VI. STRUCTURE AND ENZYME REACTIONS.

III. THE SYSTEM POLYSACCHARIDE-AMYLASE-PROTEIN.

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IN Part II of this series [Przylecki, Niedzwiecka and Majewski, 1927] it was shown that amylase adsorbed upon charcoal has a considerably weaker hydrolytic action than in the absence of the adsorbent; this was explained as being due to the spatial separation of the substrate from the enzyme by fixation on different parts of the adsorbent surface. This model is, however, but a crude imitation of biological conditions, and can serve only as an illustration of the above phenomenon of spatial separation, and as a basis for further research upon the physiology of enzyme reactions.

In this paper only hydrolases of the amylase group will be considered, with the object of establishing that conditions may exist within the living organism which would allow of the spatial delimitation of the enzyme from its substrate. Further, the consequences of the existence of such conditions will be considered.

Lesser explains differences in the kinetics of amylase reactions due to the addition of various substances as being caused by the latter influencing the state or degree of adsorption of the enzyme. In this way he explains the action of adrenaline, and also the influence of alcohol upon the secretion of ptyalin by the submaxillary glands.

As far as the action of adrenaline is concerned, Lesser's [1920] experiments are not entirely satisfactory, since they were carried out upon a perfused liver, in which differences in the permeability of the cell walls might easily take place. Further, other authors have been unable to confirm his results.

The action of alcohol upon the submaxillary glands, causing augmented secretion of amylase, has, on the other hand, been established beyond doubt [Lesser, 1927], but this case is not in any way comparable with conditions such as obtain in the liver, muscles and other organs, in which the ferment is not secreted externally but is used exclusively intracellularly. The conclusions drawn by Lesser from this research cannot, therefore, be applied to organs other than those of external secretion.

No researches have up to the present been undertaken with a view to the elucidation of this problem in organs which metabolise carbohydrates intracellularly, and it is our opinion that this problem should be first investigated upon models before commencing complicated *in vivo* experiments, in which it is difficult to establish the mechanism of the reactions and to be aware of all the factors entering into play. The component parts of these models should be as far as possible identical with those of the living organism.

The first step towards the realisation of these models was to ascertain upon which substances enzyme and substrate could be adsorbed. The spatial delimitation mentioned previously may be greater within the organism as a result of specific differences in adsorption; thus the enzyme and substrate may be adsorbed upon different substances. The possibility of the adsorption of the enzyme is supported by the researches of Hedin [1915], of Starkenstein [1910], who demonstrated the adsorption of amylase by liver components, and of Chrzaszcz and Goralowna [1925] who showed that milk amylase is adsorbed on the fat globules. We have, however, been unable to find any systematic researches on this subject, or upon the similar problem of adsorption of polysaccharides.

It was necessary to find under which conditions and with what facility elution of the adsorbed enzyme and substrate could take place. Further, the degree of activity of the adsorbed enzyme had to be determined and, finally, the kinetics of hydrolysis in the presence of different adsorbents had to be investigated.

All the experiments described in this paper were conducted upon amylase and glycogen or starch. As adsorbents proteins such as egg-white or caseinogen were taken, and minced liver, which is mainly protein, was also used.

EXPERIMENTAL.

Przylecki's [1918] modification of Pflüger's method for the determination of small quantities (0.2–2.0 mg.) of glycogen or starch was used. A given volume of 1–2 % polysaccharide was shaken for from 2 to 4 hours with a fixed quantity of protein, after which the mixture was filtered. The filtrate was treated with two volumes of 60 % caustic potash, and further treated as for glycogen.

Adsorbed amylase was determined in the following way. Two equal portions of a solution of amylase in 1 % sodium chloride were taken; to one protein was added, and to the other an equal volume of water. Both portions were then shaken for from 2 to 4 hours, the containers being as far as possible completely filled, in order to avoid foaming. Both portions were then filtered, and starch solution was added to equal volumes of the filtrates, which were then placed in a thermostat at 37° for 6–12 hours, when the quantity of reducing sugars formed was determined by Bertrand's method.

For the determination of the velocity of reaction in the presence of

adsorbent, care had to be taken that, before glucose was determined, the protein was completely precipitated. This was done by adding an equal volume of absolute alcohol, and filtering after 4 hours, the residue of protein being then washed three times with 50 cc. of absolute alcohol. The united filtrates were then concentrated to 20–40 cc., *i.e.* until all alcohol had been driven off, made up to a known volume and their glucose content determined by Bertrand's method. In some cases protein was precipitated by Michaelis's method, using colloidal ferric hydroxide; precipitation by means of sodium tungstate and sulphuric acid was also applied. The coagulated egg-white used was in all cases washed for 2 days in running water, in order as far as possible to remove glucoproteins, the prosthetic group of which might interfere with sugar determinations.

As Pringsheim and Winter [1927] have shown that proteins may combine with carbohydrates, an equal portion of protein was, at the end of every experiment, added to those portions to which it had not been added at the beginning, thus minimising this possible source of error.

All experiments were carried out at the same p_H .

RESULTS.

I. Adsorption of substrate.

Glycogen and starch were taken as the substrates, which were adsorbed upon egg-white, caseinogen or liver coagulum (horse or ox). The dispersing medium was either 1 % sodium chloride solution, or the same with the addition of propyl or butyl alcohol.

10 g. of powdered caseinogen adsorbs 20 % of the starch from 50 cc. of a 1 % solution of the latter. Alcohols considerably reduce adsorption, and elute already adsorbed starch, so that in their presence adsorption attains barely 5–7 % (Table I).

Table I. *Adsorption (%) of polysaccharides on caseinogen.*

No. of determinations	Adsorption		Adsorption in			
	Mean	Limits	0.45 M propyl alcohol		0.075 M butyl alcohol	
			Mean	Limits	Mean	Limits
10	20	11.7–32.1	6.5	4.9–7.4	7.1	5.1–7.6

Table II. *Adsorption (%) of polysaccharides on coagulated egg-white.*

Substrate	No. of deter- mina- tions	Quantity of egg- white g.	Adsorption		Adsorption with			
			Mean	Limits	Propyl alcohol		Butyl alcohol	
					Mean	Limits	Mean	Limits
Glycogen	6	10	31.5	27.8–34.3	12.9	11.8–19.1	12.6	11.0–19.5
Starch	10	10	34.0	29.7–38.9	14.1	12.3–21.6	14.7	11.8–21.0
"	6	20	55.0	49.8–57.1	—	—	—	—

10 g. of coagulated egg-white adsorbs from 30 to 40 % of the starch from 50 cc. of its 1 % solution, whilst in the presence of alcohols equilibrium is attained at from 10 to 20 % (Table II). The values obtained using various concentrations of butyl and propyl alcohols (Table III) are of particular importance, since the curves obtained by plotting these values are of the adsorption type, showing that in these phenomena Traube's law is followed. The action of alcohols may therefore be explained as being due to elution, caused by diminution of the adsorbent surface of the protein, which preferentially adsorbs alcohols. Starch and glycogen gave identical results.

Table III. *Elutive action of alcohols.*

Alcohol	Concentration of alcohol	% eluted starch
<i>n</i> -Propyl	0.225 %	41
"	0.450 %	59
<i>n</i> -Butyl	0.038 %	30
"	0.075 %	57

50 cc. of a suspension containing 10 g. of liver coagulum was mixed with an equal volume of 1 % starch solution. Under these conditions 50-70 % of the starch is adsorbed, so that after 2-4 hours the concentration of the latter falls to 0.25-0.45 % (Table IV). In the presence of alcohols adsorption is greatly diminished, and here, too, alcohols have an intense elutive effect upon adsorbed starch, the concentration of which falls from 0.5-0.7 % to 0.2-0.4 %.

Table IV. *Adsorption (%) on liver coagulum.*

Substrate	No. of determinations	Quantity of coagulum taken g.	In aqueous solution		With propyl alcohol		With butyl alcohol	
			Mean	Limits	Mean	Limits	Mean	Limits
2 % glycogen	6	20	74.1	70.3-80.1	31.6	23.3-50.8	30.9	26.1-49.9
1 % starch	10	10	71.3	50.3-74.6	29.9	21.2-40.0	28.1	20.8-39.4

Similar experiments upon glycogen gave identical results. 25 g. of liver coagulum in a volume of 50 cc. adsorbs 70-80 % of a 2 % solution of glycogen; in the presence of alcohols this value falls to 23-50 % (Table IV).

The concentration of free starch, on the addition of quantities of alcohol such as were used by Lesser (0.47 *M* propyl alcohol, 0.075 *M* butyl alcohol) rises by 260 % of that present in solutions containing protein.

II. *Adsorption of enzyme.*

A solution of purified pancreatic amylase was divided into two equal portions. To one was added 20 cc. 1 % sodium chloride solution, and to the other 20 g. of coagulated egg-white. After 2-4 hours, both portions were filtered and 20 cc. of 2 % starch solution were added to equal volumes of the filtrates, which were placed in a thermostat at 37°, and the reducing sugars were then determined. The results, given in Table V, show that 70-95 % of the amylase is adsorbed under these conditions. Alcohols only affect this adsorption to a negligible extent, the maximum elution found being only 5 %.

Table V. *Adsorption (%) of amylase on egg-white.*

No. of determinations	In aqueous solution		With propyl alcohol	With butyl alcohol
	Mean	Limits	Mean	Mean
8	79.0	70.5-95.3	74.2	75.3

Liver coagulum was prepared in the following way. Liver was passed through a mincing machine, and for every 100 g. of pulp were added 60-150 cc. of glycerol, 50 cc. of chloroform and 500 cc. of water. The whole was left for 2 days, shaking every 12 hours, after which a further 500 cc. of water were added, and the whole was filtered. The filtrate was placed in a flask plugged with cotton-wool, and left at 37° for 4 days; then for 10 days at room temperature, after which the supernatant fluid had separated from the coagulum which had formed. Amylase was found in both solution and coagulum, the partition being 20-40 % and 60-80 % respectively. Amylase could not be removed from the coagulum by washing, as after prolonged washing the filtrates no longer contained amylase, which was still present in the residue (Table VI) and which remained active even after 10 months. A number of experiments carried out upon the coagulum show that the addition of alcohols leads to only a negligible elution of amylase, barely 3-8 % of which passes into solution.

Table VI. *Amylolytic action.*

Mg. starch decomposed in 24 hours.

No. of determinations	By ppt.		By filtrate in aqueous solution	With alcohols	
	Mean	Limits	Mean	Mean	Limits
5	14.1	11.9-17.4	0	0.75	0.47-0.92

It appears from the above that the passage of a protein from sol to gel may lead to corresponding changes in the dispersion of enzymes present, which, previously uniformly distributed throughout the medium, are now adsorbed upon the protein gel.

Pancreatic amylase was now added to such carbohydrate-free liver coagulum, the whole shaken and filtered after 2-6 hours. Simultaneously the same quantity of amylase as before was added to an equal volume of water, this portion serving as a control. Under such conditions from 50-95 % of the added amylase, according to its concentration, underwent adsorption. This adsorbed amylase was not elutable by alcohols, ether or chloroform to an extent of more than 3-8 % (cf. Table VII).

Table VII. *Percentage of amylase adsorbed upon coagulum.*

No. of determinations	Aqueous solution		With butyl alcohol	
	Mean	Limits	Mean	Limits
5	61.0	59.0-75.6	58.1	54.7-70.2

Similarly, glycogen causes a negligible elution of amylase from egg-white or from liver coagulum (Table VIII).

Table VIII. *Elutive action of glycogen.*

No. of determinations	% adsorption of amylase.			
	Filtrate without glycogen		Coagulum + glycogen, filtered	
	Mean	Limits	Mean	Limits
3	48.1	39.9-50.3	46.3	36.8-49.2

III. *Influence of protein upon the velocity of hydrolysis of carbohydrates.*

If the velocities of enzymic hydrolysis of starch in aqueous solution in the presence and absence of coagulated egg-white be compared, it is apparent that the protein retards the velocity of reaction, and experiments made in the presence of buffer solutions show that the observed differences cannot be ascribed to differences in p_H .

Table IX shows that the percentage retardation increases with the degree of adsorption. Hence it follows that, in systems containing protein, on which the whole of the amylase present is adsorbed, the velocity of hydrolysis of polysaccharides is a function of the concentration of unadsorbed starch or glycogen, the adsorbed portion of the latter substances taking no part in the reaction.

 Table IX. *Influence of coagulated egg-white upon action of amylase.*

No. of determinations	Quantity of protein g.	Mean values.		Decrease in hydro- lysis in % of that in absence of protein
		% adsorbed starch	Mg. starch hydrolysed	
6	0	0	76.2	0
6	10	34	51.8	32
6	20	55	35.4	53

If curves be plotted representing velocity of hydrolysis with and without protein, it will be seen that, while in the latter case the velocity of reaction diminishes with time, the curve in the former case is much closer to a straight line. This difference is probably due to the more constant concentration of starch in the presence of the adsorbent, which gives up adsorbed starch to the solution as fast as it is there used up to form glucose. This phenomenon will, however, be more closely investigated in a future research.

Taking into consideration that no elution of enzyme takes place, we see that the addition of alcohols to systems containing coagulated egg-white leads to an acceleration of reaction proportional to the degree of elution of adsorbed polysaccharide. This is well illustrated in Table X from which curves may be constructed representing percentage elution and acceleration of reaction: these curves are very nearly coincident.

Identical results were obtained with liver coagulum and with coagulated egg-white.

Table X. *Action of alcohols.*

% retardation of reaction with respect to that in aqueous solution without protein.
Same with alcohols

No. of deter- mina- tions	Aqueous solution with protein		Propyl alcohol				Butyl alcohol			
			0.225 M		0.450 M		0.038 M		0.075 M	
	% retarda- tion	Mg. starch decom- posed	% retarda- tion	Mg. starch decom- posed	% retarda- tion	Mg. starch decom- posed	% retarda- tion	Mg. starch decom- posed	% retarda- tion	Mg. starch decom- posed
5	32	51.8	18.8	61.7	13.1	66.1	22.4	59.0	13.7	65.5

If fresh egg-white be taken, the effect is quite different from that of coagulated albumin. Scarcely any retardation of reaction is here observed, and the addition of small quantities of alcohols has no accelerative effect upon the velocity of hydrolysis (Table XI).

Table XI. *Influence of uncoagulated egg-white upon the action of amylase.*

Mg. starch decomposed in 50 cc. reaction solution containing 5 or 10 cc. egg-white solution, 1 % starch and 0.5 % amylase solution, in 0.5 % NaCl. Kept 24 hours at 37°.

No. of deter- mina- tions	Without egg-white		5 cc. egg-white		Same with propyl alcohol		10 cc. egg-white		Same with butyl alcohol	
	Mean	Limits	Mean	Limits	Mean	Limits	Mean	Limits	Mean	Limits
	6	68.3	62.5-71.9	66.8	62.2-73.4	67.0	63.1-69.8	65.4	61.9-70.6	66.1

DISCUSSION.

The above results show that the velocity of reaction in the system polysaccharide-amylase-protein gel is regulated exclusively by the concentration of unadsorbed substrate. It is a matter of the greatest interest that uncoagulated egg-white present as a sol in the above system does not to any appreciable extent retard the hydrolysis.

It would follow from this that the retardation of reaction depends upon the state of dispersion of the adsorbed enzyme. This may be due either to the non-adsorption by protein sols of the enzyme or substrate or of both, or it may be that adsorption takes place, but does not always retard reaction. In order that reaction should be retarded, the number of collisions between enzyme and substrate must be diminished. An active adsorbed enzyme may combine with the substrate as easily as when unadsorbed: in solution, therefore, in spite of both or either of the constituents, *i.e.* enzyme and substrate, being adsorbed, the number of collisions taking place in a given time need not necessarily be diminished. Such a diminution could take place if the state of the adsorbent changes from that of a sol to that of a suspension. Passage from the liquid to the solid phase and changes in the size of the particles of the adsorbent may also influence the reaction.

This problem is now being investigated in our laboratory.

Adsorption of amylase upon protein does not affect its activity, while the action of alcohols is confined almost exclusively to elution of the polysaccharide, that of the enzyme being negligible.

These results are identical with those obtained using charcoal as the adsorbent (Part II).

Alcohols produce elution of polysaccharides, and are, in all probability themselves strongly adsorbed, this adsorption following Traube's law. It is therefore extremely probable that the accumulation of lipin-soluble narcotics in the cells is due not only to their collection on lipin surfaces but also to their adsorption upon protein surfaces.

From the above data, obtained exclusively from biological material, similar to that of which cells are composed, the following conclusions may be drawn. Starch and glycogen, present normally in cells in small quantities, are mainly adsorbed upon proteins, which are present to the extent of 10 % in the cell fluid. The concentration of these polysaccharides in the solvent itself is probably less than 20 % of their total amount. This estimate of 20 % may in all probability be still further reduced, if the presence of colloidal adsorbents other than proteins be taken into consideration.

Whilst this view is not universally accepted, yet it would appear that the concentration of unadsorbed amylase in the cell must be almost negligible, as is shown by Lesser's observations as well as by our observation that, where concentrations of amylase and of protein comparable to those of the cell are taken, over 90 % of the enzyme undergoes adsorption.

Under such conditions, changes in the velocity of hydrolysis of polysaccharides may be ascribed to elution of substrate, of enzyme, or of both, to the release of the enzyme or substrate from some part of the cell where it is not free to enter into reaction, or to changes in the state of dispersion of the protein. The last mentioned factor, without any changes in the degree of adsorption of the constituents, may, *in vivo*, appreciably affect the kinetics of the reaction.

It should also be borne in mind that all the above factors may exert their influence simultaneously, and that, in all attempts at the explanation of the reasons for differences in the velocity of the biological hydrolysis of polysaccharides they must be taken into consideration, as well as other factors which catalyse reaction in homogeneous systems, such as hydrogen and other ions.

Further, if the reversibility of the reaction glycogen $\rightarrow$ glucose be accepted, the degree of hydration of the sphere of action of the enzyme must be an important factor in the reaction. It is very probable that the degree of hydration of the absorbent decides the direction of enzymic reaction; should this be the case, this would be a further factor influencing the velocity of hydrolysis without affecting the degree of adsorption. In this connection, it is of interest that, according to Chick [1914], 1 g. of protein may bind as much as 2.1 g. of water, and it is conceivable that, within the cell, salts or other substances may affect this degree of hydration.

The acceleration of the velocity of hydrolysis caused by the addition of narcotics such as chloroform, ether or alcohols might on the basis of this

research be explained as being due exclusively to elution of polysaccharide. While such an explanation appears to fit in very well with experimental findings, it should not be forgotten that in our case we are dealing with a system simpler than that present within the cell, as it contains only one of the components of the latter, namely, protein. The possibility remains that within the cell the enzyme is adsorbed not only on its protein elements, but also upon lipin or nucleoprotein surfaces, from which elution by narcotics may be much greater than from proteins.

Dauwe [1905] has shown that pepsin, rennin and β -glucosidase may be adsorbed upon cholesterol and lecithin (see also Chrzaszcz). This problem may, therefore, be completely elucidated only after all possible enzyme adsorbents present in the cell have been investigated.

There can, however, be no doubt that elution of substrate from protein, or change in the state of dispersion of the latter, plays an important part in the acceleration of the velocity of enzymic hydrolysis of polysaccharides due to the addition of narcotics.

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