SERUM FERMENTS AND ANTIFERMENT AFTER FEEDING.

STUDIES ON FERMENT ACTION. XXI.

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Considerable progress in our understanding of the metabolism of protein digestion has been made possible in recent years due largely to microchemical investigations carried out by Van Slyke (1) and by Folin(2), and by means of the ingenious vividiffusion apparatus of Abel, Rowntree, and Turner (3).

There has been, however, no corresponding investigation of the serum ferments which might be involved during the process, possibly because of the lack of a suitable quantitative method of estimating the amount of protease in the serum. Neither the optical nor the dialysis method of Abderhalden are of value for such work, for apart from several objections of a theoretical nature, which we have reviewed in a former paper (4), they are inconveniently cumbersome, and the dialysis method particularly is open to the serious objection that it is not the introduced protein (placenta, tumor tissue, etc.) which is digested, but that the ferment acts on the serum proteins. The introduced substrate therefore adds an unnecessary complication and confusion.

Method of Determining Protease Action.

The method which we have used is based on the following considerations. When serum antiferment is removed by means of lipoid solvents, chloroform, ether, etc., whatever protease is present can become active and digest the serum proteins (5). If, there-

fore, the amount of non-coagulable nitrogen is determined both before and after incubation, the difference in amount will be an index of protease action. The nitrogen determinations can be made with great facility by the Folin or Van Slyke method. We have noticed, however, that under chloroform the increase in amino-acids need not be in proportion to the amount of non-coagulable nitrogen found, the chloroform evidently offering no insult to the true proteases, while the ereptase action may be retarded.

The normal activity of protease has been assumed to be lytic, while the synthetic action of the ferment has not been demonstrated. Few observations have been made in this direction, the idea being held that the synthetic process is more intimately connected with the function of the individual cell. Theoretically there is, of course, no reason why proteosynthesis should not occur in the serum; indeed, it seems rational that such ferment activity must be functionating when the serum proteins are formed.

The technique used is as follows: The clear hemoglobin-free serum is measured with an accurate 1 cc. pipette into a rather wide test-tube (about 18 mm.). To the tube 0.5 to 0.75 cc. of chloroform is added and the tube is sharply shaken, at intervals, until a milky emulsion is formed. We prefer chloroform because the emulsion is more stable than with ether or other lipoid solvents. A control tube is inactivated at 60° C. for 30 minutes and a drop of toluol is then added in place of chloroform. Both tubes are then incubated over night (15 to 16 hours at 37°). In the morning about 1 cc. of a mixture of 10 per cent acetic acid plus 20 per cent salt solution is added, and the tubes are then gently warmed in a water bath until the chloroform has been evaporated. About 2 or 3 cc. of distilled water are then added slowly and the tubes boiled for at least 10 minutes. The coagulated protein is filtered off by means of hard filter papers, previously moistened, the filtrate being permitted to filter directly into the large tubes used for oxidizing. The tubes are then oxidized and Nesslerized according to the usual Folin method, the readings being made against varying dilutions of the 1 mg. standard, so that test readings are made against a standard of apparently equal color.

Whenever possible we made duplicate determinations of each serum to obviate any error. When properly made the difference in any two readings should not exceed a few hundredths of a mg. per cc. of serum, a degree of accuracy sufficient for the work. If, for example, the total non-coagulable nitrogen per cc. of serum is proved to be 0.32, and after incubation, has increased to 0.45 mg., the protease activity can be expressed as 0.13 mg. per cc., and has

been so designated in the accompanying text-figures. The proteins are not precipitated by alcohol, as in the usual Folin method, because we wish to determine the increase of higher splitting products of the proteins as well as of the amino-acids. By precipitating with alcohol the higher split products would, of course, be retained on the filter. The non-coagulable nitrogen which we determine is therefore not quite analogous to the non-protein nitrogen determined by the Folin method.

Method of Determining Serum Esterase.

Serum esterase has been determined as follows: To 1 cc. of the serum, 1 cc. of neutral, redistilled ethyl butyrate and 0.5 cc. toluol are added, the volume being brought to 10 cc. with physiological salt solution. The flasks are then shaken 100 times and incubated for 4 hours. 25 cc. of neutral 95 per cent alcohol are then added to each flask and the acidity which has developed is titrated with N/50 sodium hydrate (alcoholic) to a faint pink with phenolphthalein. After deducting the proper controls, *i. e.*, serum alone, ethyl butyrate alone, etc., the esterase index is expressed in terms of cc. of N/100 sodium hydrate used to neutralize the acidity developed by 1 cc. of serum from 1 cc. of ethyl butyrate.

The antiferment has been determined by the method described in our previous papers (5).

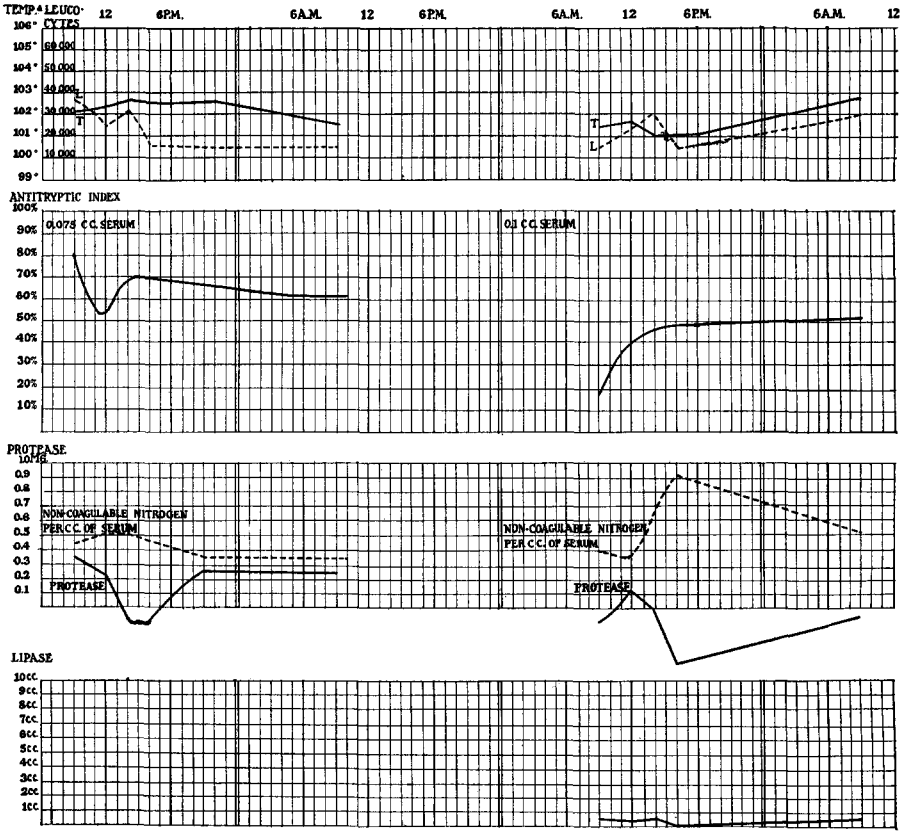
Healthy adult dogs have been used exclusively. Blood was taken from the ear veins before feeding, and the animals were then fed with chopped boiled meat and bread. They were permitted to eat as much as desired. No water was given until the following day. Blood samples were usually taken at noon, at 2 and 4 p. m., and the following morning.

EXPERIMENTAL.

Dog 22.—(Text-fig. 1.) Fed at 9.30 a. m. Bled at 8.30 a. m., noon, 2 and 4 p. m., 9 p. m., and the following morning. It will be noted from the chart that the antiferment index dropped rather sharply, rose again by 2 p. m., and remained fairly constant after that time. The non-coagulable nitrogen increased from 0.45 mg. per cc. to 0.5 mg. at noon and at 2 p. m., after which time it continued to decline.

The protease action which was 0.35 mg. per cc. before the meal had decreased to 0.25 mg. at noon, and at both 2 and 4 p. m. was negative. The serum on incubation at this time showed an actual decrease in non-coagulable nitrogen. The serum taken at 9.00 p. m., and that of the morning showed almost the original protease index. Lipase was not determined.

Dog 17.—(Text-fig. 2.) Fed at 9.45 a. m. Bled at 9.00 a. m., 2 and 4 p. m., and the following morning. In this animal there was a well marked rise in the antiferment index, which persisted till the following morning. The non-coagulable nitrogen showed a striking increase, reaching more than twice the original amount at 4 p. m. In view of this result we repeated the experiment at a later date on the same animal with quite similar results. Thinking that possibly a



TEXT-FIG. 1. TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. Serum ferments and antiferment index after feeding.

kidney lesion might be at fault, in view of the repeated observation of renal pathology reported in dogs, the urine was tested for albumin and casts; and the kidneys were examined histologically with entirely negative results.

The serum showed an increase of protease at noon, after which no protease action was found. The blood drawn at 4 p. m., and containing 0.9 mg. of non-coagulable nitrogen per cc., when incubated with chloroform, showed a decrease

to 0.5 mg. per cc.; *i. e.*, a loss of 0.4 mg. per cc. The lipase showed a slight decrease.

Dog 26.—(Text-fig. 3.) Fed at 9.30 a. m.; relatively small feeding. Bled at 9 a. m. and 1.30 p. m. Killed at 4 p. m.

The antiferment index remained constant for the first two samples and showed a slight decline at 4 p. m. The lipase remained constant throughout. The non-coagulable nitrogen showed a marked increase, as will be noted in Table I.

TABLE I.

Serum.	Non-coagulable nitrogen per cc.	After incubating under chloroform 16 hrs.	Increase of non-coagulable nitrogen.	After incubating under toluol.	Decrease in non-coagulable nitrogen.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
9 a. m.....	0.23	0.72	0.49	0.23	0
	0.22				
1.30 p. m.....	0.31	0.66	0.35	0.27	−.04
	0.31				
4 p. m.....	0.36	0.70	0.31	0.29	−.10
	0.39				

The protease action decreased progressively, although it did not fall to quite so low a level as in other experiments.

When the serum, instead of being inactivated at 60°, was permitted to remain under toluol (0.5 cc.), it will be noted that for the first sample there was no change, while in both the other specimens of serum a distinct decrease in non-coagulable nitrogen occurred.

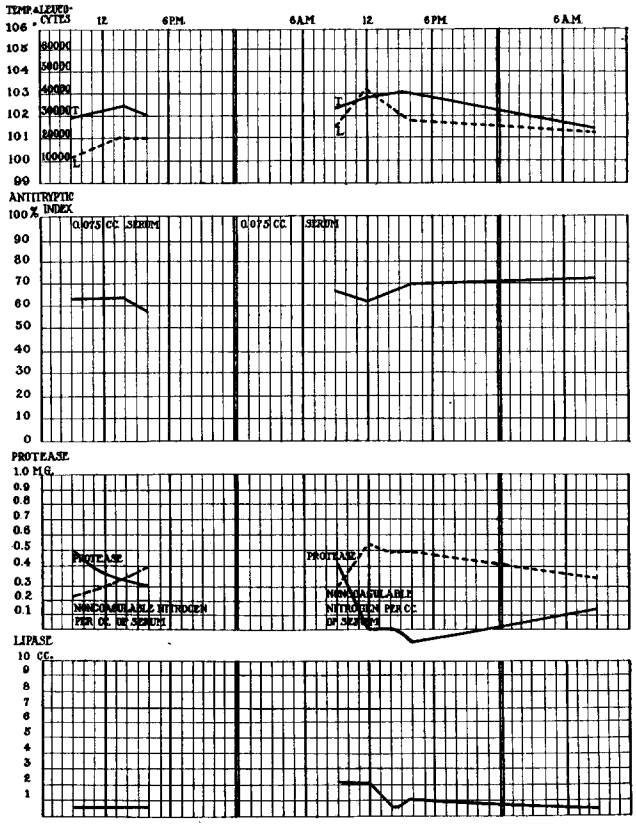
Estimation of Proteoses.

In order to determine the nature of the increase in the non-coagulable nitrogen, which was not due to an increase in urea, we estimated the amount of proteoses as follows:

To 10 cc. of serum, acetic acid and salt were added and the coagulable proteins removed by filtration after acidifying and boiling for 30 minutes. The clear fluid was now neutralized and permitted to stand until whatever acid albuminates were present had been precipitated. It was again filtered and the material placed in a dialyzing bag under toluol. After 24 to 48 hours, depending upon the rate of dialysis, and having been freed from all urea and other diffusible nitrogenous substances, nitrogen determinations were made on the material remaining in the dialyzing membranes.

In place of dialyzing we have made direct determinations by precipitating all the proteoses by saturating the boiling filtrate with sodium sulphate. The precipitate collected on the filter while hot is washed with a saturated solution of sodium sulphate and is then

washed through the filter with water made slightly alkaline with sodium carbonate. Nitrogen determinations are made on the proteoses so dissolved. As a rule, the figures obtained after dialyzing are slightly higher than those obtained by this method.



TEXT-FIG. 3. TEXT-FIG. 4.

TEXT-FIG. 3. Serum ferments and antiferment index after feeding.

TEXT-FIG. 4. Serum ferments and antiferment index after feeding in dog with pancreatic insufficiency.

In the following experiments somewhat larger amounts of blood were withdrawn by means of the suction pump from the ear veins (about 60 cc. of blood) before and after feeding. The animal was usually anesthetized after about six or seven hours and blood samples

were removed from various parts of the circulation. In these experiments it frequently happens that the terminal blood so collected may contain proteases unless great care is taken to prevent handling of the intestine and any unnecessary trauma, for blood taken during such conditions, as well as agonal blood under any circumstances, always contains proteases. This is shown in the following experiment.

Dog 42.—Small feeding at 9.00 a. m. Bled before feeding and at 1.15 p. m. Killed at 3.30 p. m. (Table II).

TABLE II.

Serum.		Non-coagulable nitrogen per cc.	After incubating under chloroform.	Increase in non-coagulable nitrogen.	Non-dialyzable nitrogen per 5 cc. of serum.
		mg.	*mg.*	*mg.*	*mg.*
9 a. m.		0.32	0.32	0	0.8
1.15 p. m.		0.3	0.31	0.01	0.71
3.30 p. m.	Arterial	0.21	0.5	0.29	0.44
	Inferior vena cava	0.22	0.5	0.28	
	Portal	0.22	0.45	0.23	0.45
	Hepatic	0.22	0.35	0.13	

This experiment differs from the others in that there is a decrease in non-coagulable nitrogen, made evident also by the decrease in the non-dialyzable nitrogen, as shown in the last column. The animal, furthermore, was without protease in the serum at the beginning of the experiment, but showed the presence of the ferment when killed. Inasmuch as the ferment was found in all the samples and not only

TABLE III.

Serum.		Non-coagulable nitrogen per cc.	Amino nitrogen in 2 cc. of serum (direct determination).	Increase in non-coagulable nitrogen after incubating under chloroform.
Dog 50		*mg.*	*mg.*	*mg.*
9 a. m.		0.34	1.05	
Noon		0.34	0.96	
3.00 p. m.	Arterial	0.35	0.99	
	Portal	0.45	1.35	
	Hepatic	0.25		
Dog 41			Amino nitrogen per 5 cc. of serum. Protein precipitated by alcohol	
9 a. m.		0.25	0.23	0.55
3.00 p. m.	Arterial	0.34	0.215	0.43
	Portal	0.45	0.283	0.55
	Hepatic	0.38		0.45

in the portal blood, in which the concentration, as the following experiment will show, is usually greatest, we believe that the ferment present was due to trauma incident to the collection of the samples and possibly to an agonal condition.

The non-coagulable nitrogen is usually greatest in the portal blood, as is shown in Table III, taken from specimens of two dogs.

It is evident that only part of the increase in non-coagulable nitrogen is due to an increase in amino-acids.

The following experiment (Table IV) shows that there is no increase in the higher split products (primary and secondary proteoses) in the serum.

Dog 52.—No food for 24 hours. Large feeding of meat and milk at 8.30 a. m.

TABLE IV.

Serum.	Non-coagulable nitrogen per cc.	Incubated under chloroform.	Increase of non-coagulable nitrogen under chloroform.	Incubated under toluol.	Decrease in non-coagulable nitrogen.	Proteoses per 1 cc. of serum.	Lipase per cc.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
8.30 a. m.	0.31	0.50	+0.19	0.25	0.06	0.17	0.0
12 noon	0.38	0.30	−0.08	0.3	0.08	0.15	0.3
3.00 p. m. Arterial	0.45	0.43	−0.02	0.38	0.07	0.09	0.5
3.00 p. m. Inferior vena cava	0.37	0.34	−0.03	0.34	0.03		0.3
3.00 p. m. Hepatic	0.41	0.34	−0.07	0.3	0.11		0.0
3.00 p. m. Portal	0.45	0.51	+0.06	0.25	0.2	0.11	

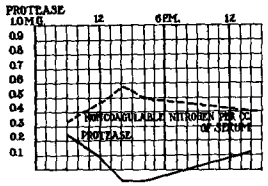
As will be noted, there is a distinct increase in non-coagulable nitrogen after the feeding, while the serum protease, originally 0.19 per cc., drops in all the samples, although the portal blood still shows some protease action. When preserved under toluol this serum shows the greatest decrease in non-coagulable nitrogen. The proteoses, estimated by the direct precipitation with sodium sulphate, are decreased throughout. It is interesting to note that the hepatic blood contained no lipase.

The increase in amino-acids after feeding has previously been noted, and Van Slyke, Cullen, and McLean (6) have recently demonstrated an increase in urea in the blood after feeding. We are rather inclined to assume that some of the increase in non-coagulable nitrogen must be due to the lower dialyzable split products other than amino-acids. In certain pathological conditions Pribram (7) has

noted an increase in non-dialyzable protein split products; this is especially true during anaphylactic shock, as we shall show in a later paper.

Occlusion of the Pancreatic Duct.

Dog 26.—(Text-fig. 4.) The animal was operated on Feb. 11, 1915, the pancreatic duct ligated, and bile salts were injected into the pancreatic duct. The animal recovered from the acute pancreatitis so produced. On Feb. 18 it was used for the feeding experiment, and was killed the following day. The pan-



TEXT-FIG. 5. Average change in non-coagulable nitrogen and serum protease after feeding.

creatic duct was found completely occluded and the pancreatic tissue showed marked evidences of a chronic inflammation. The animal was fed at 9.30 a. m., and bled at 9.00 a. m., noon, 2 and 4 p. m., and the following morning. The animal ate a very large meal and showed obvious evidence of discomfort and distress after the feeding.

The antiferment index showed a slight drop and a following recovery. The esterase showed rather a marked fall. The relation of the non-coagulable nitrogen and the protease are typical and similar to those of normal animals.

The Non-Coagulable Nitrogen of the Serum.

A composite chart (Text-fig. 5) made from eight feeding experiments shows a very distinct increase in the non-coagulable nitrogen of the serum, reaching a maximum about five to six hours after the feeding. Holweg (8) has recently shown that this increase is due partially to an increase in urea, while the change in amino-acid and albumoses is not constant. Abderhalden and numerous other workers with the dialysis method have called attention to the fact that for the Abderhalden test the serum should be drawn before a meal in order to overcome the presence of split products and non-specific ferments in the blood stream. In several previous papers Abderhalden had denied the presence of split products of proteins in the serum. The increase in amino-acids as determined by the Van Slyke apparatus is very slight, except in the portal blood, but nevertheless we have

found it constant. On the other hand, there is no increase in proteoses. Whether or not there is an increase in peptone we have not determined.

Serum Protease.

The protease curve in Text-fig. 5 indicates a marked fall in the amount of ferment present in the serum after feeding. In view of the fact that proteolytic ferments have been demonstrated in the urine after feeding, the tryptase reaching its maximum in from six to seven hours, these results were quite contrary to our expectations. Inasmuch as the serum reaction under chloroform is slightly acid, we have several times altered the reaction to one slightly alkaline, but have not been able to show an increased proteolysis in this way. It is to be remembered, however, that we are here dealing with true protease and not an ereptase, the latter being the ferment most frequently tested for in the urine.

While this decrease in protease has usually been noted in the peripheral circulation, the portal blood may at the same time show an increase in ferment, as is illustrated in the following experiment (Table V).

Dog 34.—Fed at 8.30 a. m. Bled at 8.45 a. m. and 3.30 p. m.

TABLE V.

Total Non-Coagulable Nitrogen per Cc.

	mg.	
Before feeding	0.22	(Peripheral.)
After feeding	0.32	(Peripheral and portal.)

Protease. Increase of Non-Coagulable Nitrogen on Standing.

Before feeding	0.22	(Peripheral.)
After feeding	0.18	(Peripheral.)
After feeding	0.42	(Portal.)

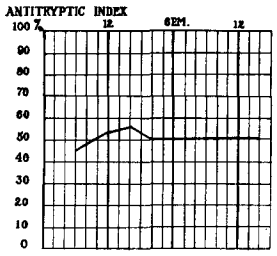
The increased amount of ferment in the portal blood evidently does not pass the liver. It is possible that in the portal blood a large amount of split products of proteins are present which might offer an available substrate for ereptase rather than tryptase action. The absorption of free tryptic ferment from the normal intestinal tract must be small in amount, for the ferment is rapidly bound by the proteins present, so that even in the actively digesting intestine the amount of free ferment is not large.

Whether the decrease of non-coagulable nitrogen noted on incubation in various of these experiments depends on any actual synthesis to coagulable proteins or upon purely experimental manipulation will be discussed fully in a later paper.

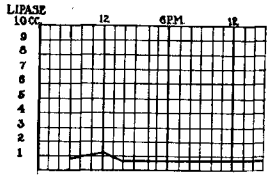
Serum Antiferment.

The average antiferment index following feeding is shown in Text-fig. 6. The increase, previously noted, being coincident with an increase in split products in the serum was supposed to lend support to the view of Rosenthal that the antiferment was due to protein split products.

This increase is not uniform in all animals. While in some there is a very sharp rise, others may remain unaltered or even show a



TEXT-FIG. 6.



TEXT-FIG. 7.

TEXT-FIG. 6. Average change in antiferment titer after feeding.
TEXT-FIG. 7. Average change in lipase after feeding.

decrease. The increase is probably due to an increase in unsaturated lipoids, a subject which we have previously discussed (5).

Serum Esterase.

The increase in serum esterase is very slight in amount, the average for the experiments (Text-fig. 7) rising from 0.9 cc. N/100 sodium hydrate to 1.2 cc. after three hours. When samples are collected at various parts of the circulation, the blood from the hepatic veins usually contains less ferment than from other sources, indicating that the source of the ferment is probably not in the liver.

CONCLUSIONS.

1. After feeding, an increase in non-coagulable nitrogen of the serum can be determined, reaching a maximum in about six hours.

2. This increase is greatest in the portal blood and is partially due to an increase in amino-acids. There is no increase in proteoses.

3. There is usually a progressive decrease in serum protease, reaching a minimum after from five to seven hours.

4. The portal blood may show an unaltered or an increased amount of protease.

5. The serum antiferment shows a slight increase, but is subject to considerable fluctuation.

6. The serum lipase (esterase) shows a slight increase, reaching a maximum after three hours. The hepatic blood usually contains the lowest concentration of lipase.

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