STUDIES IN ENZYME ACTION.

Part I.—Amylase from Cholam (Sorghum vulgare).

By V. N. Patwardhan and Roland V. Norris.

Since the discovery by Kirchoff in 1813 that a crystalline sugar is produced by the action of 'vegetable albumin' on starch and the observation of Musculus in 1840 that sugar and dextrins are produced simultaneously during this reaction by a process of hydration, no enzyme has received closer study than diastase or, to use the modern nomenclature, amylase. The results obtained by various workers have been very contradictory in regard to some particulars however, and even in the more recent work the same divergencies persist. Among the points in doubt has been the question as to whether amylase is an individual enzyme or a mixture. Marker early stated that at 60° four molecules of starch yield three of maltose and one of dextrin; hydrolysis at 65° gave a reduced yield of maltose and at higher temperatures he found the proportion of maltose to dextrin to be as two to one. He therefore considered the process to involve two enzymes, of which the one chiefly responsible for sugar production was more sensitive to heat than the other. Brown and Morris (*J.C.S.*, 1890, **57**, 458) also considered that two enzymes were concerned and these were named by them 'translocation diastase' and 'secretion diastase' respectively; the former acted only on soluble starch but did not hydrolyse starch paste. Other workers have opposed this view considering that variations in the property of the enzyme could be explained by physical changes induced in the latter by the treatment to which it had been subjected.

It has long been known that starch is not a single substance, but is composed of at least two constituents, amylopectin and amylose (Gatin-Gruzewska, *Compt. rend.*, 1908, **146**, 540; Maquenne and Roux, *Compt. rend.*, 1905, **140**, 1303); this fact alone might be considered to lend some support to the two enzyme theory. Ling and Nanji (*J.C.S.*, 1923, **123**, 2666) have shewn that while the enzyme of unmalted barley can hydrolyse amylose it is without action on amylopectin, suggesting that the enzyme capable of hydrolysing amylopectin is only developed on germination of the grain. In other words, it corresponds to the secretion diastase of Brown and Morris.

Further evidence in favour of two enzymes is forthcoming when amylase from sources other than barley is compared with barley enzyme. Work on such lines has not been extensive, though the results of Stone and Wright (*J. Amer. Chem. Soc.*, 1899, **20**, 637) and

of Waksman (*J. Amer. Chem. Soc.*, 1920, 42, 293) on taka diastase, of Chrzasez (*Bied. Zentr.*, 1910, 39, 641) and of Baker and Hulton (*J.C.S.*, 1921, 119, 805) on the amylase of rye are of much interest.

In 1923, while examining various S. Indian grains, Norris and Viswanath (*Agric. J. India*, 1923, 18, 3[illegible]) compared the action of barley diastase with the enzyme derived from cholam (*Sorghum vulgare*) and shewed that their relative activity varied according to whether the saccharifying or liquefying power was under consideration, cholam enzyme bringing about much more rapid liquefaction of starch than barley enzyme, though the formation of sugar was much slower. This observation supports the idea that two enzymes are concerned and that the proportion in which the two components are present varies in different grains.

In the present paper the results of a more detailed examination of cholam diastase are given, the direct object of the investigation being to obtain more definite evidence of two enzymes being present. Though the results afford but little fresh evidence of this view, several facts of interest have emerged. These will be discussed at a later stage but we may point out here that the enzyme of unmalted cholam differs in at least one important respect from the enzyme of unmalted barley. Ling and Nanji shewed that the latter is without action on amylopectin and we confirm this result. In the case of cholam, however, the enzyme from the unmalted grain appears to resolve amylopectin into a mixture of two dextrins.

To investigate whether amylase is an individual or a mixture two lines of inquiry suggested themselves: (1) If there are two enzymes in amylase the conditions of reaction might be changed in such a manner that the two constituents would be affected unequally; hence the experimental conditions have been widely varied and the results of such changes closely studied: (2) A comparison of the products obtained by the action of cholam amylase on potato starch paste with those formed by the barley enzyme. These results will be discussed in a subsequent paper.

Experimental Methods.

Preparation of enzyme.—The process consists of two steps: (1) Malting the grain from which amylase is required; (2) Extraction and precipitation.

(1) In their researches on the malting of cholam, Norris and Viswanath (1923) have given the optimum conditions. Before germination the grains were steeped in cold running water for 30–36 hours and were spread on a bed of moist sand, the germination being

conducted at 20–25°. In three days the seedlings showed nearly half an inch of plumule when the germination was arrested by washing out the sand and drying the seedlings in the sun. The air-dried seedlings were maintained at 50° during 72 hours and then at 70° during 24 hours, being finally dried at 100°. The dried plumules and radicles were removed by light crushing and blowing, and the grain powdered.

(2) The method of extraction and precipitation suggested by Ling and Baker (1895) was used for obtaining the enzyme. The powdered malt (200 gms.) was extracted with 600 c.c. of cold 20 per cent. ethyl alcohol for 24 hours and filtered; 1,500 c.c. of 95 per cent. alcohol was slowly added with stirring and the precipitate allowed to settle during 24 hours when the alcohol was siphoned off, the precipitate being washed and dehydrated with stronger alcohol and finally with ether. It was dried in a vacuum over phosphorus pentoxide at the ordinary temperature. Enzyme preparations were obtained in this way from barley and cholam, both malted and unmalted.

Sometimes when using amylase from unmalted barley considerable difficulty was experienced in filtering the cuprous oxide precipitate owing to starch remaining in the reaction mixture. In such cases we used Peter's method of estimating unreduced copper by standard thiosulphate (*J. Amer. Chem. Soc.*, 1912, **34**, 422), filtration being thus avoided.

The degree of liquefaction was determined by one of the following methods:—(*a*) The reaction mixture (10 c.c.) was boiled and treated with 20 c.c. of 95 per cent. alcohol to precipitate the non-saccharine substances; the precipitate was allowed to settle, washed, dehydrated with stronger alcohol and absolute ether, dried at 100° and weighed. (*b*) The change in viscosity of the reaction mixture itself was measured at intervals, Ostwald's simple viscosimeter being used; this gave results identical with those obtained by method (*a*).

EXPERIMENTAL.

1. Comparison of Amylases from Malted Barley and Malted Cholam.

The reaction mixture was composed of 40 c.c. starch paste (2 per cent. potato starch), 10 c.c. enzyme solution (0·251 gm. enzyme in 50 c.c.), 30 c.c. water and 1 c.c. toluene.

After mixing the solutions, 10 c.c. was immediately transferred to a viscosimeter maintained at 30° and viscosity readings taken at

regular intervals. The remainder was kept in a thermostat at 30° and in 10 c.c. portions the sugar was determined at regular intervals.

TABLE I.

Barley Malt Amylase.

Time in mins.	Sugar formed ; mgms. of maltose in 10 c.c.	Viscosity ; time of flow in secs.
0	0	165·5
15	39·4	109·2
30	58·0	107·5
60	69·5	106·2
100	76·8	106·0
240	83·7	...

TABLE II.

Cholam Malt Amylase.

Time in mins.	Sugar formed ; mgms. of maltose in 10 c.c.	Viscosity ; time of flow in secs.
0	0	177·5
15	3·3	136·0
30	5·5	128·8
60	11·4	117·8
100	18·4	112·0

The results are plotted in Figs. I and II, showing that the amylase from malted cholam is less active in sugar production than the amylase from malted barley ; but for the same amount of sugar produced in the same solution much more starch is liquefied by malted cholam amylase than by the amylase from malted barley under strictly comparable conditions. On comparing the conditions existing in both solutions after hydrolysis for 30 minutes, it will be seen that ten times as much maltose has been produced by the barley enzyme as by that from cholam. The viscosities of the two solutions are not, however, widely different showing that the degree of liquefaction has been of the same order in both cases.

This indicates that two enzymes, amylase and amylopectinase exist in different proportions in the extracts from malted cholam and malted barley and is confirmed when the enzyme from unmalted grain

Fig. I.

Sugar production by Barley and Cholam Enzymes.

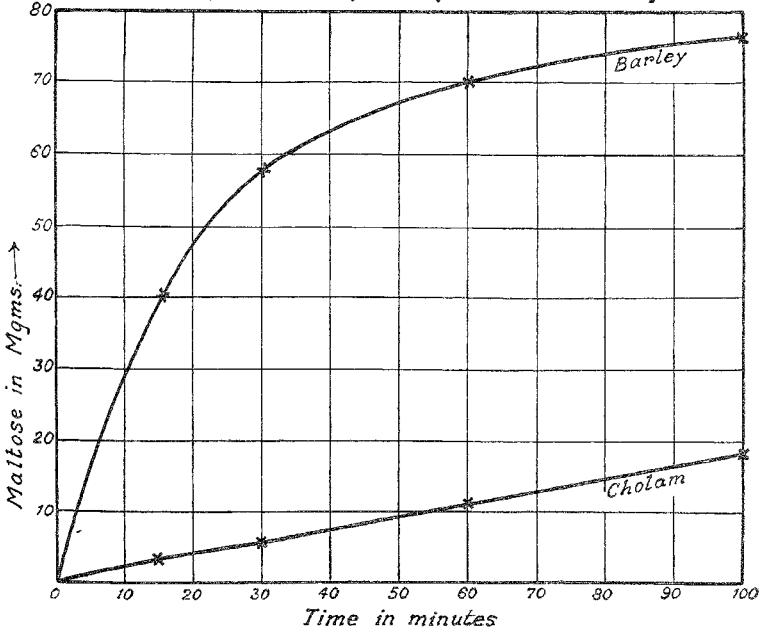
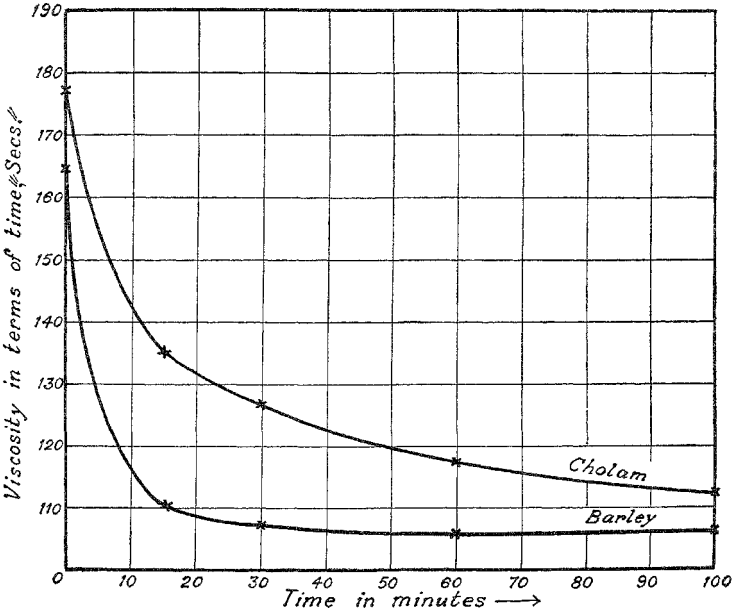


Fig. II.

Liquefaction by Barley & Cholam Enzymes.



is examined, sugar-production with cholam being much less than when enzyme from unmalted barley is used. Under identical experimental conditions the enzyme from unmalted barley produced nearly 1·66 times as much sugar from soluble starch as the enzyme from unmalted cholam did in the same period. These two results appear to indicate that in cholam the proportion of amylase to amylopectinase is less than in barley.

2. Effect of Time on a Solution of Malted Cholam Enzyme.

Before conducting the hydrolysis for prolonged periods it was considered advisable first to ascertain the extent to which activity of the enzyme was affected when kept at 30° in solution.

Starch paste was prepared from 0·869 gm. with 150 c.c. of water; 0·0243 gm. of precipitated enzyme from malted cholam was shaken with water and made up to 50 c.c. Mixtures A and B were made up as follows:—

A contained 75 c.c. of starch paste together with 25 c.c. of fresh enzyme solution brought to 30° with 2 c.c. toluene.

B was identical with A except that the enzyme solution had been kept for 24 hours at 30° in presence of a few drops of toluene, prior to usage.

The mixtures were kept in a thermostat at 30° and the sugar formed in them at different intervals determined. The results are given in Table III.

TABLE III.

Time in mins.	Maltose in mgms.	Time in mins.	Maltose in mgms.
30	4·53	30	4·53
60	8·5	60	6·8
140	10·76	120	9·63
245	...	180	11·33
1415	25·49	1680	20·96

The results show that the activity of the enzyme is affected only slightly by keeping it in solution for reasonable periods at 30°.

3. Effect of Temperature on the Hydrolysis of Starch by Cholam Malt Amylase.

Eight portions of 10 c.c. 2 per cent. starch paste with 7·5 c.c. water were made up and brought to the respective desired temperatures; 2·5 c.c. of 25 per cent. cholam malt extract were added to each.

The figures representing the sugar production and liquefaction after 30 minutes of hydrolysis at the temperatures studied are represented in Figs. III A and III B. The results show that if the temperature is raised the rate of hydrolysis first increases, then attains a maximum and finally falls again in the normal manner. Both liquefaction and saccharification appear to be almost equally affected by the change of temperature. The optimum stage is between 50° and 55°.

4. Influence of Salts on the Hydrolysis of Starch by Cholam Malt Amylase.

In all the experiments described under this heading the enzyme was shaken with water and the extract dialysed against distilled water either for 24 or 48 hours and then made up to a known volume for use. The fact that the enzyme was dialysed will not therefore be mentioned in every experiment.

It is well established that amylases of animal origin are almost completely inactivated by dialysis and that the activity is readily restored by the addition of certain salts, particularly chlorides. In the case of the enzyme derived from malted grains, however, there is considerable disagreement as to whether the activity is lowered in the same way by dialysis. Some authors (e.g., Eadie, *Biochem. J.*, 1926, **20**, 1016) maintain that this process has no influence at all on the activity of barley malt amylase. The present experiments do not support the latter view as dialysis has always resulted in a reduction of activity which could be restored by the addition of chlorides. The activity, however, is not reduced to the same extent as with amylases of animal origin. The following experiment was carried out to show the effect of adding sodium chloride in various proportions to a dialysed preparation of cholam amylase.

Seven flasks were arranged each containing 15 c.c. of starch paste. Quantities of water and sodium chloride solution as given in the table were added and the mixtures kept in a thermostat of 30°, 5 c.c. of the enzyme (0·08 per cent.) being then added to each. Saccharification and liquefaction were measured after 30 minutes.

Fig. III.(a)

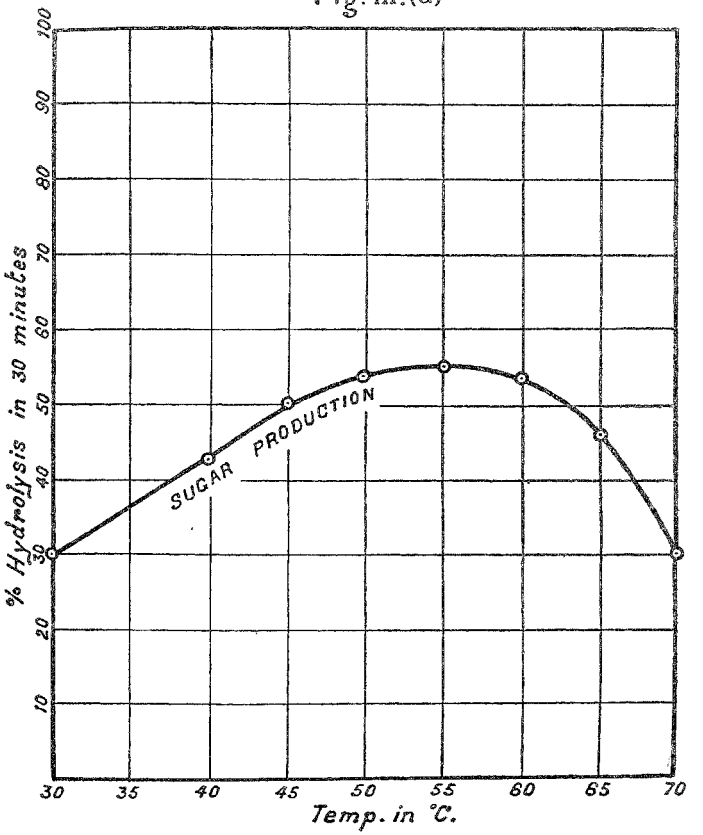

% Hydrolysis in 30 minutes
100
90
80
70
60
50
40
30
20
10
SUGAR PRODUCTION
30
35
40
45
50
55
60
65
70
Temp. in °C.

Fig. III. (b)

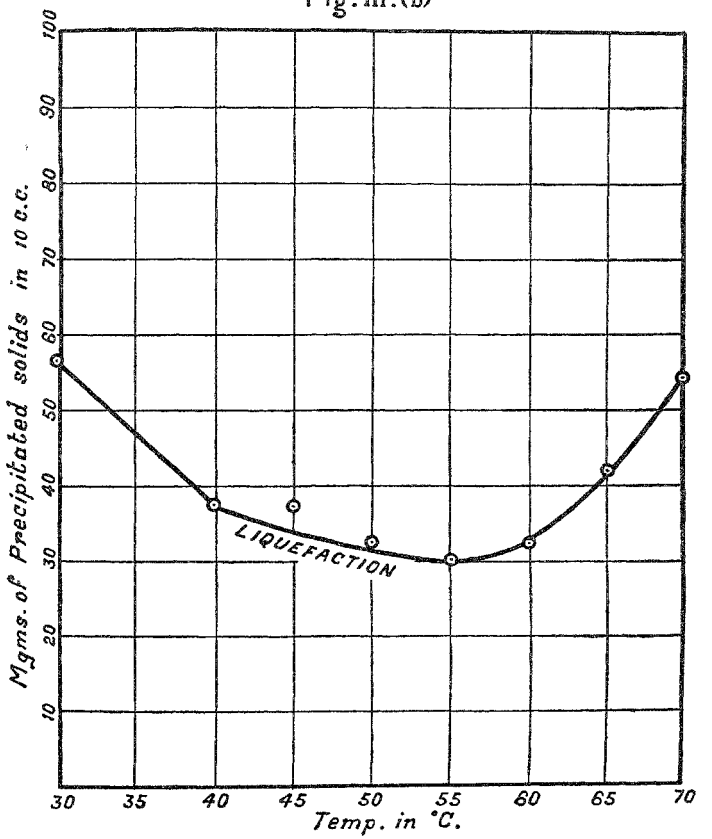

Mgms. of Precipitated solids in 10 c.c.
100
90
80
70
60
50
40
30
20
10
LIQUEFACTION
30
35
40
45
50
55
60
65
70
Temp. in °C.

TABLE IV.

Effect of Sodium Chloride on Cholam Malt Amylase.

Sodium chloride: c.c. of 0·311 per cent.	Water, c.c.	Mgms. of maltose in 20 c.c.	Mgms. of ppt. in 10 c.c.
0·0	5·0	12·8	65·6
0·5	4·5	14·0	61·8
1·0	4·0	20·4	...
1·5	3·5	19·4	94·2
2·0	3·0	16·2	84·2
3·0	2·0	16·2	61·0
4·0	1·0	15·2	85·0

The results show that under the conditions of this experiment the effect of sodium chloride is most pronounced at $N/500$.

Besides sodium chloride other chlorides were studied, the salts being added in their gram-molecular proportions, namely, barium chloride, 1·0000; cupric chloride, 0·6457; potassium chloride, 0·3580; sodium chloride, 0·2808; ammonium chloride, 0·2568.

The reaction mixture was composed of 10 c.c. starch paste (2 per cent.), 10 c.c. enzyme (0·1726 per cent.) and 5 c.c. water with the respective salt. The results are given in Table V.

TABLE V.

Comparison of Chlorides; Cholam Malt Amylase at 30°.

Salt	Mgms. of maltose in 20 c.c.	Mgms. of ppt. in 10 c.c.
$BaCl_2$	11·3	66·1
KCl	12·5	65·5
NaCl	12·5	65·5
NH_4Cl	12·5	65·6

Cupric chloride poisons the enzyme and the hydrolysis does not occur.

It will thus be seen that there is little difference between the various chlorides, and that they affect liquefaction and saccharification equally.

The experiments with sodium chloride were repeated with amylase from unmalted cholam, for which purpose a reaction mixture was prepared from 10 c.c. starch paste (2 per cent.), 5 c.c. enzyme solution, the salt solution (0·4408 per cent.) in the quantity tabulated and water to make the total volume 20 c.c. in each case.

The reaction was conducted at 50° and is extremely slow; the readings were taken after one hour and the results are shown in Table VI.

TABLE VI.

Influence of Sodium Chloride on Unmalted Cholam Amylase.

NaCl in c.c.	..	0	0·5	1·0	2·0	3·0	4·0	5·0
Mgms. maltose in 20 c.c.	...	16·2	17·6	17·6	16·2	14·2	11·7	...

Hence the effect of salt is very small, and increasing amounts depress the rate of hydrolysis. There is considerable evidence that the influence of salts on dialysed enzyme solutions is modified to a marked degree by the hydrogen-ion concentration and the nature of the impurities present. Further work on this point is in progress in this laboratory.

Influence of Amino-acids.

A number of experiments have been made by various workers on the effect of amino-acids on the rate of hydrolysis of starch by amylases (Sherman and Walker, *Amer. Chem. Jour.*, 1921, **43**, 2461), and show that to obtain consistent results three factors are involved, (*a*) purity of the enzyme, (*b*) quantity of sodium chloride and phosphate, and (*c*) control of the hydrogen-ion concentration by means of buffer solutions.

We have observed all these conditions and have obtained results with malt barley amylase in agreement with those of Sherman. We then tried the same experiments with malt cholam amylase in the same concentration of amino-acids.

The reaction mixture was composed of 20 c.c. starch paste (2 per cent.), 20 c.c. McIlvaine buffer solution, P_H 7·0, 10 c.c. enzyme, 10 c.c. sodium chloride (0·055 per cent.) and 10 c.c. water containing the required quantity of the amino-acid. The reaction was allowed to proceed at 30° and the sugar determined as usual at the end of one hour. The results are given in Table VII.

TABLE VII.

Influence of Amino-acids on Malted Cholam Amylase.

(C.c. of $KMnO_4$ required for 20 c.c.)

	Asparagine	Alanine	Glycine	*l*-Leucine	Hippuric acid
Amino-acid ...	2·5	2·6	1·4	0·25	2·2
Control ...	3·4	3·4	3·4	0·4	0·4

The experiments show that hippuric acid alone had any accelerating influence, all the other acids examined having an inhibiting influence on the rate of hydrolysis of starch by cholam malt enzyme. Asparagine, alanine and glycine when used in the same concentrations with barley malt amylase accelerated the rate of hydrolysis. An extensive series of experiments is necessary to explain this anomaly.

Influence of Bye-products ; Maltose.

It is well known that the rate of hydrolysis of starch by amylase diminishes considerably after a certain period, one explanation being that accumulation of bye-products may inhibit the hydrolysis. The influence of this factor in the case of cholam amylase was therefore investigated.

Two solutions were examined at 30°, namely, (*a*) the control, composed of 50 c.c. starch paste (2 per cent.), 15 c.c. enzyme solution (0·32 per cent.), 35 c.c. water and 1 c.c. toluene ; and (*b*) the same solution with 0·55 gm. of maltose dissolved in the water.

The results are given in Table VIII.

TABLE VIII.

Influence of Maltose on Cholam Malt Amylase.

Time in hours	Mgms. of maltose in 10 c.c.	
	A	B
2	20·3	5·18
4	32·0	12·08
24	66·7	35·1
48	78·6	49·88
95	90·3	64·8

The figures in the third column have been corrected for the amount of maltose originally present, and show that there is a decided inhibiting action produced by maltose as with the barley enzyme.

5. Effect of Hydrogen-ion Concentration on the Hydrolysis of Starch by Cholam Malt Enzyme.

Experiments on the effect of hydrogen-ion concentration have been made by other workers and a P_H of 4·5 found to be the optimum reaction for barley malt amylase. Experiments on similar lines upon the cholam malt enzyme have yielded interesting results.

(*a*) *Cholam Malt Extract* (*unpurified*).—Solutions were prepared containing 25 c.c. starch paste (2 per cent.), 5 c.c. extract and 20 c.c. buffer mixture with varying P_H.

These were examined at 30° and the reaction continued during 30 minutes with the results given in Table IX.

TABLE IX.

Influence of P_H on Sugar Production by Cholam Malt Extract.

P_H	5·26	5·07	4·86	4·66	4·48	4·34
C.c. $KMnO_4$ for 10 c.c. ...	5·3	4·95	5·7	6·3	5·8	5·9

The figures in the second line are corrected for the reducing power of the malt extract added. The P_H value was determined electrometrically and the buffer solution used was prepared according to McIlvaine, e.g., a mixture of 0·2 molar disodium phosphate solution and 0·1 molar citric acid.

(*b*) *Precipitated Amylase from Malted Cholam.*—Amylase was prepared by precipitating a 20 per cent. alcoholic extract with 95 per cent. alcohol and was used dry. Solutions were prepared containing 50 c.c. starch paste (2 per cent.), 25 c.c. enzyme (0·1028 per cent.) and 25 c.c. water containing the desired amount of *N*/100 hydrochloric acid. Hydrolysis was carried out at 30° and readings taken after fifteen minutes.

The P_H values were determined colorimetrically. Normally the curve would be expected to show a slow rise, a flat maximum and then a slow fall, but in the present case the sudden irregularity between P_H 5·5 and 4·5 is noteworthy, and was repeatedly confirmed. To eliminate any possible effect of the action of the chlorine ion, *N*/100 sulphuric acid was compared with *N*/100 hydrochloric acid as the acidifying agent with the following results.

Fig. IV.

Effect of H-ion Concentration on Saccharification

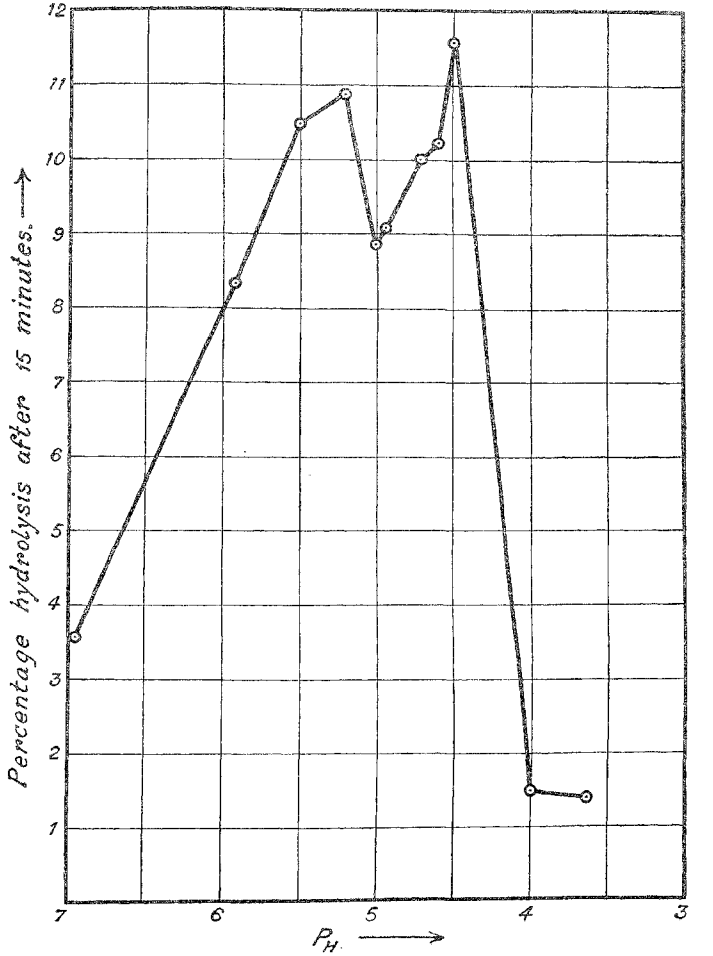


Fig. V.

Effect of H-ion Concentration on Liquefaction by Malted Cholam

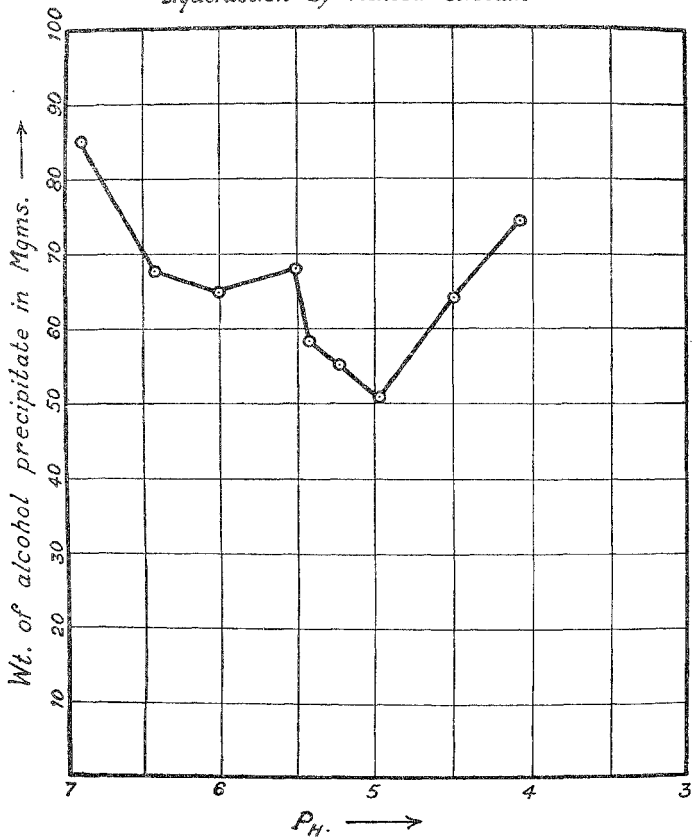


TABLE X.

P_H	HCl c.c. $KMnO_4$ for 10 c.c.	H_2SO_4 c.c. $KMnO_4$ for 10 c.c.
5·5	2·10	2·15
5·0	1·75	1·85
4·8	1·80	1·80
4·7	2·00	1·50
4·6	2·05	1·40
4·5	2·30	1·70
4·4	.....	1·60

The readings were taken after fifteen minutes. Considering that the method gives results accurate to half a drop of permanganate, it may be concluded that the sulphuric acid series show the same abnormality, but the range of P_H value over which it occurs differs a little from that of the hydrochloric series.

Standard buffer solution prepared according to McIlvaine was then used and solutions prepared from 20 c.c. buffer solution of varying P_H, 30 c.c. starch paste (2 per cent.), 7·5 c.c. enzyme (0·1028 per cent.) and 2·5 c.c. water.

The reaction was conducted at 30° and, readings taken after fifteen minutes with results given in Table XI.

TABLE XI.

Effect of P_H on Hydrolysis by precipitated Cholam Malt Amylase.

0. 2/M Na_2HPO_4 c.c.	0. 1/M citric acid c.c.	P_H	Saccharification (per cent. hydrolysis)	Liquefaction (gm. of ppt. in 10 c.c.)
16·5	3·5	7·05	5·21	....
13·5	6·5	6·62	10·20	0·1336
12·1	7·9	6·15	13·75	0·1420
11·6	8·4	5·96	14·03	0·1416
11·2	8·8	5·82	14·96	0 1128
10·9	9·1	5·60	16·37	0·0766
10·4	9·6	5·26	16·79	0·1400
9·9	10·1	5·07	13·38	0·1154
9·4	10·6	4·86	13·38	0·1180
8·9	11·1	4·67	11·23	0·1330
8·4	11·6	4·50	13·28	....
7·8	12·2	4·34	13·75	0·0858
7·3	12·7	4·10	13·75	0·0884
6·8	13·2	3·92	9·16	0·0910
6·3	13·7	3·78	5·21	0·1008
5·8	14·2	3·65	2·73	0·1046

These results show most clearly that an abnormality does exist between the P_H intervals 5·26 and 4·5, and it was therefore necessary to dialyse the enzyme before use. After dialysis the enzyme was added to the reaction mixture in such quantities that the concentration was the same as in the previous experiment. The results are given in Table XII.

TABLE XII.

Effect of P_H on Hydrolysis by dialysed Cholam Malt Enzyme.

P_H	Saccharification (c.c. of $KMnO_4$ after 15 mins. for 10 c.c.)	Liquefaction (gm. of ppt. in 10 c.c.)
5·26	3·1	0·1108
5·07	3·4	0·1066
4·86	3·55	0·1012
4·66	3·55	0·0860
4·48	3·05	0·0790
4·34	2·90	.....

A comparison of these results with those obtained from the once precipitated enzyme is illustrated in Fig. VI, and shows that the enzyme does not exhibit abnormal behaviour after purification by dialysis. The curious point arising from these three series of experiments is that this anomaly between P_H 5·2 and 4·6 is only shown in the partially purified enzyme. Both the crude malt extract and the enzyme purified by dialysis gave normal curves with an optimum P_H lying between 4·86 and 4·66. Another point of interest is that in the case of the precipitated enzyme, the abnormality was exhibited equally by both liquefaction and saccharification curves, a fact of importance in considering the two-enzyme theory. The phenomenon is being further investigated.

6. Effect of Enzyme concentration on Rate of Hydrolysis.

The experiment was conducted at 30°, with 5 c.c. portions of 2 per cent. starch paste and varying quantities of a 0·2162 per cent. solution of cholam malt amylase, the reaction mixture being diluted to 20 c.c. and the time allowed for the reaction being 60 minutes. The results are given in Table XIII and illustrated in Fig. VII.

Fig. VI.

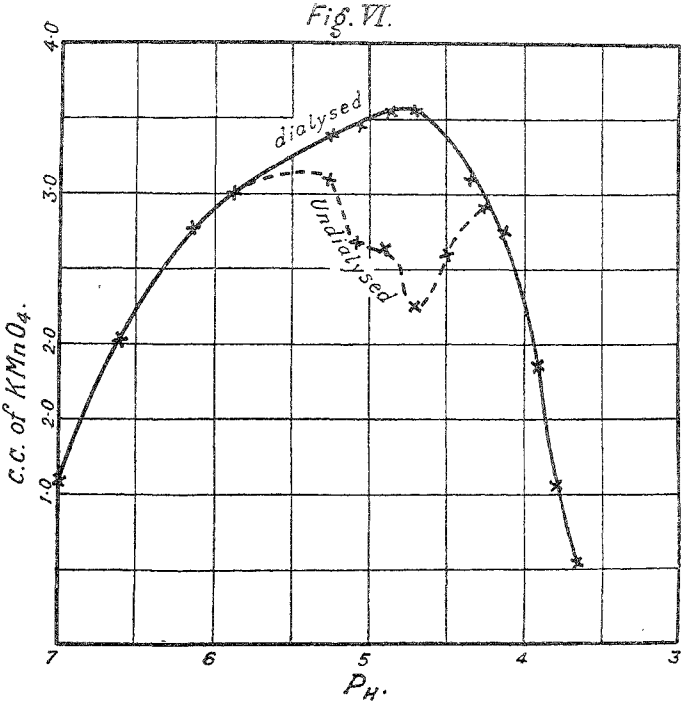


Fig VII

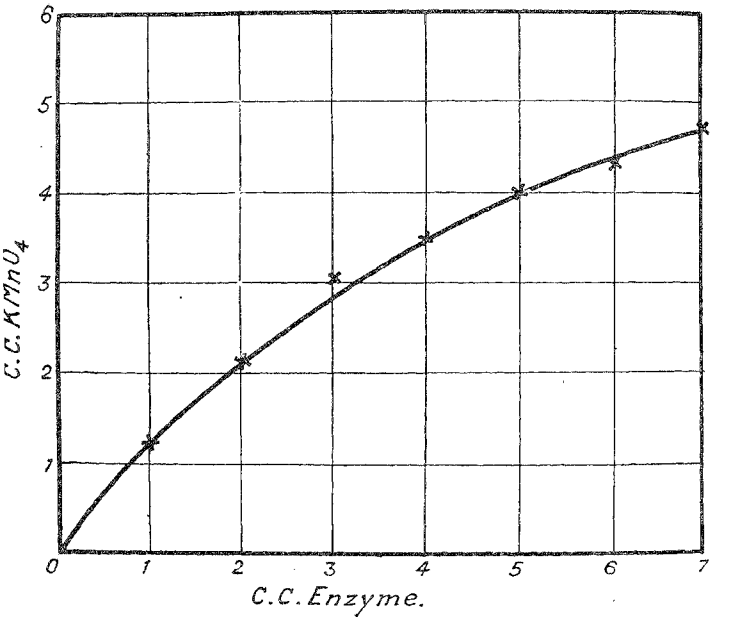


TABLE XIII.

Influence of Enzyme-concentration.

No.	Enzyme soln. c.c.	Water	c. c. of $KMnO_4$ for 20 c.c.
1	1	14	—
2	2	13	2·2
3	3	12	3·1
4	4	11	3·45
5	5	10	4·00
6	6	9	4·35
7	7	8	4·75

The rate of hydrolysis increases with increasing concentration of enzyme, the curve being, as one would expect, almost a straight line in the earlier stages when there is excess of substrate.

SUMMARY AND CONCLUSIONS.

1. A detailed comparison of malted cholam amylase with the enzyme from malted barley has been made. The results obtained confirm the conclusion of Norris and Viswanath that the cholam enzyme, while relatively inactive in sugar production, is more active than the barley enzyme in regard to liquefying power.

2. The variation in the ratio of the saccharifying and liquefying powers of the enzymes obtained from different sources supports the view that each action is brought about by a separate enzyme, the proportion in which the two enzymes occur varying in different grains.

3. Attempts to effect a change in this ratio, i.e., to bring to bear a selective influence on either enzyme, by varying the experimental conditions have given negative results. Variations, in temperature of reaction and in salt-content, within the limits investigated, have affected both liquefaction and saccharification to the same degree.

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