[Chem. Pharm. Bull. 24(10)2294—2300(1976)]

UDC 615.225.1.011.5 : 547.96.05.09

Isolation of an Acute Hypotensive Substance from Bovine Brain Lipid Fraction[1)]

Hiroaki Tsukatani, Sadaji Yamada, Akira Tokumura, Tsumoru Miyamoto, and Kenkichi Takauchi

Faculty of Pharmaceutical Sciences, University of Tokushima[2)]

(Received October 31, 1975)

A depressor substance was found in acetone extract of bovine brain, greatly purified with the use of various chromatographic techniques, and tentatively named as D-I.

It caused a sharp fall in arterial blood pressure of anesthetized cats with intravenous injections of 10 μg level of purified sample per kg animal body weight on a weight basis.

Its hypotensive activity was not affected by previous treatment of cats with atropine, diphenhydramine, or hexamethonium.

In the enzymic assay with the use of prostaglandin dehydrogenase (PGDH), neither prostaglandin E (PGE) nor prostaglandin F (PGF) compound was found.

In the test of an incubation with a proteinase (nagase), the depressor activity was recovered almost completely.

This depressor substance is definitely distinguishable from other depressor compounds and relatively resemble to lysolecithin, in the comparison of its attitude on TLC or in the course of purification process.

Martini and his coworkers have reported that the acetone extracts obtained from brains of various species of animals cause a fall of mean arterial blood pressure when injected intravenously in anesthetized cats or guinea-pigs, and named the hypotensive substance as "Neuroketon."[3-5)]

More recently, the same workers have shown that it is possible to obtain a hypotensive substance from the papain-treated brain lysate, whereas when the brain homogenate is hydrolyzed with trypsin or chymotrypsin, the extract possesses a clearly hypertensive action.[6,7)]

They have also claimed that the hypotensive activity is observed in the oxidized form of the substance while hypertensive activity in the reduced form.[6,7)]

Besides, such biologically active substances have been reported obtainable also from dog cerebrospinal fluid,[6,8)] dog peritoneal dialysate,[8,9)] and so on.[10-12)] In some of these materials have been found several other physiologically or biochemically interesting significances.[3,6,7,9,13,14)] Martini and his colleagues, however, did not purify these substances extensively, and it seems that they consider the active principles in these extracts are possibly

1) Part of this work was presented at the 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, Apr., 1971.
2) Location: *Shomachi-1-chome, Tokushima.*
3) E. Martini, A. Marzorati, and E. Morpurgo, *Experientia*, **8**, 225, (1952).
4) F. Chiesa, *Ist Lombardo* (Rend. Sc.), **93**, 7 (1959).
5) F. Chiesa, *Ist Lombardo* (Rend. Sc.), **93** 26 (1959).
6) E. Martini, F. Chiesa, A. Spiga, and R. Oberosler, *Enzymologia* **29**, 1 (1965).
7) F. Chiesa, E. Martini, R. Oberosler, and A. Spiga, *Enzymologia*, **29**, 38 (1965).
8) E. Martini, F. Chiesa, R. Oberosler, G. Maffeo, and C. Secchi, *Enzymologia*, **33**, 100 (1967).
9) C. Secchi and E. Martini, *Ital. J. Biochem.*, **20**, 98 (1971).
10) E. Martini, F. Chiesa, R. Oberosler, G. Maffeo, C. Secchi, and E. Seren, *Atti. Soc. It. Sc. Veter*, **20**, 235 (1966).
11) F. Chiesa, E. Martini, R. Oberosler, C. Secchi, G. Maffeo, and E. Seren, *Atti, Soc. It. Sc. Veter.*, **20**, 232 (1966).
12) E. Martini, F. Chiesa, R. Oberosler, E. Seren, and V. Beghelli, *Enzymologia*, **38**, 189 (1970).
13) F. Chiesa, *Enzymologia*, **21**, 61 (1959).
14) F. Chiesa and V. Cima, *Enzymologia*, **22**, 47 (1960).

the same compound in spite that they were obtained widely from various animals and different tissues with different extraction procedures. Thus, the isolations of these substances have not been attained, and their chemical constitutions have been remained undefined.

In the previous paper, we have reported the presence of 2,2,6,6-tetramethylpiperidon-(4) as a main depressor compound in the extract of the pronase or papain hydrolysate of bovine brain, and assumed that it may be formed from ammonia and acetone bodies during hydrolytic procedure.[15]

However, the hypotensive substance contained in the acetone extract of brain still remains unidentified.

This paper reports the extraction and purification for a depressor substance in the acetone extract of bovine brain, and the partially purified substance is tentatively named as "Depressor I" (D-I).

Result

Purification procedure of the hypotensive substance is briefly outlined in Chart 1.

bovine brain
↓ extracted with acetone, evapd. under reduced pressure
acetone extract I
↓ partitioned between EtOH–H_2O (7:3) and petroleum ether; aqueous ethanol layer was evapd. under reduced pressure
ethanol extract II
↓ dissolved in $CHCl_3$, washed with 0.1N HCl, water, 5% $NaHCO_3$, and water successively, dried over anhyd. Na_2SO_4 and evapd. under reduced pressure
crude extract (Martini's extract) III
↓ silicic acid chromatography
active fraction IV
↓ "Sephadex LH-20" gel filtration
active fraction V
↓ cellulose chromatography
active fraction VI
↓ preparative TLC on a silica gel plate, $CHCl_3$–MeOH–H_2O (65:25:4 v/v/v)
active fraction VII

Chart 1. Procedure for the Separation of the Hypotensive Principle D-I from Bovine Brain

Each fraction was assayed on the arterial blood pressure of the anesthetized cat with an intravenous injection, and the injection into the peritoneal cavity was also applied if necessary.

Extraction procedure of active principle from bovine brain was carried out according to the method proposed by Martini and his coworkers.[4]

The crude extract III (Martini's extract) thus obtained was subjected to a silicic acid chromatography. The chromatographic pattern was shown in Fig. 1.

The weights of successive fractions from the column and the relative depressor activity of fractions are shown. On this chromatography, the depressor activity was concentrated mainly in chloroform–methanol (40: 60) fraction. This active fraction IV was further submitted to

15) H. Tsukatani, T. Itami, T. Awaji, and K. Takauchi, *Chem. Pharm. Bull.* (Tokyo), **19** 2201 (1971).

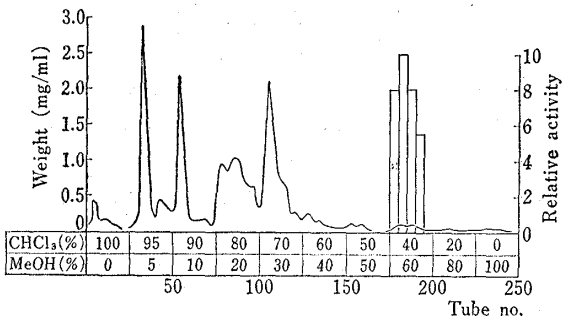

Fig. 1. Silicic Acid Column Chromatography

column: Silicic acid (Mallinckrodt, 100 mesh, activity II B) 40 g, hyflosuper-cel 10 g
one tube: 20 ml
sample: crude extract (Martini's extract) III, 1.16 g, corresponding to 500 g of bovine brain
bioassay: Relative depressor activities of the extracts injected *i. v.* in cats anesthetized with amobarbital (60 mg/kg *i.p.*)
□ relative activity —— weight

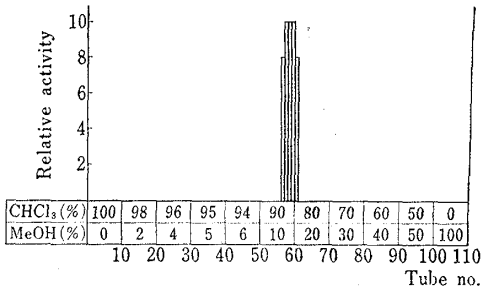

Fig. 2. Cellulose Column Chromatography

column: cellulose (Toyoroshi, 300 mesh) 7.0 g
one tube: 5 ml
sample: active fraction V, 8 mg, corresponding to 1 kg of bovine brain.
bioassay: In the same manner as shown in Fig. 1.

"Sephadex LH-20" column, eluting with chloroform–methanol (1: 1), the active principle was located in the eluates from 140 to 170 ml (V).

Nextly, the active fraction V was applied on a cellulose column.

The chromatographic pattern was shown in Fig. 2.

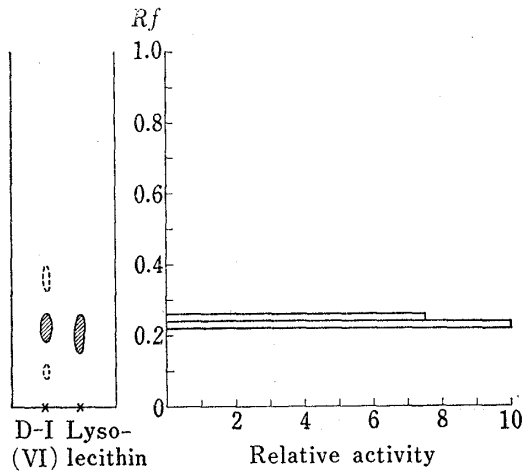

Fig. 3. Distribution of Depressor-activity of D-I on TLC

sample: Depressor-active fraction VI
plate: PLC plate silica gel 60 (Merck)
solvent system: $CHCl_3$–MeOH-H_2O (65: 25: 4)
detection: iodine vapour
bioassay: intravenous injections in urethane anesthetized guinea-pigs (1.8 g/kg *i. p.*)

The active principle was found in the eluates from chloroform–methanol (90: 10) to chloroform–methanol (80: 20).

For further purification the active fraction VI was purified with thin-layer chromatography (TLC) on a silica gel plate, with $CHCl_3$–MeOH–H_2O (65: 25: 4, v/v/v) as a solvent system. Fig. 3 shows the distribution of the depressor-activity of the active fraction VI on the silica gel plate when injected intravenously into the anesthetized guinea-pigs.

The hypotensive principle D-I in acetone extract of bovine brain was highly purified approximately 7000 fold at the step VI, although the recovery of biological activity was not good at the final step VII. The effective concentration of the hypotensive principle D-I on each step was summarized in Table I.

Only a small amount of the final product being available, it was decided to investigate its chemical properties without any further attempts in purification.

D-I is relatively unstable in the purified state when allowed to stand at room temperature, its depressor-activity was diminished in a few days, although relatively stable in the crude state.

However, it can be stored for several months in the dark at −20° under an atmosphere of nitrogen in a syrup form.

In the investigation with TLC on silica gel plates with other solvent systems and several indicators, it was demonstrated that this final product is almost pure and contained only

Table I. Effective Concentration of the Hypotensive Principle D-I

Step	Weight (g/kg wet tissue)	Recovery of hypotensive activity[a] (%)	Effective concentration
Acetone extract I	41.1	—	1
Ethanol extract II	8.4	—	4.28
Crude extract III (Martini's extract)	2.32	—	17.72
Silicic acid chromatography IV	0.041	almost quantitatively	1002
"Sephadex LH-20" gel filtration V	0.008	90	4623
Cellulose chromatography VI	0.005	90	6658
PLC: $CHCl_3$–MeOH–H_2O (65: 25: 4) VII	trace	10	—

a) The total hypotensive activity on each step was measured on the blood pressure responses in amobarbital anesthetized cats (60 mg/kg) or urethane anesthetized guinea-pigs (1.8 g/kg).
Aliquot portion of the fraction which shows the equal effect with 0.05 μg/kg of acetylcholine was determined on each step in animals with the intravenous injection, and the recovery of the hypotensive activity compared to the preceding step was calculated. In the comparison between step III and IV, the fractions were dosed intraperitoneally. Each value is the average of two experiments.

small amounts of impurities. The main spot can be detected with iodine vapour, sulphuric acid, antimony trichloride, ammonium molybdate, or phosphomolybdate. Dragendorff's and 2,4-dinitrophenylhydrazine reagents gave reddish orange colours, while with ninhydrin detectable colour was not observed with this substance.

The relative mobility to other compounds including lipid- and water-soluble hypotensive substances are shown in Table II.

Table II. *Rf* Values of the Hypotensive Principle D-I

Substance	(A) $CHCl_3$–MeOH–AcOH–H_2O (60: 35: 1: 8)	(B) $CHCl_3$–MeOH–H_2O (65: 25: 4)	(C) $CHCl_3$–MeOH–2.5 N NH_4OH (60: 35: 8)	Detection
D-I (VII)	0.50	0.20	0.38	a
DL-α-Lecithin	0.69	0.47	0.58	a
Lysolecithin	0.48	0.20	0.25	a
PGE_1	front	front	front	b
PGE_2	front	front	front	b
$PGF_{1\alpha}$	front	0.92	front	b
$PGF_{2\alpha}$	front	0.92	front	b
ATP	0.11	origin	origin	c
ADP	0.11	origin	origin	c
AMP	0.11	origin	origin	c
Acetylcholine	0.33	0.21	0.26	d
Serotonin	0.58	0.03	0.32	e
Histamine	0.21	0.08	0.24	e
Lactic acid	0.05	0.04	0.38, 0.18, 0.06	c
2,2,6,6-Tetramethyl-piperidone-(4)	0.80	0.58	front	f

plate: Merck TLC plate silica gel 60
detection: a) Iodine vapour, b) phosphomolybdate, c) bromocresolgreen, d) Dragendorff's, e) ninhydrin, f) potassium permanganate

In the comparison of *Rf* values, this depressor substance is definitely distinguishable from not only prostaglandins (PGs) but also water-soluble depressor substances such as acetylcholine, histamine, serotonin, adenosine derivatives and so on.

Considering the data obtained here and attitudes of the hypotensive principle in the course of the purification process, this hypotensive principle named as D-I relatively resembles to lysolecithin, except depressor-activity, *Rf* value with solvent system (C), and the colour reaction on the TLC plate.

As stated above, the recovery of biological activity at the last step of purification process was so poor that the pharmacological assays were performed with the active fraction VI. The intravenous injection of the extract was found to produce a sharp fall in blood pressure lasting about 5 minutes in anesthetized cats, and the dose of the extract obtained from 2 to 4 g wet tissue per kg animal body weight is sufficient to evoke the minimum blood fall, which corresponds to 10 μg level of purified D-I (VI) on a weight basis. The blood pressure responses of purified D-I in anesthetized cats comparing to other hypotensive substances are shown in Fig. 4.

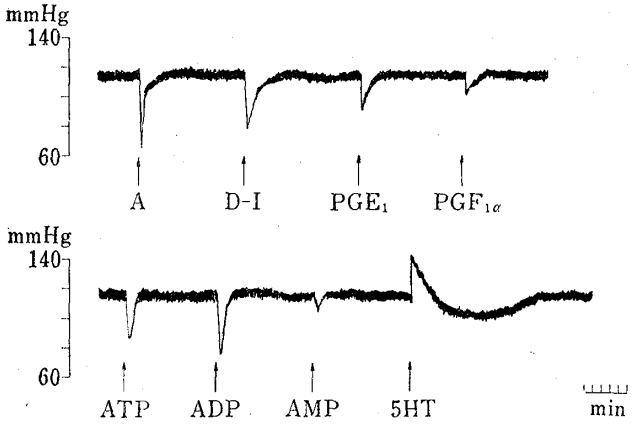

Fig. 4. Effects of the Hypotensive Principle D-I and other Hypotensive Substances on Blood Pressure in Anesthetized Cats

The cats were anesthetized with amobarbital (60 mg/kg *i.p.*). Intravenous injections were made at points indicated by arrows.
dose: acetylcholine (A, 1 μg/kg), D-I (VI, 25 μg/kg, corresponding to 5 g of bovine brain /kg), PGE_1 (3 μg/kg), $PGF_{1\alpha}$ (3 μg/kg), ATP (300 μg/kg), ADP (300 μg/kg), AMP (600 μg/kg), and serotonin (5HT, 600 μg/kg)

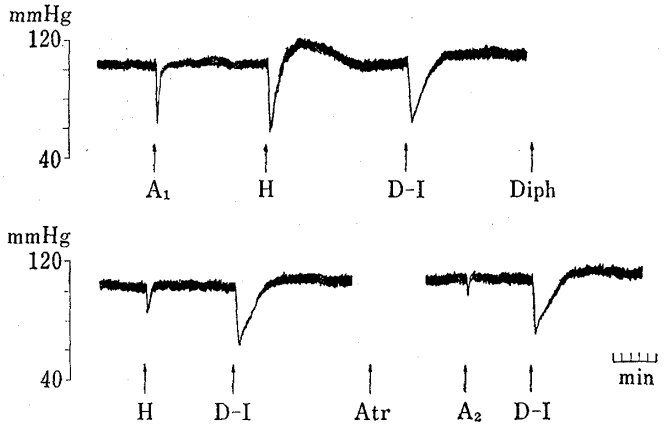

Fig. 5. Effects of Diphenhydramine and Atropine on the Blood Pressure Responses to D-I in Anesthetized Cats

Cats were anesthetized with amobarbital (60 mg/kg, *i.p.*), and received diphenhydramine (Diph, 5 mg/kg, *i.p.*), and atropine (Atr, 3 mg/kg, *i.p.*) respectively. Thirty min later after the injection of diphenhydramine or atropine next reagent was given. Intravenous injections were made at points indicated by arrows.
dose: acetylcholine (A_1, 1 μg/kg, A_2, 40 μg/kg), histamine (H, 5 μg/kg), D-I (VI, corresponding to 5 g of bovine brain/kg, 25 μg/kg)
See diphenhydramine or atropine failed to block the depressor-action of D-I.

As the hypotension of D-I was unaffected by previous treatment with atropine, diphenhydramine, or hexamethonium bromide in cats, it may be concluded that the hypotension of D-I is not derived from acetylcholine, histamine, and so on. The effects of atropine and diphenhydramine on blood pressure responses to D-I in anesthetized cats are shwon in Fig. 5.

In order to distinguish between D-I and PGs, additionally, we examined D-I by a method of enzymic separatory determination of PGE and PGF which utilizes PGDH connecting with enzymic cycling of NAD^+-NADH system and resazulin, recently reported from our laboratory,[16] and neither PGE nor PGF compound was found.

Lastly, we tested D-I with a proteinase. When D-I was incubated with nagase (1 mg enzyme/ml, phosphate buffer (pH 7.0), 37°, 1 hr), this hypotensive substance was not destroyed.

From this result, it may be concluded that this active principle is not a peptide such as substance P, bradykinin, kallikrein, and so on.

Discussion

The main purpose of the present investigation is to establish a method of purification and make clear parts of the chemical and pharmacological properties of this physiologically interesting substance, which was obtained first from acetone extract of brains of various animals.

16) T. Itami, K. Fukuzawa, M. Takahashi, and H. Tsukatani, *Chem. Pharm. Bull.* (Tokyo), **23**, 1152 (1975).

By the purification procedure, the hypotensive principle D-I in the acetone extract of bovine brain was highly purified.

The purified D-I thus obtained relatively resembles to lysolecithin in the comparison of its behaviour on TLC or column chromatographies. Of course, there are definite differences in their natures between D-I and lysolecithin otherwise hypotension, namely, D-I shows a positive reaction by the spray of 2,4-dinitrophenylhydrazine reagent, and a higher *Rf* value in the solvent system (C). D-I shows a sharp fall in blood pressure of anesthetized cats or guinea-pigs.

It is not well known with endogenous hypotensive substances which are lipid-soluble and chemically defined other than PGs.

PGs were first isolated from human seminal plasma and sheep seminal vesicles,[17] have been found in other tissues, and attracted great interests owing to their potent biological significances. PGs consist of many groups of compounds, among them PGE and PGF series compounds play main roles in the biological functions and are found in various organs and fluids.

In addition, PGE and prostaglandin A series compounds have powerful and acute vasodepressor effects.

If we focused on the comparison of D-I with PGs, it is difficult to compare to each other, and satisfy every request, since the naturally occurring PGs which are known in the literature at present are so numerous. However, broadly speaking, D-I which was found in the phospholipid fraction is a polar substance compared to PGs, from the attitudes on TLC and column chromatographies. In addition, naturally occurring PG derivatives which belong to phospholipid have not been revealed in the literature.

In the investigation on enzymic assay using PGDH, D-I differs apparently from PGEs and PGFs. Furthermore in the test on the isolated guinea-pig ileum preparations, D-I shows no contraction. From these findings, it is difficult to consider that D-I may be a certain PG compound. Judging the results of TLC, purification procedure and pharmacological examinations, it is suspicious that a water-soluble hypotensive substance such as acetylcholine, histamine, adenosine derivatives, or substance P, *etc.* is responsible to the depressor-activity of D-I by the contamination in the phospholipid fraction.

It is still difficult to decide at present whether this hypotensive principle is a new compound or not, since its chemical constitution remains undefined. However, the question was solved to some extents by the evidences obtained in this investigation, and they suggest that the purified principle D-I which belongs to phospholipid fraction may be a new short-lasting depressor substance.

Recently, a number of new components of phospholipid fraction have been reported from the various sources, including biologically active compounds such as dinogunellin, which is a toxin of nothern blenny roe.[18] It is considered that these facts also support above estimation.

Of course, there are many points which should be solved and these data which were obtained in this investigation will be a clue to elucidate D-I, the hypotensive principle in the phospholipid fraction, in future.

Experimental

Materials——PGs and PGDH[16] were kindly gift from Ono Pharmaceutical Industries, LTD.

17) *a*) R. Kurzrok and C.C. Lieb, *Proc. Soc. Exptl. Biol. Med.*, **28**, 268 (1930); *b*) U.S. von Euler, *Arch. Exptl. Pathol. Pharmkol. Naunyl Schmiedebergs*, **175**, 78, (1934); *c*) U.S. von Euler, *Klin. Wochschr.* **14**, 1182 (1935); *d*) U.S. von Euler, *J. Physiol.*, **88**, 213 (1936); *e*) M.W. Goldblatt, *J. Soc. Chem. Ind.*, **52**, 1056 (1933); *f*) M.W. Goldblatt, *J. Physiol.*, **84**, 208 (1935); *g*) J.R. Cockrill, E.G. Jr. Miller, and R. Kurzrok, *Am. J. Physiol.*, **112**, 577 (1935); *h*) R. Eliasson, *Acta Physiol. Scand.*, **46**, Suppl. 158, (1959).

18) M. Hatano and Y. Hashimoto, *Toxicon*, **12**, 231 (1974).

Amobarbital and other drugs used *in vivo* study were of commercial quality. TLC Plate: Merck TLC plate silica gel 60 and PLC plate silica gel 60 were purchased. All other materials were of reagent grade and purchased commercially.

Solvents were redistilled before use. Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure.

Extraction and Preliminary Separation——Fresh bovine brain, 1 kg, obtained at the slaughterhouse and kept in ice, was cut into portions of about 200 g each.

These were homogenized for 2 min each with 2 liters of acetone.

The homogenate is centrifuged for 10 min, and the supernatant was decanted off. The sediment was resuspended in 2 liters of acetone and again centrifuged.

The acetone extracts were combined, and evaporated under reduced pressure (I). The syrup was partitioned between equal volume of ethanol–water (7: 3) and petroleum ether, and aqueous ethanol layer was separated, and evaporated under reduced pressure to the syrup (II). The syrup thus obtained was dissolved in chloroform, washed with 0.1 N HCl, water, 5% $NaHCO_3$ solution, and water successively, dried over anhydrous sodium sulfate, and evaporated to dryness. These procedures give the crude extract III (Martini's extract).

Silicic Acid Chromatography——Silicic acid (Mallinckrodt: 100 mesh) was activated at 100° for at least 72 hr, and partially deactivated (activity II B) before use. Silicic acid (40 g) and hyflosuper-cel (10 g) were washed twice in 500 ml of chloroform and poured into 1.5 × 70 cm glass column.

Crude extract III corresponding to 500 g of bovine brain, 1.16 g, was dissolved in 10 ml of chloroform, and applied to the top of the column. The column was eluted successively with 500 ml of each of chloroform, $CHCl_3$–MeOH (9: 1), (8: 2), (7: 3), (6: 4), (5: 5), (4: 6), (2: 8), and methanol respectively at the room temperature. At 22°, flow rate of approximately 40 ml/hr were achieved, and each 20 ml of the eluate from the column was collected in a tube. An aliquot of each tube was taken, dried and weighed. An aliquot for each 100 ml of eluate was taken, dried and redissolved in 2% tween 80 containing saline, and applied for the assay of blood pressure response in anesthetized cats. The depressor-activity was found mainly in the eluates of $CHCl_3$–MeOH (4: 6).

The active fractions were combined and evaporated under reduced pressure, and the active fraction IV was given.

"Sephadex LH-20" Gel Filtration——A hundred gram of "Sephadex LH-20" (Pharmacia) was washed with each 500 ml of methanol, chloroform, and $CHCl_3$–MeOH (1: 1), successively. "Fines" were removed in the solvents, and poured into 2.7 × 90 cm glass column.

The active fraction IV (16.4 mg) corresponding to 400 g of bovine brain was dissolved in the same solvent. The depressor activity was located in the eluates from 140 to 170 ml (V).

Cellulose Chromatography——Seven gram of cellulose (Toyoroshi, 300 mesh) was suspended in 20 ml of 95 % methanol and poured into 1 × 35 cm glass column.

Active fraction V (8 mg) corresponding to 1 kg of bovine brain was dissolved in a small volume of chloroform and applied to the column. The column was eluted successively with each 50 ml of chloroform, $CHCl_3$–MeOH (98: 2), (96: 4), (95: 5), (94: 6), (90: 10), (80: 20), (70: 30), (60: 40), (50: 50), and methanol respectively. Each 5 ml of the eluate from the column was collected in a tube, and the flow rate of approximately 30 ml/hr were achieved. The bioassay was carried out as above with an aliquot of each tube. The depressor-activity was located in the eluates from $CHCl_3$–MeOH (90: 10) to (80: 20).

The active fractions were combined and dried, and the active fraction VI was obtained.

Preparative TLC——Lastly, the hypotensive substance from active fraction VI was isolated with preparative TLC.

The sample was applied on line on the plate (Merck PLC plate silica gel 60, layer thickness 2 mm), and the same sample and lysolecithin were spotted on the both sides as reference species.

The plate was developed with $CHCl_3$–MeOH–H_2O (65: 25: 4 v/v/v).

After cutting off of the regions of reference species, the reference spots were detected with iodine-vapour. The remaining region was scraped off with the range of *Rf* value 0.02, and each region was transferred to the test tube. Each sample was extracted with $CHCl_3$, $CHCl_3$-MeOH (2: 1), and MeOH successively, and the extracts were combined.

An aliquot portion of combined extract was taken, dried, and dissolved in 2% tween 80 containing saline, and applied for the assay of the blood pressure responses in urethane anesthetized guinea-pigs (1.8 g/kg *i.p.*). The depressor fractions were combined, and dried, and this procedure gives the active fraction VII.

Bioassay——Measurements of blood pressure responses were carried out on cats anesthetized with amobarbital (60 mg/kg body weight *i.p.*) or guinea-pigs anesthetized with urethane (1.8 g/kg body weight *i.p.*). The trachea was canulated and arterial blood pressure was measured by means of mercury manometer.

Acknowledgement The authors wish to express their appreciation to late professor Seishi Takagi for his continuous encouragement, and also to Ono Pharmaceutical Industries, LTD for the presentation of prostaglandins and PGDH.