Physiology. — *"On the Movement of Pepsin in a protein-containing or protein-free Gel of Agar-agar"*. By Prof. C. A. PEKELHARING.

(Communicated at the meeting of November 26, 1921).

In an earlier paper [1]) presented to this Academy I dwelt on a peculiar protein obtainable from the gastric mucous membrane, which could be procured in a purer condition from a dog's gastric juice that is neither contaminated with swallowed matter nor with the constituents of the intestinal contents, so that the elementary analysis did not bring forth greater differences than are generally found with purified proteins. This peculiar protein evinced the properties of pepsin in such a marked degree and the digesting power was in different preparations so constant, that I felt justified in supposing that this protein could be the enzyme itself. Subsequent investigations have repeatedly confirmed this view.

However, in discussing the nature of enzymes with our fellow member BEIJERINCK he raised an objection against this conception. According to his experience pepsin, or chymosin (which enzymes I hold to be identical) diffuses in agar-agar about as quickly as albumoses.

This was, indeed, a serious objection. The pepsin, as I prepare it, is split while being rapidly heated in an acid solution to the boiling point, so that albumoses which remain in solution, are liberated, while a considerable precipitate is being formed, from which, on heating with potassium hydrate, part of the sulphur is freed together with substances yielding a biuret-reaction. With acid a precipitate of a new protein can now be obtained from the alkaline fluid, which protein possesses comparatively energetic acid properties and is soluble in alcohol. This pepsin, then, is of a much higher composition than the simple proteins grouped under the name of albumoses. If my conception were correct, pepsin would surely not diffuse so easily as albumose in a gel of agar-agar.

However, I put myself the question whether the movement of the enzyme is indeed to be ascribed entirely to diffusion. Might

[1]) Proceedings of the Meeting of 25 Jan. 1902, p. 450,

there not be another cause, if the pepsin were to find in the gel protein which it could attack?

It has long been known that enzymes are capable of binding other substances, not only substances which can be decomposed under the influence of the enzyme, but also substances of quite a different nature, on which the enzyme does not exert any influence at all. Pepsin e.g. combines not only with proteins but also with carbon.

In a concise review on the nature and the action of enzymes[1]) I have endeavoured to show that this combination is effected in various ways. First of all there is adsorption. If, as in the case of pepsin, the enzyme and the substance bound by it are both colloidal substances and consequently a difference in surface-tension is of little importance for the adsorption, it is especially the difference in the electric charge of the molecules that comes into play. Owing to this the particles of one substance aggregate as closely as possible on the periphery of those of the other substance. In an acid solution pepsin is charged negatively, protein positively.

The compound thus formed is to a large extent independent of the nature of the two substances. Just as finely divided carbon can bind pepsin as well as all sorts of other enzymes, trypsin also combines not only with protein but also with starch and compounds have been obtained of amylase not only with starch, but also with casein. Adsorption promotes the action of the enzymes by increasing the concentration of the substrate in the immediate neighbourhood of the enzyme or the concentration of the enzyme in the immediate environment of the particles of the substrate. This action is, however, only of a promotive character. For a chemical change the enzyme must combine with it in a manner that depends on the molecular constitution of the substrate as well as of the enzyme. As E. FISCHER has put it: the enzyme must fit to the substrate, or what BEYERINCK terms the "zymotele", like a key to a lock. Only when this kind of combination is effected, can the decomposition of the substrate, generally with addition of water, take place. In this process the enzyme is detached from the substrate in order to combine again with other still intact particles of it. Consequently a small amount of the enzyme can continually decompose new particles of the substrate, unless the enzyme itself is destroyed by noxious influences, as e.g. is the case with trypsin by alkaline reaction of the solution, which however aids the action of the enzyme.

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[1]) Some Remarks on Enzymes. Recueil des Trav. Bot. néerl. XVI, 207.

In this connection we are induced to suppose that the particles of the enzyme are continually moving, while incessantly particles of the substrate are being decomposed. So, when a certain amount of pepsin is going to spread into the protein-containing gel through diffusion, the particles of the pepsin will first be bound to the protein-molecules by adsorption, through the difference in the charge. If only adsorption should come into play, a large number of the pepsin-molecules would be detained, without undergoing or causing any change, while the subsequent diffusion would be inhibited rather than accelerated. It makes a great difference, however, if in virtue of their constitution the molecules of the enzyme also grasp the protein-molecules and they attach themselves to new intact protein-molecules with which they come into contact, after the splitting of the protein and, consequently, because free protein-particles are lying on the periphery, they move towards the periphery, and — seemingly — quicken the diffusion.

Now in the experiments which gave rise to BEIJERINCK's objection the agar-gel contained protein. The question, therefore, was, whether in such a gel, ceteris paribus, the spreading of the pepsin would be quicker in the presence of protein than in a gel without protein, in which only true diffusion would take place.

— To my friend and successor Prof. W. E. RINGER I feel greatly indebted for his highly appreciated help in my endeavours to find an answer to this question in his laboratory. The inquiry was conducted as follows:

25 mgrs of purified pepsin from the pig's gastric mucous membrane was put in a test-tube, dissolved at body-temperature in 2,5 cc. 0,2 % HCl, and subsequently 2,5 cc. 3% agar-agar in water was added. By rapid shaking the pepsin was evenly mixed up with the heated agar-agar and immediately after cooled down in melting ice. The small clots of coagulated agar sticking to the wall of the tube consequent on the shaking, were whisked cautiously away and, in order to destroy all the pepsin that might be left behind in the tube above the coagulated column, the tube was filled with 1 % NaHO, then emptied after some moments and washed out a couple of times with water and afterwards with 0,1 % HCl. After this 10 cc. of a mixture of 5 cc. agar-agar 3% and 5 cc. 0,2 % of HCl to which protein was added or was not, was put into the tube. Then the tube was cooled down again in ice. In each experiment four tubes were filled in this way, two with and two without protein. They were then closed with a cork stopper, placed vertically in an incubator that was kept at 27° C.

The amount of pepsin in the lower part of the tube was very considerable (25 mgr.), while 0,1 mgr. of this enzyme in 10 cc. 0,2% HCl dissolves in METT's tubes from 5 to 6 mm. of coagulated white of a hen's egg in 24 hours at 37° C. What was lost of it in mixing the pepsin with the heated agar (which was directly after cooled down in ice) and what was lost in the washing of the tubes with sodium-hydrate, through which of course also a little of the pepsin at the surface of the pepsin-agar column was attacked, could only be very insignificant in relation to that considerable amount of pepsin. It was assumable, therefore, that the concentration of the enzyme in the reservoir sufficed to prevent in the several tubes considerable differences in the degree of the rise of the enzyme in the agar-column above the pepsin-agar.

After a few days every time two tubes were opened, one with and one without protein. To this end a circular incision was made into the glass just on a level with the boundary between the pepsin-agar and the column above it and the glass was broken by touching it with a heated rod. The lower part of the tube could then readily be removed and the whole content be slid out and put on filter-paper.

It might be that the fluid in the capillary spaces between the agar and the glass should have taken up more or less pepsin from the pepsin-agar: a possibility which deserves the more consideration as occasionally it could be observed at the free surface of the column that some fluid had been pressed out, which could dissolve fibrin, though it be in a very small degree. That is why after the reservoir of pepsin had been cut off from the agar-column, this column was immersed for some moments in 1% Na_2CO_3, then washed immediately in 0,1 % HCl and dried by cautiously rolling it along filterpaper.

We now had to determine the level to which the pepsin had penetrated into the agar-column. With a view to this we proceeded as follows: after cutting off a layer of 2 mm. thickness, there where the column had been in direct contact with the pepsin-agar, the column was divided into three cylinders of equal length, mostly 13 mm. in length, sometimes 15, if the diameter of the tube had been somewhat smaller, and if the whole column had consequently been somewhat longer. In this division we started from the bottom, so that the layer nearest to surface could be rejected. The cylinders were weighed, rubbed down in a mortar with 5 cc. 0,1% HCl. For every one of these fluids we now determined the time in which 1 cc. coagulated 5 cc. of milk at 27° C. We ascertained the com-

parative proteolytic power, after the method of GRÜTZNER [1]) by mixing 1 cc. of the fluid with 9 cc. 0,1% HCl to which, at least 10 minutes before, 50 mgrs of finely divided carmin fibrin had been added, then turning the tube once every minute, filtering the contents after a certain time through glasswool and establishing the intensity of its colour with the aid of GRÜTZNER's colorimeter against a solution of carmin fibrin in pepsin hydrochloric acid.

First of all the proceeding of the enzyme was compared in agar-agar with and without fibrin. With a view to this in each of two tubes, containing agar-agar, 10 CC. was added of a mixture of equal portions of 3% agar and 0,2% HCl, and in two others 10 CC. of a mixture of 3% agar and 0,2% HCl with carmin-fibrin that had been rubbed down very finely and had swollen in this acid.

After three days one of each couple of tubes was opened and examined in the manner described. Just as in all the following experiments **I** designates the lowermost cylinder, the one nearest to the pepsin-agar; **II** the one next to it and **III** the topmost cylinder.

The result was to this effect:

	Weight (grms)	Milk clots in	Division mark Colorimeter	Weight (grms)	Milk clots in	Division mark Colorimeter	Weight (grms)	Milk clots in	Division mark Colorimeter
with fibrin	I 2.04	2 min.	1.2	II 2.14	15 min.	0.5	III 2.0	30 min.	not measurable
without »	I 1.8	4 »	1.2	II 1.5	no clotting	0	1.5	no clotting	0
After 6 days the other two tubes were opened. Now we found:									
with fibrin	I 2.48	3½ min.	1.7	II 2.54	10 min.	0.8	III 8.24	90 min.	not measurable
without »	I 2.04	4 »	1.0	II 2.04	no clotting	0	III 2.0	no clotting	0
The 2nd experiment was conducted in the same way. Result after 4 days:									
with fibrin	I 1.80	2 min.	4.1	II 1.95	17 min.	1.1	III 2.00	40 min.	0.5
without »	I 2.10	2½ »	3.1	II 2.00	18 »	0.5	III 2.00	no clotting	not measurable
After 13 days:									
with fibrin	I 1.20	1¾ min.	2.8	II 1.54	4 min.	2.4	III 1.60	17 min.	1.2
without »	I 1.66	2¼ »	2.6	II 1.94	14 »	1.2	III 1.90	60 »	0.1

[1]) Vide GESELSCHAP, Zeitschr. f. Physiol. Chem. XCIV, 205 and Onderz. Physiol. Laborat. Utrecht, 5e R. XVI, 198.

I have desisted from a determination of the absolute pepsin amount of the several columns, though this could be done by comparison with a solution of pepsin of known strength, since the pepsin in each column was not divided evenly, but lessened considerably from the bottom upwards. The values given show distinctly enough, that the enzyme rose in the agar without protein as well as in the agar with protein, but in the latter more considerably. Only after 13 days could it be demonstrated that the enzyme had reached the upper column in the protein-free gel.

As regards the absolute value of the figures it will not do to compare the results obtained on various days, because the milk used was different every time and for comparison in the colorimeter every time another solution of carmin-fibrin was taken.

Similar results were achieved with clotted white of a hen's egg:

White of a hen's egg, diluted with 10 times its volume of water, was beaten up and coagulated by boiling under addition of acetic acid to a very weak acid reaction. The flaky precipitate was filtered off and washed with water. Part of this was put in 0,2 % HCl and evenly distributed in the fluid by rapid shaking. In each of two tubes with pepsin-agar was added 5 CC. of this protein-containing acid, mixed with 5 CC. 3 % agar in two other tubes 5 CC. 3 % agar with 5 CC. 0,2 % HCl.

Two tubes examined after 3 days:

	Weight	Clotting	Colori-meter	Weight	Clotting	Colori-meter	Weight	Clotting	Colori-meter
with protein	I 2.64	1¾ min.	2.2	II 2.56	22 min.	0.4	III 2.35	40 min.	very light red
without »	I 2.70	2 »	2.0	II 2.56	130 »	not measurable	III 2.40	none	0

The second set of two tubes got lost.

In every tube so much hydrochloric acid had been put, that the content of the gel was 0,1 % over the whole tube. Here, however, we had to consider that in the tubes containing the protein, the acid was partly bound, so that the concentration of the H-ions in the agar-protein gel was undoubtedly lower than in the agar gel without protein. The observed differences could, however, hardly be attributed to it. If the movement of the enzyme depended exclusively upon diffusion, it might presumably be promoted by an acid reaction, considering that, during the sojourn of the tubes in an environment of 27° C., the acid attacks and softens the agar. In every experiment therefore, the protein-containing agar was more solid than the protein-

free agar, nay in some experiments the protein-free agar had become so soft that it could not be divided into three small cylinders so that comparison with the protein-containing agar was impossible.

In accordance with this we also found, that the enzyme proceeds in agar with casein more rapidly in acid-, than in neutral reaction.

A 3% neutral solution was made of pure casein prepared after HAMMARSTEN by addition of NaHO. A part of this was diluted with an equal volume of water, another part with an equal volume of 0.4% HCl. The precipitate arising primarily on the addition of hydrochloric acid was dissolved again in the excess of acid.

Of this neutral solution 5 CC was put in two tubes filled with pepsin-agar, and was mixed with 5 CC 3% agar. In two other tubes 5 CC of the acid solution mixed with 5 CC 3% agar.

After 3 days:

	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.
acid	I 2.27	3 min.	4.5	II 2.30	none	0.6	III 2.24	none	not measurable
neutral	I 2.25	2 „	5.6	II 2.30	none	0.3	III 2.30	none	0
After 4 days:									
acid	I 2.32	1¼ min.	4.4	II 2.30	10 min.	1.6	III 2.33	13 min.	1.3
neutral	I 2.30	1½ „	4.2	II 2.33	none	0.3	III 2.30	none	0

In both sets of tubes, then, there was a balance in favour of the acid solution. After 4 days pepsin could even distinctly be observed in the top cylinder of the acid gel.

Also with a neutral reaction the movement of the enzyme through the gel was aided by the presence of protein, as appeared from an experiment with milk.

Of 4 tubes containing pepsin-agar two were supplied with 3 CC milk mixed with 7 CC 3% agar; the other two with 3 CC 1% NaCl mixed with 3 drops of 1% $CaCl_2$ and 7 CC 3% agar.

We observed that also globulin from blood-serum and from edestin had a favourable action on the movement of pepsin through the agar-gel.

Finally I report some more experiments which I carried out to verify the supposition from which I started, viz. that the advance of the pepsin in the protein-containing gel is promoted, because besides

After 3 days:

	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.
with milk	I 2.24	2 min.	3.0	II 2.24	4 min.	2.1 not	III 2.20	9 min.	0.5
without „	I 2.20	4 »	2.1	II 2.40	2¾ hour	measurable	III 2.30	none	0

After 4 days:

with milk	I 2.25	2 min.	1.7	II 2.36	3 min.	1.5	III 2.40	20 min.	0.8
without „	I 2 50	2½ »	1.6	II 2.50	1 hour	0.4	III 2.25	none	not measurable

through adsorption it combines with the protein still in another manner in consequence of the chemical structure of the molecules. When this compound breaks down, in which process the action of the enzyme manifests itself, the liberated enzyme is supposed to attach itself to other still intact protein molecules which are lying on the periphery and to advance in this way in the direction of the diffusion-current. If this supposition is correct, the movement of the pepsin must also be promoted by albumoses, which it is still able to attack; not, however by animo-acid freed from the protein which pepsin cannot attack and which, in contradistinction to albumoses, it cannot grasp[1]) in an electric field.

To this end we mixed, in the manner described above, first, primary and secundary albumoses, prepared by digestion of fibrin with gastric juice, and then a mixture of pure amino-acids approximately in the relation in which they are contained in fibrin, in a solution of 0.2 % HCl, with the same volume of 3 % agar-agar.

The primary albumoses contained a considerable amount of heteroalbumose, the secondary ones were freed as much as possible from primary ones by repeated half-saturation with ammonium-sulfate and by filtration.

Primary albumoses. Two tubes each with 100 mgrs. of albumose, two without albumose, prepared as usual.

It appears then that, while the primary albumoses largely promote the movement of the enzyme, the action of the secondary ones, which are much less attacked by pepsin, though it cannot be entirely denied, is much less significant.

Nothing, however, could be detected of an action of the amino-acids, as is shown by the following experiment:

[1]) Vide RINGER, Zeitschr. f. Physiol. Chem. XCV, 195 etc. Onderz. Physiol. Laborat. Utrecht. 5de R. XVI, 252.

After 3 days:

	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.
with album.	I 2.35	1 min.	3.3	II 2.40	7 min.	2.1	III 2.40	22 min.	1.0
without »	I 2.20	1¼ »	3.2	II 2.20	35 »	1.0	III 2.15	none	0
After 4 days:									
with album.	I 2.05	50 sec.	3.5	II 1.95	2 min.	3.2	III 1.98	4 min.	2.5
without »	I 2.04	1 min.	3.1	II 2.00	6 »	1.2	III 2.08	none	0
Deutero-albumoses, 100 mgrs.									
After 2 days:									
with album.	I 2.40	1¼ min.	5.5	II 2.44	15 min.	2.5	III 2.44	1 hour	1.0
without »	I 2.40	1½ »	4.5	II 2.34	1 hour	1.0	III 2.30	2 hrs	0.6
After 3 days:									
with album.	I 2.24	1 min.	3.0	II 2.10	2½ hour	0.5	III 2.14	none	0
without »	I 2.20	1¼ »	3.0	II 2.34	none	0.2	III 2.20	none	0

The solution contained in 11 CC 0.2 % HCl, 75 mgrs. tryptophan, 7.5 mgrs. of cystin, 40 mgrs. of histidin, 70 mgrs. of tyrosin and 30 mgrs. of alanin. On heating to 40° C. the solution was almost clear. Of this solution 5 cc. and 5 cc. of 3 % agar was put in each of 2 tubes. In the other 2 tubes 5 cc. of 0.2 % HCl was put, together with 5 cc. of agar.

In the tube heated for 4 days at 27° C. the gel without amino-acids, which, therefore, had been more exposed to the action of the acid, was very soft. Perhaps it is owing to this that the pepsin has penetrated farther than is generally the case in the agar without protein.

It might be surmised, that from the experiments described it does not even follow that pepsin is indeed competent to diffuse in pure agar, seeing that a gel of this agar prepared in the usual way, will always contain nitrogenous substances, which may belong to the group of proteins. I believe this is an unjustifiable assumption. It is difficult to ascertain whether the agar-gel or sol contains protein' because sensitive reactions on protein cannot be successfully applied

After 3 days:

	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.	Weight	Clotting	Colorim.
with am.-acids	I 2.27	1 min.	5.5	II 2.28	22 min.	0.5	III 2.80	none	0
without »	I 2.10	2½ »	3.1	II 2.20	none	0	III 2.16	none	0
After 4 days:									
with am.-acids	I 2.35	1½ »	3.5	II 2.30	2 hour	0.3	III 2.30	none	0
without »	I 2.16	1 »	4.5	II 1.85	8 min.	1.7	III 1.70	25 min.	0.8

here, owing to the dark colour caused by the action of strong mineral acids on the carbonhydrate. It is possible, however, to remove the greater part of the nitrogenous substances by warming the agarsol during 24 hours at about 50° C. The nitrogenous substances will then separate in flakes, so that they can be filtered off. In this way I obtained from an agarsol a sol which was scarcely opalescent and remained almost clear also after the clotting. The agarsol had been prepared in the usual way by warming it just sufficiently and then filtering it through cottonwool. It contained 1.6 % N of the solid substance. In the sol there was only 0.39 % N after heating during 24 hours and filtering through compressed paper pulp at about 50° C. This gel was now compared in the usual way with the one that had been filtered only once, to the effect that there was no difference to be observed in the advance of the pepsin. In the gel containing only very little N we could make out in the lowermost cylinder, which had been situated at a few millimeters distance from the pepsin-agar, as much enzyme as in the gel which contained four times that quantity of nitrogen.

That pepsin had no doubt advanced through diffusion. But this movement is very slow.

Whereas after a few days a rather considerable amount of pepsin has penetraded from the reservoir at the lower portion of the tube into the adjoining agar, there is none or hardly any to be made out at a few centimeters distance, anyhow, if the gel has preserved its compactness. If, however, the gel contains protein, the enzyme has proceeded much further in the same space of time.

In my opinion the foregoing warrants the conclusion that the movement of pepsin through a gel which contains protein it is able

to attack, does not entitle us to doubt that the size of the pepsin-molecule is as great as previous observations have assigned to it.

On the other hand it seems to me, that the bearing of protein on the movement of pepsin through a gel, favours the hypothesis that the combination of an enzyme with the "zymotele" is not to be ascribed only to adsorption, but also to a totally different action depending on the structure of the molecules.

On starting this inquiry I purposed to extend it in various directions and over more enzymes, notably invertin and emulsin, which can attack various carbonhydrates of known structure. But I understand that my time for Laboratory work is passed. I must now leave this to younger workers who consider this subject interesting enough to investigate it further in the indicated direction or in their own way.