Purification and characterization of metabolically active capillaries of the blood–brain barrier

Lise DALLAIRE, Lise TREMBLAY and Richard BÉLIVEAU*

Laboratoire de Membranologie Moléculaire, Département de Chimie-Biochimie, and Groupe de Recherche en Transport Membranaire, Université du Québec à Montréal, C.P. 8888, Succ. A, Montréal, Québec, Canada H3C 3P8

Microvessels were isolated from bovine and rat cerebral cortex by simple procedures involving mechanical homogenization, differential and density-gradient centrifugation, and chromatography on a column of glass beads. The preparations were composed of short capillaries with a diameter of 1–10 μm . Both purifications were monitored by assaying the activity of the marker enzyme γ -glutamyl transpeptidase (γ -GTase). The final bovine and rat preparations were enriched 20- and 14-fold over the homogenate respectively. γ -GTase activity was measured in different fractions after bovine and rat membranes were solubilized with 0.5% and 0.3% Triton X-100 respectively. Measurement of 5'-nucleotidase and acetylcholinesterase activities indicated very low levels of contamination of the microvessel preparations by glial cells and neurons. The integrity of the capillary membranes was confirmed by the assay of a cytosolic marker enzyme, lactate dehydrogenase. Viability of the microvessels was demonstrated by the presence of detectable levels of adenylates and by tissue respiration induced by glucose and succinate. Comparison of the proteins of homogenized bovine and rat brain cortex with those of purified capillaries separated by SDS/PAGE revealed enrichment of at least three predominant proteins of 14, 16 and 18 kDa in the capillary preparations. It is concluded that these methods allow rapid isolation of small blood vessels of the blood–brain barrier which are suitable for metabolic and structural studies *in vitro*.

INTRODUCTION

Brain capillary endothelial cells together with pericytes, astroglial cells and the basal membrane maintain a unique selective barrier between blood and brain [1]. These endothelial cells lack fenestrations and are linked together by tight junctions, forming continuous belts. They differ from the capillaries of muscle and mesentery by showing a paucity of pinocytosis and transendothelial channels [2,3]. The blood–brain barrier regulates all exchanges which occur between the brain and blood circulation by transport systems and receptors located on the luminal and anti-luminal membranes. Carrier-specific transport of nutrients such as glucose [4,5], amino acids [6,7], monocarboxylic acids [6] and choline [8,9] have been described. Receptors for neurotransmitters [10,11], insulin [12] and other hormones [13] have also been demonstrated.

The biochemical events controlling these activities remain relatively unclear, due to the difficulty of obtaining brain microvessels suitable for studies *in vitro*. Several laboratories have reported methods for isolating brain capillaries from human, bovine, rabbit, dog and rat [14–23]. However, the yields obtained are generally very low and there has been no detailed biochemical characterization of the fractions obtained by these procedures, a microscopic examination often being the sole criterion used for the evaluation of the purification. An optimization of these methods is desirable, in order to avoid artifactual interference from contaminating neuronal and glial cells.

In this paper, we describe rapid and simple isolation procedures, involving mechanical homogenization, purification with dextran or Ficoll, and use of glass beads columns, for the preparation of bovine and rat brain capillaries with high yields. The small blood vessels prepared by these techniques were morphologically intact and their diameters ranged from 1 to 10 μm . They appear free of neuronal and glial contamination

and are highly enriched for the marker enzymes γ -glutamyl transpeptidase (γ -GTase) and alkaline phosphatase.

MATERIALS AND METHODS

Dextran T-70, dextran T-500 and Ficoll T-400 were obtained from Pharmacia LKB Biotechnology (Montréal, Québec, Canada). Glass beads were purchased from Chromatographic Specialties (Brockville, Ontario, Canada). Reagents for the electrophoresis were products of Bio-Rad (Mississauga, Ontario, Canada). NADH and phosphoenolpyruvate were from Boehringer (Laval, Québec, Canada) and [^{14}C]glucose was from NEN–Dupont (Boston, MA, U.S.A.). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Isolation of bovine brain capillaries

Fresh bovine brains were obtained from a local abattoir on the morning of the capillary isolation and were transported to the laboratory in ice-cold physiological buffer (PB) composed of 147 mM-NaCl, 4 mM-KCl, 3 mM- CaCl_2 , 1.2 mM- MgCl_2 , 5 mM-glucose and 15 mM-Hepes, pH 7.4. Microvessels were isolated using modifications of the mechanical homogenization technique of Pardridge *et al.* [22]. To minimize proteolysis, all steps were carried out at 4 °C. Bovine brain cortex (approx. 100 g) was freed of the pial membrane and minced in 500 ml of PB containing 5 mM-EDTA, pH 7.4. Samples (150–200 ml) were homogenized with a Polytron (Brinkman Instruments, Rexdale, Ontario, Canada) run at 6000 rev./min for 15 s. The combined homogenates were mixed with an equal volume of 26% (w/v) dextran T-70 in PB. The suspension was centrifuged at 25000 *g* for 10 min. The pellet was resuspended in 30 ml of PB and passed through a 250 μm nylon mesh screen (Tekto, Elmsford, NY, U.S.A.). The nylon mesh screen was rinsed with approx. 75 ml of

Abbreviations used: γ -GTase, γ -glutamyl transpeptidase; PB, physiological buffer; LDH, lactate dehydrogenase.

* To whom all correspondence should be sent.

PB and the filtrate was concentrated by centrifugation at 25000 *g* for 10 min. The pellet was resuspended in 30 ml of PB and passed through a 2.5 cm × 4.0 cm glass beads column [40/60-mesh (0.25 mm) glass beads]. The column was washed twice with 25 ml of PB over a 30 min period. The glass beads were transferred to a beaker and swirled vigorously (15 min at 4 °C) in PB to separate the microvessels from the beads. The beads were allowed to settle and the supernatant was decanted and kept at 4 °C. The beads were swirled for another 15 min in PB. The supernatants were pooled and the microvessels were collected by centrifugation at 25000 *g* for 10 min and kept at -180 °C in a cryopreservation solution (PB with 14 % glycerol and 1.4 % sorbitol, pH 7.4). The procedure is outlined schematically in Fig. 1(a).

Isolation of rat brain capillaries

Fifteen male Sprague-Dawley rats were killed by decapitation. The brains were removed immediately and cleared of meninges, superficial large blood vessels and choroid plexus. The cerebral cortex was minced in 4 vol. of PB. The present method represents a modification of the technique described by Lidinsky & Drewes [21] for isolating cerebral capillaries from dog brain cortex. Brain tissue was homogenized with a Potter-Elvehjem homogenizer by 7 up-and-down strokes at 400 rev./min of a Teflon pestle (clearance 0.15–0.23 mm). The homogenate was centrifuged at 3500 *g* for 10 min. The pellets were resuspended in 4 vol. of PB containing 20 % Ficoll T-400 and homogenized by 20 up-and-down strokes of a loose-fitting Teflon pestle (clearance > 0.23 mm). After centrifugation at 25000 *g* for 10 min, the myelin floating on top of the supernatant was removed. In order to form a discontinuous gradient, the pellets were resuspended in 18 ml of 15 % dextran T-500 and 9 ml was applied on to 3 ml of 20 % dextran T-500 and centrifuged in a swinging-bucket rotor at 25000 *g* for 10 min. The pellets were resuspended in 20 ml of PB and passed through a 2.5 cm × 3 cm glass beads column. The column was washed with 2 × 20 ml of PB. The isolated capillaries were collected by resuspension of the beads in 50 ml of PB, agitation, decantation and centrifugation at 3500 *g* for 10 min. The final pellet was resuspended in PB at a concentration of approx. 4 mg of protein/ml and stored in liquid nitrogen. Fig. 1(b) summarizes schematically the purification procedure.

Enzyme assays

All enzymic reactions requiring a preincubation at 25 °C were assayed in the presence of a cocktail of proteinase inhibitors (2 µg of aprotinin/ml, 10 µg of pepstatin A/ml, 10 µg of chymostatin/ml and 100 µg of bacitracin/ml).

γ-GTase. γ-GTase activity was assayed using a slight modification of the method described by Orlowski & Meister [24]. Bovine fractions (0.1 ml) were preincubated for 60 min at 25 °C with 0.5 % Triton X-100, and rat fractions were preincubated for 5 min at 25 °C with 0.3 % Triton X-100. Enzymic activity was assayed at 37 °C by adding 0.9 ml of a solution containing 5.56 mM-L-γ-glutamyl-p-nitroanilide, 11 mM-MgCl₂ and 111 mM-Tris/HCl, pH 9. The reaction was allowed to proceed for 5–60 min and was terminated by the addition of 0.5 ml of 3 M-acetic acid. The solution was centrifuged at 14000 *g* for 5 min at 25 °C and the absorbance of the supernatant was measured at 410 nm.

Alkaline phosphatase. Alkaline phosphatase activity was measured by the method of Kelly & Hamilton [25] with some modifications. Samples (0.05 ml) of bovine brain homogenate (5 µg of protein) or capillaries (20 µg of protein) were preincubated for 45 min at 25 °C with 0.2 % SDS before starting the reaction with 0.45 ml of 32 mM-p-nitrophenyl phosphate in 1.5 M-propanol buffer, pH 10. The assay was run for 20 min (homogenate) or 5 min (capillaries) and terminated by the

Fig. 1. Capillary isolation schemes

Preparation of microvessels fraction from (a) bovine brain cortex and (b) rat brain cortex.

addition of 0.5 ml of 450 mM-NaOH. The samples were centrifuged for 5 min at 14000 *g* at 25 °C and the absorbance of the supernatant was measured at 410 nm.

Acetylcholinesterase. Acetylcholinesterase activity was assayed as described by Bergmeyer [26]. Samples containing 0.2 mg of protein were incubated for 30 min at 37 °C with 1.33 mM-acetylcholine in 0.1 M-Veronal buffer, pH 8.6.

5'-Nucleotidase. 5'-Nucleotidase activity was assayed according to the method described by Aronson & Touster [27], with the following modifications: 0.05 ml of bovine brain homogenate (0.2 mg of protein) or capillaries (0.4 mg of protein) was preincubated for 30 min at 25 °C with 0.2 % SDS, before the addition of 0.45 ml of a solution containing 50 mM-AMP (sodium salt), 0.1 M-MgCl₂ and 0.5 M-glycine, pH 9.1. After 20 min at 37 °C, the assays were stopped with 2.5 ml of 8 % trichloroacetic acid. The suspension was centrifuged at 3000 *g* for 10 min at 4 °C, and the supernatant (0.15 ml) was acidified with 0.15 ml of 2 M-HCl. Inorganic phosphate content was measured by the molybdate/ascorbic acid method described by Chen *et al.* [28].

Lactate dehydrogenase (LDH). LDH was assayed by the method of Moldéus & Högborg [29]. Capillaries were preincubated for 30 min with or without 0.5 % Triton X-100 at 37 °C. Samples containing 0.1 mg of protein were tested in a modified Krebs-Henseleit buffer (118 mM-NaCl, 4.8 mM-KCl, 0.96 mM-KH₂PO₄, 2.5 mM-MgSO₄, 2.9 mM-CaCl₂, 25 mM-NaHCO₃, 25 mM-Hepes, 1 % bovine serum albumin, pH 7.4), with 1.36 mM-pyruvate and 0.2 mM-NADH at 25 °C.

Adenylate content

The cellular adenylates (ATP and ADP) of bovine capillaries were measured by the method described by Goswami & Pande [30]. Bovine capillary proteins were first precipitated with 4 % HClO₄ and the ATP+ADP content was determined in the supernatant, which was adjusted to pH 7.0 by the addition of KOH.

Polyacrylamide-gel electrophoresis

PAGE was performed on a Bio-Rad apparatus. The gels (1.5 mm thick) were cast according to the method of Laemmli [31] with a 11 % (w/v) separating gel and 4 % (w/v) stacking gel. Proteins (60 µg) were solubilized in a sample buffer containing 1.25 % SDS, 12.5 % glycerol, 1.25 % mercaptoethanol and 0.001 % Bromophenol Blue in 0.063 M-Tris/HCl, pH 6.8, and boiled for 2 min. Following electrophoresis, the proteins were stained with 0.1 % Coomassie Blue in 30 % methanol/7 % acetic acid.

Measurement of respiration

Tissue respiration was measured by incubating bovine microvessels (2 mg of protein/200 µl) in a 1.8 ml thermostatically controlled (37 °C) and stirred chamber fitted with an oxygen electrode. The chamber was filled with freshly gassed modified Krebs-Henseleit buffer composed of 118 mM-NaCl, 4.8 mM-KCl, 0.96 mM-KH₂PO₄, 2.5 mM-MgSO₄, 2.9 mM-CaCl₂, 25 mM-NaHCO₃, 25 mM-Hepes and 5 mM-glucose, pH 7.4. The rate of oxygen consumption by the microvessels was measured in the presence of 5 mM-glucose and 10 mM-succinate.

RESULTS

Bovine microvessels isolated by the procedure described in the Materials and methods section were obtained with a high yield (660 µg of protein/g of cortex). Complete removal of the pial membrane was found to be essential for the successful isolation of these capillaries by mechanical homogenization. Centrifugation of the homogenate in dextran T-70 removed neuronal and glial material and gave a pellet (P₁, Table 1a; see Fig. 1 for definition of fractions) composed of capillaries, erythrocytes, nuclei and cell debris. P₁ was enriched 7-fold over the homogenate for the marker enzyme γ -GTase. Some capillaries were lost with the glial and neuronal material during this centrifugation, as S₁ and M₁ retained 21 and 19 % of the total γ -GTase activity respectively (Table 1a). The centrifugation with

dextran removed contaminant proteins, since S₁ and M₁ contained 85 % of the total proteins and were de-enriched for γ -GTase. The passage of P₁ through a nylon mesh screen was required to remove vessels with a diameter larger than 250 µm. The resulting preparation (P₂; Table 1a) was enriched 8.1-fold. P₂ was further purified on a glass beads column. The capillaries adhered to glass beads and the contaminants were eluted with the washings. As can be seen in Table 1(a), 70 mg of contaminant proteins, with minimal enrichments for γ -GTase of 1.44 and 0.06 respectively, were removed in the first and second washings of the column (E₂ and E₃). The purification of P₂ on the column more than doubled its γ -GTase specific activity and enrichment. The final preparation retained 23 % of the total γ -GTase activity and was enriched 20-fold for a total yield of 660 µg of protein/g of cortex (Table 1a).

Application of this purification procedure to isolate rat brain capillaries has had limited success (results not shown). Rat brain microvessels were thus obtained by a method modified from that of Lidinsky & Drewes [21], with a high recovery of total proteins (500 µg/g of cortex). The total yields of protein and γ -GTase activity and the specific activities and enrichment factors for γ -GTase are shown in Table 1(b). The low-speed centrifugation of the initial homogenate removed 25 % of the protein. These proteins, which remained in the supernatant (S₁), had a low γ -GTase specific activity. The pellet (P₁) was resuspended and homogenized in PB containing 20 % Ficoll T-400, and myelin and neuronal material were removed by centrifugation. At this step, mild homogenization conditions were required in order to minimize damage to capillaries. For this reason, the microvasculature could not be freed completely from glial cells, and 31 % of the total γ -GTase activity remained associated with the myelin and neuronal material (S₂). The enrichment factor for this fraction was nevertheless small compared with that of the pellet (P₂), which contained 22 % of the total activity and 5 % of the total protein of the initial homogenate (Table 1b). A discontinuous gradient of 15 % and 20 % dextran T-500 was then used to remove myelin and neuronal material from P₂. In contrast with the method described by Lidinsky & Drewes [21], the present procedure yielded most of the activity (80 %) in the resulting pellet (P₃) instead of at the 15 %/20 % dextran interface. The enrichment factor, which was 5-fold in P₂, was increased to 8.2-fold in P₃. Mild conditions of homogenization were again required to avoid complete disruption of the tissue; the latter increased the amount of protein at the 15 %/20 % dextran interface and resulted in low capillary yields (results not shown). After the purification of P₃ on the column, 78 % of the activity present in P₃ was found in the final fraction. The γ -GTase specific activity was 14-fold higher in the purified microvessels than in the brain cortex homogenate. The capillary fraction contained 13.5 % of the total γ -GTase activity and 1 % of the total protein, or about 6 mg of protein from 12 g of cortical tissue (Table 1b).

Examination of bovine and rat purified capillaries by phase-contrast microscopy revealed that both preparations were composed of twisted plexuses of microvasculature and were devoid of large blood vessels. The microvessels exhibited external diameters ranging from 1 to 10 µm (Figs. 2a and 2b).

γ -GTase was chosen as the marker enzyme to monitor each step of the capillary purification. However, because γ -GTase is a membrane-associated enzyme with its catalytic domain facing the cytoplasm of the endothelial cell, the measurement of its activity had to be optimized for each fraction. γ -GTase activity of intact cells from bovine microvessels was thus studied in the presence of different detergents to solubilize the membranes and to facilitate the access of the substrate to the enzyme. Bovine homogenate and capillary fractions were preincubated for 5 min

Table 1. Distribution of protein and γ -GTase activity during the purification of brain capillaries

Fractions were preincubated (a) for 60 min at 25 °C with 0.5 % Triton X-100, or (b) for 5 min at 25 °C with 0.3 % Triton X-100 before measuring γ -GTase activity. Enrichment was the ratio of specific activities in a fraction compared with the cortex homogenate. N.D., not detectable. Enzymic assay was linear with regard to protein concentration and incubation time. Values represent the means $\pm$ S.E.M. for three preparations. See the Materials and methods section for details (fractions are those defined in Figs. 1a and 1b).

Fraction	Specific activity (nmol/min per mg)	Enrichment	Total protein (mg)	Total activity (μ mol/min)	Protein yield (%)	Activity yield (%)
(a) Bovine						
Homogenate	5.2 $\pm$ 0.4	1.00	5560 $\pm$ 330	28.4 $\pm$ 0.6	100	100
S ₁	1.7 $\pm$ 0.5	0.33 $\pm$ 0.09	2930 $\pm$ 15	5.9 $\pm$ 0.9	51 $\pm$ 4	22 $\pm$ 5
M ₁	3.6 $\pm$ 1.1	0.7 $\pm$ 0.2	1800 $\pm$ 600	5.5 $\pm$ 0.8	31 $\pm$ 8	19 $\pm$ 3
P ₁	36.1 $\pm$ 2.3	7.0 $\pm$ 0.3	435 $\pm$ 60	16 $\pm$ 1	7.7 $\pm$ 0.6	55 $\pm$ 6
A ₁	67.6 $\pm$ 5.5	13.3 $\pm$ 1.7	7 $\pm$ 1	0.51 $\pm$ 0.07	0.14 $\pm$ 0.00	1.8 $\pm$ 0.3
P ₂	41.9 $\pm$ 4.4	8.1 $\pm$ 0.3	322 $\pm$ 29	13.3 $\pm$ 0.3	5.8 $\pm$ 0.2	47 $\pm$ 1
E ₁	2.3 $\pm$ 2.5	0.4 $\pm$ 0.3	4 $\pm$ 2	0.02 $\pm$ 0.01	0.08 $\pm$ 0.05	0.2 $\pm$ 0.2
E ₂	7.2 $\pm$ 0.4	1.44 $\pm$ 0.04	62 $\pm$ 5	0.45 $\pm$ 0.05	1.1 $\pm$ 0.2	1.6 $\pm$ 0.2
E ₃	2.1 $\pm$ 2.1	0.06 $\pm$ 0.06	8 $\pm$ 1	0.02 $\pm$ 0.02	0.15 $\pm$ 0.03	0.07 $\pm$ 0.07
Capillaries	101 $\pm$ 6	19.6 $\pm$ 0.3	66 $\pm$ 6	6.6 $\pm$ 0.4	1.18 $\pm$ 0.07	23 $\pm$ 1
(b) Rat						
Homogenate	4.5 $\pm$ 0.5	1.0	603 $\pm$ 32	2.6 $\pm$ 0.5	100	100
S ₁	0.9 $\pm$ 0.2	0.20 $\pm$ 0.04	150 $\pm$ 20	0.14 $\pm$ 0.02	25 $\pm$ 4	4 $\pm$ 2
P ₁	3.8 $\pm$ 0.3	1.0 $\pm$ 0.20	410 $\pm$ 20	1.6 $\pm$ 0.2	67 $\pm$ 2	65 $\pm$ 19
S ₂	3.0 $\pm$ 0.7	0.7 $\pm$ 0.20	355 $\pm$ 9	1.1 $\pm$ 0.2	60 $\pm$ 2	31 $\pm$ 15
P ₂	21 $\pm$ 6	5 $\pm$ 1	28 $\pm$ 9	0.56 $\pm$ 0.01	5 $\pm$ 2	22 $\pm$ 4
S ₁₅ %	2 $\pm$ 1	0.4 $\pm$ 0.2	6 $\pm$ 2	0.01 $\pm$ 0.01	1.0 $\pm$ 0.2	4.0 $\pm$ 0.2
Interface	7.0 $\pm$ 0.6	1.7 $\pm$ 0.4	3 $\pm$ 1	0.02 $\pm$ 0.01	0.4 $\pm$ 0.1	0.7 $\pm$ 0.3
S ₂₀ %	10 $\pm$ 4	2.2 $\pm$ 0.8	2.0 $\pm$ 0.6	0.02 $\pm$ 0.00	0.3 $\pm$ 0.1	0.6 $\pm$ 0.2
P ₃	35 $\pm$ 6	8.2 $\pm$ 0.4	13 $\pm$ 3	0.45 $\pm$ 0.07	2.0 $\pm$ 0.5	18 $\pm$ 4
E1	N.D.	N.D.	2 $\pm$ 1	N.D.	0.2 $\pm$ 0.1	N.D.
E2	N.D.	N.D.	2 $\pm$ 1	N.D.	0.4 $\pm$ 0.1	N.D.
E3	N.D.	N.D.	0.5 $\pm$ 0.4	N.D.	0.12 $\pm$ 0.01	N.D.
S ₄	N.D.	N.D.	2 $\pm$ 1	N.D.	0.5 $\pm$ 0.2	N.D.
Capillaries	61 $\pm$ 7	14 $\pm$ 2	6 $\pm$ 1	0.35 $\pm$ 0.07	1.0 $\pm$ 0.2	14 $\pm$ 1

at room temperature with 0.2 % or 1 % of Triton X-100, SDS or deoxycholate (Table 2). Triton X-100 at 1 % was the only detergent to increase the γ -GTase specific activity in both fractions. γ -GTase activities of the homogenate and capillaries were increased by 17 and 26 % respectively. Although 0.2 % deoxycholate had no influence on the activity of γ -GTase in the capillary fraction, it inhibited γ -GTase in the homogenate by 20 %. SDS at 0.2 % and 1 % and deoxycholate at 1 % inhibited the capillary enzyme activity by 28 % and 60 % respectively.

The γ -GTase activity of bovine and rat homogenate and capillaries was analysed further as a function of Triton X-100 concentration and preincubation time at 25 °C. Bovine enzyme activity was maximal after 60 min of preincubation in the presence of 0.5 % Triton X-100. The γ -GTase specific activity increased by 30 % in the homogenate fraction and by 55 % in the capillary fraction (Fig. 3a). The maximal γ -GTase activity in the rat preparation was observed after 5 min of preincubation with 0.3 % Triton X-100. A large increase in the enzyme activity was obtained in the rat cortex homogenate (67 %), and a much smaller stimulation occurred in purified rat capillaries (17 %) (Fig. 3b). Consequently, the enrichment for bovine and rat capillaries in each fraction of the purification was determined under those standard conditions. Under these experimental conditions, the bovine final capillary enrichment increased from 16.5- to 19.6-fold (Table 3a), while rat final capillary enrichment decreased from 20- to 14-fold (Table 3b).

The contamination of capillaries by larger vessels, neurons and glial cells was examined by the analysis of alkaline phosphatase, acetylcholinesterase and 5'-nucleotidase activities respectively (Table 4). Alkaline phosphatase, 5'-nucleotidase and acetylcholinesterase were also studied in the presence of different

Fig. 2. Phase-contrast photomicrograph of isolated brain microvessels

Occasional branches and bifurcations characterize bovine and rat preparations (a and b respectively). The vessel diameter ranged from 1 to 10 μ m. The bar represents 10 μ m.

Table 2. γ -GTase activity of bovine brain cortex homogenate and capillaries in the presence of detergents

Bovine cortex homogenate and capillaries (500 μ g of protein/100 μ l) were preincubated for 5 min at 25 °C with 0.2 and 1 % Triton X-100, SDS or deoxycholate, before measurement of γ -GTase activity. The assay was terminated after 35 or 4 min of incubation for the homogenate and capillaries respectively. The absorbance of the supernatant was read at 410 nm. Enzymic assay was linear with regard to protein concentration and duration of incubation. Specific activities are expressed in nmol/min per mg of protein. Enzymic activities of controls without detergent were 5.24 ± 0.02 (homogenate) and 69.7 ± 0.8 (capillaries) nmol/min per mg.

Detergent	Concn. ...	γ -GTase activity (nmol/min per mg)			
		Homogenate		Capillaries	
		0.2 %	1 %	0.2 %	1 %
Triton X-100		4.90 ± 0.07	6.32 ± 0.04	69 ± 1	90.2 ± 0.9
SDS		4.62 ± 0.01	4.0 ± 1.7	50 ± 1	50.4 ± 0.4
Deoxycholate		4.18 ± 0.03	3.5 ± 0.4	73 ± 2	28 ± 1

concentrations of detergent (Triton X-100, SDS and deoxycholate) and with different preincubation times, as described for γ -GTase. In summary, bovine brain cortex and capillaries had to be preincubated for 45 and 30 min with SDS to obtain maximum specific activities of alkaline phosphatase and 5'-nucleotidase respectively. Acetylcholinesterase activity was not increased by any of the three detergents tested (results not shown). Alkaline phosphatase, which is present in the luminal membrane of both microvessels and larger vessels [15], was enriched 10-fold in our preparation. 5'-Nucleotidase showed an identical low enrichment of 1.5-fold in the myelin fraction (M_1) and the capillary fraction. Acetylcholinesterase was not significantly enriched (0.5-fold) in the capillary fraction.

Fig. 3. Effect of Triton X-100 concentration of γ -GTase activity in brain cortex homogenate and capillaries

(a) Bovine homogenate and capillaries (200 μ g of protein) were preincubated for 60 min at 25 °C with Triton X-100. The reaction was carried out for 50 or 10 min at 37 °C for the homogenate ($\square$) and capillaries ($\blacksquare$) respectively. (b) Rat homogenate ($\square$) and capillaries ($\blacksquare$) (90 μ g of protein) were preincubated for 5 min at 25 °C with Triton X-100. The reaction was terminated after 15 and 60 min. The absorbance of the supernatant was read at 410 nm. Assays were linear with regard to protein concentration and incubation time. Values represent means $\pm$ S.E.M. for three assays done in triplicate.

The physical integrity of the bovine and rat capillaries was evaluated by measuring LDH activity. LDH is a cytosolic enzyme; detection of its presence outside the cells indicates major damage to the cell structure. In order to measure the activity of extracellular LDH, the bovine microvessel preparation was filtered through a 0.45 μ m-pore-size filter to retain the intact capillaries. The filtrate of the capillary fraction gave a low LDH activity of 0.04 μ mol/min, which represents only 0.15 % of the total activity (Table 5). The release of LDH activity by detergent, to allow access of exogenous substrate to the enzyme, is another test to measure the integrity of a cell preparation. As shown in Table 5, solubilization by Triton X-100 caused 2.4- and 6.1-fold increases in the activity of LDH of bovine and rat capillaries respectively. The high activity detected in the bovine preparation in the absence of detergent was probably due to passive diffusion or carrier-mediated transport of pyruvate to the cytosol, since there was very little extracellular LDH present (Table 5).

Capillary viability was demonstrated by their ability to produce adenylates (ATP+ADP) and by the respiratory activity of mitochondria. Bovine endothelial capillary cells were found to contain 1.3 ± 0.2 nmol of adenylates/mg of protein. Basal O_2

Table 3. γ -GTase specific activity of homogenate and capillary fractions with and without Triton X-100

(a) Fractions (500 μ g of protein) were preincubated for 60 min at 25 °C with or without 0.5 % Triton X-100 before measuring γ -GTase activity. (b) Fractions (90 μ g of protein) were preincubated for 5 min at 25 °C with or without 0.3 % Triton X-100. Enrichment factors are the specific activities of the capillary fractions relative to the homogenate. Specific activities are expressed in nmol/min per mg of protein. Results are means $\pm$ S.E.M. for three experiments performed in triplicate.

Fraction	γ -GTase specific activity (nmol/min per mg)	
	Control	Triton X-100
(a) Bovine		
Homogenate	3.95 ± 0.04	5.15 ± 0.04
Capillaries	65.0 ± 0.5	101 ± 6
Enrichment	16.5 ± 0.5	19.6 ± 0.3
(b) Rat		
Homogenate	2.7 ± 0.3	4.5 ± 0.5
Capillaries	52 ± 4	61 ± 7
Enrichment	20 ± 2	14 ± 2

Table 4. Enzyme activities in bovine cortex homogenate, capillaries and M₁ fractions

Fractions were preincubated at 25 °C with 0.2 % SDS for 45 min for alkaline phosphatase and for 30 min for 5'-nucleotidase. Acetylcholinesterase was assayed without detergent. Fraction M₁ was obtained after centrifugation in 13 % dextran and contained glial and neuronal materials. Values represent means $\pm$ S.E.M. for 3–8 assays done in triplicate. Each assay was linear with regard to protein concentration and incubation time. n.d., not done. Values in parentheses indicate enrichment (fold) in the specific activity of the enzyme compared with that in the homogenate.

Fraction	Alkaline phosphatase		5'-Nucleotidase		Acetylcholinesterase	
	Specific activity (nmol/min per mg)	Total activity (μ mol/min)	Specific activity (nmol/min per mg)	Total activity (μ mol/min)	Specific activity (nmol/min per mg)	Total activity (μ mol/min)
Homogenate	78 $\pm$ 4	340 $\pm$ 20	100 $\pm$ 10	420 $\pm$ 70	46 $\pm$ 1	200 $\pm$ 20
M ₁	n.d.	n.d.	130 $\pm$ 30 (1.5 $\pm$ 0.2)	180 $\pm$ 30	60 $\pm$ 10 (1.2 $\pm$ 0.4)	80 $\pm$ 12
Capillaries	768 $\pm$ 93.8 (10 $\pm$ 2)	29 $\pm$ 6	140 $\pm$ 10 (1.5 $\pm$ 0.1)	5.7 $\pm$ 0.9	45 $\pm$ 7 (0.5 $\pm$ 0.3)	1.5 $\pm$ 0.1

Table 5. Assessment of functional integrity of microvessel fractions prepared from bovine and rat brain cortex

A and D, bovine and rat capillaries were assayed as described in the Materials and methods section. B, bovine capillaries were preincubated at 37 °C for 30 min with 0.5 % Triton X-100 and were then assayed as in A. C, bovine capillaries were filtered on a 0.45 μ m nitrocellulose filter. The analyses were done with the filtrate, as in A. E, rat capillaries were preincubated at 25 °C for 60 min with 0.5 % Triton X-100 and were then assayed as in D. Data are means $\pm$ S.E.M. of three different capillary preparations assayed in triplicate.

Preparation	LDH		
	Specific activity (nmol/min per mg)	Total activity (μ mol/min)	Total activity (%)
Bovine			
A. Capillaries	180 $\pm$ 20	11.5 $\pm$ 0.2	41
B. Capillaries + 0.5 % Triton	440 $\pm$ 60	28 $\pm$ 2	100
C. Capillary filtrate	1140 $\pm$ 130	0.04	0.15
Rat			
D. Capillaries	37.2 $\pm$ 0.2	0.21 $\pm$ 0.03	16
E. Capillaries + 0.5 % Triton	225.6 $\pm$ 0.6	1.3 $\pm$ 0.1	100

consumption (with 5 mM-glucose) by fresh and previously frozen bovine brain capillaries was 2.9 $\pm$ 1.0 and 1.2 $\pm$ 0.2 nmol of O₂/min per mg of protein, respectively. O₂ consumption was stimulated 3.5- and 8-fold by the addition of 10 mM-succinate to fresh and frozen bovine capillaries respectively.

PAGE of bovine and rat brain cortex homogenate and microvessel fractions revealed several major proteins (Fig. 4). Electrophoretic separation of bovine and rat cortex capillaries showed three major proteins of low molecular mass (14, 16 and 18 kDa). Bovine microvessels were also enriched in a 47 kDa protein which has been previously reported by Pardridge *et al.* [32], and in at least three other proteins, of 34, 58 and 116 kDa. Rat capillaries were also enriched in three major proteins of low molecular mass described above, and two other proteins of 34 and 116 kDa. Both bovine and rat protein patterns were unaffected by whether a preincubation was performed in the presence or absence of proteinase inhibitors.

DISCUSSION

The procedures described in this paper have proved to be rapid and reliable methods for the purification of bovine and rat brain microvessels. The total time required to isolate microvessels from

two bovine brains (100 g of cortex) was 5 h and that from 15 rats (12 g of cortex) was 3 h.

The use of a Polytron homogenizer to disperse bovine tissue considerably decreased the time required for sufficient disruption of the brain cortex and minimized the proteolytic damage done to the tissue. The softer texture of the rat cortex compared with bovine cortex required a milder homogenization procedure to minimize cell damage and fragmentation of rat brain capillaries.

High-speed centrifugation in dextran T-70 and Ficoll T-400 adequately separated bovine and rat capillaries from the non-vascular material, leaving a microvessel aggregate virtually free of myelin and other neuronal elements. High-speed centrifugation (25 000 *g*) gave a better separation than the low-speed centrifugation (3500 *g*) used by Pardridge *et al.* [22] (results not shown). Dextran or Ficoll replace advantageously sucrose and serum bovine albumin used in other methods, because they do not interfere with the protein assay. The protein contamination from bovine serum albumin or the high concentrations of sucrose, used in previously published methods, were a major problem for the measurement of the recovery of capillary protein. Furthermore, the use of high-molecular mass polysaccharides allows one to avoid possible damage caused by resuspending the cells in solution of high osmolarity.

Fig. 4. PAGE of bovine and rat brain cortex homogenate and microvessels

Bovine proteins are shown in (a) and rat proteins in (b). Homogenate (lanes 1 and 2) and capillaries (lanes 3 and 4) were separated on an SDS/11% polyacrylamide gel using the conditions of Laemmli [31]. Lanes 2 and 4 show fractions of homogenate and capillary proteins preincubated for 30 min at 25 °C with a cocktail of proteinase inhibitors (aprotinin; 2 µg/ml; pepstatin A, 10 µg/ml; chymostatin, 10 µg/ml; bacitracin, 100 µg). Molecular mass markers are: myosin, 200 kDa; β -galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

In order to remove contaminating material that would have otherwise interfered with the glass beads column, bovine preparations were filtered through a nylon mesh screen. Nylon mesh screens were used to enrich the bovine preparation. Furthermore, the washings of the nylon mesh screen increased γ -GTase total activity by 20% (results not shown). For the rat preparations, a discontinuous dextran gradient was used to increase the γ -GTase enrichment 1.6-fold. In contrast with the procedure previously described by Lidinsky & Drewes for the isolation of microvessels from canine brain cortex [21], our protocol prevents the rat microvascular material from staying at the 15%/20% interface of the discontinuous dextran gradient. This difference can be explained by the mild homogenization used in the present method. Most of the microvessels were recovered in the pellet, whereas contaminating material stayed at the interface.

Marker enzyme (γ -GTase) analysis of fractions obtained during the purification of bovine and rat microvessels demonstrated the usefulness of the centrifugation with dextran and Ficoll and the chromatography on glass beads. They increased the specific activity of bovine γ -GTase 7- and 2.5-fold respectively, to give isolated bovine microvessels enriched 20-fold. These same procedures increased rat γ -GTase specific activity by 5- and 1.7-fold, to enrich the final preparation 14-fold. These methods compare advantageously with previously published procedures in term of protein yield (660- and 500 µg of capillary protein/g of bovine and rat cortex respectively) and enrichment factor. Others have reported γ -GTase enrichments for bovine brain microvessels or microvessel plasma membranes ranging from 8.2- to 16-fold [15,32]. Other groups [17,20] have reported higher γ -GTase enrichment for rat capillary preparations, but the estimate of enrichment was based on enzymic studies done without detergent, and comparisons with our method are therefore difficult to make.

The importance of using a suitable detergent to solubilize the

membranes of intact cells, when analysing the activity of membrane marker enzymes (γ -GTase and alkaline phosphatase) from capillaries, should be pointed out. As noted in this report, the enrichment of microvessel marker enzymes, compared with the homogenate, changed dramatically depending on the animal species (bovine versus rat), the homogenization procedure (Polytron versus Potter) and the treatment of the resulting fractions with a suitable detergent. For example, rat capillaries were enriched 20-fold in γ -GTase activity when the assay was performed in the absence of detergent, but only 14-fold in the presence of Triton X-100 (Table 2b). The decrease in enrichment found in the presence of detergent can be explained by the stronger increase of γ -GTase specific activity in the homogenate fraction compared with the capillary fraction. This is probably caused by sequestration of the enzyme due to mild homogenization conditions.

The higher enrichment observed for bovine γ -GTase indicated that alkaline phosphatase was not a very good marker for capillaries. Indeed, Orlowski *et al.* [15] have shown that, although this enzyme is present in brain capillaries, it is predominant in larger vessels (arterioles and venules). Furthermore, Kreutzberg & Toth [33] reported intracellular activity of this enzyme in glial cells and some neurons.

5'-Nucleotidase was used as a marker enzyme for glial cell contaminants [33] in bovine microvessel preparations. The total activity of the 5'-nucleotidase in the bovine capillary fraction, which was only 1.4% of that of the homogenate, and its low enrichment strongly suggest no significant contamination by glial cells. However, the enrichment of 5'-nucleotidase in the capillary fraction was similar to that found in the M_1 fraction, confirming that the enzyme was present both in the glial cells and in the capillaries, as suggested earlier [33].

Acetylcholinesterase was used as a marker enzyme for contamination of neuronal origin [1,33]. The low enrichment measured in bovine capillary preparation indicates very little contamination by neurons. Acetylcholinesterase activity in the microvessel fraction was only 0.75% of that of the homogenate.

Separation of bovine and rat capillary proteins by SDS/PAGE revealed an enrichment of proteins in the 14–18 kDa range. These capillary proteins were also observed by Lidinsky & Drewes [21] and by Pardridge *et al.* [34]. The gels of the bovine capillaries also showed a 47 kDa protein which was not found in neurons, glial cells or capillaries of other tissues. This protein could be a component of the tight junctions [35]. Our bovine microvessel preparations were enriched for a protein of 58 kDa which could correspond to one of those described by Pardridge [35] as two phosphoproteins (53 and 55 kDa) specific for brain capillaries. The lack of significant proteolysis of the homogenate during the purification of the capillaries is suggested by the similarity in the protein patterns of the homogenate and the capillaries in the presence or absence of proteinase inhibitors during a 30 min incubation at 25 °C.

The integrity of the capillary membrane was studied by measuring the activity of the cytoplasmic marker LDH. The low LDH activity in the untreated rat capillaries corresponds to a high microvessel integrity of 84%. The absence of LDH activity in the bovine capillary filtrate indicates that the microvessels were sufficiently intact (> 90%) to sequester LDH. Consequently, the high LDH activity observed in intact bovine capillaries in the absence of Triton X-100 appears to be due to transport of pyruvate across the membrane. In fact, it was suggested that, since glial and endothelial cells are closely associated, removal of the astrocytes during the purification could make the microvessels more permeable to small molecules such as pyruvate without losing their main metabolic functions [36,37].

We were also able to detect significant quantities (1.25 nmol/mg of protein) of adenylates (ATP and ADP) in bovine capillaries. This strongly supports the conclusion that the isolation procedure gives intact and viable microvessels. Indeed, porous or leaky cells would destroy ATP with their membrane ATPases and make adenylates undetectable. These are the first ATP and ADP measurements reported for a brain capillary preparation. The strong stimulation of respiration observed with succinate lead us to conclude that bovine microvessels have the capacity to regenerate ATP. Indeed, metabolic activity was also demonstrated in these preparations by their ability to consume oxygen in the presence of adequate substrates. The stimulation observed in the presence of succinate was similar to that reported by Brendel *et al.* [16]. Tissue respiration demonstrated the integrity of the citric acid cycle, as the capillaries exhibited an endogenous rate of oxygen consumption which was markedly stimulated by succinate.

Bovine brain capillaries isolated by the procedure described herein are highly purified, metabolically active and viable. Purified rat capillaries, isolated by a different procedure, are obtained in large amounts and show functional and structural integrity. These methods for preparing microvessels may be useful to elucidate further the unique structural and metabolic features of the blood-brain barrier.

Special thanks are given to Dr. Vincent Vachon for his critical reading of the manuscript, to Mr. Michel Marion for technical support, to Mr. Denis Gingras for helpful advice and Mr. Ben Sulsky for his stimulating discussions. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR).

REFERENCES

1. Algers, G., Karlsson, B. & Sellström, A. (1986) *Neurochem. Res.* **11**, 661–670
2. Reese, T. S. & Karnovsky, M. J. (1967) *J. Cell Biol.* **34**, 207–217
3. Brightman, M. W. (1977) *Exp. Eye Res.* **25** (Suppl. 1), 1–25
4. Betz, A. L., Gilboe, D. D. & Drewes, L. R. (1978) in *Transport Phenomena in the Nervous System* (Levi, G., Batistin, L. & Lajtha, A., eds.), pp. 133–149, Plenum Press, New York
5. Dick, A. P. K., Harik, S. I., Klip, A. & Walker, D. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7233–7237
6. Pardridge, W. M. (1983) *Physiol. Rev.* **63**, 1481–1535
7. Hargreaves, K. M. & Pardridge, W. M. (1988) *J. Biol. Chem.* **263**, 19392–19397
8. Cornford, E. M., Braun, L. D. & Oldendorf, W. H. (1978) *J. Neurochem.* **30**, 299–308
9. Shimon, M., Egozi, Y., Kloog, Y., Sololovsky, M. & Cohen, S. (1988) *J. Neurochem.* **50**, 1719–1724
10. Peroutka, S. J., Moskowitz, M. A., Reinhard, J. F., Jr. & Snyder, S. H. (1980) *Science* **280**, 610–612
11. Hardebo, J. E. & Owman, C. (1980) *Ann. Neurol.* **8**, 1–11
12. Pardridge, W. M., Eisenberg, J. & Yang, J. (1985) *J. Neurochem.* **44**, 1771–1778
13. Pardridge, W. M. (1988) *Annu. Rev. Pharmacol. Toxicol.* **28**, 25–39
14. Joò, F. & Karnushina, I. (1973) *Cytobios* **8**, 41–48
15. Orlowski, M., Sessa, G. & Green, J. P. (1973) *Science* **184**, 66–68
16. Brendel, K., Meezan, E. & Carlson, E. C. (1974) *Science* **185**, 953–955
17. Goldstein, G. W., Wolinsky, J. S., Csejtek, J. & Diamond, I. (1975) *J. Neurochem.* **25**, 715–717
18. Mrsulja, B. B., Mrsulja, B. J., Fujimoto, T., Klatzo, I. & Spatz, M. (1976) *Brain Res.* **110**, 361–365
19. Betz, A. L., Firth, J. A. & Goldstein, G. W. (1980) *Brain Res.* **192**, 17–28
20. Hwang, S. M., Weiss, S. & Segal, S. (1980) *J. Neurochem.* **35**, 417–424
21. Lidinsky, W. A. & Drewes, L. R. (1983) *J. Neurochem.* **41**, 1341–1348
22. Pardridge, W. M., Eisenberg, J. & Yamada, T. (1985) *J. Neurochem.* **44**, 1178–1184
23. Kasanicki, M. A., Cairns, M. T., Davies, A., Gardiner, R. M. & Baldwin, S. A. (1987) *Biochem. J.* **247**, 101–108
24. Orlowski, M. & Meister, A. (1965) *J. Biol. Chem.* **240**, 338–347
25. Kelly, M. H. & Hamilton, J. R. (1970) *Clin. Biochem.* **3**, 33–43
26. Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis*, (Bergmeyer, H. U., ed.), pp. 840–844, Academic Press, New York
27. Aronson, N. N. & Touster, O. (1974) *Methods Enzymol.* **31**, 92
28. Chen, P. S., Jr., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
29. Moldéus, D. & Högborg, J. (1978) *Methods Enzymol.* **57**, 60–71
30. Goswami, T. & Pande, S. V. (1984) *J. Biochem. Biophys. Methods* **9**, 143–151
31. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
32. Pardridge, W. M., Yang, J. & Eisenberg, J. (1985) *J. Neurochem.* **45**, 1141–1147
33. Kreutzberg, G. W. & Toth, L. (1983) *Acta Neuropathol. Suppl.* **8**, 35–41
34. Pardridge, W. M., Yang, J., Eisenberg, J. & Mietus, L. J. (1986) *J. Cereb. Blood Flow Metab.* **6**, 203–211
35. Pardridge, W. M. (1986) *Endocrine Rev.* **7**, 314–330
36. Bradbury, M. (1979) *Trends Neurol. Sci.* **2**, 36–38
37. Betz, A. L. & Goldstein, G. W. (1986) *Annu. Rev. Physiol.* **48**, 241–250

Received 15 January 1991; accepted 13 February 1991