bittern produced by salt manufacturing factories are shown in Fig. 4 by "S". The range "S" is included in the crystallization field of NS salt B, but not in those of langbeinite, therefore, NS salt B could be directly separated from bittern, while langbeinite could not be separated directly.

From these experimental data, in order to separate potassium dissolved in bittern as sulfate directly at 100°, it should be concluded that the only one method is to separate it as NS salt B. Calculating the yield of NS salt B from bittern at 100° by these phase diagrams, about fifty per cent of dissolved potassium is separated as NS

salt B.

From the equilibrium diagram of the quaternary system, eliminated magnesium hydroxide from the quinary system, our experimental data hardly agreed with results obtained by A. N. Campbell and others³⁾.

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Studies on Taka- α -amylase (8)*

On the Mechanism of EDTA** Inactivation of TAA***

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This paper deals with some properties of reversibly inact.**** TAA effected by means of EDTA. The mechanism of EDTA inactivation was discussed from the view point that EDTA has a conspicuous suppressive effect on the reactivation of heat-inact. enzyme, while it has no effect on the active enzyme itself. Particular attention was paid to the problem of calcium transfer along with the progress of inactivation.

INTRODUCTION

The role of metal-binding reagents such as EDTA, on the inactivation of a variety of α -amylases has been ascribed to the removal of some calcium ions which are essentially incorporated into enzyme proteins. Stein and Fischer¹⁾

have however reported that α -amylases of different origins including *Aspergillus oryzae* are quite stable towards the EDTA action if the enzyme preparations are completely free from the contaminated proteases.

The reversible inactivation of α -amylases by means of EDTA has been reported earlier by Fischer and Haselbach employing malt- α -amylase²⁾.

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** Ethylenediamine tetracetate-Na.

*** Taka- α -amylase

**** inactivated

1) E. A. Stein and E. H. Fischer, *J. Biol. Chem.*, **232**, 867 (1958).

2) E. H. Fischer and C. H. Haselbach, *Helv. Chim. Acta*, **34**, 325 (1951).

In the previous report, the reactivation phenomena of EDTA-inact. TAA was studied, and the equilibrium between the active and inact. TAA was described³⁾.

Recently, Yamamoto and Hukumoto have succeeded in obtaining the EDTA-inact. pancreas-, or bacterial- α -amylase in a completely reversible state, and have emphasized the importance of metal ions for restoring activity⁴⁾.

This report deals with one assumptive schema regarding the role of EDTA which plays a part during inactivation process.

Yamamoto et al. have also pointed out on the special behavior of TAA towards EDTA and suggested a similar schema for the EDTA inactivation of TAA⁵⁾.

METHODS AND MATERIALS

Crystalline TAA was prepared from Taka-diestase Sankyo according to Akabori's method⁶⁾. After recrystallization for 3 times, inert calcium was removed by the author's method⁷⁾; Rivanol-amylase complex was crystallized by shaking a concentrated solution of the complex in M/2 acetate buffer (pH 6.0) at 20°C⁸⁾. Rivanol was removed by caolin, and crystallization was executed in M/5 acetate buffer (pH 6.0) by means of acetone. The crystals thus obtained contained about 1 mol of calcium⁷⁾.

The enzymes were always diluted to M/25,000 before use. The determination of enzyme concentrations was based on the assumption that the molecular weight of TAA should be 53,000⁹⁾.

The relative change of amylase activity was followed by the percentage of saccharifying power remaining during the course of incubation with EDTA. Sacchari-

fying power was assayed by a slightly modified method of Willstätter and Schudel¹⁰⁾; one ml of M/25,000 TAA was diluted to 200 ml, and this solution was used as a standard for comparison.

Assayed samples were previously controlled to have almost equal activity to the standard (error within $\pm 5\%$) and then the iodine consumption was compared as a basis for calculation. Sampling and measurement of activity were always carried out at pH 5.0 in order to prevent the reactivation of activity³⁾. The assay system employed was: 2% soluble starch solution 20 ml containing M/50 acetate buffer of pH 5.0, enzyme solution 5 ml, 35°C, ten minutes.

RESULTS

1. The Reversibility of the Inactivation.

Fig. 1 illustrates the reversible inactivation of TAA induced by incubation with EDTA. One ml of each M/5,000 EDTA solution of various pH (M/20 veronal buffer) was added to a series of tubes containing 1 ml of the enzyme solution, and then incubated at 32.5°C*. The reactivation was effected by adding 2 ml of M/100 calcium acetate solution and M/10 veronal buffer (pH 8.5), respectively, and incubating them at the same temperature from one to two days. The dotted lines show the course of restoring activity.

As a further example of showing the reversibility of the enzyme, inactivation was performed in almost the same way as that given in Fig. 1 (37°C, pH 8.5, 100 mol equivalent of EDTA), and reactivation was merely effected by exposure to a lower temperature (Fig. 2).

After restoration of the activity reached a maximum level, the mixture was again warmed up to 37°C. The enzyme lost its activity again almost in accordance with the first course of inactivation (dotted line).

Salts of bivalent metals, such as calcium acetate, magnesium sulfate and barium chloride were of considerable potency in regenerating the enzyme and, to a lesser extent, manganese, cobalt, and zinc were also effective. The degree of potency ran parallel with the metal complex strength of EDTA (i.e., $\text{Ca} > \text{Mg} > \text{Ba}$), but, as

10) R. Willstätter and G. Schudel, *Ber.*, **57**, 780 (1918).

* In all of the following experiments, controls without added EDTA were quite stable.

- 3) A. Tanaka, *J. of Japanese Biochem. Soc.*, **30**, 431 (1958).
- 4) T. Yamamoto and J. Hukumoto, This Bulletin, short communication, **23**, 68 (1959).
- 5) T. Yamamoto and J. Hukumoto, Presented at the 155th Meeting of the Agri. Chem. Soc. Japan, held in Osaka, Dec. 13, (1958).
- 6) S. Akabori, T. Ikenaka and B. Hagihara, *J. Biochem.*, **41**, 577 (1954).
- 7) A. Tanaka, *Science Report of the Hyogo University of Agriculture*, **2**, 84 (1956).
- 8) A. Tanaka, *J. of Japanese Biochem. Soc.*, **28**, 529 (1956).
- 9) S. Akabori et al., *J. of Biochem.*, **41**, 803 (1954).

FIG. 1. The Reversibility of Inactivation (I)

Dotted lines do not show the time course of restoring the activity, but they only show the maximum levels that can be reached by incubation with calcium acetate solutions.

for heavy metals, this relation was disturbed by the denaturing action of heavy metals themselves.

2. Effect of the Dilution upon the Rate of Restoring Activity.

Partially inactivated enzyme which was prepared in almost the same way as described in Exp. 1 was diluted to a various extent with $M/40$ veronal buffer (pH 8.5), and then kept in a refrigerator to effect reactivation. The progress of restoring activity was measured by the passage

of time (Tables Ia and Ib). In the latter case, calcium acetate solution was added previously prior to dilution in order to eliminate the influence of excess EDTA. In both cases, the rate of reactivation was not affected practically by the degree of dilution, suggesting that the reactivation is of a type of monomolecular reaction.

3. Effect of EDTA on the Reactivation of EDTA-inact. TAA and Heat-inact. TAA.

One ml aliquot of $M/250$ EDTA solution (pH

8.5, M/20 veronal buffer) was added to a series of tubes containing 1 ml of the enzyme stock solution, and then the mixture was incubated at 37°C. After three hours, the reaction mixtures

were withdrawn, were kept at 6°C to effect reactivation, 1 ml of EDTA or calcium solution being added beforehand to control their concentrations in the solution (Table II).

In cases where heat-inact. enzymes were employed, EDTA solutions were added to the heat-

FIG. 2. The Reversibility of Inactivation (2)

Barium- and Calcium-salt solutions (1ml, M/100, pH 8.5) were added merely for comparison. Dotted lines at the right hand side was displaced leftwards at the intact form

TABLE I. EFFECT OF THE DILUTION UPON THE RATE OF RESTORING ACTIVITY

a. diluted in the presence of unreacted EDTA incubation periods at 6°C (hrs.)

dilution	0	12	24	48
1	21.2	52.5	61.5	70.4
5	21.2	52.5	61.5	70.4
10	21.2	51.2	62.5	70.0

Inactivation condition; 37°C, pH 8.5 (veronal buffer M/40) 4 hrs, EDTA 100 mol equivalent

b. diluted after cancelling the unreacted EDTA with calcium acetate.*

dilution	incubation periods at 6°C.			
	0	5	24	48
1	47.7	72.0	79.5	85.0
10	47.7	73.4	81.5	85.9
50	47.7	73.5	80.0	85.9

Inactivation condition; 37°C, pH 8.5, 2 hrs, EDTA 100 mol equiv.

* Calcium acetate solution was added so as to make the final concentration of Ca** to M/1,000.

TABLE II. INFLUENCE OF THE ADDITION OF EDTA AND CALCIUM UPON THE REACTIVATION OF EDTA-INACT. TAA

additions	incubation period at 6°C (days)				
	0	3	5	7	check*
M/100 Ca(Ac) ₂	32.3	90.0	86.5	83.5	86.9
H ₂ O**	32.3	63.0	57.2	55.9	82.6
M/50 EDTA	32.3	41.5	27.7	20.4	76.5
M/10 EDTA	32.3	32.5	21.5	15.5	76.0

* After seven days 1 ml of 1M Ca(Ac)₂ solution was added and was stood at 6°C for 3 days.

** The net concentration of EDTA is calculated to be about M/1,000.

TABLE III. THE SUPPRESSIVE ACTION OF EDTA TOWARDS THE REACTIVATION OF HEAT-INACT. TAA

a. incubated at 5°C

EDTA added*	incubation periods (hrs.)			
	0	25	50	check**
0.0	34.0	63.5	68.0	69.1
2.5	34.0	59.0	67.5	69.2
25.0	34.0	48.0	50.7	69.2
250.0	34.0	40.0	42.8	69.9

b. incubated at 20°C

EDTA added*	incubation periods (hrs.)			
	0	2	4	check**
0.0	33.0	56.0	63.0	66.0
2.5	33.0	55.0	61.8	67.7
25.0	33.0	36.5	36.8	67.0
250.0	33.0	33.5	31.8	67.1

c. incubated at 28°C

EDTA added*	incubation periods (hrs.)			
	0	1	2	check**
0.0	32.5	52.3	60.0	64.1
1.0	32.5	45.0	59.2	65.9
2.5	32.5	41.5	51.5	66.0
5.0	32.5	35.5	39.0	66.0

* Expressed by mol equivalent of TAA.

** Occurrence of irreversible inactivation was checked up. 2 ml of M/100 Ca(Ac)₂ solution was added and was incubated at 28°C for 20 hrs.

TABLE IV. pH DEPENDENCE OF THE SUPPRESSIVE ACTION OF EDTA TOWARDS THE REACTIVATION OF HEAT-INACTIVATED ENZYME.

pH	activities after 25 hrs. incubation at 5°C*		difference (B-A)	check**	
	EDTA added (A)	not added (B)		(A')	(B')
6.0	49.1	52.5	3.4	58.7	57.3
8.0	45.5	55.5	10.0	62.9	60.0
8.5	43.0	58.3	15.3	64.7	62.0
9.0	38.0	56.5	18.5	62.9	62.8
9.5	37.9	57.3	19.4	64.1	65.5

* Activity at zero hour was 29.4.

** 2 ml aliquots of M/100 Ca(Ac)₂ solution were added to A and B, and held at 6°C for 2 days.

inact. enzymes (inactivated by 50°C, 1 hr., pH 8.5, M/20 veronal buffer), and incubation was carried out at different temperature stages (Table IIIa~c).

It is of special interest to note the temperature dependency of the suppressive ability of EDTA. As for heat-inact. enzyme, for example, as much as 250 M EDTA was required to stop the reactivation at 5°C, and 25 M at 20°C, but only 5 M EDTA was enough to stop their self-reactivation at 28°C.

Table IV shows the pH dependency of the suppressing ability of EDTA. One ml aliquot of the enzyme stock solution was adjusted to pH 8.5 by 1 ml of M/20 veronal buffer, and incubated at 50°C for 1.5 hrs. This procedure destroyed about 70% of the activity. After cooling, 1 ml of M/100 EDTA solution was added, pH being adjusted beforehand by 5 ml of M/10 veronal buffer, and incubated at 5°C for twenty-five hours. Controls without added EDTA were simultaneously run.

The difference may give the measure of the suppressing power of EDTA at that pH. Enhanced suppression can be observed as the pH value rises.

Throughout these experiments, no loss of activity due to the reversible and irreversible denaturation was observed. For example, incubation of active amylase with 100 mol equivalent of EDTA at pH 8.5 deprived only 5.0 and 6.5% of their activities upon incubation at 5°C for seven days and at 20°C for four hours, respectively. As for irreversible inactivation,

note the checks given in the Tables.

4. Calcium Content of the Enzyme reactivated by means of Magnesium Ion.

TAA solution (8×10^{-5} M, 8.5 ml, about 360 mg dry matter) was incubated at 33°C for seven hours with 10 mol equivalent of EDTA (M/50 solution 3.4 ml), pH of the solution being adjusted by the addition of 10 ml of veronal buffer of pH 9.5. By this procedure about 80% of activity was destroyed. Seventeen ml of M/50 MgSO₄ solution was then added to this solution, and it was kept at 28°C overnight, about 88% of the original activity being recovered thereby.

Enzymes were collected by precipitation with acetone, and were then converted into a crystalline Rivanol-amylase complex⁸⁾ (166.6 mg dry matter). The complex was then subjected to assay of calcium and magnesium. Results of flame-photometrical analysis of mineral components of the specimen after being dried over P₂O₅ at 56°C in vacuum till a constant weight was obtained, was as follows:

Found: Calcium 82.4γ (which corresponds to about 0.75 mol equiv. per 1 mol equiv. of amylase protein).
Magnesium trace (below 10γ)

5. Effect of EDTA on the Rate of Irreversible Inactivation.

One ml aliquot of the enzyme stock solution was mixed with each 1 ml of M/10 veronal buffer (pH 8.5) and M/1,000 EDTA solution (25 M equiv. of EDTA), held at 38°C for 13.5 hours, the enzyme almost completely losing its activity thereby. One ml of each ascending concentra-

tion of EDTA solutions was then added anew to the inact. enzyme solution, and incubation was continued again at the same temperature. The reaction mixtures were then withdrawn at intervals of several hours, and to these was added 1 ml of M/100 calcium acetate solution, and incubated at 28°C overnight to effect reactivation.

The results obtained are shown in Fig. 3.

FIG. 3. Effect of the Concentration of EDTA on the Rate of Irreversibilization

Under curve: TAA activities were diminished by incubation with 25 M equiv. of EDTA. After being almost completely destroyed, 0, 25, and 250 M equiv. of EDTA were added, and the incubation was continued.

Upper curve: Enzymes were reactivated by incubation with an excess calcium acetate solution at 28°C.

Three curves show the corresponding decrease in the amount of the remaining reversibly inact. TAA during EDTA digestion.

The results show that the concentration of EDTA is of a secondary significance in regard of the rate of irreversibilization, at least in so far as the concentration of EDTA does not overwhelmingly predominate. Looking at the matter having these facts in mind it may be suspected that the irreversibilization much more depends on a reaction of a self-decomposing nature.

DISCUSSION

In the last report, a kinetic scheme for a partially reversible system has been presented as the mechanism of EDTA inactivation of TAA³⁾.

Let k_1 , k_2 and k_3 be the velocity constants of the forward, reverse and irreversibly denatured reactions, respectively, then:

An example of a further confirmation of schema (1) may be found in the following data showing the formation of inflection points. To follow the net change in activity after complete restoration, i.e., $(A+B)$ with time, the partially inactivated enzyme which was withdrawn at time-intervals shown in Fig. 4, was incubated with a slight excess of the calcium acetate solution, and activity recovered was measured. The corresponding graphical analysis is shown in Fig. 4. Apparent inflection points can be seen in curves A', B', and C'.

FIG. 4. Formation of the Inflection Point

Curves A, B, C and A', B', C' show the course of EDTA-inactivation before or after restoring their activities by incubation with Ca-acetate solution. Inactivation conditions are

- A) 100 M equiv. of EDTA, 37°C, pH 8.5 (M/40 Veronal buffer)
- B) EDTA 2.5 M, 37.5°C, pH 8.0.
- C) EDTA 5.0 M, 35°C, pH 8.0.

A mathematical analysis of this phenomenon will be demonstrated as follows:

From the schema (1) we get

$$A = \frac{A_0}{K_2 - K_1} \{ (K_2 - k_1)e^{-K_1 t} + (k_1 - K_1)e^{-K_2 t} \}$$

$$B = \frac{k_1 A_0}{K_2 - K_1} (e^{-K_1 t} - e^{-K_2 t})$$

then $A + B = \frac{A_0}{K_2 - K_1} (K_2 e^{-K_1 t} - K_1 e^{-K_2 t})$

where, A_0 : initial amount of active amylase

$$K_1 = \frac{\sum_{i=1}^3 k_i - \sqrt{\left(\sum_{i=1}^3 k_i\right)^2 - 4k_1 k_3}}{2}$$

$$K_2 = \frac{\sum_{i=1}^3 k_i + \sqrt{\left(\sum_{i=1}^3 k_i\right)^2 - 4k_1 k_3}}{2}$$

In order to follow the time-change of $(A+B)$, put

$$f(t) = K_2 e^{-K_1 t} - K_1 e^{-K_2 t}$$

and differentiating it successively with respect to t gives:

$$f'(t) = K_1 K_2 (e^{-K_2 t} - e^{-K_1 t}) < 0$$

$$f''(t) = K_1 K_2 (K_1 e^{-K_1 t} - K_2 e^{-K_2 t})$$

Let $f''(t)$ be zero, then we get the inflection point at

$$t_{\text{infl.}} = \frac{1}{K_2 - K_1} \log \frac{K_2}{K_1} \quad (\because K_2 > K_1)$$

Focussing our attention upon the convexity of the curve—

let $t < t_{\text{infl.}}$ then $f''(t) < 0$ (upper convex)

let $t > t_{\text{infl.}}$ then $f''(t) > 0$ (under convex)

The shape of the curve expected from these results is quite in agreement with the tendency of the curve shown in Fig. 4, and it can thus also be taken as evidence of showing reliability of the mechanism.

As our next step in analysis, let us enter into some details concerning the mechanism of EDTA inactivation.

If we consider the most probable process which can be expected from schema (1), then

- a) Amylase·Ca (active)
 $+ \text{EDTA} \rightleftharpoons \text{amylase (inact.)}$
 $+ \text{Ca} \cdot \text{EDTA} \longrightarrow \text{irreversibly inactivated}$
- b) Amylase·Ca (active)
 $+ \text{EDTA} \rightleftharpoons \text{amylase} \cdot \text{Ca} \cdot \text{EDTA (inact.)}$
 $\longrightarrow \text{irreversibly inactivated}$

Assuming that the mechanism of inactivation is either of the two types pictured above, the loss of activity would then have to follow the course of a bimolecular reaction.

As it has been already shown the enzyme lost its activity, quite unexpectedly being due to monomolecular reaction, for example, raising the concentration of EDTA over a certain limit did not accompany a corresponding increase in the rate of inactivation¹¹⁾. Such mechanisms must therefore be excluded, and further studies are required to obtain a better schema compatible with the experimental data.

It is now a well-known fact that some parts of the enzyme in the solution are present in reversibly-inactivated state (probably inactivated by heat), and are in an equilibrium state with the remaining active enzyme.

In cases where the temperature is low enough so as to stabilize the enzyme, the equilibrium is thought to be shifted overwhelmingly towards the active side, and much of the largest part of the enzyme in the solution exists in the active form.

Here, in this case the results of Exp. 3 have showed that the reactivation of heat-inact. enzyme is predominantly inhibited by the presence of EDTA. This indicates that the addition of EDTA to this equilibrated system (active $\rightleftharpoons$ heat inact.) may result in stopping the backward (reactivating) reaction, followed by an equivalent loss in enzyme activity. Such a speculation may lead us to one plausible hypothesis that it is only the heat-inact. enzyme in solutions which is influenced by the sequestering action of EDTA.

Supporting evidence can be obtained by comparing the pH dependences of deactivation with the ability of EDTA to stop the regeneration (Table IV).

A rough proportionality can then be observed between them, that is, in the pH range so far as EDTA can act effectively, the degree of deactivation is highly correlated with the ability to stop the regeneration of heat-inact. enzyme.

11) A. Tanaka, *J. of Japanese Biochem. Soc.*, **28**, 567 (1956).

However, the question still remains open as to whether some addition compounds of heat-inact. enzyme with EDTA may occur or whether or not the Ca atom may be removed from the heat-inact. enzyme by EDTA.

Experiments designed to explore these points have shown that the reactivated enzyme, which was effected without the addition of calcium salts, still contains at least 1 mol of calcium (Fig. 2 dotted line and Exp. 4), and neither calcium nor some other essential factor is required in the process of regeneration (Exp. 2). In other words, this type of regeneration is of a self-reactivating nature, and 1 mol of calcium still remains to be removed, at least at the stage of reversible inactivation.

The results of Exp. 4 may give satisfactory evidence to illustrate the role of bivalent metal ions for restoring activity: that is, the addition of bivalent metal ions including magnesium ion have no direct interaction with the protein moiety of amylase, but only play a part in can-

celling the EDTA from their surroundings, thus facilitating the self-reactivation of the enzyme.

It would therefore conclusively be postulated that the mechanism of EDTA inactivation might mainly consist in the accumulation of heat-inact. enzyme through the sequestering action of EDTA. On this basis, a formulation of the following mechanism is suggested:

The complex formation of the heat-inact. enzyme with EDTA would indeed be expected to be the cause of accumulation of the heat inact. enzyme, but no clear evidence was obtained in this work.

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