LXXVIII. STRUCTURE AND ENZYME REACTIONS.

IX. THE SYSTEMS AMYLASE-STARCH-GELATIN AND UREASE-UREA-GELATIN.

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THE previous parts of this series were devoted to the study of the influence of adsorption on the velocity of enzymic reactions in heterogeneous systems. In the present paper experiments on the influence of the state of the sorbent, of its viscosity, occlusion and aggregation (flocculation) will be described.

Increase in the viscosity of the medium in which an enzymic reaction is proceeding in a heterogeneous system should lead to retardation of the velocity of reaction in two ways, by reduction of the number of collisions taking place in unit time between the free enzyme and substrate, and between the enzyme and substrate adsorbed on different colloidal particles. The effect of increasing viscosity was studied in the systems amylase-starch-gelatin and urease-urea-gelatin, differing in that the first system contains a colloidal, slowly diffusing substrate, whilst the substrate of the second system is a rapidly diffusing crystalloid. The phenomenon of occlusion in relation to the velocity of reaction was next studied in the above systems. As the state of aggregation of gelatin increases, monons, together with adsorbed enzyme and substrate particles, cohere, yielding polyons, which may then further aggregate, finally to yield either a coagulum or a jelly. As a result of this process the enzyme or substrate or both may be enclosed within the aggregates, and, should the structure of these be such as to exclude entry of larger particles, may be excluded from reaction. The system amylase-glycogen-caseinogen, described in the preceding part of this series [Przyłęcki and Gurfinkel, 1930] affords an example of occlusion with consequent inhibition of reaction.

EXPERIMENTAL.

I. THE SYSTEM AMYLASE-STARCH-GELATIN.

Starch solution was prepared from starch paste (Merck's soluble starch), which was poured into boiling water and boiled for 30 min. under a reflux condenser. Gelatin solution was prepared by dissolving gelatin (Coignet Père et Cie, Paris) at 40°; the p_H of the solution was 5.89. Merck's amylase ("Diastase absolut") dissolved in saline solution and filtered was used in

these experiments. The p_H of all systems was fixed by addition of Sørensen's phosphate buffer solutions (p_H 5.89). Reducing sugar was determined by Bertrand's method and starch by Pflüger's method for glycogen.

(i) *Determination of sugar in the presence of gelatin.*

In view of the observation that the results obtained by Bertrand's method for reducing sugars are greater in aqueous than in gelatin solution, it was first necessary to determine whether the difference depended on the concentrations of sugar and of gelatin, on the time during which these were in contact, or on the temperature at which the systems were maintained.

0.3 % solutions of glucose or maltose, containing 1, 2, 3 or 4 % of gelatin, were left for 2 hours at 30°, after which the sugar content was compared with that of similar systems which had been allowed to stand for 10 min. at 15°; the results indicate that neither the duration of contact nor the temperature affects the results obtained.

The effect of varying the concentrations of sugar and of gelatin was studied in the following way (Table I). The sugar contents of 20 cc. of 0.1, 0.2, 0.3 and 0.4 % glucose or maltose in 5 % gelatin solution, and of 0.2, 0.4, 0.6 and 0.8 % maltose in 0.5 % gelatin solution were determined; in all cases the values found were lower than in aqueous solution, and the difference varied irregularly with the concentration of sugar, from 51.5 % for 0.1 % maltose

Table I. *Influence of concentration of sugar on the values found using Bertrand's method in the presence of gelatin.*

20 cc. were taken for the determination.															Conc. of gelatin %
% concentration of sugar															
0.1		0.2		0.3		0.4		0.6		0.8					
mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%		
Sugar found	diff.	found	diff.	found	diff.	found	diff.	found	diff.	found	diff.	found	diff.		
Maltose	9.7 51.5	30.5 23.7	50 16.6	64 20	— —	— —	— —	142 11.2	5						
"	— —	39 2.5	— —	75 6.25	83 30	— —	— —	— —	0.5						
Glucose	12.5 37.5	26.7 33.3	48 20	52 35	— —	— —	— —	— —	5						

in 5 % gelatin to 20 % for 0.4 % maltose. In systems in which the concentration of sugars was maintained constant (0.3 %), but that of gelatin was varied (1.25–7.5 %), it was found that the proportion of sugars found by Bertrand's method diminished with the concentration of gelatin, the difference increasing in the case of maltose from 1.8 % in 1.25 % to 30.4 % in 7.5 % gelatin; the corresponding figures for glucose were 7.5 % and 28.3 % (Table II).

The following experiments were next performed in order to determine whether the above effect was due to combination of gelatin with sugar, with the production of a compound of lower reducing capacity, or to combination with cuprous oxide, with consequent prevention of precipitation. 15 cc. of 0.3 % maltose solution were added to 40 cc. of Bertrand's solution, and the mixture was heated to boiling. As soon as the precipitate of cuprous oxide

Table II. *Influence of concentration of gelatin on the reducing sugar content as determined by Bertrand's method.*

The sugar content was 0.3 % and 20 cc. were taken for the determination.

Conc. gelatin	mg. glucose found	% difference	mg. maltose found	% difference
0	60.0	—	57.5	—
1.25	55.5	7.5	56.5	1.8
2.5	53	11.7	55	4.3
5	48	20	50	11.1
7.5	33	28.3	40.5	30.4

had commenced to form, 5 cc. of 8 % gelatin were added to one series of flasks, and 5 cc. of water to another, after which the solutions were boiled for a further 3 min., and cuprous oxide determined as usual. Practically identical results were obtained in all cases (42 mg. where gelatin was added, and 41.8 mg. in its absence). It follows that cuprous oxide does not combine with gelatin, nor is its precipitation prevented by gelatin, under the conditions of the above experiment.

Solutions containing maltose and gelatin were next subjected to ultra-filtration at 30° through parchment membranes, using a Zsigmondy apparatus. The membranes used were not completely impermeable to gelatin; for the purpose of this experiment it was considered sufficient if the filtrate gave no precipitate on addition of alcohol, as harder filters were partially impermeable to maltose, with consequent dilution of sugar in the filtrate. The results obtained, using 0.3 % maltose in 2 % gelatin solution and taking 10 cc. of filtrate or solution for each determination, indicate that compound-formation does not take place, 34.8 mg. of maltose being found in 10 cc. of the filtrate of the gelatin solution, and 33.3 mg. in the filtrate from solutions not containing gelatin, whilst the corresponding values for unfiltered solutions are 32.0 and 38.7. The cause of the lower values obtained by Bertrand's method for reducing sugar in the presence of gelatin is not therefore apparent.

(ii) *Inhibition of action of amylase on starch.*

(a) *Kinetics of starch-gelatin-amylase systems.* It was at first attempted to determine the velocity of decomposition of starch by amylase, in the presence of various concentrations of gelatin, by comparing the reducing sugar content of such systems with that of systems not containing gelatin. The results obtained were, however, not reproducible, in spite of the application of every precaution, and it was found necessary in certain cases to determine not reducing sugar produced but residual starch, using Pflüger's method for glycogen.

Series of systems, containing 10 cc. of 0.5 or 1 % starch solution, 5 cc. of gelatin solution, and 1 cc. of 0.1 % amylase or water were prepared, and the starch content was determined after keeping for 2 hours at 30°. The amount of starch decomposed was 37–38 mg. in systems containing 0.4 % of

gelatin, 30.3 mg. in 5 %, 29.3 mg. in 6 %, and 24 mg. in 9 % gelatin, representing a percentage retardation of respectively 20, 22 and 36 (Table III).

Table III. *Concentration of gelatin and velocity of enzymic hydrolysis of starch.*

Conc. starch %	Conc. gelatin %	mg. starch decomposed	
		In absence of gelatin	In presence of gelatin
0.63	1	83	83
		89	88
	2	83	81
		89	90
	3	83	85
		89	89
	4	83	79
		89	89
	5	83	81
		89	87
0.32	6	76	70
		84	79
	7	76	72
		84	78
	8	76	74
		84	78
	9	76	76
		84	79
	10	76	71
		84	76
0.32	1	38	38
	2	"	38.6
	3	"	38
	4	"	37
	5	"	30
	7	"	29
	9	"	24

(b) *Diffusion of starch and of amylase through gelatin gel.* Starch-gelatin gels were prepared by cooling mixtures of 15 cc. of 8 % gelatin and 10 cc. of 1 % starch to 0°, 15 cc. of water were then poured on the gels, which were left for 2, 4, 6 and 12 hrs. at 0°, after which the aqueous layer was examined for starch. This was in all cases found to be absent, indicating that starch does not diffuse through gelatin gels under the above experimental conditions. Analogous experiments in which 0.2 % amylase was taken in place of starch solution and in which the amylolytic power of the aqueous layer was determined showed that similarly amylase does not diffuse through gelatin gel.

(c) *Velocity of reaction in the presence of starch or amylase contained in gelatin gel.* Flasks were prepared containing 15 cc. of 3 % gelatin and 10 cc. of water or 1 % starch solution, and the solutions were left for 1, 2, 8 and 24 hrs. at 16°. 10 cc. of 1 % starch solution were then added to those flasks to which it had not previously been added, and 10 cc. of water to the remainder, and all flasks were allowed to stand for 1 hr. at 16°, after which 10 cc. of 0.1 % amylase were added to all systems. Half of the systems were stirred before addition of amylase, so as to break up the gel, which was left intact in the remainder. The starch content of all systems was determined after

standing for 1 hr. at 16°; the results, given in Table IV, show that considerable retardation (about 70 %) results from occlusion of starch within the gel, and that this retardation was considerably smaller (about 40 %) in those systems in which the gel had previously been broken up.

Table IV. *Effect of gelation of starch solution on velocity of reaction.*

Time of gelation hrs.	mg. reducing sugar found					
	Systems not stirred before addition of amylase			Systems stirred before addition of amylase		
	Starch and gelatin solutions mixed before gelation			Starch and gelatin solutions mixed before gelation		
	Starch added to gel	% inhibition		Starch added to gel	% inhibition	
24	26.6	4.9	81.2	26.3	15.4	45.2
8	28	4.3	84.6	28.3	15.6	44.8
2	25	12	52	29.3	17.3	40.9
1	25.3	7.2	71.1	28	19.6	30.0

Similar experiments were next performed on occlusion of amylase by gelatin gels. Solutions containing 15 cc. of 3 % gelatin and 1 cc. of 0.1 % amylase or water were left for 24 hrs. at 16°, after which 10 cc. of 1 % starch solution or water were added; as in the previous experiment, the gel in half of the flasks was broken up by stirring before the addition of starch. The reducing sugar content was determined after standing for 90 min. at 16°, and amounted to 46 mg. in the control systems, to 7.6 mg. in the stirred systems, and to zero in those systems consisting of unbroken gel and amylase solution.

The velocity of reaction in gels containing both starch and amylase together was studied in the following way. A series of flasks was prepared; each contained 10 cc. of 1 % starch, 15 cc. of 0.5 or 5 % gelatin, and 1 cc. of 0.1 % amylase and was immersed in ice-water for 5 min., when the 5 % gelatin solutions set to a jelly. The flasks were then left in water at 16° for 4 hrs., after which reducing sugars were determined using Bertrand's method. 54 mg. of sugar were found in 0.5 % gelatin solutions, and 47 mg. in 5 % gelatin gels (5 experiments). This result points to the absence of retardation of reaction to any considerable extent by conversion of the medium from the liquid state to a jelly; the possibility existed, however, that this effect was only apparent, and that the reaction proceeded with great velocity in its initial phases, before gelation was complete. That this was not the case was shown by experiments in which reducing sugars were determined immediately on gelation, *i.e.*, 10 min. and 150 min. after addition of amylase, 1.8 and 40 mg. being found respectively (5 experiments).

(d) *Velocity of reaction in gels.* The experiments described above indicate that the velocity of reaction is chiefly influenced by the physical state (sol or gel) of one part of the system at the moment of addition of the other. The influence of mechanical breaking up of the gel was examined in the following way.

A number of systems containing 10 cc. of 1 % starch and 15 cc. of 2 % gelatin were allowed to stand for 20 hrs., during which time all systems set to a jelly. Two flasks were then shaken for 210 min., and 1 cc. of 0.1 % amylase was added, whilst amylase was immediately added to two other flasks, which were then shaken for 210 min. Amylase was also added to a third pair of flasks, which were not shaken at all and to the control systems not containing gelatin. Reducing sugar was determined in all systems, using Bertrand's method, 19.3 mg. being found in systems shaken before the addition of gelatin, 19.0 in those shaken after, 0.0 in unshaken systems, and 24 mg. in the control systems, both shaken and unshaken.

A number of similar systems were now set up, with the difference that gelation was not allowed to take place. Half the solutions were shaken for 2 hrs., and all flasks were then left for 20 hrs. at 30°, after which 1 cc. of 0.1 % amylase was added to all flasks, and reducing sugar was determined after 1 hr. at 30°. Practically the same values were found in all systems, *viz.* 46.8 and 46.0 mg. in shaken and unshaken gelatin solutions respectively, and 44 and 46 mg. in shaken and unshaken control solutions respectively.

(e) *Effect on amylolysis of inhibition of gelation due to addition of alcohol.* A number of systems were prepared, containing 10 cc. of 1 % starch, 5 cc. of 9 % gelatin solution or phosphate buffers at the same p_H , and 4 cc. of water or 96 % ethyl alcohol. Gelation is to a large extent inhibited by addition of alcohol. 1 cc. of 0.1 % amylase was added to all systems, and reducing sugar was determined after 4 hrs. at 17° and after 1 hr. at 30°. At 17° the control flasks without alcohol contained 59.7 mg., whilst with alcohol 42.7 mg. of sugar were found, indicating a certain inactivation of amylase by alcohol. In systems containing gelatin, those to which alcohol had not been added contained 5.3 mg., whilst in the presence of alcohol 40.2 mg. were found. At 30° gelation did not take place, and alcohol has consequently an exclusively inactivating action, 44 and 31.4 mg. of reducing sugar respectively being found in control systems without and with alcohol, and 44.9 and 32.6 mg. in the corresponding systems with gelatin.

II. ACTION OF UREASE IN MEDIA CONTAINING GELATIN.

A number of systems were set up containing 10 cc. of 1 % urea, 5 cc. of phosphate buffer (p_H 5.9), 5 cc. of water or gelatin of the requisite concentration and 1 cc. of 0.1 % urease. The flasks were left for 1 hr. at 30°, after which ammonia was determined by Folin's aeration method. The velocity of reaction was the same in all cases, 66–68 mg. of urea being found in all systems containing 0–8 % of gelatin.

DISCUSSION.

The velocity of diffusion is, as follows from Einstein and Smoluchowski's formula, inversely proportional to the viscosity of the medium, whence it might be expected that in certain heterogeneous systems increase in viscosity

would lead to retardation of reaction. In the case of the system amylase-0.3 % starch-gelatin, however, no retarding effect is observed below 5 % gelatin, in spite of the considerable viscosity of such systems, whilst using 0.6 % starch retardation of reaction is inconsiderable even in 10 % gelatin. This difference might be ascribed to the relatively sparser distribution of starch particles in the former case than in the latter, as a consequence of which the average distance travelled through the solution before collision with amylase particles is greater; since the time required to travel this distance is proportional to the viscosity of the medium, factors connected with diffusion have a greater influence in more dilute than in more concentrated solutions. The above results are not in agreement with the experiments described in Part VIII of this series, in which 1 % gelatin exerted a considerable inhibitory action on similar systems; the p_H of the systems studied earlier was, however, different from that maintained in the above reactions, and hence the apparent discrepancy.

In the case of the system urease-urea-gelatin, the velocity of reaction appears to be independent of the viscosity of the medium.

The experiments on jellies indicate that diffusion of colloidal particles such as starch or amylase is practically nil, *i.e.* that on the addition of a solution of one substrate to a jelly containing the other practically no reaction takes place unless the jelly is broken up. Where both enzyme and substrate are enclosed within the same jelly, the reaction velocity is little inferior to that in solutions. It is possible approximately to calculate spatial separation of the individual amylase particles; this calculation can be, of course, only relatively correct as to the order of magnitude. The systems in question contained 100 mg. of starch and 10 mg. of amylase in 16 cc. of jelly. Assuming that the given sample of amylase contains 1 % of pure enzyme, and that the mol. wt. of the latter is about 20,000, it follows that about 2×10^{16} molecules are present in the system. Taking the mol. wt. of starch to be 40,000, there should at the same time be present 10^{20} starch molecules, whence it follows that the system contains 5000 starch molecules per molecule of amylase, and that the average distance between individual amylase molecules is about 100μ . According to Hardy [1928] the sphere of action of colloidal particles may exceed 100μ , whence it would follow that the immobilisation of the above system need not necessarily inhibit reaction. An alternative explanation would be that amylase particles combine immediately with the entire number of starch molecules corresponding to one enzyme particle, and that subsequent gelation of the system would hence be without effect on the velocity of reaction. This explanation would, however, be incompatible with Michaelis and Menten's [1913] theory of formation of enzyme-substrate compounds, and with the values obtained for the dissociation constant of this compound. A third possible explanation would be the possibility of local thixotropic [Freundlich, 1928] liquefaction of the jelly in the vicinity of amylase particles, with consequent mobilisation of the enclosed starch.

Finally, the possibility exists that gelatin jelly is not a homogeneous mass, but that it contains lacunae filled with more liquid substance, and that amylase and starch are chiefly concentrated in these spaces. Should both amylase and starch be present in these spaces, they would be able to move in the same way as in gelatin solution, so that the reaction could proceed with considerable velocity. The next part of this series will be devoted to a closer investigation of this phenomenon.

SUMMARY.

1. The quantity of sugar found by Bertrand's method in the presence of gelatin is smaller than in its absence. The apparent diminution in sugar content increases with concentration of gelatin.
2. The velocity of reaction in the system amylase-0.6 % starch-gelatin sol is unaffected below 9 % gelatin, and appears to be little dependent on the viscosity of the medium. Where 0.3 % starch is taken, no retardation of reaction is observed below 5 % gelatin, whilst at higher concentrations of gelatin increasingly great retardation of reaction takes place.
3. The velocity of the reaction between urease and urea is the same in the presence or absence of gelatin.
4. The rate of diffusion of starch and amylase in gelatin jellies is considerably smaller than in similar sols; as a consequence the velocity of reaction in systems containing one substrate in solution and the other enclosed in a jelly is very small. It may be greatly accelerated by destruction of structure, either mechanically or by the addition of alcohol.
5. The velocity of reaction between amylase and starch in gelatin solution is little greater than in gelatin jelly.

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