[Chem. Pharm. Bull. 22(3) 663—668 (1974)]

UDC 547.92.09 : 615.357.015.4

Occurrence of Cysteine Conjugate of 2-Hydroxyestrone in Rat Bile, with Special Reference to Its Formation Mechanism[1]

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(Received July 12, 1973)

The biliary excretion of the water-soluble metabolites in the rat administered with 2-hydroxyestrone-6,7-^{3}H and estrone-4-^{14}C has been investigated. A polar metabolite separated from the collected bile was characterized to be a conjugate of 2-hydroxyestrone with cysteine at C–1 or C–4 through a sulfide linkage. The *in vitro* experiment with the rat liver preparation also demonstrated the formation of the cysteine conjugate. Based upon the ^{3}H/^{14}C values of 2-hydroxyestrone and its cysteine conjugate the probable mechanism involved in conjugation has been discussed.

In recent years considerable attentions have been focused on *in vivo* and *in vitro* formation of the estrogen conjugate linked with sulfur-containing amino acid or peptide. The structure of the water-soluble product formed from estradiol with the rat liver preparation was elucidated to be the conjugate of 2-hydroxyestrogen with glutathione or cysteine at C-1 or C-4 through a sulfide linkage.[3-5] Similarly, estradiol was transformed into the analogous conjugate, when incubated with the human liver homogenate.[6] It was also reported that the glutathione conjugate of catechol estrogen was converted into the corresponding mercapturic acid along the known pathway associated with the exogenous substances[7,8] and was capable of being an acceptor for transmethylation catalyzed by catechol O-methyltransferase.[6] The formation mechanism of the conjugate was explained in such a way that a possible intermediate derivable from the catechol estrogen, that is quinone or semiquinone, would react with the sulfhydryl group nonenzymatically.[3,9] The metabolism of estrogen has a characteristic feature of enterohepatic circulation, which is distinctly different from that of other steroid hormones. However, the significance of biotransformation, in particular conjugation, in the biliary excretion still remains unsolved.[10-13] A great interest in these respects prompted us to explore the biliary excretion of the water-soluble metabolite of estrogen and its formation mechanism by *in vivo* and *in vitro* studies.

1) This paper constitutes Part LXIX of the series entitled "Analytical Chemical Studies on Steroids"; Part LXVIII: T. Nambara and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **22**, 687 (1974). In this paper the following trivial names were used: estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; 2-hydroxyestrone, 2,3-dihydroxyestra-1,3,5(10)-trien-17-one; 2-methoxyestrone, 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one; 2-hydroxyestrone 3-methyl ether, 2-hydroxy-3-methoxyestra-1,3,5(10)-trien-17-one; 3-deoxyestrone, estra-1,3,5(10)-trien-17-one.
2) Location: *Aobayama, Sendai.*
3) P.H. Jellinck, J. Lewis, and F. Boston, *Steroids*, **10**, 329 (1967).
4) E. Kuss, *Z. Physiol. Chem.*, **348**, 1707 (1967); *ibid.*, **350**, 95 (1969); *ibid.*, **352**, 817 (1971).
5) H.O. Hoppen, L. Siekmann, H. Breuer, and R. Knuppen, *Acta Endocrinol.*, Suppl. **152**, 56 (1971).
6) R. Knuppen, P. Ball, O. Haupt, and H. Breuer, *Z. Physiol. Chem.*, **353**, 565 (1972).
7) J.S. Elce, *Biochem. J.*, **116**, 913 (1970); *ibid.*, **126**, 1067 (1972).
8) J.S. Elce and J. Harris, *Steroids*, **18**, 583 (1971).
9) F. Marks and E. Hecker, *Biochim. Biophys. Acta*, **187**, 250 (1969).
10) A.A. Sandberg and W.R. Slaunwhite, Jr., *J. Clin. Invest.*, **36**, 1266 (1957).
11) A.A. Sandberg, R.Y. Kirdani, N. Back, P. Weyman, and W.R. Slaunwhite, Jr., *Am. J. Physiol.*, **213**, 1138 (1967).
12) H. Watanabe, *Biochim. Biophys. Acta*, **231**, 399 (1971).
13) A. Bartke, R.E. Steele, J.G. Williams, and K.I.H. Williams, *Steroids*, **18**, 303 (1971).

2-Hydroxyestrone-6,7-^{3}H and estrone-4-^{14}C were intravenously administered to the rat with cannulation of the bile duct, and the bile was then collected. After removal of the free steroids by extraction with ether, the residue was subjected to hydrolysis with β-glucuronidase. A mixture of the liberated steroids was submitted to thin-layer chromatography (TLC), by which 2-methoxyestrone (I), 2-hydroxyestrone 3-methyl ether (II), and 2-hydroxyestrone (III) were separated and unequivocally characterized by usual criteria. These metabolites were recrystallized repeatedly until the constant $^{3}H/^{14}C$ value was attained as collected in Table I.

The non-hydrolyzable fraction was adsorbed on Amberlite XAD-2 resin, washed thoroughly with distilled water and then eluted with methanol. The eluate was submitted to gel filtration on Sephadex G-25, whereby the radioactive metabolites were separated into three fractions, G-I, G-II, and G-III, as shown in Fig. 1. Of these three G-I was further divided into two predominant fractions, A-I and A-II, accompanied with fraction A-III when chromatographed on DEAE-Sephadex, as illustrated in Fig. 2. Being subjected to enzymatic hydrolysis, followed by solvolysis, both A-I and A-II did not yield any detectable amount of the free steroid.

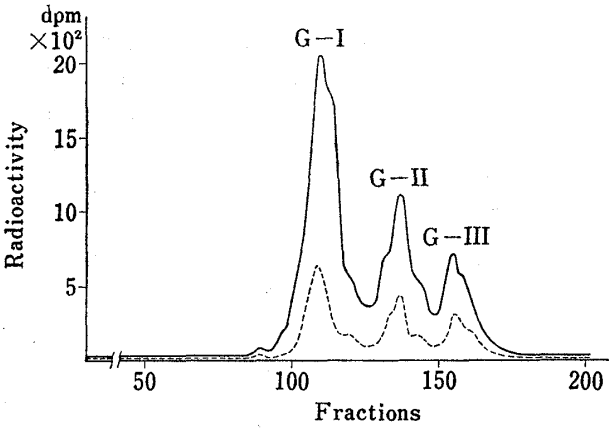

Fig. 1. Chromatography of Water-soluble Fraction on Sephadex G-25

——: ^{3}H ------: ^{14}C

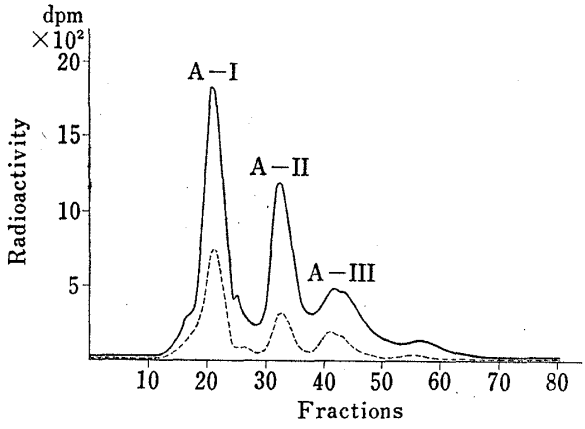

Fig. 2. Chromatography of Fraction G-I on DEAE-Sephadex A-25

——: ^{3}H ------: ^{14}C

A single metabolite derived from the fraction A-I showed red coloration with conc. sulfuric acid and the positive reaction with both potassium dichromate-silver nitrate[14] and Folin-Ciocalteu reagent indicating the conjugate of catechol estrogen with sulfur-containing amino acid. Desulfurization with Raney nickel afforded a steroidal aglycone, whose structure was definitely characterized as 2-hydroxyestrone by reverse dilution analysis (Table II). On the other hand hydrolysis with hydrochloric acid resulted in formation of cysteine, which was identified by two-dimensional TLC. These results permitted us to assign the structure S-[2,3-dihydroxy-17-oxoestra-1,3,5(10)-trien-1(or 4)-yl]cysteine (2-hydroxyestrone 1(or 4)-cysteine conjugate) (IV) to this metabolite.[15] It is to be noted that the $^{3}H/^{14}C$ value of 2-hydroxyestrone was obviously higher than that of cycteine conjugate.

The presence of two estrogen conjugates in fraction A-II was indicated from the results on the color reactions, chromatographic behaviors, and enzymatic hydrolysis. Unfortunately these metabolites were not sufficiently available for further examination. Fraction G-II underwent enzymatic hydrolysis to yield the radioactive metabolites in the rate of *ca.* 60% and hence appeared to consist of the glucuronides principally.

14) R.H. Knight and L. Young, *Biochem. J.*, **70**, 111 (1958).
15) Whether the conjugated amino acid was the N-acetate or not remains unclear since acid hydrolysis was performed prior to analysis.

In order to clarify the mechanism involved in the formation of the cysteine conjugate, *in vitro* study was then undertaken by means of the double isotope technique. 2-Hydroxyestrone-6,7-^{3}H, estrone-4-^{14}C, and cysteine were incubated aerobically with rat liver microsomes in the presence of NADPH. Separation of the cysteine conjugate from the incubation

Table I. Identification of Metabolites Derived from Glucuronide Fraction by Reverse Isotope Dilution

Compound	Crystallization No.	Crystallization from	Specific activity (dpm/mg) ^{3}H	Specific activity (dpm/mg) ^{14}C	$^{3}H/^{14}C$
2-Hydroxyestrone	1st	MeOH	8.71×10^{4}	10.1×10^{3}	8.1
	2nd	acetone	7.63	9.89	7.7
	3rd	MeOH–H_2O	7.76	9.95	7.8
2-Methoxyestrone	1st	MeOH	6.55×10^{4}	1.15×10^{4}	5.8
	2nd	MeOH	7.01	1.08	6.5
	3rd	acetone–hexane	7.02	1.08	6.5
2-Hydroxyestrone 3-methyl ether[a)]	1st	MeOH	8.89×10^{3}	2.14×10^{3}	4.1
	2nd	MeOH	8.23	2.06	4.0
	3rd	MeOH	8.15	2.12	3.8
	4th	acetone–H_2O	8.16	2.13	3.8

a) Nonradioactive 2-hydroxyestrone 3-methyl ether (10 mg) was added as a carrier.

Table II. The $^{3}H/^{14}C$ Value of 2-Hydroxyestrone formed from Cysteine Conjugate by Desulfurization[a)]

Crystallization[b)] No.	Crystallization[b)] from	Specific activity (dpm/mg) ^{3}H	Specific activity (dpm/mg) ^{14}C	$^{3}H/^{14}C$
1st	MeOH	4.50×10^{2}	1.90×10^{2}	2.4
2nd	MeOH	4.15	1.20	3.5
3rd	MeOH–H_2O	4.20	1.12	3.7

a) 2-Hydroxyestrone-6,7-^{3}H (1.36×10^{8} dpm) and estrone-4-^{14}C (3.62×10^{7} dpm) were administered.
b) Nonradioactive 2-hydroxyestrone (10 mg) was added as a carrier.

Table III. The $^{3}H/^{14}C$ Values of 2-Hydroxyestrone and Its Cysteine Conjugate derived from Incubation Mixture[a)]

	Exp. No.	Specific activity (dpm/mg) ^{3}H	Specific activity (dpm/mg) ^{14}C	$^{3}H/^{14}C$
2-Hydroxyestrone	1	5.28×10^{3}	1.47×10^{3}	3.6
	2	6.80	1.28	5.3
	3	6.83	1.20	5.7
	mean			4.9
Cysteine conjugate[b)]	1	2.83×10^{3}	0.98×10^{3}	2.9
	2	2.05	0.70	2.9
	3	2.15	0.80	2.7
	mean			2.8

a) 2-Hydroxyestrone-6,7-^{3}H (1.70×10^{6} dpm) and estrone-4-^{14}C (1.13×10^{6} dpm) were employed for incubation.
b) Crystallization was carried out on 2-hydroxyestrone obtained by desulfurization.

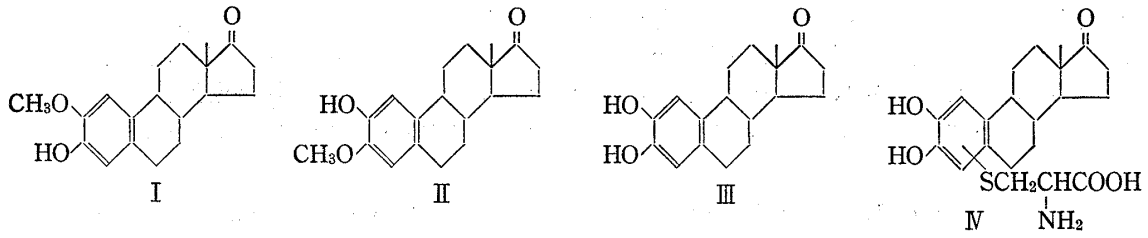

Chart 1

mixture was processed in the manner as described above. Extraction of the free steroids with ethyl acetate, followed by chromatographic purification afforded 2-hydroxyestrone. The remaining water-soluble fraction was subjected to TLC using *n*-butanol–acetic acid–water (4: 1: 1) as solvent, whereby a radioactive product appeared at *Rf* 0.46 exhibiting the positive reactions with ninhydrin, potassium dichromate-silver nitrate, and Folin-Ciocalteu reagent. In addition desulfurization with Raney nickel provided 2-hydroxyestrone, which was identified by TLC and reverse dilution analysis. As listed in Table III the isotope ratio of 2-hydroxyestrone was found to be higher than that of the cysteine conjugate.

Discussion

The occurrence of 2-hydroxyestrone and two isomeric monomethyl ethers as the glucuronide in the bile has been demonstrated. This result is in accord with the previous finding that these metabolites were excreted in the rat bile following administration of estrone.[1)]

It was reported that the glutathione conjugate of catechol estrogen would be transformed into the corresponding mercapturic acid in the rat. However, the definite evidences for *in vivo* formation of the conjugate of this kind have not yet been available. The present work has confirmed the biliary excretion of the cysteine conjugate of estrogen, which may be possibly derived from the glutathione conjugate. Explanation for the mechanism involved in the biosynthesis of the conjugate was offered in such that quinone or semiquinone would serve as an intermediate to condense with the thiol compound. At present both *in vivo* and *in vitro* experiments have demonstrated that the $^{3}H/^{14}C$ value of 2-hydroxyestrone formed was rather higher than that of the cysteine conjugate. These data indicate that the cysteine conjugate would not necessarily be produced by way of 2-hydroxyestrone, and in another word an alternative formation mechanism should be operative.

It is sufficiently substantiated that hydroxylation of the aromatic compound proceeds through the arene oxide, which also serves as an intermediate leading to the glutathione conjugate.[16,17)] In the previous paper we reported the occurrence of the glutathione conjugate whose steroidal moiety was not catechol estrogen but estradiol or estrone, in the rat urine after administration of 3-deoxyestrone.[18)] The present results together with the previous findings strongly imply that a hitherto unknown biosynthetic route other than that proposed by Jellinck, *et al.*, that is glutathione or cysteine conjugation without preceding C-2 hydroxylation, may be also participated.

It is hoped that further work in progress in this laboratory will provide the more precise knowledge on the formation mechanism of the amino acid or peptide conjugate of estrogen.

Experimental

Animal——Male Wistar rats weighing 220—250 g were used.

Material——Estrone-4-^{14}C (specific activity 50 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, and purified by TLC prior to use. 2-Hydroxyestrone-6,7-^{3}H (specific activity 2.2 mCi/

16) D.M. Jerina, J.W. Daly, and B. Witkop, *J. Am. Chem. Soc.*, **90**, 6523 (1968).
17) D.M. Jerina, J.W. Daly, B. Witkop, P. Zaltzman-Nirenberg, and S. Udenfriend, *J. Am. Chem. Soc.*, **90**, 6525 (1968).
18) T. Nambara and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 855 (1971).

mmole) was prepared by the method of Marks, *et al.*[19] employing estrone-6,7-^{3}H (500 mCi/mmole) (the Radiochemical Centre, Amersham) as a substrate. The radiochemical purity was more than 95% according to TLC.

Thin-Layer Chromatography (TLC)——The chromatogram was run on the plate with a layer (0.25 mm) of silica gel G or HF (E. Merck, AG, Darmstadt) activated at 110° for 90 min.

Radiocativity Counting——Counting was carried out on a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. Samples were counted in a Bray's scintillator, composed of naphthalene (60 g), 2,5-diphenyloxazole (4 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (200 mg), MeOH (100 ml), ethylene glycol (20 ml), and sufficient dioxane to make the total volume 1 liter.[20]

Administration of Steroid and Collection of Bile——Ten rats were anesthesized with sodium pentobarbital, cannulated to the bile duct with polyethylene tube PE 10 (Clay Adams, Parsippany) by surgical operation, and housed in a Bollman cage for collection of the bile. A solution of estrone-4-^{14}C (1.6 μCi) and 2-hydroxyestrone-6,7-^{3}H (6.0 μCi) dissolved in 95% EtOH (0.1 ml)–saline (0.9 ml) was injected intravenously and at the same time a suspension of 2-hydroxyestrone (10 mg) and estrone (10 mg) in Tween 80 was given to each rat orally. The bile was collected for the following 24 hr, whereby ^{3}H and ^{14}C were recovered at the rate of 68% and 58%, respectively.

Separation of Biliary Metabolites——The pooled bile (85 ml) was combined, diluted ten-fold with distilled water, and extracted with ether (850 ml$\times$2) to give the free steroid fraction (^{3}H: 5.9×10^4 dpm, ^{14}C: 3.6×10^4 dpm). The aq. phase was adjusted to pH 4.8 with 0.1 M acetate buffer (85 ml) and incubated with beef-liver β-glucuronidase (Tokyo Zōkikagaku Co., Tokyo) (500 Fishman units/ml) at 38° for 5 days. The incubation mixture was extracted with ether (800 ml$\times$3) to separate the glucuronide (^{3}H: 4.3×10^6 dpm, ^{14}C: 9.9×10^5 dpm) and water-soluble (^{3}H: 2.1×10^6 dpm, ^{14}C: 6.7×10^5 dpm) fractions. A gummy substance (280 mg) obtained from the glucuronide fraction was submitted to further purification by preparative TLC. The water-soluble fraction was percolated through a column packed with Amberlite XAD-2 resin (Roam and Haas Co., Philadelphia) (70$\times$5 cm i.d.) and then washed with H_2O (2 liters). The conjugate was eluted with MeOH (3 liters) and the eluant was evaporated *in vacuo* below 50° to give a brownish yellow syrup. An aq. solution of this residue was chromatographed on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala) (110$\times$3 cm i.d.) using H_2O as eluent and fractions of 4 ml were collected as illustrated in Fig. 1. The effluent of fraction 110—115 (G-I) was combined and liophilized. The residue was dissolved in 0.5 M AcOH (0.5 ml) and chromatographed on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala) (45$\times$2.5 cm i.d.). Gradient elution was carried out with 0.5 M AcOH (350 ml)–5 M AcOH (350 ml) and fractions of 4 ml were collected as shown in Fig. 2.

Identification of Metabolites——2-Methoxyestrone (I): Purification by preparative TLC, followed by recrystallization from MeOH gave I (23 mg) as colorless needles. mp 188—191°. Mixed melting point on admixture with the authentic sample showed no depression and infrared (IR) spectra of two samples were entirely identical. TLC: *Rf* 0.37 (hexane–AcOEt (5: 1)).

2-Hydroxyestrone 3-Methyl Ether (II): Purification by preparative TLC, followed by recrystallization from MeOH gave II (3 mg) as colorless needles. mp 174—178°. Mixed melting point on admixture with the authentic sample showed no depression and IR spectra of two samples were entirely identical. TLC: *Rf* 0.31 (hexane–AcOEt (5: 1)).

2-Hydroxyestrone (III): Purification by preparative TLC, followed by recrystallization from MeOH gave III (15 mg) as colorless plates. mp 190—193°. Mixed melting point on admixture with the authentic sample showed no depression and IR spectra of two samples were entirely identical. TLC: *Rf* 0.35 (hexane–AcOEt (2: 1)).

2-Hydroxyestrone 1 (or 4)-Cysteine Conjugate (IV): i) A portion of fraction A–I (^{3}H: 2.3×10^3 dpm, ^{14}C: 5.6×10^2 dpm) was dissolved in 0.01 M acetate buffer (3 ml) and incubated with β-glucuronidase (500 Fishman units/ml) at 38° for 48 hr. Upon removal of the free steroids by extraction with ether (5 ml$\times$2), the water-soluble metabolites corresponding to 92% of the radioactivity remained in the aq. layer. The aq. phase was brought to pH 1 with 50% H_2SO_4, saturated with NaCl, and extracted with AcOEt (10 ml) and the organic phase was then allowed to stand at 38° for 12 hr.[21] Hereupon any radioactive free steroid could not be detected on TLC.

ii) A portion of fraction A–I (^{3}H: 7.9×10^3 dpm, ^{14}C: 2.0×10^3 dpm) and 2-hydroxyestrone (2 mg) were dissolved in 0.5 N AcOH (2 ml). To this solution was added Raney Ni W-6[22] (100 mg) and stirred at 4° for 3 days. The resulting solution was saturated with NaCl and extracted with AcOEt (5 ml$\times$3). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. To the residue thus obtained was added 2-hydroxyestrone (10 mg) as a carrier and recrystallized repeatedly to the constant specific activity.

19) F. Marks and E. Hecker, *Biochim. Biophys. Acta*, **114**, 690 (1967).
20) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
21) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).
22) L.F. Fieser and M. Fieser, "Reagents for Organic Synthesis," Vol. 1, John Wiley and Sons, New York, 1967, p. 729.

iii) A portion of fraction A–I (^3H: 1.5×10^4 dpm, ^{14}C: 3.8×10^3 dpm) was dissolved in 6 N HCl (1 ml) and heated at 110° for 15 hr in a sealed tube. The hydrolyzate was submitted to two-dimensional TLC using $CHCl_3$–MeOH–17% NH_4OH (2: 2: 1) and C_6H_5OH–H_2O (3: 1) as developing solvent, whereby cysteine (*Rf* 0.16, 0.75) and cystine (*Rf* 0.26, 0.28) were identified.

Incubation Study——Enzyme Preparation: Liver was obtained from decapitated rat and washed with 0.25 M sucrose. A 10 g piece was homogenized with 0.25 M sucrose (40 ml) and centrifuged at $8000 \times g$ for 30 min and the supernatant was then centrifuged at $105000 \times g$ for 60 min. The microsomal pellets were washed with 0.25 M sucrose and suspended in 1/30 M Sörensen phosphate buffer (pH 7.4).

Incubation: Each incubation mixture contained the following: microsomal preparation (equivalent to 1 g of tissue), 2-hydroxyestrone-6,7-^3H (0.35 μmole, 0.75 μCi), estrone-4-^{14}C (0.4 μmole, 0.5 μCi), nicotinamide (1.3 mmole), NADPH (12 μmole) and sufficient 0.05M Tris-HCl buffer to make the total volume 10 ml. The mixture was incubated at 38° for 20 min in a shaker-incubator with air as the gas phase.

Separation of Cysteine Conjugate: To the incubation mixture was added 1 N HCl (5 ml) to stop the reaction and extracted with AcOEt (15 ml × 3). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue thus obtained was submitted to TLC using hexane–AcOEt (5: 3) as solvent. The remaining aq. phase was diluted with H_2O (50 ml), percolated through a column packed with Amberlite XAD-2 resin (10 × 1.5 cm i.d.), washed with H_2O (50 ml), and then eluted with MeOH (100 ml). The effluent was evaporated *in vacuo* below 50° to give the residue (recovery rate 86%), which in turn was submitted to TLC in the manner as described above.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.