STUDIES ON THE ACTIVATION OF SERUM PROTEASE BY AN ANTIGEN-ANTIBODY SYSTEM

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Serum protease, also known as plasmin and fibrinolysin, has been shown to be present in an inactive state as a natural constituent of mammalian blood. Numerous activating agents have been recorded for this enzyme since Delezenne and Pozerski (1) first demonstrated the proteolytic activity of serum treated with chloroform. This observation has been confirmed by other investigators (2–4). Jobling and Petersen (5–6) had reported that adsorption of serum protease inhibitors by various materials, kaolin, starch paste, agar, and bacteria, resulted in demonstrable proteolytic activity of the serum. More recently, activators from tissue and serum have been recorded (7–12). The most rapid and potent activators of serum protease have been prepared from bacterial sources, notably the so called streptokinase, from streptococcal culture filtrates (4, 13) and staphylokinase from staphylococcal cultures (14–15). The activation pattern of the bacterial products, however, varies with the species of animals used as a source of inactive protease. Streptokinase has been regarded as the only efficient activator of human serum protease and staphylokinase as the best activator of the proteases of rabbits, guinea pigs, dogs, and monkeys (15–19).

Antigen-antibody combinations also have been reported as activators for serum protease. In 1915, Jobling *et al.* (20) concluded that activation of serum proteolytic enzymes resulted if a precipitin reaction occurred in serum or if particulate matter was added to the serum. Bronfenbrenner (21, 22) in similar studies, concluded that proteolytic enzymes of fresh serum were released either by the non-specific mechanical adsorption of inhibitors or by the inactivation of serum inhibitors as a result of the specific combination of antigen and antibody. Ungar and Mist (23) reported that the protease of guinea pig immune serum could be activated by the addition of the specific antigen. Recently, Geiger (24) demonstrated that the protease of immune rabbit serum could be activated by some but not all, of the various antigens he employed as sensitizing agents.

Some of the earlier investigators referred to above (5, 6, 20–22) had regarded the activation of serum protease to be the result of the removal of inhibitors from the serum. It has repeatedly been shown that the inhibitory power of whole serum for active protease is associated with the albumin fraction (25–27). However, the accumulated evidence now indicates that serum protease is not in combination with the known protease inhibitors of serum but is in the form of an inactive protease

precursor which can be converted to the proteolytic state (4, 10, 19, 25, 28–30). The serum fractionation methods of dilution and acidification, or of dialysis and salting-out have shown the precursor to be in the resulting crude euglobulin fraction. This fraction can be further purified by acid extraction (*cf.* Christensen and Smith (31) for references). That inhibitors of the bacterial activating agents may occur in serum has been indicated by the work of Kaplan (30) who reported an antistreptokinase factor distinct from that of the normal inhibitor found in the albumin fraction. Characterization of the serum protease indicates it to be a non-dialyzable protein enzyme, water-insoluble and saline-soluble with an optimal pH range of 7.2–7.6, an optimal temperature of 37°C., and with the ability to effect proteolysis of fibrin, fibrinogen, gelatin, and casein, all commonly employed substrates (28, 29, 32, 33).

Because antigen-antibody reactions have been reported as serum protease activators, attempts have been made to assign a role to the active enzyme in systemic or local allergic reactions. The suggestion has been made that it plays a part in anaphylactic shock, (*a*) through its direct activation (23, 34, 35), (*b*) through the production of toxic substances (5, 6, 20–22), or (*c*) as the cause of a release of histamine (36, 37). Some investigators also have implied that protease may be involved in certain types of local hypersensitivity (38–41).

Grob (26) had demonstrated that subcutaneous injections of trypsin produce local necrotic lesions. Dragstedt and Wells (42) showed that Arthus-like lesions are produced by intracutaneous injections of trypsin. Ungar and Damgaard (41) suggested that activated serum protease may be involved in the Arthus reaction.

In following the line of reasoning just outlined our inability to demonstrate any *in vitro* activation of immune serum protease with its antigen in preliminary experiments has led us to a reinvestigation of the problem of protease activation by an antigen-antibody system. Since egg albumin readily elicits anaphylaxis and the Arthus phenomenon in rabbits and guinea pigs, this was used as the sole antigen in our investigations. The present report deals with our inability to activate serum protease through the agency of this antigen-antibody system.

Materials and Methods

Buffered Saline.—The solution used as solvent or diluent in all of the experiments was prepared by adding 1 volume of 0.15 M phosphate buffer of pH 7.4 to 9 volumes of 0.9 per cent saline. No change in pH resulted from this dilution.

Antigens.—Four times crystallized egg albumin was prepared according to the procedure of Cole (43) and used as antigen in many of the experiments. Merck's soluble egg albumin was prepared in sterile solution and was also used as antigen in guinea pig experiments.

Immune Sera.—Rabbit antisera were prepared against the crystalline egg albumin and the approximate antibody content of such antisera was determined by the method of Culbertson and Seegal (44).

Immune guinea pig sera were prepared as described elsewhere in the text. The presence of precipitable antibodies was determined by the qualitative ring test technic.

Sources of Serum Proteases.—Fresh, whole sera or the euglobulin fractions thereof were utilized as the two sources of the protease precursor fraction. In all instances, the precursor

sources were stored at −20°C. until used. Fresh, whole normal human serum was obtained from donors whenever required. Human euglobulin protease precursor fraction was prepared from plasma less than 24 hours old. The plasma was recalcified and the fibrin removed. The resulting serum was dialyzed for 2 days against running tap water and 1 day in frequently changed distilled water at 4°C. The euglobulin precipitate obtained in this manner was redissolved in an amount of buffer equal to one-tenth of the original plasma volume. Spontaneous activation of the protease never occurred in any of the samples prepared in this manner.

Rabbit euglobulin precursor fractions were prepared from fresh rabbit antiovalbumin serum. In initial experiments it was found that the euglobulin fraction contained no appreciable antibody as compared with the original immune serum. To overcome this difficulty, the whole globulin fraction of the immune serum was precipitated by half saturation with ammonium sulfate. The resulting precipitate was collected, dialyzed, and redissolved in a volume of buffered saline to give a concentration approximating that of the original serum. With this procedure there was only a negligible decrease of the original serum precipitin titer.

Whole guinea pig serum was used according to the procedures described by Ungar and Mist (23). Two sets of animals were employed, the first all males averaging 250 gm., while the second set consisted of animals of both sexes, all above 500 gm. in weight.

Protease Activators.—Streptokinase[1] was used as the activator for human and rabbit whole serum as well as for the human euglobulin precursor fraction. 250–350 units of streptokinase were added per milliliter of the sample to be activated.

Staphylokinase was obtained by the method of Gerheim *et al.* (14). The crude precipitate isolated by this procedure was redissolved in buffered saline and used to activate guinea pig serum and the euglobulin fraction. 0.1 ml. of the staphylokinase solution was added for every 0.5 ml. of the sample to be tested.

Protease Assay Method.—Serum protease activity was determined by measuring the increase of acid-soluble nitrogen produced by this enzyme from a purified casein substrate. Since it was found, in agreement with Remmert and Cohen (29), that commercial casein was unsuitable for this purpose, their method of preparing milk casein was employed. This six times reprecipitated substrate gave reproducible results with different batches.

The actual assay method consisted of adding 0.5 ml. of the protease sample to 2 ml. of a 4 per cent casein solution with a drop of toluene added as a preservative. Immediately, 1 ml. of the complex was removed and this was regarded as the zero hour blank. The remainder of the enzyme-substrate mixture was incubated in a 37°C. water bath for 18 hours. 1 ml. of the mixture was then removed and added to 0.5 ml. of distilled water and precipitated with 4 ml. of 10 per cent trichloroacetic acid. After a half-hour minimum contact period at room temperature, the solution was filtered through a No. 5 Whatman paper. The acid-soluble nitrogen content of the filtrate was determined by the technic of Koch and McMeekin (45). The nesslerized solution was read within 7 minutes in a Klett-Summerson photoelectric colorimeter using a No. 54 green filter. The previously obtained zero hour blank reading was subtracted from the 18 hour reading. This corrected reading was then interpolated from a previously calibrated curve to obtain the measure of proteolytic activity in terms of units per total digest.

A standard curve was obtained through the use of a streptokinase-activated human euglobulin fraction. Serial dilutions of this preparation were tested for activity on the casein substrate. The means of at least three determinations at each protease concentration were plotted against dilutions of protease. A linear relationship between the relative enzyme concentration and non-protein nitrogen liberated was obtained over a limited range. Values at

[1] Obtained through the courtesy of Lederle Laboratories as streptokinase-streptodornase.

each end of the linear portion of the curve were expressed as < 0.5 or > 15.0 units. Under these conditions, one proteolytic unit was arbitrarily defined as that amount of protease producing an increment of 100 μg. of acid-soluble nitrogen in a medium of 4 per cent casein in 18 hours at 37°C.

For the determination of serum protease activity the fibrinogenolytic method of Ungar and Mist (23) was employed in some instances also. Bovine fibrinogen, fraction I (Armour and Co.), and bovine thrombin (The Upjohn Co.) were utilized as the clot-producing agents in this method. Evidence of fibrinogenolysis was recorded here merely as the highest dilution of protease which resulted in failure to clot upon the addition of 0.1 ml. of the thrombin solution containing 25 units per ml. Appropriate saline and thrombin controls were used with each sample run as a check of the efficacy of the system.

TABLE I

Comparison of Protease Assay Methods

Protease dilutions	Fibrinogenolytic method	Casein proteolysis method
	Clot formation*	Proteolytic units
Undiluted	−	>15
1-4	−	>15
1-8	−	>15
1-16	−	>15
1-32	−	12.7
1-40	−	10.3
1-64	+	7.8
1-80	+	5.3
1-160	+	3.7
Saline control	+	0

* − = no clot formation, indicating fibrinogenolysis; + = clot formation, indicating no fibrinogenolysis.

Protease Controls.—As a control for the presence and availability of the protease precursor, streptokinase or staphylokinase was added to the appropriate samples. Saline was included as a control in order to detect spontaneous or non-specific activation of the samples.

EXPERIMENTAL RESULTS

Comparison of Protease Assay Methods.—Some of the results obtained in this study were contrary to the results obtained by other workers who employed the fibrinogenolytic method for assaying serum protease (23). This method was compared with our casein proteolysis assay procedure for protease. The comparative data shown in Table I were obtained on a streptokinase-activated human euglobulin fraction. Although proteolysis was evident with the higher concentrations of protease, using both methods, the casein procedure appeared to be the more sensitive for detection of smaller amounts of activated protease. This can be explained by the longer incubation period of the casein method.

However, when the fibrinogenolytic test mixtures were incubated 2 hours or more after the thrombin had been added, complete dissolution of the clot was apparent in all tubes including the saline control. This probably indicated the presence of protease precursor or protease contaminants in the components of this particular system. That the sodium chloride, *per se*, had no effect on clot lysis or protease activation has been made plain by the work of Lewis and Ferguson (19). This was also demonstrated by some of our experiments in which both methods of assay were employed. When the euglobulin fraction of guinea pig serum was added to fibrinogen, a clot formed in some instances prior to the addition of thrombin. This again suggested that contaminants may be found in the materials used in the fibrinogenolytic assay method. By employing the casein digestion methods, these variables are apparently obviated because the substrate is obtained from a source unlikely to contain either blood-clotting

TABLE II

Inhibition of Human Protease by Various Fractions

Fraction tested*	Inhibition of active protease‡
	per cent
Normal rabbit euglobulin	0
" " albumin	48
" human "	0–4
" " " (4 × concentration)	0–5
Crystalline egg " (4.2 per cent)	41

* Active protease incubated with equal volume of test fraction for 1 hour at 20 C°.

‡ Data obtained with casein digestion method.

factors, protease, or protease-inhibitor fractions. The heating of the substrate during its preparation also would tend to inactivate such contaminants. It was also noted that in no case did the protease samples ever show fibrinogenolysis without demonstrating definite casein proteolysis.

Effect of Various Albumins as Protease Inhibitors.—The possibility that the egg albumin antigen was a protease inhibitor was investigated. Some serum albumin fractions were also assayed for protease-inhibiting activity. The serum fractions were prepared by ammonium sulfate fractionation. Streptokinase-activated human euglobulin protease was incubated at room temperature for 1 hour with equal amounts of the fraction to be tested. Samples of the protease-inhibitor mixture were then assayed for casein proteolysis. The results shown in Table II indicate the percentage of protease inhibition by the various fractions. Although the crystalline egg albumin exhibited significant inhibition of the protease, it is to be noted that the concentration of albumin used in this experiment was at least 100 times greater than any concentration employed in the subsequent protease activation experiments. The lack of inhibition by the

human albumin fractions and the marked inhibition by the rabbit and egg albumin fractions suggest a species specificity of antiproteases. Although Lewis and Ferguson (25) could not demonstrate any species specificity of whole serum antiproteases for their homologous proteases, Guest *et al.* (46) reported marked species variations in the inhibitory effects of sera on bovine protease. Duthie and Lorenz (47) reported marked species variation in the antitryptic power of different animal species with little variation in the sera of individual members of the same species. It is apparent that results obtained with serum protease and its inhibitors will vary, depending on the experimental conditions employed as well as on the origin and manner of preparation of the protease and the protease inhibitors.

Studies on Human Protease.—Experiments were carried out to determine whether precipitin reactions or the addition of preformed precipitates as a form of particulate matter to serum would activate the protease as had been reported by earlier investigators. These reports could not be substantiated when either whole immune or normal serum was used as protease sources. Since whole serum normally contains protease inhibitors, it was deemed necessary to perform the activation experiments under as ideal conditions as possible. Therefore serum fractions were employed which were known to be free of any normal protease inhibitors. These precursor fractions were also shown to be easily converted to active protease by a known activator, streptokinase. Even under these conditions no activation of the protease could be demonstrated such as was evidenced by the following observations:—

A mixture of immune rabbit globulin and its antigen at the equivalence point and human euglobulin protease precursor was incubated for 15 minutes at 37° C. followed by overnight incubation at 4° C. Saline and streptokinase controls were run simultaneously. The supernates and the precipitates that formed were then tested separately for casein proteolysis. No proteolytic activity could be demonstrated by the antigen-antibody euglobulin supernatant mixture, the precipitates, or the saline control. The human euglobulin preparation incubated with streptokinase under identical conditions exhibited strong casein proteolysis.

Since a known precursor fraction could not be activated during the occurrence of an antigen-antibody reaction, attempts to activate the human protease precursor fraction with a preformed antigen-antibody precipitate were carried out for two reasons: first, to determine whether particulate materials such as preformed precipitate could activate the precursor fraction and, second, because of the opinion of Opie (48) that an antigen-antibody precipitate is essential for the Arthus phenomenon.

Antigen-antibody precipitates were prepared aseptically and washed three times with sterile saline before use. In most of the experiments the precipitates were obtained at the equivalence point of the precipitin reaction but in some

the precipitates obtained from the antigen and the antibody excess zones were employed. In every instance the supernate from the precipitate obtained in this manner consistently yielded negative results when assayed for protease activity. Portions of these washed precipitates were then mixed with human euglobulin protease precursor and incubated under various conditions. The variables tested in these experiments were the temperature (4° C., 20° C.), duration of contact (5 minutes to 24 hours), and the protease precursor itself. The precursor was derived from six separate blood samples obtained from four normal human beings. Aliquots of the supernate mixtures and of the precipitate mixtures were then tested for casein proteolysis. No proteolytic activity could be demonstrated in any experiment with either of them. The control tubes consisting of saline in place of the precipitate were also negative while the streptokinase control tubes always showed the conversion of the precursor to an extremely active protease.

Since activation is dependent on whether the whole serum or euglobulin fraction is used, the preformed precipitates also were tested on various samples of fresh human serum. Owing to the presence of protease inhibitors in whole serum, the 3 minute contact time of Ungar and Mist (23) was employed. Using their procedure for isolating the euglobulin fraction which they found contains the protease, the fraction was tested for casein proteolytic activity. To check the possibility of any activated protease being adsorbed onto the precipitate, the precipitates in some of the experiments were centrifuged off after the stated contact period and also tested for casein proteolysis. The usual controls of saline and streptokinase were included in the experiments. On assay, the precipitates, the isolated euglobulin fraction of the precipitate-treated serum, and saline-treated serum were shown to be devoid of proteolytic activity. The euglobulin fractions from the streptokinase-treated sera always exhibited marked casein proteolysis.

As an incidental part of this study, the possibility that active protease could be adsorbed onto the preformed precipitate was investigated. Preformed precipitates were added to various concentrations of a streptokinase-activated human euglobulin protease and incubated at different time intervals. The supernates were separated from the precipitates as completely as possible and both preparations were tested for casein proteolysis. In no case did the precipitates ever show any proteolytic activity beyond that attributable to the small amount of supernate left in contact with the precipitate. The supernates from these tests, as well, remained unchanged in their proteolytic activity.

Studies on Immune Rabbit Serum Protease.—Initial experiments consisted of attempts to demonstrate the activation of the protease of an immune rabbit serum or immune rabbit globulin with its homologous antigen. The rabbit sera and the globulins prepared from the sera were always titered for the presence and the amount of precipitable antibody. The sera were obtained only from

rabbits which had exhibited a strong Arthus reaction at the end of the course of immunization.

Crystalline egg albumin was added to fresh rabbit antisera and/or the immune globulin fraction. The antigen was added in the zones of antigen and antibody excess and at the equivalence point. The incubation periods for the reactants varied from the 3 minute contact time technic to overnight. Incubation temperatures were 37° C., 20° C., and 4° C. in different experiments. These combinations all resulted in failure to activate the protease precursor of the rabbit immune serum or globulin as determined by our casein digestion assay method. The saline controls were also negative. That these sera did contain a protease precursor was demonstrated by adding streptokinase to aliquots of each sample and incubating under the same conditions. These sera treated in this manner yielded protease activity of 7 to 13 units. However, streptokinase did not activate the corresponding rabbit globulin fractions in confirmation of the findings of other investigators (16, 18).

Since some activators of serum protease are known to possess species specificity, experiments utilizing preformed precipitates also were carried out with immune rabbit globulin and immune rabbit serum. Again, to exclude the possible effect of protease inhibitors in the experiments involving whole rabbit serum, the 3 minute contact technic was employed. These experiments with the rabbit globulin and whole serum were performed under the same conditions as previously described in the studies on human protease. In these experiments as well, no protease activation could be detected by the use of preformed precipitate as an activator. The streptokinase controls showed positive casein digestion only with the whole serum. Negative results were obtained when a fourfold concentrated rabbit globulin fraction was used, irrespective of the time of incubation of the reactants. Thus, regardless of the presence or absence of possible protease inhibitors of the serum, or of the duration of the incubation period, no protease activation could be demonstrated by preformed antigen-antibody precipitates, or by antigen-antibody complexes formed in the presence of the precursor, even under conditions thought to be best suited for activation.

Studies on Guinea Pig Serum Protease.—Since the results with human serum and with the rabbit antibody-antigen system resulted in no protease activation, investigations proceeded on a guinea pig antibody-antigen system, which had been reported to result in protease activation (23).

Preliminary experiments consisted of treating five guinea pigs with Merck's soluble egg albumin exactly as described by Ungar and Mist (23). The treatment of five other guinea pigs varied from their technic only in that crystalline ovalbumin replaced the crude egg albumin. In addition, five more animals received the same volume of saline in place of the antigen to serve as controls. After the time interval of 25 days as recommended by the above authors, the serum of each animal was obtained by cardiac puncture. Antigen and saline were added to separate aliquots of the sera of treated and control animals, and the euglobulin fraction was isolated as prescribed by these authors. However, assay for possible protease

activation by antigen or saline was done with our casein proteolytic method instead of the fibrinogenolytic method used by Ungar and Mist. A further aliquot of each serum was tested for precipitable antibodies by the ring test technic.

TABLE III

Effect of Antigen and Saline on Guinea Pig Serum Protease Activation

Treated* guinea pigs		Proteolytic units‡			Fibrinogenolytic method§		Precipitin test	Normal guinea pigs		Proteolytic units‡			Fibrinogenolytic method§	
		Saline	Soluble egg albumin	Crystalline egg albumin	Saline	Soluble egg albumin				Saline	Soluble egg albumin	Crystalline egg albumin	Saline	Soluble egg albumin
Males	1	15		11.8			−	Males	11	>15		>15		
	2	>15		>15			−		12	>15		>15		
	3	>15		14			−		13	>15		>15		
	4	< 0.5		< 0.5			+		14	>15	>15			
	5	>15		>15			−		15	>15	>15			
	6	1.8	1.5				−		26	>15	>15		1–50	1–50
	7	>15	>15				−		27	5.4	6.6		0	0
	8	>15	>15				−		28	0	0		0	0
	9	< 0.5	1.0				−		29	1.0	0		0	0
	10	>15	>15				−		30	0	0		0	0
	16	>15	>15		1–50	1–50	−							
	17	2.6	3.5		0	0	−							
	18	0	0		0	0	−							
	19	0	0		0	0	−							
	20	0	0		0	0	−							
Females	21	0	0		0	0	−	Females	31	2.7	2.3		1–10	0
	22	<0.5	<0.5		0	0	−		32	<0.5	<0.5		0	0
	23	0	0		0	0	−		33	0	0		0	0
	24	0	0		0	0	−		34	0	0		0	0
	25	<0.5	<0.5		0	0	−							

* Pigs given a single injection of antigen.

‡ Data obtained by casein proteolysis method.

§ Highest dilution which indicated fibrinogenolysis by failure to clot upon addition of thrombin.

The results obtained with these animals (Nos. 1 to 15) are shown in Table III. It can be observed that no significant differences occurred in the degree of activation of serum protease for normal and treated animals. Although the lack of serum prevented measuring titers beyond 15 units, it should be noted that three of the treated pigs showed less activation with the antigen than when saline was added to the sera. In treated guinea pig 4, there was no activation at all, regardless of the activating agent used. It also is of interest that with this

particular set of animals, the members of the control group all showed as much (or more) protease activation as those of the treated groups. It therefore seems apparent that there was no significant difference in protease activation whether antigen or saline was added to the serum of either treated or normal animals.

Because these results were divergent from those of Ungar and Mist who had reported significant protease activation with the antigen-treated serum as compared to their saline controls, the experiments were repeated with twenty new guinea pigs obtained from a different source.

Ten males and ten females were placed in separate cages. Five animals of each sex were randomly selected and injected with the soluble egg albumin antigen. The remaining animals were treated with saline and regarded as the control animals. The technic of Ungar and Mist was again carried out in as exact detail as possible. Since the possibility existed that their fibrinogenolytic method of protease assay might be measuring a different enzyme than the casein digestion method generally used here, both methods of protease assay were used on all of the serum samples from these guinea pigs.

The comparative data for animals 16 to 34 are shown in Table III. It is worthy of note that whereas the first set of guinea pigs exhibited high values of protease activation, the second set of animals, except for animal 16 demonstrated negative or low values of activity. The data obtained agree with the results obtained with the first set of guinea pigs 1 to 15 in that no difference in protease activity could be shown whether antigen or saline was added to serum samples from either the albumin-injected or saline-injected animals. The instances of demonstrable proteolytic activity may be explained by either the acidification dilution method of obtaining the euglobulin fraction which at times leads to spontaneous activation (17), or possibly to shock due to trauma or hemorrhage (10, 49, 50) occurring during cardiac puncture of the animal.

The concurrent use of both assay methods also precludes the possibility that the failure to duplicate the results of Ungar and Mist may have been due to a substrate specificity of the protease activated.

A possible explanation for the discrepancy of our results compared with those of the above authors may rest on the lack of sensitization of our animals as only in guinea pig 4 could precipitable antibodies be demonstrated (see Table III). To explore this possibility, the animals were reimmunized with the soluble egg albumin.

With the first subcutaneous reinjection, one of the animals died of anaphylactic shock (No. 16) while another (No. 17) exhibited anaphylactic symptoms with recovery. The remaining animals were desensitized with an 0.05 ml. (20 mg./ml.) injection of the antigen. Succeeding subcutaneous inoculations were made without any further symptoms or fatalities. The injections were continued until positive precipitin titers were obtained. These animals also developed Arthus reactions during the course of immunization.

Table IV records the results of assaying the attempted protease activation of the immune sera by antigen and by saline. Again the results show no significant difference in the protease activation whether antigen or saline was added to the serum of sensitized guinea pigs. The activity of the sera of treated animals in this experiment corresponded rather closely to that previously obtained and shown in Table III, with the exceptions that pig 17 showed some decrease in

TABLE IV

Activation of Immune and Normal Guinea Pig Serum Protease

Guinea pig serum (1.5 ml.)		Proteolytic units*			Precipitin test
		Activators added to serum‡			
		Saline	Merck egg albumin	Staphylokinase	
Sensitized animals					
Males	17	0	0		+
	18	0	0		++
	19	2.2	1.4		+
	20	0.7	0	>15	+
Females	21	0	0	>15	++
	22	0	0	>15	++
	23	0	0	>15	++
	24	<0.5	0	>15	+
Controls					
Males	27	11.8	4.6		−
	28	0	0		−
	29	>15	>15		
Females	31	1.5	4.1	>15	−
	32	2.2	13.5		−
	33	0	0	>15	

Results obtained with these sera by the fibrinogenolytic assay method were all negative except where staphylokinase was added.

* Data obtained by casein proteolysis method.

‡ 0.1 mg. antigen or 0.1 ml. saline or staphylokinase added per ml. serum.

activity and pig 19 some increase in activity. The serum of the control animals, however, seemed to show greater variability although with no definite pattern. The activation of serum 31 and 32 would seem to be confirmatory evidence that the amount of egg albumin employed did not exhibit any inhibitory action on the possible activation of serum protease.

As a control for the potential protease precursor in the serum, staphylokinase was used as an activator wherever enough serum was available. Strong proteolysis of the casein substrate was always demonstrable in these instances.

As a further indication for the presence of the precursor, most of the euglobulin samples derived from these experiments were treated with staphylokinase. Although the activity of these fractions did not yield as high an activity as the original whole serum, each sample thus treated exhibited significant protease activity compared with that of the saline-treated aliquots. The staphylokinase-treated samples ranged from 2 to 5 units while every saline-treated sample yielded zero units.

DISCUSSION

The experiments and results described in this study indicate that activation of serum protease by an antigen-antibody combination does not occur to any significant extent, at least *in vitro*. Although only one antigen was used in this study, the fact that negative results were obtained by the use of the sera of three different species would tend to discredit this type of reaction as being a general phenomenon. Our results obtained with the rabbit antiovalbumin-ovalbumin system are in direct conformity with the results of Geiger (24) employing this combination as one of his immune systems. Furthermore, Geiger had shown that even the activation that can be effected with another immune system generally falls within the upper limits of spontaneous activation obtained with untreated and normal animals.

The divergence of results obtained in this work from those of Ungar and Mist, involving activation of guinea pig serum protease with antigen cannot be explained on the available evidence. Although Geiger confirmed the results of Ungar and Mist with mucopolysaccharides as guinea pig serum activators, he could not demonstrate the activation of rabbit serum with these preparations. He did not report any experiments employing guinea pig serum sensitized to egg albumin, although his results with rabbit serum sensitized to this antigen were negative. Furthermore, McIntire and Roth (51) reported that the addition of antigen to sensitized rabbit blood did not activate enough protease to cause detectable lysis of a plasma clot.

Although Ungar and Mist reported their guinea pigs to be sensitized to egg albumin, they did not record any serological studies to demonstrate this sensitivity. In our study only one of twenty animals showed any precipitable antibodies (Table III) when immunized according to their method, and even this animal exhibited no specific protease activation. Thus, even though these authors did demonstrate the activation of the serum of their treated animals with antigen, one can not state unequivocally that this activation was due strictly to the antigen-antibody combination. It must be recognized, however, that there is great variability in the activation pattern of protease in individuals of the same species as seen in Tables III and IV.

The early evidence previously cited on which much of the protease theory of allergic reaction is based deserves comment. Although Jobling *et al.* (5, 6, 20) had

concluded that particulate matter or a precipitin reaction resulted in serum proteolytic activity which presumably yielded substances causing anaphylaxis, in none of their papers do they describe the employment of an actual precipitin test as a confirmatory procedure. Their conclusions could not be substantiated here as shown by the negative results obtained with the numerous precipitin tests carried out in this investigation. Negative results, as well, were always obtained in the attempts to activate serum protease with preformed precipitates.

The similar conclusions of Bronfenbrenner (21, 22) have also to be reevaluated. He immunized animals against raw egg white and demonstrated precipitins had formed against the antigen. He reported that following the mixing of the fresh immune serum with homologous antigen, previously boiled for 30 minutes, toxic substances formed through the release of proteolytic "ferments" by this antigen-antibody union. Thus, Bronfenbrenner in considering this a specific antigen-antibody reaction assumed that the specificity of the antigen was not altered by the boiling process. However, Furth (52–53) reported profound changes in the antigenic structure of heated proteins. He demonstrated that antisera to native crystalline egg albumin would no longer react with egg albumin heated to 100°C. for only 5 minutes. He concluded a new specificity was produced by heating as demonstrated by anaphylaxis, Arthus phenomenon, and precipitin reactions. There is still the possibility that heat-stable antigens exist in egg white and were responsible for the results obtained by Bronfenbrenner.

Another point in opposition to the protease theory of allergy is that although protease activation could not be demonstrated by our rabbit or guinea pig antiovalbumin-ovalbumin systems, severe Arthus reactions and precipitable antibodies were readily demonstrated in the first mentioned animals. Anaphylaxis as well as the Arthus phenomena and precipitable antibodies was also observed in the guinea pigs. If the release of protease was the significant factor involved in these reactions, it would seem, from a physiological point of view, that in general all antigen-antibody systems should be capable of activating significantly large amounts of protease. This apparently does not hold true for it can be observed in Tables III and IV that no consistent differences occur in the content of protease precursor in the normal or sensitized animals. Euler and Heller (54) could find no difference in the amount of protease activation, using staphylokinase as an activator, between normal and hypersensitive rabbit euglobulin fractions.

The results of this study therefore are further evidence against the activation of serum protease by an antigen-antibody combination. It would also seem that the protease content of serum during anaphylaxis is not primarily dependent upon an antigen-antibody union.

SUMMARY

The results obtained in this study indicate that serum protease is not activated by either a rabbit or guinea pig antiovalbumin-ovalbumin system, *in vitro*.

A precipitin reaction occurring in the presence of a serum protease precursor of three species (human, rabbit, and guinea pig) failed to activate the protease precursor. Furthermore, particulate material as preformed precipitates could not be shown to activate the protease of either human or rabbit serum or their euglobulin fractions.

The material presented seems to be further evidence against the postulated role of serum protease in immunologic systems.

BIBLIOGRAPHY

1. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, **55,** 690.
2. Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942, **27,** 1119.
3. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Inv.*, 1942, **21,** 525.
4. Christensen, L. R., *J. Gen. Physiol.*, 1945, **28,** 363.
5. Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, **19,** 480.
6. Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, **20,** 37.
7. Astrup, T., and Permin, P. M., *Nature*, 1947, **159,** 681.
8. Astrup, T., *Nature*, 1947, **160,** 571.
9. Tagnon, H. J., and Petermann, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70,** 359.
10. Tagnon, H. J., and Palade, G. E., *J. Clin. Inv.*, 1950, **29,** 317.
11. Lewis, J. H., and Ferguson, J. H., *J. Clin. Inv.*, 1950, **29,** 1059.
12. Lewis, J. H., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1951, **78,** 184.
13. Milstone, J., *J. Immunol.*, 1941, **42,** 109.
14. Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66,** 525.
15. Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyles, P. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68,** 246.
16. Gerheim, E. B., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 261.
17. Cliffton, E. E., and Downie, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1950, **73,** 559.
18. Cliffton, E. E., and Cannamela, D. A., *Proc. Soc. Exp. Biol. and Med.*, 1951, **77,** 305.
19. Lewis, J. H., and Ferguson, J. H., *Am. J. Physiol.*, 1951, **166,** 594.
20. Jobling, J. W., Eggstein, A. A., and Petersen, W. J., *J. Clin. Inv.*, 1915, **21,** 239.
21. Bronfenbrenner, J., *J. Exp. Med.*, 1915, **21,** 221.
22. Bronfenbrenner, J., *J. Exp. Med.*, 1915, **21,** 480.
23. Ungar, G., and Mist, S. H., *J. Exp. Med.*, 1949, **90,** 39.
24. Geiger, W. B., *J. Immunol.*, 1952, **68,** 11.
25. Lewis, J. H. and Ferguson, J. H., *J. Clin. Inv.*, 1950, **29,** 486.
26. Grob, D. J., *J. Gen. Physiol.*, 1943, **26,** 405.
27. Loomis, E. C., Ryder, A., and George, C., Jr., *Arch. Biochem.*, 1949, **20,** 444.
28. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28,** 559.
29. Remmert, L. F., and Cohen, P. P., *J. Biol. Chem.*, 1949, **181,** 431.

30. Kaplan, M. H., *J. Clin. Inv.*, 1946, **25,** 331.
31. Christensen, L. R., and Smith, D. H., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 840.
32. Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Inv.*, 1942, **21,** 533.
33. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12,** 1.
34. Burdon, K. L., *Fed. Proc.*, 1949, **8,** 401.
35. Burdon, K. L., Guthrie, R. R., and Rich, D. F., *Fed. Proc.*, 1951, **10,** 404.
36. Scroggie, A. E., Jaques, L. B., and Rocha e Silva, M., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66,** 326.
37. Rocha e Silva, M., Bier, O., and Aronson, M., *Nature*, 1951, **168,** 465.
38. Schlamowitz, S. T., and De Graff, A. E., *Circulation*, 1950, **1,** 816.
39. Schlamowitz, S. T., De Graff, A. C., and Schubert, M., *Circulation*, 1950, **1,** 833.
40. Schlamowitz, S. T., De Graff, A. C., and Schubert, M., *Circulation*, 1951, **3,** 413.
41. Ungar, G., and Damgaard, E., *J. Exp. Med.*, 1951, **93,** 89.
42. Dragstedt, C. A., and Wells, J. A., *Quart. Bull. Northwestern Univ. Med. School*, 1944, **18,** 104.
43. Cole, A. G., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30,** 1162.
44. Culbertson, J. T., and Seegal, B. C., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29,** 909.
45. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46,** 2066.
46. Guest, M. M., Daly, B. M., Ware, A. G., and Seegars, W. H., *J. Clin. Inv.*, 1948, **27,** 785.
47. Duthie, E. S., and Lorenz, L., *Biochem. J.*, 1949, **44,** 167.
48. Opie, E. L., *J. Immunol.*, 1924, **9,** 259.
49. MacFarlane, R. G., and Biggs, R., *Lancet*, 1946, **2,** 862.
50. Cliffton, E. E., *J. Lab. and Clin. Med.*, 1952, **39,** 105.
51. McIntire, F. C., and Roth, L. W., *Fed. Proc.*, 1952, **11,** 104.
52. Furth, J., *J. Immunol.*, 1925, **10,** 777.
53. Furth, J., *J. Immunol.*, 1926, **11,** 215.
54. von Euler, H., and Heller, L., *Ark. Kemi.*, 1951, **2,** 581.