Alpha-Amylases as Calcium-Metalloenzymes. I. Preparation of Calcium-free Apoamylases by Chelation and Electrodialysis*

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Two methods leading to the complete removal of calcium from the α-amylases of *B. subtilis* and human saliva are described, namely, chelation by ethylenediaminetetraacetate (EDTA) and electrodialysis. In contrast to earlier procedures, these techniques do not bring about irreversible denaturation, and thus yield calcium-free amylases that can be fully reactivated upon restoration of the metal. Electrodialysis proved to be a much more efficient procedure than chelation; whereas removal of calcium from salivary amylase required 60 hours of dialysis vs. EDTA, it could be achieved in 2–4 hours by electrodialysis. Calcium-free human salivary amylase could be crystallized. The rate at which calcium was released from α-amylases varied markedly according to the biological origin of these enzymes, decreasing in the order mammalian > bacterial > fungal.

All α-amylases so far investigated have been found to contain at least one atom of calcium firmly and specifically bound to the enzyme molecule (Fischer *et al.*, 1960; Vallee *et al.*, 1959). Mounting evidence[1] indicates that the metal innate to α-amylases plays a decisive role in preserving the integrity of the enzyme. Indeed, calcium might be visualized as the "locking-pin" of the entire molecular structure, accounting for the compactness and rigidity, remarkable stability, immunity to proteolytic enzymes, and perhaps the catalytic activity of α-amylases.

In order to investigate further the role of calcium, it was necessary to devise methods by which amylase preparations could be obtained completely free of calcium. Earlier reports, however, indicated that removal of the metal could be achieved only under relatively drastic conditions which led to irreversible inactivation and, presumably, denaturation of the protein (Yamamoto, 1956). The present paper describes conditions whereby EDTA[2] can be used to remove all calcium from *Bacillus subtilis* and human saliva α-amylases without irreversible denaturation. Also reported are preliminary results concerning the removal of calcium from *Aspergillus oryzae* α-amylase.

In order to ascertain the role of calcium in the structure and function of the enzyme, the properties of calcium-free apoamylase must be compared with those of the native calcium-containing enzyme or, better yet, with the properties acquired by the apoenzyme upon restoration of the metal, provided of course that the removal of calcium and all related modifications are fully reversible. The former approach is not entirely unequivocal, since the behavior of the enzyme upon removal of calcium could be a reflection of the procedure used to eliminate the metal, rather than the result of genuine structural modifications caused by loss of calcium. For this reason, the need arose for a second technique, completely different from chelation, that would also yield calcium-free amylases. Electrodialysis, as described below, proved to fulfill these requirements most adequately.

The accompanying paper (Hsiu *et al.*, 1964) reports on the role of calcium in the catalytic activity of α-amylases. The importance of intramolecular calcium cross links in the over-all stabilization of the amylase molecule will be examined in a later publication.

Experimental Procedures

Enzyme Preparations.—Endoamylases from *B. subtilis* and human saliva were purified and crystallized four times in the presence of diisopropylphosphorofluoridate. The purification procedures and assay method have been described elsewhere (Stein and Fischer, 1961; Fischer and Stein, 1961). A molecular weight of 50,000 was used in the calculation of calcium content (Fischer and Stein, 1960). Protein concentrations were determined spectrophotometrically at 280 mμ in 0.005 M glycerophosphate buffer, *p*H 6.8, using the factor $E^{1\%}_{1\,cm}$ = 25.3, 23.3, and 19.7 for bacterial, human, and fungal amylases, respectively (Hsiu *et al.*, 1964).

Water was redistilled in an American Sterilizer Co. steam-operated water still and further purified by passage through a Plexiglas column, 10 × 100 cm, three-fourths full of "analytical reagent" Amberlite MB-1 (monobed mixture of IR-120 and IRA-400 ion exchangers, purchased from Mallinckrodt). A 5-cm high layer of Amberlite IR-120 was placed at the bottom of the column to retain material released from the anionic resin. Water was stored in polyethylene carboys. When 2 liters of the deionized water was evaporated to dryness in an acid-washed Pyrex flask, and the presumed residue was taken up in concentrated redistilled nitric acid, no calcium could be detected by the analytical procedure described below.

Glassware.—Pipets, crucibles, glass covers, and the like used for the metal analyses were treated for several hours with redistilled, hot, concentrated HNO_3, then thoroughly rinsed with distilled water, dried in an oven, and stored in dust-proof plastic containers. All analytical reagents were kept in polyethylene bottles.

Reagents.—All reagents were Baker Analyzed Reagents, unless otherwise stated; their calcium content was checked before use. The 0.01 M NaCl used in connection with the electrodialysis procedure was further purified by passage through a column of Dowex A-1 chelating resin to remove calcium and other multivalent cation contaminants.

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[1] For a review of the literature, see Fischer and Stein (1960).

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

Metal Analyses.—Spectrographic analyses performed on a variety of crystalline amylases of different biological origins (Vallee *et al.*, 1959) had shown that calcium was the only cation found in significant amounts in the samples prepared for this study.[3] It was therefore quite permissible to employ an analytical procedure far less specific than emission spectroscopy and restricted to the analysis of calcium. Titration with EDTA at *p*H 12–13 in the presence of "Calcein" as indicator (Diehl and Ellingboe, 1956) was used, according to the method of Socolar and Salach (1959). The only metal ion demonstrated to interfere significantly with this analytical procedure was strontium, which was shown to be absent from the crystalline enzyme preparations. The titration was modified for the analysis of amounts of calcium ranging from 2.5 to 40 μg: the EDTA solution was 12.5 mM in 0.2 N NaOH. One μg of Ca^{++} consumed 20 μl of this solution. For the stock indicator solution, 50 mg Calcein (fluoresceinmethyleneiminodiacetic acid, purchased from G. Frederick Smith Chemical Co., Columbus 22, Ohio) was dissolved in 50 ml of 0.1 N NaOH; this solution could be stored at 4° in the dark for a few months. Before use, a 1:10 dilution in water was prepared.

Mineralization.—Aliquots from dialyzed amylase solutions were evaporated to dryness in porcelain crucibles (Coors Size 0, glazed, white) on a sand bath. Usually there was no tendency to foam. After cooling, 2 ml of redistilled nitric acid and 8 drops of 72% perchloric acid were added to the film of dried protein. The crucible was covered with a weighted watchglass (30–35 g), convex side down, to slow down the evaporation of the oxidizing agents as much as possible, and heated on the sand bath for about 4 hours at approximately 140° (electric hot plate on "low"), then for about 2 hours at 210° (hot plate on "medium"). The watchglass was removed and the remainder of the oxidant was driven off by turning the hot plate on the "high" position until all acid fumes had disappeared. At this point, the residue (if any) should be white or colorless; otherwise, the wet digestion should be repeated. EDTA was fully oxidized during this treatment and did not interfere with the subsequent titration. Destruction of the organic material was more efficient in the presence of 2 drops of concentrated H_2SO_4, but the complete elimination of this acid required for the titration proved to be tedious and detrimental to the precision of the analyses.

Titration.—Water (2.4 ml) was added to the cold crucibles and stirred for 30 minutes with a small magnetic stirrer embedded in polyethylene. One-half ml of 1 N NaOH and 0.1 ml of the Calcein solution were then added. When the sample was suspected of containing traces of heavy metals, the alkali solution was made 0.1 M in NaCN.

Titration was carried out with EDTA delivered from a 1-ml Gilmont microburet connected by means of a polyethylene capillary tubing to the bottom of the crucible, which was placed on a "Mag-Mix" motor. Appropriate illumination was required for optimum detection of the shift from yellow-green (Calcein-Ca complex) to pale orange-pink (Calcein in alkaline solution). Blurring of the end point by the fluorescence of the indicator was at a minimum since the titration vessel was opaque and white.

Reagent blanks, calcium standards, and unknowns were all run in triplicate. The calcium content of amylase was expressed by the following relation: g-atoms Ca/mole enzyme = 0.0625 × (μl EDTA/mg amylase), in which the factor 0.0625 gives the proper dimensions to the analytical data. The levels of calcium prior to sequestration or electrodialysis were of the order of 2 and 5 g-atoms/mole salivary and bacterial amylase, respectively. Removal of calcium was considered complete when the level dropped below 0.05 g-atom/mole.

It should be stressed that all steps of this analytical procedure were carried out in the same container; a transfer would have been required if the titration had been carried out in a spectrophotometer (Herrmann, 1958).

Dialysis versus EDTA.—Amylase solutions were enclosed in seamless cellulose tubings (Visking Co., Division of Union Carbide Corp., Chicago 38, Illinois) that were treated for 10 minutes in boiling water before use. The dialysis bags were suspended from a shaft of a slowly rotating (10-rpm) motor and immersed in a minimum of 100 volumes of 0.01 M EDTA (4°) which was renewed at least once every 24 hours. Although the sequestration of calcium by EDTA is optimum at alkaline *p*H values, dialysis must be performed between *p*H 6.0 and 7.0 in view of the very narrow *p*H-stability range of calcium-free apoamylases (Fischer *et al.*, 1960).

Electrodialysis.—A custom-made Lucite electrodialyzer (Medical Instrument Shop, University of Washington School of Medicine) was used (Fig. 1). The instrument consisted of two outer chambers, A and A′, fitted with platinum electrodes B, 23 mm in diameter, and a center chamber C separated from A and A′ by two membranes cut from a Visking casing (1-5/8 in. inflated diameter). These membranes, 0.00155 in. thick, also served as gaskets to keep the three compartments watertight. The inner chamber consisted of interchangeable cylinders 15, 20, 30, or 50 mm long, permitting the treatment of amounts of solution up to 50 ml. The distance between cathode and anode was 10 mm greater than the length of the center cell. The inside diameter of all three compartments was 38 mm. The electrodialyzer was equipped with a nylon stirrer D (fitted with a plastic blade E) connected to a motor with variable speed; this was important to prevent electrodecantation phenomena from taking place and to minimize local *p*H variations in the protein solution. Two holes were provided on top of the center compartment, one for the stirrer (F), the other (G) to accommodate the syringe needle used to introduce or withdraw protein solution from the center cell. The latter hole, which could also be used to introduce a needle-probe electrode for *p*H or isoionic point measurements, could be closed by a nylon screw (H).

Prior to electrodialysis, which was performed in the cold room, the ionic strength of the amylase solution was reduced by overnight dialysis vs. distilled water; the solution was then diluted to 2–3 mg of protein per ml. Cold water flowed constantly upward (from I to J) through the electrode chambers, to remove secondary products of electrolysis and to minimize current and temperature. At the beginning of the electrodialysis the rate of flow was set to about 20 ml/minute in each outer chamber; it could be diminished progessively to 5 or 10 ml/minute towards the end of the operation. By proper adjustment of voltage and waterflow in the electrode chambers, the current was kept below 10 ma. The voltage provided by a Spinco "Duostat" power supply was manually increased from 50 v to 400 v, usually within a period of 1 hour, then kept constant at this value, which was the normal operating voltage.

The transparency of the instrument made it easy

[3] The traces of zinc present in *B. subtilis* α-amylase (0.5 g-atom per mole) are far less firmly bound than calcium (Stein and Fischer, 1960). When amylase preparations are exposed to metal-sequestering procedures, zinc is released by the enzyme before calcium (Vallee *et al.*, 1959).

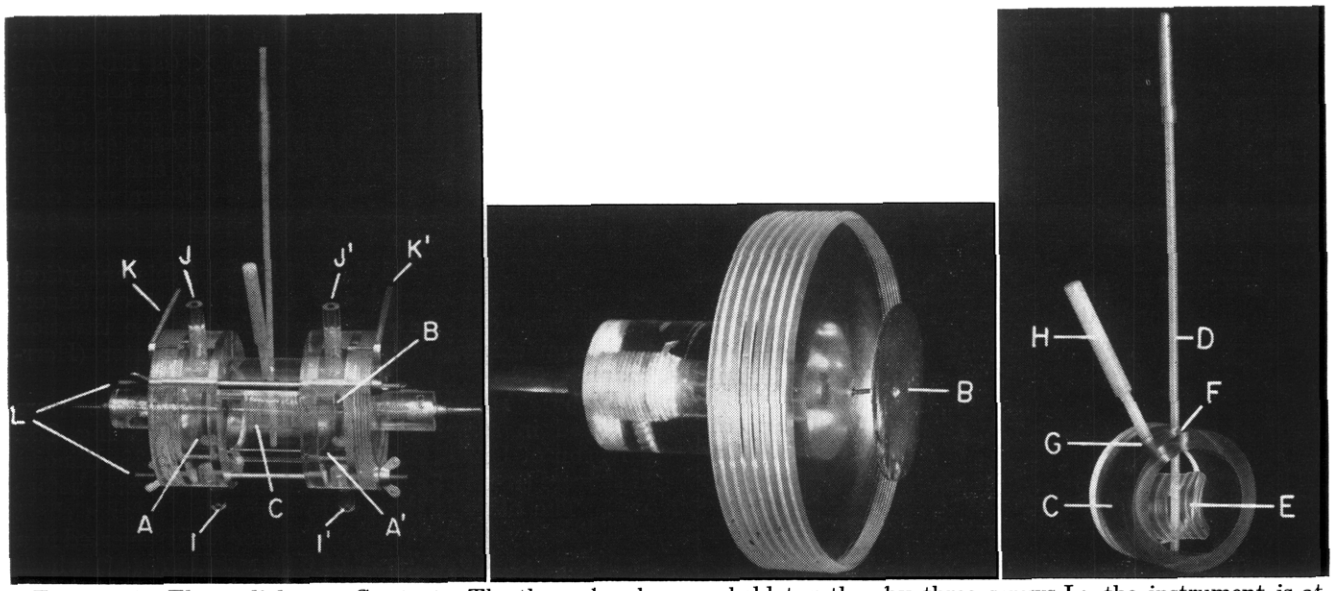

FIG. 1.—(a) Electrodialyzer. See text. The three chambers are held together by three screws L; the instrument is attached to a rack (not shown) by means of two aluminum slotted plates K-K'. (b) Electrode. (c) Center chamber (30 mm) with stirrer and plug.

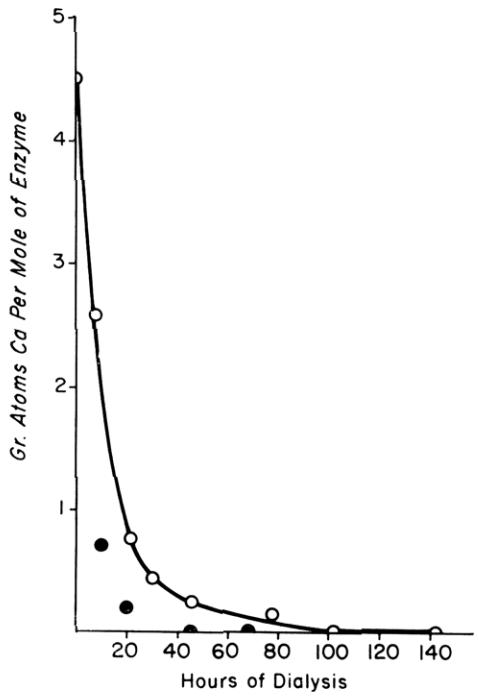

FIG. 2.—Removal of calcium from *B. subtilis* α-amylase by dialysis against EDTA, 0.01 M, *p*H 6.2, 4°. —○— Na-EDTA; —●— NH_4-EDTA.

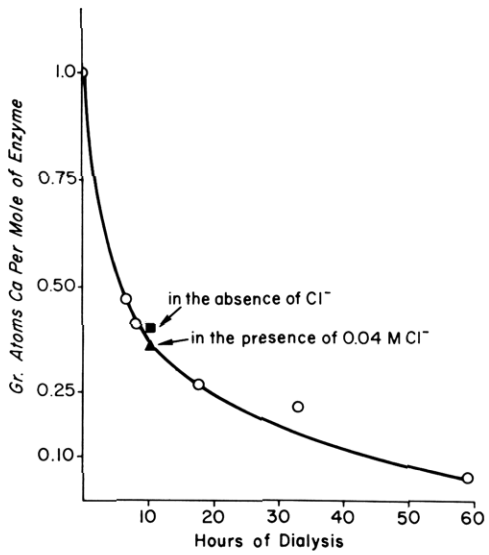

FIG. 3.—Removal of calcium from human salivary α-amylase by dialysis against Na-EDTA, 0.01 M, *p*H 6.9, 4°.

to detect any increase in turbidity of the protein solution or precipitation occurring on the cathodic or anodic membrane. At the end of the electrodialysis it was sometimes necessary to dissolve some material which had precipitated. This could be done by raising the ionic strength (and possibly the *p*H) by small injections of NaCl or NH_4OH solutions into the center chamber after the outer compartments had been emptied. Stirring was continued until the solution cleared (approx. 30 minutes). Traces of insoluble material could be removed by centrifugation. Finally, the solution was tested for protein concentration, specific activity, and calcium content.

RESULTS AND DISCUSSION

Prior to the use of dialysis against EDTA and electrodialysis, several procedures were examined in an attempt to remove calcium from amylase. Dialyses were carried out at 4°, *p*H 7.0, for at least 48 hours against large volumes of 0.02 M sodium fluoride, oxalate, citrate, polyphosphate,[4] and the like. These experiments were consistently unsuccessful, yielding preparations with a molar ratio of calcium to enzyme always greater than one. Column chromatography of the protein solutions through Dowex A-1 chelating resin also proved to be most inefficient.

A. Dialysis versus EDTA.—The successful removal of calcium from *B. subtilis* and human salivary α-amylase is illustrated in Figures 2 and 3. In spite of the high affinity of EDTA for calcium, the transfer of metal from enzyme to chelating agent is very slow. The process, of course, can be accelerated by working under conditions at which amylases are less stable, i.e., at higher temperatures or *p*H values (Yamamoto, 1956), but then irreversible denaturation of the enzyme obtains. In contrast, the conditions described above

[4] Ethylene glycol bis(β-aminoethylether)*N,N*-tetraacetic ether was not available at the time these experiments were performed.

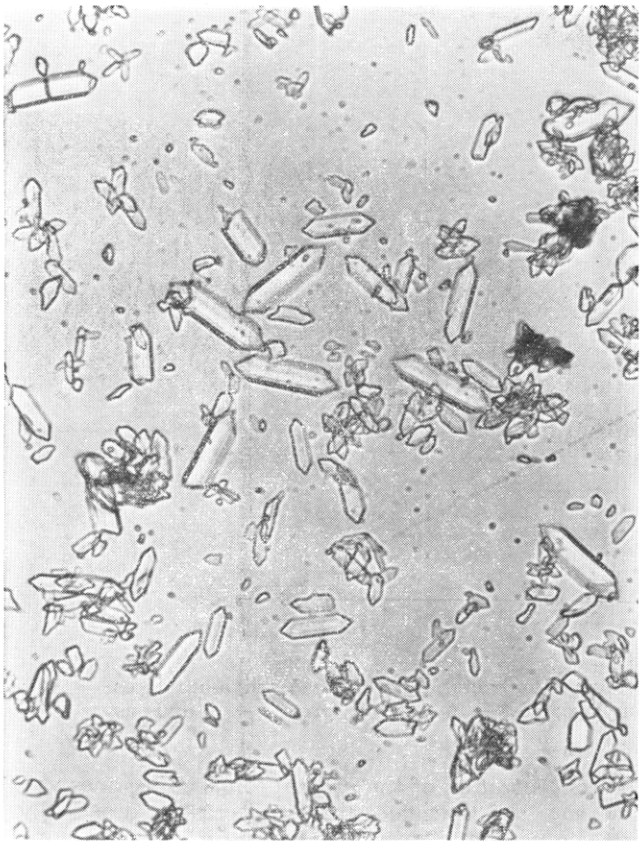

FIG. 4.—Crystals of calcium-free human salivary α-amylase (570×).

yield apoamylases that can be reactivated 90–100% in the presence of Ca^{++} ions as described in detail in the following paper (Hsiu *et al.*, 1964).

It is of interest that Na^{+} and NH_4^{+} ions seem to affect the binding of *B. subtilis* α-amylase in opposite ways: whereas the binding appears to be reinforced by Na^{+}, it is weakened by NH_4^{+} (Fig. 2). These two ions have no influence in the instance of salivary amylase. Thus, when NH_4-EDTA is used the rate of calcium removal is no longer significantly slower for bacterial than for human amylase.

Dialysis of salivary amylase against Na-EDTA always resulted in heavy crystallization of the enzyme within 6–10 hours, in spite of the low concentration of the enzyme solution (5 mg/ml). This was unexpected in view of the observation reported earlier (Vallee *et al.*, 1959), according to which crystallization of the enzyme was greatly enhanced by the presence of certain divalent metal ions, in particular Ca^{++} and Ni^{++}. Calcium analyses performed on the crystals of human salivary amylase obtained during these dialyses demonstrated the absence of this metal, indicating that calcium-free human salivary apoamylase can indeed be crystallized[5] (Fig. 4). However, it was difficult to dissolve the crystals without causing irreversible inactivation, since the apoenzyme no longer tolerates the alkaline conditions (*p*H 10–11) required to dissolve the protein (Fischer and Stein, 1961). Dialysis versus EDTA did not bring about the crystallization of the bacterial amylase. As reported earlier, this enzyme crystallizes as a dimer cross-linked by an atom of zinc which is readily chelated by EDTA (Stein and Fischer, 1960).

[5] Dilute solutions (≤3 mg/ml) of human salivary amylase made calcium-free by electrodialysis also yielded crystals, after overnight storage in the refrigerator.

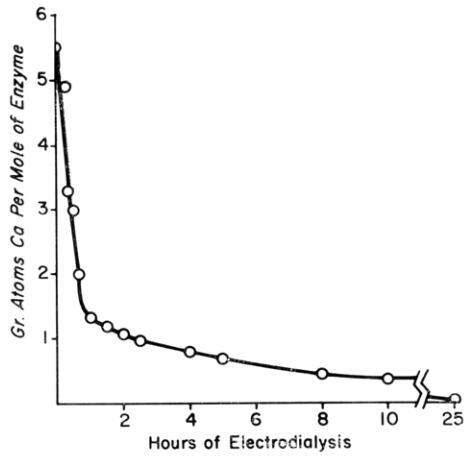

FIG. 5.—Removal of calcium from *B. subtilis* α-amylase by electrodialysis (50 mm center chamber).

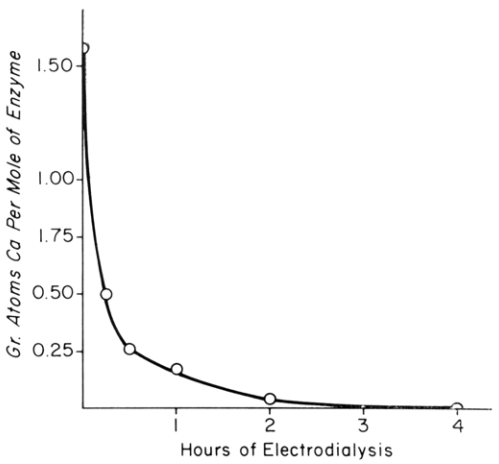

FIG. 6.—Removal of calcium from human salivary α-amylase by electrodialysis (30 mm center chamber).

Chloride ions affect many properties of salivary amylase (activity, stability, isoionic point, crystallizability, etc.); therefore the rate of calcium removal was studied both in the presence and absence of NaCl (Fig. 3). No significant difference could be detected.

B. Electrodialysis.—The effect of electrodialysis on the calcium content of amylases is shown in Figures 5 and 6. Here again, specific activities upon reactivation (Hsiu *et al.*, 1964) after electrodialysis were 90–100% of the original values. Protein recoveries were also 90–100% except for 24-hour runs, where losses varied from 10 to 20%.

In Figure 7 the rates of calcium removal by dialysis vs. EDTA and by electrodialysis are compared; it can be seen that the latter procedure (particularly in the case of salivary amylase) is far more effective than the former. It is also of interest that the removal of the metal appears to proceed at two different rates (illustrated by the broken curves observed on the semilogarithmic plot). In spite of the fact that the experiments were not performed under conditions of equilibrium, transition to a slower rate is believed to be a significant feature of the over-all process, related to the difficulty of removal of the last, most firmly bound atom(s) of calcium. Extrapolation of these slower rates to zero time of dialysis yields intercepts somewhat below 1 g-atom of calcium per mole of enzyme in the case of human salivary amylase, and slightly above one for *B. subtilis* amylase.

Rather large variations were observed in the ease with which the metal could be removed when *different*

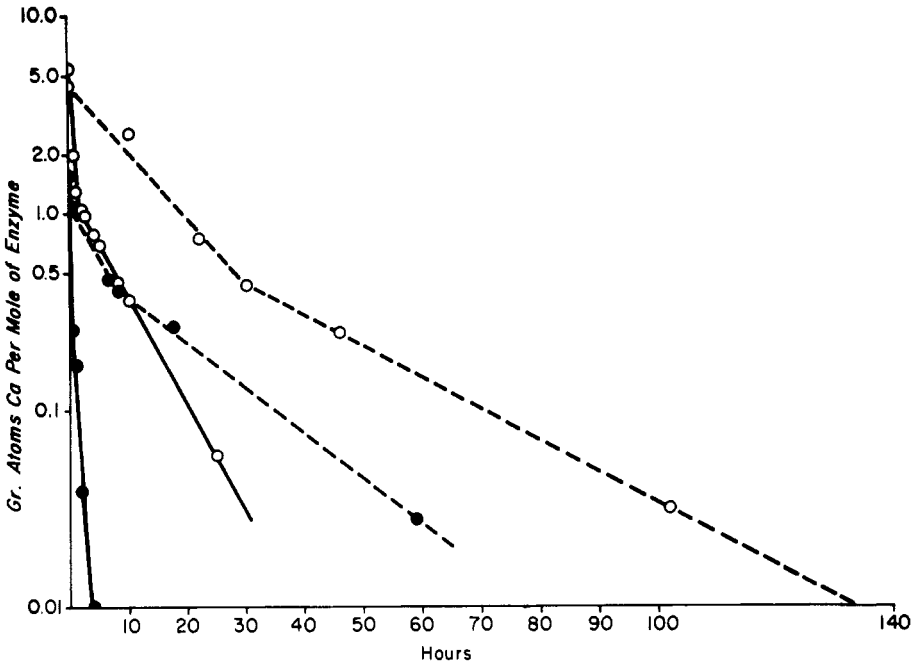

FIG. 7.—Comparison between the rates of calcium removal from bacterial and human saliva amylases by sequestration and electrodialysis. —○— bacterial amylase; —●— human amylase; - - - - dialysis vs. EDTA; ——— electrodialysis.

preparations of the same amylase were investigated. Thus, complete elimination of calcium from salivary amylase could require from 2 to 6 hours, according to the sample used. Similarly, runs of 8 to 25 hours could be required in the instance of different preparations of bacterial amylase. Although no systematic studies were made to correlate these observations with specific properties of the samples involved, it is clear, nonetheless, that the strength with which an enzyme preparation binds calcium is related to its general stability (e.g., resistance to proteolysis, extreme *p*H values, high temperature, etc.). It appears that binding strength are influenced by the kind of treatment to which the enzyme preparation had been exposed, i.e., its "previous history." Therefore, precise data on association constants would not be very meaningful unless strictly qualified.

C. Removal of Calcium from A. oryzae α-Amylase.— Previous experiments designed to yield calcium-free mold amylase without permanent damage to the enzyme molecule were consistently unsuccessful. Whereas the several extrinsic calcium atoms bound to this enzyme could be readily removed, 150 hours' dialysis vs. Na-EDTA or NH_4-EDTA at *p*H 7.0 or even 9.0 failed to remove the single intrinsic atom of calcium (see also Oikawa and Maeda, 1957). In view of the efficiency of electrodialysis, attempts were made to extend this method to the removal of calcium from this amylase. Forty-eight-hour runs at 400 v could not bring the calcium level below 0.5 g-atom/mole; moreover, this treatment was accompanied by a 20% irreversible loss of activity. Ninety-six hours of electrodialysis at 800 v brought the calcium content down to about 0.3 g-atom/mole, but recovery of activity was no greater than 20% (J. G. Falbriard and E. A. Stein, unpublished results). There are two apparent reasons for this failure. (1) *A. oryzae* amylase has a low isoionic point, 4.2, as compared to 5.2–5.4 for human and bacterial amylases (Stein *et al.*, 1960). When solutions of this enzyme are kept at such *p*H for prolonged periods of time, the enzyme undergoes irreversible denaturation and comes out of solution. Thus, when the operation is pursued for more than 48 hours, the concentration of the protein solution becomes very low and the specific activity decreases drastically. (2) The apparent association constant of the enzyme-metal complex being extremely high (perhaps of the order of 10^{15}), removal of calcium proceeds very slowly. When the concentration of calcium in *A. oryzae* α-amylase reaches 0.3 g-atom/mole, the rate of removal of the metal falls to approximately 0.002 g-atom/hour. Assuming that the inner chamber contains 20 mg of enzyme and that the quartz-redistilled water which is used to flush the anode chamber flows at a rate of 5 ml/minute, it can be calculated that as little as 1 part/10 billion Ca^{++} ions in the water would be sufficient to maintain a steady-state concentration of metal at the above level. Use of permselective ion-exchange membranes might have improved the situation somewhat. A more promising approach would be to attempt to remove calcium from a reversibly denatured preparation of *A. oryzae* α-amylase, possibly after rupture of disulfide bridges (Isemura *et al.*, 1963).

D. Conclusions.—The strength with which calcium is held by α-amylases varies markedly according to the biological origin of the enzyme. The binding is rather weak for plant (barley) amylase (Schwimmer and Balls, 1949; Fischer and Haselbach, 1951), stronger for mammalian (human saliva or pig pancreas) amylases, and stronger yet for bacterial amylase. As for mold amylase, its binding strength has thus far defied evaluation, since the metal has not yet been removed without denaturation of the enzyme.

As has been pointed out previously, release of calcium from the amylase molecule is difficult only as long as the enzyme is maintained within its range of optimum stability; denaturation of the amylase molecule will greatly enhance the dissociation of the protein-calcium complex. Unfortunately, removal of calcium from amylase results in a labile, "spineless" structure (Fischer and Stein, 1960), and precautions must be taken to prevent irreversible damage to the calcium-depleted protein. Therefore, when dialysis against EDTA is used, one has little choice but to work under very mild conditions of *p*H and temperature, which preclude rapid removal of the metal.

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.

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REFERENCES

Diehl, H., and Ellingboe, J. L. (1956), *Anal. Chem. 28*, 882.
Fischer, E. H., and Hàselbach, C. H. (1951), *Helv. Chim. Acta 34*, 325.
Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.
Fischer, E. H., and Stein, E. A. (1961), *Biochem. Prep. 8*, 27.
Fischer, E. H., Sumerwell, W. N., Junge, J. M., and Stein, E. A. (1960), *Proc. Intern. Congr. Biochem. 4th Vienna (1958) 8*, 124.
Hermann, R. G. (1958), *Proc. Soc. Exptl. Biol. Med. 99*, 777.
Hsiu, J., Fischer, E. H., and Stein, E. A. (1964), *Biochemistry 3*, 61 (this issue).
Isemura, T., and Fujita, S. (1957), *J. Biochem. (Japan) 44*, 797.
Isemura, T., Takagi, T., Maeda, Y., and Yutani, K. (1963), *J. Biochem. (Japan) 53*, 155.
Oikawa, A., and Maeda, A. (1957), *J. Biochem. (Japan) 44*, 745.
Schwimmer, S., and Balls, A. K. (1949), *J. Biol. Chem. 179*, 1063.
Socolar, S. J., and Salach, J. I. (1959), *Anal. Chem. 31*, 473.
Stein, E. A., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 867.
Stein, E. A., and Fischer, E. H. (1960), *Biochim. Biophys. Acta 39*, 287.
Stein, E. A., and Fischer, E. H. (1961), *Biochem. Prep. 8*, 34.
Stein, E. A., Junge, J. M., and Fischer, E. H. (1960), *J. Biol. Chem. 235*, 371.
Vallee, B. L., Stein, E. A., Sumerwell, W. N., and Fischer, E. H. (1959), *J. Biol. Chem. 234*, 2901.
Yamamoto, T. (1956), *Bull. Agr. Chem. Soc. Japan 20*, 188.

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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