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

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It has been shown that normal serum contains a characteristic protease whose activity is revealed through the action of certain chemical activators.[1] The seroprotease shows a thermal resistance similar to that of certain proteolytic enzymes, but is peculiar in its ready destruction by the action of acetone or alcohol, to which other ferments manifest a high degree of resistance.

In the present paper we have considered the relation which this ferment bears to the various fatty and lipoidal substances and also the existence of an antiferment in serum and its relation to the seroprotease. The material and method of study have been described in the previous paper.[1]

Relation of Neutral Fats, Fatty Acids, and Lipoids to the Serum Protease.

Since all the reagents, the activating effects of which have been discussed in the previous paper, belong to the group of so called fat solvents, it is not out of place to consider what part the fat or lipoid bodies may play in the autodigestion of serum caused by these reagents. The experiments were carried out partly by adding excessive amounts of fats or lipoids to the digesting mixtures, and partly by removing the native fats and lipoids from the serum by the use of fat solvents.

Several preparations of neutral fats and lipoid bodies were dissolved in acetone in high concentration, with the exception of lecithin, which, on account of its insolubility in acetone, was dissolved in methyl alcohol. Each substance was added to 2 cc. of the dialyzed

[1] Yamakawa, S., The autodigestion of normal serum through the action of certain chemical agents. I, *J. Exp. Med.*, 1918, xxvii, 689.

guinea pig serum in two different concentrations. Some of the solutions precipitated particles of the substance when mixed with the serum and formed a layer near the surface. After standing for 30 minutes at room temperature, the contents of each test-tube were transferred into a dialyzing thimble and incubated at 37°C. for 16 hours (Table I).

That cholesterol, lecithin, and the neutral fats such as triolein and tripalmitin, even when they are added in excess to the serum, are indifferent to the process of autodigestion is proved by these experiments. The weakness of digestion in cases where fatty acids are added to the serum may be explained in various ways. As was stated in the previous paper,[1] the serum ferment is extremely sensitive to an acid reaction and is undoubtedly influenced by the fatty acids. The inhibiting power of the oleic acid was found to be much stronger than that of the palmitic acid (Tests 13, 14, 17, and 18), when they are allowed to act upon the serum ferment in equal concentration. It is not improbable that the weakness of the latter is chiefly due to its inferior solubility in a medium containing much water and to its higher melting point.

The phenomenon might be explained in another way; namely, by a specific inhibiting power of an unsaturated fatty acid such as oleic against the serum ferment. Jobling and Petersen[2] found that the unsaturated fatty acids in serum act as antitrypsin, and that they can be removed by extraction with ether or chloroform. But their results with trypsin do not find an analogy with the serum protease. As has been said, acetone or chloroform can impart their activating power to the serum ferment, and they do so without eliminating any of the native elements from it; a subsequent removal of the reagents from the activated serum does not restore the original resistance to autodigestion. Moreover, ether, toluene, benzene, and petroleum ether do not act as activators for the serum protease. The following experiment was undertaken in order to determine the effect of complete removal of the fats, fatty acids, and lipoids from the serum upon the phenomenon of autodigestion.

[2] Jobling, J. W., and Petersen, W., The nature of serum antitrypsin, *J. Exp. Med.*, 1914, xix, 459.

TABLE I.

Effect of Fatty Substances on the Autodigestion of Serum.

Test No.	Acetone solution of fats added to 2 cc. of dialyzed guinea pig serum.		Acetone.	Acid reaction.		Ninhydrin test.
				In thimble.	Of dialysate.	
		cc.	*cc.*			
1	10 per cent oleic acid.	0.8	0	+	−	<+
2		0.08	0.72	+	−	++
3	10 per cent triolein.	0.8	0	−	−	+++
4		0.08	0.72	−	−	+++
5	0.3 per cent palmitic acid.	0.8	0	<+	−	++
6		0.08	.72	<+	−	+++
7	0.3 per cent tripalmitin.	0.8	0	−	−	+++
8		0.08	0.72	−	−	+++
9	Cholesterol saturated.	0.8	0	−	−	+++
10		0.08	0.72	−	−	+++
11	Guinea pig serum alone (controls).		0.8			+++
12			0			±

Test No.	Chloroform solution of fats added to 2 cc. of dialyzed guinea pig serum.		Chloroform.	Acid reaction.		Ninhydrin test.
				In thimble.	Of dialysate.	
		cc.	*cc.*			
13	10 per cent oleic acid.	1.0	0	+	−	<+
14		0.1	0.9	+	−	++
15	10 per cent triolein.	1.0	0	−	−	+++
16		0.1	0.9	−	−	+++
17	10 per cent palmitic acid.	1.0	0	+	−	++
18		0.1	0.9	+	−	++
19	10 per cent tripalmitin.	1.0	0	−	−	+++
20		0.1	0.9	−	−	+++
21	Guinea pig serum alone (controls).		1.0			+++
22			0			±

TABLE I—*Concluded.*

Test No.	Dialyzed guinea pig serum.	1 per cent ovo-lecithin emulsion in salt solution.	Salt solution.	Methyl alcohol.	Acid reaction.		Ninhydrin test.
					In thimble.	Of dialysate.	
	cc.	*cc.*	*cc.*	*cc.*			
23	2.0	1.0		1.5	−	−	+++
24	2.0	0.1	0.9	1.5	−	−	+++
25	2.0	1.0	0	0	−	−	±
26	2.0	0	1.0	1.5	−	−	+++

10 cc. of guinea pig serum were completely dried in the desiccator by means of vacuum. The residue was ground into powder, placed in a flask, and treated with 100 cc. of absolute ether. The flask was kept for 48 hours in the refrigerator, repeatedly shaken at intervals, and the solvent three times renewed. At the expiration of this period the ether was decanted, the precipitate was washed with another 100 cc. of ether, and the trace of the solvent was removed *in vacuo*. The dried powder was then dissolved in 10 cc. of sterile distilled water and dialyzed in salt solution to remove the dialyzable substances. After dialysis the serum was diluted to 20 cc. with salt solution and used for the tests (Table II).

TABLE II.

Autodigestion of the Serum Delipolyzed with Ether.

Test No.	Extracted guinea pig serum.	Further treatment.	Ninhydrin test.
	cc.		
1	2.0	No further treatment.	±
2	2.0	Acetone 0.8 cc. added.	+++
3	2.0	Shaken with 1 cc. of chloroform.	+++
4	2.0	Methyl alcohol 1 cc. added.	+++
5	2.0	Substrate (chicken liver) added.	+++

The experiment shows that the extraction of fatty substances from the dried serum with ether causes no change with regard to the phenomenon of autodigestion of the serum.

The Inhibitory Substance in Native Serum against the Serum Protease.

It is generally known that human or animal serum has an inhibitory effect upon various proteolytic ferments, such as pepsin, trypsin, leukoprotease, and autolytic ferment. The results of the investiga-

tions on the influence of serum on the serum protease will be described here.

The investigation divided itself into two parts: (1) the digestion of heterologous substrate by the guinea pig serum ferment, and (2) the autodigestion of serum caused by the chemical reagents already mentioned. In the latter case particular care was taken to remove the chemical reagents completely from the treated serum before the sample of native serum which was to be tested for its inhibitory power was added, because, should any trace of the activators still be present, it would lead to an activation of the serum thus introduced. Acetone was used throughout the experiment because of the ease with which it can be completely removed from the serum mixtures. The dialyzed serum, acetonized and then deacetonized, will be designated, for the sake of brevity, as "activated serum."

The result shown in Table III indicates that the larger the amount of the dialyzed guinea pig serum added, the greater is the digestion of the substrate. On the other hand, the addition of a dialyzed horse serum caused neither increase nor decrease of digestion by guinea pig serum (Table IV). The horse serum itself was inactive.

The result of the autodigestion test with activated serum distinctly shows the presence of an inhibitory substance in a dialyzed but otherwise unmodified serum (Table V). The contradictory results in both cases will be discussed later.

The serum antienzymes directed against various proteolytic ferments disappear from the serum when the latter is heated to a certain temperature. The two following experiments were undertaken to determine the thermal resistance of the antiseroprotease.

1 cc. of dialyzed guinea pig serum was heated in the water bath at different temperatures for varying periods of time. The heated sera, after having been cooled, were added to 2 cc. of the activated serum in tubes, allowed to stand at room temperature for 30 minutes, and transferred as usual into thimbles for incubation and dialysis (Table VI).

According to this experiment, the inhibitory substance in unmodified or native serum withstands heating at 55°C. for 30 minutes, whereas it is destroyed by exposure at 60°C. for the same period. The thermal resistance of the antiseroprotease coincides with that

TABLE III.

Digestion of a Substrate with Guinea Pig Serum in Increasing Quantities.

Dialyzed guinea pig serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate Ninhydrin test.
cc.	*cc.*		
2.0	2.0	+++	±
3.0	1.0	++++	±
4.0	0	++++	<+

TABLE IV.

Effect of a Heterologous Serum on the Digestion of a Substrate by Guinea Pig Serum.

Dialyzed guinea pig serum.	Dialyzed horse serum.	Salt solution.	Digested in thimble with chicken liver. Ninhydrin test.	Digested in thimble without substrate. Ninhydrin test.
cc.	*cc.*	*cc.*		
2.0	0	2.0	+++	±
2.0	1.0	1.0	+++	±
2.0	2.0	0	+++	±
0	2.0	2.0	±	—

TABLE V.

Inhibitory Power of the Homologous Serum against the Autodigestion of an Activated Guinea Pig Serum.

Test No.	Activated guinea pig serum.*	Homologous guinea pig serum.*	Salt solution.	Digested in thimble. Ninhydrin test.
	cc.	*cc.*	*cc.*	
1	2.0	2.0	0	<+
2	2.0	1.0	1.0	<+
3	2.0	0.5	1.5	<+
4	2.0	0.25	1.75	++
5	2.0	0.1	1.9	+++
6	2.0	0.05	1.95	+++
7	2.0	0	2.0	+++
8	0	2.0	2.0	±

* Both sera were previously dialyzed, and the mixture of both had been allowed to stand for 30 minutes at room temperature before being placed in the incubator at 37° C.

TABLE VI.

Inactivation of the Antiseroprotease of Guinea Pig Serum by Heating.

Test No.	Activated guinea pig serum.	Guinea pig serum 1 cc. exposed to various temperatures.	Ninhydrin test.
	cc.		
1	2.0	Not heated. Clear.	<+
2	2.0	30 min. at 55°C. Clear.	<+
3	2.0	30 " " 60° " Slightly turbid.	+++
4	2.0	30 " " 65° " Opalescent.	+++
5	2.0	30 " " 70° " "	+++
6	2.0	5 " " 100° " Coagulated.	+++
7	2.0	Salt solution 1 cc.	+++
8	Salt solution 2 cc.	Not heated. Clear.	±

TABLE VII.

Thermal Resistance of the Protease and Its Antisubstance in Serum.

Tests for protease.				Tests for antisubstance.				Heated serum alone.		
Test No.	Native guinea pig serum 1 cc. heated at 55°C. for.	Acetone added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.	Activated guinea pig serum added.	Ninhydrin test.	Test No.	Native guinea pig serum 2 cc. heated at 55° C. for.	Ninhydrin test.
	min.	*cc.*			*min.*	*cc.*			*min.*	
1	30	0.8	+++	6	30	2.0	<+	12	30	±
2	60	0.8	+	7	60	2.0	++	13	60	±
3	120	0.8	±	8	120	2.0	+++	14	120	±
4	240	0.8	−	9	240	2.0	+++	15	240	−
5	Guinea pig serum not heated, 2 cc.	0.8	+++	10	Guinea pig serum not heated, 1 cc.	2.0	<+	16	Guinea pig serum not heated, 2 cc.	±
				11	Salt solution 1 cc.	2.0	+++			

of the serum protease itself.[3] This fact was proved again in the next experiment.

The unmodified or native guinea pig serum, 1 cc., was exposed to a temperature of 55°C. for various periods of time. Each heated serum was mixed

[3] Yamakawa,[1] Table V.

with acetone or activated serum respectively, to be tested for its proteolytic and antiproteolytic power. After standing for 30 minutes at room temperature the mixtures were transferred into thimbles and placed in the incubator (Table VII).

Exposed to a temperature of 55°C., both the ferment and the antisubstance remain unimpaired for 30 minutes, but their activity gradually diminishes after a longer time, finally disappearing after 2 hours. A dissociation of the ferment from its antisubstance through heating was found to be impossible.

Effect of the Adsorbing Substances on the Serum.

Certain inorganic substances, which had been previously sterilized by heating, were put into the dialyzed guinea pig serum in a proportion of 5 gm. to 10 cc. The mixtures were allowed to stand at room

TABLE VIII.

Digesting Power of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.	Further treatment.	Ninhydrin test.
1	Serum treated with kaolin 2 cc.	No further treatment.	−
2		Acetone 0.8 cc. added.	−
3		Substrate added.	−
4	Serum treated with charcoal 2 cc.	No further treatment.	−
5		Acetone 0.8 cc. added.	±
6		Substrate added.	−
7	Serum treated with talc 2 cc.	No further treatment.	−
8		Acetone 0.8 cc. added.	±
9		Substrate added.	−
10	Serum treated with silicious marl 2 cc.	No further treatment.	−
11		Acetone 0.8 cc. added.	±
12		Substrate added.	−
13	Serum treated with barium sulfate 2 cc.	No further treatment.	−
14		Acetone 0.8 cc. added.	±
15		Substrate added.	−
16	Untreated serum 2 cc. (controls).	No further treatment.	±
17		Acetone 0.8 cc. added.	+++
18		Substrate added.	+++

temperature for an hour, with repeated shakings, and then centrifuged. The clear supernatant fluids were used for the experiment (Table VIII).

As may be seen from Tables VIII and IX, the proteolytic ferment can be easily removed from serum by adsorbents, but the antisubstance, on the other hand, still remains in the treated serum.

TABLE IX.

Antienzymic Action of the Serum Treated with Adsorbents.

Test No.	Kind and amount of guinea pig serum.		Activated serum added.	Acetone added.	Ninhydrin test.
		cc.	*cc.*	*cc.*	
1	Guinea pig serum treated with kaolin.	2.0	0	0	−
2		2.0	0	0.8	−
3		1.0	2.0	0	<+
4	Guinea pig serum treated with talc.	2.0	0	0	−
5		2.0	0	0.8	±
6		1.0	2.0	0	<+
7	Salt solution.	1.0	2.0	0	+++
8	Guinea pig serum.	1.0	2.0	0	<+

Occurrence of the Proteolytic Ferment and Its Antisubstance in the Sera of Different Animals.

It would surely have been an advantage if we could have found larger animals which would furnish us with a serum as rich in the

TABLE X.

The Proteolytic Ferment and Its Antisubstance in the Sera of Different Animals.

Kind of serum.	No. of tested specimens.	Dialyzed serum alone 2 cc.	Dialyzed serum 2 cc. + acetone 0.8 cc.	Dialyzed serum 1 cc. + activated guinea pig serum 2 cc.
Human serum.....	2	+	<+	<+
Dog "	5	±	<+	<+
Cat "	2	±	<+	<+
Rabbit "	8	±	<+	<+
Horse "	2	−	<+	<+
Guinea pig serum.....	Over 100	±	+++	<+

serum protease as that of the guinea pig. The results of examinations of various animal sera, however, showed that the guinea pig is the only animal whose serum is exceedingly rich in the proteolytic ferment. On the other hand, the sera of other animals, while poor in their content of protease, contain a considerable amount of the antisubstance capable of counteracting the action of the autolytic ferment of guinea pig serum. The result of the digestion tests with the sera of different animals is shown in Table X.

Mode of Digestive Action of the Serum Ferment.

It has been stated in a previous paragraph that the proteolytic ferment of serum, when it is incubated with substrate, can produce the dialyzable substances despite the presence of native serum, while in the autodigestion of activated serum, the ferment action is inhibited by the addition of native serum. There seems to be a certain difference in the mode of action in the two instances.

The explanation of the autodigestion of the activated serum may probably be sought in the destruction or paralysis of the antienzymic substance through the treatment. Reagents such as certain ketones and alcohols, when their optimal concentration for activation is reached, may destroy the antienzyme, but not the enzyme, thus enabling the latter to exert its full activity upon the serum proteins. The concentration of reagent which dissociates the ferment from its antisubstance lies between narrow limits, and when it exceeds the upper limit, the ferment itself is also destroyed.

In autodigestion the protein in the treated serum must serve as substrate, because there is nothing else present to be hydrolyzed. But what is the origin, then, of the dialyzable substance produced when the serum is incubated with various tissue substrates? There are two possibilities for the source of the protein derivatives: first, the substrates may be directly digested by the serum ferment; second, it may be assumed that the homologous tissues are not really digested, but that they act only as an adsorbing agent which removes the antienzyme and leaves the freed autolytic ferment to digest its own serum protein. The latter explanation was advanced by Bron-

fenbrenner[4] in the Abderhalden reaction, in which pregnant human serum, when incubated with placenta tissue, gives a positive ninhydrin test. He states that pregnant serum is able to show autodigestion in the incubator when allowed to remain in contact with

TABLE XI.

Antienzymic Action of Normal Serum after Treatment with Substrate at 0.5°C.

Test No.	Dialyzed guinea pig serum.	Further treatment.			Digested in thimble. Ninhydrin test.
	cc.				
1	2.0	Substrate added. Tubes left at 0.5° C. for 16 hrs.	Centrifuged. Substrate removed. Supernatant fluid alone used for tests.		±
2	2.0			Boiled.	−
3	2.0			Acetone 0.8 cc.	+++
4	1.0			Activated guinea pig serum 2 cc.	<+
5	2.0		Substrate *in situ*.		+++
		Control tests.			
6	2.0	Without any treatment.			±
7	2.0	Acetone 0.8 cc. added.			+++
8	2.0	Substrate added.			+++
9	1.0	Activated guinea pig serum 2 cc. added.			<+
10	Salt solution 1 cc.	Activated guinea pig serum 2 cc. added.			+++

placenta tissue for 16 hours on ice and then separated from the substrate. He ascribes the phenomenon to adsorption of the antienzymic substance by the substrate impregnated with a specific antibody contained in the serum of a pregnant subject.

The next experiment was undertaken to determine whether this

[4] Bronfenbrenner, J., On the present status of the Abderhalden reaction, *J. Lab. and Clin. Med.*, 1915–16, i, 79.

mode of interpretation was applicable in our case, in which a heterologous, non-specific substrate is treated with normal serum (Table XI).

Tests 1 and 4 show that normal guinea pig serum, when it is kept on ice with substrate, is neither activated nor deprived of its antienzymic substance. In other words, the normal serum is indifferent to the treatment, contrary to the result which Bronfenbrenner reported to have obtained with human pregnant serum and placenta tissue. But this result does not exclude the possibility of the adsorption of the antisubstance by ordinary substrates in the incubator at a temperature of 37°C.

To determine the fate of the antienzymic substance in serum, after digestion, the serum was treated according to four methods, as follows:

Serum A.—8 cc. of the dialyzed guinea pig serum were kept in the incubator with substrate in four thimbles for 16 hours, 2 cc. being placed in each thimble. At the expiration of this time, when the dialysate showed a ninhydrin reaction of +++, the serum in the thimbles was separated from the substrate layer and every trace of the latter removed by means of centrifugation. The serum was then dialyzed in a celloidin sac against salt solution to eliminate the split products of protein contained in it.

Serum B.—8 cc. of the activated guinea pig serum were kept in thimbles in the incubator for 16 hours. The ninhydrin test of the dialysate showed a reaction of +++. The sera in the thimbles were reunited and dialyzed as mentioned above.

Serum C (Control 1).—The dialyzed guinea pig serum, 8 cc., without any substrate and without being activated, was treated in the same way as the other two sets of serum; *i.e.*, incubated in thimbles and afterwards dialyzed.

Serum D (Control 2).—The dialyzed guinea pig serum without any preliminary treatment.

These sera were further treated as shown in Table XII and digested in thimbles at 37°C. for 16 hours. The dialysates were tested as usual.

The result of this experiment indicates that the serum, the proteolytic power of which has already been exhausted by treatment with substrate, still contains its antienzymic substance (Serum A, Test 4), while the latter is no longer found in the activated serum after digestion (Serum B, Test 4). There is no doubt that in the former case the digestion can take place in spite of the presence of

the antienzymic substance. We have no more reason in this instance to assume the occurrence of an indirect digestion of serum protein, due to the absorption of the antienzymic substance through the substrate, because the substrate does not absorb the antienzyme under the experimental conditions here recorded. It seems justifiable, therefore, to conclude that the serum ferment directly digests the protein of the heterologous substrate, while in the case of the activated serum, the ferment splits its own serum protein after the antienzymic substance has been removed by the treatment with ketones or alcohols.

TABLE XII.

Fate of the Antienzyme Substance in Serum after Digestion.

Test No.	Amount of serum.*	Further treatment.	Serum A	Serum B	Serum C	Serum D
	cc.					
1	2.0	No further treatment.	−	−	−	±
2	2.0	Acetone 0.8 cc.	−	±	++	+++
3	2.0	Substrate.	−	+	++	+++
4	1.0	Activated guinea pig serum 2 cc.	<+	+++	<+	<+
5	1.0	Dialyzed guinea pig serum 2 cc. + substrate.	+++	+++	+++	+++

* The volume of the serum was increased after secondary dialysis by about one-fourth.

SUMMARY AND CONCLUSIONS.

1. The neutral fats, fatty acids, and lipoid bodies of serum seem to play no part in autodigestion. Neither the addition of fats or lipoids in excess to the serum, nor their removal by extraction with ether influences the phenomenon of autodigestion.

2. There is present in native serum an antienzymic substance which is closely related to the autolytic ferment of serum.

3. The antiseroprotease of normal serum has almost the same thermal resistance as the seroprotease; that is, it survives heating at 55°C. for 30 minutes but is completely inactivated at 60°C. for the same length of time.

4. The ferment can be removed from the serum by means of inorganic adsorbents, but the antienzymic substance remains in the treated serum.

5. The autolytic power of the sera of man and other animals is much weaker than that of guinea pig serum, but they contain as much as does the latter of the antisubstance which inhibits the digestion of the activated guinea pig serum.

6. The autodigestion of the activated serum is due to the splitting of the serum protein by the proteolytic ferment of the same serum and is brought about by the destruction of the antienzymic substance by the chemical reagents. On the other hand, the digestion products in a mixture of a foreign substrate and guinea pig serum are derived from the direct digestion of the substrate by the serum ferment. This digestion takes place in spite of the presence of the antiseroprotease. The serum separated from the substrate can no longer produce a split product, but is as actively antienzymic as the original serum and undergoes autodigestion only when treated with acetone or other chemical activators.

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