

# *Acta Medica Okayama*

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*Volume 44, Issue 1*

1990

*Article 3*

FEBRUARY 1990

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## Abstract

Peptides and proteins in the extracellular space in the central nervous system were investigated in vivo using an intracerebral microdialysis probe. The molecular cut-off of the hollow fiber which was used for the probe was approximately 100 kDa. We examined recovery rates of several compounds in vitro. The recovery rates of proteins and peptides were between 7-28%, with the exceptions of substance P and insulin-like growth factor I. The recovery rates of monoamines and their metabolites were 22-40%. In in vivo studies, two major proteins with apparent molecular weights of 62 kDa and 12 kDa, and several minor proteins (28 kDa, 43 kDa, 52 kDa and 70 kDa) were detected by SDS-polyacrylamide gel electrophoresis in the dialysate from a probe implanted in the striatum of anesthetized rats. These results suggest that the newly developed, intracerebral microdialysis probe might be useful for investigating the dynamic changes of peptides and proteins in the central nervous system.

**KEYWORDS:** protein, peptide, microdialysis, extracellular space, probe

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\*PMID: 2330841 [PubMed - indexed for MEDLINE]

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## **In Vivo Analysis of Extracellular Proteins in Rat Brains with a Newly Developed Intracerebral Microdialysis Probe**

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Peptides and proteins in the extracellular space in the central nervous system were investigated *in vivo* using an intracerebral microdialysis probe. The molecular cut-off of the hollow fiber which was used for the probe was approximately 100kDa. We examined recovery rates of several compounds *in vitro*. The recovery rates of proteins and peptides were between 7-28 %, with the exceptions of substance P and insulin-like growth factor I. The recovery rates of monoamines and their metabolites were 22-40 %. In *in vivo* studies, two major proteins with apparent molecular weights of 62 kDa and 12kDa, and several minor proteins (28kDa, 43kDa, 52kDa and 70kDa) were detected by SDS-polyacrylamide gel electrophoresis in the dialysate from a probe implanted in the striatum of anesthetized rats. These results suggest that the newly developed, intracerebral microdialysis probe might be useful for investigating the dynamic changes of peptides and proteins in the central nervous system.

**Key words :** protein, peptide, microdialysis, extracellular space, probe

There are many neural tissue-specific proteins in the brain, which may be classified into soluble acidic, cytoskeletal, receptor, and ion channel proteins (1). Some of the proteins in the extracellular space might be released from neurons or glial cells as certain signal transmitters and/or neural trophic factors. To investigate the dynamic changes of peptides and proteins in the extracellular space of brains *in vivo*, the push-pull perfusion technique and intracerebral microdialysis method have been applied (2-5). However, these methods have some limitations. In the push-pull technique, there is the possibility of tissue damage by the pressured-stream of

experimental fluid. In the microdialysis method, the molecular size of neurotransmitters which can be analyzed is limited. Very few papers on the analysis of extracellular proteins in the brain by microdialysis have been communicated (3).

In order to study the dynamic changes of peptides and proteins in the extracellular space *in vivo*, we developed a new probe made of hollow ultrafiltration fiber for intracerebral microdialysis. Both *in vitro* recovery rates of peptides and proteins using the new probe and tissue damage due to the implantation of the probe were studied as a basic control. The probe was implanted unilaterally in the striatum of anesthetized rats, and then peptides and proteins in the dialysates obtained from the probe were analyzed.

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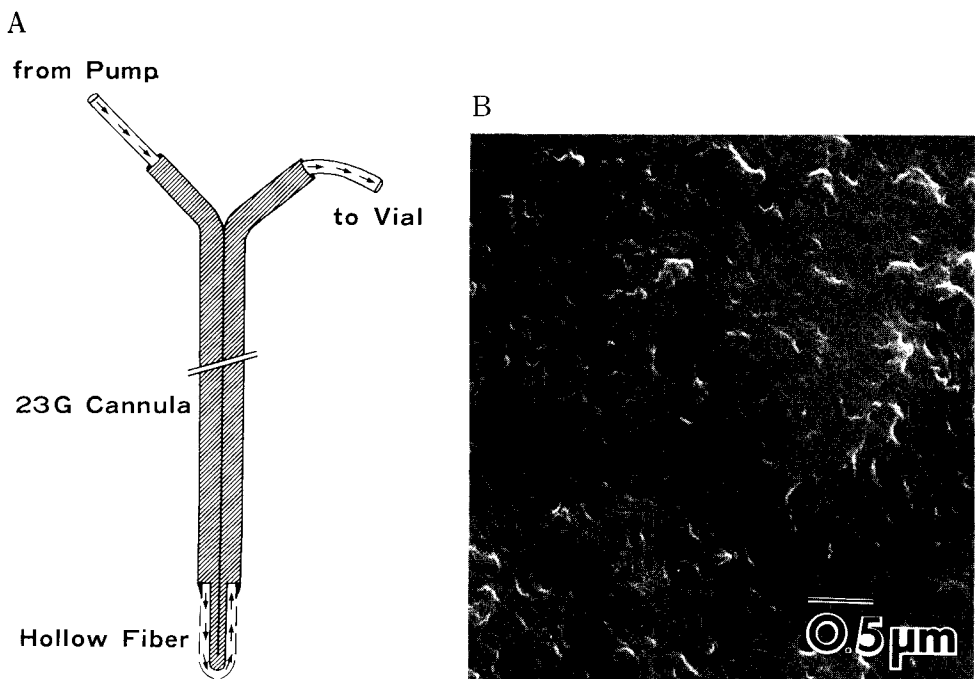
## Materials and Methods

**Materials.** A new hollow fiber made of ultrafiltration membrane for microdialysis, Evaflex 4A, was courteously supplied by Kuraray Medica, Kitaku, Osaka, Japan. Molecular weight markers, and phenylsepharose for the purification of S-100 protein were purchased from Pharmacia-LKB Biotech., Uppsala, Sweden. Prestained molecular weight markers and Coomassie Brilliant Blue (CBB) solution for the determination of protein concentration were obtained from Bio-Rad Labs., Richmond, CA, USA. Proteins and peptides as basic controls were obtained from Peptide Institute Inc., Minoh, Japan. All chemicals were special reagent grade and were obtained from Wako Pure Chemical Ind., Ltd., Osaka, Japan. Twenty-three gauge cannulae were purchased from Top, Tokyo, Japan.

**Making the probe.** Fig. 1 is a diagram of the probe (A) and an electron micrograph of the surface of the hollow fiber (B)(Hitachi S-550 scanning electron microscope, accelerating voltage of 15 KV). The microdialysis probe was made of a hollow fiber and a 23 gauge cannula. The hollow fiber was made from ultrafiltration membrane,

composed of an ethylenevinyl alcohol polymer, with a molecular cut-off of approximately 100,000 daltons. The outer diameter of the hollow fiber was 0.3mm, and the inner diameter was 0.2mm. The 23-gauge cannula was 8 cm in length. Half of the circumference of the cannula was longitudinally cut away for 4 mm at the middle of the cannula, and the cannula was bent back to back into a U-shape, with the cut-away portion on the outside. The hollow fiber, which was supported with a nylon fiber in the lumen, was pushed through the cannula and secured with cement.

**Determination of recovery rates of peptides and proteins in vitro.** The probe was held with a small gripper mounted on a stereotaxic instrument. It was immersed into a solution containing known amounts of several peptides and proteins (1mg/ml). One end of the probe was connected to a microinfusion pump (IP-2 microinjection pump, Bio-Research Center, Nagoya, Japan). Perfusion was conducted by delivering physiological saline at  $2\mu\text{l}/\text{min}$  through the microinfusion pump. The dialysate was collected every 10 min, and the concentrations of peptides and proteins in the dialysate were determined by the dye-binding method (6) or the fluores-



**Fig. 1** Diagram of the U-shaped probe for microdialysis and an electromicroscopic image of the surface of the hollow fiber. A : U-shaped probe. B : The surface of the hollow fiber (Kuraray Evaflex 4A, molecular cut-off of 100kDa) glued into a 23 gauge cannula.

camine binding method (7). Dopamine, its metabolites and 5-hydroxyindoleacetic acid were measured using a high pressure liquid chromatographic apparatus (655-12 A, Hitachi, Tokyo, Japan) with an electrochemical detection device system (HPLC/ECD)(Eicom, Kyoto, Japan). The column was octadecyl sulfate, and the elution buffer contained 0.1M potassium phosphate (pH 3.5), 30 % methanol, 1mM EDTA and sodium octanesulfonate (160mg/l). The analytical voltage of the ECD was set at +0.75mV. Noradrenaline was measured by fluorometry using diphenyl ethylenediamine (automatic catecholamine analyzer, HLC-8030, Tosoh, Tokyo, Japan).

*Analysis of the dialysate in vivo.* Adult Sprague-Dawley rats (250–350 g) were anesthetized with diethylether, and fixed in a stereotaxic instrument. A hole was drilled in the skull. The probe was implanted unilaterally into the striatum (co-ordinates of the tip of the dialysis probe were: rostral, +1.0mm; lateral, 2.5mm; ventral, 5.0mm relative to the bregma and dura surface)(8). Perfusion was done by the delivery of physiological saline using a microinjection pump. The dialysate was collected for approximately 500min after a washout period of 3h. The dialysate was analyzed by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (9), and gel permeation chromatography (10). The column was TSK 3,000SW (Tosoh). The elution buffer contained 50mM Tris-HCl (pH 7.2), 150mM NaCl and 2mM EDTA. The flow rate was 0.8ml/min, and the protein peaks were detected at 280nm with a UV monitor.

*Serum and cerebrospinal fluid (CSF).* Blood samples (1–2 ml) were taken from the rat hearts, and were left for 60min at room temperature. The samples were centrifuged for 10min at 3,000rpm. The clear supernatants were stored at  $-80^{\circ}\text{C}$  until analysis.

The rats were anesthetized, and their heads were placed downward to achieve flexion at the atlanto-occipital joint. A midline incision was performed to expose the atlanto-occipital membrane below the neck muscles. CSF was drawn from the cerebello-medullary cistern. The sample was frozen and stored at  $-80^{\circ}\text{C}$ .

*SDS-PAGE and immunoblotting.* The proteins in the dialysates from the striatum, CSF and serum were analyzed by SDS-PAGE. The proteins of the dialysate and CSF were precipitated with 10 % trichloroacetic acid. The amount of protein in each samples of dialysate, CSF of serum was 16  $\mu\text{g}$ . The precipitates were dissolved in sample buffer (10  $\mu\text{l}$ ) containing 2 % SDS, 2.5 %  $\beta$ -mercaptoethanol, 11 % glycerol and 0.005 % bromo-

phenol blue in 50mM Tris-HCl buffer (pH 6.8). After being boiled for 2min in a water bath, the mixtures were applied to the slots of a 15 % polyacrylamide gel. Electrophoresis was carried out in 25mM Tris buffer (pH 8.3) containing 0.1 % SDS. A constant current of 30mA per gel was applied until the dye front reached the bottom of the gel. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue.

After the electrophoresis, the gels were briefly washed, and the proteins were transferred to a nitrocellulose membrane electrophoretically. The blotted nitrocellulose membranes were blocked with gelatin, and then incubated with an anti-rat albumin mouse antibody as the first antibody. The blotted membrane was thoroughly washed in order to remove any unbound antibodies. The membrane was then incubated with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (second antibody), and washed again. The nitrocellulose membrane was immersed into the HRP color development solution containing 0.0015 %  $\text{H}_2\text{O}_2$ , 0.01 % 4-chloro-1-naphthol and 16.7 % methanol.

*Purification of rat brain S-100 protein.* S-100 protein was semi-purified from the rat brains according to the procedure described elsewhere with modifications (11). In brief, the rat brains were homogenized with 4 volumes of buffer containing 100mM potassium phosphate (pH 7.1), 1mM EDTA and 35 % ammonium sulfate. The homogenate was centrifuged at  $10,000 \times g$  for 30min to get a supernatant. Ammonium sulfate was added up to 85 % saturation. The sample was centrifuged at  $10,000 \times g$  for 30min to get a precipitate. The precipitate was resuspended in the homogenizing buffer, and isoelectric precipitation was done with the addition of orthophosphate at pH 4.7. After centrifugation at  $10,000 \times g$  for 30min, the precipitate was again dissolved in buffer containing 50mM Tris-HCl (pH 7.4), 1mM  $\text{CaCl}_2$  and 1mM  $\beta$ -mercaptoethanol. The solution was then dialyzed against the same buffer overnight. The sample was applied to phenylsepharose affinity chromatography. S-100 protein was eluted from the column with 50mM Tris-HCl (pH 7.4) containing 2mM EDTA and 1mM  $\beta$ -mercaptoethanol.

*Enzyme immunoassay of S-100 protein.* S-100 protein  $\beta$  subunit in the dialysate from the striatum was assayed by means of a sandwich-type (two sites) immunoassay system according to the procedure described elsewhere (12).

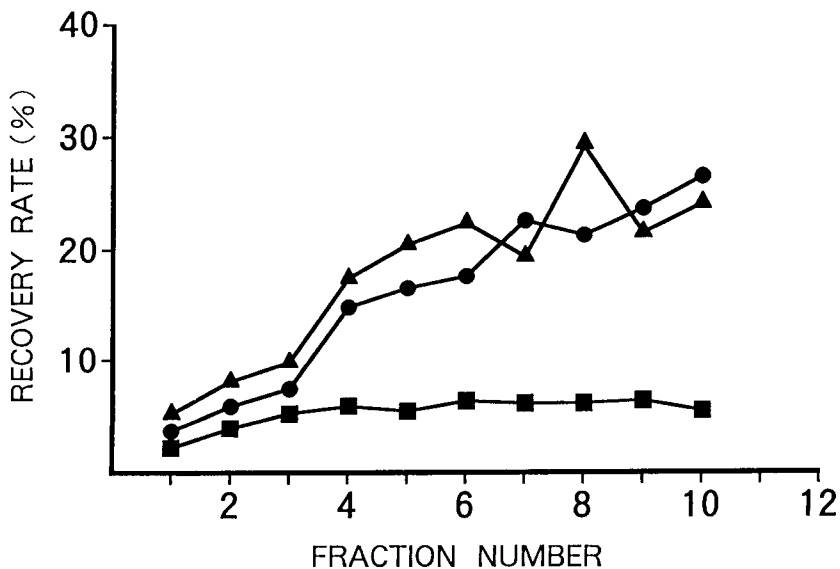
## Results

*Recovery rates of peptides and proteins in in vitro systems.* Fig. 2 shows the change in the recovery rate of cytochrome C at several flow rates. At flow rates of  $1\ \mu\text{l}/\text{min}$  for 20 min,  $2\ \mu\text{l}/\text{min}$  for 10 min and  $4\ \mu\text{l}/\text{min}$  for 5 min, the recovery rates of cytochrome C were 26.2 %, 22.7 % and 5.3 %, respectively. The recovery rate was not so good at the flow rate of  $4\ \mu\text{l}/\text{min}$  as at slower flow rates, but it only took a short time to reach a steady state at  $4\ \mu\text{l}/\text{min}$ . Though we chose  $2\ \mu\text{l}/\text{min}$  in this study, higher flow rates could be applicable depending on the purpose of the experiment. Table 1 indicates the recovery rates of several peptides, proteins and monoamines. The recovery rates varied from 2.3 % (insulin-like growth factor I : IGF-I) to 27.4 % (somatostatin). There were no clear relationships between their recovery rates and their molecular weights. Besides molecular weight, there may be other factors that could influence recovery rates, such as hydrophobicity or electrostatic properties. Monoamine and its

**Table 1** *In vitro* recovery rates of proteins, peptides and amines<sup>a</sup>

	Molecular weight	Recovery rate (%)
Bovine serum albumin	66,000	18.2 ± 0.9
Cytochrome C	13,000	16.9 ± 1.8
IGF-1	6,800	2.3 ± 1.0
ACTH	4,500	7.8 ± 0.3
Somatostatin	1,638	27.4 ± 2.7
Substance P	1,348	4.4 ± 3.9
CCK-8	1,143	12.5 ± 1.7
Vasopressin	1,084	13.1 ± 2.3
Dopamine	153	40.6 ± 6.9
DOPAC	168	33.7 ± 4.0
HVA	182	22.0 ± 5.8
Noradrenaline	169	22.0 ± 3.7

*a* : *In vitro* recovery rate was defined as the ratio between the concentration of a particular substance in the dialysate and the concentration of the standard solution. The concentration of each protein and peptide in the standard solution was 1 mg/ml. The concentration of each amine and metabolite in the standard solution was  $2\ \mu\text{M}$ . Recovery rates are expressed as the mean ± SD of 4 samples at steady state. The flow rate was  $2\ \mu\text{l}/\text{min}$ , and the collection time was 10 min. Abbreviations: IGF-1, insulin-like growth factor 1; ACTH, adrenocorticotrophic hormone; CCK-8, cholecystokinin C-terminal octapeptide; DOPAC, dihydroxy-phenylacetic acid; HVA, homovanillic acid.



**Fig. 2** The change in *in vitro* recovery rates of cytochrome C at several flow rates (▲, 1 ; ●, 2 ; ■,  $4\ \mu\text{l}/\text{min}$ ). The amount of each fraction is  $20\ \mu\text{l}$ .

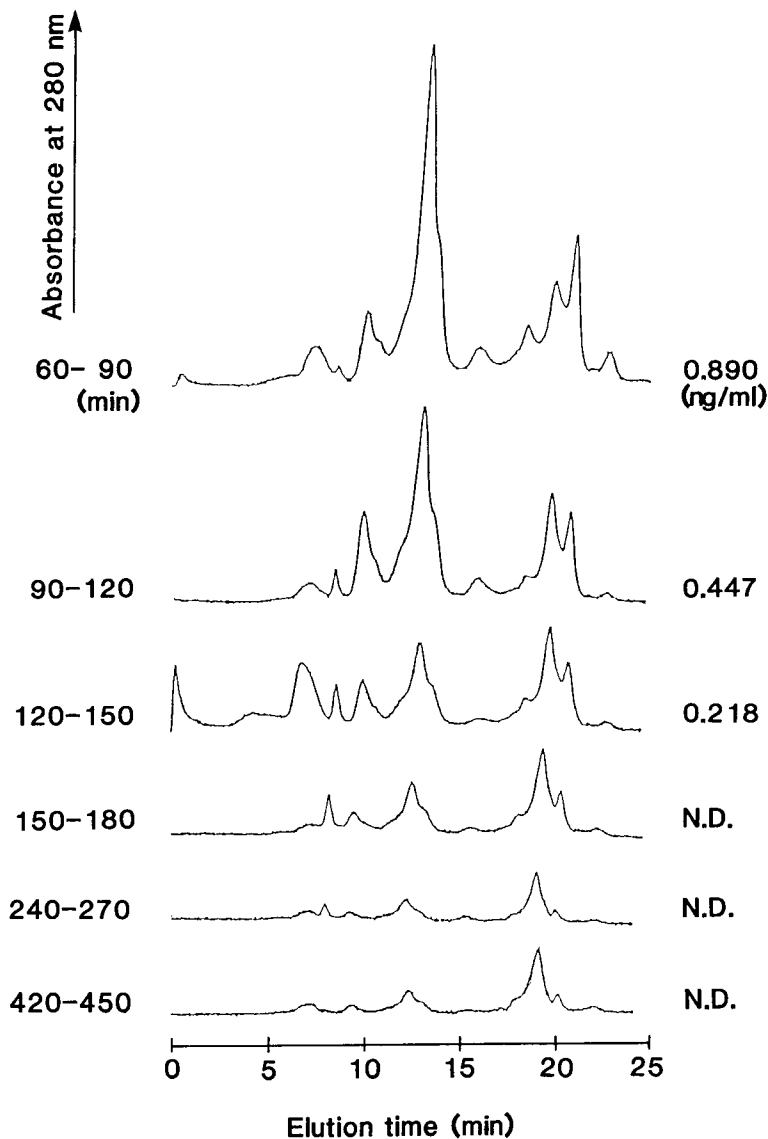
metabolites showed high recovery rates (22–40 %). These *in vitro* results showed that this probe reliably detected peptides, proteins and catecholamines.

*In vivo studies.*

*Tissue damage by the implantation of the probe.*

Fig. 3 shows the HPLC patterns of dialysates

from rat striatum. These patterns show the change in absorbance at 280 nm (every 30 min) after the implantation of the probe. At 60 min, one large peak was detected in the middle of the eluant. Its height was found to decrease as time elapsed. At 240 min, this peak was only a trace. To study the damage to glial cells, we determined

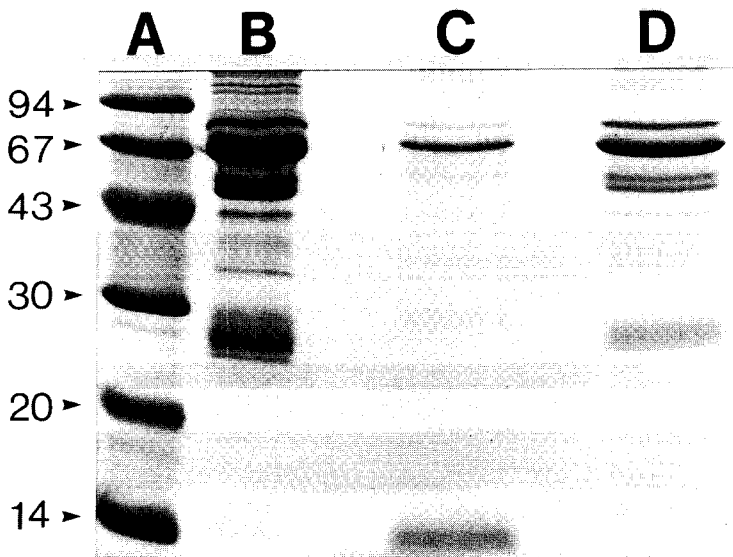


**Fig. 3** Chromatograms of the dialysate from the striatum and the contents of S-100 protein  $\beta$  subunit. The dialysates were collected for every 30 min continuously from the time of probe implantation, and were applied to a TSK gel filtration column 3,000 SW, and the absorbance at 280 nm was monitored. The contents (ng/ml) of S-100 protein  $\beta$  subunit of the dialysates were determined by enzyme immunoassay, and values are shown on the right of the chromatograms. N.D., not detected.

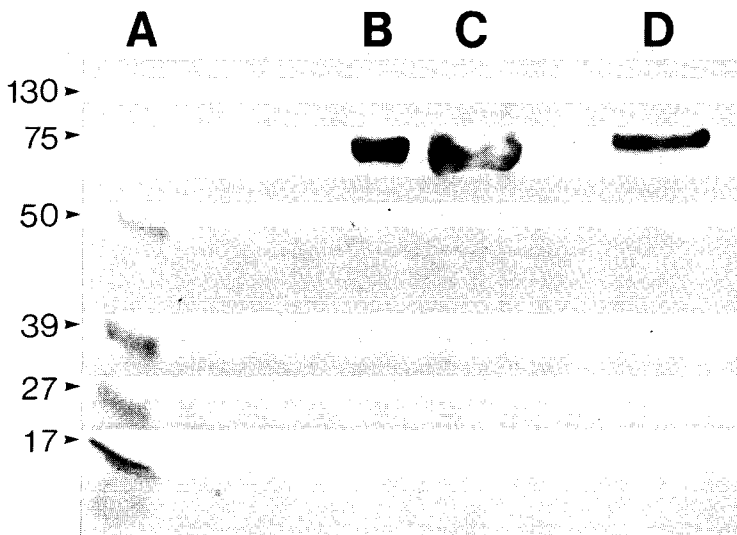
the contents of S-100 protein in the dialysates. After 150 min, we could not detect any S-100 protein. These results suggested that washing for 3 h is necessary to eliminate the proteins present due to tissue damage.

*SDS-PAGE of the dialysates.* Fig. 4 shows

CBB-staining patterns of the dialysates from the striatum, CSF and serum. In the dialysate pattern, two major bands with apparent molecular masses of 62 kDa and 12 kDa, and several minor bands (28 kDa, 43 kDa, 52 kDa and 70 kDa) were present. In the CSF and serum patterns, a 62



**Fig. 4** Coomassie Brilliant Blue-staining patterns of the dialysates from the striatum, cerebrospinal fluid (CSF) and serum. The amount of protein in each lane was  $16\mu\text{g}$ . Lane A, molecular weight markers (molecular weights  $\times 10^{-3}$  are shown on the left of the figure); B, serum; C, the dialysate; D, CSF.



**Fig. 5** Immunoblotting of proteins in the dialysates with an anti-rat albumin mouse antibody. Lane A, prestained molecular weight markers (molecular weights  $\times 10^{-3}$  are shown on the left of the figure); B, serum; C, CSF; D, the dialysate from the striatum.



kDa band was also a major band. This band was shown to be albumin by immunoblotting with an anti-rat albumin antibody (Fig. 5). The 12kDa band was a major band in the dialysate pattern, but it was a very minor band in the CSF or serum patterns.

## Discussion

The microdialysis technique is very useful for determining dynamic changes in the concentration of neurotransmitters in the cerebral nuclei (13), but it has certain limitations. The analysis of large molecular weight materials such as peptides and proteins is difficult using conventional dialysis membranes. On the other hand, the push-pull perfusion technique can potentially damage tissues (13). To overcome these faults, we developed a thin hairpin-shaped probe for microdialysis using a hollow fiber made of ultrafiltration membrane.

It is necessary to perform a fundamental study of the properties of hollow fibers and to determine *in vitro* recovery rates in order to estimate the real *in vivo* concentrations of target compounds in the extracellular space. We clarified the permeability of the hollow fiber to several materials *in vitro*, and found that it was suitable for the analysis of peptides, proteins and catecholamines, and that its permeability might be influenced by the molecular size of target materials, molecular cut-off of the membrane, and hydrophobicity or electrostaticity between the membrane and materials.

We used a flow rate of 2  $\mu$ l/min, since the recovery rate was best. However, it took about 100 min to reach a steady state at this flow rate. In the case of 4  $\mu$ l/min, it took only about 15 min to reach a steady state. However, the recovery rate was low, about 4.5 % with cytochrome C as the target.

Tissue damage due to implantation of the probe may change the contents of dialysates. Extracellular levels of many substances included in the blood and CSF were high just after probe

implantation. Continuous measurement of S-100 protein and chromatography of the dialysates indicated that washing for 3 h was necessary before a stable pattern was established.

SDS-PAGE analysis showed that the dialysate pattern was different from the serum and CSF patterns, although there were several bands in common. The amount of 12kDa protein was more in the dialysate than in the serum or CSF. Twelve kDa protein might be specific to extracellular fluid of the brain. There was a certain degree of similarity between serum and CSF. The major fraction of CSF is secreted at the choroid plexus from the blood. In addition, there is also communication between CSF and extracellular fluid. Therefore, it is reasonable that they should share some components.

The SDS-PAGE pattern of the dialysates is similar to that communicated by Nyström *et al.* (3) and Fazeli *et al.* (4). Fazeli *et al.* investigated the extracellular proteins in the rat dentate gyrus using the push-pull technique, and found that 76.5kDa and 30kDa proteins increased after high frequency stimulation of the perforant path. According to the SDS-PAGE pattern of the dialysate, Fazeli *et al.* suggested that 14.5kDa protein might be S-100 protein of NGF. S-100 protein was reported to be released into the extracellular spaces of brains (14). We examined the possibility that the 12kDa protein in our study was S-100 protein by immunoblotting with an anti S-100 protein antibody, but the 12kDa protein band was not immunoreactive (data not shown).

In summary, an intracerebral microdialysis method was developed and applied to the detection of extracellular proteins in the striatum. Two major bands (12kDa and 62kDa) and several minor bands were detected by SDS-PAGE. The 62kDa protein was albumin, and the 12kDa protein was not S-100 protein. These results indicate the potential usefulness of the newly developed probe for microdialysis for monitoring the dynamic changes in the concentrations of peptides and proteins in cerebral nuclei.

Acknowledgment. We wish to thank Dr. K. Kato for his valuable suggestions and help with the quantification of S-100 protein.

Part of this work was presented at the 31st Annual Meeting of the Japanese Neurochemical Society and the International Symposium on Information Transduction and Processing in Biological Systems from Cell to Whole Body, held in Takamatu, March 12-16, 1989.

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Received September 20, 1989; accepted December 5, 1989.