THE AUTODIGESTION OF NORMAL SERUM THROUGH THE ACTION OF CERTAIN CHEMICAL AGENTS. I.

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INTRODUCTION.

In contrast with the so called defensive ferment, or "*Abwehrferment*" of Abderhalden, which has been recently much studied and discussed, comparatively little attention has been paid to the proteolytic ferment in normal serum, to which only occasional brief references can be found.

Abderhalden[1] has stated that he sometimes found a proteolytic ferment in the sera of guinea pigs and rabbits, which he held to have arisen through the introduction of foreign proteins, such as those due to the ingestion of plants, or to infectious diseases, especially coccidiosis. Stephan[2] reported that guinea pig serum shows an apparently polyvalent proteolytic power. Fuchs[3] found that rabbits inoculated with serum gave a positive ninhydrin reaction with other kinds of substrates, and he explained this result by assuming that the sera of herbivorous animals contain a comparatively large amount of dialyzable substance. Michaelis and von Lagermarck[4] obtained a positive Abderhalden reaction not only with pregnant serum but also with non-pregnant and even male serum, and they came to the conclusion that they could not confirm the existence of the specific ferment in Abderhalden's sense. Van Slyke, Vinograd-Villchur, and Losee[5] also

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[1] Abderhalden, E., Abwehrferment. Das Auftreten blutfremder Substrate und Fermente im tierischen Organismus unter experimentellen, physiologischen und pathologischen Bedingungen, Berlin, 4th edition, 1914, 53–54.

[2] Stephan, R., Die Natur der sogenannten Abwehrfermente, *Münch. med. Woch.*, 1914, lxi, 801.

[3] Fuchs, A., Tierexperimentelle Untersuchungen über die Organspezifität der proteolytischen Abwehrfermente (Abderhalden), *Münch. med. Woch.*, 1913, lx, 2230.

[4] Michaelis, L., and von Lagermarck, L., Die Abderhaldensche Schwangerschaftsdiagnose, *Deutsch. med. Woch.*, 1914, xl, 316.

[5] Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., The Abderhalden reaction, *J. Biol. Chem.*, 1915, xxiii, 377.

found proteolytic ferment in non-pregnant human serum by means of Van Slyke's method of amino nitrogen determination.

The existence, then, in normal human and animal serum, of a non-specific proteolytic ferment which digests certain proteins other than the serum has often been proved, but little investigation into the nature of this ferment has hitherto been made. The question of autodigestion of normal serum has received some attention from a few investigators, Delezenne and Pozerski[6] having observed the autolysis of the serum under the influence of chloroform.

The present paper deals with a phenomenon of the autodigestion of normal serum brought about with certain chemical agents under various conditions.

Materials and Methods of Study.

Guinea pig serum was used chiefly in the present investigation, because it possesses advantage over other sera in its constancy and its richness in the ferment in question. Since, to secure uniformity of results, it was necessary to provide a sufficiently large quantity of serum for each series of experiments, with small animals a pool had to be made of many specimens from animals killed at the same time. When guinea pigs were used, the blood was withdrawn from the heart under general anesthesia by means of a sterile test-tube provided with a sharp cannula. The blood was collected in a sterile paraffined centrifuge tube, and upon coagulation it was centrifuged to separate the serum from the clot. By this method a clear serum, absolutely free from any trace of hemolysis, may be obtained. It is important to note that for the demonstration of autodigestion of normal serum through the intervention of certain chemical substances no specimen which contains hemoglobin should be employed, since, as will be shown later, the presence of hemoglobin and stroma, whether homologous or alien, leads to the appearance of digestive products and renders the issue of the self-digestion of the serum indecisive. The experiments were carried out with fresh active serum, although it was found that the activity of the serum is not perceptibly impaired by standing at a temperature of 6°C. for many days.

The amino substances normally contained in serum were previously removed by dialysis. The serum was placed in sterile celloidin sacs and was allowed to dialyze for 5 hours at room temperature in a sterile salt solution which renewed itself from a flow from another bottle placed above the level of the dialysis vessel. The celloidin sacs were preserved in sterile distilled water with a layer of toluene

[6] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327, cited by Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, xix, 460.

and before use were washed repeatedly with sterile salt solution. A layer of toluene protected the serum from bacterial interference during dialysis. The volume of the serum at the completion of dialysis was increased from one and a half times to twice its original volume. To secure a constant concentration the dialyzed serum was diluted with sterile salt solution until the volume became twice that of the original serum; that is, the dialyzed serum was made one-half of the original concentration. The dialyzed serum thus obtained, when kept in the refrigerator at 6°C., does not lose its proteolytic power for a long time, at least not for 3 or 4 weeks. We therefore kept in this way a sufficient supply of serum to complete many successive experiments with the same material.

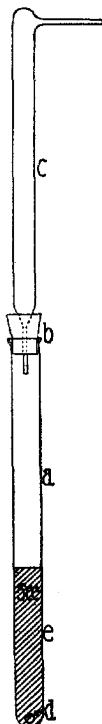
The technique for dialysis was somewhat similar to that recommended by Abderhalden. The dialyzing thimbles used were those made by Schleicher and Schüll bearing the mark of 579A. In order to select perfect thimbles, it was necessary to test beforehand their permeability and intactness by means of solutions of silk peptone (Höchst) and egg white. Those which leaked or showed unusual porosity or retardation of dialysis were discarded as unsuitable. A serum to be tested for digestion was measured into a thimble which stood inside a sterile Jena glass wide mouthed flask. The height of the thimble and that of the flask were about the same, and the former was held upright by the edge of the latter. 15 cc. of sterile distilled water were poured into the flask outside the thimble. At the termination of dialysis, the fluid outside the thimble, representing the dialysate, was removed for determination of the amount of dialyzable proteins diffused out of the serum contained within the thimble. For this purpose the ninhydrin reaction was resorted to.[7] Since this reaction requires a temperature above 100°C. maintained for at least 1 minute, it was not easy to obtain a uniform and constant result, owing to rapid evaporation and frequent loss of the fluid incidental to the violent bubbling caused by the application of a direct flame to the test-tube containing the dialysate and ninhydrin solution. A few previous workers have attempted to eliminate errors arising from this source by using a liquid paraffin bath instead of a direct flame. The overboiling of the fluid from the test-tube placed in the paraffin oil bath at a temperature above 100°C. was greatly reduced by a specially devised stopper,[8] but we have found this device of little value, since it fails to prevent the loss of fluid by explosive escape of vapor, which forces out the fluid gathering about the narrow exit for steam.

After experiments with various devices a satisfactory result was obtained with the use of one suggested by Dr. Noguchi and illustrated in Text-fig. 1. With this

[7] The Van Slyke apparatus for the determination of amino nitrogen was also used in certain series of experiments where the amounts of the split products were sufficiently large to use this apparatus, but in ordinary experiments the amounts were too minute to permit its use.

[8] Oeller, H., and Stephan, R., Technische Neuerungen zur Dialysiermethode, *Deutsch. med. Woch.*, 1913, xxxix, 2505.

apparatus only occasionally does a small amount of the fluid escape. It consists of a hard glass test-tube (Pyrex), 1 cm. in diameter and 20 cm. in height (*a*), connected through a perforated rubber stopper (*b*) with another, somewhat narrower test-tube (*c*), the mouth of which is drawn into a long narrow neck to fit the stopper, and which has a narrow side arm near the bottom. When connected, the smaller test-tube, with narrow openings at both ends, stands in-



TEXT-FIG. 1. Apparatus used for the ninhydrin test. *a*, test-tube connected through a perforated rubber stopper (*b*) with another, narrower test-tube (*c*). In the lower test tube is a glass bead (*d*) which facilitates uniform diffusion of heat during the boiling of the mixture of dialysate and ninhydrin solution (*e*).

verted. The stopper may be lifted out with the upper tube attached and the mixture (*e*) of the dialysate (5 cc. as a rule) and ninhydrin solution (1 cc. of a 1: 1,000 solution for 5 cc. of dialysate) placed in the lower test-tube, with a glass bead (*d*), which facilitates uniform diffusion of heat during the boiling. The upper portion is then tightly refitted, and the fluid is ready for boiling. The paraffin oil bath is made by filling an enameled pan with a sufficient amount of

the oil to give a depth of about 12 cm., which will cover nearly two-thirds of the height of the lower test-tube containing the fluid for heating. The bath should have width enough to hold a metal rack containing several tubes, as it is a great advantage to heat the entire series of tubes used in the experiment at the same time. It may be mentioned that the heating period is an important factor in relation to the intensity of the ninhydrin reaction. The color which manifests itself on cooling is gradually increased as the heating period is prolonged, although it was impossible to ascertain definitely, on account of the rapid evaporation of the fluid, at what rate and how long the increase proceeded. It was found, however, that the reaction at the end of 1 minute was much weaker than that of 5 minutes, and that after 10 minutes much stronger than that of 5 minutes' duration. A comparison of the intensity of the reaction was, of course, made after the volume of the fluid had been restored to the original standard by adding distilled water to the 5 cc. mark in the tube. It is easily seen, therefore, that a reaction which increases in intensity through minute errors due to inaccurate time limits would be greater during the preliminary few minutes than at the end of 5 minutes or longer. For this reason, throughout the entire experiment, instead of the 1 minute period of other investigators, we heated the fluid for exactly 5 minutes at a temperature of 150°C., or as near 150°C. as possible, the temperature being maintained by means of an oil bath in a wind-proof hood. At the end of 5 minutes the tubes were taken out of the bath and left at room temperature for 30 minutes before the reaction was read. In order to obtain a uniform and comparable result the content of each tube, which was reduced almost one-half through evaporation, was filled with distilled water up to the original volume of the dialysate; namely, 5 cc. in our experiment. The intensity of the reaction varied from a mere nuance to a distinct violet, with many intermediate grades. It was therefore necessary to prepare a standard by which different degrees of the reaction could be determined. Alanine was selected for producing the required color reaction by ninhydrin. 0.01 cc. of this substance, in 0.1 N solution, gives a distinct violet color, while 0.0025 cc. gives only a faint violet, when present in 5 cc. of distilled water. It was therefore possible to prepare a series of tubes in which color scales, based upon the gradually increasing amounts of 0.1 N alanine solution, were obtainable.

In the present study the reaction produced by 0.01 cc. of a 0.1 N solution of alanine in 5 cc. of distilled water was chosen as the standard. In an estimation of color intensity there may be two procedures. One is to have many grades of the color for comparison with a given reaction. The other, which is the one adopted in the present work, is to have one standard and to estimate the intensity of a given reaction by noting the amount of distilled water necessary to reduce the color exactly to correspond with the standard. If a given reaction requires a quadruple dilution to reach the standard, its intensity must be considered quadruple the standard; a reaction requiring triple or double dilution would be triple or double in strength.

For practical purposes, we have arbitrarily designated the reaction ++++ when the standard was attained by diluting with 3 to 3.9 cc. of water, +++ with 2 to 2.9 cc., ++ with 1 to 1.9 cc., and + with 0.9 cc. or less. Reactions weaker than this were recorded as <+ and ±, which corresponded with a mixture of 1 cc. of the standard and water up to 1 cc. and that containing more than 1 cc. of water, respectively. The reactions may be briefly summarized as follows:

++++ for a reaction requiring 3 to 3.9 cc. of water to make it correspond with the standard.
+++ for a reaction requiring 2 to 2.9 cc. of water.
++ for a reaction requiring 1 to 1.9 cc. of water.
+ for a reaction requiring 0 to 0.9 cc. of water.
<+ for a reaction corresponding with standard 1 cc. + water up to 1 cc.
± for a reaction corresponding with standard 1 cc. + water more than 1 cc.

The ninhydrin reaction with amino-acid undergoes, within a day or so, a rapid discoloration, which cannot be prevented even by preserving the tubes in a refrigerator at 6°C. A suitable substitute was sought, therefore, among various violaceous aniline dyes, and it was found that a certain high dilution of crystal violet resembles very much the ninhydrin reaction, when carefully adjusted to the standard color of the latter, and remains unaltered for a long time, provided it is kept in a dark refrigerator. The standard color solution of crystal violet was utilized for the reading of the reaction because of its stability. It should be added, however, that when subjected to further dilution, the relative color values and effect no longer run parallel.

The proteolytic activity of the serum was tested not only for the autodigestion caused by chemical reagents, but also by using as substrates some pure preparations of plant or animal proteins and various animal tissues or blood corpuscles. When animal tissues were used, they were freed from blood, boiled, and emulsified exactly as in the procedure recommended by Abderhalden. All these substrates were dialyzed in a celloidin sac before use in order to remove any dialyzable protein substances which might be contained in some of the preparations.

Occurrence of the Proteolytic Ferment in Normal Guinea Pig Serum.

To 2 cc. of the dialyzed guinea pig serum various substrates, as shown in Table I, were added and digested in thimbles for 16 hours at 37°C. The control tests done with each substrate alone gave no color reaction, whereas those done with 2 cc. of dialyzed guinea pig serum gave a reaction of only ±.

As will be seen from the table, the guinea pig serum, when incubated with some animal and plant proteins, produces dialyzable substances which show a positive ninhydrin test. Whether the serum in this case really digested the substrates, or whether the former was

TABLE I.

Effect of the Proteolytic Ferment of Normal Guinea Pig Serum on Different Substrates.

Substrate boiled.	Ninhydrin test.	Substrate boiled.	Ninhydrin test.	Substrate boiled.	Ninhydrin test.
Guinea pig liver.	+++	Rabbit placenta.	+++	Cat serum.	±
" " corpuscles.	+++	" serum.	±	" fibrin.	±
" " placenta.	+++	" fibrin.	±	Sheep corpuscles.	+++
Chicken liver.	+++	Dog corpuscles.	+++	" fibrin.	±
" corpuscles.	+++	" serum.	±	Egg white.	±
" serum.	±	" fibrin.	±	Casein (Hammersten).	+++
Rabbit liver.	+++	Cat liver.	+++	Edestin (Merck).	+++
" corpuscles.	+++	" corpuscles.	+++	Ricin (Merck).	++
Guinea pig serum.	±	Dog liver.	+++	Sheep serum.	±

brought to autodigestion only by the influence of the substrates, is not shown by this experiment. The question will be discussed in more detail below. Among the substrates tested, the serum and fibrin of various animals and egg white remained indifferent to the proteolytic ferment of serum. The presence of such a polyvalent proteolytic ferment in normal serum is already known.

Autodigestion of Normal Serum through the Action of Certain Chemicals.

Quite distinct from the proteolytic phenomenon already described is the autodigestion of normal serum brought about through the intervention of non-nitrogenous chemicals such as acetone, alcohols, and chloroform. Table II gives the results obtained when these

TABLE II.

Autodigestion of Normal Serum as a Result of Treatment with Certain Chemical Reagents.

Test No.	Dialyzed guinea pig serum.	Chemical reagents.	Digested.	Ninhydrin test.
	cc.			
1	2.0	Acetone (Kahlbaum) 0.8 cc.	In thimble at	+++
2	2.0	Methyl alcohol (Kahlbaum) 1.0 cc.	37°C. for 16	+++
3	2.0	Chloroform (Kahlbaum), shaken, 2.0 cc.	hrs.	+++
4	2.0	Salt solution 1.0 cc.		±

chemicals were added to the dialyzed guinea pig serum, and the mixture was incubated at 37°C. for 16 hours. This phenomenon seems to suggest a sort of activation of the serum ferment by these chemicals.

The object of the following experiment was to determine the optimal concentration of acetone for a given volume of serum in order to cause autodigestion. The dialyzed guinea pig serum (2 cc.) was mixed in test-tubes with 1 cc. of acetone of various concentrations. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a thimble and digested at 37°C. for 16 hours. The dialysates outside the thimbles were tested with ninhydrin (Table III).

TABLE III.

Optimal Concentration of Acetone to Activate Serum.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Concentration of acetone mixture.	Ninhydrin test.
	cc.	*cc.*	*cc.*	*per cent*	
1	2.0	0	1.0	$33\frac{1}{3}$	–
2	2.0	0.3	0.7	$23\frac{1}{3}$	+++
3	2.0	0.5	0.5	$16\frac{2}{3}$	+
4	2.0	0.7	0.3	10	±
5	2.0	0.8	0.2	$6\frac{2}{3}$	±
6	2.0	0.9	0.1	$3\frac{1}{3}$	±
7	2.0	1.0	0	0	±

As will be seen from the table the optimal concentration of acetone is very limited, and, according to repeated tests, it lies between 23 and 28.5 per cent. Either a lower or a higher concentration than this causes less effect, and no digestion takes place beyond a certain point. An amount of acetone which is sufficient to produce a strong turbidity or precipitation in the serum destroys the serum ferment at the same time, and there is no means of securing an active ferment by precipitating it from the serum with acetone. The same is true when the serum is precipitated with alcohol. The extreme lability of the serum ferment against acetone and alcohol presents a striking contrast to pepsin, trypsin, and other ferments, which, as is well known, withstand treatment with these reagents.

In autodigestion the serum no doubt plays the part of the ferment solution as well as of the substrate; hence, the more serum is used,

ceteris paribus, for digestion, the more split products are to be found in the dialysate. The relation of varying amounts of serum to digestion, under constant acetone concentration, was considered in the next experiment (Table IV). The result shows that under the same conditions of total liquid volume and acetone concentration the concentrated serum solution produces more dialyzable substances than the diluted one.

TABLE IV.

Relation between Various Amounts of Serum and the Degree of Autodigestion under Constant Acetone Concentration.

Test No.	Dialyzed guinea pig serum.	Salt solution.	Acetone.	Ninhydrin test.
	cc.	*cc.*	*cc.*	
1	2.0	0.2	0.8	+++
2	1.0	1.2	0.8	+
3	0.5	1.7	0.8	<+
4	0.25	1.95	0.8	±
5	0.1	2.1	0.8	–
6	0.05	2.15	0.8	–
7	2.0	1.0	0	±

Influence of Higher Temperature on the Serum Protease.

The test-tubes, each containing 2 cc. of the dialyzed guinea pig serum, were placed in the water bath regulated at 37°, 55°, and 60°C., respectively. After 30 minutes the tubes were taken out of the baths and allowed to cool at room temperature. An adequate amount of acetone or substrate (boiled chicken liver) was then added to the serum to permit detection of the presence of the ferment (Table V). The proteolytic ferment of serum, as the result shows, survives exposure to 55°C. for 30 minutes, but it is completely destroyed by heating at 60°C. for 30 minutes. The serum to which a suitable amount of acetone or a substrate had been added did not undergo autodigestion when placed in the incubator at 55°C. The optimal temperature for the action of this ferment seems to be 37°C.

TABLE V.

Resistance of the Serum Protease to Temperature.

Test No.	Dialyzed guinea pig serum.	Temperature applied.	Further treatment.	Ninhydrin test.
	cc.	°*C.*		
1	2.0	37	Acetone 0.8 cc.	+++
2	2.0		Substrate.	+++
3	2.0		Salt solution 0.8 cc.	±
4	2.0	55	Acetone 0.8 cc.	+++
5	2.0		Substrate.	+++
6	2.0		Salt solution 0.8 cc.	±
7	2.0	60	Acetone 0.8 cc.	±
8	2.0		Substrate.	±
9	2.0		Salt solution 0.8 cc.	±

Digestion Experiment in Test-Tubes without Simultaneous Dialysis.

In the preceding experiment autodigestion proceeded simultaneously with dialysis, since the serum and substrates or chemical activators were placed in a dialyzing thimble from the beginning. The question naturally arose whether or not the rate of digestion would be equally great if the mixture were put in a test-tube instead of a dialyzing thimble. There was reason to think that certain chemical activators such as acetone or alcohols would exert in test-tubes an injurious effect upon the serum ferment when added in proportions optimal for a dialyzing thimble, because in the latter a continuous reduction of the chemicals through osmosis must constitute a factor for yielding a maximum hydrolysis. In other words, the amounts of the reagents for digestion in thimbles would be too large for an optimal action of the ferment in test-tubes. This proved to be the case, as may be seen from the experiment recorded in Table VI.

Test 1 is a control test, showing the digesting power of the serum alone, without any treatment. Test 2 is another control, which demonstrates positive autodigestion caused by acetone. Test 3 shows that the serum loses its proteolytic power when mixed with acetone and kept at a temperature of 37°C. for 30 minutes. Test 4 shows that the acetonized serum which stands at room temperature for 30

minutes and then at 37°C. for 30 minutes is also inactivated. Test 5 shows that inactivation also takes place when the serum is acetonized immediately after being taken out of the water bath. Test 6 shows that a previous incubation of the serum for 30 minutes at 37°C. has no injurious effect upon the ferment action if acetone is introduced after the serum has been sufficiently cooled by standing 30 minutes after the bath.

The foregoing experiments indicate that the quantity of acetone inducing an optimal digestion of the serum in a dialyzing thimble destroys the ferment in 30 minutes when the mixture is kept at 37°C.,

TABLE VI.

Effect of Acetone upon the Serum Protease in the Test-Tube at Different Temperatures.

Test No.	Dialyzed guinea pig serum 2 cc. in test-tube.					Transferred into thimble; digested at 37°C. for 16 hrs.
	At 37°C. in water bath.	At room temperature.	Acetone.	At room temperature.	At 37°C. in water bath.	Ninhydrin test.
	min.	*min.*	*cc.*	*min.*	*min.*	
1						±
2		30	0.8	30		+++
3			0.8		30	±
4			0.8	30	30	±
5	30		0.8	30		±
6	30	30	0.8	30		+++

while no injurious effect can be detected when it is kept at room temperature for half an hour.

That the use of the dialyzing thimble is an important factor in attenuating the destructive action of acetone upon the ferment through rapid exosmosis of the reagent is shown by the presence in the dialysate of some acetone soon after dialysis began. Prevention of exosmosis of acetone from the dialyzing thimble by the addition to the outside water of acetone in exactly the same proportion as that contained in the serum within the thimble results in total inactivation of the serum protease, as will be seen from the following experiment.

In Test 7, 2 cc. of dialyzed guinea pig serum were placed in a thimble with 0.8 cc. of acetone. Instead of the usual distilled water 15 cc., a mixture of dis-

tilled water 10.7 cc., and acetone 4.3 cc., was placed outside the thimble. The concentration of acetone was then equal on both sides of the thimble. As was expected, at the end of the usual incubation period, no digestion was found to have taken place.

Unlike acetone, chloroform and tissue substrates exert no injurious action upon the ferment, even when employed in excessive quantities; hence autodigestion by means of these substances can be carried out in test-tubes.

Removal of the Activating Reagents from the Mixture with Serum.

The phenomenon of autodigestion of serum through the intervention of certain reagents, belonging chiefly to the group of so called fat solvents, arouses interest as to the causes underlying this interaction. With acetone and the simpler alcohols it was noticed that a faint opalescence appears when the reagents are mixed with serum in the optimal proportion. Whether or not this slight physical change has any relation to autodigestion is not apparent. Moreover, in the case of chloroform, which is an excellent activator, no perceptible change, except the emulsification of the serum, takes place. One might assume that autodigestion is brought about by the extraction of fatty and lipoidal substances from the serum proteins, thus enabling the serum protease to act upon the delipolyzed proteins. But ether, benzene, toluene, or petroleum ether, in spite of their delipolyzating powers, have no activating property. At all events, it seemed important to ascertain what would happen if the chemicals once mixed with the serum were extracted from the mixture. As will be shown in the following experiments, it was found that serum once acetonized or treated with other suitable chemical activators in proper proportions remains autodigestive even after the activators are completely removed. The continued presence of the activating reagents in the serum is not necessary in order to induce autodigestion.

The chemical activators can be eliminated from the mixture with serum either by (1) evaporation, (2) dialysis, or (3) treatment with other indifferent substances which free the serum from the activating chemicals.

Evaporation Method.—Evaporation by means of vacuum is preferable, because it can be done at a lower temperature and with the least risk of bacterial contamination. A temperature above 15°C. should be avoided, since, in the mixture with activators, the activity of the ferment is highly sensitive to higher temperatures.

A mixture of serum with an adequate amount of a chemical activator is put into a large sterile Petri dish, the cover replaced, and the whole placed in a desiccator, which is then exhausted by means of vacuum. As soon as the pressure drops below a certain point, the

TABLE VII.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.		Digested in thimble. Dialysate.	
					Test for acetone.	Ninhydrin test.
	cc.	*cc.*				
1	2.0	0	Controls. No further treatment.		–	±
2	2.0	0.8			++	+++
3	2.0	0.8	Acetone evaporated to the point when bubbling ceased. Volume restored to 2 cc.		+	+++
4	2.0	0.8		Acetone 0.8 cc.	++	+++
5	2.0	0.8	Acetone completely removed by desiccation *in vacuo.* Volume restored to 2 cc.		–	+++
6	2.0	0.8		Acetone 0.8 cc.	++	+++
7	2.0	0.8		Boiled.	–	–

contents of the dish begin to bubble. During the bubbling care must be taken to avoid loss of the liquid by overflow by regulating the speed of evaporation. The liquid ceases to bubble in a few minutes, as much of the activating reagents is already driven out of the mixture, but the odor reveals the presence of the small amount remaining. For complete removal of the reagents, evaporation must be continued until the contents of the dish are quite or nearly dried up. The residue obtained is then redissolved in sterile distilled water of a volume equal to that of the original dialyzed serum.

Acetone, chloroform, and methyl alcohol can be easily removed by this method, on account of their having a lower boiling point than the higher alcohols. But the higher series of alcohols, having a higher boiling point than that of distilled water, cannot be satisfactorily eliminated by this method.

TABLE VIII.

Effect of the Removal of Chloroform from the Serum Mixture by Evaporation in Vacuo.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble. Ninhydrin test.
	cc.				
1	2.0	Control. No further treatment.			±
2	2.0	Shaken with chloroform repeatedly; stood at room temperature for 30 min.			+++
3	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		±
4	2.0			Shaken again with chloroform.	+++
5	2.0	Shaken with chloroform repeatedly; stood at 6°C. for 30 min.			+++
6	2.0		Chloroform completely evaporated. Volume restored to 2 cc.		+++
7	2.0			Shaken again with chloroform.	+++
8	2.0			Boiled.	–

The behavior of the serum ferment after it has been freed from its activators by evaporation is shown in Tables VII and VIII. These tables show that acetone and chloroform can be completely removed from the mixture without any loss in the autodigestive activity of the serum, since it had already been activated by the reagents (Table VII, Test 5; Table VIII, Test 6). There is, however, a slight difference between the two reagents in their mode of action. The activating action of acetone is rather rapid, while that of chloro-

form is much slower (Table VIII, Test 3), requiring nearly 2 hours to insure an activation which will endure after the evaporation of the chemical.

Dialysis Method.—This method can be used only for the elimination of water-soluble substances, such as acetone and the lower alcohols. It is unavailable for chloroform and certain higher alcohols which are insoluble or less soluble in water. In order to utilize

TABLE IX.

Effect of the Removal of Acetone from the Serum Mixture by Evaporation and Dialysis.

Test No.	Dialyzed guinea pig serum.	Acetone.	Further treatment.			Digested in thimble. Dialysate.	
						Ninhydrin test.	Test for acetone.
	cc.	*cc.*					
1	2.0	0	Controls. No further treatment.			±	–
2	2.0	0.8				+++	++
3	2.0	0.8	Acetone partly evaporated immediately after having been mixed with serum.			+++	+
4	2.0	0.8		Residual acetone removed by dialysis for 2 hrs.		±	–
5	2.0	0.8			Acetone 0.8 cc.	+++	++
6	2.0	0.8	Acetone partly evaporated after standing with serum for 30 min. at room temperature.			+++	+
7	2.0	0.8		Residual acetone removed by dialysis for 2 hrs.		+++	–
8	2.0	0.8			Acetone 0.8 cc.	+++	++

celloidin membrane for dialysis it was necessary to remove, by a brief preliminary evaporation *in vacuo*, much of the reagent from the mixture, as the presence of acetone in such a concentration may affect the membrane. Complete removal of the reagent is then effected by dialysis in celloidin sacs for 2 hours in running salt solution. This combined method was used for the serum employed in Table IX.

As Test 4 shows, digestion cannot take place when the acetone is removed immediately after being mixed with the serum. If removal is begun after the mixture has already been allowed to stand at room temperature for 30 minutes, however, there is no difference in the ultimate outcome (Test 7). It is therefore advisable, in order to insure a thorough activation, to keep the mixture of serum and acetone at room temperature for at least 30 minutes before further treatment is started.

Extraction with Indifferent Fat Solvents.—As indifferent substances for the removal of chloroform or acetone from the serum by extraction, ether and petroleum ether were used, since they were found to possess neither an activating nor an injurious effect upon the serum

TABLE X.

Effect of Extraction by Means of Petroleum Ether of the Acetonized Serum.

Test No.	Kind of serum.	Further treatment.	Digested in thimble. Ninhydrin test.
1	Extracted serum 2 cc.	Alone.	+++
2		Acetone 0.8 cc.	+++
3		Emulsion of residuum 0.5 cc.	+++
4	Unextracted dialyzed guinea pig serum 2 cc. (controls).	Alone.	±
5		Acetone 0.8 cc.	+++
6		Emulsion of residuum 0.5 cc.	±

ferment. It was understood from the beginning that even by repeated and renewed extractions the acetone or alcohols cannot be completely exhausted from the serum admixtures. However, a point of interest in this mode of extraction lies in the fact that by it not only the added chemicals, but also the native fats and lipoids are removed, as is not the case in the evaporation or dialysis methods. Methyl alcohol is far less amenable to extraction from its mixture with serum, either by ether or by petroleum ether. An experiment in which this method was used follows.

4 cc. of acetone were mixed with 10 cc. of dialyzed guinea pig serum in a large centrifuge tube. After the mixture had been standing for 30 minutes at room temperature 10 cc. of petroleum ether were added to the liquid, which was then shaken energetically. The

emulsified liquid was centrifuged and the clear upper layer, consisting of petroleum ether and acetone, separated with a pipette. The extraction procedure was repeated five times and the extracted serum subsequently placed in a vacuum apparatus in order to remove the petroleum ether. The portions of petroleum ether containing fractions of acetone and representing several renewed extractions were reunited and evaporated *in vacuo*. The residue was emulsified in 1 cc. of salt solution (Table X).

As far as the experiment is concerned, the extraction of the acetone from the acetonized serum with petroleum ether makes no difference in the digesting process (Test 1). In other words, the absence of the substances of serum soluble in petroleum ether and acetone has no influence on the autodigestion of serum. It is interesting to note further that the addition of the lipoidal emulsion had neither an inhibitory action nor an accelerating influence upon the ferment activity of either the extracted (Test 3) or the unextracted (Test 6) serum. There was no antiferment in this fraction against the serum protease in question.[9]

Influence of Reactions upon the Serum Protease.

It is well known that the activity of a ferment is greatly influenced by the reaction of the medium in which it is found. In order to ascertain the optimal reaction for the serum protease, experiments were performed in which the digestion of the serum was carried out in various reactions. For this purpose amounts ranging from 0.01 to 1 cc. of a 0.1 N solution of hydrochloric acid or sodium hydroxide were added to a number of test-tubes, each containing a mixture of dialyzed guinea pig serum 2 cc., and acetone 1 cc. The total volumes of the mixtures were made uniformly 4 cc. by adding salt solution in the necessary amounts. The mixtures were allowed to stand at room temperature for 30 minutes and then were transferred

[9] The inactivity of the lipoid and fatty constituents of serum as an antiferment is attributed by Jobling and Petersen (*J. Exp. Med.*, 1914, xix, 549) to an imperfect dispersion after they are once extracted. By saponification they found them to be highly antienzymic. It seems open to discussion whether the antienzymic property of an unsaturated soap can explain the original antiferment of the serum.

to a corresponding number of dialyzing thimbles for incubation. The thimbles were placed in dialyzing flasks containing distilled water to which such quantities of acid or alkali were added as would make the reaction correspond exactly with the acidity or alkalinity of the contents of each thimble. The digestion was continued for 16 hours at 37°C. On account of the disturbing effect of acid or alkali upon the ninhydrin reaction, the acidity or alkalinity of the

TABLE XI.

Effect of Acid and Alkali on the Autodigestion of Serum.

Test No.	Dialyzed guinea pig serum.	Acetone.	Acid or alkali.	Salt solution.	Concentration of reaction in medium.*	Digested in thimble. Ninhydrin test.
	cc.	*cc.*	*cc.*	*cc.*		
			0.1 N hydrochloric acid.			
1	2.0	1.0	1.00	0.00	N/40 hydrochloric acid.	−
2	2.0	1.0	0.50	0.50	N/80 " "	−
3	2.0	1.0	0.25	0.75	N/160 " "	−
4	2.0	1.0	0.10	0.90	N/400 " "	±
5	2.0	1.0	0.05	0.95	N/800 " "	+++
6	2.0	1.0	0.01	0.99	N/4,000 " "	+++
7	2.0	1.0	0	1.00	0	+++
			0.1 N sodium hydroxide.			
8	2.0	1.0	0.01	0.99	N/4,000 sodium hydroxide.	+++
9	2.0	1.0	0.05	0.95	N/800 " "	++
10	2.0	1.0	0.10	0.90	N/400 " "	−
11	2.0	1.0	0.25	0.75	N/160 " "	−
12	2.0	1.0	0.50	0.50	N/80 " "	−
13	2.0	1.0	1.00	0.00	N/40 " "	−

* The figures under this heading give the resulting degrees of the reaction in the mixtures. The alkalinity of the serum itself after dialysis is weaker than $\frac{N}{1,000}$ sodium hydroxide and is therefore ignored in the calculation (Test 7).

dialysates was neutralized upon the completion of digestion (Table XI). A parallel series of experiments was carried out with alanine solution as controls.

As may be seen from Table XI, the serum protease is highly sensitive to the change in the reaction of the medium. The optimal re-

action for the ferment action is that of the dialyzed serum, or at least is within the narrow limits on each side of it, either toward acid or alkaline. Even a slight deviation in the reaction beyond these limits affects the activity of the serum ferment.

Certain Chemical Reagents as Activators of the Serum Protease.

In addition to acetone, chloroform and some alcohols were found to be ferment activators, and there may be others which behave similarly. On the other hand, ethyl ether, petroleum ether, benzene, and toluene have neither an activating nor a paralyzing action. They are indifferent towards the serum protease.

Chloroform as a ferment activator has been much discussed in preceding sections. Chloroform has as much activating power as acetone. However, the simple addition of chloroform to serum does not have much effect. The mixture must be energetically and repeatedly shaken in order to insure activation. For digestion, the emulsion of the mixture as a whole may be placed in the incubator; or one may use only the upper semitransparent layer which appears when the emulsion is allowed to stand for a few minutes at room temperature, while the greater part of the clear transparent chloroform settles at the bottom of the tube. With chloroform there is no optimal proportion to be added to the serum; the ferment is not affected at all, even when the chloroform is added in excess to the serum. That chloroform requires a longer time for activating the serum ferment than does acetone has already been noted (Table VIII).

In the following experiments some monovalent saturated alcohols and ketones were tested for their activating property.

Varying amounts of different ketones and alcohols were added to 2 cc. of the dialyzed guinea pig serum in test-tubes. Before the addition of the reagents adequate amounts of salt solution were added to the serum in order that the total volume in each test should be 3 cc. With substances which are less soluble or insoluble in serum, the mixtures were repeatedly shaken. All the tubes were allowed to stand for 30 minutes at room temperature and then were transferred into dialyzing thimbles to be placed in the incubator at 37°C. for 16 hours (Tables XII and XIII).

The ketones and alcohols behave similarly towards the serum ferment. A certain optimal concentration activates ferment, and an excess injures it. Moreover, it seems to be a rule among the reagents that the higher molecular substances of the series are generally more active than the lower ones. The optimal concentration, therefore, for activating ferment was found to be approximately 33 per cent for methyl alcohol, 23 to 27 per cent for ethyl alcohol, and 20 per cent for isopropyl alcohol. This rule seems to apply also to

TABLE XII.

Activating Power of Ketones on the Serum Protease.

Dialyzed guinea pig serum.	Salt solution.	Reagent.		Test No.	Acetone.		Test No.	Methylethyl ketone.	
		Amount.	Concentration.		Appearance of mixture.	Ninhydrin test.		Appearance of mixture.	Ninhydrin test.
cc.	*cc.*	*cc.*	*per cent*						
2.0	0	1.0	33⅓	1	Turbid.	–	7	Emulsified.	–
2.0	0.3	0.7	23⅓	2	Slight turbidity.	+++	8	"	–
2.0	0.5	0.5	16⅔	3	Clear.	+	9	"	+*
2.0	0.7	0.3	10	4	"	±	10	Slight turbidity.	+
2.0	0.8	0.2	6⅔	5	"	±	11	Clear.	±
2.0	0.9	0.1	3⅓	6	"	±	12	"	±

* The optimal concentration of methylethyl ketone for digestion +++ lies between 10 and 16.7 per cent.

ketones, though the tested substances were very few. The rule is well defined only for the lower series of substances which can be mixed with water in any proportion. In the case of the higher series, which are less soluble in water, the relation is not so constant, as will be seen from the results of tests with butyl and amyl alcohols.[10] Finally, the still higher series, such as octyl alcohols, which are not soluble in water, have no activating power for the serum ferment.

[10] The isobutyl alcohol is soluble in 10.5 parts of water at 18°C., and the isoamyl alcohol in 39 parts of water at 16.5°C.

TABLE XIII.

Activating Power of Alcohols on the Serum Protease.

Reagent.		Methyl alcohol.		Ethyl alcohol.		Isopropyl alcohol.		Isobutyl alcohol.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	*per cent*								
1.0	$33\frac{1}{3}$	Opalescent.	+++*	Strong turbidity.	–	Strong turbidity.	–	Emulsified.	–
0.7	$23\frac{1}{3}$	"	+	Opalescent.	+++	Opalescent.	+	"	–
0.5	$16\frac{2}{3}$	"	<+	"	+	"	++†	"	–
0.3	10	Clear.	±	Clear.	±	Clear.	±	Strong turbidity.	<+
0.2	$6\frac{2}{3}$	"	±	"	±	"	±	Slight turbidity.	+++
0.1	$3\frac{1}{3}$	"	±	"	±	"	±	Clear.	±
0.07	$2\frac{1}{3}$							"	±
0.05	$1\frac{2}{3}$							"	±

Reagent.		Isoamyl alcohol.		Amyl alcohol, active		Octyl alcohol, normal.		Octyl Alcohol 2.	
Amount.	Concentration.	Appearance of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.	Appearance, of mixture.	Ninhydrin test.	Appearance of mixture.	Ninhydrin test.
cc.	*per cent*								
1.0	$33\frac{1}{3}$	Emulsified.	<+	Emulsified.	<+	Emulsified.	±	Emulsified.	±
0.7	$23\frac{1}{3}$	"	+	"	+	"	±	"	±
0.5	$16\frac{2}{3}$	"	+	"	+++	"	±	"	±
0.3	10	"	+++	"	+++	"	±	"	±
0.2	$6\frac{2}{3}$	"	+++	"	+++	"	±	"	±
0.1	$3\frac{1}{3}$	Moderate turbidity.	+++	Slight turbidity.	+++	"	±	"	±
0.07	$2\frac{1}{3}$	Slight turbidity.	±	Clear.	+	"	±	"	±
0.05	$1\frac{2}{3}$	Clear.	±	"	±	"	±	"	±
0.03	1	"	±	"	±	"	±	"	±
0.02	$\frac{2}{3}$	"	±	"	±	"	±	"	±
0.01	$\frac{1}{3}$	"	±	"	±	"	±	"	±

* The methyl alcohol, when added in a 1.5 cc. amount to 2 cc. of dialyzed serum, destroys the ferment.

† The concentration of isopropyl alcohol giving the maximal digestion is about 20 per cent.

SUMMARY AND CONCLUSIONS.

1. By means of certain chemical reagents, normal guinea pig serum can be brought to autodigestion without the presence of any foreign substrate. There exists in normal sera a highly characteristic protease.

2. The serum ferment survives heating at 55°C. for 30 minutes, but is completely inactivated at 60°C. for the same length of time.

3. The autodigestion of serum requires a temperature of about 37°C., and no noticeable digestion takes place at a temperature of 16°C. or lower.

4. Autodigestion of the serum may be brought about by chloroform and various saturated monovalent ketones and alcohols of the lower series.

5. The ketones and alcohols have a certain narrow limit of concentration for activating serum, beyond which the ferment is destroyed, even at room temperature.

6. The ketones and alcohols in concentrations regulated to activate serum at room temperature destroy the ferment when allowed to act on serum at 37°C. for 30 minutes. The elimination of the concentrated reagents from serum by evaporation or dialysis protects the ferment from their destructive action.

7. A certain length of time is required for the chemical activators to complete their action. In this respect chloroform is much slower than acetone.

8. The chemical activators may be removed from the activated serum by means of vacuum, dialysis, or extraction with certain indifferent chemicals without causing a return of the serum to its original non-autolytic state. Once activated by these reagents, the serum remains in the activated state, in spite of the removal of the activators.

9. The ferment is highly sensitive to the reaction of the medium, being readily inactivated when the reaction exceeds a certain narrow limit towards acid or alkaline. The optimal digestion is obtained with a faintly alkaline or neutral reaction.

This work was done in the laboratory of Dr. Hideyo Noguchi, under his direction.