I.—STUDIES IN ENZYME ACTION—PART V.

AGEING OF AMYLASES IN AQUEOUS SOLUTION.

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Although it is well known that preparations of enzymes lose their activity under a variety of conditions, the processes leading to such inactivation have not, so far, been properly understood. Age, light and other radiations, prolonged dialysis, mechanical agitation, accumulation of reaction products, and poisonous chemicals lead to marked fall in activity resulting in complete inactivation. The adverse effects due to most of the above can be prevented by proper control of conditions, but no suitable method has, so far, been devised to thwart the effect of age with the result that large amounts of originally valuable preparations have to be rejected from time to time, thereby involving manufacturer and consumer in considerable loss.

Sherman and Schlesinger (*J. Amer. Chem. Soc.*, 1911, **33**, 1195; 1912, **34**, 1100) observed that solutions of pancreatic amylase undergo rapid deterioration on keeping. Fenger and Hull (*J. Biol. Chem.*, 1921, **46**, 431) noted that all pancreatic enzymes excepting trypsin become inactivated on storage. Sumner and Hand (*J. Biol. Chem.*, 1928, **76**, 149) observed that urease is readily inactivated to the extent of 80 per cent. in aqueous solutions.

Sherman and Schlesinger (*loc. cit.*) attributed the inactivation of pancreatic amylase to hydrolysis of the enzyme by the associated proteases. This view is not, however, supported by the observation of Willstätter and his co-workers (*Z. Physiol. Chem.*, 1926, **126**, 143) who find no evidence for the presence of protein in their purified preparations. Abderhalden and Guggenheim (*Z. Physiol. Chem.*, 1910, **68**, 317) and other workers, who noted that certain enzyme preparations are rapidly inactivated on vigorous shaking, attributed their observation to certain physical changes undergone by the enzymes. Working with crystalline pepsin, Northrop (*J. Gen. Physiol.*, 1930, **13**, 740) observed that the inactivation of the enzyme by heat or alkali is quantitatively proportional to the denaturation of the enzyme protein. Falk (*J. Biol. Chem.*, 1917, **31**, 97) who studied the inactivation of lipolytic enzymes found no evidence of protein hydrolysis, but obtained indications to show that under such conditions the enzymes undergo tautomeric changes. Sumner and Hand (*loc. cit.*), on the other hand, found that the rapid inactivation of aqueous solutions of crystalline urease was due to traces of lead present in the distilled water used by them.

The foregoing observations show that the mechanism of enzyme inactivation under different conditions has not yet been fully understood; an investigation was therefore undertaken to throw further light on the problem. The present paper deals with the changes that accompany the ageing of amylases in aqueous solutions.

Fig. I Effect of age on the saccharifying component - Barley amylase

Fig. II Effect of age on the Saccharifying Component - Cumbu amylaze

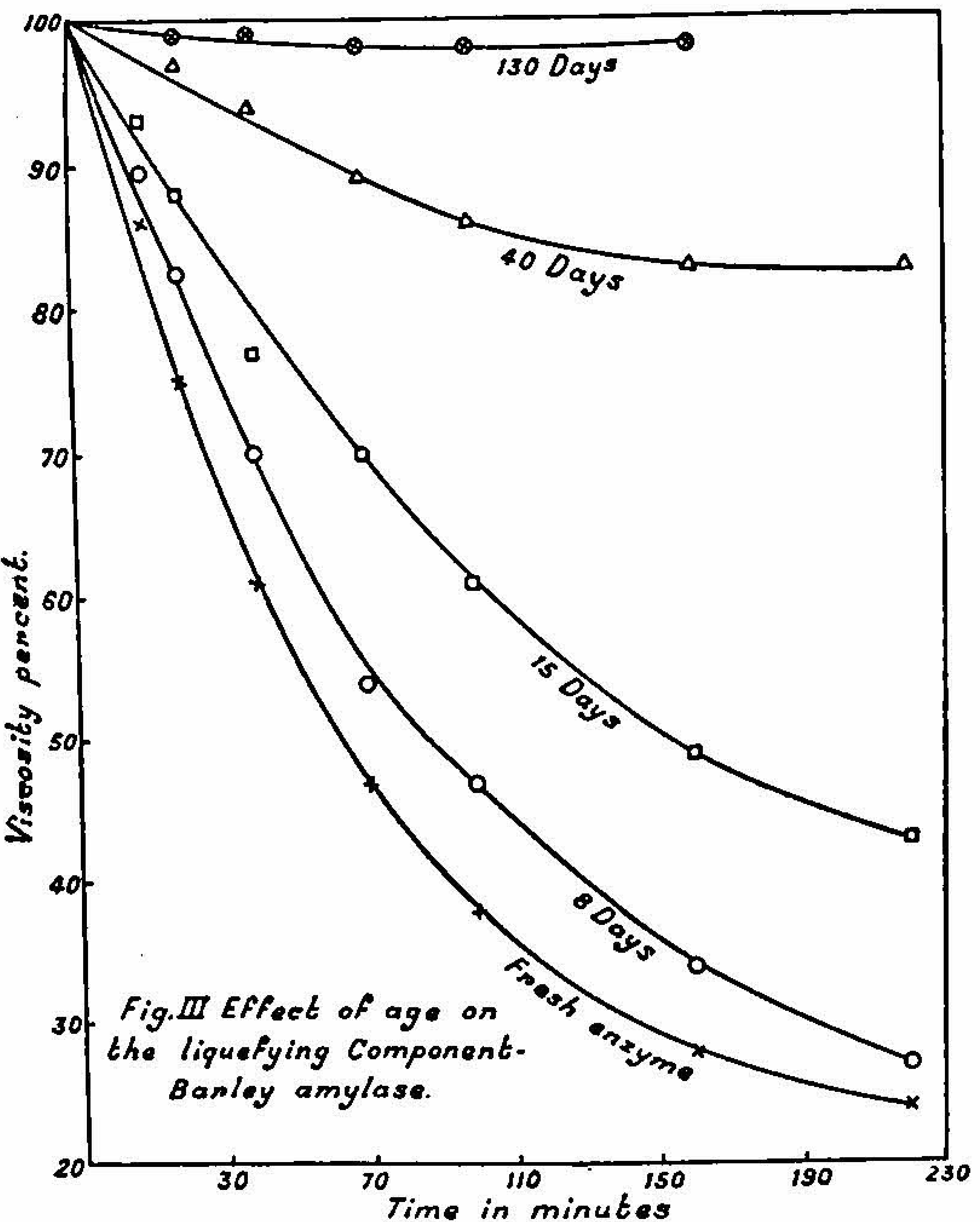

Fig. III Effect of age on the liquefying Component-Barley amylase.

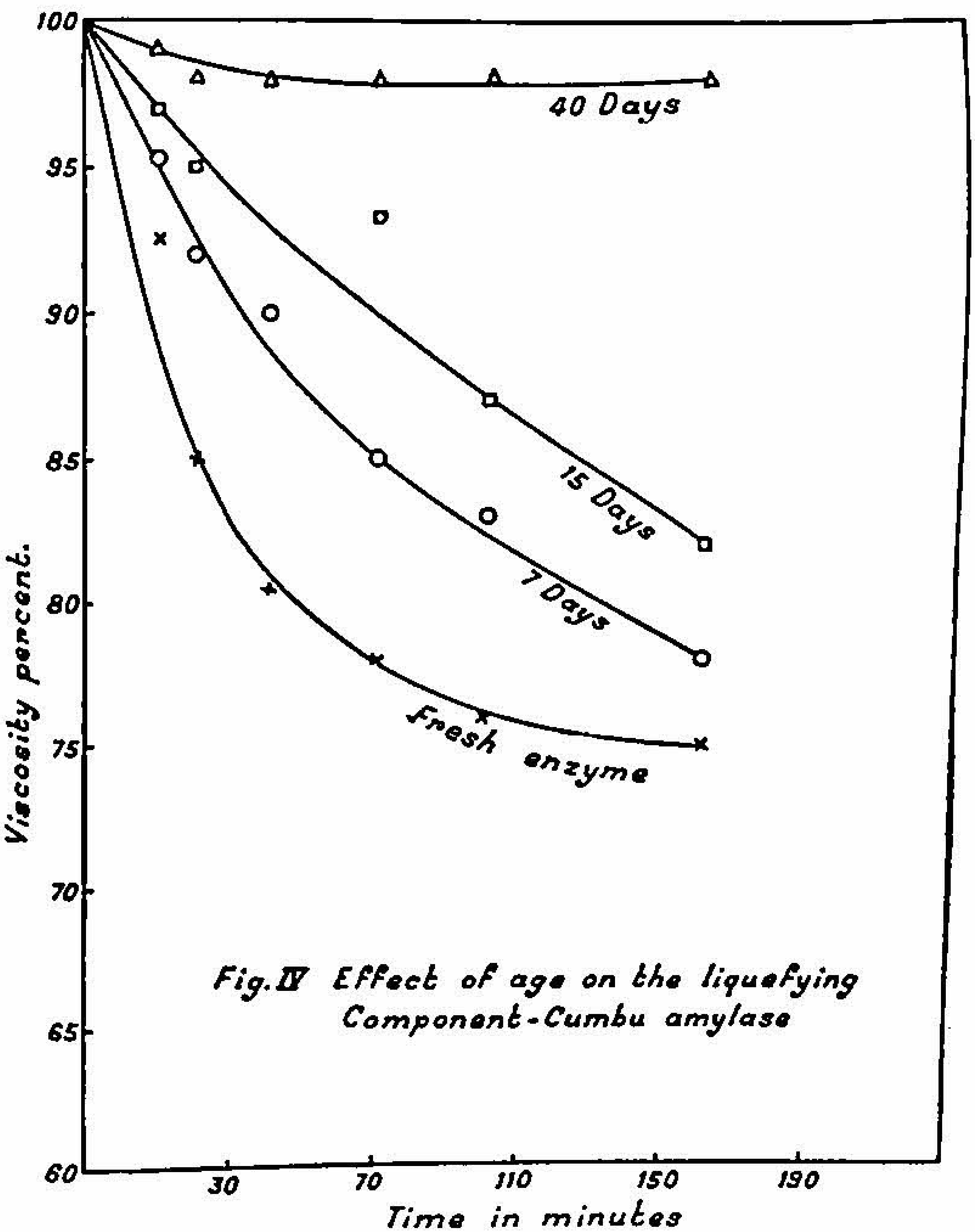

Fig. IV Effect of age on the liquefying Component-Cumbu amylase

Experimental.

The preparations of amylases used in the present study were those derived from barley and cumbu (*Pennisetum typhoideum*). Some experiments were also conducted with Taka-diastase (Parke-Davis).

Preparation of Enzymes.—The grains were steeped in running water for 36–48 hours and then spread out to germinate on moist sand for about 3 days at 27–29°. The germination was then arrested, the grains freed from sand and dried first in the sun and then under reduced pressure at 40° for 4–6 hours. The dried plumules were then removed by light crushing and the malts powdered.

The extracts were prepared by treating 400 g. of powdered malt-meal with 1200 c.c. of distilled water in presence of toluene for 3 days at 27–29°. They were then filtered through paper pulp and dialysed in collodion bags against distilled water for a week, when all the proteins were precipitated and the sugar removed. They were then centrifuged to remove suspended matter, the resulting clear liquid being treated with toluene and maintained at constant temperature (27–29°) in a dark chamber and free from dust.

Effect of age on saccharifying component.—Previous work (particularly, Fricke and Kaja, *Ber.*, 1924, **57**, 310, 313; Narayanamurti and Norris, *J. Indian Inst. Sci.*, 1928, **11A**, 134) having established that vegetable amylases are made up of liquefying as well as saccharifying components, experiments were made to determine the relative effects of ageing on the two components.

The changes in saccharogenic power were determined in the following manner:—The reaction mixture containing 40 c.c. of starch solution (2 per cent.), 10 c.c. of Walpole's acetate buffer at the optimum P_H for the enzyme, 5 c.c. of enzyme solution and 25 c.c. of water with a few crystals of thymol as antiseptic, was maintained at 34°. Aliquots were removed at intervals and the sugar-content determined by the method of Schaffer and Hartmann (*J. Biol. Chem.*, 1920–21, **45**, 365), the results being assembled in Figs. I and II. Although both the preparations lost the major part of their activity at the end of 130 days, the rate of inactivation was slower in the case of barley amylase than in that of the cumbu enzyme. In the latter case, there was a distinct fall in activity even at the end of 40 days.

Effect of age on liquefying component.—This was determined by the viscosimeter method under conditions similar to those adopted in the previous experiment; the results have been plotted in Figs. III and IV. There was distinct fall in the liquefying power even at the end of 40 days. The effect was more marked in the case of the cumbu enzyme than in that of barley. In both the cases the liquefying power was almost entirely lost at the end of 130 days.

A comparison of the above with the previous observations shows that the liquefying power is more readily lost than that of saccharification, thereby suggesting that the saccharifying component is less affected by age than the liquefying one under the conditions of the present investigation.

Change in optimum P_H.—Experiments were conducted with solutions of Taka-diastase (0·2 per cent.) in addition to those with barley and cumbu enzymes to determine whether the optimum P_H of the enzymes is affected as the result of ageing. Reaction mixtures composed of 10 c.c. of soluble starch (2 per cent.), 10 c.c. of Walpole's acetate buffer adjusted to varying P_H and 5 c.c. of enzyme at each stage of ageing were maintained at constant temperature and the activities determined at the end of half an hour. In the case of malt amylases the temperature of reaction was 35° while with Taka-diastase it was 40°: in the former, sugar was estimated by the method of Schaffer and Hartmann (*loc. cit.*) and in the latter gravimetrically, by the cuprous oxide method. The results have been given in Tables I*a*, I*b* and I*c*.

TABLE I *a*.

Change in Optimum P_H *—Barley Amylase.*

P_H	Activity in terms of c.c. of Thiosulphate (0.005*N*)				
	Fresh enzyme (control)	Age of enzyme in days			
		7	15	40	130
6·2	6·4	4·9	4·1	3·5	0·4
5·9	8·4	..	5·3	3·5	0·9
5·2	8·6	8·0	6·7	5·6	*1·7*
5·1	8·9	9·5	6·8	*6·0*	*1·7*
5·0	9·2	9·7	*7·0*	5·8	1·6
4·8	11·0	*9·9*	6·8	5·8	1·6
4·6	*11·4*	9·7	6·6	5·5	1·6
4·4	11·2	9·3	6·4	5·4	..
4·2	10·6	9·0	..	5·2	1·5
4·0	10·3	8·5	6·2	5·2	1·45
3·0	8·5	8·5	6·0	5·2	1·5

TABLE I *b*.

Change in Optimum P_H *—Cumbu Amylase.*

P_H	Activity in c.c. of Thiosulphate (0.005*N*)				
	Fresh enzyme (control)	Age of enzyme in days			
		7	15	40	126
6·0	7·0	6·0	4·0	1·0	0·6
5·8	7·2	6·8	4·4	2·0	1·6
5·5	7·2	7·2	5·0	3·4	*2·4*
5·3	7·6	7·4	*6·6*	*4·4*	2·2
5·2	8·6	8·0	5·6	4·0	2·2
5·1	8·6	*8·4*	5·2	3·8	2·2
5·0	*10·0*	7·8	5·4	3·8	2·2
4·8	8·8	7·4	5·8	3·6	2·4
4·6	8·0	7·4	6·4	..	2·2
4·4	8·0	7·2	6·4	3·2	2·4
4·0	8·8	7·4	6·0	3·4	..
3·7	8·0	7·2	5·6	3·4	2·4

TABLE I *c*.

Change in Optimum P_H—*Taka-diastase.*

P_H	Activity in terms of mg. of Cu_2O		
	Fresh enzyme (control)	10 days	25 days
6·2	271·6	235·2	205·0
5·4	323·6	274·8	240·0
5·2	328·2	279·0	240·0
5·0	*332·6*	*281·2*	*240·0*
4·8	323·6	276·6	238·6
4·6	319·8	272·4	233·0
4·3	295·4	254·6	219·2
3·6	209·6	181·2	158·8
3·1	154·2	135·6	..
Without buffer	285·8	237·6	207·4

It may be noted that (1) the optimum P_H in the two malt amylases is changed to a less acid one, and (2) the zone of optimum P_H tends to become wider as the result of ageing. There is no perceptible shift of optimum P_H in the case of Taka-diastase though, as in the case of the other enzymes, the zone becomes wider with ageing.

Similar changes in the optimum P_H of enzyme preparations have been recorded by Nelson and Bloomfield (*J. Amer. Chem. Soc.*, 1924, **46**, 1025) who studied the effect of temperature on invertase, and by Sherman and co-workers (*J. Amer. Chem. Soc.*, 1928, **50**, 2529) who investigated the effect of neutral salts on pancreatic amylase. The evidence so far obtained is not sufficient to explain the shift in optimum reaction in the case of the malt amylases, but in view of the previous observations it appears that the saccharifying enzyme, which is the only component left in the later stages, has a wide range of optimum reaction, particularly on the acid side.

Stability at different H-*ion Concentrations.*—Solutions of the two malt amylases were dialysed against distilled water for a week. They were then made up to constant volume and aliquots (50 c.c.) transferred to bottles each containing 20 c.c. of Walpole's acetate buffer of a particular reaction. After adding toluene, the mixtures were maintained at 27–29° and the activities determined, from time to time, after adjusting the final reaction, in each case, to the optimum P_H of the enzyme concerned. Control experiments were also conducted with enzyme solutions to which buffers had not been added. The results have been presented in Tables II *a* and II *b*.

TABLE II *a*.

Stability of Cumbu Amylase at different H-ion Concentrations.

P_H	Activity in c.c. of Thiosulphate (0.005*N*)			
	Fresh enzyme	Age in days 7	22	40
Without buffer	5·3	5·0	2·7	1·8
6·50	5·4	5·0	2·7	1·3
5·57	5·3	4·9	2·6	1·3
4·99	5·4	4·9	3·2	2·1
4·60	5·2	5·0	3·5	2·6
3·70	5·3	5·0	2·7	1·3
2·69	5·4	5·1	2·5	1·0
Undialysed enzyme	12·6	12·4	11·8	11·0

TABLE II *b*.

Stability of Barley Amylase at different H-ion Concentrations.

P_H	Activity in c.c. of Thiosulphate (0.005*N*)		
	Fresh enzyme	Age in days 15	40
Without buffer	17·0	15·8	10·9
6·50	17·1	15·5	10·0
5·57	17·0	15·7	10·2
4·99	17·1	16·1	11·0
4·62	17·1	16·2	11·0
3·72	17·0	*nil*	*nil*
2·69	17·0	*nil*	*nil*

It may be noted that all the dialysed preparations lost their activity more readily than the undialysed ones though the reactions were subsequently maintained constant by addition of buffers. The observations suggest that during dialysis certain components essential for the stabilisation of the enzymes are lost. In the case of the buffered preparations, inactivation proceeded more slowly in the neighbourhood of the optimum reaction (P_H 4·6) than at others. The barley enzyme was more sensitive to reactions on the acid side of the optimum P_H than the cumbu preparation.

A similar experiment with undialysed Taka-diastase (0·2 per cent.) gave the results in Table III showing that the enzyme is most stable at P_H 6·2 which is less acid than the optimum (P_H 5·0) for the enzyme. The enzyme deteriorates readily as the medium becomes more acid.

TABLE III.

Stability of Taka-diastase at different H–*ion Concentrations.*

P_H	Activity in mg. of Cu_2O			
	Fresh enzyme (control)	Age of enzyme in days 8	19	50
6·2	333·4	309·0	302·6	292·6
5·6	,,	299·0	269·8	236·2
5·1	,,	253·8	209·4	142·4
4·2	,,	39·8	29·8	27·6
3·4	,,	28·0	26·2	24·0
2·7	,,	25·6	25·0	20·2
Unbuffered	,,	285·2	220·6	140·4

The relation of the stabilising reaction of an enzyme to its optimum P_H is comparatively obscure. Although many of the commoner enzyme preparations resist heat inactivation more effectively at their optimum P_H than at any other reaction, cases of exceptions are also known. Thus Northrop (*J. Gen. Physiol.*, 1922, **4**, 261) observed that trypsin is most resistant to heat at P_H 6·0 though its optimum reaction is P_H 8·0–10·0. Ege (*Z. Physiol. Chem.*, 1925, **143**, 159) noted that pepsin is most stable at P_H 3·6–4·6 though its optimum reaction is P_H 1·6.

Effect of concentration on ageing.—Solutions of cumbu amylase of two different concentrations (A and B) were dialysed, treated with toluene and their activities determined, suggesting that the enzyme solution is more stable concentrated than when dilute (Table IV).

TABLE IV.

No. of days	Activity in c.c. of thiosulphate A	B
0	16·3	9·2
10	15·7	8·0
40	9·2	3·5

Changes in the P_H *of the enzyme solution.*—Solutions of barley and cumbu amylases were dialysed, treated with toluene and maintained at 27–29°. Changes in P_H were determined colorimetrically at intervals (Table V).

TABLE V.

P_H

Source of Amylase	Before dialysis	After dialysis—days 0	7	20	40
Barley	6·0	6·1	5·4	..	4·6
Cumbu	5·9	6·2	5·5	4·4	4·2

The results show that the reaction of the enzyme solution tends to become more acid on keeping; a similar experiment conducted with Taka-diastase showed that the reaction of the solution changed, in a like manner, from P_H 6·2 to 5·2. This might have been due to either production of fresh acid or removal of buffering materials originally present in the enzyme solution: further work is necessary to elucidate the point, but the observations of Graber (*Indus. Eng. Chem.*, 1914, 6, 403) who detected lactic acid in glycerine extracts of malt, are rather suggestive in this connection.

Attempts to reactivate aged enzymes.—The observations of Onodera (*Biochem. J.*, 1915, 9, 544) and others having shown that the activities of enzyme preparation can be restored under certain conditions, some experiments were conducted to ascertain whether inactive amylases can be revived under similar conditions.

To determine whether fresh or boiled enzymes could provide the necessary activators, mixtures of the following compositions were prepared and their activities determined:—(1) Boiled enzyme+water, (2) boiled enzyme+aged enzyme, (3) aged enzyme+fresh enzyme, (4) aged enzyme+water, and (5) fresh enzyme+water. Equal volumes (10 c.c.) of the different components were used to prepare the mixtures. The activities as represented by the equivalents of sugar formed at the end of half an hour, in each case were as follows.— (1) 0·2 c.c.: (2) 0·3 c.c.: (3) 16·3 c.c.: (4) 0·1 c.c. and (5) 16·7 c.c. of standard thiosulphate, respectively.

In another experiment, diluted fresh enzyme was added to the aged preparation and the activity measured at the end of 24 hours (Table VI).

TABLE VI.

Time in minutes	Activity in c.c. of thiosulphate			
	Fresh	Aged	Fresh + Aged	
			Expected	Found
5	3·1	2·1	2·6	2·4
10	3·5	..	..	..
20	4·4	2·4	3·4	2·9
40	5·4	3·1	4·3	3·3
60	5·6	3·7	4·7	3·9
90	7·1	4·4	5·8	4·5
120	7·7	5·3	6·5	5·5

Thus the activity of the mixture was in every case less than it would have been if the components had functioned independently. The results show that instead of activating the aged enzyme the fresh enzyme was itself partially inactivated as the result of standing in contact with the aged preparation. Since the fresh enzyme would not by itself have lost any of its activity as the result of standing for 24 hours it appears from the above that the aged preparation catalyses the inactivation of the fresh enzyme.

To ascertain whether inactivation of the aged enzyme is due to certain crystalloids present in the aged preparation, specimens of the latter were dialysed at different stages and their activities determined. In no case, however, was the activity appreciably improved as the result of dialysis.

In order to determine whether the electrolytes present in the aged enzyme were responsible for the inactivation of the fresh preparation added, some experiments were conducted repeating the addition of aged enzyme after dialysis. The results were the same as those stated in Table III thereby showing that the electrolytes were not responsible for the inactivation of the fresh enzyme.

Effect of dialysis and age on Taka-diastase.—Taka-diastase (1 g.) was dissolved in 300 c.c. of distilled water and dialysed in collodion bags for 3 days, then made up to 500 c.c., treated with toluene and the activity noted at intervals. The control experiments were with undialysed enzyme. Results showed that both dialysed and undialysed preparations lost their activity readily on standing, the former at a slightly faster rate than the latter. The effect is not, however, so marked as in the case of the malt amylases.

Further work on the mechanism of inactivation is in progress. Effects of various stabilisers on the keeping qualities of enzyme preparations are also being studied.

Summary.

As the result of age, (1) both the liquefying and the saccharifying components of the malt amylases became steadily inactivated, the former at a faster rate than the latter, (2) the optimum P_H is shifted to a less acid reaction and (3) solutions of malt amylases tend to become more acid on standing.

Dialysed malt enzymes are most stable when maintained at their optimum P_H. Undialysed Taka-diastase is best preserved at a less acid reaction than the optimum for its activity. Solutions of Taka-diastase lose their activity rapidly on standing; the effect is more marked in dialysed than with undialysed preparations. Attempts to reactivate aged enzymes have not so far been successful.

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[Accepted, 27-10-32.]