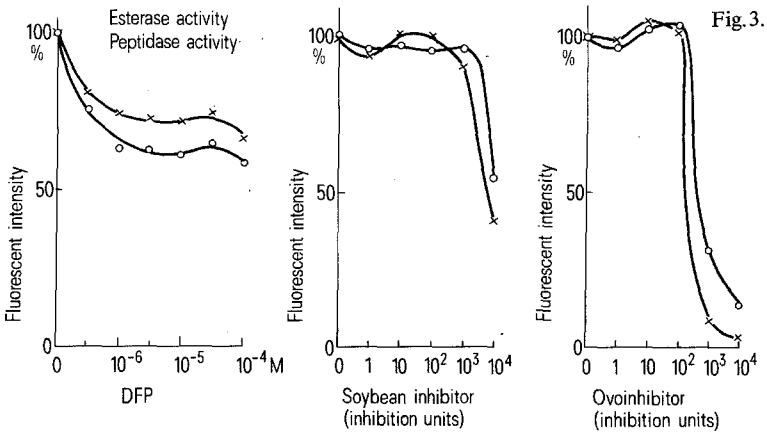


Fig. 3. Effect of addition of various inhibitors on elastase activity.

possible, only 1 activity of elastase, but both activities were inhibited simultaneously (figure 3). Amino acid residues involved in the active center of elastase may be the same for ester and peptide hydrolysis, but a slight conformational change might affect the nature of hydrolytic cleavage of C–N and C–O bonds. It might also be suggested that a more critical spatial conformation is required for the peptidase activity of elastase, and esterase activity may be exerted by a rather loose conformational relation of the various amino acid residues in the active center.

1 To whom reprint request should be directed.

2 R.A. Reisfield, U.J. Lewis and D.E. Williams, Nature *195*, 281 (1962).

## Brain capillary guanosine triphosphatase: A distinction from adenosine triphosphatase[1]

B.M. Djuričić, T. Stojanović and B.B. Mršulja[2]

*Laboratory for Neurochemistry, Institute of Biochemistry, Faculty of Medicine, Belgrade (Yugoslavia), 10 April 1979*

*Summary.* Differences in kinetic properties, pH response, sensitivity to ouabain, and disc-acrylamide electrophoresis resolution, are observed when GTP and ATP are used as the substrates for triphosphohydrolases in isolated rat brain microvessels. In brain parenchyma there are no such differences. It is concluded that substrate-specific GTPase exists in brain microvessels.

The essential role of adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3; ATPase) in the maintenance of the ionic distribution on the cell surface is well documented[3]. The importance of the high potassium and low sodium intracellular concentrations in nervous tissue can hardly be overemphasized, for this unequal distribution is the physicochemical basis for the formation of the membrane (synaptic) potential[4]; this potential and its changes are conditio sine qua non for brain function to exist.

It is considered that 2 compartments exist in brain: neurons and glia[5]. Recently, we postulated that the brain microvessels, representing the anatomical and functional basis of the blood-brain barrier[6], are a separate one, since microvessels have a specific enzymatic organization[7,8] indicative of a difference in metabolic fate of the substances in them; this is a characteristic of the compartment[5].

During our studies on enzymatic organization in isolated brain microvessels, noticeable ATPase and guanosine triphosphatase (GTPase) activities were found[7]. It is known that all the nucleotide triphosphates (i.e. ATP, ITP, GTP, CTP and UTP, in order of their supply ability) can be used as the energy fuel for the ionic pump[9,10].

*Materials and methods.* Adult male Wistar rats were sacrified by decapitation, the skulls were quickly removed, and the forebrains homogenized in 5 vol. of ice-cold homogenizing medium[11]. Capillary and parenchymal specimens were obtained as described by Djuričić and Mršulja[11]. Triphosphohydrolase activities were determined in the assay mixture, which contained the appropriate substrate (ATP or GTP) in the concentration indicated, 90 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, and N-2-hydroxyethyl-N-2-ethane sulfonic acid-NaOH (HEPES-NaOH) buffer (50 mM, pH 7.1). Phosphorus liberated after the proper incubation time at 37 °C was determined according to Lowry and Lopez[12]. The technique employed is not applicable for substrate concentrations lower than 0.1 mM, so $K_i$'s were obtained from a Lineweaver-Burk plot rather than $K_m$'s.

When the pH was varied, the same HEPES buffer at the pH indicated was used. The effects of ouabain were investi-

Rat brain microvessels and parenchymal ATPase and GTPase activities, the ratios (microvessels activity/parenchymal activity) and the half-maximal inhibitory constants ($K_i$) for ouabain

| Triphosphohydrolase | Activity In microvessels | In parenchyma | Ratio | $K_{i_{ouabain}}$ (mM) In microvessels | In parenchyma |
|---|---|---|---|---|---|
| ATPase | 196 ± 18 | 260 ± 17 | 0.75 | 0.068 | 0.062 |
| GTPase | 154 ± 12 | 170 ± 18 | 0.91 | 0.073 | 0.063 |

* The activities of triphosphohydrolases are expressed in nmoles of the liberated phosphorus $min^{-1}$ mg $protein^{-1}$, 37 °C. Mean values ± SEM for 4–6 duplicate measurements are given. Concentrations of ATP (or GTP) were 3 mM.

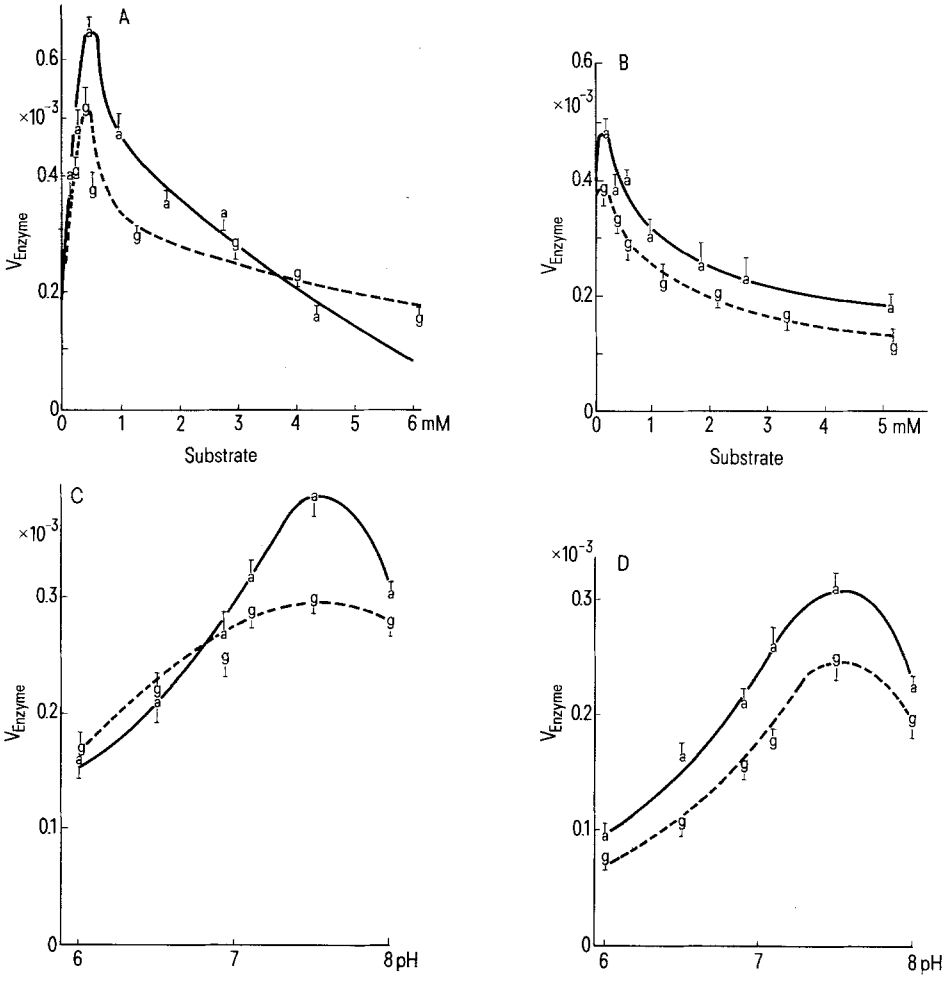


Fig. 1. The effects of different substrate concentrations and pH on ATPase (a——a) and GTPase (g——g) activities in isolated rat brain microvessels (A and C) and rat brain parenchyma (B and D). $V_{enzyme}$ is given as nmoles of phosphorus liberated $min^{-1}$ mg $protein^{-1}$, 37 °C. Vertical bars indicate SEM for 4–6 measurements, done in duplicate.

gated with glucoside concentrations varying from 0.1 mM to 3 mM. Protein concentrations were estimated according to Lowry et al.[13], using bovine serum albumin as the standard.

Electrophoresis was performed in 5% acrylamide gel for 20 h at 4 °C, with glycine-NaOH buffer (200 mM, pH 8.6), with a current of 1.5 mA/gel. After the cessation of current, gels were incubated for 30 min in mixtures that were essentially the same as described above, and afterwards stained for the phosphatase activities[14].

*Results and discussion.* Brain microvessels have noticeable GTPase activity (91% of parenchymal) compared to ATPase (table). It should be pointed out that GTPase activity in the capillaries is about 80% of that of ATPase, while in parenchyma it represents 65% of ATPase activity. Although sensitivity to ouabain is similar both for GTPase and ATPase in microvessels and parenchyma (table), microvessel GTPase is less sensitive to glucoside inhibition in the 0.1–1.0 mM range compared with ATPase (not shown). Strict parallelism between the two phosphohydrolases activities exists in the parenchyma.

When the nucleotide triphosphate concentrations are varied, a difference is noted in microvessels (figure 1,A) compared with parenchyma (figure 1,B). While in microvessels a clear distinction in ATPase and GTPase response is seen (figure 1,A), a strict parallelism in parenchyma is obvious (figure 1,B). Half-maximal inhibitory constants ($K_i$) are 0.350 mM for ATP and 0.232 mM for GTP in capillaries; corresponding values in parenchyma are 0.132 mM and 0.100 mM. 2 conclusions can be drawn from these observations: first, the microvessel enzyme(s) show less sensitivity to substrate inhibition, compared with parenchyma, and second, while $K_i$'s in microvessels are quite different from each other, values in parenchyma are similar.

Further evidence that a difference does exist between ATPase and GTPase in microvessels is obtained when the enzymatic activities are measured within the pH range 6–8. GTPase activity is increased to about 180% (at pH 7.5) from the lowest (at pH 6.0) in the capillaries (figure 1,C); ATPase activity increases to 240% from the lowest (same pH range as for GTPase). Also, the activity sharply decreases when the pH is changed from 7.5 to 8.0 in the case of ATPase; little change occurs in the microvessel GTPase activity in this pH region (figure 1,C). Parenchymal ATPase activity is more sensitive to the lowering of the pH (figure 1,D) as the activity is lowered to one-third (at pH 6.0) of the maximal (pH 7.5). GTPase activity runs parallel to that of ATPase (figure 1,D).

Figure 2 shows a rat brain microvessel preparation subjected to disc-acrylamide electrophoresis and subsequently stained for ATPase and GTPase activities; 2 bands of activity appear with GTP as the substrate and only one with ATP. 1 band, with slightly higher electrophoretic mobility, is at the same position regardless of the triphosphate used; this is indicative of 1 enzyme which is not strictly specific,

while the second is observable only with GTP (figure 2). In parenchyma tissue subjected to the same electrophoresis and staining conditions, a sole band of enzyme activity could be seen either with ATP or GTP (not shown), and it can be proposed that this ATPase is similar to the microvessel one. Protein staining (fast green) shows homogeneous protein bands at the sites of the enzymatic activities.

The kinetic studies indicate that in microvessels GTPase activity is distinct from ATPase. If ATPase is the nonspecific enzyme (i.e. uses either GTP or ATP as substrate) differences are to be attributed to the substrate-specific enzyme (or enzyme-like activity), GTPase.

On the other hand, in parenchyma, ATPase 'works' with either substrate, but is probably less active with GTP. This ATPase is perhaps similar to the capillary ATPase. Raison d'être of the separate GTPase in brain microvessels is unclear. One can speculate that 2 phosphohydrolase activities have to be separated in this brain compartment for the sake of the normal blood-brain functioning. Namely, ATPase is essential for the maintenance of the large potassium gradient between plasma and brain tissue[15], which occurs at the brain capillary surface[16]. Therefore, ATPase is to be strictly regulated according to its role in potassium transport. But GTP is very potent inhibitor of hexokinase (EC 2.7.1.1)[17], and there are indications that this enzyme participates in glucose transport from blood to brain[7,8]. It is obvious that hexokinase must be operative and regulated according to the energy demands of the brain tissue as a whole, and all 'locally-born' inhibitors such as GTP have to be removed as quickly as possible, but without interference with other systems (i.e. the ATPase-related transport system).

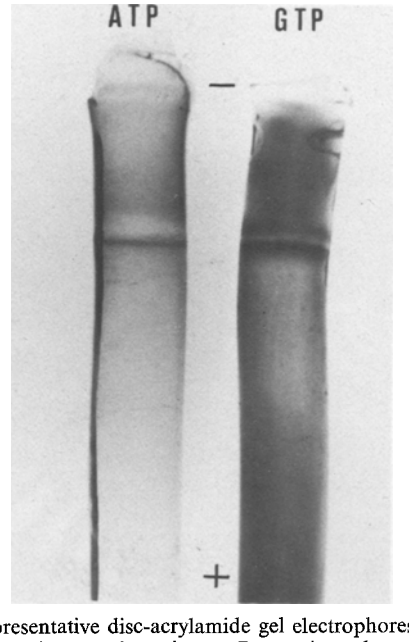


Fig. 2. Representative disc-acrylamide gel electrophoresis of isolated rat brain microvessel specimens. Respective substrates are indicated on the picture.

1 This work was supported by the research grant No. 214 from ZMNU of Serbia.
2 To whom reprint requests should be addressed.
3 J.L. Dahl and L.E. Hokin, A. Rev. Biochem. *43*, 327 (1974).
4 D. Desaiah and I.K. Ho, Biochem. Pharmac. *26*, 2029 (1977).
5 R. Balàsz and J.E. Cremer, in: Compartmentation in the brain, p. 361. Ed. R. Balàsz and J.E. Cremer. Wiley & Sons, New York 1972.
6 R. Katzman, in: Dynamics of brain edema, p. 373. Ed. H.M. Pappius and R. Feindel. Springer, Berlin 1976.
7 B.M. Djuričić, Lj. Rogač, M. Spatz, Lj.M. Rakić and B.B. Mršulja, in: Advances in Neurology, vol. 20, p. 197. Ed. J. Cervós-Navarro, E. Betz, G. Ebhardt, R. Ferszt and R. Wüllenweber. Raven Press, New York 1978.
8 B.M. Djuričić and B.B. Mršulja, Experientia *35*, 169 (1979).
9 W. Schoner, R. Beusch and R. Kramer, Eur. J. Biochem. *7*, 102 (1968).
10 Y.E. Shamoo and W.A. Brodsky, Biochim. biophys. Acta *203*, 111 (1970).
11 B.M. Djuričić and B.B. Mršulja, Brain Res. *138*, 561 (1977).
12 O.H. Lowry and J.A. Lopez, J. biol. Chem. *162*, 421 (1946).
13 O.H. Lowry, N.J. Rosebrough, A.L. Farr and J.P. Randal, J. biol. Chem. *193*, 265 (1951).
14 E.N. Goren, A.H. Hirsch and O.M. Rosen, Analyt. Biochem. *43*, 156 (1971).
15 G.W. Goldstein, in: Advances in Neurology, vol. 20, p. 11. Ed J. Cervós-Navarro, E. Betz, G. Ebhardt, R. Ferszt and R. Wüllenweber. Raven Press, New York 1978.
16 J.A. Firth, Experientia *33*, 1093 (1977).
17 D.L. Purich and H.J. Fromm, J. biol. Chem. *246*, 3456 (1971).

## Evidence for the existence of two carbonic anhydrase isozymes in rabbit ciliary body

K. Kishida and N. Ochi

*Department of Ophthalmology, Osaka University Medical School, Osaka 533 (Japan), 17 April 1979*

*Summary.* Two carbonic anhydrase isozymes were found in the rabbit ciliary body. Their isoelectric points were determined to be pH 6.6 and pH 7.4, respectively. The total activity of the higher isoelectric point isozyme was higher than that of the lower isoelectric point isozyme.

Carbonic anhydrase (E.C. 4.2.1.1) is believed to play an important role in aqueous humor formation in the ciliary body[1]. Although 2 isozymes of the enzyme (CA I and CA II) have now been isolated from many mammalian tissues[2], no one has ever succeeded in isolating the isozyme(s) from the rabbit ciliary body. Using the gel electrofocussing technique[3], the authors have isolated 2 carbonic anhydrase isozymes from the rabbit ciliary body.

*Materials and methods.* Erythrocyte-free ciliary body was obtained as follows: An albino rabbit was anesthetized with sodium pentobarbital and 5000 units of heparin sodium was i.v. injected. After the heparinization the rabbit was perfused (via the carotid artery) with 5 l of physiological saline (the 1st l of saline containing 10000 units of heparin sodium, the 2nd l containing 5000 units of heparin sodium and the last 3 l containing no heparin sodium). The absence of red cells in the perfusate was checked by the determination of iron by atomic absorption photometry (Model 170-50 Atomic Absorption Spectrophotometer, Hitachi, Japan). After the perfusion the eyeball was enucleated and the ciliary body was excised under a microscope. 100 mg of the ciliary body was homogenized with 100 µl of 0.6 M sucrose solution. After centrifugation the homogenate was subjected to gel electrofocussing. The gel electrofocussing was