Synthesis of Platelet Activating Factor by Cholinephosphotransferase in Developing Fetal Rabbit Lung

Dennis R. Hoffman, Maureen K. Bateman and John M. Johnston*
Departments of Biochemistry and Obstetrics-Gynecology and The Cecil H. & Ida Green Center for Reproductive Biology Sciences, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235

Developing fetal lung is a possible source of the platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) present in amniotic fluid of women in labor. We have assayed the microsomal activities of a specific enzyme for the de novo synthesis of PAF in developing fetal and neonatal rabbit lung, 1-alkyl-2-acetyl-glycerol-dependent dithiothreitol-insensitive cholinephosphotransferase. The specific activity of this enzyme increased from 0.92 to 3.60 nmol × min^{-1} × mg^{-1} protein between day 21 and day 31 of gestation. In contrast, during this same period the activity of the PAF-biosynthetic cholinephosphotransferase in developing rabbit kidney did not change significantly. The specific activity of the diacylglycerol-dependent, dithiothreitol-sensitive cholinephosphotransferase that catalyzes the final step in phosphatidylcholine biosynthesis was not altered during late gestation in either fetal lung or kidney. Previously, increased amounts of pulmonary PAF had been found during the latter stages of gestation (Hoffman, Truong and Johnston (1986) *Biochim. Biophys. Acta 879,* 88–96) and may be attributed to increased activity of the PAF biosynthetic enzymes found in this investigation. This elevated level of PAF in fetal lung may serve to facilitate breakdown of glycogen that provides, in part, the carbon and energy source for surfactant biosynthesis. In addition, PAF may be secreted in association with surfactant into amniotic fluid in which it may interact with amnion tissue and subsequently participate in the events associated with the initiation of parturition.
***Lipids 23,* 96–100 (1988).**

The potent biologically active phospholipid, platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) first was identified with various pathological states such as inflammation, asthma and anaphylaxis (1,2). More recently, it is evident that PAF may have important normal cellular functions. We have proposed two potential physiological roles for PAF in reproductive biology: PAF may initiate glycogenolysis in fetal lung, the products of which provide energy and a carbon source for biosynthesis of the glycerophospholipids of surfactant (3–5), and PAF may be secreted from the fetal lung into amniotic fluid, in which it may interact with amnion to enhance the formation of prostaglandins by activation of the arachidonic acid cascade. Both prostanoids and PAF may facilitate the biochemical events of parturition (5,6).

PAF is only found in significant amounts in the amniotic fluid obtained from women in labor (7) and in part is associated with a lamellar body-enriched fraction containing surfactant. Since lamellar bodies are a primary secretory product of type II pneumonocytes of lung, we considered that fetal lung may be a potential source of the PAF present in amniotic fluid.

*To whom correspondence should be addressed.

Abbreviations: CPTase, cholinephosphotransferase; DTT, dithiothreitol; PAF, platelet activating factor; PC, phosphatidylcholine.

In previous investigations, we have reported a three-fold increase in the concentration of PAF in vivo in developing fetal rabbit lung during the latter stages of gestation (4) and also in rapidly differentiating human fetal lung explants cultured for six days (3). In both of these model systems, a three-fold increase was found in the activity of 2-lysoPAF:acetyl CoA acetyltransferase (3,4), a major regulatory enzyme of the remodeling pathway of PAF biosynthesis (8).

PAF also can be synthesized by 1-alkyl-2-acetyl-*sn*-glycerol:CDP-choline cholinephosphotransferase (CPTase) (9,10). This enzyme catalyzes the last step in the de novo pathway for PAF biosynthesis (11). In this investigation, we have examined the specific activity of this enzyme in developing fetal and neonatal rabbit lung and kidney.

MATERIALS AND METHODS

Chemicals. Cytidine 5′-diphospho[methyl-^{14}C]choline (56 μCi/μmol) and [^{3}H]acetyl CoA (500 mCi/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). Phospholipid standards were from Avanti Polar Lipids (Birmingham, AL). Fatty acid–free fraction V bovine serum albumin, 1-hexadecyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and other general chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Unlabeled 2-lysoPAF was obtained from Calbiochem (La Jolla, CA).

Diradylglycerol preparation. 1-Alkyl-2-acetyl-*sn*-glycerol was synthesized as described by Woodard et al. (10). Briefly, 1-hexadecyl-*sn*-glycerol was chemically acetylated, the *sn*-3 acetate was cleaved by porcine pancreatic lipase treatment. The 1,2-isomer was separated from the 1,3-isomer on boric acid impregnated thin layer chromatography plates developed in chloroform:methanol (98:1.5, v/v). 1,2-Dioleoyl-*sn*-glycerol was purified chromatographically as above.

Tissue preparation. Timed pregnant New Zealand white rabbits (Hickory Hills, Flint, TX) were the source of fetal and adult tissues. Tissues were excised and rapidly homogenized (4 C) in 6:1 (vol/g tissue) with sucrose (0.25 M)/Tris-HCl (10 mM, pH 7.4)/EDTA (1 mM) using a Potter-Elvehjem vessel and a Teflon pestle. The suspension was centrifuged at 600 × g for 10 min to remove unbroken cells, cell debris and nuclei. The supernatant fraction was centrifuged at 18,000 × g for 15 min to obtain a pellet rich in mitochondria and lamellar bodies (12). The remaining supernatant fraction was centrifuged at 105,000 × g for 60 min to obtain the microsomal (pellet) and cytosolic fractions (supernatant). Protein concentrations were quantitated by the Bradford procedure (13).

Cholinephosphotransferase assay. The PAF biosynthetic activity of dithiothreitol (DTT)-insensitive CPTase (EC 2.7.8.16) was determined employing Tris-HCl (100 mM, pH 8.0 at 37 C), DTT (10 mM), EGTA (0.5 mM), 0.1% bovine serum albumin, $MgCl_2$ (10 mM), 1-hexadecyl-2-acetyl-*sn*-glycerol (0.2 mM in ethanol, final

ethanol concentration equals 2.5%), 25 μg microsomal protein and CDP-[methyl-^{14}C]choline (100 μM, 1 μCi/mmol) according to the procedure of Renooij and Snyder (9). Assay of phosphatidylcholine (PC) biosynthetic activity of CPTase (EC 2.7.8.2) was as above except the reaction was at pH 8.5, contained no DTT, and utilized 1,2-dioleoyl-*sn*-glycerol (0.2 mM) as substrate. The incubation was for 10 min at 37 C. PAF and PC were separated by thin layer chromatography on Kieselgel 60 plates (E. Merck, Darmstadt) developed in chloroform:methanol: concentrated ammonium hydroxide (60:35:8, v/v/v). Radioactivity comigrating with authentic PAF and PC added as carrier (20 μg each) was determined, and the values for both CPTase assays were corrected for the incorporation of [^{14}C]choline in the absence of diradylglycerol substrate. The assay was linear to 75 μg microsomal protein and for 20 min.

LysoPAF:acetyl-CoA acetyltransferase assay. The activity of this enzyme was determined using a modification of the procedure described by Wykle et al (8). Briefly, the reaction mixture includes Tris-HCl (30 mM, pH 7.4 at 37 C), DTT (1 mM), $CaCl_2$ (10 μM), 2-lysoPAF (40 μM), 0.025% bovine serum albumin, [^{3}H]acetyl CoA (250 μM, 6.4 μCi/μmol), 50 μg microsomal protein, and was incubated for 15 min at 37 C. PAF was separated and radioactivity determined as described. The assay was linear to 120 μg microsomal protein and for 20 min.

RESULTS

The PAF biosynthetic CPTase reaction is distinguished from the PC biosynthetic activity primarily by the inhibitory action of the sulfhydryl agent, dithiothreitol, on the latter activity (9). As illustrated in Table 1, when PAF biosynthesis was assayed utilizing 1-alkyl-2-acetyl-*sn*-glycerol as substrate the addition of DTT (10 mM) only slightly stimulated (19%) its biosynthesis. In contrast, PC biosynthesis with the same fetal rabbit lung microsomal preparation is inhibited by 95% in the presence of DTT. When alkyl-acetyl-glycerol was the substrate for the CPTase reaction, negligible amounts of [^{14}C]choline from CDP-[^{14}C]choline were incorporated into PC. Similarly, when dioleoyl-glycerol was utilized as substrate, [^{14}C] incorporation into PAF was less than 2% of that incorporated into PC.

The PAF- and PC-biosynthetic CPTase activities primarily were (66–68%) localized in the 105,000 × *g* (microsomal) pellet of adult rabbit lung (Table 2). This subcellular distribution of the DTT-insensitive CPTase is similar to that reported in rat liver and spleen (9). The distribution of CPTase activity nearly was identical to that of acetyltransferase in rabbit lung (Table 2).

Substrate specificity characteristics of the PAF and PC biosynthetic reactions were investigated in adult rabbit lung microsomal preparations. Reaction mixtures at pH 8.0 containing DTT favor PAF biosynthesis, however, inclusion of dioleoyl-glycerol at an equimolar concentration as the alkyl-acetyl-glycerol substrate (0.2 mM) causes a 36% decrease in PAF biosynthesis when compared to that

TABLE 1

Dithiothreitol Sensitivity of PAF and PC Biosynthetic Cholinephosphotransferases in Fetal Rabbit Lung Microsomes

Substrate	Specific activity (nmol × min^{-1} × mg^{-1} protein)	
	−DTT	+10 mM DTT
Alkyl-acetyl-glycerol	5.48 ± 1.08	6.54 ± 0.67
Dioleoyl-glycerol	1.49 ± 0.18	0.07 ± 0.04

Values (±S.E.M.) are from duplicate analysis in at least three separate samples.

TABLE 2

Subcellular Distribution of DTT-insensitive and DTT-sensitive Cholinephosphotransferases (CPTase) and LysoPAF:Acetyl-CoA Acetyltransferase Specific Activities in Adult Rabbit Lung

Enzyme	Subcellular fraction			
	Supernatant (600 × *g*)	Pellet (18,000 × *g*)	Pellet (105,000 × *g*)	Supernatant (105,000 × *g*)
	nmol × min^{-1} × mg^{-1} protein			
DTT-insensitive PAF-CPTase	1.06 ± 0.03	2.82 ± 0.07 (33)	5.83 ± 0.43 (67)	0
DTT-sensitive PC-CPTase	0.77 ± 0.13	1.07 ± 0.06 (34)	2.10 ± 0.07 (66)	0.01 ± 0
Acetyltransferase	0.54 ± 0.10	0.73 ± 0.11 (28)	1.78 ± 0.30 (69)	0.07 ± 0.01 (3)

Values are means (±S.E.M.) from four (CPTase) or three (acetyltransferase) separate experiments.

Values in parentheses are percent of total activity present in each subcellular fraction. Average recovery of the PAF-CPTase, PC-CPTase and acetyltransferase activities were 119, 63 and 98%, respectively.

containing only alkyl-acetyl-glycerol (i.e., 4.07 vs 6.33 nmol × min^{-1} × mg^{-1} protein, Table 3). Similarly, in a corresponding experiment alkyl-acetyl-glycerol inhibited PC biosynthesis by 36% (2.72 to 1.74 nmol × min^{-1} × mg^{-1} protein) in incubation mixtures at pH 8.5 and from which DTT was omitted. In the presence of equimolar concentrations of both substrates, total product formation, (2.13 nmol PAF formed × min^{-1} × mg^{-1} protein plus 1.74 nmol PC formed × min^{-1} × mg^{-1} protein) approaches a rate observed using only alkyl-acetyl-glycerol as substrate (i.e., 4.05 nmol PAF formed × min^{-1} × mg^{-1} protein). Although these results would be consistent with the view that both diradylglycerols are substrates for a single enzyme, the precise mechanism must await purification and characterization of the enzymes involved.

During late gestation in the rabbit (term = 31–32 days), only the activity of the PAF biosynthetic (DTT-insensitive) CPTase of fetal lung increased significantly (Figure 1). In contrast, this enzymatic activity was not altered significantly in fetal kidney. During neonatal development, the PAF biosynthetic activity increased in both lung and kidney to reach the adult level. The PC

TABLE 3

Cholinephosphotransferase Diradylglycerol Substrate Specificity in Adult Rabbit Lung Microsomes

Substrate added (200 μM)		Alkyl-acetyl glycerol	Dioleoyl glycerol	Both
Reaction conditions	Product assayed	nmol × min^{-1} × mg^{-1} protein		
+DTT pH 8.0	PAF	6.33 ± 0.54	0.02 ± 0.01	4.07 ± 0.52
	PC	0.03 ± 0.03	0.02 ± 0.01	0
−DTT pH 8.5	PAF	4.05 ± 0.08	0.09 ± 0.04	2.13 ± 0.35
	PC	0	2.72 ± 0.93	1.74 ± 0.91

Values are the means (± SEM) of three separate experiments.

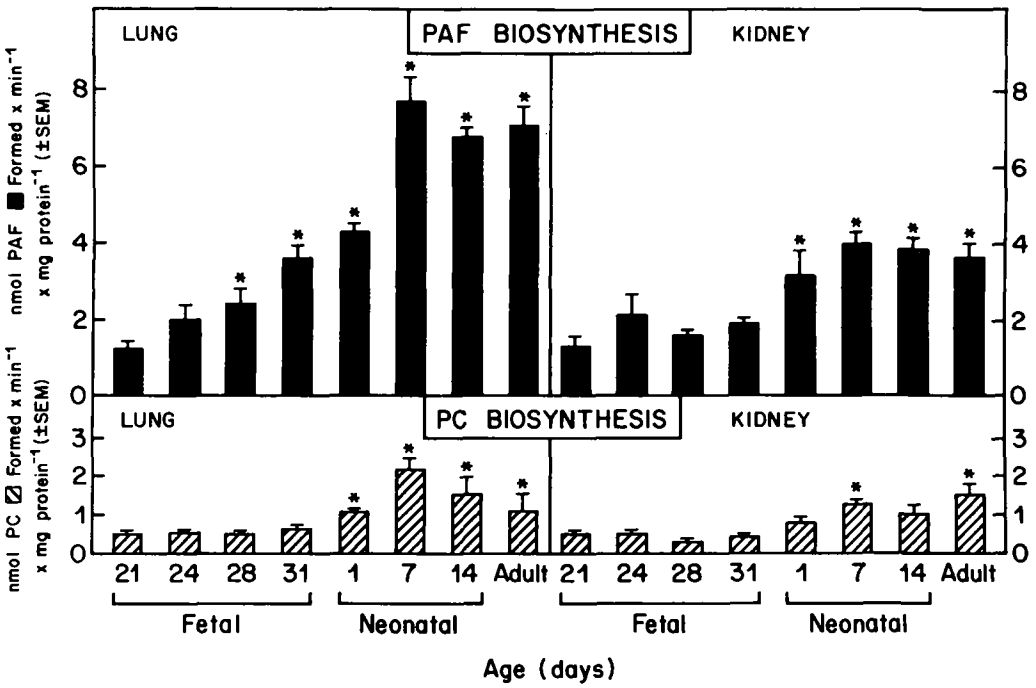

FIG. 1. Specific activity of the PAF (DTT-insensitive) and PC (DTT-sensitive) biosynthetic cholinephosphotransferases in microsomes of fetal, neonatal and adult rabbit lung and kidney. Biosynthetic activities were determined as described in Materials and Methods. Bars represent the mean ± SEM for a minimum of three separate experiments. * Indicates a significant difference ($p > 0.01$) from the day 21 value as determined by Student's t-test.

biosynthetic (DTT-sensitive) activity of CPTase was not altered during gestational development in the lung or kidney. In both neonatal lung and kidney, the PC-biosynthetic CPTase was elevated over the fetal level. In kidney, an adult level of enzymatic activity was reached and maintained; however, in neonatal lung, a peak of activity is reached at seven days followed by a return to an adult level comparable to that in the fetal lung.

We previously had found (4) that the microsomal 2-lysoPAF:acetyl CoA acetyltransferase specific activity in fetal rabbit lung microsomes increased significantly between days 21 and 24 of gestation (from 116 to 392 pmol PAF formed $\times$ min^{-1} $\times$ mg^{-1} protein) and remained elevated through day 31. The acetyltransferase activity in adult rabbit lung microsomes was further increased to 1780 $\pm$ 300 (S.E.M.) pmol $\times$ min^{-1} $\times$ mg^{-1} protein. In kidney (4), the enzyme activity also increased during the final 10 days of fetal development (from 68 to 187 pmol $\times$ min^{-1} $\times$ mg^{-1} protein). The adult rabbit kidney acetyltransferase (305 $\pm$ 50 [S.E.M.] pmol $\times$ min^{-1} $\times$ mg^{-1} protein) was elevated over fetal activities; however, neither the fetal nor adult rabbit kidney acetyltransferase activities reach the level of that in the lung at the corresponding age of development.

DISCUSSION

The insensitivity of the PAF biosynthetic activity of CPTase to DTT (Table 1) and localization in the 105,000 $\times$ *g* pellet (microsomal) fraction in rabbit lung (Table 2) are indicative of the presence of a similar enzymatic activity in this tissue to that described in adult rat kidney (9). The PC biosynthetic activity of CPTase in lung markedly was inhibited by DTT (Table 1) and each substrate, alkyl-acetyl-glycerol and diacyl-glycerol, influenced the ability of CPTase to utilize the other substrate (Table 3). The PAF and PC biosynthetic activities of cholinephosphotransferase(s) are thought to be two distinct enzymes (10). However, a distinction between the presence of one or two enzymes in lung tissue must await purification of the enzyme and further evaluation of the physical properties of the substrates utilized.

In this investigation, a stepwise increase in the activity of alkyl-acetyl-glycerol-dependent PAF-biosynthetic CPTase was found in fetal rabbit lung microsomes. This developmental pattern of the de novo PAF biosynthetic pathway is consistent with that found previously for a major regulatory enzyme of the remodeling pathway, lysoPAF:acetyl CoA acetyltransferase (4). However, the specific activity of the PAF biosynthetic CPTase (Figure 1) was found to be approximately 10-fold higher than the acetyltransferase activity in fetal lung and kidney and thus may contribute more significantly to the total pool of PAF in these tissues. A similar relationship between the activities of these two enzymes has been reported in adult rat kidney (10). Lipid substrate availability, however, may limit the in vivo activity of the PAF-biosynthetic CPTase as this substrate, alkyl-acetyl-glycerol, is derived from numerous enzymatic steps of the de novo ether lipid pathway. In contrast, we have quantitated the acetyltransferase substrate, lysoPAF, in the developing fetal lung and found it in great excess as compared with PAF concentrations (3,4).

The PC biosynthetic specific activity of CPTase did not change significantly between day 21 and day 31 in fetal rabbit lung (Figure 1) and confirms our earlier observations (14) of the ontogenic development of this enzymatic activity. Although it originally was suggested that CPTase was one of the primary enzymes involved in regulation of surfactant PC biosynthesis during fetal development (15,16), in rabbit it appears not to be the case (14,17–19). The PAF biosynthetic specific activity of CPTase was three- to six-fold higher than the PC biosynthetic activity throughout fetal lung maturation (Figure 1). This same relationship of enzymatic activity was observed in adult rat kidney (10) and may reflect a preferential synthesis of PAF under certain physiological circumstances. However, this paradox of higher PAF biosynthetic capacity compared to PC biosynthesis also may be attributable to the substrate availability in the developing fetal lung.

The activities of the PAF biosynthetic CPTase (Figure 1) and acetyltransferase (4) both increase at a time (starting at day 24 of gestation) when the epithelial lining of fetal rabbit lung rapidly is differentiating into type II pneumonocytes (20). It is well-established from both morphological and biochemical studies that during differentiation of type II pneumonocytes, the concentration of glycogen rapidly decreases (21–23) in association with increased synthesis of the glycerophospholipids unique to the surfactant fraction of these cells (23–26). We have demonstrated that increased concentrations of PAF also are found in fetal lung during this time period (3,4), possibly reflecting the increase in the activities of CPTase and acetyltransferase. We have proposed (3–5) and recently obtained experimental evidence (Hoffman and Johnston, unpublished data) that PAF may stimulate glycogenolysis in fetal lung in a manner similar to that originally reported by Shukla et al. (27) in isolated, perfused rat liver. Thus, PAF initiates or potentiates the breakdown of glycogen that may supply in part the energy and carbon moieties necessary for synthesis of surfactant glycerophospholipids. Other investigators have suggested that this is the case in fetal lung during the latter stages of gestation (24,25) because it receives only a small fraction of the cardiac output and primarily will utilize glycogen for surfactant biosynthesis rather than circulating blood glucose (25,28). The increased amounts of PAF in fetal lung also may become associated with surfactant and be secreted out into the amniotic fluid during late gestation just prior to parturition. This increased level of PAF in amniotic fluid is found in women at term and in labor (7). Thus, the presence of PAF in amniotic fluid only at the time of parturition is indicative of its role in the initiation of this process (6,29). The PAF that is present in amniotic fluid may interact with amnion tissue to stimulate prostaglandin synthesis (30), which in turn may participate in the events of parturition. PAF also directly can elicit the contraction of rat (31) and human (32) myometrium. Thus, it appears that the PAF synthesized in fetal lung, the last major fetal organ system to develop, also may be involved in the events of labor.

ACKNOWLEDGEMENTS

This work was supported in part by USPHS grant 5-PO1-HD13912 and The Robert A. Welch Foundation, Houston, TX. D. R. Hoffman

is a recipient of a fellowship from the Chilton Foundation. The authors are indebted to Dolly Tutton for editorial assistance in the preparation of this manuscript.

REFERENCES

1. Hanahan, D.J. (1986) *Annu. Rev. Biochem. 55,* 483-509.
2. Lee, T-c., and Snyder, F. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J.F., ed.) Vol. 2, pp. 1-39, CRC Press, Boca Raton, FL.
3. Hoffman, D.R., Truong, C.T., and Johnston, J.M. (1986) *Am. J. Obstet. Gynecol. 155,* 70-75.
4. Hoffman, D.R., Truong, C.T., and Johnston, J.M. (1986) *Biochim. Biophys. Acta 879,* 88-96.
5. Hoffman, D.R., and Johnston, J.M. (1987) in *New Horizons in Platelet Activating Factor Research* (Winslow, C.M., and Lee, M.L., eds.) pp. 51-55, John Wiley and Son, New York, NY.
6. Johnston, J.M., Bleasdale, J.E., and Hoffman, D.R. (1987) in *Platelet-Activating Factor and Related Lipid Mediators* (Snyder, F., ed.) pp. 375-402, Plennum Press, New York, NY.
7. Billah, M.M., and Johnston, J.M. (1983) *Biochem. Biophys. Res. Commun. 113,* 51-58.
8. Wykle, R.L., Malone, B., and Snyder, F. (1980) *J. Biol. Chem. 255,* 10256-10260.
9. Renooij, W., and Snyder, F. (1981) *Biochim. Biophys. Acta 663,* 545-556.
10. Woodard, D.S., Lee, T-c., and Snyder, F. (1987) *J. Biol. Chem. 262,* 2520-2527.
11. Lee, T-c., Malone, B., and Snyder, F. (1986) *J. Biol. Chem. 261,* 5373-5377.
12. Okazaki, T., Johnston, J.M., and Snyder, J.M. (1982) *Biochim. Biophys. Acta 712,* 283-291.
13. Bradford, M.M. (1976) *Anal. Biochem. 72,* 248-254.
14. Schultz, F.M., Jimenez, J.M., MacDonald, P.C., and Johnston, J.M. (1974) *Gynecol. Invest. 5,* 222-229.
15. Farrell, P.M., Lundgren, D.W., and Adams, A.J. (1974) *Biochem. Biophys. Res. Commun. 57,* 696-701.
16. Oldenborg, V., and van Golde, L.M.G. (1976) *Biochim. Biophys. Acta 441,* 433-442.
17. Tsao, F.H.C., and Zachman, R.D. (1977) *Pediatr. Res. 11,* 858-861.
18. Heath, M.F., and Jacobson, W. (1984) *Pediatr. Res. 18,* 395-401.
19. Gluck, L., Sribney, M., and Kulovich, M.V. (1967) *Pediatr. Res. 1,* 247-265.
20. Snyder, J.M., Mendelson, C.R., and Johnston, J.M. (1985) in *Pulmonary Development: Transition from Intrauterine to Extrauterine Life* (Nelson, G.H., ed.), pp. 19-46, Marcel Dekker, New York, NY.
21. Kikkawa, Y. (1975) in *Pulmonary Physiology of the Fetus, Newborn and Child* (Scarpelli, E., ed.), pp. 37-60, Lea and Febiger, Philadelphia, PA.
22. Shelley, H.J. (1961) *Br. Med. Bull. 17,* 137-143.
23. Snyder, J.M., Johnston, J.M., and Mendelson, C.R. (1981) *Cell Tissue Res. 220,* 17-25.
24. Maniscalco, W.M., Wilson, C.M., Gross, I., Gobran, L., Rooney, S.A., and Warshaw, J.B. (1978) *Biochim. Biophys. Acta 530,* 333-346.
25. Bourbon, J.R., Rieutort, M., Engle, M.J., and Farrell, P.M. (1982) *Biochim. Biophys. Acta 712,* 382-389.
26. van Golde, L.M.G. (1976) *Am. Rev. Respir. Dis. 114,* 977-1000.
27. Shukla, S.D., Buxton, D.B., Olson, M.S., and Hanahan, D.J. (1983) *J. Biol. Chem. 258,* 10212-10214.
28. Maniscalco, W.M., Finkelstein, J.N., and Parkhurst, A.B. (1982) *Biochim. Biophys. Acta 11,* 49-58.
29. Bleasdale, J.E., and Johnston, J.M. (1984) *Rev. Perinatal Med. 5,* 151-191.
30. Billah, M.M., Di Renzo, G.C., Ban, C., Truong, C.T., Hoffman, D.R., Anceschi, M.M., Bleasdale, J.E., and Johnston, J.M. (1985) *Prostaglandins 30,* 841-850.
31. Nishihira, J., Ishibashi, T., Imai, Y., and Muramatsu, T. (1984) *Lipids 19,* 907-910.
32. Tetta, C., Montrucchio, G., Alloatti, G., Roffinello, C., Emanuelli, G., Benedetto, C., Camussi, G., and Massobrio, M. (1986) *Proc. Soc. Exptl. Biol. Med. 183,* 376-381.

[Received July 10, 1987; Revision accepted September 9, 1987]