LXXVI. THE RELATION OF THE ENZYMES TRYPSIN AND PEPSIN TO THEIR SUBSTRATES.

By GEORGE EDWARD BRIGGS.

From the Botany School, Cambridge.

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THE subject referred to in the title of this note is one upon which different authorities have different views. Northrop believes that no combination exists between them and has in various papers put forward the evidence which he considers supports his view. It is not our intention to review the whole of the evidence at the present time but merely to discuss that recorded in two of Northrop's papers [1924, 1; 1925].

This author states that, when trypsin is added to a suspension of gelatin in water, the ratio of the concentration of the enzyme in the gelatin to that in the liquid phase outside is the same as the ratio of the concentrations of chlorine ions outside and inside the gelatin. This equality holds from p_H 2-10 except near the isoelectric point of the gelatin where the ratio for the enzyme is much greater than the other, which is unity. As Northrop points out, this equality can be explained, if Donnan's theory of membrane equilibria is applicable, by assuming that the enzyme is a mono-acid base which throughout the region mentioned is completely dissociated. In the case of pepsin a similar relation of ratios exists from p_H 1-7 with the exception that here it is the ratio of the concentration of chlorine ions in the gelatin to that outside, and consequently the enzyme is assumed to be a mono-basic acid. Further, the author states that a combination of enzyme with gelatin would disturb this equality and that his results are such as to be incompatible with such a combination, or, in his own words, "The results further show that the removal of trypsin from solution by *insoluble*¹ proteins is not evidence of the existence of a compound between the enzyme and the protein, as the writer formerly supposed, but, on the contrary, proves that there is no combination between the two²."

¹ Our italics.

² That Northrop does not distinguish between the type of compound referred to here and the hypothetical compound which is assumed by many workers to be involved in the enzymic hydrolysis of protein solutions is shown by the statement [1924, 2] "Experimental evidence has already been found which contradicts such an assumption." Here the assumption refers to the hypothetical compound, and the experimental evidence is that in the earlier paper [1924, 1] on the distribution of trypsin between gelatin and water.

We do not know on what grounds Northrop assumes gelatin to be an insoluble protein (probably he means undissolved). In any case we shall assume with others that, not only is it soluble, but that some of it is dissolved. The rate of the solution and the final concentration attained depend upon the concentration of hydrogen ions and upon the concentration and nature of the salts present. If the dissolved molecules and ions of gelatin can pass out of the gelatin, the final state will be that the concentration of the molecules will be the same inside and out. If they cannot pass, the molecules from the outside of the particle will dissolve until equality is reached, assuming that the distribution of the ions is such as predicted by Donnan's theory. On account of the bound gelatin ions inside the particle there will be a smaller concentration of other ions of the same sign inside the particle than in the liquid outside¹.

Let s be the concentration of gelatin molecules and s' that of the ions, e and e' of the molecules and ions of the enzyme, e_s , $e_{s'}$, e'_s and $e'_{s'}$ of the compound of enzyme and substrate and the ions of this compound. The subscript i will denote those inside and o those outside. Then

$$\begin{aligned} e'_i &= k_b e / OH_i, & e'_o &= e k_b / OH_o, \\ e_{s_i} &= s e / k_s, & e_{s_o} &= s e / k_s, \\ e_{s'_i} &= s'_i e / k_s = e s k_A / H_i k_s, & e_{s'_o} &= e s k_A / H_o k_s, \\ e'_{s_i} &= e'_i s / k_s = e s k_b / OH_i k_s, & e'_{s_o} &= e s k_b / OH_o k_s, \\ e'_{s'_i} &= e'_{s_i} k_A / H_i = e s k_A k_b / H_i OH_i k_s, & e'_{s'_o} &= e s k_A k_b / H_o OH_o k_s, \end{aligned}$$

where k_A is the acidic dissociation constant of the gelatin and k_b the basic constant for the trypsin and k_s for the compound, whatever its form, into enzyme and gelatin. There is evidence for the combination of the enzyme and dissolved gelatin, some of which we shall produce presently.

The total enzyme inside, E_i , is equal to $e + e_{s_i} + e'_i + e'_{s_i} + e_{s'_i} + e'_{s'_i}$. If the enzyme is a strong base, as Northrop supposes, then e , e_s and $e_{s'}$ can be neglected, and we have

$$\frac{E_i}{E_o} = \frac{OH_o}{OH_i} \cdot \frac{\{1 + s/k_s(1 + k_A/H_i)\}}{\{1 + s/k_s(1 + k_A/H_o)\}}.$$

Any other anion can be substituted for OH and any other cation for H . The greater H , *i.e.* the nearer the isoelectric point of the gelatin, the more nearly will E_i/E_o approximate to OH_o/OH_i . These ratios will approximate whatever the value of H as s becomes small relative to k_s . With H_i greater than H_o , as it is in the region being considered, the enzyme ratio should be smaller than the ratio OH_o/OH_i , and more so the greater s . With increasing alkalinity s becomes greater [Cohn, 1925]². It may be noted that, in as far as the concentration of the molecules e and e_s is not negligible, the enzyme ratio should approach more closely to unity and hence, since E_i is greater than E_o , this

¹ Of course *inside* and *outside* are not to be taken literally.

² It is not clear as to whether it is s which is increased or only the total, s plus the ions.

factor also will tend to make the ratio smaller. When we eventually reach the point where the enzyme is practically un-ionised we shall have

$$E = e + e_{s_i} + e_{s_o}$$

and then our ratio E_i/E_o will become

$$\left[1 + \frac{s}{k_s} \left(1 + \frac{k_A}{H_i}\right)\right] / \left[1 + \frac{s}{k_s} \left(1 + \frac{k_A}{H_o}\right)\right]$$

and if H is small compared with k_A

$$\left[1 + \frac{sk_A}{H_i k_s}\right] / \left[1 + \frac{sk_A}{H_o k_s}\right].$$

This will approach to H_o/H_i or OH_i/OH_o , the reciprocal of the previous limiting value, unless s is small compared with k_s , the actual value being between H_o/H_i and unity.

If the gelatin molecule has, not one, but several acid groups ionising over the range of p_H investigated, our expression needs modification. The titration curve, since it is practically identical with that of a mono-basic acid, shows that the dissociation constants of the groups must be practically all the same and the groups sufficiently far apart to act independently. If there are x such groups we shall have $e'_{s''}$, $e'_{s'''}$, etc. as well as e'_s , and since

$$e'_s = \frac{e'_s k_A x}{H} \text{ and } e'_{s''} = \frac{e'_s k_A^2 x (x-1)}{H^2 \cdot 2} \text{ etc., } \Sigma e'_s = e'_s \left(1 + \frac{k_A}{H}\right)^x,$$

and our expression for E_i/E_o becomes

$$\frac{OH_o}{OH_i} \cdot \frac{[1 + s/k_s (1 + k_A/H_i)^x]}{[1 + s/k_s (1 + k_A/H_o)^x]}.$$

Hence with H small compared with k_A , E_i/E_o should be yet smaller.

If the gelatin molecule can combine with as many as y molecules or ions of enzyme instead of one, as we have supposed above, then our expression becomes further modified to

$$\frac{E_i}{E_o} = \frac{OH_o}{OH_i} \cdot \frac{[1 + ys/k_s (1 + k_A/H_i)^x (1 + e'_i/k_s)^{y-1}]}{[1 + ys/k_s (1 + k_A/H_o)^x (1 + e'_o/k_s)^{y-1}]}$$

if the points of combination are sufficiently far apart to act independently. If, as seems likely, the enzyme concentration is small compared with k_s , the new terms will approximate to unity. If not, then since e'_i is greater than e'_o , this factor will tend to increase the ratio E_i/E_o as compared with OH_o/OH_i .

Instead of approaching to OH_i/OH_o when the enzyme is practically un-dissociated, etc., the ratio will approach to $(OH_i/OH_o)^x$.

On the acid side of the isoelectric point of the gelatin the expression for E_i/E_o differs from that given above in having k_B/OH instead of k_A/H . As we remove from the isoelectric point, OH_i tends to become greater than OH_o and s tends to become greater, and hence the enzyme ratio should again

become smaller. In this region there is less likelihood of any enzyme remaining undissociated, a complication which would increase the ratio in this case and bring it nearer unity.

Table I. *Distribution of chloride ions and trypsin, inside and outside of gelatin particles (after Northrop).*

p_H	2.0	2.5	3.0	3.5	4.0	4.7	5.5	6.0	6.5	7.0	8.0	8.5
Cl_o/Cl_i	0.6	0.4	0.24	0.13	0.38	1.0	1.3	1.6	2.0	1.9	1.9	1.8
E_i/E_o	0.5	0.3	0.23	0.17	0.35	1.5) 11.0 1.6 20.0)	1.5) 4.0 6.0 1.2)	1.4	2.5	1.9	1.7	2.1
p_H	9.0	9.2	9.4	9.6	9.8	10.0	10.2	10.4	10.6	10.8	11.0	
Cl_o/Cl_i	2.0	2.4	2.6	2.5	2.2	2.3	2.2	1.8	2.0	2.0	2.2	
E_i/E_o	2.0	2.6	2.5	2.6	2.0	1.9	1.0	0.38	0.43	0.43	0.30	

Examining the results obtained by Northrop (see Table I) we notice that, apart from the region of the isoelectric point which we shall consider presently, the majority of the enzyme ratios are smaller than the ratios for chlorine ions, and increasingly so as more alkaline regions are approached. It is not much use to take the average of all the experiments and show that the enzyme ratio is the same as the chlorine ion ratio, as does Northrop, particularly as he says that each figure in the table is the average of ten. This worker's interpretation of the results from p_H 10 onwards is that the enzyme is an ampholyte with its isoelectric point at 10.2, where the ratio is unity¹, and afterwards the ratio ought to be equal to Cl_i/Cl_o instead of Cl_o/Cl_i .

As we have seen above, our theory, which does not assume any amphoteric properties for the enzyme, explains an approach to equality of the ratios E_i/E_o and Cl_i/Cl_o . Actually the former becomes smaller than the latter, thus indicating further complications; possibly further ionisation of the gelatin.

So far we have assumed that the system is in equilibrium. The experiments were carried out at 0° to restrain hydrolysis of protein, and it is quite possible that the water was not saturated with gelatin, although the suspension had been standing for eighteen hours. Since the ratio of surface of gelatin to volume of liquid was greater inside than outside at the beginning of the experiment and remained so in the region of the isoelectric point of the gelatin, the concentration of the gelatin in solution outside is likely to be further removed from the equilibrium than that inside and particularly so nearer the isoelectric point. Consequently we shall have to insert s_i in the numerator of our expression and s_o in the denominator. With s_i greater than s_o , the value of E_i/E_o will tend to become greater, thus counteracting the disturbing tendency of the other factors considered above. Eventually the ratio will be greater than Cl_o/Cl_i when the isoelectric point is reached and the other factors k_A/H and k_B/OH become negligible. Further Loeb [1922] has shown

¹ Any combination with ions of gelatin such as we suggest would make the ratio greater than unity.

that the rate of solution of gelatin at 38° is much less at the isoelectric point than elsewhere. If the same holds at 0° and solution follows the usual logarithmic course ($dx/dt = K - cx$) then, when t is sufficiently small, s_i and s_o will both be practically zero and hence the ratio $(s_i + k_s)/(s_o + k_s)$ will be unity; with t finite the ratio will be greater than unity, and, with the rate increasing as we depart from the isoelectric point, the ratio of s_i/s_o will decrease and our ratio, which will increase at first, will eventually decrease until we approach the point at which the rate of solution is so great that the system is practically in equilibrium in the finite time and s_i equal to s_o when the ratio is again unity.

With trypsin, as we have seen, the enzyme ratio becomes much greater than the chlorine ion ratio as the isoelectric point is reached. As to whether the whole discrepancy is due to the above cause, it would be rash for us to say. Possibly there is a combination with the gelatin particle in all regions and hence a tendency to increase the ratio E_i/E_o as compared with Cl_o/Cl_i on the alkaline side and Cl_i/Cl_o on the acid¹. Northrop suggests that the large numbers may be due partly to experimental errors. "From p_H 4.5 to 6.0 all the trypsin solutions showed a slight precipitate, especially in the presence of gelatin. This precipitate contains trypsin and is probably the cause of the irregular results contained in this range." Further on he remarks "it is possible that a small amount of trypsin is combined with the gelatin and that this is the disturbing factor." He is referring, presumably, to the gelatin particle itself and not to gelatin in solution, which, indeed, he never mentions. As to the nature of the combination, he makes no further suggestion in this paper, in fact he stoutly denies its existence except where he cannot ignore the anomalous results.

In the experiments with pepsin, he again found the ratio for the enzyme to be much greater than unity at the isoelectric point. Our expressions for pepsin are the same as those for trypsin with the substitution H_o/H_i for OH_o/OH_i . The ratios found were 1, 5, 10, 9 and 1 for the following concentrations of NaCl; 0, 0.0016, 0.008, 0.04 and 0.2 *M* respectively. With increasing concentrations of NaCl, Loeb [1922] has shown that gelatin at the isoelectric point dissolves at an increasing rate when the temperature is 35° . If the same holds at 0° , we should expect, from what we have said previously, that $(s_i + k_s)/(s_o + k_s)$ or E_i/E_o would increase up to a certain concentration and then decrease. We have not the requisite data to forecast the concentration for the maximum value of the ratio when the time is eighteen hours and, indeed, there are probably other complications.

Table II. *Comparison of chloride and pepsin ratios with gelatin at various p_H and salt concentrations (after Northrop).*

p_H	4.7	5.1	5.6	6.0	6.5	6.5	6.5	7.2
CV_i/CV_o	1.01 ± 0.01	0.95 ± 0.01	0.70 ± 0.01	0.62 ± 0.02	0.65	0.74	0.88	0.50 ± 0.013
E_i/E_o	1.01 ± 0.03	1.06 ± 0.05	0.61 ± 0.04	0.51 ± 0.04	0.53	0.57	0.80	0.51 ± 0.05

¹ This may be a combination of gelatin ion with oppositely charged enzyme ion to form a salt.

It will be seen also (Table II) that, except near the isoelectric point, the enzyme ratio is smaller than Cl_i/Cl_o , just as in the case of trypsin it was less than Cl_o/Cl_i . Northrop was not able to carry out experiments in solutions more alkaline than p_H 7 on account of the instability of the enzyme. One wonders whether the ratio would have fallen still further in this region.

With pepsin he attributes the abnormalities at the isoelectric point to "adsorption" of the enzyme and states that it is irreversible. He does not give a reason for these statements. The point of great interest is that this adsorption could be prevented by the addition of *dissolved* gelatin to the system. This is just what our theory predicts: as to whether the ratio could be reduced to less than unity, he does not say.

Finally, this adsorption takes place with egg-albumin, freshly precipitated and washed, but not if it is dried for 24 hours at 70°. The adsorption appears again, however, after this albumin stands in water for several days. This suggests that the albumin dissolves but very slowly (or perhaps very rapidly) after heating, so that both s_i and s_o are negligible (or equal).

In conclusion we may say that, not only is the evidence considered not antagonistic to a theory involving the combination of gelatin and similar proteins with enzymes such as trypsin and pepsin, but much of the evidence points to such a combination. Further investigation is necessary to test whether a picture of the type we have suggested is more nearly correct. We propose to consider the other evidence elsewhere.

REFERENCES.

- Cohn (1925). *Physiol. Reviews*, 5, 349.
 Loeb (1922). *Proteins and the Theory of Colloidal Behaviour*. (New York.)
 Northrop (1924, 1). *J. Gen. Physiol.* 6, 337.
 — (1924, 2). *J. Gen. Physiol.* 6, 417.
 — (1925). *J. Gen. Physiol.* 7, 603.