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Effect of acceptor membrane phosphatidylcholine on the catalytic activity of bovine liver phosphatidylcholine transfer protein

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Protein-mediated transfer of phosphatidylcholine (PC) by bovine liver phosphatidylcholine transfer protein (PC-TP) was examined using a vesicle-vesicle assay system. Donor and acceptor membranes were prepared from *Escherichia coli* phospholipids and limiting amounts of egg yolk PC. PC transfer between vesicles of *E. coli* lipid / egg PC was markedly higher than transfer of PC from vesicles of *E. coli* lipid / egg PC to vesicles of *E. coli* lipid. Kinetic parameters of the interaction between PC-TP and *E. coli* lipid vesicles with or without PC was investigated. The apparent dissociation constants of the complex formed between PC-TP and these vesicles were determined kinetically and from double-reciprocal plots of intrinsic PC-TP fluorescence intensity increase versus vesicle concentration. The magnitude of the dissociation constant decreased as the PC content of the vesicles increased from 0 to 5 mol%. In addition, kinetic analysis revealed that the presence of PC in acceptor vesicles increased both the association and dissociation of PC-TP from vesicles. The effect of membrane PC molecules on transfer rates was examined using bis-phosphatidylcholine, a dimeric PC molecule which is not transferred by PC-TP. Rates of PC transfer to acceptor vesicles comprised of *E. coli* lipid / bis-PC were virtually identical to rates observed with acceptors vesicles prepared from *E. coli* lipid. The results suggest that transfer of PC by PC-TP is enhanced only when insertion of protein-bound PC occurs concurrently with the extraction of a molecule of membrane PC, i.e., a concerted, one-step catalytic mechanism for phospholipid exchange.

Introduction

Phosphatidylcholine transfer protein isolated from bovine liver catalyzes the transfer of PC between donor and acceptor membranes in vitro. Transfer of PC by PC-TP can result in either an exchange of PC between donor and acceptor membranes or a net transfer of donor PC to acceptor membranes. Under conditions of PC exchange, PC-TP behaves as a freely diffusable PC vector by forming a soluble one to one molar complex with PC. In the next transfer of PC, PC-TP is delipidated of PC as it leaves an accep-

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Abbreviations: PC-TP, bovine liver phosphatidylcholine transfer protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; BHT, butylated hydroxytoluene; bis-PC, 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoyl-*sn*-glycero-3-phosphocholine).

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tor membrane initially lacking PC [1,2].

Earlier work has addressed the kinetics of protein-mediated PC transfer. Transfer of PC has been shown to be influenced by the concentration of negatively charged phospholipids in the membrane [3–7], the phase properties of the membrane [8–10], and membrane curvature [11,12]. In addition, protein-mediated transfer of PC to acceptor membranes appears to be enhanced by acceptor membrane PC. Previous investigators have shown that the rate of PC transfer to acceptor membranes lacking PC is 4–16 times slower than to acceptor membranes containing 80–98 mol% PC [4,13]. These studies clearly indicate that the physical and chemical properties of the membrane can greatly influence protein-mediated PC transfer. However, it is not yet understood what factors are involved in determining whether PC-TP will catalyze an exchange or a net transfer of PC.

The goal of the present work is to address the role of membrane PC and its relationship to the dual catalytic activity of PC-TP. We approach this question by examining PC transfer between vesicles of limited PC content, conditions in which PC-TP could potentially mediate either PC exchange, net PC transfer, or both. We present evidence that the rate of PC transfer from donor to acceptor vesicles containing 5 mol% PC is significantly enhanced compared to acceptor vesicles initially lacking PC. We attempt to account for this difference by exploring possible mechanisms by which PC, either as a transferable lipid or as a membrane lipid component, could enhance protein-mediated transfer. We demonstrate that the apparent dissociation constants for acceptor vesicles prepared with or without PC cannot explain the stimulatory effect of PC. In addition, rates of transfer to PC acceptor membranes containing a non-transferable PC molecule, bis-PC, are similar to rates observed with acceptor membranes initially devoid of PC. A mechanism for protein-mediated PC exchange is constructed from these experimental findings; its relationship to the dual catalytic activities of PC-TP is discussed.

Methods and Materials

Materials. *trans*-Parinaric acid was obtained from Molecular Probes, Inc., Junction City, OR, and lactosyl-*N*-palmitoyldihydrosphingosine (lactosylceramide) was a product of Calbiochem-Behring, La Jolla, CA. *N,N*-Dimethylaminopyridine and dicyclohexylcarbodiimide were products of Aldrich Chemical Co., Milwaukee, WI, and BHT was obtained from Sigma Chemical Co., St. Louis, MO. Cholesterol [1-^{14}C]oleate, [9,10-^{3}H]palmitic acid, and [9,10-^{3}H]oleic acid were purchased from New England Nuclear, Boston, MA, while [^{3}H]choline chloride was obtained from Amersham Corp., Arlington Heights, IL, and [1-^{14}C]myristic acid was bought from Research Products International, Mount Prospect, IL.

Lipids. Lipids were stored in chloroform/methanol (2 : 1, v/v) at −20 °C, while the fluorescent lipids were stored at −70 °C. Phosphorus analysis was used to determine phospholipid concentration as described by Rouser et al. [14]. Crude egg PC (type X-E) was obtained from Sigma, St. Louis, MO, and purified by column chromatography on silica gel G using chloroform/methanol/water (65 : 25 : 4, v/v) as the elution solvent [15]. Lipid extracts of *Escherichia coli* were obtained from Sigma (PE, type IX) and were used directly. That *E. coli* lipid extract lacks PC was verified by two-dimensional TLC on silica gel G plates (Analtech, Newark, DE), developed first with chloroform/methanol/35% ammonium hydroxide (65 : 25 : 5, v/v) followed by acetone/methanol/acetic acid/water (30 : 40 : 10 : 10 : 5, v/v) in the second dimension. Phospholipid analysis of the lipid extracts was determined by one-dimensional TLC; silica gel G plates (Analtech) developed in chloroform/methanol/glacial acetic acid (65 : 25 : 8, v/v) and visualized with I_2 vapor. Individual spots were identified with appropriate standards and scraped for phosphorus determination [14]. Fatty acid methyl esters of the *E. coli* lipids were prepared by transmethylation using 1.5 M methanolic HCl [16] and analyzed by GLC with a Hewlett Packard model 5830 A instrument. A fused silica capillary column of SP-2330 (Supelco, Inc., Bellefonte, PA) was operated between 185 and 220 °C. The relative weight composition of the fatty acid species was determined from the peak areas.

[^{3}H]PC was prepared from egg PC, which was first hydrolyzed with phospholipase A_2 and then reacylated with [9,10-^{3}H]oleic acid [8]. Di-

[^{14}C]myristoylPC was synthesized from [^{14}C]myristoyl anhydride and 1-myristoyl-lyso-PC, essentially as described elsewhere [17]. Cholesteryl[^{3}H]-oleate was synthesized from [9,10-^{3}H]oleic acid and cholesterol [18], and [^{3}H]dipalmitoylPC was prepared by choline exchange, using Savoy cabbage phospholipase D and [^{3}H]choline chloride [19]. ^{3}H-Labelled bis-PC was synthesized from [^{3}H]palmitoyl anhydride and 1-(16-(*S*-methyldithio)hexadecanoyl)-lyso-PC using the procedures described in the preceding paper [20]. The product co-migrated with authentic bis-PC, and the specific activity was 0.43 μCi/μmol. The mixed acyl chain intermediate in the synthesis of radiolabelled bis-PC, 1-(16-(*S*-methyldithio)hexadecanoyl)-2-[9,10-^{3}H]palmitoylPC, was also recovered and used in phospholipid transfer experiments.

1-Palmitoyl-2-*trans*-parinaroylPC and 1-(17,18-dithiatetratriacontandioyl)-bis(2-*trans*-parinaroyl PC) were prepared by the following method. To lysoPC (85 μmol), previously dried under vacuum over phosphorus pentoxide, was added dimethylaminopyridine (85 μmol), *trans*-parinaric acid (250 μmol), BHT (250 μmol), freshly distilled chloroform (2 ml), and dicyclohexylcarbodiimide (250 μmol). The reaction mixture was flushed with nitrogen and stirred, protected from light, for 48 h at room temperature. The products were purified on a dry silica gel G column, protected from light, using a nitrogen-saturated eluent of chloroform/methanol/water (65 : 25 : 4, v/v) containing 0.5 mg/l BHT. The product, 1-(16-(*S*-methyldithio)-hexadecanoyl)-2-*trans*-parinaroylPC, was then converted to bis-PC using the procedures described for 1-(16-(*S*-methyldithio)hexadecanoyl)-2-palmitoylPC [20]. Both purified products, 1-palmitoyl-2-*trans*-parinaroylPC and 1-(17,18-dithiatetratriacontandioyl)-bis(2-*trans*-parinaroylPC) gave single spots on TLC which co-migrated with PC and bis-PC, respectively. Based on the molar absorption coefficient of $8.9 \cdot 10^{+4}$ for the *trans*-parinaroyl chromophore in ethanol [21], 1-palmitoyl-2-*trans*-parinaroylPC was found to contain 0.92 mol parinaroyl residues per mol phosphorus. The other product, 1-(17,18-dithiatetratriacontanoyl)-bis(2-*trans*-parinaroylPC), was found to have 0.83 mol parinaroyl residues per mol phosphorus, assuming a molar absorption coefficient of $7.6 \cdot 10^{+4}$ for the *trans*-parinaroyl group in chloroform [21]. The absorption spectra of both products were nearly identical to the starting parinaric acid; bis(2-*trans*-parinaroylPC) had a slightly increased absorption in the 260–280 nm region, which may be attributed to some conversion of the parinaroyl group to trienoic material [22]. 1-Oleoyl-2-*trans*-parinaroylPE was a generous gift from Dr. Ruth Welti, Kansas State University, Manhattan, KS.

Vesicle preparation and characterization. E. coli lipid vesicles for the phospholipid transfer assay were prepared by ethanol injection [23]. Unilamellar vesicles (2 mM lipid phosphorus) were prepared at 48° C by injection of 30 mM phospholipids, dissolved in ethanol, into 10 mM Hepes, 50 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3 buffer (pH 7.4). Mixtures of *E. coli* lipids and bis-PC were also prepared in this manner. Vesicles of *E. coli* lipid/bis-PC (5 mol% bis-PC, lipid phosphorus) were passed over a small column (0.9 ml) of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ); approx. 90% of the radiolabelled bis-PC eluted in the void volume, where the vesicles were found to co-elute, indicating an efficient incorporation of bis-PC. The size of *E. coli* lipid vesicles containing either egg PC or bis-PC was determined by electron microscopy [20]. Vesicles composed of *E. coli* lipid/5 mol% egg PC had an average diameter of 51 ± 2 nm, while *E. coli* lipid/5 mol% bis-PC vesicles had an average size of 61 ± 13 nm.

Phospholipid dispersions used in the fluorescence experiments were also prepared by ethanol injection [23]. Prior to injection lipids were dissolved in either ethanol or ethanol/dimethylsulfoxide (3 : 1, v/v) to a phospholipid concentration of 30 mM; they were then injected into warm (48° C) 10 mM Hepes, 50 mM NaCl buffer (pH 7.4). BHT was included in these dispersions to a concentration of approximately 0.1 nmol per nmol parinaroyl phospholipid); buffers were saturated with nitrogen to minimize exposure of the parinaroyl groups to oxygen.

Phosphatidylcholine transfer protein. Transfer protein from bovine liver was purified 1900-fold, essentially as described by Westerman et al. [24]. Purity of the protein was greater than 95% when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by silver

staining. The specific activity of PC-TP was 1600 units · mg^{-1}. One unit is defined as 1 nmol PC transferred per min from donors (200 nmol phospholipid) to acceptors (200 nmol phospholipid) using the vesicle-vesicle assay system described previously [8]. The protein (0.15 mg · ml^{-1}) was stored at −20 °C in 50% glycerol containing 10 mM sodium citrate/20 mM disodium hydrogen phosphate (pH 5.0). Protein concentration was based on a molar absorption coefficient of $3.7 \cdot 10^{+4}$ at 280 nm [25].

Vesicle-vesicle assay of phosphatidylcholine transfer activity. Protein-mediated PC transfer from donor to acceptor vesicles was performed as described by Welti and Helmkamp [19]. Donor vesicles used in the present study contained *E. coli* lipid/PC/lactosylceramide (89 : 5 : 6, mol%), unless indicated otherwise; the indicated amount of PC included both radiolabelled (either ^{3}H or ^{14}C) and non-radiolabelled PC of the same molecular species. The bulk phase lipid component of acceptor vesicles was *E. coli* lipids, and radiolabelled cholesteryl oleate (either ^{3}H or ^{14}C) was included as a non-exchangeable marker. Unless indicated, 200 nmol (lipid phosphorus) each of donor and acceptor vesicles were incubated in the absence or presence of the indicated amount of PC-TP at 37 °C in a final volume of 0.5 ml. In samples without PC-TP an equivalent amount of 10 mM sodium citrate/20 mM disodium hydrogen phosphate (pH 5.0) in 50% glycerol was used. In no case was the final glycerol concentration in excess of 0.5%, a concentration known to interfere with the vesicle agglutination process. Transfer determined in the absence of protein was subtracted from transfer in the presence of protein to yield proteinmediated transfer; corrections were made for the recovery of acceptor vesicles (usually 85–100%).

Fluorometric measurements. Fluorescence anisotropy measurements were recorded on an SLM 8000 spectrofluorometer. Temperature scans were performed by cooling or heating the cuvette at a rate of 1 C° · min^{-1} as the sample was stirred. A Neslab Endocal RTE 5 bath, a Neslab ETP-3 temperature programmer, and an Instrulab digital thermometer with a thermocouple were used to regulate the temperature. Excitation was in the vertical plane. Vertical and horizontal intensities were collected by computer. No corrections for scattering depolarization were used, as all the samples prepared as described had absorbances of less than 0.05 at 320 nm [26]. Static fluorescence experiments were performed at 37 ± 1 °C on a Perkin-Elmer MFP-44 fluorometer equipped with a thermostatted cuvette holder. A water bath (Brinkmann Lauda RC 20) was used to regulate the temperature. The final volume of ethanol/dimethylsulfoxide (3 : 1, v/v), used to prepare the vesicles, did not exceed 0.05% and had no effect on the fluorescence spectrum of the protein. Fluorescence intensities were corrected for volume changes and scattering due to vesicles.

Kinetic treatment. The apparent rate at which PC-TP mediates the transfer of PC from donor vesicles (D) to acceptor vesicles (A) is expressed by the following theoretical equation [3]

$$v_{app,D/A} = \frac{k_D k_A [D][A][PC\text{-}TP]}{(k_D[D] + k_A[A])(1 + [D]/K_D + [A]/K_A)} \quad (1)$$

In Eqn. 1, k_D and k_A are the rate constants describing the association of PC-TP with donor and acceptor vesicles, respectively; K_D and K_A are the apparent dissociation constants of the PC-TP vesicle complex for the donors and acceptors, respectively; and [D] and [A] are the donor and acceptor phospholipid concentration, respectively. The experimentally determined apparent transfer rates were fitted with the theoretical rate equation (Eqn. 1) to determine the kinetic constants. A computer program was used which minimized the total deviance, i.e., the sum of the squared differences between the experimental (v_{exp}) and calculated (v_{calcd}) rates, by varying k_D, k_A, K_D, and K_A. The standard deviation of v_{calcd} is the square root of the total deviance divided by the number of degrees of freedom ($n - 4$). The same kinetic treatment is also valid for assay systems in which acceptor vesicles lack PC, if the following assumptions are made: (1) PC-TP does not dissociate from donor vesicles without a donor PC molecule; and (2) PC-TP containing a donor PC, in the phospholipid binding site, does not dissociate from acceptor vesicles with donor PC. If events other than these were to occur, net transfer of PC would not be a consequence in the transfer protein-membrane system.

Results

Composition and characterization of E. coli lipid vesicles

In order to examine the effect of membrane PC on rates of protein-mediated PC transfer and to minimize phospholipid compositional differences between vesicles with and without PC, it was necessary to employ a bulk phase lipid common to both. Accordingly, *E. coli* lipid extract was utilized as a background lipid phase in the subsequent experiments. Table I summarizes the major phospholipid composition of this commercially obtained lipid extract. The major phospholipid species was PE. Palmitate was detected as the major fatty acid; relatively little of the two naturally occurring mono-unsaturated fatty acids, palmitoleate and *cis*-vaccenate, was found. Rather, considerable cyclopropane fatty acid was observed, implying that the culture had been harvested in stationary phase [27]. Evidence of an unusual level of lipid oxidation was not apparent.

Due to the heterogeneity of the *E. coli* lipid mixture and the high proportion of saturated fatty acids detected, the thermotropic phase behavior of vesicles derived from this mixture was examined. The gel-fluid transition was investigated by fluorescence polarization using parinaroyl phospholipids. Parinaroyl phospholipids were utilized because these lipids preferentially partition into the lipid rather than the aqueous phase and the fluorescence of the parinaroyl group is sensitive to the gel-fluid bilayer transition [21]. The temperature dependence of the polarization of 1-palmitoyl-2-*trans*-parinaroylPC in *E. coli* vesicles is shown in Fig. 1. A broad gel-fluid phase transition was observed between 18.5°C and 29.5°C, with a midpoint of 24°C. Similar thermotropic behavior was observed when bis(2-*trans*-parinaroylPC) was used as a probe (Fig. 1B), indicating that the PC analog, bis-PC, is also capable of detecting the *E. coli* lipid transition. Further, it appears to cluster to no greater extent than the 1-palmitoyl-2-*trans*-parinaroyl PC probe. Thus, at 37°C, the temperature used in all subsequent experiments, *E. coli* lipids generate and maintain a fluid phase in the donor and acceptor vesicles.

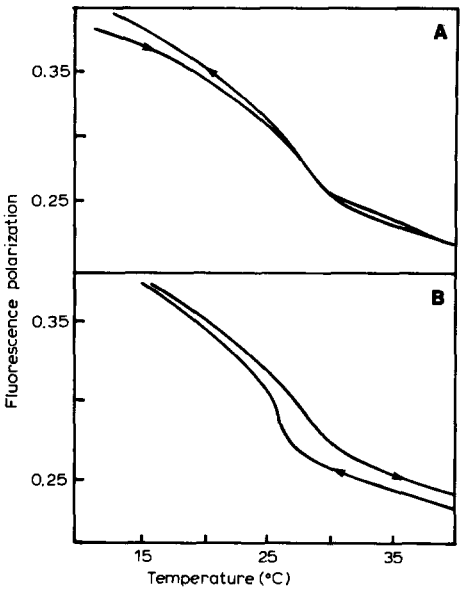


Fig. 1. Phase behavior of *E. coli* lipid monitored by fluorescence polarization. Vesicles were prepared at a concentration of 0.05 mM and contained 1-palmitoyl-2-*trans*-parinaroylPC (A) or *trans*-parinaroyl-bis-PC (B) at a probe to lipid ratio of 1:200. Excitation and emission wavelengths were 320 nm and 420 nm with slit widths of 2 and 16 nm, respectively.

TABLE I

COMPOSITION OF *E. COLI* LIPID EXTRACT [a]

Phospholipid	mol% [b]
Phosphatidylethanolamine	75.4±0.6
Phosphatidylglycerol	14.5±2.3
Cardiolipin	10.0±1.8
Fatty acid	wt%
Myristate	2.4
Palmitate	51.6
Palmitoleate	1.6
9,10-Methylpalmitoleate	26.8
Stearate	1.5
Oleate	4.5
cis-Vaccenate	11.5

[a] Mean±S.D. of three determinations.

[b] Mol% of phospholipid was determined from phosphorus analysis; one mol of cardiolipin was assumed to contain two mol of phosphorus.

Transfer to acceptor vesicles of varying PC concentrations

The protein-mediated transfer of radiolabelled PC from *E. coli* lipid/PC donors (5 mol% PC) to acceptors comprised of *E. coli* lipid with varying

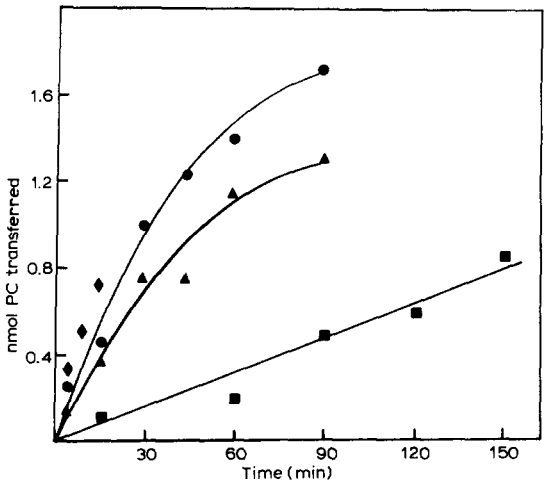


Fig. 2. Effect of acceptor PC on protein-mediated transfer of PC. Donor vesicles (200 nmol) comprised of *E. coli* lipid/5 mol% egg PC were incubated with acceptors (200 nmol) comprised of *E. coli* lipid/10 mol% egg PC (◆), *E. coli* lipid/5 mol% egg PC (●), *E. coli* lipid/2.5 mol% egg PC (▲), or *E. coli* lipid only (■) and 2 pmol protein.

amounts of PC is shown in Fig. 2. The initial rate of PC transfer from donors to acceptors containing 5 mol% PC was 0.037 nmol PC per min compared to 0.005 nmol PC per min for acceptors initially lacking PC. Acceptors with 2.5 mol% PC had an initial rate of 0.018 nmol PC per min. Increasing the acceptor PC concentration to 10 mol% gave an initial rate of 0.040 nmol PC per min, suggesting that the rate of transfer becomes maximal when the PC content in both donors and acceptors is the same. The observed curvature of the kinetic profiles at higher levels of phospholipid transferred can be attributed to a reverse flux of PC from acceptor vesicles to donor vesicles in this assay system where identical amounts of donor and acceptor membranes are employed. The dramatically slower rate of transfer to acceptors lacking PC was not significantly altered by increasing the donor PC content (Table II). Since the composition of the donor and acceptor vesicles used in these experiments differs only by small quantities PC (0–10 mol%), the observations clearly demonstrate that acceptor membrane PC enhances catalytic activity.

TABLE II

EFFECT OF DONOR PC CONCENTRATION ON PROTEIN-MEDIATED TRANSFER OF PHOSPHATIDYLCHOLINE

Acceptor vesicles (200 nmol) comprised of *E. coli* lipid were incubated with donor vesicles (200 nmol) of varying PC content. The total mol% of PC in the donor vesicle was varied, but the ratio of labelled to unlabelled PC was held constant. Transfer determinations were performed in triplicate and reported as a mean ± S.D.

Donor vesicle PC content (mol%)	Transfer activity (nmol PC transferred per h)	
	0.5 μg [a]	1.0 μg [a]
5	0.58 ± 0.01	1.30 ± 0.05
10	0.57 ± 0.04	1.70 ± 0.01
20	0.70 ± 0.06	2.04 ± 0.32

[a] Amount of bovine liver PC-TP assayed.

Kinetic effect of acceptor PC concentration on PC transfer

In an attempt to account for the stimulatory effect of acceptor PC on protein-mediated transfer,

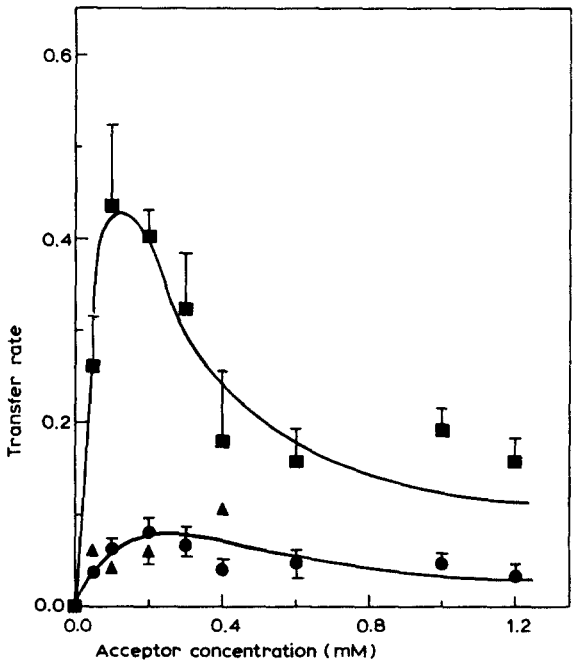


Fig. 3. Effect of acceptor phospholipid concentration on the apparent velocity of PC transfer from donor to acceptor membranes. Donor vesicles (50 nmol) comprised of *E. coli* lipid/5 mol% egg PC and acceptor vesicles comprised of *E. coli* lipid only (●), *E. coli* lipid/5 mol% egg PC (■), and *E. coli* lipid/5 mol% bis-PC (▲) were incubated with 2 pmol protein for 15–45 min. The experimentally determined rates, nmol PC/min per μg protein, are plotted (means ± S.D., 3–6 values at each vesicle concentration) and used to generate the computer-calculated curves, as described in Results.

TABLE III

EFFECT OF MEMBRANE PHOSPHATIDYLCHOLINE ON KINETIC PARAMETERS

Values obtained from computer analysis of the data presented in Fig. 3, using the theoretical rate equation (Eqn. 1) described in Materials and Methods.

PC content of acceptor vesicles	k_A (nmol·mM^{-1}·min^{-1}·μg^{-1})	K_A (mM)	k_{-A} [a] (nmol·min^{-1}·μg^{-1})
None	3.4	0.10	0.34
5 mol% egg PC	30	0.06	1.80
5 mol% bis-PC	1.4	0.30	0.42

[a] Calculated from the relationship, $K_A = k_{-A}/k_A$.

the rate of transfer (v_{exp}) to acceptor membranes with or without PC as a function of acceptor concentration was examined. When the bulk phase acceptor composition was *E. coli* lipid alone and the donor concentration was held constant, the data obtained are shown in Fig. 3. The curves drawn have been calculated by fitting values of v_{exp} to the theoretical rate equation (Eqn. 1). In one case both donor and acceptor membranes contained 5 mol% PC, while in a second case the donor PC content was 5 mol% and the acceptor membranes lacked PC. The average standard deviation in v_{calcd}, which defines the curve, was 0.37 for the first case and 0.017 for the second. The estimated dissociation constant, K_A, was found to be slightly decreased for acceptor vesicles containing 5 mol% PC compared to acceptor vesicles comprised solely of *E. coli* lipids, whereas the estimated association rate constant, k_A, increased approximately 9 times (Table III). Dissociation constants of 0.6 mM and 0.4 mM have been reported for PC vesicles containing 13 and 16 mol% phosphatidylglycerol, respectively [25]; the *E. coli* lipid vesicles used in the present studies contain a similar proportion of phosphatidylglycerol (Table I). The dissociation rate constants are also summarized in Table III. As shown , k_{-A} is approximately 5 times greater for *E. coli* lipid vesicles containing 5 mol% PC compared to vesicles lacking PC. Thus, it appears that the presence of PC in acceptor vesicles enhances both the association and dissociation of PC-TP from the vesicle.

Apparent dissociation constants for the protein-vesicle complex were also determined spectrophotometrically using the intrinsic fluorescence of the protein [25]. The fluorescence of PC-TP was enhanced in the presence of *E. coli* lipid vesicles and was not accompanied by a shift in the emission maximum. A double-reciprocal plot of the intensity charge and vesicle concentration was constructed (Fig. 4); the data could be fitted by straight lines (correlation coefficients of 0.94, 0.93, and 0.96 for *E. coli* lipid vesicles, *E. coli* lipid/PC vesicles, and *E. coli* lipid/bis-PC vesicles, respectively). The absolute value of the reciprocal of the *x*-axis intercept is equal to the apparent dissociation constant of the protein-vesicle complex [28]. Values of 0.04 mM, 0.59 mM, and 0.31 mM were obtained for *E. coli* lipid/PC (95 : 5, mol%), *E. coli* lipid/bis-PC (95 : 5, mol%), and pure *E. coli* lipid vesicles, respectively, using least mean squares analysis. These numbers agree reasonably well with the kinetically determined values of the acceptor vesicle dissociation constant, K_A (Table III).

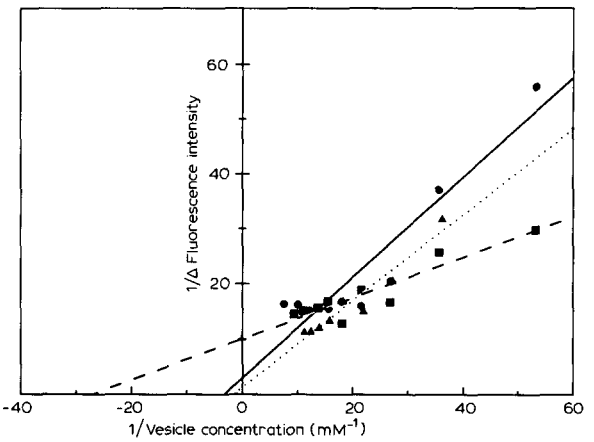


Fig. 4. Double-reciprocal plot of the increase in intrinsic PC-TP tryptophan fluorescence and vesicle concentration. 1 ml of PC-TP (2 μM protein) in 10 mM Hepes/50 mM NaCl (pH 7.4) was titrated with increasing amounts of vesicles (2 mM phospholipid). Excitation at 280 nm (slit width, 8 nm) and emission at 327 nm (slit width, 8 nm) was used to determine the enhancement of the protein's tryptophan fluorescence increase in the presence of vesicles. The vesicles compositions were *E. coli* lipid (●, solid line), *E. coli* lipid/5 mol% egg PC (■, dashed line), and *E. coli* lipid/5 mol% bis-PC (▲, dotted line).

Involvement of membrane PC

Kinetic analyses indicate that the presence of

TABLE IV

PROTEIN-MEDIATED TRANSFER OF PC SPECIES BETWEEN DONOR AND ACCEPTOR VESICLES

Donor and acceptor vesicles (200 nmol each prepared with *E. coli* bulk phase lipids and 5 mol% of the indicated PC molecular species) were incubated with 2.5 pmol of bovine phosphatidylcholine transfer protein for 20 min at 37 °C; values are the mean ± S.D. of triplicate determinations.

Membrane PC	PC transferred (nmol)
Egg PC	1.32 ± 0.31
1,2-DimyristoylPC	1.43 ± 0.29
1,2-DipalmitoylPC	1.38 ± 0.49
1-(16-(*S*-Methyldithio – hexadecanoyl)-2-palmitoylPC	1.50 ± 0.36
Bis-PC	−0.01 ± 0.02

PC in acceptor membranes enhances the rate constants describing both the association and dissociation of PC-TP with vesicles. While this conclusion offers some explanation for the overall enhancement effect of PC observed in Fig. 2, it is not clear whether these effects obtain because PC in the membrane may be exchanged for a protein-bound PC molecule or because PC is simply a membrane component. A non-transferable, choline-containing phospholipid is required to distinguish between these possibilities. Accordingly, bis-PC, 1-(17,18-dithiatetratriacontandioyl)-bis(2-hexadecanoylPC), has been synthesized and partially characterized [20]. The ability of PC-TP to transfer bis-PC from *E. coli* lipid donor vesicles to acceptors of the same composition was compared to the transfer of other PC species (Table IV). Under conditions where good transfer of an otherwise poorly transferred substrate, dipalmitoylPC [29], was observed virtually no transfer of bis-PC was detected. This results implies that bis-PC is not a transferable substrate and thus is incapable of being removed from the membrane by PC-TP. Interestingly, transfer of the 'half-molecule' of bis-PC, 1-(16-(*S*-methyldithio)hexadecanoyl)-2-palmitoylPC, was comparable to that measured for other limiting saturated or unsaturated PC substrates (Table IV). It, therefore, appears that the presence of a bulky disulfide moiety in a diacylPC structure in no way impedes its binding to PC-TP and subsequent intermembrane transfer.

To determine whether PC-TP interacts physically with bis-PC in any manner, a series of fluorescent phospholipids was synthesized. The interaction of PC-TP with three such phospholipids, 1-palmitoyl-2-*trans*-parinaroylPC, 1-oleoyl-2-*trans*-parinaroylPE, and bis(2-*trans*-parinaroylPC), in *E. coli* lipid vesicles was examined by monitoring the intrinsic tryptophanyl fluorescence spectrum of PC-TP. Incubation of PC-TP with *E. coli* lipid vesicles containing *trans*-parinaroyl phospholipids, in which the protein/probe and probe/lipid ratios were virtually the same, resulted in a decrease in the tryptophanyl fluorescence intensity (Fig. 5). It is obvious that the extent of quenching was similar for all the phospholipid fluorescent probes, including *trans*-parinaroylPE which has been shown not to be transferred by PC-TP [13]. Although previous

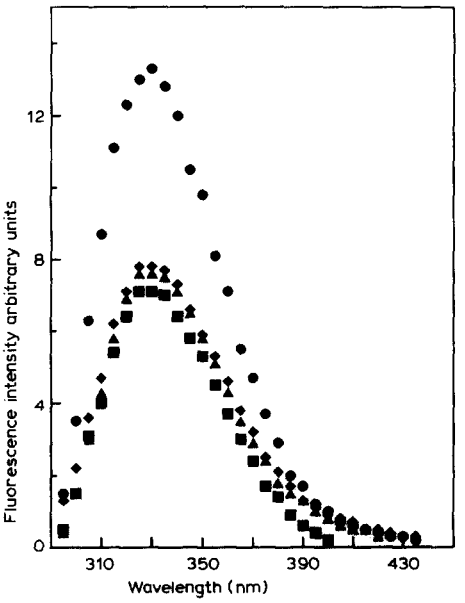


Fig. 5. Fluorescence characteristics of PC-TP in the presence of *E. coli* lipid vesicles containing *trans*-parinaroyl phospholipids. PC-TP concentration was $5 \cdot 10^{-7}$ M and lipid vesicle concentration was $2.5 \cdot 10^{-5}$ M in a final volume of 1 ml; the probe to lipid mol ratio was 1 : 100. With excitation at 280 nm (slit width, 6 nm), emission spectra (slit width, 10 nm) were recorded on solutions of PC-TP in the presence of *E. coli* lipid vesicles containing no *trans*-parinaroyl phospholipids (●), *trans*-parinaroylPC (◆), *trans*-parinaroylPE (■), or bis-(*trans*-parinaroylPC) (▲). Spectra have been corrected for scattering and emission of the vesicles and but not for the inner filter effect, as absorbances at the emission and excitation wavelengths were less than 0.1.

studies using *cis*-parinaroyl PC derivatives have demonstrated binding of PC to PC-TP by radiationless energy transfer quenching of tryptophan fluorescence [13], the nature of the quenching demonstrated here and its relationship to the substrate specificity of PC-TP requires further investigation. Despite an apparent lack of specific interaction with the choline moiety, the quenching of bis-PC fluorescence by PC-TP, nevertheless, suggests that the protein encounters the non-transferable bis-PC at a distance close enough to induce chromophore interaction.

The effect of membrane PC molecules on PC-TP activity was investigated by measuring the rate of PC transfer to acceptor membranes composed of *E. coli* lipids only and *E. coli* lipids with 5 mol% and 10 mol% bis-PC (Table V). The non-transferable bis-PC had no significant effect on PC transfer: the rate was neither enhanced nor diminished compared to acceptor membranes which contained no PC. In addition, bis-PC did not alter the rate of PC transfer when the acceptor and donor vesicles had equal concentrations of transferable PC (egg PC) present. These results imply that the low levels of bis-PC used in these experiments influenced the overall membrane packing minimally or to an extent that protein-mediated transfer is not modified. These results further suggest that the mere presence of a phosphorylcholine polar headgroup in the membrane does not enhance PC transfer by fascilitating PC-TP–membrane interaction.

Bis-PC, a non-transferrable PC, was also used to examine the role of membrane PC molecules in the catalytic activity of PC-TP. Rates of transfer (v_{exp}) at varying concentrations of acceptor membrane were measured for acceptor vesicles prepared from *E. coli* lipids/5 mol% bis-PC. These data are shown in Fig. 3, where the standard deviation in v_{calcd} was 0.084; the estimated kinetic parameters are summarized in Table III. It is noted that the presence of bis-PC in the acceptor membrane leads to association and dissociation rate constants more similar to those for *E. coli* lipid membranes rather than *E. coli* lipid/5 mol% egg PC membranes. The apparent dissociation constant for the complex of PC-TP and *E. coli* lipid/bis-PC vesicles was also determined by quenching of intrinsic protein fluorescence (Fig. 4); a value of 0.59 mM was calculated. Reasonable agreement between the kinetically and spectrophotometrically calculated dissociation constants was seen. Both values provide further evidence that bis-PC does not significantly alter PC-TP–membrane interactions and support the earlier results that PC-TP does not interact specifically with PC molecules at the lipid/water interface.

Discussion

We have examined the role of PC in the catalytic function of PC-TP by developing a vesicle-vesicle assay system in which the level of PC in both donor and acceptor vesicles was limiting, i.e., 10 mol% PC or less. Unlike other systems previously used, this membrane system provided a unique means to explore PC exchange and net transfer. Under conditions when PC is absent from the acceptor membrane, a net transfer of PC is the only allowable catalytic process between donor and acceptor membranes [7,13,30]. Conversely, when PC is present in both membrane populations, a true exchange occurs [31,32]. Rates of protein-mediated PC transfer from donor vesicles (5 mol% PC) to acceptor vesicles increased as the PC content of the acceptor membrane increased from 0 to 5 mol%, demonstrating clearly that PC enhances PC-TP activity. This enhancement may reflect a shift in the catalytic activity of PC-TP from net transfer to exchange, or it may

TABLE V

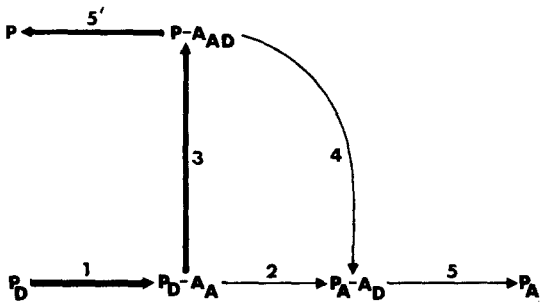
EFFECT OF NON-TRANSFERABLE PC ON INITIAL RATES OF PROTEIN-MEDIATED PC TRANSFER FROM DONOR TO ACCEPTOR VESICLES

Donor vesicles (200 nmol of *E. coli* lipid and 5 mol% egg PC) were incubated at 37 °C with acceptor vesicles (200 nmol of *E. coli* lipid and varying PC content). Initial rates were determined from slopes of nmol PC transferred per μg protein against time plots using mean values obtained from three determinations.

PC content of acceptor vesicles	Transfer rate (nmol PC per min per μg)
None	0.087
5 mol% bis-PC	0.084
10 mol% bis-PC	0.088
5 mol% egg PC	0.98
5 mol% egg PC/5 mol% bis-PC	0.97

reflect the involvement of the PC recognition site on PC-TP in the transfer process. To distinguish between these possibilities, i.e., whether the enhanced activity of PC-TP was attributable to PC acting as a transferable phospholipid or as an acceptor membrane phospholipid component, a non-transferable PC analog was investigated.

We propose a kinetic model (Scheme I) of protein-mediated PC transfer which incorporates the previous work of others (for reviews, see Refs. 1 and 33) and provides a framework for examining our observations in relation to the exchange and net transfer catalytic functions of PC-TP. The exchange of protein-bound PC for acceptor membrane PC or a net insertion of protein-bound PC occurs after PC-TP associates with that membrane. Since monolayer studies suggest that PC-TP does not significantly penetrate the membrane to



Scheme I. Kinetic paths of protein-mediated, intermembrane phospholipid transfer. Protein containing a non-covalently bound donor (acceptor) PC molecule is designated P_D (P_A); protein devoid of PC in the inding site is represented as P. P_D-A_A, P_A-A_D, and P-A_{AD} describe complexes of transfer protein with an acceptor membrane in which the lipid binding site of the protein contains a donor PC molecule, acceptor PC molecule, or is vacant; A_D, A_A, and A_{AD} refer to acceptor membranes containing either a donor PC, acceptor PC molecule, or both. Step 1 represents the association of the soluble protein with an acceptor membrane; Steps 5 and 5′ describe the dissociation of the protein from the acceptor. Steps 2 and 3 describe the process by which endogenous PC is inserted in the acceptor vesicle: Step 2 indicates that this process occurs concurrently with the extraction of acceptor PC into the protein's lipid binding site while Step 3 shows that insertion occurs independently of membrane extraction. Step 4 represents the process by which PC in the acceptor membrane moves into the empty lipid binding site on the protein. The thickened arrows represent net transfer steps, i.e., those events which can occur with acceptor membranes initially devoid of PC. Even though the arrows are all unidirectional in order to focus on the measured flow of phospholipid molecules between membranes, it is important to keep in mind that each of the indicated steps is theoretically reversible.

mediate PC transfer [31], this association may be limited to the interface. Scheme I depicts two mechanisms for phospholipid exchange. In one, exchange is envisioned as a two-step process: the insertion of protein-bound PC into the membrane (step 3) and the extraction of membrane PC (step 4) occur sequentially. In the other, exchange is viewed as a concerted insertion and extraction of PC (step 2). The net transfer activity of PC-TP is described by steps 1, 3 and 5′ which represent an insertion of protein-bound PC into the acceptor membrane without subsequent binding of membrane PC to the protein.

Referring to Scheme I, explanations of the increased rates of PC transfer in the presence of acceptor membrane PC may be argued in the following ways:

(i) Insertion of protein-bound PC into the vesicle interface may require membrane PC to compete for the lipid binding site on the protein in a concerted process (step 2).

(ii) Exchange of protein-bound PC for membrane PC may occur by a sequential, two-step process, with the overall rate-limiting step being dependent on PC extraction from the vesicle surface (step 4); a net transfer of protein-bound PC to a membrane may occur when the rate-limiting step becomes the dissociation from a vesicle surface of PC-TP devoid of PC in the lipid binding site (step 5′).

(iii) PC, as an acceptor membrane lipid component, may enhance PC-TP activity in that the association of P_D with a membrane interface (step 1) may involve recognition of a phosphorylcholine polar headgroup, as suggested by Wirtz et al. [2].

(iv) Exchange of protein-bound PC for membrane PC may proceed by a sequential, two-step process in which the rate-limiting insertion (step 3) involves a PC molecule which effectively competes for the protein's phosphorylcholine recognition site [2].

Coupled to these arguments are certain predictions. According to arguments (i) and (ii), acceptor membrane PC, as a transferable lipid, should enhance protein-mediated PC transfer from donor to acceptor vesicles. In contrast, arguments (iii) and (iv) would contend that acceptor membrane PC, as a membrane lipid component should enhance transfer. In a sequential, two-step exchange of

protein-bound PC for vesicle PC, argument (ii) would predict that protein-vesicle dissociation constants for PC vesicles compared to vesicles lacking PC should be significantly greater. Likewise, both arguments (iii) and (iv) would predict that the rate of PC transfer to acceptor membranes containing either transferable or non-transferable PC molecules should be similar but significantly greater than the rate to acceptor vesicles lacking PC. On the other hand, in the concerted, one-step mode of PC exchange (argument (i)), both the magnitude of the protein-membrane dissociation constants for vesicles with or without PC and the rate of transfer to non-transferable PC acceptors and acceptors lacking PC should be similar.

Apparent dissociation constants for transfer protein-membrane complexes indicate that slightly more protein is associated with membranes containing 5 mol% egg PC compared to membranes comprised of *E. coli* lipids or a mixture of *E. coli* lipids and 5 mol% bis-PC. Although there are some limitations to the kinetic model (see previous section on 'Kinetic treatment' and assumptions 1 and 2), the kinetically obtained dissociation constants are consistent with the fluorescence binding data (Table III, Fig. 4), both of which indicate that dissociation from vesicles containing transferable PC is, in fact, decreased compared to vesicles which lack transferable PC. These results, coupled with data demonstrating that acceptor membrane PC enhances phospholipid transfer (Fig. 2), suggest that neither the extraction of membrane PC in the overall transfer of donor vesicle PC to acceptor vesicles (step 4, Scheme I) nor the dissociation of PC-TP from a membrane devoid of PC (step 5′, Scheme I) are reasonable rate-limiting steps. Consequently, an enhancement of transfer activity through a sequential, two-step process, in which PC acts as a transferable phospholipid, is unlikely, and argument (ii) may be eliminated.

Kinetic analysis of protein-mediated phospholipid transfer revealed that the rate constants describing PC-TP association with vesicles (step 1, Scheme I) are increased significantly with vesicles containing small amounts of PC (Table III). These findings may indicate that the enhanced PC-TP activity with PC acceptor vesicles involves PC, as a transferable lipid, in a concerted, one-step mechanism, according to argument (i). On the other hand, the results may indicate that PC, participating as a membrane component, affects the catalytic process (argument (iii) and (iv)). To discriminate between these two roles of membrane PC, bis-PC was designed as a structural analog of a typical diacyl, transferable PC-TP substrate [20]. Bis-PC differs chemically from a diacylPC molecule by the presence of a disulfide bond at the distal carbon of the two *sn*-1 acyl chains. The dimeric phospholipid was not transferred by PC-TP in a vesicle-vesicle assay system in which bis-PC was the sole donor membrane PC (Table IV). Furthermore, the presence of bis-PC in the acceptor vesicle membrane had virtually no effect on the transfer of donor PC (Table V).

The above observations suggest several possible conclusions concerning the relationship between bis-PC and PC-TP: (1) PC-TP simply does not 'see' membrane bis-PC; (2) the choline moieties of bis-PC are not accessible to PC-TP; or (3) PC-TP does not interact specifically with bis-PC because the molecule is not a transferable substrate. The first possibility can be ruled out, as the intrinsic fluorescence of PC-TP is readily quenched by bis-(*trans*-parinaroylPC). We have also shown, in the preceding paper [20], that bis-PC is not only 'seen' but hydrolyzed by snake venom phospholipase A_2, another example of a soluble protein which interacts with phospholipid molecules at membrane interfaces. Although the orientation of the choline moieties of bis-PC has not been definitively established, the fact that vesicular structures may be formed from bis-PC [20] would predict an exposure of the choline groups to PC-TP. Moreover, good transfer of the disulfide-containing 'half-molecule' of bis-PC was noted (Table IV). Concluding that the mere presence of a choline-containing glycerophospholipid as an acceptor membrane lipid component does not facilitate the protein-mediated transfer of PC, arguments (iii) and (iv) may be ruled out.

The rate of PC transfer from a donor vesicle to an acceptor vesicle is dependent on the concentration of transferable PC in the acceptor vesicle. However, slow rates of transfer to acceptor vesicles lacking transferable PC is not a result of altered PC-TP-vesicle binding equilibria. Our experimental observations can most consistently be explained by a concerted, one-step mechanism for

the exchange of protein-bound PC for membrane PC, as depicted by step 2, Scheme I, and described by argument (i). Such a mechanism would explain both the increased association and dissociation of PC-TP from PC vesicles compared to vesicles lacking PC (Table III) and the inability of the non-transferable bis-PC to enhance transfer, or, at the very least, to participate in any specific, measurable interaction with PC-TP (Table V). Although it is well known that PC-TP has an absolute specificity for the binding and transfer of PC [13,29], we could find no data in the literature to suggest any type of interaction between PC-TP and PC at a membrane/water interface other than binding and transfer. A concerted, one-step mode for PC exchange implies that the insertion of a protein-bound PC molecule into a membrane is the overall rate limiting step in protein-mediated transfer. Thus, in the absence of PC in acceptor vesicles, the insertion of protein-bound PC into the vesicle (step 3) must become rate-limiting and be slower than the insertion of phospholipid which occurs with PC exchange (step 2), as quantitated in Fig. 2. For membranes containing PC as the major lipid component, the exchange reaction (step 2, Scheme I) will be the predominant catalytic activity of PC-TP [31,32].

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