VI. ON THE BEHAVIOUR OF TRYPSIN IN THE PRESENCE OF A SPECIFIC PRECIPITATE.

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In a former paper on amylase [1913], I discussed the possibility of finding a substitute for complement in the recognition of antibody as well as the advantages which would follow if such a substitute were found.

Certain animal ferments are rather similar to complement in their general behaviour, each being a hydrolyst of a particular substrate by which it is first absorbed. Each is also readily taken up by different substances such as collodion and gelatin membranes [Porter 1909, 1910, Pribram 1910], or even inorganic precipitates [Hailer 1908, p. 283]. Pepsin absorbed into egg-white [Dauwe 1905, p. 426], or pepsin, ptyalin etc. into collodion [Porter 1910, p. 382] can be recovered in a small degree by a solution of the particular substance which they digest. Pepsin absorbed into collodion was however unable to act on a solid protein suspended in water [Porter 1910, p. 379]. As it is only by producing digestion that these ferments are recognised,—through the introduction of their particular substrate,—this possibility of partial recovery presents the chief difficulty in the use of ferments as substitutes for complement.

The first attempt to absorb a ferment by means of a specific precipitate was made by Hailer [1908, p. 280]. His choice of rennet was unfortunate, as it loses hardly at all on dilution, being active at a dilution of 1 in 340,000 [Porter 1911, p. 394]. Still, though unsuccessful at stronger concentrations, his results do show with diluted rennet that some absorption had taken place. Ptyalin [Porter 1913, p. 601] displays only a very slight affinity for a precipitate of this nature, while taka diastase shows itself quite indifferent.

Ferments may be divided into two classes in respect to their behaviour with serum. In the one class are the proteolytic ferments which combine with serum proteins in the dissolved condition so firmly that they are inhibited by normal serum. On the other hand non-proteolytic ferments, the action of many of which is accelerated by serum, form much less stable compounds

with precipitated serum proteins. In search of a suitable ferment, I treated a number of ferments by adding diluted serum and saturating with carbon dioxide. Next morning the precipitate was removed, after centrifuging, and the upper fluid tested for ferment. Emulsin and lipase were unaffected by this treatment, except that insoluble lipase is removed. On turning to proteolytic ferments, pepsin was found less influenced by the globulin precipitate than might have been expected, possibly because the acidity of the carbonic acid enabled it to produce some hydrolysis. A rennet was accidentally examined in the form of a mere trace in a sample of trypsin for which I have to thank Dr Cramer. After precipitation with carbon dioxide the upper fluid differed from the control in not being able to curdle milk at 56°, although it increased its viscosity figure.

Trypsin alone, of all the ferments which I have studied, is absolutely absorbed by a diluted serum precipitated with carbon dioxide, while it is only partly absorbed by the same dilution of the same serum before precipitation. If the upper fluid is left in contact with the precipitate only for one night, and then removed from it, it will be found by every test applied to it devoid of trypsin.

METHOD.

A trypsin solution was made by dissolving Armour's pancreatin in water. The powder did not perfectly dissolve in the proportion of 1 gram to 100 cc., so that after standing for a night in the cold room, the upper fluid was separated off. The solution was kept in the cold room. Several methods were employed of testing tryptic action:

- (1) by dissolving coagulated serum contained in Mett's tubes, as recommended by Dauwe [1905] in the case of pepsin,
- (2) by dissolving fibrin,
- (3) by forming hollows on serum plates, as recommended by Mueller and Jochmann [1906],
- (4) by rendering dilute caseinogen incapable of being precipitated by 0.25 % acetic acid,
- (5) by lowering the viscosity of caseinogen according to Golla [quoted from Eve 1910].

The most delicate and rapid method is undoubtedly the digestion of caseinogen. This cannot be tested in the presence of serum by means of the ordinary method of slight acidulation, as the globulins of the serum are as easily precipitated by acid as is the caseinogen itself. The viscosity method of Golla, as described by Eve [1910], is very simple and accurate, very small

differences indeed being estimated with the help of a stop-watch. This method presents a very severe test of trypsin absorption because, as I have pointed out, an absorbed ferment is much more easily reached by a solution of its corresponding "substrate" (the specific substance on which it acts) than where this substrate is in solid form, suspended in a medium which is in contact with the absorbed ferment. The form of caseinogen used was 2, 3, and 4% casumen, a soluble preparation of caseinogen (that is to say easily soluble with heat and shaking), which was made up fresh on each occasion. Digestion was carried out at 56° to prevent bacterial action. The method of using serum tubes was found rather slow, but fibrin was a good deal used, being either digested in the presence of the serum precipitate or else treated with it and afterwards washed and transferred to an alkali. Digestion in this case was carried on at 37° under toluene. The method is somewhat rough so that fine differences are not distinguishable, but it is quite serviceable.

Trypsin in serum precipitated with CO₂.

A. *Examination of upper fluid alone.*

The following solutions were made up.

- (1) To 3 cc. ox serum were added 15 cc. trypsin solution and 12 cc. water (i.e. 50 % trypsin, and 10 % serum).
- (2) A control trypsin solution 50 % in water.
- (3) N/10 NaOH containing 1.6 % NaCl, called NaCl—NaOH.
- (4) 10 % ox serum in NaCl—NaOH.
- (5) N/5 Na₂CO₃.
- (6) 10 % ox serum in N/5 Na₂CO₃ (containing no NaCl as this rendered the serum too cloudy).

No. 1, the mixture containing trypsin and serum, was saturated with CO₂, and left over night in the cold room. Next morning the upper fluid was separated from the precipitate after centrifuging.

This upper fluid was then tested for trypsin by the following methods:

<i>With Fibrin.</i>				5 hrs.	1 day	2 days
(1)	0.75 cc. control trypsin + 0.75 cc. NaCl—NaOH	...	...	—	gone	—
(2)	" " " + " serum—NaCl—NaOH	...	...	—	0.5	—
(3)	" " " + " serum—Na ₂ CO ₃	...	...	0.5	—	0.75
(4)	" absorbed trypsin upper fluid + 0.75 cc. NaCl—NaOH	...	...	—	0	—
(5)	" " " " + " Na ₂ CO ₃	...	...	0	—	0

Fibrin in NaOH and Na₂CO₃ also negative, except of course for swelling.

With Mett's Tubes (Serum).

Absorbed serum with Na₂CO₃ or NaCl and Mett's tubes was negative after a week, while control trypsin with serum under similar conditions was partly digested in a day or two.

With Serum Plates.

(1) Equal parts control trypsin + 10 % serum in NaCl—NaOH	24 hrs. at 56°
(2) " absorbed trypsin upper fluid + NaCl—NaOH	Faint hollow
	Negative

With the Viscosity Test.

(Each contained 5 cc. 2 % casumen; 10 hours at 56°.)

	Mins.	Secs.
(1) 0.75 cc. control trypsin + 0.75 cc. 10 % serum—NaCl—NaOH	1	30
(2) " " " + " " serum—Na ₂ CO ₃ ...	1	25
(3) " absorbed trypsin upper fluid + 0.75 cc. NaCl—NaOH...	1	46
(4) " " " " + " " Na ₂ CO ₃ ...	1	38
(5) 1.5 cc. H ₂ O	1	43

With Caseinogen (tested by acid).

Serum containing globulins cannot be used with this test, so very dilute trypsin was substituted for trypsin serum.

(Each contained 2 cc. 0.1 % pure caseinogen, 24 hours at room temperature.)

	Precipitate with 0.25 % acetic
(1) 0.75 cc. 1.25 % trypsin + 0.75 cc. Na ₂ CO ₃	0
(2) " 50 % absorbed trypsin upper fluid + 0.75 cc. Na ₂ CO ₃	+

By the above tests it is clear that trypsin is only partially absorbed by 1/10 normal serum, but is wholly absorbed by the same strength of serum which has been precipitated by saturation with carbon dioxide. That is to say, if the precipitate bearing the trypsin is removed out of the serum the remaining fluid is found to be without trypsin.

It is however necessary to discover whether the absorbed ferment is so fixed that it cannot be recovered by the addition of more protein, especially in solution and in an alkaline medium.

B. Examination of upper fluid plus precipitate.

The following mixtures were made, in water, and all precipitated by saturation with CO₂.

- (1) Ox serum 10 %, trypsin solution 50 %.
- (2) " " 5 %, " " "
- (3) " " 2.5 %, " " "
- (4) " " 1.25 %, " " "
- (5) No serum " " "

Next day each mixture was shaken up, and the whole, containing the precipitated globulin, was tested for trypsin by the following methods:

With Fibrin.

(In this case the alkali was added to the precipitate before the fibrin.) Time, over night at 37°.

	Result
(1) 0.75 cc. control trypsin + 0.75 cc. 10 % serum in Na ₂ CO ₃	0.5 dissolved
(2) " " " + " 5 % " "	all "
(3) " " " + " 2.5 % " "	" "
(4) " " " + " 1.25 % " "	" "
(5) " " " + " Na ₂ CO ₃	" "
(6) absorbed trypsin (10 % serum) + 0.75 cc. Na ₂ CO ₃	Slightly "
(7) " " " (5 % ") + " "	Almost "
(8) " " " (2.5 % ") + " "	Dissolved
(9) " " " (1.25 % ") + " "	"
(10) " H ₂ O " + " "	0

If the fibrin be added to the precipitated serum mixture while it is neutral, and after an hour removed, washed and placed in an alkaline solution, less of the ferment is recovered.

		Next day
(1)	0.75 cc. control trypsin + 0.75 cc. 10 % serum in NaCl	0.5 dissolved
(2)	,, absorbed ,, + ,, 1.6 % NaCl	0

With the Viscosity Test.

(Each + 5 cc. 3 % casumen, containing 20 % N/5 Na_2CO_3 .) Time 4 hours at 56°, then room temperature for 24 hours.

		Hours			
		4		24	
		min.	secs.	min.	secs.
(1)	0.75 cc. control trypsin + 0.75 cc. 10 % serum in Na_2CO_3	1	42	1	42
(2)	,, ,, ,, + ,, 5 % ,, ,,	1	36	1	36
(3)	,, ,, ,, + ,, 2.5 % ,, ,,	1	37	1	37
(4)	,, ,, ,, + ,, 1.25 % ,, ,,	1	32	1	32
(5)	,, ,, ,, + ,, Na_2CO_3	1	30	1	27
(6)	,, absorbed trypsin (10 % serum) + 0.75 cc. Na_2CO_3	1	47	1	41
(7)	,, ,, ,, (5 % ,,) + ,, ,,	1	47	1	45
(8)	,, ,, ,, (2.5 % ,,) + ,, ,,	1	45	1	41
(9)	,, ,, ,, (1.25 % ,,) + ,, ,,	1	40	1	37
(10)	,, H_2O + ,, ,,	1	56		

Time in Viscosimeter

The above experiments show clearly that some trypsin can free itself from the serum precipitate and enter the casumen or fibrin. Still this tendency to recovery is not sufficient to obliterate the difference of the effects of original and precipitated serum upon trypsin, which is still marked enough to permit an expectation that a similar difference may be found in the case of a specific precipitate.

Trypsin with a specific precipitate.

In this case more protein was present,—in the form of antigen,—than in the last, where the influence of serum alone was under investigation. The antigen used was egg-white. The extent to which egg-white absorbs trypsin was next investigated, both with fibrin and by the viscosity test. To 0.15 cc. normal ox serum, or 0.15 cc. saline solution, 0.15 cc. egg-white, in dilutions of from 1/10 upward, was added, followed by 0.75 cc. trypsin solution and 0.75 cc. N/5 Na_2CO_3 , and after an hour's contact, fibrin, or 5 cc. 3 % casumen. At these dilutions egg-white exerted no visible influence upon the results, other than by its presence diluting the serum by one-half, and so lessening its inhibitive effect.

Serum was obtained from rabbits which had been treated as described on p. 55, and used in the following condition :

- (1) Thrice injected, precipitation at 1/10,000 (absorption of trypsin positive).
- (2) Thrice injected, precipitation negative (absorption negative).
- (3) Once injected, precipitation at 1/1000.
Twice injected, precipitation not tested higher.
Four times injected, precipitation at 1/10,000 (absorption in all positive).
- (4) Normal (absorption negative).
- (5) Normal (absorption negative).
- (6) Normal (absorption negative).
- (7) Normal (absorption negative).
Twice injected, precipitation at 1/5000.
Thrice injected, precipitation at 1/5000, not tested higher (absorption in both positive).
- (8) Normal (absorption negative).

Experiment I.

Rabbit serum 1, to each tube added 0.75 cc. trypsin solution + 0.75 cc. Na_2CO_3 N/5, after 0.5 hour 5 cc. 3 % casumen. Time 2 hours at 56°.

					Viscosity min. secs.
(1)	0.15 cc. Immune serum	1 + 0.15 cc. 1/10 egg-white			1 45
(2)	"	"	+	" 1/100 "	1 46
(3)	"	"	+	" 1/250 "	1 46
(4)	"	"	+	" 1/500 "	1 46
(5)	"	"	+	" 1/1000 "	1 40
(6)	"	"	+	" 1/2500 "	1 40
(7)	"	"	+	" 1/5000 "	1 40
(8)	"	"	+	" NaCl	1 40

(All later viscosity experiments from this point were tested with a stop-watch.)

Experiment II.

Rabbit 3 injected once, positive, Rabbit 2 injected thrice, negative, and Rabbit 4 normal. To each tube added 0.75 cc. trypsin solution, after 3 hours added 5 cc. 3 % casumen and 0.75 cc. N/5 Na_2CO_3 . Digestion at 56° 0.75 hour, then 0° over night.

					Viscosity min. secs.
(1)	0.15 cc. Immune serum	3 + 0.15 cc. 1/10 egg-white			1 32.5
(2)	"	"	3 +	" 1/100 "	1 31
(3)	"	"	3 +	" 1/250 "	1 31
(4)	"	"	3 +	" NaCl	1 30.5
(5)	"	"	2 +	" 1/10 "	1 31.5
(6)	"	"	2 +	" 1/100 "	1 31.5
(7)	"	"	2 +	" 1/250 "	1 31
(8)	"	"	2 +	" NaCl	1 31.5
(9)	"	Normal	4 +	" 1/10 "	1 29.5
(10)	"	"	4 +	" 1/100 "	1 29
(11)	"	"	4 +	" 1/250 "	1 29
(12)	"	"	4 +	" NaCl	1 29

Experiment III.

Rabbit 3 twice injected, Rabbits 5, 6, 7 and 8, all normal. Each tube received 0.75 cc. trypsin solution, after half an hour's contact 5 cc. 4 % casumen and 0.5 cc. Na_2CO_3 were added. Digestion at 56° for ½ hour, then cold room till next day.

						Viscosity min. secs.
(1)	0.15 cc. Immune serum	3+0.15 cc.	1/10 egg-white			1 40
(2)	"	"	3+ "	1/100 "		1 40
(3)	"	"	3+ "	1/500 "		1 40
(4)	"	"	3+ "	1/1000 "		1 39
(5)	"	"	3+ "	NaCl		1 36
(6)	"	Normal	5+ "	1/10 "		1 37.5
(7)	"	"	5+ "	1/100 "		1 37.5
(8)	"	"	5+ "	1/500 "		1 38
(9)	"	"	5+ "	NaCl		1 37.5
(10)	"	"	6+ "	1/10 "		1 40
(11)	"	"	6+ "	1/100 "		1 40
(12)	"	"	6+ "	1/500 "		1 41
(13)	"	"	6+ "	NaCl		1 40
(14)	"	"	7+ "	1/10 "		1 40.5
(15)	"	"	7+ "	1/100 "		1 40.5
(16)	"	"	7+ "	1/500 "		1 40
(17)	"	"	7+ "	NaCl		1 40
(18)	"	"	8+ "	1/10 "		1 39
(19)	"	"	8+ "	1/100 "		1 38.5
(20)	"	"	8+ "	1/500 "		1 38.5
(21)	"	"	8+ "	NaCl		1 39.5

Experiment IV.

Rabbit 7, twice injected. Each tube received 0.75 cc. trypsin solution, after $\frac{1}{2}$ hour 5 cc. 3% casumen and 0.75 cc. N/5 Na₂CO₃. Digestion at 56° for 3 hours.

						Viscosity min. secs.
(1)	0.15 cc. Immune serum	7+0.15 cc.	1/10 egg-white			1 32
(2)	"	"	7+ "	1/500 "		1 32
(3)	"	"	7+ "	1/1000 "		1 32
(4)	"	"	7+ "	1/5000 "		1 31.5
(5)	"	"	7+ "	NaCl		1 28.5
(6)	"	"	7+ "	"		1 28.5

Experiment V.

Comparison of some rabbits' sera with fibrin. Immune sera 3 and 7, both twice injected, normal sera, 4, 5, 6 and 7. Each tube received 0.75 cc. trypsin solution, after a night's contact in the cold room, fibrin was added to each mixture next morning, and after half an hour removed, washed, and placed in N/5 Na₂CO₃ at 37° over night.

						Days	
						1	3
(1)	0.15 cc. Immune serum	3+0.15 cc.	1/10 egg-white			Frayed	Almost gone
(2)	"	"	3+ "	NaCl		Gone	Gone
(3)	"	Normal	4+ "	1/10 "		Frayed	"
(4)	"	"	4+ "	NaCl		"	"
(5)	"	"	5+ "	1/10 "		"	Almost gone
(6)	"	"	5+ "	NaCl		"	Gone
(7)	"	"	6+ "	1/10 "		"	Frayed
(8)	"	"	6+ "	NaCl		"	"
(9)	"	"	7+ "	1/10 "		"	"
(10)	"	"	7+ "	NaCl		"	"
(11)	"	Immune	7+ "	1/10 "		Untouched	Untouched
(12)	"	"	7+ "	NaCl		Frayed	Frayed

Experiment VI.

Immune sera 3 and 7, and normal serum 7, were tested with fibrin and casumen. Rabbit 3 had been injected four times, Rabbit 7 thrice.

With Fibrin.

Hitherto the trypsin had been added to a mixture of serum and egg-white. Here 0.75 cc. trypsin solution was added to serum, and the egg-white added immediately afterwards. After three hours of contact fibrin was immersed for 0.75 hour and then washed and placed in N/5 Na₂CO₃, covered with toluene, at 37° over night.

						Days	
						1	2
(1)	0.15 cc. Immune serum	3 + 0.15 cc. 1/10 egg-white				Whole	Whole
(2)	"	"	3 + "	1/100 "		Gone	Gone
(3)	"	"	3 + "	1/500 "		"	"
(4)	"	"	3 + "	NaCl		"	"
(5)	"	"	7 + "	1/10 "		Whole	Whole
(6)	"	"	7 + "	1/100 "		0.5 gone	Gone
(7)	"	"	7 + "	1/500 "		"	"
(8)	"	"	7 + "	NaCl		Gone	"
(9)	"	Normal	7 + "	1/10 "		Almost gone	"
(10)	"	"	7 + "	1/100 "		Gone	"
(11)	"	"	7 + "	NaCl		Almost gone	"

With Casumen.

To 0.15 cc. of serum were added 0.75 cc. trypsin solution and 0.15 cc. of egg-white. After three hours 5 cc. 3% casumen and 0.75 cc. N/5 Na₂CO₃ were added. Digestion at 56° for 0.75 hour, then all were transferred to the cold room for the night, and their viscosity determined next morning.

						Min. Secs.	
(1)	0.15 cc. Immune serum	3 + 0.15 cc. 1/10 egg-white				1	30
(2)	"	"	3 + "	1/100 "		1	29
(3)	"	"	3 + "	1/500 "		1	29
(4)	"	"	3 + "	NaCl		1	28
(5)	"	"	7 + "	1/10 "		1	32.2
(6)	"	"	7 + "	1/100 "		1	31.4
(7)	"	"	7 + "	1/500 "		1	29
(8)	"	"	7 + "	NaCl		1	28
(9)	"	Normal	7 + "	1/10 "		1	32
(10)	"	"	7 + "	1/100 "		1	32.4
(11)	"	"	7 + "	NaCl		1	32.2

The above results show clearly that trypsin can be inhibited by a specific precipitate in a greater degree than by immune serum alone or by normal serum with antigen. This result is, however, only relative. Trypsin is partly inhibited by untreated serum, and it is not wholly inactivated by precipitated serum. Judging from the analogy of the carbonic acid precipitate where trypsin is wholly absorbed, but is in part recoverable, a total inhibition in the presence of a specific precipitate could hardly have been expected. A similar recovery of trypsin—from the antitrypsin of Weinland—has been described by Dastre and Stassano [1903]. Time, which would favour fixation, or inactivation, of absorbed trypsin through aeration [Levy 1905],

also favours the recovery of trypsin from antitrypsin (Dastre and Stassano), a tendency which can also be seen in the above results. The recovery of trypsin is therefore an unfavourable factor which cannot be entirely avoided. It can be diminished by using a solid protein as a test for the ferment, especially in a neutral or slightly acid medium, to avoid tryptic action, as any such action would produce a soluble protein which could combine with the absorbed trypsin. After contact with the serum-trypsin mixture, the solid protein would be removed from the sphere of action of the precipitate into an alkaline medium suitable for promoting the action of any trypsin which it might have taken up. If such precautions are taken—and even without these precautions—trypsin-absorption might well be used in the recognition of antibody. At least, as far as my experience goes, trypsin is the most suitable ferment for the purpose.

CONCLUSIONS.

Trypsin is only partially inhibited in the presence of 10 % ox serum.

It is however completely bound by the precipitate produced in 10 % ox serum by saturation with carbon dioxide and if this precipitate be removed the trypsin is completely removed with it.

The absorbed ferment is partly recoverable from the precipitate by protein, especially in solution.

This recovery is not however sufficient to obliterate the difference between the inhibition exerted by 10 % untreated ox serum and that by 10 % precipitated ox serum.

In the presence of a specific precipitate (immune rabbit serum and egg-white) trypsin is more inhibited than by immune serum alone or by normal serum and egg-white. This is especially the case where free trypsin is tested for by a solid protein, but also occurs if the test is carried out with protein in solution.

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