CLXIX. STRUCTURE AND ENZYME REACTIONS.

X. ACTION OF SALTS ON THE SYSTEMS: AMYLASE-STARCH-PROTEINS.

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IN the present paper the results obtained from a study of the effect of addition of electrolytes (NaCl , MgCl_2 , CaCl_2) to systems containing malt-amylase, starch, and gelatin, ovalbumin, or egg-white will be described.

EXPERIMENTAL.

Methods.

Solutions of Merck's starch were prepared by boiling with water. The amylase used was Merck's "Diastase absolut." Solutions not containing protein were buffered either by Sørensen's phosphate solutions or by glycine-sodium chloride-sodium hydroxide solutions. p_{H} was determined potentiometrically, using a calomel electrode. Gelatin purchased from Coignet Père et Fils (Paris) was used, and Merck's ovalbumin, solutions of which were prepared by dissolving in water, leaving 12 hours and filtering. The p_{H} of protein solutions was adjusted by addition of the requisite quantities of 0.1 N NaOH or HCl . The salts used were Merck's analytical reagents. The experiments were in most cases performed at 20° , and the velocity of reaction was determined either from the final reducing sugar content, determined by Bertrand's method, or from the final starch content, determined by Pflüger's glycogen method.

RESULTS.

I. *Effect of salts in the absence of proteins.*

Action of sodium chloride. This was studied at p_{H} 3.6 and 5.2, i.e. above and below the optimal p_{H} 4.5. The results (Table I) show that at p_{H} 5.2 no effect is observed in concentrations of less than N ; in $2N$ solutions of NaCl hydrolysis amounts to 53 % and in $3N$ solutions to 8 % of the normal value. At p_{H} 3.6 the inhibitory action of sodium chloride appears at lower concentrations, hydrolysis being 88.3 % in 0.1 N , 63 % in 0.2 N and 40.7 % in 0.3 N NaCl .

Table I. *Action of amylase in presence of NaCl.*

Systems contain 10 cc. Sørensen's phosphate buffers, 10 cc. 1.5 % starch, 10 cc. NaCl solution, and 1 cc. 0.1 % amylase. Temp. 20°. Duration of reaction: 4 hours for systems at p_H 5.2, 5 hours for systems at p_H 3.6. Maximum yield of maltose 120 mg.

Conc. NaCl <i>N</i>	Maltose produced			
	At p_H 3.6 mg.	% of yield without NaCl	At p_H 5.2 mg.	% of yield without NaCl
0	36.5	100	77.9	100
0.02	35.9	—	77.4	99.4
0.05	34.2	—	83.1	107
0.10	30.2	88.3	83.6	
0.20	21.4	63.0	83.4	
0.30	13.9	40.7	83.8	
0.60	—	—	84.4	
0.80	—	—	84.0	
1.0	—	—	83.7	53
2.0	—	—	41.3	
3.0	—	—	6.1	8
4.0	—	—	6.3	8

Action of calcium chloride. Similar results were obtained with calcium chloride, in solutions buffered to p_H 3.6, 5.2 and 6.8 (Table II). The velocity of reaction fell at p_H 3.6 to 90 % of normal in 0.072 *N* $CaCl_2$, 51.8 % in 0.144 *N*, 8 % in 0.2–0.9 *N*, and 4.2 % in 1.1 *N* salt. At p_H 5.2 the inactivating effect was observed first in 0.444 *N* solutions of $CaCl_2$, in which the velocity of hydrolysis was diminished by 18 %, and further increase in concentration of $CaCl_2$ led to a further slow decline. At p_H 6.8 three periods were observed: in the first, the addition of $CaCl_2$ enhanced hydrolysis by about 40 %, this optimal value being maintained over a range of 0.018–0.222 *N* $CaCl_2$, whilst in higher concentrations the velocity of hydrolysis again fell. Thus in 0.444 *N* solutions it amounted to 90 % of the original value, in 0.666 *N* $CaCl_2$ to 32 %, and in 0.888 *N* solutions to 1.6 %. At both p_H 5.2 and 6.8 the inhibitory action of $CaCl_2$ was first manifested in 0.444 *N* solutions.

Table II. *Effect of $CaCl_2$.*

Systems contain 15 cc. of 1.5 % starch, 1 cc. 0.1 % amylase without NaCl, and 5 cc. $CaCl_2$ or H_2O . The control systems yielded 37.7 mg. glucose on hydrolysis.

Conc. $CaCl_2$ <i>N</i>	Starch decomposed (expressed as glucose)					
	At p_H 3.6 mg.	% of yield without $CaCl_2$	At p_H 5.2 mg.	% of yield without $CaCl_2$	At p_H 6.8 mg.	% of yield without $CaCl_2$
0	16.6	100	17.7	100	8.6	100
0.018	17.4	106	18.2		12.7	147.6
0.086	17.9		17.7		12.4	144.2
0.072	14.9	90.0	18.5		12.6	146.5
0.144	8.6	51.8	17.7		12.4	144.2
0.222	1.4	8.4	17.9		13.2	153.5
0.444	1.4		14.5	82.0	11.4	132.0
0.666	2.6		8.1	46.0	4.1	47.9
0.888	1.4		4.7	26.6	0.2	1.6
1.100	0.7	4.2	4.8	26.8	0.2	

Action of aluminium chloride. The action of this salt was studied at p_H 5.2, and the solution of $AlCl_3$ was previously reduced to this p_H by addition of

0.1 *N* NaOH. The velocity of amylolysis was reduced to 65.3 % of the original value by 0.025 *N* AlCl₃, and to 44.4 % by 0.05 *N* aluminium chloride (Table III).

Table III. *Effect of AlCl₃.*

Systems contain 10 cc. 1.8 % starch, 15 cc. AlCl₃ or H₂O, 1 cc. 0.1 % amylase (without NaCl), and 6.7 cc. H₂O or 0.1 *N* NaOH for neutralisation of AlCl₃. Systems kept 4 hours at 20° *p_H* 5.2.

Conc. AlCl ₃ <i>N</i>	Maltose produced mg.	% of yield without AlCl ₃
0	34.0	100
0.025	22.2	65.3
0.050	15.1	44.4
0.100	11.6	34.1
0.200	5.1	15.0

II. *Action of salts in systems containing gelatin.*

The systems contained 0.6–1.2 % gelatin, warm solutions of which were added to starch solutions with or without electrolyte, and the mixtures were left for 12 hours, after which 1 cc. of 0.01 % amylase in 0.1 % NaCl was added with stirring to the semi-gelated mass. All systems were at *p_H* 5.7. Systems containing sodium chloride exhibited a feeble Tyndall effect; this was much more evident in the presence of calcium chloride.

Action of sodium chloride. The results obtained (Table IV) indicate that sodium chloride has, after a certain concentration, an activating effect, 15 % more maltose being produced in 0.02 *N*, and 23 % more in 0.04 *N* NaCl than in the absence of this salt.

In view of Freiburger's observation [1931] that the apparent reducing sugar content of solutions as determined by Bertrand's method is smaller in the presence of gelatin than in its absence, experiments were performed on solutions containing maltose and gelatin, with and without salts. The values found were the same in both cases, indicating that the activation found is not due to methodical errors, but to an actual increase in the activity of amylase.

Table IV. *Action of NaCl in systems containing gelatin.*

10 cc. 0.6 % starch, 10 cc. 1.2 % gelatin, 1 cc. 0.01 % amylase in 0.1 % NaCl, and 2 cc. of salt solution or H₂O. Systems kept 4 hours at 20° (*p_H* 5.7). Maximum yield of maltose 45 mg.

Conc. NaCl <i>N</i>	Maltose produced			
	Exp. 1		Exp. 2	
	mg.	% of yield without NaCl	mg.	% of yield without NaCl
0.0	9.0	100	7.3	100
0.002	8.6	95.5	7.6	
0.004	8.6	95.5	7.3	
0.008	8.7	96.7	6.9	
0.012	8.7	96.7	7.2	
0.016	9.3	103.3	7.4	
0.020	11.3	126.6	8.4	115
0.032	11.4	126.7	9.0	123.3
0.040	11.4	126.7	9.0	123.3

Action of potassium chloride. The results obtained with KCl are identical with those found for NaCl.

Action of calcium chloride. Calcium chloride at certain concentrations diminishes velocity of reaction at 18°, but not at 30°. The percentage retardation varies from 5 to 23 % in the initial stages of the reaction; given sufficient time, the reaction proceeds in all cases to completion. The addition of potassium chloride to systems containing calcium chloride diminishes the inactivating effect of the latter salt.

Table V. *Action of CaCl_2 in systems containing gelatin.*

10 cc. 0.6 % starch, 10 cc. 1.2 % gelatin, 1 cc. 0.01 % amylase in 0.1 % NaCl, and 2 cc. of salt solution or H_2O . Systems kept 4 hours at 20°; p_{H} 5.7.

No. of exp.	Time of reaction (hours)	Starch decomposed		
		Without CaCl_2 mg.	In presence of 0.032 N CaCl_2 mg.	% retardation
1	1	15.0	11.6	22.6
	2	40.4	35.7	11.6
2	0.5	4.4	3.8	13.6
	1	5.5	4.4	20.0
3	0.5	8.7	7.0	19.5
	1.0	15.1	12.9	14.6
4	12.0	45	45	0
	0.5	6.1	5.8	5.0
	1	11.0	11.0	9.1
	24	44.8	45.1	0

III. *Action of salts in systems containing ovalbumin.*

Amylase was added to the following systems: (a) 5 cc. 1 % starch solution and 5 cc. of about 4 % Merck's ovalbumin; (b) the same, boiled; (c) ovalbumin solution first boiled, and starch solution then added; (d) starch solution added

Table VI. *Action of dissolved and heat-coagulated ovalbumin in presence and absence of CaCl_2 .*

The starch present was equivalent to 42.4 mg. glucose.

System	Starch hydrolysed (mg.) at			
	p_{H} 5.2		p_{H} 6.1	
	0.01 % amylase		0.1 % amylase	0.01 % amylase
	1 hour	2 hours	4 hours	4 hours
(a) Ovalbumin + starch + amylase	11.2	19.7	35.7	8.2
(b) Ovalbumin + starch, heated at 100°, then amylase	12.7	20.1	35.5	8.2
(c) Ovalbumin heated at 100°, then starch and amylase	11.2	19.7	35.3	8.3
(d) Ovalbumin + starch + CaCl_2 + amylase	12.7	20.1	35.5	8.7
(e) Ovalbumin + starch + CaCl_2 heated at 100°, then amylase	11.2	20.1	35.5	8.3
(f) Ovalbumin + CaCl_2 , heated at 100°, then starch and amylase	11.2	20.1	35.3	8.7

to ovalbumin solution containing CaCl_2 ; (e) the same, boiled, and (f) starch added to boiled ovalbumin- CaCl_2 solution. The experiments were carried out at p_{H} 5.2 and 6.06. 1 cc. of 0.2, 0.1 or 0.01 % amylase was added to all systems, and 1 cc. of water or of 1.2 N CaCl_2 solution. The results (Table VI) indicate that the velocity of hydrolysis is independent of the state of the ovalbumin (sol or gel) and of the presence of CaCl_2 (0.1 N).

IV. Action of salts in systems containing egg-white.

Coagulated egg-white. 1 cc. of water or CaCl_2 in various concentrations and 10 cc. of 1 % starch solution were added to 5 g. of heat-coagulated egg-white and the systems were shaken for 1 hour, after which 1 cc. of 0.1 % amylase in 0.1 % NaCl solution was added, and residual starch was determined after 5 hours by Pflüger's method. The results (Table VII) indicate that 0.01 N CaCl_2 accelerates hydrolysis by 5.2 %, and 0.2 N CaCl_2 by 32 %. Considerably greater activation was observed when the time of shaking before addition of amylase was prolonged to 4 hours, when the acceleration of reaction due to 0.1 N CaCl_2 was not 24 %, as after 1 hour's shaking, but 55 %, whilst in 0.2 N CaCl_2 it was now 60 %. The activating effect of the addition of calcium chloride was evident in concentrations at which no effect was observed in the absence of protein.

Table VII. Action of CaCl_2 in systems containing egg-white.

Starch present equivalent to 74.0 mg. glucose. Residual starch determined by Pflüger's method.

Conc. CaCl_2 N	Shaken 1 hour		Shaken 4 hours	
	Starch decomposed mg.	% acceleration due to CaCl_2	Starch decomposed mg.	% acceleration due to CaCl_2
0	23.2	—	27.0	—
0.01	24.4	5.2	—	—
0.02	24.6	5.3	—	—
0.05	27.3	18	34.7	28.5
0.10	28.8	24	41.5	54.0
0.20	30.6	32	44.2	64.0

The p_{H} of the systems was originally 8.8, but fell to 8.1 on addition of calcium chloride; this change in p_{H} leads to an acceleration of reaction amounting to about 43 %.

Sodium chloride (0.1 N) has no influence on the velocity of hydrolysis in analogous systems. It was, however, observed that the addition of NaCl to systems containing CaCl_2 diminished the activation due to the latter salt. Thus we see (Table VII) that in the presence of 0.1 N NaCl the acceleration due to 0.1 N CaCl_2 was only 23 %, whilst with 0.05 N CaCl_2 no acceleration was observed.

The addition of sodium sulphate (0.05–0.2 N) has no influence on the system starch-amylase-coagulated egg-white (Table VIII).

Comparison of the action of dissolved and coagulated egg-white. 5 cc. of egg-white were added to 5 cc. of water, 10 cc. of 1 % starch, 1 cc. amylase in

0.1 % NaCl, and 1 cc. of water or 2.2 *N* CaCl₂ were added. Egg-white was coagulated in certain of the systems by heating for 2 minutes at 100°, in the presence or absence of CaCl₂, and amylase was added to the cooled systems. The results (Table IX) show that in the absence of electrolytes the coagulation of egg-white reduces the velocity of reaction by 41–54 %. CaCl₂ in concentrations up to 0.1 *N* accelerates reaction both in the presence of dissolved and of coagulated egg-white; this acceleration amounted respectively to 40–56 % and 86–142 %, the velocity of reaction in the presence of coagulated egg-white and CaCl₂ being greater than in the presence of dissolved egg-white without CaCl₂.

Table VIII. *Systems as in Table VII, but different salts.*

Salt	Starch decomposed (expressed as mg. glucose)
—	30.4
0.1 <i>N</i> NaCl	28.9
0.1 <i>N</i> NaCl + 0.05 <i>N</i> CaCl ₂	29.7
0.1 <i>N</i> NaCl + 0.1 <i>N</i> CaCl ₂	37.3
—	30.8
0.05 <i>N</i> Na ₂ SO ₄	30.8
0.10 <i>N</i> Na ₂ SO ₄	30.2
0.20 <i>N</i> Na ₂ SO ₄	30.2

Table IX.

Duration of reaction 4 hours; *p_H* without CaCl₂ 8.8, with CaCl₂ 8.1.

System	Starch decomposed mg.	% decomposition as compared with system (a)
(a) Dissolved egg-white + starch	29.2–31.5	100
(b) " " " + CaCl ₂	42.5–46.1	135–158
(c) Egg-white + starch, heated at 100°	14.1–17.0	44.8–58.6
(d) Egg-white + starch + CaCl ₂ , heated at 100°	31.7–34.8	109–111

DISCUSSION.

The action of salts in systems containing amylase and starch is quantitatively different, according to whether the systems are above or below the optimal *p_H* of the amylase; in the former case the same effect is achieved by 10 times higher concentrations than in the latter. At *p_H* 7 CaCl₂ accelerates reaction when present in small concentrations. At *p_H* 5.2 the action of salts appears to be a function of the valency of the cation; thus the minimal retarding normal concentrations of the salts AlCl₃:CaCl₂:NaCl are as 1:18:60. At *p_H* 3.6 this dependence is not observed, the actions of the salts being roughly equal at equivalent concentrations. At *p_H* 5.2 and 3.6 no acceleration of reaction was observed in the presence of low concentrations of salts, and this may be ascribed to the fact that the enzyme preparation was not previously freed from salts by dialysis, and to the relative insensitiveness of vegetable amylase to mineral salts [Sherman and Tauberg, 1916; Saito, 1906]. The action of salts is very different according to the variety of protein present, and to its physical state. In systems containing gelatin, the addition of

sodium chloride leads to acceleration of reaction (23 % acceleration in 0.04 *N* solution). The causes of this effect are not clear; it may be due to combination with gelatin of the NaCl added with the amylase solution, and necessary for its action, and hence the addition of further NaCl accelerates reaction. Experiments with CaCl_2 , however, do not support this hypothesis, but suggest that the action of NaCl is rather in the direction of affecting the degree of aggregation of gelatin, thereby facilitating the penetration of amylase into protein aggregates containing occluded starch.

Calcium chloride exerts a retarding action, in contradistinction to NaCl. Since the retarding concentrations of calcium chloride in such systems are considerably smaller than the smallest active concentrations in systems not containing protein, the explanation of its action should be connected with the presence of protein, and this view is further supported by the fact that at 30° no retardation due to the presence of CaCl_2 can be observed, and that, given sufficient time, the final yield of maltose is the same in the presence or absence of CaCl_2 . Observations of the Tyndall effect and of viscosity in jellified systems indicate that CaCl_2 enhances the compactness of the jelly (Targońska, unpublished work), and the possibility may therefore exist that the penetration of amylase within starch-containing aggregates is rendered more difficult in the presence of this salt.

The results of experiments conducted on systems containing ovalbumin or egg-white are even more difficult of interpretation than are those for gelatin. Filipowicz (unpublished work) has found that the velocity of reaction of amylase with starch is accelerated by the addition of proteins, in systems having p_{H} values considerably higher than the optimal for amylase; this acceleration is considerable, and may under suitable conditions amount to over 40 %. A second factor influencing the velocity of reaction is the change in p_{H} due to addition of neutral salts; this, as has been shown by Przyłęcki and Giedroyć [1931], may be quite considerable in alkaline systems in the presence of protein. Filipowicz has, however, found that changes in p_{H} have a much feebler effect on the activity of amylase in systems containing protein than in its absence. In this case, we see (Table X) that 0.1 *N* CaCl_2 reduced the p_{H} of a system containing protein and starch from 8.8 to 8.1, and that this change in p_{H} itself leads to an acceleration of reaction of 43.3 %.

Table X.

Systems contain 5 cc. egg-white, 5 cc. water, 10 cc. 1 % starch, 1 cc. 0.1 % amylase, and 1 cc. of CaCl_2 , HCl, or water.

System	p_{H}	Starch decomposed mg.	% acceleration
As above, with water	8.8	33.3	—
„ HCl	8.1	47.7	43.3
„ CaCl_2	8.1	50.0	50.1

As to possible changes produced by salts on the state of combination (this we shall term “sorption,” without entering into the question of the chemical,

physical, or mechanical nature of the forces to which the phenomenon is due) of starch or amylase with protein, our knowledge is very inadequate, at least in so far as systems containing protein sols are concerned. Both NaCl and CaCl₂ diminish sorption, to a greater extent when added before coagulation of the sol than when added after. The reversal of the process of sorption, where it has already taken place, requires considerably higher concentrations of sodium chloride than of calcium chloride, and the process of desorption proceeds very slowly in both cases, equilibrium being attained in about 4 hours. In systems to which CaCl₂ has been added after coagulation of protein 64 % of the total starch remains in solution after shaking for 1 hour, whilst in the absence of CaCl₂ only 55 % remains, a difference of 17 %. After a further 3 hours, the corresponding figures are 51 and 27 %, and the difference now amounts to 51 %. When CaCl₂ (0.1 *N*) is added before coagulation of protein, 55 % of the total starch remains in solution, as compared with 20 % in the absence of CaCl₂, or 175 % more. Sodium chloride, in the concentrations taken (up to 0.05 *N*), had very little influence on the proportion of starch remaining in solution (22 % with NaCl, 15 % without).

Coagulated ovalbumin combines with starch to a much smaller extent than does coagulated egg-white. Neither dissolved nor coagulated ovalbumin retards the velocity of amylolysis, either in the presence or absence of CaCl₂, whence it appears that in such systems the reduction in concentration of the substrate in solution is without effect on the velocity of reaction, in spite of the fact that in homogeneous systems the latter is reduced by about 50 % by a similar change in concentration. The velocity of reaction is, further, independent of whether the ovalbumin was coagulated in the presence or the absence of starch. Occluded starch is liberated in alkaline solution in the same way as sorbed starch, in contradistinction to acid systems (*p_H* 3), in which both occlusion and sorption are irreversible [Przyłęcki and Gurfinkel, 1930; Przyłęcki and Majmin, unpublished work].

In systems containing egg-white quite different results were obtained, the velocity of reaction being considerably smaller in the presence of coagulated than of soluble protein. This relative retardation amounts in the systems studied to about 50 %, whilst at the same time sorption amounts to about 80 %. The disproportionality between these figures may be ascribed to the partial combination of starch or enzyme with protein sol; alternative explanations would be that it is due to the greater viscosity of systems containing soluble proteins, or that the reason for the smaller velocity observed in the presence of coagulated egg-white lies not in sorption but in change in concentration of activating protein. The effect of addition of CaCl₂, however, supports the view that sorption is the cause of retardation of reaction, as this salt accelerates reaction in the presence of dissolved and coagulated egg-white respectively by 50 and above 100 %.

This activation may be due largely to reduction in *p_H* of unboiled systems, in which a change from *p_H* 8.8 to 8.1 accelerates reaction by 43 %; this effect

does not, however, apply equally to systems containing coagulated egg-white. Here the following facts support the view that CaCl_2 acts by desorption, with consequent increase in active concentration. The same concentration of CaCl_2 has more than twice as great an effect in systems containing coagulated than dissolved egg-white. The percentage acceleration of reaction is more than doubled by shaking systems containing coagulated egg-white and CaCl_2 for 4 hours instead of 1 hour, whilst where egg-white is coagulated in the presence of CaCl_2 it is about four times as great. These changes in the velocity of reaction vary roughly in parallel with the variations in the proportion of dissolved to sorbed starch in the above systems. In systems containing soluble egg-white, CaCl_2 may, apart from its usual effect of lowering the p_{H} of the systems, diminish the proportion of starch combined with protein particles. No obvious explanation is forthcoming as to the reason why the velocity of reaction in the presence of coagulated egg-white and CaCl_2 is greater than in systems containing dissolved egg-white without CaCl_2 . It is possible that certain of the effects observed are due to variations in the active concentration of enzyme due to its adsorption on dissolved or coagulated protein; work on this aspect of the question is now being conducted.

The above observations may possess certain biological applications, chiefly in plant physiology. It is evident that vegetable cells possess agents, such as CaCl_2 , which are able to affect the quantitative relation between free and sorbed starch in the cytoplasm, and that such influences may determine the velocity of enzymic hydrolysis; in systems in which the reaction of synthesis is in equilibrium with that of hydrolysis the presence of mineral salts may even determine the direction of reaction.

SUMMARY.

1. Electrolytes retard the velocity of enzymic hydrolysis of starch. Systems of p_{H} below the optimal are more sensitive to the action of salts than are those of p_{H} higher than the optimal; in the latter case the active concentration of ions is a function of their valency.
2. The velocity of enzymic hydrolysis in systems containing gelatin is accelerated by the addition of NaCl , and retarded by the addition of CaCl_2 .
3. In systems containing Merck's ovalbumin neither heat-coagulation nor CaCl_2 affects the velocity of reaction.
4. In systems containing crude egg-white heat-coagulation in the presence of CaCl_2 accelerates reaction. The velocity depends on the time during which the system containing coagulated egg-white and CaCl_2 is shaken before addition of amylase. NaCl and Na_2SO_4 are without effect in this system. In the absence of CaCl_2 heat-coagulation reduces velocity of reaction by 50 %.
5. The velocity of reaction in the presence of dissolved egg-white is increased by about 50 % by addition of CaCl_2 . This effect is ~~due to reduction~~ ^{due to desorption}.

of p_H , as the same acceleration is obtained by addition of the appropriate quantity of HCl.

6. In systems containing heat-coagulated egg-white and CaCl_2 , the acceleration observed is due probably to desorption of starch from protein.

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