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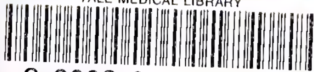
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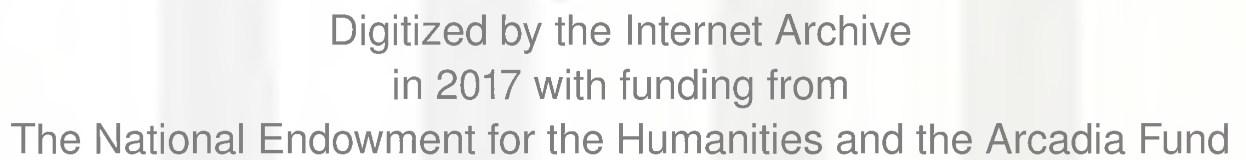

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STUDIES ON THE DEGRADATION OF SEVERAL IgG PREPARATIONS.

Submitted by

Richard Bockman

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I would like to express my deep appreciation to those people whose contribution to the present work was essential for its completion: To Dr. B. Waksman who initiated, administrated and corrected this paper, I am most indebted. To Drs. B. and L. Robert whose collaboration formulated a continuous teaching experience and developed a permanent friendship, I am most grateful. To Dr. B. Halpern who provided the liberal environment in which this work was carried out, I am most appreciative.

My graditude is also extended to Dr. W. Konigsberg for allowing me to use his laboratory facilities and to Mrs. E. Mac Kinnon for typing the final manuscript.

I dedicate this cornerstone of five years' work to Marie-France In order that we may share equally in the future building.

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I. INTRODUCTION

The immunoglobulins are a group of glycopeptides which appear to be present in every tissue and secretion of the animal body. To date, antibody activity has been demonstrated only within this group of proteins. The carbohydrate moiety of the immunoglobulins is an extremely minor fraction comprising only 2-10% of the entire molecule and has been shown to be unnecessary for the specific antibody activity of the molecules (Porter, R. R., 1959; Fleischman, I. B. et al, 1963). It does, however, appear to be involved in making immunoglobulins antigenically specific (Chaudiere, H., 1964; Robert, B. et al, 1964). The ability of antibody to fix complement (Taranta, H. et al, 1961; Amiraian, K. et al, 1961) to fix to skin (Ovary, Z. et al, 1961) and to cross placental membranes (Brambell, F., 1960) has, to date, only been generally localized to the major carbohydrate containing polypeptide chains of the immunoglobulins.

As a result of the deepening confusion in the nomenclature of the major classes, subclasses, chains and proteolytically produced fragments of the immunoglobulins, standardization of terminology was necessary. In 1964, a committee convened by the World Health Organization drew up a series of recommendations which have since been accepted by the majority of investigators in the field. The major

classes designated by the committee to replace the many synonyms being used were respectively:

Synonyms	Proposed Usage
γ, 7sγ, 6.6sγ, γ_2, γ_{ss}	IgG, γG
B_2A, γ_2A	IgA, γA
γ_1M, B_2M, 19sγ, γ Macroglobulin	IgM, γM
----	IgD, γD (Rowe, D., Fahey, J., 1964)

IgG comprises about 90% of all the serum immunoglobulins, IgA about 7% and the rest consists primarily of IgM. All of the three major classes contain common antigenic sites (Franklin, E. C. et al, 1957; Heremans, J. F. et al, 1960). These sites probably determine the species specificity. In addition, there are class (Franklin, E. C., 1960) and chain specific antigenic sites which compose the minor antigenicities of the molecules. A summary of data on the three classes of immunoglobulins is given in Table I (Press, E. M., Porter, R. R., unpublished).

IgG, the most prevalent and most studied of the immunoglobulins has provided the basis for most current speculations on the structure of these groups of proteins. In 1959, Edelman (Edelman, G. M., 1959) demonstrated that reduction of human IgG in 6 molar urea led to a fall in the molecular weight to about one third that of the starting material. Similar findings were soon extended to other species

(Edelman, G. M. and Poulik, M. D., 1961; Phelps, R. A. et al, 1961; Franek, F., 1961; Ramel, A. H. et al, 1961). This reductive cleavage of an estimated 7-9 disulfide bonds in human IgG produced two polypeptide fragments which were resolved by starch gel electrophoresis in 8M urea-formate pH 3.5 and on carboxymethyl cellulose in 6M urea. The first peak on the carboxymethyl cellulose represented the smaller component with an estimated molecular weight of 17,000 and corresponded to the faster migrating band by starch gel electrophoresis. The second peak, corresponding to the several slower migrating starch gel bands, had an estimated molecular weight of 103,000. Partial reduction of IgG in mercaptoethanol gave similar results. No appreciable amount of diffusible peptides was released nor was there any evidence of the loss of carbohydrate following the complete reduction of human IgG. No effect on the molecular weight of fraction II starting material could be shown after the addition of streptokinase in an attempt to activate any contaminating plasminogen (Edelman, G. M., Poulik, M. D., 1961). These findings and the corroboration of these results in other species gave evidence that all 7s gamma globulins have the same number of chains per molecule and that some of the N-terminal amino acids must be similar or unreactive.

It was subsequently shown that milder reduction of IgG (rabbit) with 0.2M mercaptoethanol, pH 8.0, would cleave five

disulfide bonds with no change in either the molecular weight or its precipitating ability as antibody. If, however, these ten sulfhydryls (the products of the reductive cleavage) were blocked with iodo-acetamide, then dialysed against n-acetic acid, or n-propionic acid, two components could be recovered after chromatography on sephadex G-75 in n-propionic acid (Porter, R. R., 1962; Fleischman, J. B. et al, 1963). These two components were called A and B (Porter, R. R., 1962) and had molecular weights of 50,000 and 20,000 respectively (Pain, R. h., 1963). Their behaviour on starch gel during electrophoresis in acid urea closely paralleled that of the Heavy and Light components described by Edelman from totally reduced IgG (Edelman, G. M. and Benacerraf, B., 1962).

These preliminary findings with mildly reduced IgG in addition to earlier work with papain hydrolysis (Porter, R. R., 1959) led to the postulation of a four chain structure for IgG (Porter, R. R., 1959) (see Figure 1). Further investigation has virtually confirmed these speculations as well as elucidated some important features of this four chain structure (Fleischman, J. B. et al, 1963; Cohen, S., 1963 a, b; Weir, R. C., 1964).

Studies on enzymatic hydrolysates of IgG have provided a great deal of information on the molecular structure and have provided a means of localizing several of the known biological activities of

the molecule. Most notable of the enzymatic digests of IgG is that by papain , which in the presence of cysteine cleaves the IgG into three components I, II and III, (Porter, R. R., 1959; Edelman, G. M. et al, 1960; Stiehm, E. R. et al, 1960; Fahey, J. L and Askonas, B. A., 1962; Hsiao, S. and Putnam, F. W., 1961) with a concomitant release of 8-10% of diffusible glycopeptides (Robert, B. et al, 1965). The cysteine, essential for the reaction, has been shown to preactivate the proteolytic activity of the papain as well as to reduce the disulfide bond which exists between two pieces I or II. Piece III which is released simultaneously by this reduction appears to be non-covalently linked to piece I and II until the latter fragments are separated (Cebra, J. J. et al, 1961). Pieces I and II, each with a molecular weight of 42,000, are considered to be identical in their general structure (Palmer, J. L. et al, 1962), but differ in charge. This difference in charge is expressed by differences in electrophoretic mobility (Palmer, J. L. et al, 1962). The correspondence between the two fragments of the partial reduction of IgG and the three fragments of papain-cysteine treatment was elucidated by comparing the minor antigenicities of the molecule. (Fleischman, J. B. et al, 1962: Fleischman, J. B. et al, 1963; Olins, D. R. et al, 1962; Cohen, S., 1963a). The above findings can be best appreciated by referring to Figure 1.

Efforts to localize the biological activities of IgG in terms of the components produced by partial reduction or by papain-cysteine hydrolysis have revealed much about the functional properties of the whole molecule. By a variety of techniques, the antibody site has been shown to be contained in pieces I and II (Porter, R. R., 1959; Karush, F., 1959; Nisonoff, A. et al, 1960a, Velick, S. F. et al, 1960) although it still remains unclear whether both the A and B components are essential for the complete activity. Complement fixation is a complex reaction requiring the participation of both pieces I or II and III (Schur, P. M. and Becker, E. C., 1963). The ability of IgG to fix to skin (Ovary, Z. et al, 1961) and to pass through the placental membranes (Brambell, F. R. et al, 1960) is attributed to structural features of piece III. Piece III also contains the major species specific antigenic site (Porter, R. R., 1959; Goodman, J., 1964). Group specific (allotypic) antigenic sites are associated with pieces I and II (Kelus, A. et al, 1960; Dubiski, S. et al, 1961; Leskowitz, S., 1963). The fact that these hydrolyzed and reduced pieces of the original molecule maintain biological activity is remarkable and has been interpreted to mean that the proteolytic cleavage site is quite small, leaving the major portion of the molecule unaffected (Porter, R. R., unpublished).

Recently, it has been shown that IgG releases a significant amount of diffusible peptides and glycopeptides with up to a 46% loss

of sialic acid from the non-diffusible fraction following dialysis at $4^{\circ}C$ (see Table II; Robert, B. et al, 1965). This liberation of peptides is temperature dependent; the quantity released at $37^{\circ}C$ greatly exceeds that at 4-$6^{\circ}C$ (see figure 2). If the IgG is preheated to $60^{\circ}C$ for 40 minutes, this liberation process is virtually halted. A similar arrest of this process can be effected by the addition of epsilon-aminocaproic acid or parachloromercuribenzoic acid to the dialysis solutions (Bockman, R. et al, 1965). On the basis of these findings, it has been postulated that proteolytic enzymes accompanying the IgG through the various preparative and purification procedures are responsible for degrading the IgG during dialysis or storage with the subsequent release of small peptides and glycopeptides (Robert, B. et al, 1965a). In the present paper, further evidence will be presented in support of the latter hypothesis. Efforts to characterize the enzymes involved will be described and discussed. In addition, several interesting chemical, immunological and biological activities of the diffusible fraction will be described in as much detail as is possible.

II. MATERIALS AND METHODS

A. Materials: Immunoglobulins:

Human and bovine Cohn Fraction II were purchased from Mann Biochemicals, New York; Nutritional Biochemicals, Cleveland, Ohio; Armour and Company, Chicago, Illinois and Squibb, New Brunswick, New Jersey. Rabbit Cohn Fraction II was purchased from Pentex, Kankakee, Illinois. Human IgG Lot #208 came from Immunology Incorporated, New York.

Human and bovine immunoglobulins were also prepared as described by Strauss (Strauss, A. J. et al, 1964) and also by the method of Kekwick (Kekwick, R. A., 1940). IgG was purified in some cases from Cohn Fraction II preparations on DEAE cellulose columns after the method of Sober (Sober, H. A. and Peterson, E. A., 1958). All blood samples were obtained under aseptic conditions. In some preparations, epsilon-aminocaproic acid was added to the freshly drawn blood, prior to the precipitating procedures.

Antisera: Antisera to human Cohn fraction II and bovine Cohn fraction II were prepared by immunizing rabbits with these antigens in Freund's complete adjuvant (Press, E. M. and Porter, R. R., 1962).

Other Proteins:

a) Thrombin (Thrombase 500) was purchased from Laboratoires de L'I. S. H., Paris, Lot #341P2.

b) Fibrinogen was purchased from the Laboratoires de Bio-Hematologie, Paris.

c) Fibrinolysin (bacterial) Lot #16877, was purchased from Koch and Lights, Colnbrook, Bucks, England.

d) Streptokinase (Veridase[R]) Lot #2200-906A, was purchased from the Lederle Company, New Jersey. This preparation contained 28 μg of dialyzable peptides/1000 units. The appropriate corrections were made in calculating peptide release when streptokinase was used as an activator.

e) Antiprotease peptide, sold in France as Zymofren[R], was a gift of Specia of Paris. This polypeptide substance is considered to be an inhibitor of several known proteases (Frey, K. K. et al, 1950). Lot #ES 1402 was used.

Synthetic substrates:

Benzoyl-L-Arginine amide, (BAA) Lot #400314 was obtained from Calbiochemicals, Los Angeles.

Tosyl-L-Arginine-Methyl ester (TAME), Batch #7492, was purchased from Koch and Lights Ltd., Colnbrook, Bucks, England.

Glycyl-L-Tyrosine amide acetate (GTAA), Lot #C2009 was obtained from Mann, New York.

Reagents:

p-chloromercuribenzoic acid (pcmb), sodium salt, was purchased from Nutritional Biochemicals, Cleveland, Ohio. Cysteine came from Koch and Lights Ltd., Colnbrook, Bucks, England. Epsilon-aminocaproic

acid was a gift of the Laboratoires Delagrange, Paris. All other reagents were analytical grade.

Dialysis Sacs:

Two kinds of tubing were employed: Visking tubing 18-23 mm in diameter, purchased from Membranefiltergesellschaft, Gottingen, Germany and Visking tubing from Sophic, Levallois, France.

B. Methods:

Dialysis rate studies were carried out at 4-6°C in the cold room and at 37°Cin a waterbath. The rate of escape of the dialyzable peptide material was estimated by the Lowry test for protein (Lowry, O. *et al*, 1950). 0.5 - 20.0 grams of IgG dissolved in saline or double distilled water were placed in dialysis sacs, which had previously been washed for several hours in double distilled water. The outer fluid was double distilled water alone, or various buffer mixtures containing 0.1M acetate-acetic acid to achieve a pH between 3-6, 0.05M veronal-HCl for a pH between 6-7, and 0.1M tris HCl for the pH range between 7-10. Whenever the dialysis experiments lasted longer than 6-8 hours, sterility tests were performed with agar plates and nutrient broth. Toluene was routinely added as a preservative to the diffusate in the experiments carried out at 37°C. The diffusate was concentrated in a rotary evaporator at 35°C, then stored at -20°C. The porosity of the dialysis sacs was calibrated by dialyzing 10 ml of a sucrose solution containing 150 mg/ml

against 250 ml of twice distilled water. Sucrose appeared in the diffusate at a rate of 125 mg/hour at 4°C (determined by the Orcinol method of Weimer, H. and Moshin, J. 1952). From 36 mg of a commercial sample of insulin, 1.5 mg escaped in the first hour under conditions identical with that of the sucrose run.

Self Digestion of the IgG was also studied by incubating 34.0 to 100 mg/ml solutions of IgG at 37° for 24 hours in the above mentioned buffer mixtures. These solutions were then treated with trichloroacetic acid (TCA) to a final concentration of 2% w/v. After centrifugation at 1000 G in a refrigerated centrifuge at 6°C, the amount of TCA soluble material was estimated by the Lowry method. A 0 minute control was kept at -20°C until the end of the experiment, at which time it was treated with TCA in a manner identical to the incubated sample and the amount of Lowry positive TCA-soluble material determined. A 0 minute value was subtracted from each experimental sample.

In order to check the presence of intact IgG in the TCA supernate, the following experiment was carried out. 300 mg of sulfate-prepared IgG in 8 ml of 0.1 M tri-HCl buffer at pH 8 was precipitated by adding 8 ml of 4% TCA and allowed to stand for one hour at 4°C. It was then centrifuged and the supernate washed 3 times with ethyl ether and

concentrated in a flash evaporater to 2 ml. The peptides were determined by the Lowry method. A solution of 1 mg/ml of this material was deposited on a 0.8% agarose plate along with the original untreated IgG against a specific rabbit anti IgG of the same species. No precipitation line was observed with the TCA soluble material. This and the evidence from the amino acid studies indicate that the TCA precipitated all the IgG.

Fibrin Plates were prepared according to the method of Astrup (Astrup, T. et al, 1952) modified in the following way: 280 mg of bovine fibrinogen was dissolved in 35 ml of a 0.1M barbiturate-HCl buffer, then filtered through medium mesh fiber glass. 0.5 ml of a 0.1% thrombin solution was added. The mixture was then poured into 9.5 x 25 cm plastic fibrin plate molds (see Figures 5 and 6) and allowed to coagulate undisturbed at room temperature. 20 μl of each sample or standard was placed directly on the fibrin. The plates were then incubated at 37° for 18 hours. The average product of the mean perpendicular diameters of the lysis plaque was recorded after this incubation. The results were expressed as percent equivalents of a fibrinolysin standard, or as percent inhibition compared to an appropriate control.

Labeling of Proteins with I^{131}. Human Cohn fraction II and bovine fibrinogen were labeled with I^{131} according to the method of Biozzi (Biozzi, G., et al, 1957). The specific activity for the Cohn fraction II preparation varied between 2,350-2,570 cnt/mn/mg protein

and for the bovine fibrinogen, between 5,670-6,580 cnts/mn/mg protein. The radioactivity was estimated on a Tracerlab Scintillation well counter employing small hemolysis tubes containing 2-5 ml of sample. The rate of escape of diffusible peptides was measured by determining the radiocativity of the diffusate at given intervals. The specific activity of the peptides was calculated by relating the radioactivity to Lowry positive material. The specific activity of the diffusible peptides could then be compared to that of the original protein, and dialysis rate curves constructed by plotting the mg of peptide released (calculated from the radioactivity measurement)as a function of time. (These calculations assume uniform I^{131} labeling of the IgG)

Enzymatically released peptides were measured according to the method of Lowry (Lowry, O.H., 1951). Ninhydrin tests for amino nitrogen were performed according to the method of Rosen (Rosen, H., 1957).

Hexoses were determined by the Primary cysteine-sulfuric acid (PCY_1) method (Dische, Z. et al, 1949).

Sialic Acid was measured according to the method of Svennerholm (Svennerholm, L., 1957).

The rate of release of sulfate from the sulfate precipitated preparations was measured on a Societe Biolyon C-60 conductimeter, and compared to the appropriate standards.

The rates of hydrolysis of the synthetic substrates BAA and GTAA were determined by the alcohol titration method as given by Davis

(Davis, N. C. and Smith, E. L., 1955), modified in the following way: 1.0 ml of 0.1-0.125 M of BAA or GTAA was brought to 1.5 ml with the appropriate buffer (see Methods on Self Digestion), the tube containing the substrate was then placed in a constant temperature water bath at 37°C for 3-5 minutes, 1.0 ml of the sulfate-precipitated IgG or Cohn fraction II preparation was added and the mixture incubated at 37°C with constant agitation. An initial sample of 0.2 ml was withdrawn immediately to which was added 1.8 ml of absolute alcohol. This initial sample and the subsequent samples were allowed to stand for ten minutes in the cold after which they were centrifuged at 1000 g for 10 minutes. 1.0 ml of the clear supernate was placed in a separate tube with two drops of a phenophthalein indicator, 1.0% phenophthalein in 95% alcohol, and was titrated with 0.019 M KOH in 95% ethyl alcohol. Constant mixing was achieved by bubbling nitrogen gas through the solution. Additional aliquots of the incubation mixture were withdrawn at appropriate intervals and titrated in the same way. The rate of hydrolysis of TAME was estimated spectrophotometrically (Roberts, P., 1958).

Immunoelectrophoresis was performed according to the methods of Grabar (Grabar, P., et al, 1960).

Paper Chromatography was carried out on Whatman #1 paper using phenol and water (8:2) as the solvent of migration. The ninhydrin

stain for amino acids was composed of a 0.5% ninhydrin solution in water-saturated butanol, which was sprayed on the paper following the migration. A periodic acid stain was used to reveal the glycopeptides according to the method of Gordon (Gordon, H. T., 1956).

Passive hemagglutination was performed according to the method described by Halpern (Halpern, B. N. et al, 1961) with rabbit erythrocytes coated with IgG and rabbit anti-IgG sera. Inhibition of the agglutination reaction by peptides derived from IgG was tested in the following manner: a dilution of the antiserum, two times that pretested to give a strong agglutination was made, 0.25 ml of this dilution was placed in several holes in the plaque. 0.25 ml of various dilutions of the peptides to be tested was added and thoroughly mixed. After 10 to 15 minutes, the sensitized red cells were added and the results were read 2 to 18 hours later. Failure to agglutinate was interpreted as an inhibition.

Augmentation of vascular permeability was performed according to the method of Davies (Davies, G. E. and Lowe, J. S., 1961). 0.5 ml of a 0.5% solution of Evan's blue dye was injected intravenously into each guinea pig. After 5 minutes 0.1 ml of various concentrations of the peptides or IgG, previously made isotonic, was injected intradermally into the shaved dorsal skin. Fifteen minutes after the initial injection, the animals were exsanguinated, their skin removed, and the size of

the blued spot at the site of injection estimated after the skin had been pressed against a glass plate.

Amino acid analysis was carried out on a Beckman Model 120B auto analyzer with an automatic integrater. The peptide samples were hydrolyzed with 6N HCl for 24 hours prior to analysis.

Preliminary purification of the diffusible peptides was carried out on a DEAE-Sephadex G 50 column using a gradient elution of pyridine-acetate 0.008 - 1.0 M pH 7.5. Sixty mgm of peptide material obtained from incubation and TCA precipitation was placed on a 1.5 x 40 cm column, with a flow rate of 3 ml per 15 minutes.

III. RESULTS

A. Rates of release of diffusible peptides from dialyzing IgG preparations:

With freshly precipitated IgG, it is possible to measure the rate of diffusion of small peptides and glycopeptides released during dialysis. At a dialysis temperature of $4^{\circ}C$ to $6^{\circ}C$ this rate was quite low, being for the sulfate-precipitated preparations tested about 0.139 mg peptide/g IgG/24 hours. At $37^{\circ}C$ the rate is much higher, giving values from 2.8 to 3.75 mg peptide/g IgG/24 hours (see Figure 2). These experiments were carried out at a pH of 8.6 and are described in more detail in a previously published work (Bockman, R. et al, 1965). When a similar

dialysis was carried out at pH 7.6, the rates of release were somewhat higher, being up to 4.25 mg peptide/g IgG/24 hours at a dialysis temperature of 37°C. The estimated energy of activation calculated from the following formula:

$$E = 2.3\ RT\ \frac{\log K_1 - \log K_2}{1/T_1 - 1/T_2}$$

with K_1 and K_2 derived from the slopes of the curves at the two different temperatures, is about 15 K Cal.

The dialysis of Cohn fraction II preparations under similar conditions revealed significantly lower rates of release of diffusible material. At 4° C this rate was 0.09 mg peptide/g protein/24 hours, while at 37°C the rate was 1.25 mg peptide/g protein/24 hours. The estimated energy of activation for this system is also about 15 K Cal.

Trichloroacetic acid precipitation of previously incubated IgG preparations greatly augmented the yields of peptide material. Up to 15 mg peptide/g human IgG were recovered after incubation at 37°C for 24 hours at pH 7.9. More than 32.0 mg peptide were released by a bovine preparation under exactly the same conditions. Both preparations were obtained by the sulfate-precipitation method. Cohn fraction II preparations gave much lower yields, 2.7 to 3.0 mg peptide under similar conditions of incubation. Unlike the sulfate preparations, the Cohn fraction II preparation yielded more peptide material after the addition of streptokinase to the incubation mixtures at pH 8.

B. Rate of release of peptides from several external substrates:

For the purpose of studying the specificity of the proteolytic activities of the IgG preparations, several external protein substrates were tested. In one set of experiments, I^{131} labeled human Cohn fraction II was mixed with sulfate precipitated bovine IgG and the rate of release of diffusible, labeled peptides was measured during the course of dialysis against twice distilled water at pH 7.8 with a few drops of ammonium formate added as buffer. Under these experimental conditions, an initial rate of release of 3.07 mg peptide/g total protein/24 hours was achieved for the first ten hours of dialysis, after which the rate fell to 2.59 mg peptide/g protein/24 hours (curve #3 in Figure 3). If the sulfate preparation was heated to 60°C for 45 mn prior to being mixed with the I^{131} labeled human Cohn fraction II, a steady rate of 1.73 mg peptide/g protein/24 hours was obtained (curve #2). A control sac containing only I^{131} labeled human Cohn fraction II showed a six-hour lag in the release of labeled peptides, then released labeled material at a rate of 1.68 mg peptide/g protein/24 hours (curve #1). The labeled peptides that were recovered showed a specific activity of 2600 cnts/mn/mg peptide, which was identical to that of the starting mixture. This could be interpreted to mean that the mixture of human and bovine IgG behaved as a homogenous pool of substrate protein for the accompanying proteases. Similar experiments were carried out with

I^{131} labeled bovine fibrinogen as substrate and a sulfate-precipitated bovine IgG preparation as the source of enzyme. The results closely parallelled those of the previous experiment in that the straight mixture released labeled peptides at a rate of 0.270 mg peptide/hour, while the mixture containing the heated IgG released peptides at a rate of 0.107 mg peptides/hour, less than half that of the unheated mixture. These results would indicate that the proteolytic activities of IgG preparations were capable of attacking heterologous IgG as well as other groups of proteins. Further, this activity could be greatly retarded by heating to 60°C for 45 minutes.

C. pH dependence of proteolytic activity:

Estimation of the proteolytic activity as a function of pH was determined by the TCA-precipitation technique. IgG preparations were incubated at 37°C for 24 hours in acetate, veronal and tris buffers covering a wide range of pH values. Following the incubation, the IgG was precipitated with TCA, at a final concentration of 2% w/v, and the quantity of TCA soluble material estimated by the Lowry test for protein. A separate zero minute value was determined for each pH studied and was subtracted from the experimental (incubated) value. Figure 4 shows the results from such experiments. It is evident that there are at least two sharp pH optima, one sharp peak at pH 7.9 the other at pH 4; the latter was evoked only in the presence of cysteine.

D. Inhibitors:

Several well known enzymatic inhibitors were subsequently tested in an attempt to obtain more information on the nature of these proteolytic acitvities. The results of these experiments, using the incubation-TCA precipitation technique as the test system are presented in Table III. Relatively high concentrations of epsilon-Aminocaproic acid were found to be necessary to give significant inhibition. At pH 8, 0.1 M epsilon-aminocaproic acid produced a 41.4% inhibition, while 10^{-3} M p-chloromercuribenzoic acid yielded only a 16% inhibition. These values are considerably lower than those reported using the dialysis rate technique as the test system (Bockman, R. et al, 1965). No inhibition by epsilon-aminocaproic acid was observed in the cysteine activated preparation at pH 4.

Similar tests were carried out with the fibrin plate method. Estimates of the inhibition were obtained by comparing the area of lysis produced by the mixtures of IgG with epsilon-aminocaproic acid or the antiprotease peptide to the appropriate standards. These results are presented in Table IV. These data show that 4000 moles of epsilon-aminocaproic acid per mole of IgG were required to effect a 100% inhibition. Much less of the antiprotease peptide was necessary to produce the same result.

Heating of the IgG preparations at 60°C for 45 mn has previously

been mentioned to diminish its intrinsic proteolytic activity. Up to 1.72 mg of a heated sulfate preparation failed to produce any detectable lysis on the fibrin plates. Similar results were obtained when the trichloroacetic acid precipitation method was used to study heat inactivation. Table V shows the results of several such experiments. 75 to 100% inactivation was obtained by heating for 40 minutes at $60^{o}C$, whether the activity was measured at pH 3.8 or at pH 7.8, in the presence or in the absence of cysteine. This table also shows that the addition of cysteine did not increase the peptide yield at pH 7.8; in fact, in several experiments a decrease was noticed at this pH when cysteine was added (Robert, B. et al, 1967).

E. Activators:

As was made evident in the pH dependence studies (see Figure 4), the low pH activity could only be evoked by the presence of cysteine. A sulfate-preparation of bovine IgG when incubated alone at $37^{o}C$ for 24 hours at pH 4 was found to release 0.235 mg TCA soluble peptides/g IgG. In the presence of 0.005 M cysteine, and under the same conditions a similar preparation released 5.4 mg of TCA soluble peptides/g IgG after subtracting the contribution due to cysteine. A human Cohn fraction II preparation when treated in the same way released 0.38 mg TCA soluble peptides/g protein in the absence of cysteine and 2.3 mg TCA soluble peptides/g protein when cysteine was present during the incubation period.

Particularly strong activation of the alkaline proteolytic activity could be evoked by the addition of streptokinase to several of the preparations tested. Striking increases in the fibrinolytic activity were manifested by human, bovine and rabbit Cohn fraction II preparations as well as a commercially purified human IgG preparation following the addition of streptokinase (Figure 5). A semiquantitative estimate of the preoteolytic activity was obtained by using a bacterial fibrinolysin at different concentrations as a comparative standard on the same plate as the samples (see Figure 5). A double log curve (i.e. log of the concentration expressed in percent of the fibrinolysin standard versus the log of the area of lysis) provides a means of describing the proteolytic activity of IgG preparations on fibrin in terms of fibrinolysin equivalents. A typical sulfate IgG preparation, measured under these conditions produced a lysis plaque equivalent to 3.2 mg of fibrinolysin/g IgG in the absence of streptokinase and 11.5 mg fibrinolysin/g IgG in the presence of streptokinase. A human Cohn fraction II preparation showed no lytic activity by itself, but demonstrated in the presence of streptokinase a fibrinolytic activity equivalent to 14.0 mg fibrinolysin/g sample. This same activator accelerated the rate of hydrolysis of one of the synthetic substrates by a Cohn fraction II preparation; this is discussed in the next section.

F. Synthetic Substrates:

Employing the alcohol titration technique or a spectrophotometric method, as described in the section on methods, the rates of hydrolysis of several synthetic substrates by various preparations of IgG were estimated. It was found that at pH 5, and in the presence of cysteine, BAA was rapidly hydrolyzed both by a sulfate precipitated IgG as well as by a human Cohn fraction II preparation. Very little activity was manifested at the higher pH values. GTAA proved to be a poor substrate and was only slowly hyrolyzed by the Cohn fraction II preparation in the presence of cysteine. TAME proved to be a good substrate for both IgG preparations at pH 9, with some augmentation of the activity of the Cohn fraction II preparation being evoked by the addition of streptokinase during the incubation period. See Table VI for the composite results.

G. Peptide Analysis:

Concomitant with the studies to characterize the enzymatic activities of the various IgG preparations, work was begun to elucidate the character and composition of the diffusible material. Chromatography on paper was carried out to study the gross composition of the products. The physical and chemical as well as biological properties were examined. These results are only preliminary and can only be briefly presented.

Early studies performed on the diffusible material recovered from dialyzing bovine Cohn fraction II material at pH 6.0 revealed from 5 to 10 ninhydrin spots, two of which were shown to be glycopeptides. When these glycopeptides were isolated by preparative chromatography and hydrolyzed in 2N HCl at 100° for 2 hours then resubjected to chromatography, galactose, mannose, glucose and fucose were identified. After hydrolysis in 6N HCl at 105° for 16 hours, thin layer chromatography revealed 5 to 6 amino acid spots which were identified as leucine, glycine, alanine, lysine and aspartic acid (Robert, B. et al, 1965).

An extremely rough estimate of the average size of the peptide material recovered after dialysis of bovine IgG at pH 8 was attempted by comparing the relative amounts of Lowry positive material to ninhydrin positive material. This value turned out to be about 500. This is probably greatly below the average weight for this material with the error resulting from the heterogeneity of the peptide population as well as the presence of free amino acids. Large quantities of tyrosine have been reported to be released during the dialysis of IgG (Zakrzewski, K., personal communication), although no such findings were noted by others (Robert, B., 1965). A preliminary ultracentrifugation run of a human Cohn fraction II preparation incubated at pH 8, revealed trimers and dimers as well as undegraded IgG and some material with a molecular weight of 2000. This latter material is presumed to be the diffusible peptide material.

The primary cysteine sulfuric acid test, corrected for the fucose contribution and calculated with galactose as an internal standard was used to estimate the hexose content of these diffusible and TCA soluble glycopeptides. Employing this technique, the material obtained by dialysis at pH 7.8 could be shown to contain up to 7.05 mg hexose/100 mg peptide. For the TCA soluble material, glycopeptides recovered after incubation at pH 4 and in the presence of cysteine could be shown to contain up to 6.1 mg hexose/100 mg peptide, while the material obtained after incubation at pH 8 contained only 1.74 mg hexose/100 mg peptide. In all these samples, the amount of hexose present was so low that the significance of the data awaits further study. If prior to dialysis, human Cohn fraction II was incubated with trypsin, chymotrypsin or papain, the amount of Lowry positive material released could be greatly augmented (i.e. up to 8% of the original material), but never did the hexose-to-peptide ratio approach the value obtained for the peptides recovered after dialysis alone (Robert, B., unpublished). This is interpreted as an augmentation of the proteolytic attack on the molecule resulting in a hydrolysis deeper into the molecular chain beyond the carbohydrate moiety to the adjacent amino acids. This represents a more profound hydrolysis than that which spontaneously seems to result when IgG is dialyzed or stored.

Sialic acid determinations on the peptide material released after dialysis showed only trace amounts to be present. Measurements on the non-diffusible fraction after dialysis demonstrated losses of up to 46% of the sialic acid compared to the starting value (Robert, B., et al, 1965). It is believed that much of the free sialic acid is destroyed during the evaporation procedures (Dische, Z. personal commun.).

A preliminary effort to effect a separation of the trichloroacetic acid soluble peptides obtained after incubation of human IgG in the presence of cysteine was attempted on DEAE-Sephedex G-50. The effluent pattern revealed one large peak with a trailing shoulder, followed by a small wide peak. Amino acid analysis revealed the following composition for the major peak: (Asp_4 Thr_4 Ser_8 Glu_4 Pro_4 Gly_8 Ala_2 Cys_{18} Leu) with the shoulder and the broader peak being smaller fragments of the major peak. None of the peaks resembled the starting intact IgG. The more acidic amino acids were not studied.

H. Biological Activity of the Peptides:

Turning from the chemical properties of these peptides, several interesting observations were made on their biological properties. It was discovered that the diffusible material released from dialyzing bovine IgG, when injected into the dorsal skin of guinea pigs, would greatly increase the vascular permeability at the site of injection. This effect was shown to be concentration dependent. The injection

of bovine IgG also increased the vascular permeability at the injection site; this finding was similar to that already described with IgG of another species (Davies, G. E. et al, 1961). The ability of bovine IgG to augment vascular permeability was not effected by the injection, im. of 0.4 mg mepyramine, 30 minutes before the skin test, or by 5000 units of the Antiprotease peptide injected ip. 10 minutes before the skin test was made (Halpern, B. N. et al, unpublished); see Table VII.

I. Immunological Studies of the Peptides:

Perhaps the most intriguing property of these peptides is their ability to inhibit the reaction between IgG and its specific antiserum, as tested in the passive hemagglutination system. For example, 0.37 μg of peptide material, recovered during the dialysis of bovine IgG at pH 8.0, was able to stop the reaction between red cells coated with bovine IgG and a specific rabbit antibovine IgG serum. When the TCA soluble materials were tested in this same system, the peptides which had been released after incubation at pH 4 in the presence of cysteine were able to inhibit at a concentration of 2.8 μg. The TCA soluble peptides released after incubation at pH 8 could inhibit at a concentration of 1.0 μg. The inhibition effected by the dialysis produced peptides has been shown to be species specific in that these peptides inhibited the reaction between their parent molecule and its specific antiserum, but virtually not at all the reaction between heterologous

IgG and the specific antiserum to it (Robert, B. et al, 1965). Inasmuch as the inhibition produced by the peptides is much more specific than that produced by the entire parent molecule, the possibility of contamination of peptide material by the parent molecules as the mechanism of this activity is unlikely (Robert, B., Unpublished). Additional evidence of the absence of intact IgG molecules in the diffusate, or TCA soluble peptides, is the failure of the peptide materials to produce a line on Ochterlony plates with anti-IgG serum.

An immunological study of several IgG preparations following extensive dialysis gave interesting results. Immunoelectrophoresis of three sulfate preparations following 200 hours of dialysis at 4°C and 37°C with the release of up to 14 mg of peptide/g IgG revealed that no changes occurred in the immunoelectrophoretic precipitin line. However, significant changes in the antigenicity of the molecules are known to occur following dialysis. One such alteration was manifested by the change in the precipitation curves produced by these dialyzed samples; there was a marked flattening and a shift to the right in comparison to the curves produced by undialyzed samples (Robert, B., 1964). This loss in antigenicity was made much more evident if previously dialyzed samples were stored as a solution. This loss of specificity, or antigenicity, which occurs during dialysis and during storage probably represents a continual phenomenon, that of the degradation of IgG preparations by its accompanying proteases.

IV. DISCUSSION

From the data presented here, and in earlier publications (Robert B. et al, 1965, and 1965a), there can be little doubt that a great number of IgG preparations, commercial as well as laboratory-prepared, release diffusible peptides upon incubation and storage. Several machanisms could be responsible for this release, for example: the diffusion of low molecular weight material absorbed during the purification procedure, the splitting of peptides by some non-enzymatic reaction, and finally, the cleavage of peptides by accompanying proteolytic enzymes.

The first possibility is unlikely because of the kinetics of release (Bockman, R. et al, 1965). These kinetics reveal graphically purified IgG. The rate of release of diffusible peptides at 4°C and 37°C gives an activation energy value of about 15 Kcal/mole, which is of the same order of magnitude as those found for other proteases (Laidler, K., 1958). Such a high value argues against the possibility that peptide release could represent the mere freeing of peptides trapped in the IgG preparations.

Several non enzymatic mechanisms, such as SH-S-S interchange with the rupture of labile bonds may have yielded peptide material. The inhibition by pcmb acid of peptide release at pH 8 could be taken as an argument in favor of a Sh-S-S exchange mechanism. The pcmb

acid having reacted with the SH groups of the IgG prevented them from interrupting essential S-S bridges. Against such a SH-S-S exchange mechanism is the finding that the addition of cysteine did not increase peptide release at pH 8. In fact, the addition of cysteine decreased peptide yield at pH 8. This is especially interesting since it has recently been shown that the enzyme plasmin, of which more will be said, is greatly inactivated following the reduction of some of its S-S bridges (Robbins, K. et al, 1966). Hong and Nisonoff (Hong, R. et al 1965) have reported the appearance of half molecules with the release of light chains equivalent to 5% of the total protein following the incubation of IgG at pH 8 in the presence of mercaptoethanol. Such an amount of peptide material would have easily been detected in our experiments. The findings in the present paper would indicate that such material was either precipitated with the TCA, did not pass through the membrane, or that such splitting did not occur under these experimental conditions. There is no doubt that the addition of cysteine greatly augmented peptide release at pH 4, but this activity is probably not explained by the reduction of S-S bonds. Such a conclusion is based on the strong inhibition of peptide release after heating of the IgG and from hydrolysis rate studies done on synthetic substrates at the acid pH. Other experimental work has shown the decreased reducibility of S-S bonds in IgG at acid pH (Hong, R., Nisonoff, A., 1965).

So, it would appear that the splitting of S-S bonds was not essential for the release of peptide material; however, it can not be excluded that such a reaction could have contributed to the production of a preferential substrate for the enzymes.

The most probable explanation for these results would be the presence of proteolytic enzymes in the IgG preparations. The enzymatic nature of peptide release is shown by its kinetics, the action of IgG on external as well as synthetic substrates, the action of inhibitors and activators, as well as the inactivation of IgG preparations after heating at 60°C for forty minutes. On the basis of data presented here, the nature of the proteases involved can be more clearly visualized than before.

The alkaline proteolytic activity seems from all the experimental data to act very much like plasmin. This conclusion is founded on the following criteria: first, that the pH optimum is within the range of that for plasmin (Christensin, L. R. et al, 1945), second, that the samples would hydrolyze fibrin and the synthetic substrate TAME at an alkaline pH, both being good plasmin substrates (Christensen, L. R. et al, 1945; Troll, W. et al, 1954); third, that epsilon-aminocaproic acid, a known plasmin inhibitor (Ablondi, F. B. et al, 1959; Alkjaersig, N. et al, 1959) interfered with the degradation process. The effect of streptokinase, a known activator of plasminogen (Christensen, L. R. et al, 1945), may be put forward as further evidence for the presence of this enzyme-precursor system. As has already been mentioned, the decrease in peptide yield

following the addition of cysteine at pH 8 is of interest in that it has recently been shown to diminish the activity of plasmin by reducing some of its S-S bridges. The possibility of the presence of plasmin in sulfate preparations of IgG has already been noted (Skvaril, F. et al, 1962). It is of interest to note that further purification of the IgG preparations by Cohn fractionation or chromatography diminished and even abolished the spontaneous proteolytic activity at pH 8, but the streptokinase induced activity remained quite active.

The other proteolytic activity which is manifest in the acid pH range in the presence of cysteine has been identified tentatively as being like cathepsin-B. This conclusion was arrived at on the basis of the facts that the samples would hydrolyze BAA at an acid pH only in the presence of cysteine, BAA being a specific cathepsin B substrate under these conditions (Tallan, H. H. et al, 1952). In addition, it should be noted that only in the presence of cysteine would the self degradation process occur at the acid pH range (see Figure 4). It can be seen from the latter figure, that the ratio of plasmin and cathepsin B like activities was roughly 6 to 1 in the bovine sulfate-precipitated sample tested. This ratio was inconstant and lower ratios were found for most other IgG preparations.

It has already been shown (Davies, G. E. and Lowe, J. S, 1966) that guinea pig IgG prepared by DEAE and zone electrophoresis

would hydrolyze the synthetic esters TAME and BAME but would not hydrolyze caesin or fibrin. On the basis of these and other findings, Davies and Lowe attributed this activity to a Kallifrein type of enzyme. As has already been demonstrated in the present paper, the fibrinolytic activities of Cohn fraction II preparations as well as commercially prepared IgG preparations is made manifest only after the addition of streptokinase.

Similar to the results reported by Davies and Lowe (Davies, G. E. and Lowe, J. S., 1961), the injection of bovine IgG into the shaved skin of guinea pigs greatly augmented the vascular permeability at the injection site. Of great interest was the finding that a similar augmentation of the vascular permeability could be effected by the injection of the peptide material obtained during the course of dialysis of a sulfate precipitated bovine IgG preparation, (Halpern, B.N. et al, 1965). This would mean that the proteases present in IgG preparations were capable of releasing peptide material possessing pharmocological activity.

The occurrence of a 'splitting' in the immunoelectrophoretic precipitin line following a prolonged storage of IgG has been noted (Grabar, P., 1958). Since Grabar's observation, this phenomenon has been attributed to be the result of a proteolytic process effecting IgG (Skvaril, F., 1960; 1962). Subsequent investigations (James, K. et al, 1964) have shown that dimerization of IgG molecules occurring during

incubation at 37°C is the first stage in the denaturation of the molecules. This dimerization is thought to render certain labile bonds susceptible to proteolytic hydrolysis, resulting in a major cleavage made manifest in the splitting of the immunoelectrophoretic precipitin line. These authors have shown that this splitting becomes evident after the twentieth hour of incubation concomitant with the appearance of a 5 s component. This is in contrast to the results presented in the present paper, where it was found that after 210 hours of incubation at 4° and 37°C, a release of up to 15 mg of peptides/g IgG no splitting of the immunoelectrophoretic precipitin line was observed in the several IgG preparations tested. It is thought that the phenomenon manifested by the release of diffusible peptides is different from that represented by a major splitting of the IgG chains. The degradative process described in the present paper releases glycopeptides as well as peptides; it is reasonable to assume that the source of the carbohydrate is from the major carbohydrate moiety of the IgG at the C-terminal portion of the Heavy chain. Such an assumption would imply that the hydrolysis responsible for producing the diffusible material was occurring at some site other than that hydrolyzed during papain digestion. Therefore, the hydrolysis described in the present paper would be contained within the Fc portion of the Heavy chain and would be incapable of producing major splits in the molecule.

Unfortunately, the quantity of peptides released during the degradation of IgG preparations is very small. This greatly restricts the confidence that one can have in these preliminary results on the chemical properties. The immunological data are tentative; yet, it seems unlikely that the inhibiting capabilities of these peptides can be dismissed as mere contamination by parent molecules. This inhibition could be effected with far less peptide material than parent molecules, indicating the greater specificity of the former molecules. Further, glycopeptides, purified by paper chromatography, were capable of producing a similar inhibition (Robert, B., unpublished). Also, from the incubation-TCA precipitation experiments, a more specific inhibition could be demonstrated with the peptides recovered after incubation at pH 8 than by the peptides recovered after incubation at pH 4. Surely, if it were merely a question of contamination with the parent IgG molecules, such contaminants would not be more frequent at any single pH.

The correspondence between the loss of the major antigenicity of IgG, and appearance of glycopeptides (shown to contain galactose, mannose, fucose and aspartic acid) and the ability to specifically inhibit the reaction between IgG and its specific antiserum is such that they must in fact be causually related. As a more complete hypothesis, it is postulated that the dialysis or storage of IgG results in the proteolytic release of peptides and glycopeptides from the C-terminal end of

the Heavy chains of the IgG and these glycopeptides carry the major species specific antigenic site. Previous experiments have shown that the loss of up to 46% of the sialic acid in IgG following dialysis resulted in a graded loss of antigenicity directly dependent on the quantity of sialic acid lost (Chaudiere, H., 1963; Robert, B. et al, 1965). On the basis of the current speculations the sialic acid in IgG is probably in a terminal position attached through galactose to the carbohydrate moiety which is in turn attached through aspartic acid at the C-terminal end of the A chain (Nolan, C. and Smith, E., 1962; Rothfus, J. and Smith, E., 1963; Clamp, J. and Putnam, F., 1964). This latter hypothesis would indicate that sialic acid is the index of the integrity of the C-terminal carbohydrate moiety. However, it is the adjacent glycopeptide grouping which is probably responsible for determining the major species specific site of IgG. The fact that sialic acid is not recovered with the diffusate implies that it is being destroyed during the evaporation procedures. The finding that the diffusible material is quite capable of inhibiting the reaction between IgG and its specific antiserum implies that the sialic acid plays little or no role in determining the major antigenicity of IgG.

REFERENCES

ABLONDI, F. B., HAGAN, J. J., PHILIPS, M., DeRENZO, E. C. (1959) Arch. Biophys. Biochem. 82, 153.

ALKJAERSIG, N., FLETCHER, A. P., SHERRY, S., (1959) J. Biol. Chem. 234, 832.

AMIRAIAN, K., LEIKHIM, E. J., (1961) Proc. Roy. Soc. Exp. Biol. 108, 454.

ASTRUP, T., MULLERTZ, S., (1952) Arch. Biochem. Biophys. 40, 346.

BIOZZI, G., BENACERAFF, B., STIFFEL, C., HALPERN, B. N., MOUTON, D. (1957) Annales de l'Institut Pasteur 92, 89.

BOCKMAN, R., CREPIN, Y., ROBERT, B., (1965) C. R. Acad. Sci. (Paris) t 260, 3515.

BRAMBELL, F., HEMMINGS, W., OAKLEY, C., PORTER, R. R., (1960) Proc. Roy. Soc. (London) B151, 476.

CAMPBELL, D. H., GARVERY, J. S., CREMER, N. E., SUSSDORF, D. H., Methods in Immunology, W. A. Benjamin, New York, 1963, pp. 90-95.

CEBRA, J. J., GIVOL, D., SILMAN, H. I., KATCHALSKI, K., (1961) J. Biol. Chem., 236, 1720.

CHAUDIERE, H., (1963) Etude de l'Hydrolyse Acide et Enzymatique de l'Acide Sialique de Quelques Glycoproteines, Diplome d'Etudes Superieures, Faculte des Sciences, Paris.

CHRISTENSIN, L. R., MacLEOD, C. M., (1954) J. Gen. Physiol. 28, 559.

CLAMP, J., PUTNAM, F. (1964) J. Biol. Chem. 239, 3233.

COHEN, S. (1963 a) Nature, 197, 253.

COHEN, S. (1963 b) Biochem. J. 89, 334.

DAVIES, G. E., LOWE, J. S. (1961) Immunol. 4, 289.

DAVIS, N. C., SMITH, E. L., in (Glick, D. ed.) Methods of Biochemical Analysis, Vol. II, Interscience Publishers, New York, 1955, pp. 224-227.

DISCHE, Z., SHETTLES, L. B., OSNOS, M. (1949) Arch. Biochem. 22, 164 and personal communication.

DUBISKI, S., DUBISKI, A., SCALBA, D. (1961) Immunol. 4, 236.

EDELMAN, G. M. (1959) J. Am. Chem. Soc. 81, 3155.

EDELMAN, G. M., HEREMANS, J. F., HEREMANS, M., KUNKEL, H. G. (1960) J. Exp. Med. 112, 203.

EDELMAN, G. M., POULIK, K., (1961) J. Exp. Med. 113, 861.

EDELMAN, G. M., BENACERRAF, B. (1962) Proc. Nat. Acad. Sci. U. S. 48, 1035.

FAHEY, J. L., ASKONAS, B. A. (1962) J. Exp. Med. 115, 623.

FLEISCHMAN, J., PAIN, R. H., PORTER, R. R. (1962) Sup. I, 174.

FLEISCHMAN, J., PORTER, R. R. PRESS, E. M. (1963) Biochem. J. 88, 220.

FRANEK, F. (1961) Biochem. Biophys. Res. Commun. (Prague) 4, 28.

FRANKLIN, E. C., KUNKEL, H. G. (1957) J. Immunol. 78, 11.

FRANKLIN, E. C. (1960) J. Immunol. 85, 138.

FREY, K. K., KRAUT, H., WENLE, E. in (Erike, F. ed.) Kallikrein Vol II, Stuttgart, 1950.

GOODMAN, J (1964) Biochem. 3, 857.

GORDON, H. T. (1956) Anal. Chem. 28, 849.

GRABAR, P. (1958) Adv. Protein Chem. 13, 17.

GRABAR, P., BURTIN, P., Analyse Immuno-Electrophoretique, Masson et Cie Editeurs, Paris, 1960.

HALPERN, B. N., JACOB, M., BINAGHI, R., PARLEBAS, J. (1961) Rev. Francaise d'Allergie 4, 201.

HALPERN, B. N., BOCKMAN, R., In preparation.

HEREMANS, J. F., HEREMANS, M., SCHULTZE, H. E. (1959) Clin. Chim. Acta 4, 96.

HONG, R., NISONOFF, A. (1965) J. Biochem. 240, 3883.

HSIAO, S., PUTNAM, F. W. (1961) J. Biol. Chem. 236, 122.

JAMES, K., HENNEY, C. S., STANWORTH, D. R. (1964) Nature 202, 563.

KARUSH, F. (1959) Fed. Proc. 18, 577.

KEKWICK, R. A. (1940) Biochem. J. 34, 1248.

KELUS, A., MARRACK, J. R., RICHARDS, C. B. (1960) Biochem. J. 76, 73.

LAIDLER, K. J. (1958) in the Chemical Kinetics of Enzyme Action, Table 26, p. 202, Clarendon Press, Oxford.

LESKOWITZ, S. (1963) J. Immunol. 90, 98.

LOWRY, O. H., ROSEBROUGH, N. J., FAR, A. L., RANDALL, R. I. (1951) J. Biol. Chem. 193, 265.

NISONOFF, A., WISSLER, F. C., WOERNLEY, D. L. (1960 a) Arch. Biochem. Biophys. 88, 241.

NOLAN, C., SMITH, E. (1962) J. Biol. Chem. 237, 453.

OLINS, D. R., EDELMAN, G. M. (1962) J. Exp. Med. 116, 635.

OVARY, Z., KARUSH, F. (1961) J. Immunol. 86, 146.

PAIN, R. H. (1963) Biochem. J. 88, 234.

PALMER, J. L., MANDY, W. J., NISONOFF, A. (1962) Proc. Nat. Acad. Sci. (US) 48, 49.

PHELPS, R. A., NEET, K. A., LYNN, L. T., PUTNAM. F. W. (1961) J. Biol. Chem. 236, 96.

PORTER, R. R. (1959) Biochem. J. 73, 119.

PORTER, R. R. in (Gellhorn, A. and Hirschberg, E. eds.) Basic Problems in Neoplastic Disease, Columbia University Press, New York 1962, p. 177.

PRESS, E. M., PORTER, R. R. (1962) Biochem. J. 83, 172.

PRESS, E. M., PORTER, R. R., Unpublished.

RAMEL, A., STELLWAGEN, R., SCHACHMAN, H. K. (1961) Fed. Proc. 20, 387.

ROBBINS, K. C., SUMMARIA, L. HSIEH, B., SHAH, R. (1966) Fed. Proc. 25, 194.

ROBERT, B. in (Peters, H., ed.) Protides of the Biological Fluids, Vol. III Elsevier, Amsterdam, 1964, pp. 151-152.

ROBERT, B., DENES, Y., CREPIN, Y. (1965) C. R. Acad. Sci. (Paris) t260, 734.

ROBERT, B., BOCKMAN, R., CREPIN. Y. (1965 a) Biochem. J. 95, 21p.

ROBERT, B., BOCKMAN, R. (1967) Biochem. J. 102, 554.

ROBERTS, P. (1958) J. Biol. Chem. 232, 285.

ROSEN, H. (1957) Arch. Biochem. Biophys. 67, 10.

ROTHFUS, J., SMITH, E. (1963) J. Biol. Chem. 328, 1402.

STELOS, P., RADZIMSKY, G., PRESSMAN, D. (1962) J. Immunol. 88, 572.

ROWE, D., FAHEY, J. L. (1965) J. Exp. Med. 121, 171.

SCHUR, P. M., BECKER, E. C. (1963) Science 141, 360.

SKVARIL, F. (1960) Nature 185, 475.

SKVARIL, F., GRUNBERGER, D. (1962) Nature 196, 481.

SOBER, H. A., PETERSON, E. A. (1958) Fed. Proc. 17, 1116.

STELOS, P., RADZIMSKY, G., PRESSMAN, D. (1962) J. Immunol. 88, 572.

STIEHM, E. R., MORTON, J., DEUTSCH, H. F. (1960) J. Immunol. 85, 337.

STRAUSS, A. J. L., KEMP, P. S., VANNIER, E., GOODMAN, H. (1964) J. Immunol. 93, 24.

SVENNERHOLM, L. (1957) Biochem. Biophys. Acta 24, 604.

TALLAN, H. H., JONES, M. E., FRUTON, J. S. (1953) J. Biochem. 194, 793.

TARANTA, A., FRANKLIN, E. (1961) Science 134, 1981.

TROLL, W., SHERRY, S., WACHMAN, J. (1954) J. Biol. Chem. 208, 85.

VELICH, S. F., PARKER, C., EISEN, H. N. (1960) Proc. Nat. Acad. Sci., U. S. 46, 1470.

WEIMER, H. E., MOSHIN, J. R. (1952) Ann. Rev. Tuberculosis 68, 594.

WEIR, R. C. (1964) In Press.

ZAKRZEWSKI, K. (1965) Personal communication.

TABLE I

SUMMARY OF THE DATA ON THE MAJOR CLASSES OF IMMUNOGLOBULINS*

Immunoglobulins	Approximate Molecular Weight	Approximate content in human sera g/100 ml	Approximate carbohydrate content g/100 g protein
IgG	150,000	1.3	2.5
IgM	1,000,000	0.05	10
IgA	150,000	0.10	5 - 10

* From Press, E. M. and Porter, R. R., unpublished.

TABLE II

QUANTITY OF DIFFUSIBLE PEPTIDES, THEIR HEXOSE CONTENT AND THE CHANGE IN SIALIC ACID CONTENT FOLLOWING EXTENSIVE DIALYSIS AT $0^{o}C$ OF SEVERAL IgG PREPARATIONS. *

IgG Preparation	Quantity of diffusible peptides mg/g IgG	Mg hexose per 100 mg diffusible peptides**	Percent of diffusible sialic acid***†
Fraction II[1]	0.83	26.7	10.2
Fraction II[2]	0.88	48.3	30.2
Fraction II[3]	2.6	13.6	46
Fraction II[4]	0.81	--	30.0
Fraction II[5]	4.85	--	14.7
Fraction II[6]	0.84	79	--

1) Cohn Fraction II, bovine, Armour Company, Chicago, Illinois.
2) Cohn Fraction II, bovine, Nutritional Biochemicals, Cleveland, Ohio.
3) Cohn Fraction II, bovine, Mann Biochemicals, New York.
4) Cohn Fraction II, human, Nutritional Biochemicals, Cleveland, Ohio.
5) Cohn Fraction II, human, prepared by Squibb for the American Red Cross.
6) Cohn Fraction II, rabbit, Pentex, Kankakee, Illinois.

* Taken from Robert, B. et al, 1965.
** By the method of Dische, Z. et al, 1962.
*** By the method of Svennerholm, L., 1957.

† $\frac{\text{Mg Sialic Acid before dialysis - Mg Sialic Acid in non-diffusible fraction after Dialysis}}{\text{Mg Sialic Acid Before Dialysis}}$

TABLE III

Effect of various inhibitors on the proteolytic activity of a sulfate precipitated preparation of bovine IgG incubated for 24 hours at 37° and at pH 4 with an acetate buffer, or at pH 8 with tris, the final concentration of the IgG being 34-36 mg/ml. The quantity of released peptides was determined following TCA precipitation at a final TCA concentration of 2% w/v.

pH	Activator	Inhibitor	Molar ratio Inhibitor/IgG*	mg Peptide per g IgG	Percent Inhibition
4	-	-	-	0.235	-
4	0.005 M cysteine	-	-	5.41	-
4	-	epsilon-amino-caproic acid 0.1 M	465	0.176	(Not significant)
4	0.005 M cysteine	epsilon-amino-caproic acid 0.1 M	465	6.00	0
8	-	-	-	6.34	-
8	-	epsilon-amino-caproic acid 0.1 M	440	3.72	41.4
8	-	p-chloromercuri-benzoic acid 10^{-3} M	4.4	5.34	16
7**	-	epsilon-amino-caproic acid 0,1M	25	-	95.3
7**	-	p-chloromercuri-benzoic acid 1.64×10^{-3} M	0.73	-	62.4

* Molecular weight of IgG taken as 160,000

** By dialysis rate studies (Bockman, R. *et al*, 1965).

TABLE IV

Effect of various inhibitors on the fibrinolytic activity of a sulfate precipitated bovine IgG preparation. 20.0 µl of a 2 mg % solution of IgG mixed with various concentrations of an antiprotease peptide or epsilon-aminocaproic acid was placed on fibrin plates, which were then incubated for 18 hours at 37° before being read.

Inhibitor	Concentration	Molar ratio Inhibitor/IgG*	Percent Inhibition
Antiprotease Peptide	5 units/100 mg IgG	-	0
	10 units/100 mg IgG	-	7.5
	20 units/100 mg IgG	-	18
	80 units/100 mg IgG	-	100
Epsilon-amino-caproic acid	0.01	4	18
	0.02	8	23
	0.10	40	32
	0.20	80	41
	2.00	800	61
	10.00	4000	$>$ 80

* The molecular weight of IgG as 160,000.

TABLE V*

Action of heating on the release of peptides from bovine sulfate precipitated IgG. 4 ml of IgG at a final concentration of 40 mg/ml were heated in a water bath at 60°C for 40 minutes, either in tris HCl buffer at pH 7.8, or in collidine-acetate buffer at pH 7.0. After heating at pH 7 the sample was acidified to pH 3.8 with acetic acid and the concentration adjusted to 20 mg/ml. The control experiments were performed on the same solution of IgG diluted to 20 mg/ml and kept at pH 7.8 or acidified to pH 3.8 without previous heating. The heated and unheated IgG's were further incubated at 37°C with and without cysteine. The diffusible peptides were isolated by TCA precipitation technique.

pH	Cysteine M	mg peptide per g unheated	IgG heated to 60°C for 40 min	Percent Inhibition
7.8	-	9.70	2.00	79.5
7.8	5	9.16	2.20	75.6
3.8	-	1.51	0.17	88.8
3.8	5	10.63	0	100.0

* (Robert, B. et al, 1967)

TABLE VI

Rates of hydrolysis of the synthetic substrates by a sulfate precipitated bovine IgG and a human Cohn Fraction II preparation. The hydrolysis of BAA and GTAA was estimated by titration in alcohol (See Methods). The cleavage of TAME was estimated spectrophotometrically (Roberts, P., 1958). The positive volues are given as μM substrate split/60 mn/100 mg sample.

Sample	Activator	BAA			GTAA	TAME	
		pH 5	pH 7.4	pH 9	pH5	pH 5	pH 9
IgG bovine	-	0	3.96	0.23	0	0	298.0
	Cysteine 0.005 M	136.5	-	-	0	-	-
	Strepto-kinase	-	-	-	-	-	305
Cohn fraction II, human	-	0.1	3.20	0	0	-	29
	Cysteine 0.005 M	232.0	-	-	10.5	-	-
	Strepto-kinase	-	-	-	-	-	95.0

TABLE VII

Test of the augmentation of vascular permeability. 0.1 ml of various concentrations of diffusible peptides or IgG were injected intracutaneously into the shaved dorsal skin of guinea pigs which had been previously injected intravenously with 0.5 ml of 0.5% Evans blue dye in saline. Histamine standards were injected simultaneously on the opposite side. After 10 minutes, the animals were exsanguinated and the mean diameter of the blue spot on the underside of the skin was estimated.

Sample Injected	Mean Diameter	Histamine equivalents
Diffusible Peptides		
11.5 μg	6.9 mm	(0.1) μg
23.0 μg	8.2 mm	(0.24) μg
Bovine IgG		
1.0 mg	13.5 mm	0.35 μg
2.0 mg	18.0 mm	0.90 μg
Heat Aggregated IgG		
2.0 mg	15.0 mm	0.50 μg

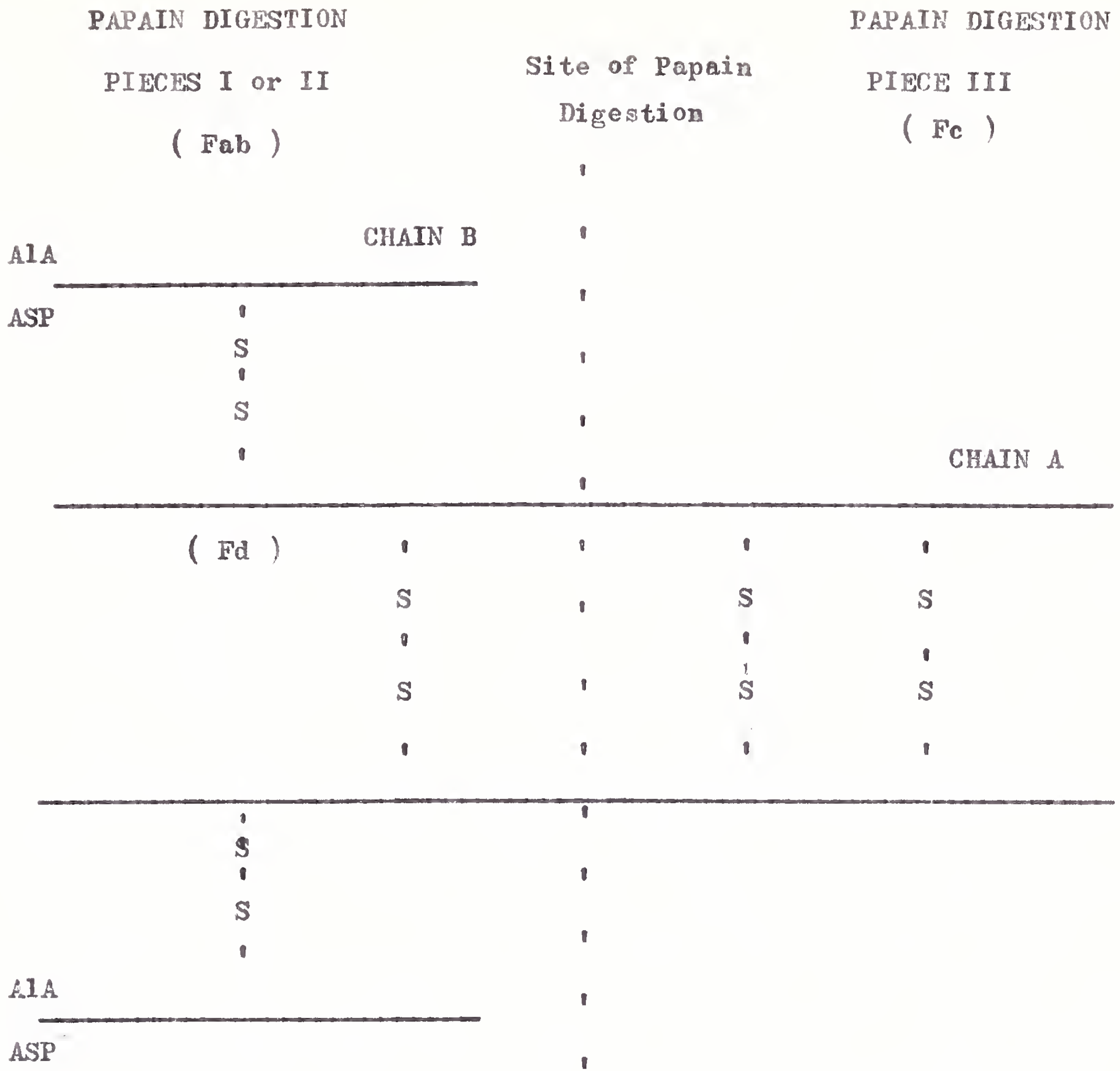

FIGURE 1. Postulated four chain structure of rabbit IgG showing the probable site of attack by papain and the resulting hydrolysis pieces (Porter, R. R., 1962).

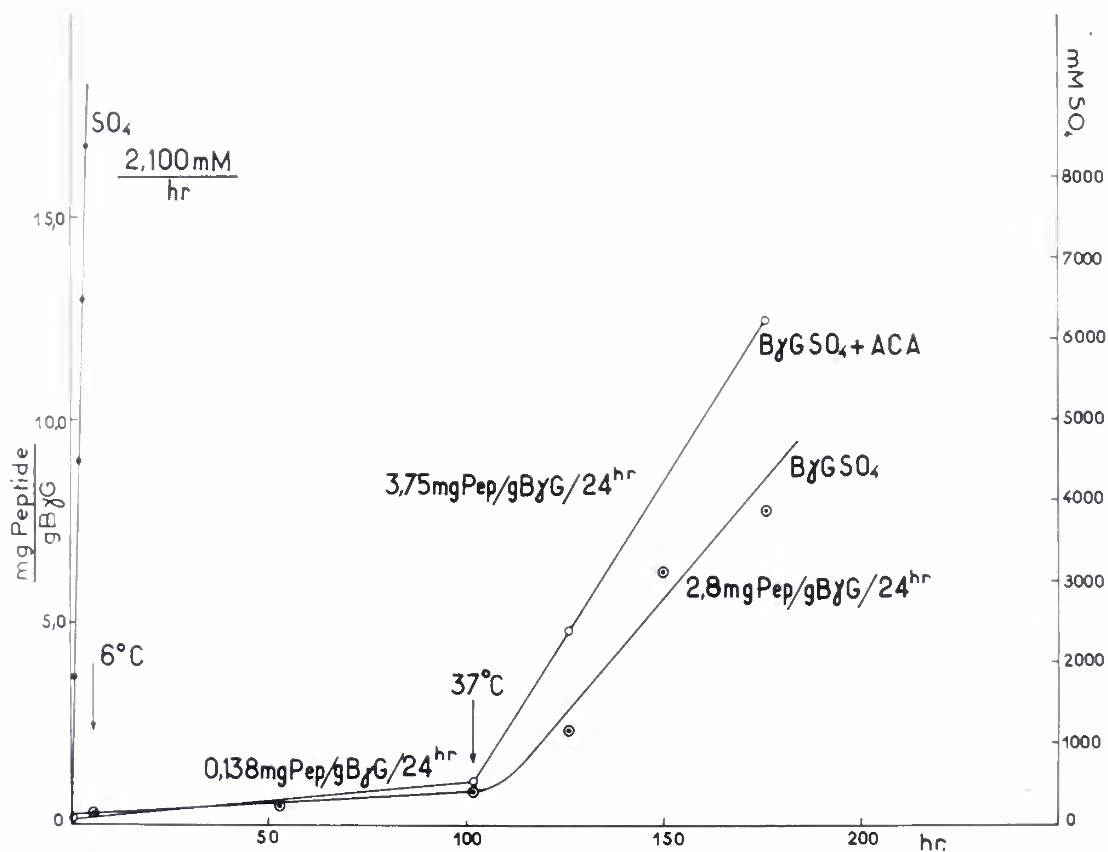

FIGURE 2. Rates of release of diffusible peptides from a bovine, sulfate-precipitated preparation of IgG dialyzed against 0.04 M tris, pH 8.6 at 4 to 6°C and then at 37°C (Bockman, R. ~~E~~. et al, 1965).

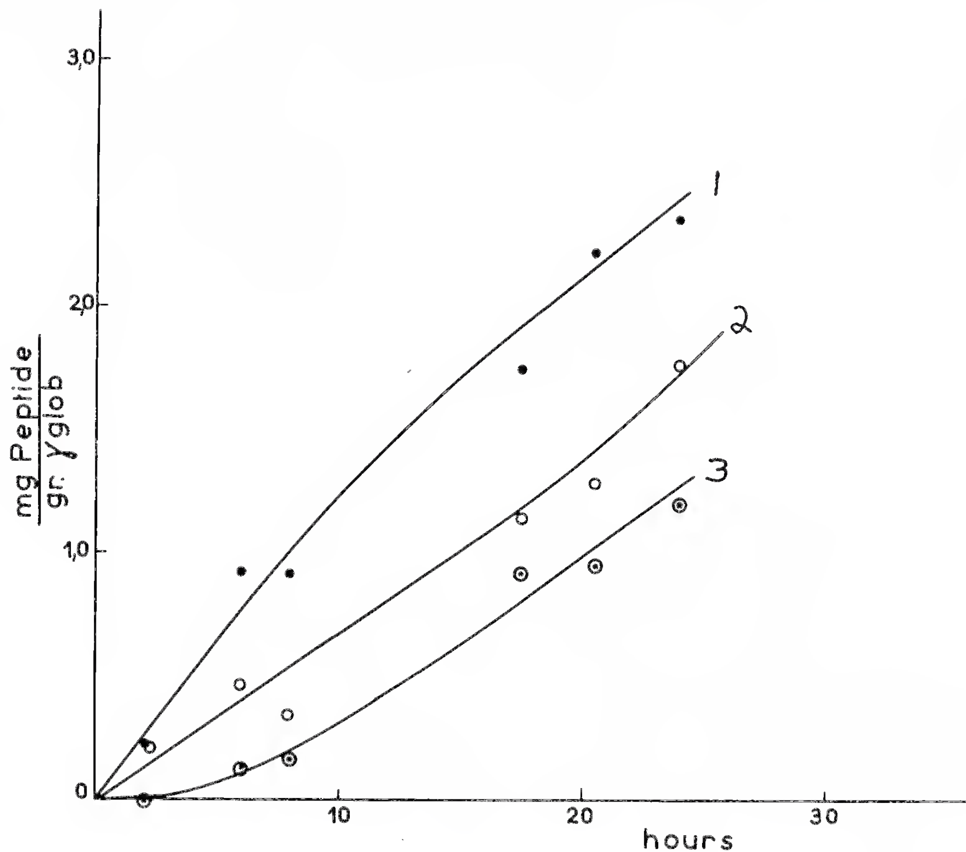

FIGURE 3. Rates of release of diffusible peptides at a dialysis temperature of 37°C, pH 7.8 with formate buffer calculated from the cnts/mn of the diffusate and the Specific Activity (S.A.) of the starting mixture. Curve #1 represents the mixture of 1225 mg of I^{131} labeled human Cohn fraction II plus 400 mg of bovine sulfate-precipitated IgG, S.A. = 2570 cnts/mn/mg protein. Curve #2 is similar to #1, however, the bovine IgG was heated to 60°C for 45 mn prior to being mixed with the labeled Cohn fraction II, S.A. = 2490 cnts/mn/mg protein. Curve #3 represents 1645 mg of I^{131} labeled human Cohn fraction II, S.A. = 2350 cnts/mn/mg protein.

FIGURE 4. [illegible] of [illegible]
65°C, [illegible] fractions [illegible]
[illegible] specific activity [illegible]
represents [illegible] of [illegible]
II plus 400 [illegible] of bovine [illegible]
[illegible] is similar to [illegible]
to 65°C for 15 min prior to [illegible]
S.A. = 24 [illegible] protein [illegible]
[illegible]

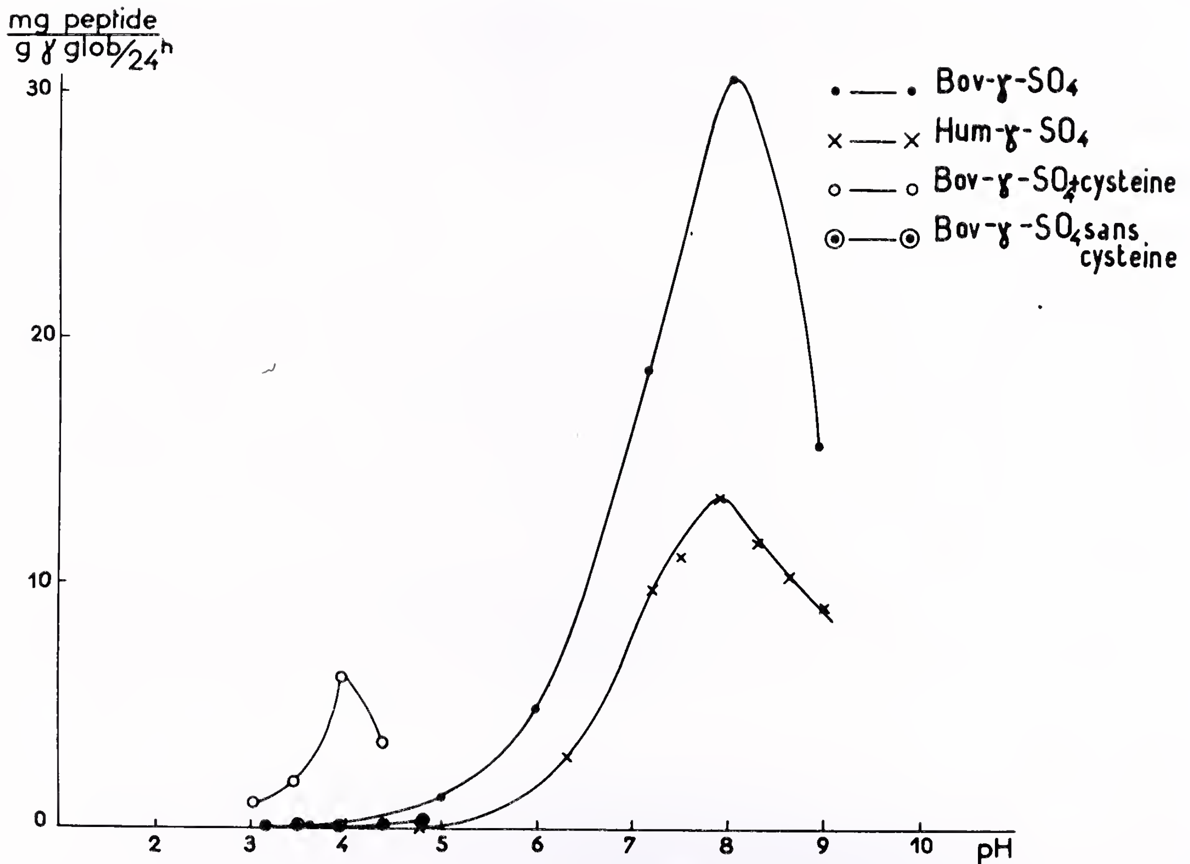

FIGURE 4. Proteolytic activity as a function of pH. Sulfate-precipitated bovine and human IgG were incubated for 24 hours at 37°C at several Ph values. After incubation, the preparations were precipitated with TCA at a final concentration of 2% w/v; the quantity of TCA soluble peptides was determined. •—• Bovine IgG; x-x Human IgG; 0-0 Bovine IgG in the presence of 0.005 M cysteine; ⊙-⊙ Bovine IgG.

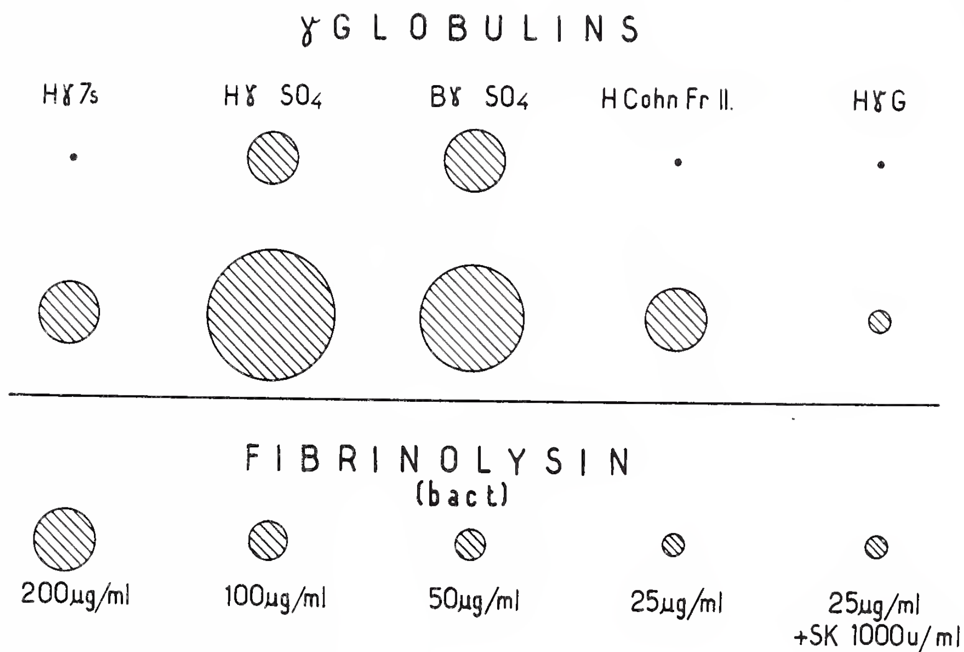

FIGURE 5. A comparison of the fibrinoloytic activity of several IgG preparations. 0.4 mg of each IgG preparation was placed on coagulated fibrin at a concentration of 2.0 mg %. The effect of streptokinase at a final concentration of 1250 units/ml is noted. Bacterial fibrinolysin is used on the same plate to enable calculation of proteolytic equivalents.