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ISOLATION AND CHARACTERIZATION OF LIPID *N*-METHYLTRANSFERASE FROM DOG LUNG

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SUMMARY

A soluble protein with lipid *N*-methyltransferase activity has been isolated from microsomes of dog lung. This protein catalyzes the transfer of methyl groups from *S*-adenosylmethionine to phosphatidyl ethanolamine with the formation of phosphatidyl choline. The partially purified protein was stable at pH greater than 8.2 in the presence of cysteine. It contained 2–5% lipid and had optimal activity at pH 8.0–9.0. Reaction velocities were markedly increased by exclusion of O_2 from the reaction system. When saturating levels of *S*-adenosylmethionine were used, highest reaction velocities were obtained with disaturated phosphatidyl ethanolamine and lower rates with more unsaturated substrates. Reaction rates in the liver particulate-bound system are higher than lung but are the same with saturated or unsaturated phosphatidyl ethanolamine as substrate.

In sucrose density-gradient centrifugation experiments the transferase protein was shown to be associated with lamellated structures peculiar to lung pneumocytes. These results suggest that the *N*-methyltransferase of lung has special properties and may be important in the synthesis of disaturated surface-active phosphatidyl choline.

INTRODUCTION

Enzymes capable of catalyzing a variety of N-methylations have been described in various animal tissues and bacteria. Particular attention was directed by BREMER and co-workers to the *N*-methyltransferase activity of rat liver[1–3]. Microsomes isolated from rat liver were shown to catalyze the transfer of methyl groups from *S*-adenosylmethionine to lipid precursors with phosphatidyl choline as a final product. Lipid methyltransferases have now been identified in liver, muscle, kidney and lung of several animal species, and attempts have been made to compare the contribution of the transferase pathway with other synthetic routes for phosphatidyl choline[4].

Our interest in the properties of *N*-methyltransferases of lung was stimulated by the finding in dog lung of phosphatidyl *N*,*N*-dimethylethanolamine[5]. This intermediate was of particular interest because it contained acyl groups whose composition closely resembled that of highly surface-active dipalmitoyl phosphatidyl choline. In both phosphatidyl dimethylethanolamine and phosphatidyl choline, palmitate accounted for 65–80% of fatty acyl groups, and both lipids possessed the ability to

lower surface tension markedly when tested by the Wilhelmy dynamic surface tension apparatus. These findings suggested that the highly surface-active phospholipids might be synthesized in lung by N-methylation of phosphatidyl ethanolamine rather than by incorporation of preformed choline and that N-methylation in lung may be a selective process leading to the specific concentration of surface-active lipids in lung tissue[5].

In studying the role of lung *N*-methyltransferases in the formation of surface-active phosphatidyl choline in lung, a soluble protein fraction with *N*-methyltransferase activity has been isolated from dog lung. The isolation, purification and characterization of this protein with regard to substrate specificity and other properties particularly related to the biosynthesis of surface-active lipids are the subject of this report. Previous work also suggested that lipid biosynthesis is affected by exposure to high O_2 concentration for prolonged periods[5] and led us to study the effect of various gas mixtures on transferase activity.

MATERIALS AND METHODS

(i) Isolation

Adult mongrel dogs were anesthetized by the intravenous administration of 10 mg pentobarbital per kg body weight. The chest was opened and the dog exsanguinated. The lungs were removed rapidly *en bloc*, and the pulmonary arteries were irrigated with cold 0.9% NaCl solution to remove blood. The lungs were then weighed, homogenized in 2 vol. 0.1 M Tris buffer (pH 8.2) for 1 min in a chilled Waring blender and the homogenate strained through two layers of gauze to remove connective tissue. All subsequent operations were carried out at 4° in the same buffer unless otherwise noted. The homogenate was centrifuged at 1000 × *g* for 15 min to remove nuclei and fragmented cells. The supernatant was decanted and centrifuged at 21 000 × *g* for 15 min. Streptomycin sulfate was added to the new supernatant (final concn., 10 mg/ml) to precipitate nucleic acids and prevent bacterial growth. After 30 min the mixture was centrifuged at 100 000 × *g* for 90 min. The reddish-brown microsomal sediment was washed with Tris buffer and then resuspended to a protein concentration of 5–10 mg/ml as estimated by the method of LOWRY *et al.*[6].

Granular $(NH_4)_2SO_4$ was added to 30% satn. After standing 12 h, the solution was clarified by centrifugation at 35 000 × *g* for 20 min. The supernatant was adjusted to 60% satn. with $(NH_4)_2SO_4$ and centrifuged to yield a reddish precipitate which redissolved on addition of 2 vol. Tris buffer. $(NH_4)_2SO_4$ fractionation was repeated on this solution as before.

The second 30–60% $(NH_4)_2SO_4$ precipitate was then dissolved in Tris buffer and dialyzed overnight against 10 vol. of buffer containing 2 μM cysteine. This protein solution was diluted to a protein concentration of 2–3 mg/ml with 0.02 M KCl. Chromatography was carried out at 4° on a 2 cm × 30 cm DEAE-cellulose column packed in 0.02 M KCl in 0.01 M Tris–HCl buffer (pH 8.2) and eluted with 0.1, 0.2, 0.3, and 0.5 M KCl in 0.01 M Tris–2 μM cysteine buffer (Fig. 1). Absorbance at 280 nm was monitored using a Gilson ultraviolet monitor. 10-ml fractions were collected which were then recombined on the basis of the absorbance at 280 nm, dialyzed against Tris buffer and assayed for activity as described below. Active fractions were lyophilized and stored at −10°.

(ii) Assay for phospholipid methyltransferases

The reaction mixture consisted of transferase protein (or homogenate); 100 μ-moles of Tris–HCl buffer (pH 8.2), 10 μmoles cysteine, 10 μmoles $MgCl_2$, 0.14–0.30 μ-mole *S*-adenosyl[*Me*-^{14}C]methionine ($6.6 \cdot 10^8$ or $9.2 \cdot 10^8$ disint./min ^{14}C per μmole) and 0.5 μmole of dispersed phospholipid in a total volume of 1.50 ml. Phosphatidyl ethanolamine was isolated from dog lung using silicic acid and ammonium silicate column chromatography[5] and carefully dispersed by dialysis[7,8]. The phospholipid after repeated chromatography was estimated to contain only trace amounts of plasmalogen, lysophosphatidyl ethanolamine and methylated derivatives of phosphatidyl ethanolamine. Synthetic phospholipids were dispersed by dialysis or by ultrasonic irradiation[6]. *S*-Adenosyl[*Me*-^{14}C]methionine was obtained from Tracerlab, Waltham, Mass., and rechromatographed before use. Incubation was carried out for 60 min at 30° in a Dubnoff metabolic incubator. The reaction was stopped by adding 0.15 ml concentrated HCl and chilling. Each sample was extracted as described by KANESHIRO AND LAW[7] and the chloroform washes pooled and dried under N_2 were spotted on silica gel G thin-layer chromatography plates. Phospholipid standards were also applied to the plates which were developed in chloroform–methanol–water (95:35:4; by vol.) and exposed briefly to iodine vapor. Lipid fractions identified by comparison with the standards were scraped into scintillation vials filled with Cabosil (Thixotropic gel, Packard Instrument) and counted in 0.4% 3,5-bis-(2-(5-*tert.*-butyl-benzoxazolyl))-thiophene (scintillation grade) in toluene using a Packard Tri-Carb Liquid Scintillation Spectrometer. Quench correction was applied using automatic external standardization. Efficiency of the system was 73.0%.

(iii) Identification of products of enzymatic activity

A reaction mixture comprised as described in the preceding section, but containing 5 μmoles dispersed phosphatidyl ethanolamine, 30 mg partially purified *N*-methyltransferase (DEAE-cellulose, Fraction 3 (Fig. 1)) and 5-fold amounts of all other components, was incubated for 2 h at 30°. The reaction was stopped and lipids were extracted as described above. 1 aliquot of the lipid extract was hydrolyzed by refluxing in 3 M methanolic HCl overnight. Water was added and the mixture extracted with 20 vol. diethyl ether. The remaining water-soluble components were separated by paper chromatography using a solvent system of 1-butanol–phenol–80% formic acid–water (50:50:3:10; v/w/v/v) saturated with KCl. These water-soluble components were identified under ultraviolet light and by their color reactions with ninhydrin and Dragendorf spray reagents, and counted with a Nuclear-Chicago paper strip gas-flow counter. Ethanolamine, mono- and dimethylethanolamine and choline chloride (obtained from Eastman–Kodak) were chromatographed concurrently as standards. R_F values obtained are given in Table IV. Another aliquot of the lipid was hydrolyzed as above, and the identity of the water-soluble bases was confirmed by gas chromatography using a method previously described[5]. A third aliquot of the lipid extract (containing approx. 2 μmoles phosphatidyl ethanolamine and its biosynthetic products) was chromatographed on a 3-g Al_2O_3 column[5]. The ethanolamine and choline phospholipid fractions separated by chromatography were deacylated and fatty acid composition was determined by gas chromatography[5].

(iv) Subcellular distribution of lipid methyltransferases

A sample of lung was subjected to differential centrifugation as described in Section (i) above except that streptomycin was omitted. The material sedimenting at 100 000 × *g* for 90 min was resuspended in 0.1 M Tris–HCl buffer (pH 8.2) containing 24.2 mg sucrose/ml ($\varrho = 1.100$) and centrifuged for 90 min in a swinging bucket rotor at 100 000 × *g* in a Spinco Model L ultracentrifuge. Aliquots of all sedimenting fractions and of the final sucrose supernatant were removed for assay of protein content and methyltransferase activity. The remaining portions of each fraction were stirred with 1.2% OsO_4 in S-collidine buffer (pH 7.4) and fixed for 4 h. Samples were then dehydrated and embedded in Epon 812, as described by LUFT[9], centrifuging briefly in a table centrifuge where necessary to sediment fixed samples. 1-μ sections were cut and examined by Dr. G. L. Huber using an RCA EMU-2A electron microscope.

RESULTS

Isolation and purification of *N*-methyltransferase activity from dog lung was carried out by a scheme similar to that employed by KANESHIRO AND LAW for the bacterial enzyme[7]. Results of the purification are summarized in Table I. Overall

TABLE I

PURIFICATION OF PHOSPHATIDYL ETHANOLAMINE *N*-METHYLTRANSFERASE

Step	*Enzyme specific activity**	*Enzyme recovery (%)*	*Phospholipid content***
Whole lung homogenate	0.007	100	11.6
Sediment (100 000 × *g*, 90 min)	0.047	28	26.0
$(NH_4)_2SO_4$, 30–60%	1.270	3	27.3
DEAE-cellulose chromatography:			
Fraction 2	2.180	0.34	2.5
Fraction 3	1.720	0.08	4.6

* nmoles [^{14}C]methyl incorporated into phospholipid per mg protein per h.
** (Phospholipid weight/protein weight) × 100.

purification using $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography was 300-fold. All DEAE-cellulose fractions showed transferase activity, but maximal enzyme activity was obtained by elution with 0.1 M KCl (Fig. 1). Chromatography was carried out rapidly in the cold, and even brief exposure to pH less than 7.0 led to almost complete loss of activity. The protein retained more than 50% activity, however, when lyophilized at pH 8.2 and stored at −10° for several weeks.

Phospholipid content and composition was assayed in each step of the purification procedure (Table I). Phospholipid was 27% of the microsomal fraction by weight, but the final enzyme fractions were low in phospholipid content. The phospholipid distribution in DEAE-cellulose Fractions 2 and 3 was phosphatidyl choline 57%, phosphatidyl ethanolamine 20% and lysophosphatidyl choline *plus* sphingomyelin 12%. These values were averages of five determinations, spots being identified as previously described[4].

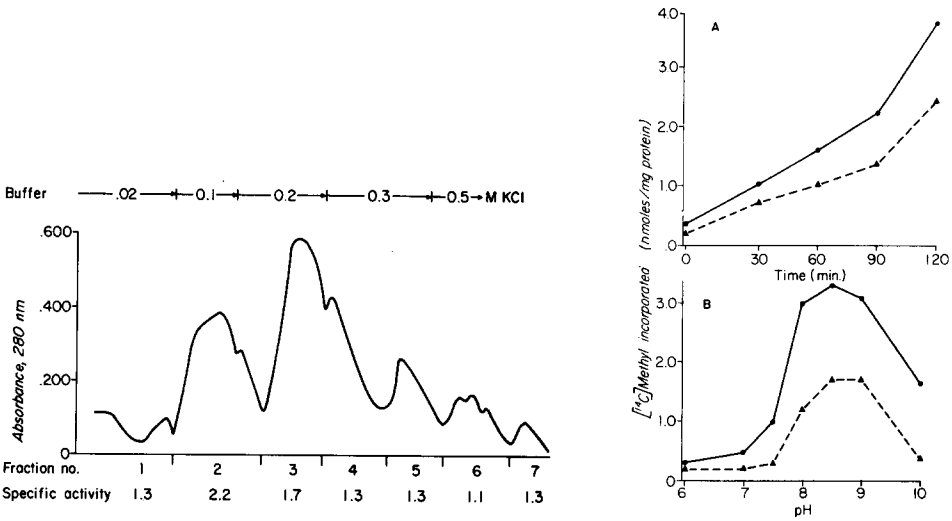

Fig. 1. DEAE-cellulose chromatography of 30–60% $(NH_4)_2SO_4$ fraction from dog lung microsomes. DEAE-cellulose chromatogram indicating the elution of ultraviolet-absorbing material at 280 nm (———). Details of the conditions are given in the text. Incubation of aliquots containing 0.1 mg protein was carried out for 1 h at 30° (pH 8.2) as described in the text. Specific activity: nmoles [^{14}C]methyl incorporated into total phospholipid per mg protein per h.

Fig. 2. Effect of time and pH on incorporation of [^{14}C]methyl into lipid fractions. Mixtures for incubation at 30° contained 0.3 mg enzyme protein (isolated by DEAE-cellulose chromatography), 100 μmoles Tris–HCl buffer (pH 8.2 or as indicated), 0.5 μmole dispersed dog-lung phosphatidyl ethanolamine, 10 μmoles $MgCl_2$ and 0.3 μmole *S*-adenosyl[*Me*-^{14}C]methionine in a total volume of 1.5 ml. After various intervals (1 h in pH experiment), the reaction was stopped and the lipids separated as described in the text. ●—●, incorporation into the total phospholipid fraction; ▲— — —▲, incorporation into phosphatidyl choline. Values given are means of duplicate determinations.

Properties

Temperature dependence. *N*-Methyltransferase activity was assayed using DEAE-cellulose fraction 2 and exogenous phospholipid obtained from dog lung. Incubation at 35° frequently produced aggregation of protein, therefore, 30° was selected for the routine assay system. Raising the reaction temperature to 35° approximately doubled the reaction rate and lowering to 25° halved the reaction rate. There was no transferase activity after heating 30 min at 50°.

pH. *N*-Methyltransferase activity at pH greater than 8.0 was approximately three times that at lower pH values (Fig. 2). There was no appreciable activity in the pH range 2–6, while at pH greater than 9, decomposition of *S*-adenosylmethionine occurred, and the results were unreliable.

Cofactors and inhibitors. Transferase activity was 30–50% of maximal activity in the absence of Mg^{2+}, and normal activity was rapidly lost during incubation or chromatography unless cysteine was added (Table II). Sulfhydryl inhibitors (10 μM $HgCl_2$ and *p*-chloromercuribenzoate, 100 μM *N*-ethylmaleimide) effectively blocked lipid incorporation of [^{14}C]methyl but CN^-, F^- and maleate (at 1 mM) were without effect. The addition of ethanolamine or choline to the assay system had no effect on the rate of [^{14}C]methyl incorporation into phospholipids.

TABLE II

COFACTOR AND INHIBITOR EFFECT ON *N*-METHYLTRANSFERASE ACTIVITY

The complete assay system contained 0.1 mg protein (DEAE-cellulose fraction 2), 0.5 μmole dog lung phosphatidyl ethanolamine dispersed by dialysis[7,8], 10 μmoles cysteine, 10 μmoles $MgCl_2$, 0.14 μmole *S*-adenosyl [*Me*-^{14}C]methionine and 100 μmoles Tris-HCl buffer (pH 8.2) in a volume of 1.5 ml. Incubation was 1 h at 30°. Incorporation was assayed as described in the text A.

	*N-Methyltransferase activity**
Cofactor requirements	
Complete system	2.08
Complete system, less dispersed phosphatidyl ethanolamine	0.48
Complete system, less $MgCl_2$	0.91
Complete system, less cysteine	1.31
Complete system, *plus* ethanolamine (1 μmole) and choline (1 μmole)	2.04
Sulfhydryl inhibitors	
Complete system	1.92
Complete system, *plus* $HgCl_2$ (10 μM)	0.96
Complete system, *plus* *p*-chloromercuribenzoate (10 μM)	0.53
Complete system, *plus* *N*-ethylmaleimide (0.1 mM)	0.20
Metabolic inhibitors	
Complete system	1.98
Complete system, *plus* KCN (1 mM)	1.90
Complete system, *plus* NaF (1 mM)	2.02
Complete system, *plus* sodium maleate (1 mM)	1.85

* nmoles [^{14}C]methyl incorporated in phospholipid per mg enzyme protein per h.

Substrate. The effect of various exogenous phospholipids on transferase activity was estimated using phosphatidyl ethanolamine obtained from dog lung by chromatography or from yeast (gift of D. J. Hanahan) and synthetic L-α,β-dipalmitoyl phosphatidyl ethanolamine (obtained from International Chemical and Nuclear, Los Angeles). Saturating levels of *S*-adenosylmethionine were employed to determine a saturation curve for dog-lung phosphatidyl ethanolamine dispersed by dialysis. When 0.1 mg enzyme protein and 0.5 μmole phospholipid were used, approx. 0.05 μmole *S*-adenosyl-[*Me*-^{14}C]methionine was required to saturate the system (Fig. 3). The yeast and synthetic phospholipids could not be dispersed by the dialysis method of FLEISCHER AND KLOUWEN[8]; therefore, all substrates in this experiment were suspended in buffer by ultrasonic irradiation with a 20-kcycle Branson instrument (0.5 A, 30 min, 4°). Following irradiation, the suspension was clarified by brief centrifugation, and an aliquot was removed for phosphorus assay and thin-layer chromatography. Incubation was then carried out in the usual way. Results of this study are presented in Fig. 4. An apparent Michaelis constant of 0.35 mM was calculated for dipalmitoyl phosphatidyl ethanolamine and higher constants for the more unsaturated yeast and lung phosphatidyl ethanolamine. For comparison dog liver microsomal methyltransferase was isolated by the method of GIBSON *et al.*[10], and a similar attempt was made to demonstrate methyltransferase substrate specificity. The liver microsomal fraction was generally more active than lung (2.3–2.9 nmoles [^{14}C]methyl incorporated per mg enzyme protein per h), but the reaction rate was the same with all phosphatidyl

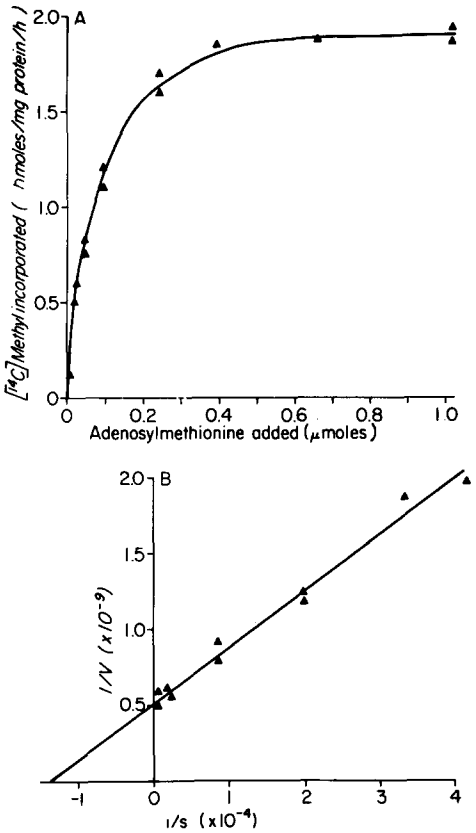

Fig. 3. A. Curve of methyltransferase saturation with *S*-adenosylmethionine. Each flask contained 0.1 mg enzyme protein and 0.5 μmole dog-lung phosphatidyl ethanolamine dispersed by dialysis. Incubation was 1 h at 30°. B. Lineweaver–Burk plot.

ethanolamine substrates regardless of acyl composition. This result could have been due to saturation of the enzyme with endogenous substrate. Phosphatidyl serine and phosphatidyl choline derived from dog lung by silicic acid column chromatography were also dispersed by dialysis and tested for substrate activity in the lung-enzyme system. With phosphatidyl choline as substrate incorporation was less than the endogenous rate. A 10% augmentation in rate of incorporation occurred with phosphatidyl serine.

Subcellular distribution of methyltransferase

A systematic identification of the intracellular components isolated by differential centrifugation was carried out so as to ascertain which subcellular fractions were associated with methyltransferase activity. Centrifugation at 100 000 × *g* for 90 min sedimented microsomes and fine tubular structures which possessed high specific activity (Table III). When this microsomal sediment was resuspended in 24.2% sucrose ($\varrho = 1.100$) and again centrifuged for 500 min at 100 000 × *g*, the sediment now had low specific activity. Under the electron microscope, this sediment contained microsomes and some pale vesicular structures (Fig. 6C). By contrast, the

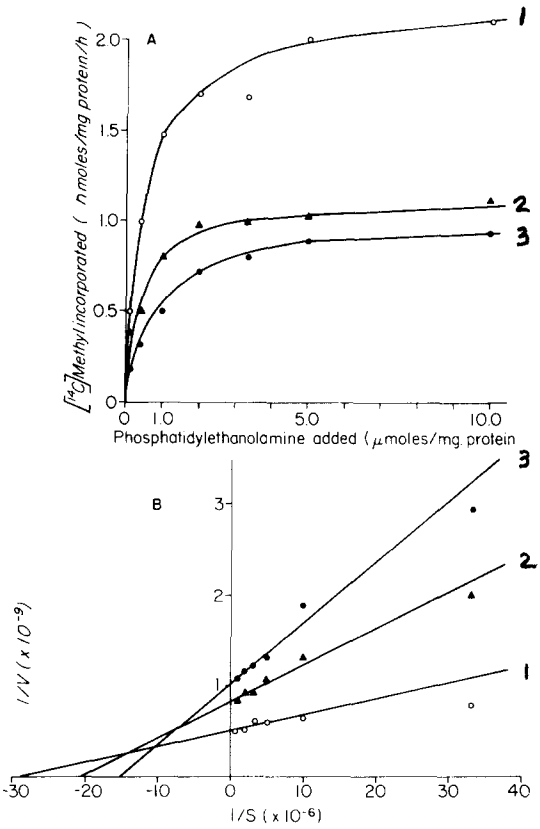

Fig. 4. A. Saturation curves of methyltransferase (0.1 mg protein) with phosphatidyl ethanolamine and 0.04 μmole *S*-adenosyl[*Me*-^{14}C]methyl methionine. (1) synthetic dipalmitoylphosphatidyl ethanolamine; (2) dog-lung phosphatidyl ethanolamine (acyl composition: palmitate 36%, stearate 12%, unsaturated C18, 20 fatty acids 48%); (3) yeast phosphatidyl ethanolamine (palmitate 4%, palmitoleate 61%, stearate 10%, unsaturated C18,20 fatty acids 20%). All phospholipids were dispersed by ultrasonic irradiation (see text) and reactions were maintained 1 h at 30°. B. Lineweaver–Burk plot.

opalescent supernatant fraction (6B) showed high enzyme activity. Morphologically, this supernatant fraction was shown to contain a few microsomes and numerous structures which had the appearance of lamellated bodies in various stages of degeneration. Total protein content was relatively low, but there was high enzyme activity and a large amount (approx. 40% by wt.) of lipid was present. The lipid was found to be 60% phosphatidyl choline and 30% phosphatidyl ethanolamine after thin-layer chromatography and phosphorus assay. By contrast, all *N*-methyltransferase fractions isolated by DEAE-cellulose chromatography contained less than 5% phospholipid.

Lipid products of methyltransferase activity

The formation of labeled *N*-methyl derivatives from phosphatidyl ethanolamine was confirmed by the method of BREMER *et al.*[1]. The results given in Table IV indicate that a considerable amount of radioactivity was found in the ether-soluble fraction

TABLE III

INTRACELLULAR LOCALIZATION OF *N*-METHYLTRANSFERASE ACTIVITY

*Procedure**	*Microscopic identification***	*Specific activity****
(1) Homogenization	—	0.007
(2) Homogenate (1), centrifuged at 1000 × *g*, 15 min: Sediment	Nuclei, fragments of tissue	0.05
(3) Supernatant (2), centrifuged at 15 000 × *g*, 30 min: Sediment	Mitochondria	0.09
(4) Supernatant (3), centrifuged at 100 000 × *g*, 90 min:		
(a) Sediment	Microsomes, tubular structures and vesicles	2.92
(b) Supernatant	—	0.03
(5) Sediment (4a) resuspended in 24.2% sucrose and centrifuged at 100 000 × *g*, 10 h:		
(a) Sediment	Microsomes, occasional pale vesicles	0.64
(b) Supernatant	Lamellated bodies	3.51

* For details, see text.

** Samples were collected, fixed, dehydrated and embedded for electron microscopy as described in the text.

*** nmoles [^{14}C]methyl incorporated into phospholipid per mg protein per h.

after acid hydrolysis presumably due to incorporation into other lipid components. Similar ether-soluble activity was found in nonincubated controls, but there was no significant incorporation into N-methylated bases. Separation of the N-methylated bases by paper chromatography was not consistent, and base identity was confirmed by gas chromatography (choline excepted)[5]. Although specific activity of the *N*-methylethanolamine or *N*,*N*-dimethylethanolamine fractions could not be accurately measured, these fractions are quantitatively minor in amount and appear to have high specific activity. Choline, qualitatively present in the greatest amount, was estimated to have lower specific activity.

TABLE IV

BASE PRODUCTS OF *N*-METHYLTRANSFERASE SYNTHESIS OF PHOSPHOLIPIDS

A mixture containing 30 mg transferase protein (DEAE-cellulose fraction 2), 1.5 μmoles *S*-adenosyl [*Me*-^{14}C]methionine ($1.1 \cdot 10^9$ disint./min), 5 μmoles dog-lung phosphatidyl ethanolamine dispersed by dialysis, 50 μmoles cysteine, 50 μmoles Mg^{2+} and 100 μmoles Tris–HCl buffer at pH 8.2 was incubated 2 h at 30°. The reaction was stopped and lipids were extracted. Aliquots of the liquid extract were hydrolyzed overnight in methanolic HCl. Ether-soluble products (16.2% of total radioactivity) were removed, and the water-soluble components were chromatographed on paper. The components were identified and counted using a gas-flow paper strip counter. Total radioactivity in the lipid fraction was $8.8 \cdot 10^8$ disint./min. Details of the procedure are given in the text.

Product	R_F	*Radioactivity (%)*
Ethanolamine	0.14	2.4
N-Methylethanolamine	0.39	15.0
N,*N*-Dimethylethanolamine	0.55	43.0
Choline	0.70	23.3

When the acyl composition of the phosphatidyl choline formed by N-methylation of dog lung phosphatidyl ethanolamine was determined, there was a relative increase in esterified saturated fatty acids. The product phosphatidyl choline contained 8.6% more myristate, 23.3% more palmitate and 10% more stearate than the precursor phosphatidyl ethanolamine. Also, the phosphatidyl ethanolamine contained 49% saturated fatty acids and the product phosphatidyl choline 69% saturated fatty acids.

Effect of composition on enzyme activity

There was marked effect of variations in gas composition on the rate of *N*-methyltransferase synthesis of phospholipids (Fig. 5). Usually assays of methyltrans-

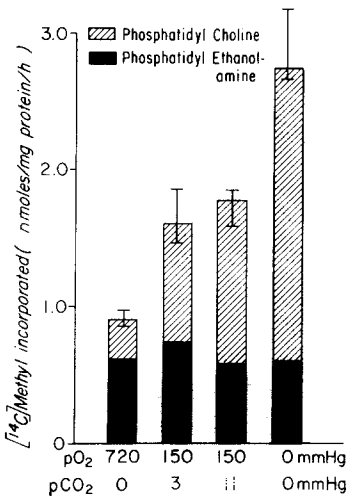

Fig. 5. Effect of gas composition on *N*-methyltransferase activity. Samples were gassed 10 min with the gas mixtures shown and for 2 min at 10-min intervals during incubation. Total incubation was 1 h at 30°, pH 8.2. Incubation mixtures and other details as in Fig. 2. Values given are means of three experiments, vertical lines indicate range of values obtained.

ferase activity were carried out with incubations at an O_2 partial pressure of 150 mm Hg and pCO_2 of 3 mm Hg. Incubations were also carried out in atmospheres of 0 or 720 mm pO_2 but with pCO_2 less than 3 mm Hg. An atmosphere composed of pO_2 150 mm and pCO_2 increased to 11 mm Hg was also used. Varying the gas composition had little effect on the rate of incorporation of [^{14}C]methyl into phosphatidyl *N*-methyl- and *N*,*N*-dimethylethanolamine, but increased O_2 partial pressure significantly depressed incorporation of radioactivity into phosphatidyl choline. Variations in pCO_2 were without effect when the pH was controlled to prevent changes in the assay system.

DISCUSSION

Of the various biosynthetic pathways known for the formation of phosphatidyl choline the cytidine diphosphocholine pathway appears to be the most important

for the formation of plasma and liver phosphatidyl choline[4]. From the data of BJORNSTAD AND BREMER[4], we may also conclude that *N*-methyltransferase-mediated phosphatidyl choline synthesis from phosphatidyl ethanolamine proceeds at a lower but significant rate in most organs. In this study, our attention has been focused on the problem of the enzymatic synthesis of the highly saturated phosphatidyl choline which appears in greatest quantity in the lung, and we have not dealt with the relative quantitative importance of various pathways of phosphatidylcholine synthesis. The transferase system isolated from lung may represent, as in microorganisms[11], two enzymes, one of which methylates phosphatidyl ethanolamine and a second which carried out subsequent methylations. Since phosphatidyl choline is the final product of this system, we assume that both enzymes, if present, have been isolated together and since we have no direct evidence whether one or more enzymes are present, we assume that a partially purified system of enzymes has been obtained.

We have compared some of the properties of the *N*-methyltransferases isolated from liver as well as lung and agree with BJORNSTAD AND BREMER[4] that hepatic transferase activity is greater than lung. There are, however, several differences between the properties of the liver and lung transferases. First, the liver subcellular fraction with greatest activity was bound to microsomes and was obtained at less than 30% satn. with $(NH_4)_2SO_4$, but the lung transferases appear to dissociate from the microsomes on prolonged standing in 30% $(NH_4)_2SO_4$ and to precipitate at between 30 and 60% satn. Second, enzymes derived from lung had reasonable stability at pH above 7.5 on DEAE-cellulose chromatography, but liver-enzyme activity was rapidly lost on chromatography. In these respects the *N*-methyltransferases of lung more closely resembled the soluble bacterial enzyme described by KANESHIRO AND LAW[7], although strictly comparable studies are not available for the particulate liver enzymes. Finally, when methyltransferases from liver and lung were incubated with substrates varying in acyl composition, saturated substrates gave high reaction velocities with the lung system. Acyl composition had no effect on the reaction velocities obtained with the liver enzymes.

The acyl composition of phosphatidyl choline isolated from lung differs markedly from that of liver or plasma. The lung shows a higher saturated acyl composition with a preponderance of palmitate. Further, previous studies have shown that phosphatidyl *N*,*N*-dimethylethanolamine contains the same high proportion of palmitate and shows the same high surface activity as does phosphatidyl choline isolated from lung. Other lung phospholipids do not show this composition or surface activity[5]. Two methods of investigation were used in this study to determine whether the *N*-methyltransferase system might operate selectively to produce the high degree of saturation which characterizes surface-active lung phosphatidyl choline. The first method employed standard enzyme kinetics and the determination of reaction velocities at saturating levels of *S*-adenosylmethionine. When the transferase system was incubated with a variety of exogenous phosphatidyl-ethanolamine substrates varying in the degree of saturation of the esterified acyl groups, reaction rates were highest in the presence of dipalmitoyl substrates and lower when unsaturated groups were present. We were able to obtain Michaelis constants for the lung transferase, but the results obtained must be interpreted with caution. First, it is likely that more than one enzyme is active, second, such velocity measurements cannot be strictly equated with substrate affinity and, third, solubility problems made it necessary to disperse substrates in

aqueous solutions by ultrasonic irradiation. Sols thus obtained may bear little relation to *in vivo* conditions, and lipid–protein interactions in this system may not be comparable to those found in the intact cell[12]. A second method was also used as a further test of the selectivity of the enzymatic system. In this experiment, phospha-

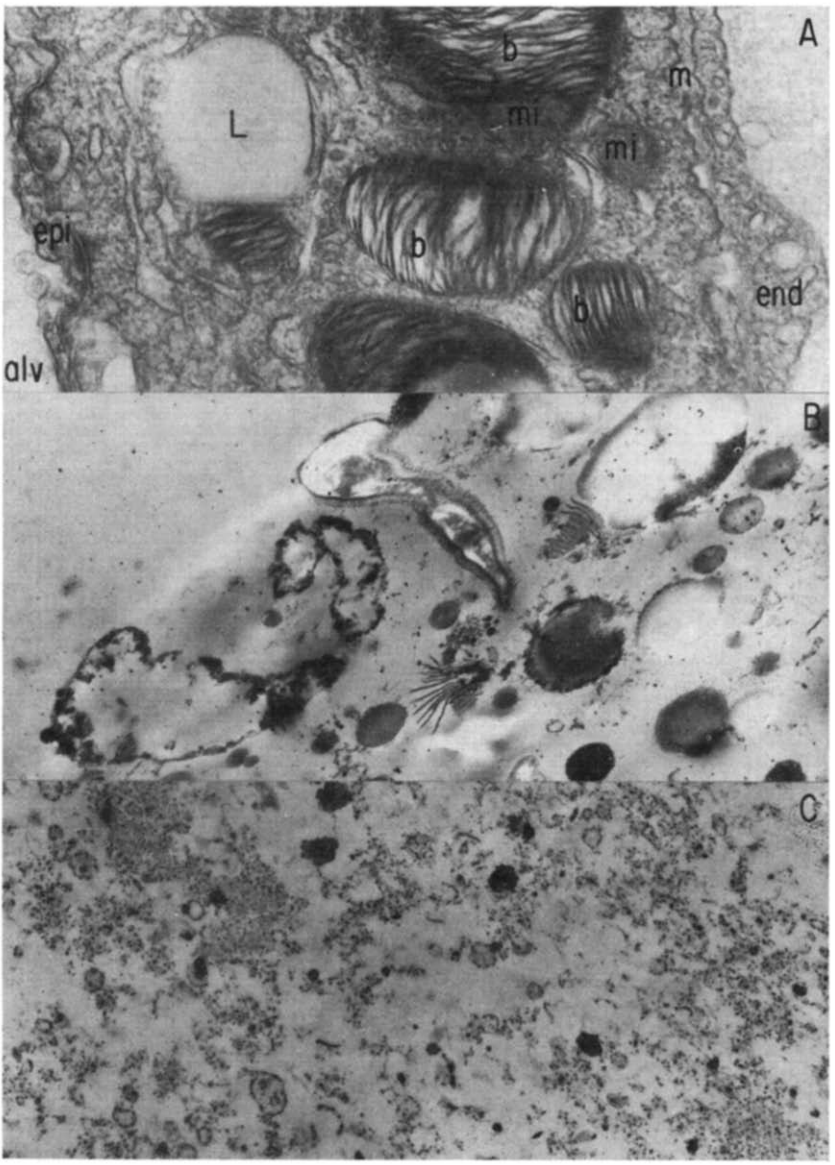

Fig. 6. Electron micrographs of dog lung. A. Section through alveolar pneumocyte, Type II, showing: alv, alveolar space; b, lamellated bodies; end, endothelial cell; epi, epithelial cell; m, basement membrane; mi, mitochondria. The space, L, is presumed to be a lipoid droplet. Section fixed and stained as described in text. B. Supernatant fraction, derived from dog-lung microsomal pellet suspended and centrifuged in 24.2% sucrose (Table III, Step 5). C. Sediment (Table III, Step 5). Magnification: (A) ×25 000; (B) and (C) ×20 000.

tidyl ethanolamine isolated from dog lung and containing acyl groups of various chain lengths and degrees of saturation was incubated with transferase protein and co-factors. The product phosphatidyl choline was isolated and found to contain a significantly higher proportion of palmitic acid and other saturated fatty acids than the starting material. Together, the results lend support to the hypothesis that disaturated phosphatidyl choline is formed in lung by *N*-methyltransferase activity. Whether dipalmitoyl phosphatidyl choline is also selectively formed by CDP-choline or by a reacylation mechanism such as that suggested by LANDS AND MERKEL[13] has not yet been determined, but work is now in progress to investigate this possibility.

An interesting and unsuspected susceptibility of *N*-methyltransferase activity to high concentrations of O_2 was encountered. Transferase activity appears to be facilitated by exclusion of O_2 from the incubation environment. The presence of high O_2 tension apparently depresses the last step of transmethylation. Since cysteine protects against loss of activity during chromatography and sulfhydryl inhibitors effectively block methyltransferase activity, it appeared possible that the effect of O_2 was due to oxidation of sulfhydryl groups important in tertiary enzyme protein structure or, possibly, in lipoprotein enzyme–substrate interaction. However, O_2 inhibition could be reversed by subsequent flushing of the incubation atmosphere with N_2, and the O_2 inhibition could not be prevented by addition of cysteine to the incubation media. In a study which may have relevance to the present work, MEYER AND BLOCK[14] have noted differing rates of esterification of saturated and unsaturated fatty acids into phospholipids of yeast grown under anaerobic or aerobic conditions. These workers have not identified the exact mechanism by which anaerobiosis effects these changes.

Circumstantial evidence suggests that the surface-active lipid or lipoprotein appears in peculiar organelles of the alveolar pneumocyte[15]. These organelles, the so-called lamellated bodies, probably contain a large amount of lipid material (Fig. 6). Our attempts to localize *N*-methyltransferase activity, however, showed it to be in the 'microsomal' fraction. That this operationally defined microsomal fraction did indeed contain microsomes was confirmed by electron microscopy. The microsomal fraction was about 27% lipid by weight and also contained lipoid droplets and vesicles with lamellated structure. On recentrifugation in a sucrose density gradient, two fractions were obtained. The sediment contained microsomes; the supernatant was rich in lipid–*N*-methyltransferase activity and was composed of lipoid droplets and lamellated bodies with but few microsomal elements. On subsequent DEAE-cellulose chromatography *N*-methyltransferase activity was highest in lipid-poor (less than 5% lipid) fractions. These findings suggest that lipid is intimately associated with, but not an integral part of the enzyme. The association of lamellated bodies isolated by differential centrifugation and *N*-methyltransferase activity makes it tempting to assume that surface-active phosphatidyl choline is synthesized in the lamellated bodies. However, this assumption cannot be considered proved and must be tested by further experiments.

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