AN ANTITRYPTIC EFFECT OF CHARCOAL AND A COMPARISON BETWEEN THE ACTION OF CHARCOAL AND THAT OF THE TRYPTIC ANTIBODY IN THE SERUM.

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By chance I recently observed that charcoal, when added to a digesting fluid of trypsin and casein, produces a very marked diminution of the tryptic effect. There can hardly be any doubt that this depends upon the trypsin being taken up by the charcoal, thus being prevented from acting upon casein. It is a well known fact that charcoal readily condenses gases on its surface, and likewise takes up colouring matters from solutions. That other solid substances and especially hydrogels, have got the power of taking up crystalloids from their solutions has been pointed out by several investigators (v. Bemmelen,¹ Schmidt,² Biltz,³ Bayliss,⁴ and others). This process has been termed adsorption (Du Bois Reymond, Ostwald).

As to the laws of adsorption very little is known. Henry's rule, which has been found to be valid for the distribution of a soluble substance between two fluids, which implies that the ratio between the concentration in the two solvents is a constant ($\frac{C_1}{C_2} = k$), when equilibrium has been reached, does not apply to the adsorption. In the case of adsorption, a state of equilibrium seems to be arrived at sooner or later; if C_{ads} is the concentration in the solid substance and C_f the concentration in the fluid, then $\frac{C_{ads}}{C_f}$ is not a constant, but diminishes as the total amount of the originally dissolved substance is increased. This means that the solid takes up relatively more from

1. *Zeitschr. anorg. Chem.*, Vol. XXIII, p. 321, 1900.
2. *Zeitschr. physik. Chem.*, Vol. XV, p. 60, 1894.
3. *Chemiker Zeitung*, Vol. XXIX, No. 25, p. 325, 1905.
4. *Bio-Chem. Journ.*, Vol. I, p. 175, 1906.

a dilute than from a concentrated solution. The way in which the ratio $\frac{C_{\text{ads}}}{C_{\text{fl}}}$ varies with the total amount of dissolved substance is different in different cases, and no general rule has been found. As to other factors which influence the adsorption v. Bemmelen points out the following :—(1) the adsorbing substance, (2) the solvent, (3) the substance to be adsorbed, (4) the state of its molecules, (5) the temperature, without being able to give any general rules as to their influence.

In the cases where the adsorption of colloids has been investigated, the influence of the amount of colloid upon the ratio $\frac{C_{\text{ads}}}{C_{\text{fl}}}$ has been found to be roughly the same as for crystalloids (Bayliss). In addition, v. Bemmelen observed that the adsorption of colloids (e.g., As_2S_3) may sometimes be complete. Bayliss found that the total amount of congo-red taken up by paper is less the higher the temperature, and he also observed that the congo-red can be completely removed from the paper by repeated extraction with water.

Adsorption of enzymes by solids, and particularly by charcoal, has been observed by Glaessner,¹ Dauwe,² and others, but no investigations as to the conditions and quantitative relations of the adsorption have so far been published.

My experiments on the adsorption of trypsin by animal charcoal were carried out with a view to make out whether there exists any analogy between the action of the charcoal upon trypsin and the action of the tryptic-antibody in the normal serum. The animal charcoal used had been prepared from bones, and the phosphates had been removed by repeated heating with HCl. The trypsin had been obtained as given in a previous paper³. The digestive power of the trypsin was estimated as described in the same paper, and the figures given represent the number of cubic centimetres of decinormal acid required to neutralise the ammonia obtained from equal volumes of the tannic acid filtrates.

1. *Hofmeisters Beiträge*, Vol. I, p. 1, 1902.

2. *Ibid.*, Vol. VI, p. 427, 1906.

3. Hedin, 'Observations on the action of trypsin,' *Journ. of Physiol.*, Vol. XXXII, p. 486, 1905.

ADSORPTION WITHOUT THE PRESENCE OF PROTEID

In the first place it was found that the presence of a proteid (*e.g.* casein) materially influences the process of adsorption, and therefore some experiments were carried out in which only the small amount of proteid contained in the trypsin solution was present during the process of adsorption. Thereupon the charcoal was filtered off, and the digestive power of the filtrate was tested with casein.

Experiment I.—Two mixtures containing, the one 2 gr. charcoal + 80 c.c. trypts. (A), and the other 0.1 gr. charcoal + 80 c.c. trypts. (B), were kept at 20° as recorded below. Thereupon, equal volumes of the filtered samples were made to act upon 50 c.c. of 2.5% casein solution at 37° for 24 hours. 25 c.c. of 10% tannic acid solution were added, and 50 c.c. filtrate were taken for each estimation. The trypsin used for each estimation would, without previous treatment with charcoal and with a sufficient amount of casein, have given the result 240.

	A.	B.
1 hour	0	55.4
4 hours	0	53.75
24 „ ...	0 ...	46.75
48 „ ...	0 ...	43.5

The influence of the amount of charcoal is evident, the adsorption in A with 2 gr. of charcoal being complete after one hour, and in B with 0.1 gr. equilibrium being reached in 24 hours. The small drop observed after the expiration of this time may depend upon destruction of enzyme, as shown by the fact that samples of the same enzyme having been kept without charcoal for two days at 0°, gave 38.6, and at 20° 34.2.

The influence of the temperature was examined by putting the mixture B (after 48 hours at 20°), at 37° for 24 hours. The result was :

After 48 hours at 20°		43.5
After 48 hours at 20° and 24 hours at 37°		2.3

Therefore, if the temperature is raised after equilibrium has been reached at a low temperature, then more trypsin is adsorbed.

Experiment II shows the influence of the temperature and of the time, low temperatures being used in order, as far as possible, to prevent destruction of the trypsin. Samples of the same mixture were kept at 0° and at 20°, as given below. Then the filtrates were tested as above. Without charcoal 120.

		0°		20°
24 hours	...	22.3	...	4.5
48 "	...	7.95	...	0.2

The experiment shows that more trypsin is adsorbed at the higher temperature. The equilibrium was not reached in 24 hours.

Experiment III was carried out at 20°, and only the amount of charcoal was varied.

0.5 gr. charcoal	...	0	0.05 gr. charcoal	...	25.75
0.1 gr. "	...	3.75	Without "	...	750

This experiment verifies the rule that $\frac{C_{ads}}{C_A}$ diminishes as the total amount of enzyme is increased, or (which comes to the same) as the amount of charcoal is diminished. A consequence of this rule is, that when the amount of trypsin is the same and the amount of charcoal is diminished, then the charcoal approaches by degrees a state of saturation, where no more trypsin is adsorbed. I have been able to verify this by keeping mixtures of charcoal and trypsin at 20° for 24 hours, whereupon fresh trypsin was added to equal volumes of the fluids before (A) and after (B) filtration. If the charcoal is not saturated, then A gives less digestion than B, and at saturation both give the same result.

0.025 gr. charcoal	...	...	(A. 28
			(B. 36.3
0.0125 gr. "	...	...	(A. 49.8
			(B. 52.65

In this experiment, 100 c.c. of casein solution was added as substrate. The result shows that saturation was nearly reached in the mixture with 0.0125 gr. charcoal.

The above results are remarkable as showing how readily charcoal effects a complete adsorption of trypsin. Similar observations have

been made before by v. Bemmelen (page 485), but to my knowledge complete adsorption has not been observed for crystalloids.

According to the above experiments, more trypsin is adsorbed the higher the temperature. In the case of the adsorption of congo-red by paper, the influence of the temperature is the reverse (Bayliss, p. 485).

INFLUENCE OF THE PRESENCE OF THE CHARCOAL DURING THE DIGESTION

In all the above experiments, the charcoal used for the adsorption was removed before the casein was added. The result of the digestion becomes somewhat different if the charcoal is not removed, as proved by the following experiment.

Experiment IV.—The trypsin and the charcoal were allowed to act upon each other as recorded, then equal volumes of the fluids were digested with 50 c.c. of casein solution in set A, after filtering off the charcoal, and in set B, in the presence of the same.

				A.	B.
0.5	gr. charcoal	24 hours at	37° ...	0	11.7
0.25	"	"	37° ...	0	18.45
0.5	"	"	17° ...	0	44.1
0.5	"	48 hours at	37° ...	0	5.6
0.25	"	"	37° ...	0	8.5

The amount of trypsin rendered inactive by the charcoal was very considerable, since the trypsin used for each digestion, without charcoal and with an excess of casein, would have given the result 960. No sample of set A showed any tryptic effect, which implies, that before the addition of the casein the adsorption was complete in all samples. The digestion taken place in the samples of Set B, therefore, must depend upon the casein being able to deprive the charcoal of a small amount of the trypsin adsorbed. This is confirmed by the following experiment.

Experiment V.—An amount of trypsin, which corresponded to the effect 2400, was kept with 0.5 gr. charcoal at 37° for 24 hours.

Then the adsorption was complete, 5 c.c. of filtrate, in 24 hours,

with 50 c.c. of casein solution, giving the effect 0·7. The charcoal was thoroughly washed and digested with 50 c.c. of casein solution. The effect was 30·6.

That the trypsin is attached to the casein before the digestion, is proved by the following experiment.

Experiment VI.—A mixture of 0·5 gr. charcoal + 80 c.c. tryps., was kept at 20° for five hours. The adsorption was then complete. To 50 c.c. of the mixture 300 c.c. of casein solution at 37° were added, and the whole was kept at 37° for one hour. Then part of the mixture was repeatedly filtered in order to remove the charcoal. 50 c.c. of the casein-charcoal-trypsin mixture and 50 c.c. of the filtrate were digested with the following result :—

With charcoal	...	...	...	...	25·1
Without „	...	...	...	...	23·2

Another experiment with other proportions gave

With charcoal	...	...	...	...	65·8
Without „	...	...	...	...	64·4

The experiments show that all the trypsin available had been transferred from the charcoal to the casein during one hour at 37°.

Therefore, the casein undoubtedly is capable of taking back some of the trypsin already adsorbed by the charcoal. The amount taken back in the above experiments was 1·15% of the amount originally adsorbed. The amount of trypsin rendered active by addition of casein after complete adsorption by charcoal depends upon

- 1.—The amount of charcoal used, less trypsin being rendered active the more charcoal present.
- 2.—The temperature at which the adsorption has taken place, less trypsin being rendered active the higher the temperature.
- 3.—The time of interaction between the trypsin and the charcoal, less trypsin being rendered active the longer the time.

This is all borne out by Experiment IV.

The influence of the temperature was tried in another experiment, where lower temperatures were used, in order, as far as possible, to prevent destruction of enzyme.

Experiment VII.—Two equal mixtures were kept for 48 hours, the one at 0°, the other at 20°, whereupon the digesting power was tested after filtering off the charcoal (A) and in the presence of the same (B). The amount of trypsin used, would, without treatment, with charcoal, have given the effect 93.

			A		B
48 hours at	0°	...	0	...	29·8
48	„	20°	0	...	9·8

According to the above investigations, the process, by which trypsin is rendered inactive by charcoal, can be divided up into two different stages: (1) the *taking-up*, or adsorption of the trypsin by the charcoal, and (2) the *fixation* of the same. The amount of trypsin taken up by the charcoal is represented by the difference in digesting power between the trypsin not treated with charcoal, and the filtrate after treatment with charcoal. The process of fixation can most conveniently be studied in experiments with rather a large amount of charcoal, in which case all the trypsin very soon is taken up, whilst the fixation is still in progress. In such cases the amount of trypsin taken up and not fixed is obtained by direct estimation of the digestive power of the mixture.

If very little charcoal is used, then both processes come to a standstill before all trypsin is rendered inactive. This shows that the trypsin is not merely destroyed, because in that case the process would not finish till all trypsin were destroyed. It is very difficult to form a clear view of what is taking place. At present I am inclined to think that the trypsin is attached to the surface of the charcoal by molecular attraction, and subsequently proceeds further into the charcoal by diffusion, thus, by degrees, becoming inaccessible for the proteid. As long as the trypsin is attached to the surface of the charcoal, or merely adsorbed, it can be transferred to added casein, but not when it has entered deeper into the same. This view would account for the fixation going faster the more charcoal is present, *i.e.*, the larger the surface through which the diffusion takes place. It would not be difficult to conceive that a process of diffusion would depend upon the temperature and the time, as found above, for the

process of fixation. In this way it would be easy to understand that only part of the trypsin taken up by the charcoal can be rendered active by addition of casein.

As pointed out, charcoal, if present in sufficient amount, adsorbs trypsin completely, leaving no trypsin in the filtrate when filtered off. On the other hand, if casein is added to the mixture of charcoal and trypsin before filtration, then a considerable amount of the trypsin already adsorbed appears in the filtrate together with the casein. This fact affords a very strong support for the view that trypsin is attached to proteids previous to its action, and it does not seem unlikely that this is a process of adsorption. This would be quite consistent with the fact that proteids do not form any real solutions with water, but suspensions of larger or smaller particles. If the taking-up of trypsin by proteids is a process of adsorption, then it might be expected that the proteids, if present in sufficient amount and in absence of other adsorbing substances, could, like charcoal, completely adsorb the trypsin. In a previous paper I have pointed out that complete combination of the trypsin is required for the explanation of the following two observations :—

- 1.—The effect of trypsin upon certain proteids is independent of the dilution, and
 - 2.—The effect is the same after the same number of trypsin time units.¹
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COMPARISON OF THE NEUTRALISING POWER OF CHARCOAL WITH THAT OF THE TRYPTIC ANTIBODY IN THE SERUM

From the above experiments it is evident that charcoal exercises a very marked checking influence upon the action of trypsin, which influence is very much like the effect produced by the tryptic antibody contained in normal serum. In the case of charcoal, the process of neutralisation can easily be distinguished from the process of digestion, by filtering off the charcoal before the proteid to be digested is added. In the case of the antibody, the two processes cannot be strictly

1. *Journ. of Physiol.*, Vol. XXXII, p. 480, 1905.

distinguished, because the neutralising substance cannot be removed. In order to compare the effect upon trypsin of charcoal and of antibody, one, therefore, has to carry out the digestion in the presence of the neutralising substance in both cases. Investigations of that kind I have previously carried out as far as the antibody is concerned.¹ Corresponding observations with charcoal can be obtained from Experiments IV and VII. Some additional observations are given below. Investigations have been carried out with reference to :—

- 1.—The time of interaction of trypsin and the neutralising substance.
- 2.—The temperature.
- 3.—The dilution.
- 4.—The saturation of the neutralising substance with trypsin, and
- 5.—The complete neutralisation of trypsin.

From the above experiments it follows that the *amount of trypsin neutralised is larger, the longer the time and the higher the temperature*. Complementary conclusions can be drawn from the following experiments.

Experiment VIII.—Mixtures of 1 gr. charcoal + 20 c.c. trypts. were kept as given below. Then the digesting power of the mixtures was determined with 50 c.c. of casein solution at 37° for 24 hours.

		17°		37°
3 hours without charcoal	...	43·65	...	43·4
5 min. with charcoal	...	30·5	...	22·9
30 min. ,,	...	25·5	...	lost.
1 hour ,,	...	21·4	...	9·2
3 hours ,,	...	16	...	2·95
3½ hours ,,	...	14·35	...	2·15

Equilibrium was not reached in three hours.

With another enzyme, the following results were obtained.

			20°		37°
18 hours	...	...	40·6	...	11·85
19 ,,	...	...	39·9	...	9·8

1. See page 474.

The amount of enzyme neutralised was very considerable, since the trypsin used, with an excess of casein, would have given the figure 470. One sample, which had been kept at 37° for 18 hours (corresponding to 11·85), was kept at 20° for 24 hours, and gave then the result 9·6. Another sample, which had been kept at 20° for 18 hours (corresponding to 40·6), lost in digesting power during eight hours at 37° (result = 25·1).

This experiment, and others which have not been recorded, show that a very long time is wanted for reaching the state of equilibrium, where no change takes place. This makes it very difficult to decide whether a diminution in activity be due to adsorption or to destruction of enzyme. Considering the enormous amount of enzyme rendered inactive by the charcoal the fall after 24 hours is however rather insignificant, and I think one is justified in saying that equilibrium is arrived at, although very slowly.

Experiment IX.—In order to find out whether the *dilution* has any influence upon the amount of trypsin rendered inactive by charcoal, specimens were made up as follows :—

0·05 gr. charcoal + 20 c.c. trypsin	...	...	...	A
0·05 " "	+	20 c.c. H ₂ O	...	B
0·05 " "	+	40 c.c. H ₂ O	...	C.

After 24 hours at 20°, 40 c.c. of water were added to A, and 20 c.c. to B, whereupon the digestive power of the specimens was estimated as usual. The result was :—

$$A = 78\cdot4 \qquad B = 80\cdot3 \qquad C = 80\cdot5$$

and consequently *the dilution had no influence*. Control specimens, which were filtered before the casein was added, showed that the adsorption was not complete.

Experiment X.—Whether charcoal can be *saturated* with trypsin was tried as follows. 0·01 gr. charcoal + 50 c.c. trypts. was kept at 20° for 24 hours, and then at 37° for 24 hours. The digestive power was determined with an excess of casein (200 c.c.), in which case the results obtained are proportional to the amount of active trypsin present.¹

1. *Journ. of Physiol.*, Vol. XXXII, p. 471-474, 1905.

Three estimations were carried out, namely, of 5 c.c. of the above charcoal-trypsin mixture, of 5 c.c. of this mixture in the presence of 1 c.c. of trypsin, not previously acted upon by charcoal, and of this amount of trypsin alone. The sample, with mixture and trypsin, was kept at 37° for one hour before the casein was added. The results were :—

5 c.c. of mixture	...	...	...	12·1
5 c.c. „	+	1 c.c. of trypts.		35·3
1 c.c. of trypsin	...	...	...	23·4

Therefore, the effect of the trypsin, by itself, (23·4) was the same as the effect of the trypsin in the presence of the charcoal-trypsin mixture (35·3 — 12·1 = 23·2), which proves that the charcoal in the mixture had not affected the trypsin, and that the *charcoal was saturated with trypsin* before the fresh trypsin was added.

As to the question, *whether trypsin can be completely neutralised* by charcoal, it is sufficient to refer to the above investigations, according to which it is very easy completely to remove all trypsin from a solution, by using a large amount of charcoal and filtering it off before the casein is added, but that *always some tryptic effect has been observed, when the digestion has been carried out in the presence of the charcoal which had absorbed the trypsin.*

The above results, obtained with trypsin and charcoal, as compared with corresponding results obtained with trypsin and its antibody in the serum,¹ show that in all respects tried, there exists a very close agreement between charcoal and the antibody, with regard to their effect upon trypsin. In consequence of this agreement, I am very much inclined to believe that the neutralisation of the trypsin is brought about in the same way in both cases. As I have pointed out above, the neutralisation of trypsin by charcoal implies, not only the *adsorption* or the taking-up of trypsin by the charcoal, but also the *fixation*, or the process by which the trypsin is rendered inaccessible for proteids. In all my experiments with charcoal some trypsin has remained merely adsorbed, and therefore transferable to added casein

1. See page 483.

and active. Accordingly, it is only reasonable to think that the same might have been the case in experiments with the antibody, and, in my opinion, this is a very acceptable explanation of the fact, that it has been found impossible completely to neutralise trypsin by aid of the antibody.

CONCLUSIONS

1.—The process of neutralisation, exercised by charcoal upon trypsin, can be divided in two consecutive stages :—

(a) The taking-up, or *adsorption* of the trypsin by the charcoal.

A complete adsorption of the trypsin can easily be effected by using a sufficient amount of charcoal. The trypsin merely adsorbed is readily transferred to added casein.

(b) The *fixation*, or the process by which the trypsin is rendered inaccessible for added casein. The amount of trypsin fixed is larger, the larger the amount of charcoal, the longer the time of interaction and the higher the temperature.

2.—The action of charcoal has been found to agree with that of the tryptic antibody in all respects tried, and therefore the neutralising effect, in all probability, is brought about in the same way in both cases.