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

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Stability of D-I preparation on standing at low temperature was examined. Standing in saline solution at 4°, the hypotensive activity was stable for the first 45 days, and lost completely within a following week. Standing as $CHCl_3$–MeOH (2: 1, v/v) solution at 4°, the decomposition began at 75 days later, and it reached a plateau maintaining about 20% of the initial activity. At −20° as a syrup form, the activity could be stored unaltered for 120 days.

In the partition experiments, *n*-hexane and ethylether could extract little active component at all pH values tested (pH 4, 7 and 10). While *n*-butanol or Folch's solvent could extract unilaterally without any pH effects.

The nuclear magnetic resonance spectrum of D-I strongly resembles that of lysophosphatidyl choline (LPCh). Specific optical rotation was also similar to that of LPCh. In the assessment of molecular weight on Sephadex LH-20 column eluting with 80% EtOH, the active substance was eluted in the neighbourhood of L-dipalmitoylphosphatidyl choline or L-1-palmitoyl-LPCh and its molecular weight was estimated to be about 500 to 800.

In the analysis, the ratio of phosphorus, glycerol, choline and fatty acid was approximately 1: 1: 1: 1, and oleic acid shared about half of the fatty acid composition.

D-I might be a LPCh or closely related compound to it.

Keywords——D-I; hypotensive; lysophosphatidyl choline; brain acetone extract; stability; partition behaviour

Recently, we have reported the occurrence of an acute hypotensive factor from acetone soluble fraction of bovine brain and tentatively designated the substance as D-I.[3)] More recently in precending paper,[4)] we established a modified procedure for preparation of further purified material showing a single spot on thin-layer chromatography (TLC) through partition and repeated column chromatographies. In addition, the chemical characteristics of D-I were examined by means of assessment of functional groups. From these investigations it became clear that D-I was apparently distinguishable from not only already known water soluble hypotensive substances such as acetylcholine, histamine, serotonin, adenosine derivatives but also naturally occurring hypotensive lipids, such as arachidonic acid,[5)] prostaglandins (PGs)[6)] and so on.

D-I was considered to be a trace component of bovine brain lipid fraction, generally resemble lysophosphatidyl choline (LPCh) and possibly may play an important role in the

1) Part of this work was presented at the 97th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 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).
4) H. Tsukatani, S. Yamada, M. Fujii, T. Awaji, M. Okamoto, and T. Itami, *Chem. Pharm. Bull.* (Tokyo), **26**, 3271 (1978).
5) J.C. Rose, M. Johnson, P.W. Ramwell, and P.A. Kot, *Proc. Soc. Exptl. Biol. and Med.*, **147**, 652 (1974).
6) *a*) J. Nakano and B. Cole, *Amer. J. Physiol.*, **217**, 222 (1969); *b*) M.C. Koss, J.W. Gray, M. Davidson, and J. Nakano, *Eur. J. Pharmacol.*, **24**, 151 (1973); *c*) S. Bergstrom, L.A. Carison, and J.R. Weeks, *Pharmacol. Rev.*, **20**, 1 (1968).

regulation of the blood pressure in cardiovascular system.

In the present investigation we carried out physico-chemical studies and some chemical analysis of D-I.

Materials and Methods

Materials——All chemicals were of reagent grades and purchased commercially. Solvents were redistilled before use.

The preparation VII of D-I was obtained by modified extraction and purification procedures through repeated column chromatographies according to the method described in preceding paper.[4] The preparation VII showed a single spot on TLC and was used throughout this study.

Bioassay——Most biological experiments were conducted by intrafemoralvenous injections into urethane anaesthetized rats (1.8 g/kg, *i.p.*) in the same manner as reported[4] unless otherwise stated.

Stability——1) A solution of preparation VII was prepared in physiological saline. The hypotensive activities of aliquots of the solution kept at 4° after different days were measured. The mean of two experiments was calculated.

2) A Folch's solvent [$CHCl_3$–MeOH (2: 1, v/v)] solution of preparation VII was prepared and kept at 4°. Aliquots of the solution were dried and redissolved in saline for assay.

3) A certain amount of the preparation VII was transferred into an ampule, dried *in vacuo*, sealed and stored in a freezer at −20°.

The ampule was cut and an aliquot of the content was used for assay.

Partition Behaviours——To the solution of preparation VII dissolved in respective organic solvent was added equal volume of distilled water adjusted to various pHs. After shaking vigorously for 10 min at room temperature, organic layer was separated from aqueous layer. Both of the organic and aqueous layers were neutralized and evaporated *in vacuo*. Partition between Folch's solvent and one fifth of its volume of aqueous media adjusted to various pHs were performed in the same manner as described above.

Assessment of Molecular Weight——On a Sephadex LH-20 column was applied D-I preparation VII and the column was eluted with 80% ethanol.

Analytical Procedures——Phosphorus was determined by microanalytical method described by Chalvardjian and Rudnicki.[7]

Glycerol content was measured by the procedure descrived by Renkonen.[8] Choline was determined according to the method of Glick[9a] utilizing microanalytical method described by Argiydekusm and Tobias.[9b]

Fatty acid methyl esters were prepared by methanolysis,[10] and determined by a Shimadzu Gas Chromatograph GC-5A.

The GC–MS analysis of the methyl esters was carried out on a Hitachi GC–MS Model RMU-6M equipped with datalyzer system. The inlet system was equipped with a glass column (3 mm id × 2 m) packed with 1.5% SE-30 on 60—80 mesh Gas Chrom Q. Column temp. 150°—260° at a rate 5°/min, injection temp. 260°, carrier gas He 40 ml/min. The molecular separator was maintained at 260° and the ion source of the mass spectrometer at 270°. Accelerating voltage was 3.2 kV. Spectra were recorded at ionizing energies of 20 and 70 eV.

The Dawson's preparative procedure[11] was also used in ethanolic medium for the acids and followed by methylation by diazomethane. But it resulted in formation of a mixture of ethyl and methyl esters. Therefore, no longer this procedure in ethanol was used in the recent study.

Nuclear magnetic resonance (NMR) was recorded on a JEOL NMR Spectrometer Model JNM-PS-100. The preparation VII (15 mg) was dissolved in 0.35 ml of $CDCl_3$ and measured using tetramethylsilane as a reference. Optical rotation was measured on a Yanaco Automatic Polarimeter Model OR-50.

Results

Stability

In order to examine the biological stability the preparation VII of D-I was permitted to stand under three different conditions at low temperatures. The remaining hypotensive activity *vs.* time after its exposure to various conditions were depicted in Fig. 1.

7) A. Chalvardjian and E. Rudnicki, *Anal. Biochem.*, **36**, 225 (1970).
8) C. Renkonen, *Biochim. Biophys. Acta*, **56**, 367 (1962).
9) *a*) D. Glick, *J. Biol. Chem.*, **156**, 643 (1944); *b*) C.A. Argiydekusm and J. Tobias, *Anal. Biochem.*, **64**, 276 (1975).
10) Y. Kishimoto and N.S. Radin, *J. Lipid Res.*, **6**, 435 (1965).
11) R.M.C. Dawson, *Biochem. J.*, **75**, 45 (1960).

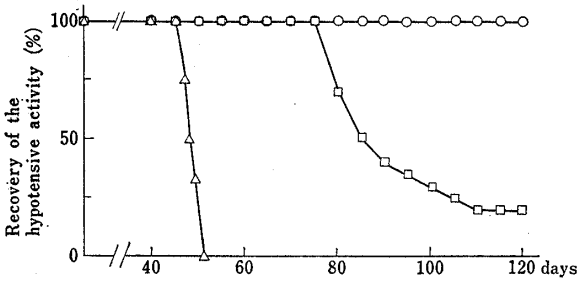

Fig. 1. Stability on Standing of the Hypotensive Factor D-I

△—△: in NaCl solution, at 4°.
□—□: in $CHCl_3$–MeOH (2: 1, v/v), at 4°.
○—○: as a syrup form, at −20°.

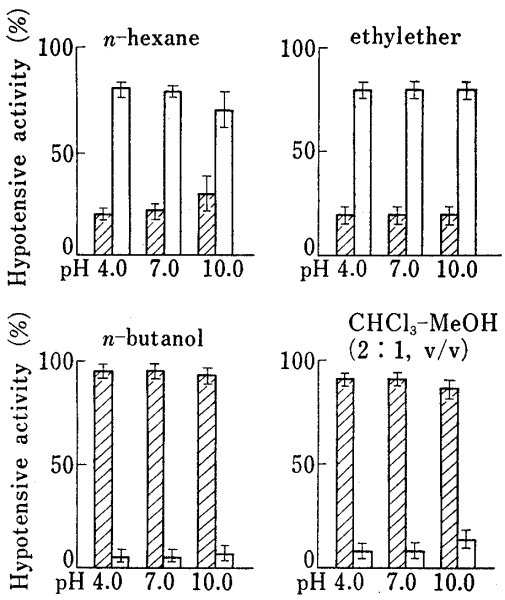

Fig. 2. Partition Behaviours of the Hypotensive Factor D-I

▨: organic layer □: aqueous layer
The data were shown as means ± standard errors of the means. $n=5$.

1) On standing in a saline solution at 4,° the activity was stable for the first 45 days, and lost completely within a following week suggesting autocatalysis.

2) In Folch's solvent at 4,° the pattern of destruction was somewhat different from that in saline solution. The decomposition started at 75 days later, and its activity fell down to approximately 30% of the initial potency. Ninety days later it reached a plateau where was maintained approximately 20% of the depressor activity at the initial stage.

3) In a storage at −20° as a syrup form, the biological activity was unaltered for 120 days.

When the decomposition started, velocities were very fast in every conditions and half-life periods (days required for 50% destruction of the active factor) were about 3 and 10 days in saline and Folch's solvent respectively. Considering these results, storage at −20° as a syrup form is the most preferable way for reservation of hypotensive activity of D-I. In comparison between aqueous and organic solvent for the reservation, the latter system was superior than the former.

Partition Behaviours

We examined partition behaviours of D-I between several organic solvents (*n*-hexane, ethylether, *n*-butanol and Folch's solvent) and aqueous media at different hydrogen ion concentrations (pH 4, 7 and 10). The hypotensive activity extracted in each phase was illustrated in Fig. 2.

At all hydrogen ion concentrations, no detectable amounts of the active factor could be extracted with *n*-hexane, the least polar solvent of the tested.

Ethylether also behaved in the similar manner to *n*-hexane.

Whereas the hypotensive activity was unilaterally partitioned at any pH range of aqueous medium with *n*-butanol, the most polar solvent among three. In the case of Folch's solvent, the tendency was similar to that of *n*-butanol.

It seems that with augmentation in polarity of organic solvent the hypotensive activities in organic phases were increased without any pH effects. From these findings it seems that D-I might be a polar and possibly neutral or amphoteric substance.

The distribution of organophosphorus was parallel to that of depressor-activity in each partition experiment. These partition behaviours of D-I seem to be identical to that of LPCh as reported by Kristian and others.[12]

12) S. Kristian, B. Jerve, N. Ludvig, W. Daae, and J. Bremer, *Anal. Biochem.*, **58**, 238 (1974).

NMR Spectrum

The NMR spectrum of D-I preparation was shown in Fig. 3 and the assignment of each signal was summarized in Table I. It shows general characteristics of LPCh.

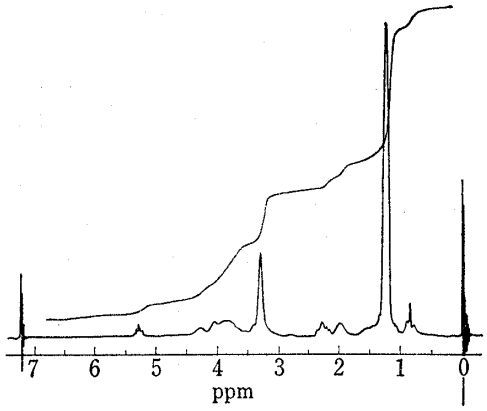

Fig. 3. NMR Spectrum of the Hypotensive Factor D-I

Table I. NMR Analysis of the Hypotensive Factor D-I

Chemical shift (δ) (ppm)	Group
0.88	$-C\underline{H}_3$
1.3	$-C\underline{H}_2-$
2.0—2.04	$-C\underline{H}_2-CH=$
2.26	$-C\underline{H}_2-CO-$
3.3	$-\overset{+}{N}(C\underline{H}_3)_3$
3.6—4.6	$>C\underline{H}-OH$, $-C\underline{H}_2-OP-$, $-C\underline{H}_2-OCOR$
5.3	$-C\underline{H}=C\underline{H}-$

Specific Optical Rotation

The specific optical rotation of D-I was $[\alpha]$ 3.3 ($CHCl_3$), and that of LPCh (from egg yolk lecithin) was $[\alpha]$ 3.3 ($CHCl_3$). Any fundamental difference was not observed between them.

Charge Characteristic

D-I was examined for its electrophoretic mobility. In view of presumptive evidence that D-I was unstable in strong alkali or acid, the active factor was examined for its electrophoretic mobility only in a neutral condition. The active factor hardly migrated at the constant current 1 mA/cm [0.2 M phthalate (pH 5.9), initial voltage 600 V, 120 min, Toyo Roshi No. 51] by the detection with phosphomolybdate reagent.

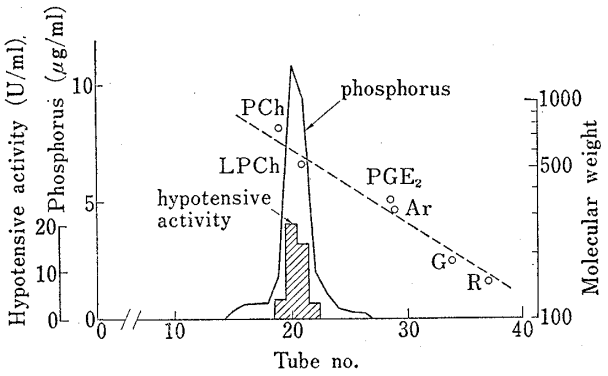

Fig. 4. Elutional Profile of the Hypotensive Factor D-I on Sephadex LH-20 Column Eluting with 80% Ethanol

Column: Sephadex LH-20 100 g (Pharmacia), 2.7×90 cm).
One tube: 10 ml.
Sample: D-I preparation VII 4.77 mg corresponding to 900 g of bovine brain.
PCh: L-Dipalmitoylphcsphatidyl chlone (M.W. 751)
LPCh: L-1-Palmitoyllysophosphatidyl choline (M.W. 514)
PGE_2: Prostaglandin E_2 (M.W. 352)
Ar: Arachidic acid (M.W. 313)
G: D-Glucose (M.W. 183)
R: D-Ribose (M.W. 150).

Table II. Fatty Acid Composition of the Hypotensive Factor D-I

Fatty acid	%
$C_{14:0}$	trace
$C_{15:0}$	trace
$C_{16:0}$	26.6
$C_{16:1}$	1.2
$C_{17:0}$	0.2
$C_{17:1}$	0.2
$C_{18:0}$	6.4
$C_{18:1}$	56.1
$C_{18:2}$	0.4
$C_{19:1}$	0.4
$C_{20:1}$	8.5

Fatty acid methyl esters obtained by methanolysis of D-I preparation VII were analyzed by a Shimadzu Gas Chromatograph GC-5A equipped with a glass column (3 mm id×1.5 m) packed with 15% DEGS on 60—80 mesh Gaschrom Q; column temp. 160°, injection temp. 210°, nitrogen flow rate 25 ml/min.

From this result, D-I might be a neutral or amphoteric substance, and it was in excellent agreement with findings of mobilities on TLC under three different conditions[3] or partition behaviours.

Assessment of Molecular Weight

The preparation VII was subjected on a Sephadex LH-20 column and filtered with 80% ethanol according to the method described by Orange and his coworkers,[13] who applied the procedure for assessment of molecular size of slow reacting substance of anaphylaxis (SRS–A). The elutional profile of D-I was depicted in Fig. 4.

Application of the preparation on the column and development yielded D-I of apparent molecular size between about 500 and 800 as a single peak. It was closely related to L-dipalmitoyl-phosphatidyl choline (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) or L-1-palmitoyl-LPCh (1-palmitoyl-*sn*-glycero-3-phosphocholine).

Analysis of Constituents of D-I

Analysis of D-I preparation VII was performed. The molar ratio of glycerol or choline to phosphorus was indicated as 0.99 or 0.97 respectively.

In fatty acid analysis with a sample obtained by methanolysis the molar ratio to phosphorus was 0.98, that is 1:1 indicating D-I might be LPCh. The fatty acid composition was shown in Table II.

Neither fatty acids obtained by alkaline hydrolysis according to Dawson's method[11] nor methyl esters by methanolysis[10] from preparation VII had definite detectable biological activity.

The most predominant fatty acid was $C_{18:1}$ acid other than saturated acid, and its content was 56.1%. The contents of saturated fatty acid such as $C_{16:0}$ and $C_{18:0}$ were 26.6 and 6.4% respectively, which were known to be main components of LPCh derived from animal tissues or fluids.[14]

The contents of unsaturated fatty acids involving $C_{18:1}$ and $C_{20:1}$ (8.5%) attained to about 65%. This value seems to be somewhat higher than those found already in LPCh obtainable from mammalian sources. Whether such unusual high contents of unsaturated fatty acids is associated with the biological function or not remained to be examined further.

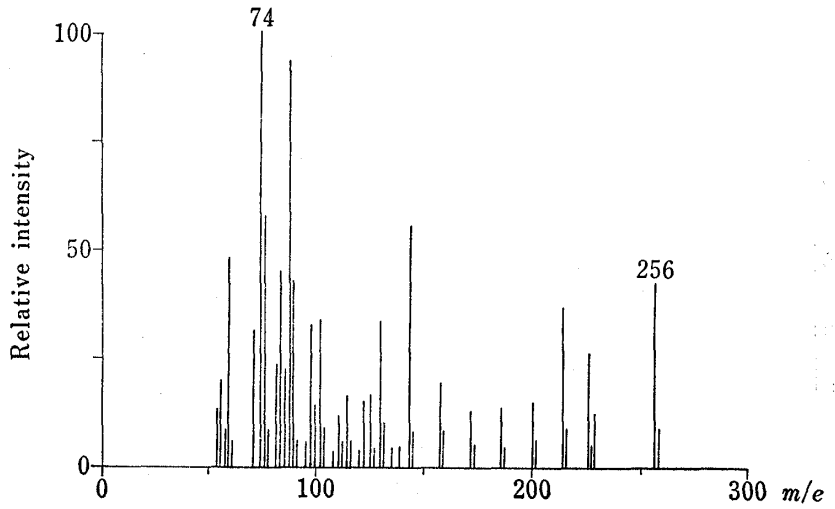

Fig. 5. Mass Spectrum of $C_{15:0}$ Fatty Acid Methyl Ester

13) R.P. Orange, R.C. Murphy, M.L. Karnovsky, and K.F. Austen, *J. Immunol.*, **110**, 760 (1973).
14) N.H. Tattrie and R. Cyr, *Biochim. Biophys. Acta*, **70**, 693 (1963).

The $C_{18:1}$ acid was confirmed as oleic acid by GC–MS spectroscopy, and unlikely so-called "G-acid," namely $\Delta^{3,4}$-octadecenoic acid.[15a,b]

Besides such characteristic pattern in the distribution of fatty acid composition of preparation VII, the presence of odd number fatty acids such as $C_{15:0}$, $C_{17:0}$, $C_{17:1}$ and $C_{19:1}$ were confirmed by GC–MS spectroscopy, although their contents among fatty acids were very poor. The GC–MS spectra of $C_{15:0}$, $C_{17:0}$ and $C_{19:1}$ fatty acid methyl esters were illustrated in Fig. 5, Fig. 6 and Fig. 7 respectively.

The content of arachidonic acid ($C_{20:4}$) which might be able to have hypotensive activity[5] was very poor.

During the fatty acid analysis by Dawson's preparative procedure[11] in ethanol and followed by methylation with diazomethane, we obtained a mixture of methyl and ethyl esters. By replacing the medium to methanol instead of ethanol, contamination of ethyl group was eliminated.

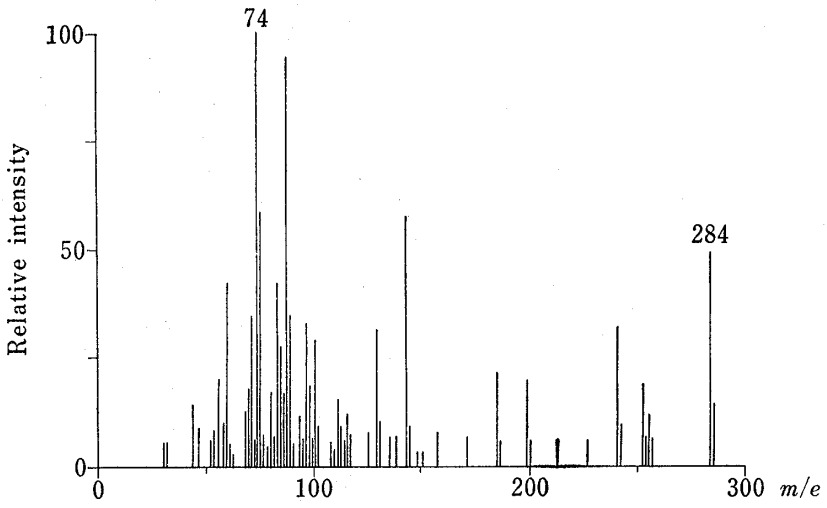

Fig. 6. Mass Spectrum of $C_{17:0}$ Fatty Acid Methyl Ester

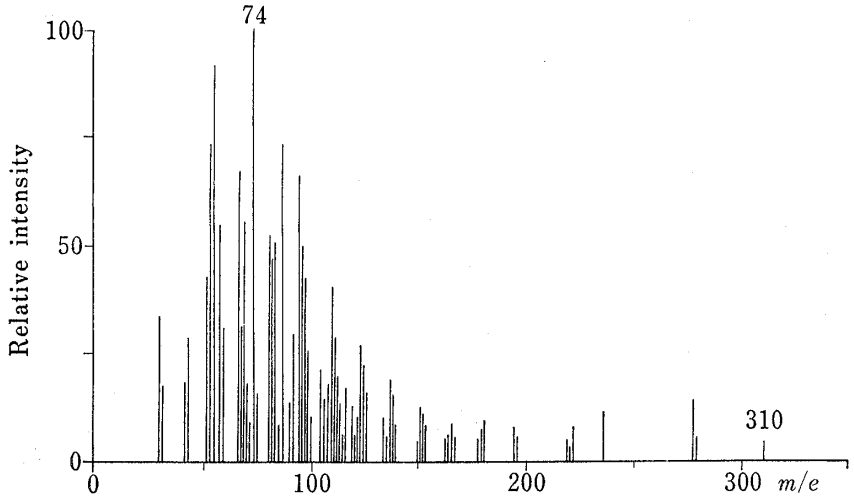

Fig. 7. Mass Spectrum of $C_{19:1}$ Fatty Acid Methyl Ester

15) *a*) Y. Gabr, *Brit. J. Pharmacol.*, **11**, 93 (1956); *b*) Y. Gabr, *ibid.*, **17**, 51 (1961).

Discussion

Enormous amounts of literatures with regard to biologically active lipids have revealed. Vogt[16] and Erspamer[17] reviewed with pharmacologically active lipids occurred in animal sources.

Thereafter, the chemistry and pharmacology of PGs have been developed extensively involving studies on PG endoperoxide intermediates.[18a-g] Among other fatty acids, arachidonic acid attracted our attentions as a bio-active acid which had a role as a precursor for biosynthesis of PG endoperoxide intermediates,[18a] though arachidonic acid itself elicited only a low hypotensive response.[5] So called G-acid[15a,b] was also notable, though its occurrence has not been confirmed by other investigators.

With respect to pharmacological activities of phospholipids especially with vaso-activity, there have not been abundant informations. No report with PG phospholipid derivative have been published. It seems that vaso-activity was focused mainly on lyso-type derivatives among phospholipids.

A few reports concerned with the vaso-activity of LPCh; Khairallah and Page[19] reported the appearence of pressor active substance from incubated dog plasma, and ascribed it to LPCh possibly produced from phosphatidyl choline or others. On the other hand, they observed that synthetic stearoyl-LPCh elicited depressor responses in rats. They ascribed the contradictory vaso-activities to differences in fatty acid compositions and spatial arrangements between two.

In our preliminary investigation (unpublished data) the hypotensive activity of LPCh (L-1-stearoyl-LPCh, by intravenous injections in urethane anaesthetized rate) was not so intensive compared with D–I preparation. Furthermore, we observed no hyper-tensive action with LPChs so far as tested in anaesthetized rats. Judging from our recent observations, we are apt to consider that the hyper-tensive actions in rats observed by Khairallah and Page might be due to L-1-lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate, LPA[20a-c] possibly formed during incubation of plasma by activated phospholipase A_2 or D from phosphatidyl choline, LPCh and others.

In regard to D-I upon its analysis, the ratio of phosphorus: glycerol: choline: fatty acid was approximately 1: 1: 1: 1, and it was suggested that the factor might be LPCh in which oleic acid occupied about half of the total fatty acids. This profile in fatty acid composition seemed to be a specific character of D-I.

In addition, the observations in examining partition behaviour, charge character, NMR spectrum, specific optical rotation and estimation of molecular weight support the view that D-I might be a sort of LPCh. The results obtained from IR spectrum or mobilities on TLC reported in the preceding papers[3,4] also support the view. On the other hand, in the examinations of stability at low temperatures, apparent differences were recognizable between D-I preparation and stearoyl-LPCh. This was another distinguished character. It also true that there were clear-cut evidence which suggested the presence of a certain ketonic

16) W. Vogt, *Pharmacol. Rev.*, **10**, 407 (1958).
17) V. Erspamer, *Ann. Rev. Pharmacol.*, **1**, 175 (1961).
18) *a*) M. Hamberg and B. Samuelsson, *Proc. Nat. Acad. Sci. USA.*, **71**, 3400 (1974); *b*) M. Hamberg and B. Samuelsson, *ibid.*, **70**, 899 (1973); *c*) M. Hamberg, J. Svensson, T. Wakabayashi, and B. Samuelsson, *ibid.*, **71**, 345 (1974); *d*) M. Hamberg, J. Svensson, and B. Samuelsson, *ibid.*, **71**, 3824 (1974); *e*) D.H. Nugteren and E. Hazelhof, *Biochim. Biophys. Acta*, **326**, 448 (1973); *f*) R.R. Gorman, M. Hamberg, and B. Samuelsson, *J. Biol. Chem.*, **250**, 6460 (1975); *g*) M. Hamberg, P. Hedqvist, K. Strandberg, J. Svensson and B. Samuelsson, *Life Sci.* **16**, 451 (1975).
19) P.A. Khairallah and I.H. Page, *Amer. J. Physiol.*, **161**, 561 (1960).
20) *a*) A. Tokumura, Y. Akamatsu, S. Yamada, and H. Tsukatani, *Agric. Biol. Chem.*, **42**, 515 (1978); *b*) A. Tokumura, K. Fukuzawa, Y. Akamatsu, S. Yamada, T. Suzuki, and H. Tsukatani, *Lipids*, **13**, 468 (1978); *c*) A. Tokumura, K. Fukuzawa, and H. Tsukatani, *Lipids*, **13**, 572 (1978).

group in the molecule of D-I as a decomposition product as described in the preceding papers.[3,4] If one assumed that D-I might be LPCh constituted with common fatty acids, it is difficult to understand evidence mentioned above. Of course, there remains the possibility that the component convertible to ketone might be only a contaminant carrying no hypotensive activity.

Because the cardiovascular effect of LPCh has been not well characterized at present, we are unable to draw the conclusion about chemical structure of D-I.

Our D-I is a trace component of phospholipid fraction and is undoubtedly a lyso-type phosphatide, possibly LPCh.

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