LXI. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. II. THE INFLUENCE OF SALTS ON THE RATE OF HYDROLYSIS. (Preliminary Communication.)

By ROLAND VICTOR NORRIS, *Beit Memorial Research Fellow.*

From the Biochemical Laboratory, the Lister Institute.

(Received Nov. 17th, 1913.)

In a recent communication [Norris, 1912] it was pointed out that samples of glycogen prepared from different animals were hydrolysed at different rates by pancreatic amylase. In view, however, of the fact that these preparations contained varying amounts of salts it seemed desirable to examine how far this might influence the rate of hydrolysis, and a quantitative study has therefore been made of the action of neutral salts on diastatic action. The results quickly showed that the experiments referred to were in no way invalidated by the varying salt content, the latter in every case being sufficient to produce the maximum degree of hydrolysis and not great enough to cause any inhibition.

The experiments have however been continued in the hope of finding some explanation of the manner in which salts exert their influence.

It has been known for some time that a dialysed amylase solution when added to a starch solution free from salts produces but little hydrolysis, and that on adding certain salts in small quantities the activity of the enzyme is restored. In spite of a considerable amount of work on this point, the explanation of this fact is not by any means clear. It is agreed that the most active salts are those of the halogen acids. With regard to sulphates, however, very divergent results have been obtained. Cole [1906, 1], for example, states that sulphates accelerate the action while Grützner [1902] maintains that magnesium and sodium sulphate are "specific poisons" for the ferment. In this connection however it must be pointed out that in one at least of Cole's experiments the addition of sulphate produced no acceleration although the concentration of the salt was similar to that used in previous experiments in which an acceleration had been obtained. These and other divergencies

may perhaps be partly explained by one of the following reasons. Firstly, the source and method of preparation of the enzyme has differed with nearly every worker. Hence while some investigators have employed solutions containing only traces of proteins, in other cases these have been present in considerable quantity. Again, in the few cases where the action has been followed quantitatively, the diastatic activity has been estimated either by Roberts' achromic point method [Roberts, 1891] or by the method of Wohlgemuth [1908, 1], and it has been shown by Evans [1912, 1] that neither of these is satisfactory. Finally, in some cases, the enzyme and starch solution employed have been by no means free from salts, the controls all showing a marked degree of hydrolysis, that is to say the observed effect was really due to a mixture of salts and not alone to the particular salt under investigation.

In the following experiments glycogen has been employed instead of starch, while the enzyme has consisted of an extract of pigs' pancreas.

Preparation of glycogen. This was obtained from dogs' liver by Pflüger's method. The crude glycogen was purified by repeated precipitation of its solution by alcohol and was finally dialysed for a week, the last three days' dialysis being against running distilled water.

Preparation of enzyme. This consisted of a Buchner extract of pigs' pancreas which was dialysed for three days, in the course of which a certain amount of protein usually separated out. The dialysed extract was then filtered till perfectly clear and diluted from ten to twenty times with distilled water.

Experimental methods. The following may be described as typical of the method employed.

A 1 per cent. or 2 per cent. solution of glycogen was as a rule used and to this was added a suitable concentration of the salt under investigation. The mixture was then brought to a temperature of 37° in a thermostat and the enzyme added. After 15 minutes and 30 minutes hydrolysis, 20 cc. of the mixture were removed and the sugar immediately estimated by Bertrand's method. The sugar solution was added directly to the alkaline copper mixture and hence the hydrolytic action stopped instantaneously. The strength of enzyme used was adjusted so that the readings taken fell on the linear portion of the hydrolysis curve. [See Evans, 1912, 2 and Norris, 1912.] The salts employed were nearly all Kahlbaum's "for analysis with certificate of guarantee."

Effect of dialysis on glycogen hydrolysis.

As in the case of starch the result of dialysis of both enzyme and glycogen resulted in almost complete inactivation. Similarly the hydrolytic power was again restored by the addition of certain salts.

Influence of salts.

Under this heading neutral salts only are considered. The rate of hydrolysis is of course greatly influenced by any change in the reaction of the medium, but this point has been dealt with in a previous communication [Norris, 1912].

Hydrogen ion determinations made on glycogen solutions containing varying concentrations of sodium chloride showed that the reaction of the medium was not altered by the presence of this salt in the concentrations employed, hence the accelerating effect of sodium chloride is not due to this cause.

Sodium chloride.

A series of mixtures was made up each containing 1 per cent. glycogen and a concentration of sodium chloride ranging from zero to 0.003 N. These were in turn incubated with 1 cc. of a dilute enzyme preparation and hydrolysis allowed to proceed for 15 minutes. The sugar in 20 cc. of each solution was then estimated.

The results are shown in Fig. 1 where the abscissae represent the cc. of 0.1 N NaCl in 100 cc. of the mixture and the ordinates the cc. of KMnO_4 used in the sugar estimation.

Fig. 1.

The results show that there is a rapid increase in the hydrolysis with increasing NaCl content until the latter reaches a concentration of about 0.002 N (0.012 per cent.). This value is in close agreement with Cole's results with starch and ptyalin.

On further addition of salt there is at first no change in rate, but with high concentration a slight retardation may be produced.

An investigation was next undertaken to decide whether the value of this optimum concentration (0.002 N) would be changed by alteration in the concentration of either glycogen or enzyme. On this point results have been somewhat contradictory but it seems probable that the glycogen concentration has but little influence on the amount of salt required to produce the maximum rate of hydrolysis. The optimum salt content for 2 per cent. glycogen has usually been about 0.002 N, that is to say the same as for 1 per cent. glycogen.

On increasing the amount of enzyme, however, a higher concentration of NaCl is usually required. This is in agreement with the results of Cole [1906, 1] and Starkenstein [1910]. The latter, working with starch, states that the amount of NaCl necessary to give the maximum rate of hydrolysis varies *directly* with the concentration of enzyme and has even based on this a method for the estimation of diastase in animal organs [Starkenstein, 1912]. The results of my experiments do not point to such a simple relationship, though as already stated more NaCl is usually required with increased concentration of enzyme. It must be remembered that it is usually in the enzyme that impurities such as proteins etc. will be found and the latter may begin to play an important part when the amount of enzyme present is not small.

The following table shows the results of one experiment in which the concentration of enzyme was twice that usually employed.

TABLE I.

Determination of NaCl optimum with 1 per cent. glycogen and high enzyme concentration.

Experiment	Concentration of NaCl	Percentage hydrolysis	
		15 mins.	30 mins.
A	0	5.5	9.20
B	0.002 N	15.1	26.5
C	0.004 N	16.5	28.0
D	0.006 N	18.3	29.8
E	0.01 N	18.3	29.5

In this case therefore by doubling the concentration of enzyme the optimum salt concentration was raised from 0.002 N to 0.006 N, that is to say three times.

In other experiments however very much lower results were obtained and there seems to be some unknown factor concerned. The point is still under investigation.

Comparison of sodium chloride with other salts.

Chlorides of different metals.

Table II shows the accelerating effect of the chlorides of sodium, potassium, calcium, barium and magnesium.

These were added in insufficient quantity to produce the optimum rate of hydrolysis, so that any variation in their accelerating power could be detected.

TABLE II.

*Accelerating power of various chlorides. 1 per cent. glycogen.
Concentration of salt = 0.0005 N.*

Salt	Percentage hydrolysis	
	15 mins.	30 mins.
NaCl	10.83	18.76
KCl	10.61	18.8
CaCl ₂	10.70	—
BaCl ₂	10.61	17.6
MgCl ₂	10.61	18.52
Control	0	0.68
		1.14

All the above salts are therefore of equal accelerating power, that is to say the kation exerts practically no influence on the reaction. This is in agreement with the results of Starkenstein [1910]. Cole [1906, 1] considered that the anion accelerated while the kation depressed the action. If the latter were correct, however, one would expect the chlorides containing a divalent kation to be less active than sodium or potassium chloride and as shown this is not the case. The view that the anion is the more important factor is further strengthened by a comparison of the accelerating power of chlorides, bromides and iodides. The results given in Table III confirm those of previous investigators, namely, that the acceleration decreases in the order given, the drop from bromide to iodide being much greater than that from chloride to bromide.

TABLE III.

Comparison of KCl, KBr and KI. (Concentration of salt = 0.0005 N.)

Salt	Percentage hydrolysis	
	15 mins.	30 mins.
KCl	10.61	18.80
KBr	8.31	15.60
KI	2.5	3.70
Control	0.68	1.40

Influence of sulphates.

The influence of three sulphates has been investigated but it has been found that they have no accelerating power at all; these results are therefore in agreement with those of Wohlgemuth [1908, 2] but in opposition to those of Cole [1906, 1]. The latter however found that sulphates were much less effective than the halogen compounds. These results are of some interest, as one would expect if the anion were the factor concerned, that the divalent anion would be extremely potent. The situation is complicated, however, as pointed out by Cole, by the fact that sodium sulphate in moderate dilutions chiefly dissociates into Na^+ and NaSO_4^- in which case the anion is monovalent.

On the other hand it has not been found that sulphates have any depressing action [cf. Grützner, 1902], nor do they hinder the acceleration produced by NaCl etc.

TABLE IV.

Influence of sulphates. 1 per cent. glycogen.

		Concentration of salt	Percentage hydrolysis	
			15 mins.	30 mins.
1.	0	Control	1.60	5.04
	Na_2SO_4	0.002 N	1.65	5.0
	Na_2SO_4	0.01 N	1.55	5.0
	MgSO_4	0.001 N	1.60	—
2.	MgSO_4	Each		
	+ NaCl	0.001 N	9.10	16.04
	NaCl	0.001 N	9.20	16.10

For the sake of comparison the results obtained with different salts have been collected in Table V.

TABLE V.

Comparison of various salts. 1 per cent. glycogen containing 0.0005 N salt.

Salt	Percentage hydrolysis		Remarks
	15 mins.	30 mins.	
0 (Control)	0.68	1.14	
NaCl	10.83	18.76	Halogen salts. Both ions monovalent.
KCl	10.61	18.8	
KBr	8.31	15.6	
KI	2.5	3.7	
CaCl ₂	10.70	—	Halogen salts with divalent kation.
BaCl ₂	10.61	17.6	
MgCl ₂	10.61	18.5	
KNO ₃	2.75	5.08	Both ions monovalent.
La(NO ₃) ₃	2.75	4.94	Trivalent kation.
Na ₂ SO ₄	0.55	1.0	Divalent anion.
K ₂ SO ₄	0.64	1.20	" "
MgSO ₄	0.70	—	Both ions divalent.

It will be seen from the results tabulated above that the only salts of those tried which have a powerful accelerating action are those of the halogen acids, although nitrates have a small influence. It is also clear that the anion is much more concerned in the reaction than is the kation. Further it is probable that the action of the salts is chiefly confined to the enzyme, for it has been shown by Cole [1906, 2] that in the action of invertase, where the substrate is not a colloidal solution, salts have again a powerful influence. In this case, however, the action is reversed, that is to say, the hydrolysis is retarded by chlorides.

If the function of the anion be simply to alter the charge on the enzyme, it is difficult to understand why sulphates have no accelerating effect, for sulphates discharge a ferric hydroxide solution much more readily than chlorides. The fact that the number of salts producing an acceleration is so restricted, however, points to some other explanation.

In the meantime it seems desirable to examine separately the effect of salts on the glycogen and enzyme respectively from the point of view of adsorption and charge, and experiments on these lines are in progress but are not as yet sufficiently advanced to furnish an explanation. The action of a further series of salts is also being investigated.

SUMMARY.

- (1) A dialysed glycogenase (pancreatic) solution has practically no hydrolysing action when added to a dialysed glycogen solution.
- (2) The activity of the enzyme is restored by the addition of small quantities of certain salts.

(3) The most powerful of these are the salts of the halogen acids, the activity diminishing in the order chlorides, bromides, iodides; nitrates have also a slight accelerating action.

(4) Sulphates do not restore the activity of a dialysed enzyme solution, neither are they inhibitors [cf. Grützner, 1902].

(5) The concentration of salt required to produce a maximum degree of hydrolysis rises with increasing enzyme concentration but appears to be independent of the glycogen concentration within the limits tried.

(6) The anion is probably the part of the salt concerned in the acceleration, the nature of the kation (valency) having no influence.

REFERENCES.

- Cole (1906, 1), *J. Physiol.* **30**, 202.
— (1906, 2), *J. Physiol.* **30**, 281.
Evans (1912, 1), *J. Physiol.* **44**, 220.
— (1912, 2), *J. Physiol.* **44**, 191.
Grützner (1902), *Pflüger's Archiv*, **91**, 195.
Norris (1912), *Biochem. J.* **7**, 26.
Roberts (1891), *Digestion and Diet*, London, p. 68.
Starkenstein (1910), *Biochem. Zeitsch.* **24**, 210.
— (1912), *Biochem. Zeitsch.* **47**, 800.
Wohlgemuth (1908, 1), *Biochem. Zeitsch.* **9**, 1.
— (1908, 2), *Biochem. Zeitsch.* **9**, 10.