LIX. FURTHER OBSERVATIONS ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN.

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(Received June 18th, 1921.)

In a previous paper [Edie, 1919] it was shown that the activity of trypsin as measured by its digestive action on fibrin and on caseinogen is affected to such a different degree by alcohol as to make it seem either that two enzymes are concerned, or, if only one enzyme, that the two substrates are acted on by different groups or side chains, one group being much more sensitive than the other. The effect of heat on the digestion of fibrin and caseinogen by trypsin was next studied. It had been found previously [Edie, 1914] that trypsin when boiled in acid solution still retains much or in some cases all of its power of hydrolysing caseinogen, but in only one case was its action on fibrin tested after heating in this way. Digestion of the fibrin was still noticed, but the amount even in the case of the unheated trypsin was so small that a fresh series of experiments was undertaken.

The trypsin solutions were generally prepared by extracting finely minced sheep's pancreas with water and a little chloroform for 10 to 14 days and filtering. A little chloroform was then added as a preservative. The experiments were carried out as previously detailed.

In the experiments described in the previous paper it had been found that in presence of $N/25$ to $N/50$ HCl the trypsin solutions used retained 60 to 100 % of their original digestive power, as tested on caseinogen, after being heated to 100° for three minutes. The same treatment was applied to the trypsin solutions in this series of experiments and the results are shown in the table.

In making these extracts, one part of pancreas was extracted with two parts of water in each case.

In each experiment 1 cc. trypsin + 40 cc. 0.5 % Na_2CO_3 + 1 g. fibrin were used on the one hand, digestion being for three hours, and 1 cc. trypsin + 40 cc. 1.5 % caseinogen in 0.5 % Na_2CO_3 on the other hand, digestion being for one hour.

The amount of digestion was estimated by precipitating unchanged caseinogen with tannic acid, or by filtering off the undissolved fibrin in the different sets of experiments respectively, and determining the nitrogen in

the filtrate by Kjeldahl's method. Control experiments were always carried out at the same time.

No.	Reaction (HCl)	Treatment	Fibrin digested (in cc. of N/10 nitrogen)	Caseinogen digested
1	N/55	3 min. at 100°	0.0	1.2
		Control	20.1	28.6
2	N/20	1.5 min. at 60°	0.0	3.1
		Control	21.9	34.7
3	N/20	1.5 min. at 60°	0.0	5.1
		Control	22.1	30.9
4	N/30	1 min. at 85°	0.0	1.9
		Control	7.1	22.4
5	N/30	1 min. at 65°	0.0	3.2
		Control	8.6	22.9
6	N/20	0.75 min. at 100°	0.0	3.2
		Control	21.2	34.6
7	N/20	0.75 min. at 66°	2.2	19.5
		Control	16.6	34.3
8	N/20	1.5 min. at 66°	1.1	8.3
		Control	16.2	31.8
9	N/40	1.5 min. at 75°	3.4	17.0
		Control	17.4	37.4

In the above experiments it will be seen that even in acid solution the pancreatic extracts used generally lost practically all their power to digest fibrin even when heated only to 60° for a minute and a half. The power to digest caseinogen was not so completely lost, especially in the last three experiments, but it was so markedly reduced, compared with what had been previously found, that the matter merited further investigation.

The extracts used above were aqueous, so a few experiments were carried out to compare the effect of heat on aqueous and alcoholic (20 %) extracts of the same pancreas. The experiments were carried out in the same way as those previously noted.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested cc.	Caseinogen digested cc.
10	N/40	Aqueous	1.5 min. at 75°	3.4	17.0
		"	Control	17.4	37.4
		Alcohol	1.5 min. at 75°	17.3	29.3
		"	Control	31.0	47.2
11	N/50	Alcohol	1 min. at 100°	23.4	34.4
		"	Control	27.9	42.8
12	N/40	Aqueous	1 min. at 100°	0.0	—
		"	Control	11.8	—
		Alcohol	1 min. at 100°	21.1	—
		"	Control	23.1	—
13	N/40	Aqueous	1 min. at 100°	0.4	—
		"	Control	11.7	—
		Alcohol	1 min. at 100°	14.0	—
		"	Control	21.5	—

It will be seen that the alcoholic extracts retain very much more of their power to digest fibrin after being heated than do the corresponding aqueous extracts. The amount of protein and other nitrogenous substances was practically the same in the two sets of extracts. It might be supposed that the alcohol itself in some way protected the trypsin from destruction when

heated, the lower boiling point of alcohol preventing the alcoholic extract from reaching such a high temperature in the water-bath as the corresponding aqueous extract would reach. No such protection was found to be afforded by alcohol, however. Corresponding aqueous and alcoholic extracts were diluted with alcohol and water respectively so that the percentage of alcohol was the same in both. They were then heated to the same extent and tested on fibrin.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested cc.
14	N/40	Alcoholic	1 min. at 100°	21.1
		Control		23.1
		Aqueous, alcohol added afterwards	1 min. at 100°	0.0
15	N/40	Control		18.9
		Alcoholic	1 min. at 100°	14.0
		Control		21.5
		Aqueous, alcohol added afterwards	1 min. at 100°	0.0
		Control		7.9

These experiments show that the addition of alcohol to an aqueous extract of pancreas does not afford any protection against heat to the trypsin as measured by its action on fibrin.

In the original experiments on the resistance of trypsin solutions to heat, the solutions, when heated, contained only a very small amount of nitrogen, not more, in some cases, than 0.02 %. The pancreatic extracts used in the experiments now described were very much richer in nitrogen and generally contained 15 to 20 times as much as the older extracts. This corresponds to a considerable amount of protein in the solution and as protein is known to form a loose compound with hydrochloric acid it was decided to try the effect of considerably higher amounts of acid in the solutions to be heated.

It was now found that the protection afforded to trypsin solutions when heated depends on the amount of acid present, and the more protein there is in solution, the more hydrochloric acid must be added to prevent the trypsin being destroyed by heat.

The following experiments show this increasing protection with increase of acid:

No.	Reaction (HCl)	Treatment	Fibrin digested cc.	Caseinogen digested cc.
16	N/50	1 min. at 100°	5.6	21.1
		Control	16.9	39.1
17	N/23	1 min. at 100°	—	25.1
		Control	—	35.7
18	N/17	1.5 min. at 100°	7.4	26.8
		Control	7.6	26.7
19	N/15	3 min. at 100°	10.9	26.2
		Control	10.9	26.4
20	N/15	2 min. at 100°	3.5	19.2
		Control	11.6	28.7
21	N/12	2 min. at 100°	8.4	14.8
		Control	12.2	15.6

Experiments 16 to 19 show the effect of using increasing amounts of hydrochloric acid with the same trypsin preparation, and experiments 20 and 21 show this again with another sample of trypsin.

Similar results have been obtained with many aqueous extracts of pancreas prepared in the laboratory, and it may be stated generally that the higher the proportion of nitrogen contained in such an extract the larger the amount of hydrochloric acid which must be added in order to prevent destruction of the trypsin by heat.

In a few cases, as for example experiments 18 and 19, it was found that the protection against heat afforded by a certain amount of acid was the same as regards digestion both of fibrin and of caseinogen. Usually, however, the destruction of the fibrin digesting power was considerably greater than that of the caseinogen digesting power.

It has been pointed out by Mellanby and Woolley [1913] that in acid of the strength of 0.05 *N* (HCl) trypsin is slowly destroyed at 16° and more rapidly at 35°. Apparently at room temperature about half the trypsin is destroyed in four hours, and two-thirds is destroyed in a day. The activity of the trypsin in their experiments was measured by its power of coagulating calcified milk. Other references to the effect of hydrochloric acid on trypsin at moderate temperatures have been mentioned in a previous paper [Edie, 1914]. It was also found by Lénard [1914] that if trypsin is rendered inactive by addition of acid, only a trace of its activity is restored by neutralising and then adding alkali. These observers appear only to have tested the activity of the trypsin on one substrate, but in the following experiments the action of hydrochloric acid on trypsin at room or body temperature has been tested as regards the power to digest both fibrin and caseinogen. In these experiments alcoholic (15 %) extracts of pig's pancreas were used. These were practically neutral. In every case 1 cc. of the original trypsin was compared with that quantity of the trypsin + acid which would contain 1 cc. of trypsin originally, and the solutions so adjusted as to contain the same amount of sodium chloride. Digestion both of fibrin and of caseinogen was carried on in presence of 0.5 % sodium carbonate, 1 g. fibrin or 0.6 g. caseinogen being used, in about 40 cc. of fluid. The amount of digestion is expressed, as usual, in cc. of *N*/10 nitrogen.

22.	10 cc. trypsin + 20 cc. <i>N</i> HCl. Kept at 36° for 12 min.	20 cc. <i>N</i> NaOH then added.
	Digestion by control	21.6 cc. fibrin, 38.2 cc. caseinogen.
	„ treated trypsin 0.0 cc.	„ 8.8 cc. „
23.	20 cc. trypsin + 10 cc. <i>N</i> HCl. Room temperature for 4 days.	10 cc. <i>N</i> NaOH added.
	Digestion by control	12.1 cc. fibrin, 31.6 cc. caseinogen.
	„ treated trypsin 0.0 cc.	„ 5.4 cc. „
24.	40 cc. trypsin + 20 cc. <i>N</i> HCl. Room temperature for 11 days.	20 cc. <i>N</i> NaOH added.
	Digestion by control	11.7 cc. fibrin, 38.7 cc. caseinogen.
	„ treated trypsin 0.0 cc.	„ 5.6 cc. „

It will be seen from these experiments that the power of trypsin to digest fibrin is destroyed considerably more readily in acid solution at moderate

temperatures than is the power to digest caseinogen. It is also seen that the power to digest caseinogen withstands a considerably higher percentage of hydrochloric acid than has generally been supposed, the strength of acid being $N/3$ in these experiments and several others with similar results. In one experiment, after 24 hours at room temperature in $N/3$ hydrochloric acid, the trypsin still retained about 10 % of its original fibrin digesting power, but otherwise no fibrin was digested at all after treatment of the trypsin with acid of this strength for a day or upwards.

On the whole, then, treatment of trypsin solutions with hydrochloric acid either at high or low temperatures shows that the power to digest fibrin is more readily destroyed than the power to digest caseinogen. This bears out the theory discussed previously [Edie, 1919] that in some respects the fibrin digesting power is the more subject to outside influences and again points to the hydrolysis of fibrin and of caseinogen being carried out by different side chains, those digesting caseinogen being the more stable.

In my previous paper [1919], the work of Fermi was referred to as showing that after treatment with various reagents trypsin would no longer digest fibrin but would still digest gelatin. Pollak was also mentioned as finding that with different enzyme preparations the relative amounts of serum and gelatin digested varied enormously. I have also found that the relative amounts of fibrin and caseinogen digested by different trypsin solutions vary very much, and this without subjecting the enzyme to treatment of any kind.

Thus, three enzyme solutions were prepared in exactly the same way, by extracting minced sheep's pancreas with three times its weight of water for 14 days and filtering. These were compared at the same time. 1 cc. of each trypsin was taken, with 40 cc. 0.5 % Na_2CO_3 and 1 g. fibrin on the one hand, and 1 cc. trypsin with 40 cc. 1.5 % caseinogen in 0.5 % Na_2CO_3 on the other hand. The amounts of digestion in the three cases were as follows:

Enzyme	Fibrin digested in 2 hours. cc.	Caseinogen digested in 1 hour. cc.
1	12.4	33.7
2	16.1	32.9
3	3.3	17.6

The differences in the relative amounts of fibrin and caseinogen digested by these enzymes, especially Nos. 2 and 3, are very marked. Similar results were frequently noticed in other cases, the general rule being that considerable amounts of caseinogen were digested even though a particular enzyme solution had little or almost no action on fibrin.

When enzyme solutions were kept for some time it was found that the fibrin digesting power as a rule diminished to a very much greater extent than the caseinogen digesting power. For example, a freshly prepared trypsin, under the usual conditions, digested 16.1 cc. fibrin and 32.9 cc. caseinogen. In 15 months, under the same conditions, this trypsin digested 4.8 cc. fibrin and 18.5 cc. caseinogen.

In one extreme case I examined a solution of trypsin which had been in the laboratory for over ten years. It had no digestive action on fibrin at all, but still digested caseinogen to the extent of 26.6 cc. under the usual conditions.

These facts afford further evidence that the digestion of fibrin and of caseinogen by pancreatic extracts is either due to different enzymes or at least to different side chains if only one enzyme is involved. In my previous paper [1914] I mentioned that the power to digest caseinogen seemed to be less affected by heat than the power to coagulate milk, which was taken as the measure of activity of trypsin by Mellanby and Woolley. I further suggested that different sets of side chains might be responsible for these different functions.

In a later paper [1914] Mellanby and Woolley take exception to my suggestion and say "Pancreatic rennin and trypsin are identical. In fact the coagulation of milk by trypsin is an expression of a general law that all proteolytic ferments coagulate milk provided sufficient calcium be contained in it." These authors further say "The unique fact that the ferment or ferments in pancreatic juice which digest protein and coagulate milk should withstand boiling in acid solution is practically conclusive proof that the two actions are produced by one and the same substance." If this assumption of Mellanby and Woolley is correct, however, then the milk coagulating power and the power to hydrolyse both fibrin and caseinogen should presumably be quite parallel in their behaviour. The experiments detailed in the present paper, and those described previously [Edie, 1919], however, tend to show that the digestion of fibrin and of caseinogen, if carried out by one enzyme, involves at least two sets of groups of the enzyme molecule, and therefore cannot be said really to be produced by the same substance in the sense evidently meant by Mellanby and Woolley.

I have also carried out some experiments comparing the milk coagulating power of pancreatic extracts with their proteolytic power, and shall now deal with these.

25. To 20 cc. of pancreatic extract (alcoholic) was added 0.5 cc. *N* HCl. Half of this was then heated to 100° for 1 minute and filtered.

1 g. fibrin. Digestion 2.75 hours at 37°.

(a) 1 cc. trypsin, 40 cc. 0.5 % Na_2CO_3 . Digestion 21.0 cc. *N*/10 nitrogen.

(b) 1 cc. trypsin (heated), 40 cc. 0.5 % Na_2CO_3 . Digestion 10.2 cc. *N*/10 nitrogen.

To 20 cc. milk was added 1 cc. of trypsin (1) fresh trypsin.

" " " " " (2) heated trypsin.

(1) Complete coagulation in 6 minutes at 37°.

(2) No coagulation in 3 hours.

26. Similar to last experiment.

1 g. fibrin. Digestion 2.5 hours.

(a) 1 cc. trypsin, 40 cc. 0.5 % Na_2CO_3 . Digestion 26.2 cc.

(b) 1 cc. trypsin (heated), 40 cc. 0.5 % Na_2CO_3 . Digestion 8.1 cc.

(1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.

(2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

From these two experiments it will be seen that though the heated trypsin is still able to digest a considerable amount of fibrin, its milk coagulating power, if any, is now quite negligible.

27. 20 cc. trypsin + 0.5 cc. *N* HCl. Half kept at 100° for 1 minute and filtered.

(a) 1 cc. trypsin, 20 cc. milk. Digestion 39.3 cc.

(b) 1 cc. trypsin (heated), 20 cc. milk. Digestion 4.6 cc.

Digestion 1 hour. Tannic acid added and digestion estimated as in the usual caseinogen experiments.

(1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.

(2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

28. Similar to last experiment.

(a) 1 cc. trypsin, 20 cc. milk. Digestion 38.4 cc.

(b) 1 cc. trypsin (heated), 20 cc. milk. Digestion 3.9 cc.

(1) 20 cc. milk, 1 cc. fresh trypsin. Complete coagulation in 5 minutes.

(2) 20 cc. milk, 1 cc. heated trypsin. No coagulation in 2 hours.

These two experiments confirm Nos. 25 and 26 in showing that the milk coagulating power of pancreatic extracts is more readily destroyed by heat than the proteolytic power.

It was noticed that the coagulated casein gradually dissolved under the influence of the fresh trypsin, digestion of this protein taking place rapidly even in neutral solution.

More striking differences are found between the milk coagulating power and the proteolytic action of pancreatic extracts under certain conditions without subjecting these to any such drastic treatment as heating to 100° involves. Edkins [1891] found that fresh, active pancreatic extracts were not so active in altering milk so as to produce Roberts' "metacasein" reaction as were older extracts, but that the proteolytic action was greater in the fresh extracts. Edkins suggested that the production of the metacasein reaction might be an aspect of the proteolytic enzyme of the pancreas. Halliburton and Brodie [1896] confirmed what had been pointed out by Benger, that freshly prepared extract of pig's pancreas had very little curdling action on milk, but acquired this property on being kept a considerable time. They accounted for this fact by supposing that the trypsin at first masks or hinders the milk curdling enzyme, but that the former enzyme deteriorates more quickly and so finally allows the rennin to reveal its presence. Vernon [1901] obtained similar results and found that the ratio of rennin value to tryptic value varied largely in different extracts of pancreas and also in the same extracts at different times. In alcoholic extracts the ratio usually became higher as the extract became older, the tryptic value deteriorating more rapidly than the rennin. In glycerol extracts, however, the ratio diminished after say nine weeks, owing to the trypsin being liberated more slowly from its zymogen than the rennin. Some glycerol extracts which were very rich in trypsin gave practically no clot at all, as though the clot were dissolved nearly as fast as it was formed. In a later paper, Vernon [1903] found that rennin and trypsin were precipitated from pancreatic extracts to practically

the same extent when excess of alcohol was added. He considered that in the case of trypsin some groups have the property of coagulating milk and others have the proteolytic power.

I have tested a number of pancreatic extracts at different stages and record some of the principal results below.

In each case sheep's pancreas was used. It was finely minced and extracted with two and a half times its weight of water and a little chloroform. The experiments were similar to Nos. 25 to 28 in technique.

29. Pancreas extracted for two days and extract then tested.
 - 1 cc. trypsin, 40 cc. 0.5 % Na_2CO_3 , 1 g. fibrin. 14.9 cc. digested in 3 hours.
 - 1 cc. trypsin, 40 cc. caseinogen (1.5 % in 0.5 % Na_2CO_3). 44.6 cc. digested in 1 hour.
 - 1 cc. trypsin, 40 cc. milk. 23.3 cc. digested in 0.5 hour.
 - 1 cc. trypsin + 20 cc. milk. No coagulation in 0.5 hour. No coagulation on now adding an active coagulating extract, as the caseinogen had been changed (as can be seen above) into products which no longer give a coagulum with rennin.
30. Pancreas extracted for 1.5 hours and extract then tested.
 - 26.4 cc. fibrin digested in 3 hours.
 - 41.8 cc. caseinogen digested in 1 hour.
 - No coagulation of milk, but much digestion (tannic acid).
31. Pancreas extracted for 3 days and extract then tested.
 - 26.7 cc. caseinogen (milk used) digested in 0.5 hour.
 - No coagulation of milk.
32. Pancreas extracted for 3 days and extract then tested.
 - 31.0 cc. caseinogen digested in 0.5 hour.
 - No coagulation of milk, but much digestion.

These four experiments show that freshly prepared aqueous extracts of pancreas generally do not coagulate milk, but are very active proteolytic agents, both on fibrin and on caseinogen.

In a few cases I have found that the filtrate after three hours' extraction coagulated milk rapidly, but this was exceptional.

It has already been mentioned that Halliburton and Brodie accounted for the fact that fresh extracts have very little curdling action on milk by supposing that trypsin deteriorates more quickly and in a few days or weeks allows the rennin to reveal its presence. Vernon seems to suggest that a clot is formed but is dissolved almost at once. In the extracts which I used, the coagulating power seemed to be fully developed within 15 days, but I never found the proteolytic power to diminish as rapidly as would have to be the case if the above suggestion accounted for all the facts.

The extract used in experiment 30 was tested again for proteolytic power when five weeks old. Complete coagulation of milk now took place within four minutes, and the amount of caseinogen digested was now 44.7 cc., this being actually slightly more than it digested at first. If in the first case a clot were formed but almost immediately redissolved, this should have been still more the case when the proteolytic power had increased, instead of which complete coagulation rapidly took place. In other cases the milk coagulating power seemed to have developed completely within four days, the proteolytic

power being practically the same as at first and there was no evidence whatever in support of the view that with extracts a few hours old coagulation really takes place but the clot is redissolved almost instantly. These last experiments once more show that pancreatic extracts differ greatly in their milk coagulating and proteolytic powers. This again points to there being either two or more separate enzymes present, which develop at very different rates from their zymogens, or at least the groups which are responsible for the different functions develop their properties quite independently.

SUMMARY.

1. The amount of acid required in order to protect trypsin from destruction by heat depends on the amount of protein present. The more protein in solution, the more acid required. If not enough acid is present to afford complete protection to the trypsin, the fibrin digesting power is usually destroyed by heat to a considerably greater extent than the power to digest caseinogen.

2. Hydrochloric acid at moderate temperatures also destroys the fibrin digesting power considerably more rapidly than the caseinogen digesting power.

3. The relative amounts of fibrin and caseinogen digested vary very much in different pancreatic extracts.

4. The milk coagulating power of pancreatic extracts is more easily destroyed by heat than the proteolytic power.

5. Generally, but not always, freshly prepared pancreatic extracts have no milk coagulating power. These extracts are always actively proteolytic, but the proteolytic power does not fall off so rapidly as to justify the assumption that the non-appearance of a coagulum with milk is due to the coagulum being really formed but instantly redissolved. All these facts point to the proteolytic and milk coagulating powers of pancreatic extracts being due to a number of distinct enzymes, or, if only one enzyme is concerned, to the different functions being due to different groups of the molecule.

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