Comparative Physiology. — *The presence of α- and β-amylase in the saliva of man and in the digestive juice of Helix pomatia.* I. By L. ANKER and H. J. VONK. (From the laboratory of Comparative Physiology, University of Utrecht.) (Communicated by Prof. G. KREDIET.)

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In 1889 WIJSMAN showed that malt-extract contains two enzymes causing cleavage of starch. These are now called α-amylase and β-amylase, according to KUHN. These names were introduced by KUHN, because with the activity of α-amylase the maltose first is formed in the α-form, with that of the β-amylase in the β-form [1]). We know chiefly also through WIJSMAN that with the activity of α-amylase the residual product is not coloured by iodine, while with that of β-amylase the residual product is coloured with iodine. For the separation of these enzymes WIJSMAN used the separation-method of two substances discovered by BEIJERINCK, by means of a gelatine-plate often used in bacteriology. When he brought a drop of malt-extract on such a plate which contained soluble starch, after a few days, by colouring with a solution of iodine in potassium-iodide, he found at the place of the drop an uncoloured field surrounded by a purple ring, round which a very narrow clear-blue ring formed the transition to the dark blue colour of the rest of the medium. This observation led him to suppose that at the height of the purple ring, by a quicker diffusion, there was only one enzyme, which decomposes starch, not only into maltose, but also into a product that still gives a purple colour with iodine. He proved this, because part of this ring not yet coloured with iodine, transferred to another plate, there only gave a diffusion-field coloured purple by iodine. This enzyme is the β-amylase and the coloured disintegration-product is erythro-granulose which is decomposed further by α-amylase into products (chiefly maltose) that can no longer be coloured with iodine.

In the same way in 1934 GIESBERGER examined, among other things, the saliva of man. He found a weak purple ring round the uncoloured field and thought that by this he had demonstrated the presence of β-amylase beside that of the α-amylase, chiefly present (as appeared from the big uncoloured field). However he could not carry out the critical experiment, the transferring of part of the ring to another plate, the ring being too narrow for this purpose.

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[1]) This is shown with the polarimeter. After some time the normal equilibrium mixture of 36 % α-maltose and 64 % β-maltose arises. KUHN found that pancreasamylase (at least, chiefly) is an α-amylase. Some experiments with the polarimetric method will be discussed in a following communication.

In the same year, however, PURR, with a method which makes use of the connection between the degree of saccharification and the colouring with iodine, confirmed the current opinion that saliva-amylase is the purest α-type which is known; in this investigation he also showed that pancreas-juice of the pig contains β-amylase, but in an inactive form, which he could activate with vitamin C.

In the following year GIESBERGER published a rectification to his work of 1934. For when repeating the diffusion-experiments, he had only succeeded a few times in getting a purple ring. In these cases also the starch in the rest of the plate was not coloured dark-blue, but purple-blue. In most of the cases he found a clear blue ring round the colourless diffusion-field. Because of these new results he thought he could not maintain his original statement that saliva-amylase is a mixture of two enzymes. The clear blue ring would mean that as a consequence of a weak enzyme-concentration, unchanged starch is still to be found there, while the purple ring would point to a product of disintegration already present in the starch and not to an activity of β-amylase from the saliva.

That this ultimate conclusion is insufficiently founded may appear from the following theoretical considerations and from experiments taken to test them.

GIESBERGER does not mention a p_H at which he made his experiments, so that we may suppose that he worked at the acidity of the gelatine-starch plate. In such weakly acid surroundings (as appeared from our measurements 5,8) according to the p_H-optimumcurves of OHLSSON and those of VAN KLINKENBERG, the activity of β-malt-amylase has already been reduced by the p_H which is high for this enzyme, while α-malt-amylase acts about optimally here [2]). For the amylases of pancreas-juice and saliva (the former of which is doubtless and the latter probably α-amylase) the optimum lies between p_H 6.2 and 6.8 (dependent on the buffer used). Though the situation of the p_H-optima of enzymes is influenced by additional mixtures (the optimum for saliva-amylase does lie a little higher than for that of malt), it is not unreasonable to suppose that also for a β-amylase if present in the saliva or the pancreas-juice, the optimum will lie lower than that of the α-amylase. If this supposition is right, we may expect that under the circumstances of GIESBERGER's experiments the activity of a β-amylase if present in a very small quantity, becomes less clearly visible; for the few residual products of this disintegration that can be coloured with iodine will be changed relatively quickly into the products that are not coloured any more, by the α-amylase.

A comparison of the p_H-optimumcurves of α- and β-amylases will no more make us expect a purple ring at a higher p_H. On the other hand, the possibility of discovering β-amylase in the saliva at p_H-values lower than

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[2]) The optimum for α-malt-amylase lies at p_H 5.75, that for β-malt-amylase at p_H 4.9. For the former enzyme the optimum-curve runs steeply upward, for the latter it is flatter.

those of an unbuffered gelatine-plate, increases, in the first place because this enzyme becomes then more active and secondly because the resulting residual products will be disintegrated more slowly by α-amylase. Anyhow, it is wrong to conclude that β-amylase is absent, if the p_H was not sufficiently varied when the experiments were made.

The following experiments confirm that these expectations are reasonable (Table I). The experiments 1—3 of this table are repetitions of those made by GIESBERGER. The plates [3]) remained resp. 2, 3 and 4 days in the thermostat (we shall say more presently about the importance of this indication of time). In these experiments the purple ring was also absent; the white diffusion-field was surrounded by a clear blue ring. By this a reproduction of GIESBERGER's last results was obtained. In experiment 4 a weak NaOH-solution was added to the saliva and in experiment 5 a weak HCl-solution. In the letter case the colourless field was indeed surrounded by a narrow purple ring. Round this there lay a clear blue ring, about as broad, the presence of which can also be known in advance, as the residual products formed when the β-amylatic disintegration takes place can be coloured blue with iodine a considerable time after the beginning of the enzyme-activity (SAMEC, HANES) and only become purple via blue-violet and violet towards the time that the disintegration-limit is reached. So this result of experiment 5 is a first argument for β-amylase being present in the saliva. In order to be as certain as possible that this β-amylase did not come from food-remnants or other pollutions of the mouth we always used saliva which had been produced before breakfast after thorough cleansing of the mouth-cavity.

TABLE I.

Experiment	Digestive juice	Diffusiontime in days	Result
1	Saliva	2	—
2	„	3	—
3	„	4	—
4	„ with NaOH	2	—
5	„ „ HCl	2	+

GIESBERGER is right in not ascribing the purple ring found by him in some cases, to the activity of β-amylase, because in those cases the unchanged soluble starch also had a purple-blue colour in the plate, which might point to disintegration-products already being present. But he is wrong in taking the blue ring for the place where there is starch still unchanged as a consequence of a weak concentration of α-amylase. For if one lets the diffusion-experiments take place on an alkaline medium (at p_H 8

[3]) They contain 8 % gelatine (powder-gelatine Twee Torens, Delft) and 0.25 % soluble starch (amylum solubile of KAHLBAUM) without addition of buffer. For the preparation cf. the dissertation of VAN KLINKENBERG.

to 9) the uncoloured field is immediately surrounded by the blue of the unchanged starch.

It follows from this that α-amylase by itself (the β-amylase of saliva is probably also inactive at this p_H), does not cause a clear blue ring round the uncoloured diffusion-field; in other words the blue ring is an indicator for the presence of β-amylase.

This inequality of the diffusion-field appearing after colouring with iodine, which must be based on the fact that the α- and the β-amylase affect the starch-molecule differently, is in complete accordance with the characteristics that have been given of these enzymatic changes. The α-amylatic disintegration is characterised by a quickly disappearing colouring with iodine, because according to modern views this enzyme causes the starch-molecule to break up into fragments that can no longer be coloured, already in the first stage of the development. With the β-amylatic disintegration the colouring blue with iodine continues to exist a long time and only changes into purple when the disintegration-limit is reached. For this enzyme separates one maltose-molecule each time from the extremities of the starch-molecule, so that the disintegration is more gradual than that caused by the α-amylase.

WIJSMAN also mentions a blue ring round the purple one. In view of what was known at that time of enzymatic starch-disintegration it can be understood that he takes this ring for the place where the amylum, by the β-amylase, is made more suitable to be coloured by iodine.

At present, according to us, the most likely explanation of the phenomena is this:

a) a colourless diffusion-field, surrounded immediately by the blue of the unaffected starch, points to exclusive presence or activity of α-amylase,

b) a colourless diffusion-field, surrounded by a clear blue ring, points to a low concentration or little activity of β-amylase, present in the solution of α-amylase.

c) a colourless diffusion-field, surrounded by a purple and a blue ring, points to a higher concentration or a higher activity of β-amylase present in the solution of α-amylase.

In order to determine the p_H where the ring is clearest the experiments 6—17 were made (Table II) [4]). When in the following we speak of a ring, we mean by this the purple ring. From this series of experiments appears that the ring is clearest at $p_H = 4{,}5$—$4{,}7$ both for saliva and for the digestive juice of HELIX.

This confirms the supposition expressed above that β-amylase will be demonstrated best at a weakly acid reaction.

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[4]) The plates contain 8 % gelatine and 0.25 % soluble starch as in the experiments of table I. They were here buffered with potassium-biphtalate and NaOH (according to CLARK and LUBS) in various proportions.

A factor which may have a considerable influence on the results of the experiments is the time during which one allows the diffusion to proceed. In order to get the rings sharply divided from one another, it appeared necessary to let the diffusion take place at a low temperature (in a thermostat at 3° C) viz. during two days. Also at room-temperature one can make the phenomenon appear, but then iodine should be added sooner, as the diffusion takes a quicker course at a higher temperature. It is easy to see that when the period of diffusion too short, there is no sufficient differentiation. But also if one makes the period too long, the phenomenon becomes unclear. This is illustrated by the experiments 6—11 of Table II. It follows,

TABLE II.

Experiment	Digestive juice	Diffusiontime in days	Results					
			3.8	4.5	4.7	4.9	5.1	5.3
6	Saliva	2		+	+	+?	+?	—
7	„	3		+?	+?	—	—	—
8	„	4		+?	+?	—	—	—
9	dig. juice Helix	2		++	++	++	++	+?
10	„	3		+	+	+	+	+?
11	„	4		+	+	+	+	+?
12	„	2	+		++			
13	„	2	+		++		+	
14	„	2		++			++	
15	„	2		++	+	+	—	
16	Saliva with 60 % aethanol	2	+	+	+?			
17	Saliva (filtered)	2	+?	+	—			

— = no purple ring.
\+ = purple ring.
++ = broad purple ring.
+? = hardly visible purple ring.

among other things, from these experiments

a) that the diffusion at 3° C should not be allowed to take longer than two days,

b) that the digestive juice of *Helix pomatia* gives a clearer blue ring than the saliva of man, from which follows that the former probably contains more β-amylase.

When these experiments were carried out, care was taken that the gelatine-plates were of exactly the same thickness (equally large Petri-dishes with an equal number of cm^3 of gelatine). As it was often very difficult to judge the breadth of the ring (so that mostly we had our observations checked by two persons), it is important to compare the rings at different p_H-values only when this requirement that the plates are equally thick, is met. The p_H values were measured with the Hellige-comparator. In the whole studied p_H-region the degree of acidity of liquid gelatine

appeared to lie 0,2 higher than that of solid gelatine. The influence of saliva and digestive juice of Helix (the p_H of which appeared to lie resp. at 6,8 and 5,3) on the degree of acidity of the plates was not examined.

In view of the results of PURR, who, it appeared, could activate the β-amylase from the pancreas of pigs by the addition of vitamin C, we examined what influence this vitamin has on the ring. A tablet of "Cebion" of E. MERCK, Darmstadt, was dissolved in 2, 4 and 6 cm^3 of water. Such a tablet contains 0,05 gr. of crystalline ascorbic acid. To one drop of saliva one drop of vitamin-solution was added. In all the three cases the ring was clearer than in the controls. If the vitamin C was brought into the gelatine-media in equally strong concentrations, it appeared to have no influence. These experiments took place at p_H 4,5. In an experiment following upon this at p_H 6,4 a broad purple-blue ring arose with gradual transitions, both in- and outside. No opinion can as yet be offered on the interpretation of this phenomenon.

Finally, in imitation of WIJSMAN, the enzyme-solution (the saliva) was heated for *ten* minutes at 70° C in order to render (analogous to WIJSMAN's experiments) the β-amylase, if present, inactive. It appeared that the activity of the α-amylase was also so strongly reduced by this treatment that a light-blue field at the place of the drop was the only visible result. The diffusion-field of a saliva-solution heated for *five* minutes appeared to agree much with that on an alkaline medium (so only activity of α-amylase). This is a new argument for the opinion, formed because of the diffusion-experiments, that in the saliva beside the α-amylase a β-amylase is present, the latter of which is rendered inactive by 5 minutes' heating, while the former remains intact. The transition between the colourless field and the blue of the uneffected starch was somewhat less sharp than in the experiments on an alkaline plate, but there was hardly any blue ring left.

The facts stated in the preceding experiments form together a very strong indication for the presence of a small quantity of β-amylase in the saliva.

Summary.

According to the diffusion-method introduced by WIJSMAN in enzymology we examined whether β-amylase was present in the saliva of man and in digestive juice of *Helix pomatia*. The following results:

1) the presence of a purple and a clear-blue ring round the colourless diffusion-field in acid surroundings (optimal at $p_H = 4,5$) and their absence in alkaline surroundings,

2) the broadening of the purple ring by the addition of vitamin C,

3) the absence of the rings after 3 minutes' heating at 70° are all in favour of the supposition that β-amylase occurs beside the chiefly present α-amylase in the saliva of man, while a corresponding, even clearer diffusion-picture at a lower p_H points to the presence of β-amylase beside the chiefly present α-amylase in the digestive juice of *Helix pomatia*.

The presence of β-amylase in the digestive juices can have a certain biological significance. For the starch is decomposed further and especially more quickly by the α-amylase, which is chiefly present, in the presence of β-amylase. The activity of vitamin C to render β-amylase active might play a part here, as vitamin C is always present in the intestinal canal of herbivorous animals.

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