In rabbit platelet membranes, the contents of alkylacyl phospholipids (plasmalogen) were 56% of phosphatidylethanolamine and 3% of phosphatidylcholine. This uneven distribution of plasmalogens in each phospholipid class could be attributed to the different substrate specificity of ethanolaminephosphotransferase (EC 2.7.8.1) and cholinephosphotransferase (EC 2.7.8.2). The properties of the enzymes were studied, using endogenous diglycerides and CDP-[3H]ethanolamine or CDP-[14C]choline as substrates. The newly formed phospholipids were mainly diacyl and alkylacyl and only rarely alkylacyl type. The ratios of the labeled alkylacyl to diacyl type of phospholipids clearly varied with the concentrations of CDP-ethanolamine or CDP-choline. When 1, 10, and 30 μM CDP-[3H]ethanolamine were used, the labeled phospholipids contained 53, 37, and 27% of the alkylacyl type, respectively. The apparent Kₘ for CDP-ethanolamine to synthesize alkylacyl and diacyl types were 2.2 and 8.1 μM. On the other hand, when 1, 10, and 30 μM CDP-[14C]choline were used, the labeled lipids contained 10, 17, and 24% alkylacyl type, respectively. The apparent Kₘ for CDP-choline to synthesize alkylacyl and diacyl types were 24 and 4.3 μM. Further, the syntheses of diacyl type of phosphatidylethanolamine and the alkylacyl type of phosphatidylcholine were markedly inhibited by unlabeled CDP-choline and CDP-ethanolamine, respectively. The two enzymes had opposite substrate specificities, and ethanolaminephosphotransferase showed a high preference to plasmalogen synthesis, especially in the presence of CDP-choline.

Phosphatidylethanolamine and phosphatidylcholine are major constituents of glycerophospholipids in platelets. Phosphatidylethanolamine and phosphatidylcholine are synthesized mainly by the CDP pathway (1, 2). The final reaction step of the pathway is catalyzed by ethanolaminephosphotransferase (EC 2.7.8.1) or cholinephosphotransferase (EC 2.7.8.2). The properties of these enzymes have been investigated extensively in the mammalian liver (3–6), brain (7–9), intestine (10), fat cells (11), lung (12), and platelets (13). Although in most mammalian tissues the linkage to the 2-position of the glycerol in glycerophospholipids is only the acyl type, three linkage types are known to be acyl, alkyl, and alkenyl bonds, in the 1-position. These three types of glycerophospholipids have different distributions of phosphatidylethanolamine and phosphatidylcholine in various mammalian tissues including platelets (14), neutrophils (14, 15), brain, kidney, lung, and testis (16).

In platelets, the alkylacyl type of phospholipids (plasmalogens) is found dominantly in phosphatidylethanolamine and scarcely in phosphatidylcholine (14, 17). Alkenylglycerophosphoethanolamine (alkenylacyl-GPE) contains a large amount of arachidonic acid in the 2-position of glycerol and may play an important role as a major source for arachidonic acid releasing during platelet activation (18).

In an attempt to explain the uneven distribution of alkylacyl phospholipids in phosphatidylethanolamine and phosphatidylcholine, the activities of both transferases in the syntheses of alkylacyl and diacyl type of phospholipids in rabbit platelet membranes were examined, using endogenous diglycerides and labeled CDP-ethanolamine or CDP-choline as substrates. We found that the transferases have different substrate specificities for diglycerides and different Kₘ values for CDP-bases to synthesize the alkylacyl and diacyl type of phosphatidylethanolamine or phosphatidylcholine.

**EXPERIMENTAL PROCEDURES**

**Materials**—CDP-ethanolamine and CDP-choline were purchased from Sigma. [1-3H]Ethanol-1-ol-2-amine hydrochloride (8.8 Ci/mmol) and CDP-[methyl-3H]choline (56 mCi/mmol) were from The Radiochemical Centre (Amersham, England). High-performance silica gel 60, cellulose, polyethyleneimine cellulose precoated glass plates were obtained from E. Merck, Darmstadt. All other chemicals were of analytical grade.

**Preparation of Radioactive CDP Ethanolamine**—CDP-[3H]ethanolamine was prepared enzymatically using partially purified ethanolamine kinase (EC 2.7.1.82) and phosphorylethanolamine cytidyltransferase (EC 2.7.7.14) from the rat liver. The partially purified ethanolamine kinase (DEAE-cellulose step) (19) and phosphorylethanolamine cytidyltransferase (ammonium sulfate fractionation step) (20) were extensively dialyzed in 50 mM Tris-succinate buffer (pH 8.5) and 50 mM Tris-acetate buffer (pH 7.5), respectively, to remove contaminant free ethanolamine and its derivatives. The reaction mixture (4 ml) contained ethanolamine kinase (2 units), 50 mM Tris-succinate (pH 8.5), 3 mM ATP, 15 mM MgCl₂, and 1 μCi of [3H]ethanolamine. Incubation was carried out at 37 °C for 1 h and terminated by boiling at 90 °C for 2 min. After centrifugation (10,000 × g for 10 min), the supernatant (0.5 ml each) was applied on a column (0.7 × 1.5 cm, Dowex 50W-X8, H⁺ form, 100–200 mesh). The

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1 The terms of "phosphatidylethanolamine," "phosphatidylcholine," and "diglycerides" in the text are used as general names of alkylacyl, alkenylacyl, and diacyl types.
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column was washed with 1.2 ml of distilled water, and then phospho-
ryl-[1-\text{H}]ethanolamine was eluted with 4 ml of distilled water. The
fractions containing labeled phosphorylethanolamine were lyophi-
лизированные. The labeled phosphorylethanolamine was utilized for the syn-
thesis of labeled CDP-ethanolamine, using partially purified phos-
pholipidase A2, which was kept in the organic phase after the stepwise
methanolysis, were less than 3% of the total labeled phosphatidyleth-
anolamine and less than 2% of the total labeled phosphatidylcholine
under our assay conditions (0.5-50 \text{\mu M} CDP-ethanolamine or CDP-
choline, and incubation for 5–20 min). Therefore, the thin layer
chromatography system was routinely used for separation of alke-
nylacyl phospholipids, and diacyl plus alkenylacyl fractions were re-
garded as diacyl phospholipids.

RESULTS

Contents of Plasmalogen in Phosphatidylethanolamine and Phosphatidylcholine of Rabbit Platelet Membranes—The
phospholipids extracted from the platelet membrane prep-
eration were separated by two-dimensional thin layer chro-
matography with HCl fume treatment. As indicated in Table
I, the distribution of alkenylacyl glycerophospholipids in platelet
membranes was totally different and the contents of the
alkenylacyl type in each phospholipid class were 56.4 and
3.3%, respectively, by phosphorus analysis. These values are
not identical to but are essentially similar to reported data
(14, 17).

pH Dependence of Ethanolaminephosphotransferase and Cholinephosphotransferase Activities—The activities of the
transfereases were examined at various pH, using HEPES and
glycine buffer systems. As shown in Fig. 1, the pH dependence of the two enzymes was slightly different. The optimal pH of
ethanolaminephosphotransferase was around 7.5 and that of
cholinephosphotransferase was around 7.0. However, the op-
timal pH for the alkenylacyl and diacyl types of phospholipid
synthesis was much the same in each enzyme, and the ratios
of alkenylacyl and diacyl phospholipid syntheses were fairly
constant at various pH values.

Effects of the Concentrations of CDP-Ethanolamine or CDP-
Choline on Alkenylacyl and Diacyl Phospholipids Syntheses—
As shown in Fig. 2, the time courses of synthesis of both
labeled alkenylacyl and diacyl types of phosphatidylethanol-
amine and phosphatidylcholine were similar, and the synthe-
sis rates were linear at least up to 5 min. The rates of synthesis
of alkenylacyl and diacyl phospholipids varied with the con-
centrations of CDP-ethanolamine or CDP-choline. When 1
\text{\mu M} CDP-[\text{H}]ethanolamine was used, 53% of the radioactivity
in newly formed phosphatidylethanolamine was found in al-
kenylacyl-GPE. However, at higher concentrations (10 and
30 \text{\mu M}) of CDP-ethanolamine, the percentage of alkenylacyl-
GPE formation in total labeled phosphatidylethanolamine
was decreased to 37 and 27%, respectively. On the other hand,
when 1 \text{\mu M} CDP-[\text{C}]choline was used, only 10% of the
radioactivity in synthesized phosphatidylcholine was found

| TABLE I |
| Contents of phosphatidylethanolamine and phosphatidylcholine in rabbit platelet membranes |

Phospholipids were extracted from the platelet membrane prep-
eration and separated by two-dimensional thin layer chromatography,
as described under "Experimental Procedures." Values are mean ±
S.D. of four determinations.

| phospholipid class | nmol/mg protein | % of the phospholipid class |
|--------------------|----------------|---------------------------|
| Alkenylacyl-GPE    | 80.6 ± 0.2     | 56.4 ± 1.0                |
| Diacyl-GPE plus alkenylacyl-GPE | 63.6 ± 4.3 | 43.6 ± 1.0                |
| Alkenylacyl-GPC    | 4.2 ± 0.4      | 3.3 ± 0.2                 |
| Diacyl-GPC plus alkenylacyl-GPC | 147.8 ± 4.5 | 96.7 ± 0.2                |
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![Image](https://example.com/image.png)

**FIG. 1.** pH dependence of ethanolaminephosphotransferase and cholinephosphotransferase. The reaction mixture contained 150 μg of platelet membranes, 5 mM MgCl₂, and 30 μM CDP-[³H]ethanolamine (A) or CDP-[³H]choline (B). After 5 min of incubation at 37°C, the reaction products were extracted and separated as described under “Experimental Procedures.” C, total labeled phospholipids; △, alkenylacyl type; □, diacyl type. Open symbols, 50 mM HEPES buffer; Closed symbols, 50 mM glycine–NaOH buffer.

**FIG. 2.** Time course of ethanolaminephosphotransferase and cholinephosphotransferase activities. Platelet membranes (150 μg) were incubated with 1, 10, and 30 μM CDP-[³H]ethanolamine (A, B, and C) or CDP-[³H]choline (D, E, and F) in the presence of 50 mM HEPES buffer (pH 7.5) and 5 mM MgCl₂. After the incubation as indicated, the labeled lipids were extracted and separated by two-dimensional thin layer chromatography as described under “Experimental Procedures.” CDP-ethanolamine and CDP-choline: 1 (A and D), 10 (B and E), and 30 μM (C and F). Open circles, diacyl type of phospholipids; closed circles, alkenylacyl type of phospholipids.

in the alkenylacyl-GPC. At higher concentrations (10 and 30 μM) of CDP-choline, the percentage of alkenylacyl-GPC formation was increased to 15 and 24%, respectively.

Mg²⁺ and Mn²⁺ are effective metal cofactors for both ethanolaminephosphotransferase and cholinephosphotransferase (1). Therefore, kinetic analyses of both transferases were carried out using Mg²⁺ and Mn²⁺ as metal cofactors. As shown in Fig. 3, A and C, the apparent Kₐ values for CDP-ethanolamine to synthesize alkenylacyl-GPE were clearly lower than the values to synthesize diacyl-GPE, with both Mg²⁺ and Mn²⁺. The apparent Kₐ values for CDP-ethanolamine to synthesize alkenylacyl-GPE and diacyl-GPE were 2.2 and 8.1 μM with Mg²⁺ and 1.0 and 4.5 μM with Mn²⁺, respectively. Whereas, as shown in Fig. 2, B and D, the apparent Kₐ values for CDP-choline to synthesize alkenylacyl-GPC were clearly higher than the values to synthesize diacyl-GPC. The apparent Kₐ values for CDP-choline to synthesize alkenylacyl-GPC and diacyl-GPC were 24 and 4.3 μM with Mg²⁺ and 11 and 3.6 μM with Mn²⁺, respectively. The synthesis rates of alkenylacyl-GPC were evidently lower than those of diacyl-GPC at all concentrations of CDP-choline tested. However, the synthesis rates of alkenylacyl-GPE were clearly higher than those of diacyl-GPE, at least at lower concentrations of CDP-ethanolamine (≤ 1 μM with Mg²⁺ and ≤ 2 μM with Mn²⁺). When Mn²⁺ but not Mg²⁺ was used as a cofactor, a definite substrate inhibition was observed at higher concentrations of CDP-ethanolamine (≥ 25 μM).

**FIG. 3.** Lineweaver-Burk plots of ethanolaminephosphotransferase and cholinephosphotransferase activities. Platelet membranes (160 μg) were incubated with various concentrations of CDP-[³H]ethanolamine (A and C) or CDP-[³H]choline (B and D), and 5 mM MgCl₂ (A and B) or 2 mM MnCl₂ (C and D). Other assay conditions and separation of products by thin layer chromatography were as described under “Experimental Procedures.” △, alkenylacyl and □, diacyl type of phospholipid formation.

**DISCUSSION**

To investigate enzymic properties of ethanolaminephosphotransferase and cholinephosphotransferase diglycerides were added exogenously to the assay mixtures as lipid acceptors in most of the previous reports. Three analogous diglycerides, diacyl, alkenylacyl, and alkylacyl glycerol, were demonstrated to be the substrates for ethanolaminephosphotransferase and cholinephosphotransferase (3, 7, 8, 13). Although the enzyme activities were fairly low, some workers found in various tissues, including platelets, significant activities of the transferases to form diacyl, alkenylacyl, or alkylacyl phospholipids, detected without addition of exogenous diglycerides.
Phospholipids; products were separated on thin layer chromatography as described under “Experimental Procedures.”

Inhibitory effects of CDP-choline of the formation of diacyl-GPE, and CDP-ethanolamine to synthesize diacyl-GPE, alkenylacyl-GPC, and alkenylacyl-GPC were 290, 270, 350, and 200 μM, respectively, in the presence of corresponding diglycerides. In human platelet homogenates, K_m for CDP-ethanolamine was 160 μM, with the addition of diacyl glycerol (13). All these values are much higher than those determined in the present study. However, since the concentrations of these endogenous diglycerides were not determined and those in isolated membranes might be insufficient for both transferase activities, the K_m values for CDP-ethanolamine and CDP-choline determined in this study were apparent K_v values.

The addition of CDP-choline or CDP-ethanolamine to other phosphotransferase assay mixtures demonstrated the preferential synthesis of alkenylacyl and diacyl type of phospholipids (Fig. 4). It has been reported that CDP-choline and CDP-ethanolamine inhibit competitively other transferases in rat liver (5) and Plasmodium knowlesi-infected erythrocytes (30). In fat cells, the inhibitory effect of CDP-choline on ethanolaminephosphotransferase was noncompetitive (11). In those reports, the enzyme activities were examined with exogenously added diglycerides. In the present study, diacyl-GPE and alkenylacyl-GPC formations were noncompetitively inhibited by CDP-choline and CDP-ethanolamine, respectively. Surprisingly, alkenylacyl-GPE and diacyl-GPC formations were stimulated by the other CDP-bases, and the ratio of the two glycerol derivatives in the microenvironment of the transferases as well as the different affinity of two glycerol derivatives for the enzymes determines the ratio of the products. In rat liver, Kanoh and Ohno (5) demonstrated not only that ethanolaminephosphotransferase and cholinephosphotransferase were different enzymes but also that cholinephosphotransferase was partially separated into Mg²⁺-requiring and Mn²⁺-requiring components. Therefore, the different ratio of the two products (diacyl to alkenylacyl) with the co-existence of CDP-choline and CDP-ethanolamine, may be attributed to two or more different transferases in rabbit platelet membranes.

In many mammalian tissues (18), including platelets (17), alkenylacyl phospholipids are found mainly in phosphatidylycholine, phosphatidylethanolamine, and phosphatidylserine. Many of these phospholipids are preferentially utilized for the syntheses of phosphatidylethanolamine and phosphatidylcholine, respectively. It is possible that the ratio of the two glycerol derivatives in the microenvironment of the transferases as well as the different affinity of two glycerol derivatives for the enzymes determines the ratio of the products. In rat liver, the

Addition of diglycerides with some detergents may alter the enzyme conformation and lipid environments, and the choice of detergent and its concentration is most critical for enzyme activities (6, 10, 29). Therefore, in order to clarify which endogenous diglyceride, diacyl, alkenylacyl, or alkylacyl glycerol, is utilized as the substrate for ethanolaminephosphotransferase and cholinephosphotransferase in rabbit platelet membranes, diglycerides or detergents were not added to the assay mixture in the present study.

Ansell and Metcalfe (7) reported that with brain microsomes, K_m for CDP-ethanolamine in the presence of diacyl glycerol (260 μM) was almost the same as that in the presence of alkenylacyl glycerol (220 μM). Strosznajder et al. (8) also reported that in brain synaptosomes, K_v values for CDP-ethanolamine and CDP-choline to synthesize diacyl-GPE, alkenylacyl-GPE, diacyl-GPC, and alkenylacyl-GPC were 290, 270, 350, and 200 μM, respectively, in the presence of corresponding diglycerides. In human platelet homogenates, K_m for CDP-ethanolamine was 160 μM, with the addition of diacyl glycerol (13). All these values are much higher than those determined in the present study. However, since the concentrations of these endogenous diglycerides were not determined and those in isolated membranes might be insufficient for both transferase activities, the K_m values for CDP-ethanolamine and CDP-choline determined in this study were apparent K_v values.

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two transferases were demonstrated to have different specificities for diglycerides depending on the fatty acid side chains not only at the 2', but also at the 1-position of the glycerol (4, 6). However, it has not been demonstrated that the enzymes have different specificities for alkenylacyl and diacyl glycerols in mammalian tissue. Recently, Smith (28) demonstrated the different selectivity of the phosphotransferases of tetrahymena for diacyl glycerol and alkenylacyl glycerol and suggested that this selectivity accounted for the high content of alkylacyl-GPC in tetrahymena. Although the content of alkylacyl-GPC has been reported to constitute more or less the same percent as that of alkenylacyl-GPE in platelet membranes (13, 14), the incorporation of [14C]choline into alkylacyl-GPC was found only slightly under our assay conditions. This discrepancy is not clear now, but the presence of endogenous alkylacyl glycerol in rabbit platelet membranes may be very low as compared to alkenylacyl glycerol. More experiments are needed to clarify whether or not the uneven distribution of alkenylacyl phospholipid in phosphatidylethanolamine and phosphatidylcholine is attributed to the different enzymic properties of ethanolaminephosphotransferase and cholinephosphotransferase.

It is not known whether alkenylacyl glycerol is formed by de novo pathways in platelets. Possible sources of diglycerides are de novo synthesis, degradation of pre-existing phospholipids by phospholipase C, phosphatidic acid phosphatase, or reverse reactions of ethanolaminephosphotransferase and cholinephosphotransferase. If diglycerides are supplied from degradation of phospholipids, these phosphotransferases do not catalyze the net synthesis of phospholipids. However, as described above, ethanolaminephosphotransferase preferentially synthesized the alkenylacyl type and cholinephosphotransferase preferentially synthesized the diacyl type, in particular with the co-existence of CDP-choline and CDP-ethanolamine. In the rabbit platelet membrane preparations used, the content of alkenylacyl type was 56% of the phosphatidylethanolamine and was only 3% of phosphatidylcholine. The preference of the enzymes no doubt plays some important role in regulating the uneven alkenylacyl phospholipid distribution in rabbit platelets.

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