CCLX. OBSERVATIONS ON THE STABILITY OF XANTHINE OXIDASE

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In the course of work on the isolation of xanthine oxidase from milk, much inconvenience arose from the instability of the preparations, which increased with each stage of purification. Rapid inactivation occurred when the bulk of the casein had been removed by precipitation with Na_2SO_4 and the diluted supernatant liquid was incubated at 38° for purposes of testing. The inactivation could be prevented by restoring the casein. This led to the hope that a non-protein stabilizer might be found, and, as described below, this hope has been fulfilled.

Experimental

All tests were carried out by the Thunberg technique using the following quantities: 0·1 *M* phosphate buffer, *p*H 7·0, 2·5 ml.; 1 in 5000 methylene blue, 0·5 ml.; 1·0 *M* glycine, 0·5 ml.; 0·0132 *M* xanthine, 0·2 ml.; the final volume was made up to 5·0 ml. Unless otherwise mentioned, the xanthine was added from the hollow stopper after temperature equilibration at 38°.

Results

Table I shows that two-fold dilution of a whey preparation greatly affects its stability. Table II shows the restoration of stability by glycine. Table III shows that reactivation by glycine is also dependent on the time of incubation with glycine.

Table I. *The effect of dilution*

Incubation time in min.	Reduction time: 0·5 ml. whey (min. sec.)	Reduction time: 0·25 ml. whey (min. sec.)
1	5 45	11 40
2	6 05	11 10
3	6 00	15 00
4	5 55	19 00
5	6 05	20 05
10	5 58	23 00
20	6 20	35 00

Table II. *Stabilization by glycine*

1·0 *M* glycine ml.	Enzyme ml.	Incubation time in min.	Reduction time (min. sec.)
—	0·25	2	7 0
—	0·25	10	120 0
0·5	0·25	10	5 30
0·5	0·25	60	7 55
1·0	0·25	60	6 50

The glycine was added to the tubes before incubation.

Table III. *Reactivation by glcyine*

Incubation time (min.) enzyme	Incubation time (min.) enzyme + glycine	Reduction time min. sec.
0	2	5 30
10	0	16 00
10	10	10 15
10	20	7 50

The enzyme was first incubated anaerobically with buffer and methylene blue, then glycine was added and the tubes re-evacuated and incubated, and finally the xanthine was added from the stoppers.

Table IV. *Protection by glycine against copper*

1·0 *M* glycine ml.	Enzyme ml.	0·500125 *M* $CuSO_4$ ml.	Incubation time (min.)	Reduction time min. sec.
—	0·25	—	2	11 10
0·5	0·25	—	2	5 30
—	0·25	0·05	2	150 0
0·5	0·25	0·05	2	5 45
0·5	0·25	0·05	23	5 30

The $CuSO_4$ was added to the other reagents in the tube before incubation.

As the distilled water then being used came from a copper still, it was suspected that the inactivation was due to copper poisoning [Wieland & Mitchell, 1931; Andersson, 1936], and that glycine was acting by forming a copper complex (Table IV). It was shown that glycine protected against the inactivation caused by added copper. Thenceforward all activity tests were done in presence of 0·1 *M* glycine.

In later work it was found that on dialysis at 0°, against large volumes of dilute buffer solutions, there was a loss of activity which could only be partially prevented by adding glycine (0·1 *M*) to the outer liquid and testing in presence of

Table V. *Activation and protection by KCN*

Batch of milk	Preparation	ml.	KCN (final conc.) *M*	Reduction time min. sec.
46	Fat-free milk	1·0	—	2 25
			0·001	1 50
			0·03	1 30
			0·075	2 30
46	Decalcified milk	1·0	—	6 00
			0·015	2 45
			0·03	1 55
			0·045	1 55
			0·075	No reduction
46	Decalcified milk 0·000125 *M* $CuSO_4$	1·0	—	No reduction
			0·015	1 45
			0·03	2 05
			0·045	4 00
48	Fat-free milk	1·0	—	1 45
			0·015	0 45
			0·03	0 40
			0·045	0 48
49	$(NH_4)_2SO_4$ fraction	1·0	—	9 00
			0·0075	4 00
			0·015	3 15

Incubation time 2 min. in all cases.

a high concentration of glycine (1·0 *M*). However, on testing a dialysed preparation in presence of KCN, which has a greater affinity for copper than glycine, it was found that the activity could be not only restored, but actually increased above the original level. A number of different preparations were tested and all, including whole and fat-free milk, were found to be activated, the optimum KCN concentration lying between 0·01 and 0·03 *M*. At lower concentrations the activation is submaximal and at higher concentrations destruction of the enzyme occurs, as found by Dixon & Thurlow [1925]. KCN was also found to protect against inactivation by added copper. The KCN and xanthine were added together to the other reagents from the stopper in order to avoid the destruction produced by KCN alone [Dixon & Keilin, 1936]. As with glycine, the reactivation by KCN, after incubation at 38°, appears to be dependent on time. If tests are carried out in absence of glycine it is found that the reduction time depends on the length of the preliminary incubation. The interpretation of these results is, however, complicated by the fact that the enzyme cannot be incubated alone with KCN without being destroyed.

Discussion

Wieland & Mitchell [1931] stated that KCN does not protect the enzyme against copper poisoning, but they did not test concentrations higher than 0·001 *M*. On the other hand, the high concentration required here suggests that the poison concerned is not silver, gold or mercury (cf. Wieland & Mitchell).

It appears from this that, during the process of purification, xanthine oxidase easily becomes inactivated by traces of copper, but it is not certain where the copper comes from. In the earlier stages of the work the distilled water contained traces, but since then a change has been made to a pyrex glass still. Boyden & Potter [1937] mention that their cellophane dialysis sacks contained copper. The cellophane used here does not contain enough to inactivate more than one batch of 50 ml. of enzyme preparation, and the same sack was used several times over, dialysis producing the same inactivation each time. Since there are traces of copper in milk [Davies, 1936], it seems more probable that the inactivation on dialysis is due to the removal of some substance which normally protects the enzyme against copper poisoning.

The inactivation of the enzyme does not appear to be due to the disappearance of —SH groups, for the following reasons.

(1) Milk has no detectable —SH groups.

(2) The concentration of KCN which activates milk instantaneously has only a very slow reducing action on the —SS— groups in milk.

(3) Addition of cysteine, when testing the activity of milk, causes no activation.

From the practical point of view, if accurate activity measurements are required, it is recommended to use both glycine (final conc. 0·1 *M*) and KCN (final conc. 0·01–0·03 *M*). Since KCN can only be added together with the xanthine, after temperature equilibration is complete, it is advisable to retain glycine to avoid the inactivation which may occur during that time.

Summary

1. Glycine and cyanide can increase the activity of xanthine oxidase in whole milk and concentrates.

2. This is probably due to removal of copper inhibition.

3. The loss of activity of some preparations on dialysis or incubation can be wholly or partly reversed in the above way.

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