STUDIES IN ENZYME ACTION.

Part II.—The Nature of Amylase.

By D. Narayanamurti and Roland V. Norris.

It is well established that the diastatic hydrolysis of starch to maltose is no simple process but rather a series of reactions. Knowing that enzymes are specific to a high degree, it is very improbable that all these different processes (Oppenheimer, *Die Fermente*, Vol. I, 665) are catalysed by one and the same enzyme. It is no wonder, therefore that during the past fifty years the view has been repeatedly put forward that diastase, from whatever source, consists of at least two enzymes.

Grützner (*Pfluger's Archiv*, 1876, **12**, 285) found that heating salivary diastase for a short period at 80° inhibited the saccharifying activity without affecting its power of liquefying starch. Wijsman (*Rec. Trav. Chim.*, 1890, **9**, 1) was the first to express the two-enzyme theory in a definite form. From evidence based on some diffusion experiments, he concluded that diastase is a mixture of two enzymes, one hydrolysing starch to maltose and 'erythro-granulose', and another which converts this erythrogranulose to leuco-dextrin and starch to malto-dextrin.

Pottevin (*Ann. Inst. Pasteur*, 1899, **13**, 665) arrived at conclusions very similar to those of Grützner. He found that malt-diastase, when heated for a short period at 80°, lost its saccharifying power while retaining its capacity to liquefy starch. This has recently been confirmed by E. Ohlsson (*Compt. rend. Soc. Biol.*, 1922, **87**, 1183). Fränkel and Hamburg (*Hofm. Beitr.*, 1906, 8, 389) claimed that they had separated malt diastase into two components by dialysis, the sugar-producing enzyme diffusing out and the liquefying enzyme remaining behind in the dialyser. Reinitzer (*Z. physiol. Chem.*, 1909, **61**, 352) working with acacia gum diastase made the observation that the saccharifying enzyme was held back by Pukall filters, while the liquefying enzyme passed through. This is of some interest with reference to our observation.

Chrzasez (*Woch. Brau.*, 1911, **28**, 510; *ibid.*, 1912, **29**, No. 41–44; *ibid.*, 1913, **30**, 41–42; *Zeit. Spiritus Ind.*, **31**, 52; **32**, 520; **33**, 66; **34**, 545; **35**, 630 and *Biochem. Z.*, 1923, **142**, 417; *ibid.*, 1924, **150**, 60) also recognised three distinct stages in starch hydrolysis, namely, liquefaction, dextrin-formation and sugar-production. He could effect

a selective alteration of any of these processes by alcohol precipitation, application of high temperature, or ageing of the enzyme. He claims to have effected a separation of the enzymes by fractional precipitation with ammonium sulphate. He also observed that the relation between saccharification, liquefaction and iodine coloration varied with the origin of the malt. Effront (*Compt. rend.*, 1922, **174**, 18) found the ratio

$$\frac{100 \times \text{saccharification power}}{\text{liquefaction power}} \left(\frac{SP}{LP}\right)$$

to vary from 0·1 to 2,400, depending on the source of the enzyme. The experiments of Windisch, Dietrich and Beyer (*Woch. Brau.*, 1923, Nos. 40, 49, 55, 61, 67) also suggest that the ratio SP/LP is not constant for all malts.

Holmbergh (*Z. Physiol. Chem.*, 1924, **134**, 68; *Biochem. Z.*, 1924, **145**, 244) found that the iodide-ion accelerated dextrin formation by liver diastase, but suppressed the saccharification. The experiments of Hizume (*Biochem. Z.*, 1924, **146**, 52) indicated on the contrary that the ions Cl, Br, NO_3, SO_4 . . . affect liquefaction and saccharification by diastase in the same manner. Fricke and Kaja (*Ber.*, 1924, **57**, 313) found that during electro-dialysis of barley malt diastase, with increasing purification the liquefying power decreased with an increase in the saccharifying power.

It was observed by one of us (*Agric. J. India*, 1923, **18**, 362) that cholam malt diastase is a more powerful liquefier and a weaker saccharifier than the corresponding barley enzyme. The experiments lend further support to the two-enzyme theory and indicate the presence in cholam malt of the liquefying enzyme in a greater proportion than the saccharifying enzyme.

In view of all these observations, sometimes conflicting, efforts have been made to arrive at some definite conclusion regarding the nature of diastase, and a separation of the two or more enzymes by some physico-chemical method has been attempted. Of such methods electro-dialysis, electro-osmosis, ultra-filtration, electro-ultrafiltration and adsorption seem to be the most promising. In this paper some preliminary experiments on the purification of cholam malt diastase by electro-dialysis and the separation of the component enzymes by electro-osmosis are described.

EXPERIMENTAL.

Electro-Dialysis.

An electro-dialyser very similar to that of Fricke and Kaja (*loc. cit.*) was used. In principle this consists of three compartments, of

which the middle, bounded by parchment membranes, held the enzyme solution. Through the outer cells distilled water was allowed to flow continuously in a thin stream. The main laboratory supply (110 volts, D.C.) was used as the source of current. Three thick arc carbons served as anode and a platinum foil (1½ sq. in.) was used as cathode. An aqueous extract of cholam malt prepared according to Euler (*Z. physiol. Chem.*, 1920, **112**, 193) was used and electro-dialysis allowed to proceed for four days. The conductivity had then almost reached that of the distilled water, but the solution still reduced Fehling's solution. The anode cell solution also showed reducing properties, while the cathode liquid was non-reducing. The P_H of the middle cell solution also changed during the course of the electro-dialysis. Too great an acidification was however avoided by the addition of a few c.c. of very dilute caustic soda, when necessary.

After the completion of the electro-dialysis, the solution was taken out, filtered from precipitated globulin and its activity compared with that of a solution dialysed in an ordinary parchment beaker under exactly the same conditons but without the electric current. The results are represented in Fig. I.

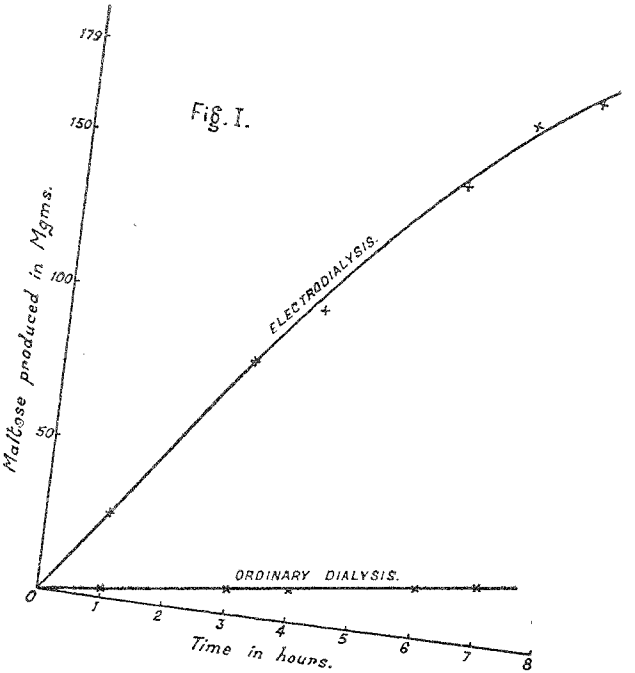
Regarding the chemical nature of the enzyme it is premature to speculate; the solution of the electro-dialysed enzyme gave none of the protein reactions but was strongly positive to the Molisch test.

Electro-Osmosis.

Fricke and Kaja used electro-osmosis for the purification of enzymes from other colloids. We have used this method in our attempts to separate the two enzymes in cholam malt diastase. It was observed by Fricke and Kaja (*loc. cit.*) that the liquefying power of malt diastase decreased while the saccharifying power increased with the progressive purification by electro-dialysis, suggesting that the liquefying enzyme might have migrated. Our experiments seem to confirm these expectations, for on electro-osmosis we found the ratio SP/LP to be different for the different fractions. The experimental arrangement was as follows :—

The same electro-dialyser was used but it now consisted of five cells. A_1 and A_2 (see Fig. II) were parchment membranes, C_1 and C_2 membranes made of 6 per cent. collodion. The enzyme solution (dialysed cholam malt diastase prepared by Euler's method) was put in the first chamber and distilled water in the other cells. To avoid the possible destruction of the enzyme electro-osmosis was carried out at about 20 volts, tapped by means of a potentiometric arrangement as shown in the diagram. The electro-osmosis was allowed to proceed

Fig. I.


179
150
100
50
0
Maltose produced in Mgms.
ELECTRODIALYSIS.
ORDINARY DIALYSIS.
1
2
3
4
5
6
7
8
Time in hours.

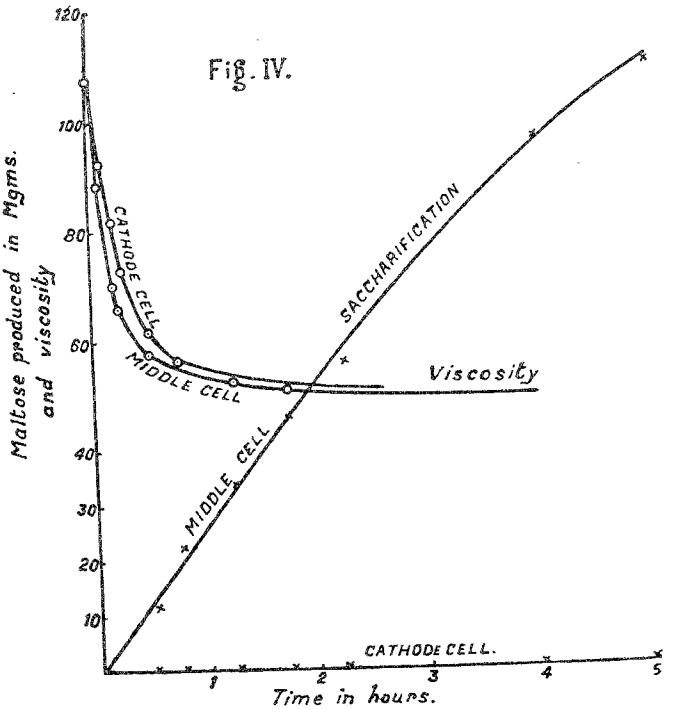


Fig. IV.
Maltose produced in Mgms. and viscosity
120
100
80
60
40
30
20
10
CATHODE CELL
MIDDLE CELL
SACCHARIFICATION
Viscosity
MIDDLE CELL
CATHODE CELL.
1
2
3
4
5
Time in hours.

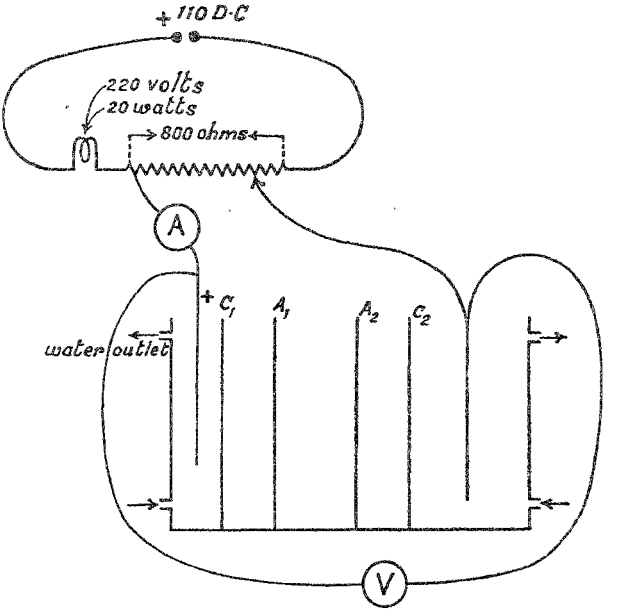
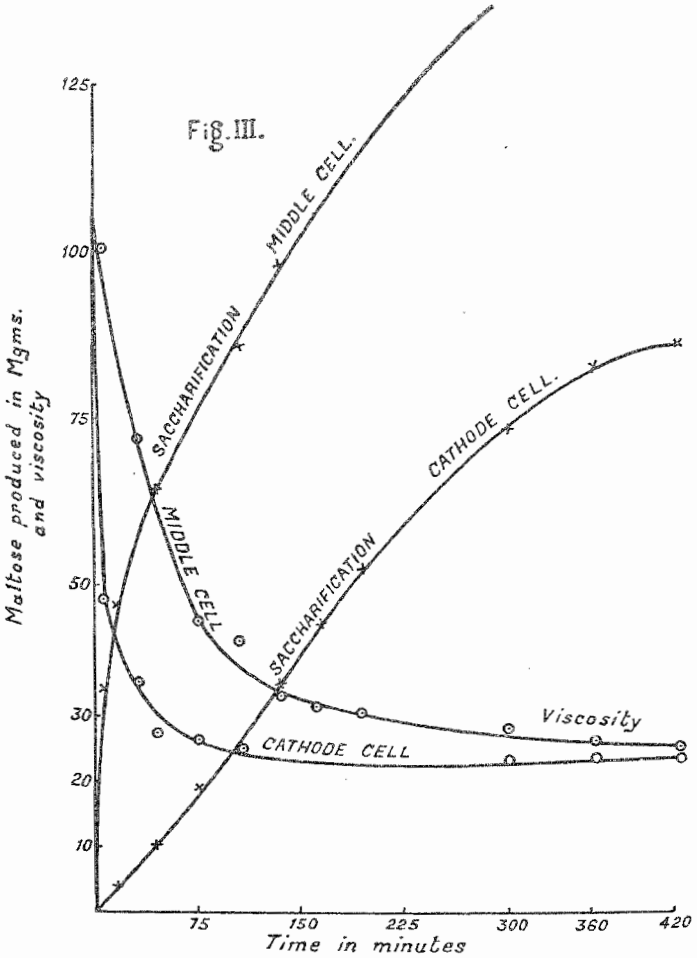


Fig. II.

Fig. III.


125
100
75
50
30
20
10
Maltose produced in Mgms. and viscosity
MIDDLE CELL.
SACCHARIFICATION
MIDDLE CELL
SACCHARIFICATION
CATHODE CELL.
Viscosity
CATHODE CELL
75
150
225
300
360
420
Time in minutes

for three days after which the several fractions were examined for their liquefying and saccharifying activities. The liquid in the middle cell was predominantly saccharifying, that in cell 3 (cathode cell) was essentially liquefying. This is well illustrated by the accompanying curves (Fig. III).

Since the cathode cell liquid has a greater liquefying power and a lower saccharifying power than the middle cell liquid it is impossible to explain the different activities of the two solutions on the ground of mere difference in enzyme concentration, for if both the properties are due to one and the same enzyme they should be affected to the same extent and in the same direction. It is but reasonable to assume the existence of two enzymes, one mainly responsible for the liquefaction, and the other for saccharification. This view gains further support from another series of experiments in which we were able to obtain a cathode-wandering enzyme almost completely devoid of saccharifying power but still able to liquefy starch (see Fig. IV).

It is well known that neutral salts, especially chlorides, activate animal diastase. We therefore tried to ascertain whether it would be possible to activate the cathode side solution (separation I); but our experiments (see Fig. V) indicate that if there is any action at all it is one of slight inactivation. Fricke and Kaja observed inactivation by neutral salts in the case of their electro-dialysed enzyme. Patwardhan and Norris (Pt. I of this series) found activation with malted cholam diastase and inactivation at high salt concentration with unmalted cholam diastase. Eadie (*Biochem. J.*, 1926, **20**, 1016) found slight activation at high salt concentration (1·62 *N*). Hahn and Hapruder (*Z. Biol.*, 1919, **71**, 287, 302) distinguished the effect of neutral salts at three ranges (*a*) optimal P_H, (*b*) acid side of optimal P_H and (*c*) alkaline side of optimal P_H. At the optimal P_H salts were without any effect, on the acid side they had an inhibiting action, and on the alkaline side acceleration was caused. Our experiments were made without buffer and the P_H was 5·4. According to Patwardhan and Norris (this *Journal*, 1928, 11A, 132) the optimal P_H of cholam diastase is about 4·6. If this be true in the case of our electro-dialysed enzyme also, our results agree with those of Fricke and Kaja, disagreeing with those of Hahn and Hapruder. Further experiments are also in progress to test the observations of Hahn and Hapruder at various P_H values as also their observation that the electrical nature of the enzyme is of no consequence.

We consider that in our experiments at least a partial separation of the enzymes has taken place. It will be interesting to ascertain whether (*a*) complete separation of the enzymes can be effected and

(*b*) the saccharifying enzyme can carry on its activities in the absence of the liquefying component.

Oppenheimer (*Die Fermente*, Vol. I, 640 and 677) believes the liquefying enzyme to be responsible for the breaking down of the colloidal aggregate, a depolymerisation, which is probably nothing more than the loosening (dissolution) of subsidiary valence bonds. According to him the enzyme is not a 'hydrolase' but a 'hydratase' which brings about the breakdown of molecular linkages by wedging in of water molecules.

Karrer (*Helv. Chim. Acta*, 1921, **4**, 678; *Polymere Kohlenhydrate*, 1925, p. 48) also inclines to the same view while he postulates, as a logical consequence of the present conception of starch-constitution, the existence of two enzymes one depolymerising and the other anhydride-splitting. Others believe that the function of the liquefying enzyme is to convert starch into dextrins which are then easily hydrolysed to maltose while some believe that it is mainly concerned with the splitting of amylopectin. It is generally recognised that starch consists of two components, amylose and amylopectin; and it is known that amylopectin is mainly responsible for the viscosity of starch. The investigations of Samec and his collaborators show that the ratio of amylose to amylopectin varies with the origin of the starch. According to Ling and Nanji this is always 2 : 1, others give various ratios differing from Samec. Experiments are in progress in these laboratories to standardise the methods of separation and arrive at some definite conclusions on this point.

Samec believes that the viscosity is associated with the phosphorus content of the amylopectin. This suggests that the enzyme splits off this organically bound phosphorus thus lowering the viscosity. Adler's work (*Biochem. Z.*, 1915, **70**, 1) indicates the presence of phosphatases in malt; based on these researches Euler (*Chemie der Enzyme*, Part II, 1922, p. 59) postulates an amylophosphatase (starch-phosphatase) which will liberate phosphoric acid from amylopectin. Compare Ling and Nanji (*J.C.S.*, 1925, **127**, 639; Oppenheimer, *Lehrbuch der Enzyme*, 1927, p. 310); but according to Samec (*Kolloid-chemie Beihefte*, 1914, **6**, 23) while the lowering of viscosity in amylopectin solutions on boiling proceeds with the cleavage of phosphoric acid from the carbohydrate molecule, the fall in viscosity during the hydrolysis of starch by diastase is not similar.

With a view to elucidating the mechanism of liquefaction, experiments have been initiated in continuation of our studies on the kinetics of diastatic action.

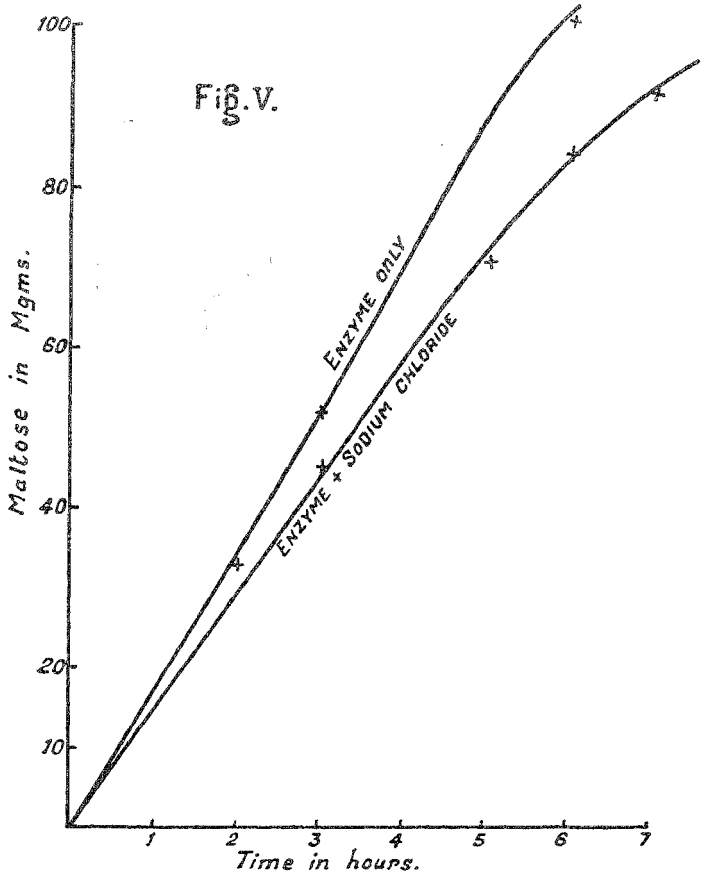


Fig. V.
100
80
60
40
20
10
Maltose in Mgms.
ENZYME ONLY
ENZYME + SODIUM CHLORIDE
1
2
3
4
5
6
7
Time in hours.

SUMMARY.

1. Cholam malt diastase is considerably increased in activity on electro-dialysis.

2. Electro-dialysed cholam malt diastase gives none of the usual protein reactions, but is strongly positive to the Molisch test.

3. By electro-osmosis cholam malt diastase is separated into two fractions, a good saccharifier and a powerful liquefier.

4. The work in progress to decide between the various theories on the mechanism of starch-liquefaction by diastase is described.

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