I. STUDIES IN NEUTRAL SALT ACTION.

Part I.—Diastase.

By D. Narayanamurti.

It is known that neutral salts are often of importance in enzyme action. Enzymes being colloidal systems will naturally respond to the action of electrolytes. Starkenstein, Bierry and Giaja (*Compt. rend.*, 1906, **143**, 300) and others have shown that animal diastase is inactive in the absence of neutral salts. Höber (*J. Gen. Physiol.*, 1928, **8**, 265) attributes the ionic antagonism observed by him in enzyme systems to the colloidal part of the complex. Neuschloss (*Kolloid Z.*, 1920, **27**, 292) has drawn attention to the great similarity between enzymes and lecithins. It is evident that electrolytes might act on enzymes in various ways, namely, change in degree of swelling, electro-kinetic potential, adsorption power, degree of dispersion, surface conductance, etc., and as remarked by Bayliss it is difficult to formulate statements of general application. This communication forms part of an extended series of investigations undertaken by the author to elucidate the mechanism of neutral salt action.

Influence of neutral salts on malt diastase.—There has been much controversy regarding the action of neutral salts on malt diastase. Hahn and Hapruder (*Z. Biol.*, 1919, **71**, 287, 302) distinguished the effect of neutral salts on malt and salivary diastase at three ranges, (*a*) optimal P_H, (*b*) acid side of optimal P_H and (*c*) alkaline side of optimal P_H. In buffered solutions salts were without any effect at the optimum P_H, on the acid side at great dilution of the buffer and neutral salt, acceleration was caused; at higher concentrations of buffer, inhibition was observed. On the alkaline side of the optimal P_H at great dilution of buffer and salt pronounced activation was caused, at higher concentration this was less marked, but inhibition was never observed. The decrease of the activating effect can, at higher buffer concentration, pass into inhibition in the case of nitrates. They further observed that the optimum P_H is not changed (cf. however Sherman, *J. Amer. Chem. Soc.*, 1928, **50**, 2532), that the enzyme wanders to the cathode at the optimum P_H and that on the addition of salt it wanders to the anode, when the isoelectric point is shifted to the acid side. Hence they consider that the electrical nature of the enzyme is of no consequence, and that the electrochemical dissociation theory of Michaelis finds no application to malt and salivary diastase. Fricke and Kaja (*Ber.*, 1924, **57**, 310, 313) found that addition of neutral salts inactivated their electro-dialysed enzyme. Patwardhan and Norris (*J. Indian Inst. Sci.*, 1928,

11A, 127) found activation with malted cholam diastase (alcohol precipitated) and inactivation with unmalted cholam diastase at high salt concentration. Eadie (*Biochem. J.*, 1926, **20**, 1016) observed slight activation at salt concentrations of about 1·62*N*. Narayanamurti and Norris (*J. Indian Inst. Sci.*, 1928, **11A**, 134) noticed inactivation with electro-dialysed cholam diastase at the concentration of salt found most beneficial by Patwardhan and Norris.

In view of the above observations it was deemed necessary to investigate the matter in more detail and the results so far obtained are of sufficient interest to place on record.

EXPERIMENTAL.

The enzyme was prepared by Euler's method (*Z. physiol. Chem.*, 1920, **112**, 193) from cholam malt the dialysis in collodion bags against flowing distilled water being continued for ten days. A distinct advance was also made by using electrolyte-free amylose prepared according to Samec (*Kolloid Beihefte*, 1920, **12**, 281) in place of ordinary starch as substrate. The sodium chloride used was Kahlbaum's analytical reagent dissolved in conductivity water. All reactions were carried out in silica vessels washed with hot dichromate-sulphuric acid mixture and steamed before use.

Order of adding components in preparing the reaction mixture.—It is quite evident from the results shown in Fig. I., that the order of addition has a marked influence on the activity of the enzyme. The neutral salt was most pronounced in action when added to the enzyme before the amylose, thus recalling similar behaviour with colloidal systems. This is to be expected from the nature of diastase action, there being ample evidence to show that enzyme action ought to be considered as a case of contact catalysis. It is well known that adsorption has much to do with contact catalysis, and Frumkin (*Nature*, 1926, **117**, 790; *Biochem. Z.*, 1927, **182**, 220) has investigated the influence of electric fields on the adsorption of neutral molecules. His experiments indicate that adsorption is greatest when the adsorbent is almost uncharged. He found maximum adsorption at a small negative charge in the case of a mercury surface and a small positive charge in the case of a silver surface. Later work by Obretschewa (*Biochem. Z.*, 1929, **207**, 25) however, has shown that the relations are not so simple and that the concentration of the adsorbate must also be taken into account. With higher concentrations the maximum is slightly diverted from the isoelectric point. The adsorption depends not only on the electrical nature but also on the degree of dispersion of the adsorbent. This indicates that the decomposition of the neutral

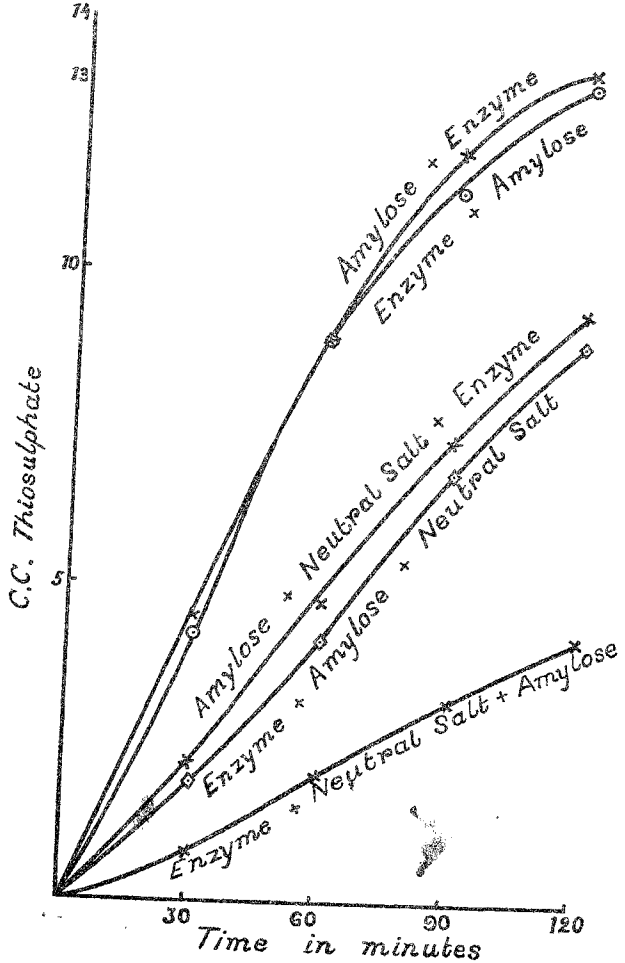
C.C. Thiosulphate
14
13
10
5
Amylose + Enzyme
Enzyme + Amylose
Amylose + Neutral Salt + Enzyme
Enzyme + Amylose + Neutral Salt
Enzyme + Neutral Salt + Amylose
30
60
90
120
Time in minutes

Fig. I

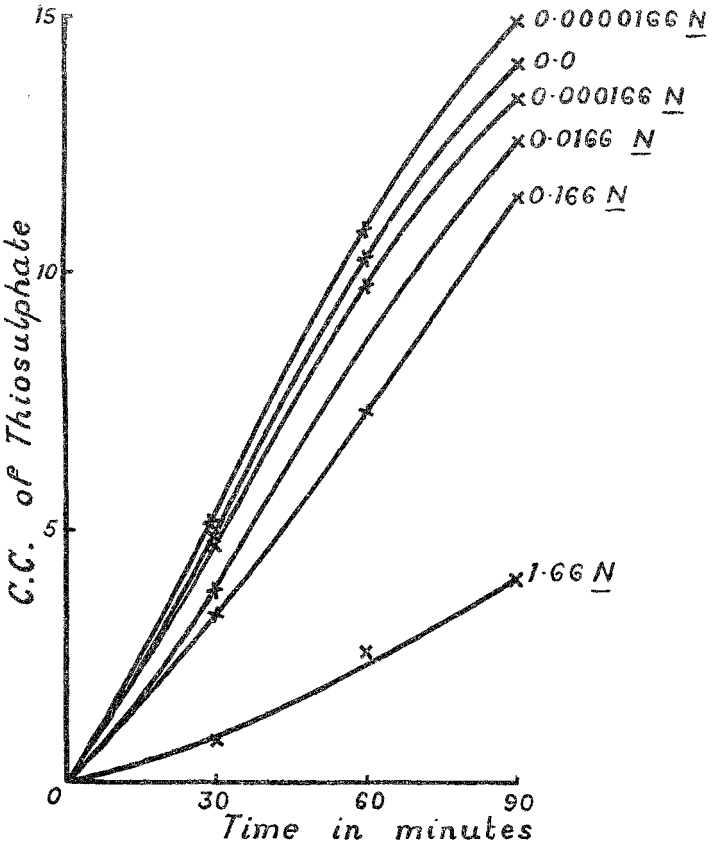
15
10
5
0
C.C. of Thiosulphate
30
60
90
Time in minutes
0·0000166 N
0·0
0·000166 N
0·0166 N
0·166 N
1·66 N

Fig. II

molecules should be greatest near the isoelectric point of the surface concerned, which has been found to be the case in hydrogen peroxide decomposition at a variety of surfaces (Wright and Rideal, *Trans. Faraday Soc.*, 1928, **24**, 530).

Influence of neutral salt concentration.—The foregoing observations suggest that the action of neutral salts on enzymes alters their electro-kinetic potential, and consequently the adsorption and decomposition of neutral molecules at enzyme surfaces. This receives support from the results shown in Fig. I and also from the influence of neutral salt concentration. As will be seen from Fig. II, where the order of addition was enzyme, neutral salt, amylose, the action of sodium chloride on diastase varies with the concentration of the salt. At concentrations varying from 1·66*N* to 0·000166*N* it had an inhibiting action, while at 0·000016*N* it had a slightly accelerating action. The possible explanation of the different concentration effects seems to be that the neutral salt at very low concentration reduces the charge on the enzyme particle; this does not involve any great decrease in stability, because before coagulation it must not only be discharged but also desolvated. The adsorption of electrically neutral amylose is thereby increased, thus accelerating the speed of hydrolysis. On the other hand, with increasing concentration of the salt, probably the charge on the particle is neutralised and reversed in sign, and at still higher concentrations increased in magnitude thus cutting down the power of adsorption. Though what has been outlined above seems probable, it should not be forgotten that electrolytes at very high concentrations may coagulate the enzyme particles and so decrease the active surface. It has also been observed in the case of other colloidal systems that electrolytes which at high concentrations act as discharging agents may increase the charge at low concentrations thus increasing the stability and probably the dispersion also. It is hoped that electro-kinetic potential and other physico-chemical measurements in progress will throw light on this question. It must also be mentioned that adsorption alone cannot account for the hydrolysis. The activation of water has been a matter of speculation. Recently Bancroft (*J. Physical Chem.*, 1926, **30**, 1194) has suggested that the action of neutral salts shifts the water-equilibrium, and has adduced indirect evidence in favour of this hypothesis by which L. E. Bowe has explained the beneficial action of neutral salts on acid hydrolysis. It has been suggested that at the interface, an oriented layer of molecules (Harkins, *J. Amer. Chem. Soc.*, 1925, **47**, 2083) may be largely composed of monohydrol. King and Lassieur (*Compt. rend.*, 1923, **117**, 109) have suggested that the monohydrol is a conductor of electricity while polyhydrols are not; this indicates that water in the monohydrol form is more reactive. Briggs (*Colloid Symposium monograph*, 1928, **6**, 41) finds that with alkali salts the surface conductance of cellulose

increases with increasing concentration while the zeta potential falls; in the case of thallium and aluminium salts, however, the surface conductance also falls. He believes that the surface conductance is to be attributed to the monohydrols and thus thinks that the water equilibrium is favourably shifted with alkali salts.

Influence of neutral salt at various P_H ranges.—The results given in Tables I and II indicate that the neutral salt is practically without any effect at the two P_H optima, has an accelerating effect on the acid side of the optimum P_H and that inactivation is caused on the alkaline side.

TABLE I.

The optimum P_H.

P_H.	...	6·02	5·57	5·3	5·1	4·99	4·8	4·63	4·43	4·05	3·42
Activity in c.c. thiosulphate	...	4·4	5·3	6·0	5·4	6·2	5·7	5·5	4·8	4·1	0·4

The reaction mixture contained 15 c. c. of 0·58 per cent. amylose, 5 c. c. of enzyme, 5 c. c. of buffer of required P_H and 5 c. c. of either water or 0·0000166*N* sodium chloride. 1 c.c. of toluene was added as antiseptic.

TABLE II.

P_H.	Maltose in c.c. thiosulphate		
	Without neutral salt.	With neutral salt.	Change in activity.
6·02	8·9	8·1	− 0·8
5·37	9·3	9·2	− 0·1
4·99	9·3	9·4	+ 0·1
4·27	6·2	7·3	+ 1·1

Further support to the theory of diastase action outlined above is given by the observations recorded by several other workers. Höber observed that salts were without effect on the acid side of the isoelectric point of the colloidal part of the enzyme complex, and had an inhibiting action on the alkaline side. Sherman found that the isoelectric point of barley malt diastase lies near its optimum P_H. Impure invertase wanders to the anode, and after kaolin adsorption it wanders to both poles. According to Michaelis the undissociated

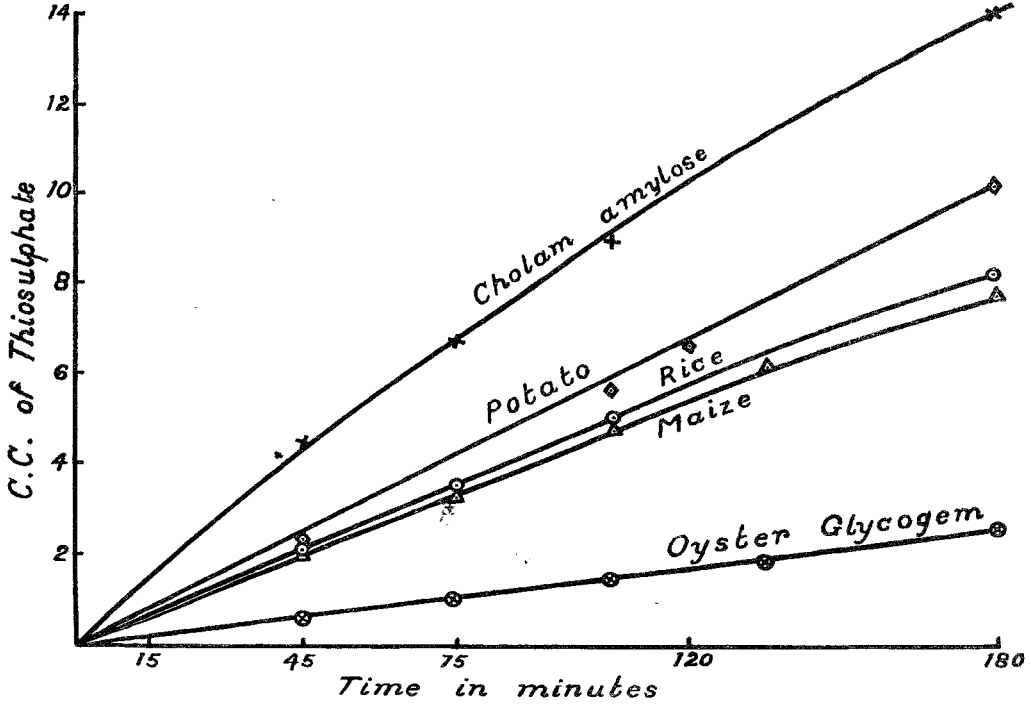
C.C. of Thiosulphate
14
12
10
8
6
4
2
Cholam amylose
Potato
Rice
Maize
Oyster Glycogem
15
45
75
120
180
Time in minutes

Fig. III

molecule is active in the case of invertase. It has been shown by the author (*J. Indian Inst. Sci.*, 1929, **12A**, 109) that tyrosinase acts best near its isoelectric point. Reiner has shown that frequently the end reaction in electro-dialysis is the isoelectric point of the colloid concerned; this probably explains partly the increase in activity on electro-dialysis and also the shift of the optimum P_H which frequently varies with conditions. It was noticed by the author that the optimum P_H of cumbu diastase varies with age, and a similar shift has been observed by Sherman (*J. Amer. Chem. Soc.*, 1928, **50**, 2532) on addition of salts. Hahn and Hapruder found that the optimum P_H lies in a more alkaline P_H with phosphate buffer than with acetate. Rohman (*Kolloid Z.*, 1926, **39**, 159) found that the isoelectric point with a citrate buffer lies between 4·4 and 4·6, in the case of a phosphate buffer between 5·4 and 5·7.

Divergent results have been obtained with different starches. Stone (*U. S. Dept. Agric. Bull.*, 1896, **34**, 29) found potato starch to be the most easily hydrolysed, then wheat and last maize. Sigmond (*Woch. Brau.*, 1897, **14**, 412) found a similar sequence, but according to Barnatzky (Karrer, *Polymere Kohlenhydrate*, 1925, 43), rice-starch is the most easily hydrolysed. Sherman and Baker (*J. Amer. Chem. Soc.*, 1916, **38**, 1885) noticed that amylose is more easily hydrolysed than amylopectin. Many have tried to correlate the varying amylopectin content with the varying resistance towards diastase, but according to Samec potato starch is richest in amylopectin and according to most investigators it is the most easily hydrolysed. Moreover, Sherman (*J. Amer. Chem. Soc.*, 1919, **41**, 1123) found that the different starches on washing with dilute alkali were hydrolysed at the same rate. It was found by the author that the hydrolysability of the different starches decreased with increasing ash-content. Thus cholam amylose was most easily hydrolysed, then potato starch, next rice and last maize. So there is hardly any meaning in the assumption that the different starches vary in composition. The differences may be entirely attributed to the inorganic and other impurities accompanying them and the physical characteristics of the starch. The results are given in Fig. III. Some unpublished experiments of the author also seem to support the same view. Amylose was found to be hydrolysed at the same rate as amylopectin. On another occasion amylopectin was hydrolysed more easily than amylose and yet in a third separation amylose was more rapidly hydrolysed. A systematic investigation of the question has been undertaken.

In this connection it is interesting to record the results of some experiments on the hydrolysis of amylopectin prepared by two different methods. Samec's method is well known. The other method devised by the author was based on the following considerations. Weimarn

has shown that by mere grinding of substances (sometimes with the addition of an indifferent substance) at low temperatures even denatured protein can be brought back into solution. Low temperatures, especially that of liquid air confer brittleness on the substance so that it can be ground very fine, and the meal is easily peptised by water. Starch has to be gelatinised to effect a thorough disintegration of the granule before amylopectin is separated from it. For gelatinisation either heating under pressure or chemical additions have been tried and have the disadvantage of effecting chemical changes. It was interesting therefore to effect the disruption of the granule by a purely mechanical process. Applying Weimarn's idea the starch was exposed to the temperature of liquid air, ground finely in an agate mortar and the treatment repeated several times; it was then dissolved in hot water and the amylopectin separated by electro-dialysis. The results, given in Fig. IV, clearly indicate that the amylopectin prepared by the liquid air method is more easily hydrolysed, it being immaterial whether the comparison is made with or without buffer.

SUMMARY.

The order of adding the reaction mixture components has a marked influence on the activity of the enzyme; the maximum effect of the neutral salt is obtained when it is added to the enzyme before adding the substrate.

Sodium chloride at a concentration of $0{\cdot}000166N$ has a slightly accelerating action on cholam malt diastase. At concentrations of $0{\cdot}000166N$ and above it has an inhibiting action. Sodium chloride of $0{\cdot}000166N$ in buffered solutions has an accelerating action on the acid side of the optimum P_H, no action at the optimum P_H and a retarding effect on the alkaline side.

Experiments on the hydrolysis of different starches, and amylopectin prepared by two different methods are described.

The mechanism of diastase action has also been discussed.

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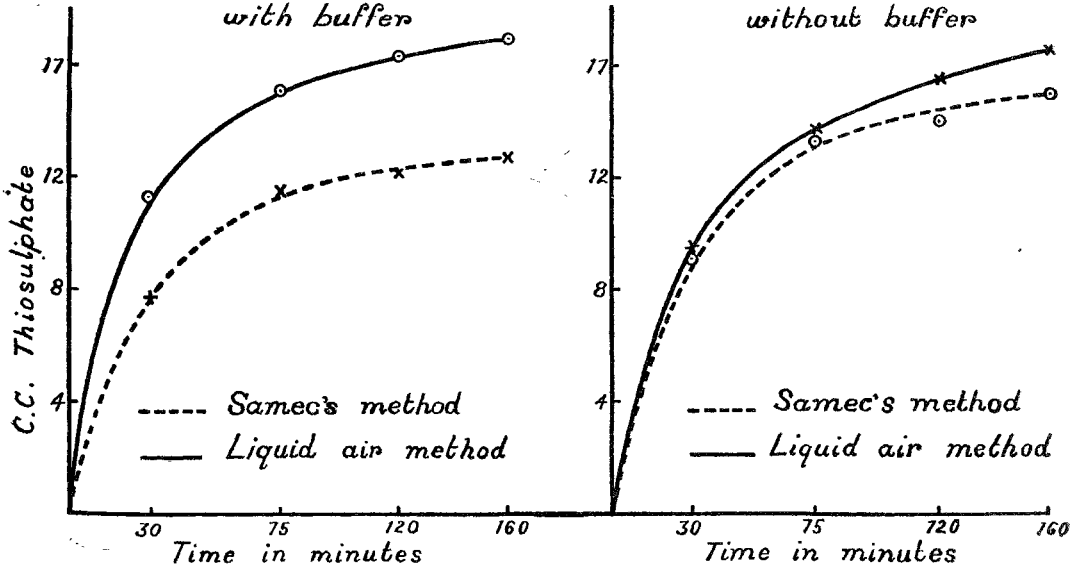
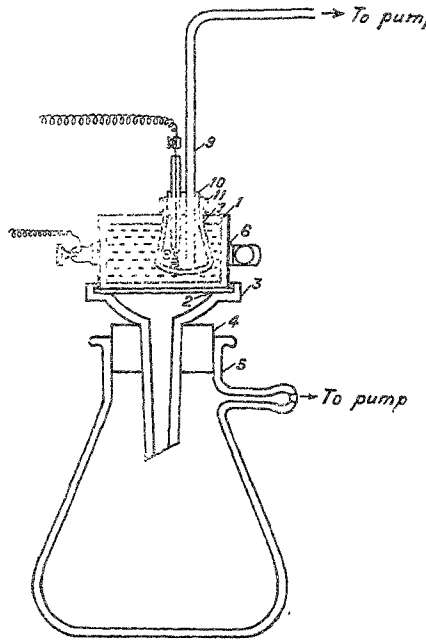

with buffer
without buffer
C.C. Thiosulphate
17
12
8
4
Samec's method
Liquid air method
30
75
120
160
Time in minutes

Fig IV



After Bechhold

1. *Cylindrical dish.*
2. *Rubber washer.*
3. *Funnel.*
4. *Rubber stopper.*
5. *Suction flask.*
6. *Brass collar nickel-plated.*
7. *Flask.*
8. *Spiral platinum electrode.*
9. *Glass tube.*
10. *Rubber stopper.*
11. *Clamp.*

Fig. I.