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THIAMINE TRIPHOSPHATASE ACTIVITY IN BOVINE KIDNEY

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SUMMARY: Properties of soluble thiamine triphosphatase (ThTPase), adenosine triphosphatase, nucleoside triphosphatase and alkaline phosphatase activities in bovine kidney were compared. ThTPase and the other phosphatases differed clearly in their pH-dependences, K_m and molecular masses. Apparent K_m and pH optimum for ThTPase were determined to be 45.5 μM and 8.9, respectively. Molecular mass of the enzyme was 29.1 kDa as estimated by Sephadex G-100 gel filtration. The results obtained show bovine kidney to contain a specific soluble ThTPase, this enzyme being the only one hydrolyzing low concentrations of ThTP.

KEY WORDS: Bovine kidney, thiamine triphosphatase.

INTRODUCTION

The cleavage of the phosphoanhydride bond between β- and γ-phosphate residues in thiamine triphosphate molecule is catalyzed by homogenates of various mammalian tissues [1,2]. In the brain the reaction proceeds under the action of thiamine triphosphatase (EC 3.6.1.28) [1] having an absolute substrate specificity [3]. ThTPase was found to be localized in the supernatant fraction obtained after removal of nuclei, mitochondria and microsomes during differential centrifugation of brain homogenate [2]. The protein with properties similar to those of brain ThTPase was also found in rat liver [4]. There is good reason to believe specific ThTPase to be present in other organs viz. heart, kidney, spleen, muscle [4]; however, the nature of the ThTPase activity observed has not been fully established. The problem is that, apart from ThTPase, ThTP can serve as a potential substrate for nucleoside triphosphatases (NTPases) (EC 3.6.1.15) and ATPases with a comparatively broad substrate specificity. Thus, for instance, myosin ATPase (EC 3.6.1.32) and NTPase from *E.coli* were shown to display ThTPase activity [5,6]. The hydrolysis of ThTP is also catalyzed by some nonspecific phosphatases: apyrase(EC 3.6.1.5), alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatases [4]. Most phosphatases hydrolyzing various nucleoside phosphates are membrane-bound proteins sedimenting during high-speed centrifugation of the homogenate. Soluble forms of

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alkaline phosphatase [7,8] and NTPase [9-11] are also known as well as cytosolic enzymes with ATPase activity - mechanochemical ATPases of the cytoskeleton, dynein [EC 3.6.1.33] and kinesin.

The aim of this work was to study the relationship of ThTPase activity in bovine kidney extract to activities of soluble enzymes catalyzing the hydrolysis of ATP, ITP and *p*-nitrophenyl phosphate.

MATHERIALS AND METHODS

Chemicals. Sephadex G-100 and papain were from Loba Feinchemie, Fischamend, Austria; Servacel DEAE-32, α-chymotrypsinogen, human serum albumin, lactate dehydrogenase, pyruvate kinase, alcohol dehydrogenase, ATP, ITP were from Reanal, Budapest, Hungary; ovalbumin, thiamine diphosphate, Coomassie brilliant blue G-250 were from Serva, Heidelberg, Germany; *p*-nitrophenyl phosphate was from Chemapol, Praha, Czech Republic; all the other reagents were from Reachim, Moscow, Russia. ATP and ITP were purified by ion-exchange chromatography on a Servacel DEAE-32 column (∅ 2.8 x 22 cm). Thiamine triphosphate was synthesized by the method described previously [12].

Extract preparation. Bovine kidneys were cut into pieces, frozen and stored at -20 °C before use. To prepare the extract thawed samples were homogenized with a glass homogenizer in 10 volumes of 50 mM Tris-HCl, pH 7.3, cooled to 4 °C, containing 0.15 M KCl, 0.2 mM EDTA and then centrifuged at 105000 x g for 60 min. The resulting supernatant was used as the extract for enzyme activity assays. ***Determination of phosphatase activities***. All the assays were performed at 37 °C. The standard reaction mixture for ***ThTPase*** contained 50 mM Tris-HCl, pH 8.9, 10 mM $MgSO_4$, 0.1 mM ThTP and 20-90 μg of protein in a final volume of 0.5 ml. After incubation for 20-30 min the reaction was stopped by dilution of the reaction mixture with 2 ml of 0.5 M Na-phosphate buffer, pH 6.7. ThDP was determined enzymatically after recombination of 0.1 ml aliquots with apopyruvate decarboxylase from brewer's yeast as described previously [13]. When studying pH optimum the reaction was terminated by boiling. ***Alkaline phosphatase*** activity was determined by the rate of *p*-nitrophenyl phosphate hydrolysis. The reaction mixture of 0.5 ml containing 50 mM Tris-HCl, pH 8.9, or glycine, pH 10.0, 10 mM $MgSO_4$, 1 mM substrate and 20-200 μg of protein was incubated for 40-60 min. The concentration of *p*-nitrophenol formed was calculated from ε_{405}=18500 after diluting the incubation mixture with 5 ml of 0.02 M NaOH. ***ATPase*** and ***NTPase*** assays were carried out by measuring the amount of inorganic phosphate liberated during 30-60 min incubation. The assay mixture of 0.5 ml consisted of 10 mM $MgSO_4$, 1 mM ATP or ITP, 20-200 μg of protein and 50 mM Tris-HCl, pH 8.9, or 20 mM Tris-maleate, pH 7.0. The amount of P_i formed was determined by the method of Sapru et al. [14]. ***One unit of activity*** (U) was defined as the enzyme amount catalyzing the formation of one μmol of product in one minute under the assay conditions. The concentration of phosphatases studied was expressed in units per litre (U/l). ***Protein*** was determined according to Bradford [15] or from the absorbance at 280 nm. ***Molecular mass*** determinations were done at 4 °C on a Sephadex G-100 column (∅ 2.8 x 61 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and calibrated with the following standard proteins: papain (20.7 kDa), α-chymotrypsinogen (25.7 kDa), ovalbumin (45 kDa), peroxidase (55 kDa), human serum albumin (67 kDa), lactate dehydrogenase (135 kDa) and pyruvate kinase (228 kDa). The elution volumes (V_e) of the phosphatases studied were determined by activity assays. Their apparent molecular masses were calculated from the plot of log M_r versus log V_e/V_o ratio.

RESULTS

pH optima. The effect of pH on ATPase, NTPase, ThTPase and *p*-nitrophenyl phosphatase activities in bovine kidney extract was investigated in the pH range from 6.0 to 10.0, using maleate (pH 6.0–6.5), Tris-maleate (pH 7.0), Tris-HCl (pH 7.5–9.0) and

glycine (pH 9.2–10.5) to buffer the assay mixture. As can be seen in Fig. 1 both ATPase and NTPase exhibited two maxima of activity, one in the neutral and one in the alkaline zone. While ATPase activity had distinct pH optima at pH 7.0 and 9.2, NTPase showed a pronounced maximum at pH 7.0–7.2 and a less distinct one at pH 8.9–9.0. The rate of ATP hydrolysis in alkaline optimum was 1.3 times higher than at neutral pH. Unlike ATPase the highest NTPase activity was observed in neutral medium, the reaction rate at pH 7.0 being more than twice as high as that at pH 8.9. Alkaline phosphatase displayed two essentially equal activity peaks corresponding to pH values 8.9 and 10.0. In contrast with the other phosphatases ThTPase exhibited a bell-like peak of activity with a maximum at pH 8.9.

Michaelis constants. K_m determinations for all substrates were carried out at a fixed Mg^{2+} concentration of 10 mM and pH 8.9. The double-reciprocal plots of ATPase, NTPase, ThTPase and alkaline phosphatase initial reaction velocities against the substrate concentration are depicted in Fig. 2. Both for ThTP and ATP the plots gave straight lines permitting one to calculate the apparent K_m values of 45.5 and 49.8 μM, respectively (Fig. 2A,B). With ITP the Lineweaver-Burk plot approximated to a hyperbola (Fig. 2C) indicating the presence of at least two enzymes differing in affinity and attacking the same substrate. In such a case the extrapolation of a linear portion of the curve permits the K_m for the high affinity enzyme to be calculated. The apparent K_m for ITP was calculated in this way to be 104.3 μM. The plot for

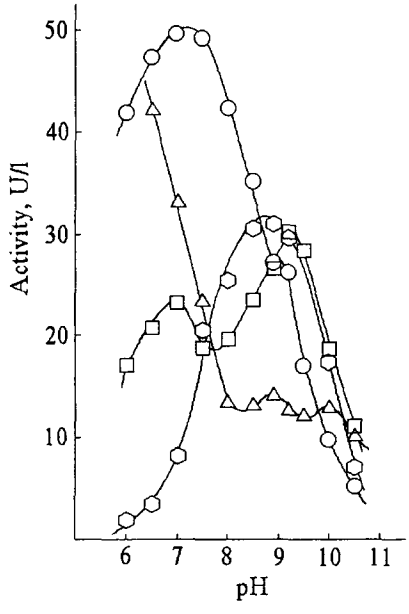

Fig. 1. Phosphatase activities in bovine kidney extract as a function of pH. ○,–ThTPase; ○,– NTPase; □,– ATPase; △,– alkaline phosphatase. The values of NTPase activity are reduced five-fold in the Figure. Each curve is the mean of 2-4 animals.

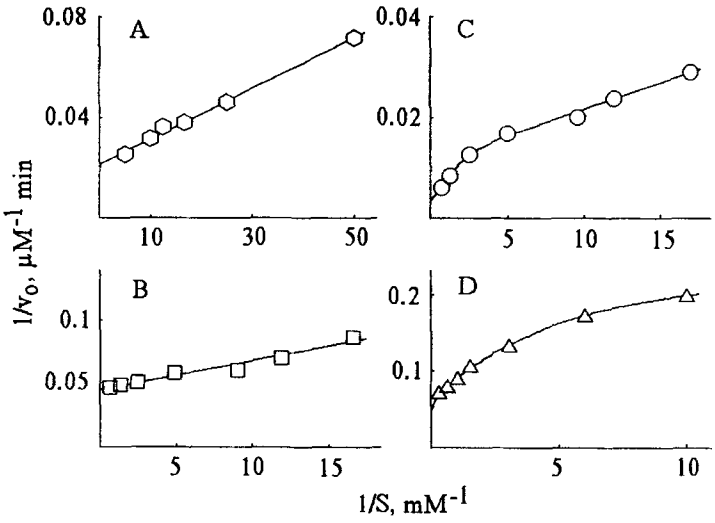

Fig. 2. The double-reciprocal plots of bovine kidney extract ThTPase (A), NTPase (B), ATPase (C) and alkaline phosphatase (D) initial reaction velocity against substrate concentration. Means for 3 animals.

alkaline phosphatase over the range of used p-nitrophenyl phosphate concentrations from 0.1 to 3.5 mM was non-linear (Fig. 2D). The K_m for the enzyme was failed to determine.

Gel filtration. Gel filtration experiments were done with extracts of two animals. Samples of 10 ml were fractionated on a Sephadex G-100 column at a flow rate of 5 cm/h and ThTPase, ATPase, alkaline phosphatase and NTPase activities were determined in the eluate at pH values corresponding to their pH optima, i. e. at pH 8.9 with ThTP as substrate, pH 7.0 and 9.2 with ATP, pH 8.9 and 10.0 with p-nitrophenyl phosphate, pH 7.0 and 8.9 with ITP. The location of the activity peaks obtained is shown in Fig. 3. Both experiments revealed ThTPase activity to be eluted as a single symmetrical peak clearly separated from the peaks of the other phosphatases assayed. NTPase activity was divided into three peaks. They were distinct in the chromatographs of one of the extracts (Fig. 3A) but were less distinguishable in the other case (Fig. 3B). The location of all the three peaks was identical when the activity was determined both at alkaline and neutral pH. The elution pattern for alkaline phosphatase depended on assay conditions. At pH 8.9 three activity peaks were obtained (Fig. 3A), a major peak eluted nearly in the void volume of the column and two less active ones corresponding to lower molecular masses. When being assayed at pH 10.0, alkaline phosphatase gave another pattern. A peak was found corresponding to the high molecular form of the enzyme and a smaller one coming later (Fig. 3B). Both peaks differed in their location from those brought out at pH 8.9. ATPase activities were excluded from the column.

Molecular mass determinations. The molecular masses of the phosphatases under investigation were obtained from their elution behaviour. The standard proteins run in the same

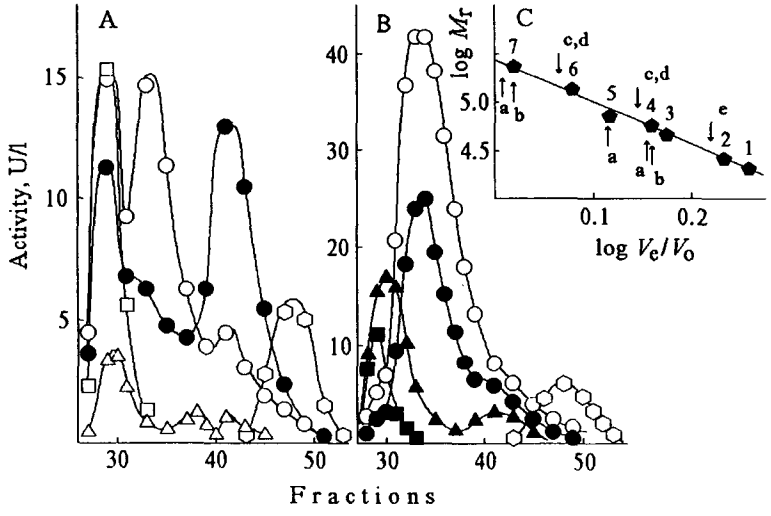

Fig. 3. Fractionation of bovine kidney extract phosphatases on Sephadex G-100 and their molecular mass determination . A,B: ○,– ThTPase; ○,– NTPase, pH 7.0; ●,– NTPase, pH 8.9; □,–ATPase, pH 7.0; ■,– ATPase, pH 9.2; △,– alkaline phosphatase, pH 8.9; ▲,–alkaline phosphatase, pH 10.0. C: 1 - papain, α-chymotrypsinogen, 3 - ovalbumin, 4 - peroxidase, 5 - human serum albumin, 6 - lactate dehydrogenase, 7 - pyruvate kinase; a - alkaline phosphatase, pH 8.9; b - alkaline phosphatase, pH 10.0; c - NTPase, pH 7.0; d - NTPase, pH 8.9; e - ThTPase. The values of alkaline phosphatase activity in Fig. 3B are increased five-fold.

Sephadex G-100 column gave a linear plot of log M_r versus log V_e/V_0 (Fig. 3C). Apparent molecular masses were calculated from this plot for the components presented in Fig. 3A,B. The positions of the phosphatases peaks are marked in Fig. 3C with arrows. The molecular masses were: 29.1 kDa for ThTPase; >280 kDa, 146 kDa and 60 kDa for NTPase (activity for ITP both at neutral and alkaline pH); 220 kDa, 54 kDa (activity for *p*-nitrophenyl phosphate at pH 10.0), ≅260 kDa, 84 kDa and 57 kDa (activity for *p*-nitrophenyl phosphate at pH 8.9) for alkaline phosphatase. ATPase peaks were eluted in the void volume corresponding to molecular mass greater than 280 kDa.

DISCUSSION

ThTP has been shown to be widely distributed in living cells. The exact physiological significance of this compound has remained unclear. A specific neurochemical function of ThTP has been proposed [16]. It is thought that ThTP plays a specific role in neuronal excitability or membrane permeability. There is strong evidence that ThTP may be involved in the regulation of the maxi Cl^- channels but the mechanism of its action is obscure [17]. The processes of nerve activity were shown to be gone with ThTP hydrolysis, supposing the involvement of specific phosphatases [16]. Soluble ThTPase exhibiting an absolute specificity for its substrate has been purified and characterized from bovine brain [3].The enzyme with

properties like those of brain ThTPase was found in rat liver [4]. ThTPase activity was also detected in other mammalian tissues [1,2] but the nature of this activity is to be elucidated. In this paper we described some properties of bovine kidney soluble ThTPase activity in comparison with those of ATPase, NTPase and alkaline phosphatase.

As the first step pH optima for these reactions were examined. The shape of pH dependence for ThTPase activity differed from the other phosphatases assayed (Fig. 1) but the values of pH optima in the alkaline region for ThTP, ITP and *p*-nitrophenyl phosphate were identical. Unlike this, the positions of pH optima for ThTP and ATP were clearly distinguishable. In eukaryotic cells cytosol ATP hydrolysis is known to be coupled to various process of cell motility fulfilled by cytoskeleton ATPases, myosin, kinesin and cytoplasmic dynein. Myosin filaments are normally soluble at high concentrations of salt (0.5–0.6 M KCl); on lowering ion strength they form insoluble high-molecular-mass aggregates consisting of 15–20 myosin molecules [18]. In contrast to myosin, both cytoplasmic dynein and kinesin can be extracted with low-ionic-strength solutions. In all probability the hydrolysis of ATP observed here in the kidney extract was due to dynein ATPase activity, since kinesin exhibits a very low Mg^{2+}-dependent activity in the absence of microtubules [19]. The K_m for ATP was calculated to be 49.8 μM, being in reasonable agreement with the K_m for dynein ATPases [20,21].

One may assume that pH optima for p-nitrophenyl phosphate activity observed in the alkaline pH region (Fig. 1) corresponded to two different forms of alkaline phosphatase. Most of well-known alkaline phosphatases from animal tissues are membrane-bound proteins; the general way of the enzyme isolation consists in treating the homogenate with butanol [22]. On being extracted with aqueous buffer solutions and then centrifuged at a high speed only a little part of total activity, corresponding to the soluble form, may be detected in the supernatant [7,8]. Three forms of soluble alkaline phosphatase were found by electrophoresis in rat kidney [4]. A part of the activity that we observed in bovine kidney extract seems to originate from membranes due to acting endoprotease, as was shown for the liver [23]. Another soluble form apparently is genetically determinated [7]. In spite of identical positions of pH optima for ThTP and *p*-nitrophenyl phosphate (Fig. 1), one may conclude, proceeding from the ratio observed, that these activities do not belong to the same enzyme, since the rate of *p*-nitrophenyl phosphate hydrolysis by known alkaline phosphatases is higher that with triphosphate esters. On the other hand, specific ThTPase purified from the brain has an absolute specificity for ThTP [3].

NTPase displayed two maxima of activity - at pH 7.0–7.2 and at pH about 8.9–9.0 (Fig. 1). There have been described several soluble NTPases from mammalian sources. Rat liver contains two cytosolic NTPases, one with M_r 125 kDa and pH optimum 8.6–9.6 [9], the other with M_r 65000 and pH optimum 4.0–4.5 [11]. Metal-independent NTPase with pH optimum 3.0 has been isolated from human serum [24]. Another acid NTPase displaying a maximal

activity at pH 5.0–5.5 was found to be present in the lysosomes of rat liver and kidney[10]. The activity of the above enzymes was not tested toward ThTP so that no evidence indicating the ability of known soluble NTPases to hydrolyze ThTP is available. Alternatively, membrane-bound NTPase purified from *E. coli* was shown to have an equal activity with NTPs and ThTP [6]. On examining the pH profiles depicted in Fig. 1 it was difficult to conclude whether the hydrolysis of ThTP and ITP was catalyzed by the same or different enzymes since the positions of pH optima for both substrates were essentially identical. Michaelis constants and molecular masses of the enzymes under investigation were determined.

Kinetic study revealed the hydrolysis of ThTP over the concentration range tested to obey Michaelis-Menten kinetics. A linear double-reciprocal plot was obtained (Fig. 2A) indicating the presence in the extract either of only one enzyme catalyzing the reaction or several with closely similar kinetic properties. The K_m value was calculated from this plot to be 45.5 μM being in a very good agreement with that for brain specific ThTPase [3]. Unlike ThTP, the Lineweaver-Burk plot for ITP hydrolysis was non-linear showing an apparent activation with substrate excess (Fig. 2B). Such behaviour is known to be typical for systems which contain two or more enzymes attacking the same substrate, at least two of them having strong differences in their affinity for the substrate. The K_m for ITP calculated from a linear portion of the curve was 104.3 μM exceeding more than two times that for ThTP.

Gel chromatography experiments clearly separated ThTPase activity from the other phosphatases assayed (Fig. 3).The peak of ThTPase activity corresponded to a protein with M_r 29.1 kDa . Previously we purified from bovine brain low-molecular-mass (M_r 33.9 kDa) high affinity (K_m 43.7 μM) ThTPase with an absolute specificity for ThTP [3]. The protein described in the present work has closely similar properties with those of brain ThTPase being apparently a kidney version of that enzyme. Lacking ThTPase activity in the fractions with the other phosphatases location indicates ThTPase to be the only enzyme in the kidney extract which catalyzes the hydrolysis of ThTP at relatively low concentrations of this compound (below 0.1 mM).

According to Fig. 3 we found three different soluble enzymes with NTPase activity. Their molecular masses were >280 kDa, 146 kDa and 60 kDa (Fig. 3C). One may suggest that the activity peak corresponding to M_r >280 kDa belongs to 19S NTPase which was initially detected in calf brain [25] and then identified as cytoplasmic dynein. It is not improbable that all the peaks with ATPase and NTPase activities registered in the exclusion volume belong to this protein. We failed to find in the literature any mention of animal soluble NTPases with M_r 146 kDa or 60 kDa and pH optima respectively 7.0–7.2 and 8.9–9.0. These soluble NTPases which we revealed appears to be novel enzymes undescribed before. Molecular masses corresponding to p-nitrophenyl phosphatase activity location in the Sephadex G-100 effluent were about 260 kDa, 84 kDa, 57 kDa when the assays were carried out at pH 8.9, and 220 kDa, 54 kDa - at pH 10.0 (Fig. 3C). Most alkaline phosphatases isolated from mammalian

tissues have molecular masses of 120–190 kDa and consist of two or four identical subunits [22]. Lower and higher molecular-mass forms as well as alkaline phosphatase consisting of different subunits are also known [26]. Such variety in molecular mass values in many cases may be due to differences in carbohydrate content [22]; the same cause often leads to the formation of "isoenzymes" separated by ion-exchange chromatography or electrophoresis [27]. Without additional experiments it is difficult to account for simply the elution patterns that we obtained for alkaline phosphatase. The appearance of smaller peaks with low molecular masses seems to be due to conditions of extract preparation harmful for alkaline phosphatase. The important role in stabilizing the native structure of the enzyme is known to be fulfilled by zinc which can be eliminated by metal-chelating agents leading to dissociation of the enzyme; the monomers formed were shown for some alkaline phosphatases to keep a low activity [22]. When preparing the extract we used the buffer solution containing 0.1 mM EDTA; therefore, the dissociation of the enzyme in large quantities appears likely. It is not impossible that we dealt with two high-molecular-mass forms of alkaline phosphatase (M_r 220 kDa and about 260 kDa) and their dissociation products into subunits of a low activity.

The results presented in this paper show bovine kidney to contain a specific ThTP hydrolyzing enzyme, thiamine triphosphatase (EC 3.6.1.28), which clearly differs in its properties from the enzymes with ATPase, NTPase and *p*-nitrophenyl phosphatase activities. ThTPase activity observed in bovine kidney extracts at low ThTP concentrations is fully due to this enzyme activity.

REFERENCES

1. Hashitani,Y., and Cooper,J.R. (1972) J.Biol.Chem. 247, 2117-2119.
2. Makarchikov,A.F., and Chernikevich,I.P. (1993) Vesti AN Belarus, Ser. Biol. Nauk. N 3, 43-48.
3. Makarchikov,A.F., and Chernikevich,I.P. (1992) Biochim. Biophys. Acta. 1117, 326-332.
4. Penttinen,H.K., and Uotila,L. (1981) Med. Biol. 59, 177-184.
5. Murai,A., and Katsura,E. (1975) J. Nutr .Sci. Vitaminol. 21, 169-183.
6 .Nishimune,T., Ito,S., Abe,M., Kimoto,M., and Hayashi,R. (1987) Biochim. Biophys. Acta. 923, 74-82.
7. Yong,G.P., Yedlin,S.T., and Alpers,D.H. (1981) Biochem. J. 200, 645-654.
8. Castle,A.G., Ling,A., and Chibber,R. (1984) Int. J. Biochem. 16, 411-416.
9. Lewis,M., and Weissman,S. (1965) Arch. Biochem. Biophys. 109, 490-498.
10. Brightwell,R., and Tappel,A.L. (1968) Arch. Biochem. Biophys. 124, 333-343.
11. Naryzhnyi,S.N., and Krutiakov,V.M. (1982) Biokhimia 47, 569-574.
12. Chernikevich,I.P., Gritsenko,E.A., Luchko,T.A., and Zabrodskaya,S.V. (1990) Dokl. Akad. Nauk BSSR 34, 274-278.
13. Chernikevich,I.P., Gritsenko,E.A., Makarchikov,A.F., and Voskoboev A.I. (1991) Prikl. Biokhim. Mikrobiol. 27, 762-771.
14. Sapry,M.K., Geetha,H., and Shetty,T.K. (1987) Ind. J .Biochem. Biophys. 24, 340-343.
15. Bradford,M.M. (1976) Analyt. Biochem. 72, 248-254.
16. Cooper,J.R., and Pincus,J.H. (1979) Neurochem. Res. 4, 223-239.
17. Bettendorff, L. (1994) Metab. Brain Dis. 9, 183-209.
18. Citi,S., and Kendrick-Jones,J. (1987) Bioessays 7, 155-159.
19. Kuznetsov,S.A., and Gelfand,V.I. (1986) Proc. Nat. Acad. Sci. USA. 83, 8530-8534.

20. Hisanaga,S., and Sakai,H. (1983) J. Biochem. 93, 87-98.
21. Belles-Isles,M., Chapeau,C., White,D., and Gagnon C. (1986) Biochem. J. 240, 863-869.
22. McComb,R.B., Bowers,G.N., and Posen,S. (1979) Alkaline phosphatase. 986 pp. Plenum Press, New York.
23. Seiffert,U., Siede,W.H., and Oremek,G. (1989) Clin.Biochem. 22, 103-107.
24. Dahlmann,N., and Kirchgesser,M. (1990) Biochem. Int. 20, 317-327.
25. Baccetti,B., Burrini,A.G., Dallai,R., and Pallini, V. (1980) Eur. J. Cell Biol. 22, 292.
26. Portmann,P., and Dudler,N. (1990) Experientia. 46, Abstr. C. 39.
27. Cathala,G., Brunel,C., Chappelet-Tordo,D., and Lazdunski,M. (1975) J. Biol. Chem. 250, 6040-6045.