THE COMPLEX NATURE OF ALKALINE PHOSPHATASE*

by

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It has been reported repeatedly[1,2,3,4] that the activity of alkaline phosphatase, if the enzyme acts in the absence of Mg, rapidly declines, much more than can be accounted for by a first order reaction in respect to substrate concentration, by the inhibitory effect of products of hydrolysis or by a reverse reaction, and can be explained only by inactivation of the enzyme proper. However, if hydrolysis is followed over a sufficiently long period of time, it becomes obvious that this inactivation follows a very unusual pattern. Since no studies could be found in which the rate of hydrolysis was analyzed beyond a period of 5 hours, it was decided to investigate the inactivation of alkaline phosphatase in the course of much longer periods of hydrolysis, in the hope that clues as to the mechanism of inactivation will be found. In the course of these experiments many other properties of alkaline phosphatase, on which data are already available, were re-examined.

EXPERIMENTAL

The enzyme

Alkaline phosphatase was prepared mainly from dog and calf intestine by fractional precipitation with alcohol. The mucosa of the intestine (preferably proximal jejunum) was scraped off, ground with sand and a few crystals of camphor, and extracted with 2 to 3 volumes of water at room temperature for 2 days. After this time it was strained through cheesecloth and placed in the refrigerator. When thoroughly chilled, 5 ml of a *M* acetate buffer of p_H 5 and 90 ml of ice-cold 95% alcohol was added to each 100 ml of the crude filtrate (final concentration of alcohol, 43 to 45%). After a few hours' standing in the ice box, about 5 g of Hyflo Supercel per 100 ml was stirred into the mixture, which was then filtered under suction. The clear amber filtrate was chilled once more, and 60 to 80 ml of ice-cold 95% alcohol was added to each 100 ml of the filtrate (final concentration of alcohol, 60 to 65%). In the ice box a flocculent white precipitate settled out which was centrifuged off, dissolved in about 1/20 of the original volume of distilled water. A somewhat turbid solution was obtained which was subsequently dialyzed for 3 to 6 (in some cases up to 30) days against repeated changes of large volumes of distilled water in the refrigerator. The solution was then filtered through a Seitz filter. The crystal clear, colorless or slightly amber fluid represented the final product; it could be stored in the refrigerator, with the addition of some camphor, for several years, without any significant loss in activity, although some properties of the enzyme, to be mentioned later, did change on storage. The yield was about 80% of that of the first crude extract.

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In a few cases it was attempted to effect further purification by repeating the fractional precipitation procedure but increase in specific activity (activity/mg of N), as a rule, was only minimal. The maximum specific activity obtained routinely in this fashion was about 35.000 to 40.000 μM units[5] (about 1300 BODANSKY units) under optimal conditions (see below). This compares favorably with activities of the best preparations on record[6,7].

Properties of the enzyme

Activity of the enzyme under various conditions will be stated in terms of μg of P or phenol liberated in 1 hour at 37° C. This measure of activity (proportional to the absolute amount of substrate hydrolyzed per unit of time) was chosen in preference to others (percentage of substrate hydrolyzed in a specific length of time; Q values) because values proportional to the amount of enzyme can be reproduced easily and obtained directly within a fairly wide range of substrate concentration as long as the enzyme remains saturated, while Q values, as a rule, must be determined graphically. In most experiments the concentration of the substrate was 0.005 M, that of the buffer, 0.05 M; when Mg was used as an activator, its concentration was 0.05 M. The activity of the enzyme was determined by adding 1 ml of enzyme dilution to 10 ml of buffered substrate and incubating the mixture for 1 hour at 37° C. Phosphate was determined by one of the molybdenum blue methods[8]; phenol either with the FOLIN-CIOCALTEU reagent or by the azo-dye method[5].

1. *Effect of* p_H. Since it was found that the nature of the buffer had an effect on activity, in the first experiments a single buffer was used over the entire range of p_H investigated. 1,3-diaminobutane* was found to be an efficient buffer between p_H 8 and 11.

TABLE I

1,3-DIAMINOBUTANE BUFFER

10 ml of a 0.5 M solution (51.25 ml/l) of diaminobutane + x ml of 0.5 N HCl diluted to 100 ml p_H values at 26° C

x	p_H	x	p_H
1	11.1	10	9.3
2	10.83	11	9.08
3	10.7	12	8.85
4	10.5	13	8.73
5	10.32	14	8.53
6	10.15	15	8.33
7	9.97	16	8.1
8	9.75	17	7.7
9	9.5		

As seen from Table II, the p_H optimum of the enzyme depends both on the substrate and on the presence or absence of Mg; the presence of Mg lowers the optimum by about 0.3 p_H unit. This point is important in studies on the activation by Mg; also, it is clearly shown that the extent of Mg activation greatly depends on p_H.

2. *Effect of the buffer.* The nature of the buffer also influences enzymatic activity, and the effect of the buffer may vary in the case of different substrates. Table III shows the effect of various buffers at p_H 9.3. Na barbital, the buffer most often used in the

* Obtainable from Sharples Chemicals, Inc., Philadelphia 9, Pa.

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TABLE II

PH CURVE OF ACTIVITY

Enzyme R; 1 ml of a 1:100 dilution to 10 ml of 0.005 *M* substrate in 0.05 *M* diaminobutane buffer. Temperature 37° C

Substrate	Glycerophosphate						Phenylphosphate					
pH	8.3	8.8	9.3	9.55	9.8	10.2	8.8	9.3	9.55	9.8	10.2	10.5
μg of P/h without Mg	11.5	20	29.5	32.5	29.5	15	13	30.5	41.5	50.5	53.5	41.5
μg of P/h 0.005 *M* Mg	22.5	40	48	45.4	35.5	9.5	26	49	66	68.5	43	24

past with alkaline phosphatase, is not included because its buffering capacity at p_H 9.3 (1.5 units above its pK) is poor. The different effect especially of borate buffer on the hydrolysis of glycerophosphate and phenylphosphate is shown distinctly. In addition, in a few experiments it was found that the buffer also influences the p_H optimum, but this point was not investigated in detail. In all the experiments, to be described later, the buffer used was 2-amino-2-methyl-1,3-propanediol[9] (Commercial Solvents Corporation), referred to henceforth as ammediol.

TABLE III

THE EFFECT OF VARIOUS BUFFERS

Enzyme K; 1 ml of a 1:1000 dilution to 10 ml of 0.005 *M* substrate in 0.05 *M* buffer at p_H 9.3
A ammediol; B borate; C diaminobutane; D carbonate; E piperazine

Substrate	Glycerophosphate					Phenylphosphate				
Buffer	A	B	C	D	E	A	B	C	D	E
μg of P/h without Mg	14	12	14	21	15	14	31	14.5	23	13.5
μg of P/h 0.005 *M* Mg	135	87.5	167.5	122.5	150	177.5	208	144	162	118

3. *Effect of the nature of substrate.* The rates of hydrolysis for a few selected substrates are given in Table IV.

TABLE IV

RATES OF HYDROLYSIS OF VARIOUS SUBSTRATES

Enzyme o; 1 ml of a 1:250 dilution to 10 ml of 0.005 *M* substrate in 0.05 *M* ammediol buffer at p_H 9.3

Substrate	Glycero PO_4	Phenyl PO_4	Phenolphth. PO_4	Hexosedi PO_4	Aminoethyl PO_4
μg of P/h without Mg	33	32	13	46	33
μg of P/h 0.005 *M* Mg	65	62	31	90	34

4. *Effect of substrate concentration.* The effect of substrate concentration in the case of glycerophosphate and phenylphosphate at p_H 9.3 is shown in Table V. From the data

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presented it is evident that high concentrations of phenylphosphate inhibit the enzyme somewhat. The MICHAELIS-MENTEN constant for both substrates is around $4 \cdot 10^{-4}$; the presence of Mg seems to depress it slightly in the case of glycerophosphate and to have no effect on it in the case of phenylphosphate.

TABLE V

EFFECT OF SUBSTRATE CONCENTRATION

Enzyme K; 1 ml of a 1:250 (in the presence of Mg, 1:1250) dilution to 10 ml of substrate of varying molarity in 0.05 *M* ammediol buffer at pH 9.3

Substrate	Glycerophosphate									Phenylphosphate								
q substrate	1.7	2.0	2.3	2.6	2.9	3.2	3.5	3.8	4.1	1.7	2.0	2.3	2.6	2.9	3.2	3.5	3.8	4.1
μg of P/h without Mg	30	28	30	28.5	27	21	15	12.5	8	25	28.5	31	35	35	33	22.5	15	7
q ^{K}MM	3.5									3.7								
μg of P/h 0.005 *M* Mg	55	52.5	48	40	32.5	22.5	14	11	6.5	52.5	60	63	64.5	66	60.5	47.5	27.5	17
q ^{K}MM	3.15									3.65								

5. *Effect of enzyme concentration.* The rate of hydrolysis under the conditions of these experiments is proportional to the amount of enzyme only if hydrolysis does not exceed about 50 μg of P per sample. Above this amount of enzyme the relative rate of hydrolysis gradually declines. In case of readings above 50 μg the actual rate can be read from the empirical curve given in Fig. 1.

6. *Effect of temperature.* Rates of hydrolysis were determined at 27, 37 and 47° C. Within this range, the effect of temperature appears to be minimal in the absence of Mg, and slight in its presence.

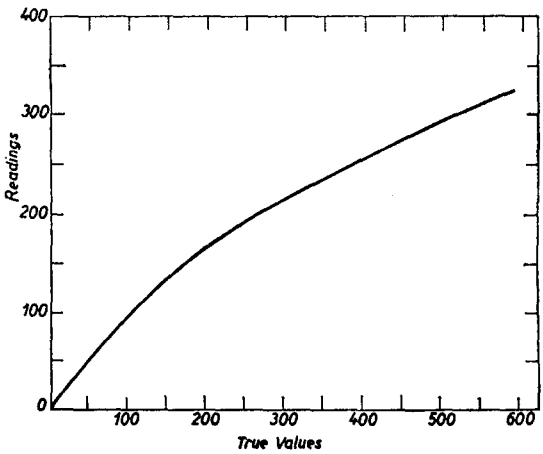

Fig. 1. Correction for high readings μg of P/h. Volume, 11 ml; substrate concentration, 0.005 *M*; buffer, ammediol 0.05 *M*, pH 9.3

7. *Activators and inhibitors.* The effects of Mg, Mn, Ca, Be and alanine, besides those of products of hydrolysis, were investigated. Mg activated all preparations but to a varying extent. Fresh enzymes were activated by Mg 30 to 60%, whereas old ones, stored in the refrigerator for 1 to 9 years, 200 to 1000%. The activating effect also depended on both the substrate and the buffer (see Tables III and IV). Mn activated to about the same extent as Mg. Ca also had some activating effect, although much less marked than Mg (fresh enzyme, about 15%; old ones, 150 to 200%). The combined effect of Ca and Mg was more than the activation by Mg alone but less than the sum

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of the two effects. Be was found to be an extremely powerful inhibitor; even $6 \cdot 10^{-7}$ *M* caused an inhibition of about 40%; $4 \cdot 10^{-5}$ *M* an inhibition of about 93%; further increase in Be concentration up to $2 \cdot 10^{-3}$ *M* did not show any additional effect. The presence or absence of Mg did not affect inhibition by Be.

TABLE VI

THE EFFECT OF TEMPERATURE

Enzyme H: 1ml of a 1:7500 dilution to 10 ml of 0.005 *M* glycerophosphate in 0.05 *M* ammediol buffer at p_H 9.3.

Temperature	27° C	37° C	47° C
μg of P/h without Mg	42.5	51	50
μg of P/h 0.005 *M* Mg	71	93	118

Since it has been shown by ROCHE and associates[10] that activation by alanine has a period of latency, its effect was studied by first incubating the enzyme for 2 hours at 37° C, in the presence of 0.05 *M* alanine buffered to p_H 9.3 but in the absence of substrate. This pre-incubated enzyme was then added to the substrate containing the same concentration of alanine. Fresh enzymes were found to be activated by alanine only to the extent of 20 to 30%, while old enzymes were activated 100 to 400%. In the case of fresh enzymes, the alanine-treated enzyme was further activated by Mg, and activity in the presence of both alanine and Mg was higher than in the presence of Mg alone; in the case of old preparations the activation of the alanine-treated enzyme by Mg was considerably less than that of the plain enzyme (Table VII).

TABLE VII

ACTIVATION BY Mg, Ca AND ALANINE

Enzyme R, 1:100 dilution, and enzyme K, 1:1000 dilution; 1 ml of enzyme to 10 ml of 0.005 *M* glycerophosphate in 0.05 *M* ammediol buffer at p_H 9.3. Concentration of additions, 0.05 *M*. In case of alanine, enzyme pre-incubated for 2 hours with the same concentration of alanine at p_H 9.3

Addition	None	Ca	Mg	Alanine	Mg + alanine
Enzyme R μg of P/h	28	33	47	35	59
Enzyme K μg of P/h	13.5	36	125	53	70

As for the products of hydrolysis, glycerol did not inhibit even at concentrations as high as 0.5 *M*. Phenol at 0.001 *M* caused an inhibition of about 8%. The inhibitory effect of phosphate was studied by using phenylphosphate as a substrate and determining the amount of phenol liberated, in order to avoid inaccuracy resulting from the determination of relatively small amounts of enzymatically liberated P in the presence of large amounts of free P added before incubation. The effect of phosphate was moderate and depended on the ratio of free P/substrate concentration. Inhibition did not exceed 6% below ratios of 1:10 and reached 40% at a ratio of 1:2.

8. *The course of hydrolysis in time.* This was followed for 48, occasionally for 72 or

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96 hours. The substrate concentration was raised to 0.01 *M*. The amount of enzyme was so chosen that not more than 10% of the substrate was hydrolyzed by the end of 48 hours. This arrangement necessitated the use of smaller amounts of enzyme in the presence of Mg than in its absence; however, in Fig. 2 and Table VIII the values given for the Mg-activated enzyme have been corrected for the same amount of enzyme as used in the absence of Mg. The interesting observation was made that while in the presence of Mg hydrolysis follows a fairly uniform pattern throughout, in its absence there is a progressive inactivation up to about 12 hours, and from then on the reaction continues linearly, at a uniform rate, until the end of the experiment. The course of hydrolysis in the presence of Mg is compatible with the assumption that there is a slow inactivation of the enzyme which varies between 3 and 17% per hour with different preparations. These rates are initial and apply to the first 2 hours only; on continued incubation the inactivation rate gradually drops to less than 2% per hour (by the end of 36 hours). In a number of experiments, when high phosphate concentrations were reached, a Mg-amine phosphate precipitated, which partly stuck to the walls of the flask, making exact determinations of P in subsequent samples illusory.

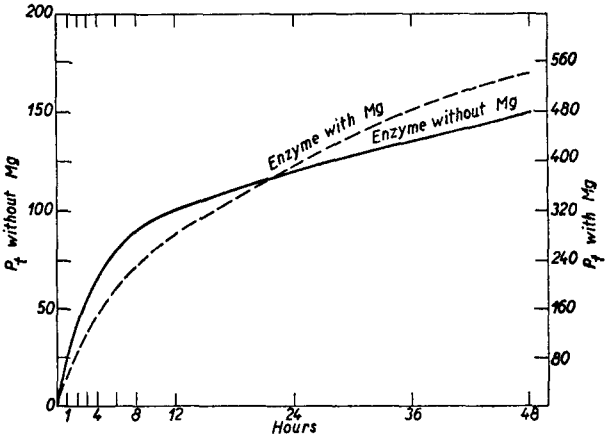

Fig. 2. Course of hydrolysis in time
Enzyme B, 1:1000 dilution; 4 ml to 250 ml of 0.01 *M* glycerophosphate in 0.05 *M* ammediol buffer at pH 9.3

TABLE VIII

COURSE OF HYDROLYSIS IN TIME

Enzyme B, 1:1000 dilution; 4 ml to 250 ml of 0.01 *M* glycerophosphate in 0.05 *M* ammediol buffer at pH 9.3

a. Plain enzyme

Time (hours)	1	2	3	4	6	8	12	24	36	48
P_t Exper. values	26.5	43	56	66	81	90.5	100	118.5	134	149
Theor. values if $P_y = 1.4$; $\lambda = 0.26$	23.2	42	55	66	80	88	100	117	134	151

b. Alanine-activated enzyme
(Not shown in Fig. 2)

P_t Exper. values	39.5	66	85	99	119	130.5	138	146	150	154
Theor. values if $P_y = 0.33$; $\lambda = 0.275$	38.3	65.7	85	99	118	127	139	146	150	154

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On the other hand, the time-curve of hydrolysis in the absence of Mg is strongly suggestive of the superimposition of two independent curves of activity, one of which is due to an enzyme rapidly inactivated and completely lost by the end of 24 hours, the other one to an enzyme the action of which continues in a linear fashion from the beginning to the end of the experiment (Fig. 2). The apparent ratios between these two hypothetical enzymes varied greatly with different preparations. As a rule, fresh preparations showed a preponderance of the rapidly inactivated fraction (to be called, for the sake of brevity, fraction X), whereas preparations preserved for long periods of time contained more of the fraction which is not inactivated (fraction Y).

In Table VIII an attempt is made to fit the experimental data into a theoretical scheme in which it is assumed that the two fractions are actually present.

According to this scheme the total amount of P liberated by the combined action of the two fractions by the end of t hours (P_t) would be:

$$P_t = P_x \frac{1-(1-\lambda)^t}{\lambda} + tP_y$$

where P_x stands for the amount of P liberated by fraction X at the end of the first hour, λ for the inactivation fraction (per hour) of fraction X, and P_y for the amount of P liberated per hour by fraction Y. P_x is calculated by subtracting P_y from P_1 (total P liberated by the end of the first hour). For λ the value of 0.25 to 0.30 is obtained. As shown in the table, experimental data agree remarkably well with those of the theoretical scheme, except for the first hour, when in some cases a slight discrepancy is observed. This could possibly be accounted for by the presence of a small amount of a third component which is inactivated even more rapidly than fraction X.

Calculations on the basis of this scheme were applied to a number of enzyme preparations, both fresh and old, and a good agreement between experimental data and the theory was obtained in all cases. The values of λ were within a narrow range for all preparations although the ratios Y/X varied widely. Fresh preparations invariably contained a much higher percentage of fraction X than old ones; the relative amount of Y seemed to increase with age of the preparation. The results obtained seemed to justify the conclusion that alkaline phosphatase of the intestine is actually made up of two main components, with the possible admixture of a small amount of a third component.

Attempts at isolating fraction Y were successful. Hydrolysis was allowed to proceed for 24 hours, at which time the enzyme-substrate mixture was dialyzed against large amounts of distilled water in the ice box until inorganic phosphate was completely removed (usually 3 to 4 days). When fresh buffered substrate was added to this dialyzed enzyme, hydrolysis was resumed at a rate practically identical with that observed during the last hours preceding dialysis, and this rate was maintained for several days, even in the absence of Mg. The activation by Mg was 500 to 900%, the same as observed in regular experiments by the end of 24 hours.

These interesting initial observations gave the impetus for a more detailed study of the mechanisms of spontaneous inactivation, of activation by Mg and alanine, and also of a possible interdependence between the various forms of the enzyme.

Since, on account of the relatively small increment in P against a high background of pre-existing inorganic phosphate, exact determination of hourly changes in the rate of activity became increasingly difficult as incubation was extended beyond a few hours,

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TABLE IX

INACTIVATION OF THE ENZYME IN THE COURSE OF HYDROLYSIS

Enzyme B, 1:250 dilution; 4 ml to 250 ml of 0.01 *M* glycerophosphate in 0.05 *M* ammediol. At intervals stated, 5 ml samples transferred to test tubes containing 1 ml of 0.03 *M* phenylphosphate, with or without 0.005 *M* $MgCl_2$ added. Phenol determined 45 minutes later. On account of rapid inactivation of fraction *X* during the first few hours, values were graphically adjusted by plotting the 1 hour value at 0.5 hour, the 2 hour value at 1.5 hours, etc., up to 8 hours, and by reading the corrected values at 1, 2, etc., from the graph

(Ph_t and Ph_y = values corresponding to P_t and P_y in terms of phenol)

Time (hours)	0	1	2	3	5	7	11	24	36	48
Plain enzyme; no Mg										
Exper. values	98	84	76	71	61.5	56.5	52.5	51	50	51
Ph_t–Ph_y	47.5	33.5	25.5	20.5	11	6	1.5	0.5	—	—
Graphically corrected	39	29	23	18.5	10.5	6	1.5	0.5	—	—
Theor. values if $Ph_y = 50.5$; $\lambda = 0.215$	37	29	23	18.5	10.8	7.5	3.1	0.2	—	—
Plain enzyme with Mg										
Exper values	254	175	163	154	144	137	132	128	129	128
Ph_t–Ph_y	126	48	35	26	16	9	4	—	—	—
Graphically corrected	67	40	30	23	14	8	4	—	—	—
Theor. values if $Ph_y = 128$; $\lambda = 0.25$	52 5	40	31	23	13 5	7 5	2 5	—	—	—
Alanine-activated enzyme; no Mg										
Exper values	150	108	86	68.5	50	38.5	27	19.5	16	16
Ph_t–Ph_y	134	92	70	52.5	34	22.5	11	3.5	—	—
Graphically corrected	105	80	60	46.5	30	20	11	3.5	—	—
Theor. values if $Ph_y = 16$; $\lambda = 0.23$	103	79	60.5	46.5	27.5	15.8	5.6	0.2	—	—
Alanine-activated enzyme with Mg										
Exper. values	205	150	125	102.5	75	57.5	44	33	27.5	28
Ph_t–Ph_y	197	122	97	74.5	47	29.5	16	5	—	—
Graphically corrected	135	107	84	66	42	27.5	16	5	—	—
Theor. values if $Ph_y = 28$; $\lambda = 0.215$	138	107	84	66	40	24.5	10	0.8	—	—

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the following technique was devised. The enzyme was incubated in a flask with 250 ml of buffered 0.005 or 0.01 *M* glycerophosphate, and at intervals 5 ml samples were transferred into test tubes containing 1 ml of 0.03 *M* phenylphosphate buffered to the same p_H, with or without 0.005 *M* $MgCl_2$ added. The tubes were incubated, and phenol was determined 30 to 60 minutes later. In this way, even a low enzymatic activity could be determined accurately on account of the zero background.

The results of these experiments are given in Tables IX and X.

Table IX clearly shows that, in the absence of Mg, inactivation of the enzyme takes place according to the same trend as shown in Table VIII. In this case the *Y* fraction (constant after 12 hours) represents about 51% of the total activity, which is very much higher than the value given in Table VIII. The same phenomenon was subsequently found in similar experiments with other enzyme preparations; its cause is not clear. The λ of fraction *X* is about 0.22, with the exception of the first hour when activity found experimentally is about 8% in excess of the value obtained by extrapolation from later values. In the presence of Mg, the same phenomenon is greatly accentuated, the first hour experimental value being 100% (with other preparations, 0 to 180%) in excess of the extrapolated value. Activation ratio by Mg of fraction *Y* is 2.5, with other preparations up to 3.5; that of fraction *X*, 1.5. If the excess activity during the first hour is attributed to a third component (to be designated fraction *Z*), its activation ratio by Mg is found to be 20 or over.

The actual existence of fraction *Z* could be proven by the following experiments:

1. 0.005 *M* Mg was added at the start to the flask containing the glycerophosphate-enzyme mixture, and 5 ml samples were transferred to test tubes containing phenylphosphate. Under such conditions (Mg present from the start), the activity of the enzyme showed a slow gradual decline (inactivation fraction about 0.03 per hour) for over 24 hours, without any marked difference between the 1 hour and 2 hour values.

2. Experiment given in Table IX was slightly modified by using a relatively large amount of enzyme and by incubating samples transferred at short intervals for only 10 minutes. Results given in Table X conclusively show that a fraction of the enzyme is inactivated at the rate of about 50% per 10 minutes, and that this fraction is practically gone by the end of 75 minutes. The persistence of fraction *Z* under the conditions of the experiment given in Table VIII explains the high ratios of activation by Mg observed especially between 12 and 36 hours (500 to 900%, depending on the enzyme preparation). Such high ratios are never found in experiments where Mg is not added at the start.

TABLE X

INACTIVATION OF FRACTION *Z*

Enzyme K, 1:25 dilution; 1 ml to 120 ml 0.01 *M* glycerophosphate in 0.05 *M* ammediol at p_H 9.3. Five ml samples transferred to test tubes containing 1 ml of 0.03 *M* phenylphosphate at intervals stated; phenol determined 10 minutes later.

Time (minutes)	0	10	20	30	40	50	75
μg of phenol in 10 minutes	51	39	29.5	24.5	22.5	20	19.5

The effect of alanine as used in the experiment resulted in the transformation of a certain percentage of the enzyme from fraction *Y* into fraction *X*. The activation ratio

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of fraction X by Mg remained around 1.5; that of fraction Y was reduced to about the same level. Fraction Z has disappeared completely. If it is assumed that fraction X is formed at the expense of fraction Y by some kind of loose combination with alanine, the number of enzyme molecules remaining unchanged; calculations show that the intrinsic activity of fraction X is 2 to 5 times that of fraction Y, depending on the individual enzyme preparation.

It must be remarked that in no case has it been possible to transform fraction Y by alanine completely into fraction X; about 7 to 12% of the enzyme always remained in form Y. Another point of importance is that alanine is by no means the only substance causing this transformation; ammonium salts and a number of amines, ammediol included, were found to exhibit an essentially similar but, in most cases, a slower and less marked effect.

DISCUSSION

The kinetics of alkaline phosphatase, its activation and inhibition by various substances, has been the subject of such a large number of publications that giving a full bibliography of the subject appears impractical. Only papers with a direct bearing on the results presented will be referred to.

The MICHAELIS-MENTEN constant found in the present experiments is almost identical with that reported by NEUMANN[11] but much lower than the values found by ROCHE AND SARLES[12] or by AEBI[13].

The effect of temperature was found to be distinctly less marked than reported by BODANSKY[14].

Among the activators studied, Mg and Mn were found to be powerful activators by all workers; the activating effect of Ca is mentioned by THOAI and co-workers[15].

The peculiar curve of the course of hydrolysis (progressive decline in rate for a number of hours, followed by a continued constant rate of activity) has been observed by HOVE, ELVEHJEM, AND HART[16] (in fact, these authors give curves identical with that given in Fig. 2) but not subjected to mathematical analysis. That inactivation of the enzyme cannot be due to decrease in substrate concentration or to the accumulation of inhibitory products of hydrolysis is obvious since the rate of inactivation is highest in the early stages of hydrolysis and becomes less later. It can be shown that the enzyme is not inactivated by the buffer (at least, not by the one used in these experiments) but only by action on a substrate. The data presented indicate that the enzyme is composed of three fractions, two of which are inactivated rather rapidly (half life of fraction Z, about 10 minutes; that of fraction X, about 2½ hours) in the absence of Mg while the third component (Y) is quite stable. The inactivating effect of glycerophosphate is more marked than that of phenylphosphate, as could be shown by experiments of the type presented in Fig. 2, using phenylphosphate as a substrate; however, the actual rate of inactivation was not computed.

The effect of alanine and of some other amines is to convert part of the stable fraction Y into the unstable, but more active, fraction X; hence the activating effect. In the case of three enzyme preparations with very high Y/X ratios (over 8:1), activation by the buffer alone was so high as to outstrip the rate of spontaneous inactivation, with the net result of increasing rates of activity for 3 to 5 hours. In the presence of alanine (but without previous incubation of the enzyme with it) this effect was even more

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marked. In several other preparations slow activation by buffer or alanine partly or completely counterbalanced inactivation for as long as 3 hours. This phenomenon must have led BODANSKY[17,18] to assume that alanine accelerates hydrolysis by slowing down the rate of spontaneous inactivation.

Activation by Mg is due to two mechanisms: first, the prevention of inactivation, especially fo fraction *Z*, and second, increase of the efficiency of the enzyme. The three fractions possess their specific ratios ot activation by Mg.

SUMMARY

Alkaline phosphatase of the intestine consists of three fractions present in variable proportions in different preparations. Two fractions are rapidly inactivated while acting on the substrate in the absence of Mg; the third is stable for several days. The three fractions have different and specific rates of activation by Mg. The effect of alanine consists in transforming part of the stable fraction into one of the unstable ones.

RÉSUMÉ

La phosphatase alcaline de l'intestin est composée de trois fractions, de proportions variables. Deux fractions sont rapidement inactivées au cours de l'hydrolyse en absence d'ion Mg; la troisième est stable pendant plusieurs jours. Les trois fractions ont des taux d'activation (par Mg) différents et spécifiques. L'alanine transforme partiellement la fraction stable en une des fractions instables.

ZUSAMMENFASSUNG

Die alkalische Darm-Phosphatase besteht aus drei Fraktionen, welche in verschiedenen Präparaten in wechselnden Verhältnissen vorkommen. Zwei Fraktionen werden bei der Hydrolyse in Abwesenheit von Mg rasch inaktiviert; die dritte Fraktion ist mehrere Tage haltbar. Die Geschwindigkeit der Aktivierung durch Mg ist für die drei Fraktionen verschieden und spezifisch. Die Wirkung von Alanin besteht darin, dass ein Teil der beständigen Fraktion in eine der unbeständigen umgewandelt wird.

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