Comparison of Two Precipitating Derivatives of Rabbit Antibody: Fragment I Dimer and the Product of Pepsin Digestion*

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ABSTRACT: This report concerns "fragment I dimer," the product of the consecutive action of insoluble papain and dodecylsulfate on rabbit γ-globulin, and "pepsin product," the product of exhaustive pepsin proteolysis of γ-globulin. Improved procedures are reported for the preparation of both these immunologically divalent products free of any detectable contamination with antigenic determinants associated with fragment III. The measured $s^{\circ}_{20,w}$ and $D^{\circ}_{20,w}$ for fragment I dimer were 4.96 S and 5.63 × 10^{-7} cm^2/sec, and for pepsin product were 4.86 S and 5.11 × 10^{-7} cm^2/sec. The $mw_{s,D}$ of fragment I dimer was 84,000 and of pepsin product was 90,700. The fragment I dimer and pepsin product were compared by a number of other criteria, including antibody activity, content of antigenic determinants, electrophoretic mobility in agar gel, and ability to re-form after reduction by reassociation of univalent fragments. The two products differed most when compared by the latter criterion. The fragments produced by reduction of pepsin product recombined in good yield while under the same conditions the two moieties of fragment I dimer did not. Brief treatment with insoluble papain, either before pepsin proteolysis, or before or after reduction of the pepsin product, resulted in loss of ability of the univalent fragments to recombine under standard conditions.

The limit product obtained by peptic digestion of antibody-active rabbit γ-G globulin has been characterized by Nisonoff and co-workers. This moiety of γ-globulin retained its ability to precipitate homologous antigen (Nisonoff *et al.*, 1960b), was shown to contain two antibody combining sites per molecule, and had a sedimentation coefficient ($s^{\circ}_{20,w}$) of 5.25 S and molecular weight ($mw_{s,D}$) of 106,000 (Nisonoff *et al.*, 1960a). Furthermore, this immunologically divalent product could be converted to two univalent fragments by reduction of a single disulfide bond (Nisonoff *et al.*, 1961). It has been generally accepted that pepsin proteolysis removes or degrades a portion of the γ-G globulin similar or identical with fragment III obtained by papain proteolysis (Taranta and Franklin, 1961; Ovary and Taranta, 1963; Schur and Becker, 1963). Indeed, Goodman (1964) has shown that isolated fragment III is markedly susceptible to peptic digestion. Thus a great number of studies have been made comparing various biologic activities of intact antibody with those of pepsin-digested antibody with the intention of elucidating the role of fragment III in a number of complex antigen-antibody reactions (Taranta and Franklin, 1961; Ovary and Taranta, 1963; Schur and Becker, 1963; Amiraian and Leikhim, 1961; Baxter and Small, 1963).

More recently another immunologically divalent product has been isolated from antibody-active rabbit γ-G globulin after the consecutive action of water-insoluble papain and sodium dodecylsulfate (Cebra, 1964). This moiety of γ-globulin precipitated with homologous antigen, had a $mw_{s,D}$ of 78,000–86,000, and lacked antigenic sites common to fragment III derived from γ-globulin by papain digestion and reduction (Cebra, 1964).

This present paper reports improved procedures for obtaining both precipitating derivatives of rabbit antibody described in a more immunologically and physicochemically homogeneous form than has thus far been reported. Furthermore, these two moieties derived from γ-globulin have been compared using the following parameters: sedimentation and diffusion coefficients, electrophoretic mobility in agar gel, ability to precipitate antigen, ability to be reoxidized to dimers following reduction, and antigenic cross-reactivity with various antisera.

Experimental Procedure

Materials. The antigen ovalbumin (twice crystallized) and the enzyme pepsin (twice crystallized) were obtained from Worthington Biochemical Corp. Water-insoluble papain was prepared as described by Cebra *et al.* (1961). DEAE- and CM-cellulose were products of C. Schleicher and Schuell Co., Keene, N.H. Dowex 1, in its chloride cycle, was obtained from Sigma

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Chemical Co. and transformed to its acetate cycle as previously described (Cebra, 1964). Sephadex was obtained from Pharmacia, Uppsala, Sweden. Sodium dodecylsulfate was recrystallized from the detergent sodium lauryl sulfate, USP (Fisher Scientific Co.), by the method of Crestfield *et al.* (1955). Thioglycolic acid and 2-mercaptoethylamine were purchased from Sigma.

Preparation of Antiovalbumin Rabbit γ-Globulin. Rabbits were immunized with ovalbumin by a single intramuscular injection of 2 mg of alum-precipitated protein in each hind leg followed by intravenous injections of 1 mg of soluble protein begun 4 weeks after primary stimulation and continued for 5 consecutive days. Bleedings were taken from 9 to 12 days after the last injection.

The γ-globulin was precipitated from pooled sera at 4° with 0.37 M saturated ammoniumsu lfate and subsequently chromatographed on DEAE-cellulose according to Levy and Sober (1960).

That fraction of γ-globulin which was not absorbed by the exchanger equilibrated in *p*H 6.3, 0.0175 M sodium phosphate buffer was concentrated by pressure dialysis against 0.1 M phosphate buffer, *p*H 6.8, to approximately 20 mg protein per ml and kept frozen at −20°. The concentration of γ-globulin or derived products, such as fragment I dimer or the pepsin digest product, was determined from optical density measurements at 280 mμ, using the extinction coefficient ($E_{1cm}^{1\%}$) of 14.0 found for intact γ-globulin and fragment I (Porter, 1959). The γ-globulin prepared by these procedures served as starting material for all following preparations.

Enzymatic Hydrolysis with Pepsin or Water-insoluble Papain. The enzymatic digestion of antiovalbumin γ-globulin in acetate buffer, *p*H 4.5, was performed according to the procedures described by Nisonoff *et al.* (1960a,b). The immunoglobulin preparation was used at a concentration of 10 mg protein per ml and 0.02 part by total weight of crystallized pepsin was added. The reaction mixture was incubated for 20 hours at 37°. The digested protein was precipitated with 19% sodium sulfate, dissolved in 0.9% saline, and dialyzed against 0.1 M phosphate buffer, *p*H 6.8.

For hydrolysis with water-insoluble papain, subsequent incubation in 0.5% sodium dodecylsulfate, and removal of detergent on Dowex 1 (acetate), the procedures described by Cebra (1964) were followed. Modifications and further steps in purification of both the above-mentioned preparations are given under Results.

Reduction and Reoxidation of Antiovalbumin γ-Globulin after Either Pepsin or Water-insoluble Papain Proteolysis. Portions of both products prepared by hydrolysis with either pepsin or water-insoluble papain were reduced by incubation with 0.05 M or 0.02 M thioglycolate in 0.1 M phosphate buffer, *p*H 6.8, for 90 minutes at 37° or with 0.01 M mercaptoethylamine as described by Nisonoff *et al.* (1960a). To stop reduction, the reaction mixture was passed through a column of Dowex 1 (acetate) in 0.05 M sodium acetate, *p*H 5.0, to remove thioglycolate or through a column of Dowex 50 in 0.05 M sodium acetate, *p*H 5.0, to remove mercaptoethylamine. Eluted protein-containing fractions were pooled and pressure-dialyzed against 0.15 M phosphate-borate buffer, *p*H 8.0, at 2° for 24 hours, conditions favorable for reoxidation. The extent of reoxidation of univalent fragments was assessed by sedimentation analysis after equilibration of the protein in 0.1 M phosphate buffer, *p*H 6.8.

Titration of Sulfhydryl Groups. The concentration of the reducing agents thioglycolate and 2-mercaptoethylamine and of the free sulfhydryl groups present in the various reductively fragmented derivatives of γ-globulin was determined by titration using dithio-bis-nitrobenzoic acid (Aldrich Chemical Co.) according to Ellman (1959). The reaction was carried out in 0.1 M Tris buffer, *p*H 8.0. When protein-bound sulfhydryl groups were determined the reaction mixture also contained 0.5% dodecylsulfate.

Immunodiffusion Analysis. Gel diffusion and immunoelectrophoresis were carried out in agar gel made up in 0.05 M barbital buffer, *p*H 8.6, according to the methods described by Ouchterlony (1953) and Scheidegger (1955), respectively. For comparisons of relative electrophoretic mobilities of the various products, high-voltage electrophoresis in agar gel, according to a modified procedure of Wieme (1959) as described by Ballieux (1963), was used.

Molecular Weight Determinations. Molecular weights were calculated according to Svedberg and Pederson (1940) from sedimentation and diffusion coefficients. A Spinco Model E ultracentrifuge was used to measure sedimentation coefficients while diffusion coefficients were determined in the same centrifuge by the method of Ehrenberg (1957). A value of 0.745 (Kabat, 1939) for the partial specific volume ($\bar{v}$) of products of γ-globulin was used in all calculations.

Antisera against Rabbit γ-Globulin and Its Fragments. A specific sheep antirabbit γ-globulin serum was kindly provided by Dr. Zoltan Ovary and a goat antirabbit γ-globulin serum was donated by Dr. John Robbins. The preparation of goat sera with anti-fragment I or anti-fragment III specificity has been previously described (Jaquet *et al.*, 1964).

Guinea pigs were immunized with the pepsin digest product by the method of Benacerraf *et al.* (1963). A 2-mg amount of protein in complete Freund's adjuvant was injected into the footpads of each of four guinea pigs. Ten days after the primary stimulation the guinea pigs were injected intradermally at four sites with a total of 40 μg of protein in a saline solution. The intradermal injections were repeated daily for 5 days, at which time moderate Arthus reactions were developing. Bleedings were taken at this time and the resultant sera had precipitating antibody which reacted with the pepsin product. After a 7-day period of rest the guinea pigs were rechallenged with 40 μg given intradermally at four sites and again with 20 μg given 5 days later. Large bleedings were taken on the day following the last injections and the resulting sera were used for gel diffusion studies.

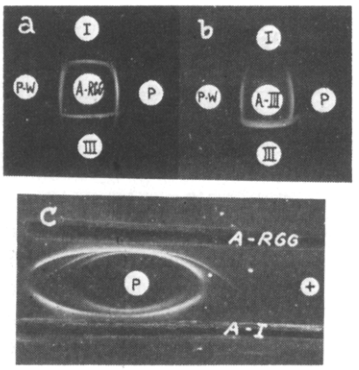

FIGURE 1: Antigenic analysis of pepsin-digested γ-globulin, purified only by precipitation with 19% Na_2SO_4. The symbols used indicate: A-RGG, sheep anti–whole γ-globulin; A-I and A-III, goat anti–fragment I and anti–fragment III, respectively; I and III, the corresponding antigens, fragments I and III; P, pepsin-digested γ-globulin after 19% Na_2SO_4 precipitation and dialysis against 0.9% saline; P-W, the former preparation after further proteolysis with water-insoluble papain. In the immunoelectrophoresis experiment shown in (c) a current of 5 ma was passed for 3 hours (slide was 2.5 cm in width) before development with antisera.

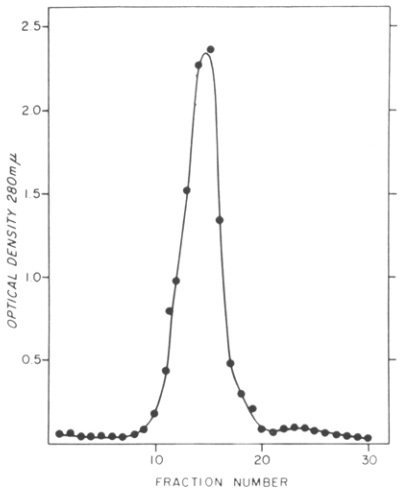

FIGURE 2: Final purification of pepsin-digested rabbit γ-globulin on Sephadex G-100. The protein precipitated from pepsin digests of γ-globulin with 19% Na_2SO_4 was equilibrated with 0.1 M phosphate buffer, *p*H 6.8. A 15-ml sample of this protein solution (165 mg) was applied to a Sephadex G-100 column (2.5 cm i.d. × 40 cm) equilibrated in the same phosphate buffer. Fractions of 7.0 ml were collected. The major protein fraction, eluted first from the column, contained the pepsin product.

Results

Improved Procedures for the Preparation of Both Fragment I Dimer and the Product of Pepsin Digestion for Comparative Studies

Since both fragment I dimer and the peptic digest product prepared by the standard procedures described under Methods contained small amounts of fragment III, or its degradation products, both preparative methods were modified. Antiovalbumin γ-globulin (680 mg immunoglobulin) at a concentration of 10 mg/ml was incubated with pepsin (13.6 mg) for 20 hours at 37°. The digested product was precipitated with 19% sodium sulfate and 55% of the protein (350 mg) was recovered. This protein, called "5 S product" by Nisonoff and co-workers, retained its ability to precipitate antigen and appeared to sediment in the ultracentrifuge as one homogeneous boundary. When this "5 S product" was allowed to diffuse toward antisera specific for fragment III, however, a distinct precipitation band formed in agar gel (Figure 1b), indicating that the foregoing procedures had not removed all parts of fragment III from the preparation. In gel diffusion, as well as in immunoelectrophoresis (Figures 1a,c), clear separation of the major precipitation band from that formed by molecules containing fragment III–type antigenic sites seemed to indicate that the two different antigenic moieties were located on different molecules. To purify further such an immunologically heterogeneous mixture, fractionation by gel filtration seemed appropriate and the product (350 mg) was passed through Sephadex G-100 in phosphate buffer (Figure 2). The main component (295 mg) eluted from the column contained no detectable antigenic sites related to fragment III (as shown in Figure 5e). The small amount of retarded material eluted after the main component did not have any visible antigenic sites related to fragment I and contained some, but not all, of the antigenic sites present in crystallizable fragment III. Further characterization of what were apparently degradation products of fragment III was not carried out. The principal product, freed of these contaminating polypeptides, was used in all comparative studies described and is called the *pepsin product*.

To prepare fragment I dimer in an equally homogeneous form, antiovalbumin γ-globulin (350 mg) as a solution 10 mg/ml after proteolysis with water-insoluble papain was incubated in 0.5% sodium dodecylsulfate as described previously (Cebra, 1964). A major soluble product (230 mg) was recovered upon removal of the detergent on Dowex 1 (acetate) (Cebra, 1964). This material was also antigenically heterogeneous and contained small amounts of protein having fragment III–type antigenic sites even after 19% sodium sulfate precipitation. To remove those molecules bearing fragment III–type antigenic sites from the dissociated immunoglobulin, the pooled clear effluent from the Dowex 1 (acetate) column (230 mg) was further fractionated on a column of Sephadex G-200 (Figure 3). Gel filtration resolved the detergent-free dissociated globulin into aggregated material, fragment I dimer, and some smaller components. A generous pool of

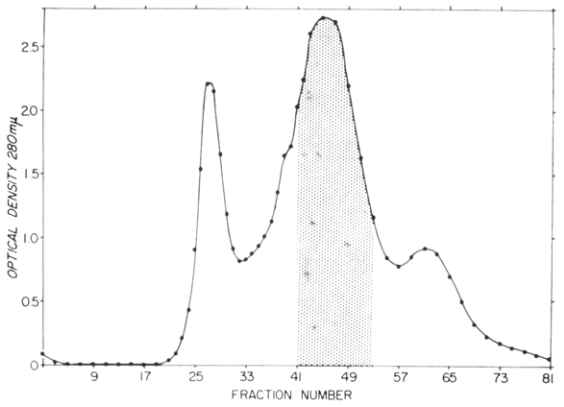

FIGURE 3: Gel filtration of dissociated γ-globulin after detergent removal. A 12-ml sample of dissociated γ-globulin (230 mg), freed of dodecylsulfate by passage through a column of Dowex 1 (acetate) and equilibrated with 0.1 M sodium phosphate buffer, *p*H 6.8, was applied to a column of Sephadex G-200, 2.5 cm i.d. × 80 cm. Fractions containing 4.5 ml were collected. The fractions indicated by the shaded area were pooled and concentrated for chromatography on CM-cellulose.

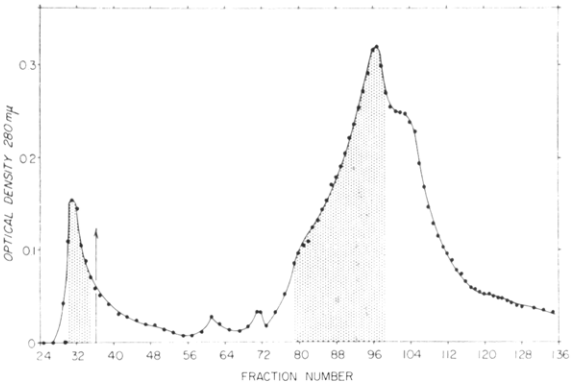

FIGURE 4: Final purification of fragment I dimer by chromatography on CM-cellulose. A 24-ml sample of the fragment I dimer fraction prepared by gel filtration (shaded area of Figure 3), containing 87 mg, was applied to a column of CM-cellulose, 2.5 cm i.d. × 32 cm. The sample and column were equilibrated in 0.01 M sodium acetate buffer, *p*H 5.5. Fractions of 5.0 ml were collected. At the point indicated by the arrow, gradient elution was initiated using the 0.01 M acetate buffer in a 1200 mixing chamber (filter flask) into which a 0.6 M acetate buffer, *p*H 5.5, was introduced. The fractions indicated by the shaded areas were pooled and concentrated.

material containing fragment I dimer (shaded area in Figure 3) was made and the protein was concentrated by pressure dialysis (90 mg). Fragment I dimer, at this stage of purification, may still contain a very small proportion of molecules which react with an anti–fragment III serum. These molecules, which still contain fragment III–type antigenic sites, appear to be undissociated γ-globulin. Undissociated γ-globulin is not well separated from fragment I dimer on the Sephadex G-200 column. For the comparative studies, fragment I dimer was completely freed of such undissociated molecules by chromatography on CM-cellulose under conditions similar to those described by Porter (1959) for the resolution of papain digest products of γ-globulin (Figure 4). The protein obtained by pooling the effluent indicated by the shaded area in Figure 4 was fragment I dimer, free of fragment III–type sites, as shown in Figure 5d. The proportion of fragment I dimer eluted before and after initiation of gradient elution varies with different preparations in a manner similar to fragment I itself. A sedimentation pattern of fragment I dimer prepared by these procedures is also shown in Figure 9d.

A Comparison of the Properties of Fragment I Dimer and the Limit Product of Pepsin Digestion

Antigenic Composition. If papain and pepsin had hydrolyzed different peptide bonds to give rise to their respective digest products of γ-globulin it would seem possible that one of these products might retain more antigenic sites than the other. The antigenic compositions of the two products, however, thus far seems identical as judged by analysis with the immune sera now available. Figure 5c,d,e indicates that both

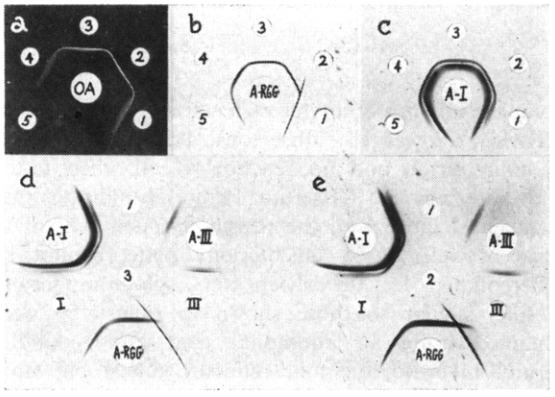

FIGURE 5: Comparative immunologic analysis of pepsin-digested immunoglobulin and fragment I dimer after the purification steps. Symbols used indicate: A-RGG, A-I, and A-III, antisera prepared against intact rabbit γ-globulin, fragment I, and fragment III, respectively; I and III indicate fragments I and III, respectively; OA indicates ovalbumin. In (a) an analysis for antibody activity is shown and the central well contains ovalbumin at 500 μg/ml. The contents of all numbered wells are as follows: (1) intact immunoglobulin, (2) pepsin product, (3) fragment I dimer, (4) pepsin product after papain hydrolysis, (5) fragment I.

fragment I dimer and the pepsin product were antigenically indistinguishable from both fragment I and intact γ-globulin when allowed to diffuse toward an anti–fragment I serum. Both these products also showed

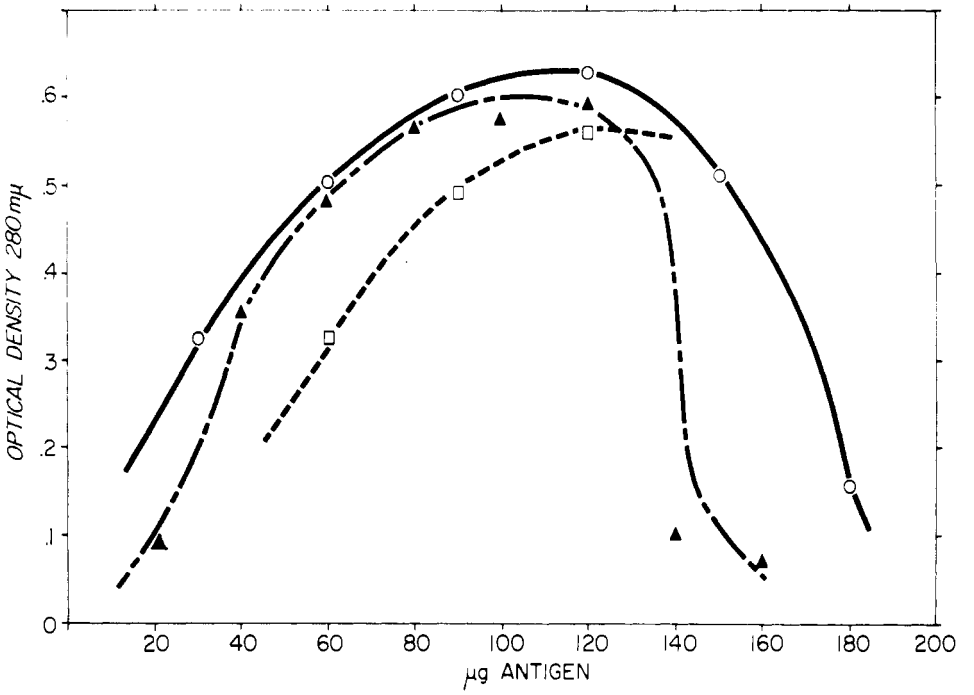

FIGURE 6: Precipitation of ovalbumin by intact immunoglobulin and its derivatives, the pepsin product and fragment I dimer. Increasing amounts of antigen were added to 4.3 mg of each of the various antibody preparations in 0.2 ml. The precipitates were incubated for 90 minutes at 37° and kept at 2° for another 12 hours. The twice-washed precipitates were dissolved in 1.0 ml of 0.1 N NaOH and the protein was measured by optical density measurements at 280 mμ. O—O, intact rabbit antiovalbumin γ-globulin; ▲—▲, pepsin product; □—□, fragment I dimer.

complete absence of any cross-reactivity with fragment III when allowed to diffuse toward an anti-whole γ-globulin serum and no reaction at all when tested with anti-fragment III serum. Figure 5b indicates that fragment I dimer and the pepsin product, present in adjacent wells of an Ouchterlony plate, cannot be differentiated by the sheep anti-γ-globulin serum. Results similar to those shown in Figure 5b were obtained using an additional goat anti-γ-globulin serum. Likewise, the four different guinea pig anti-pepsin product sera gave results similar to those shown in Figure 5b except that no spur was present at the juncture of the precipitin lines formed by intact γ-globulin and the pepsin product. Thus the fragment I dimer and the pepsin product could not be differentiated by an anti-fragment III serum, an anti-fragment I serum, two anti-γ-globulin sera, and four anti-pepsin product sera.

Ability to Precipitate Specific Antigen. Both the pepsin product and fragment I dimer have been shown to precipitate with antigen (Nisonoff *et al.*, 1960b; Cebra, 1964). Figure 6 shows typical quantitative precipitation curves obtained for intact γ-globulin and the two products, both derived from this same globulin. In general, antigen seemed to precipitate less fragment I dimer than pepsin product in the region of antibody excess. About the same amounts of precipitate were obtained at immunologic equivalence by reacting antigen with similar amounts of each of the two products. Thus, as shown graphically by the Ouchterlony plate portrayed in Figure 5a, preparation of either the pepsin product or the fragment I dimer did not result in a selective gain or loss in specific antibody activity by either product.

Sedimentation and Diffusion Coefficients and Molecular Weight. From sedimentation and diffusion coefficients of 5.25 S and 4.7 × 10^{-7} cm^2/sec, respectively, Nisonoff *et al.* (1960a) calculated a molecular weight of 106,000 for the "5 S product" of pepsin digestion. We have attempted a redetermination of these parameters in parallel with making similar measurements on preparations of fragment I dimer. Figure 7 shows plots of $s_{20,w}$ and $D_{20,w}$ versus concentration for the two products. Extrapolated values of $s^\circ_{20,w}$ = [4.86 ± 0.07] S and $D^\circ_{20,w}$ = [5.11 ± 0.11] × 10^{-7} cm^2/sec were obtained for the pepsin product. The corresponding extrapolated values for fragment I dimer were $s^\circ_{20,w}$ = [4.96 ± 0.12] S and $D^\circ_{20,w}$ = [5.63 ± 0.15] × 10^{-7} cm^2/sec. The molecular weights calculated from these data ($mw_{s,D}$) were 90,700 ± 2300 for the pepsin product and 84,000 ± 3000 for the fragment I dimer.

Electrophoretic Mobility in Agar Gel. One of the principal characteristics of γ-globulin is its charge polydispersity (Alberty *et al.*, 1948). The various enzymically and chemically produced fragments and peptide chains of γ-globulin likewise show considerable charge polydispersity. Nevertheless, the distributions of electrophoretic mobilities of the molecules which

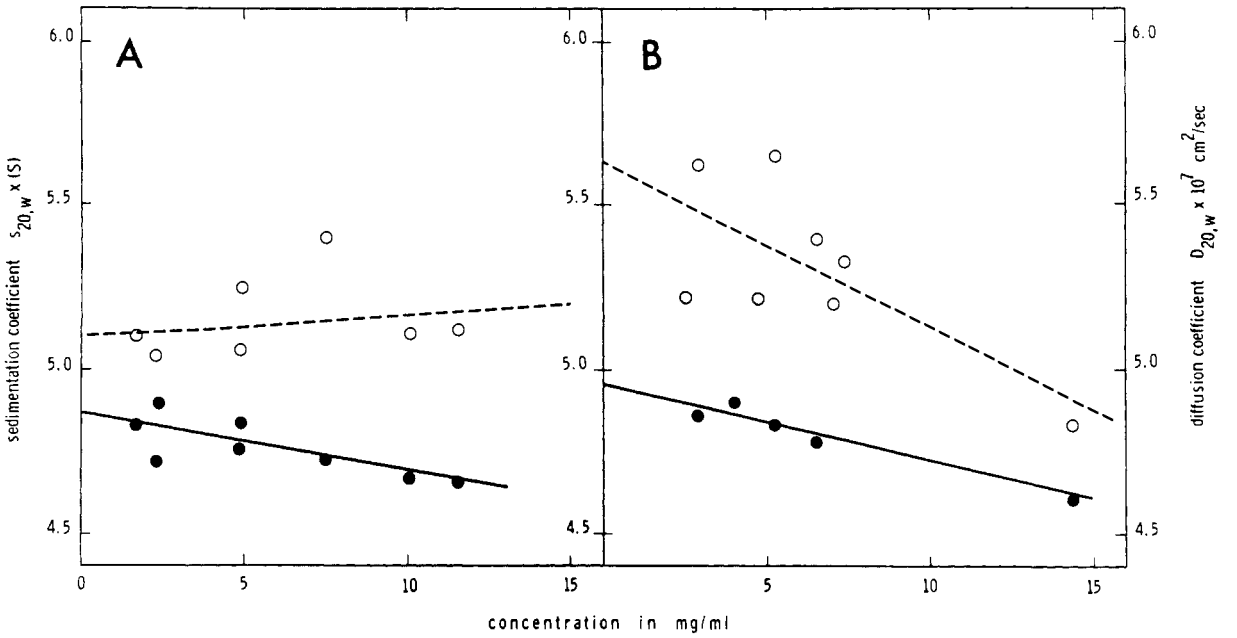

FIGURE 7: Determination of sedimentation and diffusion coefficients of fragment I dimer and the pepsin product. (A) Data for the pepsin product; (B) data for fragment I dimer. ● = $s_{20,w}$; ○ = $D_{20,w}$. Straight lines were calculated from the data by the method of least squares.

comprised either fragment I dimer or the pepsin product seemed somewhat different. Comparison of the two products, both by immunoelectrophoresis and high-voltage gel electrophoresis, as shown in Figure 8, indicated that the average mobility of fragment I dimer toward the anode was about the same as that of fragment I itself and slightly greater than that of the pepsin product. Reduction of both products to univalent fragments resulted in a slight increase in average electrophoretic mobility toward the anode.

Ability to Be Reoxidized after Reduction to Univalent Fragments. Mandy *et al.* (1961) have reported that the "5 S product" of pepsin digestion, derived from purified antibody, could be reduced and then be partially reoxidized to "5 S product." The reoxidized protein had the ability to precipitate antigen and yields of approximately 60% recombined antibody fragments were obtained. Thus, for this present study, ability to be reoxidized was taken as a criterion by which the fragment I dimer and the pepsin product could be compared.

First, the pepsin product was shown to be reoxidizable to the extent reported by Mandy *et al.* (1961) by using two different procedures. The pepsin product (15 mg in 2.0 ml) was reduced with 0.01 M 2-mercaptoethylamine in sodium acetate buffer, *p*H 5.0, according to the procedure of Nisonoff *et al.* (1960a). The positively charged reducing agent was removed by passing the reduction mixture through a column of Dowex 50 cation-exchange resin equilibrated with sodium acetate buffer, *p*H 5.0. The eluted protein was concentrated by pressure dialysis against 0.15 M phosphate-borate buffer, *p*H 8.0, for 24 hours to facilitate reoxidation. Ultracentrifugal analysis of the concentrated sample indicated that 60–70% of the fragments had recombined to a product with $s_{20,w} \cong 5$ S. A second procedure for reduction and reoxidation was adopted for most subsequent experiments. This procedure utilized thioglycolate as reducing agent to facilitate the analysis for and isolation of any peptide material released on reduction of the products to be described. The pepsin product (15 mg in 2.0 ml) was reduced by incubation with either 0.02 M or 0.05 M thioglycolate for 90 minutes at 37° in 0.1 M phosphate, *p*H 6.8. A portion of the reduction mixture was taken for ultracentrifugal analysis to demonstrate the complete conversion of the pepsin product to fragments having $s_{20,w} = 3.4$ S (Figure 9b). The balance of the mixture (12 mg in 1.6 ml) was freed of negatively charged reducing agent by passage through a Dowex 1 anion exchanger (1 cm i.d. × 9 cm) in the acetate form, equilibrated with 0.05 M sodium acetate or suspended in distilled water. After pressure dialysis of the protein-containing effluent against phosphate-borate buffer, *p*H 8.0, as described, the extent of recombination was assessed by sedimentation analysis. Figure 9c indicates that reoxidation of the fragments derived from the pepsin product occurred to an extent comparable to that observed using 2-mercaptoethylamine as the reducing agent.

The fragment I dimer was reduced using thioglycolate under conditions identical with those described. Figure 9d,e shows that the dimer, having $s_{20,w} = 4.9$ S, was completely converted to fragments having $s_{20,w} = 3.4$ S. However, upon removal of reducing agent from the fragmented dimer and concentration against phosphate-borate buffer, *p*H 8.0, no appreciable recom-

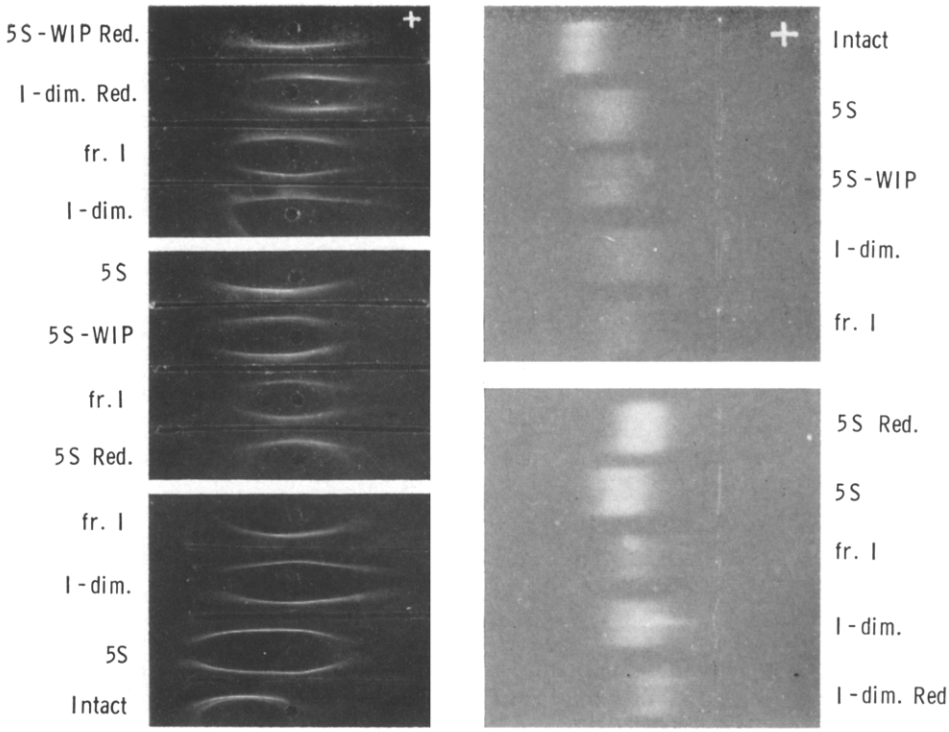

FIGURE 8: Comparative electrophoretic mobilities in agar gel of pepsin product, fragment I dimer, and various related preparations. At the left, immunoelectrophoresis in agar gel is shown. The 3-mm layer of agar was prepared in 0.05 M Veronal buffer, *p*H 8.6. A current of 5 ma per 2.5 cm of slide width was passed 2.25 hours. At the right, high-voltage electrophoresis in agar gel is shown. The 1-mm layer of agar was prepared in 0.05 M Veronal buffer. A current of 70 ma was passed per 2.5 cm of slide width (220 volts) for 10 minutes. Symbols used indicate: intact, untreated γ-globulin; 5 S, pepsin product; I-dim, fragment I dimer; fr I, fragment I; "Red" indicates that the preparation was reduced with 0.05 M thioglycolate in phosphate buffer, *p*H 6.8, for 1.5 hours; "WIP" indicates that the product was treated with water-insoluble papain for 30 minutes.

bination occurred and the bulk of the protein sedimented with $s_{20,w} = 3.5$ S (Figure 9f).

In order to assess whether the papain-cleavage step was critical in predetermining that the immunologically divalent product could not be reoxidized after reductive fragmentation, exposure to insoluble papain was introduced at three separate stages in the preparation of reduced pepsin product. Figure 10 schematically presents the stepwise manipulations performed on the γ-globulin. Reduction and reoxidation of the final products were in all cases carried out using thioglycolate exactly as described before. The protein was exposed to insoluble papain at one of the following stages in the preparation of reduced pepsin product: (1) immediately before proteolysis with pepsin, (2) immediately following proteolysis with pepsin, or (3) immediately after reduction of the pepsin product. In none of the foregoing procedures did insoluble papain detectably change the sedimentation properties of its substrate or appear to effect the subsequent course of peptic digestion. However, in all cases where the protein was exposed to insoluble papain, the final pepsin product did not appreciably recombine or reoxidize after reductive fragmentation.

Figure 11a–d shows the sedimentation analysis of four preparations which were reduced with 0.05 M thioglycolate, freed of reducing agent, and pressure-dialyzed under conditions suitable for reoxidation of the pepsin product. These preparations, the pepsin product (a), the pepsin product hydrolyzed with water-insoluble papain either before (c) or after reduction (d), and water-insoluble papain–hydrolyzed γ-globulin (b), were all manipulated simultaneously. Only the pepsin product showed appreciable reoxidation. However, after pressure dialysis all other products seemed to contain a very small amount of material which sedimented faster than the univalent fragments and which was absent following reduction alone.

Figure 11e,f shows similar results obtained by reducing the pepsin product before and after water-insoluble papain proteolysis with a lower concentration of thioglycolate, 0.02 M. The two samples, reduced in parallel, were each titrated for the presence of free sulfhydryl groups immediately after removal of reducing agent. The pepsin product contained 1.84 free sulfhydryl groups per original molecule while the water-insoluble papain–treated pepsin product contained only 0.78. Again only the reduced pepsin product showed appreciable recombination.

Figure 11g,h shows results typical of attempts to

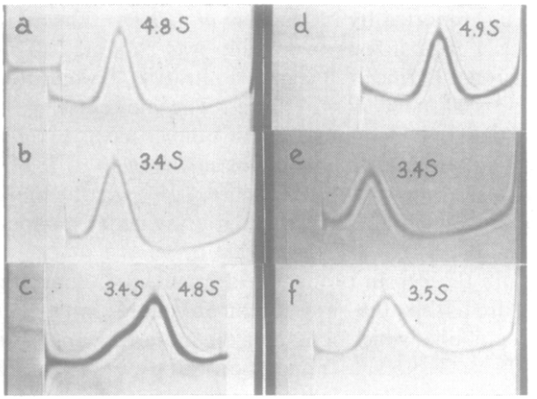

FIGURE 9: Sedimentation analysis of pepsin product and fragment I dimer, before and after reduction and after subsequent exposure to conditions favorable for reoxidation. Speed, 56,100 rpm. All samples were equilibrated in 0.1 M phosphate buffer, *p*H 6.8. Protein concentration was 4–6 mg/ml. Values for $s_{20,w}$ are indicated in the figure. The sedimentation patterns are of (a) pepsin product, (b) reduced pepsin product, (c) reoxidized pepsin product, (d) fragment I dimer, (e) reduced fragment I dimer, (f) reduced dimer after attempted recombination. Pictures a, b, d, and e were taken 68 minutes, and c and f taken 96 minutes after attainment of the indicated rotor speed.

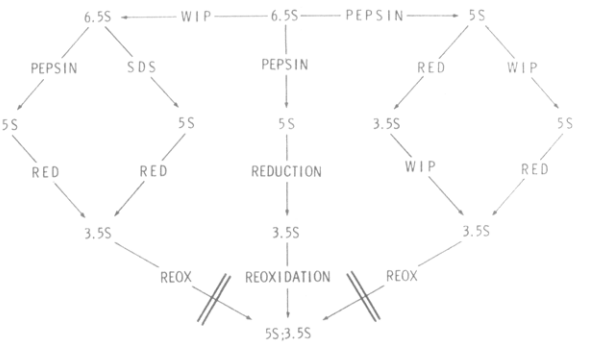

FIGURE 10: Schematic diagram showing the various consecutive manipulations performed on γ-globulin to determine their effect on the ability of univalent fragments to recombine readily. The sedimentation coefficients given in the chart are approximate. Figure 11 shows some of the sedimentation analyses used to construct this chart. Abbreviations used: WIP and RED as in Figure 8; SDS is sodium dodecylsulfate; REOX indicates reoxidation.

recombine papain fragments I. The mixed fragments (I and III) were obtained by minimal reduction of water-insoluble papain–hydrolyzed γ-globulin in distilled water with 0.0075 M 2-mercaptoethylamine. Incubation with this concentration of reducing agent, at 37° for 1 hour at *p*H 6.8, gives almost complete reductive fragmentation of the hydrolyzed globulin (Figure 11h). After reduction, the reaction mixture (2.5 ml containing 25 mg of protein) was passed rapidly onto a column of CM-cellulose (1.8 cm i.d. × 14 cm), equilibrated at *p*H 5.5 with 0.01 M acetate buffer (Porter, 1959). The protein which passed directly through the column was fragment I, free of reducing agent. This protein was immediately pressure-dialyzed against phosphate-borate buffer, *p*H 8.0. Sedimentation analysis, shown in Figure 11g, indicated that no detectable dimerization of fragment I had occurred.

To summarize the foregoing results it appeared that the loss of reoxidizability of the various products could be correlated with exposure to papain and did not appear to result from the exposure of fragment I dimer to detergent or from some other pecularity of its preparation.

Discussion

In view of the extensive use made of pepsin-digested rabbit-immune globulin (Taranta and Franklin, 1961; Ovary and Taranta, 1963; Schur and Becker, 1963; Amiraian and Leikhim, 1961; Baxter and Small, 1963) and more recently of fragment I dimer (Cebra *et al.*, 1963) as precipitating antibodies which lack the class-specific part of the heavy chain (fragment III) it seemed useful to: (1) compare these two products with respect to a variety of properties; and (2) to improve the procedures used in their preparation so that these products would indeed be free of all fragment III antigenic sites detectable by immunologic means. The existing procedures for the preparation of the pepsin product (Nisonoff *et al.*, 1960a,b) and fragment I dimer (Cebra, 1964) have provided derivatives of a homogeneity adequate for many previous studies. For instance, no intact globulin could be detected after 20 hours of pepsin proteolysis and the degree of contamination of pepsin product with pieces of fragment III never exceeded 3%. However, should the activity of either pepsin product or fragment I dimer be measured in very sensitive biologic systems, which detect opsonization, complement fixation, or passive sensitization, it would appear worthwhile to eliminate all residual pieces of fragment III from the antibody derivative.

The two purified products, fragment I dimer and the pepsin product, have been found to be strikingly similar in their ability to precipitate quantitatively antigen and indistinguishable on the basis of gel-diffusion analysis of their antigenic sites using the several immune sera at our disposal. At present, additional heterologous species are being intensively immunized with the pepsin product to determine whether the resulting immune sera can be used to distinguish between the two products antigenically.

The two products likewise seem to behave similarly in two biologic systems. Both fail to sensitize guinea pig skin for the demonstration of passive cutaneous anaphylaxis (Ovary and Taranta, 1963; Cebra *et al.*, 1963) and both show a marked decrease in specific ability to "fix" hemolytic complement compared with intact globulin. About the same residual complement-

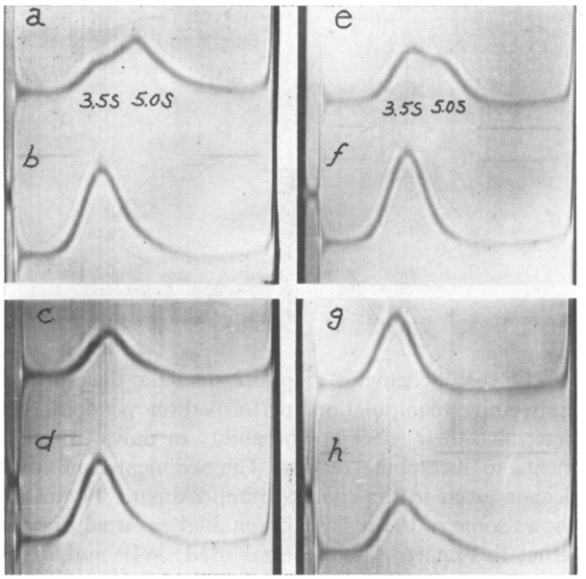

FIGURE 11: Sedimentation analysis of various univalent fragments after attempted oxidative recombination. Speed 56,100 rpm. All samples were equilibrated in 0.1 M phosphate buffer, *p*H 6.8, before centrifugation. Protein concentration was 4–7 mg/ml. Pictures were taken 95 minutes after attainment of the indicated rotor speed. The sedimentation patterns are of the following preparations, after removal of reducing agent and pressure dialysis against 0.15 M phosphate-borate buffer, *p*H 8.0, for 24 hours to promote recombination: (a) pepsin product, reduced with 0.05 M thioglycolate; (b) water-insoluble papain–hydrolyzed γ-globulin, reduced with 0.05 M thioglycolate; (c) pepsin product treated as in (a) except also treated with water-insoluble papain before reduction; (d) same as (c) but treated with water-insoluble papain after reduction; (e) pepsin product, reduced with 0.02 M thioglycolate; (f) same as (e) except treated with water-insoluble papain before reduction; (g) fragment I prepared from water-insoluble papain–hydrolyzed γ-globulin shown in (h); (h) water-insoluble papain–hydrolyzed γ-globulin after reduction with 0.007 M 2-mercaptoethylamine for 1 hour at 37° at *p*H 6.8.

fixing activity, approximately 40% with respect to intact immunoglobulin, is retained by both pepsin product and fragment I dimer (H. Jaquet and J. J. Cebra, unpublished data).

The molecular weight of the pepsin product appears to exceed that of the fragment I dimer by 6700 units at most. The experimental points used in the plot of $D_{20,w}$ versus concentration show some scatter within a few tenths of a unit, making precise extrapolation to a value for $D^{\circ}_{20,w}$ somewhat uncertain. However, all values obtained for three different preparations have been plotted and the values for $D_{20,w}$ of the dimer seem to be indeed higher than those obtained for the pepsin product. The molecular weight of 90,700 calculated for the pepsin product is somewhat less than the value of 106,000 reported by Nisonoff *et al.* (1960a). The pepsin product used in our own study was subjected to one additional purification step, gel filtration, beyond those used by Nisonoff *et al.* (1960a). The molecular weight of 84,000 obtained for fragment I dimer seems in agreement with the approximate molecular weight of 78,000–86,000 previously reported (Cebra, 1964) and the molecular weight of 40,700 obtained for fragment I monomer by Pain (1963).

Both the pepsin product and fragment I dimer were prepared from the same chromatographic fraction of γ-G globulin, which contained the more basic molecules in the γ-G globulin population. However, slight differences in the electrophoretic mobility of the two products in agar gel at *p*H 8.6 were observed. The slightly lower anodal mobility of the pepsin product could reflect the presence of a different or additional peptide portion with respect to fragment I dimer. The slight difference in mobilities could also have resulted from selectivity in the diverse preparatory procedures used. Nevertheless, both products show a slight but definite increase in mobility toward the anode upon reduction.

Perhaps the most striking difference between the pepsin product and fragment I dimer is the ability of the former to be reoxidized after reductive fragmentation, while the latter, upon reduction, shows almost no tendency for recombination of fragment I moieties to form dimer. Palmer and Nisonoff (1964) have shown that a majority of the rabbit γ-globulin half-molecules are apparently held together by a single disulfide bond. Even when this bond is reduced and the sulfhydryl groups are blocked the half-molecules show a tendency to reassociate noncovalently (Stein *et al.*, 1964). Recently, Nisonoff and Dixon (1964) have presented circumstantial evidence that the single disulfide bond which joins the half-molecules is the same one which holds the two component parts of the pepsin product together. Whether or not the disulfide bond which joins two fragment I moieties to form dimer is also this same bond is still an open question. Fragment I dimer is formed from γ-globulin by the consecutive action of insoluble papain and detergent. However, recent evidence has indicated that the ability of detergent to dissociate the components of fragment III from the whole molecule depends on the presence of trace amounts of protein-bound sulfhydryl groups (J. J. Cebra, 1964, unpublished data). If these free sulfhydryl groups are blocked, dissociation in the presence of detergent can then be initiated by trace amounts of γ-globulin fragments which contain free sulfhydryl groups. Thus fragment I dimer appears to arise through a disulfide interchange reaction (see Cebra, 1964). The disulfide bond joining the fragment I moieties to form dimer could therefore have been present in the original molecule or could have been created during this dissociation.

On the basis of the above-mentioned characteristics of the two products, one can suggest several explanations for the lack of appreciable oxidative recombinations of fragment I moieties to form dimer: (1) the pepsin product may contain an additional peptide section of the heavy chain not present in the dimer which facilitates

reassociation of the fragments in such a way that the disulfide bond readily reforms; (2) owing to the particular bond cleaved by papain the free sulfhydryl group on fragment I may become inaccessible because of a chemical or conformational change which does not occur upon reduction of the pepsin product; and (3) the naturally occurring disulfide bond between the half-molecules is not included in the papain-produced dimer. This last explanation must also include the assumption that the disulfide bond which is present has been artificially created and will not re-form in a solution of fragment I moieties. Our own experiments have indicated (J. J. Cebra, 1964, unpublished data) that fragment I, freshly prepared by the action of papain and reducing agent, does contain one free sulfhydryl group per molecule. This sulfhydryl group seems to disappear upon storage at *p*H 6.8 at 5°.

An analysis of antigenic determinants of human γ-globulin which appear to be in the neighborhood of the critical cleavage points of papain and pepsin has been made by Grey and Kunkel (1964). The antigenic sites which were studied were those which form the basis for classifying human γ-globulin into Vi, Ge, and We subgroups. The antigenic specificities of the Vi, Ge, and We subgroups appear to be present in the heavy chains. Two antisera, anti-Zu and anti-Vi, absorbed to react specifically with molecules of the Vi subgroup, react differently with the fragments produced by enzymic digestion. Anti-Vi gives no reaction with either papain fragment (S or F) from Vi-myeloma globulins, while anti-Zu reacts with the F fragment alone. The anti-Vi serum did react with the pepsin digestion product obtained from Vi-myeloma globulins, while the anti-Zu serum did not. Reduction of the pepsin digest product led to loss of antigenic reactivity and reoxidation to restoration of reactivity with the anti-Vi serum. These findings suggest that a restoration of antigenic sites occurs concomitantly with reoxidation of reduced pepsin product.

The difference in reoxidizability between the pepsin product and fragment I dimer and the ability of insoluble pppain to convert the pepsin product at various stages of its preparation to a nonreoxidizable form seem to suggest that the critical cleavage points of pepsin and papain are different. Thus far preliminary experiments indicate that the consecutive treatment of pepsin product with insoluble papain and a low concentrations of reducing agent, used in either order, results in the release of dialyzable peptides. Attempts to isolate and characterize particular peptides are now being made.

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