SUMMARY

Alanine is the principal N-terminal group in the cell walls of *M. lysodeikticus*, *S. lutea* and *B. megaterium*. Most of the ε-amino groups of lysine in the cell walls of *M. lysodeikticus* are available for reaction with FDNB and about one third of the diaminopimelic acid residues of *B. megaterium* cell walls have one amino group free.

Lysozyme digests DNP-cell walls at about the same rate as untreated walls and the digests show the same number of electrophoretic components.

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TRUE SUBSTRATES FOR ALKALINE PHOSPHATASE*

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INTRODUCTION

It has been reported[1, 2, 3] that the optimum pH for alkaline phosphatase increases with an increase in the concentration of the substrate accompanied by a concurrent increase in the hydrolysis rate. Since a third order rate-controlling reaction involving the hydrogen-ion activity is extremely unlikely, this could be interpreted to imply that the active form of the enzyme varies with different substrate concentrations, and that this change is dependent on pH in some manner. Ross *et al.*[3] use this hypothesis to calculate pH optima for cellular alkaline phosphatase from the total hydrolyzable phosphate available to the enzyme. Another possibility lies in the change of "true" substrate concentration with pH. JOHNSON[4, 5] has analyzed data on the hydrolysis of triglycine by intestinal aminopolypeptidase to demonstrate reaction rates proportional to the concentration of the zwitterion form of the substrate, which concentration is in turn controlled by the pH. However, JOHNSON advances no hypothesis which can be used to reconcile (a) a decrease in rate when substrate concentration is increased by pH change with (b) an increase in rate when substrate concentration is increased

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by addition of substrate. The present paper advances the hypotheses that (1) pH does not influence the reactivity of the active sites of the enzyme over the range studied; (2) there is an optimum substrate concentration for a given enzyme; and (3) there are specific "true" substrates on which the enzyme acts, which substrates are not necessarily the most abundant ion species of the substrate present. To investigate the validity of these hypotheses, kinetic studies of the alkaline phosphatase catalyzed hydrolysis of β-glycerophosphate were made.

METHODS

A. *Preparation of alkaline phosphatase*

Alkaline phosphatase from the intestinal mucosa of adult rats was prepared according to the method of Ross *et al.*[3]. The preparation was then lyophilized, resuspended in 1/100 of the original volume, and dialyzed against glass-distilled water at 2°C for 24 hours. This solution contained 675 μg of nitrogen per ml and was stable for at least one month at 2°C.

B. *Preparation of* ^{32}P*-labelled* β*-glycerophosphate*

^{32}P-labelled β-glycerophosphate was prepared by a modification of the procedure employed by SPINKS *et al.*[6]. 2.76 g $NaH_2PO_4 \cdot H_2O$ containing 29 mc ^{32}P was heated with 6 ml glycerol for 2 hours at 180°–190°C at reduced pressure. 40 ml 10% NaOH was added and the mixture was refluxed 2 hours at 135°–145°C. After cooling, 20 ml 2.0 *M* $Ba(OOCCH_3)_2$ was added to precipitate the inorganic phosphate. The $Ba_3(PO_4)_2$ was centrifuged down; the supernatant was concentrated to 30 ml, cooled, and separated from the small amount of additional precipitate by centrifuging. The supernatant was then heated to 80°C and held at that temperature for 2 hours, with precipitation of a large portion of the glycerophosphate as the barium salt. This was centrifuged down, washed twice with 5 ml absolute ethanol, dried and weighed. This residue was leached with 30 ml H_2O and the amount which dissolved was determined. To this was added an equivalent amount of 0.2 *M* Na_2SO_4. The $BaSO_4$ was removed by centrifugation. The sodium β- glycerophosphate was crystallized from H_2O at 2°C. with a yield of 0.97 g. This was diluted with 9 parts of Eastman sodium β-glycerophosphate and recrystallized with a final yield of 0.506 g. The final preparation was analyzed for α-glycerophosphate by the method described by TOAL AND PHILLIPS[7] and found to contain 0.3% α-form. 3.4% of the ^{32}P was in the α-glycerophosphate form, 0.08% in the inorganic form, and the remainder in the β-glycerophosphate form. The final preparation had a specific activity of 15 mc per mole. A duplicate preparation was made from unlabelled NaH_2PO_4 for the experiments in which counterparts of labelled and unlabelled substrate were to be used.

C. *Analysis procedures*

Each hydrolysis reaction was stopped after the incubation period by the addition of an equal volume of chilled 10% trichloroacetic acid; and the denatured protein was removed by centrifugation at 2,000 $\times$ *g* for 10 minutes. Inorganic phosphate was determined by the method of KING[8] using a Beckman DU spectrophotometer. To each specimen to be analyzed for inorganic ^{32}P, NaH_2PO_4 containing 1 mg P was added and 10 minutes allowed for equilibrium to be established. Then 0.05 ml 2 *M* barium acetate was added and the pH brought above 10 by the addition of 6 *M* NaOH. The solution was made to 3 ml with water and allowed to stand at least 1 hour. The precipitate was filtered off, washed well, dried and counted in a gas flow counter to determine inorganic ^{32}P. The supernatant and washings were made to a standard volume and an aliquot was evaporated to dryness on a planchet where β-glycerophosphate ^{32}P activity was desired. All counts were corrected with instrumental calibrations and for decay. Control blanks were run for all analyses and the the results substracted from the sample results. In the barium phosphate precipitation, the radioactivity precipitated in the control blanks (which included the inorganic ^{32}P in the preparation plus any coprecipitated organic ^{32}P) varied by 1%, 5% and 3% respectively of the reaction inorganic ^{32}P values in the three experiments; this was taken as evidence of relatively little error from coprecipitation in the procedure.

D. *Reaction procedures*

For the various experiments, the reactants were all brought to the reaction temperature before starting the reaction. Two types of experiment were run: (1) reactions using mixed tagged and untagged substrates to assess transient reaction rate differences, and (2) reactions using stable isotopes to determine the effect of enzyme con-

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centration on rates at different pH values. The rationale of these approaches is considered in the DISCUSSION.

In the first set of experiments, 0.25 ml each of equimolar (0.0060 *M*) concentrations of ^{32}P-labelled sodium β-glycerophosphate, pH 6.80, and unlabelled sodium glycerophosphate, pH 7.23 were simultaneously injected into 0.50 ml of enzyme suspended in doubly concentrated Mg^{++} and tris (2-amino-2-hydroxymethyl-1, 3-propandiol) buffer. The final concentrations in the reaction mixture were 0.0030 *M* β-glycerophosphate, 0.01 *M* Mg^{++}, 0.020 *M* tris, and pH 6.93. The reaction was allowed to run 30 seconds at 38° C. A "mirror" reaction was run in which the acid substrate was unlabelled and the basic substrate was labelled, but all other conditions were the same. A similar pair of reactions was run for 15 seconds at pH 8.0 in which the injected substrates were at pH 6.80 and pH 10.23 respectively. The acidity of these solutions was such that one volume of each of them mixed with two volumes of water would give pH = 8.0. A third pair was also run at 38° C and pH 8.0; it was identical to the second pair except that the substrates were mixed 10 minutes before incubation with the enzyme and the incubation time was 50 minutes. The specific activity of all labelled substrate in the three experiments was the same, 231 counts/sec/μmole; the specific activity of the combined substrate being one-half of the above. The results are shown in Table I.

TABLE I

PRODUCT OF β-GLYCEROPHOSPHATE HYDROLYSIS UPON SIMULTANEOUS MIXING OF LABELLED AND UNLABELLED SUBSTRATE INTO PHOSPHATASE

Expt.	*pH of β-glycerophosphate*		*Reaction conditions*		*Inorganic P liberated*	
	^{32}P-labelled	*Unlabelled*	*Time*	*pH*	*μmoles/ml*	*Counts/sec/μmole*
1A	6.80	7.23	30 sec	6.93	0.203	157
B	7.23	6.80	30 sec	6.93	0.219	77
2A	6.80	10.23	15 sec	8.00	0.362	128
B	10.23	6.80	15 sec	8.00	0.391	91
3A	8.00	8.00	50 min	8.00	2.55	118
B	8.00	8.00	50 min	8.00	2.58	123

TABLE II

EFFECT OF VOLUME OF INCUBATION MEDIA ON HYDROLYSIS OF β-GLYCEROPHOSPHATE WITH A CONSTANT AMOUNT OF PHOSPHATASE

pH	*Reaction time*	*Volume of medium*	*Inorganic P released μmoles*
8.5	0.50 min	1 ml	0.519 ± .013
	0.50 min	2 ml	0.728 ± .018
7.0	3.00 min	1 ml	0.803 ± .009
	3.00 min	2 ml	0.818 ± .018

In the second set of reactions the same amount of enzyme was placed in one and two ml of the same reaction medium: 0.0030 *M* β-glycerophosphate in 0.01 *M* Mg^{++} and 0.02 *M* tris at 38° C. Duplicate experiments were run at each of two pH's, 8.5 and 7.0. The results are recorded in Table II.

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DISCUSSION

The observation has been repeatedly made of enzyme systems with an optimal substrate concentration below which or above which the rate of reaction falls. To explain this, HALDANE[9] uses the concept of an activated complex first emphasized by VON EULER AND JOSEPHSON[10] in which the atoms of the substrate on both sides of the bond to be broken bind strongly with the enzyme. Then, as substrate concentration increases beyond the optimum, the active sites necessary for the second bonding of any substrate molecule are reduced in number by competitive first binding with other substrate molecules. If the assumption is made that this is the case in the alkaline phosphatase-β-glycerophosphate system, a satisfactory explanation must still be made for the change in this optimal concentration with pH. If we designate the double-charged β-glycerophosphate ion by $R^{=}$, then β-glycerophosphate exists in these systems in 3 species: H_2R, HR^- and $R^{=}$. The first set of experiments was intended to determine qualitatively if there was any increased specificity for the acid forms H_2R or HR^- as compared to $R^{=}$. The ideal way to accomplish that would be to have one solution with H_2R and HR^- and another solution with $R^{=}$ in such abundances that when mixed these three species would be in ionic equilibrium. As a practical approach, the pH of the unlabelled solution and the pH of the labelled solution were adjusted so that upon mixing with each other and the enzyme the species closely approached ionic equilibrium at the resulting pH. It was hoped that there would be sufficient time after mixing of the substrates before isotopic equilibrium to allow enough hydrolysis to occur to differentiate between the possible substrates. This was considered to be a feasible approach since the driving force to isotopic equilibrium in the presence of ionic equilibrium is entirely a matter of the entropy of isotopic mixing rather than the energy of ionic equilibration, so that the isotopic equilibration time may be relatively long[11].

From the results shown in Table I, it is evident that the hydrolysis occurs to a greater extent on the acid forms, whether labelled or unlabelled. Whether or not ionic equilibrium was reached does not alter this conclusion, since the ionic composition of parts A and B are identical in each experiment. How extensive the hydrolysis difference is may be estimated by referring to the abundance of each species in the reaction media assuming rapid ionic equilibrium. The labelled abundances may be obtained by multiplying the specific activity in the labelled portion by the ratio of labelled to unlabelled species before mixing; and, where equilibrium after mixing requires a greater total species concentration, combining this abundance with the necessary addendum from the species which must be converted to it for the equilibrium. Calculated values of these abundances using the ionization constants for β-glycerophosphoric acid of KIESSLING[12], $K_1 = 4.3 \cdot 10^{-2}$ and $K_2 = 4.6 \cdot 10^{-7}$, and the data from Table I, are assembled in Table III.

It may be seen that the specific activity of the product is compatible with the hydrolysis of the HR^- and H_2R present and only that amount of $R^{=}$ which must be converted to the acid forms to replace that hydrolyzed, even though the $R^{=}$ is in much greater abundance. This gives no clue, however, as to whether the substrate used is HR^-, H_2R or both.

On the assumption that there is an optimal substrate concentration a selection may be made between HR^- and H_2R as the true substrate by determining which

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TABLE III

COMPARISON OF SPECIFIC ACTIVITY OF THE PHOSPHATE IN THE PRODUCT TO THE SPECIFIC ACTIVITIES OF THE POSSIBLE SUBSTRATES IN β-GLYCEROPHOSPHATE MEDIA

Expt.	Substrate after ionic equilibrium						Product	
	μmoles/ml			Counts/sec/μmole			μmoles/ml	Counts/sec/μmole
	$R^=$	HR^-	H_2R	$R^=$	HR^-	H_2R		
1A	2.4	0.61	$17 \cdot 10^{-7}$	125	167	199	0.203	157
B	2.4	0.61	$17 \cdot 10^{-7}$	105	74	32	0.219	77
2A	2.9	0.064	$0.15 \cdot 10^{-7}$	118	231	231	0.362	128
B	2.9	0.064	$0.15 \cdot 10^{-7}$	113	<0.1	<0.1	0.391	91

maintains the more constant concentration at optimum pH for various β-glycerophosphate concentrations. For this study the values reported by Ross *et al.*[3] for optimum pH and the values of KIESSLING[12] for the ionization constants of β-glycerophosphoric acid were used. The calculations showed the β-glycerophosphoric acid concentrations to remain invariant at $(8.1 \pm 1.6) \times 10^{-13}$ *M* while the singly-charged ion concentration varied by a factor of 60 and the doubly-charged ion varied by a factor of 3,600. The invariance of its optimum concentration with widely varying concentrations of the ionized forms suggests a very high degree of enzyme specificity for the unionized form and permits the postulate that β-glycerophosphoric acid is the true substrate. The wide variance in concentrations of the ionized forms suggest that neither of these species exhibits competitive inhibition.

The second group of experiments was run to investigate the effect of enzyme dilution on the hydrolysis rate. If, as suggested, the true substrate is the unionized form, H_2R, then the rate of hydrolysis will be dependent on its being furnished rapidly enough to the enzyme. With sufficiently rapid enzyme action, the ionization reactions

$$R^= + H^+ \rightleftharpoons HR^-$$

and

$$HR^- + H^+ \rightleftharpoons H_2R$$

may become rate-controlling. Under these conditions, provision of twice the volume of substrate for the same amount of enzyme should decrease this limiting action, so that the rate of reaction per unit of enzyme would increase with dilution. That is noted in Table II for the higher pH. At greater hydrogen ion activities, however, the rates of formation of HR^- and H_2R from $R^=$ are correspondingly increased and the dilution effect becomes less. This is also demonstrated in the data.

The mechanism postulated may not be the only mechanism which could cause an increase in hydrolysis rate with enzyme dilution; for example, an alternative hypothesis is that an inhibitor, recalcitrant to the enzyme preparation procedure, is diluted out. However, the work of Ross *et al.*[3] provides data which are compatible with the postulate that the formation of the unionized form is rate-controlling. These authors have reported phosphate production values at optimum pH for different β-glycerophosphate concentrations. Using these data, and postulating formation of the substrate to be rate-limiting, hydrolysis rates were calculated assuming an optimum concentration of H_2R at $8.1 \cdot 10^{-13}$ *M* (see above). These agreed with the rates observed over a 28-fold increase in rate with a standard deviation of 12%, but diverged above

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0.016 *M* β-glycerophosphate where it might be assumed that substrate formation is fast enough so that it is no longer rate-controlling.

In addition to the cases above, Ross and his associates[3] have reported variation in pH optima for alkaline phosphatase activity with substrate concentration for phenyl disodium phosphate, adenylic acid, fructose-1,6-diphosphate, fructose-6-phosphate, and ribonucleic acid. The same analysis may be applied to these; and the "true" substrate may be selected as that which demonstrates an optimum concentration invariant with pH change. It was found that the optimum concentration was constant when the un-ionized acid form was picked as the substrate for all cases but ribonucleic acid, where the monovalent ion showed invariance.

SUMMARY

In the enzymic hydrolysis of β-glycerophosphate, the species on which the alkaline phosphatase acts is the un-ionized acid. Supporting evidence for this includes: (a) ^{32}P specific activity of the hydrolysis product corresponding to the specific activity of the un-ionized acid in the reaction medium; (b) an increase in reactivity with enzyme dilution which disappears with lower pH; and (c) experimental data from the literature.

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FLUORESCENCE SPECTROPHOTOMETRY OF REDUCED PHOSPHOPYRIDINE NUCLEOTIDE IN INTACT CELLS IN THE NEAR-ULTRAVIOLET AND VISIBLE REGION

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INTRODUCTION

It is necessary to study enzymic systems *in vivo* in order to verify or correct conclusions obtained with *in vitro* systems. Such a study may also reveal unknown reactions and mechanisms.

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