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Chemical Characteristics of D-I, a Hypotensive Factor Occurred in Acetone Extract of Bovine Brain[1]

Hiroaki Tsukatani, Sadaji Yamada, Masanobu Fujii, Toshikazu Awaji, Masanori Okamoto, and Takafumi Itami

Faculty of Pharmaceutical Sciences, Tokushima University[2]

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A modified procedure for preparation of D-I was offered, and became possible preparation of highly purified material showing a single spot on thin-layer chromatography in a more effective manner.

Further examinations on chemical characteristics of D-I involving assessments of functional groups by chemical inactivations treating with various agents were carried out. In Girard's separation, about 30% of depressor-activity was recovered in nonketonic fraction. By treatment with sodium borohydride, the hypotensive activity disappeared completely. It was abolished completely with treatment by hydrogen peroxide (0.1%) or permanganate, while the activity was recovered almost completely by treatment with ferric chloride. It was also damaged with bromine.

D-I became substantially less active by treatment with succinic anhydride, and inactive by phenylisocyanate. By treatment with triphenylphosphine or diazomethane at cold D-I was unaffected.

The attitudes of D-I against several spray reagents on TLC were further examined, and it was found that 2,4-dinitrophenylhydrazine-ferricyanide, 2′,7′-dichlorofluorescein and others were useful for detection of D-I.

Generally, absorptional pattern of D-I in infrared spectrum resembled that of lysophosphatidyl choline. D-I might be a choline-containing phospholipid having double bond, free hydroxyl, ester, whereas it has no free amino, free carboxyl, amide, peroxide nor vicinal hydroxyl group, and it might have no carbonyl group other than ester. However, it might be convertible readily to a ketonic compound by some causes.

Keywords——D-I; hypotensive; brain; lysophosphatidyl choline; phospholipid; acetone extract

Recently, we reported isolation of a depressor-active factor from acetone extract of bovine brain in an almost pure grade, and tentatively designated the substance as D-I.[3]

The intravenous injection of some 10 μg/kg of D-I caused a sharp fall in arterial blood pressure of an anaesthetized cat.

Its hypotensive activity was unaffected by pretreatment of a cat with atropine, diphenhydramine or hexamethonium. The active substance was resistant against proteases and prostaglandin dehydrogenase. D–I might be distinct from prostaglandins (PGs) and other naturally occurring hypotensive compounds, and was considered to be a phospholipid since it could be detected with Dittmer's and Dragendorff's reagents.

In the present paper, modifications of extraction and purification procedures of the active factor D–I, and further examinations on chemical characteristics will be reported.

Experimental

Urethane and other drugs used *in vivo* study were of commercial quality. All other materials were of reagent grades and purchased commercially. Solvents were redistilled before use.

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1) Part of this work was presented at the 93rd Annual Meeting, Tokyo, Apr., 1973, and 97th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, Apr., 1977.

2) Location; *Shomachi-1, Tokushima.*

3) H. Tsukatani, S. Yamada, A. Tokumura, T. Miyamoto, and K. Takauchi, *Chem. Pharm. Bull.* (Tokyo), 24, 2294 (1976).

Biological Assay——Wistar strain male rats weighing 240 to 260 g were mainly used throughout this study. The rats were anaesthetized with urethane (1.8 g/kg body weight, *i.p.*). Sometimes Hartley strain guinea pigs and cats were also used under pentobarbital anaesthesia (40 mg/kg, *i.p.*). Animals was catheterized on a trachea and a left carotid artery or a left femoral artery. The arterial blood pressure was recorded on a smoked drum by means of a mercury manometer. The materials to be tested were dissolved or suspended in 0.15 ml of physiological saline solution. If necessary saline containing 2% Tween 80 was used, and samples were injected into the right femoral vein through cannula. In the case of crude samples, materials suspended in saline containing 2% Tween 80 or dissolved in olive oil were injected intraperitoneally.

Modified Extraction and Purification Procedures——In our preceding paper,[3] bovine brain was homogenized and extracted with acetone, and the resultant extract was dissolved in $CHCl_3$ and washed with acidic and alkaline solutions, applying Martini's procedure precisely.[4,5] A modified scheme for the extraction was illustrated in Chart 1.

	Weight (g/kg wet tissue)	Recovery of hypotensive activity
bovine brain	1000	—
│ extracted with $CHCl_3$–MeOH (2:1, v/v). │ washed with 0.2 vol. of 0.7% NaCl soln. │ under layer was evapd. *in vacuo*.		
$CHCl_3$-MeOH extract I (total lipids)	230	—
│ extracted with acetone. │ evapd. *in vacuo*.		
acetone extract II	52	Almost quantitatively
│ partitioned between 70% EtOH and hexane. │ aqueous ethanol layer was evapd. *in vacuo*.		
aqueous ethanol extract III	1.2	Almost quantitatively

Chart 1. Modified Procedure for Extraction of the Hypotensive Factor D-I from Bovine Brain

Bioassays were carried out in urethane anaesthetized rats by intraperitoneal injections.

Fresh bovine brain was cut into pieces and homogenized with 10 l of Folch's solvent [$CHCl_3$–MeOH (2:1, v/v)] per kg wet tissue on the blender and centrifuged. The residue was resuspended in Folch's solvent and centrifuged again. The $CHCl_3$–MeOH extracts combined were washed by mixing with one fifth of its volume of 0.7% NaCl solution (Folch's washing procedure).[6] The upper phase and fluff were removed and the lower phase was evaporated *in vacuo*.

The $CHCl_3$–MeOH extract I (total lipids fraction, 230 g/kg wet tissue) suspended in a small volume of $CHCl_3$ was poured into 5 liters of cold acetone. The mixture was centrifuged and the supernatant was decanted off. The sediment was resuspended in $CHCl_3$, extracted with cold acetone in the same manner

aqueous ethanol extract III
│ chromatographed on the first silicic acid column
active fraction IV ($CHCl_3$–MeOH (4:6, v/v))
│ chromatographed on the second silicic acid column
active fraction V ($CHCl_3$–MeOH (5:5, v/v))
│ filtered on the first Sephadex LH-20 column with $CHCl_3$–MeOH (1:1, v/v)
active fraction VI
│ filtered on the second Sephadex LH-20 column with acetone–EtOH (1:1, v/v)
active fraction VII

Chart 2. Modified Procedure for Purification of the Hypotensive Factor D-I

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4) F. Chiesa and E. Martini, *Arc. Int. Physiol. Biochim.*, 66, 210 (1958).
5) F. Chiesa, *Ist. Lombardo* (*Rend. Sc.*), 93, 7 (1959).
6) J. Folch, M. Lee, and G.H. Sloan-Stanley, *J. Biol. Chem.*, 226, 497 (1957).

and finally washed twice with 500 ml of acetone. The acetone extracts were combined and evaporated *in vacuo* (acetone extract II, approximately 50 g). The extract II was further partitioned between equal volume of 70% ethanol and hexane. Aqueous ethanol layer was separated and evaporated to dryness *in vacuo* (crude extract III, approximately 1.2 g).

The First Silicic Acid Chromatography——The crude syrup III was applied to the first silicic acid column as indicated in Table I and an active fraction IV was afforded.

TABLE I. Separation of the Hypotensive Factor D-I on the First Silicic Acid Chromatography

Fraction	Weight (mg)	Phosphorus (μg)	Hypotensive activity (U)
Hexane	709	n.d.	—
Hex.–$CHCl_3$ (1: 1, v/v)	2506	n.d.	—
$CHCl_3$	82	n.d.	—
$CHCl_3$–MeOH (8: 2, v/v)	964	13202	—
$CHCl_3$–MeOH (6: 4, v/v)	331	29010	—
$CHCl_3$–MeOH (4: 6, v/v)	314	3674	+2770
MeOH-I	62	1365	—
MeOH-II	14	157	—
MeOH–H_2O (1: 1, v/v)	261	n.d.	—

Column: silicic acid (Mallinckrodt, 100 mesh, IIB) 100 g and hyflosuper-cel 50 g. 4.1×45 cm.
Sample: aqueous ethanol extract III (5.28 g corresponding to 4.40 kg of wet tissue).
Elution: discontinuous gradient elution with Hex.-$CHCl_3$ and $CHCl_3$–MeOH systems. one liter on each step.
Phosphorus: method described by Chalvardjian and Rudnicki.7)
Bioassay: in urethane anaesthetized rats. One unit(U) of D-I activity was defined as the amount required to produce a depressor response equal in amplitude to that produced by intravenous injection of 5×10^{-8} g/kg of acetylcholine into an urethane anaesthetized rat weighing 250 g.

TABLE II. Relationship between Weight, Phosphorus and Hypotensive Activity of D-I in the Modified Procedure for Purification

Stage	Weight (mg/kg wet tissue)	Phosphorus (μg/kg wet tissue)	Hypotensive activity (U/kg wet tissue)
Aq. EtOH extract III	1.2×10^3	11.34×10^3	Almost quantitatively[a]
First silicic acid chromatography IV	71.3	835	630
Second silicic acid chromatography V	11.0	576	610
First gel filtration on Sephadex LH-20 ($CHCl_3$–MeOH, 1:1, v/v) VI	9.4	482	600
Second gel filtration on Sephadex LH-20 (acetone–EtOH, 1: 1, v/v) VII	5.3	271	540

Each data is the mean of two experiments. Bioassays were carried out in the same manner as indicated in Table I.
a) Recovery of the hypotensive activity obtained by intraperitoneal injection compared with the preceding stage, acetone extract II.

The Second Silicic Acid Chromatography——The active fraction IV was further chromatographed on the second silicic acid column. The active fractions were collected, dried and weighed (46.2 mg, organophosphorus7) 2419 μg, active fraction V).

The First Gel Filtration on Sephadex LH-20 Column——The active fraction V (41.8 mg) was applied to the Sephadex LH-20 column and eluted with $CHCl_3$–MeOH (1: 1, v/v) in the same way as reported3) (35.7 mg, organophosphorus 1832 μg, Active fraction VI).

The Second Gel Filtration on Sephadex LH-20 Column——The active fraction VI (corresponding to 3.4 kg of bovine brain, 32.0 mg) was filtered on another Sephadex LH-20 column (Pharmacia, 100 g, 2.7×90 cm) with acetone–ethanol (1: 1,v/v) and resulted in active fraction VII (18.0 mg, organophosphorus 921 μg).

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7) A. Chalvardjian and E. Rudnicki, *Anal. Biochem.*, 36, 225 (1970).

Examinations on Chemical Characteristics——Treatment with Girard's Reagent T:[8] An aliquot of the active fraction VII (2.7 mg, corresponding to 500 g of bovine brain, organophosphorus 136 μg, hypotensive activity 270 U) and 50 mg of Girard's reagent T were dissolved in a mixture of 5 ml of ethanol and 0.1 ml of acetic acid, and was refluxed for 30 min on a steam bath in the stream of nitrogen. The mixture was cooled, neutralized with 5% $NaHCO_3$ solution, diluted with 2 ml of water and subjected to extraction with 3×5 ml of $CHCl_3$. The $CHCl_3$ extracts were combined, washed with 1 ml of water twice and evaporated *in vacuo* (non ketonic fraction).

The aqueous solution was acidified to pH 4.0, allowed to stand for 10 min and extracted with 3×10 ml of $CHCl_3$. The aqueous solution was further refluxed at 90° for 2 hr in the stream of nitrogen and extracted with $CHCl_3$ as described above. The $CHCl_3$ extracts were combined and dried *in vacuo* (ketonic fraction). The nonketonic or the ketonic fraction was placed on the silicic acid column and fractionated respectively.

Treatment with Sodium Borohydride: To 10 ml of methanolic solution containing the active fraction VII (1.06 mg) was added 50 mg of sodium borohydride at $-20°$ and stirred for 5 hr. The mixture was brought to 0° and kept for 10 hrs. The reaction mixture was acidified to pH 3.0 with 1 N HCl, evaporated *in vacuo* and extracted with 3×8 ml of $CHCl_3$. The $CHCl_3$ extracts combined were washed with 2×3 ml of water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*.

Treatment with Triphenylphosphine: To 5 ml of $CHCl_3$ solution containing 1.06 mg of the active fraction VII was added 2 mg of triphenylphosphine, and permitted to stand at room temperature for 2 hr. The mixture was evaporated and assayed.

Oxidation with Hydrogen Peroxide: Hydrogen peroxide solutions in different concentrations were prepared from the pool which was determined by titration with 0.01 N $KMnO_4$ preliminary. To 268 μg of the active fraction VII was added 10 ml of respective hydrogen peroxide solution and incubated at 25° for 30 min. An aliquot was evaporated *in vacuo* for assay.

Oxidation with Permanganate: To the solution of the preparation VII 1.06 mg dissolved in 5 ml of water was added 0.8 ml of diluted permanganate solution (diluted a saturated $KMnO_4$ solution with two folds volume of water) in excess. The mixture was left stand at room temperature for 24 hr in the dark. A small volume of ethanol was added to the mixture and centrifuged. To the sediment was added a small volume of water and centrifuged. The combined supernatants were extracted and washed by Folch's procedure. The extracts were dried over anhydrous Na_2SO_4 and evaporated *in vacuo*.

Treatment with Ferric Chloride: Ten ml of ether solution containing the active fraction VII (1.06 mg) and excess ferric chloride was left to stand at room temperature for 6 hr and concentrated to dryness *in vacuo*. The residue was extracted and washed by Folch's procedure.

Addition of Bromine: To 10 ml of $CHCl_3$ solution containing 1.06 mg of the active fraction VII was added 50 ml of bromine solution (diluted bromine with 10 folds volume of $CHCl_3$). The mixture was permitted to stand at room temperature overnight and evaporated *in vacuo*.

Treatment with Succinic Anhydride: A mixture of preparation VII (2.7 mg), 70 mg of succinic anhydride, 0.6 ml of pyridine and 2 ml of CCl_4 was refluxed at 110—115° for 3 hr under nitrogen atmosphere. The reaction mixture was condensed *in vacuo* to the syrup. The residue was redissolved in 15 ml of $CHCl_3$ and filtered. The remaining materials were washed with 2×2 ml of 1 N HCl and extracted with 3×6 ml of 10% $NaHCO_3$ solution.

a) The aqueous extracts combined and acidified to pH 2.0 with 1 N HCl in cold was extracted and washed by Folch's procedure.

The extract was dried over anhydrous Na_2SO_4 overnight, evaporated *in vacuo* and chromatographed on a silicic acid column.

b) The $CHCl_3$ layer was neutralized, dried over anhydrous Na_2SO_4, evaporated *in vacuo* and chromatographed.

Treatment with Phenylisocyanate: A mixture of 530 μg of the active fraction VII, 1.2 ml of $CHCl_3$, 10 mg of phenylisocyanate dissolved in 5 ml of dry benzene was allowed to stand for 24 hr at room temperature, added 0.2 ml of ethanol, and allowed to stand for 5 hr. The reaction mixture was evaporated *in vacuo*, added 5 ml of $CHCl_3$, centrifuged and the sediment was washed with 2×1 ml of $CHCl_3$. The supernatants combined were evaporated *in vacuo* for assay. The sediment was dried *in vacuo* and suspended in saline containing 2% Tween 80 for assay.

Treatment with Diazomethane: The active fraction VII (1.06 mg) dissolved in 20 ml of $CHCl_3$ was treated with etheral solution of diazomethane in large excess at 0°, kept at the same temperature for 24 hr and evaporated *in vacuo*.

Infrared Spectroscopy——Infrared spectrum of D-I (the active fraction VII, 1.0 mg) was measured on a Hitachi Grating Infrared Spectrophotometer Model EPI-GII by liquid film. The wave numbers were calibrated at 2850.7, 1601.4 and 1028.0 cm^{-1} using a polystyrene film.

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8) A. Girard and G. Sandulesco, *Helv. Chim. Acta*, 19, 1095 (1936).

Results and Discussion

Modified Extraction and Purification Procedures

By the use of Folch's solvent as the first extracting solvent and acetone as the second, the procedure became more efficient.

By introduction of Folch's washing procedure[6] to eliminate non-lipids instead of using acidic and alkaline solutions it became feasible to avoid possibility that the active principle might be decomposed by acid or alkali. Partition between 70% ethanol and hexane was very effective to remove a large amounts of inactive simple lipids from the acetone extract II.

Further improvement in the procedures was an application of the second silicic acid chromatography. By the first silicic acid chromatography the lipids (crude syrup III) were roughly fractionated into certain categories of lipids (Table I). The hypotensive activity was mainly recovered from the eluate of $CHCl_3$–MeOH (4: 6, v/v) (active fraction IV). The remaining simple lipids and most parts of polar lipids such as phosphatidic acid, phosphatidyl inositol, phosphatidyl ethanolamine and phosphatidyl choline were removed upon the procedure.

The active fraction IV was further fractionated on the second silicic acid column by discontinuous gradient elution with $CHCl_3$–MeOH mixtures and MeOH successively as depicted in Fig. 1.

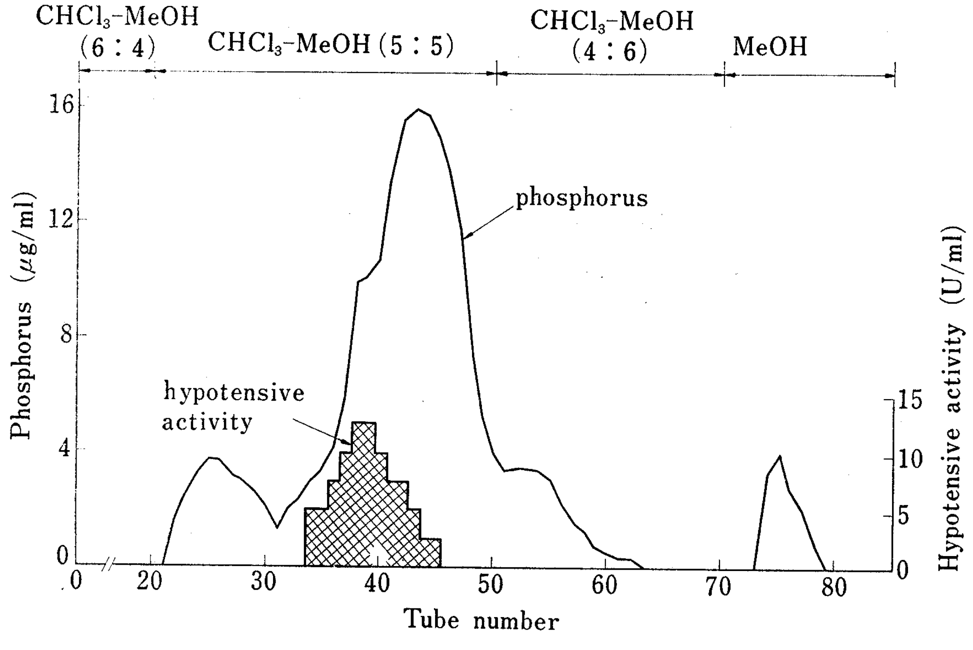

Fig. 1. Elution Pattern of the Second Silicic Acid Chromatography of the Hypotensive Factor D-I

Column: silicic acid (Mallinckrodt, 100 mesh IIB) 40 g and hyflosuper-cel 10 g, 2.5 × 26 cm.
Sample: active fraction IV (299.7 mg corresponding to 4.20 kg of wet tissue).
Elution: discontinuous gradient elution with $CHCl_3$–MeOH (6: 4, v/v) (400 ml), $CHCl_3$–MeOH (5: 5, v/v) (600 ml), $CHCl_3$–MeOH (4: 6, v/v) (400 ml) and MeOH (400 ml). Fractions of 20 ml were collected in tubes.
Phosphorus: carried out in the same manner as indicated in Table I.
Bioassay: carried out in the same manner as indicated in Table I.

The hypotensive activity was distributed in the tube number 35—45 together with a shoulder of the second organophosphorus peak (active fraction V).

Consequently, the first gel filtration was performed in the same way as reported (active fraction VI).[3]

Further modification consisted of the repeated gel filtration using acetone–ethanol (1: 1, v/v) instead of $CHCl_3$–MeOH (1: 1, v/v) as an eluant. The active principle was located in the eluates of 180 to 220 ml (active fraction VII). This gel filtration enabled us to eliminate minor contaminants such as sphingomyelin or phosphatidyl choline without significant loss of biological activity of pure material.

The final material VII obtained by improved procedure was highly purified and showed a single spot on thin-layer chromatography (TLC) with three developing solvent systems used in the previous paper.3)

In the former procedure, the preparation of pure material was first attained by preparative TLC which was accompanied with a great loss of hypotensive activity (approximately 90%). By these modifications the preparation of highly purified material became possible only by column chromatographies in a larger scale and in a more effective manner with relatively good reproducibility.

The preparation VII is estimated to be pure as high as about 200 times compared with crude extract III, and the content of the active factor was so small and estimated to be approximately 0.002% of the total lipids ($CHCl_3$–MeOH extract I).

For evaluating hypotensive potency of the compound, we settled unit value comparing with acetylcholine as a standard agent. In preparation VII, weight of one unit (U) was about 9.8×10^{-6} g, and its phosphorus content was approximately 5.0×10^{-7} g. The minimum effective dose was approximately 1.5 U/kg in an urethane anaesthetized rat.

Examinations of Chemical Characteristics

In order to analyze functional groups involved in the molecule of D–I, *in vivo* experiments were carried out to examine whether the depressor activity of D–I was inactivated or not by treatment with various chemical agents. The results were summarized in Table III.

The preparation VII was examined with Girard's reagent T.9,10)

Upon the bioassay, the hypotensive factor was not detected in ketonic fraction but in nonketonic fraction (approximately 30%) which was eluted mainly in the eluate of $CHCl_3$–

Table III. Recoveries of the Hypotensive Activity of D-I after Treatments with Various Agents

Agent	Recovery of hypotensive activity a) (%)	Martini and others
Girard's reagent T b)		
ketonic fraction	0	Hypotensive9)
non ketonic fraction	30	Inactive
Sodium borohydride	0	Hypertensive11)
Triphenylphosphine	Approximately completely	—
Hydrogen peroxide (0.1%)	0	—
Potassium permanganate	0	—
Ferric chloride	Approximately completely	Hypotensive11)
Bromine	50	—
Succinic anhydride b)	10	Hypotensive (water-soluble)8)
Phenylisocyanate	0	—
Diazomethane	Approximately completely	—

a) Each value is an average of two experiments.
b) After treatment with the agent, the reaction product was chromatographed on a silicic acid column.

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9) E. Martini and A. Marzorati, *Bull. Soc. It. Biol. Sper.*, 29, 436 (1953).
10) E. Martini, A. Marzorati, and E. Morpurgo, *Experientia*, 8, 225 (1952).

MeOH (4: 6, v/v) on silicic acid chromatography. Although it was accompanied with a significant loss of activity possibly because of drastic condition during reaction. In comparison of organophosphorus contents, its 95% was recovered from the non ketonic fraction.

The results obtained here were re-examined using more crude material III to coordinate purification stage of Martini's material, but the results were unchanged.

By treatment with sodium borohydride,[11] the activity of D-I disappeared completely. The preparation was treated with triphenylphosphine, however, no decrease of depressor activity was observed.

The preparation of D-I was allowed to stand with hydrogen peroxide in various concentrations for 30 min at 25°, and the results were shown in Fig. 2.

The hypotensive activity of D-I was inactivated completely with 0.1% of hydrogen peroxide solution, while with 0.001% of the agent the activity suffered little change. The hypotensive activity was also abolished entirely by standing with potassium permanganate (neutral) solution, while with ferric chloride solution[11] the activity was restored almost completely. Standing the preparation with bromine solution resulted in a loss of about half of depressor activity.

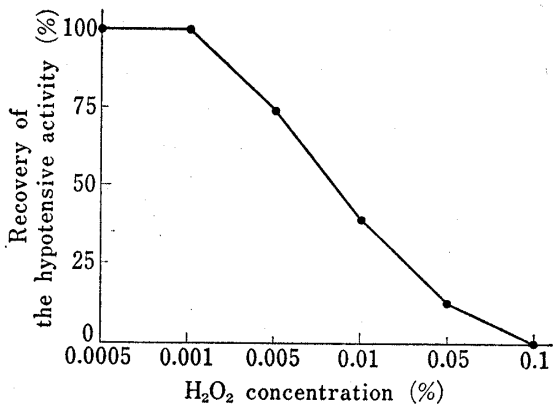

Fig. 2. Recoveries of the Hypotensive Activity of D-I by Treatments with H_2O_2 at Different Concentrations

Samples were incubated at 25° for 30 min at different concentrations of H_2O_2. Each data is a mean of two experiments.

In the reaction of D-I preparation with succinic anhydride, the potency was substantially diminished (approximately 90%), which were located in the eluates of $CHCl_3$–MeOH (4: 6, v/v) and $CHCl_3$–MeOH (2: 8, v/v) mixtures on the silicic acid chromatography in the similar manner to the starting material. With the crude extract III the same result was

Table IV. Attitudes of D-I against Various Reagents on TLC

Reagent		Martini and others
Ammoniac silver nitrate	—	+[10]
α-Naphthol	—	
Resorcinol	±	
Anthrone	±	
Diphenylamine	—	
Blue tetrazolium	—	+[12]
2,4-Dinitrophenylhydrazine-potassium ferricyanide	+ (blue)	
Mercuric chloride-Schiff's	—	
Potassium permanganate	+	
Osmic acid	+	
Periodate-Schiff's	—	
2′,7′-Dichlorofluorescein	+	
Ferric chloride	Brownish green	
Acetic acid–sulfuric acid	Brown	
Ferrous hydroxamate	+	
Benzidine	—	
Phosphoric acid–ethanol	+	

The plates and developing solvent systems on TLC were the same ones as reported in preceding paper.[3]

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[11] E. Martini, F. Chiesa, A. Spiga, and R. Oberosler, *Enzymologia*, 29, 1 (1965).

obtained as preparation VII. On the other hand, Martini and others[5] reported that a water-soluble hemisuccinyl derivative of the active material was obtainable retaining its original activity.

The activity of D–I was completely disappeared by the reaction with phenylisocyanate. By treatment with diazomethane,[12] the hypotensive activity of D–I suffered little alteration.

In our previous paper,[3] the behaviours of D–I against several spray reagents on TLC were described. In the present investigation, further examination was carried out and the results were summarized in Table IV. The colour reactions observed by Martini and his coworkers were also listed up for comparison.[10,13]

Particular attention should be focused on the fact that D–I developed blue colour against the spray of 2,4-dinitrophenylhydrazine followed by potassium ferricyanide reagent suggesting the presence of ketonic group which should be different from aldehyde (greenish). In addition, it is also notable that D–I was positive against the spray of 2′,7′-dichlorofluorescein reagent, and developed a certain colour by the spray of ferric chloride or acetic acid-sulfuric acid reagent.

Infrared (IR) Spectroscopy

Infrared spectroscopy of D–I (preparation VII) was shown in Fig. 3.

D–I shows general characteristics of phospholipid, and especially that of choline-containing phospholipid judging from the strong absorption at 970 cm^{-1}.

The presence of hydroxyl group was confirmed from an absorption at 3400 and the weak one at 1135 cm^{-1} which might be attributed to O–H stretching and C–O stretching, respectively. The strong absorption at 1730 and the weak one 1180 cm^{-1} may be compatible with a C=O stretching and C–O stretching respectively, but any absorption which suggested the presence of carboxyl, aldehyde nor ketonic group was not observed. The strong absorption at 1085 and feeble at 1050 cm^{-1} might be attributed to P–O stretching of phosphate ester group.

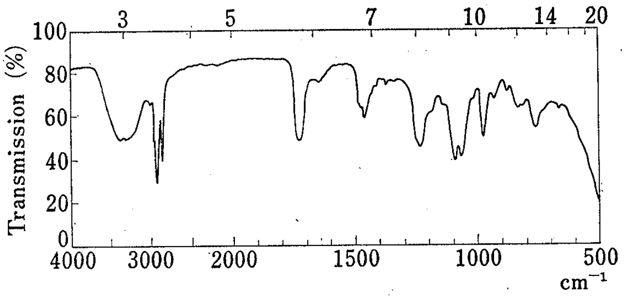

Fig. 3. IR Spectrum of the Hypotensive Factor D-I (Liquid Film)

Furthermore the absorptions which show the presence of CH$_3$ and CH$_2$ were observed as follows: strong absorption at 2920 and 2840 cm^{-1} (asymmetrical stretching of C–H), strong at 1470 cm^{-1} (C–H bending of symmetrical deformation of CH$_2$), weak at 1380 cm^{-1} (C–H bending of symmetrical deformation of CH$_3$) and strong at 1230 and 3030 cm^{-1} (C–H wagging of CH$_2$ and C–H stretching of CH=CH group respectively).

It can be said with these data that absorptional pattern in IR resembled strongly that of lysophosphatidyl choline (LPCh).

Although the presence of ester moiety was recognizable in the preparation VII of D–I from the facts obtained from TLC and IR, however, the question whether D–I contained carbonyl group other than ester group or not remained undissolved, since the results obtained were somewhat conflicting to define: a) having been the hypotensive activity of D–I preparation recovered from only nonketonic fraction in the treatment with Girard's reagent T and no evidence of aldehyde nor ketonic group from IR spectrum obtainable, it is difficult to deduce the presence of the aldehyde or ketonic group in the preparation. From the results on TLC, it may be concluded that D–I has no reducing power due to aldehyde nor sugar

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[12] H.D. Crone, *Biochim. Biophys. Acta*, **84**, 665 (1964).

[13] M. Marmori, E. Martini, and L. Zanaria, *Enzymologia*, **16**, 117 (1953).

moiety. Possibility containing long chain aldehyde group will be also excluded from results on TLC, and this fact was further confirmed by combined gas chromatography–mass spectrometry (GC–MS). These findings support a view that D–I might have no such group in its molecule.

b) It seems proper ascribing disappearance of depressor activity of D–I by sodium borohydride to strong alkaline of the agent not to its reducing power, when we considered the instability of D–I to alkaline obtained from our experiences in the previous investigations.

c) As a proof for the presence of a ketonic group in the D–I preparation was colour development (blue) by the spray of 2,4-dinitrophenylhydrazine-ferricyanide reagent on TLC.

Judging from results obtained above and in preceding paper,³⁾ it is likely that D–I might have no carbonyl group otherwise ester group concerning the hypotensive activity but as one of modified or degraded product, namely, by the spray of reagents or some alterations in conditions the active factor converts readily to ketonic compound possibly resulting in the loss of hypotensive activity.

The depressor factor contains a part in the structure sensitive to attacks by oxidizing agents (permanganate, hydrogen peroxide) which affect on essentially active sites to produce the biological activity. In addition D–I might have unsaturated bond since the hypotensive activity was damaged by absorption of bromine, and it was further supported by the observation in IR spectrum. From result of treatment with succinic anhydride or phenylisocyanate, D–I might have free hydroxyl group and this is in accordance with the observations in IR. However, it might have no vicinal hydroxyl group, since it was negative to periodate-Schiff's spray reagent on TLC. D–I might have no corticoid type moiety in the structure because it was negative against the spray of blue tetrazolium reagent on TLC. Having unaffected the activity by treatment with diazomethane, D–I has no free carboxyl group as PGs in agreement with the observation in IR spectrum. D–I also has no peroxide group, since its activity was recovered after treatment with triphenylphosphine.¹⁴⁾ D–I contains no amide group from the results on TLC and IR spectrum. As a conclusion for the chemical constitution of D–I, this vaso-depressor active principle resembles LPCh, except the potent hypotensive action and several characteristic reactions on TLC against spray reagents such as 2,4-dinitrophenylhydrazine and so on. D–I might be a choline containing phospholipid having double bond, free hydroxyl, ester, while it has no free amino, free carboxyl, amide, peroxide nor vicinal hydroxyl group.

In comparison between Martini and his coworkers' results and ours, several differences in chemical inactivations and colour reactions by the agents were observed as indicated in Table III and IV. The reason why such discrepancies between Martini and others and us has occurred might be mainly due to difference in purities of materials used. With the colour reactions by phosphomolybdate and 2,4-dinitrophenylhydrazine reagents,³⁾ however, the results obtained by us agreed with those by Martini's.

Vogt¹⁵⁾ and Erspamer¹⁶⁾ reviewed on the lipids of pharmacological interest and having mammalian origin. In regard to LPCh the reports which investigated on the vaso-activity of LPCh are not so many.¹⁷,¹⁸⁾ Recently, we have reported the occurrence of a potent pressor-active factor in anaesthetized rats, L-1-lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate) from crude soybean lecithin.¹⁹,²⁰⁾

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Our hypotensive principle D–I is obviously a phospholipid with some special characters, and might be a naturally-occurring vasoactive factor which possibly plays an important role in the regulation of blood pressure in cardiovascular system of animal body.

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