). The extremely slow dissociation of carbon monoxide from horseradish peroxidase reported here stands in marked contrast to the behavior of the other protoheme proteins so far studied.

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Extensive Degradation of Antibody by Pepsin*

Edgar Haber and Marcia Stone

ABSTRACT: Digestion of antibody by pepsin at pH 2.5 and 37° results eventually in degradation to small peptides and loss of antibody activity. When digestion is interrupted intermediate products of mol wt 97,000 and 47,000 can be isolated which retain antigen binding activity. The molecular weights of these products in 6 M guanidine and in the same solvent following reduction or oxidation of disulfide bonds indicate that peptide cleavages within the constituent chains have not occurred. Pepsin apparently can cleave antibody on both sides of its inter-heavy-chain disulfide bond, releasing both a dimer and monomer of the Fab region of the molecule. Further peptic digestion results in rapid degradation of these fragments to small peptides. Lower molecular weight intermediates with antibody activity could not be isolated.

γG-Globulin (γG)[1] can be cleaved by several proteolytic enzymes as well as by cyanogen bromide (Porter, 1959; Nisonoff *et al.*, 1960; Cahnmann *et al.*, 1965) in such a manner as to separate the part of the molecule associated with antigen binding activity from that related to biologic function and major antigenic identity. It appears that these enzymatic cleavages all occur at approximately the same place, about midway in the heavy polypeptide chain. Papain produces three fragments of approximately equal molecular weight. Two of these are identical, comprising the N-terminal half of the heavy chain and an intact light chain (Fab). Each carries an antibody combining site. The third fragment (Fc) is a dimer of the carboxy-terminal half of the heavy chain. The action of pepsin at pH 4.5–5 (Nisonoff *et al.*, 1960) produces a dimer of the Fab portion $(Fab')_2$ held together by a single disulfide bond and cleaves Fc in several places, producing peptides of lower molecular weight. $(Fab')_2$ has a molecular weight of approximately 100,000, contains the two antibody combining sites of the original molecule, and is capable of precipitating with polyvalent antigens. The purpose of the present investigation is to explore the further action of pepsin on $(Fab')_2$ in order to determine whether or not subfragments of lower molecular weight which are still capable of binding antigen can be isolated, and then to examine these fragments for cleavages within their polypeptide chains.

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[1] The abbreviations used are in accordance with the recommendations of the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)).

Materials and Methods

γG was prepared either by sodium sulfate precipitation (Kekwick, 1940) from normal rabbit serum or purchased from Pentex. Dinitrophenyllysyl antibody was produced in rabbits and isolated according to the method of Cheng and Talmadge (1965). Antibody directed against the azoarsanilate determinant was raised in rabbits by the injection of azoarsanilic acid coupled with bovine γG, and purified from hyperimmune serum by antigen precipitation and hapten elution. The general procedure for the dinitrophenyllysyl antibody was followed except that the separation of antibody from soluble antigen was performed on a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate (pH 7.4) and 0.15 M sodium chloride followed by dialysis and finally separation of the remaining hapten (arsanilic acid) on a column of Dowex 1 equilibrated with 0.05 M sodium borate (pH 9.5). Pepsin was the twice-recrystallized product obtained from Worthington. Guanidine·HCl was prepared from guanidine carbonate according to the method of Anson (1941).

Antibody combining activities of antibodies directed against dinitrophenyllysyl and azoarsanilate determinants were measured by fluorescence quenching according to the method of Velick *et al.* (1960) with modifications described by Haber and Richards (1966). An Aminco-Bowman fluorimeter was employed with a thermospacer at 25°. Dinitrophenyllysine (Mann) and mono(*p*-azobenzenearsonic acid)chloroacetyl-L-tyrosine, synthesized according to the procedure of Tabachnick and Sobotka (1959), were the haptens employed. Enzymic digestions were monitored in a Radiometer pH-Stat equipped with jacketed reaction vessel. Sedimentation velocity coefficients and molecular weights were determined in Beckman-Spinco Model E analytic ultracentrifuge, equipped with schlieren, interference, and ultraviolet-scanning optics.

Results

Unlike digestion of γG or antibody by pepsin at pH 4.5–5 (Nisonoff *et al.*, 1960), which produces even at the end of 48 hr an intact $(Fab^1)_2$ fragment, without appreciable loss of antibody combining activity, hydrolysis at pH 2.5 proceeds until all parts of the molecule are reduced to small peptides and no activity remains. A typical relationship between peptide-bond cleavage and loss of combining activity is seen in Figure 1. At the end of 1 hr, when approximately 25 peptide bonds/mole have been cleaved, about one-half the antibody combining activity is lost. Peptic digestions were stopped by neutralization with NaOH after varying times and the components of the mixture were separated by filtration through Sephadex G-100. Typical separations following 4 and 18 hr of digestion are shown in Figure 2. Four major components can be resolved, but by the end of 18 hr most of the material is included in the last peak, which represents a mixture of small peptides, with an average sedimentation value

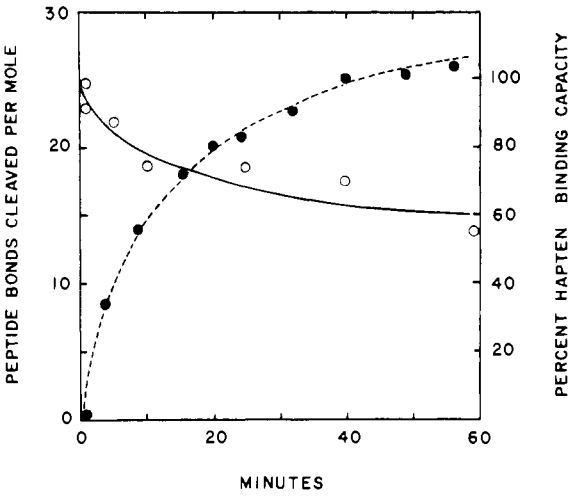

FIGURE 1: Pepsin hydrolysis of antidinitrophenyl antibody at pH 2.5 and 37°. Antibody concentration, 1%; pepsin, 0.05%. Peptide bonds cleaved per mole (●——●) computed from acid uptake, assuming a p*K* of 3.5 for the liberated carboxyl. Per cent hapten binding capacity (○——○) computed by fluorescene quenching.

of <0.5 S. Refiltration of the major high molecular weight components, an example of which is shown in Figure 8, effectively separates them so that detailed examination of each can be carried out. Table I indicates the distribution of the four peaks and their sedimentation velocities following varying times of digestion. It is apparent that early in the digestion, the major products are the 4.9S fragment and peptides, analogous to what has been reported after digestion at pH 4.5 or 5 (Nisonoff *et al.*, 1960). As digestion proceeds, the amount of 3.5S fragment increases until it becomes as prevalent as the 4.9S products. At the end of 8 hr, both 4.9S and 3.5S products have been degraded largely to peptides, though the amount of 3.5S product remaining now exceeds the amount of 4.9S material. Varying amounts of undegraded 6.5S

TABLE I: Distribution of Fractions from G-100 Column after Varying Times of Digestion (as determined by absorbance at 280 mμ).

Hr	I 6.3 S[a]	II 4.9 S	III 3.5 S	IV <0.5 S
0.5	2	28	10	60
3	3	20	14	63
4	4	17	19	60
8	4	2	6	88

[a] The roman numerals represent the fraction numbers; $s_{20,w}$ values given in Svedberg units.

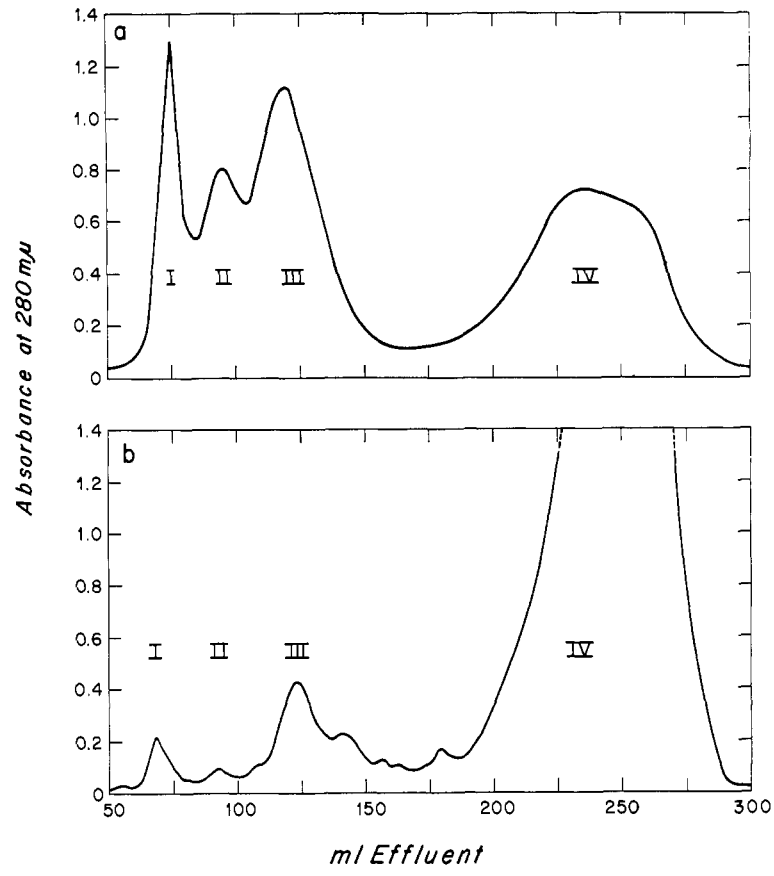

FIGURE 2: Separation of products of pepsin hydrolysis at pH 2.5 and 37° by gel filtration on a 2.5 × 45 cm column of Sephadex G-100, equilibrated with 0.1 M Tris·acetate (pH 7.5). (a) 4-hr digestion; (b) 18-hr digestion.

material remain throughout digestion, depending on the preparation used.

The molecular weights of the products were examined, employing the high-speed equilibrium method of Yphantis (1964) using interference optics. $\bar{v}$ for antibody fragments in dilute salt solutions was taken to be 0.738 (Marler *et al.*, 1964). Samples were taken from the peak tubes of a secondary gel filtration as in Figure 8, and the typical plots shown in Figure 3 indicate by their relative linearity the high degree of homogeneity of these materials. The molecular weight of the 4.9S product produced at pH 2.5 is very similar to that of the 4.9S product produced at pH 5, and corresponds to more recent values reported (Utsumi and Karush, 1965; Jaquet and Cebra, 1965). The 3.5S product is of somewhat greater molecular weight than that of Fab produced by papain digestion (Pain, 1963; Marler *et al.*, 1964).

In order to ascertain whether or not any peptide-bond cleavages had occurred within these fragments, molecular weight determinations were performed in 6 M guanidine·HCl and also in the same solvent following performic acid oxidation (Hirs, 1956). Some samples were run in a solvent consisting of 6 M guanidine·HCl, 0.1 M mercaptoethanol, and 0.1 M Tris·acetate (pH 7.5). The low-speed short-column equilibrium method (Yphantis, 1960) using interference optics was selected for these determinations in order to be certain that any peptides released by denaturation and disulfide cleavage be detected. Such peptides may prevent meniscus depletion in high-speed equilibrium runs. Columns of 1 mm or less were employed and a speed was chosen according to criteria described by Van Holde and Baldwin (1958) so that at equilibrium the concentration at the center of the column is equal to the initial concentration (C_0). The conventional means for obtaining C_0 by synthetic boundary determination could not be employed here because the possibility of dialyzable peptides made equilibration with solvent impossible. Consequently C_0 was determined spectrophotometrically, using $E_{280}^{1\%}$ of 15.0 for Fab, 11.8 for light chain, 11.05 for performic acid oxidation products, and 6.7 for ribonuclease. Absorbance at 280 mμ was related to fringe numbers by a series of synthetic boundary determinations at varying concentrations, employing the undegraded protein. Weight-average molecular weight (M_w) was computed according to the formula

$$M_w = \frac{2RT\dfrac{C_b - C_m}{C_0}}{(1 - \bar{v}\rho)\omega^2(r_b^2 - r_m^2)}$$

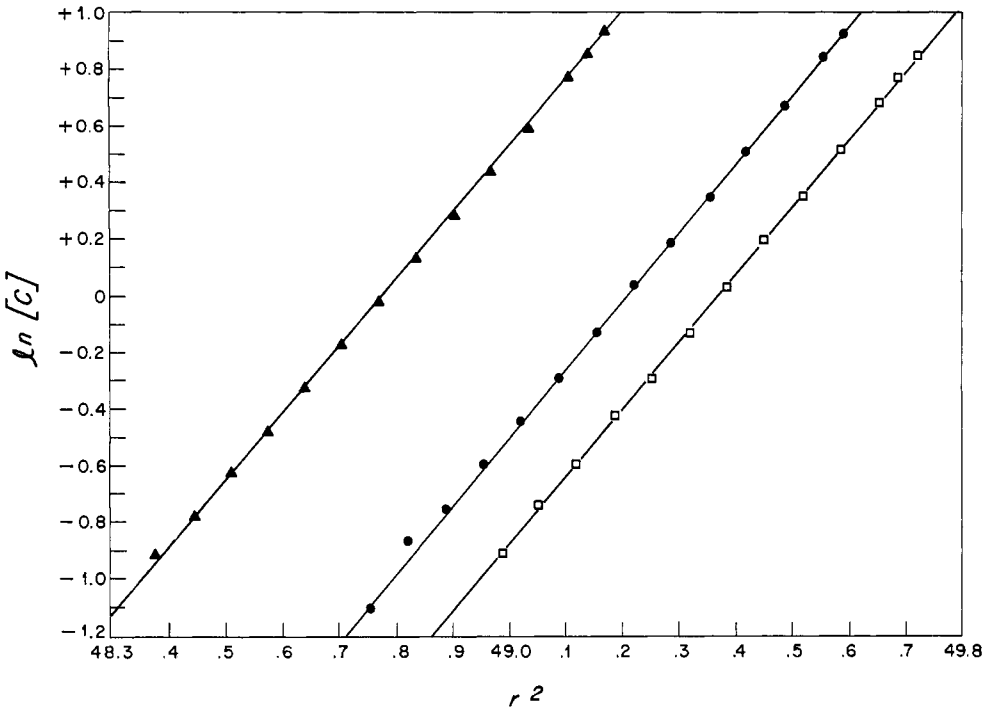

FIGURE 3: High-speed equilibrium molecular weight determination of products of pepsin digestion in 0.1 M Tris acetate (pH 7.5). C is the vertical fringe displacement and r the distance from the center of rotation. A 4.7S fragment is produced at pH 5.0 (●– – –●); 4.9S fragment produced at pH 2.5 (▲– – –▲); 3.5S fragment produced at pH 2.5 (□– – –□). Molecular weights are summarized in Table II.

TABLE II: Sedimentation Data.

Material Pepsin Digest at pH (hr)	$s_{20,w}$ 0.1 M Tris·Acetate (pH 7.5)	M_w (high-speed equil) 0.1 M Tris·Acetate	M_w (low-speed short-column equil) 6 M Guanidine·HCl	6 M Guanidine·HCl after Performic Acid Oxidation	6 M Guanidine·HCl–0.1 M Tris·HCl (pH 8.2)–0.1 M Mercaptoethanol
5.0 (18)	4.7	98,300	–	–	28,200
2.5 (4)	4.9	95,300	97,600	24,300	–
2.5 (4)	3.2	46,700	47,400	–	27,700
2.5 (18)	3.5	–	–	21,600	–
Light chain	–	–	24,800	–	–

where R is the gas constant, T the absolute temperature, ω speed in radians per second, r_b the distance from the center of rotation to the bottom of the column, r_m the distance to the meniscus, $\bar{v}$ in guanidine·HCl was taken as 0.72 (Marler *et al.*, 1964), ρ (the density) was measured for each solution, C_b the concentration at the bottom of the column, and C_m the concentration at the meniscus were determined by extrapolating experimental points in a plot of log C *vs.* r^2. Molecular weight determinations were performed at several different concentrations and an extrapolation to zero concentration was made for each sample.

A typical short-column equilibrium determination for the 4.9S fragment in 6 M guanidine·HCl is shown in Figure 4. Linearity and lack of upward curvature are indicative of the relative molecular weight homogeneity of this material.

This variation of the short-column equilibrium method was validated experimentally by a molecular weight determination on γG light chain. The result

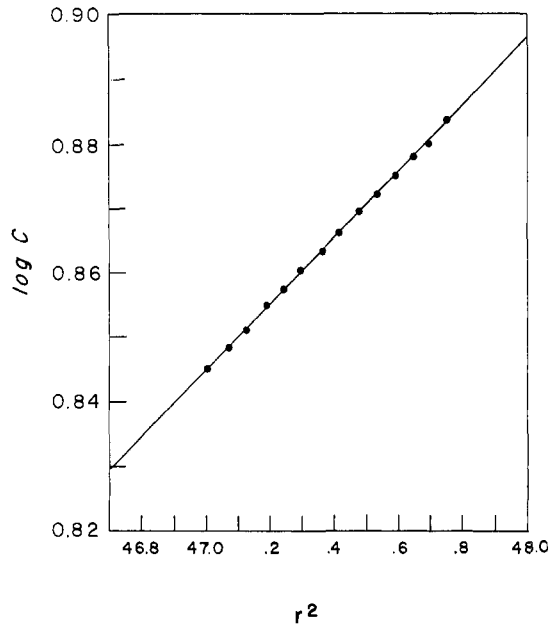

FIGURE 4: Sedimentation equilibrium of 4.7S pepsin product in 6 M guanidine·HCl in a 0.9-mm column at 9341 rpm. Protein concentration is 2.1%. C and r are explained in text. Line drawn by the method of least squares.

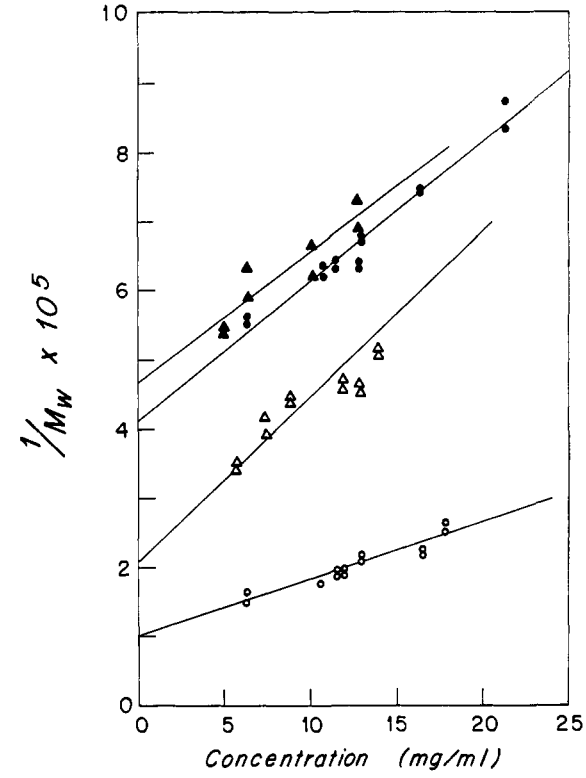

FIGURE 5: Concentration dependence of M_w in 6 M guanidine·HCl determined by the low-speed short-column equilibrium method for the 4.9S (○) and the 3.5S (△) products of pepsin digestion at pH 2.5, and the 4.9S (●) and 3.5S (▲) products after performic acid oxidation. Lines drawn by the method of least squares. Results are summarized in Table II.

is detailed in Table II. The value falls within 4.8% of that determined by Marler *et al.* (1964).

Figure 5 shows the concentration dependence of molecular weights in guanidine·HCl for the various pepsin degradation products produced at pH 5 and 2.5. Results are summarized in Table II. It is of interest that in the presence of 6 M guanidine·HCl there is no appreciable change in molecular weight from the values obtained in aqueous salt solution. After either reduction or performic acid oxidation, the molecular weight of the 4.9S fragment produced at either pH 5 or 2.5 falls to one-fourth of the previous value, while the 3.5S fragment falls to one-half. These results are in concord with the presently accepted model for the structures of $(Fab^1)_2$ and Fab, the former being composed of two light chains, and two N-terminal halves of the heavy chain, while the latter has one light chain and one-half heavy chain. Upon disulfide cleavage, the expected average molecular weights of these fragments should be about 25,000. Since the experimental values approximate what is expected in the presence of full unfolding of structure (Tanford *et al.*, 1966) and the rupture of all disulfide bonds, it is highly unlikely that any significant internal peptide-bond cleavages have occurred.

In order to determine whether the 3.5S fragment was produced through the hydrolytic action of pepsin, or through disulfide interchange, or protonation of the disulfide bond, the purified 4.9S fragment produced at pH 2.5 was incubated for 18 hr at pH 2.5 at 37° and the sedimentation velocity was examined (Figure 6). $s_{20,w}$ of this sample prior to incubation was 4.90 and thereafter 4.82, which is within the limits of experimental error of this method. Consequently, it is likely that cleavage of a C-terminal peptide in the 4.9S product containing the inter-heavy-chain disulfide bond is responsible for the production of the 3.5S fragment.

The hapten-combining properties of fragments produced by pepsin digestion of arsanilate and dinitrophenyl antibodies were examined. As determined by fluorescence quenching, employing methods of computation described by Eisen and Siskind (1964) with the aid of a PDP-1 time-sharing computor, K_A for anti-arsanilate was 4.5×10^6, and for both the 4.9S and 3.5S fragments, 1.3 ± 10^6. A typical Sipps plot for the 3.5S fragment is shown in Figure 7.

Figure 8 illustrates the second gel filtration of components II and III of a 4-hr pepsin digest at pH 2.5 of dinitrophenyl antibody. Sedimentation velocities at the points indicated on the graph show that the first peak consists of the 4.9S product, and the second of the 3.5S product. Fluorescence quenching determinations done at various points along both curves with ϵ-dinitrophenyllysine indicate that binding activity must be uniform through both peaks, only falling off toward

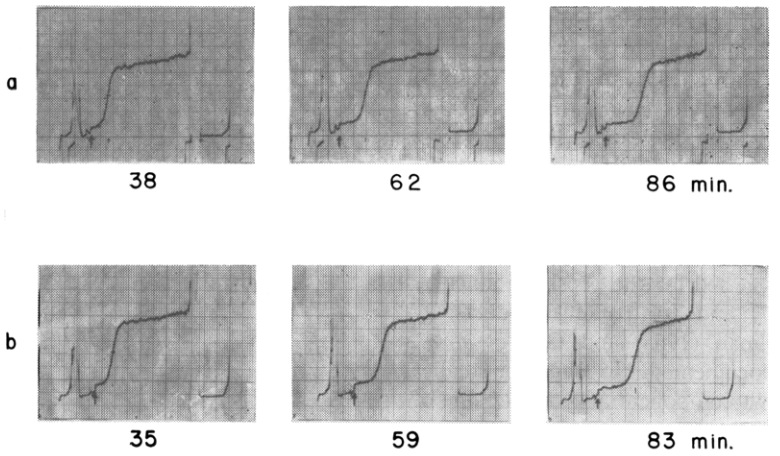

FIGURE 6: Sedimentation velocity determination at 60,000 rpm and 8° employing the ultraviolet scanner at 280 mμ. (a) 4.9S peptic fragment produced at pH 2.5; (b) same sample after 18-hr incubation at 37° (pH 2.5). ↑ indicates the meniscus and the direction of sedimentation is from left to right. Frames are timed after reaching speed.

the down slope of the second peak. These results, as well as those obtained for arsanilate antibody, indicate that both the 4.9S and the 3.5S fragments produced as a result of pepsin degradation are reactive in binding specific antigen, and indeed little changed from native antibody.

Discussion

Pepsin, acting at pH 2.5, releases two macromolecular products from immunoglobulin G, first one of mol wt 97,000, then another of mol wt 47,000. Both have antibody combining activity similar to that of the parent material. The polypeptide chains comprising these fragments appear to be intact. Molecular weight does not decrease in 6 M guanidine, and following cleavage of all disulfide bonds, either by oxidation or reduction, the molecular weight is reduced to the average of the expected constituent polypeptide chains. Major internal peptide-bond cleavages are consequently unlikely, though a detailed analysis of end groups would be required to completely exclude this possibility. This is likewise true for the 3.5S fragment

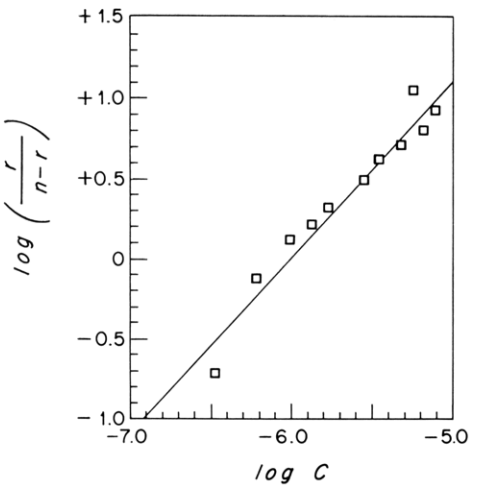

FIGURE 7: Sipps plot for 3.5S pepsin fragment of anti-azoarsanilate as determined by fluorescence quenching. r, moles of mono(p-azobenzenearsonic acid)chloroacetyl-L-tyrosine bound per mole antibody; c, free concentration of the hapten; $n = 1$. K_A is given in text.

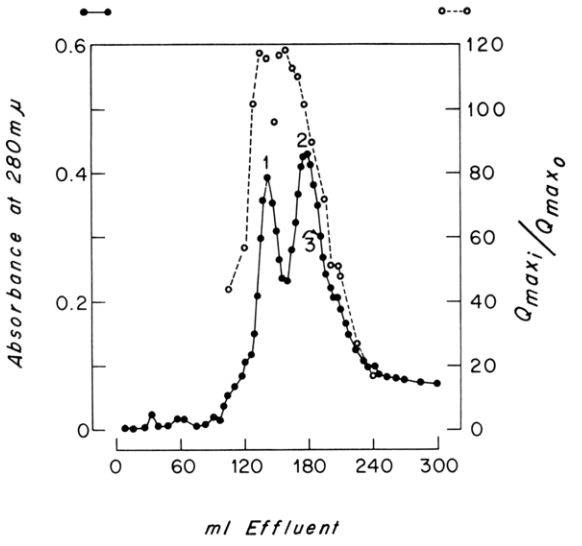

FIGURE 8: Second gel filtration of a 3 × 75 cm column of Sephadex G-100 in 0.1 M Tris·acetate (pH 7.5) of peaks II and III from a pepsin digest of antidinitrophenyl antibody. At fractions marked: 1, $s_{20,w} = 4.9$ S; 2, $s_{20,w} = 3.6$ S; and 3, $s_{20,w} = 3.2$ S. Q_{max_1}, maximal quenching of fluorescence in indicated fraction; Q_{max_0}, maximal fluorescent quenching of original antibody preparation.

isolated after 18 hr of digestion, when it comprised only 5% of the total products. A preliminary report of these experiments (Haber, 1965) suggested that some cleavage of the polypeptide chains had occurred. Review of the earlier data indicates that the strong concentration dependence of molecular weight of the chains in guanidine·HCl was not appreciated, and consequently an erroneous conclusion was drawn. Under the conditions of these experiments, the presence of internal cleavages suggested for the pepsin product by Heimer *et al.* (1967) and for papain products by Fougereau and Edelman (1965) and Gyenes *et al.* (1966) could not be documented. No fragment smaller than the 46,000 mol wt component appears capable of binding hapten. The most reasonable interpretation of these findings is that the Fab portion of the molecule is relatively resistent to proteolytic attack, even at pH 2.5, except at its C-terminal region. However, once a single peptide-bond cleavage has occurred within Fab, further proteolysis is exceedingly rapid. The life of intermediates must be exceedingly short for they cannot be isolated.

The larger fragment is probably identical with $(Fab^1)_2$ of Nisonoff *et al.* (1960). The smaller fragment's molecular weight appears somewhat greater than that of Fab produced by papain digestion. This may indicate that the second pepsin cleavage occurs closer to the C terminus of the heavy chain than the papain cleavage which releases Fab. Disulfide interchange or protonation of the disulfide cannot be invoked since conversion does not occur at pH 2.5 in the absence of pepsin.

Acknowledgments

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