SEROTOXIN.

Studies on Ferment Action. XIV.*

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In a previous paper (1) we have demonstrated the lipoidal nature of serum antitrypsin and have discussed the various methods which may be employed to remove the antitrypsin from the serum; *i. e.,* by extraction, or by the saturation or oxidation of the unsaturated carbon bonds of the fatty acids on which the antitryptic property depends. In view of the fact that practically all sera contain proteolytic ferments, we undertook to determine whether or not sera from which the protective substance had been removed, with resulting exposure of the serum proteins, would be toxic for the homologous animal; and if so whether complete removal of the lipoids was necessary to produce toxic effects. Apart from the interest that such a study would have in relation to the production of the so called anaphylatoxins, we felt that numerous pathological conditions, which at present are not wholly understood, might possibly have their basis in a protein intoxication,—a true auto-intoxication in the sense that the toxic substances were formed from the serum or cellular protein of the host, and without reference to the gastro-intestinal tract. We have in mind particularly such conditions as arteriosclerosis, nephritis, asthma, and acute acidosis in infants. The work of Longcope (2) supports this idea. He observed that repeated injections of egg albumen into sensitized dogs and cats caused nephritis and other organic lesions.

TOXICITY OF SERA WHICH HAVE BEEN RENDERED ACID AND THEN FILTERED.

Our first experiments were made with serum from which the lipoids had been removed by acidifying the serum and then filter-

ing it through hard paper filters and kaolin, or through a Berkefeld filter. We have shown that part of the antitrypsin can be recovered from such filters by saponification.

0.2 of a cubic centimeter of N/10 sulphuric acid was added to each cubic centimeter of fresh guinea pig serum, and the mixture was filtered several times through a hard paper filter in the evening. It was then placed on ice over night, and on the following morning it was neutralized before injection. In all instances unless otherwise stated the injections were given intravenously.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
4 c.c.	250 gm.	0.016 c.c.

This dose given intravenously caused the immediate symptom complex of anaphylaxis with death of the animal in two minutes. The autopsy findings were typical,—lungs emphysematous, heart beating, and blood fluid.

In the next experiment 4 c.c. of N/4 hydrochloric acid were added to 20 c.c. of guinea pig serum and left over night in the ice box. Before injection it was passed through a Berkefeld filter.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
5 c.c. (not neutralized).	330 gm.	0.015 c.c.

There were marked respiratory convulsions, defecation, scratching of the nose, and the animal was weak for several hours, though it finally recovered.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
5 c.c. (neutralized).	250 gm.	0.02 c.c.

Typical death in three minutes. At autopsy the lungs were emphysematous, the heart was beating, and there were no intravascular clots.

In both of these experiments the serum from which the lipoids had been removed—not merely rendered inactive by acidifying—had become toxic for its own species in doses from 0.016 to 0.02 of a cubic centimeter per gram of weight. The simple acidification of the serum, which inactivates its antitryptic effect, does not seem to make the sera toxic in the same degree, probably because on reinjection the lipoids are again brought into a more active condition. Some toxic symptoms are, however, elicited by such sera in large doses (five cubic centimeters).

TOXICITY OF SERA AFTER EXTRACTION WITH ETHER OR CHLOROFORM.

We have shown that serum antitrypsin can be removed by ether extraction, and that the antitrypsin can be recovered in an almost quantitative relation from the ether extract by saponification of the extracted lipoids. That such sera are toxic is shown in the following experiments.

Guinea pig serum was placed under ether on Jan. 6 at room temperature and was left for two days. The flask was shaken occasionally.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
4 c.c.	330 gm.	0.012 c.c.

Death was immediate. The lungs were emphysematous, the heart had ceased beating, there was a firm clot in the right ventricle, and the gall bladder was distended.

A second preparation of serum was placed under ether on January 8 and permitted to stand at room temperature until January 13. The effect on intravenous injection was as follows:

Dose in c.c.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	330 gm.	0.012 c.c.	Immediate death. Lungs emphysematous. Heart filled with a large clot.
2 c.c.	245 gm.	0.008 c.c.	Immediate death. Findings as above.
1 c.c.	320 gm.	0.003 c.c.	Some respiratory spasms. Looked sick. Complete recovery.

In like manner chloroform will remove the serum antitrypsin, the result being even more rapid than with ether. It is best to mix about two volumes of pure chloroform with one volume of serum and to shake the mixture thoroughly, so that a thick emulsion is formed. After incubation or standing at room temperature the emulsion is centrifuged at high speed, and the supernatant serum is pipetted off and filtered through a coarse filter paper until the serum is quite clear and all the chloroform has evaporated. In the next experiment the serum was extracted with chloroform for two days at room temperature and the serum then removed.

Amount injected.	Weight of guinea pig.	Dose per gm. of weight.
3.5 c.c.	185 gm.	0.018 c.c.

Death was almost instantaneous. The lungs were emphysematous, the heart beating, and the blood fluid.

A similar preparation was kept at room temperature for five days and then tested.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4.0 c.c.	280 gm.	0.014 c.c.	Immediate death.
2.0 c.c.	310 gm.	0.006 c.c.	Immediate death.
1.0 c.c.	320 gm.	0.003 c.c.	Immediate death.
0.5 c.c.	365 gm.	0.0013 c.c.	Respiratory spasm. Looked sick. Complete recovery.

TOXICITY OF IODIZED SERA.

We have shown that the antitryptic effect of the serum can be completely destroyed by agents that oxidize or saturate the unsaturated bonds of the fatty acids, as for instance with potassium or sodium iodide, free iodin, or hydrogen peroxide. The iodide can not be used for the preparation of serotoxin because to lower the antitryptic index relatively large amounts must be used and these salts in themselves injected intravenously in small doses will produce toxic effects similar to the toxic effects of the serum.

Free iodin dissolved in serum containing only a trace of potassium iodide can be readily used for our purpose, according to the method described by von Dungern and Hirschfeld (3). With an excess of iodin a heavy precipitate is thrown out of the serum, but this is redissolved on the addition of an excess of sodium hydroxide, and the serum can then be brought back to a neutral reaction and will remain clear. It requires about two days at incubator temperature to remove completely the antitryptic action. Such sera remain sterile and will not autolyze if an excess of iodin is added. The results of an intravenous injection of an iodized serum which no longer had any antitryptic effect were as follows:
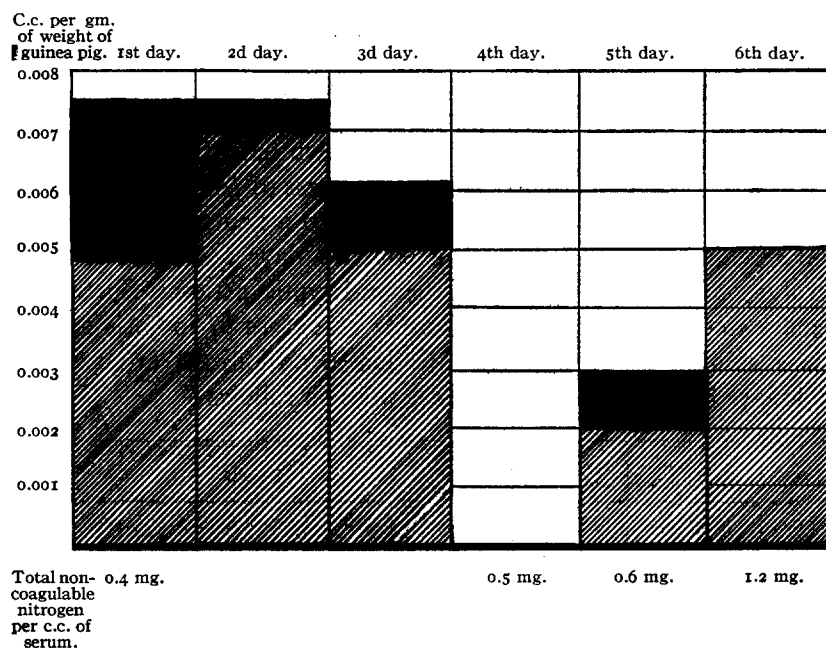
Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
3.2 c.c.	320 gm.	0.01 c.c.	Death in 2 min. Lungs emphysematous. Heart blood fluid. Heart beats strongly.
1.6 c.c.	310 gm.	0.005 c.c.	No effect.

Typical anaphylactic shock was produced with immediate fatal result in a guinea pig which had not been sensitized. This result is of course not comparable to the work of Bruck (4) and Friedberger and Ito (5), who have attempted to show that animals may be sensitized to iodized proteins. It is interesting to note that von Dungern and Hirschfeld (3) observed that their iodized sera underwent autolysis readily, but they could find no explanation for this fact, because they found no difference in the digestion of casein when iodized. They entirely overlooked the fact that the iodin might act on other bodies besides proteins. We have observed no increase in autolysis if an excess of iodin is present because the iodin

itself interferes with ferment action. The serum proteins are, however, digested more easily by trypsin after such treatment. We have been unable to secure toxic sera by treatment with strong hydrogen peroxide.

INFLUENCE OF TIME ON CHLOROFORM EXTRACTION.

We next determined the length of time required for serum kept at room temperature under chloroform to become toxic. For brevity the result has been charted in text-figure 1. The serum before
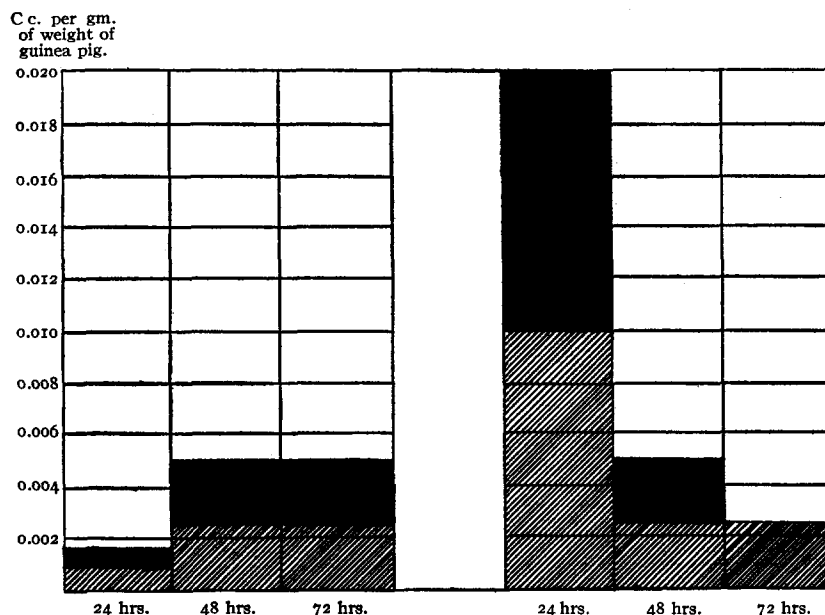

Black = minimum lethal dose per gm. of weight of guinea pig.
Shaded = sublethal dose.

TEXT-FIG. 1. Toxicity of guinea pig serum treated with chloroform at room temperature.

treatment was not toxic in large doses (5 c.c.), and the total non-coagulable nitrogen per cubic centimeter amounted to 0.2 of a milligram. As will be seen from the chart there was a progressive increase in toxicity after the first day until a maximum was reached on the fifth day, when the minimum lethal dose was 0.003 of a cubic centimeter per gram of weight, or 0.75 of a cubic centimeter for a guinea pig weighing 250 grams. There then follows a rather

sudden loss of toxicity, so that 0.005 of a cubic centimeter per gram of weight was no longer toxic. During this time there had been a constant autolysis of the serum, as indicated by the increase in the total non-coagulable nitrogen (Folin's method) from 0.2 of a milligram per cubic centimeter to 1.2 milligrams per cubic centimeter on the sixth day. In each case the minimum lethal dose caused the typical anaphylactic complex, although it was found that excessive


C c. per gm.
of weight of
guinea pig.

24 hrs.   48 hrs.   72 hrs.   24 hrs.   48 hrs.   72 hrs.

Activated serum under chloroform at   Inactivated serum under chloroform
37° C.   at 37° C.

Black = minimum lethal dose in c.c. per gm. of weight.
Shaded = sublethal dose.

TEXT-FIG. 2. Toxicity of guinea pig serum treated with chloroform at 37° C.

doses, two minimum lethal doses, caused thrombosis in the larger vessels and heart.

When left at 37° C. the serum becomes toxic much earlier, and the atoxic stage is correspondingly produced at an earlier period of time (text-figure 2). The active serum so prepared has reached a very toxic stage in twenty-four hours, so that 0.0015 of a cubic centimeter per gram of weight of guinea pig is toxic,—0.31 of a cubic centimeter killed a guinea pig weighing 210 grams,—while the

inactivated serum (fifteen minutes at 54° C.) is toxic only in a much larger dose, 0.02 of a cubic centimeter per gram of weight. After forty-eight hours, however, the active serum has become less toxic, while the inactivated serum has become more toxic than before. Here, as in the previous experiment, there is a constant increase in the amount of non-coagulable nitrogen. In other experiments we have observed that the toxicity will reach its maximum within eight to twelve hours and will then rapidly decline. This depends entirely upon the amount and the rate of removal of the lipoid protective substances. Thus the inactivated serum becomes toxic less rapidly owing to the fact that the antitrypsin is removed more slowly than from the active serum at 37° C.

Per cent.
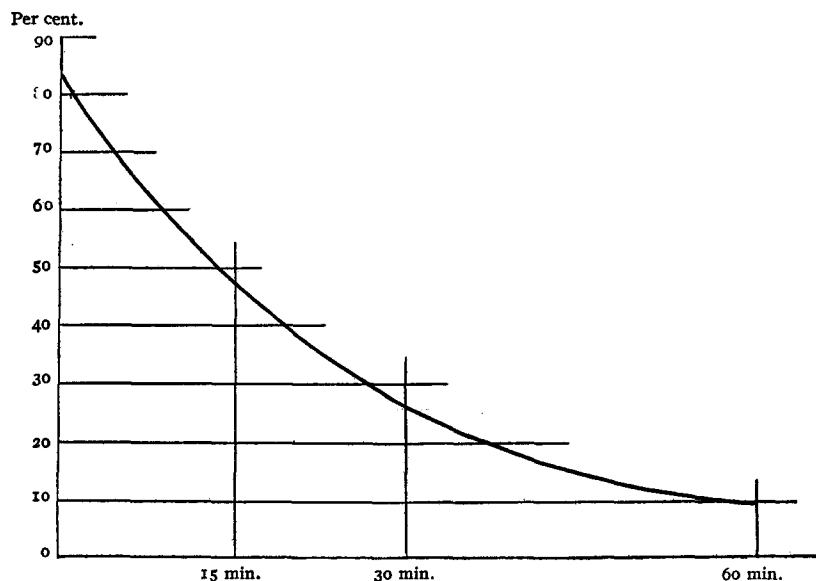
TEXT-FIG. 3. Curve showing the rate of extraction of serum antitrypsin by chloroform at 37° C.

The rate of removal of the antitrypsin from the serum by chloroform is shown in text-figure 3. In this experiment samples of serum were removed from the incubator at fifteen minute intervals, and the antitrypsin was determined by the method described in our previous papers (6).

0.075 c.c. of normal serum inhibited 84 per cent. of digestion.

After 15 minutes under chloroform it inhibited 48 per cent. of digestion.

After 30 minutes under chloroform it inhibited 27 per cent. of digestion.

After 1 hour under chloroform it inhibited 10 per cent. of digestion.

In one hour practically all the antitrypsin has been extracted. When made strongly alkaline this extraction is markedly accelerated, while the inactivated serum is more resistant to the chloroform extraction during the first half hour, although after about an hour's extraction the lipoids in this case are also removed. The serum is toxic, however, before all the antitrypsin is removed. This is shown by removing a sample after one half hour's incubation. These sera kill in a dose of about 0.01 of a cubic centimeter per gram of weight, and in this case the immediate cause of death is intravascular coagulation. There is no increase in the non-coagulable nitrogen at this time.

PROTOCOL I.

One Hour Chloroform Serum.

Dose.	Weight.	Dose per gm. of weight.	Result.
4.5 c.c.	300 gm.	0.015 c.c.	Immediate death.
3.2 c.c.	270 gm.	0.012 c.c.	Respiratory spasms. Prostrated for 30 min. Recovery.

Two Hour Chloroform Serum.

| Dose.	Weight.	Dose per gm. of weight.	Result.
4.0 c.c.	260 gm.	0.015 c.c.	Immediate death.
1.6 c.c.	215 gm.	0.0075 c.c.	Death in 15 min. Lungs distended. Heart blood fluid. Heart beats.

Four Hour Chloroform Serum.

Dose.	Weight.	Dose per gm. of weight.	Result.
1.7 c.c.	280 gm.	0.006 c.c.	Immediate death. Heart blood clotted. Heart does not beat. Lungs emphysematous.
1.0 c.c.	340 gm.	0.003 c.c.	Death in 30 min. Heart beats. Lungs somewhat distended.

Six Hour Chloroform Serum.

Dose.	Weight.	Dose per gm. of weight.	Result.
1.1 c.c.	370 gm.	0.003 c.c.	Immediate death. Heart blood clotted. Lungs distended.
0.62 c.c.	310 gm.	0.002 c.c.	Immediate respiratory spasms. Prostrated. Death in 20 min. Small clot in right heart. Blood fluid. Lungs slightly distended.

In text-figure 4 is shown the rate of increase of toxicity observed at 1, 2, 4, 6, 24, and 48 hour intervals of serum kept under chloroform. As will be observed there is a rapid increase during the first six hours, after which the toxicity remains at a constant
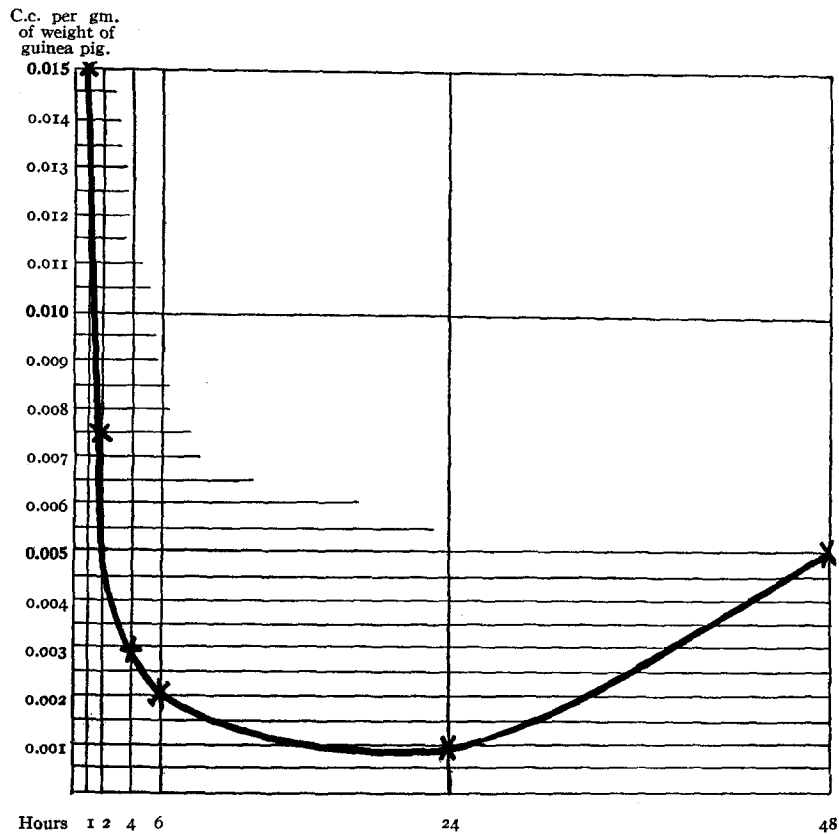
Text-Fig. 4. Curve showing the increase of toxicity of guinea pig serum treated with chloroform at 37° C. Minimum lethal dose in c.c. per gm. of weight.

level for a period of twelve or more hours, and then rapidly decreases. The minimum lethal doses have been charted from protocol I. The twenty-four and forty-eight hour tests were made at a different time, though with the same lot of serum.

INTRAVASCULAR CLOTTING.

The minimum lethal dose of the toxic sera when given intraperitoneally is much larger, at least five to six times the intravenous dose being required. The immediate cause of death observed in the minimum lethal dose has been either a typical anaphylactic complex, with the lungs emphysematous, the heart beating, and the blood fluid,—observed in the acidified and filtered serum, in the iodized serum, and in the chloroform serum, especially when kept at room temperature; or death has been due to immediate intravascular coagulation with a secondary emphysema, observed in the ether sera, and in the chloroform serum prepared at $37°$ C. In order to rule out the effect of coagulation we have made the following experiments. The serum used in this experiment had been incubated for two hours at $37°$ C. The minimum lethal dose was then determined.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Remarks.
1.0 c.c.	340 gm.	0.003 c.c.	Weak. Prostrated. Recovered.
1.1 c.c.	220 gm.	0.005 c.c.	Immediate death. Intravascular coagulation.

Some of this serum was mixed with saline solution to which had been added 1 per cent. sodium citrate, and injected.

Dose.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1.25 c.c.	1.25 c.c.	250 gm.	0.005 c.c.

Immediate death. Heart found beating. Lungs emphysematous. Small clot in right heart.

Inasmuch as there was a small clot present we increased the amount of citrate.

Serum.	Citrate.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	2 c.c.	200 gm.	0.005 c.c.

Death in 2 min. Blood fluid. Lungs emphysematous. Heart beating.

Even without a trace of clotting, we find the serum prepared in this way toxic in the same dose, and post-mortem examination shows a typical lung finding.

We next prepared some hirudinized animals and injected them five minutes after treatment with hirudin. In this case each guinea pig received two minimum lethal doses of the toxic sera.

Dose.   Weight of guinea pig.   Amount of serum per gm. of weight.
2.25 c.c.   225 gm.   0.01 c.c.
Typical death in two minutes. No clotting. Lungs emphysematous.

We then used a single minimum lethal dose and also one and one fourth minimum lethal doses, as will be seen below.

Dose.   Weight of guinea pig.   Dose per gm. of weight.
0.825 c.c.   165 gm.   0.005   c.c.
1.0   c.c.   160 gm.   0.0062 c.c.
No immediate result. The animals seemed well and were placed in the cage over night. The next morning both were found dead; in each case the blood was fluid, the lungs emphysematous, although not so markedly as in acute anaphylactic shock.

INHIBITION OF TOXIC ACTION.

Dilution of a single minimum lethal dose with an equal volume of normal guinea pig serum, and incubation for thirty minutes before injection does not alter the toxicity, but heating to 70° C. for thirty minutes greatly decreases it.

If the assumption that we are removing a protective substance from the serum is correct, then by returning the extracted substance to the serum it should become non-toxic. We have previously shown (1) that by saponifying the ether and chloroform extracts of sera we can recover all the antitrypsin. These protective lipoids are not originally present in the serum as soaps, but inasmuch as it is practically impossible to get the lipoids back into solution in the serum in the fine state of dispersion in which form only they are active, we must saponify them, and in the form of soaps attempt to reintroduce them into the serum. Twenty cubic centimeters of guinea pig blood were placed under chloroform for two hours, the serum was then separated and tested for toxicity.

Dose.	Weight of guinea pig.	Dose per gm. of weight.	Result.
0.9 c.c.	180 gm.	0.005 c.c.	Immediate death with intravascular clotting, etc.
0.51 c.c.	170 gm.	0.003 c.c.	Scratches; no symptoms.

The minimum lethal dose was therefore 0.005 of a cubic centimeter per gram of weight. The chloroform extract was mixed with an equal volume of alcohol and water and made strongly alkaline with sodium hydrate. This was heated in the water bath for an hour. The mixture was then acidified, and the fatty acids were

taken up in ether and resaponified by neutralization with alcoholic sodium hydrate. These soaps were now mixed with 1.5 cubic centimeters of the serotoxin and incubated for fifteen minutes.

Dose injected.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	215 gm.	0.005 c.c.

There were no immediate symptoms and the animal remained well. The toxicity had therefore been completely neutralized. In like manner one can completely neutralize the serotoxin by adding soaps of the unsaturated fatty acids, which, as we have shown (6), have marked antitryptic properties, although they are less active when mixed with serum than when dissolved in salt solution. The serotoxin here used was freshly prepared and autolysis had not occurred, so that preformed toxic split products of proteins (primary proteoses) were probably not present. If on the other hand we use a serotoxin which has been prepared at room temperature by standing for several days, in which there is a marked increase in the total non-coagulable nitrogen, and from which one can isolate toxic proteoses, then the neutralization by the soap is no longer complete, the effect depending of course on the relative amount of the proteins and their toxic split products already formed.

A chloroform serum standing for five days at room temperature caused immediate death in doses of 0.003 of a cubic centimeter per gram of weight. Soap was prepared from serum lipoids and mixed with the dose.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	280 gm.	0.0035 c.c.

There were immediate respiratory convulsions, after which the animal recovered for ten minutes; then clonic convulsions occurred at frequent intervals, with death in thirty minutes. In a similar way a dose was prepared by adding 10 mg. of linseed soap (iodin value 110), and in this case, also, while immediate death was prevented, the ultimate result was fatal.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1 c.c.	330 gm.	0.003 c.c.

If the toxicity of the serotoxin depends on the exposure of the proteins, so that they can be easily split to toxic products in the injected animal, then the reduction of the normal antiferments in the injected animal should render the serotoxin more toxic than for a normal animal. A three hour serotoxin (chloroform at

37° C.) was used, the minimum lethal dose of which was 0.005 of a cubic centimeter per gram of weight. Four guinea pigs were prepared in the morning by shaving the abdomen and painting with tincture of iodin, so that each animal received about 0.2 of a gram of iodin. In this way one can reduce the serum antitrypsin in an amount shown in text-figure 5, in which the black line represents the
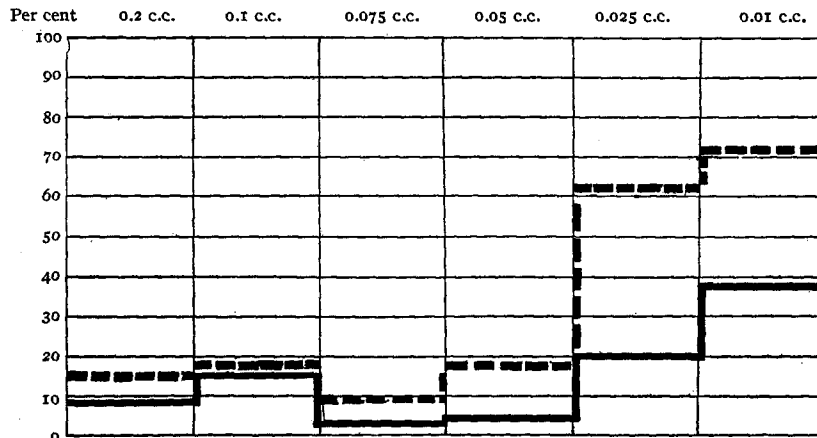
Black line = before treatment (A. M.).
Dotted line = after being painted with tincture of iodin (P. M.).
    TEXT-FIG. 5. Effect of iodin on antitrypsin of guinea pig.

amount of digestion of casein in the presence of varying amounts of the guinea pig serum drawn in the morning before treatment, and the dotted line represents the digestion in the serum of the same guinea pig in the afternoon. This decrease in antitryptic activity is probably due to the saturation by the iodin of the unsaturated carbon bonds of the fatty acids, as we have stated elsewhere (1).

A sublethal dose was first injected into an iodized guinea pig.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.
0.54 c.c.	180 gm.	0.003 c.c.

Immediate death. Heart blood clotted. Lungs slightly distended. Gall bladder markedly distended.

Next a half sublethal dose was given.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.
0.27 c.c.	185 gm.	0.0015 c.c.

The animal was immediately prostrated, sick, with occasional convulsions. Death in thirty minutes. Heart beating. Blood fluid. Lungs slightly distended.

The experiments indicate that the toxicity of the serotoxin is greater for animals in which the antitrypsin has been reduced, thus permitting the ferments normally present to be more active. A single injection of a sublethal dose, given intravenously or intraperitoneally, increases the antitrypsin in the guinea pig; the effect can be demonstrated within twenty-four hours, and persists for at least two weeks. A similar increase has been noted following anaphylaxis (Meyer (7), Pfeiffer (8, 9)). Zinsser (10) has recently noted an increased resistance to a minimum lethal dose of a strong anaphylatoxin prepared from typhoid bacilli, following a single sublethal dose given seven to twelve days previously.

If the serotoxic effects are due to an intravital splitting of the serum protein, then during the period of increased antitrypsin fol-
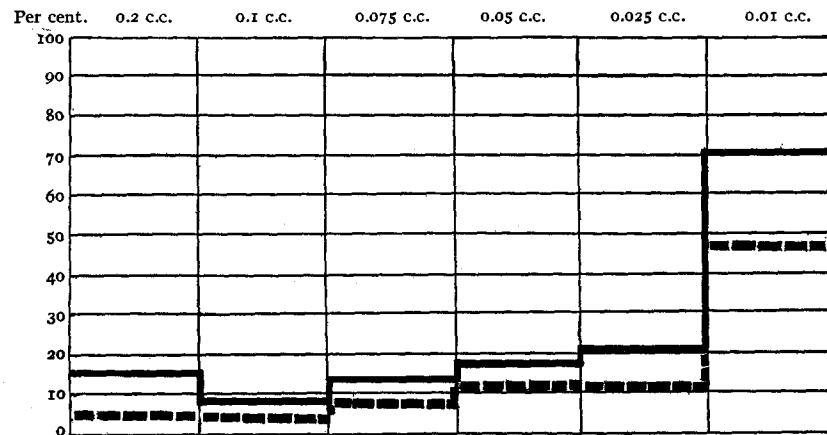
Black line = original antitrypsin of guinea pig serum drawn on Feb. 24, 1914.
Dotted line = serum drawn on Feb. 28, 1914.

TEXT-FIG. 6. Increase of antitrypsin following a single injection of a sublethal dose of serotoxin.

lowing a sublethal dose there should be an increased resistance to the serotoxin. The effect of a sublethal dose on the guinea pig antitrypsin is shown in text-figure 6. The black line indicates the percentage of casein digestion as influenced by the normal guinea pig serum drawn on February 24, 1914. A sublethal dose of serotoxin was given intraperitoneally on the same day. A second

sample of blood was drawn on February 28, and is shown in the dotted line. As will be seen, there is a well marked increase in inhibiting power.

The increased resistance to serotoxin is shown in the following experiment (February 24).

Dose of serotoxin.	Weight of guinea pig.	Dose per gm. of weight.
1.9 c.c.	190 gm.	0.01 c.c.

Guinea pig sick and prostrated.   Complete recovery.

The following day the guinea pig received 0.006 of a cubic centimeter, per gram of weight, of a serotoxin preparation of which the minimum lethal dose was 0.005 of a cubic centimeter. There were no symptoms. After several days following the original sublethal dose we have secured animals that are resistant to two minimum lethal doses.

EXPERIMENTS WITH PURIFIED HORSE SERUM ALBUMEN.

In order to compare the results which we had so far obtained with a pure protein intoxication we prepared some horse serum albumen in the following manner: The globulins of the serum were thrown out of solution by half saturation with ammonium sulphate, and after filtration, the albumens were removed from the filtrate by precipitation with acetic acid. This precipitate was redissolved in water made slightly alkaline with sodium carbonate, treated with an equal amount of a saturated solution of ammonium sulphate, filtered, and the filtrate again treated with acetic acid. The final precipitate was dialyzed against running water until free of acid and ammonium sulphate, and was then evaporated to dryness. It was made up in a 1 per cent. solution in normal saline. Of this solution a dose of 0.02 of a cubic centimeter per gram of weight was not toxic for the guinea pig. On testing its antitryptic property it was found that two cubic centimeters of such a solution inhibited 86 per cent. of tryptic digestion, and on digesting two cubic centimeters with a strong trypsin solution for forty-five minutes, only 0.1 of a milligram of non-coagulable nitrogen was obtained. If we now extract the solution by mixing with chloroform at 37° C. for two hours we find the toxicity well marked in the dose which was not at all toxic for the unextracted serum.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
4 c.c.	200 gm.	0.02 c.c.	Immediate death. Heart blood clotted. Lungs distended.
2 c.c.	190 gm.	0.01 c.c.	No effect.

After extraction for twenty-four hours the toxicity had still increased.

Amount of serum.	Weight of guinea pig.	Dose per gm. of weight.	Result.
2.4 c.c.	240 gm.	0.01 c.c.	Killed in 2 min. Heart blood clotted.
0.9 c.c.	180 gm.	0.005 c.c.	No effect.

The last dose, which was sublethal, was now injected into an iodized guinea pig. The method of preparing the guinea pig has been already described.

Dose.	Weight of guinea pig.	Dose per gm. of weight.
1.1 c.c.	220 gm.	0.005 c.c.

Immediate death. Heart blood fluid. Lungs distended. Gall bladder distended.

As with the serotoxin, there was a lessened resistance to the toxic effect in the iodized guinea pig. When the antitryptic effect of the extracted (twenty-four hour) horse serum solution was tested, it was found that it now inhibited only 33 per cent. of casein digestion, and was itself digested by trypsin much more easily (0.35 of a milligram for 2 cubic centimeters of solution). After extraction for seventy-two hours the toxicity had increased so that a dose of 0.005 of a cubic centimeter per gram of weight caused marked toxic symptoms but did not kill. At this time the serum albumen was no longer antitryptic. Inasmuch as there are no ferments present in the serum albumen solution, and no trace of autolysis can be obtained when such solutions are incubated, there is no preformation of toxic split products, and whatever increase in toxicity occurs must be due to the increased availability of the serum albumen for attack by the ferments of the injected animal.

In the serotoxin we have a somewhat different condition due to the presence of quite active proteolytic ferments. It would therefore seem reasonable that the ferments would attack the serum proteins as soon as the antitrypsin had been completely removed, and

produce toxic split products,—primary proteoses. This condition actually obtains. If one fractionates a serotoxin which has been under chloroform at 37° C. for only two hours, only faint traces, or no evidence at all, of primary proteoses can be obtained. From twenty-four hour serotoxin we have obtained toxic proteoses, as is shown in the following experiment.

Ten cubic centimeters of normal guinea pig serum were mixed with chloroform and incubated for twenty-four hours. The chloroform was then removed, the native protein removed from the serum by the heat and acid method, and the filtrate made faintly alkaline to throw out the acid albuminates, and again filtered through kaolin. The primary proteoses were then precipitated by half saturation with ammonium sulphate. These were removed, dialyzed until free from all traces of ammonium sulphate, and then made up to the original volumes. When injected intravenously in 0.1 per cent. salt solution the primary proteoses are toxic in a dose of about 0.01 cubic centimeter per gram of weight. When so prepared the toxicity is unaltered by boiling.

DISCUSSION.

In the preliminary experiments we observed the marked similarity to anaphylactic death which our animals showed on the injection of the serotoxin, and later, while working with the rapidly prepared chloroform sera, the paradoxical similarity to cytotoxic death—immediate coagulation—was noted. In view of the fact that the usual method of death is intravascular coagulation, we have used the term serotoxin to designate the entire toxic effect of the extracted sera, although we wish to emphasize the importance of the protein cleavage phenomena.

The toxicity of organ extracts first noted by Brieger and Uhlenhuth (11), was shown by Blaizot (12), Gley (13), and by Dold and Ogata (14) to depend on the intravascular coagulation, and could be rendered completely inert by the treatment of animals with hirudin. Ichikawa (15) has recently shown that sodium citrate will also render a toxic dose non-toxic. Serotoxin differs fundamentally from the cytotoxin in that the toxic effects persist when coagulation is prevented by hirudin or citrate. The toxicity is due to

factors quite apart from the altered coagulation balance, although we believe that it will be found that the unsaturated lipoids play an important part in the mechanism of coagulation and its prevention.

From our experiments we believe that we are justified in concluding that the exposed homologous serum proteins are capable of being split when injected into an animal, and produce the observed toxic effect. In many of our experiments the exact picture of acute anaphylactic death was observed; in sublethal doses a marked fall in temperature and extreme prostration were noted, the latter being most marked in sublethal doses of sera which had been permitted to autolyze. The toxicity of serum protein is well illustrated in the experiment with purified horse serum albumen which was not toxic in doses of 0.02 of a cubic centimeter of a 1 per cent. solution per gram of weight, but when extracted with chloroform it gradually became more and more toxic until a final dose of 0.005 of a cubic centimeter per gram of weight (or 0.00005 of a gram of serum albumen per gram of weight) was sufficient to cause marked toxic effects. The length of time required to extract the lipoids from this solution indicates how intimate the relation of the lipoid to the serum albumen complex must be.

That the exposed serum proteins are the substances which produce *in vivo* the toxic effects when split, is shown by the fact that in the rapidly prepared serotoxin no increase in non-coagulable nitrogen is demonstrable, and that no toxic split products (primary proteoses) can be isolated by fractioning the serum proteins at this time. The antiferments having once been extracted, there is of course no further impediment to ferment action, such sera become actively proteolytic, as was first shown by Delezenne and Pozerski (16), and undergo autolysis as shown by the marked increase in the non-coagulable nitrogen. Under these conditions we have always been able to isolate preformed toxic split products. The effect of the soaps, too, in not completely neutralizing the serotoxin at this period is confirmatory evidence, since the soaps act by preventing the splitting of native proteins (true antitryptic effect, rather than anti-ereptic). With continual autolysis we should expect lessened toxicity, and this is found to be the case. That the

normal inhibitory mechanism of the serum markedly influences the
intravital splitting, as it does in the test-tube, is shown by the great
increase of toxicity of the serotoxin for animals with lessened anti-
ferments and by the greater resistance of animals with increased
antiferments. We believe that the effect of the iodin is due to a
saturation of the unsaturated bonds of the fatty acids of the
lipoids, rendering the substituted product less active as an anti-
tryptic agent. The effect of a single sublethal dose of serotoxin
in increasing the antiferment can possibly be explained by a lowered
oxidation of the organism due to the toxin, with a resulting lower-
ing of the physiological oxidation of these unsaturated compounds,
or it may be due to a breaking up by the toxic action, of pro-
tein-lipoid combinations with a resulting increase in the lipoidal
elements in the blood. From our studies we are even more
firmly convinced of the biological importance of the protein-
lipoid combination in the organism, and the correlated neces-
sity of according to lipolytic action greater emphasis in the part
of the adjunct which breaks up these combinations before actual
proteolytic changes can take place. The small amount of purified
horse serum albumen (after complete chloroform extraction) re-
quired to kill a non-sensitized animal is significant from this point
of view.

A large number of investigators, notably Vaughan (17), Fried-
berger (18), and Pfeiffer (9), have, during the past few years, em-
phasized the importance of the split products of proteins in the pro-
duction of the toxic effects in the various infections, but the basic
idea has always been held that the substrate which was acted upon
was the foreign protein concerned, and that the homologous proteins
might be neglected, although the work of Keysser and Wassermann
(19), Doerr (20), Bordet (21), and Ritz and Sachs (22) indicate that
adsorption phenomena and colloidal changes might render homol-
ogous sera toxic. The idea, however, that the adsorbed substances
were the antiferments, or protective agents, has not been expressed,
and no efforts have been made to remove such antiferments, pos-
sibly because of the unfortunate view that the antiferments repre-
sented immune bodies or split products of the protein molecule.

From a clinical point of view we are inclined to believe that the

proof of the toxicity of exposed serum proteins may be of importance. Longcope (2) has recently shown that repeated injections of heterologous proteins can cause marked nephritic changes. The profound prostration shown by leukemics in the terminal stages when the serum ferments are known to increase greatly, so that such sera may become proteolytic, suggests the profound prostration observed in experimental animals injected with sublethal doses of autolyzed sera. Further experiments are now in progress to determine the results from chronic sero-intoxication.

CONCLUSIONS.

1. Sera from which the protective lipoids (unsaturated fatty acids) have been removed are toxic for the homologous animal.

2. The toxicity is due to three factors: (a) an alteration in the mechanism of coagulation, with resulting intravascular coagulation; (b) the exposure of the native serum proteins; (c) the formation of toxic split products (primary proteoses) by autolysis.

3. A definite maximum of toxicity can be determined, with a final stage of atoxicity due to continued autolysis.

4. Hirudin and sodium citrate do not protect animals.

5. Heating to 70° C. destroys, or greatly lessens, the toxicity of the serotoxin, although the isolated proteoses are toxic after boiling.

6. The return of the extracted lipoids (saponified) neutralizes the toxicity.

7. Unsaturated soaps also neutralize the toxicity.

8. Sublethal doses produce extreme prostration, marked fall in body temperature, no eosinophilia, and an increase of antitrypsin.

9. Sublethal doses of rapidly prepared chloroform sera cause a decrease in coagulation time; sublethal doses of autolyzed sera cause an increase in coagulation time.

10. Previously injected animals are more resistant (increased antiferments).

11. Iodized animals are less resistant (decreased antiferments).

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