# A rapid biosynthetic method for the preparation of radioactive phosphatidyl-CMP(CDP-diacylglycerol) of high specific activity

Joseph Eichberg and George Hauser

*Department of Biophysical Sciences, University of Houston, Houston, TX 77004; Ralph Lowell Laboratories, Mailman Research Center, McLean Hospital, Belmont, MA 02178; and Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115*

**Summary** A procedure is described for the preparation of [$^{32}$P]phosphatidyl-CMP(CDP-diacylglycerol) from rat pineal glands incubated with [$^{32}$P]orthophosphate and DL-propranolol. The product is 95% radiopure and of high specific activity. The yield of liponucleotide is 0.4–0.9 $\mu$Ci/mCi of [$^{32}$P]orthophosphate in the medium. The same method can also be used for the biosynthesis and purification of [$^{3}$H]phosphatidyl-CMP when [$^{3}$H]cytidine is the precursor.

**Supplementary key words** pineal gland · propranolol · liponucleotide · [$^{32}$P]phosphatidyl-CMP · [$^{3}$H]phosphatidyl-CMP

The importance of Ptd-CMP as an intermediate in the biosynthesis of phosphatidylinositol, polyglycerophosphatides, and (in bacteria) phosphatidylserine has been well documented (1–8). However, studies of the metabolic role of the liponucleotide have been hampered by its extremely low concentration in microorganisms and mammalian tissues. Recently, Thompson and MacDonald (9, 10) isolated Ptd-CMP from bovine brain and liver in sufficient quantities to permit its complete chemical characterization. Their findings concerning the nature of the molecular species have focused attention on the precise metabolic relationships between Ptd-CMP and other acidic phospholipids. For these reasons, the availability of a simple method for the preparation of radioactive Ptd-CMP which could be utilized in metabolic studies would be useful. Aside from a relatively tedious chemical synthesis (11, 12), no such preparative procedure has been described.

Previously, we reported (13, 14) that incubation of rat pineal glands in vitro with the $\beta$-adrenergic receptor blocking agent, DL-propranolol, caused enhanced de novo synthesis of phosphatidylinositol and phosphatidylglycerol as well as an accumulation of Ptd-CMP. We also briefly noted that the recovery of the liponucleotide from a $CHCl_3$–$CH_3OH$ extract of pineal lipids was dependent on the acidity of the aqueous phase used to wash out water-soluble contaminants. In this report, we describe how these observations have been utilized to develop a rapid method for the preparation of nearly radiopure Ptd-CMP of high specific activity.

Abbreviations: Ptd-CMP, phosphatidyl-CMP(CDP-diacylglycerol); TLC, thin-layer chromatography.

## Materials and methods

*Materials.* [5-$^{3}$H]Cytidine (6–22 Ci/mmol) was obtained from Schwarz-Mann, Orangeburg, NY. [$^{32}$P]-Orthophosphate was purchased from New England Nuclear, Boston, MA. Unisil silicic acid (220–325 mesh) was a product of Clarkson Chemical Co., Williamsport, PA. Silica Gel H plates for TLC were obtained from Analtech, Inc., Newark, DE. DL-Propranolol-HCl was purchased from Sigma Chemical Co., St. Louis, MO. Female rats, 125–150 g, were provided by Texas Inbred Mice Co., Houston, TX, or Charles River Breeding Laboratories, Wilmington, MA. Ptd-CMP hydrolase from *Escherichia coli* was purified through the acetone precipitation step according to Raetz et al. (15).

*Biosynthesis of radiolabeled Ptd-CMP.* Rat pineal glands were incubated with carrier-free [$^{32}$P]orthophosphate as described previously (14) in 100 $\mu$l of Krebs-Ringer bicarbonate medium, pH 7.4, from which calcium chloride and orthophosphate were omitted and which contained 5.5 mM glucose and 0.1 mM propranolol. Incubations with [$^{3}$H]cytidine were carried out under identical conditions except that complete Krebs-Ringer bicarbonate–glucose medium containing 0.1 mM propranolol was used. All incubations were performed for 3–6 hr at 37°C and terminated by the addition of 2 ml of ice-cold isotonic NaCl solution. Each gland was immediately removed and washed successively in two 2-ml portions of isotonic NaCl. Up to 10 glands were then transferred to 5.0 ml of $CHCl_3$–$CH_3OH$ (2:1)–5% $H_2O$ for extraction of lipids.

*Purification of phosphatidyl-CMP.* To the lipid extract was added 1.0 ml of 1 mM sodium citrate, pH 8. The mixture was shaken, the phases were allowed to separate, and the upper phase was collected. The lower phase was washed with two-thirds its volume of $CHCl_3$–$CH_3OH$–1 mM sodium citrate 3:48:47, pH 8. The pooled upper phases were adjusted to pH 2 by dropwise addition of $CHCl_3$–$CH_3OH$–1N HCl 3:48:47 (ca. 0.2 ml) and back-extracted with two 5 ml portions of $CHCl_3$–$CH_3OH$–$H_2O$ 86:14:1.

The $CHCl_3$-rich extract was taken to dryness under nitrogen, taken up in 0.5 ml of $CHCl_3$–pyridine–formic acid 50:30:7, and applied to a 0.5 × 5 cm column of Unisil silicic acid which had previously been equilibrated with the same solvent. The column was

TABLE 1. Recovery of radioactivity in phosphatidyl-CMP after neutral or acid extraction procedures

| | Precursor | Additions | Phosphatidyl-CMP Recovered: Neutral Wash | Acid Wash | Acid/ Neutral |
|---|---|---|---|---|---|
| | | | *pmol/gland* | | |
| Exp. 1 | [$^3$H]cytidine | None | 0.05 | 0.36 | 7.2 |
| | [$^3$H]cytidine | 0.3 mM propranolol | 2.48 | 14.53 | 5.9 |
| Exp. 2 | [$^3$H]cytidine | 0.1 mM propranolol | 1.03 | 5.98 | 5.8 |
| | $^{32}$P | 0.1 mM propranolol | 2.80 | 17.91 | 6.4 |

Rat pineal glands were incubated for 1 hr in Krebs-Ringer-bicarbonate-glucose medium containing [$^3$H]cytidine (sp act 3.8 × $10^3$ cpm/pmol) or $^{32}$P (171 cpm/pmol) and then transferred to 5 ml of $CHCl_3$–$CH_3OH$ (2:1)–5% $H_2O$. The neutral wash procedure was carried out according to Folch, Lees, and Sloane Stanley (16). The acid wash procedure and other details are described in Materials and Methods. Each value is the average of results from two to four incubations.

eluted with 15 ml of $CHCl_3$–pyridine–formic acid 50:30:7, followed by 5 ml of $CHCl_3$. The radioactive Ptd-CMP was then eluted with 15 ml of $CHCl_3$–$CH_3OH$ (1:3 or 3:2).

*Analytical procedures.* When the distribution of radioactivity present in pineal phospholipids after incubation was to be determined, a washed total lipid extract was prepared from a portion of the initial $CHCl_3$–$CH_3OH$–$H_2O$ solution. The aliquot was brought to 5 ml with $CHCl_3$–$CH_3OH$ (2:1)–5% $H_2O$ and the mixture was shaken with 1.0 ml of 0.01 N HCl. The resulting upper phase was discarded and the lower phase was washed twice with two-thirds its volume of $CHCl_3$–$CH_3OH$–0.01 N HCl 3:48:47, the interface was then rinsed with $CHCl_3$–$CH_3OH$–$H_2O$ 3:48:47 and $CH_3OH$ was added to make one phase.

When [$^3$H]cytidine was the precursor, an aliquot of the washed total lipid extract was taken for radioassay. Phospholipids labeled with $^{32}$P were separated by two-dimensional TLC, located by radioautography, and appropriate areas of silica gel were counted as previously described (13). Radioactive measurements were performed using a Packard liquid scintillation spectrometer.

## Results

*Development of the method.* The use of acidified aqueous phases to wash a Ptd-CMP-containing lipid extract caused retention of far more of the liponucleotide in the $CHCl_3$ layer than if neutral aqueous phases were employed (**Table 1**). The data indicate that about six times as much Ptd-CMP was recovered following a sequence of HCl washes as compared to a series of KCl washes, as judged from both [$^3$H]cytidine and $^{32}$P incorporated into the compound in the presence of propranolol. These observations suggested that the effect of pH on the partition of Ptd-CMP between an aqueous and an organic phase might be made the basis of a method for its purification.

In developing such a method, the first step was to define conditions in which Ptd-CMP would partition to the maximum extent into an aqueous phase from a $CHCl_3$-rich layer. To investigate this, citrate buffers of different pH values and concentrations were equilibrated with an extract of rat pineal lipids containing [$^3$H]Ptd-CMP (**Table 2**). As the pH was raised from 3 to 6, progressively more liponucleotide passed from the organic phase to an aqueous phase containing 10 mM citrate buffer. No more than about 60% of Ptd-CMP was transferred even when the pH increased to 8. In the presence of 1 mM citrate buffer at a pH near neutrality, over 90% of the liponucleotide was extracted into the aqueous layer. In contrast, when the aqueous phase contained 1 mM Tris buffer, pH 8.0, 61% of Ptd-CMP was retained in the $CHCl_3$ phase. Thus, the observed partition characteristics do not seem to be due simply to a salt effect (16).

After acidification of the citrate upper phase, from 70 to 90% of Ptd-CMP was transferred into an organic phase. When $^{32}$P was the precursor, the radiopurity of liponucleotide after this back-extraction step varied from 60 to 85%, the principal labeled contaminant being phosphatidylinositol.

Upon silicic acid column chromatography of the lipid back-extracted into an organic phase, nearly all

TABLE 2. Effect of pH and citrate buffer concentration on retention of phosphatidyl-CMP in $CHCl_3$-rich phase

| Aqueous Phase | Phosphatidyl-CMP Not Partitioned into Aqueous Phase |
|---|---|
| | % |
| 0.01 N HCl | 100 |
| 10 mM Na citrate buffer | |
| pH 3.3 | 108 |
| 4.0 | 83 |
| 5.0 | 34 |
| 6.0 | 33 |
| 7.0 | 45 |
| 8.0 | 39 |
| 1 mM Na citrate buffer | |
| pH 3.3 | 99 |
| 6.0 | 7 |
| 7.0 | 4 |
| 8.0 | 4 |

Five rat pineal glands were incubated for 3 hr with 10 $\mu$M [$^3$H]cytidine (6 Ci/mmol) and 0.1 mM propranolol as described in Materials and Methods. Aliquots which contained 6000–10000 cpm were removed from the $CHCl_3$–$CH_3OH$ (2:1)–5% $H_2O$ extract and made to 5.0 ml with the same solvent. Each solution was washed with 1.0 ml of a designated aqueous phase and the resulting lower phase was then washed with two-thirds its volume of $CHCl_3$–$CH_3OH$–aqueous phase 3:48:47. Portions of the final lower phase were taken for radioassay.

TABLE 3. Purification of [$^{32}$P]phosphatidyl-CMP

| Step | Propranolol | | No Propranolol |
|---|---|---|---|
| | *Total cpm* | *cpm in phosphatidyl-CMP* | *Total cpm* |
| Acid washed lower phase | 203,000 | 89,200 | 103,000 |
| Back-extraction from citrate upper phase | 73,200 | | 13,000 |
| Unisil column fractions | | | |
| $CHCl_3$-pyridine-HCOOH 50:30:7 | 6,780 | | 3,720 |
| $CHCl_3$ | 325 | | 350 |
| $CHCl_3$–$CH_3OH$ 3:2 | 41,560 | 40,150 | 960 |
| $CHCl_3$–$CH_3OH$ 1:9 | 12,830 | 10,850 | 2,150 |
| Not transferred to column | | 1,670 | 5,060 |

Five rat pineals were incubated in either the presence or absence of propranolol (0.1 mM) for 3 hr. Each group of glands was pooled and extracted with 5.0 ml of $CHCl_3$–$CH_3OH$ (2:1)–5% $H_2O$. A 0.25-ml aliquot of each extract was removed and washed with HCl-containing aqueous solutions to prepare acid washed lower phase. Aliquots of this phase were taken for radioassay as well as for two-dimensional TLC of phospholipids and determination of radioactivity in Ptd-CMP. The remainder of the initial extract was carried through the Ptd-CMP purification procedure. Aliquots for radioassay were removed from the $CHCl_3$-rich phase obtained in the back-extraction step and from Unisil column fractions. Additional portions of the $CHCl_3$–$CH_3OH$ 3:2 and $CHCl_3$–$CH_3OH$ 1:9 fractions from the column were utilized to separate phospholipids in order to assay their content of Ptd-CMP radioactivity. The cpm are expressed per pineal gland and are normalized to $10^8$ cpm of $^{32}$P in the medium. Further experimental details are described in Materials and Methods.

residual phosphatidylinositol, together with trace amounts of other less polar phospholipids, such as phosphatidic acid, were eluted by $CHCl_3$–pyridine–formic acid. The bulk of Ptd-CMP could then be eluted by $CHCl_3$–$CH_3OH$ 3:2 or $CHCl_3$–$CH_3OH$ 1:3. Subsequent elution of the column with $CHCl_3$–$CH_3OH$ 1:9 yielded a fraction containing a small quantity of Ptd-CMP of somewhat lower radiopurity.

The results of a representative preparation of [$^{32}$P]-Ptd-CMP are summarized in **Table 3** (columns 1 and 2). After incubation of several pineal glands with propranolol, 44% of the total $^{32}$P in phospholipid was present in Ptd-CMP as determined on aliquots of the initial solvent extract. When the remainder of the extract was subjected to the purification scheme, nearly half of the Ptd-CMP radioactivity was recovered in the $CHCl_3$–$CH_3OH$ 3:2 fraction from the Unisil column and an additional 12% was eluted by $CHCl_3$–$CH_3OH$ 1:9.

*Identity of the product.* The presence of Ptd-CMP in lipid extracts prepared from pineal glands that had been incubated with propranolol has been conclusively established by chromatographic, chemical, and enzymatic techniques (14). The purified $^{32}$P-labeled material behaved exactly like Ptd-CMP when chromatographed in the two-dimensional TLC system employed previously. When treated with Ptd-CMP hydrolase (14), more than 75% of the final product was converted into a substance that comigrated with phosphatidic acid on silica gel G in $CHCl_3$–pyridine–formic acid 50:30:7.

*Radiopurity and yield of $^{32}$P-labeled product.* The data in **Table 4** indicate that more than 40% of the $^{32}$P incorporated into pineal lipids was present in Ptd-CMP. Much smaller and approximately equal proportions of radioactivity were present in other acidic phospholipids, namely phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid; these four

TABLE 4. Distribution of $^{32}$P in washed total lipid extract and in purified phosphatidyl-CMP

| Phospholipid | Radioactivity in Washed Total Lipid Extract | Purified Phosphatidyl-CMP | | |
|---|---|---|---|---|
| | | Preparation 1 | Preparation 2 | Preparation 3 |
| | % | | % | |
| Phosphatidyl-CMP | 42.3 ± 0.6 | 92.2 | 94.1 | 96.6 |
| Phosphatidylinositol | 12.9 ± 1.0 | 3.2 | 0.8 | 2.1 |
| Phosphatidylcholine | 4.7 ± 1.4 | | | |
| Phosphatidylethanolamine | 7.1 ± 1.2 | | | |
| Phosphatidylglycerol | 11.9 ± 2.0 | | | |
| Phosphatidic acid | 11.5 ± 0.9 | | | |
| Diphosphatidylglycerol | 2.0 ± 0.3 | | | |
| Other | 7.8 ± 3.3 | 4.6 | 5.0 | 1.3 |

Five pineal glands were labeled with $^{32}$P in the presence of propranolol. A portion of the initial lipid extract was washed and the compounds were separated by two-dimensional TLC. The values for the washed total lipid extract are averages ± SEM derived from four experiments. Phosphatidyl-CMP was purified and portions of the preparation were subjected to two-dimensional TLC. For further details, see text.

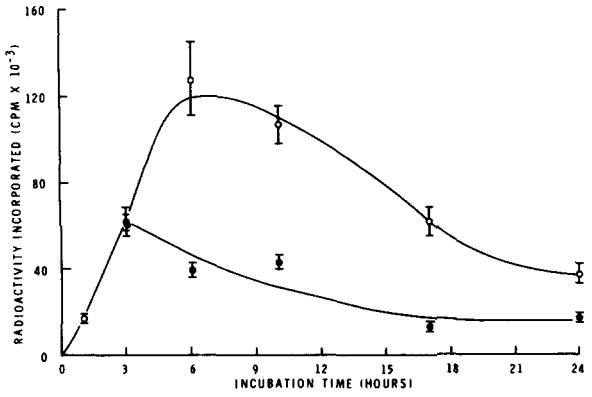


**Fig. 1.** Time course of incorporation of radioactive precursors into phosphatidyl-CMP. Incubations with $^{32}P$ (15 $\mu$Ci) were carried out in 100 $\mu$l of Krebs-Ringer–bicarbonate–glucose (no $CaCl_2$ or orthophosphate) that contained 0.1 mM propranolol. Lipids were extracted and separated and radioactivity in Ptd-CMP was quantitated as described in the text and in (14). Incubations with [$^3$H]-cytidine (6 $\mu$Ci) were performed in 100 $\mu$l of Krebs-Ringer–bicarbonate–glucose medium that contained 0.1 mM propranolol. Lipids were extracted and portions of the washed total lipid extract were taken for radioassay. [$^3$H]Cytidine, O——O; $^{32}P$, ●——●.

lipids contained over 75% of the incorporated radioactivity. The Ptd-CMP purification procedure gave preparations of liponucleotide that were consistently greater than 90% radiopure. A small amount of phosphatidylinositol (1–3%) could not be readily removed. Other impurities consisted of radioactive substances that migrated more slowly than Ptd-CMP in the two-dimensional TLC system employed and are apparently more polar than the liponucleotide. These compounds, some of which may be lysophosphoglycerides, are presumably trace lipid components which are selectively concentrated during Ptd-CMP purification.

The radiopurity of the product was further evaluated by carrying out the purification procedure starting with pineal glands that had been labeled with $^{32}P$ in the absence of propranolol. Since negligible amounts of Ptd-CMP are formed under these conditions (Table 1), virtually no radioactivity should be recovered. Data in Table 3 (column 3) show that only 5.7% of the counts present in Ptd-CMP containing column fractions was recovered in those same fractions in the experiment in which propranolol was omitted. If contaminants are considered to account entirely for the residual counts, this figure is in good agreement with the extent of impurities as determined by direct analyses of Ptd-CMP preparations.

The yields of purified radioactive Ptd-CMP based on the amount of liponucleotide estimated to be present in the total $^{32}P$-labeled lipid extract as determined by counting the appropriate TLC spot were 37, 57, and 57% for the three preparations summarized in Table 4. Losses occurred principally in the back-extraction and column chromatography steps; the latter losses were apparently due to the elution of varying amounts of Ptd-CMP by $CHCl_3$–pyridine–formic acid. The yield of final product in these preparations, expressed per millicurie of $^{32}P$ present in the incubation medium, ranged from 0.37 to 0.89 $\mu$Ci. The variability is a reflection not only of preparative losses, but also of the extent of $^{32}P$ uptake into lipids during pineal gland incubations.

*Yield of [$^3$H]cytidine-labeled product.* In two experiments in which pineal glands were incubated with [$^3$H]cytidine and 0.1 mM propranolol, the amount of label incorporated into lipids was 8,000 and 15,000 cpm, respectively, per $10^6$ cpm of precursor. Previous findings had established that the sole radioactive product obtained under these conditions is Ptd-CMP (14). The $^3$H-labeled material behaved in the same manner as [$^{32}$P]liponucleotide when carried through the purification procedure and was recovered in similar yield.

An additional experiment was performed to confirm that $^3$H- and $^{32}$P-labeled lipids were copurified in the method. To do this, pineal lipids were labeled separately from [$^{32}$P]orthophosphate and [$^3$H]cytidine and washed lipid extracts were prepared. The radioactivity in [$^{32}$P]Ptd-CMP was determined after separation of lipids by two-dimensional TLC whereas the $^3$H incorporated was measured by counting an aliquot of the extract. The $^3H/^{32}P$ ratio in this instance was 1.91. Equal aliquots of each lipid extract were combined and the mixture was subjected to the purification procedure. The corresponding ratio of counts eluted from the Unisil column by $CHCl_3$–$CH_3OH$ 3:2 was 2.13.

*Time course of isotope incorporation into Ptd-CMP.* When incubations were performed for different lengths of time, the incorporation of $^{32}P$ into Ptd-CMP was highest after 3 hr of incubation and then declined (**Fig. 1**). Indeed, radioactivity in the liponucleotide as a percentage of that in total phospholipid fell from 42% after 3 hr to 14% after 24 hr. The incorporation of [$^3$H]-cytidine followed a similar time course, rising to a peak at 6 hr and then falling steadily to a value at 24 hr that was 30% of the maximum.

## Discussion

The practice that gave impetus to this investigation, namely the use of acidified aqueous phases during lipid extraction to recover maximal quantities of Ptd-CMP, has been employed previously by several investigators in studies of Ptd-CMP biosynthesis (17, 18). The dependence on pH of the extent of partition

between an aqueous and organic solvent of the more polar acidic phospholipids, such as acyldihydroxyacetone phosphate and lysophosphatidic acid, has also recently been demonstrated (19, 20). Investigators who do not take this pH effect into account may only partially recover biosynthesized Ptd-CMP.

The present report describes for the first time a biosynthetic procedure for the isolation of nearly radiopure Ptd-CMP. Since the product is formed enzymatically, it has the natural steric configuration in contrast to the racemic mixture obtained when the liponucleotide is synthesized chemically. Our previous studies established that the $^{32}P$ in Ptd-CMP formed in the pineal gland during a 3-hr incubation is distributed such that 40% is in the CMP moiety and the remainder is in the phosphatidyl group (14).

The preparative method offers the particular advantage of speed since numbers of pineal glands can be dissected within minutes and, after incubation, the purification of $^{32}P$-labeled Ptd-CMP can be accomplished within a few hours. A limitation of the approach is that only relatively small quantities of liponucleotide may be obtained with this system. Besides the pineal gland, only pancreatic islets in the presence of tetracaine (21) and iris muscle and retina in the presence of propranolol (22, 23) have so far been reported to accumulate Ptd-CMP. If a means can be found to deplete other tissues of endogenous inositol, it may be possible to obtain larger amounts of Ptd-CMP after incubation in the presence of propranolol or other cationic amphiphilic amines (24).

The small quantity of liponucleotide present in the pineal gland after incubation precluded direct determination of the specific activity of the product. However, an estimate of the specific radioactivity of the $^{3}H$-labeled Ptd-CMP formed may be made as follows. In one experiment using [$^{3}H$]cytidine (sp act 22 Ci/mmol) as precursor and assuming that external cytidine equilibrates rapidly with nucleotide in the pineal gland, approximately 10 pmol of liponucleotide was biosynthesized. If the level of endogenous Ptd-CMP is the same in pineal tissue as in the liver, namely 10 $\mu$mol/kg (9), and the weight of a rat pineal gland is 1 mg, the specific activity of the product is roughly one-half that of the precursor. Using carrier-free [$^{32}P$]orthophosphate, Ptd-CMP of much higher specific activity may be obtained.

Recently, a biosynthetic method was described for the preparation of many $^{32}P$-labeled phospholipids of high specific activity using cultured cells (25). The present method is a useful complement to this procedure.

We thank Patricia Click, Ellen Hauge, Robert Murray, Sanford Schupper, and Alicja Skupny for skillful technical assistance. This work was supported by NIH grants NS12493 and NS06399.

*Manuscript received 24 August 1977; accepted 24 February 1978.*

## REFERENCES

1. Agranoff, B. W., R. M. Bradley, and R. O. Brady. 1958. The enzymatic synthesis of inositol phosphatide. *J. Biol. Chem.* **233:** 1077–1083.
2. Paulus, H., and E. P. Kennedy. 1960. The enzymatic synthesis of inositol monophosphatide. *J. Biol. Chem.* **235:** 1303–1311.
3. Thompson, W., K. P. Strickland, and R. J. Rossiter. 1963. Biosynthesis of phosphatidylinositol in rat brain. *Biochem. J.* **87:** 136–142.
4. Kiyasu, J. Y., R. A. Pieringer, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238:** 2293–2298.
5. Stanacev, N. Z., J. B. Davidson, L. Stuhne-Sekalec, and Z. Domazet. 1973. Biochemistry of polyglycerophosphatides. The mechanism of cardiolipin biosynthesis in isolated mitochondria. *Can. J. Biochem.* **51:** 286–304.
6. Hirschberg, C. B., and E. P. Kennedy. 1972. Mechanism of the enzymatic synthesis of cardiolipin in *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **69:** 648–651.
7. Hostetler, K. Y., H. van den Bosch, and L. L. M. Van Deenen. 1972. The mechanism of cardiolipin biosynthesis in liver mitochondria. *Biochim. Biophys. Acta.* **260:** 507–513.
8. Kanfer, J., and E. P. Kennedy. 1964. Metabolism and function of bacterial lipids II. Biosynthesis of phospholipids in *Escherichia coli*. *J. Biol. Chem.* **239:** 1720–1726.
9. Thompson, W., and G. MacDonald. 1975. Isolation and characterization of cytidine diphosphate-diglyceride from beef liver. *J. Biol. Chem.* **250:** 6779–6785.
10. Thompson, W., and G. MacDonald. 1976. Cytidine diphosphate-diglyceride of bovine brain. *Eur. J. Biochem.* **65:** 107–111.
11. Agranoff, B. W., and W. D. Suomi. 1963. Cytidine diphosphate-DL-dipalmitin. *Biochem. Prep.* **10:** 47–51.
12. Prottey, C., and J. N. Hawthorne. 1967. The biosynthesis of phosphatidic acid and phosphatidylinositol in mammalian pancreas. *Biochem. J.* **105:** 379–392.
13. Eichberg, J., H. M. Shein, M. Schwarz, and G. Hauser. 1973. Stimulation of $^{32}P_i$ incorporation into phosphatidylinositol and phosphatidylglycerol by catecholamines and $\beta$-adrenergic receptor blocking agents in rat pineal organ cultures. *J. Biol. Chem.* **248:** 3615–3622.
14. Hauser, G., and J. Eichberg. 1975. Identification of cytidine diphosphate-diglyceride in the pineal gland of the rat and its accumulation in the presence of DL-propranolol. *J. Biol. Chem.* **250:** 105–112.
15. Raetz, C. H. R., C. B. Hirschberg, W. Dowhan, W. T. Wickner, and E. P. Kennedy. 1972. A membrane-bound pyrophosphatase in *Escherichia coli* catalyzing the hydrolysis of cytidine diphosphate-diglyceride. *J. Biol. Chem.* **247:** 2245–2247.
16. Folch, J., M. B. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
17. Petzold, G. L., and B. W. Agranoff. 1967. The biosyn-

thesis of cytidine diphosphate-diglyceride by embryonic chick brain. *J. Biol. Chem.* **242:** 1187–1191.
18. Bishop, H. H., and K. P. Strickland. 1976. Studies on the formation by rat brain preparations of CDP-diglyceride from CTP and phosphatidic acids of varying fatty acid compositions. *Can. J. Biochem.* **54:** 249–260.
19. Hajra, A. K. 1974. On extraction of acyl and alkyl dihydroxyacetone phosphate from incubation mixtures. *Lipids.* **9:** 502–505.
20. Bjerve, K. S., L. N. W. Daae, and J. Bremer. 1974. The selective loss of lysophospholipids in some commonly used lipid-extraction procedures. *Anal. Biochem.* **58:** 238–245.
21. Freinkel, N., C. El Younsi, and R. M. C. Dawson. 1975. Inter-relations between the phospholipids of rat pancreatic islets during glucose stimulation and their response to medium inositol and tetracaine. *Eur. J. Biochem.* **59:** 245–252.
22. Abdel-Latif, A. A., and J. P. Smith. 1976. Effects of DL-propranolol on the synthesis of glycerolipids by rabbit iris muscle. *Biochem. Pharmacol.* **25:** 1697–1704.
23. Bazán, N. G., M. B. Ilincheta de Boschero, N. M. Giusto, and H. E. Pascual de Bazán. 1976. De novo glycerolipid biosynthesis in the toad and cattle retina. Redirecting of the pathway by propranolol and phentolamine. *Adv. Exp. Med. Biol.* **72:** 139–148.
24. Eichberg, J., J. Killion, G. Guerrant, and G. Hauser. 1978. Modification of phosphatidylinositol metabolism by propranolol and local anesthetics *In* Cyclitols and Phosphoinositides. W. W. Wells and F. Eisenberg, Jr., editors. Academic Press, New York, N.Y. In press.
25. Margraff, W. D. 1976. A biosynthetic method for the preparation of high specific activity. $^{32}$P-labeled phospholipids. *J. Lipid Res.* **17:** 622–628.