DEPENDENCE OF THE p_H-OPTIMUM OF THE PHOSPHOMONOESTERASE I ON THE SUBSTRATE CONCENTRATION AND ON INHIBITORS AND ACTIVATORS

by

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INTRODUCTION

In previous publications we pointed to the bearing of p_H activity curves on the determination of phosphatase in extracts of organs and serum[1, 2, 3]. We demonstrated that small differences in p_H might cause great differences in activity. Hence for the accurate determination of phosphatase it is better to establish activity curves than to content oneself with one determination at optimum p_H. Moreover these curves give an indication of the presence of isodynamic phosphatases. For the various organs we found specific activity curves. Fig. 1 shows the p_H curve for the kidney, Fig. 2 that for the intestine of the rat.

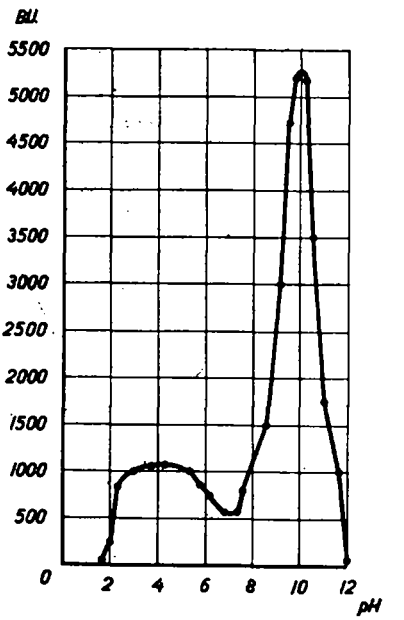

Fig. 1. p_H-activity-curve of the phosphatase(s) of the kidney of the rat. (B.U. = BODANSKY Unit)

It is notable that the optimum p_H for phosphomonoesterase I (the so-called alkaline phosphatase) is so high. Although we are rather in the dark regarding the value of the p_H in the living cell or part of the cell, yet a p_H of 9.8 must be considered to be unphysiological. Further it is remarkable that the ferment deploys so little activity at more physiological p_H values.

The presence of accompanying substances can influence the optimum p_H of an enzyme reaction. This is the reason that identical p_H optima are not always found for one and the same ferment in unpurified extracts of various organs. For various organs these differences are very slight as far as the alkaline phosphatase is concerned. Phosphomonoesterase I in intestine extract has its maximum action at $p_H = 9.6$; in kidney extract at $p_H = 9.9$. FRANKENTHAL[4] has found that an acid phosphatase from sarcomas shows another optimum when Mg^{++} and Mn^{++} ions are present than when these ions are absent. We have now investigated the influence of various substances on the alkaline phosphatase.

When ferments are purified, which evidently causes many accompanying substances to be removed, it sometimes happens that the optimal action of the purified

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enzyme is found at another p_H than that of the initial material. FROMAGEOT[5] describes this phenomenon for glucosulphatase. We have subjected phosphomonoesterase I to a purification, examining whether also in this case the p_H optimum changed.

The p_H optimum is dependent on the substrate used. DELORY AND KING[6] stated that the optimum p_H for phosphomonoesterase I is higher for phosphoric acid esters with a low degree of dissociation than for esters with a higher degree of dissociation.

With one particular substrate the optimum p_H of a ferment reaction sometimes depends on the substrate concentration.

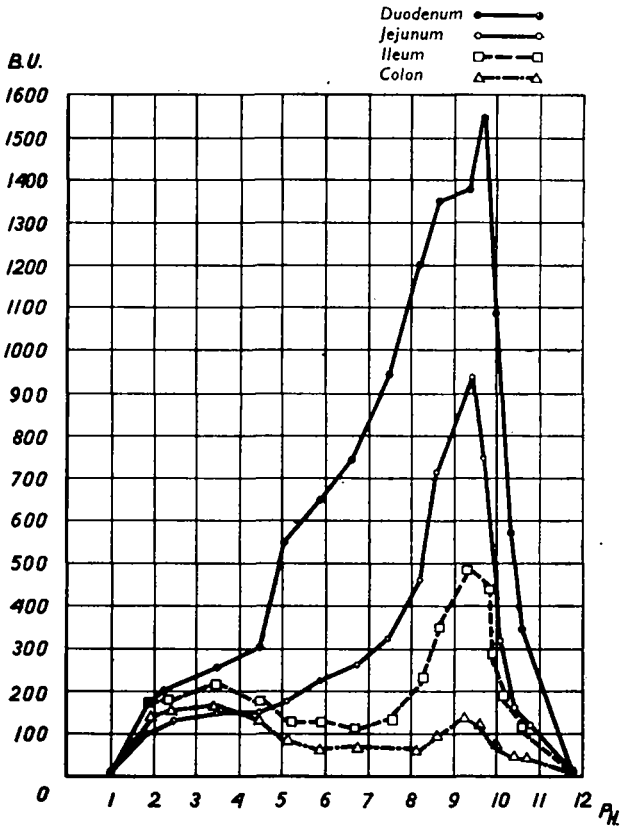

Fig. 2. p_H activity-curve of the phosphatase(s) of the intestine of the rat

It is assumed that the enzyme reaction passes through various phases. First there is a combination of enzyme and substrate, forming a complex.

$$E + S \rightarrow ES \qquad (1)$$

Then there is a disintegration:

$$ES \rightarrow E + p_1 + p_2 \qquad (2)$$

Both reactions depend to a different extent on the p_H. Finally the equilibrium

$$\text{Holo E} \rightleftarrows \text{Apo E} + \text{Co E} \qquad (3)$$

is also in many cases influenced by the p_H.

VAN SLYKE[7] assumes that with a low concentration of the substrate reaction 1

predominates. With a high concentration of the substrate the enzyme is fairly constantly combined with substrate molecules and reaction 2 predominates. For urease and arginase the optimum p_H was shown to be dependent on the concentration of the substrate[7]. We have examined whether with the alkaline phosphatase the optimum also varies with the concentration of the substrate.

EXPERIMENTAL PART

I. METHODS OF PHOSPHATASE DETERMINATION[1, 2, 3]

For these we refer to our previous publications.
Na-β-glycerophosphate served as a substrate.

II. INFLUENCE OF ACCOMPANYING SUBSTANCES IN THE EXTRACTS

For the preparation of extracts of organs, as this was done for determining activity curves[1], the organ is ground up with powdered quartz, consequently the environment in which the phosphatase reaction takes place may be quite different from that of the cells which contain the phosphatase in the living organ. The low-molecular substances are removed from the extract by dialysis. We could confirm the observation described by ALBERS[8, 9] that in the kidney extract a substance occurs which inhibits the phosphatase. In intestine extracts activity was the same before and after dialysis. The optimum p_H did not vary. The extracts were dialysed in a collodion bag for 24 hours against running tapwater and afterwards once more against distilled water till no more PO_4''' ions were demonstrably present.

TABLE I

Organ extract	P.U.*	p_H optimum
Kidney before dialysis	19	9.9
Kidney after dialysis	25	9.9
Intestine before dialysis	15	9.6
Intestine after dialysis	16	9.6

III. PURIFICATION OF PHOSPHOMONOESTERASE I

The organ extract was brought to $p_H = 4.5$ with the aid of 1 n. acetic acid. Per ml of extract 25 mg kaolin was added and the mixture shaken. After 15 minutes centrifugation took place and the centrifugate was poured off. The precipitate was eluted with 33% ethanol, which had been brought to $p_H = 9$ with ammonia. After centrifugation the p_H of the centrifugate was reduced to 7 and ethanol was added drop by drop, the centrifugate being stirred continuously till the ethanol concentration amounted to 70%. The result was a precipitate from which phosphomonoesterase I could be extracted at p_H 8.7. A large proportion, however, remains insoluble (we found that when precipitation takes place at −10° C readily soluble preparations are obtained.**

* P.U. = Phosphatase-Unit (ALBERS[8])
** To be published shortly

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The solution of phosphomonoesterase I obtained by this method no longer contained any other phosphomonoesterase, on the other hand it still contained pyrophosphatase. By bringing the liquid to $p_H = 5.0$ and adding a solution of diphenylmethanol in 96% ethanol the diphenylmethanol onto which the ferments had been adsorbed was precipitated. After centrifugation the phosphomonoesterase I could be eluted at $p_H = 9.0$ and so freed from pyrophosphatase I. With this purified preparation a p_H activity curve was determined (Fig. 3). It showed that the optimum had not shifted.

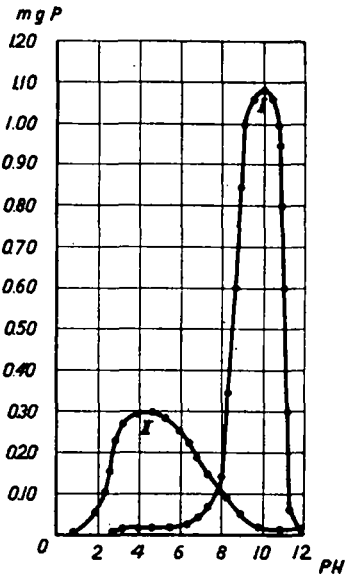

Fig. 3. Separation of phosphomonoesterase I and phosphomonesterase II of the kidney of the rat

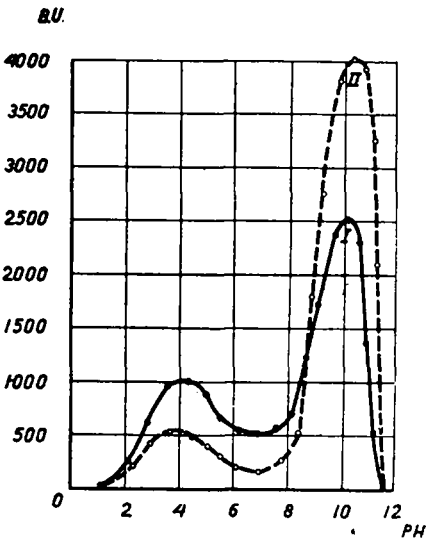

Fig. 4. Kidney of the rat; I p_H-activity-curve of the phosphatase without Mg^{++}; II p_H-activity-curve of the phosphatase with Mg^{++}

IV. INFLUENCE OF VARIOUS IONS

Of a number of cations and anions we tested the influence on the activity and on the p_H optimum of phosphomonoesterase I. The inhibitory anions such as CN′, F′, CO_3', oxalate and citrate ions hardly shift the optimum. No more do the activating No'_3 and tartrate ions.

The activating cations Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} and the inhibitory Sr^{++}, Ba^{++}, Al^{+++}, Ag^{+}, Pb^{++}, Cu^{++} and Hg^{++} produce no change in the optimum p_H. Mg^{++} is the most strongly activating; Fig. 4 shows an activity curve with and without the addition of $MgSO_4$ (10^{-3} mol).

V. INFLUENCE OF THE SUBSTRATE CONCENTRATION

Fig. 5 gives a curve showing the influence of the substrate concentration on the liberation of phosphate. The non-enzymatic hydrolysis of β-glycerophosphoric acid presents a reaction of the first order.

The curve showing the influence of the substrate concentration on the enzymatic

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hydrolysis consists of a linear part (a) which then curves (b), following by a horizontal part (c), while the end dips (d). In part a the reaction is still of the first order, for which

$$-\frac{dS}{dt} = k_1 SE$$

hence $k_1 E = \frac{1}{S} \ln \frac{S_o}{S_t}$ in which S_o represents the initial substrate concentration and S_t

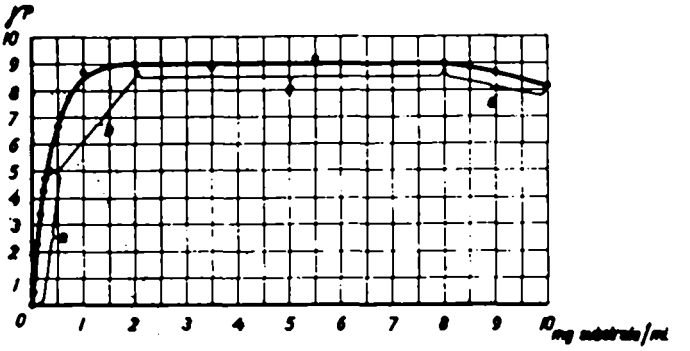

Fig. 5. Influence of the substrate concentration on the liberation of phosphate

the final substrate concentration. Table II shows that $\frac{S_o}{S_t}$ is constant for low substrate concentrations.

In all cases the reaction time was 45 minutes. For part c of the concentration curve

$$-\frac{dS}{dt} = k_2 E \text{ is found.}$$

Now the rate of reaction has become independent of the substrate concentration (reaction of zero order with respect to the substrate).

TABLE II

S_o	S_t	S_o/S_t	S_o	S_t	S_o/S_t
0.079	0.063	1.25	0.790	0.637	1.24
0.158	0.126	1.25	0.948	0.788	1.20
0.316	0.244	1.29	1.58	1.356	1.16
0.474	0.369	1.28	3.16	2.88	1.10
0.632	0.495	1.27			

Substrate: $C_3H_7O_6PNa_2.5\frac{1}{2}H_2O$
Concentrations: millimol l

As the assumption of VAN SLYKE concerning the reaction of urease and arginase reaction has proved also to apply to phosphomonoesterase I it was desirable to establish a p_H activity curve of the alkaline phosphatase with varying substrate concentration.

Fig. 6 shows p_H curve of the alkaline phosphatase of the kidney of the rat with the following substrate concentration: 4 mg/ml; 1 mg/ml; 0.2 mg/ml and 0.1 mg/ml.

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It is seen that at low substrate concentration the curve shifts to the neutral region. That this is also the case for the phosphatase of the intestine is proved by Fig. 7.

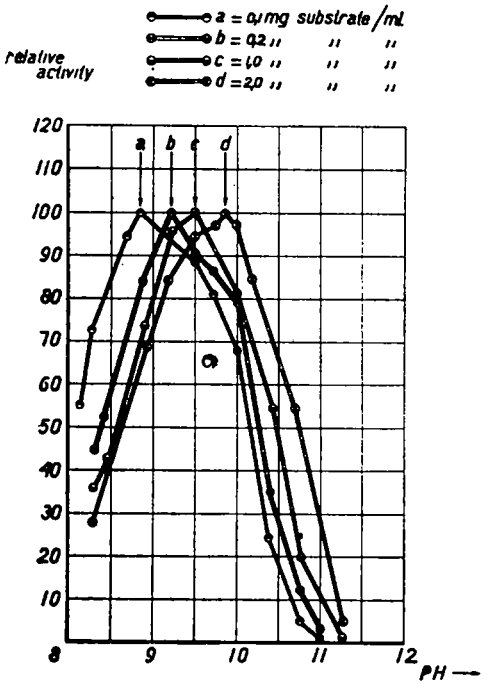

Fig. 6. Kidney of the rat p_H-activity-curves of phosphomonoesterase I with different substrate concentrations

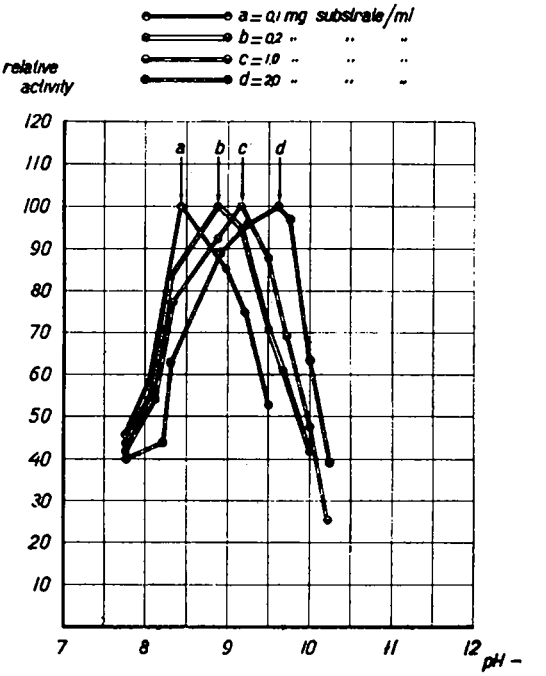

Fig. 7. Intestine of the rat p_H-activity-curves of phosphomonoesterase I with different substrate concentrations

CONCLUSION

When determining p_H activity curves for phosphomonoesterase I we always choose a substrate concentration of 4–6 mg/ml (Na-β-glycerophosphate) in the reaction liquid (buffer + enzyme solution + substrate solution). Then we are on the horizontal part of the substrate concentration curve, which is desirable for the determination; the rate of reaction has then become independent of the substrate concentration. The hydrolytic reaction turns out to pass through several phases. At lower substrate concentration the p_H curve shifts to the neutral region. In the living cell, where substances are continuously arriving or from which they are removed, the substrate concentrations will be low. In the cells the optimum p_H for phosphomonoestease I will be lower than would follow from the activity curve.

We wish to express our thanks to the ROCKEFELLER FOUNDATION for helping us to carry out this research.

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SUMMARY

1. The fact is pointed out that according to the p_H activity-curve of phosphomonoesterase I from animal organs the ferment is so highly active at an unphysiological p_H and so little active in the physiological p_H region.

2. For the alkaline phosphatase it was shown that the substrate concentration influences the position of the p_H-activity curve. At a low substrate concentration the curve shifts to the neutral region. It is expounded that in the cell the substrate concentrations will be low. For the determination of the p_H-activity curves the substrate concentration chosen is generally such as will not affect the rate of reaction. These are comparatively high concentrations, so that inferences about the optimum p_H for the phosphatase activity in the cell cannot be drawn from them.

3. By dialysis of kidney extracts it was possible to remove an inhibitory substance. Before and after dialysis the optimum is found at the same p_H.

4. Phosphomonoesterase I could be adsorbed to kaolin and could afterwards be eluted again. Subsequently the enzyme is precipitated with ethanol. The preparations purified in this way contain no other phosphomonoesterases; however, they do contain pyrophosphatase I. By adsorption to diphenylmethanol and a subsequent elution phosphomonoesterase I could be freed from pyrophosphatase. The purification did not affect the position of the optimum p_H.

5. The influence of a number of cations and anions on the situation of the optimum p_H of the alkaline phosphatase was tested. They were shown to have practically no influence.

RÉSUMÉ

1. L'auteur souligne le fait que, d'après la courbe d'activité en fonction du p_H, de la phosphomonoestérase I des organes animaux, l'enzyme est extrêmement active à des p_H non-physiologiques et, au contraire, fort peu aux p_H physiologiques.

2. La concentration du substrat influence la position du p_H optimum de la phosphatase alcaline. Pour une faible concentration en substrat, la courbe d'activité en fonction du p_H est décalée vers la neutralité. Il est vraisemblable que, dans la cellule, la concentration en substrat est faible. La détermination des courbes d'activité en fonction du p_H se fait habituellement en présence de concentrations en substrat telles qu'elles n'influencent pas la vitesse de la réaction. Ce sont là des concentrations relativement élevées; aussi ne peut-on rien en déduire quant au p_H optimum des phosphatases dans la cellule.

3. La dialyse d'extraits de rein permet d'éliminer une substance inhibitrice. Le p_H optimum reste le même avant et après dialyse.

4. La phosphomonoestérase I peut être adsorbée sur kaolin, puis en être éluée. L'enzyme est ensuite précipitée par l'éthanol. Une telle préparation ne contient pas d'autre phosphomonoestérase, mais contient encore la pyrophosphatase I. Celle-ci peut être éliminée par adsorption de la phosphomonoestérase I sur le diphényl méthanol, suivie d'une élution. Cette purification ne modifie pas le p_H optimum.

5. L'influence d'une série de cations et d'anions sur le p_H optimum de la phosphatase alcaline s'est révélée nulle.

ZUSAMMENFASSUNG

1. Die Kurve der Aktivität der Phosphomonoesterase I in Abhängigkeit vom p_H lässt erkennen, dass das Enzym bei nicht physiologischen p_H-Werten sehr aktiv, bei physiologischen Werten dagegen sehr wenig aktiv ist.

2. Für die alkalische Phosphatase hängt die Lage des p_H-Optimums von der Substratkonzentration ab; ist diese niedrig, so ist die Kurve gegen neutrale p_H-Werte hin verschoben. Wahrscheinlich ist die Substratkonzentration innerhalb der Zelle gering. Bei der Bestimmung der p_H-Aktivitätskurven werden die Substratkonzentrationen im allgemeinen so gewählt, dass sie die Reaktionsgeschwindigkeit nicht beeinflussen. Diese Konzentrationen sind aber verhältnismässig gross, sodass sie keine Rückschlüsse auf das in der Zelle für die Aktivität der Phosphatase herrschende p_H-Optimum erlauben.

3. Durch Dialyse konnte aus Nierenextrakten eine, die Wirkung der Phosphatase hemmende Substanz entfernt werden. Das p_H-Optimum wurde durch die Dialyse nicht verändert.

4. Die Phosphomonoesterase I konnte an Kaolin adsorbiert und danach wieder eluiert werden. Das Enzym wurde dann mit Äthanol ausgefällt. Auf diese Weise gereinigte Präparate enthalten keine anderen Phosphomonoesterasen, wohl aber Pyrophosphatase I. Diese konnte durch Adsorption der Phosphomonoesterase an Diphenylmethanol bei saurem p_H und Eluieren bei alkalischem p_H entfernt werden.

5. Es wurde gezeigt, dass eine Anzahl von Kationen und Anionen auf die Lage des p_H-Optimums der alkalischen Phosphomonoesterase keinen Einfluss haben.

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