II.—DIALYSIS OF SOME CEREAL AMYLASES.

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Investigations on the properties of cereal amylases carried out in this laboratory have already shown that these enzymes lose activity when subjected to continued dialysis. Attempts to ascertain the cause of this phenomenon have led to the conclusion that it is due to the progressive destruction of the enzymes themselves owing to certain hitherto obscure causes. The present investigation has shown that the deterioration is not due to the relatively high room temperature, since it has been found that aqueous solutions of similar enzyme preparations retained more than 50–75 per cent. of their original activity even after more than two months in glass containers, while on prolonged dialysis nearly all their activity was lost within about three weeks.

Sherman and his co-workers (*J. Amer. Chem. Soc.*, 1919, **41**, 1867; 1921, **43**, 2461) suggested that amylase loses its activity owing to the hydrolysis of enzyme protein by proteases accompanying the enzyme. The present investigation has however shown that such a hypothesis is untenable. Supposing that the enzyme proteins were hydrolysed during dialysis, they would pass out of the collodion bag and thus bring about a steady decrease in the total nitrogen of the enzyme solution. Figures given in the experimental portion of the text show that with the progress of dialysis the nitrogen content reached a minimum and remained subsequently constant, while the activity continued to diminish to the very end. A direct proof of the untenability of Sherman's hypothesis was also obtained by testing the action of proteolytic enzymes on amylase solutions. The experimental details of such an experiment have been given in a previous paper (*J. Indian Inst. Sci.*, 1930, **13A**, 159), where it was shown that pepsin inactivated wheat amylase in aqueous solution, the rate depending upon the concentration of pepsin; papain and trypsin were without effect. All three proteolytic enzymes were allowed to act on amylase solutions at neutral reaction, since it was observed that loss of activity during dialysis took place in the neighbourhood of P_H 6·7. Similar experiments carried out with amylases from rice, barley, cholam (*Sorghum vulgare*), maize, ragi (*Eleusine coracana*) and bajri (*Pennisetum typhoideum*) failed to show any inactivation due to the presence of proteolytic enzymes. These results do not support Sherman's hypothesis regarding the destruction of the enzyme nor do they supply evidence for the proteid nature of amylase as advocated by him.

Possibility of enzyme adsorption by collodion membranes was also tested by trying to elute the enzyme from the membrane, but no trace of adsorbed enzyme could be obtained by elution.

The dialyses were conducted carefully and sterile, toluenated water was used throughout. The bacterial count actually decreased with the progress of dialysis, and the possibility of bacterial decomposition of the dialysing enzyme was thus eliminated.

As a preliminary to experiments on dialysis, it was found that membranes made with two coats of 6 per cent. collodion did not allow the enzyme to pass through them. The same type of membranes were also used for electro-dialysis. Membranes prepared according to Frick and Kaja (*Ber.*, 1924, **57**, 310) were found to be unsatisfactory.

Some of the previous workers found electro-dialysis to be a rapid means of purifying amylases. Thus Narayanamurti and Norris (*J. Indian Inst. Sci.*, 1928, **12A**, 134) purified cholam malt amylase by electro-dialysing it through parchment membrane. Similar attempts to purify other amylases by electro-dialysis led, however, to almost complete destruction of the enzymes concerned. The details of our experiments were the same as those described by Narayanamurti and Norris except that rigorously tested collodion membranes were used in place of parchment ones. Of the seven amylases subjected to electro-dialysis, only wheat amylase retained its activity, all others being rendered inactive.

Rice amylase was first dialysed in the electro-dialyser at room temperature without passing the current. After three days, the terminals were connected to the main (110 D.C.) with a resistance of 2800 ohms in series. It was observed that the inactivation began at once, and at the end of four hours the activity was reduced by fifty per cent. It is possible that the destruction of the enzyme was due to the high potential between the terminals, because the enzyme could not have diffused out, or adsorbed on the membrane, or been precipitated with protein: it is however difficult to reconcile the above observation with those of Fricke and Kaja on commercial amylase (*loc. cit.*) Narayanamurti and Norris on cholam amylase (*loc. cit.*) and our own on wheat amylase.

In the present investigation, the activities of the enzyme solutions were compared on the volume basis rather than that of solid content as is usually done. Thus if a given solution of enzyme shows an activity x remaining unchanged when a large amount of inert solids have passed out of the dialysing membrane, the activity can be said to have remained unaffected, even though according to the solid content estimations it must have increased. During dialysis a stage is reached when solid content remains constant while the activity continues to

diminish till it can be said to have been practically lost. This would mean that the solids undergo changes leading to the destruction of the hydrolytic properties of the enzymes and thereby converting them into inert substances. The mechanism of that process can be understood only by a physico-chemical study of the enzyme in solution. Investigation of such a nature has been undertaken in this laboratory and the results will be published shortly.

EXPERIMENTAL.

The methods of preparing malts and enzymes are the same as those described in the previous communications (*loc. cit.*).

I.—*Estimation of total and soluble nitrogen content of cereal malts and their enzymes.*

Since it is believed that proteins act as carriers of the enzyme and since there is considerable fall in the nitrogen content during dialysis, it was considered that a study of total and soluble nitrogen of these enzymes might be useful.

A weighed quantity of malt (3–4 g.) was digested with 100 c.c. of cold water during 1 hour, filtered and made up to 150 c.c. Concentrated sulphuric acid (5 c.c.) was added and the contents evaporated nearly to dryness on the water bath. Nitrogen was then determined by the kjeldahl method.

TABLE I.

Malts				Precipitated enzymes	
	Percentage		Days required for inactivation	Percentage	
Malt	Total N	Soluble N		Total N	Soluble N
Wheat ...	2·43	0·58	15	...	5·35
Barley ...	1·76	0·28	35(*ca.*)	...	5·85
Rice ...	1·32	0·17	15	6·85	2·30
Maize ...	1·77	0·26	3–4	...	4·34
Ragi ...	1·33	0·27	9	...	2·78
Bajri ...	1·74	0·43	13	...	5·08
Jawari ...	1·53	0·21	...	...	1·56

II.—*Prolonged dialysis of cereal amylases.*

Portions of 20 c.c. of the malt extract (30 per cent.) were dialysed in a collodion bag, several being in one dialyser. At known intervals bags were removed, the contents filtered and made up to 100 c.c. Nitrogen, solid content and activity were determined. The results are given in tables II, III and IV.

TABLE II.

Rice Amylase.

Days	P_H	Solids per cent.	Nitrogen in c.c. of 0·1071 N alkali	Maltose in c.c. of N/20 thiosulphate
0	5·9	2·13	6·75	15·00
1	6·4	0·62	1·50	14·00
3	6·7	0·04	1·00	13·90
6	6·5	0·05	0·50	6·80
8	6·7	0·05	0·50	6·10
15	6·7	0·05	0·50	1·70
Control after 15 days ...	5·9	2·12	6·75	15·0

TABLE III.

Bajri Amylase.

Days	P_H	Solids per cent.	Nitrogen in c.c. of 0·1071 N alkali.	Maltose in c.c. of N/20 thiosulphate
0	5·7	4·20	15·50	14·70
2	6·2	0·49	3·50	11·70
5	6·5	0·10	1·50	4·20
7	6·7	0·08	1·00	3·30
10	6·9	0·08	1·00	1·80
13	6·9	0·08	1·00	0·70
Control after 13 days ...	5·7	4·20	15·00	13·50

TABLE IV.

Barley Amylase.

Days	P_H	Solids per cent.	Nitrogen in c.c. of 0·1071 N alkali	Maltose in c.c. of N/20 thiosulphate
0	5·6	5·12	16·75	16·70
3	6·3	0·20	3·50	15·00
6	6·7	0·13	1·75	15·60
8	6·9	0·12	1·0	14·80
11	6·9	0·08	1·0	14·50
14	6·9	0·08	1·0	14·20
35	...	...	...	0·30
Control after 35 days ...	...	5·11	16·50	17·30

III.—*Effect of ageing on dialysed amylase.*

Portions of 20 c.c. of the malt extract (30 per cent.) of wheat and rice were dialysed during three days when the enzymes were practically free from electrolytes and reducing sugars. The solutions were preserved for over two months with added toluene, and their activities determined at intervals.

TABLE V.

Date	Maltose in c.c. of N/20 thiosulphate	
	Wheat	Rice
14—11—1930	15·4	12·1
3—12—1930	15·3	12·2
10—12—1930	15·5	11·9
18—12—1930	13·4	9·5
23— 1—1931	11·9	6·3

IV. *Testing for possible adsorption of enzyme by the collodion membrane.*

Four collodion bags, each containing 20 c.c. of the amylase from wheat enzyme solution (0·1 per cent.) were preserved for dialysis as

usual. After 15 days when the amylase was almost inactivated, the bags were removed, contents separated and the bags washed with small quantities of distilled water. Two bags were then cut to pieces and eluted with 20 c.c. of the elutive agent (100 c.c. consisting of 57 c.c. of 1 per cent. $(NH_4)_2HPO_4$, 3 c.c. of N ammonia and 40 c.c. of 87 per cent. glycerol) at P_H 7·8. Two other bags were similarly treated with another elutive agent at P_H 4·6 (100 c.c. consisting of 40 c.c. or McIlvaine's buffer of P_H 4·6 and 60 c.c. of 40 per cent. glycerol). After four hours, 10 c.c. were taken from each flask and dialysed to remove glycerol. The activity was then tested against starch, and in no case could any activity be detected showing that the enzyme was not adsorbed on the membrane used for dialysis.

V. *Electro-dialysis of amylases.*

(*a*) An electro-dialyser very similar to that of Fricke and Kaja (*loc. cit.*) was used. Laboratory supply (110 volts, D.C.) with a resistance of 2,000 ohms in series was used as a source of current.

A stock solution of 500 c.c. of malt extract (15 per cent.) was prepared, 20 c.c. being made up to 50 c.c. and kept as control ; 400 c.c. was electro-dialysed till the conductivity approached that of distilled water. The solution was then removed, made up to 1000 c.c. and filtered, the solids being inert. The activity of the filtrate was then determined and the results are given in Table VI.

TABLE VI.

Malt	Control		Electro-dialysis		
	P_H	Maltose in c.c. of N/20 thiosulphate	Days	P_H of middle cell	Maltose in c.c. of N/20 thiosulphate
Rice ...	5·8	15·0	3	4·5	1·3
Wheat ...	5·9	14·8	2	4·6	14·7
Barley ...	5·6	16·1	3	4·4	0·9
Maize ...	5·6	8·4	3	4·6	1·2
Ragi ...	5·6	12·2	3	4·4	2·0
Bajri ...	5·7	12·9	3	4·5	0·2
Cholam ...	5·6	6·8	3	4·7	0·3

(*b*) Rice-malt extract was dialysed in the electro-dialyser without the passing current, which was then switched on and the enzyme electro-dialysed, in one case for four hours and in the other for six hours. Their respective activities are given in Table VII.

TABLE VII.

Activity before electro-dialysis	Activity after electro-dialysis		
Maltose in c.c. of N/20 thiosulphate	Electro-dialysis hours	P_H	Maltose in c.c. of N/20 thiosulphate
14·2	6	4·5	6·4
12·9	4	4·5	6·5

SUMMARY.

1. All the cereal amylases hitherto investigated have been found to lose their activity on prolonged dialysis.

2. Electrolyte-free amylase in aqueous solution retained more than 50 per cent. of its original activity even after two months.

3. On electro-dialysis through collodion membranes, all cereal amylases excepting that from wheat lost their activity.

4. Probable causes of destruction of the enzymes have been investigated.

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[Accepted, 18-5-31.]

PRINTED AND PUBLISHED

AT THE DIOCESAN PRESS, POST BOX 455, VEPERY,

MADRAS—1931. C2175.