A FINE-STRUCTURE GENETIC AND CHEMICAL STUDY OF THE ENZYME ALKALINE PHOSPHATASE OF *E. COLI*

I. PURIFICATION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE

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SUMMARY

A procedure for purification of alkaline phosphatase from *E. coli* is described, and several physical-chemical properties of the purified enzyme are reported. When the cells are grown under the condition of phosphate deprivation, which is optimal for the synthesis of alkaline phosphatase, about 6 % of the total protein synthesized is alkaline phosphatase.

INTRODUCTION

The main physiological role of genetic material appears to be the determination of the specificity of protein molecules. This generalization is based on the many examples known of mutations which cause the loss or change of an enzymic function[1], and particularly on those cases for which there is direct evidence that a mutation causes the formation of an altered protein molecule[2–6]. Studies of genetically controlled alterations in haemoglobin by INGRAM, using the technique of comparative fingerprinting[7,8], have shown that a single mutation can result in the replacement of one amino acid at a particular position in the polypeptide chain by a different amino acid. This finding indicates that alterations in protein specificity brought about by mutations can involve changes in the amino acid sequence of the molecule. Several mechanisms have been proposed to account for the genetic control of amino acid sequences in proteins[9,10].

The aim of the present work is the development of a system with which it will be possible to do fine-structure genetic analysis[11–13] of mutations affecting a single enzyme, and at the same time to identify chemical alterations in the enzyme molecule resulting from the mutations. For this purpose the enzyme alkaline phosphatase produced by the bacterium *Escherichia coli* strain K12 was selected. The following factors influenced this choice:

1. The bacterium is suitable for genetic analysis, since crosses can be performed with large populations by pairwise mating between cells[14,15] or by transduction with phage P1[16]. It will be shown later in the course of this work that phosphatase-negative mutants can be isolated and, in crosses between these negative mutants, rare phos-

phatase-positive genetic recombinants can be detected, thereby satisfying the requiring for fine-structure mapping.

2. Recent work on the alkaline phosphatase of *E. coli* by Dr. A. M. TORRIANI[17] (who kindly informed us of her results before publication) shows that the enzymic activity in a culture can be regulated over a wide range by control of the concentration of inorganic phosphate in the growth medium. The maximal level of alkaline phosphatase activity is obtained with a medium in which the concentration of phosphate is sufficiently low so that it becomes the growth-limiting constituent. This finding suggested that under conditions of phosphate limitation the cells might produce the enzyme in high enough concentration to make the purification relatively simple.

3. Alkaline phosphatase is a reasonably stable enzyme, and its activity is easily assayed by following the color change of the substrate *p*-nitrophenyl phosphate[17].

This paper will report the methods that have been developed for purifying alkaline phosphatase from *E. coli*, and also some physical-chemical properties of the purified material.

MATERIAL AND METHODS

A prototrophic Hfr strain of *E. coli* K12 was used in all experiments. This strain was isolated as a spontaneous revertant from the Hfr strain CS101 which requires methionine for growth[18]. CS101 derives from the Hfr originally isolated by CAVALLI[19].

The composition of the growth medium used to obtain high yields of alkaline phosphatase is as follows: $8 \cdot 10^{-2}$ *M* NaCl; $2 \cdot 10^{-6}$ *M* $FeCl_3$; $2 \cdot 10^{-2}$ *M* KCl; $1.2 \cdot 10^{-1}$ *M* Tris buffer pH 7.5; $2 \cdot 10^{-2}$ *M* NH_4Cl; $1.2 \cdot 10^{-2}$ *M* glucose; $1 \cdot 10^{-3}$ *M* $MgCl_2$; $1.4 \cdot 10^{-4}$ *M* sodium glycerophosphate; $2 \cdot 10^{-4}$ *M* $CaCl_2$; 0.04% Bacto-peptone (Difco); $5 \cdot 10^{-4}$ *M* Na_2SO_4. It was found convenient to prepare 20-l cultures in a glass carboy vigorously aerated through several sintered-glass filters. The medium was inoculated with about $2 \cdot 10^7$ cells/ml and incubated at 37° for 20 h.

For preparing cultures labeled with ^{14}C proline, the growth medium was modified by eliminating the Bacto-peptone and sodium glycerophosphate, and adding KH_2PO_4 in the required amounts. This medium will be called P medium.

Alkaline phosphatase activity was assayed by incubating the enzyme with 0.2 mg/ml of the substrate *p*-nitrophenyl phosphate (NPP) in 1.0 *M* Tris buffer pH 8.0, which is the pH optimum for the reaction. The increase in absorption at 410 mμ, resulting from the dephosphorylation of NPP to yield nitrophenol, was followed spectrophotometrically with a cell of 1-cm path length. Under these conditions the enzymic activity is linearly dependent on the enzyme concentration in the range of 0.001 to 1.0 O.D. units/min at 25°, and in this range is independent of substrate concentration when it is in excess of 0.1 mg/ml. In this assay the enzymic activity is not affected by the addition of 0.01 *M* Mg^{++} to the medium. However, if the concentration of Tris buffer is lowered to 0.01 *M*, full enzymic activity is not obtained without the addition of Mg^{++}. A Mg^{++} activation effect has generally been found with other phosphatases[20].

N,N-Diethylaminoethyl-cellulose[21] (DEAE) (obtained from Distillation Products Industries, Rochester, New York) was washed extensively with 0.1 *M* Tris buffer at pH 7.4 before use. It was packed to a height of about 10 cm under eight pounds per square-inch pressure into a chromatography tube containing a sintered-glass disk

at the bottom. The packed DEAE column received a final wash with 0.01 *M* Tris buffer pH 7.4. The enzyme material, which had been dialyzed against 0.01 *M* Tris buffer pH 7.4, was applied to the column and elution was carried out with a linearly increasing concentration of salt. The eluting solution was passed through the column at a rate of about 0.5 ml/min, and was collected in separate tubes on a fraction collector.

RESULTS

Synthesis of alkaline phosphatase protein in cultures starved for phosphate

The fact that the amount of alkaline phosphatase in *E. coli* is optimal when the cells are starved for phosphate, and is markedly reduced when there is an excess of phosphate available[17], suggested that the enzyme protein might be identified as a component which is found only in cells starved for phosphate. The following experiment shows a comparison of protein fractions obtained from two cultures, one of which was starved for phosphate while the other was provided with an excess.

A log-phase culture grown in P medium containing $1.6 \cdot 10^{-3}$ *M* KH_2PO_4 (excess phosphate) was centrifuged and resuspended in P medium without added phosphate at a density of $5 \cdot 10^8$ cells/ml. The resuspended cells were divided into two cultures. A and B, with 100 ml in each; culture A remained without added phosphate and culture B was adjusted to $1.6 \cdot 10^{-3}$ *M* KH_2PO_4. The cells were aerated on a shaker at 37° for 20 min, and then 5 μC of labeled L-[^{14}C]proline (specific activity 13 μC/μmole) were added to each culture. Aeration was continued for 4 h, after which the cultures were centrifuged and the cells resuspended in 10 ml of a medium containing 0.05 *M* Tris buffer pH 7.4 and 0.02 *M* $MgSO_4$. The cells were then ruptured in a French decompression press[22] by release from a pressure of 10,000 pounds/sq. in. The presence of $MgSO_4$ in the medium serves to preserve intact the ribosomal particles during this treatment[23]. The ruptured cells were exposed to 20 μg/ml of DNAase for 20 min at 37°, and were then centrifuged at 35,000 rev./min in a Spinco head 40 for 1 h at 5°. The pellet material was discarded and the supernatants were dialyzed for 20 h at 5° against a solution containing 0.01 *M* Tris buffer at pH 7.4 and 0.001 *M* $MgSO_4$. Of the total ^{14}C activity that had initally been incorporated in the cells, 61 % was present in the dialysate of culture A and 52 % in the dialysate of culture B. At this stage there was added to the dialysate of B unlabeled carrier alkaline phosphatase from a culture which had been prepared in the same manner as culture A, except that [^{14}C]proline had not been added. The dialysates were then passed through a column of DEAE; under these conditions all of the alkaline phosphatase activity attached to the DEAE. Elutions from the column were carried out with a linear gradient of NaCl ranging from 0 to 0.14 *M*, buffered at pH 7.4 with 0.01 *M* Tris. Eluate fractions were collected and assayed for ^{14}C and alkaline phosphatase activities.

The results for cultures A and B are plotted in Figs. 1 and 2. Culture A, which was starved for phosphate and consequently produced alkaline phosphatase at an optimal rate, shows a single prominant peak of ^{14}C activity which corresponds to the peak of enzymic activity. The ^{14}C peak represents 8.4 % of the total [^{14}C]proline initially present in the cells; all of the alkaline phosphatase activity initially present in the cells is accounted for in the peak of enzymic activity. In culture B, which was provided with an excess of phosphate and consequently produced only 1/200 as much

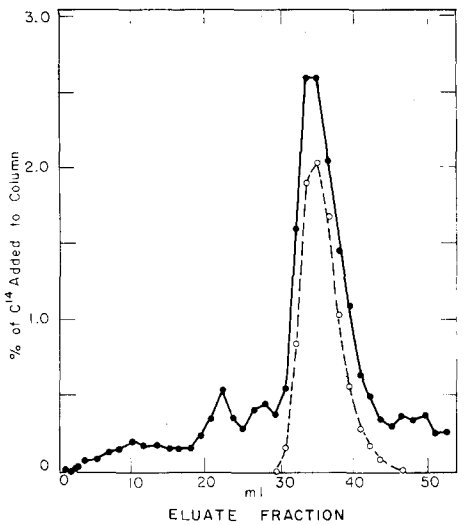

Fig. 1. Synthesis of alkaline phosphatase protein by cells grown in phosphate-limited medium. A cell extract was prepared by the procedure described in the text and adsorbed to a column of DEAE. Elution was achieved by a linear gradient of NaCl ranging from 0 to 0.14 *M* buffered at pH 7.4 with 0.01 *M* Tris. The eluate fractions were assayed for [^{14}C]proline, which was used as a specific label for the cellular protein (solid line curve), and for alkaline phosphatase enzymic activity (broken line curve).

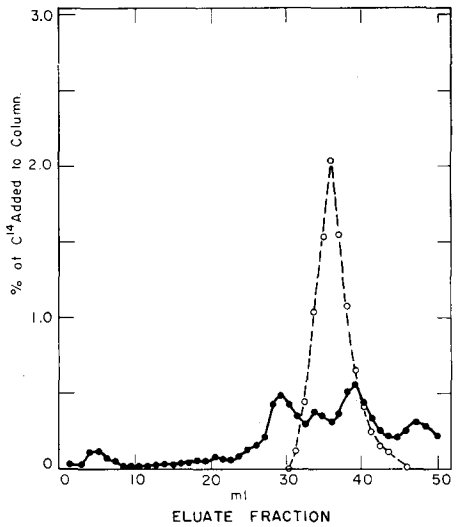

Fig. 2. Lack of synthesis of alkaline phosphatase protein by cells grown in a medium containing excess orthophosphate. The experimental procedure was the same as for Fig. 1.

alkaline phosphatase activity as culture A, there is no prominent ^{14}C peak in the region where the enzymic activity (added as unlabeled carrier) comes off the column. Within the region containing the carrier enzymic activity, 2.4 % of the ^{14}C initially present in the cells is found. This ^{14}C represents the amount of non-enzyme protein material that comes off the column along with the alkaline phosphatase. Subtracting this amount from the amount found in the enzyme peak in culture A, we obtain the result that 6 % of the proline incorporated into cellular protein, at the time the culture was producing alkaline phosphatase at an optimal rate, was alkaline phosphatase proline. Since proline constitutes 4.8 moles percent of the amino acids in alkaline phosphatase protein, which is close to the value of 4.6 moles percent reported for the total protein of *E. coli* grown in synthetic medium[32], we conclude that about 6 % of the total protein synthesized was alkaline phosphatase protein.

Larger-scale purification of the enzyme

A 20-l culture was grown in a medium containing limiting amounts of phosphate by the procedure described in the section on methods. Purification of the alkaline phosphatase was achieved as follows. The cells were centrifuged and resuspended in 100 ml of 0.1 *M* Tris buffer pH 7.4; all of the enzymic activity remained with the cells. The cells were equilibrated at 82° for 15 min (alkaline phosphatase is stable at this temperature—*cf.* section on properties of the enzyme) and then were dialyzed against 0.1 *M* Tris buffer 7.4 for 20 h at 5°. The dialysate was centrifuged at 20,000 rev./min in a Spinco head 20 for 20 min, and the pellet material was put through three successive cycles of centrifugation and resuspension in 50 ml of 0.1 *M* Tris pH 7.4. All super-

natants were pooled and given a final centrifugation for 1 h. The final supernatant contained 90 % of the initial enzymic activity. This supernatant was dialyzed against 0.02 *M* Tris buffer pH 7.4 for 20 h at 5°. The dialysate was passed through a column of DEAE which was one inch in diameter and three inches in height; all of the enzymic activity was retained on the column. The enzyme was eluted with a linear gradient of Tris buffer pH 7.4 ranging from 0.02 to 0.20 *M*. The eluate was collected in tubes containing 4-ml samples, and each tube was tested for absorption at 280 mμ and for alkaline phosphatase activity. The results are plotted in Fig. 3. It is evident that the first major peak of 280 mμ-absorbing material coincides with the peak of enzymic activity. All of the enzymic activity added to the column was recovered in this peak.

The samples which showed alkaline phosphatase activity were pooled into two fractions, fraction 1 consisting of tubes 17 through 22 and fraction 2 of tubes 23 through 36. Fraction 1 contained 85 % of the total alkaline phosphatase activity and fraction 2 the remaining 15 %. Each fraction was dialyzed against a solution of 0.01 *M*.

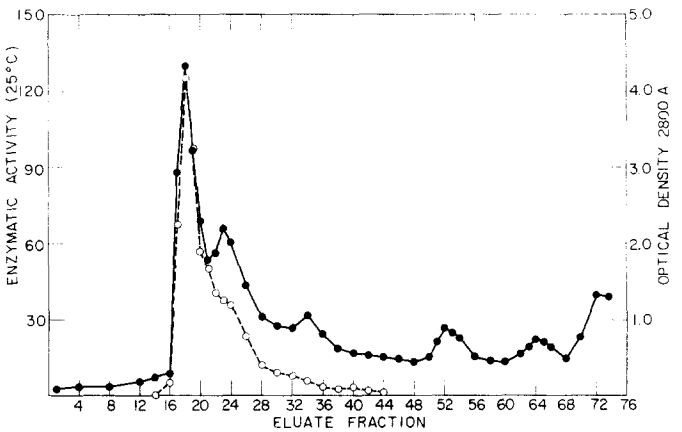

Fig. 3. Fractionation on DEAE of alkaline phosphatase protein from a crude cell extract. The extract was prepared from a 20-l culture by the procedure described in the text and adsorbed to a column of DEAE. Elution was achieved with a linear gradient of Tris buffer ranging from 0.02 to 0.20 *M* at a constant pH of 7.4. The eluate fractions were assayed for O.D. at 280 mμ (solid line curve) and for alkaline phosphatase enzymic activity (broken line curve).

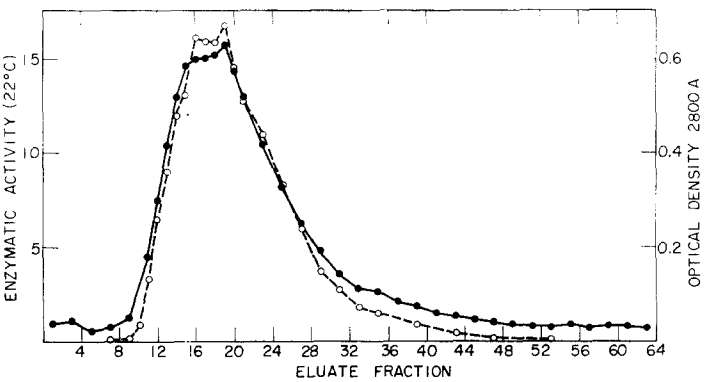

Fig. 4. Re-fractionation on DEAE of the alkaline phosphatase protein obtained from the enzyme peak in Fig. 3. Elution was achieved with a linear gradient of NaCl ranging from 0.04 to 0.10 *M* buffered at pH 8.6 with 0.01 *M* Tris.

Tris buffer pH 8.6 + 0.04 *M* NaCl, and then was passed through a column of DEAE. All of the enzymic activity was retained on the column. Elution was carried out with a linear gradient of NaCl ranging from 0.04 *M* to 0.10 *M* buffered at pH 8.6 with 0.01 *M* Tris. The results for fraction 1 are plotted in Fig. 4. There is only a single peak of 280 mμ-absorbing material and this peak corresponds to the peak of alkaline phosphatase activity. Similar results were obtained for fraction 2. The fact that the enzymic activity of both fractions 1 and 2 eluted as a single peak in the same range of NaCl concentration is an indication of homogeneity of the enzyme. Tubes 12 through 31 of Fig. 4, which contained 80 % of the alkaline phosphatase activity that had been added to the column, were pooled and this material used in all the succeeding tests for purity. The absorption spectrum of this material is shown in Fig. 5. The absence of any peak at 260 mμ indicates the lack of significant contamination from nucleic acid.

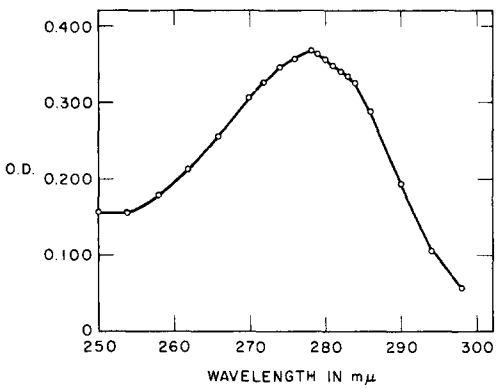

Fig. 5. Absorption spectrum of purified alkaline phosphatase protein.

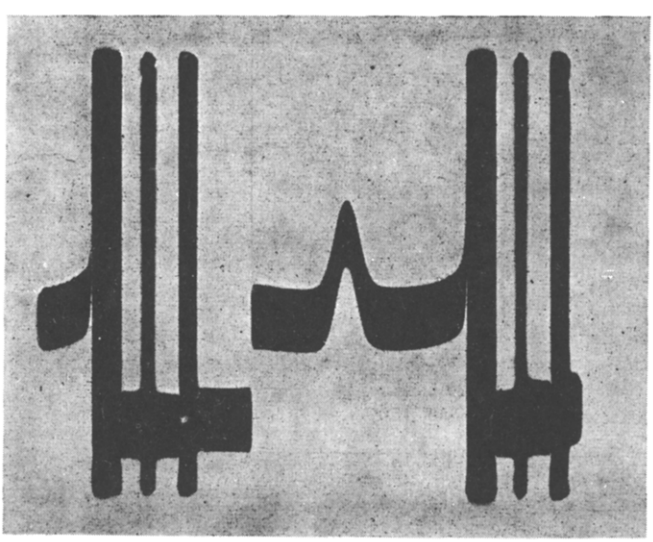

Fig. 6. Sedimentation pattern of purified alkaline phosphatase protein. The enzyme concentration was 2 mg/ml in a medium of 0.15 *M* NaCl buffered at pH 7.4 with 0.01 *M* Tris. The centrifugation speed was 59,780 rev./min. and the picture was taken 48 min after the start of the run.

Further evidence for the purity of the enzyme and some of its properties

Sedimentation constant and molecular weight: When the purified material was examined in the analytical ultracentrifuge there was a single schlieren peak which appeared symmetrical as shown in Fig. 6. The sedimentation constant, obtained at 20° in 0.15 *M* NaCl buffered at pH 7.4 with 0.01 *M* Tris, was 6.3S. This value agrees with the result reported for hog kidney phosphatase[24].

The molecular weight was determined by the ARCHIBALD method as described by EHRENBERG[25]. Using a partial specific volume of 0.73 which was estimated from a preliminary amino acid analysis of the purified enzyme, a molecular weight of 75,000 to 80,000 was calculated. Taking this molecular weight value to calculate the frictional constant f_0 of the equivalent sphere[26], and the sedimentation constant and molecular weight values to calculate the true frictional constant f, a value of f/f_0 of 1.05 is obtained. This indicates that the molecule is approximately spherical and is not greatly hydrated.

Electrophoresis: Electrophoresis was carried out in a Tiselius type cell at three different pH's, 7.6, 6.5, and 4.4, at an ionic strength of 0.10. The results from each of these runs showed only a single schlieren peak which appeared to be symmetrical, as shown in Fig. 7. The electrophoretic mobility at pH 7.6 was $-3.3 \cdot 10^{-5}$ cm²/V sec. (The mobility of swine kidney phosphatase[24] in pH 7.2 phosphate buffer of ionic strength 0.10 has been found to be $-1.8 \cdot 10^{-5}$ cm²/V sec.) The isoelectric point was estimated to be 4.5. Electrophoresis was also carried out in starch gel at pH 8.0, 4.5, and 3.6, using the method of SMITHIES[33]. After electrophoresis the gel was sprayed with NPP in order to locate the alkaline phosphatase. All of the enzymic activity was

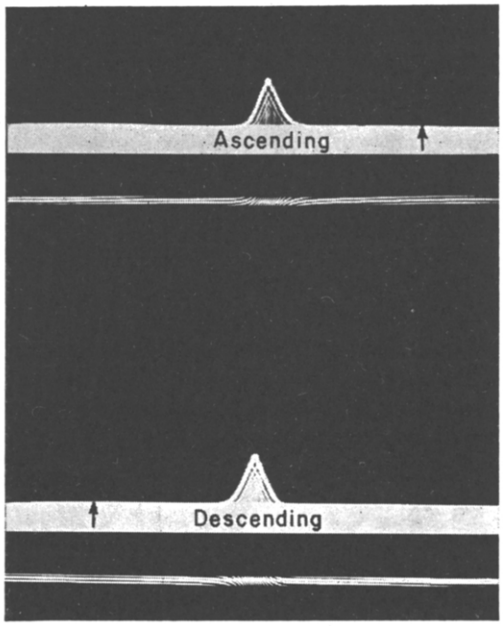

Fig. 7. Electrophoresis pattern of purified alkaline phosphatase protein at pH 7.6. Electrophoresis was carried out in the micro-cell of the Spinco model H at a current of 5 mA. The protein concentration was 1.5 mg/ml, in a solution of 0.01 *M* Tris + 0.10 *M* NaCl.

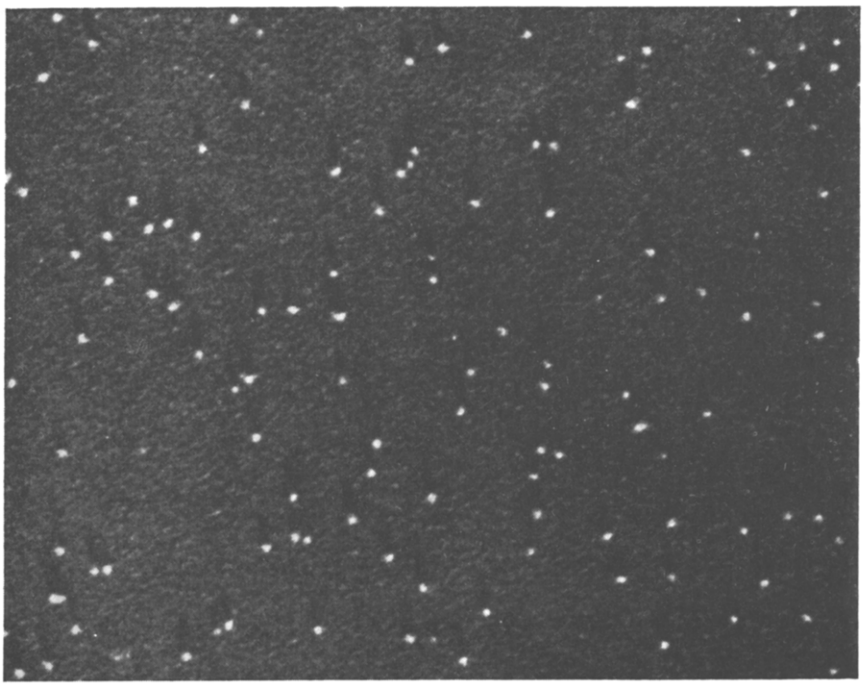

Fig. 8. Electron micrograph of purified alkaline phosphatase protein, magnified 163,000 times (taken by Prof. C. E. HALL[31]).

found localized in a single band, indicating the presence of only a single electrophoretic species of alkaline phosphatase. The isoelectric point again occurred at pH 4.5*.

Electron micrography: Fig. 8 shows the appearance of the purified enzyme preparation in the electron microscope. The particles appear to be of uniform size and approximately spherical in shape, and are not contaminated with a larger component. The diameter of the particles is 60 Å, in reasonable agreement with the value of 57 Å calculated for a spherical unhydrated protein particle of molecular weight 80,000. (The diameter was estimated from the length of the shadow—Prof. C. E. Hall, personal communication).

pH optimum: The effect of pH on the velocity of enzymic activity against NPP is shown in Fig. 9. The maximum velocity occurs at pH 8.0, and there is no detectable activity below pH 6.

Activation energy: The effect of temperature on the velocity of the enzymic activity as measured against NPP is shown in Fig. 10. The activity increases by a factor of 1.5 for a 10° increase in temperature. The activation energy for the reaction is 6880 cal./mole.

Heat inactivation: Crude cell extracts containing alkaline phosphatase can be maintained at 85° for at least 30 min without loss of enzymic activity, whereas the purified enzyme is unstable at the same temperature. It was found that the heat

* In mammalian cells several electrophoretically different alkaline phosphatase molecules have been observed[30], whereas in *E. coli* we have detected only the single electrophoretic species after starch gel electrophoresis of crude cell extracts.

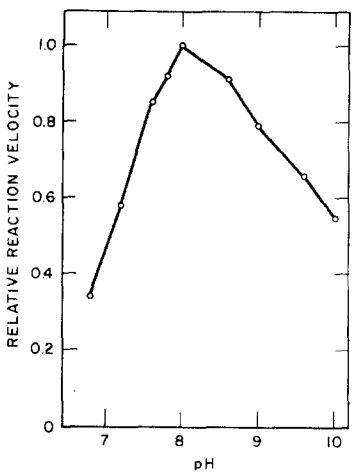

Fig. 9. Effect of pH on the rate of alkaline phosphatase activity against *p*-nitrophenyl phosphate The solutions were buffered with 1 *M* Tris and measurements were made at 25°. The enzyme concentration was 1 μg/ml.

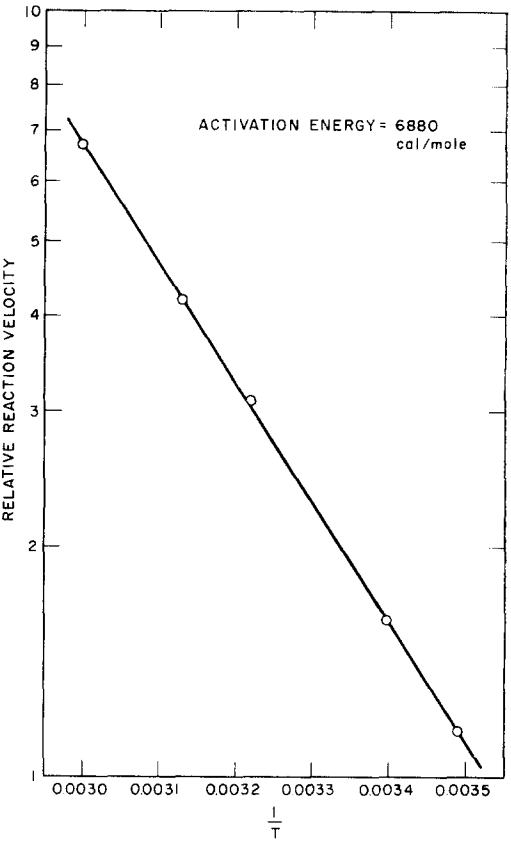

Fig. 10. Effect of temperature on the rate of alkaline phosphatase activity against *p*-nitrophenyl phosphate. The calculated activation energy is 6880 cal./mole. Measurements were made in 1.0 *M* Tris buffer at pH 8.0.

TABLE I

ACTIVITY OF ALKALINE PHOSPHATASE AGAINST DIFFERENT COMPOUNDS

The reaction mixtures contained 10^{-2} *M* substrate buffered at pH 8.0 with 1 *M* Tris (at this concentration the reaction velocity is independent of substrate concentration). Enzymic activity was measured by the release of orthophosphate.

Compound tested as substrate	*Relative reaction velocity*
p-nitrophenyl phosphate	1.0
Sodium β glycerophosphate	0.9
Glucose 1 phosphate	0.9
Adenosine 3′ phosphate	1.0
Adenosine 5′ phosphate	0.8
Cytidine phosphate	1.2
Guanosine phosphate	1.0
Uridine phosphate	1.3
Riboflavin 5′ phosphate	0.7
L-histidinol phosphate	0.9
Creatine phosphate	0
Adenosine triphosphate	0
Sodium pyrophosphate	0

stability for the purified enzyme could be raised to a level comparable to that of a crude extract by the addition of $MgCl_2$ or $MgSO_4$ to the medium. Fig. 11 shows the effect of Mg^{++} on the rate of inactivation of purified enzyme at 95°. With 0.01 *M* Mg^{++} in the medium, the rate of inactivation is about 15 times slower than that observed in the absence of Mg^{++}. Furthermore, in 0.01 *M* Mg^{++} medium the kinetics of inactivation is exponential over the range tested, which extended to 0.3 % residual activity. However, at lower concentrations of Mg^{++} the slope of the inactivation curve is not constant; initially the slope is greater than, and ultimately becomes equal to, the slope in 0.01 *M* Mg^{++} medium. This behavior indicates that in 0.01 *M* Mg^{++} medium the entire population of enzyme molecules is maximally stabilized to heat, while at lower Mg^{++} concentrations only a fraction of the population is stabilized.

Substrate specificity: The rate of phosphorolytic activity of the enzyme against several compounds, as measured by the release of orthophosphate, is shown in Table I.

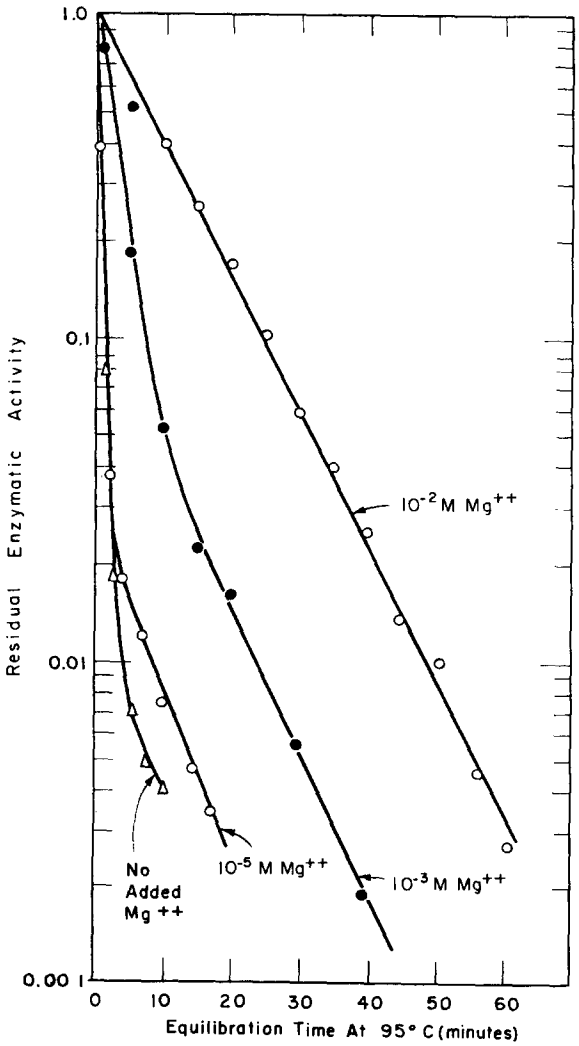

Fig. 11. Effect of Mg^{++} on the rate of thermal inactivation of alkaline phosphatase at 95°. The enzyme was equilibrated at 95° for the indicated times, after which the enzymic activity against *p*-nitrophenyl phosphate at 25° was measured. The solutions were buffered at pH 8.0 with 1 *M* Tris. The enzyme concentration was 0.1 mg/ml.

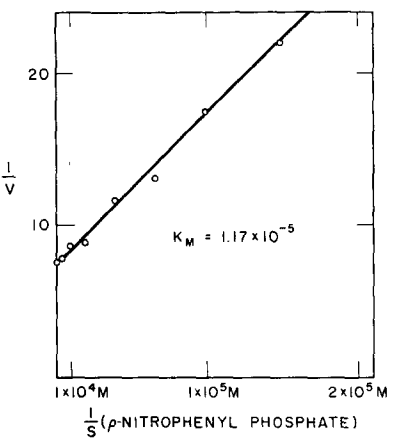

Fig. 12. Measurement of the Michaelis constant K_m of alkaline phosphatase with *p*-nitrophenyl phosphate. K_m was calculated from the equation:

$$\frac{1}{V} = \frac{1}{S}\frac{K_m}{V_{max}} + \frac{1}{V_{max}}$$

where V is the velocity of enzymic activity at a substrate concentration S, and V_{max} is the maximum velocity attainable with an excess of substrate. The experiments were carried out at 25° in 1 *M* Tris buffer at pH 8.0. The enzyme concentration was 0.2 μg/ml.

Activity was observed against all the phosphomonoesters tested, but the enzyme did not liberate orthophosphate from ATP, sodium pyrophosphate, or creatine phosphate. The organic moieties of these phosphomonoester substrates have only a small effect on the rate of enzymic activity. Alkaline phosphatase from cow's milk and calf intestinal mucosa have also been found to act as general phosphomonoesterases[27]. However, in contrast to the bacterial enzyme, these phosphatases were reported to be active against creatine phosphate.

Michaelis constant: A determination of the Michaelis constant with the substrate NPP is shown in Fig. 12. The value obtained at a pH of 8.0 and a temperature of 25° is $1.2 \cdot 10^{-5}$ (moles of NPP/l).

Turnover number: The turnover number calculated for a molecular weight of 80,000 is 2700 molecules of NPP cleaved/enzyme molecule/min at 25°. This value is lower, by a factor of about twenty, than the turnover number calculated for mammalian alkaline phosphatases[24,27] assuming a molecular weight of 80,000.

Competitive inhibitors: Several compounds were tested as competitive inhibitors of alkaline phosphatase activity against the substrate NPP by the procedure described in Fig. 13. In this procedure the substrate concentration was maintained constant at $10^{-3}\,M$ and the inhibitor concentration was varied. A compound was classified as a competitive inhibitor if the value of $1/V$ was found to be linearly proportional to I, according to the equation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} + I\,\frac{K_m}{K_I\,V_{max}\,S}$$

where V is the rate of cleavage of NPP in the presence of the inhibitor, V_{max} is the maximum rate without inhibitor, S and I are the concentrations of NPP and inhibitor respectively, K_m is the Michaelis constant for NPP and K_I is its equivalent for the inhibitor. The compounds listed in Table II were found to be competitive inhibitors by this criterion. The ratio K_I/K_m, which is a measure of the affinity for the enzyme of NPP relative to that of the competitive inhibitor, has been evaluated for each compound. Orthophosphate and arsenate have the highest affinity for the enzyme,

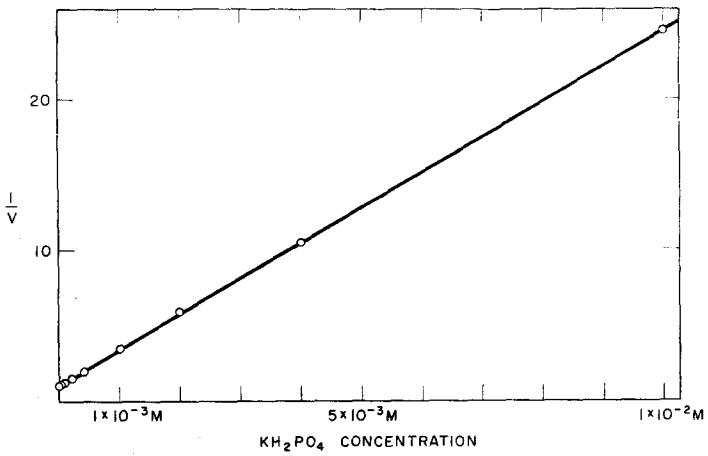

Fig. 13. Competitive inhibition by orthophosphate of the activity of alkaline phosphatase against *p*-nitrophenyl phosphate. The concentration of *p*-nitrophenyl phosphate was kept constant at $1 \cdot 10^{-3}\,M$. Measurements were made at 25° in 1 *M* Tris buffer at pH 8.0.

three times greater than the affinity of NPP. Six other compounds tested, five of which are substrates for the enzyme (*cf.* Table I), have a lower affinity than NPP.

Evidence for a metal at the active site: Four metal chelating agents, EDTA (ethylenediaminetetraacetic acid), cyanide, 8-hydroxyquinoline, and 1,10-*o*-phenanthroline, were found to inhibit enzymic activity, as shown in Table III. The measurements reported in Table III were carried out immediately after diluting the inhibitor one hundred-fold, and under this condition EDTA was the only inhibitor whose effect was largely irreversible. However, the inhibition by EDTA could be reversed by exhaustive dialysis against Tris buffer.

It has been reported that an alkaline phosphatase from swine kidney is inhibited by EDTA and cyanide, and that this enzyme contains one atom of zinc per molecular weight of 37,000[24]. Assuming that the *coli* enzyme also contains a metal atom, and that the chelating agents act by binding to the metal, the following experiment suggests that the metal is located at the active site of the enzyme. If orthophosphate

TABLE II

COMPETITIVE INHIBITORS OF ALKALINE PHOSPHATASE ACTIVITY AGAINST *p*-NITROPHENYL PHOSPHATE

K_I/K_m was determined by the procedure described in Fig. 13. The value of K_I/K_m indicates the relative affinities of the inhibitor and NPP for the enzyme; the inhibitors which yield values of less than 1.0 have a greater affinity than NPP, and those which yield values in excess of 1.0 have a lower affinity than NPP.

Competitive inhibitor	*Inhibitor constant* K_I/K_m *at 25°*
Sodium phosphate	0.32
Sodium arsenate	0.32
Uridine phosphate	3.7
Guanosine phosphate	3.8
Sodium β glycerophosphate	4.2
Glucose-1-phosphate	5.3
Adenosine 5′ monophosphate	7.8
Adenosine triphosphate	8.0

TABLE III

INHIBITION BY METAL-CHELATING COMPOUNDS

Inhibitor and enzyme (0.5 mg/ml) were equilibrated together in 1 *M* Tris buffer pH 8.0 at 23° for the period indicated. Enzymic activity was assayed using the substrate NPP at a concentration of 10^{-3} *M*. The assays in which an inhibitor was present contained the same concentration of inhibitor as was used for equilibrating with the enzyme. Removal of the inhibitor before assaying was achieved by diluting the equilibration mixture by a factor of 100, and the assay was carried out immediately after dilution.

Inhibitor	*Inhibitor concentration*	*Minutes of equilibration of inhibitor with enzyme*	*% of initial enzymic activity*	
			Inhibitor present	*Inhibitor removed*
EDTA	$2.5 \cdot 10^{-3}$ *M*	20*	20	42
Sodium cyanide	$1 \cdot 10^{-2}$ *M*	10	0.7	90
	$1 \cdot 10^{-3}$ *M*	10	90	
8-hydroxyquinoline	$1 \cdot 10^{-3}$ *M*	10	2	88
1,10 *o*-phenanthroline	$1 \cdot 10^{-3}$ *M*	10	74	100

* The extent of inhibition by EDTA was found to depend on the time of equilibration; with longer equilibration the inhibition eventually reaches completion.

is present when the enzyme is equilibrated with EDTA, the enzyme is protected against the inactivating effect of EDTA. For example, equilibration of the enzyme with 0.09 *M* EDTA for 3 min at 30° reduces enzymic activity, as measured immediately after diluting away the EDTA, to 10 % of the initial value, but if 0.0005 *M* orthophosphate is present in the equilibration mixture the activity is reduced only to 70 %. It has been shown (*cf.* Table II) that the orthophosphate is capable of competitively inhibiting enzymic activity against the substrate NPP, which is an indication of the affinity of orthophosphate for the active site of the enzyme. Therefore, the protective effect of orthophosphate against inhibition by EDTA probably results from competition between the two substances for the active site.
the protective effect of orthophosphate against inhibition of the enzyme by EDTA probably results from competition between orthophosphate and EDTA for the active site.

DISCUSSION

It has been shown for several bacterial enzymes that the rates of enzyme synthesis are subject to regulation either by specific "inducer" substances, such as galactosides for the enzyme β-galactosidase[28], or by specific "repressor" substances, such as orthophosphate for the enzyme alkaline phosphatase[17]. The maximal rate of enzyme synthesis is obtained when the inducer is added to the growth medium, or when the repressor is removed. There is evidence from recent work on β-galactosidase that an inducer acts by competing with an intracellular repressor[29]. Therefore, both inducible and repressible enzymes may be controlled by a similar basic mechanism involving enzyme-specific repressors which reduce the rate of enzyme synthesis.

The maximal rate at which a bacterial cell synthesizes one of its enzymes, relative to the total rate of protein synthesis, has been measured for two of the enzymes of *E. coli*: for β-galactosidase[29] and, as reported in the present paper, for alkaline phosphatase. The β-galactosidase rate was determined with an inducer added to the medium, and the alkaline phosphatase rate with the repressor orthophosphate removed from the medium. The result in both cases is that the single enzyme constitutes about 6 % of the mass of cellular protein. The similarity between the two cases suggests that an *E. coli* cell may have the potentiality to synthesize any one of its proteins at a differential rate of about 6 % of the total, although it is unlikely that more than a few proteins could be synthesized simultaneously at this rate.

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MITOCHONDRIAL SWELLING AND ELECTRON TRANSPORT

I. SWELLING SUPPORTED BY FERRICYANIDE

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SUMMARY

The swelling of rat-liver mitochondria in presence of phosphate is inhibited by cyanide but restored by further addition of ferricyanide. The effects on swelling of inhibitors of electron transport (amytal, antimycin A) and of compounds which prevent phosphorylation (2,4-dinitrophenol, oligomycin) have been studied in presence and absence of ferricyanide and cyanide. It is concluded that, under defined conditions, swelling is supported by electron transport, even through a restricted portion of the respiratory chain, by a mechanism not directly involving the coupled phosphorylation process.

INTRODUCTION

That the swelling of isolated rat-liver mitochondria in the presence of phosphate or thyroxine is dependent on respiration is indicated by the following considerations.