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Purification and Crystallization of the Alkaline Phosphatase of *Escherichia coli**

MICHAEL H. MALAMY† AND BERNARD L. HORECKER‡

From the Department of Microbiology, New York University School of Medicine, New York City

A new procedure is described for the purification and crystallization of alkaline phosphatase of *Escherichia coli*. The starting material for this procedure is the supernatant solution obtained when cells are converted to spheroplasts. The crystalline enzyme preparations are stable, contain little or no diesterase activity, and are homogeneous in the analytical ultracentrifuge. Three protein bands, all with phosphatase activity, are obtained in agar-gel electrophoresis. The purified protein shows a sedimentation coefficient of 6.15 and appears to break up in acid solution to yield smaller fragments with a sedimentation coefficient of 2.6. The enzyme is sensitive to inhibition by thiol-carboxyl compounds such as cysteine and thioglycolic acids. This inhibition is reversed by the addition of zinc ions.

Although procedures for the purification of alkaline phosphatase from *Escherichia coli* have been described (Garen and Levinthal, 1960; Schwartz and Lipmann, 1961), the enzyme has never been crystallized nor have purified preparations been demonstrated to be completely free of other contaminating proteins. Contaminating phosphodiesterase activity hampers the application of the enzyme to the study of nucleotide sequences in nucleic acids (Harkness and Hilmoe, 1962). Previous purification procedures have used as starting material either broken-cell preparations obtained by treatment in the French press (Garen and Levinthal, 1960; Garen and Echols, 1962; Schwartz and Lipmann, 1961), or extracts of acetone powders prepared from whole cells (D. R. Harkness and R. J. Hilmoe;[1] Schwartz and Lipmann, 1961; Plocke *et al.*, 1962). These extracts contain much of the protein and nucleic acid of the cell and extensive purification, involving heat treatment as well as addition of RNAase and DNAase, is required. The use of such drastic steps introduces certain complications that may possibly alter the chemical and physical properties of the protein and confuse the interpretation of multiple electrophoretic bands seen in vertical starch-gel electrophoresis (Bach *et al.*, 1961). Treatment with RNAase and DNAase introduces contaminating proteins which are difficult to remove completely in subsequent steps.

These difficulties can be avoided by using as starting material the supernatant fraction obtained during spheroplast formation with lysozyme and EDTA in an osmotically protective medium (Malamy and Horecker, 1961, 1964). By this procedure alkaline-phosphatase activity is completely liberated from the cells, while the bulk of the other proteins is retained. Few purification steps are required to yield a crystalline preparation which possesses high specific activity and which is free of detectable phosphodiesterase. A single sedimenting species is seen in the ultracentrifuge.

MATERIALS AND METHODS

Bacterial Strains.—A strain of *E. coli* K12, designated C_4F_1, was obtained from Dr. A. M. Torriani of the Massachusetts Institute of Technology. This strain produces a constitutive alkaline phosphatase. It was tested by streaking the cells on nutrient agar and examining the resulting colonies for alkaline phosphatase according to the method of Echols *et al.* (1961). Colonies which turned yellow quickly after the addition of a drop of nitrophenylphosphate solution were considered to be constitutive and were used as the initial inoculum for growth.

Media.—The peptone-glucose-salts medium of Levinthal *et al.* (1962) was utilized. The composition of this medium was: 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.003 M Na_2SO_4, 0.001 M $MgCl_2$, 0.0002 M $CaCl_2$, 2×10^{-6} M $ZnCl_2$, 0.5% glucose, and 0.5% Difco peptone. The medium was adjusted with HCl to a final *p*H of 7.4. The glucose solution was prepared separately as a 20% sterile solution and added to the sterile medium at the time of inoculation.

* Taken in part from a doctoral dissertation submitted by Michael Malamy to the Graduate School of Arts and Sciences, New York University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Supported by grants from the National Institutes of Health and the National Science Foundation.

† National Science Foundation Cooperative Fellow, 1961–1963. Present address: Institut Pasteur, Service de Biochimie Cellulaire, Paris, France.

‡ Present address: Department of Molecular Biology, Albert Einstein College of Medicine, Bronx 61, New York.

[1] Personal communication.

Assay of Alkaline Phosphatase.—The assay mixture (1 ml) contained 1 μmole of *p*-nitrophenylphosphate in 1 M Tris buffer, *p*H 8. Enzyme solution was added to start the reaction. The liberation of *p*-nitrophenol was followed at 420 mμ in a Beckman DU spectrophotometer with the cell compartment kept at 27°. The molar absorbancy index of *p*-nitrophenol under these conditions is 1.32×10^4. A unit of enzyme was defined as the quantity required to liberate 1 μmole of *p*-nitrophenol in 1 hour. The specific activity was expressed as μmoles of *p*-nitrophenol liberated per hour per mg of enzyme protein at 27°.

Protein Assays.—For assays of protein concentration in purified phosphatase solutions the method of Bücher (1947) was employed. The protein concentration in highly purified enzyme preparations was determined spectrophotometrically, using the absorbancy coefficient at 278 mμ as determined with the crystalline protein. To standardize this method enzyme crystals were dialyzed exhaustively against several changes of distilled water and an aliquot was dried to constant weight at 105°. Another aliquot was taken for measurement of absorbance at 278 mμ. The value obtained, 0.72 absorbancy units per mg protein, compared favorably with the value of 0.71 determined by Plocke *et al.* (1962).

Sedimentation Velocity Determinations.—These were performed in the Beckman Spinco Model E ultracentrifuge equipped with a temperature regulator set for 20°. Samples were run at 59,780 rpm in a cell holder with a 12-mm Kel-F centerpiece. The protein samples, at a concentration of about 3 mg/ml, were dialyzed against 0.01 M Tris buffer, *p*H 8, before analysis.

Results

Enzyme Purification Procedure (summarized in Table I).—Five 2-liter flasks containing 500 ml of peptone-glucose-salts medium were inoculated with 2 ml of an exponential growing culture of *E. coli* K12-C4Fl. The cultures were incubated at 37° on a high-speed rotary shaker for 16–20 hours. The cultures reached a final turbidity of about 1.3 at 590 mμ in the Beckman DU spectrophotometer. The intact bacteria contained 14.4 units of phosphatase per ml of culture, for a total of 36,200 units. The cells were harvested by centrifugation at 4°, and the pellets were washed with several 100-ml portions of 0.01 M Tris buffer, *p*H 8.

The washed cells were resuspended to a final volume of 1 liter with a solution of 20% sucrose, which was also 0.033 M with respect to Tris buffer at *p*H 8. The cell suspension had a turbidity of 3.4 at 590 mμ. The suspension was kept in a 2-liter flask at room temperature and was stirred slowly with a magnetic stirring bar. Ten ml of 0.01 M EDTA, *p*H 8, and 10 mg of crystalline egg white lysozyme (Nutritional Biochemicals Corp.) were added in succession. Samples (0.1 ml) were tested at intervals for completeness of spheroplast formation; each was diluted to 1 ml with distilled water and turbidity at 490 or 590 mμ in the Beckman DU spectrophotometer was measured. This reading was compared with that obtained before the addition of lysozyme. More than 90% of the cells had become osmotically sensitive at 9 minutes. The suspension was centrifuged at 8500 rpm for 15 minutes at 4°. The residue remaining when the supernatant fluid had been removed was washed with small portions of the sucrose-Tris buffer solution and recentrifuged. The supernatant solution contained a total of 234,000 units of enzyme and 260 mg of protein (Table I). The wash solution, containing 23,000 units of enzyme, was discarded.

Table I
Summary of Enzyme Purification Procedure

Fraction	Total Enzyme Units (μmoles/hr/ml)	Volume (ml)	Specific Activity (μmoles/hr/mg)
(1) Intact cells	36,200[a]	2500	
(2) Sucrose-Tris buffer supernate	234,000	1000	910
(3) DEAE-cellulose eluate	142,000	18	2190
(4) Crystals[b]	4,700[b]	1[b]	2900[b]

[a] The alkaline-phosphatase activity of intact cells was generally only a fraction of that obtained with cell extracts. [b] These values were obtained with an aliquot (1 ml) of fraction 3.

A suspension of DEAE-cellulose (Bio-Rad Co.-Calbiochem) was prepared by stirring 20 g of the dry powder into 1 liter of 0.05 M NaCl. After the slurry had been stirred for several minutes, the material was allowed to settle and the fine particles were removed by decantation. After repeated washing with 0.05 M NaCl, the suspension was acidified by adding a quantity of HCl equivalent to the exchange capacity of the gel. For 20 g of DEAE-cellulose, 17.4 ml of 1 N HCl was required. This suspension, which was slightly acid (about *p*H 5), was used to pack a column 2.6 × 5.5 cm. Pressure was maintained in the system by means of a constant-pressure cautery bulb. The packed column was washed with 0.05 M NaCl under pressure.

The entire supernatant fraction was allowed to drip slowly onto the column from a reservoir supported above the top of the column. The flow rate was slow and the process was allowed to continue overnight in the cold room (4°).[2] When the entire fraction had been adsorbed, the column was washed with 20 ml of 0.05 M NaCl to remove the remaining sucrose-Tris buffer solution. Fractions (4 ml) were collected on an automatic fraction collector at 10-minute intervals. The phosphatase activity was eluted from the column with 0.125 M NaCl and appeared in 4 tubes, between 20 ml and 36 ml of eluent (DEAE-cellulose eluate). These 4 tubes contained a total of 65 mg of protein, as calculated from the absorbancy readings at 278 mμ.

These column fractions were then made 0.01 M with respect to $MgCl_2$ and brought to 50% saturation by the addition of solid ammonium sulfate, 0.29 g/ml. The solution was centrifuged in the clinical centrifuge at room temperature to remove gas bubbles and adjusted to about *p*H 8 with 2 N NaOH. Saturated ammonium sulfate at *p*H 8 was added dropwise until the solution became faintly turbid (about 61% saturation). The turbid suspension was left at room temperature for 1 hour; the suspension increased in turbidity. It was then placed in an ice bath for several minutes, whereupon the turbidity was found to disappear almost entirely. The solution was then allowed to warm slowly to room temperature by placing the tube in a 5-liter insulated water bath at 0°. As the solution warmed the turbidity reappeared and the suspension developed

[2] It has been found possible to increase the dimensions of the column and thereby increase the flow rate during the adsorption of the supernatant fraction to the column. In subsequent experiments a column 4 cm in diameter by 4.5 cm high, containing a total of 8 g of DEAE-cellulose suspension, has been employed. A 2-liter supernatant fraction was put onto this column in 8 hours. Elution from this column is not always as sharp as with the smaller column and an additional ammonium sulfate (90% saturation) concentration step may be necessary.

a silky sheen when viewed against a dark background. The temperature of the water bath after 16 hours was 22°.

To complete the crystallization process, the suspension was kept at room temperature for several days. Because crystals dissolved rapidly when the solution cooled, all subsequent operations were carried out at room temperature. The precipitate, which contained all of the enzyme units, was collected by centrifugation and suspended in a 50% saturated ammonium sulfate solution at *p*H 8, and the crystallization procedure was repeated. The specific activity of the enzyme obtained after crystallization was 2900 units/mg, and the specific activity after recrystallization was 3250 units/mg. When concentrated solutions of the enzyme were used for crystallization (greater than 5 mg/ml), the turbidity which reappeared on warming was often amorphous, but became crystalline after several hours at room temperature.

Microscopic Observation of Crystals.—Microscopic observation, using dark-field or phase-contrast illumination, revealed a uniform suspension of fine needles (Fig. 1). Observation of a preparation stored at room temperature for more than 1 month, without loss in enzymatic activity, revealed that the individual crystals had grown in size and had become pointed at either end, with a measurable thickness. The crystals were about 20 μ long and less than 1 μ in width.

Chemical and Physical Properties of Crystalline Alkaline Phosphatase.—SEPARATION FROM NUCLEASES.—Alkaline phosphatase is a valuable reagent for the study of RNA structure, but for this purpose it must be free of nucleases and diesterases. Sensitive tests have been applied (Heppel, *et al.*, 1962; Harkness and Hilmoe, 1962), to detect this activity. On the basis of two such tests, the crystalline preparations isolated by the present method show little or no nuclease activity (Tables II and III). In the direct assay for release of acid-soluble nucleotides, only preparation 2 showed significant ribonuclease or marked diesterase activity. This preparation was isolated from an experiment in which lysis of the spheroplasts had occurred during their preparation.

TABLE II
COMPARISON OF FOUR CRYSTALLINE PREPARATIONS FOR ALKALINE PHOSPHATASE AND NUCLEASE ACTIVITY

Assay for:	Preparation Number			
	1	2[a]	3[b]	4[b]
	(units/ml)			
Alkaline phosphatase[c]	490	1600	1280	500
Ribosomal ribonuclease[d]	0	28.9	0	1.3
E. coli phosphodiesterase[e]	7.8	284	0	16

[a] This preparation was isolated in an experiment in which some lysis of spheroplasts occurred before separation of the sucrose-Tris supernatant solution. [b] These preparations were prepared by Dr. Harold C. Neu at the National Institutes of Health. The assays reported in Tables II and III were carried out by Dr. Neu. [c] Alkaline-phosphatase assays were as described in the text except that incubation was at 23° and absorbancy readings were at 410 mμ. The unit of enzyme activity is the amount required to produce a change in absorbancy of 1.0/minute at 410 mμ and room temperature. [d] The incubation mixture for ribosomal ribonuclease, 0.1 ml, contained 0.1 mg s-RNA from *E. coli*, 0.02 M EDTA, 0.1 M potassium phosphate buffer, *p*H 7.0, and 0.015 ml of enzyme solution. After 120 minutes at 37°, 0.35 ml of 3% perchloric acid was added and the mixture was centrifuged. An aliquot (0.1 ml) of the supernatant solution was mixed with 0.9 ml of water and the absorbancy was measured at 260 mμ. A unit of activity is the amount required to release 0.45 absorbancy unit of acid-soluble nucleotide per 0.1 ml reaction mixture per 40 minutes. [e] For phosphodiesterase activity the assay mixture contained 0.25 mg poly-A, 1 M Mg^{2+}, 0.1 M potassium phosphate, *p*H 7.0, and 0.015 ml of enzyme solution. Incubation and precipitation were as above. A unit of activity is the amount required to release 0.70 absorbancy unit at 260 mμ of acid-soluble nucleotide per 0.1 ml of reaction mixture.

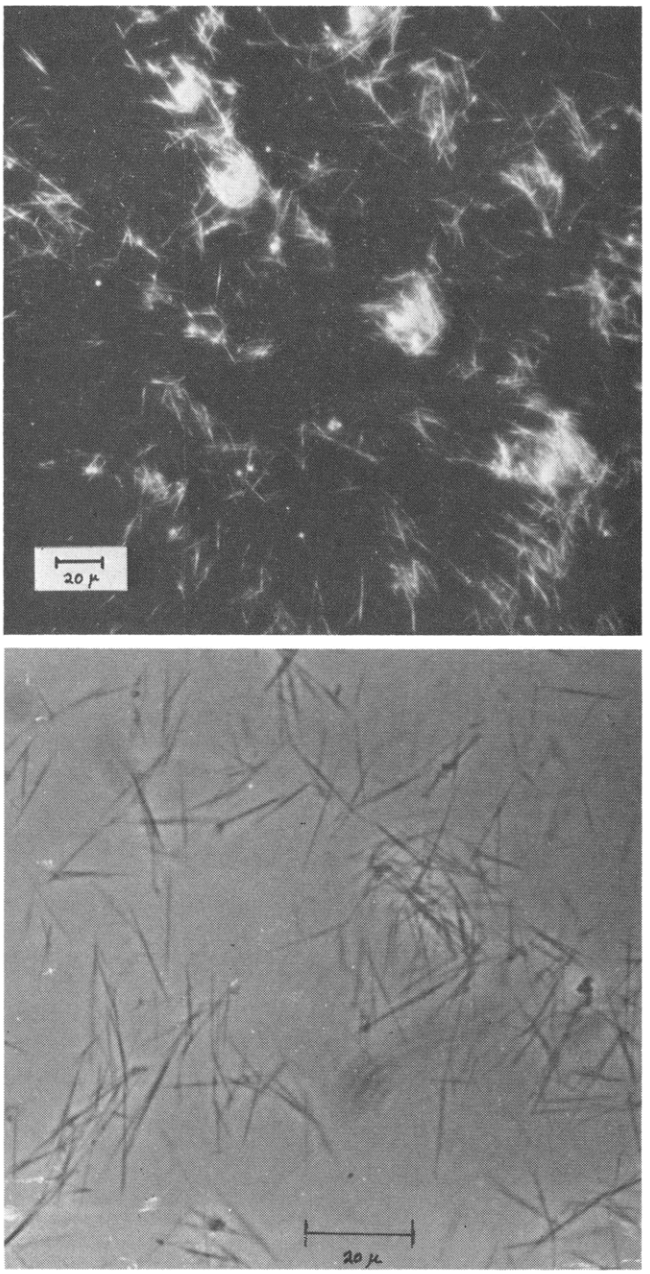

FIG. 1.—Crystalline preparation of alkaline phosphatase with dark-field (left) or phase-contact (right) illumination.

The most sensitive assay for phosphodiesterase contamination is the test of the biological activity of the s-RNA after incubation with the enzyme preparation. It has been established (Harkness and Hilmoe, 1962) that treatment of s-RNA with a phosphatase preparation which removes only the terminal 5′-phosphate does not destroy the ability of the s-RNA to act as an amino acid acceptor. With all of the crystalline alkaline phosphatase preparations except that in which cell lysis had been observed (no. 2), the loss of amino acid acceptor activity of s-RNA in 4 hours was not significantly greater than in the control without added enzyme (Table III).

STABILITY.—Suspensions of crystals in 61% ammo-

nium sulfate, 0.01 M $MgCl_2$, *p*H 8, were stable, losing less than 5% of the original activity when stored at room temperature for more than 6 months. Microbial contamination did not occur at this high salt concentration. Refrigeration of the suspension of crystals caused a decrease in the turbidity of the sample although all enzymatic activity was retained. Freezing the sample at −20° resulted in a loss of more than 90% of the activity.

ELECTROPHORESIS.—Crystalline enzyme preparations were subjected to agar-gel electrophoresis (Crowle, 1961) and the slides were developed for enzymatic activity (Boyer, 1961). In every case, a pattern consisting of three bands was obtained. These are probably the three major bands observed in vertical starch-gel electrophoresis (Bach *et al.*, 1961; Levinthal *et al.*, 1962; Signer *et al.*, 1961).

TABLE III
LOSS OF AMINO ACID ACCEPTOR ACTIVITY OF S-RNA INCUBATED WITH CRYSTALLINE ALKALINE PHOSPHATASE[a]

Preparation[b]	Hours of Incubation			
	0	1	2	3
	(^{14}C counts of amino acid acceptor RNA)			
No enzyme	10,800	9,900	9,200	7,800
1	13,000	12,600	11,100	9,100
2	8,100	6,400	4,300	3,700
3	7,600	7,100	5,100	5,300
4	7,300	5,500	5,800	5,100

[a] *E. coli* s-RNA, previously tested for nuclease contamination, was incubated with alkaline phosphatase in 0.1 M Tris, *p*H 8.5, at 60° for the times indicated. The s-RNA was precipitated by the addition of ethanol to 80% in 0.3 M NaCl, and the precipitate was dried and the traces of ethanol were removed by evaporation in a vacuum desiccator. The s-RNA was then incubated with ^{14}C-pooled amino acids in 0.05 M Tris, *p*H 7.4, 0.013 $MgCl_2$, 0.03 M KCl, 0.01 M GSH, 0.0006 M ATP, and amino acid acceptor enzymes for 20 minutes at 37°. The reaction was stopped with cold 20% trichloroacetic acid and the precipitates were collected on a Millipore filter. The precipitates were washed with cold 20% trichloroacetic acid and dried, and radioactivity was assayed in a Packard scintillation counter. In each instance the comparison of counts was made with the zero-time sample because the amount of [^{14}C]amino acids used was different in different experiments. [b] The numbers correspond to the same preparations described in Table II. Tests were carried out with 0.005 ml of preparations 1 and 2 and 0.01 ml of preparations 3 and 4.

SEDIMENTATION ANALYSIS AND ACID DISSOCIATION.—The sedimentation coefficient obtained for crystalline alkaline phosphatase under the conditions described under Materials and Methods was 6.15. Below *p*H 3.5 this changed to a single sedimenting peak with a sedimentation coefficient of 2.6. This treatment resulted in the loss of all enzymatic activity. Schlesinger *et al.* (1963) have recently reported that acid treatment causes the dissociation of the enzyme into subunits.

TREATMENT WITH THIOL COMPOUNDS.—Cox and MacLeod (1963) have reported that cysteine and cystine caused both repression of the synthesis and inhibition of the activity of the alkaline phosphatase of HeLa cells in tissue culture. At a concentration of cysteine of 2×10^{-4} M, *p*H 8.5, we observed a complete inhibtion of activity (Table IV). The inhibition caused by cysteine was completely and instantaneously reversed upon the addition of stoichiometric amounts of zinc ions. Thioglycolic acid caused a similar inhibition of enzymatic activity. In the concentration range between 2×10^{-3} and 5×10^{-3} M, thioglycolic acid acted as a competitive inhibitor of alkaline-phosphatase activity. Again, inhibition could be reversed by the addition of zinc ions. The requirement for Zn^{2+} has been studied in detail by Plocke and Vallee (1962).

Other thiol compounds tested resulted in some stimulation of the enzymatic activity. With reduced glutathione or 2-mercaptoethanol a 15–40% stimulation of activity was observed.

TABLE IV
EFFECT OF THIOL COMPOUNDS ON ALKALINE PHOSPHATASE ACTIVITY

Additions to Standard Assay[a]	Activity μmoles/hour/ml
None	46.2
2×10^{-4} M cysteine	0
2×10^{-4} M cysteine + 3×10^{-4} M Zn^{2+}	47.3
1×10^{-3} M reduced glutathione	53.0
1×10^{-3} M 2-mercaptoethanol	66.7
2×10^{-3} M thioglycolic acid	42.4
2×10^{-3} M thioglycolic acid + 3×10^{-4} M Zn^{2+}	50.0
5×10^{-3} M thioglycolic acid	10.0
5×10^{-3} M thioglycolic acid + 3×10^{-4} M Zn^{2+}	51.5

[a] Each determination was performed in 1 ml of 1 M Tris buffer, *p*H 8.5, containing 1 μmole of *p*-nitrophenylphosphate, 0.16 μg of crystalline alkaline phosphatase, and the compound tested. The cuvet contents was incubated at 27° in the Beckman DU spectrophotometer, and the liberation of *p*-nitrophenol was followed at 420 mμ. After the initial activity was determined, zinc ions were added to the sample by rapidly pipetting an aliquot of $ZnCl_2$ into the cuvet, mixing, and replacing the cuvet in the spectrophotometer.

DISCUSSION

Our previous observation that alkaline phosphatase is liberated quantitatively into the supernatant fluid when cells of *E. coli* containing this enzyme are converted to spheroplasts has now been employed as the basis for the purification of this enzyme. The procedure is rapid and convenient and can be carried out with large quantities of material. It has the great advantage that the starting material is relatively uncontaminated with other proteins and enzymes derived from the cell sap. As a consequence, only a 3- to 4-fold purification is required in order to obtain a crystalline enzyme preparation. The recrystallized protein is free of detectable quantities of other proteins.

The original spheroplast supernatant solutions contained appreciable quantities of ribonuclease and deoxyribonuclease. It has recently been reported (Neu and Heppel, 1964) that this ribonuclease activity is specifically liberated along with the alkaline phosphatase on formation of spheroplasts. This observation is of considerable interest, since it suggests that alkaline phosphatase is not the only enzyme to be located in the extramembranal compartment of the cell. It has not been excluded, however, that these enzymes are loosely attached to components of the cell membrane and are liberated by the procedures used in preparation of spheroplasts.

The availability of preparations of alkaline phosphatase free of diesterase should provide a powerful tool for structural studies of phosphate-containing polymers. The great stability of the enzyme permits incubation for long periods of time at elevated temperatures. In such cases it is particularly important that diesterase activity be completely absent.

ACKNOWLEDGMENT

We are grateful to Drs. H. C. Neu and L. A. Heppel of the National Institutes of Health for carrying out the tests for nuclease and diesterase activity, and for making these data available to us. A detailed account of their work has been submitted for publication in the *Journal of Biological Chemistry*.

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The Reaction of Carboxypeptidase A with Hippuryl-DL-β-Phenyllactate*

WILLIAM O. MCCLURE,† HANS NEURATH, AND KENNETH A. WALSH

From the Department of Biochemistry, University of Washington, Seattle

Received August 21, 1964

The action of carboxypeptidase A on hippuryl-DL-β-phenyllactate has been examined over a 2860-fold range of substrate concentration. The system is characterized by a complex inhibition by excess substrate, which suggests either that the enzyme binds substrate to form several inactive complexes or that the enzyme exists as several differentially susceptible species. Initial velocities measured at substrate concentrations low enough to avoid substrate inhibition yield maximum velocities which are the highest yet reported for any substrate for carboxypeptidase A. In this range of substrate concentrations the rates are independent of *p*H between *p*H 7.5 and 9.7, indicating that the unusual *p*H rate profile previously reported for the action of carboxypeptidase on hippuryl-DL-β-phenyllactate (J. F. Riordan and B. L. Vallee [1963], *Biochemistry 2*, 1460) is not due to substrate inhibition. Measurements of the effect of temperature on the reaction velocity yield activation parameters which are of the same order of magnitude as those previously reported for peptide substrates.

In 1948, Snoke and Neurath reported that bovine carboxypeptidase A hydrolyzes hippuryl-DL-β-phenyllactate, the ester analog of the specific substrate, hippurylphenylalanine. The rate behavior was anomalous in that the velocity increased with decreasing substrate concentration over the concentration range examined—a finding consistent with inhibition by excess substrate. Excess substrate inhibition was also noted by Lumry *et al.* (1951) for the closely related substrate carbobenzoxyglycyl-DL-phenylalanine. Because of the widespread use of HPLA[1] as a substrate for native and modified carboxypeptidase A and for bovine carboxypeptidase B, it appeared of interest to re-examine in greater detail the kinetics of the reaction over a range of substrate concentrations which extended low enough to avoid excess substrate inhibition and high enough to define the kinetic parameters over the entire concentration range. The use of a spectrophotometric method in this work permitted measurements to be carried out over a 2860-fold variation in substrate concentration.

* This work was supported by the U. S. Public Health Service (GM-04617), by the American Cancer Society (P-79), and by the Office of Naval Research (NONR 477-04).

† The data presented are taken from a dissertation submitted to the Graduate Faculty of the University of Washington by W. O. McClure in partial fulfillment of the requirements for the Ph.D. degree. Present address: Rockefeller Institute, New York, N. Y.

[1] The following abbreviations are used: HPLA, hippuryl-DL-β-phenyllactate; CGP, carbobenzoxyglycylphenylalanine; carboxypeptidase, bovine pancreatic zinc carboxypeptidase A_γ; DFP, diisopropylphosphorofluoridate; ammediol, 2-amino-2-methyl-1,3-propanediol.

EXPERIMENTAL

Bovine pancreatic zinc carboxypeptidase A_γ purified by the method of Anson (1937) as modified by Putnam and Neurath (1946) was purchased from Worthington (Freehold, N. J.) and stored as an aqueous suspension under a toluene atmosphere. Stock solutions of about 30 mg ml were made by dissolving crystals in 3 M sodium chloride, after washing three times with distilled water. Such solutions were stable at 4° for periods of at least 3 weeks, but were never used for longer than 7 days. Dilutions of these stock solutions were made as needed with 0.005 M Tris-HCl–0.1 M sodium chloride, *p*H 7.50. Diluted solutions of the enzyme were dis-