Electrodialysis is a much more efficient technique for the preparation of calcium-free apoamylase. This procedure differs from chelation on several counts. (1) The *p*H cannot be controlled. The operation is carried out by necessity at the isoionic point of the protein, whatever it may be. As a matter of fact, this provides a convenient opportunity to measure this molecular constant. (2) The ionic strength cannot be controlled either and is unavoidably at its lowest level. (3) Whereas EDTA removes exclusively multivalent cations, electrodialysis will tend to eliminate most charged substances of reasonably low molecular weight. (4) No chemical is added to the system. In particular, there is no risk of side reaction between protein and metal-binding agent or contamination by soluble material that may be released by ion exchangers.

The efficiency of electrodialysis in the instance of amylases can tentatively be ascribed to the fact that these enzymes are acidic proteins, with isoionic points close to *p*H 5. In this region of *p*H the dissociation of the calcium-amylase complex appears to increase, presumably because the highly folded structure proposed for amylase (Isemura and Fujita, 1957) is somewhat loosened by the combined effects of low *p*H and low ionic strength, and perhaps by electric and membrane phenomena. In contrast, amylase cannot be conveniently exposed to EDTA at *p*H 5.0, because the affinity of EDTA for calcium is much reduced at acid *p*H values. Moreover, amylase would not tolerate the acidity during the prolonged period of time needed for this approach.

In spite of the fact that electrodialysis may not be suitable for those proteins which are insoluble at their isoionic *p*H or do not tolerate complete absence of salts, the results obtained with amylases certainly indicate that electrodialysis may prove to be a useful technique for the study of metalloenzymes and other metal-binding macromolecules such as polysaccharides or nucleic acids.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J. G. Falbriard for his contribution pertaining to the electrodialyses of *A. oryzae* amylase and to Mrs. Lenore Wiegman for her general technical assistance.

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Alpha-Amylases as Calcium-Metalloenzymes. II. Calcium and the Catalytic Activity*

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The catalytic properties of the α-amylases from *B. subtilis* and human saliva have been studied during the progressive removal by chelation or electrodialysis of the calcium bound to these enzymes. The residual saccharogenic and dextrinogenic activities were determined at high and low enzyme concentrations. In all instances, removal of calcium was accompanied by a loss of activity that could be quantitatively reversed by restoration of the metal. Human salivary amylase required at least one g-atom of calcium per mole for full activity, whereas the bacterial enzyme needed four or more. This requirement for calcium in amylolysis is interpreted in the following manner: By forming a tight metal-chelate structure, the metal produces intramolecular cross-links similar in function to disulfide bridges, which confer to the α-amylase molecule the structural rigidity required for effective catalytic activity.

The prevailing role of calcium as a stabilizer of α-amylases has been known since the turn of the century (Wallerstein, 1909). In contrast, the participation of this metal in the enzymatic activity (Fischer *et al.*, 1960) went unnoticed for a long time, because of three misleading facts. First, calcium is bound so firmly to amylases that it cannot be removed by ordinary dialysis against most metal-binding agents. Second, when more severe conditions were applied, irreversible denaturation occurred and restoration of calcium failed to reactivate the enzyme. Finally, even when

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appropriate conditions for reversible removal of calcium were devised, the loss of enzyme activity was difficult to observe because spontaneous reactivation of the enzyme occurred during the assay, due to the ubiquity of calcium and the avidity of most α-amylases for this metal (Vallee *et al.*, 1959).

Preliminary results had indicated that a partial removal of calcium from bacterial amylase was accompanied by a corresponding decrease of enzymatic activity (Vallee *et al.*, 1959). With the recent availability of methods effecting the *complete* removal of calcium from α-amylases without causing irreversible denaturation (see preceding publication, Stein *et al.*, 1964), it has become possible to undertake a systematic study of the role of calcium in amylolysis. The present paper reports on the correlation between calcium content and catalytic activity of the α-amylases derived from *B. subtilis* and human saliva.

Experimental Procedures and Results

Several materials and methods used here have been detailed in the preceding report (Stein *et al.*, 1964), including enzyme preparation, reagents, treatment of water and of glassware, metal analyses, and removal of calcium from amylases by dialysis vs. EDTA[1] or electrodialysis.

Characterization of Human Salivary α-Amylase

Deionization.—Two batches of fourth crystals were dissolved by raising the *p*H of the suspension to 10–11 with 0.1 N NaOH; the solutions were then brought to *p*H 7.5–8.0 with 0.1 N acetic acid, incubated for 12 hours with diisopropylphosphorofluoridate[2] (10^{-4} M final concentration), and dialyzed overnight against deionized water. Thirty ml of each dialyzed amylase solution (4–5 mg/ml) was electrodialyzed for 2 hours in order to eliminate extraneous charged compounds of low molecular weight (salts and possibly amino acids) bound to the enzyme molecule, which would interfere with the determination of protein dry weight. Electrodialysis did not bring about any change in either UV absorbance or enzymic activity (when assayed in the presence of calcium), but decreased the calcium content from 1.3 to 0.1 g-atom per mole in one instance, and from 3.8 to 0.3 in the second. Since α-amylases are believed to bind their intrinsic calcium more strongly than any other ion, the loss of calcium can be taken as a measure of the efficiency of the deionization process.

Dry Weight.—Triplicate 7-ml samples of deionized amylase solutions were pipetted into weighing bottles and placed over P_2O_5 in a dessicator under a slight vacuum. After 2 days a thick film was obtained; the P_2O_5 was renewed and a strong vacuum (oil pump) was maintained until constant weight was obtained (33.7 mg). Further dessication of the sample in a vacuum oven (30 lb/sq in. of negative pressure) for 18 hours at 80° did not affect the dry weight.

Nitrogen Content.—The nitrogen content of the deionized amylase solution was determined by micro-Kjeldahl on triplicate samples; values agreed within 0.5% (see Table I).

Ultraviolet Absorbancy.—UV absorbancies at 280 mμ were determined in the Beckman DU and DK-1 spectrophotometers in dilute sodium glycerophosphate. The extinction coefficient was not significantly affected by *p*H changes in the range of 6–7 nor by variations in buffer concentration between 0.002 and 0.02 M. As can be seen in Table I, excellent correlation was obtained between the experimentally determined absorbancies of several α-amylases of different biological origins and the values calculated from the contribution of their aromatic amino acids, in spite of the fact that these amylases differ markedly in their content of tryptophane, tyrosine, and phenylalanine (Fischer and Stein, 1960).

Assay of Amylase Activity

Various assay procedures have been used to study the relationship between calcium content and amylolytic activity:

Method I (Routine Saccharogenic Assay).—The test is performed as previously described (Fischer and Stein, 1961) at *p*H 5.9 for microbial and 6.9 for mammalian amylases. The enzyme is diluted stepwise in buffer and incubated for 3 minutes at 25° with a 1% solution of buffered "Noredux" soluble starch (purchased from Siegfried Co., Zofingen, Switzerland). The buffer selected is sodium glycerophosphate hydrochloride (Eastman Organic Chemicals or Nutritional Biochemicals Corp.), because α-amylases appear to display optimum stability and activity in its presence; moreover, it does not precipitate calcium.

Method II.—To prevent spontaneous reactivation of metal-depleted amylase by extraneous calcium, the routine assay procedure is modified as follows: Glycerophosphate is replaced by a buffer less heavily contaminated with calcium, namely, maleic acid–sodium maleate (0.01 M). Moreover, calcium-free starch as a 1% solution in deionized water is used as a substrate. The latter is prepared by electrodialyzing a 5–10% solution of Noredux starch for 24–48 hours at 400 v (as described by Stein *et al.*, 1964), followed by freeze-drying. Finally, EDTA is added to the test system (2×10^{-3} M for bacterial amylase and 10^{-4} M for human amylase) in order to block trace contamination of calcium.

Method III (Rapid Assay).—This procedure involves a simplified version of a rapid-flow system. Two 2-ml graduated pipets are inserted vertically three-fourths way into a polyethylene test tube containing 1 ml of enzyme (approx 0.5 mg). The tops of the pipets are connected to a pool of mercury (serving as an air-tight plug) and to suction bulbs allowing the aspiration of 1 ml of substrate (1% calcium-free starch) into the first pipet and 2 ml of stopping reagent into the second. When the mercury reservoir is drained, the substrate is released into the enzyme solution, followed within less than a second by the stopping reagent. The assay is performed in the cold room and the tube is immersed in an ice-bath. After addition of the stopping reagent, half the reaction mixture (2 ml) is transferred to a glass test tube, diluted with 10 ml of water, and heated as in method I. In this rapid assay, production of reducing groups is directly proportional to the concentration of enzyme, the results are reproducible within ±5%, and the colorimeter readings are of the same order as those of the previous two methods.

Method IV (Dextrinogenic Assay).—The iodine test of Smith and Roe (1957) has been modified as follows: Two ml of a 0.2% starch solution (1:5 dilution of the calcium-free starch solution used in method II) is added to 1.0 ml of enzyme diluted in maleate-EDTA as above. The mixture is incubated for 3 minutes at 25° and the reaction is stopped with 1 ml of 1 N HCl. Finally, 20 ml of water and 0.5 ml of 0.01 N iodine solution prepared according to Rice (1959) are added and the absorbancy *A* is read on a Klett-Summerson

[1] Abbreviation used in this work: EDTA, ethylenediaminetetraacetate.

[2] This was done to inhibit traces of proteolytic enzymes. Unless these contaminants are thoroughly inactivated, α-amylases will undergo proteolysis and irreversible inactivation upon removal of calcium (Stein and Fischer, 1958).

TABLE I
SPECIFIC ACTIVITIES, NITROGEN CONTENT, AND ABSORBANCY INDICES

	α-Amylases from				
	Human Saliva[a]		Porcine		
	1st Batch	2nd Batch	Pancreas[b]	*B. subtilis*[c]	*A. oryzae*[d]
Specific activity[e]	1980	2080	1400	2300	450
Nitrogen content (%)	17.3	17.2	17.7	16.2	14.9
$A^{1\%}_{1\,cm}$ at 280 mμ:					
Observed		23.3	24.1	25.3	19.7
Calculated[f]		23.3	23.8	25.4	19.5

[a] This paper. [b] Yamamoto, Stein, and Fischer, unpublished results. [c] Junge *et al.*, 1959. [d] Stein *et al.*, 1960. [e] Saccharogenic activity measured according to Method I, expressed in mg maltose per mg protein. [f] The "calculated" absorbancies were computed as follows: The number of residues per 1,000 residues of tryptophane, tyrosine, and phenylalanine (Fischer and Stein, 1960) was divided by 1, 4, and 30, respectively. The sum of these weighted numbers was then multiplied by the empirical factor of 0.475.

colorimeter (red filter). The instrument is adjusted to zero reading with an iodine blank containing neither enzyme nor substrate. The dextrinogenic activity is expressed in arbitrary units as follows: $D = [(A_s - A)/A_s] \times E$, where A_s is the absorbance of the starch-iodine complex in the absence of enzyme and E is the enzyme dilution. Best results are obtained when the enzyme solution is diluted in such a manner as to make the ratio $(A_s - A)/A_s$ approach 0.20–0.25.

Dilution of the Enzyme Prior to Assay.—As reported earlier (Fischer and Stein, 1954), amylase activity is affected to some extent by the dilution procedure. Nevertheless, when comparing activities, it is preferable to remain within narrow ranges of starch degradation and colorimeter readings by diluting the enzyme to the appropriate level. For the saccharogenic assays, an amount of enzyme was used that produced oligosaccharides with a reducing power corresponding to 1 mg of maltose (hydrated) under the condition of the assay, i.e., approximately 0.5 μg of calcium-full amylase (10^{-8} M) for methods I and II, 500 μg for method III, and 0.25 μg for the dextrinogenic assay. Whenever the enzyme to be assayed had lost part of its activity, correspondingly higher amounts of protein were used; this was not always possible in the instance of the "rapid assay," for at high protein concentration addition of starch produced milkiness, which in turn gave erratic results.

Evaluation of the Various Assay Methods.—Under the experimental conditions of the routine saccharogenic assay, calcium-depleted amylases which had not undergone denaturation were fully reactivated at once by the calcium ions that contaminate both buffer and substrate. Glycerophosphate was not analytical grade; its metal content is difficult to determine because the phosphate residues interfere with the analytical procedures. Furthermore, Noredux starch contains approximately 0.4 mg of calcium per g, most of which is firmly bound. The 1% starch solution used in the assay thus corresponded to a 10^{-4} M calcium solution and provided a 10,000-fold molar excess of calcium over enzyme. Obviously, possible reductions in the activity of calcium-depleted amylases cannot be demonstrated in such a system; therefore method II was devised.

The major difficulty in attempting to correlate calcium levels with catalytic activity stems from the fact that the enzyme is assayed at a concentration several thousand times lower than that at which it is analyzed for metals. At these great dilutions, extraneous factors modify the metal content of the enzyme to a large extent. The "rapid assay" (method III), which permits the use of 1000 times more enzyme without an increase in the extent of amylolysis, was devised to minimize these difficulties (W. N. Sumerwell, unpublished results from this laboratory).

The various classes of amylases (α, β, and γ) have been characterized by comparing the rate of appearance of reducing groups and the rate of disappearance of the "iodine color," i.e., by the ratio of their saccharogenic-to-dextrinogenic activities. This ratio has been shown to vary significantly even among α-amylases of different biological origins (Tung Kung *et al.*, 1953) and to be influenced by addition of calcium in the instance of bacterial amylase (Yamamoto, 1956). Method IV was used here in order to find out whether the ratio between these two forms of activity would remain constant when the calcium content of α-amylases is modified.

Correlation between Calcium Content and Amylase Activity.—Solutions of amylase, crystallized four times, were exposed to electrodialysis or extensive dialysis vs. EDTA as previously described (Stein *et al.*, 1964). During the progressive removal of metal, samples were removed and analyzed for calcium (Stein *et al.*, 1964) and specific activity. For the latter purpose each sample was assayed at least three times, first, according to the routine assay, to ascertain that no *irreversible* inactivation had occurred during removal of the metal. Since full activity was always obtained, the data pertaining to this control have been omitted. Second, amylase activities were measured in a "calcium-free" system according to methods II, III, or IV. The residual activities thus determined were expressed as a percentage of the value obtained in a third assay identical to the second one, except that a 10,000-fold molar excess of calcium (10^{-4} M) over enzyme was added in place of EDTA. Under the latter conditions the enzyme could always be reactivated to the levels obtained in the routine assay.

Effect of Calcium Removal on the Specific Activity of B. subtilis α-Amylase

Figure 1 shows that there is a threshold at approximately 4 g-atoms of calcium per mole, below which a gradual decrease in activity accompanies the progressive elimination of calcium from bacterial amylase. The extent of inactivation is the same whether measured at high or low enzyme concentration. Also, the ratio of saccharogenic to dextrinogenic activities remains constant during removal of the metal. However, the correlation between calcium content and activity appears to depend somewhat on the experimental conditions used for the removal of the metal. Except in the instance of curve C, where the removal of calcium was quite abrupt (8 hours), the enzyme seems to lose activity more slowly than calcium. One possible explanation for this phenomenon, in harmony with the

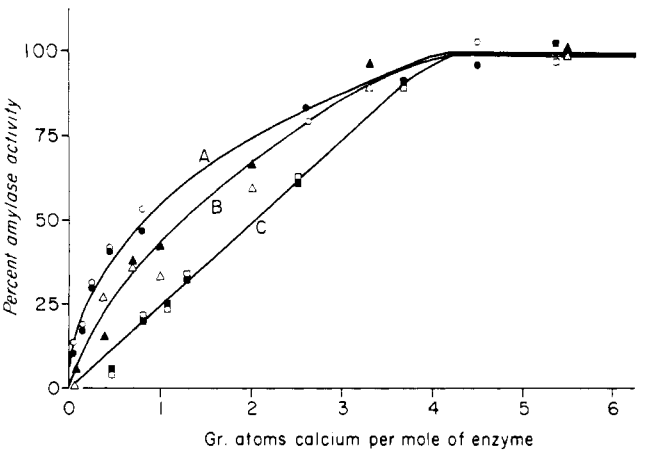

FIG. 1.—Correlation between calcium content and enzymatic activity of *B. subtilis* α-amylase. The three curves (A, B, C) refer to different procedures used for the removal of calcium: *curve A* (-●-○-), dialysis against 0.01 M EDTA, *p*H 6.2, for a total period of 6 days; *curve B* (-▲-△-), electrodialysis during 25 hours, final *p*H 5.2; *curve C* (-■-□-), electrodialysis for 8 hours, final *p*H 5.2. The concentration of the protein in solution remained at 4.5 mg/ml in experiment A, whereas it decreased from 5 to 4 and from 4.9 to 4.5 mg/ml in experiments B and C, respectively. The solid symbols (-●-, -▲-, -■-) refer to activities measured according to method II; the decreasing dilutions (9,000–400) required to obtain 1 unit of activity per ml were performed within 3 minutes at 25°. -○-, enzyme assayed according to method III after 7- to 1-fold dilutions in deionized water. For reactivation, 0.15 ml of enzyme was added to 0.15 ml of 0.02 M Ca^{++} and 0.7 ml of 0.02 M sodium glycerophosphate. -△-, enzyme assayed according to method III after a 7-fold dilution in water, irrespective of the level of residual activity. For reactivation, the enzyme was diluted 7-fold in 0.01 M calcium maleate. -□-, method IV following 18,000- to 800-fold dilution. In experiment A (-●-○-), the *p*H of the assay system was 6.2 rather than 5.9.

shape of the curves, is that the four atoms of calcium required for full activity do not contribute equally to effective amylolysis, the last atom to be relinquished by the enzyme being the most important. According to another hypothesis, calcium-free amylase might still possess a small residual activity. The fact that the rate of inactivation of amylase as a function of calcium removal was slowest when chelation (curve A) rather than electrodialysis was used could be ascribed to milder conditions more favorable to the display of a labile residual activity of calcium-free amylase: (a) the *p*H was 6.2 as compared to 5.2 in the instance of electrodialysis, i.e., closer to the *p*H of optimum stability (6.5) of calcium-free amylase (Fischer *et al.*, 1960); (b) the ionic strength, and particularly the concentration of Na^+ ions, was much higher; (c) there were no electric nor membrane phenomena; (d) the removal of calcium was very gradual, which might have allowed some slow rearrangement within the active center.

Influence of Calcium Removal on the Enzymatic Activity of Human Salivary Amylase

The correlation between calcium content and enzymatic activity was more difficult to establish in the instance of human salivary amylase than in that of the bacterial enzyme. Metal-depleted amylase showed a strong tendency to crystallize in the presence of EDTA (Stein *et al.*, 1964), and residual activities were suspiciously low, perhaps because some of the enzyme was present in the assay system as microcrystals and did not act on the high-molecular-weight substrate. Indeed, this was most obvious in the "rapid assay," where the enzyme was used at high concentrations.

At low enzyme concentration (10^{-8} M) a second difficulty arose because of the sensitivity of salivary amylase to EDTA. When this enzyme was diluted in EDTA to levels of the order of 1 μg/ml it tended to relinquish its metal readily to the chelating agent. Table II gives an idea of the relative susceptibility to EDTA of bacterial and human α-amylases at extreme dilutions and shows that bacterial amylase is much more resistant than salivary amylase to sodium EDTA, but becomes as labile (Yamamoto, 1956) as the latter enzyme in the presence of ammonium EDTA.[3] Since milder metal-binding agents such as oxalate, polyphosphates, and citrate were too weak to compete with calcium-free salivary amylase for extraneous calcium, even when used at concentrations as high as 0.04 M, the following conditions were devised under which salivary amylase could safely be exposed to EDTA: The enzyme was diluted within 2 minutes in calcium-free 0.005 M NaCl containing 10^{-4} M EDTA or, for reactivation, 2×10^{-4} M $CaCl_2$; the first steps of the dilution were performed at 0° and the last one at 25°. Then the enzyme was incubated with calcium-free starch buffered at *p*H 6.9 with 0.01 M sodium maleate–maleic acid containing 0.005 M NaCl to provide the chloride ions required for mammalian amylase activity. Under these conditions a preparation containing 1 g-atom of calcium per mole still displayed full activity.

The results are illustrated in Figure 2; a log-log plot was used in this case since most of the determinations were made at low calcium levels. It can be seen that a gradual decrease in activity accompanies the removal of the metal irrespective of whether the activity is measured at high or low enzyme concentration, by means of the saccharogenic or the dextrinogenic assay procedure. As in the case of bacterial amylase, the catalytic activity does not seem to decrease as fast as the calcium content. Assuming that the precautions taken to prevent reactivation by extraneous metal contamination were adequate, this could indicate that calcium-free salivary amylase may retain 5% or 10% activity. It is of interest that salivary amylase remains fully active as long as it still contains at least 1 g-atom of calcium per mole. Thus its calcium requirement appears to be approximately four times smaller than that of *B. subtilis* amylase.

Reactivation of Calcium-free Amylase

For all practical purposes the reactivation of calcium-free amylase is instantaneous when a large molar excess of calcium is used. A 10,000-fold excess appears to be optimum. However, reactivation can be slow and may require hours or even days if the apoenzyme has been exposed to conditions leading to denaturation. For instance, crystals of calcium-free salivary amylase brought to *p*H 10.5 for solubilization purposes and then neutralized and assayed in the presence of a 10,000-fold molar excess of calcium displayed only 25% of the original activity. Forty per cent reactivation was achieved after one-half hour's incubation at 5° with 10^{-4} M Ca^{++} in sodium glycerophosphate *p*H 6.9, and 90% after 18 hours. Similarly, a dry film of calcium-depleted salivary amylase that had been kept at 80° for 18 hours, as described for dry weight determination, could be dissolved and partially reactivated after 2–3 days' incubation with calcium. Similar experiments will be detailed in a later publication.

When reactivation was slow or incomplete, it was

[3] As reported in the preceding publication (Stein *et al.*, 1964), bacterial amylase surrenders its bound calcium faster when the enzyme is exposed to EDTA in the ammonium, rather than in the sodium, form.

TABLE II

RESIDUAL ACTIVITY OF 10^{-3} M SOLUTIONS OF NATIVE CALCIUM-CONTAINING α-AMYLASES IN 0.01 M SODIUM MALEATE AFTER A 10-MINUTE EXPOSURE TO SEQUESTERING AGENTS (*p*H 6.8, 25°)

Amylase	Metal Binding Agent		Residual Activity[a] (%)
B. subtilis	Sodium EDTA	10^{-4} to 10^{-3} M	100
		5×10^{-3} M	95
	Ammonium EDTA	2×10^{-3} M	35
Human saliva	Sodium EDTA	1×10^{-5} M	85
		2×10^{-4} M	55
		2×10^{-3} M	35
	Sodium oxalate	4×10^{-2} M	100

[a] As compared to control incubated without EDTA.

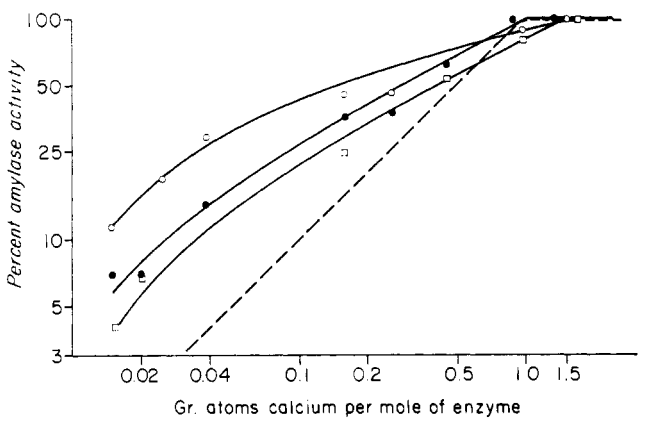

FIG. 2.—Changes in specific activity during removal of calcium from human salivary amylase. Calcium was removed by electrodialysis over a period of 4 hours during which the enzyme concentration fell from 5.5 to 2.5 mg/ml due to precipitation or crystallization. The data have been plotted on a log-log graph. The dotted line represents the theoretical correlation if one atom of calcium per mole were required for full activity. Assay: -●-, method II; -○-, method III (enzyme diluted in water to 0.8–1.0 mg/ml irrespective of the level of its residual activity); -□-, method IV.

possible to demonstrate a secondary effect of ions other than calcium. The reactivation of bacterial amylase by calcium seemed to be enhanced by Na^+ ions and very much hindered by NH_4^+ ions. In the instance of salivary amylase, chloride ions facilitated reactivation by calcium.

Attempts to achieve gradual reactivation of calcium-free amylase by limiting amounts or small excesses of calcium (0.4–10 g-atoms/mole) gave erratic results and no reproducible correlation between activity and calcium content could be obtained. Under these conditions the system was very sensitive to a variety of factors such as temperature, ionic strength, *p*H, nature of other ions present, etc.

DISCUSSION

The exact way in which calcium is involved in amylolysis is not immediately apparent. If the metal were to constitute a reactive group, inhibition of amylase activity by calcium-chelating agents should occur rapidly, for instance, through formation of a ternary complex amylase-calcium-EDTA. This has never been observed; instead, inactivation in the presence of EDTA appears to proceed at a slower rate than removal of calcium. It seems rather unlikely, then, that calcium participates directly in the formation of the enzyme-substrate complex, or that it is directly instrumental in the cleavage of the glycosidic bond. Perhaps the metal is not even located at the active site, but rather buried inside the native molecule, as suggested by the very slow rate of its sequestration (Stein *et al.*, 1964). To interpret the close relationship between calcium and amylolysis that has been demonstrated here, the following explanation is proposed. Calcium confers to the amylase molecule the structural rigidity required for effective biological activity by forming a tight, intramolecular, metal-chelate structure. It is reasonable to assume that formation of the enzyme-substrate complex produces some stress that may result in the collapse of the active center of the amylase molecule, unless the secondary and tertiary folding of the latter is stabilized by calcium cross-link(s).

If one assumes that the participation of calcium in the catalytic activity of α-amylases rests on a structural basis, then an attractive explanation becomes available for the fact that *B. subtilis* α-amylase requires roughly four times more calcium for full activity than other α-amylases of nonbacterial nature. Whereas the latter enzymes, including the α-amylases from human saliva and *A. oryzae*,[4] have so far always been found to contain cystine residues, (Stein *et al.*, 1960), *B. subtilis* α-amylase possesses no disulfide bridges (Junge *et al.*, 1959). This is to be expected from an extracellular protein of bacterial origin (Pollock and Richmond, 1962). It would seem, therefore, that calcium substitutes for missing —S—S— linkages. Further work is in progress in this laboratory to substantiate that calcium linkages are indeed instrumental in preserving the integrity of the active center of α-amylases. It is believed that intramolecular cross-links involving alkaline-earth metals and playing a role akin to that of disulfide bridges might be of general significance, rather than restricted to α-amylases. Indeed, a microbial nuclease has recently been isolated that does not possess any cystine residues but contains calcium (C. B. Anfinsen, personal communication). Moreover, the neutral protease from *Streptomyces griseus*, which requires calcium ions both for stability and activity (Nomoto *et al.*, 1960), has been found to be free of disulfide bridges.

The data indicating that amylase activities do not seem to decrease so fast as the calcium content, and that a small residual activity (<10%) might still be displayed by calcium-free amylase are consistent with the structural role proposed for calcium in amylolysis. Obviously the architecture of the active center does not rest solely on calcium cross-links; other linkages such as hydrophobic attractions and hydrogen bonds must also be involved. Indeed, under carefully controlled conditions it is possible to remove calcium from amylase without bringing about changes in optical

[4] *A. oryzae* amylase, which is fully active in the presence of only 1.0 g-atom of calcium per mole, has been shown to be stabilized by four disulfide bridges (Isemura *et al.*, 1963).

rotation (Fischer *et al.*, 1958). However, when starch is added to calcium-depleted amylase the interaction between the substrate and the enzyme molecules that have lost their rigidity is believed to bring about inactivation of the latter. On the contrary, combination with starch will protect native amylase from loss of calcium, as observed by Yamamoto (1956) and Vallee *et al.* (1959).

Since the active center of calcium-free amylase is presumably still precariously held together by weak linkages very sensitive to a variety of external factors such as *p*H, temperature, ionic strength, etc., it follows that the minimum number of calcium atoms required for full enzymatic activity will depend to some extent on the experimental conditions. The same applies to the assessment of a possible residual activity of calcium-free amylase.

Yamamoto and Fukumoto (1960) found that partial reactivation of calcium-depleted amylase could be achieved by Sr^{++}, Mg^{++}, Ba^{++}, and even Be^{++}, a strong inhibitor of mammalian amylases (McGeachin *et al.*, 1962). These alkaline-earth cations were apparently not spectroscopically pure and were used in a 20,000-fold molar excess over enzyme. Moreover, the substrate contained 0.1% of calcium; to compensate, EDTA was added to the starch. According to our own experience, however, much of the calcium that contaminates starch is so firmly bound that it cannot be readily chelated by EDTA. Under these circumstances it is difficult to assess the extent to which other multivalent metals can replace calcium within the amylase molecule. Indeed, even monovalent cations such as sodium (Yamamoto and Fukumoto, 1959), or anions such as chloride, appear to have some stabilizing action on the secondary linkages just discussed.

The participation of linkages such as hydrogen bonds and van der Waals forces in the cohesion of the active center should not obscure the major role played by calcium cross-links. All α-amylases so far investigated, namely, barley malt (Schwimmer and Balls, 1949), *A. oryzae*, *B. subtilis*, porcine pancreas, and human salivary α-amylases, have each been found to possess a small number of sites to which calcium is specifically bound (Vallee *et al.*, 1959). Whenever removal of calcium could be achieved the proteins lost their catalytic properties. On the other hand, restoration of calcium under appropriate conditions always led to a quantitative recovery of activity. Therefore it is proposed that α-amylases belong to a new class of metallo-enzymes characterized by a prosthetic group that is an alkaline-earth metal rather than a transition element, and which plays primarily a structural role, reminiscent of that of disulfide bridges.

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