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The localization of cholinephosphotransferase in the outer membrane of guinea-pig lung mitochondria

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The activity of cholinephosphotransferase was measured in the subcellular fractions of guinea-pig lung. The specific activity of the enzyme was highest in a fraction, intermediate in density between mitochondria and microsomes. Similar subcellular distribution patterns were observed for both cholinephosphotransferase and rotenone-insensitive NADH-cytochrome *c* reductase, an enzyme associated with the outer membrane of mitochondria and endoplasmic reticulum, suggesting that cholinephosphotransferase may be localized in both of these organelles. The distribution of cholinephosphotransferase activity in the subfractions of mitochondria and the intermediate fractions recovered by linear density gradient paralleled that of the mitochondrial outer membrane marker enzyme, monoamine oxidase. RNA content of a subfraction enriched in cholinephosphotransferase and monoamine oxidase was not typical to that of either rough or smooth endoplasmic reticulum. The results of this study suggest that in guinea-pig lung, cholinephosphotransferase is localized in both the outer membrane of mitochondria, and the endoplasmic reticulum.

Introduction

The stability of the lung of the newborn depends on the presence of adequate pulmonary surfactant, a highly surface-active material that lines the alveolar space of the lung [1]. The lack of adequate pulmonary surfactant system at birth results in respiratory distress syndrome, a major cause of death in the newborn [2].

The major active component of pulmonary surfactant is phosphatidylcholine (PC), especially dipalmitoylphosphatidylcholine (DPPC) [3]. PC is synthesized mainly by the CDPcholine pathway in the lung [4]. Though, cholinephosphate cytidyltransferase is generally agreed to be the regulatory enzyme in the CDPcholine pathway [5,6], CDPcholine: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2), the terminal enzyme in the pathway, regulates the species of PC that is synthesized [7].

Cholinephosphotransferase is generally believed to be localized exclusively in the endoplasmic reticulum [7]. However, we have earlier reported the presence of a significant activity of this enzyme in the mitochondria and in a fraction, intermediate in density between mitochondria and microsomes of both fetal and adult guinea-pig lungs [8,9]. Although the presence of cholinephosphotransferase has been observed in mitochondria of yeast [10], and rat liver [11–13] and intestine [14], there are several reports on rat liver [15–18], adult rabbit lung [19] and developing rat lung [20–21] which contradict these findings. However, Jelsema and Morre [11] could not account the

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mitochondrial cholinephosphotransferase activity in rat liver completely on the basis of contamination of the mitochondria by the endoplasmic reticulum. We provide here direct evidence that confirms the localization of this enzyme in both the endoplasmic reticulum and the outer membrane of mitochondria of guinea pig lung.

Materials and Methods

Materials. All radioactive compounds were purchased from New England Nuclear, Boston, MA. All biochemicals were purchased from Sigma Chemical Company, St. Louis, MO, except 1,2-diolein, which was purchased from Nuchek Prep. Inc., Elysian, MN. All organic solvents were purchased from MCB Manufacturing Chemists, Inc., Cincinnati, OH, except toluene and *n*-butanol which were purchased from Malinckrodt Inc., Paris, KY. Hydrofluor was purchased from National Diagnostics, Sommerville, NJ.

Subcellular fractionation. Hartley strain female guinea-pigs (350–400 g) obtained from Camm Laboratory Animals, Wayne, NJ, were killed by decapitation. The lung was quickly excised, washed in ice-cold saline (0.9% NaCl), blotted dry, and weighed. The excised lung was then minced with scissors, and homogenized in four volumes of 0.25 M sucrose/1 mM EDTA (pH 7.4), in a Potter-Elvehjem homogenizer.

The nuclear fraction was prepared by centrifuging the whole homogenate at $600 \times g$ for 10 min in a refrigerated Sorvall RC-5 centrifuge using an SS-34 rotor. The nuclear pellet was washed once with 0.25 M sucrose/1 mM EDTA (pH 7.4). The supernatants were combined and centrifuged at $10\,000 \times g$ for 10 min to obtain the mitochondrial fraction. The intermediate fraction was obtained from the resulting supernatant by centrifuging it at $20\,000 \times g$ for 15 min. The supernatant was centrifuged in a Beckman L8-M ultracentrifuge at $105\,000 \times g$ for 60 min using a 70.1 Ti rotor to obtain the microsomal and cytosolic fractions. Each particulate fraction was washed twice successively with 1.15% KCl and 0.1 M Tris-HCl/1 mM EDTA (pH 7.4), and suspended in a known volume of the latter buffer. All fractions were stored at $-20\,^{\circ}C$ until analysis. The protein content was determined by the procedure of Lowry et al. [22].

Fractionation of the intermediate and mitochondrial fractions. The intermediate fraction was fractionated by slight modification of the method of Schnaitman et al. [23] as outlined in Fig. 1. About 6.0 ml of 35 to 50% (w/v, in 0.1 M Tris-HCl/1 mM EDTA (pH 7.4)) linear sucrose gradient was made with a Buchler Polystatic Pump (Buchler Instruments), and 3.0 ml of the intermediate fraction (10–15 mg protein) was layered on top of the gradient and centrifuged with a Beckman L8-M ultracentrifuge at $50\,000 \times g$ for 60 min in a SW-40 rotor. Each subfraction was carefully removed with a pasteur pipette, and diluted with Tris-HCl/EDTA buffer. The diluted fraction was centrifuged at $20\,000 \times g$ for 15 min., and the resulting pellets were resuspended in a known volume of Tris-HCl/EDTA (pH 7.4).

The crude mitochondrial fraction obtained as described above was further fractionated on a linear 35 to 50% sucrose density gradient as the intermediate fraction (Fig. 1). The pellet obtained was resuspended in Tris-HCl/EDTA buffer, and centrifuged at $10\,000 \times g$ for 10 min. The resulting succinate dehydrogenase, monoamine oxidase enriched pellet was suspended in a known volume of the sucrose/EDTA buffer and used for further fractionation.

The outer mitochondrial membrane was isolated from the 'purified' mitochondria by digitonin treatment as described by Schnaitman et al. [23]. A digitonin stock solution (1.25%, w/v) was prepared by adding powdered digitonin to 0.25 M sucrose/Tris-HCl/1 mM EDTA (pH 7.4). Two drops of the non-ionic detergent, Kyro EOB (Proctor and Gamble Co., Cincinnati, OH), were added to the digitonin solution to make it soluble [24]. The digitonin solution remained clear for at least 3 h. Ice-cold digitonin was added to an aliquot of the mitochondrial fraction to a final concentration of 1.25 mg/10 mg protein, and incubated at $0\,^{\circ}C$ for 20 min. The mixture was diluted 3-fold with sucrose/EDTA buffer after the incubation period, and centrifuged at $10\,000 \times g$ for 10 min to remove intact mitochondria. The supernatant was centrifuged at $105\,000 \times g$ for 30 min to isolate the outer mitochondrial membrane.

Biochemical assays. Cholinephosphotransferase activity was assayed by measuring the incorpora-

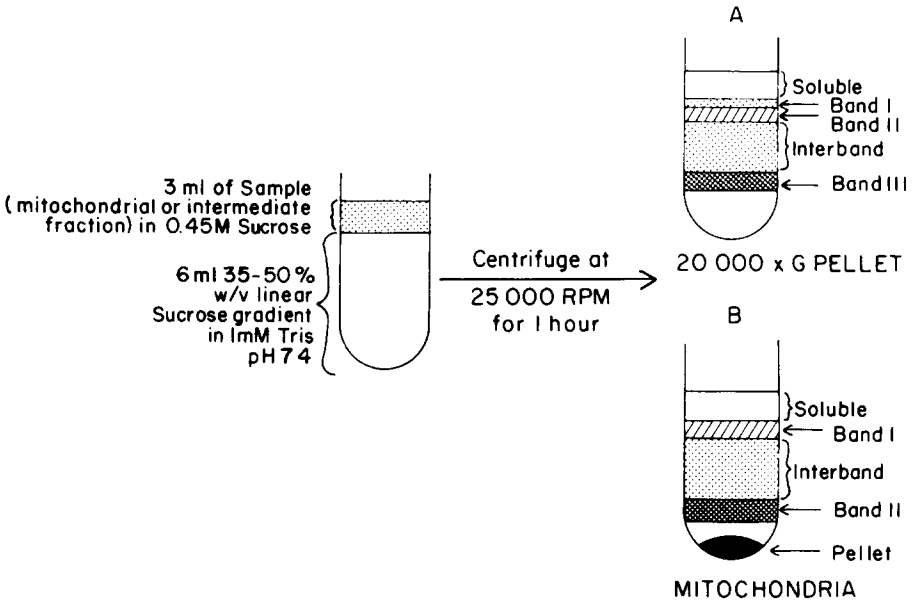

Fig. 1. Outline of the procedure used to fractionate mitochondrial and intermediate (20000 × *g* pellet) fractions on linear sucrose gradient. The pattern of separation of the intermediate fraction is shown in A, while that of the mitochondrial fraction is shown in B.

tion of radioactivity from cytidine diphospho-[*methyl*-^{14}C]choline into phosphatidylcholine as described by Stith and Das [8]. The incubation medium in a final volume of 0.1 ml, contained 10 mM $MgCl_2$, 5 mM reduced glutathione (GSH), 50 mM Tris-HCl (pH 8.5), 80 μM CDPcholine containing 0.26 μCi of CDP[^{14}C]choline (spec. act. 42.35 μCi/mmol), 6 mM 1,2-dioleoylglycerol. The dioleoylglycerol suspension was prepared by extracting 100 mg of the lipid with 0.25 ml of ethyl alcohol and added to 2.25 ml of 0.01% Triton-X 100 and sonicating the mixture for 1 min with Branson Sonifier Cell Disruptor, using the microprobe. The reaction was started by adding 20 μl (20 μg of protein) of sample, and incubated at 37 °C for 2 min. The reaction was stopped by adding 500 μl of *n*-butanol and water, respectively. The mixture was allowed to equilibrate for 10 min, and centrifuged in a Beckman Model TJ-6 table top centrifuge at 3000 rpm for 10 min. Approximately 250 μl of the butanol layer was carefully removed and placed in a counting vial. The radioactivity was determined after adding 5 ml of hydrofluor and counted in a Beckman LS-355 scintillation counter.

Succinate dehydrogenase, a mitochondrial marker, and NADPH-cytochrome *c* reductase (microsomal marker), were assayed as described by Possmayer et al. [25]. Rotenone-insensitive NADH-cytochrome *c* reductase activity was measured by the method of Sottocasa et al. [26], and catalase for peroxisomes was determined by the method of Peters et al. [27]. Monoamine oxidase, a marker for the outer membrane of mitochondria, and acid phosphatase for lysozomes, were assayed as described by Possmayer et al. [28]. The RNA content of subcellular fractions was determined as described by Munro and Fleck [29].

Results

Subcellular distribution of cholinephosphotransferase activity

The specific activity of cholinephosphotransferase and that of various marker enzymes in subcellular fractions of guinea-pig lung is shown in Table I. The enzyme activity was enriched mainly in the intermediate and microsomal fractions. In fact, the specific activity in the intermediate and microsomal fractions were about equal (2.6 and

TABLE I

SPECIFIC ACTIVITY OF CHOLINEPHOSPHOTRANSFERASE AND MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF GUINEA-PIG LUNG

The specific activities of the different enzymes are expressed in nmol/min per mg protein, except the specific activity of catalase which is expressed in units/min per mg protein. One unit of catalase activity is the amount of enzyme which causes the natural logarithm of the H_2O_2 concentration to decrease by 1. Each value represents the mean ± S.D. of six experiments.

Enzyme	Whole homogenate	Nuclear fraction	Mitochondrial fraction	20000 × *g* fraction	Microsomal fraction	Cytosol
Cholinephospho-transferase	0.4± 0.1	0.3 ± 0.1	1.3± 0.2	2.6± 0.7	2.3± 0.3	0.2± 0.1
Monoamine oxidase	0.7± 0.2	0.4 ± 0.2	3.7± 0.8	3.4± 0.7	2.1± 0.5	0.1± 0.0
NADPH-cytochrome *c* reductase	40.4±10.4	37.9 ± 7.4	43.3± 6.0	60.9± 2.2	98.3± 8.9	26.6± 8.9
Succinate dehydrogenase	12.3± 3.6	6.28± 3.9	84.2±10.1	47.9± 2.8	13.7± 1.9	6.1± 1.3
Catalase	17.0± 5.4	5.4 ± 2.0	18.0± 4.8	71.0±17.7	23.2± 3.0	27.9± 5.7
Rotenone-insensitive NADH-cytochrome *c* reductase	129.9±17.0	71.2 ±21.7	154.0±16.3	272.8±18.4	245.0±34.9	45.8±10.3
Acid phosphatase	58.7± 2.1	42.8 ± 4.8	84.4±25.3	112.4±12.7	153.4±12.2	32.9± 4.5

2.3 nmol/min per mg protein, respectively). On the contrary, the specific activity of the microsome-associated NADPH-cytochrome *c* reductase in the microsomal fraction was almost twice that of the intermediate fraction (98.3 versus 60.9 nmol/min per mg protein), indicating that at least part of the cholinephosphotransferase activity in the intermediate fraction is due to an organelle/organelles other than microsomes.

The pattern of distribution of cholinephosphotransferase activity in the different subcellular fractions resembled that of rotenone-insensitive NADH-cytochrome *c* reductase, an enzyme localized both in the microsome and the outer membrane of mitochondria [26], and differed from that of NADPH-cytochrome *c* reductase (Fig. 2). This data suggests the presence of cholinephosphotransfrease in both microsome and the outer membrane of mitochondria. It is noteworthy that the specific activity of cholinephosphotransferase in the mitochondrial fraction was relatively high. The specific activity of NADPH-cytochrome *c* reductase in the mitochondrial and nuclear fractions were about the same (43.3 and 37.9 nmol/min per mg protein, respectively). However, the former had about five times more cholinephosphotransferase activity (1.3 versus 0.3 nmol/min per mg protein). The high cholinephosphotransferase ac-

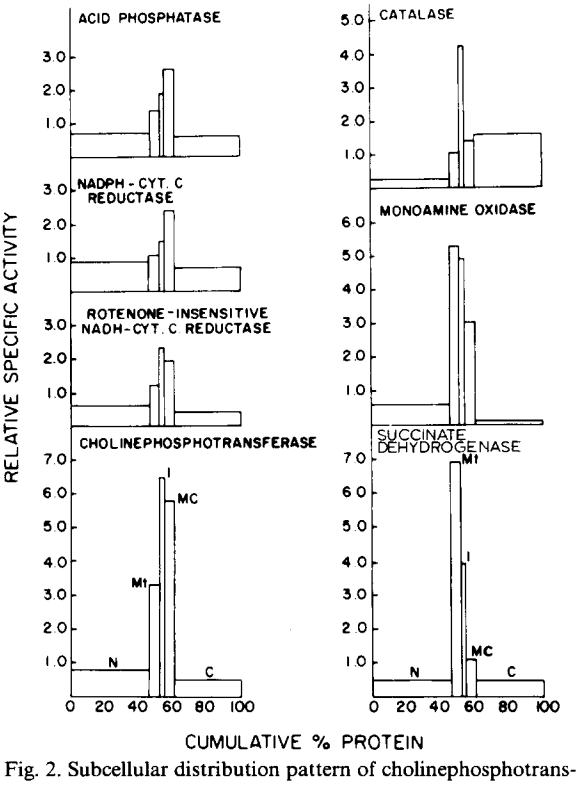

Fig. 2. Subcellular distribution pattern of cholinephosphotransferase and marker enzyme activities of guinea pig lung. Relative specific activity (the specific activity in the fraction compared to that in the whole homogenate) is plotted as a function of cumulative percent protein. Relative specific activities were determined from the data on Table I. N, nuclear fraction; Mt, mitochondria function; I, intermediate fraction; Mc, microsomal fraction; and C, cytosolic fraction.

tivity in the mitochondrial and intermediate fractions cannot be explained by microsomal contamination.

Distribution of cholinephosphotransferase activity in subfractions of the intermediate fraction

In a further attempt to localize the cholinephosphotransferase activity in the intermediate fraction, this fraction was subjected to isopynic centrifugation on a 35 to 50% linear sucrose density gradient, and the distribution of cholinephosphotransferase and various marker enzymes in the subfractions was determined (Table II). Cholinephosphotransferase, catalase, monoamine oxidase, and succinate dehydrogenase activities were enriched in the heavy subfractions (the interband and band III). On the contrary, NADPH-cytochrome *c* reductase activity was enriched in the light subfractions (bands I and II). The distribution pattern of cholinephosphotransferase activity was similar to that of monoamine oxidase, an enzyme associated with the outer membrane of mitochondria (Table II).

In order to confirm that the cholinephosphotransferase activity associated with the heavy subfractions is not of rough endoplasmic reticulum origin, the intermediate fraction was briefly sonicated prior to separation on the sucrose gradient [26]. Rough microsomal vesicles can be converted to smooth vesicles by mild sonication, due to the detachment of ribosomes from the rough vesicles [26]. Upon sonication, bands I and II that appeared as separate bands on separation of the untreated fraction, appeared as one band (corresponding to band I). the positions of the other subfractions did not change.

Sonication caused about 50% decrease in cholinephosphotransferase activity in the intermediate fraction, but did not significantly affect the activity of any of the marker enzymes (Tables II and III). The activity of cholinephosphotransferase remained mostly in the heavy subfractions, and its distribution pattern closely resembled that of monoamine oxidase as it was in the untreated intermediate fraction (Table III). There was no change in the specific activity of NADPH-cytochrome *c* reductase in bands I and III, indicating that band III is not of rough endoplasmic reticulum origin. There was a 70% enrichment in cholinephosphotransferase activity in band I. Mono-

TABLE II

SPECIFIC ACTIVITY OF CHOLINEPHOSPHOTRANSFERASE AND MARKER ENZYMES IN SUBFRACTIONS OBTAINED UPON FRACTIONATION OF 20000 × *g* PELLET BY LINEAR SUCROSE DENSITY GRADIENT CENTRIFUGATION

The specific activities of the different enzymes are expressed in nmol/min per mg protein, except the activity of catalase which is expressed in units/min per mg protein. One unit of catalase activity is the amount of enzyme which causes the natural logarithm of the H_2O_2 concentration to decrease by 1. Each value represents the mean ± S.D. of five experiments. In parenthesis is the relative specific activity, i.e. the specific activity in the fraction compared to that in the 20000 × *g* pellet.

Enzyme	Soluble	Band I	Band II	Interband	Band III	20000 × *g* fraction
Cholinephospho-transferase	0.15 ± 0.0 (0.1)	1.4 ± 0.1 (0.6)	1.9 ± 0.2 (0.8)	2.4 ± 0.6 (1.0)	2.5 ± 0.8 (1.1)	2.4 ± 0.7
Monoamine oxidase	0.2 ± 0.0 (0.1)	0.5 ± 0.1 (0.2)	1.1 ± 0.2 (0.4)	2.2 ± 0.1 (0.9)	2.3 ± 0.2 (0.9)	2.6 ± 0.2
NADPH-cytochrome *c* reductase	32.7 ± 2.6 (0.6)	62.1 ± 15.2 (1.2)	64.8 ± 6.8 (1.2)	48.0 ± 14.2 (0.9)	54.1 ± 8.2 (1.0)	54.6 ± 9.3
Succinate dehydrogenase	2.1 ± 1.5 (0.1)	6.4 ± 0.8 (0.2)	11.3 ± 1.5 (0.3)	23.9 ± 8.4 (0.6)	44.8 ± 9.0 (1.1)	40.5 ± 6.4
Catalase	15.1 ± 0.5 (0.2)	6.8 ± 5.4 (0.1)	17.5 ± 5.4 (0.3)	43.0 ± 16.5 (0.7)	70.3 ± 9.2 (1.1)	62.3 ± 10.6
Rotenone-insensitive NADH-cytochrome *c* reductase	27.1 ± 2.1 (0.2)	109.7 ± 5.2 (0.6)	170 ± 52.7 (1.0)	162.1 ± 52.0 (0.9)	180.2 ± 39.6 (1.0)	177.7 ± 6.6

TABLE III

SPECIFIC ACTIVITIES OF CHOLINEPHOSPHOTRANSFERASE AND MARKER ENZYMES IN SUBFRACTIONS OBTAINED UPON FRACTIONATION OF SONICATED 20000 × *g* PELLET BY LINEAR SUCROSE DENSITY GRADIENT CENTRIFUGATION

The specific activities of the different enzymes are expressed in nmol/min per mg protein, except the activity of catalase which is expressed in units/min per mg protein. One unit of catalase activity is the amount of enzyme which causes the natural logarithm of the H_2O_2 concentration to decrease by 1. Each value represents the mean ± S.D. of five experiments. The relative specific activity (the specific activity in the fraction compared to that in the sonicated 20000 × *g* pellet) is shown in parenthesis.

Enzyme	Soluble	Band I	Interband	Band III	Sonicated 20000 × *g* pellet
Cholinephospho-transferase	0.51 ± 0.1 (0.5)	1.27 ± 0.4 (1.3)	1.86 ± 0.5 (1.9)	1.75 ± 0.3 (1.8)	1.0 ± 0.2
Monoamine oxidase	1.1 ± 0.2 (0.5)	2.3 ± 0.2 (1.0)	2.86 ± 0.5 (1.2)	2.9 ± 0.6 (1.2)	2.4 ± 0.4
NADPH-cytochrome *c* reductase	37.8 ± 6.8 (0.8)	62.2 ± 11.5 (1.3)	46.9 ± 14.4 (1.0)	47.6 ± 13.5 (1.0)	46.8 ± 10.2
Succinate dehydrogenase	4.4 ± 2.1 (0.1)	19.9 ± 8.7 (0.6)	50.4 ± 26.9 (1.4)	67.8 ± 31.6 (1.9)	36.4 ± 10.9
Catalase	112.8 ± 6.1 (1.7)	11.6 ± 3.3 (0.2)	35.4 ± 0.6 (0.5)	29.2 ± 8.7 (0.4)	67.0 ± 20.6
Rotenone-insensitive NADH-cytochrome *c* reductase	110.0 ± 14.0 (0.6)	209.7 ± 39.4 (1.1)	207.8 ± 48.4 (1.1)	193.9 ± 35.0 (1.0)	190.8 ± 30.0

amine oxidase activity also increased by about 70% in this band, suggesting that the increase in cholinephosphotransferase activity in band I was due to increased outer mitochondrial membrane in this fraction. Catalase was recovered mostly in the soluble fraction.

The absence of rough endoplasmic reticulum in band III was further confirmed by measuring the level of RNA. As shown in Table IV, the amount of RNA in band III was approximately one-third that of band II. Also, it was only one-half that of the microsomal fraction, but comparable to that of the mitochondrial fraction. Furthermore, the electron micrograph of band III preparation (photograph not shown) from the intermediate fraction supports that this fraction represents mostly mitochondrial membrane and there was no evidence of the presence of rough endoplasmic reticulum. This data suggests that the band III cholinephosphotransferase activity is not of rough endoplasmic reticulum origin, but is from the outer membrane of mitochondria (probably light mitochondria). Since RNA content was highest in band II, this fraction most probably represents stripped rough endoplasmic reticulum. Since our homogenization medium contains EDTA, it is likely that ribosomes may have been stripped from microsomes [30], and stripped rough endoplasmic reticulum appear as smooth vesicles in band II.

Distribution of cholinephosphotransferase activity in subfractions of digitonin-treated mitochondria

It was concluded from the foregoing results

TABLE IV

RIBONUCLEIC ACID (RNA) CONTENT OF SUBCELLULAR FRACTIONS OF GUINEA-PIG LUNG

RNA concentrations are expressed as μg/mg protein and represent the mean ± S.D. of three experiments.

Fractions	RNA (μg/mg protein)
Mitochondrial 'pellet' [a]	15.1 ± 0.7
20000 × *g* pellet	21.9 ± 1.7
Band II [b]	57.5 ± 2.2
Band III [b]	16.0 ± 0.5
Microsomal pellet	31.1 ± 2.5

[a] Subfraction obtained from the fractionation of the mitochondrial fraction on linear sucrose density gradient.

[b] Subfractions obtained from the fractionation of the 20000 × *g* pellet on linear sucrose density gradient.

that cholinephosphotransferase is localized in the outer membrane of mitochondria. To further clarify this, we decided to isolate the outer membrane of mitochondria and measure cholinephosphotransferase activity. Table V shows the specific activity profile of cholinephosphotransferase, and microsomal and mitochondrial marker enzymes in subfractions of mitochondria. Cholinephosphotransferase and monoamine oxidase activities were enriched 2.2- and 1.5-fold, respectively, in the outer mitochondrial membrane-containing subfraction, and the $100000 \times g$ pellet. There was a corresponding decrease of 33 and 25 percent, respectively, of cholinephosphotransferase and monoamine oxidase activities in the $10000 \times g$ pellet, from which the outer mitochondrial membrane was isolated. Succinate dehydrogenase, a marker enzyme for the mitochondrial inner membrane was enriched in the low-speed fraction. NADPH-cytochrome *c* reductase activity was evenly distributed. The enrichment in monoamine oxidase activity was not as high as reported by Mavis and Vang [31]. However, the similarity in distribution of cholinephosphotransferase and monoamine oxidase activities, provides strong evidence for the presence of cholinephosphotransferase in the outer membrane of mitochondria.

Discussion

The data presented in this study strongly suggest that cholinephosphotransferase not only exists in the endoplasmic reticulum, but also in the outer mitochondrial membrane of guinea-pig lung. On the contrary, this enzyme has been shown to be located exclusively in the microsomes of adult lung of other species, such as rats, mice and rabbits [7]. Stith and Das [8] have reported the presence of significant levels of cholinephosphotransferase activity in mitochondria and in a fraction intermediate in density between mitochondria and microsomes of fetal guinea pig lung. Even though cholinephosphotransferase activity has also been observed in the mitochondrial fraction of rabbit lung [19] and developing rat lung [20,21], this activity has been attributed mostly to the microsomal contamination based on the NADPH-cytochrome *c* reductase activity as microsomal marker. Cholinephosphotransferase activity has also been observed in the mitochondria

TABLE V

DISTRIBUTION AND SPECIFIC ACTIVITY OF CHOLINEPHOSPHOTRANSFERASE AND MARKER ENZYMES IN SUBFRACTIONS OBTAINED BY FRACTIONATION OF THE MITOCHONDRIAL 'PELLET' SUBFRACTION BY DIGITONIN TREATMENT

Specific activities of different enzymes are expressed in nmol/min per mg protein and represent the mean ± S.D. of 3 experiments. The percentage of enzyme activity (in comparison to total activity of the untreated mitochondrial 'pellet' subfraction) is in parenthesis. The percent recovery of protein in each subfraction (60.9 for the $10000 \times g$ pellet, 9.7 for the $100000 \times g$ pellet, and 32.1 for the $100000 \times g$ supernatant) correspond to 102.7% recovery of the mitochondrial 'pellet' protein.

Enzyme	Mitochondrial subfractions				Recovery (%)
	$10000 \times g$ pellet	$100000 \times g$ pellet	$100000 \times g$ supernatant	Untreated mitochondrial pellet	
Cholinephospho-transferase	0.6 ± 0.2 (37.0)	2.0 ± 0.4 (21.6)	0.3 ± 0.1 (9.3)	0.9 ± 0.2	67.9
Rotenone-insensitine NADH-cytochrome *c* reductase	165.4 ± 37.2 (43.6)	209.2 ± 34.6 (15.3)	44.7 ± 5.0 (6.2)	228.8 ± 30.5	65.1
Succinate dehydrogenase	84.6 ± 15.5 (53.5)	38.4 ± 5.9 (4.4)	2.2 ± 0.3 (0.8)	94.5 ± 9.7	58.7
Monoamine oxidase	3.3 ± 0.1 (45.3)	6.1 ± 0.5 (18.2)	0.79 ± 0.2 (5.9)	4.4 ± 0.1	69.4
NADPH-cytochrome *c* reductase	38.5 ± 1.5 (58.2)	43.3 ± 0.4 (11.7)	37.6 ± 3.4 (30.2)	40.4 ± 3.2	100.1

of yeast [10], rat liver [11–13] and intestine [14]; however, numerous studies [15–18] did not agree that cholinephosphotransferase is present in rat liver mitochondria.

The data on the distribution of cholinephosphotransferase activity in the subcellular fractions of guinea-pig lung show that the activity in the mitochondrial and intermediate fractions cannot be totally due to microsomal contamination. The enzyme distribution profile paralleled that of rotenone-insensitive NADH-cytochrome *c* reductase, but differed from that of NADPH-cytochrome *c* reductase, indicating the localization of cholinephosphotransferase in both microsomes and the outer membrane of mitochondria. Further analysis of the mitochondrial and intermediate fractions show a close distribution profile between cholinephosphotransferase and monoamine oxidase activities in subfractions of these fractions.

The activity of NADPH-cytochrome *c* reductase in the mitochondrial fraction was relatively high. The pellet obtained on sucrose density gradient centrifugation of the crude mitochondrial fraction still retained high NADPH-cytochrome *c* reductase activity, though it was enriched in both monoamine oxidase and succinate dehydrogenase (data, not shown). Shore and Tata [32] have isolated a mitochondria-endoplasmic reticulum complex (rapidly sedimenting endoplasmic reticulum) from rat liver that sediments at low speed. It contains mitochondrial and microsomal marker enzymes and phospholipid-synthesizing enzymes [33]. We did not attempt to isolate rapidly sedimenting endoplasmic reticulum in this study. However, the cholinephosphotransferase activity should be associated with the mitochondrial portion of the complex, if such a complex is present in the mitochondrial fraction, since its distribution in mitochondrial subfractions closely resembles that of monoamine oxidase. Sottocasa et al. [26] have suggested that the presence of the NADPH-cytochrome *c* reductase activity may be due to pyridine nucleotide dehydrogenase, a mitochondrial enzyme, that converts NADPH to NADH. The NADH formed is utilized by NADH-cytochrome *c* reductase to reduce cytochrome *c*. Therefore, the presence of NADPH-cytochrome *c* reductase in the mitochondrial fraction may not represent 'true' microsomal contamination. Furthermore, it is important to emphasize that neither smooth or rough endoplasmic reticulum are homogeneous and can be fractionated [34–36]. This might explain some of the differences between cholinephosphotransferase and other endoplasmic reticulum markers. In this regard, it is important to establish that the mitochondrial fraction is not contaminated with plasma membranes and Golgi apparatus, which may contain cholinephosphotransferase activity. Preliminary studies in this laboratory have however revealed that the activities of 5′-nucleotidase, a plasma membrane marker [37] and *N*-acetylglucosamine galactosyltransferase, a Golgi marker [38] are present in very low quantities in band III (data not shown), and therefore it is unlikely that the cholinephosphotransferase activity in band III is associated with either plasma membrane or Golgi apparatus.

The dual localization of cholinephosphotransferase in both mitochondria and microsomes reported here is not unique. Glycerophosphate phosphatidyltransferase, a key enzyme in the synthesis of phosphatidylglycerol, is present in both microsomes and mitochondria [16,31,39]. CTP : cholinephosphate cytidylyltransferase which is thought to be localized in microsomes and cytosol [5] was recently reported to be present in human lung mitochondria [40]. Hunt and Postle reported that as much as 59% of human lung cytidyltransferase activity was associated with mitochondria, while the microsomal fraction accounted for only 4% of the activity. It needs to be determined if the subcellular localization site of some phospholipid-synthesizing enzymes differs between animal species. It appears that there is a difference between guinea-pig and other species, such as rats, mice and rabbits, so far the subcellular distribution of cholinephosphotransferase is concerned. Some data at least are consistent with a very small amount of cholinephosphotransferase activity being associated with endoplasmic reticulum of rat tissues. Currently, we have initiated studies to determine whether there is a species specificity and tissue specificities on the presence of cholinephosphotransferase in mitochondria.

Preliminary studies on kinetic properties of unpurified mitochondrial and microsomal cholinephosphotransferase, indicate that the two enzymes are probably different (unpublished data). It needs

to be established if the two enzymes are isozymes.

We did not address the role of the outer mitochondrial membrane cholinephosphotransferase activity here. Due to the heterogeneity of lung in regards to cell types [41], studies with whole lung does not necessarily indicate what happens in type II cells, the surfactant-producing cells in the lung [41]. We recently reported the presence of cholinephosphotransferase activity in guinea-pig lung type II cell mitochondrial fraction [42]. Batenburg et al. [39] have also reported a similar finding in fetal rat lung type II cells. Thus, it would appear that cholinephosphotransferase is also localized in type II cell mitochondria. However, it is premature to associate this activity with surfactant-PC synthesis. We are currently investigating the role of mitochondrial cholinephosphotransferase.

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References

1 Pattle, R.E. (1958) Proc. Roy. Soc. London, Ser. B. 148, 217–240
2 Gluck, L. (1972) Pediatr. Clin. North Am. 19, 325–331
3 King, R.J. and Clements, J.A. (1972) Am. J. Physiol. 224, 788–795
4 Kennedy, E.P. (1961) Fed. Proc. 20, 934–940
5 Vance, D.E. and Choy, P.E. (1979) Trends Biochem. Sci. 4, 145–148
6 Post, M., Batenburg, J.J., Schuurman, E.A.J.M. and Van Golde, L.M.G. (1982) Biochim. Biophys. Acta 712, 390–394
7 Van Golde, L.M.G. (1976) Am. Rev. Resp. Dis. 114, 977–1000
8 Stith, I.E. and Das, S.K. (1982) Biochim. Biophys. Acta 714, 250–256
9 Sikpi, M.O. and Das, S.K. (1984) Fed. Proc. 43, 1654
10 Ostrow, D. (1971) Fed. Proc. 30, 1226
11 Jelsema, C.L. and Morre, J.D. (1978) J. Biol. Chem. 253, 7960–7971
12 Van Schijndel, B.C., Reitsema, A. and Sherphof, G.L. (1973) Biochem. Biophys. Res. Commun. 55, 568–573
13 Stoffel, W. and Scheifer, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1017–1026
14 Hulsmann, W.C. and Kurpershoek-Davidov, R. (1976) Biochim. Biophys. Acta 450, 288–300
15 Dennis, E.A. and Kennedy, E.P. (1972) J. Lipid Res. 13, 263–267
16 Van Golde, L.M.G., Fleischer, B. and Fleischer, S. (1971) Biochim. Biophys. Acta 249, 318–330
17 McMurray, W.C. and Dawson, R.M.C. (1969) Biochem. J. 112, 91–108
18 McMurray, W.C. (1974) Biochem. Biophys. Res. Commun. 58, 467–474
19 Jobe, A., Ikegami, M., Sarton-Miller, I., Jones, S. and Yu, G. (1981) Biochim. Biophys. Acta 666, 47–57
20 Harding, P.G.R., Chan, F., Fasola, P.G., Frazer Fellows, G., Wong, T. and Possmayer, F. (1983) Biochim. Biophys. Acta 750, 373–382
21 Weinhold, P., Williams, S. and Miller, J. (1984) Fed. Proc. 43, 1654
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 1772–1775
23 Schnaitman, C., Erwin, V.J. and Greenawalt, J.W. (1967) J. Cell Biol. 32, 719–735
24 Janski, A.M. and Cornell, N.W. (1980) Biochem. J. 186, 423–429
25 Possmayer, F., Duwe, G., Hahn, M. and Buchnea, D. (1977) Can. J. Biochem. 55, 609–617
26 Sottocasa, G.L., Kuylenstierna, B., Ernester, L. and Bergstland, A. (1967) J. Cell Biol. 32, 415–438
27 Peters, T.J., Muller, M. and De Duve, C. (1972) J. Exp. Med. 136, 1117–1139
28 Possmayer, F., Duwe, G., Metcalfe, R., Stewart-DeHaan, P.J., Wong, C., Heras, J.L. and Harding, P.G.R. (1977) Biochem. J. 166, 485–494
29 Munro, H.N. and Fleck, A. (1966) Methods Biochem. Anal. 14, 113–176
30 Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 852–862
31 Mavis, R.D. and Vang, M.J. (1981) Biochim. Biophys. Acta 664, 409–415
32 Shore, G.C. and Tata, J.R. (1977) J. Cell Biol. 72, 714–725
33 Stuhne-Sekalec, L. and Stanacev, N.Z. (1982) Can. J. Biochem. 60, 877–881
34 Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Marriett, R. and Zacques, B. (1974) J. Cell Biol. 61, 188–200
35 Amar-Costesec, A., Beaufay, H. Wibo, M., Thines-Sempoux, D., Feytmans, E., Mariett, R. and Zacques, B. (1974) J. Cell Biol. 61, 201–212
36 Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibo, M., Mariett, R. and Zacques, B. (1974) J. Cell Biol. 61, 213–231
37 Emmelot, P. and Bos, C.J. (1966) Biochim. Biophys. Acta 120, 369–382
38 Palmiter, R.D. (1969) Biochim. Biophys. Acta 178, 35–46
39 Batenburg, J.J., Klazinga, W. and Van Golde, L.M.G. (1985) Biochim. Biophys. Acta 833, 17–24
40 Hunt, A.N. and Postle, A.D. (1985) Biochem. Soc. Trans. 13, 1203–1204
41 Sorokin, S.P. (1970) The Cells of the Lung, in Morphology of Experimental Respiratory Carcinogenesis (Nettesheim, P., Hanna, M.G. and Deatherage, J.W., eds.), pp. 3–43, U.S. Atomic Energy Commission, Oak Ridge, TN
42 Sikpi, M.O., Johns, A.E., Nair, C.R. and Das, S.K. (1986) Biochim. Biophys. Acta 877, 20–30