Biochem. J. (1967) **102**, 554

Studies on the Proteolytic Activity of γ-Globulin Preparations

By B. ROBERT and R. S. BOCKMAN
Laboratoire de Biochimie, Institut d'Immuno-Biologie, Paris 14, France

(*Received* 16 *June* 1966)

1. The proteolytic activities of several γ-globulin preparations were tested. These included sulphate-precipitated human and bovine preparations and human and bovine Cohn fraction II preparations as well as purified γ-globulin preparations. Up to 14mg. of diffusible peptides and glycopeptides/g. of γ-globulin was liberated after dialysis and up to 10mg. of peptides/g. after incubation and trichloroacetic acid precipitation, as products of the degradation process in incubated γ-globulin. 2. ε-Aminohexanoic acid and *p*-chloromercuribenzoic acid, as well as heating at 60° for 40min., were shown to inhibit strongly these proteolytic activities. Streptokinase was shown to activate strongly the proteolytic activity of all the human preparations (sulphate-precipitated, Cohn fraction II, and purified γ-globulin). 3. Two distinct pH optima were shown for human and bovine γ-globulin preparations: one at pH 8, the other at pH 3·8 (the latter activity could be demonstrated only in the presence of cysteine). 4. Both ^{131}I-labelled human Cohn fraction II and bovine fibrinogen were attacked by a sulphate-precipitated preparation of γ-globulin. Of the synthetic substrates tested toluene-*p*-sulphonyl-L-arginine methyl ester was hydrolysed by both the sulphate-precipitated and Cohn fraction II preparations, as was benzoyl-L-arginine amide at pH 5, but only in the presence of cysteine. 5. These data are interpreted to indicate that at least two enzymes are present in γ-globulin preparations, one being similar to the plasmin system, the other similar to cathepsin B.

Robert, Denes & Crepin (1965*b*) reported that diffusible peptides and glycopeptides are released from human and bovine γ-globulin incubated at 4° and 37° for several hours or days. The enzymic nature of this process, which occurs during prolonged dialysis or incubation, has been demonstrated (Bockman, Crepin & Robert, 1965; Robert, Bockman & Crepin, 1965*a*). The diffusible breakdown products inhibited in very low concentrations the reaction between the γ-globulins from which they were derived and the specific antisera against these γ-globulins. This inhibition could be shown to be species-specific (Robert *et al.* 1965*a*,*b*). The results of further investigations on the proteolytic components responsible for this degradation are presented in this paper.

MATERIALS AND METHODS

γ-Globulins. Human and bovine Cohn fraction II were purchased from Mann Biochemicals Inc. (New York, N.Y., U.S.A.), Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.), Armour and Co. (Chicago, Ill., U.S.A.) and E. R. Squibb and Sons (New Brunswick, N.J., U.S.A.). Human γ-globulin lot no. 208 came from Immunology Inc. (New York, N.Y., U.S.A.) (labelled as 7s γ-globulin). All these preparations were kept at 4° as freeze-dried powders and dissolved freshly before use.

Human and bovine γ-globulin were prepared as described by Strauss, Kemp, Vannier & Goodman (1964) and by the method of Kekwick (1940), both by using Na_2SO_4. These preparations were kept undialysed at −20° and used within a month. γ-Globulin was purified from Cohn fraction II or from sulphate-precipitated preparation on DEAE-cellulose columns after the method of Sober & Peterson (1958).

All blood samples were obtained under aseptic conditions; to some preparations ε-aminohexanoic acid was added to the freshly drawn blood before the precipitating procedures.

Other proteins. Thrombin (Thrombase 500) was purchased from Laboratoires Roussel (Paris, France). Fibrinogen (bovine) was purchased from Laboratoires de Bio-Hématologie (Paris, France). Fibrinolysin (bacterial) lot no. 16877 came from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Streptokinase (Varidase) lot 2200–906 came from the Lederle Laboratories (Pearl River, New York, N.Y., U.S.A.). This streptokinase preparation contained 28μg. of diffusible peptides/1000 units of streptokinase. Appropriate correction was made for the calculation of the yield of diffusible peptides/g. of γ-globulin in experiments where streptokinase was added as activator.

Synthetic substrates. BAA* (lot 400314) was obtained

* Abbreviations: BAA, benzoyl-L-arginine amide; TAME, toluene-*p*-sulphonyl-L-arginine methyl ester; GTAA, glycyl-L-tyrosine amide acetate.

from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). TAME (batch no. 7492) was purchased from Koch–Light Laboratories Ltd. GTAA (lot 2009) was obtained from Mann Biochemicals Inc.

Reagents. *p*-Chloromercuribenzoic acid (sodium salt) was purchased from Nutritional Biochemicals Corp., cysteine from Koch–Light Laboratories Ltd. and ε-aminohexanoic acid from Laboratoires Delagrange (Paris, France). All the other reagents were A.R. grade.

Dialysis tubing. Two kinds of sacs were used: Visking tubing of 18–23mm. diameter, purchased from Membranfiltergesellschaft (Göttingen, Germany), and Visking tubing Nojax from Sophic (Levallois, France).

Dialysis escape rates. Dialysis rate studies were carried out at 4–6° in the cold room and at 37±0·1° in a water bath. γ-Globulin preparations (0·50–20·0g.), dissolved in twice-distilled water or 0·9% NaCl, were placed in dialysis sacs which had been previously washed for 3hr. in distilled water and then rinsed with twice-distilled water. The outer fluid was twice-distilled water alone, or various buffer mixtures. Whenever the dialysis experiment lasted longer than 6–8hr., the absence of bacterial contamination was always checked by sterility tests: agar plates and nutritive broth were incubated with the γ-globulin solution. In the experiments carried out at 37° toluene was added as preservative. The diffusate was then concentrated by evaporation in a rotating evaporator at 35° and then stored at −20°. The amount of diffusible peptide material was estimated by the Lowry test for protein (Lowry, Rosebrough, Farr & Randall, 1951).

The porosity of the dialysis sacs was calibrated by dialysing 10ml. of a sucrose solution (150mg./ml.) against 250ml. of twice-distilled water. Sucrose appeared in the diffusate at a rate of 125mg./hr. at 4° (determined by the orcinol method of Weimer & Moshin, 1952). From 36mg. of a commercial sample of insulin 1·5mg. escaped in the first hour under the same conditions.

Self-digestion of the γ-globulins. This was studied by incubating solutions of γ-globulin (20·0–100·0mg./ml.) at 37° for 24hr. in various buffer mixtures. When sulphate-precipitated γ-globulin preparations were incubated at 37° the preparation was previously dialysed for 6hr. in the cold, at 4°, to eliminate the sulphate, which diffuses much faster than the peptides (Bockman *et al.* 1965). These solutions were then treated with trichloroacetic acid, to final concentration 2% (w/v), and were kept for 60min. at 4° before centrifugation. After centrifugation at 1000*g* in a refrigerated Jouan centrifuge at 5°, the amount of peptide material in the supernatant was estimated by the Lowry method. From the amount of peptides liberated during incubation we subtracted always the 0min. value, obtained by determining Lowry-positive material in the trichloroacetic acid supernatant of a non-incubated reaction mixture of identical composition, which was immediately frozen at −20° and kept at that temperature until the end of the experiment. Trichloroacetic acid was then added at the same time to the 0min. sample as well as to the incubated sample.

The amount of trichloroacetic acid-soluble material found in the 0min. sample varies with the method of preparation and the origin of the γ-globulin. The amount of trichloroacetic acid-soluble material before and after incubation of sulphate-precipitated bovine and human γ-globulin preparations is shown in Table 1. The standard error of the mean was always of the order of 10% of the average value, estimated from six separate determinations.

To check the presence of intact γ-globulin in the trichloroacetic acid supernatant the following experiment was carried out: 300mg. of sulphate-precipitated human γ-globulin in 8ml. of tris–HCl buffer (0·1M), pH8, was precipitated by adding 8ml. of 4% trichloroacetic acid, centrifuged after standing for 1hr. at 4°; the supernatant was washed three times with ethyl ether and concentrated by flash evaporation to 2ml. The peptides were determined in the concentrate by the Lowry method. A solution of this material (1mg./ml.) was deposited on an 0·8% agarose plate along with the original untreated γ-globulin against a specific rabbit antiserum to human γ-globulin. No precipitation line was observed with the trichloroacetic acid supernatant. This was taken as an indication that trichloroacetic acid at the concentration used precipitated all the γ-globulin.

Fibrin plates. These were prepared by the method of Astrup & Müllertz (1952) modified in the following manner: 280mg. of bovine fibrinogen was suspended in 35ml. of 0·1M-veronal–HCl buffer, pH7·8, then filtered through medium-mesh fibreglass (purchased from Prolabo, Paris, France). A portion (0·5ml.) of 0·1% thrombin solution was added, mixed thoroughly, then poured into plastic fibrin moulds (9·5cm.×25cm.) and allowed to coagulate undisturbed at room temperature. A portion (20μl.) of each sample or standard was placed directly on the coagulated fibrin. The plates were then incubated at 37° for 20–24hr.,

Table 1. *Estimation of peptides of sulphate-precipitated γ-globulin preparations before and after incubation*

γ-Globulin preparations were incubated at 37° for 24hr. in 0·1M-tris–HCl buffer, pH8·0; final concn. of the γ-globulins was 20–22mg./ml. Released peptides were determined quantitatively after trichloroacetic acid precipitation (final acid concn. 2%); the solutions were kept for 60min. at 4° before centrifugation. Results are given in mg. of peptide/g. of γ-globulin and represent the average of six determinations ±S.E.M.

γ-Globulin preparation	Trichloroacetic acid-soluble peptides (mg./g.) Before incubation	After incubation	Increase
Bovine	7·69±0·35	18·76±0·69	11·07
Human	7·73±0·51	16·78±0·50	9·04

and the proteolytic activity was estimated by measuring the widest diameter of the lysis plaque and the diameter perpendicular to it. The product of these two diameters was calculated and compared with a fibrinolysin standard. The results are expressed as percentage equivalents to the fibrinolysin standard or percentage inhibition compared with an appropriate control.

Labelling of proteins with ^{131}I. Human Cohn fraction II and bovine fibrinogen were labelled with ^{131}I according to the method of Biozzi, Benacerraff, Stiffel, Halpern & Mouton (1957). The specific activity for the human Cohn fraction II varied between 2350 and 2570 counts/min./mg. of protein, and for the bovine fibrinogen between 5670 and 6580 counts/min./mg. of protein. The radioactivity was estimated on a Tracerlab scintillation well counter employing small haemolysis test tubes containing from 2 to 5ml. of sample. The rate of escape of dialysable peptides was measured by determining the radioactivity of the diffusate at given time intervals. The specific activity of the peptides was calculated by relating radioactivity to Lowry-positive material. The specific activity of the dialysable peptides could then be compared with that of the original protein, and dialysis rate curves constructed by plotting amount (mg.) of peptide released (calculated from the radioactivity measurement) as a function of time.

The specific activity of the released peptides was identical or very similar to that of the starting protein solution. This would indicate that no appreciable amount of free iodine was released from the protein during dialysis.

Estimation of protein. Enzymically released peptides were measured according to the method of Lowry *et al.* (1951), twice-recrystallized bovine serum albumin being used for the calibration curve.

Estimation of sulphate. The initial rate of dialysis of sulphate from the sulphate-precipitated γ-globulin preparations was measured on a Société Biolyon C-60 conductimeter and compared against appropriate standards.

Rates of hydrolysis of synthetic substrates. BAA and GTAA were estimated by the alcohol-titration method as given by Davis & Smith (1955) modified in the following ways: 1·0ml. of 0·1–0·125M-BAA or GTAA was brought to 1·5ml. with the appropriate buffer and the tube containing the substrate then placed in a constant-temperature bath at 37° for 3–5min.; 1·0ml. (86mg. of protein) of the sulphate-precipitated bovine γ-globulin or human Cohn fraction II, or saline, was added, mixed thoroughly and then incubated with agitation at 37°. An initial sample was withdrawn immediately, to which 1·8ml. of absolute ethanol was added; the mixture was allowed to stand for 10min. in the cold, then centrifuged at 800*g* for 10min. in the cold. A portion (1·0ml.) of the clear supernatant was placed in a separate tube with 2 drops of phenolphthalein indicator (1·0% phenolphthalein in 95% ethanol) and titrated with 19mM-KOH in 95% ethanol; constant mixing was achieved by bubbling N_2 through the solution. Portions of the incubation mixture were withdrawn at appropriate time-intervals and titrated in the same way. The rate of hydrolysis of TAME was measured spectrophotometrically (Roberts, 1958). The following buffers were used for the incubation: for pH3–6, 0·1M-sodium acetate adjusted to the desired pH with 1M-acetic acid; for pH6–7, 0·1M-veronal–HCl buffer; for pH7–10, 0·1M-tris–HCl buffer; both veronal and tris buffers were prepared according to the Tables of Long (1961).

RESULTS

Yield of diffusible peptides from several preparations of γ-globulin. With sulphate-precipitated bovine γ-globulin it was possible to measure the rate of diffusion of the small peptides and glycopeptides released during dialysis. At a dialysis temperature 4–6° the yield of diffusible peptides was quite low: from 0·8–1·0mg. of peptide/g. of γ-globulin after 100hr. of dialysis (against twice-distilled water to which a few drops of 1M-ammonium formate solution, pH7·8, were added). At 37° the amounts recovered were higher, yielding from 8·1 to 14·5mg. of peptide/g. of γ-globulin after 114hr. of dialysis. This is roughly 10 times as high as the yield at 4°.

The dialysis of human Cohn fraction II preparations in exactly the same conditions resulted in significantly lower yields of diffusible material. At 4° and after 50hr. of dialysis only 0·18mg. of peptide/g. of protein was released, whereas at 37° and after 45hr. the yield was 2·5mg./g. of protein. Fig. 1 shows a comparison of the compiled results based on average 24hr. yields.

Trichloroacetic acid precipitation of previously incubated γ-globulin preparations produced higher yields of peptides. About 10mg. of peptides/g. of human or bovine γ-globulin was recoverable after

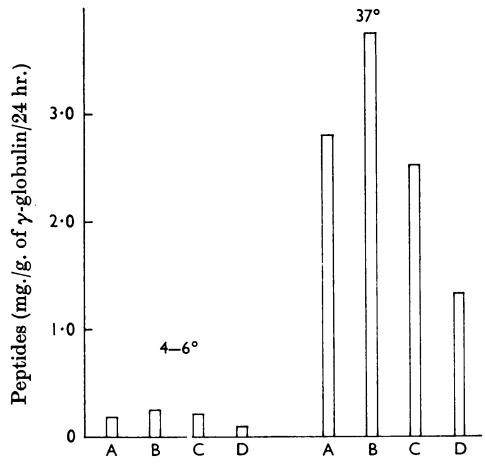

Fig. 1. Quantity of diffusible peptides released from various preparations of γ-globulin during dialysis at 4–6° and at 37°. A, Sulphate-precipitated bovine γ-globulin prepared by the method of Strauss *et al.* (1964). B, Sulphate-precipitated bovine γ-globulin prepared as in A, but from ϵ-aminohexanoic acid-protected serum. C, Sulphate-precipitated bovine γ-globulin prepared by the method of Kekwick (1940). D, Human Cohn fraction II. For the conditions of dialysis see the Materials and Methods section and the text.

24hr. incubation at 37°. Both preparations were obtained by the sulphate-precipitation method previously described.

Rate of release of peptides from several external substrates. To begin to study the nature of the enzymes present in the γ-globulin preparations an attempt was made to ascertain the enzyme's activity on several external substrates. In one series of experiments this was done by placing a mixture of ^{131}I-labelled human Cohn fraction II and a bovine sulphate-precipitated preparation in a dialysis sac and by following the rate of escape of labelled peptides. Dialysis was performed against twice-distilled water containing a few drops of 1M-ammonium formate, pH 7·8. Fig. 2 shows the results of such an experiment.

In the first control, that containing only ^{131}I-labelled human Cohn fraction II, labelled peptides were released after a 6hr. lag period and then at a rate of 0·070mg. of peptides/g. of protein/hr. (see bottom curve on Fig. 2). In the presence of sulphate-precipitated bovine γ-globulin, labelled human Cohn fraction II released at a higher rate the labelled peptides (top curve on Fig. 2). The initial rate of release was 0·128mg. of peptides/g. of protein/hr. for the first 10hr. of dialysis, slowing to 0·108mg. of peptides/g. of protein/hr. (calculated from the slope of the curve between 20 and 26hr.). If the sulphate preparation was heated to 60° for 45min. before being mixed with the labelled human Cohn fraction II, the rate of release was 0·072mg. of peptides/g. of protein/hr. (middle curve on Fig. 2), this rate being identical with that of the lower curve. However, no lag period was observed.

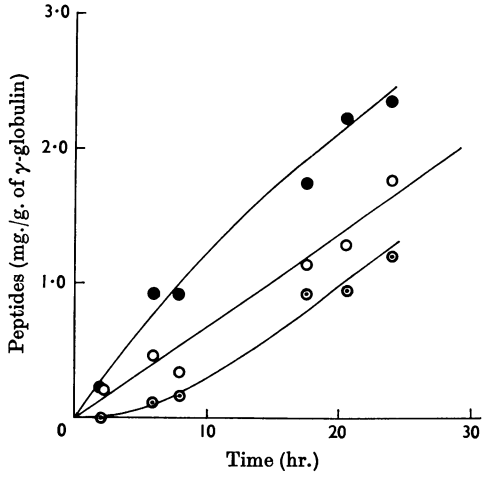

Fig. 2. Rate of release of diffusible peptides at a dialysis temperature of 37°, pH 7·8, as determined from the radioactivity of the diffusate and calculated from the specific activity of the starting material. ⊙, 1645mg. of ^{131}I-labelled human Cohn fraction II, specific activity 2350 counts/min./mg. of protein. ●, 1225mg. of ^{131}I-labelled human Cohn fraction II+400mg. of sulphate-precipitated bovine γ-globulin, specific activity 2570 counts/min./mg. of protein. ○, 1225mg. of ^{131}I-labelled human Cohn fraction II+400mg. of sulphate-precipitated bovine γ-globulin heated to 60° for 45min. before mixing, specific activity 2490 counts/min./mg. of protein.

This would seem to indicate that the enzymes present in the sulphate-precipitated preparation acted on the human Cohn fraction II, accelerating the release of labelled, diffusible peptides. The latter peptides were found to have a specific activity 2600 counts/min./mg. of peptide, which is almost identical with that of the starting material (2570 counts/min./mg. of labelled human Cohn fraction II+unlabelled bovine γ-globulin). This indicates that both the human and bovine preparations were uniformly attacked by the proteases present in both preparations. Both human and bovine γ-globulin behaved as a homogeneous pool of substrate protein towards their accompanying proteases. Heating to 60° for 45min. destroyed the enzyme activity of the bovine γ-globulin preparation: the rate of release is the same as that of the human Cohn fraction II alone, without added bovine γ-globulin. The lag phase, however, observed with the human Cohn fraction II, was not seen in the presence of the heated γ-globulin.

Very similar results were obtained by incubating a mixture of ^{131}I-labelled fibrinogen and a sulphate-precipitated bovine γ-globulin preparation. Bovine γ-globulin (400mg.) was mixed with 21·4mg. of ^{131}I-labelled fibrinogen and 10000 units of streptokinase. The specific activity of the mixture was 5670 counts/min./mg. of the total protein (counts/min./421mg. of protein). After 20hr. of dialysis at 37°, 97·44mg. of peptides was recovered in the diffusate with a specific activity 6176 counts/min./mg. of peptide. This can be interpreted as a result of a non-selective attack of the enzyme(s) present in the γ-globulin on the two kinds of protein present. The much higher rate of release (12·2mg. of peptide/hr./g. of protein) calculated from this experiment is due to the activation by streptokinase (see section on activators). In a parallel experiment the same amount of heated (60° for 45min.) bovine γ-globulin was added to the labelled fibrinogen. In this case only 16·8mg. of peptide material was recovered from the diffusate; that is, more than 80% inhibition was obtained [uninhibited value 97·44mg. of peptide/20hr.; yield with heated γ-globulin was 16·8mg./20hr., and the difference (80·64mg.) is 83% of the uninhibited value].

Proteolytic activity as a function of pH. To investigate the effect of pH on the proteolytic activity of the γ-globulin preparations, human

and bovine γ-globulin were incubated for 24hr. at 37° in acetate, veronal or tris buffers and then precipitated with trichloroacetic acid. After precipitation the quantity of trichloroacetic acid-soluble peptides was determined. A separate zero-time value was determined for every single pH studied. Fig. 3 shows the result of one experiment performed with human γ-globulin. There is a rather sharp optimum at pH 8·0. No activity or only a very weak one could be detected in the acid pH range in the absence of activator (see also Table 4). In the presence of 5mM-cysteine, significant activity became apparent around pH 4, both for the sulphate-precipitated as well as for the Cohn fraction II preparations. Further information is given in the section on activators. Bovine γ-globulin gave very similar pH-profile to the one shown in Fig. 3.

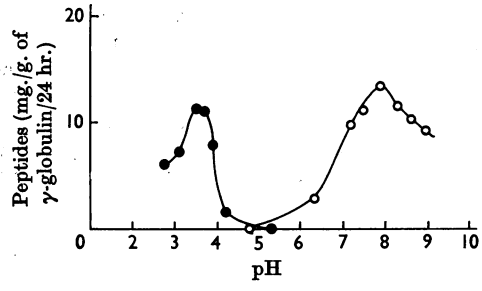

Fig. 3. Proteolytic activity as a function of pH. Sulphate-precipitated human γ-globulin was incubated for 24hr. at 37° at various pH values. After the incubation, the preparations were precipitated with trichloroacetic acid, final concn. 2% (w/v), and the peptides estimated in the supernatant. ○, Sulphate-precipitated human γ-globulin; ●, sulphate-precipitated human γ-globulin + cysteine (5mM).

Inhibitors. Several inhibitors were used to gain more information on the nature of the enzymic activity. Table 2 gives evidence that both *p*-chloromercuribenzoic acid and ε-aminohexanoic acid inhibit the proteolytic activities at pH 8, as measured by the trichloroacetic acid-precipitation method. High concentrations of ε-aminohexanoic acid are necessary to give significant inhibition; at 100mM concentration 54·6% inhibition is effected at pH 8. No inhibition was observed in the cysteine-activated preparation at pH 4. By this same method *p*-chloromercuribenzoic acid produced 42% inhibition at pH 8. These inhibitions are considerably lower than the values obtained with the dialysis-rate method (Bockman *et al.* 1965).

By the use of the fibrin-plate method, similar tests of inhibition were carried out. Table 3 gives the results of such experiments and shows the inhibition obtained with ε-aminohexanoic acid. By calculations from the areas of lysis produced by the various mixtures (see the Materials and Methods section), it is estimated that 4000moles of ε-aminohexanoic acid are required/mole of γ-globulin to produce complete inhibition of fibrinolysis.

It has already been mentioned that heating of the sulphate-precipitated preparations to 60° for 45min. resulted in a strong decrease in the intrinsic proteolytic activity, such that the rate of peptide release was greatly lowered. In the same conditions of heating in saline a sulphate-precipitated bovine

Table 2. *Effect of inhibitors on the proteolytic activity of a sulphate-precipitated bovine γ-globulin preparation*

The γ-globulin preparation was incubated for 24hr. at 37° with acetate buffer at pH 4 or with tris–HCl buffer at pH 8; final concentration of the γ-globulin was 28–36mg./ml. Released peptide was determined quantitatively after trichloroacetic acid precipitation, final acid concentration being 2%.

pH	Activator (mM)	Inhibitor (mM)	Molar ratio: inhibitor/γ-globulin*	Peptide (mg./g. of γ-globulin)	Inactivation (%)
4	Cysteine, 5	—	—	5·41	—
4	Cysteine, 5	ε-Aminohexanoic acid, 100	465	6·00	0
8	—	—	—	11·07†	—
8	—	ε-Aminohexanoic acid, 100	114	5·03‡	54·6
8	—	*p*-Chloromercuribenzoic acid, 1	11·4	6·39	42·2
7§	—	—	—	28·0	—
7	—	ε-Aminohexanoic acid, 57	25	1·3	95·3
7	—	*p*-Chloromercuribenzoic acid, 0·16	0·73	7·75	62·4

* Mol.wt. of γ-globulin taken as 160000.
† Average value of six determinations.
‡ Average value of three determinations.
§ By dialysis rate studies (Bockman *et al.* 1965) and from previously undialysed γ-globulin.

preparation at a concentration 40mg./ml. never produced any detectable lysis on the fibrin plates.

Similar results were obtained when the trichloroacetic acid-precipitation method was used for the study of heat inactivation. Table 4 shows the results of several such experiments. Inactivation (75–100%) was obtained by 40min. heating to 60°, whether the activity was measured at pH3·8 or at pH7·8, in the presence or in the absence of cysteine. This Table shows also that the addition of cysteine did not increase the peptide yield at pH7·8; on the contrary, in several experiments a decrease was noticed at this pH.

Activators. As mentioned above, cysteine activated the proteolytic activity at pH4 (see Fig. 3). One preparation of human Cohn fraction II liberated 5·0mg. of trichloroacetic acid-soluble peptides/g. of protein after 24hr. incubation at 37° at pH3·9 in the presence of cysteine. In the absence of cysteine no peptide material was released. In similar circumstances, a sulphate-precipitated preparation of bovine γ-globulin released 10·0mg. of peptides/g. of protein after 24hr. incubation at 37°, at pH3·8 in the presence of cysteine. Without cysteine only a very slight amount of peptide material was released (about 1·5%).

Streptokinase produced a strong activation of the fibrinolytic activity of the several preparations tested; Fig. 4 shows one set of results.

A very striking increase in the area of lysis was obtained by adding streptokinase to sulphate-precipitated and Cohn fraction II as well as to purified human γ-globulin preparations. Much less activation was achieved with the sulphate-precipitated bovine γ-globulin, and streptokinase alone had virtually no lytic activity in this system. Purification of the γ-globulin diminished its direct proteolytic activity but left much of its streptokinase-activatable protease activity [see the human γ-globulin 7s (extreme left of Fig. 4) and the DEAE-purified preparation (extreme right of Fig. 4)].

A semi-quantitative estimate of the proteolytic activities of the various preparations was obtained by employing a standard of fibrinolysin on the same plate as the samples. A double-logarithm curve (log concentration, expressed in per cent of the fibrinolysin standard, versus the log area of lysis, product of the perpendicular diameters) is drawn and the activity of the various preparations is estimated in terms of bacterial fibrinolysin equivalents. Such graphs usually gave straight lines. A typical sample of a sulphate-precipitated human γ-globulin gave a lysis equivalent to 6·4mg. of fibrinolysin/g. of sample, in the absence of streptokinase, and 47·0mg. of fibrinolysin/g. of sample in the presence of this activator. A human Cohn fraction II sample gave no recordable lysis alone.

Table 3. *Effect of ε-aminohexanoic acid on the fibrinolytic activity of a sulphate-precipitated bovine γ-globulin preparation*

Portions (20μl.) of a 20mg./ml. solution of γ-globulin (0·4mg.) were mixed with various concentrations of ε-aminohexanoic acid placed on fibrin plates, which were then incubated for 18hr. at 37° before being read.

Concn. of ε-amino-hexanoic acid (μM)	Molar ratio: inhibitor/ γ-globulin*	Inhibition (%)
0·01	4	18
0·02	8	23
0·10	40	32
0·20	80	41
2·00	800	61
10·00	4000	99

* Mol.wt. of γ-globulin taken as 160000.

Table 4. *Action of heating on the release of peptides from sulphate-precipitated bovine γ-globulin*

Portions (4ml.) of γ-globulin (final concn. 40mg./ml.) were heated in a water bath at 60° for 40min., either in tris–HCl buffer, pH7·8, or in collidine–acetate buffer, pH7. After heating at pH7 the sample was acidified to pH3·8 with acetic acid and the concentration adjusted to 20mg./ml. The control experiments were performed on the same solution of γ-globulin diluted to 20mg./ml. and kept at pH7·8 or acidified to pH3·8 without previous heating. The heated and unheated γ-globulin samples were then further incubated with or without the addition of cysteine.

		Peptide (mg./g. of γ-globulin)		
pH	Cysteine (mM)	Unheated	Heated to 60° for 40min.	Inactivation (%)
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

However, in the presence of streptokinase it produced a lysis equivalent to 9·5mg. of fibrinolysin/g. of sample. Further evidence of the effect of this activator could be seen in the increased rate of hydrolysis of one of the synthetic substrates. This will be discussed in the next section.

Action on synthetic substrates. The rates of hydrolysis of several synthetic substrates by various γ-globulin preparations were tested (see the Materials and Methods section for the techniques utilized), and these results are presented in Table 5. As can be seen, at pH 5, and only in the presence of cysteine, BAA was rapidly split by both the sulphate-precipitated and Cohn fraction II preparations. Much less activity was found at the higher pH values in the absence of cysteine. GTAA was only slowly attacked by the Cohn fraction II preparation, with or without cysteine. TAME proved to be a good substrate for both preparations at pH 9, with significant activation being manifested by the human Cohn fraction II preparation after the addition of streptokinase.

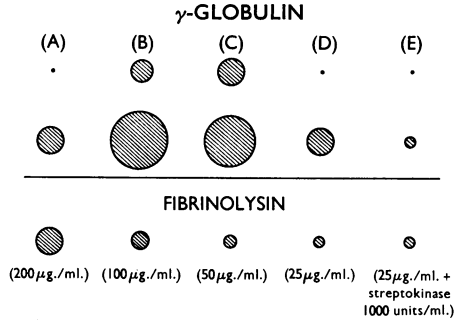

Fig. 4. Comparison of the fibrinolytic activity of several γ-globulin preparations with and without streptokinase. A portion (20μl.) of each preparation, containing 20mg./ml. of protein, was placed on the coagulated fibrin plate. The activation by streptokinase (final concn. 1250 units/ml.) is shown in the middle row. The lower row shows the lytic activity of different concentrations of a bacterial fibrinolysin. (A) Human γ-globulin 7s from Immunology Inc. (B) Human γ-globulin precipitated with sodium sulphate prepared according to Kekwick (1940). (C) Bovine γ-globulin precipitated with sodium sulphate prepared according to Kekwick (1940). (D) Human Cohn fraction II (Squibb). (E) Human γ-globulin obtained by purification on a DEAE-cellulose column from a sulphate-precipitated γ-globulin prepared according to Kekwick (1940).

DISCUSSION

The data presented here and in earlier publications (Robert *et al.* 1965*a*,*b*) leave little doubt that a great number of γ-globulin preparations, commercial as well as self-prepared, release diffusible peptides upon incubation. Several mechanisms could be responsible for such a release: e.g., the liberation by diffusion of low-molecular-weight material adsorbed during the purification process, the splitting of peptides by some non-enzymic hydrolytic reaction, or the cleavage of peptides by proteolytic enzymes present in the preparations. The first mechanism can be rendered improbable by the kinetics of reaction (Bockman *et al.* 1965), which demonstrate a slow and continuous release of peptide material from pre-dialysed or chromatographically purified γ-globulin preparations as well as by the arguments given below in favour of the enzymic mechanism.

Several non-enzymic reactions could eventually yield peptide material. Such reaction could include SH–S·S interchange and the rupture of some labile bonds. The inhibition by *p*-chloromercuribenzoic acid of peptide release at pH 8 could be taken as an argument in favour of a SH–S·S-exchange mech-

Table 5. *Rates of hydrolysis of synthetic substrates by a sulphate-precipitated bovine γ-globulin and a human Cohn fraction II preparation*

Hydrolysis of BAA and GTAA was estimated by titration in alcohol (Davis & Smith, 1955). The cleavage of TAME was estimated spectrophotometrically (Roberts, 1958). Values are given as μmoles of substrate split/hr./100mg. of sample.

		BAA			GTAA	TAME	
Sample	Activator	pH 5	pH 7·4	pH 9	(pH 5)	pH 5	pH 9
Bovine γ-globulin	—	0	3·96	0·23	0	0	298·0
	Cysteine (5mM)	136·5	—	—	0	—	—
	Streptokinase (700 units)	—	—	—	—	—	305·0
Human Cohn fraction II	—	0·1	3·02	0	0	—	29·0
	Cysteine (5mM)	232·0	—	—	10·5	—	—
	Streptokinase (700 units)	—	—	—	—	—	95·0

anism. The *p*-chloromercuribenzoic acid can in fact react with the thiol groups of the γ-globulins or with those of the enzymes present in the preparation. However, the blocking of free thiol groups eventually present in the γ-globulin preparation could also modify its susceptibility to proteolytic attack. Against such SH–S·S-exchange mechanism is also the fact that the addition of cysteine did not increase the peptide yield at pH 8. By far the most probable explanation of our results would be the presence of proteolytic enzymes in the γ-globulin preparations.

The enzymic nature of the reaction of peptide release is shown by its kinetics, by the action of the γ-globulin preparations on externally added protein substrates as well as on synthetic substrates, by the action of inhibitors and by the inactivation of the enzymes present in the γ-globulins by heating the preparation to 60° for 40 min.

With three different methods, the activity could be estimated in sulphate-precipitated, Cohn fraction II, DEAE-cellulose-purified as well as commercially purified γ-globulin preparations. Sulphate-precipitated preparations showed the highest proteolytic activity, yielding from 2·8 to 3·8 mg. of peptide/g. of protein after 24 hr. dialysis at 37° and about 10 mg. of peptide/g. of protein after incubation at 37° at pH 8 for 24 hr. and trichloroacetic acid precipitation. These same preparations could be activated by streptokinase. Human Cohn fraction II preparations showed a lower proteolytic activity, yielding only 1·2 mg. of peptide/g. of protein after 24 hr. dialysis at 37°, and a similar quantity after 24 hr. incubation and trichloroacetic acid precipitation. In the presence of streptokinase the peptide yield was higher (5 mg./g. of dialysable peptides/24 hr.), the rate of hydrolysis of the synthetic substrate TAME could be increased threefold and the fibrinolytic activity greatly increased. The purified γ-globulin preparations behaved similarly in their fibrinolytic activity to the Cohn fraction II preparation.

Some information was obtained about the nature of the enzymes involved. The activity which occurs at the alkaline pH seems from the experimental data to be very much like that of plasmin. This conclusion is based on several facts: (*a*) the pH range of the activity is close to that of plasmin (Christensen & McLeod, 1945); (*b*) the samples will hydrolyse fibrin and the synthetic substrate TAME at an alkaline pH, both being good plasmin substrates (Christensen & McLeod, 1945; Troll, Sherry & Wachman, 1954); (*c*) ε-aminohexanoic acid, a known plasmin inhibitor (Ablondi, Hagan, Philips & De Renzo, 1959; Alkjaersig, Fletcher & Sherry, 1959), will interfere with the degradation process. The effect of streptokinase, an activator of plasminogen, may be taken as further evidence of the presence of the enzyme and its precursor in the various preparations. The decrease of peptide yield in the presence of cysteine at pH 8 pleads also in favour of plasmin, this enzyme (as shown by Robbins, Summaria, Hsieh & Shah, 1966) being inactivated after the reduction of some of its S·S bridges. In the human Cohn fraction II and purified preparations the greater portion of the enzyme is present in its precursor form. The possibility of the presence of plasmin in sulphate-precipitated γ-globulins has already been noted by Skvaril & Grünberger (1962).

The rate of release of diffusible peptides at 4° and 37° gives 15 kcal./mole as an approximate value for the activation energy for the proteolytic reaction. This value is of the same order of magnitude as those found for other proteases (Laidler, 1958). This high value argues against the possibility that peptide release during prolonged dialysis could be due to the diffusion of peptides persisting and 'trapped' in the γ-globulin preparations.

The activity which manifests itself in the acid pH range has tentatively been concluded to be like that of cathepsin B. This is based on the findings that only in the presence of cysteine will the sample hydrolyse BAA at pH 5, a specific cathepsin B substrate under these conditions (Tallan, Jones & Fruton, 1952). Further, only in the presence of cysteine at pH 4 was this activity manifest in the γ-globulin preparations, as is shown in Fig. 3. The relative amounts of plasmin- and cathepsin B-like activities can be only roughly estimated to be equivalent in the sulphate-precipitated human preparation.

Though the increase of trichloroacetic acid-soluble peptides after incubation at pH 4 was observable only in the presence of cysteine (5 mM), the eventual reduction of S·S bonds by cysteine cannot explain our results. This conclusion is based on the strong inhibition of peptide release after heating of the γ-globulin preparation (see Table 4) and on the action of γ-globulin on synthetic substrates at acid pH (see Table 5). The results of Hong & Nisonoff (1965) also confirm our contention. These authors found that at pH 5 in the presence of low concentrations of mercaptoethylamine only negligible amounts of light chains were produced.

In measuring the trichloroacetic acid-soluble peptide material at pH 8 in the absence and in the presence of cysteine (5 mM) we did not observe any activation of peptide release. According to Hong & Nisonoff (1965) half-molecules are produced in similar conditions by mercaptoethanol with the release of light chains equivalent to about 5% of the total protein. This amount of peptide material could have been detected easily in our experiments.

As no such increase could be observed, we concluded that splitting of S·S bonds was not essential for the increase of the trichloroacetic acid-soluble peptides after incubation. It cannot be excluded, however, that such a reaction could have contributed to the production of a 'preferential substrate' for these enzymes. It is also possible that the proteases first split the γ-globulin in fragments similar or identical to the Fab and Fc fragments obtained with papain (Porter, 1959). These fragments would then be broken down with the liberation of peptides from the 'interior' of the molecule.

Davies & Lowe (1963) have studied the proteolytic activity of purified guinea-pig γ-globulin on several synthetic substrates as well as on casein and fibrin. They found that no activity was manifested on the protein substrates, but that significant hydrolysis of TAME and benzoyl-L-arginine methyl ester did occur. On the basis of their findings these authors attributed this activity to a kallikrein type of enzyme. As has been demonstrated in this paper, the fibrinolytic activity of human Cohn fraction II and purified γ-globulin preparations is apparent only after the addition of streptokinase. Only under these conditions could this type of proteolytic activity be shown by the methods employed.

Similar to the finding of Davies & Lowe (1961), the injection of bovine γ-globulin into the shaved skin of guinea pigs greatly increased capillary permeability at the site of injection. An identical augmentation of the capillary permeability could be effected by the diffusible peptides obtained during dialysis of sulphate-precipitated bovine γ-globulin (R. S. Bockman, B. Robert & B. N. Halpern, unpublished work). This would mean that the proteases present in γ-globulin preparations will attack the γ-globulin with the release of peptides possessing pharmacological activity.

Subsequent observations have shown that a limited proteolytic attack of the γ-globulin (release of 17mg. of peptides from 1g. of sulphate-precipitated γ-globulin during 210hr. of dialysis at 4° and then at 37°) produced no detectable changes in the precipitin line, obtained on immunoelectrophoresis. The splitting of the immunoelectrophoretic line after prolonged storage was attributed to proteolytic attack on the molecule (Skvaril, 1960; Skvaril & Grünberger, 1962). Further investigations (James, Henney & Stanworth, 1964) have provided evidence that dimerization occurring during incubation at 37° is the first stage in the denaturation of the molecule. This dimerization renders certain labile bonds susceptible to proteolytic hydrolysis resulting in a major cleavage, manifested by the splitting of the immunoelectrophoretic precipitin line. This splitting becomes apparent after 20hr. incubation, concomitant with the appearance of a 5s component. This is somewhat in contrast with the results presented in this paper, where the release of peptides is shown to start immediately upon incubation.

It is noteworthy that these diffusible peptides can inhibit the reaction between the parent γ-globulin molecule and the anti-γ-globulin immune sera produced against it (Robert *et al.* 1965*a*,*b*). Furthermore major changes in the precipitating behaviour of the γ-globulin are manifest after prolonged dialysis (Robert, 1964). This indicates that significant alterations have occurred within the molecule after incubation or dialysis.

This work was supported in part by a Public Health Service grant 5T5GM37-03, and partly by the Centre National de Recherche Scientifique. R. S. B. is on leave from the Department of Microbiology, Yale University School of Medicine, Princeton, N.J., U.S.A. We acknowledge the generous gift of human Cohn fraction II by the American Red Cross. Streptokinase was kindly provided by Dr DeRenzo, Lederle Laboratories.

REFERENCES

Ablondi, F. B., Hagan, J. J., Philips, M. & De Renzo, E. C. (1959). *Arch. Biochem. Biophys.* **82**, 153.
Alkjaersig, N., Fletcher, A. P. & Sherry, S. (1959). *J. biol. Chem.* **234**, 832.
Astrup, T. & Müllertz, S. (1952). *Arch. Biochem. Biophys.* **40**, 346.
Biozzi, G., Benaceraff, B., Stiffel, C., Halpern, B. N. & Mouton, D. (1957). *Ann. Inst. Pasteur*, **92**, 89.
Bockman, R. S., Crepin, Y. & Robert, B. (1965). *C.R. Acad. Sci., Paris*, **260**, 3515.
Christensen, L. R. & McLeod, C. M. (1945). *J. gen. Physiol.* **28**, 559.
Davies, G. E. & Lowe, J. S. (1961). *Immunology*, **4**, 289.
Davies, G. E. & Lowe, J. S. (1963). *Brit. J. Pharmacol.* **21**, 491.
Davis, N. C. & Smith, E. L. (1955). *Meth. biochem. Anal.* **2**, 224.
Hong, R. & Nisonoff, A. (1965). *J. biol. Chem.* **240**, 3883.
James, K., Henney, C. S. & Stanworth, D. R. (1964). *Nature, Lond.*, **202**, 563.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Laidler, K. J. (1958). *The Chemical Kinetics of Enzyme Action*, Table 26, p. 202. Oxford: The Clarendon Press.
Long, C. (ed.) (1961). *Biochemists' Handbook*, pp. 30–41. London: E. and F. N. Spon Ltd.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Porter, R. R. (1959). *Biochem. J.* **73**, 119.
Robbins, K. C., Summaria, L., Hsieh, B. & Shah, R. (1966). *Fed. Proc.* **25**, 194.
Robert, B. (1964). In *Protides of the Biological Fluids*, vol. 3, p. 151. Ed. by Peters, H. Amsterdam: Elsevier Publishing Co.
Robert, B., Bockman, R. S. & Crepin, Y. (1965*a*). *Biochem. J.* **95**, 21P.

Robert, B., Denes, Y. & Crepin, Y. (1965*b*). *C.R. Acad. Sci., Paris*, **260**, 734.
Roberts, P. (1958). *J. biol. Chem.* **232**, 285.
Skvaril, F. (1960). *Nature, Lond.*, **185**, 475.
Skvaril, F. & Grünberger, D. (1962). *Nature, Lond.*, **196**, 481.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Strauss, A. J. L., Kemp, P. S., Vannier, E. & Goodman, H. C. (1964). *J. Immunol.* **93**, 24.
Tallan, H. H., Jones, M. E. & Fruton, J. S. (1952). *J. biol. Chem.* **194**, 793.
Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.
Weimer, H. E. & Moshin, J. R. (1952). *Amer. Rev. Tuberc.* **68**, 594.