Pediatr. Res. 17: 362–367 (1983)

Identification and Partial Purification of Phenylethanolamine N-Methyl Transferase in the Developing Ovine Lung

JAMES F. PADBURY,[27] ROBERT W. LAM, CALVIN J. HOBEL, AND DELBERT A. FISHER

Department of Pediatrics, Harbor-UCLA Medical Center, UCLA School of Medicine, Torrance, California, USA

Summary

Phenylethanolamine N-methyl transferase (PNMT), the terminal enzyme in epinephrine biosynthesis, was identified in fetal and newborn ovine lung. The ovine lung PNMT demonstrated the appropriate substrate specificity and affinity (Km, 9×10^{-6} M). Although some homology between adrenal and lung PNMT was observed on polyacrylamide gel electrophoresis, the lung PNMT differed in its migration on ion exchange chromatography and was not inhibited by pharmacologic inhibitors active against the adrenal enzyme. Activity increased from a mean of 132 pmole/(mg protein•h) $\times 10^{-3}$ at 0 days gestation to 326 pmole/mg protein • h) $\times 10^{-3}$ in newborn animals between 1–4 days of age ($r = 0.571$, $P < 0.05$). The levels of N-methylating activity in extraadrenal tissues were relatively low; lesser but significant N-methylating activities were demonstrated in brown adipose tissue and myometrial tissues. Adrenal gland activity was 1000-fold greater than lung activity expressed on a per mg protein basis and 250-fold greater expressed per mg wet weight.

Abbreviations

DEAE, diethylaminoethyl cellulose
DSPC, disaturated phosphatidylcholine
DTE, dithioerythritol
PNMT, phenylethanolamine N-methyl transferase
SDS, sodium dodecyl sulfate

Phenylethanolamine N-methyl transferase (EC2.1.28) is the last enzyme involved in epinephrine biosynthesis (2), and until recently was thought to be restricted to the adrenal medulla. Pendleton *et al.* (17), however, demonstrated low levels of extraadrenal PNMT activity in adult rat lung, brain stem, spleen, heart, and skeletal muscle. The lung contained more activity than the brain stem or any of the other extraadrenal organs. Subsequent studies by these investigators demonstrated identifiable PNMT in lungs of adult rats, dogs, and Rhesus monkeys (18); human and rabbit lungs, however, contained a nonspecific N-methyltransferase (18). Recently we have reported significant activity in uterine tissue of pregnant humans and sheep (8, 10).

Adrenergic mechanisms are known to be important in the synthesis (11) and/or release of surfactant (1, 4, 5, 23) and possibly in the regulation of fetal lung water metabolism (12, 22). The nature of the precise regulatory control, be it humoral or neural, is unclear. Epinephrine produced locally in the lung might play a significant role in regulation of these processes. To confirm and extend the observations of Pendleton *et al.* (17–20) into the developmental period, we undertook studies to identify and characterize PNMT in the fetal and newborn ovine lung.

MATERIALS AND METHODS

Enzyme assay. PNMT activity was first detected in crude lung homogenates by a modification of the methods of Axelrod (2) and Parvez (16). This assay is based on enzymatic transfer of a tritiated methyl group to phenylethanolamine followed by solvent extraction of [^{3}H]N-methylphenylethanolamine and counting in a liquid scintillation counter. Whole lung trimmed of airways and excess connective tissue was rinsed, homogenized at high speed with a Tekmar Tissuemizer in 10 volumes 0.25 M sucrose, and centrifuged at 40,000 × *g* at 4°C for 30 min. The resultant supernatant was assayed in a 300 μl reaction volume containing a final 1.67 mM substrate concentration in 0.11 M phosphate buffer, pH 7.9 with 0.5 μCi [^{3}H]S adenosylmethionine per tube. Activity was expressed as picomoles product formed per mg protein per hour or per mg wet lung weight per hour. Protein was measured by the method of Lowry *et al.* (14) using bovine serum albumin as a standard. Activity was linear with respect to time and protein concentration.

PNMT purification. The observed N-methylating activity was purified using modifications of techniques described by several authors (13, 21, 24). Briefly, washed, minced whole lung devoid of bronchi or excess connective tissue was homogenized in 10 volumes of iced, isotonic KCl with a Tekmar tissuemizer for 30 sec. A fat free supertant fraction was recovered by centrifugation of the homogenate at 40,000 × *g* at 4°C for 1 h. The supernatant was adjusted to pH 5.0 with 1 N acetic acid, followed by centrifugation at 40,000 × *g* for 30 min to remove the precipitate. Ammonium sulfate was added slowly to 40% saturation and the precipitate removed by centrifugation and discarded. Subsequently the precipitate developed after addition of ammonium sulfate to 55% was recovered and dissolved in phosphate buffer (0.01 M, pH 6.8) plus 0.1 mM DTE and applied to a 2.5 × 80 cm Sephadex G100 column equilibrated with the same buffer. Five to ten ml fractions were collected at a flow rate of approximately 50 ml/h. The fractions with activity were pooled and dialyzed overnight against phosphate buffer (0.001 M, pH 7.2) plus 0.1 mM DTE. The dialyzed protein was applied to a DEAE cellulose column, 2.5 × 15 cm, which had been equilibrated with the dialysis buffer. After washing, the enzyme was eluted with a linear salt gradient up to 0.1 M phosphate, pH 7.2 with 0.1 mM DTE. The fractions containing activity were pooled and lyophilized. Adrenal PNMT was prepared using a similar purification scheme from whole adrenal glands.

The lyophilized preparations were applied to 10% SDS polyacrylamide slab gels with an acrylamide: bis-acrylamide ration 39.9:1. After development overnight at 10 mA, the gel was stained with Commassie blue and destained.

Enzyme characterization. The apparent Km values for fetal adrenal and fetal lung PNMT enzyme activities measured after the above purification scheme were determined for phenylethanolamine substrate at concentrations of 0.125–10 mM. Reaction velocity was normalized for protein concentration. To determine the substrate specificity of this N-methylating activity, a variety of other substrates were tested under identical assay conditions at equimolar concentrations. Activity was expressed relative to that observed using phenylethanolamine.

The PNMT selective inhibitors SKF 64139 (20) and SKF 29661

(19) were tested for their ability to inhibit purified fetal lung and adrenal PNMT under standard assay conditions. Inhibitors were tested at 10 μM and 100 μM final concentration.

Ontogenetic studies. A developmental profile of this enzyme activity was constructed from fresh tissues available from fetuses and newborns of western, mixed breed sheep. There were 13 fetal sheep from 100–140 days gestation and seven newborn sheep between 1–5 days of age available for study. For fetal studies all animals were delivered by cesarian section using spinal anesthesia. Fetuses were sacrificed immediately by pentobarbital injection and organ specimens were homogenized and frozen at −20°C for later assay as described.

Crude homogenates of brown fat and myometrium were prepared and assayed as described above for whole adrenal and lung homogenates. To demonstrate relative tissue distribution, activity was expressed either on a per milligram protein basis or a per milligram tissue wet weight basis.

RESULTS

After the observation of some *N*-methylating activity in lung homogenates, it was considered worthwhile to further characterize this activity and to study the developmental profile. The elution profiles of lung and adrenal PNMT were identical on Sephadex G100 chromatography both yielding a single peak at the same elution volume. As seen in Figure 1, the elution profiles on cellulose ion-exchange chromatography were dissimilar. In this representative experiment, the fetal adrenal preparation displayed two major peaks and the lung two peaks. In some paired experiments, the adrenal preparation demonstrated two peaks whereas the lung preparation displayed only a single peak of activity. It is possible that this is due to copurification of variable amounts of PNMT from circulating red blood cells, which have been suggested to contain measurable activity (10). Specific activity of lung PNMT on a per mg protein basis was enriched at least 250-fold in several experiments using this purification scheme.

Polyacrylamide gel electrophoresis of purified fetal lung enzyme, fetal adrenal enzyme, and adult lung enzyme submitted to the same purification scheme are shown in Figure 2. The outside lanes contain molecular weight standards. Fetal adrenal enzyme from the first major peak on DEAE cellulose chromatography and the second major peak were run separately and designated Peaks 1 and 2. Fetal lung can be seen to have a band corresponding to the major band in the adrenal preparations. A similar band is seen in this preparation of adult lung subjected to the same purification scheme. Clearly, the lung preparations were only partially purified. Each of the individual bands were not tested for activity.

Enzyme characterization. Figure 3 demonstrates the double reciprocal plot for fetal lung and adrenal PNMT activities with Km values for phenylethanolamine of approximately 9.9×10^{-6} M and 4.2×10^{-6} M, respectively. In several experiments with lung PNMT the Km ranged from $4\text{–}10 \times 10^{-6}$ M. These values are similar to those observed for the fetal adrenal enzyme.

Substrate specificity is an important criterion for identification of *N*-methyl transferase activity (2). Adrenal enzymes demonstrate high activity with phenylethanolamine and its derivatives such as normethanephrine or norepinephrine, whereas poor activity is

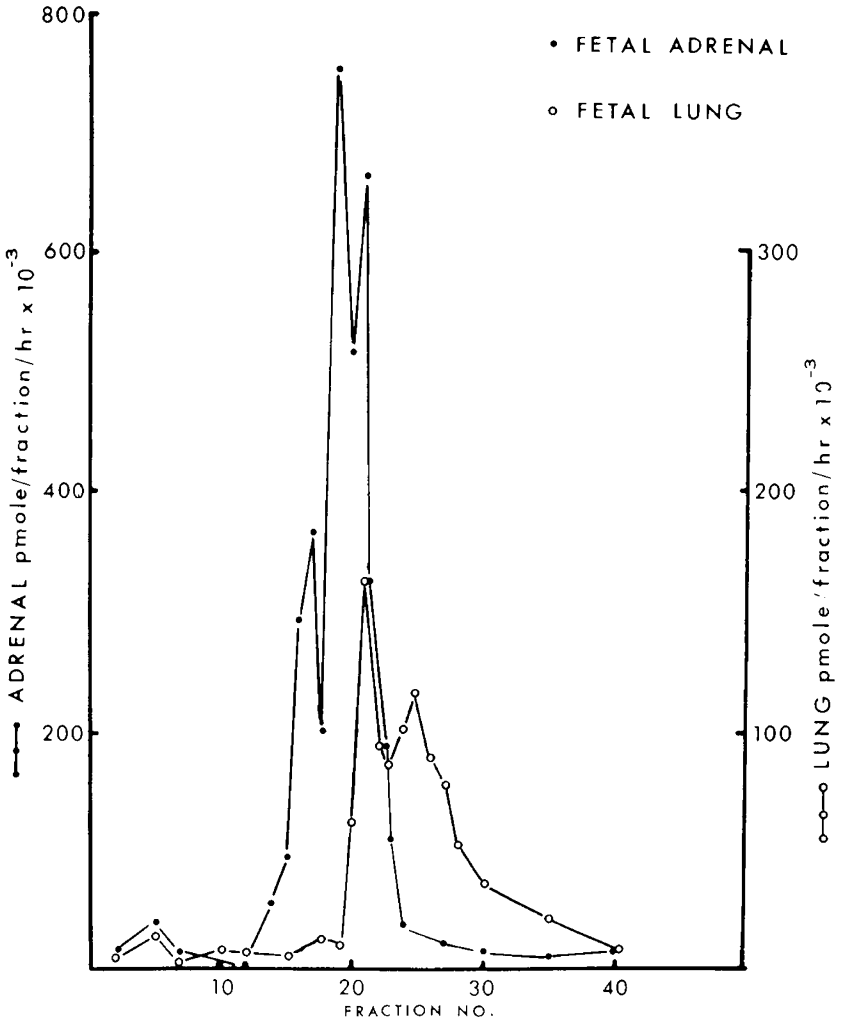

Fig. 1. Elution profile of fetal lung and adrenal phenylethanolamine *N*-methyl transferase activity on DEAE cellulose. 8.5 ml fractions were collected and 100 microliter aliquots assayed as described in "Materials and Methods." Results are expressed as pmoles product formed per fraction per hour $\times 10^{-3}$.

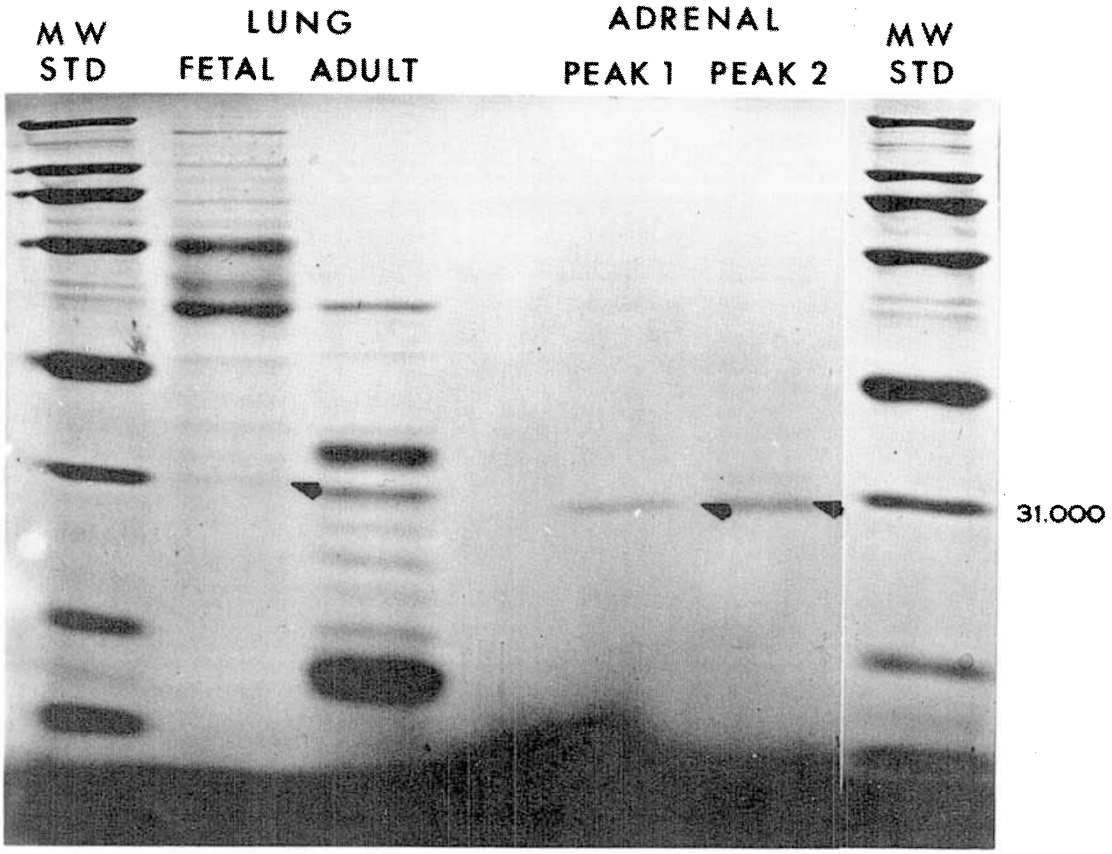

Fig. 2. Polyacrylamide gel electrophoresis of partially purified lung and adrenal phenylethanolamine N-methyl transferase. The 10% polyacrylamide gel was developed overnight at 10 mA and then stained with Commassie blue. Molecular weight standard of 31,000 is shown.

observed using phenylethylamine or its derivatives. As can be seen in Table 1, when relative activity for phenylethanolamine is held constant at 100%, phenylethylamine is a poor substrate with a relative activity of 28%; normethanephrine is a better substrate showing a relative activity of 50%.

The different enzyme preparations were tested using specific pharmacologic inhibitors of PNMT. Inhibitor SKF 64139 is a sensitive, specific *in vivo* inhibitor of both rat brain stem and adrenal PNMT, whereas SKF 29661 inhibits adrenal PNMT *in vivo*, but not the brain stem enzyme. Both compounds inhibit rat brain stem and adrenal PNMT *in vitro*. As can be seen in Table 2, substantial inhibition of adrenal PNMT was observed with SKF 64139 at 10 μM concentration. SKF 29661 was less effective but significant inhibition was observed at a final concentration of 100 μM. In contrast, lung PNMT was not significantly inhibited by either SKF 64139 or SKF 29661.

Ontogenetic studies. Results for the fetal and neonatal lung PNMT activities are shown in Figure 4. Enzyme activity was expressed as pmole/(mg protein · h) $\times 10^{-3}$. Activity increased from a mean of 132 pmole/(mg protein · h) $\times 10^{-3}$ at 100 days gestation to 326 pmole/(mg protein · h) $\times 10^{-3}$ in the newborn animals 1–4 days of age. This increase with advancing age was significant by regression analysis ($r = 0.571$, $P < 0.05$). When fetal samples alone were compared by regression analysis, the result was no longer significant ($r = 0.383$, $P > 0.1$). The pooled mean fetal values, $177 \pm 25 \times 10^{-3}$ pmole/(mg protein · h) were significantly less than the pooled newborn values, $324 \pm 31 \times 10^{-3}$ pmole/(mg protein · h) ($P < 0.001$). There were no animals studied just before delivery after spontaneous labor; however, lung PNMT activity, assayed in a single 129-day-old fetus just before delivery after ACTH-induced parturition (9), was 404 pmole/(mg protein · h) $\times 10^{-3}$. This value is at the upper limit of those observed in newborn animals.

The relative of *N*-methylating activities from adrenal, lung, brown fat, and myometrial tissues are shown in Figure 5. The bar graph demonstrates that the ovine fetal adrenal gland contains 1000-fold greater activity than fetal lung on a per mg protein basis but only 250-fold greater activity expressed per mg wet weight of tissue. These data are for 140-day gestation animals, but similar relative activities were observed at earlier gestations. Characterization of this *N*-methylating activity in brown fat has not been carried out. Partial characterization of the myometrial activity has been reported (8).

DISCUSSION

We have demonstrated a phenylethanolamine *N*-methyl transferase activity in ovine fetal and neonatal lung. This lung PNMT has many characteristics of adrenal PNMT including high substrate affinity and specificity. This enzyme displays different behavior than the adrenal enzyme on ion-exchange chromatography and is not inhibited by pharmacologic inhibitors active against adrenal enzyme. The developmental profile of this enzyme activity suggests a gradual but significant increase in activity developmental age. The pooled fetal values were significantly less than the newborn values. This suggests the possibility that the apparent developmental increase is in fact a reflection of the profound physiologic and hormonal changes occurring in the transition between fetal and neonatal life. In a single observation of a fetus delivered after ACTH-induced premature parturition (9), we observed a relatively high activity of lung PNMT. This single observation suggests the possibility that endogenous cortisol production might augment lung PNMT as has been observed for adrenal PNMT activity (3). It is also possible, but less likely, that ACTH had a direct effect. Further study is warranted.

Using pharmacologic criteria, Pendleton *et al.* (18) have char-

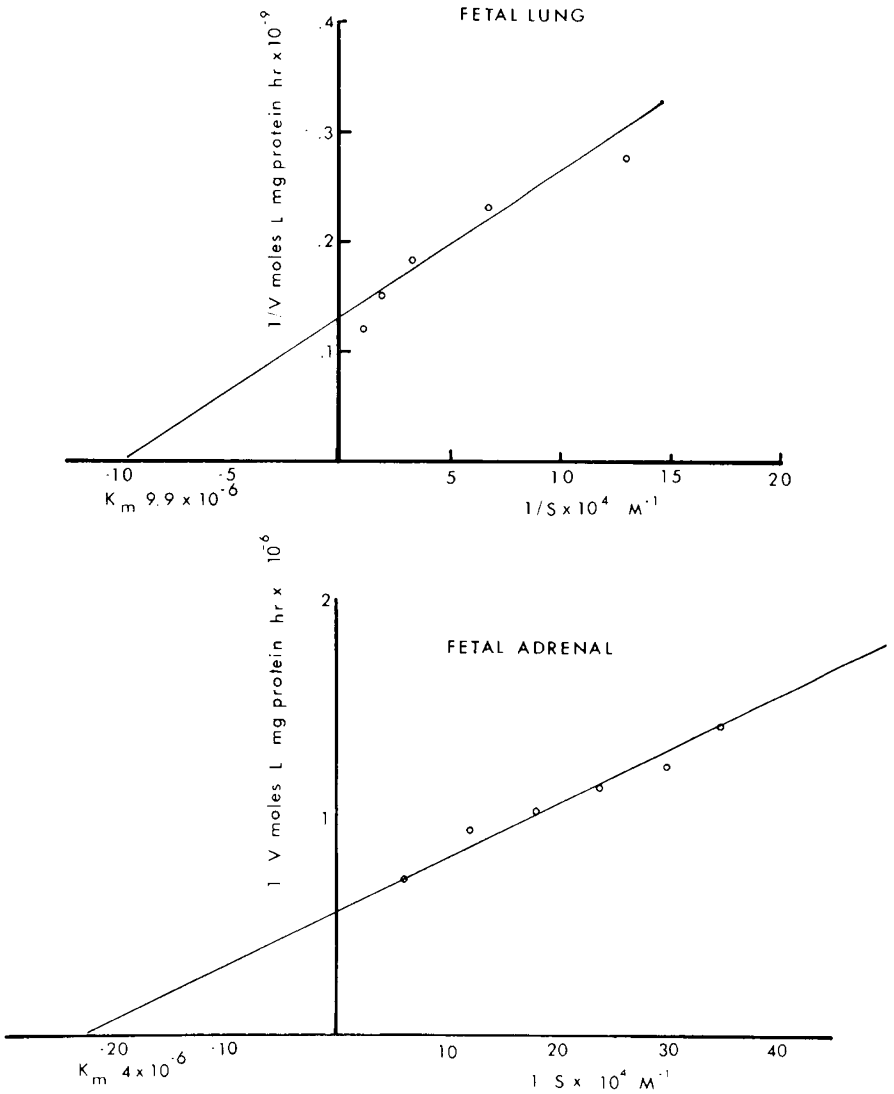

Fig. 3. Lineweaver-Burke plots for fetal lung (*upper panel*) and fetal adrenal (*lower panel*) phenylethanolamine *N*-methyl transferase. Assay as described in "Materials and Methods."

Table 1. *Fetal lung phenylethanolamine N-methyl transferase-substrate specificity*[1]

Substrate	Relative activity (x ± S.E.)
Phenylethanolamine	100%
Normetanephrine	50.2 ± 5.3%
Phenylethylamine	27.8 ± 10.4%

[1] Results represent the mean ± S.E. for three to six experiments using equimolar concentrations of substrate as described in "materials and methods"

Table 2. *Inhibition of ovine lung and adrenal phenylethanolamine N-methyl transferase (PNMT) by pharmacologic inhibitors SKF 64139 and SKF 29661*[1]

	SKF 64139[2]	SKF 29661[2]	SKF 29661[3]
Lung PNMT	83.5 ± 20.6%	94.2 ± 9.4%	95.1 ± 11.8%
Adrenal PNMT	9.1 ± 2.3%	71.5 ± 15.5%	31.0 ± 3.9%

[1] Assays as described in "Materials and Methods." Results expressed as % maximum activity observed in individual assays. Results are mean ± S.E. from three to six experiments.
[2] Final concentration inhibitor 10 μM.
[3] Final concentration inhibitor 100 μM.

acterized the lung *N*-methyltransferase activity of rats, dogs, Rhesus monkeys, humans, and rabbits. Adult rat, dog, and monkey lungs were shown to contain an *N*-methyltransferase with a high degree of selectivity for phenylethanolamine over phenylethylamine. These enzymes were likewise inhibited by SKF 64139, a demonstrated competitive inhibitor of adrenal PNMT. Humans and rabbit lungs, by contrast, contained a nonspecific *N*-methyltransferase with equivalent affinity for either phenylethanolamine or phenylethylamine; this activity was not inhibited by SKF 64139. The *N*-methyltransferase activity observed in our studies demonstrates significant substrate specificity but is not inhibited by pharmacologic inhibitors active against the adrenal enzyme; thus, our data differ from the activities reported by Pendleton *et al.* in several other species.

The precise role for local production of epinephrine in the lung is speculative. Adrenergic mechanisms are important in regulation of lung metabolism. Deflation stability studies of fetal rabbit lungs performed 3 h after direct fetal injection of isoxsuprine, a potent beta-sympatomimetic agent, showed significantly greater air retention in drug-treated than in control lungs (23). In similar studies, beta-agonists have increased surface activity and phospholipid concentrations in tracheal washes (1, 5). Concomitant decreases in concentration of lamellar bodies in type II pneumo-

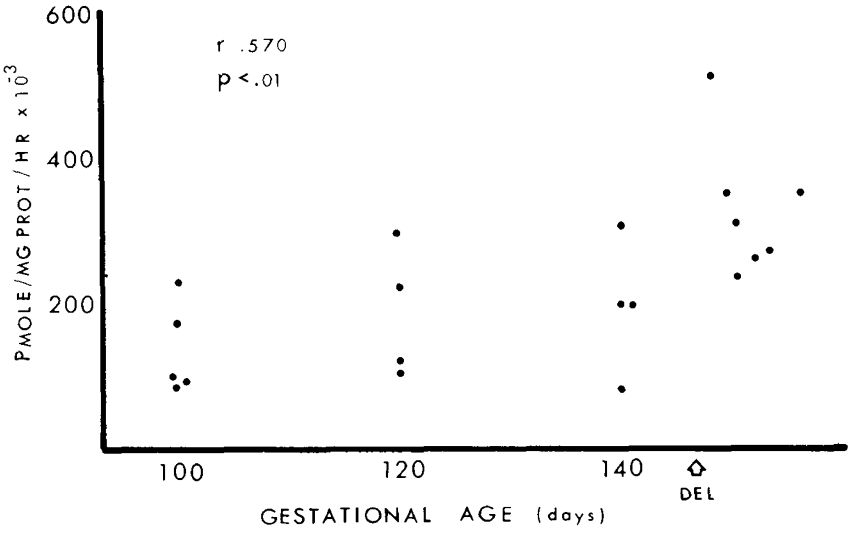

Fig. 4. Ontogenetic profile of ovine fetal and neonatal lung phenylethanolamine *N*-methyl transferase. Term in this species is 145 days. Regression analysis of activity *versus* developmental age showed a significant increase ($r = 0.570$, $P < 0.01$).

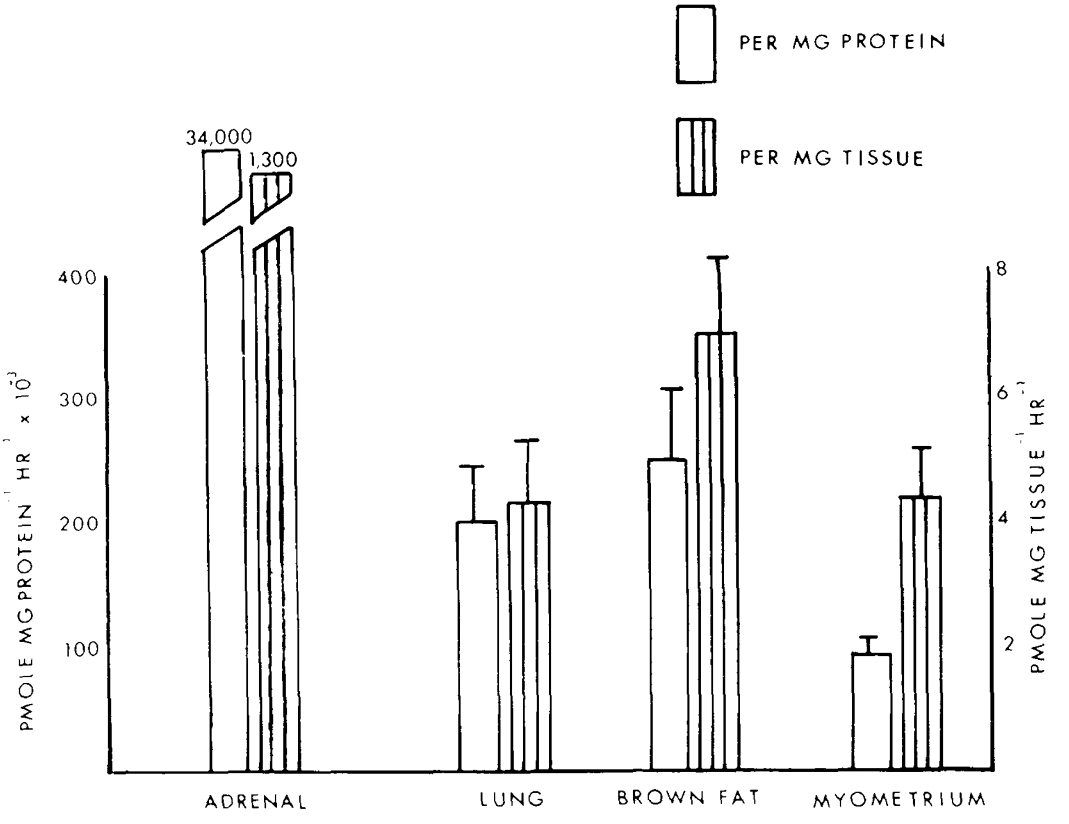

Fig. 5. Tissue distribution of ovine fetal phenylethanolamine *N*-methyl transferase at 140 days gestation. Activity is expressed as picomoles per milligram protein per hour × 10^{-3} or picomoles per milligram wet weight of tissue per hour. Myometrial tissues were obtained from the pregnant uterine horn at the time of cesarian delivery. Assay as described in "Materials and Methods."

cytes were seen but these effects were blocked by propranolol. Similar effects were induced by muscarinic agonists and blocked by phenoxybenzamine or propranolol (1, 4). *In vivo* studies have confirmed these observations: epinephrine infusion into the chronically catheterized fetal sheep results in increased surfactant flux into tracheal fluid and a decrease in the rate of tracheal fluid production (12, 22). Beta-adrenergic agonists increased the rate of choline incorporation into DSPC, the major phospholipid in surfactant, and increased the total lung DSPC, suggesting an effect on surfactant synthesis (11). Beta-adrenergic receptors have been identified in whole lung homogenates (7), lung explants (15), and cultured embryonic lung cells (6). Surfactant synthesis, processing and release seem to be increased after activation of beta-adrenoreceptor receptors on type II pneumocytes.

In summary, we have identified in ovine fetal lung a PNMT, which is similar in affinity, substrate characteristics and apparent molecular weight to the adrenal enzyme. This enzyme activity, however, differs from adrenal PNMT by its migration on ion exchange chromatography, and it is not inhibited by pharmacologic inhibitors active against the adrenal enzyme. The local production of epinephrine by this enzyme in lung tissue may be important in regulation of lung surfactant and water metabolism.

REFERENCES AND NOTES

1. Abdellif, M. M. and Hollingsworth, M.: Effect of oxotremorine and epinephrine on lung surfactant secretion in neonatal rabbits. Pediatr. Res., *14:* 916 (1980).
2. Axelrod, J.: Purification and properties of phenylethanolamine-N-methyl transferase. J. Biol. Chem., *237:* 1657 (1962).
3. Bohn, M. C., Goldstein, M., and Black, I. B.: Role of glucocorticoids in expression of the adrenergic phenotype in rat embryonic adrenal gland. Dev. Biol., *82:* 1 (1981).
4. Corbet, A. J. S., Flax, P., and Rudolph, A. J.: Role of autonomic nervous system controlling surface tension in fetal rabbit lungs. J. Appl. Physiol., *43:* 1039 (1977).
5. Enhorning, G., Chamberlain, D., Contreras, C., Burgoyne, R., and Robertson, B.: Isoxsuprine-induced release of pulmonary surfactant in the rabbit fetus. Am. J. Obstet. Gynecol., *129:* 197 (1977).
6. Fraser, C. M. and Venter, J. C.: The synthesis of β-adrenergic receptors in cultured human lung cells: Induction by glucocorticoids. Biochem. Biophys. Res. Commun. *94:* 390 (1980).
7. Giannopoulos, G.: Identification and ontogeny of β-adrenergic receptors in fetal rabbit lung. Biochem. Biophys. Res. Commun. *95:* 388 (1980).
8. Hobel, C. J., Padbury, J. F., Lam, R. W., and Marshall, C. L.: Identification of phenylethanolamine-N-methyl transferase (PNMT) in the ovine myometrium and endometrium. Its potential significance. Proc. Soc. Gynecol. Invest. (Abstract), *29:* 160 (1982).
9. Hobel, C. J., Padbury, J. F., Nathanielsz, P. W., Jansen, C. A. M., and Lam, R. W.: The effect of ACTH (1-24) on fetal ovine catecholamines (CATS) and placental β-adrenergic receptors. Proc. Soc. Gynecol. Invest. (Abstract), *29:* 50 (1982).
10. Hobel, C. J., Parvez, H., Parvez, S., Livett, M., and Papiernik, E.: Enzymes for epinephrine synthesis and metabolism in the myometrium, endometrium, red blood cells and plasma of pregnant human subjects. Am. J. Obstet. Gynecol., *141:* 1009 (1981).
11. Kanjanapone, V., Hartig-Beecken, I., and Epstein, M. F.: Effect of isoxsuprine on fetal lung surfactant in rabbits. Pediatr. Res., *14:* 278 (1980).
12. Lawson, E. E., Brown, E. B., Torday, J. S., Madansky, D. L., and Taeusch, H. W., Jr.: The effect of epinephrine on tracheal fluid flow and surfactant efflux in fetal sheep. Am. Rev. Resp. Dis., *118:* 1023 (1978).
13. Lee, H., Schulz, A. R., and Fuller, R. W.: Isolation and purification of rabbit adrenal norepinephrine N-methyl transferase isoenzymes. Arch. Biochem. Biophys., *185:* 222 (1978).
14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, A. J.: Protein measurement with the folin phenol reagent. J. Biol. Chem., *192:* 265 (1951).
15. Maniscalco, W. M., Parkhurst, A., and Shapiro, D. L.: The effect of dexamethasone on beta adrenoreceptors in fetal rat lung explants. Pediatr. Res. (Abstract), *16:* 355A (1982).
16. Parvez, H. and Parvez, S.: Micro radioisotopic determinations of enzymes catechol-o-methyltransferase, phenylethanolamine-N-methyltransferase and monoamine oxidase in a single concentration of tissue homogenate. Clin. Chim. Acta, *46:* 85 (1973).
17. Pendleton, R. J., Gessner, G., and Sawyer, J.: Studies on the distribution of phenylethanolamine N-methyltransferase and epinephrine in the rat. Res. Comm. Chem. Pathol. Pharmacol., *21:* 315 (1978).
18. Pendleton, R. J., Gessner, G., and Sawyer, J.: Studies on lung N-methyltransferases, a pharmacological approach. Naunyn-Schm. Arch. Pharmacol., *313:* 263 (1980).
19. Pendleton, R. G., Gessner, G., Weiner, G., Jenkins, B., Sawyer, J., Bondinell, W., and Intoccia, A.: Studies on SKF 29661, an organ specific inhibitor of phenylethanolamine N-methyltransferase. J. Pharmacol. Exp. Therap., *208:* 24 (1979).
20. Pendleton, R. G., Kaiser, C., and Gessner, G.: Studies on adrenal phenylethanolamine N-methyltransferase (PNMT) with SKF 64139, a specific inhibitor. J. Pharmacol. Exp. Therap., *197:* 623 (1976).
21. Pohorecky, L. A. and Baliga, B. S.: Purification and properties of rat adrenal phenylethanolamine N-methyl transferase. Arch. Biochem. Biophys., *156:* 703 (1973).
22. Walters, D. V. and Olver, R. E.: The role of catecholamines in lung liquid absorption at birth. Pediatr. Res., *12:* 239 (1978).
23. Wyzogrodski, I., Taeusch, H. W., Jr., and Avery, M. E.: Isoxsuprine-induced alterations of pulmonary pressure-volume relationships in premature rabbits. Am. J. Obstet. Gynecol., *119:* 1107 (1974).
24. Yu, P. H.: Phenylethanolamine N-methyltransferase from the brain and adrenal medulla of the rat. Neurochem. Res., *3:* 755 (1978).

25. Presented in part at the Society for Pediatric Research, 1982.
26. The authors acknowledge the generous gift of the pharmacologic inhibitors SKF 29661 and 64139 from Dr. Robert G. Pendleton, Smith Kline and French Laboratories, Philadelphia, PA 19101.

27. Requests for reprints should be addressed to: James F. Padbury, Building A-17, Harbor-UCLA Medical Center, UCLA School of Medicine, 1000 W. Carson St., Torrance, CA 90509.

28. This research was supported in part by USPHS Grant HD 11753.
29. Received for publication July 28, 1982.
30. Accepted for publication December 2, 1982.

0031-3998/83/1705-0362$02.00/0