Phosphatases VI.[1] pH Dependence of the Organ-Specific Thermostability of Alkaline Phosphatases in Tissue Homogenates

Thermostability of alkaline phosphatases (*ortho*phosphoric monoester phosphohydrolase, E.C. 3.1.3.1) in tissues has been intensively studied to determine those qualities which would enable the identification of isoenzymes in serum, particularly in liver, bone, intestinal and neoplasmic diseases[2–4]. As with other properties of the enzyme (electrophoretic mobility, L-phenylalanine inhibition, urea inhibition[5,6]), thermostability is a function of the conditions of measurement, as well as of enzyme purity[7]. These properties are well known for partly purified enzymes, but not for alkaline phosphatases in tissue homogenates, although assay of the enzyme activity in tissue homogenates (especially at alkaline pH values) is important in practice and is also necessary to compare purification steps.

In the present experiments, a marked decrease in thermostability of rat intestinal, liver, kidney and bone alkaline phosphatases has been found with increasing pH. This effect depends upon dilution of the tissue homogenate. At physiological pH values, differences in thermostability of alkaline phosphatases from various tissues were confirmed.

Material and methods. Adult male Wistar rats (180–200 g body weight), fed a standard diet and water ad libitum, were killed by decapitation under ether anaesthesia. Liver, kidney, proximal third of the small intestine and long bones were removed rapidly. The tissues were washed with cold isotonic NaCl and cut into small pieces. The mucosa was scraped away from the intestine. The tissues were then homogenized in 4 volumes of distilled water in a Waring blender, squeezed through a nylon grid and frozen at −20 °C. After thawing they were rehomogenized in 2 volumes of the appropriate buffer or water, isotonic NaCl, 0.25 *M* sucrose or a buffered sucrose (maintained at pH 7.4 with *Tris*-HCl buffer). The following buffers were used: 0.1 *M* acetate at pH 5.6, 0.1 *M* borate at pH 7.9 (borax-HCl) or 8.6, 9.5 and 10.3 (borax-NaOH). At 56 °C the pH values of these buffers were 5.6, 7.4, 8.0, 9.0 and 9.8, resp. The thawed homogenates were rehomogenized in 2, 5, 9 and 20 volumes of borate buffer at pH 7.9. All the homogenizations were carried out at 4 °C.

After rehomogenization the tissue samples were heated in an ultrathermostat for precisely 3, 6, 9, 12 and 15 min at 56° ± 0.1 °C and then quickly cooled in an ice bath. Activity of alkaline phosphatase was determined in these and in control unheated samples.

Alkaline phosphatase activity was determined for 10 m*M* *p*-nitrophenyl phosphate (B.D.H., England) in 50 m*M* carbonate-bicarbonate buffer at pH 9.8, containing 5×10^{-4} *M* $MgCl_2$. *p*-Nitrophenol released in 15 min at 37 °C was measured by optical density at 405 nm. The previously heated samples were expressed as % of the remaining activity as compared with controls. Protein concentration was determined by the LOWRY method[8].

Results and discussion. As shown in the Table the intestinal alkaline phosphatase is relatively thermostable at slightly acid and neutral pH values, as compared to the liver and particularly kidney and bone enzymes. This is in good agreement with published results[9,10]. No differences were shown regardless of rehomogenization in water, isotonic NaCl, 0.25 *M* sucrose alone or buffered or acetate buffer at pH 5.6.

Susceptibility of alkaline phosphatases in homogenates of rat tissues to heat inactivation

Organ	No. of rats	Mean ± *S.D.* (%)[a]
Intestine	10	77 ± 1.40
Liver	10	35 ± 1.58
Kidney	10	25 ± 1.30
Bone	10	25 ± 1.60

[a] % of remaining activity after 15 min of heating at 56 °C.

[1] H. BÖRNIG, J. ŠTĚPÁN, A. HORN, R. GIERTLER, G. THIELE und B. VEČEREK, Hoppe-Seyler's Z. physiol. Chem. *348*, 1311 (1967).
[2] S. POSEN, Ann. intern. Med. *67*, 183 (1967).
[3] J. OHLEN and J. RICHTER, Dt. med. Wschr. *96*, 343 (1971).
[4] J. J. FENNELLY, M. W. FITZGERALD and K. MCGEENEY, J. Br. Soc. Gastroent. *10*, 45 (1969).
[5] W. H. FISHMAN and N. K. GHOSH, Adv. clin. Chem. *10*, 255 (1967).
[6] D. W. MOSS, Ann. N. Y. Acad. Sci. *166*, 641 (1969).
[7] L. A. NAGODE, A. KOESTNER and C. L. STEINMEYER, Clin. chim. Acta *26*, 55 (1969).
[8] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDAL, J. biol. Chem. *193*, 265 (1961).
[9] J. P. MANNING, S. GREEN, N. R. INGLIS and W. H. FISHMAN, Enzymologia *37*, 262 (1969).

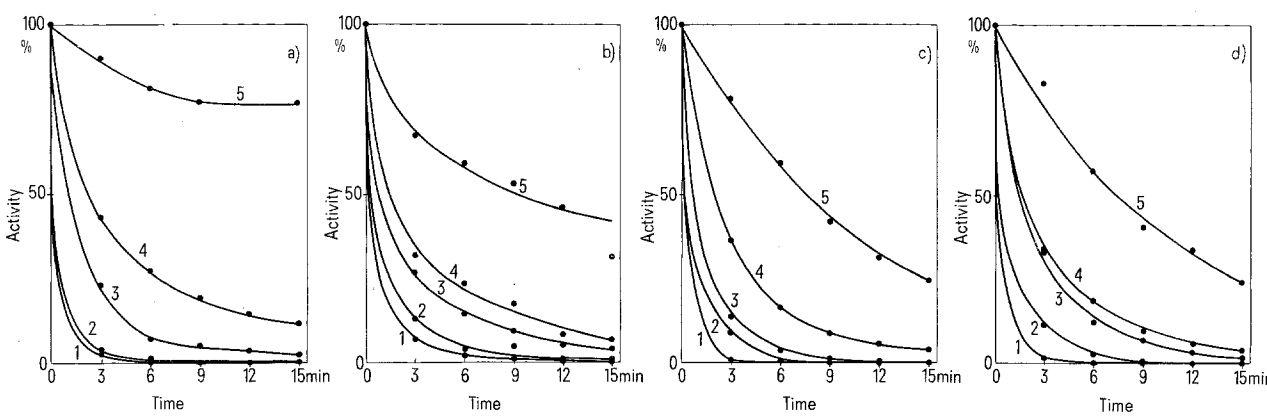

Fig. 1. Effect of pH on thermostability of alkaline phosphatases in rat tissue homogenates. a) intestine; b) liver; c) kidney; d) bone. 1. pH 9.8; 2. pH 9.0; 3. pH 8.0; 4. pH 7.4; 5. pH 5.6.

A progressive decrease in alkaline phosphatase thermostability with increasing pH values of the homogenizing media was shown. It was evident in all the tissues studied (Figure 1). As seen from the figure, bone and liver alkaline phosphatase resisted better the delirious effects of increased alkalinity. After only 3 min at 56 °C at pH 9.0 the intestinal alkaline phosphatase showed the lowest activity, as opposed to the enzyme in other organs. This difference was highly significant ($n = 10$, p 0.001). 15 min at 56 °C at pH 10.3 resulted in complete inactivation of alkaline phosphatases in all tissues. Thus, it appears that the enzyme thermostability depends upon the conditions of measurement. A comparison of tissue homogenates is complicated by the fact that the degree of enzyme purity affects its thermostability. As shown in Figure 2, the intestinal alkaline phosphatase is more thermostable in more concentrated homogenates. This finding, which requires further study, again shows the interrelationship between molecular conformation and pH, ionic strength[11] and the structure of membranes of cells[12].

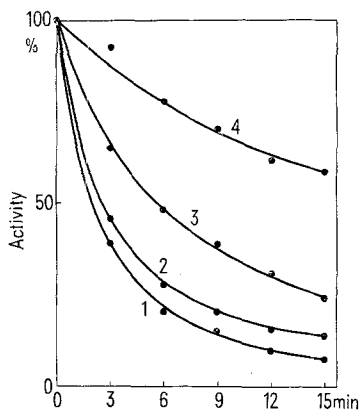

Fig. 2. Effect of dilution of tissue homogenates on susceptibility of alkaline phosphatases to inactivation by heat at 56 °C. 20% homogenate in isotonic NaCl was diluted by buffer at pH 7.4. All the activities were calculated for the initial (20%) homogenate. The resp. dilutions 1. 1:20; 2. 1:9; 3. 1:5; 4. 1:2.

The decreased thermostability of tissue alkaline phosphatases in alkaline buffers is considerable for selection of homogenizing media. Also the usual conditions for estimating alkaline phosphatase activity (dilution of an enzyme, high pH values and temperature of 37 °C) may well have an inactivating influence on the enzyme. The practical importance is demonstrated by the fact that only 80% of activity of intestinal alkaline phosphatase is preserved after 60 min of incubation at conditions used routinely for the activity estimation, i.e. 37 °C and carbonate-bicarbonate buffer at pH 9.8, even when the homogenate was diluted 1:2 (Figure 2). The factors mentioned should be considered for the interpretation of the thermostability of alkaline phosphatase, particularly under pathological conditions.

Zusammenfassung. Die Hitzeinaktivierung der alkalischen Phosphatase in Darm-, Leber-, Knochen- und Nieren-Homogenaten der Ratte erhöht sich mit steigenden pH-Werten, was für die übliche Bestimmung der Gesamtaktivität des Enzyms bedeutend ist.

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[10] A. B. Righetti and M. Kaplan, Biochim. biophys. Acta *230*, 504 (1971).
[11] J. Štěpán and B. Večerek, FEBS Lett. *4*, 151 (1969).
[12] B. F. Trump, S. M. Duttera, W. L. Byrne and A. U. Arstila, Proc. ntn. Acad. Sci. USA *66*, 433 (1970).

The Energetics of Frog Rectus Abdominis Muscle Shortening under Isotonic Load

There is considerable disagreement in the literature as to whether or not the large fraction of heat liberation during muscle shortening is accompanied by a hydrolysis of ATP or phosphorylcreatine. Particularly the data of Davies et al.[1,2], who analyzed the chemical changes in frog rectus abdominis for constant amounts of work and shortening, respectively, have been quoted as evidence for a mere correlation between mechanical work and chemical energy expenditure. In view of the importance of this kind of conclusions, both heat and chemical changes have been measured under steady state mechanical conditions, where the results are not affected by changes in the level of activation.

After dissection paired rectus abdominis muscles of *Rana esculenta* were kept in Ringer's solution containing 1 m*M* iodoacetate for 25 min at 20°C. This was followed by flushing with iodoacetate-Ringer gassed with 5% CO_2 and 95% N_2 at 1°C for another 20 min. The fresh muscle weight was obtained by hooking the tendon onto a spring balance. The individual muscle weight (M) varied between 165–180 mg. The muscles were fixed with Duco cement onto 2 anodized aluminium disks, being connected to a length and force transducer[3]. The initial free muscle length at very slight extension (l_0) varied between 4.5–4.7 cm. Isotonic experiments were carried out by reducing the maximal isometric tension at l_0+4 mm (which was initially developed by each muscle) through quick release to a level equal to any chosen external load, which in this way could be set at a well-defined fraction of the isometric tension. This fractional load then constitutes the new reference value for the servo-control mechanism of the apparatus[3].

For the heat measurements a Wilkie-type integrating thermopile[4] was used, the sensivity of which was increased by incorporating 10 chromel-constantan junctions. The gas was 99% O_2 and 1% CO_2. The temperature was maintained at 0°C in a Dewar cessel. The two muscles of the pair were stimulated simultaneously with supramaximal condenser discharges at a frequency of 10 Hz.

Chemical analyzes have been carried out in parallel experiments, so as to allow a rapid freezing in isopentane

[1] D. F. Cain, A. A. Infante and R. E. Davies, Nature, Lond. *196*, 214 (1962).
[2] A. A. Infante, D. Klaupiks and R. E. Davies, Biochim. biophys. Acta *94*, 505 (1965).
[3] R. A. Chaplain, Nature, Lond. *223*, 63 (1968).
[4] D. R. Wilkie, J. Physiol., Lond. *195*, 157 (1968).