CHANGES IN THE PROTEOLYTIC ENZYMES AND ANTI-ENZYMES OF THE BLOOD SERUM PRODUCED BY SUBSTANCES (CHLOROFORM AND PHOSPHORUS) WHICH CAUSE DEGENERATIVE CHANGES IN THE LIVER.[1]

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The observations of Salkowski,[2] which have shown that incubated liver undergoes "self-digestion," have been extended to almost all tissues of the animal body. It is well known that the autolysis of most tissues occurs with maximum activity in a weakly acid medium. Biondi[3] has found that disintegration of liver is hastened by addition of weak acid, and Hedin and Rowland,[4] using the expressed juice of various organs, have noted that spleen, kidney, and lymphatic nodes autolyze more actively in acid than in neutral or alkaline media. The trivial autolysis of voluntary muscle is not materially affected by acid or alkali, but the autolysis of heart muscle resembles that of other organs. Levene and Stookey[5] have found that autolysis of brain tissue and of testicle is favored by the presence of acid.

The pancreas is not an exception to the foregoing rule. The well-known observations of Heidenhain[6] have shown that trypsin, which exhibits maximum activity in alkali, exists in the pancreas as inactive zymogen, so that fresh extracts of the organ cause little proteolysis under conditions which favor the activity of trypsin.

[1] Received for publication, November 4, 1910.

[2] Salkowski, *Ztschr. f. klin. Med.*, 1890, xvii, Suppl., p. 77.

[3] Biondi, *Virchows Arch. f. path. Anat.*, 1896, cxliv, 373.

[4] Hedin and Rowland, *Ztschr. f. physiol. Chem.*, 1901, xxxii, 531.

[5] Levene and Stookey, *Jour. Med. Research*, 1903, x, 212.

[6] Heidenhain, *Arch. f. d. ges. Physiol.*, 1875, x, 557.

The conditions under which acid activates pancreatic extracts and their relation to the activation of pancreatic juice by enterokinase have been studied by one of us (Dochez).[7] Fresh pancreatic tissue which undergoes active autolysis in the presence of weak acid, suffers little change in neutral solutions and is almost unchanged after incubation in an alkaline medium, the concentration of which agrees with that favorable to the action of trypsin (0.2 to 0.4 per cent. sodium carbonate). Heidenhain has shown that acid converts tryptic zymogen of fresh pancreas into trypsin; pancreas subjected to the action of acid acquires the power to digest under conditions which are favorable to trypsin, namely, in neutral or alkaline media. Although splenic substance autolyzes with greater activity in acid than in alkali, Hedin[8] has obtained in relatively purified form two enzymes, one of which, designated α-lieno-protease, acts in alkali, whereas the other, designated β-lieno-protease, acts in acid. If fresh spleen (Hedin[9]) is temporarily subjected to the action of weak acid, its ability to autolyze in the presence of alkali is materially increased. Pretreatment with acid makes active an enzyme which digests in the presence of alkali.

Dochez[10] has studied the modifications of hepatic autolysis which occur as the result of changes in reaction, and he has compared autolysis of liver with autolysis of fresh pancreas. Whereas fresh liver autolyzes with greater activity in acid (0.2 per cent. acetic acid) than in neutral or alkaline media (from 0.2 to 0.4 per cent. sodium carbonate), liver which has been treated with weak acid during twenty-four hours acquires the power to autolyze or to digest casein in alkaline medium with equal or greater activity than in acid. Other evidence cited by Dochez shows that the liver contains two enzymes, one of which acts in acid, whereas the other, which acts in alkali, is active only after the tissue has been subjected to acid. Vernon[11] has shown that pancreatic extracts become active after standing; Dochez has likewise noted that liver tissue allowed to stand on ice, gradually acquires the power to autolyze in the presence of alkali.

[7] Dochez, *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 97.
[8] Hedin, *Jour. Physiol*, 1904, xxx, 155.
[9] Hedin, Festschrift für Olof Hammarsten, Upsala, 1906, vi, 1.
[10] Dochez, *Jour. Exper. Med.*, 1910, xii, 666.
[11] Vernon, *Jour. Physiol.*, 1901, xxvii, 269.

It is noteworthy that the polynuclear leucocytes of an inflammatory exudate cause proteolysis with greater activity in alkali than in acid and, unlike pancreas, liver or spleen and, doubtless, other organs, exhibit this activity in alkali when freshly obtained from the body; no pretreatment with acid is necessary in order to bring this enzyme (leucoprotease) into action. The bone marrow, unlike other tissues, exhibits the same ability to digest with greater activity in alkali than in acid.

The power of the blood serum to restrain the activity of tryptic digestion has long been known. Hahn[12] made the observation that trypsin fails to digest fibrin or gelatin when fresh blood serum is present. He found that this power to inhibit is lost if blood serum is heated to a temperature of 65° C. The inhibiting substance is apparently attached to the proteins of the serum, but is not a common property of all proteins of the blood, for the globulin fraction of the serum, precipitated by half saturation with ammonium sulphate, fails to restrain the action of trypsin, whereas the albumin fraction precipitated by complete saturation after globulin has been removed exerts a retarding influence.

Ascoli and Bezzola,[13] in 1903, doubtless influenced by the observations of Fr. Müller[14] upon self-digestion of lung consolidated by pneumonia, have sought to determine if this inhibiting property of the serum undergoes any alteration during the progress of pneumonia. They have claimed that there is at first a marked increase of anti-tryptic action; this increase is maintained for a time, and after crisis it is followed by a decrease, often occurring in association with disappearance of the local lesion. The subject attracted little attention until Brieger and Trebing[15] showed that there is an almost constant increase of the anti-tryptic activity of the blood serum in association with carcinoma. Subsequent studies have demonstrated that this increased anti-enzymotic activity is apparently dependent upon the accompanying cachexia rather than upon the new growth itself, and a similar change has been observed in association with a variety of diseases, such as pneumonia, typhoid

[12] Hahn, *Berl klin. Wchnschr.*, 1897, xxxiv, 499.
[13] Ascoli and Bezzola, *Berl. klin. Wchnschr.*, 1903, xl, 391.
[14] Fr. Müller, *Verhandl. d. 20 Cong. f. inn. Med.*, 1902, 192.
[15] Brieger and Trebing, *Berl. klin. Wchnschr.*, 1908, lxv, 1041.

fever, tuberculosis, and exophthalmic goitre, of which the common characteristic, according to K. Meyer,[16] is increased disintegration of the protein constituents of the body, perhaps referable to increased activity of proteolytic enzymes.

Finding that the serum in certain diseases associated with leucocytosis exhibits increased anti-enzymotic activity when tested with enzyme of polynuclear leucocytes, several observers (Bittorf,[17] Wiens[18]) have maintained that the enzyme set free by leucocytes neutralizes the anti-body of the serum and thus indirectly causes an excessive regeneration. Nevertheless, numerous observations have shown, on the one hand, that anti-enzymotic activity of the blood serum is not constantly increased in association with leucocytosis (K. Meyer), whereas, on the other hand, many conditions, such as malignant growth, tuberculosis, and typhoid fever, frequently accompanied by increase of anti-enzyme, usually exhibit no leucocytosis.

By a series of comparative tests, Jochmann and Kantorowicz[19] have shown that serum which exhibits high anti-enzymotic activity toward trypsin shows increased ability to restrain the activity of the enzyme of the polynuclear leucocytes, which like trypsin digests in the presence of an alkaline medium. Injection of trypsin into rabbits increases the ability of the serum to inhibit both trypsin and leucoprotease, and, furthermore, injection of leucoprotease has the same result.

The occurrence of increased anti-enzyme in the serum suggests the possibility that anti-enzyme is increased in order to balance an increased quantity of free enzyme. With this possibility in view, we have attempted to determine if the proteolytic activity of the blood serum undergoes any alteration during the course of chloroform poisoning so severe that advanced degenerative changes are produced in the liver. The possibility that such a study might prove fruitful has been suggested by the observation that increase of anti-leucoprotease accompanies prolonged intoxication with chloroform or phosphorus.

[16] K. Meyer, *Berl. klin. Wchnschr.*, 1909, xlvi, 1064, 1890.
[17] Bittorf, *Deutsch. Arch. f. klin. Med.*, 1907, xci, 212.
[18] Wiens, *Deutsch. Arch. f. klin. Med.*, 1907, xci, 456.
[19] Jochmann and Kantorowicz, *Ztschr. f. klin. Med.*, 1908, lxvi, 153.

The existence of proteolytic enzyme in normal blood serum has long been known. Delezenne and Pozerski[20] have found that blood serum mixed with chloroform acquires the power to digest gelatin and casein. Hedin has shown that the globulin fraction of the blood serum of the ox, obtained by half saturation with ammonium sulphate, contains a weak proteolytic enzyme. The albumin fraction of the serum obtained by complete saturation after removal of globulin contains anti-enzyme, which in normal serum restrains the activity of the enzyme just mentioned. One of us[21] has shown that the anti-enzymotic activity of the blood serum is lost in the presence of 0.2 per cent. acetic acid; in this medium the blood serum exhibits well marked proteolytic activity, but in neutral or alkaline media anti-body balances enzyme, and neither autolysis nor other proteolytic activity is demonstrable.

In order to determine what factors cause changes in the enzymotic and anti-enzymotic activity of the blood serum, a condition which can be produced experimentally offers obvious advantages, for the phenomena which occur can be repeatedly traced from the beginning through various stages of the process. Chloroform poisoning has proved especially favorable for this study because it reproduces the increase of anti-enzyme heretofore frequently noted with the cachexia of carcinoma, with various infections, and with other diseases.

The changes in the liver which accompany prolonged administration of chloroform by inhalation, are clearly defined by the recent studies of Howland and Richards[22] which have directed our attention to this subject. There is necrosis implicating the central part of each hepatic lobule. Disintegration of protein within the body is shown by increased elimination of nitrogen and sulphur by the urine. When intoxication is not severe, there is, instead of necrosis, fatty degeneration of the liver, kidneys, heart muscle, etc. Doyon and Billet[23] have found that the blood may become incoagulable and that incoagulability is associated with diminution of the fibrinogen content of the serum.

[20] Delezenne and Pozerski, *Compt. rend. Soc. de biol.*, 1903, lv, 327, 690, 693.
[21] Opie, *Jour. Exper. Med.*, 1905, vii, 316.
[22] Howland and Richards, *Jour. Exper. Med.*, 1909, xi, 344.
[23] Doyon and Billet, *Compt. rend. Soc. de biol.*, 1905, lviii, 852.

We have followed the coagulability of the blood during the course of chloroform poisoning produced by the daily administration by stomach of a given quantity of chloroform (one or two cubic centimeters per kilogram of body weight), and have found that the coagulation time of the blood increases during the first three days; in some instances the blood becomes wholly incoagulable. Hemorrhage into the gastro-intestinal tract or into the peritoneal cavity is not infrequent, and death often occurs at the end of three or four days. When poisoning is not acutely fatal, a reaction occurs; the coagulability of the blood rapidly regains its former activity, and even though the same daily dose of chloroform is administered, coagulation at the end of about a week after the beginning of the experiment may have become somewhat more rapid than normal. An animal which has survived the critical period of three or four days may live ten or twelve days. The animal has established some form of resistance to the poison. In such instances there is widespread fatty degeneration of the liver and other organs and comparatively little necrosis.

The phenomena which have been described have a close analogy in phosphorus poisoning. There is, it is well known, intense fatty degeneration of the liver with some necrosis, and, in some instances, as Jacoby[24] has shown, diminution of the coagulability of the blood is associated with disappearance of fibrinogen. Furthermore, Jacoby has found that the liver with phosphorus poisoning undergoes more rapid autolysis than normal liver. He suggests that enzyme set free by the liver dissolves the fibrinogen of the blood. Our observations, mentioned above, suggest that changes which occur in the blood as the effect of chloroform or phosphorus may be subject to considerable variation, dependent upon the severity of intoxication and upon the length of the period of administration.

Gradual increase of anti-enzyme for a proteolytic enzyme, namely, leucoprotease, during the progress of intoxication with chloroform and phosphorus, noted at the beginning of our experiments, has suggested the possibility that disintegrating liver tissue may set free those proteolytic enzymes which are demonstrable in normal liver. The attempt has been made to determine if at any stage of intoxi-

[24] Jacoby, *Ztschr. f. physiol. Chem.*, 1900, xxx, 174.

cation the serum exhibits increased proteolytic activity. The enzymotic content of the serum has been tested by incubating a fixed quantity of blood serum during four or five days at 37° C. and subsequently determining the degree of autolysis, or by incubating a fixed quantity of serum with a suitable protein substrate, such as heated blood serum. That hepatic enzyme, which is most readily demonstrated, digests in the presence of acid. To determine if the similar enzyme which exists in the blood undergoes any change during the progress of chloroform intoxication, a series of experiments has been performed on dogs.

A fixed quantity of blood has been subjected to autolysis in the presence of 0.2 per cent. acetic acid. Blood has been drawn at intervals of three or four days by means of a needle inserted through the skin into the jugular vein. A measured amount of serum obtained by centrifugalization of the whipped blood has been diluted with salt solution (0.85 per cent.) and acetic acid in such proportion that the final volume of twenty-five cubic centimeters contains 0.2 per cent. acetic acid. In some instances blood serum denaturalized by heat has been added to the mixture as substrate for the proteolytic enzyme of the serum. All mixtures have been incubated during four or five days at 37° C. A mixture containing the same ingredients has served as control and has been immediately heated to boiling. Since it is undesirable to withdraw repeatedly considerable quantities of blood, the quantity of serum which has been subjected to autolysis has been small and occasionally, for lack of material (*e. g.*, Experiment 9), it has been necessary to omit this control. After coagulation by heat, the coagulum has been removed by filtration, and nitrogen in the filtrate has been determined by the Kjeldahl method; the amount of nitrogen liberated by digestion of coagulable protein will be expressed in cubic centimeters of one-tenth normal sulphuric acid.

In some experiments autolytic activity of serum from an animal receiving daily doses of chloroform has been tested on different days; the first test, made before administration of chloroform, serves as a normal standard for comparison with subsequent changes. In other instances the serum of animals which have received chloroform has been compared with normal serum from another animal.

Repeated tests, some of which will be recorded, have demonstrated that autolysis in neutral or alkaline media under conditions otherwise similar to those just described fails to occur or is much less marked than autolysis in the presence of acid.

EXPERIMENT 1.—A dog (weight 6,950 grm.) received on two successive days 10 c c. of chloroform. Coagulation time of the blood before administration of chloroform was four minutes and five seconds. On the third day of the experiment the animal was sick and the blood failed to coagulate within one hour. The following figures represent autolysis in the presence of acid before and after administration of chloroform. Furthermore, serum obtained on the third day has been compared with the serum of a normal dog.

	Autolysis of 5 c.c. of serum during 4 days at 37°.			
	Control.	In neutral medium.	0.2 per cent. sodium carbonate.	0.2 per cent. acetic acid.
Serum of chloroform dog before administration of chloroform. (First day; normal serum.).....	1.35			3.0
Serum of chloroform dog on third day.....	1.5	1.7	2.5	6.45
Serum of normal dog.....	1.75	1.75	2.05	3.6

The accuracy of the figures obtained with serum of the dog which had received chloroform (3d day) and normal serum, autolyzed in the presence of acid, is confirmed by an additional test made with 3 c.c. of each serum tested in the same medium: serum of chloroform dog (3d day), 4 3; normal serum, 2.8.

The administration of chloroform has caused well marked increase in the autolytic activity of blood serum tested in acid, and little change when tested in alkaline (perhaps trivial increase) or in neutral medium.

Since the quantities subjected to autolysis are small, experiments have been multiplied with the purpose of establishing the accuracy of the result. In Experiment 2, in addition to the tests previously employed, serum and protein substrate (denaturalized serum) have been incubated in the presence of acetic acid.

EXPERIMENT 2.—A dog (weight 6,750 grm.) received on four successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time three minutes, forty seconds), on the fourth day (five minutes, twenty-one seconds), and on the fifth day (four minutes, twenty-three seconds).

	Autolysis of 3 c.c. of serum during 4 days at 37°.			1 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal).....	1.0	1.05	3.7	2.85
4th day.....	1.1	1.05	5.05	3.45
5th day.....	1.8	1.75	4.95	4.15

EXPERIMENT 3.—A dog received chloroform by mouth on fourteen successive days. The serum was tested as follows:

	Autolysis of 3 c.c. of serum during 5 days at 37°.		
	Control.	In neutral medium.	0.2 per cent. acetic acid.
1st day (normal)	1.2	—	2.4
14th day	—*	1.9	5.65

* Control on fourteenth day doubtless approximates closely digestion in neutral medium (1.9 c.c.) and certainly does not exceed this figure.

EXPERIMENT 4.—A dog (weight 7,000 grm.) received on four successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time, four minutes), on the fourth day (nine minutes, twenty seconds), and on the fifth day (six minutes, twenty-one seconds). The animal was moribund on the fifth day.

	Autolysis of 3 c.c. serum during 5 days at 37°.			1 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal).....	[1.05]*	1.05	3.2	2.65
4th day	2.1	1.9	4.6	4.3
5th day	5.85	5.9	9.2	8.15

* This control was not determined, as the available serum was insufficient; other experiments show that normal serum incubated in neutral medium gives the same figure as serum coagulated immediately. It has been assumed that this control is identical with the figure obtained after incubation with reaction unchanged.

In Experiment 4, increase of the control is noteworthy; the serum contains, in greatly increased quantity, nitrogenous substances incoagulable by heat. Nevertheless, when these controls are subtracted from the figures obtained after incubation, evidence of increased proteolytic activity is obtained.

In the following experiments, which confirm those recorded

above, there is moderate increase of proteolytic activity, which reaches a maximum on the fourth day of chloroform intoxication.

EXPERIMENT 5.—A dog (weight 5,000 grm.) received on six successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time, one minute, twenty-seven seconds), on the fourth day (coagulation time, eight minutes, fifteen seconds), and on the seventh day (coagulation time, six minutes, six seconds).

	Autolysis of 3 c.c. serum during 4 days at 37°.			3 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	0.9	1.05	2.8	2.55
4th day	0.9	1.0	3.7	2.7
7th day	1.0	1.05	3.7	2.85

EXPERIMENT 6.—A dog (weight 4,750 grm.) received on ten out of eleven days (sixth day excepted) 7 c.c. of chloroform. Coagulation time: first day, five minutes, eighteen seconds; fourth day, four minutes, thirty-four seconds; seventh day, two minutes, seven seconds; tenth day, three minutes, fifteen seconds.

	Autolysis of 4 c.c. serum during 4 days at 37°.	
	Control.	0.2 per cent. acetic acid.
1st day (normal)	1.2	3.15
4th day	1.2	3.95
7th day	1.5	3.75
10th day	1.5	4.5

EXPERIMENT 7.—A dog (weight 5,600 grm.) received on six out of seven days (none on sixth day) 7 c.c. of chloroform. Coagulation time: first day, five minutes, thirty-one seconds; fourth day, seven minutes, seventeen seconds; seventh day, seven minutes, fifteen seconds.

	Autolysis of 4 c.c. serum during 5 days at 37°.	
	Control.	0.2 per cent acetic acid.
1st day (normal)	1.2	3.45
4th day	1.45	4.85
7th day	1.55	4.35

The experiments demonstrate that the well marked increase of proteolytic activity exhibited by animals which have received chloroform, attains its maximum on the third or fourth day of intoxication, and tends to be greatest when intoxication is most severe and when delay of coagulation time is considerable. Increased activity of autolysis may be absent when the critical period of intoxication

has passed or when the injurious action of chloroform has been slight. In the following experiment there has been no evidence of increased enzymotic activity.

EXPERIMENT 8.—A dog received 10 c.c. of chloroform on six successive days. Coagulation time: first day, three minutes, forty-five seconds; fourth day, ten minutes, sixteen seconds; seventh day, two minutes, twelve seconds.

	Autolysis of 3 c.c. serum during 4 days at 37°.			3 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	1.0	1.1	4.0	2.85
4th day	1.15	1.5	4.0	3.0
7th day	2.5	2.4	3.9	3.8

The very active anti-enzymotic activity of this serum will be mentioned later.

In the following experiment there is a close relationship between proteolytic activity and severity of intoxication, well indicated by delay of coagulation time.

EXPERIMENT 9.—A small quantity of serum (1 c.c.) has been allowed to act upon coagulated protein (5 c.c. of heated blood serum) during five days at 37° C. The sera of four dogs which have received 7 c.c. of chloroform on two successive days have been drawn on the third day, and the proteolytic activity in neutral and acid media compared with that of the sera from two normal dogs.

	Coagulation time.		Serum + heated serum after 5 days at 37°.	Serum + heated serum + 0.2 per cent. acetic acid after 5 days at 37°.
	Minutes.	Seconds.		
Normal Dog A	1	15	1.7	1.65
Normal Dog B	2	30	1.75	1.75
Chloroform Dog A	7	30	1.85	2.1
Chloroform Dog B	9	30	1.65	2.15
Chloroform Dog C	16	30	2.35	2.25
Chloroform Dog D	30	0	2.65	3.55

On the fourth day Chloroform Dog C was much less sick than on the previous day, whereas Chloroform Dog D was moribund. The following data were obtained:

	Coagulation time.		Serum + heated serum after 5 days at 37°	Serum + heated serum + 0.2 per cent. acetic acid after 5 days at 37°.
	Minutes.	Seconds.		
Chloroform Dog C	12	30	1.6	2.3
Chloroform Dog D	21	0	2.8	4.45

The figures obtained are small, but the changes which occur are the same as those previously described. It is noteworthy that the coagulation time of the blood is a fair index of the intoxication caused by chloroform. There has been a close relationship between coagulation time and autolytic activity, both in an acid and in an approximately neutral medium. The close relation between the toxic effect of chloroform and autolytic activity of the blood serum is well illustrated by the tests made on the fourth day. Dog C has in part recovered from the depression produced by the first two doses, and its serum exhibits autolytic activity not far removed from normal, whereas the serum of Dog D, which has been profoundly poisoned, exhibits increased autolytic activity.

Having in view the possibility that degenerative changes in the liver are associated with increased activity of proteolytic enzymes, we have undertaken a series of experiments to determine if the blood serum of animals receiving chloroform or phosphorus exhibits any alteration of the normal power of blood serum to restrain the autolysis of liver.

EXPERIMENT 10.—A dog has been given daily doses of chloroform (2 grm. per kilo of body weight) during ten days. An emulsion has been prepared by mixing finely ground liver with four times its volume of salt solution; 20 c.c. of this mixture have been allowed to undergo autolysis during five days at 37° C. both alone and in the presence of blood serum from the same animal and from a normal animal. The volume of the mixture has been increased in every instance to 25 c.c. Autolysis of liver alone is represented by 25.7 c c. N/10 sulphuric acid.

	Normal serum.	Chloroform serum.
Liver + 1 c.c. serum	22.25	20.2
Liver + 2.5 c c serum	18.4	18.65
Liver + 5 c.c. serum	15.5	17.05

Blood serum has inhibited autolysis in slight degree, but no noteworthy change of inhibition has been caused by administration of chloroform. When a small quantity of serum has been employed, inhibition has been slightly greater with chloroform than with normal serum. It is probable that the slightly higher figure obtained with five cubic centimeters of serum of the animal which received chloroform, is referable to its greater content of nitrogenous substances incoagulable by heat.

In other experiments, fresh liver of the rabbit has been subjected to autolysis in the presence of serum of a normal rabbit and of serum of a rabbit poisoned with phosphorus.

EXPERIMENT 11.—A suspension of normal rabbit's liver (10 c.c.) has undergone autolysis represented by 5.95 c.c. N/10 sulphuric acid (control, 2.05 c.c.). The following figures represent the effect of normal serum and of serum of a rabbit receiving phosphorus upon the same quantity of this suspension:

	Normal serum.	Chloroform serum.
Liver + 0.25 c.c.	6.1	6.0
Liver + 0.5 c.c.	5.45	6.05
Liver + 1 c.c.	5.65	5.1

There has been no constant or noteworthy difference in the effect of normal serum and of serum after administration of phosphorus upon autolysis of normal liver.

In a subsequent experiment the liver of an animal which has received phosphorus has been allowed to undergo autolysis. The attempt has been made to determine if serum of an animal similarly treated with phosphorus differs from normal serum in its effect upon the autolysis of phosphorus liver.

EXPERIMENT 12.—One gram of liver exhibiting fatty degeneration as the result of phosphorus has been suspended in 25 c.c. of salt solution and allowed to autolyze alone and in the presence of serum.

	Control	After incubation during 5 days at 37°.	Digestion.
Liver	2.6	6.05	3.45
Liver + 2 c.c normal serum	3.1	5.05	1.95
Liver + 2 c.c. phosphorus serum	3.7	6.45	2.75

Both normal and phosphorus serum have inhibited autolysis of the liver of an animal which has received phosphorus. Inhibition has been less with the serum obtained after administration of phosphorus than with that of the normal animal, but the foregoing experiments offer little evidence that the anti-enzymotic activity of the phosphorus animals undergoes any significant change.

Far more constant results have been obtained when the serum of animals which have received chloroform has been allowed to act upon an enzyme which, unlike the autolytic enzyme of the liver,

acts with greatest activity in an alkaline medium. Various quantities of serum from animals which have received chloroform and of serum from normal animals have been allowed to act upon a fixed quantity of enzyme of polynuclear leucocytes (leucoprotease).

Leucoprotease (twenty milligrams) has been allowed to digest coagulated blood serum both alone and in the presence of normal and of chloroform serum.

EXPERIMENT 13.—The serum of a dog which has received 160 c.c. of chloroform during fourteen days has been employed.

	Control.	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	1 55	19.4
	Normal serum	**Chloroform serum**
Above mixture + 0.25 c.c. serum	19.25	12.1
Above mixture + 0.5 c.c. serum	15.55	4.35
Above mixture + 1 0 c.c. serum	5.45	3.3

Inhibition has been uniformly greater with the serum of the animal which has received chloroform than with the serum of a normal animal.

The experiment has been repeated with serum of a dog which has received repeated doses of chloroform administered by mouth.

EXPERIMENT 14.—

	Control.	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	1.9	20.35
	Normal serum.	**Chloroform serum.**
Above mixture + 0 25 c.c. serum	20.3	15.4
Above mixture + 0.5 c c serum	19 1	4.0
Above mixture + 1.0 c.c. serum	8.8	3.2

Small quantities (0.2 and 0.5 cubic centimeter) of normal serum produce almost no effect upon the enzyme, yet the same quantities of chloroform serum greatly diminish its activity.

Continued increase of anti-enzymotic activity of the blood serum is well seen in the following experiment in which tests repeated at intervals of three days have been made with the serum of an animal to which chloroform has been administered daily.

EXPERIMENT 15.—A dog weighing 4,750 grm. has received daily during eleven days (the sixth day excepted) 12 c c. of chloroform. From 25 to 35 c.c. of

blood have been drawn at intervals of three days. Coagulation time: first day, five minutes, eighteen seconds; fourth day, four minutes, forty-three seconds; seventh day, two minutes, seven seconds; tenth day, three minutes, fifteen seconds. On the fifth day there has been slight jaundice which has subsequently increased in intensity. The inhibition of 20 mgr. leucoprotease caused by 0.5 c.c. of serum is shown by the following figures:

	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated blood serum	17.25
Same mixture + 0.5 c.c. serum on 1st day (normal)	13.75
Same mixture + 0.5 c.c. serum on 4th day	12.45
Same mixture + 0.5 c.c. serum on 7th day	5.25
Same mixture + 0.5 c.c. serum on 10th day	2.9

The following test shows that administration of phosphorus has the same effect as administration of chloroform upon the anti-enzymotic activity of the blood serum.

EXPERIMENT 16.—A dog weighing 7,700 grm. has received 1/50 grain of phosphorus every second day during fourteen days. Its blood serum (coagulation time, four minutes, nine seconds) has been compared with two normal sera.

	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	17.25
Same mixture and 0.5 c.c. normal serum A	13.75
Same mixture and 0.5 c.c. normal serum B.	15.15
Same mixture and 0.5 c.c. phosphorus serum	7 3

One-half cubic centimeter of normal serum exhibits slight anti-enzymotic activity, whereas the same amount of serum from an animal which has received phosphorus causes strong inhibition of an equal amount of enzyme.

Increased inhibition of leucoprotease, demonstrable in the serum of dogs which have received chloroform or phosphorus, has been found in rabbits as well.

EXPERIMENT 17.—In the following series of tests, sera from a rabbit which has received phosphorus has been obtained on different days and has been compared with normal sera.

20 mgr. leucoprotease + coagulated blood serum: control, 2.8; after incubation during 5 days at 37° C., 19.45.

With serum of the animal which has received small doses of phosphorus, digestion caused by leucoprotease is constantly less than with normal sera.

		Normal serum.	Phosphorus serum.
A	Same mixture + 0.25 c.c. serum	15.2	10.75
	Same mixture + 0 5 c c. serum	7.05	5.2
	The following test has been made 4 days later:		
B	Same mixture + 0.25 c c. serum	16.95	10.9
	The following test has been made one day after last:		
C	Same mixture + 0.25 c.c. serum	16.55	11.95
	Same mixture + 0.5 c.c. serum	7.25	4.6

The foregoing experiments show that the blood serum under the influence of a poison such as chloroform, acting with intensity sufficient to cause necrosis of the liver and diminish the coagulability of the blood, acquires increased ability to cause digestion of protein. This increased proteolysis is, in part at least, referable to an enzyme which, like the autolytic enzyme of the liver and almost all other organs, digests with maximum activity in a weakly acid medium. It is not improbable that proteolytic enzyme is liberated by disintegration of parenchymatous cells and carried away by the blood.

In animals which survive the severe intoxication produced by large doses of chloroform, or in those which have repeatedly received smaller doses, the blood serum acquires increased ability to restrain the action of a proteolytic enzyme; but this anti-enzymotic action is exerted, not upon the enzyme of the liver, which acts in acid, but upon an enzyme which acts in the presence of alkali, namely, leucoprotease. There is little reason to doubt that the same serum will restrain the action of a second enzyme which acts in alkali, namely, trypsin, for, on the one hand, Jochmann and Kantorowicz have found that anti-enzyme for enzyme of leucocytes and for trypsin increases simultaneously, and, on the other hand, K. Meyer has found the anti-tryptic activity of the serum increased in animals which have received phosphorus. Anti-enzyme for one type of enzyme is increased; whereas evidence heretofore available indicates that a second type of enzyme is predominant in those tissues which are subject to the destructive action of the poison. Furthermore, our experiments have shown that an enzyme similar to that of the injured organ accumulates in the blood serum. It is difficult to correlate these observations.

Nevertheless, the experiments of Hedin and of Dochez show that

the liver contains a second enzyme which, unlike that directly obtainable from fresh liver, digests protein in the presence of an alkaline medium. These observations suggest the possibility that this enzyme may have a part in changes which increase the anti-enzymotic activity of the blood serum after administration of substances which injure the liver. Treatment of fresh liver with acid discloses an enzyme which digests in the presence of acid. Does the serum of an animal which has received repeated doses of chloroform and exhibits increased power to inhibit leucoprotease and, doubtless, trypsin, exhibit as well increased power to inhibit that enzyme of the liver which is similar to these two enzymes? The experiments which follow have been undertaken with the purpose of answering this question.

Using the method described by one of us (Dochez), we obtained that enzyme which digests protein in the presence of alkali, by subjecting fresh liver to the action of weak acetic acid during a period of twenty-four hours. Liver just after removal with aseptic precautions from an etherized animal has been finely ground by means of a hashing machine; ground liver has been mixed with twice its volume of salt solution to which acetic acid has been added in such amount that the concentration of the mixture is 0.2 per cent. After this mixture has stood at a temperature slightly above freezing during twenty-four hours, the acid has been neutralized by an equivalent quantity of one-tenth normal sodium hydroxide. Ten cubic centimeters of this neutralized mixture has been allowed to undergo autolysis and the inhibition caused by various quantities of serum has been tested. In order to determine if the inhibitory activity of serum is increased during the progress of chloroform poisoning, normal serum has been compared with serum obtained after repeated administration of chloroform. For the purpose of the present investigation, a comparative study of anti-enzymotic activity for leucoprotease and for enzyme of liver has been essential, and parallel tests of the action of each serum upon the two enzymes have been made.

The change which occurs rapidly when liver is treated with acid occurs slowly when liver in approximately neutral suspension is preserved under conditions which prevent bacterial growth. In the

following experiment a suspension allowed to stand under toluol during two months has been used.

EXPERIMENT 18.—Two dogs, Dogs A and B, have received daily 10 c.c. of chloroform by stomach. Blood has been drawn on the first and on the fourth day of the experiment. Anti-enzymotic activity of this serum has been tested by allowing a given quantity of leucoprotease (20 mgr.) to act upon substrate (5 c.c. heated blood serum) in the presence of 0.5 c.c. of blood serum obtained before administration of chloroform and on the fourth day of administration. A parallel test has been made with liver which has been allowed to stand on ice over toluol during two months in order to liberate that enzyme which, like leucoprotease, digests in alkali. This suspension of liver has been made by mixing equal volumes of liver, ground through a hashing machine, and of salt solution with sodium carbonate in the proportion of 0.1 per cent. Ten cubic centimeters of this suspension diluted with salt solution so that the total volume is 25 c.c. has been allowed to undergo autolysis; the same quantity has been autolyzed in the presence of blood serum.

Leucoprotease (20 mgr.) acting upon the substrate is represented by 16.2 c.c. N/10 H_2SO_4 (control, 1.8 c.c.). Autolysis of liver (10 c.c.) alone is represented by 7.5 c.c. N/10 H_2SO_4 (control 3.3 c.c.). The following table represents a comparison of digestion by leucoprotease and autolysis of liver in the presence of the same serum.

	Digestion with leucoprotease + 0.5 c.c. serum.	Autolysis of active liver.	
		+ 0.5 c.c. serum.	+ 1 c.c. serum.
CHLOROFORM DOG A.			
With 0.5 c.c. serum obtained before administration of chloroform ...	13.6	6.7*	8.15
With 0.5 c.c. serum obtained on 4th day of chloroform administration	3.35	5.2	—
CHLOROFORM DOG B.			
With 0.5 c.c. serum obtained before administration of chloroform ..	11.4	8.35	7.55
With 0.5 c.c. serum obtained on 4th day of chloroform administration	3.15	5.3	5.45

*This figure should be somewhat higher, since loss occurred as result of accident during Kjeldahl determination.

On the fifth day of the experiment the serum of each dog has been again tested, but as it has been necessary to use for autolysis a different suspension of liver, no comparison with the foregoing test is possible and consequently the two sera have been compared with sera from two normal dogs. Liver suspended in an equal volume of salt solution has been allowed to stand in the ice-chest under toluol during approximately two months; slight acidity of the suspension has been neutralized by addition of N/10 NaOH (4.8 c.c. to 170 c.c. of suspension). Ten cubic centimeters of the suspension have been used for each test; 5 c.c. of blood serum have been added with the purpose of supplying additional substrate for the proteolytic enzyme derived from the liver.

Digestion after incubation during five days at 37° C. is represented by 12.95 c.c. N/10 H_2SO_4 (control 4.8 c.c.).

	With 0.5 c.c. serum.	With 1 c.c. serum.
Serum of Chloroform Dog A	7.65	7.1
Serum of Chloroform Dog B	7.6	5.65
Serum of Normal Dog A	9.65	6.95
Serum of Normal Dog B	8.2	7.3

The degree of inhibition will obviously depend upon the strength of the enzyme and of the anti-enzymotic activity of the serum. In the second series of tests, one cubic centimeter of normal serum has so materially reduced the activity of the enzyme that differences between normal serum and serum after administration of chloroform are in large part obliterated.

In the first series of tests (Experiment 18), the inhibiting action of each serum upon leucoprotease has been compared with its action upon enzyme of liver; with well marked decrease of anti-enzyme for the one, there has been a parallel increase for the other. In the following experiment the serum tested has shown no noteworthy decrease of anti-enzyme for leucoprotease and there has been little, if any, change in its ability to restrain the similar enzyme of liver.

EXPERIMENT 19.—Two animals received on four successive days 10 c.c. of chloroform. Blood was drawn before administration and on the fourth day of the experiment. The effect of these sera upon leucoprotease and upon enzyme of liver is shown by the following figures:

Leucoprotease (20 mgr.) acting upon substrate is represented by 16.2 c.c. N/10 H_2SO_4 (control, 1.8 c.c.). Autolysis of liver alone is represented by 7.5 c.c. N/10 H_2SO_4 (control, 3.3 c.c.).

	Digestion with leucoprotease + 0.5 c.c. serum.	Autolysis of active liver + 0.5 c.c. serum.
Chloroform Dog C.		
With serum obtained before administration of chloroform	9.5	7.9
With serum obtained on 4th day after chloroform administration	8.5	7.55
Chloroform Dog D.		
With serum obtained before administration of chloroform	14.2	7.75
With serum obtained on 4th day of chloroform administration	13.7	7.85

In the following experiment serum of an animal which has received chloroform is compared with that of a normal dog.

EXPERIMENT 20.—A dog weighing 21 kilograms has received daily doses of chloroform increasing from 0.5 to 7 c.c. Blood has been drawn on the fourteenth day of the experiment. Inhibition of leucoprotease and of enzyme of liver are compared.

Leucoprotease (20 mgr.) acting on substrate causes proteolysis represented by 16.6 c.c. N/10 H_2SO_4, control being 1.5. Autolysis of the liver employed is represented by 27.2 c.c. N/10 H_2SO_4, control being 16.05 c.c. In order to make the degree of inhibition more conspicuous, the control has been subtracted from the figure obtained after digestion during four days at 37° C.

	Total digestion with leucoprotease + 0.5 c.c. serum	Total autolysis of liver.	
		+ 0.5 c c serum.	+ 1 c c serum.
With serum of normal dog	14.9	12.7	12.15
With serum of chloroform dog	2.8	8.45	9.75

Well marked increase of anti-enzyme for liver pretreated with acid corresponds with increased anti-enzyme for leucoprotease. In the following experiment, identical in other respects with that just described, the effect upon autolysis of pretreated liver, both of normal serum and of serum from a dog which has received chloroform, has been tested in the presence of alkali.

EXPERIMENT 21.—The foregoing experiment has been repeated by comparing serum of a normal dog with serum of a dog (weighing 21 kilograms) which has received 21 c.c. of chloroform daily (the seventh day excepted) during ten days.

The leucoprotease and suspension of liver used in Experiment 20 have been employed and the control has been subtracted from the figure obtained after digestion during four days at 37° C.

	Total digestion with leucoprotease + 0.5 c c. serum.	Total autolysis of liver.	
		+ 0.5 c.c. serum.	+ 1 c c serum.
With serum of normal dog	13.2	15.65	13.8
With serum of chloroform dog .. .	4.67	12.9	12.85

In order to obtain further information concerning the progress of the changes which effect the anti-enzymotic activity of the blood serum, comparative tests have been made with leucoprotease and with pretreated liver during the course of prolonged administration of chloroform. The method of testing inhibition is identical with

that previously employed; no alkali has been added to the mixtures prepared for proteolysis.

EXPERIMENT 22.—Serum has been obtained from a dog weighing 9 kilograms, which during nineteen days has received 9 c.c. of chloroform daily. Coagulation time, which has been two minutes and ten seconds before administration of chloroform, has been tested whenever blood has been drawn and has never risen above three minutes and thirty-five seconds. The animal has been jaundiced on the eighteenth day of the experiment.

The following table gives the results of a large series of tests made on different days with various quantities of serum added to mixtures containing either leucoprotease or liver rendered active by pretreatment with acid.

	Proteolysis with leucoprotease.			Autolysis of pretreated liver.				
	With no serum.	0.25 c.c. serum.	0.5 c.c. serum.	With no serum.	0.25 c.c. serum.	0.5 c.c. serum.	1 c.c. serum.	2 c.c. serum.
1st day (normal) .	12.9	11.65	9.5	16.2	12.5	11.2	10.5	8.9
4th day.....		7.1	4.45	15.0	9.8	8.45	7.55	6.9
7th day.....		6.5	4.4	18.2	10.1	9.0	8.05	8.0
10th day....		6.7	5.4	16.6	9.9	9.5	7.6	7.1
14th day.....	15.7	6.2	4.6	16.3	10.5	10.0	8.8	8.1
18th day.....	14.8	4.4	3.4	15.9	9.3	8.5	7.1	7.0

Although there are some irregularities in the progress of the changes represented by the foregoing figures, it is obvious that the power of the serum to inhibit the active enzyme of acid-treated liver exhibits an increase parallel with the increased power to inhibit leucoprotease. Here, as in previous experiments, larger quantities even of normal serum (one or two cubic centimeters) cause such well marked inhibition of the hepatic enzyme that differences are less conspicuous than when small quantities (0.25 or 0.5 cubic centimeter) are employed. To demonstrate more clearly the parallel increase of anti-enzyme for the two enzymes which have been compared, curves (Fig. 1.) have been plotted to represent digestion caused by hepatic enzyme (unbroken line) and leucoprotease (broken line) in the presence of 0.25 and of 0.5 cubic centimeter of blood serum.

The experiments which have been described show that chloroform given in quantity sufficient to produce profound intoxication, indicated by necrosis of the liver and loss of coagulability of the blood, causes an increase of the proteolytic enzyme normally present in the blood serum. Increase of proteolytic activity is exhibited

in the presence of weak acid, but is much less evident in an alkaline or neutral medium. This observation suggests the possibility that the proteolytic enzyme of liver tissue, which causes autolysis and has maximum activity in the presence of acid, is freed by disintegration of hepatic cells and accumulates in the blood serum.

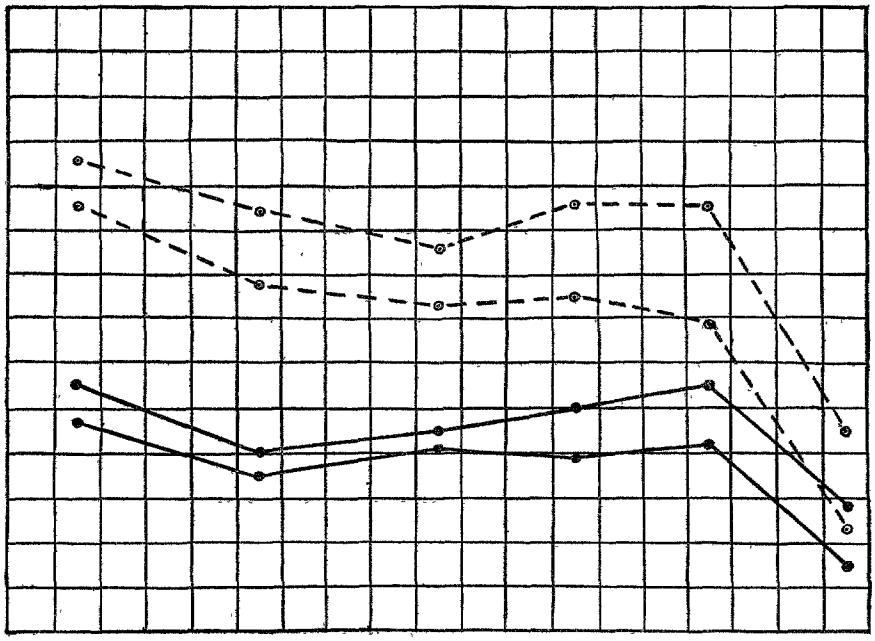

CHART 1.—Diagram showing increase of anti-enzymes in the blood serum of an animal (see Experiment 22) receiving chloroform daily. The solid lines represent digestion by enzyme of acid-treated liver in the presence of 0.25 c.c. (upper solid line) and of 0.5 c.c. (lower solid line) of blood serum. The broken lines represent digestion by leucoprotease in the presence of 0.25 c.c. (upper broken line) and of 0.5 c.c. (lower broken line) of blood serum. Changes in the two pairs of lines are an index of the activity of anti-enzyme for the two enzymes, and are, in general, parallel.

The anti-enzymotic action of the blood serum has been tested in animals which have repeatedly received chloroform in quantity insufficient to produce fatal intoxication. There is no increase of the normal power of the serum to restrain autolysis of liver. Nevertheless, when anti-enzymotic action of blood serum is tested

with leucoprotease, gradually increasing activity constantly accompanies continued administration not only of chloroform but of phosphorus as well. On the one hand, it is especially noteworthy that the enzyme of leucocytes digests protein in an alkaline or neutral medium, whereas the proteolytic enzyme of liver exhibits maximum activity in acid. On the other hand, it is now known that treatment of liver with weak acid renders active an enzyme which digests with energy in alkali in much the same way that treatment of fresh extract of pancreas with acid transforms pancreatic zymogen into trypsin. Is increase of anti-enzyme for leucoprotease an index of increase of anti-enzyme for this second enzyme of liver, which, like leucoprotease, digests protein with maximum activity in alkali, and is, perhaps, freed by disintegration of liver cells? Tests with acid-treated liver show that anti-enzyme for the hepatic enzyme which digests in alkali is increased during the progress of chloroform intoxication, and parallel tests with leucoprotease and with this hepatic enzyme have shown that serum which exhibits increased inhibition of one exhibits increased power to restrain the other.

The following scheme represents the relationship of hepatic enzymes to enzymes and anti-enzymes of the serum which is suggested by the foregoing experiments:

Proteolytic enzymes of liver.	Proteolytic enzymes of blood serum.	Anti-enzymotic action of blood serum.
(A) Enzyme digesting in acid.	(A) Enzyme digesting in acid. *Increased by chloroform.*	(A) Alkalinity of serum.
(B) Enzyme digesting in alkali. (Made active by acid-treatment of fresh liver.)	(B) Enzyme digesting in alkali. (Present in globulin fraction of serum and inhibited by anti-enzyme of albumin fraction.)	(B) Anti-enzyme for trypsin, leucoprotease, and similar enzyme of liver. *Increased by chloroform.*

Increase of anti-enzyme occurring in association with intoxication by chloroform or by phosphorus is, doubtless, similar to that which has been repeatedly observed with the cachexia of malignant growth

with various infections, and with other conditions. The increased protein disintegration which accompanies such processes is, perhaps, associated with liberation of proteolytic enzymes similar to those concerned in the postmortem autolysis of organs. The experiments which have been described suggest that formation of anti-enzyme is a means by which the body is protected from enzymes liberated by degeneration of cells.