ON THE STABILITY OF RAT ORGAN ALKALINE PHOSPHATASES

L. KADLECOVÁ and J. ŠTĚPÁN

1st Institute of Medical and Forensic Chemistry, Charles University, 121 08 Prague 2

Received August 4th, 1974

Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

The dependence of the activity of alkaline phosphatase in homogenates of rat liver, kidney, bones, and intestinal mucosa on temperature, length of heating period, pH, and dilution of homogenate was examined. The intestinal enzyme is relatively the most stable one; the enzyme from bones is thermolabile yet most stable of all these tissue phosphatases in the alkaline pH-range up to 37°C. The stability of the enzyme decreases with the dilution of the homogenate. The results obtained are of practical importance for the optimization of conditions of determination of the enzyme activity and for the differentiation of organ-specific phosphatases according to their relative thermal stability.

The thermal stability of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) is an important criterion of biochemical differentiation of the multiple molecular forms of this enzyme, synthetized in various organs[1]. It plays a role in the identification of alkaline phosphatases in the serum, especially in differential diagnostics of hepatobiliary, bone, intestinal and neoplastic disorders[2,3]. A practical diagnostic importance has also the determination of the activity of the enzyme directly in tissue homogenates, biopsy material[4], and in the process of purification of the enzyme from tissues.

The investigation of the stability of the enzyme is also essential for the optimization of conditions of activity determination. The thermal stability of alkaline phosphatase – like other biochemical properties of the enzyme (inhibition by amino acids, inactivation by urea, electrophoretic mobility)[5,6]-depends on the conditions of determination[3,7-10]. This paper describes in detail the dependence of rat tissue alkaline phosphatases on temperature and pH of the medium, on the length of the heating period, and on the dilution of the tissue homogenate.

EXPERIMENTAL

Adult male Wistar rats (weight 180–200 g), kept on a standard diet, with water *ad libitum*, were sacrificed by decapitation in ether narcosis. The liver, kidney, long bones, and the proximal part of the small intestine were excised, washed with cold isotonic NaCl solution, lightly dried by filter paper, and weighed. The liver and kidney were cut to small pieces, the bone was crushed,

the mucosa was scrubbed off the intestine. The tissues were homogenized with 4 volumes of redistilled water in a knife-type homogenizer (Type 302, Unipam), rehomogenized in all-glass Potter–Elvehjem homogenizer, and sieved through nylon gauze. All operations were carried out at 4°C. The homogenates were frozen to −20°C. After being thawed they were rehomogenized in the all-glass homogenizer with the corresponding buffers: the intestinal homogenate with 1,19, and 199 volumes of buffer, the kidney and bone homogenate with 1 and 19 volumes of buffer, and the liver homogenate with 1 volume of buffer. The activities could be easily determined at these dilutions. The buffers used were 0·1M borate, pH 7·03, 8·00, 9·32, and 10·30, and carbonate–bicarbonate, pH 9·30 and 10·30 (pH-values at 20°C). The corresponding pH-values at 37°C were 6·80, 7·74, 9·00, 9·94, 8·97 and 9·97, and at 56°C 6·54, 7·60, 8.68, 9·76, 8·66 and 9·61. The buffered homogenates were heated in thin-wall standard test tubes at 20°, 37°, and 56°C (±0·01°C) in an ultrathermostat for exactly 5, 15, 30, and 60 min. The test tubes were then quickly cooled down in a bath with crushed ice.

Determination of activity of alkaline phosphatase. The homogenate sample (0·05 ml) was added to 0·5 ml of substrate solution preheated at 37°C. The mixture was maintained at this temperature for 15 min exactly. The reaction was terminated by the addition of 5·0 ml of 0·05 val/l-NaOH. A blank experiment was carried out in the same manner except that the homogenate sample was added after NaOH. The activity of the enzyme measured was directly proportional to the incubation time under certain conditions. Composition of buffered solution: 0·1M carbonate–bicarbonate, 0·0005M-$MgCl_2$, and 0·01M 4-nitrophenyl phosphate, final pH 9·80 ± 0·05. All the chemicals used were of analytical purity. The optical density was measured in Spectromom 203 Spectrophotometer against water, at 405 nm in a 1 cm cell. The activity of the thermally treated solutions was expressed in per cent of residual enzyme activity compared to the activity of the corresponding sample not treated thermally.

RESULTS AND DISCUSSION

The dependence of the enzyme activity in individual homogenates on the time of preheating, temperature, and pH of homogenate is shown in Fig. 1. The stability of alkaline phosphatase in 10% homogenate of tissue is compared in Figs 1*a*–*d*. Fig. 1*c* shows the stability of the enzyme in 1% homogenate of intestinal mucosa. The stability of alkaline phosphatase in 1% homogenates of the remaining tissues and in 0·1% homogenate of intestinal mucosa is not expressed graphically in the figure and will be commented only.

We found that the activity of alkaline phosphatase in all tissues varies inversely with temperature, time of heating, and pH. The intestinal enzyme, however, is considerably more stable at neutral pH than the alkaline phosphatases from the remaining organs studied. This finding is in accordance with data reported so far mainly on organ-specific alkaline phosphatases of serum[1,5,7,9,11]. Whereas a 10% homogenate of intestinal mucosa still retains more than one half of the original activity after 60 min of heating at 56°C, the activity of alkaline phosphatase in equally concentrated homogenates of liver, kidney, and bone completely disappears as soon as after 30 min. The intestinal homogenate retains 77% of original activity after 15 min of heating at 56°C, the liver homogenate 35%, and the bone and kidney homogenate 28% of original activity only.

The thermal stability of alkaline phosphatase sharply decreases with increasing pH-value. The stability of the enzyme from this viewpoint has been studied so far only generally in blood serum. Bowers and McComb[10] observed that the serum alkaline phosphatase is stable at 30°C and 25°C in 2-amino-2-methyl-1-propanol buffer at pH-values above 10·0 whereas a marked inactivation of the serum enzyme can occur within 60 min at 37°C. Moss and coworkers[3] demonstrated that at serum temperature of 56°C the thermal stability of the enzyme decreases by 5% at a pH

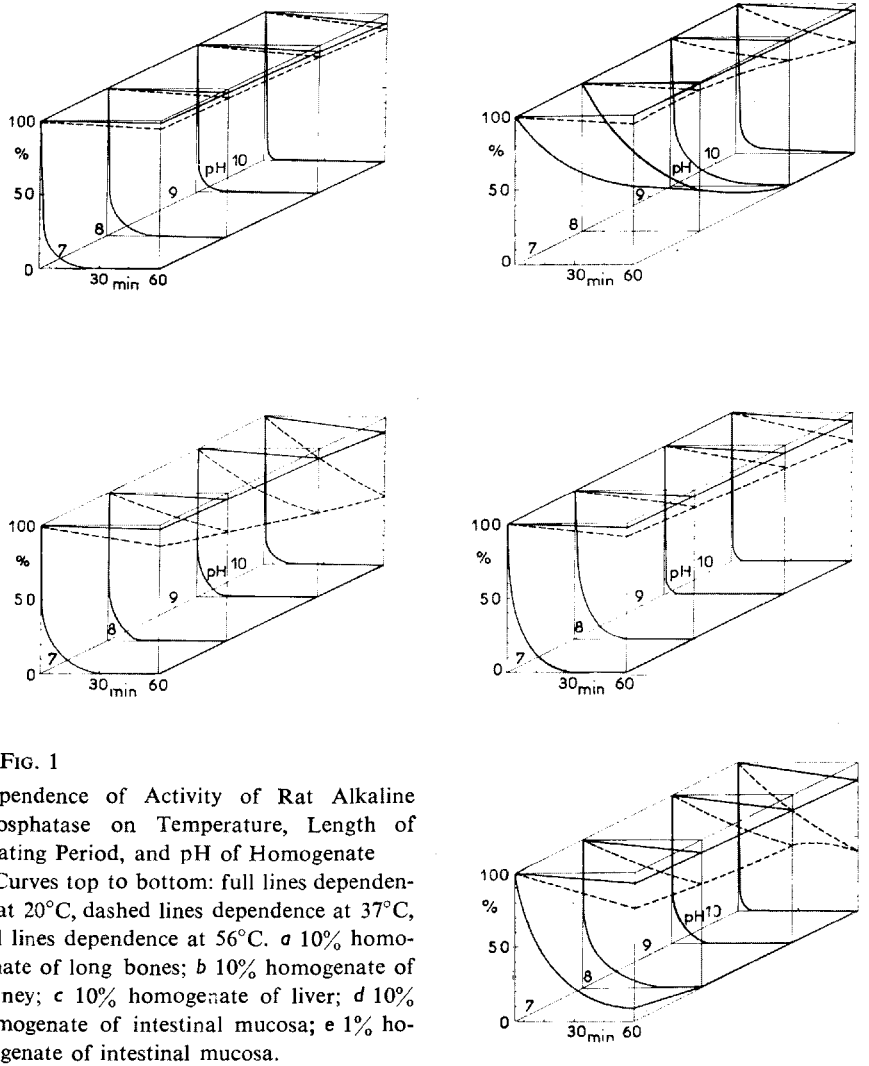

FIG. 1

Dependence of Activity of Rat Alkaline Phosphatase on Temperature, Length of Heating Period, and pH of Homogenate

Curves top to bottom: full lines dependence at 20°C, dashed lines dependence at 37°C, full lines dependence at 56°C. *a* 10% homogenate of long bones; *b* 10% homogenate of kidney; *c* 10% homogenate of liver; *d* 10% homogenate of intestinal mucosa; *e* 1% homogenate of intestinal mucosa.

approximately by one pH unit higher than that of the serum of healthy people. As can be seen in Fig. 1 *a*–*d*, the bone alkaline phosphatase, even though being thermolabile, is below 37°C the relatively most stable one at higher pH-values; by contrast, the remaining organ-specific enzymes, especially the intestinal enzyme, even though thermally stable, are partly inactivated at higher pH-values at temperatures below 37°C. This stability of bone alkaline phosphatase remained unaltered at 37°C and at higher pH-values even in 1% homogenate (the residual activity of the enzyme at neutral pH was 95%) whereas only 40% of activity remained in the intestinal homogenate (Fig. 1*e*) under similar conditions.

Hence, the thermal stability of alkaline phosphatase also depends on the dilution of the homogenate. This effect is most evident with the intestinal alkaline phosphatase, not only at 56°C but also at lower temperatures (Fig. 1*d*,*e*). The effect was even more explicit in 0·1% homogenates of intestinal mucosa. By contrast, a ten-fold dilution of the homogenate of renal and bone alkaline phosphatase did not lead to an increase of the enzyme activity at 20°C, even at the most alkaline pH-value. This was true for the bone enzyme even at 37°C whereas renal alkaline phosphatase was inactivated above pH 8·0 (at the highest pH-value and at 37°C, 50% of the activity was inactivated in 60 min).

Magnesium ions are the most important activators of alkaline phosphatase. Their protective effect manifests itself also during the heating of the enzyme[8]. The fact that magnesium no longer exists in ionized form in the solution at higher pH-values could offer one of the explanations of the observed dependence of the stability of alkaline phosphatase on pH. The stabilizing effect of Mg^{2+}-ions would then differ with the individual organ-specific alkaline phosphatases.

The data presented on the decrease of the activity of alkaline phosphatase with increasing pH-value, temperature, and increasing length of heating period are important for the choice of proper homogenization media and conditions of storage of biological material. The optimization of the conditions of determination of enzymatic activities and especially the choice of the temperature of the incubation medium have been devoted increased attention during the past few years[12]. The information presented here provides evidence that the conventional conditions of determination of alkaline phosphatase (nonphysiological pH-values, temperature 37°C, and dilution of the enzyme) are not optimal[10]. This is important for the evaluation of the activities of "isozymes" of alkaline phosphatase in terms of relative thermal stability in materials containing a mixture of alkaline phosphatases from different tissues, especially when larger quantities of intestinal alkaline phosphatase are present. Intestinal phosphatase accounts for the majority of the enzymatic activity in, *e.g.* rat serum and in the serum of patients with liver cirrhosis[2]. Eventually, additional studies on the stability of the enzyme may contribute to the knowledge of the conformation of its molecule.

REFERENCES

1. Posen S.: Ann. Intern. Med. *67*, 183 (1967).
2. Štěpán J., Volek V., Štěpán J.: Vnitřní lékařství *18*, 1003 (1972).
3. Moss D. W., Shakespeare M. J., Thomas D. M.: Clin. Chim. Acta *40*, 35 (1972).
4. Štěpán, Volek V., Balaš V., Večerek B.: Acta Hepato-Gastroenterol. *21*, 425 (1974).
5. Fishman W. H., Ghosh N. K.: Advan. Clin. Chem. *10*, 255 (1967).
6. Fernley H. N. in the book: *Mammalian Alkaline Phosphatases Enzymes.* (P. D. Boyer, Ed.) 3rd Ed., 4, 417. Academic Press, London 1971.
7. Nagode L. A., Koestner A., Steinmayer C. L.: Clin. Chim. Acta *65*, 55 (1969).
8. Iino S., Suzuku H., Oda T.: Igaku No Ayumi *85*, 593 (1973).
9. Kadlecová L., Štěpán J.: Experientia *28*, 1284 (1972).
10. Bowers G. N., McComb R. B.: Clin. Chem. *12*, 70 (1966).
11. Manning J. P., Green S., Inglis N. R., Fishman W. H.: Enzymologia *37*, 262 (1969).
12. Laue D., Hilmann G., Breuer H.: Z. Klin. Chem. Klin. Biochem. *9*, 464 (1971).

Translated by V. Kostka.