ON EXTRACTION BY CASEIN OF TRYPSIN, ADSORBED BY CHARCOAL

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According to recently published investigations of mine, charcoal exercises an anti-tryptic action, which can be divided up into two consecutive stages, namely, (1) the taking up or adsorption of the trypsin by the charcoal, and (2) the fixation of the trypsin.¹ The mere adsorption could, in absence of proteins, very readily be made complete, as shown by the fact that, after filtering off the charcoal, the filtrate showed no tryptic activity. On the other hand the fixation was never found to be complete as borne out by the observation that a mixture of charcoal and trypsin, even after complete adsorption, gave some tryptic effect with added casein. This was found to depend upon the casein extracting part of the enzyme from the charcoal. The tryptic effect obtained after complete adsorption therefore represents in a way the amount of trypsin adsorbed but not fixed. Furthermore it could be shown that, after proper treatment with casein solution and filtering off the charcoal, the filtrate gave the same tryptic effect as the non-filtered charcoal-trypsin-casein mixture. This proves that all the trypsin that is able to act upon the casein can be made to pass through the filter together with the casein—pointing to some kind of combination being formed between casein and the trypsin. This is in full agreement with the view generally accepted, according to which the action of enzymes is made up of two consecutive stages—(1) combination of the enzyme with the substrate, and (2) breaking up of the latter.

1. *Bio-Chemical Journal*, I, p. 484, 1906.

Up to the present time it has not been possible, experimentally, to distinguish between these two stages, or to study the one independently of the other. The experiments referred to open, I think, a way to studying the process of combination by itself.

If a mixture of a sufficient amount of charcoal and trypsin (approximately free from proteins) is kept for twenty-four to forty-eight hours, then the adsorption will be completed, and the fixation of the trypsin to the charcoal will be so nearly finished that the amount of trypsin adsorbed but not fixed can be considered as constant, anyhow during a short interval of time (one to two hours). By means of added casein-solution, part of the trypsin can be extracted from the charcoal and attached to the casein, and this process can be interrupted at any time by filtering off the charcoal. The tryptic effect obtained with the non-filtered charcoal-trypsin-casein mixture is produced by the whole amount of trypsin transferable under the conditions observed, and the digestive power of the filtrate is produced by the amount of trypsin combined with the casein before the filtration took place. The process of extraction is finished when filtered and non-filtered specimens give the same effect; the extraction is likewise finished when two samples, obtained by filtering after different intervals of time, show the same digestive power.

In the experiments of which I am going to give an account, the influence of the following factors upon the amount of trypsin extracted by casein from charcoal has been tried :—

1. The temperature.
2. The time of interaction.
3. Amount of water present.
4. Amount of casein.

In all the specimens digested toluol was used as antiseptic, and the effect of the trypsin extracted was determined, as described in previous papers of mine, by estimating the amount of nitrogen in equal volumes of the tannic acid filtrates.¹

1. *Journ. of Physiol.*, XXXII, p. 390, 1905.

All my experiments were carried out with charcoal-trypsin mixtures in which complete adsorption had been effected. Therefore, all the tryptic effect obtained was due to trypsin which had been transferred from the charcoal to the casein used. According to the investigations referred to, the amount of trypsin transferable is less the larger the amount of charcoal used, the longer the time of interaction between the charcoal and the trypsin before the casein is added, and the higher the temperature for this interaction.¹ Since the trypsin used was not the same in all experiments, and other conditions likewise were varied, only the figures obtained in the same experiment are comparable.

INFLUENCE OF THE TEMPERATURE

Experiment I.—The charcoal-trypsin mixture had been kept for twenty-four hours at 37°. Equal volumes of this charcoal-trypsin and of casein solution² were mixed and kept at 20° and at 0°, and after the intervals of time recorded the resulting mixtures were filtered at the same temperatures. Then equal volumes of the filtrates were *digested at* 37° for two days. At the same time one specimen which had not been filtered was digested. This specimen, therefore, showed a digestive power corresponding to the amount of trypsin transferable at 37°, and the filtered samples gave the amounts transferred at 20° and at 0°.

The results were :—

		37°		20°		0°
Unfiltered specimen	...	9.7	...	—	...	—
Filtered after ½ hour	...	—	...	4.4	...	2.4
„ „ I „	...	—	...	4.6	...	2.45
„ „ 1½ „	...	—	...	4.45	...	—

Therefore the extraction was finished after half an hour, both at 20° and at 0°, and the experiment shows that *more trypsin is transferred the higher the temperature.*

Experiment II.—The charcoal-trypsin was kept at 20° for two days. Then equal charcoal-trypsin-casein mixtures were prepared, kept and filtered at 37° and at 18°. Equal volumes of the filtrates and one unfiltered specimen, were all *digested at* 37° for two days.

		37°		18°
Filtered after ½ hour		5.85	...	5.3
„ „ I „		6.4	...	5.25
Unfiltered specimen		8.1	...	—

1. *Bio-Chemical Journal*, I, p. 495, 1906.

2. The solutions used contained 2.5 per cent. of casein in 0.2 per cent. Na₂CO₃ solution.

At 18° the extraction was completed after half an hour, but at 37° it was not finished in one hour, since the total amount of trypsin transferable at 37° is represented by the figure 8·1, obtained from the-unfiltered specimen. The experiment confirms the result of Experiment I as to the influence of the temperature upon the amount of trypsin extracted, and in addition it shows that the extraction comes to an end more speedily at 18° than at 37°.

INFLUENCE OF THE TIME OF INTERACTION

Experiment III.—The charcoal-trypsin was kept at 20° for twenty-four hours. Then mixtures were prepared and kept at 20° as follows :—

30 c.c. charcoal-trypsin + 150 c.c. cas. sol.	...	...	A
" " + 15 c.c. "	...	...	B

After the intervals of time recorded, specimens of the mixture were filtered. Of A 30 c.c. filtrate was digested by itself, and of B 7·5 c.c. filtrate was digested together with 22·5 c.c. casein solution. All specimens were digested at 37° for twenty-four hours, and the effect was determined as usual.

	A	B
Filtered at once	9·4	4·6
Filtered after $\frac{1}{2}$ hour	12	5·8

The experiment confirms the observation made in Experiment II, that *the extraction of the trypsin requires some time*. In addition it shows that more trypsin is extracted by the larger amount of casein, but this result does not seem to be quite conclusive since it was not shown in any of the cases that the extraction was finished.

INFLUENCE OF THE AMOUNT OF WATER PRESENT

Experiment IV.—The charcoal-trypsin was kept at 20° for twenty-four hours, whereupon mixtures were prepared and kept at 20°.

50 charcoal-trypsin + 50 casein sol.	...	...	A
" " + 300 H ₂ O	...	...	B
" " + 500 H ₂ O	...	...	C

After three hours, when the extraction must have been finished according to Experiments I and II, the mixtures were filtered at 20°, and specimens were prepared for digestion at 37° as follows, the results of the digestion being given at the same time :—

50 filtrate A + 250 H ₂ O + 50 cas. sol.	...	...	44·85
200 filtrate B + 100 H ₂ O + 50 cas. sol.	...	...	44·0
300 filtrate C + 50 cas. sol.	...	...	44·05

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The ratio between the volumes of A, B, C was 1 : 4 : 6. The digested specimens contained the filtrates in the same proportions and therefore the specimens contained the same amounts of the original charcoal-trypsin and of the casein solution. During the digestion the concentration was the same in all specimens; 50 c.c. casein solution was added in order to increase the effect. The results show, that *the amount of water present during the extraction was without influence.*

Experiment V was carried out with charcoal-trypsin obtained from the same reagents and in the same way as in Experiment IV. Mixtures with casein were made up as follows :—

100 charcoal-trypsin + 50 cas. sol.	...	...	...	...	A
" " "	+	450 H ₂ O	...	...	B
" " "	+	750 H ₂ O	...	...	C
100 charcoal-trypsin + 100 cas. sol.	...	...	...	...	D

After three hours at 20° the mixtures were filtered, and specimens made up as follows were digested at 37°, with the results given :—

25 filtrate A + 125 H ₂ O + 50 cas. sol.	...	...	27·6
100 filtrate B + 50 H ₂ O + 50 cas. sol.	...	...	27·45
150 filtrate C + 50 cas. sol.	...	...	27·15

The digested specimens contained amounts of the filtrates corresponding to the same amount of the charcoal-trypsin and of the casein. Moreover, the volumes and the amount of casein present were the same during the digestion. Therefore the figures obtained confirm the result obtained in Experiment IV, that *the amount of water exercises no influence.* Since it might be suggested that this result depends upon the casein having extracted the maximum of trypsin, which would not be increased by a larger amount of casein, I tried whether the larger amount of casein present in mixture D had not extracted more trypsin than the casein in the mixture A. For this purpose specimens were made up for digestion as follows, and with the results recorded :—

15 filtrate A + 5 cas. sol. + 25 cas. sol.	...	...	13·2
20 filtrate D + 25 cas. sol.	...	...	17·5

15 filtrate A correspond to the same amount of charcoal-trypsin as 20 filtrate D. In order to make the amount of casein the same in both specimens during the digestion, 5 c.c. had to be added to 15 filtrate A. To both, 25 casein sol. was added, in order to increase the effect. The figures obtained show that the larger amount of casein had extracted more trypsin, and therefore the maximum of trypsin extracted had not been reached with the lower amount of casein.

INFLUENCE OF THE AMOUNT OF CASEIN

Experiment VI.—The charcoal-trypsin had been kept at 37°. Mixtures were prepared at 37° as follows :—

50 charcoal-trypsin + 250 cas. sol.	...	...	...	A
„ „ + 100 „	...	...	...	B
„ „ + 50 „	...	...	...	C
„ „ + 10 „	...	...	...	D

The mixtures all contain the same amount of charcoal-trypsin mixture, and since the ratio between the volumes is 60 : 30 : 20 : 12, volumes of the mixtures or of their filtrates, between which the same ratio is valid, must contain trypsin obtained from the same amount of charcoal-trypsin mixture. After 1½ hours at 37° part of the mixtures were filtered and equal volumes of the non-filtered mixtures and of the filtrates were digested at 37° with the following results :—

		Non-filtered		Filtered
60 c.c. A	...	21·55	...	20·1
30 c.c. B	...	15·9	...	15·2
20 c.c. C	...	10·65	...	10·8
12 c.c. D	...	2·55	...	2·45

The figures obtained, being the same for the non-filtered and filtered specimens (except perhaps for mixture A), show that the extraction of the trypsin was finished when the filtration took place.

In order to compare the amounts of trypsin extracted by different amounts of casein, volumes of the filtrates derived from the same amount of charcoal-trypsin mixture must be digested with the same amount of casein. For this purpose, specimens prepared as follows were digested, the results of the digestion being given at the same time :—

60 c.c. filtrate A	...	...	...	...	...	20·1
30 c.c. „ B + 30 c.c. cas. sol.	...	...	...	...	...	17·8
20 c.c. „ C + 40 c.c. „	...	...	...	...	...	16·5
12 c.c. „ D + 48 c.c. „	...	...	...	...	...	8·95

The figures show that the amount of trypsin extracted increases with the amount of casein used for the extraction. Unfortunately, the amount of casein present during the digestion was not sufficient to make the results proportional to the amounts of trypsin present, and therefore no further definite conclusions can be drawn from this experiment. It is, however, obvious that the amounts of trypsin extracted were not nearly proportional to the amounts of casein used, the ratio between the amounts of casein being 25 : 10 : 5 : 1.

This being so, one must expect that for very large amounts of casein the amount of trypsin extracted should become independent of the amount of casein used for the extraction. This was the case in the following experiment :—

Experiment VII.—The charcoal-trypsin had been kept at 37°, and the following mixtures were prepared and kept at 37°.

10 c.c. charcoal-trypsin	+	50 c.c. cas. sol.	...	...	...	A
„	„	+ 100 „	+ 10 H ₂ O	...	...	B
„	„	+ 200 „	+ 30 H ₂ O	...	...	C

After one hour at 37° the mixtures were filtered, and volumes of the filtrates derived from the same amount of charcoal-trypsin were digested with the same amount of casein as follows :—

20 c.c. filtrate A	+	50 c.c. cas.	+	10 H ₂ O	...	...	23.5
40 „	B	33.3 „	+	6.7 „	...	...	23.5
80 „	C	...	...	...	...	...	23.6

That the extraction was finished when the samples were filtered was ascertained by another experiment. A mixture of 30 c.c. charcoal trypsin + 300 c.c. casein was prepared and kept as above. Then 100 c.c. unfiltered mixture and 100 c.c. filtrate were digested :—

Unfiltered mixture	...	...	65.8
Filtrate	...	...	64.4 ¹

DISCUSSION OF THE RESULTS

The above experiments give, no doubt, a very strong support to the view that the proteins combine with trypsin before they are broken up by the same. As the experiments were arranged, the casein was made to extract trypsin which had been previously adsorbed by charcoal. During the process of extraction, therefore, the trypsin was acted upon by two substances endowed with the power of taking it up—charcoal and casein. The final distribution of casein between the charcoal and the trypsin was reached in, as a rule, less than half an hour after the casein had been added to the charcoal-trypsin mixture. Both charcoal and casein take up trypsin in some proportion to the amount of substances present, and therefore the final distribution of the trypsin will be the effect of some kind of mass action

1. With regard to the above experiments, it may be mentioned that it has been found to be very difficult completely to remove charcoal from a fluid that contains casein. Even after repeated filtration traces of charcoal occur in the filtrate. Evidently these traces can be of no influence, in case the extraction was finished when the filtration took place and if at the same time the following digestion is carried out at the same temperature as the extraction and filtration (Experiment VI, VII). In other cases the figures obtained from the filtered specimens might be a trace too high, which would, however, in no instance affect the conclusions drawn. The amount of digestion taking place before and during the filtration, being the same or approximately the same for filtered and non-filtered specimens, does not influence the results.

On the other hand, the effect of this mass action can not be the same as that of Guldberg-Waage's law for real solutions, because in the case of real solutions the combinations formed are reversible and, therefore, the final equilibrium will be the same independently of the way in which it has been arrived at.

In the case of charcoal and trypsin, on the contrary, the trypsin adsorbed by degrees becomes fixed to the charcoal and only a small portion of it can be extracted by added casein, less being extracted the longer the time and the higher the temperature for the interaction between the charcoal and trypsin before the casein is added.¹ The final distribution of the trypsin, therefore, depends very much upon the way in which it has been arrived at.

SUMMARY

1. Casein is capable of extracting trypsin adsorbed by charcoal. The extraction requires some time to be finished (Experiment III). Sooner or later (as a rule in less than half an hour at 20°) the extraction comes to an end as borne out by the fact that a filtered specimen gives the same tryptic effect as the non-filtered material (Experiments VI, VII). The end of the extraction can also be recognised by the fact that specimens filtered after different intervals of time contain the same amount of trypsin (Experiments I, II).

2. The final amount of trypsin extracted is larger the higher the temperature (Experiments I, II).

3. For small amounts of casein the final amount of trypsin extracted increases with the amount of casein (Experiment VI). For large amounts of casein the amount of trypsin extracted is independent of variations in the amount of casein (Experiment VII).

4. The amount of water present is without influence upon the final amount of trypsin extracted (Experiments IV, V). This rule holds good even for small amounts of casein (Experiment V).

The results support the view that proteins combine with trypsin before they are broken up by the same.

1. *Bio-Chemical Journal*, I, 489, 1906.